Aus dem Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten

der Ludwig-Maximilians-Universität München

Dissertation

zum Erwerb des Doctor of Philosophy (Ph.D.)

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

The role of Neutrophil Extracellular Traps in hepatic immune homeostasis

vorgelegt von:

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Madrid, Spain

Jahr:

2023

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Mit Genehmigung der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

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Date of the defense:

15th March, 2023

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Abstract

The liver is a frontline immunological barrier tissue, an active detoxifying filter and the most important metabolic organ in human beings. The liver immune compartment is capable of detecting, capturing and clearing pathogens, pathogen-associated molecular patterns (PAMPs), gut-incoming antigens and diet-derived metabolites. Consequently, the hepatic immune compartment needs to ensure a delicate balance between tolerogenic and inflammatory responses, against non- and pathogenic insults, respectively. NETosis, a neutrophil-specific cell death program, is characterized by the release of web-like structures referred to as neutrophil extracellular traps (NETs). NETs are composed of extracellular DNA strands associated with modified histones and neutrophil granular proteins. NETs exert a key antimicrobial function that allows neutrophils to capture and kill pathogens. Here, we found that NET-like structures are present in murine immunological barrier tissues (e.g. spleen, lung, lymph nodes, liver) in the absence of pathology, likely supporting an alternative role of neutrophils and NETs during tissue homeostasis. In particular, NET-like structures emerged significantly during the night-time in the hepatic tissue. Absence of NET-like structures in different neutrophil-deficient knock-out mice, and their inhibition upon DNAse I or BB-Cl-Amidine treatment, confirmed the neutrophil-origin of the identified NETs and that their presence during tissue homeostasis helps maintaining a systemic anti-inflammatory condition. Homeostasis refers to the highly dynamic processes occurring within an organism internal environment in order to ensure a constant threshold of physiological parameters. Interestingly, mice are nocturnal creatures that have a night-associated active period, when they eat substantially more food compared to the diurnal phase and are exposed to an increased amount of potential insults. This brought up the idea of NETs being not just an inner circadian-regulated immunological function, but rather that food intake could work as an environmental entrainment cue for NET release. The NETosis dynamic profile in the liver was modified upon different dietary patterns, the diet´s nutritional composition and the chemical structure of the main fatty acid content of dietary fats. A diet high in fat and sugar induced the highest NETosis ratios, compared to diets with a low-fat content or fasted conditions. Fat-rich diets induce gut-permeability, intestinal dysbiosis and metabolic endotoxemia. NETs emerged as a key component of the hepatic immunological barrier system as sentinels against gut-derived PAMPs and DAMPs (reflected by a higher TLR2- and TLR4-night-associated activity), and as peacemakers within the low-profile pro-inflammatory scenario associated to postprandial conditions. Conditions gathering a higher and local hepatic presence of NETs at night were overall associated with a lower pro-inflammatory profile in the liver. Levels of IL-5 and IFN-γ (pro-fibrotic cytokines) were particularly augmented in the liver in absence of NETs. Altogether, our study spotlights NETs as key players in the maintenance of the hepatic tolerogenic and anti-inflammatory immune status and, ultimately, in the normal liver physiology.

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A

AF	Alexa Fluor
AJ	*Adherens* Junction
AJC	Apical Junctional Complex
AMP	Adenosine Monophosphate
ANCA	Anti-neutrophil Cytoplasmic Antibody
ANRT	Aryl-hydrocarbon receptor nuclear translocator
APAP	Acetaminophen
APC	Antigen Presenting Cell
ASCT	Alanine Serine Cysteine Transporter
ATP	Adenosine Triphosphate

B

BB Cl-A	BB Cl-Amidine (Hydrochloride)
BM	Bone Marrow
BMAL	Brain and Muscle ARNT-like Protein
BSA	Bovine Serum Albumin
BW	Body Weight

C

CCG	Clock Controlled Gen
CCL	CC Chemokine Ligand
CD	Cluster of Differentiation
CF-DNA	Cell Free-DNA
CGD	Chronic Granulomatous Disease
CitH3	Citrullinated Histone 3
CLOCK	Circadian Locomotor Output Cycles Kaput
CLR	C-type lectin receptor
CMP	Common Myeloid Progenitor
CpG	Cytosine-phosphorothioate-guanosine
CRIg	Complement Receptor of the Immunoglobulin superfamily
CRY	Cryptochrome
CSF	Colony Stimulating Factor
CTL	Cytotoxic T Lymphocytes
CTLA	Cytotoxic T Lymphocyte-associated Protein
CXCL	CXC-Chemokine Ligand
CXCR	CXC-Chemokine Receptor

D

DAMP	Damage/Danger Associated Molecular Pattern
DC	Dendritic Cell
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco´s Phosphate-Buffered Saline

E

E-Box	Enhancer Box
EC	Endothelial Cell
EDTA	Ethylenediaminetetraacetic
e.g.	*Exempli gratia*
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic Reticulum
ERK	Extracellular-Signal Regulated Kinase
ET	Extracellular Trap
EU	Endotoxin Units

F

FAO	Fatty Acid Oxidation
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FC	Flow Cytometry
FCS	Fetal Calf Serum
FEO	Food-entrainable oscillator

FFAR Free-Fatty Acid Receptor

FITC Fluorescein Isothiocyanate

fMLP N-Formyl-L-Methionyl-L-Leucyl-Phenylalanine

FSC Forward Scattered

G

G Gram

G-CSF Granulocyte Colony Stimulating Factor

GLUT Glucose Transporter

GM-CSF Granulocyte/Macrophage Colony Stimulating Factor

GPCR G-Protein Coupled Receptor

GPI Glycosylphosphatidylinositols

H

H Hour

H3 Histone 3

HBSS Hank's Balanced Salt Solution

HBV Hepatitis B Virus

HDN High Density Neutrophils

HFD High-Fat Diet

HFSugD High-fat and high-sucrose diet

HMGB High-mobility Group Box

HSC Hematopoietic Stem Cell

HSPC Hematopoietic Stem and Progenitor Cell

I

I-CAM Intracellular Adhesion Molecule

i.e. *Id est*

IEC Intestinal Epithelial Cell

IF Immunofluorescence

IFN-α Interferon Alpha

IFN-β Interferon Beta

IFN-γ Interferon Gamma

IL Interleukin

ILC Innate Lymphoid Cell

iNKT Invariant NK T cell

i.p. Intraperitoneal

IRF Interferon Regulatory Factor

J

K

KC Kupffer Cell

KG Kilogram

KO Knock-out

L

LAG Lymphocyte Activation Gen

LCFA Long-Chain Fatty Acid

LDN Low Density Neutrophil

LFD Low Fat Diet

LFSugD Low-fat and low-sucrose diet

LN Lymph Node

LPG Lipophosphoglycan

LPS Lipopolysaccharide

LRR Leucine-rich-repeats

LSEC Liver Sinusoidal Endothelial Cells

LXR Liver X Receptors

M

MAC Membrane Attack Complex

MCFA Medium-Chain Fatty Acid

MCP Monocyte chemotactic protein

M-CSF Macrophage Colony-Stimulating Factor

mDC Myeloid Dendritic Cell

MFI Mean Intensity Fluorescence

MHC Major Histocompatibility Complex

Min	Minute
MIP	Macrophage infiltrating protein
mL	Mililitre
mM	Milimolar
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase

N

NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAFLD	Non-alcoholic Fatty Liver Disease
NASH	Non-alcoholic Steatohepatitis
NE	Neutrophil Elastase
NET	Neutrophil Extracellular Trap
NK-kB	Nuclear factor-kappaB
NK	Natural Killer
NLR	NOD-Like Receptors
NOD	Nucleotide-binding oligomerization domain
NTP	Nucleotide Triphosphates

O

OXPHOS	Oxidative Phosphorilation

P

PAD4	Peptidylarginine Deiminase 4
PAF	Platelet Activating Factor
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid Dendritic Cell
PER	Period Circadian Protein Homologue
PFA	Paraformaldehyde
PI	Propidium Iodide
PLS	Papillon-Lefèvre Syndrome
PMA	Phorbol Myristate Acetate
PMN	Polymorphonuclear
PPP	Pentose Phosphate Pathway
PR	Proteinase
PRR	Pattern Recognition Receptor
PSGL	P-selectin Glycoprotein Ligand
PTX	Pentraxin

Q

R

RA	Rheumatoid Arthritis
RBC	Red Blood Cell
RCF	Relative Centrifugal Force
RIG	Retinoic Acid Inducible Gen
RLR	RIG-like Receptor
RNA	Ribonucleic Acid
ROR	Retinoic Acid Receptor-related Orphan Receptor
RORE	ROR Responsive Element
ROS	Reactive Oxygen Species
RPM	Revolutions per Minute
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
rTEM	Reverse Trans-endothelial Migration

S

SCN	Suprachiasmatic Nuclei
Sec	Seconds
SD	Standard Deviation
SEM	Standard Error of the Mean
SLE	Systemic Lupus Erythematosus
SPF	Specific Pathogen-free
SSC	Side Scattered
ssRNA	Single-stranded RNA

T

TEM	Trans-Endothelial Migration
TGF	Transforming Growth Factor
TJ	Tight Junction
TLR	Toll-Like Receptor
TIR	Toll/Interleukin Receptor
TNF-α	Tumor Necrosis Factor Alpha
TNF-β	Tumor Necrosis Factor Beta
T-REG	T Regulatory Cell
TTFL	Transcription-Translation Feedback Loop

U

µG	Microgram
µL	Microlitre
µM	Micromolar

V

VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth factor
Vs.	*Versus*
VWF	Von Willebrand Factor

W

WBC	White Blood Cell
WT	Wild Type

X

Y

Z

ZT	*Zeitgeber* Ti

Introduction

1.1 The Immune System

The immune system comprises a whole complex network of cell populations, proteins and cellular components that cooperate in mounting an appropriate immune response to defend the body against an insult. Failure in orchestrating a proper immune response can be categorized into 1) immunodeficiencies, a state in which one or more components of the immune system remain inactive despite a threat, and the immune system´s ability to mount a proper immune response and confront a challenge remains compromised or entirely absent; 2) allergic reactions or hypersensitivity reactions, a condition of over response of the immune system against external and typically harmless substances; and 3) autoimmune disorders, an overactive immune response where the immune system fails to discriminate between self- and non-self-antigens, and orchestrates an immune response against the host (Nicholson 2016; Iwasaki and Medzhitov 2015; Chaplin 2010; X. Wang et al. 2018; Sinha, Lopez, and McDevitt 1990). Evolutionarily, the immune system has evolved and developed from a series of defense mechanisms that secured cellular integrity and homeostasis, allowed discrimination between self and non-self-elements, and purely ensured host survival (Chaplin 2010; Marshall et al. 2018).

1.1.1 Immune Homeostasis

Homeostasis refers to the highly active and dynamic tendency of a system to maintain stable and physiological conditions within the body´s internal environment. Homeostasis ensures a constant threshold of physiological parameters by making appropriate adjustments as necessary to conditions or changes occurring both inside and outside the system. Particularly, the immune system is a tightly regulated network able to cope with a daily balance between immune tolerance and immunogenicity under physiological conditions. Immune homeostasis is sustained by a network of innate and adaptive immune populations that actively monitor the environment and keep up a balance between tolerance to self-antigens and respondence against foreign molecules (Crimeen-Irwin et al. 2005).

1.1.2 The Immune System Dichotomy

The innate immune system shares some common features found in varying forms, and is conserved in all multi-cellular organisms, from plants to invertebrates and mammals. In contrast, adaptive immunity appears to be exclusive of vertebrates and hereby evolutionarily “younger” than innate responses (Buchmann 2014; Yipp et al. 2012). The immune system shows many faces, but the main dichotomy separates adaptive or “acquired” immunity from innate or “natural-born” immunity. Briefly, innate immunity provides a non-specific and generalized response to a threat that results in the induction of a pro-inflammatory scenario. Secondly, adaptive immunity provides specific immune

protection and results in the generation of immunological memory, which allows for a more rapid, specific and robust response to subsequent challenges. On top of that, the cells of the immune system can be categorized into two main types of leukocytes or white blood cells: Phagocytes and Lymphocytes. Phagocytes mainly comprises neutrophils, monocytes, macrophages and mast cells; while lymphocytes are mainly composed of B lymphocytes, T lymphocytes and natural killer (NK) cells. Leukocytes could also be segregated according to the number of granules present in their cytoplasm. Granules are secretory vesicles that normally contain enzymes (i.e. lysozymes), cytotoxic and oxidative molecules, and antimicrobial peptides and proteins (i.e. defensis). They can mediate defense mechanisms against pathogens and digestion processes of other cellular materials. Granulocytes are cells with a multilobed nucleus that contain a high number of granules in their cytoplasm; and they include neutrophils, basophils, eosinophils and mast cells. Interestingly, granulocytes can be identified under the microscope depending on the colour of their granules when stained with a specific compound dye: neutrophil granules are pink, eosinophil granules are red and basophil granules are dark blue (Sheshachalam et al. 2014). On the other hand, mononuclear leukocytes have a big unilobed nucleus that occupies most of the cell cytoplasm and only contain few granules. They include lymphocytes, monocytes, macrophages and dendritic cells (DC) (Nicholson 2016; Marshall et al. 2018).

All immune cells arise from a precursor multipotent hematopoietic stem cell (HSC) in the bone marrow (**Fig.1-1. Hematopoietic stem cell differentiation pathways and lineage-specific markers**) (Ogawa 1993). HSCs gives rise to 1) the common lymphoid progenitor (CLP) that can be further differentiated into aforementioned lymphocytes and NK cells, and 2) the common myeloid progenitor (CMP) that can be further differentiated into megakaryocytes, erythrocytes, mast cells and myeloblasts. Myeloblasts presume the final precursor of basophils, eosinophils, monocytes, macrophages and, the most interesting cellular population throughout this doctoral thesis, *neutrophils*.

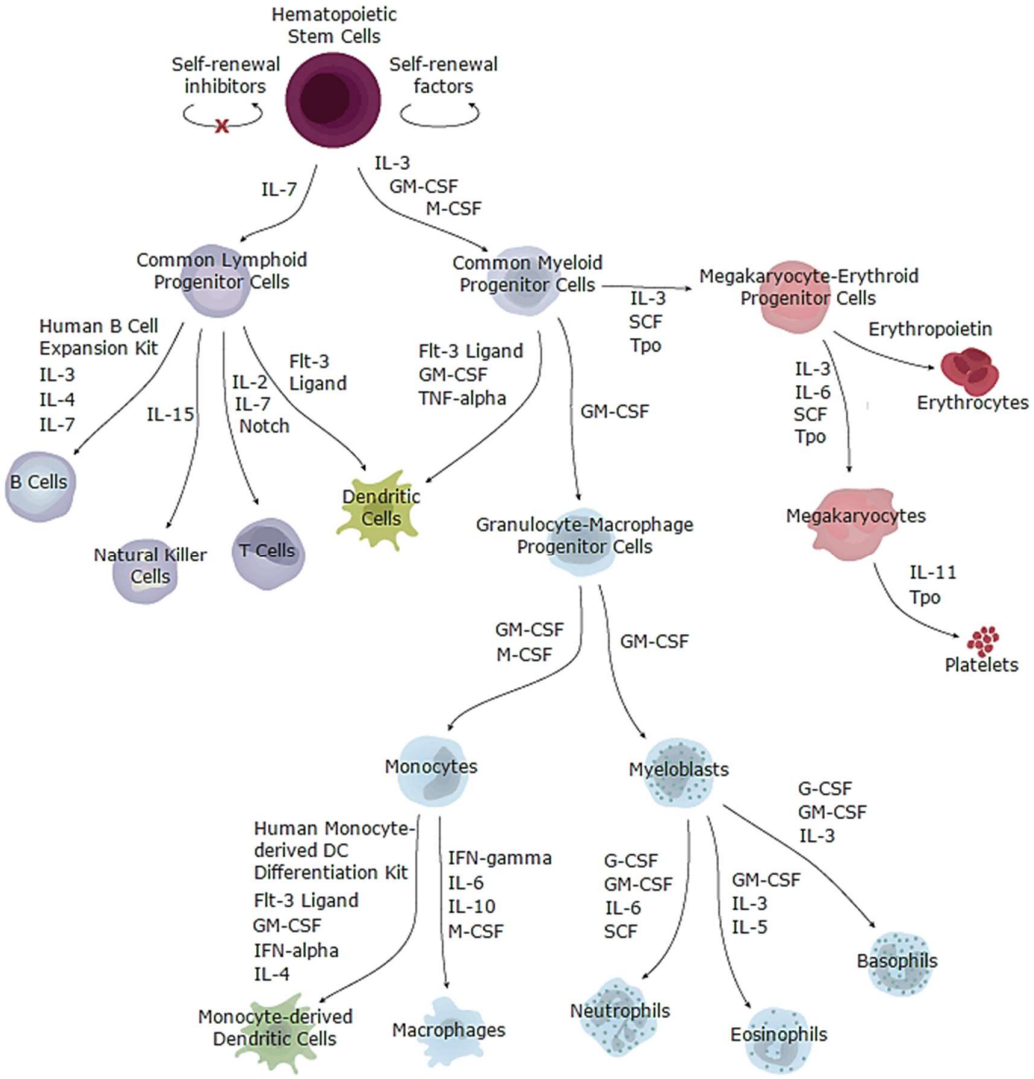


Figure 1-1. Hematopoietic stem cell differentiation pathways and lineage-specific markers. Diagram showing the development of hematopoietic stem cells (HSCs) into mature blood cells, and some of the key cytokines that determine the lineage specific maturation of each cell population. HSCs are multipotent, self-renewing progenitor cells that through the process of hematopoiesis give rise to all differentiated blood cell populations appearing in circulation. Arising cells include lymphocytes, granulocytes, and macrophages, as well as circulating erythrocytes and platelets. Differentiation of HSCs is regulated by several growth factors and cytokines. HSCs and their differentiated populations can be classified by the expression of specific cell surface lineage markers, such as cluster of differentiation (CD) proteins and cytokine receptors. IL, Interleukin; GM-CSF, Granulocyte Macrophage-colony stimulating factor; M-CSF, Macrophage-colony stimulating factor; G-CSF, Granulocyte-colony stimulating factor; TNF-α, Tumor necrosis factor-alpha; FLT-3, FMS-like tyrosine kinase 3 ligand; SCF, Stem cell factor; IFN-a, Interferon-alpha; Tpo, Thrombopoietin. **Illustration from rndsystems.com/pathways/hematopoietic-stem-cell-differentiation-pathways-lineage-specific-markers.**

1.1.3 The Innate Immune System

The innate immune system is characterized by providing a typically rapid and un-specific pro-inflammatory immune reaction that gets gradually activated in response to the stimulation of pattern recognition receptors (PRRs) *via* either damage-associated molecular patterns (DAMPs) derived from damaged or dead cells as a consequence of cellular stress; or *via* pathogen-associated molecular patterns (PAMPs) released by invading microbes (Mogensen 2009; Akira, Uematsu, and Takeuchi 2006). The innate immune system responses are mediated by cell-secreted factors including alarmins, cytokines and chemokines, antimicrobial peptides, proteases or acute-phase proteins, among others, and cell-dependent mechanisms such as phagocytosis or cytotoxicity events, mainly executed by professional phagocytes and killer cells (D. Yang, Han, and Oppenheim 2017; Gasteiger et al. 2017). Moreover, the innate immune activity does not rely on selective events such as antigen-specificity and immunological memory, typical for adaptive immune responses (Gasteiger et al. 2017). Innate leukocytes include: Basophils, DCs, eosinophils, macrophages, mast cells, monocytes, NKs and neutrophils.

1.1.3.1 Pattern Recognition Receptors

PRRs are considered main components of the innate immune system and are proteins typically expressed by tissue-resident epithelial cells, all innate immune cells and, surprisingly, some B cell subsets (Hua and Hou 2013; Kawasaki and Kawai 2014; Amarante-Mendes et al. 2018). Four major sub-families of PRRs exist, including the Toll-like receptors (TLRs), the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), the retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs) and the C-type lectin receptors (CLRs) (Amarante-Mendes et al. 2018; Noh et al. 2020). Among PRRs, the most notorious and abundant ones are the TLRs. TLRs were firstly discovered in *Drosophila* species and so far, thirteen functional members of the TLR family have been described; ten in humans (TLR1–TLR10) and up to twelve have been described in rodents (TLR1–TLR9, TLR11 and TLR12) (**Fig.1-2. Families of toll-like receptors**). TLRs share a typical structural motif, which is responsible both for the receptor appearance and their function (Botos, Segal, and Davies 2011): They are type I integral transmembrane glycoproteins composed of an extracellular region that contains leucine-rich-repeats (LRRs), and a cytoplasmatic intracellular tail which has a toll/interleukin-1 receptor (TIR) domain. TLRs mediate synthesis and secretion of different cytokines and activate both innate and adaptive immune responses (Kawasaki and Kawai 2014). In their active form, TLRs tend to dimerize: For example, TLR2 forms heterodimers with TLR1 and TLR6; and TLR4 is able to form homodimers.

When TLRs sense and interact with their specific PAMP or DAMP, it triggers downstream effector programs mediated either through the canonical MyD88-dependent intracellular signalling pathway, common to all TLRs except TLR3; or the non-canonical Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF)-dependent signalling pathway (Kawasaki and Kawai 2014; Noh et al. 2020). The MyD88-dependent signalling pathway induces the nuclear factor-kappaB (NF-κB) complex and the activation of MAP-Kinases, which modulates gene transcription and the consequent production of various inflammatory cytokines (e.g. TNF-α, IFN-α, IFN-β). PAMPs and DAMPs encompass a wide variety of molecules, such as bacterial wall components and toxins (lipopolysaccharides, mannoses, flagellin, peptidoglycans), nucleic acids (bacterial or viral RNA and DNA), extracellular matrix components, N-formyl peptides, extracellular ATP, uric acid, alarmins, lipid mediators, lipoproteins and several protein complexes such as the high-mobility group box 1 (HMGB1). For example, TLR2 is involved in the recognition of some microbial molecules, particularly those derived from Gram-positive bacteria such as peptidoglycans, lipoteichoic acid (LTA) and acylated lipoproteins, but also the lipoarabinomannan from mycobacterias, glycosylphosphatidylinositols (GPIs) from protozoans and zymosan from the yeast cell wall. TLR4, on the other hand, plays a much greater role in signalling through the lipopolysaccharide (LPS) component of the cell wall of Gram-negative bacteria; and TLR3, TLR7 and TLR9 triggers NF-κB and interferon regulatory factors (IRF)-mediated pro-inflammatory anti-viral responses upon the recognition of double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and unmethylated cytosine-phosphorothioate-guanosine (CpG) forms of DNA, respectively (Oliveira-Nascimento, Massari, and Wetzler 2012; Kumagai, Takeuchi, and Akira 2008; Doyle and O'Neill 2006; Akira and Takeda 2004).

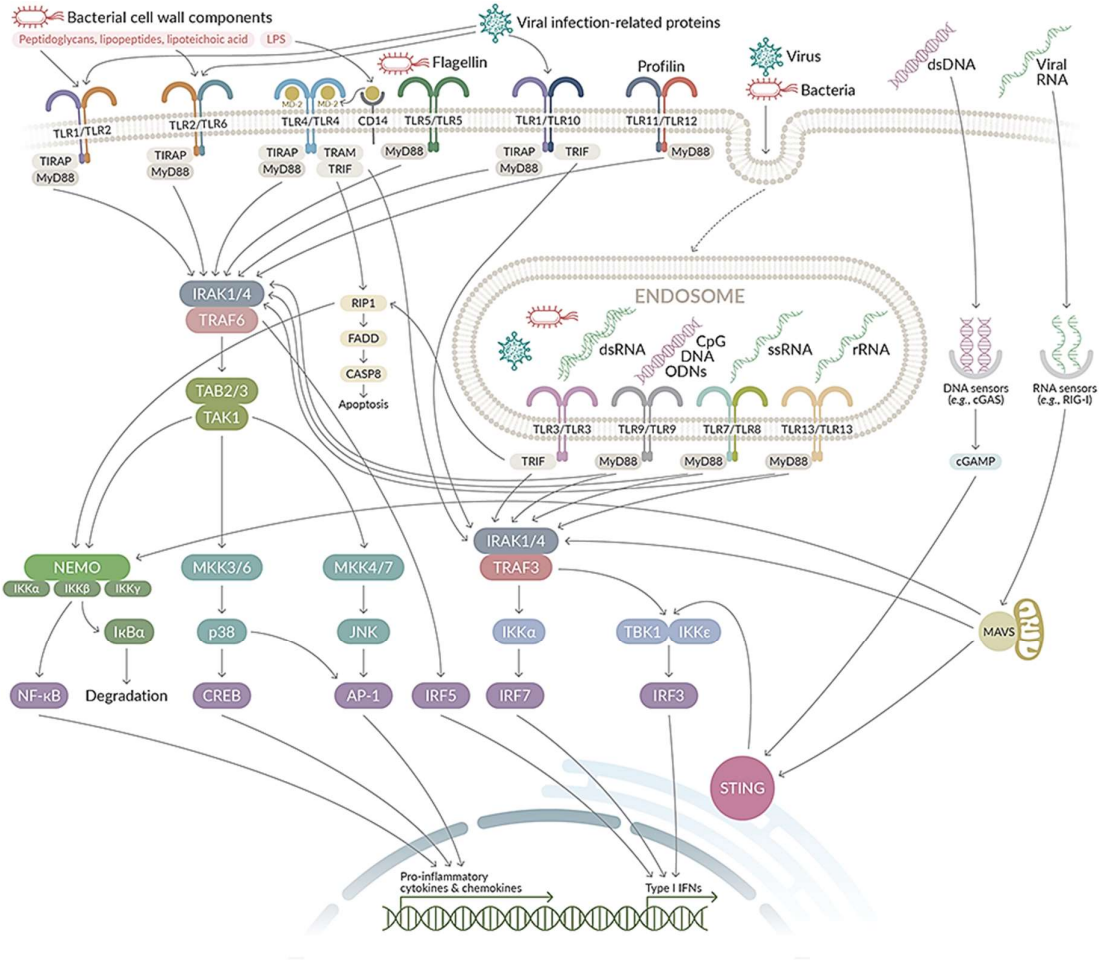


Figure 1-2. Families of toll-like receptors. Toll-like receptors (TLRs) are innate immune proteins capable of identifying invading pathogens through the recognition of specific pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) derived from tissue damage or cell death. TLRs act as sentinels, providing an immediate first line of detection of invading microbes. This lead, consequently to the activation of protective mechanisms of the innate immune system. TLR members are categorized into cell surface types (TLR1, 2, 4, 5, and 6) and endosome types (TLR3, 7, 8, and 9). TLRs can distinguish among double-stranded and single-stranded RNA (dsRNA and ssRNA), unmethylated CpG DNA, bacterial lipopolysaccharide (LPS), lipoproteins, flagellin as well as zymosan and β-glucan from fungus. Ligands of TLR can signal through MyD88-dependent and independent pathways. After ligand binding, TLRs dimerize (form homo- or heterodimers), undergo conformational re-shape and recruit downstream adaptor proteins. These proteins include the myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal), TIR domain-containing adaptor inducing IFN-*β* (TRIF)/TIR domain-containing adaptor molecule-1 (TICAM-1), and TRIF-related adaptor molecule (TRAM). AP1, activator protein 1; IRAK, IL-1R-associated kinase; IRF, interferon-regulatory factor; LPS, lipopolysaccharide; MyD88, myeloid Differentiation Primary Response 88; NF-kB, nuclear factor kappa B; TAK1, transforming growth factor-β-activated kinase-1; TIRAP, toll-IL 1 receptor domain containing adaptor protein; TLR, toll-like receptor; TRAF, tumor necrosis factor receptor-activated factor; TRAM, TRIF-related adaptor molecule; TRIF, toll/IL-1R domain-containing adaptor-inducing IFN-β. **Illustration from caymanchem.com/news/toll-like-receptors.**

1.1.3.2 Cytokines

Innate immune system responses are mediated by cell-secreted factors including alarmins, cytokines and chemokines (cytokines with chemotactic activities), antimicrobial peptides, proteases and acute-phase proteins, among others. Cytokines conform a large and diverse family of signalling molecules composed by proteins, peptides and mainly glycoproteins (usually smaller than 30 kDa). Cytokines display immunomodulatory capacities, regulate local homeostasis parameters —including cellular growth, tissue development and cell differentiation—, orchestrate cell migration, tissue remodelling, mediate haematopoiesis, angiogenesis and wound healing, and assist the innate and adaptive immune system in mounting and coordinating an effective immune response (Van der Meide and Schellekens 1996; J.-M. Zhang and An 2007). Cytokines can display autocrine, paracrine or endocrine actions. They display autocrine actions when they bind to membrane receptors and function over the same cell that secreted them. They can display paracrine actions when they function over cells that are located in close proximity to them. They can also display endocrine actions when they act over distant cells after traveling through the circulation. The production of cytokines largely relies on the stimulation (for some cytokines even the co-stimulation; e.g. LPS and IL-12) by different agonist. Among these agonists, other cytokines and bacterial endotoxins are the most potent inducers (J.-M. Zhang and An 2007). The two main producers of cytokines are T-Helper cells and macrophages, although cytokines have been reported to be found being secreted by all nucleated cells of the immune system. For example, monocytes and macrophages are the major producers of the growth factors such as colony stimulating factors (CSF). CSF are part of the haematopoietin superfamily of cytokines, which stimulate hematopoietic cells to differentiate into the eight principle types of blood cells: Macrophage-CSF (M-CSF) triggers the proliferation and differentiation of naïve bone marrow precursors into macrophages. The granulocyte/macrophage CSF (GM-CSF) mediates granulocyte proliferation (Tecchio, Micheletti, and Cassatella 2014; Tamassia et al. 2018).

Neutrophils have been long considered cells devoid of transcriptional activity and capable of performing only little protein synthesis. That is due to neutrophils hosting ten-to-twenty times less of total RNA compared to other leukocytes, which means that on a *per cell* basis, neutrophils generally produce exceptionally lower amounts of cytokines compared to monocytes, macrophages or DCs (Papayannopoulos et al. 2010b; Brinkmann and Zychlinsky 2012b; Fuchs et al. 2007a). However, growing evidence now demonstrates that neutrophils can (constitutively or upon stimulation) synthesize, express and release a wide range of pro-inflammatory, anti-inflammatory and immunomodulatory cytokines, and several growth factors both *in vitro* and *in vivo* (Rosales 2018). Cytokines expressed by neutrophils include several different interleukins (IL-1α, IL-1β, IL-3, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18), macrophage chemoattractant protein-1α (MCP-1α) and MCP-1β, TNF-α, IFN-α,

IFN-β, IFN-γ, Fas ligand (FasL), vascular endothelial growth factor (VEGF) or the monocyte chemotactic protein-1 (MCP-1) -among others-, both in response to host factors and PAMPs. (Brinkmann and Zychlinsky 2012a; Fuchs et al. 2007a).

1.1.3.3 The Complement System (Humoral Defenses)

The complement system was originally described as a branch of the innate immune system that has the ability to enhance and so "complement" the antibacterial properties of antibodies and the capacity of phagocytic cells to clear microbes, non-self-elements and remainings of cellular components from an organism (Walport 2001a; 2001b). Recently, the complement system has been suggested to bring together innate and adaptive immune responses, which grants an integrated host defence to pathogenic challenges (Bennett, Rooijakkers, and Gorham 2017; Merle, Noe, et al. 2015). The complement system is mainly composed of plasma proteins and glycoproteins that circulate in the blood as inactive precursors (zymogens) that have been synthesized mainly in the liver, by hepatocytes. The activation of these complex network of plasma and serum proteins, and plasma membrane-associated proteins leads to a proteolytic signalling reaction that starts by identifying pathogenic surfaces. Pathogen identification leads to the generation of potent pro-inflammatory mediators (anaphylatoxins, such as C3a), followed by the opsonization ("coating" or "targeting") of the pathogenic surface through complement opsonins (C4b, C3b or C3bi). It all culminates with a targeted lysis of the pathogen (opsonization) through the production of pro-inflammatory molecules, recruitment of inflammatory immune cells (macrophages, neutrophils) and the assembly of membrane-penetrating pores known as the membrane attack complex (MAC) (Ricklin et al. 2010; Ricklin and Lambris 2013; Ricklin, Reis, and Lambris 2016).

The complement system can be activated on pathogen surfaces through three major biochemical pathways (**Fig.1-3. The three pathways of complement activation: classical, lectin, and alternative pathways**). The classical pathway, the lectin pathway and the alternative pathway. The three pathways rely on different molecules to get initiated, but they all converge on the generation of the same effector molecules: anaphylatoxins (i.e. C4a/C3a/C5a), opsonins (i.e. C3b) and the MAC, a terminal assembly of the complement components C5b through C9, which can directly lyse pathogenic targeted surfaces. The classical pathway initiates by the binding of the first protein in the complement cascade C1q –in association with C1r and C1s serine proteases, they form the C1 complex–, directly to the Fc region of complement-fixing antibodies (IgG1 and IgM), attached to the surface of the targeted pathogen. The C1s protease is able to cleave C4, which binds covalently to the pathogen surface and breaks C2, leading to the formation of the C4b-C2a complex, known as the C3 convertase of the classical pathway. The generation of the C3 convertase on top of pathogenic surfaces allows this complex to cleave C3 into

the anaphylatoxin C3a and the opsonin C3b. The C3 convertase synthesis is the convergent point for all three complement activation pathways (Dunkelberger and Song 2010; Merle, Noe, et al. 2015; Merle, Church, et al. 2015; Chehoud et al. 2013). Alternatively, the lectin pathway is triggered through the binding of the mannose-binding lectin (MBL) to mannose-containing carbohydrates on the surface of typically encapsulated bacteria. This results in the formation of the MBL-associated serine proteases which again cleave the C4 and C2 proteins. C4 and C2 cleavage products associate together to form the lectin pathway C3 convertase. This molecule cleaves again into the C3a and C3b; and the C3b can associate with the C4b-C2a complex to form the C5 convertase. Finally, the alternative pathway is triggered directly on pathogen surfaces and starts when C3 undergoes spontaneous hydrolysis to form the complement effector molecules: the alternative pathway (AP) C3 convertase or the AP C5 convertase. The C5b from the C5 convertase can form consecutive complexes with C6 and C7, resulting in the assembly of the MAC, a common terminal pathway that culminates with cell lysis and pathogen death (Lesavre et al. 1979; Fishelson, Pangburn, and Müller-Eberhard 1984; Blom, Villoutreix, and Dahlbäck 2004; Beltrame et al. 2014).

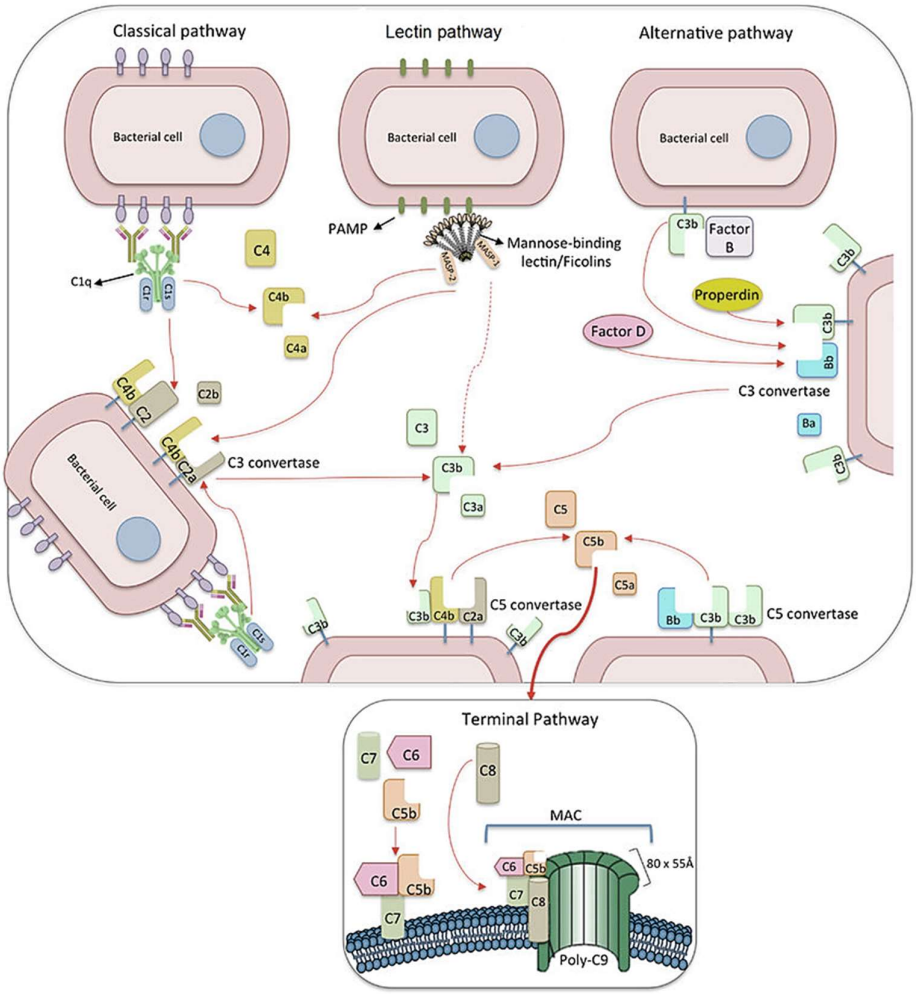


Figure 1-3. The three pathways of complement activation: classical, lectin, and alternative pathways. The classical pathway is initiated by binding of antigen-antibody complexes with the C1 complex (consisting of the C1q, C1r, and C1s molecules) on the pathogen surface. Next, C1s cleaves serum proteins C4 and C2, which forms the C4b2a complex from the complement classical pathway, also known as C3 convertase. Alternatively, the lectin pathway initiates after attachment of pattern-recognizing mannose-binding lectins (MBLs) to carbohydrate ligands on the pathogen surface. Like C1s, activated MBL-associated serine proteinase (MASP)-2 in the MBL-MASP-2 complex cleaves C4 and C2 and leads to the formation of the C4b2a complex or C3 convertase of the lectin pathway. The alternative pathway of the complement gets activated upon spontaneous hydrolysis of C3 in plasma. C3 lysis leads to the formation of C3b (homologous to C2), which binds complement factor B (CFB) and forms the C3bB complex. Complement factor D (CFD) cleaves CFB into Ba and Bb. These Bb fragments bind the C3bB complex and forms the C3bBb, also known as C3 convertase of the alternative pathway. The three pathways lead to the common activation of the lytic pathway, where opsonization, mediated phagocytosis and adaptive immune responses take place. C3 convertase cleave C3 into C3b. The addition of further C3b opsonins to the C3 convertase shapes the C5 convertase, which initiates the assembly of the membrane-attack complex (MAC) by cleavage of C5 into the C5a -an anaphylatoxin- and C5b fragments. Next, C5b binds with C6, C7, C8 and C9 to form the terminal MAC, which is inserted in the pathogen membrane. The three pathways converge to this common terminal step, ultimately mediating cell lysis and death. **Illustration from Beltrame et al.** (Beltrame et al. 2014).

1.1.4 Neutrophils

Neutrophils or polymorphonuclear (PMN) leukocytes, representing up to 70% of the total circulating leukocyte population, are the most abundant granulocytes in human blood. Notably, these cells represent about 10% of total circulating leukocytes in laboratory mice (Häger, Cowland, and Borregaard 2010). Neutrophils are essential during innate immune responses as the first line of defense against invading pathogens (Son, Kremer, and Hines 2010). Interestingly, neutrophils are also key players during sterile tissue injury (J. Wang et al. 2017; J. Wang 2018). Neutrophil physiological parameters in the bloodstream and a healthy neutrophil to leukocyte ratio are ensured by balancing the neutrophil short lifespan in circulation with their regulated release and production from myeloid precursors in the bone marrow (Casanova-Acebes et al. 2018); alongside with their migration into peripheral tissues, where they can fulfil their array of functions and ultimately be eliminated by tissue-resident phagocyting macrophages (Rosales 2018). Neutrophil daily production (up to 10^{11} cells per day) is regulated by the interleukin (IL)-23/IL-17/G-CSF axis (Wirths, Bugl, and Kopp 2014; Lieschke et al. 1994; Kolaczkowska et al. 2015; Kolaczkowska and Kubes 2013). Neutrophils are short-lived cells with an estimated half-life in circulation between 12 and 18 hours in mice and humans, respectively. However, neutrophils can increase their longevity by several folds upon activation and during inflammatory conditions (Kolaczkowska and Kubes 2013).

Upon inflammatory responses, primed and activated neutrophils are able to sense and integrate different chemotactic signals (e.g. IL-1β, CXCL8 family, CCL2, TNF, histamine, leukotriene B4, PAF) and follow the generated tissue-specific chemoattractant gradients that mediate neutrophil forward

migration into the site of damage. Neutrophil transmigration into tissues relies on the classical leukocyte recruitment cascade (**Fig.1-4. Neutrophil recruitment cascade and neutrophil effector functions**). The recruitment cascade consist on a sequence of capture, rolling, arrest, adhesion, crawling and trans-endothelial migration events that ultimately allows neutrophils to rapidly reach sites of inflammation with the aim to restore tissue homeostasis (Sadik, Kim, and Luster 2011; Adams and Shaw 1994). Briefly, free flowing neutrophils are first activated by DAMPs, derived from tissue damage or cellular stress; or PAMPs, released by invading pathogens. DAMPs and PAMPs also stimulate organ-specific sentinel cells, which release proinflammatory mediators (*e.g.* IL-1B, TNF-α) and neutrophil chemoattractants. Primed neutrophils are the first cells to reach the damaged tissue and once there, they are able to establish adhesive interactions with endothelial cells (ECs) of postcapillary venular walls of the inflamed milieu. Activation of ECs involves the upregulation of both P-Selectin and E-Selectin, two transmembrane glycoproteins; and different members of the integrin superfamily, like the intercellular adhesion molecule (I-CAM) and vascular cell adhesion molecule (V-CAM). Selectins and integrins constitute the two major adhesion receptor families that mediate the leukocyte-adhesion cascade (Phillipson et al. 2006). Neutrophil-endothelial adhesion and rolling on the vessel wall is initially mediated by the interaction of neutrophil adhesion molecules present on the neutrophil surface, such as the P-selectin glycoprotein ligand 1 (PSGL-1), L-selectin, CD44 or macrophage antigen-1 (Mac-1), with the adhesion molecules typically expressed on ECs. Transmigration and extravasation through the endothelial wall can occur *via* the paracellular route, or the transcellular route (i.e., either through tight junctions between endothelial cells or through an endothelial cell). The paracellular rout is the predominant route and requires integrins, the junction adhesion molecule 1 (JAM-1) and the platelet endothelial adhesion molecule-1 (PECAM-1 or CD31), among others (Muller et al., 1993). Finally, resolution of inflammation is key to prevent further tissue damage as part of a successful response to an acute injury. Local resolution of inflammation is an active coordinated process where an interplay of multiple events collides; including inhibition of neutrophil recruitment, promotion of neutrophil necrosis or apoptosis, macrophage-mediated neutrophil clearance and egression of infiltrated leukocytes from inflamed tissues back into the circulation, a process termed neutrophil reverse trans-endothelial migration (rTEM) (Nourshargh and Alon 2014; S. de Oliveira, Rosowski, and Huttenlocher 2016).

Neutrophils are a type of cell that is able to mediate tissue damage by producing cytokines and proteases, and through the release of factors contained in their cytoplasmic granules. Neutrophils contain three types of granules that are sequentially formed during neutrophil differentiation (Häger, Cowland, and Borregaard 2010): primary or azurophilic granules, containing hydrolytic enzymes such as myeloperoxidase (MPO) or neutrophil elastase (NE); secondary or specific granules containing

lactoferrin; and tertiary or gelatinase granules, containing matrix-metalloproteinases (MMP) such as the MMP-9 (Sheshachalam et al. 2014). Neutrophils mediate pathogen detection, trapping and killing *via* intracellular and extracellular mechanisms (**Fig.1-4. Neutrophil recruitment cascade and neutrophil effector functions**). Neutrophils have the capability to internalize pathogens through a process called phagocytosis and mediate microbial killing by delivering a whole arsenal of neutrophil granules and granule proteins into the phagosome. Neutrophils can also release pre-formed granule proteins *via* a process termed degranulation. Some of the key enzymes involved in extracellular pathogen killing are lysozymes, α-defensins and neutrophil serine proteases such as the NE, Cathepsin G or the Proteinase-3 (PR3). Phagocytosis and degranulation are processes that involve the assembly of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. NADPH oxidase is a membrane-bound enzyme complex that is able to convert oxygen molecules into superoxide radicals, which can be further catalysed into H_2O_2, hydroxyl anions or peroxynitrite anions; which ultimately mediates the production of several reactive oxygen species (ROS). Beyond the aforementioned functions (phagocytosis, ROS generation and degranulation (Papayannopoulos and Zychlinsky 2009b)), the formation of neutrophil extracellular traps (NET) structures, by a process termed NETosis, represents another antimicrobial mechanism to protect the host from damage and ensure survival (Brinkmann et al. 2004).

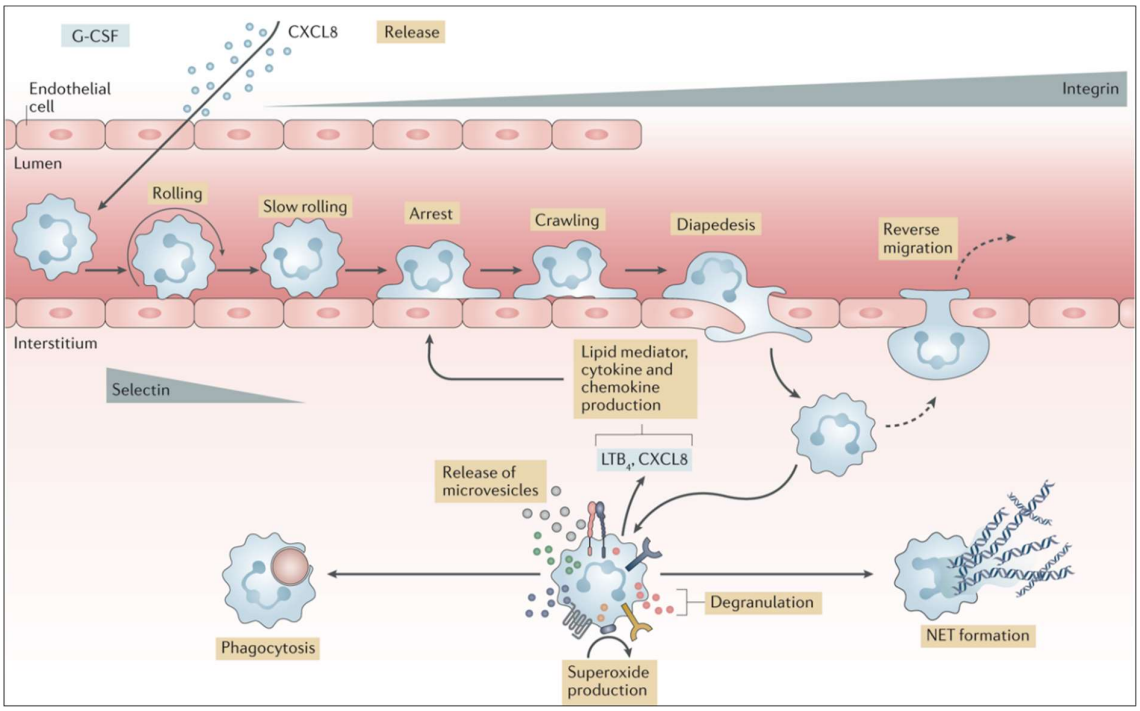


Figure 1-4. Neutrophil recruitment cascade and neutrophil effector functions. Neutrophil recruitment at sites of infection or inflammation is based on the typical leukocyte recruitment cascade. The original three steps of the typical leukocyte recruitment cascade are 1) rolling -mediated by selectins-, 2) activation -mediated by chemokines-, and 3) arrest -mediated by integrins-. Nowadays, some additional steps are considered, such as capture (or tethering), slow rolling, adhesion

strengthening and spreading, intravascular crawling and paracellular and transcellular transmigration. Briefly, upon environmental, microbial or inflammatory stimuli, circulating neutrophils are activated and slowed down in close contact to sites of infection or inflammation. This process is mediated by adhesion molecules expressed on the contiguous activated endothelium, which causes tethering and rolling of the neutrophil along the vessel wall. Interactions between endothelial cells and neutrophils result in cell adhesion and neutrophil transmigration (pericellular or transcellular) across the endothelial cell layer. Afterwards, neutrophils reach the site of inflammation following a chemotactic gradient. Once transmigrated into the peripheral tissue, neutrophils can exert different effector functions (ROS production, degranulation, phagocytosis and NET formation), fostering an inflammatory response which leads to the elimination of invading microorganisms. CXCL, CXC-chemokine ligand; LTB_4, leukotriene B_4; NET, Neutrophil extracellular trap. **Illustration adapted from Németh et al.** (Németh, Sperandio, and Mócsai 2020).

1.1.4.1 Neutrophil Extracellular Traps

NETosis, a mechanism of the neutrophil defense machinery, is a neutrophil-specific cell death program characterized by the extracellular release of web-like DNA structures (Brinkmann et al. 2004). These structures are called NETs and are mainly formed by extracellular chromatin fibers with a diameter of 15–17 nm, where DNA and modified histones represent their major components (Brinkmann et al. 2004; Kaplan and Radic 2012). NETs are also decorated with granule-derived pro-inflammatory and antimicrobial peptides and proteins, as well as different enzymes such as NE, cathepsin G, PR3, pentraxin 3 (PTX3), defensins, cathelicidin LL-37 or MPO (Papayannopoulos et al. 2010a; Brinkmann and Zychlinsky 2012b). NETs are key antimicrobial players that allow neutrophils to recognize, capture, immobilize and kill pathogens (Papayannopoulos and Zychlinsky 2009a; Brinkmann et al. 2004), including fungi, parasites, viruses as well as Gram-positive and Gram-negative bacteria. Notably, the process of NETosis was first referred to as a distinctive form of neutrophil-specific cell death (Brinkmann and Zychlinsky 2012a) wherein decondensed chromatin and associated granule proteins are released into the extracellular space resulting from the rupture of both nuclear and plasmatic membranes (Brinkmann et al. 2004).

1.1.4.1.1 NET Formation Initiators

Alongside their cellular surface, neutrophils display a wide range of receptors: G protein-coupled receptors, Fc receptors, adhesion receptors, cytokine receptors and the already mentioned PRRs, that makes them capable to sense and respond against different pro-inflammatory mediators. Through receptor activation, neutrophils can modulate self-behaviour and different functions, such as NETosis (Futosi, Fodor, and Mócsai 2013). Under physiological conditions, neutrophils can release NETs upon exposure to a wide range of stimuli (both natural and synthetic agents), several pro-inflammatory mediators and different metabolites associated with states of acute and chronic inflammation. The

most potent physiological NET stimuli inducers include bacterial fragments and bacterial components (LPS, LPG or the M1 protein), fungal products (ß-glucan), several immunological complexes, interferons (IFN-α, IFN-β, IFN-γ), neutrophil-activating chemokines and cytokines (IL-8, IL1-ß, TNF-α), growth factors (G-CSF), microRNAs, calcium ionophores, glucose and the glucose oxidase, activated platelets, endothelial cells and even cancerous cells, among many others (Hoppenbrouwers et al. 2017; S. L. Wong et al. 2015; Garcia-Romo et al. 2011; Pruchniak, Arazna, and Demkow 2013; Kaplan and Radic 2012; van der Linden et al. 2017; Kenny et al. 2017; Fuchs et al. 2007b; Saitoh et al. 2012; Papayannopoulos et al. 2010b; Rossaint et al. 2014). Yet, not all neutrophils are equally reactive to the same type of stimuli: For example, human neutrophils seem to be faster and more efficient at releasing NETs, compared to murine neutrophils (Ermert et al. 2009). In comparison to bone marrow-derived immature neutrophils, blood-derived mature neutrophils have a higher capability of releasing NETs after stimulation with type I and type II IFNs and the complement factor C5a (Martinelli et al. 2004). Altogether, this points towards an enhanced pro-inflammatory state of peripheral blood neutrophils compared to non-terminally-differentiated bone-marrow neutrophils. Interestingly, Adrover *et al.* showed that young neutrophils ($CXCR2^{high}$, $CD62L^{high}$), freshly released into circulation in the absence of pathology, contain a higher proteome content and augmented NET-forming capacity compared to aged granulocytes ($CXCR2^{low}$, $CXCR4^{high}$) (Jose M Adrover et al. 2020; José M Adrover et al. 2019). Conversely, Zhang et al. showed that the same aged murine neutrophils (primed by microbiota-derived products in steady state *via* the TLR-Myd88 signalling pathway) can exhibit an enhanced NET formation capacity compared to neutrophils newly released from the bone marrow, under conditions of sepsis (D. Zhang et al. 2015).

1.1.4.1.2 NET Formation Mechanisms

NET formation can occur *via* two different pathways (**Fig.1-5. Overview of NETosis: lytic NET-formation vs. vital NET-formation**): (1) cell lytic NET formation, also known as suicidal NETosis (Fuchs et al. 2007b; Y. Wang et al. 2009) and (2) vesicle-mediated NET formation, also known as vital NETosis (Marcos et al. 2010; Yipp et al. 2012; Pilsczek et al. 2010; Clark et al. 2007).

Suicidal NETosis was first described back in 2004 (Brinkmann et al. 2004) as a neutrophil-related phenomenon observed upon stimulation with PMA, autoantibodies or cholesterol crystals. During cell lytic NET formation, primed neutrophils up-regulate the glycolysis pathway by activating the extracellular signal-regulated kinase (ERK). ERK mediates phosphorylation and assembly of the NADPH oxidase complex. In turn, the NADPH oxidase complex regulates release of ROS and increases Ca^{2+} levels, which activates the protein-arginine deiminase 4 (PAD4) enzyme. PAD4 is a nuclear enzyme that catalyses the conversion of arginine residues into citrulline residues on proteins, autoantibodies and

H3 and H4 histones. Histone H3 and H4 citrullination is vital for NET release: citrullination leads to a reduction in histone cationic properties, which leads to chromatin decondensation (Brinkmann and Zychlinsky 2012a). ROS production mediates damage in the membrane of secretory granules and lysosomes, which permits the release of NE and MPO from the azurophilic granules into the cytosol. When granule proteins are translocated from the cytosol into the nucleus, they promote further unfolding of the chromatin. Finally, the nuclear membrane breaks, and chromatin is expelled into the cytosol and the extracellular space (Papayannopoulos et al. 2010a).

On the other hand, vital NETosis was later described and observed to be induced through complement receptors, bacterial products, TLR-2 and TLR-4 ligands or *via* TLR4-activated platelets (Clark et al. 2007). As a consequence, it has been proposed that vital NETosis might have a more fundamental role in regulating infection rather than sterile injury (Slaba et al. 2015). One of the main differences with lytic cell formation is that during vital NETosis, NET release is independent of the NADPH oxidase pathway and chromatin fibers are expelled into the extracellular compartment transported in vesicles. Hence, vital NETosis does not involve nuclear or plasma membrane rupture. Notably, DNA released during vital NETosis can also be of mitochondrial origin (Keshari et al. 2012; Lood et al. 2016a; Yousefi et al. 2009). Vital NETosis has been suggested to permit the neutrophil to still carry out further functions, including crawling, chemotaxis or phagocytosis. Interestingly, NETosis and phagocytosis are two of the main neutrophil antimicrobial functions that compete mechanistically in their response towards invading pathogens. Indeed, phagosome formation has been observed to work as a checkpoint to prevent NET formation. In line with that, neutrophils have been suggested to be able to evaluate microbe size due to a microbe size-sensing mechanism (Branzk et al. 2014) and cast a suitable immune response accordingly. For example, NETosis occurs in response to specific microbes, being large microorganisms the most effective inducers of NET release (Manda et al. 2014), while phagocytosis is intended for smaller size pathogens. Moreover, the decision that boosts NETosis over phagocytosis seems to also rely on the competition of these two cellular processes for the availability of NE (Branzk et al. 2014). However, pathogens have developed as well several strategies to evade NET capture: production and release of DNases that can trim the NET scaffold,, release of virulence factor M1 that can neutralize the activation of antimicrobial NET components, inhibition of the phagosome fusion, change of cell surface polarity, inhibition of NET binding by the development of polysaccharide capsules, or production of IL-10 chemokines (Manda et al. 2014; Ramos-Kichik et al. 2009; Marin-Esteban et al. 2012; Vitkov et al. 2009).

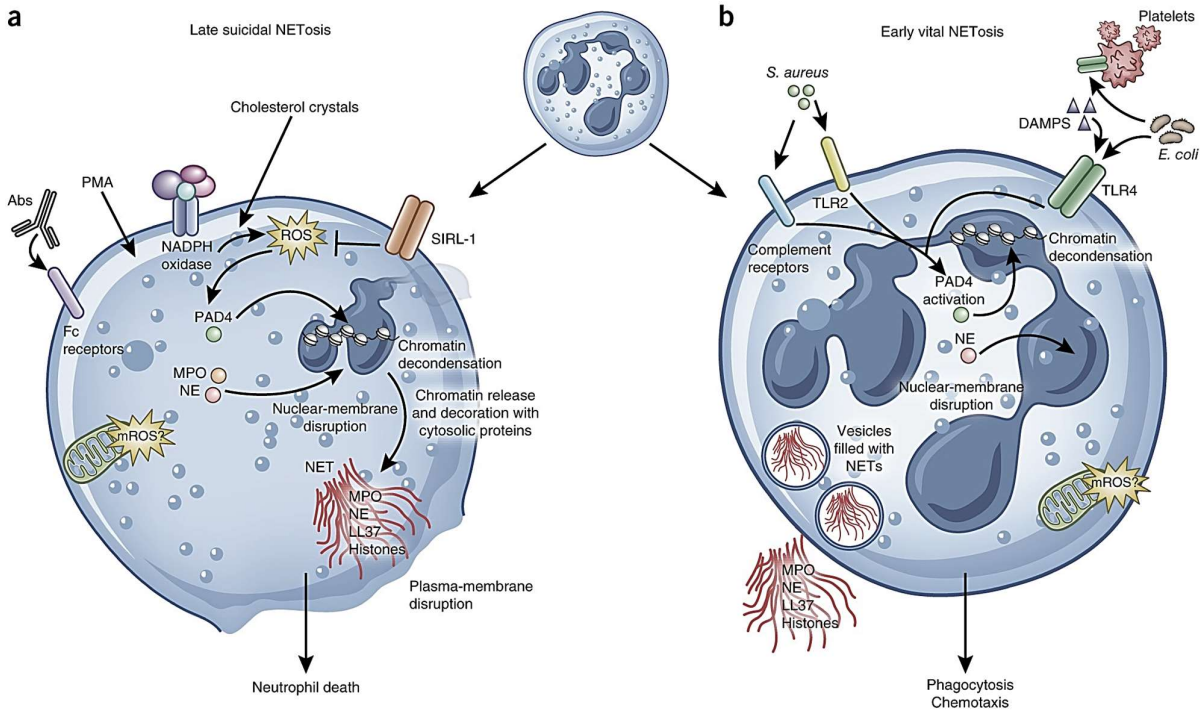


Figure 1-5. Overview of NETosis: lytic NET-formation vs. vital NET-formation. Lytic neutrophil extracellular trap (NET) formation is induced upon stimulation with typically sterile such as phorbol myristate acetate (PMA), antibodies or cholesterol crystals, among others. The NADPH oxidase, the protein kinase C (PKC) and the RAF–MEK–MAPK signaling pathway get activated and as a consequence, release of reactive oxygen species (ROS) and activation of the protein-arginine deaminase type 4 (PAD4) takes place. PAD4 activation induces histone citrullination and chromatin decondensation. Translocation of myeloperoxidase (MPO) and neutrophil elastase (NE) into the nucleus leads to further unfolding of chromatin and ultimately, the nuclear membrane breaks so chromatin can be released into the extranuclear compartment, Finally, NET DNA-fibers decorated with granular and cytosolic proteins are expelled in the extracellular space. As a consequence of NET formation, during suicidal NETosis, neutrophils undergo apoptosis (A). Non-lytic or vital NET formation is induced by typical pathogen-associated molecular patterns (PAMPs), TLR4-activated platelets or complement receptors, and it occurs independently of NAPDH oxidase activation. As in lytic NETosis, PAD4 activation induces chromatin decondensation. Here, however, chromatin is are expelled into the extracellular compartment transported in vesicles, so it does not involve plasma membrane disruption. After NET release, the neutrophil is reported to remain alive and can still display some other effector functions (e.g., phagocytosis) (B). **Illustration from Jorch et al.** (Jorch and Kubes 2017).

1.1.4.1.3 NETs in Physiopathology

Nowadays, it seems clear that NETs play a decisive role in host defense. Most of the early studies on NETs came to the conclusion that these extracellular structures have a high local concentration of very active molecules and can use their microbicidal ability to restrict systemic infection development, by catching circulating pathogens in the blood and mediating pathogen killing (Rada 2019; Kessenbrock et al. 2009). Primarily, NETs are able to extracellularly trap and kill a huge variety of microbes such as Escherichia coli (Grinberg et al. 2008), Candida albicans, Toxoplasma gondii or Streptococcus

pneumoniae and Streptococcus pyogenes (Ramos-Kichik et al. 2009; Sumby et al. 2005; Abi Abdallah and Denkers 2012; Urban et al. 2009; 2006). For example, by using intravital confocal microscopy techniques, McDonald et al. were able to in vivo image these NETs structures actively capturing *E.coli* particles that circulate alongside the hepatic sinusoids (McDonald et al. 2012).

Moreover, the effectiveness of NETs in mediating host defense is highlighted by the fact that microbes have been able to develop NET-evasion mechanisms that enable them to avoid getting trapped by these structures (Klebanoff 2005), and so avoid immune responses. For example, some microbes seem now able to suppress NET production by blocking upstream NET-release signalling pathways, whereas other pathogens can hide antigens of bacterial origin, or even encode nucleases -such as DNases- to break down and degrade the NET backbone (Sumby et al. 2005; Walker et al. 2007; S. Zhang et al. 2014). Another defense mechanism used by microbes is to develop resistance strategies against antimicrobial proteins present within the NET structure, such as suppressing MPO release (Sumby et al. 2005; Döhrmann et al. 2014; Eby, Gray, and Hewlett 2014; Storisteanu et al. 2017; Eisenbeis et al. 2018). Some bacteria have been able to develop a bacterial capsule, which contains a layer of polysaccharides similar to the sialic acid of the host in order to avoid being recognized by it. It seems that bacteria can bind to the network of DNA strands by electrostatic interactions between negatively charged chromatin strands and positively charged surface of bacteria (Storisteanu et al. 2017; Wartha et al. 2007). Lately, it has been proposed that bacteria could have been able to modify their surface charge from cation into anion, so typically-negatively charged bacterial capsules would repel NET interaction (Klebanoff 2005). All these microbial evasion strategies highlight the need for neutrophils to dispose a vast array of microbial killing mechanisms at their disposal for many and varied host defense requirements.

On the other hand, though, when NET formation is not tightly controlled (in terms of amount, location or timing of the response), NET release can mediate numerous pathophysiological processes within the organism. NET disfunction, excessive NET formation or aberrant NET removal from the system might have deleterious effects on the host and that is why NETs can be understood as double-edged swords of the innate immune responses. NET dysfunction can result in systemic infection and sustained inflammation, both associated with the exacerbation of multiple pathologic conditions. Yet, aberrant removal or accumulation of released NETs during a pathological syndrome can promote vessel and duct occlusion and thrombosis, obstruct important organ areas and facilitate both adjacent and remote tissue injury and organ damage (Rada 2019). Finally, excessive NET formation can potentiate the development of many non-infectious diseases (i.e., autoimmune disorders) or prime other immune cells in order to induce sterile inflammation, promote metastatic tumors or delay wound healing in

diabetes. Some examples of the pathophysiological processes in which aberrant NET formation is involved are briefly described next.

1.1.4.1.3.1 NETs as Mediators of Vascular Damage (Thrombosis)

NETs, as aggregated conformations, tend to be localized in body cavities, ducts (i.e. biliopancreatic ducts) and vessels. There, NETs can potentially occlude the free circulation of blood and other fluids. Indeed, some NET components have been identified within intravascular thrombi, and have been reported to display pro-thrombotic competencies, for example via thrombin activation (Fuchs et al. 2010). Mechanistically, the fibrin-like backbone present in NETs could pave the way for platelet adhesion and aggregation (McDonald et al. 2017; Fuchs et al. 2010). As a matter of fact, increased NET formation showed a positive correlation with hypercoagulability in septic patients and patients with chronic vascular pathologies (S. Yang et al. 2017). Several components of the NET backbone have been reported to display pro-thrombotic activity. NETs are able to enhance the accumulation of pro-coagulant and pro-thrombotic molecules such as von Willebrand factor (VWF), fibronectin, fibrinogen (Fuchs et al. 2010), factor XII (von Brühl et al. 2012) and tissue factor (Stakos et al. 2015). In particular, NET-derived histones H3 and H4 can recruit fibrinogen in a TLR2- and TLR4-dependent mechanism (Semeraro et al. 2011) and activate platelets (Fuchs, Bhandari, and Wagner 2011), which eventually accelerates thrombin production (Fuchs et al. 2010). Granule proteins, such as NE and cathepsin G enhance tissue factor- and factor XII-driven coagulation by mediating proteolysis of the tissue factor pathway inhibitor (TFPI) (Massberg et al. 2010). In that way, the histone-DNA backbone of NETs was proposed to participate in thrombi formation, growth and stabilization by providing a scaffold for fibrin deposition, red blood cells and platelet aggregation within thrombi (Oklu et al. 2012).

In turn, during thrombosis, activated endothelium releases P-selectin, which further promotes neutrophil recruitment and NET production (Etulain et al. 2015; von Brühl et al. 2012; Fuchs et al. 2010). Interestingly, activated platelets can also induce NET formation through mechanisms involving upregulation of TLR4 (Clark et al. 2007), HMGB1 (Maugeri et al. 2014) and P-selectin responses (Stark et al. 2016). For example, and upon activation with LPS, platelets instruct NET formation in the liver sinusoids through a TLR4-dependent mechanism (Clark et al. 2007). P-selectin, expressed on the platelet surface is able to bind to PSGL-1 expressed on the neutrophil surface and trigger NET responses (Etulain et al. 2015). Platelet-derived chemokines —such as CCL3 or the CCL5/CXCL4 heterodimer— lead the recruitment of immune cells to the inflammatory site and can instruct neutrophils to undergo NETosis (Rossaint et al. 2014). The existing complex crosstalk between NETs, activated platelets and pro-thrombotic factors is all interconnected around a positive feedback loop that mediates a pro-

thrombotic cycle of coagulation and pro-inflammatory responses that can ultimately occlude the vessel and induce organ damage.

1.1.4.1.3.2 NETs as Agitators of Immune Tolerance (Autoimmunity)

Several of the molecules and granular proteins decorating the NET scaffold (e.g., MPO, modified histones, nucleic acids), and even the extracellularly released dsDNA, are considered autoantigens in several systemic autoimmune diseases. This phenomenon has linked NETosis with the breakage of immune tolerance and the promotion of autoimmune responses in predisposed individuals. An association between increased NET formation and autoimmunity development was first described in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, and has been subsequently reported in other autoimmune conditions such as systemic lupus erythematosus (SLE) (Hakkim et al. 2010), rheumatoid arthritis (RA) (Papayannopoulos 2018; Khandpur et al. 2013), inflammatory bowel disease, ulcerative colitis or the antiphospholipid antibody syndrome (Lehman and Segal 2020; Khandpur et al. 2013; Kessenbrock et al. 2009; Villanueva et al. 2011; Leffler et al. 2014). NETs are speculated to display immune properties which can trigger a vicious cycle of immune reactions. Indeed, citrullinated histones and MPO or proteinase 3 autoantigens (Palić et al. 2007) are released into the extracellular environment during NET formation, and are presented to immunocompetent cells, which in response start producing ANCAs (R. Panda et al. 2017; Leffler et al. 2012). These ANCAs –typically autoantibodies– can potently induce NETosis in human neutrophils and usually act against the NET contained autoantigens, which starts an autoactivation positive loop that contributes to the progression of the autoimmune disorder (Hakkim et al. 2010; R. Panda et al. 2017; Leffler et al. 2012; Lood et al. 2016b; Khandpur et al. 2013; Lande et al. 2007). In addition to their antigenicity, PAD enzyme-dependent protein citrullination during NETosis increases the immune reactivity of these autoantigens, which further amplify the inflammatory response and the pathogenesis of autoimmune conditions and RA, particularly. Finally, over-excessive NETosis and a host-limited capacity to degrade NETs accounts for many of the clinical manifestations of the disease, such as host tissue damage and organ dysfunction, which ultimately supports the idea that NETs contribute to all stages of autoimmune diseases. For example, one important quality of NET-derived structures is their capacity to induce potent production of interferons (INF-α, INF-ß) or inflammatory cytokines (IL-1β and IL-18) via the activation of the nucleotide-binding oligomerization domain-like receptors protein 3 (NLRP3) inflammasome, common hallmarks of many autoimmune syndromes.

1.1.4.1.3.3 NETs as Mediators of Tumour Progression (Cancer)

Neutrophils are considered heterogeneous cell populations from the immune system. Recent studies have revealed the dual roles this cell type can play in tumor initiation, development, and progression. The multifaceted roles of neutrophils in disease are attributed to their diverse functional features and the variety of effector molecules that can be released under different conditions. For example, based on that, some terminology emerged: N1 and N2 neutrophils, or high-density neutrophils (HDNs) and low-density neutrophils (LDNs) represent neutrophil subpopulations –also termed neutrophil subsets– that display pro- and anti-tumoral activity, respectively. N1 and N2 neutrophils are defined as immunostimulating and immunosuppressive subsets, respectively, in the cancer field. It has been reported that upon activation, neutrophils can exert a targeted cytotoxic effect on cancer cells mediated by release of ROS and defensins (Souto, Vila, and Brú 2011). Conversely, neutrophils can also induce migration of cancer cells, release metalloproteinases into the extracellular matrix that stimulate cancer cells growing and facilitate angiogenesis (Souto, Vila, and Brú 2011).

Furthermore, NETs have also been found in cancer patients and suggested to be partly responsible of metastasis formation (Cools-Lartigue et al. 2013; Lee et al. 2019; Albrengues et al. 2018; Cedervall, Zhang, and Olsson 2016; Rayes et al. 2019). Although some NET components including MPO, proteinases and histones can bind cancer cells and may have a cytotoxic impact on them by inhibiting cancer growth (Souto, Vila, and Brú 2011), it has been reported that NETs main function as an inflammatory signal is the stimulation of cancer cells proliferation. NETs have been also shown to directly trigger malignant transformation of different cell types into cancer (Olsson and Cedervall 2016). For example, NET-mediated TLR-9 signalling was reported to activate cancer cell proliferation, tumor growth and metastasis (Acuff et al. 2006; Lande et al. 2007). In patients with chronic inflammatory conditions, NETs can mediate the activation of dormant cancer cells to form lung metastasis, which makes them initiators of tumor growth (Acuff et al. 2006; Cools-Lartigue et al. 2013; 2014; Masucci et al. 2020). Moreover, NETs that have been released in a tumor-free environment might also serve as physical scaffolds of the metastatic niche by facilitating the adhesion of circulating cancer cells to the tissue stroma (Acuff et al. 2006; Cools-Lartigue et al. 2013; 2014; Masucci et al. 2020; Tohme et al. 2016). When NETs are released in the already pre-metastatic niche, they can function as potent chemoattractants for disseminated cancer cells, leading to massive formation of micrometastases in the hepatic environment (Cools-Lartigue et al. 2013). In turn, neutrophil activation and NETosis are frequently induced remotely by soluble growth factors –such as G-CSF– generated by most types of cancers. This process has been particularly observed in human ovarian and breast cancer (Souto, Vila, and Brú 2011; Teijeira et al. 2020; Ireland and Oliver 2020). It has also been reported that tumors can use NETs as physical shields. In a CXCR1 and CXCR2-dependent manner, some tumors can get

surrounded by NETs that protect cancer cells through the recruitment of platelets and the activation of its protein components and that way tumors would be able to augment their resistance towards the immune response elicited by effector CD8+ T cells and NK cells (Olsson and Cedervall 2016; Teijeira et al. 2020).

1.1.4.1.4 “Silver Lining” NETs

In addition to their role in immune surveillance, NETs have been implicated in the development and maintenance of an ever-growing number of autoimmune and inflammatory conditions (Vitkov et al. 2009; Brinkmann 2018). However, the clinical phenotype of subjects with impaired or even absent NET formation does not exclusively support the hypothesis of a pro-inflammatory role of neutrophils and NETs, but rather reinforces the idea that NETs can also display downright anti-inflammatory and immunomodulatory functions. The consequences of a lack of NET formation in humans can be observed in individuals that suffer from chronic granulomatous disease (CGD) or Papillon-Lefèvre syndrome (PLS) (Bianchi et al. 2009). In CGD, ROS-dependent NET formation is impaired due to mutations in the NADPH oxidase complex. CGD patients suffer from recurrent bacterial and fungal infections and tend to develop autoimmune syndromes (Vitkov et al. 2009). Mouse strains with a normal neutrophil count but defects in NET formation also show stronger inflammatory reactions, especially in a model of heart infarction (Bonaventura et al. 2020). Furthermore, an anti-inflammatory function of NETs was proposed for gout disease. Gout is a form of inflammatory arthritis caused by high depositions of uric acid within the joints, which when crystallize into monosodium urate induce severe pain episodes. Monosodium urate crystals can form large aggregates called tophi, which have been observed to contain large numbers of neutrophils and aggregated forms of NET structures. It was hypothesized that due to the activity of enzymatic proteases and endonucleases associated to the NETosis process, these aggregated DNA structures could decrease the concentration of pro-inflammatory cytokines (IL-1β and TNF- α) in the local environment, which would explain the spontaneous resolution of the inflammatory response typical of gout (Schorn et al. 2012; Chatfield et al. 2018). That way, it brings the idea of NETs as regulators of the immune function and mediators of immune homeostasis.

1.1.4.1.5 NET Visualization Techniques

Live imaging of neutrophils is the star method to directly visualize morphology, dynamics, behaviour of NET formation and its interaction with the surroundings (De Buhr and Von Köckritz-Blickwede 2016; Alasmari 2020). A summary of the main methods and techniques used to target and describe NET formation can be found in Table 1-1: “NET visualization approaches”.

Table 1-1. NET Visualization Approaches

Identified Target in Sample [Ref]	Technique	Methodology	Dye	Strengths	Weaknesses
NETs in Animal Models[1]	Spinning disk confocal microscopy	Live-cell imaging to visualize morphology, dynamics, behaviour of NETs and interactions between NET formation and live pathogens	Fluorescent Bacteria and NET markers	Evaluation of NET formation *in situ* and *in vivo* Allows NET dynamics evaluation and kinetic studies	Invasive technique Unwanted inflammatory responses Poor temporal resolution Cardiac and Respiratory movement might compromise imaging Expensive, costly, time-consuming, technically challenging Long acquisition times
NETs in Animal Models[2]	Multi- / Two-photon microscopy		Fluorescent Bacteria and NET markers		
NETs in Animal Models[3]	Intravital Microscopy		Fluorescent Bacteria and NET markers		
Soluble NET remnants and cf-DNA in plasma, serum and fluids[4]	Fluorescent Reader		PicoGreen®	Objective Quantitative Can potentially detect NET formation *in vivo*	Low Specificity Cell-free DNA can reflect lytic cell death mechanisms (necrosis)

Identified Target in Sample [Ref]	Technique	Methodology	Dye	Strengths	Weaknesses
DNA-MPO Complexes in plasma, serum and fluids[5]	ELISA	Colorimetric measure of soluble ligands (e.g. peptides, proteins, antibodies, hormones). Target antigens are complexed with antibodies linked to a reporter enzyme that renders an activity-based colorimetric reaction.		Objective Quantitative	Low Specificity MPO (high-cationic nature) can bind to negatively charged cf-DNA MPO can reflect on neutrophil and macrophage activation not related to NET release
DNA-NE complexes in plasma, serum and fluids[6]	ELISA			Objective Quantitative	NE can reflect on neutrophil activation and degranulation not related to NET release
Citrullinated Histone H3 in human plasma[7]	Refined ELISA			Objective Quantitative Citrullination is the gold hallmark of NET formation	No consensus over a standard ELISA assay to monitor NETosis
MPO and citH3 in primary cells and cell lines[8]	Flow Cytometry	Detection of NET components attached to the neutrophil cell surface	Fluorescent antibodies against MPO and citrullinated histones	Objective Unbiased Automated Can be combined with cell-sorting techniques	Does not detect citH3-independent events Potentially able to report live NETosis events but misses lysed cells that underwent NETosis previously

Identified Target in Sample [Ref]	Technique	Methodology	Dye	Strengths	Weaknesses
DNA backbone in tissue sections[9]	IF	Co-localization of extracellular DNA, neutrophil markers, neutrophil-derived granule proteins and modified histones	DNA-intercalating dyes (DAPI, PI, SYTOX® Green)	Can differentiate between necrosis and NETosis	Biased selection of the field of view might affect results
Neutrophils in tissue sections[10]	IF		Fluorescent antibodies against Ly6G (in mice) and CD66b, NE or CD177 (in humans)		
Neutrophil granular proteins in tissue sections[11]	IF		Fluorescent antibodies against MPO, NE or LL37		Clump of NETs derived from multiple cells count as a single event
Citrullinated Histone in tissue sections[12]	IF		Fluorescent antibodies against citH3 and citH4		
NETs in primary cells[13]	IF	NET detection reagents that are based on fluorescent, chromatin binding polymers	PlaNET reagents	High Specificity Stain NETs only in activated cells Undetectable in necrotic cells	Non-specific signals reported Not suitable for NETosis kinetic studies

Table 1-1. NET Visualization Approaches. Included references as footer. [1](Yipp et al. 2012; McDonald et al. 2012), [2](Kolaczkowska et al. 2015; McDonald et al. 2012; Chèvre et al. 2014; Koji et al. 2014), [3](Yipp et al. 2012; McDonald et al. 2012), [4](S. Zhang et al. 2014; Masuda et al. 2016), [5](Rada 2019; Kessenbrock et al. 2009; Klebanoff 2005; Tatsiy and McDonald 2018), [6](Rada 2019; Kessenbrock et al. 2009; Tatsiy and McDonald 2018), [7](Thålin et al. 2017; Boeltz et al. 2019), [8](Masuda et al. 2016; Gavillet et al. 2015), [9](Fuchs et al. 2007a; Köckritz-Blickwede et al. 2010; Neumann et al. 2014; Saitoh et al. 2012; Narasaraju et al. 2011; Urban et al. 2006; Brinkmann et al. 2012; de Buhr et al. 2015; 2014), [10](Fuchs et al. 2007a; Köckritz-

Blickwede et al. 2010; Neumann et al. 2014; Saitoh et al. 2012; Narasaraju et al. 2011; Urban et al. 2006; Brinkmann et al. 2012; de Buhr et al. 2015; 2014), [11](Fuchs et al. 2007a; Köckritz-Blickwede et al. 2010; Neumann et al. 2014; Saitoh et al. 2012; Narasaraju et al. 2011; Urban et al. 2006; Brinkmann et al. 2012; de Buhr et al. 2015; 2014), [12]((Fuchs et al. 2007a; Köckritz-Blickwede et al. 2010; Neumann et al. 2014; Saitoh et al. 2012; Narasaraju et al. 2011; Urban et al. 2006; Brinkmann and Zychlinsky 2012a; de Buhr et al. 2015; 2014), [13](Tatsiy and McDonald 2018).

1.2 The Liver as a Frontline Immunological Organ

The liver is considered the most important metabolic organ in humans, as well as a frontline immunological tissue that is strategically positioned in the human body to recognize, capture, filter and eliminate pathogens and macromolecules from the circulation (Kubes and Jenne 2018). This not only includes PAMPs and DAMPs, but also many harmless molecules derived from healthy commensal microbiota and food, that enter the body and reach the liver from the intestine *via* the portal vein (Berg 1995; Lumsden, Henderson, and Kutner 1988; Son, Kremer, and Hines 2010). By default, the liver's immunological status is anti-inflammatory or immunotolerant, and its immune responses are typically tolerogenic. Maintaining a general state of immune hyporesponsiveness and immunotolerance is essential to liver function and to prevent an unwanted inflammatory response against harmless common food antigens, or the normal low levels of microbe-derived molecules that may enter the bloodstream from the gut. Nonetheless, a balance between this immune hyporesponsiveness and an effective immunity is essential to ensure not only a robust inflammatory response when the levels of microbial products are altered, but also to assure the normal functioning and homeostasis of the hepatic tissue. When this balance is ruptured, an inappropriate immune response is initiated or inflammation is chronically sustained, it all can lead to tissue remodelling, tissue damage and dysfunction, the development of pathological conditions like hepatic fibrosis, sterile liver injury and organ failure, and consequently several hepatic disorders (e.g., non-alcoholic liver disease [NAFLD] and non-alcoholic steatohepatitis [NASH]) can emerge (Kubes and Jenne 2018).

1.2.1 The Functional Anatomy of the Liver

The liver exhibits both endocrine and exocrine properties and plays an essential role during many metabolic and cytotoxic processes. The liver synthesizes and secretes bile, which in turn, ensures an excretion route for many endogenous and exogenous compounds such as bile acids, bilirubin, phospholipids, cholesterol, drugs and toxins. Due to its location, the liver plays a central role in removing toxic materials and waste products before they infiltrate into the systemic circulation. The liver is composed of 60% parenchymal cells, mainly hepatocytes; and 30% to 35% of non-parenchymal

and epithelial cells, *i.e.*, Kupffer cells (KCs), hepatic stellate cells and liver sinusoidal endothelial cells (LSECs) (Williams and Iatropoulos 2002).

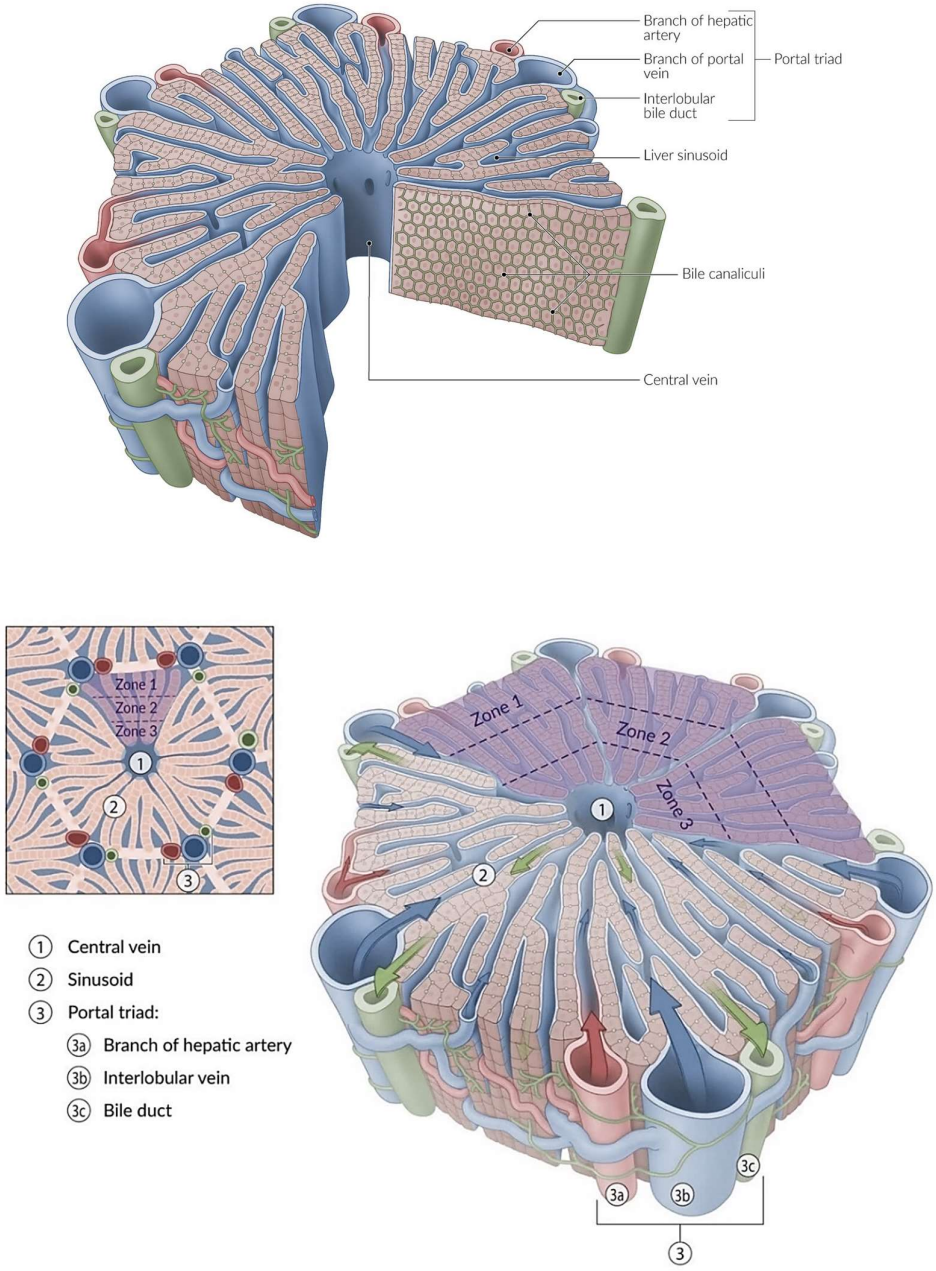


Figure 1-6. The functional anatomy of the liver. Microscopically, the liver is divided into functional units known as lobules, each with a central vein and several portal triads. Each portal triad is located at the corners between adjacent hepatic lobules and is composed of a branch of the hepatic artery, a branch of the hepatic portal vein and a branch of the bile ductule, which all together are accompanied by lymphatic vessels and a branch of the *Vagus* nerve. Collectively, portal triads transport blood into the liver and bile out of the liver. Bile canaliculi allows bile passage between adjacent hepatocytes: bile flows towards the peripheral portal triad, enter the hepatic ducts and exit the liver. Arterial and portal venous blood flow towards the central venule; from where

they are filtered, deoxygenated and re-enter the systemic circulation through the hepatic veins and the inferior vena cava. Broadly, liver parenchyma consists of hepatocytes stacked on top and next to each other, and hepatic sinusoids. Hepatic sinusoids drain into the central vein of each lobule. The liver parenchyma can be divided into zones according to their spatial location in regard to the portal triad and the central vein: Hepatocytes in zone I are closest to the portal triad and will receive relatively more oxygen and solutes, while hepatocytes in zone III are closest to the central vein, which makes this a lower oxygenated zone. **Illustration from amboss.com/us/knowledge/Liver.**

Histologically, the liver parenchyma consists of a repetitive number of microscopic functional units known as lobules (**Fig.1-6. The functional anatomy of the liver**). Each anatomical lobule has a hexagonal shape and is drained by a central vein. At the periphery, in each of the six vertices of the hexagon appear three structures (a branch of the bile duct, a branch of the portal vein and a branch of the hepatic artery), collectively known as the portal triad. Lobules can be observed and anatomically described in three different ways (**Fig.1-7. Liver lobular architecture**) (Krishna 2013):

- **Hepatic or classic lobule**: The classic lobule consists of a hexagonal composition of hepatocytes aligned one on top of each other. Within each lobule, the hepatocytes are oriented outwards from a central vein and the hepatic sinusoids travel across the hepatocyte paths, draining into the central vein. Consecutively, blood flows from the periphery to the centre, and bile flows in the opposite direction.
- **Portal lobule**: While the "classic lobule" view instructs on how the blood is supplied to the liver and how is the hepatocyte alignment; the portal lobule describes the exocrine function of the liver (i.e. bile secretion). In the portal lobule each functional unit is a triangle, which has a central axis through a portal triad and the imaginary vertices through the three closest central vein portal canals surrounding it. The triangle total area covers the hepatic regions that secrete bile into the same bile duct.
- **Acinus lobule**: The liver acinus lobule is the smallest functional unit of the liver and it has an oval or elliptical shape. The short axis is shared at the border of two adjacent lobules, together with their shared portal triad. The long axis covers an imaginary line between two adjacent central veins to the portal triad. In this structural unit, the periphery of the acinus is the central vein while the centre is the portal tract. The parenchymal region of the acinus can be divided into a periportal zone adjacent to the portal triad, a mid-zonal transition zone, and a centrilobular zone adjacent to the central hepatic vein. A liver acinus encompasses the section of hepatic tissue that is served by a single terminal branch of the hepatic artery. Therefore, blood from the portal triads flows though these zones to the venule with a decreasing oxygen and nutrient gradient.

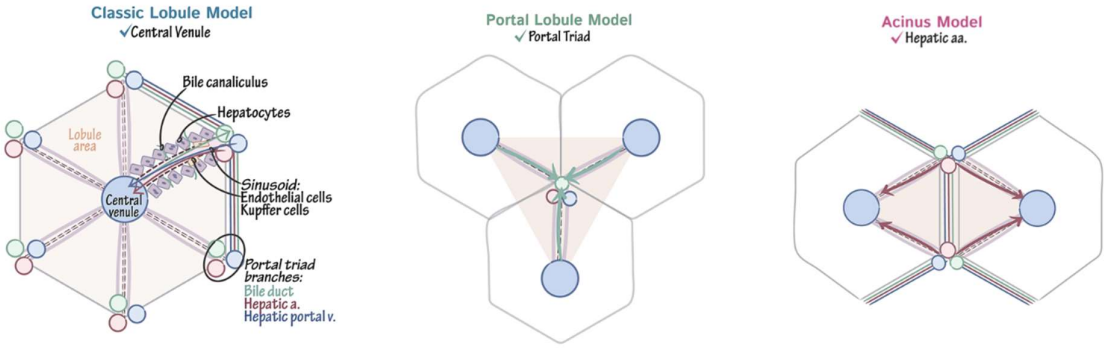


Figure 1-7. Liver lobular architecture. Representation of some of the different microanatomic and functional models of the liver: classical lobule model, portal triad model and acinus model. In the classical lobule model, the hepatocytes conform a hexagonal arrangement with a common centered central vein. Hepatocytes lobules are separated by hepatic sinusoids, which travel alongside the strips of the lobule, draining into the central vein. The area of each lobule is bound by six peripheral portal triads, located at the vertices of each hexagon. Here, blood flows from the periphery (portal triad) to the centre (central vein), and bile flows in the opposite direction. In the portal triad model, the area of the portal lobule comprises the three concomitant hepatocytes that drain into the same single bile duct. This area is a triangle bound peripherally by the central venules, with the portal triad at the center. The portal lobule model highlights the exocrine functions of the liver (i.e., pathway of bile secretion). In the liver acinus model, the area is determined by the afferent vascular blood supply (oxygenated) from the hepatic artery branches. Here the branches of arteries from the triads, running on the sides of the hepatic lobule, form the centre of the lobule. This lobule is oval and can be further divided into three zones with different metabolic functions. Some enzymes can also be preferentially expressed according to these specific microenvironments. **Illustration from drawittoknowit.com.**

1.2.1.1 The Enterohepatic Circulation

The liver is an unique organ in a way that is supplied both with arterial and venous blood (Kubes and Jenne 2018). Oxygen-rich arterial blood enters the liver *via* the hepatic artery. However, about approximately 70% of the blood-supply to the liver, enters the tissue through the portal vein. Portal blood is rich in nutrients but also in pathogens, pathogen-derived molecules and pollutants (Lumsden, Henderson, and Kutner 1988), all of them elements that get recycled into the liver after intestinal reabsorption. The solutes and substances contained in the enterohepatic circulation compose a biliary excretion containing bile acids and bilirubin –among other components–, that is excreted in the form of bile from the liver into the duodenum. Afterwards, this composition is moved into the small intestine where it is reabsorbed by enterocytes and transported back into the liver. Ultimately, when portal blood reaches the liver, it gets mixed with arterial blood flowing through the hepatic sinusoids. Therefore, the immune response in the liver must strike a delicate balance between tolerance to non-threatening substances and immunity against pathogens (Kubes and Jenne 2018).

1.2.1.2 Liver Sinusoids

Sinusoids are unique blood vessels of the hepatic microcirculation (**Fig.1-8. Liver sinusoids**). Hepatic sinusoids are dynamic microvascular structures that serve as the principal location where oxygen-rich systemic blood from the hepatic artery mixes with the nutrient-rich enterohepatic circulation from the portal vein (DeLeve 2007; Brunt et al. 2014). Sinusoidal capillaries display a discontinuous endothelium that resembles those of fenestrated capillaries, and they are mainly composed by four recognized types of non-parenchymal cells: 1) Liver sinusoidal endothelial cells, which form the sinusoid lining wall that is in contact with the blood and represent approximately 15 to 20% of liver cells, 2) Kupffer cells, intravascular liver-resident macrophages that adhere on the luminal side of the sinusoidal capillar, 3) Hepatic stellate cells, specialized pericytes that serve as myofibroblasts during hepatic injury, and 4) Pit cells, immunoreactive NK cells localized in the abluminal surface of the sinusoid.

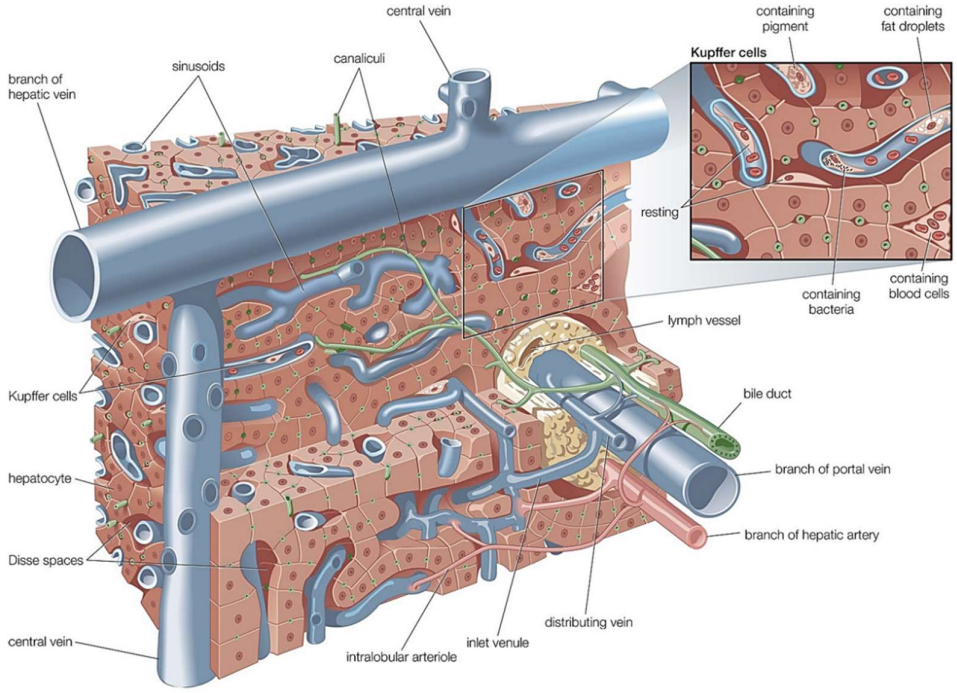


Figure 1-8. Liver Sinusoids. Hepatocytes are in direct contact with the liver´s blood supply through liver sinusoidal endothelial cells (LSECs), a fenestrated endothelium that conform the so-called sinusoid capillaries. Sinusoids allow blood, molecules and bile to pass from the tissue to the lumen. Hepatic stellate cells reside in the space defined between LSECs and hepatocytes (space of Disse), and act as the sinusoidal pericytes. Kupffer cells can also be found in the sinusoid lining. **Illustration from britannica.com/science/hepatocyte/images-videos.**

1.2.1.3 Portal Triads

Portal triads, also known as portal canals or portal tracts are located at the corners between adjacent hepatic lobules and are composed of three major tubes: 1) a branch of the bile duct (lateral location) that carries bile products away from the hepatocytes to the larger ducts and the gall bladder; 2) a venule branch of the hepatic portal vein (posterolateral location) that carries low-oxygenated blood –but rich in nutrients– from the small intestine; and 3) an arteriole branch of the hepatic artery (medial location) that supplies oxygenated blood to the hepatocytes (**Fig.1-6. The functional anatomy of the liver**). Traditionally, the portal triads definition includes only these three first structures, although they are always accompanied by lymphatic vessels and a branch of the *Vagus* nerve. Collectively, portal triads brings blood into the liver and bile out of the hepatic tissue (Abdel-Misih and Bloomston 2010).

1.2.2 The Cellular Compartment of the Liver

The liver is a complex organ composed of two main types of parenchymal cells: Hepatocytes, able to perform metabolic, regulatory and toxicological functions and accounting for approximately 60% of the total cellular compartment in the liver; and cholangiocytes, epithelial cells lining the bile ducts, that account for less than 5% of all the hepatic cells (Alpini et al. 1994). Hepatocytes are able to recycle substrates from the dual blood circulation present in the liver and secrete metabolites into the bile. Indeed, the bile is formed primarily by the hepatocyte and secreted at the bile canaliculus, and the hepatocytes are the only cell in the body that can mediate the conversion of cholesterol to bile acids. The hepatocyte primary role is metabolic; however, these parenchymal cells have been reported to mediate protein synthesis (including immune proteins such as acute-phase proteins and proteins from the complement system) and toxin neutralization, and, under specific conditions to even be able to work as antigen presenting cells (APCs) with the capability to detect pathogens and present antigens to the adaptive immune system.

1.2.3 The Immunological Compartment of the Liver

In order to regulate the influx of gut-derived immunogenic factors, the liver hosts the largest population of phagocytic cells in the body including specialized tissue-resident macrophages (e.g. Kupffer cells). Kupffer cells account for up to 80% of all body macrophages. Along with Kupffer cells, other resident and non-resident immune populations including monocyte-derived macrophages, neutrophils, liver-resident monocyte-derived DCs as well as plasmacytoid DCs (pDCs) (Kubes and Jenne 2018), NK cells, B cells and mucosa-associated invariant T cells, they all participate in the sentinel function of the hepatic tissue (Son, Kremer, and Hines 2010). Beyond protein manufacturing, diet-

derived nutrient metabolism and toxin excretion, one of the main functions of the liver is to maximize immune cell-pathogen interactions within the bloodstream. Dynamic interactions between all these hepatic immune populations are key to maintaining immunological balance, and overall hepatic tissue health.

1.2.3.1 Kupffer Cells

Kupffer cells comprise the largest population of specialized tissue-resident macrophages and are present exclusively in the liver. Kupffer cells are intravascular immobile macrophages that represent around 35% of the non-parenchymal cells in the hepatic tissue and up to 90% of all tissue macrophages (Crispe 2009). Kupffer cells are a critical component of the innate immune system and, particularly, the mononuclear phagocytic system. They play an essential role in host defense (Bilzer, Roggel, and Gerbes 2006; Blériot et al. 2015) and participate in the metabolism of multiple compounds such as protein complexes, small particles, lipids, and in the removal of apoptotic cells from the circulation (Crispe 2009; Parker and Picut 2005). In fact, a prevalent view in scientific literature is that approximately a third of senescent neutrophils are removed in the liver, such as that inhibition of Kupffer cells-mediated phagocytosis resulted in neutrophilia and the accumulation of activated neutrophils in the spleen and the lungs. The strategic localization of Kupffer cells in the hepatic sinusoids puts them in the first line of defense against gut-derived bacteria, gut bacterial endotoxins and microbial debris derived from the gastrointestinal tract (Fox, Thomas, and Broitman 1987). Kupffer cells can efficiently detect innocuous antigens, gut-derived nutrient particles, phagocytize and clear pathogens and apoptotic cells entering the hepatic tissue through the portal vein (Thomson and Knolle 2010; Y. Li et al. 2013). Most Kupffer cells can recognize pathogens due to their capacity to express a specialized pathogen receptor, the Complement Receptor of the Immunoglobulin superfamily (CRIg), which allows them to catch circulating pathogens even under shear conditions (Helmy et al. 2006). Kupffer cells' pathogen killing capacity is primarily mediated by the NADPH oxidase system. Following antigen detection and pathogen capture, the presence of pathogen-associated molecules (e.g., TLR3 and TLR9 ligands) or inflammatory cytokines can modulate Kupffer cells to act as a potent APC that express MHC-I, MHC-II, and co-stimulatory molecules required for a robust T cell activation, such as the CD1d glycoprotein (Winwood and Arthur 1993; Gül et al. 2014). Kupffer cells also express a wide number of immune receptors (FcRs, CRs), PRRs (TLRs), and scavenger receptors.

The amount of Kupffer cells in the liver is held constant. Kupffer cells have a proliferative capacity, being able to replenish themselves; which is in complete contrast to monocyte-derived macrophages that have no proliferative potential. Although Kupffer cells were initially thought to migrate from the bone marrow as monocytes and differentiate into these highly specialized tissue-resident

macrophages, their ontogeny is now becoming clearer: Kupffer cells, like microglia in the brain and pulmonary macrophages in the lung, come from the yolk sac and likely replicate locally in the liver from local intrahepatic progenitors (Yamamoto et al. 1996; Klein et al. 2007; Zigmond et al. 2014; Schulz et al. 2012). Due to its ontogeny, macrophage surface markers (F4/80, CD11b and CD68 are commonly used in mice (Sato et al. 2014)) have been used for their characterization. For example, F4/80 is an exclusive antigen of mononuclear phagocytes (Austyn and Gordon 1981; Hume et al. 1983), CD11b is a typical cytoplasmic marker of the myeloid lineage (Sanchez-Madrid et al. 1983), and CD68 antigen targets activated macrophages and activated Kupffer cells (Smith and Koch 1987). Based on these surface markers, four different populations of Kupffer cells have been proposed (Kinoshita et al. 2010): $F4/80^{+}CD11b^{-}$ and $F4/80^{+}CD68^{+}$ represent cell populations with a higher phagocytic activity and increased ROS production after LPS stimulation. On the other hand, $F4/80^{+}CD11b^{+}$ and $F4/80^{+}CD68^{-}$ populations show high levels of TNF and IL-12 after LPS stimulation.

Under homeostatic conditions, Kupffer cells display an important tolerogenic phenotype and are key in maintaining immune hyporesponsiveness and immunotolerance in the hepatic tissue. Similar to what is observed in LSECs, the continual exposure of Kupffer cells to low levels of gut-derived PAMPs is what, in homeostasis, dampens the ability of Kupffer cells to activate lymphocytes (Winwood and Arthur 1993; Su 2002). Consequently and under certain disease conditions, any alteration on Kupffer cells function shifts their tolerogenic phenotype into a pathologically activated state that is associated with various liver diseases that manifest chronic inflammation: viral hepatitis, intrahepatic cholestasis, liver fibrosis, alcoholic liver disease, NASH and NAFLD (Kolios, Valatas, and Kouroumalis 2006).

1.2.3.2 Neutrophils in the Liver

Resident immune cells in the liver are essential to cast a proper immune response. However, under certain circumstances, an effective immune response requires of the recruitment of other circulating immune populations, such as neutrophils and monocytes from the bloodstream. Traditionally, neutrophil recruitment into tissues requires a series of sequential steps that are mediated by the receptor-ligand interactions on leukocytes and the vascular endothelium in accordance with the classical recruitment cascade. In the liver, neutrophil recruitment differs per anatomical location. Like in most organs, neutrophils are recruited in a selectin-dependent mechanism in postcapillary venules, a type of vascular bed that is highly different from the sinusoidal capillaries present in the hepatic compartment. However, liver sinusoids support the majority of leukocyte trafficking (70-80%) and only the remaining traffic takes places in the post-capillary venules. Interestingly, sinusoidal capillaries – where more than 85% of the liver-neutrophil interactions occur– do not express selectins. In congruence with that, neutrophil recruitment within the hepatic sinusoids has been reported not to

require the up-regulation of the Mac-1 complex (CD11b/CD18), a member of the β_2 integrin family of adhesion molecules (H Jaeschke 2000; Hartmut Jaeschke 2003; Hartmut Jaeschke and Hasegawa 2006) and is considered a selecting and rolling-independent mechanism (J. Wong et al. 1997). In the healthy liver and under steady state conditions, a high number of neutrophils routinely patrol the hepatic sinusoids, but only a few resident cells can be found transmigrated into the hepatic tissue (N. Li and Hua 2017; Markose et al. 2018). Neutrophil infiltration in the hepatic tissue is a process reported highly influenced by the circadian clock (Casanova-Acebes et al. 2018; María Casanova-Acebes et al. 2013); *later discussed*. In line with that, daily-aged neutrophils display a higher tropism towards the liver tissue, where they can migrate to be cleared (María Casanova-Acebes et al. 2013; Furze and Rankin 2008b). Indeed, liver X receptors (LXR)-deficient mice, unable to eliminate neutrophils in the bone marrow, show an accumulation of senescent neutrophils in blood, spleen and the liver itself (Hong et al. 2012; Furze and Rankin 2008a). Furthermore, apoptotic neutrophils have been shown to accumulate in the liver sinusoids in a model of LPS-injected mice (J. Shi et al. 2001).

On the other hand, highly numbers of activated neutrophils can transmigrate and accumulate within the hepatic parenchyma in response to infection and inflammatory processes (Robinson, Harmon, and O'Farrelly 2016), as well as during many types of liver pathological conditions. Most neutrophils recruited to the liver during endotoxemia and sepsis adhere to the sinusoidal endothelium and remain within this intravascular compartment, strategically positioned there to mount an intravascular host response with the goal to eliminate incoming pathogens (McDonald et al. 2008; Menezes et al. 2009). Accumulated neutrophils in the hepatic microvasculature can transmigrate into the hepatic parenchyma following danger signals and chemotactic gradients (e.g., CXC chemokines, leukotriene β4) released by distressed or dying cells, typically hepatocytes and, once there, mediate tissue damage (Hartmut Jaeschke and Hasegawa 2006). For example, lipid peroxidation products from dying hepatocytes, but also pro-inflammatory mediators such as TNF-α and IL-1 released from Kupffer cells, can activate neutrophils and mediate their recruitment into the hepatic sinusoids (Ramaiah and Jaeschke 2007; Pedro E Marques et al. 2012; Q. Zhang et al. 2010). Once transmigrated into the liver parenchyma, neutrophils can undergo degranulation –neutrophil-derived proteinase-3 can directly cause hepatocellular injury (Markose et al. 2018)–, and can cause a respiratory burst and oxidative stress through the NADPH oxidase that mediates hepatocyte killing. Neutrophil extravasation from the hepatic microcirculation into the parenchyma is facilitated once more by the upregulation of β2 integrins (Hartmut Jaeschke and Hasegawa 2006) that interact with the ICAM-1 on distressed liver cells. Neutrophil-mediated liver injury is reported in models of hepatic ischemia-reperfusion injury (T. H. C. de Oliveira et al. 2018), alcoholic and viral hepatitis (Bautista 1997), and endotoxemia (Hartmut Jaeschke and Hasegawa 2006). An inappropriate activation of neutrophils can contribute to chronic

inflammation and aggravate liver injury (Pedro Elias Marques et al. 2015; Hartmut Jaeschke and Liu 2007). This seems clear because a depletion of neutrophils in mice prior to an acetaminophen (APAP) overdose –an experimental model of hepatotoxicity–, decreased neutrophil recruitment and, interestingly protected against liver injury (Z.-X. Liu et al. 2006). Moreover, a similar attenuation of hepatic injury was also observed when neutrophil infiltration to the liver parenchyma is blocked by the administration of CXCR2 and FPR1 antagonists (Pedro E Marques et al. 2012; Ishida et al. 2006).

1.2.3.3 NETs in the Liver

The liver is a central organ in NET formation, as the level of identified NET structures here far surpasses that in other microcirculations, where NET formation has been found to be of rare events (Yipp et al. 2012; Kolaczkowska et al. 2015). In the liver, NETs are released upon several activators through different mechanisms, including infection, ischemia and sterile damage. TLR4 dependent platelet–neutrophil interactions (*previously described*) promote NET formation in the mouse hepatic microcirculation, increasing the liver capacity of bacterial uptake by fourfold during general septic conditions (Clark et al. 2007; McDonald et al. 2012). Indeed, the neutrophil-platelet integrin-mediated cooperation axis seems to be one of the key initiators of NET release in the liver. Similar results were found in a TLR3 dependent myxomatosis viral infection, in which NETs were reported to reduce viral titers (Saitoh et al. 2012; Jenne et al. 2013). Moreover, in mouse models of septic infection and systemic inflammatory response, the liver functions to clear the majority of bacteria from the circulation, with both Kupffer cells and netting neutrophils cooperating to this process (McDonald et al. 2012; Zeng et al. 2016). Briefly, during endotoxemia neutrophils seem to accumulate in the liver microcirculation and from there, they can migrate into the liver sinusoids, where they exert protective functions by releasing intravascular NETs that line the vessel wall of the sinusoids. These NET structures ensnare bacteria from the circulation and provide intravascular immunity by preventing bacteria dissemination to remote organs (Menezes et al. 2009; McDonald et al. 2008). *E.coli*- or LPS-dependent NET production seems regulated by the β2-integrin-dependent platelet-neutrophil interactions, ocurring within the hepatic sinusoids. Using *in vivo* spinning disk confocal intravital microscopy, it was possible to visualize NET structures trapping *E.coli* particles in the hepatic sinusoids, and it was demonstrated that disruption of NET release resulted in systemic spreading of bacteria (McDonald et al. 2012). The high levels of von Willebrand factor (vWF) released by endothelial cells within the liver sinusoids is undoubtedly one of the reasons NET accumulation seems vast in that organ: Anchor molecules such as vWF and NET-associated histones show a very specific and selective receptor-ligand interaction (Sandoval-Pérez et al. 2020; J. Yang et al. 2020). Moreover, McDonald et al., identified that Kupffer cells are capable of sequestering methicillin-resistant *Staphylococcus aureus* in a model of systemic blood infection.

Staphylococcus aureus incursion in the liver induces a robust neutrophil infiltration. There, neutrophils start realising NETs in order to restrain bacterial spread, which ultimately generates ischaemia in focal areas (McDonald et al. 2012). In summary, evidence supports a key role of NETs in host liver's defense. Still, NET function comes at the expense of an inflammatory response in the host and consequent collateral tissue damage, effects that are largely attributable to their antimicrobial proteins, strong cytotoxic components of NET structures. In addition to the cytotoxic effect of NETs, they can also mediate activation of hemostasis, which leads to vascular occlusion and further tissue damage. All in all, NETs can undoubtedly contribute to the progression of liver disease and serve as main players in the development of alcoholic and non-alcoholic steatohepatitis as well as the progression of this conditions into hepatocellular carcinoma (Kolaczkowska et al. 2015; McDonald et al. 2012).

1.2.3.4 Other Immune Populations in the Liver

Similar to Kupffer Cells being the most abundant tissue-resident macrophage population, the liver also hosts the largest population of NK cells in the human body. Up to one-half of the total lymphoid population within the human liver can be defined as CD56+ NK cells (Moroso et al. 2010; Hata et al. 1991). This cellular population is essential during tissue pathology: while most immune populations screen and identify targets based on either foreignness with respect to the host (e.g., presence of PAMPs) or the presence of specific antigenic molecules, NK cells are able to screen targets and identify them based on the absence of self-identity. This results helpful because some pathogens have developed host-infection strategies that resulted in the downregulation of several antigen-presenting molecules (e.g. MHC-I), in an effort to hide themselves from the host's immune system. NK cells can take advantage of this absence of self-identity to facilitate cellular activation and cytokine production (e.g., IFN- γ) against "foreign" antigens and target cells for clearance. NK cells, alongside invariant NK-T (iNKT) cells, mucosal-associated invariant T cells, γδ T cells and innate lymphoid cells (ILCs) are part of the diverse range of innate lymphocytes in the healthy adult liver (Gregory, Sagnimeni, and Wing 1996; Kenna et al. 2004; 2003; Doherty et al. 1999).

Beyond Kupffer cells and NK lymphocytes, the liver is enriched with a diverse spectrum of other innate and adaptive immune cell populations that cooperate in maintaining the liver immunotolerogenic status but can also mediate inflammation during hepatic disease conditions. Although not a classical secondary lymphoid organ, the liver represents a unique environment for the development and function of the adaptive immune response. Adaptive immune cells that are found in the healthy liver include B cells (CD5+ B cells) and plasma cells (Norris et al. 1998), as well as different T cell populations, such as regulatory T cells (Tregs), memory T cells, cytotoxic T lymphocytes (CTLs) and Type 1, 2, 17 and 22 T-helper cells (Th1, Th2, Th17, and Th22) (Pruvot et al. 1995; Kelly et al. 2014;

Doherty et al. 1999; Norris et al. 1998). Tregs play a critical role in hepatic immunotolerance by expressing of wide range of immunoregulatory markers such as the cytotoxic T lymphocyte-associated protein 4 (CTLA-4), the CD39 glycoprotein or the lymphocyte-activation gene 3 (LAG-3) and by secreting the anti-inflammatory IL-10 cytokine [14]. In regards to innate immunity, DC populations –including both myeloid DCs (mDCs) and pDCs– can also be found in the healthy liver and seem to take part in mediating T-cell responses (Thomson and Knolle 2010; Kelly et al. 2014). Myeloid-derived suppressor cells (MDSC) are also present in the healthy liver (Chen et al. 2011) and seem to be expanded during chronic liver disease (Pallett et al. 2015). In opposition to the DC response, MDSCs are defined by their ability to suppress T-cell activation through the release of the immunosuppressive molecules such as the IL-10 cytokine or the transforming growth factor (TGF)-β (Cheng et al. 2021; Robinson, Harmon, and O'Farrelly 2016).

1.3 Physiological Modulators of NETosis

Nowadays, we know that not all neutrophils are equally prone to release NETs, as NET variability has been reported among diverse tissues and physiological states of the organism, and even across different animal species. Variations in the neutrophil capacity to undergo NETosis *in vivo*, the potentially diverse phenotype of the NET structures as well as their varied pro-inflammatory or physiological capacities, might well be influenced by physiological regulators of the neutrophil life cycle, including circadian oscillations, the timing of food intake and the quality of the diet, and even the organism commensal's microbiome.

1.3.1 Circadian Regulation of NETosis

The regular 24 hours environmental cycles generated by the planet's daily rotation have led to the evolution of daily autonomous circadian rhythms in almost all life forms on Earth. In mammals, behaviour, physiology and metabolism are all well-sustained mechanisms controlled by daily rhythms and the molecular internal circadian clock (Dibner, Schibler, and Albrecht 2010). What is more, immune responses are also highly influenced by the circadian clock: immune rhythmicity allows for a phase coherence between an organism immune condition and the normally existing factors within its environment, such as light-dark cycles. Immune rhythmicity regulates the timing and intensity of the immune response, which allows for the immune system to anticipate environmental changes and challenges, ensures the adequate respond to an insult and ultimately preserves health. Circadian regulation of innate immunity requires the integrated effort of at least three divisions: neurons of the

suprachiasmatic nuclei (SCN) and their associated endocrine organs, structural cells in peripheral tissues and, ultimately, immune cells (Palomino-Segura and Hidalgo 2021).

1.3.1.1 Circadian Rhythms

Circadian rhythms are guided by cell-autonomous biological clocks, which permit cells, tissues and organisms to anticipate and adapt to temporal changes occurring within their environment (Curtis et al. 2014). The synchronization of an organism endogenous circadian rhythm with an external environmental rhythm is called *entrainment*. In higher organisms such as mammals, all their physiological parameters are regulated by an internal circadian clock, including sleep–wake cycles, behavioural and locomotor activity, body temperature oscillations, cardiovascular and digestive processes, endocrine systems, metabolism and, to our particular interest within this chapter, immune functions (Green, Takahashi, and Bass 2008; Dibner, Schibler, and Albrecht 2010).

In mammals, the internal clock alignment is organized and regulated in a multi-oscillatory network that conforms a master central clock located in the SCN of the hypothalamus (Dibner, Schibler, and Albrecht 2010; Mohawk, Green, and Takahashi 2012; Welsh, Takahashi, and Kay 2010). The remaining clocks in the body are synchronized by what is known as rhythmically cyclical environmental cues or *zeitgebers*. The master central clock in the SCN is entrained by the light, the principal and most reliable daily environmental rhythm and therefore a universal *zeitgeber*. Ambient light signal inputs from the retinal optic nerve are transmitted into the neurons of the SCN and set the rest-activity cycle within an organism. Alongside the central clock, there exist several secondary peripheral clocks throughout the body. These peripheral clocks are located in secondary areas of the brain and in most mammalian peripheral cells (including endocrine glands) and are time- and phase-regulated by the SCN main clock (Dibner, Schibler, and Albrecht 2010). Peripheral clocks in peripheral tissues employ the same molecular components as in the SCN, display circadian oscillation of clock genes, show cellular synchronization at the tissue level and display different cellular activities between day and night. Overall, the central clock dictates a global and temporal program across an organism by synchronizing multiple peripheral clocks that oscillate in an autonomous way and that are present in all body cells. Altogether, this organisation establishes a universal common rhythm within the body. Now, while the central clock is entrained by light/dark cycles, peripheral clocks are entrained by feeding cycles –eating behaviours, timing of food intake and nutritional signals–, which are considered major *zeitgebers* of the peripheral clock and responsible of the cellular active and resting phases (Dibner, Schibler, and Albrecht 2010). However, light is indeed an indirect responsible for the time of food consumption, suggesting that the SCN entrains the phase of peripheral clocks *via* the control of feeding behaviour. In mammals,

it has been estimated that approximately 10% of the genome is under circadian control (S. Panda et al. 2002; Storch et al. 2002).

Finally, it is worth mentioning that for a biological parameter to be defined as circadian in nature, it must oscillate within a constant environment regardless external factors such as light or food intake. Oscillations that would remain rhythmic under conditions where no *zeitgebers* are present, such as in constant darkness conditions, are termed *circadian*. These criteria are fulfilled for behavioural activity, core body temperature and plasma hydrocortisone or melatonin levels; all classical markers for circadian rhythms in mammals. Oscillations that are only rhythmic under the presence of an entrainment cue are termed *diurnal.*

1.3.1.2 Molecular Circadian Clock

The mammalian circadian core clockwork genes consist of a conserved set of transcriptional factors that co-operate in a negative, autoregulatory and rhythmic transcription–translation feedback loop (TTFL) (Takahashi 2017) (**Fig.1-9. The molecular circadian clock**). The central clock brain and muscle ARNT-like 1 (BMAL1, also known as *Arntl*) and circadian locomotor output cycles kaput (CLOCK) genes encode for proteins that bind together and form the cytoplasmatic heterodimeric complex CLOCK–BMAL1, that acts on nuclear enhancer-box (E-box) elements of clock controlled genes (CCGs) (Bunger et al. 2000), driving circadian processes and their own expression by binding the promoter regions within the Clock and Arntl genes. The activation of the CLOCK–BMAL1 complex also drives the expression of other target clock genes, including period circadian protein homologue 1 (PER1) and PER2, cryptochrome 1 (CRY1) and CRY2, and REV-ERBα (encoded *Nr1d1*) and REV-ERBβ (encoded *Nr1d2*), all of them repressors of the core clockwork transcriptional activity (Rosbash 2009; Patel et al. 2015; Schibler 2006). PER and CRY proteins form a dimeric complex that accumulates in the cytoplasm and starts a second rhythmic negative feedback loop that leads to the transcriptional repression of BMAL1 and CLOCK, interfering with the complex formation. CLOCK–BMAL1 repression drops owing to the decay and degradation of the PER–CRY complex and structural changes in the BMAL1 protein, so the cycle can start anew allowing for BMAL1 and CLOCK to initiate the next cycle of transcription. Temporally, in mice, CLOCK–BMAL1 activation occurs during their resting phase in the daytime, leading to the transcription of the PER and CRY genes in the afternoon and the accumulation of the PER and CRY proteins in the late afternoon or evening. The PER-CRY complex translocates into the nucleus at night during the mice active phase, and there PER-CRY interact with the CLOCK–BMAL1 complex to repress their own transcription (Curtis et al. 2014). As repression progresses, PER and CRY transcription declines and so transcription by CLOCK–BMAL1 can begin anew to start a new round of transcription the next morning. Moreover, a second autoregulatory feedback loop is induced by the transcription of

the REV-ERBα and REV-ERBβ complexes. REV-ERBs repress BMAL1 transcription and competes with the retinoic acid receptor-related orphan receptor-α (RORα), ROR-β and/or ROR-γ –activators of BMAL1 transcription and positive cycle regulators–, for binding of ROR responsive elements (ROREs) in the BMAL1 promoter (Preitner et al. 2002). Formation, trafficking and degradation of the different clock protein complexes throughout this transcriptional cycle generate the intrinsic nature and stability of the clock oscillations.

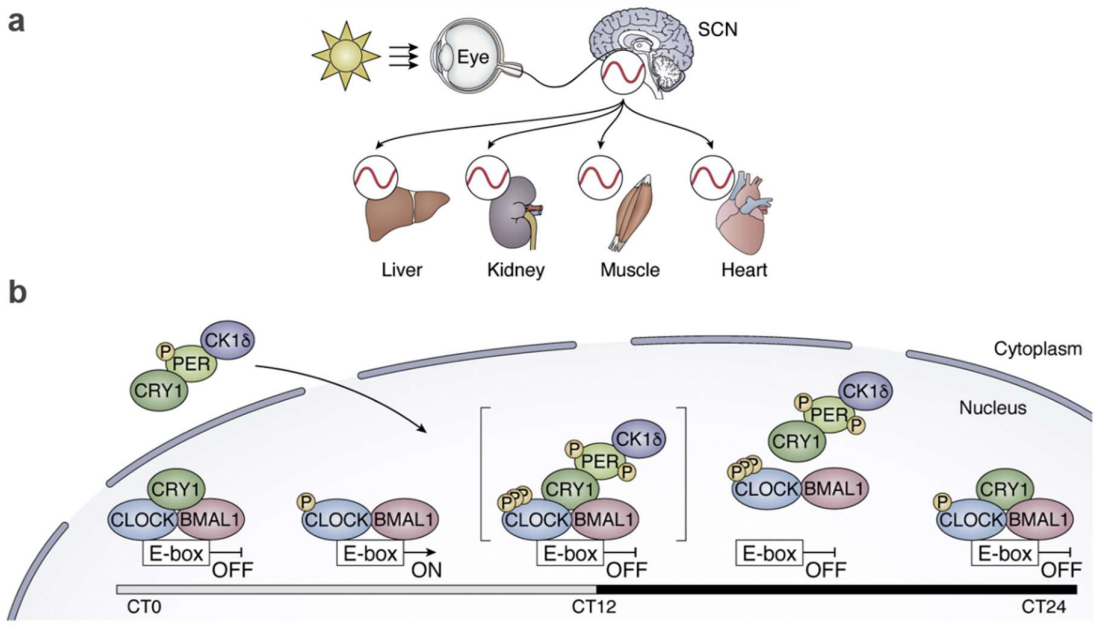


Figure 1-9. The molecular circadian clock. Schematic representation of circadian entrainment by the light. The master central clock in the suprachiasmatic nucleus (SCN) in the brain is entrained by neural inputs from photoreceptors in the eye. The master clock, in turn, aligns and maintains a coherent rhythmicity with peripheral clocks *via* hormone release, sympathetic innervation and several humoral factors (A). Transcription-translation feedback loop (TTFL) model for the mammalian circadian clock. The CLOCK–BMAL1 transcriptional activator binds to E-boxes binding sites within promoter regions to drive clock-controlled gene transcription of CRY, PER and some other clock-controlled genes (CCG). After protein synthesis in the cytoplasm, CRY1 is abundant and forms a complex with PER and CK1δ/CK2 that translocases into the nucleus. Once in the nucleus, PER-CRY bind to the CLOCK-BMAL1-E-box complex to inhibit their expression and so repress gene transcription. A next TTFL cycle begins when CRYs are degraded during the daytime and CLOCK-BMAL can activate again the transcription factor of target genes (B). **Illustration adapted from Yang et al.** (Y. Yang et al. 2021).

1.3.1.3 Circadian Rhythmicity of Immune Cell Trafficking

Multiple physiological aspects and key functions of the immune system are under the control of circadian clock genes: Trafficking of immune cells between the blood and peripheral organs (Scheiermann et al. 2018; 2012; Scheiermann, Kunisaki, and Frenette 2013), the daily influx and outflow

of progenitors and inflammatory cells between the bone marrow and the blood circulation (Maria Casanova-Acebes et al. 2018; María Casanova-Acebes et al. 2013), host-pathogen interactions and the activation of innate and adaptive responses –involving hormones production and cytokines release (Haus and Smolensky 1999)–, all seem to function in a powerful time-of-day manner. Several hematopoietic cell lineages, as well as most mature innate immune populations, including monocytes, macrophages (Keller et al. 2009), mast cells, neutrophils, eosinophils and NK cells (Boivin et al. 2003), possess intrinsic clocks that regulate the spatial-temporal function of all these cell types, such as their phagocytic activity or their cytokine release capacity. For example, in the absence of the clock protein PER1, splenic NK cells seem to maintain a normal migratory rhythmicity but show altered rhythmic expression of IFN-γ, perforins and the granzyme B. Leukocyte trafficking in the blood circulation under steady state conditions, as well as leukocyte migration into peripheral organs have been described as mechanisms of normal immunosurveillance whose main objective would be maximizing possible encounters within the host, between the immune system's patrolling cellular compartment and potential invading pathogens (Sigmundsdottir and Butcher 2008; von Andrian and Mackay 2000; Massberg et al. 2007). The release and homing of HSCs and hematopoietic stem progenitor cells (HSPCs) between the bone marrow and the blood circulation are intricately linked and exhibit robust and daily circadian fluctuations; orchestrated by the molecular clock. HSCs, HSPCs and mature immune cells –mostly all leukocyte populations, with presumably the exception of CD8+ T cells (Dimitrov et al. 2009; Bollinger et al. 2011)– are released from the bone marrow, peak in the circulation at the beginning of the resting phase (i.e., morning times in the mice) (Méndez-Ferrer et al. 2008) and find its lowest peak in circulation at the beginning of the active period (i.e., night times in the mice) (Haus and Smolensky 1999; Haus et al. 1983). Conversely, the beginning of the active period also marks the phase when leukocytes are predominantly recruited and transmigrated into tissues, in apparent anti-phase with leukocyte numbers in circulation. Furthermore, the release of HSPCs from the bone marrow depends on local sympathetic innervation, which in turn seems to also be regulated by core genes of the molecular clock. Scheiermann et al., showed that circadian HSC trafficking is actually orchestrated by the central nervous system through rhythmic noradrenaline secretion from nerve terminals in the sympathetic nervous system, in collaboration with the activation of the ß2- and ß3-adrenergic receptor, degradation of Sp1, and downregulation of the CXC-chemokine ligand 12 (CXCL12) (Scheiermann et al. 2012). Indeed, circulating HSCs and progenitor cells oscillate in anti-phase with respect to the expression of both CXCL12 (Méndez-Ferrer et al. 2008) –a major retention factor for HSCs in the bone marrow microenvironment–, as well as of its major receptor CXCR4, on HSPCs (Lucas et al. 2008). As a validation, ablation of ß2- and ß3-adrenergic receptors resulted in dysregulation of leukocyte trafficking into tissues. Therefore, the central nervous system can directly regulate the function of a stem cell

niche in peripheral tissues. Furthermore, a more recent study suggested that the cell-intrinsic clock is even sufficient to maintain the rhythmic migration pattern into tissues, of leukocyte populations. Zhao *et al.,* showed that in humanized mice, human and mouse leukocytes maintained anti-phased oscillatory patterns while in circulation, reproducing the trafficking pattern previously observed and that is consistent with their natural host species (Zhao et al. 2017).

Moreover, leukocyte extravasation into peripheral tissues is not only under clock control, but also regulated by the rhythmic expression of pro-migratory factors, such as the expression of CCL2 by endothelial cells (Winter et al. 2018). Diurnal regulated expression of adhesion molecules, such as ICAM-1 or VCAM-1 also mediates circadian leukocyte recruitment (Frenette et al. 1996). On the other hand, humoral components of the blood –complement and contact cascade proteins, naturally occurring antibodies or pentraxins, among others– seem to display opposite rhythms in comparison to cellular trafficking: Glucocorticoids levels (cortisol in humans and corticosterone in mice), epinephrine, norepinephrine, and several pro-inflammatory cytokines (TNF-α, IL-1β) peak during the onset of the active phase (Haus and Smolensky 1999; Haus et al. 1983).

To follow up, we will narrow down our point of view from the general leukocyte trafficking to just one population from the innate system: the neutrophil. In particular, circulating neutrophils undergo phenotypic changes from the time they are released from the bone marrow to their disappearance from the circulation and clearance by tissue-resident macrophages in their "fated-organs", under steady state conditions. Neutrophils are normally eliminated in the spleen, liver and the bone marrow (Furze and Rankin 2008b). This natural phenotypic shift in neutrophils is referred to as neutrophil aging and it adjusts to normal diurnal cycles (María Casanova-Acebes et al. 2013). In humans, neutrophils exhibit diurnal fluctuations in the expression of several markers: CD11a, ICAM-1, L-selectin, and CXCR4 (Niehaus et al. 2002). In mice, rhythmic expression of PSGL-1, L-selectin, CD11a, CD29, CXCR2 and CXCR4 markers has been reported as well (He et al. 2018). Murine neutrophils freshly released from the bone marrow at the beginning of the active period are phenotypically defined as $CXCR2^{high}$, $CD62L^{high}$, $CXCR4^{low}$. Due to their relatively short lifespan, neutrophils age over the course of a day in blood and young neutrophils transit into a $CD62L^{low}$ $CXCR4^{high}$ aged phenotype during daytime (José M Adrover et al. 2019; D. Zhang et al. 2015; Jose M Adrover et al. 2020). In that way, circulating neutrophils possess an intrinsic program controlled by the CXCR2 receptor and the circadian rhythm, that allows for modifications of the neutrophil proteome that leads, among other things, to a reduction of granule protein content and a reduced NET formation capacity in certain tissues such as the lungs. Other cell markers, including CD49d, TLR4, ICAM-1, CD11c, CD24 or CD45 (Pick et al. 2019), as well as transcriptional properties, nuclear morphology, granularity and cell size, also change diurnally (María Casanova-Acebes et al. 2013; D. Zhang et al. 2015). All these changes tag aged neutrophils for clearance

into tissues (He et al. 2018; María Casanova-Acebes et al. 2013; Casanova-Acebes et al. 2018). Early night represents the time of the day when neutrophil homing to the bone marrow shows its peak and when aged neutrophils can hardly be detected in blood, suggesting they have been recruited into peripheral tissues. Finally, in the bone marrow, tissue-resident macrophages phagocyte the aged neutrophil population and modulate the hematopoietic niche, allowing for the next round of cyclical release of HSPCs (María Casanova-Acebes et al. 2013; Casanova-Acebes et al. 2018). Therefore, macrophages represent a key circadian regulator of niche cell activity in the neutrophil homing process, that can sense oscillatory neutrophil populations in blood and indirectly modulate the neutrophil numbers by regulating the HSC niche.

The circadian-regulated recruitment of leukocytes into peripheral tissues has its peak during the active phase of the organism, as already described. This mechanism may be a way to replenish tissue-resident leukocyte populations, and be ready to fight potential threats –most likely to be prevalent and maximal during an organism´s active phase– and so maintain the immunosurveillance of the body. In that way, the immune system would have evolutionary adapted leukocyte trafficking to rhythmic bacterial encounters and foreign antigens inputs during the active times of the day as a mechanism that would ultimately increase the organism's own survival.

1.3.1.4 Impact of Circadian Rhythms on NETosis

It is only recently that novel scientific literature has tried to unravel and dived into the understanding of the temporal regulation of NET release. Nowadays it seems clear that neutrophils can be categorized into different subpopulations or cellular subsets depending on their diverse capacity to elicit different immunological functions (such as the previously described N1 and N2 neutrophil subpopulations in cancer) or display a differential expression of membrane surface markers. Not all neutrophils are equally prone to release NETs: Variability is present across different species, different organs and tissues, and different physiological states of the organism. Interestingly, Adrover et al. showed that the functional efficiency of released NETs varies depending on the neutrophil's maturation stage. Authors of the study demonstrated that young neutrophils ($CXCR2^{high}$, $CD62L^{high}$) in steady state, freshly released into circulation, displayed a higher proteome content and augmented NET-forming capacity compared to aged neutrophils ($CXCR2^{low}$, $CXCR4^{high}$) (Jose M Adrover et al. 2020). This mechanistic study demonstrated that autocrine signals delivered via CXCR2 can cast a progressive degranulation process in neutrophils while these cells are in circulation. Murine neutrophils, mobilized into the blood at the beginning of the night, would progressively reduce the content of their cytoplasmatic primary granules and so, by daytime their capacity to release detrimental and potentially pro-inflammatory NET structures would be reduced, which under steady state conditions and in the absence of an insult,

assures host safety. This gradual loss of granule contents, follows clear circadian oscillations and is been suggested as a neutrophil-inner mechanism subjected to the core circadian machinery. Ultimately, it can predict that NET formation would critically depend as well on the time of day. On the other hand, Zhang et al. observed that, under conditions of sepsis, the neutrophil aging process can change some properties within these cells. Daily aged neutrophils seem to represent a pro-inflammatory subset with specific functions that include a higher trans-migration capacity and a higher phagocytosis activity, and, in mice, aged neutrophils seem to be the ones exhibiting an enhanced NET formation and ROS production capacity, and are, therefore, cells more likely to initiate an inflammatory response (Uhl et al. 2016; D. Zhang et al. 2015).

1.3.2 Nutritional Regulation of NETosis

Circadian synchrony of peripheral cells is sustained by a network of parameters involving neuronal signalling, secretion of hormones and metabolic cues, all of them entrained by rhythmic feeding behaviours. Although light is the main entrainment factor for the SCN, feeding-regulated metabolic cues are now recognized as being pivotal to the regulation of peripheral clocks. In general, mice with global deletions of the main clock genes –including BMAL, PER1 and/or PER2, CRY1 and CRY2 or REV-ERBA– have an impaired clock machinery and display altered daily fasting-feeding cycles and dampened day–night variations of food intake.

1.3.2.1 Food as a Circadian Entrainment Factor

An organism's daily feeding schedule entrains the circadian clocks in multiple brain regions and most peripheral organs and tissues (Schibler 2009; Schibler et al. 2015), while reciprocally, feeding patterns are as well influenced by the master clock in the SCN (reset by daily light-dark cycles) and secondary brain clocks (reset by feeding time *via* hormonal, nutrient and visceral cues). This hierarchically organized system of circadian oscillators helps synchronizing daily circadian parameters, behavioural patterns and the host's physiological mechanisms accordingly to the moments of the day when food is most likely to be encountered. That moment mostly overlaps with the active phase of an organism; that is, a period of the day when food consumption is active, and energy stores are replenished and mobilized. All this is known as food-anticipatory behaviour and it exhibits some canonical properties of circadian clock control (Boulos, Rosenwasser, and Terman 1980; Mistlberger 1994; F. K. Stephan 2002; M. Stephan 2002). The entrainment and synchronization of peripheral and central clocks to the daily rhythm of food intake is accomplished by multiple metabolic- and feeding-related signals that modulate some arms of the circadian clock, known as food-entrainable oscillators

(FEOs), that are independent of the SCN activity. FEOs are present in these peripheral organs and tissues, such as the stomach, the liver or the pancreas, and can be modulated by the input of meal-associated nutrients (e.g., glucose, lipids, ketone bodies) and the consequent release of several metabolic hormones, such as circulating ghrelin, corticosterone (Balsalobre et al. 2000), or insulin levels (Yamajuku et al. 2012; Tahara et al. 2011; Crosby et al. 2019). As tissues become specialized in their function, the relevance of particular *zeitgebers* and the differential responsiveness of the tissue to environmental cues need to be aligned accordingly, so tissues and organs can *anticipate* their physiological expected task. For example, in metabolically active organs such as the liver, regularly constant mealtime intake is a key process in setting synchronization of the several metabolic processes that take part in it. Among peripheral organs, the liver clock phase shifts the most rapidly (a few days) in response to novel feeding patterns, such as time-restricted feeding (Damiola et al. 2000); in comparison to the heart and the lungs, that might take up to one week to become entrained. Hepatocytes are considered FEOs whose produced signals in response to food can propagate food entrainment into adjacent tissues. Interestingly, it has been reported that a large portion of the transcriptome in liver cells exhibits 24-hour rhythmicity (Kornmann et al. 2007).

A mistimed feeding pattern (that is, when feeding occurs during unusual times such as the resting phase, a period of the day when mostly sleep, fasting and energy storage occurs) can desynchronize and shift the circadian phase of peripheral organ clocks. Intriguingly, while the master clock in the SCN cannot be completely reset by a mistimed feeding schedule and is not sensitive to time-restricted feeding conditions (Stokkan et al. 2001; Damiola et al. 2000), it can yet respond and be disturbed by metabolic cues associated with unbalanced diets such as a high-fat diet (HFD), long-term fasting conditions or calorie restriction, and so promote deleterious effects on metabolic health (Stokkan et al. 2001; Hara et al. 2001; Schibler, Ripperger, and Brown 2003; Bray et al. 2013; Crosby et al. 2019). For example, high-fat diets have been reported to prolong the period of circadian behaviours, a ketogenic diet advances the onset of locomotor activity rhythms, and a high-salt diet seems to advance the phase of peripheral molecular clocks (Oike 2017; Yokoyama et al. 2020). Particularly, the liver circadian clock is presumed to be the most sensitive peripheral clock to feeding rhythms: Short-term HFD for the on-set of 1-week has been reported to phase advance the liver clock by 5 hours (Pendergast et al. 2013), and long-term HFD was shown to dampen hepatic clock gene expression (Satoh et al. 2006; Eckel-Mahan et al. 2013). Synchronization of circadian clocks by food thus appears to involve both general and tissue-specific signals that align circadian physiology with feeding rhythms.

1.3.2.2 Impact of Food Consumption on NETosis

Rhythmicity of the immune response is now clearly intertwined with rhythmic metabolic processes within an organism, and both factors influence each other with respect to circadian timing. Briefly, neutrophils have been for a long time considered as transcriptionally inactive cells that mainly depend on the glucose metabolism and the glycolytic metabolic pathway to modulate the variety of their cellular functions. Indeed, neutrophils respond to diverse stimuli by enhancing the uptake of glucose. Now, recent studies have challenged this view and have managed to identify various metabolic intermediates in the neutrophil cytosolic compartment that could only be synthesized by different metabolic routes beyond glycolysis, and that seem sufficient to meet the neutrophil energetic requirements (Rodríguez-Espinosa et al. 2015), suggesting metabolic plasticity in the neutrophil population. Different metabolic routes including Krebs cycle, oxidative phosphorylation (OXPHOS), pentose phosphate pathway (PPP) and the fatty acid oxidation (FAO) pathways are being recognized to fulfil the energetic, biosynthetic, and functional requirements of neutrophils (Kumar and Dikshit 2019), which challenges the commitment of these cells only to glycolysis. Moreover, neutrophils express a variety of receptors through which they can uptake and respond to a variety of nutrients, such as carbohydrates (by the expression of glucose transporter (GLUT)-1, GLUT-3 and GLUT-4), proteins and aminoacids (by the expression of glutamine transporters such as the alanine serine cysteine transporter-2 (ASCT-2), a neutral aminoacid transporter), and cholesterol, fatty acids and lipids (by the expression of cholesterol efflux transporter, free fatty acid receptor 1 (FFAR-1/GPR40) and FFAR-2/GPR43, or the LDL-receptor) for energy production (Futosi, Fodor, and Mócsai 2013; Maratou et al. 2007; Alvarez-Curto and Milligan 2016; Kumar and Dikshit 2019). Interestingly, some studies in humans have revealed that the postprandial phase after a meal consumption, characterized by an increased concentration in the glucose and triglyceride levels, is accompanied by a significant increase in the absolute neutrophil counts in circulation. After an oral fat load, or a mixture of fat and glucose load, researchers observed that neutrophil numbers showed its peak in circulation between 1-2 hours following meal consumption, and that the increase remained stable for at least 4 hours. The activated status of neutrophils seems to be also incremented upon a mixed bolus of glucose and fat. Neutrophils do not seem to be mobilized under controlled-fasted conditions (A. J. H. H. M. van Oostrom et al. 2003; A. J. h h m Van Oostrom et al. 2003).

Nonetheless, not many available scientific studies have unravelled yet the impact of daily feeding upon NET release. Moorthy et al. have recently showed that the neutrophils of HFD-fed BALB/c mice are more prone to spontaneous NET formation in the lungs during the course of a model of influenza virus-induced pneumonia, in comparison to neutrophils derived from low-fat diet (LFD)-fed controlled animals (Moorthy et al. 2016). Another recent study has revealed that feeding mice an obesogenic diet

induces neutrophil recruitment to adipose tissue and increases NET formation due to higher oxidative stress levels, which ultimately can lead to endothelial dysfunction. Consequently, authors concluded that targeting NETosis –through inhibition or degradation– would help restoring endothelium-dependent vasodilation, vascular damage and recover endothelial dysfunction, even when lacking improvement of other cardiometabolic complications (H. Wang et al. 2018). Some other studies have revealed that under the influence of typical diabetic microenvironment conditions such as hyperlipidemia and hyperglycemia, NET formation can also be modulated. Hyperglycemia mimics a state of constitutively active pro-inflammatory condition that primes neutrophils and activates NETosis. That way, NETs seem to be influenced by the glucose homeostasis (Rodríguez-Espinosa et al. 2015), in what seems to be an IL-6 dependent-mechanism that creates a condition of "NETosis exhaustion". NET release is compromised and reduced in response to a secondary stimuli (i.e., LPS), which leads to loss of normal immunological balance and makes diabetic subjects susceptible to infection (Joshi et al. 2013). Wong et al. corroborated that neutrophils isolated from type 1 and type 2 diabetic humans and mice were primed to produced more NETs, and that some NET-associated molecules (e.g., PAD4, citH3) were found upregulated in skin wounds in mice. The higher ratio of NET release in diabetic mice impaired and delayed secondary wound healing. Wound healing was improved and accelerated in Padi4$^{-/-}$ mice or by pharmacologically inhibition or cleavage of the NET structures, which can potentially help reducing NET-driven chronic inflammation in diabetic conditions (S. L. Wong et al. 2015). Still, it is worth emphasizing that none of these studies have been conducted in a disease-free context: Metabolic disorders –displaying common factors such as hyperglycemia, hypertriglyceridemia, dyslipidemia, and hypertension– are associated with a chronic low-grade inflammation driven primarily by the activation of several cells from the innate immune system (Wellen and Hotamisligil 2005; Gregor and Hotamisligil 2011). That so, it would be necessary to properly understand the metabolic requirements associated to NETosis and evaluate the impact upon feeding under physiological conditions on NET release.

1.3.3 Microbial Regulation of NETosis

Microbiome projects have launched worldwide through the years with the goal to elucidate the numbers, identify the populations and reach full knowledge of the microbial cells and genes that colonize a human body. The microbiome is, all in all, essential for human development, immunity and nutrition.

1.3.3.1 Commensal Intestinal Microbiome

To our particular interest here, some recent studies have revealed that the intestinal commensal microbiota exhibit circadian rhythmicity. The microbiome undergoes diurnal oscillations in terms of numbers -mostly peaking at the onset of the mouse active phase-, populations' distribution and composition, physical location alongside the intestinal epithelium, and activity -observed in terms of metabolome patterns- (Leone et al. 2015; Liang, Bushman, and FitzGerald 2015; Thaiss et al. 2014; Zarrinpar et al. 2014). These compositional and functional diurnal oscillations has been suggested to be potentially driven by the timing of food intake, alongside the composition of the diet (Leone et al. 2015; Zarrinpar et al. 2014; Thaiss, Levy, and Elinav 2015), and can be ablated by jet-lag protocols (repeated shifts in timing of light–dark cycles) or antibiotic treatment. For example, normal commensal microbiota rhythmicity is lost in mice with a double deletion of the Per1/2 genes. Interestingly, dietary restrictions such as time-restricted feeding patterns have been observed to restore the normal microbiome rhythmicity in these knockout mice. In addition, restriction of food intake to the light phase (resting phase) in mice, led to an inverted rhythm of the commensal microbiota compared to ad libitum feed animals that have a normal clock gene expression, which grants a crucial role for nutrient intake as a key mediator for the oscillations observed in commensal bacteria (Thaiss et al. 2014; Zarrinpar et al. 2014).

The preservation of a rhythmic and physiological modulation of the commensal microbiome is important for the regulation of several physiological processes in an organism, such as maintenance of the mucosal structure, metabolic processing of nutrients and xenobiotics, and regulation of a rhythmic immune response. Therefore, breaches of the natural microbiome rhythmicity and disruption of the host-gut microbiome crosstalk mechanisms, can influence the host homeostasis and impact on circadian clock-regulated activities in peripheral tissues (Leone et al. 2015; Mukherji et al. 2013; Murakami et al. 2016), particularly at the intestinal and hepatic level. This would, ultimately, increase susceptibility to disease (Thaiss, Levy, et al. 2016). As an example, alterations of the physiological gut microbial composition results in a process called dysbiosis that has been largely associated with the development of some manifestations of the metabolic syndrome, such as obesity and other cardiovascular conditions (Thaiss et al. 2015; Voigt et al. 2014; 2016).

1.3.3.2 Impact of Commensal Microbiota on NETosis

Recent evidence demonstrates that the symbiotic cooperation between the intestinal commensal microbiome and the gut epithelium is critical in maintaining a condition of immune homeostasis in the intestine, and a key regulator in modulating immune rhythmicity in the host. Based on the well-known

role that neutrophils play in coordinating an efficient immune response against invading pathogens and the large repertoire of immune sensors –aforementioned PRRs– with which these cells can recognize microbial products, it is not surprising that the microbiome indeed dictates multiple physiological aspects of neutrophil biology, and reciprocally, that the innate immune system mediates the symbiotic interactions between the host and its commensal microbiota. Along history, a co-evolution between pathogens and the host immune populations has been suggested to have emerged. Intriguingly, this interaction seems to be orchestrated by the circadian clock and entrainment cues derived from the microbiota that can activate intestinal TLRs. Rhythmic release of metabolites derived from the intestine commensal pathogens have been associated with an intimate regulation of immune development: One of the most striking examples is the commensal microbiota as a regulator of steady-state granulopoiesis by mediating the production of G-CSF. Indeed, germ-free mice have a significant reduced development of the myeloid cell compartment in the bone marrow (Balmer, Schürch, et al. 2014) compared to animals with a full microbiome community. Some other studies associated germ-free mice with a profound neutropenic condition (Bugl et al. 2013), pointing again towards the potential modulation of granulopoiesis by microbial populations. Microbiota-derived peptidoglycans can indeed regulate the lifespan of neutrophils (Hergott et al. 2016) and activate these cells to cast an efficient immune response against infections in what seems to be a NOD-1 dependent mechanism (Clarke et al. 2010). Some bacteria, through a Th17-dependent mechanism, have been reported to prime neutrophils. Neutrophil activation was ultimately linked with increased barrier permeability, systemic PAMPs dissemination, and activation of the TLR/MyD88-downstream signals. Moreover, microbiota-derived TLR agonists (e.g., endotoxins), when present in the bloodstream, have been reported to drive diurnal-associated phenotypic changes in neutrophils, associated with the physiological neutrophil aging process (D. Zhang et al. 2015). TLR ligands can also mediate the differentiation of B-cell helper neutrophils in the marginal zone of the spleen (Puga et al. 2011). Interestingly, antibiotic-induced disruption of the normal intestinal microbiome in mice dramatically reduced the number of aged neutrophils in circulation. Upon diminution of aged neutrophils, ratios of NET formation were also diminished and, altogether, this context seemed to confer protection during chronic inflammatory conditions.

Consequently, these emerging concepts might have spotlighted NET-driven inflammation as a potential therapeutical target in clinical studies. However, some other studies showed that depletion of commensal bacteria resulted, on the contrary, in an elevated amount of released NET structures within a context of ischaemia/reperfusion injury (Ascher et al. 2020), which suggested a potential inhibitory or tolerogenic action of the gut microbiome on neutrophil function. Antibiotic-mediated ablation of the microbiota disrupts immune rhythmicity, but, interestingly, it can be restored by

administration of LPS. Although there is increasing evidence recently supporting the idea of host microbiome-derived metabolites influencing NET formation mechanisms, this still remains a big open field ripe for exploration. Noteworthy, the regulation of NET release might be as well dictated as a neutrophil intrinsic mechanism dependent on the circadian clock regulation and the diurnal oscillations of the commensal microbiota. As TLR expression in neutrophils follows a circadian pattern, the coordinate expression of these receptors and the daily influx of microbial products may result in neutrophil priming and susceptibility to NETosis, potentially, also in a circadian way.

Research Rational

NETs as mediators of immune homeostasis

Neutrophils are innate immune cells specialized in early defense mechanisms against pathogens. In the last years, increasing evidence supports an alternative role of neutrophils in maintaining body homeostasis as active and functional players within healthy tissues. Earth rotation instructs circadian rhythms, which in turn govern an array of biological functions (e.g., immune response) and physiological parameters. The circadian behavior of organisms makes the likelihood of being exposed to hazard molecules, infectious insults and potential dangers to vary along the day. Interestingly, even in the absence of pathology, massive waves of neutrophils (estimated to be in the range of 10^{10} cells per day) are released from the bone marrow into the circulation in a diurnal fashion. After what has been classically considered an unproductive patrolling, neutrophils are rhythmically transmigrated into peripheral tissues just to be recycled by tissue-resident macrophages. Accumulating evidence suggest now that these to-be-cleared neutrophils could actually be functional within healthy tissues before their elimination. Therefore, the massive daily energetic expenses that are undoubtedly required to produce "needless" cells, would have an evolutionary reason to still occur in homeostasis. Notably, the diurnal fluctuations of neutrophils in the bloodstream and their recruitment into peripheral tissues in steady state correlate with the active phase of an organism. Within this scenario, timing of the immune function is essential in granting immune cells synchronization of their defense mechanisms with the higher threatening phases of the day, increasing immunosurveillance and ensuring survival. Hence, the complex regulation of neutrophil numbers in the steady-state may find a reason in the permanent need to eliminate pathogens that can potentially break through mucosal surfaces and primary physical barriers in the body, and that could entail a potential risk.

An illustrative example of an organ populated by neutrophils in the steady-state is the lung. Neutrophils appear to be strategically retained in the lung vascular niche, as this organ comprise one of the primary physical and immunological body barrier entries for invading pathogens and air-borne pollutants constantly incoming from the outside environment. Whether similar strategies are adopted by neutrophils in other barrier tissues is not known yet. NETosis is a key neutrophil-specific antimicrobial function that enables neutrophils to trap pathogens and prevent their spread within an organism. Notably, NETs accumulate in body cavities (e.g., ducts and vessels), where they offer surveillance and act as physical and chemical barriers against pathogen invasion. Here, we hypothesized that NET formation could be instrumental in containing the incoming waves of pathogens, gut-derived molecules, dietary-associated metabolic products, as well as PAMPs and DAMPs that periodically challenge a system. NET formation is evaluated to define the neutrophil capacity to contain the systemic spread of these diurnal waves of incoming insults during steady state.

This PhD research project aimed to provide basic insight into 1) the role of NETosis during tissue homeostasis as a potential contributor to the immune barrier function in peripheral tissues, 2) whether NETs contribute to immunosurveillance and ensure a balance between immune-tolerogenic responses against non-pathogenic insults (i.e., commensal microbiota) and the casting of an effective immune response against pathogens, and 3) how NETs shape the immune response by cooperation with other immune populations. Inherent to these questions, the following objectives are addressed in this PhD thesis:

1. Spatial characterization of NET release in barrier- (physical and immunological) and non-barrier organs, during the resting and active phases of an organism over the course of the day.
2. Examination of the external factors and intrinsic-neutrophil-dependent factors that regulate the physiological release of NETs. In particular:
    - Influence of the circadian clock and diurnal rhythms.
    - Influence of dietary patterns and the diet nutritional composition.
    - Influence of the commensal microbiome and pathogen-associated molecules.
3. Assessment of NET functionality as immune barrier players in homeostasis.

Materials & Methods

Table 3-1. Used Buffers, Reagents & Solutions.

Buffers & Solutions	Composition
Hank´s Buffer	1x Hank's buffered salt solution (HBSS), 0.06% BSA, 0.5 mM EDTA, pH 7.4
Blood Lysis Buffer	155mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.4
Syringe Coating Solution	0.5mM EDTA in 1x PBS
Flow Cytometry Buffer	2% mouse serum, 2% rabbit serum, 2% human serum, 2% BSA in 1x PBS
Flow Cytometry Antibody Staining Solution	Target antibodies diluted (0.1-0.2%) in 1:1 Hanks:FACS Buffer
Percol Solution	36% Pure Percol in 1x HBSS
Organ Digestion Buffer	1,25mg/mL Liberase + 1U/mL DNase I in Hank´s Buffer 0,25 U/mL Collagenase IV + 1U/mL DNase I in Hank´s Buffer
Fixation Buffer	4% PFA in 1x PBS
Blocking Solution	5% normal horse serum in 1x PBS
PBS-Tween Solution	0.02% Tween20 in 1xPBS
IHC Antibody Staining Solution	Target antibodies diluted (0.1-0.5%) in Blocking Solution
DAPI Staining Solution	0.01% DAPI in 1x PBS

Table 3-2. Used KITs

KIT	Application	Supplier
EndoLISA®	Endotoxin Detection Assay	bioMerieux Inc.
LEGENDPlex™	Soluble Analytes Quantification Immunoassay	BioLegend®

Table 3-3. Primary Antibodies

Antigen	Conjugate	Clone	Host	Target	Dilution	Application	Supplier
B220	APC-Cy7	RA3-6B2	Rat	Mouse	1:200	FC	BioLegend
CD115	BV395	T38-320	Rat	Mouse	1:200	FC	BD Biosciences
CD115	APC	AFS98	Rat	Mouse	1:200	FC	eBioscience
CD115	PE	AFS98	Rat	Mouse	1:200	FC	eBioscience
CD11b	APC-AF700	M1/70	Rat	Mouse	1:200	FC	eBioscience
CD11b	PerCP Cy5.5	M1/70	Rat	Mouse	1:200	FC	eBioscience
CD11b	BV737	M1/70	Rat	Mouse	1:200	FC	BD Biosciences
CD11b	BV395	M1/70	Rat	Mouse	1:200	FC	BD Biosciences

CD19	APC	6D5	Rat	Mouse	1:200	FC	BioLegend
CD3e	FITC	17A2	Rat	Mouse	1:200	FC	BioLegend
CD3e	FITC	145-2C1	Rat	Mouse	1:200	FC	eBioscience
CD3e	AF488	17A2	Rat	Mouse	1:100	IF	BioLegend
CD31	AF647	MEC13.3	Rat	Mouse	1:100	IF	BioLegend
CD4	APC	RM4-5	Rat	Mouse	1:100	IF	eBioscience
CD4	PE	RM4-5	Rat	Mouse	1:100	IF	eBioscience
CD4	PerCP Cy5.5	RM4-5	Rat	Mouse	1:100	IF	BD Biosciences
CD45	SB600	30-F11	Rat	Mouse	1:200	FC	eBioscience
CD45	APC-Cy7	30-F11	Rat	Mouse	1:200	FC	BioLegend
CD68	-	KP1	Rat	Mouse	1:100	IF	eBioscience
CD68	AF488	FA-11	Rat	Mouse	1:100	IF	Abcam
CD68	AF647	FA-11	Rat	Mouse	1:100	IF	Abcam
CD8a	PE	53.6-7	Rat	Mouse	1:200	FC	eBioscience
CD8a	FITC	53.6-7	Rat	Mouse	1:200	FC	BioLegend
Collagen I	-	Polyclonal	Rabbit	Mouse	1:200	IF	Abcam
Collagen III	-	Polyclonal	Rabbit	Mouse	1:200	IF	Abcam
Collagen IV	-	Polyclonal	Rabbit	Mouse	1:200	IF	Novus Bio
dsDNA	-	35I9	Mouse	Mouse	1:100	IF	Abcam
F4/80	AF488	BM8	Rat	Mouse	1:200	IF	BioLegend
F4/80	AF647	BM8	Rat	Mouse	1:100	FC, IF	eBioscience
Histone H3	-	Polyclonal	Rabbit	Mouse	1:100	IF	Abcam
Histone H3 citrulline	-	Polyclonal	Rabbit	Mouse	1:100	IF	Abcam
IgM	APC-Cy7	11/41	Rat	Mouse	1:200	FC	eBioscience
IgM	PE	11/41	Rat	Mouse	1:200	FC	eBioscience
IgD	FITC	11-26D	Rat	Mouse	1:200	FC	eBioscience
IgD	PerCP Cy5.5	11-26D	Rat	Mouse	1:200	FC	eBioscience
Ly6C	APC	HK1.4	Rat	Mouse	1:200	FC	Invitrogen
Ly6G	APC	1A8	Rat	Mouse	1:100	FC, IF	BioLegend
Ly6G	PE	1A8	Rat	Mouse	1:200	FC	BD Biosciences
Ly6G	PE-594	1A8	Rat	Mouse	1:200	FC	BD Biosciences
Ly6G	BV395	1A8	Rat	Mouse	1:200	FC	BD Biosciences
Ly6G	BV737	1A8	Rat	Mouse	1:200	FC	BD Biosciences

MHC-II	BV510	M5/114.15.2	Rat	Mouse	1:200	FC	BD Biosciences
MPO	-	Polyclonal	Goat	Mouse	1:200	IF	R&D
NE	-	Polyclonal	Rabbit	Mouse	1:200	IF	Abcam
NK1.1	PE	PK136	Mouse	Mouse	1:200	FC	eBioscience

Table 3-4. Secondary Antibodies

Host	Conjugate	Target	Dilution	Application	Supplier
Donkey	DL550	Anti-Goat	1:500	IF	ThermoScientific
Donkey	DL594	Anti-Goat	1:500	IF	ThermoScientific
Donkey	DL650	Anti-Goat	1:500	IF	ThermoScientific
Donkey	AF488	Anti-mouse	1:500	IF	ThermoScientific
Donkey	AF550	Anti-mouse	1:500	IF	ThermoScientific
Donkey	AF650	Anti-mouse	1:500	IF	ThermoScientific
Donkey	DL488	Anti-Rabbit	1:500	IF	ThermoScientific
Donkey	DL550	Anti-Rabbit	1:500	IF	ThermoScientific
Donkey	DL650	Anti-Rabbit	1:500	IF	ThermoScientific
Donkey	DL488	Anti-Rat	1:500	IF	ThermoScientific
Donkey	DL550	Anti-Rat	1:500	IF	ThermoScientific
Donkey	DL650	Anti-Rat	1:500	IF	ThermoScientific

Table 3-5. Instruments & Machinery

Instruments & Machinery	Supplier
BD LSRFortessa™ Flow Cytometer	BD Biosciences
Cytek® Aurora Northern Lights Full Spectrum Flow Cytometer	Cytek® Biosciences
ThermoMixer 5355 Comfort	Eppendorf™
Vortexer Shaker D-6012	NeoLab®
Mini-Centrifuge Sprout® Plus	Heathrow Scientific Sprout®
Mini Centrifuge 5424 R	Eppendorf™
Centrifuge 5804 R	Eppendorf™
Mastercycler EP Gradient S	Eppendorf™
NanoDrop® ND-1000 Spectrophotometer	Peqlab
Syringe Pump Programmable, PHD ULTRA™	Harvard Apparatus
Tissue Lyser LT	QIAGEN

Cryotom CM3050 S	ThermoFisher Scientific
Stereo Zoom Microscope OZL-44	Kern & Sohn
Inverted Microscope for Cell Culture DMi1	Leica Microsystems
Fluorescence Inverted Microscope DM500	Leica Microsystems
Inverted Microscope DMi8 S	Leica Microsystems
THUNDER Imager Tissue Microscope	Leica Microsystems
Spinning Disk Inverted Leica SP8X WLL Confocal Microscope	Leica Microsystems

Table 3-6. Softwares

Software	Version	Supplier
Image J/Fiji	2.0.0-rc-69/1.52p	Open-Source
Leica Application Suite X (LASX)	LAS X 5.0.2	Leica Microsystems
Huygens Professional	4.2.1p7	Scientific Volume Imaging
FACSDiva™ Software	v9.0	BD Biosciences
SpectroFlo® Software		Cytek® Biosciences
FlowJo™	V10.6.2	Becton Dickinson
GraphPad Prism	v.8.0 & v.9.0	GraphPad Software
Excel	16.54	Microsoft
NanoDrop®	ND-1000 Spectrophotometer	Peqlab
Syringe Pump, PHD ULTRA™	3.0.7	Harvard Apparatus
Mendeley Desktop	1.19.8	Mendely Ltd.

3.1 Mice

Mice have emerged over the past few decades as the preferred animal model for studying the immune system. Not least because of the high similarity between the human and murine immune systems, knowledge gained in mice can be transferred to humans. In the following animal experimental layouts, all animals referred to as wild type (WT) animals that have been used for this doctoral thesis were female C57BL/6J mice aged 8-to-12-weeks -otherwise indicated- and either purchased from Charles River Laboratories (Sulzfeld, Germany), Janvier labs (Le Genest-Saint-Isle, France) or internally bred from our own animal facility. Genetically modified animals were also used in this study. Briefly, 8-to-12-weeks-old $NE^{-/-}$, $PAD4^{-/-}$, $Ly6G^{Cre}Mcl1^{flox/flox}$, $Mrp8^{Cre}Bmal^{flox/flox}$ and $Albumin^{Cre}Bmal^{flox/flox}$ mice were used. Mice were always euthanized by 90 mg/kg Ketamine/10 mg/kg Xylazine overdose (Ketamine 10%, #N1617-03 WDT, Garbsen, Germany; Xylazine 2%, #PZN-01320422 Bayer Animal Health GmbH)

diluted in saline solution and injected intraperitoneally (i.p.). All animal experiments were approved by the local ethical committee for animal experimentation. A brief description of the used mice strains can be found below. Note that all technical data regarding mice strain generation has been transcribed from The Jackson Laboratory webpage (**jax.org**):

- **C57BL/6:** C57BL/6J is the most common used inbred laboratory mouse strain and the first one to have its whole genome sequenced. C57BL/6J mice are used in a wide variety of research areas including cardiovascular and developmental biology, diabetes and obesity, genetics, immunology and neurobiology research. C57BL/6J mice are also commonly used in the production of transgenic animals. Overall, C57BL/6J mice breed well, are long-lived, and have a low susceptibility to tumors. All animals and genetically modified mice used in this experimental project have the genetic background of the inbred strain C57BL/6J.
- **PAD4$^{-/-}$ KO:** These mice lack exons 9-10 of the peptidyl arginine deiminase, type IV (*Padi4*) gene. These exons contain part of the PAD4 active site, as well as four additional residues that are essential for Ca^{2+} binding. PAD4 is an enzyme that carries out the citrullination and deimination conversion of arginine to citrulline residues on histones. PAD4-mediated deimination of histone H3 and H4 is required for the formation of NETs. Neutrophils from PAD4$^{-/-}$ animals lack histone deimination PAD4-mediated activity and are, therefore, defective in NET formation (P. Li et al. 2010). PAD4 KO mice lose significantly more weight during influenza A infection but retain normal survival (Hemmers et al. 2011). Homozygous mice are viable and fertile.
- **NE$^{-/-}$ KO:** Neutrophil-expressed knock-out *Elane^{tm1Sds}* homozygous mice are viable, fertile and phenotypically normal in steady state. However, they have an increased susceptibility to sepsis, morbidity, and mortality following intraperitoneal injection of Gram-negative bacteria (Belaaouaj et al. 1998). Nevertheless, they don´t show an increased risk to spontaneous infection. Although neutrophil, T cell and macrophage migration to the site of infection is unaffected in homozygous mutant mice, neutrophils have impaired bactericidal activity. Further, homozygous mice treated with a broad-spectrum of inflammatory stimulus (zymosan) have impaired leukocyte firm adhesion and transmigration, as well as reduced pro-inflammatory cytokine production (Young et al. 2004). In breeding, the mice are healthy and fertile and show no pathological phenotype or distress.
- **Albumin^{Cre}Bmal1$^{Flox/Flox}$:** These animals were kindly provided by the group of Dr. Guadalupe Sabio at *Centro Nacional de Investigaciones Cardiovasculares* (CNIC), in Madrid, Spain. Albumin^{Cre} transgenic mice are useful for deletion of *loxP*-flanked genes in the liver. Here, the Cre gene is linked to the promoter for Albumin, a protein exclusively produced in the hepatic tissue. Originally, Bmal1$^{Flox/Flox}$ mice were crossed with Albumin^{Cre} mice obtained from the Jackson Laboratories, to create a Bmal1 liver-specific KO. This resulted in a deletion of Exon 8 of the Bmal1 gene in the hepatocytes,

producing a non-functional Bmal1 protein only in the hepatic compartment. Albumin^{Cre} mediated excision of the conditional Bmal^{Flox} allele does not affect the central circadian clock genes and these animals exhibit normal extrahepatic circadian physiologies (Johnson et al. 2014). Albumin^{Cre} Bmal$^{Flox/Flox}$ mice are reported to be indistinguishable from their wild-type littermates in body weight and blood glucose levels under *ad libitum* and fasted conditions (J. Li et al. 2018). Indeed, Albumin^{Cre} Bmal$^{Flox/Flox}$ exhibit normal feeding and locomotor activity patterns. In breeding, the mice are healthy and fertile and show no pathological phenotype. Albumin^{Cre} littermates were used as controls for all experiments.

- **Ly6G^{Cre} & Ly6G^{Cre}Mcl1$^{Flox/Flox}$:** Myeloid-specific deletion of the *Mcl-1* (myeloid cell leukemia 1) antiapoptotic protein in Ly6G$^{Cre/Cre}$ Mcl1$^{Flox/Flox}$ (Mcl1$^{\Delta Myelo}$) mice drives severe neutropenia, both in circulation and peripheral organs. Deletion of Mcl-1 does not affect other circulating leukocyte populations (e.g. lymphocytes, monocytes or eosinophils), as these immune cells do not rely on Mcl-1 for their survival. Surprisingly, Mcl1$^{\Delta Myelo}$ mice appear phenotypically normal, and their survival and life-span are mostly uncompromised both under specific pathogen-free (SPF) and conventional housing conditions. Mcl1$^{\Delta Myelo}$ mice are also able to breed in homozygous form. Mcl1$^{\Delta Myelo}$ mice are highly susceptible to systemic *Staphylococcus aureus* or Candida albicans infection, due to their defective capability to clear invading pathogens (Csepregi et al. 2018). In breeding, the mice are healthy and fertile and show no pathological phenotype.

3.1.1 Animal Housing

All animals were housed according to institutional regulations with *ad libitum* access to food and water, unless stated otherwise. All animals were housed under a 12-hour light/ 12-hour dark cycle (lights on at 7 am; lights off at 7 pm) in the Core Animal Facility "Central Laboratory Animal Facility (ZVH)" at the City Centre Campus of the Faculty of Medicine from the Ludwig Maximilians Universität München (LMU), in Germany and in the Core Animal Facility "Zentrale Tierexperimentelle Einrichtung (ZTE)" from the Westfälische Wilhelms-Universität Münster (WWU), in Germany. Animals were supervised by multiple care takers and veterinarians. Upon shipment, animals were given seven days to adjust to the new environmental setup before any experimental procedure was performed. All experiments were performed using age- and sex-matched groups. All animal procedures and experiments described in this PhD thesis were approved by the ministry of animal welfare of the region of Oberbayern (ROB) and the local authorities (LANUV) in Nordrhein-Westfalen (NRW), and performed in accordance with the German law of animal welfare.

Mice are nocturnal creatures that are mainly active during their dark phase. In laboratory animal housing, animal experiments are standardized to a 12 h light:12 h dark cycles in order to mimic a natural

day-night period. That so, the dark phase is standardized to 12 hours from 7p.m. to 7a.m, while the light phase lasts for another 12 hours from 7a.m. to 7p.m. Experiments assessing circadian rhythmicity use a specific timing nomenclature that is called the *Zeitgeber* time (ZT); here *zeitgeber* being a stimulus that provides a timing signal such as light onset. *Zeitgeber* time 0 (ZT0) describes the starting timepoint of the stimulus, here meaning that light is switched on inside the laboratory animal facility, which corresponds to the 7a.m. time of the day. All other times are measured in a 24-hour cycle relative to this stimulus. For example, in a cycle with 12 h light:12 h dark, ZT6 represents the middle of the light phase, ZT12 represents the onset of the dark phase –here starting 7p.m onwards–, ZT18 represents the middle of the dark phase and ZT24/0 represents the onset of the light phase once again. Oscillations that are rhythmic under these conditions will be termed *diurnal*, while oscillations that remain rhythmic under conditions where no *zeitgebers* are present, such as in constant darkness conditions, are termed *circadian*.

3.1.2 Genotyping

All mice bred in our animal facility were tail biopsied for genotypic analysis. All genotype analysis were performed by our lab technician Olga Schengel. Briefly, tail biopsies were incubated overnight at 56°C in 250 µL tissue lysis buffer supplemented with 0,2 mg/ml proteinase k solution for tissue digestion (Qiagen, Hilden, Germany) under constant shaking. Afterwards, DNA was isolated using a QIAxtractor kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For all analyzed genes, PCR reagent mixes were prepared (Table 3-7) containing either wildtype- or mutant allele-detecting primer pairs (Table 3-8). Specific PCR reaction programs were used for every analyzed gene (Table 3-9). PCR products were analyzed by gel electrophoresis with a QIAxcel Advanced System (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In every PCR reaction, wildtype and mutant material as well as water were included as positive and negative controls, respectively.

Table 3-7. PCR Reagent Mix

Reaction Component	Final Concentration
5X Green Gotaq Flexi buffer (Promega, Fitchburg, USA)	1X
25 mM MgCl2 (Sigma-Aldrich, St. Louis, USA)	2 mM
dNTPs (Sigma-Aldrich, St. Louis, USA)	0,2 mM
Forward Primer (Sigma-Aldrich, St. Louis, USA)	0,5 µM
Reverse Primer (Sigma-Aldrich, St. Louis, USA)	0,5 µM

GoTaq DNA polymerase (Promega, Fitchburg, USA)	0,05 U/µl
Genomic DNA (Promega, Fitchburg, USA)	200 ng/µl

Table 3-8. PCR Primer Pairs

Primer Name	Primer Sequence
NE common	5’ TGC ACA GAG AAG GTC TGT CG 3’
NE wildtype forward	5’ GGA ACT TCG TCA TGT CAG CA 3’
NE mutant reverse	5’ TGG ATG TGG AAT GTG TGC GAG 3’
Padi4 common	5’ CAG GAG GTG TAC GTG TGC A 3’
Padi4 wildtype forward	5’ CTA AGA GTG TTC TTG CCA CAA G 3’
Padi4 mutant reverse	5’ AGT CCA GCT GAC CCT GAA C 3’
Mcl1-Flox forward	5’ GGT TCC CTG TCT CCT TAC TTA CTG TAG 3’
Mcl1-Flox reverse	5’ CTC CTA ACC ACT GTT CCT GAC ATC C 3’
LysM-Cre common forward	5’ CTT GGG CTG CCA GAA TTT CTC 3’
LysM-Cre wildtype reverse	5’ TTA CAG TCG GCC AGG CTG AC 3’
LysM-Cre mutant reverse	5’ CCC AGA AAT GCC AGA TTA CG 3’
Bmal1 forward	5’ ACT GGA AGT AAC TTT ATC AAA CTG 3’
Bmal1 reverse	5’ CTG ACC AAC TTG CTA ACA ATT A 3’

Table 3-9. PCR Reaction Programs

Step	Temperature (°C)	Time	Reaction
NE			
1	94	2 min	Initial Denaturation
2	94	20 sec	Denaturation
3	65	15 sec	Primmer Annealing
4	68	10 sec	Polymerization
Repeat Steps 2-4 for 10 cycles			
6	94	15 sec	
7	60	15 sec	
8	72	10 sec	
Repeat Steps 6-8 for 28 Cycles			
10	72	2 min	Final Extension
11	21	5 min	
Padi4			
1	95	5 min	Initial Denaturation

2	95	30 sec	Denaturation
3	59	30 sec	Primmer Annealing
4	72	30 sec	Polymerization
Repeat Steps 2-4 for 39 cycles			
6	72	5 min	Final Extension
7	21	5 min	
Mcl1-Flox			
1	95	5 min	Initial Denaturation
2	95	30 sec	Denaturation
3	60	30 sec	Primmer Annealing
4	72	30 sec	Polymerization
Repeat Steps 2-4 for 35 cycles			
6	72	7 min	Final Extension
7	4	5 min	
LysM-Cre			
1	94	3 min	Initial Denaturation
2	94	30 sec	Denaturation
3	60	30 sec	Primmer Annealing
4	72	50 sec	Polymerization
Repeat Steps 2-4 for 37 Cycles			
6	72	3 min	Final Extension
7	21	5 min	
Bmal1			
1	94	3 min	Initial Denaturation
2	94	30 sec	Denaturation
3	60	60 sec	Primmer Annealing
4	72	60 sec	Polymerization
Repeat Steps 2-4 for 35 Cycles			
6	72	5 min	Final Extension
7	21	5 min	

3.1.3 DNAse I Treatment

In order to discern the NET-origin of the previously identified NET-like structures, mice were intraperitoneally injected so that the DNA-backbone structure could be digested, with 10U of DNAse I (Sigma-Aldrich) diluted in 200µL saline solution at 8am or 8pm, 1 hour before animals were sacrificed. Bovine pancreatic deoxyribonuclease I (DNase I) is a double-strand specific endonuclease that degrades DNA. It is a DNA minor grove-interacting nuclease, which shows relatively low specificity.

3.1.4 BB-Cl-Amidine Treatment

In order to stablish that the identified NET-like structures were truly neutrophil-borne structures, mice were subcutaneously injected at 6pm with BB Cl-amidine (1mg/Kg; Cayman Chemical Company) diluted in 200µL saline solution in order to block NET-formation 3, 7, 11 and 15 hours before animals were sacrificed. BB-Cl-Amidine is a modified version of Cl-amidine that irreversibly inactivates all four PAD enzyme subtypes by covalently modifying the active site of cysteine that is important for its catalytic activity. BB-Cl-Amidine has a significantly longer *in vivo* half-life than Cl-amidine (1.75 h versus approximately 15 min, respectively).

3.1.5 Acetaminophen Treatment

Acetaminophen (APAP) overdose was used to evaluate NET release and compare previously identified NET-like structures to those NET structures that appear in a model of APAP-induced sterile liver injury. APAP is the best characterized hepatotoxicant that induces hepatic failure that is clinically relevant, well described and can be rapidly induced *in vivo* with a single dose. Briefly, mice were deprived of food 12 hours prior to treatment with APAP. Mice were then intraperitoneally injected with 300mg/kg of APAP (Mossanen and Tacke 2015) diluted in saline solution 0, 2, 4, 8 and 24 hours before animals were sacrificed. After APAP treatment, free access to a standard chow diet was allowed.

3.1.6 Time-Restricted Feeding Experiments

For 10 hours-time restricted feeding (TRF) experiments, during a period of 2 weeks mice were granted *ad libitum* access to food and water for 14 hours (10h:14h) only during the dark phase, namely from 19:00hrs or ZT12 to 09:00hrs or ZT2. After two weeks, animals were sacrificed and all target organs were harvested. During the experimental set-up, mice control groups had *ad libitum* access to food and water and were housed under a 12h:12h light-dark conditions.

3.1.7 High-Fat Feeding Experiments

8-to-12-weeks old animals were fed short doses of different high-fat diets (**Table 3-10**) in order to evaluate neutrophil response and NETosis capacity in the liver after a food bolus input. For short-term high-fat feeding experiments, mice were granted *ad libitum* access to food, either high-fat diet or its corresponding low-fat control diet (**Table 3-10**); and water during a period of 2 hours, from 19:00hrs to 21:00hrs; otherwise stated. Next, animals were sacrificed and all target organs were harvested.

Table 3-10. Diets Composition

DIET NAME	ENERGY DENSITY	COMPOSITION /100G	CHOLESTEROL SUPPLEMENTED
Chow Diet	3.75 kcal/g	18% Kcal Protein 13% Kcal Fat (Butter Fat) 69% Kcal Carbohydrate (Starch, Sugar)	0%
High Fat-Cholesterol Diet (HF-CholD)	4.58 kcal/g	15% Kcal Protein 42% Kcal Fat (Butter Fat) 43% Kcal Carbohydrate (Starch, Sugar)	1.25%
Low Fat-Sucrose Diet (LF-SucD)	3.82kcal/g	20% Kcal Protein (Casein, Lactic, Cystine L) 10% Kcal Fat (Lard) 66% Kcal Carbohydrate (Starch, Corn, Lodex10)	0%
High Fat-Sucrose Diet (HF-SucD)	4.7kcal/g	20% Kcal Protein Casein, Lactic, Cystine L) 45% Kcal Fat (Lard) 30% Kcal Carbohydrate (Sucrose, Lodex10)	0%

3.1.8 Fasting Experiments

8-to-12-weeks old animals were fasted during a short period of time during the night in order to evaluate neutrophil response and NETosis capacity in the liver in the absence of food. For short-term fasting experiments, mice were restricted access to food -but not to water- during a period of 3 hours, from 19:00hrs or ZT12 to 22:00hrs or ZT15. Next, animals were sacrificed and all target organs were harvested.

3.1.9 Oil Gavage Administration

Oral gavage technique was used to administer a single bolus of a high-fat meal (200µL of extra virgin olive oil or extra virgin coconut oil) in combination with a controlled input of 20 x 10^6 CFU of opsonized *E.coli* particles (*Section 3.1.10*). Oral gavage was carried out by using a curved 18-gauge metal needle with a bulbed tip (B. Braun, Melsungen, Germany) 1 hour before animals were sacrificed. Mice in the

control groups received a gavage dose of 200µL of sterile water. All groups of mice continued to receive *ad libitum* food and water for the next hour before been sacrificed.

3.1.10 E.Coli Gavage Administration

In order to prove the functionality of the potentially identified NET-like structures in the liver, to assess whether these structures can really scavenge incoming bacteria and to test whether the reported capacity to trap and kill bacteria varies throughout the day, C57BL6/J mice were given 20 x 10^6 CFU of Alexa Fluor (AF) 488-conjugated *Escherichia coli* (K-12 strain) bioparticles (#E13231, ThermoFisher Scientific), in 200 µL of solution through an intragastric probe. Animals were gavaged 1-hour prior sacrifice, in order to assure bioparticles can circulate and be properly redistributed alongside the body. *E.coli* bioparticles were previously opsonized, to ensure proper uptake. Control animals received 200 µL dosage gavage of water as a control. After sacrifice, AF488-marked cells are analyzed and quantified by using cell cytometry and immunofluorescence techniques. In addition, the microbial activity of immune cells is evaluated in plasma samples and liver homogenates by luminescence determination in several TLR-modified cell lines.

3.2 Flow Cytometry

Flow cytometry is a laser-induced-fluorescence methodology that allows a rapid and simultaneous visualization and a multi-parametric analysis of physical and/or chemical characteristics of single cells by using specific fluorescent labelled antibodies. Flow cytometry provides unparalleled insight into the heterogeneity of cellular populations, analyses the expression of cell-surface and intracellular molecules of interest such as chemokines or chemokine receptors, can determine cell size and volume and assess the purity of isolated cell subpopulations. During flow cytometry analysis, fluorescently labelled single cells pass through a laser beam. Each particle is then analysed for visible light scatter (forward and side scattered light) as well as for multiple fluorescence parameters. Measurements of forward scattered (FSC) light emission provide information about cell size, whereas side scattered (SSC) light emission allows discrimination by internal complexity or cell granularity. Fluorescence measurements enable to determine molecule expression according to the fluorescence intensity. During each animal experimental condition, flow cytometry has been used in order to analyse myeloid and lymphoid populations from the liver, large intestine, lungs, bone marrow, spleen and lymph nodes, among some other tissues and organs with less particular interest.

3.2.1 Organ Processing

3.2.1.1 Blood Sample Preparation

3.2.1.1.1 Venous Blood Collection

Briefly, mice were injected i.p. with an anaesthetics' combination of ketamine/xylazine diluted in saline solution. Systemic venous blood was collected and processed according to standard protocols. Briefly, blood was collected into an Ethylenediaminetetra-acetic acid (EDTA)-containing tube to prevent blood from coagulation. Afterwards, red blood cells were hemolysed by using 3 ml of red blood cells lysis buffer (**Table 3-1**) per 100 µl of blood, for 4 min at 4°C. Erythrocyte lysis was stopped by adding 10 ml of cold HANK´s buffer solution (**Table 3-1**). Afterwards, cells were centrifuged for 5 min at 300 x G and 4 °C, cell supernatant was discharged and pelleted cells were resuspended in antibody staining solution for FACS analysis (*Section 3.2.2*).

3.2.1.1.2 Enterohepatic Blood Collection

Portal vein blood sampling (as a representation of the enterohepatic circulation) was accomplished by cannulation of the hepatic portal vein with a sterile 30G needle attached to a 1ml insulin syringe previously coated with PBS-EDTA solution (**Table 3-1**). Briefly, mice were injected i.p. with an anaesthetics' combination of ketamine/xylazine diluted in saline solution. Afterwards, the abdominal cavity was sliced and the hepatic portal vein was exposed. The 30G-needle was inserted into the portal vein against the flow, which leads to the slow but gradual filling of the syringe. Collected portal blood was transferred into an (EDTA)-containing tube to prevent blood from coagulation. Afterwards, red blood cells were hemolysed by using 3 ml of red blood cells lysis buffer (**Table 3-1**) per 100 µl of blood, for 4 min at 4°C. Erythrocyte lysis was stopped by adding 10 ml of cold HANK´s buffer solution (**Table 3-1**). Afterwards, cells were centrifuged for 5 min at 300 x G and 4°C, cell supernatant was discharged and pelleted cells were resuspended in antibody staining solution for FACS analysis *(Section 3.2.2).*

3.2.1.1.3 Peripheral Blood Collection

Peripheral vein blood sampling (as a representation of the central systemic circulation post hepatic filtering) was accomplished by cannulation of the inferior cava vein or by puncture of the heart right ventricle with a sterile 30G needle attached to a 1ml insulin syringe previously coated with PBS-EDTA solution (**Table 3-1**). Briefly, mice were injected i.p. with an anaesthetics' combination of ketamine/xylazine diluted in saline solution. Afterwards, the abdominal cavity was sliced and either the inferior cava vein was exposed or the diaphragm was opened and consequently the heart was exposed.

Collected systemic blood was transferred into an (EDTA)-containing tube to prevent blood from coagulation. Afterwards, red blood cells were hemolysed by using 3 ml of red blood cells lysis buffer (Table 3-1) per 100 µl of blood, for 4 min at 4°C degrees. Erythrocyte lysis was stopped by adding 10 ml of cold HANK´s buffer solution (Table 3-1). Afterwards, cells were centrifuged for 5 min at 300 x G and 4 °C degrees, cell supernatant was discharged and pelleted cells were resuspended in antibody staining solution for FACS analysis *(Section 3.2.2).*

3.2.1.2 Liver Sample Preparation

After mice were sacrificed and blood was collected, non-adherent cells and red blood cells were removed by flushing the vasculature through the heart left ventricle with 20 ml cold PBS-EDTA solution (Table 3-1). Liver was removed from the abdominal cavity and stored in a well-plate containing PBS solution at room temperature (RT) until further tissue processing. A cell suspension was obtained by smashing the biggest liver lobe through a 70 µm cell strainer with 5 ml of 1x Hanks' Balanced Salt Solution (HBSS). Afterwards, cells were centrifuge for 5 min at 500 x G and 4 °C degrees, cell supernatant was collected and pelleted cells were resuspended in a 36% Percol solution (Table 3-1). Suspension was further centrifuged for 20 min at 800 x G and RT in order to create a suspension gradient. Then, supernatant was discharged (hepatocytes will top in a thin layer) and pelleted leukocytes were resuspended in 1x HBSS for a quick wash. Afterwards, cells were again centrifuged for 5 min at 500 x G and 4 °C degrees, cell supernatant was discharged and pelleted cells were resuspended in antibody staining solution for FACS analysis *(Section 3.2.2).*

3.2.1.2.1 Liver Perfusates

Liver perfusion through the portal vein is a technique that allow us to purify a high viability and high yield of immune cells that have transmigrated into the hepatic vasculature and remain adhered to the hepatic sinusoids. After mice were sacrificed and blood was collected, and by using a home-made plastic catheter attached to a 30-gauge metal needle tip, the portal vein was cannulated with the help of a stereo microscope. After the portal vein was successfully cannulated, the perfusion setup was stabilized by using tissue adhesive glue (#ZG2, Surgibond) and fixed with a piece of thread in order to avoid dislocation of the catheter during buffer circulation. Carefully, the liver was removed from the abdominal cavity and transferred into a sterile Petri Dish, kept on ice. The most distant part of the catheter was attached to a 20 mL syringe filled with 5%PBS-EDTA and the solution was pumped under a constant rhythm, 50% force, for 2 minutes with the use of a syringe pump apparatus (#70-3007, Harvard Apparatus). Perfusates were collected and used for further procedures.

3.2.1.3 Lung Sample Preparation

After mice were sacrificed and blood was collected, non-adherent cells and red blood cells were removed by flushing the vasculature through the heart left ventricle with 20 ml cold PBS-EDTA solution (Table 3-1). The lung was then removed from the thoracic cavity and stored in a well-plate containing HANK´s solution at 4°Cuntil further tissue processing. A cell suspension was obtained by scything the biggest lung lobe into small pieces added to 500µl of digestion medium supplemented with 1,25 mg/ml liberase (Roche, Rotkreuz, Switzerland) + 1U/ml DNAse I and incubated for 1 hour at 37 °C in constant agitation (350rpm). Afterwards, 1 ml of RPMI solution (Table 3-1) was added to prevent further digestion and the resulting lysate was filtered through a 50 µm cell strainer (Sysmex, Norderstedt, Germany) with 3ml RPMI medium. Afterwards, cells were centrifuged for 5 min at 300 x G and 4°C, cell supernatant was discharged and pelleted cells were resuspended in antibody staining solution for FACS analysis *(Section 3.2.2).*

3.2.1.4 Spleen Sample Preparation

After mice were sacrificed and blood was collected, non-adherent cells and red blood cells were removed by flushing the vasculature through the heart left ventricle with 20 ml cold PBS-EDTA solution (Table 3-1). The spleen was then removed from the abdominal cavity and stored in a well-plate containing HANK´s solution at 4°C until further tissue processing. A cell suspension was obtained by smashing the spleen through a 70 µm cell strainer with 5 ml of HANK´s Buffer. Afterwards, cells were centrifuged for 5 min at 300 x G and 4°C and cell supernatant was discharged. Because the spleen is a highly vascularized organ, remaining red blood cells were hemolysed by using 1 ml of red blood cells lysis buffer (Table 3-1) for 1 min at 4°C. Erythrocyte lysis was stopped by adding 10 ml of cold HANK´s buffer solution (Table 3-1). Cells were again centrifuged for 5 min at 300 x G and 4°C, cell supernatant was discharged and pelleted cells were resuspended in antibody staining solution for FACS analysis *(Section 3.2.2).*

3.2.2 Antibody Staining

Each cell suspension was labelled with 50µl of corresponding fluorescence-conjugated primary or secondary antibodies staining mix (**Table 3-3 & Table 3-4**) for at least 20 min at 4 °C and under dark conditions in a 96-v-bottom well plate. After staining was completed, unbound antibodies were washed away by adding 200 µl of HANK´s buffer solution. Cells were then centrifuged for 5 min at 300 x G and 4°C. Supernatant was removed by plate inversion and cells were resuspended in 200 µl of HANK´s buffer. Cell suspensions were kept on ice until analysed in the Flow Cytometer Facility In case the samples were not going to be analysed at the time, cells were fixed in 100µl of Fixation Buffer (BioLegend, San Diego, USA) or 4% PFA solution (**Table 3-1**) for at least 15 min at 4°C and under dark conditions. After that, PFA was washed away by adding 100 µl of HANK´s buffer solution. Cells were then centrifuged for 5 min at 300 x G and 4°C. Supernatant was removed by plate inversion and cells were resuspended in 200 µl of HANK´s buffer. Samples were stored in the fridge until analysed in the Flow Cytometer Facility.

3.2.3 Gating Strategy

Different surface markers were used to discriminate among the different lymphoid (CD45, CD3, CD19, B220) and myeloid cell subpopulations (CD45, CD11b, CD115, Ly6C, Ly6G, NK1.1, F4/80) present in the blood compartment and the different analysed peripheral organs (**Fig.3-2. Gating strategy of leukocytes in peripheral tissues**). **Table 3-11** shows the cell surface markers strategy that was used to discriminate between the different leukocyte subpopulations (**Table 3-11**). **Figures 3-1** and **3-2** show the flow cytometry gating strategy that was followed to discriminate between the different leukocyte subpopulations in blood (**Fig.3-1. Gating strategy of leukocytes in blood samples**) and the aforementioned analysed peripheral organs and tissues, in mice (**Fig.3-2. Gating strategy of leukocytes in peripheral tissues**)

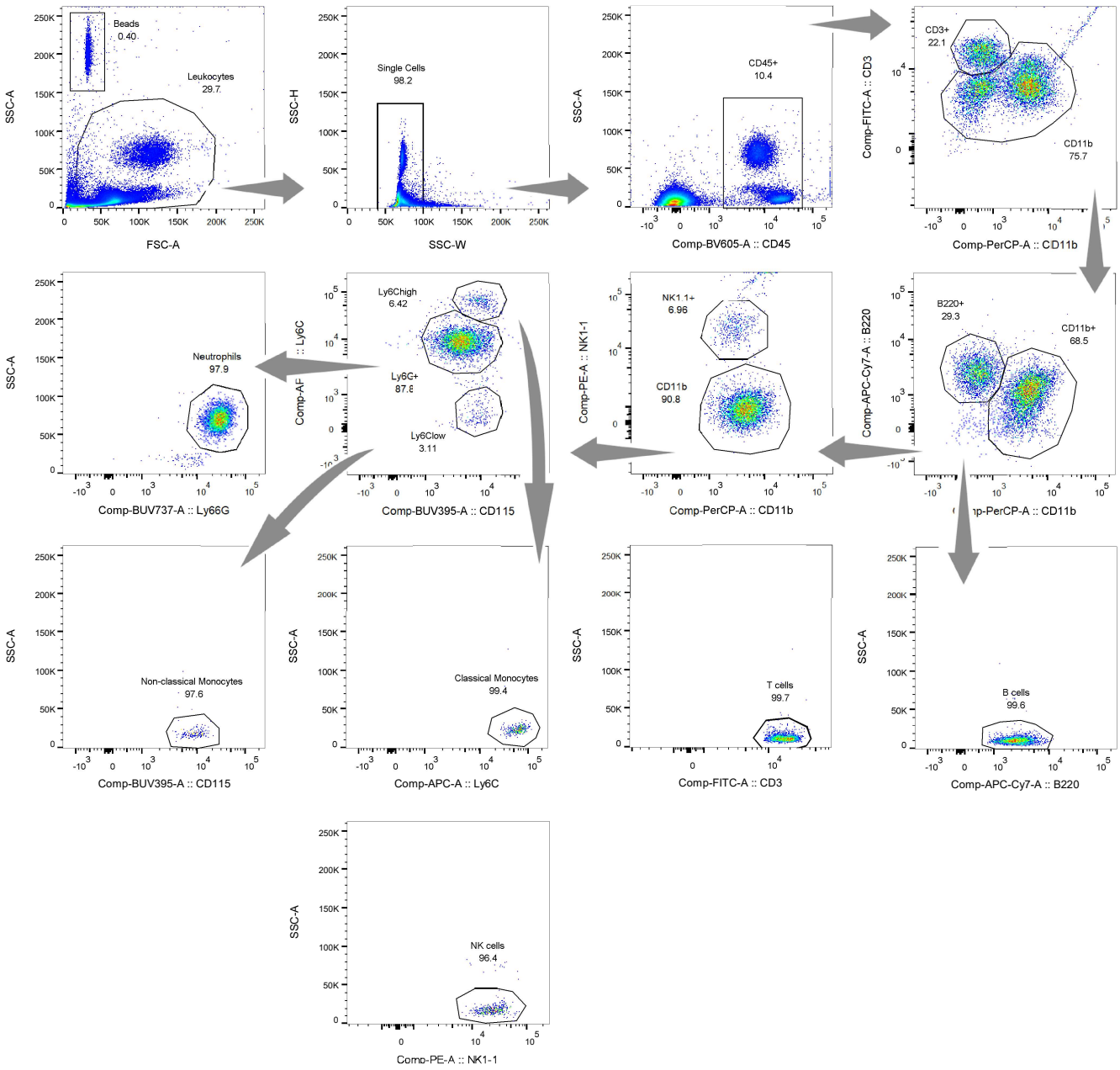


Figure 3-1. Representative gating strategy of leukocytes in blood samples. Representative flow cytometry plots of T cells, B cells, NK cells, classical Monocytes, non-classical Monocytes and Neutrophils from portal blood from C57Bl/6 animals. Briefly, blood was collected into an ethylenediaminetetra-acetic acid (EDTA)-containing tube, hemolysed with red blood cells lysis buffer and stained with an antibody mix to analyse and discriminate between the different myeloid and lymphoid populations in circulation. *Note*: "T cells" subset was obtained from CD3+ plot; "NK cells" subset was obtained from NK1.1+ plot. Single cells were excluded twice (FSC-H vs. FSH-W and SSC-H vs. SSH-W) but for representative purposes only one "single cell" gating was included.

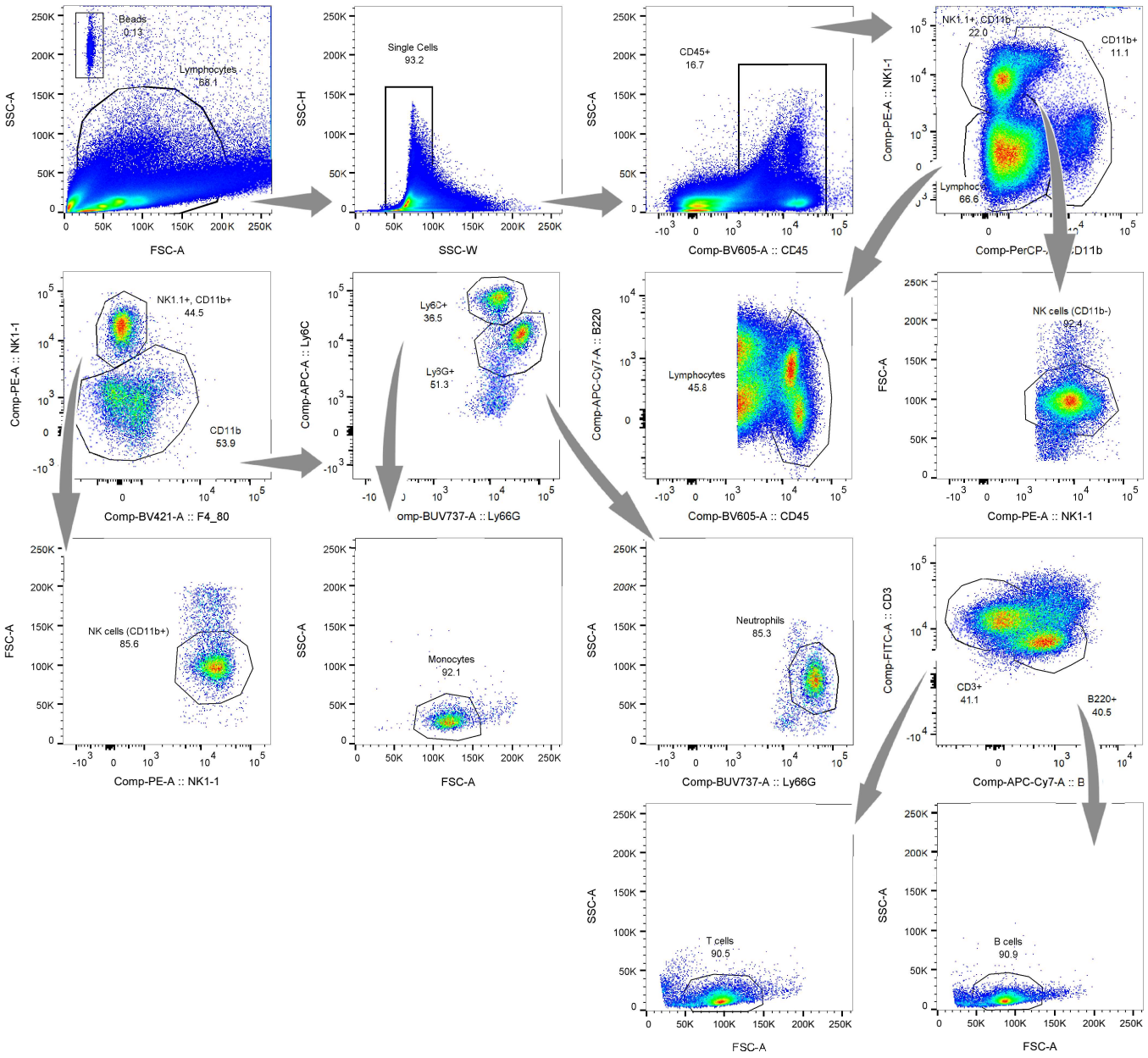


Figure 3-2. Representative gating strategy of leukocytes in peripheral tissues samples. Representative flow cytometry plots of T cells, B cells, NK cells, Monocytes and Neutrophils from livers from C57Bl/6 animals. Briefly, livers were homogenized and subsequently subjected to a Percol gradient solution (36%). Leukocytes were recovered and stained with an antibody mix to analyse and discriminate between the different myeloid and lymphoid populations in circulation. *Note*: "Comp-APC-A :: Ly6C vs. Comp-BUV737-A :: Ly6G" emerges from "CD11b+" subset. Single cells were excluded twice (FSC-H vs. FSH-W and SSC-H vs. SSH-W) but for representative purposes only one "single cell" gating was included.

Table 3-11. Immune Populations Markers

Immune Population	Identification Markers
Leukocyte Cells	CD45+
Myeloid Cells	CD45+, CD11b+
T Lymphocytes	CD45+, CD11b-, B220-, CD3+
B Lymphocytes	CD45+, CD11b-, CD3-, B220+
NK Cells	CD45+, CD11b+/-, NK1.1+
Classical Monocytes	CD45+, CD11b+, Ly6G-, CD115+, Ly6Chigh
Non-classical Monocytes	CD45+, CD11b+, Ly6G-, CD115+, Ly6Clow
Neutrophils	CD45+, CD11b+, CD115-, Ly6Cint, Ly6G+
Kupffer cells	CD45+, CD11b+/-, F4-80+
Tissue Resident-Monocytes	CD45+, CD11b+, Ly6Chigh
Tissue Resident-Macrophages	CD45+, CD11b+/-, F4-80+/-, Ly6C+

3.2.4 Gating Analysis

Flow cytometry was performed using the BD LSRFortessa™ X-20 Cell Analyzer (BD Bioscience, San Jose, CA, USA). BD FACSDiva™ Software controls the efficient setup, acquisition and analysis of flow cytometry data from the BD LSRFortessa™ Workstation. CountBright™ absolute counting beads (Invitrogen, Carlsbad, CA, USA) were used to assess the absolute cell number in each analyzed cell suspension. Flow cytometry data were analyzed with FlowJo Software (10.1 Flowjo LLC, Ashland, USA).

3.2.5 Blood retention calculation

A series of normalizations and calculations were applied in order to estimate the number of netting neutrophils within the liver. We assessed neutrophil retention in the liver (EQ1) by normalizing the amount of Ly6G+ cells in the liver by the number of Ly6G+ cells in the PV circulation (obtained by flow cytometry). The absolute number of neutrophils retained in the liver circulation (EQ2) can be obtained by multiplying the calculated ratio of neutrophil retention (in percentage) (EQ1) to the total amount of Ly6G+ cells in the liver (obtained by flow cytometry). Instead, we can

also estimate the percentage of netting neutrophils in the liver (out of the total fraction of retained neutrophils) (EQ3) by applying the percentage of identified NET-like structures (obtained by confocal imaging), to the ratio of neutrophil retention (in percentage) (EQ1) in the liver. Finally, we calculated the absolute number of netting neutrophils in the liver (EQ4.1) (out of the total fraction of retained neutrophils) by multiplying the obtained percentage of retained netting neutrophils (3), to the total amount of Ly6G+ cells in the liver (obtained by flow cytometry). Alternatively, we could obtain as well the absolute number of netting neutrophils in the liver (EQ4.2) (out of the total fraction of retained neutrophils) by multiplying the number of neutrophils retained in the liver to the percentage of of identified NET-like structures (obtained by confocal imaging).

EQ1. Retained Neutrophils in the Liver (%)

$$= \frac{\text{n° Ly6G cells in Liver (FACS)}}{\text{n° Ly6G cells in PV (FACS)}} \cdot 100$$

EQ2. Retained Neutrophils in the Liver (absolute number)

$$= \frac{\text{Neutrophil Retention (\%)} \cdot \text{n° Ly6G cells in Liver (FACS)}}{100}$$

EQ3. Netting Neutrophils in the Liver (%)

$$= \frac{\text{NET like structures (\%) (IHC)} \cdot \text{Neutrophil Retention (\%)}}{100}$$

EQ4. 1 Netting Neutrophils in the Liver (absolute number)

$$= \text{Netting Neutrophils (\%)} \cdot \text{n° Ly6G cells in Liver (FACS)}$$

EQ4. 2 Netting Neutrophils in the Liver (absolute number)

$$= \frac{\text{Retained Neutrophils} \cdot \text{NET like structures (\%) (IHC)}}{100}$$

3.3 Histology & Immunolabeling

Histology refers to the microscopic study of a tissue microstructure, whereas immunofluorescence is an analytical fluorophore-labelled antibody-based method that assists in the imaging of proteins and other biomolecules of interest in tissue sections, cultured cell lines or individual cells. High-resolution confocal imaging, THUNDER Imager systems and immunofluorescence techniques have been used in order to identify and phenotypically characterize NET-like structures in peripheral organs. Microscopic imaging techniques assist in analysing the presence and particular location with respect to vessels and sinusoids of the identified NET-like structures.

3.3.1 Tissue Preparation

All animals were euthanatized as previously stated *(Section 3.2.1).* Briefly, after mice were sacrificed under an overdose of an anesthetic's combination of ketamine/xylazine and blood was collected, non-adherent cells and red blood cells were removed by flushing the vasculature through the heart left ventricle with 20 ml cold PBS-EDTA solution (**Table 3-1**). Brown adipose tissue (BAT), Heart, Ileum, Kidney, Liver, inguinal and axillary Lymph Nodes (LN), Lung, Muscle, Spleen and Thymus were harvested, embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek, California, USA), snap-frozen on dry ice and stored at -80 °C until further processing. Before sectioning, organs were transferred for 30 minutes to 1 hour into a -20 °C freezer for temperature adjustment. Organs were sectioned in a Cryostat (Leica CM3050S, Wetzlar, Germany) at a thickness of 7µm to 10µm and were left in a drying chamber for at least 24 hours. Two to three sections from each mouse was analysed.

3.3.2 Immunofluorescence

Tissue sections were encircled with a hydrophobic acetone-resistant PAP pen (Dako, Glostrup, Denmark). The PAP pen creates a water-repellent barrier that keeps staining reagents localized on the tissue sections and prevents mixing of reagents when two different staining mixes are carried out on the same slide. Sections were fixed under RT conditions with pure acetone for 10 min, followed by a wash in PBS for 5 min. In order to prevent any unspecific antibody binding, sections were then blocked in a PBS blocking solution (**Table 3-1**) containing 5% normal horse serum (#S-2000-20, Vector Laboratories) for 1 hour at RT. Afterwards, blocking solution was removed and samples were incubated overnight (o/n) at 4°C with the specific primary antibody staining mix (**Table 3-3 & Table 3-4**) diluted in PBS blocking solution containing 5% normal horse serum. On the next day, the primary antibody mix was washed away with 3x3min PBS and 3x3min PBS-Tween20 solution (**Table 3-1**) and the samples were incubated for at least 1 hour at RT with the specific secondary antibody staining mix (**Table 3-3 & Table 3-4**) diluted in PBS blocking solution containing 5% normal horse serum. Afterwards, the secondary antibody mix was washed away with 3x3min PBS and 3x3min PBS-Tween20 solution. Nuclei were counterstained with 4',6-Diamidino-2-phenylindol (DAPI, ThermoFisher Scientific, Waltham, USA) solution (**Table 3-3 & Table 3-4**) for 15 minutes at RT. Afterwards, DAPI solution was washed away with 1x3min PBS and 1x3min PBS-Tween20 solution and slides were mounted using ProLong Gold antifade Mountant (ThermoFisher, Waltham, MA USA).

3.4 Imaging

All fluorescence immunostainings from all target organs were imaged using a spinning disk inverted Leica SP8X WLL confocal microscope or a THUNDER Imager Tissue microscope (Table 3-5). Confocal laser scanning microscopy (CLSM) allows imaging of samples by means of optical sectioning, which enables the reconstruction of three-dimensional structures within an object by capturing multiple two-dimensional images at different depths. CLSM grants increased optical resolution compared to widefield microscopes, and contrast of a micrograph by means of using a spatial pinhole that blocks and/or reduces out-of-focus light from the background or other regions of the specimen and the focal plane. That way, CLSM achieves a controlled and highly limited depth of field by using spatial filtering techniques. Meanwhile, the THUNDER Imager Tissue allows detailed, high-speed, high-quality 3D and real-time fluorescence imaging of thick tissue sections free of haze due to out-of-focus blur. THUNDER technology combines image resolving due to computational clearing procedures with the speed and fluorescence efficiency of widefield microscopes. When using CLSM, at least 5 images from every target organ section were acquired using a 20x magnification objective. Furthermore, high resolution images of -once identified- NET structures were acquired using a 63x magnification objective. Images were then submitted to a process of deconvolution using the Huygens Professional Software or a Computational Clearing process by using the Leica THUNDER Software. When using THUNDER imaging system, instead of individual fields of view liver whole sections were analysed. The number of neutrophils (co-localization of Ly6G+, MPO+ and DAPI+ staining), the number of NET-like structures (co-localization of Ly6G+, MPO+/NE+, H3+/citH3+/dsDNA+ and DAPI+ staining), the number of ET-like structures (co-localization of MPO+/NE+, H3+/citH3+/dsDNA+ and DAPI+ staining) as well as their localization in reference to the closest blood vessel (CD31+ staining) was analyzed using 3D visualization tools in Fiji.

3.5 Evaluation of Microbial Products

Pathogen recognition receptor (PRR) activity and the status of various cytokine profiles were analysed in the systemic blood compartment, the enterohepatic circulation and the liver with the aim to characterise the potential functionality of the identified NET structures in relation to microbial products and pathogen-derived molecules.

3.5.1 Blood Plasma Generation

All blood samples were collected in EDTA tubes to prevent blood from coagulation and stored on ice until further processing. Plasma isolation was achieved by centrifugation at 2500 rpm (5,2G) for 10 minutes at 4°C. Recollected supernatant was transferred into a sterile 1.5ml Eppendorf tube and all tubes were frozen at -80°C until measurements were done.

3.5.2 Liver Supernatant Generation

After mice were sacrificed and the liver was obtained from the abdominal cavity (see 2.2.1.2 “Liver Sample Preparation”), a cell suspension was prepared by disrupting the biggest liver lobe through a 70 µm cell strainer with 5 ml of 1x Hanks' Balanced Salt Solution (HBSS). Afterwards, cells were centrifuge for 5 min at 500 x G and 4 °C degrees and the 5ml of hepatic cells supernatant were collected. Recollected supernatant was transferred into a sterile 15ml falcon tube and all tubes were frozen at -80°C until measurements were done.

3.5.3 Endotoxin Detection Assay (EndoLISA®)

The EndoLISA® Endotoxin Detection Assay is a sandwich enzyme-linked immunosorbent assay (ELISA)-based detection method. In EndoLISA®, the lipopolysaccharide (LPS) specific phage derived protein is selectively pre-coated to the wells of a microtiter well plate. As the target sample is added to the well, the LPS molecules present in the sample can selectively bind to the endotoxin-specific phage binding protein. Subsequently, detection by recombinant Factor C (an endotoxin-sensitive serine protease zymogen) and a fluorescent substrate facilitates the quantification of LPS levels in the sample. EndoLISA has a measurement range of four orders of magnitude, from 0.05 EU/ml to 500 EU/ml. To evaluate the presence of endotoxins (i.e. LPS) in different blood compartments, portal blood and peripheral blood plasma samples were homogenized and incubated in a LPS pre-coated 96-well plate, according to manufacturer´s instructions (Hyglos GmbH, Bayern, Germany). Briefly, 100µL of the target sample (accordingly diluted) or standards (500 EU/ml – 0.05EU/mL) were incubated in a v-bottom 96-well plate in combination with 20µL of Binding Buffer. The 96-well plate was sealed and then incubated for 90 minutes at 37°C under constant agitation. Afterwards, the supernatant was removed by plate inversion and the samples were washed twice in 150µL of Wash Buffer. Empty wells were resuspended in 100µL of Assay Reagent and fluorescence signal was immediately acquired (time point zero). Afterwards, the 96-well plate was again sealed and incubated for 90 minutes at 37°C under constant agitation and fluorescence signal was acquired again (time point 90 minutes). Endotoxin levels (EU/ml) were acquired and analysed by using a standard curve analysis using linear regression model.

3.6 Evaluation of Innate Immune Response

3.6.1 Inflammatory Markers Detection ImmunoAssay (LEGENDplex™)

LEGENDplex™ ImmunoAssay from BioLegend is a bead-based immunoassay that follows the same basic principle of an ELISA, where a soluble analyte is captured between two antibodies. LEGENDplex™ ImmunoAssay contains different bead populations that can be differentiated by size (FSC and SSC profiles) and internal APC and PE fluorescence intensities. Each bead set is conjugated with a specific antibody on its surface and serves as the capture bead for a particular analyte. When a panel of capture beads is incubated with an unknown sample containing target analytes, each analyte will bind to its specific capture bead. Biotinylated detection antibodies will bind to its specific analyte bound on the capture beads, thus forming capture bead-analyte-detection antibody sandwiches. Subsequently, streptavidin-phycoerythrin (SA-PE) binds to the biotinylated detection antibodies, providing a fluorescent signal with proportional intensities to the amount of bound analyte. On a flow cytometer, analyte-specific populations can be segregated and PE fluorescent signal quantified. Results are analysed using the LEGENDplex™ data analysis software. For the evaluation of several different inflammatory markers (Table 3-14), extracts from liver supernatants were homogenized and incubated with the capture beads diluted in Assay Buffer (Table 3-1), according to manufacturer´s instructions (BioLegend, San Diego, CA, USA). Briefly, 25µL of the target sample or standards (4-fold serial dilutions) were incubated in a pre-washed v-bottom 96-well plate in combination with 25µL of mixed Capture Beads and 25µL of Assay Buffer. The 96-well plate was sealed and then incubated for 2 hours at RT under constant agitation. Afterwards, the sample was washed and the 96-well plate was centrifuged for 5 min at 300 x G and RT. Cell supernatant was discharged and pellets were resuspended in 25µL of Detection Antibodies. The 96-well plate was again sealed and incubated for 1 hour at RT under constant agitation and afterwards 25µL of SA-PE were directly added to the wells. The 96-well plate was again sealed and incubated for 30 minutes at RT under constant agitation and after a quick wash of the detection medium, the samples were proceeded to be read in the Flow Cytometer.

Table 3-12. Capture beads ID and panel specific inflammatory target selection

Target Analyte	Bead ID	Bead Concentration (ng/mL)
M-CSF	A3	10
IL-4	A4	10
IL-1a	A5	10
IL-5	A6	10
IL-2	A7	10

Target Analyte	Bead ID	Bead Concentration (ng/mL)
TNF-α	A8	10
IL-23	A10	50
IL-7	B2	50
IFN-γ	B4	10
IL-27	B5	50
IL-6	B6	10
IFN-a	B7	10

3.6.2 Innate Immune Sensors Activity Evaluation

PRR reporter cell lines are obtained by co-transfection of the interested target genes and the inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. As expected, PRR agonists induce the production of SEAP. For the evaluation of Innate Immune Sensors activity, extracts from liver supernatants, portal vein plasma and peripheral blood plasma were homogenized and added to the PRR reporter cell lines (**Table 3-15**), according to manufacturer´s instructions (InvivoGen, Toulouse, France). Briefly, cell suspensions of approximately 300.000 cells/ml are incubated in a v-bottom 96-well plate (50.000 cells/well) with 25µL of the target sample in HEK-Blue detection medium, a cell culture medium that allows for real-time detection of SEAP. The 96-well plate is incubated for 24hrs at 37°C in a 5% CO2 chamber. The hydrolysis of the substrate by SEAP produces a purple/blue colour that was measured with a spectrophotometer at 620-655 nm.

Table 3-13. HEK-Blue™ TLR and HEK-Blue™ NLR reporter cell lines

PRR Reporter Cell Lines	Description
HEK-Blue™ murine TLR2	HEK-Blue™-mTLR2 cells were obtained by co-transfection of the murine TLR2, and an optimized SEAP reporter gene into HEK293 cells. The SEAP reporter gene is placed under the control of the IL-12 p40 minimal promoter fused to five NF-κB and AP-1-binding sites. Additionally, the CD14 co-receptor gene was transfected into these cells to enhance the TLR2 response. Stimulation with a TLR2 ligand activates NF-κB and AP-1 which induces the production of SEAP. Levels of SEAP can be determined with HEK-Blue™ Detection Medium.

PRR Reporter Cell Lines	Description
HEK-Blue™ murine TLR4	HEK-Blue™-mTLR4 cells were obtained by co-transfection of the murine TLR4, MD-2 and CD14 co-receptor genes, and an optimized SEAP reporter gene into HEK293 cells. The SEAP reporter gene is placed under the control of an IFN-β minimal promoter fused to five NF-κB and AP-1-binding sites. Stimulation with a TLR4 ligand activates NF-κB and AP-1 which induce the production of SEAP. Levels of SEAP can be determined with HEK-Blue™ Detection Medium.
HEK-Blue™ murine NOD1	HEK-Blue™-mNOD1 cells were obtained by co-transfection of the murine NOD1 gene and an optimized SEAP reporter gene into HEK293 cells. The SEAP reporter gene is placed under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1 binding sites. Stimulation with a NOD1 ligand activates NF-κB and AP-1 which induce the production of SEAP. Levels of SEAP can be determined with HEK-Blue™ Detection Medium.
HEK-Blue™ murine NOD2	HEK-Blue™-mNOD2 cells were obtained by co-transfection of the murine NOD2 gene and an optimized SEAP reporter gene into HEK293 cells. The SEAP reporter gene is placed under the control of the IL-12 p40 minimal promoter fused to five NF-κB and AP-1 binding sites. Stimulation with a NOD2 ligand activates NF-κB and AP-1 which induce the production of SEAP. Levels of SEAP can be determined with HEK-Blue™ Detection Medium.
HEK-Blue NULL2	HEK-Blue™ Null2 cells express the SEAP reporter gene under the control of the IL-12 p40 minimal promoter fused to five NF-κB and AP-1 binding sites.
HEK-Blue NULL1	HEK-Blue™ Null1 cells express the SEAP reporter gene under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1 binding sites.
HEK-Blue NULL1V	HEK-Blue™ Null1-v cells express the SEAP reporter gene under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1 binding sites.

3.7 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 software. All data are represented as mean ± SEM. Error bars show mean ± SEM values unless stated otherwise. All statistical parameters including exact value of n, definition of centre, statistical analysis and significance are reported in the figure legends. *N* refers to biological samples unless stated otherwise. Normality was tested for each data set by D'Agostino-Pearson omnibus test. For comparisons between two groups, either an unpaired student's t-test, a paired student´s t-test or a Mann-Whitney test was performed. For comparisons including three or more groups with one variable, ordinary one-way ANOVA followed by Tukey's post hoc test was performed and Dunnett's multiple comparisons test was applied. Ordinary two-way ANOVA followed by Bonferroni's post hoc test and Šídák's multiple comparisons test were applied for multiple group comparisons with more than one variable. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

3.8 Experimental Research Contribution

All experimental designs composing this PhD thesis were carried out by the PhD candidate, Laura Pérez Olivares, and the two PhD supervisors, Oliver Soehnlein and Carlos Silvestre-Roig. Experimental procedures and animal harvestings were carried out under the main lead of the PhD candidate with the assistance and help of several lab colleagues: Celia Borja Almarcha, Chang Pan, Sanne Maas, Ariane Schumski, Patricia Lemnitzer, Kristov van Avondt and Maximilian Mauler. All genotype analysis were performed by Olga Schengel. The PhD candidate, Laura Pérez Olivares performed all microscopy image acquisition, analyzed and interpreted data and design all graphs and figures included in the PhD manuscript. Carlos Silvestre Roig assisted in data interpretation. Raphael Chevre contributed and assisted to experimental design, data acquisition, analysis and interpretation. The PhD candidate, Laura Pérez Olivares, wrote the entire PhD manuscript and Carlos Silvestre Roig, Raphael Chevre, Kristof van Avondt and Andrea Herrero Cervera proofread and ensured quality control of it.

Results

Traditionally, neutrophils have been described as a homogeneous population of short-lived and terminally differentiated cells that upon pathological challenges and inflammation can quickly get mobilized into tissues, where they exert a variety of potent and conserved antimicrobial functions, including phagocytosis, degranulation and NET release. Over the last years, this neutrophil traditional dogma has been challenged and neutrophils have recently emerged as an heterogenous population of cells with functional versatility that can infiltrate healthy tissues (Maria Casanova-Acebes et al. 2018), and where they are capable of exerting an array of homeostatic functions (Palomino-Segura and Hidalgo 2021; Aroca-Crevillén, Adrover, and Hidalgo 2020). In fact, neutrophils are periodically released from the bone marrow into the blood circulation and, in a diurnal-fashion, they are able to infiltrate multiple peripheral organs, including the lung, the liver and the spleen, among others (Maria Casanova-Acebes et al. 2018). Interestingly, accumulation of neutrophils within these organs coincides with the active phase of the organism (i.e. the night-time, in rodents). Mice are nocturnal creatures that have a night-associated active period. During their active phase, mice eat substantially more food in comparison to the diurnal phase and they are exposed to an increased amount of potential insults that could surpass and overcome typical barrier tissues and organs, which ultimately protect the host against these external incoming agents. Recently, the idea has emerged that neutrophils might contribute to host defence during these particularly challenging periods. When exposed to pathogens, damage signals, danger stimuli or pro-inflammatory cytokines, but also to metabolic-derived antigens neutrophil harbour a wide variety of weapons already mention, including ROS, degranulation and, to our particular interest, NET release. Of note, during steady state, similar hazard products periodically invade the system, especially during the active phase of an organism. Here, we hypothesize that NET formation is instrumental in containing the incoming waves of pathogens and metabolic products that periodically challenge a system in homeostasis. Based on that, this PhD research project aimed to provide basic insight into the role of neutrophils and the active process of NET formation during physiological conditions and tissue homeostasis. Our research aims to characterize the spatial-temporal repartition of NETs in different immunological and non-immunological compartments, and identify the potential physiological contributors of NET release, such as circadian factors, the timing of food intake and the nutritional composition of the diet, as well as the host´s microbiome.

4.1 Heterogeneous repartition of NET-like structures across mice tissues during conditions of immune homeostasis

Our study commenced by establishing the visualization of NETs under steady state conditions during morning (ZT2) and night (ZT14) timepoints, as a representation of resting and challenging phases of the mouse daily period, respectively, in several different tissues across the body. The presence of NET-like structures was evaluated in what was defined as non-barrier tissues: the BAT, heart, kidney, muscle (tibial) and thymus (**Fig.1**); and what was defined as physical and immunological barrier tissues: the large intestine, liver, lung, lymph nodes (axillary and inguinal) and spleen (**Fig.2**), by using quantitative immunofluorescence imaging techniques and a spinning disk confocal microscope. Preliminary observations were performed on C57Bl/6 female mice that were kept under a physiological status and *ad libitum* access to food. Cryosections from the aforementioned organs were stained for Ly6G, MPO, H3, CD31 markers and cell nuclei were observed with DAPI.

4.1.1 Neutrophil infiltration in unchallenged tissues

During the ZT2 timepoint, we observed that neutrophils –identified as co-localizing Ly6G+, MPO+, DAPI+ cells– are able to infiltrate the BAT (**Fig.1A & 3A**), heart (**Fig.1B & 3B**), kidney (**Fig.1C & 3C**), muscle (**Fig.1D & 3D**), thymus (**Fig.1E & 3E**), large intestine (**Fig.2A & 3F**), liver (**Fig.2B & 3G**), lymph node (**Fig.2C & 3H**), lungs (**Fig.2D & 3I**) and the spleen (**Fig.2E & 3J**) of unchallenged mice. Neutrophils were present in all analysed organs and tissues under homeostatic conditions (**Fig.3A-J**), in agreement with previously published data (Casanova-Acebes et al. 2018). At ZT14, during the night-time, we still observed Ly6G+ cells infiltrated in the BAT (**Fig.1F & 3A**), heart (**Fig.1G & 3B**), kidney (**Fig.1H & 3C**), muscle (**Fig.1I & 3D**), thymus (**Fig.1J & 3E**), large intestine (**Fig.2F & 3F**), liver (**Fig.2G & 3G**), lymph node (**Fig.2H & 3H**), lungs (**Fig.2I & 3I**) and the spleen (**Fig.2J & 3J**) of mice under steady state, now during their active phase. In accordance with previous reports (Casanova-Acebes et al. 2018), neutrophil numbers peak in the early morning compared to the night in mostly all analysed organs (**Fig.3A-J**). Also, the number of infiltrating neutrophils varied widely and significantly among tissues at any time of the day (**Fig.4A**).

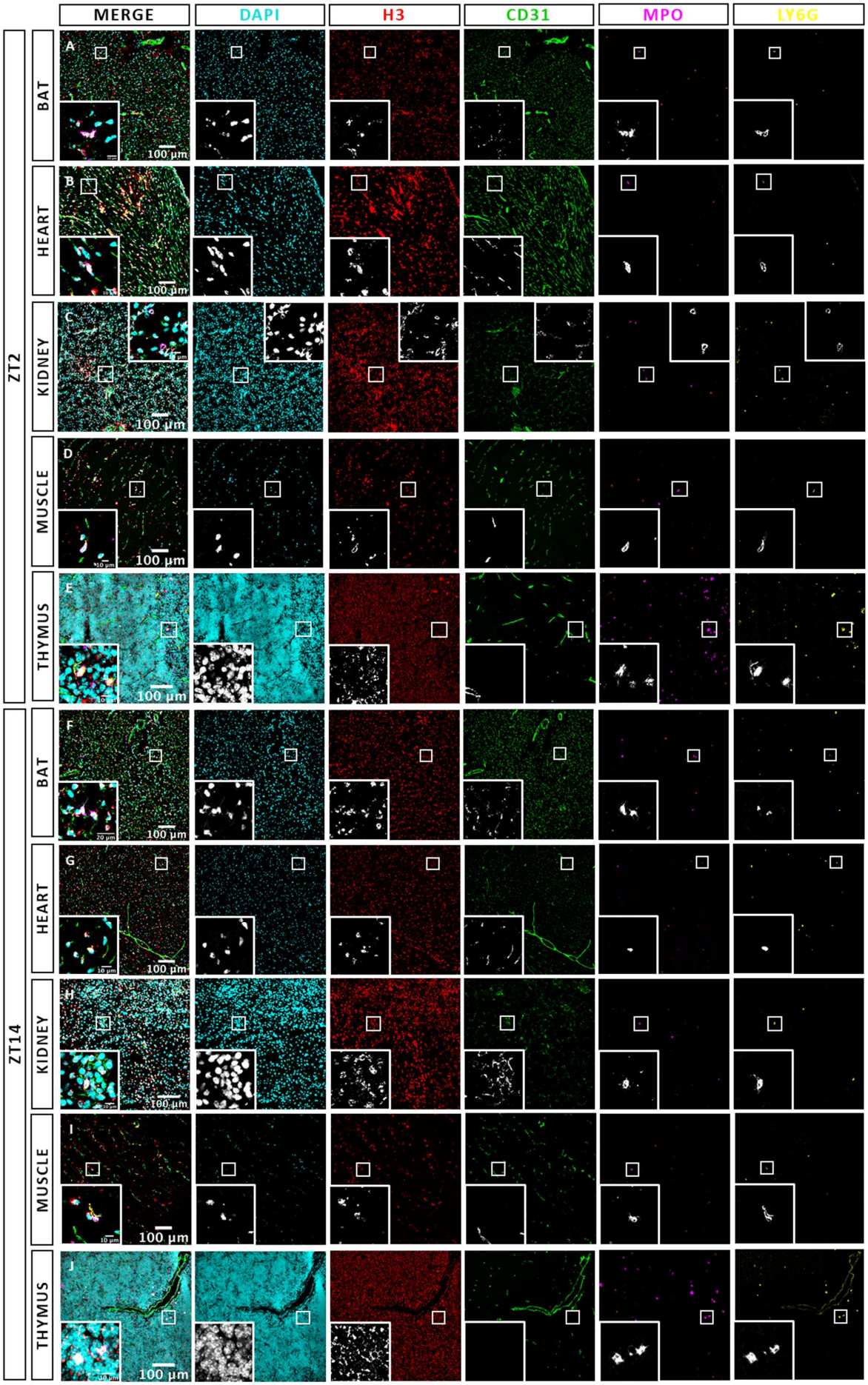

MERGE
DAPI
H3
CD31
MPO
LY6G
ZT2
ZT14
BAT
HEART
KIDNEY
MUSCLE
THYMUS
A
B
C
D
E
F
G
H
I
J
100 µm

Figure 1. Multi-organ infiltration of neutrophils and presence of NETs in non-barrier tissues during morning and night timepoints in homeostasis. Representative immunofluorescence confocal images of several organs cryosections showing stained nuclei (DAPI,cyan), histones (H3+, red), endothelial vessels (CD31+, green), neutrophils and macrophages (MPO+, magenta); and neutrophils (Ly6G+, yellow) at ZT12 and ZT14 from BAT (A,F), heart (B,G), kidney (C,H), muscle (D,I) and thymus (E,J). 20x magnification images: scale bar 100µm. 63x magnification images from BAT, heart, kidney, muscle and thymus: scale bar 10µm.

4.1.2 NETs are released at immunological barrier sites

On top of that, during the ZT2 time-point we were also able to identify the so-called NET-like structures –here defined as co-localizing Ly6G+, MPO+, H3+ and DAPI+ structures– only in the liver (**Fig.2B & 3G**), the lymph node (**Fig.2C & 3H**), the lung (**Fig.2D & 3I**) and the spleen (**Fig.2E & 3J**), but not in the BAT (**Fig.1A & 3A**), heart (**Fig.1B & 3B**), kidney (**Fig.1C & 3C**), muscle (**Fig.1D & 3D**), thymus (**Fig.1E & 3E**) or large intestine (**Fig.2A & 3F**). At ZT14 time-point, NET-like structures were consistently present only in the liver (**Fig.2G & 3G**), lymph nodes (**Fig.2H & 3H**), lungs (**Fig.2I & 3I**) and the spleen (**Fig.2J & 3J**), the same organs where they were exclusively identified at ZT2. The intestine was a tissue from which no clear conclusions could be drawn regarding NET release during the night time (**Fig.2F & 3F**). Notably, these four organs where NET release was spotted under homeostasis, are immunological compartments and neutrophil reservoirs (J. Wang et al. 2017; Christoffersson and Phillipson 2018), where neutrophils have been demonstrated to be able to execute active functions. Moreover, seems remarkable to point that beyond other physical barriers (e.g. the skin, the gut) in intimate contact with the host´s external environment, the lungs compose a major interface chronically exposed to external pathogens. Similarly, the liver, lymph nodes and the spleen also represent major immunological hubs able to contain the diurnal waves of insults that might have escaped the previously mentioned first physical lines of defence in an organism. Altogether, these physical and immunological barriers would function as sentinels regulating the entrance of a myriad of air- and food-borne pathogens, allergens and pollutants. Therefore, it would make sense that the immune compartment present there displays a unique ability to sustain tissue homeostasis, potentially through immune regulatory functions, such as the just described release of NETs.

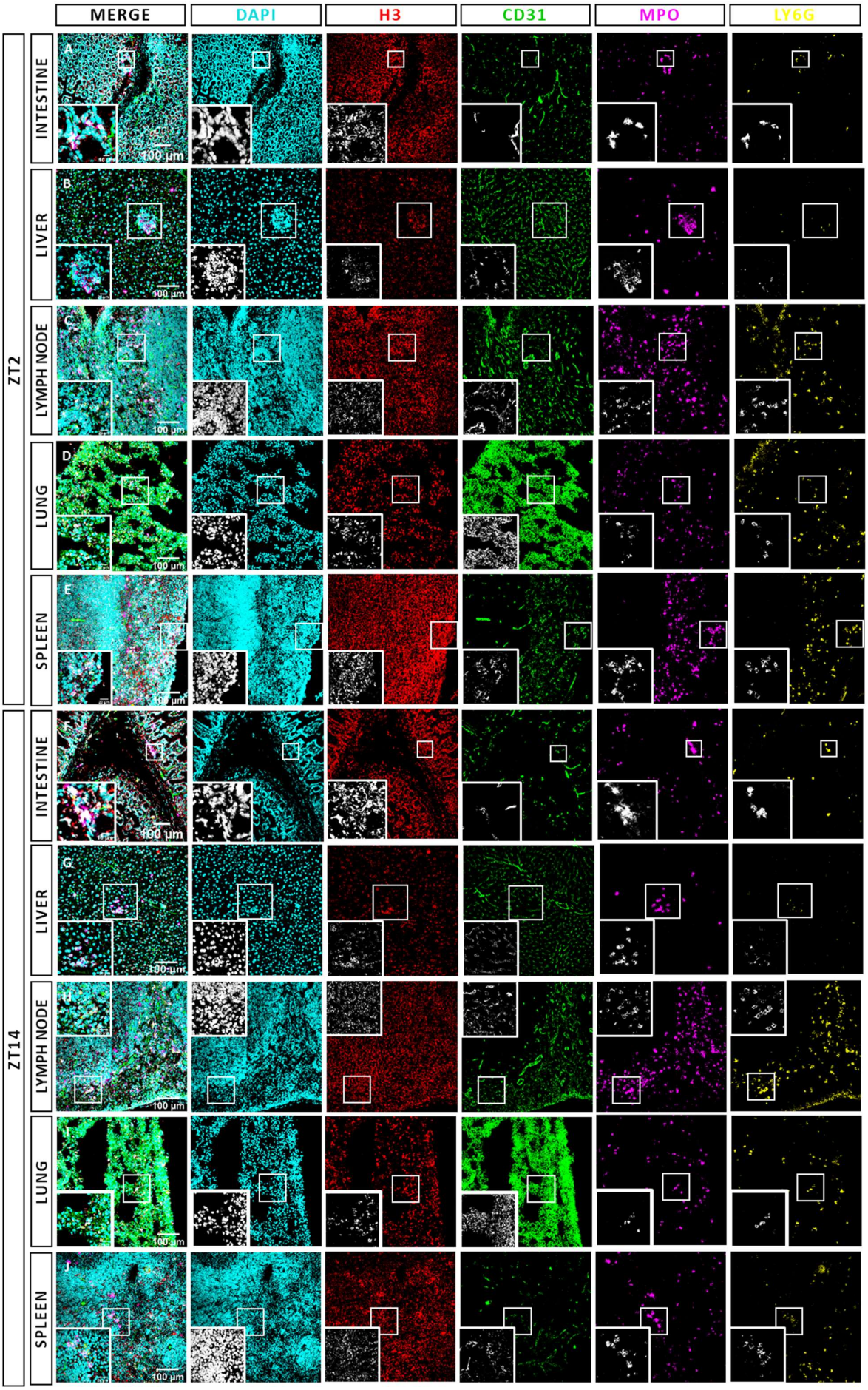

MERGE
DAPI
H3
CD31
MPO
LY6G
ZT2
ZT14
INTESTINE
LIVER
LYMPH NODE
LUNG
SPLEEN
A
B
C
D
E
F
G
H
I
J
100 µm

Figure 2. Multi-organ infiltration of neutrophils and presence of NETs in physical- and immunological-barrier tissues, during morning and night timepoints in homeostasis. Representative immunofluorescence confocal images of several organs cryosections showing stained nuclei (DAPI,cyan), histones (H3+, red), endothelial vessels (CD31+, green), neutrophils and macrophages (MPO+, magenta); and neutrophils (Ly6G+, yellow) at ZT2 and ZT14 from intestine (A,F), liver (B,G), lymph node (C,H), lung (D,I) and spleen (E,J). 20x magnification images: scale bar 100µm. 63x magnification images from intestine: scale bar 10µm. 63x magnification images from liver, lymph node, lung and spleen: scale bar 20µm.

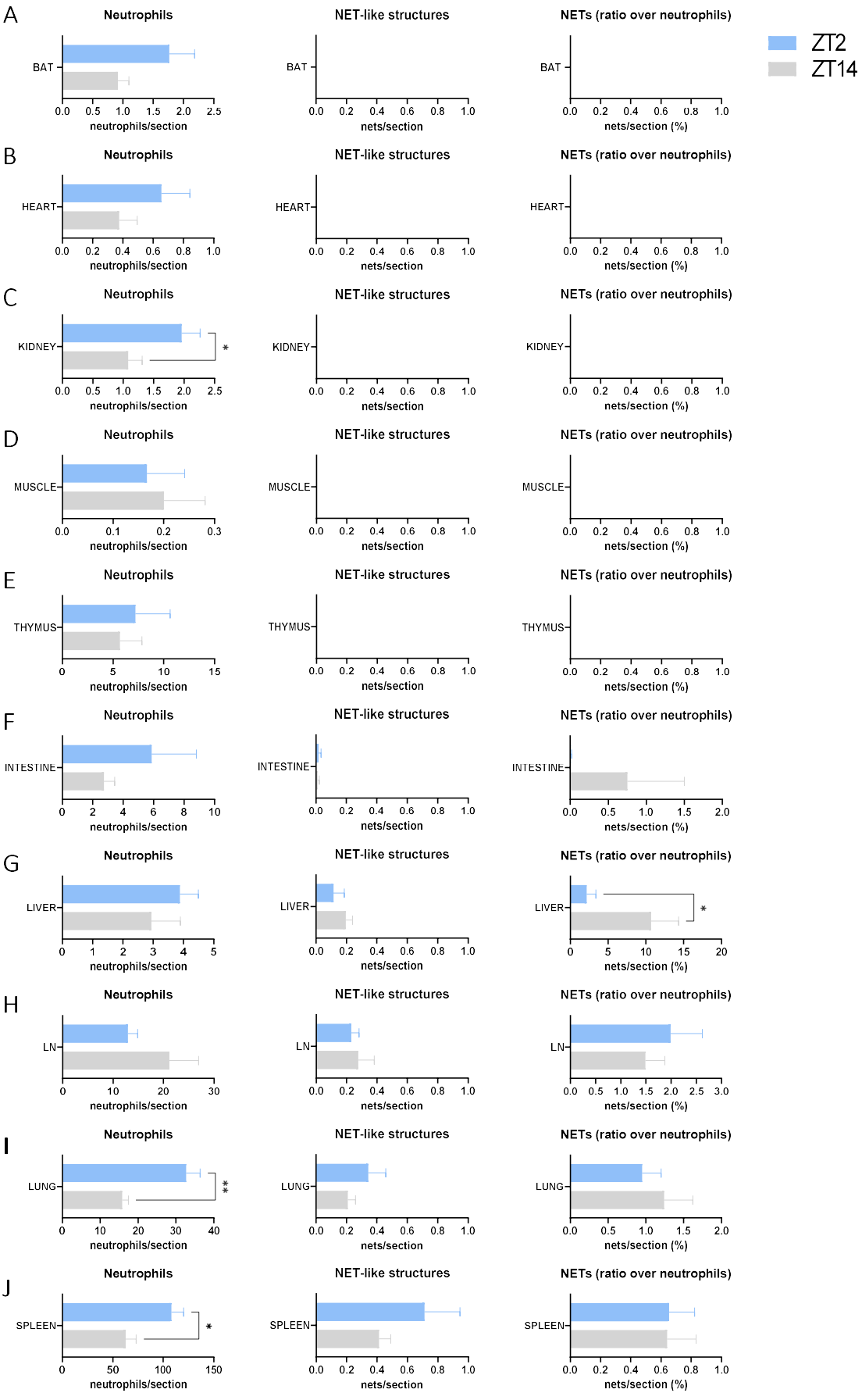

A
B
C
D
E
F
G
H
I
J
Neutrophils
NET-like structures
NETs (ratio over neutrophils)
BAT
HEART
KIDNEY
MUSCLE
THYMUS
INTESTINE
LIVER
LN
LUNG
SPLEEN
neutrophils/section
nets/section
nets/section (%)
ZT2
ZT14

Figure 3. Quantification: Multi-organ infiltration of neutrophils and presence of NETs in non-barrier tissues, physical barrier tissues and immunological barrier tissues during morning and night in homeostasis. Quantification of tissue-associated neutrophils per field of view (or analysed section), NET-like structures per field of view (or analysed section) and percentage of NETosis per field of view (or analysed section) at ZT2 and ZT14 in BAT (A), heart (B), kidney (C) muscle (D), thymus (E), intestine (F), liver (G), lymphd node (H), lung (I) and spleen (J); n = 7-8, where each n represents one biological sample that has been calculated as an average of 4-5 analysed sections or fields of view. Unpaired parametric t-test assuming Gaussian distribution was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

4.1.3 The liver shows the largest differences in NETosis capacity between morning and night

Raw quantification of acquired images shows that compared to the rest of the organs, the number of infiltrated neutrophils and NET-like structures is highest in the spleen, particularly during the morning time-point (**Fig.4A & Fig.4B**), which was not surprising considering the spleen is generally considered a neutrophil-reservoir organ (Deniset et al. 2017). Nonetheless, the ratio between the number of NET-like structures (per analysed section) and neutrophils (per analysed section) –here defining the percentage of NET-like structures per section– shows a particularly high level in the liver in comparison to the rest of analysed organs (**Fig.4C**). We observed that the percentage of NET-like structures (over total neutrophils) peaks dramatically during the night, at ZT14 timepoint (**Fig.4C**). Interestingly, the identified NET-like structures in the liver have a conformation that resemble those of aggregated NETs (**Fig.1G & Fig.2G**), a phenotype that has been already associated with inflammatory resolution properties (Schauer et al. 2014; Daniel et al. 2019). That way, the liver as one of the aforementioned immunological barrier organs, became our main target for the upcoming experimental procedures, as we recognize it now as a tissue with a population of neutrophils that shows the highest capacity to undergo NETosis under homeostatic conditions.

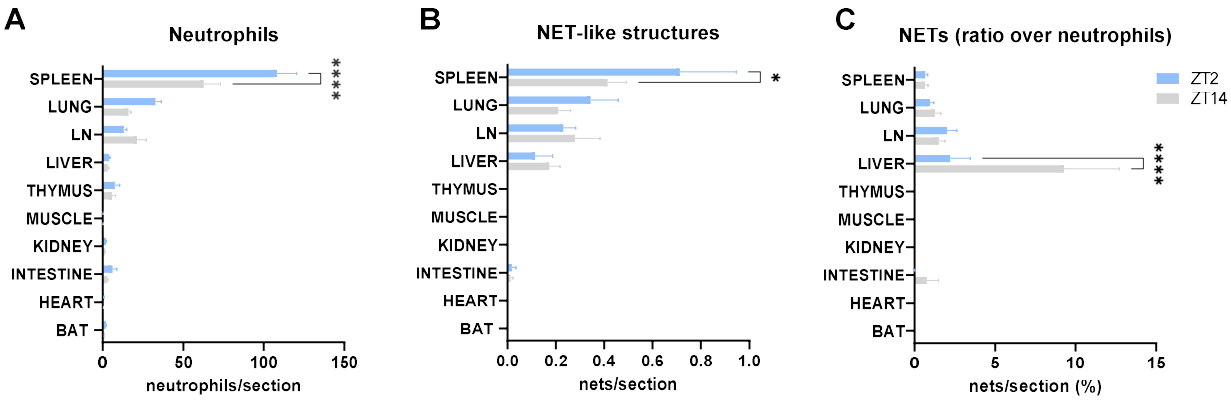


Figure 4. Overview: Multi-organ infiltration of neutrophils and presence of NETs in homeostasis during morning and night. Quantification of tissue-associated neutrophils per field of view (or analysed section) (A), NET-like structures per field of view (or analysed section) (B) and percentage of NETosis per field of view (or analysed section) (C) at ZT2 and ZT14 in BAT, heart, intestine, kidney, muscle, thymus, liver, lymph node, lung and spleen; n = 7-8, where each n represents one biological sample that has been calculated as an average of 4-5 analysed sections or fields of view. Ordinary two-way ANOVA and Šídák's multiple comparisons test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

4.2 Identified NET-like structures in the liver are neutrophil-borne

Before further research into the role in homeostasis of the identified NET-like structures in the liver and their regulation by potential physiological modulators, we decided to evaluate whether our identified NET-like structures are truly NETs conformations and have a neutrophil-origin. One of the biggest issues on the field of NET research is that there has yet not been described a systematic and categorical method to identify NETs neither *in-vivo*, *ex-vivo* or *in-vitro* (**Table 1-1. NET Visualization Approaches**). For example, every *in-vivo* imaging method involves invasive techniques that can cause unwanted inflammatory responses that might induced biased evaluation of NETosis. *In vivo* methodologies, such as spinning disk confocal microscopy, multi- / two-photon microscopy or intravital microscopy frequently entail poor temporal resolution. Absorbance and fluorescent evaluation of NET components (soluble NET-remnants, cf-DNA, MPO-DNA complexes, NE-DNA complexes, etc) trough ELISA methodologies or flow cytometry analysis render results with low specificity. As an example, MPO and NE can reflect on neutrophil and macrophage activation and degranulation not necessarily related to NET release; and cell-free DNA can reflect on other lytic cell death mechanisms such as necrosis. Even immunofluorescence of tissue sections has some weaknesses, such as a biased selection of the field of view that might compromise results, or the underestimation of NET-events due to the clump of NETs derived from multiple cells. Therefore, different staining strategies against canonical markers for NET release, several methods, as well as experimental and technical controls were implemented here in order to define a NET, to corroborate its true neutrophil-origin and to discriminate these structures from free-DNA released from damaged cells.

4.2.1 NET-like structures result positive for canonical markers of NET release

The NET-like structures previously identified were also stained for some other typical markers of the NET backbone (dsDNA) (**Fig.5A**), or typical markers that reflect on PAD4 activity and active citrullination processes (citH3) (**Fig.5B**). These experimental-control approaches allowed us to discriminate NETosis from other forms of active DNA release, such as necrosis or apoptosis. Identified NET-like structures in

the liver, both at ZT2 and ZT14 resulted positive for dsDNA staining (**Fig.5A**) and for citH3 staining (**Fig.5B**), alongside the other common markers (Ly6G, MPO and DAPI) (**Fig.5**) that were frequently used in our previous experiments. The presence of positive citH3 staining indicates that PAD4 activity and a process of histone citrullination have taken place, an undeniable signature of NET formation. DsDNA positive staining confirms that these structures are not just circulating free-DNA or DNA debris products, and so reinforce the idea that the observed NET-like conformations are a whole aggregated NET-structure.

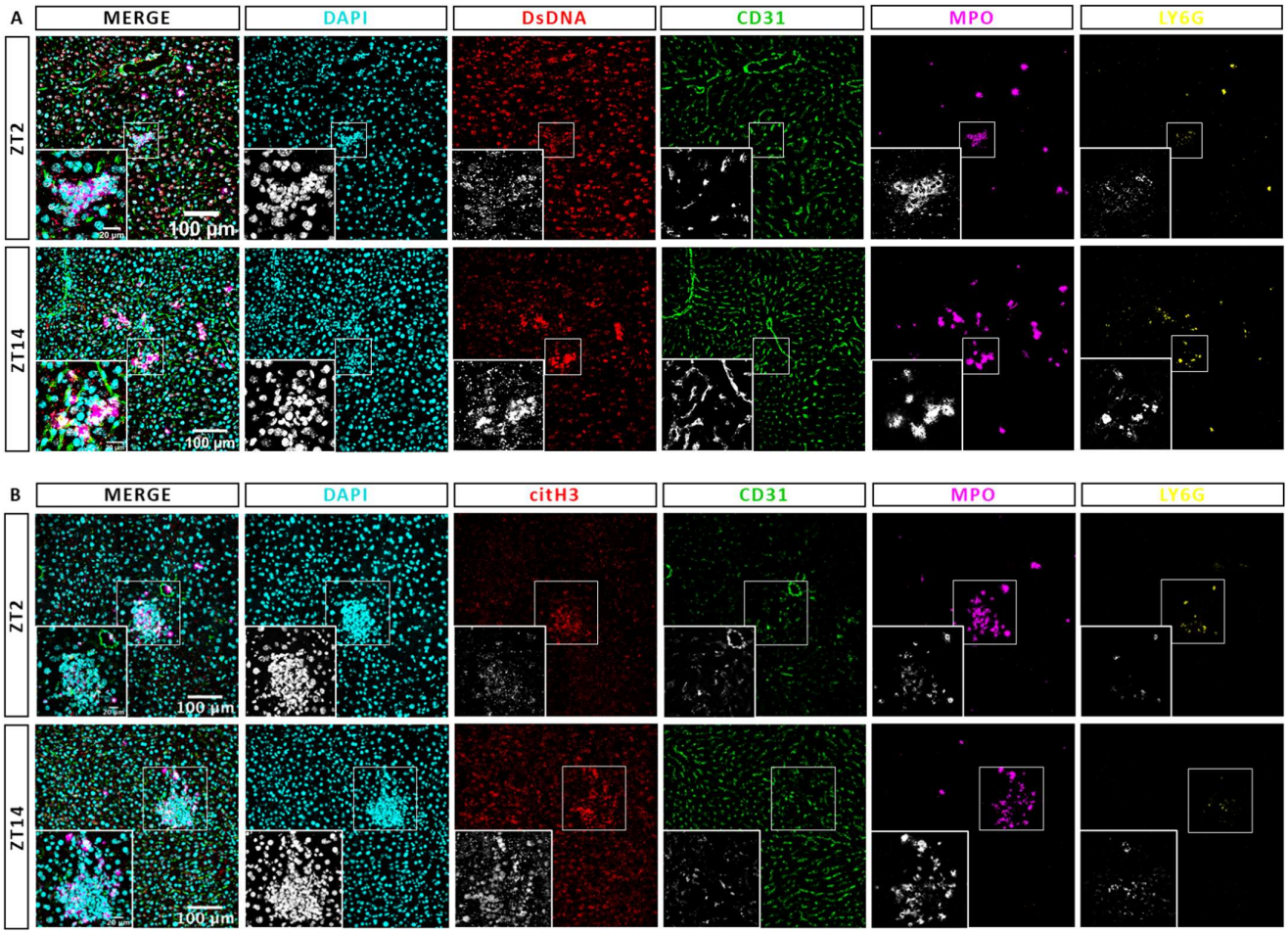


Figure 5. Characterization of canonical markers for NET release in liver sections. Representative immunofluorescence confocal images of liver cryosections showing stained nuclei (DAPI, cyan), dsDNA or citrullinated histones (dsDNA+ or citH3+, respectively; red), endothelial vessels (CD31+, green), neutrophils and macrophages (MPO+, magenta); and neutrophils (Ly6G+, yellow) at ZT2 or ZT14 timepoints. 20x magnification images: scale bar 100µm. 63x magnification images: scale bar 20µm.

4.2.2 NET-like structures are absent in genetically neutropenic and granule protein deficient mice

4.2.2.1 NET release is abolished in Elane$^{-/-}$ mice

Next, we evaluated NET release in genetically modified *Elane*$^{-/-}$ mice, an animal model that has been previously used for NETosis studies. In agreement with published literature, we were unable to detect NET-like structures under steady state conditions in liver sections from *Elane*$^{-/-}$ animals (**Fig.6**), which were described already to show abrogated NET release (Papayannopoulos et al. 2010a).

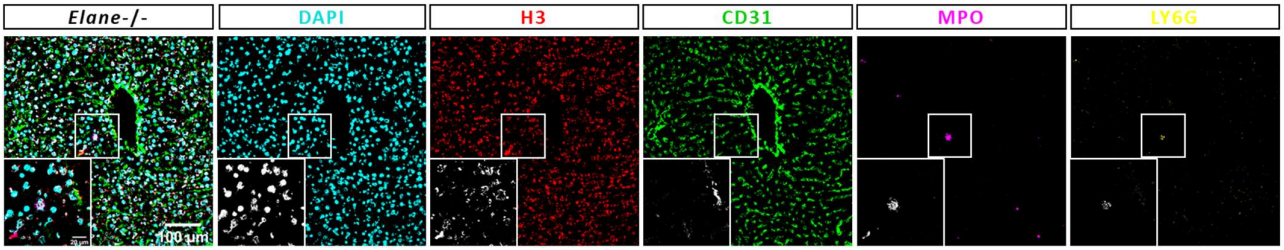


Figure 6. Characterization of NET-like structures in Elane$^{-/-}$ mice. Representative immunofluorescence confocal images of liver cryosections showing stained nuclei (DAPI, cyan), histones (H3+, red), endothelial vessels (CD31+, green), neutrophils and macrophages (MPO+, magenta); and neutrophils (Ly6G+, yellow) at ZT2 or ZT14 timepoints. 20x magnification images: scale bar 100µm. 63x magnification images: scale bar 20µm.

4.2.2.2 NET release is abolished in Ly6G$^{Cre/Cre}$ Mcl1$^{flox/flox}$ mice

To follow up, we evaluated whether NET release was absent in *Ly6g*$^{Cre/Cre}$ *Mcl1*$^{flox/flox}$ mice, an animal model that has been reported to lack neutrophils both in blood circulation and peripheral organs. In terms of NET release, some neutrophil plasma membrane and cytoplasmatic markers (MPO, NE) were evaluated in liver sections by using confocal microscopy (**Fig.7A,B**). Staining confirmed a significant reduction in the NETosis process in the *Ly6G*$^{Cre/Cre}$*Mcl1*$^{flox/flox}$ mice (**Fig.7C,D**). The number of NE+ and MPO+ cells were both significantly reduced in *Ly6g*$^{Cre/Cre}$ *Mcl1*$^{flox/flox}$ animals compared to *Ly6g*$^{Cre/Cre}$ *Mcl1*$^{wt/wt}$ controls (**Fig.7A,B**) as well as the total number of identified extracellular structures (**Fig.7C,D**).

In order to confirm induced neutropenia, CD45, CD11b, CXCR2 and Ly6C markers were evaluated in blood samples from male and female *Ly6g*$^{Cre/Cre}$ *Mcl1*$^{flox/flox}$ mice and Ly6G$^{Cre/Cre}$ *Mcl1*$^{wt/wt}$ controls (**Fig.8**). We found that *Ly6g*$^{Cre/Cre}$ *Mcl1*$^{flox/flox}$ animals show a drastic reduction in the number of neutrophils in circulation, here identified as CXCR2+, Ly6C+ cells (**Fig.8A**). Note that for the flow cytometry strategy, gating on CXCR2 and Ly6C was a more accurate strategy to evaluate neutropenia in Ly6G knock-out animals rather than evaluating Ly6G+ cells. The number of leukocytes (CD45+ cells), myeloid cells (CD11b+) and classical monocytes (CD115+, Ly6C^{high} cells) was also decreased in the blood of *Ly6G*$^{Cre/Cre}$ *Mcl1*$^{flox/flox}$ mice compared to *Ly6g*$^{Cre/Cre}$ *Mcl1*$^{wt/wt}$ animals (**Fig.8A**). Markers for other myeloid (CD115

and F4/80) and lymphoid populations (CD3, B220, NK1.1) were also evaluated to corroborate that Ly6G depletion in *Ly6g*$^{Cre/Cre}$ *Mcl1*$^{flox/flox}$ mice affects only the granulocyte cellular fraction and no other cell populations (**Fig.8A-B**). In blood, the lymphoid compartment (T cells, B cells and NK cells) as well as non-classical monocytes (CD115+, Ly6C^{low} cells) remained similar between both groups of animals (**Fig.8A**).

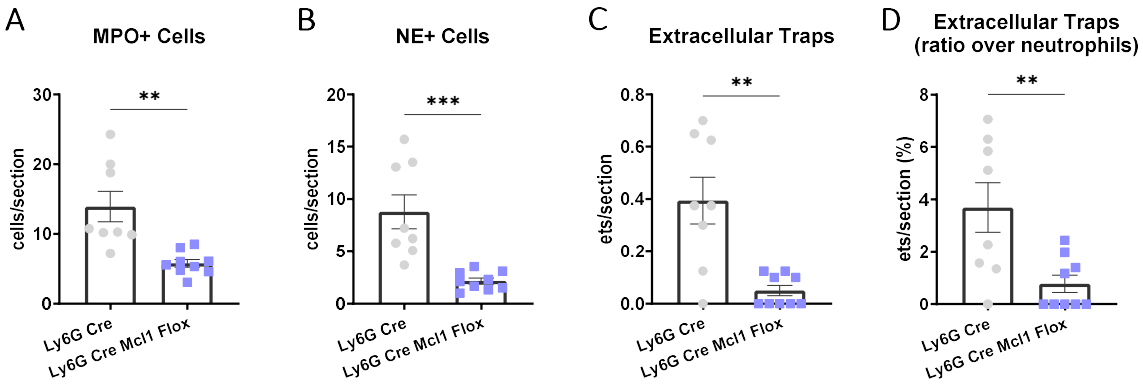


Figure 7. Evaluation of NET release in the liver in Ly6G$^{Cre/Cre}$Mcl1$^{flox/flox}$ mice. Quantification of tissue-associated MPO+ (A), NE+ (B) cells per field of view (or analysed section), ET-like structures per field of view (or analysed section) (C) and percentage of extracellular traps per field of view (or analysed section) (D) in the liver as determined by confocal imaging. n = 8-10, where each n represents one biological sample that has been calculated as an average of 4-5 analysed sections or fields of view. Unpaired t-test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

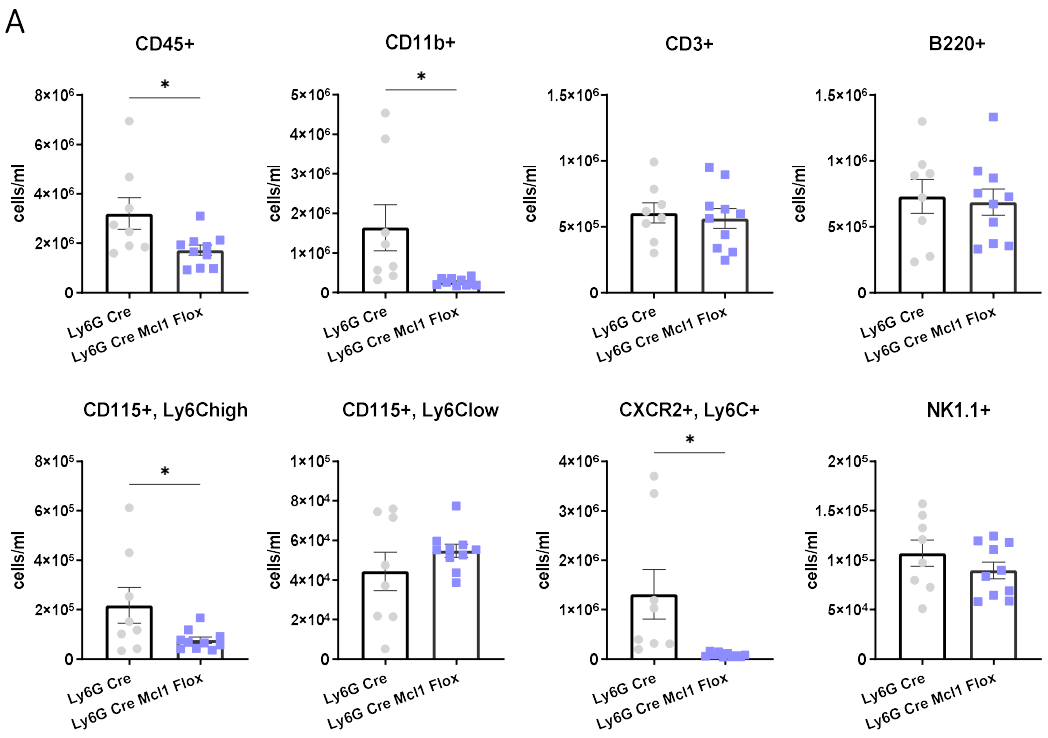


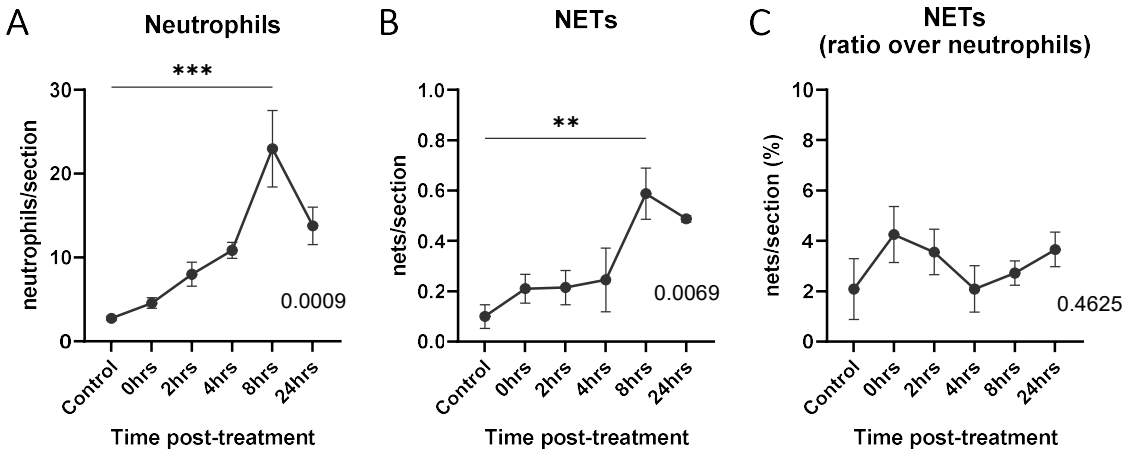
Figure 8. Flow cytometry analysis of immune populations in blood in Ly6G$^{Cre/Cre}$Mcl1$^{flox/flox}$ mice. Number of leukocytes (CD45+ cells), myeloid cells (CD11b+ cells), T lymphocytes (CD3+ cells), B lymphocytes (B220+ cells), classical monocytes (CD115+, Ly6C^{high} cells), non-classical monocytes (CD115+, Ly6C^{low} cells), NK cells (NK1.1+ cells) and neutrophils (CXCR2+, Ly6C+ cells) as determined by flow cytometry from the systemic blood in Ly6G^{Cre}Mcl1$^{flox/flox}$ or Ly6G^{Cre} mice (A). For blood samples, immune populations are represented as total number of cells in 1mL of blood. n = 8-10. Unpaired t-test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

4.2.3 Identified NET-like structures during homeostasis resemble typical NETs in a model of sterile hepatic injury

Finally, we evaluated whether our identified NET-like structures would resemble expected NET conformations typical from a model of sterile hepatic damage, which is known to involve high neutrophil recruitment into the liver. The liver can be injured by a several array of different stimuli, such as bacterial LPS (Movita et al. 2012), chemical substances, toxins or pharmacological agents (Winwood and Arthur 1993; Su 2002) such as carbon tetrachloride (CCl_4) (Luckey and Petersen 2001), endotoxins (Arthur et al. 1985), APAP (Laskin 1990) and diethylnitrosamine (DEN). Here, we used a strategy of APAP-induced liver toxicity and damage. In the liver, APAP is partly metabolized by the cytochrome P450 (CYP) (Nelson 2009; 2013), which generates the reactive metabolite N-acetyl-para-benzoquinone

imine (NAPQI) (Nelson 2009; 2013). NAPQI needs to be subsequently detoxified by the liver glutathione (GSH). An excess in NAPQI load makes the hepatocellular GSH levels to be depleted; consequently, NAPQI accumulates in the form of APAP protein adducts, which are critical for mitochondrial oxidant stress and lead to cell necrosis (Hartmut Jaeschke and Bajt 2006). APAP toxicity leads to the recruitment of neutrophils and inflammatory cytokines into the hepatic sinusoids (Lawson et al. 2000; Bajt, Farhood, and Jaeschke 2001) and the development of cell injury 4 to 24 hours after drug treatment (Lawson et al., 2000).

In our experimental set up, animals were fasted for 12-hours prior the experiment and administered a single dose (i.p.) of 300mg/kg of APAP diluted in saline solution at times 0, 2, 4, 8 and 24 hours before sacrifice. We observed a time-dependent recruitment of neutrophils into the hepatic tissue that had its peak 8 hours after APAP administration (**Fig.9A**). 24-hours after APAP administration, a decline in neutrophil numbers indicates that these cells had started to get cleared from the injured tissue (**Fig.9A**). The release of NET structures follows the same pattern as neutrophil recruitment, being highest 8 hours after APAP administration (**Fig.9B**). Ultimately, this shows that during a model of sterile hepatic injury, the NETosis capacity of newly recruited neutrophils is purely dependent on the number of neutrophils just transmigrated into the lesion tissue, where they would have casted their normal functional activity against a damage (**Fig.9C**). Identified NET-like structures in this model of hepatic toxicity also show a positive staining for previously used NET-markers (Ly6G, MPO, H3 and DAPI) and display the same phenotype that was found in the NET conformations previously identified during homeostatic conditions (**Fig.9D**). Altogether, the different animals models and experimental set ups confirmed that the identified DNA structures observed in the hepatic tissues are indeed neutrophil-borne structures, and so, true NETs.



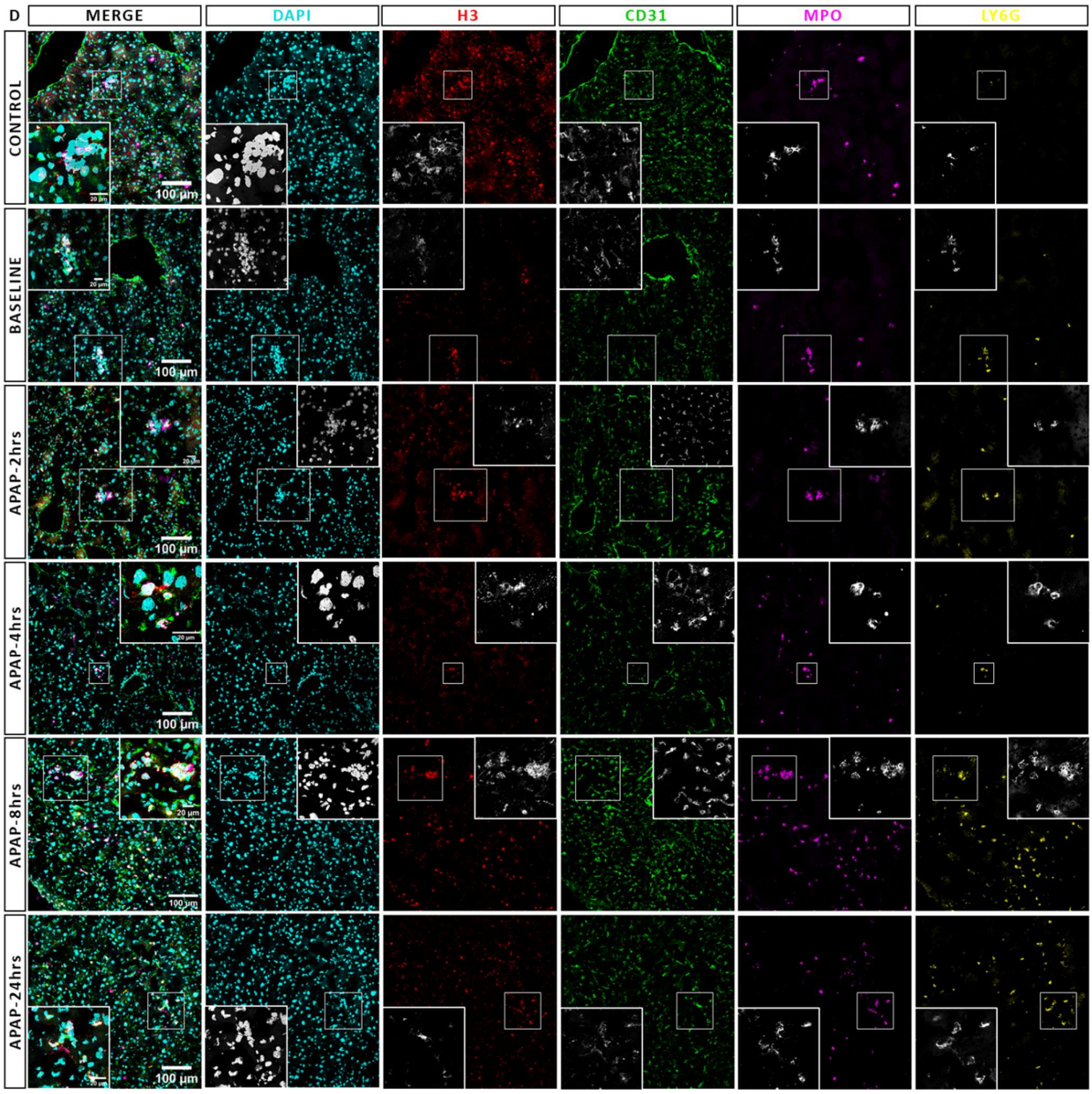


Figure 9. Characterization of NET-like structures in acetaminophen induced hepatotoxicity model in mice. Quantification of tissue-associated neutrophils per field of view (or analysed section) (A), NET-like structures per field of view (or analysed section) (B) and percentage of NETosis per field of view (or analysed section) (C) in the liver over the course of 24 hours (0hrs, 2hrs, 4hrs, 8hrs, 24hrs) after acetaminophen (APAP) administration, as determined by confocal imaging. n = 5-10; where each n represents one biological sample that has been calculated as an average of 4-5 analysed sections or fields of view. Ordinary one-way ANOVA and Dunnett's multiple comparisons test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus control. Significance was assumed with p<0.05. Representative immunofluorescence confocal images of liver cryosections showing stained nuclei (DAPI, cyan), histones (H3+, red), endothelial vessels (CD31+, green), neutrophils and macrophages (MPO+, magenta); and neutrophils (Ly6G+, yellow) at 0hrs (baseline), 2hrs, 4hrs, 8hrs, 24hrs timepoints after APAP administration (D). 20x magnification images: scale bar 100µm. 63x magnification images: scale bar 20µm.

4.3 The liver displays immune rhythmicity

Having confirmed that our identified NET-like conformations are truly NETs of neutrophil-origin, we decided next to elucidate their role in hepatic homeostasis. Absolute neutrophil counts in blood circulation oscillate with circadian frequency throughout the day (María Casanova-Acebes et al. 2013; Scheiermann et al. 2012; He et al. 2018; Pick et al. 2019). Based on our preliminary results that showed a differential capacity in NETosis between morning and night in the hepatic compartment, we wondered next whether NET release, particularly in the liver, would display similar circadian rhythmicity and whether it is a neutrophil functional mechanism intrinsically regulated by the circadian clock.

4.3.1 Myeloid cells are recruited to the hepatic tissue in a circadian way

First, we investigated whether neutrophil accumulation in the hepatic tissue and NET release in the liver follows the same circadian pattern as what has been previously reported regarding neutrophil rhythmicity in the bloodstream. We analysed, under homeostatic conditions, the levels of leukocytes (CD45+ cells), myeloid cells (CD11b+ cells), T lymphocytes (CD3+ cells), B lymphocytes (B220+ cells), classical monocytes (CD115+, Ly6C^{high} cells), non-classical monocytes (CD115+, Ly6C^{low} cells), NK cells (NK1.1+ cells), Kupffer cells (F4-80+ cells), tissue resident-macrophages (F4-80+, Ly6C+ cells), tissue resident-monocytes (Ly6C^{high} cells) and neutrophils (Ly6G+ cells) (**Fig.10**). All these cell populations were analysed in the portal vein (PV) –as a representation of the enterohepatic circulation– (**Fig.10A**), the heart right ventricle (RV) –as a representation of the systemic circulation– (**Fig.10B**), and in the liver (**Fig.10C**), through a complete 24-hours cycle represented across 7 different timepoints: ZT2, ZT6, ZT10, ZT14, ZT18, ZT22 and ZT26/2 (**Fig.10**). Neutrophil counts peaked in the early afternoon (ZT6) in the systemic circulation (**Fig.10B**) but showed a slightly later peak at ZT10 in the enterohepatic circulation (**Fig.10A**) and in the liver (**Fig.10C**). On the other hand, neutrophil counts troughed in the late afternoon (ZT14) both in the enterohepatic (**Fig.10A**) and the systemic circulation (**Fig.10B**); and past midnight (ZT18) in the liver (**Fig.10C**). We concluded that the fluctuations in neutrophil counts in the liver follow the same rhythmicity as those observed in the circulatory system. Consequently, the general CD45+ leukocyte and CD11b+ myeloid populations also exhibited rhythmic abundancies over 24h, peaking at ZT6 and troughing at the beginning of the mice active phase at ZT14, in the PV, RV and the liver (**Fig.10A-C**). The remaining analysed immune populations displayed a similar oscillatory pattern, with higher numbers during the morning both in circulation and in the hepatic tissue; and lower numbers during the night (**Fig.10A-C**).

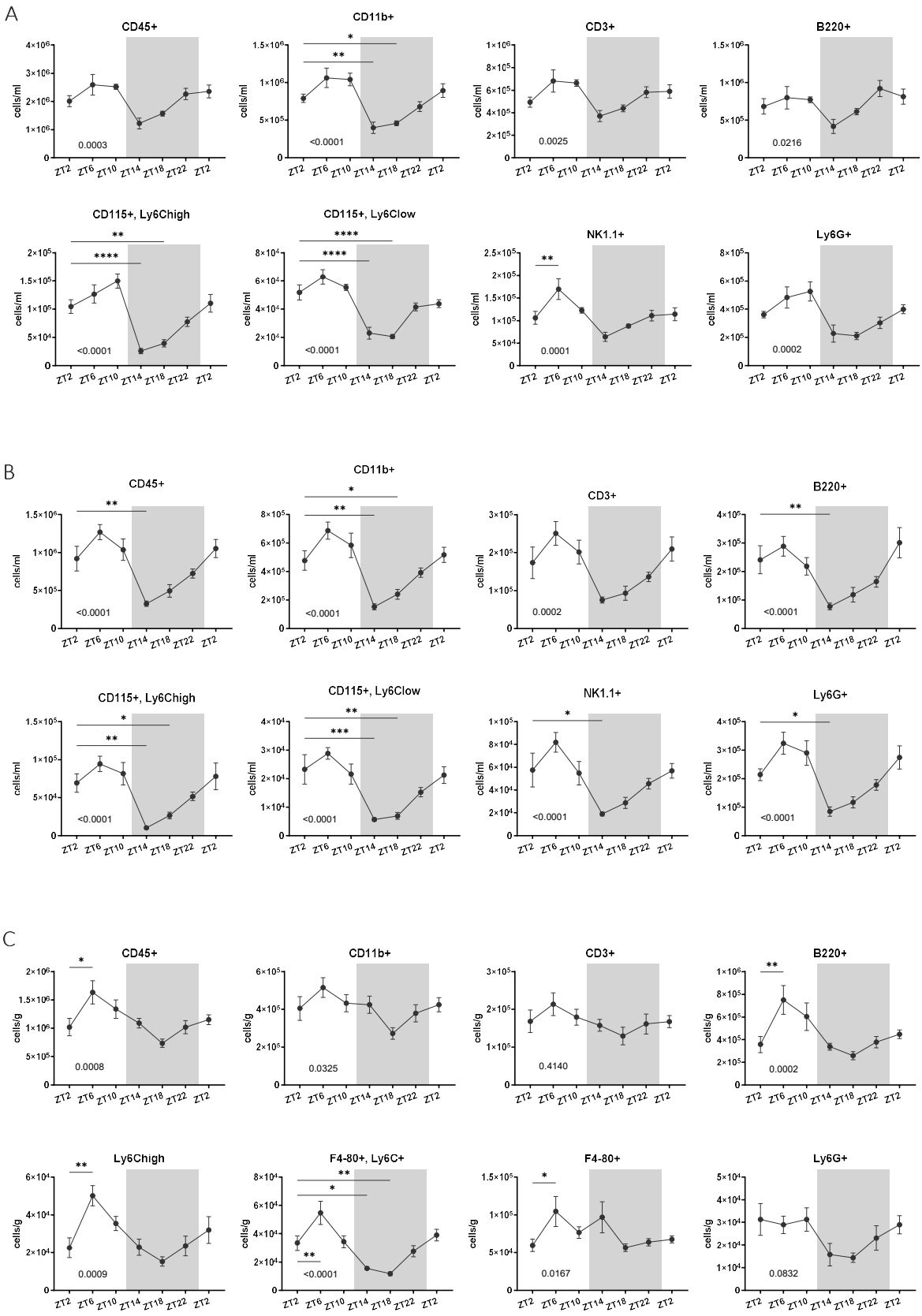

A
CD45+
CD11b+
CD3+
B220+
CD115+, Ly6Chigh
CD115+, Ly6Clow
NK1.1+
Ly6G+
cells/ml
0.0003
<0.0001
0.0025
0.0216
<0.0001
<0.0001
0.0001
0.0002
B
CD45+
CD11b+
CD3+
B220+
CD115+, Ly6Chigh
CD115+, Ly6Clow
NK1.1+
Ly6G+
cells/ml
<0.0001
<0.0001
0.0002
<0.0001
<0.0001
<0.0001
<0.0001
<0.0001
C
CD45+
CD11b+
CD3+
B220+
Ly6Chigh
F4-80+, Ly6C+
F4-80+
Ly6G+
cells/g
0.0008
0.0325
0.4140
0.0002
0.0009
<0.0001
0.0167
0.0832
ZT2 ZT6 ZT10 ZT14 ZT18 ZT22 ZT2

Figure 10. Flow cytometry analysis of immune populations in different blood compartments and liver. Curves show the number of different immune populations (leukocytes as CD45+ cells, myeloid cells as CD11b+ cells, T lymphocytes as CD3+ cells, B lymphocytes as B220+ cells, classical monocytes as CD115+, $Ly6C^{high}$ cells, non-classical monocytes as CD115+ and $Ly6C^{low}$ cells, NK cells as NK1.1+ cells, Kupffer cells as F4-80+ cells, tissue resident-macrophages as F4-80+ and Ly6C+ cells, tissue resident-monocytes as Ly6Chigh cells and neutrophils as Ly6G+ cells) as determined by flow cytometry from the PV blood (A), the RV blood (B) and the liver (C) collected every 4 hours (ZT2, ZT6, ZT10, ZT14, ZT18, ZT22 and ZT26/2) over the course of a day. For PV and RV samples, immune populations are represented as total number of cells in 1mL of blood; for liver samples, immune populations are represented as total number of cells in 1g of liver. n = 6-8. Ordinary one-way ANOVA (global p value plotted at the bottom left corner of the graphs) and Dunnett's multiple comparisons test was applied. Error bars show mean ± SEM values. Periods of darkness are shown by the shaded rectangles. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus ZT2. Significance was assumed with p<0.05.

4.3.1.1 Neutrophil retention in the enterohepatic circulation is higher at night

The blood is a compartmentalized heterogeneous system made of different cellular and plasmatic compartments. In the same way, it has been suggested that the blood molecular composition is also heterogeneous between the different vascular beds alongside the human body. Consequently, the immune cellular compartment that mediates immune tolerance and immune responses towards, for example, gut digestion-derived products in the portal circulation will potentially be heterogenous and different, compared to immune populations present in other vascular beds. In our hands, we observed that some myeloid populations get enriched differentially throughout the day in the portal vein (**Fig.11A-C**). Neutrophil retention –calculated as the ratio between the number of Ly6G+ cells in the PV blood and the number of Ly6G+ cells in the RV blood, as determined by flow cytometry (**Fig.10A-B**)–, was consistently higher in the enterohepatic circulation during the night compared to the morning time points (**Fig.11A**). The number of neutrophils in the portal blood at ZT14 was two-thirds higher than in the systemic circulation at ZT2, which conceives here the concept of blood retention (**Fig.11A**). This will be a useful concept for upcoming experiments that will help us to estimate more accurate ratios of NET release in the liver, and to conceptualize the obtained results from the evaluation of metabolic and microbial products in the different plasma samples from PV and RV blood. The same trend is observed for classical monocytes (**Fig.11B**) and non-classical monocytes (**Fig.11C**), where the same process of retention seems to occur, particularly during the early night time points (ZT14).

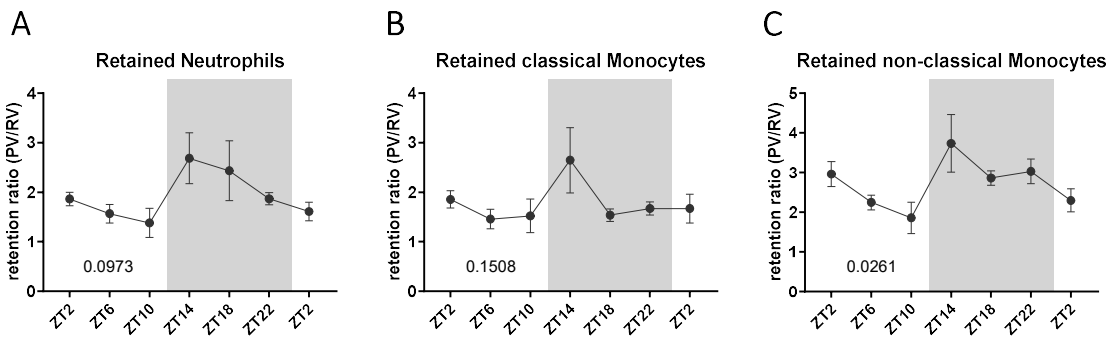


Figure 11. Retention ratios of neutrophils and monocytes in the enterohepatic blood compartment Curves show the retention ratio in the enterohepatic blood compartment of neutrophils (A), classical monocytes (B) and non-classical monocytes (C) calculated as the total number of PV blood cells divided by the total number of RV blood cells, as determined by flow cytometry over the course of a day (ZT2, ZT6, ZT10, ZT14, ZT18, ZT22 and ZT26/2). n = 6-8. Ordinary one-way ANOVA (global p value plotted at the bottom left corner of the graphs) and Dunnett's multiple comparisons test was applied. Error bars show mean ± SEM values. Periods of darkness are shown by the shaded rectangles. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus ZT2. Significance was assumed with p<0.05.

4.3.2 NETs are released in the liver in a circadian fashion

In order to evaluate the dynamics of NET structures production in the liver, sections from hepatic tissue at 7 different timepoints (ZT2, ZT6, ZT10, ZT14, ZT18, ZT22 and ZT26/2) were evaluated using IF imaging techniques. As expected, neutrophil rhythmicity in the liver follows the same infiltration pattern through a 24-hours cycle when analysed *via* confocal microscopy (**Fig.12A**), comparable with results obtained using flow cytometry analysis (**Fig.10C**). Again, we were able to identify NET structures in the liver independently of the time of the day, and we observed that the release of NET structures also fluctuated throughout the day, having its peak at the beginning of night (ZT12-ZT14) (**Fig.12B**). In agreement with previous results, the ratio of NETs over neutrophils, expressed here as the percentage of NET structures (per analysed field of view) showed again its highest peak at ZT14 (**Fig.12C**), just when animals commence their period of higher activity. Interestingly, NET release displays a likely inverse pattern with respect to neutrophil oscillations in the liver over the course of 24h, having a higher peak during the night and a troughed in the morning (**Fig.12B**). This trend is definitely different from the observed amount of NETs expelled during a model of sterile hepatic toxicity, where the amount of NET structures was consistently dependent on the number of recruited neutrophils (**Fig.9A-C**). During steady state conditions, on the other hand, there seems to be an enrichment in the NETosis capacity of the neutrophils that are present in the liver during the night times. This would suggest that "night-like" neutrophils would display an enhance capacity to undergo

NETosis compared to "morning-like" neutrophils, which could ultimately reflect on a key function to regulate tissue homeostasis, as it is not observed during purely inflammatory conditions.

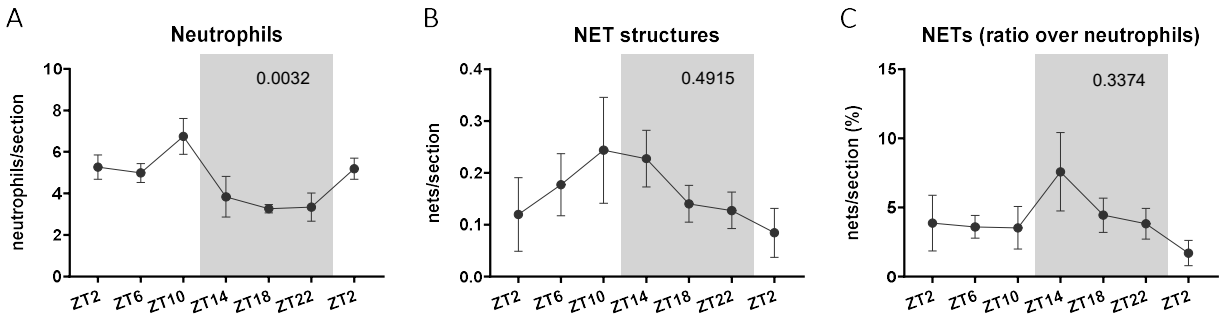


Figure 12. Evaluation of NET release in the liver over the course of a day. Quantification of tissue-associated neutrophils per field of view (or analysed section) (A), NET structures per field of view (or analysed section) (B) and percentage of NETosis per field of view (or analysed section) (C) in the liver over the course of 24 hours (ZT2, ZT6, ZT10, ZT14, ZT18, ZT22 and ZT26/2) as determined by confocal imaging. n = 6-8; where each n represents one biological sample that has been calculated as an average of 4-5 analysed sections or fields of view. Ordinary one-way ANOVA (global P value is shown plotted at the upper right corner of the graphs) and Dunnett's multiple comparisons test was applied. Error bars show mean ± SEM values. Periods of darkness are shown by the shaded rectangles. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus ZT2. Significance was assumed with p<0.05.

4.3.2.1 NET release in the liver remains constantly higher at night

Subsequently, we tried to estimate the number of "netting neutrophils" in the liver, which we believe is, in comparison to the percentage of NETs per field of view, a more accurate way to address the quantification of NET release and avoid likely underestimation of NETs. In order to do that, we need to introduce first the concept of the *marginated neutrophil pool*. Under homeostatic conditions, within special vascular beds like the bone marrow (Ussov et al. 1995), the spleen (Peters et al. 1985), the lung (Gee and Albertine 1993; Gebb et al. 1995) and the liver (Peters et al. 1985; Casanova-Acebes et al. 2018), what has been described as a marginated (i.e. slowly transiting) pool of neutrophils can be found. Margination refers to the slow and prolonged transit of neutrophils through the microvessels of specific organs, which results in discrete intravascular retained neutrophil pools. The marginated pool differs from the freely-circulating pool and the tissue-transmigrated fraction of total neutrophils. The size of an individual marginated pool is the product of the mean time taken for neutrophils to intravascularly transit through the capillary bed of the organ, and its blood flow. Whether intravascular retention is an active process mediated by adhesion molecules or a passive process due to the mechanical constriction of the cells as they move through small microvessels is still under debate. Interestingly, the major organs where neutrophil localize within blood microvessels are the lung and

the liver (Summers et al. 2010; De Filippo and Rankin 2020). Theoretically, only activated neutrophils that have been transmigrated into the tissue would be able to undergo NETosis, and not so the marginated pool of neutrophils present in the liver. Before organ harvesting, animals would have been perfusated with PBS in an attempt to remove the systemic blood that vascularize the organs. Still, we believe that a big fraction of the circulatory blood neutrophil compartment and of the marginated neutrophil pool remains within the organ even after perfusion, and so it "pollutes" the fraction of tissue-transmigrated neutrophils, making us underestimate the number of neutrophils within our tissue analysed sections that could truly cast NETs.

To calculate the number of netting neutrophils in the liver, we first assessed neutrophil retention in the liver (**Fig.13A**) by normalizing the amount of Ly6G+ cells in the Liver (**Fig.10C**) by the number of Ly6G+ cells in the PV circulation (**Fig.10A**) (obtained by flow cytometry). The absolute number of neutrophils retained in the liver circulation was then calculated (**Fig.13B**). Alternatively, the percentage of netting neutrophils in the liver (out of the total fraction of retained neutrophils) can also be estimated (**Fig.13C**). Interestingly, we observed that the percentage of the so-called "netting neutrophils" remains always higher at any time during the night (ZT14-ZT22) compared to the daylight hours (ZT2-ZT10) (**Fig.13C**). Finally, we observed that the number of absolute netting neutrophils peaks particularly at ZT14, accordingly to previous results (**Fig.13D**). A more detailed explanation of calculations can be found on *Section 3.2.5.*

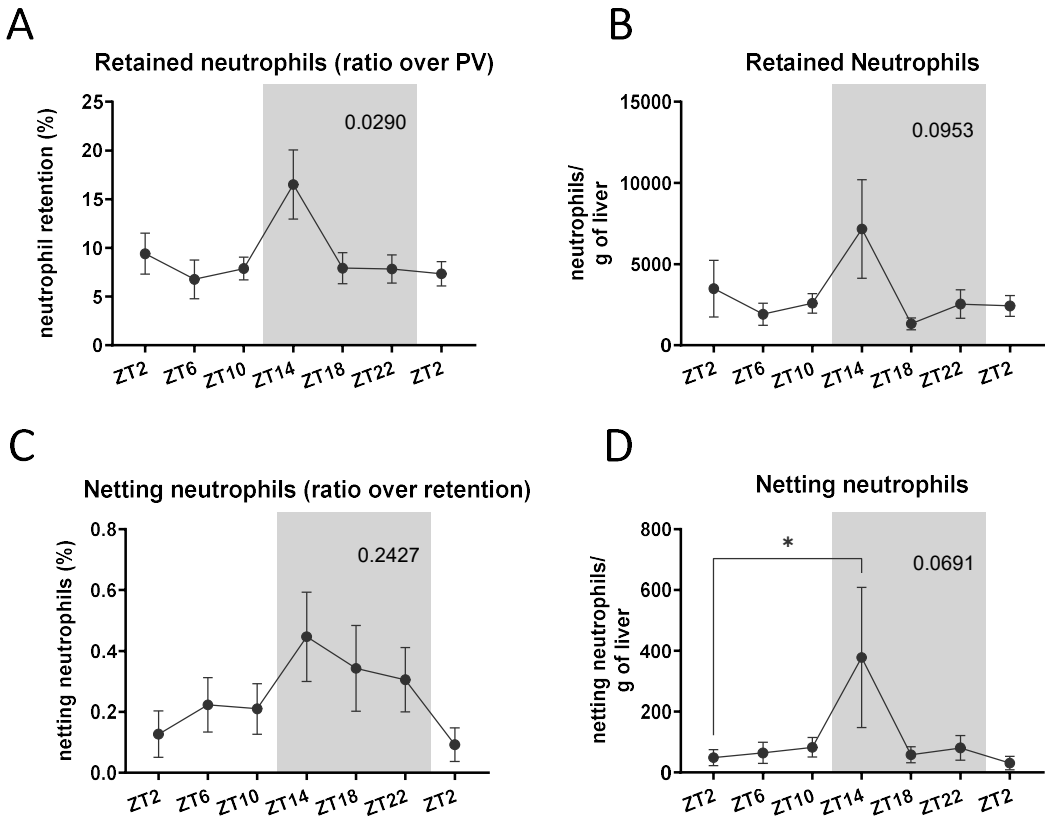


Figure 13. Estimation of the number of retained neutrophils that undergo NETosis in the liver over the course of a day. Curves show the retention ratio (in percentage) of neutrophils in the liver -calculated as the total number of liver cells divided by the total number of PV blood cells as determined by flow cytometry- (A), the number of retained neutrophils (B), the percentage of neutrophils capable to undergo NETosis -out of the fraction of retained neutrophils-, as determined by confocal imaging (C), and the number of netting neutrophils in the liver (D) evaluated every 4 hours over the course of a whole day (ZT2, ZT6, ZT10, ZT14, ZT18, ZT22 and ZT26/2). n = 6-8; where each n represents one biological sample that has been calculated as an average of 4-5 analysed sections or fields of view. Ordinary one-way ANOVA (global P value is shown plotted at the upper right corner of the graphs) and Dunnett's multiple comparisons test was applied. Error bars show mean ± SEM values. Periods of darkness are shown by the shaded rectangles. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus ZT2. Significance was assumed with p<0.05.

4.3.2.2 NET release in Albumin^{Cre} Bmal$^{flox/flox}$ mice

The up-coming experiment was carried out in collaboration with the *Centro Nacional de Investigaciones Cardiovasculares* (CNIC) and the group of Dr. Guadalupe Sabio, who kindly provided the Albumin^{Cre}Bmal$^{flox/flox}$ animals and performed the experiments in Madrid, Spain. Based on our just reported data, NET release in the liver displays circadian rhythmicity. Accordingly, NET rhythmicity could be being modulated on three different levels: The central clock level, the immune compartment

level, particularly on the neutrophil, or the niche-specific hepatic compartment level, particularly on the hepatocyte. The Albumin^{Cre}Bmal$^{flox/flox}$ animal model would help us to discern the particular effects of *Bmal1* in the liver, from the more systemic consequences of deleting Bmal1 in the central clock, which impacts the whole-body rhythmicity. If the hepatocyte clock system would indeed modulate neutrophil oscillations and NET release in the liver, then we should be able to observe differential NETosis ratios in animal models where only the hepatocyte circadian clock has been disrupted, but where the central clock, peripheral clocks and feeding rhythms remain intact. On the contrary, if food intake would be indeed the primarily entrainment factor (*zeitgeber*) in the liver and one of the major responsible of the oscillations in NET release throughout the day in the hepatic tissue, then we could anticipate that the ablation of the hepatocyte clock would have little to no effect on neutrophil and NET rhythmicity in the hepatic tissue.

In agreement with our previous data, neutrophil numbers peaked in the morning (ZT2) and troughed in the night (ZT14) regardless of the mice genotype in the RV, PV blood compartments and the liver (**Fig.14A-C & Fig.15A**). The general leukocyte population (CD45+ cells) and the classical monocyte population (Ly6C^{high}) follows a similar trend, showing higher numbers at ZT2 in the RV, the PV blood compartment and the liver compared to the ZT14 timepoint (**Fig.14A-C**). Notably, the amount of NETs/mm^2 found in the liver and the percentage of NETs/mm^2 followed a comparable trend both for Albumin^{Cre}Bmal$^{flox/flox}$ and Albumin^{Cre} littermate-control animals. NETosis augments in the liver during the night (ZT14) regardless of the mice genotype (**Fig.15B-C**). Given that both mouse genotypes display the same pattern of NET activity, we concluded that rhythmicity of NET release seems to be at least partly independent of the hepatic-specific clock gene regulation. That way, other physiological regulators of the neutrophil activity emerged as potential regulators of homeostatic NET release. Noteworthy, Albumin^{Cre}Bmal$^{flox/flox}$ show a significant increase in the percentage of NET release both in the morning and in the night compared to littermate wild-type animals (**Fig.15B-C**). This chronically higher NET activity might reflect on an inflammatory-prone scenario driven by the hepatic-specific clock disruption.

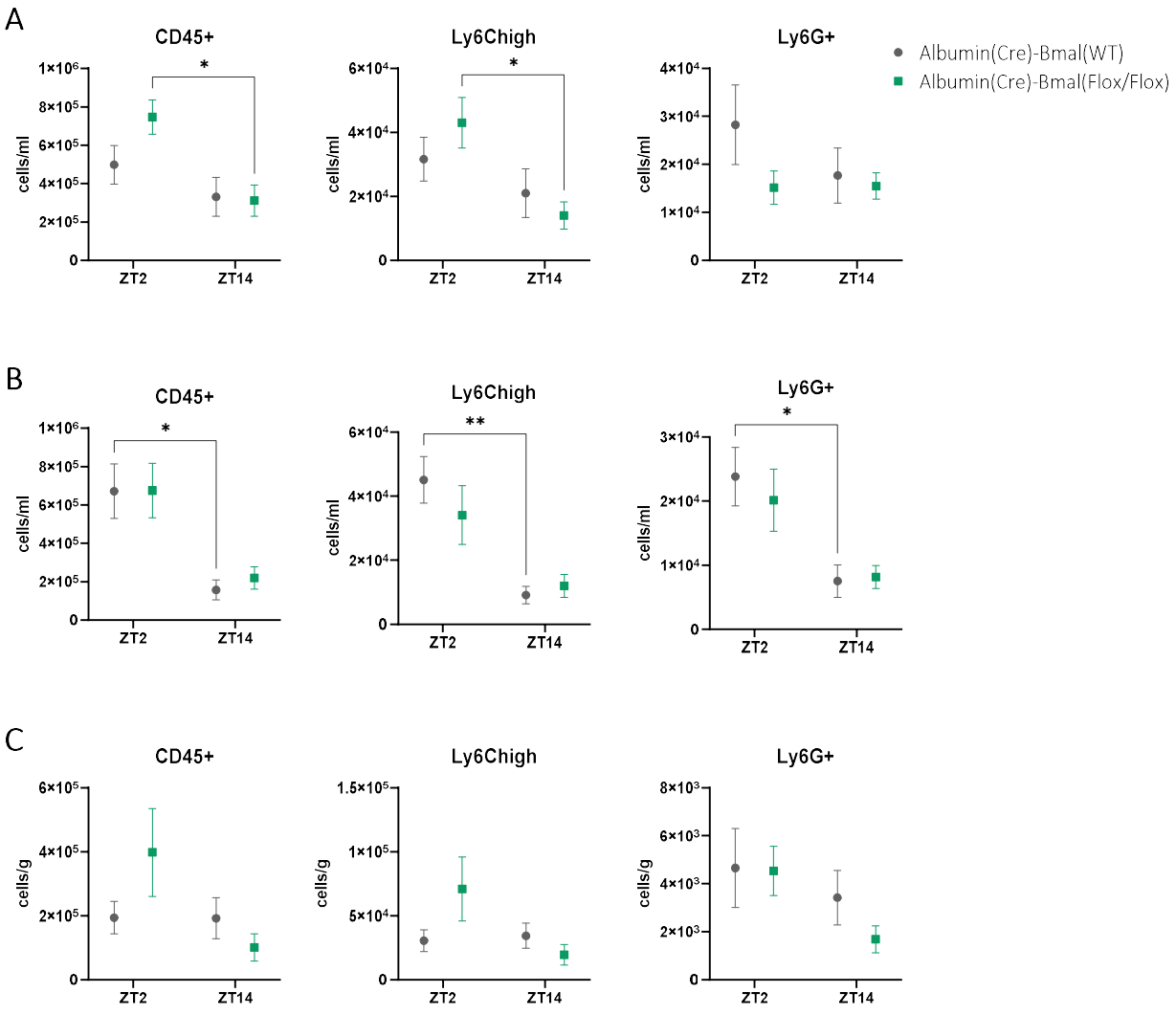


Figure 14. Flow cytometry analysis of immune populations in Albumin^{Cre}Bmal$^{flox/flox}$ mice. Number of leukocytes (CD45+ cells), myeloid cells (CD11b+ cells) and neutrophils (Ly6G+ cells) as determined by flow cytometry from the (A) PV blood, (B) the RV blood and (C) the liver in Albumin^{Cre}Bmal$^{flox/flox}$ or Albumin^{Cre} mice at 9am (ZT2) and 9pm (ZT14). For blood samples, immune populations are represented as total number of cells in 1mL of blood; for liver samples, immune populations are represented as total number of cells in 1g of liver. n = 5-6. Ordinary two-way ANOVA and Tukey´s multiple comparisons test was applied. P values represented in numbers show the results from the row effect (horizontal) and column effect (vertical) from the two-way ANOVA test. P values represented as asterisks (*) show the results from the multiple comparisons test. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

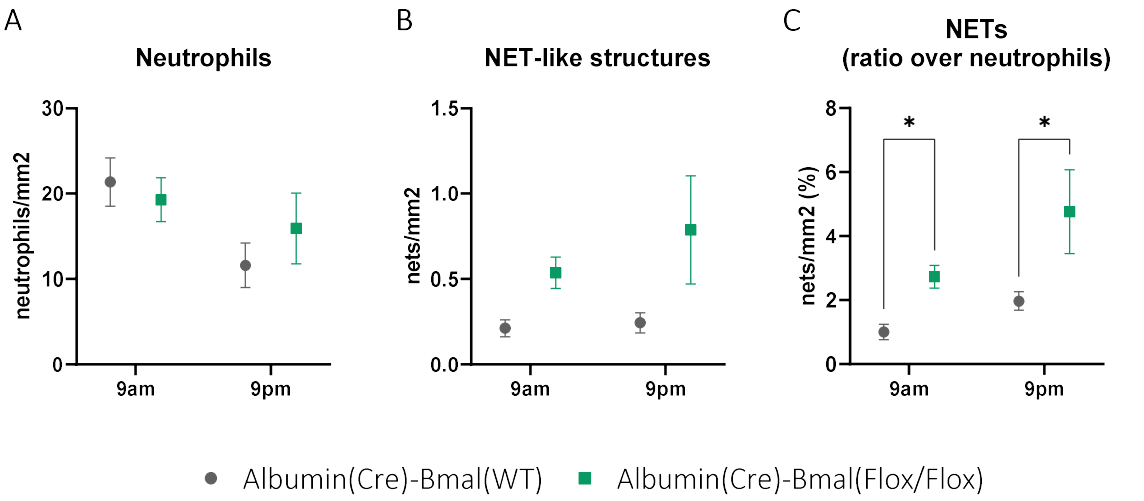


Figure 15. Characterization of NETs in Albumin^{Cre}Bmal$^{flox/flox}$ mice. Quantification of tissue-associated neutrophils per mm^2, (A), NET-like structures per mm^2 (B) and percentage of NETosis per mm^2 (C) in livers of Albumin^{Cre}Bmal$^{flox/flox}$ or Albumin^{Cre} mice at ZT2 and ZT14. n = 5-6. Ordinary two-way ANOVA and Tukey´s multiple comparisons test was applied. P values represented in numbers show the results from the row effect (horizontal) and column effect (vertical) results from the two-way ANOVA test. P values represented as asterisks (*) show the results from the multiple comparisons test. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

4.3.2.3 NETs in the liver are in close contact with Kupffer Cells

At the end of this chapter revolving around NETs in the hepatic tissue, we decided to evaluate some immune populations that might appear in closer contact to the identified NET structures. That would also allow us to discriminate between our identified NET-like structures and some tissue-resident immune populations, such as tissue-resident macrophages. Liver sections were stained for MPO, Ly6G and H3 or CD31 to again localize NET structures. In addition, we used the AF647-directly conjugated F4/80 antibody (a surface marker for mononuclear phagocytes) to target Kupffer cells. In agreement to what has been described in literature, Kupffer cells are indeed present all around the hepatic tissue and, in our hands, they also co-localize in the surroundings of the aggregated identified NET-like structures (**Fig.16**). Indeed, some authors have already revealed the existence of communication mechanisms between tissue-resident macrophages and neutrophils (Uderhardt et al. 2019). Our own findings might reflect on a capacity of NETs to establish or mediate communication with other cell types, and collaborate on functions such as antigen presentation and modulation in the development of the immune system, coordination of the immune response by mediating trapping, exposure and clearing of bacteria, or promoting anti-inflammatory mechanisms that help resolving inflammation.

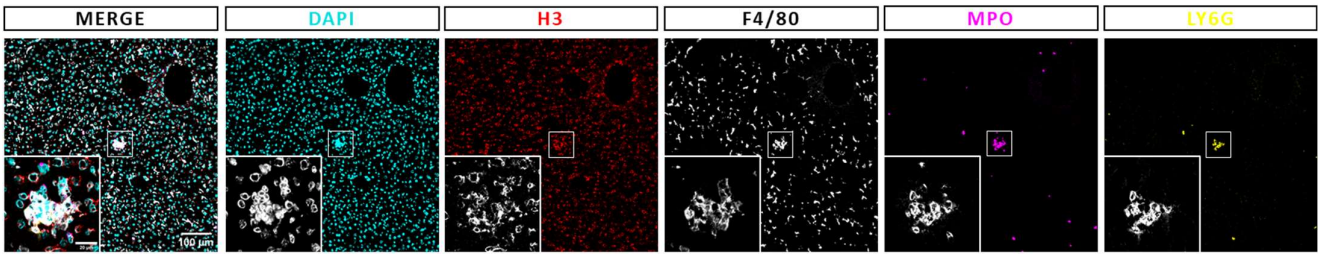


Figure 16. Identification of Kupffer Cells in close contact to NETs. Representative immunofluorescence confocal images of liver cryosections showing stained nuclei (DAPI, cyan), endothelial vessels or Histone3 (CD31+ or H3+, respectively; red), Kupffer cells (F4/80+, white), macrophages (MPO+, magenta) and neutrophils (Ly6G+, yellow) during the night. 20x magnification images: scale bar 100µm. 63x magnification images: scale bar 20µm.

4.4 NET release in the liver can be influenced by the dietary behaviour

As previously described (*Section 1.3.2*), light is the main *zeitgeiber* or entrainment cue for the central clock, while food is the most important *zeitgeiber* for the entrainment of the peripheral clock in peripheral organs. Although we cannot entirely exclude a clock-intrinsic regulation of NETosis as a neutrophil function during homeostatic conditions in the liver, the now canonically observed fact that NET release peaks during the night times, which as has been previously mentioned, coincides with the starting of the active period of mice (a moment of the day when they ingest higher amounts of food), made us speculate that NET release could indeed be regulated by food intake, dietary timing patterns, the metabolic composition of the diet and/or the diet-derived metabolic products.

When fed *ad libitum* under a regular light-dark cycle, mice show a clear daily rhythmic pattern of food intake and locomotor activity (Acosta-Rodríguez et al. 2017; Tran et al. 2018; Hatori et al. 2012; Hut et al. 2011). Mice are mainly active during the night and their feeding pattern is predominantly nocturnal: food consumption starts increasing during the last hour of the light phase at ZT12 and remains always higher during the night as compared to the daytime (Acosta-Rodríguez et al. 2017; Hut et al. 2011). It is remarkable that the peak of NET release in the liver coincides with the timing of high food consumption in mice. Aiming to evaluate the effect of feeding patterns on the release of NET structures, animal studies were carried out where a temporal dietary restriction (time-restricted feeding) applied, and different diet compositions (**Table 3-10. Diet compositions**) were used. In the upcoming experiments, mice were subjected to a 10-hours period of time-restricted feeding conditions for 14 consecutive days, where food was absent during most part of the morning time (ZT2-ZT12), was given again at the beginning of the night (ZT12) and was present for the remaining of the night time (ZT12-ZT26). We want to emphasize here that our goal was not to evaluate the impact of time-restricted feeding on NET release. Instead, we used time-restricted feeding as a methodology of eating-adaptation. Because time-restricted feeding is not a normal eating pattern in mice, as it implies non-

physiological long fasting periods (with associated stress) that animals do not typically experience, by using this feeding methodology we would ensure that when animals are provided with a new input of nutrients before the experimental procedure occurs, they would consume a sufficient amount of food to trigger an effect on NET release.

4.4.1 NET release after short-term high-fat diet consumption

Two hours before sacrifice, from ZT12 to ZT14, we granted animals *ad libitum* access to a 1.25% cholesterol-supplemented high fat diet (HFCholD) or a high-fat and high-sucrose diet (HFSugD), as a representation of a typical western-style diet. LFD was used as a control diet, and fasting was also evaluated as an extra control condition where animals have no access to food. During fasting conditions, the same time-restricted feeding pattern applies. Conversely, during the same period of two hours between ZT12 and ZT14, mice were not provided with any food stimuli and so fasting time was just prolonged. After that, we evaluated neutrophil numbers (Ly6G+, MPO+, DAPI+ cells) and NET release (Ly6G+, MPO+, H3+, DAPI+ structures) in the hepatic compartment (**Fig.17**). The number of neutrophils remained comparable between all analysed groups except for HFSugD fed animals, where neutrophil recruitment into the hepatic tissue was significantly increased (**Fig.17A**). Notably, the number of identified NET-like structures per analysed section as well as the ratio of NETosis was also highest in animals fed a HFSugD and lowest in animals under fasting conditions (**Fig.17B,C**). In general, NETosis ratios were higher in HFD fed animals compared to animals fed a LFD or animals that did not encounter food. From here we concluded that the nutritional composition of western-like diets (rich in fats and sugars) can shape neutrophil activity in homeostasis and trigger NET release.

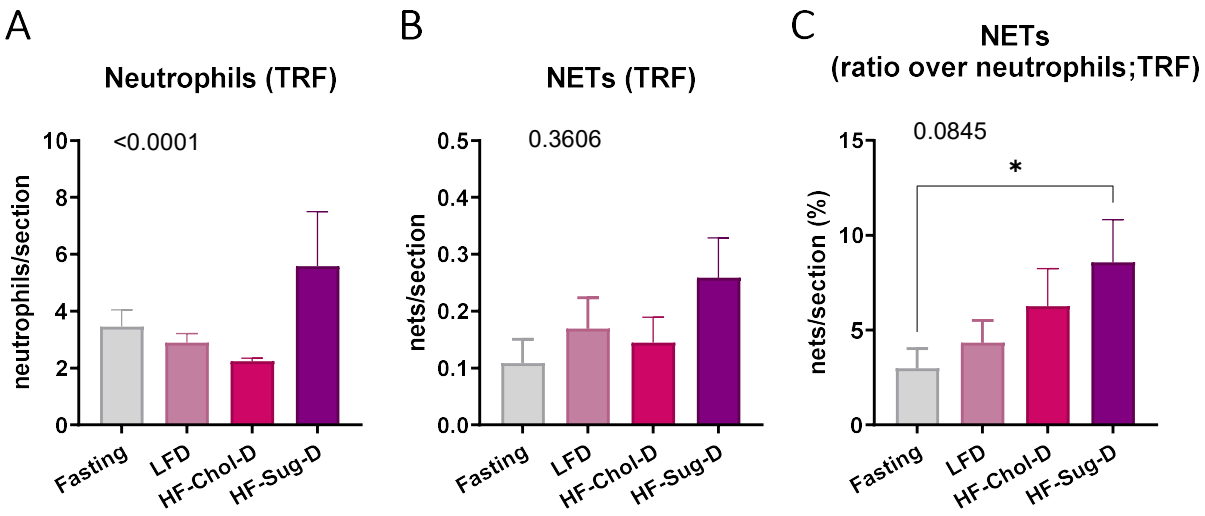


Figure 17. Characterization of NETs after short-term metabolic alterations. Quantification of tissue-associated neutrophils per field of view (or analysed section) (A), NET-like structures per field of view (or analysed section) (B) and percentage of NETosis per field of view (or analysed section) (C) in the liver after 2-hours of a high-fat-high-cholesterol meal, 2-hours of a high-fat-high-sucrose meal, 2-hours of low-fat diets or 2-hours of fasting conditions, as determined by confocal imaging. Note that mice were subjected to a 14 days pre-adaptation period of 10-hours of time-restricted feeding conditions (8hrs:14hrs) in order to ensure they ingest sufficient food when encounter a new meal. In the fasting experimental control group of mice, fasted conditions were consequently just prolonged during the experimental procedure. n = 5-19; where each n represents one biological sample that has been calculated as an average of 4-5 analysed sections or fields of view. Ordinary one-way ANOVA (global p value plotted at the top left corner of the graphs) followed by Tukey's post hoc test was performed and Dunnett's multiple comparisons test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus *Fasting*. Significance was assumed with p<0.05.

4.4.2 NET release under short-term fasting conditions in Albumin^{Cre} Bmal$^{flox/flox}$ mice

This experiment was carried out in collaboration with the *Centro Nacional de Investigaciones Cardiovasculares* (CNIC) and the group of Dr. Guadalupe Sabio, who kindly provided the Albumin^{Cre} Bmal$^{flox/flox}$ animals and performed the experiments in Madrid, Spain. Experimental approach was that, during the same period of two hours between ZT12 and ZT14, food was removed and animals were fasted until further sacrificed. Under fasted conditions during the night, neutrophil numbers remained low compared to *ad libitum* fed animals, particularly in the Albumin^{Cre}Bmal$^{flox/flox}$ mice genotype (**Fig.18A**). Notably, the amount of NETs/mm^2 found in liver sections and the percentage of NETosis/mm^2 follows a comparable trend both for Albumin^{Cre}Bmal$^{flox/flox}$ and Albumin^{Cre} littermate-control animals. As expected, NET release was increased in the liver during the night (ZT14) but was this release was abrogated in the absence of food. While fasted, the levels of NETosis resemble those at ZT2, during the morning timepoint (**Fig.18B-C**). Given that both genotypes displayed a comparable pattern in NET activity, this reinforced our idea that NET release seems to be at least partly independent of the hepatic-specific clock gene regulation but rather influenced by food consumption. Noteworthy, Albumin^{Cre} Bmal$^{flox/flox}$ animals have a significant increase in the percentage of NET release for all evaluated conditions (ZT2, ZT14, and ZT14-fasting) compared to Albumin^{Cre} control animals (**Fig.18C**). This chronic higher NET activity might reflect on an inflammatory-prone scenario driven by the hepatic-specific clock disruption.

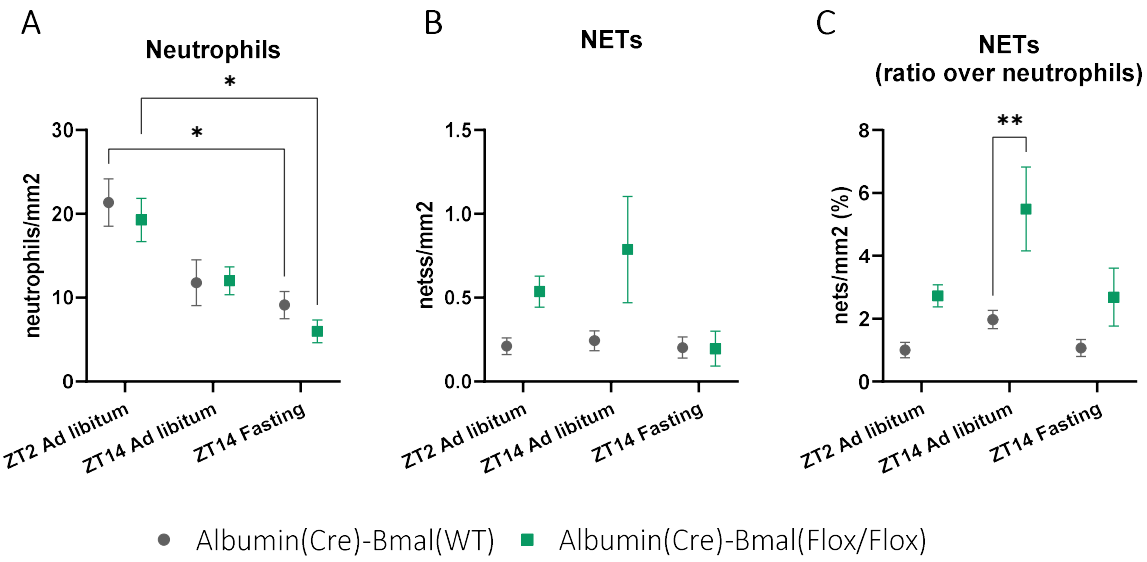


Figure 18. Characterization of NETs in Albumin^{Cre}Bmal$^{flox/flox}$ mice under fasting conditions. Quantification of tissue-associated neutrophils per field of view (or analysed section) (A), NET-like structures per field of view (or analysed section) (B) and percentage of NETosis per field of view (or analysed section) (C) in the liver at ZT2, ZT14 or after 2-hours of fasting conditions, as determined by confocal imaging. Note that here; due to technical difficulties mice were not subjected to a pre-adaptation period of time-restricted feeding. n = 5-6. Ordinary two-way ANOVA and Šídák's multiple comparisons test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

4.4.3 Evaluation of pattern recognition receptor ligands

So far, we have elucidated that NETs are present with a higher ratio in the hepatic compartment during the night time, in a mechanism that seems to be potentially dependent on diurnal rhythms modulated by food intake and the metabolic composition of the diet; being fat diets those with a higher capacity to induce NET release. Recent studies have shown that even before the onset of obesity and its derived metabolic complications, dietary fats have deleterious effects on intestinal epithelial integrity and gut barrier function (Rohr et al. 2020; Araújo et al. 2017). Fat-rich diets can lead to impaired gut permeability by altering the distribution of intestinal adherens junctions (AJs) and tight junctions (TJs), which ultimately impairs barrier function (Rohr et al. 2020). Fat-consumption-related deleterious effects seem mediated by an increase in LPS epithelial-absorption, which can induce local responses and pro-inflammatory cytokines secretion (IL-1B, IL-6, IFN-γ, and TNF-α) (Pendyala, Walker, and Holt 2012; André, Laugerette, and Féart 2019). Pro-inflammatory cytokines release creates an scenario that facilitates the passage of bacteria, bacterial components (e.g. LPS, peptidoglycan or flagellins) and nutritional metabolites (e.g. secondary bile acids) from the intestinal lumen into the circulation (Capaldo and Nusrat 2009; C. Shi et al. 2019). Particularly, LPS can diffuse from the gut to

the circulatory system either in a passive diffusion mechanism or directly incorporated into chylomicrons (Warrington et al. 2011). Indeed, a moderate increase of LPS plasmatic concentrations during postprandial conditions define metabolic endotoxemia, which has been related to immune modulation (Chassaing and Gewirtz 2014).

Having these ideas in mind, being NETs main characters in mediating immune responses against bacteria and microbes, and being nowadays undeniable that an unbalance diet impacts gut barrier function; we decided to evaluate whether microbial dispersion, microbial products and meal-derived digestion products and metabolites that travel through the enterohepatic circulation and are translocated into the liver -altogether define as PRR ligands-, could modulate NET response and immune function in the hepatic compartment in homeostatic conditions.

4.4.3.1 Evaluation of pattern recognition receptor ligands in systemic blood

Aiming to analyse the presence of specific bacterial products and PRR ligands in plasma samples from systemic blood (**Fig.19**) or peripheral (RV) and enterohepatic (PV) blood (**Fig.20**), and liver supernatant homogenates (*not shown*) from C57BL6/J mice, we used here some HEK293 PRR reporter cell lines (*Section 3.6.2*). In order to assess the potential influence of the food on NET release and intestinal barrier function, and understand how these variables are interconnected, we performed an indirect evaluation of the presence of microbes through the quantification of TLR activity and PRR ligand concentrations at different times of the day. That allowed us to evaluate whether the spreading of bacterial-products is altered in the presence or absence of NETs (night and morning timepoints), when mice encounter food. Previous studies have already reported a diurnal fluctuation of barrier function and intestine permeability based on the diurnal oscillations of PRR ligand concentrations observed in portal vein, peripheral blood and the liver (Tuganbaev et al. 2020; Thaiss et al. 2018). In our hands, we observed a trend in the oscillations of TLR2, TLR4 and NOD1 ligands in systemic blood between day (ZT2) and night (ZT14) time-points, where the night-associated plasma samples showed a higher capacity to stimulate PRRs (**Fig.19**). This might reflect on a higher accumulation of pathogen-associated antigens and gut-derived molecules present in the blood during the night.

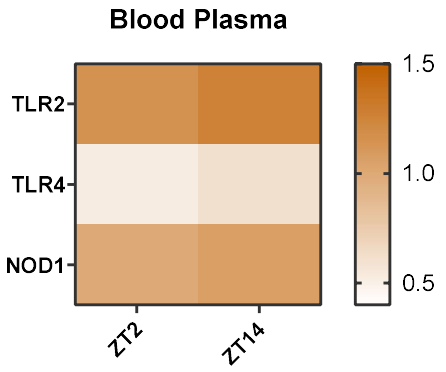


Figure 19. HeatMap of PRR ligands expression in systemic blood. HEK293 PRR reporter cells lines (HEK-Blue™-mTLR2, HEK-Blue™-mTLR4, HEK-Blue™-mTLR2 and HEK-Blue™-mNOD1) were stimulated and PRR ligand concentrations were measured utilizing a reporter cell assay examined in plasma from systemic blood. Samples were collected at ZT2 and ZT14 timepoints from *ad libitum* fed animals. n = 7-8. Data represents OD values.

4.4.3.2 Evaluation of pattern recognition receptor ligands in different vascular compartments

Based on the differences spotted in the systemic blood compartment between night and day, we decided next to evaluate PRR ligand concentrations in plasma samples from the RV or the PV blood compartments across the day (ZT2, ZT6, ZT10, ZT14, ZT18, ZT22 and ZT26/2 timepoints). TLR2 and TLR4 ligands concentration seemed slightly higher in the PV plasma samples compared to the concentration observed in RV plasma samples (**Fig.20**), consistently throughout the day. Particularly at ZT14, the concentration of PRR ligands seems to be at its highest (**Fig.20**), coinciding with the time when animals had started their active period and had commenced ingesting food.

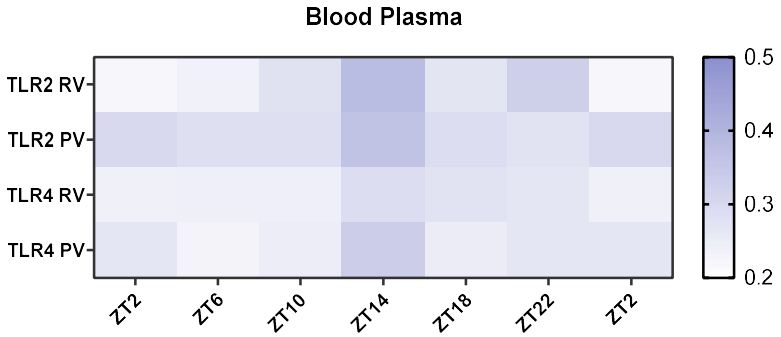


Figure 20. HeatMap of PRR ligands expression over 24 hours in PV and RV blood. HEK293 PRR reporter cells lines (HEK-Blue™-mTLR2, HEK-Blue™-mTLR4, HEK-Blue™-mTLR2) were stimulated and PRR ligand concentrations were measured utilizing a reporter cell assay examined in plasma from PV and RV. Samples were collected every 4 hours at ZT2. ZT6, ZT10, ZT14, ZT18, ZT22 and ZT26/ZT2. n = 8. Data represents OD values.

4.4.3.3 Evaluation of endotoxin levels in plasma samples

Next, we decided to perform a quantitative determination of the levels of endotoxin (i.e. LPS) in plasma samples and supernatant liver homogenates from these same experimental conditions. LPS levels were evaluated in ZT2 and ZT14 plasma samples from the systemic blood compartment. We could observe a trend showing that night-associated plasma samples tend to accumulate higher levels of LPS content (**Fig.21**). Unfortunately, plasma samples systematically contained a barely detectable amount of endotoxin, and the detection limit (0.05 EU/mL) was even not reached for most of the remaining analysed samples (supernatant liver homogenates – *not shown*). Briefly, no further conclusions have been drawn out from this experiment.

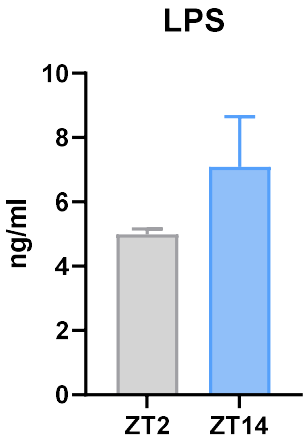


Figure 21. Endotoxin levels in plasma samples from systemic blood. Assessment of LPS determination via EndoLISA® endotoxin detection assay in plasma samples from peripheral blood (A) or from RV and PV blood (B) at ZT2 or ZT14; or from DNAse I and saline injected animals at ZT14 (C). Determination of EU levels and analysis were performed using a 4-parameters logistic regression model. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

4.5 NETs are modulators of inflammation during the mice activity phase

So far during this PhD project, NETs, during homeostasis, have emerged as potential key players in the barrier function of the hepatic compartment, as they are highly present in the liver during the mice activity phase, a moment of the day when an increased input of diet-derived metabolic products and microbial-associated antigens populate the enterohepatic system. In order to further characterize the physiological function of homeostatic NET release during the mice activity phase, we chemically disrupted these structures and evaluated their potential impact on the hepatic immunological profile.

4.5.1 NET-scaffold rupture after DNAse I administration prompts inflammation

Here, animals were injected i.p. with 10U DNase I (5mg/kg/d) 1 hour (ZT13) prior sacrifice (ZT14). Different immune cell populations were analysed in blood and liver (**Fig.22A,B**). The total number of leukocytes (CD45+ cells), myeloid cells (CD11b+), classical monocytes (CD115+, Ly6C^{high} cells), non-classical monocytes (CD115+, Ly6C^{high} cells) and neutrophils (Ly6G+ cells) increased significantly in blood after DNAse I administration compared to control saline-injected animals (**Fig.22A**). Particularly, blood neutrophilia reflects on a systemic pro-inflammatory condition. Interestingly, the analysed lymphoid compartment, here T lymphocytes (CD3+ cells), B lymphocytes (B220+ cells) and NK cells (NK1.1+ cells) were not affected upon injection of DNAse I (**Fig.22A**). In the liver, DNAse I administration also induces an increase in the number of neutrophils (Ly6G+ cells), although this trend seems more controlled as compared to the numbers observed in blood (**Fig.22B**). Indeed, when analysed using immunofluorescence techniques and confocal imaging, neutrophils are confirmed significantly increased in the liver compartment after a single shot of DNAse I (**Fig.23A,C**). B lymphocytes and NK cells appear significantly reduced in the liver after the DNAse I administration (**Fig.22B**). Treatment with DNAse I significantly reduced the amount of NET-like structures present in the liver during the night (**Fig.23A,C**), likely due to the enzymatically disruption of the NET-scaffold. Consequently, the ratio of NETosis (**Fig.23A,C**), the percentage of neutrophil retention as well as the number of netting neutrophils in the liver are dramatically decreased after DNAse I administration (**Fig.23B,C**). In conclusion, the NETosis capacity of neutrophils after a single DNAse I dose is reduced, and the absence of NETs during homeostatic conditions seems to boost inflammation in the hepatic tissue and creates a condition of neutrophilia in the circulatory system.

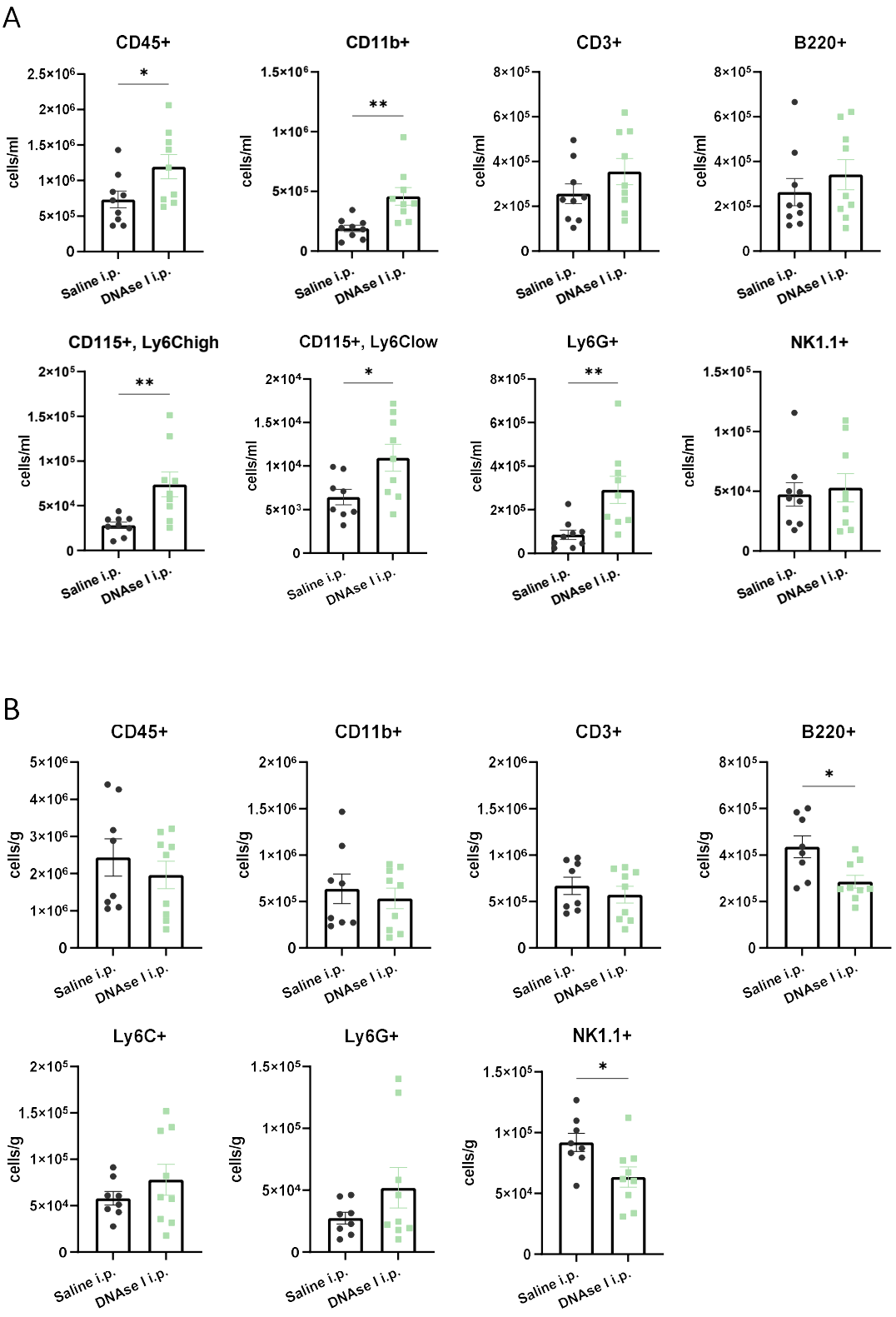
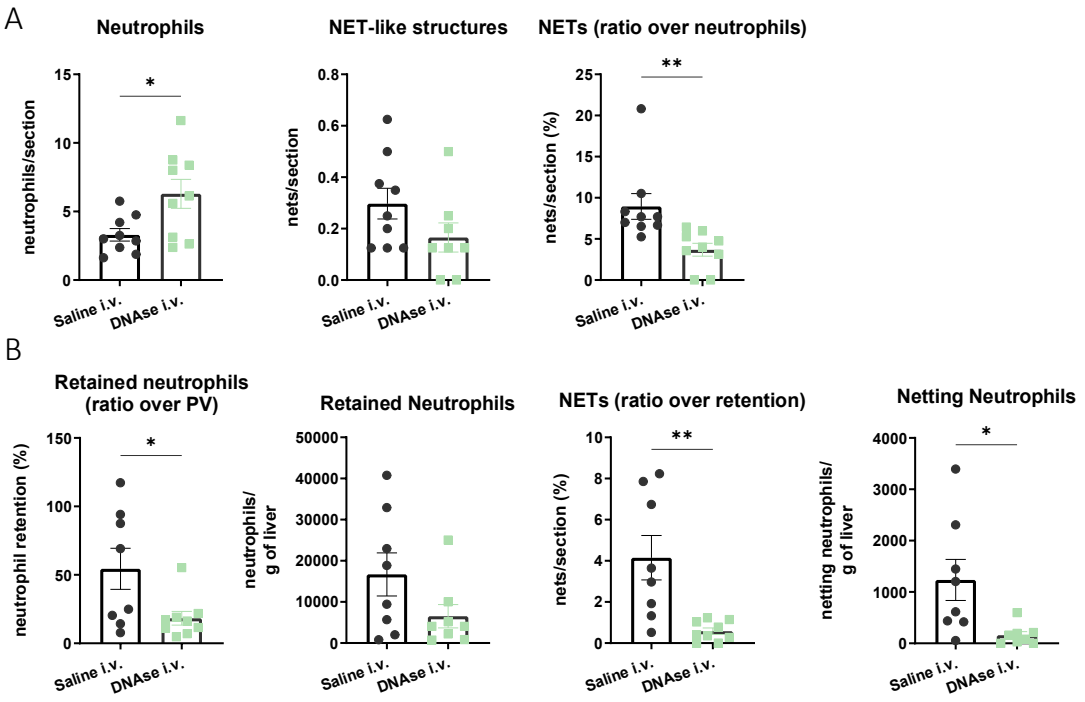

A
CD45+
CD11b+
CD3+
B220+
CD115+, Ly6Chigh
CD115+, Ly6Clow
Ly6G+
NK1.1+
cells/ml
Saline i.p.
DNAse I i.p.
B
CD45+
CD11b+
CD3+
B220+
Ly6C+
Ly6G+
NK1.1+
cells/g
Saline i.p.
DNAse I i.p.

Figure 22. Flow cytometry analysis of immune populations in blood and liver after DNAse I administration. Number of leukocytes (CD45+ cells), myeloid cells (CD11b+ cells), T lymphocytes (CD3+ cells), B lymphocytes (B220+ cells), classical monocytes (CD115+, Ly6C^{high} cells), non-classical monocytes (CD115+, Ly6C^{low} cells), NK cells (NK1.1+ cells) and neutrophils (Ly6G+ cells) as determined by flow cytometry from the systemic blood (A) and the liver (B) at ZT14 in DNAse I or saline injected animals. For blood samples, immune populations are represented as total number of cells in 1mL of blood; for liver samples, immune populations are represented as total number of cells in 1g of liver. n = 8-10. Unpaired t-test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.



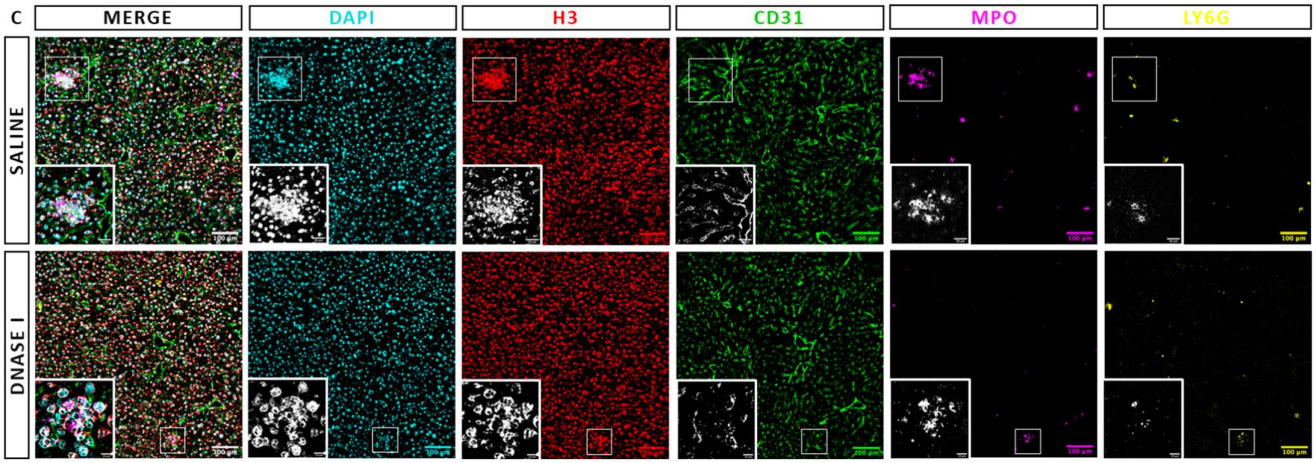


Figure 23. Evaluation of neutrophils and NET release in the liver after DNAse I administration. Quantification of tissue-associated neutrophils per field of view (or analysed section), NET structures per field of view (or analysed section) and percentage of NETosis per field of view (or analysed section) in the liver as determined by confocal imaging (A). Graphs show

the retention ratio (in percentage) of neutrophils in the enterohepatic blood compartment, calculated as the total number of liver cells divided by the total number of PV blood cells as determined by flow cytometry in saline injected or DNAse I administered animals (B). Analysis of the number of retained neutrophils, the percentage of NETosis -out of the fraction of retained neutrophils-, and the number of netting neutrophils (retained) (B). Representative immunofluorescence confocal images of liver cryosections showing stained nuclei (DAPI, cyan), histones (H3+, red), endothelial vessels (CD31+, green), neutrophils and macrophages (MPO+, magenta); and neutrophils (Ly6G+, yellow) at ZT14 in saline injected (C) or DNAse I administered animals (D). 20x magnification images: scale bar 100µm. 63x magnification images: scale bar 20µm. n = 8-10, where each n represents one biological sample that has been calculated as an average of 4-5 analysed sections or fields of view. Unpaired t-test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

4.5.2 NETs inhibition after BB-Cl-Amidine administration prompts inflammation

Treatment with BB-Cl-Amidine (10 mg/kg/d) 3 hours (ZT11) prior sacrifice (ZT14) also significantly increased the number of myeloid cells (CD11b+) and circulating neutrophils (Ly6G+ cells) compared to saline-injected control animals (**Fig.24A**), which also reflects on a situation of blood neutrophilia. The rest of the evaluated immune populations, leukocytes (CD45+ cells), T lymphocytes (CD3+ cells), B lymphocytes (B220+ cells), classical monocytes (CD115+, $Ly6C^{high}$ cells), non-classical monocytes (CD115+, $Ly6C^{high}$ cells) and NK cells (NK1.1+), remained similar in terms of numbers compared to saline injected controls (**Fig.24A**). In the liver, all the immune populations that were analysed showed no difference in numbers between BB-Cl-Amidine injected animals and control saline-injected animals, with the exception, again, of the number of neutrophils (Ly6G+ cells), that increased after a single shot of BB-Cl-Amidine (**Fig.24B**). As expected, BB-Cl-Amidine injection abolished the presence of NET structures found in the liver likely due to the inhibition of PAD4 activity (**Fig.25A,C**). As in the case of DNAse I injected animals, when analysed using immunofluorescence techniques and confocal imaging, BB-Cl-Amidine injection was confirmed to increase the number of infiltrated neutrophils in the hepatic tissue (**Fig.25A,C**). Additionally, the ratio of NETosis (**Fig.25A,C**), as well as the retained neutrophils and the number of netting neutrophils in the liver are dramatically decreased in the presence of circulating BB-Cl-Amidine (**Fig.25B,C**). In conclusion, the NETosis capacity of neutrophils after a single dose of BB-Cl-Amidine is also reduced, and again the absence of NETs during homeostatic conditions drives inflammation in the hepatic tissue and creates a condition of neutrophilia in the circulatory system. In conclusion, we have shown that the presence of NETs during homeostasis might have a beneficial role by dampening inflammation during the activity phase of an organism.

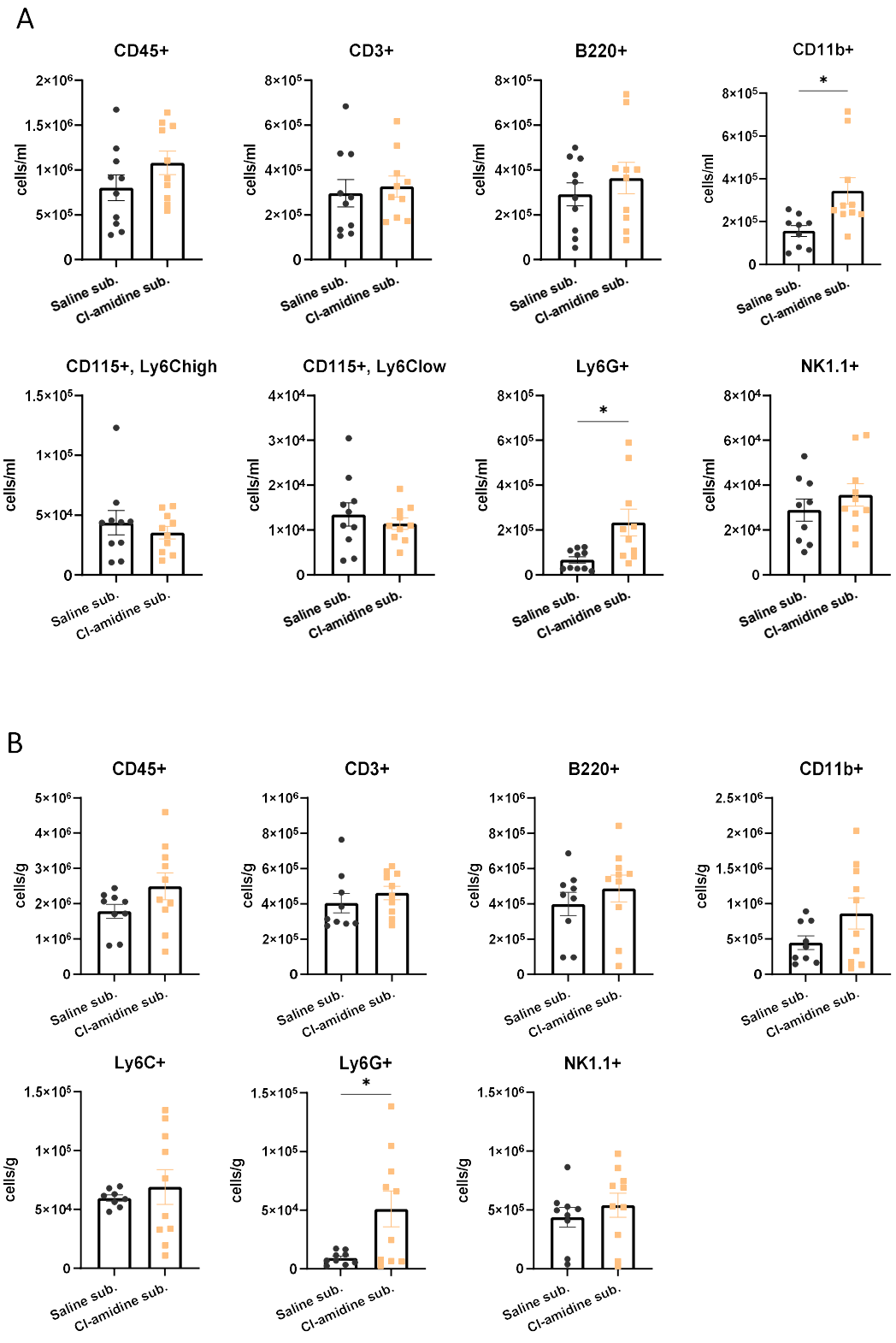


Figure 24. Flow cytometry analysis of immune populations in blood and liver after BB-Cl-Amidine administration. Number of leukocytes (CD45+ cells), myeloid cells (CD11b+ cells), T lymphocytes (CD3+ cells), B lymphocytes (B220+ cells), classical

monocytes (CD115+, Ly6C^{high} cells), non-classical monocytes (CD115+, Ly6C^{low} cells), NK cells (NK1.1+ cells) and neutrophils (Ly6G+ cells) as determined by flow cytometry from the systemic blood (A) and the liver (B) at ZT14 in BB-Cl-Amidine administered or saline injected animals. For blood samples, immune populations are represented as total number of cells in 1mL of blood; for liver samples, immune populations are represented as total number of cells in 1g of liver. n = 9-10. Unpaired t-test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

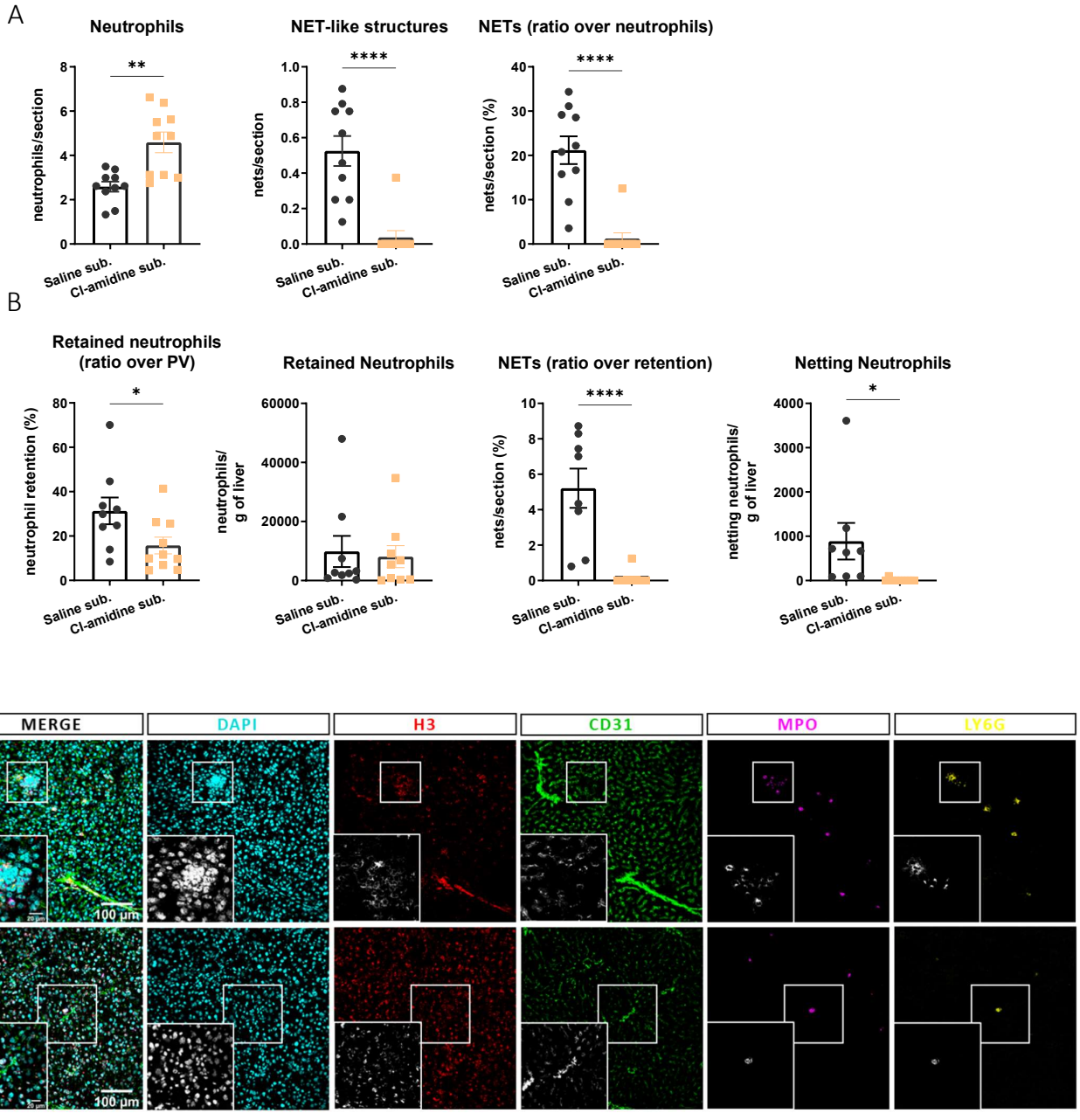


Figure 25. Evaluation of neutrophils and NET release in the liver after BB-Cl-Amidine administration. Quantification of tissue-associated neutrophils per field of view (or analysed section), NET-like structures per field of view (or analysed section) and percentage of NETosis per field of view (or analysed section) in the liver as determined by confocal imaging (A). Graphs show the retention ratio (in percentage) of neutrophils in the enterohepatic blood compartment, calculated as the total number of

liver cells divided by the total number of PV blood cells as determined by flow cytometry in saline injected or BB-Cl-Amidine administered animals (B). Analysis of the number of retained neutrophils, the percentage of NETosis -out of the fraction of retained neutrophils-, and the number of netting neutrophils (retained) (B). Representative immunofluorescence confocal images of liver cryosections showing stained nuclei (DAPI, cyan), histones (H3+, red), endothelial vessels (CD31+, green), neutrophils and macrophages (MPO+, magenta); and neutrophils (Ly6G+, yellow) at ZT14 in saline injected or BB-Cl-Amidine administered animals (C). 20x magnification images: scale bar 100µm. 63x magnification images: scale bar 20µm. n = 9-10, where each n represents one biological sample that has been calculated as an average of 4-5 analysed sections or fields of view. Unpaired t-test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance was assumed with p<0.05.

4.6 Hepatic NETs work as functional barrier filters against gut-derived metabolic and microbial products upon food intake

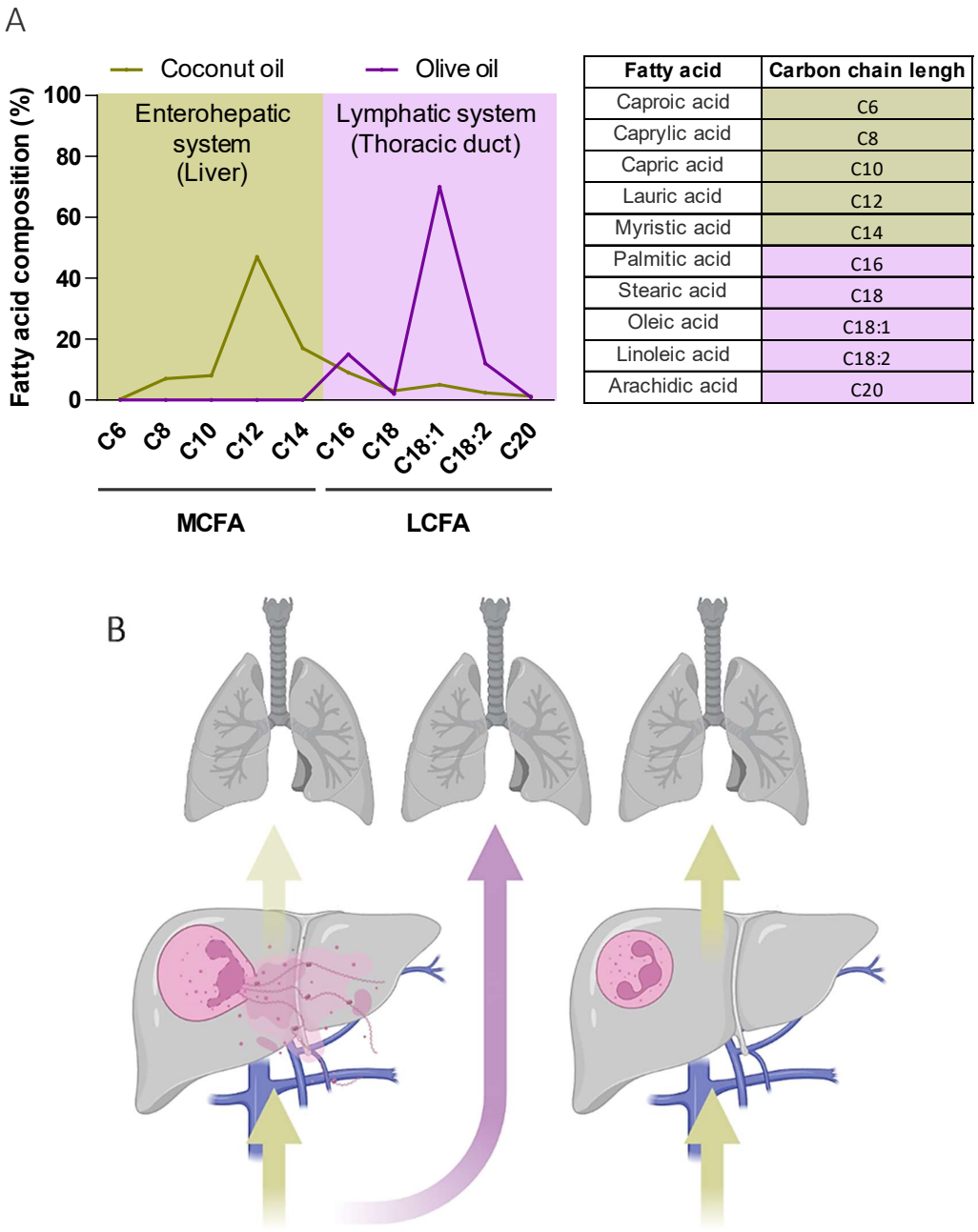
NETs, under homeostatic conditions, emerged as key peacemakers of the low-key pro-inflammatory scenario that is associated to the night-active period in mice and that defines metabolic endotoxemia in a postprandial state. Based on that, our next goal was to evaluate NET functionality as a potential physical and immunological key player of the barrier function in the hepatic compartment, and how a simulated condition of gut-derived pathogen dispersion in combination with the presence of digestion-derived products would modulate NET response and the immune function of the liver. As previously mentioned, dietary fats and its low-grade-associated intestinal inflammatory condition, have deleterious effects on intestinal gut permeability and gut barrier function (Rohr et al. 2020; Araújo et al. 2017). Microbial LPS-diffusion into the enterohepatic system, the release of TLR4 downstream-associated signals and the production of pro-inflammatory cytokines (IL-1B, IL-6, IFN-γ and TNF-α), ultimately disrupt gut-barrier integrity and promote intestinal permeability (Thaiss et al. 2018). Altogether, this facilitates the passage of bacteria, bacterial components (e.g. LPS, peptidoglycan or flagellins) and nutritional metabolites (e.g. secondary bile acids), from the intestinal lumen into the circulation (Capaldo and Nusrat 2009; C. Shi et al. 2019). Ultimately, these gut-derived pro-inflammatory microbial and metabolic products could have an impact not only in the enterohepatic compartment but also in peripheral distant organs.

Here, we tried to assess whether NETs could potentially work as a barrier mechanism in the hepatic compartment to mediate between immune responses in the liver and gut-derived products, meal-derived antigens, and commensal bacteria that would have likely translocated from the intestinal compartment into the enterohepatic circulation upon food intake. In order to mimic a condition that would resemble a compromised intestinal barrier integrity, we fed C57BL6/J mice with AF488-conjugated *E.coli* opsonized bioparticles by intragastric gavage 1 hour before sacrifice, in combination with a dosage of 200µL of oil as a representation of a high fat meal bolus. Fat-fed animals are segregated

into two groups: coconut oil- and olive oil-fed mice. The same experimental approach was also evaluated while NETs were inhibited by administration of a single dose injection of BB-Cl-Amidine (10 mg/kg/d) 3 hours before the sacrifice. Control animals were administered a dose of *E.coli* opsonized bioparticles resuspended in 200µL of water.

Coconut oil is composed of medium-chain fatty acids (MCFAs) and long-chain fatty acids (LCFAs). MCFAs represent approximately 72% of the total oil composition of coconut oil, being Lauric acid (C12:0) its most abundant component (49%) (Boateng et al. 2016). Olive oil is predominantly composed of long-chain triglycerides, being Oleic acid (C18:1) its mayor component, representing up to 75% of the total fatty acid composition in olive oil (Waterman and Lockwood 2007; Al-Bachir and Sahloul 2017) (**Fig.26A**). LCFAs are harder for the body to break down compared to smaller chained fatty acids and so tend to be slowly absorbed and transported within the organism. It has been described that fatty acids can modulate neutrophil and macrophage responses, influence the cell activation status and fate (survival or cell death), and mediate TLR-dependent responses (Rodrigues et al. 2016; Kumar and Dikshit 2019). Small-chain fatty acids (SCFAs), MCFAs and LCFAs display different absorption routes (**Fig.26B**). SCFAs and MCFAs enter the enterohepatic circulation, while LCFAs reach the systemic circulation through the thoracic duct (Bach and Babayan 1982). Thus, depending on their structure, we hypothesized that fatty acids can differentially impact innate immune cells reservoirs according to their localization. Briefly, here we took advantage of the associated chemical properties of MCFAs present in the coconut oil to rederive this fat bolus towards the enterohepatic circulation and, more particularly, towards the liver (**Fig.26B**). When these MCFAs had reached the systemic circulation, they will have done so by going through the barrier filter that we hypothesize is created by NETs in the liver. If NETs are indeed barrier filters working as sentinels against the incoming meal-derived antigenic products and gut-associated microbial components, this route of absorption of fatty acids would ensure that NETs can perform their functional physiological role in the liver under homeostatic conditions, and impede the free passage of all these molecules into the systemic circulation. That would ideally reduce inflammatory signals and the damage capacity of these molecules in peripheral and remote organs, such as the lungs (**Fig.26B**). Alternatively, the associated chemical properties of LCFAs mostly present in olive oil, would allow us to rederive them towards the thoracic duct (the largest lymphatic vessel in the human body) instead of through the enterohepatic circulation, to ultimately reach the systemic circulation in a mechanism of "hepatic by-pass" (**Fig.26B**). This route of oil absorption would indeed "by-pass" the liver circulation, and that would allow the LCFAs to enter the systemic circulation and reach peripheral organs such as the lungs while avoiding the encounter with the barrier filter that NETs potentially make in the hepatic compartment. If NETs are truly working as a main barrier component of the immunological barrier in the hepatic tissue, non-disrupted LCFAs and all associated gut-derived

metabolic and microbial components, would have a higher impact and a potentially increased damage capacity in peripheral and remote organs of the organism.



Fatty acid	Carbon chain lengh
Caproic acid	C6
Caprylic acid	C8
Capric acid	C10
Lauric acid	C12
Myristic acid	C14
Palmitic acid	C16
Stearic acid	C18
Oleic acid	C18:1
Linoleic acid	C18:2
Arachidic acid	C20

Figure 26. Experimental strategy design followed during oil gavage experiments. Representation in percentages of the main medium-chain fatty acids (MCFAs) and long-chain fatty acids (LCFAs) that compose coconut oil (caprylic acid C-8:0 (8%), capric acid C-10:0 (7%), lauric acid C-12:0 (49%), myristic acid C-14:0 (8%), palmitic acid C-16:0 (8%), stearic acid C-18:0 (2%), oleic acid C-18:1 (6%) and linoleic acid C-18:2 (2%)), and olive oil (myristic acid C-14:0 (0,02%), palmitic acid C-16:0 (15%), stearic acid C-18:0 (2%), oleic acid C-18:1 (70%), linoleic acid C-18:2 (12%) and arachidonic acid C20:0 (1%)) (A). Schematic representation of the different absorption routes of fats (hepatic-through or hepatic by-pass) after oil gavage according to

their different fatty acid composition, in the presence or absence of NETs; and their potential impact in peripheral remote organs such as the lungs (B). **Created with Biorender.com**

4.6.1 NETs are triggered according to fat redistribution between the enterohepatic system or the lymphatic compartment

In consonance with our just described hypothesis, and the previously observed results upon HFD consumption (**Fig.17**), NETosis in the hepatic tissue would be as well affected according to the predominant fatty acid composition of the fat bolus intake and how, depending on their absorption routes, these fats reach the enterohepatic system and transit through the liver, or just bypass this organ. Indeed, the NETosis capacity of the liver (**Fig.27B,C**) as well as the number of netting neutrophils present in the hepatic tissue (**Fig.27D**) were significantly augmented after a fat bolus of coconut oil compared to olive oil-fed or water-gavaged animals, in consonance with our initial hypothesis of meal as a modulator of the NET function. In accordance to previous results, BB-Cl-Amidine injected animals showed a significant reduction in their NETosis capacity in the liver (**Fig.27B,C**) as well as the number of neutrophils that can cast NETs (**Fig.27D**); even when animals had consumed a bolus of coconut oil fats. Interestingly, this last group of animals showed a significantly higher neutrophil recruitment to the hepatic tissue compared to water-gavaged or oil-fed animals that have their NETosis capacity unaltered (**Fig.27A**). This would ultimately reflect on a pro-inflammatory condition in the liver upon fat intake, when NETs are not present. Anew, NETs emerge as peacemakers and key modulators of the hepatic environment homeostasis. Moreover, this finding also supports our initial idea that the coconut oil absorption route (through the enterohepatic system) would impact the neutrophil hepatic pool, while the olive oil absorption route (through the thoracic system) would bypass the liver and hence would have no effect on the neutrophil hepatic pool. On the other hand, animals that ingested olive oil showed no significant differences in their NETosis capacity in the liver compared to water-gavaged animals: The number of neutrophils (**Fig.27A**), NETs per mm2 (**Fig.27B**), the ratio of NETs per mm2 (**Fig.27C**) and the number of netting neutrophils (**Fig.27D**) remained comparable between both groups. In conclusion, this reinforces our idea that because of the chemical properties of LCFAs present in olive oil, this fat is redistributed in the organ in a mechanism that bypasses the liver, and so has no impact on the hepatic neutrophil pool.

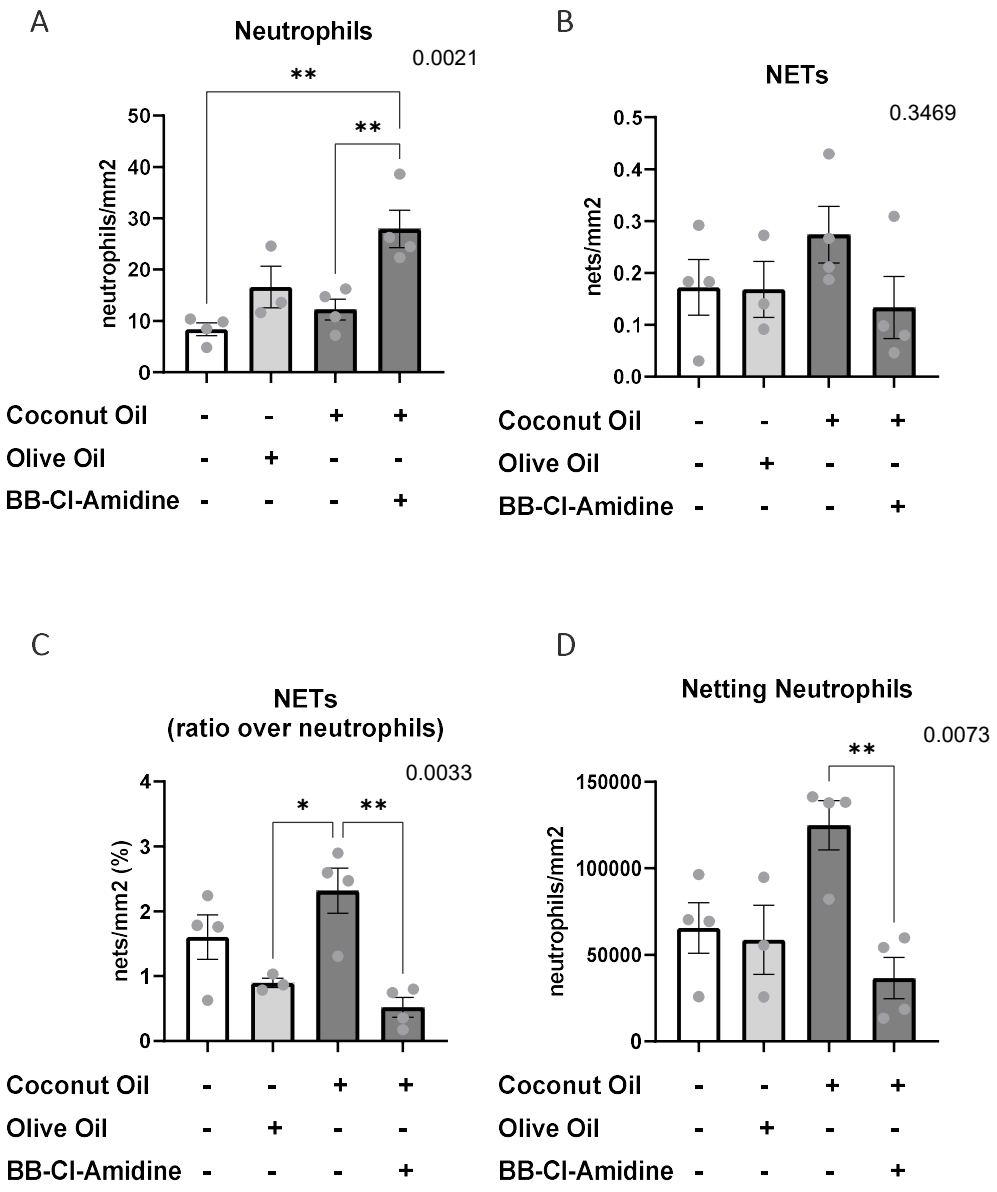


Figure 27. Evaluation of NETosis in livers from C57BL/6 mice upon oral gavage of different fat compositions. Quantification of tissue-associated neutrophils per mm^2 (or analysed section) (A), NET-like structures per mm^2 (or analysed section) (B), ratio of NETosis per mm^2 (or analysed section) over neutrophils (C) and number of netting neutrophils (D) In livers of C57BL/6 mice at ZT14 after gavage administration of different high-fat bolus (different oil compositions), as determined by confocal imaging. Note that mice were fed with AF488-conjugated opsonized *E.coli* bioparticles and administered a single dosage of BB-Cl-Amidine or a saline control. Note that here mice were not subjected to a pre-adaptation period of time-restricted feeding. Control animals were given water through a gavage probe. n = 4-5. Ordinary two-way ANOVA (global P value is shown plotted at the upper right corner of the graphs) and Tukey´s multiple comparisons test was applied (A-D). Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus water gavage. Significance was assumed with p<0.05.

4.6.2 NETs assist in the hepatic filtering capacity against gut-derived metabolites and prevent damage in remote organs

Next, in order to evaluate the damage capacity of gut-derived molecules that transit the bloodstream upon food intake, we evaluated the accumulation of Ly6G+ cells as a marker that reflects on tissue inflammation in our target organs; that is liver and lungs (**Fig.28**). Under normal conditions of NET release, a bolus of coconut oil administration had no impact on the hepatic neutrophil pool neither on the lung neutrophil pool, and did not induce an inflammatory condition in neither of these organs (**Fig.28A**). This is likely because NETs are present in the liver to counteract the negative effects of the gut-derived molecules that are released and transported upon an intake of a high-fat-meal, before they reach the systemic circulation and get rederived into peripheral organs such as the lung. As expected, the administration of olive oil had also no impact on the inflammatory profile of the liver and was not efficient at inducing a neutrophil response within the hepatic tissue, since olive oil would be redistributed within the organism in a hepatic by-pass mechanism (**Fig.28B**). Interestingly, LCFAs present in olive oil clearly reached the lung compartment after the gavage administration and induced a high neutrophil recruitment there, creating a pro-inflammatory scenario (**Fig.28B**). Upon olive oil intake, neutrophils are accumulated in the lungs up to four times more compared to coconut oil-fed animals (**Fig.28B**). In the absence of NETs, interestingly, coconut oil acquires the same damage-associated capacity as olive oil in the lungs (**Fig.28C**). Despite coconut oil being re-derived into the systemic circulation after transiting through liver, the inhibition of the immunological barrier filter that hepatic NETs compose in the liver, made that the MCFAs present in coconut oil and all associated gut-derived microbial ligands would create a pro-inflammatory scenario in peripheral remote organs (i.e. lungs) such as during conditions that would bypass the liver.

Altogether, our results indicate that in a simulated condition of altered intestinal permeability typically arising from a high-fat meal consumption, NET response in the liver is influenced and modulated by the gut-derived metabolic- and microbial-associated products. In order to modulate the potential damage-associated effects that these pro-inflammatory molecules could cause in peripheral and remote tissues once they have reached the systemic circulation, NETs in the enterohepatic system (mainly, the liver) would be working as a main component of the immunological barrier system in the hepatic compartment, and work as peacemakers by modulating the free-passage of these gut-derived antigens.

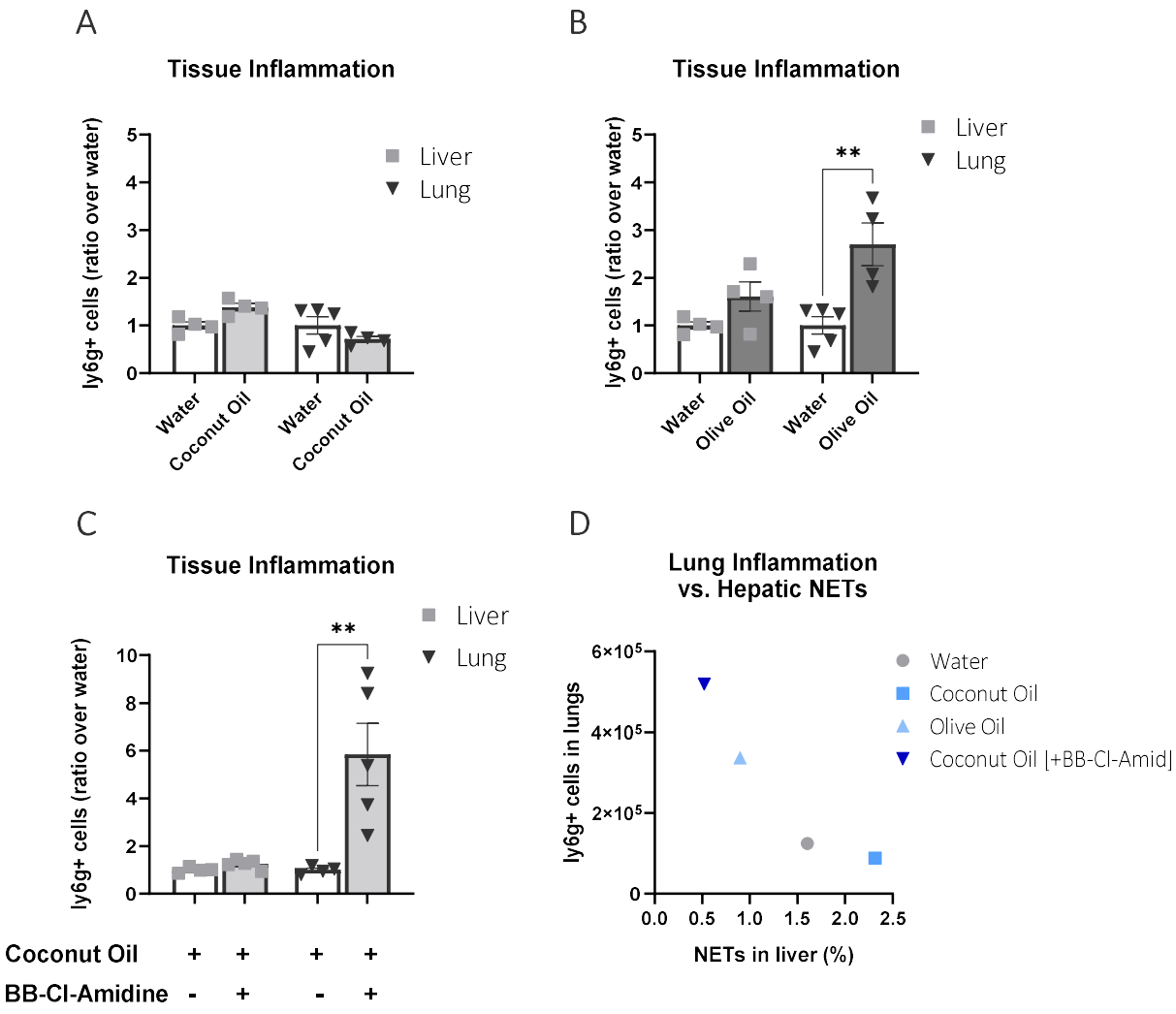


Figure 28. Evaluation of peripheral tissues inflammation upon oral gavage of different fat compositions. Graphs show flow cytometry analysis of neutrophils (Ly6G+ cells) in peripheral organs (liver and lungs) at ZT14 timepoint, after gavage administration of different high-fat bolus (different oil compositions). Inflammation scores are represented as a ratio of Ly6G+ cells per g of analysed tissue over water-fed control animals (A,B) or saline injected control animals (C). Correlation between NETosis ratios in livers (shown in percentage) and Ly6G+ cells in lungs. For representation purposes data shows the average score of each variable, per analysed group (n = 5) (D). Note that mice were fed with AF488-conjugated opsonized *E.coli* bioparticles in combination with 200µL of a fat-bolus (A-D), and administered a single dosage of BB-Cl-Amidine or a saline control (C,D). Ordinary two-way ANOVA and Tukey's multiple comparisons test was applied (A-C). n = 4-5. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus water gavage. Significance was assumed with p<0.05.

4.6.3 Evaluation of pro-inflammatory cytokines marker profile in the liver

Metabolic endotoxemia during the postprandial state, as has been already described, is associated with an increased release of pro-inflammatory cytokines that further boost intestinal permeability and facilitates the passage of these cytokines into the enterohepatic system and the liver, from where they could further migrate into the systemic bloodstream and promote both local and peripheral inflammation and potentially, damage. Intestinal barrier function in general is maintained by intercellular intestinal AJs and TJs, complexes of proteins that seal the space between adjacent epithelial cells in the intestinal membrane. Therefore, pro-inflammatory cytokine-mediated alterations of the TJs and AJs could result in an enhanced paracellular permeability and an increased presence of nutritional-derived antigens and bacterial-associated molecules into the enterohepatic circulation and peripheral tissues, such as the liver. Here, we evaluated a profile panel of pro-inflammatory cytokines in liver homogenized supernatants that would potentially reflect on gut permeabilization processes derived from meal consumption and the diet composition, which could ultimately impact the physiological release of NETs in the hepatic tissue. We evaluated the pro-inflammatory cytokine profile under conditions associated with a local-hepatic NETosis (water or coconut oil gavaged) and absence of local hepatic NETosis (BB-Cl-Amidine injected animals, olive oil gavaged or coconut oil-gavaged animals in combination with BB-Cl-Amidine). Broadly, the conditions associated to a local reduction of NETosis in the hepatic compartment show a tendency to correlate with higher levels of pro-inflammatory cytokines (**Fig.29**), particularly of M-CSF (**Fig.29A**), IL-4 (**Fig.29B**), IL-5 (**Fig.29D**) and IFN-γ (**Fig.29I**). For example, BB-Cl-Amidine injected animals (a phenotype previously associated to a higher systemic and local pro-inflammatory conditions, and neutrophilia) displayed critically higher levels of IL-5 (**Fig.29D**) and IFN-γ (**Fig.29I**) in the hepatic tissue. Once more, NETs emerged here as likely favourable components of the innate immune system, that repress a pro-inflammatory condition on the local environment during steady state conditions.

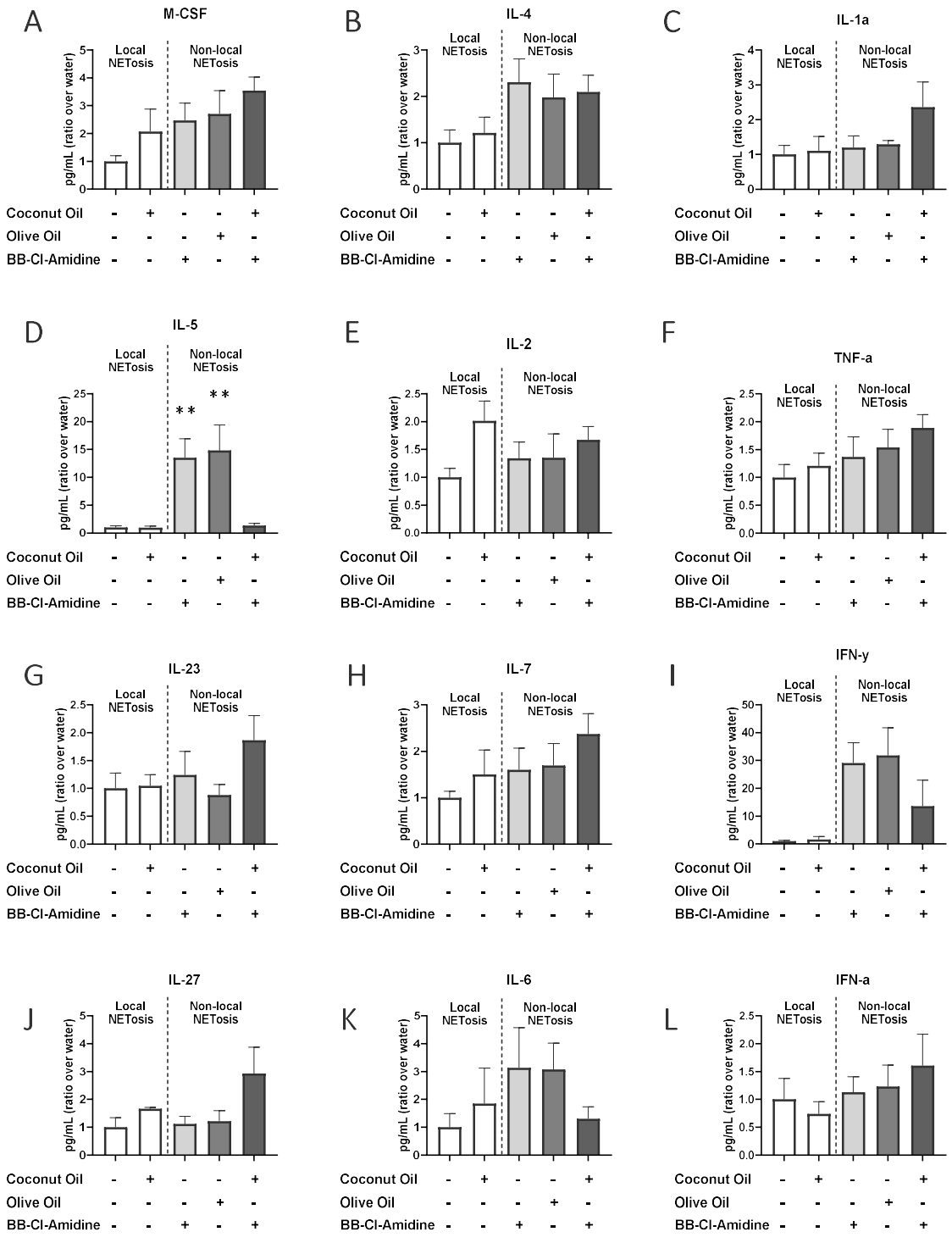


Figure 29. Cytokine pro-inflammatory profile quantification in supernatants from homogenized livers upon oral gavage of different fat compositions. Assessment of cytokine levels in the supernatants of homogenized C57BL/6 mice livers at ZT14 timepoint, after gavage administration of different high-fat bolus (different oil compositions). Note that mice were fed with AF488-conjugated opsonized *E.coli* bioparticles and administered a single dosage of BB-Cl-Amidine or a saline control. Cytokine concentrations (pg/mL) are represented as a ratio over water-fed control animals (A-L). LEGENDplex™ immunoassay was used

to measure levels of M-CSF (A), IL-4 (B), IL-1a (C), IL-5 (D), IL-2 (E), TNF-α (F), IL-23 (G), IL-7 (H), IFN-γ (I), IL-27 (J), IL-6 (K), IFN-a (L). n = 5. Ordinary one-way ANOVA and Tukey's multiple comparisons test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus water gavage. Significance was assumed with p<0.05.

4.6.4 Evaluation of pattern recognition receptor ligands in the liver

Aiming to analyse the presence of specific bacterial products and PRR ligands in the liver upon different fat intake compositions, and to evaluate how the presence of NETs, likely working as a functional barrier filter that mediates towards these gut-derived incoming pathogens; we performed an indirect evaluation of the presence of microbes and PRR ligand concentration, through the quantification of TLR activity. By using some HEK293 PRR-activity reporter cell lines (Section 3.6.2) we measured PRR activity in plasma samples from peripheral (RV) and enterohepatic (PV) blood, and supernatants from liver homogenates overnight (ZT14, ZT18, ZT22 and ZT2) during conditions where mice had consumed a normal chow diet (**Fig.31**) or alternatively at ZT14 when animals had been fed different high-fat loaded diets (**Fig.32**).

Briefly, we controlled that the group of saline and BB-Cl-Amidine injected animals displayed diurnal fluctuations in the immune compartment in blood and liver throughout the night, as previously reported. Overall, immune cell numbers are consistently lower during the night time points (ZT14, ZT18, ZT22) compared to the morning (ZT2) in blood and livers (**Fig.30A,B**). Anew, BB-Cl-Amidine injection seems to systematically modulate the myeloid compartment rather than the lymphoid populations (**Fig.30A,B**). BB-Cl-Amidine induced an increase in myeloid populations in blood (**Fig.30A**) and, particularly in neutrophils in the liver (**Fig.30B**), which potentially reflects on a local inflammatory response.

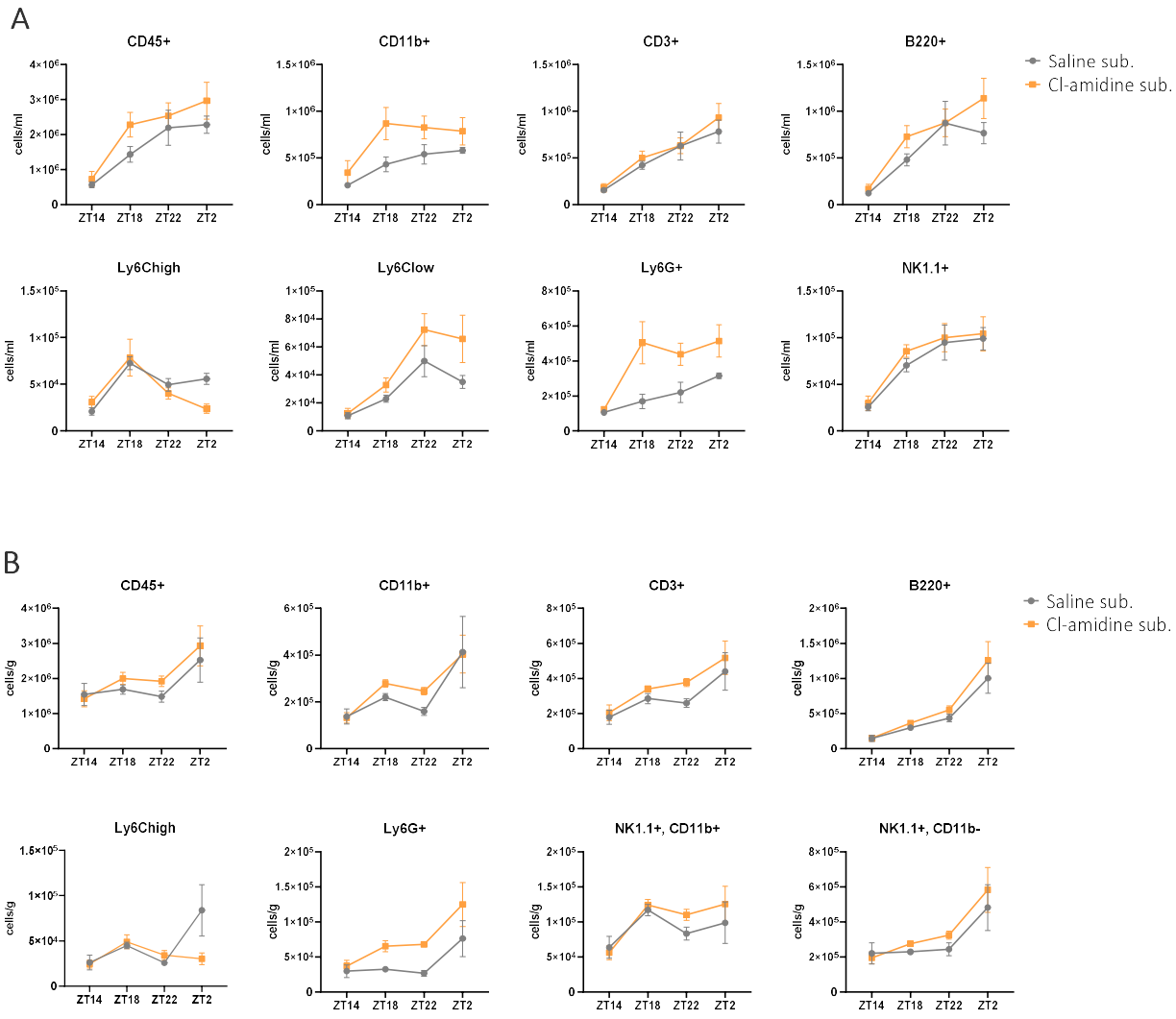


Figure 30. Flow cytometry analysis of immune populations overnight in PV blood, RV blood and liver in absence of NET release.

Number of leukocytes (CD45+ cells), myeloid cells (CD11b+ cells), T lymphocytes (CD3+ cells), B lymphocytes (B220+ cells), classical monocytes (CD115+, Ly6C^{high} cells), non-classical monocytes (CD115+, Ly6C^{low} cells), NK cells (NK1.1+ cells) and neutrophils (Ly6G+ cells), as determined by flow cytometry from blood (A) and liver (B) of C57BL/6 mice over the course of one night (ZT14, ZT18, ZT22 and ZT26/2). Note that mice were administered a single dose of BB-Cl-Amidine (orange-represented points) or a saline control (gray-represented points). For blood samples, immune populations are represented as total number of cells in 1mL of blood; for liver samples, immune populations are represented as total number of cells in 1g of liver. n = 5. Ordinary two-way ANOVA and Šídák's multiple comparisons test was applied. Error bars show mean ± SEM values. Statistical significance was assessed as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus saline. Significance was assumed with p<0.05.

Next, we evaluated the oscillations in TLR2 and TLR4 ligand concentration in PV blood plasma, liver supernatants and RV blood plasma samples overnight (ZT14, ZT18, ZT22, ZT2) (**Fig.31**). Overall, TLR2 and TLR4 activity is consistently higher during the early night timepoints compared to the morning timepoint in PV plasma, liver supernatants and RV plasma, coinciding with the moment of the day when animals are active and ingest higher amounts of food that potentially translocate gut-derived antigens into the enterohepatic system. In PV plasma, and liver supernatants, the TLR2 and TLR4 activity was overall reduced in the presence of BB-Cl-Amidine, which coincides with a reduced NETosis capacity of neutrophils, which would fail at trapping all these TLR-ligands derived from the gut and the enterohepatic circulation (**Fig31A,B,D,E**). TLR activity in RV plasma and the indirect evaluation of TLR-ligands presence necessary reflects on the filtering capacity of the liver to trap and process gut-derived pathogens and microbial-associated molecules. Seems remarkable that during the late-postprandial state (ZT18), TLR2 and TLR4 ligands concentration are increased in RV plasma (post-hepatic filtering) when NETs are inhibited (**Fig.31C,F**). Particularly, TLR4 activity is systematically higher in RV plasma in the presence of BB-Cl-Amidine (**Fig.31F**), which reflects on TLR-ligands not being trapped in the liver but rather reaching the systemic circulation. Up next, we evaluated the hepatic-filtering capacity in the presence or absence of NETs overnight, by estimating the ratio between TLR activity in RV over TLR activity in the PV. The higher the amount of TLR ligands that can trespass the liver and reach the systemic circulation, the lower it would the liver capacity work as a trapping system. That so, the lower it is the ratio between RV-TLR activity and PV-TLR activity, the better the liver is working at facing the wave of insults derived from the enterohepatic circulation. Interestingly, BB-Cl-Amidine reduces the hepatic-filtering capacity, particularly during the late-postprandial timepoints (ZT18-ZT2). In control animals, the hepatic filtering capacity remained comparable overnight (**Fig.31G,H**).

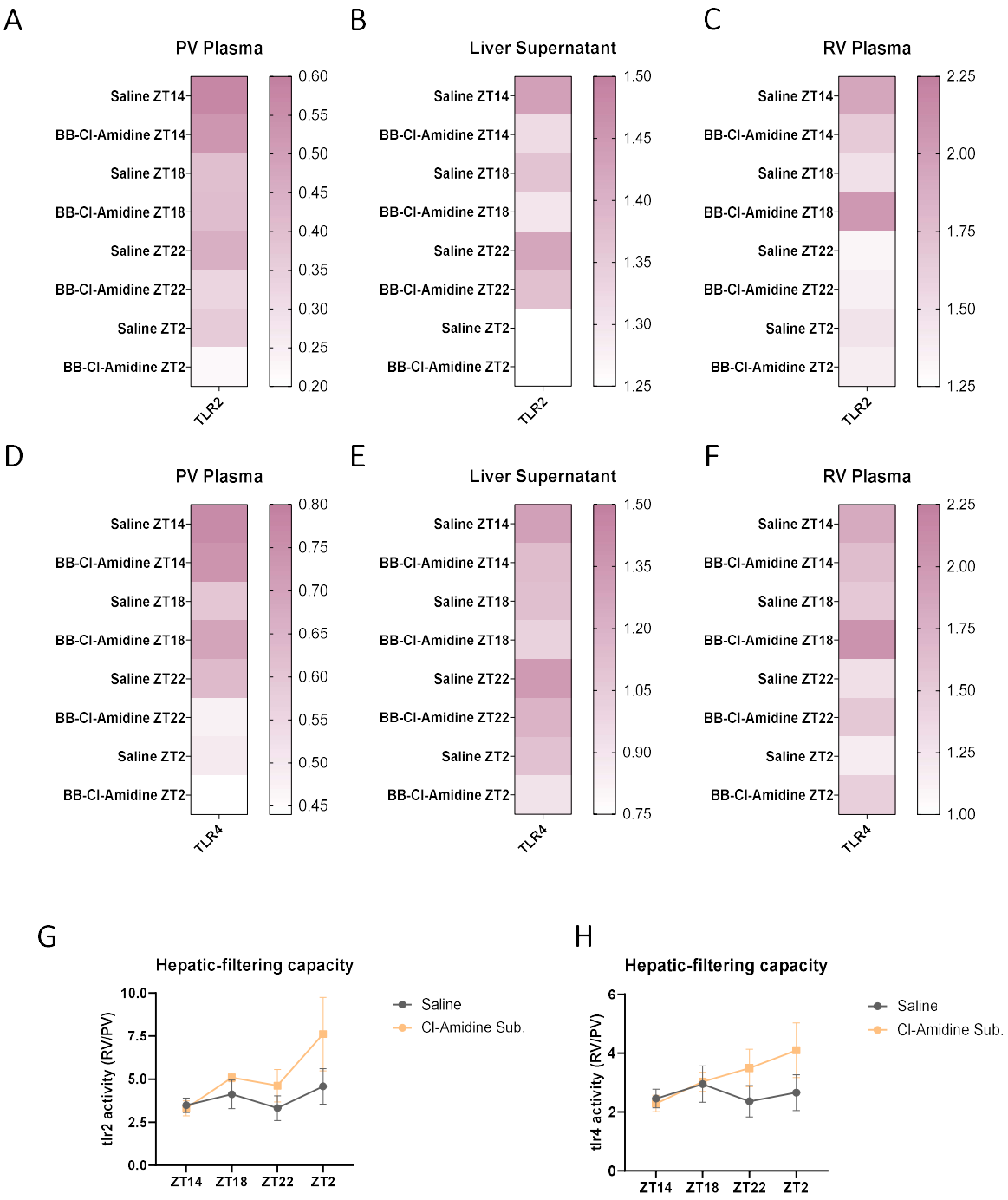


Figure 31. PRR ligands oscillation overnight in different vascular compartments in absence of NET release. HEK293 PRR reporter cells lines (HEK-Blue™-mTLR2 and HEK-Blue™-mTLR4) were stimulated and PRR ligand concentrations were measured utilizing a reporter cell assay examined in plasma from PV (A,D) and RV blood (C,F) and supernatants from homogenized liver pieces (B,E), shown as a heatmap graph. Samples were collected every 4 hours at ZT14, ZT18, ZT22 and ZT2. Hepatic-filtering capacity was calculated as a ratio between RV TLR activity and PV TLR activity (G,H). For every group, a single dose of BB-Cl-Amidine or saline was administered at ZT11. n = 5. Data represents OD values.

4.6.5 Evaluation of pattern recognition receptor ligands upon fat consumption

Finally, we tried to evaluate the dispersion of PRR-ligands into the RV blood, as a representation of the systemic circulation, during conditions associated with a local-hepatic release of NET (water or coconut oil gavaged) or alternatively during conditions where NETosis is not present on the local hepatic environment (BB-Cl-Amidine injected animals, olive oil-gavaged or coconut oil-gavaged animals in combination with BB-Cl-Amidine) (**Fig.32**). Notably, the level of TLR2 and TLR4 ligands concentration in the liver supernatants was highest in animals that received a dosage of coconut oil when NET released was inhibited, which might reflect on a potential effect of the diet in modulating PRR ligands dispersion (**Fig.32B,E**). Unfortunately, no clear matching conclusions could be taken overall from the experiment. Presumably, ZT14 timepoint was a too early timepoint to evaluate bacteria translocation from the gut into the systemic circulation. As was showed in previous experiments (**Fig.31**), later post-prandial faces (ZT18-ZT22) rather than an early one (ZT14) –what was analysed here–, are necessary to properly evaluate the hepatic filtering capacity and the transmigration of pathogen-associated ligands into the systemic bloodstream.

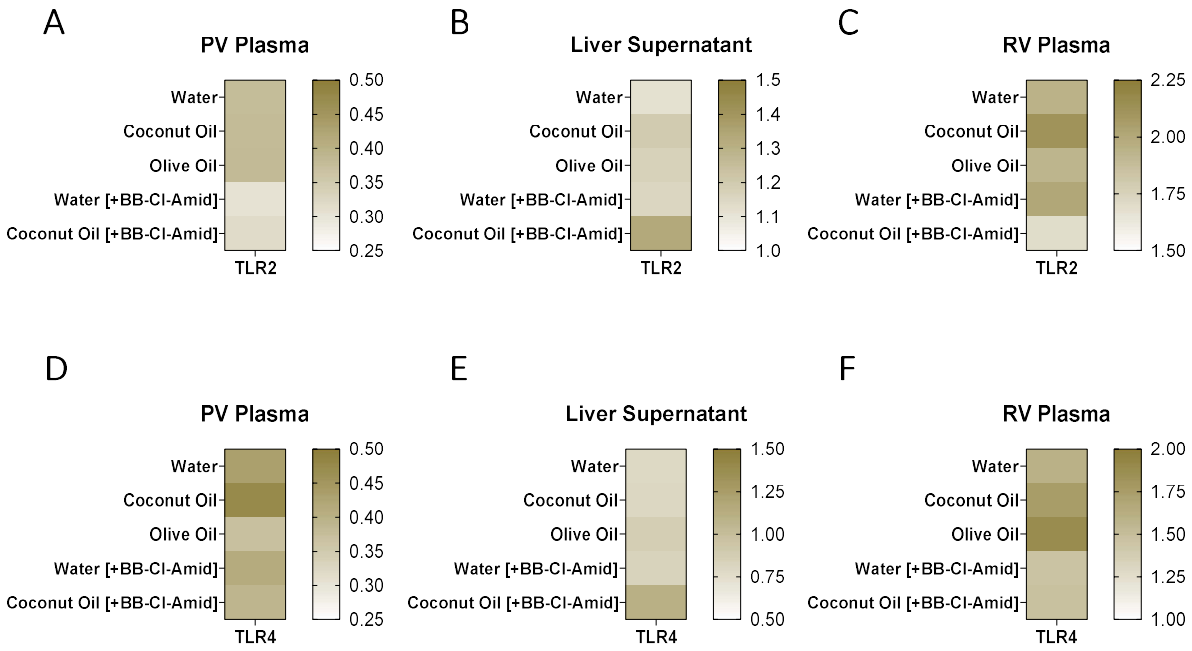


Figure 32. PRR ligands oscillation in different vascular compartments upon oral gavage of different fat compositions. HEK293 PRR reporter cells lines (HEK-Blue™-mTLR2 and HEK-Blue™-mTLR4) were stimulated and PRR ligand concentrations were measured utilizing a reporter cell assay examined in plasma from PV (A,D) and RV blood (C,F), and supernatants from homogenized livers (B,E) of C57BL/6 mice, after gavage administration of different high-fat bolus (different oil compositions). Note that mice were fed with AF488-conjugated opsonized *E.coli* bioparticles and administered a single dosage of BB-Cl-Amidine or a saline control. Samples were collected at ZT14. n = 5. Data represents OD values.

Discussion

5.1 NETs are expelled during immune homeostasis

A fundamental key in the regulation of hepatic immune homeostasis is to ensure immunotolerance and immune hypo-responsiveness towards the daily influx of harmless common food antigens and diet-derived metabolites, intestinal microbes and pathogen-associated molecules that circulate within the enterohepatic bloodstream from the gut in a diurnal fashion. The liver, as a sentinel frontline immune barrier compartment, needs to ensure a balance between tolerance towards non-threatening substances and the casting of an effective immune response towards pathological agents. Here, we have demonstrated that neutrophils infiltrate the liver in a circadian way and that these cells are able to cast NETs, one of their main effector functions, even in the absence of a pathological insult.

5.1.1 Neutrophil activation in non-pathological conditions: Getting in shape for future threats?

Within a pro-inflammatory scenario, NETs can accumulate in murine tissues due to immune activation, cell infiltration and priming, dysregulation of the NET formation machinery and/or a defective clearance of released traps. Any of these conditions lead to a persistent inflammation, tissue damage and generation of autoantibodies. NETs have been recently reported, however, to display both pro- and anti-inflammatory properties, depending on different stimuli and their context of activation (Hahn et al. 2019; Euler and Hoffmann 2019; Neubert et al. 2020; Knopf et al. 2019). In high neutrophil density sites, NETs tend to clump and form an enzymatically stable complex referred to as aggregated-NETs (Daniel et al. 2019). Interestingly, within an anti-inflammatory context, these aggregated NET conformations have been reported to contribute to the resolution of sterile inflammation, for example by targeting histones and mediating with their toxic-associated properties, which ultimately increased the viability of close-by epithelial cells in close contact to the extracellular histones (Knopf et al. 2019). In another example, monosodium urate crystals can also induce aggregations of NETs in gout disease (Schauer et al. 2014). These so-called aggregated-NETs are able to confine gout-related pro-inflammatory cytokines and degrade them, conveying an important anti-inflammatory player for the progression of the pathology (Hahn et al. 2019; Euler and Hoffmann 2019). Here, NETs limit the systemic spread of cytokines and prevents tissue peripheral-damage and further infiltration of neutrophils (Schauer et al. 2014). Aggregated NETs have also been suggested to shield viable tissues from necrotic areas (Bilyy et al. 2016), which helps preserving tissue homeostasis. Moreover, Podolska et al. reported that the presence of aggregated NETs in various healthy body fluids (e.g., eye wash fluid or human gall fluid) have a regulatory role by lowering NE activity, which would ultimately reduce NE-associated peripheral tissue damage and inflammation (Podolska et al. 2019).

Altogether, these ideas already opened the gate for NETs not only as inflammatory-associated contenders, but as active players in the maintenance of a normal physiological status within an organism.

The NET structures we reported along the different conditions during our experimental research high likely represent indeed these so-called aggregated NET conformations, which would ultimately reinforce the idea of NETs existing as modulators of tissue homeostasis. Evolutionary, seems convenient that neutrophils infiltrate peripheral tissues and organs during the active phase of an organism (*i.e.,* during the night in rodents, during the morning in humans) being this the moment of the day when a greater input of foreign antigens, external pathogens and danger-associated molecules can penetrate into the body. That so, rhythmic neutrophil infiltration into naïve tissues and augmented casting of their effector capacities (such as NETosis) would ensure anticipatory defense strategies towards invading pathogens and potential insults. In any case, the fact that we observed active neutrophil infiltration into several tissues under steady state, that neutrophil infiltration fluctuates with a circadian dynamic, and that at least some of the neutrophil effector function seems partly dependent on the time of the day, only reinforces the idea that neutrophils can influence many physiological process and temporal functions across different tissues and organs, and opens up the concept of this cells as something else beyond just effector troopers fighting an injury.

5.1.2 NETs are present in physical and immunological barrier tissues: NETs as firewalls in peripheral tissues?

Barrier tissues, categorized as physical barrier tissues (skin, lung, intestine) and immunological barrier tissues (liver, lymph nodes and spleen) are chronically and diurnally exposed to a high plethora of external insults, air pollutants, microbes, pathogen-associated molecules and diet-derived antigens and metabolites. A first physical barrier tissue is present in the body to overcome these aforementioned insults and impede their penetration within the organism. However, if these potentially-pathogenic molecules manage to surpass typical physical barrier tissues, a second line of defense compose by immunological barriers emerges. It is because of that that barrier tissues require tight control in their tissue-associated immune compartment and their resident immune populations.

We have observed that neutrophils oscillate in blood and peripheral tissues in a circadian fashion and start accumulating, particularly within the aforementioned barrier organs and tissues, at the beginning of the active phase of the organism (*i.e.* the night-time, in rodents). As already mentioned,

mice are nocturnal creatures that have a night-associated active period, when they eat substantially more food in comparison to the diurnal phase and are exposed to an increased amount of potential insults. Of note, during steady state, under homeostasis and despite non-pathological associated conditions, incoming waves of antigens, DAMPs and PAMPs still invade and circulate within the body, especially during these active and challenging times of the day. Recently, the idea has emerged that neutrophils might assist in host defense during these particularly challenging periods, by facing all the external incoming agents and so, contributing to the restoration of the normal physiological status of the body. NETosis, one of the key neutrophil defense functions, have been reported along our research to actually occur during tissue homeostasis, under steady state. As a matter of fact, we were able to spot NETs only in the liver, lung, spleen and lymph nodes from mice under non-pathological conditions, both at the morning and the night analyzed timepoints. Intriguingly, NETs were not present at any time of the day in any of the remaining analyzed tissues, all of them considered non-barrier organs. Notably, livers, lungs, spleen and lymph nodes compose indeed barrier organs, are considered immunological compartments and display neutrophil reservoirs (J. Wang et al. 2017; Christoffersson and Phillipson 2018). They are all locations where neutrophils have been demonstrated to be able to execute active functions beyond inflammatory processes. Interestingly, the NETosis capacity of neutrophils was dramatically augmented during the night-time, particularly in the liver. The liver, a key frontline immunological barrier organ emerged as a tissue within the organism where neutrophils (*via* NET release), could be functioning as sentinels and customs agents, with the main role of regulating the entrance of gut-derived pathogens and associated molecules. Overall, neutrophils and homeostatic NETs would be assisting in the immune decision within the hepatic tissue to maintain a tolerogenic status towards non-pathogenic insults or casting an immune response against pathogenic-associated antigens, and so assist in the maintenance of the normal physiological status of the body.

5.2 The Hepatic Niche

5.2.1 Can NETs display antigen presenting functions in the liver?

Sepsis, in mice models, has shown that the majority of live circulating bacteria are rapidly and primarily sequestered in the liver (Yan, Li, and Li 2014; Surewaard et al. 2016) by two key sentinel immune players: Kupffer cells, that are capable to capture pathogens directly from the blood stream; and neutrophils, that are recruited into the hepatic sinusoids at a later time point (McDonald and Kubes 2012) and upregulate the bacterial trapping capacity of Kupffer cells. Kupffer cells, the body's largest intravascular macrophage population can trap bacteria under flow conditions *via* expression of CRIg.

Interestingly, neutrophils lack this receptor (Helmy et al. 2006) and are reported to contribute to bacterial catching and killing in the hepatic tissue by casting NETs in the surroundings of infected Kupffer cells. This context of active collaboration between Kupffer cells and neutrophils brought up the idea that a similar interaction between these two cell populations might still occur in steady state. Indeed, our observations confirmed a spatial interaction between NETs and F4/80+ cells; being Kupffer cells highly present in the surroundings of the aggregated NET-conformations. In the absence of an active infection, one of the most likely scenarios for these two populations to interact would be if indeed NETs would display antigen presenting functions and would be functioning as an "antigen presenting structure" capable of communication with other immune populations in the liver. NETs could be actively collaborating with Kupffer cells in the process of discrimination between self-antigens, incoming gut-derived products, commensal microbes and metabolites; and foreign antigens, pathogenic bacteria or damage-associated molecules, and help the tissue-associated immune compartment in shaping of the immune response (tolerogenic vs. non-tolerogenic), towards these incoming external antigens. On top of that, seems particularly striking that the four organs where we were able to spot NET formation during homeostatic conditions where the liver, the lung, the lymph nodes and the spleen; all of them considered not only immunological barrier tissues, but also immune compartments where active processes of immune cross-communication and adaptive immune responses take place in a daily matter. Interestingly, Calvente et al. have recently illustrated how *via* extracellular microvesicles, neutrophils are able to deliver microRNA-223 (miR-223) particles to Kupffer cells in what seems to be a novel cell-to-cell communication mechanism, during a model of chronic liver injury in mice. This helped downregulating the activated and pro-inflammatory status of tissue-resident macrophages, promoted resolution of inflammation and partially restored liver fibrosis. The fact that neutrophil depletion led to a down-regulation of miR-223 levels in hepatic macrophages and that neutrophil-depleted animals exhibited prolonged tissue damage, inflammation and liver fibrosis –in comparison to neutrophil-complete animals–, reinforces the idea of an active process of communication in the liver between neutrophils and Kupffer cells, and spotlights these cells are crucial players in the restorage of tissue physiology (Calvente et al. 2019). In the spleen, Puga *et al.* showed that neutrophils can express a distinctive CD62-L^{lo} CD11b^{hi} I-CAM1^{hi} phenotype and are able to interact with marginal zone B cells *via* the formation of NET-like structures, interestingly, in steady state. This communication process was identified to promote B-cell maturation and survival, immunoglobulin class switching, somatic hypermutation, and B-cell mediated antibody production through a mechanism that involves the release of BAFF, APRIL, IL-21, and PTX3 (Chorny et al. 2016; Puga et al. 2011). Alternatively, Uderhardt et al. described an interesting interplay process between tissue-resident macrophages and neutrophils that goes beyond the antigenic presentation hypothesis. Here,

authors described that tissue-resident macrophages are able to respond to laser-induced damage in the liver by cloaking tissue microlesions, which prevents neutrophils from interacting with cell debris and getting activated. This would cease neutrophil swarming and further activation, downstream inflammation and neutrophil-mediated tissue damage, which ultimately would also help preserving tissue homeostasis (Uderhardt et al. 2019).

5.2.2 Can NETs assist in the liver detoxifying functions?

Our results showed that neutrophil migration to the liver entrains circadian rhythmicity, showing an increased retention of these cells within the enterohepatic circulation at night hours (during the active phase, in mice) and a decline in retention at day hours (during the resting phase, in mice). The enterohepatic circulation and the portal venous blood is highly enriched in gut-derived microbial products, toxins and food-related metabolites all derived from the digestion process in the intestine, that are then processed in the liver before being released back into the hepatic vein and the cava vein, or are alternatively stored for later use (Guerville and Boudry 2016). This process makes the composition of the portal blood necessarily different from the systemic peripheral blood that irrigates the rest of the body. Regarding microbes, the concentration of pathogen-derived molecules has been reported to drop up to 100-fold between portal venous blood and peripheral blood (Jenne and Kubes 2013). As a consequence of this blood "compartmentalization", the immune cellular compartment that mediates immune tolerance or immune responses towards these gut digestion-derived products in the portal circulation –and in the liver– will potentially be heterogenous and different, compared to immune populations in other vascular beds. Some studies have revealed a distinct composition of the venous portal blood compared to peripheral blood with respect to important innate and adaptive immune cells, such as a highly activated subtype of T cells (Wistuba-Hamprecht, Pawelec, and Derhovanessian 2014; Maecker, McCoy, and Nussenblatt 2012), which might reflect on the chronic exposure of immune cells in the portal blood to the bacterial products derived from the intestine (Queck et al. 2018). On the other hand, some reports claim that plasma markers of immune cell activation and inflammation are compartmentalized to the liver, but seem to be higher in systemic than in portal plasma. This might just reflect on the liver function to detoxify, process and metabolize gut-derived components from the portal circulation. In our hands, we observed that the portal vein appears to be a distinct immunological compartment compared to peripheral blood (at least regarding the innate immune compartment) as an enrichment tends to occur in some myeloid populations (neutrophils, classical monocytes and non-classical monocytes) in blood from the portal vein, particularly during the night time. The fact that neutrophils seem to be highly present during the night-times patrolling the liver sinusoids only reinforces our idea of these cells as key sentinels in the

hepatic tissue, that through an enriched NETosis capacity (especially during the night times), ensures the normal physiological functioning of the liver by mediating immune responses towards the aforementioned gut-derived incoming antigens.

5.3 Entrainment factors that modulate NET release in the hepatic environment

5.3.1 Circadian Regulation of NETosis: Bmal influence on NET release in the liver

Recently, Adrover et al. identified a cell-intrinsic program that mediates time-of-the-day-dependent changes in the neutrophil proteome, while these cells are in circulation. This program, governed by CXCR2 and Bmal1, causes progressive time-dependent reduction in the neutrophil-granule content and a diminished NETosis capacity over the day (Jose M Adrover et al. 2020). Interestingly, this program seems to ensure tissue protection, as authors reported that old neutrophils that transmigrate into tissues under steady state (likely to be cleared) hold low granule content and less tissue-damage capacity. In parallel, this program would also ensure that upon damage-associated activation, bone marrow young- and freshly-released neutrophils could transmigrate into tissues with their full granule-content and be efficient in mediating an immune response. Accordingly, our reported diurnally fluctuating NETosis in the hepatic tissue could be being modulated by the circadian clock on three different levels: The central clock level (which would need to evaluated on systemic Bmal1^{KO} mice), the immune compartment level, particularly on the neutrophil (which would need to be evaluated on Mrp8^{Cre}Bmal1$^{Flox/Flox}$ mice), or the niche-specific hepatic compartment level (which was evaluated on Albumin^{Cre} Bmal1$^{Flox/Flox}$ mice). While in circulation, neutrophils from Mrp8^{Cre}Bmal1$^{Flox/Flox}$, mice –that have a neutrophil-specific deletion of the *Arntl* gene (encoding Bmal1)– have been reported to show no differences in their MPO-granule content or their NET formation capacity between morning (ZT5) and night (ZT13) time-points, as opposed to the differences observed in wild-type controlled animals (Jose M Adrover et al. 2020). This spotlights Bmal1 as a potential regulator of the time-of-the-day dependent NET formation capacity in neutrophils. Nevertheless, there are no available studies –to our knowledge– that have yet unraveled NET dynamics in livers from Mrp8^{Cre}Bmal1$^{Flox/Flox}$ or systemic Bmal1^{KO} mice.

If we recapitulate previous chapters (*Section 1.3.1*), most of the biological processes happening within an organism respond to an organized temporal regulation coordinated primarily by the SCN in the hypothalamus, entrained by light cycles. Conversely, peripheral clocks in peripheral tissues (such as the liver) are entrained by feeding cycles, timing of food intake and metabolic cues, major *zeitgebers* in peripheral organs. Based on that, it would just make sense that Mrp8^{Cre}Bmal1$^{Flox/Flox}$ animals would be able to keep up on a normal NET rhythmicity in the liver as long as a normal feeding schedule (as a

peripheral environmental cue) would be available. This NETosis rhythmic profile would be lost in Bmal1^{KO} animals who, indeed, show a desynchronized eating pattern (Laermans et al. 2015), and could be so restored by administering these animals a tightly controlled eating schedule such as time-restricted feeding. Indeed, a hint to understand NETs in the liver as a *diurnal* process rather than *circadian* is reflected in the NETosis profile that was observed on Albumin^{Cre} Bmal1$^{Flox/Flox}$ animals. These animals displayed a comparable pattern in NET activity to their wild-type counterparts, also having higher NETosis ratios during the night as we have consistently observed throughout our research in C57Bl/6 mice. Interestingly, NET release is abrogated upon fasting to levels comparable to those observed during the morning-time points, a condition where animals barely consume food. This result reinforces the idea that NETs in the liver seem to be at least partly independent of the hepatic-specific clock gene regulation but rather influenced by food consumption and its derived metabolic cues.

5.3.2 Nutritional Regulation of NETosis: The composition of the diet influence on NET release in the liver

We have reported that the NETosis capacity of neutrophils is enriched during the night time in mice, which interestingly coincides with these animals' active period, a time of the day when they are awake and ingest most of their daily intake of food. Although we cannot exclude a partial influence and regulation of NET release by the inner circadian clock *perse*, we have observed promising trends indicating that NETs are likely influenced by the ingestion of food (and the absence of it), the timing of food intake and, interestingly, the composition of the meals. It seems that meals with a high content in fat and sugars are the ones most prone to induce NETosis. Eating has long been considered an entrainment factor for the inner clock regulation of peripheral tissues, which likely interconnects here the observed diurnal fluctuations of NET release in the hepatic compartment.

5.3.2.1 The "western lifestyle" impacts on NET activity

Not long ago, Moorthy et al. showed that neutrophils from HFD-fed mice are more prone to spontaneous NET formation in the lungs of BALB/c mice infected with influenza, in comparison to neutrophils derived from LFD-fed infected animals (Moorthy et al. 2016). HFD seems to induce higher neutrophil recruitment ratios into peripheral tissues, and increased NET formation due to higher oxidative stress levels (H. Wang et al. 2018). Within a clinical context of atherosclerosis disease, NET levels in murine atherosclerotic plaques and blood plasma seem to be consistently higher under HFD conditions (Silvestre-Roig et al. 2019; Y. Liu et al. 2018). In this regard, Wang et al. showed that pharmacological intervention of NET formation (*via* Cl-Amidine or DNAse administration) mitigates

endothelial dysfunction, impedes plaque progression and the endothelium-dependent vasodilation that occurs in the non-treated HFD-fed obese animals. Authors suggested that an abnormal production of NET-derived MPO would trigger the generation of ROS in close proximity to the vascular wall, which puts the spotlight on NETs as key players in obesity-related endothelial damage (H. Wang et al. 2018). To our knowledge, ours would be the first reports on NET activity in peripheral organs from short-time HFD-fed animals within a disease-free context.

Some studies report that cholesterol crystals, again within an atherosclerotic context, can function as sterile danger signals that accumulate in myeloid cells, activate the NLRP3 inflammasome (Duewell et al. 2010) and trigger neutrophils to release ROS-dependent NETs in mice atherosclerotic plaques (Warnatsch et al. 2015; Westerterp et al. 2018). During our experimental conditions, overall, NETosis ratios were higher in HFD fed animals compared to animals fed an LFD or animals under fasting conditions. Among the HFDs used, a diet high in fat and sugar content induced the highest recruitment of neutrophils to the tissue and the highest NETosis ratios. Metabolically, HFSugD represents here what we identify as the "Western-style diet" typical from modern societies, a composition of processed calorically dense meals, rich in high-saturated fats, cholesterol and refined sugar, and low in dietary fibre. Western diets are associated with a low-grade inflammatory condition that, over time, can cause cardiovascular complications (Poti, Duffey, and Popkin 2014; Christ, Lauterbach, and Latz 2019). Indeed, this low-grade inflammatory status can have detrimental consequences particularly in the grastrointestinal tract, where it can lead to impaired gut barrier integrity. This will be discussed extensively within the next chapter section (*Section 5.3.3*). In line with all this, the absence of food-borne antigens in the enterohepatic circulation during fasting conditions, cannot but noticeably reduce the amount of NETs in the liver compartment. Because NETosis ratios were lowest when animals did not encounter any food stimulus, this only reinforced our idea of NETs as mediators of the metabolic process upon food intake, and as a neutrophil inner-function that is likely regulated by the timing of food intake and the composition of the meal.

5.3.3 Microbial Regulation of NETosis: Pathogen-associated molecules influence on NET release in the liver

Based on the now identified interplay between NET release and diet composition; and the already known high-impact high-fat diets have on intestinal inflammation and gut barrier function, we hypothesized next that the intricated and balanced immune response in the liver could be disrupted by gut dysbiosis induced by a sustained high-fat intake. Alterations of the gut permeability have been described to result in the translocation of microbial products into the enterohepatic circulation, causing

hepatic damage and fibrosis upon their arrival to the liver, which would likely disrupt the physiological barrier functionality of this organ, and ultimately could cause systemic inflammation. That way, the commensal microbiome arose next as a potential candidate that could influence as well the physiological role of NETs in the liver. For example, during our investigation we observed differences in PRRs activity and responses upon meal consumption, at different times of the day and in the absence or presence of NETs. This confirmed, to a certain extent, the aforementioned potential translocation of gut-derived antigens and pathogen-associated molecules (e.g. LPS) into the enterohepatic circulation and the liver, which could potentially modulate the normal NET profile within that organ.

5.3.3.1 Gut microbiome: Involvement of PRR ligands in NET response

In the gut, HFD-induced intestinal permeability allows the translocation of luminal LPS into the circulation, a process termed *endotoxemia*. Indeed, LPS levels have been reported to differ in the fast compared with the fed state. Meals high in fat content were identified responsible for elevated serum LPS levels in a postprandial face, both in mice (Cani et al. 2007) and healthy men (Amar et al. 2008). These LPS molecules can translocate into the circulation either by passive diffusion or by enterocyte absorption as chylomicron-associated LPS, even in the absence of pathology (Ghoshal et al. 2009). The biological relevance of constant low circulating LPS in the circulation in the postprandial face, termed *postprandial endotoxemia*, is likely related to immune modulation and potentially to leukocyte training. Nowadays, most metabolic disorders that arise from an HFD-associated chronic inflammation, have a clear link to LPS diffusion, intestinal permeability and alterations in the gut microbiome that lead to metabolic endotoxemia (Cani et al. 2008). Based on the amount of bacterial cells (10^{12}) present in one gram of faeces, some studies have reported that up to 1 gram of LPS can be detected in the human intestinal lumen (Brun et al. 2007). Unfortunately, the LPS-assay kit detection limit was barely reached in most of our analysed blood plasma and supernatants from liver homogenates samples. The concentration of pathogen-derived molecules has been reported to drop up to 100-fold between portal venous blood and peripheral blood (Jenne and Kubes 2013). Because of that, we would have expected to spot vascular-compartment dependent differences at different times of the day or after the pharmacological inhibition of NET release, if homeostatic NETs would indeed be working as a filter towards gut-derived bacteria that is translocated into the enterohepatic circulation upon food intake. Nonetheless, a recent study failed to detect any bacteria in portal blood under basal conditions (Balmer, Slack, et al. 2014), although this does not necessarily mean that the liver cannot play a key role in immunity following gut bacterial dispersion. In fact, hepatic sinusoids bloodstream velocity is approximately half that of other capillary beds, which only reinforces the theory of hepatic sinusoids as

an area where the chances to detect certain molecules or trap pathogens (by NET release) is maximized (Oda, Yokomori, and Han 2003).

High-fat diets can also modulate the gut microbial composition. Western-style diets are able to induce systemic local-grade inflammation in the intestines through a variety of mechanisms linked to alterations in the intestinal microbiota composition (Moreira et al. 2012). This concept is known as *dysbiosis* or *intestinal dysbiosis*. In line with that, Cani et al. also showed that HFD-feeding significantly enriched the mice gut microflora with Gram-negative and LPS-containing bacteria (Cani et al. 2007; 2008). On top of that, gut microbes are indeed master regulators of the immune system development, as they are able to establish long-distance communications with immune cells present in peripheral organs through gut-associated antigens and metabolized products that travel alongside the bloodstream. This cross-communication has been reported to be mediated by commensal-derived D-lactate, that is transported to the liver *via* the portal vein (McDonald et al. 2020). This interaction is critical for communication with Kupffer cells, mediation of host defense against circulating pathogens, immune development and training of the immune system (Clarke et al. 2010; Khosravi et al. 2014; D. Zhang et al. 2015; Honda and Littman 2016; Thaiss, Zmora, et al. 2016). Briefly aforementioned, intestinal dysbiosis (anomalous or imbalanced gut microbial composition), compromised gut barrier integrity and increased intestinal permeability, have all been linked to increased susceptibility to disseminated bacterial infections and sepsis in humans. This happens due to the translocation of microorganisms and microbial products into the enterohepatic circulation (Crispe 2009; Pradere et al. 2010; Anand, Zarrinpar, and Loomba 2016; Seki and Schnabl 2012). All these previously mentioned gut-derived bacterial products (endotoxins, flagellin, ß-glucans and peptidoglycans or bacterial DNA, among others), known as PAMPs, have the ability to bind and activate innate immune receptors (PRRs) such as TLRs and NLRs, which are present in liver cells (Isayama et al. 2006; Seki et al. 2007). The liver is a primary site for clearance of circulating bacteria and the second firewall, after the intestines, to contain pathogen dissemination and sepsis during conditions of altered homeostasis (Hickey and Kubes 2009; Kubes and Jenne 2018). On reaching the liver, PAMPs can induce local inflammation trough activation of TLRs: endotoxins mediate activation of TLR4 (Seki et al. 2007), Gram-positive bacteria activate TLR2 responses (Hartmann et al. 2012) and ß-glucans interact with NOD1 and NOD2 receptors. TLRs are expressed by the innate immune compartment (mostly, Kupffer cells and DCs), hepatocytes, endothelial cells, biliary epithelial cells and hepatic stellate cells. Chronic activation of TLRs can contribute to the pathophysiology of several liver conditions.

Surprisingly, in a healthy liver, only minor amounts of translocated intestinal microbes have been reported to reach the tissue, likely, we hypothesized, because more than 90% of the intestinal microbiota is constituted by anaerobic bacteria (Roediger 1980). This is probably the reason why we

were barely able to detect LPS presence in liver supernatants from mice. As an alternative, the experiments where HEK293 PRR reporter cell lines were used assisted us to better understand the PAMPs and DAMPs compartmentalization between the different vascular niches (enterohepatic and systemic circulation) and the role of the liver as a potential filter towards all these gut-derived incoming antigens. Under homeostatic conditions, plasma samples from the enterohepatic and the systemic circulation compartment showed an enrichment in TLR2, TLR4 and NOD1 ligands, particularly during the night associated timepoints, which supports the initial idea of gut-derived molecules being translocated into the bloodstream in the postprandial state, coinciding with the active phase of the day in mice. Next, we tried to evaluate the “hepatic filtering capacity” during the night, both in the presence and absence of NETs. Overall, TLR activity was consistently higher during the early night timepoints compared to the morning, and a inhibited neutrophil "netting" capacity reduced the presence of TLR ligands in the enterohepatic compartment (PV blood and supernatants from homogenized livers). This shows that neutrophils unable to cast NETs would be failing at trapping and processing within the hepatic tissue, all these TLR-ligands derived from the gut. Consequently, these DAMPS and PAMPs would be freely and “unmodified” accessing the systemic circulation, and from there they would be able to reach remote peripheral organs where they could cast a potential damage. Hence, the evaluation of TLR activity upon stimulation with RV plasma samples necessarily reflects on the filtering capacity of the liver to trap and process the gut-derived pathogens and microbial-associated molecules. In fact, BB-Cl-Amidine injected animals showed a reduced hepatic-filtering capacity, particularly during the late-postprandial times (ZT18-ZT22), compared to the hepatic filtering capacity of saline-injected animals which remained comparable overnight.

5.4 NETs as a key component of the hepatic immunological barrier filter: NETs patrol in the liver to recognize gut-derived and bacterial-borne antigens?

Recent studies have shown that even before the onset of obesity and its derived metabolic complications, dietary fats can have deleterious effects on intestinal epithelial integrity and gut barrier function (Rohr et al. 2020; Araújo et al. 2017). Fat-rich diets can lead to impaired gut permeability by altering the distribution of intestinal AJs and TJs, which together comprise the apical junctional complex (AJC), a main component of the intestinal barrier system. The intestinal barrier system is composed of a mucus layer, intestinal epithelial cells (IECs), TJs, immune populations and a complex and abundant gut microbiota. The disruption of the AJC, or any of its associated components, by external factors (such as low-grade intestinal inflammation associated to fat consumption) can increase intestinal permeability and lead to pathology (Rohr et al. 2020). For example, long-term HFD has been shown to

modulate the expression and distribution of TJs alongside the gut epithelium, induce IECs oxidative stress and apoptosis, and to correlate with intestinal hyperpermeability and increased inflammatory responses in mice (Poritz et al. 2007; Cani et al. 2008; Kirpich et al. 2012). Fat-consumption-related deleterious effects seem mediated by an increase in LPS leakage, which can induce local responses and pro-inflammatory cytokines secretion (Pendyala, Walker, and Holt 2012; André, Laugerette, and Féart 2019). Moreover, dietary fats can also negatively modulate the intestinal mucus composition and can mediate a shift in the total composition of the gut microbiota towards barrier-disrupting bacteria species. This is known as intestinal dysbiosis, a condition that further disrupts epithelial integrity and increases intestinal permeability. Dysbiosis precedes the development of metabolic endotoxemia, is associated with a decrease in bacterial richness (Le Chatelier et al. 2013), promotes systemic inflammation *via* TLR ligand translocation, and ultimately leads to the development of associated metabolic disorders (C. Shi et al. 2019; Papoutsis et al. 2022).

Having all these ideas in mind, homeostatic NETs emerged as potential peacemakers within the low-profile pro-inflammatory scenario during postprandial conditions, and as likely mediators towards the augmented plasmatic concentrations of pathogen-associated molecules (i.e. LPS) meal-derived antigens and commensal bacteria at this time; all of them conditions associated to the night-active period in mice and that define metabolic endotoxemia. Based on that, our next goal was to evaluate NET functionality as a potential physical and immunological key player of the barrier function in the hepatic compartment, and how a simulated condition of gut-derived pathogen dispersion in combination with the presence of digestion-derived products (different fat compositions) would modulate NET response and the immunological response capacity of the liver.

Coconut oil is composed of MCFAs and LCFAs. MCFAs are saturated or unsaturated fatty acids that represent approximately 62% of the total oil composition of coconut oil, being Lauric acid (C12:0) its most abundant component with up to 50% of its total fat content (Boateng et al. 2016). MCFAs are quickly oxidized by the liver and thus less obesogenic than LCFAs. Due to this, medium-chain triglycerides are commonly used in parenteral nutrition, providing a rapidly accessible source of energy for the body (Mingrone et al. 1995). MCFAs can also interact with both immune and non-immune cell populations through their G-protein-coupled receptor (GPCR) GPR84. Human GPR84 is expressed in various organs, including spleen, thymus, lung, liver and colon, and on immune cells, such as granulocytes, monocytes, macrophages, and B and T cells. Interestingly, GPR84 expression is upregulated in murine and human macrophages activated with LPS. The activation of GPR84 exacerbates the inflammatory response *via* increased production of some pro-inflammatory cytokines, such as IL-12p40 (Huang et al. 2014) or IL-8 (Suzuki et al. 2013), and has been reported to increase chemotaxis of human neutrophils (Suzuki et al. 2013). The pro-inflammatory role of MCFA was

confirmed in a study showing that diets rich in the MCFA capric acid (C6:0), caprylic acid (C8:0), and capric acid (C10:0) polarized naive T cells towards Th17 and Th1 phenotypes (Haghikia et al. 2015). Olive oil, on the other hand, is predominantly composed of long-chain triglycerides, being Oleic acid (C18:1) its mayor component, representing up to 75% of its total fat composition (Waterman and Lockwood 2007; Al-Bachir and Sahloul 2017). LCFAs are harder for the body to break down compared to smaller chained fatty acids. Due to that physicochemical properties, LCFAs tend to be slowly absorbed and transported within the organism. It has been described that LCFAs can modulate neutrophil and macrophage responses, influence the activation status and fate (survival or death) of these cells, and mediate TLR-dependent responses (Rodrigues et al. 2016; Kumar and Dikshit 2019).

Thus, depending on the chemical structure of the main fatty acid content in coconut and olive oil, we hypothesized that these fats can differentially impact innate immune cells reservoirs according to their preferential route to be redistributed within the body (Bach and Babayan 1982).. Briefly, MCFAs highly present in coconut oil entered the enterohepatic circulation and reached the systemic bloodstream after transiting through the liver. On the other, LCFAs highly present in olive oil reached the systemic circulation by being rederived towards the lymphatic system, in a mechanism that avoids their transition through the hepatic tissue (**Fig.26**). This strategy allowed us to confirm the different impact these two oils compositions have upon the hepatic neutrophil reservoir and the physiological function of these neutrophils-associated NETosis capacity, under homeostatic conditions. NETs were confirmed key players within the immunological barrier system present in the hepatic compartment, working as sentinels against the incoming meal-derived antigenic products and gut-associated microbial components. The administration of a coconut oil bolus induced a significant increase in the NETosis capacity of hepatic neutrophils, which did not happen after a gavage bolus of olive oil or water. Coconut oil was confirmed to be redistributed within the body through the enterohepatic system, and consequently impacting the physiological NET profile in the liver. This route of absorption of fatty acids ensured that NETs were able to perform their functional physiological role in the liver and impede the free passage of gut-derived molecules into the systemic circulation. Moreover, we observed that this local barrier function prevented peripheral damage in remote organs, such as the lungs. LCFAs present in olive oil were confirmed to reach the systemic circulation in a “liver bypass” mechanism, because they did not only not trigger a neutrophil response in the liver but induced a highly recruitment of neutrophils in the lungs. This accumulation of neutrophils in remote tissues defines neutrophilia, which generally in the clinical context is used as a canonical marker of inflammation. Interestingly, chemical disturbance of hepatic NETs by administration of BB-Cl-Amidine had an effect on peripheral organs (the lung) that resemble those observed when animals ingested olive oil. This confirmed that when NETs are absent in the hepatic tissue, all the gut-derived metabolites, bacterial-borne antigens and the

associated pro-inflammatory cytokines can most probably freely surpass the liver, freely translocate into the systemic circulation and trigger a damage-associated effect on remote organs such as the lungs. After BB-Cl-Amidine injection, in the absence of NETs, lung tissue was highly inflamed in the same way as when animals are fed fats that do not transit through the liver. In conclusion, the presence of local hepatic NETs allows for the gut-derived DAMPS and PAMPs to be trapped in the hepatic tissue, which reduces inflammatory signals and the remote-damage capacity of these molecules. Finally, we would like to bring up the idea of the liver as an organ that would be to a certain extent "adapted" to the daily influx of metabolic inputs derived from the digestion process that occurred in the intestine. That way, it would make sense that under physiological conditions, upon a short-term high-fat input, the immunological compartment present in the liver (commanded by NETs) could cope up with the fat-derived associated damage. This is why, even after a short-term fat consumption the recruitment of neutrophils is not dramatic, and the presence of NETs ensure the maintenance of a stable physiological scenario in the liver. On the other hand, the lung is considered a barrier organ that faces chronic exposure towards air-borne antigens and external environmental pollutants, but not that much against diet-derived metabolites, such as the aforementioned fats. An acute dosage of fats freely rederived to the lungs, as well as their pro-inflammatory associated profile, would well have an acute impact in the lungs but, as observed, not in the hepatic environment.

5.5 Clinical Perspective

As a consequence of the activation of PRRs, TLR-downstream signaling induces innate immune responses. HFD-induced increased gut permeability, intestinal dysbiosis and metabolic endotoxemia are conditions that promote major inflammatory signals (primarily, TLR4-dependent) that stimulate the secretion of cytokines and pro-inflammatory mediators into the small intestine. In the liver, and upon the arrival of these inflammatory signals, Kupffer cells have been reported to be the primary cells to produce inflammatory cytokines, including TNF-α, IL-6, IL-1ß and IFN type I, several chemokines (CXCL1, CXCL2, CCL2, CCL5, CCL3, CCL4) and release ROS (Seki and Brenner 2008; Crispe 2009). In the same way, bacteria translocation from the gut can directly induce the activation of immune cells to release a collection of pro-inflammatory cytokines and chemokines (Seki and Schnabl 2012). Ultimately, this scenario may contribute to the initiation and progression of hepatic pathology (Seki and Brenner 2008). In our experimental conditions, the cytokine profile analysis of liver supernatants revealed that local release of NETs in the hepatic tissue during homeostasis can mediate the local presence of several pro-inflammatory cytokines, such as M-CSF, IL-4, IL-5, TNF- α or IFN-γ, among others. Experimental conditions that were associated to a non-local presence of NETs in the hepatic environment were overall associated with a higher pro-inflammatory profile in the livers during the night time. For

example, BB-Cl-Amidine injected animals displayed particularly high levels of IL-5 and IFN-γ. This might again reflect the capacity of homeostatic NETs to act as modulators of inflammation. Seems convenient to remind here that NETs as aggregated conformations have been previously reported to be able to degrade pro-inflammatory cytokines and act as anti-inflammatory players in the progression of pathological conditions (Hahn et al. 2019; Euler and Hoffmann 2019).

Intriguingly, IL-5 and IFN-γ have been recognized as key modulators and boosters of liver fibrosis (Reiman et al. 2006; Attallah et al. 2016). As a matter of fact, IL-5 deficient mice have been reported to have fewer formation of granulomas in the liver upon infection with *Schistosoma mansoni*. Authors reported that these results go side by side with a reduced ratio of liver fibrosis and lower numbers of infiltrating eosinophilic granulocytes, a source of IL-13 and a trigger for granulomatous responses. That way, IL-5 blockade emerged years ago as a promising target for the treatment of hepatic fibrosis (Reiman et al. 2006). On the other hand, IFN-γ is a cytokine with antiviral activity, anti-proliferative action and both pro- and anti-inflammatory properties (Horras, Lamb, and Mitchell 2011). IFN-γ is produced mainly by activated T cells and NK cells upon stimulation with IL-12 and IL-18 in fibrotic livers (Billiau and Matthys 2009). Recently, augmented levels of IFN-γ have been correlated with advanced fibrosis stages and the progression of liver disease (Attallah et al. 2016). In a steatohepatitis model induced by feeding mice a methionine- and choline-deficient HFD, animals that showed an IFN-γ deficiency displayed lower levels of pro-inflammatory markers (TNF-α), transforming growth factor-β, liver steatosis, liver fibrosis and liver fibrosis-related genes (Luo et al. 2013). IFN-γ has been reported to induce a pro-inflammatory type M1-like activation state in hepatic macrophages, which leads to the activation of hepatic stellate cells and promotes its differentiation into profibrotic myofibroblasts (Martinez et al. 2008). Luo et al. concluded that IFN-γ deficiency might inhibit this macrophage-dependent inflammatory response and suppress hepatic stellate cells differentiation (Luo et al. 2013). In addition, IFN-γ can also induce the expression of MHC-I and MHC-II in innate immune populations, promote T- and B-cell differentiation, and activate neutrophils, all known contributors of fibrosis development (van Dijk et al. 2015).

Nonetheless, IFN-γ-dependent antifibrogenic effects have also been described in former literature examples. For example, Weng et al., had reported an improvement in fibrosis scores in patients with chronic hepatitis B virus (HBV) infection after 9 months treatment with IFN-γ (Weng et al. 2005). Exogenous IFN-γ treatment has been reported to suppress liver fibrosis induced by carbon tetrachloride (CCl_4) *via* suppression of TGF-ß signaling and activation and further differentiation of the hepatic stellate cells. Noteworthy, this has only been validated in rat models of liver injury (Baroni et al. 1996). IFN-γ seemed to have direct antifibrotic effects when it interacts with these hepatic stellate cells, by reducing their proliferation (Jeong, Park, and Gao 2008; Baroni et al. 1996). However, as previously observed,

none of these results have been validated by more recent scientific literature. As a matter of fact, IFN-γ was already proposed as a potential therapeutic drug in the treatment of hepatic fibrosis and was previously examined in clinical trials as an antifibrogenic therapeutic agent for chronic liver disease. Unfortunately, clinical trials have revealed low efficacy and opposing effects of IFN-γ treatment in liver disease, most likely due to the dual role -nowadays recognized- IFN-γ exerts in liver fibrosis.

5.5.1 Context to develop hepatic fibrosis: Can NETs temper a pro-fibrotic scenario?

Liver fibrosis occurs as an ineffective wound-healing scar response in the hepatic tissue, following a condition of chronic inflammation. Liver fibrosis can have many etiologies, such as infectious diseases (e.g. viral hepatitis), metabolic complications (e.g. cholestatic liver disease, NASH and NAFLD), exposure to toxins (e.g. alcohol liver diseases) or autoimmune conditions (e.g. primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis) (Koyama et al. 2016; Gisterå and Hansson 2017; Tan et al. 2021). Liver fibrosis consist of a pathological condition of the hepatic tissue where excessive depositions of extracellular matrix (ECM) proteins, such as collagen, occur. Myofibroblasts develop from injury-activated hepatic stellate cells, they are absent in the healthy liver and are responsible for these ECM depositions, which forms the fibrous scar. Commonly, liver fibrosis is considered a risk factor and can progress into more severe pathological conditions such as cirrhosis, hepatocellular carcinoma and liver failure (Puche, Saiman, and Friedman 2013; Hoffmann et al. 2020).

To our particular interest, TLR activation in the liver and subsequent inflammatory responses have been ultimately related to hepatic injury in the form of hepatic fibrosis (Seki and Schnabl 2012; Anand, Zarrinpar, and Loomba 2016). TLR4 signaling and gut-derived LPS have been reported to promote fibrosis in a mechanism related to endoplasmic reticulum (ER) stress and subsequent liver damage (Lebeaupin et al. 2015). Not surprisingly, experimental liver fibrosis has been suppressed in mice deficient in TLR4 (Fouts et al. 2012), mice deficient in TLR4 co-receptors (CD14 and LPS-binding protein) or mice deficient in the TLR adaptors, MyD88 and TRIF (Isayama et al. 2006; Seki et al. 2007). Interestingly, a wide spectrum of liver conditions and most experimental models of liver fibrosis have been interconnected with microbial dysbiosis and increased intestinal permeability. Indeed, despite the frequent metabolic complications associated to liver failure, the most common cause of death in patients with terminal cirrhosis results from infections and general bacteremia (Bunchorntavakul, Chamroonkul, and Chavalitdhamrong 2016; Tan et al. 2021). That so, understanding the impact of liver physiology and its interplay towards host-microbial components seems highly relevant to human health. Currently, no antifibrotic drug or therapy is available during routine clinical

practice (Seki and Schnabl 2012; Koyama et al. 2016). For that reason, current clinical intervention of liver fibrosis revolves around decreasing bacterial product ligands and targeting of innate immune signaling (e.g. blocking TLR activation), which would result in decreased inflammatory signals and reduced fibrogenic deposits within the hepatic tissue. Accordingly, this clinical scenario would reinforce an initial idea of homeostatic NETs as favourable components of the innate immune system, that function as key players in maintaining the normal tolerogenic liver status during steady state conditions.

Conclusions

5.1 Future Perspectives & Concluding remarks

Altogether, the results arising from this PhD research project revealed that NET formation in steady state is inherent to barrier organs, such as lung, spleen, lymph nodes and liver; which reinforced the idea of NETs as physical and chemical barriers against diurnal pathogen invasion.

NETs accumulate preferentially in the liver at night-time, suggesting a rhythmic regulation of NET release. Nonetheless, we cannot exclude a partial influence of the clock on NET release as a clock-entrained neutrophil-intrinsic function. In order to further assess the contribution of the clock to NETosis, experimental conditions that include $Bmal1^{KO}$ mice (with a systemic genetic disruption of the circadian molecular clock) or $Mrp8^{Cre}Bmal1^{Flox/Flox}$ mice (with a neutrophil-specific genetic disruption of the circadian molecular clock) should be evaluated. Moreover, inverse light-cycle housing-conditions, where mice exposure to light is exclusively restricted to their active face, would also educate on light-entrainment in NET release.

As a matter of fact, NET release in the liver was influenced by food intake, dietary patterns and the nutritional composition of the diet. Fat-rich diets were principal modulators of this neutrophil function. Not surprisingly, food is the most important entrainment cue for the peripheral clock in peripheral organs. However, a gap of knowledge still exists in our understanding of the mechanisms controlling the neutrophil rhythmic immune responses in the liver and their synchronization with food intake. In order to address that questions, NET release evaluation over the course of a day, under time-restricted feeding conditions and in combination with either a normal light-cycle exposure or an inverse light-cycle exposure should be evaluated in the future as well. Moreover, feeding mice with different nutritional compositions (e.g., high-glucose diets, high-protein diets) would also instruct on the NETosis dependence on particular nutrients.

Fat consumption impacts gut permeability and intestinal dysbiosis, which results in the translocation of microbial-associated products into the enterohepatic bloodstream. Release of NET in the liver responds to this influx of incoming gut-derived antigens. This was reflected on a poorer hepatic filtering capacity upon NET inhibition by BB-Cl-Amidine administration and the augment of PRR ligands in the RV blood compartment. Yet, deeper assessment of the microbiome influence (by treatment of mice with broad-spectrum antibiotics, gut-microbiome transplantation and/or depletion, and evaluation of NET release on germ-free mice) will enlighten how pathogens can modulate NET release. Amplifying the bacterial gene 16S will help assessing the presence, amount and distribution of microbiota populations in mice. This will allow to better evaluate the functionality of NETs as part of the hepatic barrier filter towards incoming microbes. Finally, evaluation of NETosis (and the global inflammatory response) in wild mice, exposed to a higher amount of insults compared to animals housed under SPF

conditions, will give a much more realist idea of the true power of NETs as part of the immune defense system.

We have demonstrated that NETs contribute to the hepatic immune barrier filter and help containing the systemic spread of gut-derived molecules. NET inhibition prompted inflammation, reflected on higher numbers of neutrophils in tissues and circulation. Moreover, experimental conditions gathering a higher and local hepatic presence of NETs at night were overall associated with a lower pro-inflammatory profile in the liver. Altogether, this pointed on the capacity of homeostatic NETs to damp inflammation. A low-functional hepatic filtering capacity (by NET inhibition or lymphatic rederivation of fats) permitted PRR ligands to reach the systemic circulation and cause remote-organ damage. Lung inflammation was assessed based on neutrophil recruitment and was evaluated as an indicator of remote-organ damage. By all means, other markers of inflammation (e.g., pro-inflammatory cytokine scores evaluated through ELISA or flow cytometry) should be used in the future to confirm the inflammatory conditions observed in peripheral organs.

In conclusion, the liver is an immunological barrier organ where neutrophils, as part of the hepatic immune compartment, work as sentinels against gut-derived incoming molecules. Neutrophils, *via* NET release and their trapping capacity, are a firewall in the hepatic tissue towards insults that would have managed to surpass first physical barriers (e.g. intestine, skin, lungs). NETs are proposed here as an interconnecting link between the two cohabitant sides of innate immunity in the liver: immune hypo-responsiveness and immunity responses. On one hand, NETs would work as tolerogenic modulators towards the day-to-day influx of non-pathogenic molecules coming from the gut (diet-derived antigens and microbiota-associated molecules). On the other hand, and upon a pathogenic insult, NETs function as key players of immunity. Therefore, maintenance of a regulated NET profile in the hepatic compartment, by ensuring a physiological threshold of the here presented as NET modulating factors (diurnal rhythms, nutrients and PAMPs), would ensure a healthy liver condition and ultimately, an improvement in life quality.

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Acknowledgements

To my PhD supervisors,

To **Oliver Söhnlein**, first of all, I would like to express my deep gratitude to my PhD first-supervisor and mentor. Thank you for your blind trust in me, for betting on me, for finance me and for opening the first door into the impassable scientific world. You will have always helped me to build the foundations of my working life and taught me what a *Chef* should be.

To **Carlos**, my PhD co-supervisor and second boss – *can it get any better?* Thank you for allowing me to scientifically grow by your side. Your scientific commitment, dedication and willingness to always learn more and do better are exemplary. Thank you for being patient, for encouraging me to think by myself and to fly alone, for listening closely and always considering my ideas, and for pushing my limits. Beyond the two little ones you have back at home, I like to think I will always be your lab *firstborn*. Thank you.

To **Rapha**, a special mention goes to the unofficial *non-profit* PhD supervisor. Thank you for being always a massive support for me, either as a colleague, a mentor, a friend, a brother or even as a father, whenever I have needed each. Thank you for always finding an encouraging word for me, for believing in me as a scientist, for the shared long-night and long-day never-ending experiments, for your black humor and for being the funniest French depressed guy ever.

To the laboratory group,

Big thank you to all the past and present lab members (**Max, Bartolo, Joana, Almudena, Giovanna, Quinte, Carla, Ariane, Pati, Janine, Olga, Andrea, Claudia, Mathis…** just to mention a few!), for keeping up the laboratory environment, for the scientific discussions and project feedbacks, and for your invaluable help and support during harvestings and scientific experiments. Thank you for the beer days and all the good memories that will always persist about the scientific working life. A special mention goes to **Kristof**, for being a constant encourager especially during the last period of the PhD, for the support on key decisions, for being always there to listen my complaints, for your empathy and for your routine daily check-ups. Also, thank you for having an available sofa when I needed that!

To **Sanne** and **PC**, for sharing the rough, uncomfortable, torturing and agonizing PhD path – I looked up for synonyms here, but end up thinking they all together fit pretty well within this little adventure. It would have never been possible to walk this path without you, your help and your support.

A **Celia**, necesitaría otra tesis para agradecerte la inmensurable ayuda y apoyo, a tantos niveles, durante estos años de doctorado. Gracias por tus consejos, tu compañía, tu empatía, tu siempre disponibilidad y tu mal humor. Por ser el *perfect match*, por escucharme, por comprenderme, por complementarme, por toda la confianza compartida, los marujeos y cotilleos, y por apreciar tanto como yo que la vida sin un poco de maldad tiene menos chispa. Gracias por ser mi *co-first author* y compartir salvavidas en este océano revuelto que es el *PhD*. Ojalá encontrar alguien como tú en cada nuevo escalón de la vida profesional que está por venir. *T'estime*, amiga.

To the LMU in München,

One of the things that makes a PhD journey a more *tolerable* experience is the people you meet in the way and the experiences you make outside the lab bench. The Munich Medical Research School (MMRS) and our graduate school took a big role there. I want to thank all the members of the IRTG914

for always organizing great lectures, seminars with top speakers, training courses, social get-togethers and annual retreats across Germany and beyond the borders. Thank you to my Thesis Advisory Committee members Markus Sperandio and Peter Düwell, for finding always the time for annual reunions, being enthusiastic about my project and my progress, believing in my work and giving always valuable feedback. A special mention goes to Verena Kochan, our magnific SFB coordinator; for being always helpful and empathic, and bringing much common sense into the daily day difficulties of a PhD. I am really happy about the network of brilliant people that I luckily shared the PhD path with, through all these years: Sergi, Nikos, Stephan, Alba, Jasmin, Sophia, and many others – you all are awesome!

A los expatriados en Múnich,

Gracias por hacer que un lugar en el extranjero se sienta como estar en casa. Laura, Juan, Ángel, Nacho, Juanma, Tano, Nuria, David, Vicky, Odri… Bien en España, Alemania, Holanda, Luxemburgo, República Checa o cualquiera que sea el próximo destino, ojalá que los *Bierfest* nos sigan juntando. Una mención especial para Sandra, porque mientras escribía este proyecto de tesis doctoral me animaba pensar que, si seis meses de amistad *in situ* pueden rendir toda la vida, qué no sería capaz de optimizar tras cinco años de trabajo.

A mis amigos,

A todos, gracias por vuestra amistad altruista, por vuestro apoyo constante, por vuestros consejos, por vuestra disponibilidad, por vuestra confianza, por compartir la vida conmigo, por ser unas personas inmensamente divertidas y por hacer de la vida una circunstancia mucho más bonita. Soy quien soy, porque todos vosotros formáis parte de ello.

A Jone e Irene, por, sin conocerme, convertiros en el mejor apoyo cuando más lo necesitaba.

A Marta Calleja, a Paula Moreno y a Ricardo, los miembros de *La Cámara Secreta*. Sois los mejores *nerds* y *psychos* del internet que podría haber deseado encontrar. Gracias por convertir cualquier reencuentro en la mejor celebración.

A Jeniffer, por darme la mano al principio de nuestra vida científico-universitaria, y nunca más dejarme caminar sola. Brindo de antemano por nuestra próxima graduación juntas, *doctora*.

A Aroa, a Bea y a Paula Velasco, porque si el tiempo pone a cada uno en su lugar, con vosotras me demuestra que antes o después la vida te acaba poniendo a las personas esenciales en el camino.

A Paula Gilbert, a Berta y a Elena Marchante, por hacerme creer en las relaciones a distancia. Gracias a *El Expreso de Hogwarts*, por sentarme en el mismo vagón con personas hoy indispensables en mi vida.

A Judith y a Elena Heras, por aportarme desde hace tantísimo tiempo la amistad más pura, bonita, sincera y menos exigente que alguien pudiera soñar. Por ser mis grandes ejemplos de mujeres libres, empoderadas y capaces. Tras todos estos años, me demostráis que las amistades de verdad simplemente *son*.

A Juan Fontaniella, a Juan Moreno y a Marta Fernanz, por crecer a mi lado y ser mi hogar. Los momentos más divertidos de mi vida siempre serán con vosotros presentes. Soy inmensamente feliz cuando estamos juntos, porque me convertís en la mejor versión de mí misma. Que siempre sigamos viajando, que sigamos creciendo, que sigamos sumando y que sigamos siendo. Ojalá teneros siempre. Sois inmensos, *amigos*.

A mi familia,

A los *mayores*,

Gracias por creer en mí, por vuestro apoyo y ánimos constantes, aún en la distancia y especialmente durante estos últimos años.

A los *pequeños*,

A Alex Olivares y Sergio, aunque hayáis saqueado con todos los genes de altura de la familia. ¡Por suerte, las neuronas nos las repartimos un poco diferente! A Lara, por tu siempre disponibilidad, para escuchar, para ayudar y para apoyar. A Alex Sánchez, por los veranos en *Navaluenga* y las noches de *Perseidas*, por los reencuentros, por ser mi *gamer-partner* y mi *detective-in-crime*. A Dani, porque no existe un *Virgo* más compatible con mi *Capricornio*. Pase lo que pase, te quiero siempre. A Ana, gracias por haberte convertido en la hermana que nunca tuve.

A todos mis abuelos, sé que, aunque ya no puedan decírmelo, estarían muy orgullosos.

A mi tía Marga, por ser mi segunda madre.

A Javier y María José, mi padre y mi madre; gracias por vuestros sacrificios, por la educación que me habéis dado e inculcado y que me ha permitido llegar hasta aquí, y ser quien soy como persona. Gracias por siempre creer en que pueda lograr cualquier cosa que me proponga, por apoyarme en todas las decisiones que he tomado a lo largo de la vida, y jamás frenar ninguna de ellas. Os quiero.

A mí misma, por no abandonar*me*.

Laura

Appendix

Curriculum vitae

WORKING EXPERIENCE

2021-2022 Scientific Researcher in the “Regulatory Mechanisms of Inflammation” group, led by Prof. Dr. Oliver Söhnlein

at Centre for Molecular Biology of Inflammation (ZMBE), Münster, Germany.

2017-2021 PhD Graduate Student in the “Vascular Immunotherapy” group, led by Prof. Dr. Oliver Söhnlein

at Institute for Cardiovascular Prevention (IPEK), Munich, Germany.

2017-2022 PhD Graduate Student of the Integrate Research Training Group 914 "Trafficking of Immune Cells in Inflammation, Development and Disease" (IRTG914)

at Ludwig-Maximilians-Universität München (LMU), Munich, Germany.

2016-2017 Internship Master’s student in the “Translational Laboratory for Cardiovascular Imaging and Therapy laboratory” group, led by Dr. Borja Ibáñez Cabeza

at Spanish National Centre for Cardiovascular Research (CNIC), Madrid, Spain.

2016 Internship Bachelor’s student in the “Molecular Immunobiology” group, led by Dra. María Ángeles Múñoz Fernández

at Health Research Institute of Gregorio Marañón Hospital (IiSGM), Madrid, Spain.

2012 Employed at “Yelmo Cines Rivas Futura”.

at H2O Centro Comercial, Rivas-Vaciamadrid, Madrid, Spain.

EDUCATION

2017-2022 Ph.D. in “Medical Research” (Immunology & Cardiovascular Biology).

at Ludwig-Maximilians-Universität München (LMU), Munich, Germany.

2016 – 2017 Master’s degree (MSc) in “Biomolecules & Cell Dynamics”

at Autonomous University of Madrid (UAM), Madrid, Spain.

2012 – 2016 Bachelor of Science (BSc) in “Health biology” (Biología Sanitaria)

at University of Alcalá de Henares (UAH), Madrid, Spain.

2009 – 2011 “Baccalaureate” High school: Science modality (Awarded “Best Academic Record”).

at I.E.S. “El Carrascal”, Madrid, Spain.

2005 – 2009 High school (E.S.O.)

at I.E.S. “El Carrascal”, Madrid, Spain.

LANGUAGES

- Spanish: Native
- English: Bilingual/Full professional proficiency - Certificate in Advanced level C1
    - According to the Common European Framework of Reference for Languages (CEFR)
- German: Elementary proficiency - Certificate in Basic level A2
    - According to the Common European Framework of Reference for Languages (CEFR)

OTHER ACCOMPLISHMENTS

LICENSES

Laboratory Animal Science module (TierSchVersV) by TransMIT GmbH, Issued: Oct 2017.

Driving License (Category B), Issued: June, 2011.

COURSES

Leadership Skills *at* LMU, Munich, Germany.

Effective Visual Communication for Scientists *at* LMU, Munich, Germany.

Applied Statistics for Molecular Biologists *at* LMU, Munich, Germany.

R Workshop *at* LMU, Munich, Germany.

Scientific Writing & Presentation Skills *at* LMU, Munich, Germany.

Good Scientific Practice *at* LMU, Munich, Germany.

Models to Study Leukocyte Recruitment *at* LMU, Munich, Germany.

"Emergencias traumáticas: Atención inicial y manejo integral" *at* UAH, Madrid, Spain.

CONFERENCES & RETREATS

1st Symposium Inflammation & Imaging. *Nov, 2021.*

SFB/CCRR1123 Annual Retreat & 9th Cardiac Regeneration & Vascular Biology Conference. *Oct, 2021.*

The Neutrophil 2021 International Symposium. *June, 2021.*

IRTG 914 Annual Retreat 2019. *Nov, 2019.*

6th Munich Metabolomics Symposium: Microbiome Meets Metabolome. *Sep, 2019.*

SFB/IRTG 914 Summer Symposium. *Jun, 2019.*

IRTG 914 Annual Retreat 2018. *Nov, 2018.*

SFB/IRTG 914 Gender and Science meeting. *Sep, 2018.*

SFB/IRTG 914 International Conference 2018. *Mar, 2018.*

OTHERS

2009-2011 Student Representative (High School) in Conflicts Resolution

List of publications

1

Perez-Olivares L, Soehnlein O.

Contemporary Lifestyle and Neutrophil Extracellular Traps: An Emerging Link in Atherosclerosis Disease.

Cells. 2021;10(8):1985. doi:10.3390/cells10081985

2

Schumski A, Ortega-Gómez A, […] Perez-Olivares L, *et al.*

Endotoxinemia Accelerates Atherosclerosis Through Electrostatic Charge-Mediated Monocyte Adhesion.

Circulation. 2021;143(3):254-266. doi:10.1161/CIRCULATIONAHA.120.046677

3

Silvestre-Roig C, Braster Q, […] Perez-Olivares L, *et al.*

Externalized histone H4 orchestrates chronic inflammation by inducing lytic cell death.

Nature. 2019;569(7755):236-240. doi:10.1038/s41586-019-1167-6

Affidavit

Affidavit

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I hereby declare, that the submitted thesis entitled:

THE ROLE OF NEUTROPHIL EXTRACELLULAR TRAPS IN HEPATIC IMMUNE HOMEOSTASIS

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is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the dissertation presented here has not been submitted in the same or similar form to any other institution for the purpose of obtaining an academic degree.

Madrid, 15th March 2023 — Laura Pérez Olivares

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Place, date — Signature doctoral candidate

Confirmation of congruency

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