

Heterogeneity of skin macrophages and the impact of high-salt diet in health and disease

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Preliminary remarks

I hereby declare that I have written this dissertation independently, using only the sources cited in the text and without assistance from third parties. In accordance with common practices in English scientific writing, parts of the text are expressed in the first-person plural form. All experiments in this study were designed and conducted by me, with contributions to the presented data from the following people:

- Aya Rafea Nasr worked as well on the HSD project and mouse cohorts were planned and conducted together.
- Salt storage measurements (Figure 7) were performed by Dr. Agnes Schroeder from the University of Regensburg
- H&E staining was performed by the Histology Core Facility from the UK Bonn (Figure 9)
- IF stainings (Figure 10 and Figure 13) were conducted by Emily Thielisch under my supervision
- *C. rodentium* culture was performed by Aya Rafea Nasr (used for Phagocytosis assays in Figure 14 and Figure 26)
- Single cell RNA sequencing experiment was performed together with Dr. Nelli Blank-Stein. Data was analysed in collaboration with Dr. Lisa Steinheuer (Figure 27)

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Linda Müller

List of abbreviations

AGM	Aorta-gonad-mesonephros
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen-presenting cells
BM	Bone marrow
BSA	Bovine serum albumin
BV	Blood-vessel associated macrophage
CCL2	CC-chemokine ligand 2
CD + number	Cluster of differentiation
CD	Control diet
CHS	Contact hypersensitivity
CLP	Common lymphoid progenitor
cMoP	Common monocyte precursor
CMP	Common myeloid progenitor
CO ₂	Carbon dioxide
D	Domain
DAMP	Damage-associated pattern
DAPI	4',6-Diamidino-2-phenylindole, diacetate
DC	Dendritic cell
DNA	Desoxyribonucleic acid
DNFB	1-Fluor-2,4-dinitrobenzol
DTH	Delayed-type hypersensitivity
E	Embryonic day
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EDU	5-ethynyl-2'-deoxyuridine
EMP	Erythro-myeloid progenitor
eNOS	Endothelial nitric oxide synthase
FACS	Fluorescence activated cell sorting
FcγR	Fc gamma receptor
FcRn	Neonatal Fc receptor
GAG	Glycosaminoglycan
Gas6	Growth arrest-specific protein 6

GMP	Granulocyte-macrophage progenitor
GRC	Generic resources center
H&E	Hematoxylin and eosin
HSC	Hematopoietic stem cell
HSD	High-salt diet
I	Interstitial macrophage
IF	Immunofluorescent
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
ILC	Innate lymphoid cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LC	Langerhans cell
LT	Long-term
LT-HSC	Long-term hematopoietic stem cell
Mac	Macrophage
MDP	Macrophage-dendritic progenitor
MEP	Megakaryocyte-erythroid progenitor
MFI	Mean fluorescent intensity
MerTK	Mer receptor tyrosine kinase
MHCII	Major histocompatibility complex II
MMP	Matrix metalloproteinase
MPP	Multipotent progenitor
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NFAT5	Nuclear factor of activated T cells 5
NK cell	Natural killer cell
N	Nerve-associated macrophage
NO	Nitric oxide
Nos2	Type-2 nitric oxide synthase
NXC1	Na ⁺ /Ca ²⁺ exchanger 1
P38/MAPK	P38 mitogen-activated protein kinase
PAMP	Pathogen-associated molecular pattern

PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PI3K	Phosphoinositid-3-kinase
pMac	Pre-macrophage
PROS	Protein S
PRR	Pattern recognition receptor
PS	Phosphatidylserine
Rac1	Ras-related C3 botulinum toxin substrate 1
RBC	Red blood cell
scRNA-seq	Single cell ribonucleic acid sequencing
SGK1	Serum/glucocorticoid-regulated kinase 1
SPF	Specific pathogen free
ST	Short-term
ST-HSC	Short-term hematopoietic stem cell
TAE	Tris base, acetic acid, ethylenediaminetetraacetic acid
tdT	tdTomato
TEWL	Transepidermal water loss
TGF- β	Transforming growth factor β
Tim4	T cell immunoglobulin and mucin domain-containing protein 4
TonEBP	Tonicity-responsive enhancer binding protein
TRM	Tissue resident macrophage
TLR	Toll-like receptors
TNF- α	Tumor necrosis factor- α
Treg cell	Regulatory T cell
Th17 cell	T helper 17 cell
VEGF	Vascular endothelial growth factor
VEGF-C	Vascular endothelial growth factor-C
WBC	White blood cell
WHO	World health organization
WT	Wild type
YFP	Yellow fluorescent protein
YS	Yolk sac

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Abstract

Excessive salt consumption, particularly from processed foods, is a growing health concern in Western countries, contributing to severe health consequences like cardiovascular disease and renal dysfunction. Beyond the known effects on the heart and kidneys, high-salt intake can also impact on our skin. Previous studies have shown that excess salt can be stored in the skin connective tissue, thereby influencing the surrounding cells. Furthermore, a high-salt environment has been shown to activate immune cells, especially macrophages and T cells. Macrophages are of particular interest in this context, as they can sense elevated salt concentrations in their microenvironment and undergo transcriptional and functional adaptations in response. In this study, we investigated the effects of acute and chronic high-salt intake on the murine skin homeostasis and on the skin's resident macrophages. As a consequence of chronic high-salt intake, we discovered a psoriasis and atopic dermatitis-like phenotype in the skin, characterized by increased proliferation of keratinocytes, a thickening of the epidermal layer, elevated transepidermal water loss, as well as a reduced tissue water content. We further comprehensively described the heterogeneous macrophage populations residing in the skin, identifying five distinct macrophage subsets. Each subset occupies a specific tissual niche and differs in surface marker expression, function and developmental origin. In this study we distinguished between Langerhans cells in the epidermis as well as MHCII⁺ and MHCII⁻ blood vessel-associated macrophages, interstitial macrophages and nerve-associated macrophages in the dermis. Given this heterogeneity, the phenotypic and functional responses to high-salt conditions varied substantially across macrophage subsets. Under homeostatic conditions, acute high-salt feeding led to increased macrophage subset abundances and broad changes in surface marker expression levels. Notably, all dermal macrophages exhibited a significant upregulation of CD32b, an inhibitory receptor implicated in immune regulation. Among the skin macrophage subsets, blood vessel-associated macrophages were most affected by acute high-salt exposure, likely due to their close proximity to the vasculature. These macrophages exhibited morphological alterations, including reduced cell volume and surface area, transcriptional changes and a subset composition shift towards an increased proportion of MHCII⁻ cells, which are more active in phagocytosis and efferocytosis than their MHCII⁺ counterparts. While these phenotypic and functional changes were most pronounced under acute high-salt conditions, a similar macrophage phenotype was observed in an allergic inflammation model following chronic high-salt intake. Strikingly, CD32b upregulation was consistently observed across all dermal macrophage subsets, highlighting its potential role in macrophage adaptation to both high-salt exposure and inflammation. Overall, our findings demonstrate that chronic high-salt intake impairs skin tissue integrity, while acute high-salt exposure

leads to extensive phenotypic and functional alterations in skin macrophages. Notably, a similar macrophage response was observed under chronic high-salt conditions in combination with an inflammatory allergic skin reaction. Furthermore, we identify CD32b upregulation in skin macrophages as a potential compensatory mechanism in response to acute inflammatory high-salt conditions, highlighting it as an intriguing target for future investigations.

1. Introduction

1.1 Western diet, the role of salt and health implications

Sodium is a key dietary component, crucial for maintaining normal physiological function of our body (Kotchen et al., 2013). It is commonly included in our regular diet as sodium chloride (salt). However, in many Western countries, salt consumption has risen to levels that far exceed the recommended daily intake set by the World Health Organization (WHO, 2013). The main reason for excessive salt intake, is the increase in consumption of highly processed foods, which often lack fibers and nutrients while containing massive amounts of salt, sugars and fat (Fuhrman, 2018). Prolonged adherence to a Western-style diet has been shown to profoundly impact metabolism and overall health, contributing to weight gain, dyslipidemia, impaired energy homeostasis and chronic immune activation (Christ et al., 2019). To disentangle the specific effects of individual dietary components, such as excessive salt intake, it is essential to study each of these factors separately. Investigating a high-salt diet (HSD) allows researchers to elucidate sodium-specific mechanisms, including its effects on water retention, renal function and vascular homeostasis, without the confounding variables associated with a Western diet (Kanbay et al., 2011). Notably, a high-salt intake has been directly linked to the development of hypertension, a major risk factor for cardiovascular diseases like heart attacks and strokes, which remain the leading causes of death globally (O'Donnell et al., 2015; Rucker et al., 2018).

In recent years, research on the impact of a HSD on the immune system has gained increasing attention, as emerging evidence links excessive sodium intake to immune cell activation, inflammation and the development of immune-mediated diseases. For instance, a study demonstrated that excessive salt intake impairs neutrophil function. Volunteers who consumed an additional six grams of salt per day, an equivalent to two fast-food meals, exhibited a diminished capacity of neutrophils to effectively combat bacterial infections within just one week (Jobin et al., 2020). Another research group was able to show, that a high-salt intake promotes the development of pro-inflammatory T helper 17 cells, which are linked to autoimmune diseases. This occurs through the activation of serum/glucocorticoid-regulated kinase 1 (SGK1), a pathway enhanced by elevated sodium levels (Kleinewietfeld et al., 2013; C. Wu et al., 2018). Additionally, macrophages have gained increasing attention as a key target for studying the effects of a HSD due to their central role in immune regulation and their sensitivity to environmental changes (W. C. Zhang et al., 2015).

The current findings emphasize the significant impact of dietary sodium on immune homeostasis and suggest that excessive salt intake may play a role in the development of inflammatory and autoimmune disorders. However, the long-term effects of high-salt intake and the underlying molecular mechanisms remain poorly understood. Further research is needed to elucidate these mechanisms and their implications for immune function.

1.2 The skin tissue and physiological consequences of high-salt intake

1.2.1 Skin physiology and immune function

The skin, as the body's largest organ, serves as a vital barrier between the internal and external environments. It consists of three distinct layers, each with specific functions ranging from physical protection to sensory perception and temperature regulation (Lotfollahi, 2024). The outermost layer, the epidermis, is primarily composed of keratinocytes and acts as a waterproof barrier and protection against pathogens. Within the epidermis, melanocytes produce melanin, which shields against harmful UV radiation. Beneath the epidermis lies the dermis, which houses blood vessels, nerve fibers, hair follicles, as well as sweat- and sebaceous glands. This layer supports the epidermis while contributing to thermoregulation and sensory functions. The deepest layer, the subcutaneous tissue, consists of fat and connective tissue, playing a critical role in insulation and energy storage (Yousef et al., 2025). Beyond its role as a physical barrier, the skin functions as a dynamic immune organ equipped with specialized cells and mechanisms to defend against pathogens and maintain tissue homeostasis (Maranduca et al., 2020; Nguyen & Soulika, 2019).

The epidermis serves as the first line of immunological defense, with keratinocytes producing antimicrobial peptides and cytokines, contributing to innate immunity (Wang & Li, 2020). The primary immune cells within the epidermis include T cells and Langerhans cells. In the murine epidermis, the predominant T cell subset consists of $\gamma\delta$ T cells, which play key roles in immune surveillance, wound healing and skin homeostasis. These cells recognize stressed or damaged keratinocytes and contribute to maintaining epidermal integrity (Hu et al., 2022; MacLeod & Havran, 2011). Langerhans cells, a specialized subset of macrophages, primarily function as antigen-presenting cells (APCs). They capture antigens in the epidermis, migrate to regional lymph nodes and present these antigens to T cells, thereby initiating adaptive immune responses. In addition to their role in antigen presentation, Langerhans cells produce inflammatory mediators such as interferons (IFNs) and cytokines, which modulate immune responses and contribute to skin immune homeostasis (Kaplan, 2017).

Situated beneath the epidermis, the dermis plays a pivotal role in skin immunity, functioning as a dynamic center for immune surveillance, coordination and response. It provides structural support while housing an extensive network of blood and lymphatic vessels that facilitate immune cell trafficking and pathogen detection (Heath & Carbone, 2013). Various immune cell populations reside within the dermal layer, each contributing to skin homeostasis and defense. Key APCs are the dermal dendritic cells (DCs) taking over similar tasks as the Langerhans cells in the epidermis. Additionally, the dermis harbors multiple T cell subsets, including CD4+, CD8+ and regulatory T cells, playing essential roles in regulating inflammation, mediating cytotoxic responses, maintaining immune tolerance and preventing autoimmune reactions. Moreover, mast cells contribute to allergic reactions and pathogen defense by releasing histamines and other mediators. During infections, neutrophils rapidly infiltrate the dermis to phagocytose pathogens and release antimicrobial substances, while innate lymphoid cells (ILCs) respond to infections or tissue damage by producing cytokines, further shaping the immune response. Finally, distinct macrophage subpopulations in the murine dermis perform crucial functions such as phagocytosis, efferocytosis, cytokine secretion and supporting tissue repair and remodeling (Heath & Carbone, 2013; Nguyen & Soulika, 2019). Overall, the skin's immune system, through its complex network of resident immune cells, plays a crucial role in maintaining tissue integrity and defending against external threats.

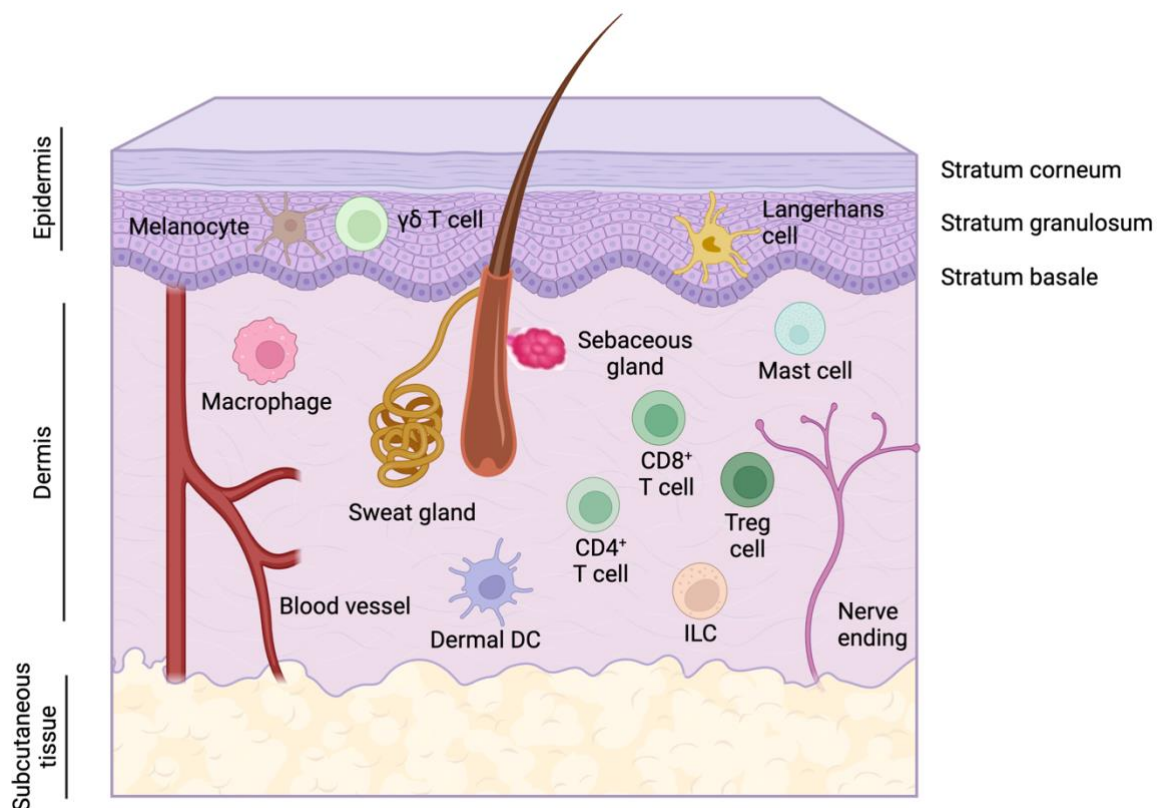


Figure 1: Immune cell composition of the murine skin.

The skin harbors a diverse repertoire of immune cells distributed across its distinct layers. In the epidermis, the dominant immune cell populations include $\gamma\delta$ T cells and Langerhans cells, primarily localized in the stratum granulosum. The dermis contains a broader variety of immune cells, including macrophages, dendritic cells (DC), mast cells, innate lymphoid cells (ILC), and multiple T cell subsets such as CD4⁺, CD8⁺, and regulatory T (Treg) cells. In addition to immune cells, the dermal compartment features an intricate network of blood and lymphatic vessels, peripheral nerve fibers, as well as skin appendages such as hair follicles, sweat glands and sebaceous glands.

1.2.2 Salt storage in the skin

The skin has emerged as a key target for research on the effects of a HSD, largely due to pioneering work by Titze and colleagues in the early 2000s. Their study introduced the concept of osmotically inactive sodium storage in the skin, revealing that sodium can accumulate at high concentrations of up to 180–190 mmol/L without proportional water retention, thereby challenging traditional models of sodium homeostasis (Titze et al., 2003). This sodium is primarily stored in the skin's interstitial space, where it binds to negatively charged glycosaminoglycans (GAGs), which are long polysaccharide chains that create a gel-like extracellular matrix and attract sodium via electrostatic interactions. However, the reason for sodium storage in the skin remains unclear.

Beyond non-osmotic sodium storage via GAGs, additional mechanisms regarding sodium homeostasis in the skin have been identified in recent years. Machnik and her colleagues demonstrated the crucial role of the immune system, particularly macrophages, in detecting elevated sodium levels and orchestrating responses to facilitate sodium clearance (further detailed in section 1.4) (Machnik et al., 2009). Another key aspect of sodium homeostasis in the skin involves the generation of local osmotic gradients, which are essential for maintaining sodium balance and regulating systemic physiological functions. These gradients arise due to differences in osmolarity between skin layers, with the epidermis exhibiting higher osmolarity than the dermis. This spatial distribution of electrolytes and urea across skin compartments enables the skin to regulate its own microenvironment independently of systemic conditions (Mai et al., 2023). Despite the hypertonic nature of the skin interstitium, both the lymph draining the skin and interstitial fluid collected from implanted wicks remain isosmotic to plasma, indicating that the skin possesses regulatory mechanisms that locally control sodium and water movement while preserving overall systemic osmotic balance (Nikpey et al., 2017).

1.2.3 The role of HSD in inflammatory skin diseases

High dietary salt intake has been increasingly associated with inflammatory skin diseases such as atopic dermatitis and psoriasis, as well as with oxidative stress and accelerated skin aging. Independent of a high-salt intake, it was shown that higher sodium levels in the skin are a significant

feature of psoriasis, particularly in patients with more severe skin disease. Higher sodium concentrations in the skin also promote the differentiation and activation of pathogenic Th17 cells, which are central to psoriasis pathology. *In-vitro* studies further discovered, that a high-sodium environment markedly induced expression of the pro-inflammatory cytokine IL-17a in naïve CD4⁺ T cells from psoriasis patients (Maifeld et al., 2022). It was also unraveled, that atopic dermatitis in mice combined with a five-week HSD feeding results in elevated sodium concentrations in the skin, alongside an amplified pro-inflammatory response of dermal fibroblasts and peripheral blood mononuclear cells (PBMCs). Additionally, HSD-fed mice exhibit impaired ECM remodeling and an overall exacerbation of disease severity (Pajtók et al., 2021). Notably, alterations of the skin morphology can also occur without the onset of inflammatory skin disease. In Wistar rats it was shown, that 8-week feeding of a HSD induces profound structural and physiological alterations in the skin, including epidermal thickening, dermal thinning and hair follicle atrophy. Furthermore, markers of oxidative stress and cell cycle inhibition are significantly upregulated, along with increased expression of inflammatory mediators and proteases, resembling key features of accelerated skin aging (Peng et al., 2024). Together, these findings highlight tissue sodium accumulation as both a hallmark and a potential driver of inflammatory skin pathology and premature skin aging, warranting further investigation into its underlying mechanisms and therapeutic implications. Given their central role in immune regulation, tissue remodeling and inflammation, macrophages likely represent key mediators in this context.

1.3 Macrophage heterogeneity

1.3.1 Macrophage ontogeny

Macrophages are essential innate immune cells found in nearly all murine and human tissues, where they adapt to their specific microenvironments and exist as distinct subtypes (Mass et al., 2023). As most immune cells differentiate from hematopoietic stem cells (HSCs) in the bone marrow (BM), macrophages were historically believed to originate exclusively from BM-derived monocytes that infiltrate tissues and differentiate in situ. Nearly a decade ago, this paradigm was challenged by the discovery of murine resident macrophages that arise from erythro-myeloid progenitors (EMPs) in the yolk sac (YS) during embryogenesis (Perdiguero et al., 2015). Notably, these fetal-derived macrophages populate tissues before the onset of definitive hematopoiesis and persist into adulthood as long-lived, self-maintaining populations (Hoeffel & Ginhoux, 2018). However, during postnatal tissue maturation, BM-derived monocytes contribute to the establishment of distinct macrophage populations across various tissues. While some tissue-resident macrophages largely maintain themselves through self-renewal, such as Microglia and Langerhans cells, others depend on

continuous monocyte replenishment throughout adulthood (Dick et al., 2022; Ginhoux et al., 2013; Kanitakis et al., 2011).

Fetal macrophage development is a complex process that occurs in distinct waves during embryogenesis, primarily originating from the YS and later involving other hematopoietic sites (Y. Wu & Hirschi, 2021). The first wave, known as primitive hematopoiesis, begins at embryonic day (E) 7.5 with the emergence of erythroid progenitors in the extra-embryonic mesoderm of the YS. This wave generates primitive erythroid progenitors which contribute to early embryonic oxygenation, as well as embryonic macrophages which provide the first line of immune defense (Palis, 2016). However, primitive hematopoiesis is a transient process and is rapidly succeeded by the second wave, known as definitive hematopoiesis, starting at E8.5. During this stage, multipotent EMPs arise through an endothelial-to-hematopoietic transition in the YS. These EMPs possess the capacity to differentiate into various hematopoietic lineages, including erythrocytes, granulocytes, mast cells, megakaryocytes, monocytes and macrophages. Between E8.5 and E10.5, EMPs migrate to the fetal liver, where they upregulate *Tnfrs11a* expression and differentiate into a distinct *Cx3cr1*-expressing pre-macrophage (pMac) population, independent of monocyte lineage commitment (Mass et al., 2016). EMPs and pMacs undergo expansion in the YS and fetal liver before entering the blood stream to migrate into developing tissues. Upon arrival, they differentiate into the majority of tissue-resident macrophages, establishing long-lived populations that contribute to tissue homeostasis and immune defense throughout life (Stremmel et al., 2018). Finally, the third wave, known as definitive hematopoiesis, begins with the emergence of HSCs in the aorta-gonad-mesonephros (AGM) region, which originates from the mesoderm germ layer. Around E12.5, the generated HSCs migrate into the fetal liver where they expand and with the potential to differentiate into all blood cell types. As development progresses, the formation of the BM cavity is completed and by E17.5 definitive hematopoiesis transitions from the fetal liver to the BM. While most cells derived from earlier hematopoietic waves are eventually replaced by HSC-derived counterparts, macrophages and a few other immune cells, such as mast cells, largely retain their fetal origins (Gentek et al., 2018; Ghosn et al., 2019). The quantity of replacement of EMP-derived tissue-resident macrophages by BM-derived monocytes varies between organs and macrophage subpopulations. Before entering tissue niches to differentiate into macrophages, HSC-derived monocytes have differentiated from *Ms4a3*-positive myeloid cells which before originated from *Cxcr4*-expressing HSCs (Liu et al., 2019).

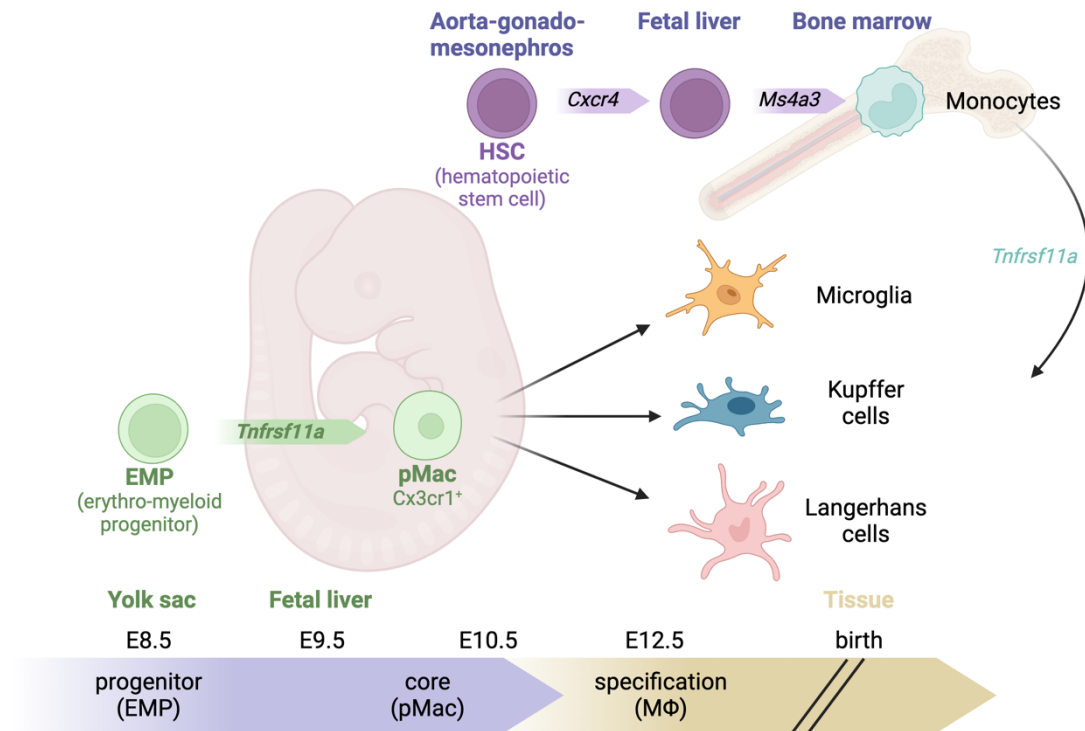


Figure 2: Ontogeny of macrophages.

Fetal-derived macrophages originate from erythro-myeloid progenitors (EMPs) in the yolk sac, which upregulate *Tnfrsf11a* expression and give rise to pre-macrophages (pMac). These pMac, characterized by *Cx3cr1* expression, migrate to the fetal liver around embryonic day (E) 9.5, where they expand and subsequently colonize developing organs. There, they differentiate into tissue-resident macrophages such as microglia in the brain, Kupffer cells in the liver and Langerhans cells in the epidermis. In parallel, definitive hematopoiesis is initiated at E10.5 in the aorta-gonadomesonephros (AGM) region, followed by the seeding of hematopoietic stem cells (HSCs) in the fetal liver. Around the time of birth, hematopoiesis shifts to the bone marrow (BM), where the *Cxcr4* expressing HSCs give rise to hematopoietic progenitors. These progenitors upregulate *Ms4a3* during monocyte commitment. In an organ-specific manner, monocytes gradually replace EMP-derived macrophages during postnatal life and adulthood.

1.3.2 Macrophage core functions

Macrophages are key players in the immune system, contributing critically to both tissue homeostasis and inflammation. Their functional roles are shaped by the tissue microenvironment and the presence of inflammatory cues (Davies et al., 2013). Under homeostatic conditions, macrophages support tissue repair by secreting growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), thereby promoting angiogenesis and stimulating fibroblast activity. For tissue remodeling, they produce matrix metalloproteinases (MMPs) and other ECM-modifying enzymes (Meizlish et al., 2024). Macrophages also regulate key metabolic processes, including iron recycling and lipid metabolism, to maintain tissue integrity (Wculek et al., 2022). Moreover, they play important roles in immune tolerance by suppressing excessive immune responses, thereby helping to prevent autoimmune reactions (Zhu et al., 2023).

One of their central functions in steady-state conditions is tissue surveillance and maintenance. In this role, macrophages continuously monitor tissues for signs of damage or infection, clearing cellular debris, apoptotic cells and misfolded proteins to prevent the accumulation of harmful materials (Davies et al., 2013). When macrophages encounter pathogens during surveillance, they recognize them via pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), and initiate an inflammatory response. Upon binding to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), macrophages engage in phagocytosis to engulf and eliminate the invading pathogens (Mukhopadhyay et al., 2009; Ozinsky et al., 2000). In addition, they secrete pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin (IL)-1 β , which amplify the immune response and recruit additional immune cells to the site of infection (Duque & Descoteaux, 2014). Macrophages also act as a bridge between innate and adaptive immunity by processing and presenting antigens to T cells via major histocompatibility complex class II (MHCII) molecules (Guerriero, 2019). To meet the energetic demands of inflammation, macrophages reprogram their metabolism, shifting from oxidative phosphorylation to glycolysis, which supports rapid energy production and the synthesis of inflammatory mediators (Viola et al., 2019). As inflammation resolves, macrophages play a pivotal role in initiating resolution processes by transitioning into reparative states, thereby contributing to tissue healing and the restoration of homeostasis (Ortega-Gómez et al., 2013).

1.3.2.1 Fc γ receptor-mediated phagocytosis

Phagocytosis represents one of the core functions of macrophages, enabling them to ingest and neutralize pathogens, apoptotic cells and cellular debris. Target recognition can occur either through PRRs or via Fc gamma receptors (Fc γ Rs), which bind to the Fc region of IgG (Immunoglobulin G) antibodies coating pathogens or immune complexes (Uribe-Querol & Rosales, 2020). There are four classes of Fc γ Rs in mice including Fc γ RI (CD64), Fc γ RIIB (CD32b), Fc γ RIII (CD16) and Fc γ RIV (CD16.2). Among these, CD64 is a high-affinity Fc γ R capable of binding monomeric IgG antibodies (specifically IgG2a and IgG2b) and plays a central role in mediating immune effector functions such as phagocytosis but also antigen presentation and cytokine production. Structurally, CD64 comprises three immunoglobulin-like extracellular domains, where domains D1 and D2 interact with the Fc portion of the IgG, while D3 stabilizes the receptor and enhances its conformation. CD64 is associated with the Fc receptor common γ -chain, which contains an immunoreceptor tyrosine-based activation motif (ITAM) critical for downstream signaling (Bruhns, 2012; Hui et al., 2023). An upregulation of CD64 on macrophages is associated with a pro-inflammatory activation, characterized by enhanced phagocytosis, cytokine production and antimicrobial activity (Akinrinmade et al., 2017). CD32b is a low-affinity Fc γ R that binds immune complexes composed of

IgG1, IgG2a and IgG2b, playing a key role in immune regulation by transmitting inhibitory signals. This type I transmembrane protein contains two immunoglobulin-like extracellular domains (D1 and D2) for Fc binding, and a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM), which mediates negative regulation of immune cell activation (Brooks et al., 1989). Upregulation of CD32b dampens immune responses by counteracting activating signals from other FcγRs (Holgado et al., 2018; Vollack et al., 2017). Another low-affinity FcγR, CD16, can bind to IgG1, IgG2a and IgG2b and plays important roles in phagocytosis, cytokine production and antibody-dependent cellular cytotoxicity (ADCC). CD16 is a transmembrane glycoprotein consisting of two immunoglobulin-like extracellular domains and a short cytoplasmic tail. It is expressed on many immune cell types including macrophages, monocytes and natural killer (NK) cells. Upregulation of CD16 enhances effector functions, including cytotoxic granule release (mainly in NK cells), antigen presentation and inflammatory mediator production (Bruhns, 2012; Junker et al., 2020). The FcγR CD16.2 shares structural and functional similarities with CD16, including its role in immune regulation and phagocytosis. It binds IgG2a and IgG2b with relatively high affinity and is implicated in autoimmune and inflammatory responses. CD16.2 is expressed on monocytes, macrophages and neutrophils (Bruhns, 2012). Although the neonatal Fc receptor (FcRn) also binds the Fc portion of IgG, it is not regarded as a conventional FcγR, as it does not elicit classical FcγR signaling. Instead, FcRn functions as a transport and homeostasis receptor. It plays a pivotal role in extending IgG half-life by protecting it from lysosomal degradation as well as in the transcytosis of IgG across epithelial barriers, including the maternal-fetal interface to mediate passive antibody transfer (Roopenian & Akilesh, 2007).

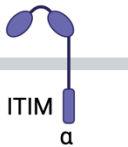
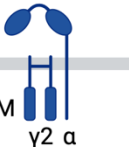
IgG					
Name	FcγRI (CD64)	FcγRIIB (CD32b)	FcγRIII (CD16)	FcγRIV (CD16.2)	FcRn (neonatal)
Structure					
Function	Activation	Inhibition	Activation	Activation	Recycling Transport Ag uptake
IgG subclass binding	- 2a 2b (3)	1 2a 2b -	1 2a 2b -	- 2a 2b -	1 2a 2b 3

Figure 3: Fcγ receptors in mice.

Murine Fcγ receptors comprise four main classes: FcγRI (CD64), FcγRIIB (CD32b), FcγRIII (CD16) and FcγRIV (CD16.2). These receptors differ in their structure, cellular distribution and functional properties, including their affinity for specific IgG subclasses. The neonatal Fc receptor (FcRn) binds as well to the Fc portion of IgG,

but it is not classified as a conventional Fcγ receptor due to its distinct structure and primary role in IgG recycling and transport.

1.3.2.2 Tim4 and MerTK-mediated efferocytosis

Another essential core function of macrophages is efferocytosis, the process by which apoptotic cells are recognized, engulfed and cleared in a non-inflammatory manner (Doran et al., 2020). This process is critically mediated by the receptors T cell immunoglobulin and mucin domain-containing protein 4 (Tim4) and Mer receptor tyrosine kinase (MerTK), which act through distinct yet complementary mechanisms. Tim4 functions predominantly as a phosphatidylserine (PS) recognition receptor, binding directly to PS exposed on the outer membrane of apoptotic cells, commonly referred to as an “eat-me” signal. However, due to the absence of intracellular signaling motifs, Tim4 is unable to initiate the cytoskeletal rearrangements required for internalization. Instead, it primarily serves as a tethering receptor, anchoring apoptotic cells to the phagocyte surface and facilitating their subsequent uptake by other receptors, such as MerTK. In addition, Tim4 collaborates with bridging molecules like protein S (PROS) and growth arrest-specific protein 6 (Gas6), which enhance apoptotic cell recognition and promote efficient phagocytosis (Moon et al., 2020). In contrast, MerTK does not directly recognize PS but requires these bridging molecules, which simultaneously bind PS on apoptotic cells and MerTK on phagocytes. Upon ligand engagement, MerTK undergoes autophosphorylation, initiating downstream signaling cascades, including phosphoinositid-3-kinase/protein kinase B (PI3K/Akt) and ras-related C3 botulinum toxin substrate 1 (Rac1) pathways. This activation promotes actin cytoskeletal rearrangement, facilitating engulfment while concurrently inducing anti-inflammatory responses, such as increased secretion of IL-10 and transforming growth factor-β (TGF-β), as well as suppressing pro-inflammatory cytokines including TNF-α and IL-12. Rac1 activation in particular, plays a key role in mediating the morphological changes required for apoptotic cell internalization. Disruption of Tim4 or MerTK function impairs apoptotic cell clearance, thereby increasing the risk of unresolved inflammation and autoimmune pathologies (Moon et al., 2020; Yang et al., 2023).

1.3.3 Macrophages in the skin

Skin macrophages are a diverse group of immune cells which take over critical functions in tissue homeostasis, immune defense and wound healing. So far, they only have been broadly classified into tissue-resident macrophages (TRMs) and monocyte-derived macrophages, with distinct subsets based on their location, surface markers and functional properties (Chakarov et al., 2019; Lee & Sacks, 2024). However, a comprehensive characterization of all dermal macrophage subsets is still lacking.

The best-described population so far are the Langerhans cells, which are the only macrophages located in the epidermis. They were first described by Paul Langerhans in 1868, who had mistaken them as nerve cells due to their dendritic shape. After the discovery of DCs over a century later, Langerhans cells were initially categorized as such due to their similar morphology and their function as APCs (Deckers et al., 2018). However, subsequent studies investigating their developmental origin led to their reclassification as macrophages, as they derive from a distinct embryonic precursor lineage. Today, Langerhans cells are recognized as a self-renewing macrophage population, with only minor replenishment by monocytes under homeostatic conditions (Ginhoux & Guillemins, 2016). They are further characterized by the expression of key surface markers such as Langerin (CD207), MHCII and EpCAM (CD326). In addition to their role in antigen presentation, Langerhans cells contribute to immune surveillance and play an important role in the promotion of immune tolerance (Becerril-García et al., 2020; Clayton et al., 2017; Nagao et al., 2009).

A common distinction among dermal macrophage subsets, which is also observed in other tissues, is the classification into perivascular and perineuronal macrophages. Perivascular TRMs are located near blood vessels in the dermis and are characterized by high expression of CD206 and Lyve1. These cells exhibit anti-inflammatory properties and are highly phagocytic, capturing blood-borne macromolecules and pathogens. In addition to their role in immune surveillance, they are implicated in tissue repair, remodeling and vascular regulation (Barreiro et al., 2016; Lee & Sacks, 2024). Perivascular macrophages have been described as being initially seeded by fetal progenitors during embryogenesis, maintaining themselves predominantly through self-renewal and displaying considerable resilience to inflammatory challenges (Lee & Sacks, 2024). In contrast, perineuronal macrophages are associated with sensory nerves in the skin. They express high levels of MHCII and Cx3cr1 and are involved in neuroimmune interactions, pain regulation, and inflammation. Their transcriptional profile shares common features with activated microglia, yet they also express a distinct set of genes linked to angiogenesis, collagen organization and axon guidance. Further, perineuronal macrophages have been shown to be seeded prenatally and to self-maintain with minimal postnatal replacement by circulating monocytes, highlighting their identity as a long-lived, TRM population (Kolter et al., 2019; Lee & Sacks, 2024). Nonetheless, a comprehensive understanding of the full diversity, ontogeny, and functional specialization of dermal macrophage populations is not yet fully established, underlining the need for further in-depth investigation.

1.3.4 The impact of high-salt conditions on macrophages *in-vitro* and *in-vivo*

High-salt conditions significantly modulate macrophage function, eliciting both pro-inflammatory and anti-inflammatory effects depending on the context and duration of exposure. Our previous *in-vitro* studies have shown that excess sodium concentrations (50–60 mM above baseline) induce chemotactic responses in bone marrow-derived macrophages (BMDMs). Moreover, high-salt environments alter the surface marker expression profile of BMDMs, notably reducing levels of the inhibitory Fcγ receptor CD32b and the efferocytosis receptor MerTK. These changes are accompanied by a dose-dependent decrease in both phagocytic and efferocytic capacity (Müller et al., 2024). Additionally, high-salt treatment of human and murine macrophages leads to increased expression of pro-inflammatory genes, such as CC-chemokine ligand 2 (CCL2), TNF-α and IL-1β, as well as a concomitant reduction in anti-inflammatory gene expression. Notably, salt exposure enhances the pro-inflammatory activation of LPS-stimulated BMDMs, further promoting an inflammatory phenotype (W. C. Zhang et al., 2015). Furthermore, a high-salt intake has been linked to several skin pathologies associated with macrophage responses *in vivo*. In this context, the influence of high-salt conditions on hypertension through a macrophage-mediated mechanism was demonstrated. Acute high-salt intake in rats results in hypertonic sodium accumulation in the skin interstitium, creating a hyperosmotic microenvironment. This, in turn, leads to recruitment of macrophages that activate tonicity-responsive enhancer binding protein (TonEBP), which binds to the vascular endothelial growth factor-C (VEGF-C) gene promoter, inducing VEGF-C secretion. VEGF-C promotes lymph angiogenesis, enhancing lymphatic drainage to buffer the hypertonic volume. Depletion of macrophages or sequestration of VEGF-C by soluble VEGF receptor-3 disrupts this buffering mechanism, leading to interstitial sodium retention, reduced endothelial nitric oxide synthase (eNOS) expression and salt-sensitive hypertension (Machnik et al., 2009). Besides hypertension, acute high-salt intake has also been shown to enhance macrophage-driven antimicrobial defense in the skin. High-sodium conditions enhance p38 mitogen-activated protein kinase (p38/MAPK) dependent nuclear factor of activated T cells 5 (NFAT5) activation in macrophages, leading to increased expression of type-2 nitric oxide synthase (Nos2) and elevated nitric oxide (NO) production, which is critical for pathogen clearance. Notably, myeloid cell-specific deletion of Nfat5 abrogates this enhanced antimicrobial response (Jantsch et al., 2016). However, most studies conducted to date have primarily focused on the effects of acute high-salt intake on skin macrophages. Given that chronic high-salt consumption better reflects modern dietary habits, it is crucial to expand future research to understand the long-term consequences of sustained high sodium exposure on macrophage function and skin homeostasis.

1.4 Aim of the thesis

Excessive dietary salt intake is a growing health concern in industrialized societies and is increasingly recognized not only for its role in promoting hypertension but also for its capacity to modulate immune function. High sodium concentrations can influence immune cell behavior, shaping both pro- and anti-inflammatory responses depending on context and duration of exposure. Among peripheral tissues, the skin presents a particularly relevant site as it serves not only as a first-line barrier against environmental insults but also as a key reservoir for sodium storage and thus represents a critical interface where dietary salt can directly alter local immune cell function. Despite this, the specific impact of increased tissue sodium levels on skin-resident immune cells, particularly macrophages, remains poorly understood. Skin macrophages constitute a highly heterogeneous immune cell compartment, occupying distinct tissue niches and exhibiting diverse functional specializations in tissue homeostasis, immune surveillance, repair and inflammation. While initial classifications have delineated subpopulations such as perivascular and perineuronal macrophages based on spatial localization, surface marker expression and transcriptional signatures, a comprehensive understanding of their phenotypic plasticity, functional roles and developmental trajectories remains incomplete. This study aims to dissect the heterogeneity of skin macrophages by integrating analyses of surface marker profiles, niche-specific localization and ontogeny. Particular emphasis will be placed on elucidating how these different macrophage subsets dynamically respond to microenvironmental perturbations, specifically elevated tissue sodium levels, as an attempt to uncover salt-induced modulatory mechanisms within the skin macrophage landscape. Given the increasing recognition of high dietary salt intake as a modulator of immune responses and a contributing factor in inflammatory skin diseases such as atopic dermatitis and psoriasis, it is critical to investigate how both acute and chronic high-salt exposure impacts distinct skin macrophage populations. While previous studies have primarily examined the effects of acute salt challenges, chronic salt exposure more accurately reflects contemporary dietary habits and may differentially shape macrophage behavior and function. Furthermore, this study seeks to determine whether high-salt conditions exacerbate inflammatory skin responses and disease severity, particularly in the presence of pre-existing immune activation. By integrating models of inflammatory skin disease with high-salt exposure, we aim to assess whether additional salt stress acts as an aggravating factor. Ultimately, this work intends to explore whether there is a unifying mechanism by which skin macrophages adapt to salt-induced changes in their microenvironment. Understanding these processes may provide novel insights into the regulation of skin immunity under both homeostatic and pathological conditions and could reveal new targets for therapeutic intervention in inflammatory skin disorders.

2. Materials and methods

2.1 Materials

2.1.1 Laboratory equipment

The following laboratory devices and equipment were used for experiments conducted for this thesis.

Device	Manufacturer
Advia 2120i Hematology System	Siemens Healthineers (Erlangen, Germany)
Axio Lab.A1	Zeiss (Jena, Germany)
Autoclave DX-150	Systec (Linden, Germany)
Centrifuge 4515R	Eppendorf (Hamburg, Germany)
Centrifuge 5810R	Eppendorf (Hamburg, Germany)
Controlled atmosphere incubator C150	Binder (Great River, USA)
Cryostat CM3050S	Leica (Wetzlar, Germany)
Dissection equipment	Fine Science Tools (Heidelberg, Germany)
Dumont '5/45 Forceps	Fine Science Tools (Heidelberg, Germany)
Electrophoresis system	VWR (Radnor, USA)
Flow Cytometer FACS Symphony A5	BD Biosciences (Heidelberg, Germany)
Flow Cytometer LSR II	BD Biosciences (Heidelberg, Germany)
Freezer -80°C	New Brunswick Scientific (Edison, USA)
Fridge 4°C	Bosch (Gerlingen, Germany)
Gel Doc Imager	BioRad (Hercules, USA)
Heating block Thermomixer Compact	Eppendorf (Hamburg, Germany)
Heating magnet stirrer ARE	VELP Scientifica (Usmate, Italy)
Laminar flow hood HeraSafe KS	ThermoFisher Scientific (Waltham, USA)
Mastercycler X50s	Eppendorf (Hamburg, Germany)
Microscope LSM 880 Airyscan (confocal)	Zeiss (Jena, Germany)
Multi Skin Test Center MC 750	Lischke Medizintechnik
Neubauer counting chamber	Marienfeld (Lauda-Königshofen, Germany)
NextSeq2000 Sequencer	Illumina (San Diego, USA)
P3 50PE flowcell	Illumina (San Diego, USA)
pHmeter MP220	Mettler Toledo (Greifensee, Switzerland)
Pipet controller accu-jet pro	Brand (Wertheim, Germany)

Pipette set (2.5 – 1000 µl)	Pipetman Classic Gilson (Middleton, USA)
Rocker Shaker WS10	Edmund Bühler (Hechingen, Germany)
Scales AG285 and JB2002-G	Mettler Toledo (Greifensee, Switzerland)
Tapestation 2200	Agilent Technologies (Santa Clara, USA)
Thermocycler C1000 Touch Thermocycler (PCR)	Bio-Rad (Munich, Germany)
Vortex	Scientific industries (Bohemia, USA)
Water bath WNE	Memmert (Schwabach, Germany)

2.1.2 Consumables

The listed consumables and plasticware were used for experiments conducted for this thesis.

Consumables	Manufacturer
Cell culture multiwell plate (6 well, 12 well)	Starlabs (Hamburg, Germany)
Cover Slip (24x60 mm)	Carl Roth (Karlsruhe, Germany)
Cryomold (Biopsy)	Weckert Labortechnik (Kitzingen, Germany)
1-Fluor-2,4-dinitrobenzol (DNFB)	Sigma-Aldrich (St. Louis, USA)
EasyStrainer (40 µm, 70 µm)	Greiner (Kremsmünster, Austria)
Eppendorf tubes (2 ml, 1.5 ml, 0.5 ml)	Eppendorf (Hamburg, Germany)
FACS tubes 5 ml	Biozol (Eching, Germany)
Falcon tubes (15 ml, 50 ml)	Sarstedt, Inc. (Nümbrecht, Germany)
Fluoromount-G™	Invitrogen (Waltham, USA)
Microcover glasses	Brand (Wertheim, Germany)
Microscope adhesive object slides	Paul Marienfeld (Lauda, Germany)
Microtome blades	Feather (Osaka, Japan)
PCR tube strips	NIPPON Genetics (Düren, Germany)
Petridish CytoOne (10cm)	Starlabs (Hamburg, Germany)
Pipet-tips (10 µl, 200 µl, 1000 µl)	Sarstedt, Inc. (Nümbrecht, Germany)
Plunger	B. Braun (Hilter, Germany)
Seal-R-film	Brand (Wertheim, Germany)
Serological pipettes (5 ml, 10 ml, 25 ml)	Sarstedt, Inc. (Nümbrecht, Germany)
Superfrost™ Plus Adhesion microscope slides	Epredia (Basel, Switzerland)
Syringe (1 ml, 10 ml)	B. Braun (Hilter, Germany)
Petridish CytoOne (10cm)	Starlabs (Hamburg, Germany)
Tegaderm™ 3M	3M (Neuss, Germany)

Tissue-Tek O.C.T. compound (OCT)	Sakura Finetek (Umkirch, Germany)
96 well plate U-bottom	VWR (Radnor, USA)

2.1.3 Cell culture reagents

The following reagents and buffers were used for experiments conducted in this thesis.

Reagent	Manufacturer
Agarose	ThermoFisher Scientific (Waltham, USA)
Anti-CD16/32 Fc blocking antibody	BioLegend (San Diego, USA)
Bovine serum albumin (BSA) Fraction V	Carl Roth (Karlsruhe, Germany)
Collagenase IV	ThermoFisher Scientific (Waltham, USA)
4',6-Diamidino-2-phenylindole, diacetate (DAPI)	BioLegend (San Diego, USA)
Dispase II powder	ThermoFisher Scientific (Waltham, USA)
Dulbecco's modified eagle medium (DMEM)	ThermoFisher Scientific (Waltham, USA)
Roswell Park Memorial Institute (RPMI) medium	ThermoFisher Scientific (Waltham, USA)
DNase I	ThermoFisher Scientific (Waltham, USA)
DRAQ-5	BioLegend (San Diego, USA)
DRAQ-7	BioLegend (San Diego, USA)
DreamTaq Green PCR Master Mix	ThermoFisher Scientific (Waltham, USA)
D-Sucrose	Carl Roth (Karlsruhe, Germany)
Dulbecco's phosphate-buffered saline (DPBS, without calcium and magnesium)	PAN-Biotech (Aidenbach, Germany)
Ethylenediaminetetraacetic acid (EDTA, 0.5 M)	Sigma-Aldrich (St. Louis, USA)
Ethanol (99.8%, molecular biology grade)	Sigma-Aldrich (St. Louis, USA)
FACS Flow Sheat Fluid	BD Biosciences (Franklin Lakes, USA)
Fetal bovine serum (FBS)	PAN-Biotech (Aidenbach, Germany)
Formaldehyde 16 % (w/v)	Fisher Scientific (Waltham, USA)
Gene Ruler 100 bp DNA ladder	ThermoFisher Scientific (Waltham, USA)
Hoechst 33258	Life Technologies (Carlsbad, USA)
H ₂ O, double-distilled for buffers	In-house preparation
iTaq Universal Probes Supermix	Bio-Rad (Herkules, USA)
Narketan, ketamine hydrochloride	Vetoquinol (Ismaning, Germany)
Normal goat serum (NGS)	VWR (Radnor, USA)
Penicillin-Streptomycin (PenStrep)	ThermoFisher Scientific (Waltham, USA)

Protein block, serum-free	Agilent Technologies (Santa Clara, USA)
Rat serum (RS)	Thermofisher Scientific (Waltham, USA)
Sodium chloride for anesthesia	B. Braun (Hilter, Germany)
SYBR Green I	Sigma-Aldrich (St. Louis, USA)
Triton-X 100	Thermofisher Scientific (Waltham, USA)
Xylazine	WDT Marktplatz (Garbsen, Germany)

2.1.4 Prepared buffers and solutions

The following buffers were prepared in order to conduct the experiments shown in this thesis.

Prepared buffers and solutions	Ingredient concentrations
Antibody solution (for IF)	1 % BSA in PBS-T
Blocking solution (for IF)	1 % BSA, 2 % NGS in PBS-T
Digestion buffer I skin	2.4 U/ml Dispase in PBS with 10 % FBS
Digestion buffer II dermis	100 U/ml Collagenase IV, 130 U/ml DNase I in PBS with 10 % FBS
Digestion buffer II epidermis	130 U/ml DNase I in PBS with 10 % FBS
DMEM for macrophage cell culture	10 % FCS, 1 % PenStrep in DMEM
FACS buffer	0.5 % BSA, 2mM EDTA in PBS
Fc block	0.5 % CD16/32 (1:100), 2 % RS in FACS buffer
Formaldehyde 4% (w/v)	16 % Formaldehyde diluted in PBS
Narcotics	20 mg/ml Xylazine, 100 mg/ml Ketamine in NaCl (0.9 %)
1 x PBS (PBS)	137 mM NaCl, 2.7 mM KCl, 10 mM Na ₂ HPO ₄ , 1.8 mM KH ₂ PO ₄ in double-distilled H ₂ O
PBS-T	0.4 % Triton-X-100 in PBS
Red blood lysis buffer	0.1 mM EDTA, 12 mM NaHCO ₃ 155 mM NH ₄ Cl in Aqua Bidest
Sucrose (5 %, 10 %, 30 %)	Sucrose powder diluted in PBS
50 x TAE buffer	121.14 g Tris-Base in 1 l double-distilled H ₂ O, adjust pH to 8.0
WAT buffer	0.5 % BSA in PBS

2.1.5 Commercial kits

The following commercial kits were used in order to conduct the experiments shown in this thesis.

Kit name	Vendor
Chromium Next GEM Chip G Single Cell Kit	10x Genomics
Chromium Next GEM Single Cell 3' Reagent Kit v3.1	10x Genomics
Click-iT® EdU kit	Thermofisher
Dead Cell Removal Kit	Miltenyi
CD45 MicroBeads Kit	Miltenyi
CD11b MicroBeads Kit	Miltenyi

2.1.6 Antibodies

The listed antibodies were used for flow cytometry antibody staining of the dermis. Grey marked antibodies were only used for wt mice and not for double fatemapper mice.

Antibody	Conjugate	Clone	Company	Dilution
CD14	BUV-395	RMC5-3	BioLegend	1:200
Tim4	BUV-563	RMT4-54	BD Biosciences	1:200
CD11b	BUV-661	M1/70	BD Biosciences	1:200
CD45	BUV805	30-F11	BD Biosciences	1:200
CD163	BV421	S15049I	BioLegend	1:200
Ly6C	BV510	HK1.4	BioLegend	1:200
CD169	BV605	3D6.112	BioLegend	1:400
CD11c	BV650	HL3	BioLegend	1:200
CD117 (ckit)	BV711	2B8	BioLegend	1:400
CD74	BV786	In-1	BioLegend	1:200
CD16.2	AF488	9E9	BioLegend	1:200
CD64	PerCP-Cy5.5	X54.5/7.1	BioLegend	1:200
MerTK	PE	2B10C42	BioLegend	1:200
F4/80	PE-Cy5	BM8	BioLegend	1:400
Cx3cr1	PE-Cy7	SAO11F11	BioLegend	1:200
CD32b	APC	AT130-2	BioLegend	1:200
MHCII	AF700	M5/113.15.2	BioLegend	1:400
CD19	APC-Cy7	6D5	BioLegend	1:200
TCRβ	APC-Cy7	H57-597	BioLegend	1:200
NK p46	APC-Cy7	PK136	BioLegend	1:200
Ly6G	APC-Cy7	1A8	BioLegend	1:200
CD24	APC-Cy7	M1/69	BioLegend	1:200
CD326	APC-Cy7	G8.8	BioLegend	1:200

The listed antibodies were used for flow cytometry antibody staining of the epidermis. Grey marked antibodies were only used for wt mice and not for double fatemapper mice.

Antibody	Conjugate	Clone	Company	Dilution
CD49	BUV-496	Ha31/8	BioLegend	1:400
Sca-1	BUV-563	E13-161.7	BD Biosciences	1:400
CD11b	BUV-661	M1/70	BD Biosciences	1:200
CD45	BUV805	30-F11	BD Biosciences	1:200
CD34	BV421	MEC14.7	BioLegend	1:200
CD3	BV510	17A2	BioLegend	1:200
CD4	BV570	RM4-5	BioLegend	1:400
MerTK	BV711	2B10C42	BioLegend	1:200
CD16.2	AF488	9E9	BioLegend	1:200
CD64	PerCP-Cy5.5	X54.5/7.1	BioLegend	1:200
CD32b	PE	AT130-2	eBioscience	1:200
F4/80	PE-Cy5	BM8	BioLegend	1:400
CD29	PE-Cy7	HMB1-1	BioLegend	1:200
CD200	APC	OX-90	BD Biosciences	1:200
MHCII	AF700	M5/113.15.2	BioLegend	1:400
CD326	APC-Cy7	G8.8	BioLegend	1:200

The following antibodies were used for flow cytometry antibody staining of the bone marrow.

Antibody	Conjugate	Clone	Company	Dilution
CD115	BUV-661	AFS98	BioLegend	1:100
CD11b	BUV-661	M1/70	BD Biosciences	1:200
CD135	BV421	A2F10	BioLegend	1:100
Ly6A/E	BV510	D7	BioLegend	1:200
CD34	BV605	RAM34	BioLegend	1:100
CD117 (ckit)	BV711	2B8	BioLegend	1:100
Nkp46	Biotin	29A1.4	BioLegend	1:200
CD3	Biotin	145-2C11	BioLegend	1:200
CD19	Biotin	6D5	BioLegend	1:400
Ly6G/Ly6C	Biotin	RB6-8C5	BioLegend	1:400
Ter119	Biotin	TER-119	BioLegend	1:100
Streptavidin	BV786		BioLegend	1:100
CD16.2	AF488	9E9	BioLegend	1:200
CD127	PerCP-Cy5.5	A7R34	BioLegend	1:100
CD32b	PE	AT130-2	eBioscience	1:200
CD150	PE-Cy7	TC15-12F12.2	BioLegend	1:200
CD48	APC	HM48-1	BioLegend	1:100
CD16/32	APC-Cy7	93	BioLegend	1:100

The following antibodies were used for immunofluorescent staining of the paw skin tissue.

Antibody	Conjugate	Company	Dilution
CD31 (hamster)	-	ThermoFisher Scientific	1:300
Iba1 (rabbit)	-	Abcam	1:800
β-3 Tubulin (mouse)	-	ThermoFisher Scientific	1:600
α-hamster	AF647	BioLegend	1:1000

α -rabbit	AF488	Thermofisher Scientific	1:1000
α -mouse	AF555	Thermofisher Scientific	1:1000

2.1.7 Mouse strains

The listed mouse strains were used for *in-vivo* experiments shown in this thesis.

Mouse strain	Background	Description
Wildtype B6	B6/Jrcc	Non-transgenic, wildtype mice
Tnfrsf11a ^{Cre} ; Rosa26 ^{LSL-YFP} ; Ms4a3 ^{FlpO} ; Rosa26 ^{LFL-tdT}	B6/JRcc	Transgenic mice, fluorescent reporter protein expression

2.1.8 Software

The listed software and online tools were used to analyze and document conducted experiments shown in this thesis.

Program name	Developer
BioRender	Science Suite Inc.
Excel	Microsoft
FACSDiva	BD Biosciencies
FlowJo	BD Biosciencies
ImageJ	NIH
Mausoleum	Hans-Eugen Stöffler
PowerPoint	Microsoft
Prism	GraphPad Software
QuPath	Dr. Pete Bankhead
Word	Microsoft
Zen (Black version)	Carl Zeiss Microscopy
Zen (Blue version)	Carl Zeiss Microscopy

2.2 Methods

2.2.1 Animal experimental techniques

2.2.1.1 Mouse colony maintenance and breeding

The used mouse lines for this thesis were C57BL/6 JRcc and *Tnfrsf11a*^{Cre}*Rosa26*^{LSL-YFP}*Ms4a3*^{FlpO}*Rosa26*^{LFL-tdT}. Mice were bred and maintained in the LIMES Genetic Resources Center (GRC) under specific pathogen free (SPF) conditions in accordance with the German Animal Welfare Act. Mice were housed in individually ventilated cages with up to five animals per cage separated by sex with a relative humidity of 55 ± 15% and at 21 ± 2°C. Breeding for colony maintenance was carried out by keeping one female and one male mouse in the same cage. Offspring was typically weaned 3-4 weeks after birth and marked with ear punches for genotyping and animal identification. Animal procedures were performed according to the project license 2021.A203, which was issued by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV).

2.2.1.2 Double-fatemapper mouse model

In the *Tnfrsf11a*^{Cre}*Rosa26*^{LSL-YFP}*Ms4a3*^{FlpO}*Rosa26*^{LFL-tdT} (double-fatemapper) model, *Tnfrsf11a*-driven Cre-recombinase activity induces the expression of the fluorescent reporter yellow fluorescent protein (YFP) combined with *Ms4a3*-driven FlpO-recombinase activity which induces the expression of the fluorescent reporter protein tdTomato (tdT) in HSC-derived progenitors. Thereby, the macrophage core gene *Tnfrsf11a* is also upregulated by HSC-derived monocytes which entered a tissue, differentiated into macrophages and stay there as resident cells. Thus, with the double-fatemapper mouse model, it can be differentiated between three distinct developmental origins of cells: Fetal-derived, tissue resident macrophages only express YFP, monocytes and monocyte-derived macrophages which are short-lived or recently differentiated only express tdT and monocyte-derived macrophages, which become resident cells express tdT and YFP (Figure 4).

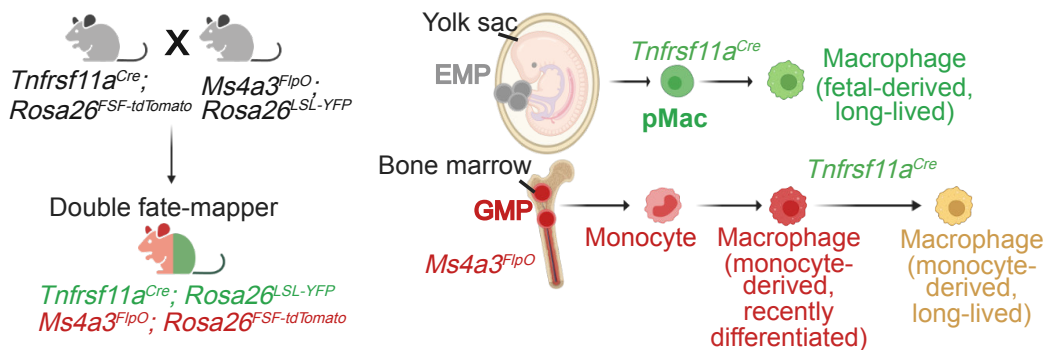


Figure 4: Double-fatemapper mouse model.

The double fate-mapper mouse line is derived by crossing of *Tnfrsf11a*^{Cre}; *Rosa26*^{FSF-tdTomato} strain with the *Ms4a3*^{FlpO}; *Rosa26*^{LSL-YFP} strain. This mouse model enables differentiation between fetal-derived and bone marrow-derived macrophages. Fetal-derived EMPs differentiate into macrophages via pMac which express the

macrophage core gene *Tnfrsr11a* (green). GMP-derived monocytes express *Ms4a3* (red). Both lineage-specific genes induce the expression of a fluorescent reporter: tdT in HSC-derived cells (red) and YFP in EMP-derived cells (green). Once GMP-derived monocytes differentiated into macrophages and stay longer in the respective tissue, they upregulate *Tnfrsr11a* and express both fluorescent reporters. Figure adapted from Makdissi et al, 2025.

2.2.1.3 Mouse genotyping

Tissue biopsies for genotyping were obtained from ear punches of adult mice. Genomic deoxyribonucleic acid (DNA) was isolated from the tissue by incubation in 200 µl 50 mM sodium hydroxide (NaOH) at 95°C for 20 min and following neutralization with 70 µl 1 M Tris (pH 8.0). The isolated DNA was stored at 4°C until further use. Genotyping was performed using polymerase chain reaction (PCR) with locus-specific primers and cycling protocols for the respective genetic loci of the mouse lines. PCR products were separated by size with a 100 bp DNA ladder on 2% agarose gels submerged in TAE buffer at 100 V for 30 min. TAE buffer and agarose gels contained SYBR Green for visualization of separated DNA under a UV transilluminator.

Table 1: Master mix for PCR.

Reagent	Volume
Primer 1	0.5 µl
Primer 2	0.5 µl
DreamTaq Green PCR Master Mix	5 µl
Sterile H ₂ O	3.0 µl
DNA	1 µl

Table 2: PCR primer for mouse genotyping.

Genotype	Primer sequence	Expected results
Ms4a3-FlpO	P1: AGA GAA ATC ATC AGG GCA GAA AT P2: GAA AGG GGA ACA AGC GAA GAT P3: TTG GCG AGA GGG GAA AGAC	517 bp: wt 412 bp: FlpO
Tnfrsr11a-Cre	P1: TCA AGG GTG ACA TCA TCG TGG T P2: ACT TCT CCA TGG TAG CCT CC P3: TAT GGG GGT GGG GTG ATA C	529 bp: wt 270 bp: Cre

Table 3: PCR program for mouse genotyping.

Step	Temperature	Time	Repeats
Initial denaturation	95 °C	3 min	
Denaturation	95 °C	30 sec	x 35
Primer annealing	60 °C	30 sec	
Elongation	72 °C	30 sec	
Final extension	72 °C	5 min	

2.2.1.4 Dissection of wild type and double fate mapper mouse HSD cohorts

Adult mice (11 weeks or 20 weeks old) were anaesthetized using ketamine/xylazine in 0.9% sodium chloride (NaCl, 1:1:8). After the loss of toe pinch reflex was noted, the thorax of the mice was opened exposing the heart. Blood for flow cytometry and hemocytometer measurement was taken from the right ventricle using a 1 ml syringe with a 0.45 mm cannula. Next, the mice were killed by transcardial perfusion, after making a small incision on the right atrium. For the perfusion, 10-20 ml of PBS was injected into the left heart ventricle using a syringe with a 0.45 mm cannula, flushing out the erythrocytes and immune cells present in the blood circulation out of the organs. Ears and bones were dissected for flow cytometry and stored in PBS on ice until further processing. Paws and back skin were dissected for histology and fixed overnight in 4 % paraformaldehyde (PFA).

2.2.1.5 Dissection of double fate mapper mice for time course experiments

Mice of different ages (1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 3 months, 6 months, 1 year) were sacrificed by inhalation of carbon dioxide (CO₂) for 3 min at 0.35 l/min. The death of the mouse was verified by respiratory arrest and the absence of a toe pinch reflex. Embryos and newborn mice were killed by decapitation. Ears (1 week and older mice) or back skin (embryos and newborn mice) were dissected for flow cytometry and stored in PBS on ice until further processing. Paws were dissected for histology and fixed for 5 hours in 4 % PFA.

2.2.2 Flow cytometry

2.2.2.1 Isolation of single cells from the ear skin tissue

For the first digestion step, the dorsal and ventral side of the ears was divided using two forceps. Next, they were placed in 12-well plates containing 1 ml of the first digestion mix, putting the inner side of the ear on the liquid. For incubation, the plate was placed in a shaking thermocycler for 1 h at 37°C and 90 rpm. Afterwards, the epidermis was peeled of the dermis under the binocular using forceps and put in a new 12-well plate containing 1 ml/well of epidermis digestion mix, while the leftover skin was put in a plate containing 1 ml/well of dermis digestion mix. The tissue was cut into small pieces using blunt end scissors and was further digested at 37°C and 90 rpm for 0.5 h (epidermis) or 1.5 h (dermis). After second digestion, the tissues were mechanically dissociated on ice by mincing the organ through a 100 µm pore strainer, which was placed on a 6-well plate. Cells were eluted from the strainer by rinsing the filter twice with 1 ml fluorescence activated cell sorting (FACS) buffer (0.5% bovine serum albumin (BSA) + 2 mM EDTA in PBS), as well as 1 ml FACS buffer, which was used to take up leftover cells from the empty 12-wells. The cell suspension was then transferred to FACS tubes and centrifuged at 400 g and 4°C for 5 min.

2.2.2.2 Antibody staining of cell surface receptors

For antibody staining of the cell surface antigens, the supernatant was decanted, followed by resuspension of the cell pellet in 50 µl FACS buffer and incubation on ice for 10 minutes. Next, 1 ml of FACS buffer was added to the tubes and they were again centrifuged at 400 g and 4°C for 5 min, the supernatant was aspirated and the cell pellet was resuspended in 50 µl of antibody mix. After 30 min incubation on ice, 1 ml of FACS buffer was added to the tubes and they were again centrifuged at 400 g and 4°C for 5 min, followed by decanting of the supernatant and resuspension of the pellet in 100 µl FACS buffer. FACS tubes with cell suspensions were stored on ice until measurement.

2.2.2.3 Flow cytometry measurement and data analysis

To prepare cells for measurement, they were filtered into new FACS tubes using a 70 µm pore strainer. In order to exclude dead cells from the analysis, 100 µl of DRAQ7 in FACS buffer (1:1000) was added to the cell suspension directly before sample recording. For measurement, the FACS Symphony flow cytometer equipped with the FACSDiva software was used. Applied combinations of cell surface markers for distinct cell population identification are listed in material section under 2.1.6.

2.2.3 Preparation of skin tissue for histological analysis

2.2.3.1 Tissue cryo-sectioning

Skin tissue was fixed using 3 ml 4% PFA in 6-well plates at 4°C overnight. Afterwards, the tissue was washed for three times, using PBS for 10 min while rotating at RT. Subsequently, the skin was placed in different sucrose solution (5 %, 10 %, 20 %). The tissue stayed in each solution for at least 6 hours at 4°C, starting at the lowest concentration. Once the tissue has sunk to the bottom of the plate in the highest sucrose solution, it was transferred into a cryo-mold and covered with OCT. After embedding the tissue, the block was placed on dry ice until the compound was solid. Cryo-mold were subsequently stored at -80 °C. For cryo-sectioning, the frozen OCT block was cut using a Cryostat. 50 µm sections were cut, collected on superfrost microscopy slides and stored at -20 °C until further usage.

2.2.3.2 Antibody staining of cryo-sections

The previously prepared and frozen skin tissue sections were thawed at RT. The slides were put in a dark wet chamber and hydrated one time with PBS for 10 min. Afterwards, PBT was added twice for 10 min in order to permeabilize the skin tissue. For blocking, the serum-free protein-block solution was added and incubated at RT for one hour. Next, the slides were rinsed once with PBT and the primary antibody mix (in PBT with 1 % BSA) was added and incubated at 4 °C overnight. The next day,

the primary antibody was removed with PBT in three washing steps of 10 min. Subsequently, the secondary antibody mix was added and incubated at 4 °C overnight. After overnight incubation, the slides were washed three times with PBT and air dried. The dry sections were mounted using Fluoromount-G™ with DAPI and a cover slip.

2.2.3.3 Microscopy and image analysis of stained cryo-sections

Skin sections, stained with immunofluorescent antibodies, were imaged using the confocal microscope LSM 880 Airyscan (Carl Zeiss) as well as the ZEN-black software. The images were subsequently analysed using the ImageJ software. For morphological analysis the MotiQ plugin was used. Based on Iba1 staining, macrophages were hand-picked using the MotiQ Cropper (v0.1.2) and further processed with the MotiQ Treshholder (v0.1.3) and the MotiQ 3D Analyzer (v0.3.0).

2.2.3.4 Tissue preparation for hematoxylin and eosin (H&E) staining

Skin tissue was fixed using 3 ml 4% PFA in 6-well plates at 4°C overnight. Afterwards, the tissue was washed for three times, using PBS for 10 min while rotating at RT. For dehydration, the skin tissue was placed in 2 ml tubes containing 70 % EtOH. Further processing was conducted by the histology core facility of the university clinic in Bonn (UKB). The processing involved embedding in paraffin, microtome cutting (4 µm), as well as staining with H&E.

Table 4: Steps of H&E staining.

Step number	Reagent	Incubation time
1	Xylene	5 min
2	Xylene	5 min
3	EtOH 100 %	2 min
4	EtOH 100 %	2 min
5	EtOH 96 %	2 min
6	EtOH 90 %	2 min
7	EtOH 80 %	2 min
8	EtOH 70 %	2 min
9	H ₂ O	1 min
10	Hemalaun	3 min
11	H ₂ O	1 min rinse
12	Eosin	3 min
13	H ₂ O	1 min rinse
14	EtOH 70 %	30 sec
15	EtOH 80 %	30 sec
16	EtOH 90 %	30 sec
17	EtOH 96 %	30 sec
18	EtOH 100 %	30 sec
19	EtOH 100 %	30 sec
20	Xylene	30 sec
21	Xylene	5 min

2.2.3.5 Microscopy and image analysis of H&E-stained skin sections

H&E-stained skin sections were imaged using the AxioLab.A1 microscope (Zeiss). The images were subsequently analyzed using the measuring tools in ImageJ and QuPath software. For epidermal thickness assessment, three areas per skin sample were imaged and five measurements were done per area. Mean of assessed epidermal thickness measurements was used for data representation.

2.2.4 Functional assays

2.2.4.1 CD11b enrichment of cell suspension from skin tissue

Ear skin tissue was digested as described in 2.2.2.1. 1 ml of FACS buffer was added to the cells, followed by centrifugation at 300 g for 5 min. Cells were resuspended in 200 μ l FACS buffer, stained with 1 μ l of biotinylated CD11b antibody (BioLegend) and incubated for 15 min on ice. Afterwards, 3 ml of FACS buffer were added and cells were centrifuged at 300 g for 5 min at 4 °C. The supernatant was aspirated and 360 μ l of FACS buffer were added to the cells, as well as 40 μ l of MojoSort streptavidin magnetic beads. After 15 min incubation time on ice, the cells were washed with 3 ml FACS buffer and centrifuged down at 300 g for 5 min at 4 °C. The supernatant was aspirated, the cell pellet was resuspended in 2 ml FACS buffer and transferred into a FACS tube. Proceeding with the magnetic enrichment, the FACS tubes were placed into the magnet for 5 min on ice. Then, the flow-through was poured off into a fresh FACS tube. Subsequently, the FACS tube was taken out of the magnet, the remaining pellet of magnetically labelled cells was resuspended in 1 ml FACS buffer and transferred into the collection tube. Now, the FACS tube containing the flow-through was placed again into the magnet, repeating the previously described procedure twice. After the final incubation, the flow-through was discarded and 5 ml of FACS buffer was added to the enriched cells, followed by centrifugation at 300 g for 5 min at 4 °C and aspiration of the supernatant. The remaining cells are CD11b enriched and can be used for functional assays.

2.2.4.2 Efferocytosis assay with skin macrophages

For quantification of efferocytotic activity of skin macrophage subsets, apoptotic thymocytes were generated. The thymocytes were isolated from 5-week-old wt mice, which were previously scarified via CO₂ inhalation. Subsequently, the thymus was taken out and was meshed through a 100 μ m strainer using a metal plunger. The strainer was flushed with 10 ml RPMI into a 50 ml Falcon tube and the resulting flow-through was meshed again through a 70 μ m strainer. Afterwards, the cells were counted and were transferred on a 6-well plate with 20×10^6 cells in 3 ml RPMI. To induce apoptosis, 5 μ M Dexamethasone was added to the cells, followed by 5 hours incubation at 37 °C. The amount of apoptotic thymocytes was determined using the Annexin V Apoptosis Detection Kit (BioLegend). The cells were washed twice with cold BioLegend Cell Staining Buffer and were resuspended in

Annexin V Binding Buffer at a concentration of 1×10^7 cells/ml. Then, 100 μ l of the cell suspension was transferred into a FACS tube, followed by addition of 5 μ l of FITC-conjugated Annexin V and 10 μ l of Propidium Iodide. Cells were gently vortexed and incubated for 15 min at RT in the dark. Afterwards, 400 μ l of Annexin V Binding Buffer was added to the cells and they were analyzed on the Flow Cytometer FACS Symphony A5. Next, the remaining cells were centrifuged at 300 g for 5 min, the supernatant was aspirated and the cells were resuspended in PBS at a concentration of 1×10^6 cells/ml. Subsequently, the cells were stained with pHrodoRed dye with a final concentration of 0.5 mM and incubated for 1 h on ice. After the incubation time, the cells were washed twice with PBS.

The CD11b enriched cell suspension from the skin was resuspended in DMEM to reach a final concentration of 0.5×10^6 cells/ml. Next, the cells were transferred on a round bottom 96-well plate (100 000 cells per well, 200 μ l) and the apoptotic thymocytes were added to the cells in a ratio of 1:10. After 2 h of incubation at 37 °C, the cells were washed and stained for 30 min with the dermal or epidermal macrophage antibody mix (2.1.6). After centrifugation at 300 g for 5 min at 4°C, the cells were resuspended in 200 μ l FACS buffer and were measured on the Flow Cytometer FACS Symphony.

2.2.4.3 Phagocytosis assay with skin macrophages

In order to quantify phagocytotic activity of skin macrophage subpopulations, the bacterial strain *citrobacter rodentium* (*c.rodentium*) was used. For *c.rodentium* culture, a vial of the strain was taken from the -80 °C freezer and a pipette tip of the bacteria was put into a glass tube containing LB medium and was subsequently incubated shaking at 37 °C overnight. The next day, the LB medium containing *c.rodentium* was spread out on LB agar plates containing 50 μ l of kanamycin. The kanamycin ensures, that only the *c.rodentium* strain grows on the plates as it is kanamycin-resistant. Once the bacterial colonies grew on the LB agar plates, 2-3 colonies were picked with a pipette tip and transferred into a glass tube containing LB medium. The glass tubes were left in a shaking incubator at 37 °C overnight. On the day of the experiment, the optical density of the bacterial suspension was measured and correlated to the *c.rodentium* growth curve, which was previously prepared by Aya Rafea-Nasr. The bacterial suspension was diluted in order to reach 1×10^{10} bacteria/ml. The *c.rodentium* suspension was centrifuged at 4000 g for 20 min and the LB medium supernatant was discarded, followed by resuspension in the same amount of PBS and centrifugation at 3200 g for 20 min. Subsequently, the supernatant was aspirated and the bacteria were resuspended in sodium bicarbonate buffer to reach again 1×10^{10} bacteria/ml. Then, the amount of bacteria needed for the phagocytosis assay was calculated, depending on the amount of wells and the ratio between *c.rodentium* and macrophages, which was 200:1. The calculated volume of bacteria suspension was transferred in a 2 ml tube and stained with pHrodoRed dye to a final concentration

of 0.5 mM. The staining was incubated for 1 h at RT on a shaker. Afterwards, the suspension was centrifuged for 20 min at 4000 g and the supernatant was aspirated. The cell pellet was resuspended in 1 ml PBS and the washing step was repeated for three times in total. Finally, the pellet was resuspended in the previously calculated amount of DMEM.

The CD11b enriched cell suspension from the skin was resuspended in DMEM to reach a final concentration of 0.5×10^6 cells/ml. Next, the cells were transferred on a round bottom 96-well plate (100 000 cells per well, 200 μ l) and *C. rodentium* was added to the cells in a ratio of 1:200. After 1 h of incubation at 37 °C, the cells were washed and stained for 30 min with the dermal macrophage antibody mix (2.1.6). After centrifugation at 300 g for 5 min at 4°C, the cells were resuspended in 200 μ l FACS buffer and were measured on the Flow Cytometer FACS Symphony.

2.2.5 Cell proliferation assay

Mice were fed with CD or HSD for 3 months, followed by intraperitoneal injection of 5-ethynyl-2'-desoxyuridin (EdU, 10 μ g/g of body weight) 1.5 h before the mice were sacrificed. Paws of the mice were collected and skin tissue was peeled off the bone. Afterwards the tissue was processed as described in 2.2.3.1. After the blocking was added to the tissue and incubated for 1 h, the EdU Click-iT® master mix (Table 5) was prepared according to the manufacturers protocol and 200 μ l was added to the cells. The tissue incubated in the staining for 30 min at RT, protected from the light. Subsequently, the slides were washed three times for 10 min with PBS and left for drying at RT. Last, the tissue was mounted with Fluoromount-G™ with DAPI and a cover slip. Slides were imaged using the confocal microscope LSM 880 Airyscan (Carl Zeiss) as well as the ZEN-black software. The images were subsequently analysed using the ImageJ software, quantifying EdU⁺ cells in three areas per sample.

Table 5: EdU click-it® master mix.

Reaction components	Amount for 10 coverslips
1x Click-iT® reaction buffer	1.8 ml
CuSO ₄	800 ml
Alexa Fluor® azide	5 μ l
Reaction buffer additive	200 μ l
Total volume	2 ml

2.2.6 Single-cell RNA sequencing

2.2.6.1 Preparation of cell solution from skin tissue

Ear skin tissue was digested as described in 2.2.2.1. Subsequently, the Dead Cell Removal Kit (Miltenyi) was used to obtain a clean, living cell solution. Buffers were prepared according to the manufacturers protocol. As a first step, the cell numbers were determined. Then, the cell suspensions were centrifuged at 300 g for 10 min and the supernatant was aspirated. The cell pellet was resuspended in 100 µl of Dead Cell Removal Microbeads per 10^7 cells, mixed well and incubated for 15 min at RT. Next, 1 x Binding Buffer was added to the cell suspension to reach a minimum volume of 500 µl for separation. For magnetic separation, the column was placed in the magnetic field of a MACS separator and rinsed with 3 ml of 1 x Binding Buffer. Afterwards, the cell suspension was applied onto the column, collecting unlabeled live cells in a 15 ml falcon tube. The column was washed 4 times with 3 ml Binding buffer and flow-through was again collected. After removal of dead cells, our cell suspension was enriched for CD45-positive cells, using CD45 MicroBeads (Miltenyi). As a first step, the cell numbers were determined. Then, the cell suspensions were centrifuged at 300 g for 10 min and the supernatant was aspirated. The cell pellet was resuspended in 90 µl of WAT buffer and 10 µl of CD45 Microbeads were added per 10^7 cells, mixed well and incubated for 15 min in the refrigerator (2-8 °C). Afterwards, cells were washed by adding 1 ml of WAT buffer and centrifuging at 300 g for 10 min. After the supernatant was aspirated completely, the cells were resuspended in 500 µl WAT buffer. For the magnetic separation, the column was placed in the magnetic field of a MACS separator and rinsed with 3 ml of WAT buffer. Next, the cell suspension was applied to the column and the flow-through, containing the CD45-negative fraction, was collected in a 15 ml falcon tube. The column was subsequently washed for three times with 3 ml WAT buffer, collecting the additional flow-through. Then, the column was removed from the separator and placed onto a new 15 ml falcon tube. 5 ml of WAT buffer were pipetted onto the column and the magnetically labelled cells were flushed out by firmly pushing the plunger into the column. The collected flow-through is the CD45-positive fraction. For further processing, the CD45-negative and -positive fraction were combined 1:1 to a final amount of 16500 cells per sample.

2.2.6.2 Single-cell library preparation and single-cell RNA sequencing

The 10x Genomics technology was used to conduct the single-cell RNA sequencing experiment. The prepared cell solution (2.2.4.1) was adjusted to a concentration of 1000 cells/µl. Processing was done using the 10x Genomics Chromium Controller in combination with the Chromium Next GEM Single Cell 3' Reagent Kit v3.1 (10 x Genomics). The kit was used according to the manufacturers protocol. Loading amounts of cells was 16 500 cells/sample, in order to target a recovered cell number of 10 000 cells/sample for library preparation and subsequent sequencing. An Agilent high-sensitivity

D5000 assay (Agilent Technologies) on a Tapestation 2200 system was used to perform cDNA integrity and concentration measurements. During the final library preparation, cDNA was fragmented, followed by usage of SPRIselect magnetic beads (Beckman-Coulter) for size selection, in order to purify for a library with a size distribution of 200 – 600 bp. The next steps involved adaptor ligation and sample indexing, subsequently measuring the final fragment size and concentration of the samples. Finally, using a sequencing depth of > 30 000 reads/cell with 50 bp paired-end reads, the samples were sequenced with the Illumina NextSeq2000 system. Single-cell library preparation and single-cell RNA sequencing was performed by Dr. Nelli Blank-Stein.

2.2.6.3 Processing and analysis of single-cell RNA sequencing data

Single-cell RNA sequencing data were processed using CellRanger (v7.1.0) for read alignment and gene quantification. Downstream analysis was conducted in Seurat (v4.1.1) with default preprocessing parameters. Cells expressing at least 200 genes in a minimum of three cells were retained. Further filtering excluded cells with <300 or >7,500 detected genes and those with >10% mitochondrial RNA content. Data were normalized and scaled using Seurat's standard pipeline. Sample integration was performed using FindIntegrationAnchors with default parameters. Uniform Manifold Approximation and Projection (UMAP) visualization was generated based on the first 20 principal components (PCs) and clustering was performed at a resolution of 0.5. Doublets were identified and removed using scDbtFinder (v1.4.0) with default settings. Cell clusters were annotated using a reference marker gene panel. For macrophage sub clustering, the first 20 PCs were used with a clustering resolution of 0.8. The over-representation analysis (ORA) utilized the clusterProfiler (v2.18.1) package, querying the Hallmark, Reactome and Gene Ontology (GO)-Biological Process (BP) databases obtained using the msigdb package (v7.5.1). Differential expression analysis was performed within macrophage subclusters, comparing the HSD group to the CD group using the Wilcoxon rank-sum test. Differentially expressed genes (DEGs) were defined using an adjusted p-value threshold of ≤ 0.1 and a log₂ fold-change (log₂FC) cutoff of ≥ 0.25 or ≤ -0.25 . Gene expression differences for selected genes across diet conditions were assessed using a Wilcoxon test, p-values were not corrected for multiple testing. Data analysis was performed by Dr. Lisa Steinheuer.

2.2.7 Skin salt and water content measurement

Measurement of salt and water content of murine skin was performed in collaboration with Agnes Schroeder from the university of Regensburg. Tissue was collected from mice fed with CD or HSD for 3 months. The weight of the tissue was taken and it was frozen down in liquid nitrogen. Afterwards

it was sent frozen to Agnes Schroeder for further processing. Salt and water content was detected by burning the tissue to ash and detection of the contents relating it to the previous weight of the tissue.

2.2.8 Skin challenge experiments

2.2.8.1 Tape stripping

For the tape stripping experiment, mice were put on either HSD or CD for 3 months. Following the diet, mice were short-term anesthetized with Isofluran (5 % Isofluran in O₂, 0,4 l/min), shaved on the back and the epidermal barrier was disrupted by sticking on and tearing off tape (Tegaderm, 3M) 12 times. The same procedure was done on the right ear of the mouse. 48 h after tape stripping, transepidermal water loss (TEWL) measurement was performed on the back skin, by using a measuring probe (Multi Skin Test Center MC 750, Lischke Medizintechnik). For non-tape stripping control, the TEWL measurement was performed before the tape stripping. Afterwards, mice were sacrificed via transcardial perfusion and ear skin was further processed as described in 2.2.2.1. Back skin was taken for histological analysis. Mastoid lymph nodes were mashed through a 70 µm strainer and was further processed as described for the epidermis (2.2.2.1-2.2.2.3).

2.2.8.2 Contact hypersensitivity assay

After three months on either a CD or an HSD, mice were sensitized with the hapten 1-Fluoro-2,4-dinitrobenzene (DNFB). For sensitization, DNFB was prepared at a concentration of 0.25 % in a solvent mixture (acetone to olive oil, 4:1 v/v). Mice were briefly anesthetized with isoflurane (5 % in O₂, 0.4 L/min) and 20 µl of the DNFB solution was applied with a pipette to the left ear, while the right ear received only the solvent. Five days later, the first challenge was administered. Under anesthesia (Isoflurane), mice received 20 µl of a 0.3 % DNFB solution on the previously sensitized ear, while the opposite ear was treated with 20 µl of solvent alone. Ear thickness was measured at 24 h, 48 h and 72 h post-challenge using a scale ruler. The second challenge was performed one week later following the same protocol. 72 h after the second challenge, mice were anesthetized using a ketamine/xylazine mixture (1:1:8 in 0.9 % NaCl). After confirming the loss of the toe pinch reflex, the thoracic cavity was opened to expose the heart. Blood was collected from the right ventricle using a 1 mL syringe with a 0.45 mm needle for analysis via hemocytometry (ADVIA). Mice were then perfused with 10 ml of PBS. Bones were harvested, flushed with 10 ml of PBS into a 50 ml Falcon tube, and cells were pelleted by centrifugation. The cells were incubated in 1 ml Red Cell Lysis (RBC) buffer for 5 min and washed subsequently with 3 ml PBS. Further processing was done as described in Sections 2.2.2.2 and 2.2.2.3. Ear tissue was digested following the protocol detailed in section 2.2.2.1 and subsequently processed according to sections 2.2.2.2 and 2.2.2.3.

3. Results

3.1 Experimental set-up

3.1.1 Feeding regimen

According to the World Health Organization (WHO), excessive salt intake is a significant health concern, particularly in Western countries like Germany. Current global daily salt consumption averages between 9 and 12 grams per individual, which is more than double the WHO's recommended limit of less than 5 grams per day (WHO, 2013). To study the effects of a high-salt diet, we used C57BL/JRcc wildtype (wt) or double fate-mapper mice, respectively, and employed specific feeding regimens (Figure 5). The control diet (CD) was supplemented with 0.5 % NaCl (per mass), reflecting the NaCl levels of standard chow diet, while the high-salt diet (HSD, abbreviation used in the following for the feeding regimen) was supplemented with 4 % NaCl (per mass). Additionally, the mice on HSD received drinking water supplemented with 0.9 % NaCl (0.9 g of salt per 100 ml tap water). Eight-week-old male and female mice were fed these diets for either 3 weeks (short-term, acute model) or 3 months (long-term, chronic model). After the feeding period, skin, blood, and bone marrow samples were collected and analyzed using flow cytometry, histology and single cell ribonucleic acid sequencing (scRNA-seq) under homeostatic conditions, as well as following inflammatory skin challenges. This approach aimed to elucidate the influence of acute and chronic high-salt intake on the macrophage compartment in the skin.

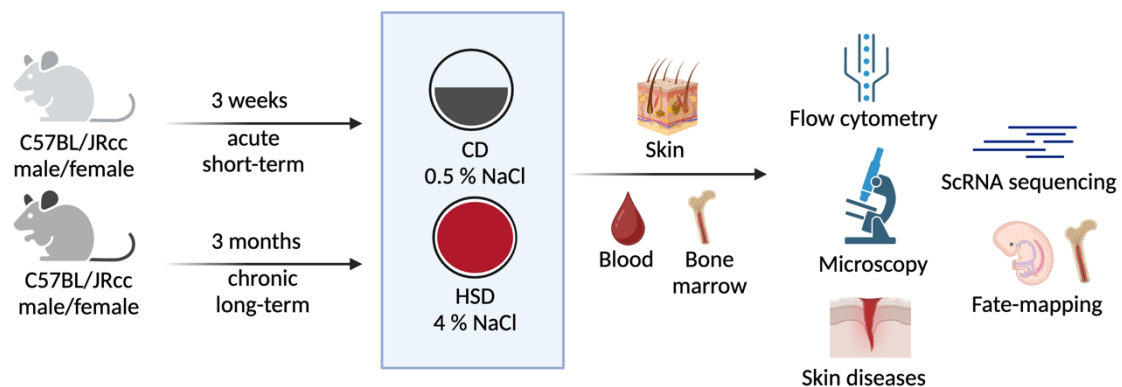


Figure 5: Experimental set-up for in vivo experiments.

Mice were either fed for 3 weeks (short-term, acute) or 3 months (long-term, chronic) with either CD or HSD. Skin, blood and bone marrow were collected and analysed using methods such as flow cytometry, microscopy, scRNA-seq and fate mapping.

3.1.2 Chronic HSD feeding increases body weight in female mice

To determine whether the long-term HSD feeding regimen impacted on the overall phenotype of the mice, we monitored their body weight on a weekly basis for 12 weeks. Analysis of body weight revealed sex-specific differences (Figure 6). While no significant differences in weight gain were observed in male mice between the CD and HSD groups over the 12-week period (Figure 6A), female mice exhibited a significantly higher increase in body weight when fed an HSD compared to a CD (Figure 6B).

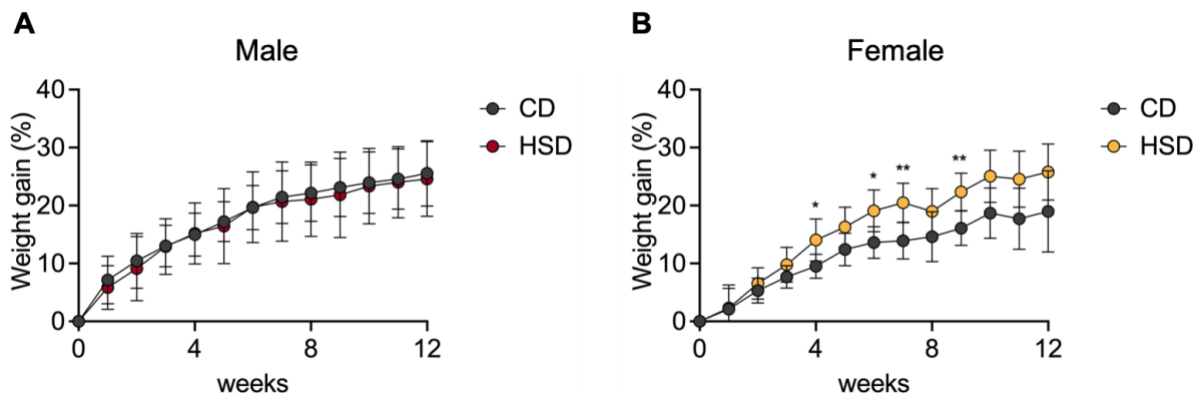


Figure 6: Percentual weight gain after chronic HSD feeding.

(A) Percentage of weight gain in male C57BL/JRcc mice over a 12-week period. Grey dots represent the CD group, and red dots represent the HSD group ($n = 19$ -20 mice per condition from 4 independent mouse cohorts). (B) Percentage of weight gain in female C57BL/JRcc mice over a 12-week period. Grey dots represent the CD group, and yellow dots represent the HSD group ($n = 9$ -10 mice per condition from two independent mouse cohorts). Data are presented as mean $\pm$ SD. Statistical significance was determined using multiple unpaired Student's *t*-tests.

3.2 Effects of high-salt intake on epidermal morphology, barrier function and fluid homeostasis

3.2.1 Region-specific alterations in skin sodium content following chronic HSD feeding

Several studies suggest that excess dietary salt may be stored in tissues such as the skin. In the Mars500 study conducted in 2010, researchers monitored various physiological parameters in humans under isolated conditions and found that not all ingested salt was excreted again (Ushakov et al., 2014). Titze and colleagues interpreted these findings as first evidence of potential salt storage within the human body. Subsequent research further indicated that salt accumulation primarily occurs in skin tissue, with macrophages playing a key role in sensing and regulating these excess salt concentrations (Jantsch et al., 2016; Machnik et al., 2009).

To determine whether salt storage also occurs in response to a HSD in our experimental setting, we quantified sodium levels in skin of different body sites, including the paws, ears and back (Figure 7). Our results revealed region-specific differences in sodium content. In the paws (Figure 7A), chronic HSD feeding led to a tendential increase in sodium levels compared to CD, though this difference was not significant. In contrast, sodium measurements in the ears showed no differences between the two diets (Figure 7B). Notably, in back skin tissue, sodium levels were significantly lower after long-term HSD compared to CD (Figure 7C).

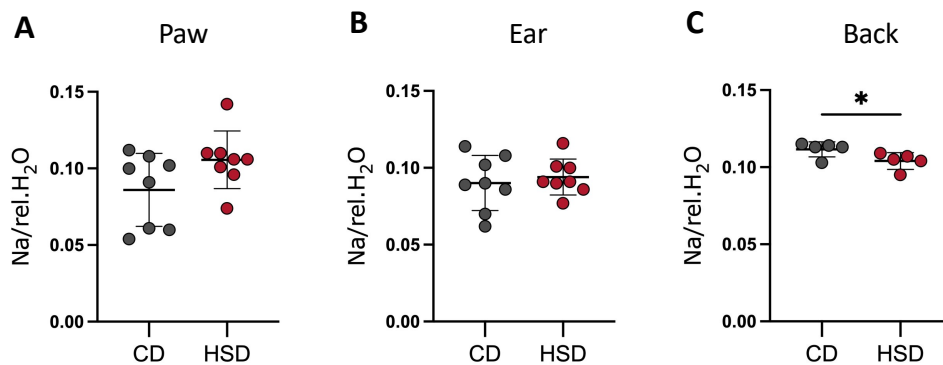


Figure 7: Sodium content in different areas of the skin following chronic HSD.

Sodium content was calculated relative to water in the skin tissue from paw (A), ear (B) and back skin (C) (n = 5-8 mice per condition from one to two independent mouse cohorts). Data are presented as mean ± SD. Statistical significance was determined using Student's t-test.

3.2.2 Long-term high-salt exposure compromises skin barrier integrity and alters tissue water homeostasis

To investigate the potential effects of altered sodium levels in skin tissue, we analyzed transepidermal water loss (TEWL) and water content following chronic HSD exposure. TEWL serves as an indicator of skin barrier integrity and is measured by assessing the amount of water lost across the stratum corneum (g/m²/h). Increased TEWL has been associated with inflammatory skin conditions, e.g. atopic dermatitis and psoriasis (Green et al., 2022).

Our measurements in back skin tissue revealed a significant increase in transepidermal water loss following chronic HSD compared to CD (Figure 8A). Additionally, we observed a significant decrease in paw water content after long-term HSD compared to CD (Figure 8B). A tendential but non-significant reduction in water content was also detected in back skin tissue of chronic HSD-fed mice, whereas no effect was observed in the ear skin (Figure 8C).

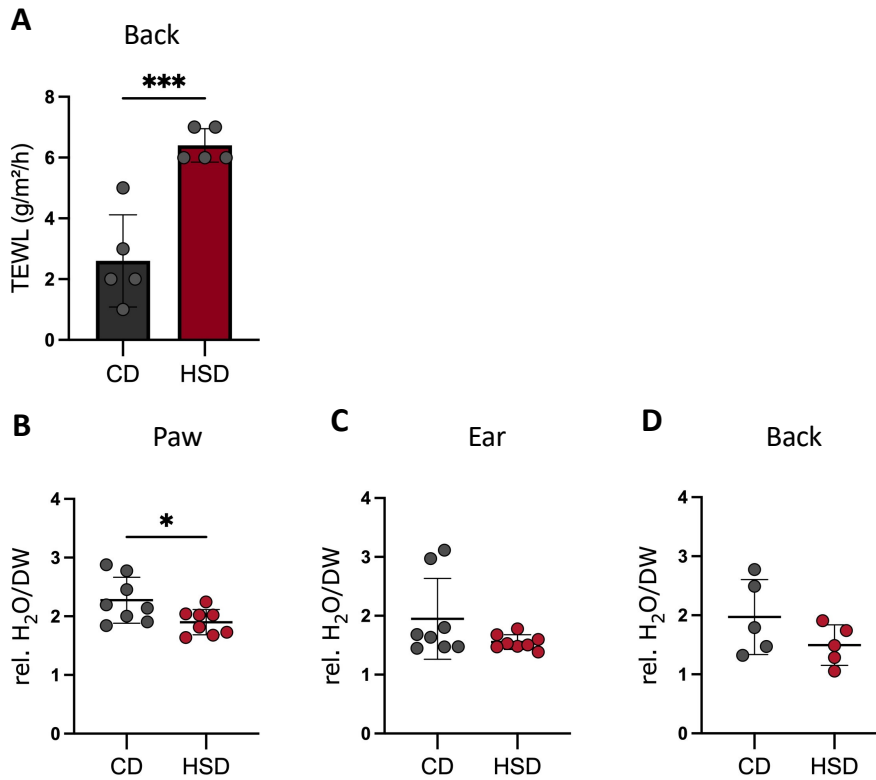


Figure 8: Transepidermal water loss and water content in different areas of the skin following chronic HSD.

(A) Transepidermal water loss of male back skin comparing CD and HSD conditions ($n = 5$ mice per condition from 1 mouse cohort). (B-C) Relative water content per dry weight (DW) of male paw (B) and ear skin (C) comparing CD and HSD conditions ($n = 8$ mice per condition from two independent mouse cohorts). (D) Relative water content of back skin comparing CD and HSD conditions ($n = 5$ mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was determined using Student's t-test.

3.2.3 Chronic HSD feeding induces epidermal thickening in a sex- and region-specific manner

In addition to examining the chemical effects of excess salt on skin tissue, we aimed to investigate potential morphological changes. For this purpose, hematoxylin and eosin (H&E) staining is a valuable method for obtaining an overview of morphological and cellular alterations within a tissue (Fischer et al., 2008).

Our analysis revealed significant changes in epidermal thickness in response to chronic high-salt exposure (Figure 9A). This effect was particularly pronounced in male mice, where a trend towards increased epidermal thickness in the paws was already observed following acute high-salt exposure compared to CD (Figure 9B). Moreover, chronic high-salt conditions resulted in a significant increase in epidermal thickness in the paw skin (Figures 9C). A similar trend was noted in ear skin; however, this difference was not statistically significant (Figure 9D). In female mice, we also observed an increase in epidermal thickness in the paws following long-term HSD feeding (Figure 9F), though this

difference was not statistically significant either and was not observed in ear skin (Figure 9G). Acute HSD feeding had no measurable effect on paw epidermal thickness in female mice (Figure 9E).

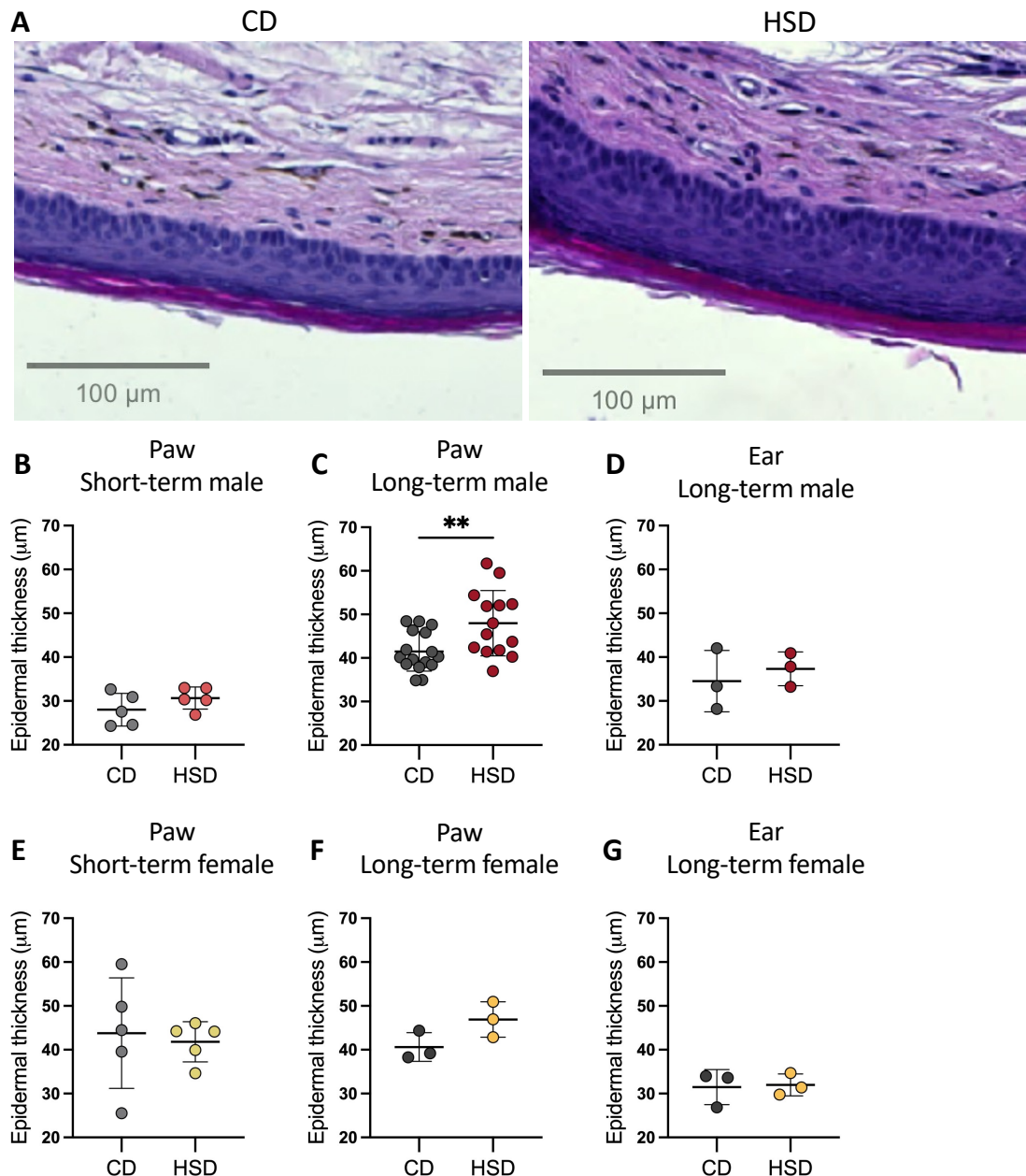


Figure 9: Epidermal thickness measurement following acute or chronic HSD feeding.

(A) Representative H&E staining of paw skin from chronic HSD-fed mice with 40x magnification. (B) Epidermal thickness of male paw skin comparing acute HSD with CD (n = 5 mice per condition from 1 mouse cohort). (C) Epidermal thickness of male paw skin comparing chronic HSD with CD (n = 14-16 mice per condition from four independent mouse cohorts). (D) Epidermal thickness of male ear skin comparing chronic HSD with CD (n = 3 mice per condition from 1 mouse cohort). (E) Epidermal thickness of female paw skin comparing acute HSD with CD (n = 5 mice per condition from one mouse cohort). (F) Epidermal thickness of female paw skin comparing chronic HSD with CD (n = 3 mice per condition from one mouse cohort). (G) Epidermal thickness of female ear skin comparing chronic HSD with CD (n = 3 mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was determined using Student's t-test.

3.2.4 Epidermal cell proliferation is enhanced following chronic high-salt conditions

To investigate the mechanisms underlying the increase in epidermal thickness following chronic HSD, we examined the effects of HSD on cell proliferation. Previous studies have shown that chronic HSD can activate tumor-initiating stem cells, promoting breast cancer proliferation (Tucker et al., 2024), and can also influence the differentiation and activation of various immune cells (Li et al., 2022).

We analyzed paw skin tissue from male and female mice following chronic HSD using 5-ethynyl-2'-deoxyuridine (EdU) staining (Figure 10A), a method that detects DNA synthesis by incorporating a thymidine analogue into newly synthesized DNA, which can then be fluorescently labelled (Zeng et al., 2010). In male mice, we observed a tendential increase in EdU-positive cells in the paw epidermis after chronic HSD (Figure 10B), though this difference did not reach statistical significance. A similar increase in EdU-positive cells was also observed in female mice, but the sample size was limited (Figure 10C).

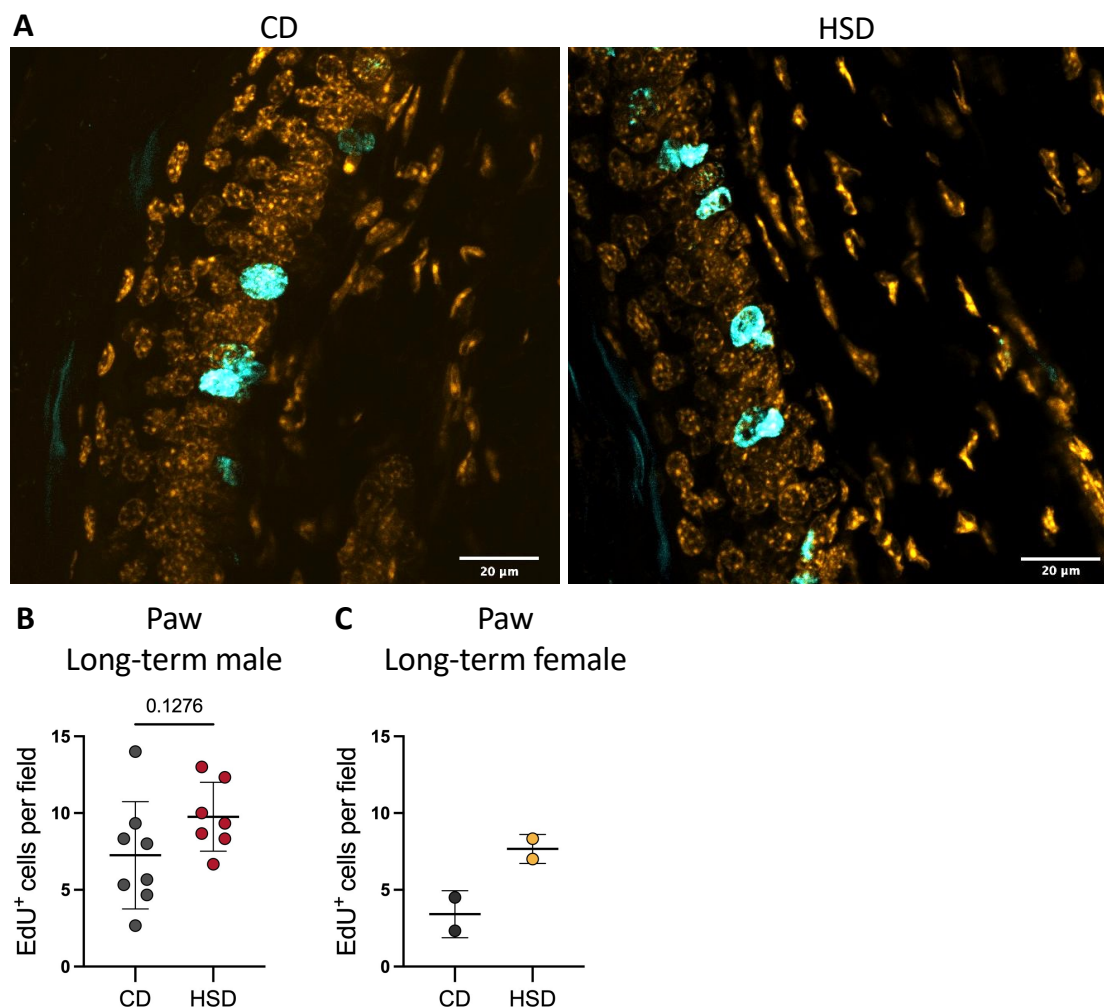


Figure 10: EdU staining of paw skin following chronic HSD feeding.

(A) Representative images of stained paw skin with DAPI (orange) and EdU (cyan) and 40x magnification. (B) Quantification of EdU-positive cells in the epidermis of male paw skin comparing chronic high-salt exposure with CD (n = 8 mice per condition from two independent mouse cohorts). (C) Quantification of EdU-positive cells in the epidermis of female paw skin comparing chronic HSD feeding with CD (n = 2 mice per condition from two independent mouse cohorts). Data are presented as mean $\pm$ SD. Statistical significance was determined using Student's t-test.

3.3 Heterogeneity of skin macrophages

3.3.1 Phenotypic characterization of skin-resident macrophage subpopulations

Macrophages are present in almost all murine and human tissue, where they exist as distinct subtypes adapted to their respective microenvironment (Mass et al., 2023). One approach to distinguish these subsets is through the analysis of specific cell surface markers, which can vary depending on both the macrophages' niche and function. Based on recent studies, as well as our own findings, we characterized the skin-resident macrophages according to their unique cell surface marker profiles (Figure 11A). Langerhans cells, the only macrophages located in the epidermis, express classical macrophage and myeloid cell markers (F4/80, CD11b, CD64) along with MHCII, CD326 and CD74. In the dermis, we can find at least three different macrophage subtypes: blood vessel-associated, interstitial and nerve-associated macrophages. Besides the classical macrophage markers, blood vessel-associated macrophages express CD206 and CD163. Interestingly we found out, that this subset can be further divided depending on their MHCII expression (Figure 11A, B). Interstitial macrophages exhibit a surface marker phenotype similar to Langerhans cells, characterized by the expression of CD74 and MHCII on their cell surface. Finally, nerve-associated macrophages can be differentiated via their expression of Cx3cr1 as well as MHCII. To facilitate flow cytometric analysis, we established a gating strategy based on the surface marker expression profiles of the different macrophages in both the dermis (Figure 11B) and epidermis (Figure 11C). Additionally, we identified a monocyte subset in the dermis, which was distinguished by CD11b and Ly6C expression but lack of F4/80 and MHCII (Figure 11B).

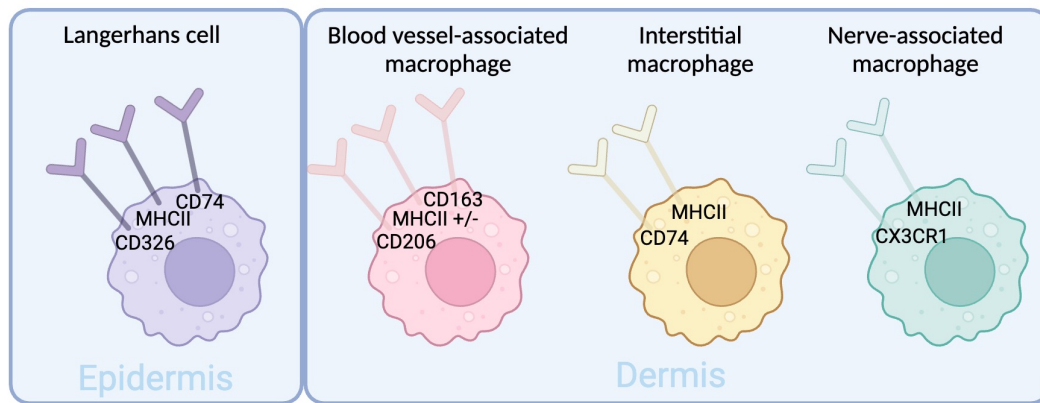
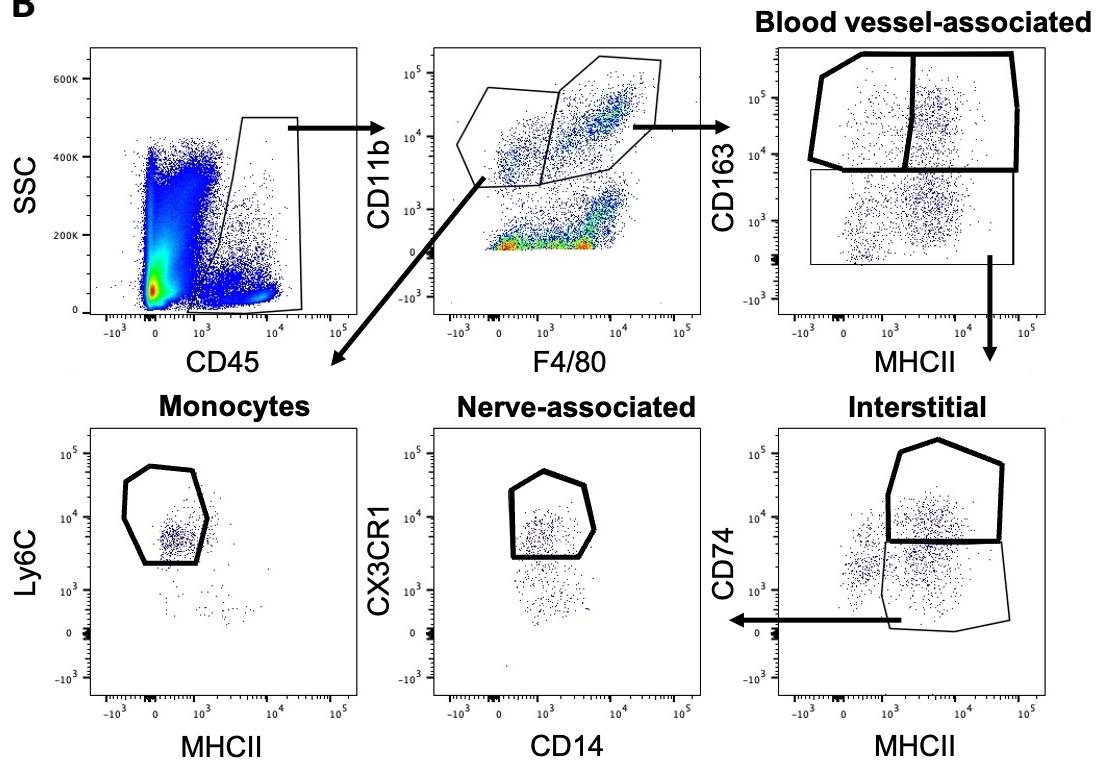
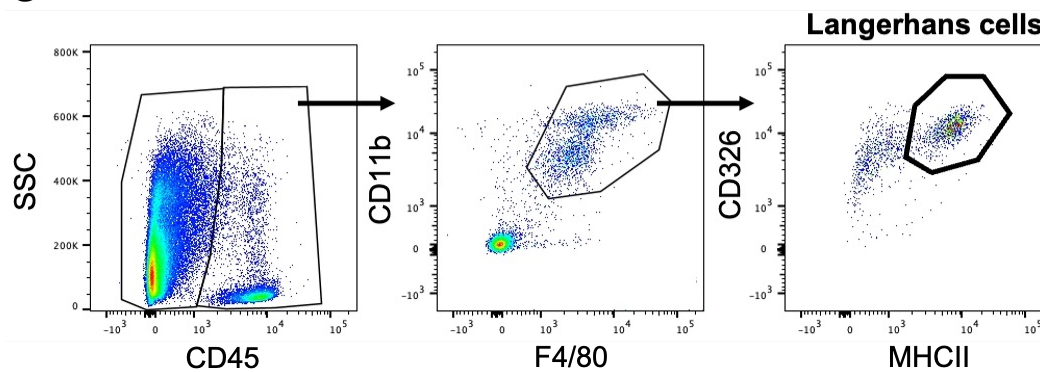
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Figure 11: Characterization of skin macrophages by cell surface marker expression.

(A) Schematic representation of skin macrophages and the respective cell surface markers used for their characterization. (B) Gating strategy for identification of dermal macrophage subsets. (C) Gating strategy for identification of epidermal macrophages.

3.3.2 MHCII expression heterogeneity in blood vessel-associated macrophages

Previous studies have described blood vessel-associated macrophages as MHCII negative cells (Chakarov et al., 2019). However, our analysis suggests the existence of two distinct phenotypes within this macrophage subset, differentiated by their MHCII expression. This distinction was evident in the flow cytometric analysis, where we could see CD163⁺ macrophages with and without MHCII expression (Figure 12A). Furthermore, we have quantified the respective subsets of blood vessel-associated macrophages in 3-month-old (ST) and 5-month-old (LT) mice showing a stable distribution of 75 % MHCII⁺ cells and 25 % MHCII⁻ cells (Figure 12B).

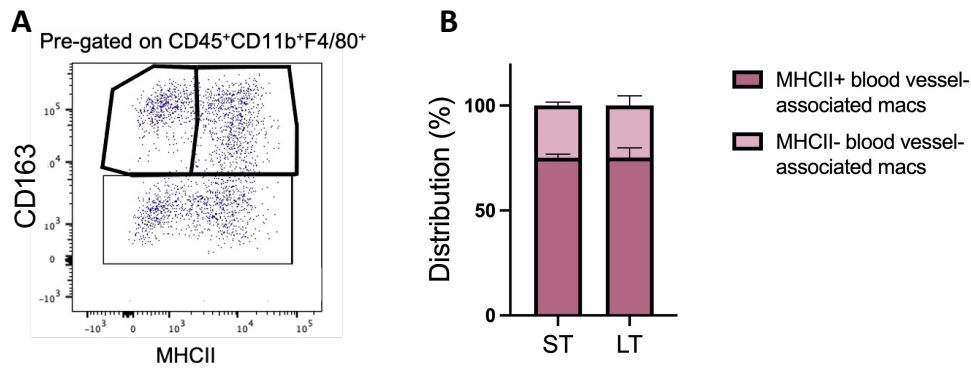


Figure 12: MHCII expression profile of blood vessel-associated macrophages in the skin.

(A) FlowJo plot showing CD163 and MHCII expression in dermal macrophages. (B) Quantification of MHCII-positive and MHCII-negative blood vessel associated macrophages in 3-month-old (ST) and 5-month-old (LT) mice (n = 5 control mice per cohort (ST/LT)).

3.3.3 Immunofluorescence-based identification of niche-specific macrophage subsets in skin tissue

To test the hypothesis that skin macrophages can be distinguished based on their niche-specific localization, we performed immunofluorescent (IF) staining of paw skin sections (Figure 13). For this analysis, we used markers to identify nerve fibers (β 3-Tubulin), blood vessels (CD31), macrophages (Iba1), and cell nuclei (DAPI). The higher cellular density in the epidermis, visualized by DAPI staining, delineates the two main anatomical compartments. Within the epidermis, Iba1-positive cells (cyan) were identified as Langerhans cells (LC). In the dermis, Iba1-positive cells in close association with nerve fibers (blue) likely represent nerve-associated macrophages (N), whereas those in proximity to blood vessels (magenta) are most likely blood vessel-associated macrophages (BV). Finally, interstitial macrophages (I) are characterized as Iba1-positive cells residing within the dermal interstitium, without direct interactions with nerves or blood vessels.

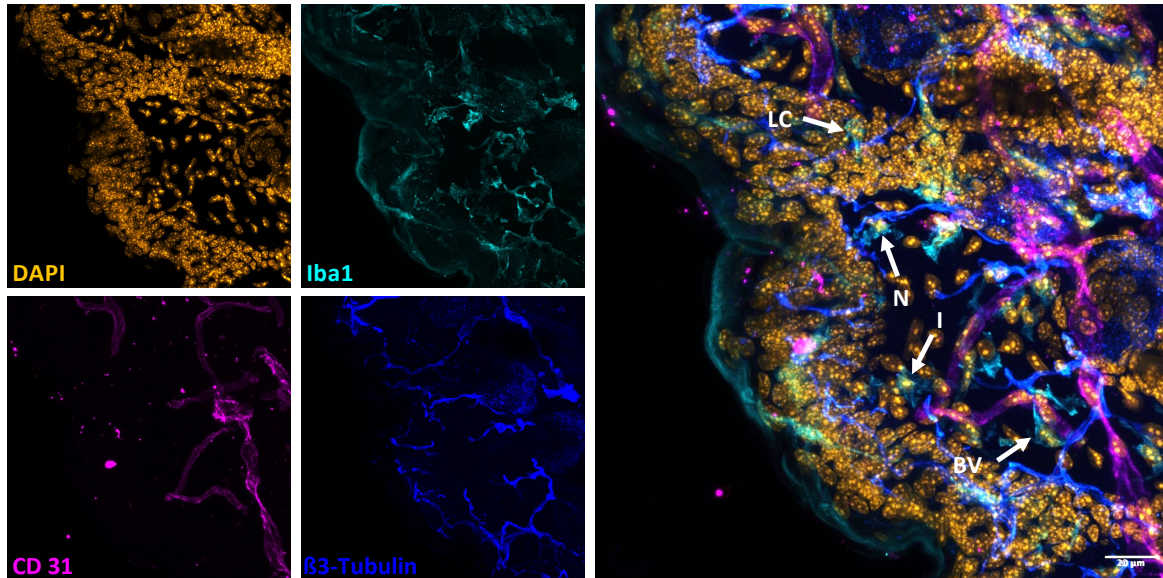


Figure 13: IF staining of skin tissue showing the different macrophage niches.

Markers included in the staining were DAPI (orange, cell nuclei), Iba-1 (cyan, macrophages), CD31 (magenta, blood vessels) and β 3-Tubulin (blue, nerve fibers). LC: Langerhans cells, N: Nerve-associated macrophages, BV: Blood vessel-associated macrophages, I: Interstitial macrophages. Pictures were taken with the confocal LSM 880 Airyscan at 40x magnification.

3.3.4 Niche-specific variations in phagocytosis and efferocytosis of skin macrophage subsets

In addition to their role as immune sentinels, macrophages perform essential homeostatic functions, including efferocytosis of apoptotic cells and phagocytosis of cellular debris. These functions are influenced by their tissue-specific niche, which imprints a distinct identity on each macrophage subset (Guilliams et al., 2020). To determine whether this niche-dependent specialization applies to our defined macrophage subpopulations, we conducted phagocytosis and efferocytosis assays using macrophages isolated from ear skin (Figure 14). Our analysis revealed a trend towards increased phagocytic activity in blood vessel-associated macrophages compared to interstitial and nerve-associated macrophages (Figure 14B), although this difference was not statistically significant. Furthermore, our 4°C control confirmed that phagocytic activity was exclusively evident at physiological temperatures Figure 14C). Notably, in the efferocytosis assay, interstitial macrophages exhibited significantly higher efferocytic activity compared to Langerhans cells, blood vessel-associated macrophages and nerve-associated macrophages (Figure 14D). The 4°C control validated that the observed efferocytosis mainly occurred under physiological conditions (Figure 14E).

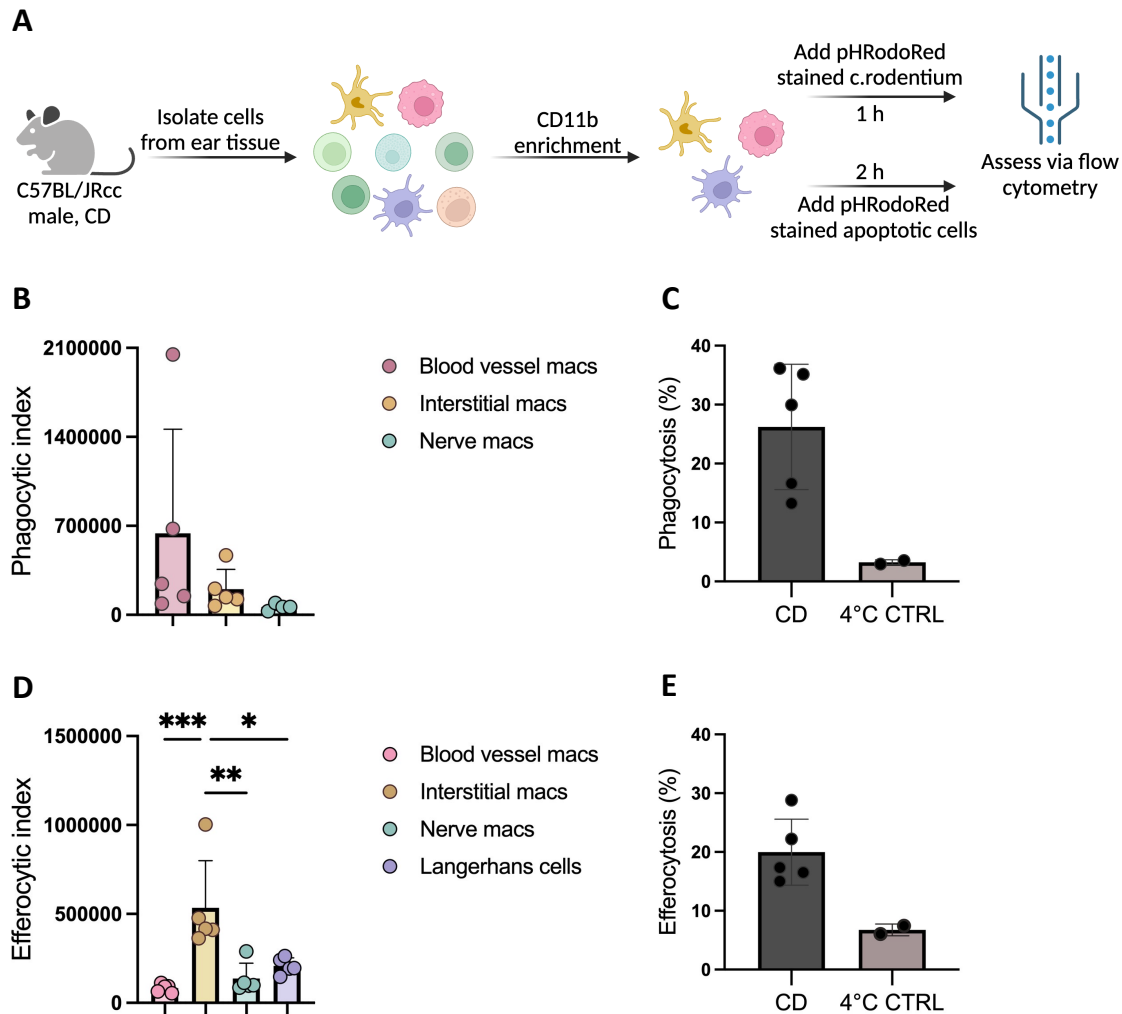


Figure 14: Functional differences in skin macrophages.

(A) Experimental set-up. (B) Phagocytic index of dermal macrophage subsets (n=5 mice per condition from one mouse cohort). (C) Percentage of phagocytosis comparing dermal macrophages with 4°C control. (D) Efferocytic index of dermal macrophage subsets and Langerhans cells (n=5 mice per condition from one mouse cohort). (E) Percentage of efferocytosis comparing skin macrophages with 4°C control. Data are presented as mean $\pm$ SD. Statistical significance was determined using one-way ANOVA.

3.3.5 Developmental origins and turnover dynamics of skin macrophage subpopulations

Macrophages differ not only in their function and tissue-specific niche but these cells may also be derived from alternative developmental origins. For decades, it was assumed that macrophages exclusively originated from hematopoietic stem cells and were continuously replenished by blood-circulating monocytes (van Furth et al., 1972). However, it is now well established that many tissue-resident macrophage populations arise from embryonic precursors, colonize their respective tissues before birth, and maintain themselves through self-renewal via proliferation (McGrath et al., 2015; Perdiguero et al., 2015). To investigate the developmental origin of skin macrophage subpopulations over time, we employed a double fate-mapping mouse model (Figure 15). This model enables the

simultaneous tracking of fetal derived pMacs and bone-marrow derived monocytes using a combination of conventional recombinase approaches. The *Tnfrsf11a^{Cre}* system efficiently labels pMacs and their progeny (Mass et al., 2016). However, since *Tnfrsf11a* is a core macrophage gene, it also becomes active when monocytes differentiate into macrophages. To address this, it was combined with *Ms4a3^{FlpO}*, which specifically targets monocytes and thus all monocyte derived macrophages (Liu et al., 2019).

Using the double fate-mapper mouse model, we analyzed mice at different time points, ranging from embryonic day 18.5 (E18.5) to one year of age. Thereby, cells of embryonic origin are labeled by yellow fluorescent protein expression (YFP⁺), whereas cells of hematopoietic origin are labeled by tdTomato expression (tdT⁺). Over time, tdT⁺ cells that persist in the tissue express YFP as well, becoming double-positive (YFP⁺tdT⁺). Our results revealed that at E18.5 and postnatal day 0 (P0), nearly all skin macrophages originated from EMPs. We further noticed, that MHCII⁺ blood vessel-associated macrophages only began expressing MHCII in the first two weeks after birth, therefore E18.5 and P0 timepoints could not be analyzed for this subset (Figure 15B, Figure 16). By one week of age, distinct differences in the developmental origins of skin macrophage subpopulations became evident. While Langerhans cells primarily maintained themselves through proliferation with minimal monocyte replenishment - even up to one year of age (Figure 15E) - dermal macrophages exhibited a higher and more rapid turnover (Figure 15A–D). Among dermal macrophages, nerve-associated macrophages underwent the fastest replacement by monocytes, with over 50% of the population expressing tdT (alone and in combination with YFP) by 3 weeks of age, increasing to nearly 100 % by one year (Figure 15D). In contrast, interstitial macrophages followed a developmental trajectory more similar to Langerhans cells, retaining approximately 80% YFP labelled, fetal derived cells after one year (Figure 15C). Blood vessel-associated macrophages experienced less frequent replenishment by circulating monocytes compared to nerve-associated macrophages, maintaining approximately 40% YFP expressing cells at one year of age (Figure 15A, B).

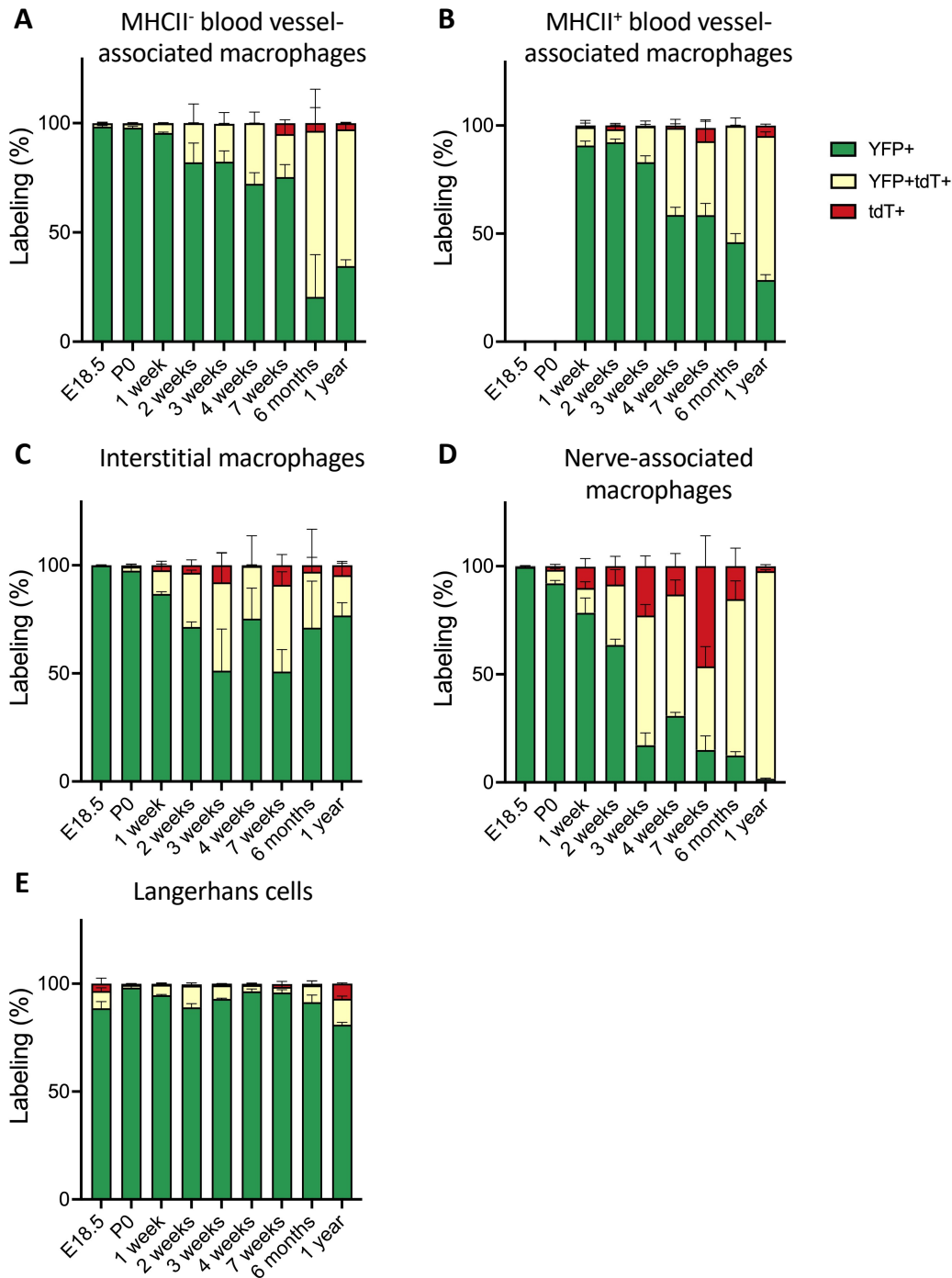


Figure 15: Developmental origin of skin macrophage over time.

(A-F) Fate-mapping of skin macrophage subsets: (A) MHCII⁻ blood vessel-associated macrophages, (B) MHCII⁺ blood vessel-associated macrophages, (C) Interstitial macrophages, (D) Nerve-associated macrophages and (E) Langerhans cells. Embryonic-derived cells are shown in green (YFP⁺), tissue-resident monocyte-derived cells are shown in yellow (YFP⁺tdT⁺) and short-lived monocyte-derived cells are shown in red (tdT⁺) (n = 2-5 mice per timepoint from nine independent mouse cohorts).

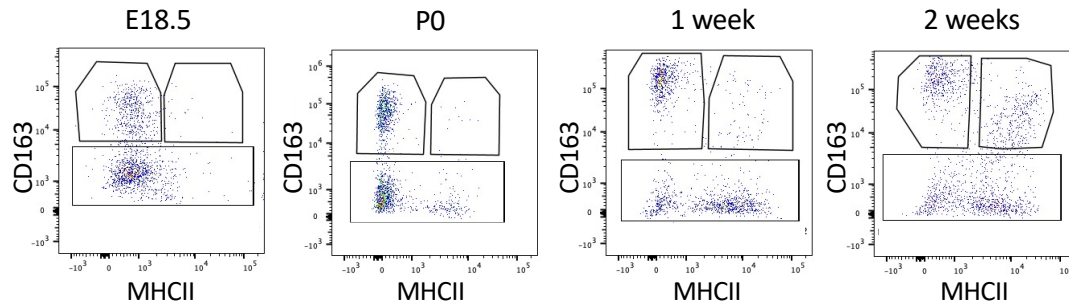


Figure 16: Development of MHCII expression on blood vessel-associated macrophages.

Double-fatemapper time course experiment showing flow cytometric data of MHCII expression in CD163⁺ macrophages. Cells were derived from mice at the age of E.18.5, P0 (day of birth), 1 week and 2 weeks. The graphs show data of one representative mice per timepoint.

3.4 Impact of acute and chronic high-salt diet on function, morphology and development of skin macrophages

3.4.1 Relative numbers of skin macrophage subsets are increased in acute high-salt conditions and decreased upon chronic high-salt exposure

After thoroughly characterizing skin macrophages in terms of function, tissue niches, surface marker expression, and developmental origin under homeostatic conditions, we next sought to investigate the impact of a HSD on macrophage subpopulations. As discussed in section 3.2.4, there is evidence suggesting that a high-salt intake may influence immune cell proliferation (Li et al., 2022). To assess this, we quantified the relative abundance of each skin macrophage subset and compared the effects of acute and chronic high-salt conditions, as well as differences between male and female cohorts (Figure 17, 18). Our results demonstrated a strong but variable impact of HSD-feeding, particularly in male mice. In the acute HSD model, blood vessel-associated macrophage numbers were significantly increased in male mice compared to the CD (Figure 17A, B), while a similar, though non-significant, trend was observed for other macrophage subtypes. However, in the chronic HSD model, the opposite effect was observed, with overall skin macrophage numbers decreasing compared to CD, reaching statistical significance for nerve-associated macrophages (Figure 18D). In contrast, female mice exhibited a less pronounced response to both acute and chronic high-salt conditions, with no consistent trend across macrophage subsets (Figure 17F-J, Figure 18F-J).

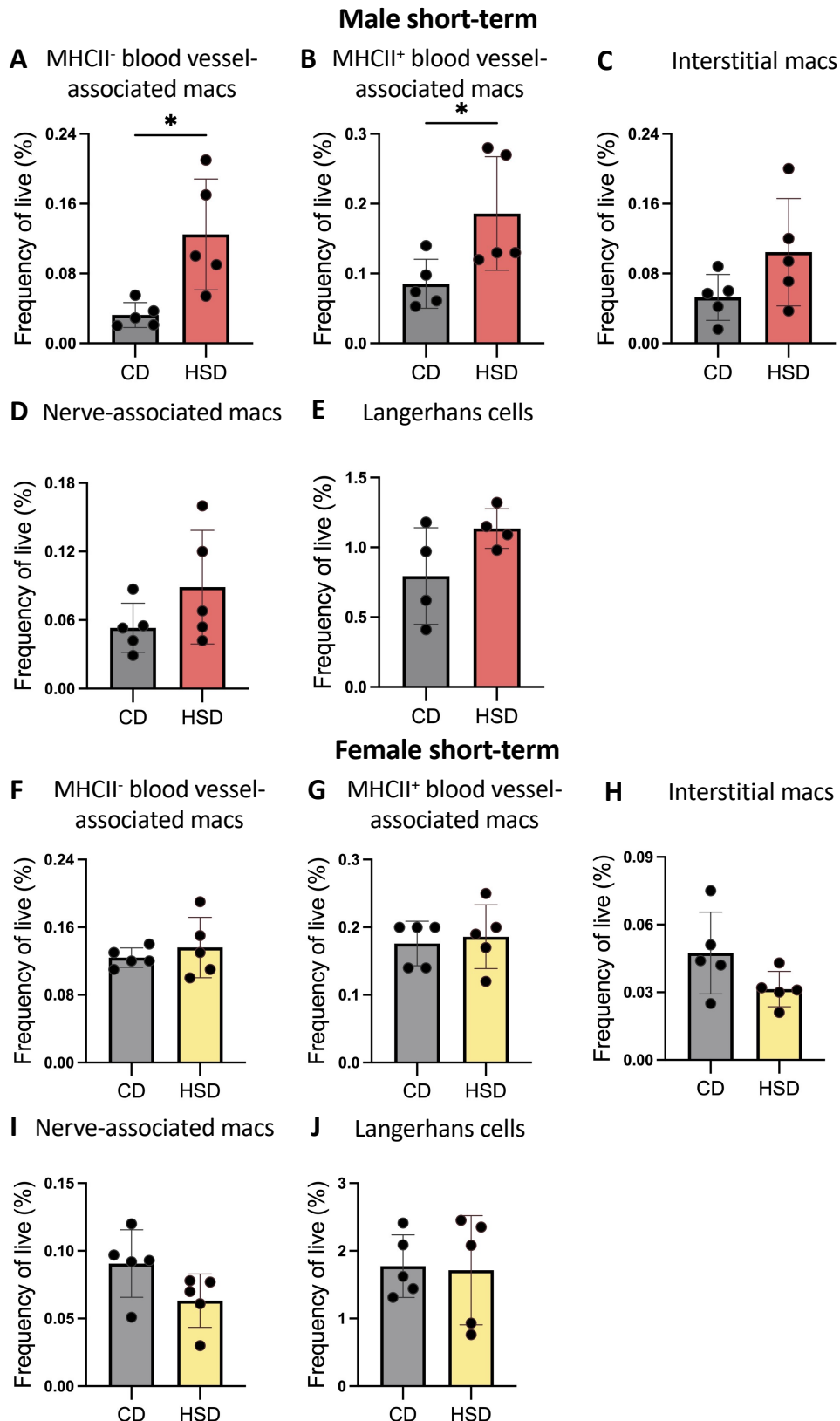


Figure 17: Frequencies of skin macrophages following acute HSD.

Flow cytometric analysis of the frequency of live of skin macrophage subsets. (A-E) Quantities of macrophage subpopulations after acute high-salt exposure in male mice (n = 5 mice per condition from one mouse cohort). (F-J) Quantities of macrophage subpopulations after acute high-salt exposure in female mice (n = 5 mice per

condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was determined using Student's t-test.

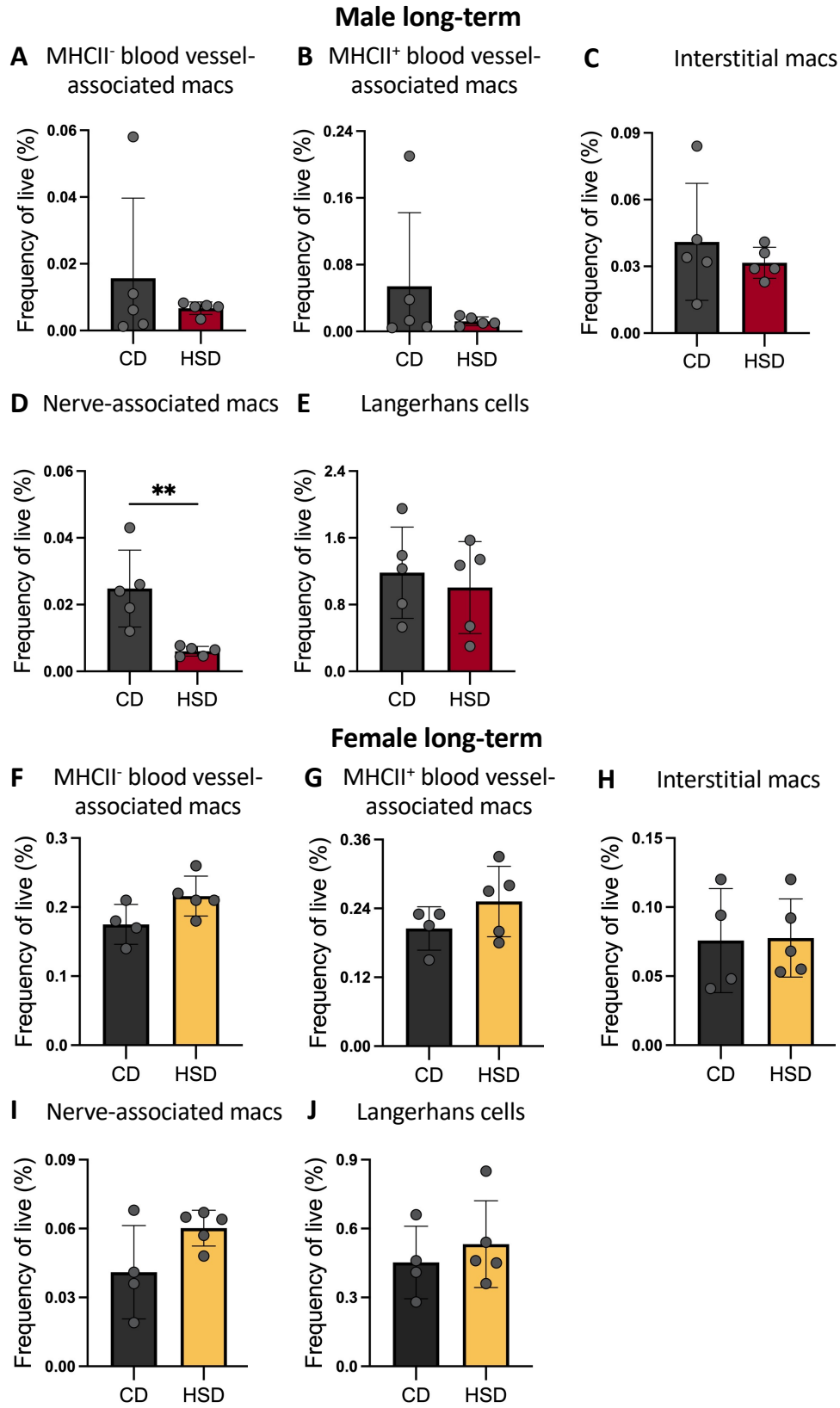


Figure 18: Frequencies of skin macrophages following chronic HSD.

Flow cytometric analysis of the frequency of live of skin macrophage subsets. (A-E) Quantities of macrophage subpopulations after chronic high-salt exposure in male mice (n = 5 mice per condition from one mouse cohort). (F-J) Quantities of macrophage subpopulations after chronic high-salt exposure in female mice (n = 5 mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was determined using Student's t-test.

3.4.2 Acute HSD feeding modulates skin macrophage surface marker expression in male mice

In addition to examining the impact of HSD on relative abundance of skin macrophage subsets, we further investigated potential changes in function-related cell surface marker expression levels following acute and chronic HSD feeding in male (Figure 19, 21) and female (Figure 20, 22) mouse cohorts. To this end, we analyzed several surface markers associated with phagocytosis (CD64, CD32b, CD16.2), efferocytosis (MerTK, Tim4), and antigen presentation (MHCII). Similar to our findings on macrophage abundances, the effects of acute HSD were more pronounced in male mice than in female mice. In male mice, there was a general trend towards increased expression levels of nearly all tested markers (Figure 19A-F). The most significant effect was observed for CD32b, which was significantly upregulated across all dermal macrophage subsets (Figure 19B). Interestingly, Langerhans cells exhibited a significant decrease in CD16.2 (Figure 19C) and MerTK (Figure 19D) expression levels following acute HSD feeding compared to CD, whereas these markers remained unchanged in dermal macrophages. Additionally, MHCII⁺ blood vessel-associated macrophages displayed significantly increased Tim4 (Figure 19E) and MHCII (Figure 19F) expression levels in response to acute HSD, while MHCII⁻ blood vessel-associated macrophages showed a significant upregulation of CD64 (Figure 19A). Nerve-associated macrophages significantly upregulated CD64 (Figure 19A) and Tim4 (Figure 19E). In contrast, female mice exhibited a far less pronounced response, with the only significant change observed in nerve-associated macrophages, which showed increased CD64 expression following acute HSD (Figure 20A). Notably, our data also revealed that, in general, most cell surface markers were expressed at lower levels in female mice compared to male mice. Interestingly, the alterations in surface marker expression observed in the acute HSD model (Figure 19) were no longer apparent following long-term HSD feeding (Figure 21). In the male cohort, no significant changes in marker expression levels were detected across all skin macrophage subsets (Figure 21A-F). In the female cohort, the only significant change observed was a reduction in MHCII expression in Langerhans cells for HSD feeding compared to CD (Figure 22B).

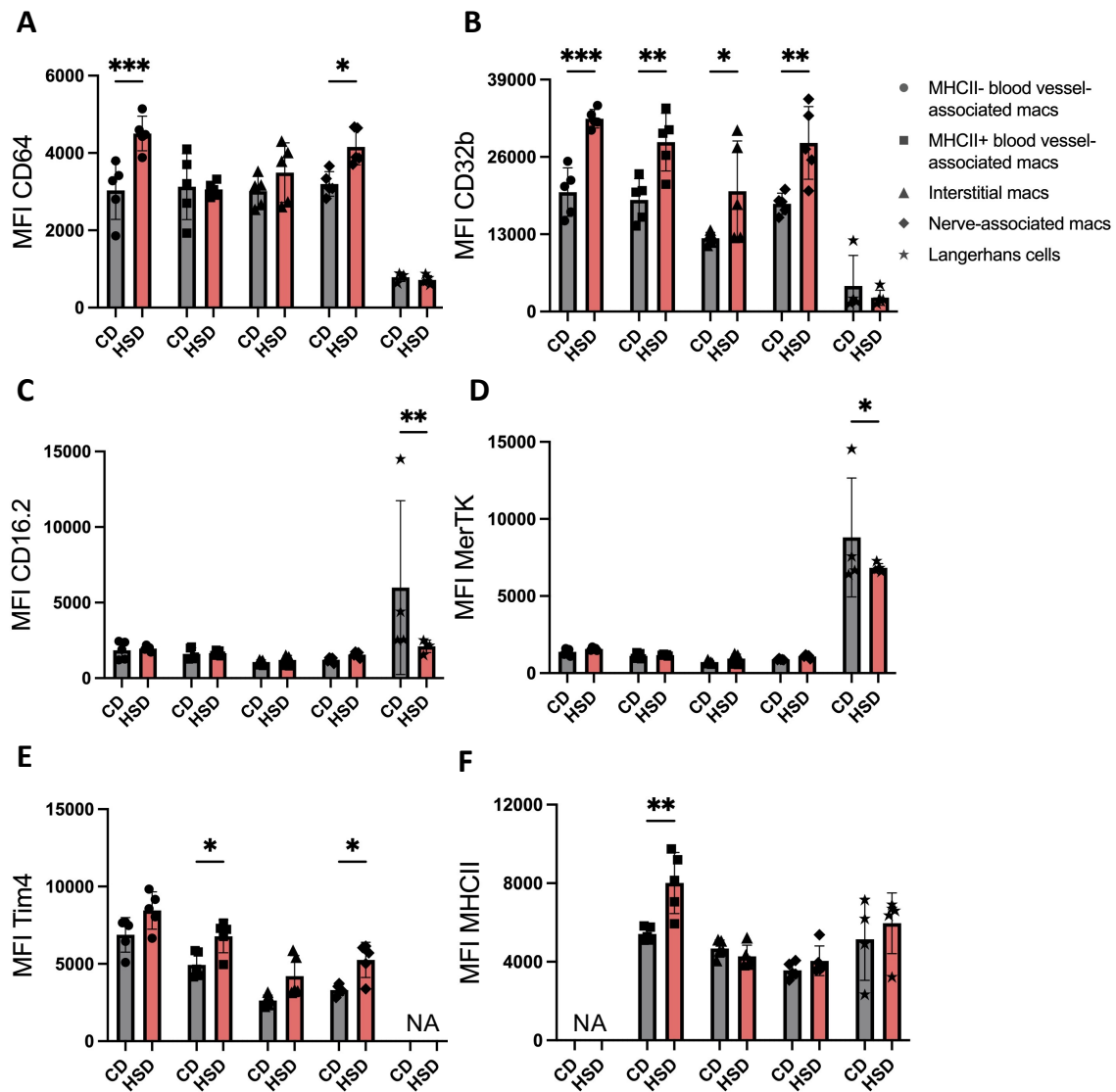


Figure 19: Cell surface marker expression levels of skin macrophages following acute HSD feeding in male mice.

Mean fluorescence intensity (MFI) of markers associated with phagocytosis (CD64 (A), CD32b (B), CD16.2 (C)), efferocytosis (MerTK (D), Tim4 (E)), and antigen presentation (MHCII (F)) in male mice following acute HSD feeding. Marker expression levels were analyzed across all skin macrophage subsets (n = 5 mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was assessed using two-way ANOVA.

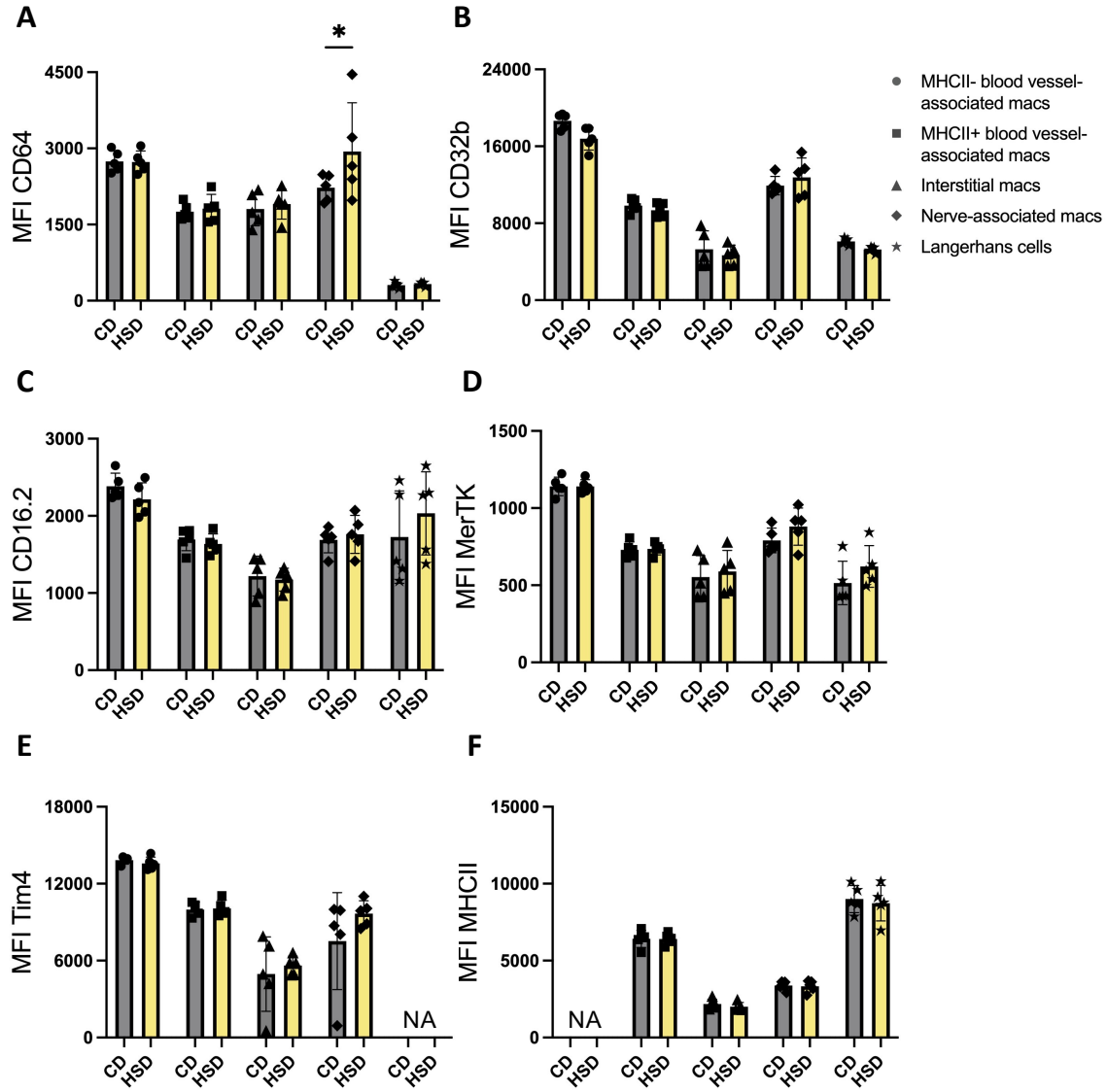


Figure 20: Cell surface marker expression levels of skin macrophages following acute HSD feeding in female mice.

Mean fluorescence intensity (MFI) of markers associated with phagocytosis (CD64 (A), CD32b (B), CD16.2 (C)), efferocytosis (MerTK (D), Tim4 (E)), and antigen presentation (MHCII (F)) in female mice following acute HSD feeding. Marker expression levels were analyzed across all skin macrophage subsets ($n = 5$ mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was assessed using two-way ANOVA.

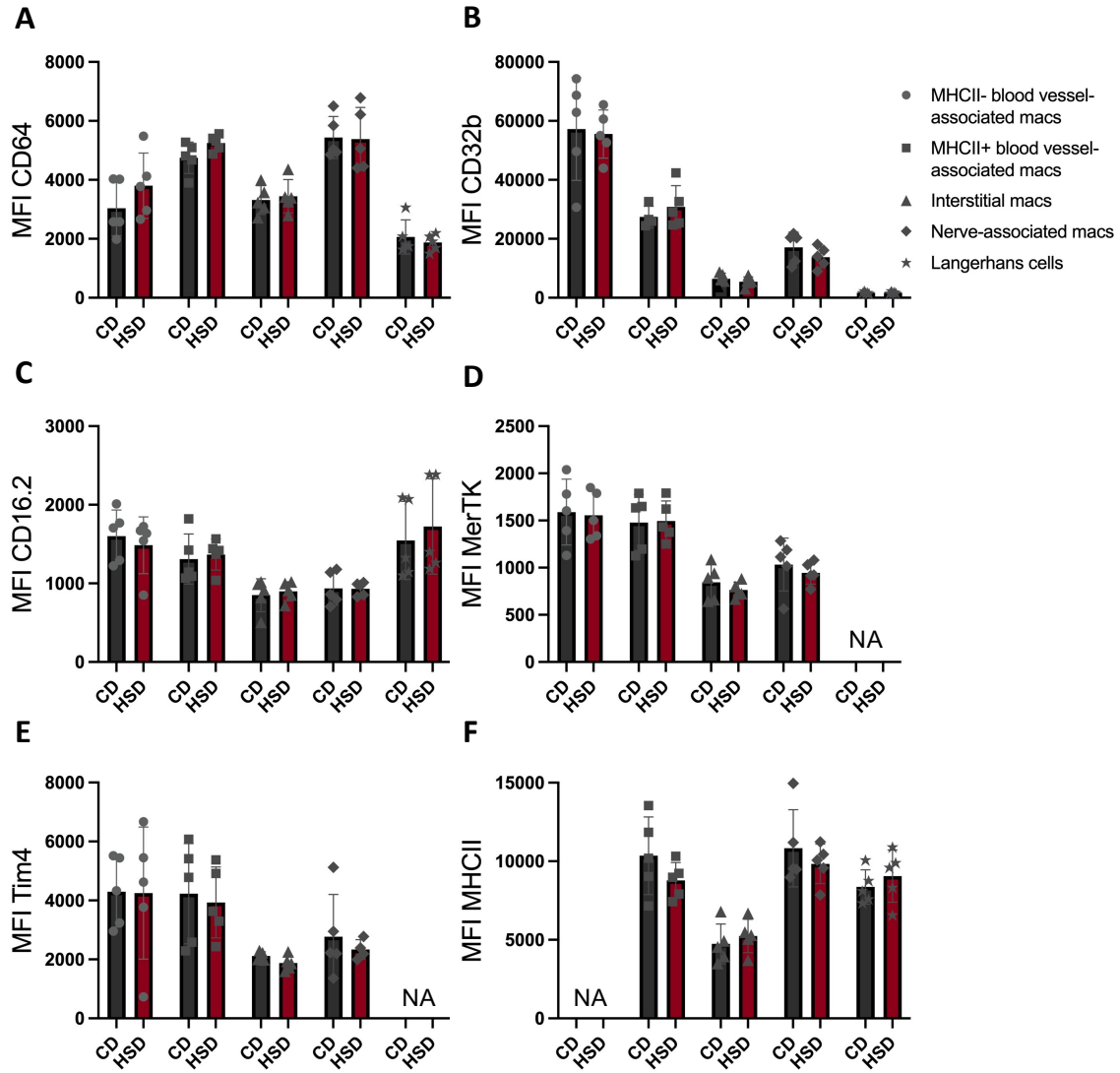


Figure 21: Cell surface marker expression levels of skin macrophages following chronic HSD feeding in male mice.

Mean fluorescence intensity (MFI) of markers associated with phagocytosis (CD64 (A), CD32b (B), CD16.2 (C)), efferocytosis (MerTK (D), Tim4 (E)), and antigen presentation (MHCII (F)) in male mice following acute HSD feeding. Marker expression levels were analyzed across all skin macrophage subsets ($n = 5$ mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was assessed using two-way ANOVA.

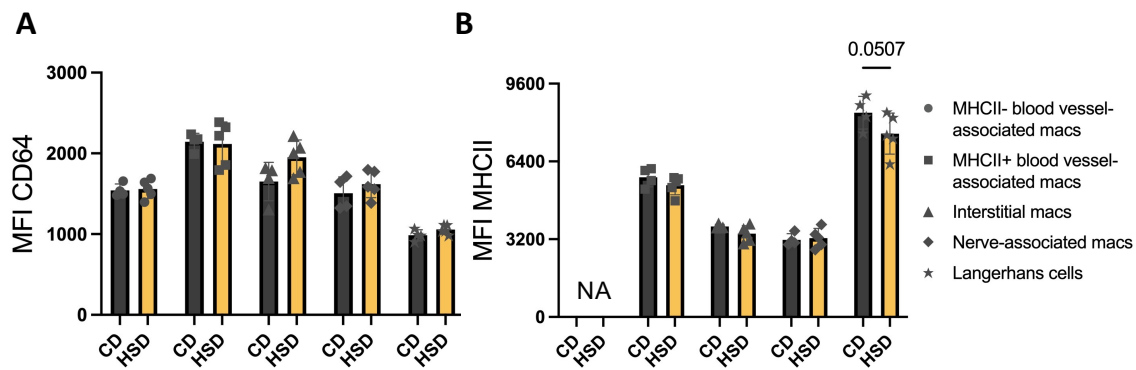


Figure 22: Cell surface marker expression levels of skin macrophages following chronic HSD feeding in female mice.

Mean fluorescence intensity (MFI) of markers associated with phagocytosis (CD64 (A)) and antigen presentation (MHCII (B)) in female mice following chronic HSD feeding. Marker expression levels were analyzed across all skin macrophage subsets (n = 5 mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was assessed using two-way ANOVA.

3.4.3 Acute and chronic HSD-feeding induces a shift in dermal blood vessel-associated macrophage populations towards less MHCII-expressing cells

Given the significant changes in both MHCII⁻ and MHCII⁺ blood vessel-associated macrophages in terms of cell numbers and surface marker expression - particularly in response to acute high-salt conditions - we sought to examine their overall distribution after short-term and long-term HSD feeding. Interestingly, we observed a significant shift in the distribution of blood vessel-associated macrophages, with a notable decrease in MHCII⁺ cells relative to MHCII⁻ cells following both acute (Figure 23A, -11.6 %) and chronic (Figure 23B, -11.8 %) HSD feeding.

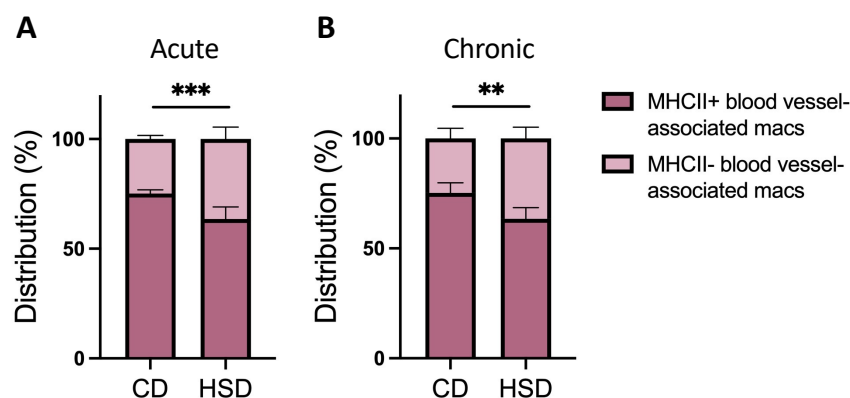


Figure 23: Distribution of MHCII⁻ and MHCII⁺ blood vessel-associated macrophages in the skin after acute and chronic HSD feeding.

Short-term (A) and long-term (B) HSD feeding, showing percentages of MHCII⁻ and MHCII⁺ blood vessel-associated macrophages for CD and HSD conditions (n = 5 mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was determined using Student's t-test.

3.4.4 Cell volume and surface area of blood vessel-associated macrophages in the skin is decreased following acute HSD

Macrophages can sense excess salt concentrations in their environment, and a recent study demonstrated that this sensing occurs via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger 1 (NCX1). Through this solute carrier, Na^+ enters macrophages while Ca^{2+} is concomitantly exported, a process that is significantly enhanced under high-salt conditions (Neubert et al., 2020). We hypothesized that increased Na^+ influx might also affect macrophage morphology, e.g., by inducing an osmotic or inflammatory reaction. To investigate this, we performed IF stainings of macrophages in paw cryosections from male mice following acute HSD and analyzed their morphology using the ImageJ plugin MotiQ (Hansen et al., 2022) (Figure 24). Interestingly, our analysis revealed that blood vessel-associated macrophages were primarily affected by acute HSD, exhibiting a significant reduction in both cell volume and cell surface area (Figure 24A, B). In contrast, other skin macrophage subsets showed a tendency toward increased cell volume and surface area, with the most pronounced effect observed in Langerhans cell volume (Figure 24J) Notably, the ramification index remained unchanged across all skin macrophage subsets following acute HSD (Figure 24A–I).

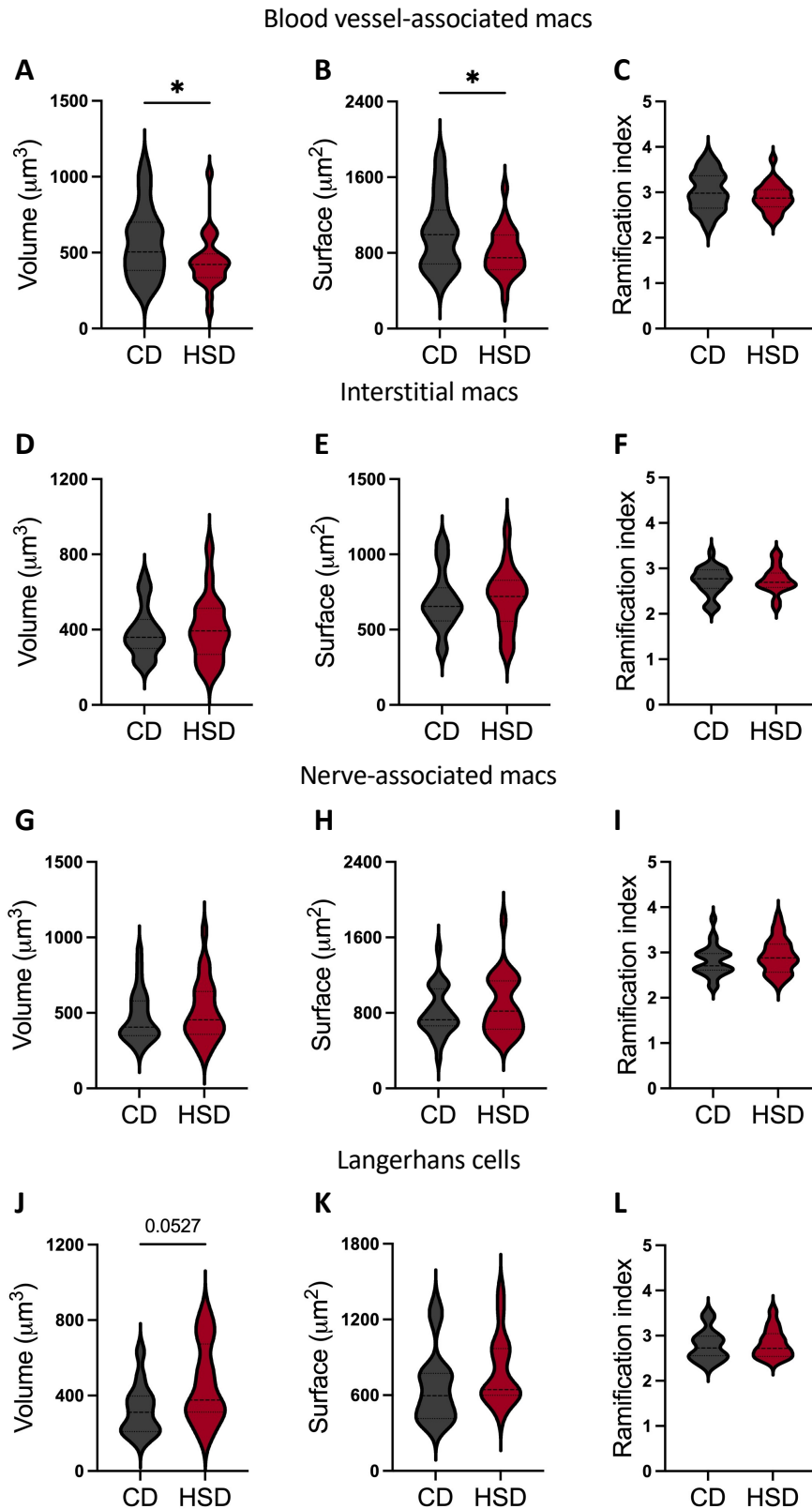


Figure 24: Cell morphology analysis of skin macrophages following acute HSD.

Measured parameters include cell volume, cell surface area, and ramification index in blood vessel-associated macrophages (A-C), interstitial macrophages (D-F), nerve-associated macrophages (G-I), and Langerhans cells (J-L) ($n = 5$ mice per condition, 3–5 cells per mouse). Morphological analysis was performed using the ImageJ plugin MotiQ. Statistical significance was assessed using Student's t-test.

3.4.5 Chronic high-salt exposure increases monocyte-derived interstitial macrophages without affecting other skin macrophage lineages

Increasing evidence suggests that HSD exerts an activating effect on the immune system, promoting tissue inflammation and monocyte infiltration (Wenstedt et al., 2019; Yi et al., 2015; X. Zhou et al., 2013). Based on these findings, we hypothesized that HSD might also influence the developmental origin of skin macrophages. Given the dynamic nature of macrophage populations, it is crucial to determine whether environmental factors, such as dietary salt intake, can alter the dynamics of macrophage development over time. To investigate this, we utilized the double fate-mapping mouse model and analyzed the distribution of tdT- and YFP-labeled cells following long-term HSD (Figure 25). Our results indicate that most skin macrophage subsets remain unaffected by chronic HSD in terms of their developmental origin. Notably, Langerhans cells exhibited a high degree of stability, with over 80 % of the population retaining single-YFP labeling after three months of either CD or HSD (Figure 25E). The only subset that showed a significant shift in developmental origin was the interstitial macrophages, which exhibited a significantly higher proportion of tissue-resident cells with a monocytic background (YFP⁺tdT⁺) and a respective reduction in fetal-derived cells (YFP⁺) following chronic HSD compared to CD (Figure 25C).

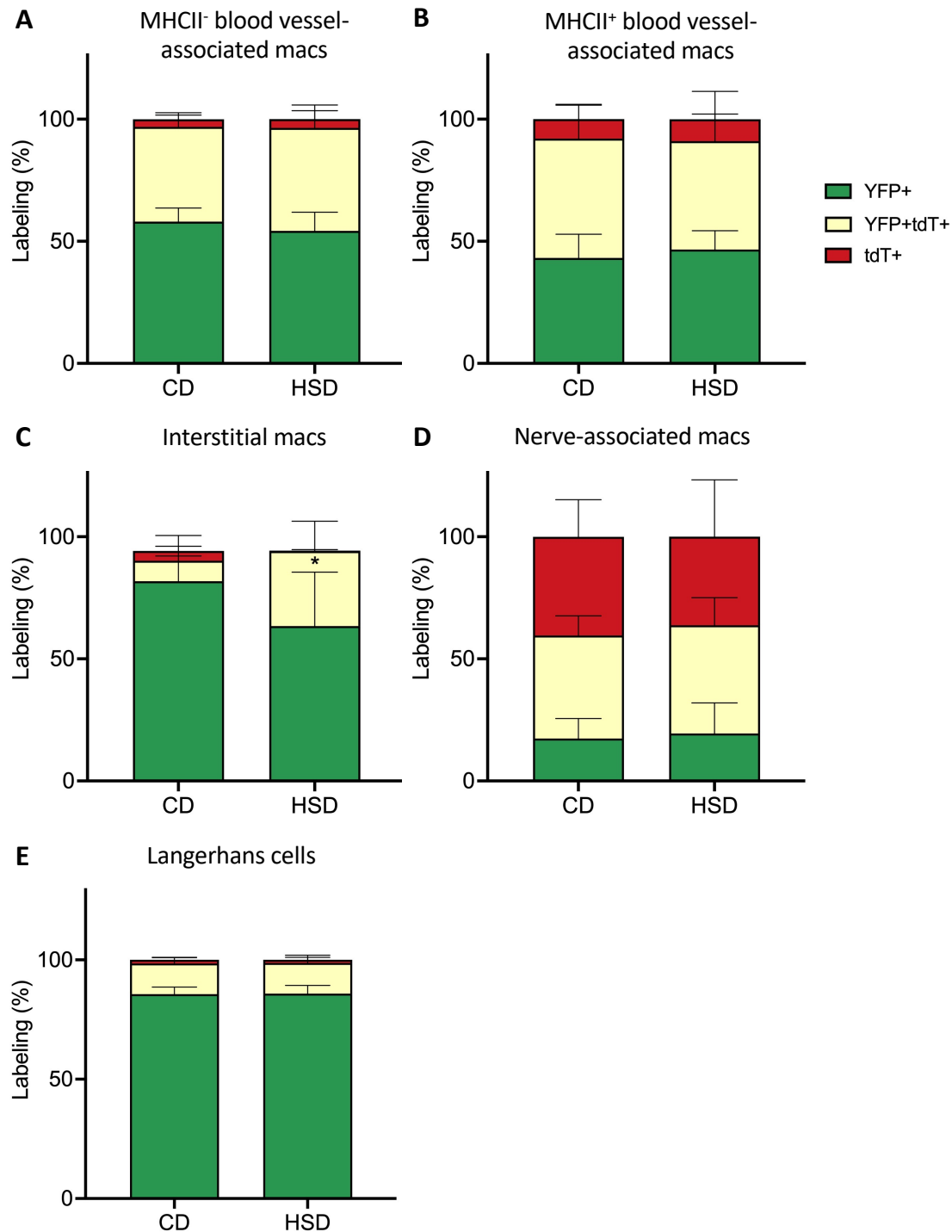


Figure 25: Fate mapping of skin macrophages following chronic HSD.

Analysis was conducted using the double fate-mapper mouse model and flow cytometry, focusing on MHCII⁻ blood vessel-associated macrophages (A), MHCII⁺ blood vessel-associated macrophages (B), Interstitial macrophages (C), Nerve-associated macrophages (D) and Langerhans cells (E) (n = 4-5 mice per condition from one male cohort). Cells of embryonic origin were identified by YFP labeling, while short-lived monocyte-derived cells were labeled with tdT, and long-lived macrophages with a monocytic background exhibited both YFP and tdT expression. Data are presented as mean $\pm$ SD. Statistical significance was determined using Student's t-test.

3.4.6 Chronic HSD feeding tends to reduce phagocytic and efferocytic capacity of skin

macrophages

Since our previous analysis revealed alterations in cell surface receptors associated with phagocytosis and efferocytosis, we next performed functional assays using isolated skin macrophages from mice subjected to either CD or HSD for 3 months (Figure 26). Phagocytosis and efferocytosis were assessed by flow cytometry, using fluorescently labeled *Citrobacter rodentium* for phagocytosis and stained apoptotic thymocytes for efferocytosis quantification. Our findings indicated a trend towards reduced phagocytic (Figure 26B) and efferocytic (Figure 26C) activity across all tested skin macrophage subsets, although these differences were not statistically significant.

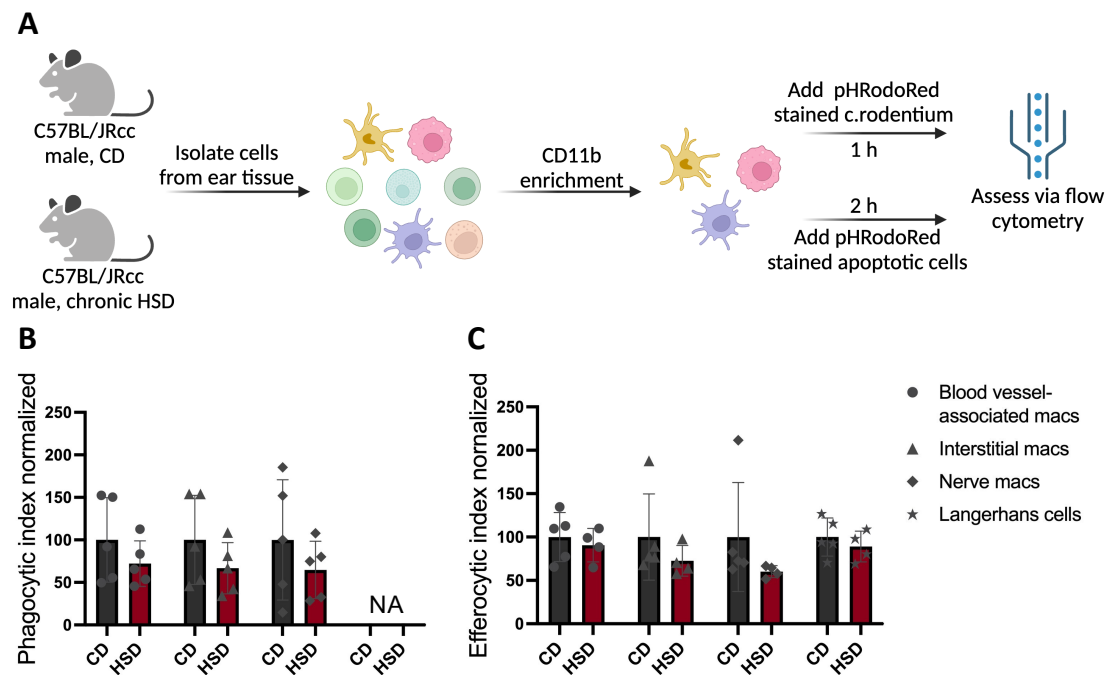


Figure 26: Phagocytic and efferocytic activity of skin macrophages following chronic HSD.

(A) Experimental set-up. (B) Normalized phagocytic index of dermal macrophages (n = 5 mice per condition from one mouse cohort). (C) Normalized efferocytic index of skin macrophages (n = 4-5 mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was determined using two-way ANOVA.

3.4.7 Characterization of the transcriptional signature of skin macrophages and alterations in functional and salt-related genes in blood vessel-associated macrophages following chronic HSD feeding

In order to characterize the transcriptional landscape of skin macrophage subpopulations and assess the impact of chronic high-salt intake on their gene expression profiles, scRNAseq was performed on cells isolated from the ears of wt mice following chronic HSD feeding. For this purpose, ear skin from four mice per dietary condition was collected, followed by tissue dissociation and enrichment of immune cells based on CD45 expression. Two biological replicates per group were pooled, resulting in approximately 16500 cells, with a targeted recovery of 10000 cells per sample for library preparation and subsequent sequencing. Although transcriptional profiling was conducted across all skin-resident cell types, the analysis presented here focuses specifically on the macrophage compartment.

To visualize macrophage subpopulations in CD and HSD samples, we performed dimensionality reduction of global gene expression data using a UMAP (Figure 27A). In this representation, cells with similar transcriptional profiles are positioned in close proximity within the two-dimensional space, whereas cells with distinct expression patterns form separate clusters. Based on this analysis, six transcriptionally distinct macrophage populations were identified in the skin, exhibiting comparable clustering patterns between CD and HSD conditions (Figure 27A).

For further characterization of the identified macrophage subpopulations in the skin, the top 20 most highly expressed genes in each cluster were evaluated (Figure 27B). To confirm the macrophage identity of the analyzed populations and exclude potential dendritic cell contamination, expression of classical dendritic cell markers such as *Itgax* and *Xcr1* was also assessed. Identified populations Mac1 and Mac6 displayed largely overlapping gene expression profiles, with the key distinction of elevated *Il1b* expression in Mac6. Based on *Cd74* and *H2-Ab1* (MHCII) expression and the absence of vascular-associated genes such as *Lyve1* and *Mrc1*, Mac1 and Mac6 likely represent distinct phenotypic variants of interstitial macrophages. Mac2 exhibited a transcriptional profile consistent with Langerhans cells, characterized by the expression of *Epcam* (CD326), *Cd207*, *H2-Ab1*, and *Cd74*. Mac3 and Mac4 were defined as blood vessel-associated macrophages, given their high expression of *Lyve1*, *Mrc1* (CD206), and *Cd163*. In accordance with our previous flow cytometry data (Figure 12), these two populations differ in MHCII expression levels and additional gene signatures, suggesting a representation of functionally distinct subsets of blood vessel-associated macrophages.

Lastly, Mac5 emerged as a macrophage subset characterized by a gene expression profile indicative of close interactions with T cells, including *Trdc*, *Cd3g*, and *Ctla2a*. Notably, *Cx3cr1* expression was low across all identified macrophage subsets, prohibiting a clear assignment of a nerve-associated macrophage population.

To assess transcriptional alterations in response to dietary salt intake, an over-representation analysis (ORA) was performed across all macrophage subpopulations comparing CD and HSD conditions (Figure 27C). This analysis revealed that Mac3 and Mac4 (both identified as blood vessel-associated macrophages) exhibited the most pronounced transcriptional changes following chronic high-salt exposure. Among the differentially expressed genes, several components of the VEGF signaling pathway were enriched, including *Elmo1*, *Nrp2*, *Prkca* and *Sos1*. Notably, *Elmo1* expression was significantly upregulated in both Mac3 and Mac4 under high-salt conditions. While *Nrp2* expression showed an increasing trend, it was not statistically significant. In contrast, *Prkca* expression was significantly elevated in Mac4, and *Sos1* expression was significantly increased in Mac3 following chronic high-salt intake.

Given the substantial transcriptional alterations observed in blood vessel-associated macrophages, we further examined the expression of selected functional and salt-regulatory genes. Among macrophage function-related genes, *MertK* expression levels were significantly reduced in Mac4 under HSD conditions, potentially indicating impaired efferocytic capacity (Figure 27D). However, there were no changes observed in *Fcgr2b* gene expression levels in both Mac3 and Mac4. Interestingly, the expression of the salt-sensing kinase *Wnk1* gene was significantly downregulated in Mac4, hinting towards an impaired salt sensing capacity of this macrophage subset (Figure 17E). The expression levels of the sodium-calcium exchanger *Slc8a1* gene remained unchanged.

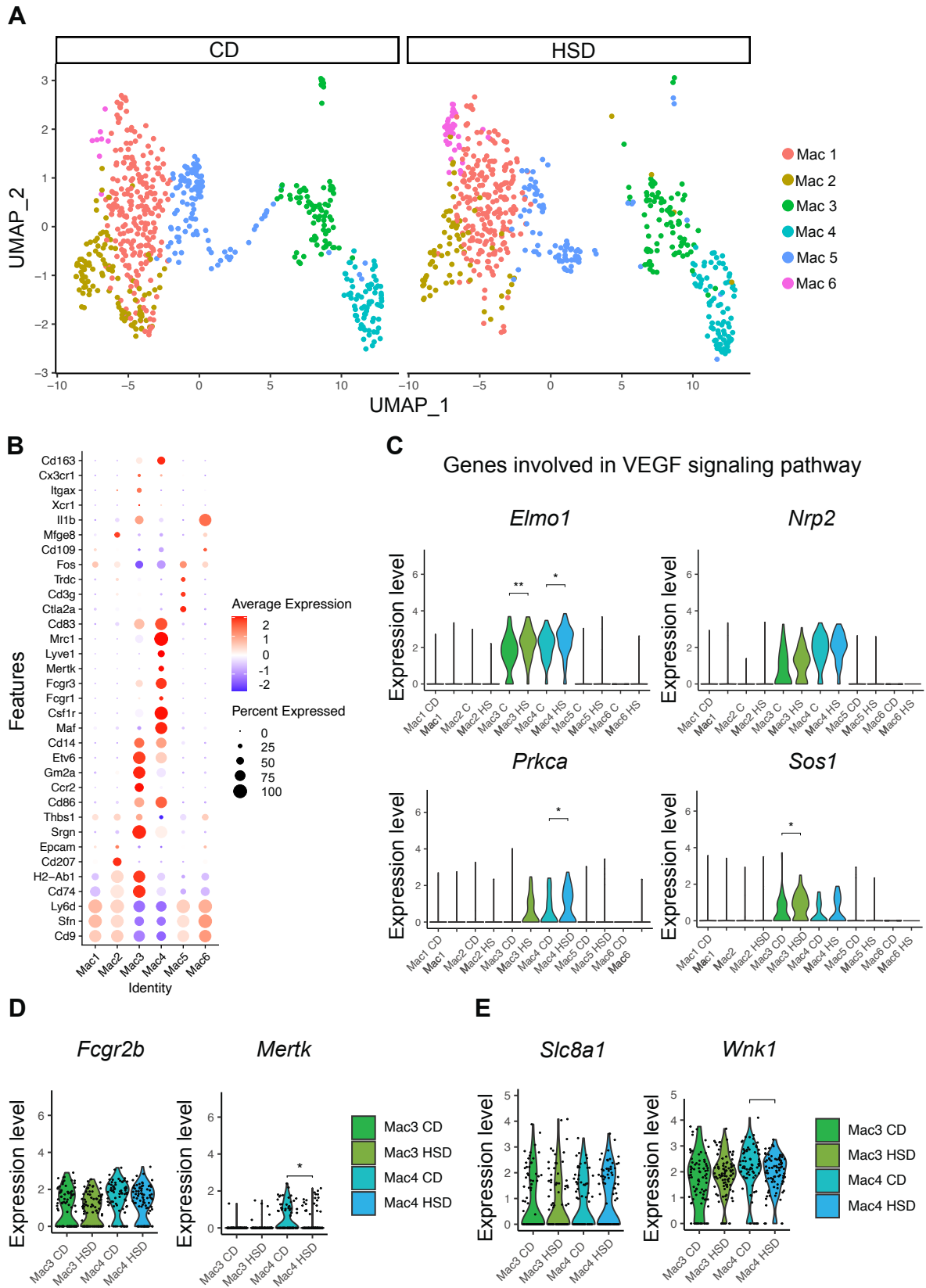


Figure 27: ScRNA-seq of skin macrophages following chronic high-salt exposure.

(A) UMAP plot of the integrated dataset showing color-coded macrophage sub-clusters for CD and HSD samples. (B) Dot plot displaying selected genes among the top 20 most highly expressed genes per macrophage subcluster in CD samples. (C) Violin plots illustrating expression levels of selected genes enriched in the over-representation analysis (ORA). (D) Violin plots showing gene expression of macrophage function-related genes in Mac3 and Mac4 subsets under CD and HSD conditions. (E) Violin plots depicting expression levels of selected salt-regulated genes in Mac3 and Mac4 subsets under CD and HSD conditions.

3.5 Severity of skin challenges after chronic high-salt diet

3.5.1 Tape stripping exacerbates skin barrier dysfunction without further increasing epidermal thickness following chronic high-salt exposure

After examining the effects of HSD feeding on skin tissue, as well as macrophage function, morphology, and development, we sought to determine whether an additional skin challenge would exacerbate the impact of chronic high-salt exposure on skin barrier function. Previous studies have demonstrated that sodium accumulation occurs in psoriatic skin and that high-salt consumption increases the risk of inflammatory skin diseases (B. M. Chiang et al., 2024; Maifeld et al., 2022; G. Zhou et al., 2024). To further investigate the connection between high-salt intake and skin inflammation, we applied tape stripping following chronic HSD feeding to induce mechanical disruption of the epidermal barrier (Figure 28). Our results (48 hours post tape stripping) revealed that chronic HSD feeding alone led to an increase in TEWL (+3.8 g/m²/h, already shown in 3.3.2), which was further exacerbated by epidermal barrier disruption (+5.4 g/m²/h, Figure 28A). Additionally, epidermal thickness measurements of back skin showed a significant increase (44.74%) following long-term HSD feeding. This thickening was only slightly further enhanced by the tape stripping intervention (51.48%, Figure 28B).

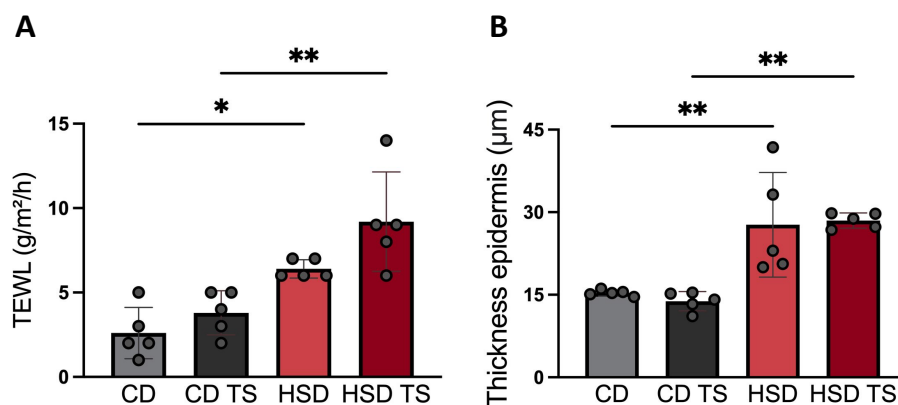


Figure 28: Measurement of transepidermal water loss and epidermal thickness of back skin following chronic HSD feeding and tape stripping.

(A) TEWL measurement of back skin from male mice comparing CD and HSD with or without tape stripping (TS) (n = 5 mice per condition from one mouse cohort). (B) Epidermal thickness measurement from male mice comparing CD and HSD with or without tape stripping (n = 5 mice per condition from one mouse cohort). Data are presented as mean ± SD. Statistical significance was determined using two-way ANOVA.

3.5.2 Abundances of skin macrophage subsets and cell surface markers are only slightly altered after tape stripping following HSD

The tape stripping model has previously been used to study the emigration of Langerhans cells to draining lymph nodes, the immigration of Langerhans cells into the epidermis from potential precursors, and their role in wound healing (Holzmann et al., 2004; Nikolić et al., 2019). We therefore aimed to assess how the abundance of macrophages changes in the dermis, epidermis, and mastoid lymph nodes following chronic HSD feeding and tape stripping (Figure 29). In the dermis, we observed a decrease in MHCII-negative blood vessel-associated macrophages; however, this reduction was not statistically significant (Figure 29A). The abundance of other dermal macrophage subpopulations remained unchanged (Figure 29B-D). We further investigated the impact of mechanical epidermal disruption on Langerhans cells. While the total macrophage numbers in the epidermis remained unaffected (Figure 29E), we observed a trend towards decrease in Langerhans cell numbers in the mastoid lymph nodes following chronic HSD feeding compared to CD conditions (Figure 29F).

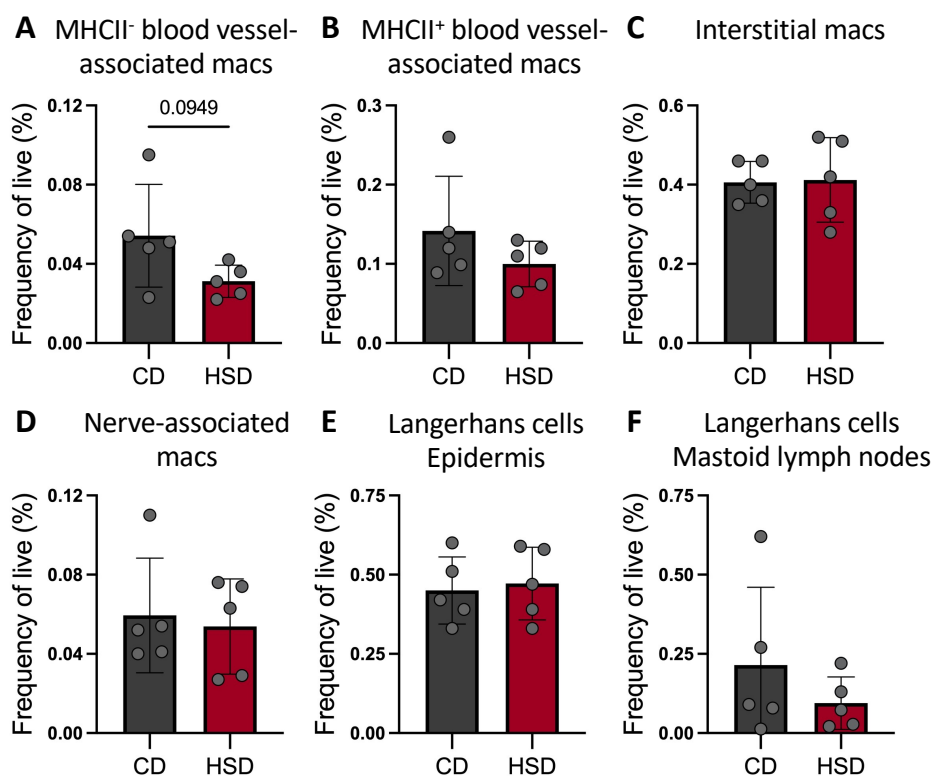


Figure 29: Changes in macrophage proportions in the dermis, epidermis, and mastoid lymph nodes following HSD feeding and tape stripping.

Frequency of live of dermal macrophage subpopulations (A), Langerhans cells in the epidermis (B), and Langerhans cells in the mastoid lymph nodes (C) comparing CD and chronic high-salt conditions 48 hours after epidermal disruption by tape stripping (n = 5 mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was assessed using Student's t-test.

Further, epidermal disruption via tape stripping did not result in significant changes in surface marker expression levels across all skin macrophage subsets (Figure 30). However, a trend towards increased CD32b expression levels was observed in Langerhans cells (Figure 30B).

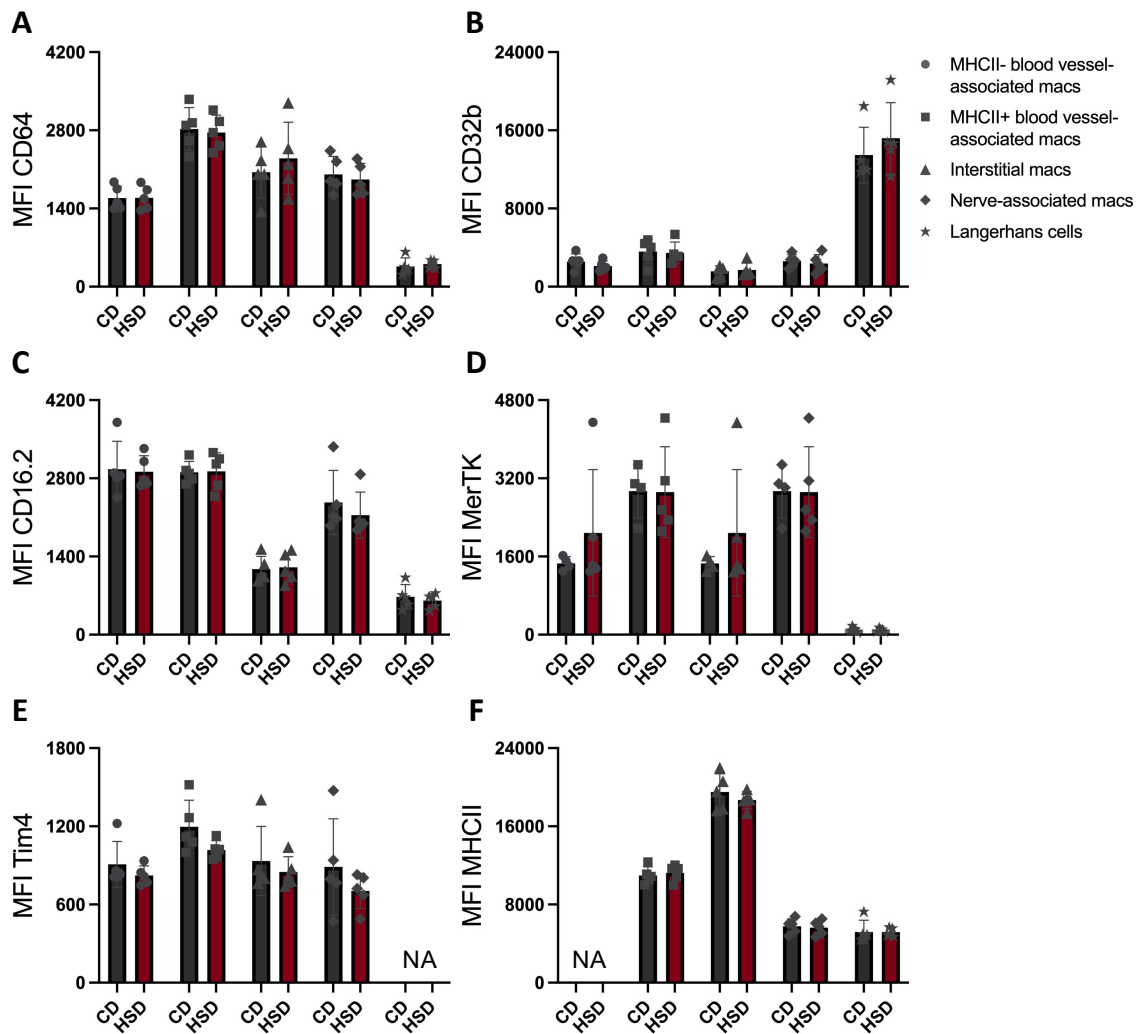


Figure 30: Surface marker profile of skin macrophage subtypes following chronic HSD feeding and tape stripping.

Mean fluorescence intensity (MFI) of markers associated with phagocytosis (CD64 (A), CD32b (B), CD16.2 (C)), efferocytosis (MerTK (D), Tim4 (E)), and antigen presentation (MHCII (F)) in male mice following acute HSD feeding. Marker expression levels were analyzed across all skin macrophage subsets (n = 5 mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was assessed using two-way ANOVA.

3.5.3 Chronic high-salt exposure alters immune reactivity in the contact hypersensitivity model

Contact dermatitis is a widespread allergic skin reaction, affecting 15–20% of the global population (Peiser et al., 2012). The murine hapten-induced contact hypersensitivity (CHS) model is a valuable tool for investigating the underlying immunological mechanisms. The CHS response occurs in distinct

phases: the sensitization phase, where the hapten 1-Fluor-2,4-dinitrobenzol (DNFB) is first introduced, followed by the elicitation phase, in which the hapten is reapplied after one week, triggering a strong inflammatory response with immune cell infiltration (Honda et al., 2013; Schwarz et al., 2023) (Figure 31A). In our study, we aimed to determine whether chronic HSD feeding influences the overall immune response, as well as the reaction and phenotype of skin macrophage subpopulations. Since ear swelling after the first and second elicitation phases serves as an indicator of immune activation, we measured ear thickness over three consecutive days following both the first (Figure 31B) and second (Figure 31C) challenges. Ear swelling was comparable between CD and HSD groups across all three measurement days following the first challenge (Figure 31B). After the second challenge, the ear swelling response exhibited greater variability among individual mice, particularly on the second day of measurement (Figure 31C). However, by the third measurement after the second challenge, ear thickening levels were again comparable between the two dietary groups.

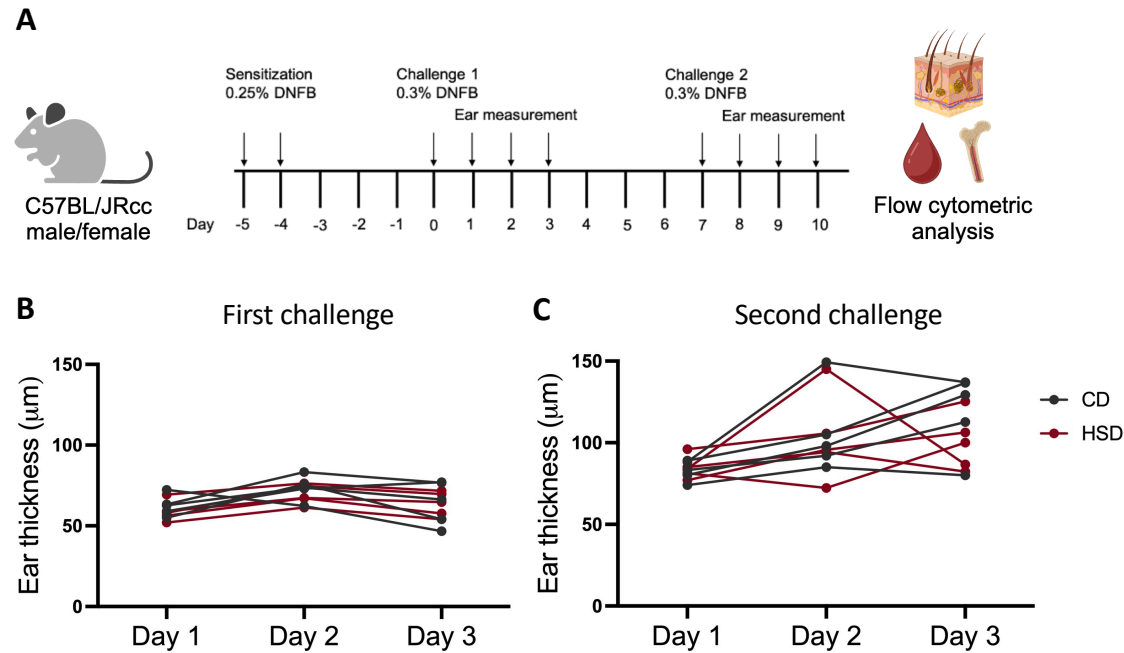


Figure 31: Hapten-induced contact hypersensitivity assay following chronic HSD feeding.

(A) Time scale of the contact hypersensitivity assay including sensitization phase and two challenges, followed by ear measurements. (B-C) Ear thickness measurement after first challenge (B) and second challenge (C). Thickness was measured on 3 consecutive days ($n = 4-5$ mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was determined using two-way ANOVA.

In addition to measuring ear swelling, blood analysis provides further insights into potential immune changes following HSD feeding and CHS challenge. Blood samples were collected on day 3 after the second challenge and analyzed for various cell populations (Figure 32). DNFB treatment resulted in

increased red blood cell (RBC) counts independent of previous exposure to CD or chronic HSD (Figure 32A). In contrast, white blood cell (WBC) counts remained largely unchanged, though there was a slight trend toward an increase in WBCs in the HSD with DNFB group compared to CD with DNFB (Figure 32B). Basophils and eosinophils are known as critical effector cells in allergic responses (Bochner, 2000). Interestingly, basophil numbers significantly increased in the HSD with DNFB group compared to both CD and HSD conditions without DNFB treatment (Figure 32C). Conversely, eosinophil numbers were slightly reduced following CHS in both HSD- and CD-fed mice (Figure 32D). Regarding other immune cell types, lymphocyte numbers showed a trend towards an increase in the HSD with DNFB group compared to CD and HSD without DNFB treatment (Figure 32E). Meanwhile, neutrophil and monocyte counts decreased in both CD- and HSD-fed mice following DNFB treatment (Figure 32F, G).

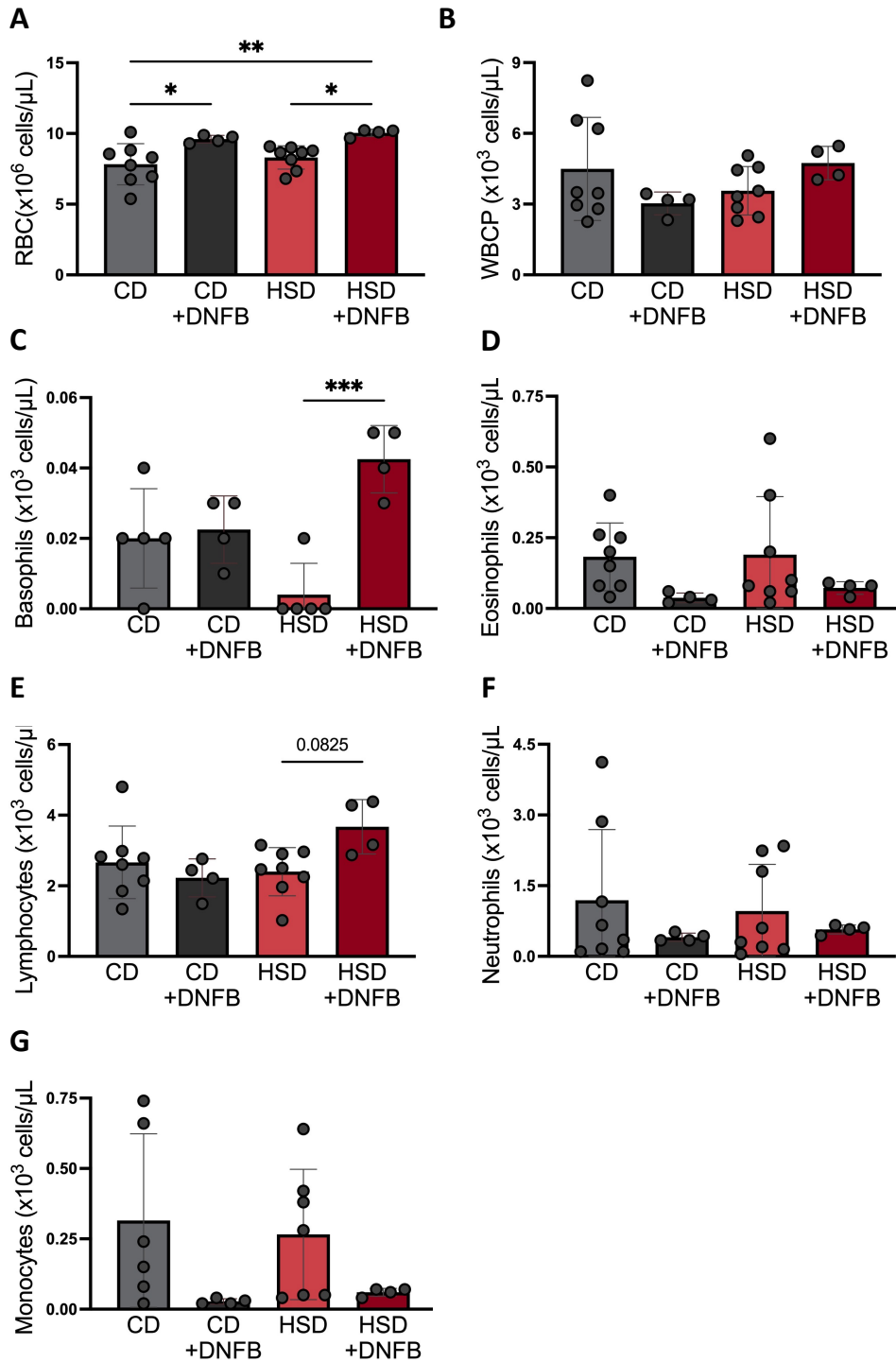


Figure 32: Blood cell counts following chronic HSD feeding and CHS challenge.

Full blood samples were analyzed using a hematology analyzer. DNFB-treated CD and HSD mice were compared to CD and HSD mice from separate cohorts without CHS challenge. (A) Red blood cell count, (B) White blood cell count (peroxidase method) (C) Basophil count, (D) Eosinophil count, (E) Lymphocyte count, (F) Neutrophil count, (G) Monocyte count (n = 4-5 male mice per condition from one mouse cohort for CHS challenge, n = 5-8 male mice per condition from 2 individual mouse cohorts). Data are presented as mean $\pm$ SD. Statistical significance was determined using two-way ANOVA.

3.5.4 CHS challenge following chronic high-salt exposure slightly alters hematopoietic stem and progenitor cell populations

As described earlier, hapten treatment during the elicitation phase of CHS triggers a strong inflammatory response characterized by immune cell infiltration (Honda et al., 2013; Schwarz et al., 2023). These infiltrating cells primarily originate from the bloodstream, where they continuously survey for inflammatory cues. Additionally, tissue-resident immune cells can proliferate or differentiate in response to local inflammatory signals (Parihar et al., 2010). The bone marrow serves as a major source of immune cells, where hematopoiesis generates progenitor populations that give rise to various immune subsets (Figure 33A). Given the ongoing debate regarding the hierarchical organization of hematopoiesis, we adapted a model from recent studies (Ng et al., 2023; Singh & Cancelas, 2020) and employed a flow cytometric gating strategy to delineate distinct progenitor populations based on specific surface markers (Figure 33B). According to the applied model, monocytes are primarily derived from granulocyte-macrophage progenitors (GMPs) but can also originate from macrophage-dendritic progenitors (MDPs) or common monocyte progenitors (cMoPs). All of these progenitors likely arise predominantly from common myeloid progenitors (CMPs), which in turn are derived from multipotent progenitors 3 (MPP3) and 2 (MPP2).

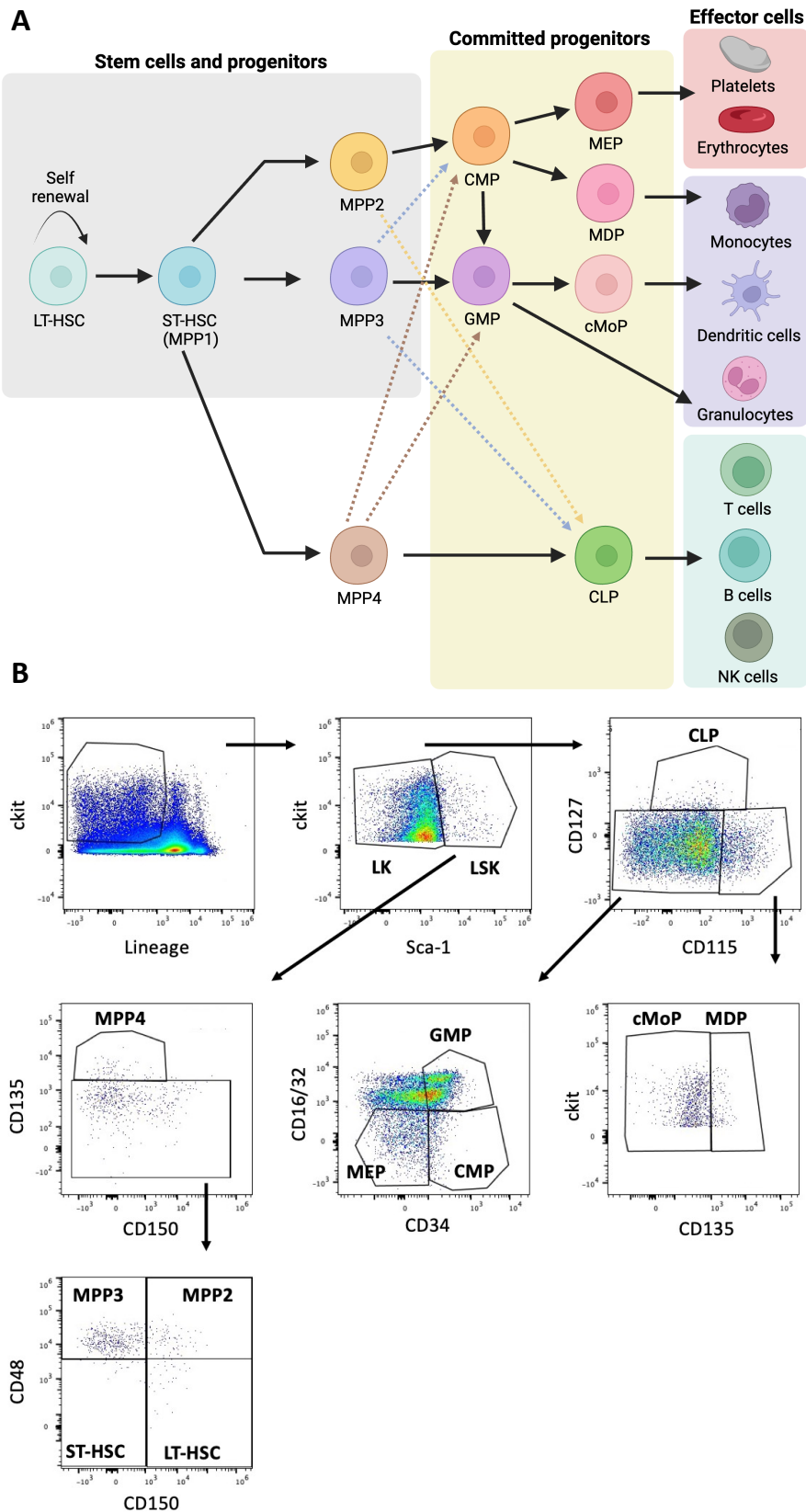


Figure 33: Identification of non-committed and committed progenitor cells in the bone marrow.

(A) Proposed model of hematopoiesis in the bone marrow adapted from Ng et al and Singh & Cancelas. (B) Gating strategy for flow cytometric analysis using specific markers to differentiate bone marrow progenitors.

Our analysis of bone marrow populations following chronic HSD feeding and CHS challenge revealed a trend toward reduced proportions of long-term hematopoietic stem cells (LT-HSCs) and short-term hematopoietic stem cells (ST-HSCs) compared to CD conditions (Figure 34A, B). Among the multipotent progenitor (MPP) subsets, MPP2 and MPP4 exhibited a similar trend of decreased numbers following HSD feeding and DNFB treatment, whereas MPP3 remained unchanged (Figure 34C–E). Within the committed progenitor populations, we observed a decrease in common myeloid progenitors (CMPs) and a comparable trend in macrophage-dendritic progenitors (MDPs) in HSD conditions with DNFB treatment compared to CD (Figure 34F, J). In contrast, common lymphoid progenitors (CLPs), granulocyte-macrophage progenitors (GMPs), megakaryocyte–erythroid progenitors (MEPs), and common monocyte precursors (cMoPs) remained unchanged (Figure 34G, H, I, K).

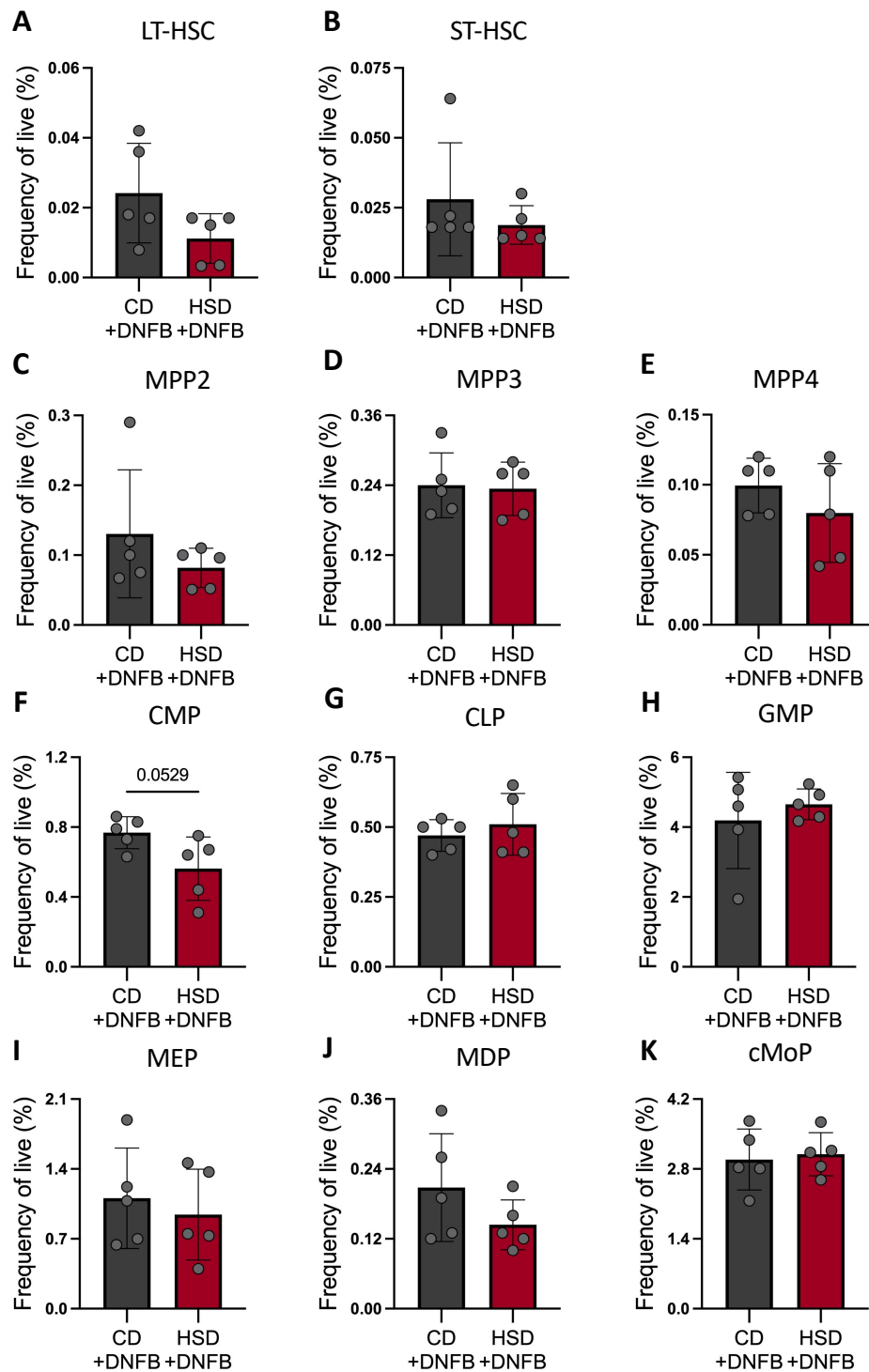


Figure 34: Changes in proportions of bone marrow populations following chronic HSD feeding and CHS challenge.

Flow cytometric analysis of frequency of live of different bone marrow populations following long-term HSD feeding and CHS challenge including LT-HSCs (A), ST-HSCs (B), MPP2 (C), MPP3 (D), MPP4 (E), CMP (F), CLP (G), GMP (H), MEP (I), MDP (J) and cMoP (K) ($n = 5$ mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was assessed using Student's t -test.

3.5.5 Relative numbers of skin macrophage populations are increased after CHS challenge following chronic HSD feeding

Following the analysis of blood and bone marrow population dynamics under chronic HSD feeding and CHS challenge, we investigated potential changes in macrophage subpopulations within the dermis and epidermis (Figure 35). Notably, we observed an increase in all macrophage subpopulations when comparing HSD and HSD + DNFB conditions, with significant differences detected for MHCII⁻ blood vessel-associated macrophages (Figure 35A), MHCII⁺ blood vessel-associated macrophages (Figure 35B), nerve-associated macrophages (Figure 35D), and Langerhans cells (Figure 35F). Interestingly, Langerhans cell numbers also significantly increased in CD + DNFB compared to CD conditions. Despite the overall increase in macrophage numbers under HSD + DNFB conditions, dermal monocyte numbers showed only a minor increase (Figure 35E).

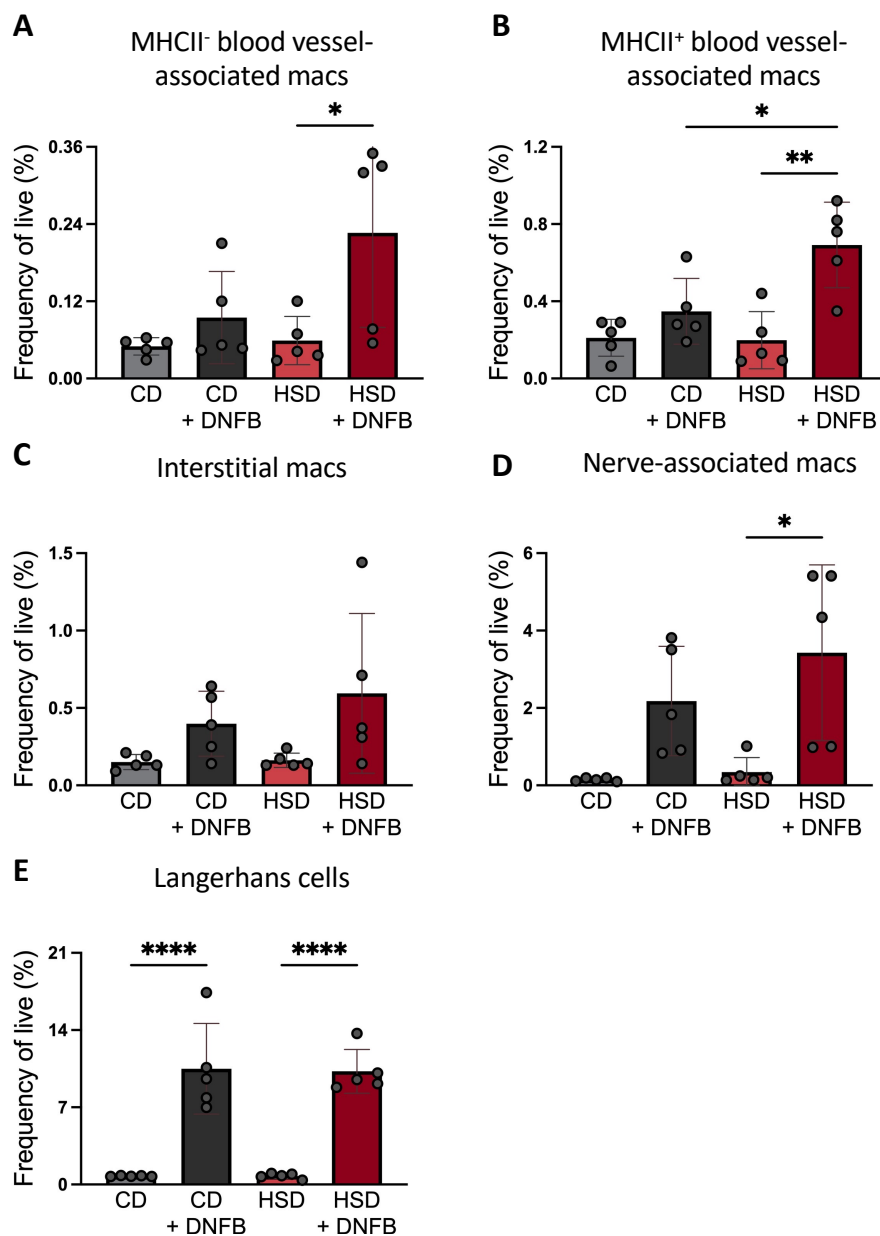


Figure 35: Altered macrophage subpopulations numbers in the skin following chronic HSD feeding and CHS challenge.

Flow cytometric analysis of frequency of live of MHCII⁻ blood vessel-associated macrophages (A), MHCII⁺ blood vessel-associated macrophages (B), interstitial macrophages (C), nerve-associated macrophages (D), dermal monocytes (E) and Langerhans cells (F). Compared conditions were CD, CD with DNFB treatment, HSD and HSD with DNFB treatment (n = 5 mice per condition from one mouse cohort). Data are presented as mean ± SD. Statistical significance was determined using two-way ANOVA.

3.5.6 Chronic high-salt exposure modulates cell surface receptor expression in skin macrophage subtypes following CHS challenge

In addition to changes in skin macrophage subpopulation numbers following chronic HSD feeding and CHS challenge, we examined alterations in the expression levels of cell surface markers associated with phagocytosis, efferocytosis, and antigen presentation (Figures 36-40). Notably, these changes varied across different macrophage subsets. MHCII⁻ blood vessel-associated macrophages exhibited a significant reduction in CD64 expression levels when comparing CD to CD + DNFB conditions, with an even more pronounced decrease under HSD versus HSD + DNFB conditions (Figure 36A). Furthermore, CD32b expression displayed a trend toward increased levels following HSD feeding and DNFB treatment compared to CD + DNFB, although this difference was not significant (Figure 36B). Expression levels of other analyzed markers remained unchanged (Figure 36C–E).

MHCII⁺ blood vessel-associated macs

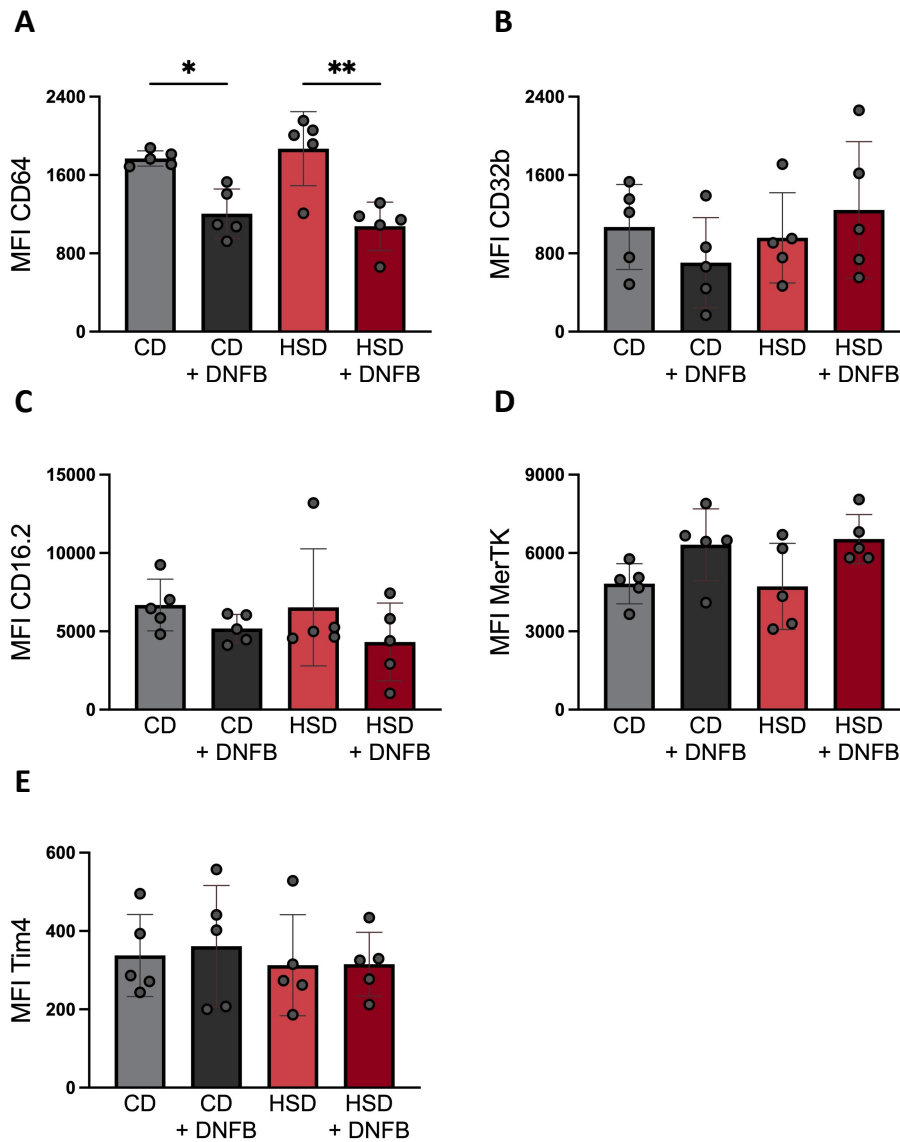


Figure 36: Cell surface marker expression levels of MHCII⁺ blood vessel-associated macrophages following chronic HSD feeding and CHS challenge.

Analyzed cell surface markers were CD64 (A), CD32b (B), CD16.2 (C), MerTK (D) and Tim4 (E). Compared conditions were CD, CD with DNFB treatment, HSD and HSD with DNFB treatment (n = 5 mice per condition from one mouse cohort). Data are presented as mean ± SD. Statistical significance was determined using two-way ANOVA.

In MHCII⁺ blood vessel-associated macrophages, we observed significant alterations in the expression of several analyzed cell surface markers (Figure 37). While CD64 and MHCII expression levels remained unchanged (Figure 37A, F), CD32b MFI was significantly increased exclusively in the HSD + DNFB condition compared to HSD and CD + DNFB (Figure 37B). Additionally, MerTK and CD16.2 expression levels were significantly elevated in DNFB-treated CD and HSD conditions compared to untreated controls, with a more pronounced increase under HSD conditions (Figure 37C, D).

Furthermore, TIM4 MFI was significantly increased under high-salt conditions, specifically in DNFB-treated mice compared to untreated controls (Figure 37E).

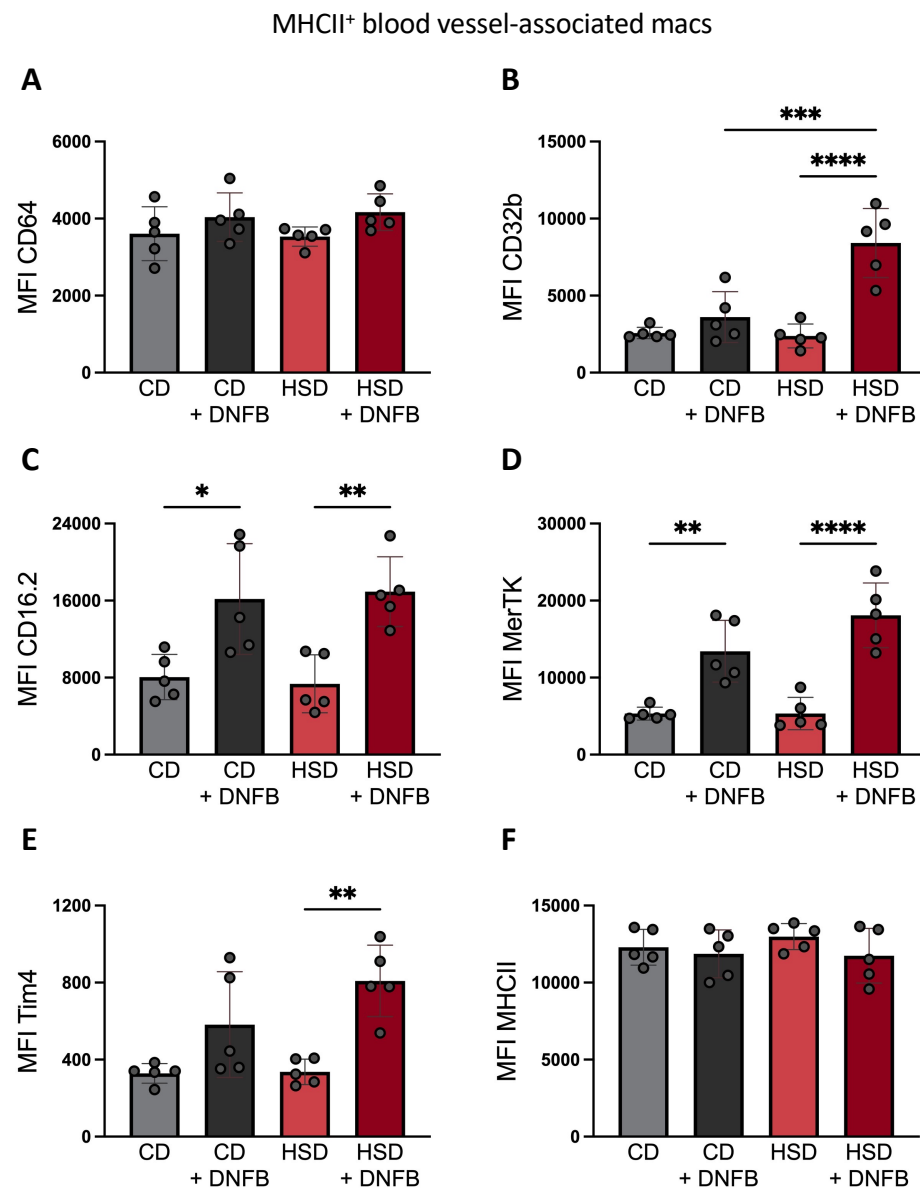


Figure 37: Cell surface marker expression levels of MHCII⁺ blood vessel-associated macrophages following chronic HSD feeding and CHS challenge.

Analyzed cell surface markers were CD64 (A), CD32b (B), CD16.2 (C), MerTK (D), Tim4 (E) and MHCII (F). Compared conditions were CD, CD with DNFB treatment, HSD and HSD with DNFB treatment (n = 5 mice per condition from one mouse cohort). Data are presented as mean ± SD. Statistical significance was determined using two-way ANOVA.

Similar to MHCII⁺ blood vessel-associated macrophages, interstitial macrophages exhibited a significant increase in CD32b expression levels when comparing HSD + DNFB to untreated HSD (Figure 38B). Additionally, the MFI of CD16.2, MerTK, and TIM4 was significantly elevated in HSD + DNFB

compared to untreated HSD, with a similar increase also observed in CD + DNFB relative to CD (Figure 38C, D, F). Furthermore, we detected a trend toward decreased MHCII expression in DNFB-treated CD and HSD conditions, with a significant reduction in MHCII levels when comparing CD to HSD + DNFB (Figure 38F).

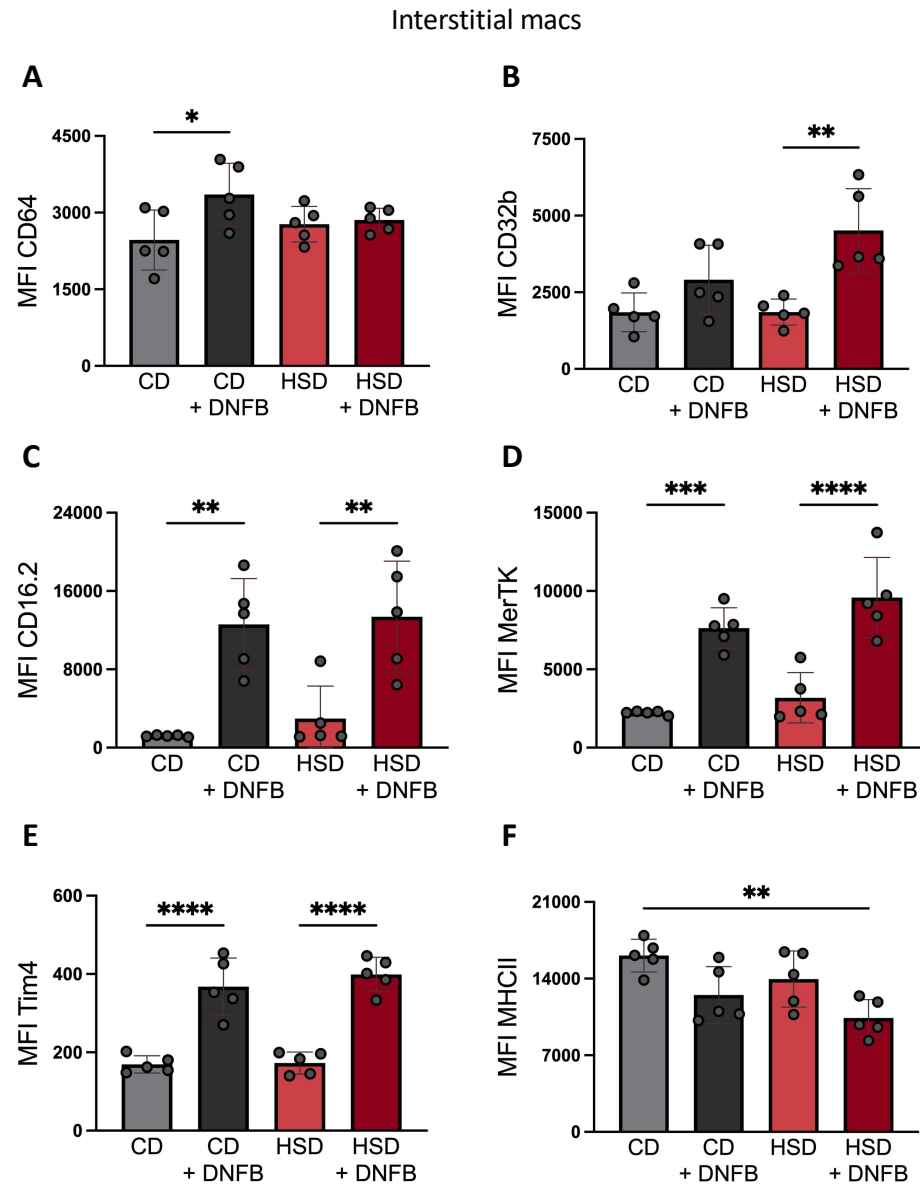


Figure 38: Cell surface marker expression levels of interstitial macrophages following chronic HSD feeding and CHS challenge.

Analyzed cell surface markers were CD64 (A), CD32b (B), CD16.2 (C), MerTK (D), Tim4 (E) and MHCII (F). Compared conditions were CD, CD with DNFB treatment, HSD and HSD with DNFB treatment (n = 5 mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was determined using two-way ANOVA.

Consistent with observations in other dermal macrophage subsets, nerve-associated macrophages exhibited a significant increase in CD32b MFI exclusively in the HSD + DNFB condition compared to

HSD and CD + DNFB (Figure 39B). Additionally, MerTK expression levels were significantly elevated in both CD + DNFB and HSD + DNFB compared to untreated controls, with a more pronounced increase under high-salt conditions (Figure 39D). In contrast to interstitial macrophages, MHCII expression levels were higher in DNFB-treated CD and HSD samples, with a significant increase observed between CD and CD + DNFB (Figure 39F). While CD64 and CD16.2 MFI exhibited only slight increases in DNFB-treated CD and HSD conditions (Figure 39A, C), TIM4 expression was significantly elevated in HSD + DNFB compared to untreated HSD (Figure 39E).

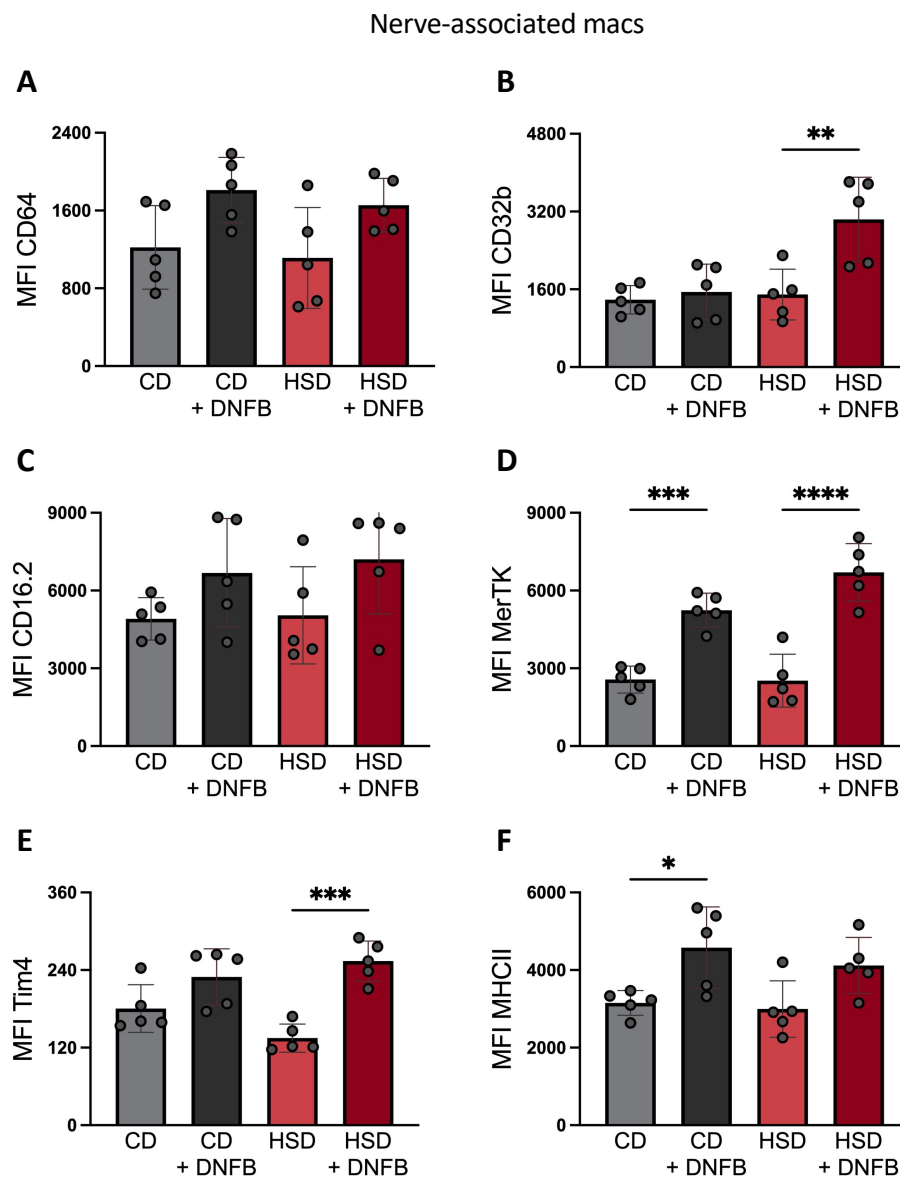


Figure 39: Cell surface marker expression levels of nerve-associated macrophages following chronic HSD feeding and CHS challenge.

Analyzed cell surface markers were CD64 (A), CD32b (B), CD16.2 (C), MerTK (D), Tim4 (E) and MHCII (F). Compared conditions were CD, CD with DNFB treatment, HSD and HSD with DNFB treatment (n = 5 mice per

condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was determined using two-way ANOVA.

In Langerhans cells, MFI levels of CD32b were elevated in both high-salt conditions, as well as in DNFB treated control mice compared to only CD (Figure 40B). Furthermore, CD16.2 expression levels were significantly increased in DNFB-treated CD and HSD samples compared to untreated conditions (Figure 40C). Additionally, we observed a trend towards elevated MerTK expression levels in DNFB-treated samples, with a significant increase in HSD + DNFB compared to CD (Figure 40D). Consistent with findings in interstitial macrophages, MHCII MFI significantly decreased following CHS challenge in both CD and HSD conditions. Notably, even in the absence of DNFB treatment, MHCII expression was significantly lower under high-salt conditions (Figure 40E). CD64 expression levels remained unchanged independent of the diet and DNFB treatment (Figure 40A).

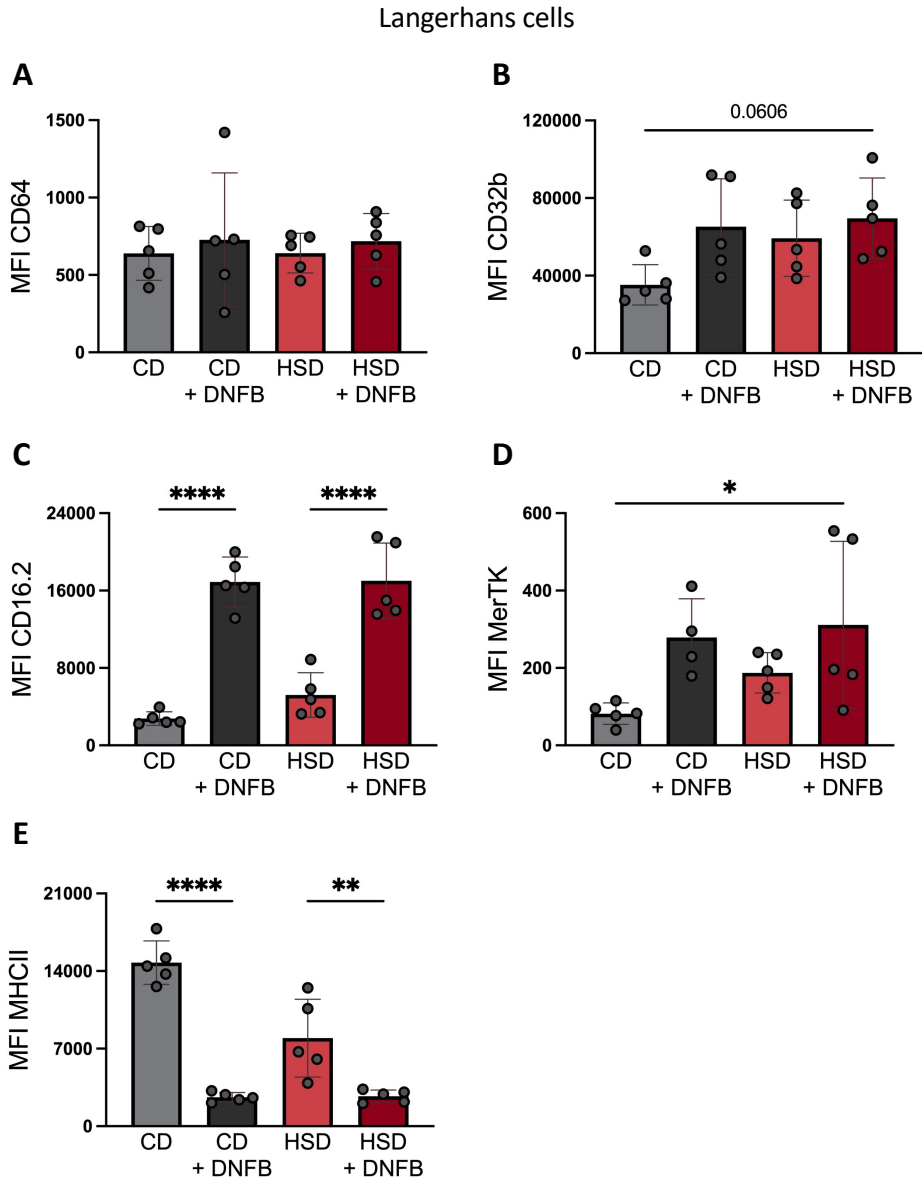


Figure 40: Cell surface marker expression levels of Langerhans cells following chronic HSD feeding and CHS challenge.

Analyzed cell surface markers were CD64 (A), CD32b (B), CD16.2 (C), MerTK (D) and MHCII (E). Compared conditions were CD, CD with DNFB treatment, HSD and HSD with DNFB treatment (n = 5 mice per condition from one mouse cohort). Data are presented as mean $\pm$ SD. Statistical significance was determined using two-way ANOVA.

4. Discussion

4.1 Chronic high-salt intake induces a psoriasis and atopic dermatitis-like skin phenotype in male mice

This study provides a deep, multilevel analysis of the effects of high-salt exposure on murine skin, with a particular focus on the heterogeneous macrophage populations residing in the dermis and epidermis. By employing distinct feeding regimens, we investigated the impact of acute and chronic high-salt conditions on skin barrier integrity, fluid homeostasis and epidermal morphology. Contrary to previous reports (Jantsch et al., 2016; Machnik et al., 2009), our chronic HSD feeding model did not reveal consistent sodium accumulation in skin tissue. Instead, sodium levels varied depending on the anatomical site which was analyzed: while paw skin exhibited a trend towards increased sodium content following chronic high-salt exposure (Figure 7A), sodium levels remained unchanged in the ear (Figure 7B) and were significantly reduced in back skin (Figure 7C). Beyond dietary intake, elevated sodium content in paw skin may result from direct exposure to external sodium sources, as mice frequently use their paws for feeding and come into contact with food particles in their environment. It is possible that externally acquired sodium penetrates the skin barrier, influencing local fluid and ion homeostasis (C. Chiang & Maibach, 2022; Portugal-Cohen et al., 2019). Furthermore, the lower sodium levels observed in the back skin may be linked to the increased TEWL following chronic HSD feeding (Figure 8A, D). Sodium plays a crucial role in maintaining epidermal hydration and skin barrier integrity by influencing keratinocyte function and stratum corneum formation, which is essential for water retention (Hołyńska-Iwan & Szewczyk-Golec, 2020; Mammone et al., 2025). A reduction in skin sodium levels, as observed in the back, could indicate altered epidermal differentiation, potentially leading to impaired barrier function and increased TEWL. Additionally, the osmotic properties of sodium contribute to tissue water retention. A decline in sodium concentrations may reduce water retention, leading to increased epidermal dehydration (Nikpey et al., 2017). Moreover, a study on human keratinocytes demonstrated that dehydration or osmotic stress serves as a signal for keratinocytes to initiate skin barrier differentiation (Mammone et al., 2025). This mechanism could explain the epidermal thickening we observed, particularly in the paw skin of male mice following chronic high-salt exposure (Figure 9B). In addition to promoting differentiation, high-salt conditions have been shown to modulate immune cell proliferation (Li et al., 2022), which may similarly affect keratinocytes. This could account for the trend towards increased numbers of EdU⁺ cells in the epidermis of mice after chronic HSD feeding (Figure 10). The skin phenotype observed under chronic HSD conditions shares key features with inflammatory skin diseases such as psoriasis and atopic

dermatitis, including keratinocyte hyperproliferation, epidermal thickening, increased TEWL and decreased water content of the skin tissue (Nikam et al., 2019; Ortiz-Lopez et al., 2022). These findings indicate that chronic high-salt intake may contribute to or exacerbate inflammatory processes characteristic of these conditions. Further investigations are necessary to elucidate whether such pathological alterations are reversible upon dietary sodium reduction.

4.2 Skin macrophages exhibit niche-specific heterogeneity in receptor expression, function and development

As key immune and homeostatic sentinels, macrophages are capable of detecting and responding to environmental changes, including elevated salt concentrations (Mosser et al., 2021). This makes them a particularly relevant cell type for investigating the impact of high-salt conditions on the skin. Given that the skin is a highly heterogeneous tissue composed of distinct microenvironments, resident macrophages also exhibit substantial phenotypic and functional diversity. To assess the potential influence of high-salt conditions on macrophage subsets in the skin, we first aimed to characterize their phenotype and function in detail. While previous studies have sought to delineate the heterogeneity of skin-resident macrophages, their findings have been quite divergent. Kolter and colleagues identified three macrophage subpopulations in the dermis, distinguished by their Cx3cr1 expression. They characterized Cx3cr1^{int} cells as interstitial macrophages and described a Cx3cr1^{high} nerve-associated subset that is prenatally seeded, self-maintaining, and exhibits minimal postnatal replenishment from hematopoietic stem cells (Kolter et al., 2019). In contrast, Chakarov and his colleagues proposed a broader classification, distinguishing only two macrophage subsets across multiple tissues: nerve-associated and blood vessel-associated macrophages (Chakarov et al., 2019).

Based on these previous findings and our own experimental data, we identified five distinct macrophage subsets in murine skin: epidermal Langerhans cells, interstitial macrophages, MHCII⁺ and MHCII⁻ blood vessel-associated macrophages, and nerve-associated macrophages (Figure 11, Figure 27). The distinction between MHCII⁺ and MHCII⁻ blood vessel-associated macrophages is a novel and surprising finding of our study. As we documented the existence of these two populations both by flow cytometric analysis (Figure 12) and by scRNA-seq (Figure 27A, B), we are confident about this new characterization of skin macrophage heterogeneity. Occupying the same niche in close proximity to blood vessels, MHCII⁺ and MHCII⁻ blood vessel-associated macrophages share many similarly expressed genes, including those encoding various important cell surface receptors. They can be broadly characterized as CD163⁺CD206⁺CD86⁺CD14⁺ macrophages, however, these two subsets differ in functionally relevant markers. MHCII⁺ blood vessel-associated macrophages exhibit high

expression of genes related to antigen presentation (CD74, H2-Ab1) as well as genes related to monocyte recruitment, migration and macrophage-mediated inflammation (CCR2, Srgn, Etv6). One might speculate that these macrophages are motile, pro-inflammatory cells with a primary role in antigen surveillance and presentation in the vicinity of blood vessels (Boniakowski et al., 2019; Xiong et al., 2022; Y. Zhang et al., 2024). In contrast, MHCII⁻ blood vessel-associated macrophages show elevated expression of genes associated with phagocytosis (Fcgr1, Fcgr3) and efferocytosis (MerTK) (Figure 27B). This suggests that this subclass of macrophages possibly consists of rather non-motile cells, which remain stationed along the blood vessels to carry out effector functions locally. While extensive research has characterized interstitial macrophages in organs like the lung, the existence of an interstitial macrophage subset in the skin is less well defined and was only briefly previously mentioned by Kolter and her colleagues (Kolter et al., 2019; Shi et al., 2021). According to our flow cytometric and scRNA-seq results, dermal interstitial macrophages can be characterized as CD74⁺MHCII⁺CD9⁺ macrophages (Figure 11B, Figure 27B). As these cells reside within the skin interstitium and are not in close proximity to blood vessels or nerves, they do not express marker genes typically associated with these structures (Figure 13, Figure 27B). Notably, our functional assays revealed that interstitial macrophages exhibit the highest efferocytic activity among all skin macrophage subsets (Figure 14C). Furthermore, their expression of CD74 and MHCII suggests a role in antigen-presentation, while our scRNA-seq results indicate that they may adopt an inflammatory phenotype characterized by a high IL1 β expression (Figure 27, Mac6). Nerve-associated macrophages likely constitute the smallest macrophage subset in the skin (Kolter et al., 2019). Flow cytometric analysis identified them by their high expression of CX3CR1, though CX3CR1 gene expression was not strongly detected in our scRNA-seq dataset (Figure 27B). According to our flow cytometric data, nerve-associated macrophages can be broadly defined as CX3CR1^{high}MHCII⁺CD14^{int}CD9⁺ cells. Our functional assay indicated that the subset shows rather low phagocytic and efferocytic activity (Figure 14, A, C) and given their MHCII expression, besides homeostatic functions, they might primarily play a role in antigen surveillance and presentation (Figure 11B). Contrary to the findings of Kolter et al. (2019), our fate-mapping analysis revealed a dynamic turnover of nerve-associated macrophages, with over 80% of the population being monocyte-derived in adulthood (Figure 15D). These divergent results may be attributed, in part, to the use of a dual fate-mapping mouse model in our study, which enables the simultaneous labeling of both EMP-derived macrophages and GMP-derived monocytes, thereby providing greater accuracy in lineage tracing. Additionally, our analysis included both, earlier developmental stages and extended time points up to one year of age, offering a more comprehensive view of macrophage ontogeny over time. Lastly, Langerhans cells are the only macrophages localized in the epidermis (Figure 13). Historically, they were mistakenly classified as

dendritic cells due to their ability to migrate to lymph nodes and stimulate T cells (Doebel et al., 2017). However, fate-mapping experiments conducted only a decade ago revealed that Langerhans cells are fetal-derived macrophages that self-maintain in the tissue with minimal contribution from cells with hematopoietic background (Gomez Perdiguero et al., 2015). This finding was also confirmed in our experiments using the double fate-mapper mouse model (Figure 15F). Overall, our results suggest a strong similarity between Langerhans cells and interstitial macrophages in the dermis, as they share several surface markers (CD74, MHCII, CD9) and primarily originate from fetal progenitors throughout life (Figure 15C, F). Still, Langerhans cells are clearly distinguishable by their epidermal niche and the expression of CD326 and CD207, which serve as defining cell surface markers (Figure 27). Overall, our findings suggest that the skin harbors multiple distinct macrophage subsets, reflecting the diversity of local niches. These subsets not only differ in localization but also exhibit distinct surface marker profiles and functional specializations. Furthermore, while all macrophage subsets predominantly originate from fetal-derived cells at birth, their degree of replenishment by cells with hematopoietic origin varies significantly over time (Figure 15). Future experiments should focus on validating flow cytometric and scRNA sequencing results through histological and functional analysis to confirm the relationship between tissue niche and the surface markers expressed by respective macrophage subsets.

4.3 Acute and chronic high-salt exposure induce phenotypic and functional remodeling of skin macrophage subpopulations

Given the heterogeneity in skin macrophage subsets, we aimed to determine whether high-salt conditions influence their phenotype, development and function in distinct ways. Comparing acute and chronic high-salt exposure, respectively, we observed phenotypical and functional differences in skin macrophages of male mice. Notably, there was a general trend towards an increase in abundances of certain macrophage subpopulations, particularly in blood vessel-associated macrophages following acute HSD feeding (Figure 17A). Previous studies have suggested that high-salt conditions influence immune cell proliferation as well as macrophage differentiation and activation (Berga-Bolaños et al., 2010; Li et al., 2022; W. C. Zhang et al., 2015). In particular, short-term high-salt exposure appears to induce inflammatory responses in immune cells, including macrophages, potentially increasing the number of circulating and infiltrating monocytes (X. Zhou et al., 2013). It would be interesting to investigate whether the observed increase in skin macrophage abundances following acute high-salt exposure results from monocyte recruitment or whether salt-induced proliferation of tissue-resident macrophages plays a role in the skin.

Interestingly we found, that chronic HSD feeding led to the opposite effect, showing a trend towards decreasing quantities for all skin macrophage subsets, particularly in nerve-associated macrophages (Figure 18C). To date, there is only little knowledge about the long-term effects of high-salt intake on the immune cells in our skin. One possible explanation for the reversed effect of a chronic high-salt intake on macrophage abundances could be, that the initial stimulatory effect of the excess salt observed under acute exposure diminishes over time as cells potentially adapt to increased salt conditions. Prolonged exposure to excess salt may also lead to exhaustion of tissue-resident macrophages as well as circulating monocytes, which could explain the reduced macrophage numbers observed after long-term HSD feeding. Our fate-mapping results support this hypothesis, as most macrophage subpopulations did not exhibit an increased proportion of monocyte-derived cells following chronic HSD feeding (Figure 25). However, interstitial macrophages appeared more sensitive to chronic high-salt conditions, showing a significant reduction in fetal-derived cells when comparing HSD to CD (Figure 25C). As previous studies showed that excess salt is potentially stored in the interstitial connective tissue of the skin, interstitial macrophages might be affected more strongly by long-term high-salt conditions than the other macrophage subsets (Titze et al., 2003). Notably, the effects of acute and chronic HSD feeding on macrophage abundances in our study were only observed in male mice, but not in females. Prior investigations by other scientists have shown that the immune system differs significantly between male and female mice, with females exhibiting an altered immune cell composition, including higher numbers of T- and B-lymphocytes as well as macrophages. This distinct immune phenotype is associated with a more efficient acute immune response in females, which may contribute to their enhanced resilience to acute high-salt exposure (Scotland et al., 2011). In addition to changes in macrophage subset abundances, we observed significant alterations in surface markers in male mice following acute HSD feeding (Figure 19). Each macrophage subset exhibited distinct changes in functional marker expression levels, yet a notable shared feature among all dermal macrophages was a significant increase in expression levels of the inhibitory phagocytosis receptor CD32b (Figure 18B). Recent studies have shown that CD32b upregulation inhibits macrophage activation and is associated with an anti-inflammatory polarization (Chuang et al., 2024; Ocaña-Guzman et al., 2018). Given that acute high-salt exposure appears to stimulate macrophage differentiation and activation, the upregulation of CD32b may serve as a compensatory regulatory mechanism to counterbalance this effect. Notably, following chronic HSD feeding, cell surface receptor expression levels remained unchanged, further suggesting that macrophages are able to adapt to prolonged high-salt conditions over time (Figure 21). Moreover, consistent with our findings regarding macrophage subset abundances, female mice exhibited only minor changes in surface marker expression levels following both acute and chronic high-salt

exposure (Figure 20,22). This supports the hypothesis that the altered immune cell composition in female mice compensates for the modulatory effects of high-salt conditions. To investigate the functional modulation of skin macrophages following chronic HSD feeding, we performed phagocytosis and efferocytosis assays using isolated macrophages from male mouse skin (Figure 26). Consistent with the minimal changes observed in surface marker expression levels, phagocytosis and efferocytosis rates decreased only slightly across all skin macrophage subsets. However, it would be interesting to explore whether more pronounced changes in macrophage functionality would occur after acute high-salt exposure.

4.4 Blood-vessel associated macrophages are most markedly affected by high-salt conditions undergoing pronounced phenotypic and transcriptional remodeling

Blood vessels extend into the dermis, playing a crucial role in immune regulation, as well as nutrient and metabolite circulation (Pappano & Wier, 2013). As salt is transported via the bloodstream, blood vessel-associated macrophages are among the first immune cells exposed to excess salt concentrations. We observed that both acute and chronic high-salt exposure shifts this macrophage population towards a higher fraction of MHCII⁺ cells, suggesting that antigen presentation may be downregulated in a high-salt environment (Figure 23). This shift could favor MHCII⁺ blood vessel-associated macrophages which, according to our scRNA-seq data, primarily exhibit effector functions like phagocytosis and efferocytosis (Figure 27B). Interestingly, following short-term HSD feeding, we detected a significant increase in MHCII expression levels among MHCII⁺ blood vessel-associated macrophages, which could represent a compensatory response to changes in population distribution (Figure 19F). Another possibility might be, that macrophages with intermediate MHCII expression shed their receptors in response to high-salt conditions, leading to an apparent increase in MHCII⁺ macrophages, while the remaining MHCII⁺ population exhibits higher MHCII expression levels. In addition to changes in macrophage distribution, blood vessel-associated macrophages exhibited altered surface marker expression following acute HSD feeding (Figure 19). Specifically, in MHCII⁺ blood vessel-associated macrophages, we observed an increase in CD64, CD32b and Tim4 expression levels, further supporting the hypothesis that acute high-salt exposure enhances phagocytic and efferocytic activity. In contrast, MHCII⁺ blood vessel-associated macrophages did not upregulate CD64 expression under acute high-salt conditions. Given that CD64 is highly expressed on activated macrophages in inflamed areas (Kiekens et al., 2000; Van de Winkel & Anderson, 1991), this finding suggests that MHCII⁺ blood vessel-associated macrophages might be more reactive to high-salt

conditions than their MHCII⁺ counterparts. In our scRNA-seq analysis, blood vessel-associated macrophage subsets exhibited the most pronounced transcriptomic alterations compared to other skin macrophage populations following chronic high-salt exposure. Over-representation analysis of differentially expressed genes revealed significant enrichment of genes associated with VEGF signaling pathways in one or both of the blood vessel-associated macrophage subsets (Mac3 and Mac4) (Figure 27C). Previous studies have linked high-salt conditions in the skin to activation of TonEBP in macrophages, which subsequently binds to the VEGF-C promoter and induces VEGF-C secretion, serving as a buffering mechanism to regulate salt-dependent tissue volume and blood pressure (Machnik et al., 2009). Notably, while earlier studies only investigated acute high-salt exposure (2 weeks), our findings suggest that this transcriptional response persists under chronic high-salt conditions. Furthermore, we found that the Mac4 subset, identified as MHCII⁻ blood vessel-associated macrophages, exhibited significantly reduced gene expression of the efferocytosis receptor *Mertk* (Figure 27D). A slight decrease in efferocytotic capacity was also observed in our functional assay (Figure 26). However, this analysis did not distinguish between MHCII⁺ and MHCII⁻ blood vessel-associated macrophages, presenting an intriguing avenue for future experiments.

Notably, we also discovered a significant downregulation of *Wnk1* gene expression (Figure 27E). WNK1 is a serine/threonine kinase that plays a central role in ion homeostasis, particularly in regulation of sodium (Na⁺), potassium (K⁺) and chloride (Cl⁻) transport (Piala et al., 2014). It is a key component of a salt-sensing signaling cascade that responds to changes in osmolarity and intracellular Cl⁻ concentrations (Yin et al., 2024). Importantly, WNK1 is also closely linked to blood pressure regulation, suggesting a potential connection to the observed enrichment of VEGF signaling-related genes in blood vessel-associated macrophages (Bergaya et al., 2011). In the context of a high-salt environment, it is known that WNK1 activity can be inhibited by high intracellular Cl⁻ and it can contribute to cell volume regulation by helping the cells to respond to hyperosmotic stress by modulating transporters and restoring osmotic balance. The significant reduction of WNK1 gene expression might be a response to increased Cl⁻ levels in the macrophages following chronic high-salt exposure, potentially leading to compromised cell volume regulation. This hypothesis is further supported by our morphological analyses, albeit conducted under acute high-salt conditions, which revealed pronounced alterations specifically in blood vessel-associated macrophages, while other macrophage subsets remained unaffected (Figure 24). In these cells, we observed a significant reduction in both, cell volume (Figure 24A) and surface area (Figure 24B). Those changes may be indicative of cellular hyperosmotic stress, a condition characterized by elevated extracellular osmolarity (>300 mOsm/kg H₂O), resulting in oxidative stress, water efflux and subsequent cellular

shrinkage (Bkaily et al., 2021). Taken together, these observations raise the intriguing possibility of a functional link between enhanced VEGF signaling, reduced WNK1 activity and macrophage morphological changes under high-salt conditions. Further mechanistic investigations are warranted to explore this interplay in more detail.

4.5 Skin barrier disruption and allergic inflammation exacerbate the effects of chronic high-salt exposure on immune responses and macrophage subset phenotypes

In this study, we utilized skin barrier disruption via tape stripping and allergic inflammation via CHS as skin disease models to examine the impact of chronic high-salt intake on skin tissue integrity and immune cell responses. Notably, the increase in transepidermal water loss (TEWL), observed after chronic HSD feeding alone, was further exacerbated following epidermal disruption by tape stripping (Figure 28A). However, this increase in TEWL did not coincide with additional changes in epidermal thickness (Figure 28B). This result is in line with, and further supports our previous findings that chronic high-salt exposure compromises skin barrier integrity, leading to reduced tissue water content (Figure 8). Combined with potential keratinocyte hyperproliferation (Figure 9, 10), these effects suggest that long-term high-salt exposure increases susceptibility to barrier disruption of the epidermis. As a consequence, we also detected a decreased abundance of Langerhans cells in the mastoid lymph nodes following chronic HSD feeding and tape stripping, while the numbers of Langerhans cells in the epidermis remained unchanged (Figure 29E, F). This result could suggest that epidermal barrier disruption following long-term high-salt conditions leads to less migration of Langerhans cells to mastoid lymph nodes and thereby possibly also results in less antigen presentation.

Finally, our study identified pronounced immune changes following chronic HSD feeding and allergic inflammation. Using a CHS assay, we assessed cell-mediated immune function by exposing epidermal and dermal cells to exogenous haptens, which triggered a delayed-type hypersensitivity (DTH) reaction, allowing for quantification and measurement (Gaspari et al., 2016). One indicator of immune response strength is ear thickness following hapten challenge. Surprisingly, after both, the first and the second challenge, ear thickness was simultaneously increased in CD and HSD-fed mice, suggesting a comparable local immune response to the CHS assay (Figure 31C). Blood analysis revealed increased basophile and lymphocyte counts following HSD feeding and CHS challenge but decreased eosinophil, neutrophil, and monocyte counts in both control and HSD groups (Figure 32). Given that CHS is T cell-mediated inflammation and basophils play a key role in allergic responses,

the observed increase in the two populations in the blood was expected (Akiba et al., 2002) although it is noteworthy that excess salt further amplified this effect. The observed decrease in blood monocyte levels following CHS challenge could be attributed to their rapid recruitment to the inflamed tissue, a process that often results in transient monocytopenia before monopoiesis in the bone marrow restores monocyte numbers (Kawamura & Ohteki, 2018). However, this transient decrease was not observed in the direct progenitors of monocytes in the bone marrow (Figure 33H, J, K). Instead, a reduction in CMPs, which can give rise to GMPs, was detected, potentially reflecting an increased demand for circulating monocytes (Figure 33F). Notably, as previously observed under acute high-salt conditions, we noticed that abundances of most skin macrophage subsets significantly increased in HSD-fed, DNFB-treated mice (Figure 35). It remains to be determined whether the increased macrophage abundance results from elevated monocyte infiltration or if it also reflects enhanced local macrophage proliferation. Moreover, functional cell surface marker expression levels varied heterogeneously across the different skin macrophage subsets (Figures 36–40), reinforcing the idea that high-salt exposure alters skin macrophage phenotype and function. Interestingly, CD32b expression was modulated again in most skin macrophage subsets, underscoring its key role in macrophage response under inflammatory conditions. Since chronic HSD alone did not elevate CD32b expression levels (Figure 21B), it is likely that CD32b upregulation in skin macrophages acts as a feedback mechanism in response to acute inflammation. Supporting this hypothesis, several phagocytosis- and efferocytosis-related receptors were upregulated across all macrophage subsets (Figures 36–40), emphasizing the stimulating effect of salt in combination with acute inflammation. Interestingly, Langerhans cells exhibited significantly reduced MHCII expression under high-salt conditions, even without additional CHS challenge, and this reduction was further exacerbated by DNFB treatment (Figure 40E). This aligns with the decreased Langerhans cell numbers in mastoid lymph nodes following tape stripping (Figure 29C), supporting the idea that chronic high-salt exposure - both following barrier disruption and during allergic inflammation - may impair Langerhans cell migration and antigen presentation. Overall, the alterations in the skin macrophage compartment observed following chronic HSD combined with allergic inflammation resembled the phenotype previously observed under acute high-salt conditions. This raises the possibility that chronic high-salt intake may prime macrophages towards a pro-inflammatory phenotype, which could be more rapidly activated upon additional acute inflammatory stimuli.

4.6 Outlook

This work describes the heterogeneity of macrophages in murine skin and explores the impact of acute and chronic high-salt intake on skin tissue integrity, macrophage phenotype and function. We

characterized macrophage subpopulations based on cell surface marker expression, tissual niche, developmental origin and functional profile, which allowed us to better interpret the changes observed under acute and chronic high-salt conditions. Our feeding regimen led to profound alterations in skin integrity, including increased TEWL, reduced water content, and thickened epidermis. These effects were further exacerbated by epidermal barrier disruption. With respect to skin macrophages, acute high-salt exposure induced notable phenotypic changes, including increased relative cell numbers, enhanced functional surface marker expression levels and a reduction in volume and surface area of blood vessel-associated macrophages. Interestingly, these changes were compensated under chronic HSD feeding but reappeared following an acute inflammatory challenge using the CHS assay. For future studies, it would be valuable to further investigate the relationship between macrophage phenotype and function in the context of high-salt exposure and inflammation. One approach could be to repeat skin challenge experiments in mice after acute HSD feeding to determine whether macrophages exhibit an even stronger inflammatory phenotype. Additionally, CD32b receptor dynamics should be explored in greater detail, as its expression was strongly modulated under high-salt conditions, particularly during acute inflammation. Moreover, it remains an open and compelling question whether the phenotypic and functional changes in macrophages observed following acute high-salt exposure are mechanistically linked to the psoriasis-like skin phenotype identified after chronic high-salt intake. Given the pronounced effects observed in blood vessel-associated macrophages, their role in the high-salt environment should be explored further, especially the connection to WNK1 and VEGF signaling. Ultimately, gaining deeper insights into how high-salt exposure influences macrophages and other immune cells in the skin could pave the way for clinical applications, such as targeted interventions for inflammatory skin diseases. If studies like this continue to raise awareness about the systemic effects of high-salt intake, we may eventually prevent the development of certain diseases through dietary and therapeutic strategies.

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