

# **Characterization of human dermal macrophages in the context of aging**

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## Summary

Macrophages are the most abundant immune cell population in the human dermis. They are critical regulators of tissue homeostasis and regeneration and play an important role in pathogen defense. Via phagocytosis they can eliminate pathogens, dysfunctional cells and other tissue components. They can enhance an immune response by the secretion of pro-inflammatory cytokines but are also crucial during inflammation resolution via the secretion of anti-inflammatory factors. Each tissue has a unique macrophage population with a tissue-specific phenotype that is shaped by the tissue environment. Skin aging is accompanied by reduced regenerative capacity and chronic low-grade inflammation. Macrophages are suggested to contribute to this imbalance of pro- and anti-inflammatory molecules, also called the inflammaging process. Alterations in macrophage number and phenotype have not yet been described for human skin. Therefore, the aim of this thesis was to study dermal macrophages in the context of skin aging to reveal a better understanding of the relation between impaired processes in the aged skin and aged macrophages and to deduce new perspectives for intervention strategies to prevent age-associated skin disorders.

In this study, scRNA-seq and immunohistochemistry analysis demonstrated that macrophage numbers increased in human aged skin. Analysis of differentially expressed genes between young and aged skin macrophages revealed that skin macrophages exhibited a more pro-inflammatory phenotype upon age. Genes shown upregulated in aged skin macrophages were reported to be highly expressed in skin MDMs defined by Reynolds et al. 2021. This led to the suggestion that MDMs replace skin-resident macrophages over time. Furthermore, the upregulated genes indicate a more pro-inflammatory M1 phenotype of dermal macrophages in aged skin. In contrast, *in vitro* aged MDMs had an impaired ability to differentiate to the M1 phenotype. Using co-culture experiments with aged or senescent dermal fibroblast, the most abundant cell type in the human dermis, it was demonstrated, that the aged microenvironment polarizes the macrophages to a more pro-inflammatory phenotype in the skin. This emphasizes the role of the aged microenvironment on the development of altered macrophage phenotype in aged skin tissue. Pro-inflammatory M1 MDMs in turn impacted the fibroblast phenotype in its metabolic age, ECM remodeling and inflammatory state. Thus, the imbalance of pro-inflammatory M1 and anti-inflammatory M2 macrophages found in aged human skin tissue could contribute to alterations of aged skin phenotype and the development of skin disorders.

## Zusammenfassung

Makrophagen sind die am häufigsten vorkommende Immunzellpopulation in der menschlichen Dermis. Sie sind entscheidende Regulatoren der Gewebekomöostase und -regeneration und spielen eine wichtige Rolle bei der Abwehr von Krankheitserregern. Durch Phagozytose können sie Krankheitserreger, funktionsgestörte Zellen und andere Gewebekomponenten beseitigen. Sie können eine Immunreaktion durch die Sekretion von entzündungsfördernden Zytokinen verstärken, sind aber auch bei der Beendigung einer Entzündung durch die Sekretion von entzündungshemmenden Faktoren von entscheidender Bedeutung. Jedes Gewebe verfügt über eine einzigartige Makrophagenpopulation mit einem gewebespezifischen Phänotyp, der durch die Gewebenumgebung geprägt ist. Die Hautalterung geht mit einer verminderten Regenerationsfähigkeit und einer chronischen, geringgradigen Entzündung einher. Es wird angenommen, dass Makrophagen zu diesem Ungleichgewicht von entzündungsfördernden und entzündungshemmenden Molekülen beitragen, das auch als Inflammaging-Prozess bezeichnet wird. Veränderungen der Makrophagenanzahl und des Makrophagenphänotyps sind für die menschliche Haut bisher nicht beschrieben worden. Ziel dieser Arbeit war es daher, dermale Makrophagen im Zusammenhang mit der Hautalterung zu untersuchen, um ein besseres Verständnis des Zusammenhangs zwischen gestörten Prozessen in der gealterten Haut und gealterten Makrophagen zu erlangen und neue Perspektiven für Interventionsstrategien zur Verhinderung altersbedingter Hauterkrankungen abzuleiten.

In dieser Studie zeigten scRNA-seq und immunhistochemische Analysen, dass die Anzahl der Makrophagen in gealterter Haut zunimmt. Die Analyse der unterschiedlich exprimierten Gene zwischen jungen und gealterten Hautmakrophagen ergab, dass Hautmakrophagen mit zunehmendem Alter einen stärker entzündungsfördernden Phänotyp aufweisen. Es wurde berichtet, dass Gene, die in gealterten Hautmakrophagen hochreguliert sind, auch in den von Reynolds et al. 2021 definierten Haut-MDMs stark exprimiert werden. Dies führte zu der Vermutung, dass MDMs im Laufe der Zeit die in der Haut lebenden Makrophagen ersetzen. Darüber hinaus deuten die hochregulierten Gene auf einen eher proinflammatorischen M1-Phänotyp der dermalen Makrophagen in gealterter Haut hin. Im Gegensatz dazu hatten gealterte MDMs *in vitro* eine geringere Fähigkeit, sich in den M1-Phänotyp zu differenzieren. Mit Hilfe von Co-Kultur-Experimenten mit gealterten oder seneszenten dermalen Fibroblasten, dem häufigsten Zelltyp in der menschlichen Dermis, konnte gezeigt werden, dass die gealterte Mikroumgebung die Makrophagen in der Haut zu einem eher entzündungsfördernden Phänotyp polarisiert. Dies unterstreicht die Rolle der gealterten



Mikroumgebung bei der Entwicklung eines veränderten Makrophagen-Phänotyps in gealtertem Hautgewebe. Die pro-inflammatorischen M1-Makrophagen wiederum beeinflussten den Fibroblasten-Phänotyp in seinem metabolischen Alter, ECM-Umbau und Entzündungszustand. Das Ungleichgewicht zwischen pro-inflammatorischen M1- und anti-inflammatorischen M2-Makrophagen, das in gealtertem menschlichem Hautgewebe gefunden wurde, könnte also zu Veränderungen des Phänotyps gealterter Haut und zur Entwicklung von Hauterkrankungen beitragen.

## List of Abbreviations

%	percent
°C	degree Celsius
μL	microliter
μM	micromolar
AMP	antimicrobial peptides
ANOVA	analysis of variances
APC	antigen-presenting cells
C1QB	complement C1q B
CD163	cluster of differentiation 163, M130
CD206	cluster of differentiation 206, mannose receptor
CD209	cluster of differentiation 209, DC-SIGN
CD68	cluster of differentiation 68, B7.1
CD80	cluster of differentiation 80
Ct	cycle threshold
CXCL1	C-X-C motif chemokine ligand 1
DAMP	damage-associated molecular pattern
DC	dendritic cell
dDC	dermal dendritic cell
DEG	differentially expressed gene
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
Fc	fragment crystallizable region
FC	fold-change
FCS	fetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony-stimulating factor
H <sub>2</sub> O	water
HDF	human dermal fibroblast
HSC	hematopoietic stem cell
HSPA1B	heat shock protein family A member 1B
IFN-γ	interferon gamma
IL-10	interleukin 10
IL-12	interleukin 12
IL-13	interleukin 13

IL-34	interleukin 34
IL-4	interleukin 4
IL-6	interleukin 6
ILC	innate lymphoid cell
LC	Langerhans cell
LPS	lipopolysaccharide
MACS	magnetic-activated cell sorting
M-CSF	macrophage colony-stimulating factor
MDM	monocyte-derived macrophage
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mL	millilitre
MMP	matrix metalloproteinase
nM	nanomolar
P/S	Penicillin-Streptomycin
PAMP	pathogen-associated pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid DC
pM1	partially polarized M1 MDM
pM2	partially polarized M2 MDM
PRR	pattern recognition receptor
qPCR	quantitative PCR
RIN <sup>e</sup>	RNA Integrity Number equivalent
RNA	Ribonucleic acid
rpm	revolutions per min
RT-PCR	reverse transcription PCR
SASP	senescence-associated secretory phenotype
SEM	standard error of the mean
TGF- $\beta$	transforming growth factor beta
TLR	Toll-like receptor
TNF- $\alpha$	tumor necrosis factor alpha
TRIIM	thymus regeneration, immunorestitution and insulin mitigation
T <sub>RM</sub>	tissue-resident memory T cell
U	unit
UMAP	Uniform Approximation and Projection method

v/v	volume per volume
x <i>g</i>	x-fold gravitational force

# 1 Introduction

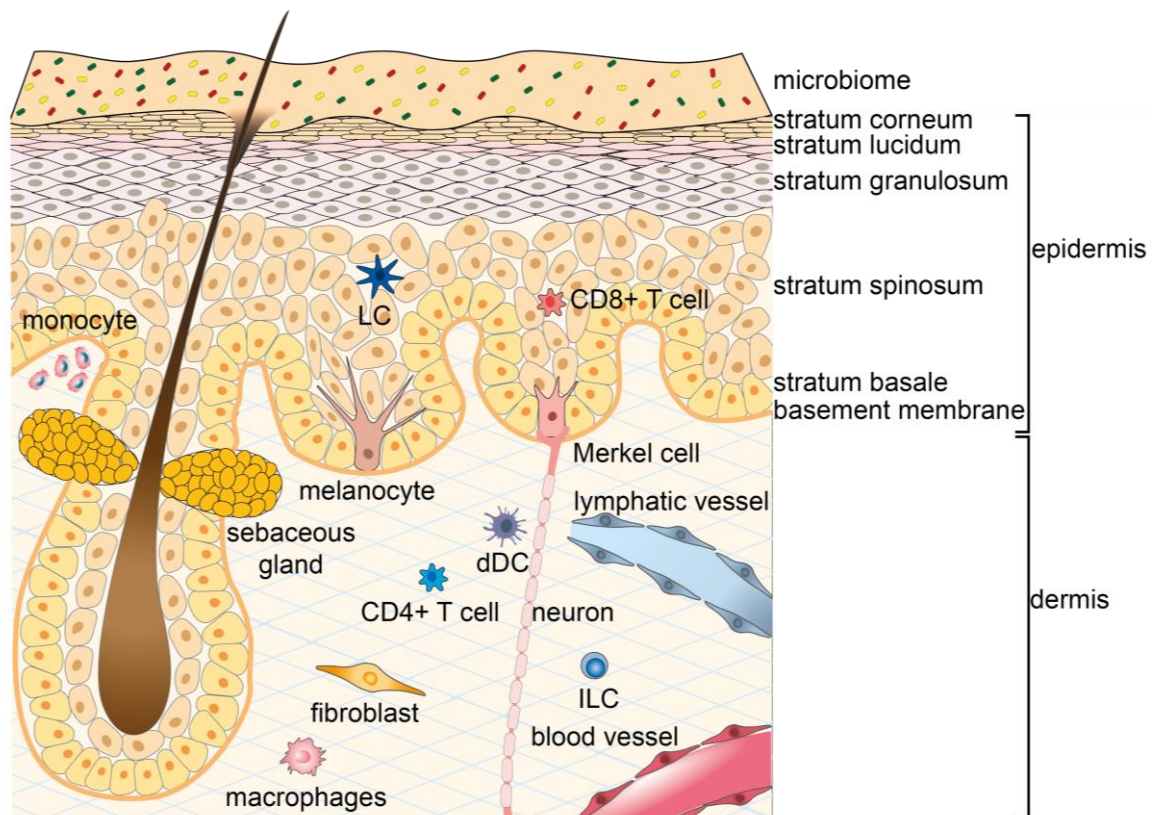
## 1.1 The structure of the human skin

The human skin is the outermost organ that forms a physical, chemical and microbiological barrier and separates the body from the outside environment. Furthermore, the skin has multiple and complex functions, such as protection against exogenous stressors like UV radiation, sensation of stimuli and regulation of water, temperature and salt balance. The skin structure provides the first barrier against potential danger (Gallo and Hooper 2012). The outermost layer of the skin is the epidermis, which is connected to the underlying loose connective tissue, the dermis, by the basement membrane. The dermis is separated from the underlying muscles by the hypodermis.

### 1.1.1 Epidermis

The epidermis is the outermost layer of the skin and is subdivided into four layers: stratum corneum, stratum granulosum, stratum spinosum and stratum basale. In palms and soles, an additional layer, the stratum lucidum, resides between the stratum granulosum and stratum corneum. Figure 1 shows the schematic structure of the human skin tissue and the skin cells. In the basal layer, keratinocytes, the major cell type of the epidermis, proliferate and differentiate gradually on their way to the outer layers while they undergo morphology changes. In the stratum corneum, they are found as nucleus-free, flat and highly keratinized squamous cells, the corneocytes, which continually build a protective layer (Eckert and Rorke 1989). Additionally, the intercellular space between the corneocytes is filled with lipids which provides a hydrophobic barrier against the environment. Thus, it forms a physical barrier to protect the body against the external environment (Murphy and Weaver 2017). Other non-keratinocyte cells of the epidermis are melanocytes, Merkel cells, CD8<sup>+</sup> T cells and Langerhans cells. Melanocytes are responsible for producing the pigment melanin inside the melanosomes and its transfer to the keratinocytes, protecting the dermis from harmful ultraviolet light. While these cells have only contact with the keratinocytes by cell extensions, Merkel cells attach to keratinocytes by desmosomal junctions (Rickelt, Moll, and Franke 2011). They are oval-shaped mechanoreceptors and essential for touch sensation. They are located in the stratum basale and are associated with nerve endings. Deformations of adjoining keratinocytes stimulate the Merkel cells to generate a signal in the adjoining afferent neuron. Langerhans cells and CD8<sup>+</sup> T cells represent two immune cell populations in the epidermis. Their functions are described in section 1.2.1 in more detail. The dermis

interacts with the epidermis via the basement membrane to maintain the properties of both layers, e.g. in tissue remodeling regarding wound healing.



**Figure 1 Schematic representation of the human epidermis and dermis.** The human epidermis is located on top of the dermis, separated by the basement membrane. The epidermis comprises five layers: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. The skin microbiome, a community of microorganisms, colonizes the outermost layer stratum corneum. The most abundant cell type of the epidermis is the keratinocyte. Other cells found in the epidermis are the melanocytes, Merkel cells, immune cell populations Langerhans cells (LCs) and CD8<sup>+</sup> T cells. In the dermis cells like fibroblasts, neurons and also immune cells such as macrophages, innate lymphoid cells (ILCs), dermal DCs (dDCs) and CD4<sup>+</sup> T cells reside in a mesh formed by ECM (blue) and blood and lymphatic vessels. Skin appendages like hair follicles and sweat glands reside in the stratum spinosum in the epidermis. Thus, they are surrounded by keratinocytes. Sebaceous glands are associated with the hair follicle.

### 1.1.2 Dermis and hypodermis

The dermis is the skin layer located beneath the epidermis. It is tightly connected to the epidermis via the basement membrane and is characterized by a high abundance of extracellular matrix (ECM) in which nerve, lymph and vascular networks, as well as skin cells, are present. The ECM consists mainly of fibrous structures like collagen and elastin fibers, which have an essential structural role in the tissue. In addition, substances such as proteoglycans, glycoproteins and glycosaminoglycans, which can bind large amounts of water, are found between the cells of the dermis. The dermis can be divided into two

structurally distinct layers, the papillary dermis and the underlying reticular dermis. The papillary dermis is adjacent to the epidermis and is characterized by a high cell density. The cell density decreases from the basement membrane to the lower reticular dermis. In addition, high content of proteoglycans is found in the papillary dermis, while collagen fibers are loosely aligned. In contrast, the reticular dermis is characterized by a high content of elastic and reticular fibers and a low cell density. Thus, the reticular dermis provides the dermis with strength, extensibility and elasticity. Fibroblasts are the most abundant cell type in both the reticular and papillary dermis. Among other things, they are responsible for producing connective tissue by producing ECM molecules (Brown and Krishnamurthy 2022). Several immune cell populations reside in the dermis or are continuously recruited to the tissue from the circulation. The recruitment takes place via the high vascular and lymphatic network throughout the ECM. Moreover, the ECM provides a scaffold for immune cell migration, ensuring a rapid immune response in the course of pathogen invasion. In addition, nutrient supply to both the dermis and epidermis is provided by the vascular network (Kabashima et al. 2019). Through the secretion of water and a change in blood pressure, the skin tissue can regulate the body temperature. Thus, the vasoactive skin vessels and the sweat glands in the dermis contribute to temperature regulation (Brown and Krishnamurthy 2022).

The dermis is separated from the underneath muscles and bones by the hypodermis, the subcutaneous fat tissue. It protects the underlying muscles and bones from injuries and plays an essential role in the thermoregulation and the supply of nutrients to the skin tissue (Kanitakis 2002).

## **1.2 Skin tissue homeostasis**

Skin tissue undergoes constant renewal during homeostatic and regenerative processes to maintain tissue homeostasis. The crosstalk between cells within the tissue, as well as with microorganisms residing on the skin, is critical for the maintenance of tissue homeostasis. The following sections provide an overview of the skin's immune system, microbiome and regeneration process.

### **1.2.1 The skin immune system**

Skin immune cells have distinct functions during tissue homeostasis and regeneration processes. In addition, the skin is continuously exposed to microorganisms, some of which are possible pathogenic. Therefore, effective crosstalk between epithelial and

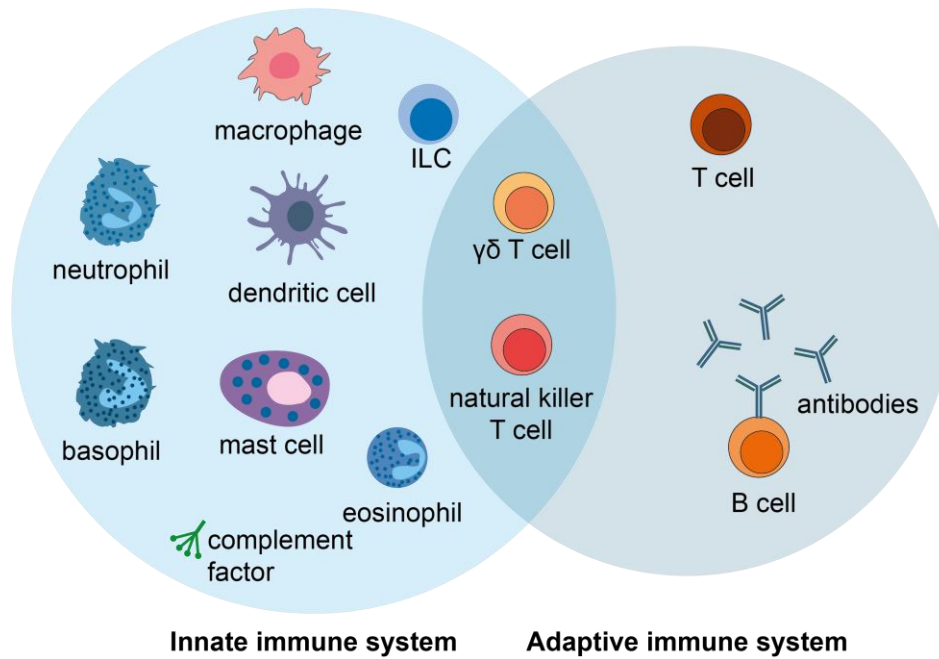
immune cells is required to eliminate pathogens and maintain tissue integrity and function.

The immune system composes two fundamental lines of defense: the innate and adaptive immunity. Physical and chemical barriers on body surfaces, such as on human skin, contribute to the initial defense against pathogens. The innate immune response is a rapid, nonspecific defense against pathogens. It is based on recognising common structures on many pathogens and harmful molecules. In contrast, the cells of the adaptive immune system recognize specific antigens and can recognize and subsequently eliminate pathogens in a highly specific and efficient manner. It also remembers the previous contact with specific pathogens and eliminates them upon re-infection. This ability of immunological memory allows the host to establish long-lasting protection against antigens and, thus, the pathogen. However, the adaptive immune system interacts with cells of the innate immune system. Specific immune cells, called antigen-presenting cells (APCs), process pathogens and present antigenic peptides on their surface to the cells of the adaptive immune system (Murphy and Weaver 2017).

According to their function, the components of the immune system are associated with either the innate or the adaptive immune system. Nevertheless, natural killer T cells and  $\gamma\delta$  T cells act in both the innate and the adaptive immune response (Figure 2). Immune cells that act during the innate immune response are macrophages, neutrophils, mast cells, innate lymphoid cells (ILCs), dendritic cells (DCs) and granulocytes (neutrophils, basophils and eosinophils). They are responsible for rapidly recognising pathogens or potentially harmful molecules and their subsequent elimination. The complement system, a part of the innate immune response, describes a biochemical cascade of small proteins, mainly serum proteins and cell membrane receptors circulating in the blood. Upon stimulation, the complements disrupt bacterial membranes and enhance pathogen elimination by recruiting cells of the innate immune system, such as macrophages and neutrophils (Murphy and Weaver 2017). The complement cascade comprises around 20 interacting soluble plasma proteins, which opsonize the pathogen to facilitate its subsequent removal via phagocytosis. Phagocytosis is an endocytosis process in which the cell membrane surrounds the material to engulf it. Subsequently, an intracellular vesicle, the phagosome, is formed around this material within the cell, which fuses with lysosomes containing antimicrobial molecules and enzymes to form a phagolysosome. These become active as the pH of the endosomes progressively decreases and degrade the engulfed phagocytes into peptide fragments. Phagocytic cells such as macrophages or neutrophils not only recognize complement components opsonizing the phagocytic surface, but also possess receptors for detecting pathogens via patterns on their surface.



Thereby, they need to distinguish the pathogenic cells from the self-cells of the host organism. Thus, the detection relies on the recognition of molecules that are common to many pathogens but absent in host cells, the so-called pathogen-associated molecular patterns (PAMPs). For example, macrophages recognize PAMPs via pattern recognition receptors (PRRs) (Murphy and Weaver 2017). Recognition of PAMPs finally leads to an inflammatory response, as it triggers a release of cytokines that can recruit and activate other immune cells. To detect a wide range of molecular structures of pathogens, macrophages and other innate leukocytes express PRRs against a large number of PAMPs that share common structural motifs or patterns. PRRs are classified according to their localization: membrane-bound PRRs such as Toll-Like receptors and scavenger receptors, cytoplasmic PRRs such as NOD-like receptors, or secreted PRRs like collectin. The best-described PAMPs are viral nucleic acids or components of the bacterial cell wall, such as lipopolysaccharides (LPS). Among several distinct classes of PRRs, the best-characterized is the class of Toll-like receptors (TLRs). TLRs are type I transmembrane receptors that recognize both viral nucleic acids and microbial structures like LPS (Medzhitov 2007). The role of Toll receptors in immunity was first discovered in 1996 for *Drosophila melanogaster* by Jules Hoffman and later extended to homologous TLRs in mice by Bruce Beutler and colleagues, for which Beutler and Hoffmann were awarded the Nobel Prize in Medicine or Physiology in 2011 (Murphy and Weaver 2017). Currently, eleven different TLRs are known for humans, whereas 13 different TLRs are described in mice. Most of them are expressed on the membrane of innate leukocytes but also on cells of the adaptive immune system and non-immune cells such as fibroblasts and keratinocytes (Mahla et al. 2013, Delneste, Beauvillain, and Jeannin 2007). After degradation of the incorporated components in the phagolysosome, the processed peptide fragments are loaded onto major histocompatibility complex (MHC) class II molecules. This complex then migrates to the cell surface to present the peptide fragments to cells of the adaptive immune system. These antigen-specific lymphocytes then develop an immunological memory against such antigen. Besides macrophages, also dendritic cells and B cells are able to present antigens bound by MHC proteins on their surface and are therefore referred to as APCs (Murphy and Weaver 2017).



**Figure 2 Illustration of innate and adaptive immune system effectors.** The innate immune response is induced by macrophages, dendritic cells, mast cells, complement factors, innate lymphoid cells (ILCs) and granulocytes (neutrophils, basophils, eosinophils). At a later time point, the adaptive immune response mediates antigenic specificity and memory by T and B cells to produce antibodies. Natural killer T cells and  $\gamma\delta$  T cells take over functions in both innate and adaptive immune responses.

All blood immune cells derive from pluripotent hematopoietic stem cells (HSCs) in the bone marrow, which differentiate into a common lymphoid progenitor or a common myeloid progenitor. The lymphoid progenitor gives rise to the lymphoid lineage, which comprises ILCs, NK cells, B and T lymphocytes. The myeloid progenitor gives rise to the myeloid lineage, including granulocytes, monocytes, mast cells, macrophages and DCs, as well as platelets and erythrocytes (Murphy and Weaver 2017). Although the immune system is divided into these two distinct systems, the adaptive immune defense relies on and interacts with the cells of the innate immune system. In the following, the skin immune cell populations are described in more detail according to their localization and their role in the skin tissue.

Immune cells either reside permanently inside or are continuously recruited into the skin tissue via the blood and lymphatic system in the dermis. In the human epidermis, two types of immune cell populations, Langerhans cells (LCs) and tissue-resident memory T cells ( $T_{RM}$ ), reside between keratinocytes inside the stratum spinosum. LC, a myeloid cell type belonging to the APCs, is characterized by a high expression of MHCII and is exclusively found in the epidermis. It has been long suggested that LCs are a subset of DCs. However, recent ontogenic studies have revealed that LCs are a population of tissue-resident macrophages with DC-specific functions to migrate to the lymph nodes

to present antigens (Hoeffel et al. 2012, Ginhoux et al. 2006). CD8<sup>+</sup> T<sub>RM</sub> cells are found in the epidermis, while CD4<sup>+</sup> T<sub>RM</sub> reside in both the dermis and epidermis. They persist long-term and serve as sentinels since they get reactivated upon reinfection with the same antigen and can recruit other lymphocytes to the skin tissue. In contrast, CD4<sup>+</sup> T<sub>reg</sub> are responsible for the resolution of inflammation, e.g. during wound healing when they suppress inflammatory monocytes (Haertel et al. 2018). In murine epidermis, an additional subset of T lymphocytes, the  $\gamma\delta$ T cells, have been discovered. During an infection, they produce high amounts of IL-17 to induce the expression of antimicrobial  $\beta$ -defensin in keratinocytes, thus they play a role in the antimicrobial defense of the skin (MacLeod et al. 2013). However, this cell type has not yet been detected in human skin (Kabashima et al. 2019). Dermal DCs (dDCs) and plasmacytoid DCs (pDCs) are both DC subsets found in the dermis. While dDCs permanently reside in the dermis and replenish every seven days, the pDCs can only be found in inflamed skin. In inflamed skin, the pDCs express interferon type I, cytokines involved in inflammation, to induce rapid inflammatory cytokine responses. Additionally, pDCs were shown to be involved in the re-epithelization of skin wounds (Gregorio et al. 2010). It has been demonstrated that dDCs play a role in the generation of commensal-specific T cells in the skin and, thus the maintenance of homeostasis between host and skin-resident commensal bacteria. They migrate to the skin draining lymph node to prime the adaptive immune response by presenting processed antigens of commensal bacteria (Naik et al. 2015). High activity of pDCs is associated with autoimmune skin disorders such as psoriasis as well as skin fibrosis (Nestle et al. 2005, Ah Kioon et al. 2018). Innate lymphoid cell populations also reside in the dermis and are essential for the recruitment of macrophages upon inflammation. Due to the expression of several PRRs on their surface, these cells play a role in the recognition of pathogens or potentially dangerous molecules. Non-immune cells in the skin, such as keratinocytes, also express PRRs on their surface and therefore play a role in pathogen recognition (Nestle et al. 2009). Especially in skin appendages such as hair follicles or sweat glands, where the stratum corneum is absent, recognition of pathogens is critical. The skin microbiome co-exists beside living keratinocytes in the stratum spinosum, which provides a potential entry point for pathogens. To handle this increased infection potential, the appendages show specific immunological properties. The binding of PAMPs to PRRs in keratinocytes leads to the synthesis of cytokines to recruit immune cell populations as well as antimicrobial peptides. Furthermore, *in vivo* studies have demonstrated close interaction of recruited monocytes with the hair follicle. It was suggested that monocytes patrol these areas to rapidly react in case of infection with a pathogen (Kabashima et al. 2019). Upon skin inflammation, monocyte clusters are formed near the hair follicle, activating T cells and enhancing the inflammatory response

(Liu et al. 2018). Not only during inflammation, but also for tissue homeostasis, the interplay of skin immune cells is critical. For instance, macrophages play an important role in the clearance of damaged or senescent cells residing via the recognition of damage-associated patterns (DAMPs). Specific PRRs are able to bind to PAMPs, resulting ultimately in the elimination of these cells or cell fragments. Elimination occurs via phagocytosis, in which components are taken up into the cell by endocytosis and destroyed within phagolysosomes within the cell (Morioka, Maueroeder, and Ravichandran 2019).

The role of macrophages during tissue homeostasis and defense against pathogens as well as their function in the skin tissue are described in detail in 1.3.

### **1.2.2 The human skin microbiome**

A community of microorganisms residing mostly commensally or mutualistically in one habitat, like the gut, lung or skin, is referred to as the microbiome of the respective habitat in its host organisms. Long-term interaction between host and microbiome can benefit one but mainly both members. The Human Microbiome Project first published the genome of human microbiota like that of the skin, mouth or gut from healthy patients in 2012 (Human Microbiome Project 2012). The skin surface, including hair follicles and glands, provides a surface area of around 30 m<sup>2</sup>, which is one of the largest areas together with the human gut (approximately 30 m<sup>2</sup>), only surpassed by the lung (approximately 50 m<sup>2</sup>). It is colonized by microorganisms such as bacteria, fungi and viruses (Gallo 2017). Depending on variations in skin temperature, pH, humidity and sebaceous gland density, the human skin sites can be classified into sebaceous, moist, or dry categories, accompanied by different growth conditions for the colonization by microorganisms. Thus, depending on the skin region, the microbiome composition is highly variable. The skin microbiome mainly functions as the first barrier against pathogens and thereby interacts with the skin tissue via keratinocytes and immune cells. Thereby, it can affect both innate and adaptive immune systems. Furthermore, colonization of the skin by the microbiome is crucial for the training of the immune system.

The colonization of the human skin starts with birth. The skin is colonized by several microorganisms recognized by the immature immune system network. This leads to the education of the immature immune system to further distinguish commensal from pathogenic microorganisms. Simultaneously, the skin can be colonized without a followed inflammatory response since the immune system is immature. Reduced

diversity of skin microbes, like after caesarean delivery, affects the later development of skin diseases associated with impaired immune response. Thus, the education of the immune system of the skin seems to depend on the early neonatal microbiome composition (Scharschmidt et al. 2015, Kobayashi et al. 2015).

In general, commensal bacteria contribute to host defense against infections but also the education of the immune system. To protect against infections, the commensal bacteria compete for sites of colonization and produce inhibition molecules such as antimicrobial peptides (AMPs). The AMPs can be produced constantly or upon binding of commensal-derived products to PRRs (Hooper and Macpherson 2010). Thus, commensal bacteria can induce neighboring cells to produce AMPs. Keratinocytes and immune cells such as macrophages or neutrophils can be induced to synthesize AMPs cathelicidin and  $\beta$ -defensins, well-known AMPs found in the skin (Frohm et al. 1997, Sorensen et al. 2003, Vandamme et al. 2012). Skin commensal *Staphylococcus lugdunensis* and *Staphylococcus hominis* are also reported to secrete AMPs, such as antimicrobial lugdunin and lantibiotic (Zipperer et al. 2016, Nakatsuji et al. 2017). The skin microbiome interacts with keratinocytes in the stratum corneum or stratum spinosum and the skin appendages, thereby influencing cutaneous immunity. Especially the microorganisms residing in the skin appendages seem to greatly impact cutaneous immunity compared to microorganisms living on dead corneocytes in the stratum corneum. Even potential pathogenic microorganisms can reside on the skin without negative effects. However, if the balance between commensal and pathogenic microorganisms is disturbed or the skin barrier is disrupted and pathogens enter the tissue, this may lead to skin or systemic diseases. During the invasion of skin appendages by pathogenic microorganisms, keratinocytes in the vicinity are stimulated to produce inflammatory cytokines to recruit immune cells, such as monocytes, and initiate an inflammatory response. Furthermore, other metabolites and structural components of microorganisms can suppress inflammatory cytokine release through keratinocytes. Inflammatory cytokine production of the keratinocytes is reported to be reduced by the inhibition of the PRR TLR3 via the secretion of lipoteichoic acid by the non-pathogenic *Staphylococcus epidermidis*. (Lai et al. 2009). Another microorganism, *Propionibacterium acnes* (*P. acnes*), is reported to stimulate sebaceous glands to produce IL-1 $\beta$ , IL-6 and TGF- $\beta$ , leading to the activation of DCs to prime T<sub>H</sub>17 cells. T<sub>H</sub>17 cells are a subset of pro-inflammatory T cells, known to be important during pathogen elimination (Mattii et al. 2018). Therefore, the microbiome is thought to influence the adaptive and innate immune system directly or via stimulation of keratinocytes or sebocytes.

Changes in the composition of the microbiome over lifetime are initiated by several intrinsic or extrinsic factors. Intrinsically, increased sebum production during puberty due to enhanced levels of hormones can result in the expansion of lipophilic microorganisms. Also, antibiotic treatment, an extrinsic factor, is reported to drive changes in the composition of the skin microbiome (Walsh, Efthimiou, and Dreno 2016).

The complex interplay between the skin, its microbiome and the immune system suggest that a disbalance can play an immense role in pathological processes. Examples of that have already been reported for skin diseases regarded to the skin microbiome, like atopic dermatitis, acne and psoriasis (Byrd, Belkaid, and Segre 2018). Also, age-related variations in the microbiome composition derived from young and aged humans have already been described (Howard et al. 2022). The association of an imbalanced skin microbiome and several dermatoses demonstrates the critical role of the microbiome in tissue homeostasis.

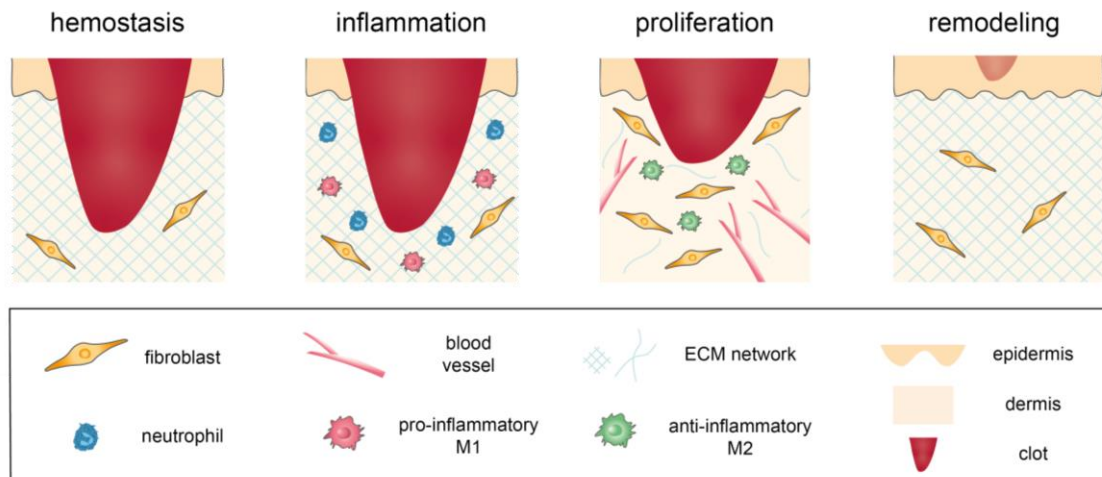
### **1.2.3 Skin tissue regeneration**

After skin tissue injury, the tissue needs to be regenerated to restore tissue integrity and barrier function. How cells within the skin layers interact to regenerate tissue can be observed during the regenerative process of wound healing (Figure 3). The wound healing process undergoes four different overlapping phases: hemostasis, inflammation, proliferation and remodeling (Velnar, Bailey, and Smrkolj 2009).

Hemostasis aims to prevent excessive blood loss and provide a matrix for invading cells involved in the regeneration process. To reduce blood loss, the smooth muscle cells of the vessels are activated to contract during an injury. Platelets from the blood vessels are exposed to ECM molecules in the damaged tissue. By binding ECM molecules, the platelets get activated to secrete clotting factors and thus initiate the formation of a blood clot (Yun et al. 2016). The blood clot provides a provisional ECM, mainly composed of fibronectin and fibrin, for later cell migration during the inflammatory phase. Growth factors and cytokines, released by platelets, attract and activate first neutrophils and later macrophages, fibroblasts and endothelial cells. Invading microorganisms activates the complement system, which in turn triggers the recruitment of neutrophils. During the inflammatory phase, activated neutrophils eliminate bacteria, foreign particles and damaged tissue via phagocytosis. The clearance from microorganisms is critical for healthy wound healing. Following blood-derived monocytes infiltrate the wound and differentiate to macrophages (MDM, monocyte-derived macrophages), which have a longer lifespan than neutrophils and continue to phagocyte microorganisms and

damaged tissue components. Lymphocytes enter at the end of the inflammatory phase to establish a memory immune response for a longer period and to specifically target later invading microorganisms (Canedo-Dorantes and Canedo-Ayala 2019).

After the inflammatory phase, the acute wound shifts towards tissue repair. In the proliferative phase, fibroblasts infiltrate the wound due to the secretion of fibroblasts growth factor 2 and TGF- $\beta$  by macrophages. In the wound, the recruited fibroblasts proliferate and produce a high amount of ECM molecules such as glycoproteins and collagen. When ECM accumulates, the provisional clot formed by fibrinogen and fibrin is deposited, and newly synthesized ECM forms a granulation tissue. The granulation tissue is composed of loosely attached ECM molecules, mainly collagen III, and functions as connective tissue on the wound's surface. Within the tissue, macrophages secrete growth factors to attract and activate keratinocytes and endothelial cells as well as fibroblasts to proliferate. In addition, endothelial cells from neighboring blood vessels migrate into the tissue and form new, intact blood vessels. This angiogenesis serves to vascularize the tissue, which is necessary to supply the tissue with nutrients and enable immune cell infiltration from the blood vessels (Velnar, Bailey, and Smrkolj 2009). After granulation tissue is formed, macrophages activate fibroblasts to differentiate into myofibroblasts, a cell type responsible for wound contraction. The myofibroblasts are located in currently existing collagen fibers and surrounding margins of the wound. By pulling newly formed collagen fibers in damaged tissue toward the center of the defect, they reduce the size of the damaged tissue (Shook et al. 2018). Macrophages stimulate keratinocytes to proliferate and thus re-epithelize the skin tissue to resurface the wound with new epithelium (Raziyeva et al. 2021). In the final phase, macrophages and fibroblasts secrete matrix metalloproteinases (MMPs), enzymes specialized in degrading ECM. Thus, the ECM of the granulation tissue undergoes degradation and a new ECM structure is synthesized. In this process, the collagen III from the granulation tissue is replaced by stronger, long-stranded collagen I (Canedo-Dorantes and Canedo-Ayala 2019, Velnar, Bailey, and Smrkolj 2009). At the end of wound healing, cell number of fibroblasts and macrophages decreases within the tissue by apoptosis (Greenhalgh 1998).



**Figure 3 The four phases of skin wound healing.** Wound healing is divided into four phases: hemostasis, inflammation, proliferation and remodeling. After an injury, a clot is formed to prevent excessive blood loss (hemostasis). Next, neutrophils and pro-inflammatory M1 macrophages are recruited into the tissue to eliminate invaded pathogens (inflammation). After inflammation, anti-inflammatory M2 macrophages drive inflammation resolution and induce ECM degradation. Cells such as fibroblasts are induced to proliferate and newly blood vessels are formed (proliferation). During remodeling the skin is re-epithelized and new ECM structure is synthesized to restore skin tissue structure.

Macrophages are the dominant cell type present in every stage of tissue remodeling. By secreting proteins to instruct other cells, they control the various steps of regeneration. They ensure both the elimination of pathogens and the rebuilding of skin tissue. During the inflammatory phase, mainly pro-inflammatory macrophages are active. They trigger an immune response and eliminate pathogens via phagocytosis. Subsequently, anti-inflammatory macrophages coordinate the resolution of inflammation by secreting anti-inflammatory cytokines. Furthermore, they stimulate the neighboring cells to proliferate. Thus, during wound healing, the macrophages first display pro-inflammatory properties with a change to anti-inflammatory properties towards the end of the process (Canedo-Dorantes and Canedo-Ayala 2019). However, it is unclear whether the macrophages change their phenotype during the process or whether anti-inflammatory macrophages replace pro-inflammatory macrophages at a later time point.

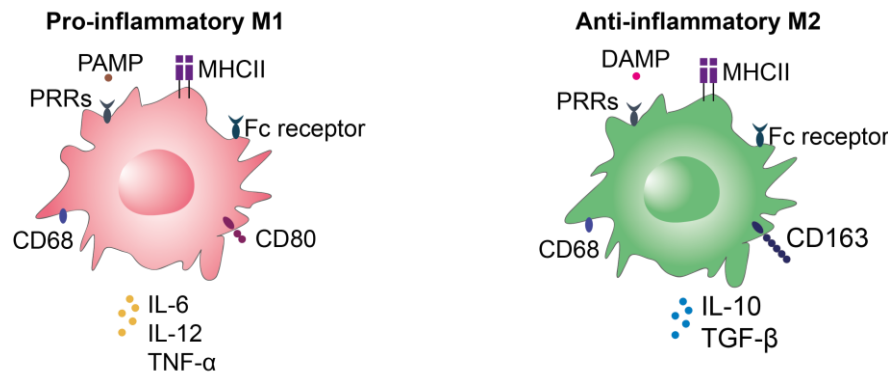
Nevertheless, the different phenotypes of macrophages during the wound healing process demonstrate how diverse and plastic these cells are. The depletion of monocytes and macrophages has been shown to result in impaired wound healing (Mori, Shaw, and Martin 2008, Goren et al. 2009, Mirza, DiPietro, and Koh 2009). This demonstrates the critical role of macrophages during the regeneration of tissues.



### 1.3 Macrophages

Macrophages are large mononuclear phagocytic cells present in most tissues. They were first identified and described for their phagocytic function by Elie Metchnikoff in 1882 (Stefater et al. 2011). They function as professional APCs, thus they can be characterized by their surface expression of MHC class II molecules, which are involved in the presentation of antigens on the cell surface. In addition, macrophages possess fragment crystallizable region (Fc) receptors on their surface that bind to the Fc region of various immunoglobulins. When cells of the adaptive immune system recognize a pathogen, lymphocytes produce antibodies to label these pathogens. By binding to the constant Fc region, which is the same for all antibodies of one class, macrophages thus bind the pathogens or fragments opsonized with antibodies (Murphy and Weaver 2017). Additionally, they can detect pathogens and damaged cells or tissue components via PRRs or scavenger receptors like CD68. The recognition of such particles leads to the activation of macrophages and the initiation of phagocytosis. Scavenger receptors that are reported to be associated with a high phagocytic activity of macrophages are e.g. CD163, CD206 and CD209 (Schulz et al. 2019). Furthermore, macrophages are the major source of cytokines, and by releasing such molecules, they can instruct other cells (Murphy and Weaver 2017). Macrophages are known for their diversity and plasticity. Although their properties are usually more complex *in vivo*, they are typically classified according to their polarization state into M1 and M2 macrophages (Figure 4). Classically activated macrophages (M1) exhibit antimicrobial and tumoricidal activity and secrete pro-inflammatory cytokines which decelerate the cellular proliferation in the surrounding tissue leading to tissue damage. In contrast, alternatively activated macrophages (M2) release anti-inflammatory cytokines contributing to tissue repair and regeneration. Currently, M2 macrophages are no longer thought to exhibit a unique activation state, but rather a functionally diverse group of macrophages. Accordingly, they can be further subdivided into M2a, M2b, M2c and M2d. They are associated with tumorigenesis, immunosuppression and elimination of parasites such as parasitic helminths (Mantovani et al. 2004, Roszer 2015). Nevertheless, based on their pro- and anti-inflammatory properties, M1 and M2 can be distinguished by several markers. Characteristic of pro-inflammatory M1 macrophages is the expression of cytokines like interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin 12 (IL-12). In contrast, interleukin 10 (IL-10) and transforming growth factor beta (TGF- $\beta$ ) serve as markers for anti-inflammatory M2 macrophages. Furthermore, the determination of the expression of surface receptors CD80 and CD163 is commonly used to distinguish and identify M1 and M2 macrophages, respectively. (Martinez et al. 2006, Martinez et al. 2008). CD80 is

crucial for the interaction of macrophages with T cell to activate them for an immune response. CD163 is a scavenger receptor known to play a role in the phagocytosis process (Murphy and Weaver 2017, Schulz et al. 2019).



**Figure 4 Overview of the *in vitro* macrophage phenotypes M1 and M2.** Pro-inflammatory M1 macrophages (M1) and anti-inflammatory M2 macrophages (M2) express both MHCII, CD68 and Fc receptors. Pro-inflammatory M1 express PRRs on their surface to recognize mainly pathogen-associated molecular patterns (PAMPs), whereas anti-inflammatory M2 express PRRs to detect damage-associated molecular patterns (DAMPs). Characteristic of M1 is the expression of lineage marker CD80 as well as the secretion of cytokines IL-6, IL-12 and TNF-α. M2 secrete high amounts of IL-10 and TGF-β and express CD163 on their surface.

According to their variable properties, they play a crucial role in tissue homeostasis and inflammation but are also involved in the pathophysiology of several diseases, including age-associated and inflammatory diseases or cancer. Gene expression pattern and, therefore, the phenotype of macrophages highly depends on their local environment. Fate-mapping studies revealed that all tissues are populated with fetal-derived macrophages at birth, but some macrophages are gradually replaced by HSC-derived macrophages. These HSCs mature into monocytes in the bone-marrow and are released into the circulation. The spleen serves as a reservoir for immature monocytes. Upon a signal, the monocytes are recruited towards a tissue to then differentiate into macrophages or dendritic cells within a tissue. However, the contribution of HSC-derived macrophages to the pool of resident macrophages differs among organs. Ginhoux et al. classified adult tissues according to their mode of monocyte recruitment into the tissue. Closed tissues are characterized by no steady-state monocyte recruitment like the brain, epidermis and lung. Therefore, the embryonic-derived macrophages are able to self-maintain during their whole lifetime. In open tissues, monocytes are recruited and differentiated into macrophages with slow or fast replacement kinetics depending on the organ. Not only does the tissue determine the origin of tissue macrophages, but also the

gene expression pattern and therefore their phenotype is shaped by the surrounding environment (Ginhoux and Guillemins 2016).

### **1.3.1 Macrophages in the first line of defense**

The immune system faces millions of potential pathogens every day and has developed a sophisticated defense strategy consisting of the innate and adaptive immune system to protect the body against infections. The innate immune response occurs rapidly on exposure to an infectious organism or upon tissue damage, while the adaptive immune response can take days to develop. Macrophages act during the innate immune response (Murphy and Weaver 2017).

Macrophages reside in tissues throughout the body and patrol to detect invading pathogens and subsequently initiate an inflammatory response to finally eliminate the pathogens. To detect pathogens, macrophages express PRRs to bind PAMPs. In mammals, LPS and other PAMPs are initially recognized by patrolling tissue-resident macrophages via TLR4, which induces the expression of pro-inflammatory cytokines such as IL-6, IL-12 and TNF- $\alpha$  via the NF $\kappa$ B pathway. In addition, the binding of PAMPs to TLR4 can activate the IRF3 signaling pathway, which induces the secretion of type-1 interferons. In turn, type-1 interferons such as interferon alpha can also induce macrophages to secrete various cytokines and chemokines through activation of the JAK/STAT pathway. Another well-characterized PRR expressed by macrophages is dectin1, a member of C-type lectins. It recognizes a variety of glucans found on the surface of fungi, which triggers the activation of NF $\kappa$ B to produce cytokines such as IL-6 and TNF- $\alpha$ . Thus, dectin1 is critical for the defense of fungus infection. Finally, the release of cytokines and chemokines is involved in the increase of blood vessel permeability and the recruitment of other immune cells to the site of infections. Furthermore, endothelial cells are induced to produce cytokines to enhance the attachment of leukocytes, such as monocytes, to the endothelium. This allows the monocytes to rapidly migrate into the tissue on the side of infection. In the tissue, monocytes can differentiate into macrophages to reinforce and sustain the innate immune response (Murphy and Weaver 2017).

Consequently, one major function of the innate immune system is the activation of phagocytes to rapidly eliminate potential pathogens or particles mainly carried out by macrophages and neutrophils. According to their efficiency professional phagocytes are distinguished from non-professional phagocytes, characterized by their expression of PRRs. Macrophages belong to the professional phagocytes, as well as neutrophils, mast

cells, monocytes and DCs (Mantovani, Rabinovitch, and Nussenzweig 1972). Consequently, macrophages play an important role in the development of a specific memory immune response, as they display peptide fragments of pathogens on their cell surface to cells of the adaptive immune system (Murphy and Weaver 2017).

### 1.3.2 Macrophages in tissue homeostasis and regeneration

However, the phagocytic function of macrophages also serves as a scavenging function to remove damaged cells and components of the extracellular matrix, which is essential for tissue homeostasis and regeneration. Every tissue comprises one or more tissue-resident macrophage populations, which are crucial for tissue development, homeostasis and regeneration, and therefore play a critical role in organ function (Stefater et al. 2011). As described in the previous chapter, macrophages are able to detect pathogens via receptors specific for pathogenic patterns, the PRRs. Several studies claim that DAMPs, components of injured tissue and damaged or apoptotic cells, bind to specific PRRs or scavenger receptors and trigger phagocytosis. In this way, macrophages can recognize and eliminate disrupted ECM after injury or dying cells. Well-known DAMPs are DNA fragments, S100 proteins, or hyaluronan fragments. To distinguish viable from dying cells, viable cells express specific proteins such as CD47. CD47 is known to bind SIRP $\alpha$ , an inhibitory receptor expressed on phagocytes. The binding of CD47 and SIRP $\alpha$  finally results in the suppression of phagocytosis (Brown and Frazier 2001, Willingham et al. 2012).

The binding of PAMPs activates the NF $\kappa$ B and mitogen-activated protein kinase and leads to the expression of various pro-inflammatory cytokines. In contrast, the binding of DAMPs activates the expression of anti-inflammatory IL-10 and TGF- $\beta$ . Therefore, the recognition of damaged tissue components and dying cells is followed by the induction of an inflammatory resolution and tissue remodeling. The importance of macrophages during dead cell clearance and tissue remodeling has been demonstrated in mice lacking functional macrophages, where both mechanisms were diminished and delayed (Wood et al. 2000). It has been suggested that macrophages not only recognize apoptotic cells but are also involved in the initiation of apoptosis. *C.elegans* lacking phagocytic receptor CED-1, a decrease in cell death with a concomitant increase in cell survival could be shown. The decrease in apoptosis during impaired phagocytic ability demonstrates the role of phagocytes such as macrophages in the initiation of apoptosis (Reddien, Cameron, and Horvitz 2001). In addition, macrophages are believed to diminish necrosis by initiating apoptosis early, e.g. in chronically inflamed wounds. Furthermore,

macrophages are suggested to play a role in the elimination of senescent cells. Senescent cells are dysfunctional cells that are prevented from continuing to proliferate by undergoing cell cycle arrest (Kale et al. 2020).

Macrophages are also thought to play an important role in leukocyte homeostasis. They have been reported to be involved in maintaining HSCs in their stem cell state. Furthermore, macrophages are believed to prevent underdeveloped leukocytes from prematurely migrating into a tissue, thus regulating leukocyte migration into tissue. Additionally, leukocytes like neutrophils undergo apoptosis with high frequency, and organs like the spleen and liver are involved in the clearance of these apoptotic cells. Interestingly, the tissue-resident macrophages in these organs phagocytose them without getting activated to secrete pro-inflammatory factors (Stefater et al. 2011).

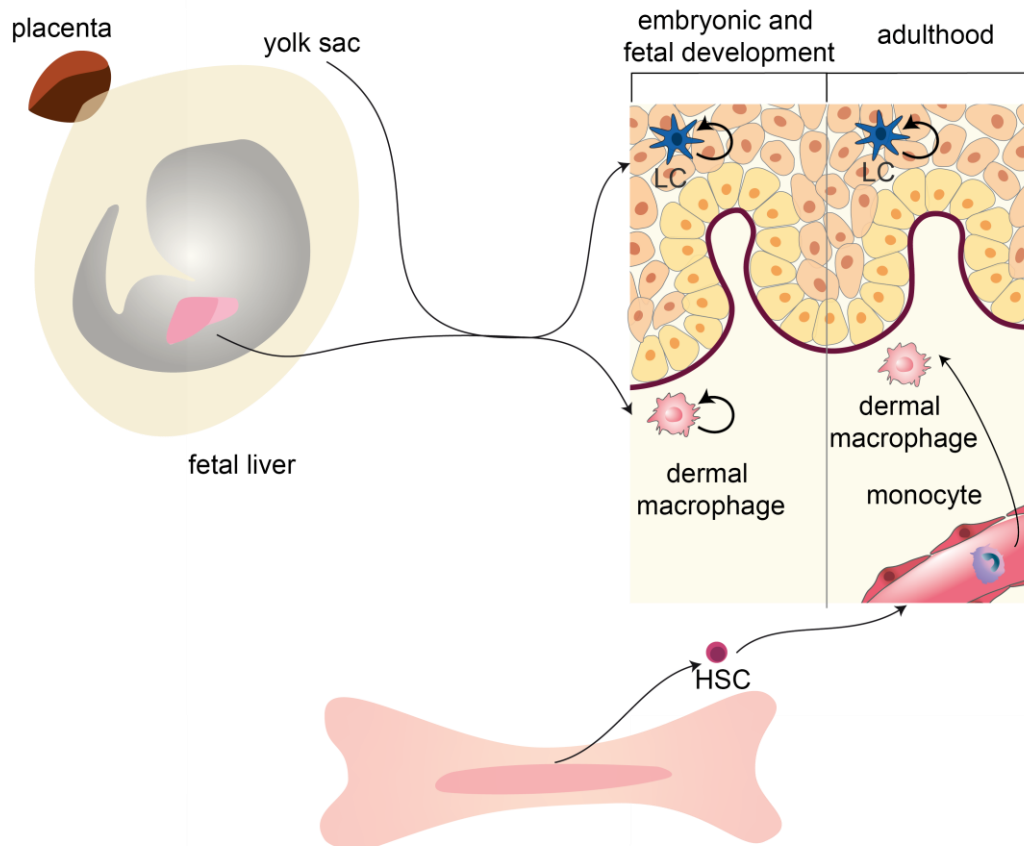
In addition, macrophages also regulate the tissue homeostasis and regeneration of tissues by apoptosis-independent mechanisms. By the secretion of pro- or anti-angiogenic factors like VEGF, the macrophages are also key players in vessel patterning. This has been demonstrated in mice lacking functional macrophages which showed an abnormal vascular patterning in the hindbrain and retina (Fantin et al. 2010, Kubota et al. 2009). Furthermore, nitric oxide (NO) is a potent vasodilator and regulator of blood pressure, mainly released by macrophages. Upon tissue injury, the macrophages thereby regulate the nutrient supply and recruitment of immune cells from circulation. Due to their expression of receptors for lipids like low-density lipoprotein receptor they are also believed to be involved in lipid homeostasis (Linton et al. 1999). The role of macrophages during tissue repair and regeneration is well-studied in skin wound repair. Mice lacking functional macrophages showed impaired healing of skin wounds, characterized by decreased ECM deposition, reduced cell proliferation and delayed re-epithelization, as well as abnormal wound vasculature (Mirza, DiPietro, and Koh 2009, Goren et al. 2009, Mori, Shaw, and Martin 2008).

### **1.3.3 Macrophages in the skin tissue**

Each tissue imprints the function of its resident macrophages. Thus, each tissue has unique macrophage populations (Ginhoux and Guilliams 2016). In skin tissue, dermal macrophages display the most abundant immune cell population. The dermal macrophages of healthy skin are involved in tissue homeostasis, e.g. they remove apoptotic and senescent cells or initiate apoptosis on dysfunctional cells (Kale et al. 2020, Covarrubias et al. 2020). In addition, skin-resident macrophages patrol to detect and eliminate invading pathogens as well as recruit other immune cells to the site of

infection by releasing several chemokines and cytokines (Murphy and Weaver 2017). Although LCs were considered a subset of dendritic cells, ontogenic studies revealed they are a subset of tissue-resident macrophages with the ability to migrate to the lymph nodes like dendritic cells (Hoeffel et al. 2012, Ginhoux et al. 2006). They are exclusively present in the epidermis. Keratinocytes express IL-34, a ligand of colony-stimulating factor-1 receptor, which is expressed on the surface of LCs. By binding IL-34 to LCs, these cells are kept within the epidermis. Thus, the keratinocytes contribute to the maintenance of LCs inside the epidermis. Furthermore, LCs anchor to keratinocytes in the tissue via E-cadherin. During inflammation and upon recognition of DAMPs or PAMPs, the expression of E-cadherin is suppressed. In addition, LCs secrete high levels of MMP9, resulting in disrupted ECM. This allows the LCs to migrate through the tissue upon stimulation like during inflammation. Thus, LCs are able to migrate throughout the tissue to the draining lymph nodes to present sampled antigens on their surface towards cells of the adaptive immune system. Furthermore, LCs not only sample microbial antigens but also self-antigens to promote tolerance during tissue homeostasis (Nguyen and Soulika 2019, Kabashima et al. 2019).

In the dermis, tissue-resident dermal macrophages function in the pathogen defense but also during tissue homeostasis. Fate-mapping technologies have revealed that LCs and dermal macrophages derive from the fetal liver and yolk sac during embryonic development. In adulthood, dermal macrophages are gradually replaced by macrophages derived from circulating monocytes from bone marrow-derived HSCs, whereas LCs maintain themselves. It is suggested that the tissue itself controls the maintenance and replenishment of the fetal-derived macrophages (Ginhoux and Guilliams 2016) (Figure 5).



**Figure 5 Origin of dermal macrophages.** Epidermal Langerhans cells (LC) and dermal macrophages develop during embryonic and fetal life from the yolk sac and fetal liver progenitor cells and populate the skin tissue. In this stage and during adulthood, the LCs maintain themselves, whereas dermal macrophages only maintain themselves during embryonic and fetal development. After birth, the dermal macrophages are gradually replaced by hematopoietic stem cell (HSC) derived bone-marrow monocytes that migrate into the tissue and differentiate into macrophages.

Reynolds and colleagues were able to identify distinct skin macrophage populations in adult skin via scRNA-seq analysis, indicating that these cells possess heterogeneous functions in the tissue. Two macrophage cell states highly express CD68. One of these two populations is characterized by higher expression of complement proteins C1QB and C1QC, indicating a pro-inflammatory phenotype. In contrast, the other population exhibits a higher expression of F13A1 and transcription factors associated with alternative activation and suppression of immune responses, suggesting to display a more anti-inflammatory phenotype. In addition, a population has been identified in adult skin that has been termed a monocyte-derived macrophage population. These are thought to be macrophages differentiated from monocytes after being recruited into the tissue (Reynolds et al. 2021).

Like in other organs, dermal macrophages function to maintain tissue integrity and characteristics. To this end, macrophages patrol the skin tissue environment to monitor any signal indicating infection, cell stress, or tissue injury. Since the skin is exposed to potential pathogens on a daily basis, a fast reaction to defend against pathogens and the activation of other immune cells is important. Upon pathogen detection, the dermal macrophages produce cytokines and chemokines to recruit immune cells like neutrophils and monocytes. Monocytes then differentiate into additional macrophages to support the defense process. Following the elimination of pathogens, the macrophages initiate the resolution of inflammation and tissue repair, e.g. through the secretion of IL-10 and TGF- $\beta$  (Murphy and Weaver 2017).

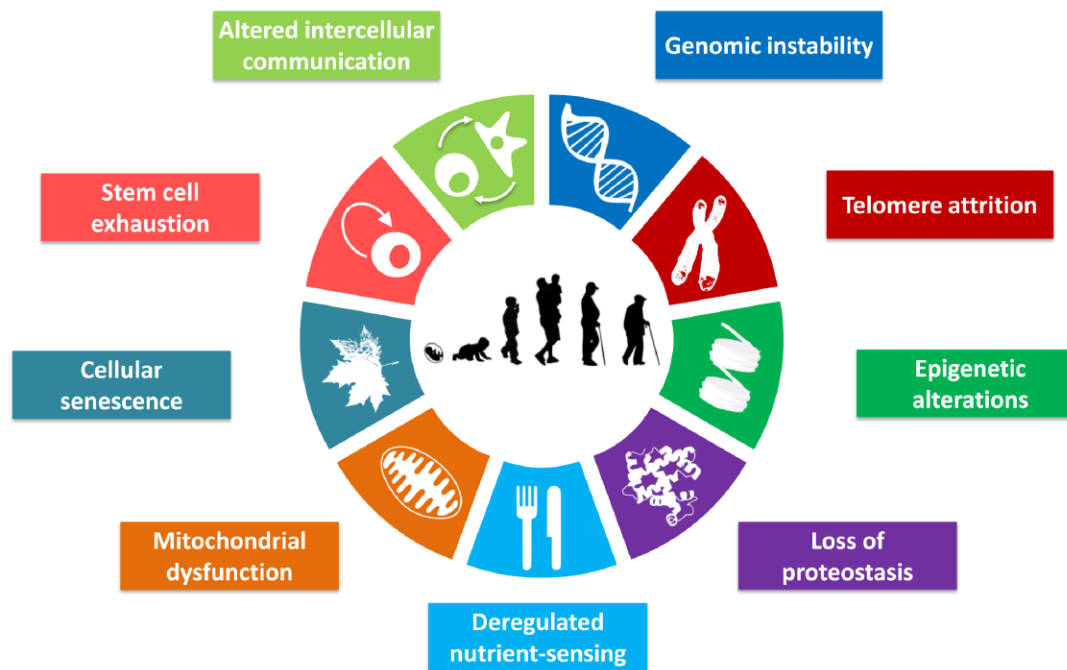
Moreover, the macrophages play a major role in tissue regeneration after injury and restoration of tissue homeostasis. The diversity and plasticity of the macrophages can be observed during skin wound healing, a well-studied process in the context of skin regeneration. During this regeneration process, the macrophages first display pro-inflammatory properties with a change to anti-inflammatory properties towards the end of the process. It is not clear whether the macrophages change their phenotype during the process or undergo apoptosis and macrophages recruited at a later timepoint acquire more anti-inflammatory properties (Canedo-Dorantes and Canedo-Ayala 2019).

In healthy skin, macrophages are also involved in hair homeostasis. While inflammatory monocyte promotes hair growth, the tissue-resident macrophages were shown to inhibit it (Amberg et al. 2016). Also, during salt homeostasis, macrophages are reported to play an important role. Infiltration of phagocytes like macrophages into the dermis after a high salt diet suggests the macrophages be involved in the cutaneous salt homeostasis (Halterman, Kwon, and Wamhoff 2012). Furthermore, the high levels of NaCl affect the macrophage activation to a more pro-inflammatory phenotype (Binger et al. 2015, Zhang et al. 2015). Dermal macrophages also play a role in skin tissue homeostasis via the elimination of damaged or dying cells as well as disrupted ECM. Furthermore, macrophages can initiate apoptosis to regulate skin tissue homeostasis. Dermal macrophages and LCs are already reported to play a critical role in limiting basal cell carcinoma (Konig et al. 2014). During homeostasis, LCs support immune tolerance to self-antigens, thus protecting from acquiring autoimmune dermatitis (Clausen and Kel 2010). Furthermore, macrophages are also associated with several skin diseases, mainly inflammatory skin diseases like psoriasis and atopic dermatitis (Malissen, Tamoutounour, and Henri 2014).



## 1.4 Aging

Aging is associated with an accumulation of molecular and cellular damage, which drives the development of age-related disorders. Lopez-Otin and colleagues defined nine hallmarks of aging (Figure 6), inspired by the hallmarks of cancer specified by Hanahan and Weinberg (Hanahan and Weinberg 2011, Lopez-Otin et al. 2013). Both cancer and aging rely on the accumulation of cellular damage. However, in contrast to the cancer scenario, cell fitness and proliferation are reduced during aging. The hallmarks of aging are subclassified into primary, antagonistic and integrative hallmarks and comprise molecular and cellular hallmarks of aging. Primary hallmarks in general show negative impacts, whereas antagonist hallmarks have beneficial effects on tissues when their levels are low but reverse when their levels increase, such as senescence. Senescence is a cellular reaction that is primarily characterized by a stable growth arrest. In this way, dysfunctional cells are prevented from continuing to proliferate. In the case of cancer, this can limit the progression of tumors. Senescent cells are also characterized by their secretion of senescence-associated secretory phenotype (SASP) factors, mainly pro-inflammatory proteins. The SASP has a beneficial effect on tissue homeostasis such as instructing tumor cell clearance, promoting tumor-suppressing cell state and preventing fibrosis (Acosta et al. 2008, Xue et al. 2007, Jun and Lau 2010). However, the number of senescent cells increases with age. Therefore, the impact of secreting factors derived from senescent cells on the surrounding cells increases with age. Furthermore, the relation of senescent cells and age-dependent diseases like diabetes has been reported already (McHugh and Gil 2018). In general, the defined primary hallmarks genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis seem to be the first cause of damage. At low levels, the inflicted damages can be compensated by mechanisms associated with the antagonistic hallmarks. But when damages occur exacerbated or chronic, they become deleterious, like cellular senescence, mitochondrial dysfunction and deregulated nutrient sensing. They display the first response to the damages caused by primary hallmarks. The integrative hallmarks, stem cell exhaustion and altered intercellular communication are thought to arise when accumulated damage caused by the primary and antagonistic hallmarks can no longer be compensated by tissue homeostasis mechanisms (Lopez-Otin et al. 2013).



**Figure 6 The hallmarks of aging defined by Lopez-Otin et al.** The scheme shows the nine hallmarks of aging described by Lopez-Otin and colleagues. Primary drivers of aging are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis. Deregulated nutrient sensing, mitochondrial dysfunction and cellular senescence are defined as antagonistic hallmarks of aging. The integrative hallmarks stem cell exhaustion and altered intercellular communication are described to be the result of damage derive from primary and antagonist hallmarks of aging. Figure adapted from Lopez-Otin et.al (Lopez-Otin et al. 2013).

### 1.4.1 Skin aging

Like other organs, the skin undergoes functional declines resulting in impaired tissue maintenance and regeneration. It is characterized by the appearance of age spots and wrinkles, loss of elasticity and a reduced ability to heal wounds (Lavker, Zheng, and Dong 1987, Swift, Kleinman, and DiPietro 1999). Skin aging is triggered by both intrinsic and extrinsic factors. More specifically, on the one hand, the tissue itself contributes to the inevitable changes, but on the other hand, environmental factors such as UV radiation also influence the aging process (McCabe et al. 2020).

Intrinsic changes in the skin tissue during lifetime occur chronologically. One remarkable change in the skin tissue is the reduction of epidermal thickness, which is caused by a decreased proliferation of cells in the basal cell layer. Cells affected by a decreased proliferation ability are fibroblasts, keratinocytes and melanocytes. These cells are thought to undergo a senescence process and are therefore in a growth arrest. This reduces the contact surface area between dermis and epidermis, the basement membrane, which results in a weakened supply of nutrients to the epidermis (Zhang and Duan 2018). Senescent fibroblasts are accumulated in aged skin and thought to drive

skin aging through the release of a SASP factors, consisting of mainly chemokines and pro-inflammatory factors (Dimri et al. 1995, Ressler et al. 2006). Endothelial dysfunction and impaired angiogenesis during aging result in declines of the microvasculature, which also contribute to decreased nutrition supply of the tissue (Scioli et al. 2014). Decreased levels of ECM molecules as collagen and elastic fibers are observed in aged compared to young skin is suggested to occur because of a downregulation of the TGF- $\beta$  signaling observed in these cells (Quan et al. 2010, McCabe et al. 2020). In addition, increased collagen and elastin degradation due to increased activity of MMPs has been observed in sun-exposed skin (Brenneisen et al. 1996, Bernstein et al. 1996). Thus, not only intrinsic aging leads to a decrease in ECM, but also external stress factors such as UV radiation promote the ECM degradation. Several molecular mechanisms are proposed to explain the basis for skin aging. These include mechanisms described in the hallmarks of aging, such as DNA damage, loss of telomeres and chronic inflammation (Lopez-Otin et al. 2013). Telomere shortening is suggested to play a role in the reduced proliferation capacity found in epidermal stem cells and, thus the proliferation of cells found in the basement membrane (Siegl-Cachedenier et al. 2007). However, telomere shortening is considered to play no role in the aging of dermal fibroblasts (Tigges et al. 2014). Chronic inflammation is one major characteristic of aged skin and is suggested to be the result of accumulated damage. Dermal macrophages are the major source of pro-inflammatory factors. It has been reported that chronic activation of dermal macrophages via repeated UV exposition resulted in high amounts of pro-inflammatory cytokines and ROS, which both promote damage to the dermis. Oxidative stress is reported to induce NF $\kappa$ B signaling, which drives the expression of several pro-inflammatory factors like IL-6 or TNF- $\alpha$ . In addition, these activated macrophages release high amounts of MMPs and therefore are involved in the degradation of ECM structure (Zhuang and Lyga 2014, Kammeyer and Luiten 2015). Within the ECM, mesh-like networks of blood and lymph vasculatures are distributed, which together provide a scaffold for sufficient immune cell migration (Kabashima et al. 2019). Both lymphatics and blood vasculatures are impaired in aged tissue (Ryan 2004). Aged skin also shows a decline in regeneration capacity, which can be observed in delayed skin wound healing upon injury (Swift, Kleinman, and DiPietro 1999). Inflammation resolution as well as remodeling of the tissue are significantly slowed down (Gosain and DiPietro 2004).

#### **1.4.2 Aging of the immune system**

Aging of the immune system is associated with low-grade inflammation and defects in the immune responses and affects both the adaptive and innate immune systems. It is

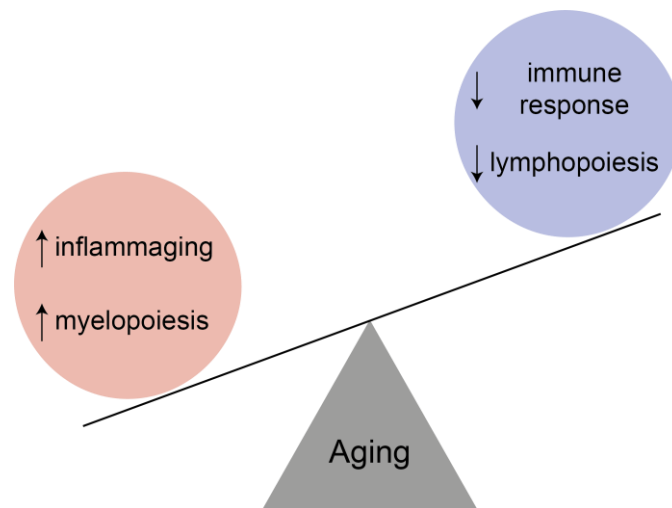
mainly characterized by an imbalance between the innate and adaptive immune system (Figure 7). The defects in the immune response, like delayed or diminished immune response, are mostly observed in the adaptive immune system and are referred to as immunosenescence. The decreased adaptive immune function is mainly attributed to insufficient naïve lymphocyte production, which is caused by thymic involution beginning after puberty (Linton and Dorshkind 2004, Palmer 2013, Rezzani et al. 2014). These diminished immune functions exhibit a decreased ability to respond to vaccination (Osterholm et al. 2012, Bondada et al. 2000) or increased vulnerability to infections, cancer and autoimmune diseases (Quandelacy et al. 2014, van der Poll and Opal 2009). The TRIIM (thymus regeneration, immune restoration and insulin mitigation) study demonstrates the critical role of immune aging in the holistic aging of the whole human organisms. In the study, Fahy and colleagues were able to regenerate the thymus through combined treatment with growth hormones and metformin and concluded that this reversed the aging of the subjects. Although the study has some limitations, such as the small number of subjects, there is evidence that thymic regeneration has beneficial effects on the immune response of cells of the innate and adaptive immune systems (Fahy et al. 2019).

However, not only the adaptive immune system is impaired with age, also the cells of the innate immune system change with age. A chronic low-grade inflammation in multiple tissues caused by unbalanced production of pro- and anti-inflammatory cytokines has been observed (Mogilenko, Shchukina, and Artyomov 2021). This so called inflammaging is characterized by increased production of pro-inflammatory cytokines, such as IL-6, IL-1 and TNF- $\alpha$  (Franceschi et al. 2000). Several mechanisms are suggested to contribute to this increased pro-inflammatory environment upon age. Macrophages are thought to play a central role in this inflammaging process, as they are the main source of pro-inflammatory factors (Franceschi et al. 2018). Upon activation, the expression of TNF- $\alpha$  and IL-6 is reported to be increased in aged tissue macrophages, while the secretion of anti-inflammatory cytokines seems to be decreased in aged macrophages (Njie et al. 2012, Thevaranjan et al. 2017). Furthermore, the resolution of inflammation is impaired in aged organisms, which can be explained by the reduced expression of anti-inflammatory molecules by macrophages. Additionally, a decrease in the number of anti-inflammatory M2 macrophages has already been shown in aged murine liver, whereas they seemed to be increased in the murine spleen, lymph nodes and bone marrow with age. Transcriptome analysis of several tissues at ten-time points of the mouse's lifetime reveals the greatest changes in immune response in the adipose tissue. This suggests that adipocytes and fat-associated macrophages, the most

abundant cells in adipose tissue, play a key role in the inflammaging process (Schaum et al. 2020). It has also been shown previously that peritoneal macrophages from aged compared to young mice exhibit reduced phagocytic activity. Aged macrophages seem to express lower levels of receptors involved in phagocytosis (Linehan et al. 2014, Wong et al. 2017). Macrophages are responsible for the clearance of senescent cells by phagocytosis. Whether the reduced phagocytosis capacity of aged macrophages could contribute to the accumulation of senescent cells during aging is not clear. Macrophages can also undergo cellular senescence, characterized by the secretion of SASP factors (De Maeyer and Chambers 2021).

Senescent cells accumulate with advanced age, and increasing amounts of secreted SASP factors can drive the microenvironment to display chronic pro-inflammatory tissue (Deleidi, Jaggle, and Rubino 2015). Furthermore, SASP promotes the infiltration of immune cells and their reprogramming to a pro-inflammatory and dysfunctional state (Mogilenko, Shchukina, and Artyomov 2021).

Additionally, studies have reported a shift from lymphopoiesis to more myelopoiesis during aging, resulting in increased numbers of myeloid cells (Cho, Sieburg, and Muller-Sieburg 2008, Melamed and Scott 2012, Wang, Geiger, and Rudolph 2011). For instance, monocyte abundance is reported to be increased in the blood of aged individuals compared to young (Nyugen et al. 2010, Costantini et al. 2018). According to their expression of CD14 and CD16, they are classified as classical (CD14<sup>++</sup>CD16<sup>-</sup>), intermediate (CD14<sup>+</sup>CD16<sup>+</sup>) and non-classical (CD14<sup>-</sup>CD16<sup>++</sup>) monocytes. Furthermore, several studies showed relations of the changed distribution of subsets with age-related diseases (Mukherjee et al. 2015). The non-classical monocyte population was reported to be increased with advanced age and is suggested to display a more pro-inflammatory phenotype (Seidler et al. 2010).



**Figure 7 Aging of the immune system.** Aging of the immune system is mainly characterized by an imbalance between the innate and the adaptive immune response. The adaptive immune response is decreased and delayed in aged individuals. In contrast, a low-grade systemic inflammation, termed inflammaging, occurs during aging. This is characterized by increased levels of pro-inflammatory cytokines produced primarily by cells of the innate immune system. In addition, a shift from less lymphopoiesis to more myelopoiesis during aging results in increased numbers of myeloid cells.

The relationship between inflammaging and immunosenescence is not well-understood. It is suggested that increased inflammatory response is a compensatory mechanism to support the decline in adaptive immunity (Dominguez-Andres and Netea 2019).

## 1.5 Objective of the study

Macrophages became of great interest in the field of aging research since they are reported to play a role in several age-dependent disorders. In healthy tissues, they are involved in tissue homeostasis but also in promoting and resolving inflammatory responses. Each tissue has a unique macrophage population. Age-dependent changes in macrophage phenotype and function have already been reported for several tissues but differ from organ to organ. However, whether and how skin macrophages change their phenotype and function with age has not yet been described. Furthermore, how aged skin macrophages are involved in the development of age-related skin diseases has not been fully elucidated.

In general, this study aims to gain comprehensive knowledge of age-related changes of macrophages in human skin tissue and their significance in the phenotypical changes of human skin upon aging. First, it was investigated whether the number of macrophages and their activity status changes in aged skin tissue *ex vivo*. In addition, the extent to which intrinsic or extrinsic factors contribute to these changes was investigated. To investigate intrinsic aging, the phenotype and function of aged compared with young

MDMs were examined in vitro. The influence of extrinsic factors on macrophage phenotype was determined using co-culture experiments or treatment of macrophages with supernatants from aged or senescent human dermal fibroblasts (HDFs). Finally, the extent to which macrophage changes affect dermal fibroblasts was assessed to determine the contribution of macrophages to age-related changes in the skin. To this end, the influence of macrophage phenotypes M1 and M2 on the expression of various genes in the HDF involved in metabolism, inflammatory status and ECM remodeling was investigated. Thus, both the influence of macrophages on skin aging and the influence of aged tissue on macrophages could be assessed.

## 2 Materials and Methods

In this section, the materials and methods, which were used throughout this work are specified. If not stated otherwise, all methods were performed as specified in this section.

### 2.1 Materials

#### 2.1.1 Standard Solutions, Chemicals and Buffers

Table 1: List of standard solutions, chemicals and buffers

Chemical	Name	Company
10% BSA stock solution	MACS® BSA stock solution	Miltenyi, Bergisch Gladbach, Germany
Bacillo® AF		VWR International GmbH, Darmstadt, Germany
BSA	Albumin Fraction V	Carl Roth GmbH & Co. KG Karlsruhe, Germany
CS&T research beads	BD FACSDiva™ CS&T Research Beads	Becton Dickinson, Franklin Lakes, USA
DMSO	DMSO Hybri-Max™	Sigma Aldrich, Saint Louis, USA
Ethanol	Ethanol absolut EMSURE®	Merck, Darmstadt, Germany
FACS Buffer	eBioscience™ Flow Cytometry Staining Buffer	Thermo Fisher Scientific Waltham, USA
FcR Blocking reagent	FcR Blocking reagent, human	Miltenyi, Bergisch Gladbach, Germany
Ficoll	Ficoll-Paque™	VWR International GmbH, Darmstadt, Germany
Fixation and permeabilization buffer	eBioscience™ intracellular Fixation & Permeabilization Buffer	Thermo Fisher Scientific Waltham, USA
Formaldehyde	Formaldehyde solution 37%	Sigma Aldrich, Saint Louis, USA
L-Glutamin	Glutamax	Thermo Fisher Scientific Waltham, USA
Liquid Nitrogen		
MACS Rinsing Buffer	autoMACS® Rinsing Solution	Miltenyi, Bergisch Gladbach, Germany
PAP Pen	ImmEdge™ Hydrophobic Pen	ACD bio-technie, Newark, USA
PBS (1x)	Gibco™ DPBS	Thermo Fisher Scientific Waltham, USA
Penicillin-Streptomycin (P/S)	Gibco™ Penicillin-Streptomycin	Thermo Fisher Scientific Waltham, USA
pHRodo Zymosan particles	pHrodo™ Green Zymosan Bioparticles™ Conjugate	Thermo Fisher Scientific, Waltham, USA
Polysorbat	Tween®20	Sigma Aldrich,
Propidiumiodid		Thermo Fisher Scientific Waltham, USA
Rainbow Calibration Beads	SPHERO™ Rainbow Calibration Particles (8 peaks)	Becton Dickinson, Franklin Lakes, USA



Chemical	Name	Company
RNA ScreenTape Ladder	Agilent RNA ScreenTape Ladder Agilent High Sensitivity RNA ScreenTape Ladder	Agilent, Santa Clara, USA
RNA ScreenTape Sample Buffer	Agilent RNA ScreenTape Sample Buffer Agilent High Sensitivity RNA ScreenTape Sample Buffer	Agilent, Santa Clara, USA
RNA ScreenTapes	Agilent RNA ScreenTape Agilent High Sensitivity RNA ScreenTape	Agilent, Santa Clara, USA
Sodium hypochlorite solution 5%		VWR International GmbH, Darmstadt, Germany
$\beta$ -Mercaptoethanol	Gibco™ 2-Mercaptoethanol	Thermo Fisher Scientific Waltham, USA

### 2.1.2 Enzymes

Table 2: List of enzymes

Name	Company
0.25% Trypsin EDTA	Thermo Fisher Scientific, Waltham, USA
DNase I	Qiagen, Hilden, Germany

### 2.1.3 Kits

Table 3: List of commercial kits

Kits	Name	Company
Accuracy & fluorescence detection kit	The Guava® easyCheck™ Kit	Merck Milipore, Darmstadt, Germany
Cell detection kit for counting cell numbers	Guava® ViaCount™ Reagent	Merck Milipore, Darmstadt, Germany
ELISA kit for detection of procollagen Type I	Procollagen Type I C-Peptide (PIP) EIA Kit	Takara Biotec Inc, Kyoto, Japan
Monocyte Isolation Kit	Pan Monocyte Isolation Kit, human	Miltenyi, Bergisch Gladbach, Germany
nCounter Sprint reagent pack	nCounter SPRINT™ Reagent Pack	NanoString Technologies, Seattle, USA
qPCR Master Mix	TaqMan® Fast Advanced Master Mix	Thermo Fisher Scientific Schwerte, Germany
Reverse Transcription Kit	Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific, Waltham, USA
RNA Isolation Micro kit	miRNeasy® Micro Kit	Qiagen, Hilden, Germany
RNA Isolation Mini kit	RNeasy® Mini Kit	Qiagen, Hilden, Germany

### 2.1.4 Stimulants

Table 4: List of stimulants and cytokines

Name	Compound	Stock	Company
GM-CSF	Granulocyte-macrophage colony-stimulating factor	50 µg/µL	Thermo Fisher Scientific Waltham, USA
IFN-γ	Interferon gamma	20 µg/µL	Promocell, Heidelberg, Germany
IL-13	Interleukin 13	20 µg/µL	Promocell, Heidelberg, Germany
IL-4	Interleukin 4	20 µg/µL	Promocell, Heidelberg, Germany
LPS	lipopolysaccharide	100 µg/µL	Enzo Biochem, New York, USA
M-CSF	macrophage colony-stimulating factor	50 µg/µL	Thermo Fisher Scientific Waltham, USA
PMA	Phorbol-12-myristat-13-acetat	20 µM	Sigma-Aldrich, St. Louis, USA

### 2.1.5 TaqMan probes

Table 5: List of commercial TaqMan probes

Name	TaqMan	Company
C1QB	Hs00608019_m1	Thermo Fisher Scientific Waltham, USA
CXCL1	Hs00236937_m1	Thermo Fisher Scientific Waltham, USA
GAPDH	Hs99999905_m1	Thermo Fisher Scientific Waltham, USA
HSPA1B	Hs01040501_sH	Thermo Fisher Scientific Waltham, USA
IL-10	Hs00961622_m1	Thermo Fisher Scientific Waltham, USA
IL-12	Hs01073447_m1	Thermo Fisher Scientific Waltham, USA
IL-6	Hs00174131_m1	Thermo Fisher Scientific Waltham, USA
TGF-β	Hs00998133_m1	Thermo Fisher Scientific Waltham, USA
TNF-α	Hs00174128_m1	Thermo Fisher Scientific Waltham, USA

## 2.1.6 Antibodies

Table 6: List of antibodies

Name	Conjugate	Host	Dilution	Company
Anti-CD14 IgG1, $\kappa$ Clone 63D3	FITC	mouse	1/100	Biolegend
Anti-CD16 IgG1, $\kappa$ Clone 3G8	APC	mouse	1/100	Biolegend
Anti-CD163 IgG1, $\kappa$ Clone GHI/61	PE	mouse	1/20	Biolegend
Anti-CD206 IgG1, $\kappa$ Clone 15-2	APC/Cy7	mouse	1/50	Biolegend
Anti-CD209 IgG1, $\kappa$ Clone DCS-8C1	BV421	mouse	1/20	Biolegend
Anti-CD68 IgG1, $\kappa$ Clone KP1	-	mouse	1/1000	Thermo Fisher Scientific Waltham, USA
Anti-CD80 IgG1, $\kappa$ Clone 2D10	APC	mouse	1/50	Biolegend
Anti-IL-10 IgG2a, $\kappa$ Clone JES3-19F1	PE/Dazzle	rat	1/20	Biolegend
anti-mouse IgG	AlexaFluor™ 546	goat	1/1000	Thermo Fisher Scientific Waltham, USA
Anti-TNF- $\alpha$ IgG1, $\kappa$ Clone MAb11	BV421	Mouse	1/20	Biolegend

## 2.1.7 Cells and cell culture media

Table 7: List of cell culture media

Name	Components	Company
Fetal calve serum (FCS)		VWR International GmbH, Darmstadt, Germany
Gibco™ DMEM	10 % FCS (v/v) 4.5 g/L D-Glucose 25 mM HEPES Buffer Sodium Pyruvate	Thermo Fisher Scientific Waltham, USA
Gibco™ RPMI 1640 Medium	10 % FCS (v/v) $\beta$ -Mercaptoethanol L-Glutamine	Thermo Fisher Scientific Waltham, USA

Table 8: List of cells

Cultivated cells	Origin	Cultivation
HDF	Isolated from human skin biopsies	DMEM 10% FCS (v/v) 1% P/S (v/v)
PBMC (peripheral blood mononuclear cells)	Isolated from human blood	RPMI 1640 10% FCS (v/v) 50 $\mu$ M $\beta$ -Mercaptoethanol

## 2.1.8 Equipment

Table 9: List of equipment

Instrument	Name	Company
Automated Electrophoresis	4200 TapeStation System	Agilent, Santa Clara, USA
Cell culture bench	HeraSafe™ KS18	Thermo Fisher Scientific Waltham, USA
Centrifuge	Heraeus® Fresco 21	Thermo Fisher Scientific Waltham, USA
	Heraeus® Multifuge 3SR+	Thermo Fisher Scientific Waltham, USA
	MiniStar silverline	VWR International GmbH, Darmstadt, Germany
CO <sub>2</sub> incubator	HERAcell 150	Thermo Fisher Scientific Waltham, USA
Dispenser	Multipette® plus Multipette® E3	Eppendorf, Hamburg, Germany
Flow cytometer	BD LSRFortessa™	BD Biosciences, Franklin Lakes, USA
	Guava easyCyte™	Merck Milipore TM, Billerica, USA
Freezing device	Corning®CoolCell®	Corning, New York, USA
Imaging Cytometer	SpectraMax® MiniMax™ 300	Molecular Devices, San Jose, USA
MACS multi stand magnet	quadroMACS™	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Microscopy	Olympus IX81	Olympus, Tokyo, Germany
Multichannel pipettes (0.5-1000 $\mu$ L)	Eppendorf Research® plus multi-channel pipette	Eppendorf, Hamburg, Germany
nCounter Sprint™ Profiler		NanoString Technologies, Seattle, USA
PCR Cycler	Eppendorf Mastercycler® nexus	Eppendorf, Hamburg, Germany
pH meter	FiveEasy™	Mettler Toledo, Columbus, USA
Pipetboy	Easypet	Eppendorf, Hamburg, Germany

<b>Instrument</b>	<b>Name</b>	<b>Company</b>
Pipette (0.1-1000 µL)		Eppendorf, Hamburg, Germany
Plate reader	Tecan Infinite M200	Thermo Fisher Scientific Waltham, USA
Precision scale	XS105	Mettler Toledo, Columbus, USA
Real Time PCR System	7900HT Fast Real-Time PCR System	Thermo Fisher Scientific Waltham, USA
Robotic workstation for purification of RNA	QIAcube	Qiagen, Hilden
Shaker	Grant-bio PMR-30	Thermo Fisher Scientific Waltham, USA
Thermomixer	Eppendorf ThermoMixer® comfort	Eppendorf, Hamburg, Germany
Tissue dissociator	gentleMACS Dissociator™	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Ultrasonic homogenizer		
Vacuum pump	VACUSAFE	Integra Biosciences, Biebertal, Germany
Vortex	IKA MS basic IKA MS3	IKA, Staufen im Breisgau, Germany
Water bath		GFL, Burgwedel, Germany

### 2.1.9 Consumables and Plastic Ware

Table 10: List of consumables and plastic ware

<b>Subject</b>	<b>Name</b>	<b>Company</b>
Cell culture flask	T25, T75, T175	VWR International GmbH, Darmstadt, Germany
Cell culture inserts	0.4 µm cell culture inserts	VWR International GmbH, Darmstadt, Germany
Cell culture plates	6-96 well	VWR International GmbH, Darmstadt, Germany
Cell strainer	100 µm	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Coverslips		VWR International GmbH, Darmstadt, Germany
Cryogenic tubes	CryoTubes™ Nunc™	VWR International GmbH, Darmstadt, Germany
Flow cytometry tube	Flow Cytometry Tube 5 mL	VWR International GmbH, Darmstadt, Germany
Magnetic columns	MACS LS columns	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Microscopy slides		Thermo Fisher Scientific Waltham, USA

Subject	Name	Company
nCounter sprint cartridge		NanoString Technologies, Seattle, USA
Optical adhesive film	microAmp®	Life Technologies™ Darmstadt, Germany
Parafilm	PARAFILM® M	VWR International GmbH, Darmstadt, Germany
Pasteur pipette		VWR International GmbH, Darmstadt, Germany
qPCR 96-well plates	MicroAmp™ Fast Optical 96-Well-reaction plate	Life Technologies™, Darmstadt, Germany
Reagent Reservoirs		VWR International GmbH, Darmstadt, Germany
Serological pipette (5 mL-50 mL)		VWR International GmbH, Darmstadt, Germany
Surgical disposable scalpel		VWR International GmbH, Darmstadt, Germany
Tips (10-1000 µL)	Combitips advanced®	Eppendorf, Hamburg, Germany
Tissue dissociation tubes	gentleMACS™ C tubes	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Tube	15-50 mL tube	VWR International GmbH, Darmstadt, Germany
Tubes (0.5-5.0 mL)	Safelock tubes	Eppendorf Hamburg, Germany
Tubes (0.5-5.0 mL)	Biopur®	Eppendorf Hamburg, Germany

### 2.1.10 Software

Table 11: List of software

Name	Version
Adobe Illustrator 2021	CC 2021
EndNote	X8
FlowJo 10.6	10
GraphPad Prism	8
ImageJ	1.53k
nSolver™ Data Analysis	4.0
RQ Manager	1.2
SDS	2.3

## 2.2 Methods

### 2.2.1 Molecular biological Methods

#### 2.2.1.1 RNA-Isolation using the RNeasy® Mini Kit

RNeasy® Mini Kit from Qiagen was used to isolate RNA from MDM or HDF according to the manufacturer's standard protocol, including DNase I digestion. The isolation was performed with the QIAcube system from Qiagen. Concentration and quality of RNA was measured by the 4200 TapeStation System.

#### 2.2.1.2 Determination of RNA concentration and quality

RNA concentration and quality were determined by using RNA ScreenTape in the 4200 TapeStation System according to the manufacturer's protocol. The RNA Integrity Number equivalent (RIN<sup>e</sup>) provides a user independent quality score for total RNA. Samples with a RIN<sup>e</sup> of >8.0 were used for further analysis.

#### 2.2.1.3 Reverse transcription

For further analysis of the mRNA expression, the RNA was first transcribed into cDNA by reverse transcription polymerase chain reaction (RT-PCR) by using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit. The reaction mixtures were prepared according to Table 12 and subsequently incubated in a thermal cycler following the program described in Table 13.

Table 12: Composition of reaction mixture for cDNA synthesis

Component	Volume	Final concentration
RNA	XX µL	500 ng
RT Buffer (10x)	10.0 µL	1x
dNTP Mix (100 nM)	4.0 µL	8 nM
RT Random Primers (10x)	10.0 µL	1x
MultiScribe™ Reverse Transcriptase (50 U/µL)	5.0 µL	-
RNase inhibitor (20 U/µL)	5.0 µL	-
Invitrogen™ RT-PCR Grade H <sub>2</sub> O	XX µL	-
Total per Reaction	50.0 µL	

Table 13: Thermal cycler protocol used for RT-PCR

Step	Temperature	Time
Primer Binding	25 °C	10 min
Reverse transcription	37 °C	120 min
Transcriptase inactivation	85 °C	5 min
Storage	4 °C	∞

#### 2.2.1.4 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) is a method to detect and quantify nucleic acids during a PCR in real-time and thus used to determine mRNA expression levels to monitor transcriptional expression changes (Weis et al. 1992). In this study, we performed a probe-based real-time PCR method with TaqMan® assays provided by Applied Biosystems. Beside reverse and forward primers it contains a short oligonucleotide complementary to the target DNA, the probe, which includes a 5' reporter fluorophore and a 3' quencher and is called the probe. During the amplification of the DNA target the polymerase degrades the probe that has annealed to the template, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Therefore, detected fluorescence is directly proportional to the amount of DNA template present in the PCR sample.

The obtained cDNA as described in 2.2.1.3 was quantified using a 7900HT fast real-time PCR system together with Applied Biosystems™ TaqMan™ Fast Universal PCR Master Mix and TaqMan® assays. The reaction mixtures for qPCRs were prepared with target specific TaqMan® assays, according to Table 14 and processed as described in Table 15.

Table 14: Reaction mixture for qPCR

Component	Volume	Final concentration
Applied Biosystems™ TaqMan™ Fast Universal PCR Master Mix (2x)	10.0 µL	1x
TaqMan® assays (20x)	1.0 µL	1x
cDNA	1.5 µL	-
Invitrogen™ RT-PCR Grade H <sub>2</sub> O	XX µL	-
Total per Reaction	20.0 µL	



Table 15: Thermal cycler protocol used for qPCR

Step	Temperature	Duration	Time
Initial Denaturation	95 °C	20 sec	HOLD
Denaturation	95 °C	1 sec	40x loop
Annealing/Elongation	60 °C	20 sec	

The cycle threshold (Ct) was determined by the RQ Manager 1.2 software (Thermo Fisher Scientific). It indicates the number of cycles at which the fluorescent signal becomes higher than the background. The higher the cDNA amount is in the sample, the sooner the threshold reaches and thus the lower the Ct. Ct values of the target genes were first normalized to the constant Ct value of an endogenous reference gene yielding the  $\Delta\text{Ct}$ . In this study Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference. Expression analysis was performed with the  $2^{(-\Delta\text{Ct})}$  method (Livak and Schmittgen 2001). When comparing treated samples to an untreated control,  $2^{(-\Delta\Delta\text{Ct})}$  method was performed.

Table 16 Overview about analysis methods for qPCR

$\Delta\text{Ct}$	Ct target gene – Ct reference gene
$\Delta\Delta\text{Ct}$	$\Delta\text{Ct}$ (treated sample) - $\Delta\text{Ct}$ (untreated sample)
$-(\Delta\Delta\text{Ct})$	log2-fold change
$2^{-(\Delta\Delta\text{Ct})}$	Linear x-fold change

### 2.2.1.5 NanoString nCounter gene expression analysis

The NanoString nCounter gene expression system has been developed for gene expression analysis by using native RNA without enzymatic reactions. It is based on the detection of a target mRNA molecule by two sequence-specific probes, via molecular barcoding by fluorescence microscopy. Both probes consist of a sequence of 35 to 50 bases complementary to the target mRNA and are coupled to either an affinity tag as biotin (capture probe) or a color-coded tag that provides the detection signal (reporter probe). The molecular barcoding technology allows the detection of up to 800 genes of interests in one reaction mixture. In a first step the mRNA is hybridized with the capture and reporter probes followed by the application on a cartridge, which is coated with a capture reagent. Thus, by flushing the reaction mixture across the surface the hybridized

probe-mRNA complexes bind to the surface and unhybridized reporter and captures probes are removed. By applying an electric field each complex is oriented in the same direction allowing them to be imaged by the integrated fluorescence microscopy. Therefore, levels of expression can then be counted by the number of codes specific for each mRNA detected by the microscope (Geiss et al. 2008).

For gene expression analysis by the NanoString nCounter system, 90 ng of RNA were diluted in Invitrogen™ RT-PCR Grade H<sub>2</sub>O in a final volume of 15 µL. The hybridization master mix was prepared by adding 70 µL Hybridization Buffer to the reporter CodeSet. The hybridization reaction mixture was then prepared according to Table 17 and incubated for 24 hours at 65 °C.

Table 17: Composition of hybridization reaction mixture

Component	Volume	Final concentration
RNA	5 µL	90 ng
Hybridization master mix	8.0 µL	-
Capture ProbeSet	2.0 µL	-
Total per Reaction	15.0 µL	

After hybridization, the samples volume was adjusted to 35 µL with RT-PCR Grade H<sub>2</sub>O, of which 30 µL were loaded into the cartridge. A transparent seal was placed over the sample loading ports and the protective seal on the reagent ports was removed before starting the run. NanoString data analysis was performed by the nSolver system and raw counts were normalized to both positive control probes and housekeeping gene probes GAPDH, GUSB, OAZ1, PUM1 and UBC.

## 2.2.2 Cell Culture Methods

### 2.2.2.1 Isolation of HDFs from skin biopsies

HDFs were isolated from skin samples from breast or abdominal reduction surgery obtained from Alphenyx, Marseille, France. The enzyme dispase cleaves fibronectin, thus the connection between epidermis and dermis is disrupted and can be separated. Furthermore, collagenase was used to break down the ECM to be able to isolate fibroblasts out of the dermis skin structure. By this technique, HDFs could be isolated dermis.

To start the isolation of primary cells, skin pieces were first washed in 70 % ethanol, followed by sterile phosphate-buffered saline (PBS). Subcutaneous fat was removed before skin samples were cut into around 0.5 cm slices. Afterwards skin slices were incubated with dispase (2.4 U/mL, 2.2 mg/mL in PBS) at 4 °C over night to separate epidermal from dermal sheets. To isolate HDFs from the dermis, the dermal sheets were cut into smaller pieces and incubated in collagenase I (312 U/mL, 4 mg/mL in DMEM 1 % P/S) for 4 hours at 37 °C. Dermal sheets were then transferred into gentleMACS™ C Tubes together with the collagenase I solution and skin protocol in gentleMACS™ Dissociator was performed. The cell suspension was filtered through a 100 µm cell strainer and centrifuged at 250 x *g* for 10 min. The cell pellet was resuspended in DMEM 10 % FCS and 1 % P/S, plated in a T175 flask and incubated at 37 °C and 5 % CO<sub>2</sub>. Medium was exchanged after 24 hours cultivation.

#### **2.2.2.2 Cell cultivation of HDFs**

HDFs were cultivated in DMEM with 10 % FCS (v/v) at 37 °C and 5 % CO<sub>2</sub> and the medium was exchanged every two to three days. At a confluence of 90 % cells were subcultured or frozen. For subculturing either 3 x 10<sup>5</sup> cells were seeded into a T75 flask or for cryopreservation 1 x 10<sup>6</sup> cells resuspended in DMEM with 20 % FCS (v/v) and 10 % DMSO (v/v) and frozen in a Corning®CoolCell® freezing device for a controlled freezing rate of -1°C/min before storage at -140 °C.

#### **2.2.2.3 Isolation of peripheral blood mononuclear cells**

Human blood consists of blood plasma and blood cells, namely erythrocytes, leukocytes and thrombocytes. Leukocytes comprise of monocytes and lymphocytes and contain a single nucleus, the reason why they are referred as PBMCs. These cells can be separated from the other components by taking advantage of the density differences between them and other cells and components of the human blood. The separation by Ficoll-Paque gradient was developed by Böyum in 1968 and has since become a standard method for the isolation of immune cells (Boyum 1968). Due to their lower density than Ficoll-Paque, PBMCs accumulate at the Ficoll layer and can be easily taken up, whereas the erythrocytes and granulocytes pass through the Ficoll layer based on their higher density.

PBMCs were isolated from human blood. Blood samples were either obtained from Institute of Transfusion Medicine, UKE Hamburg, or through a clinical study in agreement with the recommendations of the Declaration of Helsinki. The study has been approved

by the Ethics Commission (general approval Nov 24, 2020; BDF No. 70521, SGS Ref. 201-01-2057, IRB Ref.-Nr: 2020/122). The blood samples were diluted in 1:3 ratio with PBS and carefully stacked onto a cushion of Ficoll solution in a 50 mL reaction tube. After a centrifugation of the two layers for 15 min at 800 x g with minimal deceleration, the distinct leukocyte layer, representing the PBMCs, was collected. Cells were washed twice by resuspending the cell pellet in 40 mL MACS® Rinsing solution and centrifuged at 300 x g for 10 min at 4 °C. For cryopreservation,  $2 \times 10^7$  isolated PBMCs were then taken up in FCS 10 % DMSO (v/v) and frozen in a Corning®CoolCell® freezing device for a controlled freezing rate of -1°C/min before storage at -140 °C.

#### **2.2.2.4 Isolation of human monocytes from PBMCs and differentiation to macrophages**

Human monocytes were isolated from human PBMCs using the Pan Monocyte Isolation Kit via magnetic activated cell sorting (MACS) provided by Miltenyi Biotec according to the manufacturer's protocol. The kit uses a cocktail of biotin-conjugated antibodies and anti-biotin MicroBeads to magnetically label the non-monocytes like lymphocytes and dendritic cells. The differentiation from monocytes to MDMs was done by using the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF).

Volumes of reagents used for the isolation of up to  $1 \times 10^7$  cells are described in Table 18, when higher cell numbers were used in the isolation, appropriate volumes of reagents were used. For this purpose, cells were thawed in 20 mL MACS Buffer and centrifuged for 7 min at 300 x g and 4 °C. The supernatant was removed and the cell pellet was collected in an appropriate volume of MACS Buffer. To prevent unspecific binding of antibodies the Fc were blocked with FcR blocking reagent. In addition biotin antibody cocktail were added to the single cell suspension. After an incubation period of 5 min in the refrigerator at 4 °C, an appropriate volume of MACS Buffer and anti-biotin microbeads was added. While the cell suspension was incubated for 10 min at 4 °C, the LS MACS column was placed on the quadroMACS™ separator and equilibrated with 3 mL MACS Buffer. The flow-through was collected in a 50 mL reaction tube. After the incubation period, the cell suspension was applied to the column and the flow-through was collected in the same tube. The column was then washed three times with 3 mL MACS buffer and the flow-through was collected in the same tube as before. The collected flow-through contains the unlabeled cells, representing the monocytes. 30 mL of MACS buffer was added to the cell suspension and subsequently centrifuged at 300 x g for 10 min to wash the cells. Cell yield was determined as described in 2.2.2.6.

The cell amount per mL was adjusted to  $2 \times 10^6$ /mL in culture medium. For differentiation to macrophages, 500  $\mu$ L of monocyte cell suspension was seeded into one well of a 12-well plate or 125  $\mu$ L for culturing in one well of a 48-well plate. Subsequently the same volume of differentiation medium was added to the cells (M1 macrophages: 100ng/mL GM-CSF, M2 macrophages 100ng/mL M-CSF), resulting in a final differentiation stimulant concentration of 50 ng/mL. Cells were incubated for 7 days at 37 °C and 5 % CO<sub>2</sub> without media exchange. Polarization was then performed with 100 ng/mL LPS and 20 ng/mL interferon gamma (IFN- $\gamma$ ) for M1 macrophages or 20 ng/mL interleukin 4 (IL-4) and 20 ng/mL interleukin 13 (IL-13) for M2 macrophages. For later RNA analysis, cells were collected after 20 h, for flow cytometry analysis after 24 hours.

Table 18: Volumes of reagents used for magnetic labeling for up to  $1 \times 10^7$  cells

Component	Volume per $1 \times 10^7$ cells
MACS Buffer 0.5% BSA	40 $\mu$ L
FcR Blocking Reagent	10 $\mu$ L
Biotin-Antibody Cocktail	10 $\mu$ L
MACS Buffer 0.5% BSA	30 $\mu$ L
Anti-Biotin MicroBeads	20 $\mu$ L
Total per Reaction	110 $\mu$ L

#### 2.2.2.5 Phagocytosis assay

In order to investigate the phagocytotic activity in macrophages, pHrodo™ Green Zymosan Bioparticles™ conjugates were used. The pHrodo™ zymosan A BioParticles are composed of a fluorogenic dye conjugated to the polysaccharide zymosan, which can be found on the surface of fungi. Macrophages can recognize zymosan via TLR2 and Dectin-1, scavenger receptors expressed on their surface and phagocyte the particles (Sato et al. 2003, Brown et al. 2002). Phagocytosed zymosan particles fluoresce in phagosomes due to acidic pH 4 in phagosomes. These conjugates are pH-sensitive, leading to low signal at neutral pH, but a bright fluorescent emission at acidic pH such as in phagosomes at pH 4. Thus, phagocytic active macrophages were identified by their higher fluorescence intensity than controlled macrophages cultivated without pHrodo™ zymosan A BioParticles.

2,5 x10<sup>4</sup> human MDMs were cultivated in 48-well plates and polarized to either M1 or M2 macrophages. After 24 hours of polarization the medium was exchanged by 100 µL fresh media plus 20 µL pHrodo™ Green Zymosan A BioParticles™ conjugate and incubated for 1.5 hours at 37°C and 5 % CO<sub>2</sub>. Afterwards the supernatants of the cells were collected. The cells were rinsed with 100 µL of PBS, which was added to the collected supernatants. Cells were then detached with 100 µL of 0.25% trypsin/EDTA for 5 min at 37°C and then immediately resuspended with 100 µL of FCS. The cell suspension was added to the supernatants collected before. The cell plate was again rinsed with 100 µL of PBS and added to the cell suspension. Thus, PBS and cell suspensions were collected at each step and combined in one tube for each sample. Afterwards cells were centrifuged at 300 x g for 5 min. The supernatant was discarded and the cell pellet resuspended in 200 µL PBS. 20 µL of 5 µg/mL propidium iodide was added right before analysis on the Guava® easyCyte flow cytometer (Merck Millipore) to exclude dead cells. Additionally, M1 and M2 macrophages were cultivated without conjugates to define gates for phagocytic and non-phagocytic macrophages.

#### **2.2.2.6 Determination of cell number**

Cell number was determined using Guava® ViaCount™ Reagent in the Guava® EasyCyte™ flow cytometer. The reagent combines two DNA-binding dyes with different permeability to detect dying cells in PE/Cy5 and PE and the nucleus only in PE, allowing viable cells to be distinguished from apoptotic and dead cells as well as debris. Cell suspensions were diluted 1:10 in the reagent and analyzed in the InCyte program of the flow cytometer. Live cell numbers were defined by using scatter characteristics and exclusion of dead cells.

### **2.2.3 Protein biochemical Methods**

#### **2.2.3.1 Enzyme-linked immunosorbent assay**

The enzyme-linked immunosorbent assay (ELISA) is an analytical biochemical method that uses antibodies to identify the presence of ligands as secreted proteins in a liquid sample developed by Engvall and Perlmann (Engvall and Perlmann 1971). In this study, the amount of secreted procollagen in supernatants of HDFs was determined by an ELISA. Measurement was carried out with the Procollagen Type I C-Peptide (PIP) EIA Kit provided by Takara Bio. The applied test is a solid phase sandwich ELISA consisting of an anti-PIP antibody pre-coated microtiter plate and an HRP-conjugated antibody. During incubation with anti-PIP, the PIP contained in the sample, binds to the anti-PIP

antibodies on the plate and an HRP-conjugated antibody recognizes the bound PIP. The enzymatic reaction of HRP with the substrates  $H_2O_2$  and TMBZ results in a color change which allows a quantification of PIP by measuring absorbance using a spectrophotometer. Analysis was performed according to the manufacturer's protocol. Supernatants were diluted 1:50 before measuring in duplicates in the assay. Measurement of absorbance was performed with the TECAN Infinite® M200. Procollagen in supernatants of dermal fibroblasts were normalized to cell number measured SpectraMax® MiniMax™ 300 Imaging Cytometer.

### **2.2.3.2 Immunohistochemistry**

Immunofluorescence staining was used to visualize the target molecule CD68 in skin section samples and thereby analyze the quantity of CD68<sup>+</sup> macrophages within the tissue. The method is based on specific binding of a primary antibody to the target molecule and a secondary antibody conjugated to a fluorophore, which recognizes and binds to the primary antibody subsequently. The attached fluorophore can then be detected via fluorescence by a microscope. For orientation purposes, nuclei were detected via Hoechst33342 staining. Abdominal of breast skin biopsies provided by Alphenyx, Marseille, were embedded in O.C.T TissueTek® and frozen at -80°C. 10 µm sections were prepared with a cryostat and dried at RT for 2 hours before being stored at -20 °C. Skin sections were fixed with 4 % paraformaldehyde for 10 min at 4 °C and afterwards washed three times for 5 min in PBS. Before blocking with 3 % BSA in PBS (v/v) for 1 hour at RT, the sections were bordered with a PAP pen. After washing three times with 0.05 % Tween-20 in PBS (v/v) for 5 min, the sections were incubated with the primary antibody in 3 % BSA in PBS at 4 °C over night. Following another three times washing with 0.05 % Tween-20 in PBS (v/v) for 5 min, the sections were incubated with the secondary antibody, which was diluted in 3 % BSA in PBS, or 2 hours at RT. Sections were then washed twice in PBS and afterwards nuclei were stained with Hoechst33342 (1/1.000 in PBS) for 15 min at RT. Sections were washed twice with PBS for 5 min and dried properly before mounting with ProLong™ Gold Antifade Mountant and covering with a coverslip. Sections were dried over night at 4 °C before they were taken using the Olympus IX81 microscope. Images were analyzed using the Fiji software.

### **2.2.3.3 Flow cytometry**

Flow cytometry is a laser-based method to analyze cell populations in their characteristics like cell size, granularity, or expression of surface or intracellular

molecules. Expression of target molecules is determined by detecting fluorescently labeled antibodies that specifically bind to a molecule of interest. Multiple lasers and fluorescence detectors enable the detection of several parameters at the same time and thus allowing a more precisely identification of a population by phenotypic markers.

For staining of secretory proteins, cells were stimulated with 5 µg/mL Brefeldin A 3.5 hours prior to staining, in order to inhibit protein transport processes to enhance intracellular cytokine staining. Viable cells were collected and resuspended to gain a single cell suspension. FcR blocking was performed by adding FcR Blocking Buffer in a total volume of 25 µL for 5 min on ice. For the staining of surface markers, the cell suspension was incubated with different combinations of fluorophore-conjugated monoclonal antibodies for 15 min. Cells were centrifuged at 300 x g for 7 min at 4 °C before washing one time with 200 µL of Flow Cytometry Staining Buffer followed by washing with 200 µL PBS. In order to exclude dead cells in the later analysis, the cell pellets were resuspended in 100 µL Zombie Green or Zombie Violet fixable viability stain (1:1.000 in PBS) for 15 min at RT. Cell suspensions were centrifuged at 300 x g for 7 min at 4 °C, followed by two washing steps with 200 µL Flow Cytometry Staining Buffer. For fixation, the cell pellet was resuspended thoroughly in 100 µL PBS followed by adding 100 µL 4 % PFA and resuspending again. After an incubation time of 15 min at RT the cell suspensions were centrifuged at 300 x g for 7 min at 4 °C. Cells were washed twice with 200 µL Flow Cytometry Staining Buffer. For intracellular staining the cell pellet was resuspended in 200 µL permeabilization buffer and centrifuged at 300 x g for 7 min at 4 °C. Afterwards, the cell pellet was resuspended in 25 µL Perm/block buffer for 5 min on ice followed by the incubation with fluorophore-conjugated antibodies at a final volume of 50 µL. After incubation of 30 min on ice, cells were washed two times in 200 µL permeabilization buffer. Subsequently, the cell pellet was resuspended in 100 µL Flow Cytometry Buffer. Cell suspensions were analyzed with a BD LSRFortessa™ flow cytometer. The data were analyzed using FlowJo software. Living cells were defined by scatter characteristics and exclusion of Zombie Green or Violet positive cells.

All antibodies used for flow cytometry analysis were ordered from Biolegend.

#### **2.2.4 Bioinformatic methods**

The bioinformatic methods described in this chapter were either performed by Dr. Cassandra Falckenhayn, Beiersdorf AG, or in Cooperation with Prof. Dr. Stefan Simm from the Institute for Bioinformatics, University Medicine Greifswald. In the results section, it is indicated by whom the analysis was performed.



#### **2.2.4.1 Single-cell RNA sequencing, quality control and sample sequencing processing**

scRNA-Seq data from human skin biopsies were analyzed by using the Seurat package (V 3.2.157) (Stuart et al. 2019) in R (V 3.6.358). In general, the data were pre-processed and visualized according to best-practices workflow (Luecken and Theis 2019). To pre-process the data, first quality filtering steps were performed, to remove possible cell multiplets and potential apoptotic and lysing cells, followed by log normalization for each individual dataset. For batch correction each sample was integrated by feature selection of highly variable genes. Datasets were then scaled to calculate principal component analysis dimensions for dimensionality reduction. With the most important dimensions an unsupervised clustering was performed with the Uniform Approximation and Projection method (UMAP) to visualize the data. Using marker genes specific for certain cell types, the generated clusters were analyzed on a gene level and annotated. Gene expression signatures used for the definition of cell populations were: RNASE1, CD68, SELENOP and C1QA (macrophages); CD1A, HLA-DRA and CD1C (dendritic cells) (Xue et al. 2020).

#### **2.2.4.2 Differential expression of pseudo-bulk RNA-seq**

Data derived from Ahlers et al. were used to perform a pseudo-bulk RNA-seq analysis to identify differentially expressed genes between macrophages derived from young and aged human skin (DEGs) (Ahlers et al. 2022). The differential expression analysis was performed as pseudo-bulk RNA-Seq approach for each cell cluster. To create pseudo-bulk RNA-seq samples per cell type cluster the sparse count matrix ("RNA") of the Seurat objects were summed up for each gene to then perform a scaling normalization by deconvolving size factors for cell pools. In principle the pooled expression profile is normalized against an average reference pseudo-cell, constructed by averaging the counts across all cells. A log transformation was computed for the count matrix. All genes with zero expression in a specific cell cluster were removed. The differential expression analysis was performed between aged and young for each cell type cluster using default parameters for DESeq2 (V 1.32.0; (Love, Huber, and Anders 2014)), Seurat (V 4.0.3; (Hao et al. 2021, Stuart et al. 2019)) and MAST (V 1.18.0; (Finak et al. 2015)) in R (V 3.6.358), followed by Benjamini-Hochberg correction for DESeq2 and MAST and Bonferroni correction for Seurat. Differentially expressed genes with adj. p-value <0.05 in all three tools were selected as significantly differentially expressed. The calculated

log2 fold changes from DESeq2 were used to calculate the cell expression of each gene ( $\geq 1$  read in 25% of all cells in a cluster).

#### **2.2.4.3 Analysis of myeloid cell cluster in integrated scRNA-seq datasets**

Five scRNA-seq datasets of human skin were integrated together (Ahlers et al. 2022, Rojahn et al. 2020, Solé-Boldo et al. 2020, Tabib et al. 2018, Vorstandlechner et al. 2020) (Ahlers et al 2021 (PRJNA754272) Rojahn et al., 2020, (GSE153760), Solé-Boldo et al., 2020 (GSE130973), Tabib et al. 2018 and Vorstandlechner et al., 2020 (both which were kindly provided by the authors)). All datasets were processed, integrated and analyzed as described previously using Seurat package, version 4.0.0 (Hao et al., 2020), in R, version 4.0.3 (R Core Team, 2020, <https://www.R-project.org/>)

#### **2.2.5 Statistical analysis**

Statistical analysis was carried out with GraphPad Prism (Version 8). For data with multiple variables, a 2way ANOVA (Analysis of variance) was performed followed by Bonferroni correction. For comparison of two data columns, either two-tailed paired or unpaired student's t-test was performed. If samples were not normally distributed according to Shapiro-Wilk, a two-tailed Mann-Whitney U test was used. NanoString transcriptome data were analyzed using a paired two-tailed Welch's t-test with multiple error correction according to Benjamini-Hochberg. Data are represented as mean  $\pm$  standard errors of the mean (SEMs). P-values  $\leq 0.05$  were considered as indication for significance. All preformed statistical tests are specified in each figure legend.

### 3 Results

In general, this study aims to provide comprehensive insights into age-related changes of macrophages in human skin tissue and their relevance to the phenotypical changes of human skin upon aging.

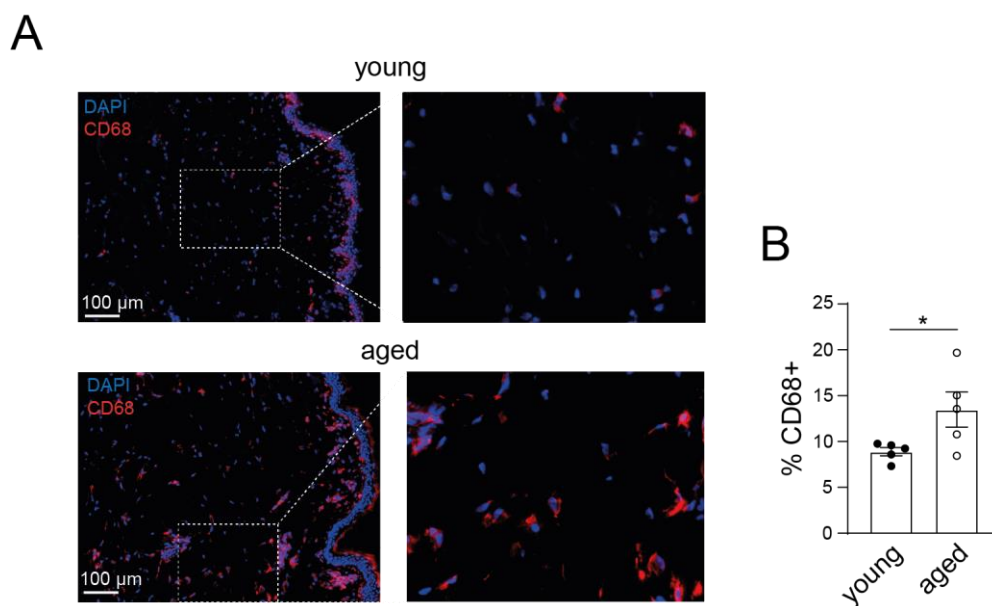
The first part focuses on age-dependent changes of macrophages in the human skin tissue. The changes of monocytes and macrophages due to intrinsic factors upon aging are described afterwards, followed by the influence of aged microenvironment on the macrophage phenotype. And last the effect of aged macrophages on the surrounding tissue is described.

#### 3.1 Age-dependent changes of macrophages in the human skin tissue

To determine age-dependent changes of skin macrophages, both their number and gene expression pattern obtained from young and aged skin were assessed.

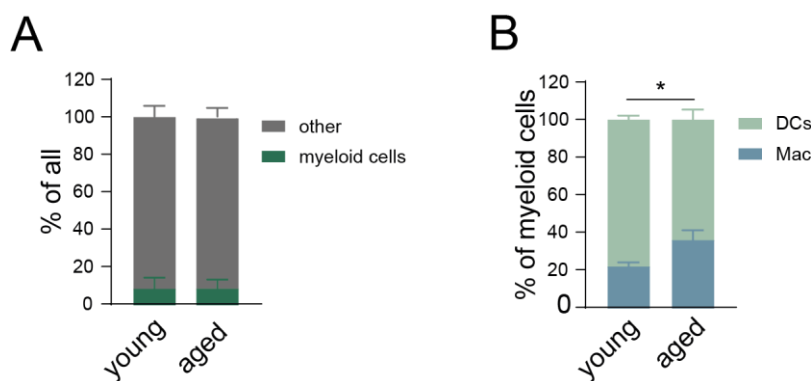
##### 3.1.1 The proportion of skin macrophages is increased in aged human skin tissue

To identify changes in the number of macrophages in the human dermis with age, the proportion of CD68<sup>+</sup> cells was determined. CD68 is a scavenger receptor expressed by tissue-resident macrophages and has been used here to identify macrophages in the skin (Chistiakov et al. 2017). Sections were prepared from human skin tissue obtained from young and aged donors and the proportion of CD68<sup>+</sup> cells was determined via immunofluorescence staining (CD68, red). The proportion of CD68<sup>+</sup> cells of all cells, identified by nuclei (blue), was determined. The analysis shows that the proportion of CD68<sup>+</sup> within all cells was significantly increased by about 50% in the aged compared to the young group (Figure 8).



**Figure 8 Proportion of CD68<sup>+</sup> cells in the human dermis.** Immunofluorescence staining of macrophages (CD68; in red) in human skin sections. (A) Representative image from n=5 aged and n=5 young donors. Scale bar 100 µm. (B) Bar graphs indicate the frequency of CD68<sup>+</sup> cells in the dermis analyzing three sections per donor of all cells identified by nuclei (Hoechst33342, blue). Dots represent individual donors and bars indicate the mean  $\pm$  SEM. Unpaired two-tailed student's t-test \* $p < 0.05$

Additionally, proportions of macrophages in whole-skin samples were determined by analysis of scRNA-seq data. Therefore, scRNA-seq data from 5 independent studies of aged and young female participants were integrated and then analyzed (Rojahn et al. 2020, Sole-Boldo et al. 2020, Tabib et al. 2018, Vorstandlechner et al. 2020, Ahlers et al. 2022). In total 25 clusters representing immune and non-immune cell types were identified, including a myeloid cluster consisting of both macrophages and DCs. The analysis of this integrated dataset showed a similar proportion of myeloid cells in both young and aged group (Figure 9 A). While the proportion of DCs was reduced in the aged group, a significant increase of the macrophage cluster within the myeloid cluster by a factor of 1.6 with age was observed (Figure 9 B).



**Figure 9 Proportion of myeloid cells analyzed in five integrated scRNA-seq datasets.** ScRNA-seq datasets of five published independent studies were integrated for analysis of the myeloid cell cluster in young (average age=25,25) and aged (average age=69,8) skin. (A) Bar graph showing the proportion of myeloid cells among all skin cells and (B) the proportion of macrophages and DCs within the myeloid cluster. Bars indicate the mean  $\pm$  SEM. unpaired two-tailed student's t-test \* $p < 0.05$ . The analysis was done by Dr. Cassandra Falckenhayn, Beiersdorf AG

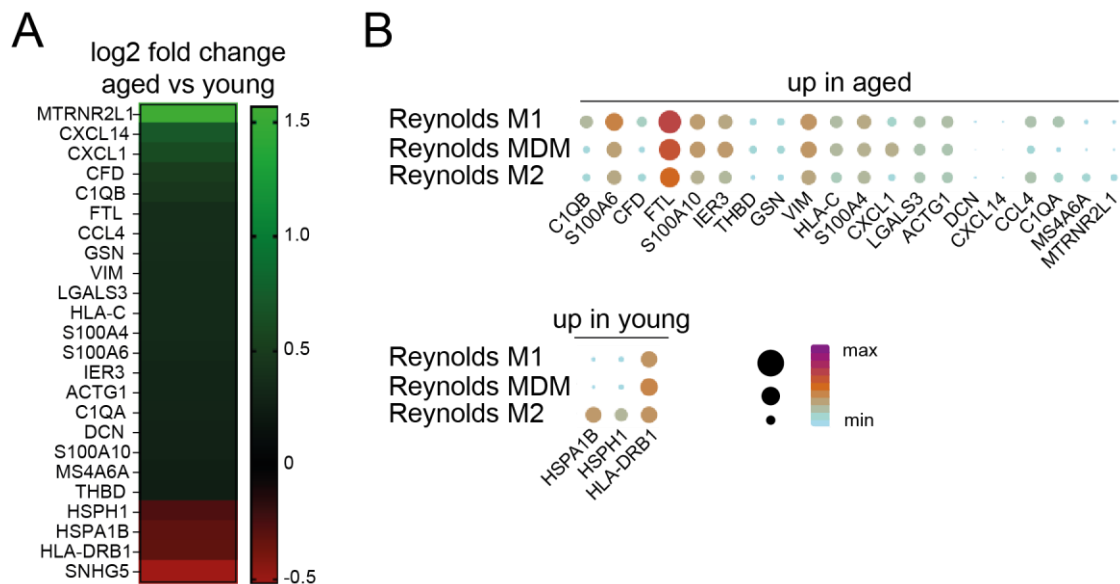
Together both the histological as well as scRNA-seq approaches indicate that the number of macrophages in human skin increases by at least 50% at advanced age.

### 3.1.2 Differentially expressed gene analysis reveals pro-inflammatory phenotype of aged skin macrophages

To examine changes in the phenotype of skin macrophages upon aging, DEGs were determined by a pseudo-bulk RNA-Seq analysis of the macrophage cluster derived from Ahlers et al. (Ahlers et al. 2022) using the aged and young macrophage clusters as described in 2.2.4.2. To identify significant DEGs, only genes with an adjusted p value of  $< 0.05$  using all three tools DESeq2, Seurat and MAST were selected. The heatmap shows DEGs that were expressed in at least 25% of all macrophages with a minimum fold-change of 0.25 calculated by the DESeq2 analysis (Figure 10 A). It was found that the majority of DEGs were upregulated in the macrophages derived from aged skin, whereas just a few were downregulated compared to macrophages derived from young skin. Among the upregulated genes were, for instance CXCL14, CXCL1 and C1QB, which are known pro-inflammatory proteins, while heat shock proteins such as HSPA1B or HSPH1 were found among the downregulated genes.

To assess whether the DEGs between aged and young skin macrophages give a hint about the macrophage M1 and M2 or MDM characteristics, the list of DEGs were compared to the development cell atlas web portal ([developmentcellatlas.ncl.ac.uk](http://developmentcellatlas.ncl.ac.uk)). This web portal comprises the expression profiles of distinct skin macrophage subsets M1, M2 and MDM of a large dataset published recently (Reynolds et al. 2021). Genes that

were upregulated in aged skin macrophages, such as C1QB, S100A6, CFD, IER3 and CXCL1 seem to be expressed at a higher level in M1 and MDMs compared to M2 according to the expression profiles provided by Reynolds and colleagues. In contrast, HSPA1B and HSPH1, both found to be upregulated in young skin macrophages, were highly expressed only in the M2 subset of the Reynolds dataset (Figure 10 B).



**Figure 10 DEGs in aged compared to young skin macrophage cluster.** (A) Pseudo-bulk RNA-seq analysis was performed on the macrophage cluster of young and aged skin macrophages. Significantly differential expressed genes (DEGs) were selected when the adjusted p value was <0.05 by all three tools DESeq2, Seurat and MAST. Heatmap shows genes with log<sub>2</sub> fold-change (FC) of >0.25 expressed in more than 25% of all macrophages. (B) Dot plot showing gene expression of DEGs in aged and young skin macrophages in the human skin M1, M2 and monocyte-derived macrophages (MDM) recently defined in a scRNA-seq study by Reynolds and colleagues, taken from the human developmental cell atlas (Reynolds et al. 2021). The pseudo-bulk RNA-seq analysis was done by Dr. Stefan Simm, University of Greifswald

Taken together, not only the number of macrophages seemed to be increased in aged human skin, but also the phenotype changes upon age. The comparison to the recently published skin macrophage profile indicates that macrophages express more pro-inflammatory characteristic for M1 and MDM skin macrophages.

## 3.2 Age-dependent changes of macrophages caused by intrinsic factors

### 3.2.1 Establishment of generating M1 and M2 MDMs *in vitro*

Age-dependent changes in macrophages caused by intrinsic factors were investigated in this study using macrophages generated from blood-derived monocytes. Human bone

marrow-derived monocytes, which circulate in the blood, can migrate into a tissue and differentiate into macrophages or dendritic cells in the tissue. Thus, these progenitor cells can be used to differentiate into MDMs *in vitro*. Furthermore, due to lack of contact of these progenitors to skin tissue, they provide a good model to assume changes caused by intrinsic aging in monocytes and their differentiation to macrophages. Although their properties are usually more complex *in vivo*, they are typically classified as pro-inflammatory M1 and anti-inflammatory M2 macrophages *in vitro* (Italiani and Boraschi 2014).

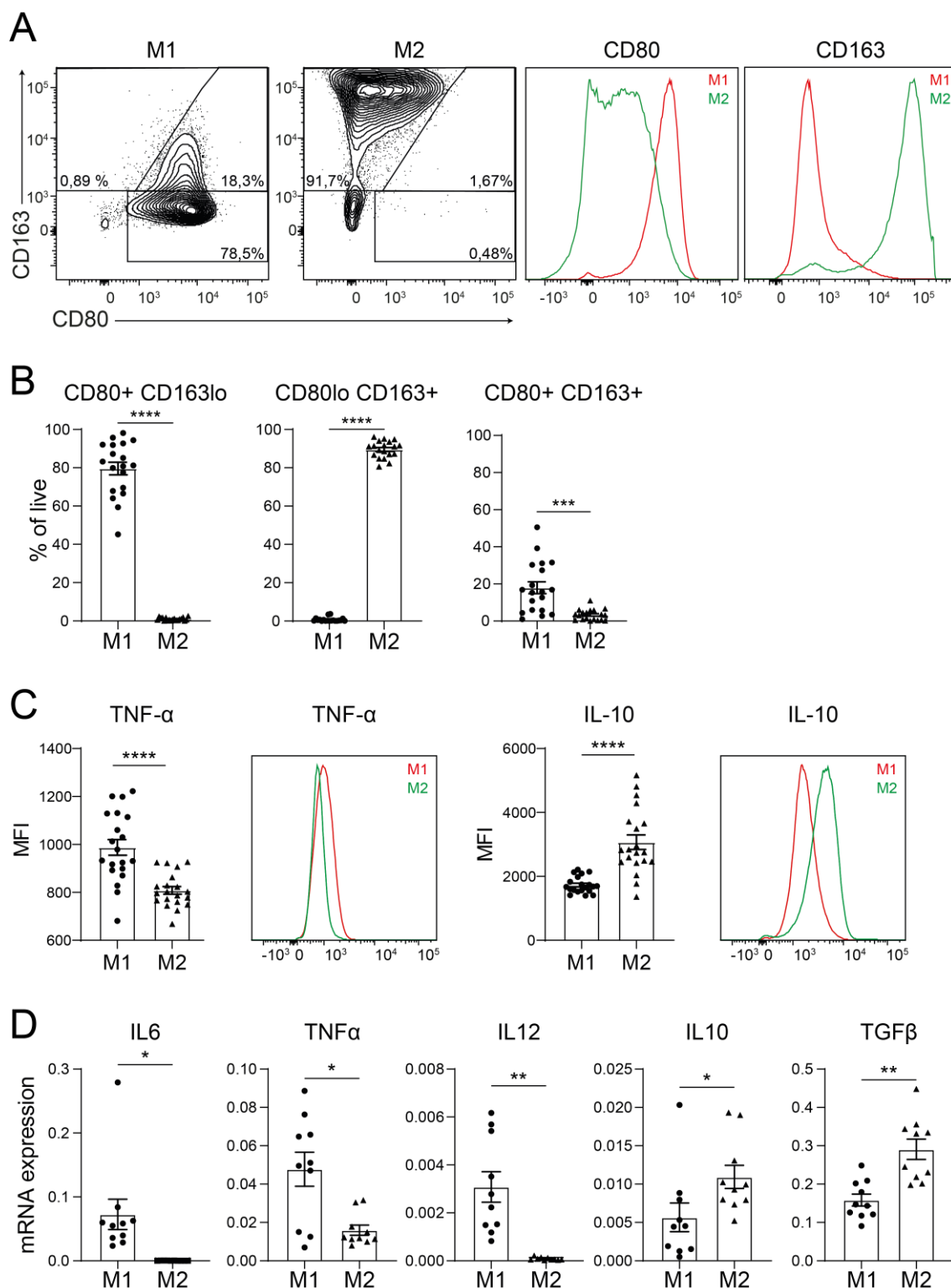
The differentiation ability to these two distinct macrophage phenotypes was used as a parameter to determine whether intrinsic factors influence the macrophage phenotype. As a first step, the differentiation towards the M1 and M2 MDMs had to be validated. Accordingly, human monocytes were isolated from PBMCs and differentiated towards the M1 or M2 MDMs as described in 2.2.2.4. The use of GM-CSF and M-CSF to differentiate monocytes towards an M1 and M2 MDMs, respectively, has become a standard method in the generation of *in vitro* macrophages (Lukic et al. 2017). However, to achieve the phenotypic endpoint, it is necessary to subsequently polarize the cells. Here, polarization to the M1 phenotype was performed by adding LPS and IFN- $\gamma$  to mimic an inflammatory scenario, while IL-4 and IL-13 were used to generate an anti-inflammatory milieu for M2 polarization. After polarization to the M1 or M2 MDMs, the cells were analyzed for their expression of macrophage lineage markers and pro- and anti-inflammatory cytokines to verify their phenotype. These markers were the basis to validate the differentiation method and were used as inclusion or exclusion criteria whether the generated MDMs are typical M1 or M2 and are distinguishable from each other.

Monocytes were isolated from PBMCs and differentiated either towards the M1 MDMs by a supplementation of GM-CSF for 7 days followed by polarization with IFN- $\gamma$  and LPS or towards the M2 MDMs with supplementation of M-CSF for 7 days followed by polarization with IL-4 and IL-13. The expression of CD80 and CD163 was analyzed via flow cytometry after polarization of 24 hours. For the analysis of mRNA expression, the RNA was isolated after incubation of 20 hours.

First the proportions of CD80<sup>+</sup> CD163<sup>lo</sup> and CD80<sup>lo</sup> CD163<sup>+</sup> were determined, representing the M1 and M2 phenotype, respectively. Additionally, a double positive CD80<sup>+</sup> CD163<sup>+</sup> population was classified to show the proportion of a remaining indistinct macrophage phenotype. Around 90% of all live M2 MDMs developed the CD80<sup>lo</sup> CD163<sup>+</sup> phenotype. Although, the proportion of the double positive CD80<sup>+</sup> CD163<sup>+</sup> population was significantly higher in the M1 compared to M2 MDMs, almost 80% of all live M1

MDMs were CD80<sup>+</sup>CD163<sup>lo</sup> (Figure 11 A and B). Furthermore, regarding the pro- or anti-inflammatory properties, the flow cytometric analysis of TNF- $\alpha$  and IL-10 indicates a significant different expression of these cytokines in M1 compared to M2 MDMs (Figure 11 C). Additionally, mRNA expression of pro-inflammatory marker IL6, TNF $\alpha$  and IL12, as well as the anti-inflammatory cytokines IL10 and TGF $\beta$  was determined in the M1 and M2 MDMs by qPCR. All pro-inflammatory markers were significantly higher expressed in M1 MDMs compared to M2 MDMs. In contrast, the expression of anti-inflammatory markers was significantly increased in M2 MDMs compared to M1 MDMs (Figure 11 D).





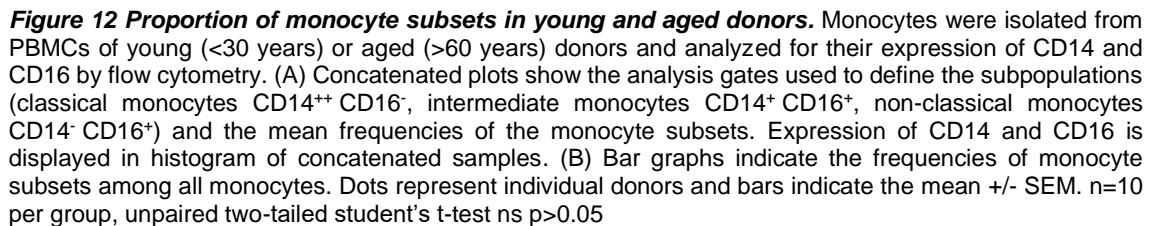
**Figure 11 Different expression of macrophage markers in M1 and M2 MDMs.** Monocytes were isolated from PBMCs, differentiated to either M1 or M2 MDMs and analyzed by flow cytometry (A-C) or qPCR (D). (A) Concatenated plots show the analysis gates used to define the subpopulations and their frequencies (M1 CD80<sup>+</sup> CD163<sup>+</sup>, M2 CD80<sup>lo</sup> CD163<sup>+</sup> and double-positive M CD80<sup>+</sup> CD163<sup>+</sup>). FACS plots are shown from one representative experiment out of two with 10 donors per group. Bar graphs indicate the frequencies of macrophage subsets among the differentiated cells. n=20 per group. (D) mRNA expression was determined via qPCR. Expression was normalized to the expression of housekeeping gene GAPDH. Bar graphs indicate mRNA expression. Dots represent individual donors and bars indicate the mean  $\pm$  SEM. (B and C) n=20 and (D) n=10 per group. Paired two-tailed student's t-test, \* p>0.05; \*\* p>0.01; \*\*\* p>0.001; \*\*\*\* p>0.0001

In conclusion, the M1 and M2 MDMs generated *in vitro* exhibited the distinct CD80<sup>+</sup>CD163<sup>lo</sup> or CD80<sup>lo</sup>CD163<sup>+</sup> pattern in M1 or M2 phenotype, respectively. Furthermore, they expressed lineage specific anti- or pro-inflammatory markers according to their properties. Thus, the differentiation method to generate M1 and M2 phenotypes was validated.

### 3.2.2 The proportion of monocyte subsets remain similar upon aging

To determine changes of macrophages caused by intrinsic aging factors, first the phenotype of the progenitor cells, the bone-marrow derived monocytes, was examined. Monocytes display a heterogeneity with respect to their phenotype and function. The differential expression of CD14 and CD16 is commonly used to define three subsets in peripheral blood monocytes: classical (CD14<sup>++</sup>CD16<sup>-</sup>), intermediate (CD14<sup>+</sup>CD16<sup>+</sup>) and non-classical monocytes (CD14<sup>-</sup>CD16<sup>++</sup>) (Hearps et al. 2012). To analyze potential effects of age-related changes in monocyte subsets on macrophage differentiation, the proportions of subsets in monocytes derived from young and aged donors were investigated.

Monocytes were isolated from PBMCs of young (< 30 years) and aged (>60 years) healthy donors and their expression of CD14 and CD16 was detected via flow cytometry in order to classify the distinct subsets (Figure 12 A). The analysis shows that the highest proportion represented the classical monocytes, followed by intermediate and non-classical monocytes. Monocytes from aged donors show a similar proportion of all three subtypes compared to young donors, with no significant differences between these two donor groups (Figure 12 B).



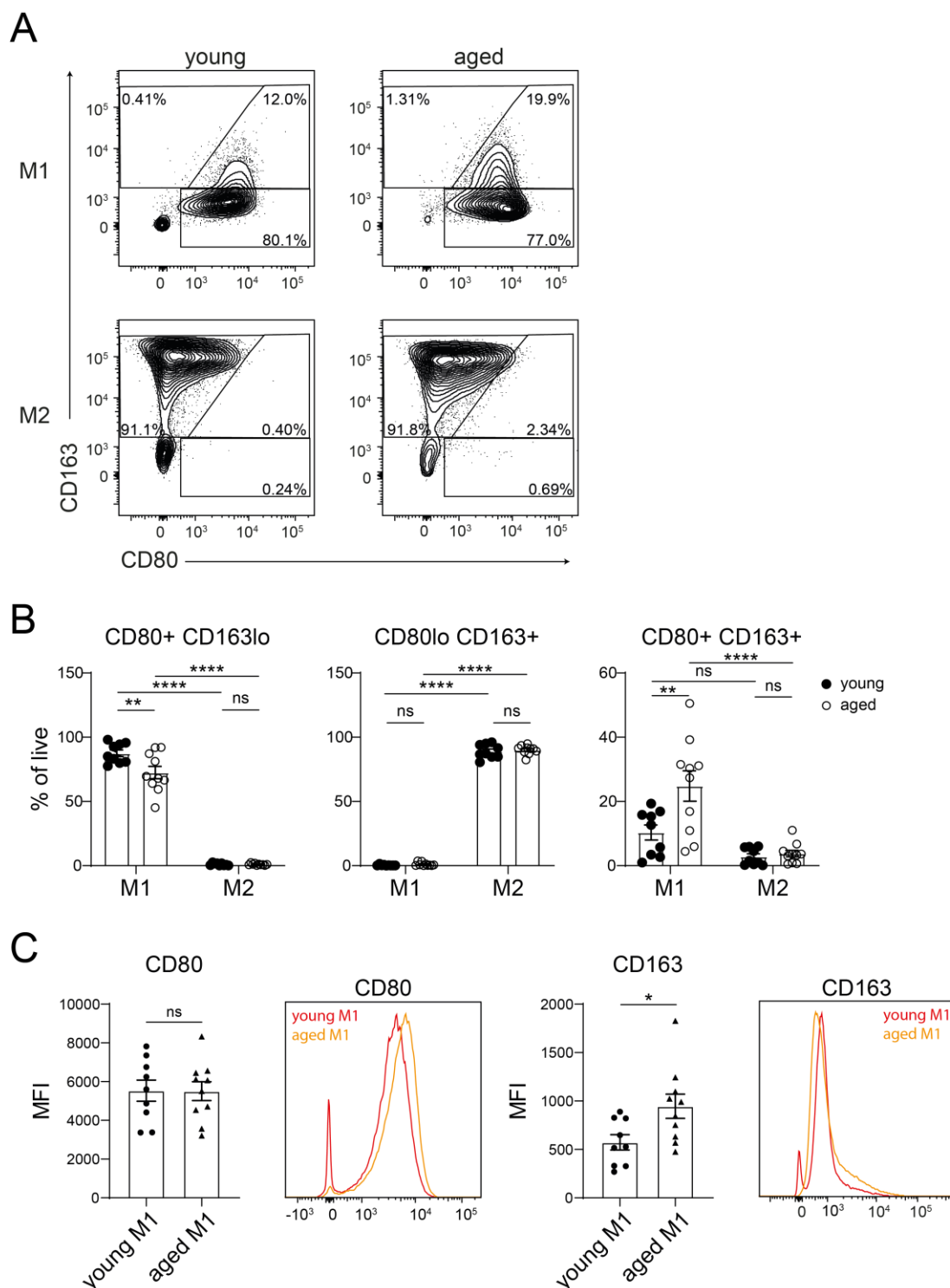
### 3.2.3 Differentiation ability towards M1 and M2 macrophages upon age

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marker CD80 and CD163 as well as the synthesis of cytokines characteristic for M1 or M2 macrophages were investigated.

### **3.2.3.1 Expression of M2 lineage marker CD163 is increased in aged M1 MDMs**

Monocytes from both young and aged donors were differentiated towards the M1 and M2 MDMs as described in 3.2.1. The proportions of CD80<sup>+</sup> CD163<sup>lo</sup> and CD80<sup>lo</sup> CD163<sup>+</sup>, as well as double positive CD80<sup>+</sup> CD163<sup>+</sup> populations were investigated by flow cytometry (Figure 13 A). The analysis shows that aged monocytes differentiated to M2 macrophages exhibit similar proportions of all three subpopulations (CD80<sup>lo</sup> CD163<sup>+</sup>, CD80<sup>+</sup> CD163<sup>lo</sup> and CD80<sup>+</sup> CD163<sup>+</sup>) compared to the young counterparts. In contrast, the M1 macrophage differentiated from aged monocytes show a significant decrease in the CD80<sup>+</sup> CD163<sup>-</sup> population as well as a significant increase of the CD80<sup>+</sup> CD163<sup>+</sup> population (Figure 13 B).



**Figure 13 Proportions of macrophage phenotypes in young and aged M1 and M2 MDMs.** Monocytes were isolated from PBMCs of young and aged donors and differentiated to either M1 or M2 MDMs. (A) Concatenated plots show the analysis gates used to define the subpopulations and their frequencies (M1 CD80<sup>+</sup> CD163<sup>lo</sup>, M2 CD80<sup>lo</sup> CD163<sup>+</sup> and double-positive M CD80<sup>+</sup> CD163<sup>+</sup>). Expression of CD80 and CD163 is displayed in histogram of concatenated samples. (B) Bar graphs indicate the frequencies of macrophage subsets among the differentiated cells. (C) Bar graphs indicate the expression of CD80 and CD163 shown as mean fluorescence intensity (MFI) in the M1 subset. FACS plots are shown from one representative experiment out of two with five donors per group, bar graphs show data of young n=10, aged n=9. Dots represent individual donors and bars indicate the mean  $\pm$  SEM. (B) 2way ANOVA (C) unpaired two-tailed student's t-test, ns p>0.05, \* p>0.05, \*\* p>0.01, \*\*\*\* p>0.0001

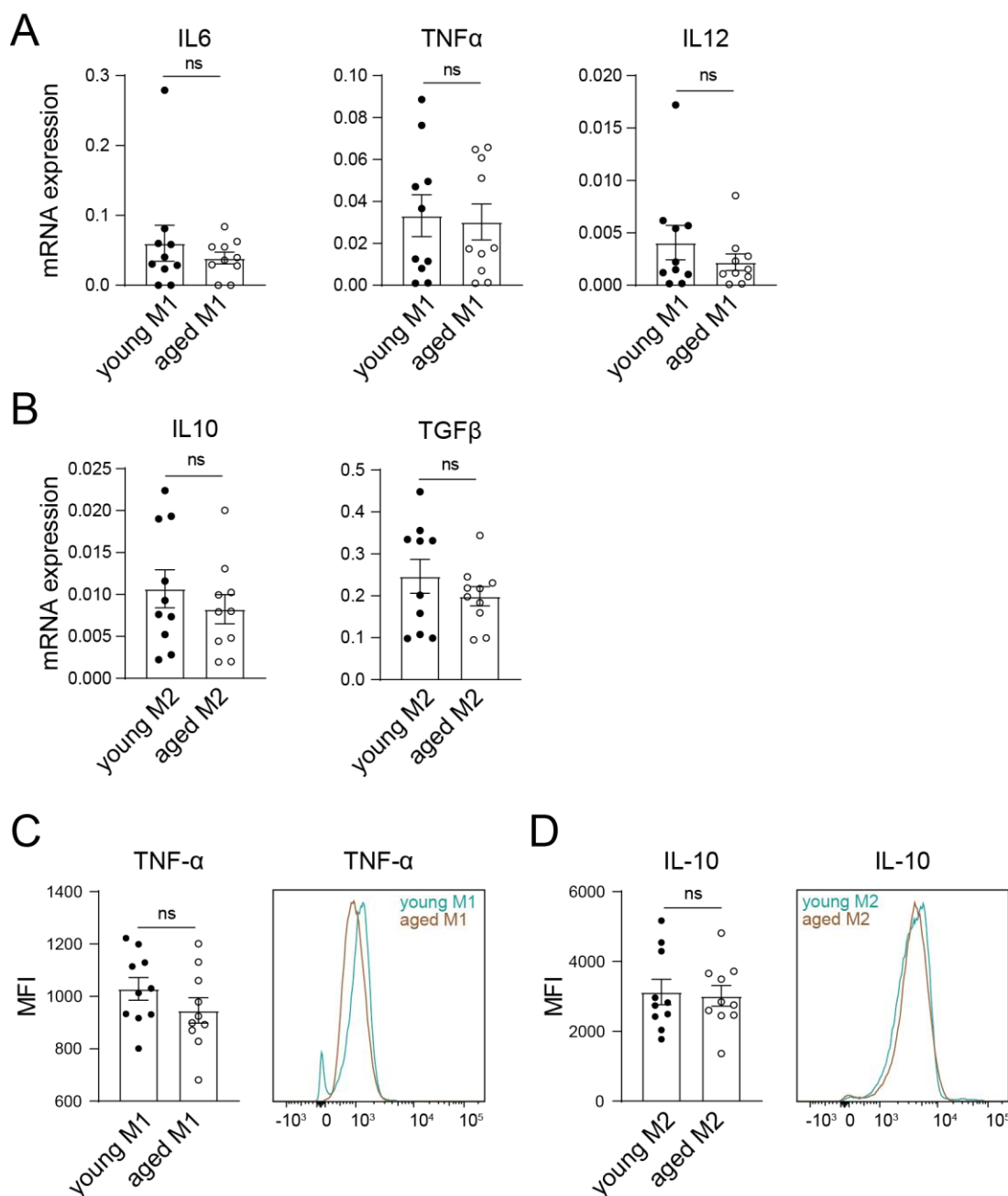
A closer look on the quantitative expression of the lineage markers in M1 macrophages revealed significant changes in cells of aged donors. The protein levels of CD163 were increased in aged M1 macrophages compared to the young counterparts, while the levels of CD80 were unaffected in the aged M1 macrophages (Figure 13 C).

In conclusion, the aged monocytes seem to be equally able to differentiate towards the M2 macrophage phenotype as the young monocytes, but the differentiation towards a M1 macrophage phenotype seems to be partly impaired upon age.

### **3.2.3.2 Cytokine expressions remain similar in MDMs upon age**

The expression of pro-and anti-inflammatory cytokines is one of the main functions of macrophages. To test whether this function is impaired in macrophages by intrinsic aging, the expression of pro- or anti-inflammatory cytokines was examined in MDMs. To this end both the mRNA expression and protein levels of pro-inflammatory (IL6, TNF $\alpha$  and IL12) and anti-inflammatory cytokines (IL10 and TGF $\beta$ ) as described in 3.2.1 were determined in MDMs derived from young and aged donors.

The expressions of IL6, TNF $\alpha$  and IL12 were determined in M1 MDMs and IL10 and TGF $\beta$  in M2 MDMs by qPCR. Although, the expression of the M1 markers IL6, IL12 and TNF $\alpha$  were slightly decreased in aged compared to young M1 MDMs, the expression was not significantly different (Figure 14 A). Additionally, the cytokines IL10 and TGF $\beta$  were slightly but not significantly less expressed in aged M2 MDMs compared to the young counterparts (Figure 14 B).



**Figure 14 Expression of cytokine markers in young and aged MDMs.** Monocytes were isolated from PBMCs derived from young and aged donors and differentiated to M1 (A and C) or M2 (B and D) MDMs. (A and B) RNA was isolated after a polarization of 20 hours, transcribed into cDNA followed by analysis via qPCR. Expression was normalized on the expression of housekeeping gene GAPDH. (C and D) Before determination of proteins by intracellular staining with flow cytometry, the cells were stimulated with 5  $\mu$ g/mL Brefeldin A for 3.5 h. Expression of TNF- $\alpha$  and IL-10 are shown as the MFI (left) and visualized in a histogram (right). Each dot represents individual donors and bars indicate the mean  $\pm$  SEM. n=10 per group, unpaired two-tailed student's t-test, ns p>0.05

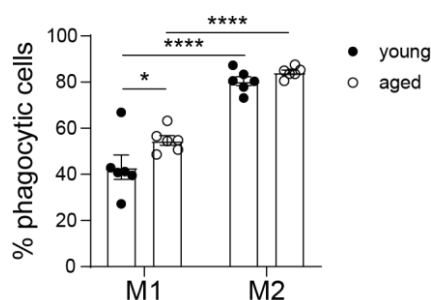
Assessment of whether mRNA levels are translated into protein expression, the protein levels of pro-inflammatory cytokine TNF- $\alpha$  and anti-inflammatory cytokine IL-10 were determined via intracellular staining in flow cytometry as described in 2.2.3.3. TNF- $\alpha$  expression was detected in M1 MDMs and IL-10 in M2 MDMs, both indicated by the

mean fluorescence intensity (MFI). Similar to their expression on mRNA level, no significant difference in cytokine protein levels between the young and aged group were observed (Figure 14 C and D).

In summary, the ability of aged monocytes to differentiate into the M1 phenotype *in vitro* was significantly decreased, demonstrated by increased expression of CD163 in these cells, while the expression of cytokines seemed not to be impaired in the corresponding derived macrophages.

### 3.2.3.3 Phagocytic activity is increased in aged M1 MDMs

To assess changes in the phagocytic activity in macrophages at advanced age, the proportion of phagocytic cells was determined in MDMs derived from young and aged monocytes. To this end, the monocytes were differentiated to either M1 or M2 MDMs as described in 3.2.1 before the phagocytosis assay was performed as explained in 2.2.2.5. The analysis shows a significantly higher percentage of phagocytic MDMs in the fraction differentiated towards M2 compared to the M1 MDMs, independently of the donors' age (Figure 15). While around 43% of the young M1 MDMs are phagocytic active, around 80% of the M2 MDMs counterpart show phagocytosis activity. Around 54% of the aged M1 MDMs compared to 84% of the aged M2 MDMs were determined as phagocytic cells. Indeed, the proportion of phagocytic macrophages was similar in M2 MDMs derived from young and aged donors. In contrast, the proportion of M1 phagocytic macrophages was significant higher in the aged group compared to the young counterparts.



**Figure 15 Proportion of phagocytic MDMs in M1 and M2 phenotype.** Monocytes were isolated from PBMCs of young and aged donors and differentiated and polarized to either M1 or M2 macrophages. Percentage of phagocytic cells after phagocytosis of pHRedo labeled Zymosan particles were determined via flow cytometry. Dots represent individual donors and bars indicate the mean  $\pm$  SEM.  $n=6$  donors per group, 2way ANOVA, \*  $p<0.05$ , \*\*\*\*  $p<0.0001$



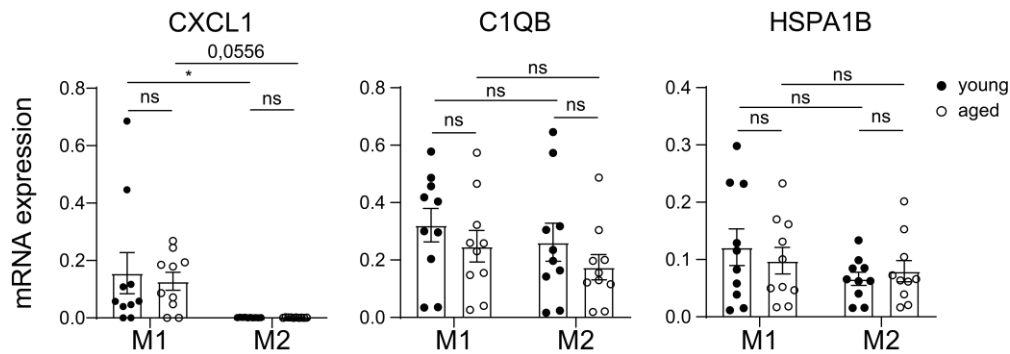
In summary, while the phagocytic activity of M2 MDMs seems to be unaffected during age, aged M1 MDMs showed an increase in their phagocytic activity compared to the young M1 MDMs.

Overall, while the cytokine synthesis seemed to be unaffected upon age, the ability of aged monocytes to differentiate into the M1 phenotype *in vitro* as well as their phagocytic activity was impaired. In contrast, M2 differentiation and phagocytic function were not altered upon aging.

### **3.2.4 Expression of skin macrophage markers is similar in young and aged MDMs *in vitro***

To get an idea of whether the changes of specific skin macrophage markers identified by Reynolds et al. in aged skin are caused by intrinsic factors, next the expression of specific skin macrophage markers was determined in young and aged MDMs *in vitro*. Regarding Reynolds et al. C1QB, CXCL1 and HSPA1B are specifically expressed in M1, MDM or M2 skin macrophages, respectively (Reynolds et al. 2021). In addition, pseudo-bulk RNA-seq analysis showed changes in the expression of these markers in aged skin macrophages (Figure 10). Thus, these three markers were chosen for the determination of their expression in MDMs *in vitro*. Thereby, it was determined whether they are specifically expressed between M1 or M2 MDMs *in vitro* and whether there are age-dependent changes in their expression.

To this end, monocytes from both young and aged donors were differentiated towards the M1 and M2 MDMs as described in 3.2.1, followed by the determination of mRNA expression of CXCL1, C1QB and HSPA1B. In general, the analysis showed a higher expression of CXCL1 in the M1 MDMs, whereas C1QB and HSPA1B were expressed at similar levels in M1 and M2 MDMs *in vitro*. Nevertheless, differences in the expression of all markers in both M1 and M2 MDMs were not significant between the young and aged MDMs *in vitro*.



**Figure 16 Expression of skin macrophage markers in MDMs *in vitro*.** Monocytes were isolated from PBMCs derived from young and aged donors and differentiated to M1 or M2 MDMs. RNA was isolated after a polarization of 20 hours, transcribed into cDNA followed by analysis via qPCR. Expression was normalized on the expression of housekeeping gene GAPDH. Dots represent individual donors and bars indicate the  $\pm$  SEM.  $n=10$  per group, 2way ANOVA, ns  $p>0.05$ , \*  $p<0.05$

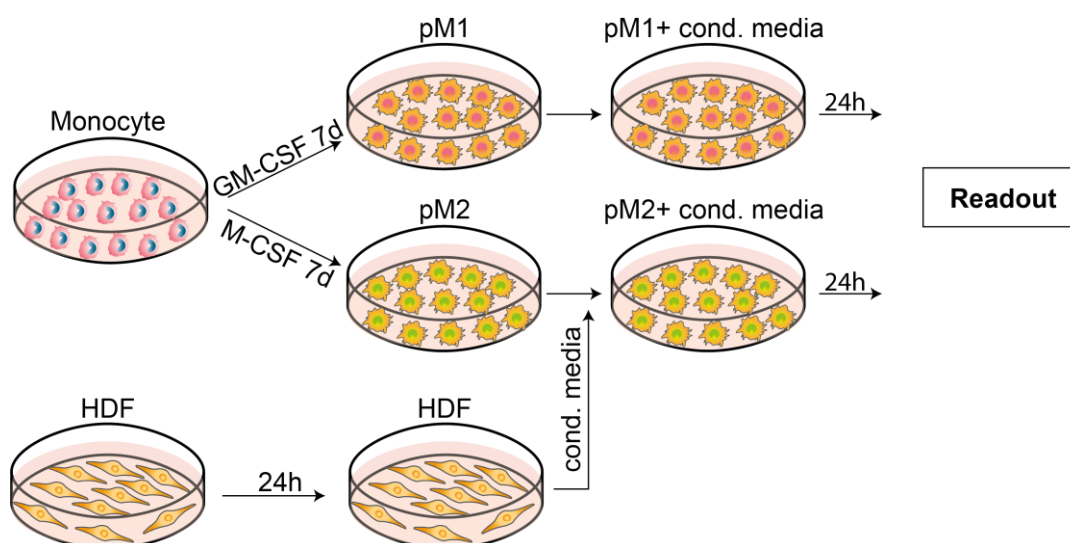
In conclusion, no age-dependent changes in the skin macrophage markers could be observed in the MDMs *in vitro*. This suggests, that changes of this specific skin macrophage markers identified by Reynolds et al. in aged skin are not caused by intrinsic factors.

### 3.3 Impact of the aged microenvironment on the macrophage phenotype

The previous results in demonstrate that intrinsic factors do not seem to induce the changes in the expression of M1, M2 or MDM skin macrophage markers in aged skin macrophages (3.1.2 and 3.2.4). To further investigate the influence of the aged surrounding tissue on the macrophage phenotype, changes of the macrophage phenotype induced by aged compared to young dermal fibroblasts as well as non-senescent compared to senescent dermal fibroblasts were assessed.

To this end, macrophages were cultured together with young or aged and non-senescent or senescent HDFs or their conditioned media to simulate the skin microenvironment. Afterwards, the expression of macrophage lineage markers CD80 and CD163, the phagocytic activity and the mRNA expression of pro- and anti-inflammatory cytokines were assessed. Figure 17 visualizes the experimental setup. Beforehand, a pool of monocytes derived from aged donors was differentiated with either GM-CSF or M-CSF without the following polarization with cytokines or LPS, to generate the partially polarized M1 (pM1) or M2 (pM2) macrophages, respectively. To be able to detect the potential effects of the fibroblasts on the macrophage phenotype, only partially polarized macrophages were used in this approach. Changes in the phenotype of pM1 and pM2

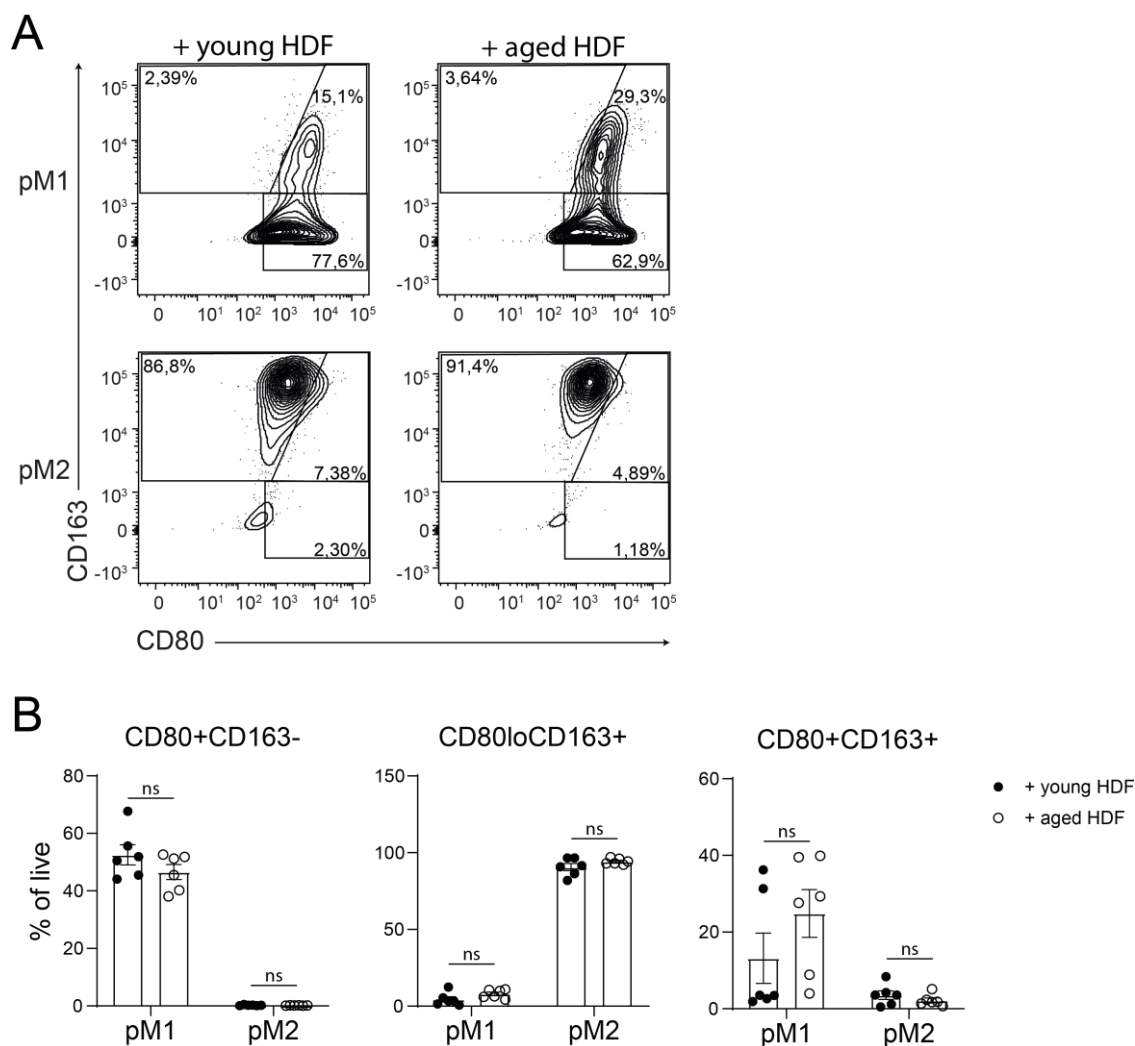
MDM phenotype were determined either after co-cultured with HDFs (3.3.1) or after using conditioned media of aged, young, senescent or non-senescent HDFs (Figure 17).



**Figure 17 Experimental scheme for examining the effect of aged or senescent fibroblasts on the macrophage phenotype.** Monocytes were isolated from PBMCs derived from 3 aged donors and differentiated with either GM-CSF or M-CSF for 7 days to generate partially polarized MDMs (pM1 and pM2, respectively). HDFs were cultivated for 24 hours (24 h) to generate conditioned media, which was harvested after 24 hours and directly used for culturing the macrophages. After 24 hours of cultivation of the macrophages with the conditioned media, surface marker expression and phagocytic activity were determined via flow cytometry and mRNA expression of cytokines and skin macrophage marker was examined by qPCR. To determine impact of aged HDFs, macrophages were cultivated with conditioned media of young compared to aged HDFs, while macrophages were cultivated with conditioned media of non-senescent compared to senescent HDFs to examine the influence of senescent HDFs on the macrophage phenotype.

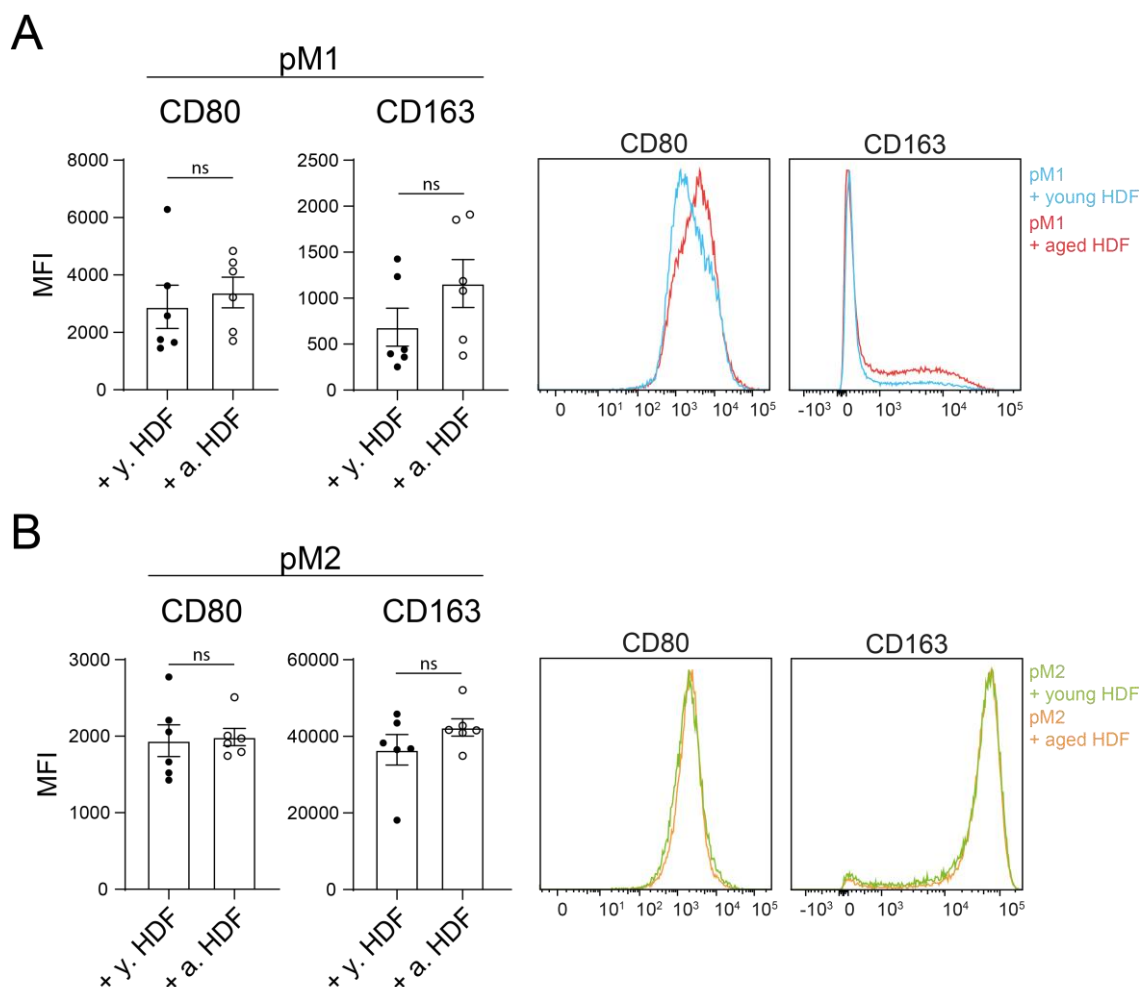
### 3.3.1 Aged HDFs induces the expression of pro-inflammatory cytokines in MDMs

First, to investigate changes in the expression of the macrophage lineage markers CD80 and CD163 induced by an aged microenvironment, their expression in pM1 and pM2 MDMs after culturing with conditioned media of young and aged HDFs were determined by flow cytometric analysis. The analysis revealed a non-significant increase in the proportion of the CD80<sup>+</sup> CD163<sup>+</sup> population and concomitant decrease in the CD80<sup>+</sup> CD163<sup>-</sup> population in the pM1 cultured with conditioned media of aged compared to young HDFs (Figure 18 A and B).



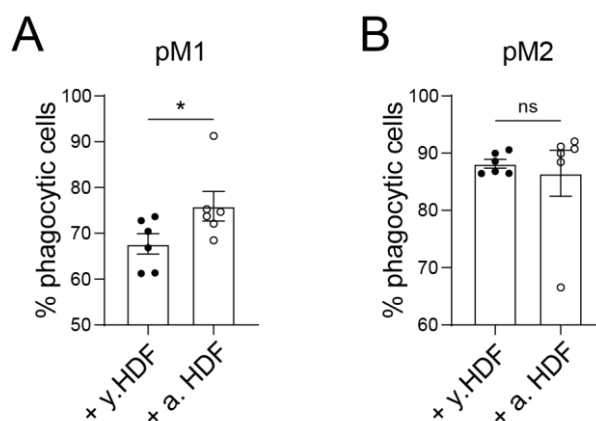
**Figure 18** Frequencies of macrophage subpopulations in pM1 and pM2 after cultivation with conditioned media of young and aged HDFs. Partially polarized MDMs were cultured with conditioned media of young (+y. HDF) and aged (+a. HDF) HDFs for 24 hours. Expression of CD80 and CD163 was determined via flow cytometry. (A) Concatenated plots show the analysis gates used to define the subpopulations and their frequencies (M1 CD80<sup>+</sup> CD163<sup>-</sup>, M2 CD80<sup>lo</sup> CD163<sup>+</sup> and double-positive M CD80<sup>+</sup> CD163<sup>+</sup>). (B) Bar graphs indicate the frequencies of macrophage subsets among the cells. Dots represent individual donors and bars indicate the mean  $\pm$  SEM. HDFs derived from n=6 per group, (B) 2way ANOVA, ns p>0.05

In contrast, the pM2 cultivated with conditioned media of aged HDFs compared to young HDFs showed similar proportions in all populations. Furthermore, no significant difference in the expression of CD80 or CD163, indicated by the MFI, was detected in both pM1 and pM2 after treatment with conditioned media of aged HDFs compared to young HDFs (Figure 19 A and B).



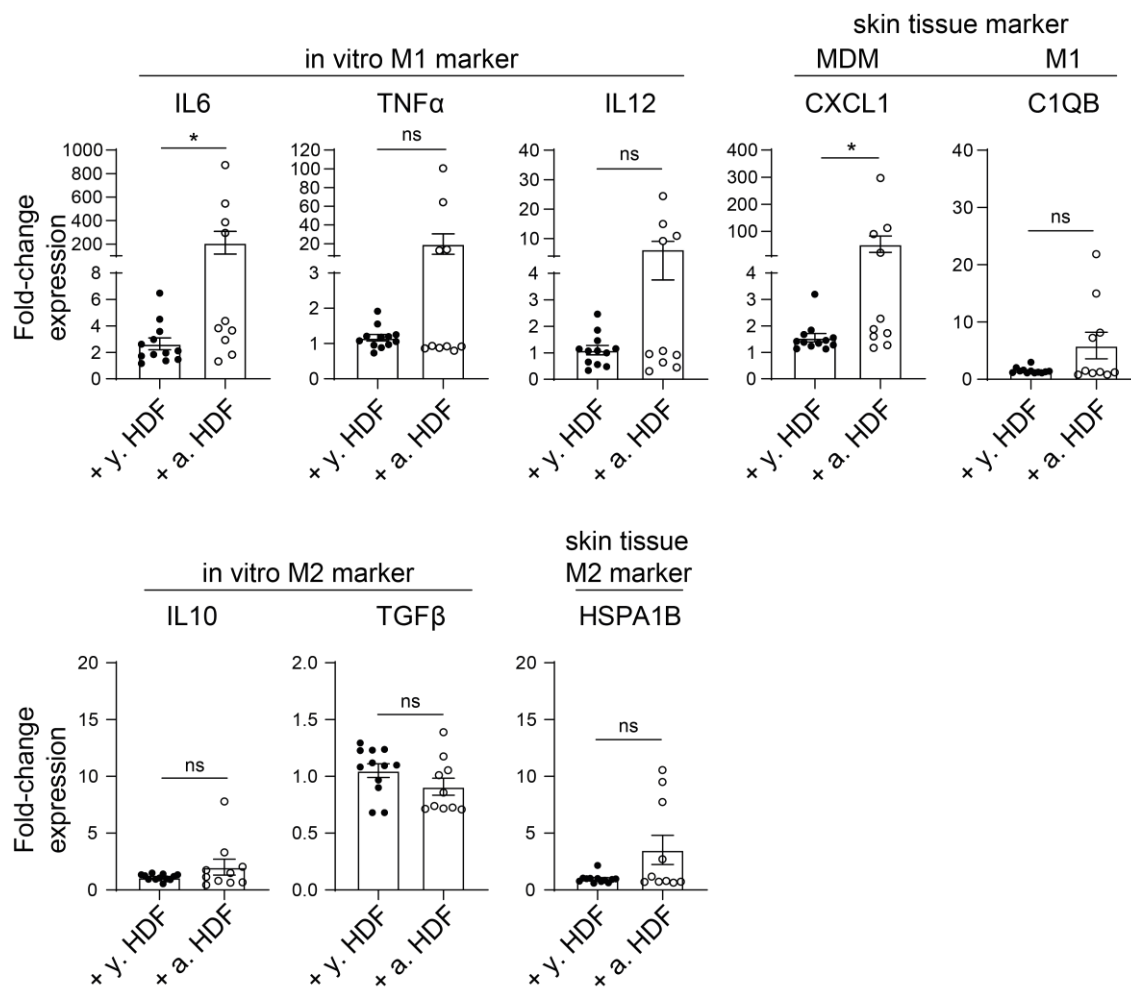
**Figure 19 Expression of macrophage lineage marker in pM1 and pM2 after cultivation with conditioned media of young and aged HDFs.** Partially polarized MDMs pM1 (A) and pM2 (B) were cultured with conditioned media of young (+y. HDF) and aged (+a. HDF) HDFs for 24 hours. Expression of CD80 and CD163 was determined via flow cytometry. Expression of CD80 and CD163 is displayed in histogram of concatenated samples. Bar graphs indicate the protein levels of CD80 and CD163 indicated by the MFI in pM1 (A) and pM2 (B). Dots represent individual donors and bars indicate the mean  $\pm$  SEM. HDFs derived from  $n=6$  per group, Two-tailed Mann-Whitney U test, ns  $p>0.05$

Additionally, the percentage of phagocytic activity was determined in these cells as described in 2.2.2.5. The pM2 treated with conditioned media of aged and young HDFs were found to be similar phagocytic active (Figure 20 B). In contrast, pM1 treated with conditioned media derived from aged HDFs showed a significantly higher proportion of phagocytic cells (75%) in comparison to pM1 treated with conditioned media of young HDFs (67%) (Figure 20 A).

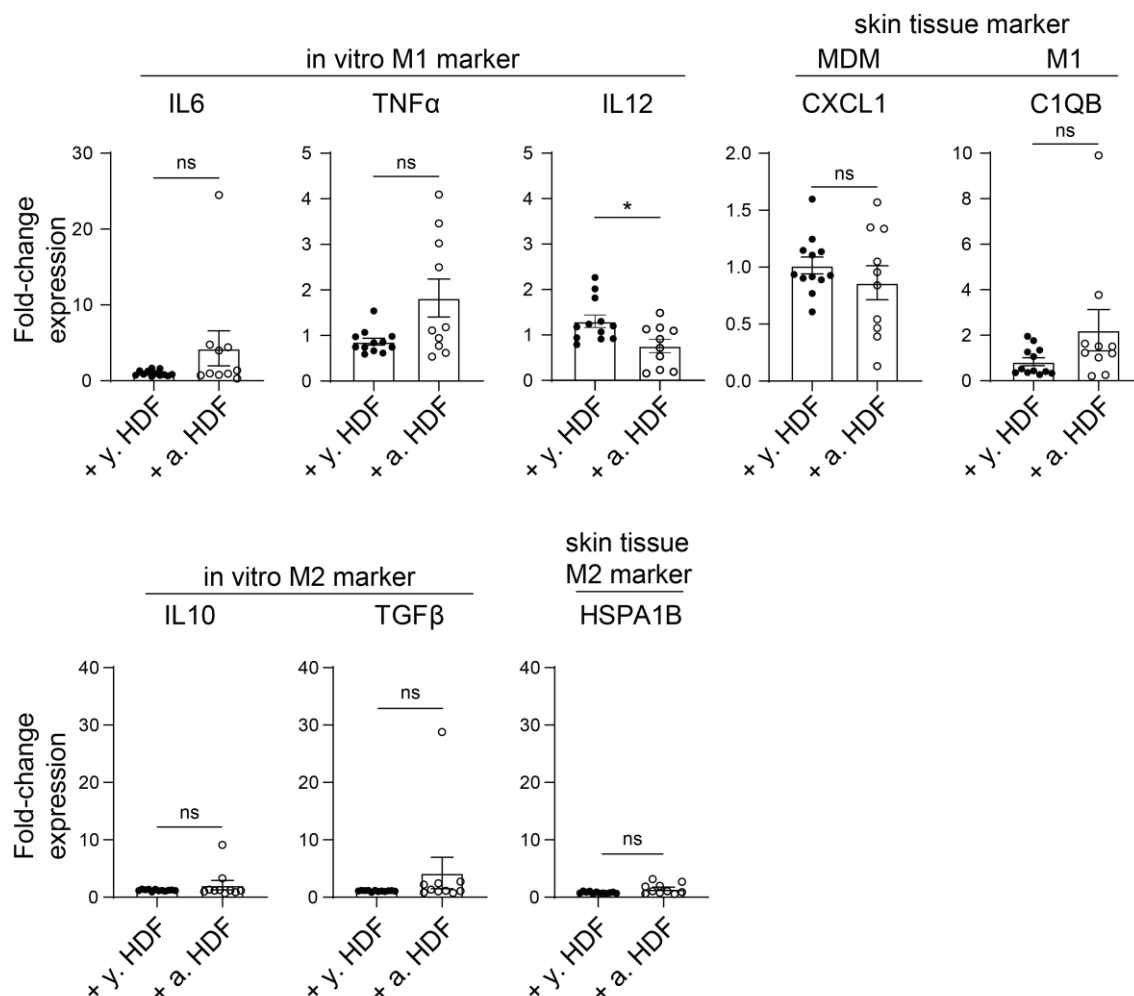


**Figure 20 Percentage of phagocytic MDMs after cultivation with conditioned media of young and aged HDFs.** Partially polarized pM1 (A) and pM2 (B) were generated by differentiation with either 50 ng/mL GM-CSF or 50 ng/mL M-CSF for 7 days, respectively. Partially polarized MDMs were co-cultured with conditioned media of young (+y. HDF) or aged (+a. HDF) HDFs for 24 hours. Afterwards phagocytosis assay was performed to identify the percentage of phagocytic cells within live cells. Bar graphs indicate the percentage of phagocytic cells, dots represent individual donors and bars indicate the mean  $\pm$  SEM. HDFs derived from n=6 per group, Two-tailed Mann-Whitney U test, ns  $p > 0.05$

In order to determine changes in the expression of pro- and anti-inflammatory markers upon cultivation with aged HDFs, the mRNA expression of these markers were determined in pM1 (Figure 21) and pM2 (Figure 22) after co-cultivation with young compared to aged HDFs. After the co-cultivation of macrophages with aged and young HDFs no significant differences in the expression of the *in vitro* M2 markers IL10, TGF $\beta$  and the skin tissue marker HSPA1B were observed in neither pM1 nor pM2 macrophages (Figure 21 and Figure 22). The expression levels of IL6 and CXCL1 was significantly upregulated in the pM1 after co-culture with aged compared to young HDFs. Although, differences in the expression of TNF $\alpha$ , IL12 and C1QB in the pM1 after co-culture with aged compared to young HDFs were not significant, they are clearly upregulated by a fold-change (FC) of 17, 5.8 and 3.9, respectively (Figure 21). Furthermore, the expression of IL6, TNF $\alpha$  and C1QB was slightly higher in the pM2 co-cultured with aged compared to young HDFs, by a FC of 4.3, 2.1 and 2.6, respectively (Figure 22). In contrast, the expression level of IL12 was significantly downregulated in the pM1 (Figure 22). Nevertheless, not all changes in the expression are significant due to the high variance of the samples.



**Figure 21 Expression of macrophage markers in pM1 MDMs after co-culture with HDFs.** pM1 MDMs were generated by differentiation with GM-CSF. Partially polarized MDMs were co-cultured with young (+y.HDF) or aged (+a. HDF) HDFs. After cultivation for 24 hours the MDMs were harvested for subsequent RNA isolation. mRNA expression of *in vitro* M1, skin tissue M1, *in vitro* M2 and skin tissue M2 marker was determined via qPCR. Expression was normalized on the expression of housekeeping gene GAPDH and control partially polarized MDMs cultured without HDFs. Dots represent individual HDF donors and bars indicate the mean  $\pm$  SEM. HDFs derived from aged (n=12) or young (n=10) donors. Two-tailed Mann-Whitney U test, ns p>0.05, \* p<0.05



**Figure 22 Expression of macrophage markers in pM2 MDMs after co-culture with HDFs.** pM2 MDMs were generated by differentiation with M-CSF. Partially polarized MDMs were co-cultured with young (+y. HDF) or aged (+a. HDF) HDFs. After cultivation for 24 hours the MDMs were harvested for subsequent RNA isolation. mRNA expression of *in vitro* M1, skin tissue M1, *in vitro* M2 and skin tissue M2 marker was determined via qPCR. Expression was normalized on the expression of housekeeping gene GAPDH and control partially polarized MDMs cultured without HDFs. Dots represent individual HDF donors and bars indicate the mean  $\pm$  SEM. HDFs derived from aged (n=12) or young (n=10) donors. Two-tailed Mann-Whitney U test, ns  $p > 0.05$ , \*  $p < 0.05$

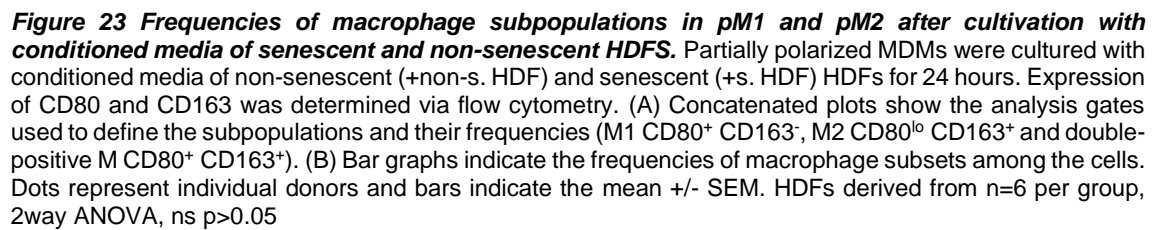
In summary, the expression of M1 markers as well as the phagocytic activity was increased after co-cultivation with aged HDFs, or treatment with conditioned media of aged compared to young HDFs, respectively. In contrast, the expression of M2 marker IL10, TGFβ and HSPA1B, as well as the expression of macrophage lineage marker CD80 and CD163 were not affected by the co-cultivation or conditioned media of aged HDFs compared to young HDFs. Thus, the presence of aged HDFs seems to both induce the phagocytosis and the expression of known pro-inflammatory markers in pM1, while anti-inflammatory cytokines, macrophage lineage markers and phagocytosis were not affected in pM2.

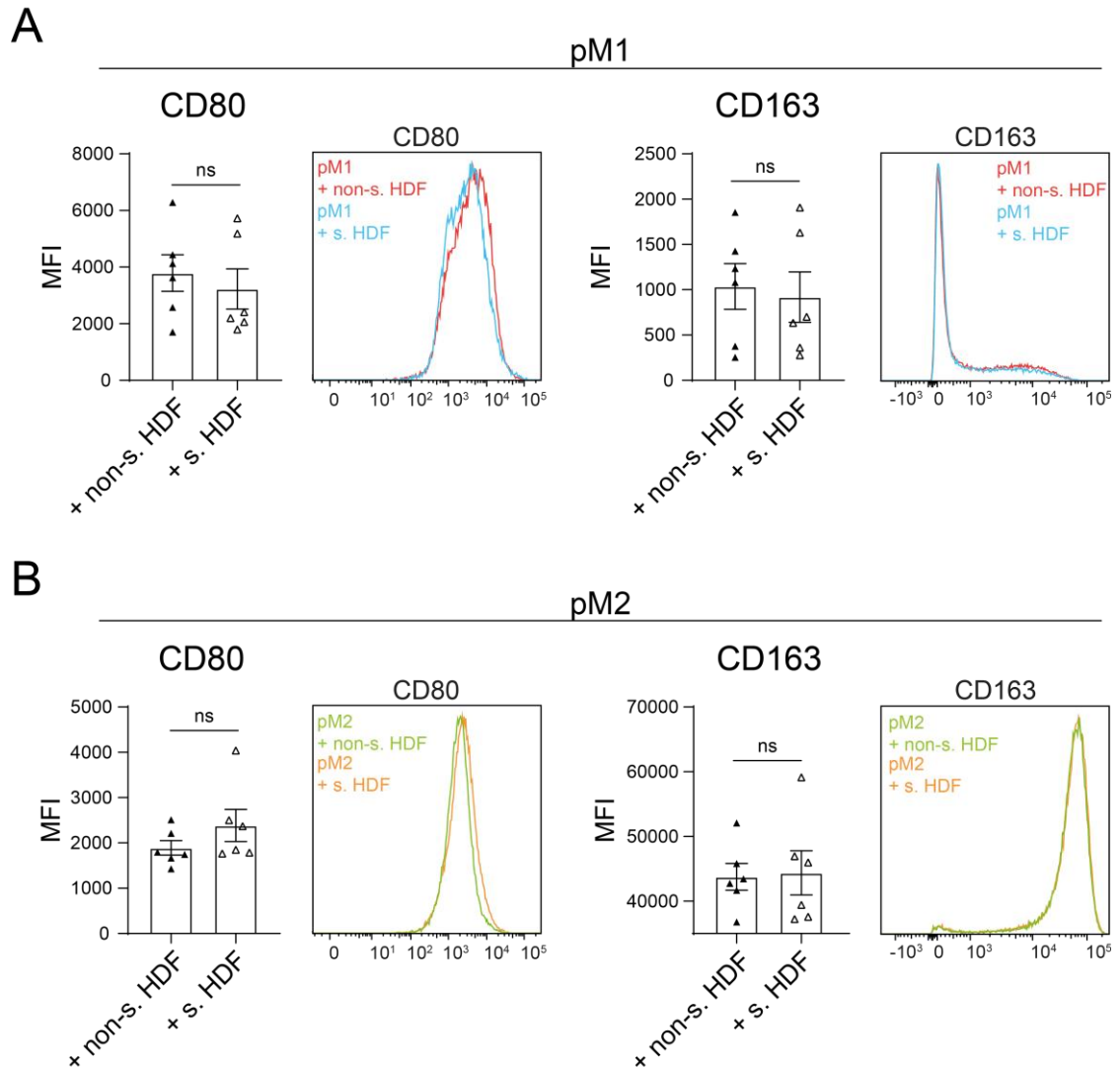


### 3.3.2 Senescent HDFs influence the macrophage phenotype

To investigate the influence of the age-related accumulation of senescent fibroblasts on the phenotype of macrophages, pM1 and pM2 macrophages were cultured with conditioned media of senescent fibroblasts and changes in the expression of macrophage lineage marker CD80 and CD163, phagocytic activity as well as mRNA expression of pro- and anti-inflammatory cytokine and skin macrophage marker were determined subsequently. Here replicative senescent HDFs were generated by subcultivation until cell cycle arrest, while HDFs cultivated until passage 8 were used as non-senescent HDFs to generate conditioned media. Conditioned media were directly used for the cultivation of pM1 and pM2 (generated as described 3.3 and Figure 17) for 24 hours and the cells were either analyzed via flow cytometry or RNA was isolated to determine mRNA expression.

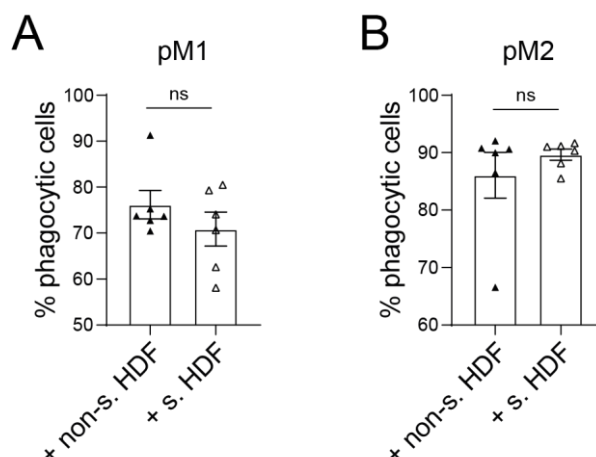
Analysis of the proportions of the subpopulations CD80<sup>+</sup> CD163<sup>-</sup>, CD80<sup>lo</sup> CD163<sup>+</sup> and CD80<sup>+</sup> CD163<sup>+</sup> revealed no significant differences in their frequencies in neither pM1 nor pM2 cultivated with conditioned media of senescent HDFs compared to non-senescent HDFs (Figure 23 A and B). Furthermore, no significant differences in the expression of CD80 and CD163 after cultivation with conditioned media from senescent compared to non-senescent HDFs, indicated by the MFI, were observed in the flow cytometric analysis (Figure 24 A and B).





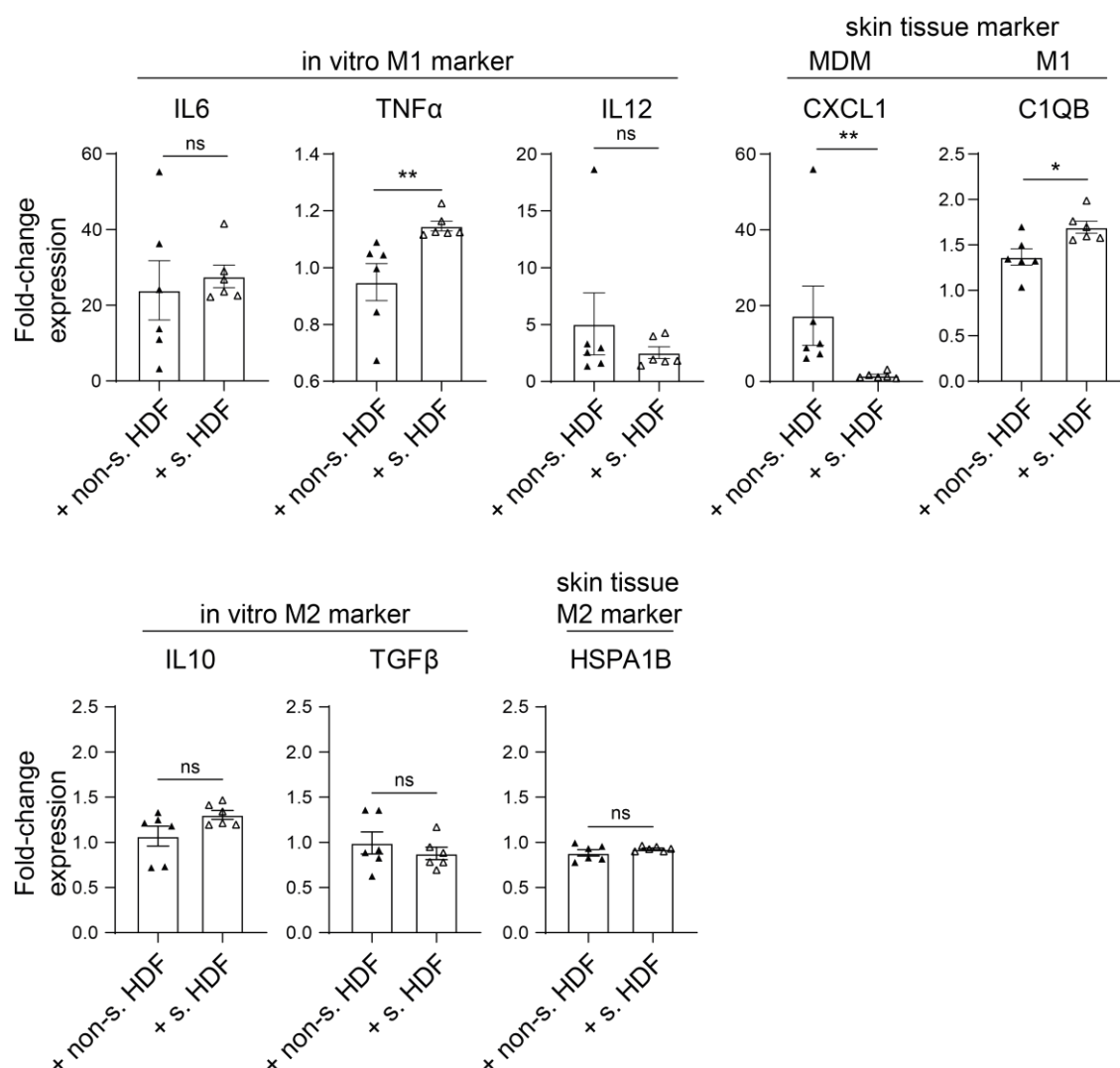
**Figure 24 Expression of macrophage lineage marker in pM1 and pM2 after cultivation with conditioned media of senescent and non-senescent HDFs.** Partially polarized MDMs pM1 (A) and pM2 (B) were cultured with conditioned media of non-senescent (+non-s. HDF) and senescent (+s. HDF) HDFs for 24 hours. Expression of CD80 and CD163 was determined via flow cytometry. Expression of CD80 and CD163 is displayed in histogram of concatenated samples. Bar graphs indicate the protein levels of CD80 and CD163 indicated by the MFI in pM1 (A) and pM2 (B). Dots represent individual donors and bars indicate the mean  $\pm$  SEM. HDFs derived from n=6 per group, Two-tailed Mann-Whitney U test, ns  $p>0.05$

In addition, the percentage of phagocytic activity in these cells was determined as described in 2.2.2.5. After culturing of pM1 and pM2 with conditioned media of senescent compared to non-senescent HDFs, no significant differences in the proportion of phagocytic cells were assessed (Figure 25 A and B).

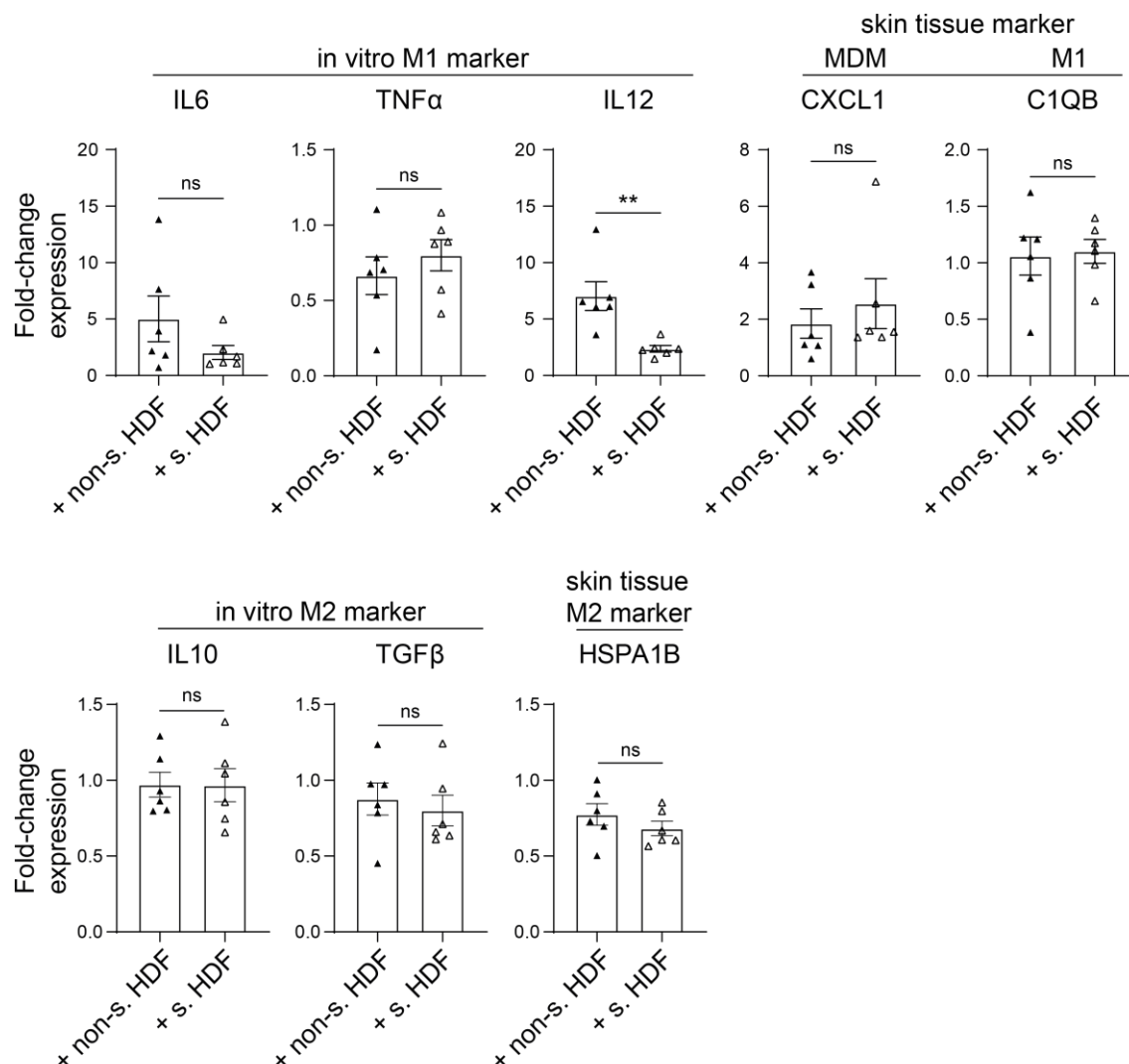


**Figure 25 Percentage of phagocytic MDMs after cultivation with conditioned media of non-senescent and senescent HDFs.** Partially polarized pM1 (A) and pM2 (B) were generated by differentiation with either GM-CSF or M-CSF for 7 days, respectively. Partially polarized MDMs were cultured with conditioned media of senescent (+s. HDF) or non-senescent (+non-s. HDF) HDFs. Afterwards, phagocytosis assay was performed to identify the percentage of phagocytic cells within all live cells. Bar graphs indicate the percentage of phagocytic cells, dots represent individual donors and bars indicate the mean  $\pm$  SEM. HDFs derived from n=6 per group, Two-tailed Mann-Whitney U test, ns  $p > 0.05$

To infer about a possible pro- or anti-inflammatory phenotype of the pM1 and pM2 after treatment with conditioned media of senescent compared to non-senescent HDFs, the expression of M1 and M2 cytokine and skin markers was determined in pM1 (Figure 26) and pM2 (Figure 27) macrophages via qPCR. The analysis shows, similar to the co-culture with aged fibroblasts, that the expression of M2 markers IL10, TGF $\beta$  and HSPA1B was not affected in pM1 and pM2 by the stimulation with conditioned media of senescent HDFs (Figure 26 and Figure 27). In contrast, the cultivation with conditioned media of senescent HDFs significantly increased TNF $\alpha$  and C1QB expression in pM1 in comparison to treatment with non-senescent HDFs. Differing from the increase of these two M1 markers, the expression of IL-12 and CXCL1 seem to be decreased in pM1 as well as IL-12 and IL6 in pM2 after cultivation with conditioned media of senescent HDFs compared to non-senescent HDFs (Figure 26 and Figure 27).



**Figure 26 Expression of macrophage markers in pM1 MDMs after cultivation with conditioned media derived from senescent or non-senescent HDFs.** pM1 MDMs were generated by differentiation with GM-CSF. Partially polarized MDMs were cultured with conditioned media of senescent (+s. HDF) or non-senescent (+non-s. HDF) HDFs. After cultivation for 24 hours the MDMs were harvested for subsequent RNA isolation. mRNA expression of *in vitro* M1, skin tissue M1, *in vitro* M2 and skin tissue M2 marker was determined via qPCR. Expression was normalized on expression of housekeeping gene GAPDH and control partially polarized MDMs cultured without HDFs. Dots represent individual HDF donors and bars indicate the mean  $\pm$  SEM. HDFs derived from n=6 donors. Two-tailed Mann-Whitney U test, ns  $p>0.05$ , \*  $p<0.05$ , \*\*  $p<0.01$



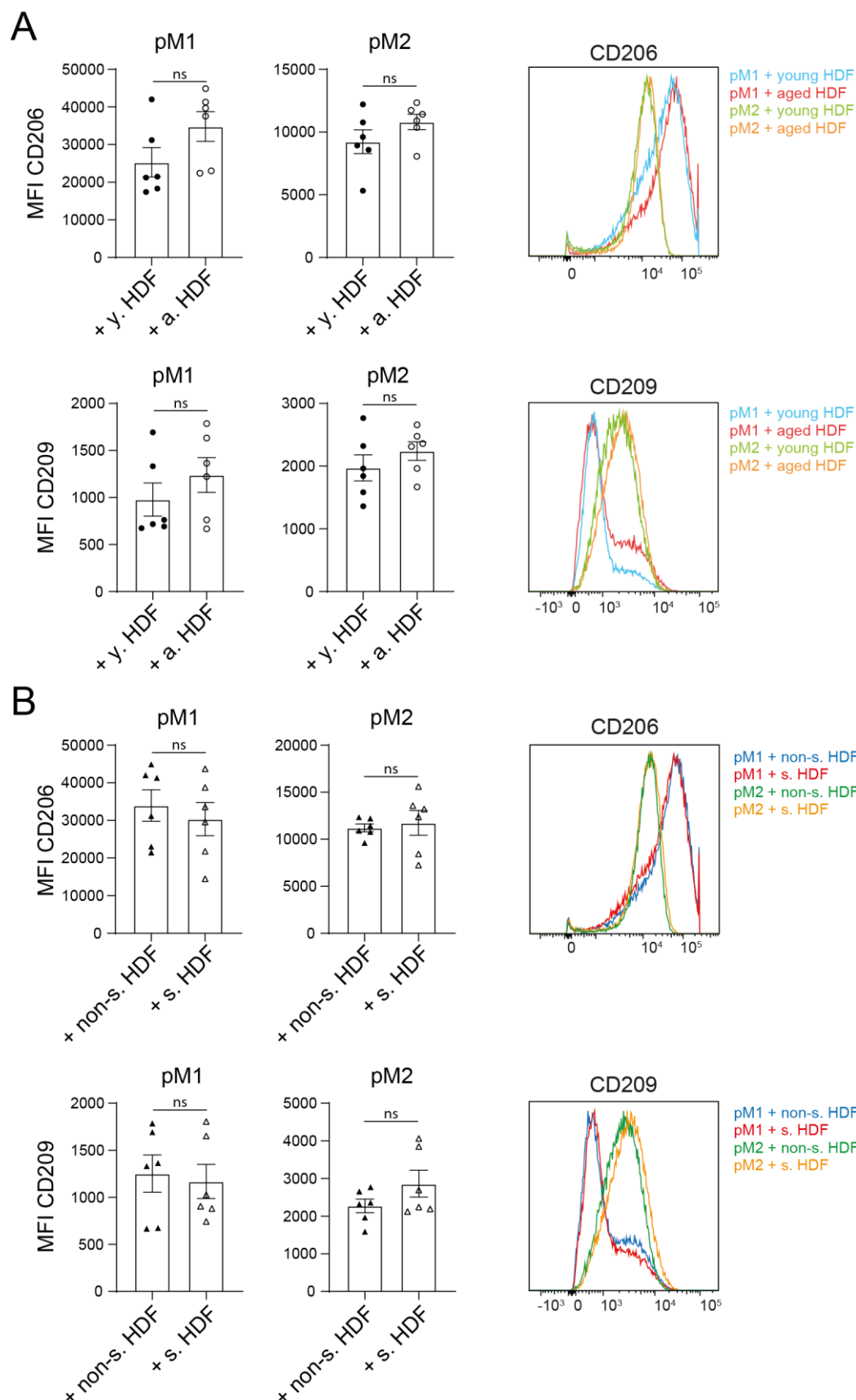
**Figure 27 Expression of macrophage markers in pM2 MDMs after cultivation with conditioned media derived from senescent or non-senescent HDFs.** pM2 MDMs were generated by differentiation with M-CSF. Partially polarized MDMs were cultured with conditioned media of senescent (+s. HDF) or non-senescent (+non-s. HDF) HDFs. After cultivation for 24 hours the MDMs were harvested for subsequent RNA isolation. mRNA expression of *in vitro* M1, skin tissue M1, *in vitro* M2 and skin tissue M2 marker was determined via qPCR. Expression was normalized on expression of housekeeping gene GAPDH and control partially polarized MDMs cultured without HDFs. Dots represent individual HDF donors and bars indicate the mean  $\pm$  SEM. HDFs derived from  $n=6$  donors. Two-tailed Mann-Whitney U test, ns  $p>0.05$ , \*\*  $p<0.01$

In conclusion, the phagocytic activity, the expression of macrophage lineage marker CD80 and CD163, as well as the mRNA expression of M2 lineage marker were not significantly affected by the cultivation with conditioned media of senescent HDFs. However, the conditioned media of senescent HDFs significantly increased the expression of M1 marker TNF $\alpha$  and C1QB. Summarized, these findings indicate that the increased pro-inflammatory characteristics observed in aged skin macrophages might be induced by the surrounding tissue comprising increased numbers of senescent cells.

### **3.3.3 CD80, CD206 and CD209 expressions correlate with phagocytic activity in pM1 MDMs**

Since the phagocytic activity was enhanced in pM1 after cultivation with conditioned media of aged HDFs compared to young HDFs, a possible relation between phagocytosis and the expression of surface markers CD206 and CD209 was investigated. CD206 and CD209 are reported to be involved in the regulation of phagocytosis mechanisms (Schulz et al. 2019).

In this regard, the expression of CD206 and CD209 was determined in the pM1 and pM2 after cultivation with supernatants of young and aged HDFs as described in Figure 17. Flow cytometric analysis reveals that both surface markers seem to be slightly increased by a FC of 1.3 in the pM1 after cultivation with supernatants of aged compared to young HDFs. Nevertheless, the expression of CD206 and CD209 in pM2 was not affected (Figure 28 A). Furthermore, the effect of conditioned media of non-senescent compared to senescent HDF conditioned media on the expression of CD206 and CD209 was determined. As shown in Figure 28 B, the expression of CD209 was slightly upregulated in pM2 by a FC of 1.3 after the treatment with conditioned media of senescent in comparison to non-senescent HDFs. Its expression in pM1, as well as CD206 expression in both pM1 and pM2 was similar after treatment with both conditioned media (Figure 28).

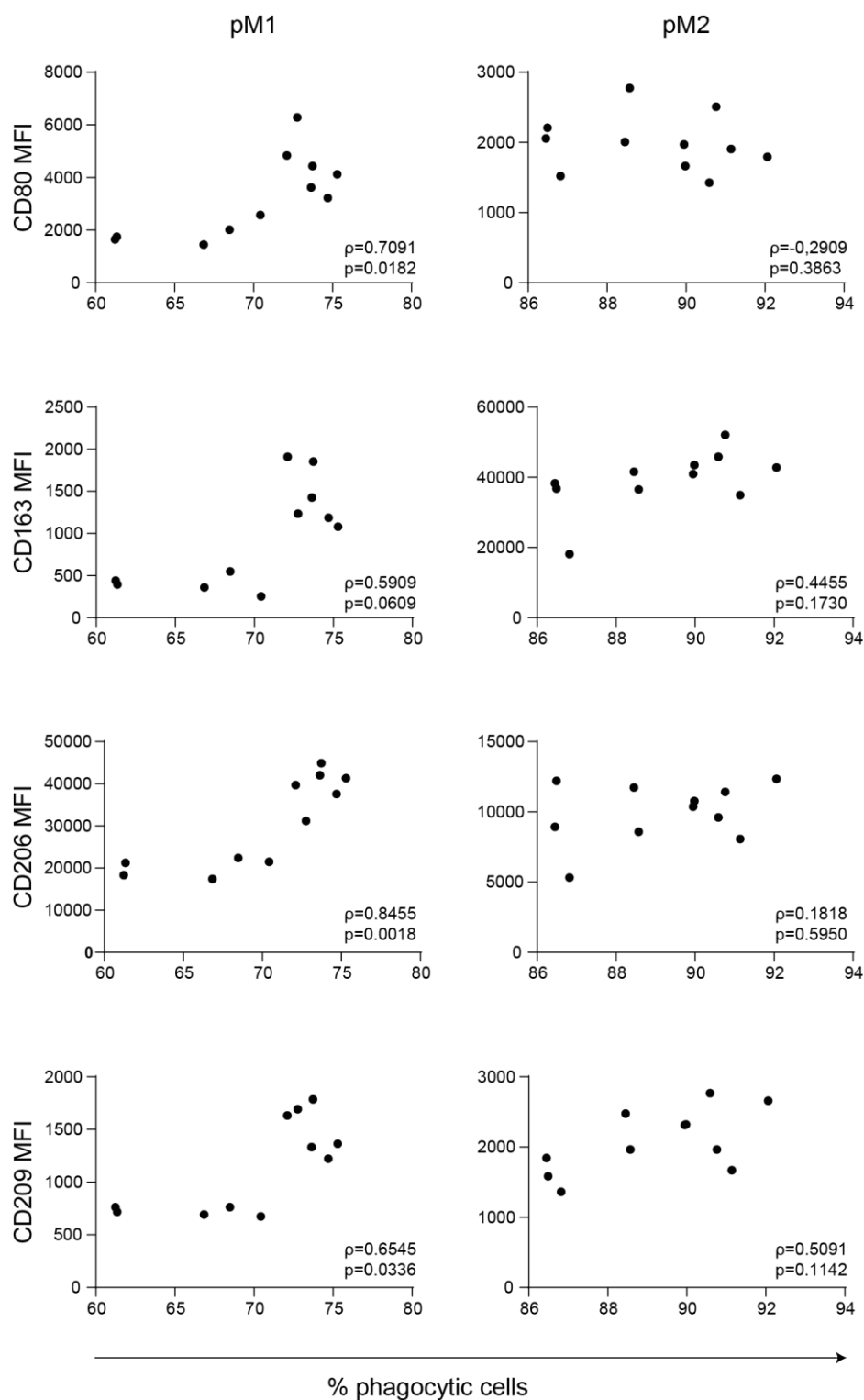


**Figure 28 Expression of surface markers CD206 and CD209 in pM1 and pM2 after cultivation with HDF supernatants.** Partially polarized MDMs were cultured with conditioned media of (A) young (+y. HDF) or aged (+a. HDF) or (B) senescent (+s. HDF) or non-senescent (+non-s. HDF) HDFs for 24 hours. Expression of CD206 and CD209 was determined via flow cytometry. Expression of CD206 and CD209 is



displayed in histogram of concatenated samples. Bar graphs indicate the protein levels of CD206 and CD209 indicated by the MFI. Dots represent individual donors and bars indicate the mean  $\pm$  SEM. n=6 per group, ns  $p>0.05$

Although the changes in the expression of the surface marker CD206, CD209 as well as CD80 and CD163 were not significant after cultivation with conditioned media of aged compared to young HDFs (3.3.1), a relation between their expression and the significantly increased phagocytic activity was determined. The phagocytic activity and the expression of the surface marker were measured in the same experiment, next a correlation analysis between those parameters was performed. The Spearman correlation analysis demonstrates that the expression of CD80, as well as CD206 and CD209 are significantly positively correlated with the phagocytic affinity in pM1, whereas no correlation in the pM2 was observed. The CD163 expression did not correlate with the phagocytic activity in neither pM1 nor pM2 (Figure 29).



**Figure 29 Spearman correlation analysis between surface marker expression and phagocytic capacity.** Expression of CD80, CD163, CD206 and CD209, indicated by the MFI, directly correlated with the phagocytic affinity in pM1 (left) and pM2 (right) after cultivation with conditioned media of HDFs. Spearman correlation analysis,  $\rho$ = spearman correlation coefficient,  $p$ =p value,  $n=11$  per group

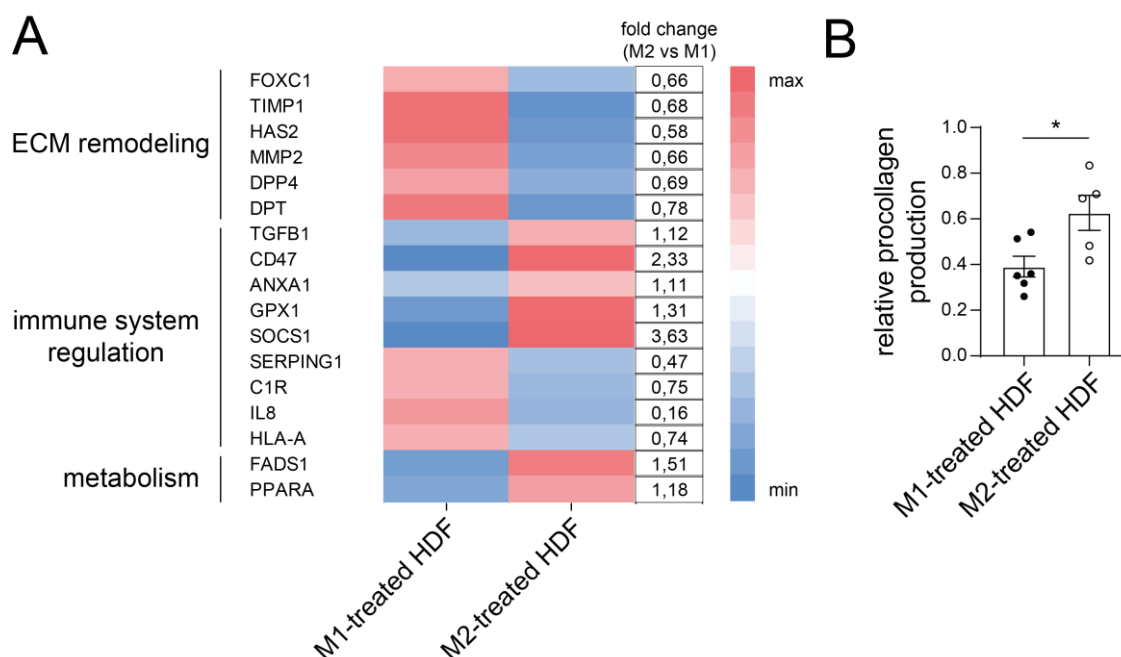
In conclusion, CD206 and CD209 were slightly upregulated in pM1 after the cultivation with conditioned media of aged HDFs. Additionally, Spearman correlation analysis demonstrates a significant correlation between the CD206, CD209 and CD80 expression and phagocytic capacity in the pM1 macrophages.

### 3.4 Macrophages subtypes affect the aged fibroblasts phenotype

To investigate how the increased M1 and the simultaneously decreased M2 characteristics in the aged skin could impact the dermal tissue, the influence of the two *in vitro* macrophage subsets on dermal fibroblasts was analyzed. Crosstalk between macrophages and fibroblasts, e.g. during wound healing, has already been reported for decades (Mescher 2017). Macrophages have been shown to activate fibroblasts to produce proteins of the ECM by secreting cytokines such as IL-1 or TGF- $\beta$  (Van Linthout, Miteva, and Tschöpe 2014).

First, the aged phenotype of HDFs during the presence of macrophage phenotype was examined by treating a pool of aged HDFs (average age = 66.3 years) with conditioned media from M1 and M2 macrophages which were generated *in vitro* as described before (2.2.2.4). The conditioned media was harvested on the second day after polarization of the M1 or M2 MDMs and directly applied to a pool of HDFs derived from 3 different donors. Subsequently, gene expression was analyzed for genes that are known to be differentially expressed in aged fibroblasts and are involved in e.g. ECM remodeling, immune system regulation and metabolism.

In comparison to the treatment with M1 conditioned media, the addition of M2 conditioned media to the HDFs significantly increased the expression of FADS1 and PPARA (Figure 30 A). Additionally, an upregulation of anti-inflammatory genes, such as TGF $\beta$  and ANXA1 as well as antioxidant enzyme GPX1 was detected. Fibroblasts treated with M1 macrophage conditioned media showed a significant increase in several genes involved in ECM remodeling, like MMP2, DPT and HAS2, compared to the M2 conditioned media treated fibroblasts. Furthermore, genes associated with the complement system like C1R and SERPING1 or the recruitment of immune cells as IL8 were increased by more than 30% in the M1-treated fibroblasts. Additionally, procollagen synthesis was investigated in the supernatant of HDFs treated with either M1 or M2 conditioned media. A 30% lower synthesis of procollagen of HDFs treated with M1 in comparison to M2 conditioned media was detected (Figure 30 B).



**Figure 30 Gene expression pattern and procollagen synthesis in M1 and M2 macrophage treated HDFs.** A pool of HDFs derived from 3 aged donors (average age 66.3 years) was treated with supernatants from *in vitro* M1 or M2 MDMs for 24 hours. (A) Expression of several genes was determined with the NanoString nCounter software. Heatmap shows genes that were significantly differentially expressed in M1- vs. M2-treated HDF pools. FCs between M2 vs. M1-treated HDFs are listed for each gene. (B) Procollagen in supernatants of dermal fibroblasts after treatment normalized to untreated fibroblasts and cell number. Dots represent individual donors and bars indicate the mean  $\pm$  SEM. (A)  $n=12$  per group, paired two-tailed t-test with Benjamini-Hochberg correction, (B)  $n=5-6$  pools per group, paired two-tailed student's t-test \*  $p<0.05$

Taken together, the M1 and M2 MDMs differentially affect the expression of genes involved in the immune response, ECM remodeling and metabolism. An imbalance of M1 to M2 macrophages in the aged human skin, could therefore lead to a changed phenotype of HDFs in the aged human skin.

## 4 Discussion

### 4.1 Changes of macrophages in aged human skin

To date, several studies reported changes in the abundance of macrophages in aged tissues. Age-related changes in the number of macrophages have been already reported for various tissues. An increase in macrophage numbers has been reported for the bone marrow, spleen, muscles and brains in aged mice (Wang et al. 1995, Duong et al. 2022, van Beek et al. 2019). In contrast, a decreased abundance of macrophages in the lung of aged mice have been described (Wong et al. 2017, van Beek et al. 2019). Furthermore, several studies reported that the aged tissue macrophage phenotype change upon age, displayed by alterations in the phagocytic activity or the production of cytokines (Mogilenko, Shchukina, and Artyomov 2021). In murine, it has been reported that the skin-resident macrophages are replaced by MDMs over time (Tamoutounour et al. 2013). But for the human skin tissue, no age-dependent changes in macrophage abundance or phenotype have been described yet.

The present study indicates an increase in the macrophage population in the aged human dermis, demonstrated by a significant higher frequency of CD68<sup>+</sup> cells in the aged compared to young human skin sections (Figure 8). This finding was confirmed by analysis of an integrated scRNA-seq dataset in which 5 independent scRNA-seq datasets were combined and analyzed. The analysis showed a significant increase in the frequencies of macrophages within the myeloid cell cluster (Figure 9). Together, these two different methods suggest an accumulation of macrophages in the aged human skin tissue. An increase in macrophage numbers has been already reported for other tissues (Wang et al. 1995, Duong et al. 2022, van Beek et al. 2019). An increase of myeloid cells during aging could be a result of increased myelopoiesis described in older individuals (Wang, Geiger, and Rudolph 2011, Cho, Sieburg, and Muller-Sieburg 2008, Melamed and Scott 2012). However, the scRNA seq data showed no increase in myeloid cell abundance in the aged compared to the young dermis (Figure 9). In the present study, pseudo-bulk RNA-seq analysis also revealed an increase in the expression of pro-inflammatory markers in aged skin macrophages (Figure 10). These included genes that are highly expressed by M1 and MDM skin macrophages which were recently classified by Reynolds et al. (Figure 10) (Reynolds et al. 2021). This suggests that the M1 macrophage phenotype increases in aged skin macrophages and MDMs are increasingly recruited to the skin tissue. In other non-lymphoid tissue such as the murine liver or white adipose tissue, a decrease of M2 macrophages has been reported. In contrast, in lymphoid tissues more anti-inflammatory M2 macrophages were detected

(Jackaman et al. 2013). Increased pro-inflammatory activity in non-lymphoid tissue such as skin tissue may compensate for reduced lymphopoiesis and adaptive immune function that has been associated with age (Melamed and Scott 2012, Linton and Dorshkind 2004, Palmer 2013, Rezzani et al. 2014). Mouse skin macrophages have been shown to acquire a memory expression profile that enables them to respond with higher pro-inflammatory reactivity to secondary infections (Chan et al. 2018). It has been suggested that these trained macrophages play an important role in the protection against pathogens reinfection of vertebrates lacking lymphocytes (Quintin et al. 2012). In the skin tissue, which is exposed to pathogens in a daily basis, this compensation mechanism could be an explanation for the increased number as well as the more pro-inflammatory phenotype of macrophages in aged human skin.

In addition, monocytes are reported to gain a pro-inflammatory imprint when they are recruited to the tissue during an inflammatory episode (Aegerter et al. 2020, Williams and Svedberg 2021). Aged skin is thought to be chronically inflamed at low levels due to the inflammaging process, as evidenced by an increase in cytokines TNF- $\alpha$  and IL-6 and other SASP factors (Franceschi et al. 2000, van Beek et al. 2019). On the other hand, CD4<sup>+</sup> T<sub>reg</sub> cells are known to suppress inflammatory mononuclear cells, and a reduced lymphopoiesis may result in a decline of these cells within the skin tissue (Haertel et al. 2018). This loss may also contribute to an accumulation of inflammatory mononuclear cells such as macrophages within the tissue.

In addition, an altered microbiome in aging may lead to altered cytokine secretion by skin cells. For aged mice, a correlation between the aged gut microbiome increased levels of circulating LPS in the gut and plasma has been already reported. This resulted in an increase in systemic inflammation followed by the activation of peritoneal macrophages (Thevaranjan et al. 2017, Kim et al. 2016).

Overall, the increased production of systemic inflammatory factors in aged human skin could result in a polarization of macrophages to a more pro-inflammatory phenotype and therefore explain the increased pro-inflammatory M1 and decreased anti-inflammatory M2 characteristics of skin macrophages. Furthermore, increased cytokine levels could drive increased recruitment of immune cells and therefore the increased number of MDMs in aged skin macrophages. However, it is not clear whether the recruited MDMs are supposed to replace the skin-resident macrophages over time or supplement them. A replacement of fetal macrophages with MDMs has been already shown for murine skin (Tamoutounour et al. 2013). This could be necessary because of an increased occurrence of senescent macrophages, that fail to become apoptotic to be able to be

removed. Accordingly, the proportion of senescent cells within the macrophages in the old skin tissue remain to be determined in future studies.

Furthermore, tissue macrophages exhibit more heterogeneous macrophage subsets *in vivo* and do not adopt distinct M1 or M2 phenotypes generated *in vitro* in tissue. Detection of changes in gene expression by pseudo-bulk RNA-seq analysis particularly regarding pro- and anti-inflammatory molecules provides only an indication of the activation state of macrophages. Future studies should include an in-depth analysis of macrophage phenotypes in the aged human skin tissue.

## 4.2 Aging of macrophages induced by intrinsic factors

Several studies reported age-dependent phenotypical and functional changes in macrophages, as evidenced by alterations in cytokine production and phagocytic activity (Mogilenko, Shchukina, and Artyomov 2021). Age-dependent changes of macrophages are mostly observed in tissue macrophages and thus in the context of tissue aging (De Maeyer and Chambers 2021). It has already been demonstrated that age-dependent changes in tissue macrophages differ from monocyte-derived macrophages *in vitro* (Linehan et al. 2014). This led to the suggestion that the changes in macrophages induced by the surrounding aged tissue manifest differently from changes induced by intrinsic aging of the macrophages themselves. Nevertheless, reports about the intrinsic aging of macrophages are rare.

To investigate the intrinsic aging of human macrophages, in this study the phenotype of primary macrophages differentiated from progenitor blood-monocytes without exposure to young or aged tissues, was determined *in vitro*. These MDMs were differentiated from blood monocytes in the presence of M-CSF or GM-CSF, followed by a polarization with LPS and IFN- $\gamma$  or IL-4 and IL-13, to generate pro-inflammatory M1 or anti-inflammatory M2 MDMs, respectively. The analysis of macrophage lineage markers CD80 and CD163, as well as the expression of pro- and anti-inflammatory cytokines proved the M1 and M2 characteristics of the generated *in vitro* MDMs (Figure 13 and Figure 14). Although tissue macrophages display more heterogeneous macrophage subsets *in vivo*, the *in vitro* M1 and M2 MDMs were chosen here as an *in vitro* model to study intrinsic aging of macrophages in terms of their differentiation ability.

Various reports demonstrated increased serum levels of pro-inflammatory cytokines such as IL-6 and TNF- $\alpha$  and a decrease in anti-inflammatory markers with age (Franceschi et al. 2007, Bruunsgaard et al. 1999, Zanni et al. 2003). In this context, macrophages are suggested to be key regulators of this imbalanced production of inflammatory factors, called inflammaging. In contrast, the present study shows similar

mRNA expression of pro-inflammatory cytokines in young and aged M1 MDMs *in vitro*. In addition, the anti-inflammatory factors, like TGF $\beta$  and IL10 were similarly expressed in young and aged M2-MDMs *in vitro* (Figure 14). Thus, intrinsic aging seems to not affect the cytokine expression in macrophages. Therefore, the imbalanced production of pro- and anti-inflammatory factors observed in aged tissues does not appear to be due to the intrinsic aging of macrophages.

In addition, CXCL1, C1QB and HSPA1B were determined to be equally expressed in young compared to aged MDMs *in vitro* (Figure 16), even though these genes were differentially expressed in aged compared to young *ex vivo* skin macrophages (Figure 10). This led to the assumption that their altered expression in aged dermal macrophages is also not induced by intrinsic aging factors. Like the increased expression of pro-inflammatory cytokines upon age, their expression seems to be affected by other factors rather than the intrinsic aging of the macrophages themselves.

Furthermore, the expression of differentiation marker in M2 MDMs did not seem to be influenced by intrinsic aging. In contrast, the differentiation to pro-inflammatory M1 MDMs appeared to be impaired in aged MDMs *in vitro*. This is evidenced by a significantly higher proportion of a population expressing both M1 and M2 lineage markers CD80 and CD163 in the aged compared to young M1 MDMs (Figure 13). Additionally, the aged M1 MDMs showed significantly higher activity in phagocytosis compared to the young M1 MDMs (Figure 15). Spearman correlation analysis revealed a significant relationship between CD163 expression and phagocytic capacity (Figure 29). It has already been reported that CD163 is involved in the phagocytic process, although it is not fully understood what function CD163 plays in this process (Schulz et al. 2019, Kneidl et al. 2012). The increased phagocytic affinity could be the result of an increased expression of CD163 in the aged M1 MDMs. With regard to phagocytosis and expression of lineage marker CD163, the aged M1 MDMs rather seem to resemble M2 MDMs. This suggests that aged compared to young MDMs are more likely to differentiate into an M2 phenotype *in vitro*. It has been already demonstrated that macrophages in lymphoid tissues such as spleen or lymph nodes of aged mice differentiate more strongly into an M2 phenotype (Jackaman et al. 2013). Whether the population of M1 MDMs that express both CD80 and CD163 show changes on other levels, such as the expression of pro- or anti-inflammatory cytokines, remains to be elucidated.

Dermal macrophages are gradually replaced by bone-marrow monocytes that migrate into the tissue and differentiate into macrophages (Ginhoux and Guillemins 2016). According to their expression of CD14 and CD16, they are classified as classical (CD14<sup>++</sup>CD16<sup>-</sup>), intermediate (CD14<sup>+</sup>CD16<sup>+</sup>) and non-classical (CD14<sup>-</sup>CD16<sup>++</sup>)



monocytes. An increase in CD14<sup>+</sup>CD16<sup>++</sup> non-classical monocytes in the peripheral blood of aged individuals is thought to contribute to an altered differentiation ability to macrophages (Hearps et al. 2012). In this study, the flow cytometric analysis showed a similar percentage of the non-classical monocyte subset in the young and aged donors. Additionally, the percentages of classical and intermediate monocyte subsets did not differ between the young and the aged group. This is in line with a publication by Costantini et al. in 2018. Although they demonstrated that healthy aging is associated with a significant increase in total monocytes, the frequencies of the three monocyte subsets showed no significant changes between young and aged subjects (Costantini et al. 2018). The present study and the study by Costantini et al. have in common that monocytes from healthy donors were examined. This suggests that healthy aging is not associated with an increase of non-classical monocytes. Considering that the distribution of monocyte subsets was similar in the young and aged donor group, a potential effect of one distinct phenotype on the differentiation to macrophage phenotype could thus be excluded.

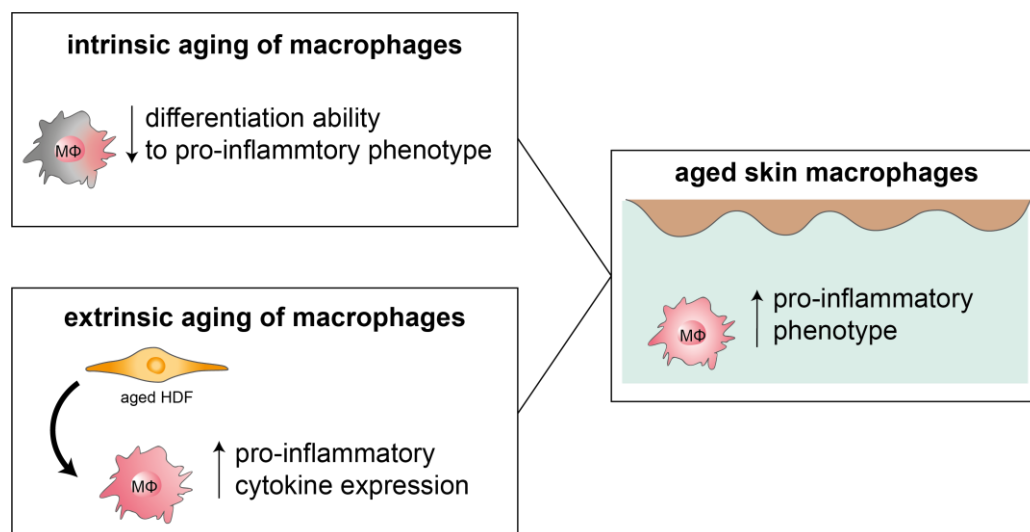
However, dermal macrophages not only originate from monocytes. During fetal and embryonic development, precursor cells of the yolk sac and fetal liver develop into macrophages and colonize the skin tissue. These cells are resident in the skin tissue and are gradually replaced over the lifetime (Ginhoux and Guilliams 2016). Thus, the dermal macrophage populations represent a heterogenic population derived from embryonic and fetal as well as HSC precursor cells. Whether the fetal and embryonic derived dermal macrophages age intrinsically remains unknown.

### **4.3 Age-dependent changes of macrophages caused by the microenvironment**

Macrophages are highly affected by tissue-specific environmental cues. For example, it has been demonstrated that aged tissue impairs the macrophage function like phagocytosis, since macrophages showed a decline in phagocytotic activity after transplantation into aged tissue (Linehan et al. 2014)

In the present study, the role of extrinsic factors during the aging of macrophages was investigated by cultivating partially polarized pM1 and pM2 MDMs with aged HDFs or their conditioned media. Although the co-cultivation with aged HDFs or their conditioned media had no effects on the expression of macrophage lineage markers CD80 and CD163, the expression of pro-inflammatory cytokines was increased in both pM1 and pM2 (Figure 19 and Figure 24). The pM1 and pM2 macrophages were already partially

polarized *in vitro*, thus they already express high levels of the lineage marker specific for M1 or M2 MDMs. Nevertheless, the high expression of pro-inflammatory cytokines suggests, that the skin microenvironment mimicked by aged HDFs causes the macrophages to display a more pro-inflammatory phenotype. Aged skin is thought to be affected by inflammaging, characterized by increased levels of pro-inflammatory cytokines (Zhuang and Lyga 2014). The increase of inflammation factors in aged human skin could result in a polarization of macrophages to a more pro-inflammatory phenotype. Therefore, the macrophages become more pro-inflammatory induced by the extrinsic stimulus. This could mean that even if the migrated MDMs have an impaired ability to differentiate to the M1 phenotype (Figure 13), they are still driven to exhibit a more pro-inflammatory phenotype by the aged HDFs, as summarized in Figure 31. Additionally, while CXCL1 and C1QB were similarly expressed in young and aged MDMs *in vitro* (Figure 16), co-culturing pM1 with aged HDFs compared with young HDFs significantly increased their expression (Figure 21). This is comparable to aged dermal macrophages, which showed significantly higher expression of these genes compared with young dermal macrophages *ex vivo* (Figure 10). This indicates that extrinsic aging induced by adjacent dermal fibroblasts contributes significantly to the increased expression of the genes.



**Figure 31 Illustration of extrinsic and intrinsic aging of macrophages versus macrophages in aged skin tissue.** Intrinsic aging results in decreased differentiation ability towards the pro-inflammatory M1 macrophage phenotype. In contrast, macrophages cultivated with aged HDFs showed increased pro-inflammatory characteristics. Similarly, macrophages in aged skin tissue also exhibit an enhanced pro-inflammatory characteristic.

In this study, the phagocytotic activity of pM1 treated with conditioned media from aged HDFs was found to be increased compared to pM1 treated with conditioned media of young HDFs (Figure 20). Additionally, these treated pM1 showed slightly higher expression of the surface markers CD206 and CD209 (Figure 28). Moreover, their expression correlated significantly positively with the phagocytic activity of these macrophages (Figure 29). In an in-depth phagocytosis assay in monocyte-derived macrophages, Schulz et al. characterized molecules involved in the phagocytosis of macrophages. Among others, CD206 and CD209 were identified as critical molecules for phagocytosis (Schulz et al. 2019). This demonstrates, that aged HDFs not only influence phagocytic affinity but also the expression of phagocytosis associated PRRs such as CD206 and CD209. However, it is possible that further PRRs not assessed in this study change upon treatment with conditioned media and contribute to altered phagocytosis. Furthermore, the phagocytosis assay performed here made use of fluorogenic dye conjugated to the polysaccharide zymosan, and therefore reflects the phagocytosis of fungi. Future studies should investigate changes in phagocytosis in the context of damaged and dead cells in the skin, which has more relevance to the regeneration and aging process.

However, studies reported decreased phagocytic activity of macrophages in aged tissues (De La Fuente 1985, Lynch et al. 2010). The development of a macrophage phenotype is influenced by the duration of exposure to a stimulus (Bleriot, Chakarov, and Ginhoux 2020). Therefore, differences in the exposure time of macrophages to the aged tissue could explain the contrary data of phagocytic affinity found in aged macrophages. While short-time cultivation with conditioned media of aged HDFs for 24 hours enhanced the phagocytic affinity in partially polarized MDMs (Figure 20), prolonged exposure to stimuli from aged tissue could impair macrophage phagocytosis function. In aged tissue, damaged or dysfunctional HDFs might permanently express 'eat-me' signals or decrease the expression of 'don't eat me' signals like CD47, to promote the elimination by macrophages (Park and Kim 2017). In addition, this study showed, that dermal macrophages exhibit a more pro-inflammatory M1 phenotype (Figure 10), and *in vitro* generated M1 MDMs showed a significant lower phagocytic activity than M2 MDMs (Figure 15). This suggests that dermal macrophages display lower phagocytic activity with age, as described already for other tissues (De La Fuente 1985, Lynch et al. 2010).

Like the treatment with aged HDFs, the conditioned medium from senescent HDFs induced the expression of pro-inflammatory cytokines (Figure 26). Nevertheless, the effect on the partially polarized MDMs was not as high as after treatment with conditioned media of aged HDFs. This might be due to the different experimental setups. pM1 and

pM2 were co-cultured with HDFs for 24 hours, whereas conditioned media of senescent HDFs were used to determine the influence of these cells on the macrophage phenotype. The difference between constant stimulation by the secretome of HDFs and one-time treatment with collected secretome of HDFs could explain the weaker effects observed in the senescent-treated macrophages. But senescent HDFs also have different properties than aged HDFs such as the secretion of SASP factors and thus could differently affect the surrounding cells (Coppe et al. 2010) .

Furthermore, it is suggested that tissue and organ failure induced by the accumulation of senescent cells is mainly caused by their long-term presence, and the development of a macrophage phenotype is also determined by the period of time it spends in a given tissue and exposure to its stimuli (Elder and Emmerson 2020, Bleriot, Chakarov, and Ginhoux 2020). This could explain why short-time cultivation with supernatants of senescent cells for 24 hours had just slight effects on macrophages.

Nevertheless, it remains unclear how long-term cultivation of macrophages with aged or senescent HDFs affects the phenotype of macrophages. Although aged HDFs were used in this study to assume the effect of aged microenvironment on the macrophages, skin tissue is more complex and comprises more cell types and components. It has been demonstrated that sensory neurons play a critical role in regulating immune responses in the skin tissue and can enhance both pro- and anti-inflammatory responses (Riol-Blanco et al. 2014, Szallasi et al. 2007, Kabashima et al. 2019). In addition, the skin microbiome is reported to influence the skin immune cells, such as dermal macrophages, and show alterations upon age (Howard et al. 2022, Byrd, Belkaid, and Segre 2018). Therefore, future studies should include other skin cells and also the microbiome when investigate age-dependent changes of skin macrophages.

#### **4.4 Influence of aged macrophages on the skin phenotype**

Macrophages and fibroblasts are reported to crosstalk within the tissue, e.g. macrophages secrete cytokines to induce fibroblasts proliferation or ECM synthesize (Velnar, Bailey, and Smrkolj 2009). Alterations of the ECM structure and its production by fibroblast were found with increasing age (Gosain and DiPietro 2004). The determination of the influence of aged macrophages on fibroblasts should provide insight into to which extent macrophages and their imbalance of M1 and M2 phenotype are involved in the development of the aging phenotype of the skin.

The data shown in this study indicate that the distinct macrophage subtypes have different influences on fibroblast metabolism, ECM remodeling and inflammatory status

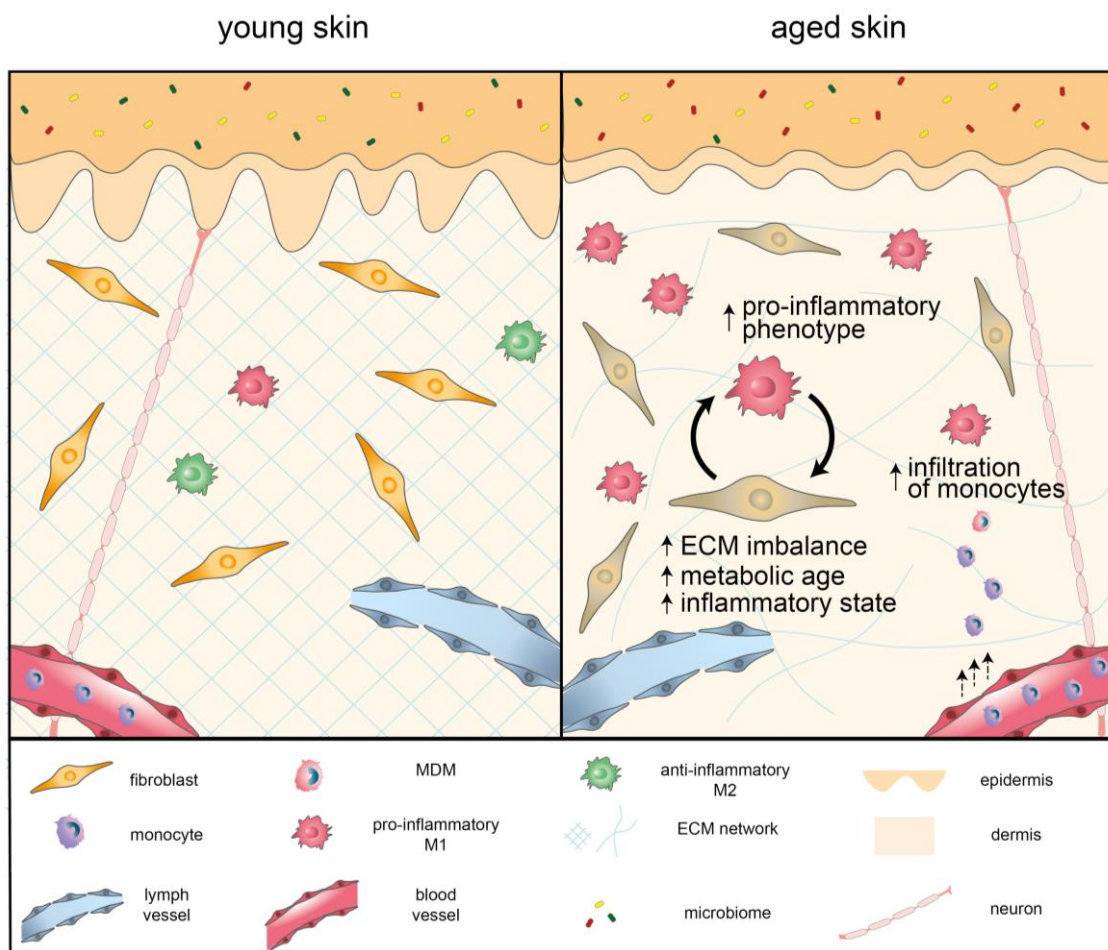
(Figure 30). M1 macrophages particularly enhanced the expression of genes involved in ECM remodeling, such as MMP2. Enhanced MMP2 expression in dermal fibroblasts induced by treatment with M1 conditioned media could potentially lead to the degradation of collagen IV and consequent loss of this molecule in aged skin, which has been reported previously (Feru et al. 2016). On the other hand, the treatment with M2 conditioned media increased the TGFB1 expression (Figure 30). TGF- $\beta$  is reported to increase procollagen synthesis (Bettinger et al. 1996) and suppress MMP2 (Risinger et al. 2010). Additionally, procollagen synthesis was significantly lower in M1-treated HDFs compared to M2-treated HDFs (Figure 30). Overall, this suggests that the increased M1 characteristics and simultaneous loss of the M2 phenotype in human aged skin have detrimental effects on collagen formation and the ECM in aged skin and might thereby contribute to the age-associated loss of tissue integrity and stability.

It was also observed that the expression of PPARA was decreased in M1-treated fibroblasts (Figure 30 A). PPARA is involved in lipid metabolism and its expression is reported to be decreased upon aging. Furthermore, PPARA is thought to slow down aging and prevent age-associated metabolic disorders (Erol 2005, 2007). Reduced expression of PPARA could indicate increased metabolic age of the cells.

In addition, PPARA has been reported to play a role in resolving inflammation by inhibiting NF $\kappa$ B signaling and IL-6 expression (Poynter and Daynes 1998). Moreover, the expression of anti-inflammatory genes TGF- $\beta$  and ANXA1 as well as antioxidant enzyme GPX1 was increased in the M2-treated fibroblasts compared to the M1-treated fibroblasts. In contrast, the pro-inflammatory cytokine IL-8 was higher expressed in the M1-treated fibroblasts compared to the M2-treated fibroblasts. IL-8 belongs to the SASP proteins and is therefore highly expressed by senescent cells such as senescent skin fibroblasts (Dimri et al. 1995). Recently, it was shown, that fibroblasts treated with M1-conditioned media exhibit an increased senescence characteristic manifested by a high percentage of protein 16, protein 21 and  $\beta$ -galactosidase positive cells (Horiba et al. 2022). This led to the hypothesis that M1 macrophages enhance the development of senescence fibroblasts, which are accumulated in aged skin (Dimri et al. 1995, Ressler et al. 2006). In turn, the enhanced SASP in dermal fibroblasts could promote the recruitment as well as a polarization of immune cells such as macrophages towards a pro-inflammatory phenotype.

Taken together, the increased M1 and the concomitant reduced M2 phenotype of macrophages found in human aged skin is likely to contribute to an aged metabolic as well as a more pro-inflammatory and senescent state of the fibroblasts. In addition, this change of macrophage phenotypes in aged human skin may contribute to an imbalanced

ECM remodeling that results in a loss of tissue integrity and stability. Furthermore, the ECM network provides a scaffold for immune cell migration within the tissue and therefore imbalanced ECM remodeling could result in dysregulated immune cell migration. Aged fibroblasts in turn polarize the macrophages to display a more pro-inflammatory phenotype (Figure 21). Overall, the results of this thesis suggest that over time this leads to a vicious circle of inflammation and chronic tissue damage, as shown by the proposed model in Figure 32.



**Figure 32 Illustration of age-related changes in the human skin.** The number of macrophages in human skin increases with age. Macrophages appear to have more MDM characteristics, suggesting that the infiltration of monocytes into the skin tissue is increases with age. Aged skin macrophages also exhibit increased pro-inflammatory M1 characteristics. Aged microenvironment, such as aged HDF, appears to polarize macrophages to display a more pro-inflammatory phenotype. In turn, the M1 macrophages influence the HDF phenotype in terms of metabolic age, ECM remodeling behavior and inflammatory state. Overall, this leads to a vicious circle of inflammation and chronic tissue damage over time. Overall, this leads to a vicious cycle of inflammation and chronic tissue damage over time.

Other components of the skin, such as the microbiome, neuronal network and the vasculature are likely to influence the macrophage phenotype and contribute to the aging

and inflammaging process as well. Therefore, future studies are necessary to investigate the influence of the macrophage phenotype on the aging process of these cells and components. Although studying the interplay of macrophages and fibroblasts by using *in vitro* generated MDMs provides evidence of age-related phenotypic changes, MDMs do not fully resemble the skin macrophages found in young and aged skin *in vivo*. Consequently, future studies are needed to in-depth characterize age-dependent changes in dermal macrophages and their impact on neighboring cells.

## 4.5 Relevance and perspectives

Macrophages are the most abundant immune cell population of the human dermis. They are known for their regulatory function during skin tissue homeostasis and regeneration. Additionally, they are important in the defense against pathogens via eliminating such pathogens and enhancing the inflammatory response by secreting pro-inflammatory factors. The aging of skin tissue is accompanied by phenotypical changes such as impaired regeneration as well as increased levels of pro-inflammatory factors. The increased level of pro-inflammatory factors within the skin tissue, is thought to derive from macrophages. Thus, studying macrophages in the context of aged skin tissue was of great interest, as this could reveal the relation between impaired processes and components in the aged skin tissue and aged macrophages.

In this study, it has been demonstrated that macrophages are mainly affected by extrinsic factors derived from the aged surrounding skin tissue. Impairment of dermal macrophage phenotype and function induced by the aged microenvironment, in turn, influences the surrounding cells like dermal fibroblasts. Chronically increased levels of pro-inflammatory factors secreted by aged macrophages inside the skin tissue could lead to tissue damage, like degradation of the ECM, as this study demonstrated an impaired ECM remodeling in M1-treated dermal fibroblasts. Chronically damaged tissue in turn needs to be permanently regenerated. Since macrophage phagocytic function is critical during tissue homeostasis and regeneration, alterations in the phagocytic activity can influence these processes. In this study, it has been demonstrated, that M1 MDMs in general are less phagocytically active than M2 MDMs. An increase of M1 characteristics in aged skin tissue could lead to loss of phagocytosis.

Reports from the past showed controversial changes in the phenotype and function of macrophages. These studies investigated this process either in tissue macrophages or in animal experiments. It is important to mention that laboratory mice are kept under specific pathogen-free conditions, whereas humans are usually exposed to repeated

infections throughout their lives. These reshape the immune system over time and contributes to immune aging. Thus, the different influences of the environment on the immune system must not be neglected.

Macrophages are associated with many age-associated diseases, like rheumatoid arthritis, atherosclerosis or cancer (Ardura et al. 2019). Nevertheless, the beneficial function of macrophages has been demonstrated, since depletion of macrophages has been shown to result in impaired wound healing or immunosuppression (Patel and Janjic 2015). It has been hypothesized, that the polarization state is the key determinant of disease development. Macrophages represent an attractive therapeutic target especially targeting the polarization state is a strategy of several novel therapies (Ardura et al. 2019). Therefore, revealing that an imbalance of M1 to M2 macrophage phenotype occurs during skin aging, is an interesting point for anti-aging strategies.

Each tissue has a unique macrophage population, suggesting the tissue environment imprints the function and phenotype of macrophages (Ginhoux and Guillemins 2016). As already reported, age-dependent changes of tissue macrophages differ from organ to organ, especially between lymphoid and non-lymphoid tissues. Therefore, it was important to study macrophages in the human skin tissue in order to get insight how dermal macrophages change upon age and to reveal the role of macrophages in age-dependent disorders of the skin. This will provide new approaches for dermal macrophages to prevent or reverse age-associated skin disorders. Future studies should focus on the effects of other skin cell types, such as neurons, immune cells, or even components of the ECM, on the aging of dermal macrophages. In addition, an examination of macrophages in their skin tissue environment across the lifespan should provide insight into the process of skin inflammation and macrophage aging.



## 5 References

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## 8 Eidesstattliche Erklärung

Hiermit erkläre ich, Leonie Katharina Gather, geboren am 21.08.1992 in Neuss, dass die hier vorliegende Arbeit unter dem Titel „Characterization of human dermal macrophages in the context of aging“, abgesehen von der wissenschaftlichen Beratung meines Doktorvaters und meiner Betreuerin in der Beiersdorf AG in Hamburg, nach Inhalt und Form meine eigene Arbeit ist und nur mit den angegebenen Hilfsmitteln und Quellen verfasst wurde.

Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule im Rahmen eines Prüfungsverfahrens vorgelegt. Teile der Arbeit wurden in dem Journal „Journal of Investigative Dermatology“ veröffentlicht. Nicht von mir erstellte Ergebnisse sind als solche ausgewiesen. Weiterhin bestätige ich, dass die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden ist. Ich erkläre, dass ich noch keinen Promotionsversuch unternommen habe und dass mir kein akademischer Grad entzogen wurde.

Hamburg, Oktober 2022

Leonie Katharina Gather