Modulation of Neutrophil Phenotype in Myocardial Infarction and Diabetes

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by

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List of Abbreviations

7-AAD	7 Amino-Actinomycin D
Arg1	Arginase-1
AKT	Protein kinase B
AMI	Acute myocardial infarction
BM	Bone marrow
BSA	Bovine serum albumin
CD	Cluster of Differentiation
cDNA	complementary DNA
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
Cre	Causes recombination
CVD	Cardiovascular disease
DIO	Diabetes induced obesity
dNTPs	Desoxyribonucleosidtriphosphate
dsDNA	Double stranded DNA
ECG	Echocardiography
ER	Endoplasmatic Reticulum
ERK	extracellular-signal-regulated kinase
FC	Fold change
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
gDNA	genomic DNA
GMP	Granulocyte-monocyte progenitor
GRB2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase-kinase 3
GTT	Glucose Tolerance Test
HBSS	Hanks` Balanced Salt Solution
HPC	Haematopoietic progenitor cell
HSC	Haematopoietic stem cell
LT-HSC	Long term haematopoietic stem cell
ST-HSC	Short term haematopoietic stem cell
ICAM1	Intracellular adhesion molecule 1
iNOS	Inducible nitric oxide synthase
IL-4	Interleukin-4

IL-6	Interleukin-6
i.p.	Intraperitoneal injection
IP	Immunoprecipitation
IRS-1/2	Insulin-receptor substrate 1/2
ITT	Insulin Tolerance Test
JAK1/2/3	Janus kinase 1/2/3
KO	Knock-out
LAD	Left anterior descending
loxP	Locus of crossing over
LT-HSC	Long term haematopoietic stem cell
NaCl	Sodium Chloride
NE	Neutrophil Elastase
NET	Neutrophil extracellular trap
NGS	Next-generation sequencing
Nudc	Nuclear distribution protein
MAPK	Mitogen-activated protein kinase
MEP	Megakaryocytic-erythroid progenitors
MI	Myocardial infarction
MFI	Mean fluorescence intensity
MM	Master Mix
MOI	Multiplicity of Infection
MPO	Myeloperoxidase
MPP	Multipotent progenitor
mTOR	Mechanistic target of rapamycin
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDK1	phosphoinositide-dependent kinase 1
PIP2	Phosphatidylinositol-biphosphat
PIP3	Phosphatidylinositol-trisphosphat
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-Myristat 13-Acetat
PRR	Pattern recognition receptor
Raf	Rapidly accelerated fibrosarcoma
Ras	Rapidly accelerated sarcoma

ROS	Reactive oxygen species
RT	Room temperature
s.c.	Subcutaneous injection
SDS	Sodium dodecyl sulfate
SD	Standard deviation
Shc	Src homology collagen
SOCS	Suppressors of cytokine signalling
SSC	Side scatter
T1D	Type 1 diabetes
T2DM	Type 2 Diabetes mellitus
TAM	Tumour-associated macrophages
TAN	Tumour-associated neutrophils
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor α
TPS	Total protein stain
T_{reg}	Regulatory T cells
VEGF	Vascular endothelial growth factor

Abstract

A common cause of death in the western world are cardiovascular diseases (Roth, Huffman et al. 2015). Acute myocardial infarction (AMI) causes a profound sterile inflammation, and neutrophils are the first cells to massively infiltrate the infarcted area. This inflammatory response critically influences outcome after myocardial infarction. Until recently, neutrophils were thought to play a detrimental role in ischaemic heart disease since they enhance inflammation and tissue damage. However, it was lately shown that neutrophils also play an essential role in cardiac remodelling and repair post-MI, thus can have beneficial effects on myocardial healing (Ma, Yabluchanskiy et al. 2016). Comparable to macrophages, at least two distinct phenotypes exist for neutrophils (Fridlender, Sun et al. 2009). Pro-inflammatory N1- neutrophils are contra productive in healing and scar formation after a myocardial infarction, whereas anti-inflammatory N2 neutrophils carry out reparative effects and are involved in the resolution of inflammation, angiogenesis and tissue remodelling (Ma, Yabluchanskiy et al. 2016, Hasan, Luo et al. 2016). It is well known that Type 2 Diabetes Mellitus (T2DM) strongly predisposes to cardiovascular diseases and it was reported that obesity and the following development of hyperglycaemia and hyperlipidaemia, often leading to T2DM and metabolic syndrome, results in chronic inflammation (Lumeng, Bodzin et al. 2007, Lackey and Olefsky 2016).

The major goal of this work was to investigate the modulation of neutrophil polarization towards an anti-inflammatory phenotype and the functional differences in the subtypes of neutrophils in wildtype and hyperglycaemic mice and after MI. The first part of the thesis "Modulation of Neutrophil Phenotype in Myocardial Infarction and Diabetes" concentrates on the characterization of murine bone marrow neutrophils and their polarization *in vitro* in presence of cytokines in a time frame of four hours. It was found that Interferon-γ/Lipopolysaccharide (IFN-γ/LPS) treatment strongly stimulated activation of murine BM neutrophils and led to the induction of a pro-inflammatory neutrophil phenotype. It was as well demonstrated that IL-4 skews neutrophils *in vitro* towards a N2-like neutrophil phenotype. Further, it was shown that neutrophils of male mice were more prone to pro-inflammatory polarization than neutrophils of female mice. Additionally, it was assessed that IL-4 and LPS/IFN-γ induced polarization is dependent on the JAK/STAT signalling pathway. It was demonstrated that, to a large extent N1 polarization by LPS/IFN-γ is dependent on JAK1 activation, which induces phosphorylation and activation of STAT1. In contrast, N2 polarization is mainly dependent on JAK2 activation, which leads to activation and phosphorylation of STAT6. Further, the effect of IL-4 on different neutrophil functions was investigated. This analysis revealed, that IL-4 was able to significantly reduce the formation of NETs and substantially increase the phagocytic capacity of neutrophils. However, IL-4 treatment had no effect on migration and degranulation of neutrophils.

In a second part, the effect of anti-inflammatory IGF-1 on neutrophils in the context of myocardial infarction *in vivo* was investigated and it was shown that IGF-1 treatment suppressed the pro-inflammatory neutrophil phenotype three and seven days after myocardial infarction.

In a last part, the effect of hyperglycaemia on neutrophil phenotype and function was investigated. This analysis revealed that severe hyperglycaemia substantially altered neutrophil phenotype towards a more pro-inflammatory phenotype with upregulation of, amongst others, genes involved in Nfκb signalling. Hyperglycaemia also affected neutrophil functions, as phagocytic capacity was reduced and the formation of NETs was slightly increased.

Thus, this study indicates that neutrophils might have a beneficial effect on myocardial healing by polarizing towards an anti-inflammatory phenotype and that hyperglycaemia induces a more pro-inflammatory neutrophil phenotype.

Zusammenfassung

Eine häufige Todesursache der westlichen Welt sind kardiovaskuläre Erkrankungen (Roth, Huffman et al. 2015). Ein akuter Myokardinfarkt (MI) verursacht eine sterile Entzündung und Neutrophile sind die ersten Zellen, welche die Infarktregion infiltrieren. Diese Entzündungsantwort beeinflusst kritisch den Heilungsprozess nach Myokardinfarkt. Bis zum jetzigen Zeitpunkt wurden Neutrophile als schädigend in ischämischen Herzerkrankungen angesehen, da sie die Entzündung fördern und das Gewebe beschädigen. Jedoch zeigten neueste Daten, dass Neutrophile eine wichtige Rolle in kardialen Remodeling-Prozessen haben und zur Reparatur nach einem Myokardinfarkt beitragen und somit nutzbringend in myokardialer Heilung sind (Ma, Yabluchanskiy et al. 2016). Wie bei Makrophagen, existieren auch bei den Neutrophilen zwei verschiedene Phänotypen (Fridlender, Sun et al. 2009). Proinflammatorische N1-Neutrophile sind kontraproduktiv in Heilung und Narbenbildung nach einem Myokardinfarkt, wohingegen antiinflammatorische N2-Neutrophile reparative Effekte aufweisen und an der Auflösung der Entzündung, Angiogenese und Geweberemodelierung beteiligt sind (Ma, Yabluchanskiy et al. 2016, Hasan, Luo et al. 2016). Außerdem ist bekannt, dass Patienten mit T2DM stark für kardiovaskuläre Erkrankungen prädisponiert sind. Auch wurde berichtet, dass Adipositas und die sich daraus entwickelnde Hyperglykämie und Hyperlipidämie, was wiederum oft zu T2DM führt, in chronischen Entzündungsprozessen im Körper resultiert.
Das Ziel dieser Arbeit war die Untersuchung der Regulierung der Neutrophilenpolarisation zu einem anti-inflammatorischen Phänotypen und die funktionalen Unterschiede der verschiedenen Neutrophilen-Subtypen, sowohl in wildtypischen als auch in hyperglykämen Mäusen und nach MI. Der erste Teil dieser Thesis befasst sich mit der Charakterisierung von murinen Knochenmarksneutrophilen und deren Polarisation *in vitro* in der Anwesenheit von Zytokinen in einem Zeitraum von vier Stunden. Es wurde gezeigt, dass eine LPS/IFN-γ Behandlung Neutrophile aktiviert und einen pro-inflammatorischen Phänotypen induziert. Außerdem wurde gezeigt, dass eine IL-4 Behandlung einen anti-inflammatorischen N2-ähnlichen Phänotypen erzeugt. Zudem wiesen Neutrophile von männlichen Mäusen, im Vergleich zu weiblichen Mäusen, eine verstärkte pro-inflammatorische Polarisation auf. Zusätzlich konnte gezeigt werden, dass die N1- und N2- Polarisation abhängig von dem JAK/STAT Signalweg ist. Die N1-Polarisation, induziert durch LPS/IFN-γ Behandlung, ist hauptsächlich von JAK1-Aktivierung, was wiederum STAT1-Phosphorylierung und -Aktivierung induziert, abhängig. Im Gegenzug ist die IL-4 induzierte N2-Polarisation hauptsächlich von der JAK2-Aktivierung, welche zu STAT6-Phosphorylierung und -Aktivierung führt, abhängig. Außerdem wurde der Effekt der IL-4 Behandlung auf verschiedene Neutrophilen-Funktionen untersucht. Diese Analyse zeigte, dass IL-4 die Formation von NETs (neutrophile extrazelluläre Fallen) signifikant reduzieren kann und die Phagozytose-Kapazität deutlich erhöht. Jedoch hatte die IL-4 Behandlung keine Auswirkung auf die Migration und Degranulation von Neutrophilen.
In einem zweiten Teil der Arbeit, wurde der Effekt von anti-inflammatorischem IGF-1 auf Neutrophile in dem Kontext eines Herzinfarktes *in vivo* untersucht. Es wurde gezeigt, dass IGF-1-Behandlung den pro-inflammatorischen Phänotypen von Neutrophilen drei und sieben Tage nach Infarkt unterdrückt.
In einem letzten Teil der Arbeit wurde die Auswirkung von Hyperglykämie auf die Polarisation und Funktion von Neutrophilen untersucht. Dies zeigte, dass schwere Hyperglykämie wesentlich zu einem pro-inflammatorischen Phänotypen von Neutrophilen führt, was unter anderem durch die Hochregulierung von Nfκb Genen charakterisiert ist. Zudem konnte gezeigt werden, dass Hyperglykämie auch die Neutrophilen-Funktionen beeinträchtigt, da die Phagozytose-Kapazität reduziert war und die Formation von NETs leicht erhöht war.
Zusammenfassend weist dies darauf hin, dass Neutrophile einen positiven Einfluss auf die Heilung nach Herzinfarkt haben können, indem sie zu einem anti-inflammatorischen Phänotypen polarisieren und dass Hyperglykämie einen eher pro-inflammatorischen Phänotypen induziert.

1. Introduction

1.1. General elements of immunity

In general, immune responses may be subdivided into innate and adaptive immunity. The innate immunity was described as a fast, non-specific, but efficient response. However, this type of immunity was believed to have no long-lasting memory. The cells of the innate immune response, as macrophages and granulocytes, recognise invading pathogens and tissue damage through pattern recognition receptors (PRR) (Medzhitov and Janeway 2000). In contrast, adaptive immune responses of lymphocytes are slow, antigen-specific and develop a long-term memory (Bonilla and Oettgen 2010). In some cases, as for example in the event of infection, inflammation is essential and beneficial for the protection from exogenous pathogens and to repair the damaged tissue. However, the inflammatory response is often also detrimental, as it can damage the host and lead to tissue dysfunction.

In the absence of pathogens, a sterile inflammation can be initiated by the recognition of damage-associated molecular patterns (DAMPs). One of the leading causes of morbidity and death in Europe is acute myocardial infarction (AMI) (Roth, Huffman et al. 2015). Death of cardiomyocytes and thus loss of cardiac tissue, induced by the lack of oxygen and nutrient supply during AMI, initiates an inflammatory response, a sterile inflammation, and a cascade of events leading to cardiac repair processes (Jennings 1969). The immune system plays a substantial role in the regulation of damage and repair processes after AMI, as an exaggerated inflammatory response worsens outcome after MI. Neutrophils are one of the first cells to massively infiltrate the infarcted area and are attracted by cell debris and inflammatory signals, such as danger-associated molecules, and recruit further inflammatory cells (Yan, Anzai et al. 2013).

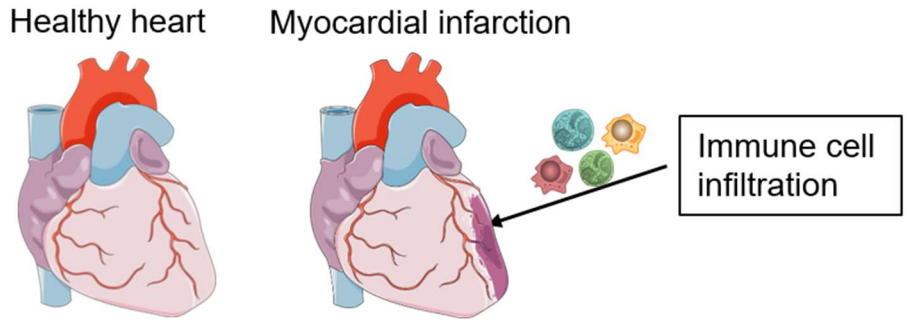

Figure 1: Neutrophils in Myocardial Infarction.

The immune system plays a crucial role in cardiac remodelling after AMI. Neutrophils are one of the first cells to infiltrate the infarcted area and attract more immune cells to the site of injury, such as monocytes/ macrophages**.**

However, a temporary sterile inflammation can also segue into chronic inflammation. Type 2 Diabetes Mellitus (T2DM) for example was, until recently, widely accepted as a metabolic disease, though it is more and more often characterised as an immune-mediated inflammatory disease. It was reported that obesity and the following development of hyperglycaemia and hyperlipidaemia, often leading to T2DM and metabolic syndrome, results in chronic inflammation. Immune cells increasingly secrete pro-inflammatory cytokines, thereby leading to inflammation and also impairment of the insulin signalling (Lumeng, Bodzin et al. 2007, Lackey and Olefsky 2016). Here, the inflammatory response does not lead to a return to tissue homeostasis, and consequently results in chronic inflammation.

Neutrophils play an important role in inflammation and substantially contribute to progression and outcome of diseases such as MI and T2DM, therefore the main focus was put on this cell type. Thus, in the following I will bring focus on immune cell signalling in general and neutrophil phenotypes and their functions in detail. Further, neutrophils in the context of diseases as myocardial infarction and diabetes will be described in more detail. In regards to diabetes, also the new but widely discussed topic of trained immunity will be addressed.

1.2. Haematopoiesis

All blood cells derive from multipotent haematopoietic stem cells (HSC) in a process called haematopoiesis, which predominantly takes place in the bone marrow, spleen and thymus (Seita and Weissman 2010). HSC`s are multipotent, meaning they can differentiate into all blood cells, and are capable of self-renewal. Thus, they can generate identical daughter HSC´s without differentiation (Seita and Weissman 2010). It was shown that there exist three multipotent cell types, the long-term HSC`s (LT-HSC), the short-term HSC`s (ST-HSC`s) and the multipotent progenitors (MPP) (Morrison and Weissman 1994). The LT-HSC`s can generate both, an identical daughter LT-HSC´s and a progenitor cell with differentiation potential, the ST-HSC´s. These cells are able to self-renew for a defined time interval of around eight weeks and afterwards lose their self-renewal capacity and generate the MPP`s (Morrison and Weissman 1994, Morrison, Wandycz et al. 1997). The MPP`s have low self-renewal capacities and differentiate into two different oligo-lineage progenitors, the common lymphoid progenitors (CLP`s) or the common myeloid progenitors (CMP`s) (Kondo, Weissman et al. 1997, Akashi, Traver et al. 2000, Karsunky, Inlay et al. 2008). The CLP`s give rise to T-, B- and NK-cells, which are responsible for adaptive and innate immune responses. The CMP´s evolve to the granulocyte-monocyte progenitors (GMP`s) or the megakaryocytic-erythroid progenitors

(MEP). The GMP`s in turn can differentiate into monocytes/macrophages or to granulocytes, which are divided into neutrophils, basophils and eosinophils. The MEP´s induce the erythroid-megakaryocyte fate and develop into erythrocytes or megakaryocytes (Seita and Weissman 2010). Interestingly, CLP`s as well as CMP`s can give rise to dendritic cells (Traver, Akashi et al. 2000, Manz, Traver et al. 2001). All of these progenitor and mature cell populations can be distinguished by using specific cell markers.

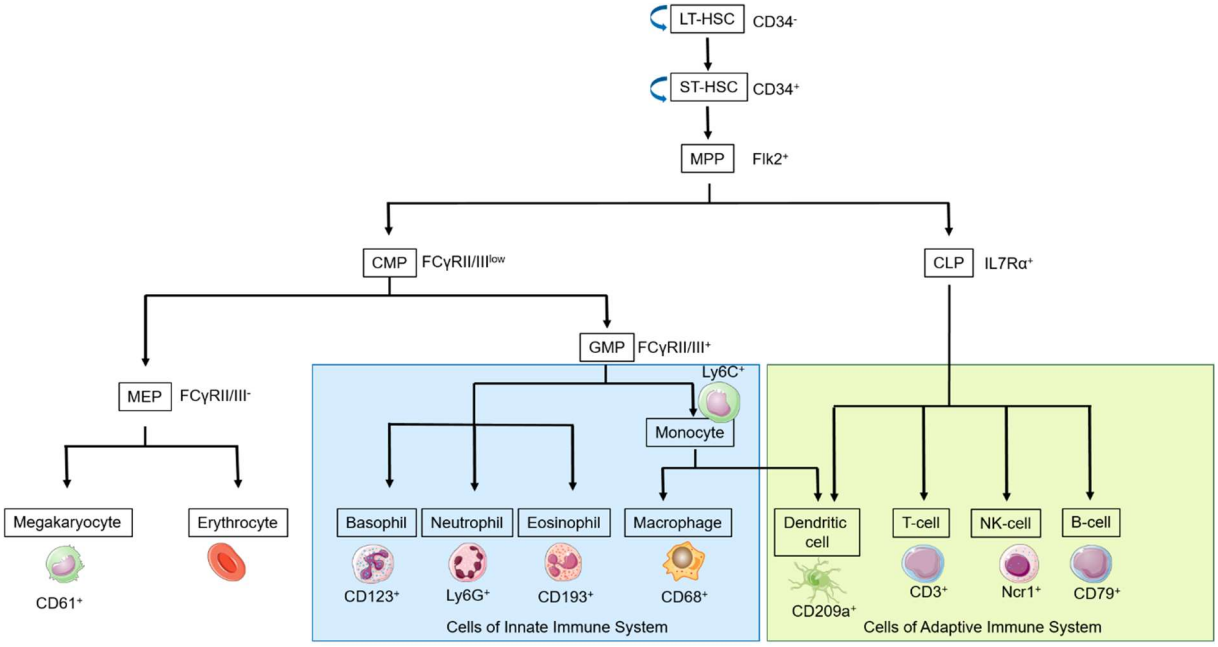

Figure 2: Haematopoiesis.

The self-renewable haematopoietic stem cell (HSC) develops into the multipotent progenitor cells (MPP), which evolves either to the common myeloid progenitor (CMP) or the common lymphoid progenitor (CLP). The CMP further differentiates to the granulocyte-monocyte progenitor (GMP) or the megakaryocytic-erythroid progenitors (MEP). The GMP`s can than evolve to granulocytes (basophil, neutrophil, eosinophil) or to monocytes/macrophages or dendritic cells. The MEP´s develop to erythrocytes or to megakaryocytes. On the other side the CLP`s evolve to T-, B-, NK- or dendritic cells. Created with BioRender.com.

1.3. Neutrophils

Neutrophils are polymorphnuclear leukocytes, which are continuously generated in the bone marrow from myeloid precursors and around 10^9 neutrophils per kilogram of body weight are generated in the bone marrow of healthy humans per day (Dancey, Deubelbeiss et al. 1976). Neutrophils are short-lived cells with a circulating half-life of 6 to 12 hours in mice and humans (Price, Chatta et al. 1996, Basu, Hodgson et al. 2002). Neutrophils are important effectors during acute inflammation, they are crucial for the activation and coordination of innate and adaptive immune cells, as for example macrophages and lymphocytes (Mantovani, Cassatella et al. 2011, Chen, Wu et al. 2014).

1.3.1. Polarization of neutrophils: “N1” versus “N2”

In literature, different cytokines are described to induce different immune cell phenotypes. In macrophages, it was shown that Interferon-γ/Lipopolysaccharide (IFN-γ/LPS) treatment leads to the induction of a M1-like macrophage phenotype, identified by the up-regulation of the M1-marker tumour necrosis factor α (*Tnfα*), whereas Interleukin-4 induces a M2-like macrophage phenotype, characterised by the induction of Arginase 1 (*Arg1*) (Martinez, Gordon et al. 2006, Jablonski, Amici et al. 2015, Roszer 2015).
Also we could show in previous experiments, in the course of my master thesis and the publication (Nederlof, Reidel et al. 2022), by RNA sequencing transcript expression analysis and qPCR analysis, that murine bone marrow neutrophils polarize at least to a pro-inflammatory N1-like and to an anti-inflammatory N2-like phenotype. As those results are the basis of this work, I will shortly summarise these data here. The RNA-sequencing revealed, that IL-4 and LPS/IFN-γ induced major transcript expression alterations when compared to basal neutrophils, however insulin did not, being concordant to the results of expression analysis by qPCR (Figure 3). LPS/IFN-γ treatment induced a pro-inflammatory N1-like phenotype, characterised by an upregulation of the known M1-marker genes *Tnfα, IL12a, Nos2* (Figure 3 [B]). In contrast, IL-4 treatment induced an anti-inflammatory N2-like phenotype, identified by the upregulation of the known M2-marker genes *Arg1, Retnlα* and *Chi3l3* (Figure 3 [C]). However, insulin treatment did induce neither upregulation of pro- nor of anti-inflammatory genes. Thus, it was shown that there exist at least two neutrophil subtypes, characterised as a pro- and anti-inflammatory phenotype. In further experiments novel proper phenotypic markers can be identified with the help of this data set to better distinguish pro- and anti-inflammatory neutrophils.

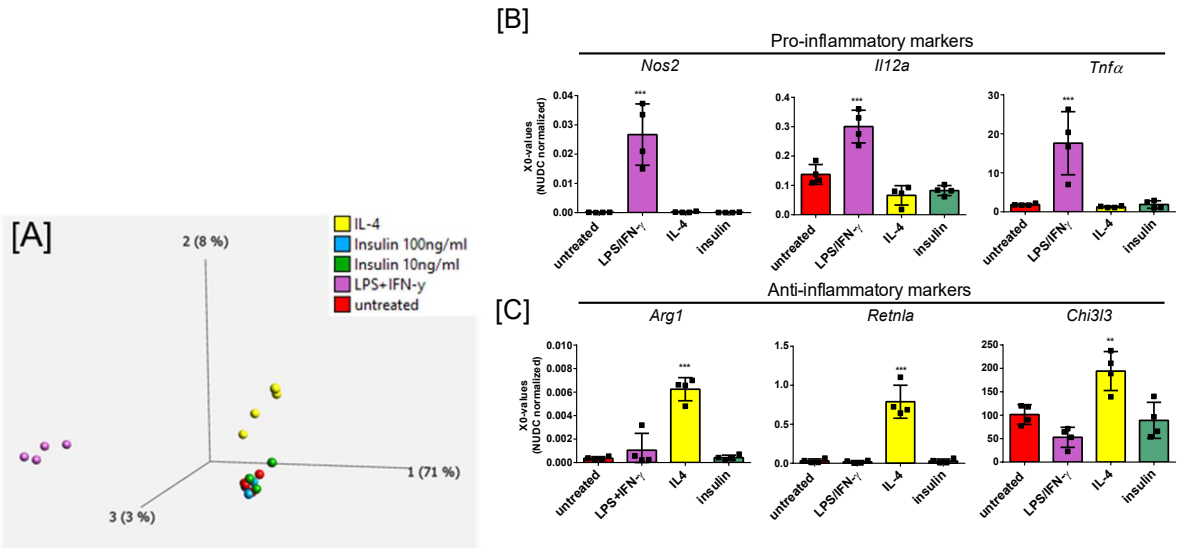

Figure 3: Summarised data of previous experiments.

RNA sequencing transcript expression analysis revealed that IL-4 and LPS/IFN-γ treatment induced transcriptional changes when compared to untreated neutrophils, whereas insulin did not **[A]**. qRT-PCR results confirmed that neutrophils polarized at least to a pro- and anti-inflammatory phenotype, as LPS/IFN-γ treatment induced upregulation of inflammatory genes *Nos2, IL12a, Tnfα* **[B]** and IL-4 treatment induced upregulation of *Arg1, Retnlα* and *Chi3l3* **[C]**. X0 values are normalized to *Nudc*. Expression of single measurements are shown. Bars represent mean ± SD. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test), (*p<0.05, **p<0.01, ***p<0.001). LPS, Lipopolysaccharide; IFN-γ, interferon-gamma; IL-4, Interleukin 4; *Nos2*, nitric oxide synthase 2; *IL12a*, Interleukin 12a; *Tnfα*, Tumor Necrosis Factor α; *Arg1*, Arginase 1; *Retnla*, resistin-like molecule alpha; *Chi3l3*, Chitinase 3-like 3. (Nederlof, Reidel et al. 2022)

Interestingly, the phenotype of neutrophils can change over time. It was shown, that during tumour development the phenotype changes from an anti-tumorigenic N1 phenotype to a tumour-promoting N2 phenotype in later stages (Mishalian, Bayuh et al. 2013). This indicates that neutrophil phenotype and function is influenced by the different surrounding microenvironments. In the event of cardiac diseases, pro-inflammatory N1 neutrophils are detrimental for healing and scar formation. In contrast, anti-inflammatory N2 neutrophils are rather reparative and are crucial for the resolution of inflammation, angiogenesis and tissue remodelling (Horckmans, Drechsler et al. 2014, Ma, Yabluchanskiy et al. 2016, Horckmans, Ring et al. 2017). Thus, it was of interest to analyse neutrophil phenotypes and function in different environments.

1.3.2. Neutrophils in Myocardial Infarction

Death of cardiomyocytes and thus loss of cardiac tissue, induced by the lack of oxygen and nutrient supply during acute myocardial infarction (AMI), initiates an inflammatory response and a cascade of events leading to cardiac repair processes (Jennings 1969). The immune system plays a substantial role in the regulation of damage and repair processes after AMI. Neutrophils are among the first cells to massively infiltrate the infarct area and are attracted by cell debris and inflammatory signals, such as danger-associated molecules (Yan, Anzai et al. 2013).

Until recently, neutrophils were considered to be detrimental in ischaemic heart disease since they enhance inflammation and tissue damage. Different neutrophil functions, as degranulation of matrix-degrading enzymes, reactive oxygen species (ROS) production and formation of NETs cause further cell death and infarct expansion (Brinkmann, Reichard et al. 2004, Chia, Nagurney et al. 2009, Kolaczkowska and Kubes 2013). However, it was recently described that neutrophils are crucial in cardiac remodelling and can have beneficial effects on myocardial healing (Ma, Yabluchanskiy et al. 2016). One positive effect is that phagocytosis of apoptotic neutrophils by macrophages induces the release of reparative chemokines. It was demonstrated that depletion of neutrophils worsened outcome after AMI, as macrophage polarization towards an anti-inflammatory phenotype failed due to the missing stimuli released by neutrophils, finally leading to increased fibrosis and thus to heart failure (Huynh, Fadok et al. 2002, Horckmans, Ring et al. 2017).

Remodelling after AMI can be divided into several phases. It begins with the acute inflammatory phase, which involves the recruitment of inflammatory cells, in particular Ly6G^{high} neutrophils, which reach their peak on day 1 after AMI. On the one hand, those neutrophils clear the cell debris and on the other hand aggravate injury, as they produce inflammatory cytokines and ROS (Ma, Yabluchanskiy et al. 2013). The released cytokines attract inflammatory Ly6c^{high} monocytes and macrophages, inducing a sustained inflammatory environment (Soehnlein, Zernecke et al. 2008). The second sub-acute phase is dominated by the recruitment of reparative, alternatively-activated neutrophils, which disperse the inflammatory response and initiate tissue replacement and myocardial repair (Nahrendorf, Swirski et al. 2007, Ma, Yabluchanskiy et al. 2016). In a final step, the inflammation is resolved due to phagocytosis of dead cardiomyocytes and neutrophils by anti-inflammatory monocytes/macrophages and a stable collagen-rich scar is formed (Nahrendorf, Swirski et al. 2007).

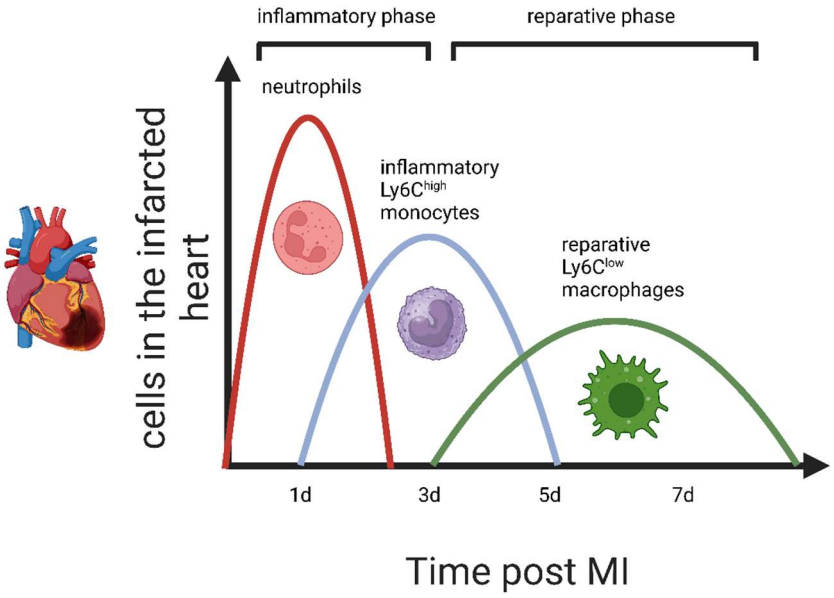

Figure 4: Immune response in Myocardial Infarction.

Inflammatory neutrophils are among the first cells to be recruited to the infarct area. Neutrophils release chemokines and thereby attract further inflammatory cells, as for example macrophages, and induce a sustained inflammatory environment. Phagocytosis of apoptotic neutrophils by macrophages induces the release of reparative chemokines, thus initiating the polarization of macrophages towards an anti-inflammatory phenotype. Created with BioRender.com.

Consequently, the efficiency of repair after AMI seems to be dependent on a well-coordinated balance between the inflammatory and reparative functions of the infiltrating immune cells. If the initial inflammatory phase is suppressed, the clearance of cellular debris is hampered and if the second reparative phase is lacking, angiogenesis is disturbed and fibrosis inefficient (Frodermann and Nahrendorf 2017). However, uncontrolled and excessive infiltration of inflammatory cells, accompanied by the release of pro-inflammatory cytokines and ROS, into the infarct area also results in an impaired remodelling (Hasan, Luo et al. 2016). Hence, the regulation of this process by altering neutrophil phenotype could offer a potential therapeutic strategy to improve outcome after MI.

1.3.3. Neutrophils in Type 2 Diabetes Mellitus

Cardiovascular diseases are the leading cause of death in western societies and Type 2 Diabetes Mellitus (T2DM) strongly predisposes to these diseases (Wild, Roglic et al. 2004, Lopez, Mathers et al. 2006). T2DM is rising to a global epidemic, estimated 537 million people suffered from diabetes in 2021 and the number is predicted to increase to

783 million people by 2045. Shockingly, around 6.7 million humans died from diabetes in 2021 (International Diabetes Federation 2021). Thus, it is of importance to also analyse this disease.

T2DM is caused by both, poor lifestyle and genetic factors. The disease is defined by high blood glucose levels, hyperinsulinaemia, insulin insensitivity and increased monocyte counts. Typical symptoms are fatigue, increased thirst and frequent urination (Defronzo, Ferrannini et al. 1989, Groop, Bonadonna et al. 1989, Magnusson, Rothman et al. 1992, Ginter and Simko 2013).

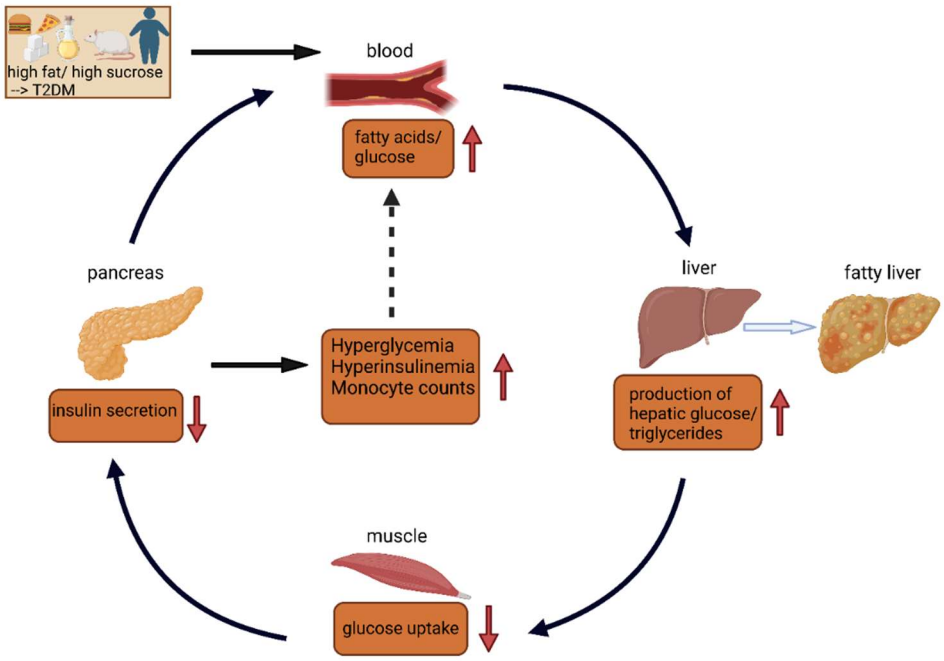

Figure 5: Pathophysiology of Type 2 Diabetes Mellitus.

Type 2 Diabetes Mellitus leads to an impaired insulin secretion in the pancreas, which induces hyperglycaemia and thus increased production of hepatic glucose and decreased uptake of glucose in the muscle. Subsequently, this leads to hyperinsulinaemia, insulin insensitivity and increased monocyte counts. Created with BioRender.com.

Obesity is often accompanied by and a cause of T2DM. Until recently, T2DM was widely accepted as a metabolic disease, however it is more and more often characterised as an immune-mediated inflammatory disease, accompanied by chronic inflammation. In mouse models of diabetes, a shift from anti-inflammatory adipose tissue macrophages in lean mice to more pro-inflammatory macrophages in obese mice was demonstrated. Those pro-inflammatory macrophages in adipose tissue are thought to play a major role in the development of inflammation in obesity; they secrete pro-inflammatory cytokines, thereby leading to chronic inflammation and also impairment of the insulin signalling (Lumeng, Bodzin et al. 2007, Lackey and Olefsky 2016). Interestingly, it was reported, that several pro-inflammatory cytokines, as for example IL-1β, CXCL1 and CXCL5 are

able to trigger pancreatic β-cell dysfunction through ER stress and thus can contribute to insulin resistance in patients establishing T2DM (O'Neill, Lu et al. 2013, Nunemaker, Chung et al. 2014). The role of macrophages in the context of hyperglycaemia and T2DM is well known, whereas the contribution of neutrophils has not been examined in detail. However, as described above, neutrophils are among the first cells to arrive at the site of inflammation and recruit further inflammatory cells, as for example macrophages, thereby helping to sustain the inflammatory state. It was reported that proteases secreted by neutrophils, as for example elastase, further enhance inflammation and induce insulin resistance. Lack of neutrophil elastase reduced inflammation and ameliorated glucose tolerance and insulin sensitivity (Talukdar, Oh et al. 2012).

In some cases, as for example in the event of infection, inflammation is essential and beneficial for the protection from exogenous pathogens and to repair the damaged tissue. However, the inflammatory response is often also detrimental, as it can damage the host and lead to tissue dysfunction. Here, the inflammatory response does not lead to a return to tissue homeostasis, and consequently results in chronic inflammation.

1.3.4. Trained immunity

In general, as described above, immune responses may be subdivided into innate and adaptive immunity. However, only recently it has been shown that, besides the adaptive immunity with induced memory of B- and T-cells, also innate immune cells, mostly macrophages, may be associated with some kind of memory, which is now known as “trained immunity” or “innate immune memory” (Netea, Quintin et al. 2011). It is assumed, that trained immunity is independent of adaptive immunity, but is rather generated by epigenetic changes, which affect gene expression of innate immune cells, such as monocytes, macrophages or natural killer cells (Kleinnijenhuis, Quintin et al. 2012). These epigenetic changes and immunometabolic programs are activated by cytokines or pathogen-associated molecular patterns (PAMP`s) and therefore paves the way for a sensitized and stronger, non-specific response to a secondary stimulation (Quintin, Saeed et al. 2012).

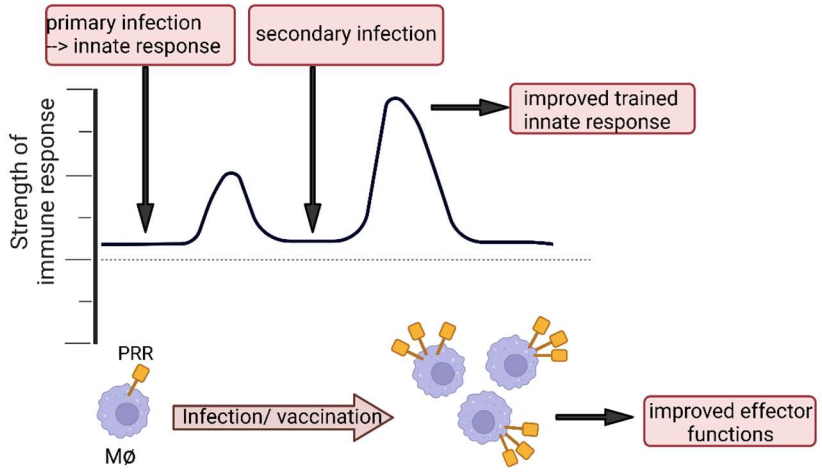

Figure 6: Exemplary process of trained immunity.

Innate immune cells achieve an immune memory and thereby enhance a non-specific response to ensuing infections, as they have increased inflammatory properties. (modified after (Netea, Joosten et al. 2016)) Created with BioRender.com.

However, due to the short lifespan of monocytes and granulocytes, it is still unidentified how the memory is conserved. One possible mechanism may be due to epigenetic changes occuring already in HSC`s in the bone marrow. During haematopoiesis, HSC`s and HPC`s express PRR`s and proinflammatory cytokine receptors and therefore are also able to recognise inflammation and react with emergency myelopoiesis, leading to increased numbers of GMP`s and thus of myeloid cells (Nagai, Garrett et al. 2006, Trumpp, Essers et al. 2010, Chavakis, Mitroulis et al. 2019, Schultze, Mass et al. 2019, Chavakis, Wielockx et al. 2022). Mitroulis et al. for example demonstrated, that progenitor cells in the bone marrow adapt to different primary signals and thus induce advantageous adaptations in myelopoiesis. This finding supports the hypothesis that trained immunity arises on the level of haematopoietic stem and progenitor cells (Mitroulis, Ruppova et al. 2018).

Trained immunity can have beneficial effects in response to infections, however, in diseases with chronic inflammation, as for example atherosclerosis or diabetes, a permanent activation of the innate immune system might be destructive. In this context, training of progenitor cells in the bone marrow may be maladaptive and lead to hyper-responsive and trained myeloid cells, which infiltrate the tissue and aggravate inflammation. Thereby, a mechanism that is advantegous in fighting infections may lead to inflammatory comorbidities in diseases associated with sterile inflammation (Kalafati, Hatzioannou et al. 2022).

In the context of AMI and diabetes, trained immunity in neutrophils has not been studied in detail yet, most likely due to their limited life span. However, one can assume that

epigenetic changes in the bone marrow are transferred to neutrophils as well, as they descend from HSC`s (see Figure 1). This raises the question of the role of neutrophils in trained immunity and if they are as well affected by epigenetic remodelling.

1.3.4.1. Trained immunity in T2DM

Interestingly, it has been demonstrated that diabetic patients who become due to treatment normoglycemic again, still have a higher risk for cardiovascular diseases. This phenomenon is called “hyperglycemic memory” and can be explained by trained immunity occuring also in diabetes (El-Osta, Brasacchio et al. 2008). Nagareddy et al. investigated in diabetic mouse models, that glucose enhanced proliferation and inflammation of innate immune cells, and their progenitors, thus promoting diabetes-induced atherosclerosis *in vivo* (Nagareddy, Murphy et al. 2013). Permanent inflammation induced by hyperglycaemia initiated long-term functional reprogramming of innate immune cells (El-Osta, Brasacchio et al. 2008). Gallagher et al. found that in mice with T2DM epigenetic changes occurred in the bone marrow, which might be responsible for the hyperglycaemic memory (Gallagher, Joshi et al. 2015). Moreover, a study of Lee et al. demonstrated, that monocytes of T2DM patients show increased inflammasome activation and cytokine secretion. This study indicates, that a diabetic environment reprograms the function of circulating cells, causing a more pro-inflammatory phenotype of these cells (Lee, Kim et al. 2013). Thus, more “trained” proatherogenic macrophages might be recruited to atherosclerotic plaques, inducing a hastened development of CVD. In line, it was shown that macrophages cultivated in high glucose medium evince a more pro-inflammatory phenotype (Edgar, Akbar et al. 2021). In addition, it was demonstrated that bone marrow-derived macrophages of diabetic mice maintained this phenotype, even if returned to physiological glucose or if transplanted into normoglycaemic mice, indicating a training of macrophages in diabetes (Edgar, Akbar et al. 2021).

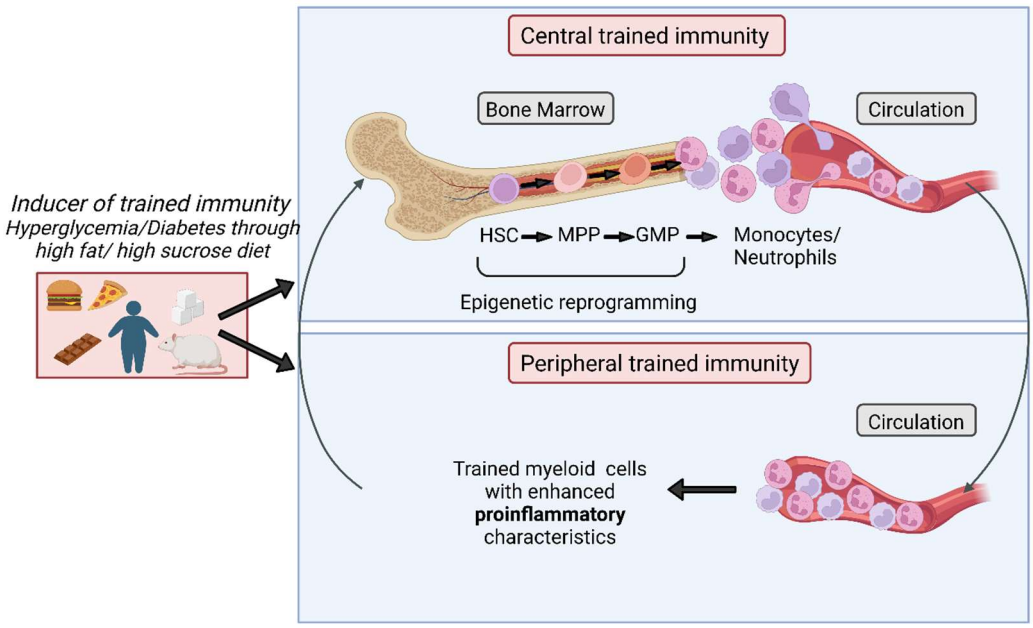

Figure 7: Diabetes-induced hyperglycaemia causes epigenetic reprogramming and changes cellular metabolism.

Hyperglyacemia induces epigenetic rewiring in the bone marrow, which results in trained myeloid cells in the circulation with more pro-inflammatory characteristics (Kalafati, Hatzioannou et al. 2022). Created with BioRender.com.

1.3.5. Neutrophil immune responses

During inflammation, neutrophils become activated and their longevity increases, thus the presence of neutrophils at the site of inflammation is ensured. Neutrophils can eliminate pathogens by different mechanisms, such as phagocytosis, degranulation, or by neutrophil extracellular traps (NETs). However, subsequently neutrophils die by apoptosis and are then eliminated by macrophages and dendritic cells (Colotta, Re et al. 1992, Hotta, Niwa et al. 2001, Pillay, den Braber et al. 2010).

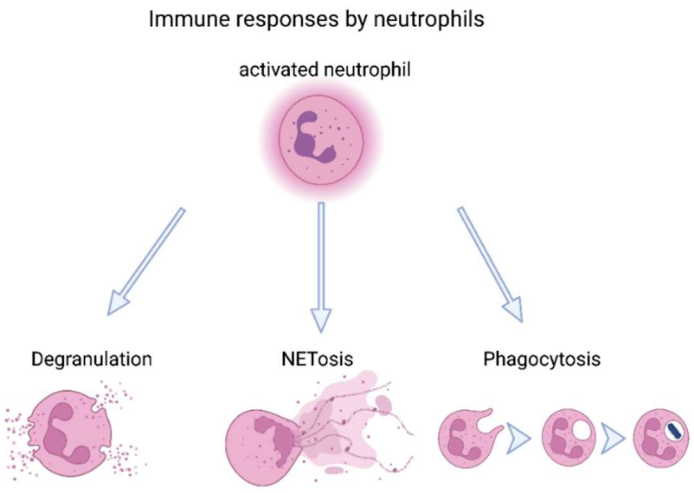

Figure 8: Immune responses by neutrophils.

Neutrophils eliminate and immobilize pathogens by three main mechanisms. Neutrophils can react in the form of degranulation, by releasing antibacterial proteins. Another mechanism is NETosis, by releasing nuclear material they can catch and immobilise the pathogens. Alternatively, they can react by phagocytosis, by phagocytosing the pathogens and killing them intracellularly. Created with BioRender.com.

1.3.5.1. Formation of neutrophil extracellular traps

Neutrophils play a central role in immune defense. One mechanism that is used by neutrophils is the formation of neutrophil extracellular traps (NETs). Upon activation, as for example by bacteria, neutrophils release NETs, which are extracellular net-like structures composed of histones, granule proteins and chromatin. Those NETs trap and bind, and thereby immobilize and kill, bacteria, viruses and parasites extracellularly (Brinkmann, Reichard et al. 2004). Brinkmann et al. showed, that NETs are threads, which are not surrounded by membranes, with diameters up to 50nm, that consist of smaller smooth stretches and globular domains (Brinkmann, Reichard et al. 2004). Those NETs contain proteins from azurophilic (primary), specific (secondary) and tertiary granules, however do not contain various cytoplasmic proteins and CD63, which is a granule membrane protein (Brinkmann, Reichard et al. 2004).

It was shown recently, that reactive oxygen species (ROS) production is necessary for the release of NETs. ROS production induces the translocation of neutrophil elastase (NE) and myeloperoxidase (MPO) to the nucleus, where it causes severe chromatin decondensation, finally resulting in cell rupture and NET formation (Papayannopoulos, Metzler et al. 2010). Brinkmann et al. showed *in vivo*, that NETs are abundantly present at the sites of inflammation. They have antimicrobial characteristics and additionally prevent unimpeded spreading of bacteria. Another function of NETs is, that potentially

detrimental proteins, such as proteases, are captured by the NETs and are thereby impeded to cause harm in nearby tissues, close to the site of inflammation (Brinkmann, Reichard et al. 2004).

However, the formation of NETs can also be detrimental, as for example in the case of autoimmune diseases or myocardial infarction. It was for example reported that NETs also play an important role in the development of thrombosis (Fuchs, Brill et al. 2010) and that the intensity of NETs directly correlates with infarct size and are predictors of adverse cardiovascular events (Helseth, Shetelig et al. 2019).

1.3.5.2. Phagocytosis

Phagocytosis is an important mechanism in host defense and the resolution of inflammation. A group of different cell types, including monocytes, macrophages, dendritic cells and neutrophils, are known as professional phagocytes (van Kessel, Bestebroer et al. 2014). However, neutrophils are the prevailing phagocytes in the human blood (van Kessel, Bestebroer et al. 2014). Phagocytes can engulf pathogens, cells or cell fragments into the phagosome, which is a plasma membrane-derived vacuole (Kobayashi, Malachowa et al. 2018).
In general, the process of phagocytosis can be divided into four phases. At first, the target has to be detected, subsequently the process of internalisation of the target is activated. Then the phagosome is formed and lastly the phagosome matures into a phagolysosome (Levin, Grinstein et al. 2016). Neutrophils recognise the pathogens by receptors, such as pattern recognition receptors and opsonic receptors, which are on the cell surface of neutrophils. Those receptors recognise specific proteins that are expressed on the microbial surface in specific molecular patterns. Binding by target molecules initiates a signalling cascade that leads to extension of the membrane in order to cover the target (Freeman and Grinstein 2014, Kobayashi, Malachowa et al. 2018, Uribe-Querol and Rosales 2020). Subsequently, the phagosome is formed as the membrane closes and the target is internalised (Freeman, Goyette et al. 2016). Once formed, the phagosome fuses with lysosomes, to give rise to phagolysosomes containing lytic enzymes, which are active in an extremely acidic and oxidative environment and thus can inactivate and destruct the target (Levin, Grinstein et al. 2016). Interestingly, phagocytes can induce pro- or anti-inflammatory reactions. They induce a pro-inflammatory response if they interact with foreign material, whereas anti-inflammatory mediators are released to reduce tissue destruction, when opposed to apoptotic cells (Flannagan, Jaumouille et al. 2012). In the context of myocardial infarction, neutrophils are amongst others important

for the resolution of inflammation, as they phagocyte cellular debris and necrotic myocardium (Daseke, Valerio et al. 2019).

1.3.5.3. Neutrophil degranulation

Another immune mechanism exerted by neutrophils is degranulation. Degranulation is necessary for killing of pathogens, but also plays a role in the immune response during (non-) infectious diseases (Mortaz, Alipoor et al. 2018). Neutrophils contain different types of granules, which are released in a specific order upon stimulation (Kolaczkowska and Kubes 2013). At first, the tertiary granules, also known as gelatinase granules, are released, which contain for example cathepsins and gelatinase. Those tertiary granules induce the expression of CD11b/CD18 on the cell surface. Subsequently, secondary granules, also known as specific granules, are released. Those secondary granules contain, among others, lactoferrin, collagenase and NADPH oxidase. Lastly, primary granules, also known as azurophilic granules, are released, which are the largest and contain for example elastase, MPO, cathepsin G and defensins. Primary granules are the most pro-inflammatory ones and contain the highest amount of antimicrobial proteins (Faurschou and Borregaard 2003). Lastly, a fourth type of granule exists, the secretory vesicles. Those vesicles contain, amongst others,serum albumin and CD35, known as the complement receptor 1 (Hughes, Hollers et al. 1992, Kjeldsen, Sengelov et al. 1994, Deree, Lall et al. 2006, Amulic, Cazalet et al. 2012). Also in the context of myocardial infarction, neutrophil degranulation plays a crucial role. Excessive degranulation aggrevates tissue injury post MI, whereas some degradation of the extracellular matrix by released proteases is necessary for wound healing and removal of necrotic debris post MI (Hoenderdos, Lodge et al. 2016, Chalise, Becirovic-Agic et al. 2021, Daseke, Chalise et al. 2021). Thus, regulating the process of degranulation could be important in myocardial infarction.

1.3.5.4. Neutrophil migration

Neutrophils are the first cells to be recruited to the site of inflammation and build the first line of defense. Therefore, they must rapidly cross the endothelial cell barrier from the blood to the inflamed tissue. Pro-inflammatory stimuli, such as chemokines, are necessary for the recruitment, as they direct circulating cells to the inflamed tissue. The chemokine CXCL1 for example is a known and important modulator of neutrophil migration by binding to and activating the CXCR2 receptor on neutrophils (Girbl, Lenn et al. 2018). Again, some neutrophil infiltration is necessary for healing processes and

elimination of pathogens, whereas extensive infiltration of neutrophils is detrimental (Choi, Santoso et al. 2009). Also in the context of myocardial infarction, neutrophils are among the first cells to migrate to the infarct area. The neutrophils can either migrate between endothelial cells, which is called the paracellular route and is predominantly used by neutrophils, or directly through endothelial cells, which is described as the transcellular route (Marchesi and Florey 1960, Williamson and Grisham 1961, Carman and Springer 2004). For the migration process several adhesion receptors are necessary, the selectins, the integrins and the immunoglobulin superfamiliy. The whole process starts with selectin-mediated rolling, followed by chemokine-induced activation and adhesion and lastly the transendothelial migration. Therefore, neutrophils have to leave the circulation, this happens mainly in post-capillary venules, as the shear forces are alleviated and the vessel walls are thinner (Ma, Yabluchanskiy et al. 2013). There, they start rolling by binding of neutrophil P-selectin ligand 1 and L-selectin to endothelial P-selectin, E-selectin, ICAMs and vascular cell adhesion molecules (Bruehl, Moore et al. 1997, Buscher, Riese et al. 2010). In a next step, neutrophils adhere to the endothelium by binding of CD11b/CD18 with ICAM-1 and ICAM-2 on endothelial cells, followed by paracellular or intracellular migration through the nearest endothelial cell junction. If those integrins (CD11b/CD18, ICAM) are blocked, cells will not reach the junctions and thus are not able to migrate (Schenkel, Mamdouh et al. 2004, Borregaard 2010).

1.4. Immune cell signalling

Many immune cell functions and polarization are initiated by cytokines (IL-4, IFN-γ, LPS) and growth factors (insulin, IGF-1) and are transduced by signalling pathways, as the JAK/STAT, AKT and ERK pathways.

1.4.1. The JAK/STAT pathway

The signalling pathway of the Janus kinases and signal transducers and activators of transcription (JAK/STAT) mediate the responses of cells to many cytokines, hormones and growth factors, as for example to IL-4, IL-6, GM-CSF, IL-13, IFN-γ and many more (Morris, Kershaw et al. 2018). The JAK/STAT pathway is an essential mediator for many cellular mechanisms, as for cell survival, differentiation, proliferation, migration and apoptosis (Ghoreschi, Laurence et al. 2009). The JAKs are intracellular non-receptor tyrosine kinases and comprise four different JAKs, namely JAK1, JAK2, JAK3 and Tyk2. The STATs are transcription factors and seven different members are known, namely

STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Bousoik and Aliabadi 2018). The JAK-STAT-mediated signal transduction was described in detail by James Darnell et al (Stark and Darnell 2012). This revealed that a cytokine utilizes three components to induce a response, a receptor, a kinase and a transcription factor. The cytokine binds to its specific receptor, the long intracellular domains of this receptor provide sequence-specific docking sites and are associated with the JAKs, which are a family of tyrosine kinases, and the STATs (Firmbachkraft, Byers et al. 1990, Wilks, Harpur et al. 1991). The JAKs bind to membrane proximal regions, known as Box1 and Box2 motif, and the STATs bind to membrane distal regions, which are located towards the C-terminus (Morris, Kershaw et al. 2018). Which JAK binds to which receptor is defined by sequence differences in the Box1 and Box2 motifs, and some receptors can even bind multiple JAKs (Stahl, Boulton et al. 1994, Morris, Kershaw et al. 2018). The initially inactive Janus kinases are auto-activated by transphosphorylation upon binding of the cytokine to its receptor (Feng, Witthuhn et al. 1997). The activated JAKs then phosphorylate the intracellular tails of the receptors on specific tyrosines, which act as docking sites for the STATs (Argetsinger, Campbell et al. 1993, Morris, Kershaw et al. 2018). Additionally, the activated JAKs phosphorylate the STATs, which are, at this point, localized at the receptor. Subsequently, the STATs disassociate from the receptor and translocate to the nucleus, where they induce gene expression (Schindler and Darnell 1995). Interestingly, there exist also specific negative feedback inhibitors of the JAK/STAT-signalling. The suppressors of cytokine signalling (SOCS), which can bind between the JAK and STAT binding sites and are induced by the STATs, can switch off the pathway (Morris, Kershaw et al. 2018).

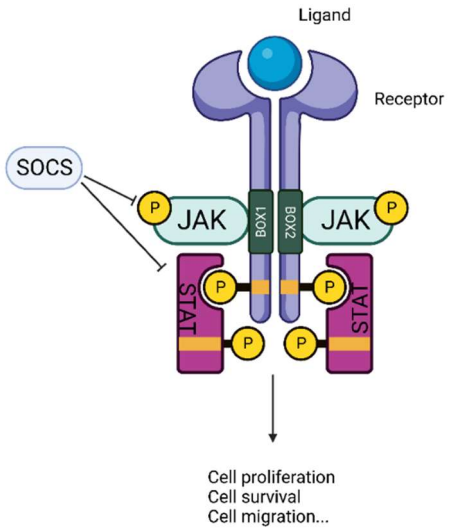

Figure 9: Simplified JAK/STAT signalling.

The Janus kinases are auto-activated by transphosphorylation upon binding of the cytokine to its receptor. The activated JAKs then phosphorylate the intracellular tails of the receptors on specific tyrosines, which act as docking sites for the STATs. Additionally, the activated JAKs then phosphorylate the STATs, which disassociate from the receptor and translocate to the nucleus, where they induce gene expression. There also exist specific negative feedback inhibitors of the JAK/STAT-signalling, the suppressors of cytokine signalling (SOCS) can switch off the pathway. Created with BioRender.com.

As described above, the JAK/STAT signalling has multiple important roles and mutation or loss of elements of this signalling cascade is linked to many diseases, as for example tumours and autoimmune diseases. But there is rising evidence that the JAK/STAT pathway also plays an important role in cardiovascular diseases and diabetes mellitus.

The JAK/STAT pathway is a crucial regulator of inflammation, which essentially contributes to cardiovascular diseases. Myocardial infarction, and also angiotensin II treatment, are known to activate the JAK/STAT signalling (Peeler, Conrad et al. 1996, Krishnamurthy, Rajasingh et al. 2009). However, more detailed analyses are needed, as there are data indicating a detrimental role of JAK/STAT signalling in cardiovascular diseases, but also a beneficial and cardioprotective role (Yu, Kennedy et al. 2003, Krishnamurthy, Rajasingh et al. 2009, Bolli, Stein et al. 2011, Szczepanek, Chen et al. 2011).

In the context of diabetes, several therapeutical approaches comprising the JAK/STAT pathway have been described. For instance, it was reported that STAT3 activation is involved in weight loss and decreased food-intake by compensating leptin deficiency (Matthews and Febbraio 2008). Additionally, IL-22, another activator of STAT, was shown to reduce inflammation and ER-stress in β-cells and enhance insulin sensitivity, thus possibly reversing hyperglycaemia and insulin resistance and offering a potential

therapeutical target (Hasnain, Borg et al. 2014, Wang, Ota et al. 2014). However, due to the broad and pleiotropic spectrum of the JAK/STAT signalling molecules, undesired effects of JAK/STAT inhibitors are likely and deeper analysis is required.

1.4.2. The AKT pathway

A second pathway is the intracellular PI3K/AKT signalling pathway, which plays a crucial role in diverse cellular processes, such as metabolism, proliferation, cell growth and survival, transcription and protein synthesis. Dysregulation of the AKT signalling is also implicated in diseases, such as cancer, cardiovascular diseases, and diabetes. Thus, AKT might be an important therapeutic target for the treatment of diseases, but as for JAK/STAT, the central role of the AKT kinases may lead to substantial side effects when activated or inhibited (Hixon, Muro-Cacho et al. 2000, Lawlor and Alessi 2001, Ackah, Yu et al. 2005, Tucka, Bennett et al. 2012).
Three different isoforms of the protein kinase B exist, namely the PKBα isoform, known as AKT1, the PKBβ isoform, known as AKT2, and the PKBγ isoform, known as AKT3. The three isoforms share a 80% sequence homology and the same structural organisation (Cheng, Godwin et al. 1992, Brodbeck, Cron et al. 1999).
The AKT1 isoform is broadly expressed and is crucial for growth, as AKT1 knock-out results in growth defects, persisting from the foetal stage to adulthood. AKT1 is the most important isoform in the heart, as it regulates somatic growth (Cho, Thorvaldsen et al. 2001). The AKT2 isoform is highly expressed in liver, muscles and adipocytes, which are typical insulin target tissues (Jones, JAKubowicz et al. 1991, Konishi, Shinomura et al. 1994, Cho, Mu et al. 2001, Matheny, Geddis et al. 2018). This isoform plays an important role in metabolic regulation, especially glucose homeostasis, as AKT2 deficiency results in glucose and insulin intolerance (Cho, Mu et al. 2001). The AKT3 isoform is crucial for brain development, as deficiency results in reduced brain size and mainly a neurological phenotype (Easton, Cho et al. 2005).

The AKT signalling pathway can be induced by multiple receptors, as by different cytokine receptors, G-protein-coupled receptors, receptor tyrosine kinases or integrins. PI3K is bound to the receptor and induces, upon activation of the receptor, phosphorylation of PIP2 into PIP3 (Myers, Backer et al. 1992, Kabuyama, Nakatsu et al. 1996, Manning and Cantley 2007, Hemmings and Restuccia 2012, Kearney, Norris et al. 2021). PIP3 induces the 3-phosphoinositide-dependent protein kinase-1 (PDK1), which phosphorylates AKT at Thr308, and thereby partially activates AKT (Alessi, James et al. 1997). The mTOR complex 2 (mTORC2) phosphorylates AKT at Ser473 and induces full activation of AKT

(Sarbassov, Guertin et al. 2005). In turn, AKT is inhibited by tumour suppressor phosphatase and tensin homolog (PTEN), which dephosphorylates PIP3 (Li, Yen et al. 1997, Steck, Pershouse et al. 1997, Maehama and Dixon 1998).

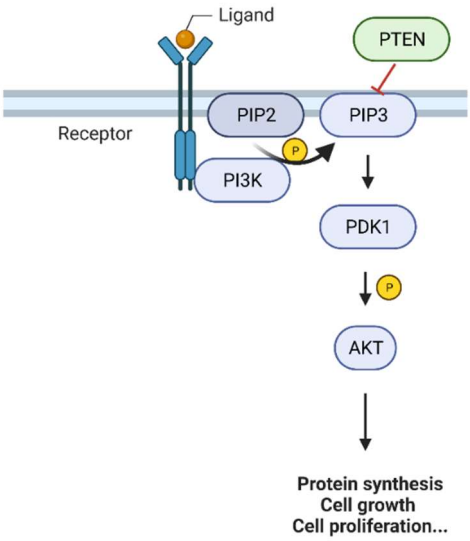

Figure 10: Simplified PI3K/AKT signalling.

After binding of a ligand to its receptor, PI3K gets activated. PI3K is bound to the receptor and induces phosphorylation of PIP2 into PIP3. PIP3 induces PDK1, which phosphorylates AKT at Thr308, only partially activating AKT (Alessi, James et al. 1997). In turn, AKT is inhibited by PTEN, which dephosphorylates PIP3. Created with BioRender.com.

1.4.3. The ERK pathway

The ERK-pathway belongs to the mitogen-activated protein kinase (MAPK) signal transduction pathways. There exist four different MAPK cascades, namely the ERK, JNK/stress-activated protein kinase, p38 MAPK and ERK5 signalling cascade (Morrison 2012). The JNK and p38 MAPK pathways are more associated with stress and apoptosis (Xia, Dickens et al. 1995, Nagata and Todokoro 1999). The ERK-pathway is an important regulator of a number of cellular processes, as cell survival, differentiation, proliferation, growth, metabolism and migration (Tamemoto, Kadowaki et al. 1992, Dikic, Schlessinger et al. 1994). Thus, an imbalance of ERK signalling can be malicious to cells and the body. Profuse activation of the ERK pathway causes a number of diseases, as developmental or neurological malfunction, inflammation and cancer (McCubrey, Steelman et al. 2007, Schubbert, Shannon et al. 2007, Kim, Lee et al. 2011, Garcia-Gomez, Bustelo et al. 2018).

The pathway can be activated by multiple ligands, as for example by a growth factor, cytokine or hormone. Binding of the ligand to the receptor results in activation of RAS, which is a small G protein (Overbeck, Brtva et al. 1995, Peyssonnaux, Provot et al. 2000).

RAS activates the protein kinase B-RAF (B-rapidly accelerated fibrosarcoma), which induces phosphorylation of the protein kinase MEK (Mitogen-activated protein kinase kinase) (Macdonald, Crews et al. 1994, Marais, Light et al. 1995, Reuter, Catling et al. 1995, Yamamori, Kuroda et al. 1995, Marshall 1996). MEK then activates the Map kinase ERK by phosphorylating a tyrosine and threonine residue. Upon activation, ERK translocates to the nucleus, where it induces transcription factors and thus, gene expression (Kyriakis, App et al. 1992, Seger, Ahn et al. 1992, Marshall 1996, Roskoski 2012, Eblen 2018).

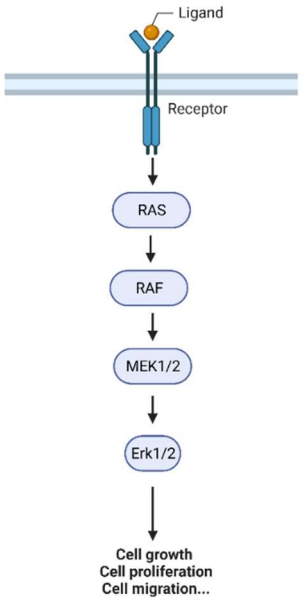

Figure 11: Simplified RAS/RAF/ERK pathway.

After binding of a ligand to its receptor RAS gets activated. RAS activates RAF, which induces phosphorylation of the protein kinase MEK. MEK then activates the Map kinase ERK by phosphorylating a tyrosine and threonine residue. Upon activation, ERK translocates to the nucleus, where it induces transcription factors. Created with BioRender.com.

1.4.4. Important growth factors and cytokines in immune cell signalling

1.4.4.1. Insulin-like growth factor 1

1.4.4.1.1. Structure and signal transduction

Insulin-like growth factor 1 (IGF-1) is a hormone, which enhances cell growth and differentiation and is mainly secreted by the liver. In addition, local release of IGF-1 in many other organs was observed (Zapf, Schoenle et al. 1978, Dercole, Applewhite et al. 1980, Guler, Zapf et al. 1987, Baltensperger, Kozma et al. 1993). Tissue-specific factors regulate the expression of the IGF-1 gene, hereby the major factor is growth hormone,

which induces IGF-1 gene transcription. The IGF-1 receptor is a heterotetramer that is composed of two extracellular α-subunits linked by disulphide bonds and two β subunits. The α-subunits have binding sites for specific ligands and the receptor undergoes autophosphorylation upon ligand binding. The two transmembrane β-subunits are composed of a short extracellular, a transmembrane and an intracellular domain, the latter holding a tyrosine kinase domain (Steeleperkins, Turner et al. 1988, Kato, Faria et al. 1994). IGF-1 specifically binds to the α-subunit of the IGF-1 receptor, thereby inducing a conformational change of the β-subunit, which consequentially activates the receptor tyrosine kinase activity. After binding of IGF-1 to its receptor, the IGF-1 receptor interacts with and activates insulin receptor substrate 1/2 (IRS1/2) and Src, which are important adaptor molecules (Izumi, White et al. 1987, Hakuno and Takahashi 2018). Phosphorylated IRS activates the PI3K-AKT pathway (Myers, Backer et al. 1992). Activated AKT induces several metabolism processes, including glycogen synthesis, glucose transport and gene transcription (Shepherd, Withers et al. 1998). Besides the AKT pathway, binding of IGF-1 to its receptor also activates the ERK pathway, which mainly promotes proliferation and differentiation (Baltensperger, Kozma et al. 1993).

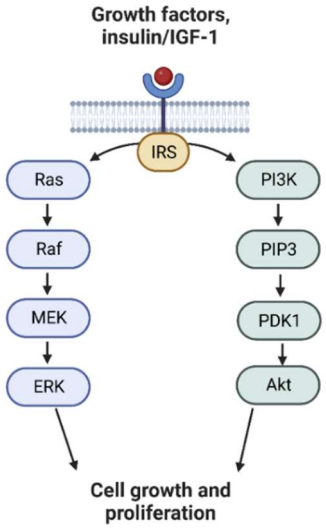

Figure 12: Signal transduction of Insulin/IGF-1.

After binding of insulin or IGF-1 to its receptor, two distinct pathways are activated. Phosphorylation of IRS1/2 induces activation of PI3K and thus activation of AKT, leading to induction of metabolism. Insulin/IGF-1 can also activate the ERK pathway. Here, ERK is activated via Shc and RAS, mainly triggering proliferation and differentiation. (modified after (Hakuno and Takahashi 2018)). Created with BioRender.com.

Interestingly, IGF-1 and insulin share a high homology. Insulin is an anabolic hormone which is solely expressed and secreted in pancreatic β-cells in the islets of Langerhans in response to increased blood glucose levels (Melloul, Marshak et al. 2002). Insulin is crucial for the control of glucose homeostasis, as it stimulates the uptake of glucose into

skeletal muscle and, to a lesser degree, liver and adipose tissue (James, Brown et al. 1988, Zorzano, Wilkinson et al. 1989). The insulin receptor is, like the IGF-1 receptor, a heterotetramer linked by disulphide bonds, composed of two extracellular α- and two transmembrane β-subunits (Ebina, Ellis et al. 1985). Further, insulin signalling is mediated by binding of insulin to the receptor, which induces, similar to IGF-1, AKT and ERK signalling (DeFronzo, Bonadonna et al. 1992). IGF-1 and insulin, both promote growth and metabolism, and are structurally highly homologous, both share a 50% amino acid homology (Rinderknecht and Humbel 1978). Interestingly, when IGF-1 and insulin receptors are expressed in the same cells, they are also able to form a hybrid receptor composed of insulin receptor α-β-'hemireceptor' and an IGF-1 receptor α-β-hemireceptor (Moxham, Duronio et al. 1989). Despite their structural and signalling similarities, functional differences seem to exist. It is assumed that the role of insulin and its receptor is focussed on metabolic responses, whereas IGF-1 and its receptor induce growth (Leroith, Werner et al. 1995, Siddle, Urso et al. 2001). However, there is as well evidence that those two hormones can exhibit similar responses, as IGF-1 can have metabolic effects and insulin can initiate growth and differentiation by substituting IGF-1 (Froesch 1993).

1.4.4.1.2. IGF-1 in diseases

IGF-1 is an essential hormone that regulates the somatotropic axis and is involved in muscle regeneration, neuronal survival and adipose tissue function. Additionally, some studies indicate that IGF-1 as well influences the immune system (Lu, Huang et al. 2011, Saclier, Yacoub-Youssef et al. 2013, Ueno, Fujita et al. 2013, Chang, Kim et al. 2016). Moreover, a balance of the growth hormone system is crucial for the preservation of a normal heart function, as an imbalance enhances the risk of cardiovascular diseases (Rosen and Bengtsson 1990). This imbalance can manifest as a reduced cardiac output and risk for ischaemic heart diseases, as IGF-1 is crucial for cardiac muscle growth and function and suppresses apoptosis of cardiomyocytes (Markussis, Beshyah et al. 1992, Ito, Hiroe et al. 1993, Li, Li et al. 1997). Interestingly, chronic transgenic overexpression of IGF-1, as well as short-term treatment with IGF-1, after MI in mice led to a reduction of scar formation, increased vascularization and an improvement of cardiac function (Santini, Tsao et al. 2007, Heinen, Nederlof et al. 2019). Moreover, an increased expression of IGF-1 in the viable part of the myocardium after MI was observed, which correlated with less ventricular dilatation and improved ventricular function, indicating that IGF-1 is involved in myocardial remodelling (Isgaard, Kujacic et al. 1997, Lee, Chen et al. 1999).

There is also rising evidence, that IGF-1 is a key hormone in the development of metabolic syndrome and T2DM. It was reported in the literature, that low circulating IGF-1 levels correlated with insulin insensitivity, glucose intolerance and T2DM (Sandhu, Heald et al. 2002, Succurro, Andreozzi et al. 2009, Mannino, Greco et al. 2013). Amongst other things, IGF-1 facilitates fatty acid transport in muscle and consequently its inhibition provokes insulin resistance and diabetes, as the liver is then taking up all the circulating fatty acids (Mauras, O'Brien et al. 2000, Fernandez, Kim et al. 2001, Heron-Milhavet, Haluzik et al. 2004). Additionally, diabetes is more and more often characterised as an inflammatory disease and interestingly it was shown that inflammatory cytokines are able to reduce IGF-1 levels, as they initiate phosphorylation of serine residues on IRS and thereby prevent binding to the IGF-1R (Efstratiadis, Tsiaousis et al. 2006, Smith 2010).

Due to the versatile role of IGF-1, it would be of interest to closer investigate the role of IGF-1 in diseases and especially in neutrophils.

1.4.4.2. Interleukin-4

1.4.4.2.1. Structure and signal transduction

Interleukin-4 (IL-4) signalling plays a central role in immune responses. The pleiotropic cytokine is mainly secreted by $CD4^+$ T-helper cells, but also by basophils, mast cells and eosinophils (Seder 1994). IL-4 is a crucial regulator of the adaptive immunity and is primarily known to trigger differentiation of T-helper (T_h) cells into protective T_h2-cells (Nelms, Keegan et al. 1999). Additionally, it was reported that IL-4 induces macrophage differentiation into M2 cells, which reduce inflammation and induce repair (Sica and Mantovani 2012, Jablonski, Amici et al. 2015). However, excessive production of IL-4 is associated with allergies (Tepper, Levinson et al. 1990).

The IL-4 receptor is expressed on various cell types, including haematopoietic, endothelial and epithelial cells (Lowenthal, Castle et al. 1988, Nelms, Keegan et al. 1999). The IL-4 signalling is typically mediated by heterodimerization of two different receptor chains. IL-4 binds to the IL-4Rα-chain, which is expressed on almost all cells and is also described as the cytokine-binding receptor chain (Mosley, Beckmann et al. 1989, Harada, Castle et al. 1990). The complex of IL-4 and IL-4Rα binds a second receptor chain, which can be the IL-2Rγc or the IL-13Rα1 chain (Kondo, Takeshita et al. 1993, Obiri, Debinski et al. 1995, Miloux, Laurent et al. 1997, Obiri, Leland et al. 1997). Lymphocytes express only marginally the IL-13Rα1 chain, but express high levels of IL-2Rγc chain, therefore the predominant IL-4 receptor in lymphocytes is the type I IL-4

receptor composed of the IL-4Rα-chain and the IL-2Rγc chain (Kondo, Takeshita et al. 1993, Russell, Keegan et al. 1993, Andrews, Rosa et al. 2001). In contrast, non-haematopoietic cells express nearly no IL-2Rγc, but high levels of IL-13Rα1, thus the IL-4 receptor in those cells is composed of the IL-4Rα-chain and the IL-13Rα1, and is called the type II IL-4 receptor (Aman, Tayebi et al. 1996). Myeloid cells are an exception, as they express the IL-13Rα1 as well as the IL-2Rγc chain (Nelms, Keegan et al. 1999, Junttila 2018). The binding of IL-4 to the IL-4Rα-chain and the following recruitment of the secondary chain initiates conformational changes in the intracellular receptor domain, subsequently leading to activation of the JAKs. Each receptor chain is specifically associated with a JAK, the γc chain is associated with JAK3, the IL-4Rα-chain is associated with JAK1 and the IL-13Rα1 chain with JAK2. The JAK kinases auto- and cross-phosphorylate each other and lead to activation of IRS (type I IL-4R) and STAT6 (type I and II IL-4R) (Nelms, Keegan et al. 1999, Heller, Qi et al. 2008). Activated STAT6 translocates to the nucleus, where transctiption of specific genes is enabled. Activated IRS induces other IL-4 signalling pathways, as PI3K/AKT signalling and mTOR signalling (Nelms, Keegan et al. 1999, Junttila 2018).

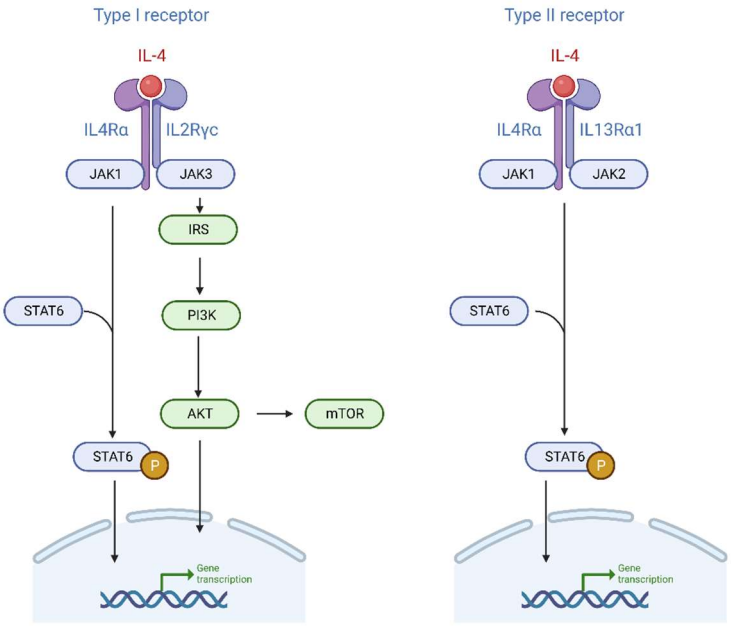

Figure 13: Signal transduction of Interleukin-4.

Two distinct IL-4 receptors exist. The type I receptor, composed of the IL4Rα chain and the IL2Rγc chain, and the type II receptor, composed of the IL4Rα chain and the IL13Rα1 chain. Each receptor chain is specifically associated with a JAK, the γc chain is associated with JAK3, the IL-4Rα-chain is associated with JAK1 and the IL-13Rα1 chain with JAK2. The JAK kinases auto- and cross-phosphorylate each other and lead to activation of IRS (type I IL-4R) and STAT6 (type I and II IL-4R). Activated STAT6 translocates to the nucleus, where transcription of specific genes is enabled. Activated IRS induces other IL-4 signalling pathways, as PI3K/AKT signalling and mTOR signalling. Created with BioRender.com.

1.4.4.2.2. Interleukin-4 in diseases

The cytokine IL-4 has pleiotropic effects, but it is well known that IL-4 is able to attenuate inflammatory processes induced by T_H1 and T_H17 cells, thus being more anti-inflammatory (Ansel, Djuretic et al. 2006, Luzina, Keegan et al. 2012). In the context of myocardial infarction, IL-4 has cardioprotective effects post MI, as IL-4 treatment dampened inflammatory neutrophils and increased anti-inflammatory macrophages, thereby initiating resolution of inflammation (Daseke, Tenkorang-Impraim et al. 2020). Song et al. demonstrated, that myeloid-specific IL4Rα deficiency led to a dysregulated inflammation and thus to an impaired cardiac function and deficient fibrotic remodelling. This is attributed to the fact, that the anti-inflammatory cytokine production is reduced and collagen 1 deposition is decreased due to an increase of matrix metalloproteinases (Song, Frieler et al. 2021). Therefore, IL-4 might be an important therapeutical target during and after MI.

As mentioned above, IL-4 exerts anti-inflammatory functions by reducing pro-inflammatory cytokines, such as IL-1β and TNFα, which are elevated in inflammatory diseases as T2DM (Cheung, Hart et al. 1990, Tevelde, Huijbens et al. 1990). Moreover, IL-4 induces lipolysis and inhibits lipid accumulation in fat and adipocytes and attenuates insulin resistance and glucose intolerance (Shiau, Chuang et al. 2019). Mice with overexpressed IL-4 displayed an improved glucose tolerance and insulin sensitivity by upregulating insulin – AKT signalling (Chang, Ho et al. 2012). Additionally, it was reported that IL-4 increased GLUT2 expression, thereby enhancing glucose uptake, and promoting glycogen synthesis in hepatocytes (Yang, Shiau et al. 2018). Thus, there is accumulating evidence that IL-4 as well has a regulatory effect on metabolism and has beneficial effects on diseases such as T2DM.
However, dysregulation of IL-4 can as well be detrimental and is described to play a pivotal role in the development of allergies and asthma (Tepper, Levinson et al. 1990, Punnonen, Yssel et al. 1997, Chipps and Marshik 2004).

1.4.4.3. LPS & IFN-γ signalling

1.4.4.3.1. LPS signal transduction

Lipopolysaccharide (LPS) is defined as a pathogen-associated molecular pattern (PAMP), as it is on the surface of gram-negative bacteria and thereby inducing a substantial host immune response by release of pro-inflammatory cytokines up to systemic inflammation and sepsis (Sassi, Paul et al. 2009, Bertani and Ruiz 2018).

LPS is located in the outer membrane of bacteria, plays a pivotal role as a barrier for small, hydrophobic molecules, thereby making them resistant to antimicrobial compounds (Galloway and Raetz 1990, Nikaido 2003, Zhang, Meredith et al. 2013). LPS is composed of three domains, the core olgosaccharide, lipid A and the O antigen (Raetz and Whitfield 2002).

Lipid A of LPS binds to host TLR4, thus the immune system mainly responds to lipid A (Sassi, Paul et al. 2009, Scott, Oyler et al. 2017, Bertani and Ruiz 2018). The toll-like receptors (TLR) are expressed on cells of the innate immune system and are stimulated by LPS as PAMP, eventually leading to expression of pro-inflammatory proteins, such as TNFα and iNOS, as well as maturation of antigen-presenting cells. Upon LPS binding to TLR4, it induces TLR4 oligomerization and recruitment of signal transduction adaptor proteins, namely the myeloid differentiation primary response gene 88 (MγD88), TIR domain-containing adaptor protein (TIRAP), TRIF (TIR domain-containing adaptor inducing IFN-β) and TRAM (TRIF-related adaptor molecule) (Lu, Yeh et al. 2008). The LPS-induced signalling involves an early MγD88-dependent response or a delayed MγD88-independent response. The MyD88-dependent pathway results in the activation of NF-κB and the MAPK pathways, leading to the expression of several pro-inflammatory genes (Palsson-McDermott and O'Neill 2004). The MyD88-independent pathway leads to activation of NF-κB or interferon-responsive genes, as IFN-β (Palsson-McDermott and O'Neill 2004).

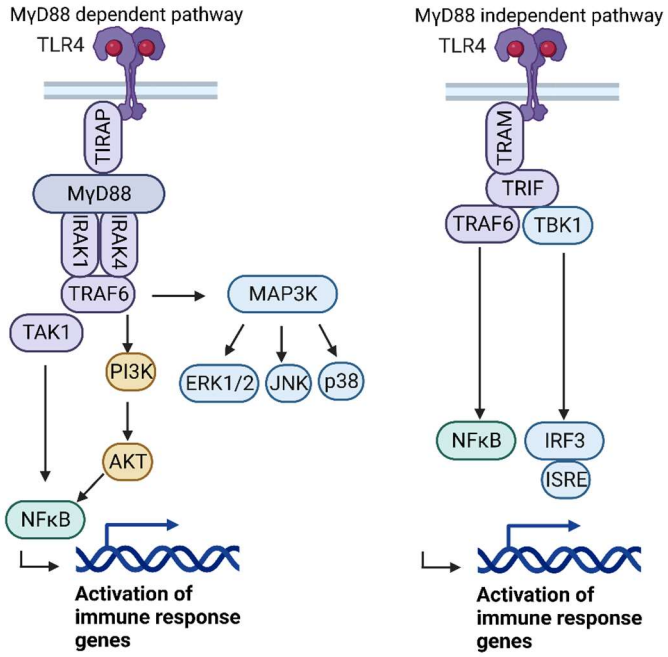

Figure 14: Signal transduction of LPS.

In general, two distinct LPS-induced pathways exist. The MγD88-dependent and the MγD88-independent pathway. Created with BioRender.com.

1.4.4.3.2. IFN-γ signal transduction

The interferons (IFN) exist in three different types, namely the type I, consisting of IFNα, IFNβ, IFNδ, IFNϵ, IFNκ, IFNτ, IFNω, and IFNζ; the type II, consisting of IFN-γ; and type III, consisting of IFNλ (Mazewski, Perez et al. 2020). Here I will focus on the type II interferon IFN-γ. Interferons are mainly produced by natural killer cells, but also by lymphocytes, and antigen-presenting cells, as monocytes and dendritic cells, during virus infection and are crucial for the regulation of immune responses (Schroder, Hertzog et al. 2004, Lee, Chen et al. 2017). IFN-γ is an important stimulus of T_h1 responses, as it enhances clearance of the infection and generates memory for future infections (Martin-Fontecha, Thomsen et al. 2004, Goldszmid, Caspar et al. 2012). The type II cytokine increases antigen presentation by increasing MHC II expression and DC maturation (Steimle, Siegrist et al. 1994, Lee and Ashkar 2018). Further, IFN-γ induces NO production and elevates ROS production and phagocytosis in macrophages, thereby inducing release of the pro-inflammatory cytokines TNFα and IL-1β (Karupiah, Xie et al. 1993, Schroder, Hertzog et al. 2004). Therefore, IFN-γ is defined also as a M1 polarizer of macrophages (Kang, Park et al. 2017, Lee and Ashkar 2018, Wang, Zhang et al. 2018).

IFN-γ binds to the IFN-γ receptor, composed of two IFNγR1 and two IFNγR2 chains. The intracellular domain of the IFNγR1 comprises binding motifs for JAK1 and STAT1, the activation of JAK1 initiates recruitment of STAT1 to the receptor and induces signal transduction (Farrar, Fernandez-Luna et al. 1991, Greenlund, Farrar et al. 1994, Kaplan, Greenlund et al. 1996). The intracellular domain of the IFNγR2 comprises a binding motif for JAK2 (Kotenko, Izotova et al. 1995).
The IFNγR1 chains bind the ligand, whereas the IFNγR2 chains are responsible for signal transduction (Schroder, Hertzog et al. 2004). IFN-γ signalling can be divided into a canonical and non-canonical signalling pathway. The canonical signalling pathway of IFN-γ signalling is the JAK-STAT pathway. Binding of IFN-γ to its receptor induces autophosphorylation and activation of JAK2, which in turn transphosphorylates JAK1. Activated JAK1 then induces phosphorylation and activation of STAT1 (Farrar, Fernandez-Luna et al. 1991, Greenlund, Farrar et al. 1994, Kotenko, Izotova et al. 1995, Briscoe, Rogers et al. 1996, Kaplan, Greenlund et al. 1996). Activated STAT1 dissociates from the receptor and travels to the nucleus, where it induces or inhibits transcription of IFN-γ-regulated genes, such as *Irf-1, iNos* and *IL-1β* (Schroder, Hertzog et al. 2004).

Besides the canonical pathway, IFN-γ can, in the absence of or simultaneously to STAT1, also signal via non-canonical pathways. For instance, IFN-γ can also activate MAPKs, as ERK, JNK or AKT (Ramana, Gil et al. 2001, Matsuzawa, Fujiwara et al. 2014, O'Donnell, Henkins et al. 2015).

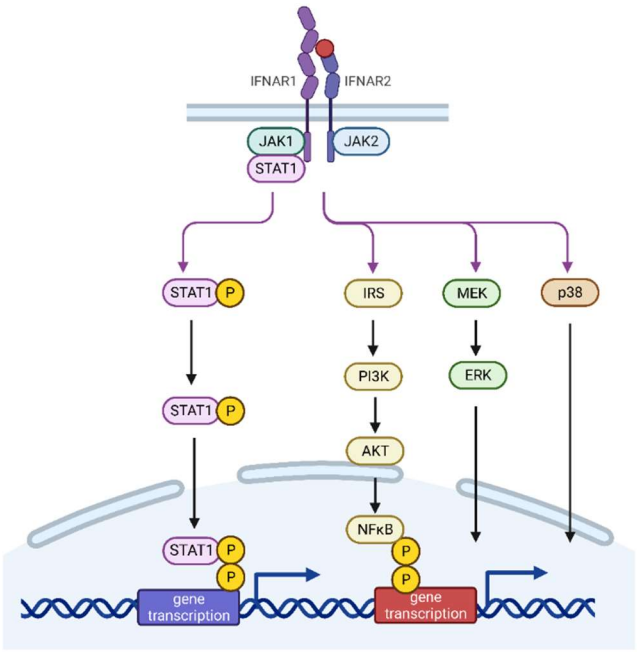

Figure 15: Signal transduction of IFN-γ.

The IFN-γ signalling consists of a canonical and a non-canonical pathway. The canonical signalling pathway of IFN-γ signalling is the JAK-STAT pathway. Besides the canonical pathway, IFN-γ can, in the absence of or simultaneously to STAT1, also signal via non-canonical pathways. For instance, IFN-γ can also activate MAPKs, as ERK, JNK or AKT. Created with BioRender.com.

1.5. Objective of the project

A common cause of death in the western world are cardiovascular diseases (Roth, Huffman et al. 2015). Acute myocardial infarction causes a profound sterile inflammation, and neutrophils are among the first cells to massively infiltrate the infarct area. This inflammatory response critically influences outcome after myocardial infarction. Further, T2DM strongly predisposes to cardiovascular diseases and it was reported that obesity and T2DM often result in chronic inflammation. Neutrophils are important effectors during acute inflammation, they are crucial for the activation and coordination of innate and adaptive immune cells. Until recently, the diversity of neutrophils is only poorly understood and the modulation of neutrophils to an anti-inflammatory phenotype could offer potential therapeutic strategies. Therefore, the main goal was to investigate neutrophil polarization capacity towards an anti-inflammatory phenotype and the underlying mechanisms. Further, the effect of modulation of neutrophils to an anti-inflammatory phenotype in the context of MI and the effect of hyperglycaemia on neutrophil phenotype was analysed. To this end, experiments with murine bone marrow and heart neutrophils were accomplished with the following aims:

I. Neutrophil polarization capacity

The first aim of this thesis was the analysis of polarization capacity of murine BM-derived neutrophils and the identification of novel phenotypic markers.

II. Analysis of underlying signalling mechanisms of neutrophil polarization

Based on the finding that neutrophils polarized to an anti- and pro-inflammatory phenotype, it was of interest to analyse possible signalling pathways responsible for polarization. Known IL-4 and LPS/IFN-γ signalling pathways are the AKT, RAS/RAF/ERK and JAK/STAT pathway. Therefore, the dependency of neutrophil polarization on those pathways were analysed.

III. Investigation of the effect of neutrophil phenotype on neutrophil functions

Neutrophils are important players during acute inflammation; they are crucial for the coordination and activation of other immune cells and are also key players in host defence by using different immune responses. Thus, the third task was to analyse if polarization of neutrophils also alters neutrophil function. Therefore, different immune response mechanisms of neutrophils, such as phagocytosis, degranulation, NET formation, migration and cytokine release in presence or absence of different polarizers were analysed.

IV. Analysis of neutrophil phenotypes in the context of Myocardial Infarction

After defining neutrophil phenotypes *in vitro*, it was of interest to analyse neutrophil phenotypes also *in vivo*. Neutrophils arrive early at the infarct area and it was recently reported that they play a crucial role in cardiac remodelling. Further, it was shown that IGF-1 mediates cardioprotective effects post MI and that this effect was mediated by myeloid cells. Therefore, the effect of IGF-1 on neutrophil subtype distinction in the time course of myocardial infarction was investigated.

V. Assessment of neutrophil phenotype and function in the context of hyperglycaemia

T2DM is a considerable comorbidity of cardiovascular diseases and is widely accepted as a metabolic disorder. However, it is now more and more often as well characterised as an immune-mediated inflammatory disease, accompanied by chronic inflammation. It was reported that this chronic inflammation leads to long-term functional reprogramming of innate immune cells. Therefore, the effect of hyperglycaemia on neutrophil polarization and function *in vitro* and *in vivo* was assessed.

At the end, this work should give novel insight into neutrophil phenotypes and function *in vitro* and *in vivo*. This could enhance our understanding of the role of different neutrophil phenotypes. Therefore, this work aimed at elucidating if modulation of neutrophil phenotype could offer a potential therapeutic strategy to improve outcome of diseases such as myocardial infarction or T2DM.

2. Materials

2.1. Laboratory equipment

Table 1: Applied Devices.

Product name	Manufacturer
Centrifuges	Eppendorf (5415R), Eppendorf (5402), VWR (Mega Star 1.6R), Labnet (Spectrafuge Mini Centrifuge), Roth (Rotilabo)
Cell scraper	VWR, Germany
Corning® Transwell® Polycarbonatmembran-Zellkultureinsätze (#CLS3421)	Merck
EASYstrainer™ 40/100µM (#542040,#542000)	Greiner bio-one
BD FACS Canto™ II	Beckman Coulter
FACS	MoFlo XDP, Beckman-Coulter
FACS tubes (#24222019)	Falcon
Fluorescence microscope	Kyence (BZ 9000)
Glomax Multiplate reader	Promega
Incubator	Heraeus (B6120), Infors HAT (Ecotron)
Light microscope	Leica (M60)
McIlwain tissue chopper	Cavey Laboratory Engineering Co. Ltd.
Neubauer chamber	Laboroptik
NextSeq 550 system	Illumina Inc.
PCR plate centrifuge	VWR (521-1648E)
Photometer	Bio-Rad (iMark, Microplate Absorbance Reader)
Pipettes	Gilson (Pipetman 10-1000 µl)
Power Blotter	Thermo Scientific (Pierce™ G2 Fast Blotter)
Real Time PCR System	Applied Biosystems (StepOnePlus)

Spectrophotometer	Peqlab (Nanodrop ND-1000 Spectrophotometer)
STATStrip Xpress2 Glucose Teststreifen	Nova Biomedical
Ultracentrifuge	Thermo Scientific (Mx120)
Ultrasound bath	Elma Transsonic (TS540)
Ultrasound device	VisualSonic (Vevo 2100 Imaging System)
Water purification system	Merck Millipore (MilliQ)
1/10/50 ml syringe	Braun AG
6-well cell culture plates	Greiner Bio-One

This table lists all materials applied during the experiments.

2.2. Chemicals

Table 2: Chemicals applied for the experiments.

Chemicals	**Manufacturer (Cat. No.)**
Acrylamid-Bisacrylamid-stock (40%)	Roth (#7876)
Actinomycin D	Sigma-Aldrich (#01817)
APS	Roth
Bromphenol blue	Roth (#*A5121*)
BSA Fraction V	Sigma-Aldrich (#10775835001)
Cell- & tissue adhesion, Corning Cell TAKT^{M}	VWR (#*734-1081*)
Collagenase Type I	Worthington (#LS004197)
DNAse	Roche Diagnostics (#10104159001)
DTT	Roche (#708984)
Endotoxin free FCS	Gibco (#10270)
Endotoxin free H_2O	Sigma-Aldrich (#TMS-011-A)
Ethanol, 99%	Roth (#9065.3)
Fetal Bovine Serum (FBS)	Biochrom (#S0615)
Glycerol	Sigma-Aldrich (#G7757)

Glycin	Sigma-Aldrich (#4034)
Hank´s Balanced Salt Solution (HBSS)	Gibco (#14025)
HEPES	Biochrom (#4027269840959)
Igepal CA-630	Sigma-Aldrich (#I3021)
n-Butanol	Roth (#7724.1)
Paraformaldehyd (PFA 4%)	Sigma Aldrich (#*16005*)
Percoll Plus	Cytiva (#17544501)
Phosphatase Protease Inhibitor Cocktail	Thermo Scientific (#*78442*)
SDS	Sigma-Aldrich
Sodium chloride	VWR Chemicals (#27.810.364)
TEMED	Roth (#2367)
Triton X-100	Sigma (#*T8787*)
Tricin	Roth (#6977.4)
Tris	Roth (#AE15.3)
Trypan blue	Sigma-Aldrich (#93595)
Trypsin	Sigma-Aldrich (#T8003)
Tween 20	Merck (#822184)

This table lists all chemicals applied during the experiments.

2.3. Buffers

Table 3: Buffers used for the experiments.

Buffer	**Composition (Concentration)**
Anode buffer, pH 8.8	300 mM Tris HCL 100 mM Tricin
Cathode buffer, pH 8.7	30 mM Tris HCL 300 mM Aminocaproicacid
Facs buffer (PEB)	PBS 0.5% BSA 2 mM EDTA
HBSS-prep for 1 mouse (50 ml)	5 mL 10x Ca-Mg-free HBSS 5 mL 200 mM HEPES stock (final concentration 20 mM)

	250 µL endotoxin free FCS (final concentration 0.5%) 40 mL endotoxin free water
Laemmli buffer (4x)	250 mM Tris-HCl (pH 6.8, 12.5 mL) 25% Glycerol (v/v, 10 mL) 8% SDS (w/v, 15 mL) 0.005% Bromphenolblau (w/v, 5mg) 100mM DTT
Polarization medium	500 mL VLE Dulbesco DMEM medium 3 % (15 mL) heat-inactivated FCS 1 M (5 mL) Hepes 1% (5mL) PenStrep
Phosphate-buffered saline (PBS), pH 7.4	NaCl (137 mM) KCl (2.7 mM) $Na_2PO_4*2H_2O$ (8.1 mM) KH_2PO_4 (1.76 mM)
SDS-Page running buffer	25 mM Tris 250 mM Glycin 0.1% SDS (w/v) 2 L H2O
Separation-gel (10%)	5 mL H_2O 2.5 mL 40% Acrylamid 2.5 mL 1.5 M Tris pH 8.8 100 µL 10% SDS 20 µL TEMED 25 µL 10% APS
Stacking-gel (2.5%)	3.75 mL H_2O 625 µL 40% Acrylamid 625 µL 1 M Tris pH 6.8 50 µL 10% SDS 10 µL TEMED 15 µL 10% APS
Tris-buffered saline (TBS), pH 7.5	Tris-Cl (50 mM) NaCl (150 mM)
Tris-buffered saline + Tween (TBS-T)	Tris-Cl (50 mM) NaCl (150 mM) Tween 20 (0.1%)
0.2% NaCl	1.25 mL 1.6% NaCl 8.75 mL endotoxin free H_2O
1.6% NaCl stock	1.6g NaCl per 100 ml endotoxin free H_2O, filter sterile
3T3-L1 growth medium	89% DMEM 12.5 mM glucose 10% FBS 1% PS 4 mM L-glutamine
3T3-L1 starving medium	99% DMEM 12.5 mM glucose

	1% PS 4 mM L-glutamine
62.5% Percoll	3.6 ml Percoll 0.4 ml 10x HBSS
100% Percoll	3.6 ml Percoll 0.4 ml 10x HBSS
200 mM HEPES stock (pH 7.4)	2.358g HEPES in 50 ml, set pH 7.4 and filter sterile

This table lists all buffers applied during the experiments.

2.4. Antibodies

2.4.1. Primary antibodies

Table 4: Required primary antibodies for Western Blot analysis.

Primary Antibody	animal	dilution	Cat. No.	Manufacturer
AKT	mouse	1:2000	2920	Cell signalling
phospho-AKT	rabbit	1:1000	9271	Cell signalling
AKT1 (9Q7)	mouse	1:1000	AHO1112	Thermo Fisher Scientific
AKT2 (D6G4) XP	rabbit	1:1000	3063	Cell signalling
p44/42 MAP Kinase	mouse	1:2000	9107	Cell signalling
phospho-p44/42 MAP Kinase	rabbit	1:1000	4307	Cell signalling
Phospho-STAT6	rabbit	1:2000	700247	Thermo Fisher Scientific
Phospho-STAT1	rabbit	1:1000	9167	Cell signalling

This table lists all primary antibodies applied during the experiments.

2.4.2. Secondary antibodies

Table 5: Required secondary antibodies for Western Blot analysis.

Secondary Antibody	animal	dilution	Cat. No.	Manufacturer
Goat-antiRabbitIGg Dye®800CW	rabbit	1:10000	926-32211	LI-COR
Goat-antiRabbitIGg Dye®680RD	rabbit	1:10000	926-68071	LI-COR
Goat-antiMouseIGg Dye®800	Mouse	1:10000	926-32210	LI-COR

This table lists all secondary antibodies applied during the experiments.

2.5. Antibodies Flow Cytometry

Table 6: Required antibodies and fluorophores for flow cytometric determinations.

Antibody	Clone	dilution	Abbreviation	Manufacturer
CD11b (#101235)	M1/70	1:100	Brilliant violet	BioLegend
CD18 (#101414)	M18/2	1:100	AF647	BioLegend
CD115 (#565249)	T38-320	1:100	PE	BD Biosciences
CD35/CD21 (#123410)	7E9	1:100	PE	BioLegend
Fc-block (#101302)	93	1:20		BioLegend
Ly-6G (#127654)	1A8	1:100	PerCP Cy5.5	BioLegend

This table lists all antibodies and fluorophores applied during flow cytometry experiments.

2.6. Chemokines

Table 7: Chemokines applied for the experiments.

Product Name (Cat. No.)	Concentration	Source	Manufacturer
D-Glucose (#G7528)	2g/kg of body weight		Sigma-Aldrich
Interleukin-4 (#214-14)	20 ng/ml	*murine*	Peprotech
Insulin (#2526396)	10 ng/ml + 100 ng/ml 0.75 U/kg of body weight	*human*	Lilly Deutschland GmbH, Germany
Insulin like growth factor 1 (IGF-1) (#130-093-887)	10 ng/ml	*human*	Miltenyi
Interferon-γ (IFN-γ) (#I4777)	2 ng/ml	*mouse*	Sigma-Aldrich
Lipopolysaccharide (LPS) (#L4391-1MG)	10 ng/ml	*E.coli*	Sigma-Aldrich
Phorbol 12-Myristat 13-Acetat (PMA) (#P8139)	100 ng/ml		Sigma-Aldrich
Recombinant mouse Cxcl1/Kc Protein (#453-Kc-010)	0,02 ng/µl	*mouse*	R&D Systems
Staphylococcus aureus BioParticles™, Fluorescin conjugate (#S2851)	MOI 10	*S.aureus*	Thermo Fisher Scientific

This table lists all chemokines applied during the experiments.

2.7. Inhibitors

Table 8: Inhibitors applied for the experiments.

Product Name (Cat. No.)	Manufacturer
InSolution™ JAK inhibitor I (#420097-500UG)	Sigma Aldrich
Ruxolitinib (#INCB018424)	Selleckchem
BMS-911543 (#S7144)	Selleckchem

This table lists all inhibitors applied during the experiments.

2.8. qPCR primer

Table 9: qPCR-Primer.

Primer	Direction	Sequence 5`-3`	Manufacturer
AKT1 Exon 6	Fwd	ACCATGAACGAGTTTGAGTACC	Invitrogen
AKT1 Exon 7	Rev	TAGAGTTCTGCAGGACACGG	
AKT2 Exon 5	Fwd	GGTAGCTGTCAACAAGGCAC	Invitrogen
AKT2 Exon 6	Rev	CAATGATGACCTCCTTGGC	
Arginase 1	Fwd	CTGACCTATGTGTCATTTGG	Sigma-Aldrich
	Rev	CATCTGGGAACTTTCCTTTC	
Car4	Fwd	GCATTTATGATTGAGGTAGGAG	Sigma-Aldrich
	Rev	AATGGGGTTTGGAGATACTG	
Cxcl9	Fwd	GAGGAACCCTAGTGATAAGG	Sigma-Aldrich
	Rev	GTTTGATCTCCGTTCTTCAG	
Fam19a3	Fwd	AAAACCAGACAACACCTAAG	Sigma-Aldrich
	Rev	CCATCTGATGAACAATATGGC	
IL-1ß	Fwd	GGATGATGATAACCTGC	Sigma-Aldrich
	Rev	CATGGAGAATATCACTTGTTGG	
IL-6	Fwd	AAGAAATGATGGATGCTACC	Sigma-Aldrich
	Rev	GAGTTTCTGTATCTCTCTGAAG	
IL12a	Fwd	GAAGACATCATGAAGAC	Sigma-Aldrich
	Rev	CTCTTGTTGTGGAAGAAGTC	

IL-23	Fwd	AATAATGCTATGGCTGTTGC	Sigma-Aldrich
	Rev	CTTAGTAGATTCATATGTCCCG	
Mcp-1	Fwd	CAAGATGATCCCAATGAGTAG	Sigmal-Aldrich
	Rev	TTGGTGACAAAAACTACAGC	
Nos2	Fwd	CATCACCAGTATTATGGCTC	Sigma-Aldrich
	Rev	TTTCCTTTGTTACAGCTTCC	
Nudc	Fwd	AGAACTCCAAGCTATCC	Sigma-Aldrich
	Rev	CTTCAGGATTTCCTGTTC	
Resistin-like molecule α	Fwd	GATGAAGACTACAACTTGTTCC	Sigma-Aldrich
	Rev	AGGGATAGTTAGCTGGATTG	
Slc28a3	Fwd	CATTTAAGATCCTGCCCATC	Sigma-Aldrich
	Rev	CCAATAAATATGTTGCCAGC	
Tnfα	Fwd	CTATGTCTCAGCCTCTTCTC	Sigma-Aldrich
	Rev	CATTTGGGAACTTCTCATCC	
Ym1/Ym2 ≙ Chi3l3	Fwd	TCACAGGTCTGGCATTCTTCTG	Invitrogen
	Rev	TTTGTCCTTAGGAGGGCTTCCTCG	

This table lists all qPCR-Primer applied during the experiments.

2.9. Kits and mastermixes

Table 10: Kits

Product Name (Cat. No.)	Manufacturer
Chromium Single Cell 3' NextGEM Reagent Kit v3.1	10X Genomics, Pleasanton, CA
Fix & PERM™ Cell Permeabilization Kit (#GAS004)	Invitrogen™
Halt Protease & Phosphatase Inhibitor Cocktail (#78443)	Thermo Fisher Scientific
LegendplexTM MU Cytokine Release Syndrome Panel (#BLD-741024)	Biolegend
LegendplexTM MU Inflammation Panel (#BLD-740446)	Biolegend
Maxima SYBR green/ROX qPCR master mix (#K0221)	Thermo Fisher Scientific
Odyssey Blocking Buffer in TBS (#927-40000)	LI-COR Bioscience

Page Ruler Prestained Protein Ladder (#26616)	Thermo Fisher Scientific
Pierce BCA Protein Assay Kit (#23225)	Thermo Fisher Scientific
Quantitect Reverse Transcription kit (#205311)	Qiagen
Quant-iT™ PicoGreen® dsDNA Reagent and Kits (#P7581)	Thermo Fisher Scientific
Revert 700 Total Protein Stain (#926-11011)	LI-COR Bioscience
RNeasy Kit (#74104)	Qiagen

This table lists all Kits applied during the experiments.

2.10. Softwares

Table 11: List of applied softwares.

Software	Manufacturer
BD FacsDiva Software v.8.0.2	BD Biosciences
BioLegend`s Legendplex™ Data Analysis Software	Biolegend
Microsoft Office	Microsoft Corporation, Redmond, USA
GraphPad Prism 7	GraphPad Software, Inc., La Jolla, USA
Endnote X8	Clarivate Analytics, Philadelphia, USA
SigmaPlot 13	Systat Software GmbH
StepOne Software v2.1	Applied Biosystems
Image Studio Lite Ver 5.2	LI-COR Biosciences
Microsoft Excel	Microsoft Corporation, Redmond, USA
Microsoft Word	Microsoft Corporation, Redmond, USA
Microsoft PowerPoint	Microsoft Corporation, Redmond, USA

This table lists all softwares applied during this work.

3. Methods

3.1. Transgene mouse models

All mouse experiments were conducted according to the national guidelines of the National Institute of Health (NIH) and were authorized by the local animal care and use committee (LANUV, Recklinghausen, 81-02.04.2020.A171, 81-02.04.2017.A401).

Dr. rer. nat. Stefanie Gödecke established the knockout mouse strains in the Institute of Cardiovascular Physiology, Heinrich-Heine-University Düsseldorf.

Felasa B certificate (ID: F048/16_#_0342) was obtained at the central institution of animal research and scientific animal welfare functions (ZETT) of the Heinrich-Heine-University Düsseldorf in March 2019 (22.03.2019).

All mice (*mus musculus*) were kept under controlled temperature (20-22 °C) and in a 12 -hour light/dark cycle. The mice received water and food *ad libitum.* The mice were kept at the animal stables of the ZETT of the Heinrich-Heine-University Düsseldorf. In all experiments, mice with an inbred C57BL/6J mouse line background were used.

3.1.1. Inducible gene knockouts

The *Cre/loxP* system was used to generate the knockout animals, this system has already been used for decades to delete, insert or inverse specific genes in the DNA of cells or animals. The enzyme *Cre recombinase*, which is derived from P1 bacteriophages, cuts between two 34 bp spanning *loxP* sites and thus can induce deletion, insertion or inversion of a *loxP*-flanked (“floxed”) DNA sequence (Sternberg, Hamilton et al. 1981).

3.1.1.1. Tie2 AKT1 knockout mouse model

The Tie2 AKT1 KO mice were used to analyse the effect of the absence of AKT1, specifically in neutrophils, on neutrophil phenotype.

Therefore, $AKT1^{WT}$ (C57BL/6J;Cg-TgH(AKT1)^{tm44AG}, strain 1461) mice were crossbred with Tie2-Cre mice (strain 1084) to generate conditional knockout mice. The Tie2-Cre mice conditionally express the Cre recombinase under the control of the Tie2 promotor. This results in a deletion of AKT1 in endothelial cells, haematopoietic cells and in germ line during embryogenesis, as well as in adulthood (Kisanuki, Hammer et al. 2001, Tang, Harrington et al. 2010).

3.1.1.2. Tie2 AKT2 knockout mouse model

The Tie2 AKT2 KO mice were used to analyse the effect of the absence of AKT2, specifically in neutrophils, on neutrophil phenotype.

Therefore, $AKT2^{WT}$ (C57BL/6J;Cg-AKT-2^{tm87AG}, strain 1472) mice were crossbred with Tie2-Cre (strain 1084) mice. The Tie2-Cre mice conditionally express the Cre recombinase under the control of the Tie2 promotor. This results in a deletion of AKT2 in endothelial cells, haematopoietic cells and in germ line during embryogenesis, as well as in adulthood (Kisanuki, Hammer et al. 2001, Tang, Harrington et al. 2010).

3.1.1.3. Global ΔAKT2 KO

The global ΔAKT2 KO mice were used to analyse the effect of the absence of AKT2 on neutrophil phenotype.

Therefore, $AKT2^{WT}$ (C57BL/6J;Cg-AKT-2^{tm87AG}, strain 1472) mice were crossbred with PGK-Cre (strain 1250) mice. The PGK-Cre mice conditionally express the Cre recombinase under the control of the PGK promotor in all cells (Lallemand, Luria et al. 1998). This results in a deletion of AKT2 in all cells. Those mice show no apparent defects, however develop insulin resistance, hyperglycaemia and hyperinsulinaemia over time (Cho, Mu et al. 2001, Garofalo, Orena et al. 2003).

3.1.1.4. Global ΔAKT1 KO

The global ΔAKT1 KO mice were used to analyse the effect of the absence of AKT1 on neutrophil phenotype.

Therefore, $AKT1^{WT}$ (C57BL/6J;Cg-$AKT1^{tm44AG}$, strain 1461) mice were crossbred with PGK-Cre (strain 1250) mice. The PGK-Cre mice conditionally express the Cre recombinase under the control of the PGK promotor in all cells (Lallemand, Luria et al. 1998). This results in a deletion of AKT1 in all cells. Those mice show impaired growth from fetal to adult state, however have no glucose intolerance (Cho, Thorvaldsen et al. 2001).

The genotypes of the mice were identified by tissue biopsies (about 3 weeks after birth, after being separated from their mother) and following DNA analysis by PCR, using different allele-specific primers.

3.1.2. DIO mouse model

In order to analyse the effect of hyperglycaemia and T2DM on neutrophil polarization, a diet-induced obesity mouse model (DIO) was established. Therefore, male C57BL/6J mice of 8 weeks were fed a high fat/ high sucrose diet (BioServ F1850) composed of 36% fat, 20.5% protein and 36.2% carbohydrates for 12 weeks. This protocol induced a pre-diabetic status with significantly increased body weight, but only slightly increased

plasma glucose and insulin levels (Figure 54). Control mice received a standard chow diet.

3.1.3. DIO-STZ mouse model

To induce a more advanced status of diabetes, the DIO-STZ (Streptozotocin) model was established. This non-genetic mouse model was originally developed by Luo et al., and then refined by Gilbert et al., to analyse a more severe state of non-insulin dependent type 2 diabetes mellitus (Luo, Quan et al. 1998, Gilbert, Fu et al. 2011). Therefore, 8 weeks old male C57BL/6J mice were fed with the high fat/ high sucrose diet, as was used for DIO mice, for 12 weeks. Additionally, mice were given 5 consecutive daily intraperitoneal injections of STZ (40mg/kg) in the 5th week of feeding, whereas control mice were injected with a vehicle (citrate buffer). Streptozotocin is a ß-cell toxin, which induces destruction of the pancreatic ß-cells, thus resulting in a strongly reduced functional ß-cell mass and low insulin secretion (Rakieten, Rakieten et al. 1963, Arison, Ciaccio et al. 1967, Schein, Cooney et al. 1967). Those mice showed strong hyperglycaemia, with fasting blood glucose levels of over 400 mg/dl, but no obesity (Figure 57).

3.2. *In vitro* methods

3.2.1. Isolation and polarization of murine bone marrow neutrophils

3.2.1.1. Murine BM neutrophil isolation by percoll gradient-centrifugation

The murine bone marrow neutrophil isolation was established according to the protocol of Mocsai et al. (Mocsai, Ligeti et al. 1999).

The mice were sacrificed by cervical dislocation and the femurs and tibias were removed by dislocating the acetabulum from the hip joint. Hereby, special care was taken not to break the femur head while dislocating, to avoid activation of neutrophils by circulating bacteria in the air or by causing a wound. The remaining muscles were removed and the bones were incubated in 70% ethanol for one minute to sterilize them and to remove possible contaminants. The bones were kept in HBSS-prep to prevent dry-out. All following steps were then executed under the laminar airflow to prevent activation of neutrophils by an unsterile environment, as these cells are very sensitive and easily activated.

The femur was disconnected from the tibia at the knee joint, again special care was taken to not break the bone ends. The ends of the bones were cut as far towards the end as possible and the joint surface of the distal femur and proximal tibia was dislocated. The

bone marrow cells were flushed with HBSS-prep from both ends of the bone shafts into a 50 ml falcon tube, using a 23G needle and a 10 ml syringe. Subsequently, the isolated bone marrow was sucked through a 20G needle to disaggregate larger bone marrow pieces. The suspension was centrifuged at 400 xg at room temperature for 5 minutes and the pellet was resuspended for 30-40 seconds in 10 ml 0.2% NaCl to lyse all the red blood cells. Afterwards, the osmolarity was restored with 10 ml of 1.6% NaCl and the suspension was filtered through a 100-micron cell strainer to remove remaining bone parts and clots. Again, the suspension was centrifuged at 400 xg at room temperature for 5 minutes and the lysed bone marrow was resuspended in 5 ml HBSS-prep. For the density centrifugation 5 ml 62.5% Percoll was poured in a 15 ml Falcon tube and the lysed bone marrow, resuspended in 5 ml HBSS-prep, was slowly layered on top. Thereby, it was important to avoid mixing of the layers. The suspension was centrifuged at 1000 xg at room temperature for 30 minutes and the run was stopped with brakes switched off. After the gradient centrifugation a sharp interface was visible atop the 62.5% Percoll and on the bottom of the 15 ml Falcon. The bottom layer contained the neutrophils and the interface above the 62.5% Percoll included immature cells and non-granulocytic lineages. First, the interface with the non-granulocytic lineages was removed and discarded or transferred into a 15 ml Falcon tube for use in other experiments. Subsequently, the interface containing the neutrophils was transferred into another 15 ml Falcon tube and washed twice with HBSS-prep and centrifuged at 400 xg at room temperature for 5 minutes. The pellet was then resuspended in 1 ml polarization medium and the cell count was determined in an improved Neubauer chamber.

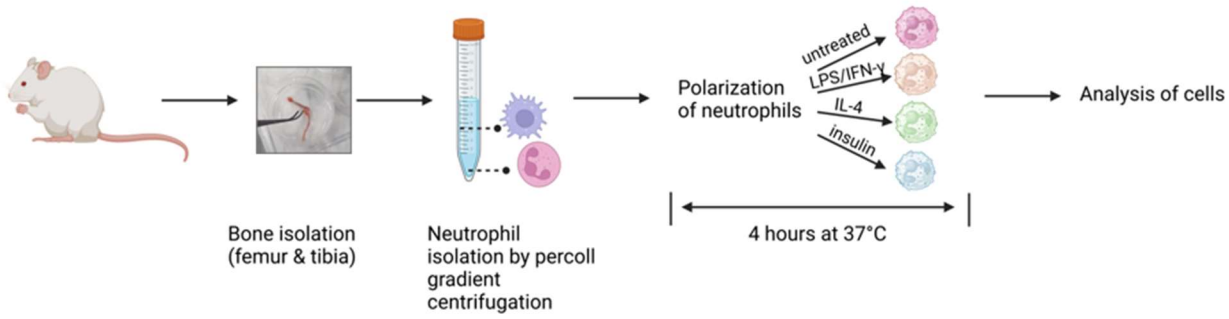

Figure 16: Flow chart of the murine BM neutrophil isolation and polarization process.

The murine BM was extracted by cutting out the femurs and tibias and disconnecting the femur from the tibia at the knee joint. The neutrophil isolation was performed by lysing all the red blood cells and separating the immature cells and non-granulocytic lineages from the neutrophils by a 62.5% percoll gradient centrifugation. The cell-count was determined by the improved Neubauer chamber. The neutrophils were cultivated and polarized for 4 hours at 37°C and 5% CO_2 and were treated with 20 ng/ml IL-4, 10 ng/ml LPS plus 2 ng/ml IFN-γ, 10 ng/ml insulin. Created with BioRender.com.

3.2.1.2. Polarization of murine BM neutrophils

For the analysis of neutrophil polarization 5 to 6 million cells were seeded per well within a total of 2 ml per well. Neutrophils were left untreated or treated with 20 ng/ml IL-4, 10 ng/ml LPS plus 2 ng/ml IFN-γ or with 10 ng/ml insulin. Untreated cells served as a negative control and contained only neutrophils in polarization medium (Figure 17). The samples were incubated in a 6-well plate for 4 hours (transcript expression analysis) or 10 minutes (protein analysis) at 37°C. Afterwards, the non-adherent cells were collected in 15 ml Falcon tubes and centrifuged at 400 xg at RT for 5 minutes. The cell pellet was resuspended in 750 µl Trizol or cell lysis buffer and frozen at -20/ - 80 °C for further investigations. It has to be noted, that the neutrophils cultured with LPS and IFN-γ needed to be scraped, as they were sticking to the bottom of the well due to their activated status. In contrast, cells of all other conditions were non-adherent and could be aspirated without scraping.

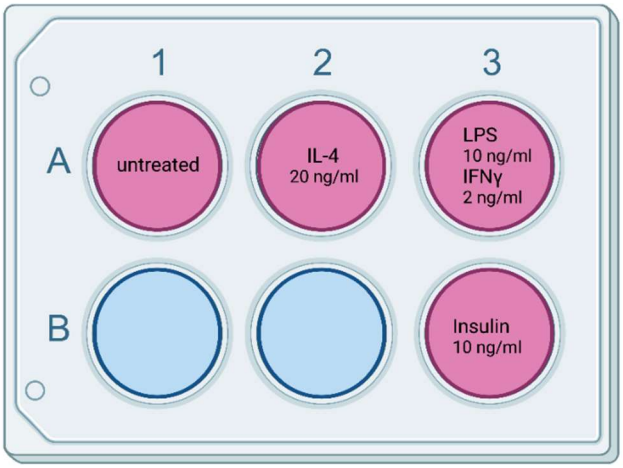

Figure 17: Experimental setting of the murine BM neutrophil polarization.

Illustration of the pipetting scheme on a 6-well plate. Created with BioRender.com.

In additional experiments, different inhibitors of the Janus kinase family (JAK1, JAK2, JAK3, Tyk2) were applied to analyse if IL-4 and LPS/IFN-γ induced polarization is dependent on the Janus kinases. Therefore, a combination of different JAK inhibitors was used. Neutrophils were left untreated or treated with 20 ng/ml IL-4, or 10 ng/ml LPS plus 2 ng/ml IFN-γ +/- the JAK inhibitor for four hours or 10 minutes. Three different JAK inhibitors were used. InSolution™ JAK Inhibitor I has a higher IC_{50} value for JAK1 than for JAK2, JAK3 or Tyk2 (Table 12). To narrow down which JAK isoform is responsible for N1 or N2 neutrophil polarization, we additionally used the JAK inhibitor Ruxolitinib, which has a 150-times lower IC_{50} for JAK1 and JAK2 than for JAK3, and the JAK2 inhibitor BMS-911543.

Table 12: IC_{50} values of the different JAK inhibitors used.

Inhibitor	IC50 values [nM]				High conc. [nM]	Low conc. [nM]	Inhibited at low conc.
	JAK1	JAK2	JAK3	Tyk2			
InSolution™	15	1.0	5	1	250	5	JAK2/3/Tyk2
Ruxolitinib	3.3	2.8	>390		2500	30	JAK1/2
BMS-911543	75	1.1	360	66		10	JAK2

This table shows the IC_{50} values and used concentrations of the different JAK inhibitors. The indicated high concentration inhibits all JAKs and Tyk2, whereas at the lower concentrations only the indicated JAKs are inhibited, which are shown in the last column.

3.2.2. Flow cytometry

3.2.2.1. General information

Flow cytometry was used to investigate different functions of isolated BM neutrophils according to their granularity, size and their specific cell surface proteins.

Flow cytometry enables the fast screening of large amounts of cells and makes quantitative analysis of protein expression and DNA content at the single-cell level possible. A fluorescent dye-labelled antibody, which is specific for a particular cell surface molecule, is added to the cells. The cells are sucked through a capillary into the flow cell, where the cells pass a laser beam in single file. Thereby, the fluorescence of each cell is measured via photomultipliers, which amplify the fluorescent light emitted at different wave lengths. Following the simultaneous measurement of multiple molecules, different cell types scatter light, depending on their size and structure. Granulocytes show a rough cell surface; hence, they scatter light more than B- and T-cells, which have a plain cell surface. The two light scatters depend either on the cell size and volume (Forward Scatter, FSC) or on granularity (Side Scatter, SSC) (Sutherland, Keating et al. 1994, Sutherland, Anderson et al. 1996). Thus, several different cell types can be identified and analysed precisely within one sample.

For data analysis and presentation, BD FACS DIVA version 8.0.2 software was used. Results for cell populations are shown as dot plots or density plots and each dot is equivalent to one measured cell in the flow cell. The individual dots were combined to populations with the aid of gates.

3.2.2.2. Gating strategy for characterization of murine BM neutrophils

In order to investigate differences in neutrophils upon different stimulations, cells were labelled with monoclonal antibodies, which were conjugated to fluorescent dyes.

Neutrophils are positive for Ly6G, which belongs to the Ly-6 family and is expressed on granulocytes, a subset of eosinophils, and transiently during developmental stages of monocytes (Nagendra and Schlueter 2004). Further, neutrophils are negative for CD115, which is a macrophage colony-stimulating factor receptor and is expressed on macrophages, monocytes, dendritic cells and osteoclasts, but not on neutrophils (Haegel, Thioudellet et al. 2013). Strongly activated neutrophils express high levels of CD11b and CD18. CD11b, also known as integrin α-M, is an adhesion molecule expressed on monocytes and granulocytes. CD11b forms a complex with CD18, also known as integrin β-2, which is expressed in leukocytes and binds to ICAM-1. The chemotactic stimulation of neutrophils induces the translocation of these integrins from intracellular milieu to the cell surface, thus they can be used as markers of degranulation. CD35 is a secretory vesicle and is used as a marker of exocytosis (Hughes, Hollers et al. 1992, Deree, Lall et al. 2006). Hence, samples were stained with Ly6G to identify neutrophils and with CD11b, CD18 and CD35 to identify degranulation of neutrophils. In favour of characterising isolated neutrophils, 100 µl of isolated cells and 1 µl (1:100) of each antibody was added to 5 ml polypropylene round-bottom tubes and incubated for 15 minutes at 4 °C in the darkness. Subsequently, the samples were washed for 5 minutes at 400 xg and were measured with an eight-colour flow cytometry instrument (FACS Canto). Neutrophils were gated for a high-density expression of Ly6G, size and granularity.

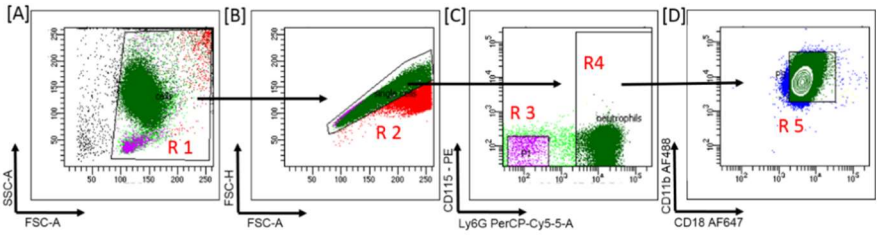

Figure 18: Exemplary gating strategy for flow cytometric analysis of murine BM neutrophils.

[A] Dot plot displaying cell size (forward scatter ≙ FSC) vs. cell granularity (sideward scatter ≙ SSC) for all events (R1), in the lower left corner the debris is excluded from further analysis. **[B]** Dot plot to define single cells. All cells were analysed by FSC-H vs. FSC-A. The gate R2 defines all single cells and excludes all doublets, thus avoiding false-positive results. **[C]** Dot plot representing the population of all single cells (R2) and analysing for CD115 (CD115 PE-A) and Ly6G (Ly6G PerCP-Cy5.5-A). Gate R3 represents all $Ly6G^-$ $CD115^-$ cells, whereas gate R4 displays the $Ly6G^+$ $CD115^-$ neutrophils. **[D]** Dot plot representing the R4 population gated on CD11b and CD18 expression, thus showing the activation state of neutrophils. It is determined by CD11b expression (CD11b Alexa Fluor 488) vs. CD18 expression (CD18 APC-A).

3.2.3. Protein analysis

3.2.3.1. Protein isolation from cells

The neutrophils were lysed in 200 µl 4% SDS lysis buffer supplemented with protease- and phosphatase inhibitors and centrifuged for 10 minutes at 16000 g at 16 °C. Subsequently, the supernatant was transferred into a new Eppendorf tube and was used for the following determination of protein concentration (BCA Assay).

3.2.3.2. BCA Assay

Determination of protein concentration was achieved by performing the bicinchoninic acid (BCA) assay, based on a protocol of Smith et al. (Smith, Krohn et al. 1985). A standard series with ascending concentrations of BSA in lysis buffer (0.125-2 mg/µl) was used for normalisation. Samples, diluted with 200 µl of BCA reagent, were pipetted as doublets onto a 96-well plate. This plate was then incubated for 30 minutes at 37 °C and subsequently the absorption of the samples at 577 nm was measured via ELISA-Reader SpectraCount and analysed with the program PlateReader V3.0. Based on the standard curve the protein concentration was determined and samples were adjusted to a specific protein concentration (usually 1 µg/µl).

3.2.3.3. Polyacrylamidgelelctrophoresis with SDS

In order to separate the proteins, the samples were mixed with 4x Laemmli buffer and heated at 95 °C for 5 to 10 minutes to denature the proteins. The proteins were then separated electrophoretically according to the protocol of Laemmli et al. (Laemmli 1970). Therefore, the Hoefer-electrophoresis tanks, which were filled with SDS-running buffer, were used at 100-200 V. To achieve an uniform transition of the proteins into the separation gel, a stacking gel with 2.5% (pH 6.8) was used and the separation of the proteins, based on their molecular size, followed in a 10% or 7.5% separating gel (pH 8.8). The PageRuler Prestained Protein Ladder (10-170 kDa) was used to determine the estimated size of the proteins.

3.2.3.4. Western Blot Analysis

Following electrophoretical separation, the proteins were transferred to a nitrocellulose membrane with the help of Pierce™ G2 Fast Blotter with a pre-programmed setting for mixed molecular weight (25 V, 1.3 mA, 10 minutes). After the transfer was completed,

the membrane was blocked for a minimum of 60 minutes in a blocking solution (TBS 1:1 LI-COR Odyssey solution), in order to avoid unspecific binding of antibodies. The primary antibody, diluted in 5% BSA and TBS-T (concentration according to distributor), was then incubated over night at 4 °C on a shaker. Subsequently, the membrane was washed three times for 10 minutes with TBS-T. The secondary fluorescent antibody (LI-COR antibodies, 1:10000 in LI-COR odyssey solution) was incubated for 45 to 60 minutes at RT in the dark on a shaker. Again, the membrane was washed two times for 10 minutes with TBS-T. To remove the Tween, the membrane was washed once for fifteen minutes with only TBS. Finally, the fluorescent-coupled dye antibodies were detected via LI-COR Odyssey 9120 Infrared Scanner. The scanner can detect wavelengths of 700 nm (red) and 800 nm (green), thus it is possible to detect two proteins simultaneously. For analysis, the computer program LI-COR Image Studio was used.

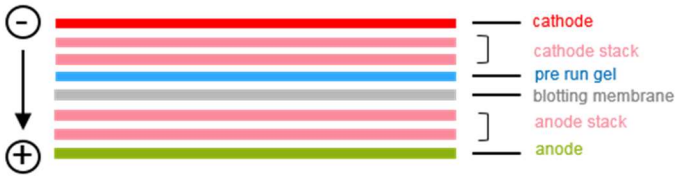

Figure 19: Transfer of proteins to nitrocellulose membrane.

The transfer stack contains, beginning from the bottom, two sheets of pre-wet filter paper (anode stack) and the blotting membrane equilibrated in anode buffer, then the gel, and lastly two sheets of filter paper equilibrated in cathode buffer (cathode stack). Negatively charged proteins move away from the cathode (negatively charged pole) and towards the anode (positively charged pole) by applying a voltage.

3.2.4. Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

3.2.4.1. Isolation of RNA

RNA was isolated according to a modified protocol, combining Trizol and the RNeasy kit (Qiagen). Following neutrophil isolation and polarization, the cells were lysed in 750 µl Trizol. After lysis, 0.2 ml chloroform was added per 1 ml Trizol and a centrifugation step followed. After centrifugation, the upper aqueous phase, which contains the RNA, was transferred to a new Eppendorf tube and 0.5 ml isopropanol was added. The samples were then transferred to a RNeasy Mini spin column and RNA was purified by several washing steps without or with genomic DNA digestion (RNA sequencing analysis). In a last step, the RNA was eluted with 20 µl RNase-free water according to the manufacturers protocol (Qiagen). The concentration of the isolated RNA was assessed with the help of Nanodrop ND-1000. The absorbance ratio at 260/280 mm determines the purity of the RNA relative to proteins and should be above 1.8 and under 2.1.

3.2.4.2. cDNA transcription

Following RNA isolation, RNA has to be transcribed into complementary DNA (cDNA). Up to 1 µg mRNA was transcribed into cDNA, using QuantiTect Reverse Transcription Kit, according to the manufacturers protocol. mRNA was diluted with RNase-free water to a final concentration of 1 µg per 20 µl reaction volume and genomic DNA (gDNA) was eliminated by using gDNA buffer. The mRNA was reverse transcribed while heating the samples at 42 °C for 15 minutes and the reverse transcriptase was then inactivated at 95 °C for 3 minutes. The resulting cDNA could be used immediately or stored at -20 °C.

3.2.4.3. Polymerase Chain Reaction

For the quantitative polymerase chain reaction (qPCR) the Maxima SybrGreen/ROX qPCR Master Mix was used to detect transcript expression. The Master Mix (MM) contained Maxima Hot Start Taq DNA Polymerase and desoxyribonucleosidtriphosphates (dNTPS). cDNA and primers were used as indicated below:

SybrGreen MM	10.0 µl
ddH_2O	7.2 µl
cDNA (20-40 ng/µL)	1.0 µl
Fwd Primer	1 µM
Rev Primer	1 µM
Total	20 µl

cDNA of neutrophils was used at a final concentration of 25 ng/µl. Pre-designed primer pairs (see chapter 2.8) were used, which were specifically designed for cDNA based qPCR, as at least one primer is exon-spanning. Thus, gDNA could not be amplified, as it still contained introns and exons, compared to cDNA. The melting curve analysis at the end of every qPCR run indicated the primer specificity, as the melting point of each amplicon is defined by its length and nucleotide composition. Upon stepwise heating, the double strands opened and the intercalated fluorophores were released and the fluorescent signal was lost. The melting temperature is defined by the maximum of the first derivative of the melting curve. The correct transcript was amplified if there is only one peak, as it indicates the same size of all amplicons. Each gene was measured in technical duplicates and compared to *Nudc*, a nuclear movement protein, which was used as a reference gene. Furthermore, a negative control for each primer was included (MM without cDNA).

3.2.4.4. Settings

All experiments were performed with the Step One Plus Real-Time PCR Detection System (Applied Biosystems). The following setting was used for gene amplification: The first cycle, to activate Taq-polymerase, lasted 10 minutes at 95 °C and was followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds. The specificity of amplicons was monitored by melting curve analysis, starting with 15 seconds at 95 °C and subsequent 60 seconds at 60 °C. Afterwards, the temperature was increased by 0.3 °C every 15 seconds until the final temperature of 90 °C.

3.2.4.5. Analysis

In order to analyse the genes of interest the X_0 method was used. This method depends on an endogenous reference gene to compare different conditions.

The X_0 method is based on the general equation of PCR amplification according to Sasse et al. (Sasse, Wallich et al. 2003) and was used in this work:

$X_n = X_0(1 + E_{amp})^n$

X_0 = amount of transcript in the sample at cycle 0

X_n = amount of transcript after n-cycles (defined by Ct-value)

E_{amp} = efficieny of amplification (usually 1)

n = number of cycles to reach X_n

If E_{amp} equals 1, which is equivalent to 100% efficiency of the qPCR, the equation can be simplified and solved for X_0:

$X_0 = \frac{x_n}{2^n}$

In order to normalise samples and to Statistically compare different conditions, the X_0 value of a gene of interest is divided by the X_0 value of a reference gene.

3.2.5. RNA sequencing Transcript Expression Analysis

RNA sequencing transcript expression analysis (RNAseq) was performed to identify the transcript profile of murine BM neutrophils. RNAseq is based on sequencing by synthesis, as millions of different DNA fragments are sequenced in parallel by one sequencing run.

The mRNA of murine BM neutrophils was isolated as described in 3.2.4.1. Hereby, it was crucial to eliminate gDNA contamination by the on-column digest with DNase. The mRNA concentration was measured by Nanodrop ND-1000 and, if applicable, was diluted to a final concentration of 100 ng/µl. The quantity (Qubit RNA HS Assay, Thermo Fisher Scientific, MA, USA) and quality of the mRNA were measured by capillary electrophoresis using the Fragment analyzer and the “Total RNA standard sensitivity assay” (Agilent Technologies, Inc. Santa Clara, CA, USA) by the “Biologisch-Medizinisches Forschungszentrum” (BMFZ by P. Petzsch) of the Heinrich-Heine-University by defining the RNA quality number (RQN). All samples in this study showed RNA Quality Numbers (RQN) with a mean of 10.0. The RQN value indicates the integrity of the RNA, the RNA is classified by their 18S to 28S ribosomal subunit ratio, as the decrease in the 18S to 28S ribosomal band ratio indicates the degradation of RNA. The RQN values rank from 1 to 10, while one equals the most degraded RNA and 10 marks the most intact RNA (Schroeder, Mueller et al. 2006). For a transcript expression analyis the mRNA has to be of high quality, with RQN values of 8 to 10 (Figure 20). After confirmation of high quality of the samples, the BMFZ started to perform the RNA sequencing transcript expression analysis by preparing DNA libraries. Therefore, DNA was fragmented and adapters were ligated to both ends of the DNA fragments. Those adapters contained sequence motifs, as binding sites for the flow cell, and PCR primer, which are required for subsequent steps, using ‘VAHTS™ Stranded mRNA-Seq Library Prep Kit for Illumina®’. 500 ng total RNA were used as input for mRNA capturing, fragmentation, the synthesis of cDNA, adapter ligation and library amplification. These libraries were purified with beads and were normalised and finally sequenced on the NextSeq2000 system (Illumina Inc. San Diego, CA, USA) with a read setup of SR 1x100 bp. In order to convert bcl files to fastq files, and for adapter trimming and demultiplexing, the BCL convert tool (version 3.10.11) was used.

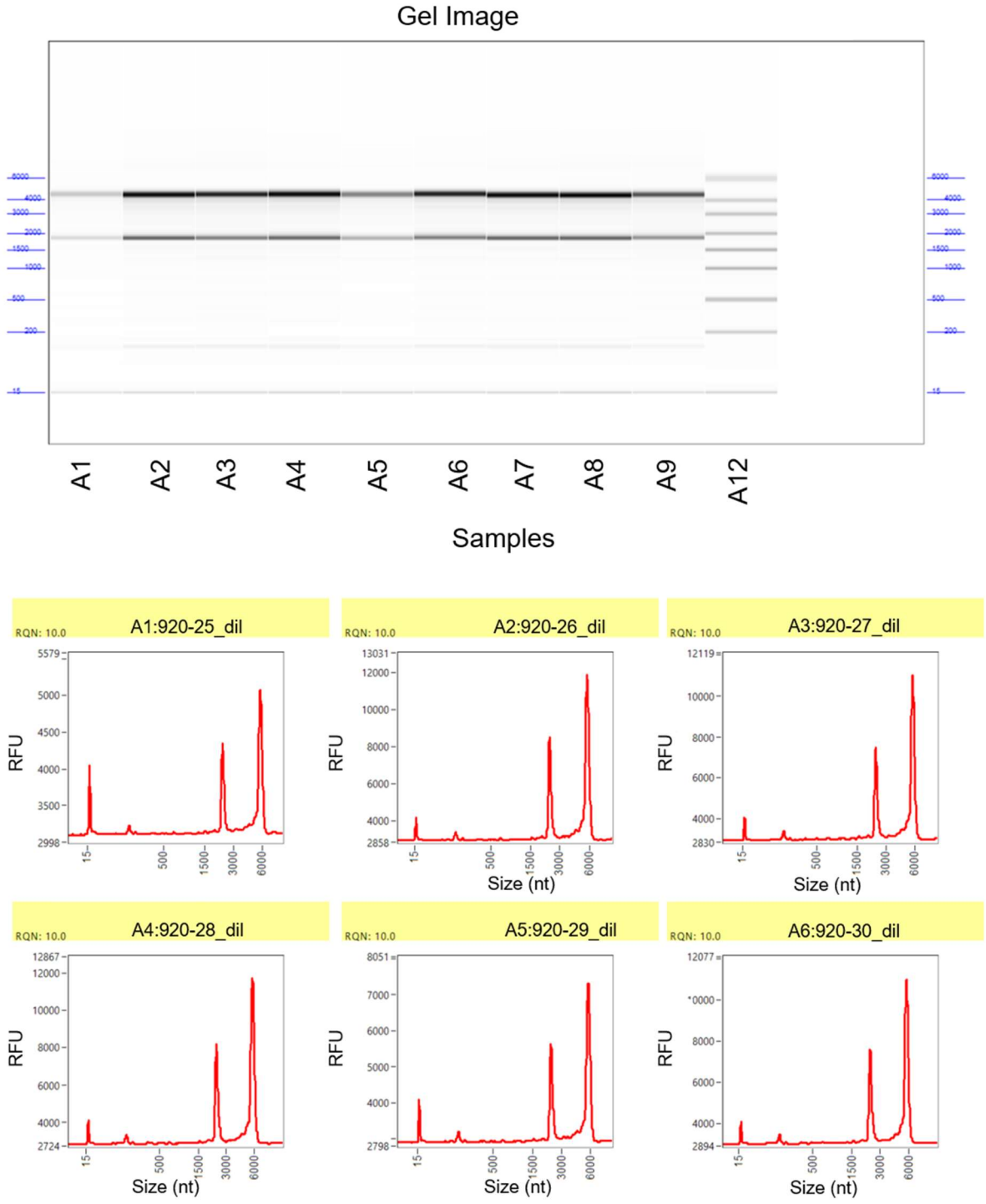

Figure 20: Exemplary quality control results of mRNA isolates.

Exemplary quality control results of mRNA isolated from murine BM neutrophils are shown. The length of 18S and 28S mRNA is displayed in nucleotides [nt], while 18S is about 2000nt in size and 28S about 4000nt. The amount of both ribosomes is calculated based on their fluorescence [RFU].

3.2.5.1. Ingenuity Pathway Analysis (IPA) of RNA sequencing Data

Core analysis of RNA sequencing data on fastq files was done with CLC Genomics Workbench (version 23.0.4, Qiagen, Venlo. NL). Reads were UMI (Unique Molecular Identifier) filtered and all remaining reads of all probes were adapter trimmed and quality trimmed (using the default parameters: bases below Q13 were trimmed from the end of the reads, ambiguous nucleotides maximal 2). Mapping of the data was done against *Mus musculus* (mm39; GRCm39.107) (July 20, 2022) genome sequence. The samples were grouped for each biological replicate according to their respective experimental condition and subsequently Statistical differential expression was determined using the Differential expression for RNA-Seq tool (version 2.7). P-values, resulted from this analysis, were then corrected for multiple testing by FDR and Bonferroni-correction. A listing of significantly altered transcripts (fold change ≥ 1.5, $p \leq 0.05$) of murine BM neutrophils after different treatments for four hours was provided online by the BMFZ. The Gene Set Enrichment Test (version 1.3) was done with default parameters and based on the GO term “biological process” (*M. musculus*; October 13, 2022) (information of method provided by BMFZ, Medical Faculty, Heinrich-Heine-University).
Core analysis of RNA sequencing data was done to identify significantly altered canonical pathways using the IPA software (Ingenuity Pathway Analysis, Quiagen) and significantly altered transcripts using Qlucore Omics Explorer.

3.2.6. Neutrophil extracellular trap formation assay

Neutrophil extracellular trap formation assay was performed to analyse the effect of different polarizers on the formation of NETs. Therefore, murine BM neutrophils were isolated as described in 3.2.1. Afterwards, 150000 neutrophils per well (100 µl) were seeded in a 96-well plate in polarization medium without FCS, as FCS is known to inhibit NETosis. Neutrophils were then treated with the different polarizers IL-4 (20 ng/ml), insulin (10 ng/ml), LPS (10 ng/ml) & IFN-γ (2 ng/ml) and additionally 100 ng/ml PMA for 4 hours at 37°C. After polarization, a standard series of 2 µg/ml, 1 µg/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml and a blank was added to the 96-well plate. Then, 50 µl Quant-iT™ PicoGreen®dsDNA reagent in a dilution of 1:100 was added to each well and incubated for 10 minutes at 37°C. Quant-iT™ PicoGreen®dsDNA reagent is able to quantitate dsDNA in solution, as it is a very sensitive fluorescent nucleic acid stain. After incubation, the fluorescence is measured by using a fluorescence microplate reader and standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nm).

3.2.7. Phagocytosis Assay

The phagocytosis capacity of murine BM neutrophils was measured by flow cytometry using fluorescently labelled (FITC) *Staphylococcus aureus (S. aureus)*. Murine BM neutrophils were isolated and 150000 neutrophils per well (100 µl) were seeded in polarization medium in a 96-well plate. Neutrophils were then treated with the polarizer IL-4 (20 ng/ml) or were left in media only as a control for 4 hours at 37°C. Afterwards, neutrophils were incubated for 15 minutes with fluorescently labelled *S. aureus* at a concentration of MOI 10. Thereafter, neutrophils were collected and washed with PBS. The fluorescence intensity of each cell was then measured by flow cytometry.

3.2.8. Degranulation Assay

Degranulation assay was performed to analyse the effect of different treatments on degranulation of neutrophils. In order to assess this, murine BM neutrophils were isolated and treated with IL-4 (20 ng/ml) or LPS (10 ng/ml)/ IFN-γ (2 ng/ml) for 4 hours at 37°C and 5% CO_2. Additionally, it was of interest to analyse if IL-4 treatment is able to reduce degranulation of neutrophils. Therefore, neutrophils were left untreated or prestimulated with IL-4 for one hour and than LPS/ IFN-γ was added for three more hours. After incubation, neutrophils were collected and analysed by flow cytometry. To analyse degranulation, CD11b (BV), CD18 (APC) and CD35/CD21 (PE) antibodies were used. It is known that strongly activated neutrophils express high levels of CD11b and CD18. CD11b, also known as integrin α-M, is an adhesion molecule expressed on monocytes and granulocytes. CD11b forms a complex with CD18, also known as integrin β-2, which is expressed in leukocytes and binds to ICAM-1. The chemotactic stimulation of neutrophils induces the translocation of these integrin's from the intracellular milieu to the cell surface. CD35 is a secretory vesicle and is used as a marker of exocytosis (Hughes, Hollers et al. 1992, Deree, Lall et al. 2006).

3.2.9. Migration Assay

In order to assess the effect of different stimulations on migration capability of neutrophils, murine BM neutrophils were isolated and left untreated or treated with IL-4 (20 ng/ml) for 90 minutes at 37°C and 5% CO_2. Therefore, Corning Transwell Polycarbonat Culture Inserts were used. The lower compartments were filled with polarization media (with 10% FCS) containing 0.02 ng/µl CXCL1, which induces chemoattractive movement, or without CXCL1 to determine spontaneous migration, serving as a negative control. The upper compartments were seeded with 0.5 x 10^4

neutrophils per 150 µl and the corresponding treatment. After incubation, neutrophils were collected from the lower compartment, which are the neutrophils that migrated from the upper to the lower compartment. The number of migrated neutrophils was evaluated by flow cytometry. Each condition was run with technical duplicates and to allow comparison between experiments, the treatments were normalised to the mean positive control value (untreated + CXCL1).

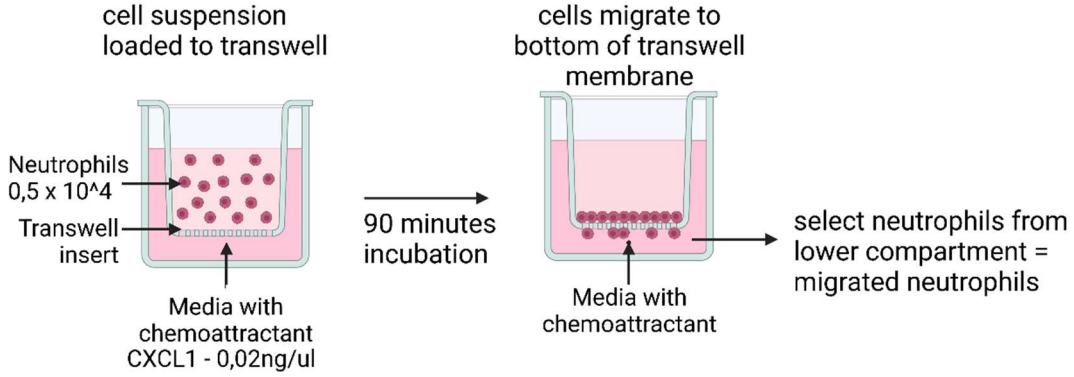

Figure 21: Experimental setting of migration assay.

0.5 x 10^4 neutrophils per 150 µl were seeded with the corresponding treatment in a transwell polycarbonate culture insert. The lower compartment (well) was filled with the chemoattractant CXCL1 (0.02 ng/µl) in polarization media. The plate was then incubated for 90 minutes at 37°C with 5% CO_2. Afterwards, neutrophils from the lower compartment were collected, which correlate with the migrated neutrophils, and cell count was determined by flow cytometry. Created with BioRender.com.

3.2.10. Cytokine Release Assay

The release of cytokines by neutrophils under different stimulations was analysed, using a Legendplex mouse cytokine release syndrome panel and mouse inflammation panel. With the combination of both panels, we were able to simultaneously detect various cytokines, such as IFN-γ, IL-10, CCL4 (MIP-1β), IFN-α, CXCL9 (MIG), CXCL10 (IP-10), TNF-α, IL-6, VEGF, IL-4, CCL3 (MIP-1α), CCL2 (MCP-1), IL-23, IL1-α, IL-12p70, IL-1ß, IL-27, IL-17A, IFN-ß and GM-CSF, using fluorescence-encoded beads, which can be analysed by flow cytometry.

Murine BM neutrophils of chow or DIO/STZ mice were isolated and left untreated or treated with IL-4, LPS/IFN-γ, LPS/IFN-γ + IL-4, LPS, LPS + IL-4, IFN-γ or IFN-γ + IL-4 for 4 hours. Afterwards, the cells were centrifuged and supernatant, containing the released cytokines, was stored at -20°C. 12.5 µl of the cell supernatant was incubated with 12.5 µl of assay buffer and 12.5 µl of antibody-immobilized beads for 2 hours at RT on a shaker in the dark. Afterwards, the plate was washed and centrifuged at 400 g for 5 minutes, using a swinging bucket rotor with microplate adaptor. Next, 12.5 µl of detection antibody

was added and incubated for one hour at RT on a shaker in the dark. After incubation, 12.5 µl of SA-PE was added and incubated for 30 minutes at room temperature on a shaker in the dark. Then, the plate was washed and centrifuged at 400g for 5 minutes twice. Finally, 150 µL of 1x wash buffer was added to each well and the samples were measured by flow cytometry. The data was then analysed using BioLegend`s Legendplex™ Data Analysis Software.

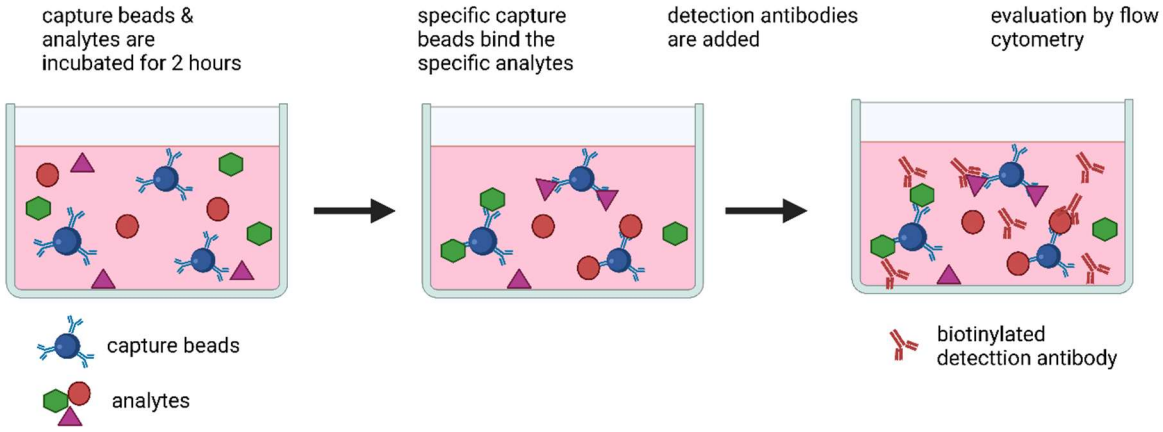

Figure 22: Assay procedure of cytokine release assay.

Antibody-immobilised capture beads are incubated with the analytes and subsequently incubated with the detection beads. The samples are then evaluated by flow cytometry (Modified after Biolegend). Created with BioRender.com.

3.3. *In vivo* methods

During all *in vivo* experiments, mice were under anaesthesia, using isoflurane inhalation at a flow rate of 1-3 % (v/w) (isoflurane vaporizer, *VetEquip*). Mice were placed on a warm pad (temperature set to 40-42°C) to keep the core temperature at 37.5°C. Body temperature of mice was monitored during all experiments with the aid of a thermometer, heating platform and heater lamp. Electrocardiography (ECG) was recorded during the complete surgery.

3.3.1. *In vivo* Myocardial Infarction

In order to access the surgery spot, mice were shaved (*Contura*) and hair remover lotion (*Veet*) was used. 2% isoflurane was used for anaesthesia, then mice were intubated and ventilated with oxygen-enriched gas (40% oxygen) with the help of a minivent microventilator. Buprenorphine (0.1 mg/kg body weight, subcutaneously (s.c.)) was given for analgesia. Then, thoracotomy was executed, the pericardium was dissected and a 7-0 surgical prolene suture

was conducted underneath the LAD coronary artery 1 mm from the tip of the left atrium, tightening of the snare induced myocardial ischaemia. Ischaemia was confirmed by blanching of the myocardium and a ST-elevation in ECG. Mice received a bolus of IGF-1 (40 ng/g) or vehicle (0.1% BSA) after 43 minutes of ischaemia and reperfusion was induced after 45 minutes. The suture was removed and the chest closed. Subsequently, micro-osmotic minipumps (Alzet, 1003D) were implanted s.c. to administer IGF-1 or BSA (as a control) (1 µg/g/day) for a maximum of three days. Mice obtained buprenorphine (0.1 mg/kg s.c.) every 4 hours and in drinking water (0.009 mg/mL) over night for 3 days for analgesia. This operation was executed by Dr. Rianne Nederlof.

3.3.2. Implantation of osmotic mini pumps

Osmotic mini pumps are used for a continuous administration of an ingredient over a defined time period. According to the manufacturer, pumps were filled 12-16 hours before implanting and were stored overnight in PBS at 37°C. Before implanting the pumps, mice were anaesthetised and placed on a warm pad to keep the body temperature at 37.5°C. A small incision was made in the neck and the pumps were inserted subcutaneously. Afterwards, the skin was sewed and the scar was treated with an antiseptic (Betaisodona). This operation was executed by Dr. Rianne Nederlof.

3.3.3. Single Cell Sequencing

For the purpose of reducing the influence of daily variability, on each experimental day, one control (BSA) and one IGF-1 treated animal was prepared. Mice were exposed to either BSA or IGF-1 for a maximum of 3 days, starting at reperfusion. Cardiac cells, except cardiomyocytes, were isolated one, three and seven days after an induced MI. Therefore, the murine heart was removed, the aorta cannulated and perfused with PBS, heparin and Actinomycin D, to avoid blood contamination. In order to achieve a single-cell suspension, a retrograde perfusion with digestion buffer (collagenase I (450 U/mL), DNAse I (60 U/mL), Actinomycin D in HBSS) was performed. Subsequently, the atrium was discarded and the heart was cut into ~1 mm^3 pieces by a tissue chopper. For the purpose of digestion, these small tissue pieces were again incubated in digestion buffer for 30 minutes at 37°C and were dissolved by pipetting 12 times using a 10 mL serological pipet after 10 and 20-minute incubation, and 30 times using a 1 mL pipette at the end of digestion. To remove cardiomyocytes, the digested cell suspension was filtered through a 100-micron cell strainer and centrifuged for 1 minute at 50 g at 4 °C. Again, the supernatant was filtered through a 40-

micron cell strainer and was centrifuged for 10 minutes at 300 g at 4 °C. The pellet was then dissolved in Facs buffer and incubated for 10 minutes with Fc-Block, which ensures antigen-specific binding. To obtain information of all different cell types on the different days after myocardial infarction, we sorted leukocytes ($CD45^+$), endothelial cells ($CD45^-CD31^+$) and all other cells ($CD45^-CD31^-$) in the ratio 2:1:2. Additionally, to differentiate the conditions, in this case IGF-1 and control mice, different TotalSeq hashtag antibodies were added and incubated for 15 minutes. Again, cells were washed and propidium iodid was added to identify dead cells. Ensuing, living and single cells were sorted by FACS (MoFlo XDP, Beckman-Coulter). After sorting, an equal amount of cells from each sample was combined and quality of cells was reviewed with trypan blue staining and visual inspection. If the cells were of good quality, single-cell RNA sequencing was started, which was performed in cooperation with the BMFZ (Dr. Tobias Lautwein).

For the generation of the single-cell droplet libraries a total of ~16.000 cells per sample were applied as an input on the 10X Chromium Controler system using the Chromium Single Cell 3' NextGEM Reagent Kit v3.1 (10X Genomics, Pleasanton, CA, USA), according to manufacturer's instructions. Sequencing was executed on a NextSeq 550 system (Illumina Inc. San Diego, USA) with a mean sequencing depth of ~50.000 reads/ cell.

The raw sequencing data was analysed with the 10X Genomics CellRanger software (v3.1) and raw BCL-files were demultiplexed and processed to Fastq-files using the CellRanger *mkfastq* pipeline. In order to generate a gene-barcode matrix, reads were aligned to the mm10 genome and UMI counting was carried out by the CellRanger *count* pipeline. All samples were combined and normalized for sequencing depth using the cellranger *aggr* pipeline.

The Seurat v3.2 R package was used for further analysis (Butler, Hoffman et al. 2018, Stuart, Butler et al. 2019, Hao, Hao et al. 2021). Cells with less than 200 detected genes and genes expressed in less than 3 cells were removed from the analysis. Additionally, dead or damaged cells were identified, by a mapping rate of > 10% to the mitochondrial genome, and removed. Seurat was also used for demultiplexing, based on cell labelling with hashtagging antibodies. DoubletFinder v2.0 was used to remove cell doublets from the dataset and SC Transform was utilized for normalization (McGinnis, Murrow et al. 2019). Principal Component Analysis (PCA), based on identified variable genes and subsequent UMAP embedding, was applied for the dimensional reduction of the data set. Again, Seurat was used to cluster cells, according to its graph-based clustering approach, and Wilcoxon Rank Sum test was utilized to calculate markers, determining each cluster as well as differential gene expression between different clusters. Subsequent to the analysis of all cells, only leukocytes, endothelial cells and

fibroblasts were identified and an individual reclustering analysis, with either cell type, was executed.

3.3.4. *In vivo* glucose and insulin tolerance test

In vivo glucose tolerance test was performed to analyse how fast exogenous glucose was cleared from the blood following glucose injection. Insulin tolerance test was performed to examine insulin sensitivity, by measuring how fast endogenous glucose was cleared after insulin injection.

Mice were starved for 5 hours and baseline blood glucose levels were measured from tail vein with the help of a blood glucose monitor. Subsequently, mice received i.p. injections of D-glucose (2g/kg body weight) or of Insulin (0.75 U/kg body weight). Blood glucose levels were then measured after 15, 30, 60 and 120 minutes.

3.4. Statistics

All data are presented as mean ± standard deviation (SD) and statistical analysis, except for RNA transcript expression sequencing and sc-RNAseq, was performed using SigmaPlot 13.

Parametric student *t*-test (Brown Forsythe) was used to compare between two groups. Parametric one-way ANOVA with Bonferroni`s Multiple Comparison post-test was used to compare more than two experimental groups. If an apparent non-gaussian distribution was observed, normal distribution was tested by Shapiro Wilk Test and if a non-gaussian distribution was confirmed, a non-parametric t-test (Mann Whitney Rank Sum Test) or one-way ANOVA (Kruskal-Wallis, Dunn`s Method) was used. Repeated measurements were analysed by two-way ANOVA followed by Bonferroni post test and data were considered statistically significant for p values ≤ 0.05 (* $p < 0.05$*, ** $p < 0.01$, *** $p < 0.001$).

4. Results

4.1. Polarization of neutrophils: “N1” versus “N2”

Based on the finding that myeloid cells, to which belong neutrophils and macrophages, play an important role in myocardial remodelling after acute myocardial infarction, it was the aim of this work to analyse to what extent different treatments modulate neutrophil function *in vitro* and therefore could potentially positively affect outcome after MI or diseases as T2DM. I could show in previous experiments, as described in chapter 1.3.1, that there exist at least two neutrophil subtypes, characterised as a pro- and anti-inflammatory phenotype.

4.1.1. Further characterisation of neutrophil polarization

With the help of the RNA sequencing data, possible new N2 and N1 marker genes were identified. *Carbonic anhydrase 4 (Car4), Solute carrier family 28 member 3 (Slc28a3)* and *TAFA Chemokine Like Family Member 3* (*Tafa3)*, were among the most upregulated genes upon IL-4 treatment. These genes might be interesting novel N2 marker genes, which could be used in future experiments and thus were analysed by qPCR to confirm these results (Figure 23). *IL-6* and *IL1ß* were analysed as possible novel N1 marker genes, as they are as well known to be involved in diseases as T2DM and MI (Figure 23). Therefore, murine BM neutrophils were isolated and polarized for 4 hours with IL-4 or LPS/IFN-γ or left untreated with media only as a control. Anti- and pro-inflammatory genes were next analysed by qPCR analysis. This analysis confirmed the significant upregulation of the N2-markers *Car4* (by 59 fold)*, Slc28a3* (by 17 fold) and *Fam19a3* (by 22.7 fold) upon IL-4 stimulation but not upon LPS/IFN-γ stimulation (Figure 23). As expected, *IL-6* (by 18.2 fold) and *IL-1ß* (by 21.5 fold) were significantly upregulated upon LPS/IFN-γ but not upon IL-4 treatment (Figure 23).

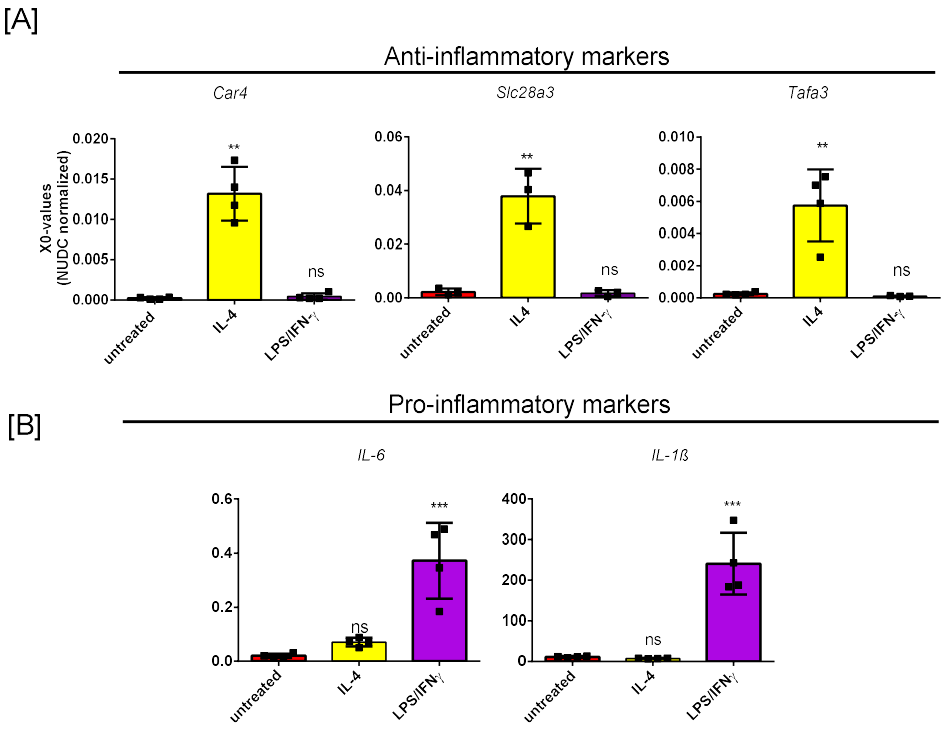

Figure 23: LPS/IFN-γ induces a pro-inflammatory phenotype, whereas IL-4 induces an anti-inflammatory phenotype.

Murine BM neutrophils were harvested untreated, or treated with LPS (10 ng/ml) + IFN-γ (2 ng/ml) or IL-4 (20 ng/ml) after a polarization time of 4 hours and qPCR analysis was performed. IL-4 treatment induced upregulation of anti-inflammatory genes *Car4*, *Slc28a3* and *Tafa3* **[A]**, whereas LPS/IFN-γ treatment induced upregulation of pro-inflammatory genes *IL-6* and *IL1β* **[B]**. X0 values are normalized to *Nudc*. Expression of single measurements for n=4 experiments are shown. Bars represent mean ± SD. Statistical significance between two groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated). In parts also published in (Nederlof, Reidel et al. 2022).

After verifying that neutrophils polarize towards a pro- and anti-inflammatory phenotype, it was of interest to analyse if an additional pro-inflammatory stimulus is able to reduce N2 polarization, and vice versa, if an anti-inflammatory stimulus alleviates polarization towards a pro-inflammatory phenotype. In addition, only LPS and IFN-γ treatment was used to identify if single treatment augments upregulation of pro-inflammatory genes (Figure 24).

As shown in Figure 24 [A], LPS and IFN-γ treatment did not alter the basal expression level of the N2-marker gene *Retnla.* However, LPS attenuated the IL-4-induced upregulation of *Retnla* by 1.7 fold, whereas IFN-γ reduced *Retnla* expression by 50.8 fold. This reduction was similar to the effect of the combined treatment of LPS and IFN-γ (by 63.6 fold). Thus, IFN-γ is a strong antagonist of the IL-4-induced N2 polarization. In line, as shown in Figure 24 [B], IL-4 treatment did not change the basal expression level of the N1 marker gene *Tnfa*. However, LPS alone induced a significant upregulation of

Tnfa expression by 20.7 fold, which was similar to the effect of the combined treatment of LPS and IFN-γ. In contrast, IFN-γ alone did induce only a minor upregulation of the N1 marker *Tnfa* (by 2.4 fold). Interestingly, IL-4 treatment attenuated the LPS-induced upregulation of *Tnfa* by 1.4 fold. Despite the minor upregulation of *Tnfa* expression upon IFN-γ treatment, IL-4 still attenuated this upregulation by 1.2 fold (Figure 24 [B]). Further, LPS alone, but not IFN-γ alone, was able to slightly induce *Nos2* expression, but to a lesser extent than the combination of LPS and IFN-γ (Figure 24 [B]). IL-4 treatment was not able to significantly reduce LPS-induced *Nos2* expression (Figure 24 [B]). Thus, IL-4 is partly an antagonist of LPS/IFN-γ-induced N1 polarization, however not as strong.

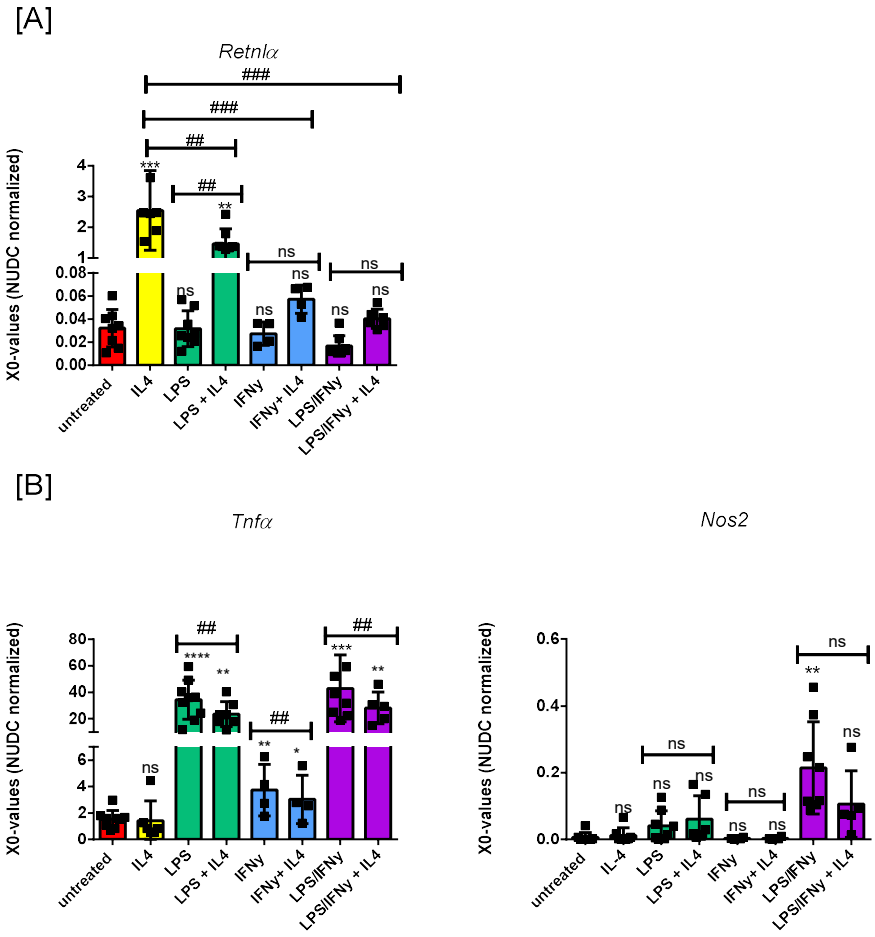

Figure 24: LPS/IFN-γ inhibits N2 polarization, whereas IL-4 is able to only slightly reduce *Tnfα* expression.

Murine BM neutrophils were left untreated, or treated with IL-4, LPS/IFN-γ, LPS or IFN-γ only or plus IL-4 for a polarization time of 4 hours and qPCR analysis was performed. LPS/IFN-γ, LPS or IFN-γ treatment only reduced upregulation of anti-inflammatory gene *Retnlα* **[A]**, whereas IL-4 treatment induced a slight reduction of pro-inflammatory gene *Tnfα* but not of *Nos2* **[B]**. X0 values were normalized to *Nudc*. Expression of single measurements for n=4-8 experiments are shown. Bars represent mean ± SD. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated; #p<0.05, ##p<0.01, ###p<0.001 when compared to the same treatment with additional treatment).

Furthermore, it is well documented that men have an increased risk for several diseases, as cardiovascular disease and stroke, but also for infectious diseases, as SARS-CoV-2 (Bots, Peters et al. 2017, Takahashi, Ellingson et al. 2020). As there is rising evidence, that biological sex seems to be a contributing factor to the severity and progression of several diseases, it was of interest to analyse if the biological sex has an impact on neutrophil polarization and therefore would be an important consideration for following experiments and the development of possible therapies. Thus, neutrophils of male and female mice were isolated and left untreated or treated with IL-4 or LPS/IFN-γ for a polarization time of four hours and subsequently analysed for pro-inflammatory (*Nos2, IL-6*) and anti-inflammatory (*Retnlα, Arg1*) marker genes (Figure 25).

This analysis revealed that sex had no effect on upregulation of N2 marker genes *Retnlα* and *Arg1* (Figure 25 [A]). Interestingly, N1 marker genes *Nos2* (by 2.9 fold) and *IL-6* (by 2.1 fold) were less upregulated in neutrophils of female mice (Figure 25 [B]), thus, indicating sex-specific differences in neutrophil polarization. Due to these differences, I continued to work with only male mice in the following experiments.

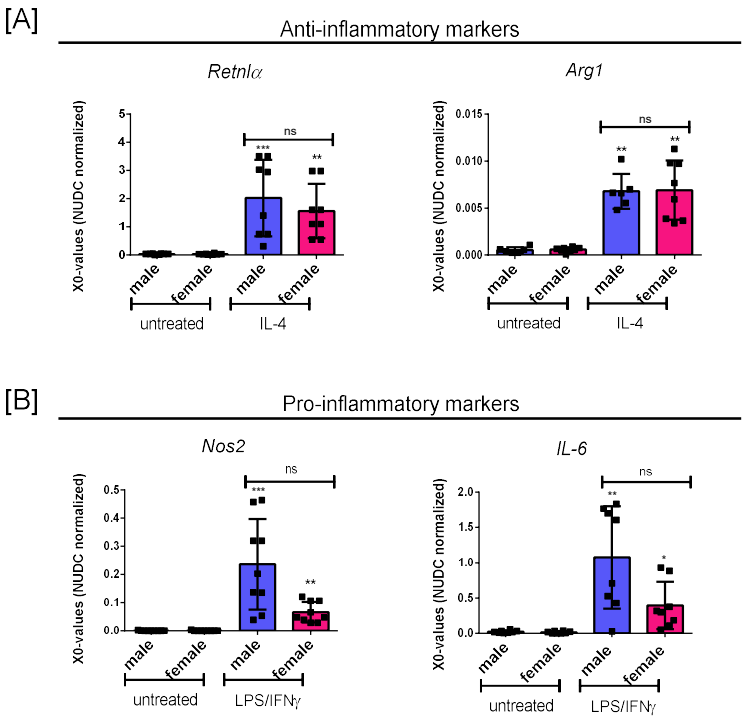

Figure 25: Female mice show reduced N1-polarization capacity.

Female mice upregulated anti-inflammatory genes *Retnlα* and *Arg1,* upon IL-4 treatment, to the same extent as male mice **[A]**, whereas female mice upregulated pro-inflammatory genes *Nos2* and *IL-6*, upon LPS/IFN-γ treatment, to a lesser extent than male mice **[B].** X0 values were normalized to *Nudc*. Expression of single measurements for n=8 experiments are shown. Bars represent mean ± SD. Statistical normal distribution was tested by Shapiro Wilko test. Statistical significance was calculated using non-parametric One-way ANOVA (Kruskal-Wallis One-way ANOVA, Dunn`s method) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated of the same sex; #p<0.05, ##p<0.01, ###p<0.001 when compared to the same treatment of different sex).

4.2. Signalling pathways induced by N1- & N2- polarization

4.2.1. The AKT pathway

Based on the finding that neutrophils polarized to an anti- and pro-inflammatory phenotype, it was of interest to analyse possible signalling pathways responsible for polarization. Known IL-4-dependent signalling pathways are the AKT, Ras/Raf/ERK and JAK-STAT pathways. As described in chapter 1.4, binding of IL-4 to its receptor leads to activation of different JAKs, which in turn activate STAT or IRS. Activated IRS induces other IL-4 signalling pathways, as PI3K/AKT signalling and mTOR signalling (Nelms, Keegan et al. 1999, Junttila 2018). Thus, at first we took a closer look at the canonical AKT signalling pathway. The intracellular PI3K/AKT signalling pathway plays a crucial role in several cellular processes, such as metabolism, proliferation, cell growth, and dysregulation of the AKT signalling is also implicated in several diseases (Hixon, Muro-Cacho et al. 2000, Lawlor and Alessi 2001, Ackah, Yu et al. 2005, Tucka, Bennett et al. 2012). The AKT1 isoform is broadly expressed, is crucial for growth and is the most important isoform in the heart (Cho, Thorvaldsen et al. 2001). The AKT2 isoform plays an important role in metabolic regulation, especially glucose homeostasis (Cho, Mu et al. 2001). Therefore, murine BM neutrophils of global ΔAKT1 and ΔAKT2 knockout mice, and additionally of Tie2-Cre AKT1$^{FL/FL}$ and Tie2-Cre AKT2 $^{FL/FL}$ mice were analysed. It was reported, that in Tie2-Cre mice deletion occurs not only in endothelial cells, but also in the haematopoietic lineage, including myeloid-, B- and T-cells (Tang, Harrington et al. 2010).

Knock-out efficiency in neutrophils was analysed on transcriptional (Figure 26 [A, B]), as well as translational level (Figure 26 [C]). The analysis of the knock-out efficiency of neutrophils of global ΔAKT1, ΔAKT2, Tie2-Cre AKT1$^{FL/FL}$ and Tie2-Cre AKT2$^{FL/FL}$ mice showed an absence of AKT1 in neutrophils of global ΔAKT1 and Tie2-Cre AKT1$^{FL/FL}$ mice and an absence of AKT2 in neutrophils of global ΔAKT2 and Tie2-Cre AKT2$^{FL/FL}$ mice on transcriptional, as well as translational levels.

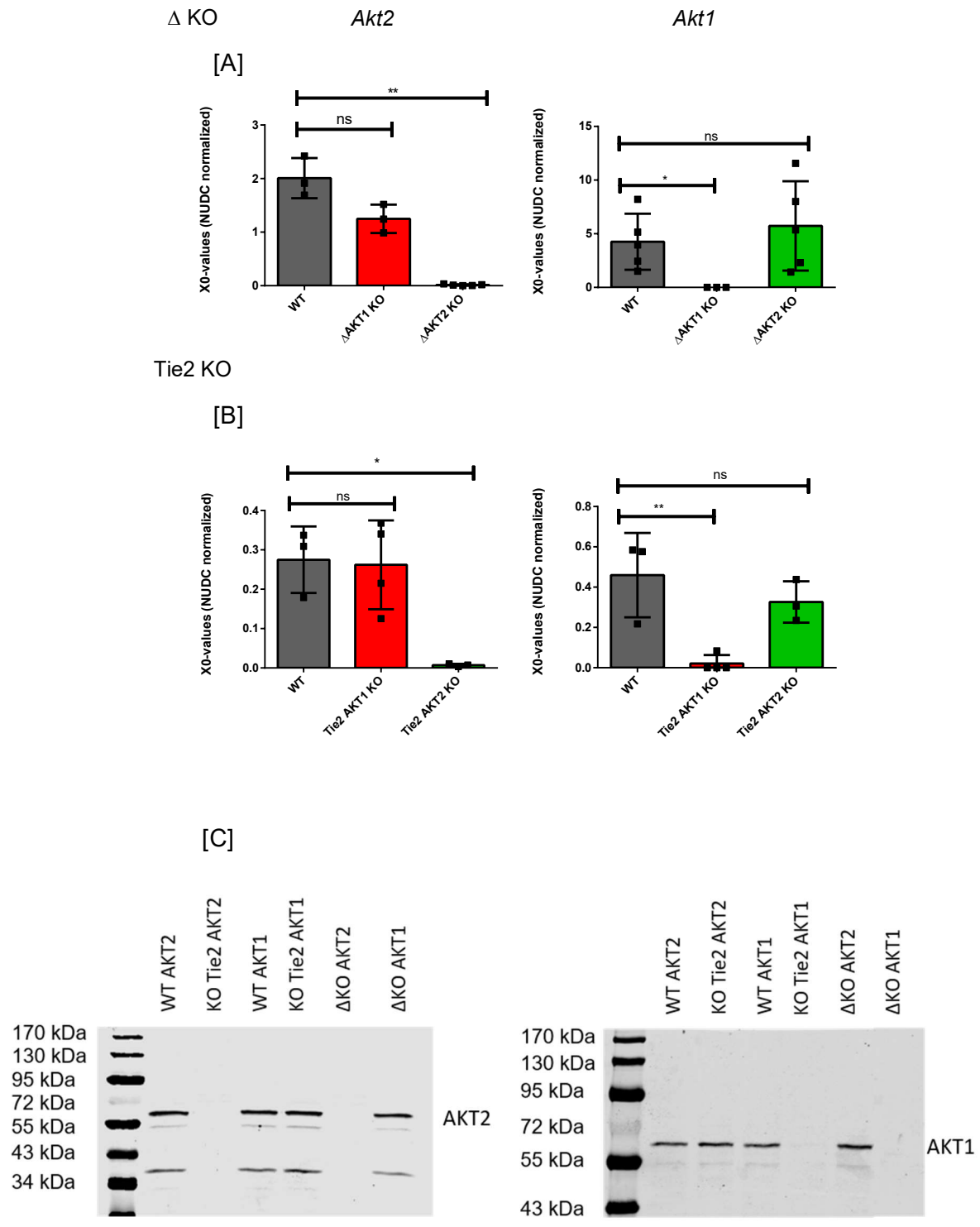

Figure 26: KO efficiency of neutrophils of ΔAKT2, ΔAKT1, Tie2 AKT1 and Tie2 AKT2 KO mice.

AKT1 and AKT2 expression in ΔAKT2, ΔAKT1, Tie2-Cre AKT1$^{FL/FL}$ and Tie2-Cre AKT2$^{FL/FL}$ mice was assessed by qRT-PCR **[A, B]** and Western Blot analysis **[C]**. Total protein stain was used as loading control. Expression of single measurements for n=3-5 experiments are shown. Bars represent mean ± SD. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to WT).

Next, it was investigated to what extent deficiency of AKT1 or AKT2 affects neutrophil polarization. Therefore, neutrophils of ΔAKT1, Tie2-Cre AKT1$^{FL/FL}$, ΔAKT2 and Tie2-Cre AKT2$^{FL/FL}$ mice were polarized for 4 hours or 10 minutes with IL-4 or LPS/IFN-γ or were left untreated with media only as a control. Additionally, insulin treatment was analysed here, as the AKT pathway is a canonical signalling pathway of insulin. Subsequently, qPCR and western blot analysis were performed. *Nos2* was utilized as a pro-inflammatory marker gene, evincing a polarization towards the N1 neutrophil phenotype. *Arg1* and *Retnlα* served as anti-inflammatory marker genes, indicating a N2 neutrophil phenotype. The investigation of the effect of AKT1 or AKT2 deficiency in neutrophils on polarization capacity revealed an upregulation to normal extents of *Nos2* after LPS/IFN-γ treatment and of *Arg1* and *Retnlα* upon IL-4 treatment, in all, global ΔAKT1 (Figure 27 [A]), Tie2-Cre AKT1$^{FL/FL}$ (Figure 27 [B]), global ΔAKT2 (Figure 27 [C]) and Tie2-Cre AKT2$^{FL/FL}$ (Figure 27 [D]) mice. Again, insulin had no effect on neutrophil polarization. This indicates an independency of N1- and N2- polarization on the AKT1 and AKT2 isoforms.
Additionally, murine BM neutrophils of wildtype mice were harvested untreated or treated with LPS/IFN-γ, IL-4 or insulin after a polarization time of 10 minutes and western blot analysis was performed. Neither treatment induced phosphorylation of AKT in neutrophils (Figure 27 [E]). As a control, 3T3 cells were harvested untreated, or treated with insulin, after an incubation time of 10 minutes. Insulin treatment induced phosphorylation of AKT in 3T3 cells, indicating again that neutrophil polarization seems to be independent of AKT1 and AKT2 (Figure 27 [E]).

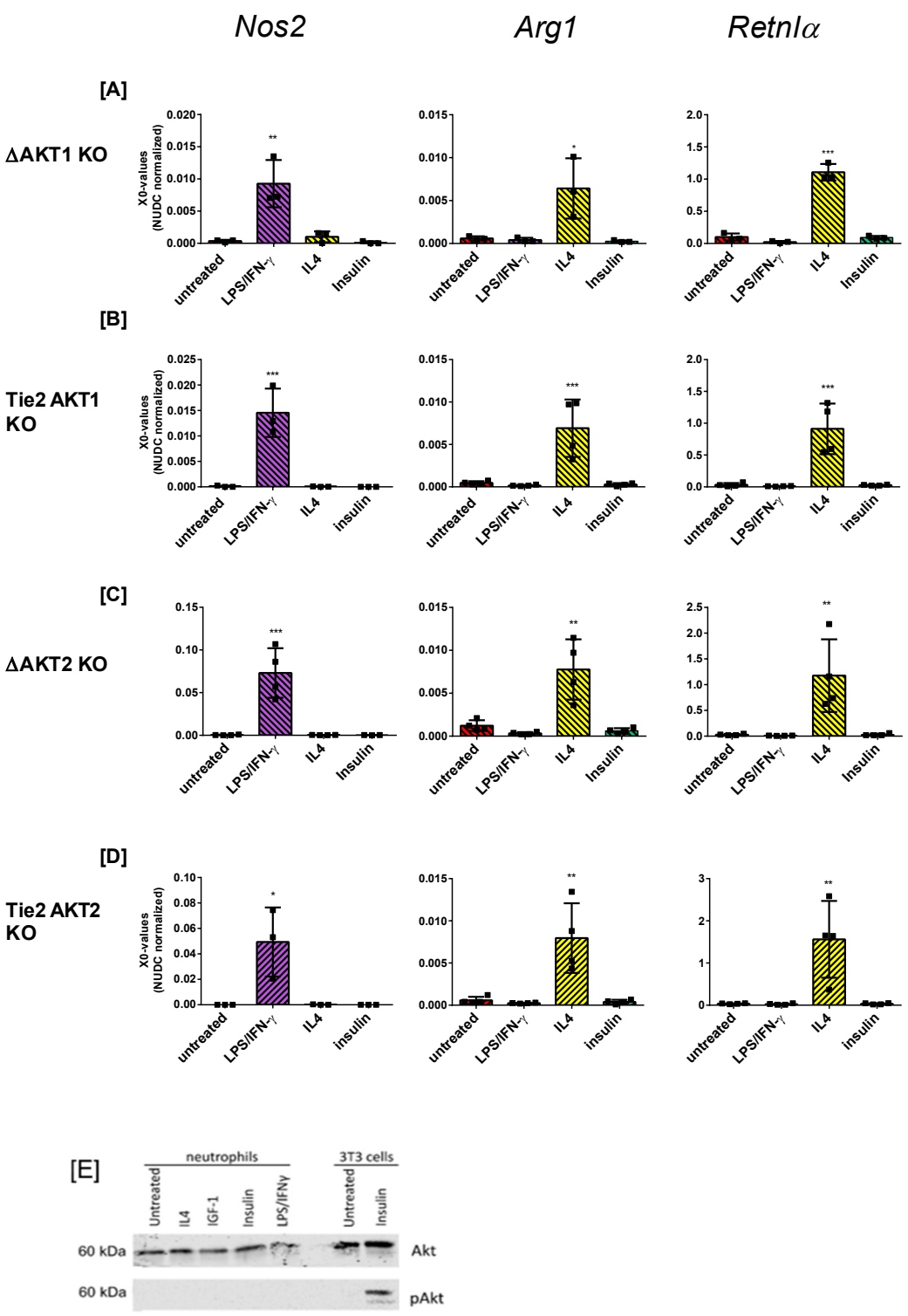

Figure 27: The effect of AKT deficiency in neutrophils on N1- and N2-polarization.

N1 and N2 polarization was not affected in ΔAKT1 **[A],** Tie2-Cre AKT1 $^{FL/FL}$ **[B],** ΔAKT2 **[C]** and Tie2-Cre AKT2 $^{FL/FL}$ mice **[D]**. Additionally, neither treatment induced phosphorylation of AKT in neutrophils. Insulin treatment induced phosphorylation of AKT in 3T3 cells **[E].** X0 values are normalized to *Nudc*. Expression of single measurements are shown. Bars represent mean ± SD. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated). Experiments were stopped after n=3/4 due to clear effects. In parts also published in (Nederlof, Reidel et al. 2022).

4.2.2. The RAS/RAF/ERK pathway

As the AKT pathway was excluded to be responsible for N1- and N2- neutrophil polarization, next the RAS/RAF/ERK pathway was assessed. This pathway is an important regulator of a number of cellular processes, as cell survival, differentiation, proliferation, growth, metabolism and migration (Tamemoto, Kadowaki et al. 1992, Dikic, Schlessinger et al. 1994). Furthermore, the RAS/RAF/ERK pathway was reported to be activated by IL-4, as well as LPS and IFN-γ signalling (Palsson-McDermott and O'Neill 2004, O'Donnell, Henkins et al. 2015, Shi, Song et al. 2021). Therefore, the activation of ERK was analysed in murine BM neutrophils after stimulation with LPS/IFN-γ, IL-4 or insulin for 10 minutes (Figure 28). Neither LPS/IFN-γ treatment, nor IL-4 treatment induced phosphorylation of ERK. However, insulin, which does not induce an anti- or a pro-inflammatory phenotype, induced phosphorylation of ERK. Therefore, neutrophil polarization towards an anti- and pro-inflammatory phenotype is independent of the activation of the RAS/RAF/ERK pathway.

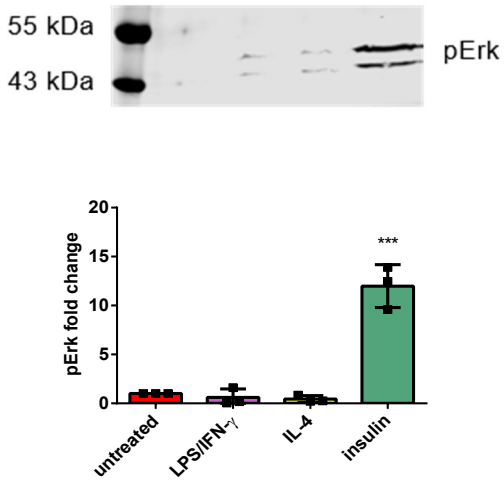

Figure 28: N1- and N2-polarization is independent of the activation of ERK.

Murine BM neutrophils were either left untreated or treated with LPS (10 ng/ml) + IFN-γ (2 ng/ml), IL-4 (20 ng/ml) or insulin (10 ng/ml). After a 10-minute treatment, western blot analysis was performed. Insulin, but not LPS/IFN-γ and IL-4 treatment, induced phosphorylation of ERK. Total protein stain was used as loading control. Values were normalised to control, which was set equal to 1. Expression of single measurements for n=3 experiments are shown. Bars represent mean ± SD. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (***p<0.001). In parts also published in (Nederlof, Reidel et al. 2022).

4.2.3. The JAK/STAT pathway

4.2.3.1. Neutrophil polarization is dependent on JAK activation

In the previous experiments, it was shown that the AKT and Ras/Raf/ERK pathways are not responsible for N1- and N2- polarization. Thus, the next step was to analyse the JAK/STAT signalling pathway. The JAK/STAT pathway is an essential mediator for many cellular mechanism as for cell survival, differentiation, proliferation, migration and apoptosis (Ghoreschi, Laurence et al. 2009). The Janus kinases (JAKs) are intracellular non-receptor tyrosine kinases and comprise four different JAKs, namely JAK1, JAK2, JAK3 and Tyk2. JAK is known to be an important signalling molecule of IL-4 and IFN-γ signalling in macrophages, as its activation results in the phosphorylation and activation of PI3K and STAT (Reichel, Nelson et al. 1997, Nelms, Keegan et al. 1999, Liang, Tang et al. 2017, He, Gao et al. 2020, Runtsch, Angiari et al. 2022). In order to analyse to what extent the different JAK isoenzymes contribute to neutrophil polarization, neutrophils were polarized with IL-4 or LPS/IFN-γ in the presence or absence of the individual JAK inhibitors as listed in Table 13. Non-polarized cells served as control. Since the JAK inhibitors show isoform preferences, this approach should lead to identification of the JAK isoform(s) mediating N1/N2 polarization. InSolution™ JAK Inhibitor I (CAS-457081-03-7) has a higher IC_{50} value for JAK1 than for JAK2, JAK3 or Tyk2, whereas Ruxolitinib has a 150 times lower IC_{50} for JAK1/2 than for JAK3, and the JAK2 Inhibitor BMS-911543 has a low IC_{50} value for JAK2 (Table 13). Thus, high and low concentrations of JAK inhibitors should identify the JAK isoforms involved in polarization.

Table 13: IC_{50} values of the different JAK inhibitors used.

Inhibitor	IC50 values [nM]				Used High conc. [nM]	Used Low conc. [nM]	Inhibited at low conc.
	JAK1	JAK2	JAK3	Tyk2			
InSolution™	15	1.0	5	1	250	5	JAK2/3/Tyk2
Ruxolitinib	3.3	2.8	>390		2500	30	JAK1/2
BMS-911543	75	1.1	360	66		10	JAK2

This table shows the IC_{50} values and concentrations used of the different JAK inhibitors. The indicated high concentration inhibits all JAKs and Tyk2, whereas at the lower concentrations only the indicated JAKs are inhibited, which are shown in the last column.

Analysis of neutrophils treated with a high dose (250 nM) of InSolution™ JAK Inhibitor I, which inhibits all JAKs and Tyk2, revealed a substantial inhibition of LPS/IFN-γ-induced upregulation of *Nos2* and also prevented an IL-4-induced upregulation of *Arg1* and *Retnlα* (Figure 29 [A]). Thus, polarization towards an anti- and pro-inflammatory phenotype, respectively, is dependent on JAK activation. By using the low dose of the InSolution™ JAK Inhibitor I (5 nM), with only JAK1, and minimally JAK3, being active, *Nos2* upregulation (N1 polarization) was partially reduced (65%), but still significantly higher than untreated cells (Figure 29 [A]). Thus, JAK1, and maybe partially JAK3, appears to be involved in LPS/IFN-γ-induced upregulation of pro-inflammatory *Nos2.* Also, upregulation of IL-4-induced anti-inflammatory genes *Arg1* (73%) and *Retnlα* (61%) was significantly reduced, when only JAK1, and minimally JAK3, was active, but not completely inhibited, revealing a contribution of JAK1, and maybe partially JAK3, in an IL-4-induced anti-inflammatory phenotype (Figure 29 [A]). Next, the Ruxolitinib inhibitor was used to analyse the importance of JAK3 for N1- and N2- neutrophil polarization, because Ruxolitinib at the low concentration should block JAK activity except for JAK3 (Table 13). Again, using a high concentration (2.5 µM) of Ruxolitinib, which inhibits all the JAKs, showed substantial inhibition of N1- and N2- polarization (Figure 29 [B]). When only JAK3 was active, which was achieved by using a lower concentration (0.03 µM) of Ruxolitinib, LPS/IFN-γ as well as IL-4 treatment were not able to induce significant upregulation of pro- and anti-inflammatory genes (Figure 29 [B]). Therefore, JAK3 plays only a negligible role in N1- and N2- polarization. Lastly, a JAK2 inhibitor (BMS) was used to analyse dependency of neutrophil polarization on JAK2. This revealed, that in the absence of JAK2, a signifcant upregulation of N1- and N2- marker genes was prevented (Figure 29 [C]). Combining these data, one can assume that N1- and N2- polarization are mainly dependent on JAK2 activation, with some contribution of JAK1.

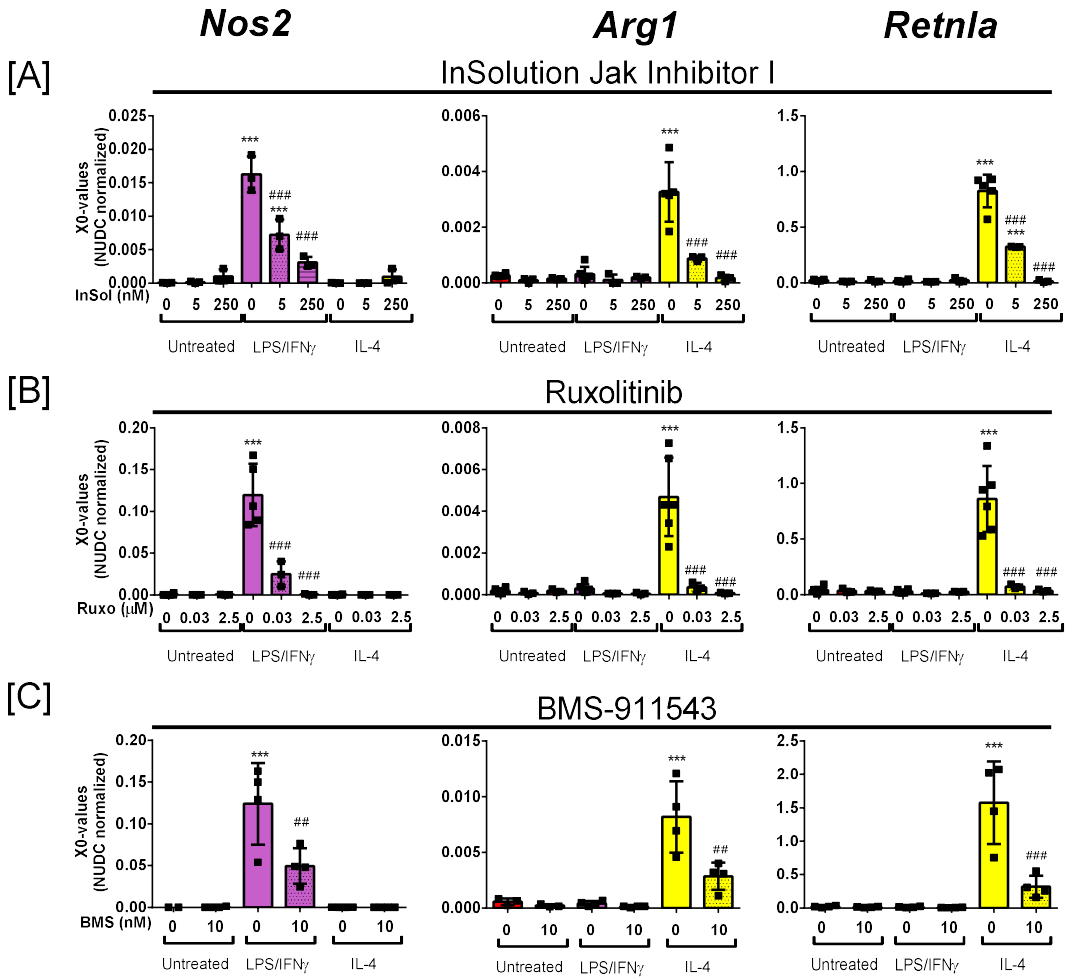

Figure 29: N1- and N2-polarization is dependent on the activation of Janus kinases. Murine BM neutrophils were harvested untreated or treated with LPS (10 ng/ml) + IFN-γ (2 ng/ml) or IL-4 (20 ng/ml) in the presence or absence of JAK inhibitors after a polarization time of 4 hours and qPCR analysis was performed. By using InSolution™ JAK Inhibitor I (InSol) **[A]**, Ruxolitinib (Ruxo) **[B]** or BMS-911543 (BMS) **[C]**, it was shown that N1- and N2- polarization is dependent on JAK activation. IC_{50} values of the different inhibitors are shown in Table 13. X0 values are normalized to *Nudc*. Expression of single measurements are shown for n=3-6 experiments. Bars represent mean ± SD. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001, when compared to untreated without inhibitor); ($^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001, when compared to the same treatment without inhibitor). In parts also published in (Nederlof, Reidel et al. 2022).

4.2.3.2. LPS/IFN-γ induces STAT1 phosphorylation, whereas IL-4 induces STAT6 phosphorylation

The STATs are transcription factors and seven different members are known, namely STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Bousoik and Aliabadi 2018). The activated JAKs phosphorylate and activate the STATs, which subsequently

disassociate from the receptor and translocate to the nucleus, where they induce gene expression (Argetsinger, Campbell et al. 1993, Schindler and Darnell 1995, Morris, Kershaw et al. 2018). Therefore, the effect of IL-4 and LPS/IFN-γ treatment on STAT1 and STAT6 activation in neutrophils was assessed by western blot analysis. LPS/IFN-γ, but not IL-4 treatment, induced phosphorylation of STAT1, whereas STAT6 was phosphorylated upon IL-4, but not upon LPS/IFN-γ treatment (Figure 30).

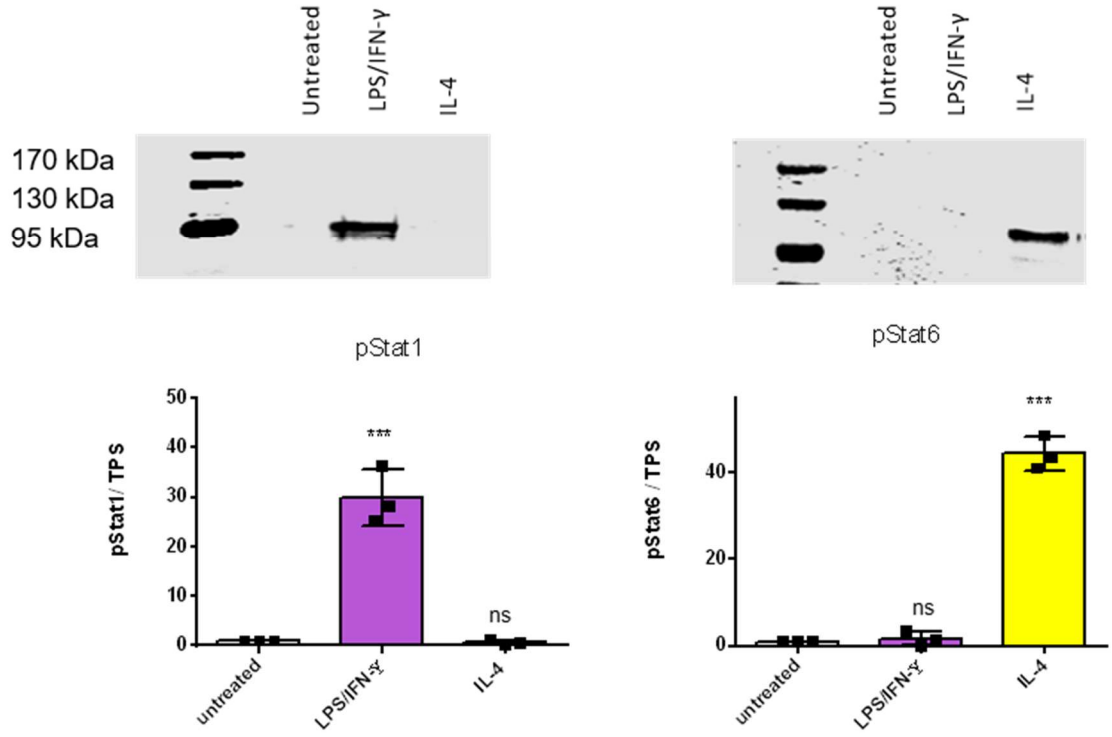

Figure 30: **LPS/IFN-γ induces STAT1 phosphorylation, whereas IL-4 induces STAT6 phosphorylation in neutrophils.**

Murine BM neutrophils were left untreated or treated with LPS (10 ng/ml) + IFN-γ (2 ng/ml), or IL-4 (20 ng/ml). After a polarization time of 10 minutes, western blot analysis was performed. LPS/IFN-γ, but not IL-4 treatment, induced phosphorylation of STAT1. Only IL-4 treatment induced phosphorylation of STAT6. Representative Western Blots for n=3 experiments are shown. Total protein stain was used as loading control. Values were normalised to control, which was set equal to 1. Expression of single measurements are shown. Bars represent mean ± SD. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated). In parts also published in (Nederlof, Reidel et al. 2022).

Additionally, the effect of JAK inhibition on STAT activation was analysed. Therefore, neutrophils were left either untreated or polarized for 10 minutes in the presence or absence of the different JAK inhibitors (Table 13) as before. With a high concentration of InSolution™ JAK Inhibitor I (250 nM) or Ruxolitinib (2.5 µM) all JAKs and Tyk2 were inhibited, leading to complete inhibition of both, LPS/IFN-γ and IL-4-induced, STAT

activation (Figure 31 [A], [B]). Mainly JAK1, and minimal JAK3, activity, which was achieved by using a low dose of InSolution™ JAK Inhibitor I (5 nM), was sufficient to induce STAT1 phosphorylation (Figure 31 [A]), however to a minor extent. Only JAK3 activity (low dose of Ruxolitinib) was not sufficient to induce STAT1 phosphorylation (Figure 31 [B]) and inhibition of JAK2 (BMS) had no significant effect on STAT1 phosphorylation (Figure 31 [C]). In contrast, only JAK1 (InSolution JAK Inhibitor) or JAK3 (Ruxolitinib) activity, did not induce significant phosphorylation of STAT6 (Figure 31 [A], [B]). JAK2 inhibition (BMS) prevented phosphorylation of STAT6 (Figure 31 [C]). Taken together, mainly JAK1 activity is necessary for high-level STAT1 activation leading to N1 polarization, whereas mainly JAK2 activity mediates STAT6 activation and thereby promotes the N2 phenotype.

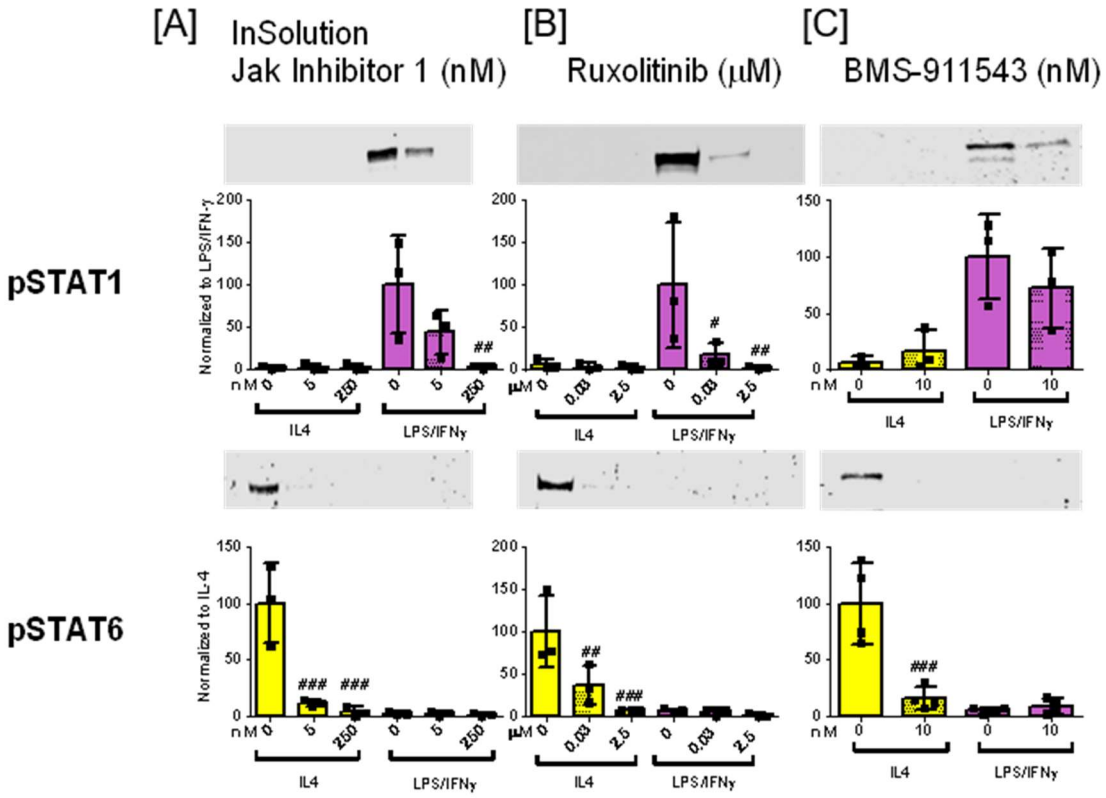

Figure 31: **LPS/IFN-γ induced STAT1 phosphorylation is JAK1 dependent, whereas IL-4 induced STAT6 phosphorylation is JAK2 dependent.**

Murine BM neutrophils were left untreated, or treated with LPS (10 ng/ml) + IFN-γ (2 ng/ml), or IL-4 (20 ng/ml) in the presence of different concentrations of InSolution JAK inhibitor **[A],** Ruxolitinib **[B]** or BMS-911543 **[C].** After a polarization time of 10 minutes, WB analysis was performed. Representative Western Blots of n=3-4 experiments are shown. Total protein stain was used as loading control. Mean signals for pSTAT1 LPS/IFN-γ without inhibitor and for pSTAT6 IL-4 without inhibitor were set to 100. Expression levels of single measurements are shown. Bars represent mean ± SD. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test); ($^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ when compared to the same treatment without inhibitor). In parts also published in (Nederlof, Reidel et al. 2022).

4.3. N1- & N2- polarization induces different neutrophil functions

During inflammation, neutrophils become activated and their longevity increases, thus the presence of neutrophils at the site of inflammation is ensured. Neutrophils play a central role in immune defence and can eliminate pathogens by different immune responses, as for example by neutrophil extracellular traps (NETs), phagocytosis or degranulation. Therefore, it was of interest if modulation of neutrophil phenotype also alters neutrophil functions and thereby being able to dampen inflammation.

4.3.1. N2-polarization is able to attenuate the formation of NETs

Upon activation, neutrophils release NETs (neutrophil extracellular traps), which are extracellular net-like structures composed of histones, granule proteins and chromatin (Brinkmann, Reichard et al. 2004). As IL-4 treatment induced upregulation of anti-inflammatory genes, the question arose if IL-4 treatment could also attenuate inflammatory functions of neutrophils or augment resolution of inflammation. Therefore, in a first step, representative fluorescence staining with Ly6G antibody (red), labelling neutrophils, and citrullinated histone H3 (C3) (green), which is a marker of NETs, was performed (Figure 32 [A]). Citrullination of histone H3 was reported to be essential for the formation of NETs and the binding of this modified histone to cell-free DNA was described as a hallmark of NETs (Li, Lin et al. 2020). Neutrophils were either left untreated or treated with N2-polarizer IL-4 or Phorbol 12-Myristat 13-Acetat (PMA), a known stimulator of NETosis (Hoppenbrouwers, Autar et al. 2017). Interestingly, the representative fluorescence imaging data showed a reduction of C3 signals upon IL-4 treatment and an increase of C3 signals upon PMA treatment, when compared to untreated neutrophils (Figure 32 [A]). Next, to get more quantitative measures of the effect of the different polarizers on the formation of NETs, neutrophils were treated for 4 hours with IL-4, LPS/IFN-γ or insulin. Additionally, neutrophils were treated with PMA, which served as a positive control. QuantIT PicoGreen was added and subsequently fluorescence was measured, indicating the amount of released dsDNA (Figure 32 ([B], [C]). This analysis revealed as well, that under basal conditions IL-4 treatment attenuated the formation of NETs (35.9%) when compared to untreated neutrophils (Figure 32 ([B]). PMA only, but also high concentrations of LPS (25 µg) + IFN-γ, significantly induced the formation of NETs when compared to untreated neutrophils (Figure 32 [B]). In contrast, Insulin treatment showed no effect on the formation of NETs, when compared to untreated neutrophils (Figure 32 [B]).

In order to assess, if N2- polarization by IL-4 is able to reduce inflammation, simultaneous treatment of the different polarizers with PMA (PMA + IL-4 or PMA + LPS/IFN-γ) was

analysed (Figure 32 [C]). Interestingly, IL-4 was also able to reduce PMA-induced NETosis, as simultaneous treatment of IL-4 plus PMA significantly reduced (43.8%) the formation of NETs when compared to only PMA. In contrast, additional LPS/IFN-γ treatment even slightly increased PMA-induced NETosis (Figure 32 [C]). Therefore, an anti-inflammatory stimulus is able to attenuate inflammatory functions of neutrophils.

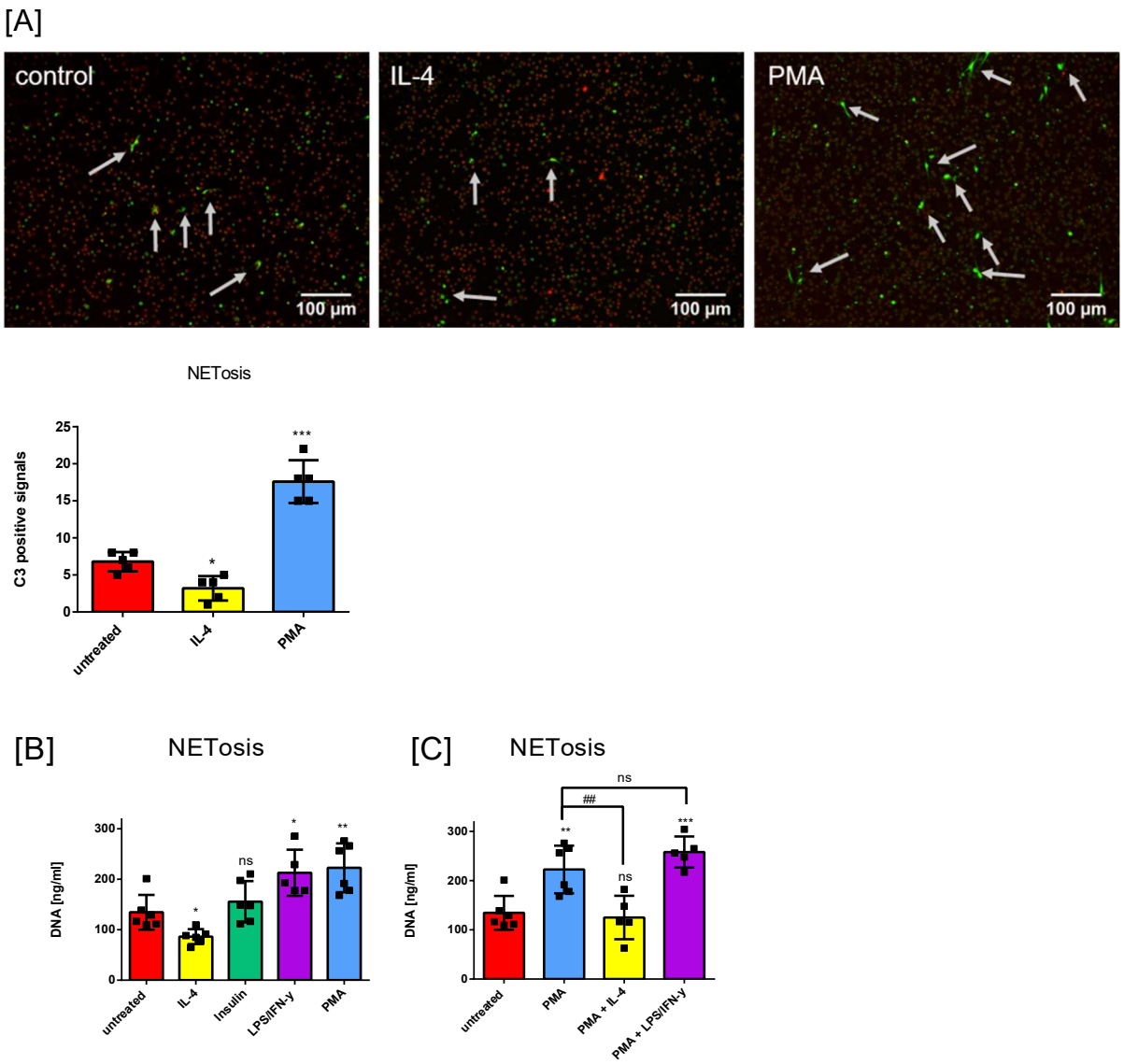

Figure 32: **IL-4 is able to attenuate the formation of NETs, whereas LPS/IFN-γ increases the formation of NETs.**

Murine BM neutrophils were stained untreated or after IL-4 or PMA treatment, with Ly6G (red), as a neutrophil marker, and C3 (green) as a NETosis marker **[A]**. Further, murine BM neutrophils were left untreated, or treated with different polarizers only **[B]** or additionally PMA (100 ng/ml) **[C]** for 4 hours. The untreated and PMA samples in [B] and [C] are the same. Samples are normalised to DNA standard concentrations. Bars represent mean ± SD, n=5-6 experiments are shown. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 when compared to PMA). PMA, Phorbol 12-Myristat 13-Acetat. In parts also published in (Nederlof, Reidel et al. 2022).

4.3.2. N2-polarization augments phagocytic capacity of neutrophils

As phagocytosis is an important defense mechanism of neutrophils and plays an important role in the resolution of inflammation, it was also of interest to analyse the effect of anti-inflammatory IL-4 on phagocytosis. Therefore, neutrophils were polarized for four hours with IL-4 or medium only as a control. Subsequently, neutrophils were challenged with fluorescently labelled *Staphylococcus aureus (S. aureus)* for 15 minutes, then phagocytosis was analysed by flow cytometry. As shown in Figure 33, IL-4 treatment significantly enhanced the phagocytic capacity of neutrophils by 45%, indicating that IL-4 treatment alters the function of neutrophils.

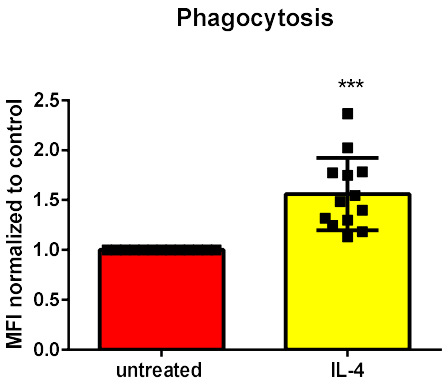

Figure 33: **IL-4 treatment increases phagocytic capacity of fluorescently labelled *S.aureus.***

Murine BM neutrophils were left untreated or treated with IL-4 for 4 hours and subsequently challenged with fluorescently labelled *S. aureus* for 15 minutes. Mean fluorescence intensity (MFI) of control is set to 1 and IL-4 is normalised to control. Bars represent mean ± SD, n=13 experiments are shown. Statistical significance between two groups was calculated using Mann Whitney Rank Sum test (*p<0.05, **p<0.01, ***p<0.001). In parts also published in (Nederlof, Reidel et al. 2022).

4.3.3. N2-polarization is not able to diminish neutrophil degranulation

Another immune mechanism of neutrophils is degranulation. Degranulation is necessary for killing of pathogens, and is involved in the immune response during (non-) infectious diseases (Mortaz, Alipoor et al. 2018). Neutrophils are densely packed with different kinds of granules, which they release upon stimulation and thereby induce inflammation. Regulating the process of degranulation could also be important in myocardial infarction healing. Neutrophils contain at least four different types of granules. Here, I analysed CD11b, a marker for tertiary granules which translocates from the intracellular milieu to the cell surface upon activation (Hughes, Hollers et al. 1992). I also took a closer look at the tertiary granule CD18, which forms a complex with CD11b.

At first, it was analysed if the isolation process activates neutrophil degranulation. This analysis revealed, that immediately after isolation, under basal conditions, neutrophils were activated, which is shown in an increased CD11b and CD18 surface expression. This stress level was reversed after an incubation period of 4 hours, as CD11b (by 43.3%) and CD18 (by 16.6%) surface expression were reduced, when compared to basal conditions.

As IL-4 treatment induced upregulation of anti-inflammatory genes, the question arose if IL-4 treatment could also attenuate degranulation. Therefore, neutrophils were treated with IL-4, LPS/IFN-γ or simultaneously with IL-4 plus LPS/IFN-γ. As shown in Figure 34, IL-4 treatment did not reduce CD11b or CD18 expression when compared to control neutrophils (untreated). In contrast, LPS/IFN-γ-treatment significantly enhanced CD11b and CD18 surface expression (by 49.2% and 13.4%, respectively). Additional IL-4 treatment was not able to diminish LPS/IFN-γ-induced degranulation of CD11b and CD18 (Figure 34).

Further, I analysed CD35, which is contained in secretory vesicles and is a marker of exocytosis (Hughes, Hollers et al. 1992, Deree, Lall et al. 2006). CD35 expression was not altered due to the isolation process (Figure 34). As shown in Figure 34, IL-4 treatment slightly increased CD35 expression (by 5.9%) when compared to control neutrophils (untreated). In contrast, LPS/IFN-γ-treatment did not alter CD35 surface expression and additional IL-4 treatment had as well no effect on CD35 surface expression (Figure 34).

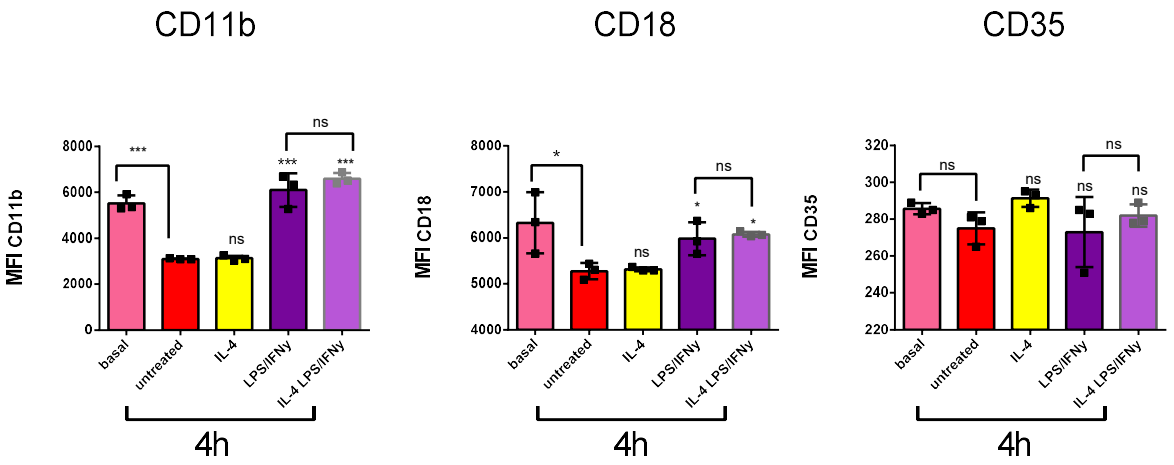

Figure 34: **IL-4 treatment is not able to reduce neutrophil degranulation, but LPS/IFN-γ augments neutrophil degranulation.**

Murine BM neutrophils were analysed immediately after isolation (basal) or were left untreated or treated with IL-4 or LPS/IFN-γ alone, or in combination, for 4 hours and subsequently analysed for CD11b, CD18 and CD35 expression by flow cytometry. Mean fluorescence intensity (MFI) is shown in the graphs. Bars represent mean ± SD, n=3 experiments are shown. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001).

4.3.4. N2-polarization has no effect on neutrophil migration

Neutrophils are the first cells to be recruited to the site of inflammation and build the first line of defense. Also in the context of myocardial infarction, neutrophils are the first cells to arrive at the infarcted area (Ma, Yabluchanskiy et al. 2016). The chemokine CXCL1 is an important modulator of neutrophil migration by binding to and activating the CXCR2 receptor on neutrophils (Girbl, Lenn et al. 2018). Thus, it was of interest to analyse whether N2-polarization modulates neutrophil migration. Therefore, neutrophils were left untreated or treated with IL-4 for 90 minutes and chemoattractive movement of neutrophils was induced by the chemokine CXCL1 (0.02 ng/µl) in a transwell assay. Samples without CXCL1 were used to determine spontaneous migration, serving as a negative control. The number of migrated neutrophils after stimulation was analysed by flow cytometry. As shown in Figure 35, IL-4 treatment did not alter the migration capacity of neutrophils.

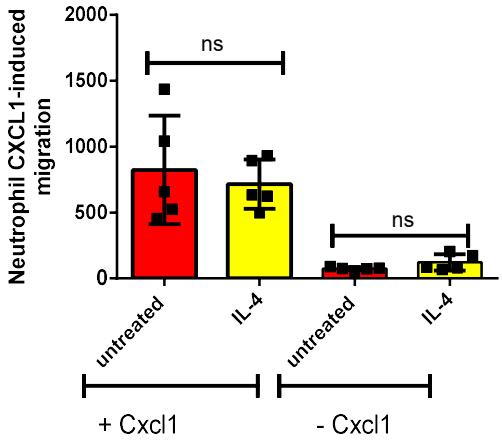

Figure 35: **IL-4 treatment has no effect on neutrophil migration.**

Murine BM neutrophils were seeded in the upper compartments in a transwell membrane and left untreated or treated with IL-4 for 90 minutes. The lower compartments were filled with polarization media with or without 0.02 ng/µl CXCL1, which induces chemoattractive movement. Number of migrated neutrophils of control (-CXCL1) and chemokine-induced (+CXCL1) neutrophils is shown. Bars represent mean ± SD, n=5 experiments are shown. Statistical significance was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001).

4.3.5. N1- & N2-polarization induce different cytokine secretion profiles

Cytokines, produced by neutrophils or other immune cells, are crucial regulators of inflammation, but also of repair processes. In general, there exist pro- and anti-inflammatory cytokines. Thus, the regulation of cytokine secretion by neutrophils is a crucial target in many diseases.

Hence, it was investigated which cytokines are secreted by anti-inflammatory neutrophils, induced by IL-4, or by pro-inflammatory neutrophils, induced by LPS/IFN-γ. As a combination of cytokines can induce different responses, the secretion profile of neutrophils treated with only LPS or only IFN-γ was as well analysed. Additionally, it was examined if anti-inflammatory IL-4 can dampen secretion of pro-inflammatory cytokines induced by LPS, IFN-γ or LPS/IFN-γ (Figure 36, Figure 37). Therefore, murine BM neutrophils were cultivated in media only as a control or polarized with LPS/IFN-γ ± IL-4, LPS ± IL-4, IFN-γ ± IL-4 or only IL-4 for 4 hours and subsequently, the supernatant was analysed for a set of cytokines by flow cytometry, using a legendplex assay. Measured cytokines and their predicted functions are shown in Table 14.

Table 14: Measured cytokines and their functions.

Cytokine	Function	Induced by
IFN-β	Pro-or anti-inflammatory (downregulation of inflammatory response)	IL-4
IL-27	Pro- or anti-inflammatory (suppressed production of inflammatory cytokines)	IL-4
GM-CSF	Pro-or anti-inflammatory	IL-4
IL-23	Production of inflammatory mediators	LPS/IFN-γ, IFN-γ
TNFα	Pro-inflammatory, cytotoxic	LPS/IFN-γ, LPS
IL-1β	Pro-inflammatory	LPS/IFN-γ, LPS
IL-17A	Pro-inflammatory, upstream of IL-6	LPS/IFN-γ, LPS
IL-6	Pro-inflammatory	LPS/IFN-γ
IL12p70	Pro-inflammatory	LPS/IFN-γ
CCL3	Recruitment and activation of leukocytes	LPS/IFN-γ, LPS
MCP-1/CCL2	Chemoattractant, recruitment of cells	LPS/IFN-γ
CCL4	Proliferation, migration	LPS/IFN-γ, LPS
CXCL9	Migration, differentiation	LPS/IFN-γ
CXCL10	chemotaxis, cell growth, mediation of angiostatic effects	LPS/IFN-γ
IFN-a	Anti-viral, activation of immune cells	LPS/IFN-γ, IFN-γ
IL-10	Anti-inflammatory	LPS/IFN-γ

This table lists the measured cytokines, their functions and by which stimulus they were induced.

At first, IL-4-induced cytokines were analysed (Figure 36 [A]). Treatment of neutrophils with IL-4 induced significant upregulation of IFNβ (1.53 fold), IL-27 (2837 fold), and GM-CSF (2.41 fold) (Figure 36 [A]). Combined (LPS/IFN-γ), as well as single treatment with LPS and IFN-γ treatment, had no effect on IFNβ and GM-CSF secretion. However, IFN-

γ treatment, but not LPS treatment, induced IL-27 secretion, but to a lesser extent than IL-4 treatment (Figure 36 [A]).

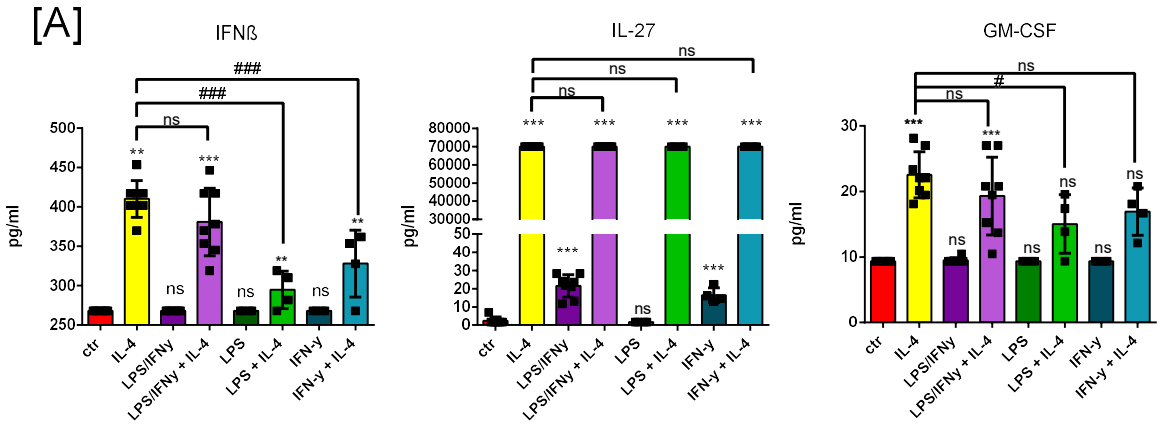

Figure 36: IL-4 induced cytokine secretion.

Murine BM neutrophils were left untreated or treated with LPS (10 ng/ml) + IFN-γ (2 ng/ml), LPS only, IFN-γ only, IL-4 only (20 ng/ml) or additional IL-4 for 4 hours and supernatant was analysed for different cytokines by flow cytometry, using a legendplex assay. IL-4-induced cytokines are shown **[A]**. Bars represent mean ± SD, n=4-8 experiments are shown. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001; #p<0.05 when compared to untreated, ##p<0.01 and ###p<0.001 when compared to the same treatment with an additional stimulus).

Next, LPS/IFN-γ-induced cytokines were analysed (Figure 37 [B]). Pro-inflammatory polarization, induced by combined treatment with LPS/IFN-γ, induced strong upregulation of pro-inflammatory cytokines IL-23 (2092 fold), TNFα (276.16 fold), IL-1ß (86.68 fold), IL-17A (38.77 fold), IL-6 (13.04 fold) and IL-12p70 (50.23 fold) (Figure 37 [B]). Sole treatment with only LPS induced significant release, but to a lesser extent when compared to combined LPS/IFN-γ treatment, of TNFα (105.4 fold), IL-1ß (32.68 fold) and IL-17A (25.39 fold) when compared to untreated neutrophils. Sole treatment with IFN-γ only induced significant release, but also to a lesser extent compared to combined LPS/IFN-γ treatment, of IL-23 (1936 fold). The cytokines IL-6 and IL-12p70 were induced only by combined treatment of LPS and IFN-γ. Further, an additional IL-4 treatment was not able to dampen pro-inflammatory cytokine secretion induced by LPS and IFN-γ alone or in combination (Figure 37 [B]).

As shown in Figure 37 [C], LPS/IFN-γ treatment as well upregulated cytokines mainly responsible for migration and chemotaxis. Combined treatment with LPS/IFN-γ induced CXCL10 (69.68 fold), CCL4 (53.42 fold), CCL3 (49.68 fold), MCP-1 (5.8 fold) and CXCL9 (5.8 fold) upregulation. Sole treatment with only LPS induced significant release, but to

a lesser extent when compared to combined LPS/IFN-γ treatment, of CCL3 (32.2 fold) and CCL4 (23 fold). Sole treatment with only IFN-γ was not sufficient to induce upregulation of these cytokines. Again, an additional IL-4 treatment was not able to reduce pro-inflammatory cytokine secretion induced by sole or combined treatment with LPS and IFN-γ (Figure 37 [C]).

Further, LPS/IFN-γ-induced upregulation of rather anti-inflammatory IL-10 (9.31 fold) and IFNα (2.6 fold) (Figure 37 [D]). Sole treatment with LPS did induce neither IFNα, nor IL-10 secretion. However, sole treatment with IFN-γ induced significant release, but also to a lesser extent compared to combined LPS/IFN-γ treatment, of IFNα (2 fold). Interestingly, an additional IL-4 treatment significantly enhanced IFN-γ-induced IFN-a secretion (Figure 37 [D]).

Thus, these data combined indicate, that different environments induce different cytokines. However, additional IL-4 treatment was not able to dampen pro-inflammatory cytokine secretion induced by LPS and IFN-γ.

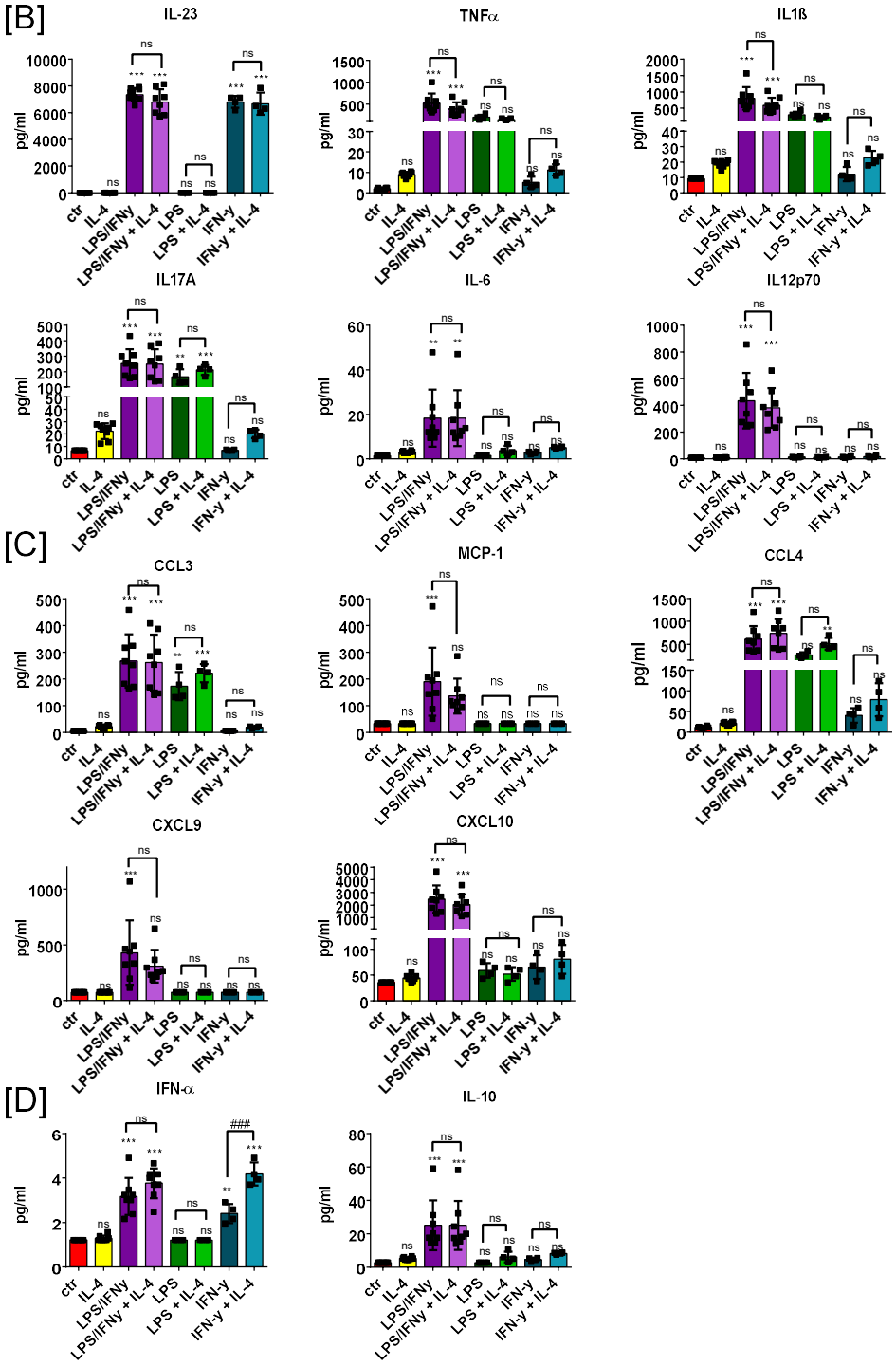

Figure 37: IL-4 treatment cannot reduce pro-inflammatory cytokine secretion.

Murine BM neutrophils were left untreated or treated with LPS (10 ng/ml) + IFN-γ (2 ng/ml), LPS only, IFN-γ only, IL-4 only (20 ng/ml) or additional IL-4 for 4 hours and supernatant was analysed for different cytokines by flow cytometry, using a legendplex assay. Cytokines were divided into LPS/IFN-γ-induced inflammatory cytokines **[B]**, LPS/IFN-γ-induced chemotaxis associated cytokines **[C]** and LPS/IFN-γ-induced anti-inflammatory cytokines **[D]**. Bars represent mean ± SD, n=4-8 experiments are shown. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001; #p<0.05 when compared to untreated, ##p<0.01 and ###p<0.001 when compared to the same treatment with an additional stimulus).

4.4. Neutrophils in the context of Myocardial Infarction

The immune system plays a crucial role in the repair processes after myocardial infarction, and neutrophils arrive early at the infarcted area (Carbone, Nencioni et al. 2013). Neutrophils were long thought to only negatively affect cardiac remodelling since they enhance inflammation (Soehnlein, Zernecke et al. 2008, Ma, Yabluchanskiy et al. 2013). However, there is now increasing evidence that neutrophils can also have beneficial effects on remodelling of the heart after MI (Serhan and Savill 2005, Horckmans, Ring et al. 2017).

In the context of myocardial infarction, human and animal studies demonstrated a cardioprotective effect of IGF-1 (Santini, Tsao et al. 2007, Heinen, Nederlof et al. 2019). Further, it was shown that this cardioprotective effect was mediated by myeloid cell lineages and that IGF-1 induced an anti-inflammatory macrophage phenotype (Heinen, Nederlof et al. 2019). To examine the effect of IGF-1 on neutrophil phenotype in the context of myocardial infarction *in vivo*, mice were exposed to 45 minutes of LAD coronary artery occlusion followed by reperfusion. Beginning with reperfusion, mice were treated with IGF-1 or BSA, which served as a control, for three days. After this time period, bone marrow and cardiac neutrophils were isolated and qPCR analysis was executed (Figure 38). Classical N1- and N2- marker genes were analysed to assess if the treatment with IGF-1, not only improves function after myocardial infarction (Heinen, Nederlof et al. 2019), but has also an effect on the bone marrow and cardiac neutrophil phenotype. For cardiac neutrophils, only the pro-inflammatory marker genes *Tnfa* and *IL12a* (Figure 38) were measured, due to limited material. For BM neutrophils, however, the N1 marker genes *Tnfa*, *Nos2*, *IL12a*, *IL1ß*, and the N2 marker genes *Arg1*, *Retnla* and *Car4* were analysed by qPCR (Figure 38). All analysed N1- and N2- marker genes showed no significant differences in BM neutrophils between IGF-1 and BSA treated mice. However, cardiac neutrophils showed a significant reduction of *Tnfα*, but not *IL12a*, after IGF-1 treatment when compared to BSA treated mice.

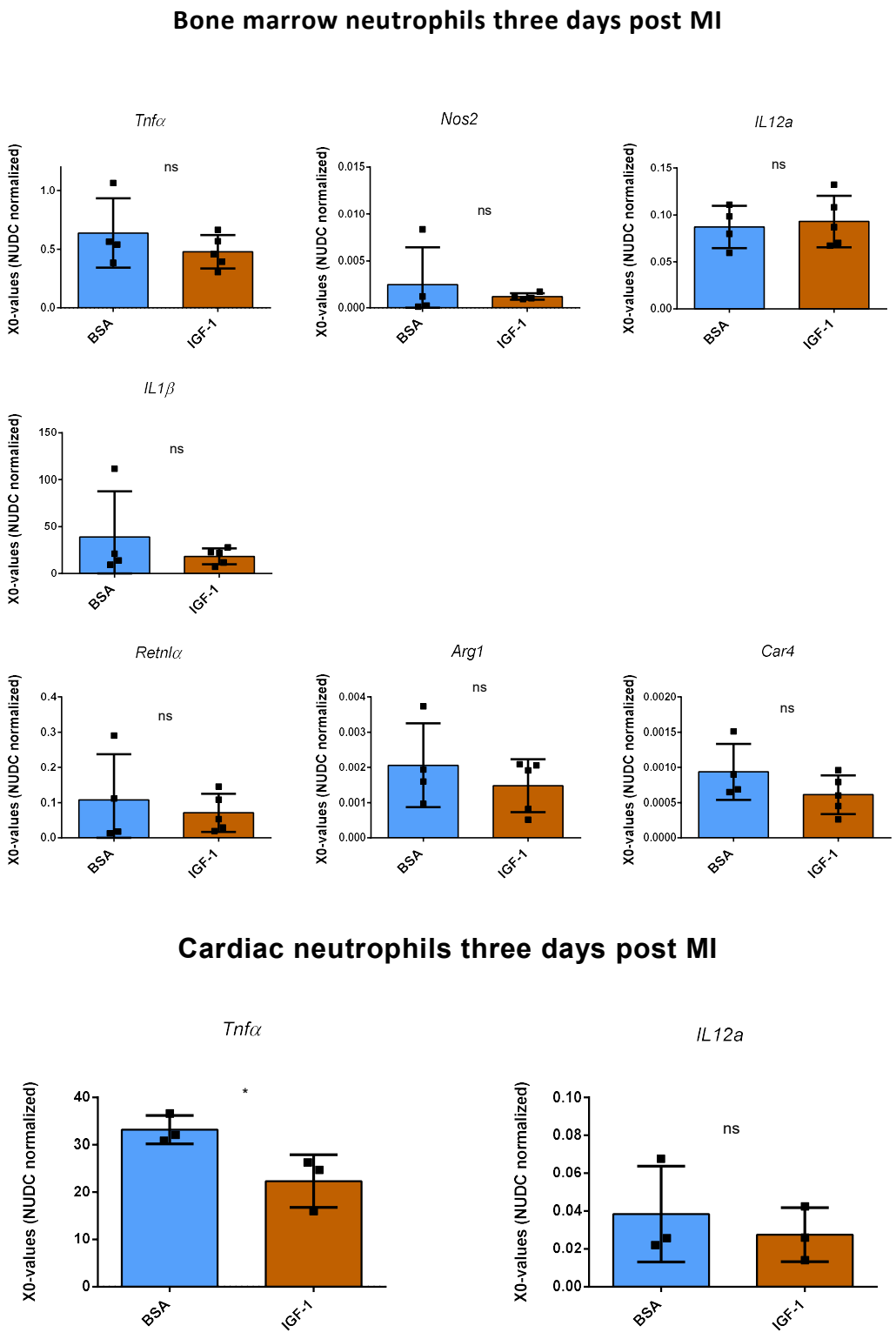

Figure 38: IGF-1 treatment after MI does not alter pro- or anti-inflammatory gene expression of BM neutrophils but reduces *Tnfα* expression of cardiac neutrophils.
Murine BM and cardiac neutrophils of mice, which received IGF-1 (1µg/g/day) or BSA as control via s.c. osmotic minipumps for three days post MI, were isolated and analysed for pro-inflammatory marker genes *Tnfα*, *Nos2*, *IL12a and IL1ß* and for anti-inflammatory marker genes *Retnlα, Arg1* and *Car4*. X0 values are normalized to *Nudc*. Expression of single measurements are shown, n=4/3 experiments are shown. Bars represent mean ± SD. Statistical significance between two groups was calculated using Mann Whitney Rank Sum test (*p<0.05, **p<0.01, ***p<0.001).

4.4.1. Characterisation of cardiac cells post MI

Since *Tnfα* expression appeared to be suppressed in cardiac neutrophils by IGF-1, further experiments were performed, because the number of independent experiments was quite low. To get a more detailed insight into the effect of IGF-1, the time course of immune cell infiltration and the cellular phenotypes, in particular of neutrophils, post MI were investigated by single-cell RNAseq. Again, mice were exposed to the standard 45 minutes of LAD coronary artery occlusion followed by reperfusion and were treated maximally for three days with IGF-1 or BSA, beginning at the start of reperfusion. On day one, three or seven post MI, hearts were isolated and enzymatically dissociated. All cardiac cells, except for cardiomyocytes, were used for single-cell RNA sequencing (scRNA-seq) (cooperation with the BMFZ of Heinrich-Heine-University, Dr. Tobias Lautwein). Since individual cell populations are present in the heart in substantially different numbers, with endothelial cells and fibroblasts exceeding other less frequent cell types (e.g. B-/T-cells) it was decided to fractionate the isolated cells into leukocytes ($CD45^+$), endothelial cells ($CD45^-CD31^+$) and other cells (mostly fibroblasts; $CD45^-CD31^-$). Then, all cells were mixed prior to scRNA-seq in a ratio 2:1:2 with a total of 10000 cells per analysis (Figure 39). This protocol minimizes the risk of losing a minor cell population of leukocytes and fibroblasts by increasing their numbers relative to abundant endothelial cells. However, this approach limits the quantitative assessment of cells to relative amounts within the three groups.

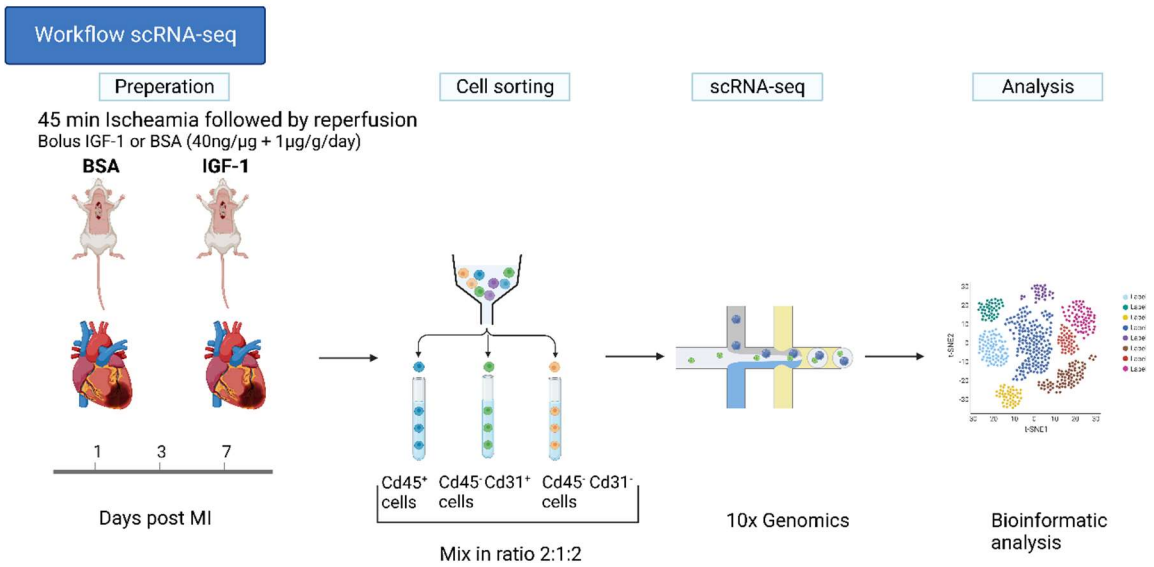

Figure 39: Workflow of scRNA-sequencing analysis.

Mice were exposed to 45 minutes of LAD coronary artery occlusion followed by reperfusion and were treated maximally for three days with IGF-1 or BSA, beginning at the start of reperfusion. One, three or seven days post MI, hearts were isolated and digested and all cardiac cells, except for cardiomyocytes, were used for single-cell RNA sequencing (scRNA-seq). Leukocytes ($CD45^+$), endothelial cells ($CD45^-CD31^+$) and all other cells ($CD45^-CD31^-$) were sorted and mixed in the ratio 2:1:2. Created with BioRender.com.

Following scRNA-seq, cardiac cells were clustered with low resolution, depicting 22 different clusters (Figure 40, UMAP). This analysis showed that neutrophils clearly differed from other cardiac cells, and also from other myeloid cells, as for example macrophages (Figure 40). In total 75685 transcripts were detected, 32509 transcripts derived from control mice and 43176 transcripts derived from IGF-1 treated mice. In a first step, identity of the observed cell clusters was determined by characteristic marker genes. Table 15 summarises all identified cell clusters with the common marker genes per cell type and also the differential marker genes per cluster, specifying subgroups. In summary, fibroblasts (Cluster 1, 3, 6, 8, 9, 16, 22), endothelial cells (Clusters 0, 7, 12, 20), neutrophils (Clusters 2, 15, 17, 18, 19), macrophages (Cluster 4, 5, 14, 21) including dendritic cells (DCs) (Cluster 13), B-cells (Cluster 11) and T-cells (Cluster 10) were identified (Chiba, Nakagawa et al. 1999, Medvedovic, Ebert et al. 2011, Wang, Wei et al. 2012, Lee, Wang et al. 2013, Sauteur, Krudewig et al. 2014, Cochain, Vafadarnejad et al. 2018, Muhl, Genove et al. 2020, Sprenkeler, Zandstra et al. 2022, Miranda, Janbandhu et al. 2023). Defined clusters and characteristic genes per defined cell types are depicted in Figure 40 [A] and [B].

Table 15: Summary of identified cell clusters of the heart post MI.

Cell type	Cluster	Common marker	Differential marker	Comment	Literature
Fibroblasts	1	*Pdgfra* *Col1a1* *Acta2* *Postn*	*Ly6a*	Sca1 low/high	(Cochain, Vafadarnejad et al. 2018, Muhl, Genove et al. 2020)
	3		*Ifit, Isg15, Rsad2*	Ifn stimulated	
	6		*Lgals1, Hspa5, Nme2*	Cancer associated?	
	8		*Mki67, Bub1, Mybl2*	Proliferating	
	9		*CD36, Fabp4*	SMC/Pericytic	
	16		*Wif1, Wnt5a, Dkk3*	Wnt expressing	
	22		*??*	??	
Endothelial cells	0	*Pecam1* *Cdh5*	*Sox17, Sema3, Hey1*	Arterial EC	(Chiba, Nakagawa et al. 1999, Sauteur, Krudewig et al. 2014)
	7		*Mki67, Bub1, Mybl2*	Proliferating EC	
	12		*Col3a1, Col1a1, S100a4*	Mesenchymal EC	
	20		*Vwf, Npr3, Cdh11*	Endocardial EC	
Macrophages	4	*Fcgr1* *CD68* *Mrc1* *Adgre1*	*Cxcr3, Siglec1, Timd4*	Tissue resident	(Cochain, Vafadarnejad et al. 2018)
	5		*Arg1, Ifit, Isg15, Rsad2*	Ifn stimulated/Arg1^{+}	
	13		*Cd209a, Itgax*	DCs	
	14		*Cxcr3, Mrc1, Siglec1*	Tissue resident	
	21		*H2-Aa, H2-Ab1*	Infiltrating (antigen presenting)	
Neutrophils	2	*S100a8* *S100a9* *Ly6g*	*Retnlg, Slpi, Wfdc17*	Young, blood derived	(Lee, Wang et al. 2013, Sprenkeler, Zandstra et al. 2022)
	15		*Ifit, Isg15, Rsad2*	Ifn stimulated	
	17		*Actc2, Tnnt, Myl3*	Cardiomyocyte expressing (phagocytosed)	
	18		*Tnf, Nfkbia, Dusp2*	Pro-inflammatory	
	19		*Pecam1, Cdh5, Sox17*	EC expressing (phagocytosed)	
B-cells	11	*Ly6d* *CD79* *Pax5* *CD19*			(Medvedovic, Ebert et al. 2011, Wang, Wei et al. 2012, Cochain, Vafadarnejad et al. 2018)
T-cells	10	*CD3*	*CD4*	T-helper cell	(Cochain, Vafadarnejad et al. 2018)
			CD8	Cytotoxic T-cell	
			Klrb1c/ Ncr1	Nk-cell	

This table lists the identified cell clusters with the common marker genes per cell type and the differential marker genes per cluster.

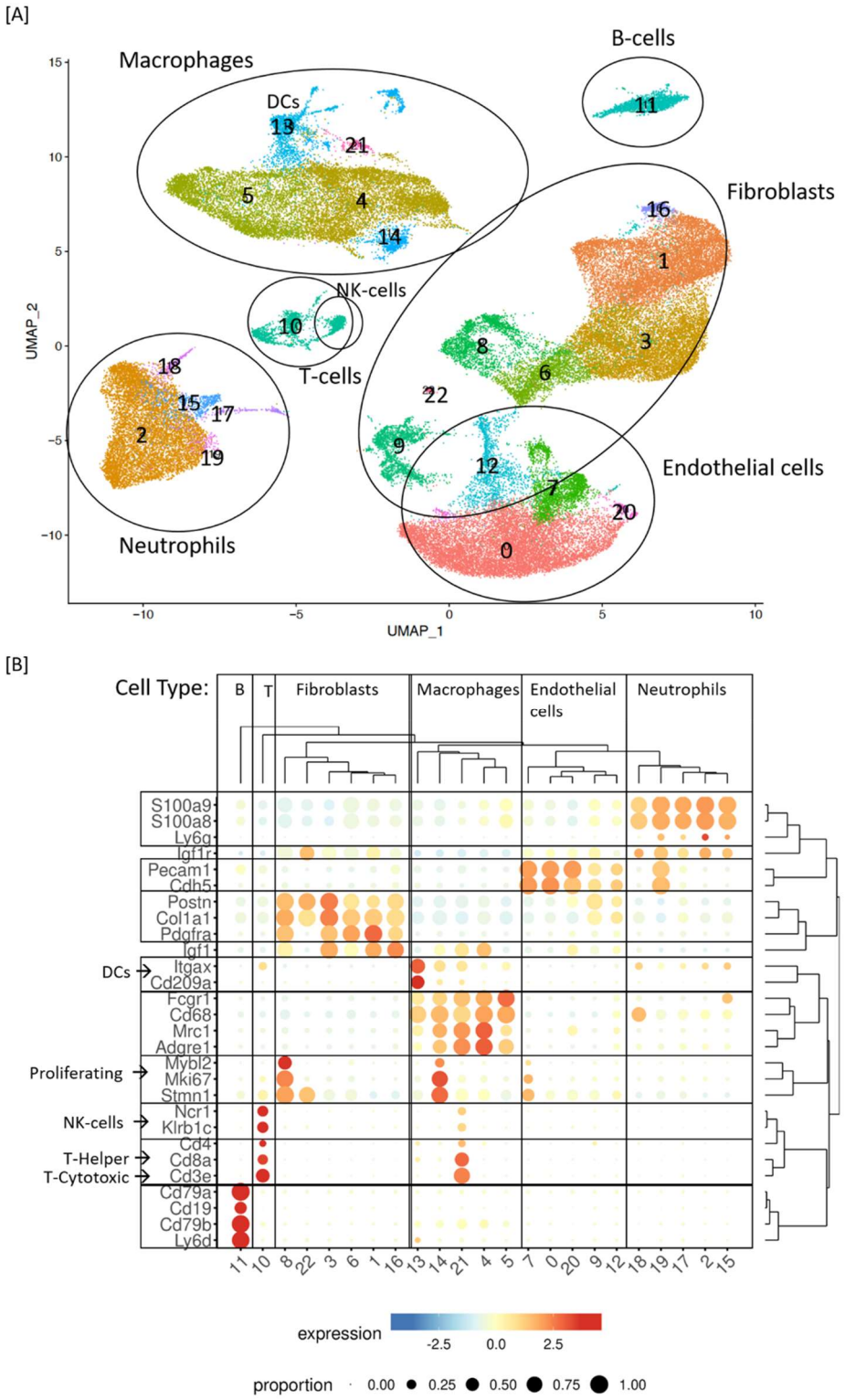

Figure 40: Characterisation of the identified cell clusters of hearts post MI.

Cardiac cells were sorted for scRNA-sequencing and in total, 22 different clusters were identified. Cardiac cells were divided into macrophages, dendritic cells, neutrophils, B-cells, T-cells, fibroblasts and endothelial cells **[A]**. Characterisation of each cell type with specific marker genes is visualised in a bubble plot **[B]**.

As the first clustering included cells from control and IGF-1 treated mice, it was next analysed if IGF-1 treatment creates specific “IGF-1 clusters”, by separating IGF-1 and control cells in UMAP plots (Figure 41). As shown in Figure 41, the analyses revealed no clear differences in the cell distribution over the clusters between control (blue) and IGF-1 (brown) treatment on each day, thus IGF-1 treatment created not specific “IGF-1 clusters”, as visualised in UMAP (Figure 41).

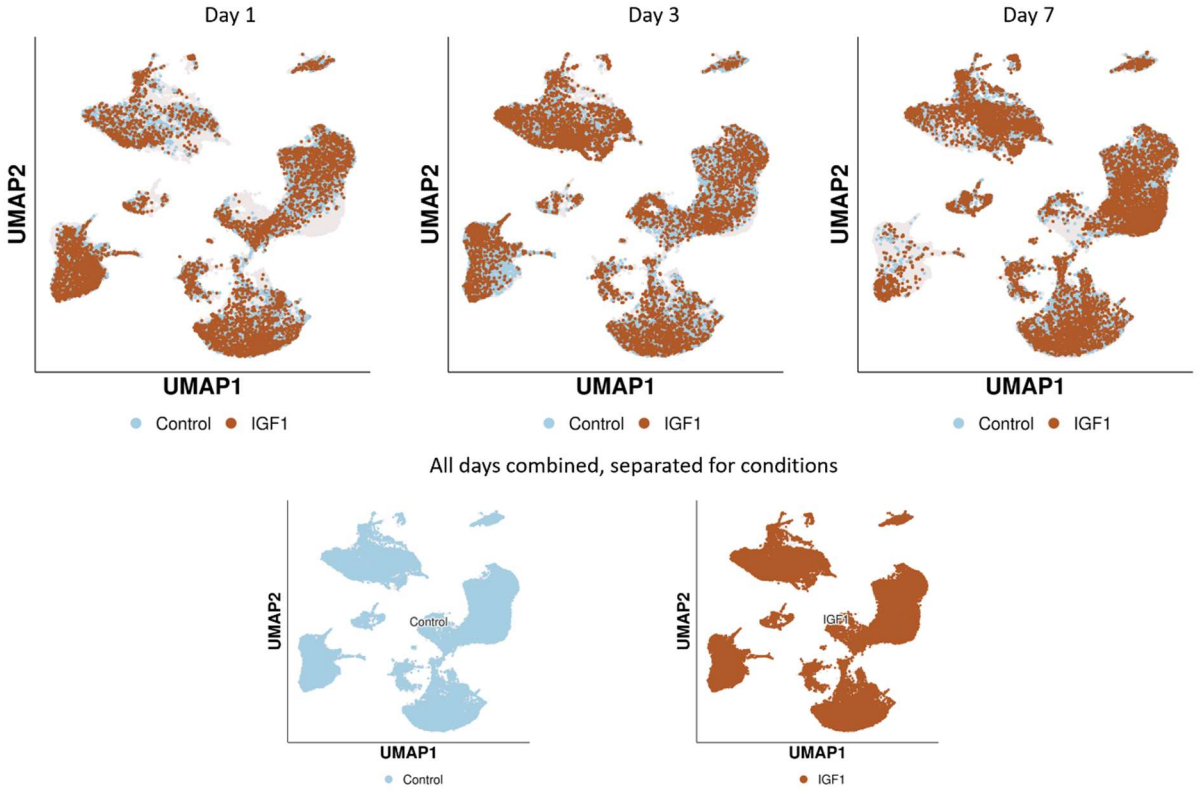

Figure 41: IGF-1 does not create a specific IGF-1 cluster.

IGF-1 treatment does not create a specific IGF-1 cluster, as shown in UMAP depicting control (blue) and IGF-1 treated (brown) cardiac cells.

4.4.1.1. Cardiac cells over the timecourse post MI

Next, the distribution of the cardiac cell types in the heart on days one, three and seven post MI was analysed (Figure 42). This revealed that the majority of fibroblasts increase over time, with the lowest amount on day 1 and highest number on day 7 post MI. Endothelial cells are high on day 1, decrease minimally on day 3 and increase again on day 7 post MI. Neutrophil numbers are highest on day 1 post MI, decrease on day 3 and are nearly vanished on day 7 post MI. Macrophages are lowest at day 1 and increase over time, with their peak on day 7. Those data show that the state obtained in these experiments conform with the general model of cellular dynamics after MI.

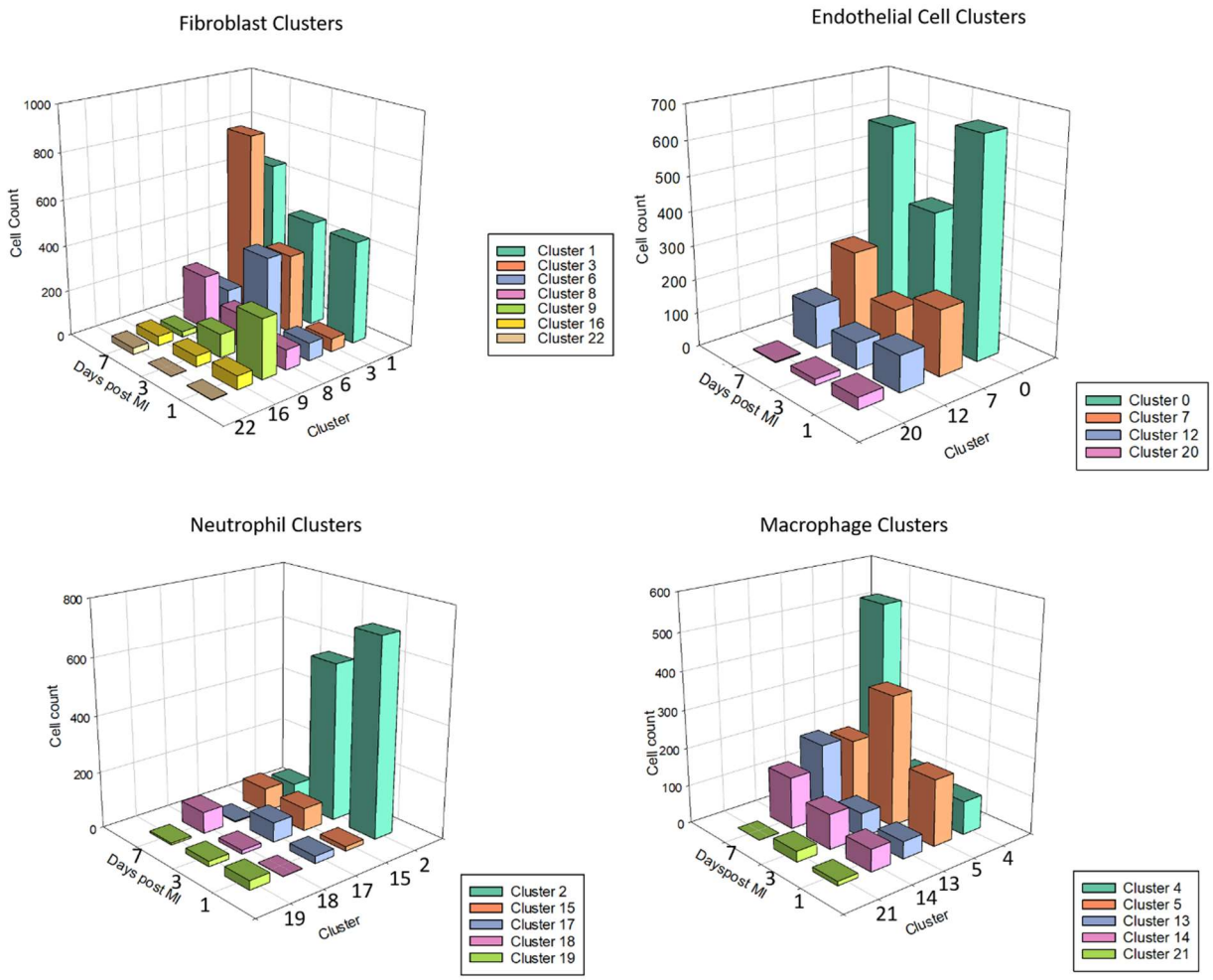

Figure 42: Distribution of cardiac cell types over the time post MI.

There is a dynamic shift of cell types in the heart over time post MI.

4.4.2. Characterisation of leukocytes post MI

In a next step, the leukocytes were reclustered to achieve a higher resolution and to enable a closer look at neutrophils. In total 28156 transcripts were detected, with 13510 transcripts derived from control mice and 14646 transcripts derived from IGF-1 treated mice. Again, clusters were defined by the expression of specific marker genes and Table 16 summarises all identified cell clusters with the common marker genes per cell type and the differential marker genes per cluster. As before, neutrophils (Cluster 0, 2, 10, 16 and 17), macrophages (Clusters 1, 3, 5, 6, 9, 11, 12, 13, 15), DCs (Cluster 7, 18), B-cells (Cluster 4), T-cells (Cluster 8) and NK-cells (Cluster 14) were identified (Medvedovic, Ebert et al. 2011, Wang, Wei et al. 2012, Lee, Wang et al. 2013, Cochain, Vafadarnejad et al. 2018, Marzaioli, Canavan et al. 2021, Zhang, Chopin et al. 2021, Sprenkeler, Zandstra et al. 2022). Cluster 19 and 20 could not be clearly allocated, however might be related to macrophages and specifically to dendritic cells. Defined clusters and

characteristic genes per defined cell types are depicted in Figure 43 [A] and [B]. Further, heat map shows the top 5 differentially expressed genes for each leukocyte cluster (Figure 43 [C]).

Table 16: Summary of identified leukocyte cell clusters of the heart post MI.

Cell type	Cluster	Common marker	Differential marker	Comment	Literature
Neutrophils	0	*S100a8* *S100a9* *Ly6g*	*Retnlg, Slpi, Wfdc17*	Young, blood derived	(Vafadarnejad, Rizzo et al. 2020, Calcagno, Zhang et al. 2021, Nederlof, Reidel et al. 2022)
	2		*Siglecf, Nfκbia, Tnf, Ccl3*	Old, pro-inflammatory	
	10		*Pecam1, Cdh5, Cd36*	EC expressing (phagocytosed)	
	16		*Ifit1, Ifit3, Rsad2*	Ifn stimulated	
	17		*Tnnt2, Myl3, Actc1*	Cardiomyocyte expressing (phagocytosed)	
Macrophages	1	*Fcgr1* *CD68* *Mrc1* *Adgre1*	*Arg1*	Arg1 expressing	(Cochain, Vafadarnejad et al. 2018)
	3		*Hif1a*	Hif1a Macrophages	
	5			??	
	6		*Timd4, Igf1, Retnla*	M2/ Timd4 Macrophages	
	9		*Mki67, Bub1, Mybl2*	Proliferating	
	11			??	
	12			??	
	13		*Ifit1, Ifit3, Rsad2*	Ifn stimulated	
	15		*Acta2, Col1a1, Postn*	MØ mesenchymal transition (Fibroblast expressing)	
	19			??	
	20		*Ccl22, Il12b, Ccr7*	Inflammatory	
DCs	7		*Itgax, Cd209a*	Mo derived DCs	(Cochain, Vafadarnejad et al. 2018)
	18		*Itgae*	DC1	
B-cells	4	*CD79* *CD19*			(Medvedovic, Ebert et al. 2011, Wang, Wei et al. 2012, Cochain, Vafadarnejad et al. 2018)
T-cells	8	*CD3*	*CD4*	T-helper cell	(Cochain, Vafadarnejad et al. 2018)
			CD8	Cytotoxic T-cell	
Nk-cells	14	*Nkg7* *Ncr1*			(Cochain, Vafadarnejad et al. 2018)

This table lists the identified leukocyte cell clusters with the common marker genes per cell type and the differential marker genes per cluster.

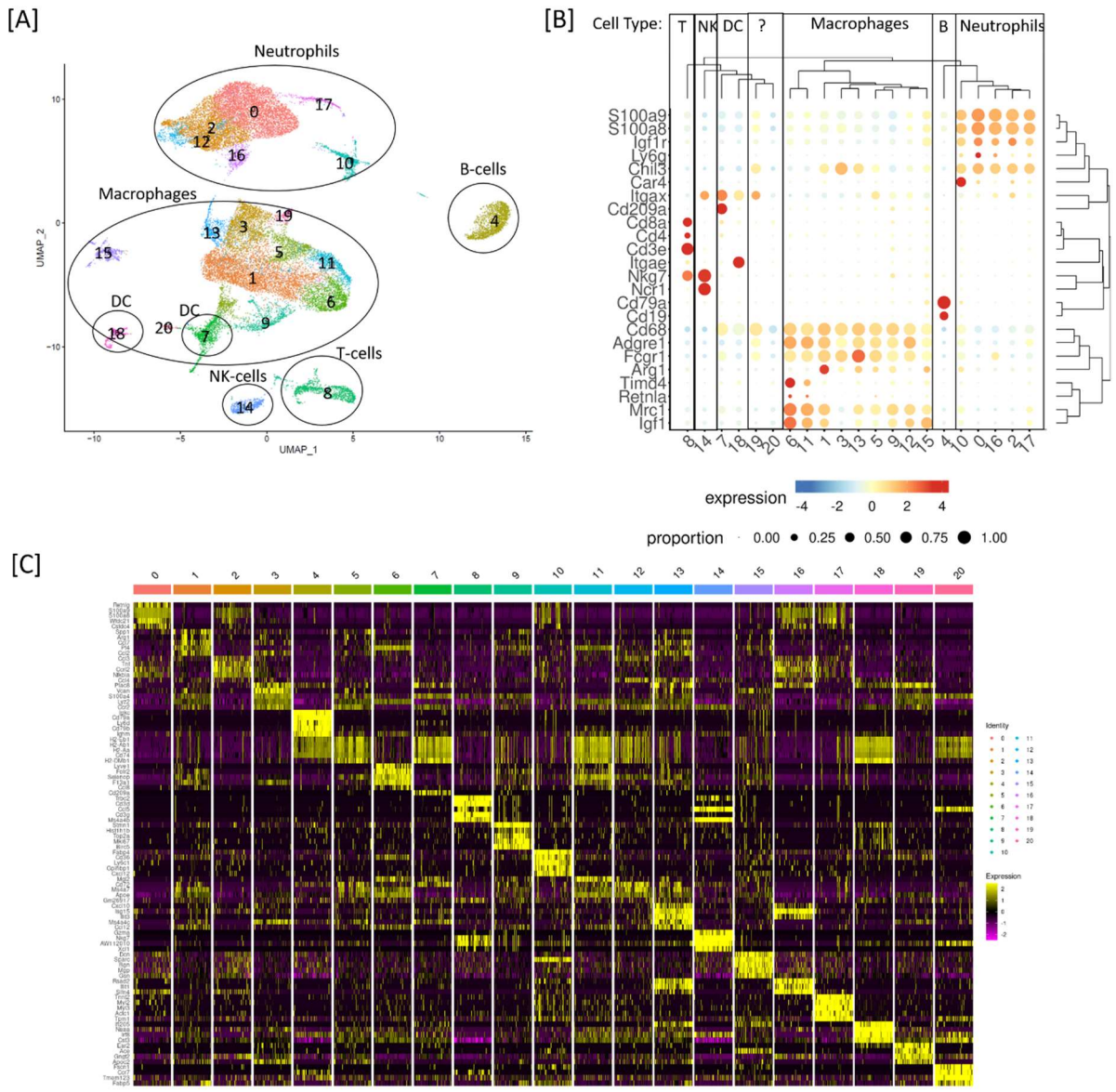

Figure 43: Characterisation of leukocyte populations.

In total 20 cell clusters were identified after reclustering of only leukocytes in a higher resolution. Leukocytes were divided into macrophages, DCs, neutrophils, B-cells, T-cells and NK-cells, as visualised in UMAP **[A]**. Bubbleplot shows characteristic marker genes of each cell type **[B]**. Heatmap showing the top 5 differentially expressed genes for each leukocyte cluster **[C]**.

4.4.2.1. Leukocytes over the timecourse post MI

Following myocardial infarction, neutrophils are one of the first cells to rapidly infiltrate the heart, whereas macrophages are recruited by, amongst others, neutrophils and arrive only later (Soehnlein, Zernecke et al. 2008). Therefore, it was of interest to analyse the dynamics of leukocytes post MI. In line with Figure 42, this revealed, that among all leukocytes, the neutrophil fraction was highest on day 1 post MI in all clusters, stayed

roughly the same on day 3 and nearly vanished from the heart on day 7 (Figure 44). Macrophages, including DC´s, contributed only low cell numbers on day 1, but increased over time, generally reaching the highest proportion on day 7. In Figure 44 only exemplary macrophage clusters are shown, which reflect the overall picture. B-, T- and NK-cells were lowest on day 1 and 3 post MI and slightly increased over time until day 7 post MI (Figure 44). Thus, these data state clearly that there is a dynamic shift of leukocytes in the heart over time post MI.

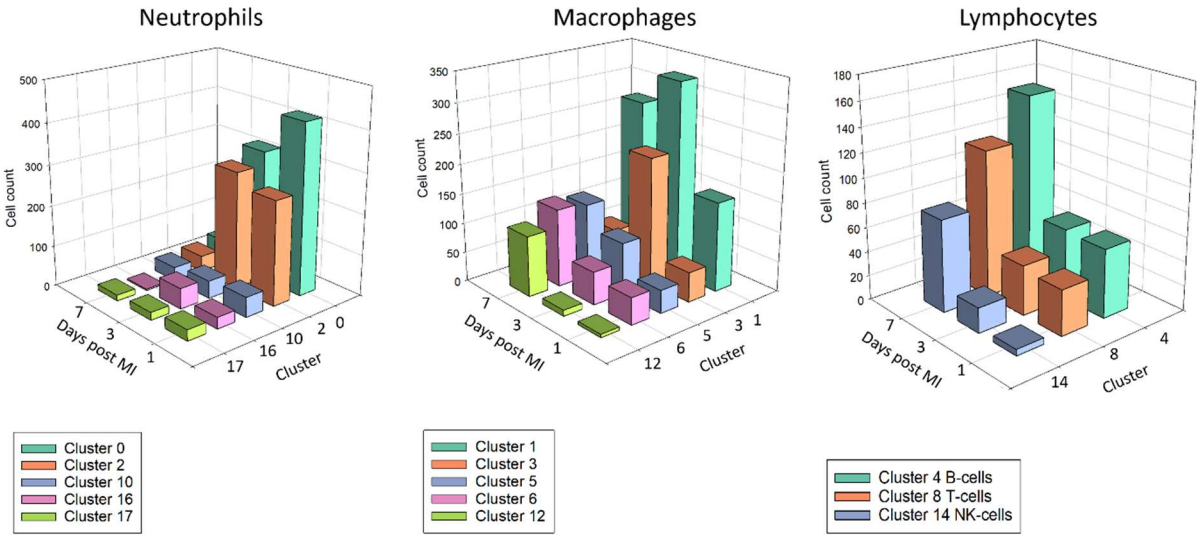

Figure 44: Presence of leukocytes in the heart over the time post MI.

Neutrophil number in the heart was highest on day 1 post MI, stayed roughly the same on day 3. On day 7 neutrophils nearly vanished from the heart. Exemplary macrophage clusters are shown. Macrophage number, including DC´s, was lowest at day 1 and increased over time on day 3 and day 7. B-, T- and NK-cells were lowest on day 1 and 3 post MI and slightly increased over time until day 7 post MI.

Interestingly, the gene expression profile of leukocytes changes over time post MI as well, which is shown in violinplots of exemplary genes (Figure 45). Exemplary N1 (*Tnf*) and N2 (*Arg1, Chil3*) marker genes, which were identified in *in vitro* experiments, were analysed. Most cell clusters abundantly expressed *Chil3* on day 1, whereas on day 7 post MI only 2 macrophage cell clusters remained expressing *Chil3*. *Arginase 1* was expressed by 7 macrophage cell clusters on day 1, sparsely expressed by four macrophage cell clusters on day 3 and was not expressed on day 7. Also, the expression of pro-inflammatory *Tnf* varies over time, being highest on day 1 and reduced, but still present in most clusters on day 7 (Figure 45). Thus, not only immune cell infiltration changes over time, but as well expression pattern of immune cells shifts from day 1 to day 7.

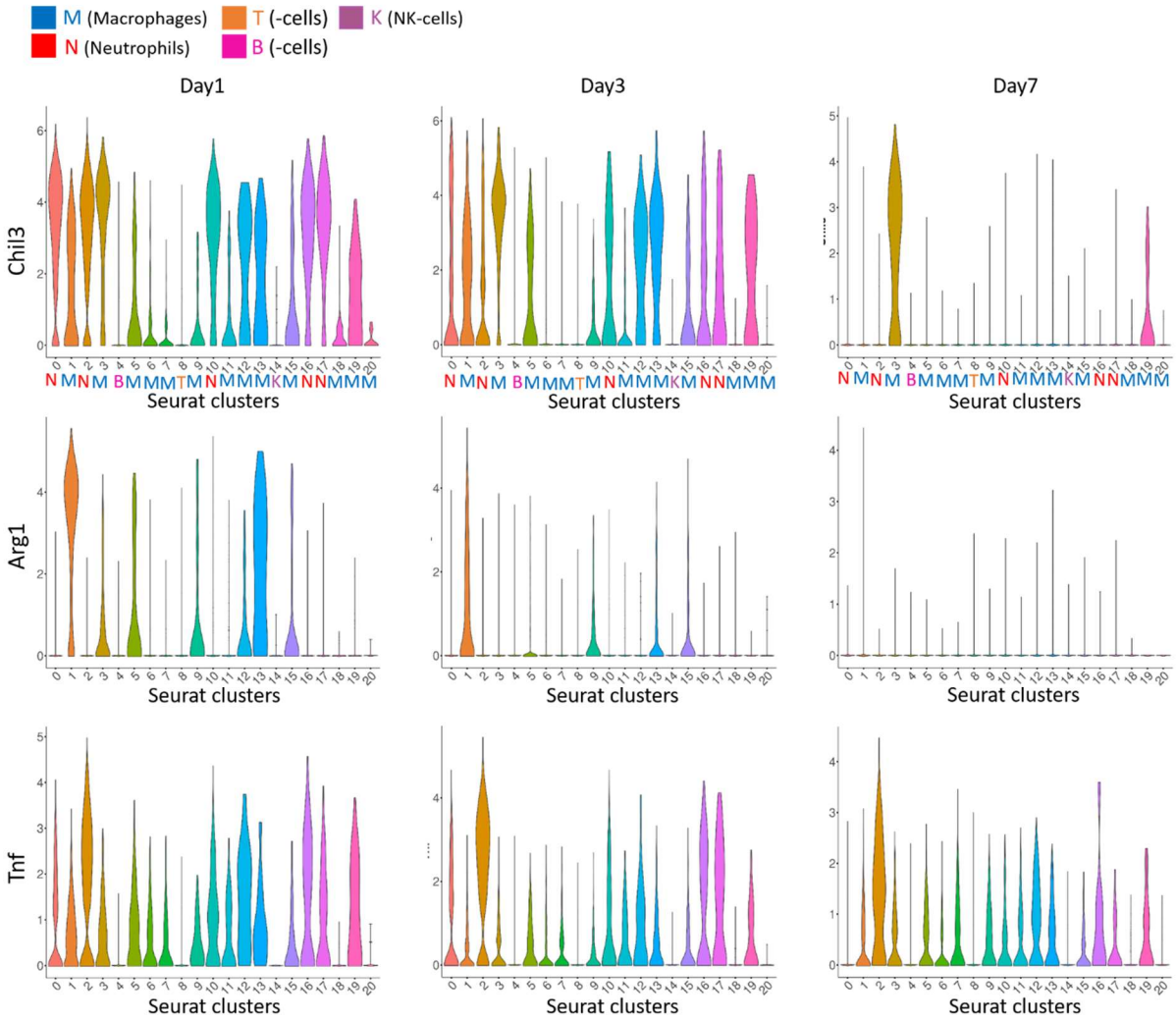

Figure 45: Gene expression changes over the time post MI.

Violinplots show gene expression of *Chil3, Arg1* and *Tnf* per cluster on day 1, day 3 and day 7 post MI.

4.4.3. Characterisation of neutrophils post MI

As the focus of this thesis is on neutrophils, in a next step, neutrophil clusters were defined in more detail. Neutrophil Cluster 0 is the largest neutrophil cluster and is defined by high expression of, amongst others, *Retnlg, Wfdc21* and *Slpi,* as visualized in the bubble plot, which depicts the top differentially regulated genes per cluster (Figure 46). This neutrophil cluster might represent young neutrophils derived from blood, which infiltrated the heart only recently (Vafadarnejad, Rizzo et al. 2020, Nederlof, Reidel et al. 2022). In contrast, neutrophil cluster 2 may be old pro-inflammatory neutrophils, as they show high expression of, inter alia, *Siglecf, Nfκbia, Tnf* and *Ccl3* (Vafadarnejad, Rizzo et al. 2020, Calcagno, Zhang et al. 2021, Nederlof, Reidel et al. 2022). Neutrophil cluster 16 showed high expression of type I interferon genes, as for example *Ifit1, Ifit3* and

Rsad2. Two of the clusters, namely cluster 10 and 17, express genes characteristic for neutrophils but also for endothelial cells (Cluster 10) and cardiomyocytes (Cluster 17), respectively. In cluster 10 for example the endothelial cell markers *Cdh5, Pecam1, CD36, Ly6c1* and *Fabp4* were found. In addition, cluster 10 is the only cluster showing high expression of *Car4,* which was identified as a N2-marker in our *in-vitro* polarization studies. Characteristic transcripts for cardiomyocytes found in neutrophil cluster 17 are amongst others *Tnnt2, Myl3* and *Actc1*. Thus, one can assume that neutrophils from cluster 10 and 17 have phagocytosed other cells, explaining the "co-expression" of endothelial and cardiomyocyte-derived transcripts.

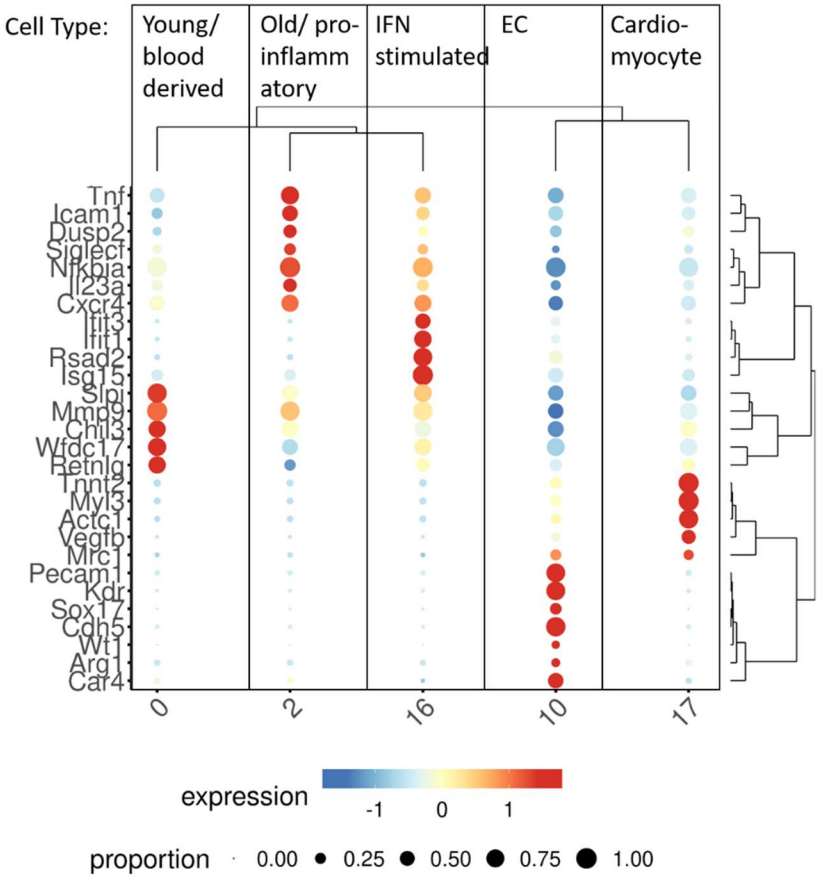

Figure 46: Characterisation of neutrophil clusters.

Bubble plot depicting the defining genes for each neutrophil cluster.

As IGF-1 was reported to have cardioprotective properties and to induce an anti-inflammatory macrophage phenotype it was as well analysed if IGF-1 has an effect on neutrophil number and phenotype (Santini, Tsao et al. 2007, Heinen, Nederlof et al. 2019). As the first clustering (Figure 41) revealed no specific "IGF-1 clusters", it was analysed, if specific "IGF-1 clusters" can be found after reclustering with a higher resolution. However, again IGF-1 treatment did not induce a specific IGF-1 cluster, as

cells were equally distributed over the clusters at all measured time points (Figure 47). However, UMAP of leukocytes nicely visualizes again that the neutrophil cluster vanished over time, whereas in contrast, macrophage, NK- and B- and T-cell clusters increased over time post MI (Figure 47).

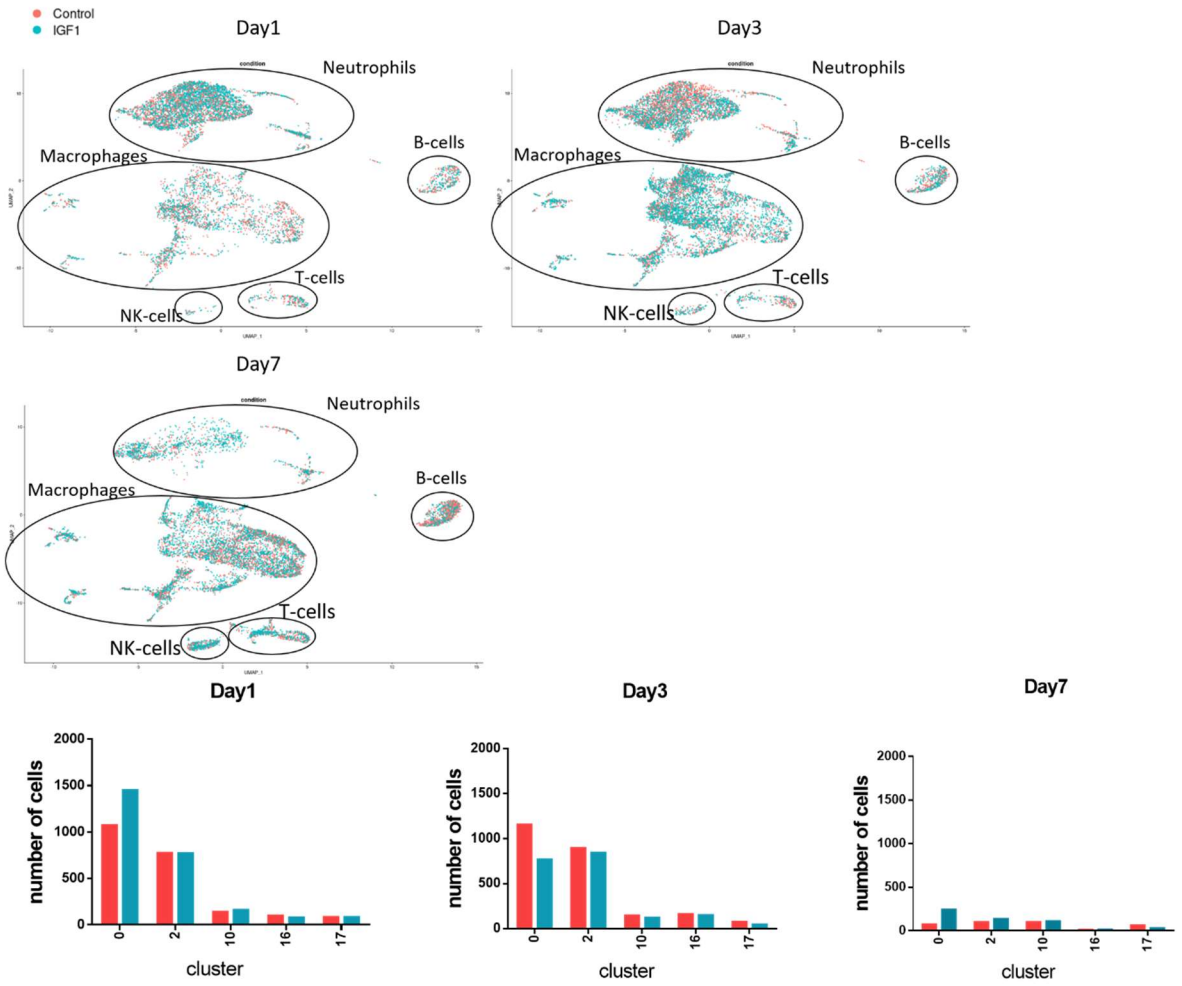

Figure 47: IGF-1 treatment has no effect on neutrophil number and does not create a specific IGF-1 cluster.

Control (red) and IGF-1 (blue) treated neutrophils are equally distributed over the different clusters and the number of neutrophils per cluster is equally distributed between control (BSA) and IGF-1 treated neutrophils.

4.4.3.1. IGF-1 treatment post MI attenuates a pro-inflammatory neutrophil phenotype

Next, it was analysed if IGF-1 treatment modulates neutrophil phenotype and reduces inflammation. Therefore, a differential gene expression analysis of all neutrophil clusters was performed (Figure 48). This analysis revealed, that IGF-1 treatment slightly induced

upregulation of *Chil3, Serinc3, Lcn2, Retnlg* and *Crispld2* expression and downregulation of *Ifitm1, Pgk1, Dusp5, Isg15* and *Ccl3* expression in a minimum of two neutrophil clusters one day post MI (Figure 48).

On day 3 post MI, IGF-1 treatment induced upregulation of anti-inflammatory *Fth1* and *Aldoa* and downregulation of rather inflammatory *Il1r2, Hp, Klhl6, Lcn2, Il23a, Ccl6* and *S100a8* expression+ (Figure 48).

On day 7 post MI not a lot of differentially regulated genes between IGF-1 and control treated mice were detected. Only *Wfdc21* was upregulated upon IGF-1 treatment on day 7 post MI and *Hnrnpa2b1* was downregulated in 2 neutrophil clusters (Figure 48).

Interestingly, this analysis revealed that there was a twist of gene expression on day 1 and 3. *Chil3* expression was upregulated in all 5 neutrophil clusters on day 1 upon IGF-1 treatment, but on day 3 post MI *Chil3* was downregulated in all 5 neutrophil clusters upon IGF-1 treatment. Also *Lcn2* expression was upregulated in four neutrophil clusters on day 1 and downregulated in three neutrophil clusters on day 3 post MI upon IGF-1 treatment.

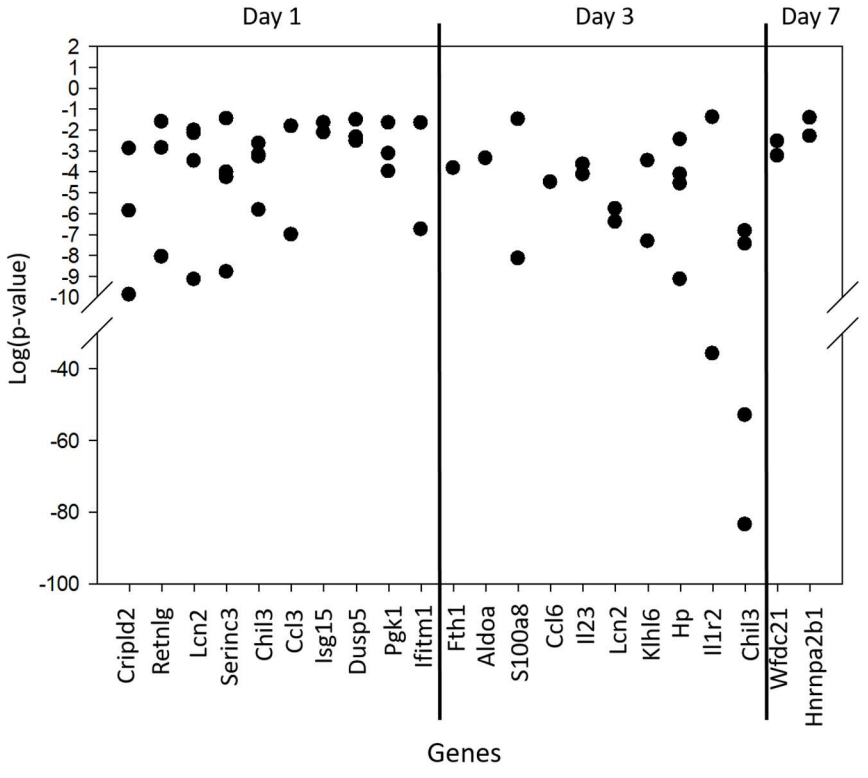

Figure 48: Differential gene expression between control and IGF-1 treatment on day 1, 3 and 7 post MI.

The top differentially expressed genes in a minimum of two neutrophil clusters after IGF-1 treatment on day one, three and seven post MI are displayed.

In order to get deeper insight into coordinate measures of the effect of the IGF-1 treatment, also the upstream regulator function (Figure 49) and pathway function (Figure 50) of IPA was used, which could help to identify regulators responsible for the alterations in gene expression. By using the differential gene expression of neutrophil clusters, IPA analysis showed a downregulation of *Klf6* and upregulation of *Ppara* and *Ezh2* on day 1 post MI (Figure 49).

On day 3 post MI the *granulocyte-macrophage colony stimulating factor (Csf2*), *Rictor* and *Cpt1b* were downregulated in three of five neutrophil clusters upon IGF-1 treatment. Further, IPA analysis showed a downregulation of the *Nfκb* complex and of the pro-inflammatory cytokines *IL1b, IL1* and *IL-6* on day 3 post MI. In contrast, IGF-1 treatment upregulated *Myc* and the repressor of toll-like receptor gene *Tead1*, *ZFP36, PPARGC1α, Insr, Stk11* and *Mycn*.

Also on day 7 post MI, IGF-1 treatment downregulated *Csf2* and inflammatory *Ifng* and *Nfat5*. Only *Prdm1*, a repressor of inflammatory cytokines as IL-6, was upregulated upon IGF-1 treatment on day 7 post MI.

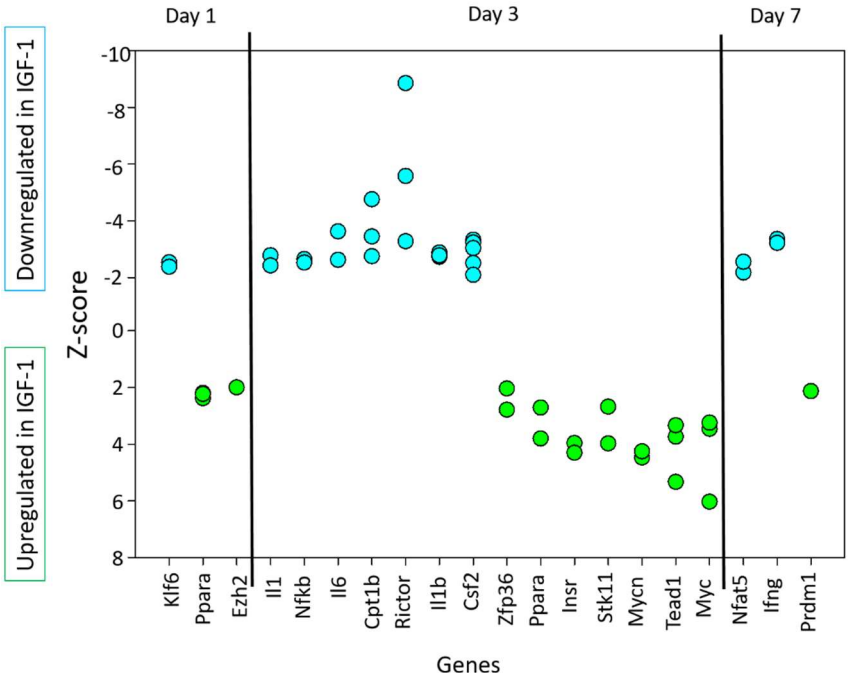

Figure 49: Major upstream regulators that are affected by the IGF-1 treatment on day 1, 3 and 7 post MI.

Upstream regulators that are affected by the IGF-1 treatment with a Z-score >2.0 in a minimum of two neutrophil clusters on day 1, day 3 and day 7 post MI.

Further, differentially regulated pathways after IGF-1 treatment were analysed (Figure 50). On day one post MI, there were no significant differences in pathway activation between IGF-1 and control treatment. However, on day three post MI, several alterations in pathway activation

were observed. IGF-1 treatment induced downregulation of inflammatory cytokine signalling, as IL-6, IL-17, LPS/IL-1-mediated signalling and also the pathogen-induced cytokine storm signalling pathway in several neutrophil clusters. Further, the inflammation-associated granzyme A signalling pathway and the p38 Mapk signalling pathway, which activates Nfκb, were downregulated in IGF-1 treated neutrophils.

Additionally, IGF-1 treatment induced upregulation of several pathways, as the necroptosis signalling, insulin secretion, NETosis, Eif2 and oxidative phosphorylation signalling in a minimum of two of five neutrophil clusters. Interestingly, IGF-1 treatment also slightly increased anti-inflammatory IL-10 signalling in three neutrophil clusters on day 3, though not of importance, as the Z-score was only low (≤ 0.8).

However, on day 7 post MI the anti-inflammatory IL-10 signalling was considerably upregulated in 2 neutrophil clusters of IGF-1 treated mice, and interestingly, the NETosis pathway which was upregulated on day 3 post MI was downregulated in one neutrophil cluster on day 7 in IGF-1 treated mice.

Further, still on day 7 rather inflammatory pathways were downregulated by the IGF-1 treatment, as the pro-inflammatory Th1 pathway and Nfκb signalling.

Thus, these results demonstrate that the IGF-1 treatment attenuates the pro-inflammatory neutrophil phenotype three and seven days post MI. This demonstrates that IGF-1 is not only able to modulate macrophage phenotype, but also neutrophil phenotype.

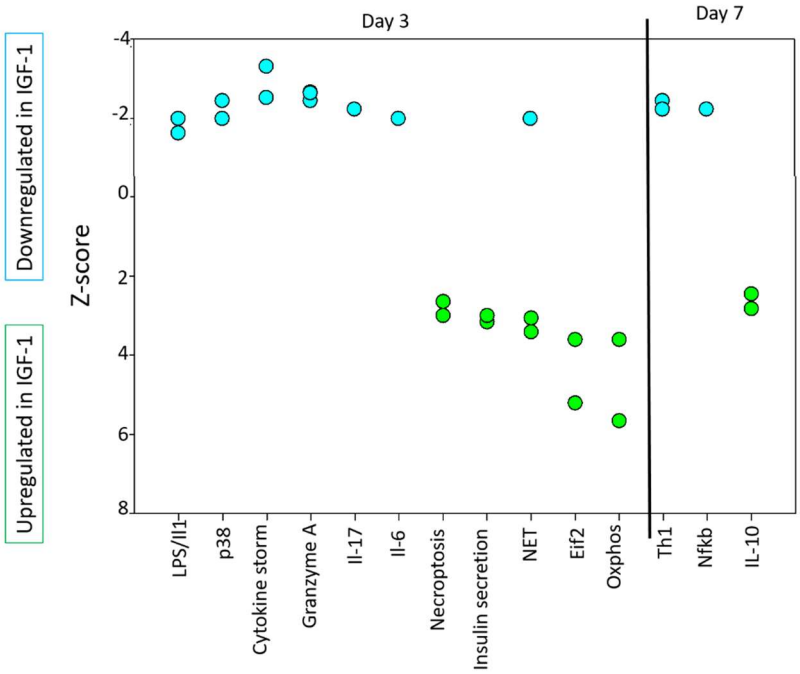

Figure 50: Pathways that are affected by the IGF-1 treatment.

Pathways that are affected by the IGF-1 treatment with a Z-score >2.0 in a minimum of two neutrophil clusters on day 1, day 3 and day 7 post MI.

4.4.3.2. Neutrophil phenotype *in vitro* versus *in vivo*

In previous experiments we were able to show *in vitro* that neutrophils polarize to an anti- or pro-inflammatory phenotype upon IL-4 or LPS/IFN-y treatment. Therefore, it was of interest to analyse if characteristic N1 and N2 neutrophil marker genes identified *in vitro* in BM neutrophils, were as well expressed in cardiac neutrophils post MI and if so, were up- or down-regulated by the anti-inflammatory IGF-1 treatment (Figure 51). Thus, we examined if a comparable N1- or N2- like neutrophil phenotype could be observed in cardiac neutrophils post MI.

This analysis demonstrated, that most N1 (*Nos2, IL12b, IL-6*) and N2 marker genes (*Retnlα, Arg1, Car4*) used for phenotype characterisation in BM cells *in vitro* were only sparsely expressed in cardiac neutrophils post MI, indicating that those marker genes, which are substantially regulated in N1/N2 polarization, are not applicable for the characterisation of anti- or pro-inflammatory neutrophils in this setup. However, when analysing the top upregulated genes upon LPS/IFNγ treatment, identified by RNAseq of BM neutrophils *in vitro,* in cardiac neutrophils post MI, neutrophils of cluster 16 most likely resemble pro-inflammatory N1-like neutrophils (Figure 51). In line, neutrophil cluster 16 was identified as a cluster with high expression of Ifn-stimulated genes. When analysing the top upregulated genes upon IL-4 treatment, identified by RNAseq of BM neutrophils *in vitro,* in cardiac neutrophils post MI, no cluster showed abundant expression of N2 genes, but rather each cluster expressed scattered N2 genes. However, neutrophil cluster 10 showed expression of N2 genes *Arg1, Car4 and Tafa3*. Thus, these data demonstrate, that *in vitro* experiments are a helpful tool to identify phenotypic changes under well-defined conditions, but have only a limited transferability to more complex *in vivo* situations.

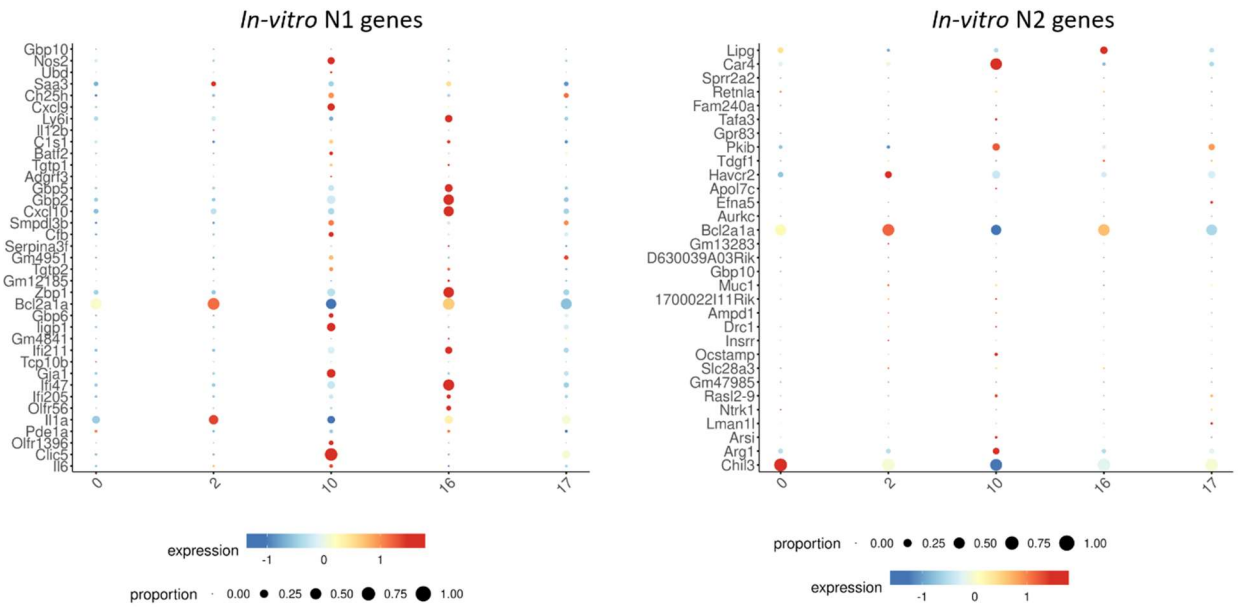

Figure 51: Most genes that are highly upregulated in BM N1 or N2 neutrophils *in vitro*, are only sparsely expressed in cardiac neutrophils post MI *in vivo*.

Characteristic N1 and N2 marker genes that were highly upregulated in BM neutrophils upon LPS/IFN-γ treatment are sparsely expressed in cardiac neutrophils post MI.

4.5. Neutrophils in a hyperglycaemic environment

Cardiovascular diseases are the leading cause of death in western societies and Type 2 Diabetes Mellitus (T2DM) strongly predisposes to and exacerbates these diseases (Wild, Roglic et al. 2004, Lopez, Mathers et al. 2006). Until recently, T2DM was widely accepted as a metabolic disease, however it is nowadays more and more often characterised as an immune-mediated inflammatory disease. It was reported that obesity and the following development of hyperglycaemia and hyperlipidaemia, often leading to T2DM and metabolic syndrome, results in chronic inflammation. The role of macrophages in the context of hyperglycaemia and T2DM is well known, whereas the contribution of neutrophils has not been examined in detail. Thus, it was of importance to also analyse the effect of hyperglyceamia and obesity on neutrophil polarization and function.

4.5.1. Effect of short-term high glucose treatment on neutrophil polarization *in vitro*

Previously, it was shown that macrophages cultivated in high glucose medium evince a more pro-inflammatory phenotype (Edgar, Akbar et al. 2021). To also analyse if

hyperglycaemia modulates neutrophil polarization *in vitro,* murine BM neutrophils isolated from standard chow fed mice were polarized with LPS/IFN-γ or IL-4, either in low (1 g/L) or high (4.5 g/L) glucose medium for 4 hours. Non-polarized cells cultivated in low and high glucose media were used as controls. Subsequently, qPCR analysis of pro- and anti-inflammatory marker genes was performed. Figure 52 displays, that both, neutrophils polarized in low or high glucose medium, showed the expected upregulation of pro- and anti-inflammatory genes without quantitative differences between low and high-glucose cultured cells. Thus, in this case the high glucose medium had no effect on neutrophil polarization, at least on the marker genes that were studied here, possibly due to the limited polarization time of four hours.

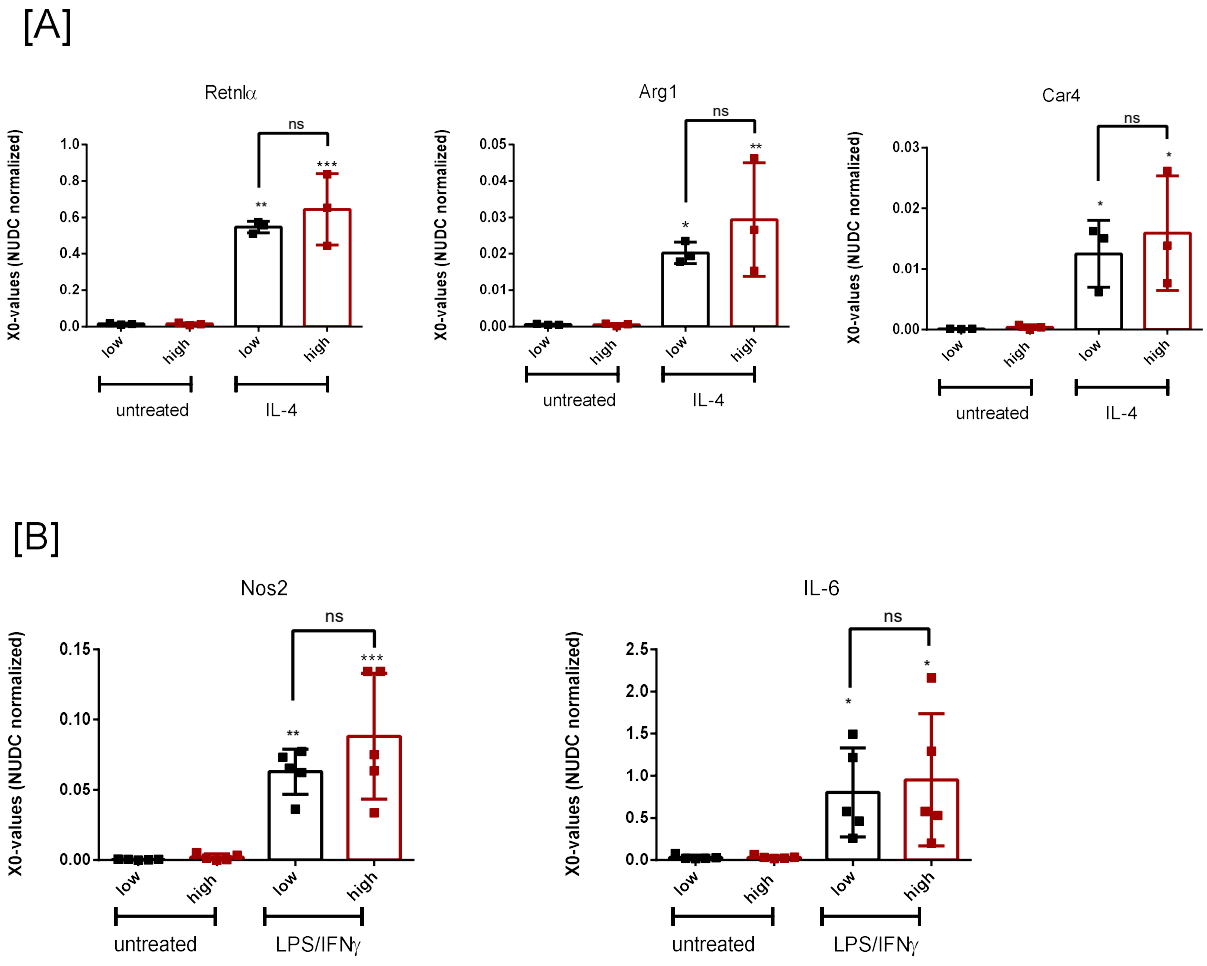

Figure 52: Hyperglycaemia *in vitro* has no effect on neutrophil polarization.
Murine BM neutrophils of standard chow fed mice were left untreated, or treated with LPS (10 ng/ml) + IFN-γ (2 ng/ml) or IL-4 (20 ng/ml), either in low (1 g/L) or high (4.5 g/L) glucose medium, for a polarization time of 4 hours and qPCR analysis of anti-inflammatory (*Retnlα, Arg1, Car4*) **[A]** and pro-inflammatory (*Nos2, IL-6*) **[B]** genes was performed. Expression of single measurements are shown; n=3-5 experiments are shown. Bars represent mean ± SD. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated of the same condition).

4.5.2. Characterisation of the Diet Induced Obesity (DIO) model

As no changes in pro- or anti-inflammatory polarization capacity due to high glucose *in vitro* were observed (Figure 52), the question arose if a continuous hyperglycaemic environment and obesity *in vivo*, induced by a high fat/ high sucrose diet, primes neutrophils towards a more pro-inflammatory phenotype. Therefore, the diet-induced obesity (DIO) mouse model was established and characterised (Figure 53). 8 weeks old C57BL/6J mice were fed a high fat/ high sucrose (HFHS) diet for 12 weeks and subsequently body weight and metabolic changes (Figure 54) were analysed.

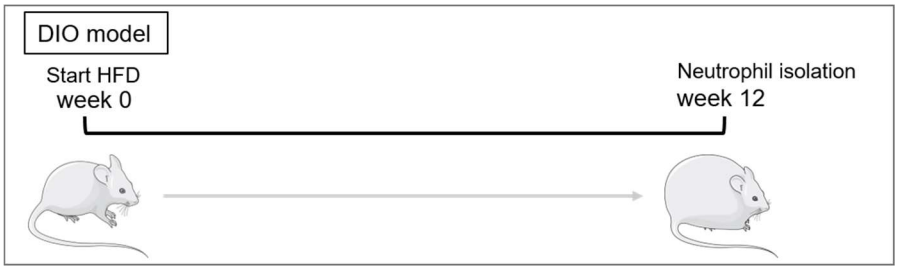

Figure 53: Hyperglycaemic mouse model.

Mice were fed for 12 weeks with a high fat/ high sucrose diet (HFHSD) and control mice a standard chow diet.

Glucose and insulin tolerance tests were performed to analyse changes in glucose metabolism. Glucose tolerance test measures the clearance of exogenous glucose, and can be linked to diseases such as diabetes or metabolic syndrome. Insulin tolerance tests are used to analyse insulin sensitivity. When insulin is administered, blood glucose levels should drop as glucose is then transported into tissues and gluconeogenesis is suppressed.

Figure 54 [A] displays that DIO mice evinced an increased body weight, with a mean body weight of over 40 grams, at the end of the feeding period. Standard chow fed mice had a mean bodyweight of 32 grams at the end of the 12 weeks feeding period. However, DIO mice develop only mild hyperglycaemia with mean starved blood glucose levels of 190.7 ± 12.07 mg/dl, control mice fed a standard chow diet evinced mean starved blood glucose levels of 133.0 ± 8.6 mg/dl (Figure 54 [B]). But, DIO mice exhibited a distinct glucose intolerance, as blood glucose rose above 400 mg/dl in GTT and also the decline of plasma glucose levels was slower than in control mice (Figure 54 [C]). In addition, DIO mice showed no insulin resistance, as blood glucose levels dropped slightly after insulin administration (Figure 54 [D]). Thus, the DIO model rather represents a prediabetic state with mild hyperglycaemia and advanced obesity.

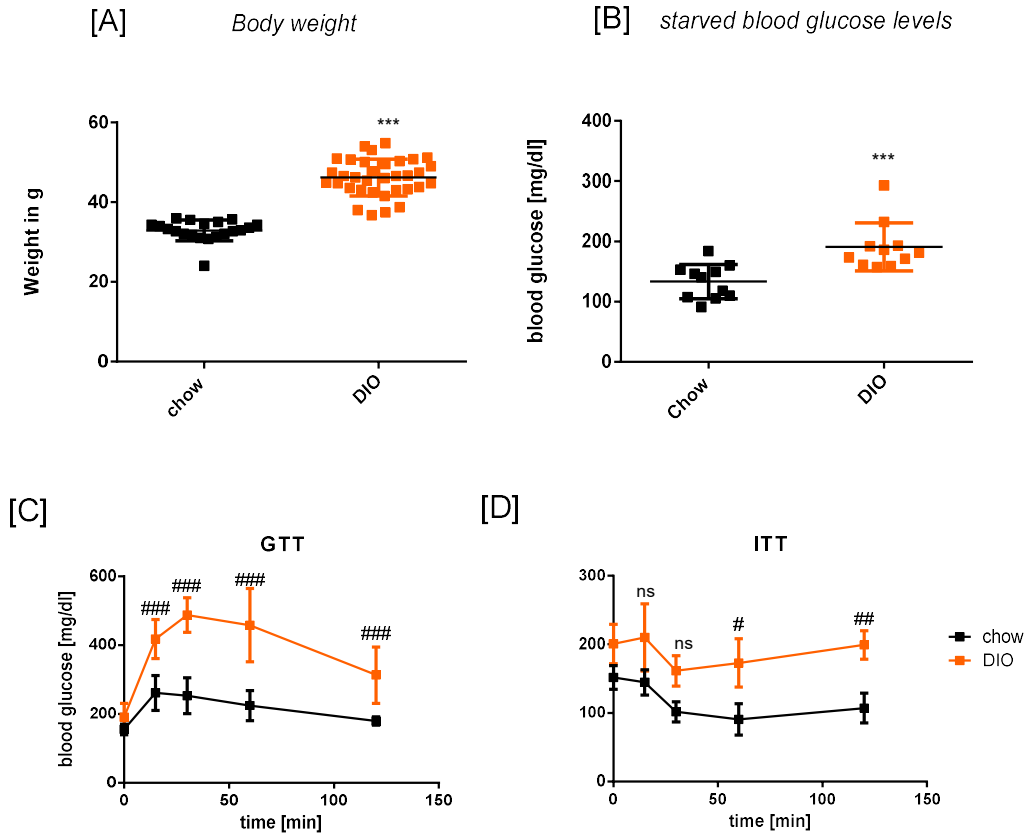

Figure 54: **Analysis of the effect of a high fat/ high sucrose diet on metabolism.**

Summarised data for body weight of mice fed a high fat/ high sucrose diet (DIO) or standard chow diet for 12 weeks **[A]** and at week 12 starved blood glucose levels of chow and DIO mice were measured **[B].** Additionally, summarised data of glucose **[C]** and insulin **[D]** tolerance test of DIO and chow diet fed mice at week 12 are shown. Mice were injected 2 mg glucose per gram body weight or 0.75 U insulin per kg bodyweight. For GTT and ITT the area under the curve was calculated. Graphs represent mean ± SD. Statistical significance between two groups was calculated using Mann Whitney Rank Sum test (*p<0.05, **p<0.01, ***p<0.001) or two-way ANOVA (#p<0.05, ##p<0.01, ###p<0.001).

4.5.2.1. Mild hyperglycaemia slightly alters neutrophil phenotype

As described before, it was demonstrated that glucose augments pro-inflammatory actions of macrophages (Lee, Kim et al. 2013, Nagareddy, Murphy et al. 2013, Edgar, Akbar et al. 2021). However, short-term high glucose treatment for four hours showed no effect on neutrophil polarization (Figure 52). In the next set of experiments, it was analysed if continuous mild hyperglycaemia, with reduced glucose tolerance in DIO mice, has an effect on neutrophil phenotype. Hence, neutrophils from DIO mice and standard chow fed (control) mice were isolated and were cultivated in media only for 4 hours, before RNA was prepared for RNA transcriptome analysis (cooperation with Patrick Petzsch, BMFZ HHU). In order to identify differences in transcript expression, normalized

RNA transcript expression data (TPM, log2 transformed) were analysed with the Qlucore Omics Explorer software package followed by Ingenuity Pathway Analysis (Qiagen, IPA). This analysis revealed that mild hyperglycaemia in DIO mice caused few changes in transcript expression profile, as visualised in heat map and PCA plot (Figure 55 [A], [B]). In total, 98 genes were differentially regulated when comparing basal neutrophils of control mice to neutrophils of DIO mice (FC 1.5, q=0.05).

In order to identify pathways modulated by the mild hyperglycaemia in neutrophils, the upstream regulator function and canonical pathway function (Figure 55 [C]) of IPA was used. Analysis of the differentially expressed genes (DEGs) by the canonical pathway function in IPA identified xenobiotic metabolism (Z-score 2), Nrf2-mediated oxidative stress response (Z-score 1.89) and the ostheoathritis pathway (Z-score 1) as upregulated and the ferroptosis signalling pathway (Z-score -0.8) as downregulated in basal neutrophils of DIO mice. However, a Z-score of ± 2 is considered to indicate an altered gene expression pattern that is consistent with an up/downregulation of a pathway. Thus, only the xenobiotic metabolism was considerably upregulated.

The upstream regulator function in IPA was used to infer which upstream activator might modulate the observed changes in gene expression. This analysis indicated that pro-inflammatory signalling was upregulated in neutrophils of DIO mice, as the most upregulated upstream regulators were inflammatory cytokines as *Tnf* (Z-score 3)*, Infγ* (Z-score 2.5) and *Il1b* (Z-score 2.8), as well as *Nrf2* (Z-score 3.37), *Myc* (Z-score 3.37) and *Cebpa* (Z-score 2.6). On the other hand, regulators which attenuate oxidative stress, *Gluthatione reductase (Gsr)* (Z-score -2.5), *Thioredoxin reductase (Txnrd1)* (Z-score -2.4) as well as *Kelch-like ECH-associated protein* 1 *(Keap1)* (Z-score -2.2*)* were downregulated in neutrophils of DIO mice*.* Furthermore, anti-inflammatory *Cish* (Z-score -2.2) was downregulated in neutrophils of DIO mice. Therefore, mild hyperglycaemia slightly altered transcript expression in neutrophils, which is consistent with an incipient chronic inflammation in DIO mice, although the differences were not very prominent.

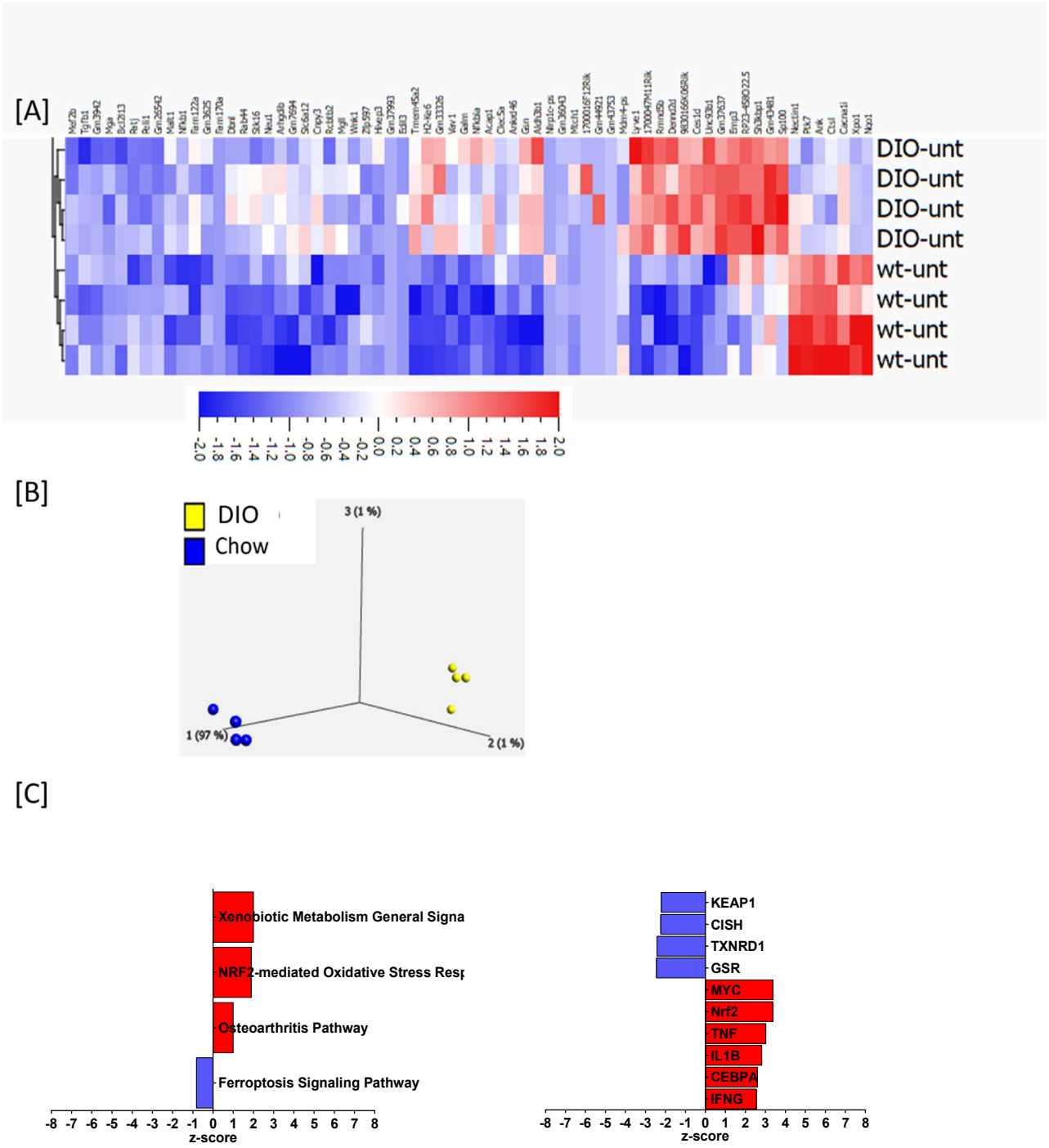

Figure 55: Mild hyperglyceamia in DIO mice alters gene expression profile of murine BM neutrophils.

Analysis of RNAseq data of murine BM neutrophils of standard chow fed and DIO mice revealed slight basal differences, as visualised in heatmap **[A]** and PCA plot **[B]**. IPA analysis of differentially regulated pathways and upstream regulators revealed upregulation of rather inflammatory processes under basal conditions **[C]**. Fold change (FC)=1.5; q=0.05; n=4 experiments.

4.5.3. Characterisation of a mouse model with severe hyperglycaemia

As mild changes in neutrophil phenotype were observed in the prediabetic DIO model with mild hyperglycaemia (Figure 55), it was further analysed if severe hyperglycaemia has a stronger effect on neutrophil polarization and function. Therefore, the DIO/STZ (Streptozotocin) model was established and characterised (Figure 56, 57). 8 weeks old C57BL/6J mice were fed a high fat/ high sucrose diet, as was used for DIO mice, for 12 weeks and were given 5 consecutive daily intraperitoneal injections of STZ (40 mg/kg) in the 5th week of feeding. Control mice were injected with a vehicle (citrate buffer). Streptozotocin is a ß-cell toxin, which induces destruction of the pancreatic ß-cells, thus resulting in a strongly reduced functional ß-cell mass and low insulin secretion (Rakieten, Rakieten et al. 1963, Arison, Ciaccio et al. 1967, Schein, Cooney et al. 1967).

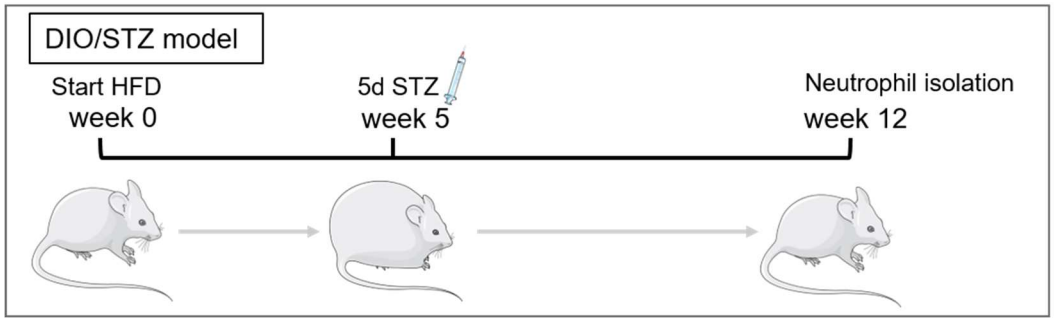

Figure 56: Protocol of the DIO/STZ mouse model.

Mice were fed for 12 weeks with a high fat/ high sucrose diet (HFHSD) and control mice a standard diet. DIO/STZ mice were additionally injected with 40 mg/kg STZ for 5 days in the 5th week of feeding and control mice were injected with citrate buffer.

Subsequently, body weight and metabolic changes, shown in Figure 57, were analysed in those mice. Glucose and insulin tolerance tests were performed to analyse changes in glucose metabolism. DIO/STZ mice had a mean body weight of 28.62 ± 0.3 grams, whereas standard chow fed mice had a mean body weight of 30.13 ± 0.2 grams at the end of the feeding period (Figure 57 [A]). Figure 57 [B] displays that DIO/STZ mice gain weight until STZ injection and afterwards start to loose weight again. With the start of STZ injection, blood glucose levels of those mice increased rapidly from under 200 mg/dl to over 400 mg/dl (Figure 57 [C]). At week 5 post STZ injection DIO/STZ mice had mean starved blood glucose levels of 388.4 ± 7.8 mg/dl, wheras standard chow fed mice had mean starved blood glucose levels of 145.8 ± 5.0 mg/dl (Figure 57 [D]). Thus, in contrast to control and DIO mice, DIO/STZ mice displayed a substantial hyperglycaemia over a 7-weeks period. Further, DIO/STZ mice exhibited a distinct glucose intolerance, as blood glucose rose above 600 mg/dl upon injection and was not lowered as fast as in standard chow fed mice (Figure 57 [E]). However, those mice showed no insulin resistance, as

blood glucose levels rapidly dropped after insulin administration (Figure 57 [F]). Thus, the DIO/STZ model showed strong hyperglycaemia without obesity and insulin intolerance.

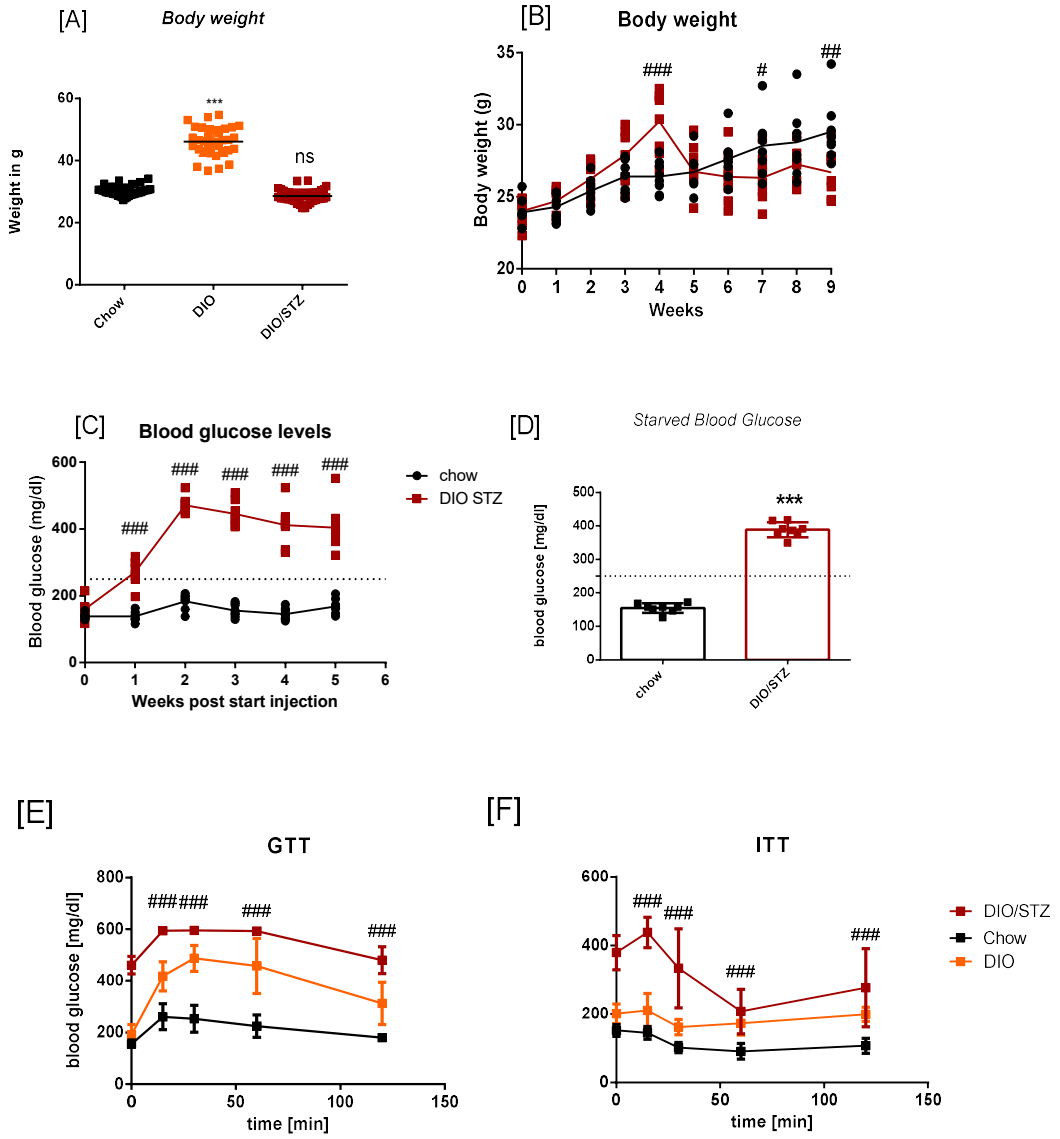

Figure 57: **Analysis of the effect of a high fat/ high sucrose diet and STZ injection on metabolism.**

Summarised data for body weight of mice fed a high fat/ high sucrose diet plus additional STZ injection or standard chow at week 12 **[A].** Body weight of standard chow fed and DIO/STZ mice was measured weekly **[B].** Basal blood glucose levels of standard chow fed and DIO/STZ mice were monitored weekly from the beginning of STZ injection **[C]** and at week 12 starved blood glucose levels were measured **[D].** Additionally, summarised data of glucose **[E]** and insulin **[F]** tolerance test of DIO/STZ and standard chow fed mice at week 12 are shown. Mice were injected with 2 mg glucose per gram body weight or 0.75 U insulin per kg bodyweight. For GTT and ITT the area under the curve was calculated. Graphs represent mean ± SD. Statistical significance between two groups was calculated using Mann Whitney Rank Sum test (*p<0.05, **p<0.01, ***p<0.001) or two-way ANOVA (#p<0.05, ##p<0.01, ###p<0.001).

4.5.3.1. Severe hyperglycaemia in DIO/STZ mice substantially alters neutrophil phenotype

The observation of a prolonged hyperglycaemia in DIO/STZ mice raised the question if the high fat/ high sucrose diet with additional STZ injection, induced stronger alterations in neutrophil phenotype and function. Thus, N1 and N2 marker genes were analysed by qPCR (Figure 58). To this end, neutrophils were left untreated or treated with IL-4 or LPS/IFN-γ for 4 hours and qPCR analysis was performed to determine transcriptional changes triggered by polarization of 4 hours. As shown in Figure 58 [A], this analysis revealed for anti-inflammatory marker gene *Retnla* a trend towards a reduced upregulation (by 31.8%) in response to IL-4 in DIO/STZ mice compared to standard chow fed mice, although the significant level was not reached. However, no changes were observed between standard chow fed and DIO/STZ mice for the anti-inflammatory marker gene *Car4* after IL-4 stimulation (Figure 58 [A]). LPS/IFN-γ had no effect on *Retnla* and *Car4* expression. For the LPS/IFN-γ induced marker gene *IL-6*, a signficant reduction (by 43.8%) was noted in DIO/STZ mice upon LPS/IFN-γ treatment, when compared to standard chow fed mice. Though, no changes appeared between standard chow fed and DIO/STZ mice for the pro-inflammatory marker gene *Nos2* (Figure 58 [B]).

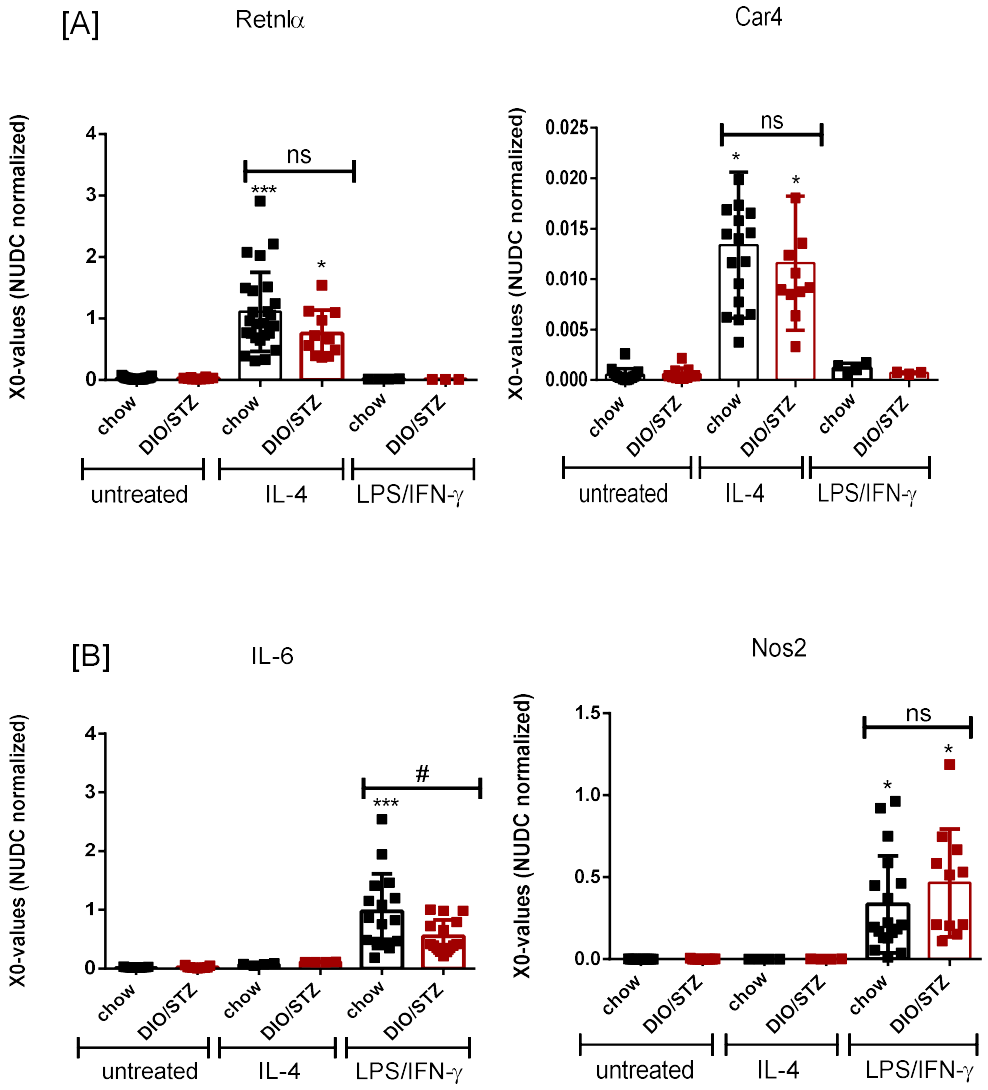

Figure 58: Severe hyperglycaemia slightly affects neutrophil polarization.
Murine BM neutrophils of standard chow fed and DIO/STZ mice were left untreated or polarized with LPS (10 ng/ml) + IFN-γ (2 ng/ml) or IL-4 (20 ng/ml) for 4 hours and qPCR analysis of anti-inflammatory marker genes *Retnlα* and *Car4* **[A]** and pro-inflammatory genes *Nos2* and *IL-6* **[B]** was performed. X0 values are normalized to *Nudc*. Expression of single measurements are shown. Bars represent mean ± SD, n=15-18. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated of the same mouse model; #p<0.05, ##p<0.01 and ###p<0.001 when compared to the same treatment in neutrophils of chow mice).

Moreover, it was of interest to analyse if an additional pro-inflammatory stimulus (LPS/IFN-γ) induced a stronger reduction of N2 polarization in neutrophils of DIO/STZ mice (Figure 59 [A]) and if an anti-inflammatory stimulus (IL-4) is still able to reduce N1 polarization in neutrophils of DIO/STZ mice (Figure 59 [B]). Therefore, murine BM neutrophils were treated with either only IL-4 or IL-4 plus additional LPS/IFN-γ. Also the inverse experiment, LPS/IFN-γ with or without IL-4, was performed (Figure 59). This analysis revealed that a pro-inflammatory stimulus was able to reduce upregulation of N2

marker genes *Retnlα* and *Car4* in both standard chow fed and DIO/STZ mice to the same extent. Additional IL-4 treatment induced a minor reduction of *Tnfα* expression also in both, neutrophils of standard chow fed and DIO/STZ mice (Figure 59). Thus, hyperglycaemia did not impair the ability to reduce pro- or anti-inflammatory polarization by an additional stimulus.

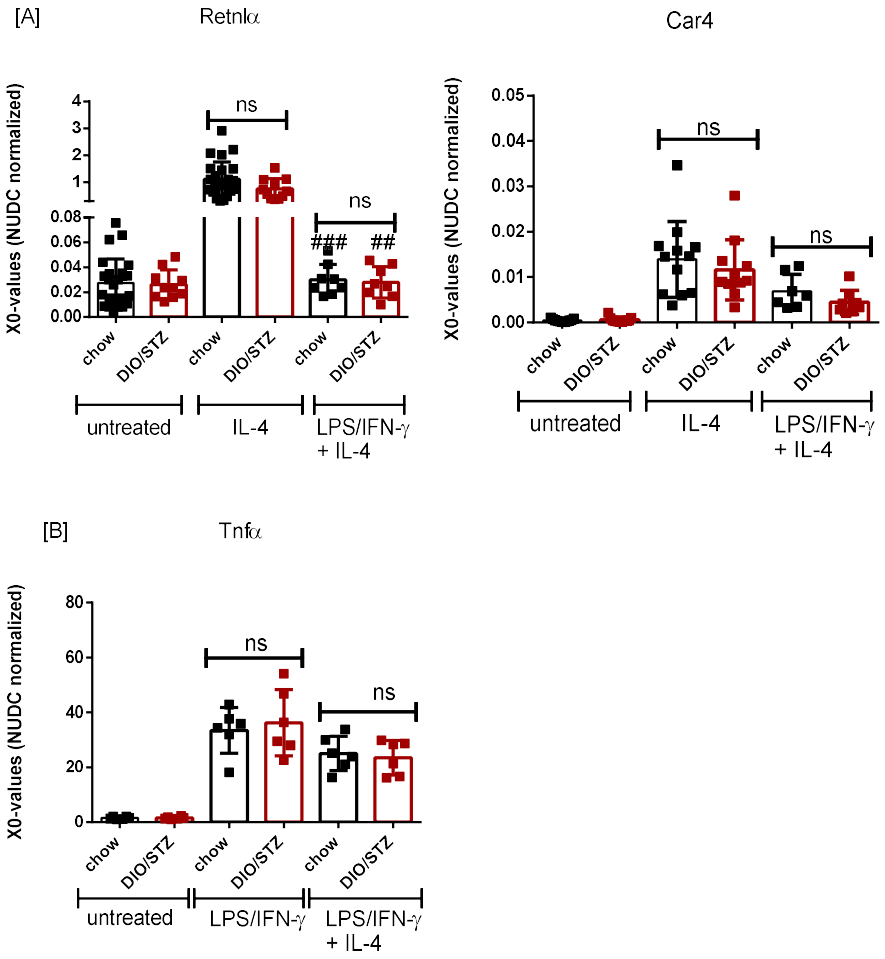

Figure 59: LPS/IFN-γ inhibits N2 polarization and IL-4 reduces *Tnfα* expression also in neutrophils of DIO/STZ mice.
Murine BM neutrophils were left untreated or polarized with IL-4 (20 ng/ml), LPS (10 ng/ml) + IFN-γ (2 ng/ml) only or plus IL-4 (20 ng/ml) for 4 hours and qPCR analysis of anti-inflammatory genes *Retnlα* and *Car4* **[A]** and of pro-inflammatory gene *Tnfα* **[B]** was performed. X0 values are normalized to *Nudc*. Expression of single measurements are shown. Bars represent mean ± SD, n=7-18. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to the same treatment in neutrophils of chow mice; #p<0.05, ##p<0.01 and ###p<0.001 when compared to IL-4 or LPS/IFN-γ treatment of the same mouse model).

As mild hyperglycaemia induced mild phenotypic differences in neutrophils (Figure 55) and severe hyperglycaemia induced some changes in gene expression of N1 and N2 marker genes (Figure 58), it was of interest to analyse the effect of severe

hyperglycaemia on neutrophil phenotype in more detail by RNA transcript expression analysis. Hence, neutrophils of standard chow fed mice and DIO/STZ mice were left untreated or treated with IL-4 or LPS/IFN-γ for 4 hours and RNA transcript expression analysis was performed (cooperation with Patrick Petzsch, BMFZ HHU) to determine transcriptional changes triggered by polarization of 4 hours. In order to identify differences in transcript expression, normalized RNA transcript expression data (TPM, log2 transformed) were analysed with the Qlucore Omics Explorer software package, followed by Ingenuity Pathway Analysis (Qiagen, IPA). This analysis revealed that severe hyperglycaemia caused pronounced differences in transcript expression profile (Figure 60). 1258 genes were differentially regulated when comparing control neutrophils of standard chow fed mice to control neutrophils of DIO/STZ mice. When comparing IL-4 treated neutrophils, 1289 genes were differentially regulated, and 1664 genes showed an altered expression when looking at LPS/IFN-γ treated neutrophils (Figure 60 [A]). When comparing all conditions, PCA analysis and heat map strikingly showed that neutrophils of standard chow fed mice and DIO/STZ mice from each treatment aligned next to each other, but separated clearly from one another (Figure 60 [B]). In order to demonstrate those differences more clearly, pairwise comparisons of only control or IL-4 or LPS/IFN-γ treated neutrophils of standard chow fed mice with neutrophils from the same conditions of DIO/STZ mice were performed (Figure 60 [C]). The heat maps displayed more clearly, that neutrophils of standard chow fed mice and DIO/STZ mice were considerably different for all tested conditions, demonstrating that severe hyperglycaemia induced major transcript expression alterations when compared to neutrophils of normoglycaemic mice.

[A]

Comparisons	Differentially regulated genes
Control DIO/STZ vs Control Chow	1258
IL-4 DIO/STZ vs IL-4 Chow	1289
LPS/IFNγ DIO/STZ vs LPS/IFNγ Chow	1664

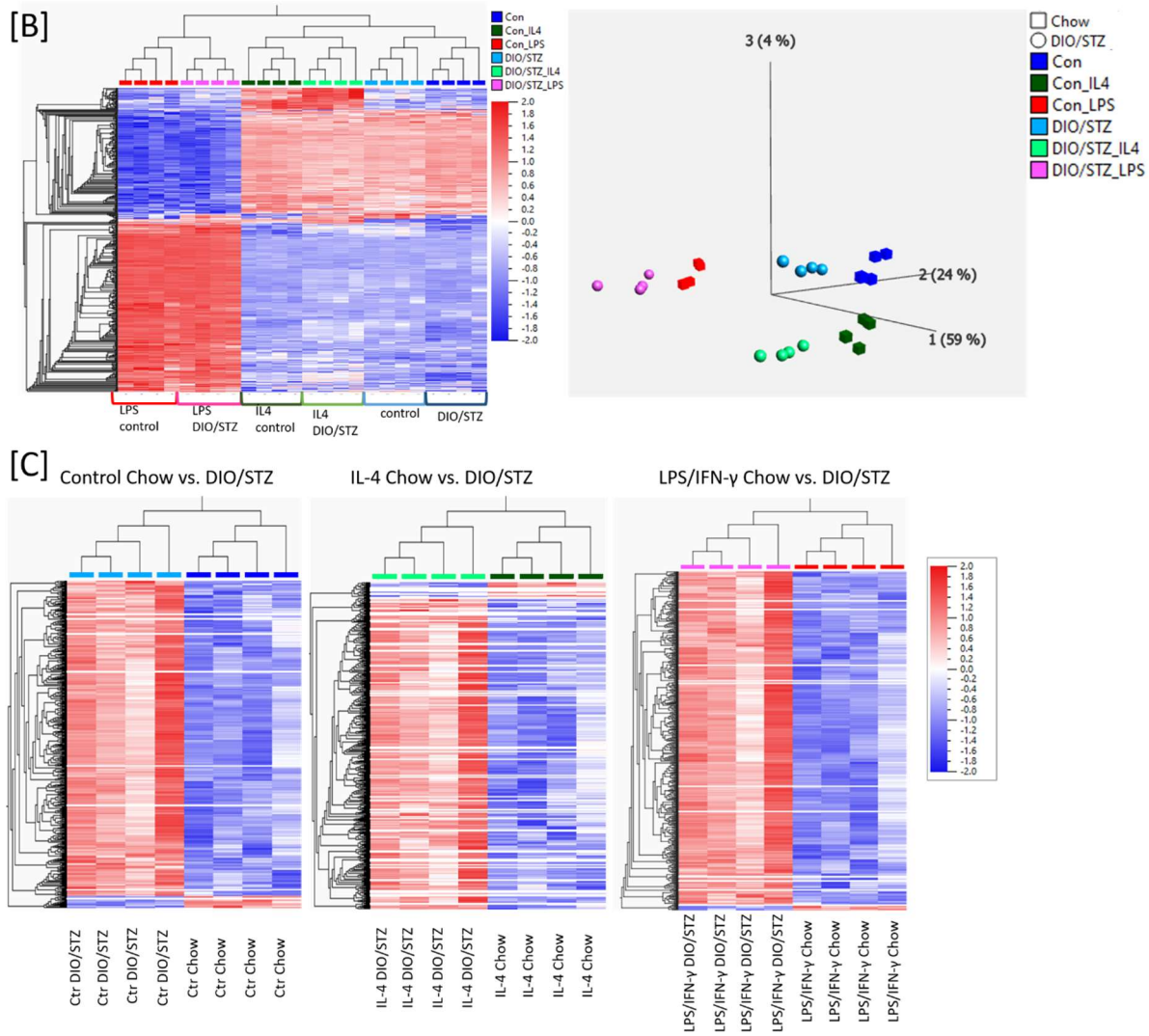

Figure 60: **Hyperglyceamia alters gene expression profile of murine BM neutrophils.**

Murine BM neutrophils of standard chow fed or DIO/STZ mice were left untreated (control) or polarized for four hours with IL-4 or LPS/IFN-γ and RNA sequencing was performed. Summary of amount of differentially expressed genes when comparing neutrophils of standard chow fed mice to DIO/STZ mice **[A]**. Heat map and PCA plot of all conditions visualises differences in the transcript expression profile of the different conditions **[B]**. Heat maps of pairwise comparisons are shown to display differences in gene expression more clearly **[C]**. Fold change (FC)=1.5; q=0.01, n=4 experiments.

In order to get more quantitative measures of the effect of severe hyperglyceamia on neutrophil phenotype, the upstream regulator and canonical pathway functions of IPA were used to identify possible regulators affected by hyperglycaemia (Figure 61). By

using the differentially expressed genes of control neutrophils of standard chow fed mice versus control neutrophils of DIO/STZ mice, IPA analysis revealed significant differences in pathway activation already under basal conditions (Figure 61). Neutrophils of DIO/STZ mice without treatment (control) showed upregulation of signalling pathways known to be increased in diabetes as Nfat, Creb, and Fak signalling. Furthermore, inflammatory signalling (S100 family and cytokine storm signalling) was upregulated in neutrophils of hyperglycaemic mice (DIO/STZ) without treatment (control).

In contrast, control neutrophils of hyperglycaemic mice (DIO/STZ) showed downregulation of anti-inflammatory signalling, as for example IL-10 and antioxidant vitamin C signalling, indicating a more pro-inflammatory phenotype of hyperglycaemic neutrophils already under basal conditions.

Analysis of IL-4 treated neutrophils revealed, that in general the same pathways as in basal neutrophils were differentially regulated. Thus, the pro-inflammatory phenotype of neutrophils isolated from DIO/STZ mice persisted after treatment with the N2 polarizer IL-4. Additionally, inflammatory NET and Phospholipase C signalling, known to mediate activation of Nfκb and interferon regulatory factors (Zhu, Jones et al. 2018), were upregulated. Furthermore, granzyme A, whose deficiency was linked to increased inflammation, and apoptosis signalling were downregulated in IL-4 treated neutrophils of DIO/STZ mice. Therefore, these data display again a pro-inflammatory phenotype of those neutrophils, however, there is no indication that additional IL-4 treatment was able to reduce basal inflammation levels in neutrophils of DIO/STZ mice.

In line, LPS/IFN-γ treated neutrophils of DIO/STZ mice showed the same alterations in canonical pathways and the treatment did not seem to aggravate inflammatory levels of those neutrophils.

The upstream regulator function in IPA was used to identify which upstream activator might modulate the observed changes in gene expression. Again, this analysis indicated that pro-inflammatory signalling was upregulated in neutrophils of DIO/STZ mice, as the most upregulated upstream regulators were inflammatory cytokines in basal neutrophils of DIO/STZ mice (*Tnf, Ifnγ, Il1b, Ifnα, Csf2, Tnfsf11*). Beside inflammatory cytokines, also Nfκb signalling (*Rela, Rel, Myc, Nfκb complex*) was upregulated in basal neutrophils of DIO/STZ mice (Figure 61).

In accordance, the most downregulated upstream regulators in basal neutrophils of DIO/STZ mice (*Trex1*, *Cited2*, *Irf2bp2, let-7*) were all known to attenuate inflammation, supporting the hypothesis of a more pro-inflammatory phenotype of hyperglycaemic neutrophils.

Again, in IL-4 treated neutrophils of DIO/STZ mice inflammatory cytokines and Nfκb related genes were the most increased upstream regulators. The most downregulated

upstream regulators (*Cited2, Socs1, Sirt, GFI1, IL10ra*) were again all linked to anti-inflammatory properties.

Also in LPS/IFN-γ treated neutrophils of DIO/STZ mice, inflammatory cytokines (*Csf2, Tnf, Il1b, Il27)* and Nfκb-related genes were the most increased upstream regulators. Further, *Egf,* which was reported to prime and even augment the inflammatory response of neutrophils, and *Ahr,* whose overactivation was reported to drive inflammation (Lewkowicz, Tchórzewski et al. 2005), were amongst the most increased upstream regulators. Again, in LPS/IFN-γ treated neutrophils of DIO/STZ mice upstream regulators with anti-inflammatory characteristics were the most downregulated upstream regulators (*Mxd1, Kdm5a, Irf2bp2* and *GFI1)*.

Taken together, these data demonstrate, that neutrophils of DIO/STZ mice show already under basal conditions pro-inflammatory characteristics, which are not substantially altered due to additional treatment with IL-4 or LPS/IFN-γ.

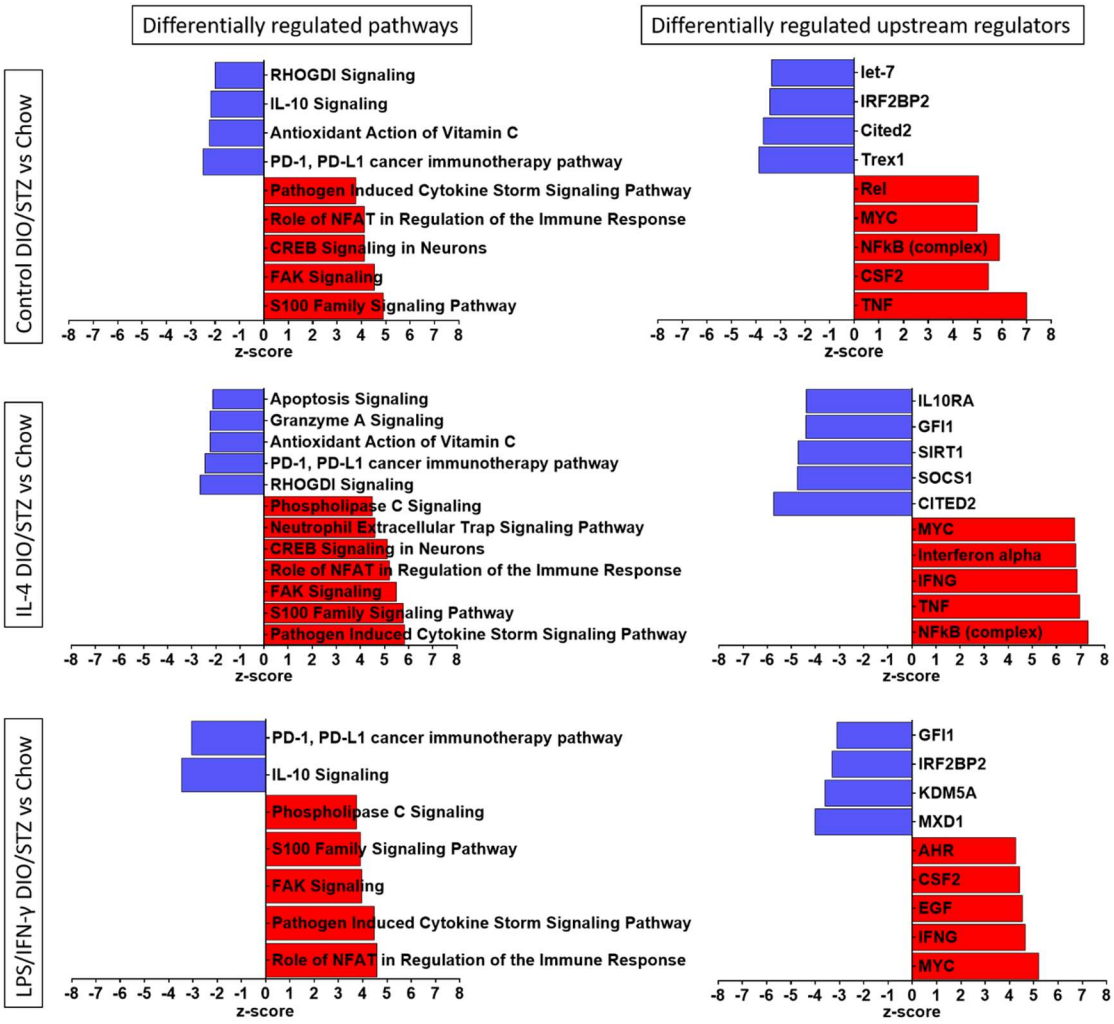

Figure 61: **Hyperglyceamia induces severe changes in neutrophil signalling.**

IPA pathway and upstream regulator analysis was performed. The top up- and down-regulated pathways and upstream regulators in control, IL-4 and LPS/IFN-γ treated neutrophils of DIO/STZ mice are depicted. Fold change (FC)=1.5; p=0.05; n=4 experiments.

As upstream regulator analysis by IPA revealed that several genes related to the Nfκb pathway and inflammatory cytokines were upregulated in neutrophils of DIO/STZ mice, a closer look at pro-inflammatory genes was taken (Figure 62). This revealed, that indeed several Nfκb-related genes (*Nfκb1, Nfκb2, Nfκbie, Rel, Relb, Myc, Jun*, *Junb, Tlr4)* and pro-inflammatory genes (*IL12a, Tnfrfs1b, Irfs, Socs*) were upregulated already under basal conditions (control) in DIO/STZ neutrophils. Figure 62 displays only exemplary genes. *Nfκb1, IL12a* and *Tnfrfs1b* were upregulated under basal conditions in DIO/STZ neutrophils (Figure 62 [A]). Also upon IL-4 treatment, those pro-inflammatory genes still showed increased expression levels in DIO/STZ compared to standard chow fed mice. However, further pro-inflammatory LPS/IFN-γ stimulation did not enhance expression of those genes in hyperglycaemic neutrophils, indicating that the maximal expression level is already reached upon LPS/IFN-γ treatment in normoglycaemic neutrophils (Figure 62 [A]).

Myc, Jun and *Rel* expression were as well upregulated under basal conditions and upon IL-4 treatment in DIO/STZ neutrophils when compared to neutrophils of standard chow fed mice. In contrast, LPS/IFN-γ treatment even enhanced upregulation of those genes in DIO/STZ neutrophils (Figure 62 [B]).

Thus, these data again indicate that hypergylcaemic neutrophils showed already under basal conditions (control) pro-inflammatory characteristics, which were partially enhanced upon inflammatory stimulation with LPS/IFN-γ.

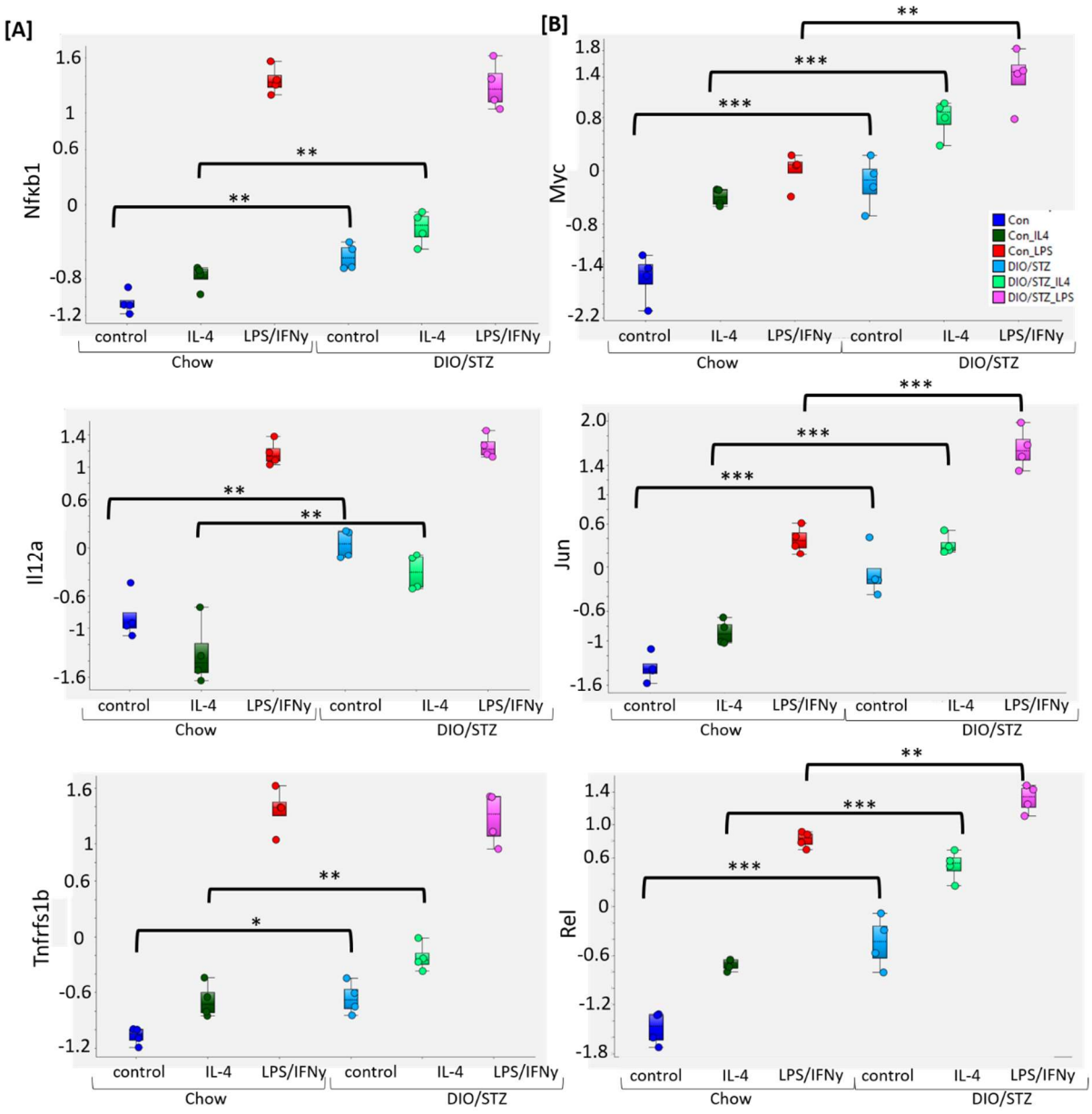

Figure 62: **Hyperglycaemia increased pro-inflammatory gene expression in murine bone marrow neutrophils.**

Expression of pro-inflammatory genes as *Nfκb1, Myc, IL12a, Jun, Tnfrfs1b* and *Rel* are depicted. Fold change (FC)=1.5; q=0.01; n=4 experiments. Statistical significance was calculated using One-way ANOVA (Tukey post hoc test) (*p<0.05, **p<0.01, ***p<0.001).

Furthermore, there seems to be a trend towards downregulation of anti-inflammatory genes, but significance levels were not reached (Figure 63). The newly identified N2 marker gene *Slc28a3* (*Solute Carrier family 28 member 3,* nucleoside transporter*)* showed a significant reduction of gene expression under basal conditions (control) in neutrophils of DIO/STZ mice. Upon IL-4 and LPS/IFN-γ treatment, expression levels were still reduced. Also, the anti-inflammatory gene *Chil3* was reduced under all tested

conditions in DIO/STZ mice. *Car4* gene expression was reduced in DIO/STZ mice only upon IL-4 treatment. However, hyperglycaemia had no effect on *Arg1* gene expression. Therefore, hyperglycaemia also negatively affects anti-inflammatory gene expression and thus pointing again towards a more pro-inflammatory phenotype of hyperglycaemic neutrophils.

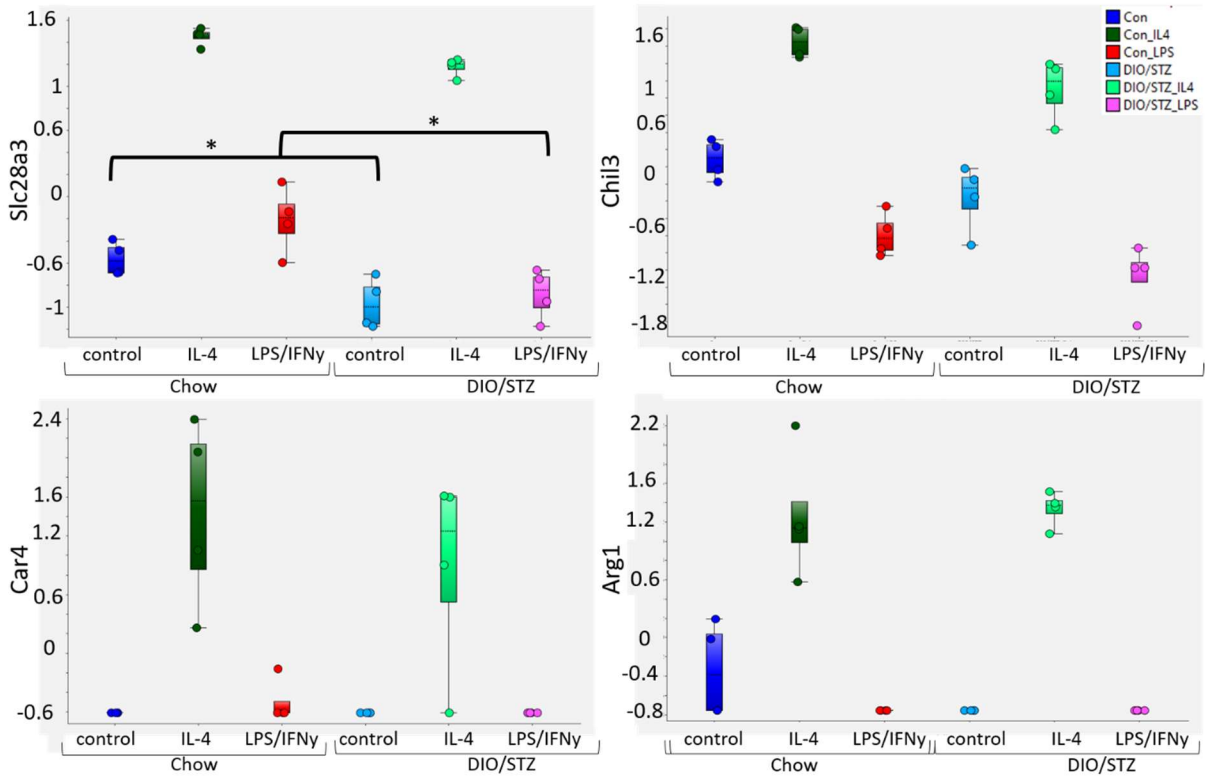

Figure 63: Hyperglycaemia tends to reduce anti-inflammatory gene expression in murine bone marrow neutrophils.

Expression of anti-inflammatory genes as *Slc28a3*, *Chil3*, *Car4* and *Arg1* are depicted. Fold change (FC)=1.5; q=0.01; n=4 experiments. Statistical significance was calculated using One-way ANOVA (Tukey post hoc test) (*p<0.05, **p<0.01, ***p<0.001).

4.5.3.2. Severe hyperglycaemia also alters some neutrophil functions

To analyse if the transcriptional changes translate into a pro-inflammatory phenotype of neutrophils from hyperglycaemic mice, functional assays were applied

Therefore, the effect of severe hyperglycaemia on the formation of NETs for neutrophils from DIO/STZ mice was analysed. Again, murine bone marrow neutrophils of standard chow fed and DIO/STZ mice were treated for 4 hours with the known polarizers IL-4 and LPS/IFN-γ. Additionally, neutrophils were treated in the presence or absence of PMA or were left untreated, which served as controls. QuantIT PicoGreen was added, and subsequently fluorescence was measured, indicating the amount of dsDNA in solution

(Figure 64 [A], [B]). This analysis revealed, that the formation of neutrophil extracellular traps in DIO/STZ mice was not significantly increased under all tested conditions, however, the formation of NETs was enhanced slightly. NET formation under basal conditions was enhanced by 11.6%, after IL-4 treatment by 24.1% and after LPS/IFN-γ treatment by 31.85% when compared to standard chow fed mice. Therefore, there might be a slight increase in inflammation capability when compared to standard chow fed mice.

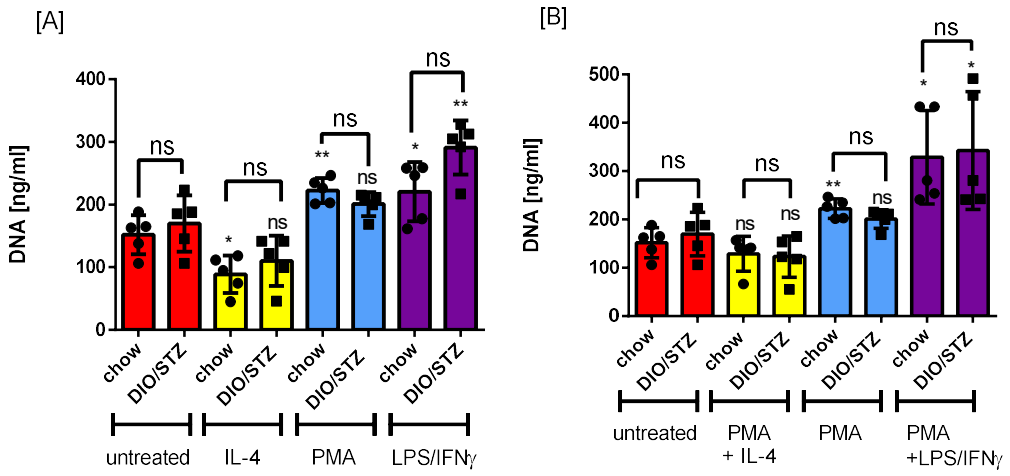

Figure 64: **Neutrophils of DIO/STZ mice show a slight increase in the formation of NETs.**

Murine BM neutrophils of standard chow fed and DIO/STZ mice were left untreated, or treated with IL-4, LPS (10 ng/ml) + IFN-γ (2 ng/ml) and only PMA (100 ng/nl) **[A]** or additionally PMA (100 ng/ml) **[B]** for four hours. Samples are normalised to DNA standard concentrations. Bars represent mean ± SD, n=5. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 when compared to PMA).

Next, it was of interest to assess if severe hyperglycaemia has a more pronounced effect on neutrophil phagocytic activity. Therefore, murine bone marrow neutrophils of standard chow fed and DIO/STZ mice were left untreated or treated with IL-4 for 4 hours and subsequently challenged with fluorescently labelled *S. aureus* to analyse phagocytic capacity by flow cytometry (Figure 65). Interestingly, IL-4 treatment in neutrophils of DIO/STZ mice was not able to significantly increase phagocytic activity when compared to untreated neutrophils of DIO/STZ mice. When comparing IL-4 treated neutrophils of standard chow fed mice to IL-4 treated neutrophils of DIO/STZ mice, there was a significant restriction in phagocytic capacity, with a decrease by 42.2% in DIO/STZ neutrophils. Thus, these data point towards an impairment of phagocytic activity due to increased blood glucose levels.

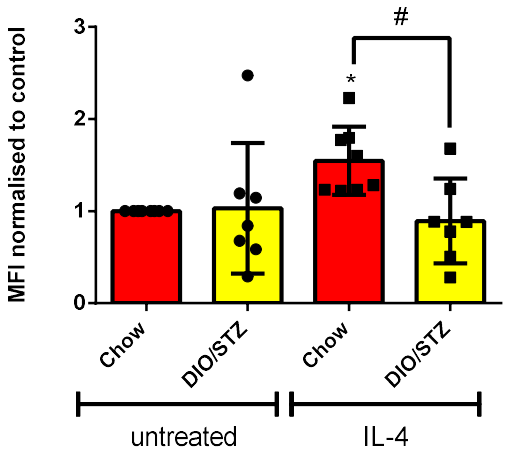

Figure 65: **Severe hyperglycaemia reduces phagocytic capacity of neutrophils.**

Murine BM neutrophils of standard chow fed and DIO/STZ mice were left untreated or treated with IL-4 for 4 hours and subsequently challenged with fluorescently labelled *S. aureus* for 15 minutes. Mean fluorescence intensity (MFI) of control chow is set to 1 and all other samples are normalised to control chow. Bars represent mean ± SD, n=8. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated of the same mouse model; #p<0.05, ##p<0.01 and ###p<0.001 when compared to IL-4 treated neutrophils of chow mice).

The regulation of cytokine secretion by neutrophils is a crucial target in many diseases. As hyperglycaemia is often linked to chronic inflammation, the question arose if hyperglycaemia also alters the cytokine secretion profile of neutrophils. Hence, it was investigated whether there are differences in cytokine secretion between standard chow fed and DIO/STZ neutrophils. Murine BM neutrophils of standard chow fed or DIO/STZ mice were cultivated in media only as a control or were polarized with IL-4, LPS/IFN-γ only or additional IL-4 for 4 hours and subsequently, the supernatant was analysed for a set of cytokines by flow cytometry, using a legendplex assay (Figure 66).

Interestingly, there are increasing reports of impaired cell chemotaxis in hyperglycaemia, and neutrophils of DIO/STZ mice showed significantly reduced cytokine levels of CXCL9 (by 63.5%), MCP-1 (by 63.5%) and CCL3 (by 49.2%) upon LPS/IFN-γ stimulation, which are essential chemokines for migration and adherence of neutrophils (Figure 66 [A]). Further, the cytokines IL-6 (by 74.5%) and IL17A (by 47.1%), which were recently reported to also exert anti-inflammatory effects, were reduced in DIO/STZ mice upon LPS/IFN-γ stimulation (Figure 66 [B]).

Additionally, anti-inflammatory cytokine IL-10 (by 69.7%) was reduced upon LPS/IFN-γ treatment, when compared to standard chow fed mice (Figure 66 [C]).

Moreover, IFNß (by 20.5%) and GM-CSF (by 26.6%) levels were significantly reduced in neutrophils of DIO/STZ mice upon IL-4 treatment, when compared to standard chow fed mice (Figure 66 [C]).

However, additional IL-4 treatment was not able to further reduce cytokine levels in both, standard chow fed and DIO/STZ neutrophils. Thus, hyperglycaemia seems to also affect cytokine secretion of neutrophils.

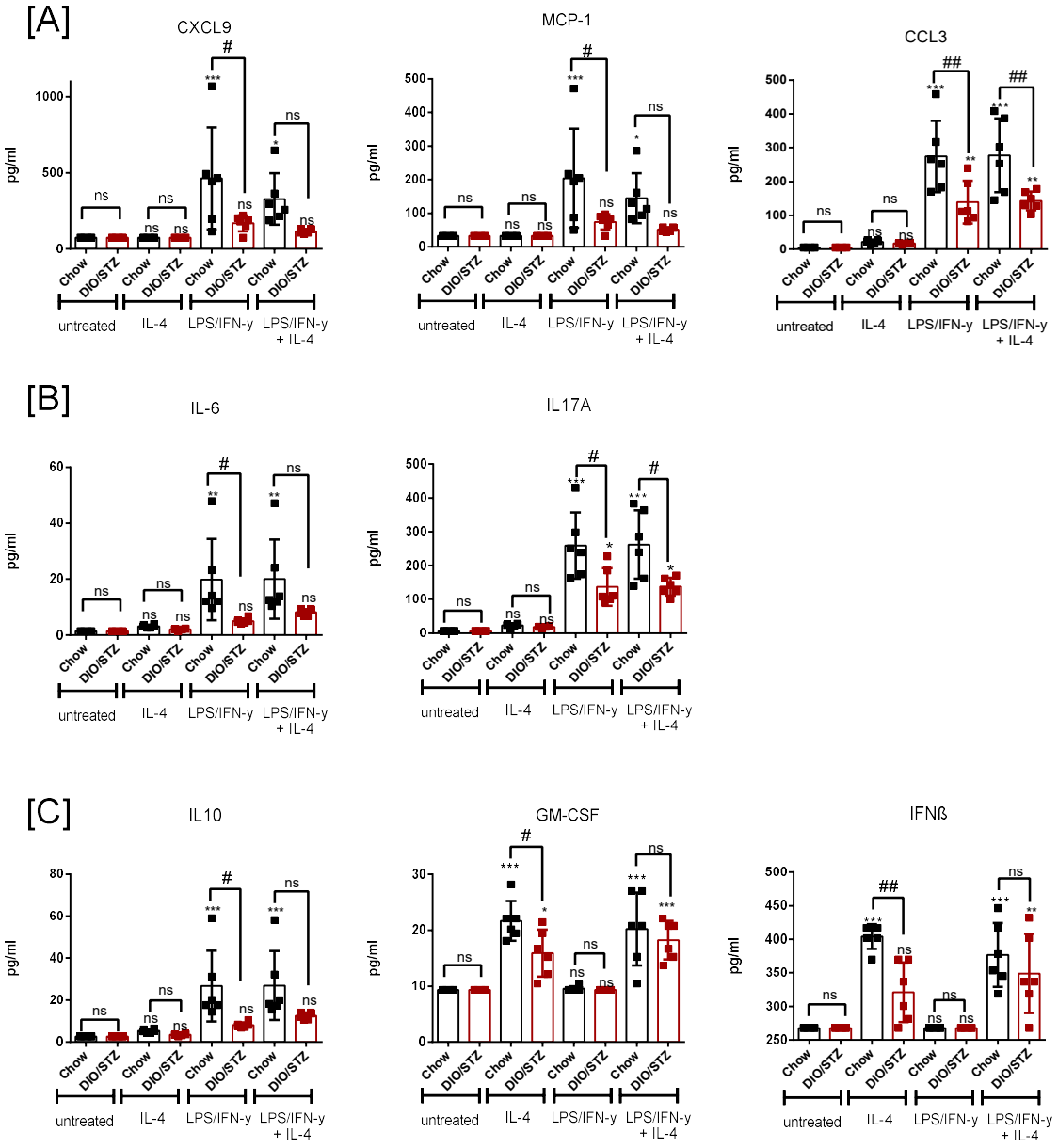

Figure 66: Hyperglycaemia affects the cytokine secretion profile of neutrophils.

Murine BM neutrophils of standard chow fed or DIO/STZ mice were left untreated or treated with LPS/IFN-γ, IL-4 only or additional IL-4 for 4 hours and supernatant was analysed for a set of cytokines by flow cytometry, using a legendplex assay. Bars represent mean ± SD, n=6. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated; #p<0.05, ##p<0.01 and ###p<0.001 when compared to the same treatment of neutrophils of chow mice).

Due to the observed changes in cytokine secretion, the question arose if those changes already appear on transcriptional levels. Therefore, polarized neutrophils of standard chow fed and DIO/STZ mice were analysed for some genes of cytokines that were altered or were unaffected by hyperglycaemia. Altered cytokines that were analysed also on transcriptional levels were for example IL-6, CXCL9 and MCP-1. As a control, also unaffected cytokines, IL-23, TNFα and IL-1ß, were analysed on transcriptional levels (Figure 67). This analysis also revealed a significant downregulation of the genes *IL-6* (by 43.8%), *Mcp-1* (by 46.4%) and *Cxcl9* (by 29.13%) in neutrophils of DIO/STZ mice on transcriptional levels. Unaltered cytokines IL-23, TNFα and IL1ß were as well unchanged on transcriptional level in neutrophils of DIO/STZ mice (Figure 67).

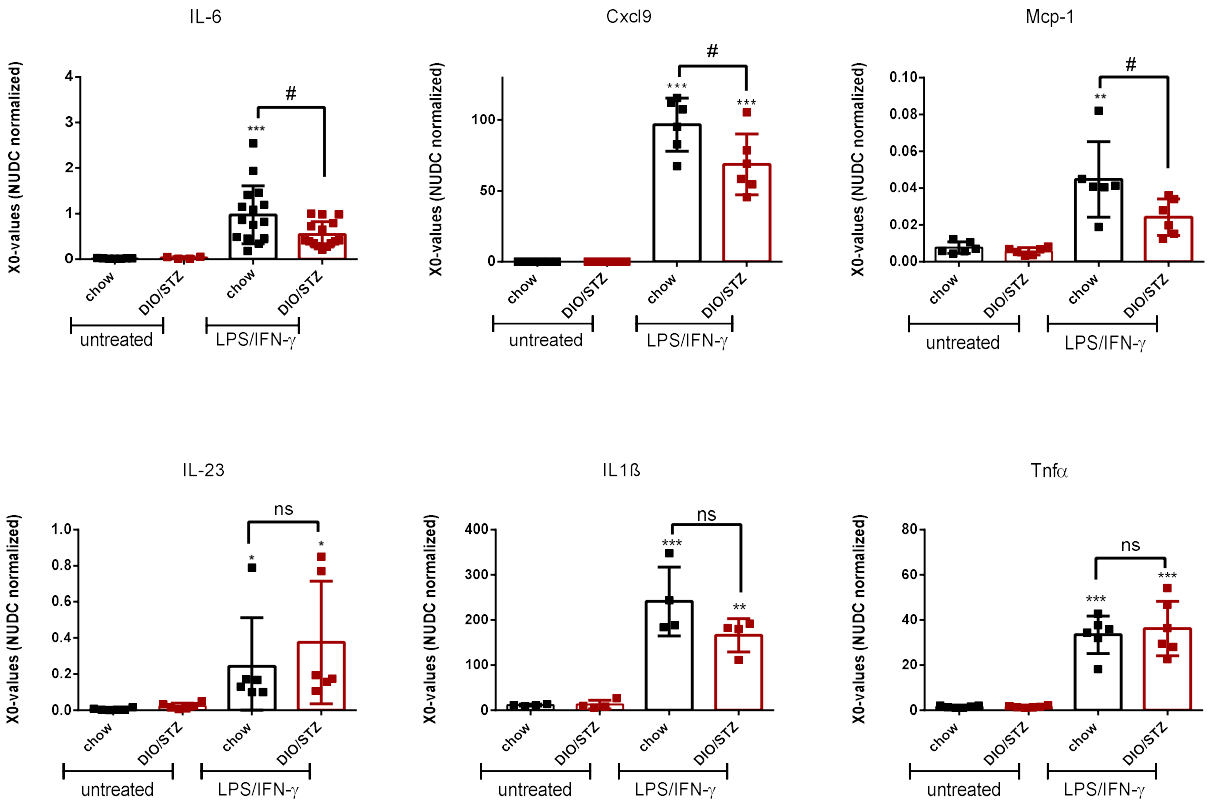

Figure 67: Changes in cytokine secretion appear already on transcriptional levels.
Murine BM neutrophils of standard chow fed and DIO/STZ mice were polarized untreated or treated with LPS (10 ng/ml) + IFN-γ (2 ng/ml) for 4 hours and qPCR analysis was performed. Neutrophils of DIO/STZ mice showed reduced upregulation of pro-inflammatory genes *IL-6, Cxcl9* and *Mcp-1,* but not of *IL-23, Tnfα* and *IL1ß.* X0 values are normalized to *Nudc*. Expression of single measurements are shown. Bars represent mean ± SD, n=4-11. Statistical significance between groups was calculated using parametric One-way ANOVA (Bonferroni`s Multiple Comparison post-test) (*p<0.05, **p<0.01, ***p<0.001 when compared to untreated; #p<0.05, ##p<0.01 and ###p<0.001 when compared to the same treatment of chow mice).

5. Discussion

The immune system plays a substantial role in the regulation of damage and repair processes. The polarization of murine BM neutrophils requires specific microenvironments *in vivo,* which are mimicked by diverse cytokines and chemokines during *ex vivo* polarization. As for macrophage polarization, LPS/IFN-γ are used to generate a pro-inflammatory and IL-4 to mimic an anti-inflammatory environment. The role of neutrophils is far from being understood, they are generally categorized into pro-inflammatory N1 and anti-inflammatory N2 subtypes.

One of the leading causes of morbidity and death in Europe is acute myocardial infarction (AMI) and until recently, neutrophils were considered to be detrimental in ischaemic heart disease, since they enhance inflammation and tissue damage (Roth, Huffman et al. 2015). However, it was recently described that neutrophils are crucial in cardiac remodelling and can have beneficial effects on myocardial healing (Ma, Yabluchanskiy et al. 2016). The efficiency of repair after AMI seems to be dependent on a well-coordinated balance between the inflammatory and reparative functions of the infiltrating immune cells. Thus, immune-modulatory therapies may represent an interesting option to improve the outcome of CVD, including heart failure and MI. Along the line, our group showed that insulin-like growth factor 1 has cardioprotective characteristics, which is mediated by myeloid cells and induced an anti-inflammatory macrophage phenotype (Heinen, Nederlof et al. 2019).

Type 2 Diabetes Mellitus (T2DM) strongly predisposes to and exacerbates cardiovascular diseases (Wild, Roglic et al. 2004, Lopez, Mathers et al. 2006). Until recently, T2DM was widely accepted as a metabolic disease, however, it is more and more often characterised as an immune-mediated inflammatory disease. It was reported that obesity and the following development of hyperglycaemia and hyperlipidaemia, often leading to T2DM and metabolic syndrome, results in chronic inflammation. Not much is known about neutrophils in T2DM, however, changes in macrophage function have been observed (Lumeng, Bodzin et al. 2007, Lackey and Olefsky 2016).

Based on this background, the mechanisms of neutrophil polarization and to what extent neutrophil phenotypes and functions are influenced by different diseases was analysed in this thesis. The major findings from the present study show that:

1) neutrophils can polarize *in vitro* at least to an anti- and pro-inflammatory phenotype and that polarization is dependent on JAK/STAT signalling,

2) IGF-1 treatment after AMI suppresses pro-inflammatory neutrophils in the heart *in vivo,*

3) a hyperglycaemic environment *in vivo* negatively affects neutrophil phenotype and

function, by suppressing rather anti-inflammatory characteristics.
These results indicate that the therapeutic modulation of neutrophil phenotype and function could offer a potential therapeutic strategy to improve outcome after AMI or to reverse the inflammatory state in hyperglycaemia. However, this could not only be of interest for the analysed illnesses, but could also be beneficial for many other diseases, accompanied by inflammation.

5.1. Neutrophils polarize *in vitro* to a pro- and anti-inflammatory phenotype

Novel proper phenotypic markers could help to further distinguish pro- and anti-inflammatory neutrophils or help to classify new neutrophil subtypes, besides the known classical N1 and N2 subtypes. In previous experiments I could show that Lipopolysaccharide/Interferon-γ (LPS/IFN-γ) treatment leads to the induction of a N1-like neutrophil phenotype, identified by the up-regulation of the known pro-inflammatory M1 macrophage markers *Tnfα*, *Nos2* and *IL-12a*, whereas Interleukin-4 (IL-4) induced a N2-like neutrophil phenotype, characterised by the induction of known anti-inflammatory M2 markers *Retnlα*, *Arg1* and *Chil3* (Figure 3, Masterthesis Sophia Reidel 2020).

RNA sequencing transcript expression analysis identified new possible neutrophil N1- and N2- marker genes, which were analysed in this thesis. *IL-6* and *IL1ß* were analysed as novel N1 neutrophil marker genes and *Carbonic anhydrase 4 (Car4), Solute Carrier family 28 member 3 (Slc28a3)* and *TAFA Chemokine Like Family Member 3* (*TAFA3/Fam19a3)* as novel N2 marker genes (Figure 23). The investigation of the new neutrophil polarization genes, induced by different cytokines, revealed a significant upregulation of the N1 markers *IL-6* and *IL1ß* (Figure 23 [B]) only upon LPS/IFN-γ treatment. In macrophages, *IL-6* and *IL-1β* were as well upregulated upon LPS/IFN-γ stimulation and were used as M1 marker genes (Orecchioni, Ghosheh et al. 2019, Zhu, Guo et al. 2021, Purcu, Korkmaz et al. 2022). Only IL-4 treated neutrophils showed upregulation of the new marker genes *Car4, Slc28a3* and *TAFA3,* which are associated with anti-inflammatory marker genes (Figure 23 [A]). *Carbonic anhydrase 4* is an enzyme which creates an acidic environment by transforming carbon dioxide to bicarbonate plus a proton (Waheed, Zhu et al. 1992). Interestingly, *Car4* is mainly expressed by neutrophils and activation and prolonged survival of neutrophils is dependent on an acidic extracellular environment (Trevani, Andonegui et al. 1999, Martinez, Vermeulen et al. 2006, Barker, Aaltonen et al. 2017). Baker et al. showed, that *Car4* is elevated immediately after wounding, thus during the early hypoxic phase of healing, and returned to baseline by day 7. They additionally demonstrated, that exogenous Car4 accelerated

wound re-epithelialization by extending neutrophil survival and function (Barker, Aaltonen et al. 2017). Therefore, it can be concluded, as an upregulation of *Car4* is induced by the N2-stimulator IL-4, that IL-4 treatment leads to an improved tissue regeneration after MI, inter alia due to elevated *Car4* expression, which induces an acidic environment and thus prolongs neutrophil survival, which is essential for proper wound repair. However, since acidosis prevails during ischaemia anyway, it is unclear to what extent this effect is caused by *Car4*. Furthermore, Shao et al. as well identified *TAFA3* as a M2 marker gene, as it promoted M2 polarization and suppressed M1 polarization of microglia and macrophages. Additionally, *Tafa3* alleviated cerebral ischaemia (Shao, Deng et al. 2015). In contrast, not much is known about the gene *Slc28a3,* but that it is involved in the homeostasis of endogenous nucleosides (GeneCards, GC09M104171, 25.08.2023). Moreover, it was not upregulated in IL-4 stimulated macrophages. These novel proper phenotypic markers could help to further characterise and distinguish pro- and anti-inflammatory neutrophils.

After verifying that neutrophils polarize towards a pro- and anti-inflammatory phenotype, it was as well of interest to analyse if an additional anti- or pro-inflammatory stimulus is able to alleviate N1- or N2-polarization (Figure 24). Further, I also analysed if only LPS or only IFN-γ treatment as well induced upregulation of pro-inflammatory genes (Figure 24 [B]). This analysis revealed that a pro-inflammatory stimulus was able to significantly reduce upregulation of N2-marker gene *Retnlα* (Figure 24 [A]). Thereby, IFN-γ treatment alone caused a stronger reduction of IL-4 induced *Retnlα* expression, than LPS treatment alone (Figure 24 [A]). Interestingly, increased Socs3 (Suppressor of Cytokine Signalling 3) was reported to dampen STAT6 activation induced by IL-4. In line, IFN-γ strongly induced Socs3 transcription, whereas LPS did not (Starr, Willson et al. 1997, Luckey, Kim et al. 2020). Thus, IFN-γ-induced Socs3 could be a possible explanation for the potent inhibition of IL-4-induced *Retnlα* expression by IFN-γ. Further, it was reported that IFN-γ can suppress anti-inflammatory M2 polarization (Kang, Park et al. 2017).

Furthermore, LPS alone, but not IFN-γ alone, was able to significantly induce *Tnfα* expression (Figure 24 [B]). It is well known that *Tnfα* is induced by LPS (Matic and Simon 1991). It was also reported that IFN-γ can induce *Tnfα* expression and release, however to a lesser extent than LPS, which is in line with the shown data here (Vila-del Sol, Punzon et al. 2008). In contrast, additional IL-4 treatment induced only a minor reduction of *Tnfα* expression and not of *Nos2* expression (Figure 24 [B]). However, it was shown in literature that IL-4 can dampen IFN-γ-, as well as LPS-induced inflammatory responses in macrophages, but it might be that it needs a longer period than four hours to develop this effect (Piccolo, Curina et al. 2017, Czimmerer, Daniel et al. 2018).

Moreover, there is rising evidence that biological sex seems to be a contributing factor to the severity and progression of several diseases. It was reported that men have an increased risk for several diseases, as cardiovascular disease and stroke, but also for infectious diseases, as SARS-CoV-2 (Bots, Peters et al. 2017, Takahashi, Ellingson et al. 2020). Based on this background, it was analysed in this thesis, whether biological sex influences neutrophil polarization and thus would be an important consideration for following experiments and the development of possible therapies. This analysis revealed that sex had no effect on upregulation of N2-marker genes *Retnlα* and *Arg1* (Figure 25 [A]). Interestingly, inflammatory N1 neutrophil polarization was substantially reduced in female mice (Figure 25 [B]). Thus, these data demonstrate sex-specific differences in neutrophil polarization. In line, it was demonstrated in literature that male mice produced more inflammatory cytokines, as TNFα and IL-1ß, whereas female mice showed reduced expression of *iNOS* and *CD80,* but no differences in *Arg1* and *Retnlα* expression (Deny, Nunez et al. 2022). Further, male macrophages showed higher *NFκB, Tnfα* and *IL-1ß* expression upon LPS stimulation, but no significant differences in *Retnlα* expression upon IL-4 stimulation (Barcena, Niehues et al. 2021).

Taken together, the present study revealed that neutrophils polarize to a pro- and anti-inflammatory phenotype *in vitro*. Anti-inflammatory neutrophil polarization can be dampened by an inflammatory stimulus, whereas the pro-inflammatory polarization could be restrained only slightly. Furthermore, it revealed a male-specific polarization of neutrophils with an increased response to a pro-inflammatory environment. Due to these differences, I continued to work with only male mice in the following experiments. However, it would be of interest to further analyse if neutrophils of male individuals are more prone to an inflammatory state and therefore be a cause of increased risk of cardiovascular diseases and inflammatory diseases in males.

5.1.1. Neutrophil polarization is dependent on the JAK/STAT signalling pathway

Based on the finding that neutrophils polarized to an anti- and pro-inflammatory phenotype, it was of interest to analyse possible signalling pathways responsible for neutrophil polarization. Known IL-4 and LPS/IFN-γ mediated signalling pathways are the AKT, RAS/RAF/ERK and JAK/STAT pathways, therefore those three pathways were analysed (see 4.2).

The JAK/STAT pathway is an essential mediator for many cellular mechanisms, including cell survival, differentiation and migration (Ghoreschi, Laurence et al. 2009). It was shown here that IL-4 enhanced phosphorylation of STAT6, whereas LPS/IFN-γ induced STAT1

activation (Figure 30). For the analysis of the dependency of neutrophil polarization on the Janus kinases (JAK), different JAK inhibitors were used and IL-4- or LPS/IFN-γ-induced gene expression and STAT-activation was analysed (Figure 29, Figure 30). The specific inhibition of single JAK isoforms was achieved by using several inhibitors at different concentrations, thereby making use of different IC_{50} values (Table 13). In doing so, the JAK2 isoform was identified as the predominant isoform mediating IL-4-induced N2-polarization and JAK1 as the predominant isoform mediating LPS/IFN-γ-induced N1-polarization (Figure 29). A specific JAK2 inhibitor prevented, to a large extent, N2-like gene expression and STAT6 phosphorylation upon IL-4 treatment. In contrast, only JAK1 activity led to N1-like gene expression and STAT1 phosphorylation, however to a lesser extent (Figure 29, Figure 30). JAK is known to be an important signalling molecule of IL-4, and also of INF-γ signalling, as its activation results in the phosphorylation and activation of PI3K and STAT (Reichel, Nelson et al. 1997, Nelms, Huang et al. 1998). It was shown in macrophages, but for neutrophils it is still unknown, that M1 polarization induces STAT1 phosphorylation, whereas M2 polarization induces STAT6 phosphorylation (Gong, Zhuo et al. 2017, Ding, Wang et al. 2019). Thus, it was shown here that N1- and N2- neutrophil polarization is dependent on the same STAT activation as in macrophages (Figure 30). In line, JAK1 and STAT1 activation was reported to be important for M1 macrophage polarization, and here it was shown that it also seems to be important for pro-inflammatory N1 neutrophil polarization (Liang, Tang et al. 2017). In human endothelial cells it was as well shown, that the IL-4 response is dependent on JAK2/STAT6 signalling, however for macrophages also a partial dependency on JAK1 was reported (PalmerCrocker, Hughes et al. 1996, He, Gao et al. 2020, Runtsch, Angiari et al. 2022), indicating that IL-4-induced signalling can vary between different cell types. This variability could depend on which IL-4R (type I or type II) is predominantly used by the different cell types.

Whereas all data indicate a dependency on the JAK2/STAT6 axis in N2-like and JAK1/STAT1 axis in N1-like neutrophil polarization *in vitro*, no indication that neutrophil polarization required AKT or ERK activation was found (Figure 27, Figure 28). N2-like, as well as N1-like gene expression, was unaffected in BM neutrophils derived from global (Δ) as well as Tie2- AKT1 and AKT2 knockout mice. Further, IL-4 and LPS/IFN-γ, in concentrations that induce N2- or N1-like gene expression, did not induce AKT phosphorylation (Figure 27). These results demonstrate a difference between neutrophil and macrophage polarization because it was reported in the literature that the AKT isoforms are involved in macrophage polarization. For macrophages it was shown, that the AKT1 isoform contributes to M2-like polarization, whereas the AKT2 isoform is involved in M1-like macrophage polarization (Androulidaki, Iliopoulos et al. 2009, Arranz,

Doxaki et al. 2012, Vergadi, Vaporidi et al. 2014).

Interestingly, insulin, a known activator of ERK signalling, activated the RAS/RAF/ERK pathway, but did not induce STAT6 or STAT1 phosphorylation or anti- or pro-inflammatory gene expression, therefore arguing against an essential role of the Ras/Raf/ERK pathway in N2- and N1-like neutrophil polarization (Figure 28). A contribution of ERK in macrophage polarization was described in literature, however contradictory reports exist. On the one hand, it was reported that ERK contributes to IL-4-induced M2-like polarization, and on the other hand, that ERK activation induces macrophage polarization to a M1-like phenotype (He, Jhong et al. 2021, Shen, Huang et al. 2021). Therefore, an involvement of ERK in macrophage polarization is questionable. Additionally, I found no indication for a contribution of ERK or AKT activation in neutrophil polarization in literature.

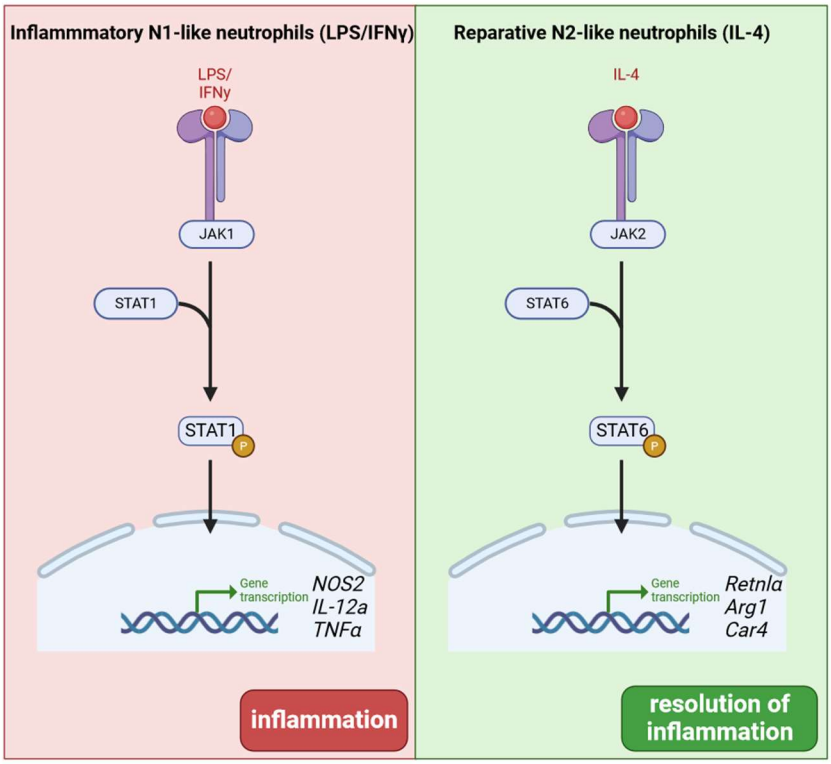

Figure 68: Summarised signalling of inflammatory N1- and reparative N2-like neutrophils.

LPS/IFN-γ treatment induced activation of JAK1, which in the following induced activation and phosphorylation of STAT1, thereby causing polarization towards a pro-inflammatory phenotype with upregulated expression of inflammatory genes (e.g. *Nos2, IL-12a, Tnfα*). In contrast, IL-4 treatment induced activation of JAK2, which in the following induced activation and phosphorylation of STAT6, thereby causing polarization towards a reparative phenotype with upregulated expression of anti-inflammatory genes (e.g. *Retnlα, Arg1, Car4*). Created with BioRender.com.

5.1.2. N2-like neutrophils exhibit inflammation-suppressing functions

Neutrophils can eliminate pathogens by different mechanisms, such as phagocytosis, degranulation, or by neutrophil extracellular traps (NETs). In some cases, as for example in the event of infection, inflammation is essential and beneficial for the protection from exogenous pathogens and to repair the damaged tissue. However, the inflammatory response is often also detrimental, as it can damage the host and lead to tissue dysfunction. Therefore, it was investigated if modulation of neutrophil phenotypes, as detected on the transcriptional level, also alters neutrophil functions.

5.1.2.1. Anti-inflammatory polarization attenuates NET formation

Upon activation, neutrophils release NETs, which are extracellular net-like structures composed of histones, granule proteins and chromatin (Brinkmann, Reichard et al. 2004). Brinkmann et al. showed *in vivo*, that NETs are abundantly present at the sites of inflammation (Brinkmann, Reichard et al. 2004). As IL-4 treatment induced upregulation of anti-inflammatory genes, the question arose if the treatment could also attenuate inflammatory functions of neutrophils or augment resolution of inflammation. Therefore, the formation of NETs of untreated neutrophils or treated with PMA, IL-4, LPS/IFN-γ, insulin ± PMA was analysed (Figure 32). PMA is a well characterised initiator of NETosis *in vitro* and was used as a positive control for the induction of the formation of NETs (Hoppenbrouwers, Autar et al. 2017). This analysis revealed, that PMA and high concentrations of LPS (25 µg) + IFN-γ induced the formation of NETs, whereas insulin treatment did neither dampen nor induce the formation of NETs (Figure 32). Also in literature, it was reported that LPS induces the formation of NETs, however only at high concentrations (Khan, Farahvash et al. 2017, Li, Ng et al. 2017). LPS/IFN-γ-induced NETosis can possibly be explained by the fact that NETosis is a potent measure against bacterial infections, as NETs trap, immobilize and kill bacteria, viruses and parasites (Brinkmann, Reichard et al. 2004, Riyapa, Buddhisa et al. 2012, Halverson, Wilton et al. 2015, Azzouz, Cherry et al. 2018). As LPS derives from gram-negative bacteria, it is presumable that neutrophils activate NETosis to combat bacterial infection.

In contrast, the formation of NETs can also be detrimental. It was for example reported that NETs also play an important role in the development of thrombosis and that the intensity of NETs directly correlates with infarction size and are predictors of adverse cardiovascular events (Fuchs, Brill et al. 2010, Borissoff, Joosen et al. 2013, Helseth, Shetelig et al. 2019, Bonaventura, Vecchie et al. 2020). In those cases, it would be of advantage to be able to dampen NETosis. In line with the above shown reduction of NETosis upon IL-4 treatment in murine BM neutrophils (Figure 32), Impellizzieri et al.

showed that IL-4 treatment significantly reduced NET formation also in human neutrophils (Impellizzieri, Ridder et al. 2019). Thus, an anti-inflammatory stimulus is indeed able to alter neutrophil function and attenuate pro-inflammatory characteristics, therefore being beneficial in the therapy of many inflammatory diseases.

5.1.2.2. Anti-inflammatory polarization augments phagocytotic capacity

Neutrophils are the prevailing phagocytes in the human blood and phagocytosis is also an important mechanism in host defence and the resolution of inflammation (van Kessel, Bestebroer et al. 2014). Interestingly, phagocytes can both, induce inflammatory or anti-inflammatory reactions. They induce an inflammatory reaction if they interact with foreign material, whereas anti-inflammatory mediators are released to reduce tissue destruction when opposed to apoptotic cells (Flannagan, Jaumouille et al. 2012). Interestingly, in this study it was shown that IL-4 treatment significantly enhanced the phagocytic capacity of neutrophils (Figure 33). Increased phagocytosis is as well a characteristic of anti-inflammatory macrophages (Schulz, Severin et al. 2019). In line, it was also demonstrated that phagocytic activity of microglia and macrophages was enhanced upon IL-4 treatment (Daseke, Tenkorang-Impraim et al. 2020, Yi, Jiang et al. 2020). In the context of myocardial infarction, neutrophils are inter alia important for the resolution of inflammation, as they phagocyte cellular debris and necrotic myocardium (Daseke, Valerio et al. 2019). Thus, increased phagocytosis could facilitate resolution of inflammation and, therefore, would have beneficial effects on disorders as myocardial infarction.

5.1.2.3. Neutrophil polarization alters degranulation of neutrophils

In order to analyse degranulation, which is another immune mechanism by neutrophils, CD11b, CD18 and CD35 expression was analysed. Neutrophils are densely packed with different kinds of granules, which are released in a specific order upon stimulation (Kolaczkowska and Kubes 2013). CD11b is a tertiary granule and is translocated from the intracellular milieu to the cell surface upon activation. I also took a closer look at the tertiary granule CD18, which forms a complex with CD11b (Hughes, Hollers et al. 1992). Further, I analysed CD35, which is contained in secretory vesicles (Hughes, Hollers et al. 1992, Deree, Lall et al. 2006). The extent of neutrophil degranulation was determined following isolation and following polarization (Figure 34). This analysis demonstrated, that the isolation process stimulated activation of neutrophils, which is shown in a significantly increased CD11b and slightly increased CD18 surface expression (Figure 34). It was

reported, that strongly activated neutrophils express high levels of CD11b and CD18, thus they can be used as markers of activated neutrophils (Hughes, Hollers et al. 1992). However, this activation level, induced by the isolation process, was reversed after an incubation period of 4 hours, as CD11b and CD18 surface expression were reduced again when compared to basal conditions (Figure 34).

As expected, treatment with LPS/IFN-γ significantly stimulated activation of neutrophils, shown by the increased mean CD11b and CD18 surface expression, whereas CD11b and CD18 levels on IL-4 treated neutrophils were not altered (Figure 34). In line, Zhou et al. displayed an upregulation of CD11b, dependent on LPS-induced TLR4 activation, which was substantial for the adhesion and transendothelial migration of neutrophils (Zhou, Gao et al. 2005). It was also shown that CD18 is necessary for the detection of *E.coli* by binding to LPS and that CD11b/CD18 is crucial for a series of LPS-induced genes, as for example the expression of inflammatory *IL-12* (Wright and Jong 1986, Wright, Levin et al. 1989, Perera, Mayadas et al. 2001). In accordance, it was shown that inhibition of CD11b/CD18 reduced secretion of inflammatory cytokines, indicating that CD11b/CD18 activation modulates inflammatory signalling (Roberts, Furnrohr et al. 2016). Furthermore, it was reported that CD11b/CD18 is involved in the inflammatory processes post MI, as inhibition of CD11b/CD18 reduced oxidative protein modifications and improved remodelling following I/R (Mollenhauer, Gesenberg et al. 2016). However, IL-4 treatment was not able to diminish LPS/IFN-γ-induced degranulation (Figure 34), indicating that a stronger or longer anti-inflammatory stimulus would be necessary to reverse LPS/IFN-γ-induced inflammation here.

In contrast, neither the isolation process nor LPS/IFN-γ treatment, but IL-4 treatment slightly increased CD35 expression (Figure 34). CD35, which is also known as complement receptor 1 (CR1), is primarily released by neutrophils into the circulation (Danielsson, Pascual et al. 1994). It was reported in literature, that CR1 inhibits complement system activation and exhibits rather anti-inflammatory properties (Hamer, Paccaud et al. 1998, Hess, Sadallah et al. 1999). Hence, this can be a possible explanation for an upregulation of CD35 upon IL-4 treatment, but not upon pro-inflammatory stimulation. Interestingly, CR1 has been described as a promising inhibitor of post MI inflammation and necrosis, by inhibiting the complement system, which mediates the acute inflammatory response (Weisman, Bartow et al. 1990, Weisman, Bartow et al. 1990, Hamer, Paccaud et al. 1998).

Degranulation is necessary for killing of pathogens, but plays also a role in the immune response during (non-)infectious diseases, as in the context of myocardial infarction (Mortaz, Alipoor et al. 2018). Excessive degranulation aggravates tissue injury post MI, whereas some degradation of the extracellular matrix by released proteases is necessary

for wound healing and removal of necrotic debris post MI (Hoenderdos, Lodge et al. 2016, Chalise, Becirovic-Agic et al. 2021, Daseke, Chalise et al. 2021). Thus, regulating the process of degranulation could be an important measure in the event of a myocardial infarction.

5.1.2.4. Anti-inflammatory polarization shows no effect on neutrophil migration

Neutrophils are among the first cells to arrive at the site of inflammation and recruit further cells. Pro-inflammatory stimuli, such as chemokines, are necessary for the recruitment, as they direct circulating cells to the inflamed tissue. The chemokine CXCL1 for example is a known and important modulator of neutrophil migration by binding to and activating CXCR2 receptor on neutrophils (Girbl, Lenn et al. 2018). Therefore, it was analysed if anti-inflammatory neutrophil polarization modulates neutrophil migration. This analysis revealed that IL-4 had no effect on neutrophil migration (Figure 35). In literature, it was reported that IL-4 stimulation rather limited neutrophil migration during inflammation via the inhibitory Fcγ receptor (FcγR2b) (Woytschak, Keller et al. 2016, Impellizzieri, Ridder et al. 2019, Panda, Wigerblad et al. 2020). However, this effect could not be observed here.

5.1.2.5. Anti-inflammatory neutrophils do not diminish inflammatory cytokine production

By secreting a wide range of cytokines, neutrophils play an important role in the regulation of damage and repair processes. In general, there exist pro- and anti-inflammatory cytokines. However, many cytokines are pleiotropic and cannot be strictly assigned to be pro- or anti-inflammatory. Thus, it was of importance to analyse if IL-4-induced N2-like neutrophils secrete rather anti-inflammatory cytokines or are able to diminish pro-inflammatory cytokine secretion. Therefore, cytokine secretion profile of IL-4 and LPS/IFN-γ treated neutrophils was analysed (Figure 36, Figure 37). As a combination of cytokines can induce different responses, the secretion profile of neutrophils treated with only LPS or only IFN-γ was as well analysed. Further, to investigate if anti-inflammatory IL-4 can dampen secretion of pro-inflammatory cytokines, neutrophils were treated simultaneously with IL-4 plus LPS or IFN-γ or LPS/IFN-γ (Figure 37).

Treatment of neutrophils with IL-4 induced significant upregulation of IFNβ, IL-27 and GM-CSF (Figure 36). In literature, it was described that IFNβ is a cytokine that cannot be

strictly assigned to be pro- or anti-inflammatory. It has been reported to cause resolution of inflammation, by inducing further anti-inflammatory cytokine secretion or reducing pro-inflammatory cytokines, and also by mediating production of pro-resolving lipids. However, depending on the activation path, it also induces increased secretion of inflammatory cytokines, as MCP-1 (Bolivar, Anfossi et al. 2018, Satyanarayanan, El Kebir et al. 2019, Sekheri, Rizo-Tellez et al. 2022). The cytokine IL-27 as well displays pro- and anti-inflammatory properties (Pflanz, Timans et al. 2002, Lucas, Ghilardi et al. 2003, Stumhofer, Laurence et al. 2006, Jouhault, Cherqaoui et al. 2023). The granulocyte-macrophage colony stimulating factor (GM-CSF), was mostly characterised as pro-inflammatory (Hamilton, Stanley et al. 1980, Fiehn, Wermann et al. 1992, Miller, Pillarisetty et al. 2002). However, there are also increasing reports of anti-inflammatory properties of GM-CSF (Vasu, Dogan et al. 2003, Cheatem, Ganesh et al. 2009, Bhattacharya, Gopisetty et al. 2011). In the context of myocardial infarction, GM-CSF is described as critical for the inflammatory response, but rather detrimental, as it triggers leukocyte recruitment (Maekawa, Anzai et al. 2004, Anzai, Choi et al. 2017). Thus, these data indicate that the cytokines IFN-β, IL-27 and GM-CSF are induced by an anti-inflammatory stimulus and might show anti-inflammatory characteristics in this setting. However, this as well demonstrates that cytokines exert pleiotropic effects and can be both, pro- or anti-inflammatory.

In contrast, combined treatment with LPS/IFN-γ induced significant upregulation of the inflammatory cytokines TNFα, IL-6, IL1ß, IL17A, IL23, IFNα and IL12p70 (Figure 37). LPS/IFN-γ treatment as well induced secretion of MCP-1 (CCL2), CCL3, CCL4, CXCL9 and CXCL10, which are cytokines mainly involved in chemotaxis, cell migration and adherence (Figure 37) (Reichel, Rehberg et al. 2009, Hasegawa, Suresh et al. 2021). LPS was reported to induce migration of neutrophils (Kesteman, Vansanten et al. 2008). Further, LPS as well triggers adhesion of neutrophils. Zhou et al. demonstrated a significant upregulation of CD11b, induced by LPS-dependent TLR4 activation, and this upregulation was crucial for the adhesion and transendothelial migration response of neutrophils (Zhou, Gao et al. 2005). The upregulation of CD11b upon LPS/IFN-γ stimulation was as well shown here (Figure 34) and in previous experiments (masterthesis, Reidel 2020) I could observe in cell culture, that LPS/IFN-γ treated neutrophils adhered to the cell culture plate, although neutrophils usually are a non-adherent cell type and cells of all other conditions did not adhere.

Similar to neutrophils, it was reported in literature, that classically activated M1 macrophages, induced by LPS and/ or IFN-γ, produce the inflammatory cytokines TNFα, IL-6, IL1ß, IL23, IL12p70 and the chemotaxis-associated chemokines CCL2, CCL3, CCL4, CXCL9 and CXCL10 (Verreck, de Boer et al. 2006, Beyer, Mallmann et al. 2012,

Italiani, Mazza et al. 2014, Vogel, Glim et al. 2014, Atri, Guerfali et al. 2018). However, not all of these cytokines show only pro-inflammatory characteristics, as it was also described that IL-6 or CCL2 are involved in the induction of alternatively activated M2-like macrophage phenotype (Mauer, Chaurasia et al. 2014, Sanmarco, Ponce et al. 2017, Deci, Ferguson et al. 2018, Xu, Li et al. 2019).
Moreover, LPS/IFN-γ treatment as well induced secretion of the anti-inflammatory cytokines IL-10 and IFN-a (Figure 37). Interestingly, it was also reported in literature that anti-inflammatory IL-10 is upregulated in response to LPS, and then strongly inhibits pro-inflammatory cytokines, as IL1β and TNFα, possibly to mitigate inflammation (Malefyt, Abrams et al. 1991).

Sole treatment with LPS induced significant release of CCL3, CCL4, IL1ß, TNFa and IL17, whereas sole treatment with IFN-γ only induced significant release of IFNα and IL-23 (Figure 37), showing which stimulus is necessary for which cytokine or chemokine secretion. In addition, this displays that a combined treatment induces a differential cytokine secretion profile compared to a sole treatment. Thus, these data demonstrate that most cytokines cannot be assigned to exert only anti- or only pro-inflammatory properties, but rather fate depends on the activating stimulus or environment.
Further, additional IL-4 treatment was not able to dampen pro-inflammatory cytokine secretion (Figure 37). Therefore, one can assume that the used IL-4 concentration was not strong enough to reduce LPS/IFN-γ-induced inflammation *in vitro*. However, these results do not completely exclude an attenuating effect of IL-4 on inflammatory cytokine release *in vivo,* and the regulation of cytokine secretion by neutrophils could still be a crucial target in many diseases.

In summary, anti-inflammatory neutrophil stimulation with IL-4 inhibited rather inflammation-promoting functions, as NET formation and CD11b/CD18 upregulation, whereas it enhanced phagocytosis and CD35 expression, which leads to resolution or limitation of inflammation. Additionally, IL-4 induced secretion of rather anti-inflammatory cytokines, but was not able to diminish inflammatory cytokine secretion. Taken together, limiting neutrophil activation, recruitment, and NETosis in inflamed tissues by an anti-inflammatory stimulus could be a therapeutic approach to suppress inflammation. However, these data again pointed out the limitations of *in vitro* experiments, due to arbitrary concentrations of cytokines and only low numbers of molecules, and as the complexity of the microenvironment *in vivo* is much higher.

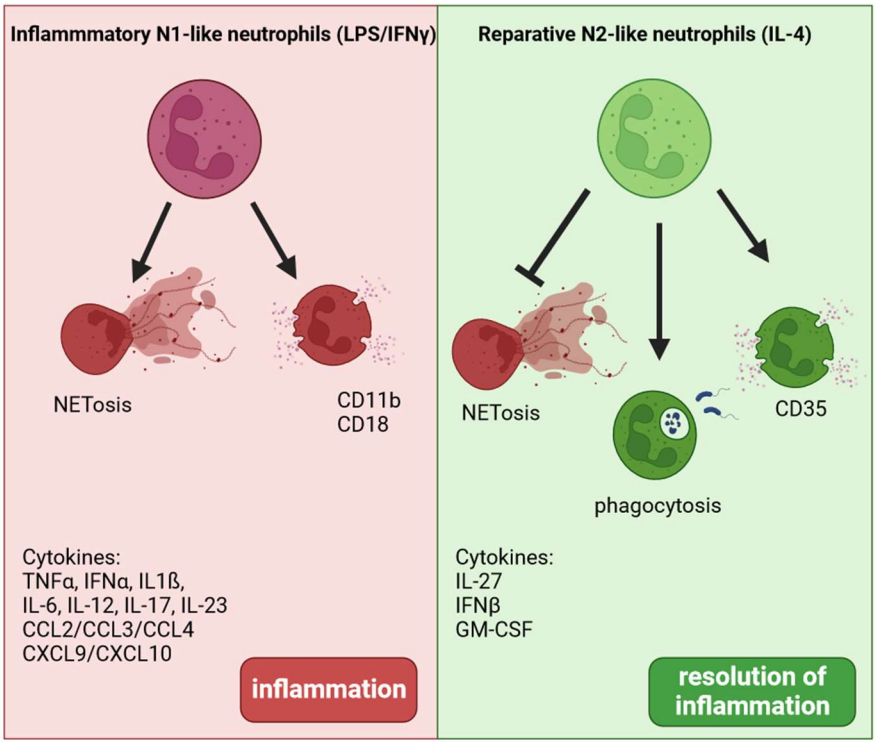

Figure 69: Summarised functions of inflammatory N1- and reparative N2-like neutrophils.

Inflammatory N1-like neutrophils induced by LPS/IFN-γ exhibit enhanced formation of NETs, degranulation of CD11b and CD18, and an inflammatory cytokine secretion profile. In contrast, IL-4-induced N2-like neutrophils evince reduced NET formation, but enhanced phagocytic capacity and CD35 degranulation, all being important for the resolution of inflammation. Created with BioRender.com.

5.2. Neutrophils in the context of Myocardial Infarction

The immune system plays a crucial role in the repair processes after myocardial infarction and neutrophils are among the first cells to arrive at the infarcted area (Carbone, Nencioni et al. 2013). Neutrophils were long thought to negatively affect cardiac remodelling since they enhance inflammation. Neutrophils increase injury by producing inflammatory cytokines and thereby attract further inflammatory cells (Soehnlein, Zernecke et al. 2008, Ma, Yabluchanskiy et al. 2013). However, there is now increasing evidence that neutrophils can also have beneficial effects on remodelling of the heart after MI, for example by inducing an anti-inflammatory M2c macrophage phenotype (Serhan and Savill 2005, Horckmans, Ring et al. 2017). In the context of myocardial infarction, human and animal studies demonstrated a cardioprotective effect of IGF-1 (Isgaard, Kujacic et al. 1997, Lee, Chen et al. 1999, Santini, Tsao et al. 2007, Heinen, Nederlof et al. 2019). Heinen et al. previously showed that IGF-1 attenuated myeloid cell lineages and induced an anti-inflammatory macrophage phenotype. Thus, one can assume that myeloid cells

play an important role in IGF-1-mediated cardioprotection. Further, it was shown here and in previous experiments, that also neutrophils can polarize at least to an anti- and pro-inflammatory phenotype *in vitro* (Nederlof, Reidel et al. 2022). Therefore, it was of interest to examine the effect of IGF-1 on cardiac and bone marrow neutrophil phenotype in the context of myocardial infarction. To address this question, mice were exposed to LAD coronary artery occlusion and were treated with IGF-1 or BSA for three days, beginning at the start of reperfusion. Subsequently, bone marrow and cardiac neutrophils were analysed by qPCR (Figure 38). This analysis revealed that the IGF-1 treatment had no effect on the expression of the classical N1- and N2-marker genes on BM neutrophils. Thus, the treatment might not be long enough to reach and induce changes in the bone marrow. However, cardiac neutrophils showed a significant reduction of *Tnfa*, but not of *IL12a* expression, after IGF-1 treatment when compared to BSA treated mice (Figure 38). This is in line with scRNA-seq data, which showed that IGF-1 treatment downregulated *Tnfα* expression in cardiac neutrophils *in vivo* (Nederlof, Reidel et al. 2022).

5.2.1. Characterisation of cardiac cells post MI

Recent scRNA-seq studies demonstrated that neutrophils can exhibit several diverse phenotypes and that the phenotype of neutrophils can change over time (Daseke, Valerio et al. 2019, Zilionis, Engblom et al. 2019, Vafadarnejad, Rizzo et al. 2020, Calcagno, Zhang et al. 2021). As a significant reduction of *Tnfα* expression, which plays a considerable role in inflammation, was observed in cardiac neutrophils of IGF-1 treated mice, we wanted to profoundly characterise cardiac cells, and in detail the neutrophil phenotype, in the time course post MI.

Therefore, mice were exposed to LAD coronary artery occlusion and were treated for three days with IGF-1, beginning at the start of reperfusion. One, three or seven days post MI, hearts were isolated and used for scRNA-seq. Following scRNA-seq, cardiac cells were clustered with low resolution, depicting 22 different clusters, which were defined by expression of specific marker genes (Figure 40, UMAP). This showed that neutrophils clearly differed from other cardiac cells, and also from myeloid cells, as from macrophages. Seven fibroblast clusters and four endothelial cell clusters were determined. Further, five different neutrophil clusters and four macrophage clusters were identified. Cluster 13 was identified as dendritic cells (DCs) and Cluster 10 and 11 as T- and B-cells.

Following characterisation of cell subsets, the behaviour of the cardiac cell types over time post MI was analysed (Figure 42). This revealed that the number of fibroblasts increased over time. Also in literature it was reported that post MI, fibroblast populations undergo phenotypic switches and increase through proliferation and migration (Cleutjens, Verluyten et al. 1995, Frangogiannis, Michael et al. 2000, Reichardt, Robeson et al. 2021).
Endothelial cells showed a reduction in cell number from day 1 to day 3 and increased again on day 7. The reduction of EC`s on day 3 can possibly be explained by apoptosis induced by increased ROS post MI, which then recovers again on day 7 post MI (Park, Shen et al. 2009, Zheng, Liu et al. 2022).
Neutrophil number was the highest on day 1 post MI and decreased on day 3 and neutrophils were nearly vanished on day 7. According to our findings here, Ma et al. described that neutrophil infiltration peaked at day 1 post MI, receded at day 5 and was nearly vanished on day 7 post MI (Ma, Yabluchanskiy et al. 2016).
In line with literature, macrophage number was lowest at day 1 and increased over time until day 7 (Yan, Anzai et al. 2013).
This states clearly that there is a dynamic shift of cell types in the heart over time post MI.

5.2.2. Characterisation of leukocytes post MI

When looking at general differences between control and IGF-1 treated cells, scRNA-seq revealed, that IGF-1 treatment does not generate an IGF-1 specific cell cluster or changes cell distribution (Figure 41). However, if IGF-1 treatment changes gene expression within each cell cluster was analysed in the subclustered leukocytes, as the focus of this thesis is on neutrophils.
Therefore, in a next step, only leukocytes were reclustered in a higher resolution, in order to take a closer look at neutrophils (Figure 43). Again, clusters were defined by expression of specific marker genes, defining five neutrophil clusters, ten macrophage clusters and three lymphocyte clusters.

Following characterisation of cell subsets, the distribution of the leukocyte cell types in the heart on day one, three and seven post MI was analysed (Figure 44). This revealed again that neutrophil number (green) was the highest on day 1 post MI and decreased on day 3 and was nearly vanished on day 7. In line with literature, macrophage number (purple), including DC´s, was lowest at day 1 and increased over time on day 3 and day 7 (Yan, Anzai et al. 2013). B-, T- and NK-cells comprised only a small part of cardiac cells, and B- (yellow), T- (orange) and NK-cells (blue) were lowest on day 1 and 3 post

MI and slightly increased over time until day 7 post MI (Figure 44). Also others reported that B-cells, and lymphoid cells in general, arrive three to seven days post MI and peak at day 7 (Yan, Anzai et al. 2013). T-cells were reported to be low in the infarct/ border zone and they were only occasionally observed in the remote area (Yan, Anzai et al. 2013).

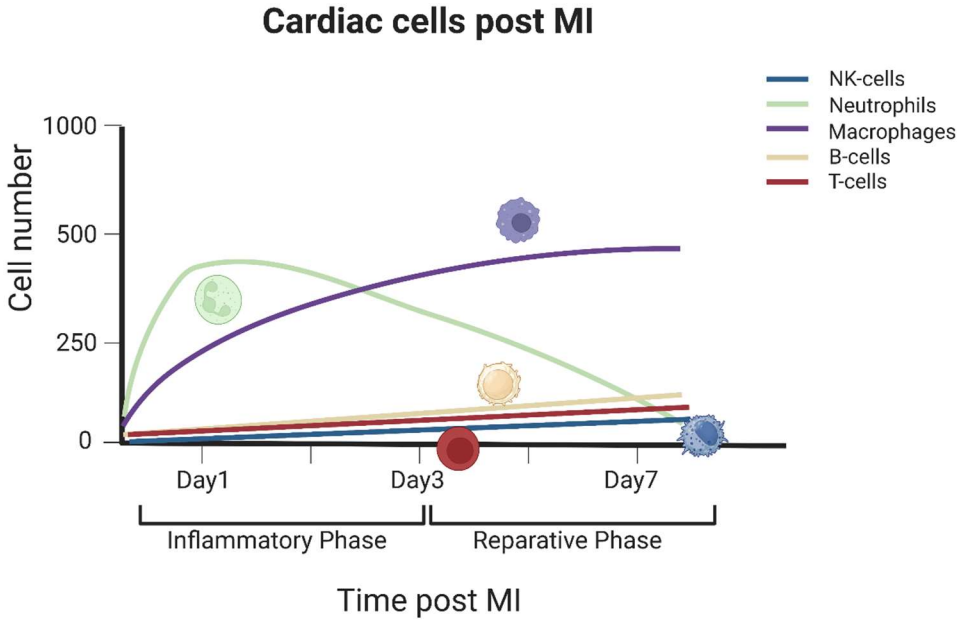

Figure 70: Distribution of cardiac leukocytes over the time post MI.

Neutrophils peak at day 1 post MI, slightly decrease until day 3 and are nearly vanished on day 7. Macrophages and DC`s are lowest at day 1 post MI and increase over time. T-, B- and NK-cells are lowest at day 1 and slightly increase until day 7 post MI. Created with BioRender.com.

Furthermore, gene expression profile of leukocytes changes over time post MI, which is shown in violinplots of exemplary genes (Figure 45). *Chil3*, *Arginase 1* and *Tnf* was abundantly expressed by most cell clusters on day 1, whereas on day 7 post MI expression was reduced (Figure 45). Thus, not only immune cell infiltration changes over time, but as well expression pattern of immune cells shifts from day 1 to day 7.

5.2.3. Characterisation of neutrophils post MI

As the focus of this thesis is on neutrophils, in a next step, neutrophil clusters were defined in more detail (Figure 46). Neutrophil Cluster 0 represents young neutrophils derived from blood, which infiltrated the heart only recently (Vafadarnejad, Rizzo et al. 2020, Nederlof, Reidel et al. 2022). In contrast, neutrophil cluster 2 was identified as old pro-inflammatory neutrophils (Vafadarnejad, Rizzo et al. 2020, Calcagno, Zhang et al. 2021, Nederlof, Reidel et al. 2022). Neutrophil cluster 16 showed high expression of type I interferon genes. Interestingly, two neutrophil clusters, namely cluster 10 and 17,

express genes characteristic for neutrophils but also for endothelial cells (Cluster 10) and cardiomyocytes (Cluster 17), respectively. In addition, cluster 10 is the only cluster showing high expression of *Car4,* which was identified as a N2-marker in our *in vitro* polarization studies. Thus, one can assume that neutrophils from cluster 10 and 17 have phagocytosed other cells, explaining the “co-expression” of endothelial and cardiomyocyte derived transcripts.

5.2.3.1. IGF-1 treatment dampens the pro-inflammatory neutrophil phenotype

Again, even for subclustered leukocyte cells, no effect of IGF-1 treatment on cell cluster size and distribution was observed (Figure 47), however, when looking at differences between control and IGF-1 treatment, a clear IGF-1 effect was observed for neutrophils in most clusters post MI. Compared to other immune cells, neutrophil subsets are only poorly determined. However, Grieshaber-Bouyer et al. uncovered, that neutrophils develop along one sequence, which they called neutrotime, and disgress from there to attain disparate polarization states (Grieshaber-Bouyer, Radtke et al. 2021). This could be a possible explanation, that no specific “IGF-1 cell cluster” exists, but rather IGF-1 affects all neutrophil clusters.

Analysis of differentially expressed genes, as well as upstream regulator and pathway functions of IPA revealed, that in most clusters, IGF-1 alleviated pro-inflammatory signalling and augmented anti-inflammatory regulators on day 3 and 7 post MI. However, analysis of all neutrophil clusters at day 1 post MI, revealed that the effect of IGF-1 at this time point is ambiguous, as down- as well as upregulation of inflammation was observed.

Upregulation of anti-inflammatory genes (eg. *Chil3, Crispld2*, *Ppara)*, as well as downregulation of inflammatory genes (eg. *Isg15, Klf6*, *Ifitm1, Ccl3, Pgk1)* was observed and associated with dampening of inflammation one day post MI. *Chil3* is a well-known anti-inflammatory M2 gene and we also identified *Chil3* as a N2-marker gene, indicating a rather inflammation-suppressing role of IGF-1 post MI (Xu, Bo et al. 2019, Boutilier and Elsawa 2021, Nederlof, Reidel et al. 2022). In line, it was shown in stroke that increased Chil3 correlated with improved post-stroke recovery and reduced infarct size (Perego, Fumagalli et al. 2016, Kang, Li et al. 2022). Thus, one can assume that Chil3 serves not only *in vitro* but also *in vivo* as an anti-inflammatory marker. Further, there exist several reports that *Crispld2* and *Ppara* attenuate inflammation by downregulation of pro-inflammatory cytokines and signalling (Takano, Nagai et al. 2000, Yue, Bao et al. 2003, Bulhak, Sjöquist et al. 2006, Wang, Xing et al. 2009, Barlaka, Ledvényiová et al. 2013,

Zhang, Kho et al. 2016, Zhang, Pei et al. 2021). In concordance, *Interferon-stimulated gene 15 (Isg15)* is associated with inflammation and heart failure, and absence of *Isg15* resulted in preserved cardiac function (Calcagno, Ng et al. 2020, Yerra, Batchu et al. 2023).

In contrast, also upregulation of inflammatory genes (eg. *Serinc3*, *Lcn2)*, as well as downregulation of anti-inflammatory genes (eg. *Dusp5)* was observed and associated with enhancement of inflammation one day post MI. *Serin incorporator 3 (Serinc3)* for example was reported to have rather inflammatory properties, as it was described to augment expression of type I interferons (IFN) and inflammatory Nfκb signalling (Zeng, Waheed et al. 2021). Also downregulation of *Dusp5* was associated with inflammation, as it was reported to evince rather anti-inflammatory properties by inhibition of ERK1/2 and Nfκb signalling and reduction of inflammatory cytokines as TNFa and IL-6 (Ferguson, Harrison et al. 2013, Seo, Cho et al. 2017). Thus, one day post MI the effect of IGF-1 is indistinct, as mainly inflammatory genes were reduced, however also some inflammation-associated genes were upregulated.

Whereas transcriptional changes on day one post MI were ambiguous with respect to pro- and anti-inflammatory genes, the IGF-1 treatment showed clearly cardioprotective effects on day 3 post MI, with upregulation of anti-inflammatory mediators (*Fth1, Aldoa, Tead1, Zfp36*) and downregulation of inflammation (Figure 48). *Ferritin heavy chain 1 (Fth1)* for example is a suppressor of ferroptosis and was significantly associated with M2 macrophages (Hu, Wen et al. 2021). Also *Tead1* and *Zfp36* were described to alleviate inflammation, by downregulating inflammatory genes and cytokines (Lai, Carballo et al. 1999, Gao, Sun et al. 2021, Makita, Takatori et al. 2021). Interestingly, IGF-1 treatment also tended to increase anti-inflammatory IL-10 signalling, though the Z-score was only low.

Further, IGF-1 treatment induced downregulation of Nfκb and pro-inflammatory cytokine signalling. For example the inhibitor of IL-1 signalling, *IL1r2,* was found to be increased during MI and to correlate with C-reactive protein and infarct size (Orrem, Shetelig et al. 2018, Zhao, Xie et al. 2020). Interestingly, *Klhl6* is upregulated upon Nfκb activation (Choi, Zhou et al. 2019), thus one can assume a reduced activation of Nfκb signalling in the IGF-1 treated mice.

Moreover, it attracted attention, that mainly genes associated with upregulation of oxidative phosphorylation (*Myc*, *PPARGC1α, Stk11)* and downregulation of fatty acid oxidation (*Rictor, Cpt1b*) were detected upon IGF-1 treatment (Angelini, Saha et al. 2021, Chu, Liu et al. 2022). Activation of the transcription factor *Myc* was shown to increase glucose uptake and utilization and reduce fatty acid oxidation, which was associated with ameliorated recovery post MI (Ahuja, Zhao et al. 2010). Further, *Pgc1a (≙ Ppargc1a)* is

a known mediator of oxidative phosphorylation and *Stk1* was reported to activate *Ampk*, which is a regulator of *Pgc1a* and thus also of oxidative phosphorylation (Mahmoud, Wilkinson et al. 2021). In line, IGF-1 treatment induced upregulation of the oxidative phosphorylation signalling pathway (Figure 50). During the onset of ischaemia, cellular metabolism in the heart switches from oxidative phosphorylation to anaerobic glycolysis, thus upregulation of oxidative phosphorylation might indicate an improved recovery post MI (Quevedo, Salinas et al. 2003, Dambrova, Zuurbier et al. 2021). Furthermore, oxidative phosphorylation has been associated with polarization of macrophages towards an anti-inflammatory M2 phenotype (Yu, Wang et al. 2020, Wculek, Heras-Murillo et al. 2023). Therefore, one can assume that the upregulation of oxidative phosphorylation upon IGF-1 treatment is also linked to an anti-inflammatory N2 neutrophil polarization, subsequently leading to dampening of inflammation. Taken together, IGF-1 treatment attenuates inflammation three days post MI.

Interestingly, this analysis also revealed that there was a twist in gene expression on day 1 and 3. *Chil3* expression was upregulated in all 5 neutrophil clusters on day 1 upon IGF-1 treatment, but on day 3 post MI *Chil3* was downregulated in all 5 neutrophil clusters upon IGF-1 treatment. Also in literature it was reported, that Chil3 expression peaked one day post ischaemia, indicating that C*hil3* expression belongs to the fast first response and is then reduced again (Perego, Fumagalli et al. 2016, Kang, Li et al. 2022). Moreover, *Lcn2* expression, which is rather detrimental in MI, was upregulated in four neutrophil clusters on day 1 and downregulated in three neutrophil clusters on day 3 post MI upon IGF-1 treatment, indicating that the protective IGF-1 effect only fully unfolds at day 2 or 3 post MI.

Analysis of day 7 revealed, that even 7 days post MI, IGF-1 treatment exerts anti-inflammatory effects. Genes associated with downregulation of inflammation were observed, as for example *Hnrnpa2b1, Nfat5* and *Prdm1* (Chan, Chiang et al. 2009, Zhao, Graber et al. 2009, Kim, Goldstein et al. 2014, Ni, Sun et al. 2020, Ulmert, Henriques-Oliveira et al. 2020). Furthermore, the anti-inflammatory IL-10 signalling, which tended to be enhanced on day 3, was considerably upregulated in IGF-1 treated mice on day seven post MI in one neutrophil cluster. Furthermore, also seven days post MI, IGF-1 treatment downregulated *Csf2,* demonstrating a persistent effect of IGF-1. *Csf2* induces growth, proliferation and recruitment of further inflammatory cells (Anzai, Choi et al. 2017, Horckmans, Bianchini et al. 2018). Thus, a diminished production and recruitment of immune cells, as neutrophils and macrophages, might point towards an improved resolution of inflammation. In line, the NETosis pathway, which was upregulated on day 3 post MI was downregulated on day 7 in IGF-1 treated mice, indicating resolution of inflammation.

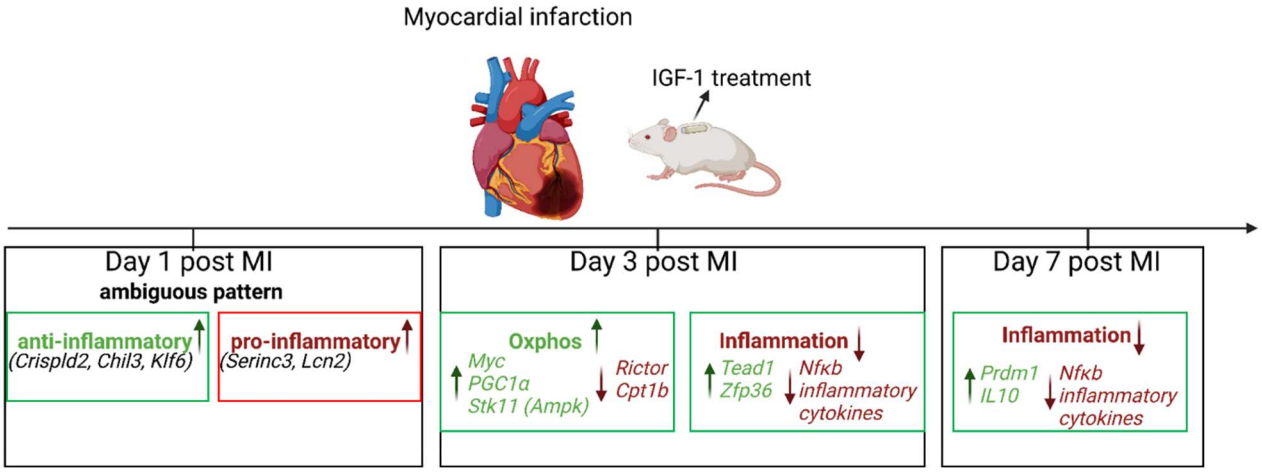

Figure 71: Regulation of expression patterns over time post MI.

Whereas expression pattern of neutrophils is ambiguous one day post MI, neutrophils show upregulation of oxidative phosphorylation and anti-inflammatory characteristics three and seven days post MI. Created with BioRender.com.

Thus, these results demonstrate that IGF-1 treatment attenuates the pro-inflammatory neutrophil phenotype in parts on day one and completely on day three and day seven post MI. Therefore, IGF-1 is not only able to modulate macrophage phenotype, but also neutrophil phenotype *in vivo*. Consequently, an interplay of anti-inflammatory macrophages and neutrophils, induced by IGF-1, might lead to an improved myocardial recovery post MI.

5.2.3.2. Neutrophil phenotype *in vitro* versus *in vivo*

In previous experiments, we were able to show *in vitro* that neutrophils polarize to an anti- or pro-inflammatory phenotype upon IL-4 or LPS/IFN-γ treatment. Therefore, it was of interest to analyse if characteristic N1 and N2 neutrophil marker genes identified *in vitro* in BM neutrophils, were as well expressed in cardiac neutrophils post MI (Figure 51). Thus, we examined if a comparable N1- or N2-like neutrophil phenotype could be observed in cardiac neutrophils post MI. This analysis revealed, that most N1- (*Nos2, IL12a, IL-6*) and N2-marker genes (*Retnlα, Arg1, Car4*) used for phenotype characterisation in bone marrow neutrophils *in vitro* were only sparsely expressed in cardiac neutrophils post MI, indicating that those marker genes are not applicable for the characterisation of anti- or pro-inflammatory neutrophils in this setup. However, when analysing the top upregulated genes upon LPS/IFN-γ treatment, identified by RNAseq of BM neutrophils *in vitro,* in cardiac neutrophils post MI, neutrophils of cluster 16 most likely resembled pro-inflammatory N1-like neutrophils. In line, neutrophil cluster 16 was identified as a cluster with high expression of Ifn-stimulated genes (Figure 46). When

analysing the top upregulated genes upon IL-4 treatment, identified by RNAseq of BM neutrophils *in vitro,* in cardiac neutrophils post MI, no cluster showed abundant expression of N2 genes, but rather each cluster expressed scattered N2 genes. However, neutrophil cluster 10 showed expression of N2 genes *Arg1, Car4 and Tafa3 (= Fam19a3)*.

Thus, these data indicate that one always has to take in consideration that *in vitro* experiments are a helpful tool to get an idea of a situation, however can not be equated to *in vivo* situations. This perception is not unexpected, as *in vivo* the environment of cells is comprised of a wide variety of cytokines, growth factors and other mediators that all influence cell function and phenotype. Further, it was shown that the M1/N1-M2/N2 paradigm of *in vitro* is a strongly simplified simulation, as there exist way more phenotypes in *in vivo* situations (Daseke, Valerio et al. 2019, Dick, Macklin et al. 2019). Therefore, the addition of only one cytokine (IGF-1) will induce a shift in gene expression but not result in a defined polarization state of N1 or N2, as shown here by scRNA-seq. However, a shift towards anti-inflammatory gene expression induced by IGF-1 might already be a beneficial therapeutic option post MI.

Summarised, Heinen et al. demonstrated that IGF-1 shows cardioprotective characteristics and that this effect was mediated by myeloid cells (Heinen, Nederlof et al. 2019). In a next step we were able to show, that IGF-1 attenuates the pro-inflammatory phenotype of myeloid cells (neutrophils and macrophages) on day three post MI (Nederlof, Reidel et al. 2022). Pursuing, in this thesis the effect of IGF-1 on neutrophil phenotype over time post MI was unveiled. This revealed, that the protective effect of IGF-1 in neutrophils fully unfolds only after one day post MI and that three days of IGF-1 treatment did not generate a specific neutrophil subtype but rather alleviated the pro-inflammatory phenotype after MI *in vivo*. Thus, demonstrating that IGF-1 acts, similar to IL-4, like an anti-inflammatory cytokine *in vivo*.

5.3. Neutrophils in the context of hyperglycaemia

Type 2 Diabetes Mellitus (T2DM) strongly predisposes to cardiovascular diseases and patients with T2DM often have a worsened outcome after myocardial infarction (Lago and Nesto 2009). Obesity is often accompanied by and a cause of T2DM. Until recently, T2DM was widely accepted as a metabolic disease, however, it is more and more often characterised as an immune-mediated inflammatory disease. In some cases, as for example in the event of infection, inflammation is essential and beneficial for the protection from exogenous pathogens and to repair the damaged tissue. However, the

inflammatory response is often also detrimental as it can damage the host and lead to tissue dysfunction. In the case of hyperglycaemia and diabetes, chronic low-grade inflammation occurs with elevated levels of circulatory pro-inflammatory cytokines, which disturb tissue homeostasis, and consequently result in chronic inflammation (Lumeng, Bodzin et al. 2007, Lackey and Olefsky 2016). For macrophages, a priming towards a pro-inflammatory phenotype by high glucose levels has been demonstrated (Edgar, Akbar et al. 2021). However, neutrophils are less well studied in the context of hyperglycaemia. Therefore, reducing inflammation by targeting neutrophils and shifting them to a more anti-inflammatory phenotype could offer a potential therapeutic strategy. Thus, it was investigated whether hyperglycaemia *in vitro* and *in vivo* affects neutrophil polarization and function.

However, short-term treatment with high glucose levels (4.5 g/L) *in vitro* had no effect on neutrophil polarization, as gene expression of analysed N1- and N2- marker genes was not altered (Figure 52). An explanation for this missing effect in neutrophils *in vitro* could be the limited polarization time of four hours due to the short life-span of neutrophils, as macrophages were cultivated in high glucose medium for seven days and then polarized for 16 hours (Edgar, Akbar et al. 2021).

5.3.1. Mild hyperglycaemia slightly affects neutrophil function and phenotype

In vivo, a shift from anti-inflammatory adipose tissue macrophages in lean mice to more inflammatory macrophages in obese mice was demonstrated. Those pro-inflammatory macrophages in adipose tissue are thought to play a major role in the development of inflammation in obesity; they secrete pro-inflammatory cytokines, thereby leading to chronic inflammation and also to impairment of insulin signalling (Lumeng, Bodzin et al. 2007, Lackey and Olefsky 2016). Thus, the next step was to analyse the effect of obesity and a persisting hyperglycaemic environment *in vivo* on neutrophil polarization and function. Therefore, the diet-induced obesity (DIO) mouse model was analysed. DIO mice evinced an increased body weight and a distinct glucose intolerance (Figure 54). However, those mice were only mildly hyperglycaemic (Figure 54). This is in accordance with literature, as a body weight of over 40 grams, mild hyperglycaemia, but distinct glucose intolerance were reported (O'Brien, Hinder et al. 2018). Thus, the DIO model rather represents a prediabetic state with mild hyperglycaemia and advanced obesity.

As short-term high glucose treatment for four hours *in vitro* showed no effect on neutrophil polarization (Figure 52), the next step was to analyse if continuous hyperglycaemia *in vivo*, induced by only a high fat/ high sucrose diet and thereby mimicking a prediabetic

state, has an effect on neutrophil phenotype and function. Indeed, RNA transcript expression analysis of neutrophils of standard chow fed and DIO mice revealed already under basal conditions mild changes in transcript expression profile (Figure 55). IPA analysis revealed, amongst others, an upregulation of Nrf2-mediated oxidative stress response. In line, *Gluthatione reductase (Gsr)* and *Thioredoxin reductase (Txnrd1)* were downregulated in neutrophils of DIO mice and both are important to reduce ROS (Sies 2014, McLoughlin, Orlicky et al. 2019). Concordant, there are several reports that hyperglycaemia is accompanied by increased oxidative stress and ROS production (Chattopadhyay, Khemka et al. 2015, Ridzuan, John et al. 2016, Lu, Liao et al. 2020). *A*nti-inflammatory *Cish*, which belongs to the suppressor of cytokine signalling family (Socs) and was reported to attenuate pro-inflammatory cytokines, was as well downregulated in neutrophils of DIO mice (Palmer, Guittard et al. 2015, Morris, Kershaw et al. 2018, Louis, Guimaraes et al. 2020). In line, inflammatory signalling was upregulated in neutrophils of DIO mice, as the most upregulated upstream regulators were inflammatory cytokines and transcription factors (*Tnf, Infγ*, *Il1β, Myc*). Also in literature it was reported that inflammatory cytokines and Nfκb are upregulated in hyperglycaemia and T2DM (Yang, Park et al. 2008, Randeria, Thomson et al. 2019). Thus, already mild hyperglycaemia induced slight changes in basal BM neutrophils, pointing towards an incipient chronic inflammation with increased oxidative stress and pro-inflammatory cytokine secretion in DIO mice. However, we could not observe severe alterations.

5.3.2. Severe hyperglycaemia induces considerable changes in neutrophil phenotype and function

The above data indicate that the DIO model, which rather represents a prediabetic state with mild hyperglycaemia and advanced obesity, evinces already minor alterations in neutrophil phenotype. Therefore, it was analysed if severe hyperglycaemia induced stronger alterations in neutrophil phenotype and function. Thus, the DIO/STZ mouse model with more severe hyperglycaemia was analysed. Streptozotocin is a ß-cell toxin, which induces destruction of the pancreatic ß-cells, resulting in a strongly reduced functional ß-cell mass and low insulin secretion (Rakieten, Rakieten et al. 1963, Arison, Ciaccio et al. 1967, Schein, Cooney et al. 1967). With the start of STZ injection mice lost weight and blood glucose levels increased rapidly from under 200 mg/dl to over 400 mg/dl (Figure 57), thus they showed severe hypergylcaemia. Further, DIO/STZ mice exhibited a distinct glucose intolerance, however showed no insulin resistance. Gilbert et al. as well observed a drop in body weight following STZ injection (Gilbert, Fu et al. 2011). In

line, it was shown by Gilbert et al. that mice on a high-fat diet, which received STZ injection, ate significantly less, thus explaining the weight loss after STZ injection observed here (Gilbert, Fu et al. 2011). It was as well shown that DIO/STZ mice evince fasting blood glucose levels of over 300 mg/dl, distinct glucose intolerance, but no insulin intolerance (Gilbert, Fu et al. 2011, O'Brien, Hinder et al. 2018).

5.3.2.1. Severe hyperglycaemia substantially alters gene expression of neutrophils

As mild hyperglycaemia induced some phenotypic differences in neutrophils (Figure 55), the effect of severe hyperglycaemia, induced by a high fat/ high sucrose diet with an additional STZ injection, on neutrophil phenotype was analysed. Therefore, at first a closer look was taken at the known N1- and N2- marker genes by qPCR analysis. This revealed a trend towards an attenuated upregulation of *Retnlα* in neutrophils of DIO/STZ mice compared to standard chow fed mice. When analysing pro-inflammatory marker genes, a significant reduction of *IL-6* was noted in neutrophils of DIO/STZ mice upon LPS/IFN-γ treatment, when compared to standard chow fed mice (Figure 58). Edgar et al. as well found decreased *Retnlα* expression upon IL-4 treatment in bone marrow macrophages of diabetic mice, indicating a reduced ability of immune cells to polarize to an anti-inflammatory phenotype (Edgar, Akbar et al. 2021). However, in contrast to the results observed here, they reported increased *IL-6* expression upon LPS/IFNγ stimulation (Edgar, Akbar et al. 2021). In line, there are conflicting reports if IL-6 has beneficial or deleterious effects in the onset of diabetes. Besides reports, that increased IL-6 levels lead to impaired insulin signalling and thus to insulin resistance, there is now increasing evidence of anti-inflammatory effects of IL-6 (Senn, Klover et al. 2002, Nakamura, Oda et al. 2012). It was reported that IL-6 KO mice developed glucose and insulin intolerance and increased fat proportions (Wallenius, Wallenius et al. 2002). Furthermore, it was shown that obesity-induced inflammation and insulin resistance were alleviated by IL-6 signalling of myeloid cells, as it induced alternative activation of macrophages (Mauer, Chaurasia et al. 2014). In line, reduced IL-6 levels were found in human blood during hyperglycaemia, additionally human peripheral blood mononuclear cells (PBMCs) exposed to high glucose showed decreased IL-6 and IL17A expression (Spindler, Ho et al. 2016), thus being concordant with the observed reduction in IL-6 expression here. Therefore, these data indicate that hyperglycaemia rather suppresses anti-inflammatory characteristics of neutrophils.

In order to get more quantitative measures of the effect of severe hyperglycaemia on neutrophil phenotype, RNAseq was performed (Figure 61). It was very evident that

neutrophils of hyperglycaemic mice (DIO/STZ) showed upregulation of inflammatory signalling already under basal conditions, which remained even after anti- and pro-inflammatory treatment. Increased S100 signalling for example is substantially involved in inflammation, and S100a8/S100a9 was reported to be increased in obesity (Mortensen, Nielsen et al. 2009, Xia, Braunstein et al. 2018). Further, it was reported that high glucose levels and hyperglycaemia in patients increase the formation of NETs, thereby driving diabetic complications (Miyoshi, Yamada et al. 2016, Ruiz-Limon, Ortega et al. 2017). Moreover, Phospholipase C was shown to be involved in inflammatory actions, as it inter alia mediates activation of Nfκb and interferon regulatory factors (Zhu, Jones et al. 2018). Increased cytokine storm signalling was in line with the identified upstream regulators, as several inflammatory cytokines were upregulated in neutrophils of DIO/STZ mice again already under basal conditions, but also after IL-4 or LPS/IFN-γ treatment (e.g. Ifnγ, Il1b, Tnf). In addition, Nfκb-related genes were upregulated in neutrophils of DIO/STZ mice (Figure 61). Also in literature, it was reported that inflammatory cytokines and Nfκb are upregulated in hyperglycaemia and T2DM (Yang, Park et al. 2008, Randeria, Thomson et al. 2019). Interestingly, *Nfκb1, IL12a* and *Tnfrfs1b* were upregulated under basal conditions in DIO/STZ neutrophils, and also upon IL-4 treatment those pro-inflammatory genes still showed increased expression levels when compared to standard chow fed mice (Figure 62 [A]). However, further pro-inflammatory LPS/IFN-γ stimulation did not enhance expression of those genes in hyperglycaemic neutrophils, indicating that the maximal expression level is already reached upon LPS/IFN-γ treatment in normoglycaemic neutrophils. In contrast, *Myc, Jun* and *Rel* expression was not only upregulated under basal conditions and upon IL-4 treatment in DIO/STZ neutrophils, but LPS/IFN-γ treatment even enhanced upregulation of those genes in DIO/STZ neutrophils (Figure 62 [B]). Interestingly, LPS/IFN-γ treated neutrophils of DIO/STZ mice showed additionally an upregulation of *epidermal growth factor (EGF)*, which was reported to even augment the inflammatory response of neutrophils activated by TNFa (Lewkowicz, Tchórzewski et al. 2005). Thus, these data demonstrate that hyperglycaemic neutrophils showed already under basal conditions (control) pro-inflammatory characteristics, which were partially enhanced upon inflammatory stimulation with LPS/IFN-γ.

Furthermore, signalling pathways known to be increased in diabetes, as Nfat, Creb and Fak signalling, were upregulated in all tested conditions in neutrophils of DIO/STZ mice (Benchoula, Parhar et al. 2021, Fang, Zhang et al. 2023, Jin, Wang et al. 2023). Nfat for example was shown to be increased by hyperglycaemia and to induce inflammatory mediators (Nilsson, Nilsson et al. 2006, Nilsson-Berglund, Zetterqvist et al. 2010, Berglund, Kotova et al. 2012).

Thus, these data repeatedly demonstrate, that neutrophils of DIO/STZ mice show

pronounced pro-inflammatory characteristics under basal conditions and after further stimulation.

In concordance, neutrophils of hyperglycaemic mice (DIO/STZ) showed downregulation of anti-inflammatory signalling already under basal conditions, which was perpetuated after anti- and pro-inflammatory treatment. The cytokine IL-10 is characterised as anti-inflammatory, and an IL-10 resistance and disrupted IL-10 downstream signalling were reported in T2DM (Barry, Shakibakho et al. 2016). In line, decreased serum levels of IL-10 in T2DM patients were reported (van Exel, Gussekloo et al. 2002, Yaghini, Mahmoodi et al. 2011, Abhilasha, Mitra et al. 2021). Also, the most downregulated upstream regulators of hyperglycaemic neutrophils were all linked to anti-inflammatory properties (Figure 61). In concordance with the above data, anti-inflammatory genes were suppressed already under basal conditions, and this effect was maintained after further treatment. *Cited2* for example was reported to augment production of inflammatory cytokines (Pong Ng, Kim et al. 2020), and the 3` repair exonuclease *Trex1* plays an important role in the regulation of inflammation, as deficiency of the exonuclease leads to systemic inflammation (Tao, Wu et al. 2019). Also, *Socs1*, *GFI1* and *Sirt1* were reported to augment production of inflammatory cytokines and oxidative stress (Karsunky, Zeng et al. 2002, Hashimoto, Ayada et al. 2009, Kitada and Koya 2013, Liu and Li 2017, Nakamura, Kageyama et al. 2017, Anderson, Murphy et al. 2020, Yang, Zhu et al. 2021). Furthermore, there seems to be a trend towards downregulation of anti-inflammatory neutrophil marker genes *(Slc28a3*, *Chil3, Car4*), but significant levels were not reached (Figure 63). Thus, all these data indicate that severe hyperglycaemia suppresses anti-inflammatory signalling of neutrophils under basal conditions, which is maintained after further stimulation.

Strikingly, these data repeatedly demonstrated, that severe hyperglycaemia induced major transcript expression alterations pointing towards a more pro-inflammatory phenotype already under basal conditions. Interestingly, additional IL-4 treatment was not able to reduce basal inflammatory characteristics of DIO/STZ neutrophils. However, additional LPS/IFN-γ treatment partially aggravated the pro-inflammatory characteristics of hyperglycaemic neutrophils. Thus, taken together a continuous severe hyperglycaemic environment induces a pro-inflammatory neutrophil phenotype. Furthermore, these data point towards a hyperglycaemic memory of neutrophils with increased inflammatory activity. Also Lee et al. demonstrated, that a diabetic environment reprograms the function of circulating cells, causing a more pro-inflammatory phenotype of these cells (Lee, Kim et al. 2013).

5.3.2.2. Severe hyperglycaemia alters neutrophil functions

As an increase of NET signalling was detected by IPA analysis in neutrophils of DIO/STZ mice (Figure 61) and as there is accumulating evidence, that obesity and hyperglycaemia accompanies with chronic inflammation, it was assessed if a strongly hyperglycaemic environment enhances the formation of NETs (Figure 64). This analysis revealed, that the formation of NETs in DIO/STZ mice was enhanced only slightly, thus, there might be a slight increase in NET formation capability. In line, it was reported that high glucose levels and hyperglycaemia in patients increase the formation of NETs, thereby driving diabetic complications (Miyoshi, Yamada et al. 2016, Ruiz-Limon, Ortega et al. 2017). Thus, inhibiting NETosis might contribute to a reduced chronic inflammation in diabetes.

Phagocytosis is, inter alia, an important mechanism in the resolution of inflammation by clearing cell debris and pathogens. Therefore, the effect of severe hyperglycaemia on neutrophil phagocytic activity was as well of interest (Figure 65). Interestingly, a significant restriction of phagocytic activity was observed in neutrophils of DIO/STZ mice (Figure 65). In line, there are reports that phagocytic activity of macrophages and neutrophils negatively correlates with fasting glucose and phagocytic activity is impaired in diabetic patients, explaining also diabetic complications as impaired wound healing (Mowat and Baum 1971, Delamaire, Maugendre et al. 1997, Lecube, Pachon et al. 2011, Dowey, Iqbal et al. 2021). Therefore, the regulation of phagocytic activity of neutrophils is an important consideration, as the impaired phagocytosis in T2DM patients also negatively effects the resolution of inflammation and wound healing in general or in a possible event of myocardial infarction.

5.3.2.3. Severe hyperglycaemia alters neutrophil cytokine production

Cytokines, produced by immune cells, are crucial regulators of inflammation, but also of repair processes. Often, the released cytokines attract inflammatory monocytes and macrophages, inducing a sustained inflammatory environment, or induce a shift towards a reparative phenotype of cells (Soehnlein, Zernecke et al. 2008). Thus, the regulation of cytokine secretion by neutrophils is a crucial target in many diseases. As hyperglycaemia is often linked to chronic inflammation, the question arose if hyperglycaemia also alters the cytokine secretion profile of neutrophils (Figure 66). This analysis revealed significantly reduced cytokine levels of CXCL9, MCP-1, CCL3, IL-6, IL17A and IL-10 upon LPS/IFN-γ treatment in neutrophils of DIO/STZ mice when compared to standard chow fed mice. Additionally, IFNß and GM-CSF levels were significantly reduced in neutrophils of DIO/STZ mice upon IL-4 treatment, when compared to standard chow fed mice (Figure

66).

The chemokines CXCL9, CCL2 and CCL3 are all linked to chemotaxis and migration (Reichel, Rehberg et al. 2009, Hasegawa, Suresh et al. 2021, Moin, Sathyapalan et al. 2021). Interestingly, impaired migration, disrupted adherence and chemotaxis of neutrophils during hyperglycaemia were reported (Mowat and Baum 1971, Bagdade, Stewart et al. 1978, Kelly, Brown et al. 1985, Mascardo 1988, Tian, Qing et al. 2016, Pahwa, Khan et al. 2020, Qiu, Zhang et al. 2020). Thus, being an explanation for the observed decrease of CXCL9, CCL2 and CCL3 transcription and secretion in neutrophils here (Figure 66, Figure 67).

As described before, IL-6 exerts pleiotropic effects, it was long regarded as pro-inflammatory, however, there is accumulating evidence, that it can have as well anti-inflammatory properties (Xing, Gauldie et al. 1998, Schieffer, Selle et al. 2004). As IL-6 is described as a critical downstream target of IL17A and elicits a positive feedback loop of IL-6 expression, the reduced IL17A levels in neutrophils observed here are only reasonable (Ogura, Murakami et al. 2008). Thus, reduced IL-6 and IL17A cytokine levels might also be a link towards suppression of anti-inflammatory characteristics.

The cytokine IL-10 is characterised as anti-inflammatory and an IL-10 resistance and disrupted IL-10 downstream signalling were reported in Type 2 Diabetes Mellitus (Barry, Shakibakho et al. 2016). In line, decreased serum levels of IL-10 in T2DM patients were reported (van Exel, Gussekloo et al. 2002, Yaghini, Mahmoodi et al. 2011, Abhilasha, Mitra et al. 2021), which was also observed here, as IL-10 secretion was reduced in neutrophils of DIO/STZ mice.

IFNβ is a cytokine which cannot be strictly assigned to be pro- or anti-inflammatory. However, it has been reported to cause resolution of inflammation, by inducing further anti-inflammatory cytokine secretion or reducing pro-inflammatory cytokines, and also by mediating production of pro-resolving lipids (Bolivar, Anfossi et al. 2018, Satyanarayanan, El Kebir et al. 2019, Sekheri, Rizo-Tellez et al. 2022). In line with the observed reduction of IFNβ secretion here (Figure 66), it was reported that high glucose levels inhibit type I Interferon production, to which IFNβ belongs (Hu, Xia et al. 2018). Furthermore, there are increasing reports of anti-inflammatory properties of GM-CSF (Vasu, Dogan et al. 2003, Cheatem, Ganesh et al. 2009, Bhattacharya, Gopisetty et al. 2011). As it is hypothesised that the anti-inflammatory properties of neutrophils induced by IL-4 are attenuated in the hyperglycaemic state, it is in line that the IL-4-induced IFNβ and GM-CSF secretion is as well alleviated.

Thus, hyperglycaemia seems to reduce a bunch of anti-inflammatory cytokines and cytokines involved in different effector functions, as migration and adhesion, of neutrophils. Further, cytokines that use the same signalling as insulin (IRS/PI3K), as for

example IL-4, IL-6 and IL-10, are possibly sensitive to the same compromised signalling seen in insulin resistance, indicating a potential “cytokine resistance” during hyperglyceamia (Hartman, O'Connor et al. 2004, O'Connor, Johnson et al. 2009).

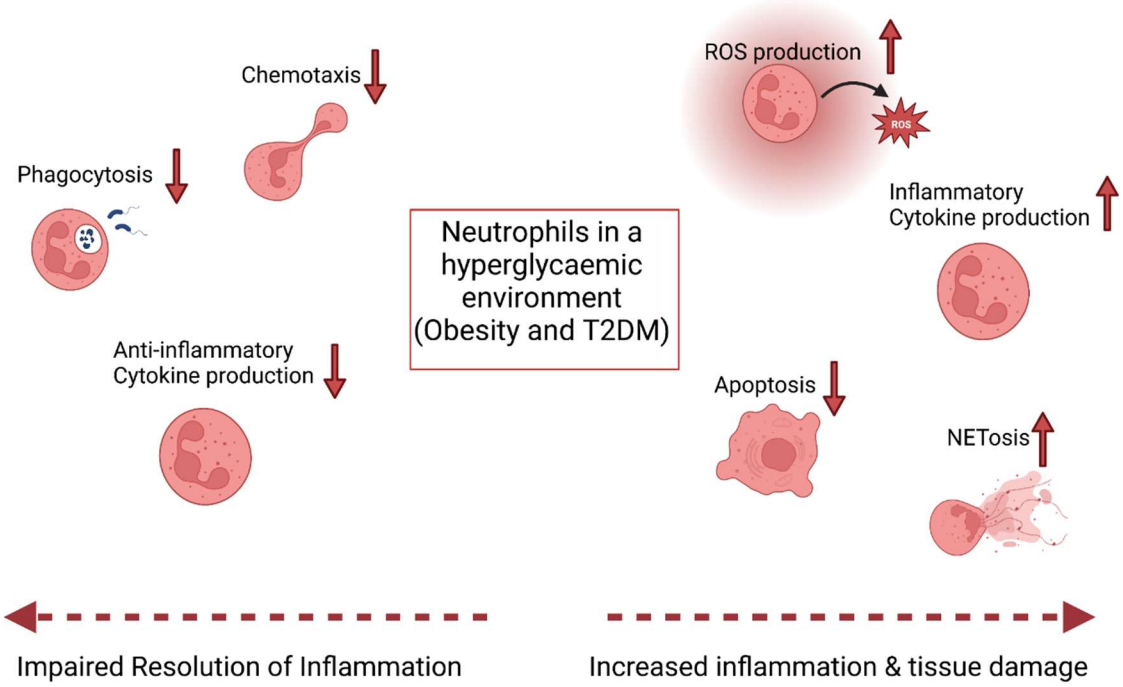

Figure 72: Impact of hyperglycaemia on neutrophil function and phenotype.

Continuous hyperglycaemia alters murine BM neutrophil function and phenotype. Hyperglycaemia leads to increased inflammatory cytokine and ROS production and NET formation. Further, continuous hyperglycaemia results in reduced apoptosis, phagocytosis, chemotaxis and decreased anti-inflammatory cytokine production. Created with BioRender.com.

Taken together, these data clearly demonstrate that hyperglycaemia affects already the phenotype and function of BM neutrophils. The above data revealed that continuous hyperglycaemia and accompanying obesity induce enhanced NET formation and pro-inflammatory cytokine production. Further, high glucose results in reduced chemotaxis, phagocytosis and anti-inflammatory cytokine production. Thus, hyperglycaemia induces a rather pro-inflammatory phenotype of BM neutrophils, consequently pointing towards a chronic inflammatory environment, and thereby impaired resolution of inflammation and enhanced tissue damage. Therefore, the modulation of neutrophil phenotype towards a more anti-inflammatory phenotype could have beneficial effects and help to reduce inflammation in the context of hyperglycaemia.

6. Outlook

Modulation of neutrophil phenotype emerged as a promising tool to reduce inflammation in several inflammation-associated diseases. However, until now, only *in vitro* approaches were conducted. All the above data indicate, that one always has to take into consideration that *in vitro* experiments are a helpful tool to identify molecular alterations under defined conditions, however can not be extrapolated to *in vivo* situations. This perception is not unexpected, as *in vivo,* the environment of cells is comprised of a wide variety of cytokines, growth factors and other mediators that all influence cell function and phenotype. Thus, those results only give a hint to how the situation might look *in vivo*. Therefore, further characterisation of neutrophil phenotype and functions in different contexts *in vitro* and *in vivo* are necessary to gain a more detailed picture of the clinical relevance. For macrophage polarization the M1/M2 concept was extended to the specimen model obtained by many different “polarizers”.

6.1 Further distinction of neutrophils of male and female mice

There is rising evidence, that biological sex seems to be a contributing factor to the severity and progression of several diseases (Bots, Peters et al. 2017, Takahashi, Ellingson et al. 2020). Initial data obtained in this study showed that neutrophils of male mice exhibited higher expression of inflammatory genes *Nos2* and *IL-6.* Therefore, it would be of interest to further analyse the impact of the biological sex on neutrophil polarization and function and thus would be an important consideration for the development of possible therapies. To address this question, RNAseq transcript expression analysis of neutrophils of male and female mice could be performed to determine differences in gene expression. Further, it should be analysed if there are also differences in neutrophil functions, by performing the functional assays used here, as for example the NETosis, phagocytosis, degranulation and cytokine secretion assay.

6.2 Measurement of ROS and oxidative stress in neutrophils of normo- and hyperglycaemic mice

A large number of signalling molecules and pathways upregulated in neutrophils of hyperglycaemic mice were related to oxidative stress and increased ROS levels. Thus, key cellular functions, such as oxygen consumption rate (OCR) and extracellular

acidification (ECAR), can be determined to gain a more concise definition of metabolic changes in neutrophils of hyperglycaemic mice.

6.3 Trained innate immunity in neutrophils of hyperglycaemic mice

Only recently it has been shown that, besides the adaptive immunity with memory capability of B- and T-cells, a memory-like innate immunity, known as trained immunity or innate immune memory, exists (Netea, Quintin et al. 2011). It is assumed that trained immunity is independent of adaptive immunity, but is rather generated by epigenetic changes, which affect gene expression of innate immune cells, such as monocytes, macrophages or natural killer cells (Kleinnijenhuis, Quintin et al. 2012). These epigenetic changes and immunometabolic programs are activated by cytokines or pathogen-associated molecular patterns (PAMP`s) and therefore pave the way to a sensitized and stronger, non-specific response to a secondary stimulation (Quintin, Saeed et al. 2012). Interestingly, it has been demonstrated that diabetic patients who become normoglycemic due to treatment, still have a higher risk for cardiovascular diseases. This phenomenon is called “hyperglycemic memory” and can be explained by trained immunity occuring also in diabetes (El-Osta, Brasacchio et al. 2008). In the context of diabetes, trained immunity in neutrophils has not been studied in detail yet, most likely due to their limited life span. However, one can assume that epigenetic changes in the bone marrow are transferred to neutrophils as well, as they descend from HSC`s (Figure 2). This raises the question for the role of neutrophils in trained immunity and if they are as well affected by epigenetic remodelling. In order to study possible “priming” of neutrophils, changes in chromatin structure can be analysed with the help of the Assay for transposase-accessible chromatin using sequencing (ATAC-seq) of bone marrow derived-neutrophils and macrophages from control and hyperglycaemic mice. Using transposon Tn5-mediated labelling (“tagmentation”), it can be assessed to what extent accessible regions within the chromatin in neutrophils and their precursors change in response to hyperglycaemia, indicating a kind of training of neutrophils.

6.4 Is IGF-1 able to reduce inflammation/ inflammatory neutrophil phenotype in diabetic mice?

The above data showed that neutrophils of hyperglycaemic mice evinced a rather pro-inflammatory phenotype. In the context of myocardial infarction, we were able to show that IGF-1 treatment reduced inflammatory characteristics of cardiac neutrophils. Thus, it would be of interest to analyse if IGF-1 administration is also able to reduce

inflammatory characteristics of BM and blood neutrophils of hyperglycaemic mice. In order to answer this question, hyperglycaemic mice could be given IGF-1 by drinking water or by i.p. injection over different time periods. Subsequently, neutrophil phenotype and function could be analysed and give a hint towards a possible therapeutic approach, under basal conditions as well as in the context of myocardial infarction.

6.5 Analysis of phenotype and function of human neutrophils

It was shown here that murine BM neutrophils polarize at least to an anti- and pro-inflammatory phenotype *in vitro*. Further, it was shown *in vivo* that an anti-inflammatory stimulus was capable to reduce pro-inflammatory characteristics of neutrophils. Therefore, it would be of interest to analyse if human neutrophils as well polarize to an anti- or pro-inflammatory phenotype upon different stimuli, and therefore modulation of neutrophil phenotype could present new therapeutic approaches in inflammatory diseases. Furthermore, the effect of polarization on human neutrophil functions could be investigated. If neutrophil polarization in humans can be confirmed, a next step would be to assess if IGF-1 modulates neutrophil polarization and function in neutrophils of diabetic patients and in the context of myocardial infarction. These analyses could give a more detailed insight into the clinical relevance of modulation of neutrophil phenotype.

7. References

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9. Statutory Declaration

Statutory Declaration

I declare under oath that I have produced my thesis independently and without any undue assistance by third parties under consideration of the ‘Principles for the Safeguarding of Good Scientific Practice’ at Heinrich-Heine-University Düsseldorf. Furthermore, I assure that I did not submit this dissertation, either in full or in part, to any other faculty and did not absolve any promotion trials before.

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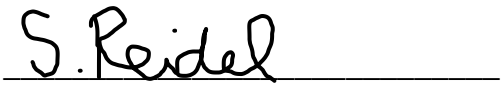

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Eidesstattliche Erklärung

Ich versichere an Eides statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der "Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf" erstellt worden ist. Darüber hinaus versichere ich, dass ich die Dissertation weder in der hier vorgelegten noch in einer ähnlichen Form bei einem anderen Institut eingereicht habe und bisher keine Promotionsversuche unternommen habe.

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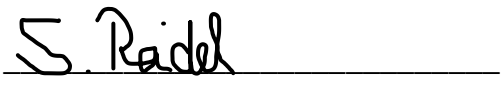

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