THESIS AND ABSTRACT

Autophagy-dependent generation of free fatty acids is essential for normal neutrophil differentiation by guiding an energy-metabolic switch

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy,

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Abstract

Autophagy-dependent generation of free fatty acids is essential for normal neutrophil differentiation by guiding an energy-metabolic switch

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Neutrophils are critical and short lived mediators of innate immunity that require constant replenishment. Their differentiation in the bone marrow requires extensive cytoplasmic and nuclear remodeling, but the processes governing these energy-consuming changes are unknown. While previous studies show that autophagy is required for differentiation of other blood cell lineages, its function during granulopoiesis has remained elusive. Autophagy was described as a critical process in HSC differentiation and in memory- and regulatory T cells, where it prevents excessive glycolysis and maintains lipid metabolic homeostasis. In the myeloid lineage, an essential role for autophagy is to prevent pro-inflammatory macrophage polarization and to limit glycolytic metabolism of acute myeloid leukemia. While these studies provide robust *in vivo* evidence for the relevance of autophagy in the differentiation of hematopoietic and immune cells, the targets and mechanisms of autophagy remain elusive. Here, we show that metabolism and autophagy are developmentally programmed and essential for neutrophil differentiation *in vivo*. *Atg7*-deficient neutrophil precursors had increased glycolytic activity but impaired mitochondrial respiration, decreased ATP production and accumulated lipid droplets. Inhibiting autophagy-mediated lipid degradation or fatty acid oxidation alone was sufficient to cause defective differentiation, while administration of fatty acids or pyruvate for mitochondrial respiration rescued differentiation in autophagy deficient neutrophil precursors. Together, we show that autophagy-mediated lipolysis provides free fatty acids to support a mitochondrial respiration pathway essential to neutrophil differentiation. Evidence presented here also contributed to studies on autophagy-mediated metabolic homeostasis in HSCs, T_{reg} cells and myeloid leukemia, suggesting that this pathway may act broadly during differentiation.

Author's Publications

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Abbreviations

4E-BP1 eIF4E-binding protein 1
AML Acute myeloid leukemia
AMP Adenosine monophosphate
AMPK AMP-activated protein kinase
ATG Autophagy-related gene
ATP Adenosine triphosphate
BC Band cell
BM Bone marrow
BMNCs Bone marrow mononuclear cells
BNIP BCL2/adenovirus E1B 19kDa protein-interacting protein 3
C/EBP Ccaat-enhancer binding protein
C57BL/6 C57 Black 6 (mouse strain)
CD Cluster of differentiation
CDK Cycling-dependent kinase
CGD Chronic granulomatous disease
CLP Common lymphoid progenitor
CMA Chaperone mediated autophagy
CMP Common myeloid progenitor
CO2 Carbon dioxide
CPT1 Carnitine palmitoyl transferase 1
CSF-1 Colony stimulating factor 1
Ct Cycle threshold
CXCL Chemokine (C-X-C motif) ligand
DMEM Dulbecco's Modified Eagle's Medium
DNA Deoxyribonucleic acid
EDTA Ethylenediaminetetraacetic acid
EM Electron microscopy
ER Endoplasmic reticulum
Erk Extracellular signal-regulated kinase
ETC Electron transport chain
FA Fatty acid
FACS Fluorescence-activated cell sorting
FAO Fatty acid oxidation
FAS Fatty acid synthesis
FCCP Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone
FCS Fetal calf serum
FFA Free fatty acid
FIP200 Focal-adhesion kinase-family interaction protein of 200kDa
FITC Fluorescein isothiocyanate
F/f Floxed LoxP-flanked
Flt-3 FMS-related kinase 3
FOXO Forkhead box O

FSC Forward scatter
Fwd Forward (primer)
g Grams/G-Force (context dependent)
GABARAP Gamma-aminobutyric acid receptor-associated protein
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
GC Gas chromatography
GFP green fluorescence protein
GM Granulocyte-macrophage
GM-CSF Granulocyte-macrophage colony stimulating factor
GMFI Geometric mean fluorescence intensity
GMP Granulocyte monocyte progenitor
GTPase Guanosine triphosphatase
H&E Haematoxylin and eosin
HCL Hydrochloric acid
HIF Hypoxia inducible factor
Hrs Hours
HSC Hematopoietic stem cell
HSPC Hematopoietic stem and progenitor cell
Cre Codon improved Cre recombinase
IFN Interferon
IL Interleukin
ILC Innate lymphoid cell
Kb Kilobase
KDa Kilo Daltons
Ki67 Kiel 67 protein KO Knockout
LAL Lysosomal acid lipase
LAP LC3-associated phagocytosis
LAMP1/2 Lysosomal-associated membrane protein 1/2
LC Liquid chromatography
LC3 MAP1LC3B
Lck Lymphocyte-specific protein tyrosine kinase
LKB1 Liver kinase B1 LMPP Lymphoid-primed multipotent progenitor
LSC Leukemic stem cell (leukemia initiating cell)
LSK Lin-Sca1+cKit+ (MPPs)
LTF Lactoferrin
LT-HSC Long-term HSC
Ly6G Lymphocyte antigen 6 complex locus G6D
Lys-M M-lysozyme
M Molar
MACS Magnetic-activated cell sorting
MAP1LC3 Microtubule-associated protein 1 light chain 3 beta
MB Myeloblast
MC Myelocyte
MDS Myelodysplastic syndrome

MFI Mean florescence intensity
Min Minute
MLL Mixed-lineage leukemia
MM Metamyelocyte
MMP9 Matrix metalloprotease 9
MPO Myeloperoxidase
MPP Multipotent progenitor
mRNA Messenger RNA
MS Mass spectrometry
mTOR(C1/C2) Mammalian target of rapamycin
mtROS Mitochondrial ROS
n.s. Not significant
NAC N-acetyl cysteine
NaCl Sodium Chloride
NAD(H) Nicotinamide adenine dinucleotide (reduced)
NaOH Sodium hydroxide
NET Neutrophil extracellular trap
NIX NIP3-Like Protein X
NK cell Natural killer cell
NKT cell Natural killer T cell
OCR Oxygen consumption rate
OXPHOS Oxidative phosphorylation
PAS Pre-autophagosomal structure
PBS Phosphate buffered saline
PCR Polymerase chain reaction
PDH Pyruvate dehydrogenase
PDK Pyruvate dehydrogenase kinase
PE Phosphatidylethanolamine
PI3K Phosphoinositide 3-kinase
PK Pyruvate kinase
PMC Promyelocyte
PMN Polymorphonuclear neutrophil
PVDF Polvinylidene fluoride
qRT-PCR Quantitative reverse transcription PCR
Rab Ras-related protein Raf Rapidly accelerated fibrosarcoma
Rag Ras-related GTPase
RAPTOR Regulatory-associated protein of mTOR
RAS Rat sarcoma (oncogene)
RBC Red blood cell
Rev Reverse (primer)
RHEB Ras homolog enriched in brain
RICTOR Rapamycin-insensitive companion of mTOR
RIPA Radioimmunoprecipitation assay buffer
RNA Ribonucleic acid

ROS Reactive oxygen species
Rpm Revolutions per minute
RPMI Roswell Park Memorial Institute medium
RT Reverse transcription
S6K p70S6 kinase
SCF Stem cell factor
SCN Severe congenital neutropenia
SD Standard deviation
SDS Sodium dodecyl sulphate
SDS-PAGE SDS polyacrylamide gel electrophoresis
Sec Seconds
SEM Standard error of the mean
SQSTM1 Sequestosome 1 (also p62)
SSC Side scatter
TCA Tricarboxylic acid
TE Tris-EDTA (buffer)
TEM Transmission electron microscopy
TFEB Transcription factor EB
T_H1 T helper 1 cell
T_H17 T helper 17 cell
T_H2 T helper 2 cell
T_{mem} memory T cell
T_{reg} Regulatory T cell
TSC1/2 tuberous sclerosis 1/2 complex
ULK Unc-51 like autophagy activating kinase 1
UPR Unfolded protein response
V Volts
VPS Vacuolar protein sorting (family)
WHIM Warts, hypogammaglobulinaemia infection and myelokathesis
WT wild-type

1 General Introduction

1.1 Overview of the autophagy pathway

1.1.1 Introduction

The term autophagy originates from the greek "αὐτόφαγος" and translates into "eating of self" [1]. In its broadest definition, it describes an universal cellular pathway that is characterized by the degradation of cellular components via lysosomes. More specifically, it can be subdivided into three major types: Microautophagy, macroautophagy and chaperone mediated autophagy (CMA). Microautophagy is defined as the direct uptake of cargo into lysosomes or endosomes, whereas CMA is restricted to proteins and depends on a molecular machine that involves uptake of these via LAMP2A [2]. Macroautophagy is arguably the best studied type of autophagy with broad relevance described in a multitude of physiological settings, and is hereafter referred to as autophagy. The molecular machinery of the autophagy process is highly conserved from yeast to mammals, and while the process was first discovered in mammalian cells [3], most of the molecules involved were discovered by extensive screening of yeast mutants [4]. This effort has led to the characterization of the "Atg genes" (AuTophaGy related genes), a family of proteins with close to 40 members to date. This work was largely led by Yoshinori Ohsumi, who received the Nobel Prize in Physiology or Medicine in 2016 for this as a representative of the entire field of autophagy researchers, that have defined not only the molecular machinery involved, but importantly also the targets and outcomes of autophagy in settings of health and disease.

1.1.2 Milestones in the autophagy field

Historically, the first observations of autophagic vacuoles were made by Clark and Novikoff in the 1950s describing the presence of degenerating cytoplasm in vacuoles by light and electron microscopy of rat and mouse kidney cells [3, 5]. They understood

that these correspond to acid phosphatase droplets, which they interpreted as support of DeDuve´s hypothesis of cellular autolysis by lysosomes. But it was not until 1963 that Christian DeDuve coined the term auto-phagy, greek for self-eating, at a conference and thereby started the field of autophagy research. Although Christian DeDuve is also criticized for his decision to end his life by legal euthanasia [6], he is celebrated as the founder of this field and received the Nobel Prize in Physiology or Medicine in 1974 for his work. Long before DeDuve however, the famous Russian cell biologist Ilya Metchnikoff described his theory of phagocytosis already at the turn of the 19th century, and from his experiments with tadpole tails he crucially extrapolated the existence of autophagy: He draw explicit parallels between the phagocytes devouring the tad pole tail at the correct time during metamorphosis and the phagocytosis of bacterial pathogens, suggesting a universal mechanism of self-eating both on an organismal but also intracellular level [7]. He believed that phagocytes essentially have the universal role of clearing dysfunctional elements, including endogenous material within the cell, and thereby postulated the existence of autophagy [8]. After these early milestones, further progress in the molecular definition of this pathway only occurred relatively recently, mostly during the last 15-20 years. Initiated by studies in yeast, the molecular characterization of the pathway has identified not only the wide range of Atg proteins [4, 9] but also further defined the cargo and substrates of autophagy. Even early studies already described the presence of mitochondria in cytosolic vesicles, essentially describing mitophagy [10, 11], while organelles such as peroxisomes were only found much later to be degraded by autophagy [12]. In parallel to the increasing understanding of the molecular events, studies found relevance of autophagy beyond simple degradation of superfluous or damaged cytoplasmic material, in the context of differentiation and development [13] as well as human health and disease [14]. To date, remaining challenges in the field are the still incompletely understood molecular events that define the selectivity and magnitude of autophagic processes, as well as strategies

to exploit our mechanistic insights to treat disease by autophagy modulation.

1.1.3 Types of autophagy

Alternative and non-canonical forms of autophagy Autophagy is often broadly categorized into macroautophagy, microautophagy and CMA, but recent studies that describe a variety of LC3-associated alternative and non-canonical pathways blur the lines of these categories and led researchers to clarify the terms used in the field [1]. In this regard, it is important to distinguish autophagy from endocytotic processes such as phagocytosis, receptor-mediated endocytosis and pinocytosis. Nevertheless, the endocytotic pathway is part of autophagy in the context of endosomal microautophagy and shares most of the molecular regulation at least during the late events of endo-lysosomal fusion [15, 16]. Similarly, LC3 is conjugated to phagosomal membranes during LC3-associated phagocytosis, but the molecular players that govern this pathway and the reason for this mostly remain to be identified [17]. Another recent study describes a non-canonical secretory autophagy pathway that can release antibacterial enzymes such as lysozyme in response to ER stress [18]. Finally, other alternative or non-canonical forms of autophagy have been recently described, including BECLIN1 independent non-canonical autophagy [19], ULK1 independent non-canonical autophagy [20], among others [21]. To avoid confusion, it was recently suggested to refrain from the terms “canonical” and “non-canonical” and instead define the specific molecular machinery that the described autophagy pathway is dependent/independent of [1]. In this regard, the only two features that are common to all forms of autophagy are 1) the cytoplasmic origin of the cargo product (as opposed to phagocytosis/endocytosis, but including exogenous cytoplasmic structures such as bacteria) and 2) the dependence on the lysosome for ultimate degradation [1].

Microautophagy Microautophagy is different to classical autophagy in that the uptake of cargo is directly mediated by invagination of the lysosomal membrane, and therefore circumvents the autophagosomal stages. Main functions of this pathway include regulation of organellar size, membrane homeostasis and cell survival under nitrogen restriction [22]. While thought to be mostly unselective initially, microautophagy can degrade multiple substrates in a selective manner, including large structures such as parts of the nucleus [23], damaged mitochondria [24] and lipid droplets [25].

Chaperone mediated autophagy In contrast to micro- and macroautophagy, CMA degradation has only been described for proteins, but not organelles or other structures, and does not require vesicles or membrane invagination for lysosomal target delivery. Instead, it relies on a protein complex bound to the lysosomal membrane which involves the oligomerization of LAMP2A monomers to form a pore complex [2]. Targeting via this machinery requires proteins to have a distinct KFERQ-motif, which can be found in up to 30 % of the proteome [26]. Therefore, it may not be surprising that this pathway is relevant in diverse processes including metabolic homeostasis [27], aging [28] and T cell activation [29].

Macroautophagy Macroautophagy may be the best described type of autophagy and can degrade both proteins as well as organelles and most other cytoplasmic structures both during bulk degradation or selective forms. It consists of three major stages: Initiation of the autophagosome formation at the phagosome assembly site (PAS), elongation of the membrane and sorting of cytoplasmic components to the double-membraned autophagosome, and finally closure of the autophagosome and fusion with the lysosome for hydrolase-dependent degradation of the cargo (*Figure* 1.1). The fused compartment of autophagosome and lysosome is referred to as autolysosome, and this is commonly measured after autophagic degradation is stopped via

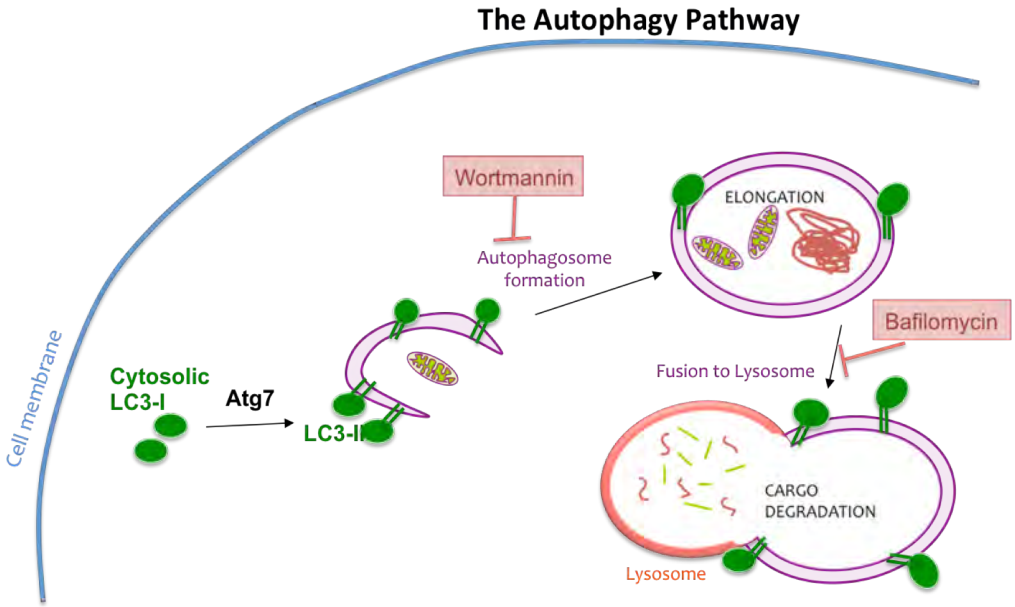

Figure 1.1: The autophagy pathway. Overview of the stages of autophagy, starting with autophagosome nucleation, followed by elongation and closure of the autophagosome and finally fusion with the lysosome to mediate the degradation of the cargo. During the first steps, LC3-I is lipidated to form LC3-II, which is anchored in the autophagosome membrane by a molecular machinery that involves ATG7. Autophagosome formation can be inhibited with the PI3K inhibitor wortmannin, while lysosomal fusion can be blocked by the ATPase inhibitor bafilomycin.

bafilomycin treatment [30]. The resulting buildup of autophagosomes allows quantification of autophagic flux [31]. One of the controversies in the field remains the source of the membrane for autophagosome formation, which has been suggested to originate from mitochondria, the plasma membrane, the golgi, and the ER [32, 33] among other sources [34, 35]. It is important to note that both the origin and the biogenesis of autophagosomes is mediated by a complex molecular machinery which is still incompletely understood despite the ever increasing network of molecular players described [36]. A brief overview of the most relevant molecular events is given in the following section, but also reviewed extensively elsewhere [37, 38].

1.1.4 Autophagy mechanism and signaling

As described earlier, the identification of *Atg* genes in yeast has originally facilitated our molecular understanding of autophagy [4], culminating in close to 40 known ATG proteins and a plethora of interacting regulatory factors [39]. Among these, the core molecular machinery is defined as those factors that facilitate the autophagosome formation and therefore are essential for various types of autophagy. They are often divided into four main categories according to the functional complexes that they form: (A) the primary initiation complex Atg1/ ULK complex (Atg1, Atg11, Atg13, Atg17, Atg29 and Atg31) is the initial stage that regulates the induction of autophagosome formation; (B) Atg9 and its cycling system (Atg2, Atg9 and Atg18) play a role in membrane delivery to the expanding phagophore after the assembly of the Atg1 complex at the PAS; (C) the PtdIns3-kinase (PI3K) complex (Vps34, Vps15, Vps30/Atg6, and Atg14) acts at the stage of vesicle nucleation, and is involved in the recruitment of PI3K-binding proteins to the PAS; (D) two ubiquitin-like (Ubl) conjugation systems: the Atg12 (Atg5, Atg7, Atg10, Atg12 and Atg16) and Atg8 (Atg3, Atg4, Atg7 and Atg8) conjugation systems play roles in vesicle expansion by allowing the conjugation of the membrane lipid phosphatidylethanolamine (PE) to the cytosolic microtubule-associated protein light chain 3 (MAP1LC3B, here referred to as LC3, and corresponding to yeast Atg8), and subsequent incorporation of LC3-PE (also called LC3-II) into the inner and outer membranes of autophagosomes [40, 41, 38]. After substrates have been packed by this mechanism, the autophagosome undergoes a series of maturation steps, and finally fuses with a lysosome to form the autolysosome, a process that involves RAB7 [42, 43]. Once fusion is complete, the contents of the autolysosome are degraded and released into the cytoplasm for recycling.

1.1.5 Autophagy substrates and specificity

While initially thought to be a bulk degradative pathway stimulated in response to cellular stress such as starvation, selectivity in the pathway for specific cargo is becoming a main feature of autophagy [44]. Studies now suggest that non-selective autophagy may be continuously active at basal level in order to maintain cellular homeostasis, but specific stimulation of selective forms of autophagy adds a dominant layer of complexity that can impact a wide area of cellular pathways and is linked to disease [27, 45]. Considering the numerous discoveries of selective autophagy forms, our view is shifting more and more towards appreciating that the autophagic forms that convey specificity and selective regulation may be the more common case. It is possible in this regard that much of the currently deemed non-selective autophagy may in fact represent part of additional but yet undiscovered selective pathways. Supporting this view, numerous studies indicate that Atg8 family proteins function as adaptors for recruitment of both selective autophagy receptors as well as regulatory proteins, which could facilitate selectivity. In mammals, seven Atg8 proteins exist (MAP1LC3A, B/B2, and C, and GABARAP, GABARAPL1, and GABARAPL2 (GATE-16)), suggesting complex diversification of their functions, which however remain incompletely characterized [46]. Selective autophagy receptors contain short linear peptide sequences that bind directly to Atg8s—the so-called LC3-interacting region (LIR) motif [47]. LIR motifs typically contain a W/F/Y-X-X-ψ sequence (ψ, hydrophobic residue: Leucine/Isoleucine/Valine, X is any residue) [44]. In the classical example of this mechanism, aggregates of aberrantly folded proteins are ubiquitylated and bound by ubiquitin-binding domain (UBD) containing receptors such as sequestosome 1 (SQSTM1, hereafter referred to as p62) for subsequent delivery to the autophagosome and lysosomal breakdown. This delivery of the aggregated proteins via p62 is facilitated by p62 oligomerization and binding of p62 to the LC3-II form on the autophagosome via its LIR domain [48]. Subsequently, several such autophagy

receptors were discovered such as optineurin (OPTN) [49], NBR1 [50], TAX1BP1 [51], NDP52 [52], TOLLIP [53], and RPN10 [54]. While p62, NBR1, OPTN and TOLLIP are classically associated with degradation of ubiquitylated proteins, other receptors bind to mitochondria (OPTN, NDP52, TAX1BP1), bacteria (p62, OPTN, NDP52, TAX1BP1) or the midbody of cells (p62, NBR1). These diverse and overlapping selection mechanisms are further complicated by the importance of phosphorylation next to LIR motifs for regulating LC3 interaction, as for example in the case of the mitophagy receptor BNIP3 [55]. Taken together, a plethora of selective autophagy receptors together with a family of Atg8 homologues mediates the selective and regulated targeting of a diverse range of substrates to the autophagosome. These substrates include most cytoplasmic organelles [56, 57, 58, 59, 60, 61, 62, 63, 64], the nucleus [65, 22, 66], glycogen [67], ferritin [68, 69], ribosomes [70, 63], specific protein complexes, and many more [71].

1.1.6 Regulation of autophagy

The magnitude of autophagy activity is highly regulated by several signaling pathways, many of which ultimately merge in the two antagonistic upstream regulatory complexes of mTOR and AMPK signaling. On top of this metabolic and energy/nutrient-dependent signaling axis, there are also transcriptional pathways that regulates autophagy genes, which include the FoxO family of forkhead transcription factors [72] as well as transcription factor EB (TFEB) mediated regulation [73]. TFEB is a master regulator of lysosomal and autophagy gene transcription that translocates into the nucleus and activates transcription in response to its activation on the lysosomal surface [73, 74].

mTOR mTOR was originally discovered as the drug target of rapamycin, a drug that induces autophagy. mTOR, the mammalian target of rapamycin, is therefore a

negative regulator of autophagy. Although mTOR forms two complexes, mTOR complex 1 is better described in its role to suppress autophagy in response to a number of environmental nutrient status signals, including amino acids, growth factors and ROS/hypoxia [75, 76]. It further consists of regulatory associated protein of mTOR (RAPTOR), proline-rich Akt substrate 40kDa (PRAS40), lethal with Sec13 protein-8 (LST8), and DEP-domain containing mTOR-interacting protein (DEPTOR) [77]. Many of these signals converge on the heterodimeric tuberous sclerosis 1/2 complex (TSC1/2), a GTPase activating protein (GAP) for Ras homologue enriched in brain (RHEB). RHEB, in its GTP-associated form, then activates mTORC1. Activation of mTOR can be mediated, among others, by class I PI3K via Akt, and extracellular-signal-regulated kinases 1/2 (ERK1/2), whereas the energy sensor AMPK is a prominent activator of TSC1/2, thereby inactivating mTOR [78]. Targets downstream of mTOR include the translational regulators S6 kinase (S6K) and eukaryotic translation factor 4E-binding protein-1 (4EBP1), resulting in increased translation and protein synthesis. It also activates glycolysis through positive regulation of hypoxia inducible factor 1α (HIF1a) [79]. Its negative regulatory action on autophagy is largely mediated by phosphorylation of the ULK1-ATG13-FIP200 complex which normally initiates autophagy [80, 81, 82] as well as through its interaction with the master regulator of autophagosomal and lysosomal biogensesis, TFEB, on the surface of lysosomes, thereby preventing TFEB nuclear translocation and autophagy gene program activation [83]. As a central metabolic regulator stimulating glycolysis and anabolism while inhibiting autophagy, mTOR has frequent and often opposing roles to autophagy in hematopoietic differentiation, as discussed later and in recent reviews [84, 85, 86, 87].

AMPK AMP-activated protein kinase (AMPK) is a heterotrimeric complex that plays a major role in regulating cellular energy balance by responding to intracellular adenine nucleotide levels [88]. It is activated by an increase in AMP/ADP relative

to ATP via binding of adenine nucleotides to its γ-subunit. Activation of AMPK increases the rate of catabolic (ATP-producing) pathways while decreasing the rate of anabolic pathways. A major function of AMPK is to monitor changes in the level of ATP and to couple this to phosphorylation of downstream substrates to maintain energy homeostasis. Calcium/calmodulin dependent protein kinase kinase (CaMKK) β [89, 90] and liver kinase B1 (LKB1) [91] have been identified as the two upstream kinases in the cascade. Its autophagy promoting function is mediated by opposing mTOR through phosphorylation of RAPTOR [92], through phosphorylation of ULK1 at Ser317/777 [93] and through VPS34 regulation [93, 94]. Similarly to and overlapping with autophagy, a number of roles have been described for AMPK in hematopoietic differentiation, most of which are opposing to mTOR [95, 96, 97, 98, 99, 100, 101].

1.2 Hematopoiesis and granulopoiesis

1.2.1 The hematopoietic tree

At the apex of the hematopoietic tree are hematopoietic stem cells (HSCs), which give rise to all blood and immune cells through differentiation and gradual lineage commitment in a highly regulated process called hematopoiesis [102]. The bone marrow environment supports this process by providing a specialized niche for HSCs, which mainly consists of endothelial cells, mesenchymal stromal cells and osteoblasts to support HSC function [103]. The large majority of HSCs are in a quiescent state and does not divide in order to maintain the reservoir of HSCs and to avoid accumulation of damage [104]. Niche cells support this state of HSCs by providing critical cytokines, including stem cell factor (SCF) [105], TGF-ß [106] and thrombopoietin [107], among others. When HSCs divide, they can either undergo symmetric self-renewal to form a new HSC, or commit to asymmetric division in order to begin the process of lineage differentiation [102]. When long term HSCs commit to differentiate, they

first form short term-HSCs and multi-potent progenitors (MPPs), which have a limited self-renewal ability and undergo expansion while increasingly forming more and more lineage biased progenitors [108, 109]. The classical separation of hematopoietic progenitors describes a common lymphoid progenitor (CLP) and a common myeloid progenitor (CMP), which give rise to myeloid and lymphoid lineages, respectively. This concept however has been refined, as for example the early lymphoid lineage includes lymphoid-primed multipotent progenitors (LMPPs), which retain their granulocyte-monocyte (GM) potential [110, 111]. Similarly, lymphoid progenitors in the thymus can also form macrophages and dendritic cells [112], leading to revised models of the hematopoietic tree.

1.2.2 Granulopoiesis

The revised hematopoietic models discussed above imply that granulocytes can arise within both the classical lymphoid and the myeloid branches. Eosinophilic and basophilic granulocytes can derive from a common erythro-myeloid progenitor, whereas neutrophilic granulocytes can also arise independently within a lympho-myeloid branch [113], and these lineage fates can be separated by expression of GATA-1 at the pre-GM stage [114]. Thus, while granulopoiesis would strictly include two arms of lineage commitment into basophils/eosinophils and neutrophils, the term is commonly, and hereafter used to describe the neutrophilic lineage differentiation. When committed progenitors undergo neutrophil generation under the direction of granulocyte-colony stimulating factor (G-CSF) or granulocyte–macrophage-colony stimulating factor (GM-CSF), myeloblasts (MBs) differentiate from a promyelocyte (PMC), myelocyte (MC) and metamyelocyte (MM) into a band cell (BC), and finally into mature, polymorphonuclear neutrophils (PMN) [115]. During this process, several morphological changes take place that make granulopoiesis an easily traceable system. The histochemical identification and description of neutrophilic granulocytes during their

various stages of differentiation is based on the nuclear invagination from a spherical towards kidney-shaped morphology, which eventually takes a banded or doughnut-shaped form and finally forms the multi-lobulated nucleus of mature neutrophils [116]. It is worth noting that in the bone marrow, PMNs often have a ring like mophology, which can become more multi-lobed (hyper-segmentation) in the context of activation and in the periphery [115]. Neutrophils have four types of granules and secretory vesicles that contain specific effector proteins which are formed during distinct developmental stages. Primary (azurophilic) granules appear during the MB to PMC stage, secondary (specific) granules appear during the MC to MM stage, tertiary (gelatinase) granules appear during the band cell to segmented cell stage and secretory granules appear in PMN [117, 118]. In general, the content of neutrophil granules can be described as a mixture of proteases, receptors and antimicrobial agents that are used to destroy invading pathogens [119]. The formation of neutrophil granules and the correct targeting of their content during maturation in the bone marrow is explained by the "targeting by timed synthesis" hypothesis [120]. Hereby, the production of granule proteins and their localization to granules or secretory vesicles occurs by timing of expression during the various stages of neutrophil development. This entails that another way of defining these neutrophil differentiation stages molecularly is by determining the stage specific granule gene signature associated with each type of granules. MBs express highest myeloperoxidase (MPO), myelocytes express lactoferrin (LTF), BCs and PMNs express gelatinase and metalloproteinase-9 (MMP-9), and so on. The stages of granulopoiesis with the associated type and content of granules is summarized in *Figure* 1.2. Some of the major transcription factors that define these populations are also noted, including CCAAT/enhancer-binding protein alpha (C/EBPα) and C/EBPε, that govern early granulocyte lineage determination [121, 122]. PU.1 is another essential transcription factor which is expressed in later stages [123]. Because C/EBPα is not expressed in HSCs at high levels, but is turned on in GMPs, its pro-

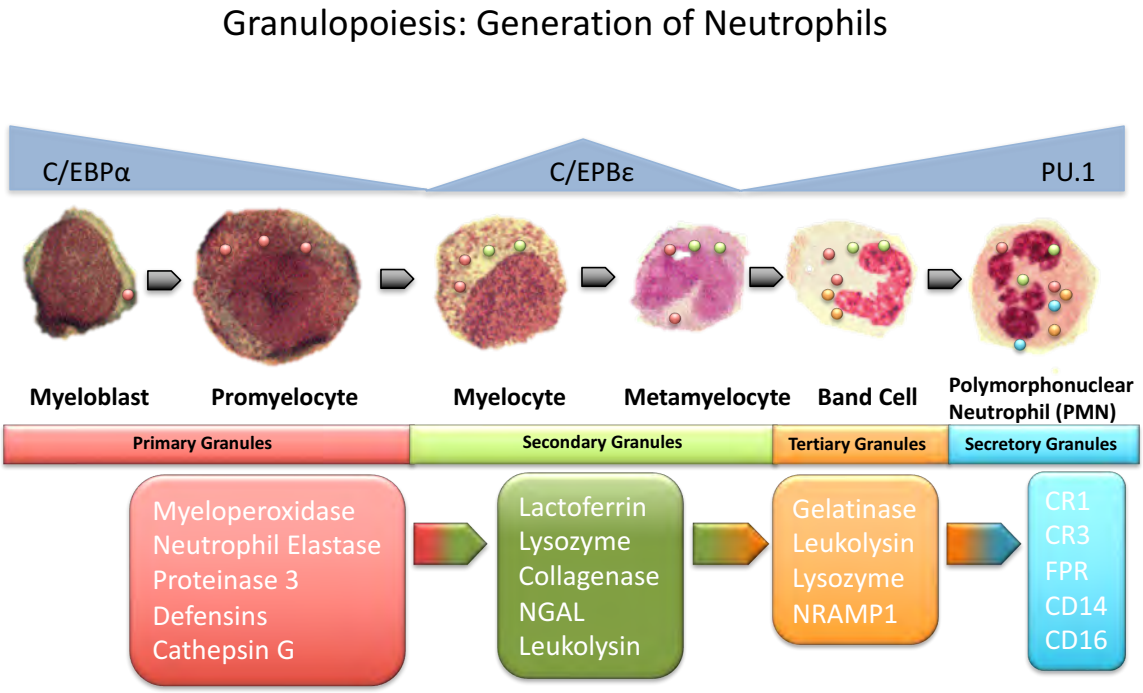

Figure 1.2: Overview of granulopoiesis. Myeloblasts and promyelocytes are the first committed neutrophil progenitors, which only contain primary granules and express high C/EBPα. They give rise to myelocytes and metamyelocytes, which form secondary granules and express C/EBPe. From these, band cells are formed, containing tertiary granules and giving rise to the polymorphonuclear mature neutrophil which also contains secretory granules.

moter can be useful to drive the expression of *cre*-recombinase in early granulocyte progenitors in transgenic mice [121].

1.3 Neutrophils

1.3.1 Neutrophil homeostasis

The neutrophil was established more than a century ago as a specialized phagocyte with a host of antimicrobial enzymes, cell surface receptors and reactive oxygen or nitrogen species to target pathogens for destruction. Ironically, Metchnikoff´s fasci-

nation with granulocytes and phagocytic cells marked not only the beginning of the field of immunology, but his postulations about intracellular degradation also set the foundations for the discovery of autophagy by Clark, Novikoff, DeDuve and others many years later. Among the subsets of granulocytes, names were assigned according to their histological staining patterns: eosinophils stain with eosin, basophils stain with basic stains and neutrophils stain with neutral stains [124]. Neutrophils have their main reservoir in the bone marrow, where they make up about 30% of cells, and are synthesized at a rate of one million cells per second in the bone marrow of humans. Their homeostasis is in part controlled by G-CSF, IL-17, and IL-23 cytokines, and retention in the bone marrow through interaction of CXCL12 with its receptor CXCR4 [125][126]. CXCR2 is the antagonistic chemokine receptor that mediates release from the bone marrow and interacts with its ligands, CXCL1 and CXCL2 [127]. The relevance of neutrophil homeostasis is underscored by a number of associated human diseases such as warts, hypogammaglobulinaemia infection and myelokathesis (WHIM). WHIM patients carry an activating mutation in CXCR4, which promotes neutrophil retention in the bone marrow through increased interaction with CXCL12 [128, 129]. A benign form of ethnic neutropenia occurs in some African populations that have up to 10-times reduced blood neutrophil counts without overt clinical symptoms [130]. Mutations in granule genes are a common course of genetic neutrophil associated disease, as well as in NADPH oxidase genes, summarized as chronic granulomatous disease (CGD). Owing to defective neutrophil bacterial killing, these diseases are characterized by frequent and recurrent life threatening infections [131, 132]. Finally, severe congenital neutropenia (SCN) is characterized by a block at the promyelocytic stage of differentiation resulting in severe and recurring infections. Mechanistically, mutations in the ELANE gene are thought to cause activation of the unfolded protein response (UPR) and endoplasmatic reticulum (ER) stress pathways, which leads to the differentiation block and eventually to apoptosis [133, 134]. Interestingly, SCN

patients often go on to develop myelodysplastic disease and myeloid leukemia later on in life, which highlights the relevance of blocked differentiation as causative contribution to this disease (Discussed in 6.4). Importantly, autophagy is a major pathway that counteracts and prevents ER stress [57, 135] and may promote differentiation, but therapeutic relevance in the context of SCN and leukemic disease has yet to be demonstrated.

1.3.2 Mobilization and trafficking

Before neutrophils encounter pathogens, they have to leave the vasculature in order to access peripheral sites of infection. Recruitment into many various organs and tissues is unique and the subject of several excellent reviews [136, 137, 138]. In brief, the common three-stage mechanism consists of rolling, adhesion and trans-endothelial migration of neutrophils. The endothelial molecules ICAM-1 and VCAM-1, as well as the selectin family (E-, P-, and L- selectin) are known as central mediators of neutrophil rolling and adhesion to the endothelium [139, 137]. Engagement of these molecules by the neutrophil integrin receptors initiates the activation of several signaling pathways within both cell types that eventually stop the rolling across the endothelium and initiate firm adhesion. After establishment of firm adhesion, neutrophils can either transmigrate trans-cellularly (penetrating through an individual endothelial cell, roughly 20 % of events) or para-cellularly (squeezing between two endothelial cells, about 80 % of events) [140].

1.3.3 Effector functions

Once arrived at the site on infection, neutrophils can rely on phagocytosis and intracellular destruction of microbes, or alternatively use their arsenal of cytotoxic granules to kill pathogens by degranulation and release of toxic agents. Neutrophils can rapidly phagocytose opsonized pathogens, a process that is mediated by a complex interplay of

membrane receptors, cytoskeletal rearrangements and intracellular signaling cascades [141]. Subsequently, fusion with intracellular granules and assembly of the NADPH oxidase for ROS generation leads to efficient destruction of the pathogen. Microbial trapping by extrusion of a meshwork of chromatin bound to granular peptides termed neutrophil extracellular traps (NETs) [142] is another more recently described concept of neutrophil function [143]. NETs expose a concentrated meshwork of antimicrobials, including DNA and histones, which can trap and kill microbes as well as activate other immune cells. Neutrophils might be triggered to respond differently to distinct microbes, and the relevance of these antimicrobial processes in specific diseases is not entirely clear [144]. Interestingly, autophagy is required for intracellular chromatin decondensation regular NET formation and NETosis (NET formation resulting in cell death) [145, 146]. This has recently been challenged by one report that found no role for autophagy in NET formation [147]. It is clear that the type of stimulus and context can lead to a number of distinct NET and NETosis pathways, which are still incompletely understood and may be the cause for this discrepancy [148]. One of these pathways depends on mitochondrial NET formation, highlighting the significance of mitochondria for mature neutrophils [149] and preserving the nucleus, and thus viability of neutrophils in the face of microbial insult. Although mitochondrial NETs enable preservation of an intact nucleus, the nucleus of a neutrophil can also be dispensable for many cellular functions [150]. Finally, the previously mentioned release of antimicrobial peptides is a major function of neutrophil mediated pathogen defense. These peptides can be released into body fluids such as sweat or mucus and play an important role at epithelial barriers [151].

1.4 Cellular energy metabolism

Energy metabolism is summarized as the cellular processes that generate energy in the form of ATP. There are several interconnected pathways that serve this function that

can operate either in the presence or absence of oxygen. Anaerobic metabolism is defined as the breakdown of glucose in the absence of oxygen, resulting in the generation of only 2 mol ATP per mol glucose. However, as a classical redox-pair, the oxidation of glucose also generates the reduced form of NAD^+, which is NADH. NADH is an important co-factor for many enzymes of central metabolism and is also recycled during oxidative phosphorylation (OXPHOS). If cells do not regenerate NADH into NAD^+ via mitochondrial metabolism, for example during anaerobic conditions, glycolysis-derived pyruvate can be reduced into lactate, thereby generating NAD^+ from NADH to drive further glycolytic metabolism [152, 153]. Aerobic metabolism converts glucose into 32 ATP molecules via oxygen dependent, mitochondrial respiration and thus seems the more efficient choice for energy production. The tri-carboxylic acid cycle (TCA cycle) inside mitochondria can be fed by glucose-derived pyruvate via the action of pyruvate dehydrogenase (PDH) to form Acetyl-CoA. The TCA cycle can also receive nutrient input from multiple other sources (termed anaplerosis), for example from amino acids such as glutamine via the action of transaminases [95]. The TCA cycle supports the electron transport across the inner mitochondrial membrane (IMM) via NADH and FADH2, which in turn generates the electron-gradient that mediates ATP production during OXPHOS. Fatty acids can be synthesized from the TCA intermediate citrate after its export into the cytosol, or can be utilized for ATP production by breakdown into Acetyl-CoA units and NADH + FADH2 co-factors, both of which enter mitochondrial respiration as described. The breakdown of fatty acids occurs inside mitochondria in order to avoid a futile circle with fatty acid synthesis (FAS). While short chain fatty acids (defined as 6 carbon atoms or less) can diffuse into mitochondria passively, the usual route requires conjugation to carnitine via the action of carnitine palmitoyl transferase I (CPT1) [95]. Etomoxir is a specific inhibitor of CPT1 often used to interfere with fatty acid oxidation (FAO) [154]. Finally, although not technically an energy-metabolic pathway, the pentose phosphate branch can re-route

metabolites from glycolysis towards synthesis of nucleotide and amino acid precursors.

1.5 Metabolism and Differentiation

A major task of metabolic pathways in the context of differentiation is to produce molecules, metabolites and energy necessary for the events associated with a new cellular state. Autophagy in this view may have similar and overlapping roles with other metabolic pathways to adapt the cellular availability of biomolecules for differentiation. Because the metabolome of immune cells is highly complex and flexible, it is difficult to deduce general principles that may explain or predict interactions between metabolic states and differentiation. However, one common feature in hematopoietic differentiation is enhanced cellular proliferation before cells undergo transition into post-mitotic quiescence (cell cycle exit). This principle is evident as hematopoietic stem and progenitor cells (HSPCs) expand but eventually undergo terminal differentiation to form the terminally maturated blood cells. In a similar way, T effector cells undergo rapid expansion and proliferation in response to antigen stimulation, but transition into memory T cell (T_{mem}) fate or regulatory T cell (T_{reg}) fate is associated with a long-lived, quiescent state [155]. In each of these cases, a hallmark of the proliferative state is aerobic glycolysis, a metabolic state originally described for cancer cells used to sustain proliferation and anabolism even in the presence of oxygen (Warburg metabolism) [156, 157]. Accordingly, metabolic changes during differentiation of healthy cells may be controlled by similar mechanisms as cellular proliferation and differentiation of transformed cells. In line with this, defective differentiation is a hallmark of some cancers, and targeting cancer cell-specific anabolic metabolism can be an effective way to treat cancer by inducing differentiation, as recently demonstrated in the context of acute myeloid leukemia (AML) [158]. A remaining major challenge, however, is to establish whether metabolic changes are causative for differentiation dynamics, an unrelated bystander, or rather a consequence, downstream of the signaling

that controls differentiation [159]. It is also unclear how these metabolic changes link in with the more established transcriptional control of differentiation. In this respect, other tissues and basic model organisms have elucidated remarkable and definitive causative links between metabolism and differentiation [159]. Evidence for this is now also emerging in the field of immunometabolism, with several examples showing that extensive metabolic remodeling may actively control lineage specification, activation and effector functions of immune cells [95]. Particularly in myeloid cells and T cells, the balance between mitochondrial and glycolytic metabolism has recently been identified as a central determinant of differentiation and phenotype, rather than merely being a bystander of differentiation [95, 97, 160, 161] (Discussed in more detail in 4.1.1 and 5.1.2). Although the link between autophagy, metabolism and differentiation has only been investigated in a few pioneering studies, the autophagic and metabolic signaling pathways are highly inter-connected, as discussed next.

1.6 Autophagy and metabolism are integrated by common nutrient-sensing pathways

When autophagy degrades cytoplasmic material, the resulting breakdown products can be directly used in other cellular metabolic pathways, thus intimately linking autophagy and metabolism. The recycled metabolites can either be used for anabolic pathways to produce new cellular building blocks, or harnessed for energy production. For example, the amino acids resulting from autophagic proteolysis can be used for translation, but over two thirds of amino acids are either glucogenic or ketogenic, meaning that they can feed directly into central metabolism for ATP production. Furthermore, by selectively degrading key metabolic organelles/compartments such as mitochondria (mitophagy), peroxisomes (pexophagy) and lipid droplets (lipophagy), autophagy has the capability to rapidly redirect cellular metabolite flow [162]. Fi-

nally, both mTOR and AMPK signaling, the two key integrators for environmental status and nutrient availability, directly control and coordinate autophagy together as discussed above (See 1.1.6). mTOR complex1 activation is a well established potent initiator of protein translation and ribosome biogenesis and also activates transcription factors, such as HIF1A and sterol regulatory element binding transcription factor (SREBF), resulting in the expression of genes controlling diverse pathways including glycolysis, inflammation and lipid synthesis [163]. While mTOR supports cell growth via these downstream metabolic pathways, it also negatively regulates autophagy by the mechanisms discussed earlier. Conversely, AMPK is a positive regulator of autophagy by suppressing mTOR activity and activating autophagy in times of ATP demand. Thus, the cellular energy-metabolic balance, autophagy, and their regulation via AMPK and mTOR signaling are intimately linked. It is not surprising that each of these pathways are crucially involved in immune cell differentiation. Particularly in effector, T_{reg} and T_{mem} differentiation as well as myeloid differentiation, recent discoveries start to reveal common principles that link interactions between these pathways to cellular differentiation, which will be discussed in the relevant thesis chapters.

1.7 Thesis summary

The focus of our lab over the past years has been to unravel the roles of autophagy in diverse settings of hematopoietic differentiation. Starting logically at the apex of hematopoiesis, an essential role was defined for *Atg7* in HSC maintenance and function [164]. Subsequently, autophagy was described as a critical process in memory T cell generation and maintenance, preventing accumulation of mitochondria and excessive glycolysis [165]. In collaboration, a similarly critical role of autophagy in regulatory T cells was described by the Maloy group, where autophagy also prevents excessive glycolysis and maintains lipid metabolic homeostasis [166]. In the myeloid lineage, work from our lab described an essential role for autophagy in preventing a

pro-inflammatory macrophage polarization and immuno-senescence [167] and to limit proliferation and transformation of acute myeloid leukemia [168]. While these studies, supported by other research groups [169, 170, 171, 172], provide robust *in vivo* evidence for the relevance of autophagy in a number of hematopoietic and immune differentiation processes, the targets and mechanisms of autophagy involvement are mostly unknown. In addition, the role of autophagy during granulopoiesis is largely unknown. While no evidence currently connects autophagy with eosinophil and basophil differentiation, there is indirect evidence that autophagy may either augment [173, 174, 175, 176] or hinder [177] neutrophil differentiation. Because this evidence is largely derived from oncogene-transformed lines or only explores terminal maturation, it has remained elusive how autophagy may affect neutrophil differentiation *in vivo*.

In this thesis, data is presented that metabolism and autophagy are developmentally programmed and essential for neutrophil differentiation *in vivo*. This dependency on autophagy is shown to occur at the first committed progenitor stage, to be independent of autophagy in HSCs, and to be cell intrinsic in its nature, both *in vitro* and *in vivo*. Further mechanistic experiments describe a metabolic remodeling that occurs during granulopoiesis, which depends on autophagy to limit glycolysis and engage oxidative mitochondrial metabolism: *Atg5/7*-deficient neutrophil precursors had increased glycolytic activity but impaired mitochondrial respiration, decreased ATP production, and accumulated lipid droplets. Inhibiting autophagy-mediated lipid degradation, or fatty acid oxidation alone, was sufficient to cause defective differentiation, while administration of fatty acids or pyruvate for mitochondrial respiration rescued differentiation in autophagy-deficient neutrophil precursors. Together, this shows that autophagy-mediated lipolysis provides free fatty acids to support a mitochondrial FAO-OXPHOS respiration pathway essential to neutrophil differentiation. The aim of this thesis is to present this new data on neutrophils in the light and context of previous findings from this lab and others, discussing the insights and common prin-

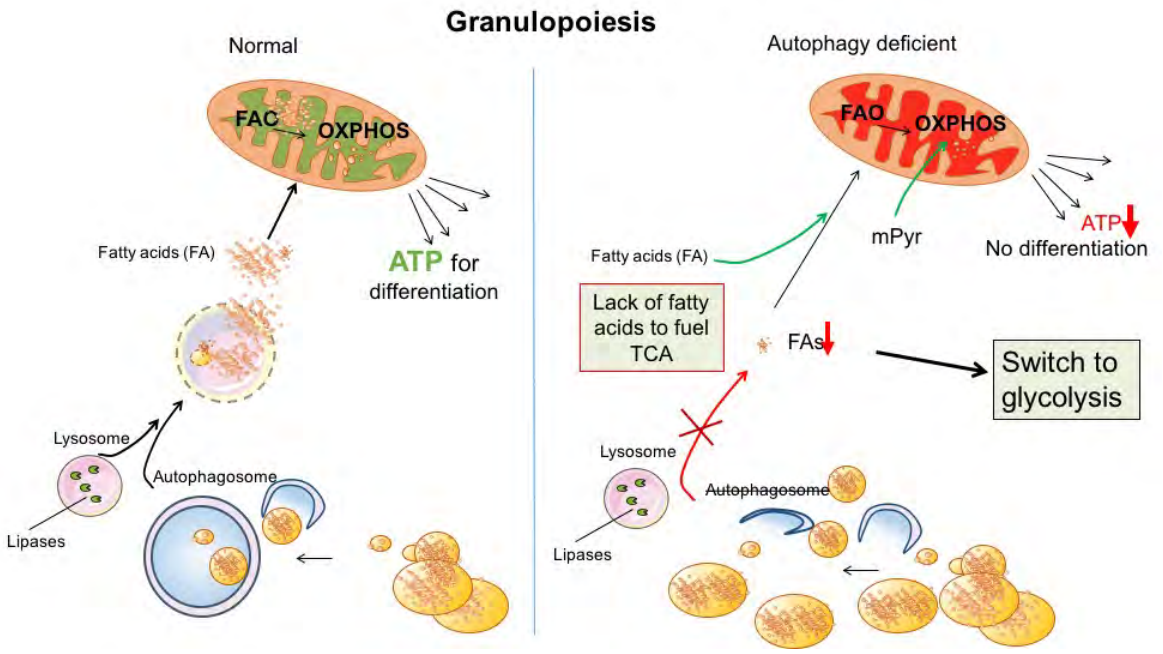

Figure 1.3: Graphical thesis summary. Neutrophil differentiation requires efficient ATP production via lipid-driven mitochondrial respiration. Left: This pathway is fed by autophagy-mediated breakdown of cellular lipid droplets through the action of lysosomal lipases. The generated free fatty acids are imported into mitochondria for fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS). Right: In the absence of autophagy ($Atg5/7^{-/-}$), lipid droplets accumulate intracellularly as a result of defective autophagy-mediated targeting for degradation. As a consequence, intracellular free fatty acid (FA) levels are depleted, interfering with FAO and OXPHOS. The shortage of ATP consequently results in arrest of neutrophil differentiation and the switch to compensatory glycolysis.

ciples evident from these studies to deduct emerging patterns of autophagy-mediated metabolic differentiation mechanisms that may be applicable in a broader setting of cellular differentiation. A summary of the main findings presented as part of this thesis is also given in section 6.5, was published recently [178], and is summarized again graphically here (Figure 1.3).

2 Methods

2.1 Mice

C/EBPa-cre mice [121] were a kind gift from Prof. Ivo Touw and crossed with $Atg7^{f/f}$ mice [179] to obtain *C/EBPa-cre x* $Atg7^{f/f}$ mice on a C57BL/6 background. *Vav-cre* [180] and *Mx1-cre* [181] mice were obtained from Charles River, UK and crossed with $Atg7^{f/f}$ and $Atg5^{f/f}$ mice, respectively. Cre^{-} *x* $Atg7^{f/f}$ mice were used as controls and were littermates where possible. C57BL/6 SJL CD45.1 mice for bone marrow chimeras were purchased from Biomedical Services, Oxford. All mice were housed under specific pathogen-free conditions in Biomedical Services, Oxford and animal experiments were approved by the local ethical review committee and performed under UK project license (PPL 30/3388).

2.2 Genotyping

2.2.1 DNA extraction

For Genotyping, mice were ear-clipped and the obtained tissue was digested for 2 hrs at 55° C in lysis buffer (5mM EDTA, 0.1 M Tris-HCl, 0.2% SDS, 0.2 M NaCl and 7.8 mg/mL proteinase K [Sigma]). Any remaining tissue was pelleted by centrifugation (16000 x g, 10 min).

The supernatant was aspirated and mixed with an equal volume of isopropanol for DNA precipitation. The precipitated DNA was pelleted (16000 x g, 10 min) and washed in 70 % ethanol. The DNA was dried at 50° C for 10 minutes, resuspended in 20-50 µl DNA/DNAse free water and stored at -20° C.

2.2.2 Polymerase chain reaction (PCR)

PCR reactions for the *Atg7 flox & wt*, *Atg5 flox & wt*, *Vav-Cre*, and *C/EBPa-cre* alleles were carried out in a total volume of 20 µL, using MyTaq™ Red Mix combined PCR mix (Applied Biosystems).

Conditions:

1. 95° C, 3 min
2. 95° C, 30 sec
3. 64° C, 30 sec
4. 72° C, 1 min
5. 34× cycles from step 2
6. 72° C for 10 min
7. Hold at 4° C

2.2.3 Primers

Vav-Cre PCR

Cre-Fwd: 5'-AGATGCCAGGACATCAGGAACCTG-3'

Cre-Rev: 5'-ATCAGCCACACCAGACACAGAGATC-3'

Atg7 Flox and *Wt* PCR (*Wt* fwd Primer)

752: 5'-CCATGCTGATGGCTAATGTCTC-3'

753: 5'-CTGCAGGAATTCGATATCATAACTTCG-3'

Atg7-14R: 5'-GTCCAGAGTCCGGTCTCTGGTTG-3'

Atg5 Flox (2. + 3.) and *Wt* PCR (1. + 2.)

1.) 5'-GAATATGAAGGCACACCCCTGAAATG-3',

2.) 5'-GTACTGCAT-AATGGTTTAACTCTTGC-3',

3.) 5'ACAACGTCGAGCACAGCTGCGCAAGG-3'

DNA analysis was performed on a 2 % agarose gel, prepared in 150 mL 1× TBE buffer (45 mM Tris-Borate, 1mM EDTA, 5 µl SYBR® Safe DNA Gel Stain. Gels were

run at 80 V for ~35 min until the bands were sufficiently separated and visualized via G-Box (Syngene).

2.3 Generation of bone marrow chimeric mice

For *Mx1-Cre x Atg5$^{f/f}$* (pIpC inducible) chimeras, BM was extracted from tibia and femur of a single 8-week old *Mx1-Cre^{neg}x Atg5$^{f/f}$ or Mx1-Cre x Atg5$^{f/f}$*(both CD45.2^{+}), and C57BL/6 SJL mouse (CD45.1^{+}). 2x10^{6} CD45.2^{+} BM cells were added to 2x10^{6} CD45.1^{+} BM cells (1:1 CD45.2^{+}: CD45.1^{+}) in a total volume of 200 µL PBS. The 1:1 BM mix was injected *i.v* 2-4 hrs after sub-lethal irradiation (450cGy twice, 4 hours apart) into C57BL/6 SJL CD45.1^{+} recipients. 6 weeks post-transplantation, mice were injected *i.p.* with 250 µg pIpC in 250 µL PBS or 250 µL PBS in order to induce expression of mx1-Cre and monitored over a time of 18 weeks by serial bleeding and evaluation of reconstitutions and hematopoietic lineages every 3 weeks. After 18 weeks, mice were sacrificed for analysis of bone marrow. Chimeric mice that received *Mx1-Cre x Atg5$^{f/f}$* CD45.2 cells were termed *Atg5*-deficient chimera (*Atg5$^{-/-}$*), while the mice that received *Mx1-Cre^{neg} x Atg5$^{f/f}$* donor type cells (CD45.2) were termed control chimera.

2.4 Cell culture and treatments

32D-cl3 myeloblast cells [182] (obtained from ATCC) were cultured in RPMI 1640 with 10% FCS, 2 mM L-glutamine,100 U/ml Pen-Strep, and 10 ng/ml IL-3. For differentiation into neutrophils during 5-9 days in G-CSF, the IL3-cultured cells were first washed twice with 50 ml PBS to remove IL-3 and resuspended at 2 x 10^{5} cells/ml in medium containing 100 ng/ml recombinant murine G-CSF (peprotech). Three independent CRISPR Atg7$^{-/-}$ clones were generated with sgRNAs as dsODN into pX458 (Feng Zhang, Addgene 48138) plasmids. These plasmids targeted several independent

regions in exons 3-4 of the *Atg7* gene. Transfection was achieved by electroporation and after three days, single cells were flow-sorted based on GFP-expression. Single cell-derived stable clones were grown in IL-3 supplemented medium and frameshift deletion confirmed by target site sequencing and western blotting. In experiments involving drug treatments, methyl-pyruvate (2 mM), di-ethyl-umbelliferyl-phosphate (DEUP, 5 µM), etomoxir (60 µM), orlistat (100 µM) linoleic acid (10 µg/ml), FFA mix (10 µg/ml each linoleic, linolenic, myristic, oleic, palmitic and stearic acids + 0.22 mg/ml cholesterol) (all from Sigma) were added to the cells in G-CSF containing differentiation medium for the time indicated. Controls were treated with the appropriate vehicle solution (H_2O, PBS or DMSO).

2.5 Magnetically-activated cell sorting (MACS)

Ly6G-specific beads were used to enrich for neutrophils and their terminal precursors from bone marrow suspensions using the MACS Ly6G isolation kit (Miltenyi Biotec) and MS or LS columns according to cell number.

2.6 Flow cytometry

Staining was performed as previously described [165]. In brief, single cell suspensions were obtained from the analysed organs by homogenization. For bone marrow, tibia and femur were crushed using a mortar and pistil and filtered through 70 µm mesh. All stainings were performed in 96 well round bottom plates in 50 - 100 µl staining volume. Fluorochrome-conjugated monoclonal antibodies were used for surface labelling (BD Bioscience, eBioscience or Biolegend). Antibody binding occurred at room temperature for 10 minutes followed by two wash steps. For mitochondrial mass analysis, cells were stained with MitoTracker Green (Life Technologies) at 150 nM in PBS, 2% FCS for 30 min at 37° C after surface antibody staining. GLUT-1

was measured through binding to its ligand, the receptor binding domain (RBD) of a recombinant glycoprotein from the human T lymphotrophic virus (HTLV) fused to eGFP (RBD-eGFP, Metaflora). To measure the consumption of glucose, cells were incubated in 50 µg/ml 2-NBDG in RPMI1640 for 20 min at 37° C. To quantify lipid content, cells were incubated in 1000 ng/ml bodipy in RPMI1640 at 37° C for 30 min. All samples were analysed on Canto III or LSR II flow cytometers (BD Biosciences).

2.7 Metabolomic analysis by LC/MS/MS and GC/GC/MS

Metabolite extraction was performed following protocols from the Chemistry Research laboratory (Courtesy of James McCullagh) and the Target Discovery Institute (Courtesy of Benedikt Kessler), recently described elsewhere [183]. In brief, $5x10^6$ cells were cultured for the indicated time in IL3 or G-CSF containing medium. 500 µL of ice cold 80 % aqueous methanol was added to the pelleted cells and the supernatants filtered with a 3KD ultrafilter (Millipore), dried by SpeedVac with vacuum and stored at -80° C. On the day of analysis, the dried extracts were re-constituted in 60 µL of ice cold 80 % aqueous methanol. A quality control (QC) sample was made by combing 5 µL of each sample. This was injected at the start of the sequence and subsequently every 10 samples throughout the LC/MS/MS analysis. Metabolites were analysed using a Thermo Scientific ICS-5000^+ ion chromatography system coupled directly to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer with a HESI II electrospray ionisation source (Thermo Scientific). The IC flow rate was 0.250 mL/min. The total run time was 37 mins and the hydroxide ion gradient comprised as follows: 0 mins, 0 mM; 1 min, 0 mM; 15 mins, 60 mM; 25 mins, 100 mM; 30 mins, 100 mM; 30.1 mins, 0 mM; 37 mins, 0 mM. For data analysis, the raw data files were processed using ProgenesisQI (Waters, Elstree). The measured criteria compared where a combination of accurate mass analysis (<5ppm), retention time (+/- 15s), fragmentation pattern and isotope pattern matching (>90%) were given in comparison to known metabolite

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PE anti-mouse CD34 Antibody, Clone HM34	BioLegend	Cat#152204; RRID:AB_313659
PE/Cy7 anti-mouse Ly-6G Antibody, Clone 1A8	BioLegend	Cat#127618; RRID:AB_1877261
APC/Cy7 anti-mouse CD117 (c-kit) Antibody, Clone 2B8	BioLegend	Cat#105826; RRID:AB_2632809
APC anti-mouse TER-119/Erythroid Cells Antibody, Clone Ter119	BioLegend	Cat#116212; RRID:AB_313713
PE anti-mouse CD11b Antibody, Clone M1/70	BioLegend	Cat#101208; RRID:AB_312790
APC anti-mouse/human CD45R/B220 Antibody	BioLegend	Cat#103212; RRID:AB_312996
APC anti-mouse CD8a Antibody, Clone 53-6.7	BioLegend	Cat#100712; RRID:AB_312751
APC anti-mouse CD19 Antibody, Clone 6D5	BioLegend	Cat#115511, RRID:AB_313646
Anti-mouse monoclonal G-CSF R/CD114 Antibody (723806); Alexa Fluor-405	R&D Systems	Cat#MAB6039, RRID:AB_10890232
APC anti-mouse CD4 Antibody, Clone GK1.5	BioLegend	Cat#100412; RRID:AB_312696
Alexa Fluor 488 anti-mouse CD36 Antibody, Clone G8.8	BioLegend	Cat#102607; RRID:AB_1134107

Table 1: List of Flow-cytometry antibodies

standards to assure correct identification. For lipidomics, 5x10^6 cells were homogenized in 400 µl of 50 % MeOH/H_2O in a beads beater tube. Myristic-acid-14,14,14-d3 (1 mg/ml) standard was spiked at ratio 1 µg/5x10^6 cells and samples were vortexed for 5 min after adding 1 ml of tert-butyl methyl ether (MTBE). After centrifugation at 13,000 RPM supernatants were collected into a 5:1 MeOH/H_2O solution. The aqueous phase was dried in a vacuum centrifuge and combined with the dried MeOH pellet. For rederivatization, dried metabolite extraction was re-suspended in 20 µg/µl MOX (volume 50 µl) and reacted at 30° C for 90 min on vortex. 30 µl of pyridine and 70 µl of MSTFA were added to react at 60° C for 60 min before direct injection into GCxGC-MS system comprising of a gas chromatograph coupled to a quadrupole mass spectrometer (Shimadzu GCMS QP2010 Ultra) and a Shimadzu AOC-20i/s auto sampler. The first dimension separation was carried out on a SHM5MS capillary column (30 mÅ~0.25 mm i.d.Å~0.25 µm film thickness) while the second dimension separation was on a BPX-50 capillary column (5 mÅ~0.15 mm i.d.Å~0.15 µm film thickness). The samples were injected at 280° C in different split ratios (between 1:1 to 1:200). The MS was operated at scan speeds between 5000 and 20,000 amu covering a range of m/z 45–600. Electron Ionization spectra were recorded at 70 eV.

2.8 q-PCR

RNA extraction was performed using the RNeasy Kit (Qiagen) and quantified with a Nanodrop spectrophotometer (Thermo Scientific). RNA reverse transcription (RT) was performed using a High Capacity RNA to cDNA kit (Applied Biosystems(AB)). The resulting cDNA was stored at −20° C. Gene expression analysis was performed using validated TaqMan probes (AB) on a 7500 Fast Real-time PCR machine (AB) set to comparative Ct method ($\Delta\Delta$Ct) . Conditions:

(1) 50° C, 2 min;

(2) 95° C, 10 min;

(3) 95° C, 15 s;

(4) 60° C, 1 min; 40 cycles of 3–4.

Normalization was performed relative to housekeeper genes *Actin, Gapdh, Hprt, B2m or 18S rRNA*.

Taqman probes: Mm00504340_m1 (*Atg5*), Mm00512209_m1 (*Atg7*), Mm01545399_m1 (*Hprt*), Mm99999915_g1 (*Gapdh*), Mm00607939_s1 (*Actin*), Mm01298424_m1 (*Mpo*), Mm00442991_m (*Mmp9*), Mm00434787_m1 (*Ltf*), Mm00514283_s1 (*C/EBPa*),

2.9 Fluidigm gene expression analysis

32D CRISPR-$Atg7^{-/-}$ and $Atg7^{+/+}$ control clones were collected by flow cytometric sorting (two hundred cells/population) and directly lysed in OneStep lysis buffer (Invitrogen). RNA reverse transcription and cDNA preamplification was performed using the CellsDirect OneStep q-RT kit (Invitrogen). 48 genes were amplified and analyzed for their expression in 48 samples (total of 2304 individual qPCR reactions) using a dynamic 48x48 array (Biomark Fluidigm) [184]. Data were analyzed using the $\Delta\Delta$Ct method, and all results were normalized to the most stable housekeeping gene identified in each context.

2.10 Immunoblotting

1×10^6 32D CRISPR-$Atg7^{-/-}$ and $Atg7^{+/+}$ cells were differentiated with G-CSF or cultured in IL-3, or 2×10^6 primary $Ly6G^+$ cells from BM were resuspended in 100 µl 1 × RIPA Lysis Buffer. BCA Protein Assay Kit (Thermo Scientific) was used for protein quantification, and after addition of reducing Laemmli Sample Buffer, SDS-PAGE electrophoresis was performed. 30–50 µg protein per lane were separated on 4%-12% SDS-PAGE, blotted to PVDF membranes (Millipore), which were subsequently blocked with 5 % skim milk. Primary antibodies: LC3 (L8918, Sigma) (1:1000), GAPDH

(MAB374, Millipore) (1:10,000), beta-ACTIN (Cell-Signaling)(1:20,000). Secondary antibodies: IRDye® 680RD Goat anti-Rabbit IgG and IRDye® 800CW Donkey anti-Mouse IgG secondary antibodies (LI-COR, Lincoln) (1:15,000) were used for fluorochrome-based band quantification using a LICOR Odyssey CLx imaging system in standard scan mode.

2.11 Microscopy

2.11.1 Transmission electron microscopy (TEM)

For transmission electron microscopy (TEM), a protocol was followed which was provided by the Dunn School of Pathology EM facility (Courtesy of Errin Johnson) and based on Reynolds et al [185]. In brief, isolation of $1x10^6$ cells by flow cytometry based on CD11b and Ly6G expression was performed and cells were fixed in 2.5 % glutaraldehyde, 4 % PFA in 0.1M PIPES buffer, pH 7.2 for approximately 1 hr at room temperature, then stored at 4° C in the fixative for 36 hrs. Pellets were washed in 0.1M PIPES buffer pH 7.2 for 3x15 mins, then in 100 mM glycine in the same buffer for 20 mins followed by 15 mins in 0.1M PIPES buffer. Cells were resuspended in warm 2.5% low melting point agarose in 0.1M PIPES and spun at 10,000 rpm for 1 min. Once set, cut agarose was incubated in 1% osmium tetroxide in 0.1M PIPES for 1 hr at 4° C, then washed with water for 20 mins. Samples were incubated in 0.5 % uranyl acetate in water overnight at 4° C and then taken through a graded ethanol series (30 %, 50 %, 70 %, 80 %, 90 % and 95 % ethanol for 10 mins each on ice, then 100 % ethanol for 90 mins on ice). Samples were gradually infiltrated with Agar100 epoxy resin, starting with 25 % resin for 1 hrs, 50 % resin for 2 hrs, 75 % resin for 1 hr and 100 % resin overnight, followed by two changes with fresh 100 % resin the next day before embedding in Beem capsules and polymerisation for 48 hrs at 60° C. Ultrathin sections (90 nm) were obtained using a Leica UC7 ultramicrotome with a

diamond knife (Diatome) and placed on 200 mesh copper grids, then post-stained with Reynold's lead citrate for 5 min, and imaged on a FEI 12 TEM operated at 120 kV using a Gatan OneView CMOS camera.

2.11.2 Confocal microscopy

For confocal microscopy, primary bone marrow neutrophil precursors were separated by flow cytometry as described and stained with bodipy at 1000 ng/ml for 20 mins at 37° C or with nile red at 1:500 dilution in PBS for 10 mins at room temperature. Cells were cytospun on glass slides and mounted with cover slips in the presence of DAPI (Vectashield). Images were acquired on a ZEISS 880 inverted confocal microscope + LSM120C with a 63x plan-apochromat objective. All images were taken with identical laser setup and voltage settings when quantifications were performed. Scale bars as indicated.

2.12 Neutrophil functional assays

2.12.1 Bacterial killing assay

Neutrophil intracellular anti-bacterial activity was measured according to a protocol courtesy of Sumeet Pandey and based on work by Tan et al and Urbanitz et al using the lysostaphin protection assay [186, 187]. Briefly, S. aureus (NTCC 6571) were freshly cultured and opsonized with mouse serum (final concentration 12.5 % in RPMI1640) at 37° C for 30 min. Mouse BM-derived $Ly6G^+$ granulocytes ($2.5x10^5$ cells/well) were infected in a 96-well round-bottom plate in 25 µl RPMI1640 + 10 % FCS at MOI of 10 bacteria / neutrophil. Following 45 min of infection, cells were washed with warm PBS and cultured in RPMI + 10 % FCS containing 100 µg/mL lysostaphin at 37° C. After 45 min, neutrophils were washed twice with PBS and lysed in 1 %-TritonX-100 in dH_2O. Lysate was serially diluted, plated on agar plates by track method and CFU

were counted next day. Results were scored as absolute numbers of CFU per ml.

2.12.2 Phagocytosis assay

Neutrophil phagocytosis was assessed by flow-cytometric quantification of fluorescent bead uptake during 1 hour incubation in the presence of 1 µg/ml LPS. pH-sensitive ph-Rhodo zymosan-beads (Invitrogen) were used except where indicated otherwise. Surface marker staining was performed after phagocytosis and data quantified as % of cells bead$^+$.

2.13 Metabolic assays

2.13.1 ATP levels

Total bone marrow cells were isolated from *C/EBPa-cre x Atg7$^{f/f}$* and littermate controls. Ly6G$^+$ cells (which include MM, BC, PMN) were purified using MACS columns (Miltenyi Biotec). Cell count was normalized to 1x10^6 cells per sample and ATP levels from cell pellets were determined by using the luciferase based ATP Bioluminescence Assay Kit HS II (Roche Applied Science). ATP concentration was calculated from intersection of sample luminescence values with ATP standard.

2.13.2 L-lactate and glucose quantification

32D CRISPR-*Atg7$^{-/-}$* and *Atg7$^{+/+}$* clones were cultured in RPMI 1640 with 1 % FCS, 2 mM L-glutamine, 100 U/ml Pen-Strep. For neutrophil differentiation, 1x10^6 cells/ml were plated in 96-well plates with medium supplemented with 100 ng/ml recombinant murine G-CSF (peprotech). At day 3, medium was supplemented with fresh 100 ng/mL G-CSF. Cells and supernatant were harvested at indicated time points. Supernatants were analysed for glucose levels and L-lactate levels following the Glucose (GO) Assay Kit (Sigma) and the Glycolysis Cell-Based Assay Kit (Cayman

Chemicals). Cells were lysed in NP40 buffer and protein was quantified using Pierce™ BCA Protein Assay Kit (Sigma). Glucose consumption and L-lactate levels were normalized to protein levels for each well individually.

2.13.3 *Seahorse* assays

Poly–Lysine (Sigma) was used for plate coating one day prior to assay by incubation at 37° C for 1 hour, washing with 200 µl PBS then aspirated dry and the plate was stored at 4° C until use. Cells were washed with 3ml unbuffered DMEM (supplemented with 200 mM L-glutamine and 200 nM sodium pyruvate) and plated at 4 x 10^4 cells/well for CRISPR cell lines or 2 x 10^5 cells/well for primary BM neutrophils. The plate was centrifuged (550rpm, 2 min) and transferred to a non-CO2 37° C incubator for 30 min then loaded onto a XF96 *Seahorse* Analyzer (Seahorse Bioscience), where oxygen consumption rate (OCR) or extracellular acidification rate (ECAR) was analysed after sequential addition of oligomycin (400nM), FCCP (400nM) and rotenone (1µM), or glucose (10 mM), FCCP (400 nM), and 2-DG (100 nM), respectively as described [188]. Following analysis, cell protein was extracted with Np40 buffer and quantified for normalization where indicated.

2.14 Statistical Analysis

p-values were determined by one-way or two-way ANOVA with Tukey´s post-tests, unpaired Student's t-test, Fisher's exact test or nonparametric Mann-Whitney test as indicated. Differences were considered statistically significant when $p<0.05$ (*<p0.05, **p<0.01, ***p<0.001, ****p<0.0001). Data are shown as mean +/− standard error of the mean (SEM) except were indicated otherwise. For all histological quantifications, the experimenter was 'blinded' to allocation of animals or experimental groups by a third person or by covering of labels. Statistics were calculated using GraphPad Prism 7 software.

3 Dynamic regulation of autophagy is essential for neutrophil differentiation

3.1 Introduction

This chapter will explore the relevance of autophagy for the differentiation of bone marrow resident hematopoietic stem and progenitor cells into neutrophils. While little is known about autophagy during granulopoiesis, important insights and lessons can be learned from the many studies and approaches that investigate autophagy in the context of HSC commitment into other myeloid or lymphoid lineages. Autophagy has been implicated in the differentiation of many hematopoietic cell types via different, but overlapping mechanisms during the last 5-8 years [189]. While it is beyond the scope of this chapter to describe all such examples, they are relevant and summarized in *Figure* 3.1. Erythropoiesis, macrophage generation and HSC differentiation are introduced individually below, as each of these examples leads to important and relevant conclusions to be considered for the study of granulopoiesis in this chapter. Several other myeloid and lymphoid cell types will be introduced in later chapters whenever respective conclusions are relevant to the appropriate chapter.

3.1.1 Autophagy in erythroid differentiation

Erythropoiesis was the first hematopoietic process identified to require autophagy for the formation of mature lineage cells almost a decade ago [190, 191]. It is also a somewhat unique differentiation process as it entails both the expulsion of the nucleus as well as removal of most cytoplasmic organelles during red blood cell generation. Active autophagy can be observed in the polychromatic erythroblast stages and deletion of autophagy genes such as *Ulk1* [192], *Atg7* [191], *Nix* (*Bnip3L*) [193, 194] and *Fip200* [195] results in defective erythroid differentiation. While several studies suggest that

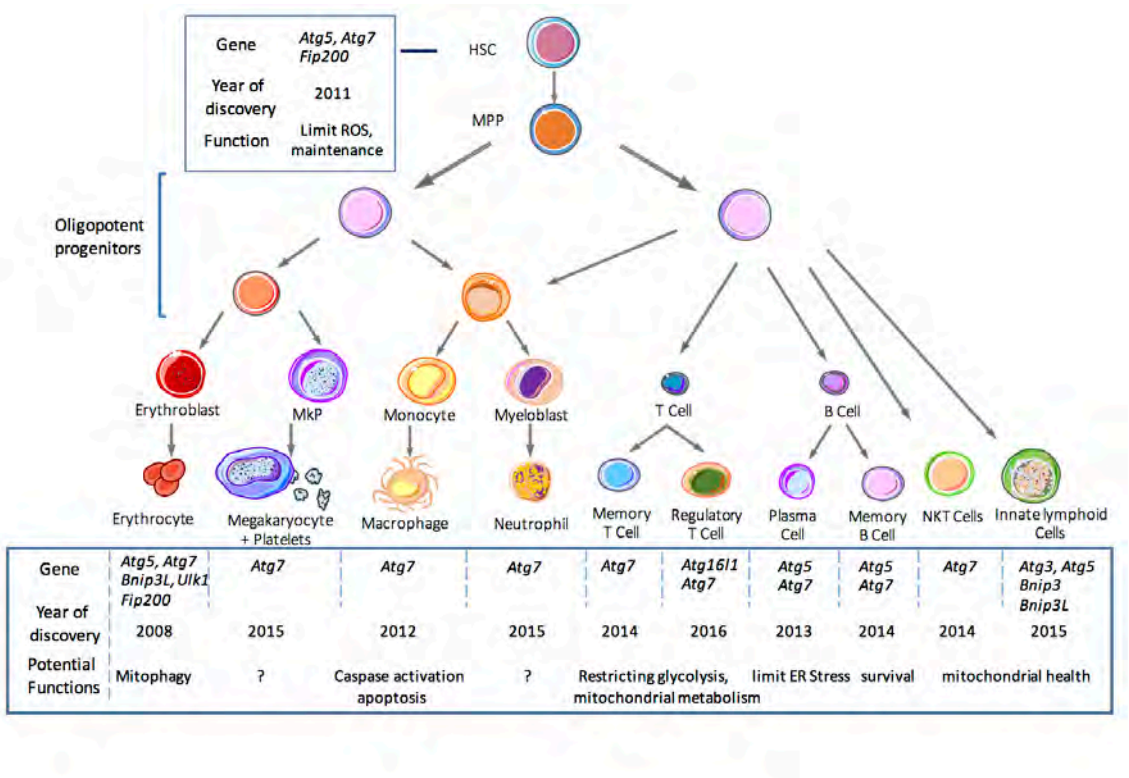

Figure 3.1: Overview of autophagy functions relevant to differentiation of hematopoietic lineages. The hematopoietic tree is labeled for each cell type with the relevant autophagy genes that have been studied, the year of discovery, and potential mechanistic functions. ROS, reactive oxygen species; ER, endoplasmatic reticulum. An adapted version of this figure was published by the author recently [189].

it is the degradation of mitochondria or other organelles that requires autophagy to allow normal maturation in this system [64, 192, 193], there also appears to be a role for alternative autophagy or *Atg5/7*-independent mechanisms that can at least in part compensate for defective removal of organelles [196, 197, 194]. The fact remains that autophagy (mitophagy) is the essential mechanism to remove dysfunctional mitochondria and other organelles, and thus this is an important mechanistic consideration also for differentiation of neutrophils. A second consideration that is relevant to granulopoiesis is the circumstances of the discovery of this phenotype: The first observations of a role of *Atg7* for erythropoiesis came from our lab in an effort aimed at exploring hematopoietic stem cell functions in *Vav-cre x* $Atg7^{f/f}$ mice, which delete *Atg7* in HSCs. During the phenotyping of these mice, Mortensen et al. noticed abnormally white paws and severe anemia developing from 6 weeks of age. This prompted researchers in our lab to look closer into erythropoiesis with more relevant models, to discover that mitochondrial degradation at the $Ter119^{+}/CD71^{High}$ stage was impaired, causing a cell intrinsic defect around this stage [198]. Thus, relevant and mechanistic observations for this specific lineage were born initially from a pan-hematopoietic deletion model of *Atg7*, an approach that will also be followed in this chapter on granulopoiesis.

3.1.2 Autophagy in macrophage differentiation

Macrophages are ontogenetically the closest relative to neutrophils that have a described role for autophagy in their generation. Just like neutrophils, monocytes are generated in the bone marrow via several intermediate maturational stages from committed precursors, which are ultimately derived from HSCs [199]. After exit of the bone marrow, monocytes can terminally differentiate into various types of dendritic cells or macrophages depending on the tissue context. If there is no signal to undergo maturation, monocytes default into programmed, caspase-dependent apoptosis.

If the cytokine GM-CSF however triggers differentiation, apoptosis is prevented and differentiation into macrophages occurs. As I recently summarized [189], several studies found that in both mouse and human, autophagy is activated during this step as observed via LC3-II formation on western blot and microscopy, and occurrence of double-membraned structures in electron micrographs [172]. This induction of autophagy is necessary to prevent caspase-3 induced apoptosis, as autophagy-inhibitors 3-MA and chloroquine as well as *Beclin1* knockdown cause caspase activation even in the presence of the survival and differentiation inducing cytokine GM-CSF. It is likely, however, that autophagy plays additional roles to promoting survival, as a significant fraction of cells survived the *Beclin1* knockdown, but retained immature features during GM-CSF treatment [200]. A problem in this study is the use of non-specific inhibitors and partial knockdown to model autophagy-deficiency, but another study has confirmed the role of autophagy in monocyte-differentiation using *Atg7*-deficient mice [172]. A separate study from our lab found autophagy was also important in the polarization of macrophages to prevent a pro-inflammatory, senescent phenotype [167]. Finally, LC3-associated phagocytosis (LAP) is a mechanism required for bacterial clearance in monocytes and macrophages that shares some of the molecular machinery with autophagy [17]. Taken together, monocyte to macrophage differentiation is an interesting model to understand the interplay of autophagy and apoptosis and how autophagy regulation can affect the differentiation into macrophages on a molecular level. Given their similar lineage origin it will be worth examining the relation between cytokine signal, survival and differentiation also in the context of autophagy during granulopoiesis, along with the potentially shared relevance of LC3-associated mechanisms for phagocytosis.

3.1.3 Autophagy in HSCs

Within defined bone marrow niches, HSCs differentiate into progenitor cells in a stepwise manner and eventually give rise to all terminally differentiated blood and immune cells, including neutrophils. It is possible that similar mechanisms act during the early HSC commitment to differentiation as occur later during committed neutrophil differentiation. Furthermore, effects on HSCs can directly determine their lineage choices downstream, such as the emergency granulopoiesis that is initiated by short term HSCs in response to TLR stimulation [201]. Stem cells must balance their own self renewal for their longevity and quiescence with continuous but infrequent asymmetric commitment to the various lineages in order to maintain hematopoietic homeostasis [104]. Because of their longevity, it is important to limit the accumulation of damage and dysfunction within this compartment by avoiding any cytotoxic stressors and actively engaging cytoprotective pathways [202]. A key role for autophagy may therefore be expected to deliver cytoprotection [203], as reviewed recently [189] and summarized here. First, FOXO3-induced autophagy gene expression including *Atg4b, Map1lc3b,* and *Bnip3* confirms active autophagy on a genetic level [171]. Functional evidence confirms highest autophagy in HSCs and generally declining levels as progenitors increasingly commit to lineages [171, 168]. High autophagy levels in murine HSCs as measured by the autophagosome-specific dye CytoID and LC3-II colocalization with lysosomal LAMP-1 in the presence or absence of flux inhibitor bafilomycinA1 in GFP-LC3 transgenic mice was also paralleled in human HSCs, which underlines the importance of robust autophagy in HSCs across species [168].

Likewise, mice deleted for autophagy in HSCs ($Atg5^{f/f}$ and $Atg7^{f/f}$ *x Vav-cre*) display an initial expansion of the hematopoietic progenitor compartment at 6 weeks of age, which progresses to severe anemia and bone marrow failure after a complete loss of the stem cell compartment and death at around 10-12 weeks of age [204, 168, 164]. Deletion via *Tie2-cre x* $Fip200^{f/f}$ is embryonically lethal and also characterized by a

loss of HSCs [195]. The difference in phenotype between these models may be due to the timing of deletion by the different promoters driving *cre* expression or additional gene specific effects on HSCs. The HSPC compartment in $Atg7^{-/-}$ mice displays an accumulation of defective mitochondria, increased proliferation as measured by Ki67 staining, DNA damage, and, at 9 weeks, also increased cleaved caspase-3 [164]. However, whether the observed proliferation is a consequence of the cytopenia or cell intrinsic is unclear. In support of the cell intrinsic hypothesis is a study showing autophagic signaling associated with ubiquitin-mediated degradation of CYCLIN-D3 in HSPCs under nutrient stress [205]. However, the evidence presented for this is solely based on pharmacological inhibition of autophagy and therefore should be interpreted with caution. Taken together, it will be important to take into account these functions of autophagy in HSCs when studying downstream granulopoiesis, highlighting the relevance of precise timing of gene deletion in animal models as well as considering whether any given phenotype such as altered proliferation is cell intrinsic or secondary to homeostatic proliferation.

3.1.4 Context and aim of this chapter

While there is plenty of evidence for autophagy roles in hematopoietic differentiation as summarized above and elsewhere [189], little evidence connects autophagy with neutrophil differentiation directly. However, there is indirect evidence that autophagy may either augment [173, 174, 175] or hinder [177] neutrophil differentiation. As these studies address differentiation in the context of acute promyelocytic leukemia (APL) cells or in oncogene-transformed lines, it currently remains unknown how neutrophil differentiation may involve autophagy. One intriguing suggestion comes from the studies that found myeloid progenitor cell accumulation and myeloid dysplasia within the bone marrow in *Vav-cre x* $Atg7^{f/f}$ mice [164, 168], which entail the possibility that these are neutrophilic in their origin. The aim of this chapter therefore was 1) to de-

termine the stages of functional autophagy activity during granulopoiesis 2) Examine the impact of autophagy deletion on these stages *in vivo* 3) Generate and characterize a cell line model of granulopoiesis with CRISPR-mediated deletion of *Atg7*. To do so, autophagic flux was first measured in neutrophil progenitors and several mouse models were used to delete core autophagy genes at various stages along the neutrophil lineage side by side with *in vitro* assays to examine the role of autophagy during neutrophil differentiation.

3.2 Results

3.2.1 Strategies to identify and isolate granulopoietic differentiation stages from bone marrow

The six stages of committed neutrophil differentiation have already been identified based on their unique histological appearance several decades ago [116]. While the characteristic changes of nuclear morphology and granule content during their differentiation can readily be visualized by histological stainings such as H+E or Wright-Giemsa based formulations, the methods to isolate these stages separately with high purity for analysis of the associated and underlying molecular events governing differentiation have remained limited. Recently, several studies have described flow cytometry based techniques to distinguish neutrophil precursors from murine bone marrow based on their surface marker expression [206, 207]. To study the mechanisms of neutrophil differentiation, we defined these differentiation stages of myeloblasts (MB), myelocytes (MC), metamyelocytes (MM), band cells (BC) and neutrophils (PMN) according to Satake et al. [207]. This flow cytometric strategy first uses a lineage marker mix containing CD4, CD5, CD8, CD19, B220 and Ter119 to exclude all non myeloid BM lineages after live cell and single cell gating. From the Lin– population, CD34 and c-kit is used to remove non granulocytic progenitors ($CD34^-$ $c\text{-}kit^+$), after

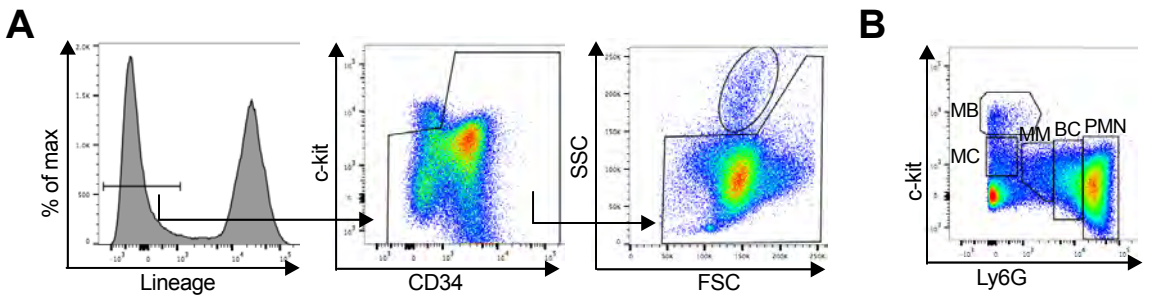

Figure 3.2: Gating strategy to define BM neutrophil precursors. Total BM cells were gated on live, single cells, lineage negative (CD4, CD5, CD8, CD19, B220 and Ter119) and excluding C-kit^{+} CD34^{-} progenitors and SSC high eosinophil gate (A). The remaining neutrophil lineage cells were separated based on c-kit and Ly6G expression into myeloblasts (MB), myelocytes (MC), metamyelocytes (MM), band cells (BC) and neutrophils (PMN) according to [207](B).

which FSC/SSC gating enables removal of a population containing mainly eosinophils. Subsequently, the c-kit positive early precursor stages can be separated from MM, BC and PMN populations which gradually upregulate the neutrophil-specific Ly6G marker (*Figure* 3.2). This staging was defined and tested for purity by Satake et al, who found up to 90 % enrichment of correct precursor stages in respective gates based on morphology and gene expression [207].

3.2.2 Purity confirmation of flow-sorted BM neutrophil precursors

We next re-confirmed identity of the five neutrophil precursor stages by morphology on Wright-Giemsa stained cytospins as well as by gene expression of relevant and stage-specific granule genes. The gradual nuclear lobularization, the lighter staining of the cytosol with higher cytoplasm:nuclear ratio and the generation of purple-staining granules are unique histological marks that are widely used to define neutrophil precursors [116]. The characteristic morphology of each of the five sorted populations confirmed separation of individual stages, although particularly in the MB and MC stage, the absence of a surface marker or morphology unique and specific for these subsets likely makes these populations more heterogenous in their composition [207]. Defining these

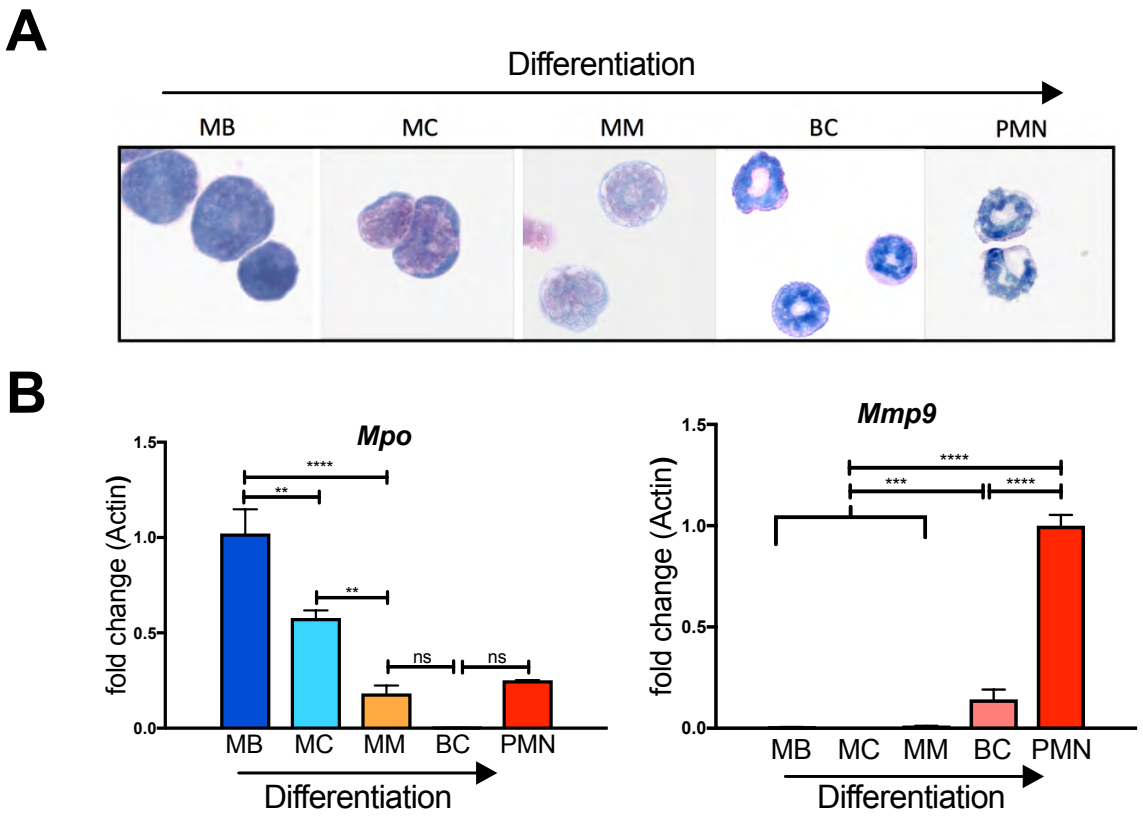

Figure 3.3: BM neutrophil precursors can be separated into 5 unique stages of differentiation. The 5 stages of MB, MC, MM, BC and PMN were flow-sorted as described above and stained with Wright-Giemsa (A). Stage-specific gene expression of the early (primary) granule gene Mpo and the late (tertiary) granule gene Mmp9 was quantified relative to β-actin (ΔΔCt) +/- SEM. Data representative of three experiments (5-6 mice/group).

stages genetically, the expression of *Mpo*, a primary granule gene, was predominant in the early stages, whereas *Mmp9*, a tertiary granule gene, was only expressed at the band cell stage and later, confirming the enrichment of successive differentiation stages genetically by their unique granule gene signature (*Figure* 3.3)

3.2.3 The 32Dcl3 culture system to study neutrophil differentiation *in vitro*

To complement our *in vivo* study of granulopoiesis, we used the well established *in vitro* culture model of 32Dcl3 myeloblasts, which were originally derived from wild-type

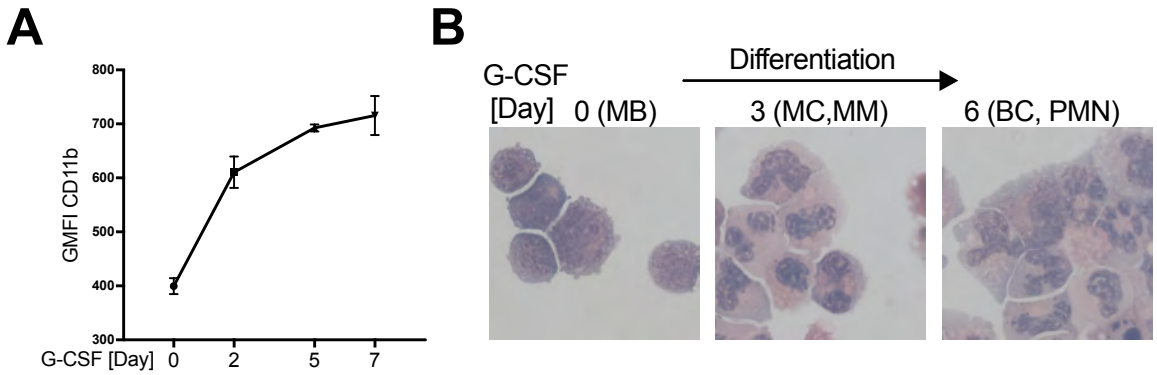

Figure 3.4: G-CSF induced neutrophil differentiation of 32Dcl3 myeloblasts. IL-3 containing myeloblast culture medium was completely removed and 32Dcl3 cells cultured in the presence of G-CSF (100 nM) for 7 days. Expression of CD11b was quantified as geometric MFI +/- SEM at the indicated days of G-CSF mediated differentiation (A). The morphological stages of differentiation were visualized by Wright-Giemsa stained cytospins in analogy to the *in vivo* staging (B).

C57/Bl6 mouse bone marrow and can be cultured with IL-3 supplemented medium to maintain their myeloblast identity [182]. In this model, uniform successive neutrophil differentiation via the same intermediate differentiation stages can be followed over 5-7 days by removing IL-3 and addition of G-CSF supplemented medium [208]. As this system only produces neutrophils but not monocyte/macrophage lineages under these conditions, expression of the myeloid maturation marker and nuclear morphology can be used to track and uniquely identify differentiation stages. We found robust up-regulation of the maturation marker CD11b which enabled tracking of differentiation progress in combination with the unique morphological maturation observed on Wright-Giemsa stained cytospins as gradual lobularization of the nucleus, increasing cytoplasma:nuclear ratio and emergence of Giemsa$^+$ granules (*Figure* 3.4).

3.2.4 Two programmed peaks of autophagy during granulopoiesis

Having established two methods to enrich separate stages of successive, committed neutrophil terminal differentiation downstream of GMPs enabled us to study the regulation of autophagy in these subsets. In order to measure stage-specific autophagy

activity *in vivo* we took advantage of LC3-GFP transgenic mice [209]. When the autophagy process is initiated, the small cytoplasmic protein LC3-I is lipidated to LC3-II, which is incorporated into the growing autophagosomal membrane and routinely used to measure autophagy. When autophagosomes fuse with lysosomes, the lysosomal hydrolases also degrade LC3-II. If lysosomal degradation of LC3 is inhibited by bafilomycin A1, the build-up of LC3-II therefore quantifies autophagic flux [31]. Using this technique combined with flow cytometric identification of neutrophil precursors, we found robust autophagic flux during 2 hours of Baf (100 nM) treatment at the MB and MC stage, which decreased at the MM and BC stage and slightly increased again in mature PMNs. Consistent with this, we found the expression of *Tfeb* (the master regulator of lysosomal and autophagy gene expression [73]) as well as the essential autophagy gene *Atg7* to follow the same dynamic expression pattern *(Figure 3.5)*.

To confirm this biphasic pattern of autophagic activity which is particularly high in earliest progenitors, we also quantified LC3-II punctae by confocal microscopy. LC3-II-positive autophagosomes were readily detectable at MB and MC stages as well as in mature PMNs and increased in number after Baf treatment, while the intermediate stages (particularly MM) had little or no autophagosomes at steady state and after flux inhibition (*Figure 3.6*).

A general approach of this study was to compare all biological observations made *in vivo* with the 32Dcl3 *in vitro* differentiation system in order to assure their validity and because the *in vitro* system offers unique scalability that overcomes cell number restrictions. We measured LC3-II levels by western blot in the IL-3 dependent culture system *in vitro* in response to G-CSF, a method that requires relatively high cell numbers and would therefore not be possible *in vivo* as MB - MM populations are rare ($<$1% of events) [182]. As expected, we found the same pattern of dynamic regulation of autophagy particularly at early differentiation stages that we observed in primary

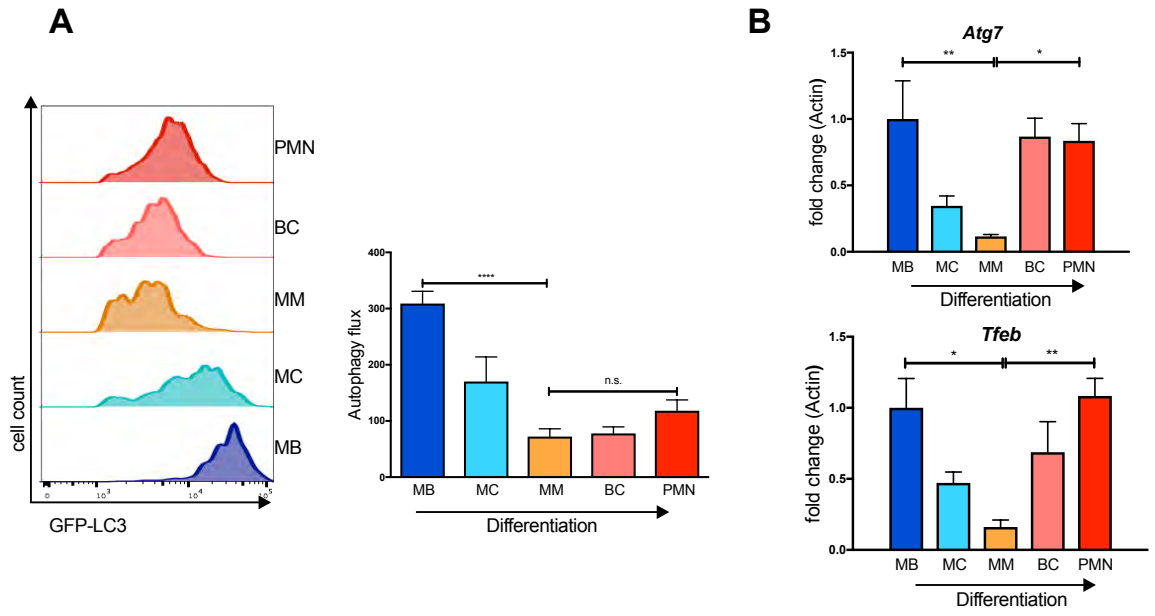

Figure 3.5: Dynamic autophagy regulation during granulopoiesis *in vivo*. BM neutrophil precursors were isolated from GFP-LC3 mice by flow cytometry and analyzed for their autophagic flux after 2 hour culture +/- Baf (10nM). Representative histograms and quantification as (MFI (Bafilomycin A1–Basal) /MFI Basal) +/- SEM (n=3 mice), representative of three experiments (A). Gene expression of *Atg7* and *Tfeb* was measured as $\Delta\Delta$Ct normalized to *Actin* and is presented as fold change +/- SEM (n=4 mice) (B).

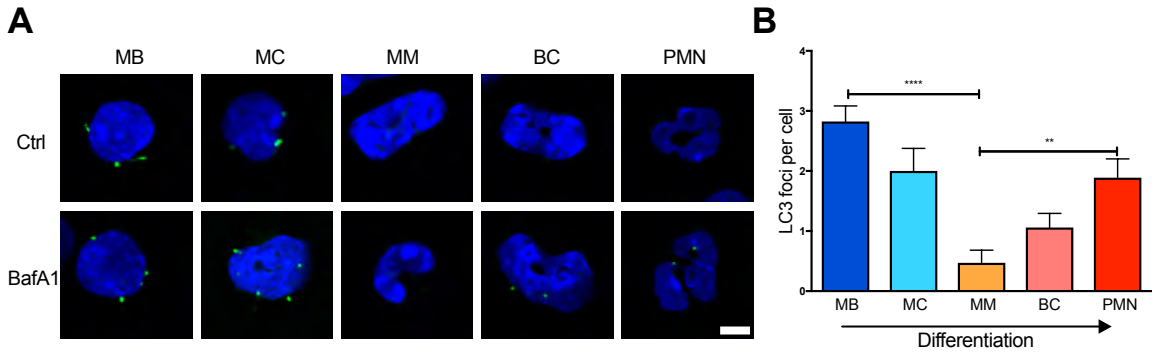

Figure 3.6: Biphasic autophagy activity during granulopoiesis *in vivo*. Confocal microscopy of GFP-LC3 fluorescence in FACS-sorted precursors after treatment with vehicle or bafilomycin A1 (10 nM, 2hours). Representative images (A) and quantification of LC3 foci per cell from 3 fields of vision including at least 20 cells (B). Data are shown as mean +/- SEM (n=3 mice), from three individual experiments.

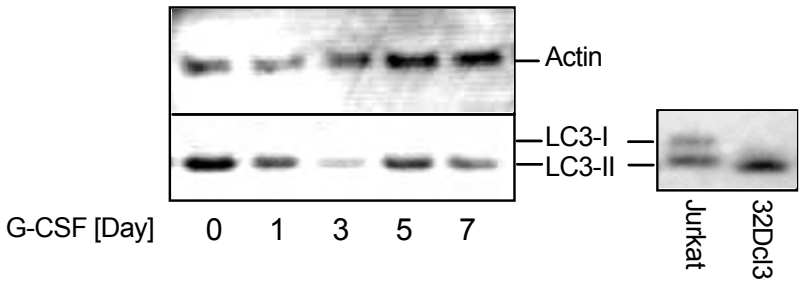

Figure 3.7: Two waves of autophagy activity during G-CSF mediated neutrophil differentiation *in vitro*. Myeloblasts were cultured with 100 nM G-CSF for the indicated duration and subjected to LC3-II western blotting. The absence of LC3-I was confirmed by comparison to Jurkat lysate.

cells *(Figure 3.7)*. Because this cell line only shows a LC3-II band at steady state, we confirmed the absence of LC3-I again by comparison with a Jurkat lysate, which has readily detectable levels of LC3-I. Autophagy was recently found to be important for neutrophil functions [210], which may explain the second peak of activity and led us to hypothesize that the first peak of autophagy may be particularly important for neutrophil differentiation at the earliest differentiation stages.

3.2.5 Accumulation of morphologically immature neutrophil precursors after pan-hematopoietic deletion of *Atg7*

Vav-cre x _Atg7_$^{f/f}$ (_Atg7_$^{-/-}$) are efficiently deleted in hematopoietic stem cells and their progeny

In order to examine the impact of autophagy deletion on neutrophil differentiation, we deleted the essential autophagy gene *Atg7* in the hematopoietic system by crossing *Atg7*$^{f/f}$ mice [179] with *Vav-cre* expressing mice [180]. *Vav-cre* is expressed after embryonic day 13 in hematopoietic stem cells (HSCs), and therefore deletes *Atg7* from early age onward and with high efficacy in the entire hematopoietic system. Indeed, the deletion efficacy was found to be 80 - 90 % already at the HSC level and in several subsequent stages of neutrophil differentiation in BM, as measured by qPCR (*Figure* 3.8).

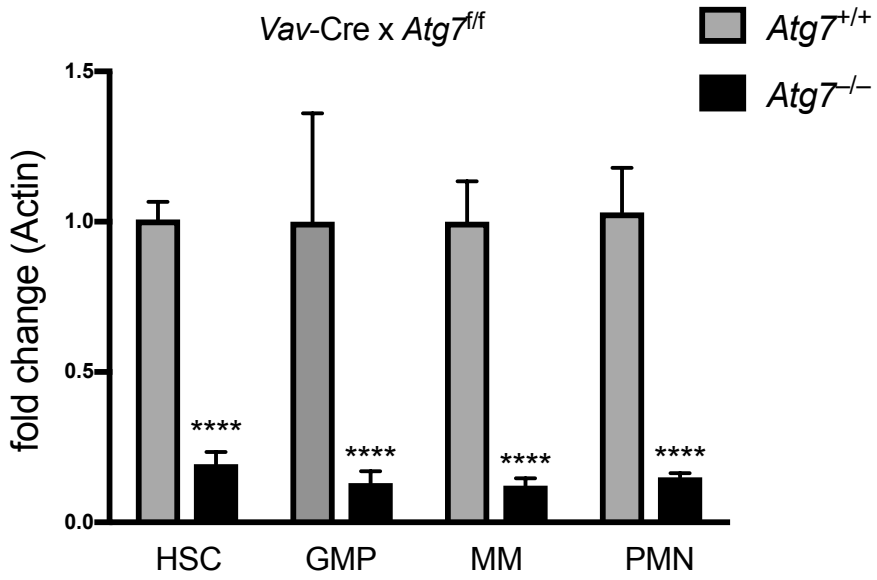

Figure 3.8: *Vav-cre x Atg7$^{f/f}$ (Atg7$^{-/-}$)* mice are efficiently deleted for *Atg7* in the granulopoietic lineage. *Atg7* deletion was assessed at four distinct stages of neutrophil generation in control and *Vav-cre x Atg7$^{f/f}$* mouse bone marrow cells. Fold change +/- SEM was normalized to *Actin* and plotted relative to wild-type ($\Delta\Delta$Ct) (n=4 mice/group). HSC, hematopoietic stem cell; GMP, granulocyte-monocyte progenitor; MM, metamyelocyte; PMN, polymorphonuclear neutrophil.

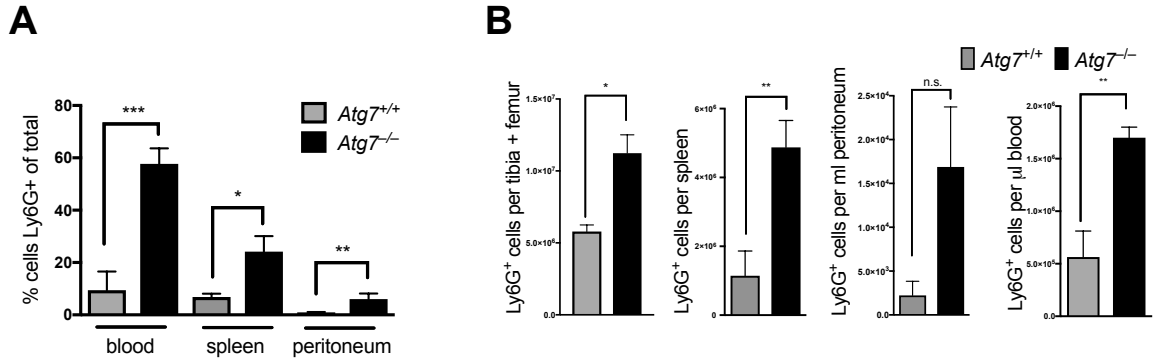

Figure 3.9: Expansion of the neutrophil lineage in *Vav-cre* x $Atg7^{f/f}$ ($Atg7^{-/-}$) mice. Relative frequencies of $Ly6G^+$ cells in 6 week old animals in indicated tissues as % of total live cells +/- SEM (n=4 mice/group) (A) and absolute cell number of $Ly6G^+$ cells in indicated tissues as mean +/- SEM (n=4-5 mice/group) (B).

Neutrophil lineage cells expand in bone marrow and tissues of *Vav-cre* x $Atg7^{f/f}$ mice

Deletion of *Atg7* at this early stage leads to dysfunctional HSCs due to the role of autophagy in HSCs, which first expand after 6-8 weeks and eventually exhaust after 10-12 weeks leading to bone marrow failure, lymphopenia and anemia [191, 190, 198, 164, 176]. Nevertheless, the high deletion efficacy and broad applicability of this model led us to examine neutrophil differentiation in this model. In addition, previous studies from our lab found an expanded population of myeloid cells with dysplastic features in these mice which could originate from the granulocytic lineage [176]. Indeed, the relative number of cells expressing the neutrophil lineage-specific marker Ly6G in the bone marrow, spleen and peritoneum was increased already at 6 weeks of age, when the HSCs are still largely unaffected [164] (*Figure* 3.9A). Because there is a mild anemia already at this age [191] and because deletion of *Atg7* in T cells leads to lymphopenia [211], it was important to also measure absolute numbers of $Ly6G^+$ cells, which, however, showed a similar picture of increased absolute numbers of neutrophil lineage cells in various tissues (*Figure* 3.9B).

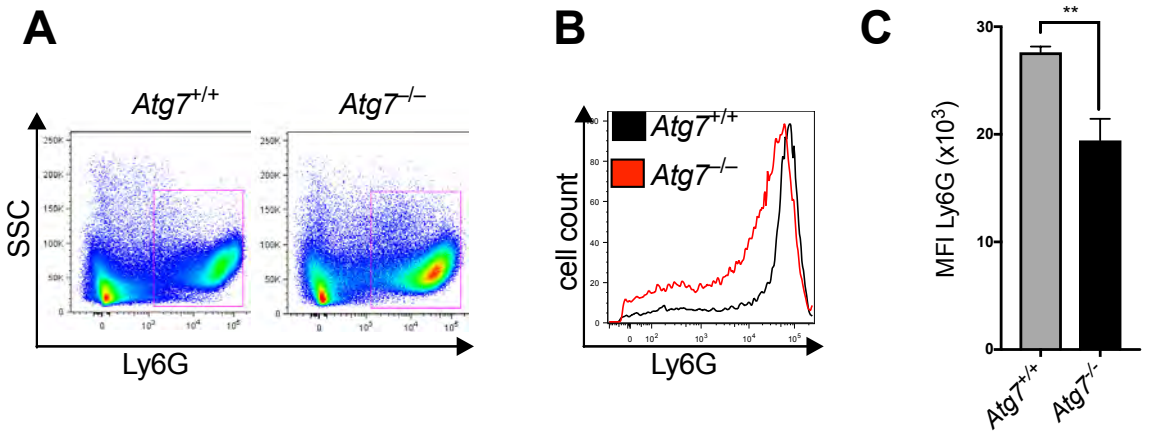

Figure 3.10: Accumulation of cells with low and intermediate expression of Ly6G. Representative FACS plots of Ly6G$^+$ SSC^{hi} BM neutrophil population (A) and representative histogram of Ly6G expression in BM (B). The overall geometric MFI of the Ly6G-expressing population was quantified as MFI +/- SEM in the total neutrophil lineage in control and *Atg7*$^{-/-}$ mice (*Vav-cre x Atg7*$^{f/f}$) (n=4 mice/group) (C).

Neutrophilia is characterized by over-representation of cells with low or intermediate Ly6G maturation marker expression

The accumulation of Ly6G$^+$ cells in *Vav-cre x Atg7*$^{f/f}$ (*Atg7*$^{-/-}$) mice suggests that defective autophagy i) is a positive signal for accelerated neutrophil generation or ii) causes a differentiation block with accumulation of immature precursors and feedback signals to expand granulopoiesis. To address this, we first evaluated the expression levels of the maturation marker Ly6G and found an increase particularly in cells that express low and intermediate levels, which was reflected in an overall significantly reduced mean intensity of Ly6G expression in the total Ly6G expressing group (*Figure* 3.10).

Atg7-deficient neutrophils have decreased nuclear lobulation and granule content

As decreased Ly6G expression suggested an enrichment of immature neutrophil precursor stages, we next assessed the nuclear morphology and cytoplasmic granule content of these cells by transmission electron microscopy. The nuclear lobularization

can be quantified from TEM micrographs as the number of nuclear lobes visible in a given cross-section of the cell. This factor was significantly reduced in *Atg7*-deficient BM Ly6G$^+$ cells compared to control. Similarly, the electron dense antimicrobial granules per cross-section are readily quantifiable by TEM. While studies suggest that specific granule types may be distinguished by conventional and immuno-EM [117], we limited our analysis to quantification of total granule content, which is an indicator of maturation [118]. In line with their immature nuclear morphology, *Atg7*$^{-/-}$ BM Ly6G$^+$ cells had significantly decreased total granule content (*Figure* 3.11). This data was kindly provided by Amanda Stranks.

Morphologically immature maturation stages are present also in the Ly6G^{high} PMN population of *Atg7*-deficient bone marrow

While the maturation marker expression, granule content and nuclear morphology suggested an accumulation of immature myeloblast and myelocyte stages reflected by the increase of total cell number within the neutrophil lineage, the question remained whether the population with highest Ly6G expression has uniformly reached terminal neutrophil maturity, resulting in an effective increase in the presence of mature neutrophils even in the *Atg7*-deficient mice. To answer this question, we next isolated the "mature" neutrophil population ("PMN" population #5 in FACS strategy) separately by using the gating strategy as previously described, sorting for single, live cells with Lineage$^-$, c-kit$^-$, SSC^{high}, Ly6G^{high} marker expression. Importantly, this excludes the MM and BC stages, which already express Ly6G and therefore are included in the total Ly6G$^+$ BM fraction as commonly isolated by magnetic separation. As expected in the isolated PMN fraction we almost exclusively found mature neutrophils in *Atg7*$^{+/+}$ mice as identified on Wright-Giemsa stained histological cytospins. In contrast, a significant proportion of cells within this gate in the *Atg7*$^{-/-}$ mice had also retained immature morphology (*Figure* 3.12). This confirms that in *Atg7*$^{-/-}$ mice,

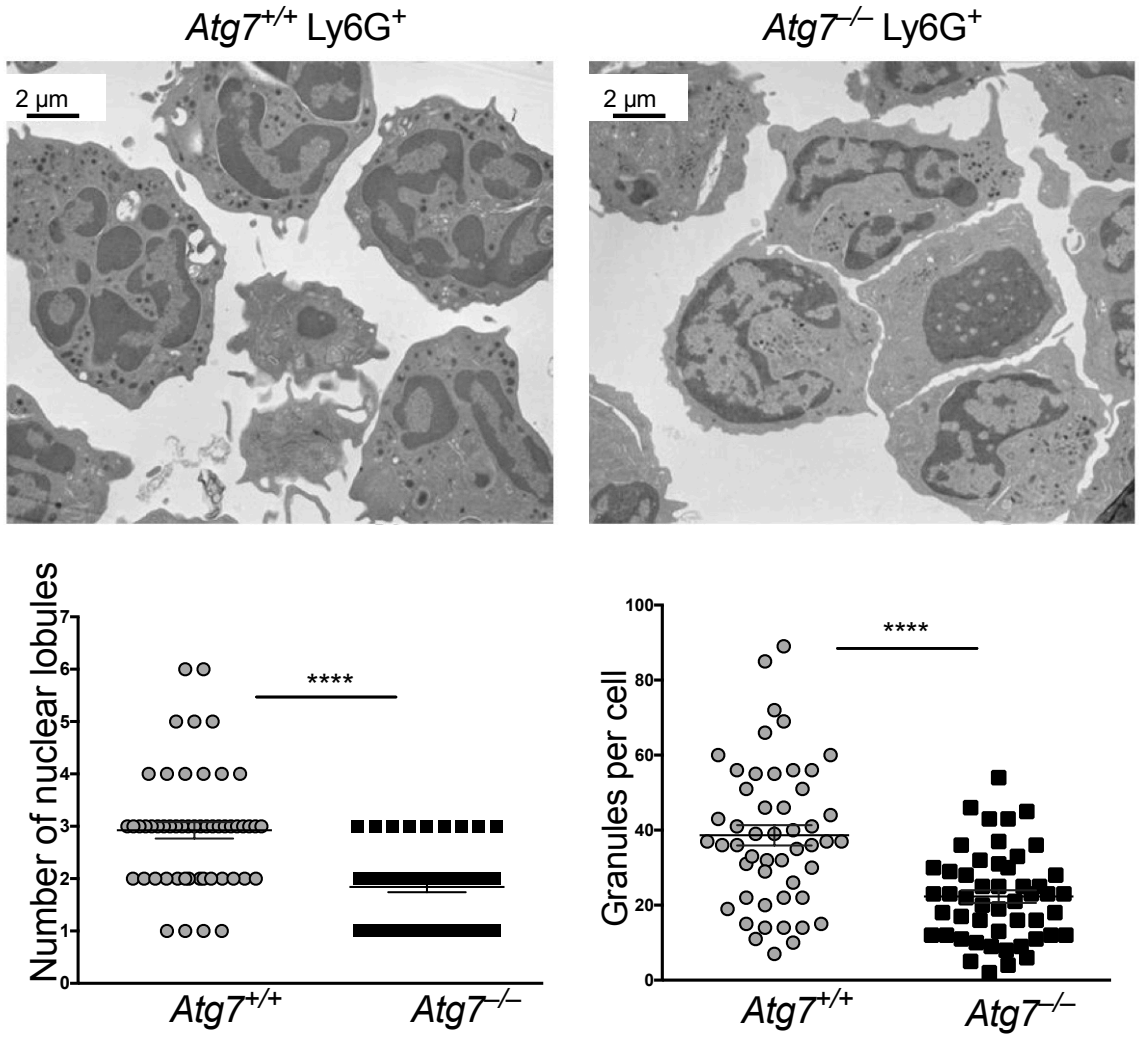

Figure 3.11: Ly6G-expressing cells retain immature nuclear morphology and granule content. Representative TEM micrographs (top) and quantification (bottom) of nuclear lobularity and granule content per cell from Ly6G$^+$ BM cells from *Atg7*$^{-/-}$ or control mice (n=4 mice/group). Data presented as mean +/- SEM. Data kindly provided by Amanda Stranks.

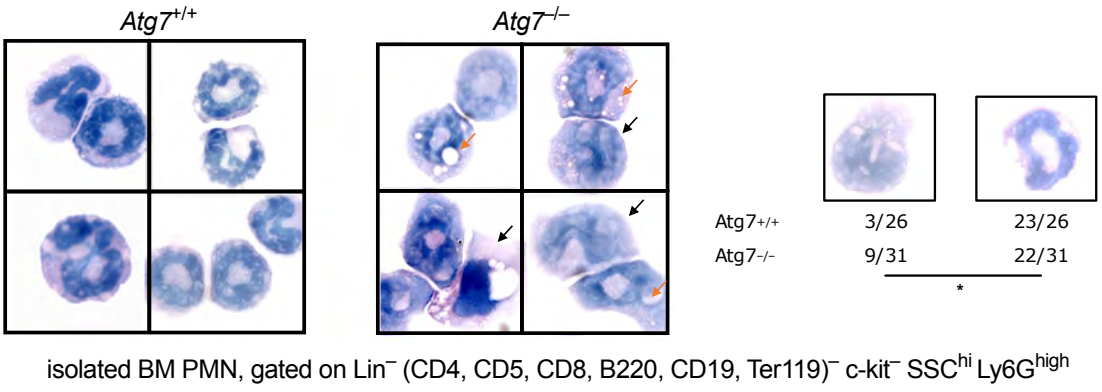

Figure 3.12: A proportion of Ly6Ghigh neutrophils of *Atg7*$^{-/-}$ mice retains immature morphology. Total BM cells were separated by flow cytometry based on single, live cells with Lineage^{-}, c-kit^{-}, SSC^{high}, Ly6G^{high} marker expression to define Ly6G^{high} mature PMN population #5 as described earlier. Wright Giemsa stained cytospins were prepared and shown are representative images (left) and scoring for terminal histological maturation stages from 26 (*Atg7*$^{+/+}$) or 31 (*Atg7*$^{-/-}$) cells assayed in total (right). Orange arrows indicate vesicles, black arrows indicate band cells and myelocytes. Data from n=2 mice/group, significance tested by Fisher´s exact test.

in addition to the accumulation of immature neutrophil precursor populations with low/intermediate Ly6G expression, a proportion of cells that express the highest levels of Ly6G also retained immature morphology.

3.2.6 Genetic and functional evidence of neutrophil immaturity in autophagy-deficient bone marrow Ly6G^{+} neutrophils

Immature granule gene signature in *Atg7*$^{-/-}$ mice

The accumulation of immature neutrophil precursors based on morphology and maturation marker expression can also be represented by functional and genetic assays. The distinct types of antimicrobial granules for example are generated in a stepwise manner during granulopoiesis, allowing us to use the gene expression of these granule proteins as a genetic mean to define their differentiation stage. Myeloperoxidase (*Mpo*) is a primary granule gene mainly expressed in MBs, while matrix-metalloprotease-9 (*Mmp9*) is a tertiary granule gene which is only expressed at the BC stage and after.

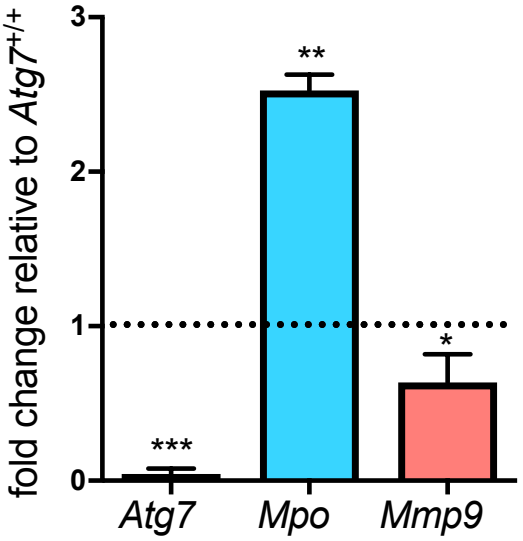

Figure 3.13: Immature granule gene signature in *Atg7*$^{-/-}$ mice. Total Ly6G^{+} BM cells were isolated from control or *Atg7*$^{-/-}$ mice and tested for *Atg7*, primary granule gene *Mpo* and tertiary granule gene *Mmp9*. Expression levels are presented as fold change +/- SEM normalized to *Actin* and relative to control ($\Delta\Delta$Ct). Data from n=3 mice/group.

We measured granule gene expression in total Ly6G expressing cells from *Atg7*$^{-/-}$ BM relative to control littermates, and found a significant increase of *Mpo* but decreased expression of *Mmp9* (*Figure* 3.13). This confirmed the left-shift in maturation which was described earlier at a genetic level.

Impaired neutrophil functional capacities in *Atg7*$^{-/-}$ mice

The functional consequence of impaired neutrophil differentiation can also be measured by phagocytosis- and bacterial killing assays. To measure phagocytosis, Ly6G^{+} neutrophils were isolated from BM and cultured in the presence of fluorescent beads (1 μm) with or without LPS stimulation (100 nM). As expected, the uptake of beads following LPS stimulation was significantly impaired in *Atg7*$^{-/-}$ neutrophils (*Figure* 3.14A). Another readout for neutrophil function is their ability to kill bacteria intracellularly after phagocytosis. The survival of intracellular bacteria can be measured

after co-culture of neutrophils with *S.Aureus* and removal of extracellular bacteria with lysostaphin, a selective and non cell-permeable antimicrobial agent. We detected a more than 8-fold increased number of colony-forming units (CFU) from $Atg7^{-/-}$ neutrophil lysates compared to control, suggesting a severely impaired intracellular killing capacity (*Figure* 3.14B). While these findings can be explained by the left-shift in maturation and the described accumulation of immature precursors that have not acquired functionality, the possibility remains that autophagy is independently required for phagocytosis and bacterial killing [172, 212, 17, 167, 213]. We therefore tested if the functional deficit in $Atg7^{-/-}$ is retained when only the PMN population #5 is isolated by flow cytometry. In the presence of LPS, phagocytosis was quantified within the $Ly6G^{high}$ population as the % of cells that took up FITC-labelled beads. The decreased ability of $Atg7^{-/-}$ neutrophils to phagocytose was conserved in this population (*Figure* 3.14C).

Taken together, these data show an accumulation of phenotypically and functionally immature neutrophil precursors after deletion of the essential autophagy gene *Atg7* within the hematopoietic system (*Vav-cre x* $Atg7^{f/f}$). These cells have mainly myeloblast and myelocyte like nuclear and cytoplasmic morphology, a decreased cytotoxic granule content, the granule gene signature of early neutrophil lineage precursors and decreased phagocytosis and bacterial killing. Because they express the neutrophil-specific marker Ly6G we can rule out that these are monocytes or other myeloid-derived populations. While this suggests an important role for autophagy in mediating differentiation into neutrophils, the excision at the HSC level also leads to functional deficits in other hematopoietic lineages in these mice, such at several T and B cell types, which depend on autophagy for their function. Thus, the possibility remains, that a cell extrinsic effect, in trans to granulopoiesis, causes the accumulation of neutrophil precursors or interferes with their differentiation. This could be caused by the pro-inflammatory milieu generated by other autophagy deficient myeloid cells,

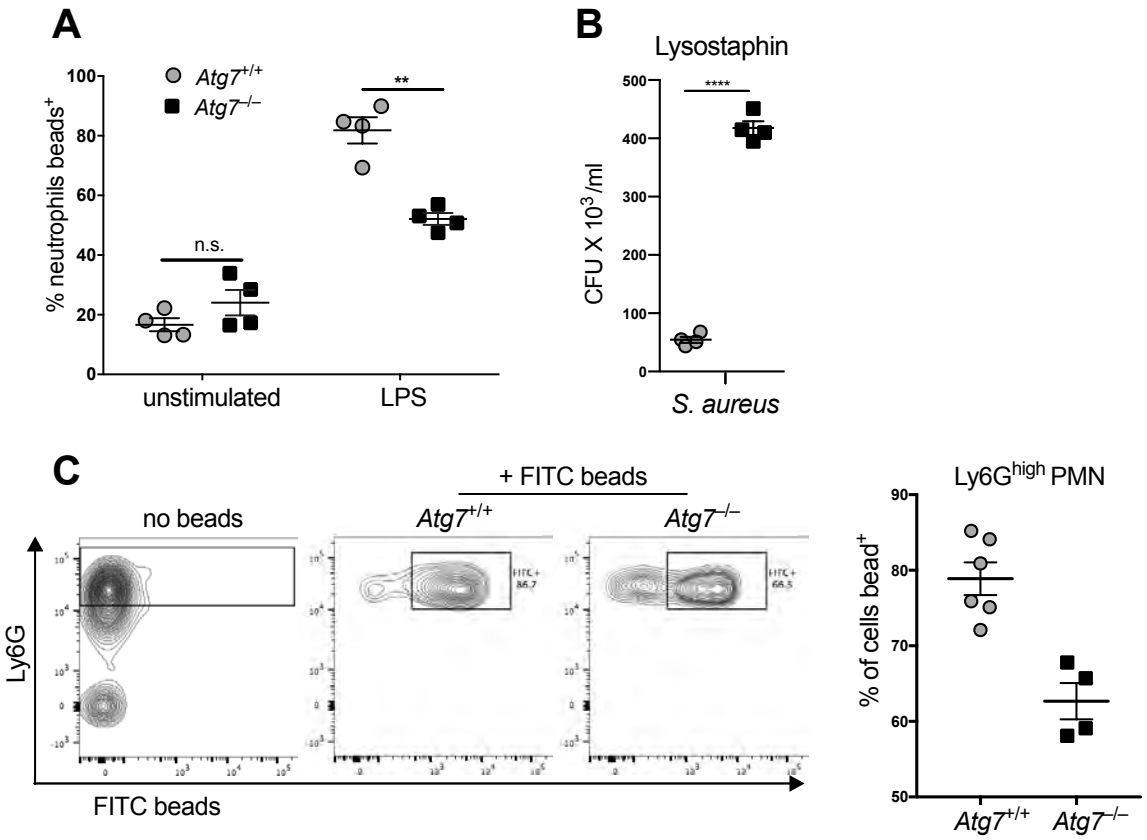

Figure 3.14: Impaired neutrophil function in *Atg7*$^{-/-}$ mice. Phagocytosis was quantified as number of Ly6G^{+} cells that took up fluorescent latex beads during a 30-minute culture period in the presence or absence of LPS. Data are shown as mean +/- SEM (n = 4 mice/group) (A). Bacterial killing with Ly6G^{+} BM cells. Extracellular bacteria were removed by lysostaphin treatment (100 µg/ml) and live intracellular bacteria which escaped killing were measured as CFU x 10^3/ml on agar plates. Data are shown as mean +/- SEM (n = 3 mice/group) (B). Phagocytosis was also quantified in the Ly6G^{high} population separately after LPS activation in the presence of FITC-labelled beads. Representative blots of the "no beads" control and control and *Atg7*$^{-/-}$ conditions (left) and quantification as % of cells bead^{+} +/- SEM (right) are shown (C). *S. aureus*, *Staphylococcus aureus*. Bacterial killing assay was done in collaboration with Sumeet Pandey.

or altered cytokine profiles which affect granulopoiesis [167, 214, 17]. Alternatively, a cell intrinsic effect within HSCs, which are known to require autophagy for efficient lymphocyte development, could imprint a differentiation bias towards myeloid or granulocytic lineages in the absence of *Atg7* [164, 176]. To investigate all of these options, a novel mouse model was obtained which drives *cre* expression by the *C/EBPα*-promotor and thus deletes at the GMP level without affecting HSCs or any lymphoid lineages, and was crossed with *Atg7flox* mice.

3.2.7 The neutrophil differentiation defect occurs downstream of the granulocyte-monocyte progenitor (GMP) and independent of trans-acting effects in stem cells or lymphoid tissues

C/EBPα-cre x Atg7$^{f/f}$ mice delete *Atg7* in earliest granulocyte precursors with high specificity

To determine the differentiation stage that requires autophagy and to rule out cell extrinsic effects, *Atg7flox* mice were crossed with *C/EBPα-cre* mice to obtain *C/EBPα-cre x Atg7$^{f/f}$* mice, which delete *Atg7* at or after the GMP stage with minimal effect on HSCs or lymphoid lineages [121]. Stage specific deletion was tested by qPCR in consecutive differentiation stages starting with HSCs, GMPs as well as in the committed MM neutrophil precursor and finally PMNs. While there was no measurable deletion in HSCs, GMPs were deleted for *Atg7* with >90 % efficacy, as were the downstream MM and PMN populations. In accordance with this, the ATG7 protein was not detectable in *C/EBPα-cre x Atg7$^{f/f}$* BM Ly6G^{+} neutrophils compared to controls (*Figure* 3.15). Taken together, *C/EBPα-cre x Atg7$^{f/f}$* mice delete *Atg7* with high efficacy in a stage specific and myeloid-restricted manner and thus present a valuable tool to examine the contribution of *Atg7*-mediated effects at the stem-cell level to the previously described neutrophil differentiation defect.

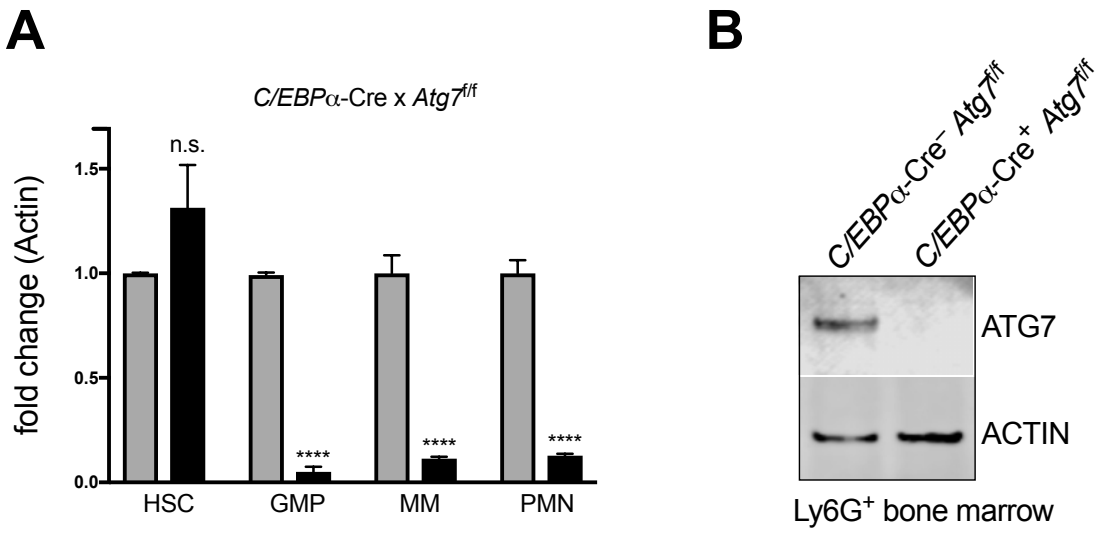

Figure 3.15: *C/EBPa-cre x Atg7$^{f/f}$* mice delete *Atg7* at the GMP level with high efficacy. *C/EBPa-cre x Atg7$^{f/f}$* BM populations were sorted by flow cytometry [215] and *Atg7* gene expression was measured by $\Delta\Delta$Ct as fold change +/- SEM relative to control (n=2-4 mice/group) (A). Deletion was also confirmed by western blot for ATG7 protein and loading controlled with ACTIN from Ly6G$^+$ BM lysate (B). HSC, hematopoietic stem cell, GMP, granulocyte monocyte progenitor; MM, metamyelocyte; PMN, polymorphonuclear neutrophil.

Expansion of neutrophil lineage and early precursor stages in *C/EBPα-cre x Atg7$^{f/f}$* mice

One of the most robust observations in *Vav-cre x Atg7$^{f/f}$* mice was the accumulation of early neutrophil precursor stages in the bone marrow and tissues as detected by flow cytometry. *C/EBPα-cre x Atg7$^{f/f}$* mice had a comparable, about 2-fold increase in Ly6G^{+} cell number as quantified by absolute counts from bone marrow. Using the same flow-cytometric strategy as in 3.2.1 to distinguish consecutive committed neutrophil precursors, it becomes obvious that particularly the early MB and MC populations expand 3-4-fold, while the BC and PMN populations are left shifted in the expression of Ly6G, confirming the observations in *Vav-cre x Atg7$^{f/f}$* mice (*Figure* 3.16). Because this expansion in the bone marrow is similar to *Vav-cre x Atg7$^{f/f}$* mice, the morphology of the expanded bone marrow population was next examined in more detail to test if these cells again retained immature features.

C/EBPα-cre x Atg7$^{f/f}$ mice accumulate morphologically immature neutrophil precursors in bone marrow

The increased presence of cells with decreased Ly6G expression in *C/EBPα-cre x Atg7$^{f/f}$* bone marrow suggested a similar maturation block as observed in *Vav-cre x Atg7$^{f/f}$* mice. To confirm this, Wright-Giemsa stained cytospins were prepared from Ly6G-expressing cells to assess morphological maturation. There was a significant increase of cells with the round or spherical nuclear morphology and small or enlarged cytoplasmic area typical of myeloblasts and myelocytes, respectively. The group of metamyelocytes and band cells which have a decreased nuclear lobulation to PMN but have invaginated or kidney-shaped nuclear morphology and the presence of granules were also slightly, but not significantly enriched. In contrast, the PMN population, characterized by a multi-lobed or thin-banded "donut" shaped nucleus was significantly decreased in the *Atg7*-deficient mice (*Figure* 3.17). Importantly, these quantifications

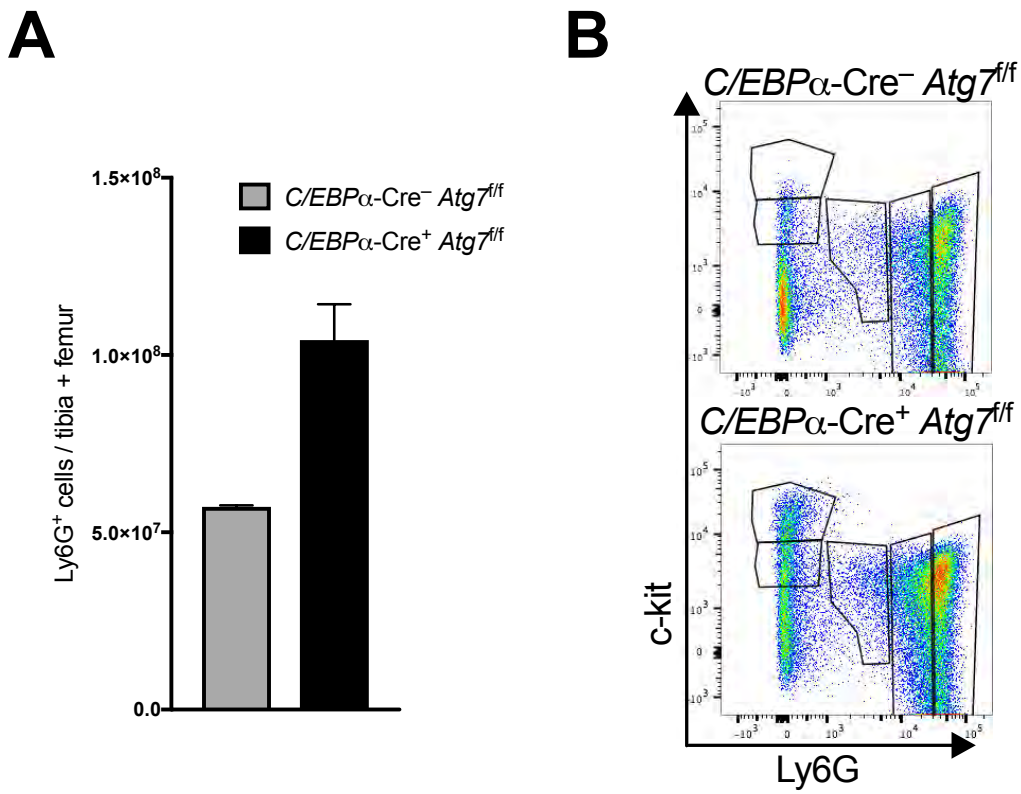

Figure 3.16: Flow cytometric characterization of Ly6G expression in *C/EBPa-cre x* $Atg7^{f/f}$ mice. Absolute number of total Ly6G-expressing cells +/-SEM was quantified per tibia+femur from *C/EBPa-cre x* $Atg7^{f/f}$ mice and littermate controls (n=4mice/group) (A). Representative images of precursor stage gating for the 5 consecutive populations MB, MC, MM, BC, PMN (from top left gate to bottom right gate, see also 3.2.1) (B).

were counted per 100 total Ly6G-expressing cells and therefore represent a relative quantification of maturation stages that is not directly affected by the absolute increase in Ly6G-expressing cells in *C/EBPa-cre x Atg7$^{f/f}$* mice. It also excludes the earliest committed precursor populations that do not yet express Ly6G. As these early populations were most significantly increased in *Atg7$^{-/-}$* as detected by FACS (*Figure* 3.16), the actual extent of the differentiation block is likely more severe than observed within the Ly6G-expressing population. However, we decided to restrict most *in vivo* characterization to Ly6G expressing cells, as even within these more terminal populations, a significant maturation defect is observable, and it excludes potentially confounding impurities of myeloid-monocytic lineages, which otherwise share similar morphology and surface marker expression to early granulocytes.

Genetic evidence for a neutrophil differentiation block in *C/EBPa-cre x Atg7$^{f/f}$* mice Following a parallel strategy to the characterization of the neutrophil defect in *Vav-cre x Atg7$^{f/f}$* mice, we next examined whether the differentiation block in *C/EBPa-cre x Atg7$^{f/f}$* mice is also evident at a genetic level as a relative increase in the total expression of early granule genes and concomitant decrease in those that are expressed later during granulopoiesis. Within BM-derived Ly6G^{+} neutrophil lineage cells, there was a 2.5-fold increase in the primary granule gene *Mpo*, while later granule genes were mildly, but significantly reduced (*Figure* 3.18). While this gene signature confirms the morphological evidence of defective maturation, this result could be explained by i) a requirement of autophagy for the generation of granules or ii) a population effect caused by the over-representation of immature maturation stages in the mixed population of all Ly6G-expressing cells.

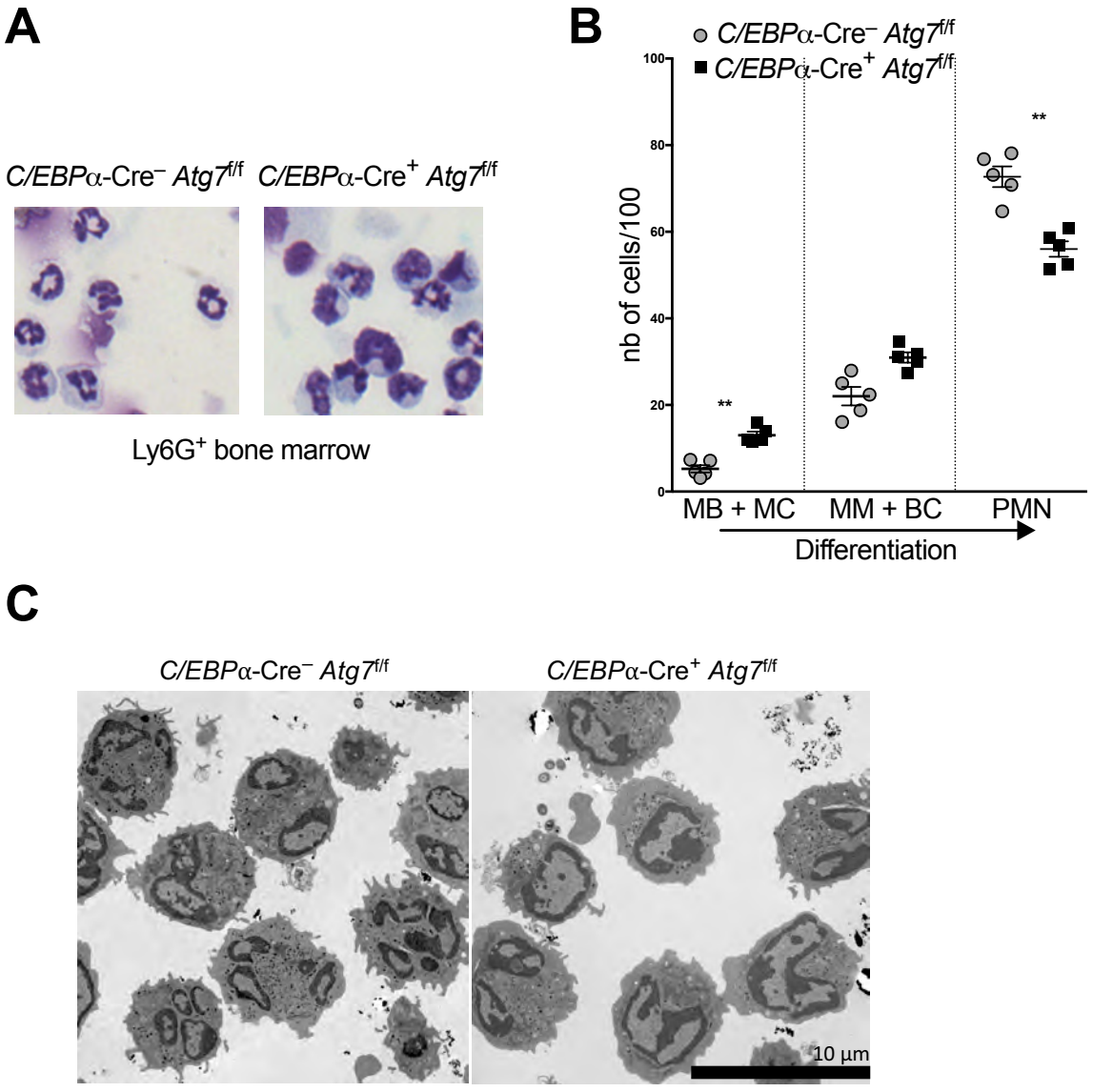

Figure 3.17: Accumulation of cells with neutrophil precursor morphology in *C/EBPa-cre x Atg7$^{f/f}$* bone marrow. Representative Wright-Giemsa stained cytospins of Ly6G$^+$ cells (A) and histological quantification (per 100 total Ly6G$^+$ cells) of indicated maturation stages by morphology (B). Representative electron micrographs are shown from Ly6G$^+$ bone marrow neutrophils (C). Grateful acknowledgements to Errin Johnson for introduction and access to the electron microscopes.

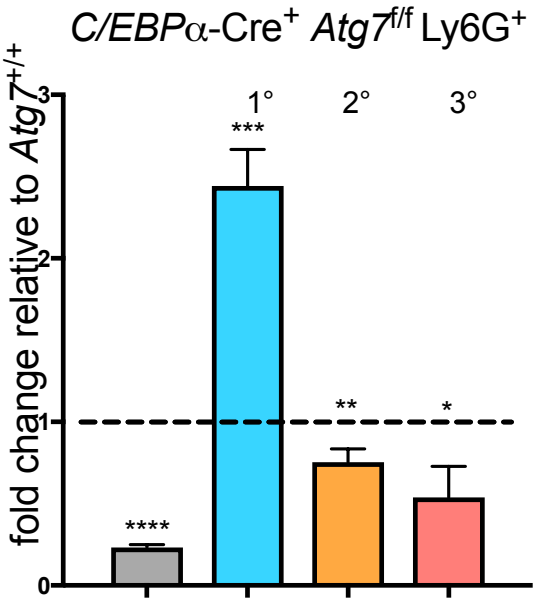

Figure 3.18: Immature granule gene signature in *C/EBPa-cre x Atg7*$^{f/f}$ mice. Total Ly6G^{+} BM cells were isolated from control or *C/EBPa-cre x Atg7*$^{f/f}$ mice and tested for *Atg7*, primary granule gene *Mpo*, secondary granule gene *Ltf* and tertiary granule gene *Mmp9*. Expression levels are presented as fold change +/- SEM normalized to *Actin* and relative to control ($\Delta\Delta$Ct). Data from n=3 mice/group. *Mpo*, myeloperoxidase; *Ltf*, lactoferrin; *Mmp9*, matrix metalloprotease 9.

3.2.8 Neutrophil granule and transcription factor expression is independent of autophagy

The finding that granule gene expression is altered in *Atg7*$^{-/-}$ mice raises the question if other transcriptional events, such as the transcription factors that govern neutrophil differentiation, are also dependent on autophagy. C/EBPα is the transcription factor that is expressed in the earliest stages of committed neutrophil differentiation (GMP-MB stage) and has a well established function in facilitating neutrophil differentiation [216, 217]. In fact, *C/EBPa* deletion causes a neutrophil-specific differentiation block and accumulation of myeloblasts similar to *Atg7*-deficient mice [218]. While there is a clear phenotypical and functional defect in the maturation of neutrophils in the bone marrow when autophagy is deleted in early granulocyte progenitors, the question remained which parameters are directly and cell-intrinsically affected by the deletion of autophagy as opposed to a secondary or downstream effect resulting from the skewed composition of predominantly immature neutrophils within the bone marrow. In this regard, the impaired nuclear lobulation as well as the functional readouts were both found in the pool of all Ly6G-expressing cells as well as after specific isolation of the mature PMN by flow-cytometric definition as in 3.2.1. In contrast, separation of the 5 consecutive maturation stages revealed that within each stage, the expression of the granulopoietic master regulator C/EBPα, as well as granule genes *Mpo* and *Mmp9*, are not directly affected by *Atg7* deletion (*Figure* 3.19). This result indicates that the previous finding of predominantly early granule gene expression in *Atg7*$^{-/-}$ Ly6G^{+} cells actually represents a population effect, i.e. the left shift and over-abundance of early maturation stages described earlier. Thus, while nuclear morphology and function are impaired in the *Atg7*-deficient Ly6G^{high} PMNs intrinsically, transcriptional events are unaffected on a per-cell basis and instead skewed as a secondary consequence of the accumulation of immature precursor cells.

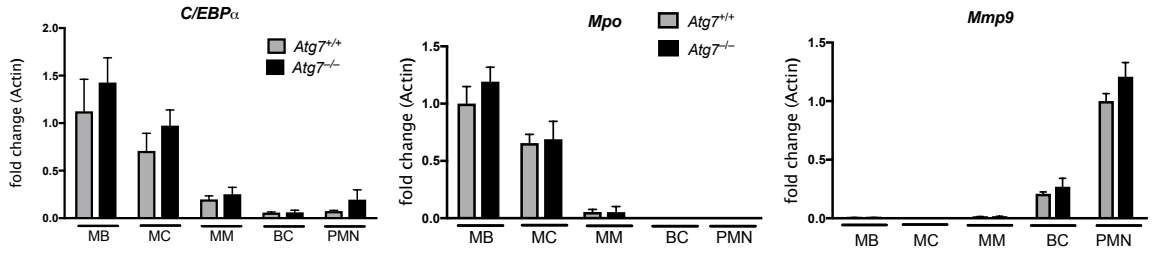

Figure 3.19: Granule gene and regulatory factor transcription is not dependent on autophagy. Gene expression analysis of the myeloid transcription factor *C/EBPa* and the early and late granule genes *Mpo* and *Mmp9* in control and *Vav-cre* x $Atg7^{f/f}$ mice (n=4 mice/group) after separation of the indicated precursor populations by flow cytometry and normalization to *Actin* relative to control ($\Delta\Delta$Ct).

3.2.9 Neutrophil cell cycle exit and cytokine receptor levels are not affected by autophagy deletion

As neutrophils mature within the bone marrow, they exit the cell cycle gradually from the myelocyte stages onward to undergo terminal differentiation into post-mitotic mature neutrophils [219, 220]. A widely used marker to measure proliferation is the DNA-binding protein Ki67 [221, 222]. As with granule gene expression patterns, the cell cycle exit occurs stepwise during granulopoiesis and therefore definition of neutrophils by Ly6G-marker only would skew the results due to over-abundance of immature cells within this population. We therefore defined the 5 consecutive stages of differentiation as in 3.2.1 and analyzed the stage specific expression of Ki67. As expected, Ki67 levels are highest in the earliest MB stage and gradually decline after the MM stage in wild-type controls. This pattern of gradual cell cycle exit, however, was not significantly affected in autophagy deficient mice (*Figure* 3.20A). Another factor that could directly explain the accumulation of immature neutrophil precursors is the expression of the respective cytokine receptors. Signaling defects in the G-CSF and GM-CSF mediated pathways that cooperate with C/EBPα signaling were previously shown to impact neutrophil differentiation [223, 224, 225]. However, G-CSF and GM-CSF cytokine receptor levels were unaffected by *Atg7* deletion (*Figure*

3.20B). Together, this data suggests that the signaling pathways through G-CSF and C/EBPα may function normally in *Atg7*-deficient animals. Similarly, the hypothesis that an increased proliferative capacity of $Atg7^{-/-}$ neutrophil progenitors, as described in other model systems [168], could cause the left shift in maturation stages, was not substantiated.

3.2.10 The neutrophil differentiation defect is cell intrinsic and conserved *in vitro*

Competitive, inducible bone marrow chimera are arrested in neutrophil maturation in the absence of *Atg5*

While *C/EBPα-cre* mediated deletion of *Atg7* is the most specific method to delete autophagy in all neutrophil committed precursors, it also affects the monocyte-macrophage lineage and the mice develop a hepatomegaly after 8 weeks due to an off-target deletion of *Atg7* in the liver [226], which could both contribute to the observed phenotype. Furthermore, the absence of mature neutrophils may likely trigger secondary feedback events that would signal for increased granulopoiesis to overcome this defect such as or similar to emergency myelopoiesis [227, 228, 229, 207]. Furthermore, while there are no non-autophagy related roles described for *Atg7*, it is best practice in the field to confirm autophagy as the underlying mechanism by deleting several core autophagy genes, particularly given the recent discoveries of alternative autophagy and LC3 associated mechanisms [196, 204, 17, 44]. To address each of these relevant issues, we generated mixed bone marrow chimeras with inducible deletion of *Atg5* (*Mx1-cre x* $Atg5^{f/f}$) in which the wild type bone marrow will give rise to a normal hematopoietic system without loss of mature neutrophils. Importantly, in order to avoid potential confounding effects on the homing to the bone marrow, *Atg5* was only deleted 6 weeks after the transplantation of mixed, competitive, hematopoietic bone marrow into recipient mice using an inducible *cre*-system. Equal numbers of congenically distinct

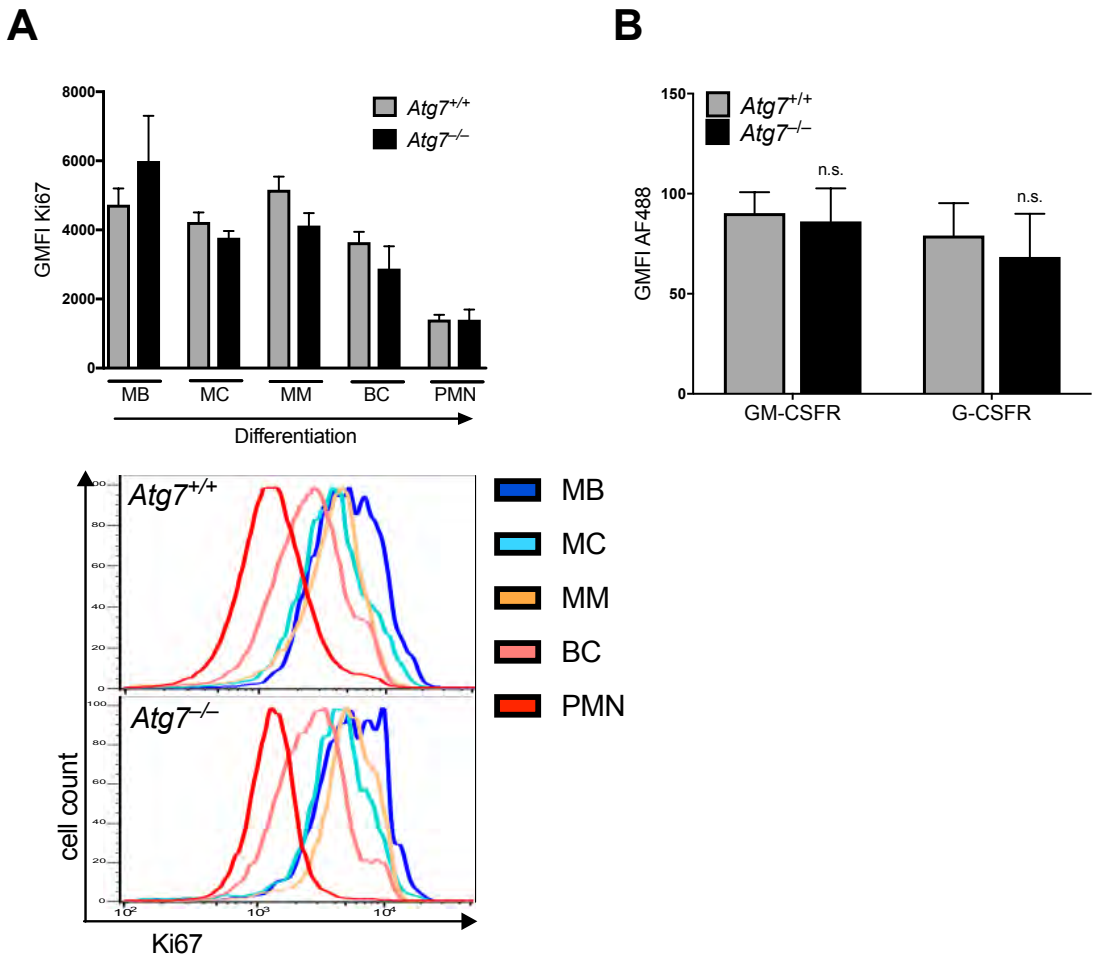

Figure 3.20: Normal cell cycle exit and cytokine receptor levels during *Atg7*-deficient granulopoiesis. Cell cycle exit was measured as geometric MFI of Ki67 expression (top left) by intracellular flow cytometry in the indicated precursor populations of control and *Vav-cre x Atg7$^{f/f}$* mice (n=3 mice/group); representative Ki67 histograms are shown for each genotype (bottom) (A). Geometric MFI +/- SEM from control and *Vav-cre x Atg7$^{f/f}$* for the cell surface expression of G-CSF and GM-CSF receptors in CD11b^{+} Ly6G^{+} PMN (n=4 mice/group) (B).

control ($CD45.1^+$) and *Mx1-cre x* $Atg5^{f/f}$ ($CD45.2^+$) bone marrow cells were injected into sub-lethally irradiated hosts ($CD45.1^+$)($Atg5^{-/-}$ chimera). Control chimeric mice were generated using equal numbers of support cells ($CD45.1^+$) and $Mx1\text{-}cre^{neg}$ *x* $Atg5^{f/f}$ ($CD45.2^+$) bone marrow (schematic outline of the experimental design: *Figure* 3.21A). Granulopoiesis was assessed by serial bleeding over 18 weeks. While control chimeric mice had roughly equal contribution of donor- and recipient-type BM cells, $Atg5^{-/-}$ chimera had progressively decreasing contribution of CD45.2 cells as expected from the fundamental role of autophagy in HSCs [164]. The progressive accumulation of $Ly6G^+$ cells with immature morphology, however, occurred only within the *Atg5* deleted CD45.2 fraction, but not in the CD45.1 wild-type cells within the same mouse (*Figure* 3.21). This suggests that the maturation defect is cell intrinsic and is not caused by feedback/compensation mechanisms, because wild-type support cells (CD45.1) were unaffected.

Neutrophil differentiation is defective in an *in vitro* culture assay of 32D $Atg7^{-/-}$ CRISPR clones

While there is a clear defect in granulopoiesis in all analyzed mouse models, that is independent of the role of autophagy in HSCs or other hematopoietic lineages, the penetrance of this phenotype is limited as some cells acquire at least a partially mature neutrophil phenotype or up regulate Ly6G and there is an overall feedback mechanism that leads to the expansion of the entire neutrophil lineage in the bone marrow. In addition, immature myeloblasts exit the bone marrow and accumulate in tissues in these mice [176], together making it difficult to understand the individual mechanistic events that cause the defect. To overcome this limitation and facilitate mechanistic studies as well as to further examine the cell intrinsic nature of this phenotype, CRISPR-Cas9 technology was used to delete several distinct early exons of the *Atg7* gene in the previously described, IL3-dependent 32Dcl3 myeloblast line (see 3.2.3) [182, 208, 230].

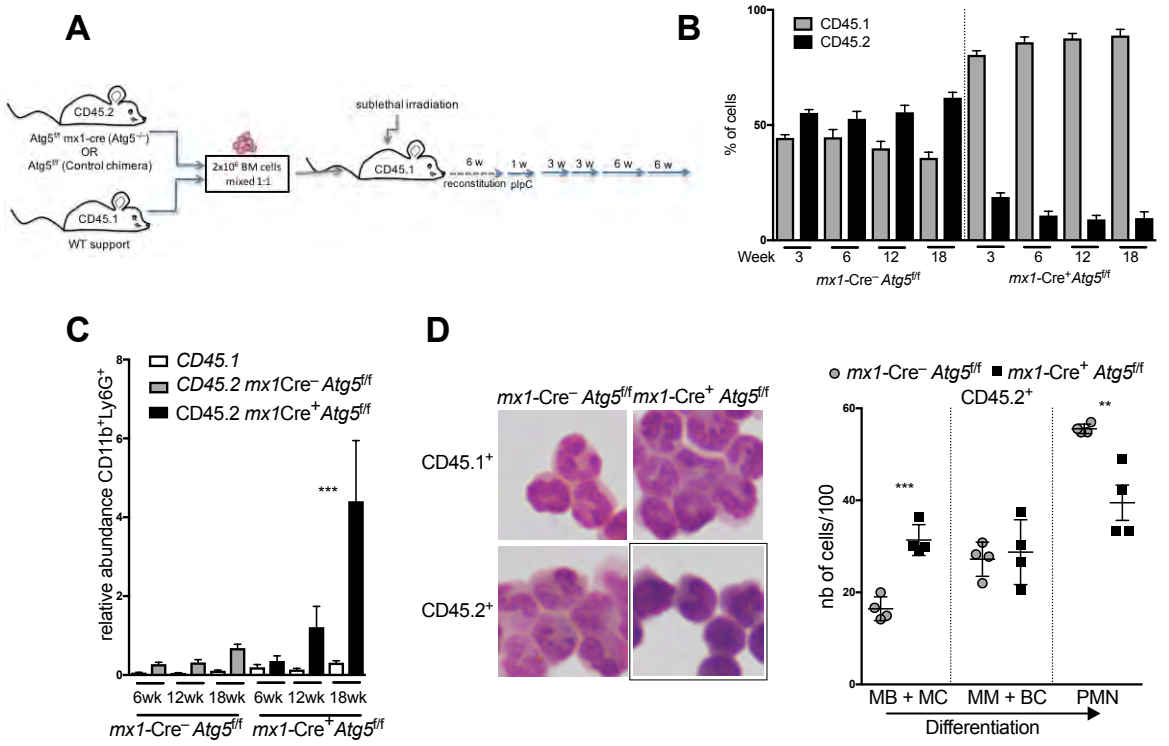

Figure 3.21: Inducible *Atg5* deletion in chimeras causes neutrophil maturation defect. Schematic of experimental set-up for mixed, inducible hematopoietic bone marrow chimeras. pIpC was injected *i.p.* (250 µg pIpC in 250 µL saline) e.o.d. for one week to induce excision (A). Peripheral blood reconstitution with donor (CD45.2) and recipient (CD45.1) type cells shown as % of total cells +/- SD at the indicated weeks after pIpC-induced *Atg5* deletion (B). Abundance of cells $CD11b^+Ly6G^+$ at surface relative to total lymphocytes (T + B cells, gated as $CD3^+$ and $CD19^+$, respectively) in PB of control and *Mx1-cre x* $Atg5^{f/f}$ chimeric mice at indicated time points (C). Data are shown as mean +/- SEM (n = 6 mice/group), representative of two experiments. ***$p < 0.001$, tested by two-way Anova. Representative Wright-Giemsa stained cytospins of $Ly6G^+$ cells (left) and histological quantification (per 100 total $Ly6G^+$ cells, right) of indicated maturation stages by morphology after flow cytometric separation of $CD45.2^+$ $Ly6G^+$ cells (D).

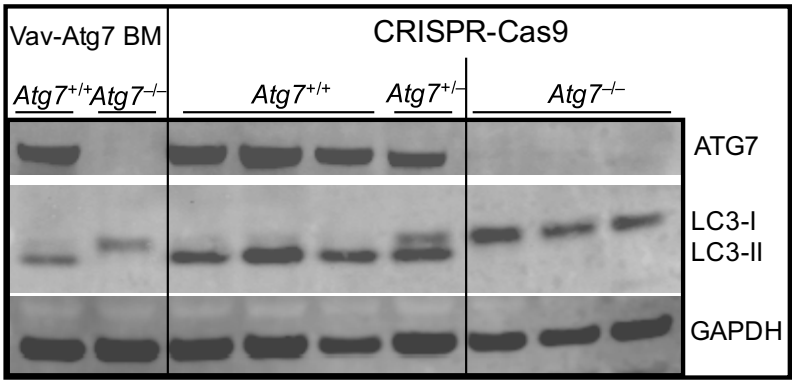

Figure 3.22: CRISPR-Cas9 mediated *Atg7* deletion in an G-CSF inducible *in vitro* system of neutrophil differentiation. Western blot for ATG7 and LC3-I/II protein expression. Ly6G$^+$ BM cells from *Vav-cre x Atg7$^{f/f}$* mice were used as control. Shown are three separate clones of wild-type (*Atg7$^{+/+}$*) and CRSIPR-Cas9-generated *Atg7$^{-/-}$* genotypes grown clonally from sorted single cells. GAPDH was used to control for equal loading.

Three clones were selected that each targeted separate regions within exon 3 or 4 of *Atg7* and have resulted in a frame shift and absence of ATG7 protein as detected by immuno blotting. *Vav-cre x Atg7$^{f/f}$* mouse BM cells were used as positive control, and the absence of autophagic flux was further confirmed by immunoblotting for LC3-I to LC3-II conversion, which was absent in all *Atg7$^{-/-}$* clones (*Figure* 3.22).

Differentiation into mature neutrophils in this system can be studied by removal of IL-3 and addition of G-CSF over the course of 5-7 days. The two most robust readouts to monitor neutrophil differentiation in this system were CD11b maturation marker up regulation and morphological characterization by Wright-Giemsa (see 3.2.3). While 32Dcl3 wild-type myeloblasts formed mature neutrophils after 5 days in G-CSF supplemented medium, all *Atg7$^{-/-}$* clones retained a myeloblast like morphology with expanded cytoplasma und absent nuclear lobularization. Similarly, the up regulation of CD11b was significantly and progressively impaired over the course of differentiation (*Figure* 3.23). Together, these data show that defective neutrophil maturation in the absence of autophagy is conserved in the 32D *in vitro* differentiation system, which thus presents a suitable model to study the underlying mechanisms in an easily tractable,

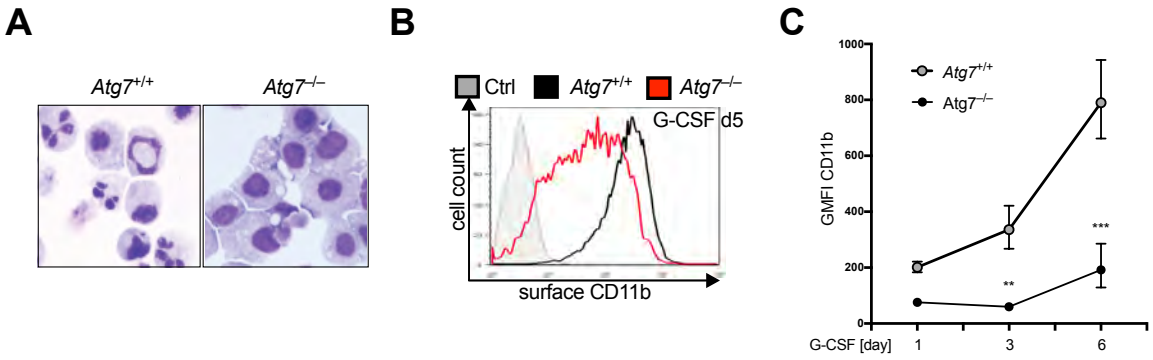

Figure 3.23: Neutrophil differentiation is defective in 32Dcl3 myeloblast *in vitro* culture of *Atg7*$^{-/-}$ CRISPR clones. Representative images of Wright-Giemsa stained cytospins of *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ cells after 5 days culture in G-CSF (100 nM) (A). Surface CD11b expression of *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ cells after 5 days in G-CSF supplemented cultures. Representative histograms with unstained control cells from day 0 shown in grey (left) and quantification of geometric MFI +/- SEM of CD11b expression at the indicated time points of G-CSF mediated differentiation. Data are representative of three independent clones tested in independent experiments. Statistics obtained using two-way ANOVA with Tukey´s post test. *p $<$ 0.05, **p $<$ 0.01, ***p $<$ 0.001, ****p $<$ 0.0001

congenic system that overcomes the problem of precursor population rarity and -isolation from *Atg7*-deficient mouse models.

3.3 Discussion

3.3.1 Dynamic regulation of autophagy during granulopoiesis correlates with functional relevance

In this study, several murine models were used to isolate neutrophil progenitor populations and characterize their autophagic activity as well as their dependency on autophagy. The committed stages of neutrophil precursors downstream of the GMP were defined already in the 1960s [116], but the functional characterization of these precursors has been limited by the ability to purify these subsets effectively. More recently, several groups have attempted to establish purification-protocols [231, 206, 207], but most of these have to date not been exploited to gain new insights into regulatory

events and the biology of granulopoiesis. Because Satake et al was able to achieve the highest degree of separation and also apply this to identify novel transcriptional regulatory events, their method was used here to identify two peaks of autophagic activity and assess their significance. The first wave of autophagy occurred early on at the MB/MC stage, and later a second wave of autophagic signaling occurred at the terminal BC/PMN stage. One might expect the second, late peak of autophagic activity in light of the functional relevance of active autophagy for a number of neutrophil effector functions [210, 232, 177, 233]. The observation that autophagy is high already in the band cell stage could be a mechanism whereby autophagic turnover is activated prematurely so that PMNs are poised for autophagy-dependent effector functions. This is somewhat reminiscent of the situation in memory $CD8^+$ T cells, where autophagy significantly peaks following the expansion phase during the effector to memory transition [169], possibly in preparation for it´s essential role in the maintenance of memory $CD8^+$ T cells [165]. Likewise, the high autophagic flux in the earliest granulopoietic stages could therefore indicate a functional relevance for autophagy during or after the MB/MC stages. Autophagy is important for the regulation of phagocytosis [212], neutrophil degranulation and NADPH-oxidase dependent ROS production [210] and therefore essential for normal neutrophil function. In line with this, both phagocytosis and intracellular bacterial killing capacity was reduced in neutrophils from *Atg5*/7-deficient animals (See 3.2.5). While these readouts are used here to support the functional consequence of impaired maturation, one would also expect them to be impaired in mature neutrophils based on this literature. In reverse however, the use of *LysM-cre* mediated excision in these functional studies may mean that incomplete maturation at the level of terminal precursors may have contributed at least in part to the described functional decline. One might conclude that it is likely a partial contribution of immaturity and functional dependence on autophagy that in combination contributes to the functional neutrophil impairment.

3.3.2 Neutrophil maturation requires autophagy at or after the GMP progenitor stage

Three murine models were used here to assess the relevance of autophagy during granulopoiesis, by deleting autophagy either in the entire hematopoietic system (*Vav-cre x Atg7$^{f/f}$*), specifically in the myeloid/granulocyte lineage at the level of GMPs (*C/EBPa-cre x Atg7$^{f/f}$* and *C/EBPa-cre x Atg5$^{f/f}$*) or in an inducible manner in competitive bone marrow chimeric mice (*Mx1-cre x Atg5$^{f/f}$*). Taken together, these results describe a differentiation defect that occurs at or after the GMP stage in a cell intrinsic manner and leads to an accumulation of myelocytic neutrophil precursor cells.

An important conclusion that can be drawn from the animal models is that although likely contributing to the severity of the *Vav-cre x Atg7$^{f/f}$* phenotype, the neutrophil defect is independent of the role of autophagy in HSCs. This is important, because autophagy genes including *Atg5/7* and *Fip200* were previously found essential for the maintenance of HSC quiescence [195, 171, 234, 164, 205] and one could imagine that in HSC or HSPC compartments, autophagy could imprint a differentiation bias towards certain myeloid lineages or facilitate processes that, if absent, cause impairment of downstream differentiation in the granulocytic lineage. However, because the differentiation defect is conserved in *C/EBPa-cre x Atg7$^{f/f}$* and the 32Dcl3 myeloblast *in vitro* system, this possibility is unlikely to have a major contribution to the described phenotype.

Furthermore, a limitation of both *Vav-cre* and *C/EBPa-cre* mediated excision is that homeostatic proliferation and compensation could cause in part the accumulation of precursors. In the absence of mature, functional lineage cells, one would expect demand adapted myelopoiesis or "emergency granulopoiesis" to compensate, much like in the context of sustained or systemic infections [235, 227]. While this likely contributes to the severity of the differentiation defect *in vivo*, the presence of immature

precursor cells even in the context of a complete and wild-type hematopoietic system in competitive bone marrow chimera experiments argues for the cell intrinsic nature of this phenotype. This is further supported by the observation of impaired differentiation in the *in vitro* culture system, and raises the obvious question as to what functional role autophagy may have in the earliest committed granulocyte precursors.

While little is known about the cell biological events occurring at this stage, one known transition that occurs immediately after the myelocyte stage is the exit of the cell cycle to undergo terminal maturation events [207]. Therefore, one idea was that autophagy may be important to limit proliferation and facilitate the exit of the cell cycle, which has been described in HSPCs [205], iNKT cell maturation [236] and embryonic fibroblasts [237]. The latter study by Lee et al. in fact found direct evidence of ATG7 binding to p53 to regulate the cyclin-dependent kinase inhibitor p21CDKN1A and mediate cell cycle exit. However, during *Atg7*-deficient granulopoiesis, the proliferation and cell cycle exit was normal in all precursors (See 3.2.9), suggesting cell type dependent variation in the relevance of this signaling axis. This observation is somewhat in contrast to a study by Rozman et al, which also found a mild increase in neutrophil precursor proliferation as well as a similar expansion of the neutrophil lineage as described here [177]. The difference in the extent of the differentiation defect and proliferative status of precursors may be explained by the use of *LysM-cre* mediated excision in this study, which is weak in GMPs (20 %) and excises in less than 60 % of the neutrophil lineage cells [238]. Taking this additional study into account means that while the requirement for autophagy during granulopoiesis is strongest when deleted already at the HSC level, it is still observable with deletion at the GMP stage, but becomes minor when autophagy is deleted partially and at the terminal stages via *LysM-cre*. A model is therefore likely whereby a cumulative effect of defective autophagy eventually leads to an increasing extent of the defect in differentiation. The nature of this cumulative effect could be manifold, such as ROS accumulation, DNA

damage, dysfunctional protein complexes, factors or organelles accumulating/failing over time, or a depletion of certain required metabolites (Summarized in Figure 3.24). While there are many studies suggesting examples of such effects in various cell types [165, 239, 172, 168, 191, 169, 166], the focus of the subsequent chapters will be to definitively identify the causative mechanism in the case of granulopoiesis.

3.3.3 Autophagy is required for differentiation in most myeloid and lymphoid lineages

It was previously described that undifferentiated myeloid cells accumulate if autophagy is deleted in murine HSCs, which was hypothesized to be either a secondary effect to homeostatic proliferation caused by the HSC exhaustion and bone marrow failure in this model, or be caused by a transformative event and accelerated proliferation in myeloid blast cells similar to a myelodysplastic syndrome [176]. What is new in this study is the identification of the granulocytic origin of these cells, based on the presence of neutrophil specific markers, their typical myeloblastic morphology, and early antimicrobial granule presence (See 3.2.5). Furthermore, the progenitor cell intrinsic nature of the defect means that it is likely a separate event to the bone marrow failure in *Vav-cre x Atg7*$^{f/f}$ and thus a novel defect linked to granulopoiesis.

While the requirement for autophagy in lymphoid lineages has been extensively studied during the last decade, it is becoming increasingly clear that most myeloid lineages are also dependent on autophagy for proper maturation or function. A recent review summarized the roles that autophagy has in the various hematopoietic lineages [189], and there is evidence that autophagy may have such roles universally during cellular differentiation and development [13]. The function of autophagy to facilitate granulopoiesis as described here supports this picture by adding a cell type to the list which was previously thought to be independent of autophagy for its generation.

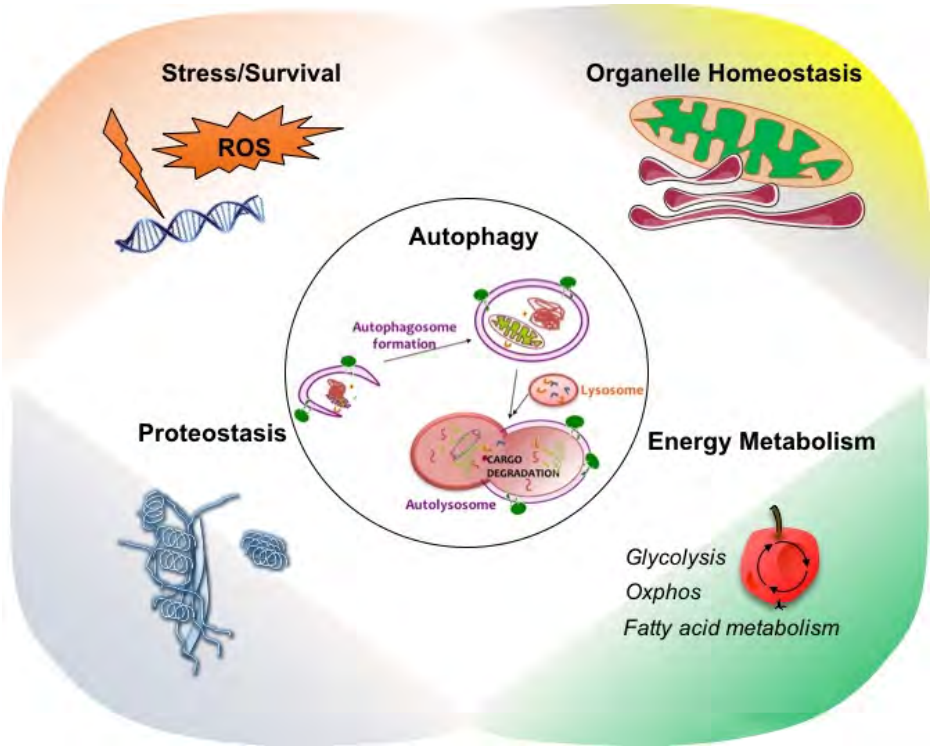

Figure 3.24: Impact of autophagy on cellular homeostasis and differentiation. Autophagy can affect the differentiation of hematopoietic cells by mechanisms summarized here in four categories. Top left: By preventing ROS, ER-stress, caspase activation and DNA damage, it may maintain survival of cells fit to differentiate. Top right: Homeostasis of organelles, particularly mitochondria, is important as cellular content undergoes rapid remodeling during differentiation. Bottom right: Energy metabolic adaption is linked to autophagy by providing nutrients and ATP necessary for differentiation. Bottom left: Protein homeostasis is maintained by regulated degradation of damaged and misfolded proteins via the proteasome and autophagy pathways. An adapted version of this figure was published by the author recently [189].

3.4 Conclusion

Employment of the recently established flow cytometric characterization of neutrophil precursor subsets enabled here the identification of two distinct phases of autophagy during granulopoiesis: A late terminal peak which may poise PMNs to be ready for autophagy-dependent effector functions, and an early peak in the earliest committed precursors, which may indicate functional relevance for differentiation. Indeed, a defect in granulopoiesis occurred around the myeloblast-myelocyte stage, which is cell intrinsic and independent of autophagy in HSCs. Autophagy acted primarily at the level of precursors, whereby an inverse correlation between the timing of autophagy deletion and the severity of differentiation impairment may point towards a gradual mechanism that leads to accumulation or depletion of a causative factor over time. In other hematopoietic cell types, such factors that are affected by autophagy and impact differentiation are: metabolic adaption (HSCs + T cell subsets), organelle homeostasis (erythropoiesis), apoptosis/cell death (macrophages and T cells) or protein homeostasis/folding and ER-stress (B-cells) (Figure 3.24). The following chapters will further define the mechanistic implication of autophagy during granulopoiesis.

At least within the hematopoietic system, most differentiation processes are thought to be mainly governed by gene regulatory networks and master transcriptional factor complexes with specific DNA binding motifs [240]. It is clear that specific lineage differentiation programs are associated with master regulators, such as the CCAT/enhancer binding family, containing C/EBPα, in the case of granulopoiesis [217, 216]. Nevertheless, recent studies also describe another, cell biological layer of regulation that is required for and capable of fine-tuning hematopoietic stem cell differentiation: The metabolic fuel choice between glycolysis, oxidative mitochondrial metabolism or glutaminolysis for nucleotide synthesis is sufficient to alter hematopoietic stem cell lineage commitment [241, 242, 243]. Further downstream, the generation and maintenance of many T cell subsets, such as T_{mem} and T_{reg} among others requires autophagy and

specific metabolic states [244, 165, 170, 245, 246, 169, 166], but the mechanistic connections are not fully understood. While comparatively little is known about how autophagy can affect the metabolic balance within neutrophil precursors, the link between metabolism and differentiation is increasingly being understood also beyond the hematopoietic system [159] and is therefore one of the mechanisms investigated in the following chapters.

4 Autophagy controls a metabolic switch in neutrophil precursors

4.1 Introduction

Defective neutrophil differentiation in the absence of autophagy was independent of transcriptional events and cell cycle regulation, leaving open the possibility that metabolic mechanisms may be mediated by autophagy similar to examples recently described in other settings of hematopoietic differentiation [189]. Metabolic regulation mediated by autophagy was recently reported in the context of macrophage differentiation and polarization, dendritic cell biology, and innate lymphocytes, among others [247]. The following chapter therefore will explore metabolic regulation during granulopoiesis and how it may be affected by autophagy.

4.1.1 Autophagy controls metabolic homeostasis during macrophage differentiation

Under inflammatory conditions, myeloid progenitor cells undergo drastic spatial and cell biological alterations to produce large number of short lived innate myeloid effector cells [248]. This highly energy-consuming process calls for a well-coordinated metabolic adaptation in monocyte and granulocyte progenitors that is able to generate both the ATP and macromolecules required to sustain extensive remodeling associated with differentiation and polarization. While the role of autophagy during macrophage differentiation was described earlier (See 3.1.2), recent literature also suggests a central metabolic contribution of autophagy in this phenotype, particularly during terminal effector polarization of macrophages. Macrophage differentiation is followed by further acquisition of a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype [249]. In this regard, T_H1-derived interferon-gamma (IFN-g) supports M1-

polarization, whereas interleukin-4 (IL4) mediates the M2 phenotype. Although this analogy to the T_H1/T_H2 paradigm may be over-simplifying [249], it reveals an interesting correlation between autophagy and the metabolic states of these lineages: a direct link of autophagy-dependent metabolism and cellular differentiation is provided by decreased potential of monocytes to differentiate into M2 macrophages in *Atg7*-KO mice [250] along with increased glycolytic activity, M1 inflammatory cytokine and reactive oxygen species production [167]. In support of this, another study found increased M1 polarization and inflammation in the liver of high-fat diet fed $Atg5^{-/-}$ mice [251]. Further differentiation into M2 macrophages requires increased fatty acid oxidation (FAO) metabolism which is mediated by the triacylglycerol importer CD36 and subsequent cell intrinsic lipolysis [252]. For this, M2 differentiation requires LAL (lysosomal acid lipase) mediated degradation of exogenous fatty acids to engage elevated OXPHOS. In line with this, constitutive activation of mTORC1 by Tsc1 deletion, which will inhibit autophagy, results in impaired M2 macrophage differentiation upon IL4 stimulation and impaired FAO and oxidative metabolism [86]. Controversially, abrogation of the mTORC2 signaling pathway also decreases levels of M2 macrophages, whereas the M1 macrophage level remains unchanged, suggesting that mTORC2 signaling is specifically important in M2 differentiation [253]. Taken together, these studies not only show that M1 vs M2 differentiation is associated with unique and opposing energy metabolic programs, but also that this metabolism can be mediated by autophagy and lysosomal breakdown. Studies that investigate *Atg*-deficient mouse models show an additional role for LC3-associated phagocytosis (LAP) in the prevention of excessive inflammation. Absence of LAP generates a pro-inflammatory cytokine milieu and impairs the removal of dying cells [17], which may further affect immune cell lineage commitment.

4.1.2 Autophagy and metabolism in other innate immune cells

Neutrophils are the most abundant innate immune cell in humans, but the role of autophagy and metabolism is better defined in innate lymphocytes. This family consists of mature NK (mNK) cells, group 1 innate lymphoid cells (ILC1s), group 2 ILCs (ILC2s), and group 3 ILCs (ILC3s). Innate lymphoid cells are derived from the common lymphoid progenitor (CLP), and ultimately from HSCs, within the bone marrow. Natural killer (NK) cells are the most abundant ILC in context of viral infections, where their roles include cytokine secretion and cytotoxic action. During their differentiation, CLP-derived natural killer progenitors (NKPs) differentiate into immature NK cells (iNKs) and further into mature NKs (mNKs) [254]. Similar to our observations in granulocytes, highly stage specific kinetic regulation of autophagy occurs also in committed NK progenitors: while NKPs and mNKs have relatively low levels of autophagy, the intermediate iNK stage has high autophagic turnover as measured by flow cytometry, immunofluorescence and immuno-EM in GFP-LC3-transgenic mice [255]. NK-progenitor specific deletion of *Atg5* in $Atg5^{f/f}$ *x NKp46-cre* mice leads to a drastic decrease in iNKs and mNKs in BM and spleen. siRNA-mediated silencing of *Atg3* confirmed that this is caused by a defect in autophagy rather than gene-specific effects [256]. The authors note increased damaged mitochondria and ROS leading to elevated apoptosis, but did not investigate the mechanism and metabolic consequence of impaired mitochondrial homeostasis on NK differentiation.

Natural killer T (NKT) cells share features of both T cells and NK cells, and are able to produce large numbers of immuno-modulary cytokines in response to their lipid-antigens [257]. They can be activated through their invariant TCR or independently via cytokine signals. Their differentiation has also been shown to require *Atg5* and *Atg7* dependent autophagy [236, 239]. Although the metabolic mechanisms are not entirely understood, NKT cells are thought to adopt a memory-like phenotype which is characterized by decreasing glycolysis. Again, autophagy is required here to limit

mitochondrial ROS and to prevent apoptosis [239]. A similar switch from glycolysis to OXPHOS that underlies this transition into a natural memory like metabolic phenotype also occurs in memory T cells, as discussed later (See 5.1.2). In line with this and as reported for NK cells, Tsc-mediated constitutive activation of mTOR also inhibits the iNKT terminal differentiation and supresses autophagy [258]. Taken together, several innate lymphocyte populations rely on stage specific autophagy activation for terminal differentiation. The fact that mTOR activation, a known metabolic regulator and activator of glycolytic metabolism via HIF1a and C-MYC [259, 260], mirrors this phenotype in all cases suggests that this mechanism may act via metabolic regulation impacted by autophagy.

4.1.3 Context and aim of this chapter

While a link is emerging between autophagy and metabolism in the differentiation of other innate and myeloid immune cells, few studies have explored this during granulopoiesis. Autophagy is activated upon G-CSF treatment in neutrophils and progenitor cells and important for neutrophil mobilization [234]. Metabolically, peripheral neutrophils that reside in low-oxygen tissues are classically associated with a predominantly glycolytic metabolism, which is further supported by the requirement of functional MTORC1 signaling and limiting autophagy during granulocyte maturation [177]. Conversely, uncoupling granulocyte progenitors from mitochondrial respiration impairs the frequency of mature neutrophils, indicating necessity of OXPHOS in terminal neutrophil differentiation [261]. Granulopoiesis also requires NAMPT, an enzyme essential for NAD^+ biosynthesis and maintaining a variety of metabolic pathways including the TCA cycle and FAO [262]. Thus, although no direct evidence exists for a role of autophagy in regulating metabolism during granulopoiesis, increasing evidence suggests links between autophagy and metabolic changes in other innate immune cells. Except for macrophage differentiation, no study has provided definite direct links be-

tween these processes. In this chapter, we aim to define the metabolic regulation that occurs during granulopoiesis and to investigate the causality between autophagy, metabolism and differentiation in this system.

4.2 Results

4.2.1 Metabolic states of neutrophil differentiation are highly distinct and impacted by autophagy deletion

Because the metabolic states of neutrophil precursors are largely unknown, a large scale gene expression profile of metabolic genes was first generated for all precursors using the Fluidigm Biomark platform. More than 40 genes from glucose metabolism, OXPHOS, FAO, and associated pathways were analyzed by unsupervised clustering based on z-scores in the five neutrophil lineage populations previously defined. All biological replicates of each population clustered together in separate groups solely based on the expression of metabolic genes. This suggests extensive metabolic remodeling during granulopoiesis and highly distinct metabolic states between precursor stages. While the gene expression analysis revealed a broad scale down regulation of many glycolytic genes, the mitochondrial content underwent a simultaneous 2-fold increase around the metamyelocyte stage (*Figure* 4.1).

Although metabolic gene expression often correlates with the activity of respective pathways, it is more informative to measure directly the cellular metabolites in the distinct stages. Because such metabolomic approaches require high cell numbers, we investigated three different timepoints of *in vitro* G-CSF driven differentiation of wild-type and $Atg7^{-/-}$ cultures that are enriched for MB + MC stages (IL-3 culture), MC and MM (2 days G-CSF) or BC and PMN (5 days G-CSF) and conducted LCxMS/MS. Principal component analysis (PCA) from the over 4000 different detected metabolites revealed highly distinct metabolomic states between the different stages (*Figure*

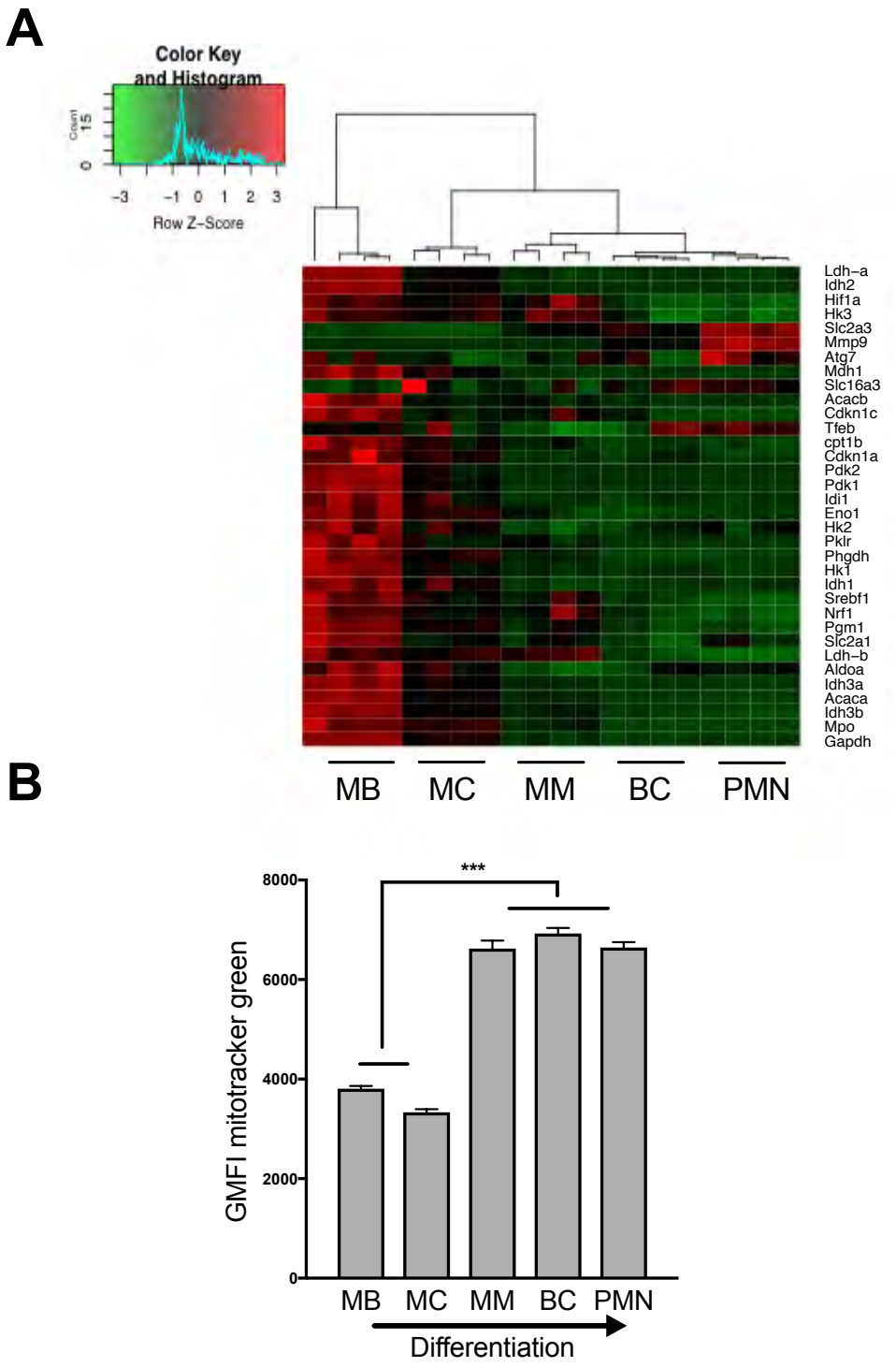

Figure 4.1: Extensive metabolic remodeling occurs during granulopoiesis. Microfluidic gene expression analysis with the Fluidigm-Biomark array for the indicated metabolic genes (100 cells/population) in five neutrophil differentiation stages sorted by flow-cytometry from wild-type mouse BM. Unsupervised clustering of replicates based on z-score (n = 4 mice) (A). Mitochondrial content was quantified by flow-cytometry from wild-type mouse BM as mitotracker green fluorescence intensity in indicated neutrophil precursor populations. Data are shown as mean +/- SEM (n=3 mice). ***$p < 0.001$, tested by one way Anova (B).

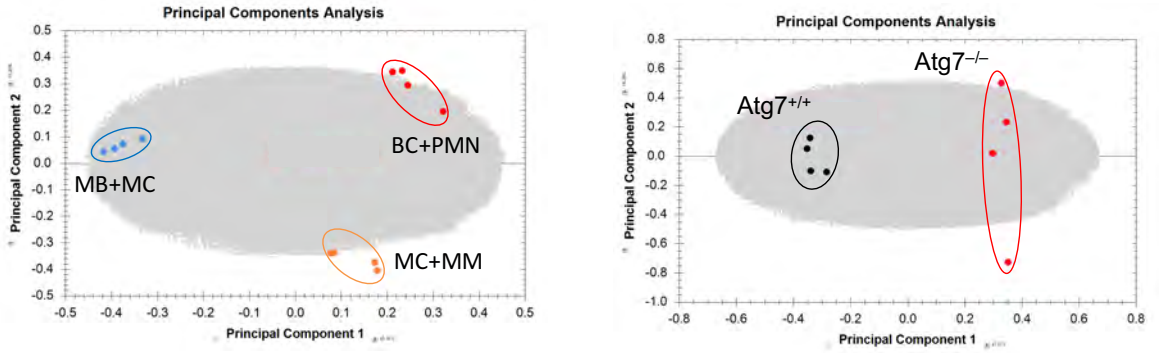

Figure 4.2: Metabolomic states of differentiation stages are distinct and impacted in CRISPR *Atg7*$^{-/-}$ clones. Metabolites were extracted from 5x10^6 cells of the indicated genotypes and metabolomic data was acquired by LCxMS/MS. Data from one experiment with 6 biological replicates. Principal component analysis of 32D myeloblasts after 2 and 5 days of G-CSF induced differentiation (100 nM), scored based on the 4215 detected metabolites (with Hotellings T2 ellipse at 0.95) (left) and principal component analysis of *Atg7*$^{+/+}$ myeloblasts and independent CRISPR *Atg7*$^{-/-}$ clones, scored based on the 4215 detected metabolites with %CV<30 on day 2 of G-CSF induced differentiation (right). Grateful acknowledgements to James McCullagh for help with sample preparation and analysis of metabolomics.

4.2, left), suggesting that a similar metabolic remodeling occurs *in vivo* and *in vitro*. Together with the gene expression profiles, this suggested a gradual alteration of the metabolome *in vivo*. Interestingly, the metabolomic composition of *Atg7*$^{-/-}$ clones was completely separated and distinct from wild-type control (*Figure* 4.2, right), indicating a role for autophagy in metabolic homeostasis during granulopoiesis.

4.2.2 Autophagy-deficiency causes excessive glycolysis in bone marrow neutrophil precursors

A surprising finding from the metabolic gene expression analysis was that most glycolytic genes were down-regulated during granulopoiesis *in vivo*, while mitochondrial content underwent a 2-fold expansion. Because autophagy-deficient precursors had a significantly altered metabolic profile, we next investigated whether glycolytic activity is limited by autophagy, as previously reported in the context of myeloid leukemias and T cell subsets [165, 168, 166]. 100 cells of each control and *Atg7*$^{-/-}$ BM neutrophil

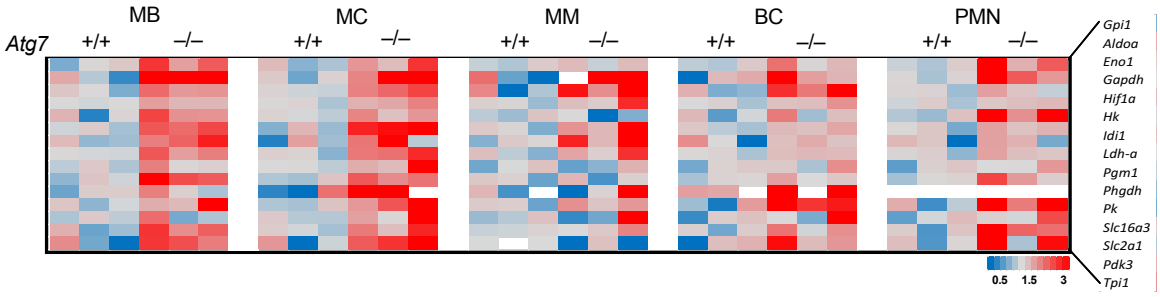

Figure 4.3: Gene expression profiling of the glycolytic pathway in primary BM neutrophil precursors. Gene expression profiling was done using the Fluidigm Biomark 48x48 dynamic array on 100 purified precursor cells of each of the indicated differentiation stages purified by flow cytometry from $Atg7^{-/-}$ and control $Atg7^{+/+}$ BM. Data from 3 separate mice per genotype. Heatmaps are shown as fold change relative to *Actin* and normalized to $Atg7^{+/+}$. All 15 glycolytic genes, except isoforms, were selected from the same gene panel as used in *Figure* 4.1.

differentiation stage were isolated by flow cytometry and analyzed for the expression of core glycolytic genes. Relative to the control group there was increased expression of almost all glycolytic genes analyzed, which was particularly significant in the earliest differentiation stages MB and MC (*Figure* 4.3). Of note, the overall decrease in expression during differentiation is not obvious here because the average wild-type gene expression is set to 1 for each stage in this representation. While the relative fold change ranged from + 1.2 to + 6 fold for most genes in $Atg7^{-/-}$, the MB and MC stage were most significantly different, as 16/30 measurements were statistically significant, as shown in *Figure* 4.4. Importantly, all glycolytic genes analyzed in the metabolic gene expression map (*Figure* 4.1) were included in this analysis, except for isoforms of the same gene, in which case the more abundant isoform is shown.

Since an altered metabolic profile was also found in the CRISPR-myeloblast cultures *in vitro*, it remained to be tested if there is evidence of glycolytic activation in the metabolomic dataset. Targeted search for glycolytic intermediates based on m/z values and chromatography retention time in comparison with known standards could uniquely identify 7 out of the 10 core glycolysis intermediates. As shown in *Figure* 4.5, all 7 intermediates were enriched in $Atg7^{-/-}$ myeloblasts compared to control,

	#1 MB, Myeloblast				#2 MC, Myelocyte				#3 MM, Metamyelocyte			
Gene name	Fold change (Actin) normalized to WT (ddCt)	SEM	p-value	Trend, significance	Fold change (Actin) normalized to WT (ddCt)	SEM	p-value	Trend, significance	Fold change (Actin) normalized to WT (ddCt)	SEM	p-value	Trend, significance
Gpi1	2.07	0.28	0.0105	↑ *	1.82	0.38		↑	1.18	0.11		→
Aldoa	3.61	0.11	0.0118	↑ *	4.41	1.39		↑	6.49	0.70		↑
Eno1	1.76	0.14		↑	2.08	0.16	0.0323	↑ *	3.81	1.58		↑
Gapdh	1.40	0.07	0.0006	↑ ***	1.55	0.14	0.0024	↑ **	1.67	0.48		↑
Hif1a	1.90	0.24	0.0266	↑ *	1.54	0.16		↑	0.72	0.20		→
Hk	2.19	0.10	0.0004	↑ ***	3.45	0.66	0.0075	↑ **	2.58	1.10		↑
Idi1	2.23	0.27		↑	1.95	0.60	0.036	↑ *	2.46	0.61	0.0298	↑ *
Ldh-a	1.87	0.19	0.0192	↑ *	2.16	0.23	<0.0001	↑ ****	1.72	0.44		↑
Pgm1	1.33	0.25		→	2.41	1.06		↑	0.97	0.20		→
Phgdh	2.52	0.29	0.0075	↑ **	1.95	0.14	0.0143	↑ *	1.03	0.20		→
Pk	1.27	0.29	<0.0001	↑ ****	5.59	0.21	0.0011	↑ **	1.77	1.09		↑
Slc16a3	2.10	0.75		↑	3.31	1.41	0.0381	↑	1.50	0.15		→
Slc2a1	1.23	0.39		→	2.94	1.66	0.023	↑	1.75	1.06	0.0076	↑ **
Pdk3	2.12	0.24	0.0027	↑ **	1.93	0.41		↑	1.32	0.24		→
Tpi1	2.21	0.19		↑	4.33	1.98	0.0452	↑	0.56	0.34		→

#4 BC, Band cell				#5 PMN, Polymorphonuclear neutrophil			
Fold change (Actin) normalized to WT (ddCt)	SEM	p-value	Trend, significance	Fold change (Actin) normalized to WT (ddCt)	SEM	p-value	Trend, significance
1.44	0.27		→	2.50	0.75		↑
2.07	0.62		↑	2.87	0.99		↑
2.66	0.47		↑	1.23	0.17		→
1.38	0.14		→	1.25	0.04		→
1.33	0.30		→	4.08	1.74		↑
1.33	0.07		→	1.37	0.09		→
1.29	0.07		→	1.22	0.23		→
1.29	0.01		→	1.32	0.10		→
1.25	0.24		→	1.21	0.20		→
1.24	0.16		→	1.70	0.37		↑
5.53	0.08		↑	-	-		
4.13	1.49		↑	2.86	0.49	0.0301	↑ *
1.63	0.74	0.0048	↑ **	1.47	0.43		→
1.54	0.29		↑	4.09	2.06		↑
1.94	0.50		↑	3.26	1.48		↑

Figure 4.4: Statistics of glycolysis gene expression in $Atg7^{-/-}$ primary neutrophil precursors. The fold change +/- SEM, p-value and trends are shown for all glycolytic genes within the 5 populations of $Atg7^{-/-}$ BM neutrophil precursors relative to control (n = 3 mice /group).

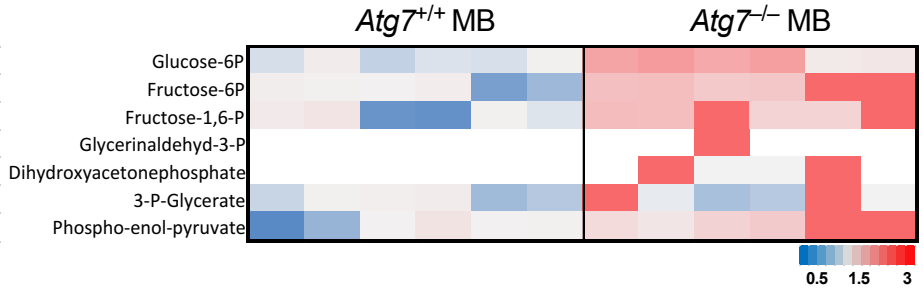

Figure 4.5: Increased glycolytic metabolites in *Atg7*$^{-/-}$ myeloblasts. Glycolysis metabolite abundance for control and CRISPR *Atg7*$^{-/-}$ clones at the myeloblast stage cultured in IL-3 is shown as heatmap of fold change normalized to wild-type abundance. Data represent 6 biological replicates per group from two independent clones. 7 of the 10 glycolytic intermediates were detectable by mass spectrometry and are presented here.

suggesting increased glycolysis or a downstream impairment of metabolite processing that could lead to secondary accumulation of these intermediates.

Since early neutrophil precursors are rare populations (<0.1% of total BM), we could not conduct metabolomic analysis in primary cells, and therefore used fluorescently-labeled glucose uptake instead to confirm the excessive glycolysis *in vivo*. As shown in *Figure* 4.6, *Atg7*-deficient precursors take up more glucose, which is again particularly significant in the early stages MB and MC. This data also confirmed the previously observed general decrease in glycolytic activity during normal differentiation (grey bars). Interestingly, *Atg7*$^{-/-}$ precursors were somewhat able to down-regulate glycolysis, but remained significantly increased in the PMN stage. The fact that MBs already had excessive glycolysis suggests that glycolytic deregulation appears independent of the metabolic switch which occurred around the MM stage (See 4.2.1). Because glycolytic metabolites accumulated in *Atg7*-deficient cells, we investigated the downstream fate of glycolysis-derived pyruvate. Pyruvate can either be converted into Acetyl-CoA from lactate by a LDH-b containing complex and feed into the TCA and OXPHOS, or alternatively can be converted into lactate via LDH-a to regenerate NAD^+ into NADH [263, 264]. The latter pathway is usually active under anaerobic conditions

and is accompanied by lactate secretion [157, 156]. In line with metabolic conversion of pyruvate towards lactate, we found high *Ldh-a* levels and reduced *Ldh-b* isoforms in autophagy deficient precursor stages. The extent of *Ldh*-isoform deregulation correlated well with the increased glycolytic flux measured in earliest MB and MC stages (*Figure* 4.6).

Taken together, autophagy limits glycolysis particularly at the earliest granulocyte differentiation stages based on gene expression data and uptake of labeled glucose *in vivo*. This is supported by the fact that excessive glycolysis was strongest at the stages when autophagy normally is most active. Metabolomic data revealed an increase of glycolytic intermediates, which, based on gene expression data, are re-routed downstream, away from the TCA and towards lactate production. Since mitochondrial oxidative metabolism is a major energy source, 18-times more efficient in producing ATP than glycolysis, we sought to further characterize the energy-metabolic pathways and their contribution to cellular ATP balance during autophagy-deficient granulopoiesis.

4.2.3 Autophagy promotes mitochondrial oxidative metabolism and limits glycolysis to facilitate robust ATP production

Glycolytic genes and glucose uptake were up-regulated in the absence of autophagy, and the expression pattern of *Ldh* isoforms suggested that this may not feed into mitochondrial metabolism, but rather convert towards lactate production. If mitochondrial respiration is impaired, cells can use retrograde signaling to compensate with other energy-producing pathways such as glycolysis [265]. To understand if this is the case in the absence of autophagy, real time *Seahorse* assays were used to measure the flux through glycolysis and oxidative metabolism, respectively. Extracellular acidification rate (ECAR) measures the proton production associated with glycolytic metabolism, while oxygen consumption rate (OCR) is indicative of mitochondrial oxygen-consuming energy production [266]. Freshly isolated $Atg7^{-/-}$ $Ly6G^{+}$

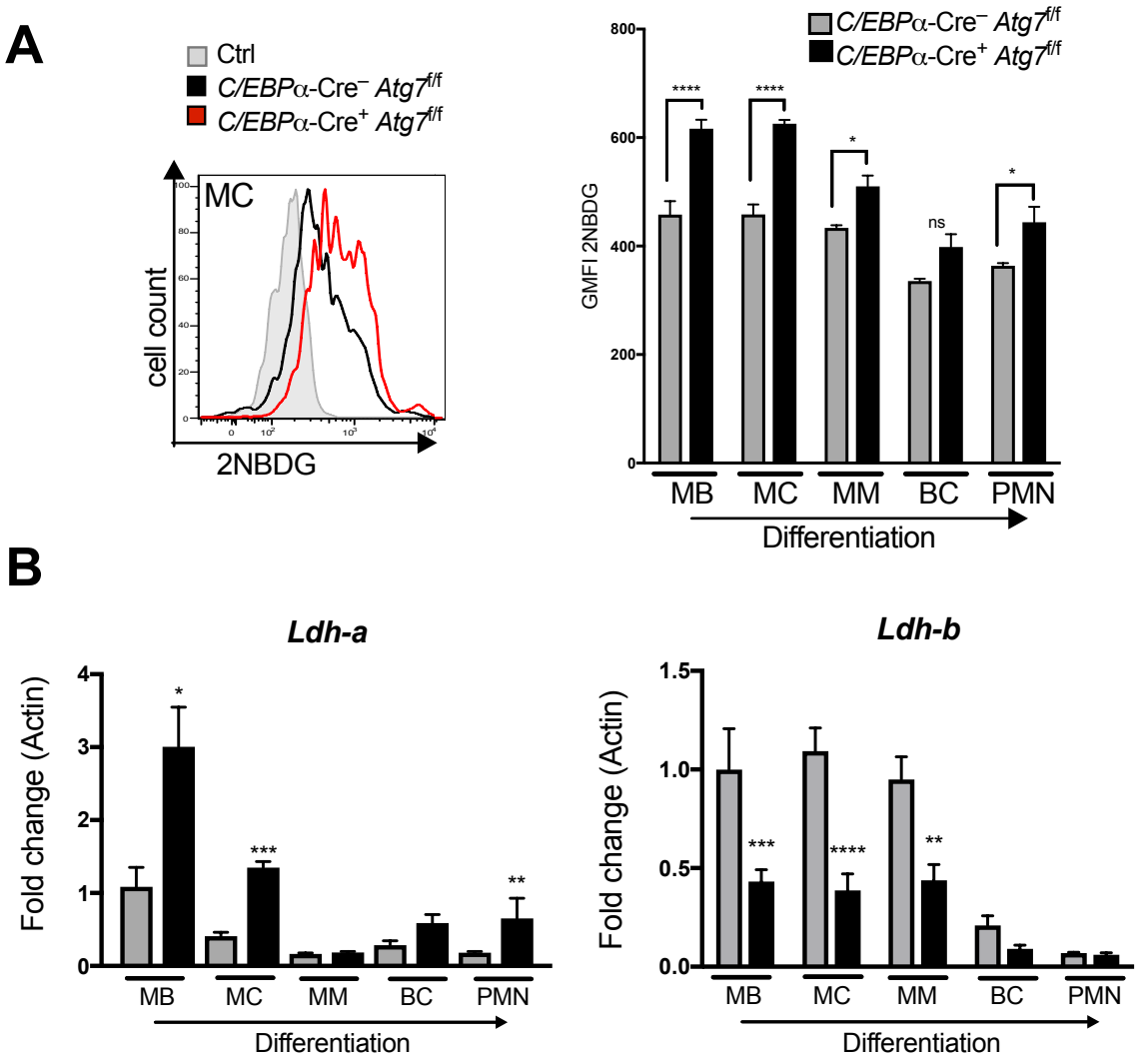

Figure 4.6: Excessive glycolysis during *Atg7*-deficient granulopoiesis. Uptake of fluorescent glucose (2NBDG) during control and *C/EBPa-cre x Atg7*$^{f/f}$ neutrophil differentiation. Representative histogram (left) shows myelocyte (MC) population. Quantification (right) as geometric MFI +/- SEM (n = 3 mice/group) from three individual experiments. *p < 0.05, ****p < 0.0001, tested by two way Anova (A). Gene expression of the glycolysis promoting lactate dehydrogenase-a (*Ldh-a*) and the OXPHOS-promoting lactate dehydrogenase-b (*Ldh-b*) isoforms as fold change +/- SEM in the indicated BM neutrophil precursor populations from control or *Atg7*$^{-/-}$ mice (n = 4 mice/group).

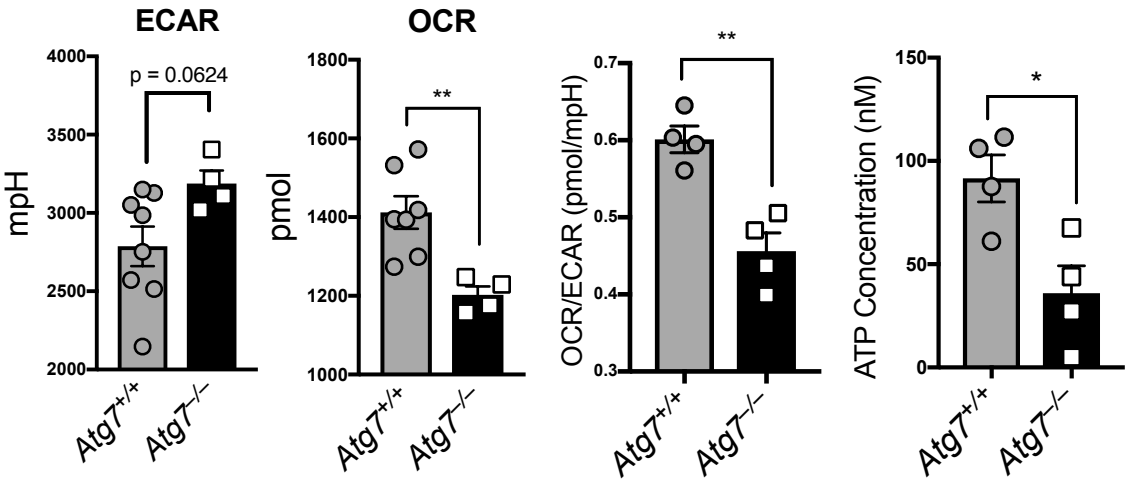

Figure 4.7: Autophagy promotes mitochondrial oxygen consumption and restricts glycolysis to facilitate ATP production. Glycolysis and OXPHOS rates measured over 80 min as extracellular acidification (ECAR) and oxygen consumption (OCR) respectively from $Atg7^{-/-}$ and control $Atg7^{+/+}$ freshly isolated Ly6G$^+$ BM neutrophils. The ratio of OCR/ECAR was measured every 20 minutes and is shown +/- SEM (n = 2 mice/group). Data representative of two experiments. ATP levels were measured with a luciferase-assay from 1x10^6 freshly isolated Ly6G$^+$ BM neutrophils from control and $Atg7^{-/-}$ mice (*C/EBPa-cre x* $Atg7^{f/f}$, n = 4 mice/group) and concentrations determined based on a standard curve.*p < 0.05, **p < 0.01. Grateful acknowledgement to Felix Richter for carrying out ATP measurements.

neutrophils from BM had significantly reduced OCR, and a mild increase of ECAR within the timeframe measured. The ratio of these two measurements is used as an indicator of fuel choice, and was significantly skewed towards glycolysis in $Atg7^{-/-}$ cells. In line with the decreased mitochondrial engagement in the absence of autophagy, cellular ATP content was significantly decreased at steady state as measured by an enzymatic, luciferase-based assay (*Figure* 4.7). While this data confirmed the metabolic re-wiring from OXPHOS towards glycolysis and suggested an underlying ATP crisis, a non-glycolytic acidification or non mitochondrial oxygen consumption cannot be excluded in these steady state measurements.

A more informative, kinetic stress-test response of glycolysis and OXPHOS can be obtained by the respective *Seahorse* assays after treatment with metabolic modulators

[266]. For glycolysis, culture can be started in a glucose-free medium to measure non glycolytic acidification. Next, addition of glucose quantifies the basal glycolytic rate, after which mitochondrial ATP synthase inhibition with oligomycin quantifies maximal glycolytic rate. The ratio of these two values then determines the glycolytic reserve. Finally, 2-DG mediated hexokinase inhibition abolishes glycolytic flux. Similarly, basal respiration is measured as OCR before mitochondrial ATP-synthase inhibition with oligomycin to determine mitochondrial ATP production. Next, FCCP-mediated disruption of the inner mitochondrial membrane integrity results in maximal oxygen consumption, and finally antimycin A and rotenone will only allow non-mitochondrial oxygen consumption to occur (for example mediated by ROS). Both the basal and maximal glycolysis were significantly increased in $Atg7^{-/-}$ myeloblasts compared to control. Interestingly, wild-type control myeloblasts had similar basal and maximal glycolytic flux, indicating that they were using glycolysis at their maximal capacity. In contrast, $Atg7^{-/-}$ cells had increased basal glycolysis but their glycolytic capacity again exceeded this, which may be mediated by the broad scale up-regulation of glycolytic pathway genes described earlier (See 4.2.2). Similarly to the basal OCR *in vivo*, the basal respiration was decreased in cultured CRISPR-$Atg7^{-/-}$ myeloblasts compared to controls, as was the spare respiratory capacity and mitochondrial ATP production (*Figure* 4.8). Taken together, this data further suggests that a defect in mitochondrial ATP production is underlying the switch to glycolysis in the absence of autophagy. These metabolic flux assays therefore support previous *in vivo* data, but were limited to characterize the myeloblast stage rather than the course of G-CSF mediated differentiation. Therefore, the question remained if the kinetics for excess glycolysis and the metabolic switch are similarly conserved in the *in vitro* culture system as the cells undergo neutrophil differentiation.

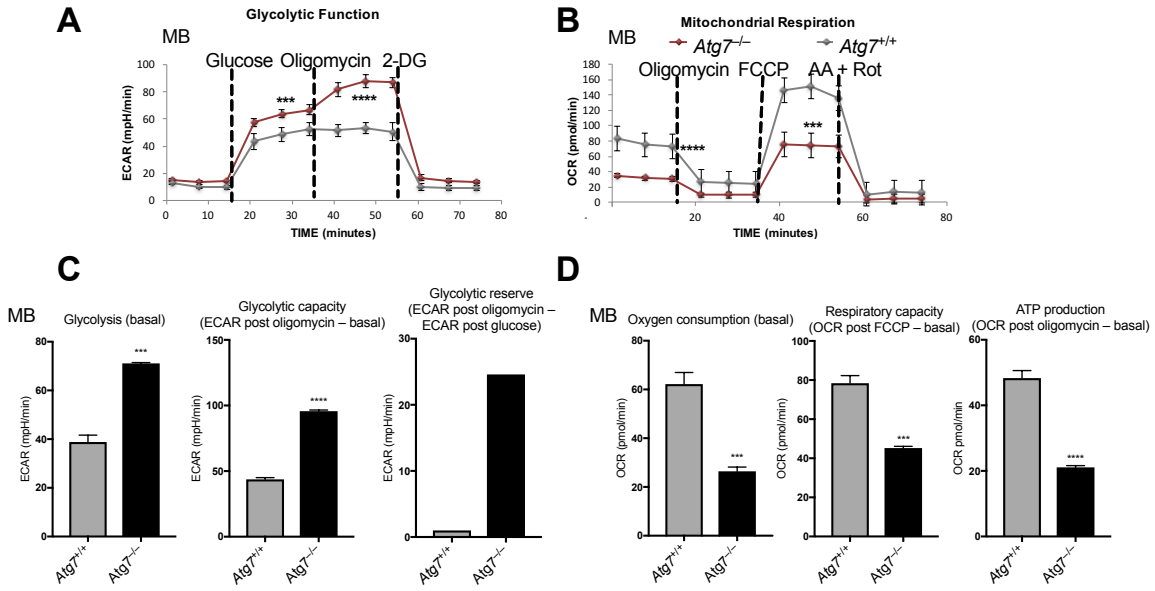

Figure 4.8: Deficient autophagy causes a metabolic switch from oxidative mitochondrial ATP production towards excessive glycolysis. Extracellular Acidification Rate (ECAR) (A) and Oxygen Consumption Rate (OCR) (B) was measured under basal conditions and in response to indicated drugs. Data represent mean of four biological replicates from two independent CRISPR $Atg7^{-/-}$ clones and $Atg7^{+/+}$ controls at myeloblast stage cultured in IL-3. Quantification of glycolytic parameters according to the "Glycolysis-Stress-test analysis package" (Seahorse Bioscience) from (A). Extracellular acidification rate is shown as mpH/min +/- SEM. Quantification of mitochondrial metabolism according to the "Mitochondrial-Stress-test analysis package" (Seahorse Bioscience) from (B). Oxygen consumption rate (OCR) is shown as pmol/min +/- SEM, ***p < 0.001, ****p < 0.0001. (C-D). The Parameters in C+D are calculated according to the equations above each graph based on the data presented in A+B.

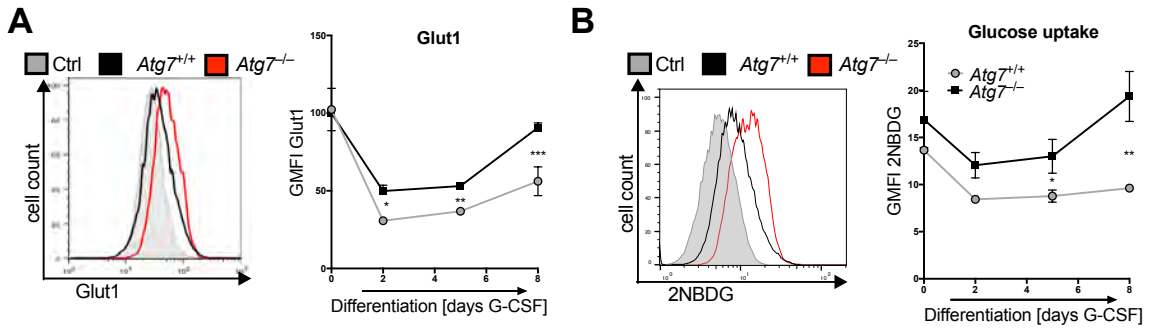

Figure 4.9: Glucose consumption is accelerated during $Atg7^{-/-}$ *in vitro* granulopoiesis. CRISPR $Atg7^{+/+}$ and $Atg7^{-/-}$ myeloblasts (derived from 32D cells) were maintained in the presence of IL-3 (10 ng/µl) or cultured in G-CSF (100 nM) for the indicated duration. A+B) Representative histogram (left) and quantification (right) of Glut-1 expression (A) and 2NBDG uptake (B) as indication of glycolytic activity. Tinted grey histograms are FMO= "fluorescence minus one" baseline FITC fluorescence control. Quantification on the right shows the geometric MFI +/- SEM in $Atg7^{+/+}$ and $Atg7^{-/-}$ cells at indicated timepoints. Data from three independent biological replicates from two experiments, *p $<$ 0.05, **p $<$ 0.01, ***p $<$ 0.001.

4.2.4 Excessive glycolysis in the absence of autophagy is conserved during *in vitro* granulopoiesis

During *in vivo* granulopoiesis in the bone marrow, glycolysis was down-regulated during differentiation, but in the absence of autophagy, precursors engaged increasingly glucose-fueled metabolism. To test if these kinetics are similar during G-CSF induced differentiation of 32D myeloblasts, the expression of the Glut1 glucose uptake receptor and the uptake of fluorescently labeled glucose (2NBDG) were measured over time. Glut1 expression can be quantified with a human T cell leukemia virus (HTLV) -derived Glut1 binding domain fused to a fluorescent reporter via flow cytometry [267, 268]. Both Glut1 and 2NBDG readouts decreased during differentiation in wild-type control cells, similar to the down-regulation of glycolytic flux *in vivo*. Further, both readouts were also significantly increased in $Atg7^{-/-}$ clones, and this phenotype became more significant over the course of differentiation (*Figure* 4.9).

To see if these functional readouts also correlate with glycolytic pathway gene

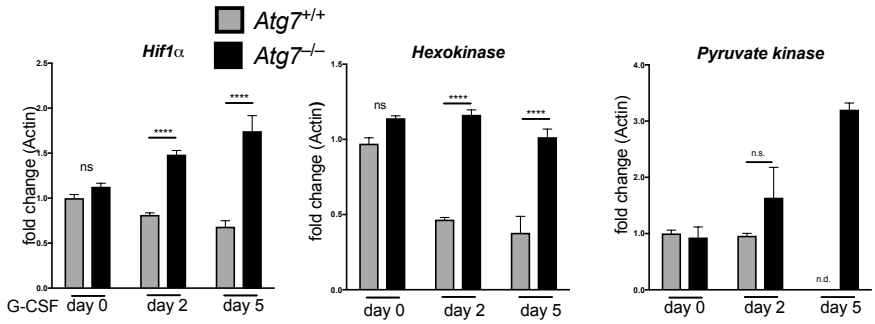

Figure 4.10: Glycolytic gene expression is reduced during normal differentiation but exaggerates in $Atg7^{-/-}$ clones. Gene expression analysis of the indicated glycolytic genes in 200 myeloblasts during G-CSF induced differentiation. Data from four biological replicates measured in technical triplicates in one experiment, representative of two experiments. Fold change +/- SEM of biological replicates is shown normalized to actin relative to day 0 control ($\Delta\Delta$Ct), ****p < 0.0001.

expression as they did *in vivo*, several key glycolytic genes were followed over time. HIF1a is a major glycolysis regulator relevant to the response to hypoxia [269, 270], hexokinase is the committing step of glycolysis [271] and pyruvate kinase is the rate limiting enzyme of the pathway [272]. The expression pattern for all three genes closely correlated with the functional activity of glycolysis during G-CSF mediated differentiation: Whereas the glycolytic genes are down-regulated over time in wild-type controls, the $Atg7^{-/-}$ cells increasingly express significantly elevated levels of all three genes over time (*Figure* 4.10). Because HIF1a was previously found to directly activate hexokinase, pyruvate kinase and lactate dehydrogenase transcription [272], it is likely that the aberrant glycolysis in $Atg7^{-/-}$ cells is at least in part mediated by HIF1a.

The question remained whether increased glycolytic activity and reduced mitochondrial respiration translates into an increased conversion of pyruvate into lactate. This may be expected, because i) lactate production is a hallmark of the Warburg-like metabolism found in $Atg7^{-/-}$ cells and required to replenish the NAD^+ pool to sustain elevated glycolysis in the absence of robust OXPHOS ii) the *Ldh* expression pattern suggested re-direction of pyruvate from the TCA towards lactate and iii) ex-

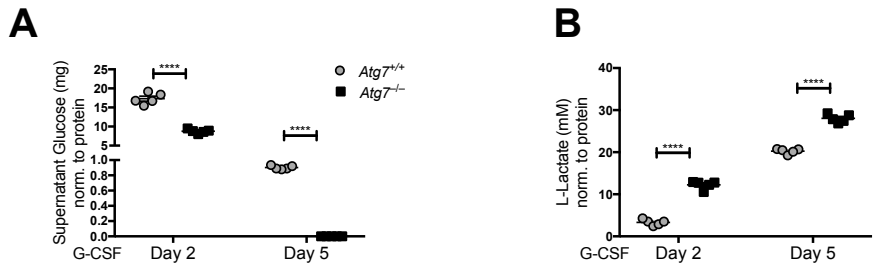

Figure 4.11: Increased glucose uptake and conversion into lactate during *Atg7*$^{-/-}$ 32D myeloblast differentiation. Enzymatic quantification of glucose consumption (A) and lactate accumulation in the supernatant (B) after the indicated time of G-CSF induced neutrophil differentiation of 32D myeloblast precursors. Concentrations were determined based on a standard curve and normalized to protein content of each culture. Grateful acknowledgements to Felix Richter for assay development and measurements.

tracellular acidification in flux assays was elevated in *Atg7*$^{-/-}$cells, which is likely due to the weak-acid property of lactate [266]. As expected, after 2 or 5 days of G-CSF mediated differentiation, there was an increased consumption of glucose from the medium in *Atg7*$^{-/-}$ cultures, which correlated well with a significantly increased production of lactate at both time points (*Figure* 4.11). Thus, the glycolysis-limiting function of autophagy is conserved during *in vitro* granulopoiesis of 32D myeloblasts and characterized by a progressive metabolic switch towards HIF1a-driven glycolysis and lactate production. Importantly, increased glycolysis can be by itself sufficient to decrease the differentiation potential of hematopoietic and embryonic stem cells by affecting epigenetic regulation [243, 273, 274]. Alternatively, glycolysis is often induced as a secondary and compensatory mechanism in the context of mitochondrial defects through ROS-mediated stabilization of HIF1a and other mechanisms [152]. Because of the described mitochondrial impairment, the second alternative appears likely, but raises the question about the cause of the mitochondrial dysfunction.

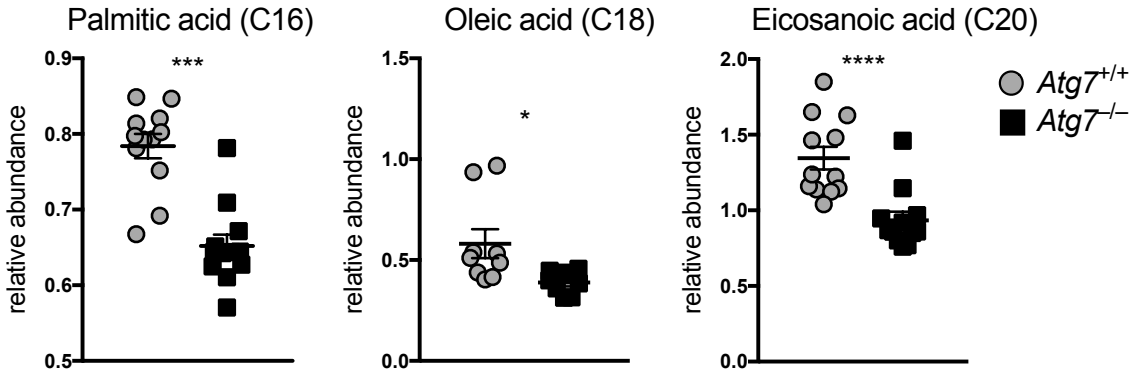

Figure 4.12: Free fatty acid abundance is decreased in the absence of autophagy. Free fatty acid levels in $Atg7^{+/+}$ and CRISPR-$Atg7^{-/-}$ clones were quantified by GCxGC/MS. Data represent 5-6 biological replicates per genotype from two independent clones. *p $<$ 0.05, ***p $<$ 0.001, ****p $<$ 0.0001. Grateful acknowledgements to Zhanru Yu for help with sample preparation and analysis of mass spectrometry.

4.2.5 Autophagy mediates lipid droplet breakdown into free fatty acids for mitochondrial respiration

During mitochondrial respiration, TCA-cycle generated metabolites help to build up a proton gradient along the inner mitochondrial membrane that drives the electron transport chain for ATP production. For this, the TCA cycle is fueled mainly by glucose derived pyruvate/Acetyl-CoA, fatty acid derived Acetyl-CoA, or amino acid derived α-ketoglutarate. Given that glycolytic intermediates were re-routed away from the TCA cycle and reduced during differentiation, we next investigated ß-oxidation derived contribution, one of the main sources of TCA-driving metabolites. ß-oxidation of lipids requires their breakdown by either cytoplasmic lipases or lysosomal lipases into free fatty acids. Autophagy can engulf lipid droplets for delivery to lysosomal breakdown, a process termed lipophagy [275, 276, 277]. Using GCxGC/MS based lipidomics, a broad decrease in various types of free fatty acids was detectable in IL3-cultured $Atg7^{-/-}$ myeloblasts compared to control myeloblasts. Both saturated fatty acids (palmitic acid) and unsaturated fatty acids (oleic acid) were reduced (*Figure* 4.12).

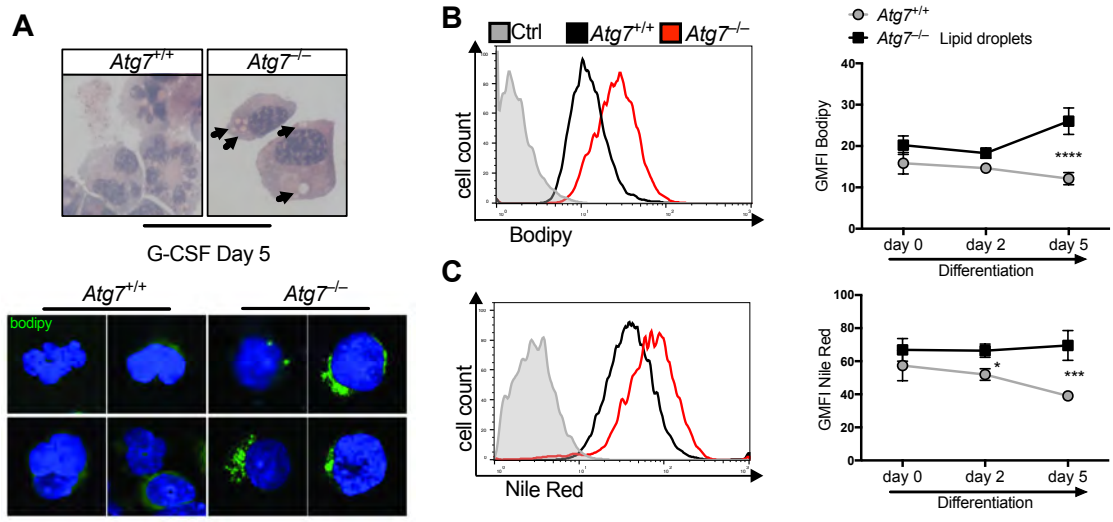

Figure 4.13: Progressive accumulation of lipid droplets during autophagy deficient granulopoiesis. Representative Wright-Giemsa stained cytospins (top) and confocal micropgraphs (bottom) showing lipid droplets stained with bodipy 493/503 (1ng/ml) in $Atg7^{+/+}$ and CRISPR-$Atg7^{-/-}$ clones after culture in G-CSF (100 nM) for 5 days, arrows indicate lipid droplets (A). Representative histograms (left) and geometric MFI (right) of (B) bodipy and (C) Nile red lipid-droplet dye in $Atg7^{+/+}$ and $Atg7^{-/-}$ myeloblasts after the indicated duration of G-CSF (100 nM) induced differentiation. Histograms are from day 5, grey is unstained control from day 0. *p $<$ 0.05, ***p $<$ 0.001, ****p $<$ 0.0001, tested by two-way Anova.

This depletion could be caused either by increased downstream breakdown of fatty acids to feed into the TCA cycle or by a defect in the generation of free fatty acids from lipid droplets. To test this, lipid droplets were quantified during control and $Atg7^{-/-}$ neutrophil differentiation in culture. After 5 days of G-CSF mediated differentiation, few lipid droplets were observable in wild-type cells, whereas $Atg7^{-/-}$ cells were filled with Giemsa-negative vesicles which stained positive for the lipid droplet-dye bodipy. Over the course of differentiation, lipid levels steadily decreased, with lowest levels in PMNs, while they instead progressively accumulated during autophagy deficient granulopoiesis, as measured by bodipy flow cytometric quantification. These results were confirmed by nile red staining, which binds neutral lipids and also cholesterols (*Figure* 4.13).

If a defect in autophagy-mediated free fatty acid generation is underlying this

accumulation of lipids, it should also be observable in primary BM-derived neutrophil precursors. To test this, isolated Ly6G$^+$ BM cells from *Atg7*$^{-/-}$ mice were stained with the nile red and bodipy lipid dyes and prepared for confocal microscopy. Large droplets of lipid were readily detectable in *Atg7*$^{-/-}$ cells and significantly increased in numbers compared to wild-type control. To exclude that this is an *Atg7*-specific effect, another essential autophagy gene, *Atg5*, was deleted using the same *C/EBPa-cre* mediated excision. In doing so, a similar increase of lipid droplet quantity confirmed a role for autophagy in balancing intracellular lipid levels (*Figure* 4.14A, B). In a parallel approach as *in vitro*, the kinetics of this accumulation were next examined using flow-cytometry to distinguish BM differentiation stages. Whereas early MB and MC precursors showed a mild increase in lipid levels in *Atg7*$^{-/-}$ mice compared to wild-type littermates, this difference further increased significantly over the course of differentiation with maximal lipid accumulation in the terminally differentiated PMN stage (*Figure* 4.14C). Taken together, this data suggests that autophagy mediated lipolysis (lipophagy) is a major pathway during granulopoiesis that mediates lipid breakdown. Although a contribution of cytosolic lipases may likely enable breakdown of some lipids in the absence of autophagy, the accumulation of lipids and depletion of free fatty acids in *Atg7*$^{-/-}$ mice suggests that autophagy mediates the bulk of lipid processing for ß-oxidation and may underly the mitochondrial defect previously described.

4.2.6 Lipophagy is active during neutrophil maturation in the bone marrow

The gradual and linear accumulation of lipids over the course of granulopoiesis indicates that lipophagy may be continuously active at all precursor stages, which suggests that it´s regulation is somewhat independent of macro-autophagy, which is active at the earliest and most terminal stages only (3.2.4). Lipophagy imaging in primary

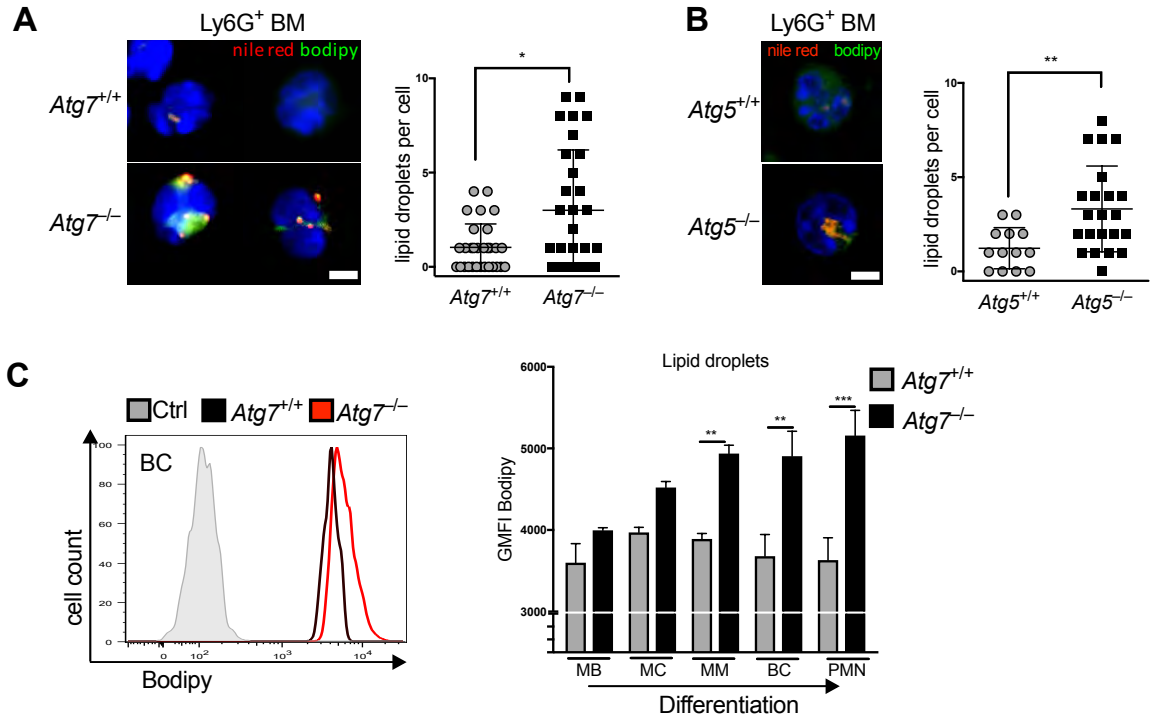

Figure 4.14: Impaired lipid breakdown in the absence of autophagy leads to progressive accumulation of lipid droplets *in vivo*. Nile red$^+$ bodipy$^+$ lipid droplets were quantified by confocal microscopy in isolated total Ly6G$^+$ BM cells from (A) *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ mice or (B) *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ mice (*C/EBPa-cre x Atg5*$^{f/f}$). Representative confocal micrographs (left; scale bar, 5μm) and quantification as lipid droplets/cell +/- SEM (right). Data in (A) is combined from two independent experiments (n=3 mice). *p $<$ 0.05, **p $<$ 0.01. Representative histograms of bodipy fluorescence in *Atg7*$^{+/+}$ and *C/EBPa-cre x Atg7*$^{f/f}$ (*Atg7*$^{-/-}$) precursors (left) and quantification of lipid droplets as the geometric MFI +/- SEM from the indicated *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ BM precursor populations (n=3 mice), representative of three independent experiments (C). **p $<$ 0.01, ***p $<$ 0.001, tested by two-way Anova.

mammalian cells has so far been done by electron microscopy [277]. Here, LC3-GFP transgenic mice were used to co-localize autophagosomes with lipid droplets, which were stained with the novel dye LD540. Because all commercially available lipid dyes exhibit some spectral overlap with GFP, LD540 is a unique dye that can be used to stain lipids and exclude any channel cross-over into the GFP signal [278]. Using this approach in combination with confocal microscopy, we detected autophagosome-lipid droplet interactions in all differentiation stages analysed. LC3-positive autophagosomes were found either in direct contact with lipid droplets or in partial or complete overlap (*Figure* 4.15A). To confirm active lipophagy with the traditional methodology, transmission electron microscopy was conducted on freshly isolated, Ly6G$^+$ BM neutrophils. The formation of double membranes (dm) and membranous structures around lipid droplets (LD) were visualized as previously described [277] (*Figure* 4.15B).

4.2.7 Uptake and synthesis of lipid occurs during granulopoiesis particularly in early precursors

The finding of active and continuous lipophagy during granulopoiesis which, if disrupted, led to significant accumulation of lipids intracellularly, raised the question about the origin of these lipids. Are they continuously synthesized in a futile cycle, or taken up from the extracellular environment? Lipids can either be taken up by plasma membrane transporters such as CD36 [279] or synthesized intracellularly by fatty acid synthase [280]. CD36 was expressed throughout granulopoiesis both in the primary precursor stages as well as during G-CSF mediated differentiation in culture with highest levels at the MB stage and a gradual decrease during differentiation. Similarly, the expression of fatty acid synthase was highest at MB and MC stage and decreased in later stages *in vivo*, which was closely mirrored in cultured 32D myelobasts during G-CSF induced differentiation (*Figure* 4.16).

While CD36 expression is the most common lipid uptake receptor in hematopoi-

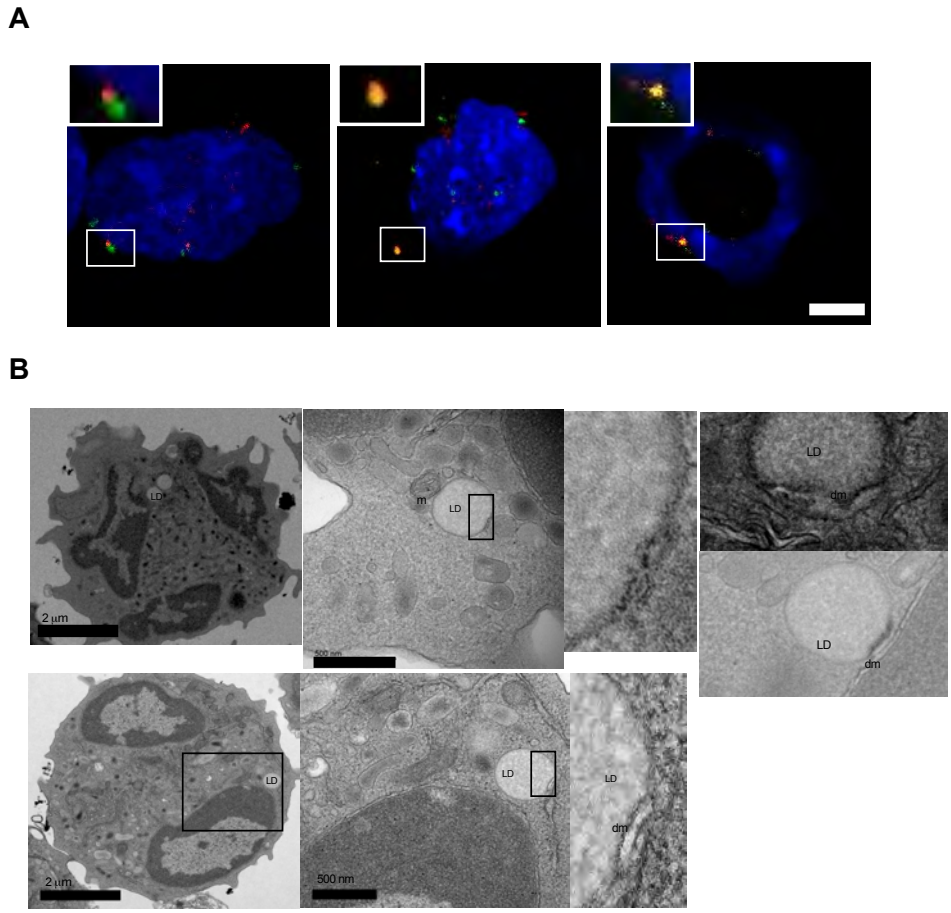

Figure 4.15: Lipophagy is active during granulopoiesis. Neutrophil precursors were purified from bone marrow of LC3-GFP transgenic mice by flow cytometry and stained with LD540 lipid droplet dye. Different representative stages of lipophagy were observed; left, direct contact with partial overlap of lipid droplet and $LC3^{+}$ vesicle; middle, complete co-localization of lipid and LC3 signal; right, dispersed lipid signal around double positive vesicle (A). Neutrophil precursors were purified from bone marrow of wild-type mice by flow cytometry and prepared for electron microscopy to visualize the formation of membraneous structures around lipid droplets (B) (See also [277]). A mix of MM, BC and PMN cells were used in this experiment isolated from BM.

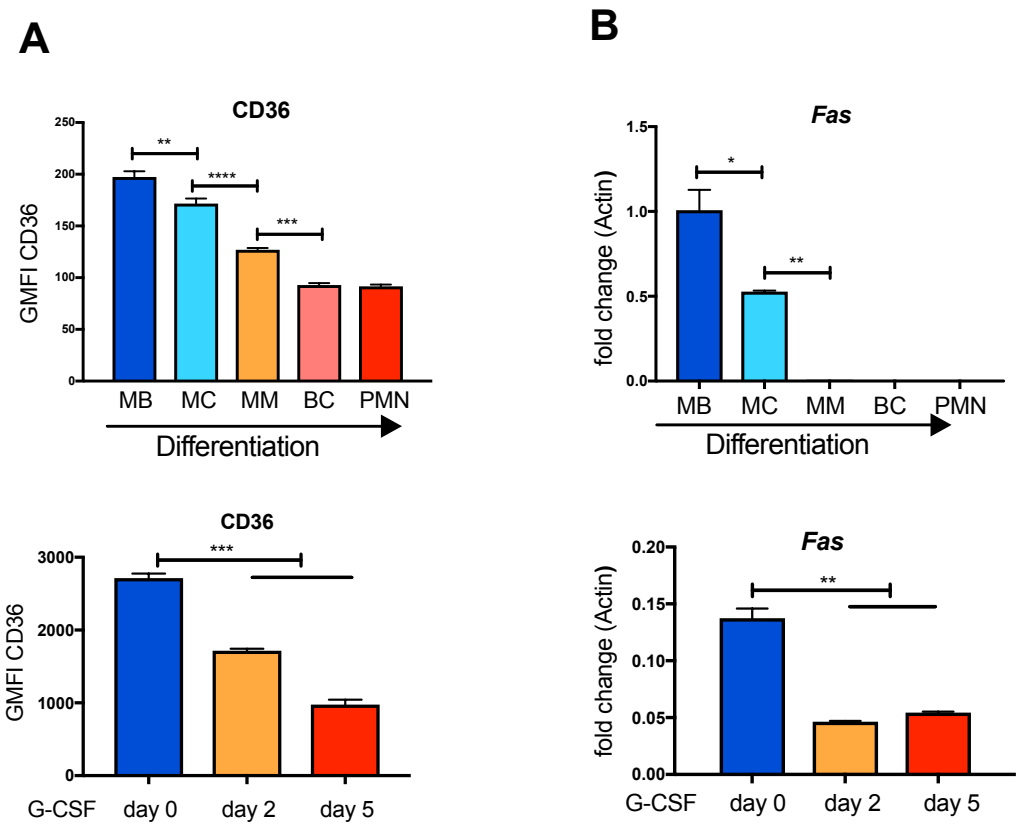

Figure 4.16: Lipid uptake and -synthesis decreases during neutrophil differentiation. Cell surface expression of the lipid uptake receptor CD36 in five consecutive neutrophil differentiation stages from wild-type mouse BM (n=3 mice/group) (top) and during 5 days of G-CSF induced neutrophil differentiation of 32D myeloblast precursors (bottom) (A). Data shown as geometric MFI +/- SEM, significance tested by one-way Anova. **p $<$ 0.01, ***p $<$ 0.001, ****p $<$ 0.0001. Expression of fatty acid synthase (*Fas*) in five consecutive neutrophil differentiation stages sorted by flow-cytometry from wild-type mouse BM (n=2 mice/group) (top) and during 5 days of G-CSF induced neutrophil differentiation of 32D myeloblast precursors (bottom) (B). Data shown as fold change +/- SEM 4 normalized to *Actin* relative to first stage ($\Delta\Delta$Ct), significance tested by one-way Anova. *p $<$ 0.05, **p $<$ 0.01.

etic cells, the possibility remained that other transport mechanisms are in place. To investigate whether CD36 expression correlates with actual lipid influx, we injected the fluorescently labeled lipid bodipy-FL-C16 into mice *i.v.* and measured how much lipid was taken up by individual BM precursor populations 2 hours later. In line with CD36 expression patterns, there was robust and high uptake of lipids in early MB and MC stages, which gradually and significantly declined during differentiation (*Figure* 4.17A). Taken together, this data suggests that the active lipid metabolism during granulopoiesis is supported by continuous uptake as well as synthesis of lipids particularly in early stages of differentiation. The obvious question remained whether inhibition of either uptake or synthesis of lipids in wild-type precursors is sufficient to cause a similar differentiation block as observed in the absence of autophagy/lipophagy. To test this, myeloblast cells were cultured in the presence of the specific Fas-inhibitor C75 [281] or with the CD36 inhibitor SSO [279]. Neither drug was able to inhibit maturation marker expression (*Figure* 4.17B), indicating that there may be sufficient lipid stores in the cell to support differentiation, or indicating redundancy between uptake and synthesis. While co-treatment with both inhibitors could answer this latter question, it caused significant apoptosis in the timeframe of this experiment. Taken together, while both synthesis and uptake of lipids occur at high rates in early precursors, inhibition of either is not sufficient to cause a similar differentiation block as observed in the absence of autophagy. One interpretation of these results is that the high levels of lipid acquisition in upstream progenitors results in sufficient lipid stores to support lipolysis and mitochondrial respiration even if further uptake or synthesis are inhibited.

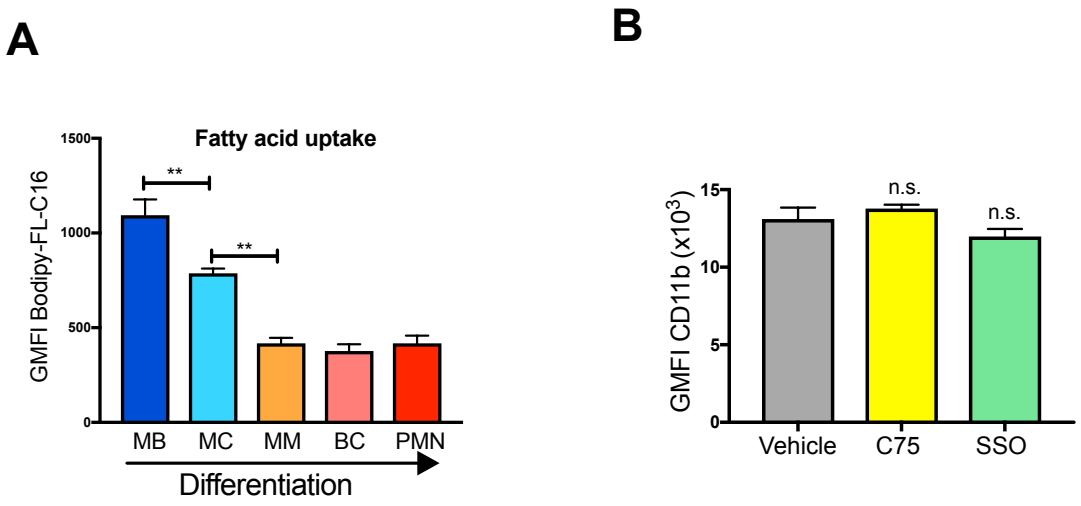

Figure 4.17: Fatty acid uptake occurs but uptake or synthesis are not essential for neutrophil maturation. Uptake of the labelled fatty acid bodipy-FL-C16 in neutrophil differentiation stages from wild-type mouse BM (n=2 mice/group) 2 hours after *i.v.* injection of bodipy-FL-C16 (25 mg/kg) (A). Data shown as geometric MFI +/- SEM, significance tested by one-way Anova. **p $<$ 0.01. Cell surface expression of CD11b maturation marker after 5 days of G-CSF induced neutrophil differentiation of 32D myeloblast precursors in the presence of the specific Fas-inhibitor C75 or the CD36 inhibitor SSO (B). n.s., not significant. Data shown as geometric MFI +/- SEM, significance tested by student´s T-test.

4.3 Discussion

4.3.1 Limiting glycolysis for mitochondrial respiration is mediated by autophagy

While autophagy has gradually become recognized as a central pathway for differentiation of immune cells (and other cells) over the last decade [13, 189], its role in metabolic control of hematopoietic differentiation is a more recent concept that has gained significant traction [87]. In this chapter, we describe a metabolic remodeling during granulopoiesis that limits glycolysis and engages fatty acid oxidation and oxidative mitochondrial metabolism. Deletion of autophagy resulted in excessive glycolysis at the genetic and functional level but impaired mitochondrial respiration. Engaging mitochondrial respiration as a necessary event during granulopoiesis is supported by the requirement for adenylate kinase 2 (AK2) to mediate ADP homeostasis for ATP generation in the inter membrane space of mitochondria [282, 261]. Deleting *Ak2* selectively impaired neutrophil but not macrophage differentiation, although evidence for this was mainly derived from cell line models [282]. Inversely to mitochondrial engagement, glycolytic activity decreased from earliest progenitors to mature neutrophils in the bone marrow, but was increased in the absence of autophagy. Excessive glycolysis as measured by acidification and glucose consumption was previously described in autophagy-deficient macrophage differentiation [167] and induces acquisition of M1-like polarization, which is inherently associated with activated mTOR and glycolysis [283]. In neutrophils however, a predominantly glycolytic metabolism has been described in early studies using human [284] and guinea pig neutrophils [285], particularly during effector functions but also in the resting state [286, 285]. Although these studies also describe oxidative contribution to ATP balance during effector functions, the mainly glycolytic phenotype is in apparent contrast to the decreasing glycolysis observed during granulopoiesis in this study. This difference may be explained by the

peripheral origin (blood or peritoneum) of neutrophils in these studies in contrast to the bone marrow phenotype described here. In keeping with this idea, peripheral sites of infection are generally hypoxic and lead to HIF1a activation [287], and this hypoxic HIF1a induction mediates neutrophil survival [288], enhanced glycolytic metabolism [289] and is essential for neutrophil effector functions [290]. Together, this suggests a second metabolic switch that may occur as cells enter peripheral tissues or encounter pathogens.

Similar to granulopoiesis, iNKT cells undergo a metabolic switch that limits glycolysis during their generation [239]. Particularly the T_H1-biased population of iNKT cells is impaired without autophagy and has constitutively activated mTOR, possibly leading to enhanced glycolysis [236]. Taken together, it is not clear if elevated glycolysis is causative or a bystander of altered lineage commitment in these models, but elevated glycolysis after autophagy impairment also occurred in our study on myeloid leukemic cells driven by the MLL-ENL fusion oncogene [168]. In this system, increased glycolysis was causing excessive proliferation of the undifferentiated, transformed myeloid progenitor cells, and enforcing normal glycolytic activity in autophagy-impaired cells by culture in galactose-medium rescued the aberrant proliferative phenotype. Thus, excessive glycolysis and impaired mitochondrial respiration in response to autophagy deletion is a common feature in myeloid lineages as well as in the innate lymphoid cells that may have direct functional impacts on the cellular phenotypes.

4.3.2 Lipophagy as a relevant pathway during neutrophil differentiation

The mitochondrial OXPHOS during granulopoiesis may be mediated by the continuous lipophagy which occurred during differentiation and was sustained by lipid uptake and synthesis. In line with this, deletion of the lysosomal lipase LAL caused accumulation of immature neutrophil-lineage precursor cells in bone marrow and tissues of mice, suggesting that autophagy may mediate a switch towards mitochondrial respi-

ration and lipid breakdown [291]. Analogously, differentiation into M2 macrophages requires cell intrinsic lipolysis mediated by LAL, which is sustained by uptake of fatty acids from the environment [252]. Although this pathway has not been linked directly to autophagy for macrophage differentiation, deleting *Atg5/7* also impairs lipophagy and blocks M2 polarization. An important activator of autophagy is AMPK, which can induce FAO through its downstream targets including PPARs [292] thereby promoting M2 polarization. Although no role for AMPK has been described in neutrophil differentiation, it regulates their survival by modulating HIF1a stability [293]. Taken together, lysosomal lipolysis may be required for neutrophil differentiation and mediated by autophagy to drive robust FAO, similar to the metabolic circuit relevant to macrophage differentiation, were both pathways were independently required for M2 polarization. In both cell types, evidence that proves this causality is currently lacking, and therefore will be the main focus of the next chapter.

4.4 Conclusion

A number of metabolic assays were employed to identify a metabolic switch during neutrophil differentiation mediated by autophagy that limits glycolysis and engages fatty acid oxidation and mitochondrial ATP production. In macrophages, evidence links autophagy mediated lipolysis to the limitation of glycolysis and mitochondrial ATP production in the context of M2 macrophage polarization, but whether a similar metabolic pathway is relevant to neutrophil differentiation remains to be established.

5 Autophagy-mediated lipolysis provides fatty acids for β-oxidation and oxidative phosphorylation to enable granulopoiesis

5.1 Introduction

The previous chapter introduced the idea that metabolic changes often accompany differentiation or polarization and can be regulated by autophagy, such as in the case of M1/M2 macrophages and innate lymphocyte populations (See 4.1.1 and 4.1.2) and described metabolic states affected by autophagy during neutrophil differentiation. The crucial question remained whether such metabolic changes are essential for differentiation, a consequence of it, or an unrelated secondary phenomenon. In this chapter, research in HSCs and T cells is introduced that proves causative relevance of metabolic states to control differentiation, and establishing causality between autophagy, metabolism and granulopoiesis is also the main goal of experiments presented here.

5.1.1 Metabolic control of HSC self renewal and differentiation

Interestingly, distinct metabolic states of HSCs can affect lineage specification in downstream myeloid progenitors, as presented in a study that found altered lineage commitment between myeloid and erythroid fates depending on glucose and glutamine metabolism activity [243]. The role of autophagy in the differentiation and self-renewal of HSCs has already been discussed previously (See 3.1.3), but more recent literature also describes a metabolic component to this phenotype, that draws important parallels to the neutrophil metabolic experiments in this chapter. While the accumulation of mitochondria has been noted across most studies in response to *Atg7* deletion [294, 164], defective HSC self-renewal was recently proposed to result from

chronic metabolic activation [294]. In this view, bulk degradation of active mitochondria through mitophagy is required to maintain a state of low metabolic activity. Because mitophagy is the only recycling pathway that can selectively remove defective mitochondria, it remains to be established how these mitochondria would maintain functionality. Another study found increased self renewal and defective differentiation of HSCs in response to a mitochondrial defect caused by *Ptpmt1* deletion [295]. The authors noted compensatory glycolysis evident as extracellular acidification, which was not able to restore differentiation but was accompanied by HSC expansion. The latter (but not the former) study therefore fits with the general view that glycolysis is the principal energy-source for HSCs, whereas mitochondrial respiration engagement is required for differentiation [296, 98]. This balance is regulated in part by pyruvate dehydrogenase kinases (PDKs) in HSCs, which phosphorylate PDH and thereby reroute glycolytic metabolites away from TCA cycle and towards lactate production. PDK action therefore enhances HSC glycolysis and self renewal capacity while preventing differentiation [242]. Taken together, self-renewing HSCs are thought to be mainly glycolytic but induce mitochondrial respiration to facilitate differentiation. Autophagy impacts HSC metabolism, self renewal and differentiation, but the cause and consequence relationship between these pathways remains unclear. The fact that altered HSC metabolism can affect downstream lineage choices will be relevant to studying granulopoiesis in this chapter.

5.1.2 T cell metabolism drives differentiation

Further examples where metabolism actively controls differentiation have been discovered in the T cell field. Contrary to most other hematopoietic cells, T cells develop in a unique organ, the thymus. After the common lymphoid progenitor stages, further differentiation requires exit of the bone marrow into the thymus, were the earliest stages are double negative thymic progenitors, which do not express CD4 or CD8 co-

receptors. This is followed by a $CD4^+$ $CD8^+$ double positive stage, and eventually, $CD4^+$ or $CD8^+$ single positive T cells are formed which are released into the periphery [297]. T cell differentiation and lineage commitment involves frequent and rapid changes between metabolic states and relies on functional autophagy in some cases but not others [298]. $CD4^+$ effector T_H1, T_H2, and T_H17 cells (T_{eff}) and $CD4^+$ regulatory T cells (T_{reg}) are maybe the best-defined $CD4^+$ T cell subsets at the metabolic level [160]. Proliferating $CD4^+$ T_H1, T_H2 and T_H17 effector cells primarily use glycolysis, while the longer lived, quiescent T_{reg} and T_{mem} cells use FAO and OXPHOS to support their catabolic lifestyle, until they are re-challenged with antigen for recall response [298, 299]. Supporting a causative role of metabolism in the control of T cell differentiation, highly glycolytic effector T_H17 cells can be reprogrammed to differentiate towards regulatory T cells (which preferentially use OXPHOS) simply by inhibiting glycolysis with 2-deoxyglucose [300], whereas mTOR activation exacerbates glycolysis and negatively affects T_{reg} differentiation and maintenance [301, 170]. Like T_{reg} cells, $CD8^+$ T_{mem} cells have enhanced respiratory capacity, abundant electron transport chain proteins and high mitochondrial content [302, 246]. The mitochondria in $CD8^+$ T_{mem} cells are present as an elongated and highly fused network mediated by the action of OPA1 (mitochondrial dynamin like GTPase), and enforced fusion of mitochondria imposes a T_{mem} phenotype by augmenting OXPHOS [246]. Conversely, inducing mitochondrial fission by knocking out OPA1 greatly reduces OXPHOS activity and is sufficient to impair $CD8^+$ T_{mem} generation, while cytotoxic T lymphocytes are unaffected [246]. In order to fuel this elongated, efficient mitochondrial network, free fatty acids must either be generated by lipolysis or taken up exogenously. Tissue-resident $CD8^+$ T_{mem} are highly dependent on lipid uptake and transport molecules such as FABP4 and FABP5 (fatty acid binding protein 4/5), as deleting the corresponding genes results in impaired mitochondrial respiration and specific loss of this population *in vivo* [303]. In contrast, central $CD8^+$ T_{mem} seem to adopt another strategy, using

cell intrinsic lipolysis of lipid droplets to generate free fatty acids from triacylglycerol [304]. Deleting the lysosomal lipase LAL accordingly results in decreased $CD8^+$ T_{mem} but normal effector T cells in mice [304]. Although no link was made to autophagy in this context, it is likely that lysosomal lipid droplet breakdown was mediated by the autophagic machinery, as previously hypothesized for macrophage differentiation (see 4.3.2). Lipophagy is a universal pathway in mammalian cells working in parallel with cytoplasmic lipases to provide free fatty acids for energy homeostasis and metabolic intermediates. While lipid-binding proteins usually shield the droplet from cytosolic lipase digestion, the autophagic machinery can robustly make a large amount of lipids available for degradation in times of metabolic demand to fuel mitochondrial respiration. In line with this idea, chaperone-mediated autophagy is able to remove the protective perilipins PLIN2 and PLIN3, which is followed by enhanced lipophagy and lipase activity [305]. The CMA-dependent degradation of lipid droplet-associated proteins is induced by AMPK and precedes and facilitates lipolysis, demonstrating the existence of an intimate crosstalk between different forms of autophagy in the control of intracellular lipid homeostasis [306]. Since lipophagy depends on the core autophagy machinery, knockout of *Atg7* will disrupt this pathway, and accordingly a strong phenotype of defective $CD8^+$ T_{mem} formation and maintenance has been reported in this context [165, 169]. Since both of these studies also found elevated compensatory glycolysis in the absence of autophagy, autophagy may be the upstream pathway controlling the metabolic switch towards OXPHOS and FAO in $CD8^+$ T_{mem} by enabling large-scale lysosomal lipolysis. T_{reg} formation mirrors many metabolic features of T_{mem} generation, such as decreased glycolysis and engagement of fatty acid metabolism and OXPHOS [307, 308]. T_{reg}cells also rely on autophagy for their formation and maintenance. Similar to other cell types, autophagy limits excessive glycolysis in T_{reg}cells [170, 166]. However, it remains to be established whether the autophagy machinery is the upstream pathway in both T_{mem} and T_{reg}cells that enables the switch

towards mitochondrial respiration. Although these studies used T cell-specific promoters to delete *Atg* genes (*Cd4-cre*, *Foxp3-cre*) as well as chimeric models to ensure that the differentiation defect is cell-intrinsic, an additional cell-extrinsic influence on T cell differentiation has to be considered. Autophagy limits pro-inflammatory cytokine production and secretion by restricting innate immune sensing mechanisms such as inflammasomes in antigen-presenting cells [309]. Autophagy-deficiency in antigen-presenting cells may also lead to an overall pro-inflammatory environment skewing T cell fate towards pro-inflammatory cell subsets such as T_H17 cells [310]. Taken together, lipid and mitochondrial metabolism is intimately linked to autophagy and can actively regulate the differentiation of T cell lineages. The following chapter will attempt an analogous approach by modulation of the FAO-OXPHOS pathway in the context of autophagy and investigate its effect on neutrophil differentiation.

5.1.3 Context and aim of this chapter

The data presented in the previous chapter identified a metabolic switch during granulopoiesis and characterized the metabolic events that are dependent on autophagy. The logical continuation of this is to investigate if these metabolic events are crucial and rate limiting for differentiation, or a bystander effect, being a consequence of the differentiation. While a bystander or consequence scenario of metabolism during differentiation has been proposed for a long time [159], recent discoveries in the T cell and stem cell fields, introduced above, have identified a central role for metabolic pathways to actively control and fine tune differentiation, which may be linked to autophagy. Because metabolic circuits such as the metabolic switch and glycolytic phenotype of *Atg7*$^{-/-}$ neutrophils described previously are also evident in *Atg7*$^{-/-}$ T cell subsets [165, 166] and HSCs [294, 171, 164], it is worthwhile to study if metabolic states are sufficient to facilitate differentiation in neutrophils. The experiments in the following chapter aimed at exploring to what degree the metabolic regulation via autophagy is

essential for the differentiation defect in neutrophils and to explore avenues to correct this deficit in granulopoiesis. Preliminary data obtained in HSCs and T_{reg}cells aims to evaluate the broader relevance of such metabolic circuits.

5.2 Results

5.2.1 Lipase inhibition or FAO impairment is sufficient to cause lipid accumulation

Data from previous chapters point towards a role for lipid metabolism in the cause for the differentiation defect of autophagy deficient neutrophils. Because there are no models that delete lipophagy without disrupting general autophagy [275], it is however difficult to dissect the lipid-specific contribution to this phenotype. To address this, the relevance for lipid breakdown and metabolism was instead investigated in autophagy competent cells. If the lipid-metabolic defect caused by autophagy-deletion leads to the differentiation block, then it may be possible to copy this phenotype by disrupting only such lipid metabolic pathways. We therefore used wild type myeloblasts and tested if lipase inhibition alone may be sufficient to cause a similar phenotype, i.e. accumulation of lipid, defective neutrophil differentiation and a switch towards glycolytic metabolism. Lipid breakdown may either be mediated by cytosolic lipases, lysosomal lipases, or hydrolysis in peroxisomes [311]. Diethyl-umbellifery-phosphate (DEUP) is a pan-lipase inhibitor that targets both cytoplasmic and lysosomal lipases [312]. Orlistat is an irreversible, selective inhibitor of serine-containing active site triglyceride lipases, thus inhibiting lysosomal lipases with some specificity [313]. Etomoxir (2[6(4-chlorophenoxy)hexyl]oxirane-2-carboxylate) is an irreversible inhibitor of carnitine palmitoyltransferase-1 on the outer face of the inner mitochondrial membrane [154] and therefore blocks the uptake of free fatty acids into mitochondria for subsequent FAO. While DEUP and orlistat target lipid breakdown, etomoxir inhibits

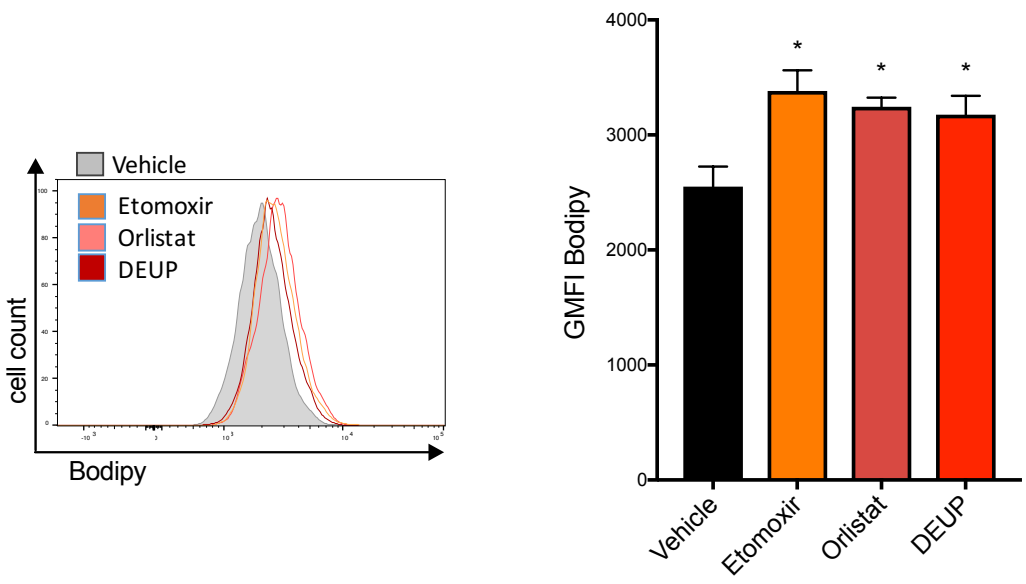

Figure 5.1: Lipid droplet content increases after lipase inhibition or FAO inhibition. Representative bodipy histogram (left) and bodipy quantification as geometric MFI +/- SEM (right) after the indicated drug treatments for five days during G-CSF induced differentiation of 32D progenitor lines (for 5 days, equals PMN stage) confirmed the accumulation of lipid droplets.

their downstream uptake and processing. To test if these drugs work on target and as expected, total cellular lipid content was measured in response to drug treatment by bodipy flow cytometry. All three drugs led to a significant increase in the neutral triglyceride and cholesterol content during G-CSF mediated differentiation (*Figure* 5.1).

To visualize directly the abundance and size of lipid droplets, bodipy fluorescence was used to mark lipid droplets for confocal microscopy. An obvious increase in both size and abundance of lipid droplets was readily observable (*Figure* 5.2), which confirms the previous flow cytometric quantification. Notably, the lipid droplets were often spatially enriched in sub-cellular cytoplasmic compartments, which may be due to their interaction with other organelles such as the ER [314]. The accumulation of cytosolic lipid droplets in response to all three inhibitors suggests that these drugs are acting on-target and are effective over the timeframe of these experiments, so that

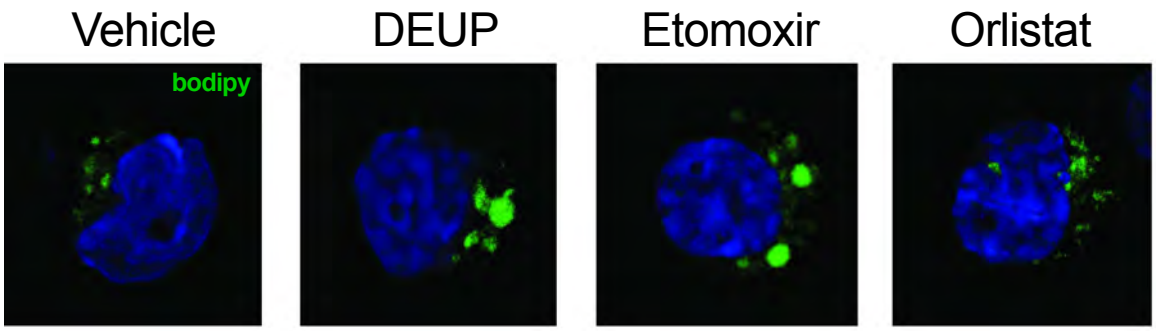

Figure 5.2: Lipid droplet size and number increases in response to lipase inhibition or FAO inhibition. Confocal micrographs of bodipy^{+} lipid droplet accumulation in response to the indicated drug treatments for five days during G-CSF induced differentiation of 32D myeloblasts.

their effect on metabolism and differentiation can be explored next.

5.2.2 Lipase inhibition shifts metabolism towards glycolysis

The notion that FAO can inhibit glycolysis is well established as part of the Randle cycle [315], which is mediated by regulatory effects of pyruvate dehydrogenase and citrate on glycolytic enzymes [316]. In this light, it is possible that the excessive glycolysis in autophagy deficient neutrophils is caused by decreased FAO/OXPHOS, resulting in compensatory glycolysis to maintain ATP balance, as previously suggested [152, 317] (See 4.6). Indeed, impaired fatty acid oxidation was reported to lead to excessive glycolysis via falling NADH and acetyl-CoA levels [316].

To test if lipase inhibition causes a metabolic shift towards glycolysis similar to autophagy deletion, myeloblasts were differentiated in the presence of orlistat or DEUP and uptake of fluorescent glucose (2NBDG) was measured by flow cytometry. Both inhibitors led to a significant increase in glucose utilization, which was strongest after pan-lipase inhibition by DEUP (*Figure* 5.3). This suggests that defective lipolysis may be in common to cause the shift to glycolytic metabolism both in *Atg7*$^{-/-}$ cells or after lipase inhibition.

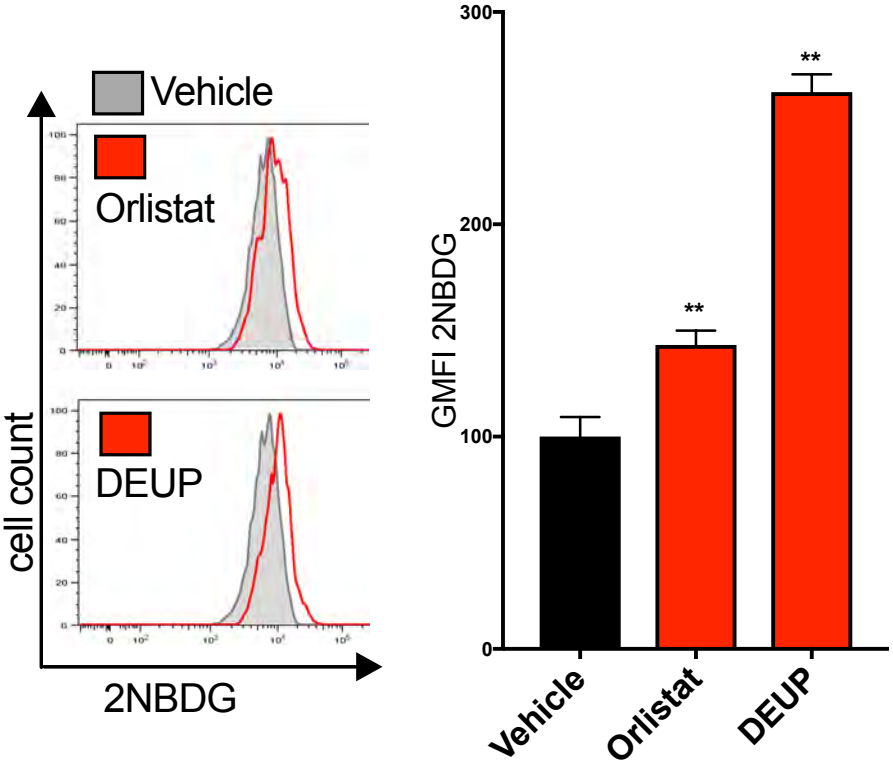

Figure 5.3: Lipase inhibition is sufficient to cause a glycolytic switch. Wild type myeloblasts were treated with LAL-inhibitor orlistat (100 µM), lipase inhibitor DEUP (5 µM), or vehicle control during 5 days of G-CSF induced differentiation. Data representative of three experiments. Histograms (left) and quantification (right) of fluorescent glucose (2NBDG) uptake as geometric MFI +/- SEM from four biological replicates on day 5 of G-CSF treatment. *$p < 0.05$, ***$p < 0.01$.

5.2.3 Lipase- or FAO inhibition impairs neutrophil differentiation

Next, we tested if impairment of lipid catabolism would also impair neutrophil differentiation in a similar manner as autophagy deletion. Engaging fatty acid oxidation and mitochondrial respiration as a necessary event during granulopoiesis is supported by previous studies of the mitochondrial adenylate kinase 2 (AK2) which is important for ATP generation to support granulopoiesis [282, 261]. We incubated wild type myeloblasts with lipase inhibitors orlistat and DEUP or FAO-inhibitor etomoxir during G-CSF mediated differentiation and quantified the maturation marker CD11b by flow cytometry. All three conditions led to a significant and similar decrease in maturation marker expression. Although DEUP mediated pan-lipase inhibition showed stronger glycolytic activation compared to orlistat, which preferentially inhibits lysosomal lipases (*Figure* 5.3), the effect on differentiation was comparable (*Figure* 5.4). This suggests that fatty acids generated by cytosolic lipases may serve other functions than supporting differentiation. In line with this, cytoplasmic lipases such as hormone sensitive lipase (HSL) primarily hydrolyze diacylglycerol, whereas lysosomal lipophagy may lead to complete breakdown into glycerol and free fatty acids for ATP generation [318] .

CD11b maturation marker expression is a good indicator for neutrophil maturation in this system, as there are no other lineages such as macrophages that develop under these culture conditions [182]. Nevertheless, autophagy could impact on receptor internalization leading to artificially low CD11b abundance independent of a differentiation defect [167]. Therefore, we also tested morphological maturation as judged by nuclear lobularization and cytoplasmic granule expression. In response to all three inhibitors (Etomoxir, orlistat, DEUP), cells maintained almost exclusive myeloblast morphology characterized by a circular nucleus and large nucleus:cytoplasma ratio. The accumulation of large non staining lipid droplets (black arrows) further confirms that the drugs are acting on target (*Figure* 5.5). Taken together, lysosomal lipid degradation and the

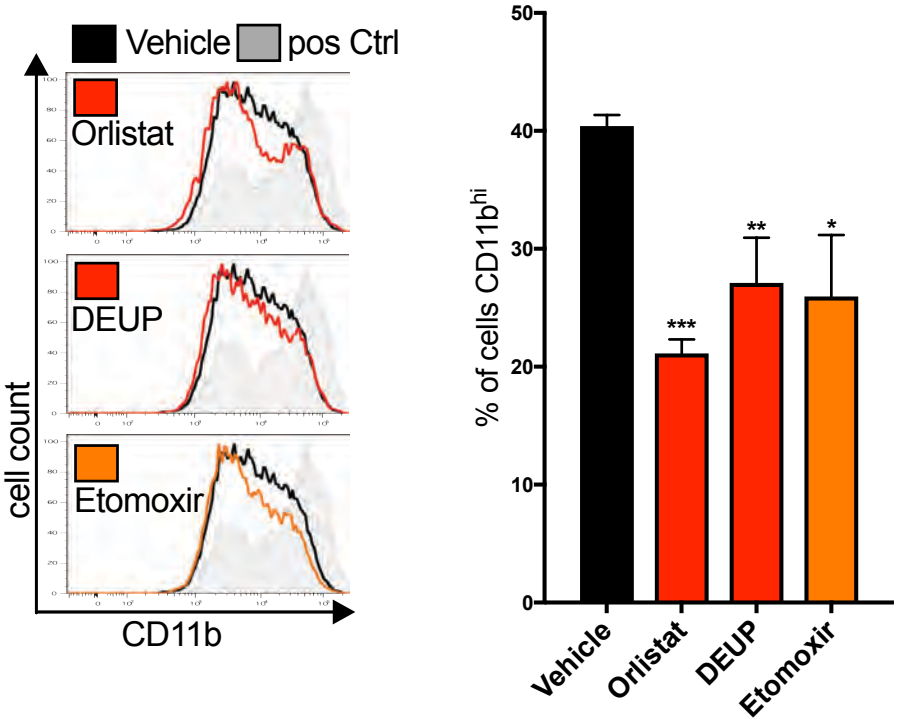

Figure 5.4: Lipase or FAO inhibition is sufficient to block neutrophil differentiation. Wild type myeloblasts were treated with LAL-inhibitor orlistat (100 µM), lipase inhibitor DEUP (5 µM), FAO-inhibitor etomoxir (60 µM) or vehicle control during 5 days of G-CSF induced differentiation. Histograms (left) and quantification (right) of CD11b maturation marker expression on day 5 of G-CSF induced differentiation. Ctrl in grey are mature control cells (G-CSF day 7). Quantification shown as mean % of cells (+/– SD) $CD11b^{high}$ from four biological replicates, representative of three independent experiments.

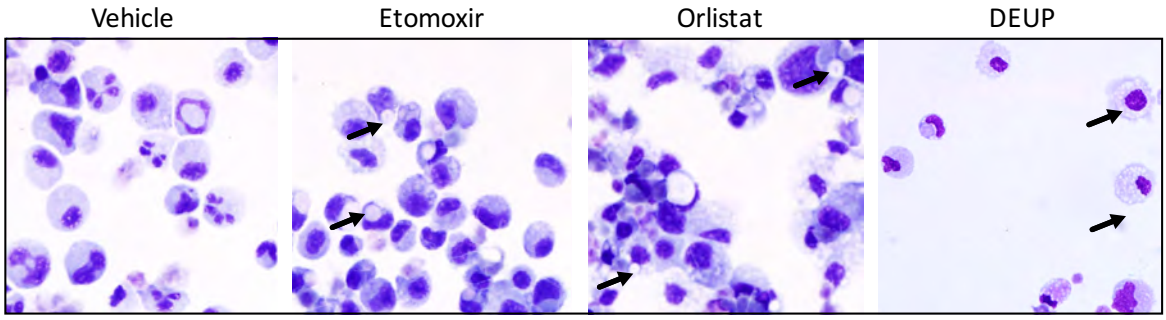

Figure 5.5: Lipase or FAO inhibition is sufficient to block neutrophil morphological maturation. Wright-Giemsa stained cytospins following inhibitor treatment over 5 days of G-CSF induced differentiation. Arrows indicate lipid droplets.

subsequent oxidation of fatty acids is required for neutrophil differentiation *in vitro*, while cytoplasmic lipases may also be actively degrading lipids for other purposes than differentiation.

Although this suggested that, in contrast to lysosomal lipolysis, cytoplasmic lipases do not directly affect differentiation, we next tested if atglistatin, a selective small molecule inhibitor for the rate limiting cytosolic triglyceride lipase ATGL [319], would affect neutrophil differentiation. During five days of G-CSF mediated differentiation, atglistatin had no significant effect on morphological differentiation at the highest dose that did not affect viability over the period of the experiment (*Figure* 5.6). This suggests that, in contrast to lysosomal lipolysis, cytoplasmic lipases are not required for neutrophil differentiation.

5.2.4 Autophagy deficient neutrophil differentiation is rescued by free fatty acids

Because lipase or FAO inhibition was sufficient to block neutrophil differentiation, we next tested if conversely, restoration of this pathway in autophagy deficient precursors may restore neutrophil differentiation and metabolism. As the compensatory glycolysis in $Atg7^{-/-}$ cells previously served as a good readout for the aberrant metabolic rewiring, we first treated $Atg7^{-/-}$ myeloblasts with free fatty acids during G-CSF

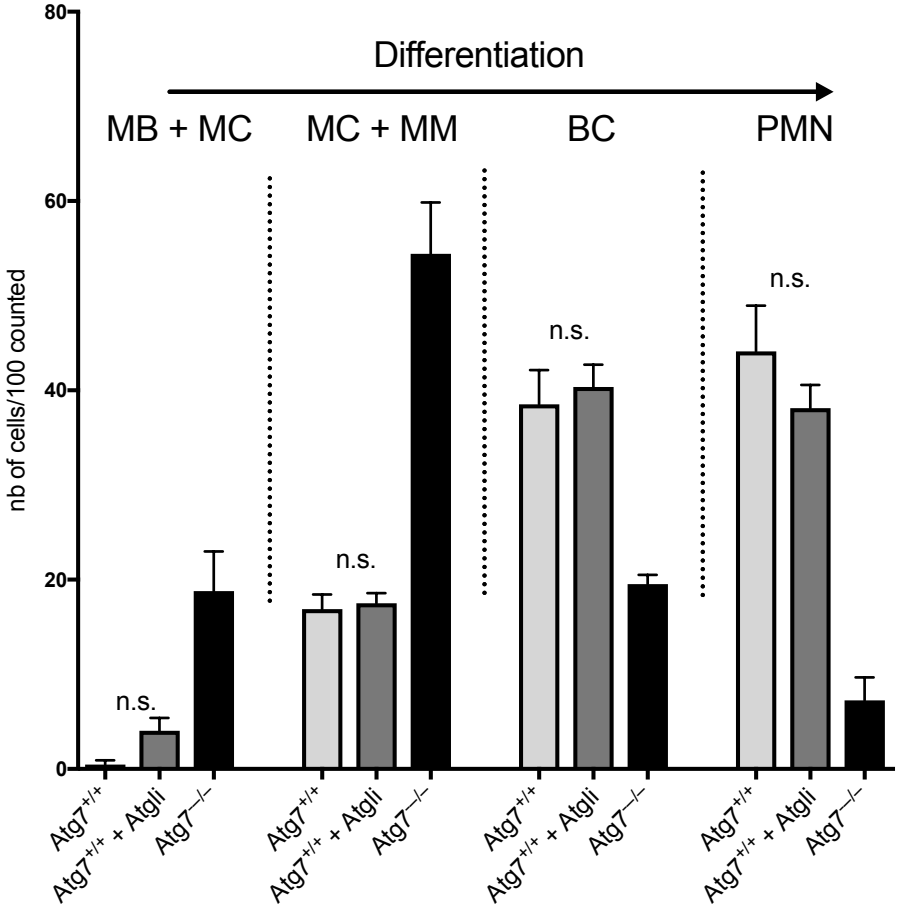

Figure 5.6: Cytoplasmic lipases are not required for differentiation. Wild type myeloblasts were differentiated in the presence or absence of the cytosolic lipase inhibitor atglistatin (Atgli) with G-CSF for 7 days. *Atg7*$^{-/-}$ for comparison. Data shown as number of cells in each morphological group per 100 cells counted +/- SEM. n.s., non-significant.

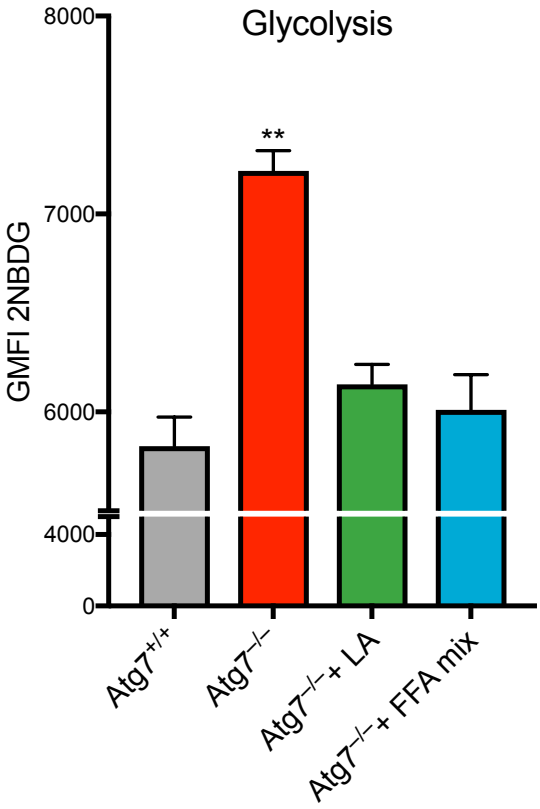

Figure 5.7: Fatty acid supplementation restores normal glycolysis in $Atg7^{-/-}$ neutrophils. CRISPR-$Atg7^{+/+}$ and $Atg7^{-/-}$ myeloblasts were cultured for 5 days in G-CSF (100 nM) to induce differentiation in the presence of Linoleic acid (LA) (10 µg/ml), a mix of saturated and non-saturated fatty acids (FFA mix) (10 µg/ml fatty acids) or vehicle control. Quantification of fluorescent glucose (2NBDG) uptake as geometric MFI +/- SEM from three replicates, representative of two experiments.

induced differentiation over 5 days and measured glycolysis. The unsaturated long chain free fatty acid linoleic acid (LA) can serve as a substrate for FAO and ATP production and improves function of mature neutrophils, but has no described effects on neutrophil metabolism or differentiation [320]. Both LA alone or a mix of cholesterol esters, saturated and unsaturated fatty acids (FFA mix) significantly restored the aberrant glycolysis in $Atg7^{-/-}$ cells to levels similar to wild type neutrophils (*Figure* 5.7).

Next, the differentiation was assessed by morphological quantification of differen-

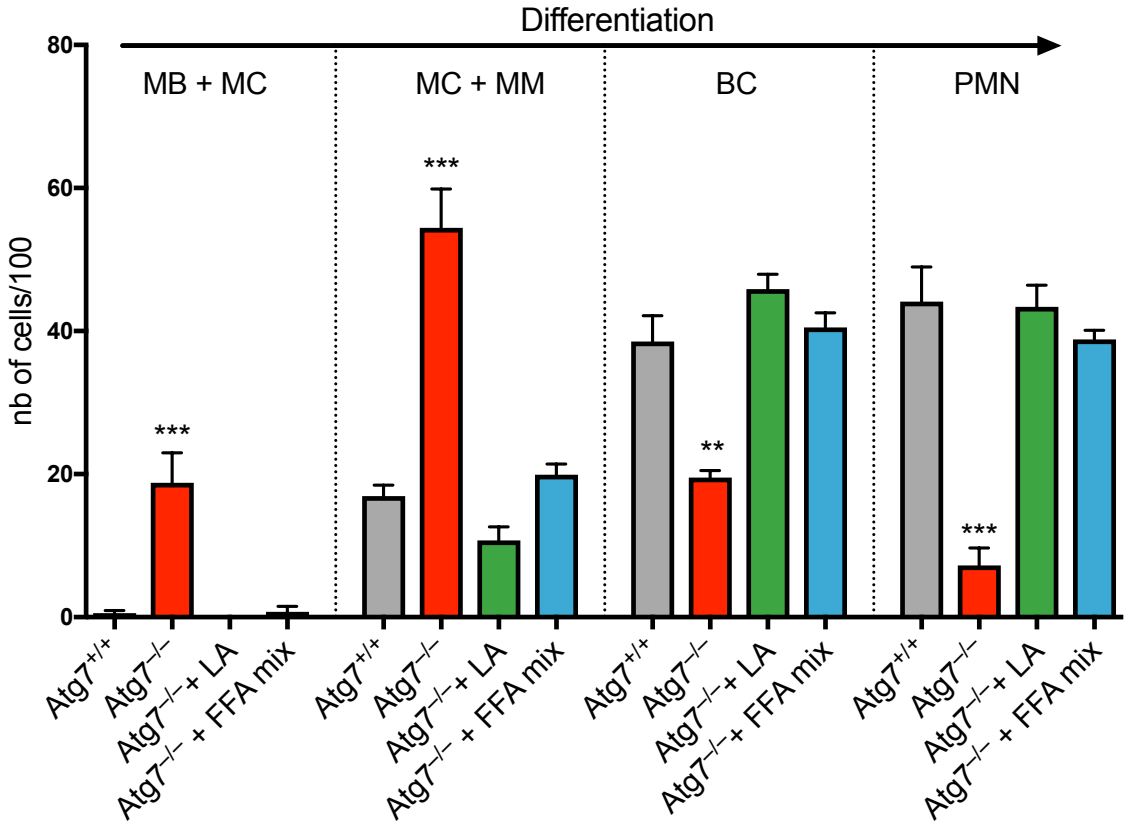

Figure 5.8: Neutrophil differentiation is restored in $Atg7^{-/-}$ myeloblasts after free fatty acid supplementation. CRISPR-$Atg7^{+/+}$ and $Atg7^{-/-}$ myeloblasts were cultured for 5 days in G-CSF (100 nM) to induce differentiation in the presence of Linoleic acid (LA) (10 µg/ml), a mix of saturated and non-saturated fatty acids (FFA mix) (10 µg/ml fatty acids) or vehicle control. Quantification of indicated maturation stages by morphology. Data are shown as number of cells per 100 cells counted combined from two individual experiments, **p < 0.01, ***p < 0.001.

tiation based on Wright-Giemsa stained cytospins. Both LA alone or the FFA mix restored maturation based on blinded quantification of four groups of consecutive differentiation stages on day 5 of G-CSF treatment. While $Atg7^{-/-}$ clones mostly exhibit MC + MM morphology at this point, the fatty acid treatment shifted this balance significantly towards the more mature BC and PMN stages in a manner similar to wild type controls (*Figure* 5.8).

In line with this, increased nuclear lobularization and cytoplasma:nucleus ratio, which was observable at this time point after fatty acid treatment, also occurred with

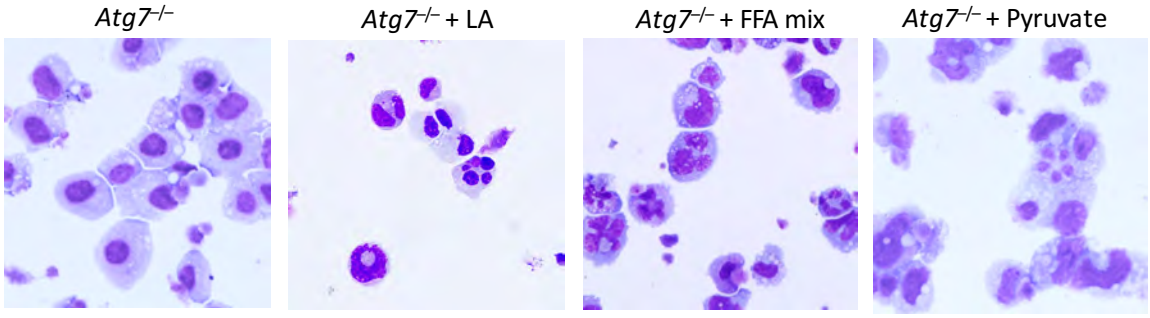

Figure 5.9: Normal neutrophil morphology is restored after free fatty acid or pyruvate supplementation in $Atg7^{-/-}$ myeloblasts. CRISPR-$Atg7^{+/+}$ and $Atg7^{-/-}$ myeloblasts were cultured for 5 days in G-CSF (100 nM) to induce differentiation in the presence of linoleic acid (LA) (10 µg/ml), a mix of saturated and non-saturated fatty acids (FFA mix) (10 µg/ml fatty acids), pyruvate (2 mM) or vehicle control. Representative of three experiments.

pyruvate supplementation (*Figure* 5.9). Pyruvate has a wide pharmacological potential that is currently assessed in several pre-clinical models [321] as it directly enters the TCA cycle after dehydrogenation by PDH into Acetyl-CoA. It can therefore overcome the need of FAO-derived Acetyl-CoA to serve as a major energy metabolite for ATP production.

In addition to rescuing the morphological and glycolytic phenotype, FFA and pyruvate treatment also restored the CD11b maturation marker expression of $Atg7^{-/-}$ neutrophils significantly and nearly back to wild type levels after five days of G-CSF induced differentiation (*Figure* 5.10 and 5.11A).

The restoration of both maturation marker expression as well as morphological features of differentiation in response to FFA treatment was therefore irrespective of the nature of free fatty acids, suggesting that it is not a specific lipid signaling pathway that is stimulated, such as eicosanoid signaling or nuclear receptors [322]. Finally, pyruvate treatment alone was also sufficient to restore differentiation based on quantification of morphological differentiation stages (*Figure* 5.11B). Taken together, saturated or unsaturated long chain fatty acids are able to restore the differentiation of $Atg7^{-/-}$ myeloblasts into neutrophils *in vitro* based on morphological classification

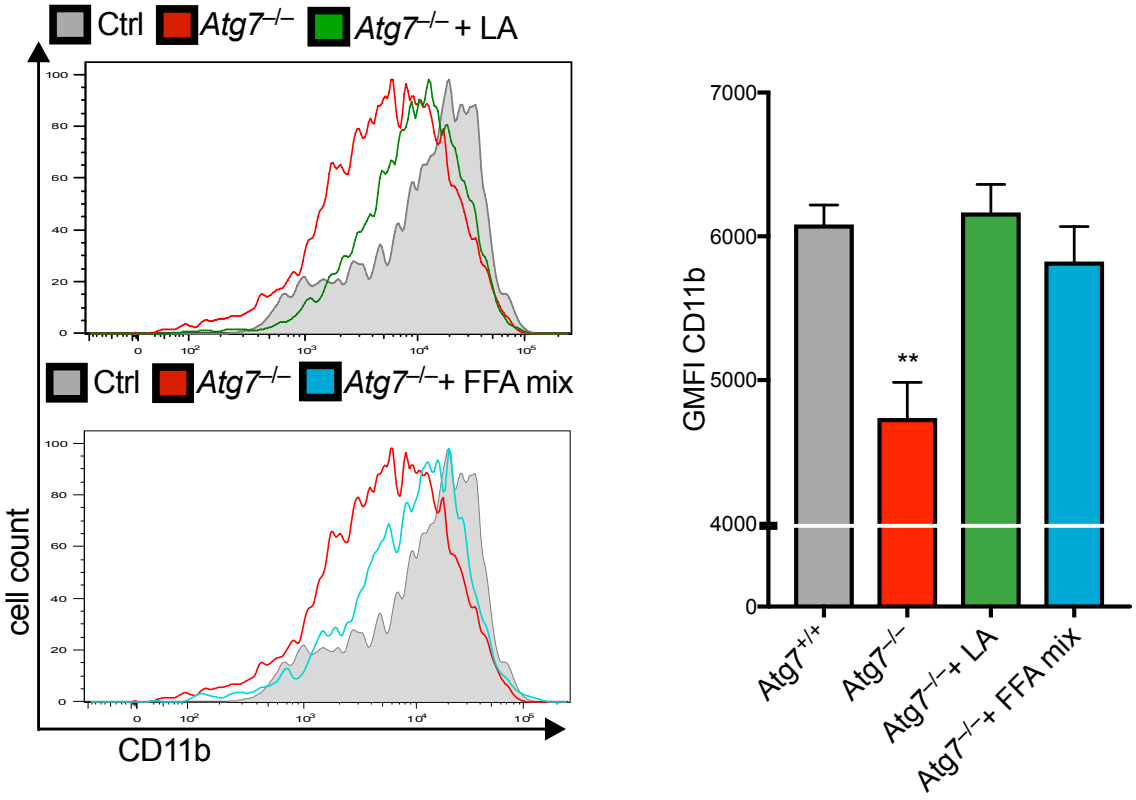

Figure 5.10: Maturation marker up regulation is restored in $Atg7^{-/-}$ myeloblasts during fatty acid supplemented differentiation. Histograms (left) and quantification (right) of CD11b maturation marker expression after day 5 of the indicated treatment in the presence of G-CSF, quantified as mean +/- SEM from three biological replicates, **p < 0.01.

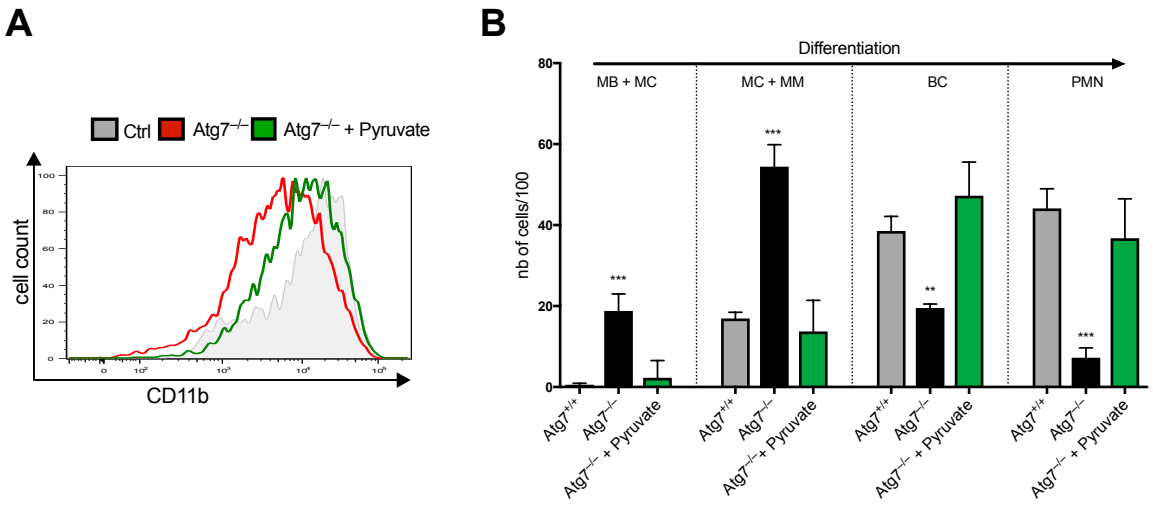

Figure 5.11: Pyruvate rescues neutrophil differentiation of *Atg7*$^{-/-}$ myeloblasts. Representative histogram of CD11b expression after 5 days G-CSF (100 nM) shows rescue of CD11b expression in *Atg7*$^{-/-}$ cells in the presence of pyruvate (A). Histological quantification of indicated differentiation stages shows a near complete rescue of neutrophil differentiation block in *Atg7*$^{-/-}$ cells in the presence of pyruvate (2 mM, green). Data shows number of cells/100 cells counted per group from Wright-Giemsa stained cytospins, from four biological replicates representative of two independent clones, representative of two experiments (B).

and quantification as well as maturation marker expression. Pyruvate, a metabolite that can directly feed into ATP generation via OXPHOS, was also able to restore differentiation in a similar manner. Both FFA and pyruvate also restored the excessive glycolytic phenotype of *Atg7*$^{-/-}$ myeloblasts, suggesting that correction of the lipid metabolic defect led to normalization of the compensatory glycolytic phenotype and restored differentiation.

5.2.5 *Atg7*-deficient chimeric mice improve granulopoiesis in response to high fat diet

The finding that FFA and pyruvate can restore *Atg7*$^{-/-}$ neutrophil differentiation *in vitro* raised the question if this could also improve the neutrophil phenotype of *C/EBPa-cre x Atg7*$^{f/f}$ mice. Because direct supplementation of free fatty acids or cholesterol can cause significant lipotoxicity via the UPR [323], high fat diet is the

safest way to induce a transient increase in free fatty acid levels in the bone marrow microenvironment. A 60 % fat diet is commonly used in mice and increases the serum concentration of free fatty acids [324]. Because of the potentially compromising hepatomegaly developing in *C/EBPa-cre x Atg7$^{f/f}$* mice (3.2.10), bone marrow chimeric mice were generated. Irradiated CD45.1 recipient mice received a non-competitive transplantation of either control or *C/EBPa-cre x Atg7$^{f/f}$* total bone marrow cells (CD45.2). After a three weeks recovery period, mice were fed a high fat diet for three weeks, which led to a significant weight gain over the control group which received standard chow (data not shown). Based on flow cytometric quantification of bone marrow populations, control mice had no measurable impact of the altered diet on granulopoietic subsets. In *Atg7$^{-/-}$* chimera, the MB precursor population was significantly expanded 1.5 - 2 fold in the bone marrow, which was restored to normal abundance in response to high fat diet. At the same time, the number of mature neutrophils (PMN) increased significantly in *Atg7$^{-/-}$* chimera after short term high fat diet treatment (*Figure* 5.12). Although this analysis is purely based on surface marker definition of these populations and cannot exclude non-specific effects of high fat diet, such as inflammation [325], it suggests that a similar mechanism of fatty acid induced metabolic rescue of neutrophil differentiation may operate *in vivo*.

5.2.6 Evidence for a broad relevance of the Autophagy - FAO - OXPHOS pathway in differentiation

The finding that rescuing the lipophagy defect can restore both differentiation and glycolytic addiction in autophagy-deficient granulopoiesis *in vitro* and *in vivo* opens the question whether this pathway is also relevant in many of the other hematopoietic cell types discussed previously, such as hematopoietic stem cells or T cells. Preliminary data obtained from mice deleted for *Atg7* at the HSC level (*Vav-cre* x *Atg7$^{f/f}$*) indicated that excessive glycolysis, as measured by the glucose uptake receptor Glut1

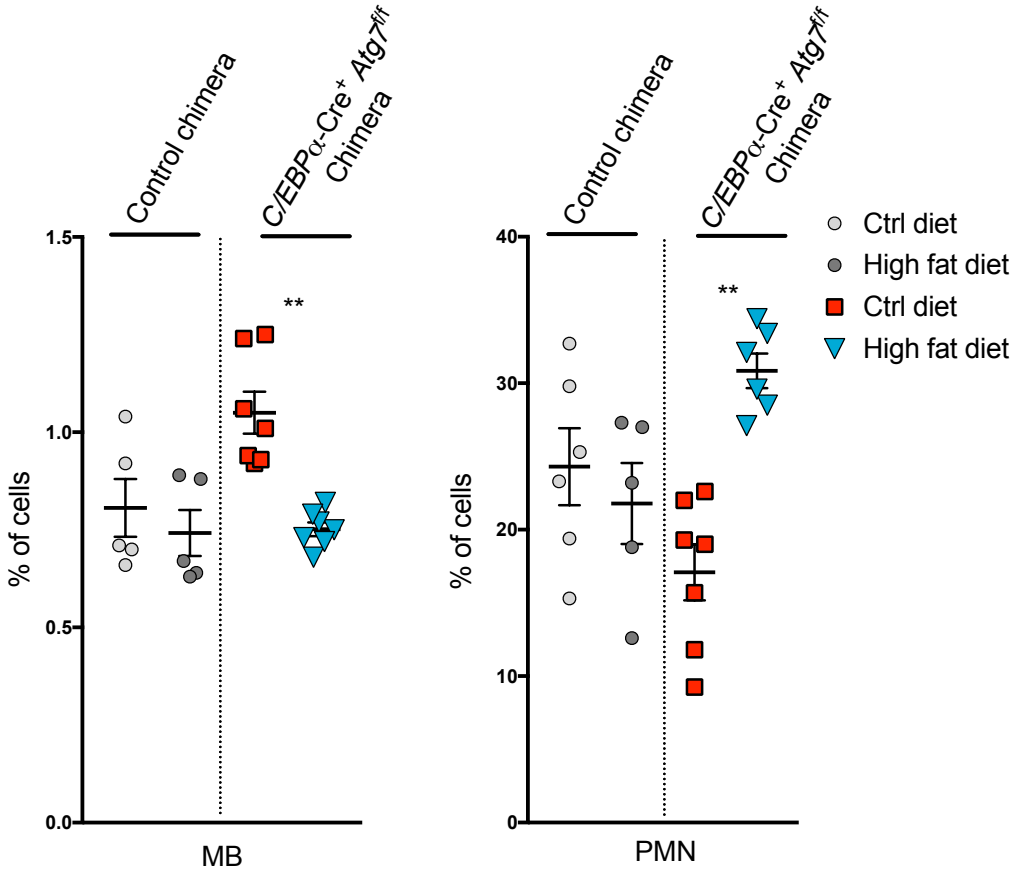

Figure 5.12: High fat diet fed $Atg7^{-/-}$ chimeric mice have improved neutrophil differentiation. CD45.1 Recipient mice received control or $Atg7^{-/-}$ total BM (CD45.2) transplantation three weeks beforehand and were fed normal chow or a 60 % fat diet for 3 weeks. The number of CD45.2$^+$ myeloblasts (MB) and neutrophils (PMN) was quantified by flow cytometry and is shown as % of cells +/− SEM. **p < 0.01.

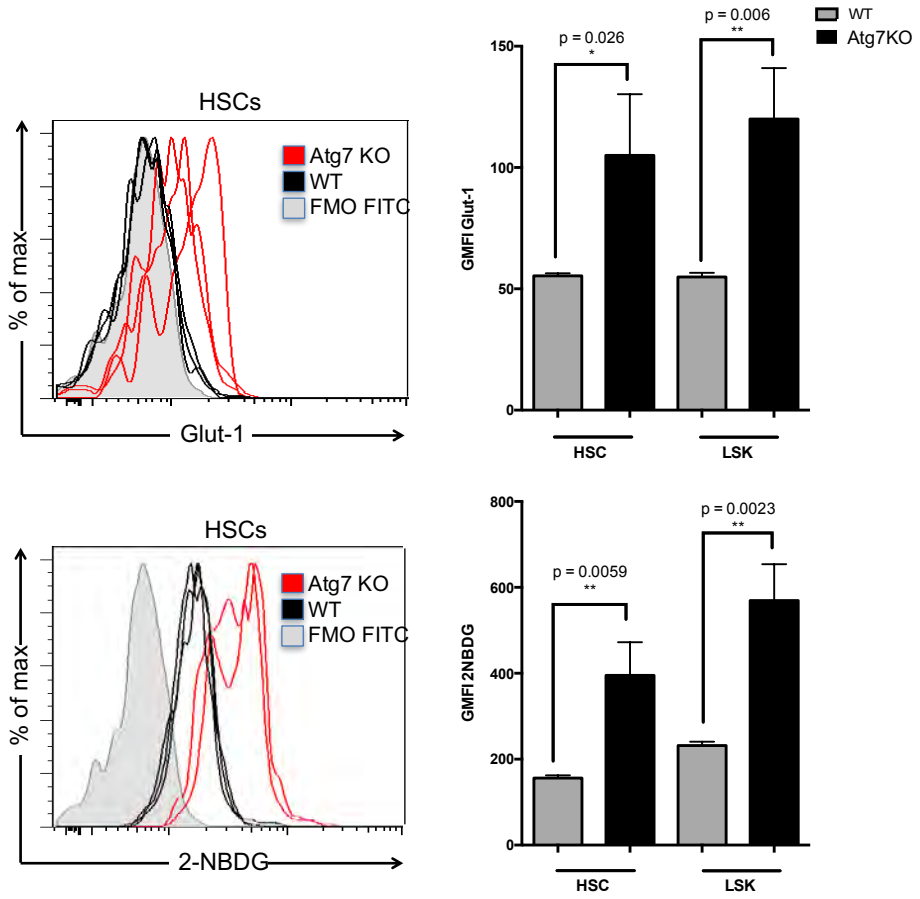

Figure 5.13: *Atg7*-deficient hematopoietic stem cells undergo excessive glycolysis. Bone marrow derived Lin$^-$ Sca1$^+$ c-kit$^+$ CD150$^+$ CD48$^-$ HSCs or Lin$^-$ Sca1$^+$ c-kit$^+$ LSK stem and progenitor cells were analyzed by flow cytometry for Glut1 receptor surface expression (top) or uptake of fluorescent glucose (bottom) over 1 hour of culture from *Vav-cre* x *Atg7*$^{f/f}$ mice or control littermates. Data shown as geometric MFI +/− SEM. *p $<$ 0.05, **p $<$ 0.01.

and quantification of fluorescent glucose uptake, is also engaged in these cells in response to the metabolic defect (*Figure* 5.13). This data was further supported by gene expression analysis that confirmed on a genetic level the up-regulation of glycolytic genes in *Atg7*$^{-/-}$ HSCs in a similar manner as previously shown for neutrophil precursors (See 4.2.2 and data not shown). This supports the idea that a similar metabolic defect is also involved in the HSC differentiation defect and eventual hematopoietic failure in these mice [164].

Robust activation both of glycolytic rate and -gene expression thus appear to be a

good marker for the response to the metabolic defects that restrict certain lineages.

In collaboration with the Maloy group, we investigated this metabolic phenotype in autophagy deficient T_{reg} cells. In their study, they found that T-cell-specific deletion of *Atg16l1* results in chronic intestinal inflammation and reduced differentiation marker expression and survival of T_{reg} cells in the intestinal mucosa [166]. In line with this, we found increased glycolytic gene expression in the *Atg16l1*$^{-/-}$ T_{reg} cells in the colon, but not in the spleen (*Figure* 5.14). Particularly, the glucose uptake receptor Glut1 (*Slc2a1*) as well as the lactate secreting transporter *Slc16a3* and the *Ldha* gene were up-regulated in the *Atg16l1*$^{-/-}$ T_{reg} cells in the colon, suggesting that increased glycolytic flux is maintained by LDHa mediated lactate generation to regenerate NAD^+, which is required to sustain this Warburg-like metabolism. The tissue specificity of this phenotype argues for an additional external factor linked to the environment, such as stress and metabolic adaption, which occurs in the context of the colon [326]. This metabolic response to autophagy deletion in the colon is remarkably similar to the observations in neutrophils (See 4.2.2) and HSCs and possibly indicates impaired fatty acid oxidation leading to excessive glycolysis via falling NADH and acetyl-CoA levels as part of the Randle cycle [316].

Indeed, preliminary data indicated impaired lipid metabolic homeostasis in colonic T_{reg}cells as shown by reduced expression of genes involved in lipid transport, synthesis and degradation as well as the mitochondrial master regulator and transcription factor for oxidative metabolism PGC1a (*Figure* 5.15). This lipid metabolic defect is further supported by metabolic profiling data [170, 166] and, together with the fact that T_{reg} cells usually switch to a metabolic program relying on lipid oxidation for energy production [160], may contribute to the functional and survival defect in *Atg16l1*-deficient T_{reg} cells. It would therefore be interesting to attempt reversal of both energy-metabolic and pathological phenotypes caused by the T_{reg} defect with direct OXPHOS substrates like methyl-pyruvate or free fatty acid supplementation.

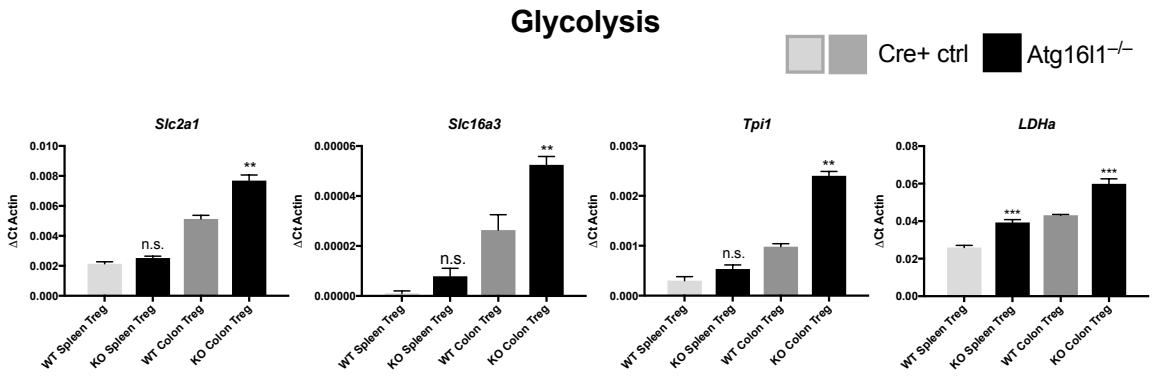

Figure 5.14: Increased glycolytic gene expression in colonic *Atg16l1*$^{-/-}$ T_{reg} cells. Fluidigm gene expression analysis of glycolytic gene levels in 200 sorted Foxp3^{+} T_{reg} cells from the spleen and colon of *Atg16l1*$^{-/-}$ and *Foxp3-cre*-Ctrl mice (sorted for CD4^{+} TCRb^{+} YFP^{+}). *Slc2a1* (Glut1), glucose transporter 1; *Slc16a3*, solute carrier family 16 member 3 (Lactate transporter); *Tpi1*: triosephosphate isomerase 1; *Ldha*: lactate dehydrogenase isoform a. Data representative of two independent experiments. This data was generated in collaboration with Agnieszka Kabat.

Taken together, preliminary data in T_{reg} cells and HSCs, together with supporting literature, indicate that a similar metabolic response of activated glycolysis operates broadly, if not universally, in many cell types in response to autophagy deletion and is accompanied, and potentially caused by, an underlying defect in lipid oxidation for energy production.

5.3 Discussion

This chapter was built on the metabolic characterization of wild-type and *Atg7*$^{-/-}$ granulopoiesis of the previous chapter and tested the causality of these metabolic alterations for the differentiation defect. The degradation of lipid droplets by lysosomal, but not cytosolic lipases in neutrophil precursors was essential for differentiation, which is the underlying defect in *Atg7*$^{-/-}$ precursors. Consequently, restoring FFA balance or TCA metabolites for mitochondrial ATP production rescued *Atg7*$^{-/-}$ neutrophil differentiation *in vitro* and *in vivo*. This suggests that the autophagy controlled FAO–OXPHOS pathway may be critical to supply sufficient ATP for the

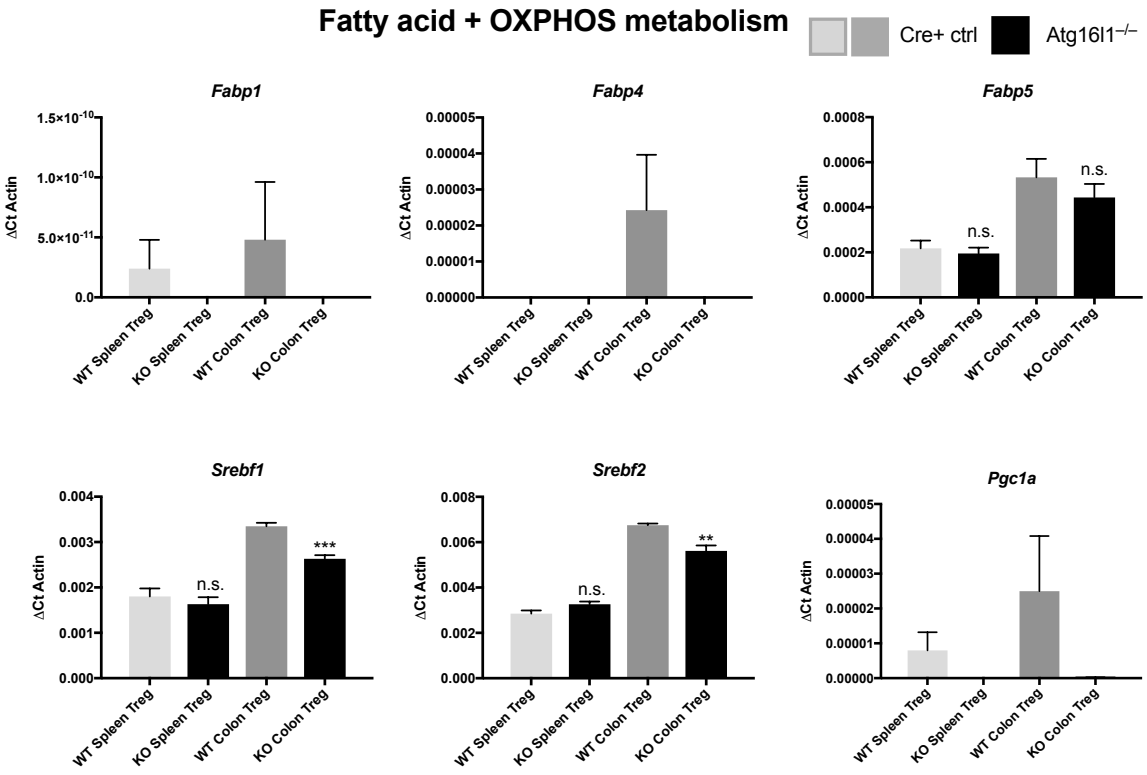

Figure 5.15: Reduced expression of genes involved in lipid metabolism in colonic *Atg16l1*$^{-/-}$ T_{reg} cells. Fluidigm gene expression analysis of fatty acid metabolism gene levels in 200 sorted Foxp3^{+} T_{reg} cells from the spleen and colon of *Atg16l1*$^{-/-}$ and *Foxp3-cre*-Ctrl mice (sorted for CD4^{+} TCRb^{+} YFP^{+}). *Srebf1*, sterol regulatory element binding transcription factor 1; *Srebf2*, sterol regulatory element binding transcription factor 2; *Fabp*, Fatty acid-binding protein; *Pgc1a*, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha. Data representative of two independent experiments. This data was generated in collaboration with Agnieszka Kabat.

energy-demanding process of differentiation. An early study on lipid metabolism during the differentiation of HL60 cells into neutrophils found rapid changes in inositol lipids for the first time [327], but the study was restricted to the human promyelocytic leukemic cell line HL60 and therefore of limited relevance. The mutual feedback regulation between glucose and oxidative metabolism is evident by the fact that high glucose levels are able to inhibit neutrophil oxidative metabolism [328], which could start a negative feedback loop that intensifies the metabolic switch from oxidative to glycolytic metabolism during $Atg7^{-/-}$ granulopoiesis. Decreased FAO and OXPHOS will activate glycolysis as soon as NADH, acetyl-CoA and ATP levels fall [316, 315]. Likely owing to these two regulatory loops, we found excessive glycolysis in response to defective neutrophil FAO/OXPHOS metabolism, but importantly this secondary glycolytic "addiction" did not provide adequate amounts of ATP, and was reversible, as FFA mediated FAO restoration normalized glycolysis.

Autophagy is critical in the transdifferentiation of hepatic stellate cells into proliferative myofibroblasts and thus required for fibrogenesis, and the addition of FFA rescued the differentiation of autophagy deficient hepatocytes [329]. This study showed accumulation of lipids in response to pharmacological or RNAi-mediated reduction of autophagy in hepatic stellate cells, as well as an ATP crisis in response to 3-MA. The differentiation defect could be rescued by oleic acid treatment *in vitro*. Despite the use of non-specific autophagy inhibitors such as 3-MA, partial autophagy knockdown via RNAi and relying mostly on *in vitro* models, this study was, to the knowledge of this author, the first to identify a physiological role for autophagy mediated lipid breakdown for differentiation. Together with the data presented here and the described studies in macrophages and T cells, it suggests that the mechanism described here operates broadly also outside the hematopoietic system to facilitate differentiation. More broadly, an alternative contribution to the differentiation defect could be defective nuclear receptor signaling pathways, which rely on lipid ligands to activate

transcriptional programs associated with differentiation. One such group of receptors are the peroxisome proliferator-activated receptors (PPAR), which bind DNA in complex with nuclear receptors to activate diverse pathways such as lipid homeostasis, proliferation and differentiation [330]. The natural ligands of these receptors are long chain fatty acids, mostly eicosanoids, and their activation is key, for example, to adipocyte differentiation [331]. Circumstantially, adipocyte differentiation also requires autophagy [277], and it is possible that absent fatty acid ligands lead to impaired nuclear receptor signaling, which would normally induce differentiation, at least in this cell type. Supporting this possibility, autophagy deletion in T_{reg} cells (via *Atg16l1*) led to a complete absence of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1a*) expression, the transcriptional coactivator of PPAR nuclear receptors and a master regulator of oxidative mitochondrial metabolism [332](See 5.2.6). Interestingly, *Pgc1a* expression was also nearly absent in *Atg7*-deleted HSCs in *Vav-cre x* $Atg7^{f/f}$ mice, and significantly reduced in CRISPR $Atg7^{-/-}$ neutrophil precursors (data not shown), again pointing to a more universal relevance of these signaling pathways. Maybe, lack of lipophagy-generated signaling molecules such as the PPAR-activating eicosanoids contribute to the lack of a differentiation-inducing signal in these cell types. It would be interesting to know the status of *Pgc1a* expression and PPAR activity in response to fatty acid mediated rescue of granulopoiesis. Maybe, agonists of nuclear receptor signaling, for example with the PGC1a-agonist clenbuterol [333], could also restore differentiation in the absence of autophagy? More generally, altered levels of free fatty acids and lipid signaling molecules such as lipid second messengers in $Atg7^{-/-}$ cells can affect diverse signaling pathways as well as the composition of intracellular membranes and membrane domains, thereby affecting differentiation [334]. In this light, the rescued differentiation in response to free fatty acids could also be explained by its ability to overcome the lack of such signaling molecules. Indeed, linoleic acid can be quickly metabolized independently of autophagy via the

peroxisome to produce diverse lipid metabolites, and can rescue the lack of PGC1a by directly activating PPAR receptors [335]. Arguing against the idea that a lack of lipid-derived signaling molecules such as eicosanoids causes the $Atg7^{-/-}$ differentiation block is a study that shows production of these molecules is relevant to neutrophilic inflammation, but mainly occurs via *Atgl* and other cytosolic lipases in neutrophils [336]. Inhibition of ATGL however did not affect differentiation of neutrophils. In addition, the fact remains that pyruvate also rescued $Atg7^{-/-}$neutrophil differentiation, which again argues in favor of mostly an energy-metabolic defect underlying defective differentiation. Pyruvate is the metabolic end product of glycolysis which can either be converted to lactate via the action of LDHa to regenerate the reduction equivalents of NAD^+ for further glycolysis, or can react into Acetyl-CoA to enter the TCA-cycle for mitochondrial respiration and ATP production. It has been reported to help overcome energy crisis as part of its oxidative mitochondrial metabolism in the context of neuroprotection [337], but can also act as a ROS scavenger. Pyruvate, sometimes referred to as metabolic "magic-bullet" [321], has diverse anti-inflammatory effects, for example healing damage to the intestinal mucosa caused by mesenteric ischemia and reperfusion in rats [338][339]. While it is possible that part of its beneficial effect on $Atg7^{-/-}$ granulopoiesis is mediated by ROS scavenging, the principal metabolic fate of pyruvate is as robust fuel-source for large scale OXPHOS-mediated ATP production. Further support for the energy-metabolic relevance of lipophagy driven ATP production also comes from another kingdom of life: Dengue virus can initiate large scale autophagy mediated processing of triglycerides in its host cells to release free fatty acids, which leads to increased ATP production via ß-oxidation and OXPHOS. This pathway, which can be replaced by exogenous free fatty acid supplementation, is essential to supply ATP for efficient dengue virus replication [340].

5.4 Conclusion

A couple of pioneering studies introduced earlier (See 4.1.1) describe a lysosomal lipolysis pathway essential for macrophage M2 differentiation [252], which requires a shift towards OXPHOS [97], and independently, autophagy is described to be important for M2 macrophage generation [250, 167]. In the T cell field, activated T cells mostly engage glycolytic metabolism, but both T_{reg} and T_{mem} switch to mitochondrial respiration for the bulk of their ATP production [341, 246], and support this metabolic reprogramming by lipid breakdown for FAO and OXPHOS [304, 302]. In line with the data presented here, immature neutrophils also accumulate in LAL-deficient mice [291], further supporting a crucial role for this pathway in neutrophils. Thus, M2 macrophages, T_{mem}, T_{reg} and neutrophil precursors have a metabolic switch in common towards FAO and OXPHOS, which depends on autophagy and lysosomal lipid breakdown, but our study is the first that directly proves lipophagy as the causative mechanism underlying the differentiation block caused by autophagy deficiency. This causal link between autophagy, lipid metabolism and differentiation has previously been suggested outside of the hematopoietic system, in hepatic stellate cells, and thus may operate broadly, if not universally, in cellular differentiation.

6 General discussion and conclusion

6.1 Autophagic flux correlates with functional relevance during granulopoiesis

Although autophagy is active at basal levels in principally all hematopoietic cells, differentiation is generally inversely correlated to autophagic flux [168]. In line with this, long term HSCs have highest autophagy levels and they uniquely rely on autophagy as a cytoprotective pathway to balance their self-renewal versus differentiation [164]. In almost all cases where autophagy has been described essential for hematopoietic differentiation further downstream, researchers also describe a strong and temporally regulated activity of the pathway at or directly before the relevant stage [342, 343, 344, 165, 236, 256, 255, 345, 169, 239]. In the case of granulopoiesis, we found high autophagic flux at the earliest and latest committed neutrophil differentiation stages, while intermediate stages had low or absent turnover of $LC3^+$ autophagosomes. It may seem likely that the induction of autophagy in the terminal stages may be relevant to neutrophil mobilization from the bone marrow and function in the periphery [210]. Indeed, autophagy is required for the mobilization of neutrophils in response to G-CSF [234], and has diverse functions in mature neutrophils including neutrophil extracellular trap formation [145], degranulation, limiting infection [210] and phagocytosis [212]. The high autophagy levels in MBs and directly downstream of GMPs in turn indicate a potential functional relevance during early neutrophil differentiation. Indeed, the differentiation defect in the absence of autophagy occurs at or directly downstream of the GMP in the earliest committed neutrophil precursors. Because in all studies that measured autophagy during differentiation, including this study, autophagy is measured as the turnover of $LC3^+$ vesicles, it is unclear if there is an increase of general turnover at these stages or rather a specific cargo preferentially targeted in each of these cases. During erythropoiesis for example, the targeted re-

moval of mitochondria is suggested as the prime function of autophagy induction at the erythroblast stage [197, 193, 64], but for most selective types of autophagy we currently lack robust assays to measure the dynamic content of autophagosomes. In this study, a novel technique was established using the novel lipid droplet specific dye LD540, which has no spectral overlap with GFP [278], in combination with GFP-LC3 transgenic mice to measure the dynamic turnover of lipid droplets during granulopoiesis (See 4.2.6). Although the current gold standard remained EM-based detection [277], confocal microscopy allows for live imaging and flux analysis of lipophagy even in rare cell types such as neutrophil precursors. From a translational perspective, the paucity in tools to discriminate the many types of autophagy cargo selection remains a major hurdle in identifying specific autophagy pathways relevant in each setting of differentiation. In this view, it is entirely possible that most of the (hematopoietic) lineages where autophagy was found critical for differentiation or survival may actually involve specific cellular components that have to be removed to facilitate this, but we currently miss this layer of complexity owing to our limited detection techniques [31]. Fast moving basic research in yeast and mammalian cells on the selective forms of autophagy has identified more than 20 different classes of cargo, including most cytoplasmic organelles [56, 57, 58, 59, 60, 61, 62, 63, 64], the nucleus [65, 22, 66], glycogen [67], ribosomes [70, 63], specific protein complexes, and many more [71]. These diverse pathways are mediated by an ever expanding autophagy interaction network which is remarkably conserved in mammals [39], suggesting that many of them serve an important physiological role in higher organisms. In the case of granulopoiesis for example, one could also imagine nuclear piecemeal or nucleophagy, the degradation of nuclear fragments [65, 66], to be required to facilitate the nuclear lobularization during differentiation. Circumstantially, the period of high LC3 turnover at the GMP-to-BC transition coincides with the stage were nuclear invagination starts [219]. It cannot be excluded that nuclear piecemeal or another type of selective autophagy partially con-

tributes to the *Atg7*$^{-/-}$ differentiation defect described in this study. Future research will undoubtedly explore the dynamic nature of the "autophagic substratome" - the autophagosome content at a given point of the differentiation processes, to identify the relevance of these specific pathways for hematopoiesis and human physiology in general. These studies will likely rely on state-of-the-art quantitative mass spectrometry techniques in combination with spatially-targeted labeling approaches, such as APEX2 proximity labeling [346]. One application of this approach recently discovered the dynamic interactions of the stress granule proteome with the autophagy network in the context of neurodegenerative disease, facilitating insight into the relevance of aggrephagy during amyloid lateral sclerosis [347]. Taken together, while the correlation between high autophagosome turnover and times of functional relevance during differentiation is remarkably synchronized, we currently have limited means to understand the type of autophagic turnover. The research presented here suggests that it is lipid droplet stores, which autophagy has to degrade, that facilitate granulopoiesis. In addition to unraveling the dynamic autophagic substratome, further research into the specific receptors that mediate cargo recognition for selective autophagy will improve our understanding of this [44]. In this view, the LC3/GABARAP family provides a regulatory network that facilitates selective targeting of substrates to the growing autophagosome via selective autophagy receptors. The best example for this may be the cargo receptors p62 and NIX, which can bind to mitochondria directly or after poly-ubiquitylation, respectively, and target them to the growing autophagosome for mitophagy [348]. Knowledge of these specific receptors enabled the discovery of mitophagy as the relevant type of specific autophagy in the case of erythropoiesis [64, 193], but also during NK cell memory formation [349]. Deletion of Nix allows to shut down mitophagy without affecting macroautophagy and most other LC3 associated pathways, giving these researchers a unique tool to discern the role of mitophagy in these differentiation pathways. Identifying such receptors for lipophagy (and other

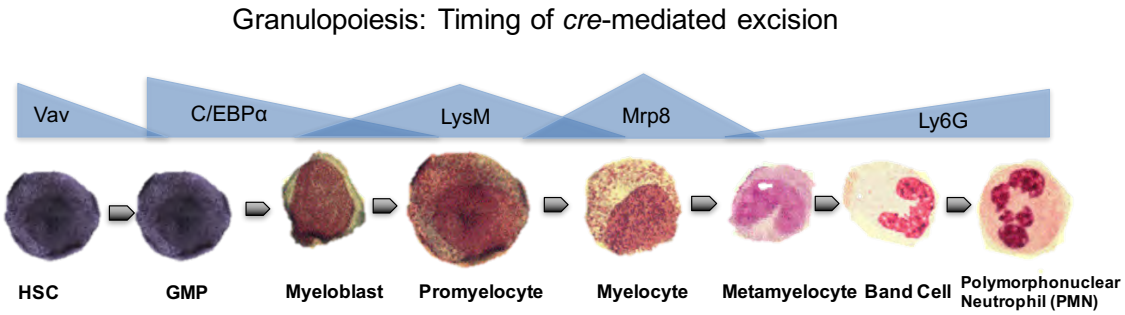

Figure 6.1: The timing of cre-mediated excision during the generation of neutrophils. The window of first expression of each of the cre-driving promotors is visualized in relation to the respective differentiation stage.

cargos) would greatly facilitate research into the relevance of such pathways, both during granulopoiesis and beyond.

6.2 Tissue and stage-specific autophagy deletion defines a cell intrinsic neutrophil differentiation defect downstream of the granulocyte-monocyte progenitor

In this study, *Vav-cre*, *C/EBPa-cre* and *Mx1-cre* mediated deletion of *Atg5* and *Atg7*, together with *LysM-cre* and *Mrp8-cre* mediated deletion of *Atg7* from the literature [177][210], was employed to define the exact nature and stage of a neutrophil differentiation defect in the absence of autophagy. Earliest deletion of Atg genes at the stem/progenitor level (*Vav-cre*) resulted in the most severe neutrophil differentiation defect; excision at the first commited precursor stage (80 % of GMPs via *C/EBPa-cre* [121]) still had a significant, but slightly milder defect; deletion at terminal precursor stages (15 % of GMPs, 60 % of PMN via *LysM-cre* [350, 351]) caused mild precursor proliferation and neutrophilia [177], and deletion in mature neutrophils (*Mrp8-cre* or *Ly6G-cre*) results in only functional neutrophil defects [210] (*Figure* 6.1).

Both *LysM-cre* and *C/EBPa-cre* mediated deletion also affect macrophages, so that feedback mechanisms and infections resulting from defective myeloid function could

also be causing progenitor accumulation and homeostatic proliferation in the bone marrow, a mechanism which is part of "emergency granulopoiesis" [248, 207, 235, 227, 229]. Therefore, the cell intrinsic nature of this differentiation defect had to be confirmed in competitive, hematopoietic bone marrow chimera experiments using pIpC-inducible deletion via *Mx1-cre* in the presence of functional wild-type HSCs and neutrophils. All these results, together with the CRISPR-$Atg7^{-/-}$ myeloblast cell culture system, form the basis of the neutrophil differentiation defect described here, which is cell intrinsic, independent of autophagy functions in HSCs, and occurs gradually around the GMP stage. Given the many studies that have described autophagy as an essential differentiation mechanism in diverse lymphoid and myeloid cell types over the last 5 years or so [189], why have neutrophils, the most abundant white blood cell in humans, been missed? The insight one may gain from this study is that spatial and temporal control of gene deletion specificity via the *cre*-LoxP system is extremely important during the study of hematopoietic differentiation, and more broadly for any study that seeks cell-type specific gene targeting. Here, *C/EBPa-cre* mediated gene deletion was the crucial tool to identify the granulopoiesis defect *in vivo*. While extensive accumulation of immature myeloid blast cells had been reported in bone marrow and tissues of *Vav-cre* x $Atg7^{f/f}$ mice already 6 years ago [176], it was presumed to be non-specific and secondary to the general hematopoietic failure and HSC dysfunction. The study presented here first defined their granulocytic lineage identity, which subsequently motivated a closer investigation of neutrophil differentiation in additional models. Similarly, when autophagy was deleted only terminally via *LysM-cre*, a mild precursor accumulation and proliferation along with neutrophilia had been noted [177], pointing towards some relevance of autophagy also late during neutrophil differentiation. Curiously, this study found no defect in the function of such neutrophils, actually leading the authors to conclude a suppressive effect of autophagy on granulopoiesis. Our results and most results in the literature agree that mature neutrophils depend on

autophagy for degranulation [210], NET formation [145], phagocytosis [212], bacterial killing [352] and inflammatory response [353]. Hematopoietic studies using different *cre*-systems to delete the same gene frequently come to slightly different conclusions, as evident for example in the B cell field: Early studies using *Beclin1*-deficient and *Atg5*$^{-/-}$ chimera models described a defect in the pro-to-pre B cell transition caused by absent autophagy[354, 355, 356], but in both these chimeric models HSCs already lack autophagy, which have been shown to critically require autophagy for their own maintenance and differentiation [164], potentially exacerbating the observed B cell phenotype. A later study used chimeric models and *Mb1-cre*-, *CD19-cre* or *CD21-cre*- mediated excision of *Atg5* at different times before and after the pro-B cell stage to suggest that autophagy is in fact dispensable for development at the pro-to-pre-B cell stage [357]. Taken together, using the best and most suitable *cre*-systems for *in vivo* studies, that delete at the correct stage and with specificity, together with relevant *in vitro* models is critical to define the cell-biological events of hematopoiesis. Having defined the neutrophil differentiation defect this way, what is the underlying causative mechanism? Deletion of the core autophagy machinery via *Atg5/7* can affect a multitude of autophagy pathways, most of which could be relevant to neutrophil differentiation.

6.3 Autophagy-mediated lipolysis to limit glycolysis and engage FAO-OXPHOS may be a broad concept of cellular differentiation

The metabolic characterization of granulopoiesis both under normal and autophagy-deficient conditions has revealed a metabolic switch that is dependent on autophagy. This switch is characterized by limiting glycolysis and engaging oxidative mitochondrial metabolism during differentiation. This metabolic reprogramming is somewhat

surprising, as peripheral neutrophils are mainly glycolytic [286]. Therefore, this may point towards a second metabolic switch that could lead to re-engagement of glycolysis when exiting the bone marrow. Interestingly, a switch from glycolysis to oxidative metabolism also underlies several other examples of hematopoietic differentiation, and these cell types typically also depend on autophagy for their generation or maintenance, pointing to a wider relevance of an autophagy mediated, oxidative metabolic pathway: During NKT cell formation, metabolism switches from glycolysis to OXPHOS to engage a "natural memory" metabolic phenotype, and the generation of NKT cells accordingly depends on autophagy [239, 257]. Both memory and regulatory T cells are another example for this, as both cell types have enhanced respiratory capacity and a preference to use OXPHOS [298], in contrast to the proliferating effector T cells such as $CD4^+$ T_H1 T_H2 or T_H17 cells, which rely on glycolysis. Accordingly, T cell specific autophagy deletion results in normal or excessive T_H1 and T_H2 response while regulatory and memory subsets are impaired [165, 166]. Finally, M1 macrophage generation is associated with glycolytic metabolism, while M2 macrophages rely on oxidative metabolism and FAO [252]. In the absence of autophagy, excessive pro-inflammatory and glycolytic M1 polarization occurs [167, 251] at the cost of defective M2 macrophage generation [250]. Thus, a picture emerges, where the pro-inflammatory, glycolytic cell types such as T_H1, T_H2, T_H17 and M1 macrophages are suppressed by autophagy, whereas the group that requires OXPHOS and FAO metabolism, including T_{reg}, T_{mem} and M2 macrophages, require autophagy (*Figure* 6.2). There are important exceptions to this general and simplifying grouping, such as the fact that M1 macrophages do not proliferate [358] and that M2 macrophages also require functional mTOR [253, 359]. Similarly, the previously described phenotype of iNKT cells does not completely fit this picture: Although they require autophagy and limit glycolytic metabolism for their "memory-like" phenotype, their response to antigen or cytokines is by producing copious amounts of inflammatory mediators such as IFNg [360], a life-style more asso-

ciated with effector T lymphocytes in group 1. Similarly, the generation of neutrophils in the bone marrow required autophagy and the oxidative metabolic axis supported by lipophagy, which generally fits into group 2. Nevertheless, once in the periphery, neutrophils are short lived effector cells characterized by glycolytic metabolism and the ability to mediate severe inflammation [220]. The fact remains that a clear correlation exists where cells that require autophagy also adopt an oxidative metabolism with limited mTOR and glycolytic activity. In this respect, linking together the relevance of lipophagy in neutrophils with the descriptions of group 2 metabolism is a key outcome of this study that may add another layer to this grouping: It may seem likely that the lysosomal lipolysis for FAO and OXPHOS described for group 2 cells [304, 252, 244] would be mediated by the autophagy machinery, and this would be causative for the differentiation defect in the absence of autophagy [165, 169, 170, 166]. However, the study presented here on neutrophil generation is the first to prove this causative link as the relevant limiting factor for hematopoietic differentiation by rescuing $Atg7^{-/-}$ differentiation with free fatty acids or pyruvate. Why is it important to prove this seemingly obvious link? Many of the arguments above were based on correlations of cellular phenotypes as a basis to define groups of cells that may help us in the future to infer and predict some of their immunologic features. It would be tempting to define group 2 instead as cells that require the aforementioned autophagy-FAO-OXPHOS axis for their metabolic maintenance, and then include BM neutrophils in this definition. The danger is that the proposed categorization in *Figure* 6.2 currently is based on signaling and metabolic pathways which are integral to the health of almost any cell to some extent: There are many examples in the literature, such as in the case of iNKT cells [258, 361], where both constitutively active as well as diminished mTOR activity results in dysfunction [362], and autophagy is a similarly universal pathway which is damaging both in excess or if absent [363, 36]. Furthermore, autophagy is the only currently known mechanism to degrade mitochondria, and thus a degree of mitochon-

drial accumulation is almost universally observed in diverse cell types[345, 349, 190]. While this study found decreased OCR as a result, there is also literature that describes increased OCR in response to autophagy impairment [168, 294, 166], making it difficult to discern effects of mitochondrial accumulation and uncoupling due to mtROS from the actual respiratory rate. Similarly, excessive glycolysis appears to be an almost universal feature of autophagy deficiency [165, 167, 168, 170, 166] which does not in all cases have to be a secondary consequence of impaired lipophagy, as proposed here. Unpublished data from this lab found activated mTOR in response to autophagy deletion in several cell types, which may represent an alternative explanation to excessive glycolysis via the mTOR-HIF1a signaling axis [364]. Interestingly, trained immunity, the non-specific protective effect in response to microbial agents that is mediated by epigenetic reprogramming of monocytes, is also marked by activated HIF1a and mTOR mediated glycolysis [365] and requires autophagy to mediate its response [366]. These examples demonstrate that both glycolytic activity and mitochondrial respiration can be affected by autophagy-deletion independently of lipophagy and represent alternative explanations for the metabolic phenotype of neutrophils and group 2 cells. Therefore, identification of the cell types that uniquely and specifically rely on lipophagy for ATP production to support their function would be the first crucial step before this pathway may be tested for pharmaceutical targeting in order to adapt immune responses in the context of human disease.

6.4 Implications for therapeutic targeting of acute myeloblastic leukemia

Focusing on the differentiation of myeloid cells, this and other studies show that autophagy plays a vital role in the physiological differentiation of hematopoietic stem cells into most myeloid cell types, including macrophages, platelets, megakaryocytes

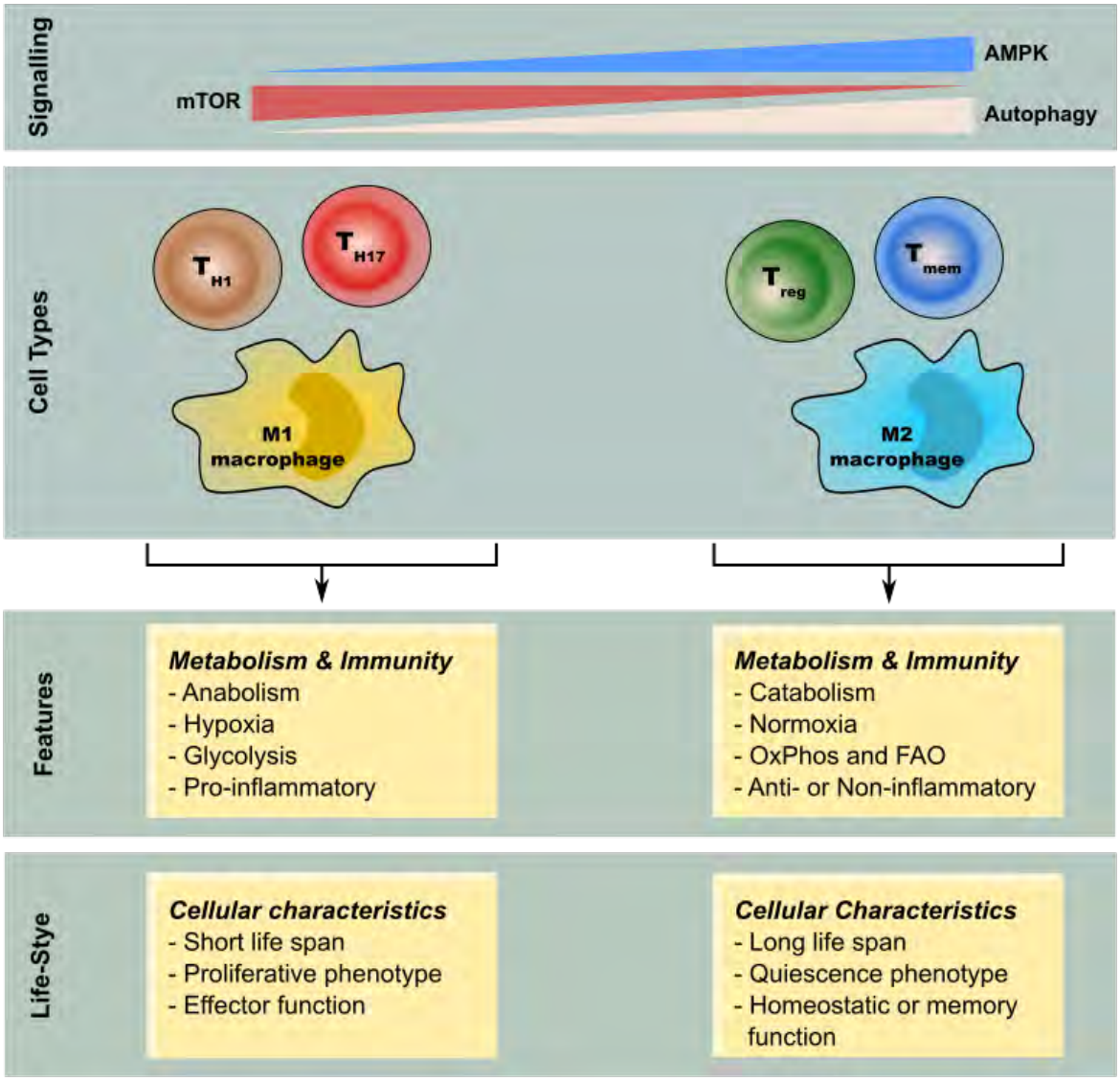

Figure 6.2: Autophagic dependence separates immune cells into two groups with opposing metabolic features. In group 1, cells that are associated with high mTOR but low autophagy activity include T_H1, T_H17 and M1 macrophages. This group mediates inflammation and adopts a life style characterized by glycolysis and anabolic metabolism to support proliferation or effector functions. In group 2, cells that require autophagy for their generation or maintenance are associated with low mTOR but high AMPK activity and include T_{reg}, T_{mem} and M2 macrophages. This group has predominantly catabolic metabolism supported by FAO and OXPHOS to mediate their long lived, immunoregulatory life style. An adapted version of this figure has been submitted for publication, artwork by Felix Richter.

and neutrophils [189]. While the underlying mechanistic role for autophagy remains to be fully understood in most of these cases, limiting aerobic glycolysis and proliferation may be one of the key functions for autophagy to facilitate terminal myeloid differentiation. When differentiation is blocked in the presence of transformation, each of these myeloid cell progenitors can cause a specific subtype of myeloid leukemia, such as myelomonocytic, megakaryoblastic, granulocytic, etc, and these are defined by the FAB and WHO classification [367, 368]. Because a differentiation block and accumulation of myeloblastic progenitor cells is therefore common to granulocytic leukemia and the $Atg7^{-/-}$ models described in this study, it is reasonable to investigate whether autophagy and the metabolic pathway described here is relevant to this disease. Acute myeloid leukemia (AML) is a common and aggressive malignancy with an incidence that increases with age, making it a major health concern in the context of our aging society [368]. The disease arises from the hematopoietic stem and progenitor pool by malignant transformation associated with multiple hits in genes for proliferation, differentiation or survival [367]. Targeting these leukemic stem cells (LSC) is presumed necessary for successful treatment, either by inducing differentiation or cell death [369]. The data presented here may point towards a potential "differentiation therapy" approach by exploiting autophagy. However, the majority of available treatments to date are non-specific, owing to our limited understanding of the biological processes occurring during physiological differentiation of myeloid cells as well as the specific events that facilitate transformation. The identification of novel drug targets that can promote the terminal differentiation of the leukemic stem cell pool is therefore highly desirable [369]. The autophagy pathway in general has already been widely associated with different forms of cancer [44] and autophagy inducers are in clinical trials for a number of these, including some types of leukemia [370]. The link between autophagy and myeloid dysplasia and leukemia specifically was highlighted in animal studies from the authors´ laboratory: Deletion of *Atg5* or *Atg7* in hematopoietic stem

cells is sufficient to block myeloid differentiation and cause a myelodysplastic syndrome with accumulation of myeloblast cells infiltrating a range of tissues with features of AML in mice [164, 176, 168]. Because *Atg5* and *Atg7* deletion resulted in identical phenotypes, normal autophagy function must be the underlying process to prevent myelodysplasia in mice. The link between low/defective autophagy and leukemia is further underscored by the fact that autophagy gene losses are often found within chromosomal regions commonly deleted in human AML [168]. One fusion-oncogene that is common in human AML is the mixed lineage leukemia (MLL) oncogene, which results from 11q23 chromosome translocations. In the genetic context of MLL, mice transplanted with MLL-$Atg5^{+/-}$ leukemic blast cells die before any control mice start to show symptoms and MLL-$Atg5^{+/-}$ cells are characterized by elevated proliferation and excessive glycolytic metabolism [168]. This striking acceleration of disease due to autophagy impairment constitutes strong genetic evidence that reduced autophagic function can drive the progression of AML, possibly by augmenting Warburg like aerobic glycolysis [371]. Indeed, inhibition of glycolysis with 3-BrOP has been reported to have a synergistic effect with the autophagy inducer rapamycin in AML therapy [372], suggesting that autophagy may act to prevent Warburg-like metabolism necessary for leukemogenesis. This synergy between limiting glycolysis and inducing autophagy could be seen as preliminary evidence suggesting that the lipophagy-FAO-OXPHOS axis described here for neutrophil differentiation may also be relevant to induce differentiation and limit glycolysis in the context of myeloid (granulocytic) leukemia. Following from this, a strategy to overcome the leukemic differentiation block would likely involve induction of autophagy and the associated FAO-OXPHOS axis. However, autophagy can both act as a tumor suppressor by enabling differentiation and limiting proliferation and glycolysis of leukemic stem cells but also facilitate the survival of leukemic blast cells, and accordingly is often termed a “double edged sword” in the context of leukemia [363]. Inhibition of autophagy via chloroquine accordingly

is tested in multiple phase I/II studies on glioblastomas in combination with standard radiation therapy [373]. On the other hand, induction of autophagy mediated by the mTOR inhibitor temsirolimus is tested in a phase II study [374] in combination with clofarabine chemotherapy [375]. Autophagy activation may also be beneficial in some cases of Flt3-ITD positive AML, as suggested by a recent study that found a synergistic effect of the autophagy inducer metformin with the classical treatment sorafenib [376]. Interestingly, an independent study found direct evidence that Flt3-ITD itself can be targeted for autophagic degradation, offering a potential alternative mechanistic explanation for therapeutic efficacy [377]. Taken together, definitive proof of concept that impaired autophagy stratifies a group of myeloid disease with accelerated progression which is susceptible to autophagy inducers as a therapeutically relevant approach is highly desirable, but currently lacking. This proof of concept would have to involve a drug-like molecule that has proven therapeutic benefit *in vivo*, which is definitively mediated by autophagy activation. It might likely only be an effective strategy in a subgroup of patients that have impaired autophagy in the leukemic blast population, which could entail the possibility of patient stratification based on autophagy levels as part of recent efforts to implement personalized medicine approaches. A major challenge however is to definitively prove autophagy as the relevant mechanism of action, as compounds such as temsirolimus, metformin and chloroquine are often pleiotropic and non-specific. In this regard, suitable genetically defined models of AML must be combined with complete genetic deletion of autophagy to prove that the mechanism of action of such compounds indeed is via autophagy. Available cell culture systems in the host laboratory of MLL-ENL and Flt3-ITD immortalized lines +/- *Atg5/7* heterozygous and homozygous ablation could be used to test both of these assumptions: First, *Atg5/7* heterozygous deletion causes a more proliferative and glycolytic leukemia, which may be particularly vulnerable to targeting with autophagy inducers. These lines could therefore be used to screen autophagy modulating drugs, with read-

outs such as blast cell survival, proliferation and differentiation by multiplexed flow cytometry. Secondly, leukemia with *Atg5/7* homozygous deletion could be used to test whether autophagy induction is the essential mechanism for the treatment benefit of such a drugs (No treatment benefit can be mediated by complete interruption of autophagic machinery, enabling "counter-screening"). The goal of this approach would be to identify small molecule compounds that have a therapeutic effect *in vitro* and *in vivo* in a subpopulation of MLL-ENL or Flt3-ITD driven AML with diminished autophagy. The general concept and approach proposed here is also summarized in *Figure* 6.3.

6.5 Thesis summary and outlook

Neutrophils are the most abundant and shortest-lived mediators of immunity and therefore require constant replenishment from hematopoietic progenitors within the bone marrow. Their differentiation requires extensive nuclear remodeling into lobulated, polymorphonuclear cells and generation of diverse and abundant granules, but what governs these energy-consuming processes is unknown. While previous studies from our lab and others show that autophagy is required for differentiation of several blood and immune cell lineages [189], its function during granulopoiesis has remained elusive. Similarly, while many studies detail diverse mechanistic roles that autophagy may have in these settings, we are only beginning to understand the mechanisms that may be shared between some of those cell types. In parallel, metabolic control of differentiation is being discovered as a broader feature underlying several hematopoietic and immune cell differentiation events in recent years, and patterns between autophagy and metabolism begin to emerge that may point to common regulatory principles [87]. Here, evidence is presented that oxidative metabolism and autophagy are developmentally programmed and essential for neutrophil differentiation *in vivo*. *Atg7*-deficient neutrophil precursors had increased glycolytic activity but impaired mitochondrial

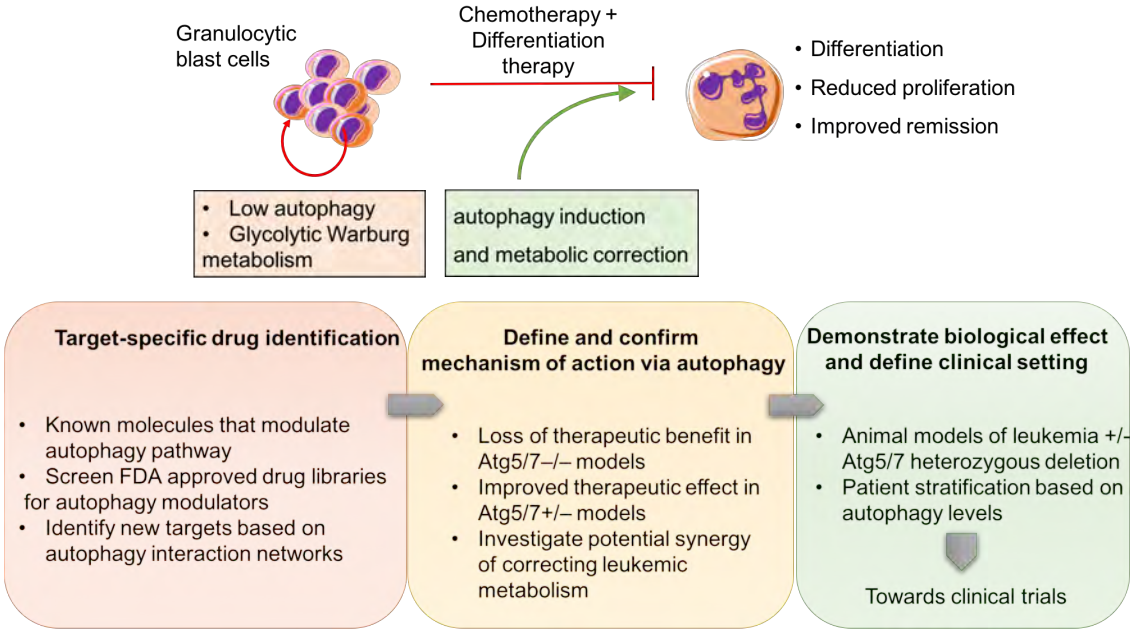

Figure 6.3: Theoretical concept of translation for leukemia therapy. Top: The simplified concept underlying this approach is a differentiation block in granulocytic precursors that is common to myeloid leukemia and *Atg5/7*-deficient animal models. In both cases, accumulation of granulocytic progenitor blasts with Warburg-metabolism is in common. To overcome this blockage, autophagy activation and metabolic correction may lead to treatments benefits such as improved/prolonged remission via LSC differentiation and reduced blast proliferation. This approach would be tested most likely in combination with current standard of practice chemotherapy. Bottom: The individual milestones of this process would be 1) The identification of suitable drug-like molecules that target the autophagy pathway to build a curated library of molecular candidates. 2) To confirm the mechanism of action through autophagy and select top-hits based on easily tractable, scalable and reproducible cell line models with autophagy gene deletions (heterozygous and homozygous) [168]. 3) To demonstrate primary/in vivo therapeutic benefit for the best-in-class molecules using leukemia patient bone marrow samples stratified for autophagy levels. Autophagy-stratified patient bone marrow samples could be transplanted into immunocompromised recipient mice to form leukemia and demonstrate (combination) treatment benefit in relation to standard chemotherapy alone. As a contingency or alternative, the leukemic lines from step 2 can be transferred directly into recipient mice to form leukemia and test drug activity. These data would form the basis for further development into clinical trials for a potential new drug that acts through autophagy and open the possibility for combination therapy approaches with metabolic modulators in current clinical trials [372].

respiration, decreased ATP production, and accumulated lipid droplets. Inhibiting autophagy-mediated lipid degradation or fatty acid oxidation alone was sufficient to cause defective differentiation, while administration of fatty acids or pyruvate for mitochondrial respiration rescued differentiation in autophagy-deficient neutrophil precursors. Together, this shows that autophagy-mediated lipolysis provides free fatty acids to support a mitochondrial respiration pathway essential to neutrophil differentiation. A simplified pattern in the myeloid and lymphoid cell types that require oxidative vs glycolytic mitochondrial metabolism has been suggested here and also by a recent literature review [161] and is in agreement with the model suggested here that also implements autophagy (Figure 6.2). Although it is tempting to speculate further about the underlying common principles of autophagy and metabolism in the control of differentiation, it is also important to highlight that there are many exceptions to this generalization. A deeper understanding of the mechanisms that connect autophagy with metabolism in the control of differentiation has the exciting potential to reveal a more sophisticated model that may eventually allow exploitation for therapeutic benefit.

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