

The contribution of bone marrow macrophages to Acute Myeloid Leukemia growth

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Cheerful break of day
The colourful flower blooms
Whilst watching the sun

Declaration of Originality

All of the work presented in here is my own, generated in Pr Cristina Lo Celso's laboratory at Imperial College London, except where clearly stated otherwise. When data was generated by or in collaboration with others, I acknowledge it within the thesis and here:

- Dr Delfim Duarte and Dr Olufolake Akinduro generated the MLL-AF9 cell batches.

Flora Birch

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Abstract

Acute myeloid leukemia (AML), is one of the deadliest forms of cancer. It has been relying on chemotherapy as its primary treatment for the past three decades. However, relapse after therapy (50-90%) leads to the development of an aggressive form of leukaemia, resistant to current treatment. While immunotherapies are on the rise to treat most cancers, its use in AML is highly challenging due to the lack of tumour cell signature and increased side effects. A better understanding of the disease and its interaction with the immune system is essential to develop targeted therapies with lower side effects and prevent relapse occurrence.

Using a combination of microscopy and high-dimension flow analysis, I identified two functionally and phenotypically distinct populations of macrophages, interacting with malignant cells. The first population named Haematopoiesis-supportive macrophage (HSM), expressed Vascular cell adhesion molecule 1 (VCAM-1), a marker associating them with the retention of healthy haematopoietic cells. These cells were found to be lost first during AML. The second population, AML-associated macrophages (AAMs), expressed Signal regulatory protein α (SIRP α) and Programmed death-ligand 1 (PDL-1), two markers important in the inhibition of the immune response in cancer. These cells were found to be enriched at early AML. Combining these results with single cell RNA-sequencing and functional *in vitro* assays, I found that AAMs were efferocytic cells, capable of phagocytosing AML cells and inhibiting activated T cells. By targeting these cells using different genetic and pharmacological techniques, I was able to show that the depletion of AAMs led to reduced AML growth while the accumulation of AAMs was associated with faster AML growth.

Together, this thesis provides an insight into the phenotype and function of different macrophage populations present in the bone marrow and their involvement in AML. This work opens up new avenues for further research and novel therapeutic targets for immunotherapy.

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Abbreviations

3D 3-dimensional.

AAM AML-associated macrophage.

AML Acute myeloid leukemia.

Ang1 Angiopoietin-1.

APC Antigen presenting cell.

APL Acute promyelocytic leukaemia.

BM Bone marrow.

BMDM Bone marrow derived macrophages.

CCL12 C-C motif chemokine ligand 12.

CCL2 C-C motif chemokine ligand 2.

CCL6 C-C motif chemokine ligand 6.

CCL9/10 Macrophage inflammatory protein 1 gamma.

CCR1 C-C chemokine receptor type 1.

CCR2 C-C chemokine receptor type 2.

CDP Common dendritic cell progenitor.

CMP Common myeloid progenitor.

CR Complete remission.

CRi Complete remission with incomplete count recovery.

CRT Calreticulin.

CSF1R Colony stimulating factor 1 receptor).

CTV Cell trace violet.

DAMP Damage-associated molecular pattern.

DAPI 4,6-diamidino-2-phenylindole.

DARC Duffy antigen/chemokine receptor.

DEG Differentially expressed genes.

DMEM/F12 Dulbecco's modified eagle medium/nutrient mixture F-12.

DMSO Dimethyl sulfoxide.

DT Diphtheria toxin.

EBI Erythroblastic island.

EDTA Ethylenediaminetetraacetic acid.

EdU 5-ethynyl-2'- deoxyuridine.

eGFP Enhanced green fluorescent protein.

Emp Erythroblast macrophage protein.

EMR1 EGF-like module containing mucin-like hormone receptor 1.

EMT Epithelial to mesenchymal transition.

FAB French-american-british.

FACS Fluorescence activated cell sorting.

FBS Fetal bovine serum.

FCS Fetal calf serum.

FDA Food and drug administration.

FlowSOM Flow self-organizing map.

FLT3 Fms-like tyrosine kinase 3-mutated.

FMO Fluorescence minus one.

GFP Green fluorescent protein.

GMP Granulocyte monocyte progenitor.

hIL-6 Human interleukin-6.

HSC Haematopoietic stem cell.

HSM Haematopoiesis-supportive macrophage.

HSPC Haematopoietic stem and progenitor cell.

i.p. Intraperitoneally.

i.v. Intravenously.

IMM Immunomodulatory Macrophage.

IRF7 Interferon Regulatory Factor 7.

IVM Intravital microscopy.

KL Kit-ligand.

KMT2A Lysine [K]-specific methyltransferase 2A.

KO Knock-out.

L-Glu L-glutamine.

LCNV Laser-induced choroidal neovascularization.

LPS Lipopolysaccharide.

LSC Leukemia stem cell.

LT-HSC Long term HSC.

LysM Lysozyme M.

LYVE-1 Lymphatic vessel endothelial hyaluronan receptor 1.

M-CSF Macrophage colony-stimulating factor.

m-SCF Murine Stem cell factor.

MaFIA Macrophage fas induced apoptosis.

mCXCL12 Murine C-X-C motif chemokine ligand 12.

MDP Monocyte-dendritic cell precursor.

MDS Myelodysplastic syndrome.

MEP Megakaryocyte-erythroid progenitor.

MFG-E8 Milk fat globule-EGF factor 8.

MGL Macrophage galactose lectin.

MHC Major histocompatibility complex.

mIL-3 Murine interleukin-3.

MLL Mixed lineage leukemia 1.

MPS Mononuclear phagocyte system.

mTmG Membrane-tomato/membrane-green.

NDD Non descanned detector.

NSCLC Non-small cell lung cancer.

NSG NOD scid gamma.

OCT Optimal cutting temperature.

PBS Phosphate buffer saline.

PDL-1 Programmed death-ligand 1.

PEG Polyethylene glycol.

PFA Paraformaldehyde.

PolyI:C Polyinosinic:polycytidylic acid.

PS Phosphatidyl serine.

PTH Parathyroid hormone.

RES Reticuloendothelial system.

RNA Ribonucleic acid.

ROI Region of interest.

ROS Reactive oxygen species.

RPMI Roswell park memorial institute.

s.e.m. Standard error of mean.

sAML Secondary AML.

SCID Severe combined immune-deficient.

scRNAseq Single-cell RNA sequencing.

SIRP α Signal regulatory protein α .

T-ALL T-cell acute lymphoblastic leukemia.

t-SNE T-distributed stochastic neighbor embedding.

TAM Tumour associated macrophage.

TGF β Transforming growth factor β .

TIL Tumour infiltrating leukocytes.

Tim4 T-cell immunoglobulin and mucin domain containing 4.

TLR Toll like receptor.

TMEM Tumour MicroEnvironment of Metastasis.

TNF α Tumour necrosis factor α .

Treg Regulatory T cell.

TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling.

UMAP Uniform manifold approximation and projection.

VCAM-1 Vascular cell adhesion molecule 1.

WHO World health organisation.

WT Wild-type.

YFP Yellow fluorescent protein.

Chapter 1

Introduction

1.1 Homeostasis of bone marrow macrophages

1.1.1 Origin and heterogeneity of macrophages

The history of macrophages

Macrophages were first identified by Ilya Metchnikoff at the end of the 19th century. Metchnikoff was a great scientist and evolutionist. His ideology applied Darwin's theory of evolution at the scale of an organism and stated that cell competition within an organ was generally favorable to the organism [2]. His interest in macrophages stemmed from the study of embryogenesis in starfish larvae, where he first observed that certain cells were capable of ingesting to feed themselves and protecting others from invaders. He named these cells phagocytes after the Greek *-phagos* "to eat/devour" and *-cyt* "cell" i.e. "the devouring cell". The phagocytosis theory was born and became his primary research focus. By studying the role of phagocytes in tadpole's tail disintegration and wound repair, Metchnikoff highlighted the importance of phagocytes in organ structure and inflammation while revealing their unchanged potential through evolution and hence their necessity for the normal development of the organism [3]. Interestingly, this indicated that right from the day of their discovery, phagocytes were described as having multiple roles in the organism. Specifically these cells were crucial for tissue re-absorption during embryogenesis and host defence against pathogen, highlighting their dual function in tissue homeostasis and inflammation.

Following this great discovery, Metchnikoff distinguished phagocytes into two categories, based on their sizes: macrophages and microphages [4] (from the Greek, *-macro* "large" or

-*micro* “small” and -*phagos* “to eat/devour” giving rise to “large eaters” and “small eaters”). However, later research in the early twentieth century revealed that the term “macrophage”, as defined by Metchnikoff, was rather vague and did not acknowledge for the differences in morphology and tissue locations of these cells. At that point in time, scientists had come up with over 30 different cell names to characterise macrophages based on their morphology and tissue locations [5].

It was in 1924 that Karl Albert Ludwig Aschoff, in an effort to systematically identify and characterise tissue cells, came up with a unifying definition of macrophages. Using a novel dye staining, this group of cells was described as the “[Reticuloendothelial system \(RES\)](#)” based on their ability to incorporate vital dyes [6], irrespective of the tissue the cells were located in. The reticulo part refers to the network formed by the cells’ cytoplasmic extensions, while the endothelial name was given based on their proximity to the vascular endothelium. The major function of the RES was thought to be the clearance of unwanted debris or particles. The main cells comprised in the RES were Kupffer cells, microglia, osteoclasts and alveolar macrophages, all considered tissue resident macrophages. As such this system characterises the first definition of tissue resident macrophages [4]. However, the origin of these cells remained a mystery.

History of the ontogeny of macrophages

Towards the last half of the twentieth century, when immunologists were able to characterise immune cells using more advanced techniques such as electron microscopy and the first flow cytometers, it was believed that the pool of tissue resident macrophages was continuously replenished from monocyte produced in the bone marrow. As such, in 1969, at a scientific symposium, Van Furth and his colleagues agreed that the term RES was outdated as it did not describe resident phagocytes and their antecedents. Following a common agreement, this definition evolved to the term “[Mononuclear phagocyte system \(MPS\)](#)” with the aim of describing tissue resident phagocyte cells and their progenitors. It comprised three main cell types, monocytes, macrophages and dendritic cells [7]. Van Furth defined the promonocyte as the earliest identifiable cell in the MPS but recognized that there must be a precursor cell feeding into this pool of cells [8]. It is only in 2006 that a clonogenic progenitor for macrophages, monocytes and dendritic cells was identified in the bone marrow [9]. In this study, Fogg et al. adoptively transferred [Monocyte-dendritic cell precursor \(MDP\)](#)s and following irradiation, found that these cells were able to give rise to monocytes, tissue resident macrophages and dendritic cells.

Following these findings, several precursors were identified, the most commonly referred to being the [Common dendritic cell progenitor \(CDP\)](#), expressing lower stem cell marker (cKit) than MDP. Overall, these results showed that monocytes, under stress, can differentiate into tissue resident macrophages however it remained unclear to what extent monocytes differentiated into tissue resident macrophages in homeostasis.

The ontogeny of tissue resident macrophages

In parallel to these findings, various studies showed that certain pools of tissue resident macrophages resisted high doses of irradiation and were capable of regenerating their own pool of macrophages independently of bone marrow precursors [10, 11, 12]. M. Merad et al. [10] demonstrated that, in lethally irradiated mice, transplanted with bone marrow cells expressing a different allele of CD45 (CD45.2), the original skin resident dendritic cells, also referred to as Langerhans cells, remained up to 18 months after irradiation and transplantation. Next, they compared the results from the skin with results from other organs and found that most dendritic cells located elsewhere were replaced with bone marrow derived cells (expressing CD45.2) within 2 months. However if the skin was exposed to ultraviolet light, Langerhans cells were immediately replaced by CD45.2+ BM derived cells indicating that, in homeostasis, Langerhans cells are capable of maintaining their pool of cells however in a stress induced environment, this pool of cells is replenished thanks to BM precursors [10]. Hence this work highlights the variability of the contribution of BM precursors to tissue resident macrophages and dendritic cells.

The development of parabiotic mice also allowed scientists to understand which pool of tissue resident macrophages was replenished by blood circulating monocytes. In this experimental model, two mice, either with different CD45 allele or expressing different fluorescent proteins are surgically attached to share the same blood system. Cells in the blood are mixed within two weeks and the contribution of monocytes to each pool of tissue resident macrophages can be calculated by measuring the percentage of cells expressing the respective mouse marker. M. Merad et al. [10] found that Langerhans cells from the [Green fluorescent protein \(GFP\)](#)- mouse did not get replaced by any GFP+ cells for at least 6 months in homeostasis. Various other studies using a similar approach have shown the same result for microglia [12] and alveolar macrophages [13] while tissue resident macrophages from the gut, dermis and heart showed some degree of mixing between GFP- and GFP+ cells indicating a contribution from BM pre-

cursors [14, 15, 16, 17].

Given the degree of diversity observed with regards to the ontogeny of tissue resident macrophages according to their location, a systematic approach understanding the contribution of BM precursors to the pool of tissue resident macrophages for each tissue was essential. S. Yona, et al. [18] developed an elegant combination of transgenic mice allowing the systematic fate mapping of all tissue resident macrophages. First, they identified a target gene, CX3CR1, highly expressed in the MPS, by BM precursors and monocytes but whose expression was absent in tissue resident macrophages. Indeed, CX3CR1 had previously been reported to be widely expressed by MPS and MDP and had a relatively low expression in tissue resident macrophages. By crossing CX3CR1-GFP mice with CX3CR1-Cre:R26-YFP, they were able to identify which macrophage population arose from CX3CR1 expressing precursor cells. Three tissue resident macrophage populations constitutively expressed CX3CR1: microglia, intestinal macrophages and kidney macrophage. The remaining tissue resident macrophages were GFP- and showed various levels of Yellow fluorescent protein (YFP) indicating that these cells were derived from precursor CX3CR1+ cells. However to find out whether these precursor cells gave rise to tissue resident macrophages during embryogenesis or during adulthood, and hence understand if the tissue resident macrophages constituted an independant pool of cells with self-renewal capacity, it was necessary to use an inducible CX3CR1 reporter mouse. The Cre-ERT2 fusion protein developed by the Jax laboratory is a synthetic system whereby the Cre recombinase is dependant on the administration of tamoxifen. Hence in the case where CX3CR1-CreERT2:R26-YFP mice were used, YFP was expressed in CX3CR1-expressing cells after tamoxifen treatment. Importantly, when mice were treated after birth, Kupffer cells, peritoneal, splenic, and lung macrophages were all YFP- indicating that these cells were established before birth and maintained themselves independently of monocytes during steady state. Interestingly, further studies have shown that macrophages are present in embryos as early as day 10.5, even before stem cells are generated [11, 19]. Hence macrophages might represent a lineage of cells independent of the HSC system. Within the embryonic precursors, it has been shown that tissue resident macrophages can derive from both yolk sac cells and fetal liver cells with the contribution of each remaining a controversy [20]. While these studies have not addressed the origin of bone marrow macrophages, bone marrow hematopoiesis does not start until the very end of gestation and as such the bone marrow environment is not colonised until birth. Therefore it is most likely that the yolk sac and fetal liver macrophages represent only a minimal contribution to bone marrow macrophages. Overall, it would interesting to perform

some fate mapping experiments to understand the contribution of the yolk sac, fetal liver and the bone marrow to bone marrow macrophages.

Altogether, in light of these studies it became apparent that two pool of macrophages existed in most tissues, tissue resident macrophages generated early on during embryogenesis and capable of self renewal during homeostasis and infiltrating macrophages generated through the differentiation of blood circulating monocyte in response to certain stress. This raised the question as to whether the varied origin of macrophages led to different macrophage functions.

Tissue resident macrophage function: phagocytosis and efferocytosis

The main characteristic identifying macrophages and more generally the MPS is the fact that these cells are considered professional phagocytes [21]. As such, they express a high number of receptors that bind to ligands absent from healthy cells but expressed by opsonized pathogens, dying cells and cell debris. This ligand-receptor interaction allows macrophages to recognize and phagocyte these particles. The receptors expressed by macrophages can be divided into 6 main families, each responsible for the recognition of one specific aspect of foreign or apoptotic bodies.

One well known family is the scavenger receptor superfamily which binds to [Damage-associated molecular pattern \(DAMP\)s](#), including [Pipopolysaccharide \(LPS\)](#) expressed on bacteria [22]. Another family, the mannose receptor family is known to bind glycoproteins expressed by certain microorganisms including but not limited to yeast and certain bacteria [23]. Finally, the MerTK family is responsible for the recognition of apoptotic cells, a process called efferocytosis. These different families of receptors lead to different signalling cascade in turn shaping the activation profile of macrophages. Indeed, thanks to these numerous receptors, macrophages are capable of distinguishing apoptotic bodies from foreign invaders, and inducing efferocytosis or phagocytosis and transmit inflammatory or anti-inflammatory signalling (See [Figure 1.1](#)) [24].

During homeostasis, the first activation step following the binding of these receptors is the triggering of the engulfment process of internalised particles. During the engulfment, the internalised particle is encapsulated and forms a phagosome. Next, the phagosome fuses with endosomes containing [Toll like receptors \(TLRs\)](#). These TLRs recognise foreign particles, nec-

essary for the pathogens. Each TLR detects a specific particle present on only one type of pathogen, TLR4 recognizes Gram- bacteria while TLR5 is able to bind flagelin and TLR3 can recognize dsRNA. The specificity and combination of these interactions allows the shaping and relay of the inflammatory signalling cascade. Eventually, following the fusion of lysosomes containing lysozymes and proteases with the phagosome, the phagosome will mature into a phagolysosome, which has been shown to be highly hydrolytic and responsible for the production of **Reactive oxygen species (ROS)** and O_2^- [25]. The pH decreases steadily towards a pH of 4.5 [26]. Collectively these factors generate a highly microbicidal environment where the pathogens will get degraded releasing ligands and antigens, which get processed and loaded on **Major histocompatibility complex (MHC)** class II molecules. Altogether through phagocytosis, the macrophage is capable of sensing the shape of the internalised particle, its viability status and the threat associated with it.

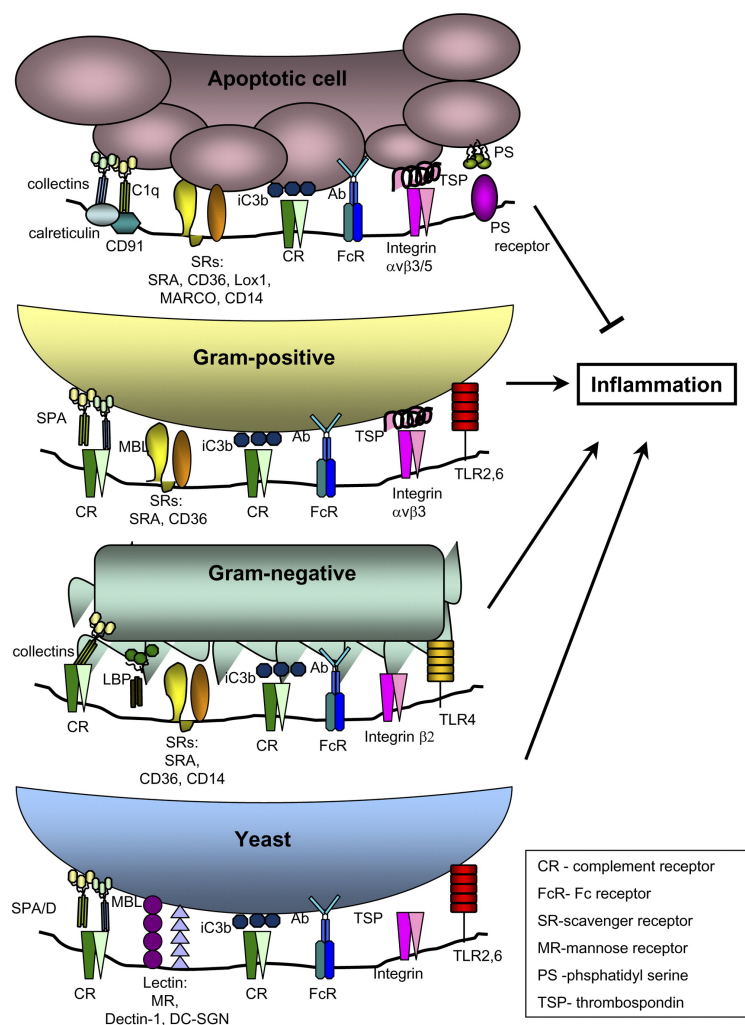


Figure 1.1: Common phagocytic receptors expressed on macrophages

The broad range of phagocytic receptors allows macrophages to recognize what type of particles is being phagocytosed. In turn the transduction of the signal will lead to either inflammatory or anti-inflammatory cytokines to be produced.

While phagocytosis is considered one of the main functions of macrophages, these cells are still capable of transmitting the presence of microorganism without phagocytosis. Indeed, they can detect fragments of degraded pathogens which will bind to receptors at the surface of their membrane and activate a signalling cascade triggering the secretion of inflammatory cytokines.

Tissue resident macrophage activation during homeostasis

As the macrophage function is defined around its phagocytic potential and its capacity to process many different unwanted particles ranging from highly toxic bacteria to cell debris, the macrophage response and activation has to be shaped accordingly. Overall, it is agreed that tissue resident macrophages, during homeostasis, have intrinsic anti-inflammatory functions, associated with efferocytosis. L. Smythies, et al. [27] showed that intestinal macrophages were capable of phagocytosis but did not release any inflammatory cytokines in response to phagocytosis. In addition, they found these cells to lack the expression of innate response receptors to LPS and other pathogens. A. Bujko, et al. [28] performed transcriptional analysis on gut macrophage subsets and found that all gut macrophages were efficient at endocytosis while displaying reduced response to proinflammatory signalling. In the liver, C. Deppermann, et al. [29] showed that Kupffer cells regulate platelet count by phagocytosing aged platelet in a [Macrophage galactose lectin \(MGL\)](#) dependant manner. Meanwhile, Q. You, et al. [30] hypothesized that Kupffer cells were important in the maintenance of an immune tolerant microenvironment in the liver during homeostasis. After evaluating the expression of antigen presentation related molecules on the surface of Kupffer cells and dendritic cells, they found that Kupffer cells not only had a lower expression of these receptors but were also capable of inhibiting dendritic cell-induced T cell activation. Together, these findings highlight the importance of reduced inflammation signalling in certain tolerogenic organs in response to phagocytosed particles during homeostasis.

However, in cases where macrophages encounter toxic pathogens, phagocytosis of the harmful pathogen leads to the activation of the macrophage and the initiation of an immune response. After engulfment of a pathogen, toll-like receptors combined with other receptors will recognize what kind of particle has been phagocytosed and transduce the signal to activate the NF-Kb pathway which will translocate to the nucleus and lead to the transcription of pro-inflammatory cytokines including $\text{TNF}\alpha$, IL-6 and IL-12 [31]. In parallel, peptides from the pathogen will be

presented on the MHCII group and co-stimulatory molecules including CD80 and CD86 will be expressed at the surface of the membrane in turn activating T cells [32].

Thus, phagocytosis can lead to different activation states of macrophages according to the phagocytosed particle and local environment. On the whole, macrophages are highly plastic and their activation can be classified along a spectrum varying from what we nowadays refer to as the classically activated (pro-inflammatory M1) or alternatively activated (previously called anti-inflammatory M2) profile. It is important to note here that the activation profile is not terminal and cells are able to switch from one activation profile to another based on the signaling present in their environment [33]. Classically activated macrophages are usually stimulated by the local secretion of $\text{IFN}\gamma$ and $\text{TNF}\alpha$, first transiently produced by NK cells and then later on by T helper cells. *In vitro*, macrophages can also adopt this activation profile in response to LPS [34]. In turn, classically activated macrophages will produce pro-inflammatory cytokines like IL-6, IL-1 and IL-17 promoting the adaptive T cell response and the recruitment of polymorphonuclear leukocytes. Alternatively activated macrophages are generated in response to IL-4 and IL-10 produced primarily by mast cells and basophil and will help resolve the inflammation and promote wound healing. In the context of parasite infection, this process is essential to control the damage generated by the parasite migration through various organs. Once activated these macrophages will help generate extracellular matrix and suppress T cell expansion [35]. In addition to IL-4, glucocorticoids are also responsible for the alternative activation of macrophages which usually reflects of a response to stress and leads to the production of TGF-beta [36]. Finally, the end of the adaptive immune response, highlighted by the presence of Tregs and secretion of IL-10 can lead to macrophage adopting an alternatively activated profile [37].

Hence, macrophages are capable of adopting many different profiles in response to different stresses. However, whether the origin of the tissue resident macrophages influences the activation profile adopted by these cells remains an unanswered question that needs to be addressed to fully understand macrophages.

Identification of macrophages

Overall, it is apparent that tissue resident macrophages exert a function specific to the need of the organ they are located in. Local cell extrinsic factors are capable of activating various transcriptional programs leading to mature tissue resident macrophages [38]. Interestingly, L. Van de Laar, et al. [39] demonstrated that the local microenvironment is a key regulator of the phenotype of tissue resident macrophages, out-weighting the origin of the tissue resident macrophages progenitor. Using *Csf2rb*^{-/-} mice which lack alveolar macrophages, they transplanted these mice with progenitors from the yolk sac, foetal liver and adult monocyte and found that all three sources led to mature functional alveolar macrophages. After analysing their transcriptional profile before and after transplantation, they found that the initial gene expression was very distinct between the three cell types but following transplantation all three sources of macrophages adopted an undistinguishable alveolar macrophage gene signature [39]. Hence macrophage progenitors are capable of remarkable plasticity and adaptation according to the local cues to generate mature functional tissue resident macrophages. In line with these results, Y. Lavin et al.[40] also performed similar studies where they transplanted peritoneal macrophages into the lung and found that the cells were shaped by the local microenvironment and were reprogrammed toward a lung macrophage profile.

By identifying the transcriptional pathways associated with tissue resident macrophage function, we are capable of using them as a reference and identifying these cells within each organ. Interestingly, when identifying these cells by flow cytometry, the markers associated with tissue resident macrophages vary greatly from one organ to the next, and even within one organ, several populations of tissue resident macrophages with different functions tend to co-exist. Z. Liu et al, generated a nice comparative analysis of flow cytometry gating of murine myeloid populations including macrophages in the liver, gut, brain, peritoneal space, blood, kidney, dermis, epidermis, lung and spleen but unfortunately, they did not include a BM analysis in this comparison. This work highlights the fact that for each organ, each myeloid population is expected to express a different combination of markers [41]. However, in the case of tissue resident macrophages, all populations no matter the organ they are located in are identified based on their expression of F4/80 and CD11b and the lack of expression of Ly6G. Certain tissue resident macrophages are also identified thanks to their expression of Tim4, CD64 and MHCII. Discovered in 1981, F4/80 is considered the pan macrophage marker, expressed by most tissue resident macrophages and absent from fibroblasts and lymphocytes

[42]. Eosinophils and monocytes also express this marker but can be excluded based on their expression of other markers [43]. The human homolog of F4/80 is [EGF-like module containing mucin-like hormone receptor 1 \(EMR1\)](#), which however was found to be expressed exclusively by eosinophils [44].

Mouse models for the study of macrophages

Different approaches are possible to study macrophages according to the experimental set-up. The two main ways to address the role of macrophages *in vivo* is either to deplete them or to label them. Depletion can be achieved either by using genetic models or using the fact that macrophages are professional phagocytes. Classified as a biological agent, clodronate liposomes were developed in 1994 by N. Van Rooijen, et al. [45], and consist of a liposomal bilayer encapsulating clodronate. Macrophages, dendritic cells and monocytes are the only cells capable of phagocytosing these particles. Once the liposomes are engulfed in a phagosome, they fuse with endosomes and lysosomes, leading to reduced pH. Next, the liposome membrane will dissociate releasing dichloromethylene-bisphosphonate (Cl2MBP or clodronate). This molecule will act as an ATP analog, inhibiting mitochondrial oxygen consumption and leading to competitive inhibition of the ADP/ATP translocase. In turn, this leads to the mitochondrial membrane potential collapse and finally the apoptosis of the cell [46]. Unfortunately, since this tool targets phagocytes, dendritic cells and monocytes will also be affected.

Concerning genetic models, due to the high level of gene expression overlap between phagocytic mononuclear cells, most genetic macrophages-ablating model also target monocytes and neutrophils [47, 48]. Most of the genetic approaches described below rely on the Cre Recombinase system coupled with Rosa 26 inducible Diphtheria Toxin Receptor (R26-DTR). The Cre gene is knocked in downstream of the promoter expressed in the cells of interest. Once translated, the Cre recombinase will remove the stop codon upstream of the DTR leading to its expression on the membrane of the cell. When the diphtheria toxin is administered, it will bind to its receptor, enter the cell and result in cell death [49]. In the case of a Cre-ER inducible system, Cre recombinase translocation to the nucleus is dependent on tamoxifen treatment generating a temporally controlled depletion system. The Cre recombinase system can also be coupled to Rosa 26 -eYFP (R26-eYFP). This allows the specific expression of eYFP in cells expressing the promoter under which the Cre is controlled and hence generating a genetic macrophage labelling system.

To target macrophages based on their preferential expression of genes, scientists considered the markers used by flow cytometry to identify these cells. Among the main markers highly expressed by macrophages are F4/80, CD11b and CD169, the latter being more specifically expressed in subsets of tissue resident macrophages. C. Abram, et al. [48] studied the various myeloid Cre deleting mouse models, their specificity and efficiency. By crossing the Cre reporter with a R26-eYFP mouse, they were able to assess which cells were being deleted. The F4/80-Cre model was rather disappointing as only 50% of peritoneal macrophages expressed eYFP and no other tissue resident macrophage population expressed the fluorescent protein. The CD11b-Cre model labelled most macrophages however, it wasn't specific for macrophages as most myeloid cells were labelled and even some lymphocytes expressed the fluorescent protein. The [Lysozyme M \(LysM\)](#)-Cre model was a promising model with the insertion of a cre cDNA following the M Lysozyme locus [50]. However, this mouse model also deletes neutrophils. Finally, the CD169-Cre mice generated by M. Tanake [51] allowed for the depletion of specific subsets of tissue resident macrophages, in particular the marginal metallophilic macrophages and marginal zone macrophages in the spleen. Paul Frenette's et al. described this model to also successfully deplete tissue resident macrophages in the BM while maintaining the pool of monocytes and neutrophils [52].

The [Macrophage fas induced apoptosis \(MaFIA\)](#) reporter mouse line generated by S. Burnett [53] was built using a different genetic approach whereby a GFP cassette and the human suicide gene, Fas, was inserted downstream of the *c-fms* promoter. The GFP cassette revealed that most phagocyte mononuclear cells but not lymphocytes were targeted in this construct. When the suicide gene is induced with the synthetic molecule AP20187, the cytoplasmic Fas fragments are activated and lead to apoptosis of the transgene-expressing cells [53]. Only the GFP+ cells were depleted using this approach.

Alternatively, macrophage depletion can be achieved by depleting monocytes or macrophages progenitors prior to their study. In the case where monocytes are targeted, only monocyte-derived macrophages will be deleted. Two possible mouse lines could be used: CSF1R-Cre or [LysM](#)-Cre mice [47]. As most monocytes and some macrophages express CSF1R (CD115), crossing these mice with R26-iDTR (CSF1RxR26-iDTR) could allow targeted depletion of both monocytes and macrophages. The [LysM](#)-Cre model, while targeting all phagocytes mononuclear cells [54, 48], has been reported to have some activity in [Haematopoietic stem and pro-](#)

genitor cells (HSPCs) and Megakaryocyte-erythroid progenitor (MEP) [55] excluding the use of this model to study the impact of macrophages on haematopoiesis. To target more generally macrophage progenitors, the CX3CR1 mice have been commonly used [18, 56, 57, 58]. Indeed, macrophages progenitors express high levels of CX3CR1 and are produced in two steps in the yolk sac and foetal liver [59]. Hence, it is important to use an inducible system like the CX3CR1-CreER mice crossed R26-iDTR to deplete the precursor cells at both stages of development. The main concern with this approach is the possible toxicity of the model as macrophages are crucial for embryonic development and liver function.

Overall, most macrophage mouse models were used to identify macrophages that were not located in the bone marrow, hence we can only hypothesize whether these models would be appropriate for the study of bone marrow macrophages. Combining these results with known flow cytometry markers, it seems that the MaFIA mice and the CD169-Cre mice are the most likely to highlight bone marrow macrophages and allow the most specific depletion of these cells. Hence, in my project, I will investigate whether the two models are appropriate models for the study of bone marrow macrophages.

1.1.2 Role of tissue resident macrophages in the BM

The BM environment and haematopoietic system

The BM is responsible for the generation of all mature blood cells. Through asymmetric division, HSCs, residing at the top of a structured hierarchy, are capable of self renewal and generating differentiated progenitors. These progenitors will be able to proliferate and generate fully differentiated haematopoietic cells. The potential of a single HSC to give rise to all mature blood cell lineages has been demonstrated in the 60s and is well accepted [60, 61]. One question remains from this theory: what controls stem cell fate and guides the cell to achieve the right balance between self-renewal or differentiation? In 1978 R. Schofield hypothesized that HSC fate was controlled by a cell-extrinsic mechanism. He introduced the notion of the niche as “the stem cell is seen in association with other cells which determines its behavior” [62]. The components of the niche, defined as the elements responsible for HSC regulation have now been extended to include stromal cells, mature haematopoietic cells, the extracellular matrix, nervous system, hormones and blood vessels [63]. Among these components are endothelial cells, found in close proximity to HSCs and who, by expressing high levels of VCAM1 interact with VLA4 expressed at the surface of HSCs and promote their quiescence [64]. Other fac-

tors including SCF1, CXCL12 and E-selectin are expressed by endothelial cells and regulate HSC fate [65]. Disruption of endothelial cells and blood vessels leads to the loss of quiescent HSCs [66, 67]. Equally important, stromal cells are known to regulate HSC proliferation and differentiation. Indeed, mesenchymal stromal cells (MSC) are capable of differentiating into adipocytes and osteoblasts. These cells, although the mechanism of their contribution remains controversial [65], have been shown to control HSC fate as depletion of several factors expressed by osteoblasts leads to the loss of HSCs [68, 69]. The third major component of the HSC niche are immune cells. Both the innate and the adaptive compartment of the immune system have been described to control HSC fate [63]. A subpopulation of regulatory T cells regulate HSC quiescence and their reduction leads to increased number of HSCs in the BM [70]. Within the innate immune compartment various macrophages have been associated with HSC regulatory functions [63]. However, the lack of studies looking at their molecular signatures limits our ability to assess the diversity of bone marrow macrophage populations and whether certain functions are associated with different macrophages subtypes. With this in mind, I will review the current knowledge on BM macrophage function and associated signatures.

Bone-lining macrophages (osteomacs)

During homeostasis, tissue resident BM macrophages are believed to play an important role in the regulation of erythropoiesis and the retention of HSCs. One macrophage population, osteomacs, located adjacently to the endosteal bone surface has been shown to regulate bone formation. These bone lining cells are found in close contact to osteoblasts. M. Chang, et al. [71] found that the systemic depletion of macrophages using the MaFIA mouse model led to the complete loss of mature osteoblasts. In addition, removing these cells from osteoblast culture led to reduced bone mineralization. Later on, another study [72] showed that early macrophage depletion using the *LysM* mouse model led to early skeletal growth retardation, reduced bone marrow mesenchymal progenitor cells and reduced bone mineral density leading to progressive osteoporosis. Interestingly, S. Cho, et al. [73] compared the effect of depleting macrophages using the MaFIA mouse model or clodronate liposomes and found that these treatment had opposite effect on bone mass and *Parathyroid hormone (PTH)*-induced trabecular bone anabolism. Indeed, in the case of macrophage depletion using the MaFIA mice model, the bone mass was reduced and *PTH* led to reduced bone anabolism. However, following clodronate treatment, the mice bone mass was increased and displayed increased *PTH*-induced anabolism. When

comparing both approaches for macrophage depletion, although the magnitude of macrophage depletion was similar, clodronate liposome led to an increase in the fraction of CD68+ and CD163+ macrophages, associated with phagocytosis. Hence they hypothesised that clodronate stimulated the MPS, in turn increasing the number of CD68+ CD163+ macrophages. [Terminal deoxynucleotidyl transferase dUTP nick end labeling \(TUNEL\)](#) staining revealed reduced apoptotic cells in clodronate treated mice compared to MaFIA mice, correlating with the hypothesis that there was increased phagocytosis taking place. Finally, the remaining macrophages in clodronate treated mice had upregulated genes associated with tissue repair and regeneration. The authors suggested that clodronate liposomes led to increased efferocytosis, and stimulation of M2 macrophage polarization responsible for microenvironmental changes including increased bone mass. Overall, this study highlights the likelihood that various macrophage populations reside in the BM space, most likely having different functions and sensitive to different depletion model.

HSC-retaining macrophages

The endosteal niche is also known to contain HSCs. The importance of osteomacs in regulating the local microenvironment including osteoblasts has pushed scientists to investigate whether osteomacs and more generally tissue resident macrophages are involved in HSC retention. I. Winkler, et al. [67] showed that following the loss of osteoblasts induced by macrophage depletion (defined as CD11b+, F4/80+, Ly6G-), there was a decrease in osteocalcin, [Angiopoietin-1 \(Ang1\)](#), and [Kit-ligand \(KL\)](#), all considered HSC supporting factors. In turn this led to increased mobilization of HSCs to the blood quantified using colony forming assay. Meanwhile A. Chow, et al. [52] focused on CD169+ bone marrow macrophages and found that depletion of these cells using the CD169-DTR mouse model led to reduced production of CXCL12 by stromal cells. This correlated with increased mobilisation of HSCs to the blood. Using clodronate liposomes, they were able to recapitulate these results and quantified the expression of HSC retention factors in a population of stromal cells, the Nestin+ cells, and found that CXCL12, Ang1, [KL](#), and VCAM1 were reduced. They proposed that BM macrophages secreted a factor inducing the secretion of HSC retaining factors by stromal cells.

Together these studies describe macrophages as a key component in the retention of HSCs in the BM. Correlating with these findings, BM macrophages have also been shown to be directly associated with HSC quiescence. Indeed, J. Hur, et al. [74] showed that through

the expression of [Duffy antigen/chemokine receptor \(DARC\)](#), macrophages can interact with the CD82/KAI1 receptor found at the surface of [Long term HSCs \(LT-HSCs\)](#) and maintain their quiescence. Depletion of [DARC](#) expressing macrophages using clodronate led to reduced expression of CD82 at the surface of HSCs, in turn leading to increased cell cycle entry, proliferation and differentiation. Finally E. Heideveld, et al. [75] co-cultured human blood CD14+ monocytes/macrophages with CD34+ HSCs and found that CD14+ cells increased the survival of HSCs but not their proliferation.

[Erythroblastic island \(EBI\) macrophages](#)

Within the BM space, macrophages have also been shown to form erythroblastic islands, a structure specific to the BM where a central macrophage is surrounded by erythroid precursors going through erythropoiesis. Erythropoiesis leads to the production and maturation of 10^{10} erythrocytes every hour in humans. Central macrophages have been shown to be essential for this process to take place. [Erythroblast macrophage protein \(Emp\)](#), a molecule involved in the contact between erythrocytes and macrophages and identified to be expressed by both cell types is essential for the proliferation and survival of erythrocytes [76]. M. Hanspal, et al. [77] showed that culturing these two cell types *in vitro* in the absence of Emp led to increased level of apoptosis by erythrocytes and aberrant erythropoiesis. Later on, S. Soni, et al. [78] showed that Emp-deficient mice died before birth due to severe anemia. Within the erythroblastic islands, macrophages have also been identified as a key player in the transfer of iron to erythroid precursors, a process essential for the growth and maturation of erythroid precursors [79].

Finally, central macrophages have been shown to promote the maturation of erythroblasts into reticulocytes. H. Yoshida, et al. showed that, pyrenocytes (nuclei surrounded by plasma membranes) from erythroid precursors, after being expelled from the precursor cell, are engulfed by macrophages [80] in a [Phosphatidyl serine \(PS\)](#) dependant manner. When the expression of Emp was masked with [Milk fat globule-EGF factor 8 \(MFG-E8\)](#) (which binds preferentially to Emp), the engulfment was prevented. S. Toda, et al. [81] analysed the molecular interaction between pyrenocytes and macrophages and found that Emp binds to a protein, Protein S which acts as a bridge with the macrophage and binds to MerTK. By transforming a fibroblast cell line incapable of engulfing pyrenocytes with the MerTK receptor, they showed that the sole expression of MerTK was sufficient to induce the binding and engulfment of pyrenocytes in the presence of Protein S.

A major challenge with all of these studies is the lack of a consensus on the identification of bone marrow macrophages and the correlation between their expression of various molecular markers and their function. Indeed, so far three bone marrow macrophage populations have been described, osteomacs important in bone mineralisation, HSC-retaining macrophages and erythropoiesis regulating macrophages. Whether these populations are actually the same cells capable of different functions or distinct populations still needs to be addressed. E. Heidevald, et al. [82] recapitulated in a table various bone marrow macrophage populations identified in the literature, their molecular markers and functions; I have adjusted the table to include more recently described populations (see Table 1.1).

One option to understand whether specific surface markers are associated with different macrophage functions is to perform [Single-cell RNA sequencing \(scRNAseq\)](#) on these cells. scRNAseq can reveal differentially expressed genes in selected cells. Based on this gene expression it is then possible to identify the function of the cell. The differential gene expression between cells can allow the identification of different cell sub-populations and hence associate cell sub-populations to function. The lack of consensus on the identification and functionality of BM macrophages will be addressed in section Chapter 4 by performing scRNAseq of all CD11b+ F4/80+ cells present in murine BM.

Category	Name	FACS markers	Function	Ref.
Bone regulating cells	Osteoclasts	F4/80 ⁻ RANK ⁺	Bone maintenance	[83, 84, 85, 86]
	Osteomacs	F4/80 ⁺ CD115 ⁺ MAC3 ⁺ CD68 ⁺	Regulate osteoblast function	[71, 87]
Erythropoiesis regulating cells	Central macrophages	F4/80 ⁺ VCAM1 ⁺ CD169 ⁺ CD163 ⁺	Promote pro-erythroblast proliferation in EBI	[88, 89, 90]
	Tissue resident SPI-C macrophages	F4/80 ⁺ VCAM1 ⁺ CD68 ⁺ CD169 ⁺ CD11b ^{lo} MHCII ^{int} TREML4 ⁺ Zbtb46 ⁻	Heme degradation and iron recycling	[91]
	Tissue resident BM macrophages	CD169 ⁺ VCAM1 ⁺ F4/80 ⁺ TRAP ⁻	Support erythropoiesis	[88]
	EBI Macrophages	F4/80 ⁺ CD169 ⁺ VCAM1 ⁺ Epdr-eGFP ⁺	Support erythropoiesis	[92]
HSC regulating cells	BM macrophages	Gr1 ⁺ CD115 ^{int} F4/80 ⁺ CD169 ⁺ MHCII ^{int} CD11c ^{int} CD68 ^{int} CD11b ^{lo} CX3CR1 ⁻	Maintenance and retention of HSCs in BM	[52]
	BM resident macrophages and monocytes	CD11b ⁺ CX3CR1 ⁺ COX2 ⁺ α -SMA ⁺ Ly6C ^{int/lo} CD115 ⁺ CD150 ⁺	Adjacent to HSPCs and support HSPCs from exhaustion	[93]
	Osteomacs	G-CSFR ⁺ CD68 ⁺	Support survival of osteoblasts and inhibit HSPCs mobilization	[94]
	Osteomacs	F4/80 ⁺ CD11b ⁺ Ly6G ⁺	Maintenance of BM niches and HSCs retention	[53, 67]
	BM resident macrophages	F4/80 ⁺ Ly6G ⁻ VCAM1 ⁺ CD169 ⁺	Promote LT-HSC engraftment	[95]
	BM macrophages	CD11b ⁺ F4/80 ⁺ VCAM1 ⁺ CD169 ⁺ Ly6G ⁻	Regulate HSCs mobilization	[96]

Table 1.1: All BM macrophage populations described in the literature present in mice, divided based on their functions and key cell surface markers

1.2 Acute myeloid leukemia and its microenvironment

1.2.1 Origin of AML

The discovery and classification of leukemias

The first documented report of leukemia was generated in 1811 by Peter Cullen [97]. He named this disease ‘splenitis acutus’ and diagnosed it as a dysregulation of white blood cells’ production in the spleen and the appearance of milky blood. Following Cullen’s publication, four other clinicians, Alfred Velpeau, Alfred Donné, John Bennett, and Rudolf Virchow reported similar conditions observed in patients [98]. While Cullen’s report is thought to be the first description of leukemia, the following publications provided accurate descriptions of the white blood cell accumulation in the spleen and blood. However, it is not until 1869 that clinicians were able to associate the symptoms of leukemia to a dysregulation of the BM [99].

By the early twentieth century, numerous cases of leukemia, defined by the accumulation of white blood cells in the BM, had been reported. It became evident that leukemia was heterogenous and a further classification of this disease was needed. Based on the speed of the development of the symptoms, leukemia was divided into two sub-categories, acute or chronic leukemia. In 1976, the [French-american-british \(FAB\)](#) Co-operative group further divided acute leukemias based on the morphology of the cells found in excess in the blood. They published a common nomenclature and classification with two main categories: lymphoblastic leukemias and myeloid leukemias [100]. The lymphoblastic leukemias, nowadays known to originate in lymphocyte precursors leading to the accumulation of immature lymphocytes, were divided into three types based primarily on the size of the cells present in the blood and their heterogeneity in terms of nuclear shape, cytoplasm and vacuolation. The myeloid leukemias were divided into six categories according to the differentiation lineage and degree of maturation of the cells. This classification has been updated several times by the [World health organisation \(WHO\)](#) as diagnostic tools evolve and it now includes neoplasms and myelodysplastic syndromes as new categories [101]. This classification also uses genetic alterations and immunophenotyping to refine the criteria for each sub-type of leukemias. Nowadays, it contains 22 categories for AML based on their genetic alteration, cell morphology and differentiation. The differentiation refers to the expression of surface markers expressed by the bulk of the AML cells. The most common types are basophilic, monocytic, erythroid and megakaryoblastic lineage AML.

Cell of origin, leukemic stem cells and the clonal evolution theory

The cell of origin is regarded as the cell from which the cancer arises. It is defined through its unlimited capacity to self renew and proliferate. As such, in the case of AML, only two options are possible for the cell of origin, either mutations arise in a primitive HSPC allowing it to proliferate or mutations accumulate in a [Common myeloid progenitor \(CMP\)](#) giving it self-renewing capacity [102]. It is still an ongoing debate which of these two scenarios is taking place in patients [103, 104]. Interestingly, it has been shown that in AML, a small fraction of the cells named [Leukemia stem cell \(LSC\)](#)s will not differentiate or proliferate and will retain their capacity to initiate the disease. It was T. Lapidot, et al. [105] in 1994, who first realised the importance of these cells. By transplanting various patient cells in [Severe combined immune-deficient \(SCID\)](#) mice, he found that a rare population of cells (1/250'000) was capable of homing in the BM and proliferating. These cells were all CD34+ CD38- (sharing markers with HSCs) and the rest of the disease was not capable of engrafting in mice. These experiments were the first to demonstrate the presence of a small population of AML-initiating cells in humans.

Overall, the presence of AML-initiating cells confirms that, within a patient, a high level of heterogeneity is present between different leukemic cells. This phenomenon, named intratumour heterogeneity can be explained through two different theories. The first theory, elaborated in 1977 by A. Hamburger and S. Salmon [106] relies on the stem cell model, and dictates that the cells are organised in a hierarchy similar to the haematopoietic system. In the case of AML, a rare population of LSCs resides at the top and can either differentiate, self renew or remain quiescent. The LSCs can generate all of the differentiated subpopulation while maintaining its own pool of stem cells [107]. Differentiated cells will constitute the bulk of the disease and are considered nontumourigenic. The heterogeneity is explained through the accumulation of differentiated populations. The second theory applies Darwin's theory of natural selection on the cancer model. Named the clonal evolution theory, it states that through proliferation, cells will naturally acquire mutations, with some mutations giving a growth advantage while others will lead to apoptosis or cell cycle arrest [108]. The most advantageous and resistant clones will then co-exist while outgrowing the weakest cells [109]. Nowadays, it is believed that the intratumour heterogeneity is responsible for chemoresistance and relapse. However, it remains unclear what regulates this heterogeneity and what is the best approach to target it.

Sequencing of AML

According to the National Institute of Health, in the last 10 years, the cost of sequencing has dropped by more than 10 folds, making it a commonly available tool for laboratories to study transcriptional differences in diseases in particular in cancer [110]. Using an estimate of sub-clone size in bulk sequencing of AML tumours, C. Grove, et al. [111] revealed that AML most likely did not evolve in a linear fashion as suspected in the clonal evolution theory but probably has a complex branching architecture. In 2012, to uncover the evolution of mutations in AML, J. Welch et al. [112] performed comparative whole genome sequencing of healthy HSPCs and various types of AML. They found that most mutations found in AML were already present in healthy HSPCs. They concluded that these mutations must be random mutational events occurring in HSPCs before acquiring disease-initiating capacity. In parallel, L. Ding, et al. [66] performed whole genome sequencing on paired primary and relapsed AML tumours from the same patient. By comparing these two sample types in eight patients, they were able to reconstitute the evolution of the leukemic cells. They found two different scenarios took place during relapse. In the first scenario, the founding clone present in the primary tumour mutated during therapy, became chemo-resistant and then expanded. In the second scenario, a subclone present in the primary tumour survived therapy, gained mutations, expanded and drove the relapse. Interestingly, in both cases, they noticed that chemotherapy failed to fully eradicate the founding clone.

Since these results, single cell [Ribonucleic acid \(RNA\)](#) sequencing has now become widely available. Transcriptional analyses at the single cell level allow scientists to understand the molecular differences between the different clones present within one patient and associate each sub-clone to a specific function such as disease progression or reduced drug sensitivity. In 2014, A. Hughes, et al. [113] compared the results from whole genome sequencing and single cell sequencing of BM samples from patients first diagnosed with [Myelodysplastic syndrome \(MDS\)](#) and then [Secondary AML \(sAML\)](#). They first modelled the clonal architecture of the leukemic cells based on the whole genome sequencing differences between the MDS and sAML results. Next, they generated a phylogenetic tree based on the single cell sequencing and found that both techniques reconstructed a similar architecture for the evolution of the leukemic cells. However, the single cell analysis revealed clonal substructure and smaller branching points. Hence, this study validated the previous studies focusing on the evolutionary process of AML using whole genome sequencing and confirmed that single cell sequencing allows a more refined analysis of

the clonal architecture of AML.

In the last 3 years, several studies have published scRNAseq of AML samples [114, 115, 116, 117]. P. Van Galen, et al. [116] performed scRNAseq on BM aspirates from AML patients and healthy donors. Using machine learning, they were able to classify and match the malignant cells according to their similarity to the HSC lineage and more precisely the HSC-myeloid axis. They found that the vast majority of leukemic sub-clones could be attributed to one of six cell types (HSC, progenitor, [Granulocyte monocyte progenitor \(GMP\)](#), promonocyte, monocyte, or conventional dendritic cell) found along that axis. Interestingly, all patients had a population of malignant HSCs or progenitors however the distribution of the cells along this axis varied greatly between patients. Finally, they found that the most differentiated AML cells with a transcriptional signature similar to that of monocytes were capable of inhibiting T cells *in vitro*, assigning an immunomodulatory function to these cells.

Mouse model of AML

Another key component in disease progression is the mutational landscape. The occurrence of mutations at diagnosis dictates the risks and therefore the treatment patients will receive. Cytogenetic abnormalities involving chromosome 11 region 2 band 3 (11q23) are among the most common cytogenetic abnormalities in haematopoietic malignancies. K. Menghrajani, et al. [118] performed a study of 63 AML cases with 11q23 mutations and their outcomes after therapy. They found that translocations at this locus led to the fusion of the [Mixed lineage leukemia 1 \(MLL\)](#) (also called [Lysine \[K\]-specific methyltransferase 2A \(KMT2A\)](#)) protein with a partner protein. Over 135 partner proteins were identified, with AF9 being the most common partner. Following treatment, 41% of patients with the MLL-AF9 rearrangement achieved [Complete remission \(CR\)/Complete remission with incomplete count recovery \(CRi\)](#). However, another study published in 2020 [119] showed that even if patients achieved CR, the 3 year survival rate for MLL-AF9 AML patient was only 41%. In addition, they found that patients with MLL rearrangement had a low number of additional gene mutations (median of 1). This finding suggests that MLL fusions require fewer cooperating mutations compared to other AML-initiating events.

Based on O. Kosmider and F. Moreau-Gachelin's observations [120], two mutations are normally necessary to generate a leukemic model representative of AML, one mutation allowing

proliferation and the second one interfering with differentiation of the progenitor cell. Using a retroviral system to insert the MLL-AF9 translocations in murine HSCs and GMP, A. Krivtsov, et al. [121] found that this mutation alone was capable of transforming both cell types into LSCs by giving proliferative capacity to HSCs and self renewing capacity to GMP. Hence the translocation of the MLL gene alone is capable of transforming progenitor cells and give them the capacity to expand into leukemia. In addition, according to which cells contained the translocation, they were able to mimic the differences in gene expression, DNA methylation and therapy response found in the clinic. Together, these results validate the MLL-AF9 translocation in progenitor cells as a good murine model to recapitulate AML, its genetic variation and therapy response in mice.

1.2.2 Current therapies in AML

Chemotherapy

For the last 50 years, chemotherapy has been the primary treatment for AML [122, 123]. Classified as a cytotoxic drug, chemotherapy is a chemical agent that will intercalate in the DNA, inhibiting cell division and finally induce cellular death. According to the aggressiveness and mutational landscape of the AML, different combinations of chemotherapies are given but overall the standard of care is usually an intensive induction therapy consisting of 3 days of one type of chemotherapy, daunorubicin, and 7 days of cytarabine, followed by consolidation therapy which can be either additional rounds of cytarabine or stem cell transplant. However, in particular in elderly patients, this drug regimen leads to therapy resistance and relapse. According to MD Anderson’s survival data, since 2010, only 16% of their patients diagnosed with AML who were over 60 years old were still alive in 2018 [124], (Figure 1.2).

Daunorubicin belongs to the category of anthracycline drugs, meaning that it inhibits cell division. This particular drug intercalates between the DNA base pairs leading to double strand breaks and inhibition of DNA synthesis [125]. Cytarabine is a pyrimidine analog which competes with cytidines during DNA synthesis. Once inserted in the new DNA strand, the DNA replication is inhibited and the cell can no longer divide [126]. A major limitation of both these drugs and chemotherapy in general is that these drugs will affect not only tumour cells but all dividing cells and many organs in homeostasis are dependant on the constant production of new cells. In particular the bone marrow and gastrointestinal tracts are known to generate trillions of new cells daily. In the gastrointestinal tract, chemotherapy has been shown to lead

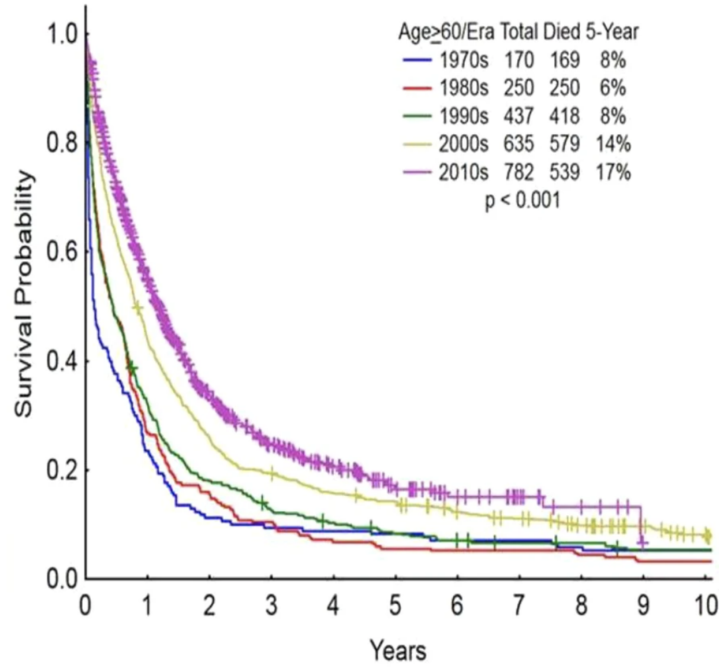


Figure 1.2: Survival of de novo AML at MD Anderson (1970-2017) *Adpated from H. Kantarjian et al., [123]*

to increased apoptosis in the crypts and crypt hypoplasia in turn generating local inflammation, ulceration and diarrhea [127, 128]. In the bone marrow, the loss of white blood cell production leads to increased risk of infection. In haematological malignancies, the combination of these two side affects, in particular the severe loss of neutrophils in the gastrointestinal tract can lead to a condition called neutropenic necrotizing enterocolitis which can be fatal [129].

Hence, while chemotherapy is considered the gold standard treatment for AML, the severity of the side effects combined with the high percentage of disease relapse reflects the limitations of this therapy. Therefore, these last 20 years, many scientists have focused their efforts into developing novel treatments, based on a better understanding of the disease. These novel therapies, also called “targeted therapies” aim to block a biological process leukemia cells are dependant on in order to induce leukemic cell death while leaving the healthy cells intact. We will discuss these novel therapies in the next section.

Novel therapies

One of the first targeted therapies developed for the treatment of AML was an anti-CD33 antibody [123]. Branded as gemtuzumab ozogamicin (GO), this anti-CD33 monoclonal antibody conjugated to a chemotherapeutic agent, targeted CD33+ cells and delivered internally

a high dose of calicheamicin, a DNA intercalating agent. CD33 is expressed by myeloid cells and leukemic blasts hence this therapy allowed a semi-specific targeting of AML cells [130]. However, the results of clinical trials were mixed as initial clinical trials did not select patients based on CD33 expression in the AML cells. The drug was therefore removed from the market and finally re-approved in 2017 in the treatment of CD33+ AML as an additional treatment in the combination regimen during induction therapy treatment [131].

In addition, a [Fms-like tyrosine kinase 3-mutated \(FLT3\)](#) inhibitor was developed in parallel as a targeted inhibitor of FLT3-expressing AML cells. Flt3 is a receptor expressed by haematopoietic stem cells and is mutated in 25-35% of AML patients [132]. This mutation leads to the constitutive activation of the receptor and the downstream JAK/STAT pathway which in turn, promotes cell proliferation and survival. Approved in 2017 for the treatment of FLT3 mutated AML cells, the Flt3 inhibitor was shown to bind to the receptor and prevent Flt3 activation, in turn stopping the cell proliferation. As a single agent, Flt3 inhibitors were shown to significantly extend the overall survival of young patients with refractory AML by approximately 4 months [133, 134]. However, as a combination therapy with chemotherapy, several trials did not lead to improved outcome [133].

Several other targeted therapies are emerging including IDH1/2 inhibitors, hypomethylating agents and immune checkpoint inhibitors. IDH1/2 inhibitors were shown to help rewire the metabolism of leukemic cells while hypomethylating agents improved the expression of tumour suppressor genes. Finally, immune checkpoint inhibitors were developed in order to help remove the break on the immune response and promote an anti-tumoural response. These include anti-PD1 and anti-CD47 antibodies. In AML, anti-CD47 has been shown to be a promising strategy as this receptor is highly overexpressed in leukemic cells. I will discuss this treatment further in the next section where I focus on the interaction of macrophages with leukemic cells and the studies performed to understand this crosstalk and target it.

To conclude, many novel therapies are being approved by the [Food and drug administration \(FDA\)](#) however their efficacy against the recurrence of AML and relapse remains limited. Interestingly, A. Burnett et al., [124] performed an analysis of the survival of >60 year old patients with AML enrolled in clinical trials over the last 50 years. Strikingly, their results show very little improvement in survival (Figure 1.3). Correlating with this fact, these survival curves are remarkably similar to the survival curves described by MD Anderson in the context

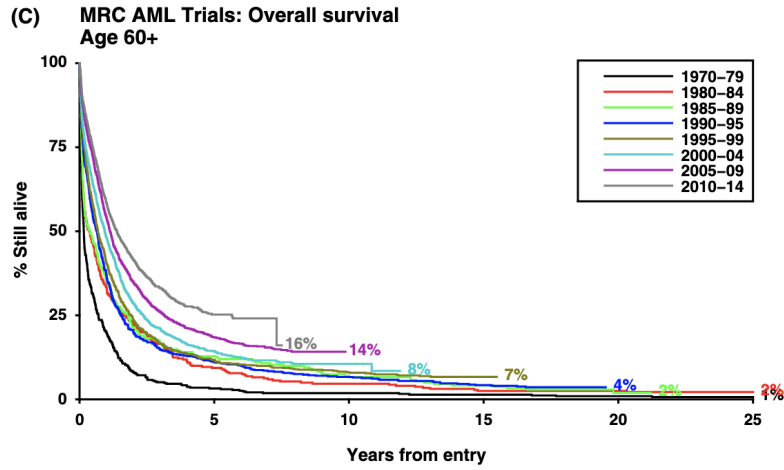


Figure 1.3: Survival improvement over time with intensive treatment. *From A. Burnett et al., [124]*

of intensive chemotherapy meaning that while these novel therapies are emerging, for now, they do not lead to increased overall survival of elderly patients. Hence it is essential to invest more energy in understanding the fundamental mechanisms allowing the development of AML and its interaction with the immune system in order to generate new immunotherapies that will help build a stronger immune response against the disease which can be combined with other therapies, including chemotherapy and targeted therapies.

1.3 Macrophages among the key regulators of cancer growth

1.3.1 Macrophages in solid tumours

In 1889, Stephen Paget hypothesised that metastasis depended on a cross-talk between tumour cells and the organ microenvironment. He called his theory the seed and soil theory where tumour cells are the seeds and the organ microenvironment are the soil [135]. Following 900 biopsies of various primary tumours, he noticed that the organ of metastasis was not random and was preferentially visceral organs and bone. Hence he concluded that the 'seed' and 'soil' had to be compatible for the tumour cells to metastasise to the new environment. This was the first time it was hypothesised that tumour cells interacted with their environment and that the healthy cells in the environment could influence tumour growth. Following Paget's work, numerous scientists focused their attention on the role of healthy cells in tumour development [136]. In particular concerning macrophages, they were found to play a dual role in cancer development, where they can either promote or inhibit tumour development by altering both the microenvironment and the immune system [137]. In the following paragraphs I will highlight the main studies demonstrating the importance of macrophages in solid tumour development.

Tumour-associated macrophages

In the late 70's, following the dissection of hundreds of tumours, it was established by several groups of scientists that macrophages infiltrated the tumour site, and could compose up to 80% of the immune cells present in the tumour microenvironment. However, their function in this environment remained uncertain [138, 139, 140]. Hence, in 1986, WH. McBride [141] decided to define the nature, role and complexity of tumour infiltrating macrophages. The tumour infiltrating macrophages were named **Tumour associated macrophage (TAM)** and described as being part of the MPS. McBride noticed that TAMs were composed of sub-populations of cells which responded differently to internal and external stimuli and their response and phenotype could be modulated. Interestingly, A. Mantovani et al., [142] wanted to address whether TAM recruitment was dependant on T cells. They measured the TAM infiltration in nude mice and normal mice and found that for most neoplasms, TAM recruitment was independant of the T cell compartment. Based on these findings, in 1992, A. Mantovani et al., [143] hypothesised that TAM recruitment and function in tumours was dependant on tumour cells and therefore

could be seen as a ‘macrophage balance’ whereby the interaction between the tumour cells and macrophages dictates the macrophage phenotype and function. Interestingly, they dictated that “ *the outcome [of the balance] depends on the sum of individual functions, dictated by the activation state of macrophages and the intrinsic properties of the tumour cell*” reflecting the high level of plasticity and ambivalence of TAM. 20 years later, the plasticity and range of activation in TAMs remains a source of contention and struggle.

Macrophages activation profile in tumours

Based on *in vitro* studies focusing on artificially differentiated and stimulated macrophages, activated macrophages were initially classified as M1 or M2 macrophages, as a parallel to the T cell responses, according to their activation profile and differentiation status. Culture of immature macrophages with LPS and IFN γ led to an M1 profile characterised by the secretion of TNF and IL-6, two cytokines leading to a strong inflammatory response [37], while the culture with IL-4, IL-10 and TLR, all led to an M2 activation profile, characterised by its important anti-inflammatory component. This M2 profile could be further divided into three types of anti-inflammatory profiles, M2a, M2b and M2c according to the activating stimuli and secreted cytokines. Given the different possible activation profiles for M2 macrophages, these macrophages are commonly called ‘alternatively activated macrophages’ (Figure 1.4)[144]. Nowadays, it has become evident that the M1-M2 classification correspond to artificially generated extreme activation profiles and many more subtle states of activation are possible as these cells are highly plastic and respond according to environmental stimuli.

In the context of tumours, the macrophage activation status plays a key role in regulating tumour growth. While TAMs were generally considered to be similar to M2 activated macrophages, and hence have an anti-inflammatory role in the tumour microenvironment, S-M Ong et al., showed that in an *in vitro* model of colorectal cancer, TAMs resembled M1 macrophages, and displayed a pro-inflammatory activation profile, capable of activating T cells towards an anti-tumour response. In addition, these macrophages inhibited the proliferation of tumour cells. Finally, in patient samples, the number of TAMs correlated with the number of tumour infiltrating T cells [145]. At the same time, R. Miguel et al., used an *in vivo* model of skin cancer and found that tumour infiltrating macrophages were capable of anti-tumoural function by producing reactive oxygen species and reactive nitrogen intermediates [146], con-

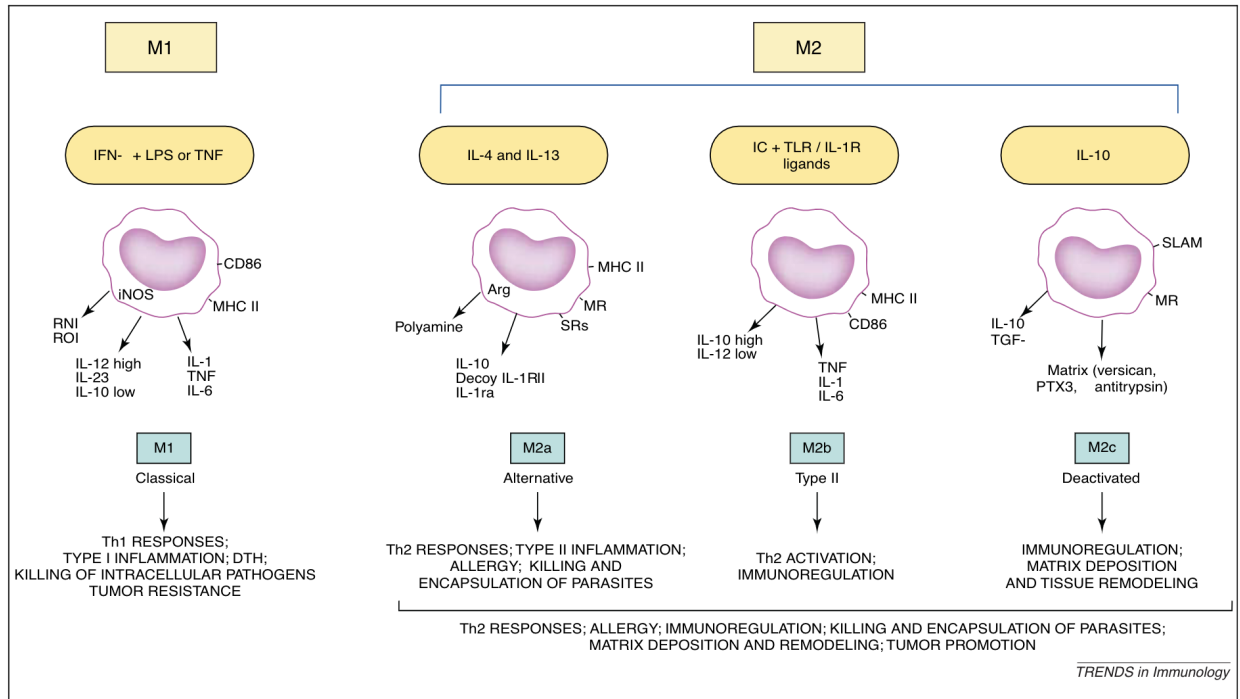


Figure 1.4: Inducers and selected functional properties of different polarized macrophage populations. *From A. Mantovani et al., [37]*

In yellow are the inducers of macrophage polarization, in blue are the adopted polarisations and black arrows represent secretion of molecules and induction of immune responses.

sidered key effector molecules in the activation of cytotoxic T cells [147].

Hence, these studies suggest that TAM can be a key component in the anti-tumour response. While these previous studies highlight the fact that TAM can help mount an anti-tumoural immune response, most studies focusing on TAMs have shed light on the important anti-inflammatory and pro-tumoural role of TAMs. Indeed, in breast cancer, Lin et al., found that over-expression of CSF1 led to increased infiltration of TAMs and in turn promoted disease progression to metastasis [148]. In addition, D. Strachan et al., showed that inhibition of CSF1R in a murine model of cervical cancer reduced the proliferation of TAM which in turn promoted the expansion of T cells [149].

Given the heterogeneity of the impact of TAMs on the tumour progression, I decided it was important to understand the mechanisms behind TAMs involvement in disease development and how these cells can affect the tumour microenvironment, the immune response and the tumour cells themselves. I will discuss these three points in the next three sections.

Macrophages in the tumour microenvironment

Among the populations of TAMs described in literature, one population named Tie-2+ macrophages, because of their expression of Tie2, have been shown to play an important role in the tumour microenvironment [150, 151]. Indeed, Tie-2, a receptor expressed mainly by endothelial cells, binds to Ang1 and this signaling pathway regulates the maintenance of vascular quiescence and the promotion of angiogenesis [152]. To maintain vascular quiescence, the Ang1/Tie-2 pathway has been shown to promote cell survival and protect endothelial cells from radiation induced damages [153]. In this context, the expression of Tie-2 by TAM was intriguing and led to numerous questions: do TAM promote tumour cell survival through the expression of Tie-2? Or does this pathway help protect tumour cell from radiation induced damage? Does Tie-2 expression by macrophages influence angiogenesis?

C. Murdoch et al., [154] showed that monocytes were recruited to the tumour site in response to the secretion of Ang-2. In addition, these cells then differentiated and upregulated their expression of Tie-2 in response to hypoxia. When cultured *in vitro* with Ang2, macrophages expressed lower levels of TNFa, an anti-angiogenic cytokine, and CCL2, a potent chemokine. Instead, secretion of IL-6, IL-4 and IL-10 was unaffected by the presence of Ang-2, hence not only is the activation profile of these cells impacted by the presence of Ang-2 but they also reduced their secretion of anti-angiogenic factors. In a model of [Laser-induced choroidal neovascularization \(LCNV\)](#) mimicking the effect of age-related macular degeneration, a disease leading to blindness generated by the formation of abnormal blood vessels in the retina, X. Yin et al., [155] found that the depletion of Tie-2 macrophages led to reduced neovascularisation, inflammatory cytokines and angiogenic factors including VEGF-A. In addition, by culturing endothelial cells with Tie-2+ macrophage supernatant they found that the endothelial cells, under hypoxic conditions, increased their mRNA level of VEGF-A and bFGF hence demonstrating that Tie-2 macrophages play an important role in angiogenesis. While this study was focusing on [LCNV](#), it does suggest that Tie-2 macrophages are capable of regulating angiogenesis in the tumour microenvironment and promoting cell survival and proliferation.

An elegant study led by John Condeelis's group has focused on understanding the role of Tie-2 expressing macrophages in breast cancer metastasis using intravital microscopy [150]. This imaging technique allowed them to identify [Tumour MicroEnvironment of Metastasis \(TMEMs\)](#), defined as the direct contact between a macrophage, tumour cell and endothelial

cell. Interestingly, Tie-2+ macrophages were enriched in these TMEMs, and also expressed high level of VEGF-A. Finally, they demonstrated that, at the TMEM site, a local loss of vascular junction took place resulting in transient vascular permeability allowing tumour cells to intravasate and in turn this led to increased metastasis. Interestingly, they found that this mechanism was dependant on the presence of Tie-2+ macrophages as depletion of macrophages led to reduced vascular permeability and a reduced number of circulating tumour cells.

In parallel to these findings, A-K. Bonde et al., [156] found that TAMs promoted the [Epithelial to mesenchymal transition \(EMT\)](#) transition in tumour cells from a teratocarcinoma model. Indeed, by depleting macrophages using clodronate liposomes and analysing the expression of key EMT related genes in the teratocarcinoma, they found that macrophage depletion led to reduced expression of EMT genes and the tumours were smaller. *In vitro* culture of teratocarcinoma cells with macrophage conditioned media revealed that macrophages secreted TGF- β which induced the EMT of teratocarcinoma cells and stimulated their invasive properties. These studies highlight the important role tumour-associated macrophages play in angiogenesis, tissue remodelling, intravasation and metastasis development.

Macrophage's direct interaction with cancer cells

In addition to their role in the tumour microenvironment, several studies have focused their attention on the direct interaction that takes place between macrophages and cancer cells. Indeed as mentioned previously, M. Ong et al. co-cultured macrophages with three different colorectal cancer cell lines and found reduced proliferation and increased apoptosis in the colorectal cancer cells. When analysing the gene expression profile of the co-cultured macrophages, they found that these cells expressed a strong inflammatory signature as well as class II antigen presentation and a phagocytic signature [145]. These results suggested that macrophages were capable of phagocytosing cancer cells and in turn reducing the proliferation of tumour cells.

The main interaction known between cancer cells and macrophages involved in phagocytosis is the CD47- SIRP α axis. This ligand-receptor interaction has been the focus of many years of research as it is considered an 'immune checkpoint'. Macrophages express SIRP α and its binding to CD47, normally expressed by healthy cells, leads to the inhibition of phagocytosis. Also referred to as the 'don't eat me' signal, CD47 is upregulated on numerous cancer types leading to the evasion of phagocytosis. In an *in vitro* co-culture setting, K. S. Chan et al,

showed that inhibition of CD47 using a monoclonal antibody on bladder cancer cells, led to increased phagocytosis by macrophages [157]. In addition, S. Willingham et al., [158] showed *in vivo* that inhibition of the CD47-SIRPα axis using an antibody reduced tumour growth in human ovarian and colorectal tumours xenografted in NOD/SCID/γ (NSG) mice which lack T cells. Similar results were obtained for mouse models of pediatric glioblastoma [159], adult glioblastoma [160], bladder and breast tumours [158]. Interestingly, since these studies were performed in NSG mice, it could be concluded that a direct interaction between macrophages and cancer cells is key in the regulation of cancer growth. Overall, these studies suggest that, in certain mouse models, the phagocytic activity of macrophages alone is sufficient to inhibit tumour growth.

However, phagocytosis is not the only mechanism by which macrophages are capable of engulfing tumour cells. Indeed, in homeostasis, an essential role for macrophages is the engulfment of apoptotic cells. This process, also known as 'efferocytosis' is defined as the identification and engulfment of apoptotic cells by macrophages or dendritic cells without inducing inflammation (Figure 1.5). Efferocytosis is essential for the clearance of old cells and the maintenance of tissue homeostasis as normal cell turnover would otherwise be disrupted and aggravated when the number of apoptotic cells increases due to disease or stress. As mentioned previously, during this process, apoptotic cells express different 'eat-me' signals from necrotic cells and these ligands bind to specific receptors on macrophages, inducing efferocytosis rather than phagocytosis and promoting tolerogenic functions. Indeed, within the macrophage, this signalling pathway leads to the increased secretion of anti-inflammatory cytokines such as TGFβ and IL-10 and reduced secretion of IL-12 and TNF [161], in turn promoting the recruitment of other anti-inflammatory cells. The most commonly studied efferocytic receptors on macrophages are MerTK, Tim4 and Axl [162].

Interestingly, in a mouse model of breast cancer where the expression of MerTK was genetically abolished (MerTK^{-/-}), R. Cook et al., [164] showed that the tumours size were smaller than in control mice and were less metastatic. In addition, in irradiated WT mice expressing MerTK, the transfer of MerTK^{-/-} bone marrow cells recapitulated the same results as seen in MerTK^{-/-} mice. Hence, they were able to conclude that the expression of MerTK in leukocytes was promoting tumour growth. Finally, they also measured the level of anti-inflammatory cytokines such as IL-10 and TGF-β and found the levels of these cytokines was significantly reduced in MerTK^{-/-} mice. These results correlated with the idea that efferocytosis-inducing

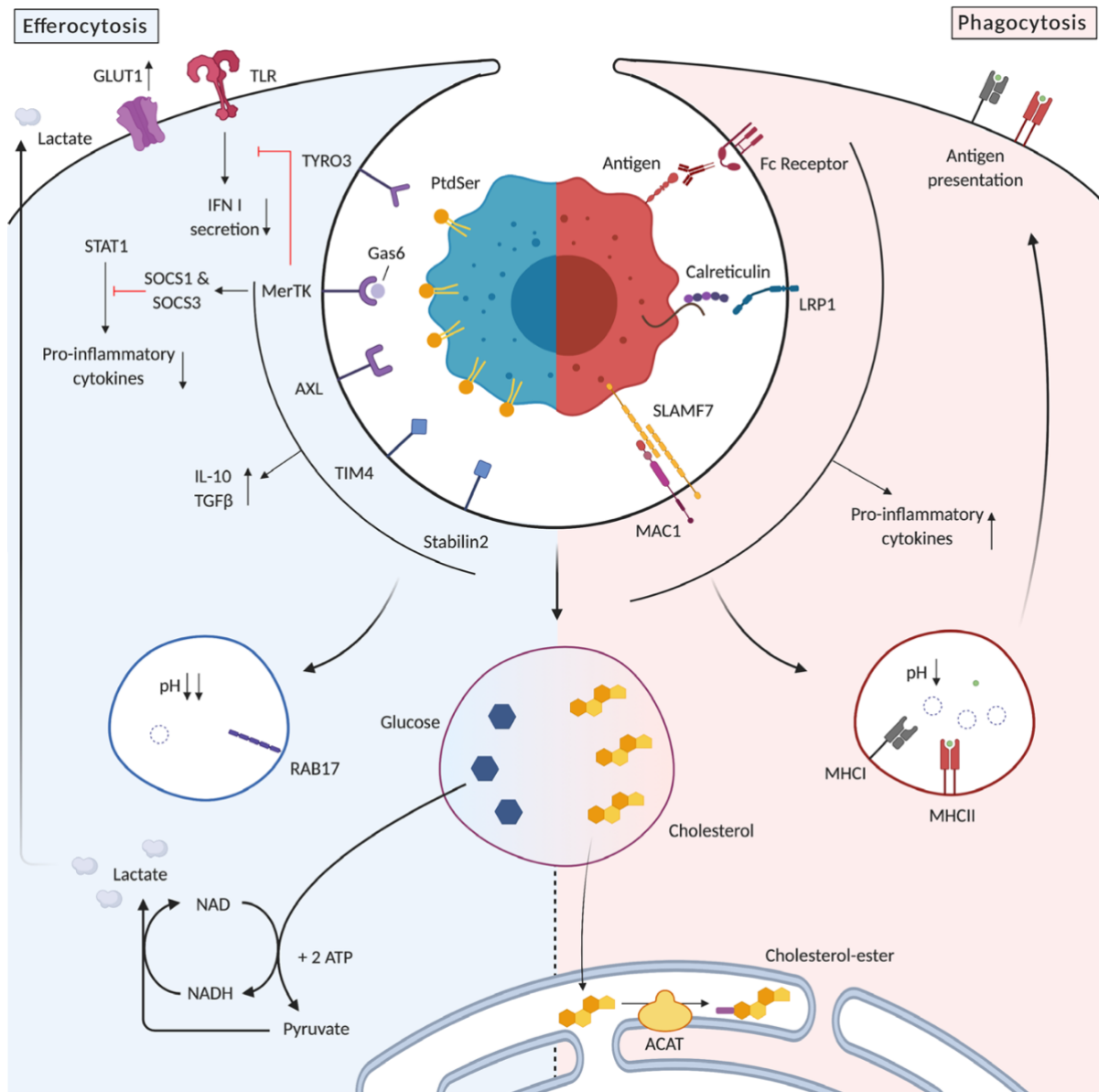


Figure 1.5: Efferocytosis promotes anti-inflammatory functions while phagocytosis promotes proinflammatory functions of tumour associated macrophages *From M. Lecoultrre et al., [163]*

signalling is mimicked by tumour cells to induce an anti-inflammatory response within the tumour microenvironment and promote tolerance.

Macrophages in the anti-tumour immune response

While I previously described successful results for the control of tumour growth by inhibition of CD47 in NSG mice, in certain tumour models however, the effect of CD47 inhibition was abolished in mice lacking T cells. Indeed, both in melanoma [165] and fibrosarcoma [166], the beneficial effect of CD47 inhibition was T cell dependant. Interestingly, K. Iribarren et al.,

found that in a mouse model of breast cancer, the treatment with anti-CD47 antibody led to reduced tumour growth. However, treatment with T cell depleting antibodies abolished the growth difference suggesting that in this mouse model, macrophages do not directly control tumour growth but rather shape the anti-tumour T cell response and with help of an anti-CD47 antibody are capable of promoting the anti-tumour T cell response. In addition, C. Roemeling et al., [167] showed that following anti-CD47 treatment, co-culture of bone marrow derived macrophages with glioma cells led to increased phagocytosis. They measured the presence of MHC-I bound glioma peptide at the surface of macrophages and concluded that the increase in phagocytosis also allowed an increase in antigen cross-presentation. When blocking the phagocytic receptor, **Calreticulin (CRT)**, this effect was abolished, indicating that the cross-presentation was dependant on the phagocytic capacity of macrophages. Finally, they co-cultured the phagocytic macrophages from the glioma cultures with antigen specific CD4 and CD8 T cells, capable of recognising the cancer antigen presented by the macrophages and found that these co-cultures led to increased T cell proliferation, which in turn displayed markers of effector T cells. Altogether, these results highlight the fact that macrophages are capable of phagocytosing cancer cells, cross presenting the cancer antigens to T cells and mounting an anti-tumour T cell response. Similar results were obtained for melanoma [168] and colon cancer cells [169]. However, all of these studies were performed *in vitro* and it will be important to find out if macrophages are also capable of generating an anti-tumoural T cell response *in vivo*.

Macrophages are also known to play an important role in the recruitment and activation of T cells through the secretion of chemokines and cytokines. In 2000, A. Sica et al., [170] showed that TAM had a defective production of IL-12 due to an autocrine production of IL-10. Inhibition of IL-10 using an antibody restored the production of IL-12. Their work suggest that the presence of IL-10 in the tumour microenvironment promote the differentiation of macrophages towards TAMs and in turn these cells contribute to the production of IL-10, which is known to promote an immunosuppressive microenvironment. In addition to their role in the secretion of anti-inflammatory cytokines, J. Liu et al., [171] showed that macrophages secreted CCL20, the sole ligand of CCR6, expressed by regulatory T cells (Tregs). Tregs migrated towards macrophages, both *in vitro* and *in vivo*, located in the tumour microenvironment. Hence, through the secretion of cytokines and chemokines, macrophages are able to recruit immunosuppressive cells and maintain the inhibitory environment.

Interestingly, in the previously mentioned study focusing on the inhibition of efferocytosis

through MerTK suppression, R. Cook et al., [164] also found that inhibition of efferocytosis led to increased T cell abundance and depletion of T cells abolished the effect of MerTK suppression as tumours grew at the same speed as in control mice. They noticed that in MerTK^{-/-} mice, the level of IL-10 was reduced while IL-12 was significantly increased. Together, their results suggest that inhibition of efferocytosis can help reduce tumour growth in a T cell dependant manner. In parallel, R. Lindsay et al., found that in a mouse model of solid tumours, they found that MerTK promoted T cell arrest in the tumours. In addition, inhibition of MerTK led to increased T cell antigen recognition and effector function. Hence, altogether we can postulate that in a tumoural context, cancer cells express high levels of CD47 to inhibit phagocytosis and promote efferocytosis which help generate a tolerogenic microenvironment. Inhibition of the efferocytic receptor MerTK leads to the accumulation of necrotic cancer cells which will in turn be phagocytosed by tumour-associated macrophages. These cells will then be able to process the cancer antigens and cross present them to T cells finally promoting the anti-tumour T cell response.

Overall, these studies highlight that tumour-associated macrophages play a pivotal role in tumour growth as these cells can modify the tumour microenvironment, immune response and the cancer growth itself. Through direct interaction and secretion of chemokines and cytokines, they are able to regulate numerous mechanisms and can be hijacked by the tumour cells to promote the disease growth at the expense of healthy cells. This led us to the questions: which of these mechanisms and processes take place in leukemia? Is the role of macrophages in disease progression different in blood cancer compared to solid tumours?

1.3.2 Interaction between AML cells and macrophages

Macrophages sub-populations involved in AML progression

In AML, macrophages are poorly characterised. Only a handful of papers focusing on the function of bone marrow macrophages in AML have been published, each focusing on a different aspect of these cells and not all of the results are comparable. In addition, a strong characteristic of this topic is the potential therapeutic interventions leading some papers to be published with the purpose of highlighting potential new pre-clinical candidates but therefore the mechanisms are not always demonstrated. In the next section, I will try and correlate these findings and highlight the importance of new studies focusing on correlating macrophage sub-populations with a function in AML.

In 2013, F. Mussai et al., [172] published a study focusing on the role of AML cells in promoting macrophage polarisation. Co-cultures of AML blasts with monocytes revealed that AML cells polarised macrophages towards an M2 phenotype, characterised by increased expression of CD206. In addition, they found that in the plasma of AML patients, the level of Arginase II activity was significantly increased. Hence, they hypothesised that macrophage polarisation was induced by Arginase II. And indeed, inhibition of Arginase II using a small molecule inhibitor (NOHA) significantly reduced the proportion of macrophages expressing CD206. Correlating with these findings, they also noticed that AML secretion of Arginase II led to the inhibition of T cell proliferation *in vitro*. Together, they concluded that AML cells were able to induce an immunosuppressive microenvironment through the secretion of Arginase II.

In 2016, Y. Al-Matary et al., [173] identified one population of monocytes/macrophages with pro-leukemogenic functions that was increased in two different AML murine models and identified the transcriptional repressor Gfi1 to regulate M2 polarization. *In vitro*, they found that bone marrow derived macrophages originating from leukemic bone marrow cells promoted leukemic cell proliferation significantly more than control bone marrow derived macrophages. Hence they hypothesised that leukemic cells polarise macrophages to support leukemia proliferation. To support this hypothesis, they checked the mRNA expression of known M1/M2 molecules including Arg1, IL-6, Nos2 and IL-10 and confirmed that leukemic cells were polarising macrophages towards an M2 profile as they had increased expression of Arg1, IL-10 and reduced expression of IL-6 and Nos2. In addition, *in vivo*, they defined two monocytes/macrophage populations, one was called AML-associated monocytes/macrophages 1 (AAM1) and the second one was named immature leukemic macrophages. The first population was characterised by flow cytometry as CD11b⁺, Ly6G⁻, Ly6C⁻ and MHCII⁻ cells while the second one was characterised by the expression of CD11b⁺, Ly6G⁻, Ly6C^{int} and MHCII^{int}. Interestingly, they found that both populations increased in the bone marrow and spleen of leukemic mice compared to control while monocytes were decreased. They repeated their *in vitro* cultures with sorted AAM1 cells and found a similar increase in AML cell count as with BMDM. They concluded that these cells were the same cells as generated *in vitro* and identified Gfi1, a transcription factor involved in macrophage development, that was upregulated in leukemic macrophages. Finally, Gfi1 Knock-out (KO) led to reduced M2 polarisation *in vitro* and *in vivo*, increased expression of IL- and reduced AML growth. Hence they concluded that the M2 polarised AAM1 were AML supportive and the absence Gfi1 repolarised these cells towards an

anti-tumour state.

Following this study, in 2017, X. Yang et al., [174] published a similar study highlighting a different population of macrophages associated with poor outcome in AML and tried to remodel them towards an anti-leukemic phenotype. Interestingly, they compared the gene expression profile of splenic and bone marrow macrophages in the context of AML, using the MLL-AF9 mutated model. Using flow cytometry, they identified and sorted macrophages as Gr1^{lo}, F4/80⁺, CD115^{int} and SSC^{lo} cells. They performed bulk RNA sequencing on sorted splenic and bone marrow macrophages from control, AML and [T-cell acute lymphoblastic leukemia \(T-ALL\)](#) mice. Hierarchical Clustering revealed that the origin of the macrophages had a stronger impact on the gene signature compared to the condition, hence splenic macrophages would cluster together regardless of the presence of leukemia. Two hypotheses could be drawn from this result, first if we think these cells are monocyte derived macrophages, recruited from the bone marrow then the microenvironment of the organ plays a stronger role in macrophages activation compared to leukemia. Else, if these cells are tissue resident macrophages then the difference between tissue resident macrophages from one organ to the other is more important than the differences induced by a disease such as leukemia. Unfortunately, the study did not address the origin of the cells so it is hard to know which one of these hypotheses is taking place .

Next, they compared side by side, the number of [Differentially expressed genes \(DEG\)](#) between control macrophages and macrophages from a leukemic mouse from the same organ. Interestingly, the bone marrow macrophages in AML were the most conserved macrophages, with 150 genes being up-regulated in AML, and 26 down-regulated. BM macrophages had 10-fold less [DEG](#) compared to all other conditions, revealing that these cells are unlikely to change with AML. Using a list of M1/M2 genes, they identified BM leukemic macrophages as ‘M1-like’ cells while splenic leukemic macrophages clustered with the ‘M2-like’ cells. Among the most up-regulated genes in the M1 macrophages and down-regulated in the M2 macrophages, was [Interferon Regulatory Factor 7 \(IRF7\)](#), a transcription factor involved in the differentiation of monocyte to macrophages [175] and in the secretion of IFN- β by macrophages [176]. With the aim to convert M2 macrophages towards M1 macrophages, they transduced IRF7 into the naive macrophage cell line, RAW264.7 and found that IRF7, through the activation of the SAPK/JNK pathway, induced M1 characteristics including the secretion of pro-inflammatory cytokines, iNOS, TNF- α and IL-6. Finally, to assess the relevance of their results on AML progression, *in vivo*, they established that [Polyinosinic:polycytidylic acid \(PolyI:C\)](#), a synthetic

analog to viral infections [177], led to the increased expression of IRF7 and induced the same changes as IRF7. Meanwhile, SP600125, a specific SAPK/JNK pathway inhibitor, led to enhanced M2 characteristics and reduced M1 gene expression. Using these two treatments, they showed that leukemic mice treated with PolyI:C has significantly prolonged survival time while SP600125 did not lead to any changes in survival time.

While this study has many limitations, for example the fact that their gating strategy used did not exclude monocytes from the analysis, two interesting points are highlighted: first is the high level of conservation in bone marrow macrophages as leukemia grows and the second point is the important differences based on the tissue where macrophages are located. The first point is slightly contradictory with the results presented in F. Mussai et al.,’s study [172] which found that AML cells were capable of inducing M2 like polarisation of macrophages *in vitro*. Given that their study cultured AML cells with macrophages, it is possible that the effect of AML cells on macrophages is magnified and *in vivo* this interaction is smaller, so when Yang et al., studied the gene expression of bone marrow macrophages from control and AML mice, no significant differences was seen. The second point highlights the important differences in the transcriptional signature of macrophages from different organs. This result correlates with the comparative flow cytometry study described in section 1.1.2 on macrophages in homeostasis, which emphasized the important differences in the identification of macrophages from different organs by flow cytometry. Together, these studies suggest that the differences in phenotypes found in macrophages are reflected at the molecular level and distinct phenotype and transcriptional signatures should be expected for bone marrow macrophages. Importantly, this means that TAM gene signatures from solid tumours will not necessarily overlap with bone marrow macrophage in AML.

In line with the idea that M2 macrophages support AML progression, Z. Xu et al.,[178] identified CD206 as a prognostic indicator for AML. Using CIBERTSORT, an algorithm that quantifies cell fractions from bulk tissue gene expression profiles and allows the estimation of the immune composition of a tumour biopsy [179], they analysed Tumour infiltrating leukocytes (TIL) in more than 2000 bone marrow samples from different haematological malignancies including AML and control bone marrow. They found that the percentage of M2 macrophages was increased in AML samples compared to control bone marrow and this increase was associated with a poor outcome. In addition, they noticed that CD206, a known M2 macrophage marker, recapitulated the same results as the results found with the M2 macrophage signatures,

and could therefore be considered a robust outcome predictive factor in AML. These studies raise the hypothesis that not only macrophages are present during AML but more importantly, they play a significant role in promoting disease progression.

Very recently, another study was published by I. Weinhauser et al., on BioRxiv [180] focusing on the interaction between M2 macrophages and leukemic cells and its importance in giving the cancer cells their growth advantage. Based on the results described by Z. Xu et al. [178] that M2 macrophages were associated with poor outcome, they hypothesized that macrophages could help leukemic cells acquire their aggressiveness. To test this hypothesis, they generated M0 and M2 macrophages *in vitro* and injected these cells with [Acute promyelocytic leukaemia \(APL\)](#) cells, which are known to fail engraftment, in the bone marrow of [NOD scid gamma \(NSG\)](#) mice. They found that APL cells were able to engraft if co-injected with macrophages. Interestingly, the difference in outcome between M2 co-injected macrophages and naive M0 macrophages was not striking, suggesting that co-injection with any macrophages, rather than a specific kind of macrophages, allowed these leukemic cells to grow. Following this result, they co-cultured the leukemic blasts with M2-macrophages and 48 hours later injected only the leukemic cells. Interestingly, the co-culture led to similar results in engraftment suggesting that the temporary interaction between macrophages and leukemic cells is sufficient to transform the leukemic cells into a more aggressive leukemia. Following phagocytosis assays, homing assays and metabolic staining, they found that macrophages co-culture resulted in the leukemic cells having improved homing capacity, evading phagocytosis through the down-regulation of the ‘eat me’ signal calreticulin and metabolic reprogramming improving their functional respiration. Finally, they showed that the effect of macrophage ‘education’ on the leukemic cells is a lasting effect as it remained after transplanting the leukemic blasts into a secondary recipient, meaning that the macrophage priming promoted leukemic growth in the primary and secondary recipient. Altogether, it is interesting that a short term interaction between macrophages and leukemic cells could influence long term disease growth and outcome.

Put together, all of these studies converge towards the idea that within the bone marrow macrophage population, there is an anti-inflammatory macrophage population and these cells are associated with poor survival. More precisely, in patients, CD206+ macrophages were associated with increased AML growth, stemness and homing capacity, and were capable of inducing an immunosuppressive environment including inhibition of T cell proliferation. Unfortunately, this population has not been described in mice. While CD206 is conserved across species, with

approx. 82% of the genes being conserved between mice and humans in the CD206 protein, no studies on murine bone marrow macrophages have highlighted the expression of CD206. One possible explanation is that this protein is not as important in mice and therefore the cells do not express it at the same level as in humans hence it has not been identified as a biomarker in mice. It would be interesting to understand whether murine bone marrow macrophages differ from human bone marrow macrophages and if so whether this also affects their response AML.

Therapeutic interventions involving macrophages in AML

One of the main characteristic scientists are trying to exploit in order to develop macrophage targeted anti-cancer therapy, is phagocytosis. Indeed, phagocytosis is the central/ main mechanism allowing macrophages to orchestrate the anti-tumoural immune response. After phagocytosing cancer cells, macrophages can process tumour antigens and present them to T cells while secreting inflammatory cytokines to activate anti-tumour T cells. However, in some cases, in particular in AML, cancer cells mimic healthy cells and up-regulate CD47 [181], known as the 'don't eat me' signal to evade phagocytosis[182].

Following the identification of this immuno-evasive mechanism, the expression of CD47 was evaluated and studied as a prognostic marker. Interestingly, using an immunohistochemistry based study, S. Galli et al., [183] found that in a majority of AML patients overexpressed CD47 in their AML cells. In addition, high CD47 expression correlated with a higher leukemic cells tumour load. Together, these results highlighted the fact that CD47 was a potential target for immunotherapy. In 2009, R. Majeti et al., [184] inhibited the CD47-SIRPα axis using a monoclonal antibody against CD47. They found that the addition of this antibody to AML cells co-cultured with macrophages enabled macrophages to phagocytose AML cells, which they were incapable of doing without the antibody. In addition, when they tested this antibody *in vivo* in NSG mice xenotransplanted with human AML, they found that daily injections led to a near complete elimination of circulating leukemic cells in the blood and a significant decrease of AML cells in the bone marrow with a few mice having no residual disease. They also found that in mice with residual disease, macrophages containing phagocytosed dying cells were present in the bone marrow suggesting that macrophages were now capable of phagocytosis *in vivo*. To confirm that macrophages were responsible for the difference in AML growth in treated mice, they depleted macrophages using clodronate liposomes and confirmed that the growth difference was abrogated. Finally, to make sure that macrophages were not phagocytosing healthy

haematopoietic stem cells, known to express CD47, they injected anti-CD47 antibody in control healthy mice for two weeks and measured the absolute number of HSCs in the bone marrow. They found that the absolute number of these cells remained unchanged after the treatment, suggesting that this treatment should not lead to any particular toxicity.

Ensuing these promising results, several studies were published highlighting the improved T cell priming and activation thanks to the restored phagocytosis following anti-CD47 treatment in xenografted mice [185, 169]. Altogether these studies constituted sufficient pre-clinical data to set-up clinical trials. Clinical trials have been ongoing since 2015, targeting either CD47 or its receptor, SIRPa [186]. While it is still early days and most clinical trials targeting this axis are still at early phases, the results are looking promising and currently 46 clinical trials are taking place in the US, targeting this axis in various cancers.

Overall, these studies highlight two main axes of research regarding macrophages in AML. The first axis is focused on understanding the heterogeneity of macrophages and their influence on the disease progression. The second axis focuses on understanding if macrophage function is impaired by AML, restoring macrophage function and studying the effect of such treatment on disease growth and the immune response. Both axes have made important progress in the last five years, however it has become evident that a gap exist between these two research poles and the link between heterogeneity and functionality is missing. Indeed, the studies focusing on the heterogeneity of macrophages aim to identify prognostic markers, such as the CD206 marker, associated with poor outcome and increased M2-polarised macrophages in the bone marrow. However, these studies do not aim to understand the function associated with increased CD206, the recruitment process of these cells, their interaction with leukemic cells and the microenvironment. On the contrary the second axis, focused on functionality bases its hypothesis on the fact that dysfunctional phagocytosis can lead to impaired anti-tumour immunity. Restoring phagocytic capacity through inhibition of CD47 reduced AML growth however it is unknown which cells are important in this process, if it is targeting several populations of monocytes and macrophages and whether this is affecting the homeostatic response of macrophages, leading to possibly increased toxicity.

Hence, in this project I hope to bridge this gap by focusing my work on understanding the heterogeneity of bone marrow macrophages and then addressing the function of the populations identified. Using a combination of high dimensional flow cytometry, intravital imaging

and single cell RNA sequencing I aim to perform unbiased analysis correlating the phenotype, molecular signature and morphology of bone marrow macrophage populations. Taking advantage of the well established MLL-AF9 AML model and macrophage depleting tools such as clodronate liposomes and CD169-DTR mice, I aim to characterise the interaction between macrophage populations and AML cells *in vitro* and *in vivo* and understand their role in AML progression and T cell response.

1.4 Project aim and research questions

The overarching hypothesis of this project is that a cross-talk between AML cells and different macrophage populations takes place in the bone marrow as AML progresses and this cross-talk promotes AML growth while impairing the anti-tumour immune response. I will test this hypothesis by focussing on three main research questions:

1. What are the different populations of macrophages in the bone marrow? (**Chapter 3**)
2. Are macrophages affected by AML, in terms of numbers, spatial distribution and transcriptional signatures and can I identify the molecular changes driving these alterations? (**Chapter 4**)
3. How do macrophages influence AML growth, especially do they directly interact with AML cells and can they influence the anti-tumour T cell response? (**Chapter 5**)

Chapter 2

Materials and methods

2.1 Mice

2.1.1 Ethics

All animal work was done in accordance with the UK Home Office standards under the Animals (Scientific Procedures) Act, 1986 and the Animal Welfare and Ethical Review Body (AWERB) at Imperial College London and the Sir Francis Crick Institute. The work described here was carried out under project licenses 70/8403 and PP9504146 and all regulated procedures were performed by me after obtaining a personal license (Reference: ICE260A07) accredited by the Society of Biology.

2.1.2 Mouse strains

All animals were bred at the Francis Crick Institute, London, UK housed and maintained there and at Imperial College London. C57Bl/6 [Wild-type \(WT\)](#) were purchased from Charles River. The [MaFIA](#) mice [53] originated from Jackson Laboratories, were imported and bred by the Francis Crick Institute (Stock No: 1002). CD169-DTR and CD169-iCre mice [51] originated from Masato Tanaka (RIKEN Research Center for Allergy and Immunology), were imported and bred by the Francis Crick Institute. CD169-Cre mice were crossed with either Rosa26-iDTR mice [187], provided by the Francis Crick Institute (Stock No: Y2292) or Rosa26-eYFP mice [188], donated by Frank Costantini (University of Columbia) and bred by the Francis Crick Institute. Life-Act [Enhanced green fluorescent protein \(eGFP\)](#) mice [189] were a gift from Roland Wedlich-Söldner (Max Planck Institute) and imported and bred by the Francis Crick Institute. [Colony stimulating factor 1 receptor \(CSF1R\)](#)-iCre mice [190] were purchased

from Jackson Laboratories (Stock No: 021024) and crossed with either either Rosa26-iDTR mice or Rosa26-eYFP mice. Rag1KO mice [191] originated from Jackson Laboratories, were imported and bred by the Francis Crick Institute. For flow cytometry experiments, cohorts of at least 8 week-old female WT, CD169-Cre x Rosa26-eYFP, CD169-DTR or CSF1R-iCre x Rosa26-eYFP, mice were used. For [IVM](#), CD169-iCre x Rosa26-eYFP, Life-Act eGFP and WT female mice older than 8 weeks were used.

2.2 AML experimental model

The [AML](#) blasts used for this work were generated by D.Duarte and described in [192]. Briefly, [GMPs](#) from WT or [Membrane-tomato/membrane-green \(mTmG\)](#) mice were sorted and transduced with MSCV-MLL-AF9-GFP retrovirus. Prior to injection, primary recipient mice were sub-lethally irradiated with 6 Gy. Then, 100'000 transduced GMPs were injected [Intravenously \(i.v.\)](#). Infiltration was monitored weekly until leukemic blasts composed 90% of blood sample, time point at which the mice were sacrificed and organs were harvested. At the time of harvest, the BM was 95% infiltrated and cells were frozen down. All cells from the BM of one mouse were grouped and labelled accordingly. Different mice of origin led to the generation of different AML clones as these cells would have a different mutational landscape. After thawing the blasts, cells were counted and injected in secondary recipients. According to the experiment, mice were sacrificed at different time- points and the BM was collected (Figure 2.1). In the case of combined clodronate treatment with AML, clodronate liposomes were injected 24 hours prior to the transplantation of AML cells.

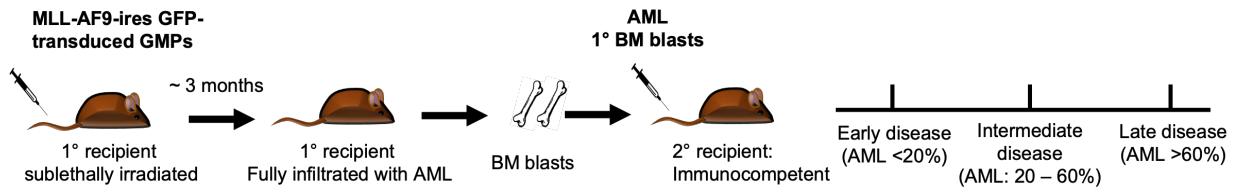


Figure 2.1: MLL-AF9 experimental model of AML

2.3 Drug administration

2.3.1 Macrophage depleting agents

For clodronate treatment, clodronate liposomes or PBS liposomes (Liposoma BV) (0.1ml/10g body weight) were injected *i.v.*. Continuous macrophage depletion was achieved by injecting clodronate or [Phosphate buffer saline \(PBS\)](#) liposomes every four days for up to 18 days. For a short term depletion, single clodronate or PBS liposome injection was performed.

For [Diphtheria toxin \(DT\)](#) treatment, 1mg diphtheria toxin (Sigma-Aldrich, D0564-1MG) was dissolved in 500 μ l of deionized water (dH₂O) to generate a stock concentration of 2mg/ml. Aliquots of 10 μ l were stored at -80°C. Working concentration of 2 μ g/ml was achieved by diluting one aliquot in 10mL of PBS and stored at -20°C. CD169-DTR mice or WT littermates were injected [Intraperitoneally \(i.p.\)](#) with DT (10ng/g body weight) for two consecutive days followed by injections every 3 days for up to three weeks.

For the activation of the MAFIA mice genetic construct, AP20187 homodimerizer (Takara Bio.) was prepared following the protocol described in S. Burnett et al. [53]. Briefly, lyophilized AP20187 was dissolved in 100% ethanol at a stock concentration of 62.5 mg/ml and stored at -20°C. Injections solutions at 0.825mg/mL were prepared in 4% ethanol, 10% [Polyethylene glycol \(PEG\)](#)-400, and 1.7% Tween in water. The drug was administered at 10mg/kg (body weight) *i.v.*. The average volume injected was 200 μ L per mouse. MAFIA mice were injected 3 days in a row with AP20187 or injection solution.

2.3.2 Chemotherapy

The chemotherapy regimen used was based on the most commonly administered drug regimen given to AML patients. Following the manufacturer's protocol [193], 100mg of Cytarabine (MedChemExpress; Cat. No. 147-94-) was dissolved in 10% [Dimethyl sulfoxide \(DMSO\)](#), 40% PEG300, 5% Tween-80 and 45% PBS to achieve a stock concentration of 100mg/ml. 100mg of Doxorubicine (MedChemExpress; Cat. No. 25316-40-9) was dissolved in 5% DMSO, 40% PEG-300, 5% Tween-80 and 45% PBS to achieve a stock concentration of 2mg/ml. When AML infiltration was greater than 10% in the blood measured by flow cytometry, 100mg/kg body weight of Cytarabine and 3mg/kg body weight of Doxorubicine were injected *i.v* for three days followed by two days of injections with only Cytarabine.

2.4 Flow cytometry

2.4.1 Bone marrow cell isolation

For analysis of BM macrophages, the legs of control and leukemic mice were harvested. Tibias, femurs and hips were cleaned and gently crushed using a mortar and pestle in FACS buffer composed of PBS + 2% heat inactivated [Fetal bovine serum \(FBS\)](#) and filtered in a 40 μ m cell strainer. The samples were centrifuged at 500 x g for 5 minutes at 4°C and resuspended in red blood cell lysis buffer (0.001g/ml Potassium bicarbonate, 0.008g/ml Ammonium chloride, 20mM [Ethylenediaminetetraacetic acid \(EDTA\)](#), all from Sigma, and 5% (v/v) FBS in Milli-Q water) for 5 minutes before being washed in FACS buffer. Following centrifugation, the supernatant was discarded and the cells were transferred to a 96 v- shape well plate for staining.

2.4.2 Cell staining and sample acquisition

One quarter of each leg sample (approx. 12 million cells) was stained in 200 μ L of FACS buffer for 30 minutes in the dark at 4°C. Next, the samples were centrifuged at 800 x g for 2 minutes and resuspended in 200 μ L. This step was repeated twice to wash the cells. To distinguish dead and apoptotic cells from live ones, cells were either stained with a fixable viability dye (Fixable Viability Stain 510, BD Biosciences, Cat No. 564406) for 10 minutes in the dark at room temperature or with [4,6-diamidino-2-phenylindole \(DAPI\)](#) (NucBlue Fixed Cell ReadyProbes Reagent; Invitrogen, Cat. No. R37606). Subsequently, cells were washed twice and resuspended at the right concentration for acquisition. Specific details for the fluorochrome-conjugated antibodies used in experiments can be found in Table 2.1. Single colour controls were generated either on compensation beads (OneComp eBeads Compensation Beads; Invitrogen, Cat. No. 01-1111-42) or WT bone marrow cells. [Fluorescence minus one \(FMO\)](#) controls were generated when necessary.

Antibody	Clone	Dilution	Source	Cat. No.
BV711 anti-mouse CD11b	M1/70	1/200	Biolegend	101241
PE/Cy7 anti-mouse CD11b	M1/70	1/500	Biolegend	101216
FITC anti-mouse CD11b	M1/70	1/500	Biolegend	101206
BUV563 anti-mouse CD11b	M1/70	1/500	Biolegend	741242
BUV395 anti-mouse Ly6G	1A8	1/200	BD Biosciences	565964
FITC anti-mouse Ly6G	1A8	1/500	BioLegend	127605
APC anti-mouse Ly6G	1A8	1/500	BioLegend	127613
PerCPCy5.5 anti-mouse Ly6C	HK1.4	1/600	BioLegend	128011
BV510 anti-mouse Ly6C	HK1.4	1/600	BioLegend	128033
APC anti-mouse F4/80	BM8	1/200	BioLegend	123116
BV605 anti-mouse F4/80	BM8	1/200	BioLegend	123133
PE/Cy7 anti-mouse F4/80	BM8	1/200	BioLegend	123114
BUV805 anti-mouse F4/80	T45-2342	1/100	BD Biosciences	749282
APC anti-mouse CD169	3D6.112	1/200	BioLegend	142418
BV605 anti-mouse CD169	3D6.112	1/200	BioLegend	142413

PEDazzle anti-mouse CD169	3D6.112	1/200	BioLegend	142424
APC/Cy7 anti-mouse SIRP α	P84	1/200	BioLegend	144018
PerCPCy5.5 anti-mouse SIRP α	P84	1/100	BioLegend	144010
APC anti-mouse SIRP α	P84	1/200	BioLegend	144014
PE anti-mouse SIRP α	P84	1/200	BioLegend	144011
BV421 anti-mouse PDL1	10F.9G2	1/200	BioLegend	124315
PEDazzle anti-mouse PDL1	10F.9G2	1/200	BioLegend	124323
PE anti-mouse PDL1	MIH6	1/200	BioLegend	153611
PE-CF594 anti-mouse PDL1	MIH6	1/200	BioLegend	153606
APC anti-mouse PDL1	MIH5	1/200	BD Biosciences	564715
BV786 anti-mouse CD11c	N418	1/200	BioLegend	117336
BV605 anti-mouse MHCII	M5/114	1/500	BD Biosciences	563413
BV421 anti-mouse CD3	17A2	1/200	Biolegend	100228
APC anti-mouse CD3	145-2C11	1/200	Biolegend	100311
BV650 anti-mouse CD4	RM4-5	1/200	Biolegend	100555
APC/Cy7 anti-mouse CD8	53-6.7	1/200	Biolegend	100714

BV786 anti-mouse PD1	29F.1A12	1/200	Biolegend	135216
PE/Cy7 anti-mouse CD25	PC61	1/200	Biolegend	102015
PacBlue anti-mouse CD69	H1.2F3	1/200	Biolegend	104524
FITC anti-mouse CD44	IM7	1/200	Biolegend	103006
PerCPCy5.5 anti-mouse CD62L	MEL-14	1/200	Biolegend	104432
APC/Cy7 anti-mouse CD19	6D5	1/200	Biolegend	115530
APC anti-mouse Tie2	TEK4	1/50	Biolegend	124010
AF647 anti-mouse TNF α	MP6-XT22	1/100	Biolegend	506314
AF488 anti-mouse iNOS	54/iNOS	1/200	Thermofisher	53-5920-80
PerCP-e7100 anti-mouse Arg1	A1ex5	1/100	Thermofisher	46-3697-80
AF700 anti-mouse RELM α	DS8RELM	1/50	Thermofisher	56-5441-80
eFluor450 anti-mouse LYVE-1	ALY7	1/20	Thermofisher	48-0443-80
APC/Cy7 anti-mouse MerTK	2B10C42	1/200	Biolegend	151519
PE anti-mouse DARC	REA 392	1/50	R&D	FAB6695P
BV480 anti-mouse MVCAM.A	429	1/100	BD Biosciences	746326
BV711 anti-mouse Tim-4	RMT4-54	1/100	BD Biosciences	745509

BUV737 VEGF-R2	Avas	1/40	BD Biosciences	741797
BUV496 anti-mouse CD86	PO3	1/100	BD Biosciences	750437
BV421 anti-mouse LAP	TW7-16B4	1/100	BD Biosciences	565638
BV750 anti-mouse CD115	AFS98	1/200	BD Biosciences	750889

Table 2.1: Details of the antibodies used for flow cytometry experiments

Calibrite Beads (BD Calibrite 3 Beads Unlabelled, FITC, PE and PerCP Beads; BD Biosciences Cat. No. 340486) were added to calculate the absolute number of cells present in the sample at the time of the acquisition following the manufacturer’s protocol [194]. Briefly, Calibrite Beads were resuspended in PBS at a concentration of 1×10^6 beads/mL. At the time of acquisition, the beads were mixed well by inverting the tube four times and 1×10^5 beads were added to each sample. The number of cells was then backcalculated based on the number of beads acquired by the flow cytometer (see Equation 2.1).

$$N_c = \frac{C_b \cdot V_b}{N_b} \cdot DF \cdot N_{cm} \quad (2.1)$$

C_b = Bead concentration, V_b = Bead volume, N_b = Number of beads measured by the FACS Fortessa, DF = Dilution factor, N_{cm} = Number of cells measured by the FACS

To analyse cell proliferation, the Click-iT EdU Pacific Blue Flow Cytometry Assay Kit (Invitrogen, Cat. No. C10418) was used. 5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen, Cat. No. A10044) was injected *i.p.* (1mg of EDU/mouse) 2 hours before harvest. After surface staining of the cells, the samples were washed and fixed in 200 μ L Fixation Buffer (BD Biosciences, Cat. No. 554655) for 20 minutes at room temperature and washed in FACS Buffer. The samples were then permeabilised in 200 μ L Permeabilization Buffer Plus (BD Biosciences, Cat. No. 561651) for 15 minutes at room temperature. To label the EDU, the Click-iT kit was used following the manufacturer’s protocol [195]. Following incubation with the Click-iT reagents in the dark for 30 minutes at room temperature, samples were washed in Permeabilisation/Wash buffer and analysed by FACS.

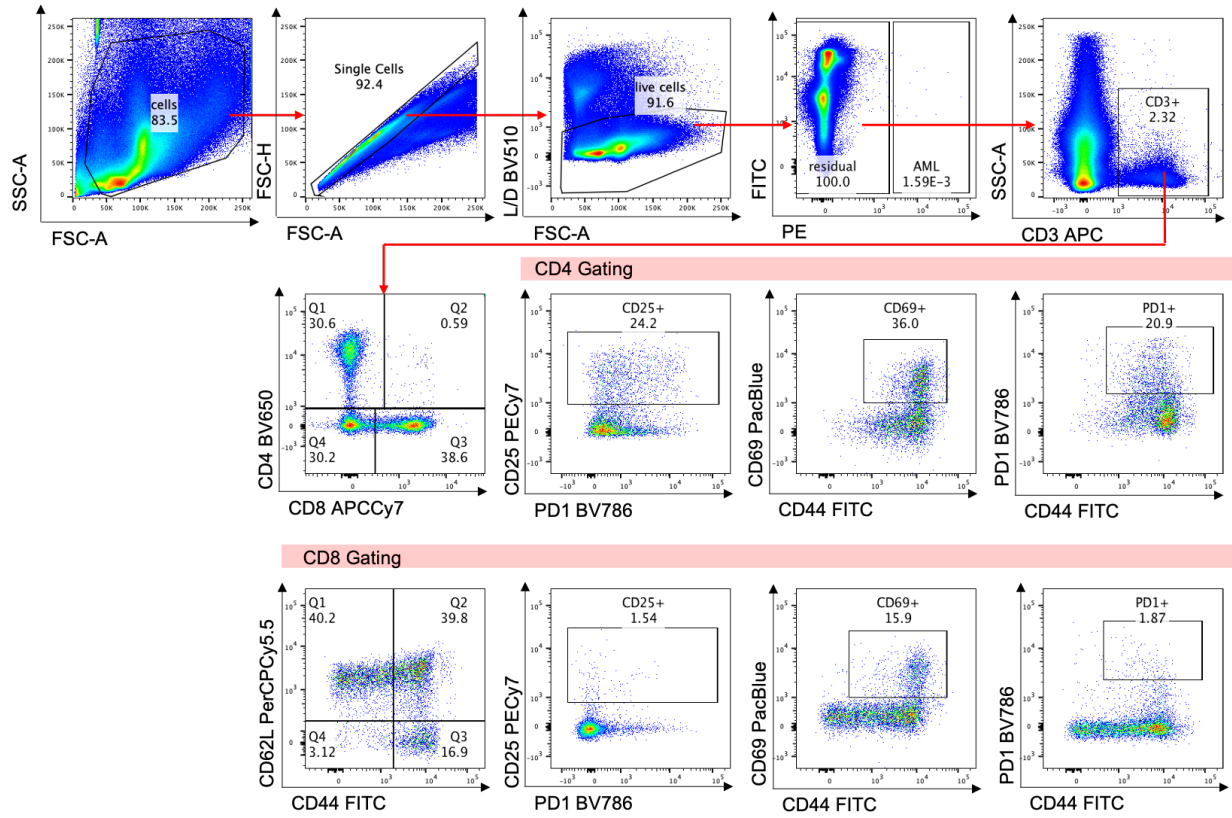


Figure 2.2: FACS gating strategy for bone marrow T cell subsets

Representative plot from the BM of a control mouse analysed by flow cytometry. Black boxes indicate gates used for gating on BM cells to exclude debris, doublets, dead cells, AML cells and gate on CD3+, CD4+ or CD8+ cells. CD25, CD69, PD1, CD44 and CD62L were used to highlight the activation profile of the various T cell subsets.

All samples were analysed on an LSR Fortessa (BD Biosciences), sort-purified using a FACSMelody (BD Biosciences) and analysed using FlowJo (Tree Star). The gating strategies used for the following group of cells can be found below:

- T cell subsets (Figure 2.2)
- Myeloid cells including neutrophils, monocytes and macrophages (Figure 2.3)
- Monocytes and macrophages for sorts (Figure 2.4)

For unsupervised analysis, single live tomato-, Ly6G^{lo}, Ly6C^{lo}, cells were first clustered and visualised using the t-sne plugin in Flowjo and clusters were indentified using heatmaps of the differential expression of markers in FlowSOM [196]. Macrophages clusters were identified based on their low expression of Ly6G, Ly6C, small SSC-A and high expression of CD11b and F4/80.

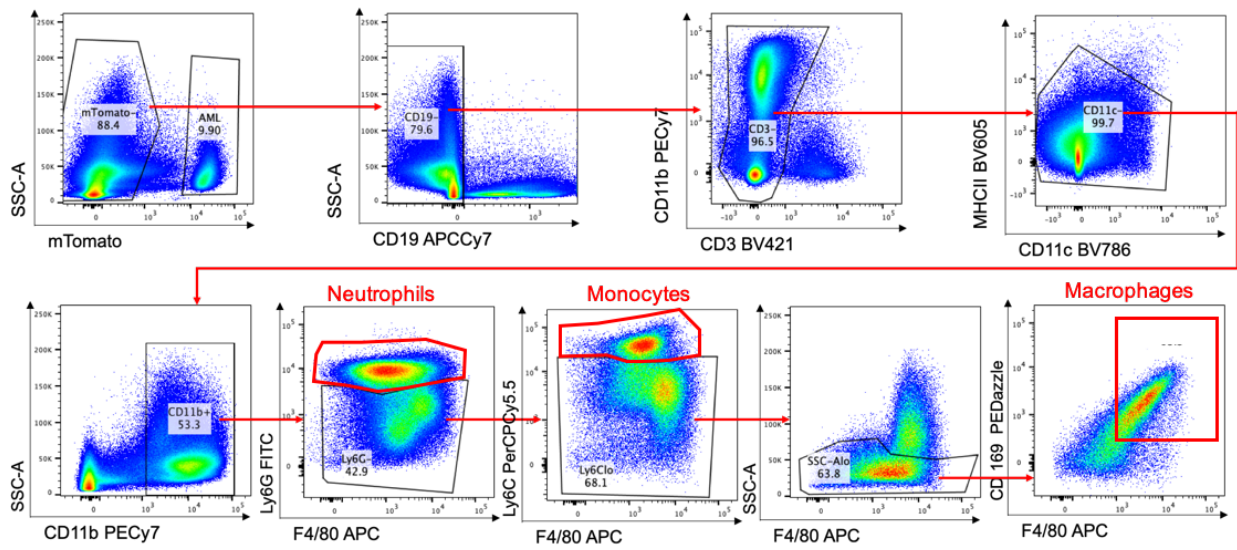


Figure 2.3: FACS gating strategy to identify monocytes, neutrophils and macrophages. *Live single cells were identified using the same strategy as for T cells. Representative plot from the BM of a mouse harvested 11 days after transplantation of mTmG AML cells. Black boxes indicate gates used on BM cells to exclude B cells, T cells and dendritic cells. Red boxes indicates gates on neutrophils, monocytes and macrophages.*

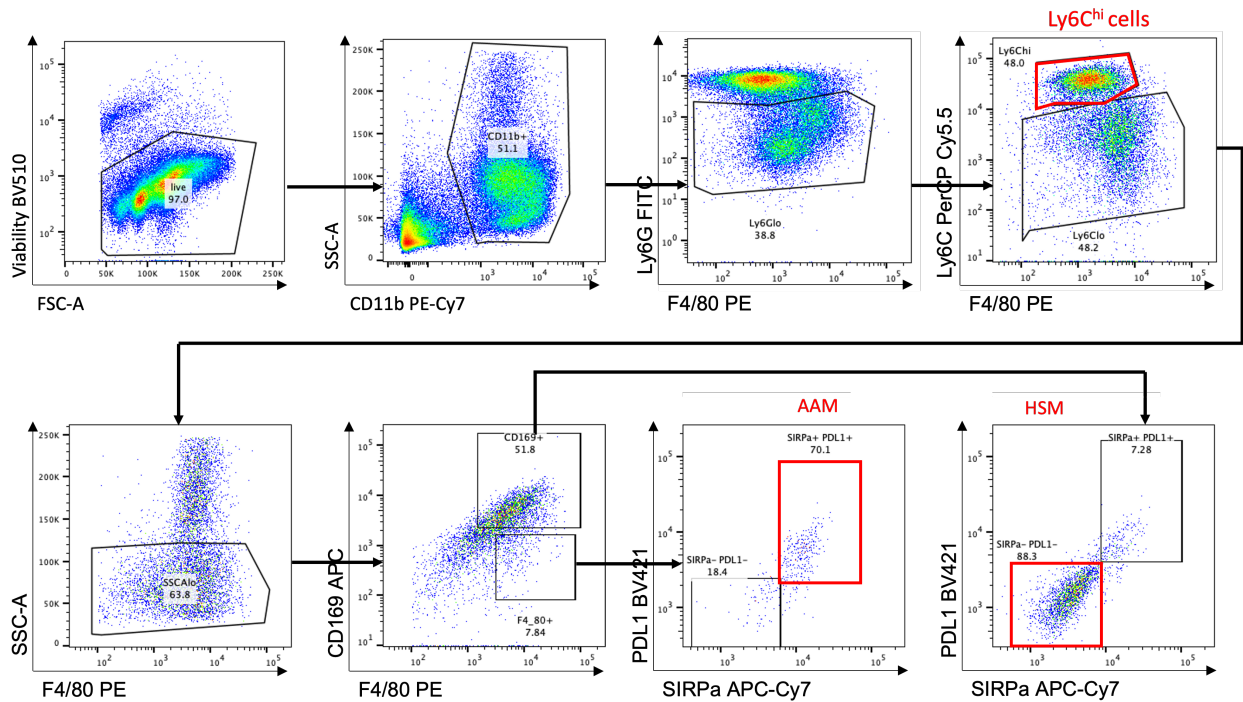


Figure 2.4: FACS sort gating strategy for bone marrow monocytes and macrophages. *Representative plot from the BM of a control mouse analysed by flow cytometry. Black boxes indicate gates used for gating on BM cells to exclude dead cells, non myeloid cells, neutrophils and eosinophils. Red boxes indicates gates used to sort the populations of interest. IMM: Immunomodulatory macrophages, HSM: hematopoiesis supportive macrophages*

2.5 Cell culture and cell migration assessment

2.5.1 BMDM cell culture

Bone marrow cells from control mice were harvested as reported previously. Cells were centrifuged at 500 x g for 10 minutes and resuspended in FACS buffer as described in [52]. Cells were counted and resuspended at 5×10^4 cells/mL for control in macrophage differentiation media composed of Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F12) (Life Technologies, Cat. No. 11320-074) with 10% FBS, 10mM L-glutamine (L-Glu) (Thermo Fisher, Cat. No. 25030-081) , 20% L929 conditioned media and 1% Penicillin/Streptomycin (Life Technologies, Cat. No. P11-010). Cells were plated in T-75 flasks in a volume of 15mL. On day 3, 7.5 mL of macrophage differentiation media was added. On day 7, the supernatant was discarded and adherent cells were harvested using a cell scraper (Greiner Bio-One Ltd. Cat. No. 541070) in 5mL fresh PBS. Cells were centrifuged and plated accordingly to the following experiments.

L929 conditioned media was generated by culturing the fibroblast cell line in DMEM/F12 (Life Technologies, Cat. No. 11320-074) with 10% FBS, 10mM L-Glu (Thermo Fisher, Cat. No. 25030-081) for 10 days and passaging them when they reached 70% confluency. On the last passage, the cells were left until they reached 100% confluency. Then the cell culture media was collected and centrifuged at 3000rpm for 5 minutes. The supernatant was finally filtered through a $0.45\mu\text{m}$ filter, aliquoted and stored at -80°C .

2.5.2 AML cells culture

Primary mTmG leukaemia cells were thawed and plated at 1×10^5 cells/mL in a 6-well tissue culture non-treated plate (VWR, Cat. No. 734-2777) in 5 mL of Roswell park memorial institute (RPMI) 1640 medium (Sigma, Cat. No. R8758), 10% FBS, 2mM L-Glu (Thermo Fisher, Cat. No. 25030-081), 10ng/mL Human interleukin-6 (hIL-6) (Peprotech, Cat. No. 200-06-100), 10 ng/mL Murine Stem cell factor (m-SCF) (Peprotech, Cat. No. 250-03-100) and 6 ng/mL Murine interleukin-3 (mIL-3) (Peprotech, Cat. No. 213-13-100). Every two days, cells were counted using a haematocytometer and plated at 1×10^5 cells/mL in fresh medium.

2.5.3 Differentiation of sorted Ly6C^{hi} monocytes into Immunomodulatory macrophages

Ly6C^{hi} cells were sorted from the bone marrow of a control mouse, at 4°C in PBS with 10% FBS, with the aim of sorting 1x10⁶ cells, following harvest and staining described in section 2.4. The cells were centrifuged at 300 x g for 10 minutes. The cells were resuspended in macrophage differentiation media and plated at 2x10⁵/mL in a flat bottom 96 well-plate. The plates were incubated for 48 hours. Following the incubation, the cells were harvested using a cell scraper (Greiner Bio-One Ltd. Cat. No. 541070) in 200µL of fresh PBS. The cells were centrifuged and stained for flow cytometry analysis of macrophage phenotyping.

2.5.4 Co-culture of AML cells and macrophages and phagocytosis assay

Ly6C^{hi}, Immunomodulatory macrophages and Haematopoiesis-supportive macrophages were sorted from the bone marrow of a control mouse, at 4°C in PBS with 10% FBS, with the aim of sorting 1x10⁶ of each population. The cells were centrifuged at 300 x g for 10 minutes. The cells were resuspended in macrophage differentiation media and plated at either 2x10⁵/mL or 1x10⁵/mL in a flat bottom 96 well-plate.

For the phagocytosis assay, mTmG+ AML cells were resuspended at a concentration of 1x10⁶ cells/mL in PBS and stained with 1 µL/mL of [Cell trace violet \(CTV\)](#) (ThermoFisher Cat. No. C34557) for 20 minutes at room temperature. Following this incubation, 5mL of macrophage differentiation medium was added to the sample and left to incubate for 5 minutes. The cells were centrifuged at 500 x g for 5 minutes and resuspended in 1 mL of pre-warmed macrophage media. The cells were incubated for 10 minutes at room temperature and finally resuspended at 2x10⁵/mL. The AML cells were plated on top of the macrophages at 3 different ratios, 2:1, 1:1 and 1:2. The co-cultures were incubated at 37°C for 24 and 48 hours. To evaluate whether macrophages promoted AML cell proliferation, the cells were counted and the percentage of macrophages (distinguished based on their absence of expression of mTmG) was measured by flow cytometry. To measure the phagocytic potential of macrophages, the incorporation of CTV dye in macrophages was measured by flow cytometry.

2.5.5 Co-culture of T cells and macrophages

On the day prior to the co-culture, the co-culture flat bottom 96 well plate was coated with 50 μ L of 1% anti-CD3 antibody (BD Biosciences, Cat. No. 553058) diluted in sterile PBS. To isolate T cells, the EasySep Mouse T cell isolation kit (StemCell, Cat. No. 19851) was used, following the manufacturer's protocol [197]. The spleen of a control mouse was harvested in ice cold PBS. Using a 70 μ m cell strainer and the plunge of a syringe, the spleen was mashed and filtered until the strainer was clear of cells. The cell suspension was centrifuged at 300 x g for 10 minutes. After centrifugation, the cells were resuspended at 1x10⁸ cells/mL in PBS and 50 μ L of Rat serum was added for each mL of sample. The sample was transferred to a 5mL FACS tube where 50 μ L of sample of isolation cocktail was added for each mL of sample. The sample was vortexed and incubated at room temperature for 10 minutes. Meanwhile, the RapidSphere were vortexed for 30 seconds. Following this incubation, 75 μ L of RapidSphere was added, mixed and incubated for 2.5 minutes. The sample was topped up with PBS to reach a final volume of 2.5mL. After pipetting up and down 3 times, the sample was placed in the EasySep Magnet (StemCell, Cat. No. 18000) for 2.5 minutes. To collect only non-labelled T cells, the tube was inverted with the magnet, in one continuous movement and the sample was collected in a new tube. Cells were centrifuged at 300 x g for 8 minutes and resuspended in PBS at 1x10⁶ cells/mL. To measure T cell proliferation, the T cells were stained with 1 μ L/mL of CTV (ThermoFisher Cat. No. C34557) for 20 minutes at room temperature. Following this incubation, 15 mL of pre-warmed T cell media consisting of RPMI media with 10% Fetal calf serum (FCS), 50U/mL penicilin, 50 μ g/mL streptomycin, 2mM L-Glu, 1% Non-Essential Amino Acids (Life Technologies, Cat. No. 11140050), 1mM of sodium pyruvate (Life Technologies Cat. No. 11360-070), 10mM HEPES (Life Technologies Cat. No. 15630-080) and 50 μ M 2-mercaptoethanol (Sigma Cat. No. M6250-100ML) was added. After a 5 minutes incubation, the cells were centrifuged at 500 x g for 5 minutes and resuspended in 3mL of pre-warmed T cell media. Following a 10 minutes incubation, the T cells were resuspended in T cell media at a concentration of 550'000 cells/mL, plated in 100 μ L/well of a flat bottom 96 well plate and left to settle for 15 minutes. To activate the T cells, 10 μ L of anti-CD28 antibody (BD Biosciences, Cat. No. 553295) was diluted in 500 μ L of T cell media. 10 μ L of antibody was distributed per well.

To isolate the macrophage populations of interest, Ly6C^{hi} and Haematopoiesis-supportive macrophages were sorted from the bone marrow of a control mouse, at 4°C in PBS with 10%

FBS, with the aim of sorting 1×10^6 of each population. The cells were centrifuged at $300 \times g$ for 10 minutes and resuspended in T cell media. The macrophages were added to the T cells at 5 different ratios (macrophage : T cell): 0.4:1, 0.2:1 and 0.1:1, 0.05:1 and 0.025:1, 0:1. As a positive control, T cells were incubated alone with anti-CD3/CD28 antibodies. As a negative control, T cells were incubated alone without antibodies. The cultures were incubated at 37°C for 72 hours.

To evaluate whether macrophages inhibited T cell proliferation, the histogram of CTV present in T cells was analysed by flow cytometry. In addition, the cells were counted with an automatic cell counter (Invitrogen, Countess II) and the percentage of macrophages (distinguished by their absence of CTV) was measured by flow cytometry.

2.5.6 Migration assay

BMDM were plated at 1×10^5 cells/well in the lower chamber of a 24 transwell plate containing $5.0 \mu\text{m}$ pore polycarbonate membrane inserts (Corning, Cat. No. 3421). The cells were plated in $750 \mu\text{l}$ DMEM/F12 (Life Technologies, Cat. No. 11320-074) with 10% FBS, 10mM L-Glu (Thermo Fisher, Cat. No. 25030-081), 100U/mL [Macrophage colony-stimulating factor \(M-CSF\)](#) (Peprotech, Cat. No. 315-02-50), 10ng/mL [hIL-6](#) (Peprotech, Cat. No. 200-06-100), 10 ng/mL m-SCF (Peprotech, Cat. No. 250-03-100) and 6 ng/mL mIL-3 (Peprotech, Cat. No. 213-13-100) and 1% Penicillin/Streptomycin (Life Technologies, Cat. No. P11-010). The cells were left to adhere overnight. As a positive control, 100ng/mL of [Murine C-X-C motif chemokine ligand 12 \(mCXCL12\)](#) (Peprotech, Cat. No. 250-20B) was added to the lower chamber without any cells. As a negative control, only media supplemented with all the cytokines but no CXCL12 was added to the lower chamber. BMDM conditioned media was also tested to evaluate whether direct contact was essential for migration of AML cells. To generate the BMDM conditioned media, the supernatant of BMDM culture was harvested 24 hours after adding fresh media and the conditioned media was produced by combining 80% of the supernatant with 20% of fresh media containing all of the above mentioned cytokines.

The following day, 1×10^5 AML cells were plated in the upper compartment of the migration chamber (See Figure 2.5) in the same media as the BMDM. 24 hours later, the cells in suspension in the upper and lower chamber were harvested using a 1mL pipette and counted with an

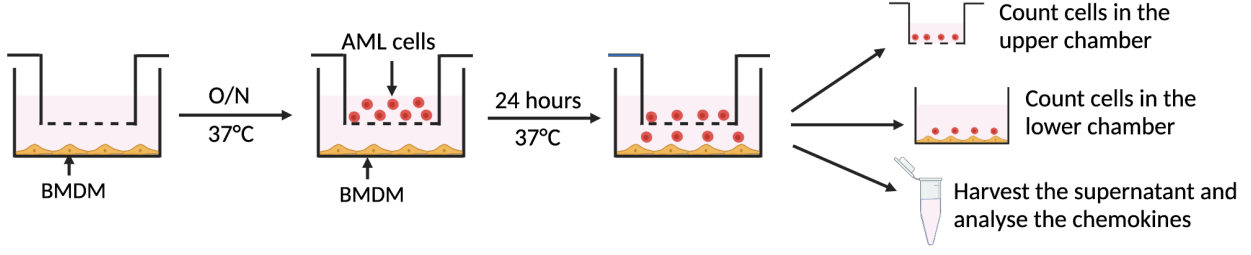


Figure 2.5: Migration assay set-up

Diagram of the experimental set-up for the AML migration assay. BMDM are first plated in the lower chamber. The next day, AML cells are plated in the upper chamber. After 24 hours the cells in the upper and lower chamber are counted using a hemacytometer and the supernatant is analysed for secreted chemokines.

automatic cell counter (Invitrogen, Countess II). To confirm BMDM were not included in the count of the lower chamber, the cells were FACSed and the percentage of mTmG⁺ cells was deduced from the total cell count. The percentage of migration was then calculated (See Equation 2.2). To check that AML proliferation was not impacted by the presence of BMDMs in the lower chamber, the total AML cell count ($N_{lc} + N_{uc}$) was also analysed.

$$\%_{migration} = \frac{\frac{\alpha}{100}(N_{lc})}{(\frac{\alpha}{100}(N_{lc}) + N_{uc})} \quad (2.2)$$

N_{lc} = Number of cells counted in the lower chamber, N_{uc} = Number of cells counted in the upper chamber, α = percentage of mTmG⁺ cells in the lower chamber measured by FACS Dilution factor, N_{cm} = Number of cells measured by the FACS

2.5.7 Chemokine array and analysis

To measure the chemokines secreted by BMDMs and AML cells in the migration assay described above, the supernatant of BMDM and AML transwell cultures were analysed using the Proteome Profiler Mouse Chemokine Array Kit (R&D, Cat. No. ARY020). This membrane based antibody array allows for a semi-quantitative measurement of the level of 25 mouse chemokines. To evaluate which chemokines were secreted by AML cells and which were secreted by BMDMs, the supernatant of single AML or BMDM cultures were also analysed. As a technical positive control, the supernatant of OP9 cell culture was also analysed. Indeed, these cells are known to secrete mCXCL12, one of the chemokine analysed in this array.

The supernatant of these 4 cell cultures were centrifuged at 500 x g for 5 minutes to remove

any cell debris. 500 μ L of the supernatant of each cell culture was analysed following the manufacturer's protocol [198]. The membranes were exposed for 30 minutes to an X-Ray film (Carestream BioMax light film, Merck, Cat. No. Z370371-50EA) using an X-Ray developer. The dots representing the levels of each chemokine, seen on the film were segmented and quantified using Fiji. The average pixel intensity of each dot was normalised thanks to the technical positive and negative dots from each membrane, removing any bias from different exposure to the X-ray (See Equation 2.3). The normalised average intensity of each chemokine for each condition was plotted using GraphPad Prism.

$$n_i = \frac{100(\bar{x}_i - \frac{\sum \bar{x}_{nd,j}}{n_{nd}})}{\frac{\sum \bar{x}_{pd,l}}{n_{pd}} - \frac{\sum \bar{x}_{nd,j}}{n_{nd}}} \quad (2.3)$$

n_i = Normalised average pixel intensity of the dot representing each chemokine, i ; $\bar{x}_i$ = average intensity of the pixels for the chemokine i ; $\bar{x}_{nd,j}$ = average pixel intensity of the negative dot, j ; n_{nd} = number of negative dots $\bar{x}_{pd,l}$ = average pixel intensity of the positive dot, l ; n_{pd} = number of positive dots

2.6 Single cell RNA sequencing and analysis

This experiment was performed with the help of the sequencing facility from the Francis Crick Institute for sample processing and sequencing. For 10x ChromiumTM (10x Genomics, Pleasanton, CA) experiments, macrophages and AML cells were analysed. Macrophages, defined as tomato- CD11b+ Ly6G- Ly6C^{lo} SSCA^{lo} F4/80+ cells were sorted from 2 control and 2 AML mice (AML = 15% of BM cells). AML cells, defined as tomato+ cells were sorted from 2 early AML mice (AML = 15% of BM cells) and 2 late AML (AML = 65% of BM cells) mice.

2.6.1 Data processing and quality control

Samples were processed and sequenced according to the manufacturer's protocol. Data processing was performed by P. Chakravarty. Briefly, after library demultiplexing, gene-expression libraries were aligned to the mm10 reference transcriptomes and count matrices were generated using the default Cell Ranger workflow, using the raw matrix output (See Appendix A for the cell ranger reports). The data was subsequently processed using Seurat 2.3.0. Cells with >10% of unique molecular identifier (UMI) counts mapping to mitochondrial genes, expressing fewer

than 500 genes, or with the total number of UMI counts further than 3 standard deviations from the mean were excluded. 9856 control macrophages, 10389 AML macrophages, 10841 early AML cells and 12023 late AML cells passed these filtering steps.

2.6.2 Clustering

After integration of all samples, initial clustering was performed at a resolution of 0.3. 16 clusters were generated. Using Immgen, clusters were identified and contaminant cell populations consisting in T cells, B cells and dying cells were excluded. Macrophage core signature was measured based on the expression of the core tissue macrophage signature genes (Appendix C). This list of genes was defined by E. Gautier et al. [199] as genes expressed specifically by macrophages (at least 2 fold higher expression than in DCs) and expressed by at least three different types of macrophages among four types of macrophages: peritoneal macrophages, alveolar macrophages, microglia and splenic macrophages. From this list, a bone marrow specific macrophage gene list was generated including all genes expressed in our scRNAseq dataset (Appendix C). Expression of phagocytosis and antigen processing and presentation genes were evaluated by importing the Gene Ontology gene lists associated with these processes and normalising the expression on Seurat. Macrophage clusters were identified based on the combination of expression of the bone marrow core macrophage signature genes and expression of phagocytosis and antigen processing and presentation genes. Clusters identified as macrophage clusters were selected for higher resolution clustering analysis using UMAP. Only the macrophage samples were included in this new analysis. 6 clusters were generated. Gene ontology analysis of the biological processes unique to each cluster and common to at least three clusters was performed. Differential expression analysis was also performed between control and AML macrophages located in cluster 2. Genes significantly differentially expressed were imported into EnrichR for gene ontology analysis.

2.6.3 Integration of our dataset with lung macrophage dataset

scRNA-seq data of macrophages from control and NSCLC lungs and accompanying cluster annotations were downloaded from the Gene Expression Omnibus (GEO) with accession code GSE127465. This dataset was integrated with our dataset and clustering was performed at a resolution of 0.5. The same analysis steps were followed as previously described in the clustering section. Cells annotated as monocytes/macrophages/DCs in the lung dataset were selected with the BM macrophages for higher resolution clustering.

2.7 Homing assay

On day 0, mice were injected with 0.1ml/10g clodronate or PBS liposomes [i.v.](#) On day 1, 1'000'000 mTmG+ AML cells were transplanted [i.v.](#). On day 2, mice were imaged using the intravital microscopy protocol described above. 1 hour-long timelapses with images acquired every 2 minutes were obtained. Following the imaging session, calvarium and legs were harvested, processed and analysed by flow cytometry following the above protocol. For the flow cytometry homing assay, the same protocol was followed but this time 2 million mTmG+ AML cells were transplanted [i.v.](#) and spleen, blood and legs were analysed.

2.8 Bone marrow chimeras

To generate chimeric mice, Life-Actin eGFP+ mice were lethally irradiated (2 x 5.5 Gy, the two doses were separated by a minimum of 3 hours). Following irradiation, mice were injected [i.v.](#) with 1×10^6 WT BM cells diluted in 100 μ L of PBS. Mice were given antimicrobial 2.5% Baytril (Elanco Animal Health) diluted in drinking water for 6 weeks following the transplantation. The percentage of chimerism was evaluated by FACS analysis of peripheral blood (% of eGFP+ cells) with >95% of chimerism obtained approximately 12 weeks after transplantation. Following complete reconstitution of the BM, the mice were injected with mTmG AML cells and imaged 11 to 15 days later using IVM.

2.9 Bone marrow histology

Femur and tibia of mice were cleaned in a similar manner as for flow cytometry analysis. Bones were then placed in 4% [Paraformaldehyde \(PFA\)](#) diluted in PBS overnight. The following day, bones were transferred through a sucrose gradient: first PBS with 10% sucrose for an hour, then PBS with 20% sucrose for another hour and finally PBS with 30% sucrose overnight. After the sucrose gradient, bones were placed in cryomolds with [Optimal cutting temperature \(OCT\)](#) and frozen at -80°C. 20 μ m sections were cut using the Leica CM3050 S Research Cryostat with Cryojane tape transfer system. Slides were stored at -80°C. For staining, slides were first re-hydrated in PBS and permeabilised in 0.1% Triton X-100. To avoid non specific staining, the sections were blocked with 5% goat serum and incubated with rat anti-F4/80(BM8, Abcam, 1:50) and rabbit anti-SIRP α (ab191419, Abcam, 1:500) overnight, at 4°C. After washing in PBS, the sections were incubated with goat anti-rat IgG Alexa Fluor

633 (Life Technologies, 1:400) and goat anti-rabbit IgG Alexa Fluor 488 (Life Technologies, 1:400) antibodies and counter-stained with DAPI. Prior to imaging, sections were mounted with Prolong Diamond antifade (Invitrogen). Consecutive z-stacks were acquired using a Zeiss LSM 780 upright confocal microscope combined to a two-photon microscope, stitched using ZEN black software (Zeiss, Germany) and analysed using Fiji/ImageJ.

2.10 Intravital microscopy

All microscopy was performed using a Zeiss LSM 780 or 980 upright confocal and two photon hybrid microscope equipped with Argon providing 450, 488, 514, 561 and 633 lasers, a tunable multiphoton laser (Spectraphysics Mai Tai eDeep See, 680-1020nm), 4 [Non descanned detector \(NDD\)](#) and an internal detector array. Signal was visualised using a Zeiss W Plan-Apochromat 20X DIC water immersion lens (1.0 NA).

The surgical procedure and imaging were completed following previously published protocols and following the Home Office regulations [200, 201, 192, 202]. Prior to the surgery, 50 μ l of APC anti-mouse F4/80 antibody (Biolegend, clone BM8) was injected intravenously to label macrophages. Mice were anaesthetised using isoflurane in medical oxygen throughout the experiment (4% isoflurane in 2L/min O₂ for the induction phase and 1-2% isoflurane in 2L/min O₂ during the surgery and imaging session). After the induction of anaesthesia, the temperature of the animal was regulated using a homeothermic monitoring system. Blephagel eye ointment (SpectrumThea) was added on the eyes to maintain eye lubrication and avoid corneal injury. Once all pedal withdrawal reflexes were absent, a small incision to remove the skin covering the calvarium of the animal was performed. The underlying tissue covering the calvarium was removed using cotton buds soaked in PBS. Using dental cement (Diamond Carve Dental Cement, Associated Dental Products; Cat. No. SUN527), a custom-made imaging window was attached to the calvarium. The dental cement was prepared by mixing 3 units of powder with 2 drops of the liquid provided by the kit. Once the headpiece was placed on the calvarium with dental cement, the cement was left to adhere for 20 minutes. Following the solidification of the cement, the mice were transferred to the microscope stage and using a lock-and-key system, the headpiece was attached to the stage. This system allows for the accurate positioning and stable imaging of the mice. To remove any dead cells, cement debris or residual tissue, the calvarium was cleaned with PBS. The lens was then immersed in PBS contained by the imaging window.

To visualise blood vessels, mice were injected i.v with 80 μ l of FITC-Dextran (2000kDa,

Sigma). GFP and FITC, mTomato and APC signals were excited at 488nm, 561nm and 633nm with the confocal microscope and detected using internal detectors. The bone signal was acquired using the second harmonic signal, excited at 860-880nm with the two-photon microscope and detected using the external detectors. 2-hour timelapses were acquired with images acquired every 2 minutes and image averaging of 4x. High resolution 30 minute time-lapses were also acquired for single cell quantification with images acquired every 30 seconds and an averaging of 16x. Finally, large 3-dimensional tile-scans of the BM space were acquired and stitched together leading to single cell resolution images of the entire calvarium cavity.

2.11 Image processing and quantification

Zen blue (Zeiss, Germany) was used to stitch the [3-dimensional \(3D\)](#) tilescans of the BM cavity. Image J/Fiji was used for all downstream analysis. To remove the central sinus and adjacent large vessels, located in between the bone marrow areas in tilescans, these large vessels were outlined and cleared from the image. The BM area was determined by merging all channels, thresholding them and applying erode and dilate four times. The area was then measured by counting the number of white pixels. The BM area was divided into 100 ROIs of 120 microns side length. Following thresholding of each channels, the total percentage of positive pixels was first calculated followed by the percentage of positive cells in each ROI, using equation [2.4](#):

$$\%_{positive\ pixel\ ROI} = 100 * \frac{Mean\ channel\ intensity\ in\ ROI}{255} \quad (2.4)$$

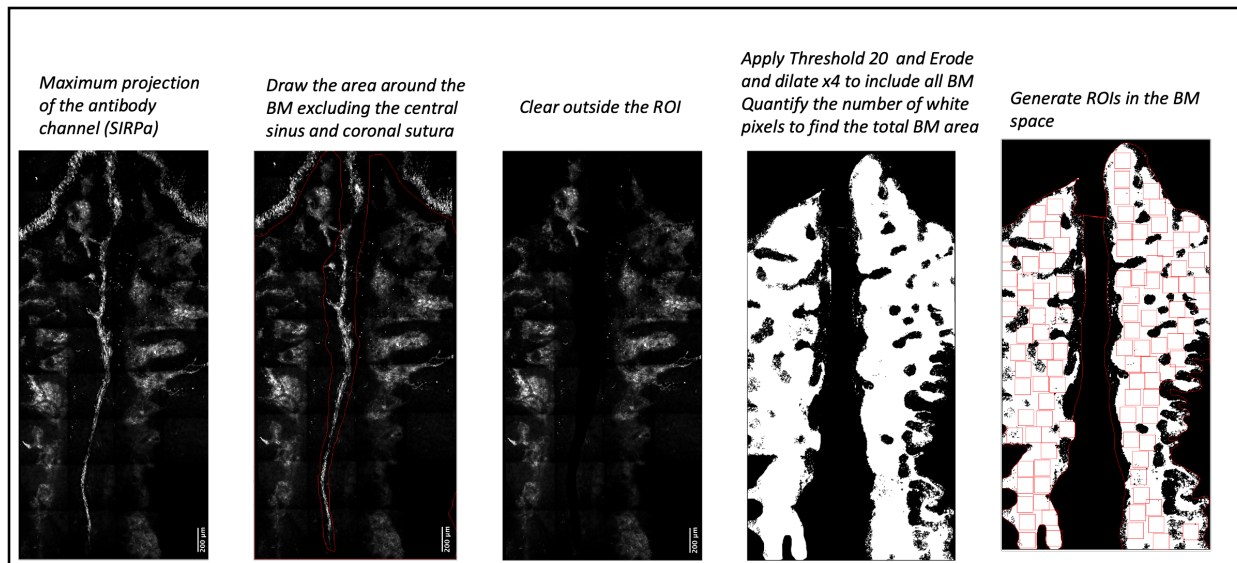
For the AML channel, ROIs were classified as single cells (% AML < 15%), small clusters (% AML > 15% & < 50%) or large clusters (% AML > 50%) according to their percentage of AML (Figure [2.6](#)).

Cell segmentation was performed by manually outlining each CD169-YFP+ cell. Cell parameters such as mean intensity, perimeter, area and circularity were calculated and exported using Fiji. For timelapses, sample movement in the x, y and z dimension occurring during the acquisition was corrected by applying a four-dimensional (4D) data protocol implemented in ImageJ which allow for the registration of the acquired time lapses [\[203\]](#). For the analysis of the homing of AML cells, Tomato+ AML cells were manually tracked in the x and y dimension using the Manual Tracking plugin in Fiji. The tracks were loaded in the Chemotaxis and Migration tool plugin in Fiji to generate Rose plots and calculate the accumulated and euclidean

distance travelled by the leukemic cells.

2.12 Quantification and statistical analysis

Raw data was analysed in Microsoft Excel and GraphPad Prism (GraphPad Software Inc.). t-tests were performed to compare group means when a single comparison was needed. In the case where statistical analysis of multiple comparison was needed, one-way ANOVA test was used. All differences were considered statistically significant when $p < 0.05$. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$ and **** represents $p < 0.0001$. Detailed statistical analysis and the number of biological and technical replicate for each experiment can be found in the figure legends.

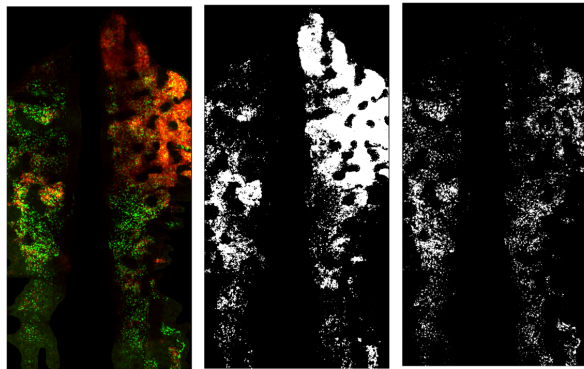


Step 2: Define the number of positive pixels for CD169-Cre YFP & AML

Define the threshold for the CD169-Cre & AML channel separately

AML mTmG
Apply threshold and quantify pixels above threshold.

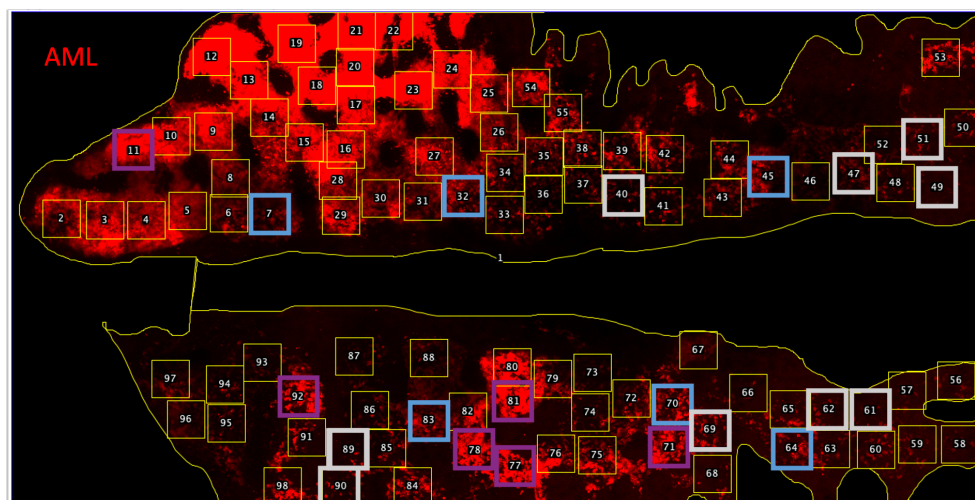
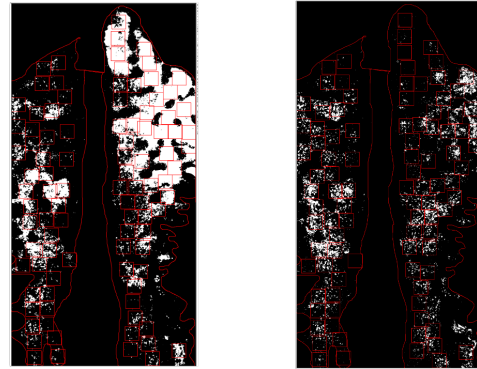
CD169-Cre x R26 YFP
Apply threshold and quantify pixels above threshold.



Step 3: Quantify the number of positive pixels in each ROI

AML mTmG
Apply the ROIs. Quantify the mean intensity of each ROI and back-calculate the % of pixels above threshold for each ROI

CD169-Cre x R26 YFP
Apply the ROIs. Quantify the mean intensity of each ROI and back-calculate the % of pixels above threshold for each ROI



Single cells: % AML < 15% Small clusters: 15% < % AML < 50% Large clusters: % AML > 50%

Figure 2.6: Pipeline for the analysis of AML tilescans

Chapter 3

Identification of bone marrow macrophage subsets

3.1 Introduction

First identified in the late 19th century, macrophages were believed to be responsible for the normal development of embryogenesis as well as endorsing a protective role against foreign pathogens, both processes taking place thanks to their phagocytic capacity. As such, right from the start of their study, macrophages were described as a heterogeneous population of cells, not only in terms of function but also in terms of origin, morphology, and phenotype. While in some organs like the spleen or the lung, scientists have come to a consensus on the different subsets, functions and phenotypes of macrophages [41], in the bone marrow however, the characterisation and classification of macrophages is far from established, as each group of scientists have studied their own population of macrophages and given them a new name, function and combination of markers used to identify them [82].

Initially designed in 1968 as an instrument capable of measuring cell characteristics in a single cell suspension [204], flow cytometry rapidly evolved to contain up to five laser beams and excite a broad range of fluorochromes to identify specific subsets of immune cells. Since 2012, a new type of cytometer has reached the market: spectral flow cytometer. This type of cytometer analyzes the full spectrum of each fluorochrome's emission and subtracts it from the other fluorochromes using an advanced mathematical algorithm, named 'spectral unmixing'. Thanks to this technology, the field of flow cytometry has evolved from a 12-15 colours staining panel to a 40 colours staining panel. This increase in panel size has allowed researchers to

perform deep phenotyping of immune cell populations and sub-populations [205].

As A. Cossariza would describe in his first edition of the Guidelines for the use of flow cytometry and cell sorting in immunological studies [206], “The marriage between immunology and cytometry is one of the most stable and productive in the recent history of science”. However, when it comes to bone marrow macrophages, many different markers have been described to identify different populations of macrophages. Several reasons can explain this discrepancy: first, macrophages are difficult to study as they are large, ramified cells which can deteriorate during single cell preparation [207]. In addition, as they have numerous functions, it is unclear whether cells like osteal macrophages, which support osteoblasts, are the same cells as central marrow macrophages described to help retain progenitor cells in the bone marrow [208]. Finally, many other myeloid cells, including monocytes, neutrophils and eosinophils, have a very similar profile and express the same markers albeit at different levels. Hence, I decided to first perform a literature review in order to identify the most commonly used markers in the study of bone marrow macrophages by flow cytometry (Table 3.1).

The only markers that were used consistently across all studies were F4/80 and Ly6G. F4/80 was first identified in 1981 as a pan macrophage marker, expressed by most tissue resident macrophages and absent from fibroblasts and lymphocytes [42]. However, since this study, it has been shown that eosinophils, monocytes and neutrophils also express F4/80 [43]. It is therefore essential to include markers which are differentially expressed to exclude these cells. Hence, Ly6G, uniformly expressed by all neutrophils, is important to eliminate these cells. In addition, Ly6C (or Gr-1 which combines Ly6-G and Ly6-C) is commonly used to identify and exclude monocytes while the side scatter helps remove eosinophils. Finally, as mentioned by A. Cassoriza, CD11b, which labels all myeloid cells, is also generally accepted as an important marker to eliminate any non-myeloid source of contamination like NK cells and B cells. This panel was first used by Taylor et al. in 2003 [43] with the addition of two markers, 7/4 and BGR to help further eliminate neutrophils and monocytes. However, these two additional markers were not used in any other articles suggesting that these are not highly specific for any population and do not allow any further discrimination between cell types. In the late 80's, Crocker et al., [209] identified the sialoadhesin receptor, also commonly referred to as CD169, which was preferentially expressed by a few tissue resident macrophage populations including bone marrow macrophages. They measured that 50-90% of bone marrow macrophages expressed this receptor. Since then, multiple studies have focused on CD169+ bone marrow

Reference	Cell surface markers								
	Negative selection markers				Positive selection markers				Other
	Ly6G	Ly6C	SSCA	CD115	CD11b	F4/80	CD169	VCAM1	
Taylor et al., 2003	Gr-1		✓	✗	✓	✓	✗	✗	7/4, βGR
Winkler et al., 2010	✓	✗	✗	✗	✓	✓	✗	✗	✗
Chow et al., 2011	Gr-1		✓	✓	✗	✓	✓	✗	✗
Kaur et al., 2018	✓		✗	✗	✗	✓	✓	✓	(GFP from MacGreen)
Yang et al., 2018	Gr-1		✓	✓	✗	✓	✗	✗	✗
Cossarizza et al., 2019	✓	✗	✗	✗	✓	✓	✗	✗	MerTK, CD64
Bisht et al., 2021	✓	✗	✗	✗	✓	✓	✓	✓	✗
Millard et al., 2021	✓	✗	✓	✓	✓	✓	✓	✓	CD11c+, B220+, CD4/8+

Table 3.1: A summary and comparison of cell surface marker combinations used to identify bone marrow macrophages in studies working on the characterisation of bone marrow macrophages.

macrophage subsets to study their function in the bone marrow microenvironment [52, 95].

Given the divergence in the combination of markers used to identify bone marrow macrophages, I decided to extend my literature review and include all markers which had previously been described to not only identify, but also characterise either bone marrow macrophages or tumour-associated macrophages; the final aim being the characterisation and classification of bone marrow macrophages in the context of leukemia.

One marker frequently used to characterise BM macrophages in the hematopoietic microenvironment is VCAM1 [95]. In hematopoietic cells, VCAM1 expression is restricted to myeloid cells and the protein is associated with the retention of progenitor cells in the BM [210]. Hence, this marker could allow the discrimination between bone marrow resident macrophages involved in [Haematopoietic stem cell \(HSC\)](#) regulation and cells important for the regulation of inflammation. In addition, [Transforming growth factor \$\beta\$ \(TGF \$\beta\$ \)](#) was shown to promote HSC quiescence in homeostasis in the BM [211, 212]. TGF β is known to be secreted by macrophages and at the same time, a regulator of macrophage phenotype which can act through an autocrine

or paracrine signaling mechanism [213]. Its secretion is generally associated with inflammation and recruitment of monocytes [214]. During cancer growth, tumour associated macrophages are found to overexpress TGF β which suppresses cytotoxic T cell function [215], promotes metastasis [216] and EMT of cancer cells .

SIRP α is known as the 'don't eat me' signaling pathway on macrophages, which allows the inhibition of phagocytosis and activation of macrophages; mechanism present to avoid the phagocytosis of healthy cells through the expression of CD47. Y. Chen et al., [217] recently published a study in humans, on follicular lymphoma where they divided patient macrophages based on their expression of CD14 and SIRP α . The two populations identified were described as having differential phagocytosis potential as well as T cell activation/suppression capacity. While CD14 does not have a mouse homologue, SIRP α does and could play an important role in leukemia growth. Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) has been described to be expressed on macrophages that help the formation of vessels and are recruited via the VEGF/VEGFR axis [218]. LYVE-1^{hi} macrophages were crucial for the inhibition of the recruitment of inflammatory cells and vessel integrity by degrading collagen through MMP9s [219, 220]. Without these LYVE-1+ macrophages, vascular leakiness was increased in tissues [220]. In AML, malignant cells are known to produce vascular factor (VEGF-A)[192] hence LYVE-1^{hi} macrophages could be recruited by AML through the secretion of VEGF-A to the BM.

MerTK belongs to a family of tyrosine kinase receptors and has been demonstrated to play a key role in phagocytosis of apoptotic cells by macrophages. A truncated version of this receptor or inhibition using antibodies resulted in deficiency in phagocytosis of apoptotic cells [221] and in the case of cancer can result in increased tumorigenicity [222]. Under oxidative stress, its expression at the surface of macrophages is associated with increased survival of these cells [223]. Hence, the expression of this protein is important to correlate a phenotype to a function. T-cell immunoglobulin and mucin domain containing 4 (Tim4) a marker of macrophage maturation, is expressed on BM macrophages but not on erythroblastic island macrophages [95]. This marker, synonym of immunoregulation and migration [224] would be a good candidate to discriminate subsets of BM macrophages. Other factors commonly used to define the activation profile of macrophages are iNOS and TNF α as pro-inflammatory markers and PDL1, Arg1 and RELM α as anti-inflammatory markers [225].

Taking advantage of the spectral flow cytometry technology, in this chapter I wished to evaluate the expression of all the markers described above in bone marrow macrophages to uncover whether different macrophage populations were present in the bone marrow and were associated with different phenotypes.

Although advanced immuno-phenotyping is possible with flow cytometry, the processing involved to generate a single cell suspension leads to the loss of all spatio-temporal and dynamic information about the cells. Intravital microscopy has been designed to perform live imaging of murine organs. In particular for the bone marrow, our group has optimised the imaging technique to allow repeated imaging of the calvarium, through minimally invasive surgery. By using a combination of reporter mouse strains and injectable substances, we are able to see the dynamics and interactions of various cells in the bone marrow in real time [226]. However, very few studies have focused on performing live imaging of bone marrow macrophages and therefore their identification and imaging protocol still requires some optimisation.

Flow cytometry allows up to 40 parameters analysis, in microscopy, this number is still limited to 3-5 parameters. While some novel technologies are starting to emerge to perform high dimensional imaging, these are still at the stage of troubleshooting and are not compatible with live imaging. To visualise interactions between macrophages and AML cells, it was important to find a macrophage labelling technique requiring one to two fluorophores maximum. In addition, another challenge for this type of imaging was photobleaching, where repeated excitation of fluorochromes leads to loss of signal. This phenomenon is much more pronounced for antibodies conjugated to fluorochromes compared to fluorescent proteins [227]. Therefore, the use of transgenic reporter animals should be favoured with in live imaging.

It is in this context that I wished to identify the most stable and specific tool to image bone marrow macrophages. By exploring all options described in literature, including reporter macrophage mouse model and injectable substance, I hoped to perform an extensive analysis of imaging options for bone marrow macrophages. However, few studies have been published on this topic therefore I broadened my search to include labelling techniques used to image macrophages in bone marrow sections (Table 3.2). In sections, two antibodies were described to label bone marrow macrophages: anti-F4/80 [67, 95] and anti-CD169 [52].

Concerning reporter mice, the MaFIA, MacGreen and MacBlue mice have been used for either *ex vivo* or *in vivo* imaging to label BM macrophages. These three mouse lines are in fact

Reference		Labelling and genetic model						
		Anti-F4/80	MaFIA	MacGreen mice	Dextran (10kDa)	Anti-CD169	Autofluorescence	MacBlue mice
Sections	Winkler et al., 2010	yes						
	Chow et al., 2011		yes			Yes		
	Kaur et al., 2018	yes		yes				
IVM	Casanova-Acebes et al., 2013				yes			
	Fried et al., 2022					Yes		
	Upadhaya et al., 2020						Yes	
	Sharma et al. 2021							yes

Table 3.2: A summary and comparison of cell surface markers and transgenic mice used to identify bone marrow macrophages with a confocal microscope.

labelling the same cells since all three mice line have a fluorescent protein inserted downstream of [CSF1R](#) promoter. However, the CSF1R receptor is known to be expressed by all cells of the mononuclear phagocyte lineage hence monocytes, neutrophils, macrophages and dendritic cells should also express the fluorescent protein. A different approach was taken by Casanova et al. [228] whereby they took advantage of the biological function of macrophages and injected low molecular weight Dextran, a polymer used to label vasculature: overtime, thanks to their phagocytic capacity, macrophages phagocytosed the polymer and became fluorescent. Finally, Upadhaya et al.[229] identified macrophages as the autofluorescent cells present in the images. In this chapter, I tested the most promising approaches to label BM macrophages: staining with F4/80 APC conjugated antibody, CD169 PE conjugated antibody, Dextran and the use of the MaFIA and CD169 reporter mice.

Altogether, to overcome the challenge linked to a lack of consensus on bone marrow macrophage phenotype, I decided to first identify bone marrow macrophage subsets using a 21-colour flow cytometry panel and then perform an unsupervised analysis using [T-distributed stochastic neighbor embedding \(t-SNE\)](#) and [Flow self-organizing map \(FlowSOM\)](#). I confirmed the correct identification of bone marrow macrophage subsets using a reduced panel on a conventionnal flow cytometer. I applied a similar approach to identify macrophages using confocal microscopy. Finally, I explored different genetic reporter mouse models and correlated the cells identified

by the reporter with the staining used to identify macrophages by flow cytometry and confocal microscopy.

3.2 Identification of BM macrophage subsets by flow cytometry

Given the number of candidates (21) I wished to stain my cells with, I had to use the newly developed spectral flow cytometer, ‘Aurora’ developed by Cytex. To apply the spectral unmixing algorithms, several details are more important compared to conventional flow cytometry: very good single colour controls were essential, preferentially using cells, correct positive controls, good titration of antibodies and the right combination of antibody with fluorochrome based on the level of expression of the targeted antigen and the strength of the fluorochrome, a highly expressed antigen was conjugated with a dim fluorochrome and inversely. Given that many of the markers in the panel were not necessarily expressed by bone marrow macrophages, I used a combination of murine cells from various organs including the peritoneal fluid, thymus and spleen to optimise the set-up of the machine and validate the panel. After titrating all the antibodies and running all single colour controls, the spectral unmixing algorithm was applied.

The first step in the analysis was to exclude all cells which I knew were not macrophages. To do so, I used an unsupervised analysis approach combining FlowSOM and t-SNE visualization. FlowSOM is a clustering algorithm which evaluates the expression of all markers expressed on all cells and clusters the cells into populations based on their resemblance. To help the user identify what population was clustered together, the algorithm returns a heatmap containing the relative expression of each marker by each population. Finally, it is possible to apply these populations to a t-SNE map hence facilitating the 2-D visualisation of all cell populations and their expression of each marker. Stringent gating on single live cells was necessary prior to presenting the compensated data to FlowSOM as debris would generate unexpected populations. The first run with the algorithm was applied to all live cells to eliminate non-macrophages. FlowSOM generated 8 main populations with a unique combination of marker’s expression. Combining the heatmap of the expression of each marker by each population (Figure 3.1A) with literature, I identified which cell population could not be macrophages and therefore should be excluded from further analyses (Figure 3.1B): monocytes were identified as population 0 given their high expression of Ly6C. Neutrophils were identified as population 4

and 6 based on their high expression of Ly6G. Finally, I also excluded population 1 and 7 as they did not express either CD11b or F4/80. Hence, I kept population 2, 3 and 5 as potential macrophages.

Given the computing power and time required to perform the FlowSOM analysis, I needed to be able to manually gate on the cells of interest. The first step to reach this aim was to compare the manual gating with the unsupervised results. Based on the heatmaps of the various populations, I excluded CD11b⁻ cells, Ly6G^{hi} cells and Ly6C^{hi} cells. Figure 3.1C shows the manual gating strategy applied to the sample. Single live cells were divided according to their expression of CD11b. A first population was identified as CD11b⁻. CD11b⁺ cells were then divided according to their expression of Ly6G. A second population was gated around the Ly6G⁺ cells. Next, CD11b⁺ Ly6G⁻ cells were divided based on their expression of Ly6C. Ly6C^{hi} cells were gated as a third population and CD11b⁺ Ly6G⁻ Ly6C^{lo} cells were gated as the fourth population. I overlayed these populations on a t-SNE plot (Figure 3.1D). In a second t-SNE plot, I overlayed the populations generated by the FlowSOM algorithm (Figure 3.1E) so I could correlate my gating strategy with the unsupervised clustering approach. The cell populations and gates from the manual analysis matched the FlowSOM analysis and therefore I could replace the unsupervised gating with this manual gating.

The following step was to identify among these cells which ones were macrophages and whether these macrophages could be divided into different populations. To do so, I applied the FlowSOM algorithm a second time on the cells identified as potential macrophages (CD11b⁺ Ly6G⁻ Ly6C^{lo}). The FlowSOM algorithm returned 6 populations (Figure 3.2A,B). Based on the lack of expression of F4/80, population 1 was rapidly excluded from further analysis. The 5 other populations could be distinguished based on their expression of F4/80, CD169, SIRP α and PDL1; two main macrophage populations could be identified as F4/80⁺ CD169⁺ and F4/80⁺ CD169⁻ and further divided based on their expression of SIRP α and PDL1. By manually gating first on F4/80 and CD169 and then on SIRP α and PDL1 (Figure 3.2C), I was able to reproduce similar cell populations visualised on the t-SNE plots (Figure 3.2D). By applying the FlowSOM populations on another t-SNE plot, I correlated the populations identified by manual gating with the FlowSOM populations and found that this gating strategy mimicked closely the unsupervised analysis. Hence, the combination of these 4 markers with CD11b, Ly6G and Ly6C was sufficient to distinguish the macrophage populations identified by unsupervised analysis.

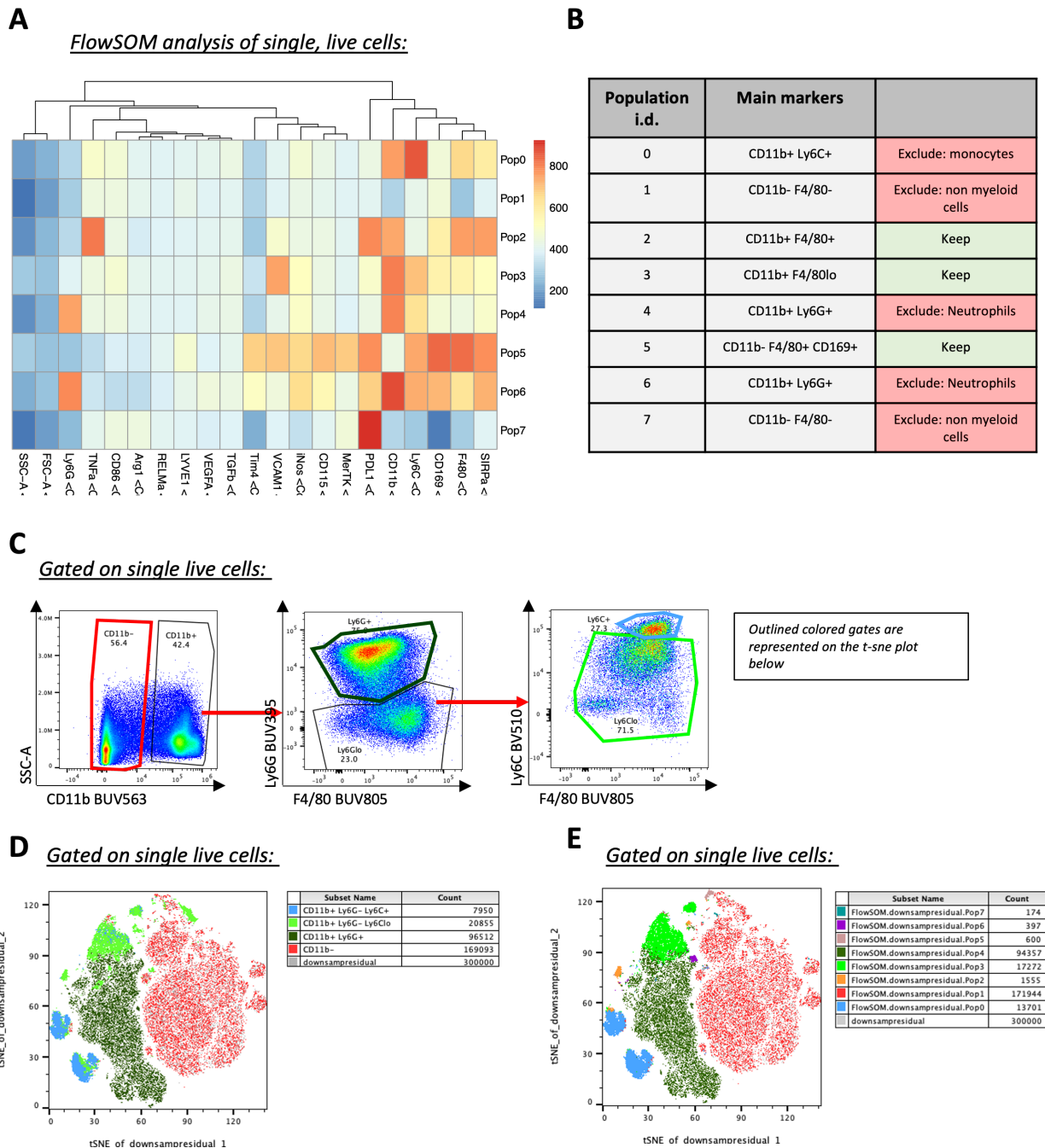


Figure 3.1: Unsupervised clustering of control murine bone marrow cells

Unsupervised analysis of flow cytometry data using a combination of FlowSOM and t-SNE algorithms. Control BM cells were stained with the 21-colours macrophage panel designed to highlight BM macrophage populations. (A) Heatmap of the expression of all 21 markers in the clusters generated with the FlowSOM algorithm. (B) Exclusion of immune clusters expressing non-macrophage markers based on the heatmap. (C) Manual gating strategy used to reproduce the clusters generated through FlowSOM. The clusters could be generated based on the expression of CD11b, Ly6G, Ly6C and F4/80. (D) t-SNE representation of the bone marrow cells with the manually gated populations overlaid. (E) t-SNE representation of the bone marrow cells with the FlowSOM clusters overlaid. The manual gating matches the clusters generated through unsupervised analysis.

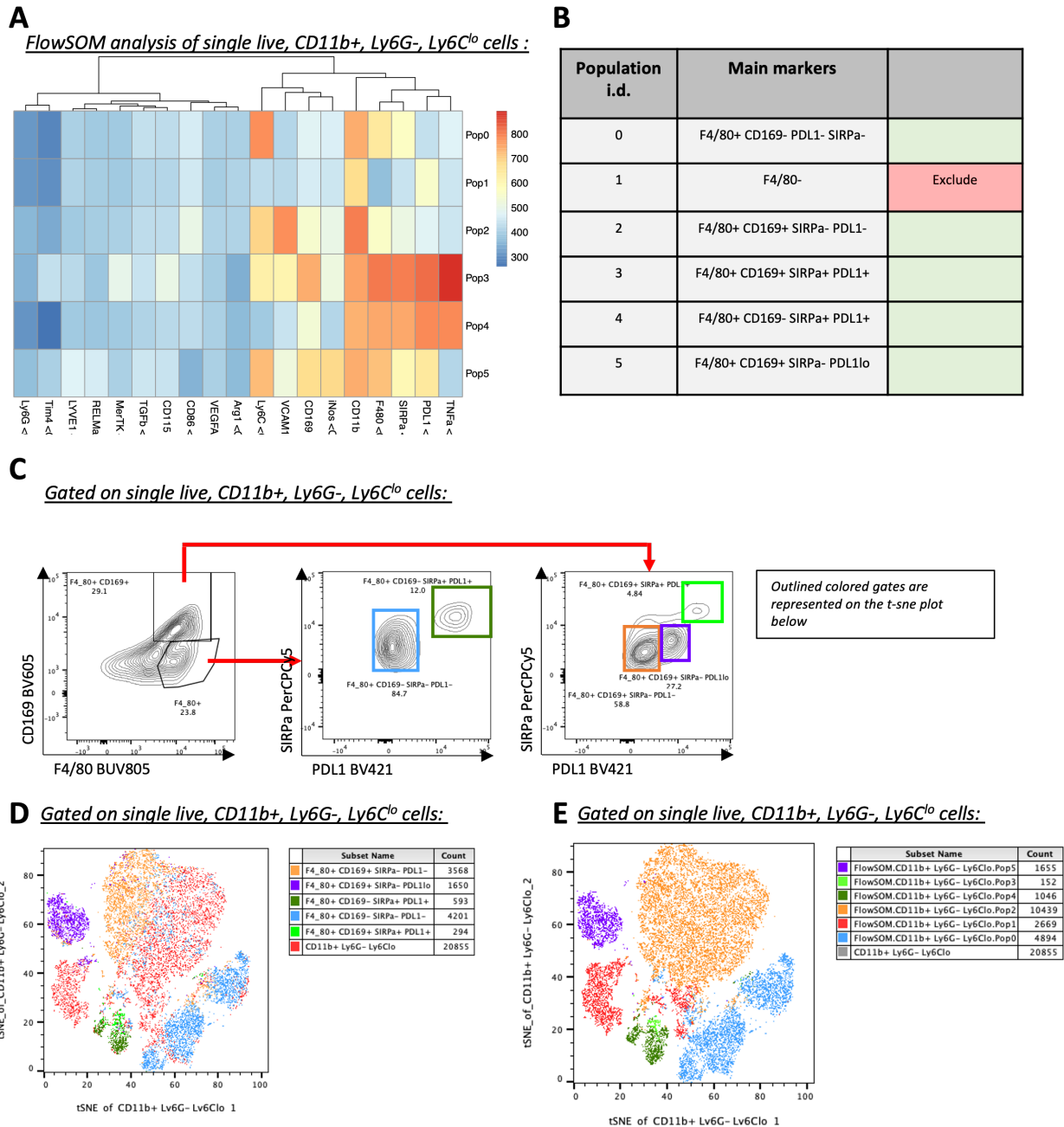


Figure 3.2: Identification of BM macrophage populations using unsupervised clustering of flow cytometry data

Unsupervised analysis of all cells previously identified as potential macrophages and expressing CD11b⁺, Ly6G⁻, Ly6C^{lo}. **(A)** Heatmap of the expression of all 21 markers in the ‘macrophage-like’ clusters generated with the FlowSOM algorithm. **(B)** Exclusion of cluster 1 based on the absence of expression of the pan macrophage marker, F4/80, in the heatmap. **(C)** Manual gating strategy used to reproduce the clusters generated through FlowSOM. The clusters could be generated based on the expression of F4/80, CD169, SIRPα and PDL1. **(D)** t-SNE representation of the CD11b⁺, Ly6G⁻, Ly6C^{lo} cells with the manual gating overlaid. **(E)** t-SNE representation of the CD11b⁺, Ly6G⁻, Ly6C^{lo} cells with the FlowSOM clusters overlaid. The manual gating matches the clusters generated through unsupervised analysis.

While, these 7 markers alone were sufficient to identify the macrophage populations generated by unsupervised analysis, the remaining markers present in the panel may still be expressed differentially by certain macrophage populations. To evaluate whether a population of macrophages preferentially expressed certain markers, I overlayed the populations identified previously on a t-SNE plot (Figure 3.3A) and generated a heatmap of each marker (Figure 3.3B).

The most striking result was that the F4/80+ CD169+ population expressed a high level of VCAM while the F4/80+ CD169- were the main cells expressing TNF α . VCAM has previously been described to be expressed on bone marrow macrophages to help retain HSCs in their niche while TNF α is a known inflammatory molecule. Together, these results suggest that the two main population of macrophages identified in the bone marrow are likely to have different functions. Most of the other markers were not highly expressed by any macrophage populations. All of the markers had been optimised with a murine positive control which confirmed that the antibodies worked on this sample. Hence, I could conclude that bone marrow macrophages did not express any of these additional markers: CD115, VEGFa, TGFb, LYVE1, Tim4, Arg1, RELM α and MerTK.

Based on this experiment, I was able to identify two main populations of bone marrow macrophages, composed of 2-3 further sub-populations each, based on their expression of SIRP α and PDL1. Hence, the staining panel could be reduced to CD11b, Ly6G, Ly6C, F4/80, CD169, SIRP α and PDL1. To confirm the identification of true populations and eliminate the risk of populations being identified based on data spread due to the large size of the panel, I stained some BM cells (containing a small percentage of mTmg+ AML cells for correct data spreading and compensation) and checked whether the same populations of macrophages could be identified using a conventional flow cytometer. While the cells could still be divided based on the same markers, fewer populations were identified. The F4/80+ CD169+ cells contained a majority of SIRP α - PDL1- cells while a majority of the F4/80+ CD169- cells expressed SIRP α and PDL1 (Figure 3.4). Of note, the F4/80+ CD169+ cells were considered negative for SIRP α when comparing the expression of SIRP α in these cells with F4/80+ CD169- cells. However, when comparing the expression of SIRP α in F4/80+ CD169+ cells to FMOs or non-myeloid cells, F4/80+ CD169+ cells were found to be positive for this marker, albeit at much lower levels compared to F4/80+ CD169-. Hence, these cells are positive for SIRP α . The absence of a SIRP α - PDL1^{lo} population suggests that, in the spectral flow cytometry data,

A

Gated on single live, CD11b+, Ly6G-, Ly6C^{lo} cells:

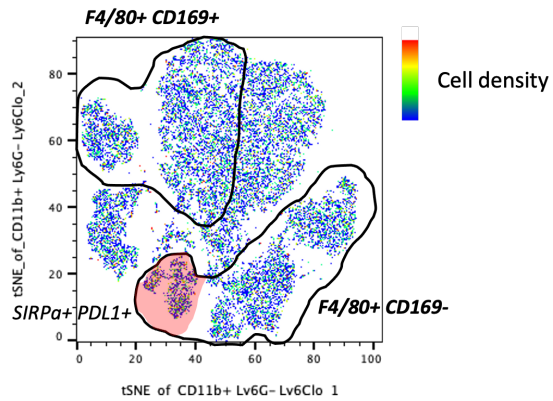
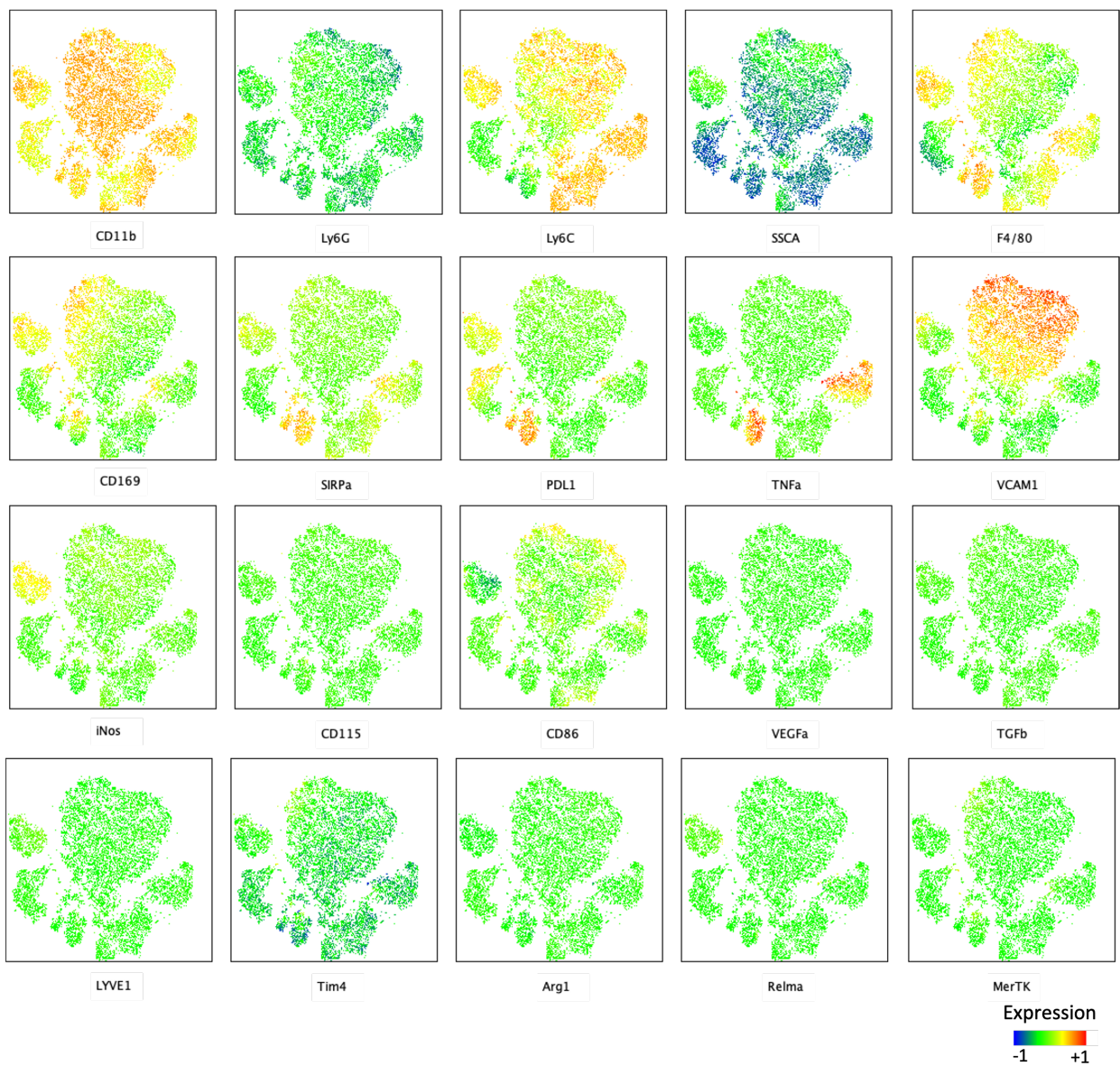
**B**

Figure 3.3: High dimensional analysis of the phenotype of macrophage populations
Density plot of the macrophage populations identified with FlowSOM, using t-SNE visualization.
(B) *Normalized expression of the 20 markers. Values range from blue to red. Most markers were not expressed by macrophages.*

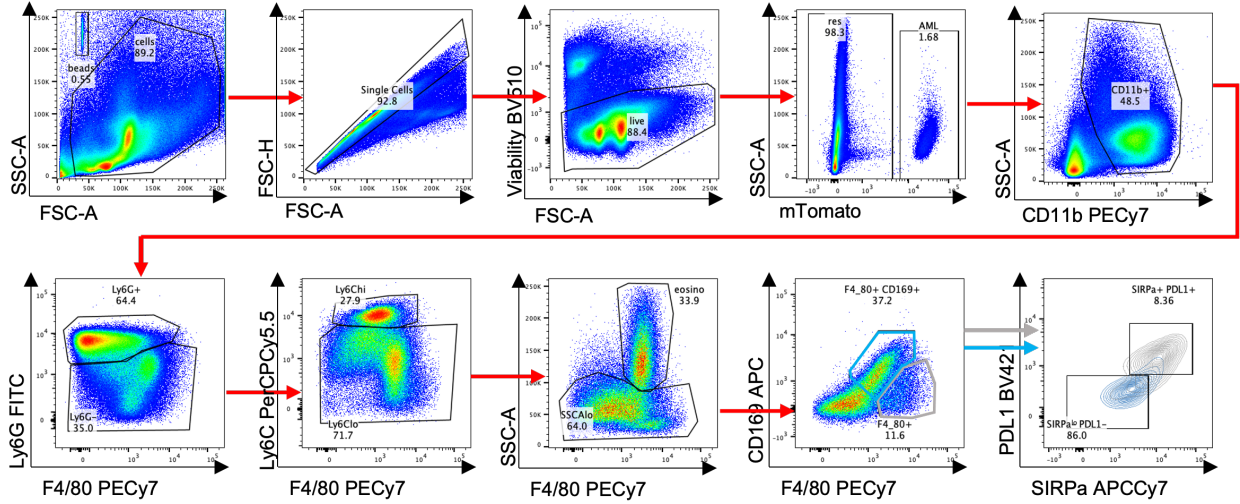


Figure 3.4: Final gating strategy to identify BM macrophage populations

Based on the unsupervised clustering, macrophage populations can be identified through the combination of these 7 myeloid markers. The first half of the gating strategy is an exclusion strategy to remove non-myeloid cells, neutrophils, monocytes and eosinophils while the second half is an inclusive strategy to identify the two main macrophage populations. The blue gated cells can be seen in blue on the SIRP α - PDL1 plot while the grey gated cells can be seen in grey on the same plot. F4/80+ CD169+ cells did not express PDL1 and expressed low levels of SIRP α while a majority of F4/80+ CD169- cells expressed SIRP α and PDL1.

this population arose from data spread in another marker, and therefore creating a false split within the population. Altogether, macrophages could be divided into two main macrophage populations, F4/80+ CD169+ SIRP α ^{lo} PDL1- and F4/80+ CD169- SIRP α + PDL1+. This is the gating strategy I will use throughout the rest of the thesis (Figure 3.4).

3.3 Identification of bone marrow macrophage by confocal microscopy

The mouse calvarium bone marrow is known to contain similar frequencies of HSCs as that of long bones and transplanted HSCs from long bones and calvaria led to similar reconstitution rates [226]. Although the composition and frequency of endogenous macrophages has not been compared, B cell and T cell frequencies were similar in steady state and acute stress [230, 231]. Hence, we assumed that the proportion of macrophages in long bones and calvarium is similar. In addition, due to its physical properties, the marrow contained in this bone can be imaged without damaging the bone itself and can therefore be studied using repeated live microscopy. Using this technique, I aimed to understand the distribution and dynamics of macrophages in the bone marrow.

I decided to try a number of labelling techniques to identify bone marrow macrophages. To compare the different cells labelled, I injected at the same time F4/80 APC conjugated antibody, CD169 PE conjugated antibody, and low molecular weight (10KDa) Dextran AF594 conjugated in a mouse and imaged the calvarium 30 minutes later. I evaluated the colocalization of the signals, two by two (Figure 3.5 & 3.6). To quantify the correlation between the F4/80, Dextran and CD169 signals, I used the colocalization algorithm from Fiji which allows us to visualise double positive pixels as a new channel in white. The 2D scatter plot shows the distribution of both signals. First, comparing CD169 and F4/80 expression, most cells appeared double positive (Figure 3.5A) and the scatter plot showed a direct correlation between both signals with an R value of 0.84 (Figure 3.5B), matching previous experiments characterising BM macrophages by flow cytometry. Next, when comparing the F4/80 and Dextran signal, I found that the colocalization of the F4/80 with the Dextran signal was very low, few pixels colocalised and the R value was 0.12, suggesting that F4/80 signal did not correlate with Dextran (Figure 3.6A-C). Hence, of these three injectable substances, it was clear that F4/80 and CD169 were more likely to be highlighting macrophages.

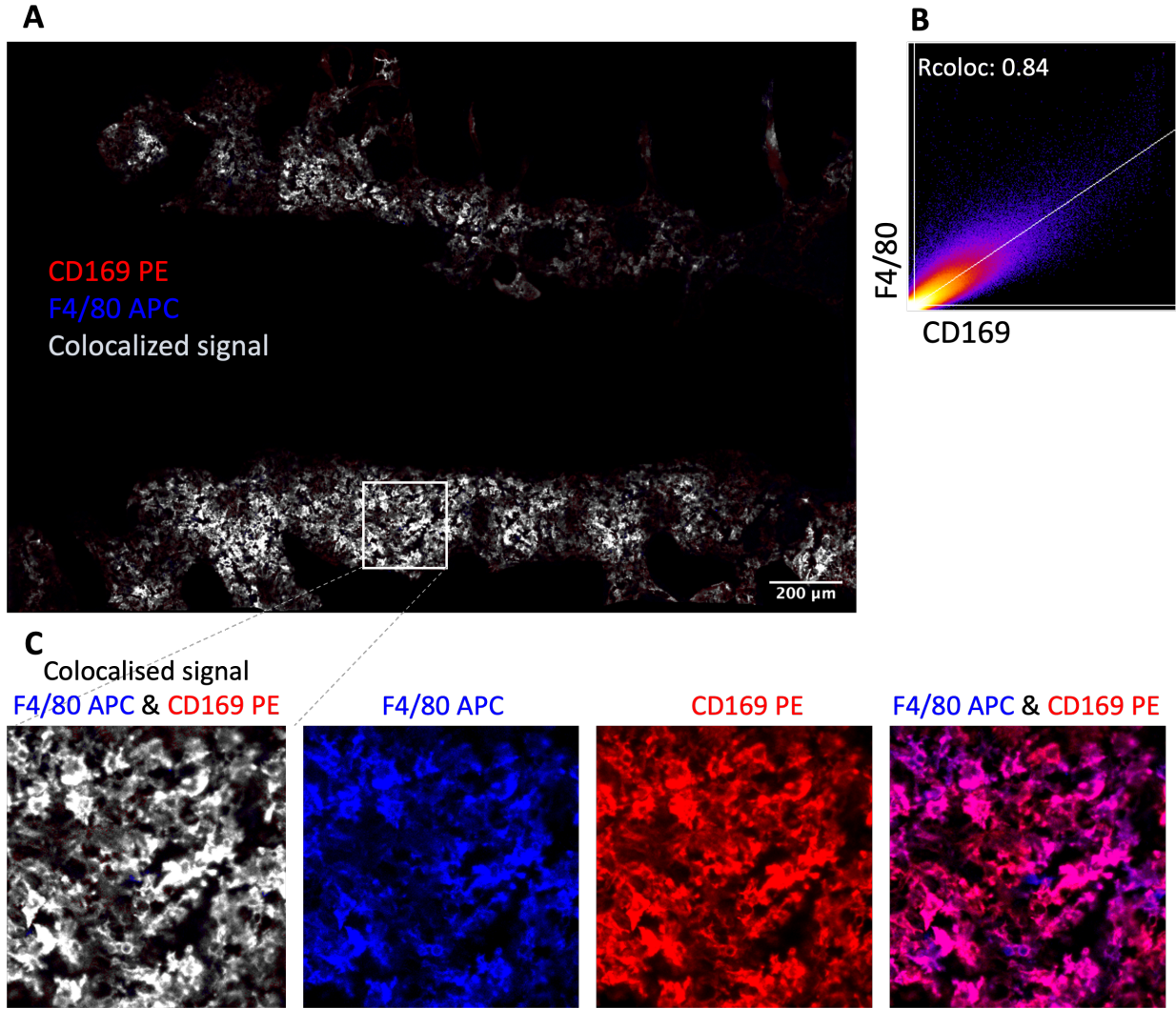


Figure 3.5: Comparison of BM macrophages labelling options for IVM
Colocalization analysis of F4/80 and CD169 signal using Coloc2 algorithm (ImageJ/Fiji) following the injection of CD169 PE and F4/80 APC in WT mice. In blue are F4/80+ cells and in red are CD169+ cells. White signal corresponds to double positive pixels. (A) Representative maximum projection of a tilescan of the calvarium BM with the colocalised analysis. (B) 2D scatter plot of CD169 versus F4/80 signal with the linear regression coefficient indicated as Rcoloc. (C) Higher magnification image of a BM area with a high density of F4/80+ cells.

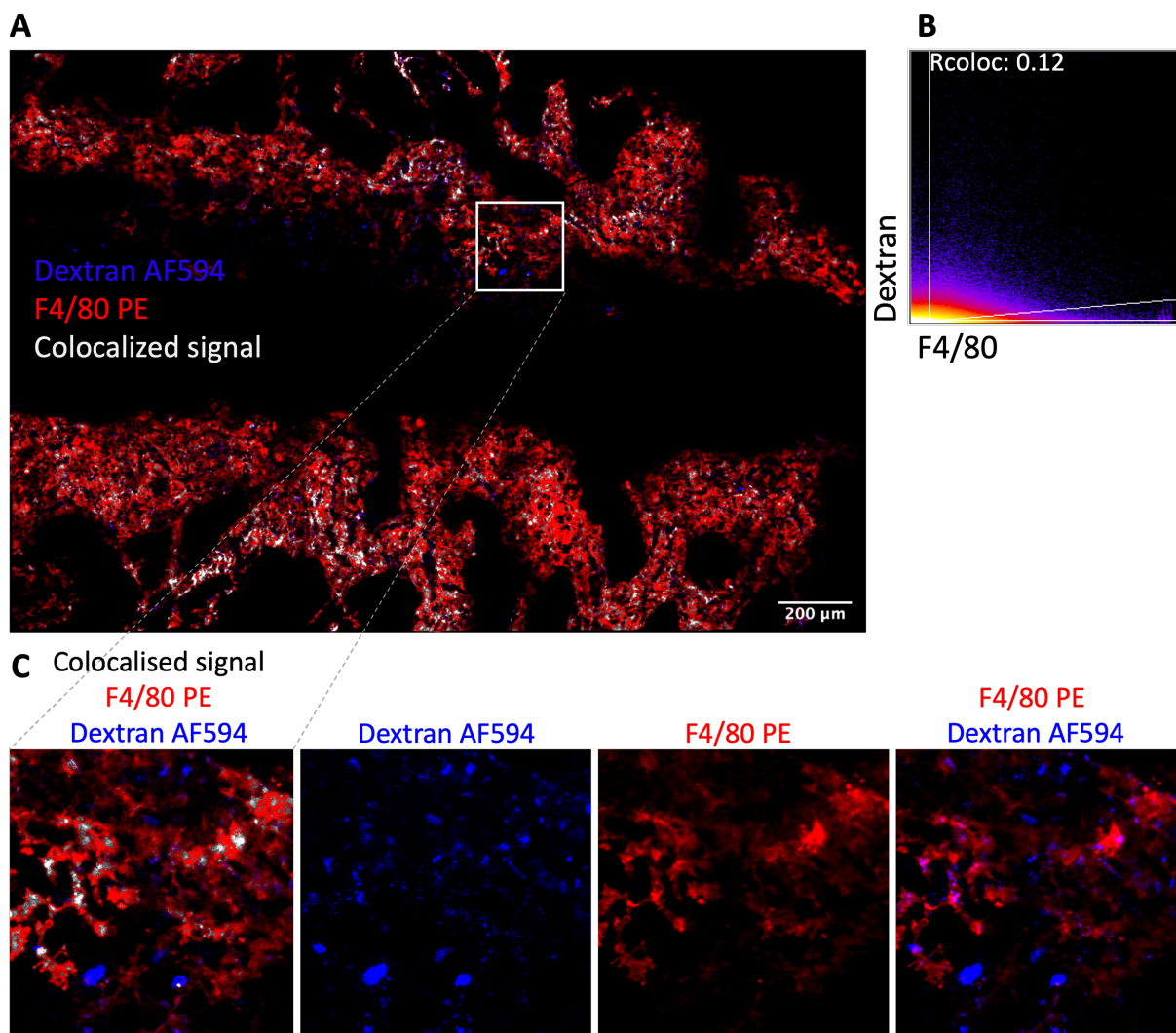


Figure 3.6: Dextran and F4/80 labelling in the BM

Colocalization analysis of F4/80 and Dextran signal using Coloc2 algorithm (ImageJ/Fiji) following the injection of F4/80 APC and Dextran AF594 in WT mice. In blue is the Dextran signal and in red are F4/80+ cells. White signal corresponds to double positive pixels. (A) Representative maximum projection of a tilescan of the calvarium BM with the colocalised analysis. (B) 2D scatter plot of Dextran versus F4/80 signal with the linear regression coefficient indicated as R_{coloc} . (C) Higher magnification image of a BM area with a high density of F4/80+ cells.

3.4 Identification of bone marrow macrophage using genetic models for confocal microscopy

Next, I evaluated two reporter mouse models, the MaFIA mice and CD169-Cre x R26-YFP mice. The MaFIA mice were my first candidate as several studies used this promoter to label macrophages [52, 95, 232]. To compare the signal from the reporter construct with the most likely macrophage labelling technique we had so far, I injected anti-F4/80 APC and measured the colocalization. While some signal did overlap, it was obvious the GFP from the reporter was present in many other cell types, including small round cells which resembled lymphocytes or monocytes (Figure 3.7A). After performing the colocalization analysis, the scatter plot revealed two single positive population, and had a linear regression coefficient of 0.13 (Figure 3.7B), indicating that there was no correlation between these two signals. Hence the MaFIA-GFP signal did not seem specific to bone marrow macrophages.

To evaluate whether the CD169-Cre x R26-YFP could be a better reporter model, I injected both F4/80-APC and CD169-PE. This time, the signals from all three channels overlapped nicely (Figure 3.8A-C). Interestingly, the antibody labelling was bound to the surface of the cell membrane, highlighting the shape of the macrophages and their dendrites, while the reporter was intracellular and therefore highlighted the nuclei and cytoplasms of these cells (Figure 3.8C). Although the signals were not completely overlapping, the scatter plot did highlight that there was a strong correlation between the antibody and the reporter, with a linear regression value of 0.67 (Figure 3.8B). In addition, given that the GFP came from a reporter mouse, the fluorescent signal would get less photobleached compared to injected antibodies. Hence, I favoured the use of this transgenic mouse line for future experiments.

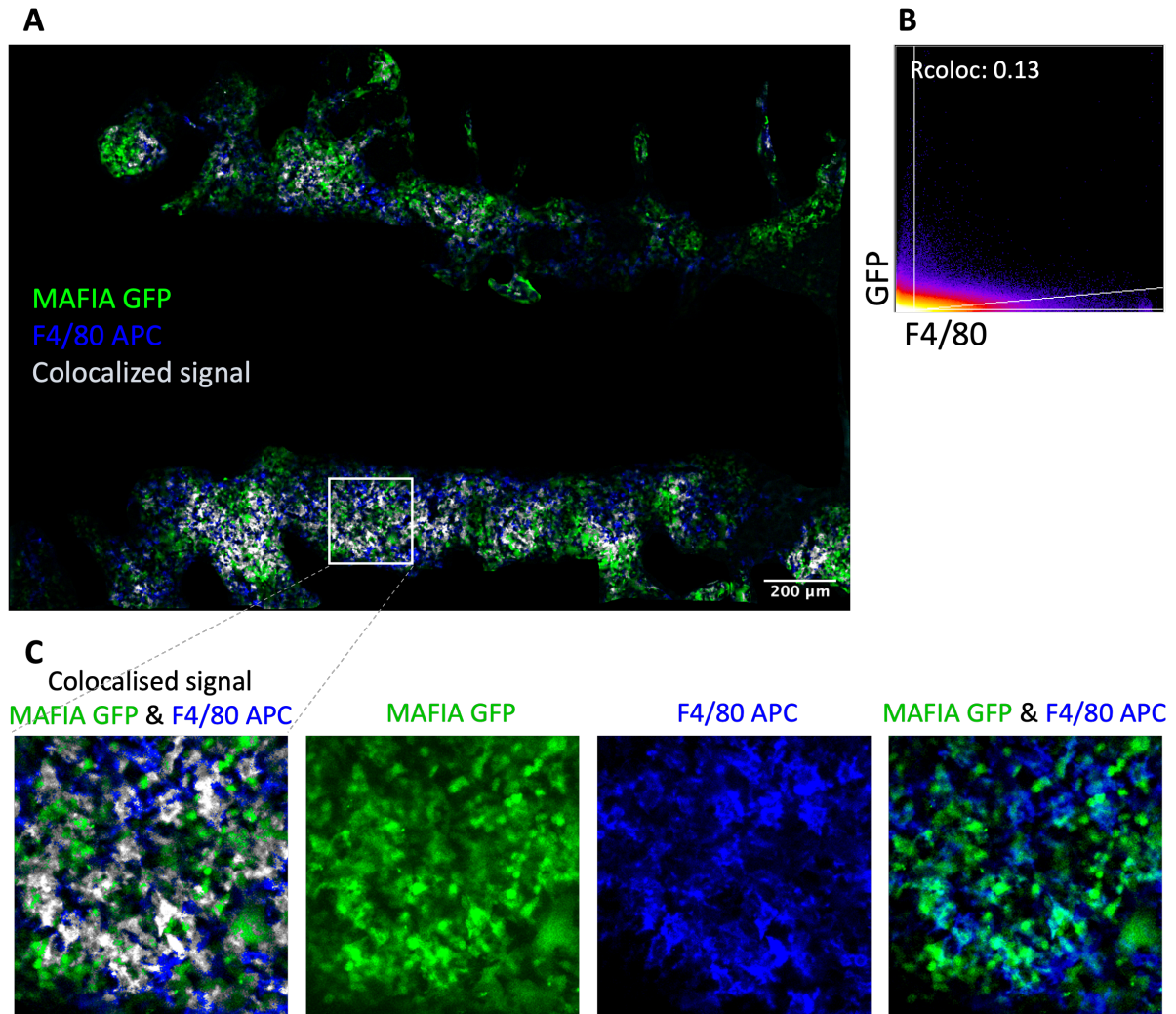


Figure 3.7: F4/80 labelling in the BM of MaFIA-GFP reporter mice
Colocalization analysis of F4/80 and MaFIA signal using Coloc2 algorithm (ImageJ/Fiji) following the injection of F4/80 APC in MaFIA-GFP reporter mice. In blue is the F4/80 signal and in green are the GFP+ cells. White signal corresponds to double positive pixels. (A) Representative maximum projection of a tilescan of the calvarium BM with the colocalised analysis. (B) 2D scatter plot of GFP versus F4/80 signal with the linear regression coefficient indicated as Rcoloc. (C) Higher magnification image of a BM area with a high density of F4/80+ cells.

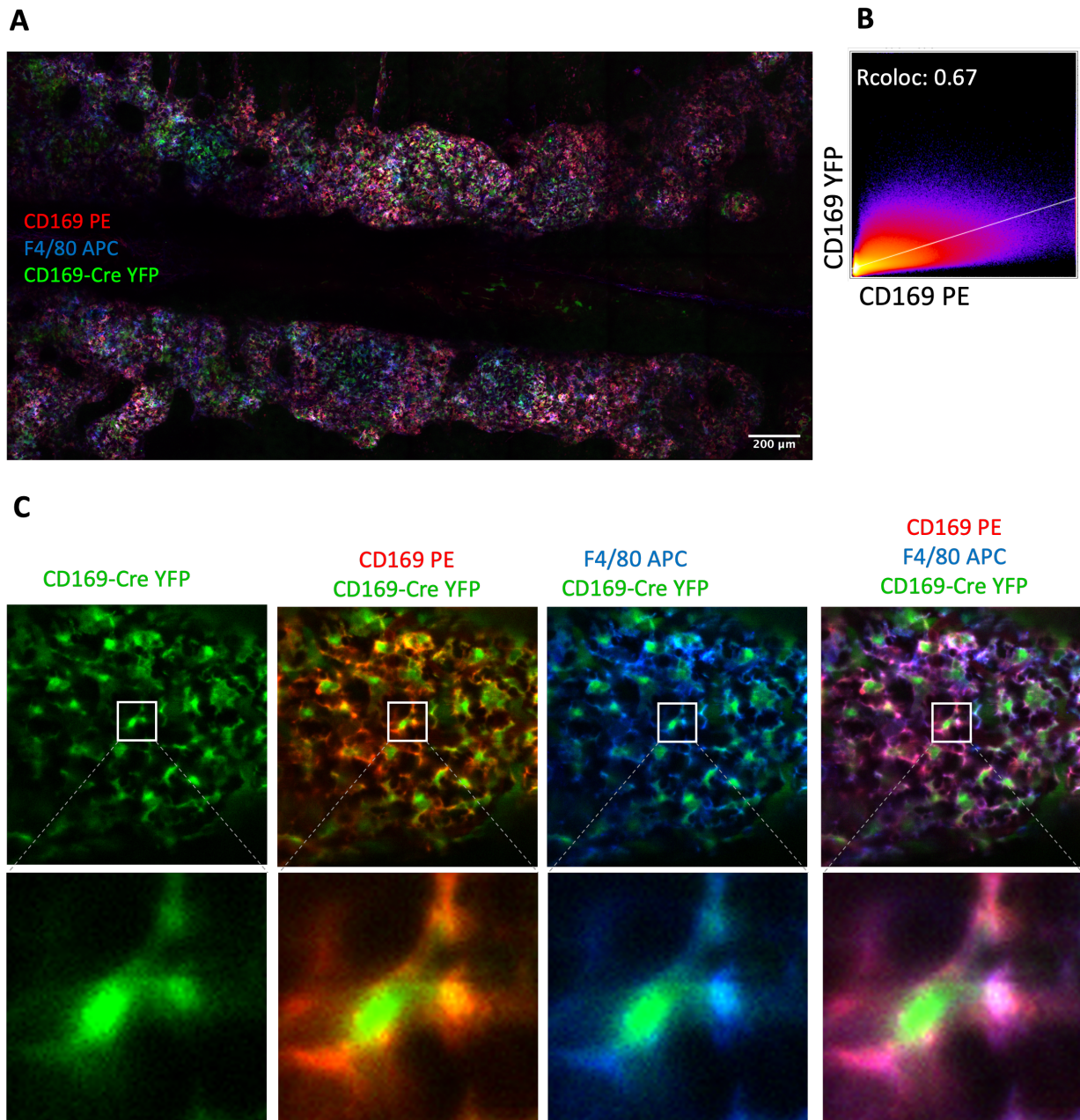


Figure 3.8: F4/80 and CD169 labelling in the BM of CD169-Cre x R26-YFP mice
Colocalization analysis of F4/80 and CD169 signals using Coloc2 algorithm (ImageJ/Fiji) following the injection of F4/80 APC and CD169 PE in CD169-Cre X R26-YFP mice. In blue are F4/80+ cells and in red are CD169+ cells, labelled with the antibody. The green signal correspond to the YFP expression from the reporter mice. (A) Representative maximum projection of a tilescan of the calvarium BM with all three signals. (B) 2D scatter plot of CD169-YFP versus CD169-PE signal with the linear regression coefficient indicated as Rcoloc. (C) Higher magnification image of a BM area with a high density of CD169-YFP+ cells. All three signals highlight the same cells, with the cytoplasm being labelled by the expression of the transgenic YFP and the membrane is highlighted by the antibodies.

3.5 Genetic models of macrophage ablation

The two reporter mice model studied for imaging have an inducible receptor leading to the ablation of the cells expressing the inserted genetic construct. The next step to validate the transgenic mouse model used during the imaging was to confirm that upon the delivery of the activating drug, macrophages were depleted. While the MaFIA mice did not show a high correlation between the GFP signal and F4/80 signal, I believed this model could still lead to a good depletion of bone marrow macrophages.

The MaFIA mice carry a human inducible apoptotic gene inserted after the common phagocyte promoter *c-fms* [53] leading to the apoptosis of the cells of interest by treatment with AP20187, a homodimerizer. Following Cho et al.'s drug regimen[73], MaFIA mice were treated with three consecutive intravenous injections of AP20187 (10mg/kg or 5mg/kg) or control vehicle followed by booster injection every three days at 1mg/kg (Figure 3.9A). Five days after the start of the treatment, mice receiving AP20187 developed nodules on their tails, preventing any further injections. On day 9, the state of the tails had deteriorated, leading to a humane endpoint. Therefore, the actual experimental protocol did not contain any booster injections and the BM harvest was performed on day 9 (Figure 3.9B). When analysing the BM of these mice, strikingly B cells were reduced by 100 fold while myeloid cells increased by 5 fold and macrophages remained in similar numbers, independantly of the dose of AP20817 injected (Figure 3.9C). Given the high depletion of B cells and lack of depletion of myeloid cells and the low correlation between the F4/80 and GFP signal when imaging these mice, I wanted to confirm that non-myeloid cells were expressing the transgene. Hence, I analysed the BM from untreated MaFIA mice by flow cytometry (Figure 3.10A-B). Two levels of GFP expression were detected in the immune compartment. GFP^{hi} cells included neutrophils, dendritic cells, eosinophils, monocytes and macrophages. B cells expressed lower levels of GFP. However, 87% of B cells expressed GFP while only 68% of macrophages were GFP+. Finally, when looking at all GFP+ cells, macrophages only represented 5.6% of these cells(Figure 3.10B). Together, these results suggest that GFP^{lo} cells are still being activated with the transgene and therefore B cells are the main cells depleted in this model. Altogether, this data revealed that the MaFIA mouse model available to us, was not satisfying as a model of macrophage.

Finally, I evaluated the CD169 reporter mouse model. A slightly different model to the imaged model was used whereby instead of the Cre cassette inserted following the CD169

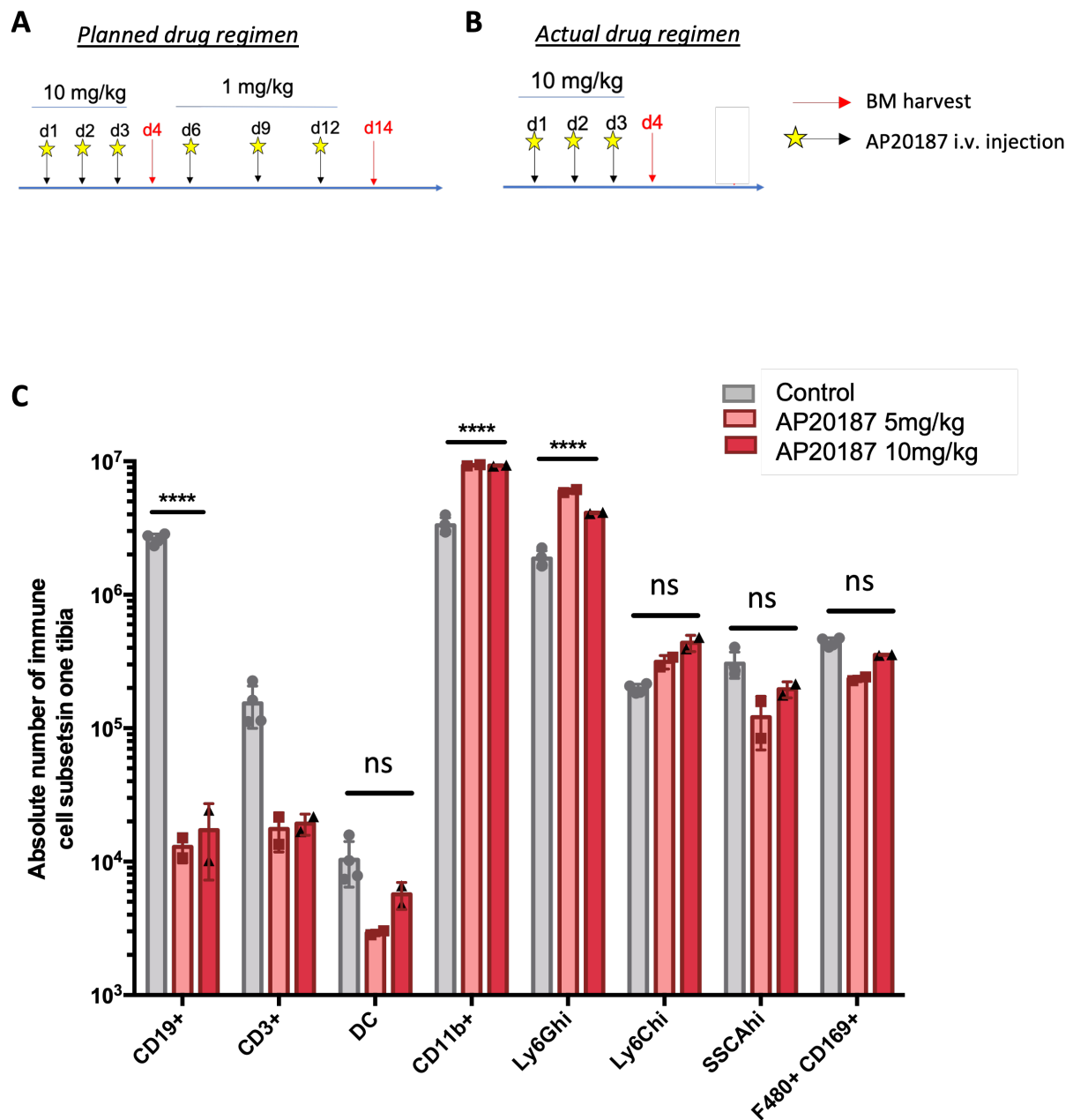


Figure 3.9: MaFIA mice cell depletion

(A) AP20187 drug administration protocol to activate the suicide gene in the MaFIA strain based on S.W. Cho et al.'s [73] drug regimen. Due to unforeseen toxicity, the experiment reached a humane endpoint and the drug regimen was adapted to (B). (C) Absolute number of the different immune cell populations in the BM of MaFIA mice treated with 5mg/kg, 10mg/kg of AP20187 or control BM. Activation of the suicide gene led to a reduction of B cells, T cells and an increase in myeloid cells. Bar graphs indicate mean ($\pm$ SD) of 2-4 mice.

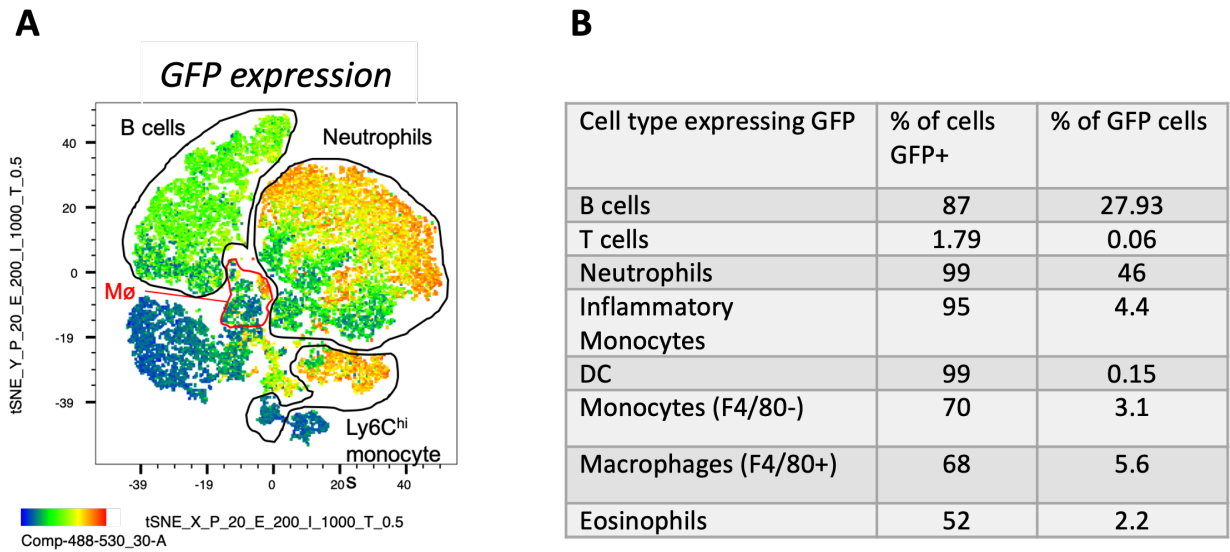


Figure 3.10: MaFIA mice characterisation by flow cytometry

(A) *t*-SNE plot of GFP expression in MaFIA mice BM. Neutrophils, monocytes, macrophages and B cells all appeared GFP+. **(B)** Percentage of GFP expressing cells among different immune cell types. The second column show what fraction of GFP cells is constituted by each immune cell subset.

promoter, a diphtheria toxin receptor was inserted. Upon the administration of DT, the toxin will bind to the receptor and lead to the conditional ablation of the CD169-expressing cells. Given that Chow et al., [52] had used this CD169-DTR mouse model to deplete macrophages in the bone marrow, I decided to follow their drug regimen. CD169-DTR^{het} and CD169-DTR-littermates were injected i.p. with diphtheria toxin at 10ng/g for two consecutive days and the bone marrow was analysed 48 hours later (Figure 3.11A). This drug regimen led to no signs of toxicity. To confirm macrophage depletion, I analysed the bone marrow of both groups by flow cytometry. The absolute number of CD11b+ Ly6G- Ly6C^{lo} SSCA^{lo} F4/80+ CD169+ cells (called ‘F4/80+ CD169+’ for simplicity) was reduced by 30 fold, dropping from 1.5x10⁶ cells in one leg of a treated control CD169-DTR- mouse to 5x10⁴ cells in a treated CD169-DTR^{het} mouse (Figure 3.11B-D). A similar drop in percentage of F4/80+ CD169+ cells was noticed with macrophages composing 2% of live cells in the bone marrow, and after depletion this number dropped to 0.1% (Figure 3.11C). In addition, when comparing control non treated WT mice with CD169-DTR- treated mice, there was no difference in macrophages, both in terms of absolute number and percentage, ruling out a possible non-specific effect of diphtheria toxin on macrophages. While I did not stain with a full panel for T cells and B cells, I grouped all cells that were CD11b- and no significant changes in number was noticed. Furthermore, the remaining cell populations of the myeloid lineage, including neutrophils, monocytes and

eosinophils were not affected by the DT treatment (Figure 3.11D). Hence, the administration of DT to CD169-DTR^{het} mice led to the specific depletion of F4/80+ CD169+ cells.

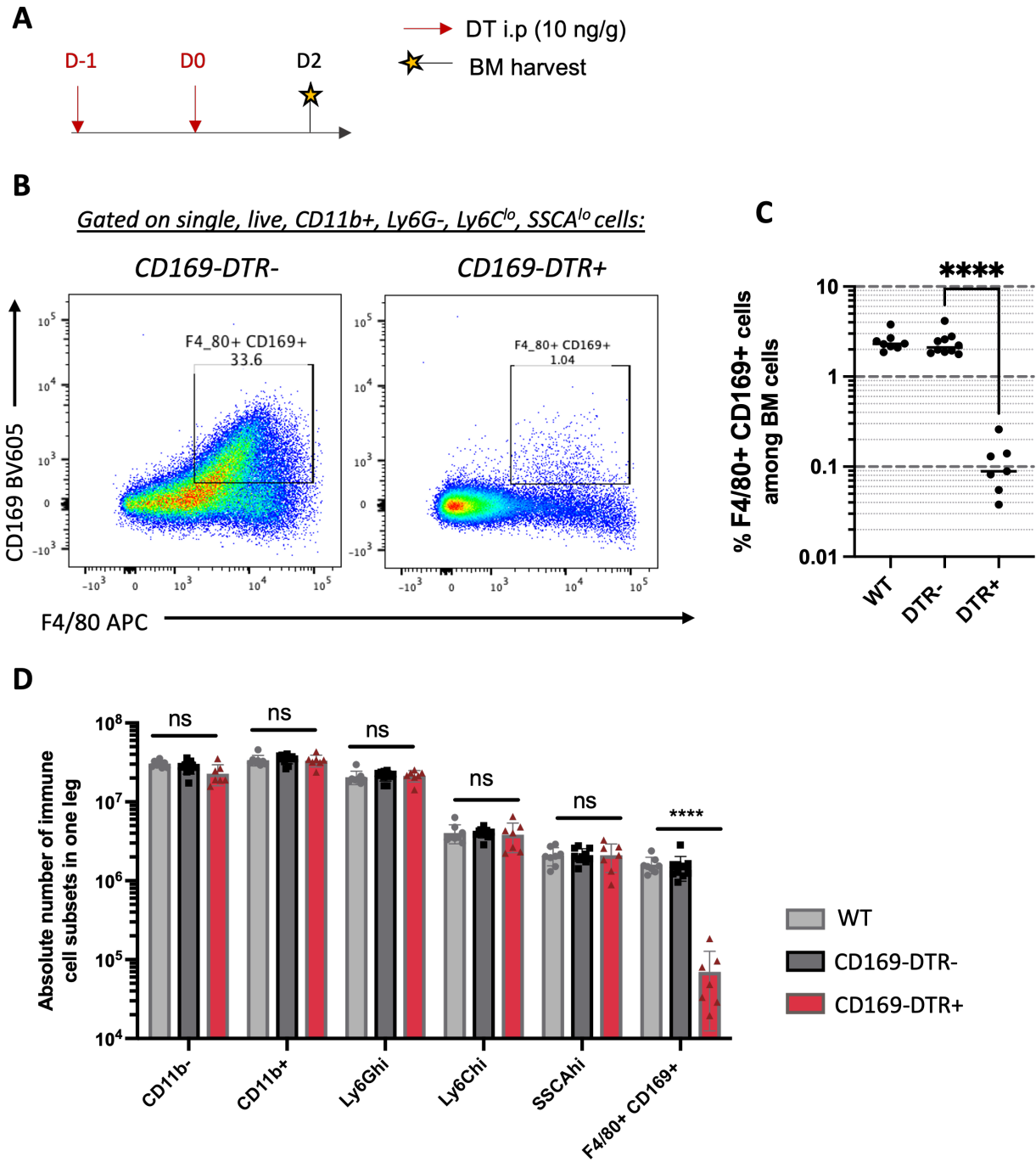


Figure 3.11: CD169-DTR mice gene activation

(A) DT administration protocol to activate the DT receptor in the CD169 expressing cells based on A. Chow et al.'s [52] drug regimen. (B) Representative FACS plots of F4/80 and CD169 cells in CD169-DTR⁻ and CD169-DTR^{het}, 48 hours after the administration of DT. (C) Absolute number of the CD11b⁺ Ly6G⁻ Ly6C^{lo} SSCA^{lo} F4/80⁺ CD169⁺ cells in the BM of CD169-DTR⁻, CD169-DTR^{het} treated mice and WT control mice. Activation of the DT receptor led to a significant reduction in F4/80⁺ CD169⁺ cells. (D) Absolute number of the different immune cell populations in the BM, in all three conditions. Only macrophages are depleted in CD169-DTR^{het} mice. Bar graphs indicate mean ($\pm$ SD) of 7-10 mice.

3.6 Discussion

The aims of this chapter were to identify bone marrow macrophages using a range of techniques and gain a deeper insight into the characteristics of the different populations of macrophages present in the bone marrow. Because of the lack of a general consensus on how to identify BM macrophages using cell surface markers, I developed my own panel of antibodies. Since numerous studies characterising bone marrow macrophages used a combination of CD11b, F4/80, CD169, Ly6G, Ly6C (or Gr1 which is a combination of Ly6G and Ly6C) and SSCa, I knew I needed to include these markers. In addition, I wanted to characterise the diversity and potential populations of bone marrow macrophages in the leukemic microenvironment hence I generated an extended flow cytometry panel including all markers described in literature to characterise both bone marrow macrophages and tumour-associated macrophages.

To make sense of the complex data arising from the 19 antibodies panel used, an unsupervised analysis approach was necessary. First published first in 2015 [196], the FlowSOM algorithm allows the clustering of cell populations based on their similarity of all markers rather than limited to two markers as it would be with conventional flow cytometry 2D scatter plots. Using this algorithm, it became clear that macrophages only constituted a small fraction of the cells present in the sample and the removal of non-macrophage cells was essential to highlight the subtle differences between bone marrow macrophage populations. Once the only cells left were potential macrophages, the FlowSOM algorithm was applied a second time. Interestingly, while the panel was composed of 21 markers, only 4 markers differentiated the 6 populations generated by the algorithm. By gating first on F4/80 and CD169 and then on SIRP α and PDL1, I was able to reproduce similar clustering. Both F4/80 and CD169 are markers commonly used for bone marrow macrophage identification and could be classified as ‘phenotypic markers’, i.e. the primary role of this receptor in today’s biology is phenotypic identification rather than function [42, 233]. However, SIRP α and PDL1 are, on the contrary, two receptors often used to define the function of the cells of interest [217]. Indeed, both of these receptors are considered to be associated with immune suppression and pro-tumoural activity [234]. Interestingly, most cells were either expressing highly both receptors or on the contrary expressing low levels or even no expression at all of either PDL1 or SIRP α . In addition, the SIRP α + PDL1+ cells were also the cells expressing the highest level of TNFa, an inflammatory cytokine. Based on these findings, I will refer to this population of macrophages as ([Immunomodulatory Macrophage \(IMM\)](#)). On the other hand, the CD169+ SIRP α ^{lo} PDL1- cells were expressing

the highest levels of VCAM1, an important receptor involved in the retention of HSCs. The combination of the expression of CD169 and VCAM1 has previously been described in tissue resident BM macrophages involved in the maintenance of erythropoiesis and haematopoiesis [52, 89, 92, 95, 96]. As such, I will be referring to these cells as Haematopoiesis-Supportive Macrophages (HSM).

Together, these results suggested that distinct populations of macrophages co-exist in the bone marrow microenvironment, with one population likely playing a role in inflammation while the second population could be involved in the maintenance of the tissue [95]. Different functions of these populations will be addressed in the next chapters. In addition, these results open the question of whether the distinct populations of macrophages arise from different origin and have different dynamics and proliferation rates. It is possible that the $\text{SIRP}\alpha^{\text{lo}}$ PDL1^- cells are tissue resident macrophages originating during embryogenesis akin to resident macrophages in other tissues, while the $\text{SIRP}\alpha^+$ PDL1^+ cells are monocyte derived cells, which can be regenerated through the hematopoietic system, in response to a pathology [235]. This however remains a hypothesis at this stage, because while macrophages ontogeny studies have been performed for the lung and the brain [11, 236] these are missing for bone marrow macrophages [20]. Because the focus of my work was to identify the function of BM macrophages during AML growth, I did not pursue this hypothesis further.

In conclusion, this analysis gave us a confident gating strategy to study bone marrow macrophages by flow cytometry. However, recently Millard et al.[207], published a study concerning the application of flow cytometry on macrophages. They reported that these cells were fragmented during the processing of the sample and therefore other cells including neutrophils and lymphocytes were stuck to macrophages fragments, hence generating false positive data for macrophages markers. In addition, they found that no macrophages remained intact after the processing of the sample and therefore weren't present in the analysed sample. While this work could potentially exclude the use of flow cytometry for the study of macrophages, currently no alternative exists, hence it is important to keep in mind their work while analysing flow cytometry data focused on macrophages.

The next experiment described in this chapter was the identification of bone marrow macrophages by confocal microscopy. Indeed, live imaging is key to study the dynamics of the cells, their interaction and location. However, the main challenge is the restriction in the

number of markers used for their identification. While I managed to reduce the flow cytometry panel to 7 markers, by confocal microscopy, the current set-up for live imaging limits us to a maximum of 3 channels. Given that one channel needs to be kept to see the interaction with other cells, this leaves us with a maximum of two channels to identify macrophages. To make things more complicated, the field of live imaging remains quite ‘niche’ and different studies have used different labelling techniques to identify bone marrow macrophages. Again, a universal, well-established approach is missing.

Among the different techniques used in the field to highlight BM macrophages, I first decided to try dextran and anti-CD169 injection as they seemed like a promising approach. I also injected anti-F4/80 antibody as the flow cytometry result indicated that the F4/80 and CD169 signal should correlate. While the correlation between these two signals was very strong, with the dextran very few F4/80+ cells were Dextran+ and inversely dextran signal was also found outside of F4/80+ cells. While these results meant that dextran was not an ideal method to label all bone marrow macrophages, it remains a technique to keep in mind if in the future I wish to label the most phagocytic macrophages sub-set. On the other hand, given the strong correlation between the F4/80 and CD169 signal, and the morphology of the cells highlighted by these antibodies, I could conclude that one of these antibody would be sufficient to image macrophages. F4/80 antibody was conjugated to APC while CD169 antibody was conjugated to PE. The biggest concern with injectable substances is bleaching. Interestingly, the CD169 signal bleached after 10 minutes of imaging while the F4/80 signal was still visible after two hours, remaining specific without spreading. Hence, I could conclude from this experiment that F4/80 in APC was the most specific and stable injectable substance to identify macrophages by confocal microscopy.

Finally, the last section of this chapter addresses the use of reporter mouse models to image and deplete bone marrow macrophages. Indeed, fluorescent protein-based reporters are known to be more resistant to photobleaching and are usually favoured for live imaging. In addition, in some models, they can be used for specific cell depletion. Hence, this option had to be explored. While many reporters have been described to study macrophages, due to the high level of gene expression overlap between phagocytes mononuclear cells many of them are not specific to macrophages but to all cells of the mononuclear phagocytic system [47, 48]. Among these reporter, the most commonly used mouse reporters are the MaFIA, MacGreen and MacBlue mice which all contain an inserted fluorescent protein following the c-fms promoter, promoter

known to be expressed by the mononuclear phagocyte system cells [53, 237].

Since these mice were well accepted to study macrophages and were available to me through at the Francis Crick Institute colony, I decided to image them in combination with anti-F4/80 APC. Unfortunately, the colocalization between the two signals was weak. While the low specificity of the reporter was disappointing, I believed that if I was able to identify what were the other cells highlighted by the reporter, I might be able to deduce which cells are true macrophages. To help me investigate this point, I performed a flow cytometry analysis of the bone marrow of MaFIA mice and stained with the panel designed previously and some markers to distinguish T cells and B cells. While phagocytes were GFP^{hi}, B cells were also expressing the GFP protein, albeit at a lower level. However, in the original study describing the MaFIA mice strain, B cells were shown to be negative for GFP [53]. The administration of the small molecule APO20187, described to activate the suicide receptor which gene was inserted following the GFP cassette, confirmed that B cells were expressing the genetic construct. In addition, the mice developed necrotic nodules on their tails leading to significant toxicity. Post-mortem analysis revealed substantial influx of neutrophils in the spleen and liver with potential signs of neoplasia. These symptoms had not been described before suggesting that this was not a common side effect of either the administration of the drug or the activation of the suicide gene. Together, the lack of depletion of any myeloid cells combined with the loss of B cells suggest a drift in the genetics compared to the original genetic construct. In conclusion, this model had to be excluded from further analyses.

The final model I was able to study was the CD169-Cre x Rosa26-YFP and CD169-DTR. These mice were designed by M. Tanaka to study marginal zone macrophage [51]. Given the high correlation between the F4/80 and CD169 expression by bone marrow macrophages, I believed these mice could be a good reporter for bone marrow macrophages. In addition, Chow et al. [52] used this strain to deplete bone marrow macrophages and showed a 5-fold reduction in the number of macrophages. By crossing the CD169-Cre with the Rosa26-YFP strain I could visualise which cells would be depleted in the CD169-DTR and at the same time check if this would be a good reporter for imaging.

By injecting both anti-F4/80 APC and anti-CD169 in PE in a CD169-Cre x R26-YFP mouse, I was able to confirm that this reporter correlated with our macrophage staining. While alone the antibody signal was hard to interpret as it seemed to highlight a mesh; combined

to the reporter, it became apparent that the antibody was labelling all the dendrites of each macrophage and was therefore highly specific to each cell. Given that the reporter was intracellular, it was easier to analyse the reporter staining and quantify macrophages in the bone marrow. However, previous work in our lab had noticed that repeated imaging of GFP reporter mice can lead to activation of the GFP+ cells and subsequently these cells would start to proliferate. This was noticed for osteoblast with the Col2.3-GFP reporter and general stromal cells with irradiated LifeAct-GFP mice. Hence, it was important to check that this was not the case for macrophages. Luckily, macrophages are known to be non-proliferative and after repeated imaging, I could confirm that the excitation of the GFP did not lead to their proliferation. Hence, this model is suitable to perform live imaging of bone marrow macrophage.

The final step to confirm this model was to activate the CD169-DTR construct by injection of diphtheria toxin and validating that macrophages were depleted. Since Chow et al. [52] had already studied the depletion of bone marrow macrophages using this model, I decided to follow their drug regimen and confirm the depletion was by flow cytometry. Not only were F4/80+ CD169+ macrophages strongly depleted but also all other cell populations were not impacted by the administration of DT. In addition, the CD169-DTR- littermates who had also been injected with diphtheria toxin did not show any differences compared to WT mice. Together, these results confirmed that the reporter was highly specific for bone marrow macrophages and DT had no direct effect on bone marrow cells. In conclusion, I have identified a strong reporter mouse model for bone marrow macrophages which allows both their identification for live imaging and their depletion to study their functionality.

Altogether this chapter describes the best approach to study bone marrow macrophages by flow cytometry and confocal microscopy. Concerning the flow cytometry analysis, a 7-colour panel and gating strategy was designed to characterise bone marrow macrophage populations. Two labelling techniques were identified to perform *in vivo* imaging of bone marrow macrophages, the anti-F4/80 APC antibody can be injected or the use of the CD169-Cre crossed with the Rosa26-YFP reporter mice can be used. For simplicity, I will refer to these mice as CD169-YFP mice in the following chapters. Finally, the reporter mouse CD169-DTR was also shown to allow specific depletion of bone marrow macrophages. In the next chapters, it will be interesting to use these techniques to address whether macrophage dynamics and functions are altered in AML.

Chapter 4

Assessing the changes in bone marrow macrophages with AML

4.1 Introduction

Since 1889, when Stephen Paget described the association between tumour cells and the organ microenvironment, it is accepted that any form of cancer including hematological malignancies leads to microenvironmental remodelling. In the case of AML, one of the major microenvironmental alteration noticed was changes in vasculature with increased secretion of VEGF-A [192]. More specifically in this study, D. Duarte et al., showed that the endosteal vessels were lost and this loss was correlated with the loss of healthy hematopoiesis, stromal cells and osteoblasts. A few years later, C. Pirillo et al., [238] found that AML cells were capable of dislodging healthy hematopoietic cells through the damaged vasculature and found an increased number of healthy cells in the blood. Macrophages, as a cell component of the healthy hematopoietic system are thought to be included in these previous results and as such, I hypothesize that bone marrow macrophages are affected by the growth of leukemia.

In 2018, X. Yang et al., [174] studied the dynamics of bone marrow macrophages with AML and found that in the bone marrow, macrophages were first increased at an early AML stage and were then lost at a later AML stage, when the disease constituted most of the bone marrow. However, the FACS gating strategy used in this study to identify macrophages includes a broad range of myeloid cells and, given that macrophages only consist of a small percentage of cells among myeloid cells, it is hard to conclude from their results whether the macrophages are affected differently compared to other healthy hematopoietic cells.

In addition, based on the results presented in Chapter 3, we know that two populations of macrophages are present in the bone marrow: the haematopoiesis-supportive macrophages (HSM) and Immunomodulatory macrophages (IMM). Given their differences in phenotype, I hypothesize that these cells are associated with different functions. Haematopoiesis-supportive macrophages express [VCAM-1](#) and CD169, two markers primarily found in macrophages involved in the regulation of erythropoiesis and haematopoiesis while Immunomodulatory macrophages strongly express [SIRP \$\alpha\$](#) and [PDL-1](#), two markers important in the inhibition of the immune response, in particular in the context of cancer where both of these receptors have been targeted to help stimulate the immune response against tumour cells. Hence, based on the differential expression of these four markers by the macrophage sub-populations, it is most likely that the function of these cells and their role in AML progression is different.

Altogether, combining the results presented by X. Yang et al., with our results from Chapter 3 we can hypothesize that [IMMs](#) are favourable to AML growth while [HSMs](#) are important to maintain a healthy environment and promote the retention of HSCs. This could explain the results observed by X. Yang et al., [174] where the increase of cells could be explained by the idea that Immunomodulatory macrophages are favourable to AML growth and as such they are preferentially selected to remain in the [Bone marrow \(BM\)](#) space and promote disease growth.

Correlating with the idea that pro-tumoural macrophages are selectively maintained in the bone marrow, a study published by F. Mussai in 2013., [172] showed that co-culture of AML cells with monocytes polarised monocytes towards an anti-inflammatory phenotype characterised by increased expression of CD206. And recently, I. Weinhauser et al., [180] published a similar study where co-cultures of CD206+ cells with AML led to increased stemness, homing and immune evasion of AML cells. Together these studies suggest that AML cells are capable of shaping the phenotype of macrophage towards a pro-tumoural phenotype in turn shaping the AML cells towards increased aggressiveness. While this hypothesis is interesting, no studies have directly addressed this possibility.

Interestingly, while in an *in vitro* setting, macrophages were polarised towards a protumoural phenotype when cultured with leukemic cells, *in vivo*, by performing bulk RNA sequencing, X. Yang et al., [174] showed that at a transcriptional level, bone marrow macrophages were highly conserved with AML compared to splenic macrophages and bone marrow macrophages

in [T-ALL](#) as they had 10-fold less differentially expressed genes. They classified the bone marrow macrophages from an AML mouse as M1 cells as they expressed strongly *Nos*, *Cxcl9* and *Cxcl11*. However, these cells also expressed several M2 genes including *Mmp9* and *Cxcr4* and did not express some classical M1 associated genes such as *Il6*, *Tgfb* and *Il12*. Finally, when they evaluated the transcriptional changes in bone marrow macrophages associated with AML, no striking gene signature indicating a polarisation by AML cells was observed. Unfortunately, no other study directly addressed the transcriptional changes in macrophages with AML, hence it is hard to conclude whether this classification is accurate, and macrophages are not significantly impacted by AML.

Hence, in this chapter, I aim to address whether the different macrophage populations identified in Chapter 3 have different dynamics with AML. As AML is known to grow in clusters, I will measure the macrophage population evolution both by flow cytometry and confocal microscopy with the hypothesis that if macrophages are promoting AML expansion, I will find them preferentially in the AML clusters. Following this analysis, I will address whether AML cells are able to shape macrophage profile by performing single cell RNA sequencing of all control and leukemic macrophages. The advantage of single cell transcriptomics being that I should be able to identify the different macrophage population in our sample while in bulk the differences would be mixed. Finally, as AML cells are known to be myeloid cells, I will also include AML cells in the transcriptional analysis and question whether AML cells are trying to hijack the macrophage phenotype and replace these cells in the microenvironment.

4.2 AML model to assess macrophage dynamics in the bone marrow

In order to investigate the effect of AML on macrophage populations, as the disease progresses, I used the well-established MLL-AF9-driven murine model of AML, which has been shown to recapitulate phenotypic and pathological features of human MLL-rearranged AML [239, 240]. To generate this model, previous members in the lab sorted [GMPs](#) from an mTmG+ donor mouse and transduced these cells with the MLL-AF9 oncogene. The transduced cells were injected into primary lethally irradiated mice and harvested once the bone marrow contained more than 90% AML cells. Each primary donor was frozen separately and would be considered a clone. Experiments were then performed using AML cells from different clones to ensure

identification of consistent features of AML growth minimizing possible primary donor-specific phenotype. To reach consistent disease engraftment and growth, 100,000 primary AML cells were injected intravenously into secondary immunocompetent mice (See Material and Methods for details). The use of immunocompetent mice was essential to our project to ensure macrophages were able to interact with the microenvironment and other immune cells, and therefore perform their full function representative of their role in humans.

I defined three main stages of AML, according to the percentage of mTmG+ AML cells in the bone marrow, measured by flow cytometry. The first stage, ‘early AML’ is defined by an AML infiltration of less than 20%, the second stage, ‘intermediate AML’ corresponds to an AML infiltration of 20-60% and the final stage is called ‘late AML’ and it corresponds to an infiltration higher than 60% (See Material and methods for details). When comparing the infiltration of AML in the bone marrow and blood, the percentage of AML cells in the blood was found to be significantly lower at all time points meaning that these cells grow first in the bone marrow and then infiltrate the blood (Figure 4.1A). This result recapitulates the growth of AML in humans, which is known to infiltrate first the bone marrow and then escape to the blood.

According to the clone injected, AML cell expansion would start to be visible in the bone marrow between day 9 and 12 post-transplantation and the disease would usually progress to late AML within a week (Figure 4.1B). The difference in speed of engraftment and growth between clones is a useful tool to adapt to the needs of different experimental set-ups and allows us to evaluate whether certain characteristics of the disease are dependant on the speed and aggressiveness of the AML. One example of this point is that the fast-growing clone, clone 1, was shown to be more chemoresistant compared to all other clones, correlating with the idea that this clone is more aggressive. For all experiments performed with this AML model, the results were generated using at least two different clones to confirm the result is a consistent feature of AML.

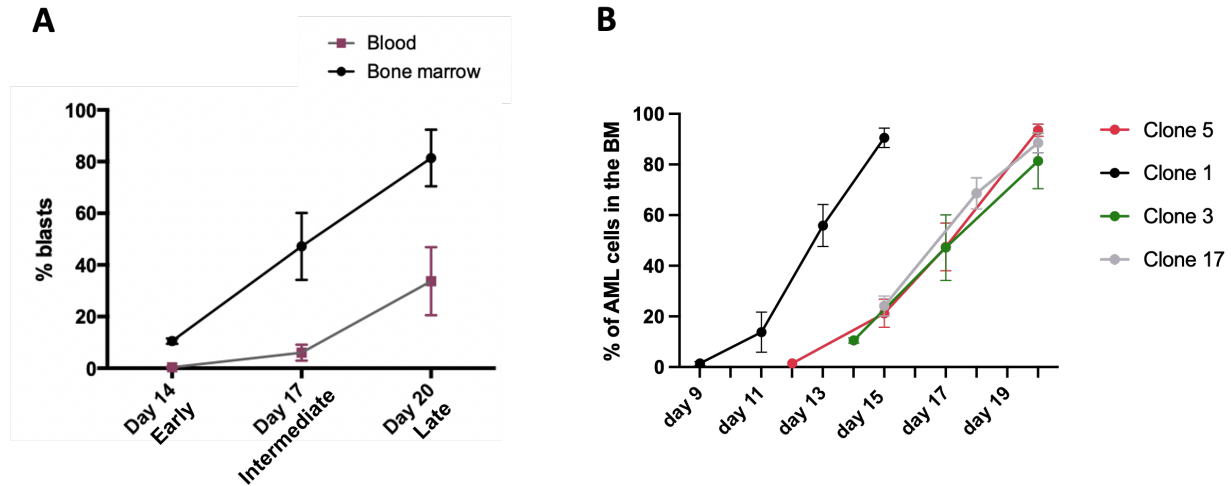


Figure 4.1: MLL-AF9 model of AML growth in the BM and blood

100'000 *mTmG* AML cells were injected into immunocompetent mice, sacrificed at early, intermediate and late infiltration corresponding to a bone marrow infiltration comprised between 0 - 20% for the early time point, 20 - 60% for the intermediate time point and 60 - 100% for the late time point. **(A)** AML growth in the BM and blood. $n = 3$ mice in each group, representative of 3 independent experiments. **(B)** Growth of different clones in secondary recipients. The steepness of the curve remain similar but the engraftment time varies according to the clone. $n = 3$ mice in each group, representative of 2 independent experiments. Error bars represent the mean $\pm$ Standard error of mean (s.e.m.)

4.3 Macrophage populations are differentially affected by AML

To characterise the dynamics of macrophages with AML, I transplanted immunocompetent mice with 100'000 AML cells and measured the absolute number of Immunomodulatory macrophages and haematopoiesis-supportive macrophages at four different time points of AML, by flow cytometry. Interestingly, the absolute number of IMMs slightly increased at the first time point and then remained close to constant until the very last time point (Figure 4.2A maroon plot). However, the HSMs were lost much faster as already by the second time point, their numbers were reduced by 2-fold (Figure 4.2A grey plot). Given these differences in dynamics, I speculated that IMMs could be preferentially maintained in the environment compared to all other healthy cells.

Hence, I decided to compare the loss of these two populations with all other healthy hematopoietic cells including progenitors and lymphocytes. By analysing each population as a percentage of their control population, I was able to normalise their number to visualise the loss of each population as AML grows. Importantly, the main read-out in this experiment is the

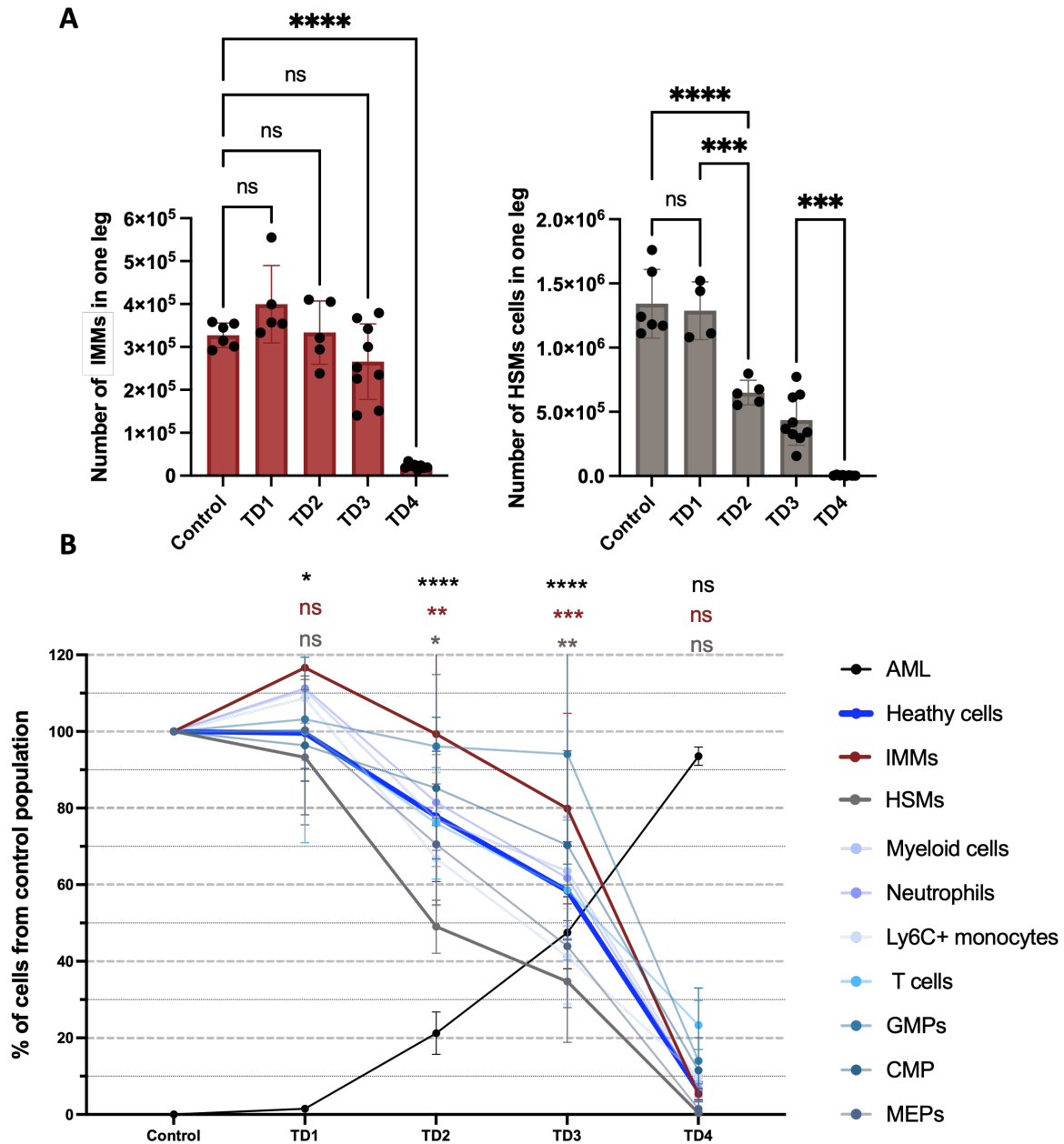


Figure 4.2: Macrophage populations are differentially affected by AML
 100'000 mTmG AML cells were injected into immunocompetent mice and sacrificed at four different time points throughout AML growth. Control aged-matched *WT* mice were sacrificed as control. **(A)** Absolute number of IMMs (maroon plot) and HSMs (grey plot) in the bone marrow. AML growth in the BM and blood. **(B)** Percentages of different immune cell populations compared to control. The steepness of the curve represent the enrichment and loss of each population. HSMs are the first cells lost with AML while IMMs are the most enriched immune cells in the BM at early AML. Myeloid cells were identified as CD11b+, T cells as CD3+, neutrophils as CD11b+ Ly6G+, Ly6C+ monocytes as CD11b+ Ly6C+, GMPs as Lin- cKit+ Sca1- CD16/32+ CD34+, MEPs as Lin- cKit+ Sca1- CD16/32+ CD34- and CMPs as Lin- cKit+ Sca1- CD16/32- CD34+, TD = take down. $n = 5 - 9$ mice in each group, representative of 3 independent experiments. Error bars represent the mean $\pm$ s.e.m. Statistically significant differences were determined using two-way ANOVA, The results are highlighted in black for the comparison between IMMs and HSMs, in maroon for IMMs and healthy cells and in grey for HSMs and healthy cells (* represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$).

steepness of each curve indicating which population is lost first. Strikingly, the IMM curve sits at the top of the plot and shows an increase in the proportion of these cells at early AML with the percentage increasing to 118% of the population from control highlighting the enrichment in these cells. In addition, when I look at the other time points, it is apparent that these cells are among the last remaining healthy cells present in the bone marrow, as they are all along among the cell population with the highest percentage of remaining cells. However, the HSM population curve is the lowest curve on this plot, meaning these cells are lost first and much faster compared to other cell population (Figure 4.2C). Of note, the GMP population curve was the flattest curve suggesting that these progenitors remain in the BM and are able to give rise to differentiated cells such as macrophages. Altogether these results reinforce the idea that IMMs are supportive of AML and therefore are preferentially maintained in the leukemic bone marrow while HSMs are eliminated much sooner suggesting these cells could be the precursors of the healthy cells dislodging. Indeed HSMs are known to secrete HSC retaining factors hence if they are no longer present to secrete these factors, healthy hematopoietic cells could be more easily dislodge to the blood.

4.3.1 Macrophages are increased inside AML clusters at early AML

To evaluate whether macrophages are preferentially maintained in the leukemic microenvironment, I decided to take advantage of the fact that AML grows in clusters [241]. Using intravital imaging of the mouse calvarium, I can evaluate whether at early AML, when AML cells clustered into patches of highly infiltrated areas, whereas the remaining BM space contained only sparse AML cells, macrophages are enriched inside the AML clusters compared to healthy areas of bone marrow. While we do not have a good reporter mouse for each macrophage population, we do have the CD169-YFP reporter mouse which highlights all macrophages that have expressed or are currently expressing CD169. This model can still allow us to assess whether macrophages are enriched inside AML clusters.

The first step to quantify the number of macrophages according to AML clusters was to identify areas containing AML and classify them as ‘single cells’, ‘small clusters’ or ‘large clusters’ in an unbiased automated way. The bone marrow was outlined in the image and divided into 100 squares [Region of interest \(ROI\)](#) of approximately 120 microns side length. The percentage of tomato+ pixels was calculated for each ROI and classified as single cells if it was lower than 15%, small clusters if it was between 15% and 50% and large clusters if it was

higher than 50%. The classification was validated visually and confirmed that the percentages of tomato+ pixels corresponded to the correct labels. Next, the percentage of YFP+ pixels was calculated for each ROI. Strikingly, the percentage of YFP+ pixels was significantly higher in ROIs containing AML clusters compared to ROIs containing only single AML cells (Figure 4.3 A-C). Two hypotheses could explain this increase:

1. The size of macrophages increases in AML areas,
2. The number of macrophages increases inside AML areas.

To answer which of these two hypotheses was taking place, every macrophage imaged in all ROIs was manually outlined. The number of macrophages was significantly higher in the ROIs containing AML clusters compared to single cell AML ROIs (Figure 4.3 D). In addition, the morphology of macrophages was altered as macrophages became more circular and both their area and perimeter was reduced in large clusters (Figure 4.3 D). Interestingly, the perimeter and area first increased in small AML clusters and then significantly reduced in large clusters suggesting that the dendrites of macrophages are retracting. One possible explanation behind these changes of morphology is that the cells are sensing the changes in the microenvironment resulting in increased intra-cellular stress-signaling and this is reflected through their changed morphology. Indeed, *in vitro* macrophages are known to become rounder and lose their dendrites as they are dying.

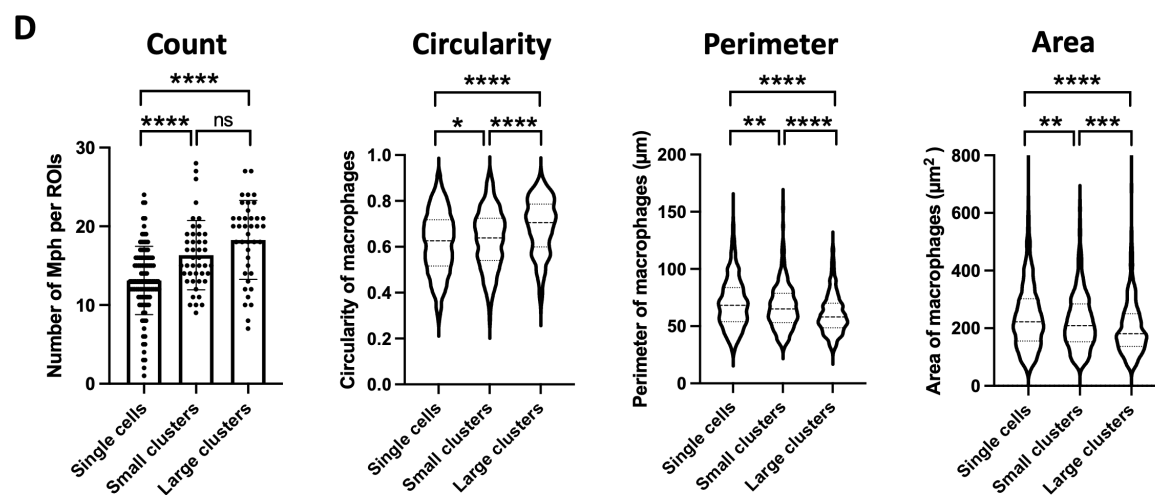
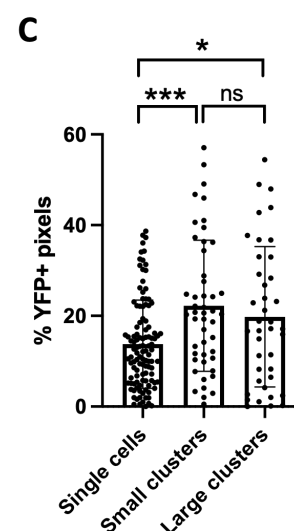
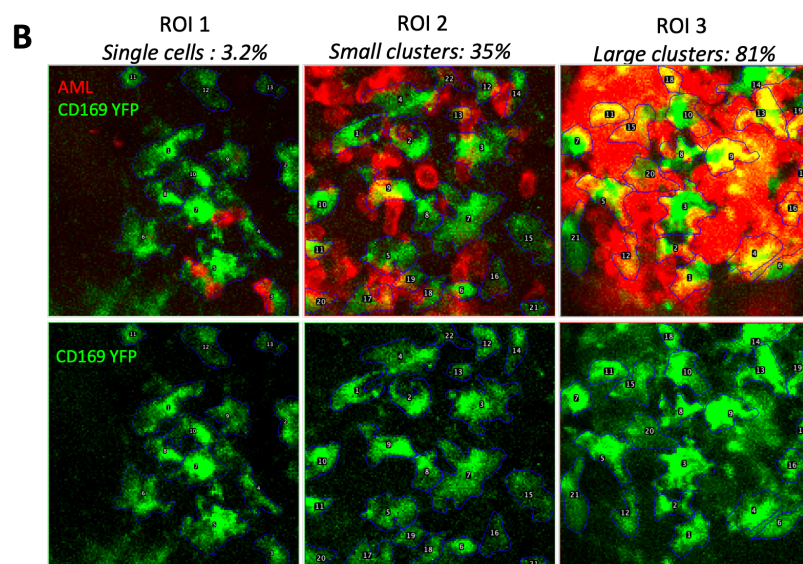
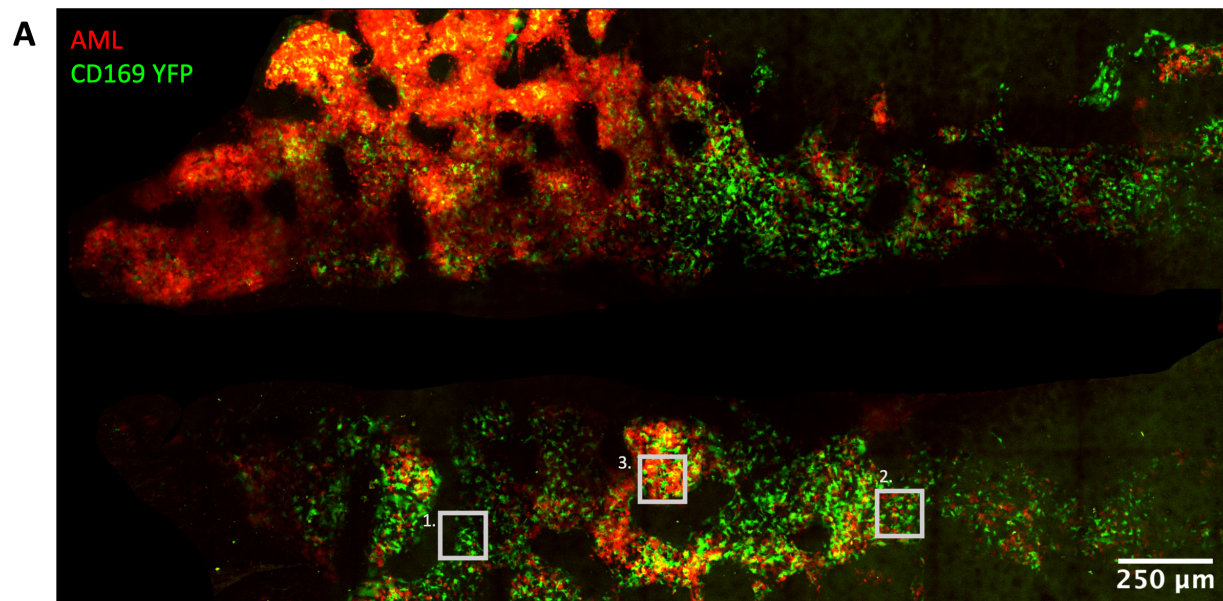


Figure 4.3 (previous page): **IVM** reveals local increase of macrophages in AML clusters
CD169-YFP mice were injected with 100'000 mTmG AML cells and imaged at early AML. (A) Representative maximum projection of a tilescan of calvarium BM with AML cells in red and CD169-YFP+ cells in green. The tilescans were divided into 100 ROIs and classified according to their AML load: ROIs were labelled as single cells when the percentage of AML+ pixels was lower than 15%. They were labelled as small cluster for AML between 15 and 60% and large clusters for AML above 60%. (B) Zoom of an ROI of each category. (C) Percentage of YFP+ pixels in ROIs classified as single cells, small clusters and large clusters. (D) CD169-YFP+ cells were manually outlined in each ROI. Number, circularity, perimeter and area of macrophages in each ROI according to the classification of the ROIs. Each dot represents one ROI, $n = 105$ for ROIs classified as single cells, $n = 44$ for small clusters and $n = 40$ for large clusters. The data was generated from 2 mice. Error bars represent the mean $\pm$ s.e.m.. Violin plots represents the mean value of all macrophages manually outlined, $n = 2813$. Statistically significant differences were determined using Student t-tests (represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$).*

In order to find out whether these cells are IMMs or HSMs, anti-SIRP α was injected (visualised in blue in Figure 4.4A). While by flow cytometry it is easy to distinguish cells expressing this receptor at different levels, by confocal microscopy, the signal is less quantitative and the signal diffuses strongly according to its location in the tissue. Two different means were used to analyse the SIRP α channel. First, the signal was thresholded and pixels were classified as either positive or negative. Interestingly, using this approach, the percentage of SIRP α + pixels was significantly increased in ROI with large AML clusters (Figure 4.4B). The second strategy consisted in measuring the mean value of the SIRP α signal for each outlined macrophage. The histogram of the mean SIRP α value was then divided into three categories, SIRP α -, SIRP α ^{lo} and SIRP α ^{hi} (Figure 4.4 C-D). The classification was validated visually and confirmed that the SIRP α ^{hi} cells corresponded to macrophages expressing high levels of SIRP α (Figure 4.4C). Interestingly, while the number of SIRP α ^{hi} was significantly higher in ROI with large AML clusters compared to ROI with single AML cells, the number of SIRP α - cells was unchanged between ROIs with single AML cells, small clusters and large clusters (Figure 4.4E). This result correlates with the flow cytometry data which showed that at early AML stage, only the IMMs were enriched. A gap in these experiments remain as IMMs do not express CD169 by flow cytometry and the imaging results characterise CD169-expressing cells however one possible explanation is that the IMMs expressed CD169 in the past and therefore are YFP+ in the imaging experiment however by flow cytometry these cells, as they are no longer expressing CD169 are CD169-. One interesting experiment would be to perform flow cytometry on these mice and characterise which cells are expressing YFP.

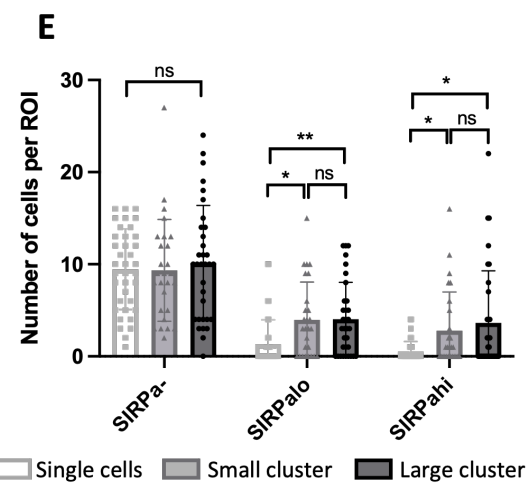
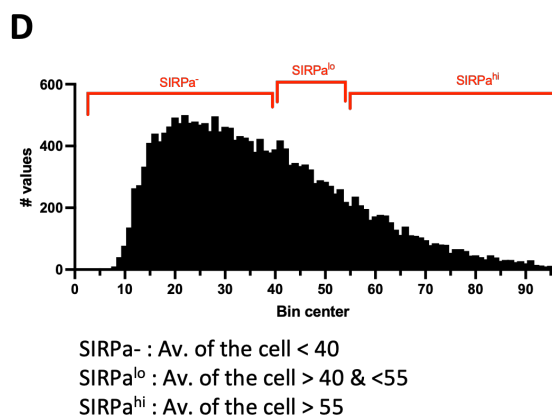
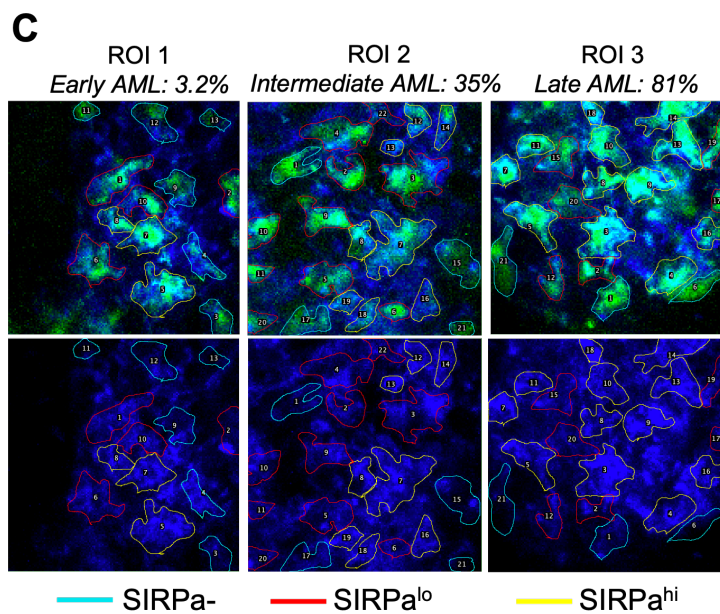
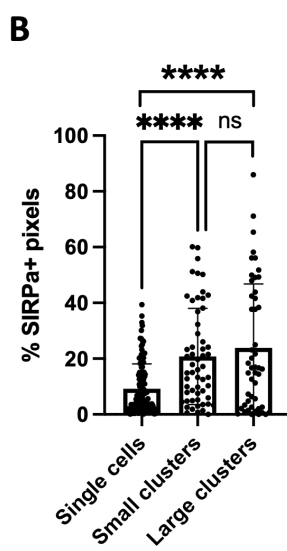
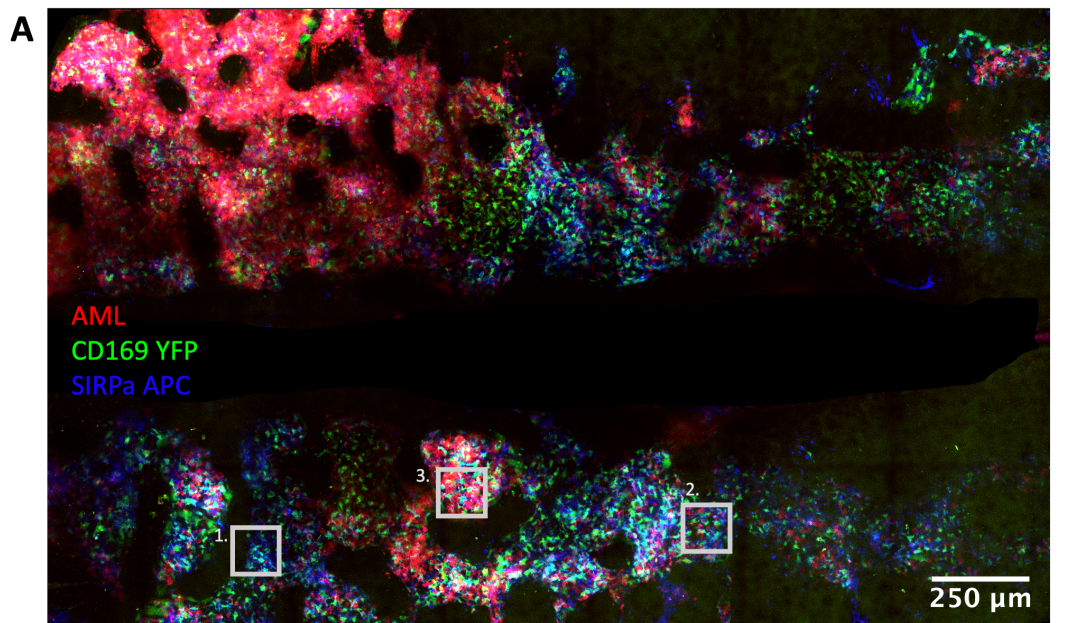


Figure 4.4 (previous page): SIRP α + cells are enriched in AML clusters

These results were generated in the same cohort as in Figure 4.3. Prior to imaging, 50 μ L of anti-SIRP α APC was injected *i.v.* to analyse the expression of SIRP α in CD169-YFP+ cells. **(A)** Representative maximum projection of a tilescan of calvarium BM with AML cells in red, CD169-YFP+ cells in green and SIRP α signal in blue. The tilescans were divided into 100 ROIs and classified according to their AML load: ROIs were labelled as single cells when the percentage of AML+ pixels was lower than 15%. They were labelled as small cluster for AML between 15 and 60% and large clusters for AML above 60%. **(B)** Percentage of SIRP α + pixels in ROIs. **(C)** Zoom of an ROI of each category. The SIRP α signal is blue and CD169-YFP is green. CD169-YFP+ cells were outlined and classified as SIRP α - (cyan line), SIRP α^{lo} (red line) and SIRP α^{hi} (yellow line). **(D)** Histogram of the mean expression of SIRP α in CD169-YFP+ cells. The histogram was divided into three categories, SIRP α - cells (mean value < 40), SIRP α^{lo} cells (mean value > 40 < 55) and SIRP α^{hi} cells (mean value > 55). **(E)** Number of macrophages classified as SIRP α -, SIRP α^{lo} and SIRP α^{hi} in ROIs classified as single AML cells, small AML cluster and large AML cluster. Each dot represents one ROI, $n = 105$ for ROIs classified as single cells, $n = 44$ for small clusters and $n = 40$ for large clusters. The data was generated from 2 mice. Error bars represent the mean $\pm$ s.e.m.. Statistically significant differences were determined using Student *t*-tests (* represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$).

Following this characterisation, I next wanted to assess whether the loss of macrophages was due to their dislodgement. As such I decided to perform intravital imaging of the calvarium of mice injected with anti-F4/80 APC and transplanted 100'000 mTmG+ AML cells. One hour long timelapses from control, early AML and late AML stages were taken and the displacement of macrophages was calculated (Figure 4.5A and Supplementary Videos 1-3). The total displacement of macrophages was significantly increased at early AML correlating with the idea that these cells are being dislodged (Figure 4.5B). While this result was interesting, the displacement over one hour was only 6 microns which remains a relatively small distance hence, it is hard to conclude whether this is biologically relevant. In addition, these results were generated before we managed to acquire the CD169 reporter mice and as such we know that the F4/80 signal is not as specific as numerous other myeloid cells could be highlighted using this technique. Finally, the injection of antibody is known to, over time, affect a number of cells including macrophages I decided to set aside this result.

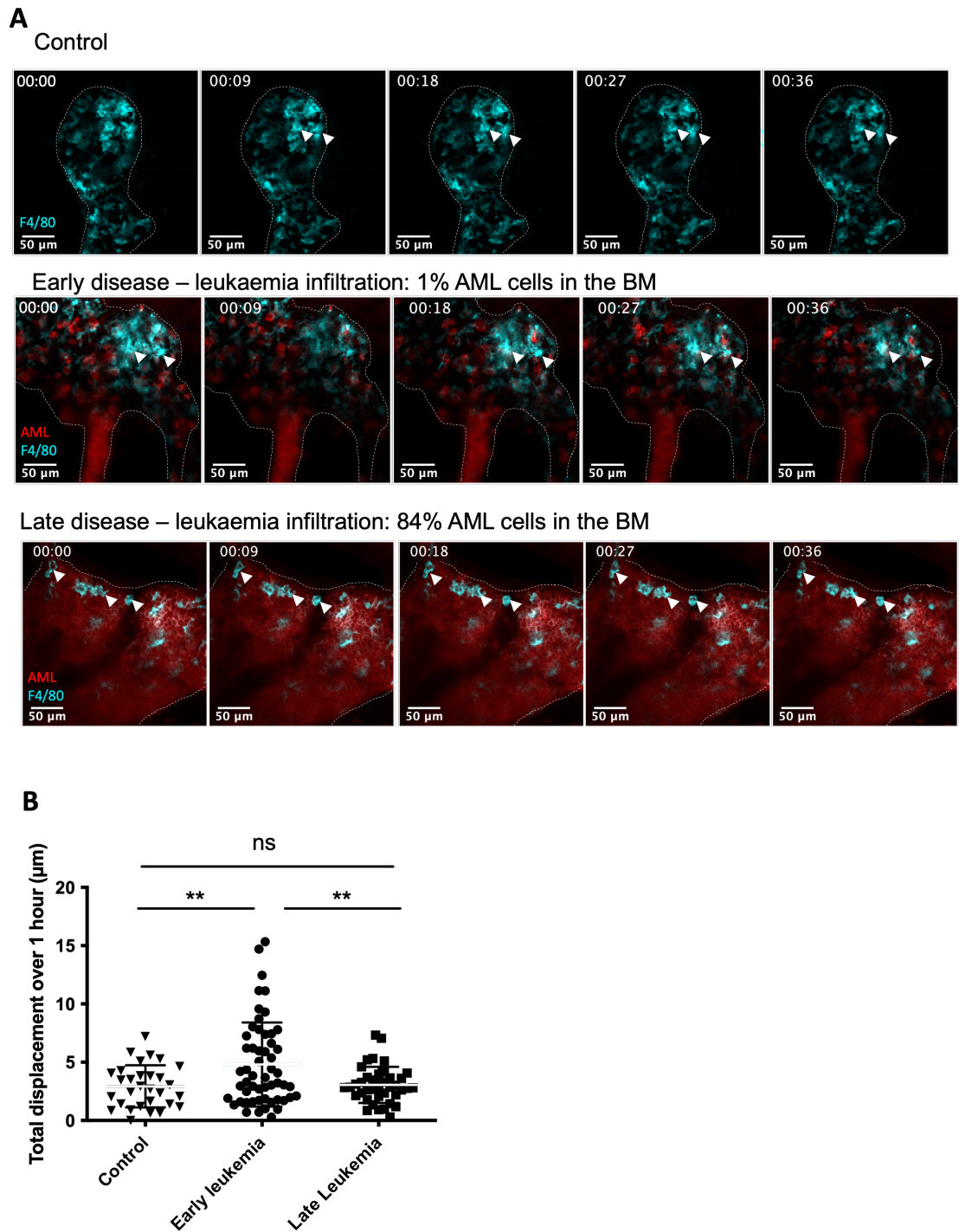


Figure 4.5: Increase in macrophage motility in early AML
Mice at different stages of disease were analysed by intravital microscopy (IVM). **(A)** Representative screenshots of maximum projections of calvarium bone marrow from 1 hour time-lapses of wild type mice injected with 100'000 tomato+ AML cells and anti-F4/80 APC. Images were acquired every 3 minutes. BM is outlined with dotted lines and arrow heads point at macrophages. **(B)** Displacement quantification of F4/80+ cells. Tracking of individual F4/80+ cells from control ($n=30$), early infiltrated AML (1%) ($n=55$) and late disease ($> 70\%$) ($n=39$) using Imaris. Macrophages from 3 mice were tracked per condition.

4.4 Single cell RNA sequencing of control and AML bone marrow myeloid cells and AML cells

In the previous chapter, I identified two populations of macrophages in the bone marrow, HSMs, expressing VCAM-1 and CD169 associated with tissue residency and HSC retention and IMMs expressing high levels of SIRP α and PDL1, two receptors important in the inhibition of the immune response. IMMs were one of the only immune cell populations that was enriched at early AML and these cells survived the longest in the bone marrow as AML grew. On the contrary HSMs were lost very rapidly in AML, before any other cell population. In combination these results indicate clear differences between the two cell populations identified. However, it remained unclear what the functional differences were and how these biological differences arose. In order to elucidate the differences at the transcriptional level, I decided to compare the populations using single cell RNA sequencing (scRNAseq) (Figure 4.6A).

To avoid the risk of biasing the experiment, I did not follow the gating strategy used for flow cytometry analyses in full and instead I decided to set up an exclusion-based sorting strategy, meaning that I excluded any cells that were known not to be macrophages and included all the cells had the potential to be macrophages. Hence, I sorted the mTmG-, CD11b+, Ly6G-, Ly6C^{lo}, SSCA^{lo}, F4/80+ cells (Figure 4.6B). I did not sort the cells based on their expression of CD169, SIRP α and PDL1 to evaluate whether the previously identified macrophage sub-populations would also cluster together based on their transcriptional profile. However, I decided to take advantage of the novel protein labelling technique, CITE-Seq to find out which cells expressed high levels of SIRP α and PDL1 in order to identify phenotypic IMMs post sequencing. In addition, I also wished to address whether certain macrophage sub-populations, like TAMs, are not initially present in control bone marrow but appear in AML. As such, I sorted macrophages from both control and early AML mice corresponding to an AML infiltration of 15%.

AML cells are myeloid derived cells which resemble monocytes and macrophages and non-malignant macrophages are lost at late disease while in most cancers; however, in most solid cancers TAMs are increased. To make sense of this discrepancy, I hypothesised that AML cells could be capable of mimicking TAM function and phenotype to generate pro-tumoural signalling and inhibit the anti-tumoural immune response. To address this hypothesis, I decided to sort AML cells from an early (15%) and late AML (65%) time point and compare

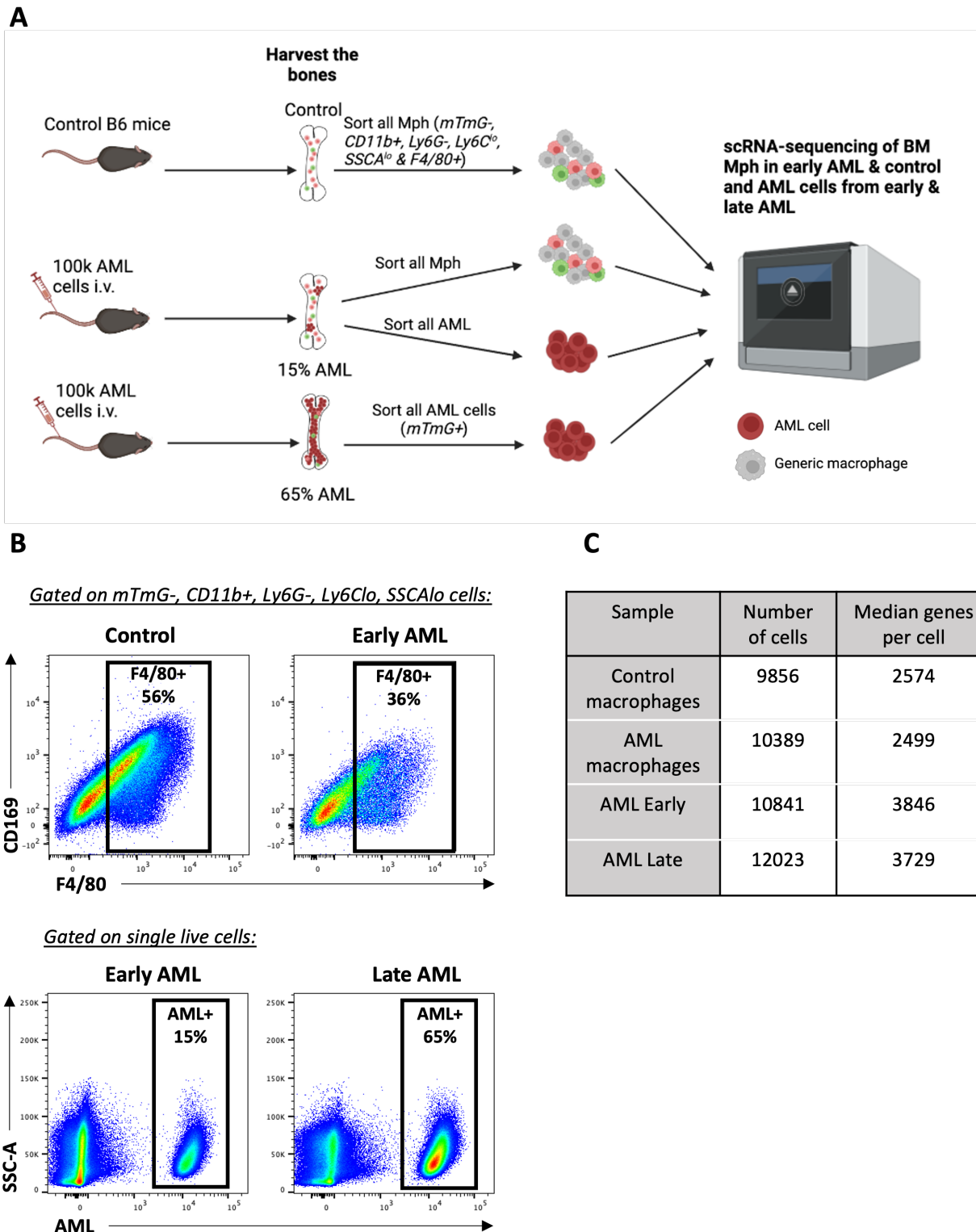


Figure 4.6: scRNAseq on macrophages and AML cells from control and AML mice
(A) Experimental set-up for scRNAseq. Macrophages were sorted from control and early AML (AML = 15% in the BM). AML cells were sorted from early AML (AML = 15% in the BM) and late AML (AML = 65% in the BM). **(B)** Macrophages were sorted based on their expression of F4/80 and AML cells were sorted based on their expression of *mTmG*. **(C)** Number of cells and median number of genes after quality control for each samples. 2 mice were pooled for each condition. Mph = macrophage

their transcriptional signature with that of macrophages (Figure 4.6 A-B). For each sample, the bone marrow of two mice were labelled and pooled together.

Similar numbers of cells were successfully analysed from control and AML animals, with 9856 control macrophages, 10389 macrophages from early AML sample, 10841 AML cells from early AML BM and 12023 AML cells from late AML BM passing quality control leading to a total cell count of 39826 cells. The median of the number of genes detected per cell was around 2500 for macrophages and 3700 for AML cells (Figure 4.6C. Detailed quality control summaries from Cell Ranger for each sample are in Appendix A). This result correlates with the fact that the leukemic cells are known to dysregulate their transcriptional network to increase proliferation [242].

After mapping the genes to the mus musculus genome and removing cells containing a high fraction of mitochondrial genes and low RNA count (see Material and Methods for details), all samples were merged together and visualised on a [Uniform manifold approximation and projection \(UMAP\)](#). 17 clusters were identified on the UMAP of the merged samples and classified according to the number of cells constituting the clusters (Figure 4.7).

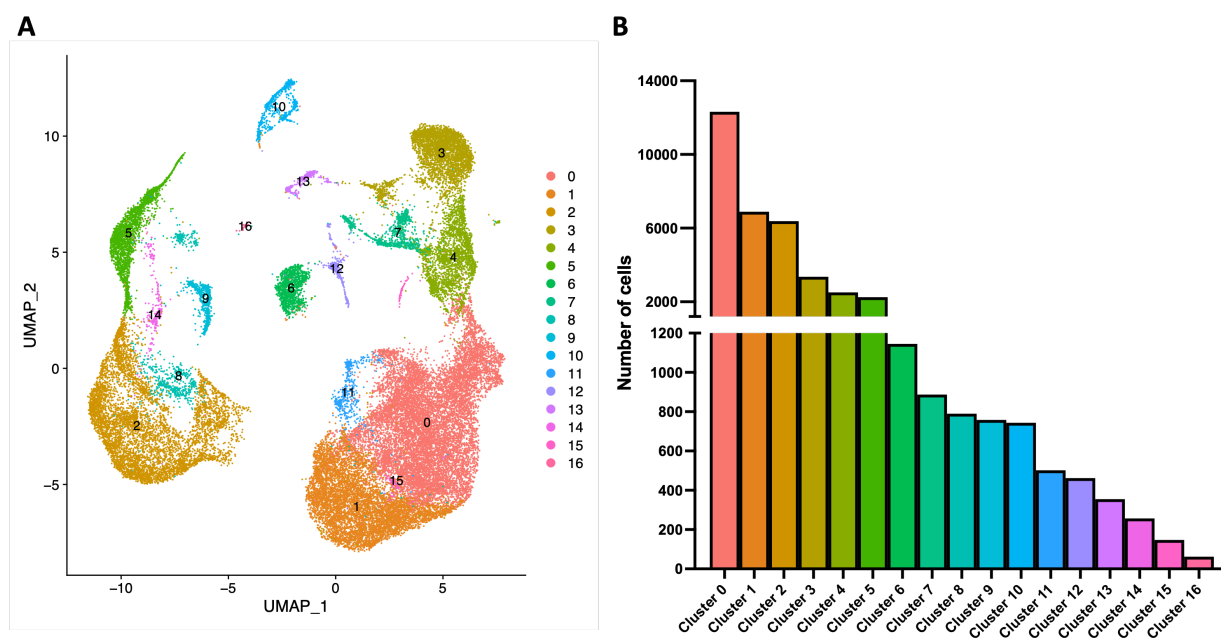


Figure 4.7: Unsupervised clustering of the merged scRNAseq samples using UMAP
All samples were analyzed using the Seurat R package. (A) UMAP visualisation of all 43'109 single cells contained in the merged analysis, coloured by clusters. (B) Number of cells in each cluster.

First, to identify what cells were present in each cluster, I analysed the top 10 differentially expressed genes for each cluster and compared their expression to the expression of each gene in all different immune cells using the Immgen platform. One small cluster containing 355 cells (less than 1% of all cells), cluster 13, could clearly be identified as NK cells while cluster 10 and cluster 16, containing in total 806 cells and representing approx. 2% of all cells were identified as B cells (Figure 4.8A-B). In addition, another cluster, cluster 6 contained a significantly higher percentage of mitochondrial genes and a low RNA count and could be classified as dying cells (Figure 4.8C). All four of these clusters were excluded from further analysis. To validate that the remaining clusters were either myeloid cells or AML cells, I analysed the expression of known myeloid genes: *Lyz2*, *Itgam* and *Ifitm3*, and known AML cells: *tdTomato*, *Myb* and *Hoxa9* (Figure 4.9A-B). The myeloid genes were found to be highly expressed in all clusters excluding the NK, B cells and leukemic cells while the leukemic genes were found in the leukemic clusters. To support these results, I split the UMAP according to the sample of origin and confirmed that the expression of leukemic genes was found primarily in the clusters present in the leukemic sample (Figure 4.9C). Interestingly, the leukemic samples contained one cluster that overlapped with the macrophage sample.

Next, I wanted to elucidate what cells were composing the myeloid clusters. Since I sorted all F4/80+ cells it is likely that some monocytes, (in particular patrolling monocytes expressing low levels of Ly6C) and neutrophils are present in the sample. To evaluate what myeloid cells were present, I assessed the expression of commonly accepted monocyte, neutrophil and macrophage genes. Monocytes are separated into two main populations, patrolling monocytes, characterised by the expression of *Cx3cr1*, *Ace* and low expression of *Ly6c2* while inflammatory monocytes express high levels of *Ly6c2*, *Ccr2* and *F13a1* [1]. During the sort, Ly6C^{hi} cells were gated out hence inflammatory monocytes should not be present in the sample however patrolling monocytes could express F4/80 and therefore be present in the sample. Strikingly, when looking at the expression of *Ly6c2*, *Cx3cr1* and *Ace*, it appears that some cells in the top right cluster express low levels of *Cx3cr1* and *Ace* but not *Ly6c2*, matching the description of patrolling monocytes while the left cluster and leukemic cells expresses *Ly6c2* (Figure 4.10A).

Next, the presence of certain neutrophil-specific genes in the most differentially expressed gene lists suggested that neutrophils were present in the samples. To understand whether neutrophils were among the myeloid cell clusters, I identified neutrophil specific genes involved in different processes of neutrophil differentiation and function. One process specific to neu-

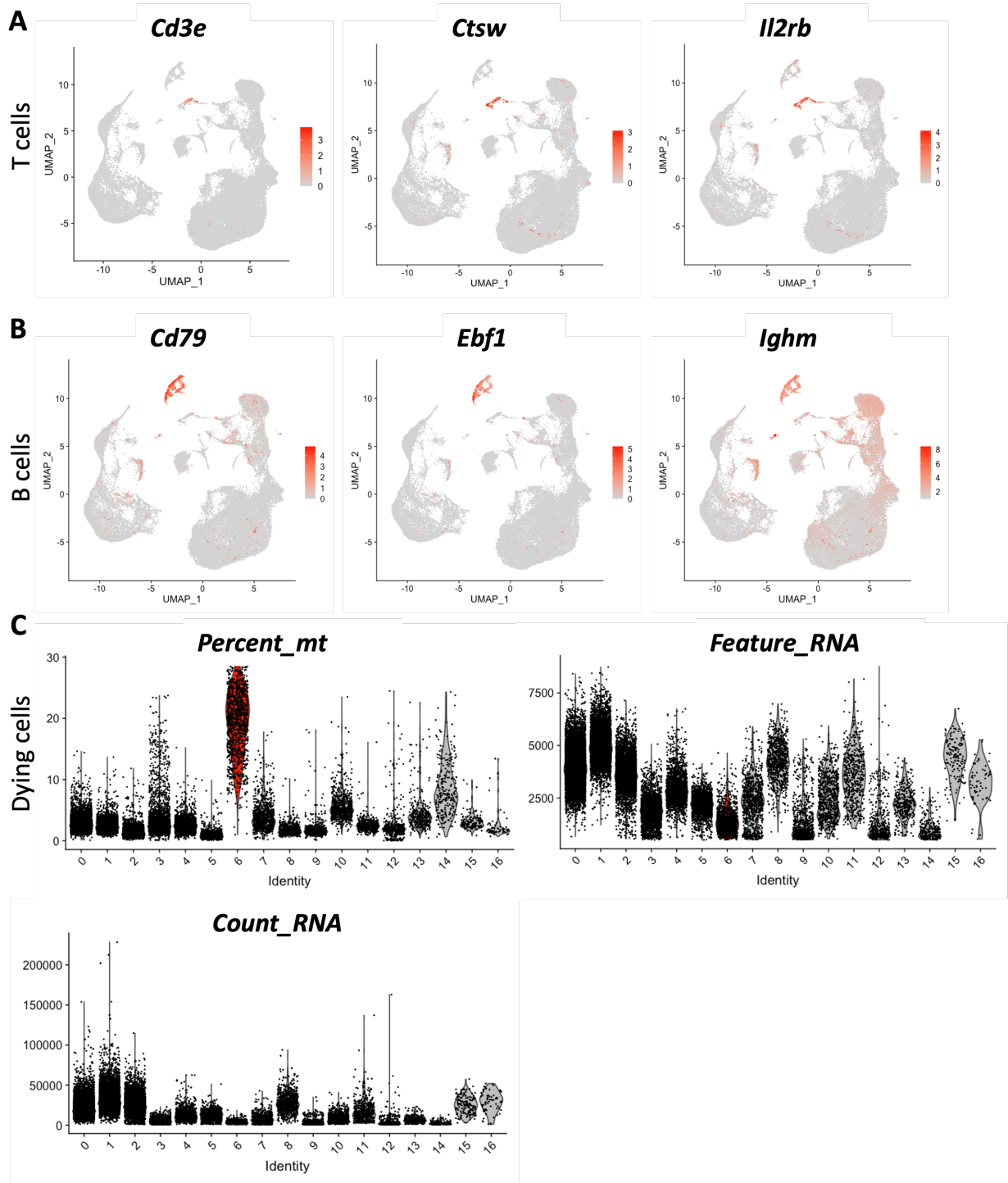


Figure 4.8: Identification of unwanted clusters in the scRNAseq analysis
UMAP representing the expression of genes used to identify (A) T cells and (B) B cells. (C) Violin plots of the percentage of mitochondrial genes, the number of genes detected and number of molecules detected within a cell for each cluster to exclude dying cells.

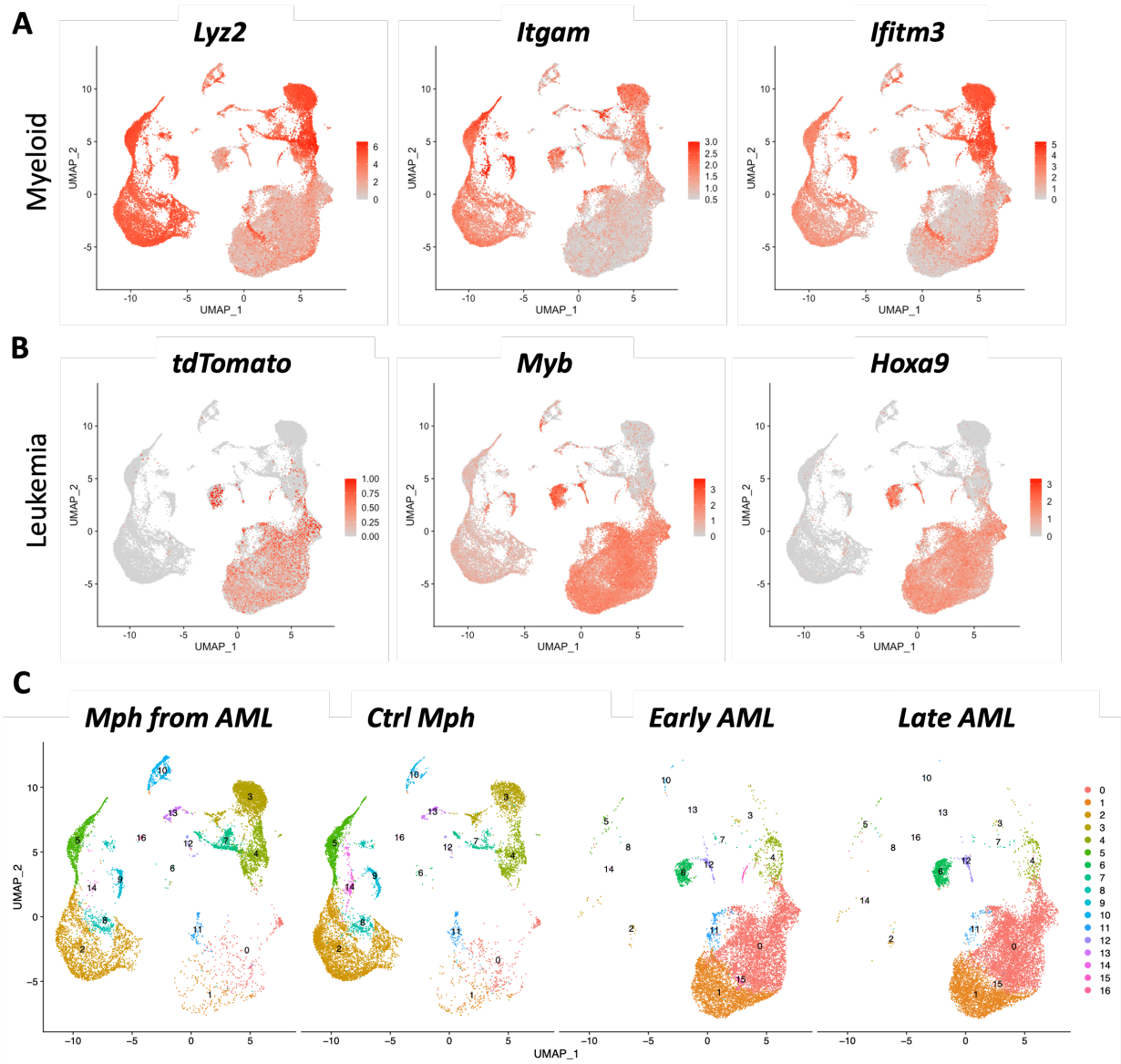


Figure 4.9: Identification of myeloid and leukemia clusters in the scRNAseq analysis
UMAP representing the expression of genes used to identify **(A)** myeloid cells and **(B)** leukemic cells. **(C)** *UMAP* split for each sample (macrophages from AML, control macrophages, early AML and late AML). Macrophage samples correlate with myeloid genes expression and AML samples correlate with leukemic genes. *Mph* = macrophage

trophils is the secretion of toxic granules. Among the genes important for neutrophils granules are *S100a9*, *Ngp* and *Camp*. Clearly, the left cluster was strongly expressing these genes and could therefore be classified as neutrophils (Figure 4.10B). Given that approximately 50% of the myeloid cells were present in this large cluster, I decided to further analyse this cluster and confirm this classification.

As mature neutrophils were eliminated during the sort based on their expression of Ly6G, I hypothesized that these cells were immature neutrophils. Since neutrophil maturation is characterised by neutrophil granule formation [243], it is possible to characterise the neutrophil differentiation stage through its granule formation. Indeed, neutrophils committed progenitors, myeloblasts, start secreting primary granules known as azurophilic granules. As differentiation occurs, these cells will be capable of secreting secondary (specific) granules, then tertiary (gelatinase) granules and finally secretory vesicles can be found only in mature neutrophils. Using the neutrophil granule formation genes lists generated to categorise neutrophils differentiation and maturation in the BM by Xie et al., [244], I analysed the neutrophils present in our sample (Appendix B).

Interestingly, all neutrophils expressed very low levels of azurophil granule genes and high levels of specific granule genes. However, based on the expression of gelatinase granule, secretory vesicles and cell proliferation genes these cells could be separated into two clusters, one, originally identified as cluster 2, expressed low levels of gelatinase granule genes, secretory vesicle genes and high levels of proliferation genes while the second cluster, identified as cluster 5, expressed higher levels of gelatinase granule genes and secretory vesicle genes and was not proliferative. Since all clusters did not express azurophil granules, the cells were necessarily more differentiated than promyelocytes. Based on these results, cluster 2 could be identified as myelocytes, known to proliferate and produce secretory granules while cluster 5 would be band cells, which are still capable of producing all forms of granules but no longer proliferate.

The presence of these two neutrophil progenitor populations can be explained by their known expression of CD11b and lack of expression of Ly6G, which appears only in terminally differentiated neutrophils [245, 246]. Hence, these cells were not excluded during the sort. However, given that my primary focus remains macrophages, I decided not to pursue further the analysis of these populations and instead characterise the macrophages present in the samples.

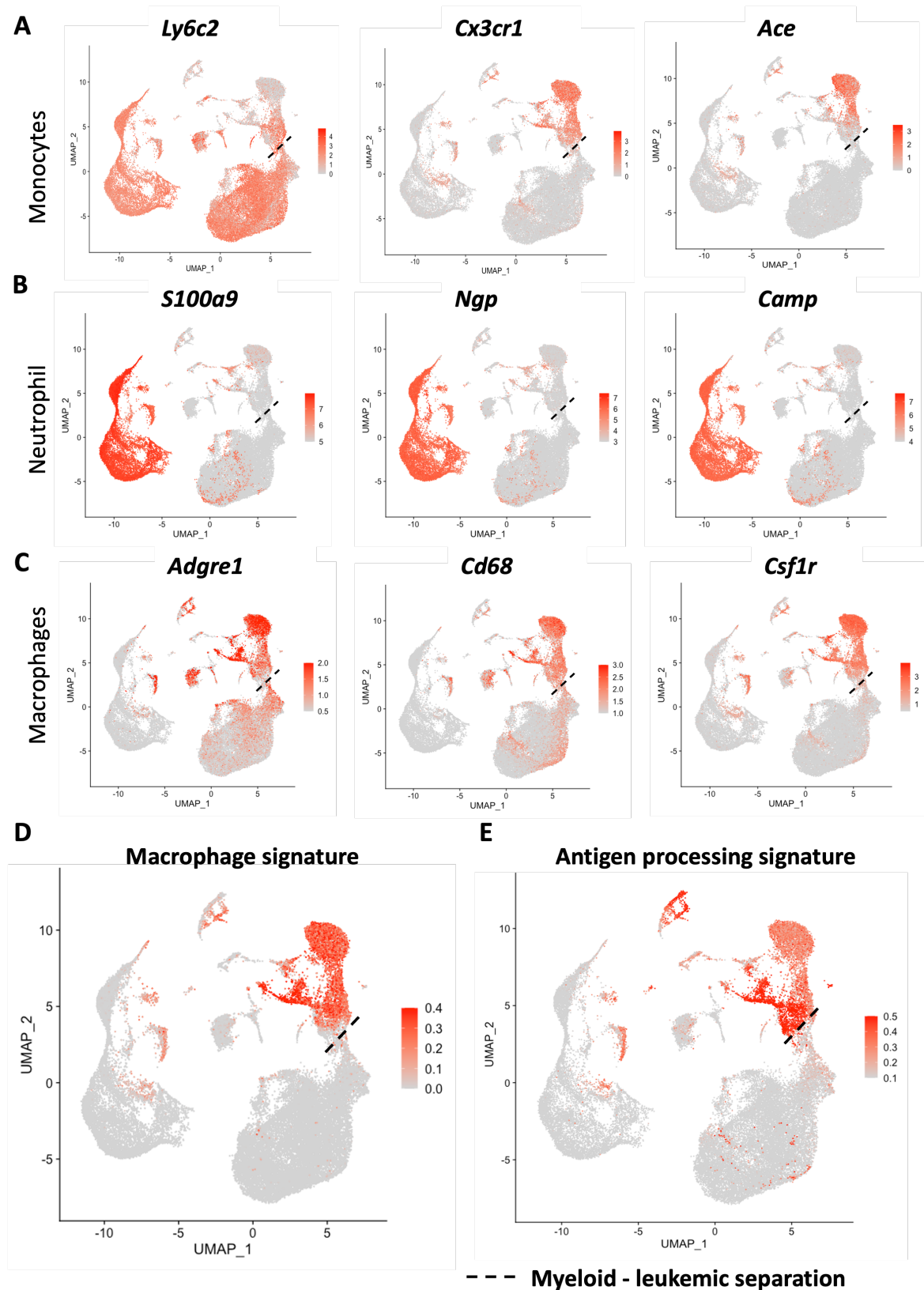


Figure 4.10: Identification of the different myeloid populations in the scRNAseq analysis
The separation between myeloid cells and AML cells was marked as a dotted line based on the previous results. UMAP representing the expression of genes used to identify (A) monocytes, (B) neutrophils and (C) macrophages. (D) Normalised expression of the core bone marrow macrophage gene signature (the list of genes can be found in Appendix C) (E) Normalised expression of antigen processing genes.

First, to identify where macrophages were located in our sample, I evaluated the expression of common macrophage genes (Figure 4.10C). Given the strong expression of *Adgre1*, *Cd68* and *Csf1r* in the top right corner I could conclude that the cells located in these clusters were most likely macrophages. Interestingly, this signal correlated with the signal for patrolling monocytes. Hence, I needed to confirm that these cells were macrophages and not monocytes. To overcome the lack of a known gene signature for bone marrow macrophage, I used the core tissue macrophage gene signature developed by E. Gautier et al., [199] , and included genes common to at least 3 different organs among macrophages from the peritoneum, lung, microglia and spleen. In this list, I only included the genes present in our sample as bone marrow macrophages were described to express few genes from this core macrophage gene signature. Altogether, these genes were primarily expressed by the top right cluster and therefore I could conclude that these cells were macrophages (Figure 4.10D). The details of the genes used for this analysis can be found in Appendix C. In addition, since macrophages are classified as Antigen-Presenting Cells (APC), these clusters should express antigen presenting genes. To validate that these cells are [Antigen presenting cells \(APCs\)](#), I evaluated the expression of antigen processing and presenting genes, classified as such in the gene ontology database. Strikingly, the clusters with the macrophage gene signature were also the clusters expressing antigen processing genes (Figure 4.10E). Hence, these results validated the fact that the top right clusters including clusters 3, 4 and 7 were macrophages, while the left cluster were neutrophils and the bottom cluster was composed of AML cells. Altogether, these results allowed me to classify the cells present in my sample in 6 major categories: T cells, B cells, neutrophils, macrophages and AML cells (Figure 4.11).

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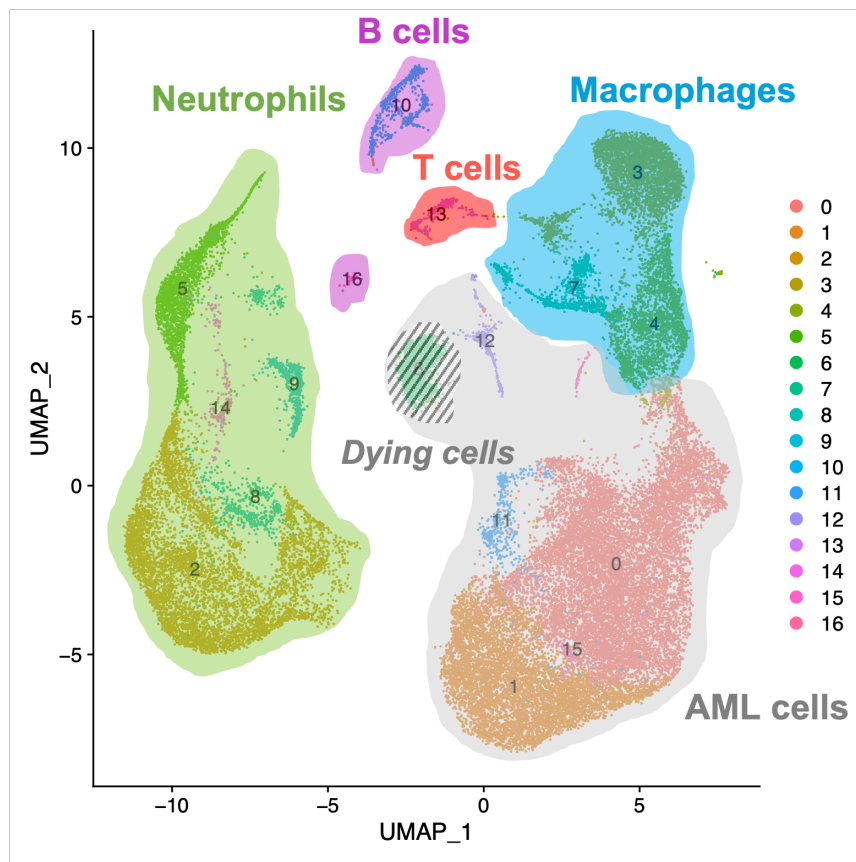


Figure 4.11: Annotated UMAP with the name of the different immune cell populations

4.4.1 Integration of the BM scRNAseq analysis with lung analysis of macrophages from control and tumour lungs

Next, to validate the identification of the different cell populations and compare their transcriptional signature, we integrated our single cell RNA sequencing dataset with a recently published macrophage dataset from healthy lung and lung tumours (Figure 4.12A). In this study, monocytes/macrophages were sorted based on the combination of markers: CD45+ Lin- (CD3/CD19/ NKP46) CD11b+ and Ly6G-. As such, this gating includes monocytes and macrophages while excluding neutrophils. Using Immgen, they identified 4 major cell types in this dataset: neutrophils, B cells, T cells and monocytes/macrophages. During the integration of both datasets, we were able to retain the lung study cluster labels. To simplify the nomenclature, from here on, our experiment dataset will be referred to as, bone marrow, while the dataset from this additional study will be referred to as, lung.

We performed clustering using UMAP dimensionality reduction and identified 21 clusters (Figure 4.12B). Strikingly, when we split the UMAP according to the experiment the cells came from, a large overlap is noticed between both samples. To identify the different clusters present on this UMAP, we visualised the labels given by the M. Casanova-Acebes et al. (Figure 4.13A). Combining their labels with the reference immune cell population database, Immgen, we were able to identify neutrophils, monocytes/macrophages/ B cells and T cells (Figure 4.13B). Surprisingly, when measuring the percentage of each population in each sample, the fraction of neutrophils was identical in both samples however the fraction of B and T cells were higher in the lung sample compared to the bone marrow (Figure 4.14A-C). Interestingly, while the fraction of neutrophils was similar in both experiments, only a small fraction of these cells overlapped on the UMAP between the two samples (Figure 4.14A-B). Using the Immgen database, the neutrophils from the bone marrow were identified as *Ltf* high neutrophils while the neutrophils from the lung were identified as *Rtnlg* high. As expected, when looking at the AML cells, these cells were found to cluster independently of any other cells from the lung suggesting that these cells are monocyte-progenitors and cluster separately from monocytes. However, most of the non-malignant monocytes/macrophage clusters overlapped (Figure 4.14A-B), consistent with these cells being indeed macrophages.

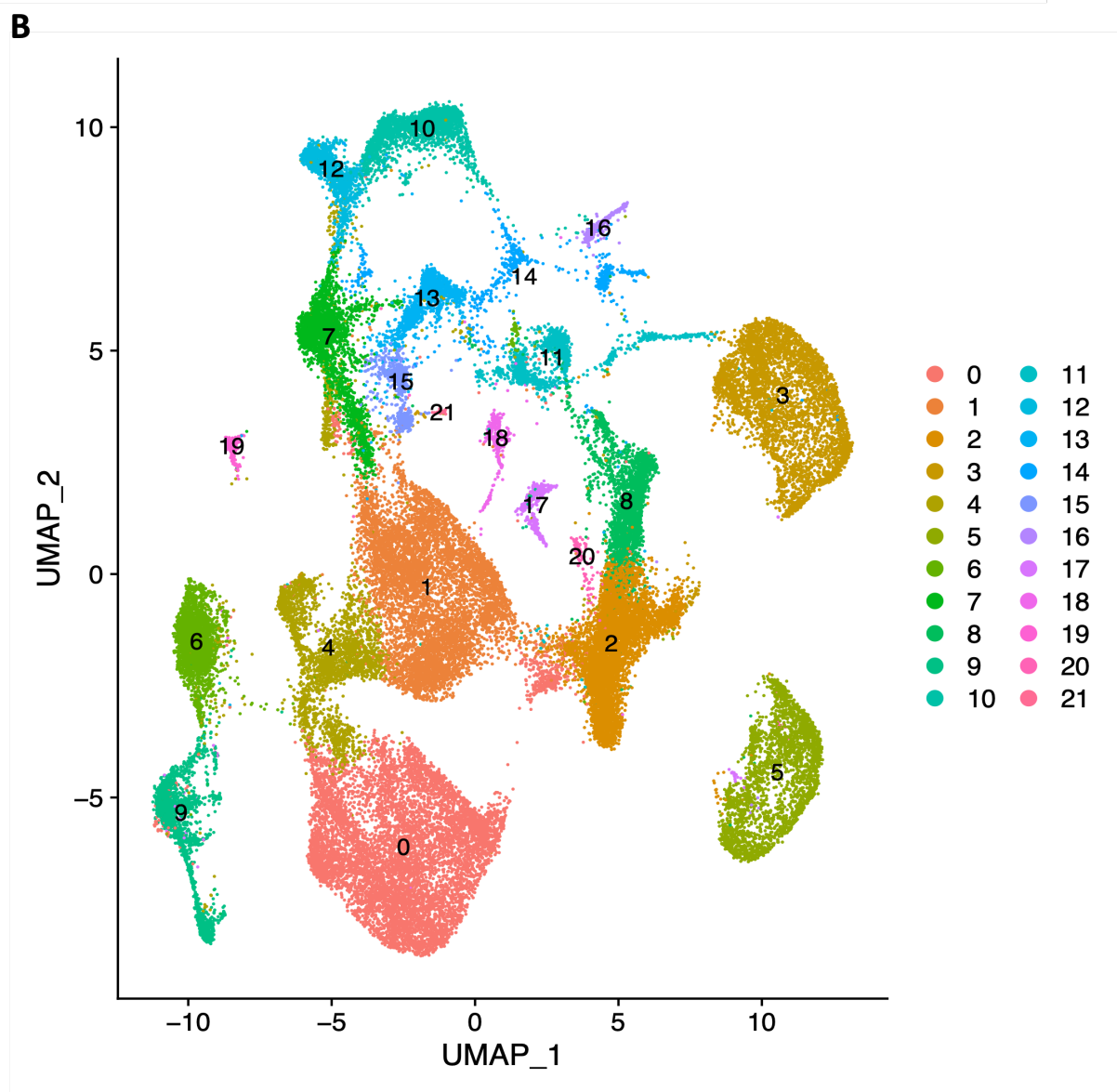
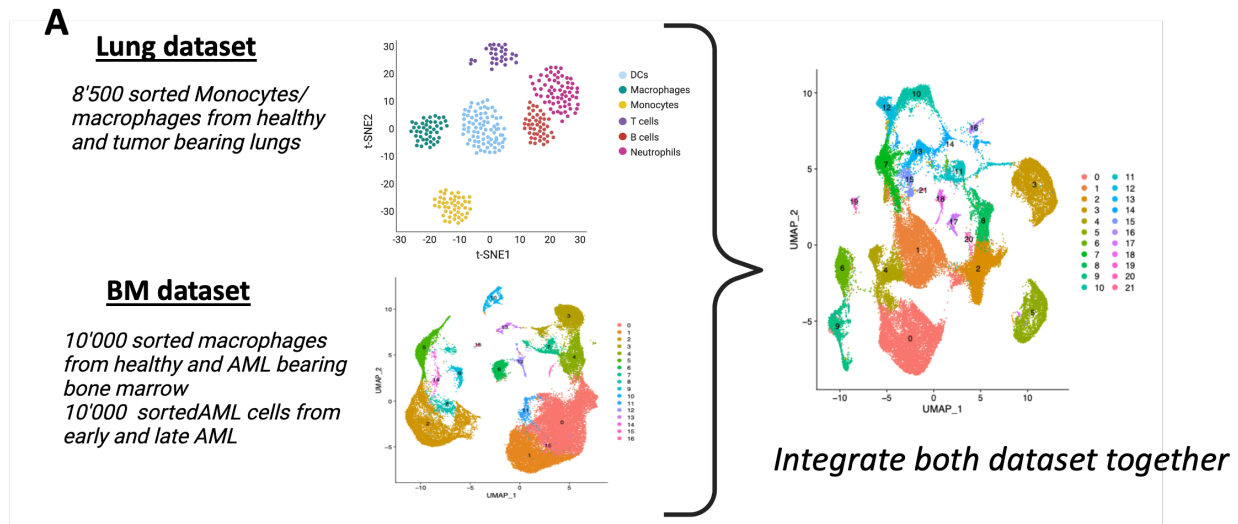


Figure 4.12: Integration of the BM scRNAseq analysis with lung analysis of macrophages from control and tumour lungs [1]

(A) Schematic of the integration of the lung dataset with our BM dataset. The lung dataset contains 8500 monocytes/macrophages sorted from healthy and tumour bearing lungs. (B) UMAP visualisation of all cells contained in the integrated analysis, coloured by clusters.

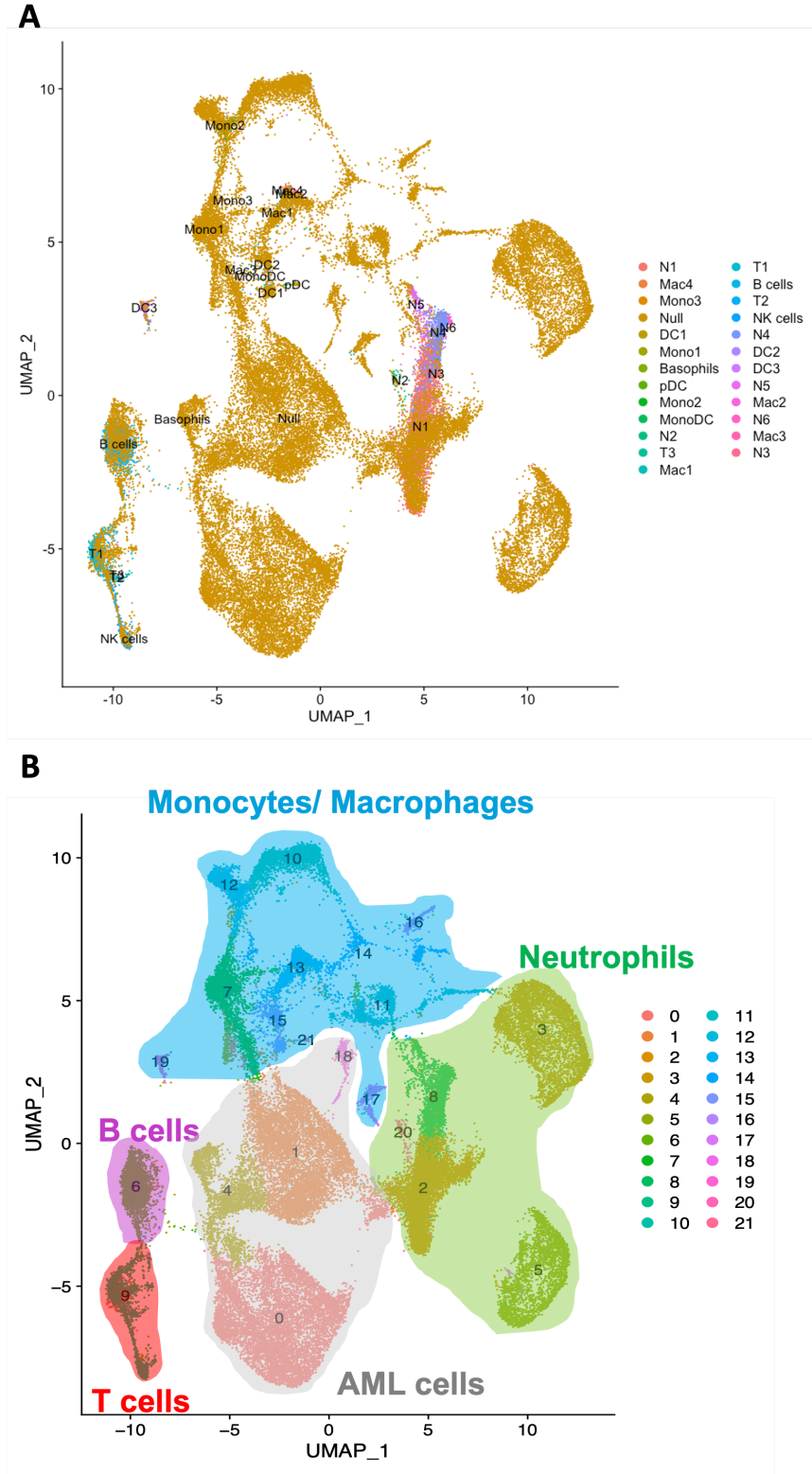


Figure 4.13: Comparison of the identification of immune cell populations in the lung and BM dataset

(A) Labelling performed by M. Casanova-Acebes et al., [1] overlayed on the integrated UMAP. Brown cells represent BM cells (B) Our own labelling performed in the previous analysis, overlayed on the integrated UMAP. Similar cell populations and identification was observed in both datasets. Mac = macrophage, pDC = plasmacytoid DC, Mono = monocyte, MonoDC = monocyte/DC, N = neutrophil.

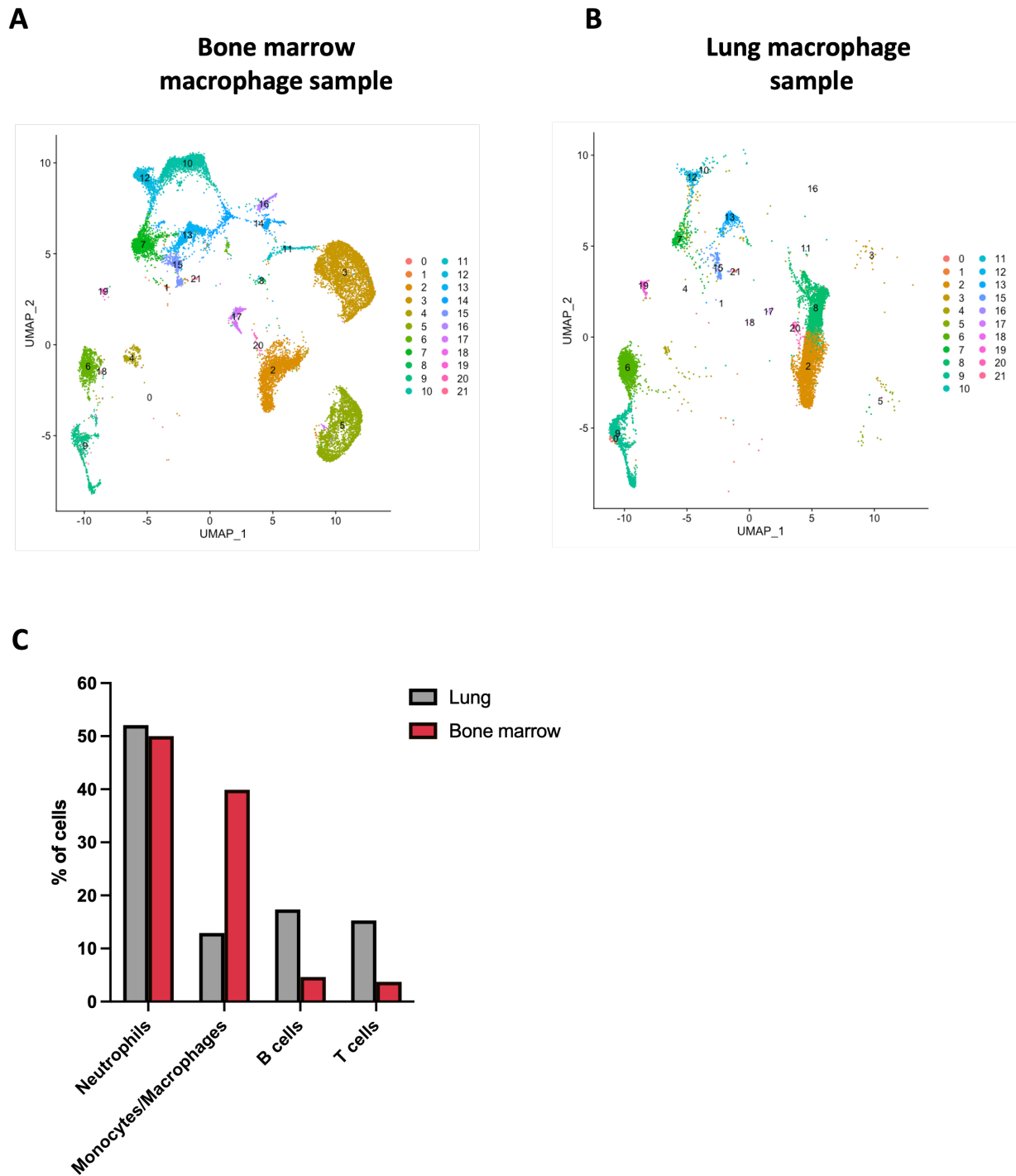


Figure 4.14: Comparison of the composition of the immune cell populations in BM and lung samples

The integrated UMAP was split into (A) bone marrow macrophage samples and (B) lung macrophage samples. The cells identified as monocytes/macrophages overlap in both samples and neutrophils primarily cluster separately. (C) Percentage of the different immune cell populations composing the bone marrow samples (red) and lung samples (grey).

4.4.2 Advanced analysis of control and AML BM macrophages

Next, to understand the different gene signatures present in bone marrow macrophage populations, and gain insight on their function, we performed a new unsupervised clustering of bone marrow cells identified as macrophages (Figure 4.15A). This time, the AML samples were not included as they would create more divergence within the cells and reduce the difference between macrophage populations

In the new UMAP, the cells were grouped in 6 main clusters. When looking at the 10 macrophage core signature genes most expressed by our cells, clear differences appeared between the 6 clusters. While cluster 0-2 expressed most genes highly, the gene expression among cluster 3-5 was much more sparse, suggesting transcriptional differences among these cells (Figure 4.15B). To highlight the differences between these macrophage clusters, we decided to perform a gene ontology analysis, with the help of a bioinformatician. Since we didn't want the AML modifications to interfere with the differences noticed between macrophage clusters, we only analysed the macrophages from the control sample. First, we analysed the uniquely expressed biological processes for each cluster. At a first glance, most processes were associated with either translation and transcription or response to different stimuli suggesting these cells are simply in a different state but do not have major functional differences. I selected the four most representative biological processes from the list of significantly different biological processes and showed the percentage of the cluster involved in this process as well as the adjusted p-value (Figure 4.16). The whole list of uniquely expressed biological processes with the associated level of expression can be found in Appendix D. Hence, I hypothesized that the lack of major functional differences was due to most functional processes being common to several different clusters and therefore would not show up in this analysis.

To test this hypothesis, we repeated the gene ontology analysis and identified all biological processes common to at least three different clusters (Figure 4.17). Surprisingly, there were no biological processes common to all six clusters meaning that no function could be common to all macrophages in the bone marrow. However, several biological processes involved in specific macrophage function were found repeatedly in this analysis. These macrophage functions were tissue remodelling and ion processing, phagocytosis and antigen processing and presentation, inflammation and efferocytosis. Strikingly, I found that all biological processes associated with each function were expressed by the same clusters. As such, clusters 0, 2 and 5 were identified

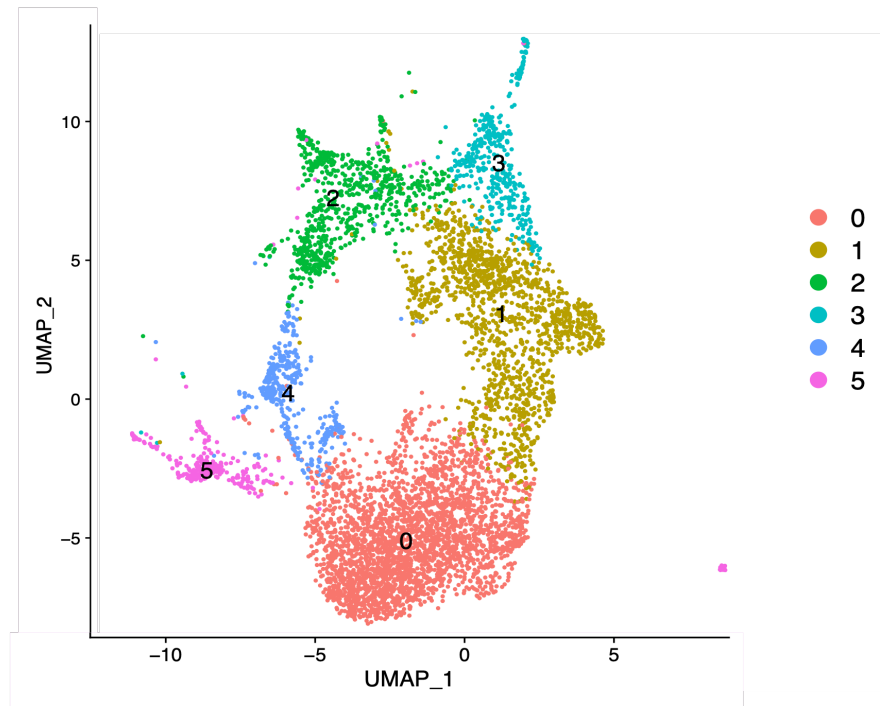
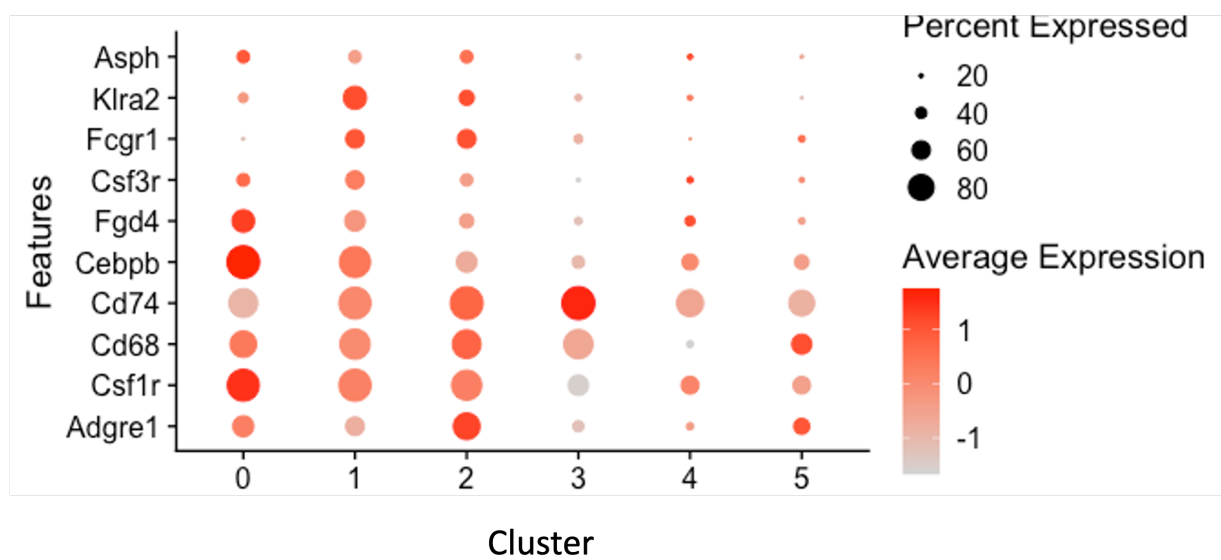
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Figure 4.15: Heterogeneity within the BM macrophage clusters

(A) Higher resolution clustering of cells classified as macrophages in the previous analysis.

(B) Dot plot of the most expressed genes from the core BM macrophage gene signature list.

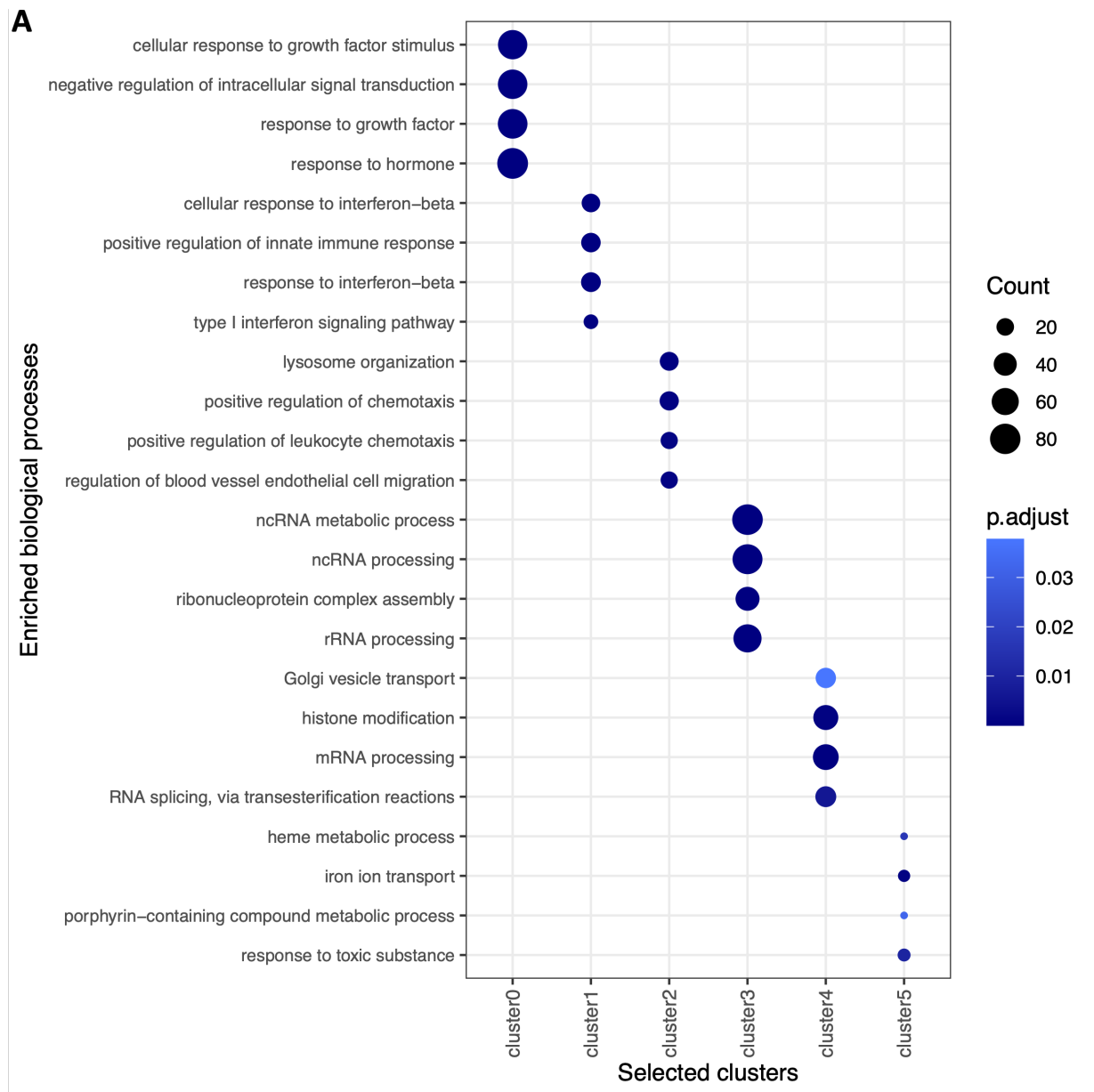


Figure 4.16: Biological processes specific to each macrophage cluster
Gene ontology analysis reveals the biological process uniquely expressed in each macrophage cluster. Top 4 processes for each clusters are plotted here. The full list of biological processes uniquely expressed by each cluster can be found in Appendix D.

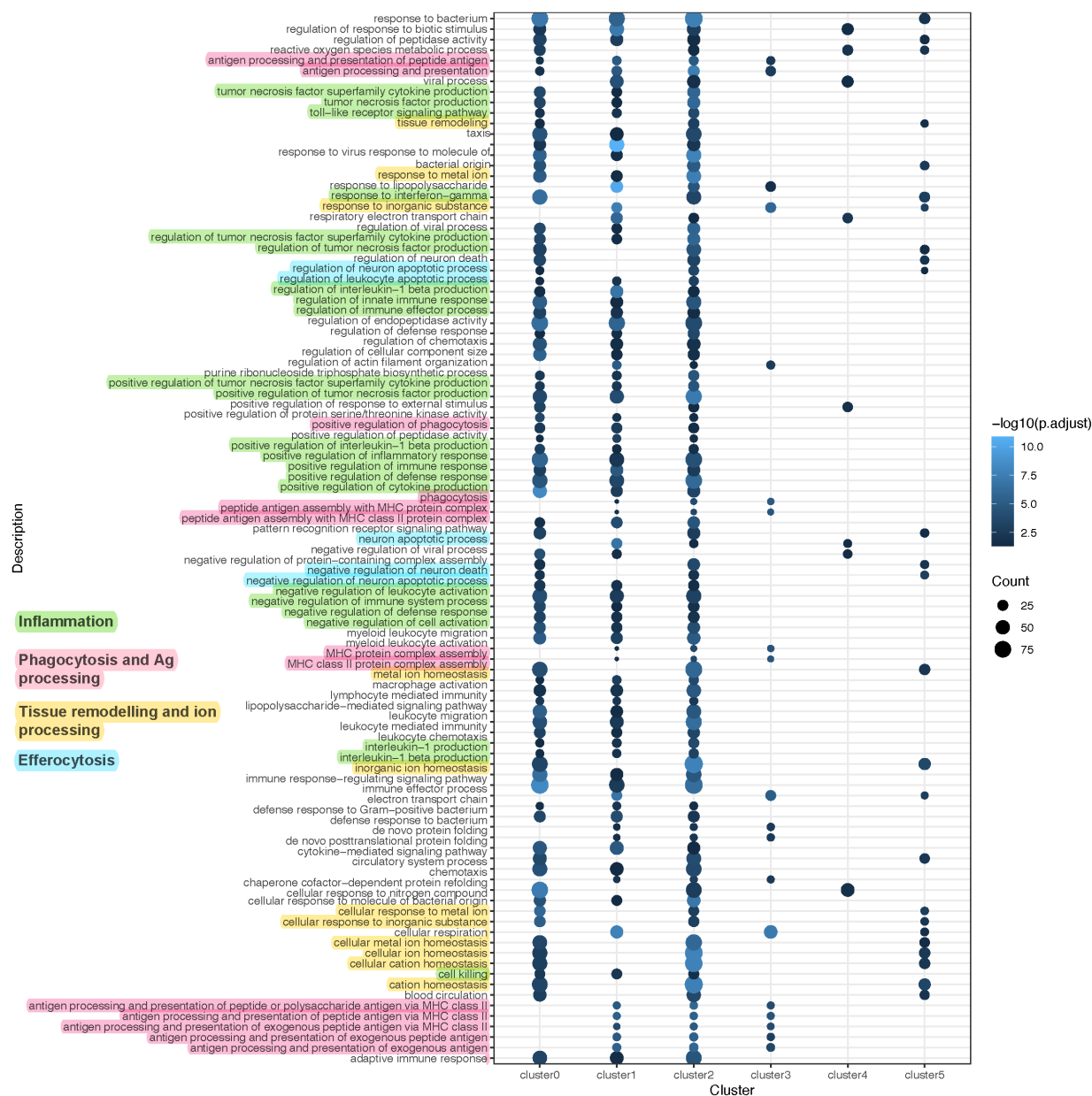


Figure 4.17: Biological processes common to three different macrophage clusters

Gene ontology analysis reveals the biological process common to at least three different macrophage clusters. Biological processes related to tissue remodelling and ion processing were highlighted in yellow. Biological processes related to phagocytosis and antigen processing and presentation were highlighted in pink. Biological processes related to inflammation were highlighted in green. Biological processes related to efferocytosis were highlighted in green.

	Biological process	Specifics/ Conditional	Clusters involved
Tissue homeostasis	Ion processing	Metal ion homeostasis	0, 2, 5
		Tissue remodelling	0, 2, 5
	Efferocytosis	Regulation of apoptotic processes	0,2, 5
Regulation of the immune response	Phagocytosis and antigen presentation	With MHC II involvement	1, 2, 3
		With phagocytosis	0, 1, 2
	Inflammation	Cytokine production (TNF and IL-1b)	0, 1, 2
		Response to IFNg	1, 2,3

Figure 4.18: Summary of the biological processes performed by the different macrophage clusters

to be involved in tissue remodelling, ion processing and efferocytosis, while clusters 1, 2 and 3 were involved in antigen processing and presentation via MHCII, inflammation and response to IFNg (Figure 4.18). These processes could be regrouped under two main roles: structural processes involved in tissue homeostasis and inflammatory processes involved in the regulation of the immune response. It is worth noting that cluster 2 was the only cluster involved in all processes.

Surprisingly, these two roles are similar to the roles of IMMs and HSMs identified by flow cytometry. Hence, I next decided to evaluate whether we were able to identify these populations in the scRNAseq data. By flow cytometry, IMMs were identified based on their expression of F4/80, SIRP α and PDL1. Since all F4/80+ cells were sequenced, I only needed to know which cells expressed SIRP α and PDL1 at the protein level to identify IMMs in the RNAseq data. Prior to the sequencing, I labelled cells expressing PDL1 and SIRP α with two antibodies conjugated to specific RNA sequences. Using the known conjugated sequences, we were able to identify which cells expressed these proteins. Surprisingly, all cells present in our macrophage analysis expressed both SIRP α and PDL1 (Figure 4.19A). Regarding SIRP α , this could be expected as, by flow cytometry, I noticed that all macrophages expressed SIRP α , however, IMMs

expressed it at much higher level. In the sequencing data, the expression levels were remarkably close between all cluster with cluster 2 having the highest level. As for PDL1, cluster 0 and 2 had the highest expression. Given the high expression at the protein level, I hypothesized that the CITE-seq antibodies might not be a good quantitative measurement of the protein expression but rather more a qualitative tool to know if cells express or not the protein. Hence to correlate the protein results, I decided to check the gene expression of these proteins. Based on the rationale that IMMs also had the highest expression of F4/80 by flow cytometry, I also included *Adgre1* (F4/80). As expected, *Adgre1* and *SIRPα* were expressed by all clusters with cluster 2 and 5 having the highest expression of both genes (Figure 4.19B). Regarding Cd274, only cluster 0 and 2 expressed this gene. Altogether these results indicated that cluster 2 was the most likely cluster to be IMM.

To uncover what function is associated with this cluster, I plotted a heatmap with the most differently expressed genes for each cluster (Figure 4.20). Cluster 0 could be classified as classical monocytes/macrophages as it highly expressed a number of genes associated with classical monocytes such as *Pglyrp1*, *Eno3* and *Nr4a1*. These cells were found to upregulate genes involved in the response to growth factors. As such I hypothesise that these cells are recruited to the bone marrow and are differentiating towards macrophages in response to local growth factors. Cluster 1 expressed high levels of *Ly6c2* and *CCR2* and could be classified as inflammatory monocytes/macrophages. In addition, a number of genes were found to be involved in the response to *Ifnb*. Interestingly, it has been shown that type 1 interferons impede Ly6C+ monocytes differentiation towards macrophages [247]. Hence, I hypothesize that these cells might be blocked in an in-between state, between an inflammatory monocyte and a macrophage phenotype. Regarding the biological processes, these cells were found to express genes involved in the regulation of the immune response, both in terms of phagocytosis and antigen processing and in terms of inflammation and cytokine production. As such, they can be seen as a highly inflammatory monocyte/macrophage cell population.

Cluster 2 was found to express the efferocytic genes, *Axl*, *Nr1h3* and *Nr1h3*. Efferocytosis is known to be hijacked by cancer cells and this could explain how IMMs are capable of helping AML growth. The gene ontology analysis revealed an enrichment for taxis and locomotion pathways, which suggest these cells have a monocytic origin. This cluster also expressed *Mrc1*, more commonly known as Cd206. Cd206 is expressed on anti-inflammatory macrophages and is known to be associated with a poor outcome in human AML. In addition, cluster 2 was also

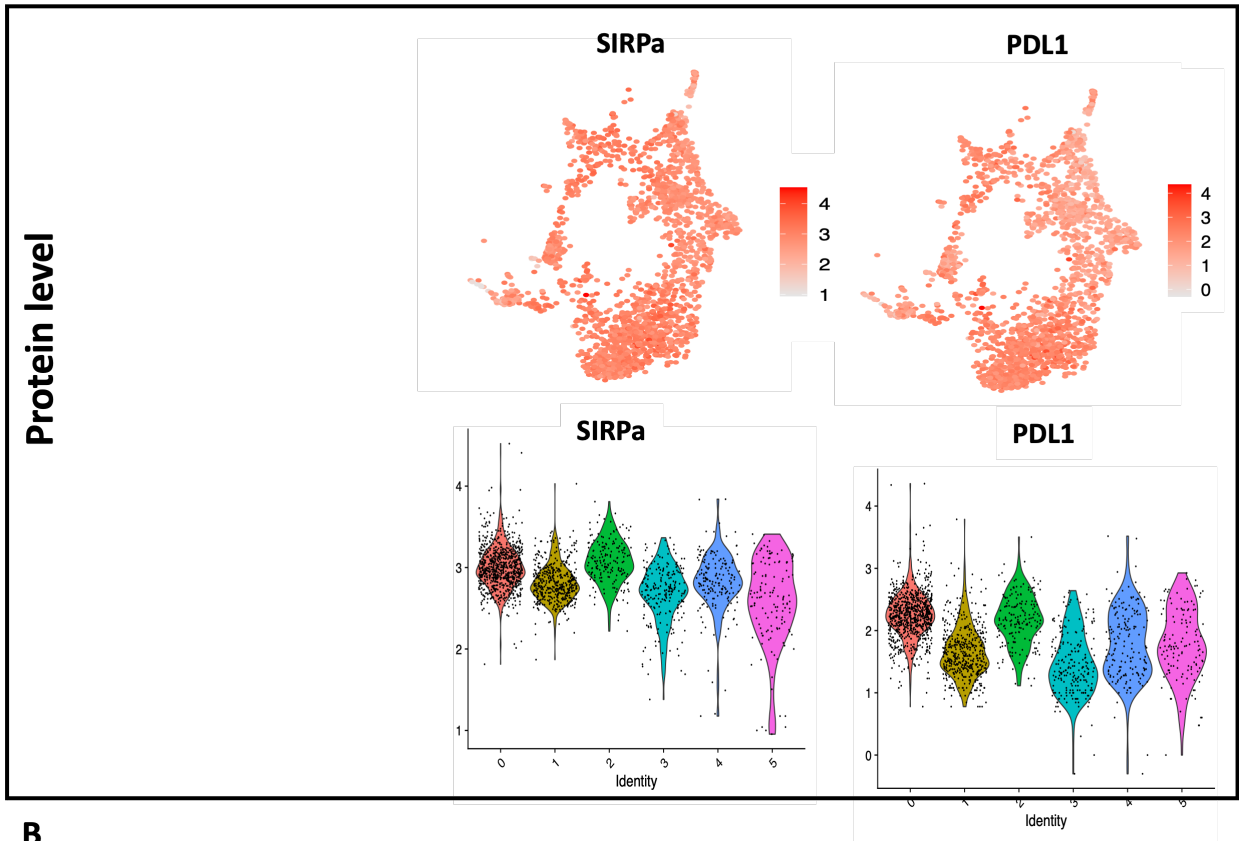
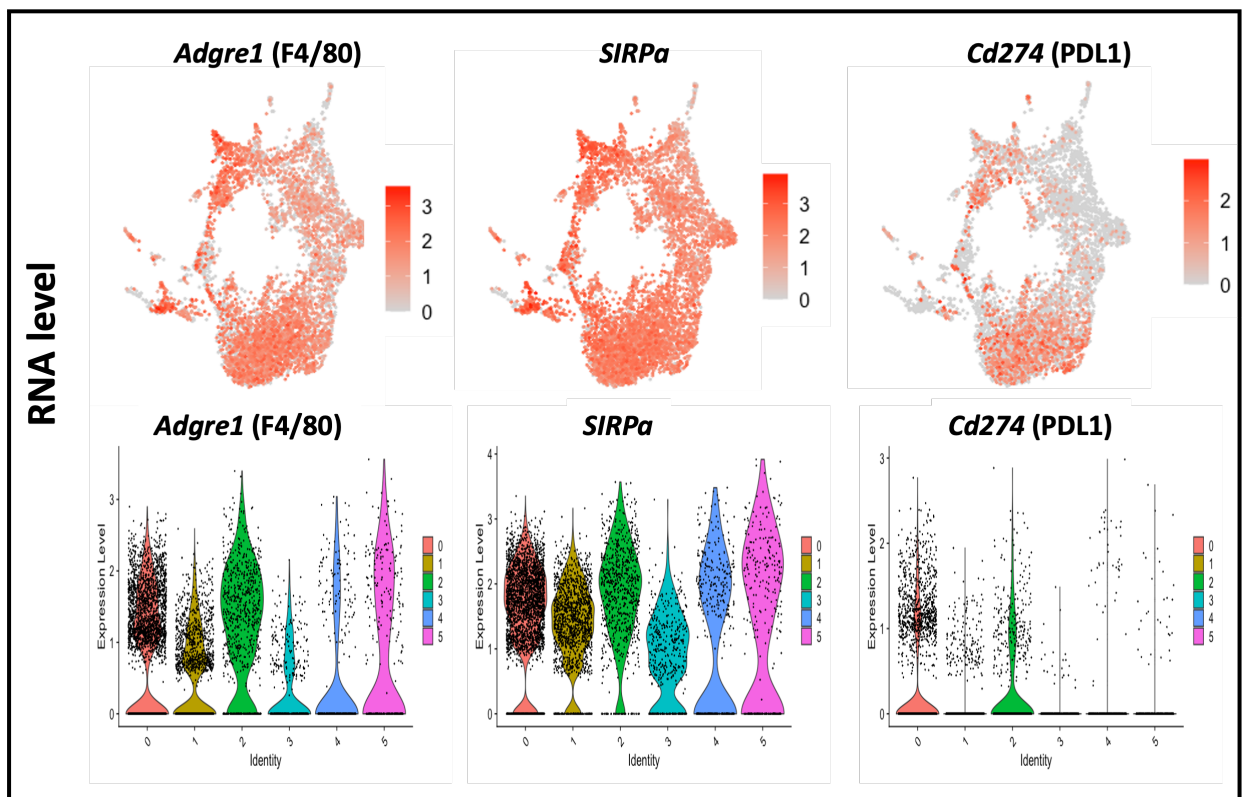
A**B**

Figure 4.19: Cluster 2 in the scRNAseq resembles IMMs
Feature plot and violin plot of the IMMs markers, SIRPα and Cd274 (PDL1) at the protein level using CITE-seq labelling technology (A) and at the RNA level (B). Expression of Adgre1 (F4/80) was also evaluated at the RNA level. Cluster 2 was found to be the only cluster expressing all three markers, at the highest levels.

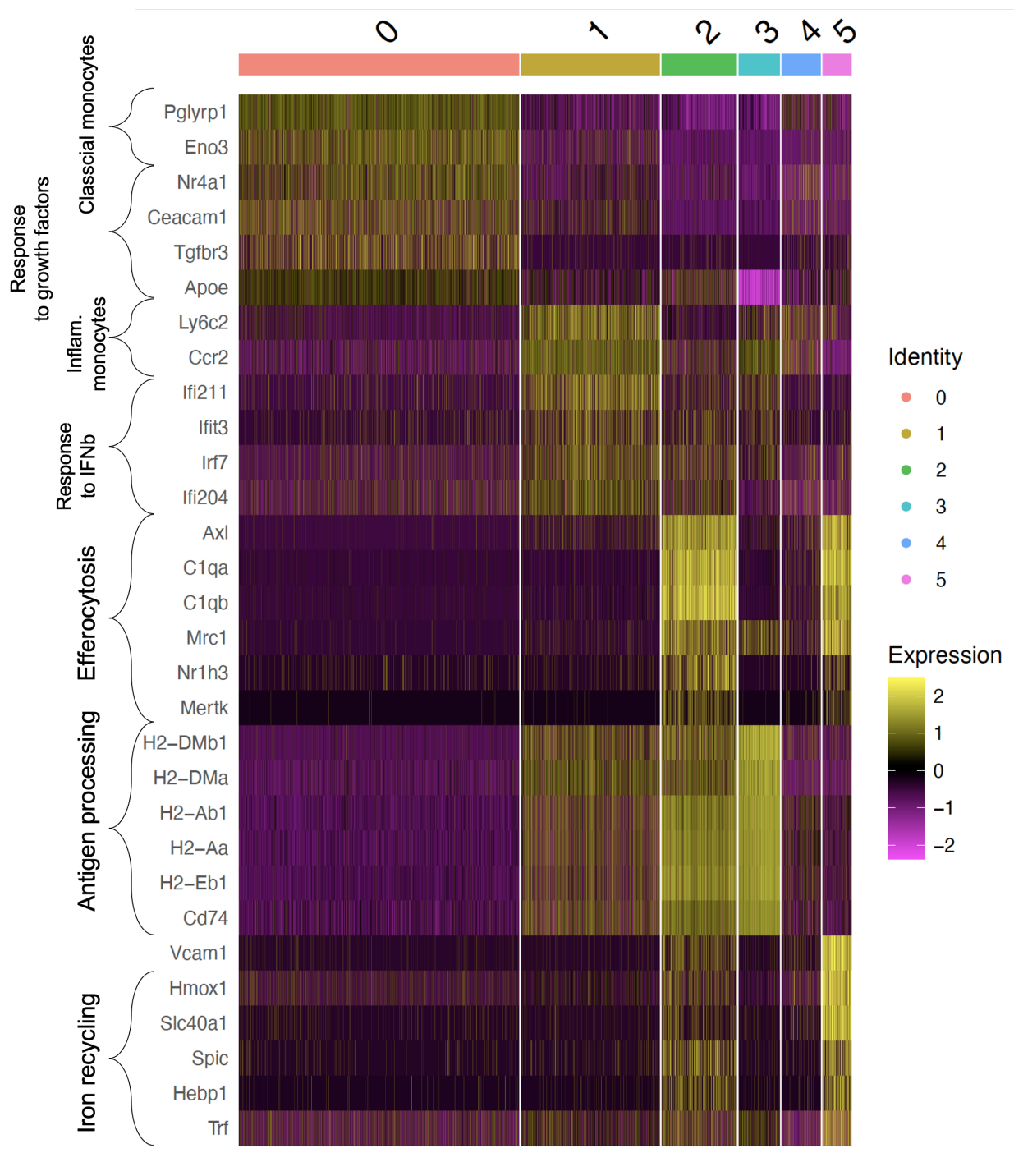


Figure 4.20: Heat maps of selected significantly differentially expressed genes for each cluster

found to express genes involved in the antigen processing and iron recycling. As such these cells are involved in both the regulation of the tissue homeostasis and the immune response. Altogether these cells could be classified as anti-inflammatory macrophages with various mechanism pointing towards the inhibition of the immune response.

Cluster 3 was found to primarily express genes related to the MHCII group and was involved in the antigen processing and presentation. Together, these results highlight the fact that these cells are important antigen-presenting cells and as such are involved in the regulation of the immune response. Cluster 4 was found to express very few specific genes and seemed to have an unspecific macrophage phenotype and I decided to not pursue further the analysis of this cluster.

Cluster 5 was found to express a number of genes associated with iron recycling. In addition, these cells expressed *Spic* and *Vcam1*. Macrophages expressing both *Spic* and *Vcam1* have previously been described as iron-recycling macrophages [91] and correlate with erythroblastic island macrophages [92]. These cells also expressed strongly the efferocytic genes suggesting there might be a link between efferocytosis and iron recycling in the bone marrow.

Surprisingly, the gene expression profile between cluster 2 and 5 was very similar, however cluster 2 had reduced expression of iron-recycling genes compared to cluster 5 and expressed antigen processing genes which were not expressed by cluster 5. Hence, these results suggest that cluster 2 is important for both tissue homeostasis and the regulation of the immune response and as such could be seen as a master regulator of the environment.

Next, I questioned whether the growth of AML affected macrophages at the transcriptional level. To evaluate whether certain macrophage clusters were enriched in the AML microenvironment, I quantified the proportion of macrophages from control or AML mice for each cluster (Figure 4.21A-B). Only cluster 3, associated with antigen processing and presentation was enriched in the control condition compared to the AML condition. All other clusters were more present in the AML condition, in particular cluster 2 was the most enriched in the AML condition, with macrophages from the AML condition composing approx. 75% of this cluster.

Given the importance of cluster 2 in all the biological processes identified previously and its increased frequency in the AML condition, I decided to explore what genes were significantly differentially expressed between both conditions in this cluster. After analysing these genes in

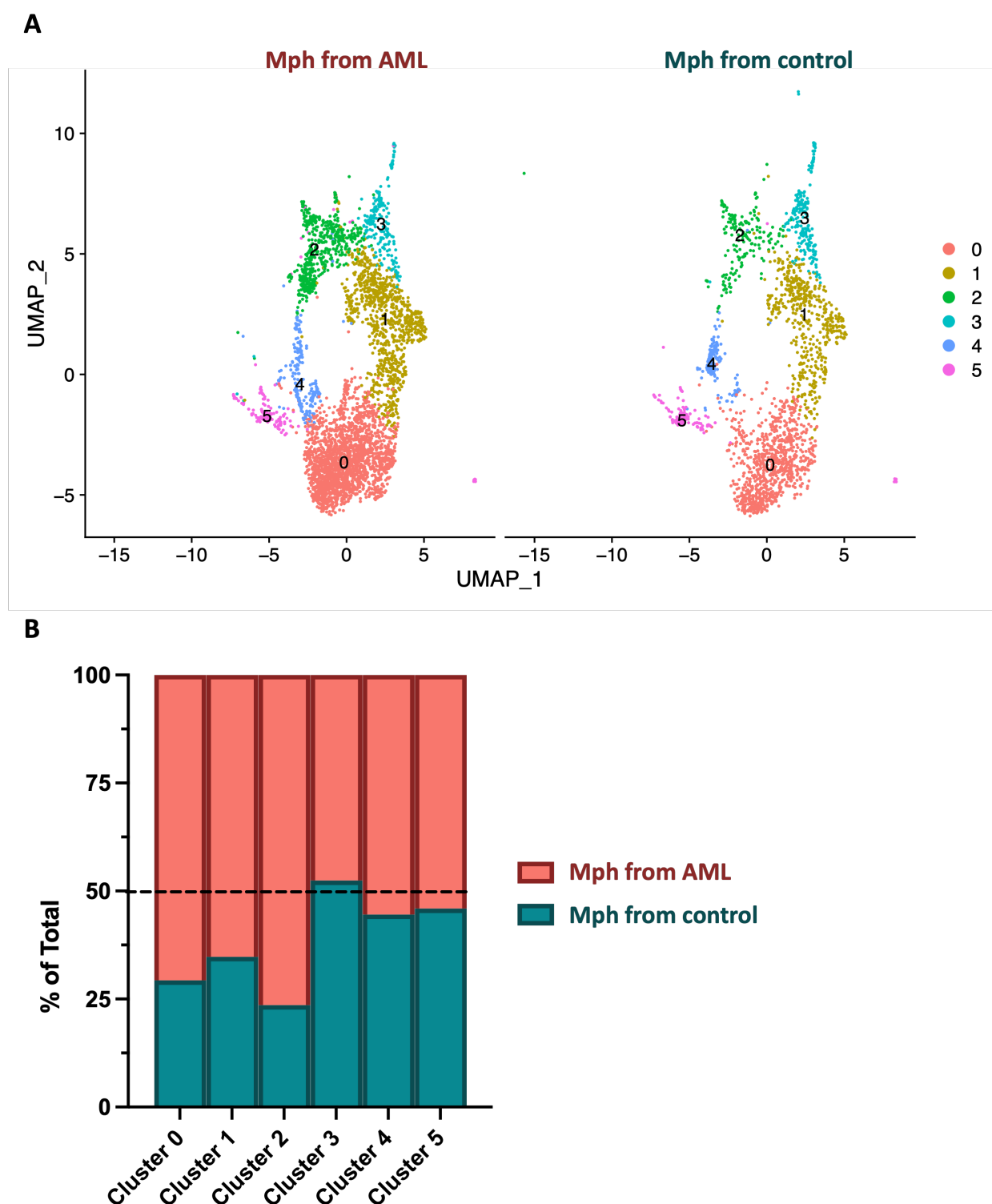


Figure 4.21: Enrichment of cluster 2 in the AML sample

(A) The higher resolution UMAP of macrophages was split into bone marrow macrophage in the AML sample and bone marrow macrophage in the control sample. (B) Proportional stacked bar graph showing the fraction of healthy macrophages and macrophages from an AML mouse composing each cluster. Cluster 2 is the most enriched in the AML sample. Mph = macrophage.

EnrichR I found that most genes were involved in the Il-1 response, regulating the inflammatory response (Figure 4.22A). The quantification of the expression level of Il-1b confirmed these results. In addition, Cd274, Il10 and Tnfa were increased in the AML condition compared to control (Figure 4.22B). While these changes suggest that AML are modifying macrophages at the transcriptional level, further investigation and confirmation of these differences by qPCR is necessary before any conclusion can be made.

A

cellular response to interleukin-1 (GO:0071347)	$p = 0.000002655$
cytokine-mediated signaling pathway (GO:0019221)	$p = 0.000003138$
regulation of inflammatory response (GO:0050727)	$p = 0.00001359$
lipopolysaccharide-mediated signaling pathway (GO:0031663)	$p = 0.00001727$
antigen processing and presentation of exogenous peptide antigen via MHC class I (GO:0042590)	$p = 0.00004654$
interleukin-1-mediated signaling pathway (GO:0070498)	$p = 0.00009655$
innate immune response (GO:0045087)	$p = 0.0001147$
positive regulation of cytokine production (GO:0001819)	$p = 0.0002015$
cellular response to lectin (GO:1990858)	$p = 0.0002104$
stimulatory C-type lectin receptor signaling pathway (GO:0002223)	$p = 0.0002104$

B

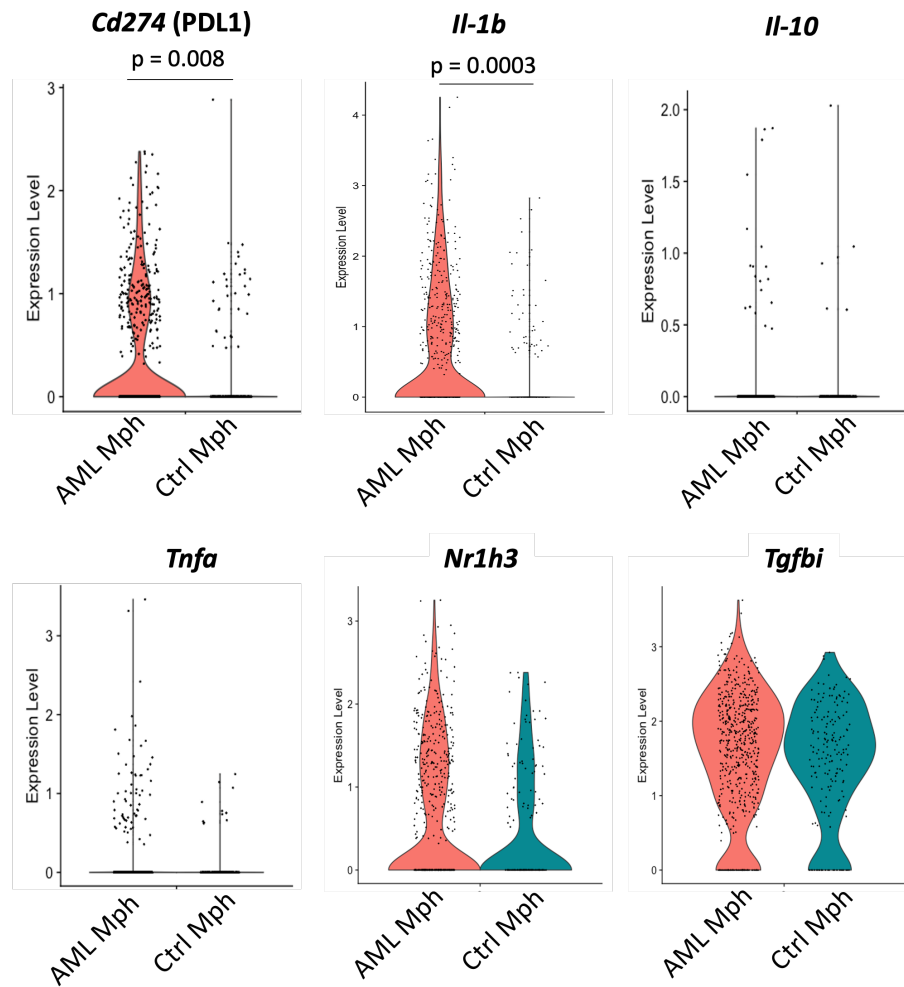


Figure 4.22 (*previous page*): Transcriptional changes in macrophages from cluster 2 with AML **(A)** *Gene Ontology analysis of biological processes enriched in cluster 2 macrophages from AML samples compared with controls cluster 2 macrophages. p-value corresponds to adjusted p-value. The analysis was performed using the EnrichR interface.* **(B)** *Violin plot of selected genes, differentially expressed in cluster 2 macrophages from AML samples compared with controls cluster 2 macrophages. Mph = macrophage.*

4.4.3 Comparison of macrophage transcriptional signature in lung and bone marrow of healthy and malignant tissue

Next, to uncover whether the transcriptional changes identified in the cluster 2 with AML were also happening in macrophages from lung tumours, I took advantage of the previously integrated lung samples with our bone marrow samples. I subsequently focused my analysis on the cells identified as monocytes/macrophages and performed a higher resolution clustering using the UMAP dimensionality reduction (Figure 4.23A). Although, a similar number of cells were sorted for each sample, the number of cells present in the macrophage analysis varied by nearly 10-fold. Indeed, in the BM samples, the number of macrophages was between 2000 cells for the healthy sample and 4000 cells for the AML, in the lung the number of cells was 484 for healthy lung and 736 for lung tumours (Figure 4.23B). Surprisingly, I found that the difference in macrophage numbers between healthy and malignant samples was primarily linked to a decrease in the immature neutrophil fraction in the samples and this decrease was similar in both organs, where the fraction of immature neutrophils composed approx. 60% of the healthy samples and dropped to 40% in the malignant samples. Altogether the fraction of macrophages remained low in all samples.

To characterise the differences between the lung and bone marrow macrophages, I performed a [DEG](#) analysis (Figure 4.23C for selected genes and Appendix E for the full list of genes) and found that most genes differentially expressed in the lung samples were also exclusively expressed by alveolar macrophages in E. Gautier’s analysis. The analysis of the expression of these genes by all immune cell types including macrophages from other organs on Immgen confirmed that these genes were specific to alveolar macrophages. When looking at the genes expressed solely by bone marrow cells, surprisingly, a number of neutrophils genes were present. However, based on the initial analysis, I know that this expression is much lower than in neutrophils as the neutrophils were excluded based on their expression of these genes. One possible explanation for the presence of these genes in macrophages is that neutrophils are known to re-

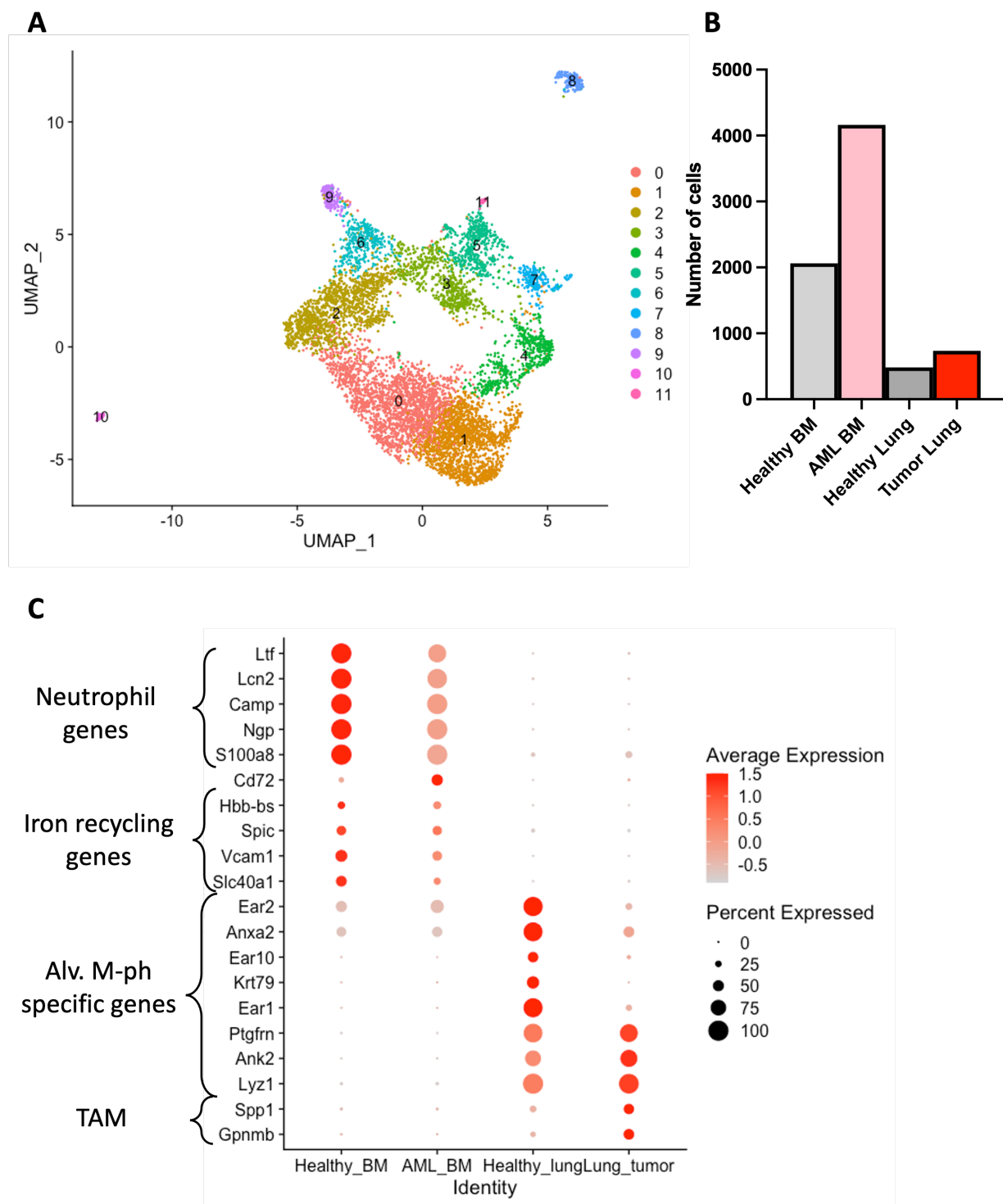


Figure 4.23: Comparison of macrophage transcriptional signature in NSCLC and AML. Cells classified as monocytes/macrophages in the lung dataset and as macrophages in the BM dataset were selected to perform a higher resolution analysis. Cells from both the healthy and malignant condition were included for both datasets. (A) UMAP of selected macrophages integrating the lung and BM samples. (B) Number of cells from each sample included in this analysis. (C) Dot plot representing the top 5 significantly differentially expressed genes for each sample. Alv. M-ph = Alveolar macrophage

turn to the bone marrow at the end of their life cycle. Macrophages are then responsible for the uptake of these dying cells. As such, through phagocytosis of dying neutrophils, macrophages could contain neutrophils genes. Finally, bone marrow macrophages were also found to be the only cells expressing iron-recycling genes, a biological process known to take place only in the spleen and bone marrow. Hence, this analysis confirmed that the most differentially expressed genes were associated with tissue-specific macrophage function.

To address what clusters were common between lung and bone marrow, in healthy and malignant conditions, I calculated the proportion of each cluster in each sample. Some clusters were found to be exclusively present in one organ such as cluster 1 which was only found in the bone marrow and cluster 8 and 9 were only found in the lung (Figure 4.24 A-B). However, a majority of the clusters were found to overlap between both organs. When looking at the core tissue macrophage signature, containing all 132 genes, defined by E. Gautier et al., [199], bone marrow macrophages were found to express more genes from this signature and at higher level compared to the lung macrophages (Appendix E).

To understand further the heterogeneity present in these samples, I mapped the labelling performed by M. Casanova-Acebes et al. onto the combined dataset (Figure 4.25A). While their samples represented only a minority of cells, I was able to extrapolate their labelling to the bone marrow thanks to the clustering algorithm. I could then identify the equivalent population in the bone marrow. One interesting result is that all the bone marrow cells present in this analysis were classified as macrophages as they expressed the core macrophage signature. However, the labelling from M. Casanova-Acebes et al., categorised most of these cells as dendritic cells and monocytes. To correlate their labelling with the clusters identified during my in-depth macrophage analysis, I overlayed the cluster numbers from the previous analysis on the combined dataset. Strikingly, I found that the cells categorised as macrophages by M. Casanova-Acebes corresponded to the cluster 2 and 5 from my previous analysis. In this analysis, I had identified these cells as IMMs (Figure 4.25B). Altogether, the similarity in classification and the presence of these cells in healthy and malignant lung and bone marrow tissue reinforces the idea that these cells are involved in disease progression. Hence, I decided to further characterise these two clusters.

In their study, M. Casanova-Acebes et al. further divided their macrophages into two clusters based on their expression of *Pparg*, *Mrc1*, *Marco*, *Siglec1* and *Siglecf* for the first clus-

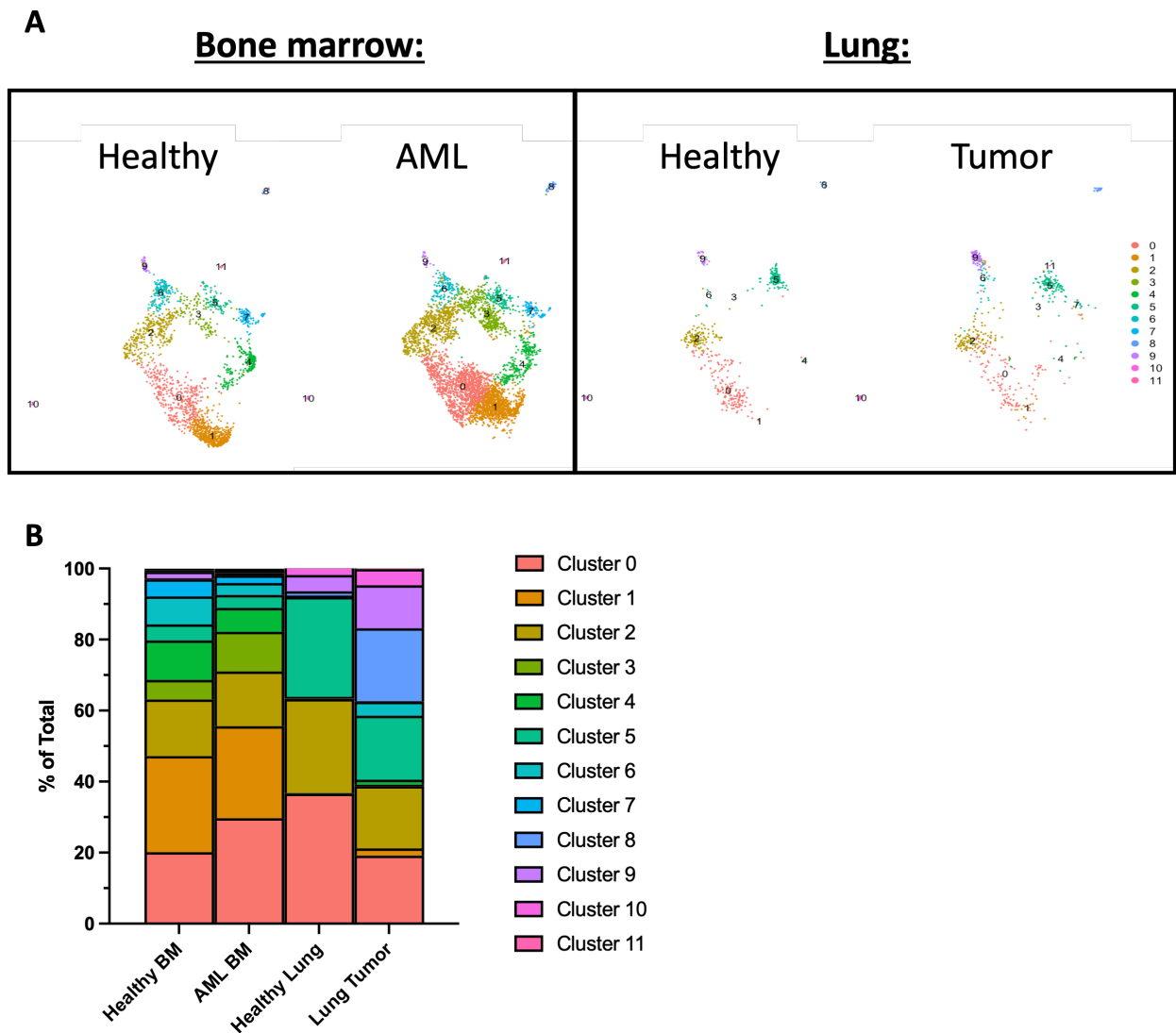


Figure 4.24: Overlap of macrophage clusters in NSCLC and AML

(A) UMAP split for each sample (healthy BM macrophages, *glsBM* macrophages from AML, healthy lung macrophages and lung macrophages from tumours) to highlight the overlap between lung and BM cells. (B) Proportional stacked bar graph showing the frequency of each cluster in each sample. Cluster 0, 2 and 5 were found in all 4 samples.

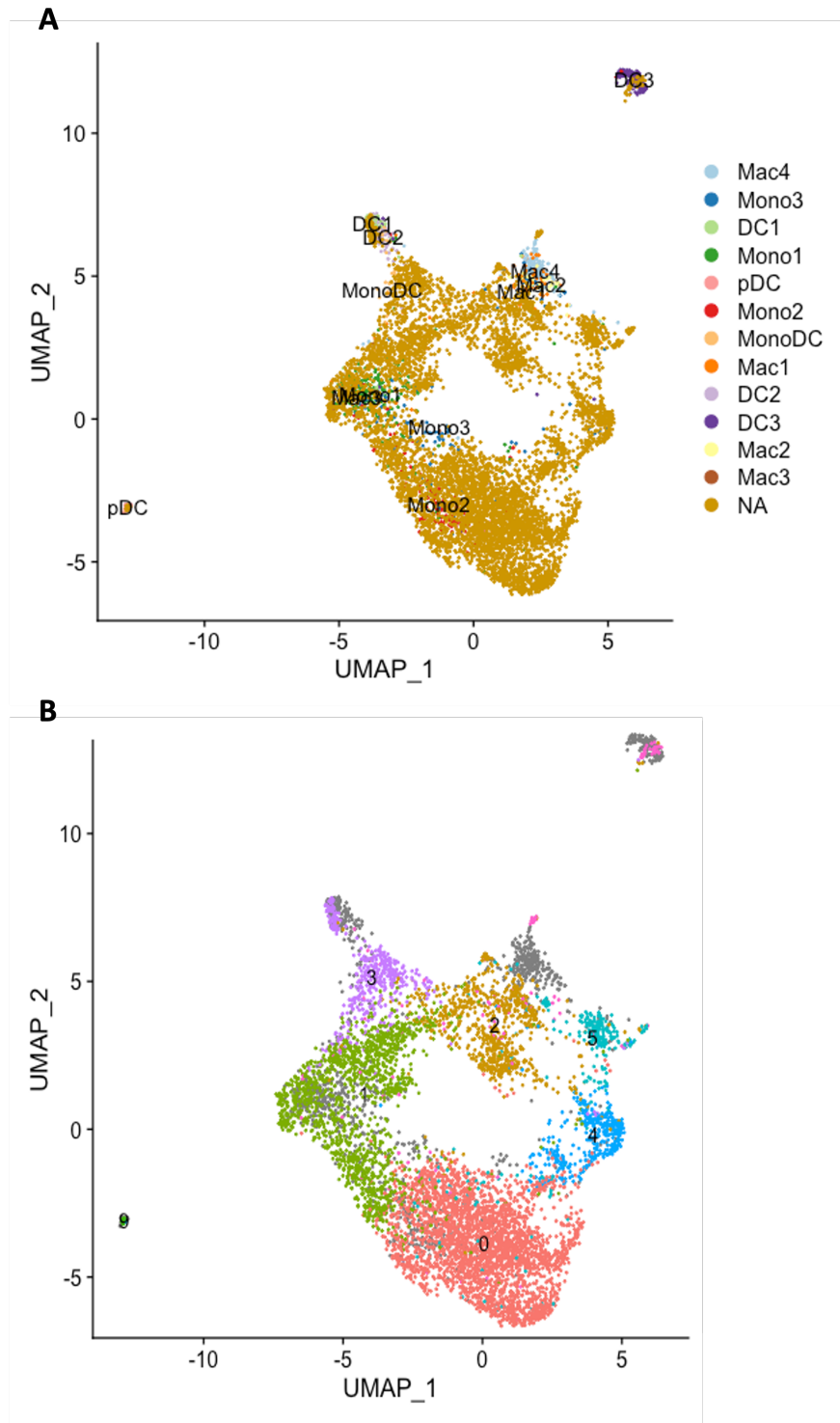


Figure 4.25: Comparison of the identification of the monocyte/macrophage populations in the lung and BM dataset

(A) Labelling performed by M. Casanova-Acebes et al., [1] overlaid on the integrated macrophage UMAP. Brown cells represent bone marrow cells. (B) Cluster numbering from our previous higher resolution macrophage analysis. Cluster 2 and 5 overlap with cells labelled as macrophages by M. Casanova-Acebes et al. Mac = macrophage, pDC = plasmacytoid DC, Mono = monocyte, MonoDC = monocyte/DC.

ter and *C1qa*, *Mafb*, *Arg1*, *Mgl2* and *Trem2* for the second cluster. To address whether our macrophages could also be sub-divided based on the expression of these genes, I compared the expression of these genes in the lung and bone marrow macrophages (Figure 4.26A-B). The expression of most of these genes was found to be relatively low in the lung macrophages and as such it was impossible to conclude on the classification of these cells.

Finally, to understand whether some lung macrophages were similar to IMMs, I assessed the expression of signature genes and functions identified in IMMs, in the lung macrophages. Previously, 3 genes were used to identify these cells as IMMs: *Adgre1*, *SIRP α* and *Cd274*. Lung macrophages were found to express only *SIRP α* (Figure 4.27A). This result correlated with the fact that in flow cytometry, lung macrophages and bone marrow macrophages are identified using different markers, therefore it was not surprising that lung macrophages did not express these markers. Next, to understand whether these populations had a similar function, I measured the gene expression of genes involved in antigen presentation or phagocytosis (Figure 4.27B). Interestingly, lung macrophages were found to express genes associated with both of these function, albeit at a lower level, in particular *Cd74*. *Cd74* is known as the gamma chain of the MHCII receptor and therefore its expression reflects the expression of MHCII. Within the phagocytic signature, previously, I found that IMMs differentially expressed a number of genes involved in efferocytosis compared to the other bone marrow macrophages. Hence, to evaluate whether lung macrophages could also be associated with efferocytosis, I compared the gene expression of *Mertk*, *Axl*, *Nr1h3* and *C1qa* (Figure 4.27C). *Axl* was found to be highly expressed by both lung and bone marrow macrophages, with a similar expression in healthy and malignant tissues. Strikingly, *Mertk* was found to be expressed only in healthy tissues while *Nr1h3* was only expressed in the bone marrow conditions. Finally, *C1qa* was highly expressed by bone marrow macrophages and lung tumour macrophages.

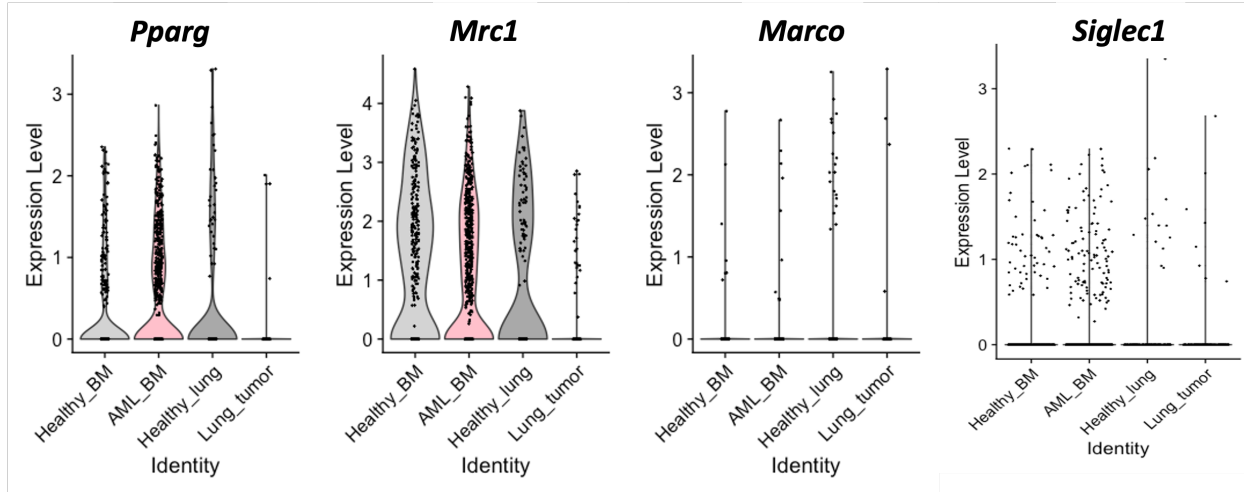
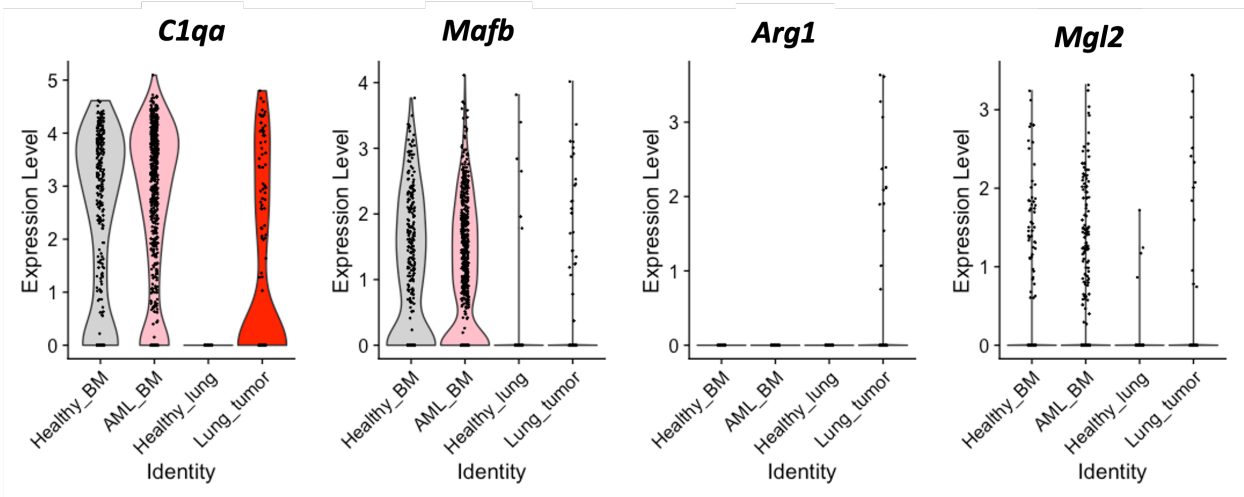
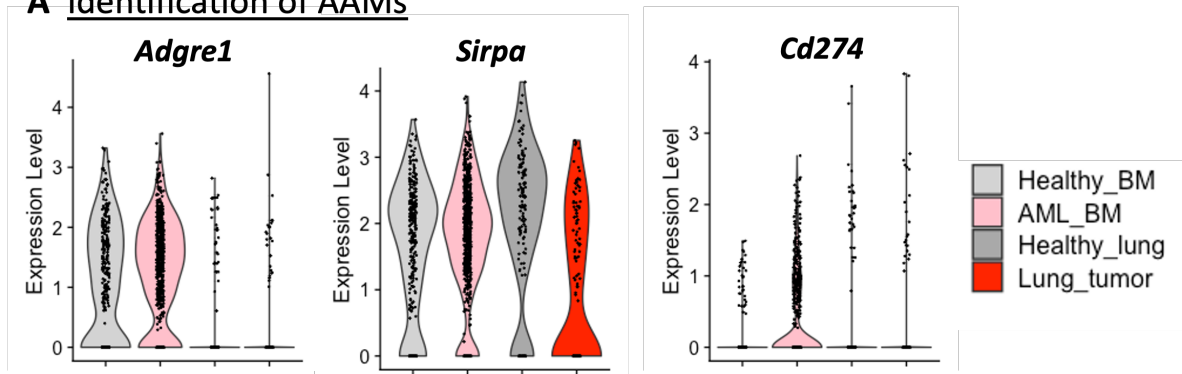
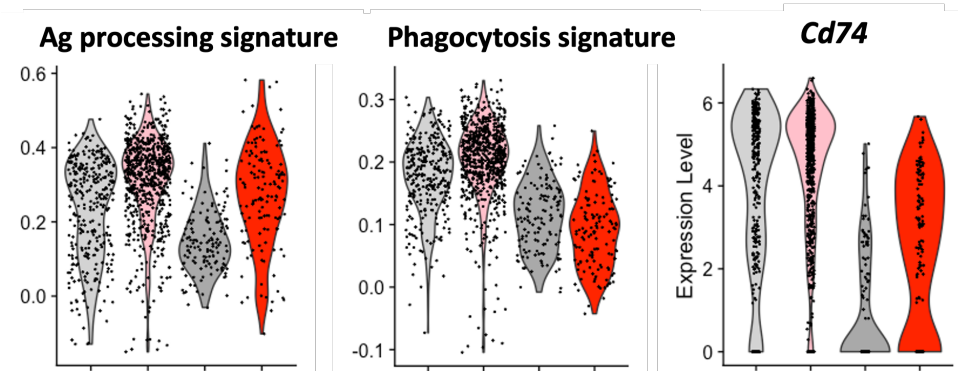
A**Cluster 1 main genes****B****Cluster 2 main genes**

Figure 4.26: Expression of signature genes characterising NSCLC macrophage clusters
 In M. Casanova-Acebes et al., [1] macrophages were divided into two clusters based on the expression of 5 signature genes for each cluster. Violin plots representing the expression of 4 of the 5 genes described to characterise cluster 1 (**A**) and cluster 2 (**B**). The low expression of these genes in the lung samples impedes us from addressing whether BM macrophages resembles one specific cluster of lung macrophages.

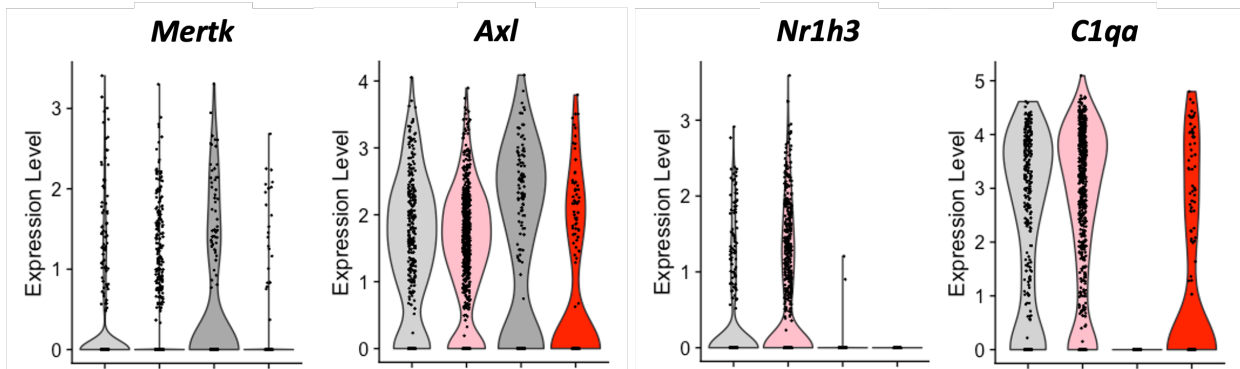
A Identification of AAMs



B AAM biological processes



C Efferocytic signature of AAM



D Genes upregulated in AML

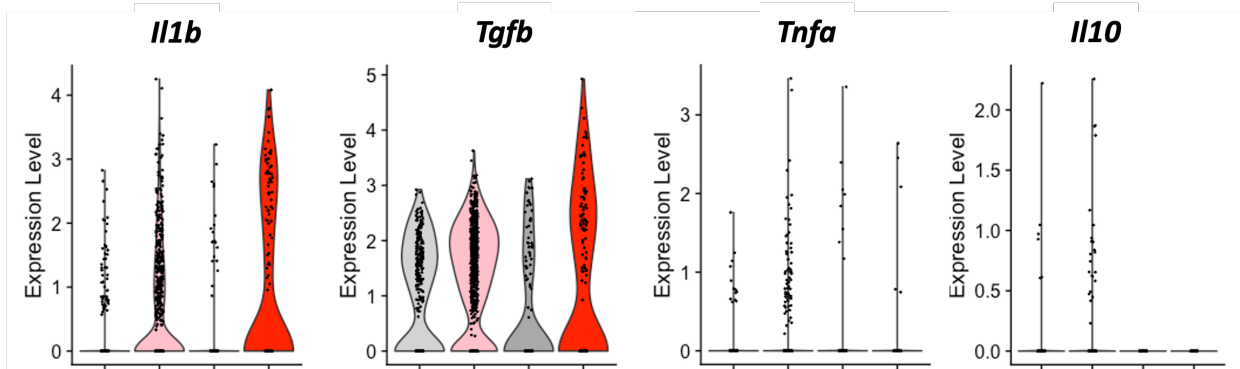


Figure 4.27 (*previous page*): Similarity in IMMs transcriptional signature between lung and BM macrophages during malignancy

(A) Violin plots representing the expression of IMMs markers in lung and BM macrophages from healthy and malignant conditions. **(B)** Violin plots of the normalised expression of antigen processing and presentation genes, phagocytosis genes and *Cd74*. **(C)** Violin plot of selected efferocytic genes in lung and BM macrophages from healthy and malignant conditions. **(D)** Violin plot of selected differentially expressed genes in AML compared to control BM macrophages.

Altogether, the expression of these genes was higher in the bone marrow compared to lung tissue however, lung macrophages still expressed a number of these genes. Hence, these results suggest that lung macrophages are also efferocytic. Finally, to address whether malignant changes influenced in a similar manner lung and bone marrow macrophages, I evaluated the expression of the genes previously found to be upregulated in bone marrow macrophages in the AML condition (Figure 4.27D). The upregulation of *Il1b* was similar in lung tumours macrophages and bone marrow macrophages while the upregulation of *Tgfb1* was higher in lung tumour macrophages compared to bone marrow macrophages. However, the upregulation of *Il10* and *Tnfa* was not seen in lung tumour macrophages. *Il1b* and *Tgfb1* were the strongest upregulation of these four changes noted and therefore it is possible that a similar process is affecting lung and bone marrow macrophages in during malignancy.

4.5 Malignant macrophages replace healthy BM macrophages

One last point I wished to address was whether AML cells were capable of hijacking the macrophage function and phenotype to replace them in the environment. Indeed, in patients, it is known that macrophage numbers are increased in AML and given that AML comes from the proliferation of the myeloid lineage, it is possible that the malignant cells are differentiating into macrophages. In addition, in humans, there are no obvious markers to distinguish malignant cells from healthy cells and therefore, macrophage numbers would include both healthy and malignant cells.

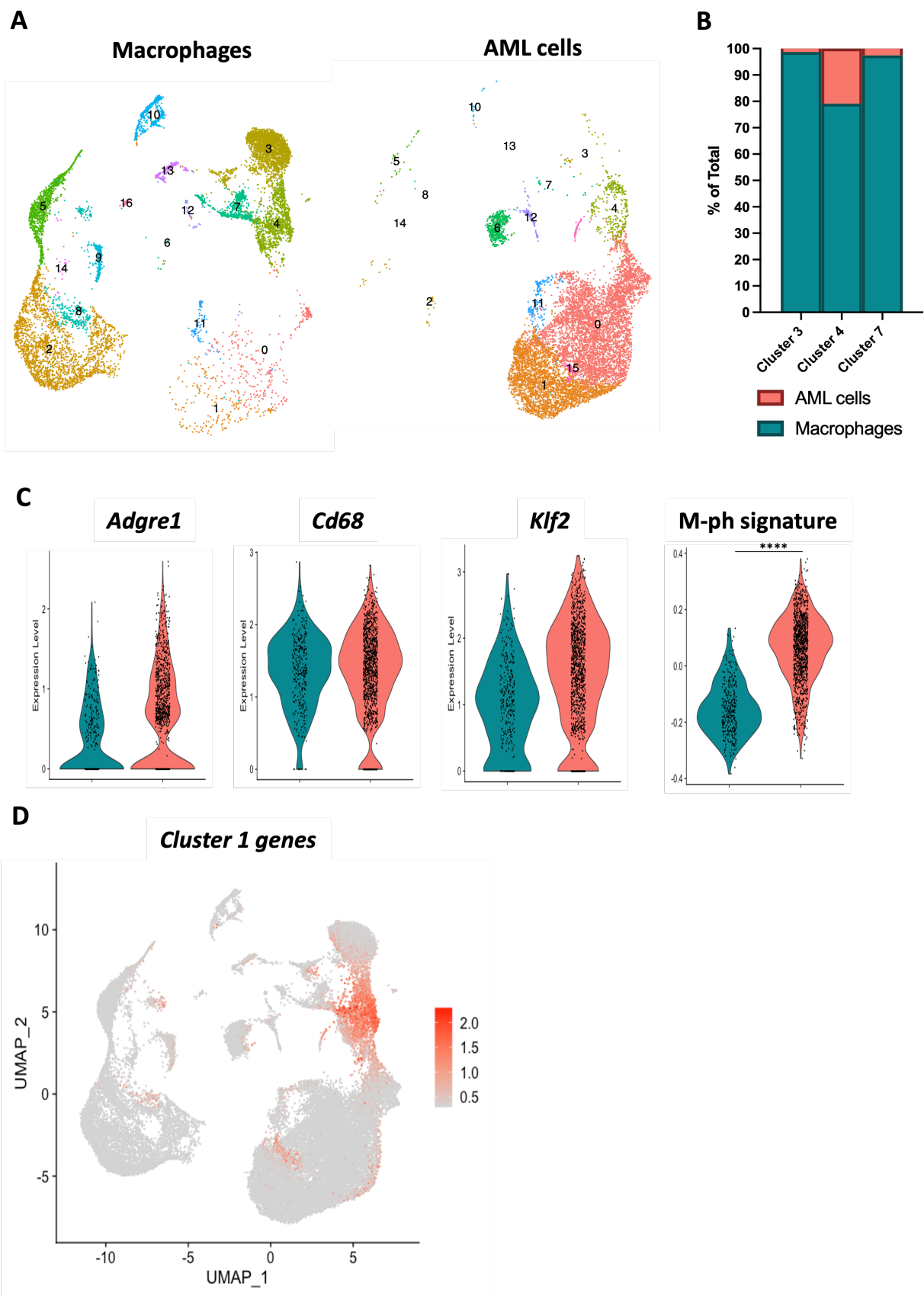


Figure 4.28 (previous page): AML cells overlap with healthy macrophages in the scRNAseq data

(A) UMAP split into macrophage and AML sample. **(B)** Proportional stacked bar graph showing the fraction of macrophages and AML cells composing each cluster. Only clusters classified as macrophage clusters were analysed. Cluster 4 was found to be the only cluster containing both macrophages and AML cells. **(C)** Violin plot of selected macrophage genes and normalised core macrophage signature genes in cluster 4 **(D)** Feature plot of the normalised expression of cluster 1 signature genes from the high resolution macrophage analysis. Cluster 4 expresses the most these genes.

To address whether AML cells are differentiating into macrophages, I reanalysed the initial scRNAseq analysis containing both the AML samples and macrophage samples. From the previous analysis we knew that macrophages were only located in cluster 3, 4 and 7 of the initial UMAP. Among these three clusters, cluster 4 was found in both the macrophage and the AML sample (Figure 4.28A-B) with the AML cells representing 25% of the cluster. To confirm that the AML cells in this cluster were macrophage-like cells, I evaluated the expression of the bone marrow macrophage core signature. Their expression was significantly lower than in the healthy counterpart, however they did still express important macrophage genes such as *Adgre1*, *Cd68* and *Klf2* and therefore can still be considered macrophage-like cells (Figure 4.28C).

Next, to uncover which macrophages, identified in the in-depth macrophage analysis, are contained in this cluster, I measured the expression of the most differentially expressed genes for all analysed macrophage clusters. The macrophage-AML cluster was found to express genes correlating with cluster 1 in the previous analysis (Figure 4.28D). Cluster 1 had previously been described as a monocyte-like macrophage cluster with high expression of *Ly6c2* and *Ccr2*. This is consistent with the MLL-AF9 AML model being monocytic and therefore a small proportion of these cells could have differentiated towards the monocyte-macrophage profile. Since healthy macrophages from cluster 1 were found to be involved in phagocytosis and antigen processing and presentation, I questioned whether malignant monocyte-macrophages also expressed genes involved in these two biological processes. Malignant monocyte-macrophages had significantly lower expression of both antigen processing genes and phagocytosis genes (Figure 4.29A). In addition, when evaluating the upregulated biological processes in the malignant monocyte-macrophages, compared to the corresponding healthy cells, most processes were found to be involved in the translation and production of proteins (Figure 4.29B). This correlates with the increased number of transcribed genes in AML cells (Figure 4.6C) and would therefore lead to increased translation. Finally, the malignant monocytes-macrophages downregulated genes

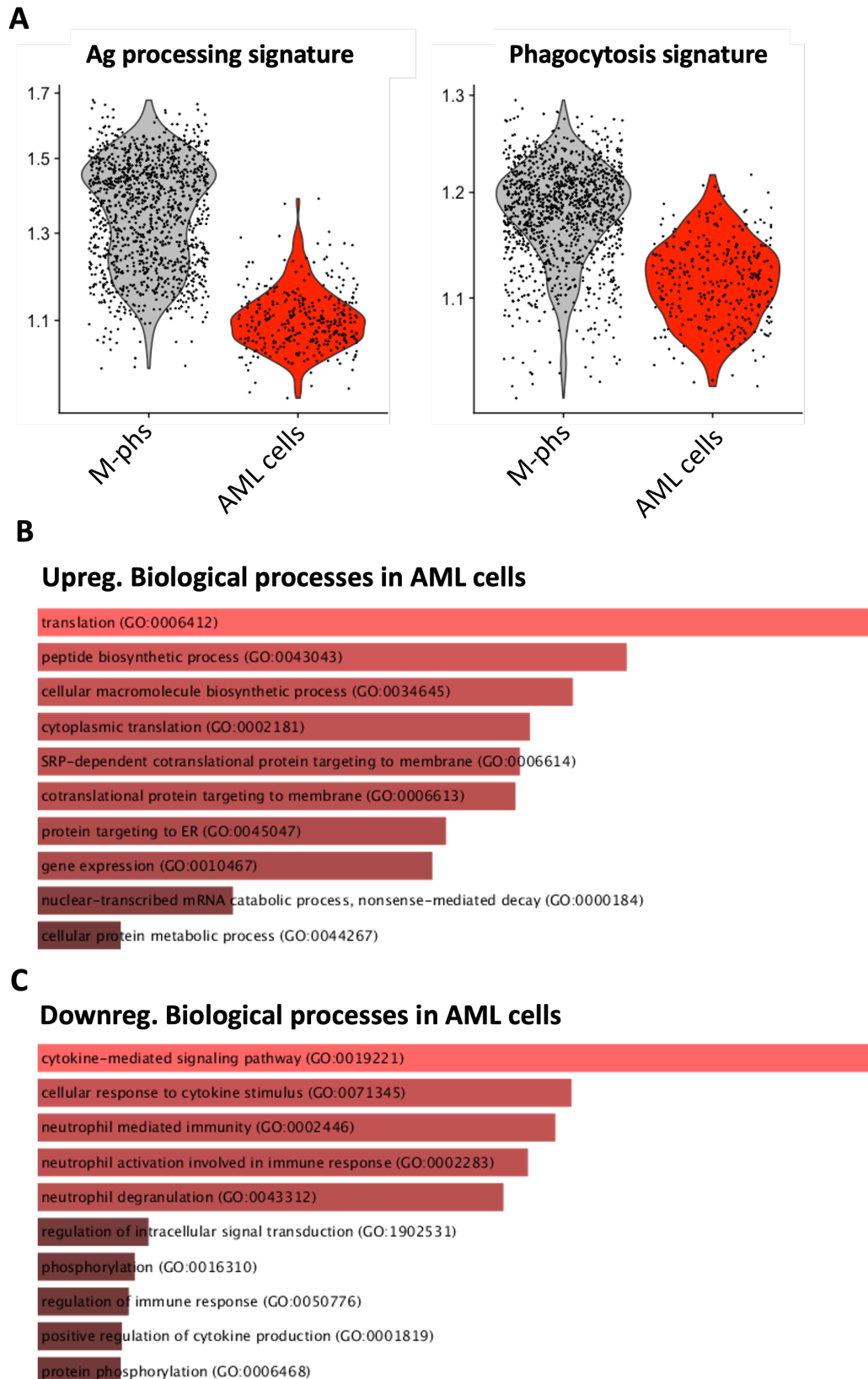
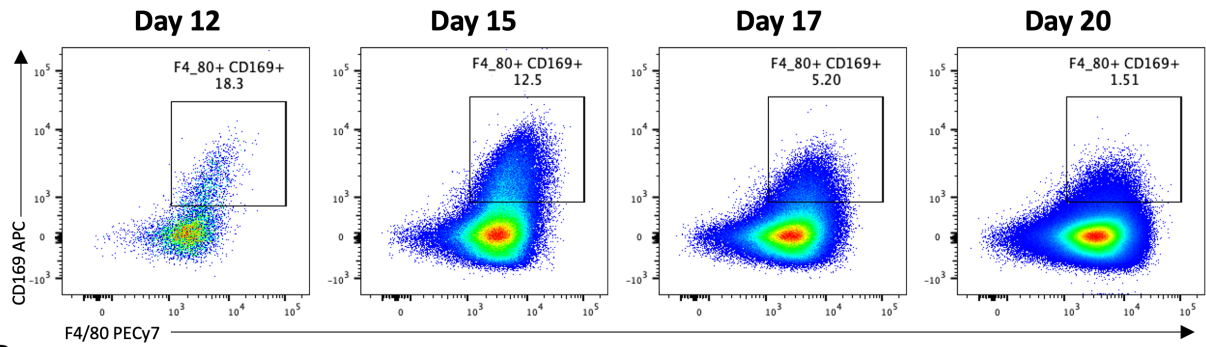


Figure 4.29: AML cells do not express genes involved in macrophage function
(A) Violin plots of macrophages and AML cells from cluster 4 representing the normalised expression of antigen processing signature genes and phagocytosis signature genes. Gene ontology analysis of biological processes **(B)** upregulated and **(C)** downregulated in AML cells from cluster 4 compared to macrophages from the same cluster.

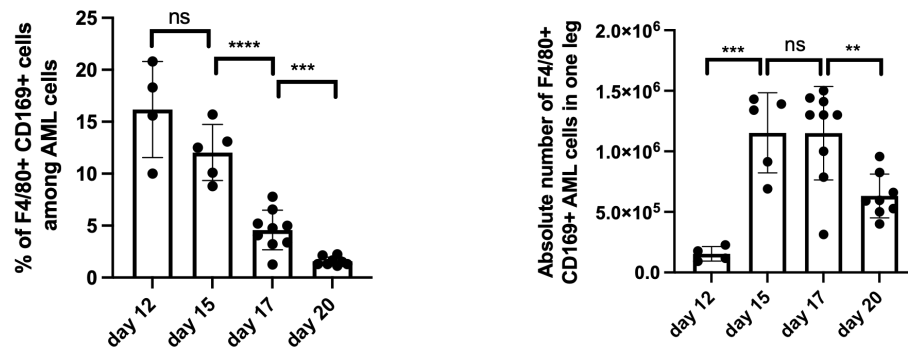
involved in the immune response in particular in the secretion of cytokines. Since cytokines are known as the primary mediator of the immune response, this result strengthens the idea that AML aims to evade the immune system. As such it could be considered that these malignant cells are hijacking the monocyte/macrophage phenotype to reduce the cytokine secretion and immune response against AML cells.

To correlate these findings with *in vivo* data, I assessed the phenotype of AML cells as the disease grew in the BM and measured their expression of F4/80, and CD169. All AML cells were found to express relatively high level of F4/80 however only a small fraction expressed CD169 (Figure 4.30A). This population was found to reduce in frequency over time, but increase in terms of absolute number in intermediate time points before decreasing at the last, late time point. (Figure 4.30B). Strikingly, when I plotted together the absolute numbers of healthy and malignant F4/80+ CD169+ cells, the total number of cells increased steadily until it suddenly dropped at the late time point (Figure 4.30C-D). These results could explain the discrepancy between the evolution of macrophages in mice and in humans where macrophages were found to increase in the bone marrow. While I don't know if this macrophage population is the same as the one identified in the scRNAseq, together, these results support the hypothesis that AML cells are replacing macrophages and modifying the monocytic-macrophage immune response in order to evade the immune system.

A Gated on single live, tomato+ cells:

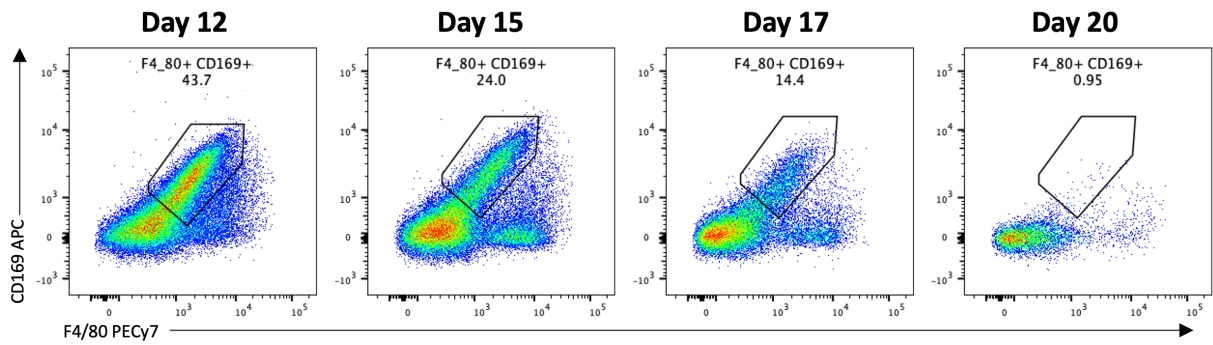


B



C

Gated on single live, tomato-, CD11b+, Ly6G-, Ly6C^{lo}, SSCA^{lo} cells:



D

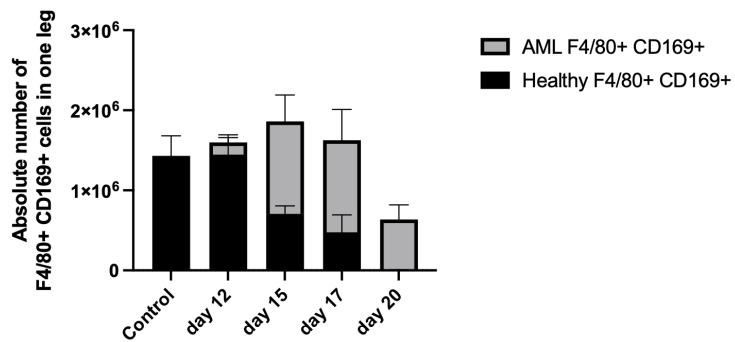


Figure 4.30 (previous page): Malignant macrophages replace healthy macrophages
 100'000 mTmG AML cells were injected into immunocompetent mice and sacrificed at four different time points throughout AML growth. Control aged-matched WT mice were sacrificed as control. **(A)** Representative *Fluorescence activated cell sorting (FACS)* plots of F4/80 versus CD169 expression in single, live mTmG+ AML cells, for each time point. **(B)** Percentage (Left plot) and absolute number (Right plot) of F4/80+ CD169+ AML cells among all AML cells. **(C)** Representative FACS plots of F4/80 versus CD169 expression in single, live, mTmG-, CD11b+, Ly6G-, Ly6C^{lo}, SSCA^{lo} cells, for each time point. **(D)** Absolute number of healthy and malignant F4/80+ CD169+ cells, stacked, for each time point. The total number of F4/80+ CD169+ cells increases at early disease and drops at very late AML infiltration. $n = 5 - 9$ mice in each group, representative of 3 independent experiments. Error bars represent the mean $\pm$ s.e.m. Statistically significant differences were determined using one-way ANOVA (* represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$).

4.6 Discussion

In this chapter, I wanted to investigate how AML affects the different macrophage populations identified in Chapter 3. First, using a combination of flow cytometry and IVM, I questioned how macrophages dynamics were affected, both in terms of distribution and numbers, during AML growth. Further to this, I examined whether AML modified the identity of macrophages at the transcriptional level. Finally, I questioned whether AML cells were capable of mimicking the macrophage transcriptional and phenotypical signature and hijack it towards an anti-inflammatory signature to favour disease growth.

I first wanted to uncover whether AML led to the loss of macrophages in the bone marrow. Due to the inherent properties of the bone as a hard, fixed size organ, I hypothesized that the growth of AML in this environment led to a competition between healthy and malignant cells, both in terms of nutrients and space. Previous studies have shown that as AML grows, healthy cells are pushed into the vasculature [238] and this is reflected in terms of number of healthy hematopoietic progenitor cells found in the bone marrow as the disease grows [248]. While macrophages are known to be part of the hematopoietic system and as such would be expected to be lost in a similar manner to the other healthy hematopoietic cells, macrophages are also known to be sessile and well attached to their environment. In addition, tissue resident macrophages are the only hematopoietic cells to survive radiation and have the capacity to replenish their pool of cells independently of progenitor cells [10, 11, 12].

Together, these different studies led me to hypothesize that macrophages could be capable of overcoming the competition with AML cells and remain in the microenvironment. While the

overall results indicated that macrophages are lost with AML, like all other hematopoietic cells, one interesting point came out of these dynamics, HSMs were the very first cells to be lost in the microenvironment while IMMs were actually enriched at early disease and were lost much later than most healthy cells. This result could be explained either by the recruitment of monocytes which then differentiated into IMMs or by the proliferating capacity of tissue resident macrophages. However, it remains unknown if IMMs are tissue resident cells and therefore if they have self-renewal capacity. Further investigation should focus on characterising the origin and proliferative capacity of IMMs, for example using lineage tracing experiments with yolk sac restricted drivers and examining BrdU incorporation, respectively.

The early enrichment of IMMs correlated with the IVM experiment showing an increased number of CD169-YFP cells in AML clusters at early disease. These cells had an increased circularity and reduced perimeter and area. Could these morphological changes reflect a difference in motility or origin? Reduced dendrites and surface contact could allow these cells to move around more or it could be a reflection of cells that have been recruited and haven't yet settled in their environment. Macrophages from different organs are known to have different morphology [249], however, the evolution of the morphology of macrophages and its link to their origin has not been investigated. It would be interesting to address whether these changes in morphology are associated with different origin, function and motility.

On another note, the CD169-YFP reporter highlights all cells that expressed CD169, or that derived from CD169+ cells through proliferation and/or differentiation, therefore it is unclear which macrophages are visualised in this reporter. The injection of SIRP α antibody can allow some correlation between the cells imaged and the populations identified by flow cytometry but the resolution of the IVM signal is inferior to that of flow cytometry and therefore there isn't a clear break between different cell populations like the one found for SIRP α by flow cytometry. To overcome this limitation, I generated a histogram containing the average value of SIRP α signal for each macrophage. By using the average value of the cell rather than the value of each pixel, I reduced the noise generated by CD169- cells expressing SIRP α , overlapping with CD169+ cells. Since the histogram did not reveal clear breaks between SIRP α -, SIRP α ^{lo} and SIRP α ^{hi} populations, a trial-and-error approach was used to set the different values for each population. By classifying the CD169-YFP cells using this approach, I found that the results correlated strongly with the flow cytometry results: SIRP α - macrophages were found in similar numbers in AML clusters and in the healthy counterpart at early disease while the SIRP α +

macrophages were increased in AML clusters, which matched the enrichment of IMMs noticed by flow cytometry. These results also correlated with a study performed on lymphoma, which characterised two populations of monocyte/macrophages in patients based on their expression of SIRP α [217]. The SIRP α^{hi} cells were found to be associated with poor survival while the SIRP α^- had no impact on outcome. Together, these results solidify the hypothesis that IMMs expressing SIRP α are promoting AML growth.

Next, I wanted to examine whether AML transformed macrophages at the transcriptional level using scRNAseq. A broad gating strategy allowed us to confirm that these cells were macrophages and resulted in a more unbiased approach. Expectedly, some minor contamination (<2%) of lymphoid cells was noticed and these cells were excluded from the main analysis. What was more surprising was that close to 50% of our sample were neutrophils although Ly6G had been used during the sort to exclude these cells.

Interestingly, when comparing our dataset with a dataset generated by M. Casanova-Acebes et al., [1] containing macrophages from the lung of naïve and lung adenocarcinoma mice, both datasets contained a similar fraction of neutrophils. In both cases, the sequenced cells were sorted based on the absence of expression of Ly6G. However, the neutrophils from the lung and bone marrow did not overlap much in terms of transcriptional signature. From the analysis of the granule formation genes and neutrophil maturation genes, it was clear that the neutrophils from the bone marrow were in fact neutrophils progenitor cells, while the neutrophils from the lung were mature neutrophils expressing *Rtnlg*. Another study performing scRNAseq on embryonic macrophages also contained 50% of neutrophil progenitor-like cells (expressing common neutrophil genes such as *Elane*, *Mmp8* and *Fcnb*) [250]. It is interesting that the fraction of neutrophil contamination is similar in all three cases.

Additionally, while in their study M. Cassanova-Acebes et al. included a lineage cocktail to exclude lymphoid cells, their fraction of contamination by B and T cells remains higher than in the bone marrow which did not include a lineage cocktail. Hence, we can conclude that a fraction of contamination by unwanted immune cells is inevitable. These cells could be the result of low sensitivity of the cell-sorting machine and weak selection of the cells of interest or stem from the challenge of harvesting intact macrophages, as described by SM. Millard et al. [207]. Still, this comparative analysis validated the identification of macrophages in our sample as most of these cells contained known monocyte/macrophage transcriptional signatures and

overlapped with the monocyte/macrophage/DC clusters from the lung study.

As very few studies have performed RNA sequencing on bone marrow macrophages, these cells are not listed on most databases like ImmGen. Indeed, up to this day, a core bone marrow macrophage signature has not been published. However, E. Gautier et al., [199] published a core tissue macrophage signature defined as macrophage genes expressed significantly more (2-fold more) in macrophages compared to all other cell types including DCs and these genes were expressed by at least three different types of macrophages among the following four macrophage populations: peritoneal macrophages, lung macrophages, microglia and red pulp macrophages. This list comprised a total of 132 genes and included most of the commonly expected macrophage genes such as *Adgre1* (F4/80), *Mertk*, *Mafb* and *Cebpb*. When applying this gene list to our sample, only 43 genes were found to be expressed by bone marrow macrophages. I defined these 43 genes as our bone marrow macrophage core signature. This result was similar to the results described by E. Gautier et al. [199] Indeed, in their study, they evaluated whether bone marrow macrophages expressed these genes and found very few genes were expressed in the bone marrow. In one figure, they showed the expression, by different macrophage populations including bone marrow macrophages, of 39 genes from this list. Of the 39 genes displayed, bone marrow macrophages were found to express only 19 and the expression was weak compared to other macrophages. Among the 19 genes expressed in the study by bone marrow macrophages, only 5 were not found in our sample, indicating that our macrophages have a similar transcriptional signature to the bone marrow macrophages analysed in that study.

When performing higher resolution clustering using UMAP dimensionality reduction specifically on the macrophage clusters, a high level of heterogeneity emerged even with regards to the expression of the core bone marrow macrophage gene signature. Combining this result with the gene ontology analysis of the different macrophage clusters, it became apparent that these cells could be divided based on two major functions: antigen processing and presentation and tissue homeostasis. Most cells were involved in either one or the other function, however one cluster appeared to be involved in both processes. This cluster, cluster 2, was also found to express the highest levels of F4/80, SIRP α and PDL1, both at the gene level and at the protein level. As such I could conclude that these cells were likely to be IMMs. Interestingly, the main genes expressed specifically by this cluster were involved in efferocytosis. Consistent with this result, C. Lantz et al., [251] analysed the different populations of peritoneal macrophages and their transcriptional signature during efferocytosis. One population named small peritoneal

macrophages (SPM), was found to have a similar pathway enrichment as cluster 2 here, with an enrichment for cell chemotaxis. In addition, these cells were found to express high levels of the MHCII genes: *H2-Eb1* and *Cd74*, again similar to the cluster 2. Interestingly, in their study they found that these cells, most likely monocyte-derived cells, had a specific transcriptional signature following efferocytosis, involving increased expression of *Il1b* and *Tgfb1*. Strikingly, these two genes were upregulated in cluster 2 in the AML condition. Altogether, these results solidify the hypothesis that these macrophages are involved in efferocytosis in particular in the AML condition.

As bone marrow macrophages remain understudied, I wished to validate the identification of bone marrow macrophages and function at the transcriptional level. Thanks to a similar, already published, analysis of lung macrophages, I could address whether bone marrow macrophages clustered together with lung macrophages. Not only did these cells cluster together, but also most of the differentially expressed genes were associated with tissue specific macrophage genes and function. As such, the most differentially expressed genes in lung macrophages were also found to be exclusively expressed by lung macrophages in E. Gautier's comparative analysis of tissue macrophages. With regards to bone marrow macrophages, the most differentially expressed genes were associated with iron recycling and neutrophils. The bone marrow and spleen are the only two organs responsible for the recycling of iron hence it is not surprising that these genes were found here. However, the presence of neutrophil genes was intriguing, one possible explanation is that aged neutrophils are known to return to the bone marrow for clearance by macrophages. As the macrophages characterised here are also known to have an efferocytosis signature, it is likely that these macrophages are responsible for the clearance of neutrophils and therefore contain some mRNA from neutrophils. One result supporting this hypothesis is the expression of *Nr1h3*, a gene which is known to be upregulated following the uptake of apoptotic bodies by macrophages [228]. However, no studies have yet addressed whether, following efferocytosis, mRNA of the apoptotic cell can be detected in macrophages. It would be interesting to investigate whether this is the reason why macrophages are expressing neutrophils genes.

Based on M. Casanova's analysis, two main macrophage clusters constituted their sample. These clusters were identified thanks to the expression of *Pparg*, *Mrc1*, *Marco*, *Siglecf*, *Cd2* and *Siglec1* for the first cluster and *C1qa*, *Mafb*, *Arg1*, *Mgl2* and *Trem2* for the second cluster. They associated the first cluster with tissue residency thanks to the expression of *Siglec1* and

self-renewal potential based on the expression of *Stmn1* and *Tuba1b* while they associated the second cluster with lipid metabolism and monocyte-identity genes. Correlating these results with fate mapping experiments they concluded that the first cluster was comprised of tissue resident macrophages and named this cluster tissue resident macrophages (TRM) while the second cluster was made of monocyte derived macrophages (named MDM). One of the questions remaining from the previous results was the origin of IMMs, hence I questioned whether using the comparative analysis, I would be able to tell which bone marrow cells clustered with TRM and MDM from the lung dataset. To answer this question, it was first essential to confirm the expression of the various genes used to identify these two clusters. Unfortunately, most lung macrophages were found to express very weakly most of the genes described. Hence, I was unable to identify the two clusters described and therefore could not conclude whether bone marrow macrophages associated preferentially with one macrophage population.

Another interesting result that came out of this comparative analysis was that both lung macrophages and bone marrow macrophages were found to be highly conserved in malignant tissues. M. Casanova-Acebes et al., following gene ontology analysis, found that only two mechanisms in macrophages were affected by the growth of lung tumours: antigen presentation and tissue remodelling. These two mechanisms were also the two main mechanisms that defined bone marrow macrophages. Interestingly, in both organs, there weren't any macrophage population present only in malignant tissues, suggesting that the pool of tumor associated macrophages analysed in these samples does not correspond to a distinct population of macrophages and most likely AML and lung carcinoma lead to minor remodelling of the macrophages already present in the tissue. Finally, similar changes were seen in *Il1b*, *Mertk* and *Tgfb1* expression in malignant samples compared to healthy macrophages. This result suggest that similar changes in macrophages are taking place in leukemia and lung tumours, promoting increased efferocytosis. Interestingly, M. Casanova et al., analysed the function of lung macrophages in tumours and found these cells to be capable of promoting invasiveness of cancer cells and induce the differentiation of Treg cells. Given the similarity in the transcriptional changes taking place during malignancy in lung macrophages and bone marrow macrophages, I hypothesise that bone marrow macrophages will also be involved in promoting disease growth through their interaction with AML cells and T cells. This hypothesis will be investigated in the next chapter.

Altogether, I can hypothesise that these results allow us to find a function to the phenotype identified: IMMs are efferocytic macrophages. To confirm this hypothesis, it will be necessary

to perform qPCRs on HSMs and IMMs and confirm the upregulation of efferocytic genes in IMMs. Among the efferocytic genes expressed by the IMMs are *Axl*, *C1q* and *Nr1h3*. Interestingly, a recent study targeted *Axl* in macrophages during AML and found that inhibition and KO of this gene led to reduced AML growth and restored the immune response against AML cells [252]. This study validates that efferocytosis is a good target in macrophages to delay AML and restore the immune response. Given the upregulation of *C1qa* and *Nr1h3*, it would be interesting to try and target these two genes in IMMs and understand whether inhibition of efferocytosis in IMMs can help delay AML growth.

Finally, the last part of this chapter focused on understanding whether AML cells were capable of mimicking macrophage phenotype and function, in order to hijack their role and promote immunosuppressive signalling. From the initial UMAP integrating both the early time point AML sample and macrophage sample, only one cluster strongly overlapped between these two types of samples. When focusing on this cluster, I found that, while these cells did express common macrophage genes such as *Cd68* and *Klf2*, a vast majority of genes remained differently expressed. Overall, when assessing the gene expression of macrophage biological processes such as antigen processing and presentation and phagocytosis, the expression of these genes was significantly lower in malignant macrophages suggesting that these cells were not capable of performing macrophage function. One interesting result was the decrease in cytokine production in the malignant macrophages. This leads us to hypothesise that the healthy macrophages are producing cytokines to mount the anti-tumour AML response, but the replacement of these cells by malignant macrophages reduces the secretion of pro-inflammatory cytokines to inhibit the immune response. While these results are only suggestive of a mechanism, it would be interesting to perform functional assays comparing side by side the healthy and malignant macrophages and confirm this phenomenon.

Altogether in this chapter, I have demonstrated that different macrophage populations are lost with different dynamics in the leukemic bone marrow. In addition to these differences in dynamics, at the transcriptional level, macrophage populations were found to be involved in two main processes, tissue homeostasis and antigen processing and presentation. IMM, which remained the longest in the bone marrow were found to upregulate genes involved in efferocytosis, a known anti-inflammatory process, essential in the clearance of apoptotic bodies. This result raises the question, does this population and process promote AML growth and if so, are they essential to AML development? Are these cells responsible for the inhibition of the

anti-tumour response? I will attempt to address these questions in the following chapter.

Chapter 5

Assessing the impact of macrophages on AML cells and the bone marrow microenvironment

5.1 Introduction

As described in the previous chapters, macrophages are a heterogeneous population of cells, involved in several processes broadly classifiable as the support of tissue homeostasis and the regulation of the immune response, both at its onset and eventually its dampening. Importantly, macrophages can be broadly classified as anti-inflammatory and pro-inflammatory according to their stimuli, environment, origin, and phenotype. The functional diversity of these cells renders the study of their role in disease, in particular cancer, very limited. In general, in solid tumours the presence of high numbers of macrophages is usually associated with a poor outcome and reduced survival, and they have been shown to contribute to cancer progression by promoting tumour cell survival, proliferation, [EMT](#) and metastasis [[137](#)].

Hence, efforts have been put into developing agents to deplete macrophages. For the last 40 years, the most commonly used biological tool to target macrophages is clodronate liposomes. Clodronate liposomes consist of clodronate molecules encapsulated in a lipid vesicle [[253](#), [254](#)]. These liposomes are phagocytosed by macrophages, leading to the apoptosis of macrophages. Importantly, free clodronate molecules are unable to cross cell membranes and are rapidly cleared by the renal system. This well-designed set-up allows specific targeting of phagocytic cells with reduced toxicity on other cell types.

In T-ALL, S. Chen et al., [255] studied the phenotype and molecular signature of bone marrow and spleen macrophages and compared these cells to the macrophages found in control healthy mice. Interestingly, depletion of macrophages using clodronate did not lead to any significant differences in disease growth. They argued that it is therefore important to target pro-leukemic macrophages and either deplete these cells specifically or convert them. Unfortunately, by RNA-sequencing they were unable to associate a transcriptional signature with these macrophages. However, they found that splenic macrophages but not bone marrow macrophages stimulated the proliferation and migration of T-ALL cells. These cells could be divided according to their expression of CD206. While this study does not answer what role macrophages play on AML growth, it does highlight the importance of the heterogeneity of macrophages in leukemia development and the complexity in their identification.

Recently, P. Pena-Martinez et al., [256] published a study where they identified that the administration of IL-4 led to an increase in IMMs which phagocytosed AML cells and in turn inhibited leukaemia development. Depletion of macrophages using clodronate abrogated the anti-tumoural effect. Finally, RNA sequencing revealed that macrophage's signature was associated with alternatively activated macrophages and increased phagocytosis in the IL-4 treated condition. In turn, this led to increased expression of CD47 on AML cells, which suppressed the macrophage activity. Interestingly, combining IL4 treatment with anti-CD47 restored the phagocytic activity of macrophages. While this study highlights an interesting function for macrophages, the clodronate administration was given i.p. and did not reach the bone marrow. Rather than seeing a decrease in bone marrow macrophages, they measured a 2-fold increase, hence it is uncertain whether correct depletion of macrophages takes place in the bone marrow, AML growth would be affected.

The phagocytosis of AML cells by macrophages is an important point studied by P. Pena-Martinez. General phagocytosis of tumour cells by macrophages can affect disease development in many ways. Three main mechanisms have been described to result from this phenomenon [163]. The first is that the engulfment of tumour cells leads to a direct interaction between these two cell types and allows tumour growth control via the direct elimination of tumour cells. The second mechanism is that macrophages are known as antigen-presenting cells and as such are capable of presenting antigens processed from phagocytosed particles and activate T cells. In turn, this can lead to tumour control. The final mechanism described depends on whether

the tumour cell is efferocytosed or phagocytosed and results in the macrophage functionality affected to acquire an anti-inflammatory or a pro-inflammatory phenotype, respectively. These changes will in turn lead to activation or inhibition of T cells. Hence, phagocytosis of tumour cells by macrophages is a key mechanism which can lead to numerous outcomes ranging from tumour growth control to enhancing the pro-tumoural microenvironment and favouring disease growth.

Based on the results presented in Chapter 3 and 4, I have identified that one macrophage population, IMMs, expressed a high level of genes associated with efferocytosis. Thus, I hypothesize that these cells are capable of phagocytosis of AML cells and in turn could inhibit T cell activation. In this chapter, I aim to address whether this hypothesis is true using a combination of *in vitro* and *in vivo* experiments.

Altogether, given the limited knowledge of the impact of macrophage depletion on AML growth, in this chapter I will establish whether macrophages promote or help control AML growth. Using two different depletion models, one pharmacological and one genetic model, I will try and define the contribution of macrophages to AML growth. In particular, I will question whether macrophages phagocytose AML cells and the consequences of this function on AML cells directly as well as the immune response. In light of this, I will explore whether macrophages directly interact with leukemic cells *in vitro* and *in vivo*. Finally, I will examine whether these cells, in their capacity of antigen-presenting cells, contribute to the shaping of the cytotoxic T cell response.

5.2 Depletion of macrophages impacts AML growth

Having identified transcriptional changes associated with efferocytosis and the regulation of the immune response in IMMs, I hypothesised that these cells must play an important role in the disease growth, especially at an early stage when these cells are enriched in the AML microenvironment.

To test this, I decided to deplete macrophages and compare AML cells' growth in treated versus control mice. Two different methods were available for macrophage depletion; pharmacological depletion using clodronate liposomes and genetic depletion with the CD169-DTR mouse line.

5.2.1 Set-up of clodronate liposomes treatment

In order to evaluate whether clodronate liposome depletion would be suitable to use in my study, I first had to check whether bone marrow macrophages were specifically depleted without causing too much toxicity. Based on the literature, the standard drug regimen with clodronate liposomes is to inject the drug every 2 days, never exceeding 4 injections as toxicity seems to occur after that [67, 257, 258]. Since I need the AML cells to have enough time to grow (15-20 days), I decided to inject clodronate or PBS liposomes at 0.1ml/10g every four days for 16 days (Figure 5.1A). During the harvest, I noticed that the spleens of treated mice were smaller and stiffer. Hence, I harvested the spleens to analyse whether these changes were linked to some toxicity.

Both the BM and spleens were harvested every other day from some of the mice in the cohort and the bone marrow was stained for myeloid and macrophage markers. By the end of the fourth injection, the size of the spleens from clodronate treated mice was significantly reduced (Figure 5.1B-C). In addition, the total number of spleen cells measured by flow cytometry was also reduced (Figure 5.1D) and this result correlated with the fact that the percentage of live cells in the spleen dropped to less than 20% (Figure 5.1E). Together these results suggest that repeated injections of clodronate lead to a high level of toxicity in the spleen. However, in the bone marrow, no signs of toxicity were noted; the absolute number of cells in the bone marrow was similar to that of control PBS liposomes treated mice (Figure 5.1F) and the percentage of live cells remained constant and similar to control mice (Figure 5.1G). Hence, clodronate can be used to study the effect of macrophages in the bone marrow, however it is important to keep in mind that these mice display a high level of toxicity in the spleen.

Next, to assess whether clodronate administration led to specific macrophage depletion, the absolute number of cells from both macrophage populations was measured by flow cytometry (Figure 5.2A). 24 hours after the first injection of clodronate liposomes, HSMs were reduced by 20-fold. Although at day 3 this number slightly increased, it dropped again at day 5 reaching a 100-fold reduction and subsequently remaining constant up to day 13 (Figure 5.2B). However, IMMs were only slightly reduced after 24 hours, their number increased by day 3 and remained similar to that of control until the end of the experiment (Figure 5.2C). This suggests that the IMMs are not very phagocytic and therefore do not pick up the clodronate liposomes from the environment. In addition, the increase in number at day 3 suggests that these cells are being

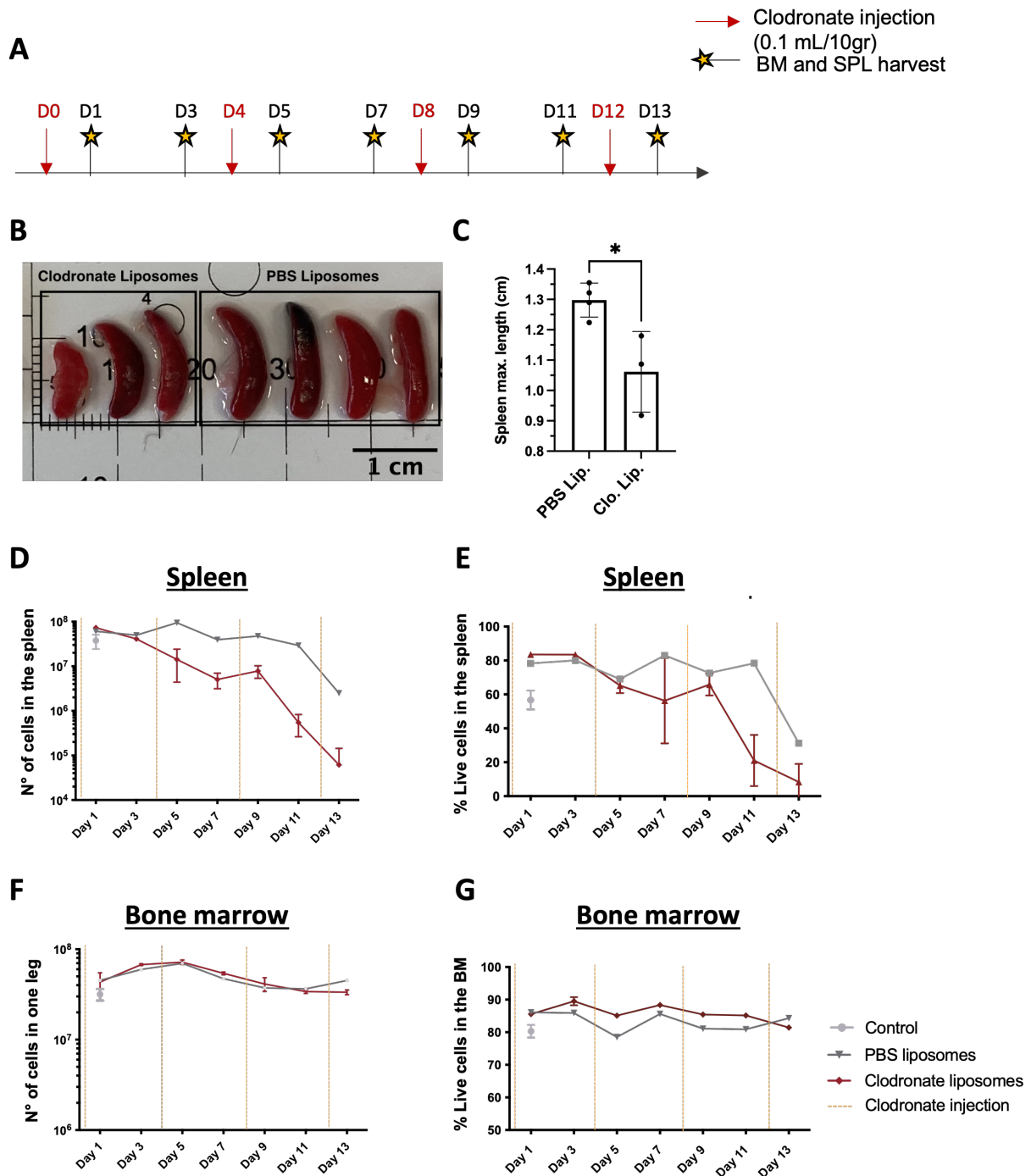


Figure 5.1: Repeated clodronate injections leads to toxicity in the spleen

Wild type mice were injected intravenously with 0.1ml/10g clodronate liposomes or PBS liposomes. (A) Experimental protocol for macrophage depletion over 13 days. Clodronate liposomes or PBS liposomes were administered every 4 days and BM was harvested every other day. (B) Pictures of spleens at day 13 of the treatment. (C) Spleen lengths of PBS liposome or clodronate liposome treated mice. (D) Absolute number and (E) percentage of live cells in the spleen. (F) Absolute number and (G) percentage of live cells in the bone marrow. Dot plot indicate mean ($\pm$ s.e.m) with 2 mice per time points were analysed for the clodronate liposome treatment and 1 mouse for the PBS liposome treatment.

replenished after the depletion of the HSMs and hence could be monocyte-derived macrophages.

Monocytes are known to be the precursors of macrophages and being part of the mononuclear phagocyte cells, they display a similar phenotype to these specific cells. Hence, it was expected that monocytes could be impacted by the off-target effect of the drug treatment. Strikingly, the opposite phenomenon was observed. The number of monocytes was increased in clodronate treated mice compared to controls (Figure 5.2D) suggesting that the depletion leads to either the recruitment of monocytes to replenish the diminished pool of macrophages or leads to increased proliferation of monocytes precursors in the BM. Another type of cells, the neutrophils, were also taken into consideration as they display a phenotype close to that of macrophages. However, their number remained similar to that of PBS liposome injected mice apart from a transient peak at day 5 (Figure 5.2E), demonstrating that these cells were not broadly impacted by clodronate treatment.

Finally, to confirm that repeated injections were necessary every four days, I compared the size of both macrophage populations at day 5 in mice that had received either a single dose of clodronate at day 0, or 2 doses, one at day 0 and one at day 4 (Figure 5.2F). There was a noticeably higher number of both the HSMs and IMMs in the single dose treatment compared to the repeated injection group (Figure 5.2G-H), suggesting that macrophages start recovering by day 5 post clodronate treatment. Hence, repeated injections are necessary to obtain an almost complete depletion of macrophages.

Before being able to question whether macrophage depletion affects AML growth *in vivo*, I first had to evaluate whether clodronate liposomes would affect the viability of leukemic cells directly. To determine the toxicity of the drug, BM derived macrophages and AML cells were treated with clodronate liposomes *in vitro*. Clodronate liposomes killed macrophages in a concentration dependent manner with an IC₅₀ of 0.3mg/mL (Figure 5.3A). When leukemic cells were treated, unexpectedly it appeared that the number of cells increased in the clodronate liposome condition compared to media or PBS liposomes conditions (Figure 5.3B). A possible explanation to this is that, since leukemic cells are non-adherent the clodronate couldn't be washed away properly and the clouds of drug could have led to a false cell count on the cell counting chamber.

To overcome this issue, I decided to take advantage of DiL-liposomes, which are built in

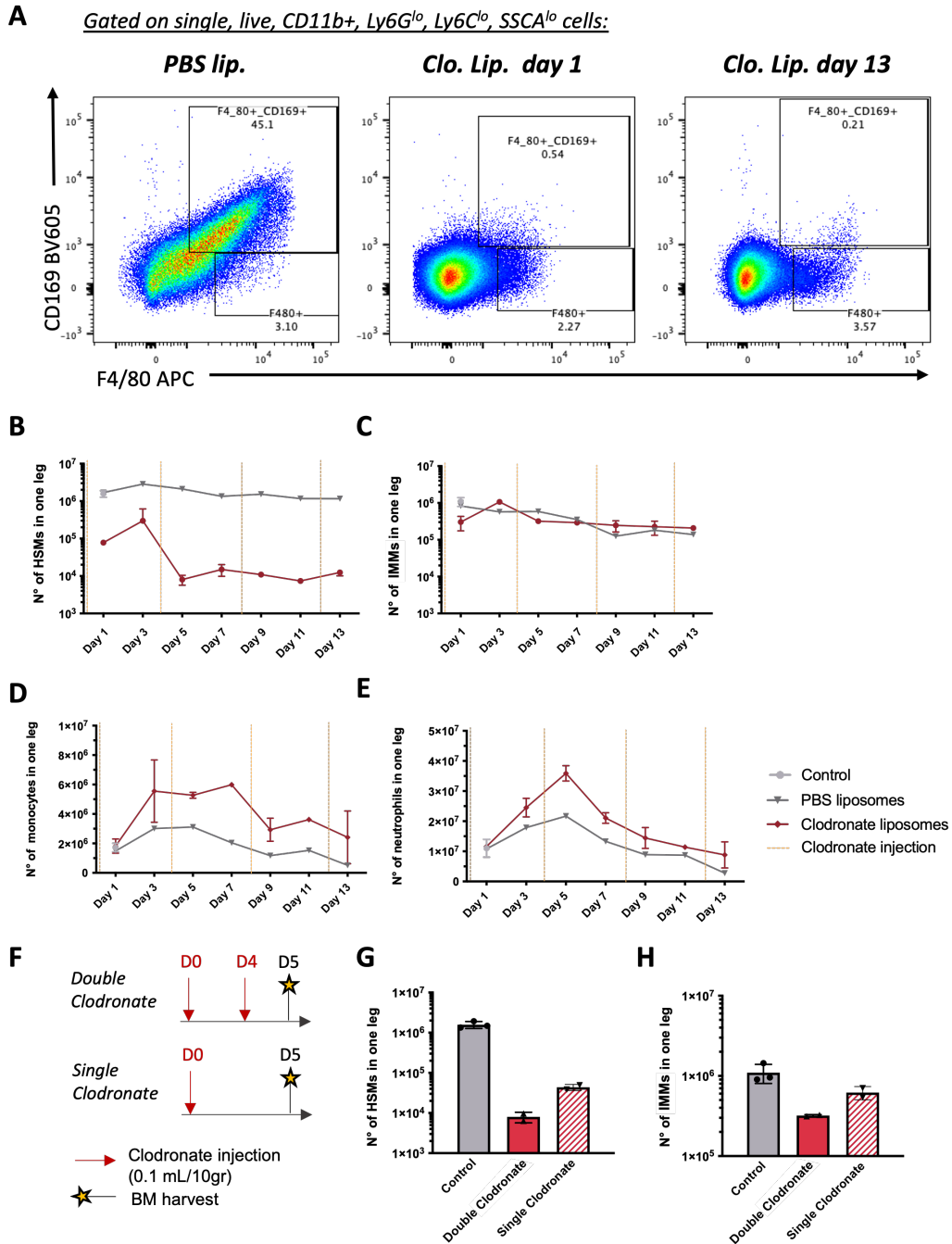


Figure 5.2: Repeated clodronate injections leads to depletion of macrophages

These results were produced with the same cohort of mice as described in Figure 5.1. (A) Representative *FACS* plots of F4/80 versus CD169 expression in single, live, CD11b⁺, Ly6G^{lo}, Ly6C^{lo}, SSCA^{lo} cells, from PBS liposomes or clodronate liposomes treated mice, one day after the first injection and 13 days after the start of the injections. Absolute number of (B) HSMs, (C) IMMs, (D) monocytes and (E) neutrophils in the BM from one leg. (F) Experimental protocol to compare the effect of single injection and repeated injections of clodronate. The repeated injection group of mice were injected with clodronate liposomes on day 0 and 4 while the single injection group of mice were injected only on day 0. The BM from all the mice was harvested on day 5. Absolute number of (G) HSMs and (H) IMMs in control, single injection and repeated injection mice group at day 5. Error bars represent the mean $\pm$ s.e.m. with 2 mice analysed for the clodronate liposome treatment and 3 mice for the control group.

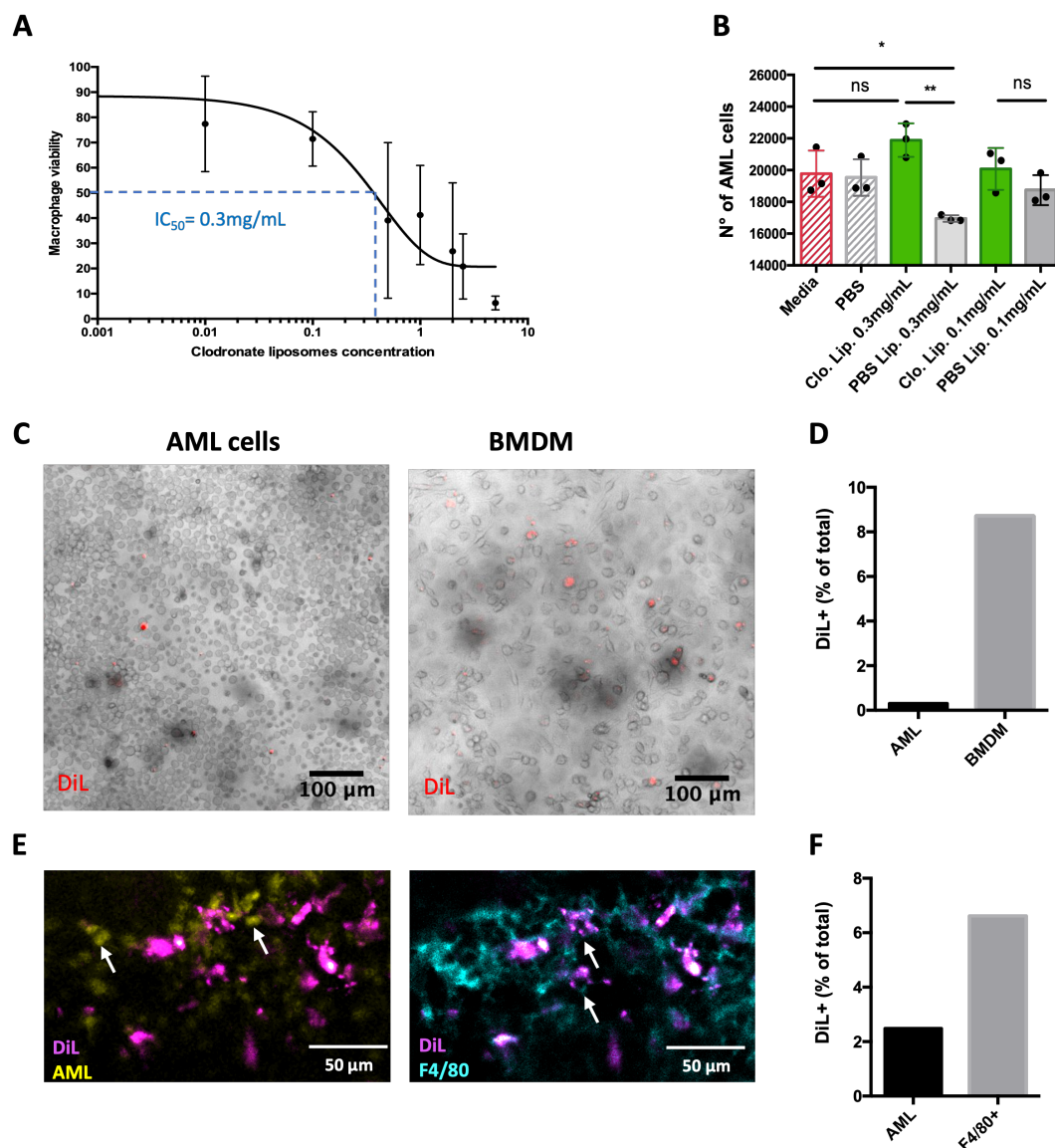


Figure 5.3: Cytotoxicity assay of clodronate liposomes.

In vitro and *in vivo* effects of clodronate liposomes on BM macrophages and AML cells. **(A)** Cytotoxicity assay of clodronate liposomes on BM derived macrophages *in vitro*. Cells were treated with clodronate liposomes at a concentration ranging from 0.001 mg/mL to 10mg/mL. 6 hours after drug addition, clodronate liposomes were removed and replaced with fresh media. The following day, viability was assessed. Killing of macrophages happened in a dose dependant manner with an IC_{50} of 0.3mg/mL. Dot plot indicate mean ($\pm$ s.e.m), ($n=3$, $N=2$). **(B)** Cytotoxicity of clodronate liposomes and PBS liposomes was assessed on AML cells. Cells were incubated for 6 hours with 0.3mg/mL or 0.1 mg/mL of PBS or clodronate liposomes. Total cell count shows a decrease in PBS liposomes treated condition and an increase in clodronate liposomes ($n=3$, $N=4$). **(C)** Images of AML cells and BMDM incubated with DiL-Liposomes for 21 hours *in vitro*. **(D)** Quantification of DiL+ cells. **(E)** IVM of a mouse transplanted with 100'000 YFP+ leukemic cells and injected with DiL liposomes 12 hours prior to imaging. F4/80 APC antibody was injected *i.v.* 30 minutes before image acquisition. Maximum projection of a representative timeframe of a 1 hour timelapse showing YFP and DiL channel (Left image) or F4/80 and DiL channel (Right image). **(F)** Quantification by FACS of the proportion of DiL+ cells among YFP+ or F4/80+ cells, ($n=1$, $N=1$).

a similar manner to clodronate liposomes but instead of encapsulating clodronate, the liposome encapsulates DiL, a red fluorophore detectable by confocal microscopy. BM derived macrophages and leukemic cells (Figure 5.3C) were treated with 0.3mg/mL DiL-liposomes, imaged 21 hours later and liposome uptake was measured as the number of DiL+ cells (Figure 5.3D). The percentage of macrophages having incorporated DiL liposomes was lower than expected as only 9% of the cells were positive, which could be due to degradation of the dye once inside the cells. However, the percentage of DiL+ AML cells was extremely low, and most of the visible red dots were located outside of the cells when the z-dimension was included in the analysis. Together, these data suggest that only a minimal proportion of AML cells are capable of phagocytosis and therefore AML cells are not targeted by clodronate liposomes.

To confirm this hypothesis, I performed one last control experiment where mice were transplanted with YFP-AML. 15 days later, the mice were injected with DiL-liposomes and the following day, they were assessed using intravital microscopy of the calvarium. F4/80-APC antibody was injected intravenously to visualize macrophages. One hour long timelapses with images acquired every minute were taken to see which cells had incorporated the dye in the BM (Figure 5.3E and Supplementary Video 4). Since AML cells are known to be motile, as previously reported in [192, 238], I was able to distinguish them easily from healthy BM cells. When looking at the dynamics of signal in the different channels, YFP+ cells did not appear to be DiL+. On the other hand, a higher degree of overlap between F4/80 and DiL signal was clear, and revealed that macrophages had incorporated the dye. Finally, the mouse's BM was analysed by flow cytometry (Figure 5.3F). 7% of F4/80+ cells were also positive for DiL while only 2% of YFP+ cells were DiL+. Among this 2% of DiL+ AML cells, it is possible that a high proportion are false positives as the *in vitro* experiment revealed that the dye could be attached to the surface of the cell but not be incorporated in the cell. Overall, this data suggests liposomes are up-taken more efficiently by macrophages and therefore should not lead to substantial depletion of leukemic cells.

5.2.2 Reduced AML growth in clodronate liposomes treated mice

Now that the drug regimen was tested and showed correct depletion of macrophages with limited impact on other cell types, macrophage depletion could be induced prior to and following transplantation of AML cells to uncover whether macrophages promote or reduce disease growth. Leukemic cells were transplanted one day after the first injection of clodronate liposomes or

PBS liposomes to make sure macrophages were depleted from the start of the disease. The BM was harvested at day 9, 11, 13 and 15 to evaluate the growth of AML (Figure 5.4A). To make sure PBS liposomes did not impact the BM microenvironment, control mice without any injection except the transplanted AML cells were also analysed. The percentage of AML cells was significantly decreased in the BM (Figure 5.4B) and the blood (Figure 5.4C) of macrophage depleted mice compared to both controls. The difference in AML infiltration increased as the disease progressed. When looking at absolute numbers, the number of AML cells in the BM of clodronate liposomes treated mice was also significantly reduced compared to both control groups (Figure 5.4D). In the blood, the absolute number of AML cells didn't reflect the percentage results, indicating an increased number of healthy cells in the blood of macrophage depleted mice must have been responsible for the decreased proportion of AML cells (Figure 5.4E). One possible explanation to this increase could be the recruitment of monocytes to replenish the macrophage pool as they are known to be recruited with clodronate treatment. Overall, these results suggest that macrophages are important for the development of AML.

Given that clodronate treatment in control mice led to the depletion of HSMs but not IMMs, I hypothesized that the depletion of HSMs led to the reduced disease. To confirm that it was only the depletion of HSMs that was responsible for the AML growth difference, I measured the absolute number of both HSMs and IMMs in clodronate and PBS liposome treated mice at early AML. To control for the impact of AML on each population, the absolute number of cells was also measured in control mice without AML or treatment. As expected, the absolute number of HSMs was significantly decreased in clodronate liposomes mice; however, the absolute number of IMMs was similarly decreased in clodronate liposomes mice (Figure 5.5). Given that clodronate did not seem to affect IMMs in control mice, it must be the combination of AML with clodronate liposomes which reduced the number of IMMs. One possible explanation is that AML leads to the recruitment of IMMs and clodronate limits this recruitment. Altogether this result reveals that both macrophage populations are affected by clodronate in the presence of AML and therefore it remains unknown which of the two populations is responsible for the delayed growth of AML.

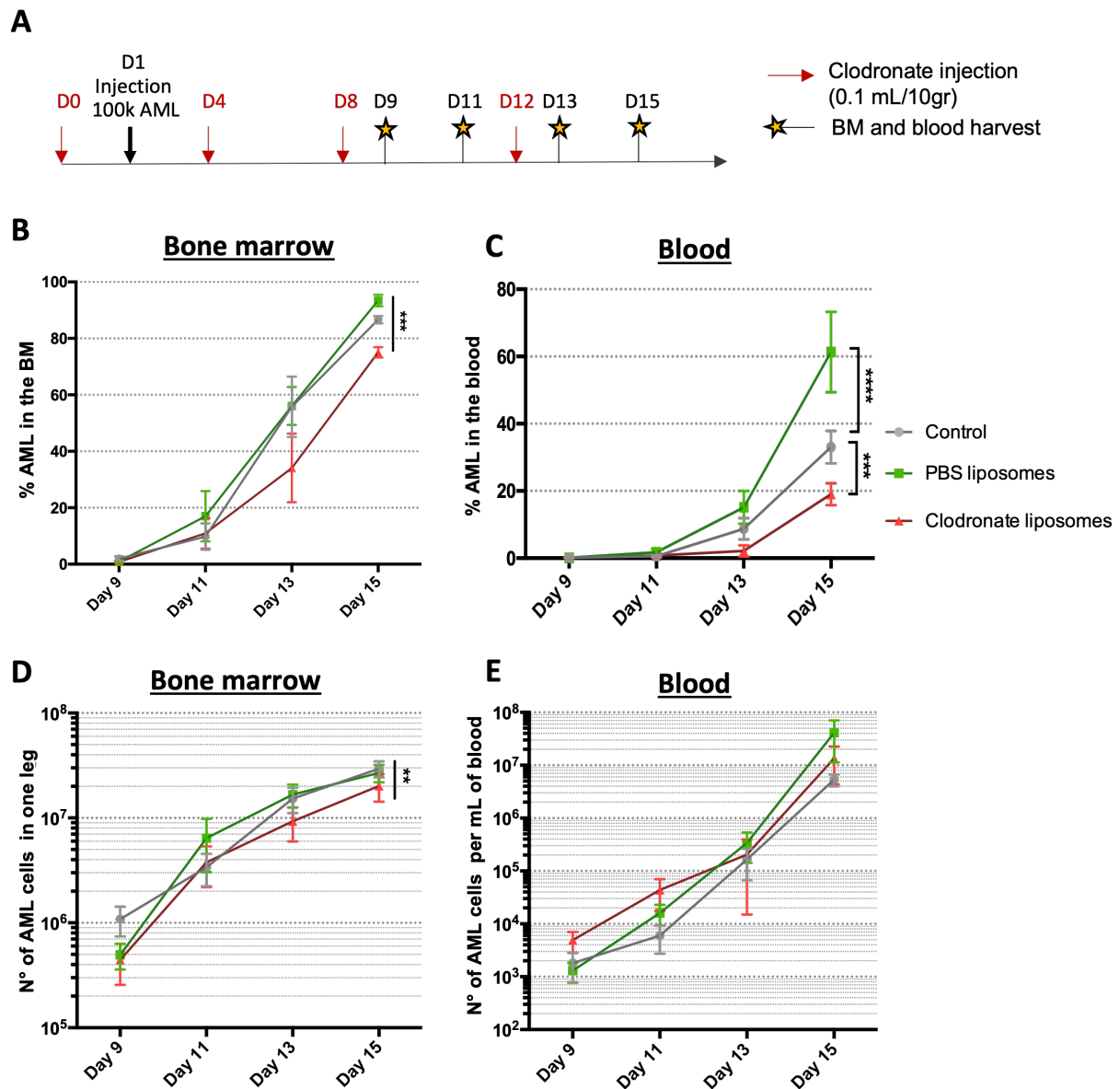


Figure 5.4: Clodronate depletion leads to reduced AML growth

Wild type mice were injected with clodronate liposomes, PBS liposomes or PBS every 4 days. At day 1, mice were transplanted with 100'000 mTmG AML cells. The BM was harvested at day 9, 11, 13 and 15 and analysed by flow cytometry to assess disease growth. (A) Experimental plan for the analysis of AML growth in clodronate liposome treated mice. (B), (C) Percentage of mTmG+ AML cells among total BM (B) and blood (C) cells. (D), (E) Absolute number of mTmG+ AML cells in the BM and blood respectively. Representative data of three independent experiments, dot plot indicates mean ($\pm$ s.e.m) with 3-5 mice per time points.

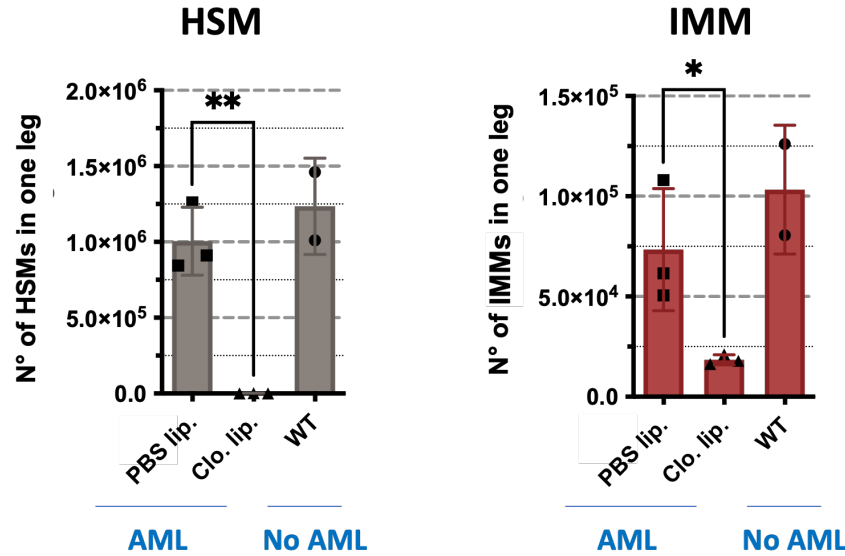


Figure 5.5: Loss of both macrophage population in clodronate treated mice with AML
Wild type mice were injected with clodronate liposomes, PBS liposomes or PBS every 4 days. At day 1, mice were transplanted with 100'000 mTmG AML cells. WT mice, not injected with AML were used as control. The BM was harvested at day 11 and analysed by flow cytometry to assess disease growth and macrophage profile. Absolute number of HSMs (grey) and IMMs (maroon) in the BM. Representative data of three independent experiments (for the AML conditions), dot plot indicates mean ($\pm$ s.e.m) with 3 mice for the AML conditions and 2 mice for the control conditions.

5.2.3 Proliferation rate is maintained but differentiation of AML is reduced in clodronate treated mice

A major concern based on the previous results is the possibility that the delayed AML growth may be the result of the clodronate treatment rather than the depletion of macrophages. Leukemogenesis is defined by a cell's acquisition of limitless proliferation potential and reduced differentiation. To address whether the reduced AML growth in clodronate treated mice was due to the loss of tumourigenic potential, I looked at the differentiation profile and proliferative state of the leukemic cells. As described in the previous chapter, this AML model contains a small proportion of 'macrophage-like' cells, defined as F4/80+ CD169+. If these cells are capable of phagocytosis, then clodronate treatment could lead to their apoptosis. The number of F4/80+ CD169+ AML cells was significantly reduced in clodronate treated mice compared to controls (Figure 5.6A - B). However, these cells represented less than 5% of total AML cells at anytime during disease progression. Hence, even if the differentiated cells were affected by clodronate treatment, this phenomenon couldn't explain the significant growth difference described previously. To understand whether the loss of macrophage-like AML cells was due to a direct effect of clodronate on the AML cells, I injected a single dose of clodronate 24 hours before injecting

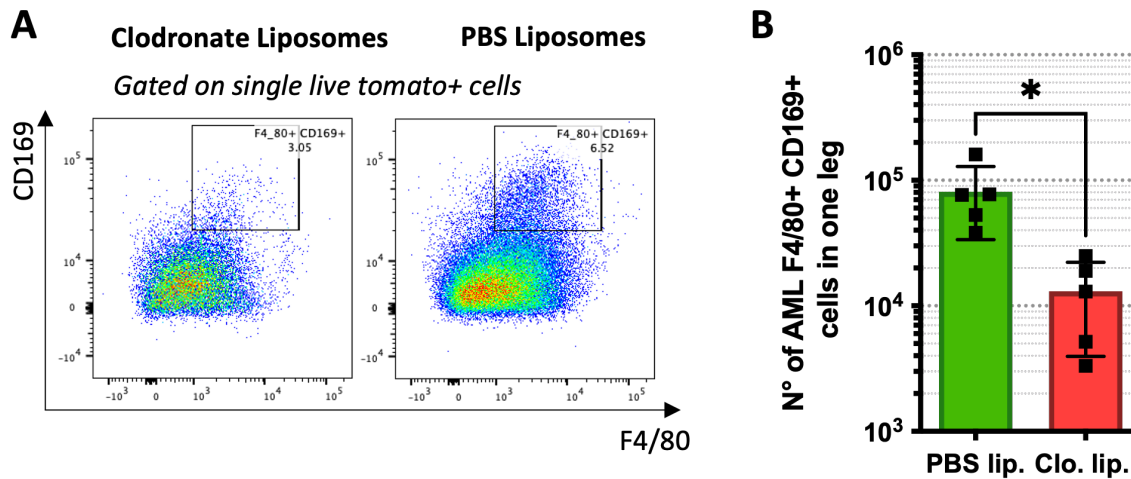


Figure 5.6: Reduced ‘malignant macrophages’ in clodronate treated mice

These results were produced with the same cohort of mice as described in Figure 5.5. (A) Representative FACS plot of the expression of F4/80+ and CD169+ among BM AML cells in clodronate treated (left) and PBS treated (right) mice. (B) Absolute number of F4/80+ CD169+ mTmG+ AML cells in the BM from one leg. Representative data of five independent experiments, dot plot indicates mean ($\pm$ s.e.m) with 5 mice per condition.

the AML cells. Indeed after 24 hours, it is known that clodronate is cleared from the system hence the drug can no longer act directly on these cells. I analysed the phenotype of the AML cells at the early disease stage (Figure 5.7A). Strikingly, the number of ‘macrophage-like’ AML cells was also significantly reduced in the single clodronate treated condition (Figure 5.7B-C), and this reduction was similar to the one noticed in repeated clodronate injection. Given that the clodronate was injected before the AML cells, the loss of macrophage-like AML cells must be linked to changes in the microenvironment rather than a direct effect on the AML cells. This result highlights the possibility that the AML differentiation towards a macrophage like phenotype does not arise from the normal differentiation of the cells but is in fact induced by the microenvironment. Hence, it is an interesting mechanism to keep in mind when developing therapies, if these cells become key in disease shaping.

Among AML cells, a loose hierarchy exist where the most progenitor-like cells, including LSCs, expressing c-Kit, a known marker of stem and progenitor cells [239]. To evaluate whether clodronate treatment affected more primitive AML cells, the fraction of cells expressing c-Kit was measured. Throughout AML growth, the percentage of c-Kit+ cells in all three groups was similar, and increased from 50% at early disease to 70% at late disease (Figure 5.8A). Since the percentage of c-Kit+ cells was unchanged in clodronate treated mice, clodronate treatment did not affect the progenitor-like population of leukemic cells.

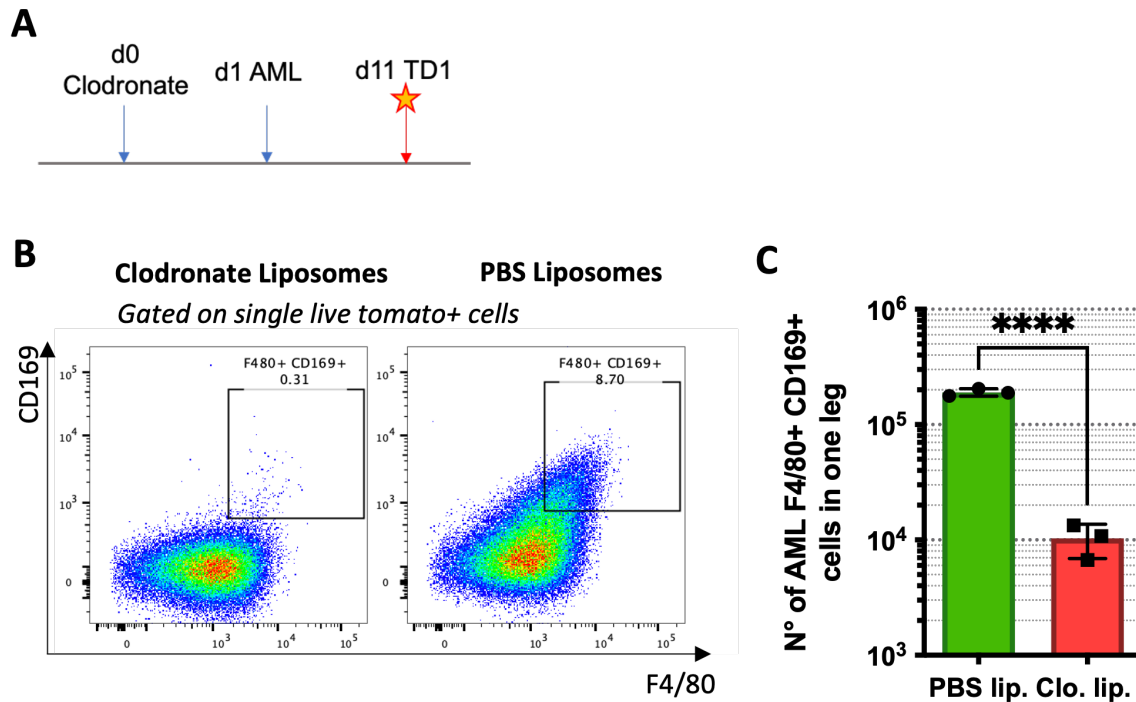


Figure 5.7: Similar change in AML differentiation in mice previously treated with clodronate. Wild type mice were injected with clodronate liposomes or PBS liposomes on day 0. At day 1, mice were transplanted with 100'000 mTmG AML cells. The BM was harvested at day 11 analysed by flow cytometry to assess disease phenotype (A). (B) Representative FACS plots of F4/80 and CD169 expression in mTmG+ AML cells in clodronate treated mice (Left) and PBS liposomes treated mice (Right). (C) Absolute number of F4/80+ CD169+ mTmG+ AML cells in the BM. Representative data of three independent experiments, dot plot indicates mean ($\pm$ s.e.m) with 3 mice per condition.

To answer the question of the loss of proliferative potential of leukemic cells, in the same experimental set-up, mice were injected with EdU two hours before tissues were harvested. EdU is a thymidine analogue which will incorporate in newly synthesised DNA strands during the S phase of the cell cycle. Through a click-it chemical reaction, EdU will be bound to a fluorescent dye and this signal will be measured by flow cytometry [195]. In this experiment, the EdU positive cells will correspond to the cells undergoing mitosis and therefore proliferating. The percentage of EdU+ cells was similar in all conditions and decreased as AML grew indifferently of the presence or not of clodronate (Figure 5.8B). Taken together, these data suggest that the leukaemia propagating capacity of AML cells was not affected by clodronate treatment. Although a small reduction in differentiated cells was noticed in clodronate treated mice, it couldn't account for the differential growth of leukemia in these mice. Hence, I hypothesized that the suppression of macrophages is responsible for the delayed AML growth.

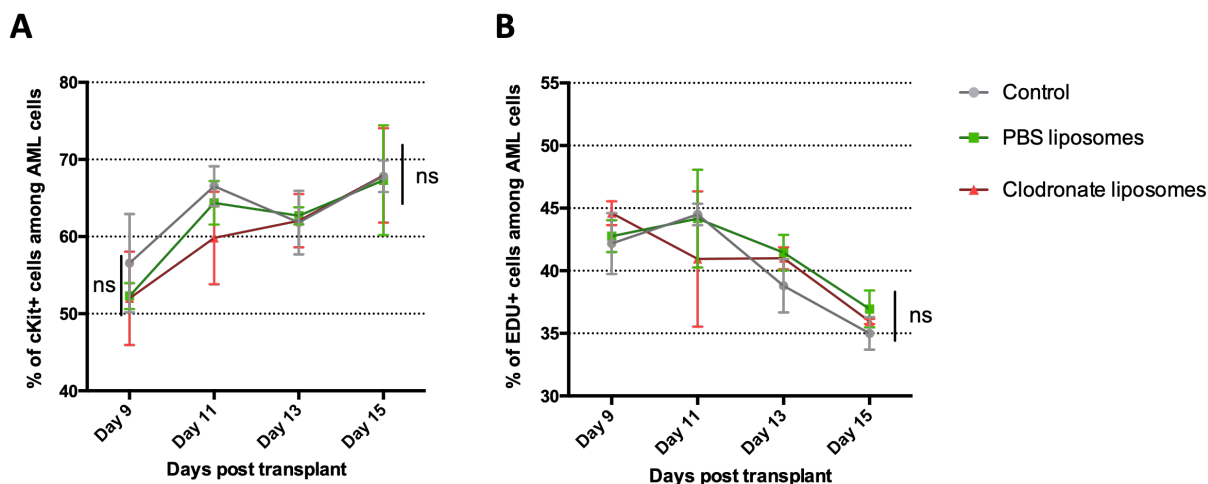


Figure 5.8: Clodronate does not affect primitive AML cells nor AML cells' proliferative state. *Wild type mice were injected with clodronate liposomes, PBS liposomes or PBS every 4 days. At day 1, mice were transplanted with 100'000 mTmG AML cells. The BM was harvested at day 9, 11, 13 and 15 and analysed by flow cytometry to assess AML cell's phenotype. (A) Percentage of cKit+ cells among mTmG+ AML+ cells in the BM. (B) 2 hours before harvesting, mice were injected with 100µl of EdU i.v.. BM proliferating cells (EdU+) were detected thanks to the click-it detection method. Percentage of EdU+ cells among mTmG AML+ cells. Dot plot indicates mean ($\pm$ s.e.m) with 3-5 mice per time points.*

5.2.4 Set-up of CD169-DTR genetic depletion model

As described in Chapter 3, the CD169-DTR model is a well-established model to study the role of CD169+ macrophages in the bone marrow. The administration of DT leads to a strong depletion of these cells in the bone marrow with little toxicity. Given that IMMs don't express CD169, I hypothesize that these cells will not be affected by DT treatment. In addition, given that this is a genetic depletion model, I can be certain that leukemic cells, generated in WT mice and therefore not expressing DTR, are not directly affected by the administration of DT. Hence, combining these results with the clodronate liposomes results could allow us to identify which macrophage population is responsible for the reduced AML growth seen in the clodronate treated mice.

Although I have already confirmed the drug regimen to deplete macrophages for 48 hours [52], in the context of AML I need to be able to deplete macrophages for an extended period of time (15-20 days). Therefore repeated injections are necessary and these can lead to toxicity. P. Gupta et al., [259] used the CD169-DTR model to deplete macrophages for 25 days in the spleen, liver, lung and kidney. Based on their drug regimen, mice were injected i.p with DT (10ng/g) for two consecutive days followed by repeated injections every 3-4 days (Figure

5.9A). CD169-DTR- littermates were also treated with DT as negative control. Given these are transgenic animals that I need to breed, fewer animals were available compared to WT mice. Hence, I was not able to sacrifice mice every other day like in the clodronate experiment. Choosing 3 strategic time points, I sacrificed mice on day 2, 7 and 16. These time points allowed me to check first that I was depleting CD169+ macrophages from the start, then that I manage to maintain this depletion through repeated injections every 3-4 days, and finally that no toxicity is generated up to the end of the treatment. By day 16, the spleens did not show any signs of toxicity, their size was similar to that of control spleens and their stiffness was unchanged (Figure 5.9B-C). Hence, I did not analyse the spleens further. The absolute number of cells in the bone marrow was also similar to that of control mice and the percentage of live cells was between 90% and 100% throughout the experiment (Figure 5.9D-E). Together, these results confirmed that no toxicity was induced by repeated injections of DT.

Next, to assess whether the administration of DT to CD169-DTR^{het} mice led to specific macrophage depletion, the absolute number of both macrophage cell populations was measured by flow cytometry. 2 days after the start of the treatment, HSMs were reduced by 40-fold and remained suppressed at this level until day 16 (Figure 5.10A-B). However, IMMs were only slightly reduced at day 2, and their number increased by day 7 and remained similar to that of control mice until the end of the experiment (Figure 5.10A,C). This correlates with the lack of phenotypic expression of CD169 in these cells and confirms that the sensitivity of flow cytometry for CD169 is similar to that of the DT treatment. Given the similarity between monocytes, neutrophils and macrophages, it was necessary to check whether these cells were affected by the activation of the DT receptor. While monocytes seemed slightly increased at day 2, their numbers reduced by day 7 and stabilised at day 16 (Figure 5.10D). As for neutrophils, their numbers were similar to control throughout (Figure 5.10E). Hence, monocytes and neutrophils were not significantly altered following the DT treatment. Altogether, following 16 days of treatment, the CD169-DTR mice did not show any toxicity and HSMs were efficiently depleted without affecting IMMs and any other myeloid cells, confirming that this a good model to study the role of HSMs in AML growth.

5.2.5 Increased AML growth in CD169-DTR^{het} mice

Now that the drug regimen was tested and showed a strong depletion of HSMs without impacting IMMs, HSMs depletion could be induced prior to and following transplantation of AML

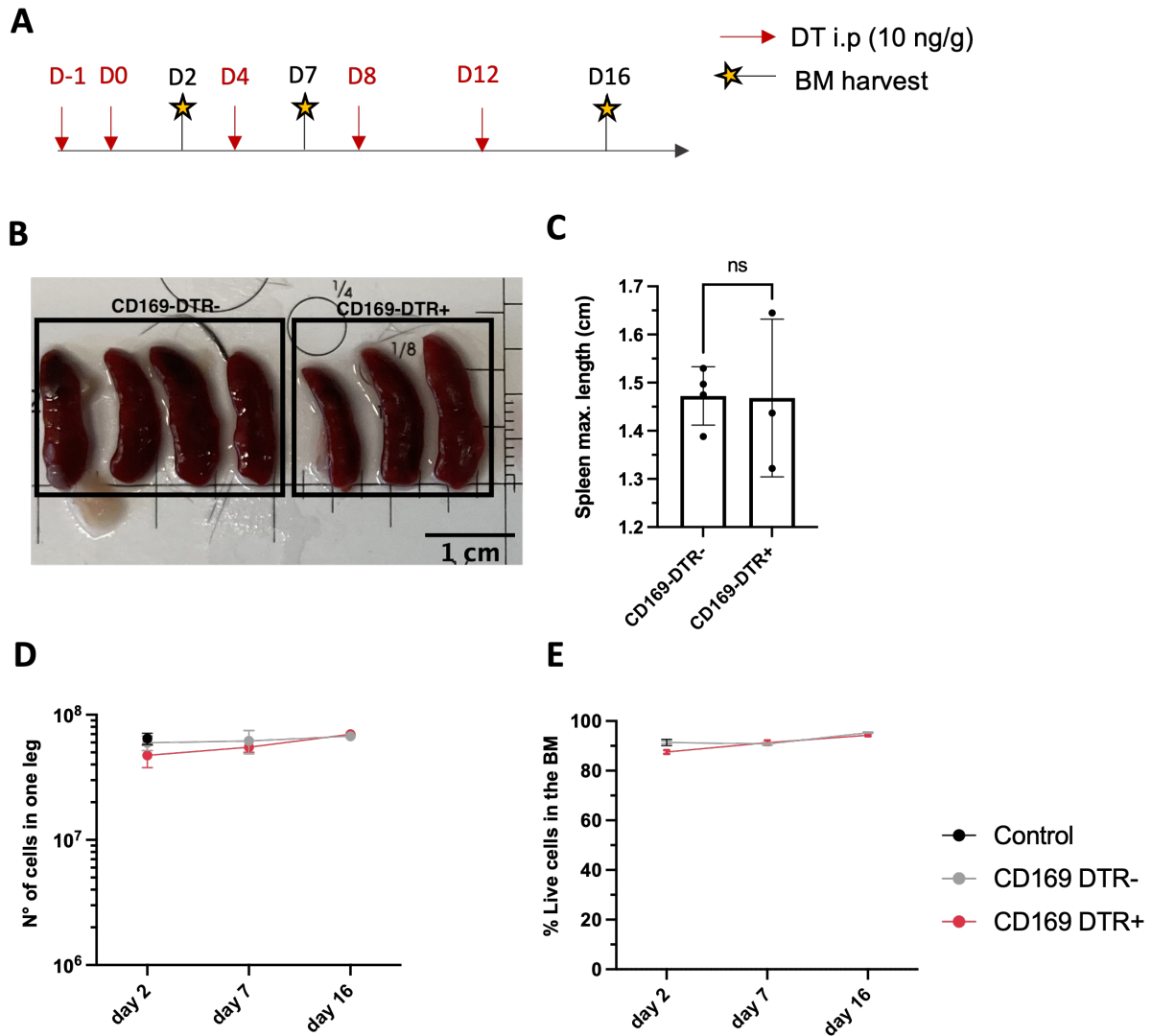
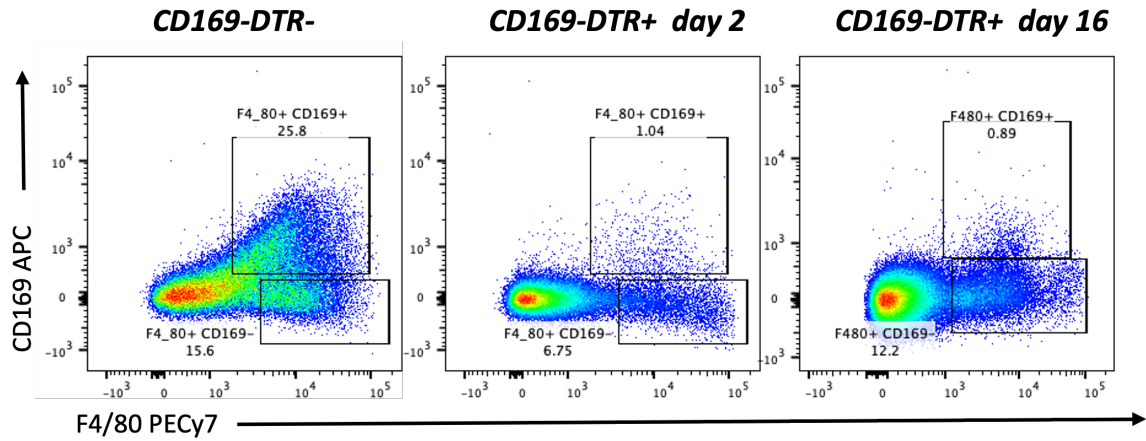


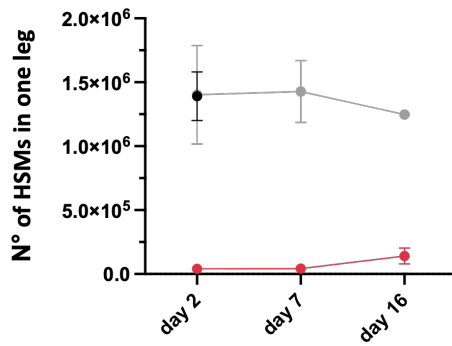
Figure 5.9: Repeated DT injections does not lead to toxicity in the spleen or bone marrow *CD169-DTR*⁻ and *CD169-DTR*^{het} mice were injected i.p. with 10ng/g DT. **(A)** Experimental protocol for macrophage depletion over 16 days. DT was administered two days in a row, followed by repeated injections every 4 days and BM was harvested on day 2, 7 and 16. **(B)** Pictures of the spleens at day 16 of the treatment. **(C)** Spleen lengths of *CD169-DTR*⁻ and *CD169-DTR*^{het} at day 16. No differences in the spleen size and stiffness was noticed hence it was not further analysed. **(D)** Absolute number and **(E)** percentage of live cells in the BM. Dot plot indicate mean ($\pm$ s.e.m) with 2-3 mice per time points.

A

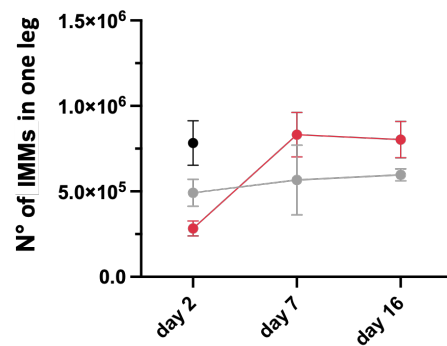
Gated on single, live, CD11b⁺, Ly6G^{lo}, Ly6C^{lo}, SSCA^{lo} cells:



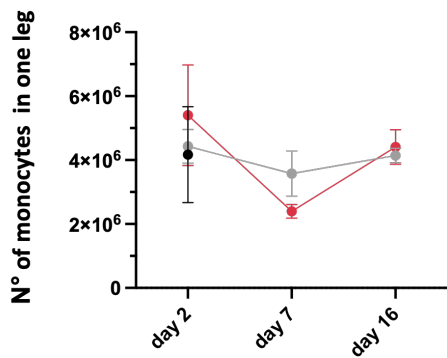
B



C



D



E

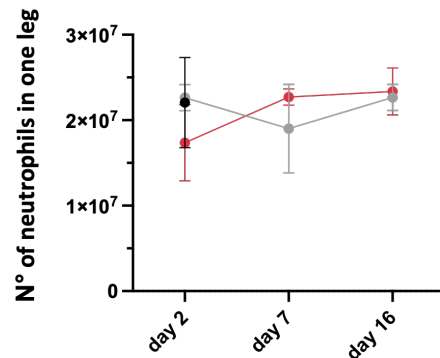


Figure 5.10: Repeated DT injections leads to depletion of macrophages in the CD169-DTR mouse model

These results were produced with the same cohort of mice as described in Figure 5.9. (A) Representative *FACS* plots of F4/80 versus CD169 expression in single, live, CD11b⁺, Ly6G⁻, Ly6C^{lo}, SSCA^{lo} cells, from CD169-DTR⁻ or CD169-DTR^{het} treated mice, two days after the first injection and 16 days after the start of the injections. Absolute number of (B) HSMs, (C) IMMs, (D) monocytes and (E) neutrophils in the BM from one leg. Error bars represent the mean $\pm$ s.e.m. with 2-3 mice analysed per time point.

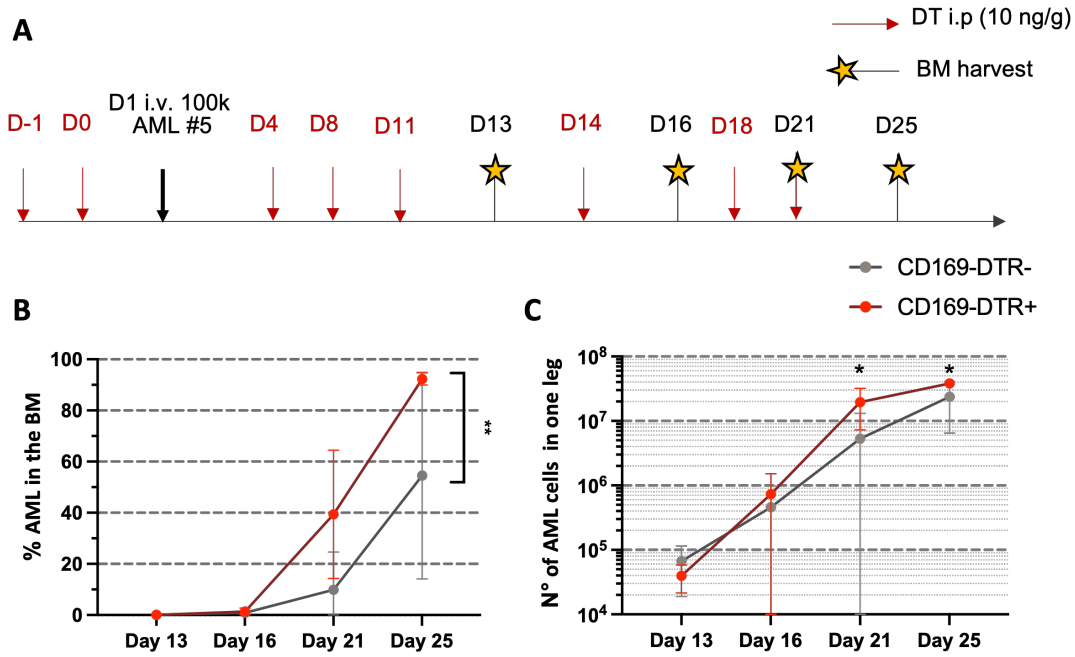


Figure 5.11: CD169-DTR depletion leads to increased AML growth

CD169-DTR⁻ and CD169-DTR^{het} mice were injected with DT on day -1, 0 and every four days after that. At day 1, mice were transplanted with 100'000 mTmG AML cells. The BM was harvested at day 13, 16, 21 and 25 and analysed by flow cytometry to assess disease growth. (A) Experimental plan for the analysis of AML growth in CD169-DTR treated mice. (B) Percentage of mTmG+ AML cells among total BM cells. (C) Absolute number of mTmG+ AML cells in the BM. Representative data of two independent experiments, dot plot indicates mean ($\pm$ s.e.m) with 5-6 mice per time points.

cells and correlate these results with the clodronate depletion model. Following the drug regimen described in section 5.2.4, CD169-DTR^{het} and CD169-DTR⁻ mice were injected with DT on day -1 and day 0. mTmG+ AML cells were transplanted on day 1 and DT was injected every 3-4 days. The BM was harvested at day 13, 16, 21 and 25 to evaluate the growth of AML cells (Figure 5.11A). Unexpectedly, the percentage of AML cells was significantly increased in the BM of CD169-DTR^{het} mice compared to CD169-DTR⁻ (Figure 5.11B). The difference in AML percentage increased as the disease progressed. This difference was also reflected in the absolute number of AML cells in the bone marrow (Figure 5.11C). To confirm that only HSMs were affected by DT in CD169-DTR^{het} mice, the absolute number of HSMs and IMMs was measured at early AML. To control for the impact of AML on each population, the absolute number of cells was also measured in control mice without AML. Strikingly, while HSMs were significantly decreased in CD169-DTR^{het} mice, the IMMs were significantly increased in these mice (Figure 5.12). Hence, DT treatment in CD169-DTR^{het} mice led to increased AML growth, depletion of HSMs and increase of IMMs.

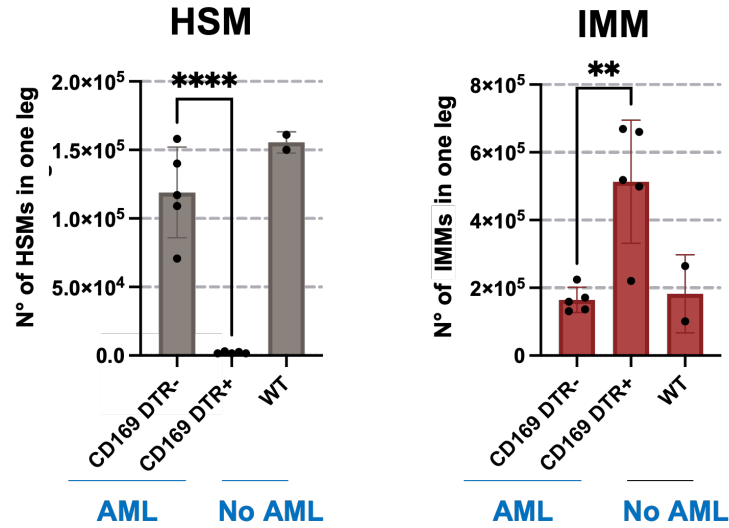


Figure 5.12: CD169-DTR depletion leads to increase in IMMs in AML *CD169-DTR⁻* and *CD169-DTR^{het}* mice were injected with DT on day -1, 0 and every four days after that. At day 1, mice were transplanted with 100'000 mTmG AML cells. WT mice, not injected with AML were used as control. The BM was harvested at day 16 and analysed by flow cytometry to assess disease growth and macrophage profile. Absolute number of HSMs (grey) and IMMs (maroon) in the BM. Representative data of two independent experiments, dot plot indicates mean ($\pm$ s.e.m) with 5 mice for the AML conditions and 2 mice for the control WT condition.

To better compare these results with the clodronate liposomes experiments, I summarised the macrophage dynamics with the two treatments in a table (Figure 5.13). The main difference lies in the IMMs population. Interestingly, when looking at the IMMs after either treatment in non-leukemic mice, this population was not altered (Figure 5.2C and 5.10C) meaning that this difference is due to the presence of the leukemic cells.

These results indicate that in the *CD169-DTR^{het}* mice, AML induces the production of IMMs, while in clodronate treated mice, AML leads to the reduction of IMMs. In general, AML was described to push healthy cells out of the bone marrow [238, 248] leading to the loss of healthy cells (Chapter 4 Figure 2A). The loss of IMMs in AML mice treated with clodronate is consistent with this observation. Hence, the unexpected result is the increase in IMMs in the *CD169-DTR^{het}* mice. One explanation for this increase is that the depletion of HSM leads to the recruitment of monocytes to replenish this pool of macrophages. However, the AML microenvironment generates some inflammatory signalling and the cells recruited may be hijacked to differentiate towards IMMs. Given their phenotype and transcriptional signature, I speculated that these cells were involved in the phagocytosis of AML cells and the regulation of the immune response.

		Macrophage populations		AML growth
		HSMs	IMMs	
No AML	Wild - type	✓	✓	
	Clodronate treated	X	✓	
	CD169-DTR, DT treated	X	✓	
With AML	Wild - type	✓	✓	-
	Clodronate treated	X	X	↓
	CD169-DTR, DT treated	X	↑↑	↑

Figure 5.13: Summary of the differences between the two macrophage depletion models
In both clodronate treatment and CD169-DTR activation, HSMs are depleted and IMMs are unaffected. In AML, IMMs are depleted with clodronate and enriched with CD169-DTR activation. These differences are associated with opposite effect on AML growth. ✓ is unaffected, X is depleted, ↑ is increased and ↓ is reduced

Given that the presence of IMMs correlated with increased AML growth, and in the single cell RNA sequencing, these cells had upregulated phagocytic and efferocytic genes, next, I questioned whether IMMs were capable of promoting AML growth through phagocytosis of AML cells and inhibition of anti-tumour signaling.

5.3 Macrophages are capable of phagocytosing AML cells

5.3.1 IVM reveals the uptake of AML cells by macrophages *in vivo*

One of the most common assay to measure phagocytosis is to fluorescently label cancer cells and measure the uptake of the dye by macrophages 24 hours later [260]. However, this type of experiment is mainly performed *in vitro*, and no equivalent protocol has been published to assess the phagocytic capacity of macrophages *in vivo*. However, given the fact that our experimental model of AML was set up to allow for intravital microscopy analyses, I wondered if through live imaging of macrophages and AML cells *in vivo*, I would be able to visualise phagocytosis of AML cells by IMMs *in vivo* in real time.

Hence, I performed intravital microscopy on CD169-YFP mice injected with mTmG+ AML

11 days before imaging to see if the YFP+ cells would phagocytose the mTmG+ AML cells. Interestingly, by imaging for two hours every two minutes, I noticed that, while CD169+ cells were not moving, they managed to engulf AML cells and the tomato signal would remain inside the YFP+ cells throughout the imaging session (Figure 5.14A for screenshots of time points and Supplementary Video 5 for the whole timelapse).

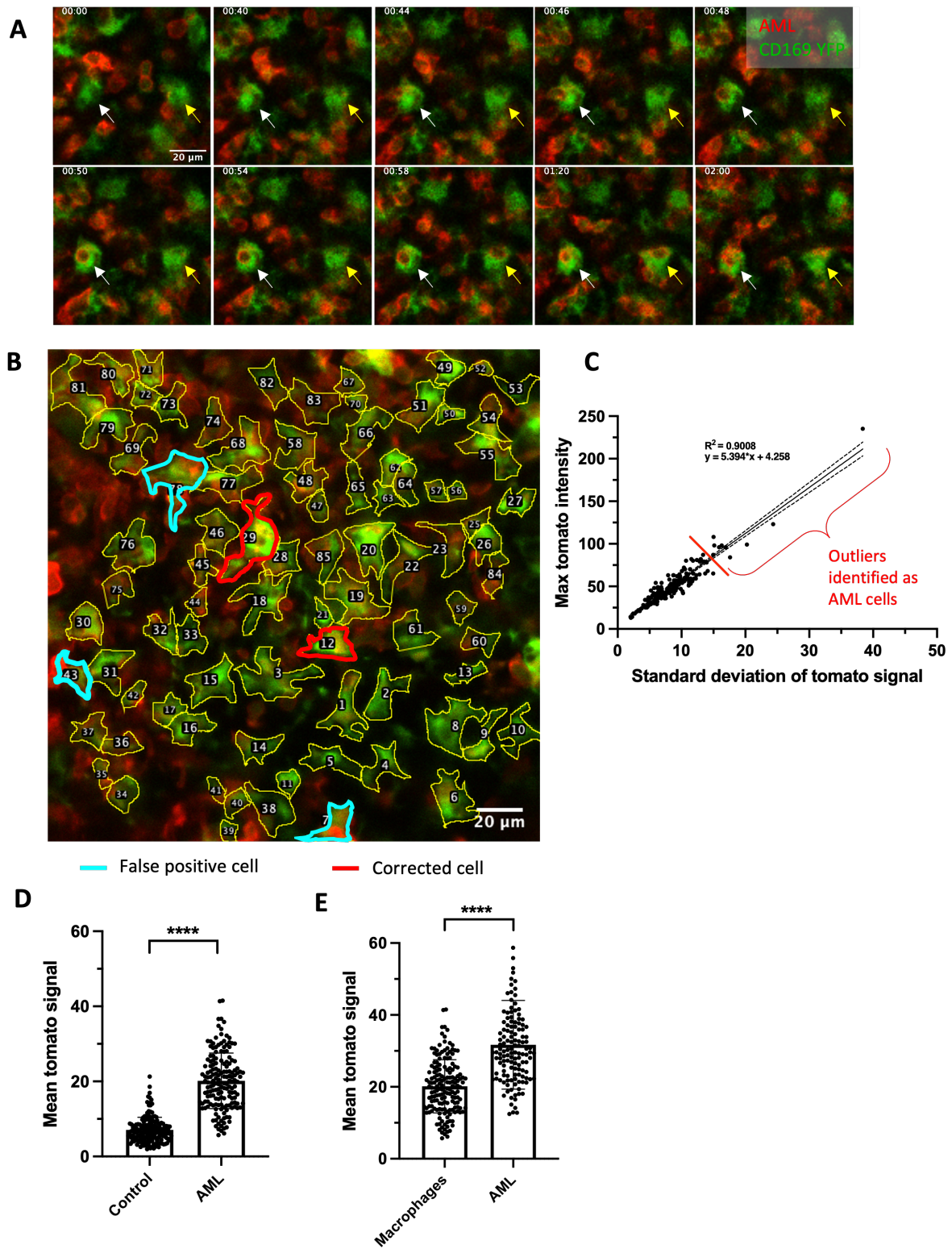


Figure 5.14 (previous page): IVM reveals macrophages uptake tomato+ cells

*IVM of CD169-YFP mice transplanted with 100'000 mTmG+ AML cells. CD169-YFP mice without transplanted AML were used as negative control. At day 11, SIRP α -APC antibody was injected i.v. 30 minutes before image acquisition. (A) Representative screenshots of maximum projections of calvarium bone marrow from 2 hour timelapse showing AML cells (red) and CD169-YFP+ cells (green). Two CD169-YFP+ cells phagocytosing tomato+ AML cells are highlighted with the white and yellow arrows. (B) Quantification of tomato signal in CD169-YFP+ cells required manually outlining each CD169-YFP+ macrophage and AML cell. (C) To exclude tomato signal from AML cells located in a different plane, the correlation of the maximum tomato intensity and standard deviation of tomato signal of each CD169-YFP+ cell was plotted. Cells that were located above the threshold outlined in red were considered false positive and excluded from the analysis. These cells were analysed to address if the signal came from a tomato cells located in a different plane (cyan line in (B)) or corresponded to bright CD169-YFP+ cells with bright tomato signal (red line in (B)). (D) Mean tomato intensity of CD169-YFP+ cells from control and AML mice. (E) Mean tomato intensity of CD169-YFP+ cells and AML cells from AML mice. 174 macrophages from the AML condition, 171 macrophages from the control condition and 132 AML cells were outlined, from 3 independent positions from 1 AML mouse and 2 control mice. Statistically significant differences were determined using Student t-tests (**** represents $p < 0.0001$).*

One concern was the fact that the AML cells could be located in a different plane and were not actually inside the cell. To overcome this, I first manually outlined each YFP+ cell and measured the maximum intensity and the standard deviation of the tomato signal within each cell (Figure 5.14B). Indeed, in the case where the cells were taken up by macrophages, the tomato signal was seen to diffuse throughout the cytoplasm and the standard deviation of the tomato intensity signal within the cell should be small. However, if the tomato signal comes from an AML cell that is superimposed, then the colocalization of both signals would be small and therefore the standard deviation should be much bigger. In addition, it was noticed that the tomato signal from the leukemic cells was higher than the signal found inside the macrophage hence, it was expected that if the tomato signal came from the superimposition of two different cells then most likely the tomato signal would be brighter. To test this hypothesis, I correlated the standard deviation of the tomato signal with the maximum intensity. As expected, the correlation was linear and the cells with the highest standard deviation were also the cells with the brightest tomato pixels (Figure 5.14C). When looking at the values plotted, I identified a break in the values (red line on Figure 5.14C) and used that as a threshold above which it was considered that the tomato signal could come from the overlay of a tomato cell with a YFP+ cell. 10 cells out of the 174 cells that were outlined were considered as false positives. Using the full length of the movie, I then checked whether the tomato signal was coming from a distinct cell (Figure 5.14B, cells outlined in cyan) or if these were just very bright cells (Figure 5.14B,

cells outlined in red). 2 cells were reclassified as real positive after this analysis. Hence, overall 8 cells were excluded from further analysis.

Finally, to address whether the tomato signal came from macrophages taking up AML cells or background signal, the mean intensity of the tomato signal contained inside each manually outlined CD169+ cell from the AML mice was compared to the signal found in control CD169-YFP+ mice. As expected, the mean intensity was significantly higher in macrophages from leukemic mice compared to control (Figure 5.14D). In addition, the tomato mean intensity was lower than the signal from mTmG+ AML cells (Figure 5.14E) confirming that the signal had been degraded and therefore suggesting that macrophages were phagocytosing the AML cells, leading to protein degradation.

Next, to elucidate which macrophage population was responsible for the phagocytosis of AML cells, I injected APC conjugated anti-SIRP α antibody and correlated the level of tomato signal with the intensity of SIRP α expression by the CD169 cells. When looking at each channel separately, it was clear that the SIRP α signal correlated with the YFP signal and more precisely the brightest tomato-containing CD169+ cells were the brightest SIRP α + cells (Figure 5.15A). To quantify the correlation between the SIRP α signal and the tomato signal in YFP+ cells, I first generated a histogram of the tomato signal contained inside the CD169+ cells and classified the cells as tomato-, tomato^{lo} and tomato^{hi} (Figure 5.15B). I then measured the mean intensity of the SIRP α signal in each population. As expected, the mean intensity of the SIRP α signal was significantly higher in the tomato^{hi} CD169+ cells compared to the other populations (Figure 5.15C). Next I performed the opposite analysis whereby the YFP+ cells were classified according to their expression of SIRP α . Again, three categories were generated, SIRP α -, SIRP α ^{lo} and SIRP α ^{hi} based on the histogram of the SIRP α signal in the YFP+ cells (Figure 5.15D). This time I compared the mean intensity of the tomato signal in these three cell populations. Consistent with the previous analysis, the mean intensity of the tomato signal was significantly higher in SIRP α ^{hi} cells compared to the other two populations (Figure 5.15E).

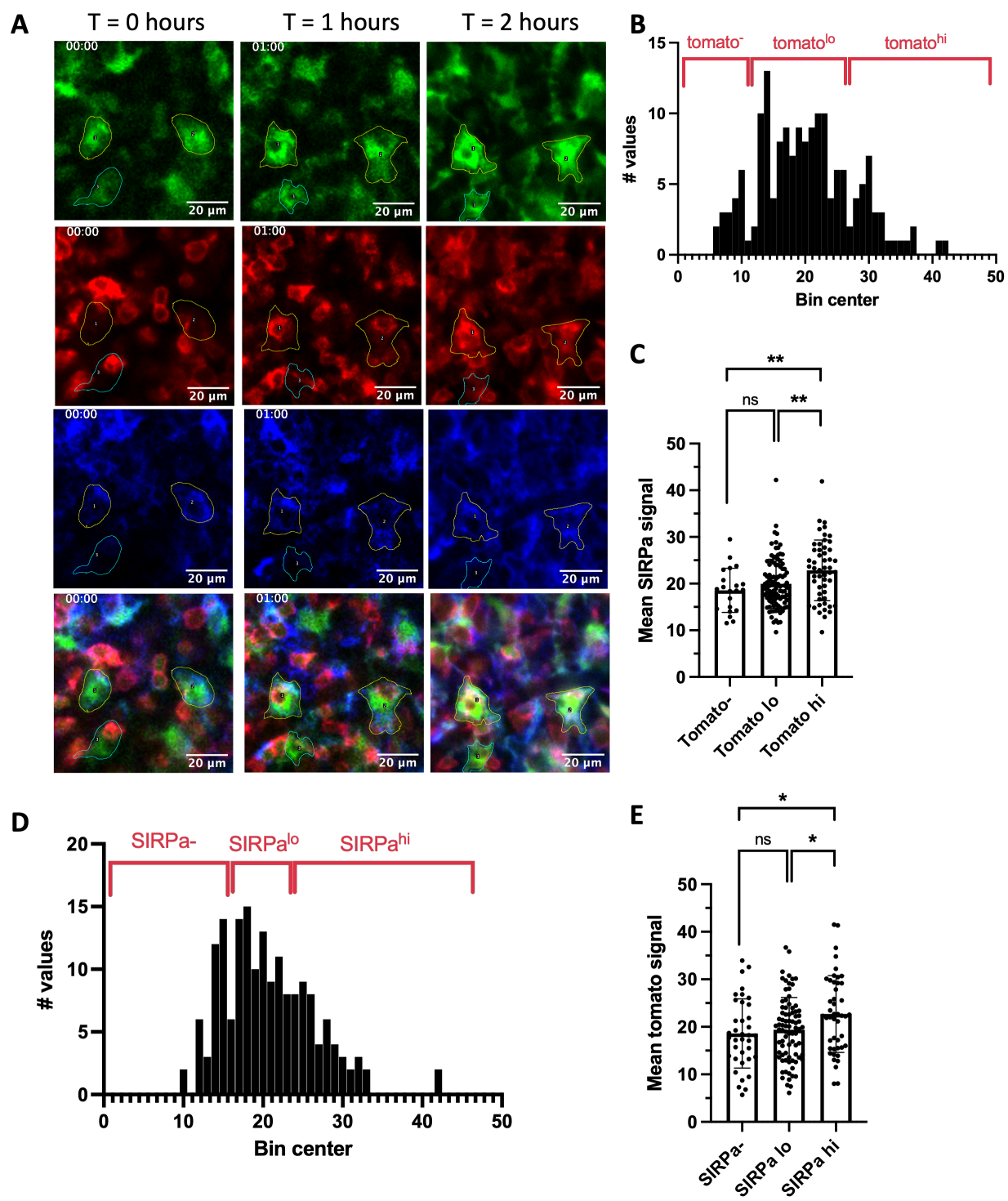


Figure 5.15 (*previous page*): IVM reveals SIRP α + macrophage as the main contributor to the uptake of tomato+ cells

*These results were produced with the same cohort of mice as described in Figure 5.14(A) Representative screenshots of maximum projections of calvarium bone marrow from the 2 hour time-lapse showing AML cells (red), CD169-YFP+ cells (green) and SIRP α signal (blue). SIRP α signal colocalised with CD169-YFP+ cells containing tomato signal. (B) Histogram of tomato mean intensity in CD169-YFP+ cells. The histogram could be divided in three sections, tomato-cells, tomato^{lo} cells and tomato^{hi} cells. (C) SIRP α mean intensity in CD169-YFP cells according to their tomato classification. The mean SIRP α intensity increased in tomato^{hi} CD169-YFP+ cells. (D) Histogram of SIRP α mean intensity in CD169-YFP+ cells. The histogram could be divided in three sections, SIRP α - cells, SIRP α ^{lo} cells and SIRP α ^{hi} cells. (E) Tomato mean intensity in CD169-YFP cells according to their SIRP α classification. The mean tomato intensity was significantly higher in SIRP α ^{hi} CD169-YFP+ cells. 174 macrophages from the AML condition were outlined, from 3 independent positions from 1 AML mouse. Statistically significant differences were determined using Student t-tests (* represents $p < 0.05$; ** represents $p < 0.01$).*

Hence, the SIRP α + CD169-YFP+ cells have a higher tomato signal than the other CD169-YFP+ cells. Several hypotheses can explain this, first it could be that SIRP α + macrophages are more phagocytic than the other sub-populations of macrophages. Another explanation is that once the mTmG+ AML cell is phagocytosed by a macrophage, this leads to the upregulation of SIRP α as this is an inhibitory receptor. The last possibility is that the SIRP α + macrophages have a reduced proteolytic capacity and therefore the tomato signal does not get degraded as quickly as in the SIRP α lo population. To evaluate which hypothesis is most likely to explain this difference, I decided to perform *in vitro* phagocytosis assays.

5.3.2 IMMs are responsible for the phagocytosis of AML cells

In order to address whether one sub-population of macrophages was more phagocytic than the others and correlate these findings with the previously identified macrophage populations, I sorted 3 populations: HSMs, IMMs and Ly6C^{hi} monocytes. Of note, the Ly6C^{hi} cells did not express F4/80, CD169, SIRP α or [PDL-1](#). These cells were sorted because certain macrophages can derive from monocytes and this would help us understand which macrophages could be generated from monocytes. I then co-cultured these cells with [CTV](#)-labelled AML cells to perform a phagocytosis assay.

Interestingly, after 24 hours of co-culture, the Ly6C^{hi} cells had differentiated towards the IMM macrophage population, had upregulated expression of F4/80, SIRP α and PDL1, but did

not express CD169 (Figure 5.16 A-F). To evaluate whether I could consider these cells as IMMs, I applied the gating strategy used to identify IMMs to all three cultures, having gated out the AML cells first. Excitingly, over 80% of the non-AML cells present in the Ly6C derived culture fell in the IMMs gating strategy, a percentage similar to the one found in the IMMs culture (Figure 15B). On the contrary, the HSMs cultures did not upregulate SIRP α or PDL1, however they did start downregulating CD169 (Figure 5.16A, D). In addition, after 24 hours, the expression of Ly6C in the Ly6C derived cells had been downregulated to the level of expression found in HSMs cultures and, after 48 hours, their expression was lower than the expression in HSMs. However, it was still significantly higher than the expression in the IMMs (Figure 5.16G, H). To understand whether this differentiation was induced by the AML cells, I compared the percentage of macrophage-like cells in the Ly6C sorted cultures alone and in co-culture with AML cells. Interestingly, I found that the percentage of macrophage like cells was similar in both cultures (Figure 5.16I), indicating that these cells would naturally differentiate towards this macrophage sub-population in this culture condition.

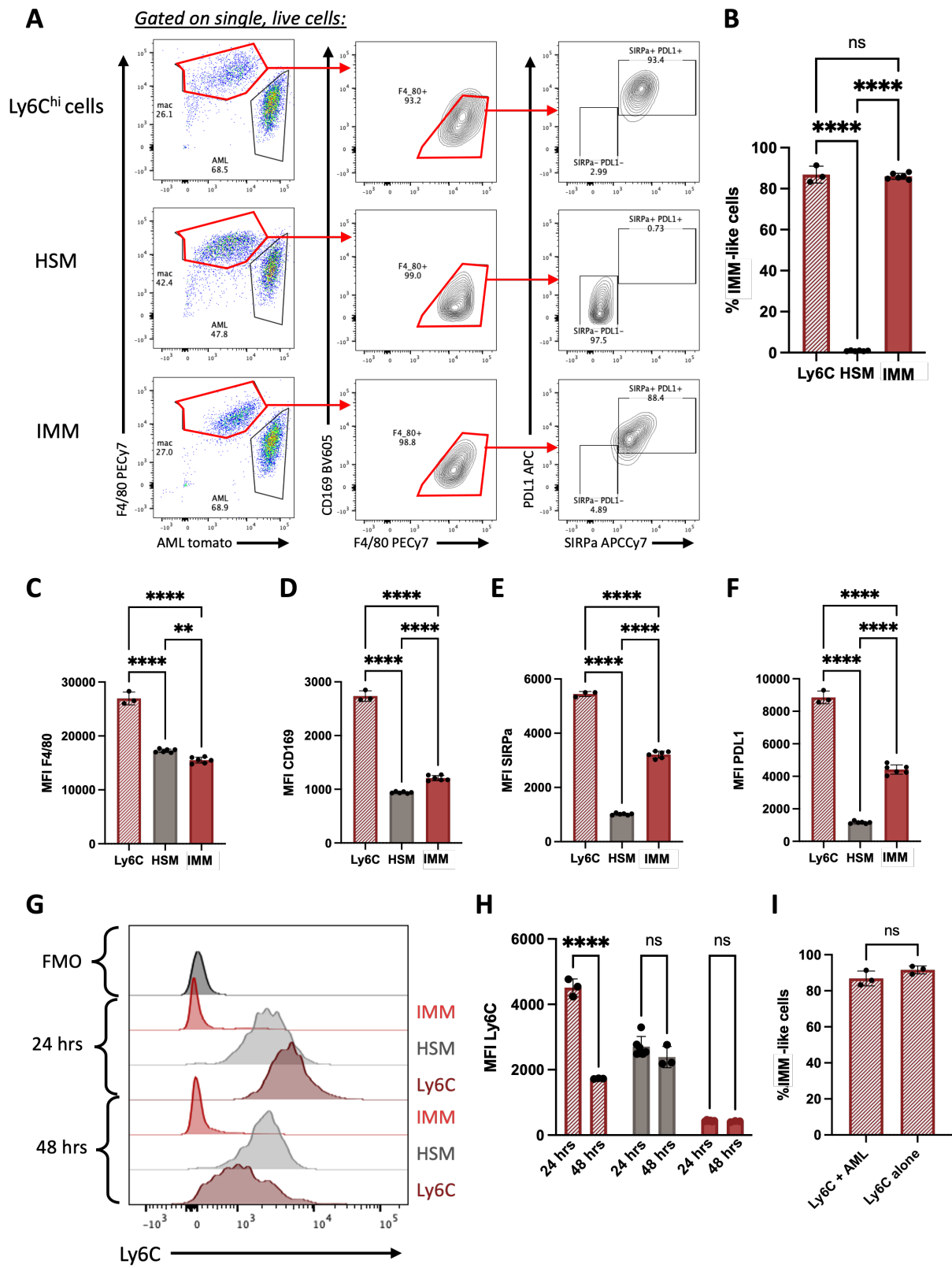


Figure 5.16 (previous page): Ly6C^{hi} monocytes give rise to IMMs *in vitro*
 Ly6C^{hi}, IMMs and HSMs were sorted from WT mice. Sorted populations were co-cultured with AML cells (or alone as a control) for 24 or 48 hours, in macrophage media. After co-culture, cells were stained and analysed by flow cytometry. **(A)** Representative FACS plot of the three sorted population's phenotype after 24 hours of co-culture. AML cells were gated out based on their expression of tomato. **(B)** Percentage of IMM-like cells (identified as F4/80+ CD169- SIRPα+ PDL1+) among the sorted cells after 24 hours of co-culture. **(C) - (F)** Mean fluorescence intensity of **(C)** F4/80, **(D)** CD169, **(E)** SIRPα and **(F)** PDL1, at 24 hours. **(G)** Histogram of the expression of Ly6C after 24 and 48 hours in the sorted populations. **(H)** Mean fluorescence intensity of Ly6C after 24 and 48 hours in the sorted populations. **(I)** Percentage of IMML-like cells in the Ly6C sorted cells, cultured alone or with AML cells. $n = 3$ for Ly6C cells, $n = 6$ for HSMs and IMMs, representative of two independent experiments. Error bars represent the mean $\pm$ s.e.m. Statistically significant differences were determined using Student *t*-tests (* represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$)

Finally, under the microscope, the Ly6C+ sorted cells had adopted an elongated morphology and developed dendrites, identical to the morphology of the IMMs. Hence, Ly6C+ monocytes differentiated towards IMMs after 48 hours. Given the difficulty to sort a decent number of IMMs as these represent a very small proportion of cells in the bone marrow, Ly6Chi cells were used as a source of differentiated IMMs. These cells will be called Ly6C-derived IMMs to distinguish them from directly sorted IMMs.

Next, I wanted to assess whether one macrophage sub-population was more involved in the phagocytosis of the AML cells than the other population. Since the AML cells had been labelled with CTV prior to co-culture, I simply needed to measure the intensity of the CTV signal in the macrophages after 24 hours of co-culture with AML cells, by flow cytometry. Interestingly, the HSMs did not contain any CTV signal while the Ly6C-derived IMMs and the directly sorted IMMs both contained some CTV (Figure 5.17 A). The percentage of CTV+ cells correlated with the ratio of AML to macrophages cells, the higher the number of AML cells relative to macrophages, the higher the percentage of CTV+ macrophages present in the culture (Figure 5.17 B); going from 40% of CTV+ cells in the culture at a ratio of 2 Ly6C derived cells for 1 AML cell all the way to 60% when the ratio was 1:2. Since this was the ratio with the highest phagocytosis, I compared the proportion of CTV+ cells from each sorted cell population at this ratio. The percentage of CTV+ cells was significantly higher in the Ly6C derived culture compared to the other conditions, with over 60% of the Ly6C derived cells being positive while in the HSMs only 1% of the cells were positive and in the IMMs approximately 10% of the cells were labelled with CTV (Figure 5.17 C). These results correlated with the mean intensity of

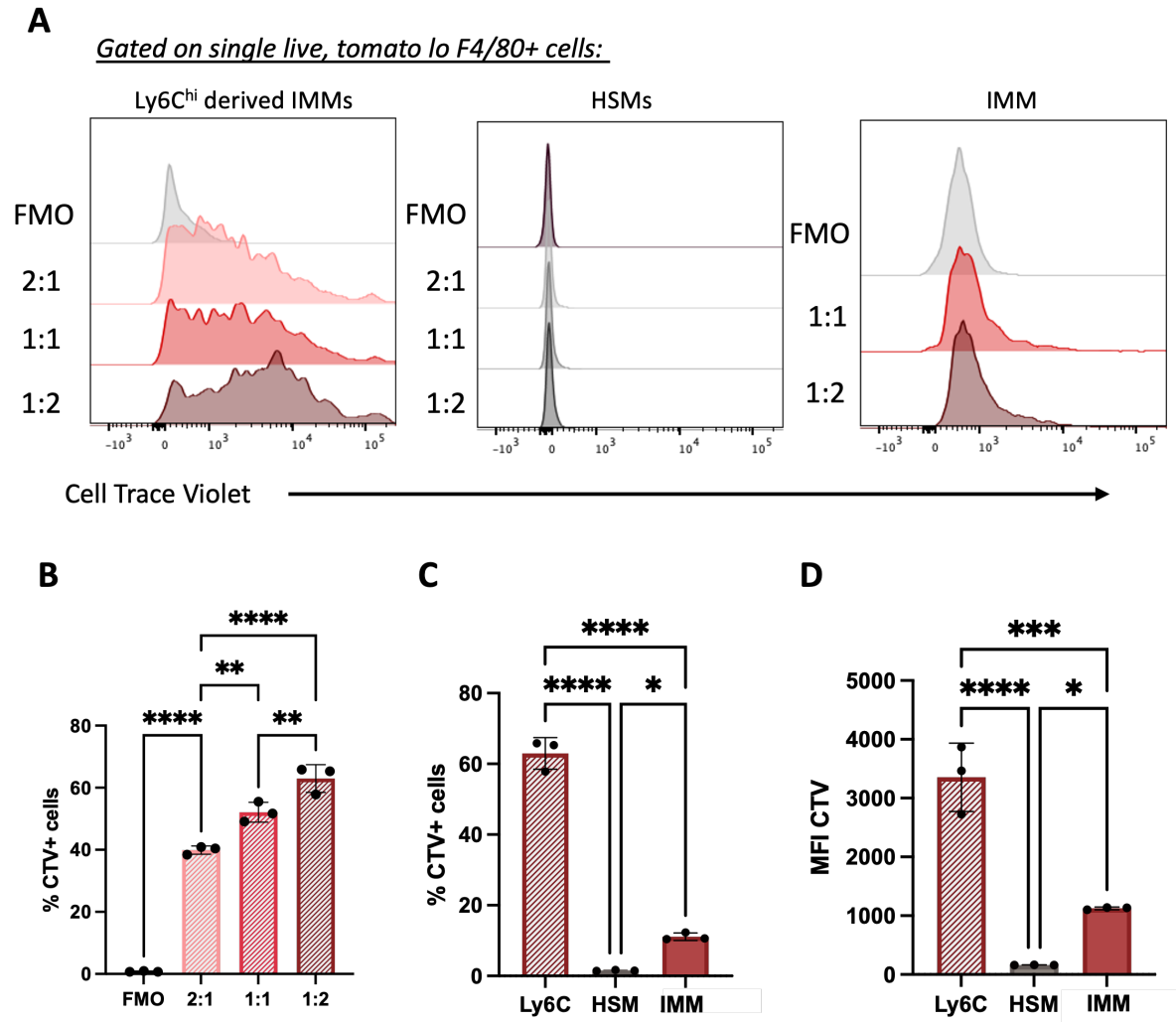


Figure 5.17: Ly6C-derived IMMs and directly sorted IMMs are capable of phagocytosing AML cells *in vitro*

Ly6C^{hi} cells, IMMs and HSMs were sorted from WT mice. Sorted populations were co-cultured with CTV labelled AML cells for 24 hours, at three different ratios (2:1, 1:1 and 1:2 sorted cells: AML cells). After co-culture, cells were analysed by flow cytometry. AML cells were gated out based on their expression of tomato. **(A)** Histogram of CTV expression in the three sorted monocytes/macrophage populations after co-culture. **(B)** Percentage of CTV+ Ly6C-derived IMMs according to the ratio of Ly6C-derived cells to AML. **(C)** Percentage of CTV+ sorted cells in the 1:2 ratio. **(D)** Mean fluorescence intensity of CTV in the sorted cells at a ratio of 1:2. $n = 3$, representative of two independent experiments. Error bars represent the mean $\pm$ s.e.m. Statistically significant differences were determined using Student *t*-tests (* represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$)

the CTV signal found in these cultures (AML cells excluded from the analysis) (Figure 5.17 D).

Together, these results show that the IMMs are the main macrophages responsible for the phagocytosis of AML cells in the bone marrow. Phagocytosis of tumour cells has been shown to impact anti-tumour immunity through three mechanisms: first it had been shown that the phagocytic activity of macrophage can directly control tumour growth. In addition, phagocytosis of tumour cell can help macrophages act as antigen presenting cell and activate CD8 T cells. Finally, both phagocytosis and efferocytosis can affect macrophage functionality and promote either anti-tumour immunity or pro-tumour immunity [163]. In the next sections I will explore which of these mechanisms takes place in AML.

5.4 Direct interaction between macrophages and AML

To evaluate whether macrophages directly influence AML growth, AML cells were co-cultured with Ly6C-derived IMMs and HSMs. As a control condition, AML cells were cultured alone. After 24 and 48 hours, the cells were counted and analysed by flow cytometry. Both macrophage populations led to a significant increase in AML count (Figure 5.18A) suggesting that either the direct contact with macrophages or a factor secreted by macrophages promotes AML growth. To uncover if the direct contact was necessary for AML growth, I set-up the co-culture in a Boyden chamber where the cells are separated by a membrane. Strikingly, in this set-up the number of AML cells was no longer increased in the presence of macrophages (Figure 5.18B). However, I noticed that the AML cells migrated significantly more towards macrophages than when the bottom chamber was empty (Figure 5.18C). To understand what chemokine secreted by macrophages was responsible for the AML cells' migration, the supernatant of the co-cultures as well as that of macrophages alone and AML cells alone was analysed using a proteome mouse chemokine array. In this array, the level of 25 most commonly secreted chemokines was analysed in a semi-quantitative manner [198]. Interestingly, AML cells did not secrete any chemokines from this array, however macrophages did secrete four chemokines, [C-C motif chemokine ligand 2 \(CCL2\)](#), [C-C motif chemokine ligand 6 \(CCL6\)](#), [Macrophage inflammatory protein 1 gamma \(CCL9/10\)](#) and [C-C motif chemokine ligand 12 \(CCL12\)](#) (Figure 5.18D-E). CCL2 and CCL12 are known to bind to [C-C chemokine receptor type 2 \(CCR2\)](#) while CCL6 and CCL9/10 bind to [C-C chemokine receptor type 1 \(CCR1\)](#). Using bulk RNA sequencing of AML cells and [GMPs](#) performed by a previous lab member, AML cells were confirmed to express both these receptors, albeit at different levels (Figure 5.18F).

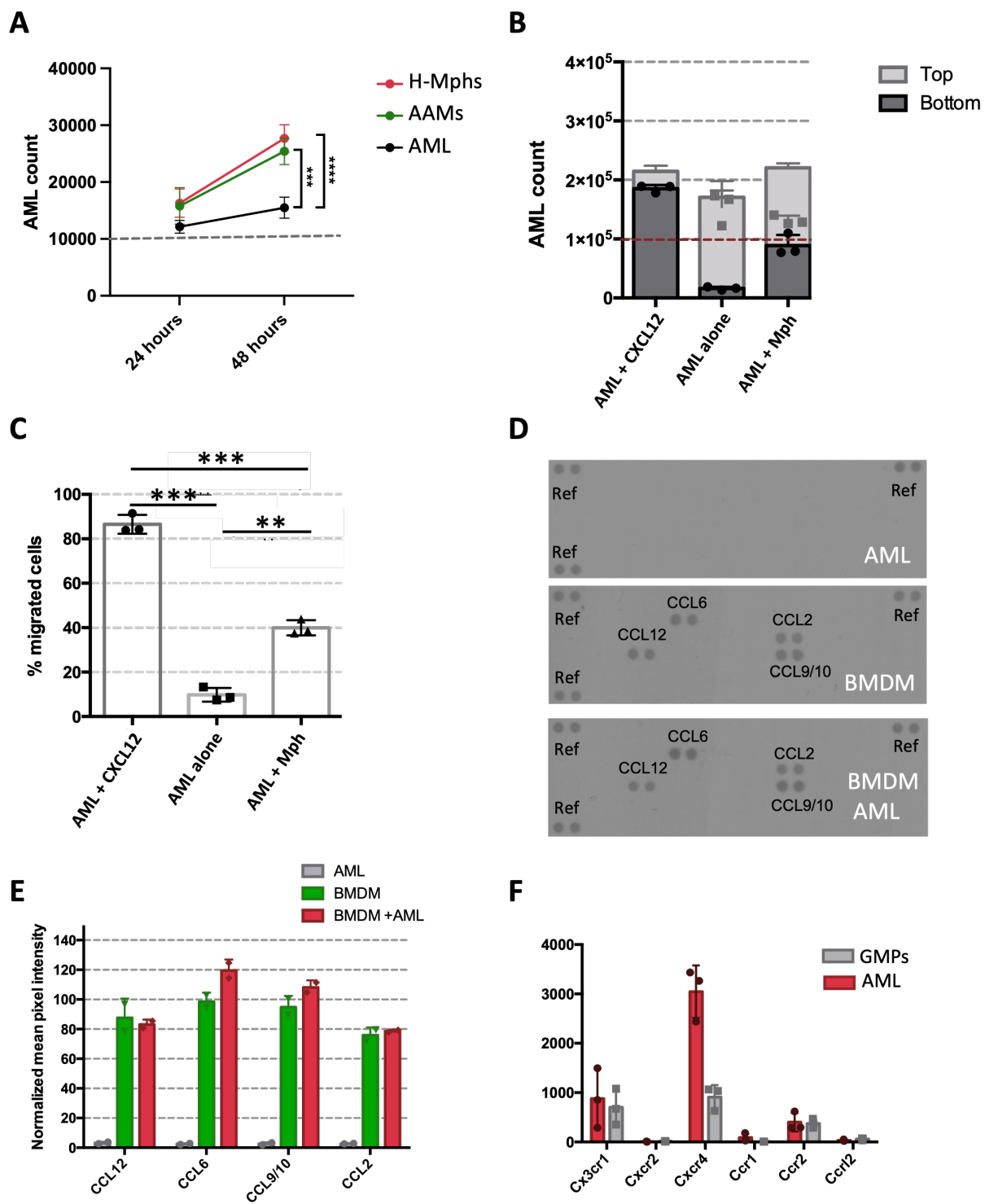
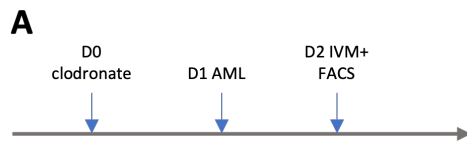


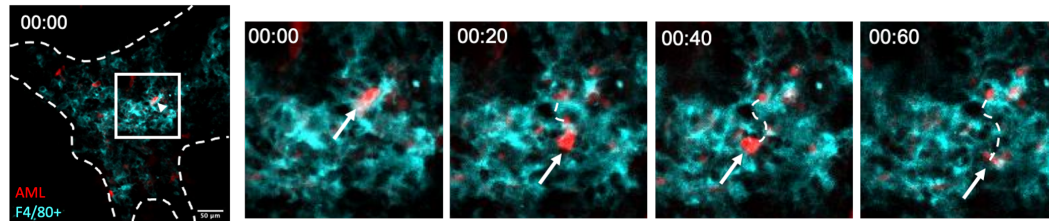
Figure 5.18 (*previous page*): Co-culture of AML cells with macrophages leads to increased AML count and migration

(A) AML count following co-cultures with Ly6C-derived IMM and HSMs for 24 and 48 hours. AML cells were cultured alone as a negative control. (B), (C), (D), (E) AML migration assay using Boyden chambers. 1×10^5 AML cells were plated in the insert (top chamber) and 1×10^5 BMDM were plated in the bottom chamber. As a positive control, CXCL12 alone was added to the bottom chamber. Negative control consisted in media alone in the bottom chamber. (B) Total AML count (including bottom and top chamber). (C) Percentage of migrated cells among AML cells, calculated as the fraction of mTmG+ cells located in the bottom compartment. (D), (E) The supernatant from the migration assay was analysed using a proteome assay [198]. As a negative control, the supernatant from BMDM culture alone was analysed. (D) Representative images of the proteome assay assessing the chemokine secretion by BMDM and AML cells. Images were acquired on an X-ray imager. BMDMs secrete CCL2, CCL12, CCL6 and CCL9/10. AML cells do not secrete any of the assayed chemokines. (E) Quantification of the chemokine secretion. (F) Analysis of bulk RNA sequencing of AML cells and GMPs (performed by D. Duarte) for the expression of chemokine receptors. $n = 3$, representative of five independent experiments for the migration assay. The proteome assay was performed once, on two samples for each condition. Error bars represent the mean $\pm$ s.e.m. Statistically significant differences were determined using Student *t*-tests (* represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$)

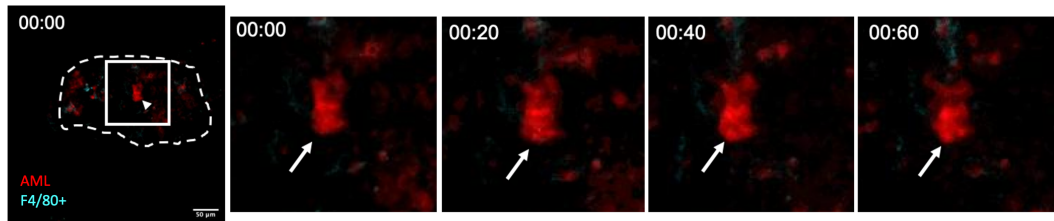
Next, to confirm that macrophages affected the migratory capacity of AML cells *in vivo*, I set up an AML homing assay in macrophage-depleted mice and control mice. I injected clodronate liposomes or PBS liposomes followed by transplantation of 2 million AML cells the next day. 16 hours later, I imaged the mice using intravital microscopy and assessed the motility and number of leukemic cells in the BM (Figure 5.19A). As visualized in the Z maximum projections from 1 hour-long timelapses, AML cells in PBS liposomes treated mice interacted with F4/80+ cells, while in the clodronate condition the malignant cells appeared sessile (Figure 5.19 B,C and Supplementary video 6 and 7). After tracking every leukemic cell found in the timelapses (Figure 5.19D, E), the average accumulated distance of AML cells was measured and the Euclidean distance and linearity were calculated (Figure 5.19F). In clodronate liposome treated mice, the accumulated distance was significantly reduced compared to control (Figure 5.19G). Interestingly, the difference in Euclidean distance was much smaller compared to the accumulated distance (Figure 5.19H). The linearity was similar in both groups with an average value of 0.2 (Figure 5.19I) indicating that the AML cells were not moving linearly, and their motility pattern was closer to random walk. Finally, the absolute number of leukemic cells found in the BM of macrophage-depleted mice was significantly reduced compared to control suggesting that macrophages are influencing both the motility and the homing of AML cells in the BM (Figure 5.19J).



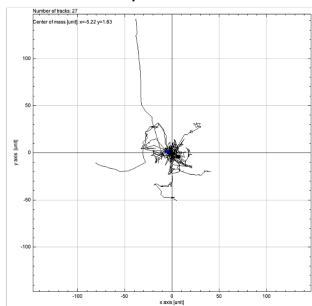
B *PBS-liposome treated mice*



C *Clodronate-liposome treated mice*



D *PBS lip. treated mice*



E *Clo. lip. treated mice*

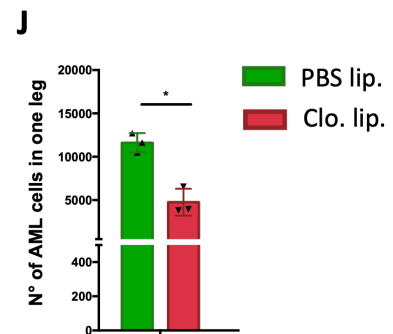
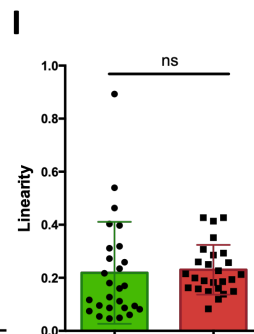
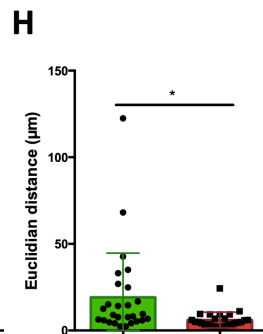
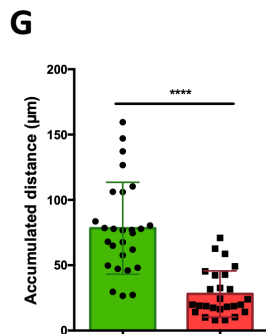
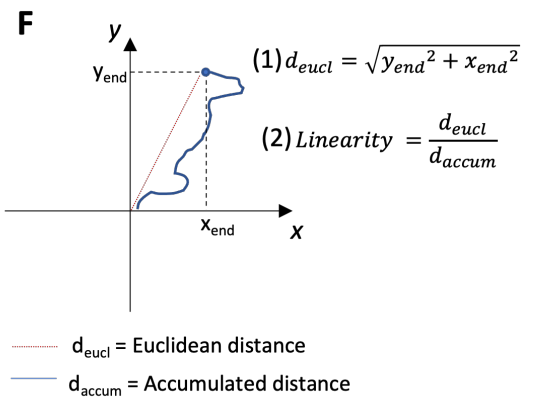
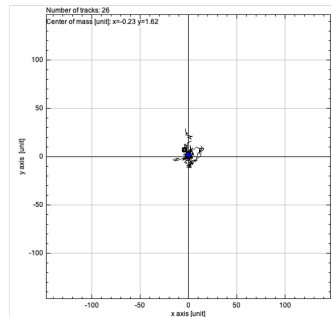


Figure 5.19 (previous page): Depletion of Mphs with clodronate leads to reduced homing of AML cells

Wild type mice were injected with clodronate liposomes or PBS liposomes. On day 1, mice were transplanted with 100'000 mTmG+ AML cells. On day 2, mice were injected with anti-F4/80 APC and imaged using intravital microscopy. The BM was then analysed by flow cytometry. **(A)** Experimental protocol for the homing assay of AML cells in clodronate liposomes or PBS liposomes treated mice. **(B),(C)** Representative screenshots of maximum projections of calvarium BM from 1 hour timelapses with images acquired every 2 minutes (4 frames) of PBS liposome **(B)** and clodronate liposomes **(C)** treated mice. Images were acquired every 2 minutes. BM is outlined with dotted lines and arrow heads point at AML cells. **(D),(E)** Rose plots of manually tracked AML cells in clodronate liposome treated mice ($n=26$) **(D)** and PBS liposome treated mice ($n=27$) **(E)**. **(F)** Schematic and equations used for the calculation of the accumulated distance, euclidean distance and linearity of tracked cells' displacements. **(G)** Quantification of the accumulated distance of tracked AML cells. **(H)** Quantification of the euclidean distance of tracked AML cells. **(I)** Calculation of the linearity of AML cells' tracks. Error bars indicate mean ($\pm SD$), 2 mice were analysed per condition, representative of two independent experiments. **(J)** Wild type mice were injected with clodronate liposomes or PBS liposomes. At day 1, mice were transplanted with 2'000'000 mTmG+ AML cells. On day 2, the BM was harvested and analysed by flow cytometry. Absolute number of mTmG+ AML cells in the BM. $n=3$ mice per condition, representative of two independent experiments. Error bars represent the mean $\pm$ s.e.m. Statistically significant differences were determined using Student *t*-tests (* represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$)

Next, to evaluate whether the reduced AML homing and motility was responsible for the growth difference noticed in clodronate treated mice, I measured AML growth in mice treated with a single clodronate treatment before AML transplantation by measuring BM AML cell numbers at day 11 and 13 post transplantation (Figure 5.20 A). The percentage of leukemic cells in the BM of clodronate treated mice was identical to the percentage measured in PBS liposome treated mice (Figure 5.20 B). In absolute numbers, AML cell growth was not significantly different either (Figure 5.20 C). Noting that, in the clodronate set-up experiment the number of macrophages was already increased at day 5 in the single clodronate treatment compared to continuous depletion, I hypothesized that repeated injections were necessary for sustained depletion of macrophages. To uncover which macrophage population repopulated the bone marrow following a single clodronate injection and AML transplantation, I compared the number of HSMs and IMMs at day 11 in PBS liposomes and clodronate liposome treated mice. The absolute number of HSMs remained significantly lower in clodronate treated mice compared to PBS liposome treated mice, however the absolute number of IMMs returned to similar levels to those observed in PBS liposome treated mice (Figure 5.20 D-E). Hence, the lack of growth difference combined with the return of IMMs suggest that IMMs are responsible for the AML growth difference and most likely through another mechanism than direct inter-

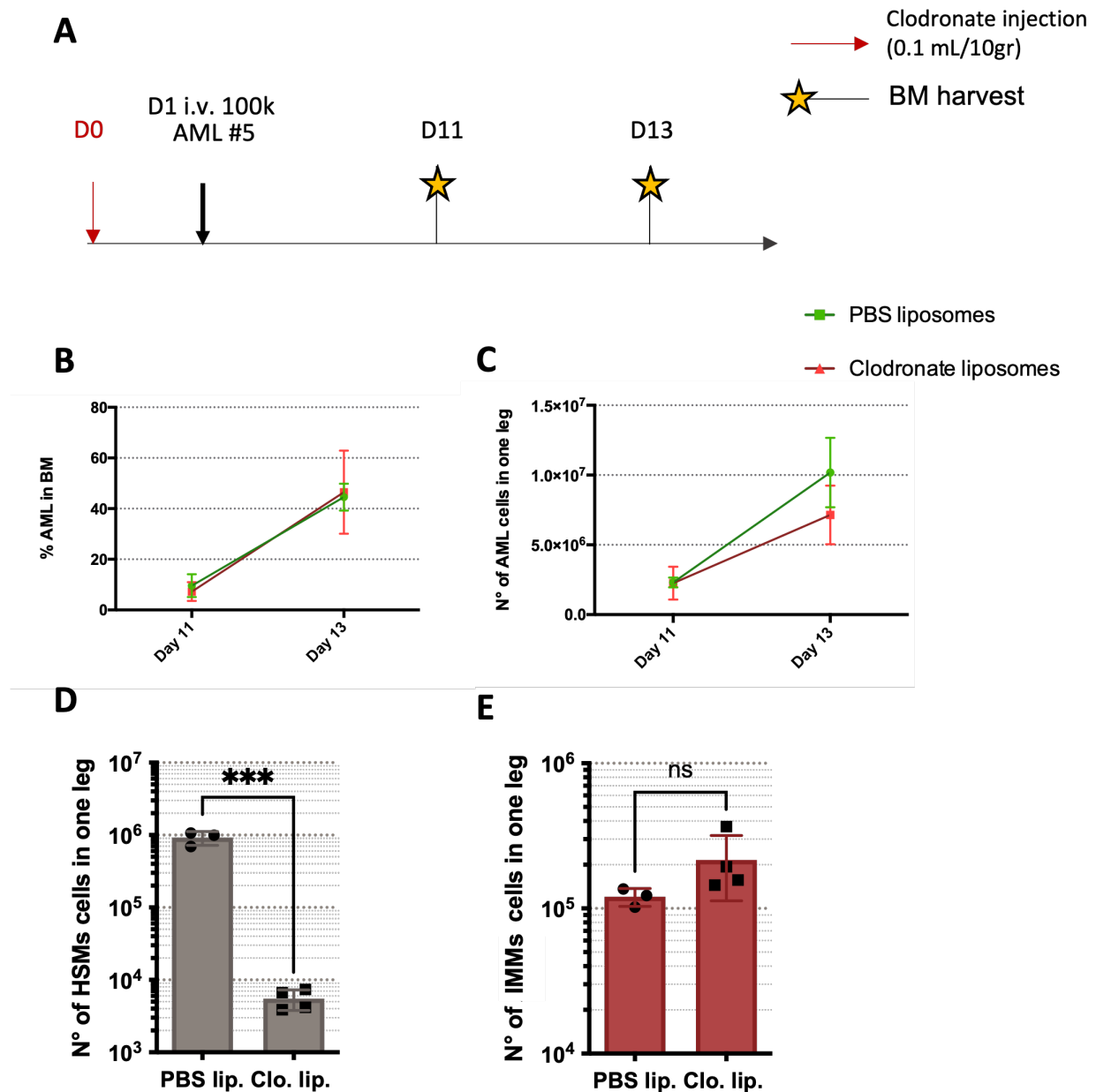


Figure 5.20: Single clodronate injection does not impact AML growth

Wild type mice were injected with clodronate liposomes or PBS liposomes on day 0. At day 1, mice were transplanted with 100'000 mTmG AML cells. The BM was harvested at day 11 and 13 and analysed by flow cytometry to assess disease growth. (A) Experimental protocol for the analysis of AML growth in clodronate liposome treated mice. (B) Percentage of mTmG+ AML cells among total BM cells. (C) Absolute number of mTmG+ AML cells in the BM. (D) Absolute number of HSMs in the BM, at day 11. (E) Absolute number of IMM cells in the BM, at day 11. Representative data of three independent experiments, dot plot indicates mean $\pm$ s.e.m with 3-4 mice per time points. Statistically significant differences were determined using Student t-tests (* represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$)

action with AML cells.

The direct interaction between cancer cells and macrophages was the first mechanism explored as macrophage role in tumour immunity. The second mechanism described in literature is linked to macrophage function as an APC and mediator of T cell activation. Indeed, following phagocytosis, macrophages degrade the proteins of the engulfed particle and present 8-10 peptides-long antigens on their MHC group. In turn, this leads to the activation of antigen specific T cells and represents the start of the T cell response. However, in the case where the tumour cell is engulfed through efferocytosis, it has been shown that macrophages will inhibit the T cell response and generate an anti-inflammatory response. Hence, I next wanted to investigate whether the phagocytic macrophages found in the bone marrow could influence T cell activation.

5.5 Impact of macrophage populations on T cells *in vitro*

To uncover whether IMMs inhibit T cell activation, Ly6C-derived IMMs and HSMs were sorted and co-cultured for 60 hours with CD3/CD28 activated T cells, following A. Doedens's et al., [261] protocol. The T cells were labelled with CTV to track their proliferation by flow cytometry as a marker of activation. Indeed, following each cell division, the amount of CTV will be divided by two generating a new peak at half the intensity of the initial peak. As a positive control, T cells alone were cultured with CD3/CD28 activating beads. As a negative control, unstimulated T cells were used, as T cells are unable to proliferate without a stimuli and therefore the CTV should not be diluted. Interestingly, Ly6C-derived IMMs led to the inhibition of T cells as the number of CTV dilution peaks was strongly reduced compared to T cells cultured alone (Figure 5.21A). In addition, this inhibition was dependent on the IMM:T cell ratio used with the highest number of macrophages to T cells (1:2.5) leading to the strongest inhibition of T cell proliferation, while the lowest ratio (1:40) did not lead to any inhibition. On the other hand, HSMs promoted T cell proliferation as additional CTV peaks were seen on the histogram meaning that T cells proliferated at least one extra cycle. To quantify this inhibition/promotion of T cell activation and compare the effect of Ly6C-derived IMMs with HSMs, the mean intensity of the CTV signal and total number of T cells in the 1:2.5 IMM:Tcell ratio were measured. The mean intensity was significantly higher in Ly6C-derived IMMs co-cultured T cells compared to control T cells, and significantly lower in HSMs co-cultured T cells (Figure 5.21 B). This result was consistent with the T cell count,

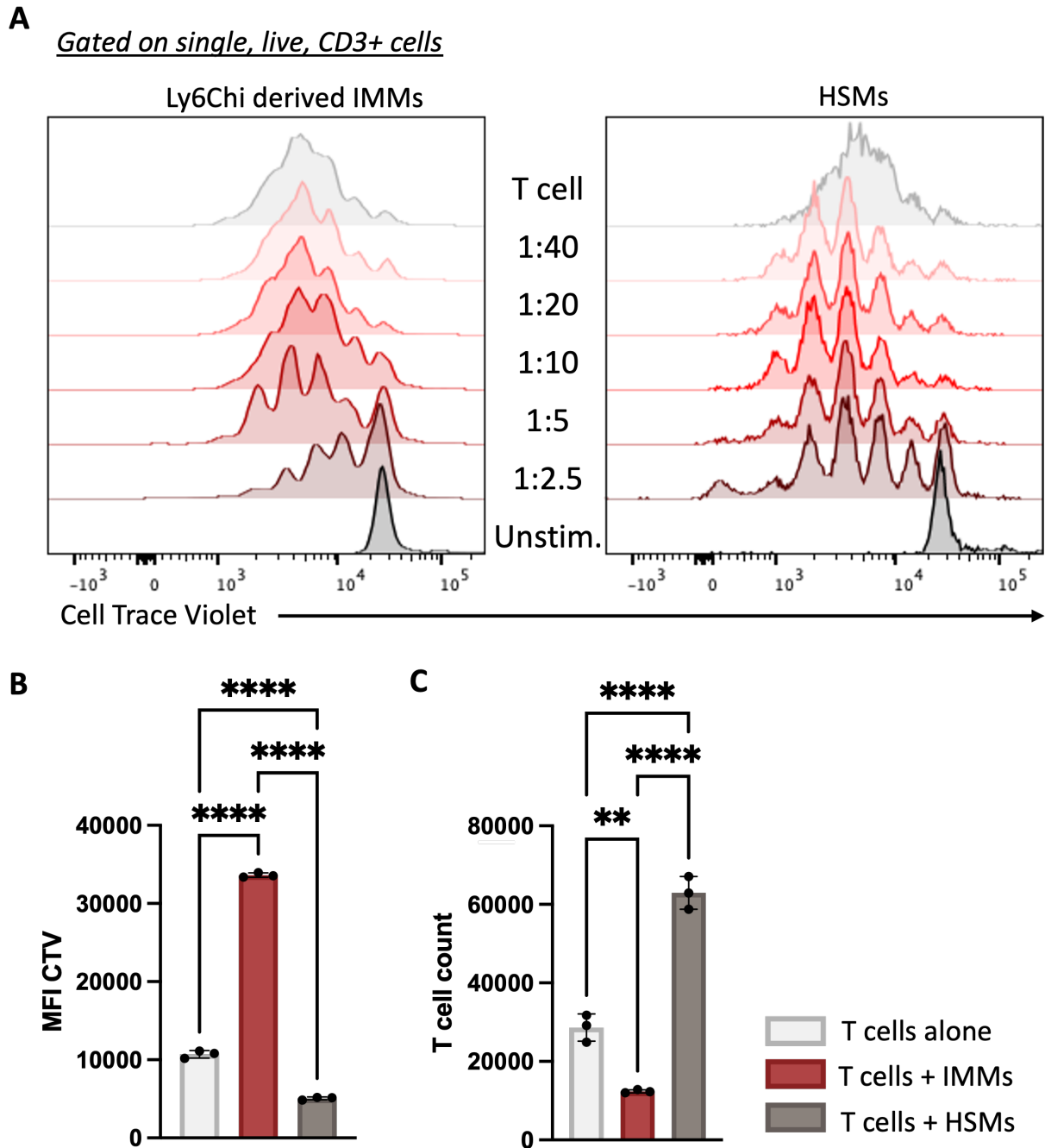


Figure 5.21: Co-culture of macrophages with T cells impacts T cell activation
Ly6C derived IMMs and HSMs were co-cultured with CD3/CD28 activated, CTV labelled T cells at 5 different ratios (1:2.5, 1:5, 1:10, 1:20 and 1:40 macrophage:T cells) for 72 hours. CTV labelling allows the visualisation of cell proliferation through reduction of the dye. Unstimulated T cells, without any CD3/CD28 activation, cultured alone were used as negative control. CD3/CD28 activated T cells cultured alone were used as baseline for T cell proliferation. At the end of the 72 hours, cells were analysed by flow cytometry and T cells were gated as single, live, CD3+ cells. **(A)** Representative histogram of CTV expression in T cells. Each peak represents a round of cell division performed by T cells. **(B)** Mean fluorescence intensity of CTV in T cells, cultured alone, with *Ly6C*-derived IMMs and HSMs. **(C)** Absolute number of T cells when cultured alone, with *Ly6C*-derived IMMs and HSMs. $n = 3$, representative data of two independent experiments, dot plot indicates mean $\pm$ s.e.m. Statistically significant differences were determined using Student *t*-tests (* represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$)

which was significantly increased in HSMs and significantly decreased in IMMs (Figure 5.21C), meaning that the HSMs promoted T cell proliferation while IMMs inhibited T cell proliferation.

In conclusion, Ly6C monocytes gave rise to IMMs *in vitro* and these cells were responsible for the phagocytosis of AML cells as well as the inhibition of activated T cells *in vitro*. While these results were very promising, it was essential to confirm the role of both macrophage populations with regards to T cells *in vivo*. Indeed, *in vitro*, T cells were artificially activated and this could mask a possible role in T cell activation (for example from the HSMs) and probably enhance the role of IMMs in T cell inhibition. Hence, next I studied the T cell phenotypes as AML grew in both models of macrophage depletion; the clodronate model was used to study the depletion of IMMs and HSMs while the CD169-DTR model was used to study the enrichment of IMMs in AML mice.

5.6 Impact of macrophage populations on T cells during AML growth *in vivo*

To understand whether T cells were activated or inhibited by macrophages in AML, BM cells from control, PBS liposomes and clodronate liposomes mice with AML as well as WT BM cells were harvested, stained with a T cell immunophenotyping panel and analysed by flow cytometry. CD69 is a common marker, expressed early on during the activation of T cells [262]. Interestingly, in AML mice, at late disease, the proportion of CD69⁺ cells among CD8⁺ T cells was increased by 7-fold compared to WT mice, going from approximately 10% in WT and reaching 70% in AML mice. However, this activation was significantly reduced in clodronate treated mice with only 30% of CD8 cells expressing CD69 (Figure 5.22B).

In addition, CD8 T cells can be classified as naive, effector, central memory and effector memory according to their expression of CD44 and CD62L [263]. With AML, the percentage of naive CD8 T cells drops drastically, from 50% in control mice to less than 10%. However, with clodronate, the proportion of naive T cells remains similar to mice without AML, suggesting that in the absence of both macrophage populations T cells do not get activated and are not capable of mounting an anti-tumoural response (Figure 5.22 C-D).

Next, I evaluated common markers of T cell inhibition and exhaustion, among which is

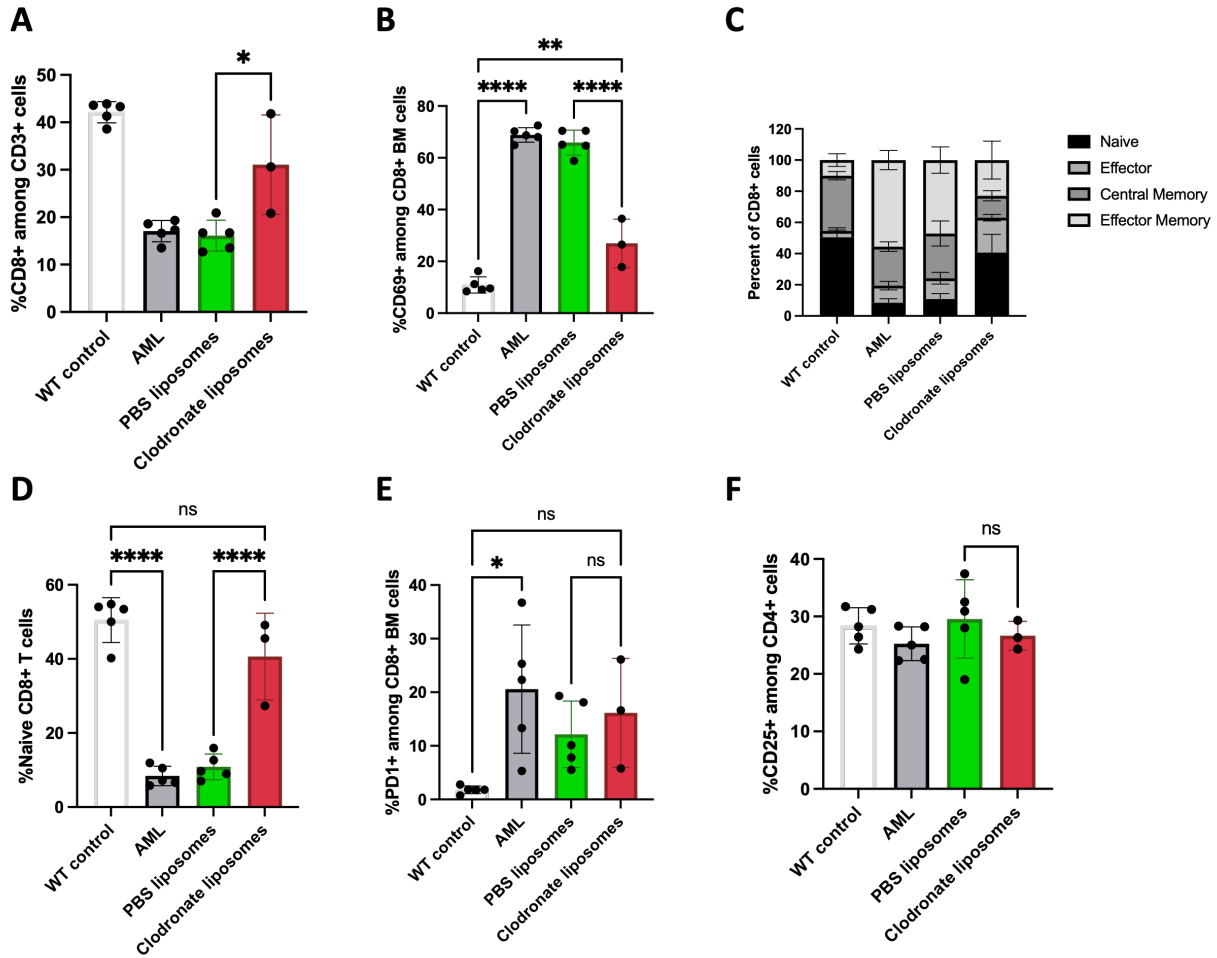


Figure 5.22: Reduced T cell activation in clodronate treated mice with AML

Wild type mice were injected with clodronate liposomes, PBS liposomes or PBS every 4 days. At day 1, mice were transplanted with 100'000 mTmG AML cells. The BM was harvested at late AML when AML represented more than 65% of the cells in the BM, and analysed by flow cytometry to assess T cell phenotype. WT control mice did not receive any AML and were used as a control for the effect of AML. **(A)** Percentage of CD8+ cells among CD3+ cells. **(B)** Percentage of CD69+ cells among CD8+ cells. **(C)** Fraction of the CD8 T cells as naive (CD44- CD62L+), effector (CD44- CD62L-), central memory (CD44+ CD62L+) and effector memory T cells (CD44+ CD62L-) T cells. **(D)** Percentage of naive T cells among CD8+ T cells. **(E)** Percentage of PD1+ T cells among CD8+ T cells. **(F)** Percentage of CD25+ T cells among CD4+ cells. $n = 3-5$ mice per group, representative of two independent experiments. Error bar indicates mean ($\pm$ s.e.m). Statistically significant differences were determined using Student t-tests (* represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$)

PD1, which in cancer is representative of dysfunctionality and ineffective T cells [264, 265]. The percentage of PD1+ cells among CD8+ T cells increased as the disease developed and was significantly higher in AML mice compared to controls (Figure 5.22 E). In clodronate treated mice with AML, the proportion of PD1+ CD8+ T cells was similar to that of untreated, AML burdened mice, indicating that exhausted T cells are present independently of macrophages. In addition, the expression of CD25 in CD4+ T cells is known to represent a fraction of T cells named **Regulatory T cells (Tregs)**, important for the resolution of the immune response. In cancer, Tregs are known to help tumour cells evade T cell surveillance by secreting anti-inflammatory cytokines, inhibiting **APCs** and consuming IL-2 [266]. Interestingly, in our model of leukemia, the proportion of Tregs is similar to control mice and remains unchanged following the administration of clodronate (Figure 5.22 F). In conclusion, clodronate treatment, known to deplete both populations of macrophages in leukemia, leads to reduced activation of T cells but does not modify the exhausted profile of T cells at late disease. Hence, macrophages are essential for the T cell activation to take place.

To understand which macrophage population was affecting T cell activation during AML development *in vivo*, I took advantage of the increase in the IMMs in CD169-DTR^{het} mice with AML and studied the T cell response in those mice. Strikingly, the proportion of CD69+ cells among CD8+ T cells was increased in the CD169-DTR^{het} mice with AML compared to CD169-DTR- AML mice (Figure 5.23B). In addition, the proportion of naive T cells was reduced compared to control mice and slightly lower than in the CD169-DTR- AML mice (Figure 5.23C-D). Hence, the activation of T cells is dependent on the IMMs, because when these cells were depleted with the clodronate liposome model the T cell activation was abrogated, while when these cells were enriched in the CD169-DTR model the T cell activation was increased. Even though in the clodronate treated mice no differences were noticed in the expression of PD1 and the proportion of Tregs, I still decided to measure these two phenotypes in the CD169-DTR^{het} mice. The proportion of PD1+ CD8 T cells was slightly increased in CD169-DTR^{het} AML mice compared to CD169-DTR- AML mice (Figure 5.23E) and a similar trend was noticed for the proportion of Tregs (Figure 5.23F). These results suggest that IMMs are responsible for the activation and inhibition of T cells in AML (Figure 5.24).

Combining these results with the previous *in vitro* study where I was able to measure a strong inhibition of T cell activation by IMMs, it is possible that the activation *in vivo* is weaker than that induced by CD3/CD28 beads and therefore the results of the inhibition,

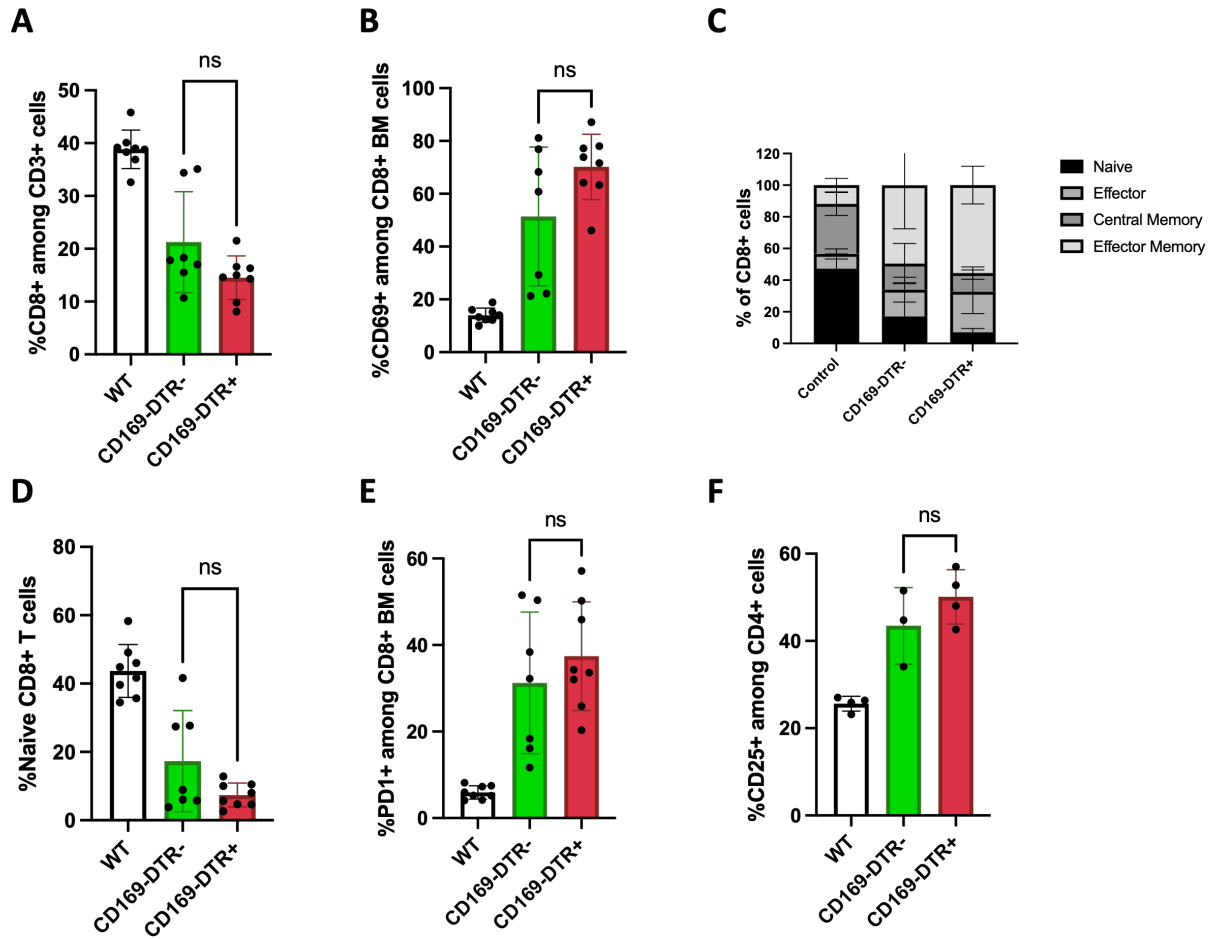


Figure 5.23: Increased T cell activation in CD169-DTR^{het} mice with AML
 CD169-DTR⁻ and CD169-DTR^{het} mice were injected with DT for two consecutive days and then every 4 days. At day 2, mice were transplanted with 100'000 mTmG AML cells. The BM was harvested at late AML when AML represented more than 65% of the cells in the BM, and analysed by flow cytometry to assess T cell phenotype. WT control mice did not receive any AML and were used as a control for the effect of AML. **(A)** Percentage of CD8+ cells among CD3+ cells. **(B)** Percentage of CD69+ cells among CD8+ cells. **(C)** Fraction of the CD8 T cells as naive (CD44⁻ CD62L⁺), effector (CD44⁻ CD62L⁻), central memory (CD44⁺ CD62L⁺) and effector memory T cells (CD44⁺ CD62L⁻) T cells. **(D)** Percentage of naive T cells among CD8+ T cells. **(E)** Percentage of PD1+ T cells among CD8+ T cells. **(F)** Percentage of CD25+ T cells among CD4+ cells. *n* = 7-8 mice per group. Error bar indicates mean ($\pm$ s.e.m). Statistically significant differences were determined using Student *t*-tests (* represents *p* < 0.05; ** represents *p* < 0.01; *** represents *p* < 0.001; **** represents *p* < 0.0001)

	Macrophage populations		T cell population phenotypes				AML growth
	HSMs	AAMs	T cell activation	Tregs	Naïve T cells	PD1 T cells	
Clodronate treated	-	-	-	=	+	-	-
CD169-DTR, DT treated	-	+	+	+	-	+	+

Figure 5.24: Summary of the effects of clodronate and CD169-DTR treatment on macrophages, T cells and AML growth

In clodronate treatment, all macrophages are depleted leading to reduced T cell activation and exhaustion in turn slowing AML growth. In the CD169-DTR treated mice, HSMs were depleted but IMMs were enriched leading to increased T cell activation and exhaustion in turn speeding up AML growth.

while present, are not striking *in vivo*. Another explanation is that many other interactions are taking place in the bone marrow microenvironment including secreted factors which can also influence T cell activation and inhibition. In conclusion, IMMs are important for the T cell activation and most likely this activation then becomes exhaustion in the AML context.

One question remains: is this mechanism responsible for the growth difference measured in both the clodronate and CD169-DTR^{het} model? Indeed, while phagocytosis is known as the main function of macrophages, in cancer, these cells were mainly studied for their secretion of pro-tumoural cytokines helping cancer progression through various mechanism including angiogenesis, epithelial-to mesenchymal transition, proliferation and survival [267]. Hence, to evaluate whether the macrophage - T cell interaction was responsible for the AML growth difference seen in the macrophage depletion models, I measured the AML growth in T cell deficient mice, (RagKO mice) treated with PBS liposomes or clodronate liposomes. In this context, if the effect of macrophage depletion on AML growth was primarily associated with the role of macrophages in the T cell response, then no growth difference should be measured between both conditions. Strikingly, the absolute number and percentage of AML cells in the bone marrow of the RagKO and clodronate treated RagKO mice were identical (Figure 5.25 A-B). Hence, the interaction between macrophages and T cells was responsible for the growth difference observed in clodronate treated mice, where both macrophage populations were depleted. Given that clodronate led to reduced AML growth, the macrophage - T cell interaction was promoting AML growth. This hypothesis correlates with the *in vitro* study

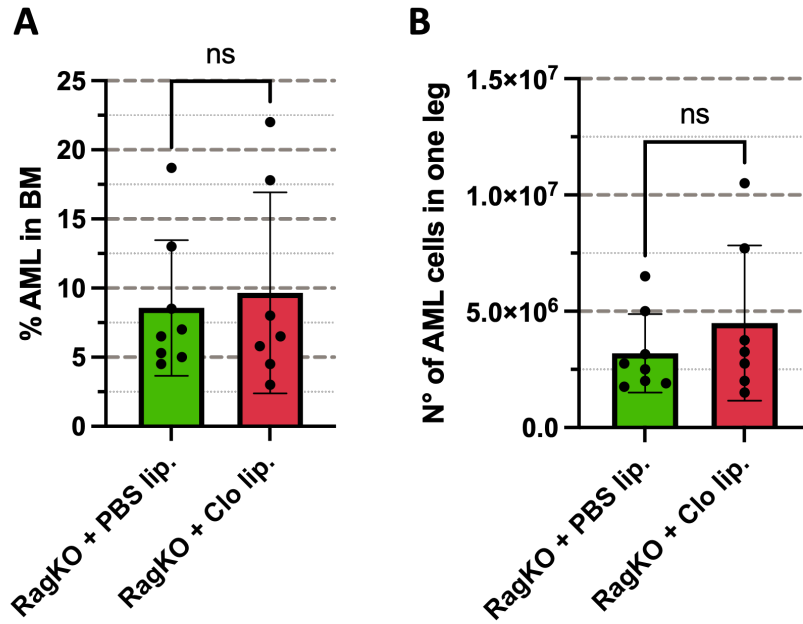


Figure 5.25: Effect of clodronate treatment on AML growth is abolished in T cell deficient mice *RagKO* mice were injected with PBS liposomes or clodronate liposomes every 4 days. At day 1, mice were transplanted with 100'000 mTmG AML cells. At day 20, BM was harvested and analysed by flow cytometry to assess AML growth. **(A)** Percentage of AML cells among all BM cells. **(B)** Absolute number of AML cells in the BM of one leg $n = 7-8$ mice per group. Error bar indicates mean ($\pm$ s.e.m). Statistically significant differences were determined using Student *t*-tests (* represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; **** represents $p < 0.0001$)

where IMMs were found to inhibit T cell activation.

5.7 Discussion

The aims of this chapter were to understand whether bone marrow macrophage promoted or inhibited AML growth and understand how macrophages interact with AML cells and lymphocytes. Overall, the key point was to understand what was the underlying mechanism tying macrophages to the AML progression.

I first wanted to uncover whether macrophages supported AML growth or helped reduce AML progression. Previous studies had used clodronate to assess the importance of macrophages in AML development, however their results showed that macrophages depletion did not alter AML growth. One study used intraperitoneal injections of clodronate and showed that their number of macrophages in the bone marrow increased rather than decreased [256]. Hence, this drug regimen cannot be considered a good depleting regimen and therefore the results didn't allow us to conclude on the role of macrophage in leukemia growth. The second paper did not detail their drug regimen or the efficiency of the depletion hence it is difficult to assess their results [255]. Surprisingly, when I injected clodronate repeatedly intravenously, AML growth was significantly reduced compared to control. Of note, while IMMs were not significantly depleted in control clodronate treated mice, in AML burdened mice, IMMs were lost right from the early stages of disease, when the fraction of AML cells in the bone marrow was lower than 5%. This striking loss was much faster than in control AML mice treated with PBS liposomes where the cells remained until the disease was relatively advanced (>50%). On the other hand, HSMs were strongly depleted by clodronate independently of the presence of AML cells. The preferential loss of IMMs at early disease suggests that the clodronate treatment combined with AML leads to the loss of the production mechanism allowing the microenvironment to maintain the pool of IMMs, a mechanism similar to the concept of synthetic lethality whereby either situation alone allow IMMs to remain in the microenvironment but when both are combined the cells are no longer produced. One possible explanation is that these cells are not directly targeted by clodronate and are monocyte derived cells. When AML engrafts, the IMMs compete with AML cells for the space and nutrients. As these cells are monocyte derived, they would naturally be replenished by the pool of myeloid cells present in the spleen, essential for emergency myelopoiesis, however clodronate induces strong spleen toxicity with more than 90% of the splenocytes dying, and therefore splenic emergency myelopoiesis can no longer take place and these cells are not regenerated.

To confirm that these results were not linked to a side effect of clodronate, I repeated these experiments using a genetic depletion approach: the CD169-DTR mice. These mice have been widely used to study the function of bone marrow macrophages as they express a high level of CD169. This model is highly specific and depletes only CD169 expressing cells, therefore IMMs were not targeted. Following the administration of diphtheria toxin, the AML grew significantly faster. While this result was highly unexpected, a striking difference was noticed with the clodronate model. Indeed, in CD169-DTR^{het} mice with AML, the IMMs increased by 3-fold. Hence, in AML, while both depletion models led to the depletion of HSMs, clodronate led to the depletion of IMMs but CD169-DTR depletion led to a significant increase in this cell population. Putting together these results, when IMMs were depleted, AML grew slower while when IMMs were enriched, AML grew significantly faster. In addition, the IMMs were identified as expressing both SIRP α and PDL1, two immune checkpoints described in literature as potential targets to remove immune inhibition. Altogether these results suggested that IMMs were the key macrophage population promoting AML growth.

My first thought when these cells were identified as Immunomodulatory macrophages was to understand whether these cells could perform their primary function: phagocytosis. Following a combination of live imaging experiments, I was able to capture the live phagocytosis of AML cells by macrophages. However, the mice used, CD169-YFP combined with SIRP α -APC injection did not allow me to identify which macrophage sub-population was responsible for the phagocytosis of AML cells. Hence, I decided to sort both macrophage populations and inflammatory monocytes, as a negative control, and culture all three populations with labelled AML cells to assess whether one specific population was responsible for the phagocytosis of AML cells. Incidentally, I found that within 24 hours, monocytes differentiated towards macrophages and adopted a macrophage-like morphology, with an elongated shape, long dendrite and a strong adherence to the culture plate. Through flow cytometry, I found that the inflammatory monocytes had differentiated towards IMMs adopting a similar expression of F4/80, SIRP α and PDL1. In addition, these cells reduced their expression of Ly6C. I found that this differentiation was dependant on the addition of M-CSF in the media (data not shown), which correlates with known macrophage differentiation protocol [268]. Finally, IMMs were found to recover poorly from the sort and the yield of IMMs was much higher when sorting Ly6C^{hi} monocytes compared to IMMs. Therefore, I decided to use Ly6C differentiated cells as a source of IMMs and named these cells Ly6C-derived IMMs to avoid any confusion with directly sorted IMMs. The co-culture of Ly6C-derived IMMs, IMMs and HSMs *in vitro* with AML cells confirmed

that IMMs were the only macrophage population capable of phagocytosing AML cells.

Following this discovery, it was essential to uncover what was the impact of phagocytosis on AML cells, macrophages and T cells. Numerous studies have focused their attention on the CD47-SIRP α pathway, more commonly known as the ‘don’t eat me’ pathway inhibiting the phagocytosis by macrophages of healthy cells. Indeed, C-Y. Huang et al., [269] have described that many cancer types including AML upregulate their expression of CD47 to evade phagocytosis. Instead, in my model AML cells still expressed CD47 (data not shown) but macrophages were clearly still capable of phagocytosing these cells. However, surprisingly this phenomenon did not help reduce AML growth *in vitro*. Indeed, when the cells were cultured together, the AML count increased significantly faster, with both macrophage populations. One possible explanation is that macrophages secrete survival factors helping AML growth. Since I previously performed scRNA sequencing on both macrophages and AML cells, it would be interesting to perform a protein-protein interaction analysis and uncover whether macrophages are responsible for the secretion of survival factors. Interestingly, these results were similar to the results published by X. Yang et al., [174] who found that splenic macrophages cultured with AML cells were capable of promoting the growth of AML cells *in vitro* and these cells were also highly phagocytic.

Meanwhile, to address whether the direct contact between macrophages and AML cells was essential to stimulate the growth of AML cells, I took advantage of the Boyden chamber assay where a membrane is placed between the top and bottom part of the culture. This membrane contains holes of 5 microns hence secreted factors can circulate creating a gradient, often promoting the migration of the cells. The size of the holes in the membrane is smaller than the size of the cells and as such to transfer from one side of the chamber to the other, the cell must actively squeeze through the membrane. This set-up limits passive diffusion of cells and allows us to study both the migration and the importance of cell to cell contact for proliferation. While the growth difference was no longer significant in this setting, AML cells were found to migrate preferentially towards macrophages. This result was consistent with the work performed by M. Casanova-Acebes et al. [1], which found that in the context of lung tumours, tissue resident macrophages were promoting the migration and invasiveness of tumour cells. In addition, I. Weihauser et al., [180] showed that co-culture of human macrophages with leukemic cells improved the engraftment of leukemic cells *in vivo*.

Hence, next I evaluated whether in our context, macrophages were also important for the homing and engraftment of leukemic cells. Depletion of macrophages using clodronate revealed that, *in vivo*, AML cells motility was significantly reduced without macrophages and the total number of AML cells found 24 hours after transplantation of these cells was also significantly reduced. Hence, I could conclude that macrophages are involved in the regulation of the motility and engraftment of AML cells. Thanks to the migration assay, I identified that macrophages secreted four main chemokines, CCL2, CCL6, CCL9/10 and CCL12, known to promote the migration of leukocytes. In addition, AML cells were found to express both receptors for these chemokines. Unfortunately, when I tried to replace the macrophages with the chemokines in the bottom well, AML cells were no longer preferentially migrating. Hence, an additional mechanism in macrophages must be responsible for the migration of AML cells.

To understand whether this mechanism is responsible for the growth difference seen in clodronate mice, I depleted macrophages with clodronate liposomes only during the homing and engraftment phase. I then let the macrophage repopulate the bone marrow environment and evaluated whether the disease growth was impacted. Interestingly, the AML in single clodronate treated mice grew at the same speed as in control mice. While this means that this mechanism is not essential in AML growth, it could still be highly relevant in a post-chemotherapy context where AML cells are known to repopulate the bone marrow. It would be interesting to address whether disruption with clodronate limits the relapse of AML after chemotherapy.

Altogether, these results show that macrophages are capable of promoting the homing and motility of AML cells, however, this mechanism alone is not sufficient to delay AML cells growth in single clodronate treated mice. Hence, macrophages must be playing an additional role to promote AML growth, most likely once the disease has engrafted and macrophages were able to repopulate the bone marrow. Thanks to the results shown in Chapter 4, I know that IMMs are enriched at early AML and these cells preferentially repopulate the bone marrow after the single clodronate treatment, hence it is most likely that these cells are responsible for the growth difference in repeated clodronate treated AML mice.

From the RNA sequencing analysis, I found that IMMs were preferentially expressing genes involved in both antigen presentation and efferocytosis, and their presence correlated with increased AML growth *in vivo* hence I hypothesized that IMMs promote AML growth through their role as an antigen-presenting cell and inhibition of T cells. Co-cultures of activated T

cells with Ly6C-derived IMMs or HSMs revealed that IMMs were capable of inhibiting T cells while HSMs supported T cell proliferation. Interestingly, X. Yang et al., [174] also performed co-cultures of activated T cells and macrophages however rather than sorting specific populations of macrophages, they performed co-cultures with all bone marrow macrophages or splenic macrophages. In their bulk RNA sequencing analysis they found different signatures of M1 and M2 phenotypes suggesting that some heterogeneity was present in these cell populations. They found that all macrophages, whether they were sorted from the spleen or the bone marrow, supported the proliferation of T cells. Since we know that IMMs represent only a small fraction of macrophages, our results are in line with their results and reinforces the idea that I have identified a small pro-tumoural population of macrophages.

Surprisingly, when I evaluated the phenotype of T cells in AML mice with clodronate depleted macrophages, I found reduced T cell activation. However, in CD169-DTR^{het} mice, which have increased IMMs, I found an increase in T cell activation. In parallel to this increase in activation, I also found an increase in the fraction of CD25+ cells among CD4+ T cells and in PD1 expressing CD8+ T cells. Hence, these results suggest that the activation of T cells is followed by an exhausted phenotype involving Tregs and PD1+ T cells. Interestingly, both steps in this process are dependent on the presence of IMMs as their depletion stops both the activation and the inhibition of T cells. Unfortunately, these results are mostly trends for the CD169-DTR model as more mice would be needed to reach significant results. Altogether, these results indicate that IMMs are playing a role in shaping the anti-tumour T cell response.

Finally, to confirm that the interaction between the macrophages and T cells was responsible for the difference in AML growth, I took advantage of the RagKO mouse model, which lack all mature lymphocytes and injected clodronate to deplete macrophages. Since no growth difference was found between the RagKO treated with PBS liposomes or clodronate liposomes, it validated that macrophages, in particular IMMs are promoting AML growth through their interaction with T cells. Altogether, I have identified that among the two macrophage populations identified in the BM, IMMs are responsible for the phagocytosis of AML cells and inhibition of T cells, in turn, promoting AML growth.

The data presented here show that a specific population of macrophages in the BM, IMMs, express a high level of efferocytic genes and are responsible for the inhibition of T cells. The depletion of these cells correlated with reduced AML growth while their increase is associated

with faster AML progression. These results highlight a number of possible targets which future work should investigate in order to find novel therapies which, combined with current therapies could help restore anti-tumour T cell response and reverse disease growth. These findings could have a significant impact for AML research and the development of therapeutic strategies which I will discuss in the final chapter.

Chapter 6

Summary, limitations and future directions

Cancer is the second cause of death worldwide. Among the different types of cancers, acute myeloid leukemia (AML), a hematological malignancy, is considered one of the most aggressive forms of cancer. [AML](#) treatment has not changed in the past three decades with intensive chemotherapy remaining the primary treatment for this cancer. However, relapse after therapy (50-90%) leads to the development of an aggressive form of leukaemia, resistant to current treatments. While novel targeted therapies are on the rise for AML, the results in elderly patients remain disappointing [\[124\]](#). A better understanding of the disease and its interaction with the immune system is essential to develop targeted therapies with lower side effects and prevent relapse occurrence.

Macrophages, as part of the innate immune system, are known as a heterogeneous population of immune cells. It has been established that a specific type of macrophages, tumour-associated macrophages (TAMs), play an important role in solid tumours. Through their interaction with the immune response, microenvironment and tumour cells, TAMs are capable of promoting tumour growth, invasiveness, immune escape and metastasis [\[137\]](#). However, in AML, these cells remain highly understudied and their role during disease progression is unclear. Several studies have shown that a sub-population of macrophages, CD206+ macrophages, were associated with a poor outcome in AML [\[178, 180\]](#), however their function remains unknown. This result highlights the possibility that different populations of macrophages are present in the bone marrow, associated with different phenotypes, functions and origins. Supporting this idea, it is known that in the bone marrow macrophages play an important role in the retention

of [HSCs](#), the enucleation of red blood cells and the regulation of the immune response, three different functions associated with different phenotypes.

In this thesis, I investigated whether different populations of macrophages are present in the bone marrow, and whether these populations play a different role during AML progression. The overarching hypothesis for this work was that:

“The cross-talk between AML cells and different macrophage populations takes place in the bone marrow as AML progresses and this cross-talk promotes AML growth while impairing the anti-tumour immune response”

Using a combination of techniques, I characterised the different populations of macrophages present in the bone marrow (Chapter 3), their dynamics with AML and the evolution of their transcriptional signature during AML (Chapter 4). Finally, I explored the function of the different populations during AML growth, their direct interaction with malignant cells and T cells to understand the impact of these interactions on disease growth (Chapter 5). To answer my research questions and gain an insight into the role and phenotype of the various bone marrow macrophage populations during AML, I studied AML transplanted mice using a combination of phenotypic, functional, molecular and IVM analyses over the course of the disease. In this final chapter, I will summarise the findings and conclusions related to each of my research questions, propose avenues for new research and highlight the wider implications of this work.

What are the different populations of macrophages in the bone marrow?

In Chapter 3, I used spectral flow cytometry to characterise the different populations of macrophages in the bone marrow. The panel, composed of 21 different markers, allowed me to distinguish the different myeloid cell populations and evaluate the expression of known bone marrow macrophage markers and [TAM](#) markers. The general consensus for the identification of bone marrow macrophages is that these cells express high levels of CD11b and F4/80 and don't express Ly6G or Ly6C at high levels [42, 43, 67, 52, 270]. Combining the expression of these four different markers and unsupervised clustering, I identified two main bone marrow macrophage populations. The first population, Hematopoiesis-supportive macrophages (HSM), expressed high levels of [VCAM-1](#) and CD169, the combination of the expression of these two receptors has been shown to be expressed in HSC-retaining macrophages [88, 89, 92, 95, 96]. The second

population, named immunomodulatory macrophages (IMM), expressed high levels of SIRP α , PDL-1 and Tumour necrosis factor α (TNF α), all three markers highlighting cells involved in the regulation of the immune response [271, 234]. Surprisingly, apart from the markers mentioned above, bone marrow macrophages did not express any of the other TAM markers included in this large panel such as iNos and Arg1, even in the AML condition, while splenic and peritoneal macrophages did. Hence, I could conclude that bone marrow macrophages are phenotypically different to tumour-associated macrophages during AML.

Next, I combined various techniques to identify bone marrow macrophages using confocal microscopy. The CD169-YFP transgenic mice had previously been described as a good reporter for bone marrow macrophages. Using intravital microscopy, I confirmed that the cells expressing the YFP protein coincided with F4/80 and CD169 expressing cells as well as some SIRP α expressing cells. The depletion of HSMs in the CD169-DTR mice finally confirmed the use of CD169 reporter mice as a good mouse model for the study of bone marrow macrophages. Thanks to these results I was able to properly question the function and phenotypes of the different bone marrow macrophage populations during AML.

Are macrophages affected by AML, in terms of numbers, spatial distribution and transcriptional signatures and can I identify the molecular changes driving these alterations?

In Chapter 4, I addressed the evolution of IMMs and HSMs, in terms of number, phenotype and transcriptional signature during AML. HSMs were found to be among the first cells to be lost in the bone marrow as AML grew while IMMs were enriched at early AML. These results correlated with findings in lymphoma, where SIRP α + macrophages characterised a sub-population of macrophages that were enriched compared to SIRP α - macrophages and were associated with worse clinical outcome [217]. Given the differences in phenotype and dynamics between HSMs and IMMs, I hypothesized that these cells were functionally and transcriptionally different. scRNAseq analysis allowed a broad and unbiased understanding of the transcriptional changes taking place between the different macrophage populations as a consequence of AML growth. Following the identification of the macrophage clusters, I was able to identify several macrophage populations, however the expression of *Siglec1* (CD169) at the transcriptional level was very low. Hence it was not possible to validate the identification of HSMs in the RNA sequencing dataset. Having said that, one population of macrophages was

found to express a number of genes involved in the recycling of iron and also expressed significantly higher levels of VCAM-1 compared to other macrophages hence these cells are likely to be HSMs [91]. Using the CITE-seq technology, I was able to confirm the identification of IMMs through the expression of *Adgre1*, *SIRP α* and *Cd274*, at the protein and gene level. In addition, these cells were found to express high levels of genes associated with efferocytosis, a process essential in the clearance of aged neutrophils [228] and often hijacked by tumour cells to promote anti-inflammatory signaling [163].

Combining this dataset with a dataset containing lung macrophages from healthy and adenocarcinoma lung tumours, I questioned whether the populations of macrophages described previously were unique to the bone marrow. Lung macrophages and bone marrow macrophages were found to cluster together and share a number of biological processes such as antigen processing and presentation and tissue remodelling. During malignancy, macrophages were similarly conserved in both organs, as malignant and healthy macrophages clustered together, without any clusters of macrophages being present in only one condition. Together these results highlight, for the first time, the similarities between bone marrow and lung macrophages and their evolution through malignancy. The lack of a “malignant-only” macrophage cluster suggests that TAMs are not a different population of macrophages but are simply the result of small transcriptional changes taking place in healthy macrophages. While few changes were noticed during malignancy in both lung and bone marrow macrophages, A. Casanova-Acebes et al., showed that lung macrophages were responsible for increased invasiveness of tumour cells and were also capable of increasing the recruitment and maintenance of Tregs in the tumour microenvironment. Hence, I questioned whether bone marrow macrophages were also involved in AML invasiveness and T cell regulation.

How do macrophages influence AML growth, especially do they directly interact with AML cells and can they influence the anti-tumour T cell response?

Based on the findings described in Chapter 3 and 4, in Chapter 5, I used a combination of macrophage depleting methods and *in vitro* assays to study the function of the different macrophage populations identified. *In vivo*, I observed that the depletion of all macrophages reduced the growth of AML while the targeted loss of HSMs and enrichment of IMMs led to increased AML growth. Using IVM of macrophage depleted mice, I identified that macrophages are an essential component of the bone marrow to promote AML cell motility and engraftment

as their depletion led to significantly reduced homing and motility of AML cells. In addition, I observed, in real time, the uptake of AML cells by macrophages. Hence, I hypothesised that a macrophage population was responsible for the phagocytosis of AML cells. Supporting this hypothesis, *in vitro* IMMs were found to be able to phagocytose AML cells and inhibit activated T cells while HSMs were unable to phagocytose any AML cells, however they supported T cell proliferation. *In vivo* analysis of the T cell compartment revealed that the depletion of all macrophages correlated with reduced T cell activation while the enrichment in IMMs was associated with increased T cell activation. Hence, IMMs are responsible for both the activation and inhibition of T cells in AML, however given the increased speed of AML in IMMs enriched mice, this process is overall supportive of leukemia growth. Finally, the use of the T cell deficient mouse model, the RagKo mice, revealed that the interaction between macrophages and T cells is responsible for the delayed growth in macrophage depleted mice.

In summary, in this thesis I have carried out a broad, unbiased analysis of macrophages in the bone marrow during AML - shedding light on the dynamics, functionality, phenotypic and transcriptional signature of the different macrophage populations present in this tissue (Figure 6.1). Based on these results, I have proposed a number of limitations and future directions for this work.

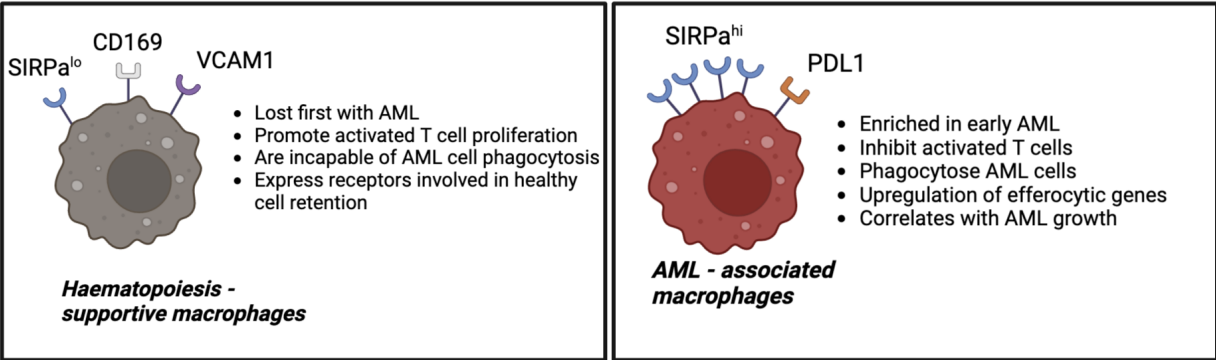


Figure 6.1: Macrophage populations identified in the bone marrow and their associated function during AML

Limitations

One surprising result in the RNA sequencing analysis was that approximately 50% of the sample was composed of neutrophils. The lack of scRNA sequencing dataset on bone marrow macrophages did not allow us to address if this was a common phenomenon in bone marrow macrophage studies. Hence, I compared this dataset with a lung dataset contain-

ing macrophages from healthy and adenocarcinoma lungs, published in Nature [1]. Not only, did their sample also contain 50% of neutrophils, it also contained a large proportion of T cells and B cells. I found a third study, focusing on embryonic macrophages, that was also composed at 50% of neutrophils [250]. One possible explanation for the high proportion of neutrophils contained in these macrophage studies, is that macrophages are fragmented during the sample processing, as described by S. Millard [207]. In their work, they described that the processing of samples towards a single cell suspension leads to the fragmentation of macrophages into subcellular remnants. These remnants were found to adhere to other cells such as neutrophils and create doubled identity cells. They conclude that no macrophage remains intact after this sort of processing and therefore the cells analysed by flow cytometry or RNA sequencing are not macrophages. Alternatively, the high percentage of contamination could correspond to a population of myeloid cells, in a differentiation state contained between neutrophils and macrophages, that have not been studied so far. Indeed, given that neutrophils and macrophages originate from the same progenitors, GMPs, it is possible that an additional outcome to the differentiation of these progenitors would be a granular macrophage, expressing a number of neutrophil genes but phenotypically similar to macrophages and as such these cells would have been sorted as macrophages. I. Winkler et al. [67] have previously described a population of Ly6G+ F4/80+ cells in the bone marrow that were sensitive to clodronate. In addition, in the unsupervised analysis of the spectral flow cytometry data described in Chapter 3, a high proportion of cells were F4/80+ but were not categorised as macrophages. These cells could probably correspond to the cells found in the RNA sequencing data. It would be interesting to further characterise these cells and understand whether an additional differentiation program is responsible for the generation of these cells.

Another discrepancy in the results presented above were the results produced in the CD169-YFP mice. In these mice I observed CD169-YFP+ cells uptaking AML cells. However, *in vitro* HSMs, which correspond to the macrophages expressing CD169, were not capable of phagocytosing AML cells. Given that the CD169-YFP mice label any cell that previously expressed or is currently expressing CD169, it is possible that IMMs, which were shown to phagocytose AML cells *in vitro*, are also the cells visualised uptaking AML cells *in vivo*. However, this would imply that IMMs previously expressed CD169 and stopped expressing this gene. This interpretation contradicts the results found in the macrophage depletion model: CD169-DTR. In these mice, all CD169+ cells were deleted, however there was an increase in IMMs in AML. If these cells derived from CD169+ cells then it would be impossible to get an increase in these cells in

CD169-DTR depleted mice. Another possible explanation is that in the reporter I visualised cells closer to the bone and these cells are known to be the hardest to extract. Preliminary flow cytometry analysis revealed that very few YFP+ cells were detected, consistent with the idea that these cells are harder to extract. Given their proximity to the bone, these cells could also be a different population of macrophages such as osteomacs. To test this hypothesis it would be interesting to analyse some sections of CD169-YFP+ bones and correlate the staining with HSMs and IMM markers.

Future directions

Altogether, these results have highlighted the role of macrophage populations in the bone marrow during AML progression. One important question to bridge the gap between this work and translational research is to uncover whether these populations are also present in AML patients. It is known that AML patients have enriched macrophages [173] and as such I can hypothesise that IMMs are responsible for this increase however it would be essential to confirm this before trying to develop any targeted therapies.

Given the transcriptional signature of IMMs and their involvement in the phagocytosis of AML cells and T cell inhibition, one interesting group of targets to revert the function of these cells would be the efferocytic genes, found to be overexpressed specifically in these cells. Indeed, it has been shown that the inhibition of the efferocytic receptor, MerTK, in macrophages from solid tumours leads to increase necrosis of tumour cells which promotes phagocytosis by macrophages [222]. Macrophages are then able to process correctly the tumour antigens to present them to T cells, in turn leading to the activation of both T cells and macrophages [222, 164]. While IMMs were found to express only low levels of *MerTK*, these cells do express high levels of other efferocytic receptors such as *Axl* and *Nr1h3*. Previous studies have shown as a proof of concept that targeting of the efferocytic gene, *Axl*, in macrophages, help restore the immune response against AML and reduce disease growth [252]. Additionally, IMMs were found to express high levels of the *C1q* genes. These genes are part of the complement cascade and were found to be associated, in macrophages, with increased pro-efferocytic phenotype and reduced TNF α response [272]. In addition, C1q production by macrophages was associated with increased tumour growth [273]. Hence, if IMMs are found to be responsible for the increase of macrophages in patients and express efferocytic genes, inhibition of these genes could allow us to restore the anti-tumour immune response and delay or ideally eliminate, AML growth.

Another important consideration to keep in mind is that, nowadays, most drugs are tested as combination therapy with chemotherapy. Incidentally, I have found that macrophages repopulate the bone marrow within 5 days after chemotherapy treatment (data not shown). Based on the fact that IMMs were the first to repopulate the bone marrow after a single clodronate injection, it is likely that IMMs would be the main macrophages to repopulate the bone marrow after chemotherapy. Hence, these cells could be playing a major role in disease recurrence. Elucidating whether IMMs are the cells repopulating the bone marrow after chemotherapy seems key to develop a macrophage targeted therapy. Ultimately, combining chemotherapy with a macrophage depleting agent such as clodronate would allow to assess the importance of IMMs in relapse.

I believe that the data presented in this thesis contributes greatly to furthering our knowledge and understanding of the role of the innate immune system, in particular macrophages, in AML growth and highlights the importance of following up this investigation with further studies, including translational work, with the ultimate aim of improving therapies for AML.

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Appendices

Appendix A

Cell Ranger data for scRNAseq quality control

Macrophages from control BM

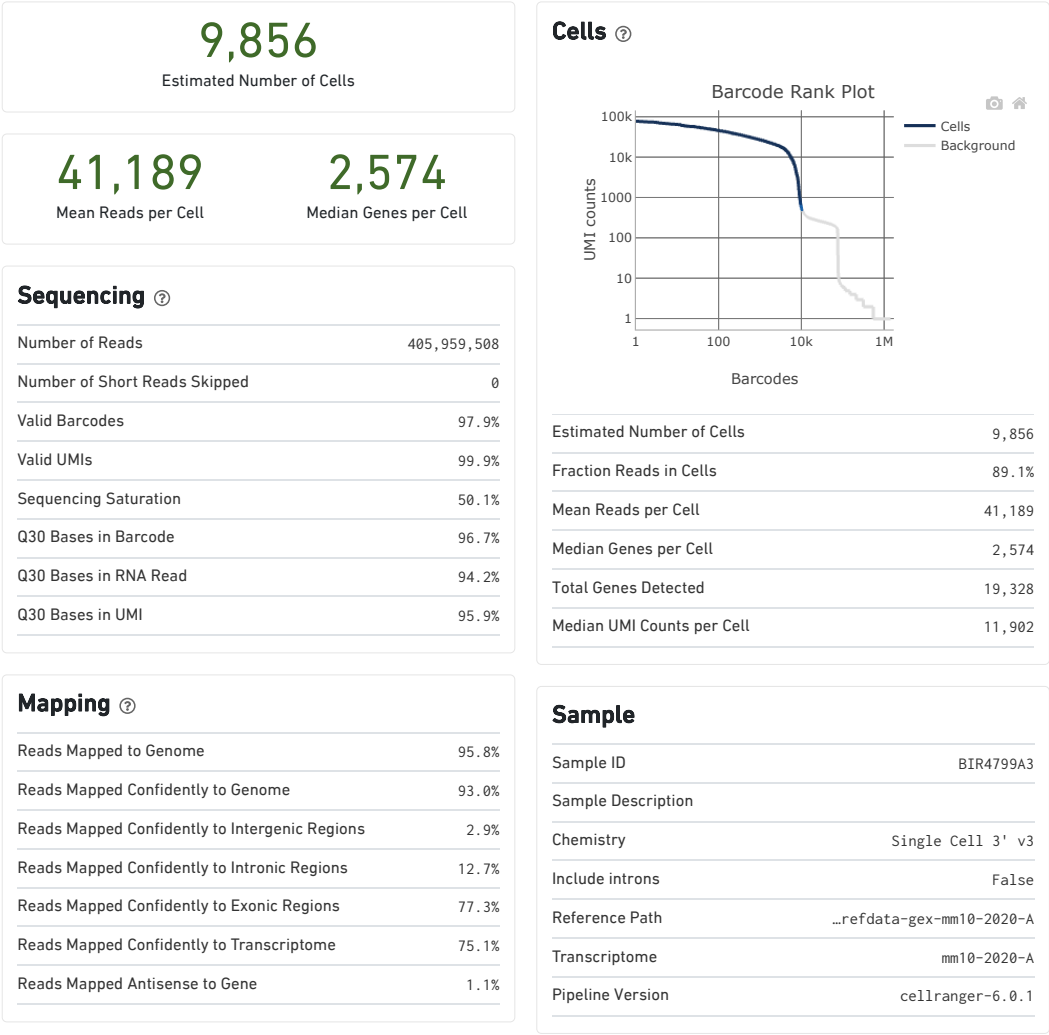


Figure A.1: Cell Ranger quality control of control macrophage sample

Macrophages from AML BM

10,389
Estimated Number of Cells

47,596
Mean Reads per Cell

2,499
Median Genes per Cell

Sequencing ⓘ

Number of Reads	494,472,937
Number of Short Reads Skipped	0
Valid Barcodes	97.9%
Valid UMIs	99.9%
Sequencing Saturation	57.7%
Q30 Bases in Barcode	96.6%
Q30 Bases in RNA Read	95.0%
Q30 Bases in UMI	95.8%

Mapping ⓘ

Reads Mapped to Genome	95.8%
Reads Mapped Confidently to Genome	92.8%
Reads Mapped Confidently to Intergenic Regions	3.1%
Reads Mapped Confidently to Intronic Regions	15.3%
Reads Mapped Confidently to Exonic Regions	74.4%
Reads Mapped Confidently to Transcriptome	72.2%
Reads Mapped Antisense to Gene	1.1%

Cells ⓘ

Barcode Rank Plot

Estimated Number of Cells: 10,389
Fraction Reads in Cells: 94.6%
Mean Reads per Cell: 47,596
Median Genes per Cell: 2,499
Total Genes Detected: 20,233
Median UMI Counts per Cell: 9,439

Sample

Sample ID	BIR4799A4
Sample Description	
Chemistry	Single Cell 3' v3
Include introns	False
Reference Path	...refdata-gex-mm10-2020-A
Transcriptome	mm10-2020-A
Pipeline Version	cellranger-6.0.1

Figure A.2: Cell Ranger quality control of AML macrophage sample

AML cells from early AML BM

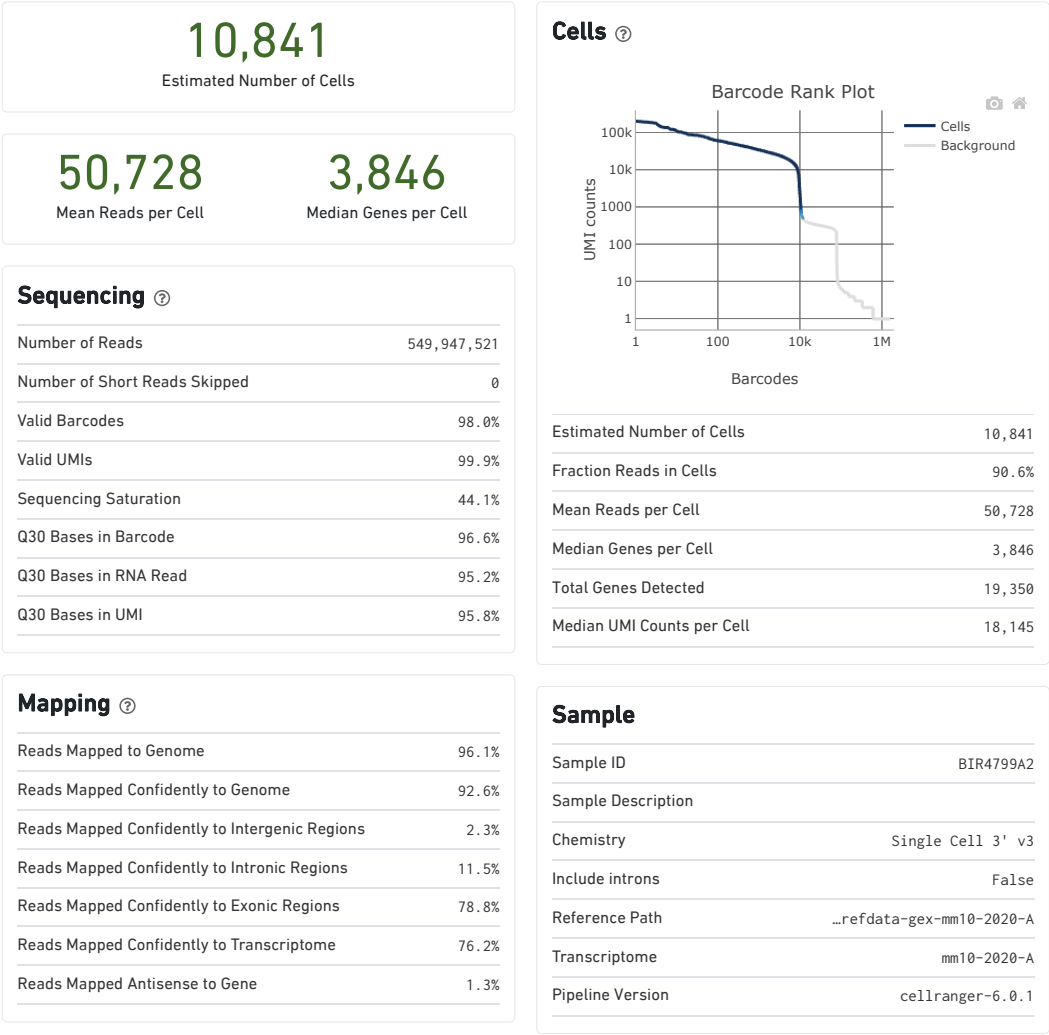


Figure A.3: Cell Ranger quality control of early AML sample

AML cells from late AML BM

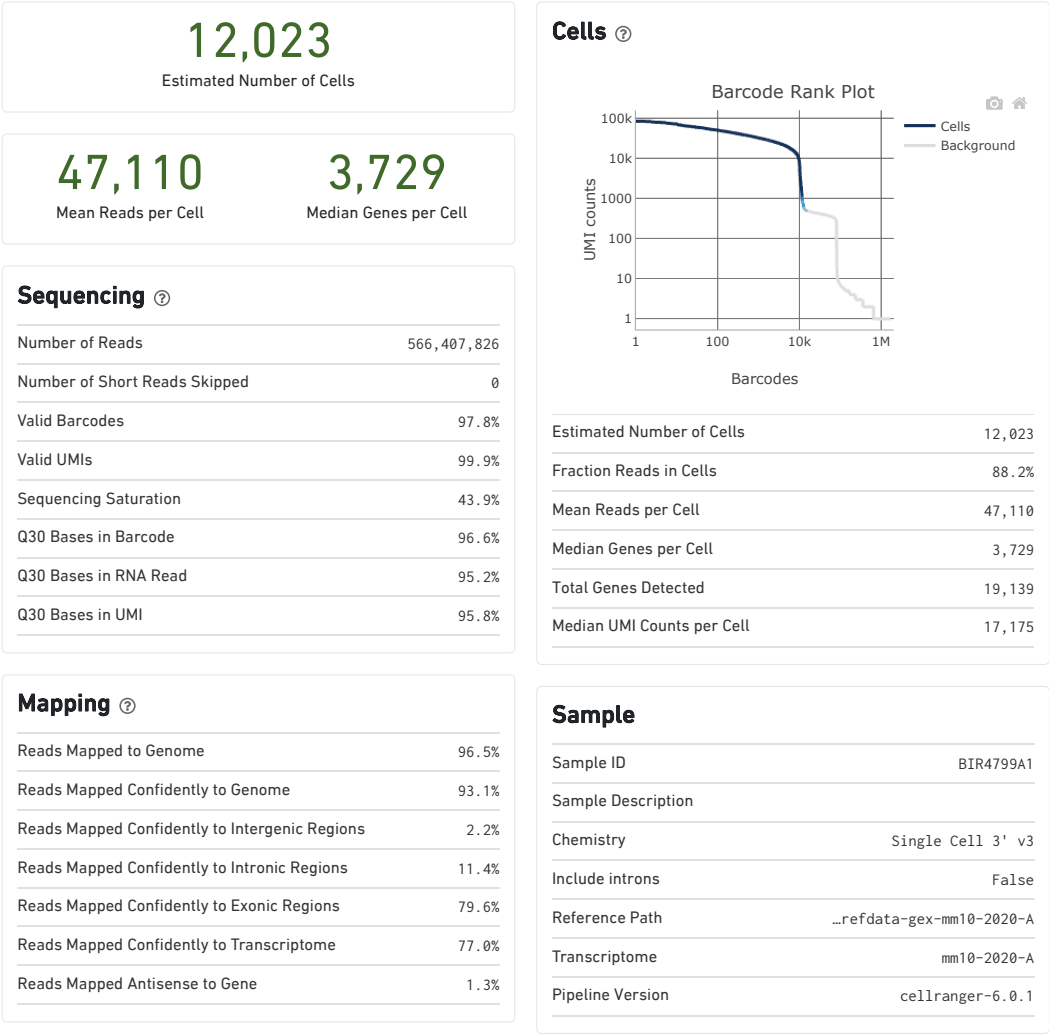


Figure A.4: Cell Ranger quality control of early AML sample

Appendix B

Neutrophils analysis in the scRNAseq

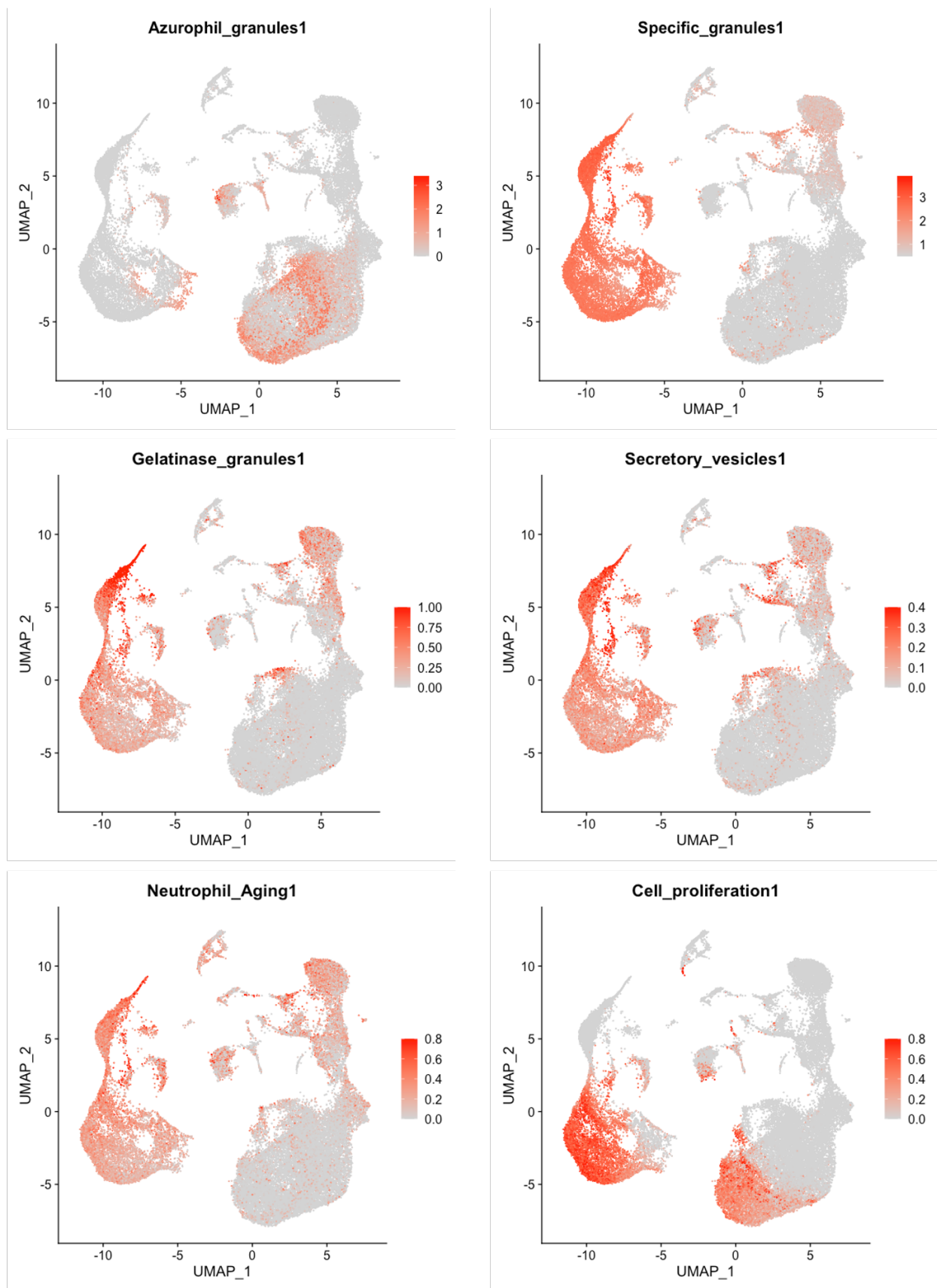


Figure B.1: Analysis of neutrophil granule formation signature
Neutrophil maturation was assessed through the expression of granule formation, neutrophil aging and cell proliferation genes, as published by X. Xie et al. [244].

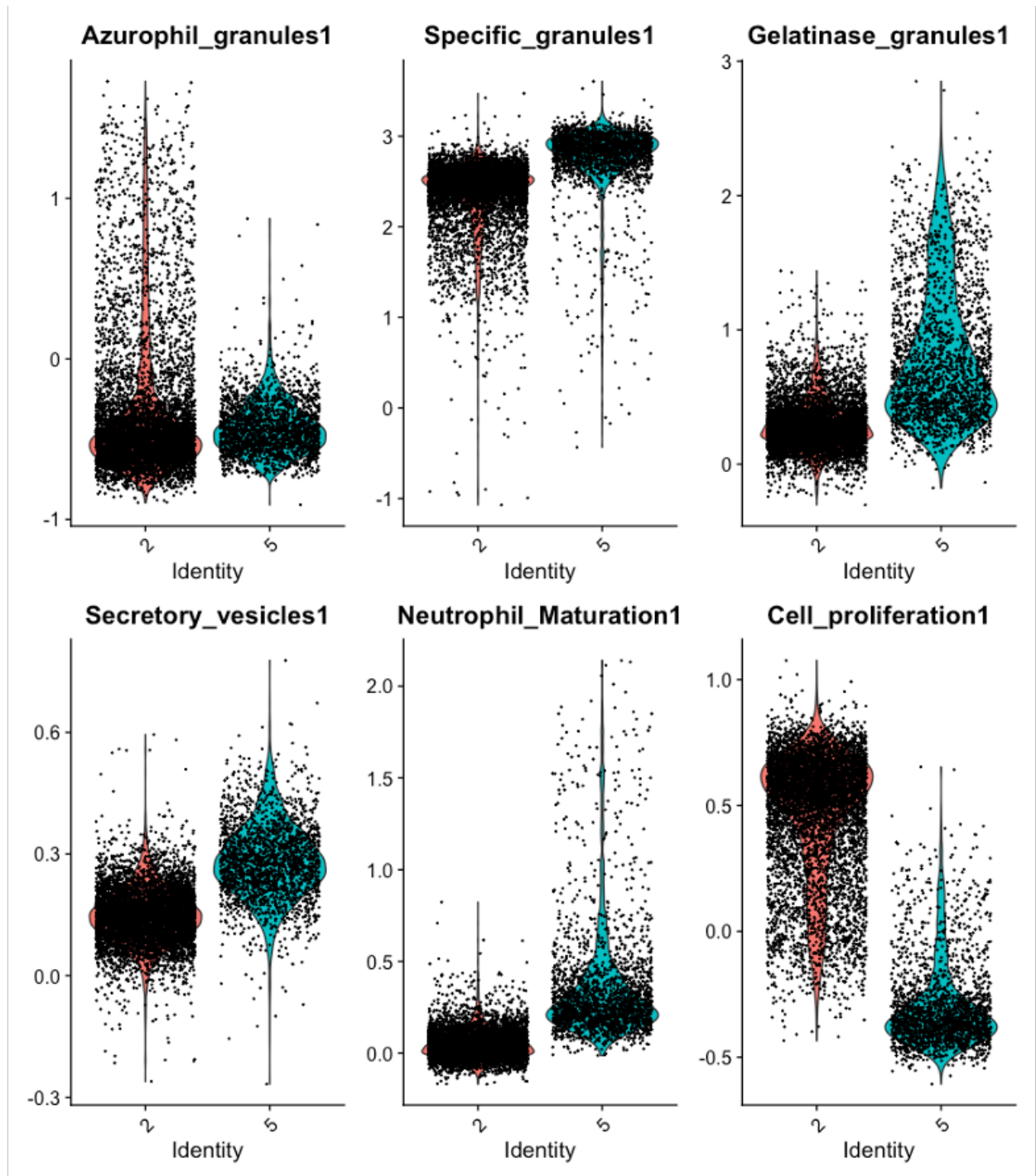


Figure B.2: Different immature neutrophils are present in the scRNAseq
Violin plots of normalised expression of granule formation, neutrophil maturation and cell proliferation genes in cluster 2 and 5. Cluster represents more immature neutrophils corresponding to myelocytes and cluster 5 correspond to band cells.

Appendix C

Gene lists used in scRNAseq analysis

Mphs core signature genes			
1810011H11Rik	Dhrs3	Mertk	Siglece
4632428N05Rik	Dnase2a	Mgst1	Slc11a1
6430548M08Rik	Dok3	Mitf	Slc15a3
A930039A15Rik	Engase	Mr1	Slc16a10
Abca1	Fcgr1	Myo7a	Slc16a7
Abcc3	Fcgr3	Nr1d1	Slc17a5
Abcc5	Fcgr4	P2ry13	Slc29a1
Acy1	Fert2	Pcyox1	Slc38a6
Adgre1	Fez2	Pecr	Slc38a7
Apoe	Fgd4	Pilra	Slc7a2
Arsg	Fpr1	Pilrb1	Slco2b1
Arsk	Gbp6	Pilrb2	Slpi
Asph	Glul	Pla2g15	Snx24
Atp13a2	Gm4878	Pla2g4a	Sqrdl
Atp6ap1	Gpr160	Pld1	St7
C130050O18Rik	Gpr177	Pld3	Tanc2
C1qa	Gpr77	Plod1	Tbxas1
C1qb	Hgf	Plod3	Tcn2
C1qc	Hgsnat	Plxnb2	Tfpi
C5ar1	Hmox1	Pon3	Tgfbr2
Camk1	Il1a	Pros1	Timp2
Cd14	Itga9	Pstpip2	Tlr4
Cd151	Itgb5	Ptgs1	Tlr7
Cd164	Klra2	Ptplad2	Tlr8
Cd302	Lamp2	Ptpm	Tmem195
Cd33	Lonrf3	Rhob	Tmem77
Cebpb	Lpl	Rhoq	Tmem86a
Cmklr1	Lrp1	Rnasel	Tnfrsf21
Comt1	Ltc4s	Rnf13	Tom1
Csf3r	Lyplal1	Sepn1	Tpp1
Ctsd	Mafb	Sepp1	Tspan14
Ctsf	Man2b2	Serpnb6a	Tspan4
Ctsl	Mavs	Sesn1	Xrcc5

Figure C.1: Macrophage core signature genes

BM Mphs core signature genes	
Adgre1	Mavs
Akr1b10	Pcyox1
Asph	Pilrb2
Atp13a2	Pla2g15
Blvrb	Pparg
Camk1	Rhob
Cd302	Rhoq
Cd68	Slc15a3
Cd74	Slc16a10
Cebpb	Slc16a7
Csf1r	Slc29a1
Csf3r	Slc38a7
Dnase2a	Tgfbr2
Fcgr1	Tlr13
Fez2	Tlr4
Fgd4	Tlr7
Glul	Tlr8
Il1b	Tnfrsf21
Itgb5	Pld3
Klra2	Dok3
Mafb	Tbxas1
Tspan14	

Figure C.2: BM Macrophage core signature genes

Phagocytosis GO0006909							
4933434E20Rik	Ighv5-15	Gm12169	Pip4p2	Cd300lf	Itga2	Ighv1-54	Slamf1
Abca1	Ighv5-16	Gsn	Pip5k1c	Cd302	Itgal	Ighv1-55	Slc11a1
Abca7	Ighv5-17	Gulp1	Pla2g5	Cdc42	Itgam	Ighv1-56	Snx3
Abl1	Ighv6-3	Havcr1	Plcg2	Cdc42se1	Itgav	Ighv1-58	Sod1
Abl2	Ighv6-4	Hck	Pld4	Cdc42se2	Itgb1	Ighv1-59	Spg11
Abr	Ighv6-5	Hmgb1	Pot1b	Cebpe	Itgb2	Ighv1-61	Sphk1
Adgrb1	Ighv6-6	Icam5	Pparg	Cfb	Itgb3	Ighv1-62-2	Spon2
Adipoq	Ighv6-7	Ifng	Prkcg	Cfp	Jmjd6	Ighv1-62-3	Srpx
Ager	Ighv7-2	Igh-8	Pros1	Clcn3	Klrl1	Ighv1-63	Stap1
Ahsg	Ighv7-4	Igha	Prtn3	Clec7a	Lbp	Ighv1-64	Syk
Aif1	Ighv8-2	Ighd	Pten	Cln3	Ldlr	Ighv1-66	Syt7
Alox15	Ighv8-4	Ighe	Ptk2	Cnn2	Lep	Ighv1-67	Syt11
Ano6	Ighv8-5	Ighg	Ptpcr	Colec10	Lepr	Ighv1-69	Tafa4
Anxa1	Ighv8-6	Ighg1	Ptprr	Colec11	Letmd1	Ighv1-72	Tgfb1
Anxa3	Ighv8-8	Ighg2a	Ptx3	Colec12	Lman2	Ighv1-74	Tgm2
Anxa11	Ighv8-9	Ighg2b	Pycard	Coro1a	Lrp1	Ighv1-75	Thbs1
Apoa1	Ighv8-11	Ighg2c	Rab5a	Coro1c	Lyar	Ighv1-76	Ticam2
Apoa2	Ighv8-12	Ighg3	Rab7	Cryba1	Lyst	Ighv1-77	Timd2
Appl1	Ighv8-13	Ighm	Rab7b	Csk	Marco	Ighv1-78	Timd4
Appl2	Ighv9-1	Ighv1-4	Rab11fip2	Cyba	Masp1	Ighv1-80	Tlr2
Arhgap12	Ighv9-2	Ighv1-5	Rab14	Dnm2	Masp2	Ighv1-81	Tlr4
Arhgap25	Ighv9-3	Ighv1-7	Rab20	Dock1	Mbl1	Ighv1-82	Tm9sf4
Arl8b	Ighv9-4	Ighv1-9	Rab27a	Dock2	Mbl2	Ighv1-84	Tmem175
Atg3	Ighv10-1	Ighv1-11	Rab31	Dppa1	Megf10	Ighv1-85	Tnf
Atg5	Ighv10-3	Ighv1-12	Rab34	Dysf	Mertk	Ighv2-2	Trbc1
Atg7	Ighv11-1	Ighv1-15	Rab39	Eif2ak1	Mesd	Ighv2-3	Trbc2
Axl	Ighv11-2	Ighv1-16	Rac1	Elane	Met	Ighv2-4	Trdc
BC053393	Ighv12-3	Ighv1-18	Rac2	Elmo1	Mex3b	Ighv2-5	Trdv4
Bcr	Ighv13-2	Ighv1-19	Rack1	Elmo2	Mfge8	Ighv2-6	Trem2
Becn1	Ighv14-1	Ighv1-20	Rap1a	Elmo3	Msr1	Ighv2-6-8	Trem14
Bin2	Ighv14-2	Ighv1-22	Rap1gap	F2rl1	Mst1r	Ighv2-7	Trex1
Bltp1	Ighv14-3	Ighv1-23	Rapgef1	Fcer1g	Myd88	Ighv2-9	Tub
Btk	Ighv14-4	Ighv1-24	Rara	Fcgr1	Myh9	Ighv2-9-1	Tulp1
C2	Ighv15-2	Ighv1-26	Rhobtb1	Fcgr2b	Myo1g	Ighv3-1	Tusc2
C3	Ighv16-1	Ighv1-31	Rhobtb2	Fcgr3	Myo7a	Ighv3-3	Tyro3
C4b	Igkc	Ighv1-34	Rhog	Fer1l5	Myo18a	Ighv3-4	Tyrbp
C4bp	Iglc1	Ighv1-36	Rhoh	Fgr	Ncf2	Ighv3-5	Unc13d
Calr	Iglc2	Ighv1-37	Scarb1	Fpr2	Ncf4	Ighv3-6	Vamp7
Camk1d	Iglc3	Ighv1-39	Sftpa1	Fpr3	Nckap1l	Ighv3-8	Vav1
Ccl2	Igll1	Ighv1-42	Sftpd	Fpr-rs3	Nod2	Ighv4-1	Washc5
Ccr2	Il1b	Ighv1-43	Sh3bp1	Fpr-rs4	Nr1h3	Ighv5-2	Xkr4
Ccr7	Il2rb	Ighv1-47	Siglece	Fpr-rs6	P2rx7	Ighv5-4	Xkr5
Cd36	Il2rg	Ighv1-49	Sirpa	Fpr-rs7	P2ry6	Ighv5-6	Xkr6
Cd47	Il15	Ighv1-50	Sirpb1a	Gas6	Pear1	Ighv5-9	Xkr7
Cd209b	Il15ra	Ighv1-52	Sirpb1b	Gata2	Pecam1	Ighv5-9-1	Xkr8
Cd300a	Irf8	Ighv1-53	Sirpb1c	Gm5150	Pik3ca	Ighv5-12	Xkr9
Gm9733	Pikfyve	Ighv5-12-4	Zp3r				

Figure C.3: Phagocytosis gene list

Antigen processing and presentation		
Abcb9	H2-K1	Nod1
Ap3b1	H2-L	Nod2
Arl8b	H2-M1	Pdia3
Atg5	H2-M2	Pikfyve
Azgp1	H2-M3	Psap
B2m	H2-M5	Psmb8
Bag6	H2-M9	Psmb9
Calr	H2-M10.1	Psme1
Ccl19	H2-M10.2	Psme2
Ccl21a	H2-M10.3	Ptpn22
Ccr7	H2-M10.4	Pycard
Cd1d1	H2-M10.5	Rab3b
Cd68	H2-M10.6	Rab3c
Cd74	H2-M11	Rab4a
Clec4b2	H2-Oa	Rab5b
Ctse	H2-Ob	Rab6a
Ctsl	H2-Q1	Rab8b
Ctss	H2-Q2	Rab10
Erap1	H2-Q4	Rab27a
Ext1	H2-Q6	Rab32
Fcer1g	H2-Q7	Rab33a
Fcgr1	H2-Q8	Rab34
Fcgr2b	H2-Q9	Rab35
Fcgr3	H2-Q10	Relb
Fcgr4	H2-T3	Rftn1
Fgl2	H2-T22	Slc11a1
Flt3	H2-T23	Tap1
Gba	H2-T24	Tap2
Gm7030	Hfe	Tapbp
Gm8909	Icam1	Tapbpl
Gm11127	Ide	Thbs1
H2-Aa	Ifi30	Traf6
H2-Ab1	Ifng	Trem2
H2-D1	Ighg2a	Treml4
H2-DMa	Ighm	Trex1
H2-DMb1	Kdm5d	Unc93b1
H2-DMb2	Marchf1	Was
H2-Ea	Marchf8	Washc1
H2-Eb1	Mfsd6	Wdfy4
H2-Eb2	Mr1	Ythdf1

Figure C.4: Antigen processing and presentation gene list

Appendix D

Gene ontology analysis of biological processes unique to each macrophage cluster

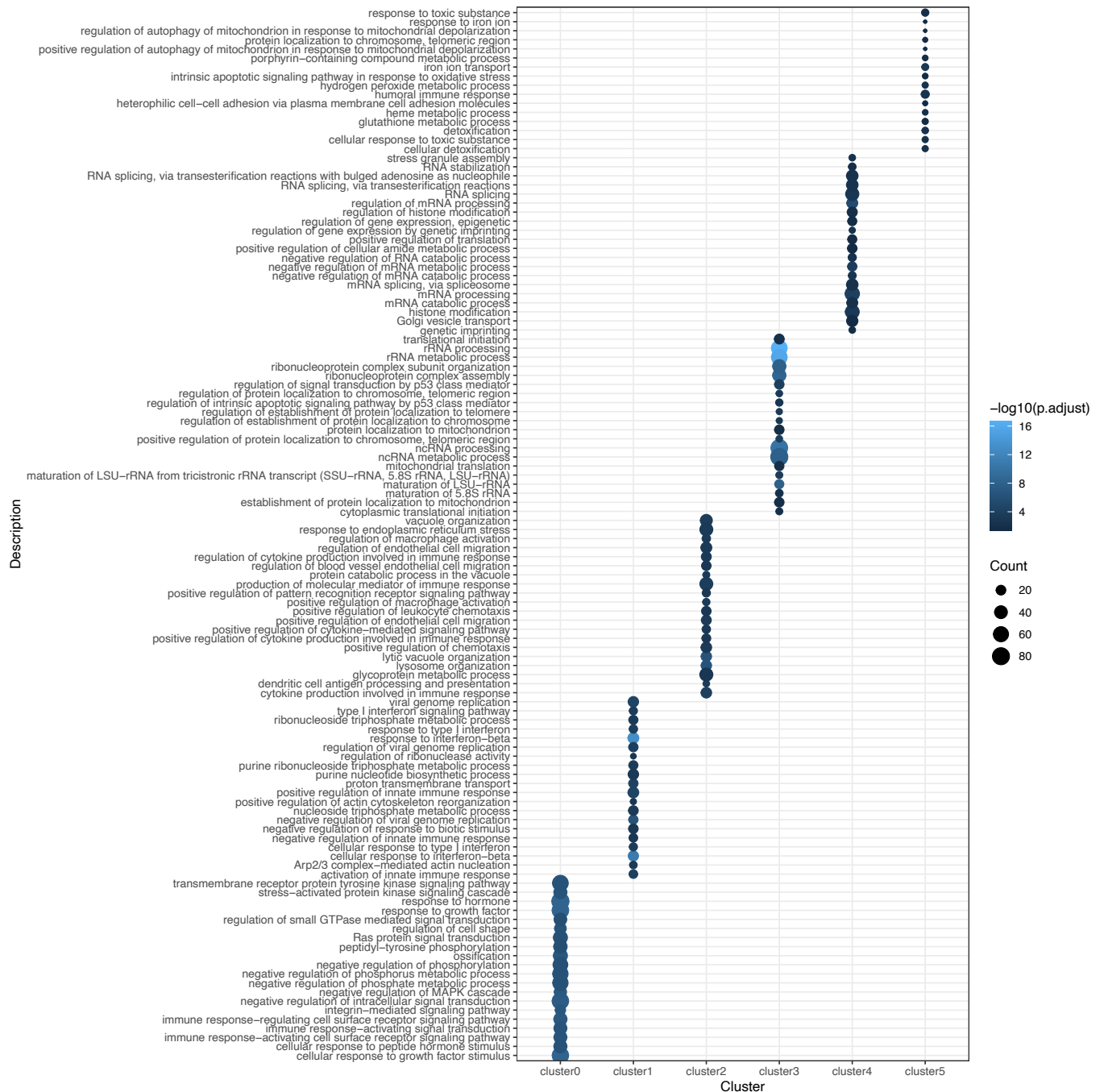


Figure D.1: Gene ontology analysis of biological processes unique to each macrophage cluster

Appendix E

DEG analysis of BM and lung macrophages

gene	p_val	avg_log2FC	pct.1	pct.2	p_val_adj	Sample
Ear1	8.83E-206	4.6847897	0.934	0.03	1.48E-201	Healthy_lung
Plet1	4.68E-173	3.54397429	0.759	0.017	7.82E-169	Healthy_lung
Krt79	2.42E-151	3.48756732	0.562	0	4.05E-147	Healthy_lung
mt.Co2	3.73E-140	3.13866464	1	0.101	6.24E-136	Healthy_lung
mt.Co1	3.34E-111	2.90632294	0.847	0.087	5.60E-107	Healthy_lung
mt.Nd4	4.29E-106	2.7616402	0.803	0.081	7.18E-102	Healthy_lung
Gm8797	5.03E-104	2.7064392	0.876	0.112	8.41E-100	Healthy_lung
mt.Atp6	1.38E-100	2.75814754	0.788	0.083	2.30E-96	Healthy_lung
Ear10	3.67E-95	2.74131696	0.467	0.015	6.14E-91	Healthy_lung
Lmna	5.01E-90	2.65105388	0.701	0.087	8.37E-86	Healthy_lung
mt.Cytb	3.26E-88	2.854399	0.737	0.088	5.45E-84	Healthy_lung
mt.Nd1	1.01E-83	2.85272332	0.672	0.076	1.69E-79	Healthy_lung
mt.Nd2	3.75E-73	2.90271169	0.628	0.076	6.28E-69	Healthy_lung
Hsd3b7	3.87E-73	2.74700192	0.38	0.015	6.47E-69	Healthy_lung
Anxa2	4.05E-69	3.22323192	0.927	0.449	6.77E-65	Healthy_lung
Cd9	5.69E-65	3.67559177	0.854	0.318	9.51E-61	Healthy_lung
Ear2	7.36E-62	3.10901671	0.942	0.54	1.23E-57	Healthy_lung
Cebpb	3.92E-54	2.8076601	0.912	0.609	6.56E-50	Healthy_lung
Cd44	1.10E-42	3.15240922	0.832	0.563	1.83E-38	Healthy_lung
Tagln2	3.39E-41	2.69847054	0.825	0.506	5.66E-37	Healthy_lung
Gm10116	1.29E-138	4.00837252	1	0.119	2.16E-134	Lung_tumor
H2.Eb1	1.11E-127	4.69692682	0.664	0.029	1.86E-123	Lung_tumor
RP23.27H19.7	1.99E-116	3.48479859	0.796	0.076	3.33E-112	Lung_tumor
H2.Aa	5.36E-115	4.45279725	0.65	0.036	8.96E-111	Lung_tumor
Cd63.ps	2.08E-111	2.84946804	0.511	0.012	3.48E-107	Lung_tumor
Ank2	3.70E-105	3.21056702	0.825	0.103	6.19E-101	Lung_tumor
Ptgfrn	5.03E-103	2.93846295	0.869	0.119	8.42E-99	Lung_tumor
Lyz1	2.12E-102	3.60114311	0.971	0.181	3.55E-98	Lung_tumor
H2.D1	5.41E-102	3.27659411	0.825	0.1	9.06E-98	Lung_tumor
H2.K1	1.66E-100	3.07395935	0.803	0.091	2.78E-96	Lung_tumor
H2.Ab1	2.39E-100	4.02395606	0.584	0.035	4.00E-96	Lung_tumor
Atp6v0c	1.96E-97	3.5843087	0.81	0.122	3.28E-93	Lung_tumor
Gm6977	3.46E-97	3.80815268	0.781	0.095	5.80E-93	Lung_tumor
mt.Co2	1.18E-86	2.69746252	0.847	0.119	1.98E-82	Lung_tumor
Ifi30	7.05E-77	2.89560846	0.62	0.071	1.18E-72	Lung_tumor
mt.Atp6	1.96E-74	2.83480148	0.701	0.094	3.28E-70	Lung_tumor
Gpnmb	3.12E-73	4.31365834	0.474	0.036	5.22E-69	Lung_tumor
mt.Nd4	5.58E-69	2.70542632	0.679	0.096	9.34E-65	Lung_tumor
Spp1	1.19E-44	3.27932209	0.46	0.076	2.00E-40	Lung_tumor
Fabp5	2.76E-36	3.12466333	0.73	0.369	4.62E-32	Lung_tumor
Igkc	6.02E-121	3.29323734	0.886	0.211	1.01E-116	AML_BM
H2-K1	7.40E-96	1.40367447	0.963	0.461	1.24E-91	AML_BM
H2-D1	7.32E-87	1.34398045	0.963	0.47	1.22E-82	AML_BM
Ighm	8.58E-84	2.7030743	0.866	0.316	1.44E-79	AML_BM
Wfdc17	4.86E-68	1.76730799	0.876	0.459	8.14E-64	AML_BM
Rpl11	6.59E-67	1.07559966	0.963	0.575	1.10E-62	AML_BM
Xist	2.12E-65	1.25934589	0.87	0.366	3.54E-61	AML_BM
Malat1	2.89E-65	1.17891624	0.969	0.506	4.83E-61	AML_BM
Cd300c2	3.77E-64	1.09781397	0.819	0.34	6.32E-60	AML_BM
Rps7	5.80E-64	1.06053651	0.958	0.561	9.71E-60	AML_BM
H2-T23	1.04E-63	1.08875266	0.753	0.295	1.74E-59	AML_BM
Grk3	1.29E-62	1.17429581	0.766	0.287	2.17E-58	AML_BM
Ms4a6c	6.90E-58	1.11264553	0.872	0.463	1.15E-53	AML_BM
Pou2f2	6.38E-56	1.5509863	0.756	0.351	1.07E-51	AML_BM
H2-Eb1	6.44E-56	1.16203741	0.907	0.396	1.08E-51	AML_BM
Fcgr4	1.01E-54	1.7661099	0.704	0.294	1.70E-50	AML_BM
H2-Aa	1.79E-53	1.08547459	0.917	0.421	3.00E-49	AML_BM
Cd72	2.79E-51	1.13426513	0.509	0.112	4.67E-47	AML_BM
Rgs1	5.41E-32	1.25148499	0.564	0.23	9.05E-28	AML_BM
Ccl5	2.77E-15	1.1630963	0.299	0.117	4.64E-11	AML_BM
S100a8	4.46E-127	2.38581389	1	0.776	7.46E-123	Healthy_BM
S100a9	1.90E-116	2.10150845	1	0.772	3.17E-112	Healthy_BM
Ngp	3.06E-111	2.12876306	0.997	0.721	5.12E-107	Healthy_BM
Camp	7.69E-106	2.00282899	1	0.72	1.29E-101	Healthy_BM
Lcn2	1.57E-101	1.97381281	0.997	0.701	2.62E-97	Healthy_BM
Ltf	4.62E-88	1.8279461	0.987	0.636	7.74E-84	Healthy_BM
Wfdc21	4.49E-62	1.495753	0.889	0.486	7.51E-58	Healthy_BM
Pglyrp1	9.48E-43	1.3076138	0.774	0.423	1.59E-38	Healthy_BM
Prtn3	4.02E-36	1.38493775	0.495	0.173	6.73E-32	Healthy_BM
Elane	4.81E-31	1.15949547	0.544	0.221	8.04E-27	Healthy_BM
Ifitm6	2.96E-30	0.99753219	0.708	0.385	4.95E-26	Healthy_BM
Mpo	5.78E-27	1.17909547	0.456	0.18	9.67E-23	Healthy_BM
Fcna	3.66E-24	1.29033059	0.4	0.138	6.13E-20	Healthy_BM
Hmgb2	6.78E-24	0.88610537	0.839	0.623	1.13E-19	Healthy_BM
Selenop	4.00E-23	0.91097592	0.839	0.562	6.68E-19	Healthy_BM
Fcnb	1.36E-22	0.86838178	0.4	0.15	2.28E-18	Healthy_BM
Slc40a1	1.74E-22	1.15149482	0.482	0.207	2.91E-18	Healthy_BM
Vcam1	1.00E-17	1.31622883	0.544	0.304	1.68E-13	Healthy_BM
Hmox1	1.60E-13	1.00120883	0.685	0.487	2.68E-09	Healthy_BM
Hbb-bs	0.00370903	1.4598755	0.302	0.234	1	Healthy_BM

Figure E.1: DEG analysis of BM and lung macrophages

Appendix F

Core macrophage signature gene
expression in lung and BM macrophage

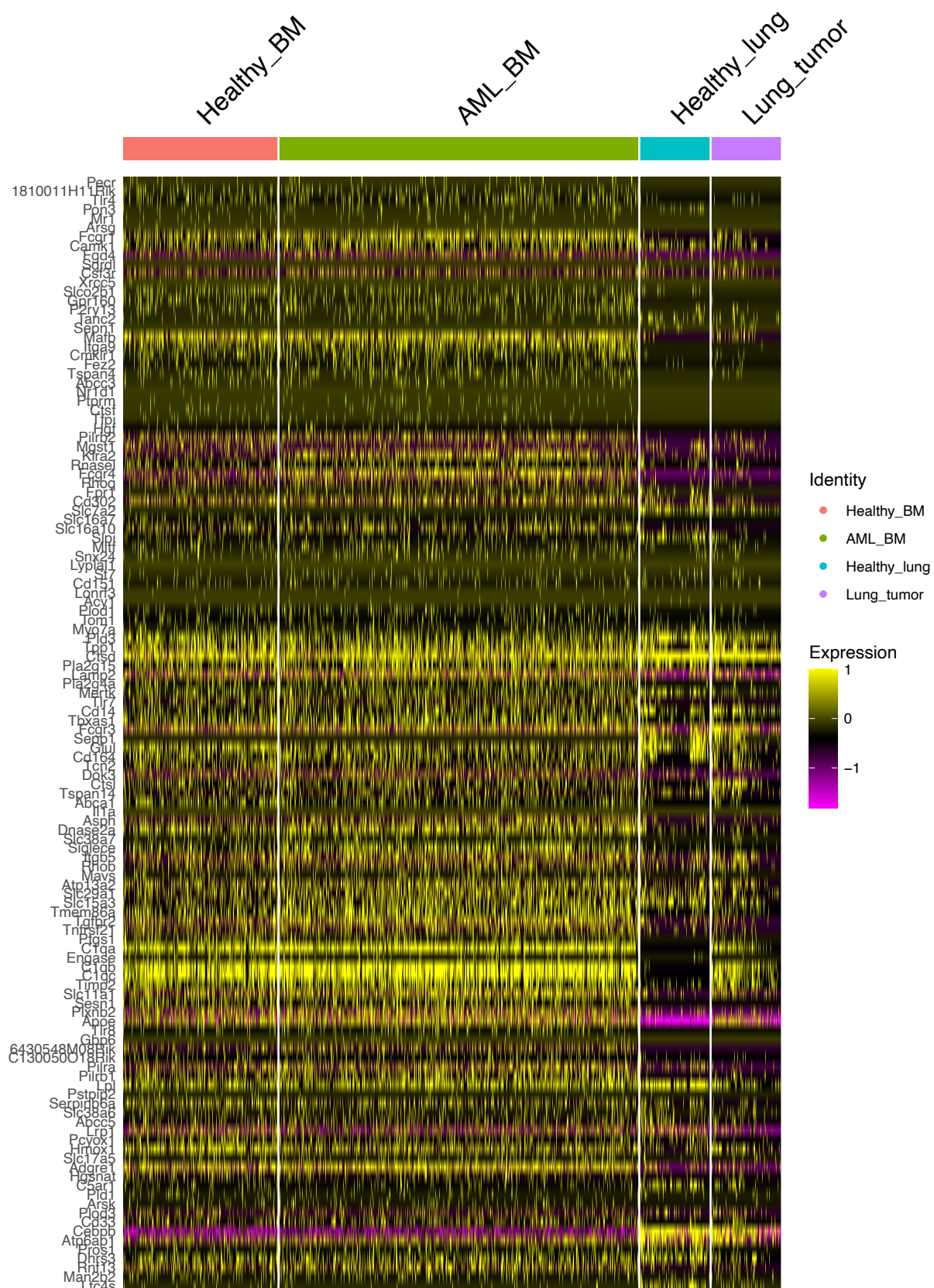


Figure F.1: Heatmap of core macrophage signature gene expression in lung and BM macrophage

Appendix G

Supplementary Video legends

Supplementary Video 1 - Macrophage motility in control BM.

Representative maximum projection of one-hour long time-lapses with images acquired every 3 minutes (8 frames per mouse). F4/80 was injected i.v. to highlight macrophages. BM is outlined with dotted lines. Cyan: Macrophages (n=2).

Supplementary Video 2 - Macrophage motility in early AML infiltration of the BM.

Representative maximum projection of one-hour long time-lapses with images acquired every 3 minutes (8 frames per mouse) from the BM of mice transplanted with 100'000 mTmG+ AML cells. AML infiltration of the BM was <1%. Anti-F4/80 APC was injected i.v. to highlight macrophages. BM is outlined with dotted lines. Cyan: Macrophages, Red: AML cells, (n=5)

Supplementary Video 3 - Macrophage motility in late AML infiltration of the BM.

Representative maximum projection of one-hour long time-lapses with images acquired every 3 minutes (8 frames per mouse) from the BM of mice transplanted with 100'000 mTmG+ AML cells. AML infiltration of the BM was 84%. Anti-F4/80 APC was injected i.v. to highlight macrophages. BM is outlined with dotted lines. Cyan: Macrophages, Red: AML cells, (n=5)

Supplementary Video 4 - Dil liposome uptake by macrophages and AML cells in the BM.

Representative maximum projection of two-hour long time-lapses with images acquired every minute (2 frames) from the BM of a mouse transplanted with 100'000 YFP+ AML cells. Dil liposomes were injected 12 hours before imaging. Anti-F4/80 APC was injected i.v. to highlight macrophages. BM is outlined with dotted lines. Cyan: Macrophages, Yellow: AML cells, Magenta: Dil Liposomes.

(n=1)

Supplementary Video 5 - Uptake of mTmG+ AML cells by CD169-YFP+ cells.

Representative maximum projection of two-hour long time-lapses with images acquired every two minutes (3 frames) from the BM of a CD169-YFP^{het} mouse transplanted with 100'000 mTmG+ AML cells. Green: CD169-YFP+ cells, Red: AML cells (n=1)

Supplementary Video 6 - Homing of AML cells in PBS liposomes treated mice.

Representative maximum projection of one-hour long time-lapses with images acquired every 2 minutes (4 frames per mouse) from the BM. PBS liposomes at a concentration of 0.1mL/10g were injected on day 0. 100'000 mTmG+ AML cells were transplanted on day 1, 18 hours before imaging. Anti-F4/80 APC was injected i.v. to highlight macrophages. BM is outlined with dotted lines. Cyan: Macrophages, Red: AML cells. (n=2)

Supplementary Video 7 - Homing of AML cells in clodronate liposomes treated mice.

Representative maximum projection of one-hour long time-lapses with images acquired every 2 minutes (4 frames per mouse) from the BM. Clodronate liposomes at a concentration of 0.1mL/10g were injected on day 0. 100'000 mTmG+ AML cells were transplanted on day 1, 18 hours before imaging. Anti-F4/80 APC was injected i.v. to highlight macrophages. BM is outlined with dotted lines. Cyan: Macrophages, Red: AML cells. (n=2)