Competition between healthy and malignant haematopoiesis: from cellular mechanisms to interventions that maintain stem cells in their bone marrow niche

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A thesis submitted for the degree of
Doctor of Philosophy

For my family and Dino that have been fundamental through this amazing journey
and for my aunt Annamaria that will be always part of me, without time and space.

Somewhere, something incredible is waiting to be known.
Carl Sagan

Declaration of Originality

The data presented in this thesis are the results of my own work, generated in Prof. Cristina Lo Celso's laboratory, at Imperial College London. 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 contributed to the generation of MLL-AF9 AML cell batches.

- Dr Delfim Duarte and Dr Edwin Hawkins generated the Notch-driven T-ALL cell batches and the RNAseq data.

- Dr Katia De Filippo (National Heart and Lung Institute, Imperial College London) performed together with me the intravital microscopy analyses of the spleen.

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Abstract

Haematopoietic stem cells (HSC) reside in the bone marrow (BM) microenvironment where, despite being very rare (<0.015% of BM haematopoietic cells), they maintain the turnover of all blood cells through a balance of quiescence, self-renewal and differentiation. Disruption of HSC function and of the BM microenvironment are key characteristics of Acute Myeloid Leukaemia (AML) progression. AML develops in both children and adults and major symptoms arise due to the loss of healthy haematopoietic cells. Although several hypotheses have been proposed to explain what factors contribute to this, the mechanisms behind HSC loss remain unknown. Here, using a murine model of MLL-AF9 driven AML, I show that AML competes against healthy haematopoietic cells for space, causing enhanced egress of haematopoietic cells from the BM into the circulation (Chapter 3). Using intravital microscopy (IVM), I show that during AML development healthy cells leave the BM in clusters and I hypothesise a role for extracellular matrix regulation and vascular leakiness in causing that. Interestingly, I demonstrate a clear association between the extent of BM infiltration and the number of HSCs found in the spleen and liver. Transplantation assays show that extramedullary HSCs are functional and able to reconstitute the BM of lethally irradiated mice (Chapter 4). Thus, extramedullary haematopoiesis (EMH) could represent a way to guarantee overall blood cell production in the short-term. I subsequently establish a novel therapeutic approach involving a metalloproteinase inhibitor, prinomastat (AG3340), which aims to delay AML progression and maintain healthy cells within their niche. Under prinomastat treatment, vascular leakiness is reduced, AML grows less in the BM microenvironment, with leukemic blasts showing reduced proliferation and migration speed as well as increased apoptosis. Most importantly, prinomastat treatment significantly enhanced residual haematopoiesis, reduced the extent of EMH in both spleen and liver, and improved survival following chemotherapy treatment (Chapter 5).

Acknowledgements

I would like to thank my supervisor Cristina Lo Celso for all the support, guidance and enthusiasm. She not only trusted me with this project but helped me to become confident as a scientist and person, giving me the freedom to follow my research interests and dreams.

I thank my project advisors Anastasios Karadimitris and Michalis Barkoulas for all their input and critical discussions. I also acknowledge all the past and current members from the Lo Celso lab involved in this project. In particular, I thank Delfim Duarte for sharing with me the beauty of leukaemia research through his support and friendship. I thank Myriam Haltalli for her scientific support and friendship, for all our 'We can do it' moments, for the crying time, the sweets and for always being there for me. She made me feel at home while being far away from my family. I thank Sara Gonzalez Anton for all the time that we had together and for always being by my side. I thank George Adams, Flora Birch, Floriane Tissot, Christiana Georgiou and Dina Pospori for their support and friendship. They were all there in my darkest moments (microscopy room included!). I thank Nicola Ruivo for her support and friendship, our afternoons at the London Glamour festival will be a nice memory of my time here. I thank Heather Ang for her friendship and her support together with Nahide Koksal, our nights in the FACS facility made me laugh in the most unworkable moments.

I am truly grateful to Edgar Ibarguen, Wendy Steel, Helen Goyal and all the Imperial College CBS facility staff, especially Julian Belulaj, for help with animal care, Jane Srivastava and Jessica Rowley for support with flow cytometry and Arwa Kocache (Imperial College Healthcare NHS Trust) for supplying cytarabine and doxorubicin.

I finally thank Bloodwise for the funding and for supporting the development of this project. This opportunity made me grow and realise, day after day, that together we can beat blood cancer.

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Abbreviations

Abbreviation	Meaning
3D	Three dimensional
4D	Four dimensional
7-AAD	7-Amino-actinomycin
ADAMS	A disintegrin and metalloprotease
ADAMTS	A disintegrin and metalloprotease with thrombospondin motif
AGM	Aorta-gonad-mesonephros
AML	Acute myeloid leukaemia
ANS	Autonomic nervous system
Ara-C	Cytarabine
B-ALL	B cell acute lymphoblastic leukaemia
BM	Bone marrow
BMA	Bone marrow adipocyte
BSA	Bovine serum albumin
c-Kit	Stem cell growth factor receptor
CAR	CXCL12 abundant reticular
CFU	Colony forming unit-spleen
CML	Chronic myeloid leukaemia
CMPs	Common myeloid progenitors
CNS	Central nervous system
CXCL12	C-X-C motif chemokine 12
CCL3	C-C-motif chemokine ligand 3
DAPI	4,6-diamidino-2-phenylindole
Doxo	Doxorubicin
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetracetic acid solution
EGFR	Epidermal growth factor receptor
EMH	Extramedullary haematopoiesis
EMT	Epithelial-to-mesenchymal transition

EVISC	Ex vivo imaging of stem cell
FAB	French-american-british
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FN	Fibronectin
GFP	Green fluorescent protein
GlyCAM	Glycosylation dependent cell adhesion molecule
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMPs	Granulocyte-monocyte progenitors
HBSS	Hank's balanced salt solution
Hif-1 alpha	Hypoxia-inducible factor alpha
HSCs	Haematopoietic stem cell
HSPCs	Haematopoietic stem and progenitor cells
IAHCs	Intra-aortic-hematopoietic cluster
I.V.	Intravenous
ICAM-1	Intercellular adhesion molecule-1
ICN	Intracellular notch 1
ITGB1	Integrin beta-1
IVCAM-1	Vascular cell adhesion molecule-1
IVM	Intravital microscopy
L-GMPs	Leukaemic GMPs
LepR	Leptin receptor
Lin	Lineage
LMPPs	Lymphoid-primed multipotent progenitors
LKS	Lin^-; c-Kit^+; $Sca\text{-}1^+$
LSC	Leukaemic stem cells
LSCM	Laser scanning confocal microscopy
LT-HSCs	Long term haematopoietic stem cells
M-CSF	Macrophage colony-stimulating factor
MEPs	Megakaryocyte-erythrocyte progenitors
miRNA	MicroRNA
MLL	Mixed leukaemia lineage
MMPs	Metalloproteinases

MMPIs	Metalloproteinase inhibitors
MP	Multiphoton microscopy
MPNs	Myeloproliferative neoplasm
MPPs	Multipotent progenitors
MRD	Minimal residual disease
MSC	Mesenchymal stem cell
MSCV	Murine stem cell virus
NDD	Non-descanned detector
NOD	Non-obese diabetic background
NSG	NOD-SCID-il2ry$^{-/-}$
OPN	Osteopontin
PBS	Phosphate buffered saline
PSGL	Platelet-selectin glycoprotein ligand
ROI	Region of interest
ROS	Reactive oxygen species
Sca-1	Stem cell antigen
SCC	Squamous cell carcinoma
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDF1	Stromal cell-derived factor 1
SEM	Standard error of the mean
SHG	Second harmonic generation
SLAM	Signalling lymphocyte activation molecule
ST-HSCs	Short-term haematopoietic
T-ALL	T-cell acute lymphoblastic leukaemia
Tcf21	Transcription factor-21
TGF	Transforming growth factor
THBS	Thrombospondins
TIMPs	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPO	Thrombopoietin

VEGF	Vascular endothelial growth factor
WHO	World health organization
YFP	Yellow fluorescent protein

List of Published Material

The papers listed here were published during my PhD and relate to the effect of AML on endosteal niches and HSCs explored further in Chapters 3 and 4.

- Duarte, D., Hawkins, E.D., Akinduro, O., Ang, H., De Filippo K., Kong IY., Haltalli M., Ruivo N., Straszkowski L., Vervoort SJ., McLean C., Weber TS., Khorshed R., **Pirillo C**., Wei A., Ramasamy SK., Kusumbe AP., Duffy K., Adams RH., Purton LE., Carlin LM., Lo Celso C. Inhibition of Endosteal Vascular Niche Remodeling Rescues Hematopoietic Stem Cell Loss in AML. *Cell Stem Cell*. 2018 Jan 4; 22 (1):64-77. e6. doi:10.1016/j.stem.2017.11.006.
- **Pirillo, C**., Haltalli, M., Gonzalez-Anton, S., Tini, V., Kong, I., Hawkins, E., Falini, B., Marra, A., Duarte, D., Lo Celso, C. The metalloproteinase inhibitor Prinomastat reduces AML growth, prevents stem cell loss and improve chemotherapy effectiveness. (under revision)

Chapter 1

Introduction

Acute Myeloid Leukaemia (AML) and T-cell acute lymphoblastic leukaemia (T-ALL) represent two classes of white blood cell cancer. Both leukaemias are characterised by aggressive cell growth, poor prognosis and lack of response to available chemotherapies. T-ALL includes a group of acute leukaemias arising from the lymphoid lineages, while AML derives from the myeloid counterparts. T-ALL is the most common type of childhood blood cancer whereas AML affects adults and, to a lesser extent, children. Patients affected by leukaemias often suffer from cytopenia as a result of disruption to the process of normal blood cell production (haematopoiesis) (Hoffrand *et al*., 2010). It has been hypothesized that leukaemia induces haematopoietic stem cell (HSC) loss by targeting the HSCs themselves and disrupting their regulatory pathways (Passegue *et al.*, 2003). On the other hand, it has been suggested that leukemic blasts alter the bone marrow (BM) microenvironment, supporting the generation of a pro-leukaemic niche and leading to the loss of HSCs and their haematopoietic function (Ishikawa *et al.*, 2007; Lane, Scadden & Gilliland, 2009; Duarte, Hawkins & Celso, 2018). Despite varying hypotheses in the field, the exact mechanism by which leukaemia overtakes hematopoietic cells remains unclear. Considering that, the overall aim of this thesis is to investigate the mechanisms that drive AML and T-ALL to overcome their healthy counterparts in order to develop better therapies for targeting leukaemia and preserving normal haematopoiesis.

1.1- Haematopoiesis

The term haematopoiesis encompasses the complex process by which all blood cells are produced during our lifespan. In adulthood, haematopoiesis occurs primarily in the BM, which contains the perfect microenvironment for HSCs. In 1961, following transplantation of fresh haematopoietic cells isolated from the BM into lethally irradiated mice, Till and McCulloch were the first to demonstrate the formation of macroscopic splenic colonies - the proportion of which was found to be in line with the number of injected haematopoietic cells (Till & McCulloch, 1961). These results allowed them to differentiate between progenitor cells, which have the ability to form multiple haematopoietic lineages, and a more specific subset, described as "self-renewing" units, able to give rise to daughter cells with identical characteristics as the parent cell. Later, these self-renewing units were identified to be HSCs (Becker, McCulloch & Till, 1963). HSCs reside at the apex of a hierarchical tree, followed by more differentiated progeny (Figure 1.1). Through asymmetric division, these cells are able to maintain their own pool and give rise to differentiating progenitors. This downstream progeny produces lineage-restricted blood cells involved in various physiological roles such as tissue repair, immune surveillance, and oxygen transport (Hoffrand *et al.*, 2010). HSCs with long-term reconstitution potential (LT-HSCs) reside at the very top of the conceptual haematopoietic tree (Figure 1.1) and give rise to HSCs with short-term reconstitution potential (ST-HSCs) and multipotent progenitors (MPPs)(Kondo *et al.*, 2003). MPPs no longer possess self-renewal ability but divide into myeloid and lymphoid lineages by differentiating into common myeloid progenitors (CMPs) (Akashi *et al.*, 2000) and common lymphoid progenitors (CLPs) (Kondo, Weissman & Akashi, 1997). CLPs give rise to a single lineage of progenitors that will generate lymphoid cells (including T cells, NK cells, B cells and innate lymphoid cells). Conversely, CMPs branch into granulocyte-monocyte progenitors (GMPs) that can produce macrophages and granulocytes and into megakaryocyte-erythrocyte progenitors (MEPs) that generate

megakaryocytes and erythrocytes. Recently, it has been suggested that lineage-commitment of HSCs might occur earlier than previously thought (Notta *et al.*, 2011; Perié *et al.*, 2015; Paul *et al.*, 2015; Karamitros *et al.*, 2017). These studies have shown that the majority of phenotypically defined MPPs and CMPs are in reality already committed to a single lineage and therefore not truly multipotent. Other studies have identified myeloerythroid and myelolymphoid progenitor populations called lymphoid-primed multipotent progenitors (LMPPs) (Adolfsson *et al.*, 2005; Lai & Kondo, 2006; Yoshida *et al.*, 2006; Arinobu *et al.*, 2007; Goardon *et al.*, 2011). These cells are able to generate B cells, T cells and myeloid cells after transplantation but have no megakaryocytic or erythroid progeny. As a consequence, the MPP compartment has now been divided into distinct subpopulations that are lineage-biased rather than restricted, tailoring together the output of myeloid and lymphoid lineages in response to haematopoietic demands (Cabezas-Wallscheid *et al.*, 2014; Pietras *et al.*, 2015).

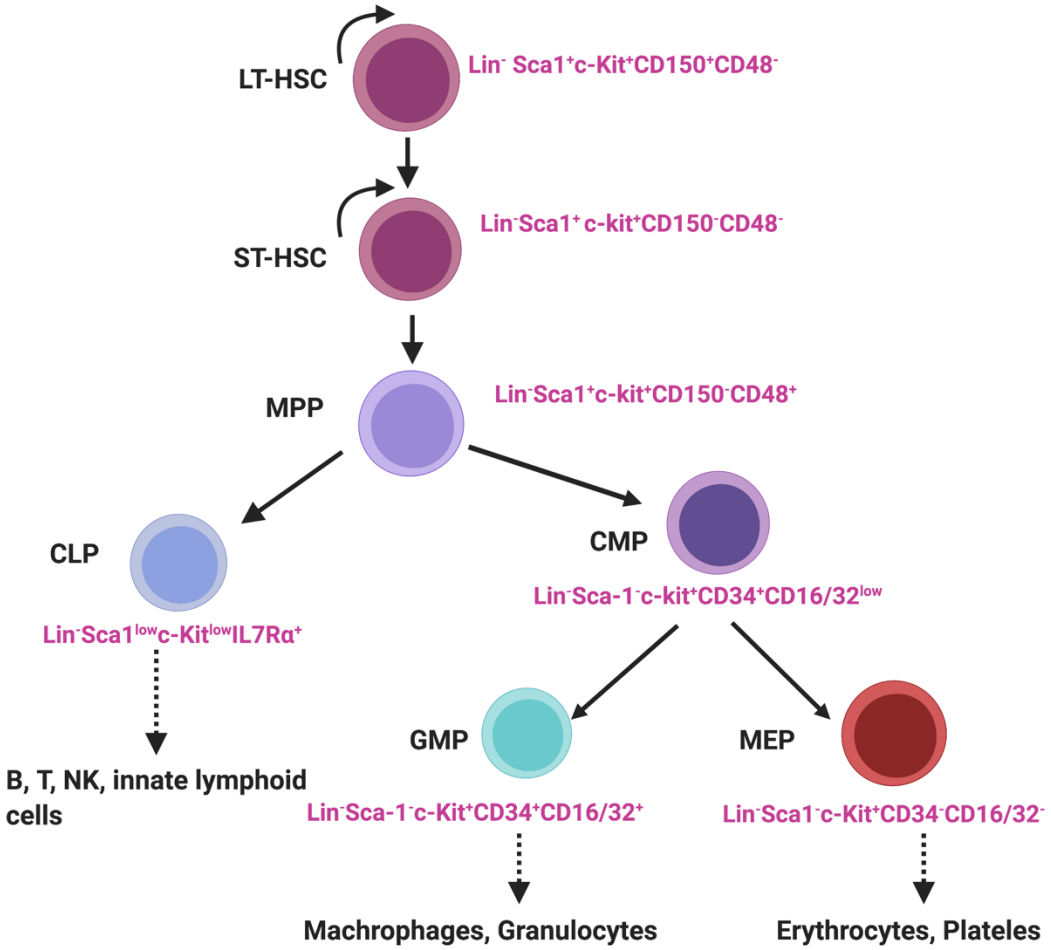

Figure 1.1-Haematopoietic tree

Using a combination of specific cell surface markers, it is possible to separate groups of haematopoietic cells characterised by different self-renewal and multipotency properties. HSC = haematopoietic stem cell (LT = Long-term; ST = short-term); MPP = multipotent progenitor; Lin = Lineage. Figure created using BioRender.com

1.1.1- Haematopoietic stem cells

Through their spleen colony assays in 1961, Till and McCulloch showed not only the clonality of colonies generated in the spleen but also the presence of haematopoietic cells from multiple lineages within them (Becker, McCulloch & Till, 1963; Till & McCulloch, 2012). At that time, this evidence led to the conclusion that the cells able to form colonies in the spleen, called CFU-S for spleen colony-forming units, were the same as those characterised by long-term reconstitution potential (Iscove 1990). However, following more accurate studies on the properties of these reconstituting cells, it appeared clear there was a certain level of

heterogeneity within the CFU-S. In fact, in 1969, it was already possible to physically isolate cells with overall greater self-renewal capacity (Worton, McCulloch & Till, 1969). Later on, other discrepancies emerged, for example the absence in the spleen colonies of lymphoid cells or their precursors as well as of cells capable of long-term multilineage reconstitution *in vivo*. Furthermore, reconstituting stem cells differed from CFU-S in their insensitivity to 5-fluorouracil (5-FU), which kills cycling cells, and in their limited capacity to regenerate themselves after transplantation into irradiated mice (Hodgson, Bradley & Radley, 1982). The establishment of competition assays led to the identification of HSC subsets based on their long-term versus short-term repopulation capacity (Harrison, 1980). In 1988, thanks to advances in cell purification methods, Spangrude *et al.* isolated and characterised mouse HSCs with the use of a variety of phenotypic markers (Spangrude, Heimfeld & Weissman, 1988). This pioneering work finally allowed detailed analysis of haematopoietic cell populations that are more or less enriched in HSCs.

In mice, haematopoietic cells are classified based on the absence of markers associated with blood cell lineage commitment (Lineage negative, Lin^-). This population is further enriched for progenitors based on the expression of stem cell antigen-1 (Sca-1) and stem cell factor receptor (c-Kit). Together, this BM fraction is known as $Lineage^-$ $c\text{-}Kit^+$ $Sca\text{-}1^+$ (LKS) and can be further divided according to the expression of CD48 and CD150, members of the signalling lymphocyte activation molecule (SLAM) family (Kiel *et al.*, 2005). Thus, this combination of markers is used to identify the LKS $CD150^+$ $CD48^{low/-}$ (LKS SLAM) cell population that is highly enriched for HSCs with an estimated frequency of 1 functional HSC in every 2.1 cells(Kiel *et al.*, 2005). Using the same marker combination, HSCs can be further divided into subpopulations (LT-HSCs, ST-HSCs, MPPs) (Figure 1.2). Other markers have been used to isolate more quiescent and functionally distinct subsets of HSCs. In 1984, Civin *et al*. identified

the importance of CD34 in relation to the most primitive haematopoietic cells. Cells expressing CD34 were able to differentiate into various haematopoietic lineages in clonal cultures (Civin *et al.*, 1984) and to generate haematopoietic progenitors in long-term cultures (Sutherland *et al.*, 1989). Following *in vivo* transplantation studies, and unlike in humans, the $CD34^{low/-}$ fraction of murine BM cells appeared to have the most long-term reconstituting ability compared to the $CD34^{high}$ (Osawa *et al.*, 1996). Another receptor, foetal liver kinase 2 (Flk2, also known as CD135 or FMS-like tyrosine kinase 3, Flt3), has heterogenous expression within the LKS compartment. Although, it was initially proposed that the most primitive HSCs were contained in the $Flk2^-$ fraction of the BM (Orlic *et al.*, 1993) , it seemed that only a low number of these cells were able to reconstitute lethally irradiated recipient mice. Later work showed that by using a combination of CD34 and Flk2 it is possible split the LKS compartment into LT-HSCs (LKS $CD34^-$ $Flk2^-$) and ST-HSCs (LKS $CD34^+$ $Flk2^-$). More recently, Flk2 has been used in combination with SLAM markers to discriminate the lymphoid-primed MPP4 subset from HSCs (and MPP1) and myeloid-biased MPP2 and MPP3 subsets (Cabezas-Wallscheid *et al.*, 2014; Pietras *et al.*, 2015). Overall, these markers provide a robust system for accurately tracking and investigating early haematopoietic stem and progenitor cells (HSPCs) experimentally in mice.

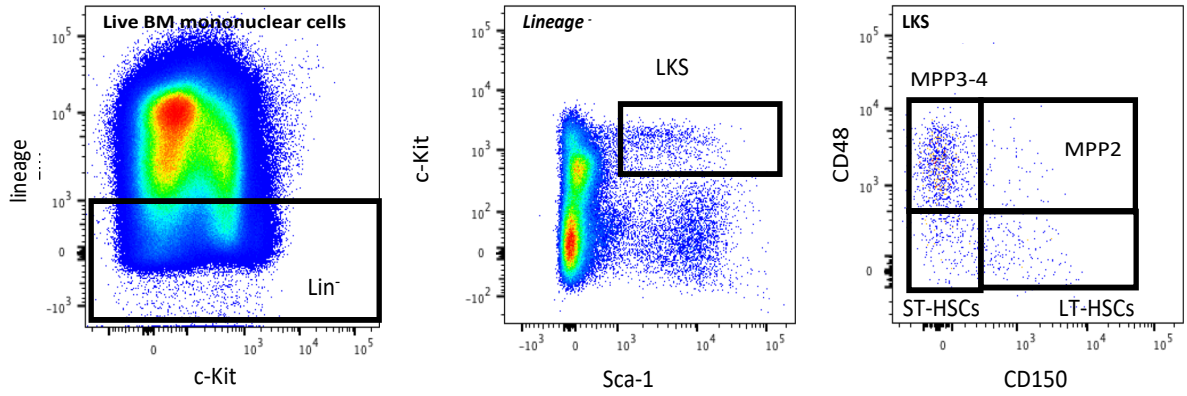

Figure 1.2-HSCs identification by flow cytometry

Gating strategy used to identify steady state hematopoietic cells by flow cytometry. Lineage- cells (left), Lin-c-Kit+Sca-1+ (LKS) progenitors (middle) and haematopoietic stem cells are $CD48^-$ $CD150^+$ (LT-HSCs), on the right

The past decades have seen the development of transgenic mouse lines in which the expression of reporter genes is driven by genes highly enriched in HSCs. Gene expression screenings of HSCs have identified *α-catulin* (Acar *et al.*, 2015), *Fgd5* (Gazit *et al.*, 2014), Pdzk1 (Upadhaya *et al.*, 2020) and *Hoxb5* (Chen *et al.*, 2016a)as useful genes to target for generating HSC fluorescent reporters. According to these studies for example, the frequency of HSCs with multi-lineage capacity was 1 in 6.7 *α-catulin*-GFP^{+} cells and 1 in 2.1 for the *Hoxb5*-tri-mCherry^{hi} cells. In early 2020, a new combination of genes including myelodysplastic syndrome 1 (*Mds1*) and *flt3*, both highly enriched in LT-HSCs, led to the development of the MDS1$^{GFP/+}$ Flt3^{Cre} reporter mouse line allowing the isolation and visualisation of endogenous HSCs by IVM (Christodoulou *et al.*, 2020). However, despite constant improvement by the field, in most cases, either other HSC subtypes express the same candidate-marker or not all HSCs express it, highlighting a lack of overlap between fluorescently-labelled HSCs and those that are phenotypically recognised by the use of flow cytometry.

1.1.2- The BM microenvironment

The concept of the stem cell "niche" was first postulated by Schoefield in 1978. He proposed that niches have a defined anatomical location and, most importantly, the removal of stem cells from these niches results in their differentiation (Schofield, 1978). In line with this hypothesis, after each division, HSCs remain in this anatomical and well-defined niche, while progenitor cells are spatially dislodged and differentiate. The first studies aiming to characterise stem cell niche properties and structure were conducted in invertebrate models including the fruit-fly *Drosophila Melanogaster* and *Caenorhabtis elegans*(Kimble & White, 1981; Xie & Spradling, 1998). These studies demonstrated a pivotal role for local signalling and niche sites in the maintenance of germ line cells, proving the existence of a spatial relationship between each niche component(Xie & Spradling, 1998; Lander *et al.*, 2012). Furthermore, *in vitro* studies

using haematopoietic cultures with stromal cells from fresh BM highlighted the evidence for a supportive stromal microenvironment *in vivo* (Dexter, Allen & Lajtha, 1977)and later work proposed a role for a BM endosteal niche(Calvi *et al.*, 2003) or a vascular niche(Kiel & Morrison, 2006)in supporting HSCs. Thus, the niche can be defined as a dynamic environment where a mixture of cells and signalling interact to influence stem cell behaviour. In this work, despite only assessing ECs in the context of AML, it is important to remember that they are only one component of the BM microenvironment and therefore I will provide a broad overview of multiple cell types that have been associated with the HSC niche. In fact, stromal cells, extracellular matrix (ECM), blood vessels and neural fibres are all conserved components and together contribute to HSC regulation and maintenance (Figure 1.3).

In adulthood HSCs reside in the BM, where their niche is located. Structurally, the BM microenvironment is a highly vascularised tissue where arteries, distal arterioles and sinusoids contribute to cell ingress and egress (Roth *et al.*, 2013; Beck *et al.*, 2014) and to the delivery of oxygen, nutrients and growth factors (Ramasamy *et al.*, 2016). In this context, the role of vascular endothelial cells in HSC maintenance has been extensively studied. Several publications have highlighted the existence of different endothelial subtypes with varying morphology, location, proliferation rate and expression of surface markers, reviewed in (Kusumbe, Ramasamy & Adams, 2014)It has been shown that a certain proportion of HSCs localise in close proximity to the sinusoidal endothelium (Kiel *et al.*, 2005; Kiel, Radice & Morrison, 2007) and that dividing HSCs are spatially separated from quiescent HSCs (Kunisaki *et al.*, 2013). In 2015, Acar *et al* showed that both dividing and non-dividing *α-catulin*-GFP^{+} HSCs reside in perisinusoidal niches within the BM and are distant from arterioles, transition zone vessels and bone surfaces (Acar *et al.*, 2015). Other studies have shown that the most quiescent HSCs are associated with small arterioles preferentially found in endosteal

BM(Kunisaki *et al.*, 2013) and that arterial vessels critically maintain HSCs in a low reactive oxygen species (ROS) state (Itkin *et al.*, 2016). Furthermore, vascular permeability has been evaluated and more permeable sinusoids were found to be associated with HSC activation(Itkin *et al.*, 2016). Several signals promoted by the endothelium are involved in HSC regulation. An example is represented by the Notch and E-selectin pathways, both important mediators of HSC maintenance in steady-state and their response to hematopoietic stress (Hooper *et al.*, 2009; Butler *et al.*, 2010; Kobayashi *et al.*, 2010; Winkler *et al.*, 2012). *In vivo* expression of Notch by endosteal vessels has been shown to be essential for HSC expansion (Ramasamy *et al.*, 2014) while *in vitro*, endothelial cell (ECs) secretion of angiogenic factors and Notch activation maintain HSCs self-renewal (Butler *et al.*, 2010). Together with Notch, the vascular niche maintains HSCs by producing factors such as vascular endothelial growth factor (VEGF), stem cell factor (SCF), and stromal derived factor 1 (SDF1, known also as C-X-C motif chemokine 12 or CXCL12) (Ding *et al.*, 2012).

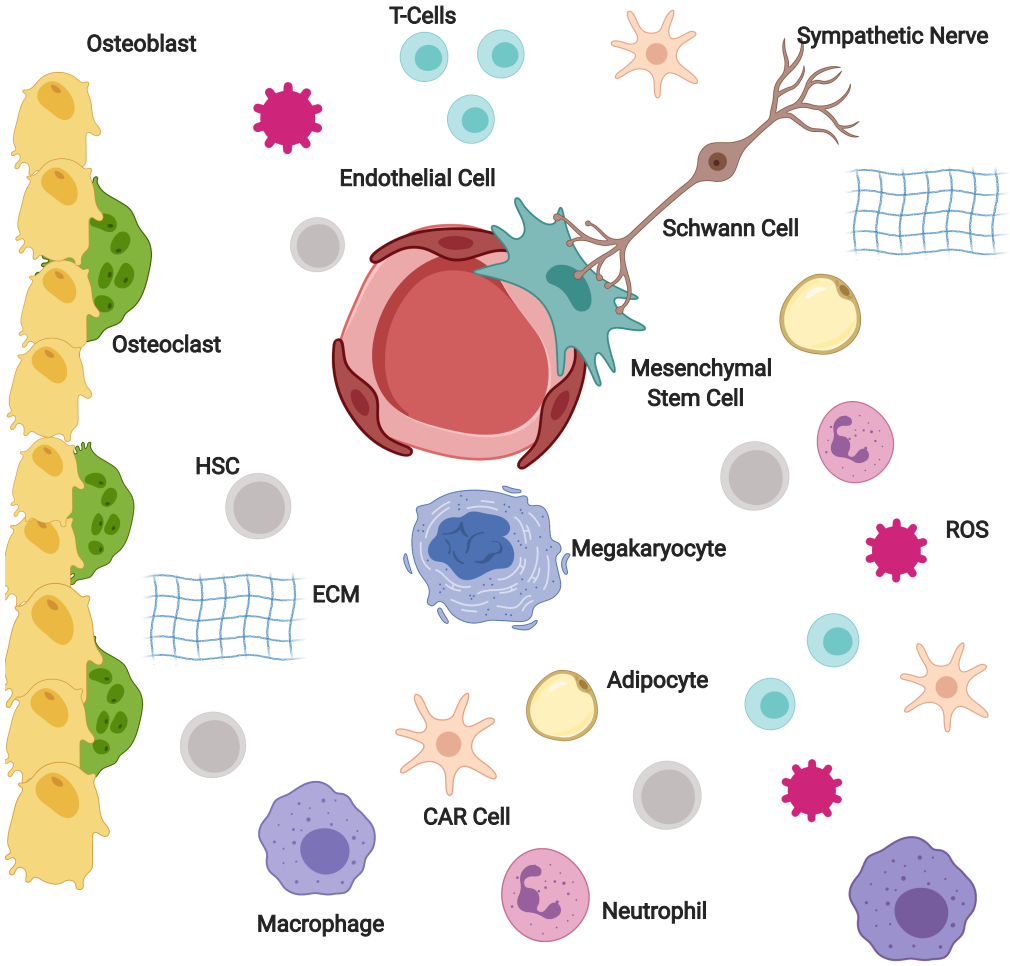

Figure 1.3-The BM niche in homeostatic states

In adulthood, HSCs reside in the BM niche where they rely on various cells for their regulation and maintenance. These cell types include stromal components such as osteoblasts, endothelial cells and mesenchymal stem cells; immune cells such as neutrophils, T cell and macrophages; and metabolic factors. Figure created using BioRender.com

Mesenchymal stem cells (MSCs) are multipotent stem cells localised in every tissue. Originally identified in the BM stroma, MSCs provide microenvironmental support for HSCs and can also differentiate into various mesodermal lineages (Pontikoglou *et al.*, 2011). In 1970, Friedenstein demonstrated that the BM contains a rare population of plastic-adherent stromal cells. *In vitro* assays showed that these cells, now called MSCs, were able to form clonal colonies which resembled fibroblastoid cells and so they were called Colony Forming Unit-Fibroblasts (CFU-F) (Friedenstein, Chailakhjan & Lalykina, 1970). As a result, Friedenstein was the first to identify the ability of MSCs to differentiate into mesodermal-derived tissue as well as uncover

their fundamental role in controlling the hematopoietic niche (Friedenstein *et al.*, 1974). Anatomically, in the BM, MSCs are tightly wrapped around arterioles and more loosely around sinusoidal vessels. It has been suggested that MSCs promote HSC regulation by producing factors like CXCL12, SCF, and Interleukin 7 (IL-7) (Mendes, Robin & Dzierzak, 2005; Méndez-Ferrer *et al.*, 2010; Ding *et al.*, 2012). MSCs are identified by a wide range of surfaces markers including Nerve/glial antigen 2 (NG2), Nestin, and Leptin Receptor (LepR). It has been shown that Nestin^{+} MSCs are spatially associated with HSCs and adrenergic fibres, highly expressing HSC maintenance genes (Katayama *et al.*, 2006; Méndez-Ferrer *et al.*, 2010). Furthermore, in lethally-irradiated mice, purified HSCs home near Nestin^{+} MSCs and *in vivo* Nestin^{+} cell depletion reduces BM homing of haematopoietic progenitors (Méndez-Ferrer *et al.*, 2010). Other work has shown that a subset of CD51^{+} Nestin^{+} cells expressing the platelet-derived growth factor receptor alpha (PDGFRα) is enriched in HSC maintenance genes and, most importantly, in human foetal BM these cells are identified as a small subset of CD146^{+} cells enriched for MSC and HSC niche activities (Pinho *et al.*, 2013). Within the Nestin^{+} MSC population, using the variable brightness of Nestin-GFP^{+} together with cell morphology, it was possible to identify two different subsets of MSCs. Anatomically, Nestin-GFP^{bright} cells, found alongside arterioles, were much rarer than Nestin-GFP^{dim} cells associated with sinusoids. Of note, the Nestin-GFP^{bright} cells were in close association with sympathetic nerves known to modulate HSC behaviour (Katayama *et al.*, 2006; Yamazaki *et al.*, 2011; Kunisaki *et al.*, 2013). Kunisaki and colleagues showed that quiescent HSCs are preferentially associated with the Nestin-GFP^{bright} cells (Kunisaki *et al.*, 2013). Other work has shown that LepR-Cre-labelled perivascular cells express high levels of SCF and, when deleted, HSCs were depleted from the BM (Ding *et al.*, 2012b). Recently, it has been shown that Nestin^{+} MSCs produce both SCF and CXCL12 and that deletion of both factors led to a reduction in HSC numbers(Asada *et al.*, 2017). However, depletion of CXCL12 in periarteriolar MSCs and specific deletion of SCF in

perisinusoidal LepR$^+$ stromal cells also caused a reduction in HSC number (Asada *et al.*, 2017). Therefore, it is possible to conclude that the stromal niche is highly heterogeneous and different factors contribute to HSC maintenance and regulation.

Osteoblasts are cells layering the endosteal bone surface, representing the first interface between bone and BM. They are derived from MSC differentiation and are responsible for the production of bone matrix components. Early studies suggested that more primitive progenitors reside closer to the bone. However, these studies were carried out using *ex vivo* labelled and transplanted haematopoietic stem and progenitor cells (HSPCs) and colony forming assays so the obtained results could not be used to prove the existence of an osteoblastic niche (Lord, Testa & Hendry, 1975; Gong, 1978; Lambertsen & Weiss, 1984). The first evidence that specific cells of the osteoblast lineage regulate HSC fate *in vivo* is attributable to two studies from 2003 by Calvi and Zhang respectively. In these studies, osteoblast manipulation was shown to have an effect on HSC self-renewal and expansion (Calvi *et al.*, 2003; Zhang *et al.*, 2003). Later on, it was shown that quiescent HSCs were in direct contact with osteoblasts (Arai *et al.*, 2004) and enriched in the endosteum (Wilson *et al.*, 2008). The idea of an endosteal niche was further confirmed in 2009 when, using intravital microscopy (IVM), Lo Celso found that transplanted HSCs tend to reside in close proximity to osteoblasts with their downstream progeny localising a distance away (Lo Celso, Wu & Lin, 2009). There has been a long debate regarding the mechanism behind HSC regulation by osteoblasts. Despite the demonstration that osteoblast deletion leads to a reduction in HSC numbers, it then appeared that the process is driven and anticipated by the depletion of more early lymphoid progenitors(Visnjic *et al.*, 2004; Zhu *et al.*, 2007). Furthermore, specific deletion of SCF (Ding *et al.*, 2012b) and CXCL12 (Ding & Morrison, 2013; Greenbaum *et al.*, 2013; Oguro, Ding & Morrison, 2013) in osteoblasts was not related with a decrease in HSC number suggesting that other niche

components play a more pivotal role in HSC regulation. Further studies have subsequently suggested that osteoblasts might have a more indirect role in hematopoietic regulation within the BM niche (Morrison & Scadden, 2014).

Together with ECs, MSCs and osteoblasts, there are a numbers of non-canonical stromal cell types in the BM microenvironment that directly or indirectly regulate HSCs through different mechanisms. In recent years, the autonomic nervous system (ANS) appeared to be an essential microenvironmental component in various tissues. Therefore, its involvement in the BM niche and HSC regulation is not surprising. In particular, the BM is highly innervated by the sympathetic branch of the ANS. Early studies proposed that bone formation was regulated by leptin signals activating the sympathetic nervous system (SNS) and acting through the β2-adrenergic receptors on osteoblasts (Takeda *et al.*, 2002). It has been suggested that sympathetic fibres and Schwann cells are not required for HSC maintenance but instead they have a role in the regeneration of stressed-impaired haematopoiesis (Lucas *et al.*, 2013) It has been highlighted that HSPC mobilization from the BM into the circulation follows a circadian rhythm in response to the oscillatory expression of CXCL12 by stromal cells regulated by nervous fibres (Méndez-Ferrer *et al.*, 2009). At the same time, Schwann cells promote HSC maintenance by modulating the activation of the transforming-growth factor (TGF)-β within the BM(Yamazaki *et al.*, 2011). It should be noted that both HSCs and stromal cells are not directly innervated by the sympathetic fibres but they might be indirectly regulated by diffusible factors produced by both Schwann cells and sympathetic fibres (Yamazaki *et al.*, 2011; Acar *et al.*, 2015).

BM adipocytes (BMA) have a pleiotropic role being mere lipid storage and filling of BM empty space (Cuminetti & Arranz, 2019). BMA arise from MSC differentiation, but they are

extremely heterogeneous and carry out their function based on their position within the marrow (Cuminetti & Arranz, 2019; Naveiras *et al.*, 2009). In the past years a role for BMA has been suggested in HSC regulation. Naveiras' work demonstrated that the number of HSCs was reduced in adipocyte-rich bones compared to adipocyte-free bones and that genetic reduction of adipogenesis accelerates HSC engraftment following irradiation (Naveiras *et al.*, 2009). These results suggested a negative role for BMA in the haematopoietic regulation. However, more recent studies have overturned this concept. Almost 30 years ago, Yamazaki and colleagues reported that BMA numbers increase after sublethal irradiation concomitantly with HSC proliferation (Yamazaki & Allen, 1991). More recently, it has been shown that, together with $LepR^+$ MSCs, BMA are the major source of SCF after irradiation and are essential for haematopoietic regeneration (Zhou *et al.*, 2017). Taking everything together, it can be argued that BMA have a role in emergency haematopoiesis but are still deleterious when present in unbalanced numbers (Cuminetti & Arranz, 2019).

Numerous evidences indicate that BM-resident immune cells contribute to HSC regulation and maintenance. Deleting macrophages from the BM niche has been associated with a reduction in CXCL12 expression and, therefore, promotion of HSC mobilization (Winkler *et al.*, 2010; Chow *et al.*, 2013). It has long been known that T cells produce cytokines that are able to affect haematopoiesis (Dent, 2008). The majority of T cells in the BM represent an activated/memory phenotype being mostly circulating T cells (Riether, Schürch & Ochsenbein, 2014). It has been shown that activated $CD4^+$ T cells positively regulate steady-state haematopoiesis maintenance (Monteiro *et al.*, 2005; Monteiro & Bonomo, 2005) while $CD4^+CD25^+$ regulatory T cells (Tregs) inhibit the differentiation of IL-3 responsive progenitors (Urbieta *et al.*, 2010). Recently, the presence of a unique BM niche-associated Treg population has been highlighted, characterised by the expression of CD150. This subpopulation seems to control HSC

quiescence and engraftment by preventing rejection during transplantation and protecting HSCs from oxidative stress (Hirata *et al.*, 2018).

Neutrophils are short-lived cells with a pivotal role in innate immunity maintenance. These leukocytes are produced in the BM and enter the circulation and tissues before getting cleared, few hours apart, in the spleen, liver and BM. It has been shown that the number of aged neutrophils, defined as $CD26^{lo}$ $CXCR4^{hi}$, oscillate in the blood following a circadian pattern and that neutrophil trafficking regulates the number of CXCL12 producing reticular cells (CAR cells) in the marrow (Casanova-Acebes *et al.*, 2013). Therefore, neutrophil depletion or a block of their recruitment to the BM, leads to increases in reticular stromal cells and reduces HSC mobilization (Casanova-Acebes *et al.*, 2013). This study was the first to demonstrate a lineage-restricted cell regulating niche size (Lucas, 2019). Recently, it has been shown that by producing the tumour necrosis factor alpha (TNF-α), neutrophils promote vascular recovery following irradiation (Bowers *et al.*, 2017). Therefore, together with HSC regulation, neutrophils seem to be able to protect other niche components.

The past decades have seen the niche concept evolving - from proposing that the niche controls HSC differentiation state and numbers (Schofield, 1978) to, more recently, HSCs being recognised as highly autonomous cells with the niche being a place of convenience for them (Baryawno, Severe & Scadden, 2017). As previously discussed, the selective modification or depletion of specific niche components could impact the HSC number and cycle status and eventually exacerbate in BM failure and other malignancies (Sánchez-Aguilera & Méndez-Ferrer, 2016). However, as the field embraces the idea that HSC differentiation state is cell-intrinsic rather than niche-dependent, it will be important to rethink the niche concept and its role, with the idea that the niche could provide more supportive than instructive input to the

HSCs. In this context, the use of microscopy to direct visualise the BM niche will allow a better understanding of the spatial organization and dynamics of HSCs within the BM.

1.1.3- Visualization of the BM niche

As previously described, several niche components and signalling pathways orchestrate HSC regulation within the BM niche. In the marrow, ECs, osteoblasts and their progenitors, CAR cells, LepR-expressing stromal cells and Nestin^{+} MSCs have been shown to affect HSC function and maintenance (Morrison & Scadden, 2014) The development of genetically modified mouse models has allowed us to study specific niche components in steady-state and disease. Through the use of the Cre-lox system, for example, it is possible to constitutively or temporarily alter expression of genes of interest in specific cells. There are a number of well-known strains that are used in HSC studies. For example, Nestin-expressing mesenchymal stem/progenitor cells can be visualised using a Nestin-GFP mouse, where the Nestin promoter drives expression of GFP (Mignone *et al.*, 2004). At the same time, CXCL12-expressing cells can be identified using the CXCL12-GFP (Sugiyama *et al.*, 2006) or the CXCL12-DsRed knock-in mice (Ding & Morrison, 2013). On the other hand, to image mature osteoblasts, the Col2.3-GFP strain is often used (Dacic *et al.*, 2001). These mice express GFP under the control of a 2.3 kb region of the rat or mouse procollagen type 1 alpha 1 (Col1a1) promoter (Calvi *et al.*, 2001), whereas their progenitors can be visualised using Osx1-CreGFP mice in which the transgene is under control of the osterix promoter (Rodda & McMahon, 2006). Furthermore, Flk1 (Xu *et al.*, 2010) and Ve-caderin reporter mice (Monvoisin *et al.*, 2006) allow the visualisation of ECs. Similar results can be obtained using appropriate antibodies for *in vivo* labelling such as anti-CD31, anti-VE-Cadherin, anti-Endomucin and anti-Laminin. These antibodies can be intravenously injected in live animals for visualisation of the endothelium when reporter lines are not available. Blood vessels are also often identified using intravascular

dyes such as dextran with various molecular weights. It should be noted that the use of antibodies is not restricted to ECs and can be applied to other niche components. Finally, the use of nonlinear optical microscopy such as second harmonic generation (SHG) signal can allow the visualisation of the bone itself by illuminating the collagen 1 fibres that compose it (Zipfel *et al.*, 2003). Therefore, using a combination of reporter mice, antibodies and SHG signals is it possible to simultaneously visualise different BM niche components both in sections or in live imaging.

1.1.4- Intravital microscopy of BM microenvironment

The use of a microscope, increasing the resolution and contrast, leads to the visualisation of images otherwise invisible to the naked eye. A combination of model organisms, fluorescent dyes and fluorochromes have been used alongside different microscopy approaches with the aim of gaining a deeper understanding of the HSC niche. Laser scanning confocal microscopy (LSCM) is suitable for imaging thick or highly scattering samples in order to obtain good quality three-dimensional (3D) information of living specimens. However, LSCM is limited to the tissue surface and exhibits a lack in signal strength and contrast for samples deeper than 100 μm. On the other hand, multiphoton microscopy (MP), and specifically two-photon microscopy, allows imaging of tissue at depths ranging from 150 μm, such as imaging of the calvarium (skull cap) BM (Lo Celso, Wu & Lin, 2009), and up to 500 μm deep in soft tissues like the lymph nodes (Halin *et al.*, 2005). Both LSCM and MP have been used in time lapse mode either alone or in combination to perform IVM, the use of which has contributed to significant discoveries in stem cell biology (Brown & Greco, 2014).

Early attempts of live imaging of the BM were made by McCuskey, McClugage and colleagues in 1971. In their studies, a metallic chamber was inserted into the tibias of rabbits and set with

a wide-field microscope allowing the same area to be revisited over time (McCuskey, McClugage & Younker, 1971). Obviously, the obtained images had low resolution and contrast and BM cells and structures could be identified based only on their morphology. Despite that, the authors were able to highlight important information regarding the haematopoietic system. They observed that bone regeneration is supported by a vascular network and, most importantly, they concluded that specifically capillaries and arterioles had an impact on maintaining haematopoiesis (McCuskey, McClugage & Younker, 1971).Recent studies, thanks to more refined techniques, have confirmed the central role of BM blood vessels in both bone formation (Kusumbe, Ramasamy & Adams, 2014) and haematopoiesis (Kobayashi *et al.*, 2010).

Several studies have been conducted to elucidate the dynamics of both phenotypically purified and endogenous HSCs in their BM niche (Lo Celso, Wu & Lin, 2009; Köhler *et al.*, 2009; Christodoulou *et al.*, 2020). In 2008, Xie and colleagues, developed a method called *ex vivo* imaging of stem cells (EVISC) in which they were imaging sagittally sectioned tibias and femurs of mice previously transplanted with HSCs (Xie *et al.*, 2008). Despite the fact that images were acquired on a damaged tissue, this study proposed that the endosteal area is able to promote HSC expansion after irradiation. On the other hand, IVM of the calvarium has been used in several studies shedding light on a number of biological processes, including platelet formation from megakaryocytes (Junt *et al.*, 2007). This technique was first implemented by the Von Adrian group using epifluorescence microscopy (Mazo *et al.*, 1998) and was later refined and expanded by the groups of David Scadden, Charles Lin, Cristina Lo Celso and Dominique Bonnet to obtain higher resolution images of the BM (Sipkins *et al.*, 2005; Lo Celso *et al.*, 2008). The mouse calvarium is a very thin bone that does not need shaving down as is required for imaging the long bones (Chen *et al.*, 2016b; Kim *et al.*, 2016). Thus, the BM can

be imaged non-invasively and without altering its architecture. Using this technique Lo Celso and colleagues performed IVM of single engrafting HSCs in the BM contained in the frontal bones of the mouse calvarium, showing that engrafting HSCs selectively localize in the proximity of osteoblastic cells and endosteal vasculature, while their progeny tend to move to more distal areas (Lo Celso, Wu & Lin, 2008). One of the main concerns raised by the use of calvarium BM for studying the haematopoietic niche is how its BM compares with the compartments in long bones. However, it has been shown that despite its nature as a flat bone, the calvarium is structural and functional representative as femur and tibia in the study haematopoiesis (Lassailly *et al.*, 2013).

Specifically, our research group has developed a protocol that enables long time lapse imaging as well as repeated cycles of imaging of the same areas by recovery of the mouse using a calvarial window and high-precision mouse holder (Scott, Akinduro & Lo Celso, 2014). The use of this approach has allowed us to uncover *in vivo* migratory patterns of infection-exposed HSCs (Rashidi *et al.*, 2014). Furthermore, Hawkins *et al.* in 2016 highlighted the migratory behaviour of T-ALL blasts throughout leukaemia progression (Hawkins *et al.*, 2016). Building on this and still using IVM, recent work uncovered the migratory behaviour of chemo resistant AML blasts (Duarte *et al.*, 2018) and a pattern whereby more infiltrated BM areas remodel endosteal vessels and lose osteoblasts (Duarte *et al.*, 2017) . Moreover, the use of new setups has adapted IVM to visualise human HSCs transplanted into NOD SCID gamma (NSG) mice (Passaro *et al.*, 2017). Using this approach, it has been demonstrated that HSCs, after being transiently migratory, arrest next to the hypothetical niche areas whereas haematopoietic progenitors remain fully motile after homing to the BM (Foster *et al.*, 2015). These data confirm previous observations and suggest that long-lasting interactions with the BM

microenvironment are essential for the reconstitution of haematopoiesis (Lapidot, Dar & Kollet, 2005).

1.2- Acute myeloid leukaemia

AML is the most common acute leukaemia among the adult population and accounts for about 80% of all cases. The incidence of AML increases with age with 12.2 cases per 100,000 people over the age of 65 (Kouchkovsky & Abdul-Hay, 2016). Despite advances in treatment, the prognosis is poor, with an average of 5-years survival due to relapses and high morbidity associated with treatment. The World Health Organization (WHO) categorises AML based on clinical, cytogenetic, molecular and morphological features. Therefore, each AML entity (e.g. AML-NPM1^{mut}, promyelocytic leukaemia with PML-RARA fusion genes, AML with t (3,3)) has different treatment strategies and prognosis. The disease is not only highly heterogeneous, but also undergoes clonal evolution. It has been proposed that leukaemia is propagated and maintained by leukaemia stem cells (LSC) characterised by an HSC-like phenotype (Lapidot *et al.*, 1994). These clonally expanded LSCs are then able to acquire additional mutations which play a key role in the development of AML. Within a leukaemia mass, blasts undergo a process - similar to Darwinian natural selection - gaining mutations that might confer or not these cells a fitness advantage. For example, studies involving the genomic analysis of leukaemia samples, have shown that the capacity of AML to relapse can be due to a dominant clone or to a sub-clone upon acquisition of new mutation during chemotherapy (Ding *et al.*, 2012). Consequently, for disease eradication, therapies aiming to target multiple clones should be implemented.

1.2.1 - Leukaemia Stem Cells

The first evidence for the involvement of LSCs in AML dates back to Lapidot's work in 1994 (Lapidot *et al.*, 1994). In fact, it was already known that the bulk of human AML had limited proliferation capacity suggesting the presence of a rare population of stem cells involved in its maintenance. Using human AML samples, xenotransplanted into immunodeficient mice (SCID), Lapidot *et al.* not only identified an AML-initiating cell able to establish human leukaemia in mice but most importantly, the presence of a hierarchy for LSCs in human AML (Lapidot *et al.*, 1994). Although in humans LSCs were believed to be part of the $CD34^+$ $CD38^-$ fraction, other studies have shown that LSCs might co-exist in all CD34/CD38 defined subpopulations, including Lin^+ (Bonnet & Dick, 1997; Hanekamp, Cloos & Schuurhuis, 2017). Despite the variation in LSC frequency due to the difference in leukaemia models studied and the assays used to calculate it, it is generally accepted that LSCs undergo both symmetric and asymmetric cell division, generate the bulk of the hierarchy of leukaemic cells, show high resistance to anti-proliferative chemotherapy drugs, have unique interactions with the BM stroma important for their homing and proliferation, and, finally, exist in different phenotypically-defined groups of cells (Stahl, Kim & Zeidan, 2016). Regarding the cell of origin, LSCs might arise following mutations to HSCs (Bonnet & Dick, 1997; Passegué, Wagner & Weissman, 2004) but they can also result, at least in experimental models, from mutations in more differentiated cells (e.g. CMPs transformed with the fusion proteins MLL-ENL or GMPs transformed with MLL-AF9) (Cozzio *et al.*, 2003; Krivtsov *et al.*, 2006). Considering the similarity between LSCs and HSCs it has been suggested that LSCs reside in a leukemic niche that supports their maintenance and chemotherapy evasion (Lane, Scadden & Gilliland, 2009).

Recently, the use of human-mouse xenograft assays allowed the assessment of the potential of a rare functional subset of AML to initiate patient-derived leukemic disease in recipient mice (leukemic-initiating cells, LICs). Thus, cells detected in LIC assays have been considered to be LSCs (Thomas & Majeti, 2017). Over the course of years, it has been proposed that LSCs preferentially resist chemotherapy constituting a cellular reservoir for leukemic relapse. This prediction was purely based on the dormant cell-cycle status of LSCs, whereas the leukemic population that selectively persist after therapy has never been tested (Jordan, Guzman & Noble, 2006; Boyd *et al.*, 2018). Despite multiple studies evaluating the functional state of relapsed AML cells, little attention has been dedicated to the initial stage of disease response to chemotherapy itself (Ding *et al.*, 2012a; Hackl *et al.*, 2014; Ho *et al.*, 2016; Shlush *et al.*, 2017). Recently, using AML patient samples and xenograft models, Broyd's group has characterised the transient leukemic-regenerating cells induced by chemotherapy and responsible for disease relapse. Their work, in line with previous observations (Farge *et al.*, 2017), revealed that LSCs are not selectively resistant to chemotherapy but proposed that a reservoir of primitive AML cells transit out of dormancy to replenish the supply of leukemic blasts. Considering that this occurs as a rapid cellular response, this work suggested that repetitive cycles of chemotherapy over brief time intervals could compromise these otherwise chemo resistant AML cells (Boyd *et al.*, 2018).

1.2.2- Acute myeloid leukaemia with the MLL-AF9 fusion gene

Structural lesions involving chromosome 11 band q23 are the most common cytogenetic abnormalities in hematopoietic malignancies and predictive of poor clinical outcomes. Rearrangement of the mixed lineage leukaemia (MLL) gene includes acquired deletions, duplications, inversions and reciprocal translocations at 11q23 (Muñoz *et al.*, 2003). The MLL-AF9 fusion results from the chromosomal translocation t(9;11) (p22;q23) resulting in the development of AML in adults (Krivtsov & Armstrong, 2007). In infants however, the MLL-AF9 fusion gene is associated with aggressive leukaemias of both lymphoid and myeloid lineages (Horton *et al.*, 2012). According to the French-American Association (FAB), the MLL-AF9 fusion is associated with myelo-monoblastic AML and classified as the M4 or M5 subtype (Swansbury *et al.*, 1998). It was shown that the cell of origin in which the MLL-AF9 oncogene is expressed, can influence gene expression, epigenetic and drug response profiles in a clinically relevant way (Krivtsov *et al.*, 2012). Studies involving the use of retroviral expression systems have shown that MLL-AF9 can drive the transformation of GMPs that as a result, acquire self-renewal capacity (Krivtsov *et al.*, 2006). Furthermore, it has been shown that the expression of MLL-AF9 oncogene in LKS (Krivtsov *et al.*, 2012) and CLP cell populations leads to the development of more aggressive leukaemias (Chen *et al.*, 2008). Recently, Stavropoulou *et al.,* using a new fully reversible doxycycline (DOX-) dependent inducible mouse model, demonstrated that the expression of MLL-AF9 in LT-HSCs resulted in a particularly aggressive AML characterized by short-latency, excessive organ infiltration, increased leukaemia-initiating potential and resistance to chemotherapy (Stavropoulou, Peters & Schwaller, 2017). Transcriptome analysis revealed the expression of many genes associated with invasion and/or epithelial to mesenchymal transition (EMT) known to be involved in solid cancers but not previously associated with acute leukaemia (Stavropoulou, Peters & Schwaller,

2017). Taking everything into consideration, the MLL-AF9 AML syngeneic mouse model provides a well-established system to study AML in all its features.

1.2.3- Current treatments for AML

Nowadays, treatments for AML involve an "induction" phase to reduce the number of circulating blasts to undetectable levels followed by a "consolidation" phase aiming to eradicate residual disease. The chemotherapy regimen consists of a combination of cytarabine with an anthracycline such as idarubicin, daunorubicin and doxorubicin (Doxo). The "induction" phase is based on the "3+7" regimen in which the cytarabine is infused intravenous (I.V) for 7 days and the anthracycline is infused on day 1, 2 and 3 (Longo *et al.*, 2015). Cytarabine (Ara-c) is an antimetabolite able to inhibit DNA synthesis while the anthracyclins block the topoisomerase II leading to DNA breaks. Different doses of cytarabine are used for the consolidation therapy, with patients younger than 60 years old receiving an intensive chemotherapy with high dose of Ara-c and allogenic haematopoietic-cell transplantation (Longo *et al.*, 2015). Patients older than 60 years old have a different response and benefit more from an intermediate-dose of cytarabine and non-myeloblative or reduced-intensity conditioning haematopoietic-cells transplantation (Longo *et al.*, 2015).

Other therapeutic strategies have proposed the use of monoclonal antibodies against CD33, CD45 and CD66 (Abutalib & Tallman, 2006). Particularly, Gemtuxumab ozogamicin, an anti-CD33 antibody has been shown to reduce the risk of relapse and increase overall patient survival when combined with induction therapy (Hills *et al.*, 2014).

1.3- T-cell Acute Lymphoblastic Leukaemia

T-cell acute lymphoblastic leukaemia (T-ALL) is a blood cancer arising from the lymphoid lineage. It accounts for about 10-15% of paediatric and 25% of adult acute lymphoblastic leukaemia cases, with males being more affected than females (Vlierberghe & Ferrando, 2012). Despite progress and improvement of treatments, the outcome for T-ALL patients remains poor, mostly due to treatment-resistance and disease relapse. The minimal residual disease (MRD) surviving treatment has been used as a prognostic factor to stratify patients and their outcome both in children and adult cohorts. Nowadays, approximately 25% of T-ALL paediatric patients develop relapse while only 40% of adult cases reach complete remission (Pui, Robison & Look, 2008).

1.3.1- Genetic alterations in T-ALL

T-ALL is an aggressive malignancy characterised by the accumulation of genomic lesions affecting the development of T cells. T cell transformation follows a multi-step process in which different genetic alterations affect homeostatic mechanisms that are critical for thymocyte cell growth, proliferation, survival and differentiation. These alterations include deletions, chromosomal translocations and gene fusions resulting in changes of the cell's gene expression profile (Vlierberghe & Ferrando, 2012). Chromosomal translocations and rearrangements are responsible for T-ALL development and are able to induce T-ALL in disease models. The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway has a key role in regulating proliferation, differentiation and function of the immune and hematopoietic system and therefore it is not surprising that its deregulation has been associated with hematologic malignancies (Schindler, Levy & Decker, 2007; O'Sullivan *et al.*, 2007; Onnebo *et al.*, 2012). The TEL-JAK2 fusion protein was originally observed following the t(9;12)(p24;p13) translocation in patients with T-ALL, B cell acute lymphoblastic

leukaemia (B-ALL) and chronic myeloid leukaemia (CML) (Lacronique *et al.*, 1997, 2000) and results in the constitutive active expression of TEL-JAK2 oncoprotein. The transformation properties of TEL-JAK2 fusion proteins have been demonstrated *in vitro* and *in vivo* where mice reconstituted with BM cells expressing the oncogene, developed a fatal mixed myelo- and T-cell- lymphoproliferative disorder (Schwaller *et al.*, 1998). These studies demonstrated the oncogenic potential of JAK/STAT pathway overactivation and it is now recognised that patients with TEL-JAK2 T-ALL have poor prognosis (Flex *et al.*, 2008).

As mentioned previously, together with chromosomal alterations, gene mutations play a pivotal role in T-ALL development. Constitutive activation of Notch1 was first demonstrated in rare leukaemia cases with the t(7;9)(q34;q34.3) translocation (Ellisen *et al.*, 1991; Palomero *et al.*, 2006)Notch receptors are involved in cell fate specification and, in the hematopoietic system, their activation is critical for T-cell development. Although the aberrant activation of Notch1 through the t(7;9) translocation seemed to be restricted to a small number of human tumours, new activating mutations leading to high levels of Notch1 signalling have been discovered in over 60% of Human T-ALL cases (Weng *et al.*, 2004). The strong oncogenic activity of the constitutive activation of Notch1 was demonstrated in studies where mice transplanted with transfected hematopoietic progenitors rapidly developed T-ALL (Aster, Pear & Blacklow, 2008; Ferrando, 2009). These studies show that it is therefore possible to design clinically relevant forms of T-ALL.

1.3.2- T-ALL treatment

As for AML, the treatment strategy in T-ALL includes an "induction" phase followed by "consolidation" treatment. The induction phase involves the administration of a glucocorticoid (dexamethasone or prednisone), vincristine, asparaginase, optional anthracycline, including an intrathecal chemotherapy to target central nervous system (CNS) disease (Pui, Robison & Look, 2008; Inaba & Pui, 2010). Glucocorticoids were the first drugs used in T-ALL treatment. Both dexamethasone and prednisone act through binding to glucocorticoid receptors that then translocate to the nucleus regulating gene expression and inducing cell apoptosis (Wyllie, 1980; Inaba & Pui, 2010). Clinically, *in vivo* and *in vitro* glucocorticoid resistance are adverse prognostic factors in ALL (Pui, Robison & Look, 2008). Vincristine belongs to a group of drugs known as vinca alkaloids. The drug targets tubulin, inhibiting microtubule formation and causing mitotic arrest resulting in cell death (Kothari, Hittelman & Chambers, 2016). Asparaginase acts by cleaving asparagine, an essential amino acid required by leukaemic cells that therefore become susceptible to asparagine depletion (Broome, 1968). Similar to AML, the aim of induction therapy in T-ALL is to eradicate the initial bulk of leukemic cells as much as possible. Normally, a 3 drug-regiment is used but 4 drugs are preferred for children and young adults with high risk ALL (Pui, Robison & Look, 2008). Unfortunately, contrary to AML, the implementation of an intensive induction therapy with higher doses of dexamethasone or asparaginase was shown to result in toxicity and death (Pui, Robison & Look, 2008). The consolidation phase can only be pursued by patients in remission after the induction therapy and has the aim to eradicate surviving T-ALL cells. Commonly, regimens include high doses of Methotrexate with mercaptopurine, high-dose asparaginase for an extended period, and reinduction treatment (Evans *et al.*, 1998; Silverman *et al.*, 2001; Longo, Hunger & Mullighan, 2015).

1.4- The Bone marrow niche in hematologic malignancies

Patients affected by blood malignancies suffer from disruption of steady-state haematopoiesis. Considering the supportive role of BM for HSCs, it has been suggested that leukaemic cells overtake healthy cells by re-shaping their niche (Boyd *et al.*, 2014). In support of this, it has been shown that transplanted human ALL cells damage the healthy BM endosteal and vascular microenvironments (Duan *et al.*, 2014). Furthermore, the BM remodelling driven by leukaemia not only promotes the loss of steady state haematopoiesis but also has a role in leukaemia survival and expansion (Méndez-Ferrer *et al.*, 2020). B-ALL blasts, for example, were shown to secrete the C-C Motif Chemokine Ligand 3 (CCL3) and create their own protective niche by recruiting $Nestin^+$ MSCs from sinusoidal niches and promoting their transition into α-SMA^+ cells (Duan *et al.*, 2014)BM remodelling happens also in CML where MSCs promote leukaemia growth rather than supporting HSCs by differentiating into aberrant osteoblasts (Schepers *et al.*, 2013). HSCs are normally supported by $NG2^+$ periarteriolar cells that arise from $Nestin^+$ MSC differentiation. In MLL-AF9-driven AML, it has been shown that the sympathetic neuropathy limits this process and promotes AML (Hanoun *et al.*, 2014)It is also known that AML damages the BM endothelium resulting in increased nitric oxide (NO) production and impaired vascular permeability (Passaro *et al.*, 2017). $Nestin^+$ MSCs are also reduced in myeloproliferative neoplasms together with non-myelinating Schwann cells and sympathetic nerve fibres (Arranz *et al.*, 2014).

The mechanisms and factors driving leukaemia BM remodelling are still poorly understood although, as a well-known hallmark of cancer, inflammation has been assumed to play a pivotal role in this process. Several cytokines are involved in the creation of a pro-inflammatory environment, including the tumour necrosis factor (TNF), CCL3 and the interleukin 1 beta (IL-1β). Together with this, deletion of the retinoic acid gamma receptor, retinoblastoma protein,

Notch signalling and the microRNA (miRNA) processing enzyme Dicer have been shown to promote aberrant myeloproliferation (Walkley *et al.*, 2007; Raaijmakers *et al.*, 2010; Wang *et al.*, 2014). More recently, particular attention has been given to exosomes, small vesicles secreted by both healthy and malignant cells that mediate cell communication (Skog *et al.*, 2008). There are several pieces of evidence that cancer-derived exosomes support cancer growth and disruption of healthy tissue (Skog *et al.*, 2008; Lee *et al.*, 2011; Melo *et al.*, 2014). Exosomes released by CML, for example, can stimulate BM stromal cells to produce IL-8 which supports leukaemia growth, while in AML the same effect is obtained by exosomes entering stromal cells and altering their function and facilitating leukaemia growth (Huan *et al.*, 2012; Corrado *et al.*, 2014). It should be noted that different leukaemia models interact and consequently re-shape the BM niche in different ways. For example, for AML (Lapidot *et al.*, 1994)and CML (Sirard *et al.*, 1996) is it known that LSCs have an altered dependency on the endosteal niche (Krause *et al.*, 2013). In this case, osteoblast expansion following parathyroid hormone (PTH) treatment promotes MLL-AF9 AML propagation while it blocks the BCR-ABL-CML-like disease (Krause *et al.*, 2013). Thus, leukaemia shifts the supportive role that BM explicates for HSCs into something that supports leukaemia growth, expansion and chemo-resistance. Understanding this hijacked role will be essential for development of new therapeutic interventions.

Recently, the use of IVM has provided a deep insight into the dynamics with which T-ALL and AML remodel the BM niche. Using this approach, work from our research group revealed that T-ALL and AML blasts interact and remodel the niche via different pathways (Hawkins *et al.*, 2016; Duarte *et al.*, 2018). IVM data acquired throughout disease progression showed that T-ALL blasts are characterised by short-lived cellular interactions and promiscuous distribution across the marrow (Hawkins *et al.*, 2016). Surprisingly, leukemic cells did not

show any preferential association with the niche sub-compartments at any stage of disease, revealing how T-ALL cells do not depend on specific niche microenvironments for propagation or the selection of chemo resistant clones. Furthermore, despite blasts not being dependent on the stroma, disease burden lead to rapid and selective remodelling of the endosteal space with the complete loss of mature osteoblasts, while perivascular Nestin-GFP^{+} cells were maintained (Hawkins *et al.*, 2016). This novel analysis of T-ALL interactions with the BM niche highlighted that future therapies aiming to eradicate invasion and blasts survival should target blast's features (e.g. migration) rather than specific niche components (Hawkins *et al.*, 2016).

On the other hand, the behaviour of AML blasts greatly differed from that of T-ALL (Duarte *et al.*, 2018). While blasts from T-ALL appeared to maintain their highly migratory behaviour at all stages of disease (including post-chemotherapy), in AML chemo resistant blasts were less migratory after treatment, revealing a more heterogeneous behaviour. Also, differently from T-ALL, AML-induced niche remodelling was local and dependent on the amount of infiltration in the related area. Specifically, our work showed a local loss of osteoblasts together with endosteal vasculature through the course of disease (Duarte *et al.*, 2017). Both components are fundamental to HSCs maintenance and therefore their disruption may contribute to HSCs loss at late disease stages (Kusumbe, Ramasamy & Adams, 2014; Itkin *et al.*, 2016b; Duarte *et al.*, 2017). Also, elimination of endosteal vessels may favour AML chemo-resistance as there is reduced drug delivery in vessel-poor microenvironments. Thus, therapies aiming to promote vessel and vascular normalization could improve AML outcome (Duarte *et al.*, 2017) .

1.4.1- Loss of steady-state haematopoiesis in AML

AML, more than T-ALL, leads to loss of healthy haematopoiesis and, as a consequence, patients develop life-threatening cytopenias involving all blood lineages. There are several hypotheses regarding the mechanisms behind HSC loss but the definitive picture remains unknown. Understanding this process is fundamental to improve patients' clinical outcome and develop new, more targeted, treatments.

It has been suggested that leukaemia growth and expansion within the marrow leads to the displacement of non-malignant haematopoietic cells from this tissue. Besides that, a poor correlation between BM infiltration and development of peripheral blood cytopenia has been noted (Rauch *et al.*, 2016). In this study it has been suggested that AML-driven thrombocytopenia and neutropenia depend on the cleavage of the thrombopoietin (TPO), a cytokine important for megakaryocyte and HSC maintenance, by AML blasts (Rauch *et al.*, 2016). However, neither erythropoiesis loss, trans-endothelial trafficking or analysis of specific haematopoietic BM populations were addressed in this study. Therefore, a model demonstrating competition between healthy and malignant cells for BM space cannot be discarded. Another study suggested that behind haematopoiesis loss, there is a disruption of the HSC differentiation to progenitors (Miraki-Moud *et al.*, 2013). Specifically, the use of a xenograft model for AML revealed that BM HSC number was not reduced whereas hematopoietic progenitors and other downstream hematopoietic cells were significantly reduced (Miraki-Moud *et al.*, 2013). Furthermore, the more quiescent HSCs surviving in the infiltrated marrow were able to successfully engraft after transplantation (Miraki-Moud *et al.*, 2013). Despite these results, this study did have some limitations which should be considered. Although several primary human samples were used, it is well known that there is limited interaction between human AML blasts, mouse microenvironment and HSCs (Miraki-Moud *et*

al., 2013). Another study proposed that leukaemic blasts induce HSC quiescence via the Early growth response 3 (*Egr3*) gene expression, a gene that inhibits proliferation (Cheng *et al.*, 2015).

Recently, our research group developed a strategy to evaluate and understand the contribution of the various proposed mechanisms to HSPC loss during leukaemia development (Akinduro *et al.*, 2018). Specifically, we determined the absolute cell number of AML and HSPCs entering S-phase per hour using a dual-pulse DNA labelling method and measured the proportion of the apoptotic cells together with the appearance of healthy and malignant cells in blood. This allowed us to understand the cellular dynamics underlying AML growth and the corresponding loss of healthy haematopoiesis. We showed that the size of the LKS SLAM population remains largely unchanged until the very late stages of disease, with some variability between the cell populations analysed. At this point, AML growth has terminated its exponential phase and undergoes a period of slower growth. This dynamic fits with the idea that AML cells proliferate but a lack of space in the marrow results in cells being displaced into the peripheral blood (PB). Similarly, HSPCs could be eliminated by leukaemia through competition for space in the BM (Akinduro *et al.*, 2018). While our data are suggestive of the ousting hypothesis, the direct confirmation that this happens was not possible at the time.

Taken together, these studies raised the hypothesis that leukaemic blasts might be able to push non-malignant cells out of the BM and into the circulation, where they could eventually colonize suitable organs and initiate extramedullary haematopoiesis (EMH).

1.4.2- Extramedullary Haematopoiesis in steady-state conditions

The term EMH refers to the hematopoietic process occurring in organs outside of the BM. As a site for haematopoiesis, in the foetus the BM microenvironment becomes functional from 4 - 5 months during human pregnancy (Kim, 2010). During its development, the foetus needs hematopoietic cells for oxygen supply and consequently non-marrow tissues, such as the yolk sac, foetal liver and spleen, become sites of haematopoiesis before the BM takes over this role (Figure 1.4). In humans, the first wave of haematopoiesis originates in the yolk sac (Silver & Palis, 1997). Within this structure, haematopoietic cells arise following the differentiation of a group of mesenchymal cells. These haematopoietic cells are surrounded by ECs which will later form the yolk sac vascular plexus (Silver & Palis, 1997; Ivanovs *et al.*, 2017).

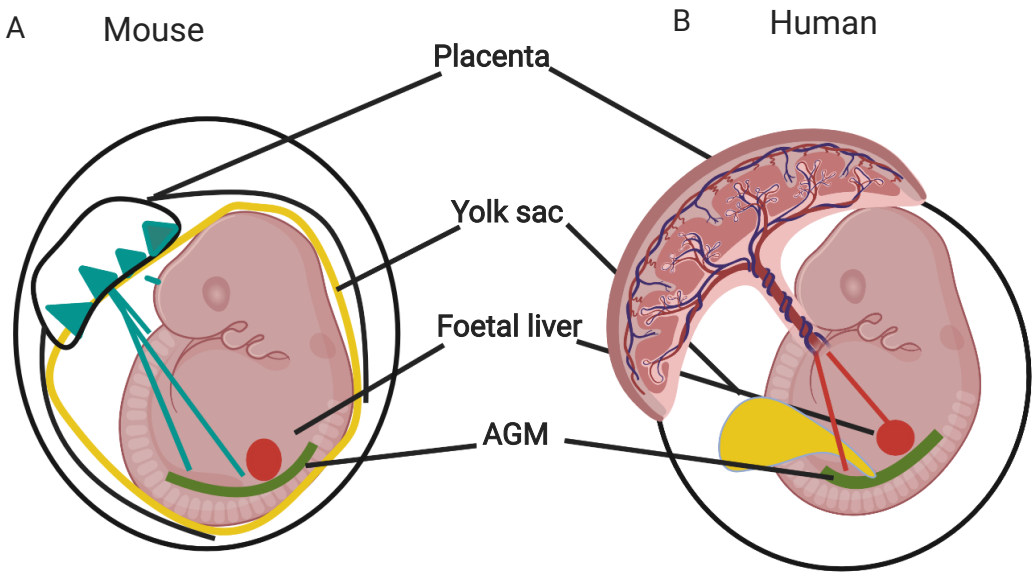

Figure 1.4-Haematopoietic organs in mouse and human embryos

Figure shows a mouse **(A)** and human **(B)** embryo highlighting the haematopoietic organs at 11 days and 5 weeks of gestation, respectively. Figure created using BioRender.com

At this stage, large erythrocytes and occasionally primitive macrophages and megakaryocytes, represent the major haematopoietic output from the yolk sac (Bloom & Bartelmez, 1940; Fukuda, 1973; Luckett, 1978). At mid-gestation stage, cells from the yolk sac colonise the umbilical cord, the aorta-gonad-mesonephros (AGM) region and subsequently the embryonic liver. The formation of an intra-aortic hematopoietic cluster (IAHC) in the AGM region determines the outset of the intra-embryonic, permanent adult haematopoietic wave in the vertebrate embryo (Anon, 1913). Thus, following the yolk sac and the AGM, the foetal liver represents the major embryonic organ for haematopoiesis before the BM becomes its preferential site during human lifespan.

In mice, HSCs appear in foetal liver around embryonic day 11.5, where a wave of expansion takes them to a maximum of ≈ 1000 HSCs by E15.5-16.5 (Ema & Nakauchi, 2000). Following that, the number of HSCs in foetal liver start to decline (Ema & Nakauchi, 2000). It has been proposed that during foetal development the foetal liver environment changes to completely fulfil lineage differentiation and HSC expansion. Regarding the spleen, haematopoiesis occurs in it at around embryonic day 14.5 and at this stage, erythropoiesis and myelopoiesis appear to be predominant and localised in the red pulp. This process seems to persist until one week after birth (Ohno *et al.*, 1993; Desanti, Cumano & Golub, 2008)Other studies have shown that the number of colony-forming hematopoietic progenitors in the spleen reach their peak of expansion at two weeks of age in mice (Wolber *et al.*, 2002), and that HSCs are recruited to the spleen during the neonatal period (Morita *et al.*, 2011). Thus, in these non-marrow organs, different cell populations are involved in haematopoiesis maintenance and regulation, representing “organ-specific” niches. The requirement of a specialised microenvironment is a restricting factor for EMH, which indeed can only happen under specific circumstances (Kim, 2010).

1.4.3- Extramedullary haematopoiesis under stress and malignancies

In mammals and rodents, EMH following birth occurs under exceptional circumstances. The spleen, liver and lymph nodes are the major non-marrow organs involved in this process, even if cases of EMH have also been reported in the thymus, skin, lungs, and brain (Kim, 2010; Tayari, Ahrar & Jafarpishe, 2013). Based on the situation in which it happens, EMH is classified as active or passive. Active EMH occurs in physiological conditions, for example during the generation of immune cells by liver and spleen through the course of infections (Plum, 1949). Infections driven by *Staphylococcus aureus*, Toll-like receptor (TLR) ligands such as lipopolysaccharides and *Plasmodium berghei* provide insight into the kinetics of hematopoietic shift from the BM to spleen. For example, it has been shown that *Plasmodium berghei* infection increases numbers of granulocyte monocyte colony forming unit (CFU-GM) in the BM in the first week of infection followed by a wave of CFU-GM in both peripheral blood and spleen at the end of the second week (Mungyer *et al.*, 1983). Therefore, it has been proposed that the final aim for EMH during infection is to generate a sufficient number of mature myeloid cells to help clear pathogens.

On the other hand, the incapacity of the BM to sustain haematopoiesis leads to passive EMH. Myeloproliferative neoplasms (MPNs) are clonal HSC disorders defined by expansion of one or more myeloid cell lines (Nangalia & Green, 2017). In myelofibrosis, for example, the BM tissue is replaced by a fibrous one and as a consequence, becomes incapable of producing blood cells. BM fibrosis can be observed in several other haematological disorders, including hairy cell leukaemia, lymphoma and multiple myeloma. Regarding leukaemia, EMH has been diagnosed in CML while it remains a rare event in AML (Kopecky *et al.*, 1986). In all these cases, the damage on the BM induces an inadequate medullary space and alters its architecture, signalling and microenvironment. As a consequence, steady-state haematopoiesis within the

marrow is halted and HSPCs are mobilised to other sites. The spleen and liver are, in haematological malignancies, common sites of passive EMH. However, organs like lymph nodes, serosal surfaces, epidural spaces and the kidneys have been documented to be involved in this process.

As already explained, there is a need for a specialised microenvironment in order for EMH to occur. It has been shown that in the human spleen the expression CXCL12 was significantly higher in cases of passive EMH, likely leading to HSCs homing to and residing in this site (Miwa *et al.*, 2013). In the same way that cellular components contribute to HSC maintenance in the BM, in extramedullary sites the microenvironment rearranges itself allowing for haematopoiesis expansion. The splenic red pulp is a prominent site of EMH in mice and humans (Lowell *et al.*, 1996; Johns & Christopher, 2012). It has been shown that CXCL12 is expressed by sinusoidal ECs in the red pulp of the human spleen raising the possibility of a perisinusoidal niche(Kiel *et al.*, 2005; Miwa *et al.*, 2013). On the other hand, the EMH niche in mouse spleen is constituted by ECs and transcription factor-21 (Tcf21)-expressing stromal cells within red pulp sinusoids (Inra *et al.*, 2015).

Despite their involvement in EMH, little is known about the niche-related environment composition for the liver, thymus and lymph nodes, opening the opportunity for future investigation. Most importantly, the functional potential of extramedullary HSCs found in these organs remains unclear, raising the fascinating hypothesis that new alternative niches may develop or be therapeutically triggered from which haematopoiesis could recover despite BM damage.

1.5- The extracellular matrix and tissue homeostasis

The extracellular matrix (ECM) is the tissue and organ component that contributes most to the physical characteristic of the cells' microenvironment and regulates processes such as morphogenesis, differentiation and homeostasis (Frantz, Stewart & Weaver, 2010). Despite the fact that the ECM was once considered an inert supportive scaffold, its pivotal role in cell biology is now extensively recognised. The ECM is a dynamic and versatile compartment that supports organ development, function and repair by modulating the production, degradation and remodelling of its components (Page-McCaw, Ewald & Werb, 2007; Lu *et al.*, 2011). The relative amounts and organization of the different ECM elements make the ECM strictly tissue-specific reflecting the cell's functions related to each tissue (Gattazzo, Urciuolo & Bonaldo, 2014). It is known that physical properties such as rigidity, porosity and insolubility influence various anchorage-related biological functions like cell division, tissue polarity and cell migration (Lu, Weaver & Werb, 2012).

From a biochemical point of view, the ECM displays both direct and indirect signalling properties since it can operate by directly binding cells surface receptors or by non-canonical growth factor presentation (Hynes, 2009). Thus, biochemical properties influence cell behaviour. For example, ECM stiffness is an essential property through which cells sense external forces and respond to the microenvironment in an appropriate manner (Kolsch *et al.*, 2007; DuFort, Paszek & Weaver, 2011; Gattazzo, Urciuolo & Bonaldo, 2014). It should be noted that cell-ECM interactions are reciprocal, whereby cells remodel the matrix and become consequently affected in their behaviour by ECM alterations (Hynes, 2009).

Expanding on previous discussion, stem cells reside in their niches where the ECM provides controlled biochemical, physical, structural and mechanical properties for each specific

microenvironment. Thus, signals deriving from cell-ECM interactions are integrated in a functional manner to permit stem cell maintenance and homeostasis (Discher, Mooney & Zandstra, 2009; Watt & Fujiwara, 2011). Kurtz and colleagues, through their *in vivo* experiments, showed that altered or aged niches lose the ability to maintain stem cells' properties, highlighting the relevance of the ECM in stem cell behaviour (Kurtz & Oh, 2012). Further studies demonstrated that natural ECM scaffolds, derived from decellularized tissues, guide stem cell differentiation into cell types residing in the tissue from which the ECM was derived (Nakayama *et al.*, 2010). As ECM composition is strictly tissue-specific, our attention will be focused on the properties and function of ECM within the BM microenvironment.

1.5.1- The Role of Extracellular matrix in the haematopoietic niche.

In the BM, the ECM is an intricate network of collagens, proteoglycans, glycosaminoglycans (called structural proteins) and glycoproteins such as fibronectin (FN), osteopontin (OPN), laminins and thrombospondins (THBS) (the so-called non-structural proteins) (Klein, 1995; Hines, Nielsen & Cooper-White, 2008). The most abundant ECM proteins in the BM are fibronectin, collagens I-XI, laminin, tenascin, THBS and elastin (Klein, 1995). Other important molecules are proteoglycans such as hyaluronic acid, chondroitin sulfate, dermatan sulfate, heparin sulfate, keratan sulfate and heparin (Rodgers, Antonio & Jacenko, 2008). Together with that, other ECM-associated molecules are the soluble or membrane bound glycoproteins of the sialomucin family such as CD34, the glycosylation dependent cell adhesion molecule 1 (GlyCAM1), mucosal vascular addressin CAM1 (MadCAM1) platelet-selectin glycoprotein ligand (PSGL1/CD162) CD45RA and CD164 (Klamer & Voermans, 2014). Within the marrow, the principal mediators of cell-cell and cell-matrix interaction are integrins and selectins (Prosper & Verfaillie, 2001), while membrane bound receptors of the immunoglobin superfamily, including the intercellular adhesion molecule-1 (ICAM1; CD54), vascular cell

adhesion molecule-1 (VCAM1; CD105) and CD166 have a role in shaping the BM niche (Bevilacqua, 1993; Klamer & Voermans, 2014).

Proteoglycans have been shown to be essential for the maintenance of long-term culture-initiating cells *in vitro* through their interactions with IL-3 and other growth factors (Gupta *et al.*, 1998). Within the marrow, an abundant proteoglycan is perlecan that, through its interaction with other molecules, is able to stabilise Collagen-X. It has been shown that mutations in perlecan or collagen-X indirectly attenuate haematopoiesis through a disruption of the endochondral ossification process, resulting in marrow aplasia and lymphopenia (Rodgers, Antonio & Jacenko, 2008).

The importance of the ECM in HSC fate decisions has been conclusively shown (Choi & Harley, 2012). Holst and colleagues demonstrated that murine BM cultured on tropo-elastin-coated plates is enriched for HSCs (Holst *et al.*, 2010). However, changes in the density of the tropo-elastin-coating negatively related with that event (Holst, et al. 2010). These results revealed that the biophysical properties of ECM regulate HSC fate. The direct impact of matrix density on HSPCs was analysed by culturing HSCs on collagen-coated polyacrylamide substrate characterised by a variable stiffness. As a result, the viability, spreading and adhesion of HSCs was increased in response to the increased collagen concentrations (Choi & Harley, 2012). It has been shown that specific inhibition of collagen secretion in the BM reduces haematopoiesis together with progenitor numbers (Zuckerman *et al.*, 1985). At the same time, direct adhesion of HSPCs to fibronectin seemed to attenuate their proliferation and differentiation, which is intergrin-mediated (Hurley, McCarthy & Verfaillie, 1995).

Integrin signalling is essential for a wide range of HSPCs functions, including HSPC differentiation and BM reconstitution. It has been shown that a block of the β2-integrins *in vitro* inhibits HSPC adhesion to BM stroma, without inducing mobilization of stem cells into the blood *in vivo* (Teixidó *et al.*, 1992). Several studies have shown that treatment of human HSPCs with antibodies against the integrin beta-1 (ITGB1) prevents HSPC engraftment in mice and sheep demonstrating that ITGB1 is essential for engraftment in the BM niche (Möhle *et al.*, 1995). Thus, adhesion of HSPCs to fibronectin, integrins and other ECM components within the marrow contribute to the BM niche and HSPC maintenance.

1.5.2- Regulation of the Extracellular Matrix in homeostasis and disease

Due to its role in tissue homeostasis, ECM dynamics are highly regulated with multiple enzymes contribute to its remodelling. All constituents of the ECM have a well-defined life-span with most of them turning over via proteolysis and synthesis (Humphrey, Dufresne & Schwartz, 2014). Computational models have shown that tissue form and function can be maintained only if the ECM's structural constituents are continuously degraded and replaced with new components with the same properties, including stiffness (Valentín & Humphrey, 2009; Cyron, Wilson & Humphrey, 2014). Thus, the dynamic remodelling of ECM is essential for development, wound healing and normal organ homeostasis and disruption of it can lead to several pathological conditions, including cancer.

The principal enzymes involved in ECM remodelling are metalloproteinases, which include matrix metalloproteinases (MMPs), disintegrins and metalloproteinases with thrombospondin motif (ADAMTS). Traditionally MMPs have been divided into different groups according to the cleaved ECM substrates, including the stromelysins, collagenases and gelatinases (Nagase & Woessner, 1999). Stromelysin-1 and stromelysin 2 (MMP-3 and MMP-10 respectively) have

a wide range of substrate specificity despite a distinct expression pattern. Both enzymes cleave proteoglycans, fibronectin and laminin while able to activate collagenases (Murphy *et al.*, 1987; Knäuper *et al.*, 1993, 1997).

Regarding collagenases, the three major enzymes in mammals are MMP-1, MMP-8 and MMP-13 (collagenase -1, -2, and 3 respectively). These enzymes actively cleave fibrillar collagens producing fragments of different sizes. MMP-2 and MMP-14 can also intervene at this site. Although these enzymes cleave collagen, they each act on a specific collagen subtype (Cawston & Young, 2009). Therefore, MMP-13 for example, cleaves type II collagen whereas MMP-1 and MMP-8 preferentially cleave collagens type III and I respectively. Macrophages, fibroblasts and chondrocytes are all able to synthesise MMP-1 and MMP-13 under inflammatory mediators. On the other hand, MMP-8 is mostly released from neutrophils upon stimulation (Cawston and Young 2009).

Finally, gelatinases act on denatured collagens, type IV and V collagen and elastin (Fosang *et al.*, 1992; Aimes & Quigley, 1995). While MMP-2 (also called gelatinase-A) is the most common of all MMPs, MMP-9 (gelatinase-B) is expressed in a variety of transformed and tumour-derived cells (Murphy & Crabbe, 1995; Li *et al.*, 2004; Mehner *et al.*, 2014). Recently, the discovery of more enzymes that do not fit easily into the MMP classification led to an alternative subdivision of these enzymes based on domain structure, activation mechanism and cellular location (Brinckerhoff & Matrisian, 2002).

MMPs regulate many biological processes and it is not surprising that they are strictly controlled at a number of critical steps, including synthesis, secretion, proenzyme activation and inhibition, localisation and clearance (Clark *et al.*, 2008). The majority of MMPs are

regulated at the transcriptional level with the exception of MMP-9 and MMP-8 that are also stored in neutrophils (Ong *et al.*, 2015). Several factors have been shown to impact the transcriptional activation of MMPs, including inflammatory cytokines, hormones and growth factors such TGFβ, the epidermal growth factor receptor (EGFR), TNFα and IL-1β (GALIS *et al.*, 2006; Imada *et al.*, 1997; Uzui *et al.*, 2002). Other studies have shown that prostaglandin 2 (PGE2) is able to upregulate transcription of MMP-1, MMP-2, MMP-9, and membrane type I-matrix metalloproteinase (MT1-MMP) in various cell types including osteoblasts and ECs (Kim *et al.*, 2005; Alfranca *et al.*, 2008). Furthermore, other studies have demonstrated that Low Density Lipoprotein (LDL) regulates MMP-9 expression in human monocyte-derived macrophages and MMP-1 in human vascular ECs (Huang, Mironova & Lopes-Virella, 1999; Xu *et al.*, 1999; Uzui *et al.*, 2002). Together with transcriptional regulation, there is also epigenetic and post-transcriptional regulation taking place. Studies have revealed that even ECM molecules affect the transcriptional and post-transcriptional mechanisms to modify MMPs expression (Gaffney *et al.*, 2015).

Alongside the mechanisms activating MMPs are those responsible for their inhibition (Gaffney *et al.*, 2015). The most prominent MMP inhibitors are those included in the Tissue Inhibitors of metalloproteinases (TIMP) protein family, secreted by various cell types. These inhibitors are able to selectively inhibit MMPs by inserting a conserved anchor into the active site of the target MMP and chelate the catalytic zinc ion (Gomis-R¨th *et al.*, 1997; Murphy, 2011) causing a decrease in the enzyme's ability to bind interfaces (Grossman *et al.*, 2010) TIMP expression is also regulated by various ECM factors, including TGF-β1 (Gomes *et al.*, 2012).

MMPs are known to contribute to tissue remodelling and migration both during developmental processes as well as during tumour progression (Page-McCaw, Ewald & Werb, 2007;

Kessenbrock, Plaks & Werb, 2010). Cellular migration and tissue invasion are driven by MMPs through their proteolytic ability to cleave ECM and basement membrane components. MMP-14 for example, is involved in the context of pericellular proteolysis which controls tumour cell traffic through the ECM (Sabeh *et al.*, 2004). Both MMP-2 and MMP-9, as gelatinases, are believed to play a crucial role in the process requiring basement membrane disruption, such tumour invasion and T lymphocytes tissue infiltration (Esparza *et al.*, 1999; Li *et al.*, 2004; Farina & Mackay, 2014; Mehner *et al.*, 2014). Other studies have highlighted an imbalance of both MMPs and TIMPs in hypertension, atherosclerotic plaque formation and instability, aortic aneurysm and varicose vein wall remodelling (Amin *et al.*, 2016).

1.5.3- MMPs deregulation in solid cancer and leukaemia.

Several studies have suggested a pivotal role for various MMPs in cancer growth, invasion and metastasis (Gialeli, Theocharis & Karamanos, 2010; Shay, Lynch & Fingleton, 2015). Tumour cells, similar to their healthy counterparts, interact with their environment. Importantly, cancer cell migration, invasion, tumour metastasis and angiogenesis seem to be supported by MMPs dysregulation (Figure 1.5). A number of studies have highlighted a positive correlation between MMPs expression and the invasive and metastatic potential of malignant tumours (Kessenbrock, Plaks & Werb, 2010; Merdad *et al.*, 2014). These include colon, lung, head and neck, basal cell, breast, thyroid, prostate, ovarian and gastric carcinomas (Westermarck & Kähäri, 1999; Gobin *et al.*, 2019). For example, MMP-1 expression correlates with colorectal and oesophageal cancer with poor prognosis (Murray *et al.*, 1996, 1998) whereas MMP-1 have been found to be over-expressed in triple negative breast cancer tissue and stroma cells compared to the non-tissue ones (Wang *et al.*, 2018). At the same time, MMP-2 and MMP-3 expression is associated with lymph node metastasis and vascular invasion in Squamous cell carcinoma (SCC) of the oesophagus (Shima *et al.*, 1992). MMP-13 expression led to the

metastatic capacity in SCCs of the head and neck and vulva whereas MMP-2 expression relates with poor prognosis of cervical SCCs. It is now recognised that angiogenesis plays a crucial role in the establishment of cancer and has a role in tumour progression. In fact, tumours require their own blood supply and as a consequence, the tumour vasculature is highly abnormal (Nagy *et al.*, 2009). In this context, MMPs participate in tumour neovascularization by actively regulate the angiogenic process. For example, MMP-1 activity promotes the expression of the vascular endothelial growth factor receptor 2 (VEGFR2) and ECs proliferation while the induction of MMP-2, MMP-8 and MMP-9 in ECs by TNF-α and other pro-angiogenic factors regulate the angiogenic process (Quintero-Fabián *et al.*, 2019). Different MMPs act differently based on cancer stage and size. It has been shown that MMP-8 expression in SCCs relates to an increase in patient survival whereas MMP-9 works as tumour promoter in carcinogenesis (Deryugina & Quigley, 2006).

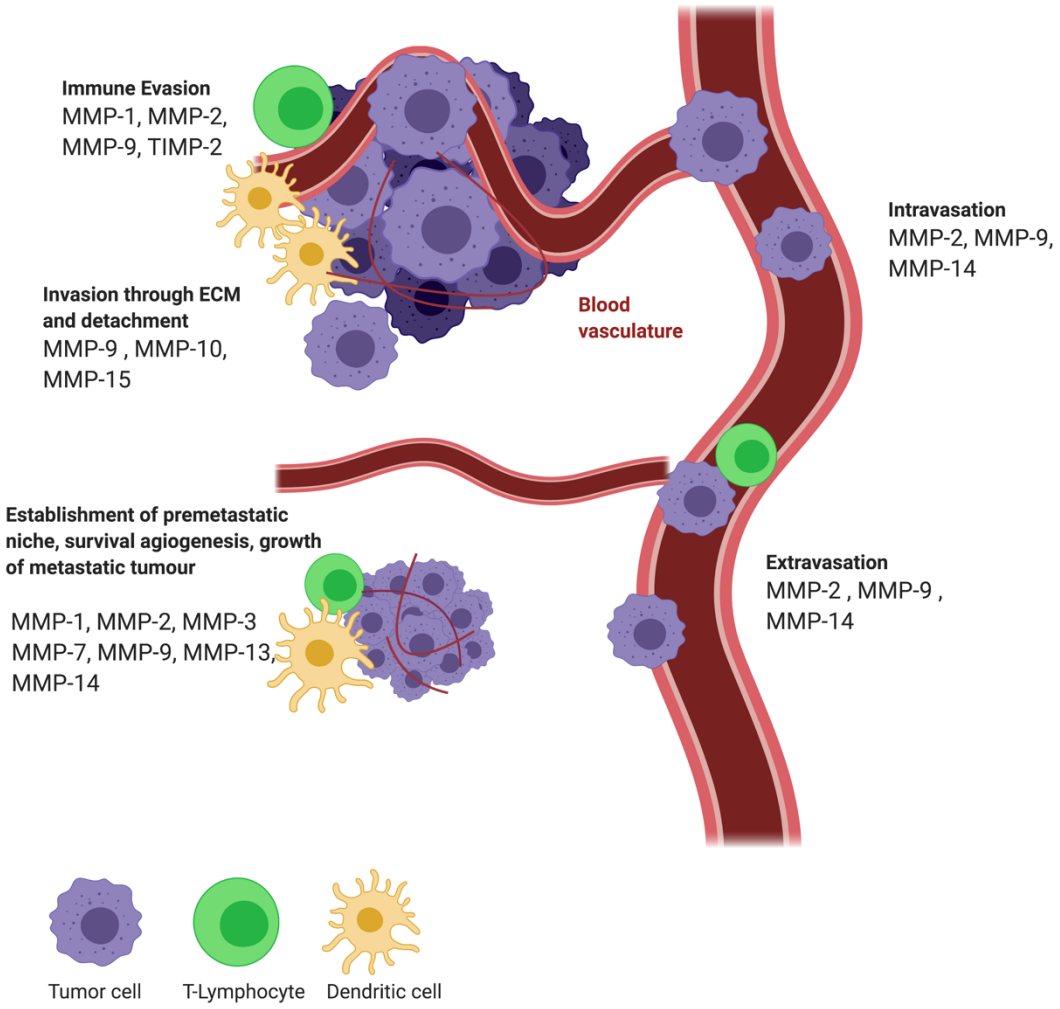

Figure 1.5-Roles of MMPs in tumour progression, invasion and metastases

Various MMPs, by digesting the ECM components, are differentially involved in the multistep process that leads to tumour progression, invasion and metastasis. In particular, MMP-2, MMP-9 and MMP-14 are involved in multiple process during cancer progression. Figure created using BioRender.com

It has been proposed that MMPs support tumour metastasis by releasing soluble factors into the circulation at the primary tumour site, helping to establish a metastatic niche in distant organs (Kessenbrock, Plaks & Werb, 2010). Surprisingly, MMPs are produced both by tumour cells and by neighbouring stromal cells in the tumour microenvironment, including in response to reciprocal paracrine interactions (Radisky & Radisky, 2007). MMP-9, for example, is strongly associated with aggressive and metastatic breast cancer with poor prognosis (Mehner

et al., 2014). It has been shown that metastatic colonization was enhanced in MMP-9 knockout mice implanted with MMP-9-expressing BM, while MMP-9-deficient mice injected with lung carcinoma or melanoma cells showed that host MMP-9 promote metastatic colonization of the lung (Itoh *et al.*, 1999; Acuff *et al.*, 2006). Furthermore, MMP-9 promotes lung metastasis in the MMTV-PyVT multistage mammary tumour model, potentially stemming from the pro-angiogenic contribution of neutrophil MMP-9 in the lung (Martin *et al.*, 2008). These results promote the idea that, in this mouse model, MMP-9 promotes metastasis when expressed by stromal cells. However, studies in humans have shown that MMP-9 is mostly produced by tumour cells with lower involvement of other cell types (Scorilas *et al.*, 2001; Li *et al.*, 2004).

While MMPs involvement in solid tumours is well established, their role in leukaemia remains unclear. In 1993, Kossakowska and colleagues proposed a role for MMPs and TIMPs in the growth and progression of lymphoid neoplasms showing an increased level of MMP-9 and MMP-7 based on tumour grade and aggressiveness (Kossakowska *et al.*, 1993). Later studies tried to address a role for various MMPs in AML and ALL focusing mostly on the expression of MMP-2 and MMP-9. For example, a study from 2002 showed that levels of TIMP-1, TIMP-2 were significantly higher while MMP-9 levels were lower in BM samples from AML and ALL patients compared to healthy controls. In particular, MMP-9 levels were significantly lower in the AML patients that achieved complete remission than in those who did not (Lin *et al.*, 2002). The AML patients with lower MMP-9 levels were shown to have longer survival time than those with higher levels (Lin *et al.*, 2002). This study proposed a close relationship between marrow MMP-9 expression level and disease status of AML aiming to use MMP-9 as a marker for disease progression and as a potential prognostic factor.

Thus, MMPs are now not only considered as diagnostic and prognostic biomarkers but most importantly, a potential therapeutic target. Nowadays, a number of MMP inhibitors have been developed and evaluated in several clinical trials with promising results (Gialeli, Theocharis & Karamanos, 2010).

1.5.4- Metalloproteinase inhibitors as a therapeutic approach in cancer

The past three decades have seen a growing interest in MMPs as a potential target for cancer treatment. From the 1990's, various synthetic inhibitors of MMPs (MMPIs) were studied and tested in clinical trials for several cancer types (Winer, Adams & Mignatti, 2018). One of the first drugs developed was Batimastat, designed to mimic collagen, the most common MMP substrate and its effect was studied in several solid tumour including pancreatic, colorectal, gastric, ovarian cancer together with mesothelioma, non-small cell lung, breast, melanoma and renal cancer (Wang *et al.*, 1994; Wojtowicz-Praga *et al.*, 1996; Prontera *et al.*, 1999; Hidalgo & Eckhardt, 2001). Regarding its specificity, Batimastat showed an ability to inhibit all MMP family members. Although early preclinical data indicated a promising anti-tumour effect, the drug unfortunately failed at the clinical stage due to its low bioavailability (Rasmussen & McCann, 1997). Despite several phase 1 studies showing the efficacy of the direct injection of Batimastat, various side effects were observed and led to the end of these studies (Wojtowicz-Praga *et al.*, 1996; Beattie & Smyth, 1998). The following years saw the development of Marimastat, an orally bioavailable drug with a similar mechanism of action to Batimastat. This time, the drug reached phase 2 and 3 of clinical trials in the metastatic setting for multiple solid tumour types including pancreatic, lung, breast, colorectal, brain and prostate cancer (Bramhall *et al.*, 2002; King *et al.*, 2003; Sparano *et al.*, 2004; Levin *et al.*, 2006). However, even in this case the drug failed to reach the final phase due to the musculoskeletal toxicity and low improvement in patients' survival (Bramhall *et al.*, 2002). The failure in clinical trials of these

two early studies pointed to two major weaknesses. First of all, it is now known that some MMPs have anti-tumour effects and therefore the use of MMPIs with a wide specificity might have blocked these MMPs and result in tumour progression. On the other hand, although MMPs are involved in the early stage of tumour progression, both MMPIs were tested in patients with advanced disease, therefore their reduced efficacy was partly expected (Winer, Adams & Mignatti, 2018). Thanks to these studies and their results, new and more specific MMPIs were developed including Tanomastat, Rebimastat and Prinomastat (Li *et al.*, 2004, 2013).

Prinomastat or AG3340 is a MMPI with selectivity for MMP-2, MMP-3, MMP-9, MMP-13 and MMP-14(Shalinsky *et al.*, 1999). Inhibition of these MMPs has been proposed to block tumour invasion and metastasis (Shalinsky *et al.*, 1999). The use of prinomastat in combination with several chemotherapeutic agents has been shown to have additive effects and inhibit tumour growth and angiogenesis in cancer models of colon, breast, lung, melanoma and glioma (Shalinsky *et al.*, 1999; Scatena, 2000). Despite this supportive evidence, the drug failed to demonstrate an increase in patient's survival and therefore studies were suspended in phase III clinical trials (Hande *et al.*, 2004; Bissett *et al.*, 2005).

Although the dysregulation of MMPs has been observed in leukaemia and the fact that AML cells often show constitutive release of several MMPs, there is still a lack of studies regarding the effect of MMPIs in this malignancy. It has been recently shown that MMP-9 function is finely regulated by its recruitment to the cell surface and that its association to the cell membrane by trans-membrane protein like CD44 or integrins favour the proteolysis of cell-associated substrate (Fridman *et al.*, 2003). Furthermore, it has been demonstrated that when the anchorage of MMP-9 to cell surface is inhibited, the invasive properties of cells are

dramatically reduced (Fridman *et al.*, 2003; Stefanidakis & Koivunen, 2006). In monocytic leukaemia (AML 4/5 types), MMP-9 is associated with the cell surface together with the membrane associated form of the DNA repair factor Ku, a heterodimer composed of Ku70 and Ku80 proteins (Monferran *et al.*, 2004; Paupert *et al.*, 2008). In leukemic blasts, Ku and MMP-9 co-localise at the leading edge of migratory cells and, when antibodies against Ku-sub-units and MMP-9 were used, the cellular invasion through collagen IV was blocked (Monferran *et al.*, 2004; Muller *et al.*, 2005). Another study showed that high BM level of MMP-9 was associated with blast crisis in myelodysplastic syndrome (MDS) and AML, suggesting its use as a possible target for treatment (Chaudhary *et al.*, 2016).

Together with MMP-9, the expression of MMP-14 correlates with progression and metastasis of multiple tumour cell types and mediates cell migration and invasion. Active MMP-14 not only degrades the ECM by pericellular proteolysis but it cleaves non-ECM molecules, including adhesion molecules and chemokines (Feng *et al.*, 2011). Furthermore, MMP-14 can also activate proMMP-2 to generate MMP-2 that, together with MMP-9, has been associated with the invasive behaviour of leukemic cells in ALL and AML (Klein *et al.*, 2004). It has been shown that MMP-14 expression in AML blasts contribute to the highly proteolytic BM microenvironment and to the AML invasive phenotype. Shirvaikar and colleagues have shown that MMP-14 is highly expressed in AML (WHO classification, AML with recurrent cytogenetic translocation, AML with multilineage dysplasia, AML not otherwise categorised) and that inhibition of MMP-14 by the epigallocatechin-3- gallate (EGCG) or by silencing it through siRNA block the migration of primary AML cells (Ahirvaikar, et al. 2008). Thus, the use of an appropriate MMPI in AML could potentially have an effect in decreasing tumour invasion (Paupert *et al.*, 2008). As previously discussed, prinomastat acts by selectively inhibiting a group of MMPs, including MMP-2, MMP-9 and MPP14 that have been shown to

be expressed in AML. Thus, prinomastat represents a good MMPI candidate to be investigated in this haematological malignancy.

1.6- Project aim and research questions

In this project, I aim to understand how leukaemia interacts with normal haematopoiesis. I will focus on three specific questions:

1. What are the cellular dynamics of the competition between AML and healthy haematopoiesis? (Chapter 3)

2. Does leukaemia induce extramedullary haematopoiesis and alternative niches for HSPCs? (Chapter 4)

3. Is it possible to delay AML progression and maintain/restore healthy haematopoiesis using MMPI? (Chapter 5)

To answer these questions, I will use a combination of flow cytometry, IVM and enzyme-linked immunosorbent assays.

Chapter 2

Materials and Methods

2.1- Mice

2.1.1- Ethics

All animal work was conducted in accordance with the UK Home Office regulations under the Animals (Scientific Procedure) Act, 1986 (ASPA) and the Animal Welfare and Ethical Review Body (AWERB) at Imperial College London and Sir Francis Crick Institute. The work described here was performed under project licenses 70/8403 and 950/4146 and all regulated procedures were conducted by me after obtaining a personal license (Reference: 16E75DD15).

2.1.2- Mouse Strains

All mice were bred and housed at Imperial College London or Sir Francis Crick Institute. C57Bl/6 wild-type (WT) were purchased from Charles River UK or obtained from the Francis Crick Institute. mT/mG mice (Muzumdar *et al.*, 2007) were purchased from Jackson Laboratories (Stock No: 007576; ME, USA). Flk1-GFP mice were a gift from Alexander Medvinsky (University of Edinburgh) (Xu *et al.*, 2010). PU1-YFP mice were a gift from Claus Nerlov (University of Oxford) (Kirstetter *et al.*, 2006). CD45.1 (B6/SJL) mice were bred at Imperial College London and Sir Francis Crick Institute. C57Bl/6 splenectomised mice were purchased from Charles River UK or obtained from the Crick institute.

For fluorescence-activated cell sorting (FACS) experiments 6-8 weeks old female WT, WT splenectomised and Flk1-GFP mice were used. For imaging experiments female WT, Flk1-

GFP, mT/mG or mT/mG chimeric mice >8 weeks old were used (refer to 2.3.5). As donors in transplantation assays, female CD45.1, WT, WT splenectomised, mT/mG mice >8 weeks old were used. As recipients in transplantation assays, 8 weeks old female WT, CD45.1 or mT/mG mice were used.

2.1.3- Genotyping

Flk1-GFP mice were genotyped by mounting a sample from the ear onto a slide and analysing the presence of GFP signal, emitted by both endothelial and perivascular cells, under the microscope. PU1-YFP and mT/mG mice were genotyped through the analysis of YFP and mTomato signals in peripheral blood by flow cytometry. CD45.1 (B6/SJL) mice were genotyped by flow cytometry when not homozygous from breeding.

2.2- Flow cytometry

2.2.1- Multiorgan cell isolation

For leukaemia and haematopoietic cell analysis, bones were crushed using a sterilized mortar and pestle, suspended in FACS buffer (Phosphate-Buffered Saline, PBS plus 2% of Foetal Bovine Serum, FBS) and filtered through a 40µm strainer. For the extramedullary haematopoiesis (EMH) experiments, spleen and liver were mashed through a 40µm strainer using the internal part of 5- or 10-ml syringes and resuspended in FACS buffer. For blood analysis, approximately 1 ml of blood was obtained by cardiac puncture after terminal anaesthesia with pentobarbital and collected in Ethylenediaminetetraacetic acid (EDTA). In all the cases, samples were then centrifugated for 5 minutes at 500 x g at 4°C, resuspended in red cell lysis buffer (0.001g/ml Potassium bicarbonate, 0.008g/ml Ammonium chloride, 20 mM EDTA, all from Sigma and 5% (v/v) FBS in Milli-Q© water) for 3-5 minutes. Samples were lysed up to three times and then washed in FACS buffer. Following that, samples were

centrifugated at 500 x g for 5 minutes at 4°C, resuspended in FACS buffer ready for subsequent staining.

For stromal cell analysis, tibias and femurs were minced with scissors and then crushed with a mortar and pestle. The resulting cells and bone fragments were then transferred directly to a 50ml Falcon tube and resuspended in FACS buffer. At the same time, spleen and liver were respectively mashed using a 40µm strainer and resuspended in FACS buffer into a 50ml falcon tube. Samples were centrifugated at 500 x g for 5 minutes at 4°C and the supernatant discarded. The cell pellets were suspended in 3ml of Collagenase I solution (0.25% (w/v) Collagenase type I powder in Hank's balanced salt solution (HBSS), both from Gibco. Following this step, tubes were sealed with parafilm and incubated at 37°C for 30 minutes with 110rpm agitation. The resulting cells were filtered through a 70µm strainer into a new 50ml Falcon tube, washed with FACS buffer and then ready to be stained for analysis.

2.2.2- Cell staining and sample acquisition

Cells were stained in FACS buffer for 30 minutes at 4°C, washed and then centrifugated at 500 x g for 5 minutes at 4°C before performing a secondary stain (if necessary) and incubating for another 30 minutes at 4°C. Cells were then resuspended in an adequate volume of FACS buffer prior to acquisition. All primary antibodies were used at 1:100 dilution, apart from anti-CD34 (1:50) and the lineage cocktail (1:20). All secondary antibodies were used at a 1:1000 dilution. Specific details for the fluorochrome-conjugated and biotinylated or streptavidin antibodies used can be found in Table 1.

Antibody	Clone	Source	Catalogue No.
Biotin anti-mouse CD3e	145-2C11	BioLegend	100304
APC/Cy7 anti-mouse CD3e	145-2C11	BioLegend	100330
Biotin anti-mouse CD4	GK1.5	BioLegend	100404
Biotin anti-mouse CD8a	54-6.7	BioLegend	100704
Biotin anti-mouse Ter119	Ter119	BioLegend	116204
APC/Cy7 anti-mouse Ter119	Ter119	BioLegend	116223
Biotin anti-mouse/human CD45R/B220	RA3-6B2	BioLegend	103204
Pacific Blue anti-mouse/human CD45R/B220	RA3-6B2	BioLegend	103227
PE/Cy7 anti-mouse/human CD45R/B220	RA3-6B2	BioLegend	103222
Biotin anti-mouse Ly6G/Ly6C(Gr-1)	RB6-8C5	BioLegend	108404
Biotin anti-mouse CD11b	M1/70	BioLegend	101204
FITC anti-mouse CD11b	M1/70	BioLegend	101206
APC/Cy7 anti-mouse CD117 (c-kit)	2B8	BioLegend	105826
APC anti-mouse CD117 (c-kit)	2B8	BioLegend	101212
PerCP/Cy5.5 anti-mouse Ly6a/E (sca-1)	D7	BioLegend	108124
Pacific Blue anti-mouse CD48	HM48-1	BioLegend	103418
PE/Cy7 anti-mouse CD48	HM48-1	BioLegend	103424
BV650 anti-mouse CD150 (SLAM)	TC15-12F12.2	BioLegend	115931
PE/Cy7 anti-mouse CD150 (SLAM)	TC15-12F12.2	BioLegend	115914
Anti-mouse CD34 FITC	RAM34	ThermoFisher Scientific	11-0341-82
APC anti-mouse CD34	HM34	BioLegend	128612
PE/Cy7 CD16/32	93	BioLegend	101318

Anti-mouse CD127 APC efluor780	A7R34	eBioscience	47-1271-82
PE/Cy7 CD31	MEC13.3	BioLegend	102524
PerCP/Cy5.5 anti-mouse CD45.1	A20	BioLegend	110728
PE anti-mouse CD45.1	A20	BioLegend	110708
APC anti-mouse CD45.2	104	BioLegend	109814
PE anti-mouse CD45.2	104	BioLegend	109808
APC/Cy7 anti-mouse CD45	30-F11	BioLegend	102524
FITC anti-mouse CD71	R17217	BioLegend	113806
Streptavidin Pacific Orange		Invitrogen	S32365

Table 1 Antibodies used in flow cytometry experiments

The lineage cocktail includes a mix of the following antibodies in 1:1 ratio: anti-CD3, CD4, CD8, B220, Ter119, Gr-1 and CD11b. Live and dead cells were identified using 4,6-diamidino-2-phenylindole (DAPI) (NucBlue® Fixed Cell ReadyProbes® Reagent, Invitrogen, Cat. #R37606). Single colour controls were always used and fluorescence-minus-one (FMO) controls were prepared when necessary.

Calibrite Beads (BD Calibrite 3 Beads FITC, PE, PerCP, Unlabelled Beads; BD Bioscences, Cat #S7579) were used to calculate the absolute cell count as described by Hawkins and colleagues (Hawkins, et al. 2007). Specifically, single colour calibrate beads were reconstituted in PBS at a concentration of 1 x 10^6 beads/ml and resuspended to reach a homogenous distribution. Following this step, a specific volume of 100µl (containing approximately 100,000 beads) was added to each sample ready to be acquired. For each sample, the number of absolute cells was then back calculated using the number of acquired cells and acquired beads.

Proliferation analysis of cells was carried out using the Click-iT™ EdU Pacific Blue™ Flow Cytometry Assay Kit (Invitrogen, Cat. # C10418). Control and AML leukemic mice were injected with 5-ethynyl-2'-deoxyuridine I.P (EdU; Invitrogen, Cat. # A10044) at a concentration of 1mg of Edu injected per mouse 2 hours prior to harvesting the BM, spleen and liver. After sample staining, cells were fixed with 200µl of Fixation Buffer (BD Bioscences, Cat. #554655) and permeabilised in 200µl Permeabilization Buffer Plus (BD Bioscences, Cat. # 561651). Next, EdU was labelled using the Click-iT™ kit following the manufacturer's instructions. Samples were then incubated for 30 minutes at Room Temperature (RT) in the dark, washed and analysed by FACS.

To identify apoptotic cells, an Annexin V kit (BD Pharmingen™ Annexin V APC; BD Biosciences, Cat. # 550474) was used. Annexin V is a calcium-dependent protein able to bind the phospholipid phosphatidylserine (PS) that is normally translocated to the outer layer of the plasma membrane in apoptotic cells. Therefore, using a combination of Annexin V and a cell-impermeable vital dye such a 7-Amino-Actinomycin (7-AAD) it is possible to identify live (Annexin V^-, 7-AAD^-), early apoptotic (Annexin V^+, 7-AAD^-) and dead (Annexin V^+, 7-AAD^+) cells. For each sample, cells were resuspended at a concentration of 1 $x10^5$ in 100µl of 1x Annexin V binding buffer (BD Biosciences; Cat # 556454) followed by the addition of 5µl of Annexin V and 5 µl of 7-AAD. Then, samples were gently vortexed and incubated at RT for 15 minutes in the dark. At the end of the incubation time, 400ul of 1x Annexin V binding buffer was added and samples analysed by FACS within 1 hour.

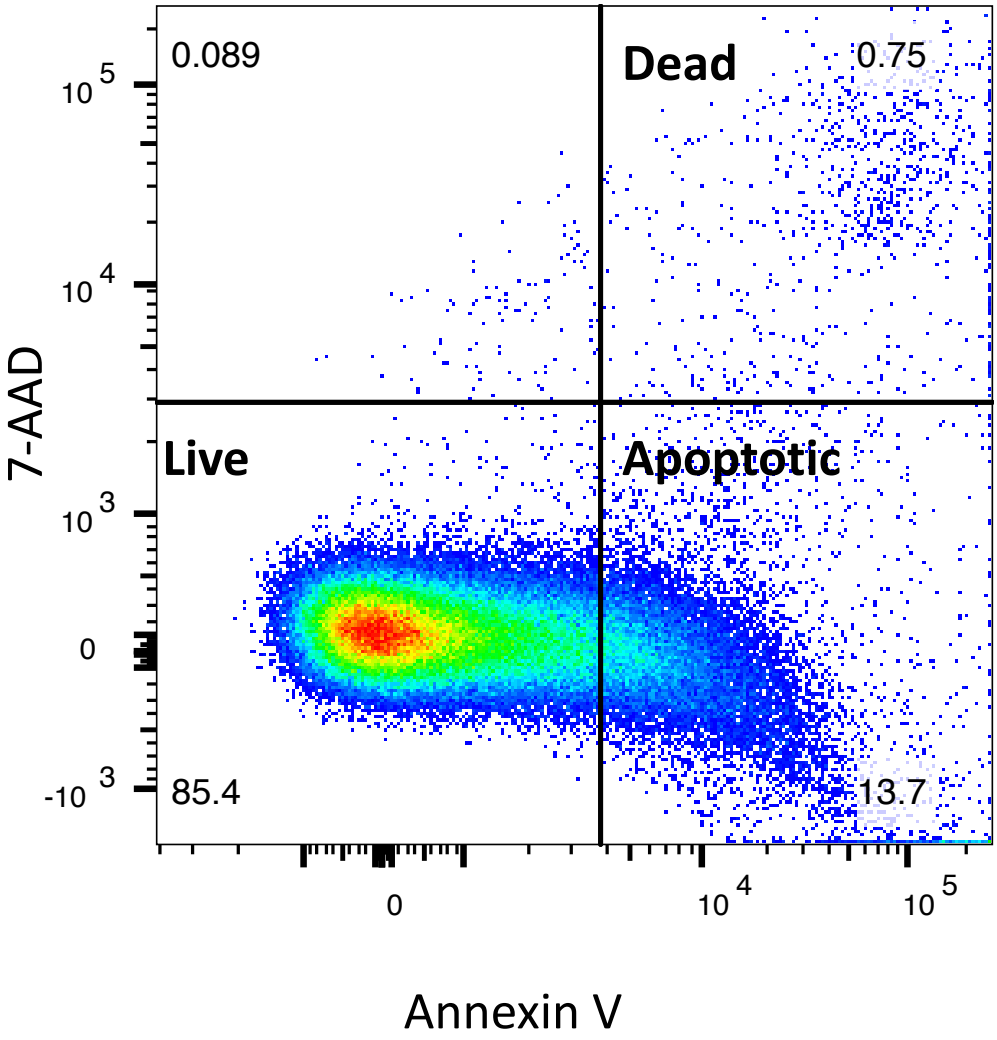

Figure 2.1-Annexin V and 7-AAD staining of BM cells

Live (Annexin V-/7-AAD-), early apoptotic (Annexin V+/7-AAd-), and dead cells (Annexin V+/7-AAD+) can be identified using flow cytometry

For all experiments, cells were analysed with LSR Fortessa and data analysed with FlowJo (Tree Star). The gating strategy used for each group is show below:

- Haematopoietic stem and progenitor cell and AML blasts (Figure 2.2)
- Stroma and ECs (Figure 2.3)

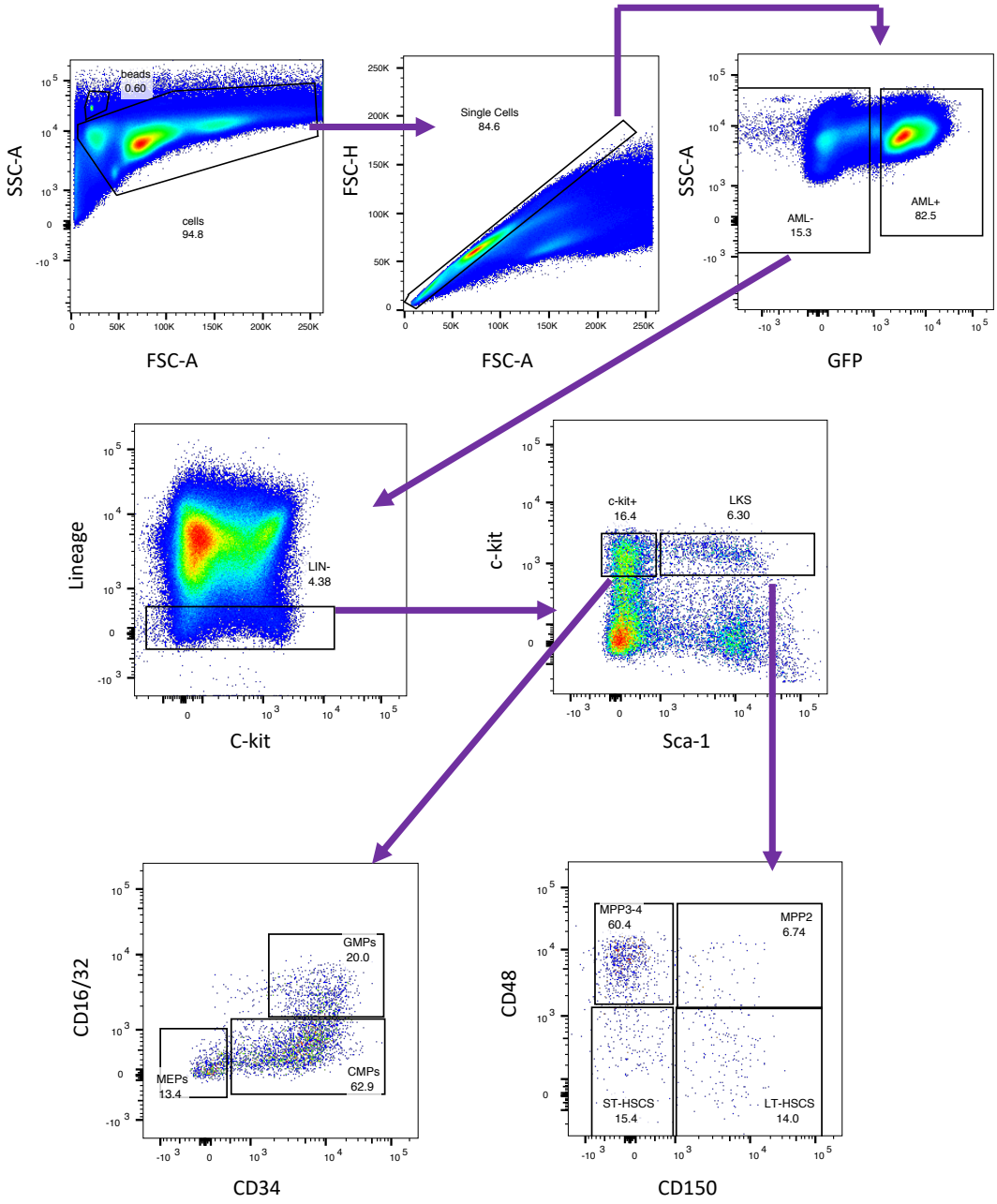

Figure 2.2-FACS gating strategy to identify HSCs ,Progenitors and Leukemic cells

Representative FACS plots from day 20 post-injection of MLL-AF9 AML blasts in non-irradiated recipient mice analysed by flow cytometry. Black boxes indicate the gates used for isolating counting beads, gating on single cells, AML$^-$ cells and lineage$^-$ population. Gating on lineage$^-$ is it possible to differentiate between the c-kit$^+$ that include the GMPs, CMPs and MEPs and the LKS population inclusive of LT-HSCs, ST-HSCs and MPPs

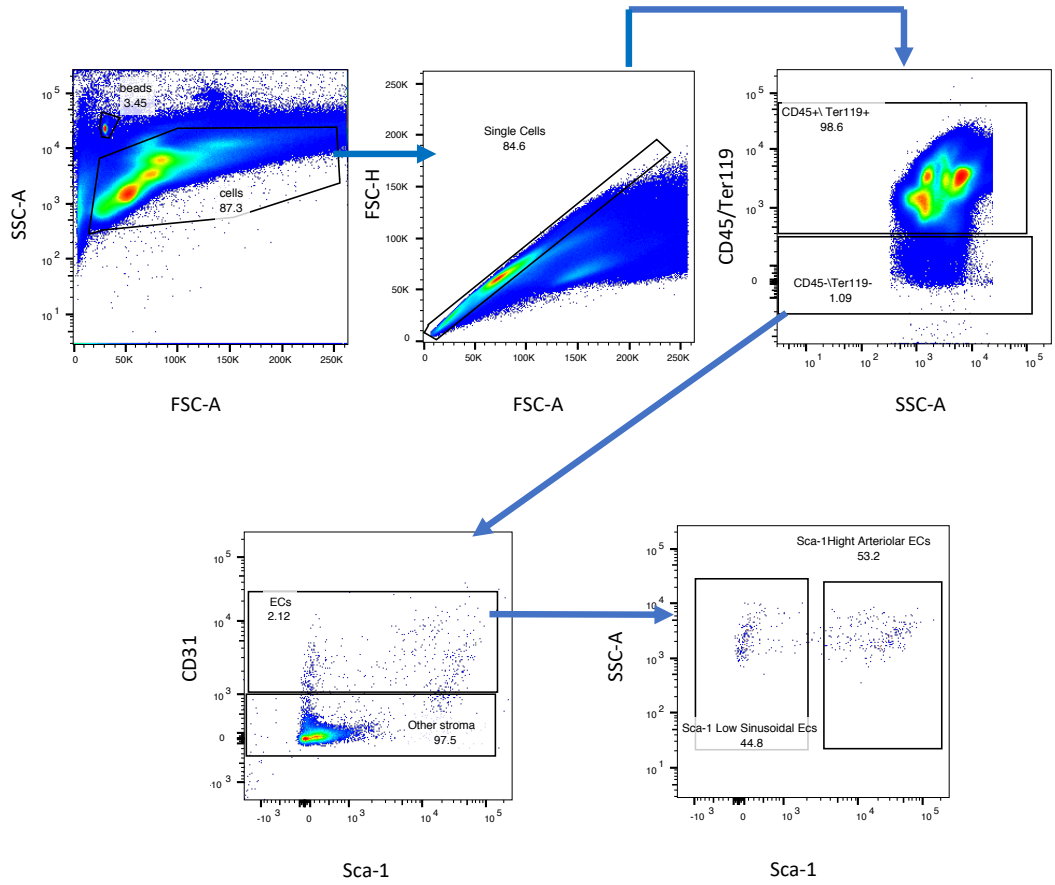

Figure 2.3-Endothelial cells gating strategy

Representative FACS plots from a BM of control mice analysed by flow cytometry. Black boxes indicate the gates used for isolating counting beads, gating on BM cells, excluding doublets by gating on single cells, $CD45^+$ $Ter119^+$ mixed stroma and haematopoietic cells, and endothelial cells

Analysis of stroma oxidative stress was performed by measuring cellular reactive oxygen species (ROS) levels using the CellROX™ Deep Red Reagent kit (Invitrogen, Cat. # C10422). Briefly, following cell surface staining, samples were incubated with CellROX™ reagent at a final concentration of 5µM for 30 minutes at 37°C. Then, samples were washed 3 times with PBS, resuspended in FACS buffer and analysed by FACS.

2.3- Transplantation assays

2.3.1- Lineage depletion

The lineage depletion protocol was performed using the MACS Column Technology (Miltenyi Biotech) before every transplantation assay and all the procedures were carried out in sterile conditions. Bones, livers and spleens (when present) were harvested from control and leukemic donor mice and cells isolated as described above (section 2.2.1). Each sample was stained with a cocktail of biotinylated lineage antibodies (CD3, CD4, CD8, B220, Gr-1, Ter119, Cd11b) in FACS buffer and incubated for 30 minutes at 4°C. Samples were then washed and resuspended in 1 ml of sterile PBS and 30µl of streptavidin magnetic Microbeads (Miltenyi Biotech, Cat. # 130-048-101) for another 30 minutes at 4°C. In the meantime, LD columns (Miltenyi Biotech, Cat. # 130-042-901) were equilibrated by running 3ml of PBS per column. At the end of the incubation time, each sample was washed and resuspended in 3 ml of PBS, then transferred to the appropriate LD columns using a 40µm strainer. At this point, each lineage depleted sample was collected in a 15ml falcon tube. After the whole sample passed through, the columns were rinsed with an additional 3 ml of PBS. As a final step, samples were stained with appropriate antibodies.

2.3.2- LKS cell transplants

Cells from BM, Liver and spleen (when appropriate) of CD45.1, mT/mG, or splenectomised C57BL/6 control and leukemic donor mice were depleted as described above. 500 Lineage$^-$ c-kit$^+$ Sca-1$^+$ (LKS) cells were transplanted into lethally irradiated recipients together with 2 x 10^5 support BM cells from an appropriate control donor of the same strain as the respective recipient mice.

2.3.3- Evaluating multilineage engraftment

The monitoring of multilineage engraftment was carried out on PB starting from week 4 post-transplantation. Approximately 10µl of blood was obtained from mice via tail vein puncture and collected in a 96 well plate containing EDTA to avoid clotting. Samples were lysed by adding 150 µl of red cell lysis buffer per well and then centrifugated at 800 x g for 2 minutes at 4°C. This step was repeated 2-3 times in order to lyse all red blood cells before washing again with FACS buffer. Following this step, samples were stained for mature lineage cell surface markers (CD3 for T cells, B220 for B cells, CD11b for myeloid cells and if appropriate, CD45.1 and CD45.2 to distinguish between donor-and recipient-derived cells). Peripheral blood was evaluated for multilineage engraftment every 4 weeks post-transplant up to week 20 followed by BM reconstitution analysis at week 20 when appropriate. Figure 2.4 below shows the gating strategy used.

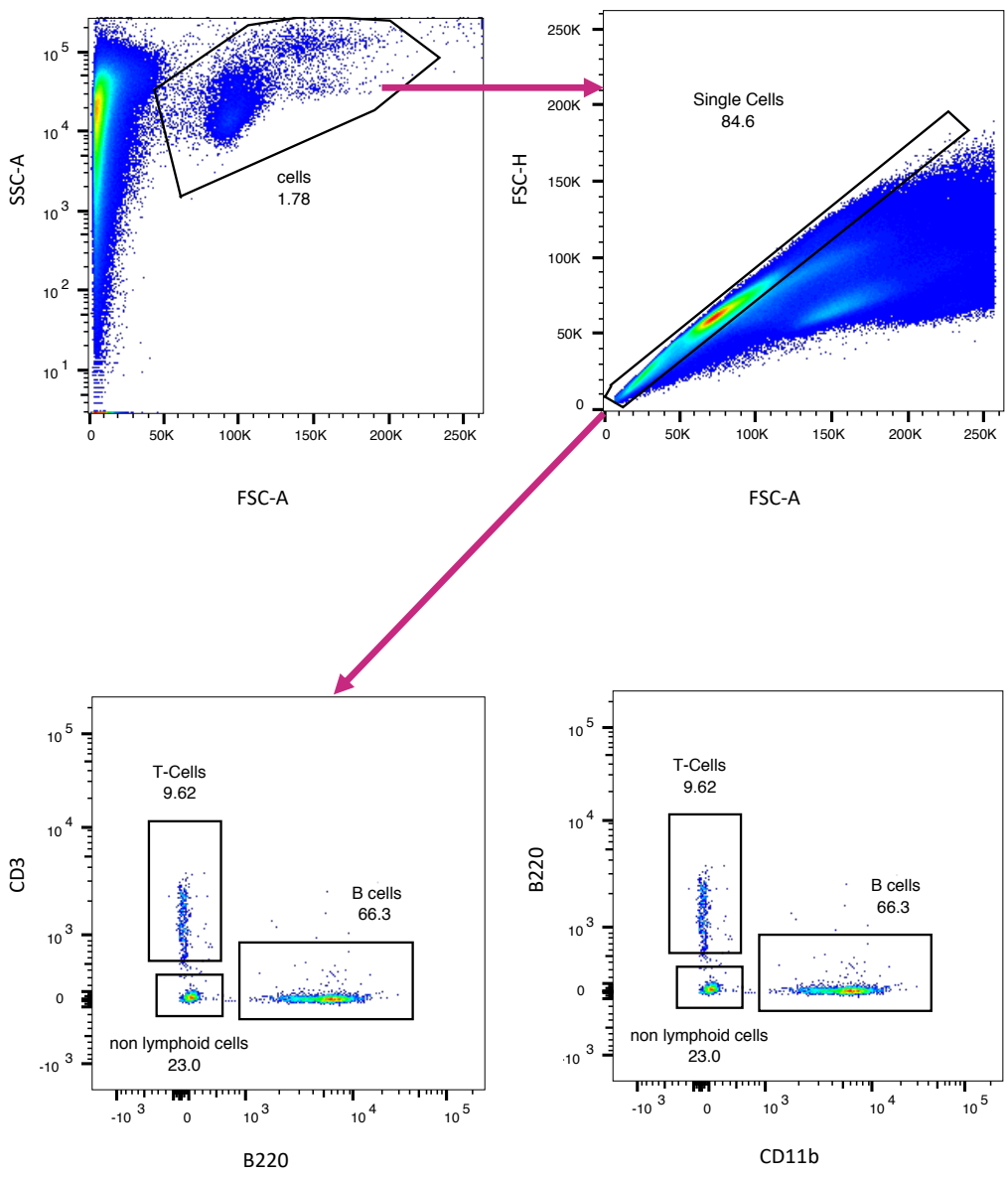

Figure 2.4-FACS gating strategy for mature cells population

Representative FACS plot from the peripheral blood of control mice analysed by flow cytometry. Black boxes indicate the gates used for gating on peripheral blood population, excluding doublets by gating on single cells, T cells, B cells and non-lymphoid cells to isolate myeloid cells. The same gating strategy was then used for the BM

2.3.4- Bone marrow chimeras

To generate chimeric mice, whole BM cells were obtained from femurs and tibias and hips of mT/mG donor mice and isolated as described before. Cells were then diluted in sterile PBS, counted and resuspended in the appropriate volume. A dose of 1 x 10^6 cells/mouse were then injected i.v in lethally irradiated WT recipient mice. Mice were kept under Baytril-treated water

for 6 weeks following transplantation. The percentage of chimerism was evaluated by FACS analysis of peripheral blood (% of mT/mG$^+$ cells) with >95% of chimersim obtained approximately 8 weeks after transplantation. At this stage, mice were ready to be injected with AML cells and used for intravital imaging experiments.

2.4- Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was carried out on supernatants obtained from BM, livers and spleens of cohorts of control, leukemic and prinomastat untreated/ treated leukemic mice. To obtain flushed BM supernatant, tibias and femurs were harvested from the appropriate mice and the metaphysis and diaphysis separated with scissors. At this point, 100µl of PBS was flushed through each diaphysis, collected in an Eppendorf tube and reflushed. Next, samples were centrifugated at 400 x g for 5 minutes in order to pellet down the cells and collect the supernatant. Any remaining cells were subsequently excluded by further centrifugation of the supernatant at 500 x g for 5 minutes. For the liver and spleen, half of each organ was mashed through a 40µm strainer using a 1ml syringe and resuspended in 100µl of PBS. The supernatant was then isolated by centrifugation as described above. All supernatants were stored at -20°C.

For analysis of the supernatants by ELISA, the following kits were used: Mouse VEGF DuoSet ELISA (R&D system, Cat #DY493), Legend MAX™ Mouse CXCL12 (SDF-1ß) ELISA kit (Biolegend, Cat # 444207), IL-6 Mouse Uncoated Elisa Kit with Plates (Thermofisher, Cat# 88-7064-22), SCF (KITL) Mouse Elisa kit (Thermofisher, Cat. # EMKITL), Mouse CSF3 (G-CSF) ELISA (Thermofisher Cat # EMCSF3). All experiments were conducted according to the manufacturer's instructions.

2.5- AML experimental model

2.5.1- AML Transplantation

The MLL-AF9 AML blasts used where generate by previous group members as described in (Akinduro *et al.*, 2018). To initiate leukaemia in experimental mice, primary blasts were thawed, resuspended in PBS, counted and 100,000 viable cells were transplanted via tail vein injection into secondary, non-conditioned, recipient mice. In some cases, secondary blasts were used. Leukaemia development was observed in peripheral blood from day 8-10 with full BM infiltration usually reached between day 22-28, followed by spleen infiltration, depending on the primary blast clones analysed. The health of transplanted mice was monitored daily and disease progression was analysed through sampling of peripheral blood (PB) by tail vain puncture followed by FACS analysis for GFP^+ cells. Mice were subsequently euthanised and experiments terminated when any one or a combination of the following signs were observed: laboured breathing, hunched posture, weight loss, enlarged lymph nodes and/or spleen.

2.6- T-ALL experimental model

2.6.1- Notch1-driven T-ALL model

The Notch1 T-ALL blasts used were generated by Edwin Hawkins and Delfim Duarte as described in (Aster *et al.*, 2000). Specifically, foetal liver cells were harvested from WT embryos at day 14.5 (E14.5) and transduced with a MigR1 plasmid containing DsRed with NotchICNΔRamΔP. After that, 1 x 10^6 $DsRed^+$ foetal liver cells were transplanted into primary lethally irradiated recipient mice (two doses of 5.5Gy administered 3 hours apart). Recipient mice were kept on Baytril-treated water to prevent infection for 6 weeks post-transplantation. Again, mice were euthanized as soon as signs of leukaemia were observed (6 to 25 weeks).

From each recipient DsRed^{+} cells were isolated from BM and spleen and stored in liquid nitrogen. Cells from each primary blasts were labelled as a separate clone.

2.6.2- T-ALL Transplantation

T-ALL cells were thawed in RPMI media containing 10% FBS (R-10) in a 15ml Falcon© tube. Cells were then centrifugated for 5 minutes at 500 x g at 4°C and resuspended in 5 ml R-10 and slowly layered in a new 15 ml Falcon© tube containing 5ml of Ficoll™. Following this step, the sample was spun down at 1,625 x g for 20 minutes at 4°C with 0 acceleration and 0 break. Then, the supernatant was discarded and T-ALL blasts isolated from the buffy coat, transferred to a new tube and resuspended in RPMI medium. Cells were centrifugated again at 1,463 x g for 5 minutes at 4°C, counted and washed with PBS alone to remove any remaining serum. After this last step, T-ALL blasts were suspended in the desired volume for injection. 100,000 cells T-ALL blasts are usually injected per sub-lethally irradiated recipient. For the titration experiment. 50,000 to 500,000 cells were injected per non-conditioned recipient. Mice were monitored daily for any signs of ill-health and leukaemia progression was analysed as described above. Mice were euthanised when any one or a combination of the following signs were observed: hunched posture, laboured breathing, weight loss and enlarged spleen and/or lymph nodes.

2.7- Drug administration

2.7.1- Prinomastat treatment

For prinomastat (AG3340) treatment, prinomastat hydrochloride ≥ 95% (Sigma Aldrich, Cat. # PZ0198) was solubilized in deionized water (dH_2O) to reach a final concentration of 15mg/ml. Mice were intravenously (i.v) injected daily with 13.5mg/kg (or 30mg/kg for intraperitoneal (i.p) injection) of prinomastat or (PBS), used as a vehicle control. Treatment was started 7 days after AML injection and continue for 15 days in total. In some experiments, Prinomastat treatment was also combined with chemotherapy regiment.

2.7.2- Chemotherapy regiment for AML

The following induction chemotherapy regimen use drugs commonly administered in AML patients. Mice showing a PB infiltration ≥ 18 %, measured by FACS, were treated I.V. for a total of 5 days. 100mg/kg Cytarabine (Ara-C; Stratech Scientific; Cat # S1208-SEL) and 3mg/kg Doxorubicin (Doxo; Stratech Scietific; Cat # S1648-SEL) were administrated together for 3 days followed by other 2 days of cytarabine only. Both ara-c and doxo were obtained from the Imperial College Healthcare NHS Trust. For experiments requiring prinomastat treatment too, mice were injected with prinomastat before, during and after chemotherapy treatment.

2.8- Intravital microscopy

IVM was performed using a Zeiss LSM 780 upright confocal/two-photon hybrid microscope equipped with Argon (458,488 and 514 nm), a diode-pumped solid-state 561nm laser and a Helium-Neon 633 nm, a tunable infrared multiphoton laser (Spectraphysics Mai Tai DeepSee 690-1020nm), 4 non-descanned detectors (NDD) and an internal spectral detector array. Signal was visualised with a Leica HCX IRAPO L 25X water immersion lens (0.95 N.A) and Zeiss W Plan-Apochromat 20X.

Intravital imaging of mouse calvarium was performed as described by Hawkins and colleagues (Hawkins *et al.*, 2016). Mice were induced and maintained anesthetized using isofluorane in medical oxygen throughout the procedure (4% isofluorane in 4L/min O_2 for the induction and 2% isofluorane in 1L/min O_2 for maintenance). To expose the calvarium, a minimally-invasive surgery was performed aiming to remove the skin and subcutaneous tissue on the top of the skull(Scott, Akinduro & Celso, 2014). The exposed part was then cleaned with a cotton swab to remove any hair that could potentially fall into the surgical area. At this point, a custom-made imaging window was attached using Diamond Carve Dental Cement (Associated Dental Products, Cat # SUN527). To prepare the cement with an appropriate consistency, 3 measured spoons of powder were combined with 2 drops of liquid provided in the kit. Following that, the obtained mixture was disposed on the top of a metallic headpiece and subsequently attached on the calvarium. The cement was left to solidify for 15-20 minutes before transferring the mouse to the microscope stage. The mouse was placed on a heat pad and its body temperature was constantly monitored throughout the procedure with a rectal probe equipped with a thermal sensor. Eye dryness and corneal injury were prevented by applying the Blephagel eye ointment (SpectrumThea) at the beginning and throughout the procedure. On the microscope stage, the accurate position and imaging stability of the mouse were obtained thanks to the lock-and-key

mechanism of the imaging window. Before starting and to improve the images quality, the calvarium was carefully cleaned with a swap allowing the removal of any leftover of subcutaneous tissue. Finally, a drop of PBS was applied on the calvarium before lowering the lens for imaging. For repeated/recovery imaging, protective intrasite gel (Smith & Nephew, Cat #7308) was applied on the surgery site to preserve the bone integrity and avoid scar formation. Then, mice were then allowed to recover from anaesthesia. After each imaging session, mice were treated with oral buprenorphine mixed in raspberry jelly at a dose of 0.8 mg/kg to alleviate any pain experienced post-procedure.

IVM of the spleen was always performed under general, terminal anaesthesia. The mice were induced and maintained under anaesthesia as described above. For spleen exposure, mice were placed in the right lateral decubitus position and a small section of fur was removed from the left flank. At this point a small incision was performed above the spleen with a part of it sequentially exposed. On the microscope stage, the surface of the exposed spleen was then mechanically stabilized with a gentle vacuum using a specific coverslip vacuum chamber similar to that used in (Headley *et al.*, 2016). Finally, a drop of water was then placed on the top of the chamber coverslip before lowering the lens for imaging.

Blood vessels were labelled using 80µl of 8mg/ml Cy5-Dextran (nanocs, MA) or 50µl of TRITC-dextran (40mg/ml 150kDA; Sigma) injected i.v. In some experiments, haematopoietic cells were labelled *in vivo* using 10µg/ml of CD45.2 antibody in PE or APC (BioLegend).

Bone signal was acquired using second harmonic signal excited at 860-880nm and detected with external detectors. GFP, mTomato/PE and Cy5 signals were excited at 488 nm, 561nm and 633 nm respectively using internal detectors. YFP signal was excited at 488nm or 514 nm

using internal detectors. Large three-dimensional (3D) tile scans of the whole BM cavity within the calvarium or the spleen were acquired by stitching adjacent, high-resolution z-stack images obtaining the visualisation of the calvarium BM or the spleen with single cell resolution. Furthermore, positions within the tilescan could be selected and imaged over a period of time to produce time lapse movies and capture cells dynamics within the marrow or spleen.

Analysis of vascular leakiness in FLK1 AML prinomastat treated/control mice was performed by adapting previous published protocols (Itkin *et al.*, 2016b; Passaro *et al.*, 2017b; Haltalli *et al.*, 2018). In brief, mice underwent surgery as described above and were positioned on the microscope stage. At this point, the appropriate positions for analysis were selected and 60µl of low molecular weight TRITC-dextran (3mg/ml, 65-80kDa; Sigma) was injected i.v. Images were captured every minute for a total of 10 minutes. The obtained time lapses allowed the analysis of vascular dye extravasating from vessel.

2.9- Image processing and quantification

Zen black (Zeiss, Germany) software was used to stich the 3D tile scans of BM and spleen. Image J was then used to visualise and process raw data. The same program was also used to manually crop out autofluorescent signal from the tissue when appropriate. For time lapse data, any displacement in the Z plane caused by artefact movement were corrected by applying four-dimensional (4D) data protocols implemented in ImageJ which allow the registration of the acquired time lapses (Preibisch, et al. 2010).

Cell and cluster counting were performed manually using the FIJI plugin cell counting while cell tracking was performed semi-automatically using Imaris. Again, time lapses were corrected using the 4D data protocols before importing the selected images to Imaris. Imaris

software was used to detect leukaemia cells and create either “surfaces” or “spots” and the semi-automatic cell tracking was done using built-in algorithms that were then manually supervised. Obtained data, including track mean speed, length and linear progression were exported for further analysis. For leaky vasculature analysis, the TRITC-dextran extravasation in time lapses movies was quantified by measuring the pixel intensity within 3 equally sized and randomly placed region of interest per time frame. Finally, the average of these values was calculated and the fold change increase in intensity for each group plotted in a graph.

2.10- Quantification and statistical analysis

All obtained data were processed and visualised using Microsoft Excel and GraphPad Prism (GraphPad Software Inc.). In some case, linear regression analysis was used. Group means were compared using the unpaired Student’s t test. For multiple comparisons, one-way ANOVA with post-hoc Tukey or Bonferroni correction was used. For all data, differences were considered significant for $p<0.05$. * $p<0.05$; ** $p< 0.01$; *** $p<0.001$****$p<0.0001$. Numbers of used animals, together with other statistical details, can be found in the figure legends.

Chapter 3

Investigating the effects of acute myeloid leukaemia on steady state haematopoiesis

3.1- Introduction

Haematopoiesis defines the process through which, every day, haematopoietic stem cells (HSCs) produce billions of new blood cells throughout the human lifespan. HSCs reside in the bone marrow (BM) microenvironment where their mutual interactions with niche components drive the regulation and maintenance of haematopoiesis. AML and T-ALL are blood malignancies both characterised by poor prognosis and low chemotherapy response. Specifically, patients suffering from AML experience cytopenia as a major symptom due to the loss of steady state haematopoiesis. Several mechanisms have been proposed as a cause for HSPC loss including a block in their differentiation (Cheng *et al.*, 2015), a reduction in stem and progenitor cell production rates and an increase in apoptosis or emigration (Miraki-Moud *et al.*, 2013). However, the exact mechanism behind AML overcoming steady state haematopoiesis remains unknown. Recently, the existence of a niche as a protective microenvironment has been proposed to be fundamental in leukaemia growth and chemo-resistance (Ladikou *et al.*, 2020). Furthermore, the combined use of IVM and genetic mouse models have highlighted that interactions between malignant cells and the BM microenvironment are specific for disease subtypes and stage (Duarte, Hawkins & Lo Celso, 2018). By using IVM our group has shown the dynamics with which T-ALL cells appear in the calvarium BM, with single cells first seen at day 10 post-injection followed by clusters at day 12-18 and then full infiltration from day 18 post- injection (Hawkins *et al.*, 2016).

Furthermore, we demonstrated that T-ALL stochastically infiltrates the BM microenvironment and remodels the endosteal niche by destroying osteoblastic cells (Hawkins *et al.*, 2016). On the other hand, in AML, the niche's role is modified based on the stage of disease and appears to be linked to disease pathogenesis in the pre-leukemic stage and it becomes permissive once blasts are established, finally transforming into a self-reinforcing structure at the later stages of disease (Lane *et al.*, 2011; Schepers *et al.*, 2013; Bernasconi & Borsani, 2019). Thus, it has been proposed that leukemic populations are able to remodel the niche environment in their favour while competing with steady state haematopoietic cells. The idea of a competition between HSCs and leukemic blasts was proposed for the first time by Colmone *et al.* when they showed that in Severe Combined Immunodeficiency Disease (SCID) mice both steady state and leukemic stem cells localise to a specific vascular niche enriched with CXCL12 (Sipkins *et al.*, 2005; Colmone *et al.*, 2008). Other studies have followed this idea revealing how increasing the dose of HSPCs transplanted together with AML cells prolongs animal survival and inhibits disease progression (Boyd & Bhatia, 2015; Glait-Santar *et al.*, 2015). These observations lead to the hypothesis that HSCs might in some cases outcompete AML for niche occupancy. Although it is now recognised that HSPCs are lost at the late disease stage (Duarte *et al.*, 2017; Akinduro *et al.*, 2018), questions remain about the effect of AML on HSPC dynamics as disease progresses. In this chapter, I explore using T-ALL as a model of competition between healthy and malignant haematopoiesis, and ultimately focus on AML and hypothesize that among all the leukemic blasts, some have distinct features that may be relevant for AML-HSPC competition. The combined use of IVM and FACS leads me to highlight that instead of being lost, HSPCs are displaced from the BM niche while leukaemia grows. Understanding the dynamics with which leukaemia impairs steady state haematopoiesis may point to novel therapeutic interventions aiming to eradicate AML by strengthening resident HSPCs in their niche.

3.2- Notch-T-ALL model establishment

Using a well-established murine model of Notch-driven T-ALL, my group had previously shown that despite being independent from the BM niche, T-ALL selectively remodels the endosteal space resulting in the total loss of mature osteoblasts, whereas perivascular cells are maintained (Hawkins *et al.*, 2016). Consequently, the disease profoundly shifts the homeostasis of the endogenous BM stroma with effects on HSC efficiency. Knowing the impact that T-ALL causes on the BM niche, I questioned how this could affect steady state haematopoiesis throughout the course of disease with the final aim to compare the effect of T-ALL on HSPC dynamics with that of AML. The Notch-driven T-ALL model is based on sub-lethally irradiated hosts and therefore presents some limitations for a study that aims to elucidate the interaction between leukaemia and steady state haematopoiesis.

It is known that irradiation causes a severe decline in bone volume, particularly in the trabecular region (Kondo *et al.*, 2009). At the same time, it has been shown that lethal total body irradiation (9 Gy) of adult mice results in profound alteration of the vasculature, including the disruption of columnar capillaries in the metaphysis, dilatation of sinusoidal capillaries in the diaphysis and expansion of the vascular area in bone (Chen *et al.*, 2019). Irradiation increases vasculature permeability and leads to an enhanced cell extravasation (Chen *et al.*, 2019)In addition, the extracellular matrix (ECM) within the bone is similarly destroyed (Gong *et al.*, 2013). Together with the damage to the niche, radiation exposure leads to a drastic reduction of haematopoietic populations (Batsivari *et al.*, 2020). As a result, the establishment of an experimental model that does not require host conditioning would be necessary in order to study an intact system in which blasts can compete with healthy and unperturbed cells. Thus, I planned to inject an increasing number of Notch blasts to evaluate whether T-ALL could grow in an irradiation-free host.

To test the capacity of T-ALL leukaemia to growth into irradiated-free mice, I used dsRed Notch T-ALL blasts previously generated in our laboratory. Specifically, foetal liver cells were harvested from WT embryos at day 14.5 (E14.5) and transduced with a MigR1 plasmid containing DsRed with NotchICNΔRamΔP. After that, 1 x 10^6 $DsRed^+$ foetal liver cells were transplanted into primary lethally irradiated recipient mice (two doses of 5.5Gy administered 3 hours apart). This step allowed the generation of several batches of DsRed primary blasts. Experiment were then performed using blasts from different batches to ensure identification of consistent features of T-ALL growth minimizing possible primary donor-specific phenotype.

In the conditioned model, 50,000 blasts are sufficient for T-ALL to develop in a well-defined manner, in time and space respectively (Hawkins *et al.*, 2016). Therefore, I used this number not only as a positive control in irradiated recipients, but as a starting point followed by the injection of an increasing number of blasts. In this experiment, a total of 12 mice were transplanted with varying numbers of $dsRed^+$ T-ALL blasts - 3 animals with 50,000 cells, 3 animals with 250,000 cells and 3 animals with 500,000 cells. The remaining 3 mice were sub-lethally irradiated (two doses of 3Gy, three hours apart) and injected with 50,000 $dsRed^+$ T-ALL blasts to be used as a positive control (Figure 3.1).

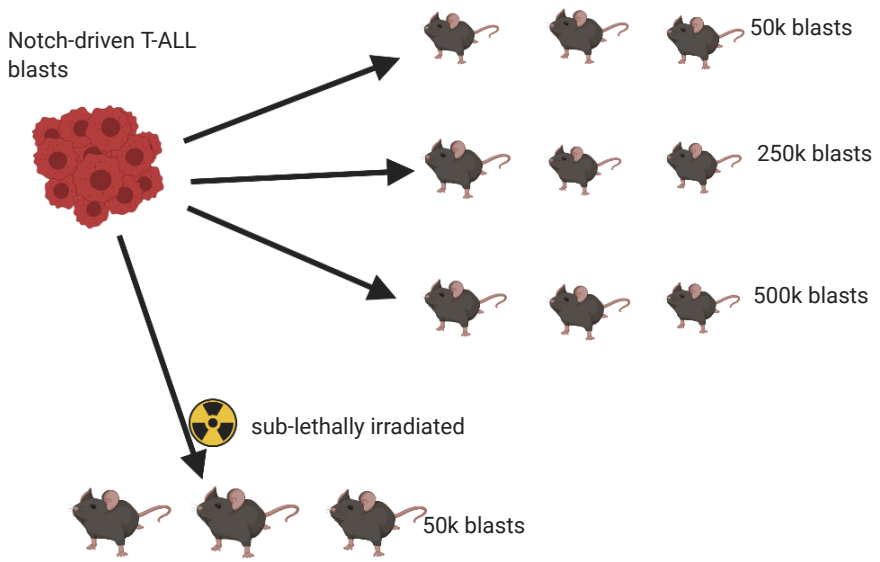

Figure 3.1-T-ALL titration experiment set up

Schematic representation of the Notch blasts titration. 12 C57BL/6 mice were injected with different numbers of dsRed^{+} Notch blasts. 3 mice were used as control, sub-lethally irradiated (two doses of 3 Gy 3 hours a part) and injected with the usual dose of blasts. The remaining animals were divided in groups of 3 and injected with 50,000, 250,000 and 500,000 cells respectively. Figure created using BioRender.com

Animals were monitored daily and T-ALL progression was evaluated by FACS analysis of peripheral blood (PB). These data revealed that, as expected, T-ALL grows consistently with a well-known pattern resulting in fully infiltrated BM by day 18 post-transplant in the sub-lethally irradiated group (Figure 3.2). However, the remaining groups showed large variability in disease development. Specifically, the group injected with 250,000 cells was more uniform in terms of BM infiltration although full infiltration was reached only 30 days after transplant compared to day 18-20 as usually observed (Hawkins *et al.*, 2016).

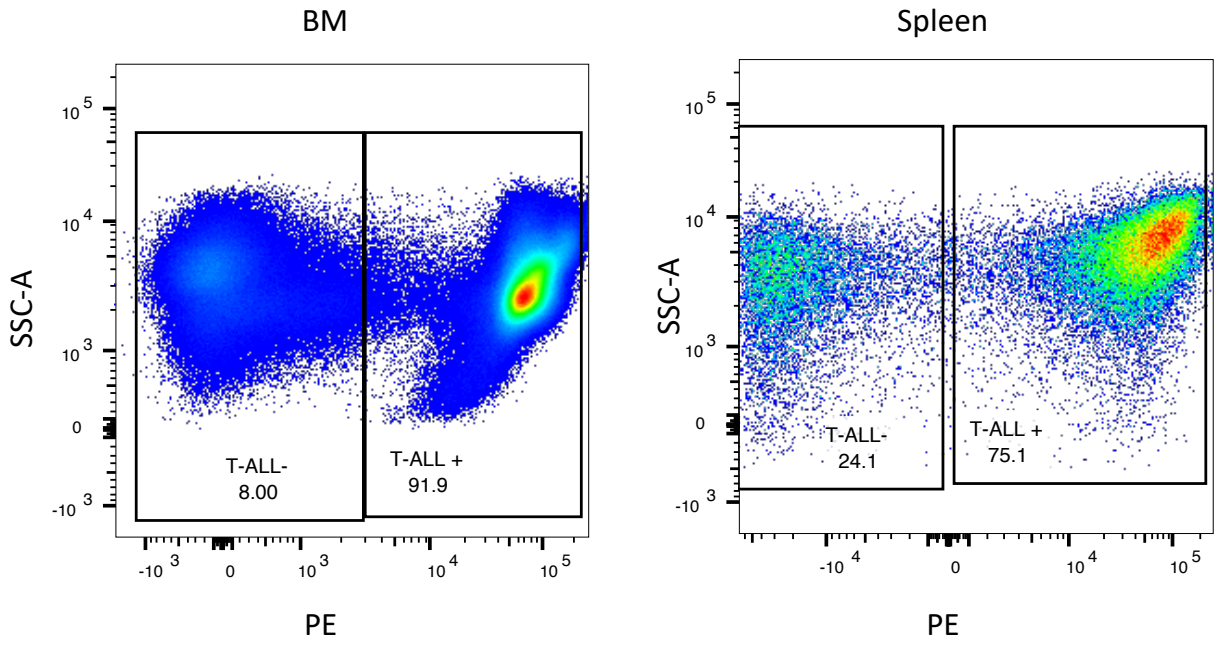

Figure 3.2-T-ALL engraftment in sub-lethally mice

Sub-lethally irradiated mice were fully infiltrated by day 18 post injection. Representative flow cytometry data show the BM and spleen fully colonised by dsRed^{+} blasts

After several other attempts at establishing this conditioning-free model, I concluded that despite the fact that T-ALL does indeed develop in non-conditioned mice (Figure 3.3), the variability seen was unpredictable. In order to produce a reliable experimental cohort, mice should present a comparable percentage of infiltration. This would allow the generation of statistically viable data from IVM and flow cytometry experiments. Instead, mice injected with Notch blasts became infiltrated at varying and unpredictable times, making this model too unreliable to work with.

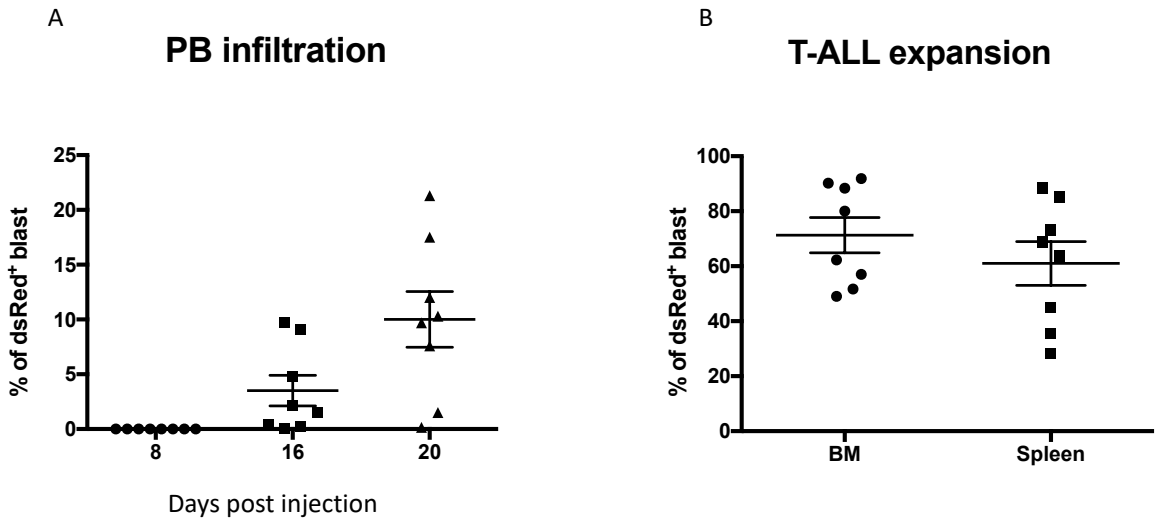

Figure 3.3-T-ALL growth in non-irradiated mice

Data shows T-ALL progression in non-irradiated mice. Mice injected with 250,000 cells showed a remarkable degree of variability in PB infiltration **(A)** as well as in BM and spleen infiltration **(B)**. Data are pooled from 2 independent experiments. Each dot represents a mouse and for each mouse the % of BM and spleen infiltration is indicated. Error bars represent mean ± s.e.m.

Therefore, even if showing that T-ALL can grow in a conditioning-free host, I decided to fully focus on AML and its interaction with hematopoietic cells.

3.3- AML model establishment

In order to investigate the effect of AML on HSPCs as disease propagates through the tissues, 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 (Krivtsov *et al.*, 2006; Somervaille & Cleary, 2006). To be able to visualise AML blasts by IVM, I used AML blasts previously generated in our laboratory. Specifically, myeloid progenitor cells from donor mice that expressed high levels of fluorescent proteins (YFP) were transduced with a retroviral vector encoding for the MLL-AF9 oncogene and GFP. After that, transduced cells were injected into sub-lethally irradiated recipient mice. This step allowed the generation of several batches of GFP^+ YFP^+ primary blasts. Experiments were then performed using blasts

from different batches to ensure identification of consistent features of AML growth minimizing possible primary donor-specific phenotype. To reach reliable disease development and progression 100,000 primary blasts were transplanted by tail vein intravenous injection into non-irradiated secondary recipients (Figure 3.4 A). In some experiments, secondary and tertiary blasts were also used to generate AML in non-irradiated mice. The use of non-conditioned recipients was critical to analyse an intact BM environment to study AML cell interaction with an unperturbed hematopoietic system. In all recipients, disease progression was monitored by evaluating the amount of blasts in the PB, which positively correlates with BM infiltration (Figure 3.4 B). At the same time, leukemic blasts start to be observed in the

BM from day 10-12 post-transplantation with full BM infiltration typically reached between day 20 and 28, with some variation depending on the source of primary blasts (Figure 3.4 C).

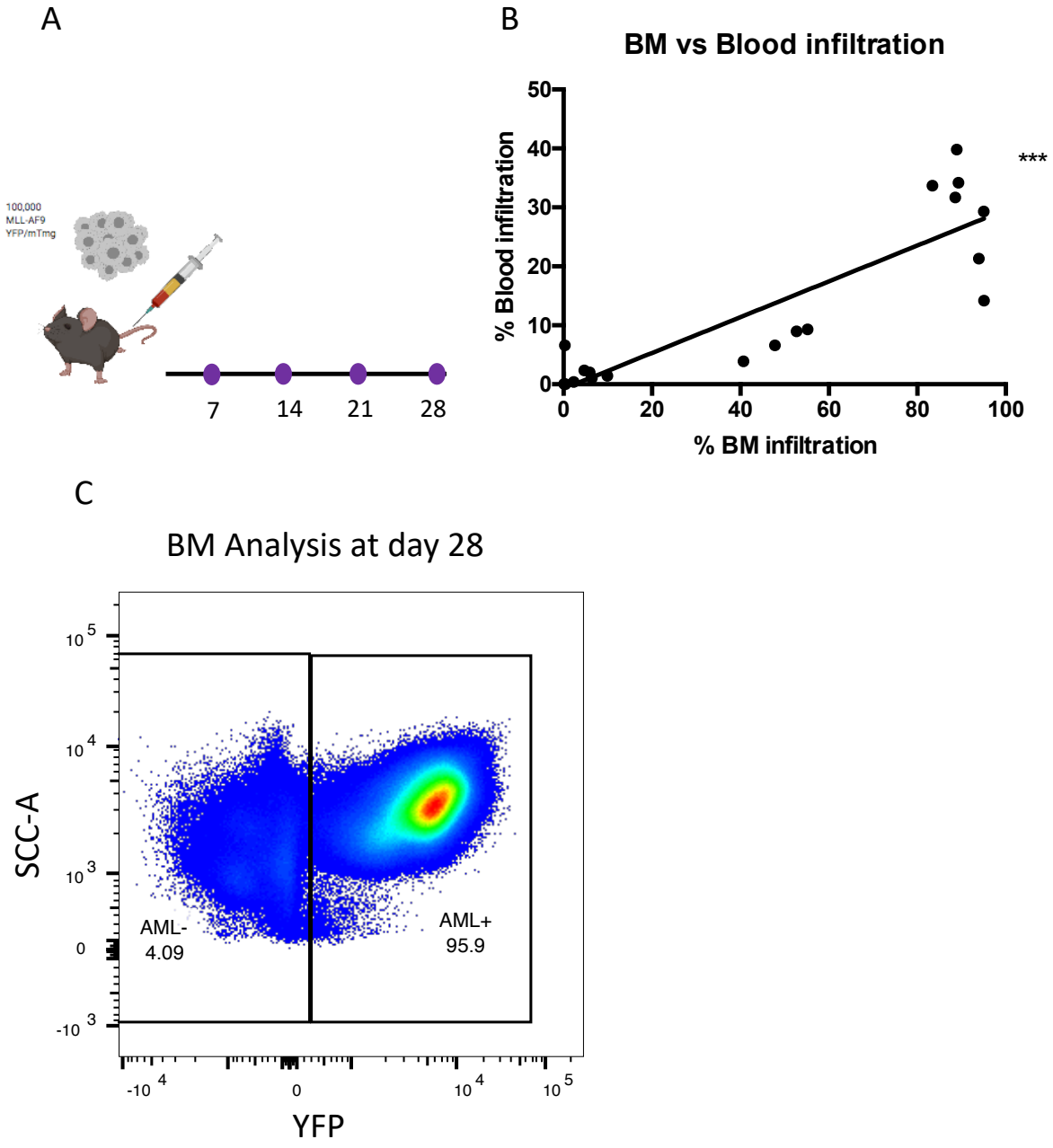

Figure 3.4-AML progression in experimental model

(A) Mice were injected with 100,000 YFP or mTmG AML cells, and disease progression was followed by checking peripheral blood infiltration. Figure created using BioRender.com. **(B)** The graph shows the positive correlation between AML blasts in PB and BM. **(C)** FACS plot shows leukaemic blasts (YFP^+) in the Bone marrow. Statistical significance obtained using linear regression (*** represents $p<0.001$)

At the same time, I was interested in understanding how AML expands outside the medullary parenchyma and I decided to evaluate blasts infiltration within the spleen and liver. Thus, I compared organ infiltration at medium (15-18 days post-injection) and late (20-26 days post-injection) AML time points. Data analysis indicated that at medium AML time point, the spleen gets infiltrated with a similar dynamic in comparison to the marrow while the liver appeared less infiltrated. Interestingly, at late AML stage, the percentage of blasts in the BM was significantly higher than that found in both liver and spleen. (Figure 3.5).

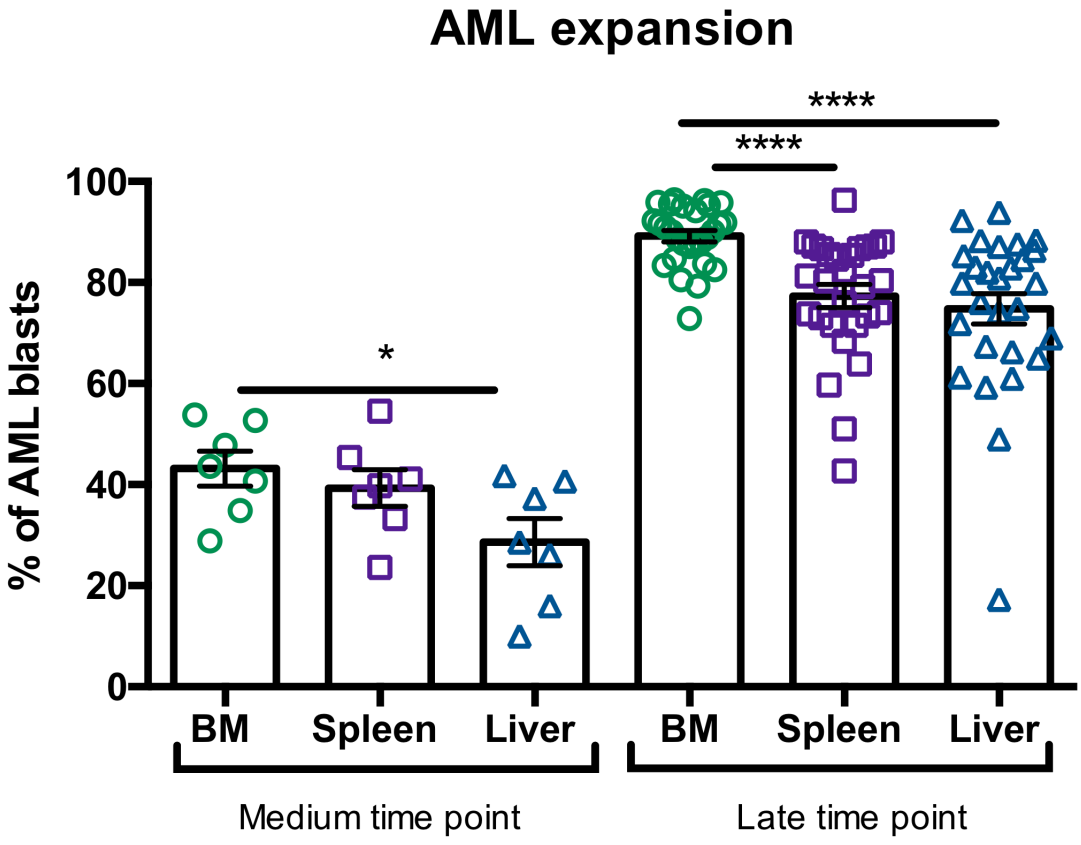

Figure 3.5-AML growth in peripheral blood, bone marrow, spleen and liver

Data show the percentage of blasts in BM, spleen and liver at medium and late AML time points. Data are pooled from 2 independent experiments and each dot represents a mouse, 35 mice in total. For each mouse multiorgan infiltration is showed. Error bars represent mean ± s.e.m. Statistical significance determined using Student's t-Test (* represents $p<0.05$; **** represents $p<0.0001$)

3.4- Intravital imaging of AML in the BM microenvironment

To get a deep insight into AML blast/healthy cell interactions and understand how the disease affects haematopoiesis, I performed intravital microscopy (IVM) analyses alongside flow cytometry experiments.

YFP^+ or mT/mG^+ AML blasts, together with healthy haematopoietic cells and BM vasculature, were visualised using IVM of mouse calvarium BM (Figure 3.6). The calvarium BM has been shown to be representative of long bones for both the niche/stroma components present within it and for its ability to support functional HSCs (Lo Celso *et al.*, 2008; Lassailly *et al.*, 2013). Specifically, tile scan images of the BM provide a comprehensive, three-dimensional single-cell resolution of the complex organization within the tissue representing an ideal tool to uncover processes that are still poorly understood. Furthermore, this approach can be extended to other organs allowing the detection of blasts outside the BM (e.g. spleen). Therefore, by combining IVM and flow cytometry it is possible to evaluate cell dynamics *in vivo* during AML progression.

While previous work aimed to demonstrate that there is a loss of haematopoietic cells from the BM using techniques such as flow cytometry (Akinduro *et al.*, 2018)I wanted to investigate whether it would be possible to uncover this mechanism *in vivo* and in real time using IVM. To do this, I needed a model in which I could clearly visualise haematopoietic cells and their interactions with the leukemic blasts. By transplanting whole BM from mT/mG donors into lethally irradiated WT recipients, I could generate chimeric mice in which the stroma was "dark" and I could analyse haematopoietic cells in red. After allowing these mice to reach steady state (in order to remove any effect due to irradiation) they could be transplanted with

YFP$^+$ AML blasts. Thus, I could perform IVM and follow blasts-steady state hematopoietic cells interaction at various disease stages.

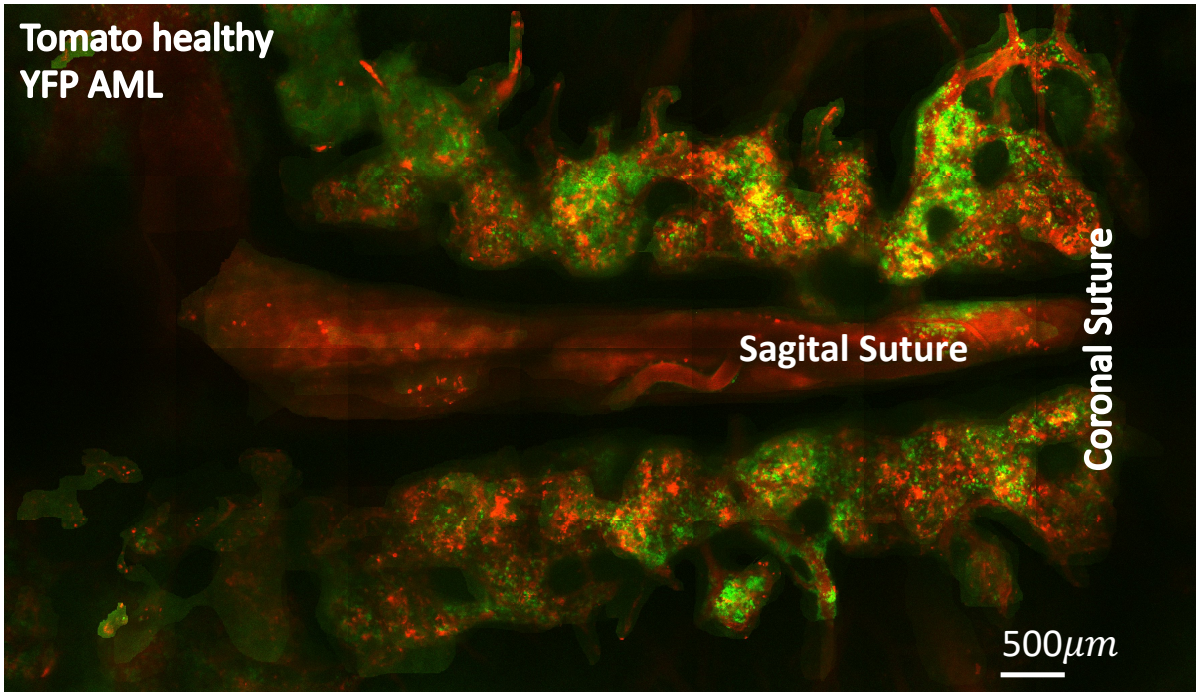

Figure 3.6-Intravital imaging of AML and healthy haematopoietic cells in the bone marrow niche

Maximum projection of IVM tile scan image showing AML cells (YFP$^+$; green) mixed with non-malignant haematopoietic cells (mTomato$^+$; red) throughout the BM space

3.5- AML blasts demonstrate highly heterogenous behaviours

To have a better chance to capture the initial stage of blasts/healthy counterpart interactions I performed IVM at various stages of disease development. Using high-resolution time lapse imaging I observed, among the AML cells, the presence of blasts, called "Pioneer blasts" from now on, characterised by distinct features when compared with the others. Comparing their morphology, I noticed that the majority of leukemic cells presented a round shape whereas

these pioneer blasts were able to form long protrusions in various directions (Figure 3.7, Supplementary Video 1). Most importantly, pioneer blasts seemed to 'touch' the healthy counterparts more than the non-pioneer cells. These observations led me to think that these cells may have a different role during AML development in comparison with the majority of other leukemic cells. The migratory capacity is a characteristic of all blood malignancies and the fact that some cells move differentially from others could have implications for disease growth. Despite moving, the majority of leukemic cells still maintain a rounded shape making these pioneer blasts easily identifiable. In fact, as it emerged from the IVM, the pioneer cells could be easily recognised by their long protrusions that expand in various directions.

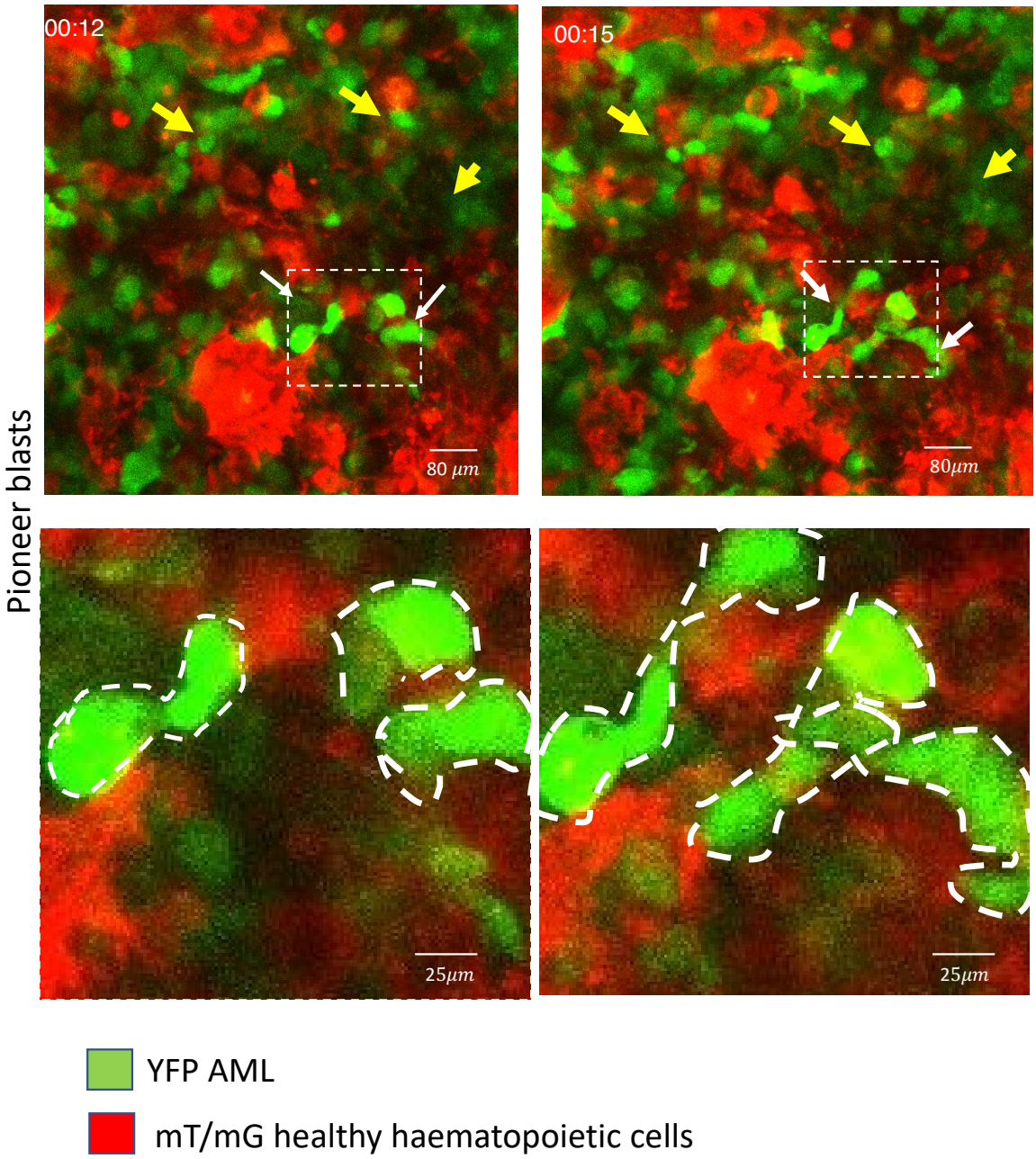

Figure 3.7-AML blasts are morphologically heterogeneous

Selected time points from representative time lapse data from a medium infiltrated chimeric mouse. Non-pioneer, bulk cells are highlighted with the yellow arrows and pioneer ones are within the white square and arrows. Bottom panels represent a higher magnification of the area containing pioneer cells at the two selected time points

Therefore, I decided to question how different the migratory behaviours of these pioneering blasts were and decided to use well-established image analysis techniques to investigate

characteristics including track length (Figure 3.8 A), total displacement (distance between the first and last cell's position, Figure 3.8 B), average speed (track length/track duration, Figure 3.8 C) and linear progression (total displacement /track length, Figure 3.8 D). This analysis highlighted that the pioneer blasts moved more than the other blasts. By measuring the track length of leukemic blasts, I discovered that pioneering blasts covered significantly more distance within the marrow when compared with non-pioneering ones. Moreover, pioneering blasts had significantly higher total displacement, average speed and linear progression, indicating they move more and have more directionally than non-pioneer blasts.

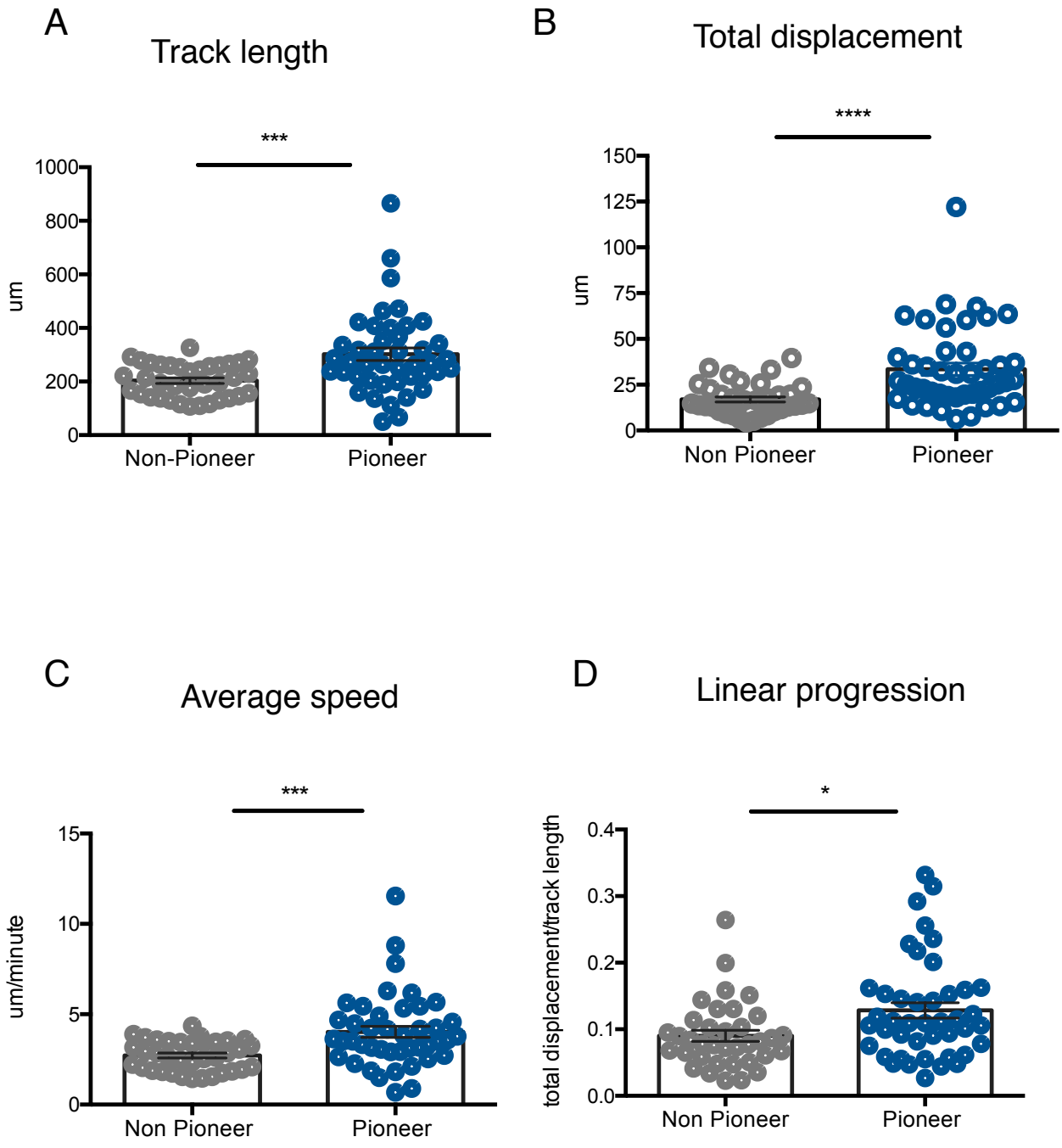

Figure 3.8-Pioneer blasts show distinct features

Pioneer blasts significantly differ from bulk AML cells in their track length **(A),** total displacement **(B)**, average speed **(C),** and linear progression **(D)**. IVM was performed and individual seeding cells were tracked for a total of 102 minutes. All graphs show the mean speed of single AML blasts located in the BM parenchyma. Each dot is a tracked cell. Data obtained from 3 mice from two independent experiments. Plotted data represent semi-automatic tracking of 68 seeding pioneer and 60 non-pioneer AML cells. Error bars represent mean ± s.e.m. Statistical significance determined using Student's t-Test (* represents $p<0.05$; *** represents $p<0.001$; **** represents $p<0.0001$)

Furthermore, these distinct blasts were mostly present at early and medium infiltration (BM< 50%) time points when healthy cells could still be detected in the BM space. Leukemic cells move fast at the early stage of disease development when they still exist as single cells and migrate within the marrow.

It has been shown that AML cells have a specific ECM signature that can also promote their migratory behaviour (Izzi *et al.*, 2017). Taking all of this into consideration, I hypothesized that pioneer blasts, thanks to their protrusions and increased migratory capacity, may be the first cells responsible for interacting with and "enveloping" healthy cells to then displace them and make space for more blasts within the BM. I decided to re-analyse the time lapse movies and specifically focus on the individual behaviours of these cells. In support of my hypothesis, I observed that the pioneer blasts were not only migrating quickly, but also often infiltrating patches of healthy cells and assuming morphologies that would encircle healthy cells (Figure 3.9). Images of low and medium infiltrated mice were consistent with previous findings that revealed AML cells form clusters in highly infiltrated areas whereas sparse AML cells were found in the remaining parts of the BM (Hérault *et al.*, 2017).

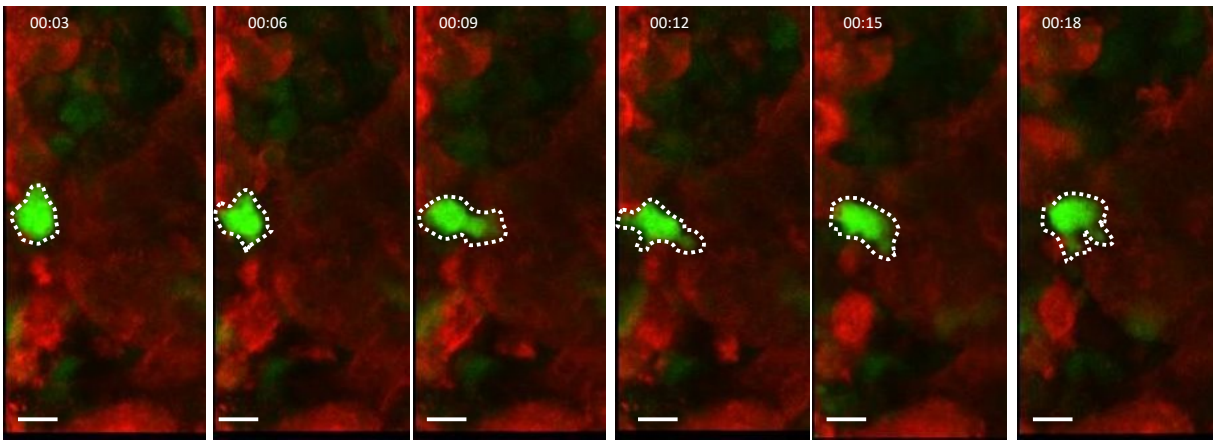

Figure 3.9-Pioneer cell enveloping healthy haematopoietic cells

Representative maximum projections of a selected time lapse. The position was selected together with others and time lapse imaged every 3 minutes for a total of 102 minutes. White dotted line highlights a pioneer cell making dynamic protrusions and contacts with healthy BM cells throughout the IVM session. Scale bar represents 80µm

3.6- Intravital imaging reveals healthy haematopoietic cells egressing from the BM niche

The BM niche represents a well-defined microenvironment in which HSPCs normally reside. However, this fine equilibrium is strongly affected by leukaemia growth. The hypothesis of competition between growing leukaemia and healthy haematopoiesis has been proposed several years ago, when it was shown that AML clusters and HSCs were localised in the same endothelial niche (Ishikawa *et al.*, 2007).

Furthermore, our research group has shown that in AML, the loss of healthy cells was consistent with neutral ousting from BM by leukaemia irrespective of cell type and cell cycle status. In this context, the HSCs expressing the lowest levels of CD48 represent an exception, being the most resistant to elimination from the BM (Akinduro *et al.*, 2018). In line with this, I questioned the possibility of visualising the ousting process in mice burdened with AML. Keeping in mind the hypothesis of pioneer cells making space for the increasing AML population, I performed IVM at various stages of leukaemia infiltration and I decided to use

the Flk1-GFP mice in which I could clearly visualise the $CD45^{-}Ter119^{-}CD31^{+}$ phenotypic ECs (Duarte *et al.*, 2017) I lethally-irradiated and transplanted these mice with whole BM from mTmG donor mice. After 8 weeks the chimeric mice were injected with YFP^{+} AML blasts, allowing the visualization of blasts (dim YFP), healthy cells (tomato) and ECs (bright GFP) within the marrow. Although, in Flk1-GFP reporter mice the ECs are labelled in GFP, this does not represent a limit for the use of YFP AML as it is easy to distinguish between the morphology of these two cell populations. High-resolution time lapse imaging allowed me to detect healthy cells intravasating in bulk into the circulation (Figure 3.10, Supplementary Video 2).

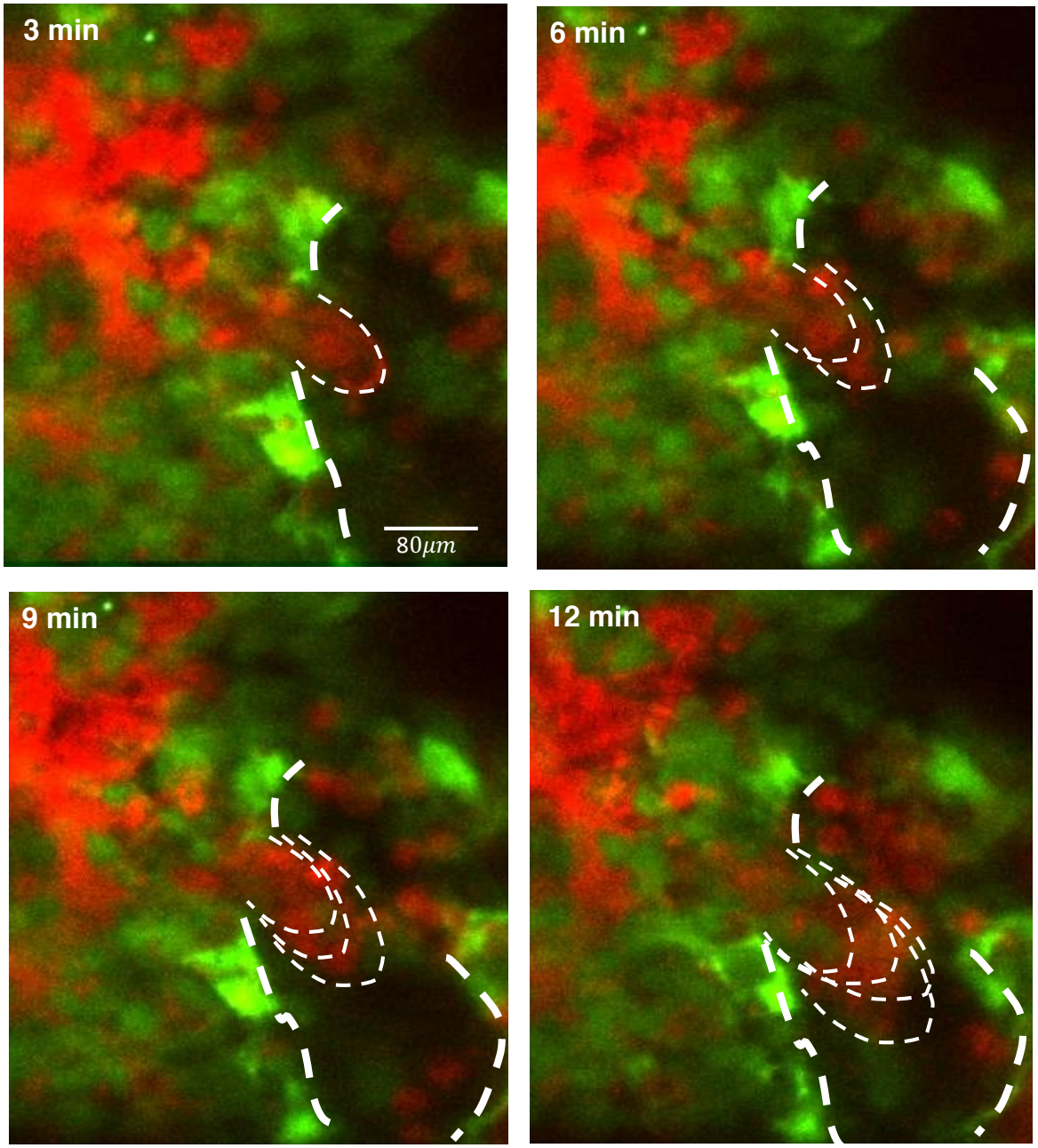

Figure 3.10-AML displaces healthy haematopoiesis

Selected frames from a representative time lapse IVM of calvarium BM in AML-burdened Flk1-GFP mice, showing endothelial cells (GFP^+, brightest green, thick dotted lines highlight vessel inner edge), YFP^+ AML cells (dimmer green), and $mTomato^+$ healthy haematopoietic cells (red) egressing from the BM as a cluster (thin dotted lines show cluster leading edge protruding into the vessel over time)

As expected, patches of residual healthy haematopoietic cells were surrounded by leukemic blasts that were organised in clusters at this stage of disease. Surprisingly, high-resolution time lapse imaging of low infiltrated areas did not show the presence of big clusters of extravasating

healthy cells. This last observation fits with the idea that steady state haematopoietic cells were forced to egress from the BM parenchyma due to the pressure of growing leukaemia. At early infiltration time points, only a few sparse leukemic blasts are seen in the BM cavity and therefore, the available space is enough for both populations. However, as soon as leukaemia grows, the competition for this limited space and resources heavily increases leading to the ultimate loss of the weakest one (Figure 3.11).

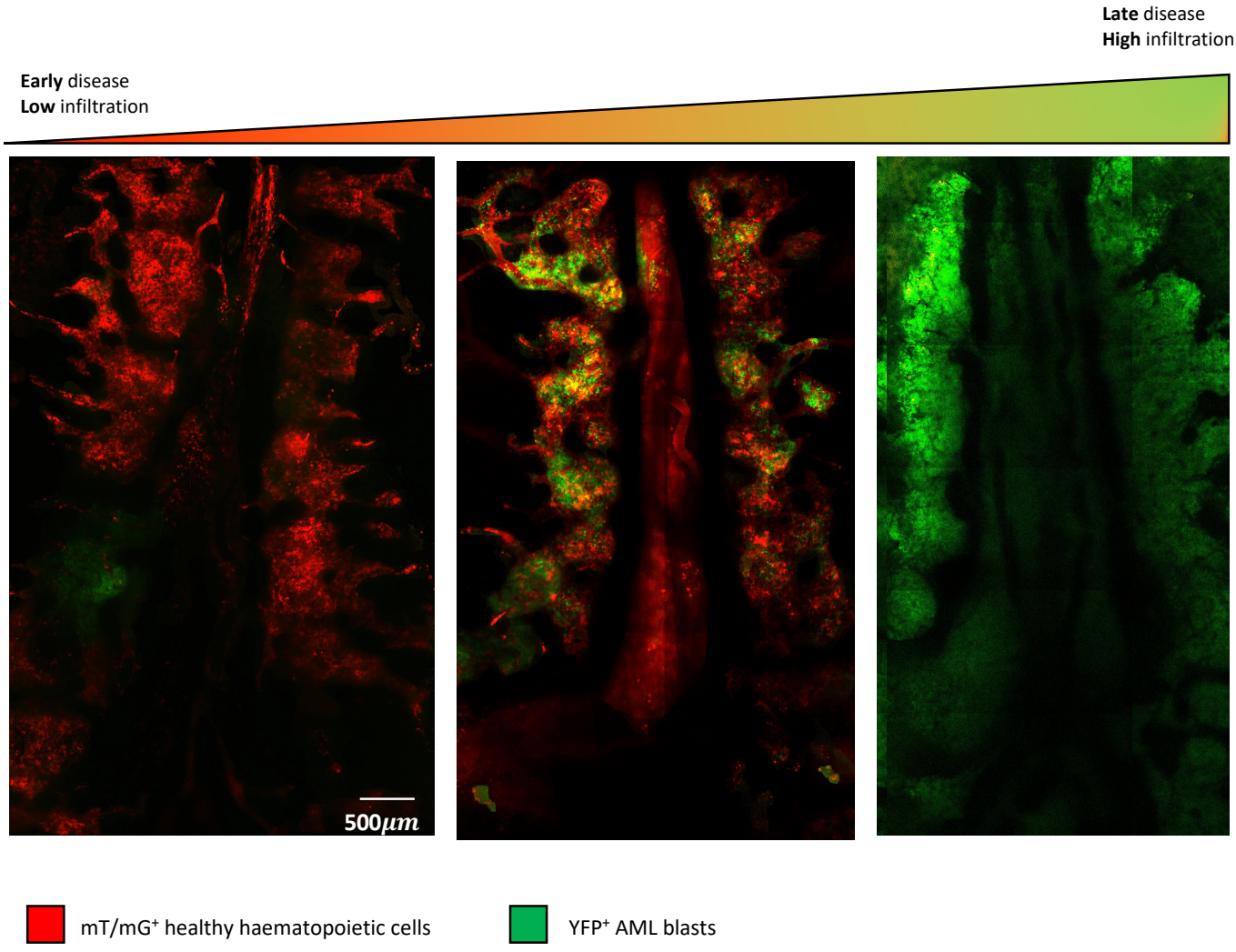

Figure 3.11-AML invasion of BM parenchyma lead to a progressive loss of steady state hematopoietic cells

Representative tile scan of BM calvarium at early, medium and late infiltration stage. At early infiltration, AML blast (YFP) are confined in a small area within the marrow surrounded by the healthy counterpart. However, as soon as disease grows, blasts progressively occupy the BM marrow niche leading to the loss of healthy haematopoietic cells

In order to test the hypothesis that the presence of extravasating cells in bulk were due to AML, I analysed healthy cells circulating in control Flk1-GFP mice (Figure 3.12A) and compared the number and size of cluster of healthy cells with the ones in leukemic mice (Figure 3.12 B).

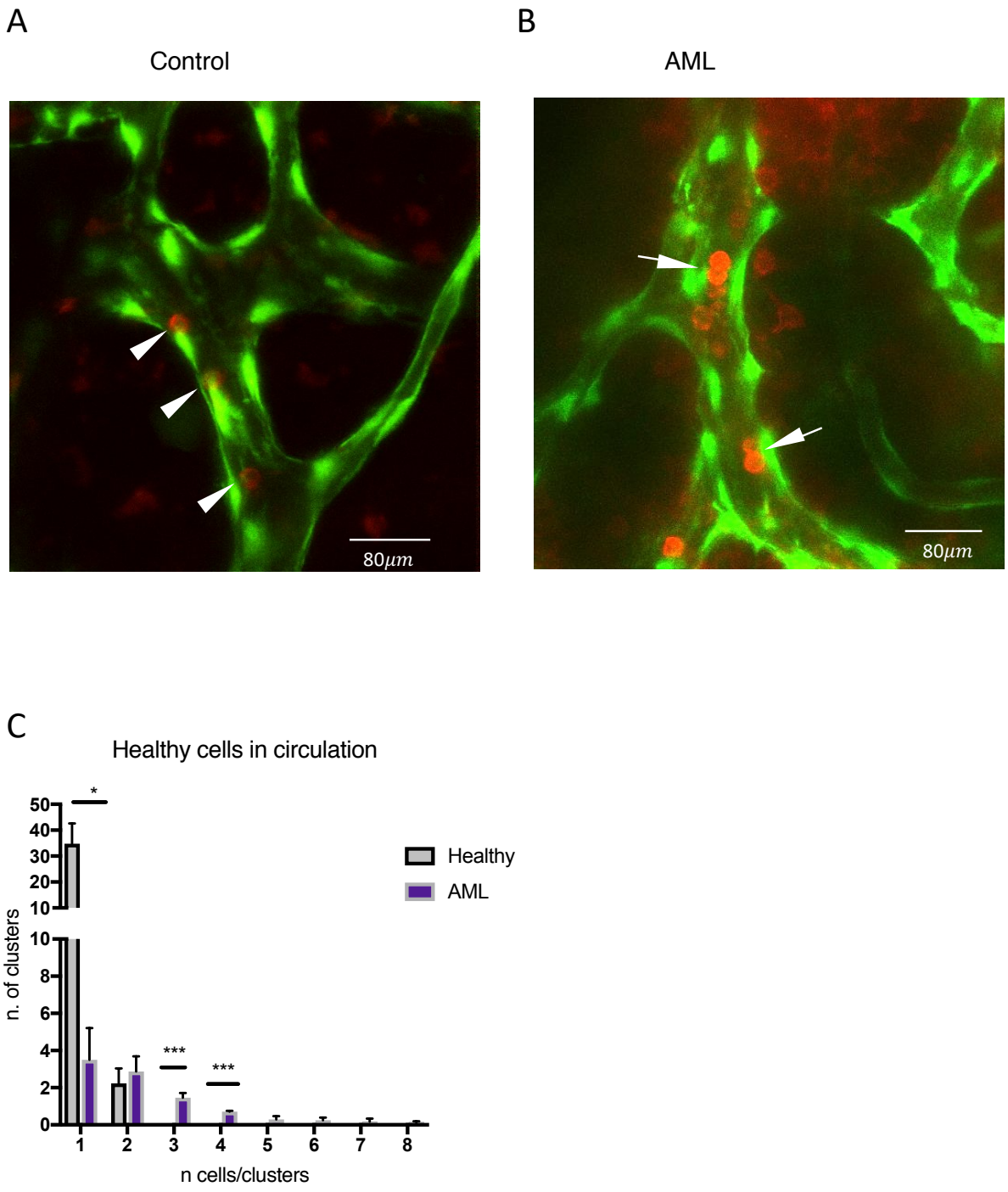

Figure 3.12-Healthy cells intravasation differ in Control vs AML

Representative maximum projection of a single 3D time frame from the time lapse of selected positions within the BM of Flk1-GFP mice. Healthy cells are found in circulation mostly as single cells in control mice **(A)** while in leukemic mice they leave the marrow in clusters of two and three cells **(B).** Single cells and clusters were manually counted using ImageJ cell count. When comparing numbers, the circulating cells are significantly different in its nature between control and leukemic mice (**C).** Data obtained from 5 control and 3 leukemic mice from two independent experiments. Errors bars represent mean± s.e.m. Statistical significance obtained using multiple t test. (* represent $p< 0.05$; *** represent $p<0.001$)

This time, IVM was performed on Flk1-GFP mice and healthy cells were labelled with PE-conjugated CD45.2 antibodies (Figure 3.12). High-resolution time lapses imaging showed that in control mice, the majority of healthy cells in circulation were single or in clusters of two whereas in AML burdened mice clusters of up to 8 cells were detected. Data analysis confirmed that there is a significant difference in terms of cluster size among the nature of cells egressing the marrow between control and leukemic mice (Figure 3.12 C). These observations led me to hypothesize that leukemic blasts may operate a certain level of extracellular matrix degradation, forcing the healthy cells to egress in groups (Chapter 5).

3.7- AML cells displace steady state haematopoiesis

The IVM data that I have presented so far suggests a progressive displacement of healthy haematopoietic cells during AML growth. The observation of clusters of healthy cells entering into the circulation raised the question whether these could be identified by other means. Therefore, I used flow cytometry to test whether I would detect an increased number of HSPCs into the blood as BM blasts infiltration progressed (Figure 3.13 A).

As hypothesised, the overall undifferentiated (Lineage$^-$) hematopoietic cell population as well as the LKS compartment expanded in the blood over the course of disease, especially at late time points (Figure 3.13 B). Beads-based counting indicated that the number of lineage$^-$ cells increased in the blood in parallel to the increase in the blasts' percentage within the marrow (Figure 3.13 C). I then focused on the stem and progenitor compartment where I observed a

similar trend for the LKS CD48^{-} CD150^{+} (LT-HSCs) population, the number of which starts to increase in the blood when blasts exceed 40% infiltration in the marrow (Figure 3.13 D).

Similar results were obtained for the LKS CD48^{-} CD150^{-} (ST-HSCs) and LKS CD48^{+} CD150^{+} (MPP3-4) (Figure 3.13 E and 3.13 F respectively). Thus, in line with the IVM results, flow cytometry data confirmed that HSPCs are detected in circulation when leukemic blasts occupy at least 40% of the BM microenvironment.

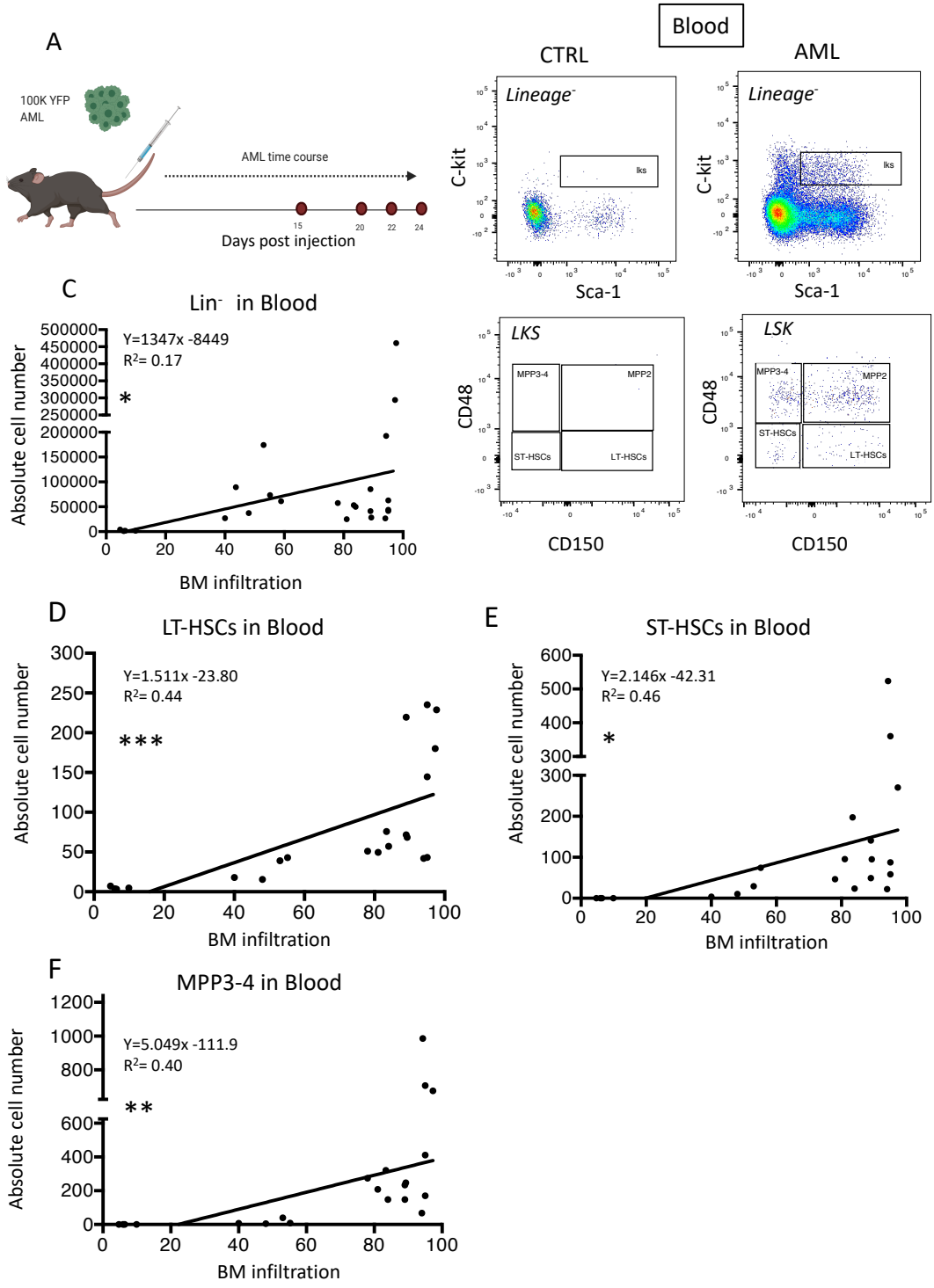

Figure 3.13-LKS population increase in the blood due to AML pressure in the marrow

(A) Experiment set up. 20 C57BL/6 mice were injected with 100,000 MLL-AF9 AML blasts. Blood was sampled at day 15, 20, 22 and 24 by cardiac puncture and BM analysed on the same days. Figure created using Biorender.com **(B)** Gating strategy, highlighting the burst in the LKS population in leukemia burdened mice compared with the control. **(C) (D)(E) (F)** show a positive correlation between the absolute number of **(C)** undifferentiated Lin^-, **(D)** LT-HSCs (LKS $CD48^-$ $CD150^+$), **(E)** ST-HSCs ($CD48^-$ $CD150^-$) and **(F)** MPP3-4 ($CD48^+$ $CD150^-$) detected in the blood, relative to BM infiltration. Each dot represents a mouse. Statistical significance determined using linear regression (* represents $p<0.05$; ** represents $p<0.01$; *** represents $p<0.001$)

To confirm that the increased number of the HSPCs detected in the blood was a direct consequence of their decreased number in the niche, I analysed HSPC dynamics within the marrow of leukemic mice at various leukaemia stages. As expected, at late AML infiltration (day 24) the bones of leukemic mice appeared white, confirming the well documented cytopenia that ensues with disease development (Figure 3.14 A). At this point, BM was fully infiltrated and leukemic cells were found in extramedullary compartments such as spleen and liver (Figure 3.14 B).

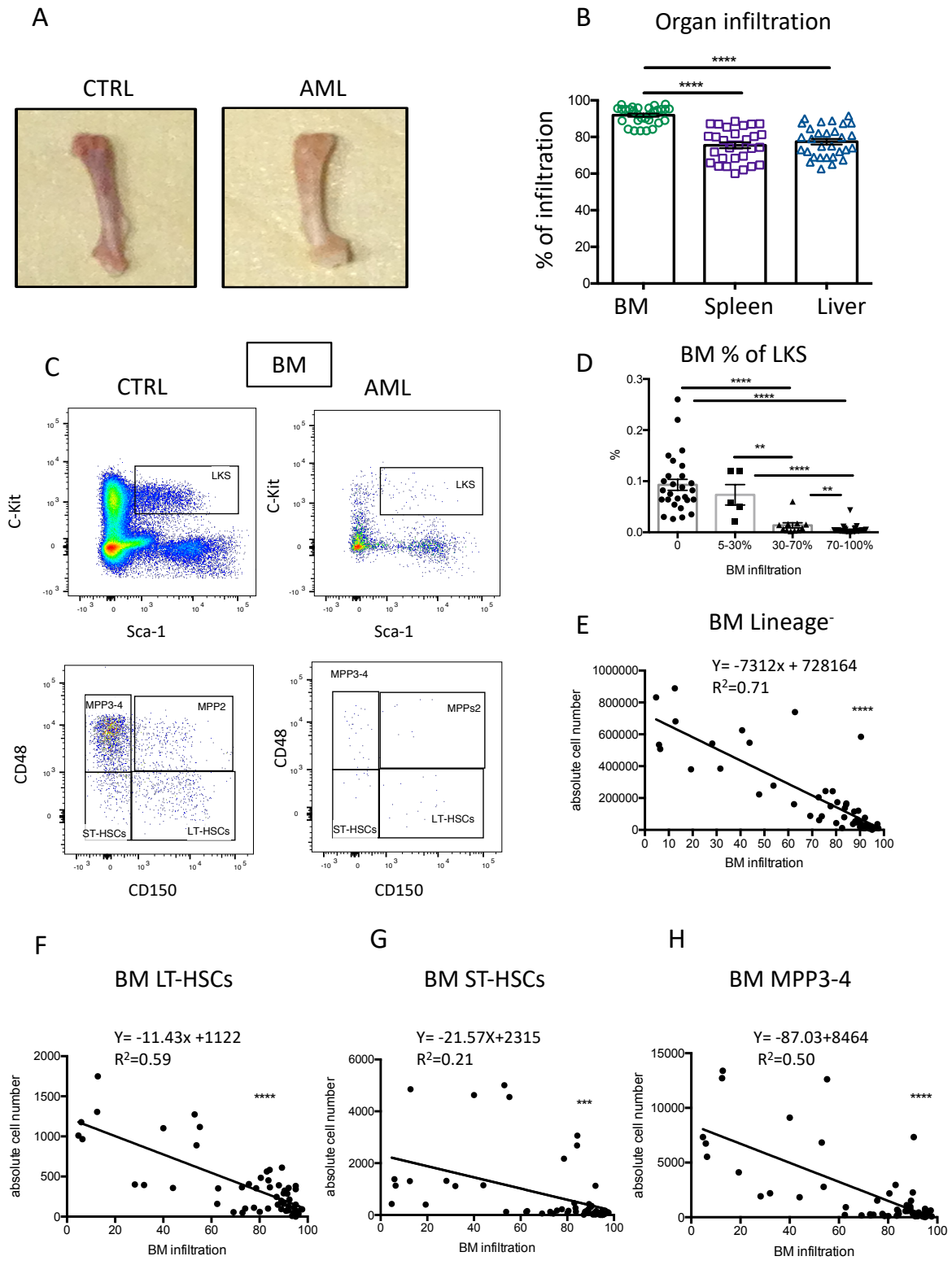

Figure 3.14-AML disrupts HSPC populations within the bone marrow

(A) Loss of healthy haematopoiesis is reflected in bones being mostly white in leukemic mice compared to control ones at late time points. A pair of representative bones from one AML and one healthy control mouse from the day 24 of AML time point is shown. **(B).** Confirming previous observations, AML expands in the extramedullary compartment, although spleen and liver are infiltrated slightly later and less compared to the bone marrow. Each dot is a mouse and from each mouse the percentage of blasts in BM, spleen and liver is showed. Data represent 30 mice in total from three independent experiments.

(C). Flow cytometry analysis of bone marrow shows a drastic reduction of the LKS population within the undifferentiated cells, including a decrease in the LKS SLAM stem and progenitor cells. **(D).** Analysis based on BM infiltration revealed a progressive reduction of the percentage of LKS fraction within the marrow. 77 mice from 6 independent experiments are shown in total, of which 27 controls, 6 early infiltrated (5-30%), 11 medium infiltrated (30-70%) and 33 at later infiltration time point (70-100%). Error bars represent the mean± s.e.m. Statistical significance determined using student t test (* represent $p<0.05$; ** represent $p<0.01$, *** represent $p<0.001$). **(E-H).** The number of residual lineage negative cells **(E)**, LKS $CD48^{-}CD150^{+}$ LT-HSCs **(F)**, LKS $CD48^{-}CD150^{-}$ ST-HSCs **(G)**, LKS $CD48^{-}D150^{+}$ MPP3-4**(H)** negatively correlate with the percentage of blasts in BM. Each dot represents a mouse data obtained from 6 independent experiments. Statistical significance examined using linear regression (** represent $p<0.01$; *** represent $p<0.001$)

Noticeable changes developed over time and were most prominent at late time points, with a notable decrease in the LKS population within the undifferentiated ($lineage^{-}$) haematopoietic cells (Figure 3.14 C). This was also confirmed by the overall percentage of LKS decreasing within the marrow through disease progression (Figure 3.14 D). Thus, these data were consistent with previous observations (Duarte *et al.*, 2017; Akinduro *et al.*, 2018), and point to the decrease in number of haematopoietic cells under increased AML growing pressure. In line with a model in which the BM space is progressively occupied by leukemic blasts, $lineage^{-}$ hematopoietic cells are slowly forced to leave their natural niche. Consistent with the data from the blood in which increased $lineage^{-}$ population could be detected for AML-burdened mice (BM infiltration >40%), I observed that the number of $lineage^{-}$ healthy haematopoietic cells was dramatically decreased in fully infiltrated BM (Figure 3.14 E).

Following these results, I wanted to understand whether the more primitive cells were also pushed out from the BM niche and if they followed a similar dynamic to that of $lineage^{-}$ cells. Therefore, I focussed my analysis on the LKS compartment, including the LT-HSCs and the progenitors ST-HSCs and MPPs. Data analysis revealed a similar trend for each population, with the (LT-HSCs) that seemed to be the most resilient ones (Figure 3.14 F, G and H). Specifically, for each of the above populations, the decrease in their number negatively and significantly correlated with the increase in BM infiltration. Therefore, this result corroborates the hypothesis that HSPCs are gradually lost while AML blasts increase in the marrow. This

finding is in line whit our recent work in which we have proposed that myeloid leukaemia ousts healthy cells in a neutral way, with the exception of the LT-HSCs expressing the lowest levels of CD48 (Akinduro *et al.*, 2018). Specifically, we showed that within the LKS compartment, there is a very rare population of cells that could be differentiated from the rest due to their very low expression of CD48 marker together with their significantly low proliferation rate. These cells, referred as $CD48^{neg}$ HSCs, represent a more quiescent population with significantly better engrafting capacity when compared to the $CD48^{low}$ HSCs (Figure 3.15 A). Therefore, I decided to investigate the dynamics of these two populations during AML progression and I observed that these are differentially impacted by leukaemia development.

Flow cytometry data revealed that the decrease in number of $CD48^{low}$ LT-HSCs significantly and negatively correlates with the increased percentage of blasts in the marrow (Figure 3.15 B). However, the impact on and the decrease of $CD48^{neg}$ LT-HSCs appeared different from the $CD48^{low}$. In fact, despite the overall impact that leukaemia has on the $CD48^{neg}$ LT-HSCs, their reduction seems less drastic in comparison with the CD48 ^{low} ones (Figure 3.15 C). To present this more clearly, I replotted the data and directly compared the number of $CD48^{neg}$ versus $CD48^{low}$ LT-HSCs. For both groups, mice with a similar range of BM infiltration were pooled and compared also with control mice (Figure 3.15 D). When compared to a steady state situation, it emerged that the $CD48^{low}$ LT-HSCs are more dramatically reduced during AML progression. Despite the number of $CD48^{neg}$ decreasing, this happens with a much slower dynamic. These data confirmed what was previously suggested, highlighting how the $CD48^{neg}$ LT-HSCs are the last cells to be displaced by leukemic blasts.

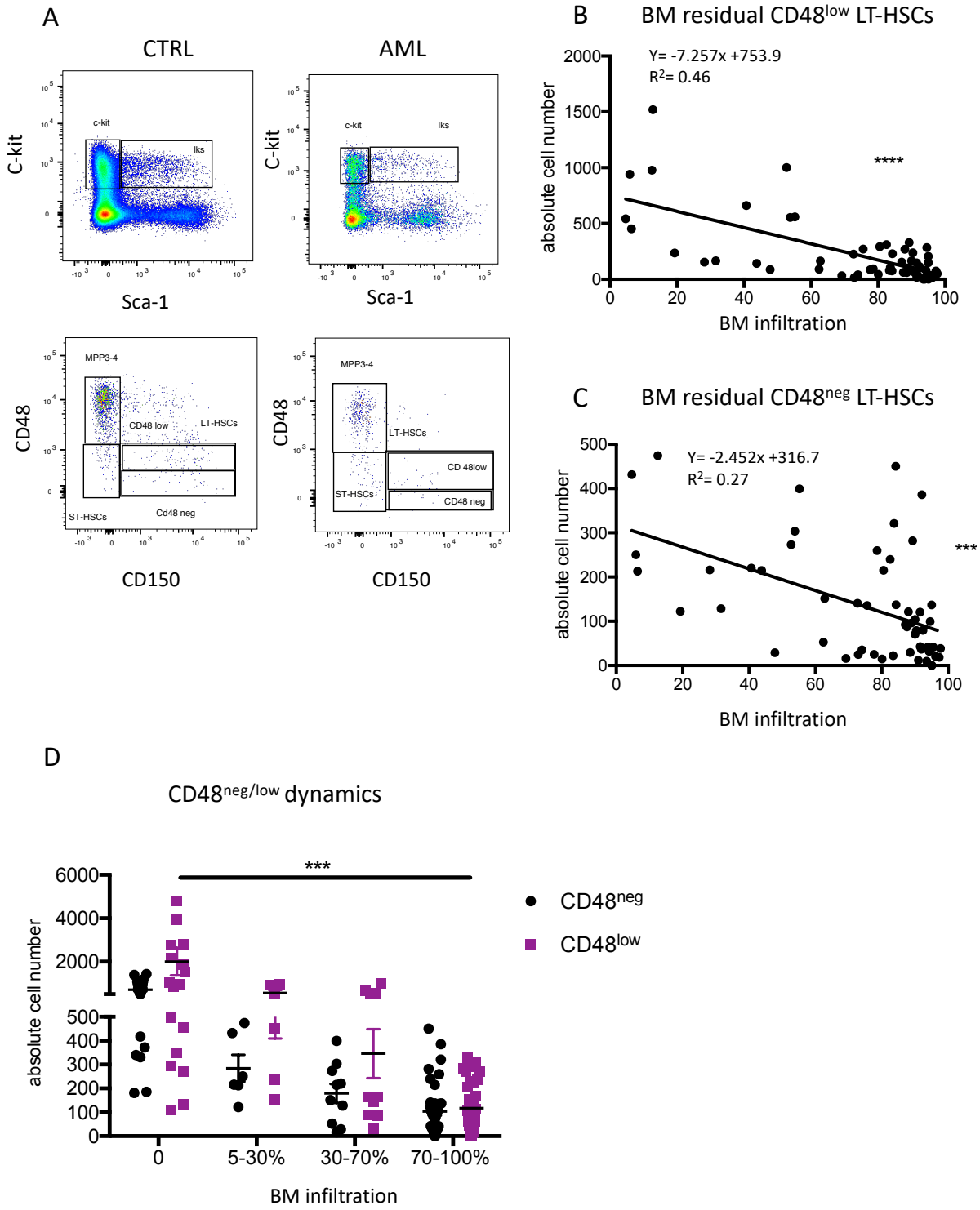

Figure 3.15-CD48 $^{neg/low}$ LT-HSCs reduction during AML growth

Differential expression of the CD48 marker allows separating two different populations within the LT-HSCs compartment **(A).** In particular, the decrease in the absolute number of LKS CD48^{low} CD150^{+} LT-HSCs negatively correlates with the percentage of blasts in the BM **(B)** while the LKS CD48^{neg} CD150^{+} LT-HSCs are being lost at a later disease stage **(C)**. Each dot represents a mouse and data were pooled from 6 independent experiments. Statistical significance analysed using linear regression (**represents p<0.01). Data were then plotted against each other and compared with control healthy mice ('0') **(D).** Mice were pooled based on similar BM infiltration rate, 18 mice used as control, 6 mice as lower infiltrated (5-30%), 10 mice as medium infiltrated (30-70%) and 40 mice at late disease stage (70-100%) data obtained from 6 independent experiments. Error bars represent the media ±s.e.m. Statistical significance obtained using student T test (*** represent p<0.001)

After having observed that AML drastically impacts the most primitive HSPCs, I wondered whether AML has a similar impact on more committed progenitors. Therefore, I followed the dynamic changes of Granulocyte-monocyte progenitors (GMPs), the Common myeloid progenitors (CMP) and the Megakaryocytes-erythroid progenitors (MEPs) contained within the Lineage^{-}c-Kit^{+}Sca-1^{-} (LK) population. According to the classical model of haematopoiesis hierarchy, the GMPs and MEPs arise from the CMPs, with the latter representing the first step in lineage commitment (Zhu & Emerson, 2002; Robb, 2007; Zhang & Lodish, 2008; Seita & Weissman, 2010). GMPs produce macrophages, granulocytes and dendritic cells while the MEPs give rise to megakaryocytes and erythrocytes. Flow cytometry plots showed a reduction in the frequency of each of the above populations at late AML stage, when the BM has between 70-90% of blasts infiltration (Figure 3.16 A). Data analysis revealed that even in this case, when compared to a healthy BM, the number of GMPs (Figure 3.16 B), CMPs (Figure 3.16 C) and MEPs (Figure 3.16 D) significantly decreased, especially at late disease stages. These populations are involved in the production of monocytes, red blood cells and platelets and their reduction, together with that of HSPCs, explain the life-threating cytopenia characterising AML. Overall these data suggest that AML causes progressive disruption of the whole haematopoietic process with cells affected at every step, from the most primitive HSCs to the committed progenitors.

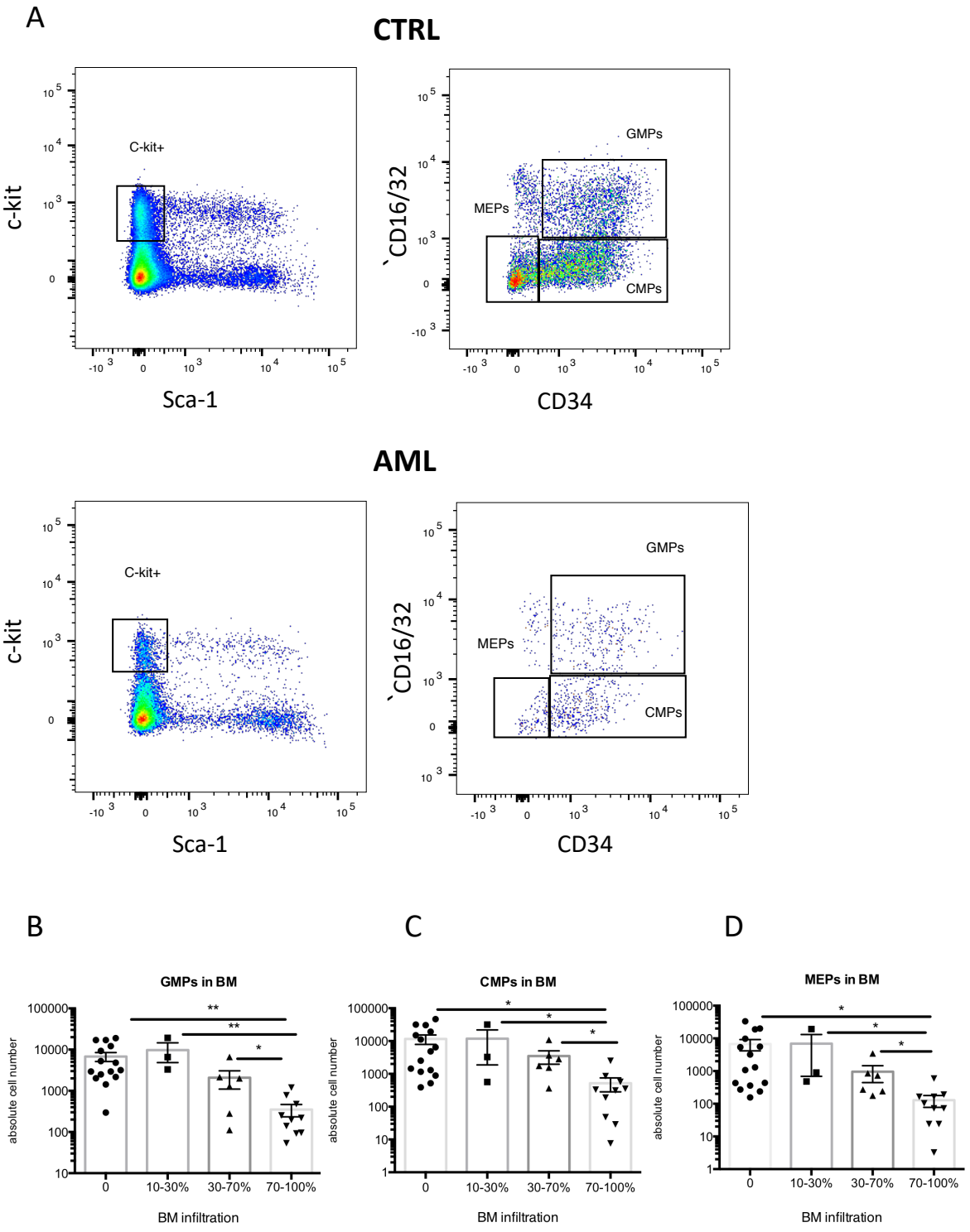

Figure 3.16-AML affects the committed progenitors

(A) Representative flow cytometry plots from a control and high infiltrated BM (80% of blasts). The Lin$^-$ c-kit$^+$ Sca-1$^-$ compartment contains the committed progenitors GMPs (CD34$^+$CD16/32$^+$), CMPs (CD34$^+$ CD16/32$^-$ and MEPs (CD34$^-$ CD16/32$^-$). **(B)(C)(D)** show changes in the absolute number of GMPs, CMPs and MEPs respectively at the indicated BM infiltration ranges. Data obtained from 3 independent experiments. Each dot represents a mouse, 35 mice in total. Error bars represent the mean ±s.e.m. Statistical significance examined using Student's T test. (* represent $p<0.05$, ** represents $p<0.01$)

3.8- HSPCs displacement is associated with a reduction of Sca-1^{high} ECs at late AML stage

Using IVM and immunofluorescence of undecalcified long bones sections we have previously shown that AML cells are able to locally remodel the stroma in highly infiltrated areas (Duarte *et al.*, 2017). These changes include a significant decrease in the endosteum and metaphysis vasculature, which both decrease over time. This feature seems to be specific for AML as endosteal vessels were maintained in the Notch-T-ALL murine model(Hawkins *et al.*, 2016; Duarte *et al.*, 2017). Furthermore, we showed that perivascular and endosteal stroma were depleted in highly infiltrated areas while maintained a normal appearance in lower infiltrated spot. Therefore, AML blasts cause a local remodelling of the stroma. It has been shown that the endosteal endothelium sustains osteoblast and that AML blasts reduce them in an infiltration-dependent manner. Therefore, as both osteoblasts and vasculature are fundamental for HSCs maintenance, changes in their homeostasis can affect haematopoiesis. As arteriolar Ecs are specifically involved in HSCs maintenance, I decided to quantify ECs numbers at late disease stages (Figure 3.17 A) and compare their changes with the observed HSPC displacement.

Flow cytometry analysis showed a decrease in the absolute number of CD45^{-}/Ter119^{-} CD31^{+} Sca-1^{high} ECs at late disease stage (Figure 3.17 B) in comparison to the healthy control. Next, I evaluated the number of residual HSPCs within the marrow associated with the decreased ECs. From this analysis it emerged that mice showing a decrease in the number of ECs were also characterised by a low amount of HSPCs (Figure 3.17 C). Although preliminary, these data agreed with previously published observations (Duarte et al., 2018) and I therefore did not pursue this line of analysis further.

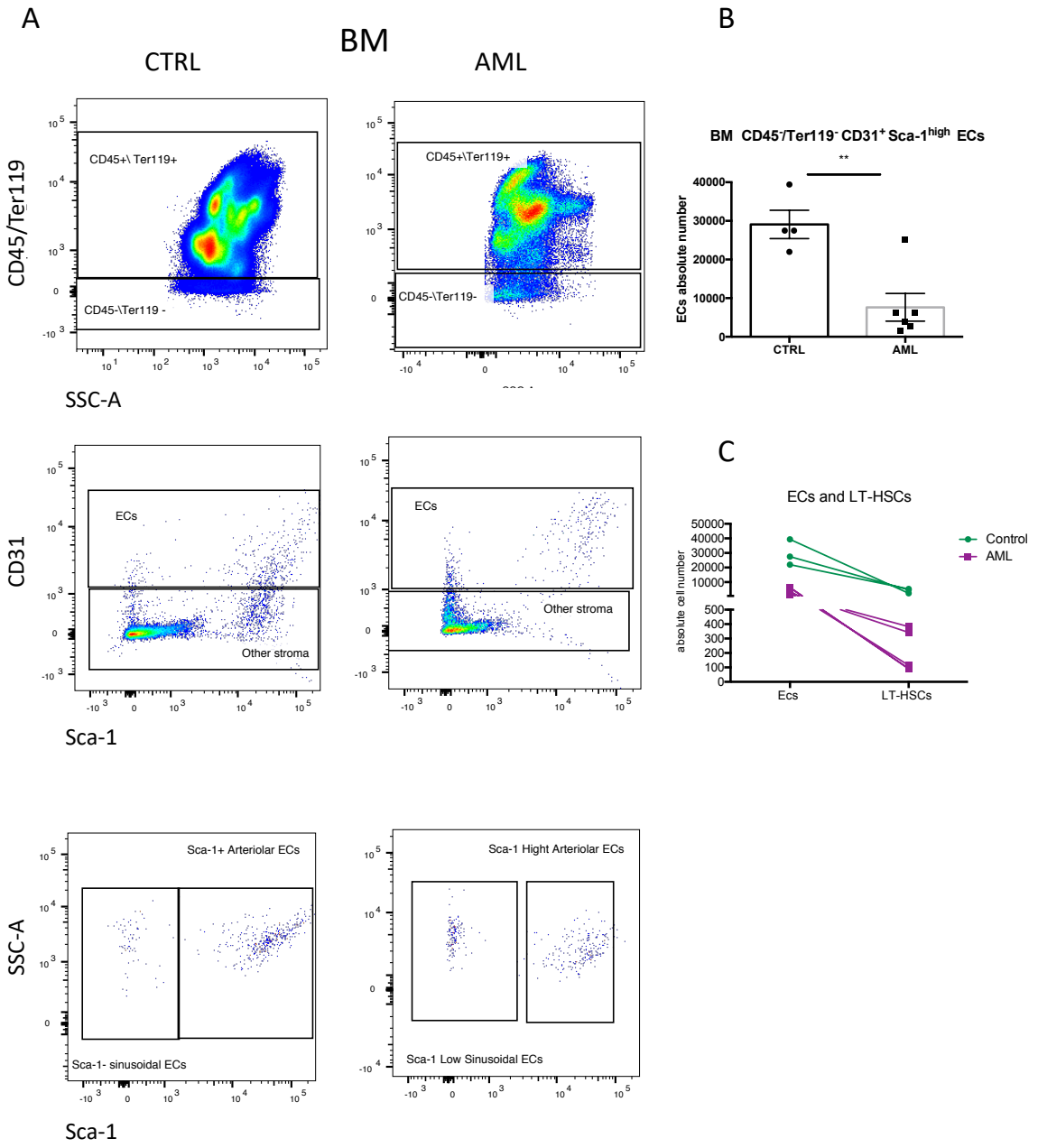

Figure 3.17-ECs are reduced at late disease stage

FACS plots showing the gating strategy used for the CD45/Ter119$^-$ Cd31$^+$ Sca-1^{hight} ECs in AML burdened mice **(A)**. In particular, among the CD45/Ter119$^-$ population it is possible to differentiate between the CD31$^+$ Sca-1^{dim} sinusoidal and the CD31$^+$ Sca-1^{hight} arteriolar ECs. Data analysis showed a significant decrease in the arteriolar ECs at late AML infiltration **(B)**. Data obtained from 6 leukemic and 4 control mice from two independent experiments. Error bars represent the mean ± s.e.m. Statistical significance analysed using Student T test (**represent p <0.01). Decreased ECs numbers corresponded to a decreased number of HSPCs at late disease stage. **(C)** shows the reduction of ECs and LT-HSCs. Data obtained from 5 leukemic and 3 control mice. Error bars represent the mean ± s.e.m.

3.9- Discussion

The aim of this chapter was to combine IVM and flow cytometry to gain a deeper insight into how leukaemia disrupts steady state haematopoiesis and, building on previous work from our research group, better understand the dynamic of HPCS loss throughout disease progression.

In the first part, I aimed to investigate if T-ALL and AML shared a common mechanism to overtake healthy haematopoietic cells within the marrow. We have previously shown that both leukaemia models profoundly remodel the BM niche (Hawkins *et al.*, 2016; Duarte *et al.*, 2017) While we showed that mature osteoblasts were systematically and dramatically lost in late T-ALL, we identified that in AML these cells were lost first in the areas within the marrow that were more highly infiltrated (Duarte *et al.*, 2017). Despite differences in their nature, both diseases disrupt haematopoietic homeostasis raising the hypothesis for a shared mechanism behind it. The Notch-driven T-ALL model established in laboratory is based on the transplant of primary blasts into sub-lethally irradiated host. However, it is well known that irradiation profoundly modify the microenvironment, giving a competitive advantage to the leukemic blasts. Thus, I tried to evaluate the chance for the T-ALL to growth in an irradiation-free host. Although the injection of 250,000 blasts appeared to induce leukaemia growth, the timing of disease development together with the high variability in organ infiltration, made the Notch-T-ALL a hard model to work with. Therefore, I decided to focus my experiments on AML and its effects on healthy haematopoiesis. In future, the use of a different model for T-ALL should be considered, for example the murine T-ALL driven by the TEL-JAK2 mutation. The TEL(ETV6)-JAK2 mutation results from the chromosomal translocation of t (9,12) (p24:p13) and is present in cases of T-ALL, B-ALL and CML (Lacronique *et al.*, 1997, 2000). Furthermore, cells from TEL-JAK2 transgenic mice injected into wild type recipients give rise

to T-ALL in a non-conditioned experimental model, representing an ideal setting to study and compare the interaction of blasts within an irradiation-free microenvironment (data not shown).

AML is associated with major cytopenias due to hematopoietic loss. Previous work has pointed to a block in HSPC differentiation, decrease of stem and progenitor proliferative rate and/or an increase in their apoptosis. However, the exact mechanism behind this loss remains still unknown (Miraki-Moud *et al.*, 2013). Colmone and colleagues were the first to propose the existence of competition between blasts and steady state cells (Colmone *et al.*, 2008), an idea pursued by other studies later on (Glait-Santar *et al.*, 2015; Colmone *et al.*, 2008). Here, by using IVM, I performed detailed, longitudinal analysis of the BM contained in the mouse calvarium, evaluating the changes that AML causes to hematopoietic cells during the course of its progression and I made a number of observations.

First, I identified heterogeneous morphologies and migratory behaviours of subsets of AML cells. While the majority of blasts exhibited a round shape, the so-called pioneer blasts presented the ability to create long protrusions in any direction. Analysis of speed, total displacement within the BM space, linear progression and track length revealed that these cells have unique features compared to the non-pioneer ones. Nowadays, heterogeneity is considered one of the greatest tumour's hallmark. This heterogeneity increases with tumour progression and includes a diverse collection of cells. The latter are characterised by distinct molecular signatures that confer, for instance, a differential level of sensitivity to treatment (Meacham & Morrison, 2013; Dagogo-Jack & Shaw, 2017). In AML, advances in sequencing technologies have revealed a high intratumor composition with multiple clones, often co-existing in the same patients (Ding *et al.*, 2012a). Specifically, the study of de Boer and colleagues defined and validated a series of leukaemia-enriched plasma membrane (PM)

proteins than have then been used to define leukaemia-associated immunophenotypes (LAIPs) (Giotopoulos & Huntly, 2018)Thus, the use of this LAIPs allowed to track leukemic sub-clones within individual AML patients (Boer *et al.*, 2018). Most importantly, it has been shown that different clones locate in different BM compartments, suggesting differential microenvironment requirements for each individual clone (Boer *et al.*, 2018). It is possible that the pioneer/non-pioneer behaviour corresponds to different immunophenotypes, which could be investigated by means of in vivo immunostainings using markers as c-kit, PDL-1, CX3CR1 and Ly6C. Whether pioneer blasts are genetically different from the bulk of the population remains an open question, however given their ubiquitous distribution, interspersed and especially at the edges of round leukaemia cell clusters, I hypothesise that their behaviour may be a result of their localisation and perhaps AML-intrinsic differentiation, rather than of genetic variability. While T-ALL has been showed to randomly migrate in the niche and to be less dependent on it, AML seems to rely more on its interactions with niche components (Hawkins *et al.*, 2016; Duarte *et al.*, 2018). In the past year several pathways have been proposed to be relevant for AML migratory capacity including the CXCR4/CXCL12 axis. However, using IVM we showed that AML migratory ability does not depend on this axis, which explains in part the failure of clinical trial for CXCR4 inhibitors (Duarte *et al.*, 2018).

Several observations lead me to the hypothesize that, due to their ability to create protrusions and move faster for a longer distance, the pioneer blasts may play a pivotal role in AML dissemination and HSC ousting from the marrow. In fact, while the non-pioneer cells were mostly moving close to each other, the pioneer ones were constantly interacting with the residual healthy marrow. Furthermore, these cells were mostly observed at early and medium infiltration stage, when individual blasts were still clearly identifiable. At this point of AML development, patches of healthy cells were still detectable within the niche and interestingly,

often, more than three pioneer cells were observed surrounding them. This observation raised the idea that leukemic blasts, thanks to their features, could displace healthy cells from their natural niche.

To test my hypotheses, more experiments should be conducted in the future. For example, qPCR or RNAseq analysis of pioneer blasts would reveal the gene signature of these cells and will allow a comparison with the other blasts that behave differentially. Before that, it would be worth evaluating their differentiation status by labelling them *in vivo* for example as their behaviour could be due to these blasts being more differentiated than others. Because purifying pioneer cells solely based on their behaviour would be challenging, an alternative to the *in vivo* labelling, could be their analysis by immunofluorescence on bones sections, where pioneer cells would be easily identified based on their morphology. Regarding the hypothesis of them interacting more with their healthy counterparts, more detailed analysis should aim to evaluate the volume of healthy cells surrounding pioneer and non-pioneer blasts.

In order to better understand how blast interact with healthy haematopoietic cells, I performed IVM at various stages of leukaemia questioning the possibility to catch the displacement of healthy cells caused by AML. Surprisingly, IVM of medium infiltrated BM revealed clusters of steady state cells, surrounded by blasts, entering the circulation and egressing from the marrow space. HSPCs seemed to continuously egress out of the marrow to the circulation under homeostatic conditions. It is now accepted that not only mature leukocytes and erythrocytes are released to the blood but also a small amount of non-cycling HSPCs (Orkin & Zon, 2008; Lapidot & Petit, 2002). Despite the steady state peripheral blood (PB) HSPCs having been considered as a failure in the system for a long time, it is now accepted that the PB HSPCs play a role in some homeostatic process such as, for example, the local production of myeloid

dendritic cells as part of host innate host innate immunity response (Massberg *et al.*, 2007). On the other hand, HSPC egression from the marrow is dramatically enhanced during stress situations and upon demand of accelerated haematopoiesis (Wright *et al.*, 2001; Abkowitz *et al.*, 2003). In my experiments, IVM of multiple control mice revealed that healthy cells intravasate mostly as a single cell with the exception of clusters formed of a maximum of two cells and only rarely of three cells. The situation is mostly inverted in leukaemia where the majority of detected intravasating cells were in cluster of two, three and up to 8 cells. As already mentioned, the finely organised BM space, differently from other organs, cannot expand under the increasing pressure from growing blasts, therefore malignant haematopoiesis ultimately occupies BM space at the expense of steady state haematopoiesis. HSPCs express on their surface an array of cell adhesion receptors that allow them to interact with the ECM components and other cell adhesion molecules expressed within the BM niche (Levesque & Winkler, 2016). Thus, the mutual interaction between these adhesion molecules and the ECM components contribute to retaining HSPCs in their niche. It is reasonable to hypothesize that while blasts colonise the marrow, they progressively advance remodelling the matrix. Based on my observations, I then conclude that this matrix remodelling could ultimately lead to bulk of steady state cells forced to egress in clusters of variable sizes. This observation raised the question of the use of inhibitors of matrix remodelling in AML aiming to reduce the healthy cells dislodgment and bolster residual haematopoiesis. Taking into consideration the recent finding about AML blasts upregulating proteases/peptidases (Izzi *et al.*, 2017), I then decided to investigate this hypothesis, aiming to propose a new treatment for AML (Chapter 5).

Considering the IVM data capturing the intravasating healthy cells, I then hypothesized that if the HSPCs were egressing in an AML-dependent manner, we should expect an increased number of HSPCs in the PB together with a decrease in the marrow. To evaluate

haematopoietic cells dynamics during AML growth, I performed flow cytometry analysis at various stages of AML, monitoring the number of undifferentiated and HSPC populations in both blood and BM marrow. As already mentioned, the mature cells are expected to be released in circulation in homeostatic conditions, while only a small amount of HSPCs should be detected. My data revealed an increase in the absolute number of lineage$^-$ cells in the blood, the amount of which related to the percentage of BM blasts infiltration. Thus, when the marrow is lower infiltrated, the presence of available space allows both healthy and malignant cell populations to co-exists. However, as cancer cells become more numerous, the lineage$^-$ cells start to be increasingly detected in the blood, while their presence decreased in the marrow with the same fashion. Similarly, my results showed that the HSPCs loss is a progressive event. As the marrow is occupied by growing blasts, the number of LKS cells decreases. The decrease of both the LKS CD48$^-$ CD150$^-$ (ST-HSCs) and LKS CD48$^+$ CD150$^-$ (MPP3-4) follow a similar kinetic. In this context, the LKS CD48$^-$ CD150$^+$ (LT-HSCs) appeared to be the more resilient ones. Looking deeper within the LT-HSC compartment, I observed that the CD48^{neg} fraction was the more resistant to being eliminated. This finding is in line with our previous study showing that AML ousts healthy cells independently on their proliferative status with the exception of the less proliferative CD48^{neg}. Thus, the niche is actively overtaken by AML blasts that compete for its own space with steady state hematopoietic cells. Interestingly, while the number of cell clusters observed in circulation by IVM decreased at the final stages of BM infiltration, arguably because no more healthy cells were left in the tissue, the number of HSPCs remained high at the same time points. This raises the hypothesis that displaced HSPCs may fail to efficiently relocate to extramedullary niches. I investigated this further with experiments described in the next chapter.

AML has been shown to depend on the BM niche, and to be able to hijack it to sustain its own growth. Within the niche, multiple cell types contribute to HSPC maintenance and regulation and some of them are affected by AML. The BM contains multiple types of ECs, with the majority of HSCs found less than 10μm from the endothelium (Nombela-Arrieta *et al.*, 2013). It has been proposed that differences in vessel integrity, permeability and oxygenation may create distinct HSC microenvironments (Perlin, Sporrij & Zon, 2017). While arteriolar ECs express higher levels of VCAM-1 that has been associated with HSCs retention, sinusoidal ECs express high level of E-selectin, important for HSC homing and proliferation (Papayannopoulou *et al.*, 2001; Ulyanova *et al.*, 2005; Winkler *et al.*, 2012; Itkin *et al.*, 2016). Considering their role in HSC maintenance within the niche, I decided to assess EC dynamics during AML, especially in relation to HSPC numbers. Flow cytometry data showed a decrease in the overall number of arteriolar (CD45/Ter119^{-} CD31^{+} Sca-1^{high}) ECs cells over disease progression and in particular at late time points, when the number of HSPCs is drastically decreased. Thus, ECs loss could also enforce HSC mobilization. A more detailed time course experiment in which the ECs loss and the HSPCs dynamic should be followed from an early time point would probably corroborate the link between the two events.

Overall, combining IVM and flow cytometry analyses, these data provide a unique insight into the dynamic interactions of AML with its steady state counterpart as disease progresses. Instead of being lost, HSPCs are progressively dislodged from the transforming marrow by competing with leukemic blasts. The increased number of HSPCs in PB together with their decrease in the marrow raises questions about their future localisations as disease persists. In particular, it raises questions about the presence of extramedullary sites within which HSCs could compensate for normal haematopoiesis in the short term and I will explore the presence of functional extramedullary haematopoiesis in the following chapter.

Chapter 4

Assessing the dynamics and functionality of AML-induced extramedullary HSC and early progenitor cells

4.1- Introduction

Extramedullary haematopoiesis (EMH) refers to the haematopoietic process occurring in extramedullary sites known to happen under various circumstances (Johns & Christopher, 2012; Inra *et al.*, 2015; Chiu *et al.*, 2015; Mohyuddin & Yacoub, 2016; Yang *et al.*, 2020). EMH occurs during embryonic/developmental periods but also as a result of pathological conditions such as insufficient BM function or ineffective haematopoiesis (Yamamoto *et al.*, 2016). During embryonic development, in mammals, HSCs are first produced from the floor of the embryonic aorta in the Aortic-Gonad-Mesonephros (AGM) region (Zovein *et al.*, 2008; Chen *et al.*, 2009; Ivanovs *et al.*, 2011) and from the vitelline and umbilical arteries (Bruijn *et al.*, 2000) during the first waves of haematopoiesis. Furthermore, human and murine studies have identified the presence of HSCs in the placenta that arise independently and in parallel with the HSCs from the AGM (Gekas *et al.*, 2005; Ottersbach & Dzierzak, 2005; Rhodes *et al.*, 2008; Gekas *et al.*, 2010). Following their production, HSCs migrate and colonise the foetal liver (FL) in response to CXCL12 signalling (Chou & Lodish, 2010; Mahony & Bertrand, 2019) where they expand before migrating to the BM. Thus, in adulthood, the BM is identified as the preferential and ultimate niche for HSCs where multiple cell components and signals participate to their maintenance and regulation. The niche concept proposed in 1978 by

Schofield defined the bone marrow (BM) microenvironment as a nest for stem cells, in which interactions with other cell types determine their function (Schofield, 1978). Therefore, although it is normal to detect a small number of HSPCs in the peripheral blood (PB), in adults the majority of these cells reside within the BM (Wright *et al.*, 2001; Massberg *et al.*, 2007; Lefrançais *et al.*, 2017). Thus, HSPC migration to and differentiation within extramedullary organs is mostly associated with haematopoietic stress. In this context, it is well documented that haematological malignancies have impacts on the niche environment and haematopoiesis itself. In some cases, this situation is exacerbated with the niche losing its ability to support HSPCs and resulting EMH.

EMH is characterized by the accumulation of hematopoietic cells in multiple locations in the body (Yamamoto *et al.*, 2016). Clinically, common sites for EMH include the spleen, liver, lymph nodes and para-vertebral regions but occasionally other organs such kidney, skin and CNS can be involved. The first evidence for the spleen as an extramedullary site dates back to 1949 when Jacobson and colleagues proposed that the HSPCs contained there were able to rescue irradiation-induced haematopoietic failure (Jacobson *et al.*, 1949). Nowadays, the spleen - specifically the splenic red pulp - is considered to be one of the most common sites of EMH in response to various haematopoietic stresses including anaemia, pregnancy, infections and myeloproliferative disorders (Bennett *et al.*, 1968; Cheshier, Prohaska & Weissman, 2007; Abdel-Wahab & Levine, 2009; Nakada *et al.*, 2014). Thus, in the short term, EMH might represent a compensatory mechanism for blood cell production following BM failures. Despite reports of EMH in MPNs and in both CLL and CML, little is known about the onset and the eventual role for it in AML.

In MPNs for example, EMH is a well-recognised secondary phenomenon occurring preferentially in organs such as spleen and liver (Georgiades *et al.*, 2002; Mohyuddin & Yacoub, 2016). Other diseases have been clinically associated with EMH including thalassemia, chronic lymphocytic leukaemia (CLL), chronic myelogenous leukaemia (CML) and other infiltrative disorders (Kopecky *et al.*, 1986; Rogers *et al.*, 2015; Bothale *et al.*, 2018). This process rarely occurs in acute myeloid leukaemia (AML)(Bothale *et al.*, 2018), however the disease profoundly remodels the BM microenvironment and outcompetes healthy haematopoiesis in that tissue. If it was possible to induce a more effective EMH, this could be a strategy to minimise AML-induced cytopenias.

In Chapter 3, I highlighted the dynamics of HSPCs throughout AML progression demonstrating that hematopoietic cells are dislodged during disease development. As leukemic blasts take over the BM space, HSPCs are forced to leave and as a consequence, the HSPCs decrease in the marrow in an infiltration-dependent manner while increasing in numbers in the blood. Little is known about the journey that HSPCs are forced to take once they enter the circulation and whether or not they colonise other organs outside the marrow. The dramatic mobilization of HSPCs to the blood caused by AML raised the hypothesis that survival of these populations may depend on seeding of extramedullary sites. In this chapter, I explore the possibility of alternative niches expanding during AML progression in mice, from where the dislodged HSPCs potentially compensate for perturbed haematopoiesis in the short term. Using IVM and flow cytometry, I follow HSPC ousting from the BM to extramedullary sites including the spleen and liver at various stages of disease. I then question the potential of these organs to be supportive niches for HSPCs, evaluating specific alterations to their microenvironment compositions (e.g. cytokines, endothelial cells (ECs) expansion) that could sustain the process of EMH. Most importantly, I test the functionality of AML-induced

extramedullary HSPCs by transplantation assays to highlight the potentially compensatory role for this process in AML.

4.2- The spleen as a site of Extramedullary haematopoiesis in AML

Given the findings presented in Chapter 3, I wanted to investigate whether AML-induced extramedullary HSPCs were seeding alternative organs outside of the marrow. This question has important implications and raises the possibility of other functional niches that might be forming or re-activate following AML development. Because the spleen has been shown to be a pivotal site of EMH in MPNs and others conditions, I decided to investigate its role in EMH during AML progression.

C57BL/6 mice were transplanted with 100,000 GFP^+ AML cells and sacrificed at different time points during disease progression. Mice developed splenomegaly during the course of disease (Figure 4.1 A) as a result of the increasing number of blasts infiltrating it. In particular, the severity of spleen enlargement was related with the percentage of blasts in the marrow. For example, the spleen's size was only slightly increased in animals with a low percentage of blasts in their BM whereas it was approximately doubled in size when the marrow reached an infiltration of around 80%. Comparison of blasts infiltration between the spleen and the marrow indicated that spleen infiltration positively correlates with that of the BM, and it is slightly delayed compared to it (Figure 4.1 B). For instance, mice with 50% infiltration in their BM presented an average spleen infiltration of 40%. Therefore, the spleen's size can be used as an indicator for the status of marrow infiltration and of disease progression in general.

As mentioned previously, the BM has a limited and defined space for which blasts and healthy cells compete. In comparison, the physiology of the spleen allows this organ to enlarge and

accommodate the co-existence of both populations. As the spleen's size relates to disease progression and considering that healthy cells are dislodged from their niche egressing into the circulation with a similar dynamic, I next decided to evaluate if the observed splenomegaly could be the combined result of hosting both blasts and extramedullary HSPCs.

I started with the analysis of the undifferentiated population (Lineage$^-$ or Lin$^-$) absolute number in the spleen in relation to BM infiltration. Data analysis revealed an increase in the absolute number of Lineage$^-$ cells, the amount of which significantly correlates with BM infiltration (Figure 4.1 C). In chapter 3, I showed that this population decreases in the marrow while increasing in the blood with a dynamic that relates to the percentage of blasts in BM. All together, these data suggest that as this population is forced to leave their niche due to the pressure of growing AML, they gradually migrate and colonise the spleen. For instance, when there is a low percentage of blasts in the BM, the amount of Lin$^-$ cells in both blood and spleen is still reduced in line with the fact that healthy cells can co-exist with blasts in their niche. However, as soon as the blast percentage increases in the BM, the active competition of leukemic cells dislodges an increased number of healthy ones that migrate through the circulation seeding in the spleen.

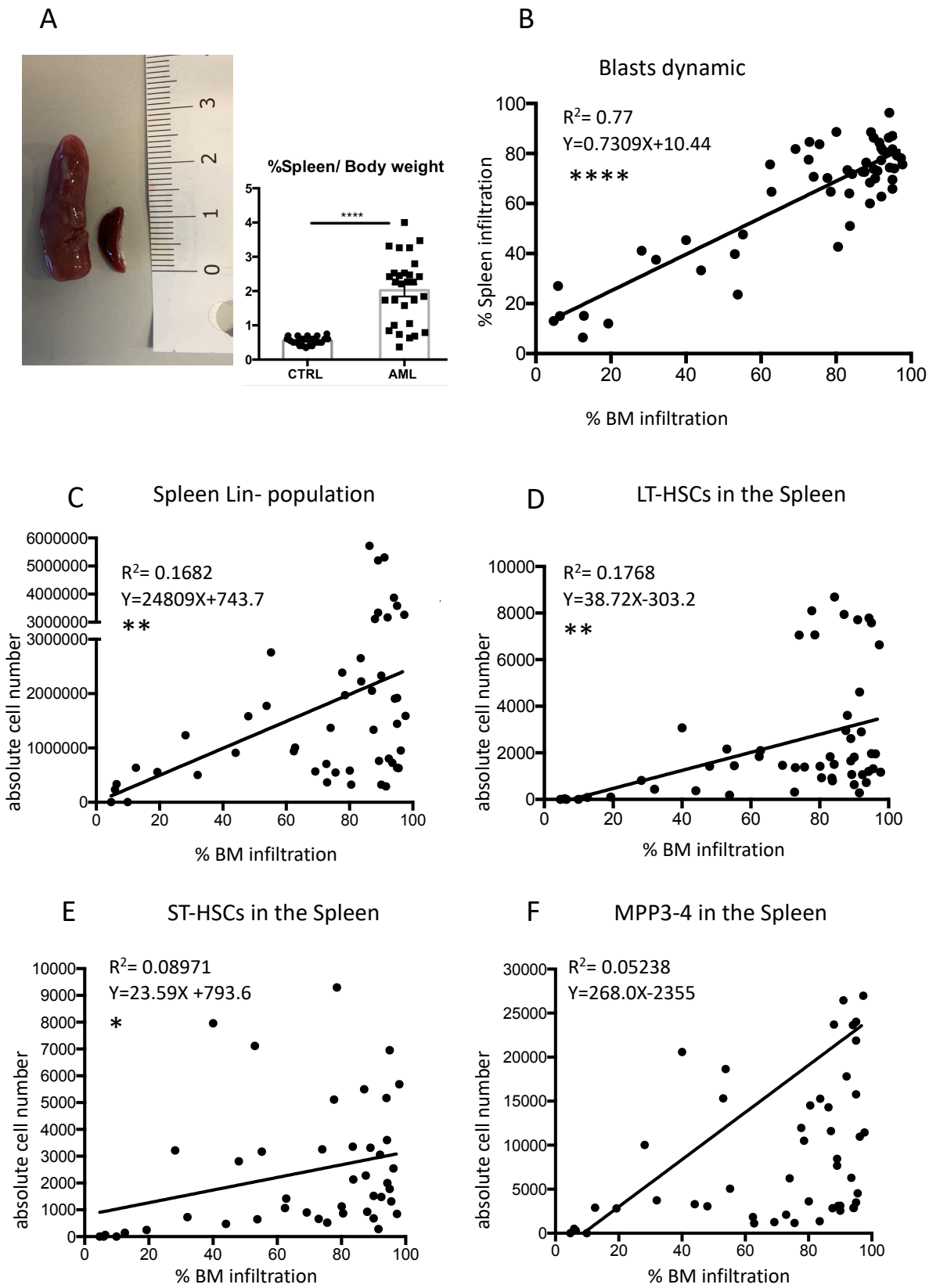

Figure 4.1-HPCs seed in the splenic niche

(A) AML progression leads to splenomegaly that correlates with the amount of blasts and EMH in it. Spleens from a heavily BM infiltrated (left) and a healthy (right) mouse are shown here togheter with the % of spleen weight/body weight. *n* =21 control and 29 AML burdened mice Statistical significance determined using Student'T-test **(B)** There is a positive

correlation between the percentage of blasts in bone marrow and spleen. Each dot is a mouse, 55 mice in total from 7 independent experiments. **(C)(D)(E)(F)** show the correlation between BM infiltration and the increase in the number of lineage- **(C),** LT-HSCs **(D**), ST-HSCs **(E)** and MPP3-4 **(F**) detected in the spleen. Each dot represents a mouse, mice pooled from 7 independent experiments. Statistical significance determined using linear regression (*represent $p<0.05$; **represent $p<0.01$, **** represent $P<0.0001$; no * represents lack of statistical significance)

With that in mind, I decided to investigate if and how more primitive hematopoietic cells were involved in this process, and I evaluated the distribution of HSPC populations at various disease stages. Data analysis indicated that the amount of HSPCs in the spleen increases during AML progression. Specifically, the increase in LT-HSC and ST-HSC numbers in the spleen statistically significantly correlated with the amount of blasts in the BM with higher infiltrated mice having more of these cells seeding in their spleens (Figure 4.1 D and Figure 4.1 E). On the other hand, despite an increase in MPP3-4 absolute number in the spleen, this is not statistically significantly correlated with BM infiltration (Figure 4.1 F).

Next, I decided to verify whether the overall increase in LT-HSC number in the spleen was due to the migration and localization of both the $CD48^{neg}$ and $CD48^{low}$ LT-HSCs or only to one of these populations. First, I plotted the overall LT-HSC spleen numbers pooled based on BM infiltration ranges of 0%, 10-30%, 30-70% and 70-100% where 0% corresponds to control mice, 10-30% are early infiltrated, 30-70% medium infiltrated and 70-100% highly/fully infiltrated mice. Again, the absolute number of LT-HSCs was significantly increased in the spleen for a fully infiltrated marrow as at this point even the more resistant primitive haematopoietic cells are dislodged from their niche (Figure 4.2 A). To be sure that the increase of LT-HSCs in the spleen was linked to their decrease in the marrow, I compared the absolute number of these cells between these two organs per each infiltration group (Figure 4.2 B). In line with my hypothesis, as disease progresses in the BM, LT-HSCs decrease in their medullary niche while significantly increasing in the spleen. As expected, this is even more pronounced for the medium-high and fully infiltrated marrow groups.

I compared the number of $CD48^{neg}$ and $CD48^{low}$ LT-HSCs present in the spleen for each infiltration range and concluded that there was no difference in the absolute number of $CD48^{low}$ and $CD48^{neg}$ HSCs seeding in the spleen - revealing that both populations equally colonise this organ after being pushed out from the BM into the circulation (Figure 4.2 D). To investigate whether each HSC population may migrate from the BM to the spleen, I evaluated the dynamics of these two fractions in the two sites. For both sub-populations, the data confirmed that cells were found in the spleen after they were lost from the BM niche. Data analysis revealed that for low BM infiltrations, both populations are still significantly higher in the BM, and start to increase in the spleen at medium/high and fully infiltrated disease stages (Figure 4.2 E and F).

Overall, these results indicate that the spleen can become a site of EMH in AML.

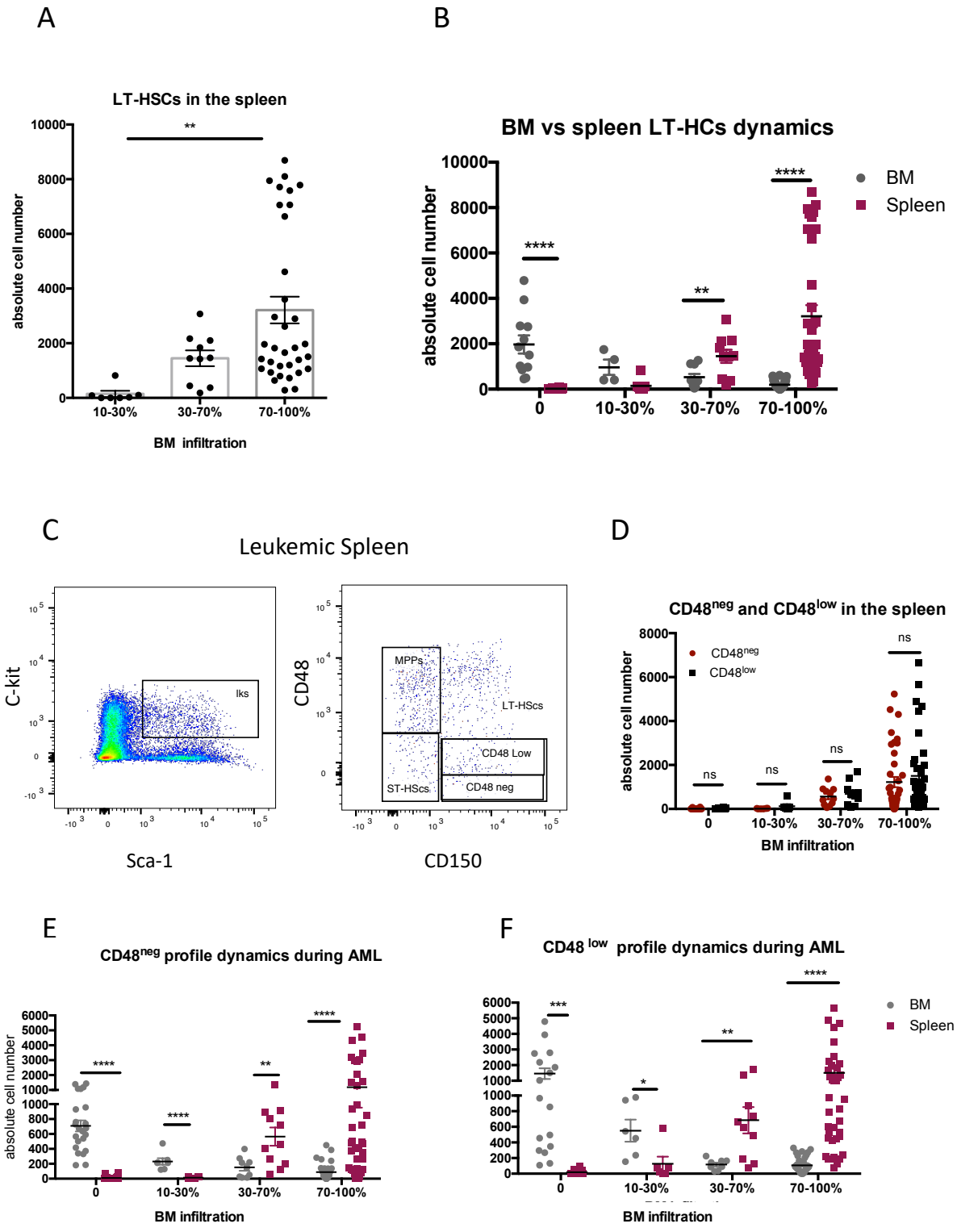

Figure 4.2 -LT-HSCs compartment dynamic in the spleen

Data show the dynamic of LT-HSCs and the two sub-populations included in it in the spleen and in comparison, to the BM. **(A)** LT HSCs number significantly increases in the spleen over the course of AML progression while decreasing in the marrow **(B). (C)** Representative FACS plots showing the gating strategy for the CD48^{low} and CD48^{neg} LT-HSCs in the spleen of AML burdened mice. **(D)** Within the spleen the abundance of CD48$^{neg/low}$ LT-HSCs is similar at each infiltration range. When compared to the BM, the number of both populations significantly increased in the spleen while significantly decreased in their natural niche **(E)** and **(F).** Each dot represents a mouse, mice pooled from 7 independent experiments. Error bars represent the mean ±s.e.m. Statistical significance determined using Student's t-test (* represent p<0.05, ** represent p<0.01*** represent p<0.001 ****represent p<0.0001)

4.3- Cytokine upregulation contributes to splenic niche formation

The role of this organ as an extramedullary site has been confirmed in mice and humans during processes such myeloablation, blood loss or pregnancy (Lowell *et al.*, 1996; Tavassoli & Weiss, 1973; Freedman & Saunders, 1981; Johns & Christopher, 2012). However, little is known about the nature of this niche, and the spleen has not been identified as a niche for HSCs in AML. Recently, in mice, it has been recognised that SCF and CXCL12 are both key players in EMH caused by the above conditions (Inra *et al.*, 2015; Miwa *et al.*, 2013; Wang *et al.*, 2014b). In particular, during EMH, HSCs have been found mainly in proximity of sinusoids in the red pulp raising the hypothesis of a perisinusoidal niche (Kiel *et al.*, 2005). CXCL12 has been found to be expressed by sinusoidal ECs in the red pulp of the human spleen (Miwa *et al.*, 2013), and the involvement of CXCL12 in the process of EMH has been demonstrated in both mice and humans in which EMH was a secondary phenomenon following a primary disease (Johns & Christopher, 2012). CXCL12 has a pivotal role in the BM niche where it interacts with the CXCR4 receptor on HSCs to facilitate their localisation and retention within the BM (Avecilla *et al.*, 2003; Sugiyama *et al.*, 2006; Greenbaum *et al.*, 2013; Beck *et al.*, 2014; Wang *et al.*, 2014b; Asada *et al.*, 2017). Furthermore, during early development CXCL12 regulates the colonisation of the BM by HSCs as it has been demonstrated that CXCL12-deficient embryos have reduced HSC numbers and function (Ara *et al.*, 2003). Thus, CXCL12 can regulate both the migration to and retention of HSPCs in extramedullary sites during EMH.

From the data presented so far, I could conclude that even in AML the spleen becomes an alternative niche for HSPCs and therefore I decided to valuated whether CXCL12, together with other factors such as SCF, would contribute to that. Bearing in mind the role that some cytokines have on HSPC regulation and maintenance, I started screening them to check

whether or not I could detect alterations in their concentrations in relation to EMH. Specifically, I analysed the concentration of CXCL12, SCF, IL-6 and G-CSF in the spleen of AML burdened mice comparing it with a steady state situation.

I performed ELISAs for CXCL12 on BM and spleen supernatants obtained from control and leukemic mice. The analysis revealed that CXCL12 concentration is significantly higher in the spleen of AML burdened mice in comparison to the control spleen (Figure 4.3 A). In parallel, CXCL12 levels significantly decreased in the BM of AML-burdened mice compared to healthy BM or leukemic spleen. Thus, CXCL12 is highly expressed only in the leukemic spleen when EMH occurs, confirming its relationship with the process of EMH. This finding is in line with previous studies in which extramedullary HSCs have been found in the spleen's red pulp where CXCL12 was highly expressed (Miwa *et al.*, 2013; Inra *et al.*, 2015)

Furthermore, the analysis of SCF levels in these samples revealed a similar situation. In the BM, HSCs are maintained in a perivascular niche in which different stromal, such as $LepR^{+}$ and ECs synthesize a number of factors, including SCF (Comazzetto *et al.*, 2019). SCF is required for the maintenance of HSCs and restricted $c\text{-}kit^{+}$ progenitors as well as other process such as erythropoiesis, mast cell development and lymphopoiesis (Russell, 1979; Huang *et al.*, 1990; Zsebo *et al.*, 1990; Flanagan, Chan & Leder, 1991; Waskow *et al.*, 2002; Comazzetto *et al.*, 2019). Furthermore, SCF has been used in combination with G-CSF in several clinical trials due to its capacity to induce peripheral blood progenitor cell mobilization (Shpall *et al.*, 1999; Usuki *et al.*, 2006). Data from BM and spleen supernatants revealed that the concentration of SCF is significantly increased in the spleens of AML burdened mice (Figure 4 B). Moreover, the comparison between leukemic BM and spleen shows a significant difference in SCF concentration between the two.

Next, I analysed the concentration of IL-6 and G-CSF. IL-6 is a mediator of haematopoietic cell growth and differentiation acting on B cells, T cells, osteoclasts and ECs (Peters *et al.*, 1997). In particular, together with other cytokines, IL-6 has been identified to regulate the proliferation and differentiation of HSPCs in a paracrine manner (Kishimoto, 1989; Zhao *et al.*, 2014). On the other hand, alongside its use in the clinic for HSC mobilization, G-CSF has been shown to induce expansion and increase quiescence of phenotypic HSCs (Schuettpelz *et al.*, 2014) and its expression has been revealed to be associated with EMH in triple negative breast cancer (Arroyo-Crespo *et al.*, 2019). Data from spleen supernatants revealed a significant upregulation of IL-6 in leukemic mice compared to healthy controls while the level of G-CSF did not differ from control (Figure 4.3C and Figure 4.3D).

Overall these data lead me to conclude that circulating HSPCs could be attracted to the spleen as a result of the combinatory increase of CXCL12 and SCF, cytokines that are both well known to be involved in HSPCs retention and regulation. Once HSPCs seed in this alternative niche, multiple signalling pathways, including the above cytokines and IL-6 could then stimulate their proliferation and differentiation in order to maintain haematopoiesis in the short term.

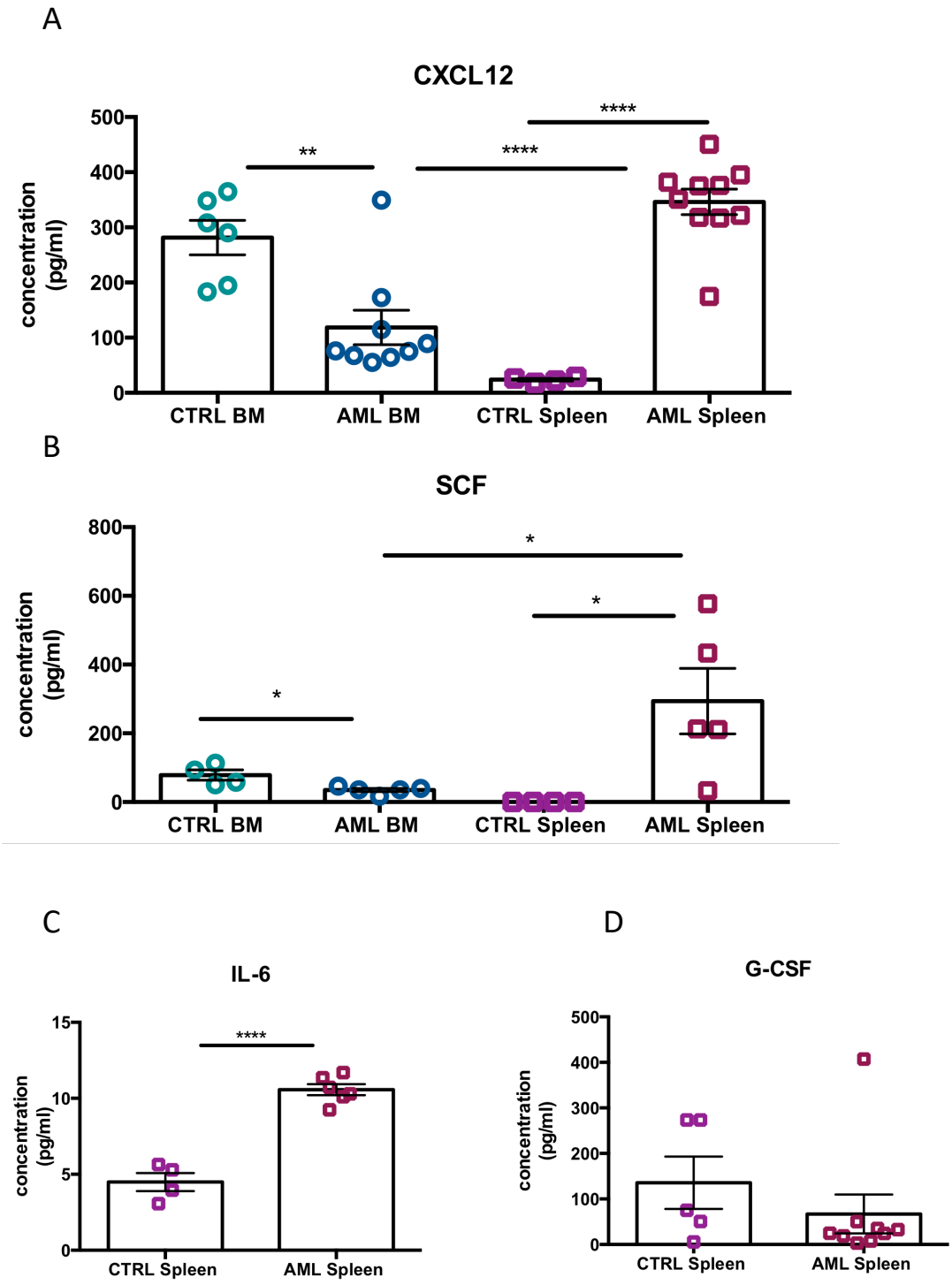

Figure 4.3-Cytokine levels in BM and spleen during AML

Data show the impact of AML on the level of CXCL12, SCF, IL6 and G-CSF. Different ELISA measurements were performed using BM and spleen supernatant from both control and leukemic mice**. (A)** CXCL12 expression is reduced in the marrow due to AML while significantly increased in the spleen of AML burden mice. At the same time SCF is significantly lower within the marrow full of blasts in comparison with its level in both control BM and leukemic spleen **(B).** the expression of IL-6 and G-CSF was evaluated only in the spleen and at both steady state and under AML. **(C)** IL-6 expression significantly increased in the AML-burden group while there was no difference in the level of G-CSF between the two condition**(D).** n= at least 3 mice per group. Statistical significance determined using Student's t-test (* represent $p<0.05$, ** represent $p<0.01$, ****represent $p<0.0001$)

4.4- Endothelial cells are expanded in the splenic niche

In the BM niche, HSC function and maintenance are supported by various factors secreted by specific niche components (Morrison & Spradling, 2008). Specifically, most HSCs have been found to reside close to sinusoidal blood vessels throughout the BM leading to the hypothesis of a perivascular niche (Kiel *et al.*, 2005; Kiel, Radice & Morrison, 2007; Kiel & Morrison, 2008). The same concept has been applied for EMH in the spleen, where HSCs have been found mainly around sinusoids in the red pulp (Inra *et al.*, 2015). Furthermore, it has been demonstrated that the production of SCF and CXCL12 from perivascular cells promote EMH in the splenic niche during myeloablation, blood loss or pregnancy (Inra *et al.*, 2015). Therefore, as this mechanism has not been investigated in AML, I wanted to evaluate if I could detect ECs expansion in the spleens of leukemic mice where EMH and cytokine upregulation were observed.

Data analysis showed an overall increase in the number of $CD45^{-}/Ter119^{-}$ $CD31^{+}$ECs in the spleen of AML-burdened mice when compared to control (Figure 4.4 A). Thus, this expansion coincides with the establishment of the EMH and therefore with the increased number of haematopoietic cells seeding in this site.

Next, I performed IVM of the spleen in AML-burdened mice to visualise the dynamics of AML and healthy cells in this organ. For this experiment, I used chimeric mice in which the haematopoietic compartment is $tomato^{+}$, allowing the visualisation of HSPCs in combination with YFP AML. High resolution time lapses and tilescan images showed patches of healthy cells surrounded by AML blasts in close proximity to the blood vasculature (Figure 4.4 B-C-D). These data suggest that dislodged healthy haematopoietic cells are indeed tightly associated

with the expanded spleen endothelium, which likely provides a "new" haematopoietic niche which is able to sustain HSCs and EMH.

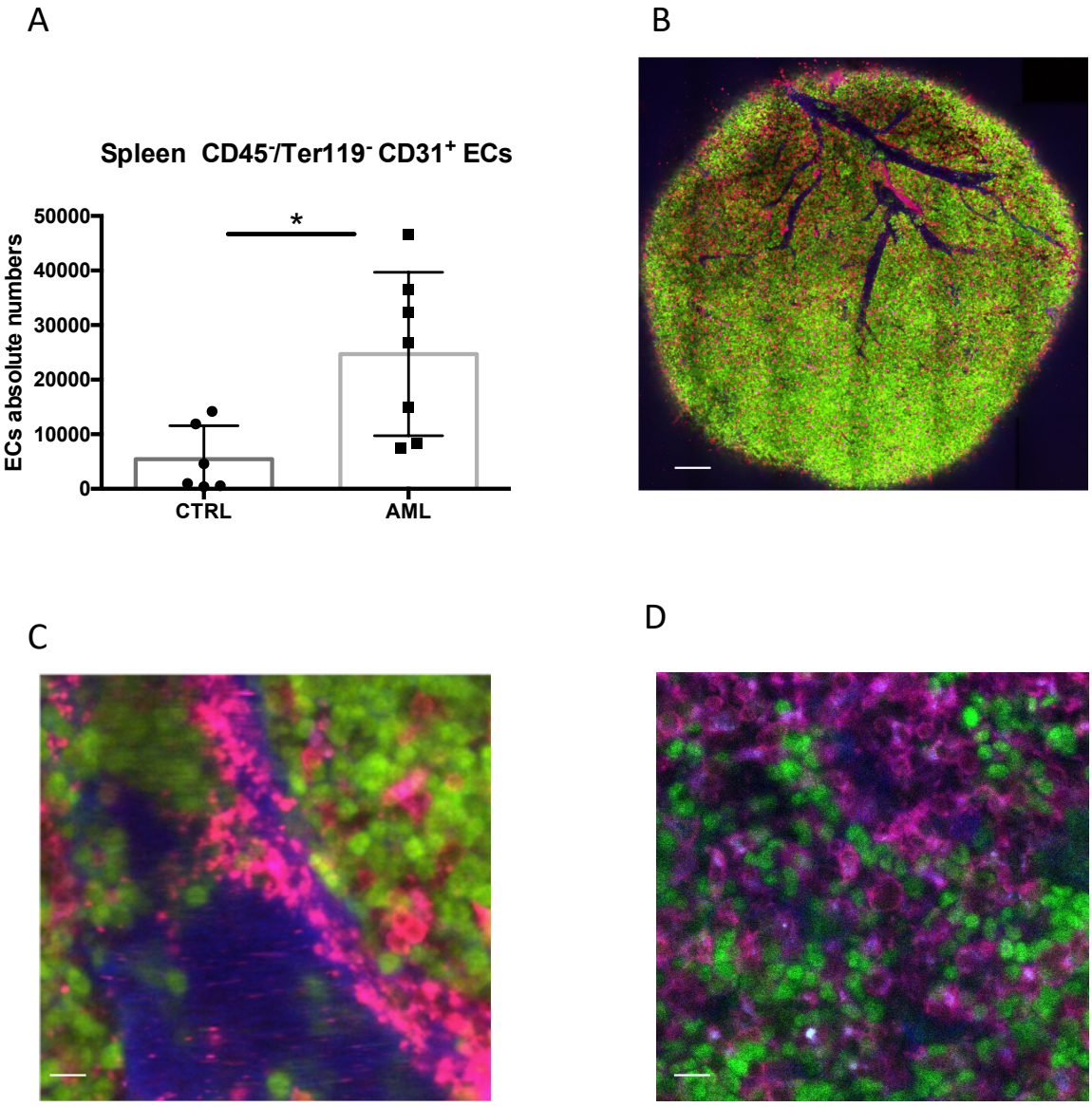

Figure 4.4-AML induces ECs' expansion in the spleens, where healthy haematopoietic cells are found near vasculature

(**A**) Flow cytometry analysis of ECs in the spleen of control and AML burdened mice. EC number is significantly increased in the leukemic spleen in comparison to control. Each dot represents a mouse, 6 mice for control and 7 mice for AML pooled from 2 independent experiments. Error bars represent the mean ±s.e.m. Statistical significance determined using Student's t-test (* represent $p<0.05$,) (**B**) Representative tilescan maximum projection of spleen IVM at a late infiltration time point. **(C)** and **(D)** Representative time frames from time lapse datasets showing healthy cells interspersed among AML cells **(D)** and localised near vasculature **(C)**. **(B) (C) (D)** Healthy cells are in magenta, AML blasts in green and blood vasculature in blue. Scale bars represent **(B)** 500μm, **(C) (D)** 80μm

4.5- The Liver as a site for Extramedullary haematopoiesis

While the spleen is the most common site of EMH, other organs have been identified as potential locations for the same process. In mammals, the foetal liver represents the first niche that expands HSCs before they migrate to the BM (Ema & Nakauchi, 2000; Ciau-Uitz *et al.*, 2014). Specifically, in the foetal liver HSCs have been found to be closely associated with ECs and stromal cells that together with cytokine secretion, promote their expansion (Tamplin *et al.*, 2015; Khan *et al.*, 2015). As mentioned above, the liver's haematopoietic output declines after embryo development and specifically as soon the BM becomes the main site of haematopoiesis. Despite is role in foetal life, it is thought that the liver does not contribute to blood cell formation during adulthood even though this organ is considered to be a maturation site for unconventional T cells (Golden-Mason & O'Farrelly, 2002; Kim, 2010). However, hepatic EMH has been identified in association with various pathological states including hepatic disorders, solid tumours (Tsamandas *et al.*, 1995; Schlitt *et al.*, 1995; Yamamoto *et al.*, 2016) and primary myelofibrosis (Mohyuddin & Yacoub, 2016). Therefore, I decided to investigate if during AML the liver can become an alternative niche and accommodate HSPCs when they have been forced out of the BM.

I carried out analysis of hepatic EMH in the same cohorts of mice analysed for the splenic EMH. For all analysed time points, hepatomegaly was not as prominent as the splenomegaly in leukemic mice when compared to control (Figure 4.5 A). This might be due to the fact that the liver is already a large organ in which multiple cell populations coexist. Even without organ expansion, the percentage of blasts colonising the liver significantly correlated with the number of leukemic cells infiltrating the BM (Figure 4.5 B). As for the spleen, liver infiltration is slightly delayed compared to that of the BM. Thus, at low BM infiltration, for example 20%, less than 5% of blast infiltration is detected in the liver, while at 95% BM infiltration there is

70% infiltration in the liver. The fact that the infiltrations of both liver and spleen appear delayed compared to the BM fits with the hypothesis that leukemic cells, due to their exponential growth, run out of space in the BM and are forced into the blood and other organs (Akinduro *et al.*, 2018).

Thus, I carried out my analysis evaluating the dynamics of undifferentiated cells (lineage$^-$) within the liver. These data revealed that, similarly to the spleen, AML causes an increase in the number of lineage$^-$ cells in the liver, the amount of which correlates with BM infiltration (Figure 4.5 C). Therefore, as blasts overtake the marrow, undifferentiated haematopoietic cells are pushed out from it through the circulation reaching the spleen and liver. Given that I could detect an increase in the Lineage$^-$ population, I questioned whether more primitive HSPCs display a similar redistribution to what I had already observed within the spleen. HSPC dynamics data analysis revealed an increase in the number of these populations in the liver during the course of disease. Specifically, the number of LT-HSCs significantly increases in relation with BM infiltration (Figure 4.5 D) with a greater number of cells detected in the liver at higher infiltration (BM >80% infiltration). Again, this fits with what was showed before about LT-HSCs being the more resistant cells to leukaemia invasion (Duarte *et al.*, 2017; Akinduro *et al.*, 2018). Similarly, the absolute number of MPPs (Figure 4.5 F) followed the dynamics observed for LT-HSCs, with a drastic increase detected when the BM is fully infiltrated (Figure 4.5F). Finally, the number of ST-HSCs increases in the liver but with more variability in terms of correlation with marrow infiltration and with respect to the other two populations (Figure 4.5 E).

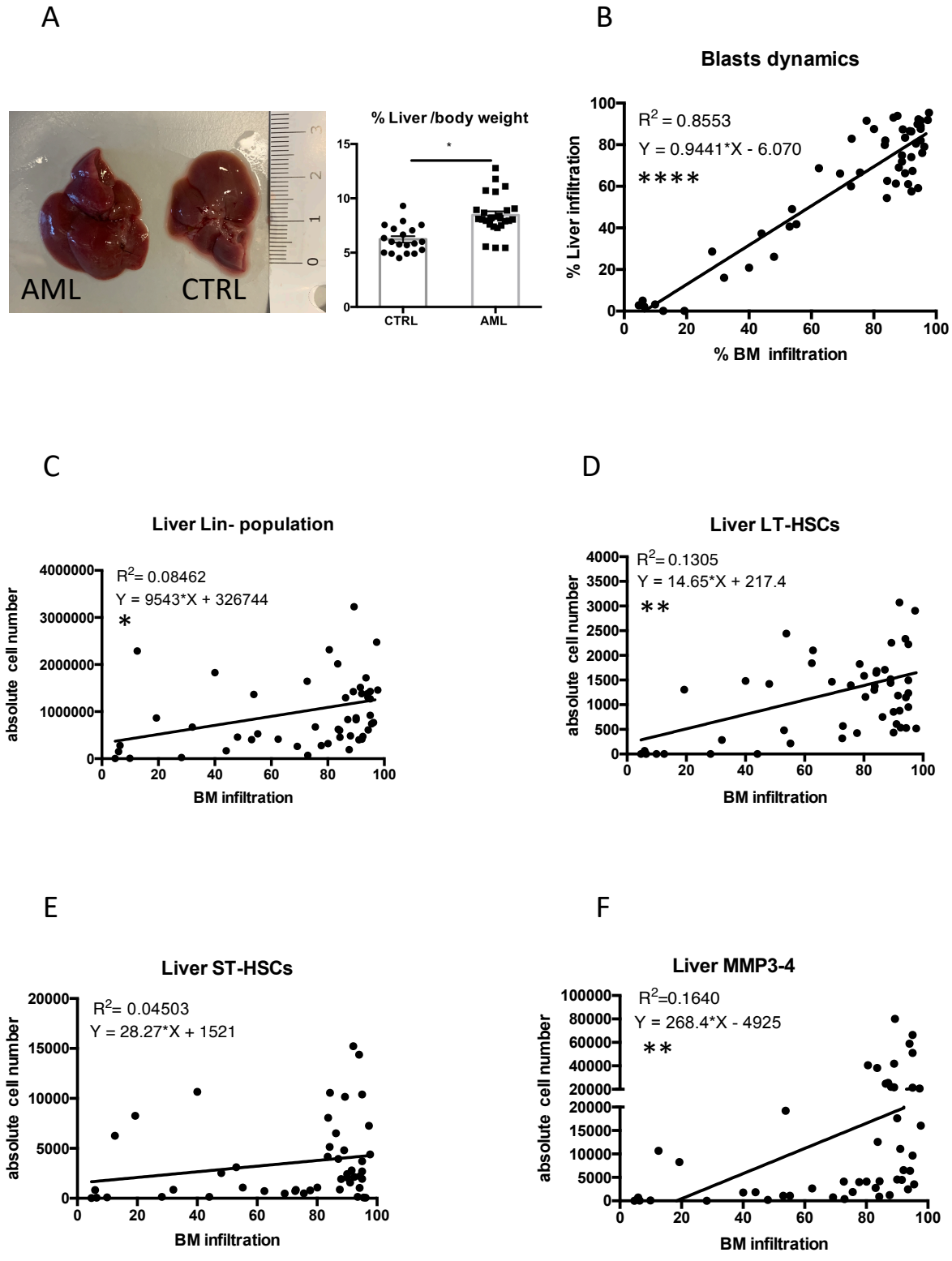

Figure 4.5-HSPCs seed in the hepatic niche

(A) AML progression leads to a slight hepatomegaly despite an increasing number of blasts and extramedullary HSPCs localising within the organ. The % of liver weight/body weight is also shown. *n*=19 control and 26 AML burdened mice Statistical significance determined using Student'T-test **(B)** graph shows a positive correlation between the percentage of blasts in bone marrow and liver. Each dot is a mouse, 54 mice in total from 7 independent experiments**. (C)(D)(E)(F)** show the correlation between BM infiltration and the increase in the number of Lin^- **(C),** LT-HSCs **(D**), ST-HSCs **(E)** and MPP3-

4s **(F)** detected in the liver. The increase is significant for Lin^-, LT-HSCs and MMPs respectively. Each dot represents a mouse, mice pooled from 7 independent experiments. Statistical significance determined using linear regression (* represent $p<0.05$; **represent $p<0.01$)

Next, I investigated the contribution of the $CD48^{neg}$ and $CD48^{low}$ HSCs in the increase of LT-HSCs within the liver. As I previously did for the spleen, I plotted the data based on the various ranges of BM infiltrations described previously. Similar to the spleen, the data highlighted a significant increase in the number of LT-HSCs when the marrow is high infiltrated (Figure 4.6 A). To be sure that the resulting extramedullary LT-HSCs in the liver were due to their migration from the BM caused by AML, I compared their cell numbers in both organs at each infiltration range (Figure 4.6 B). In steady state, the majority of HSCs, as expected, are localised within the marrow. However, through the course of disease and in line with the increased percentage of blasts in the BM, the liver starts to accommodate an increasing number of LT-HSCs. Therefore, at later time points when the BM is fully infiltrated, the LT-HSCs are significantly more abundant in this alternative environment than in their natural niche. Analysis of the $CD48^{neg}$ and $CD48^{low}$ dynamics revealed that the $CD48^{low}$ appear to be significantly higher in liver than the $CD48^{neg}$ (Figure 4.6 D). Again, the comparison of the dynamics of both populations within these two organs shows that following AML progression, they both decrease in their natural niche while raising in the liver (Figure 4.6 E and Figure 4.6 F). Together these findings indicate that in the liver there is an increased number of LT-HSCs that seems to be mostly represented by the $CD48^{low}$ compartment.

Overall, the above results demonstrate that during AML the liver is colonized by HSPCs becoming a site of extramedullary haematopoiesis. Despite both subpopulations of LT-HSCs egressing from the marrow, in the liver the $CD48^{low}$ population represents the predominant component, while the $CD48^{neg}$ HSCs migrate more efficiently to the spleen. Understanding the

journey of these phenotypically defined LT-HSCs is particularly important as they have been shown to have a greater engraftment and haematopoietic reconstitution capacity(Akinduro *et al.*, 2018). Furthermore, representing a long-term store of LT-HSCs, these cells could be involved in replenishing the BM following recovery from AML.

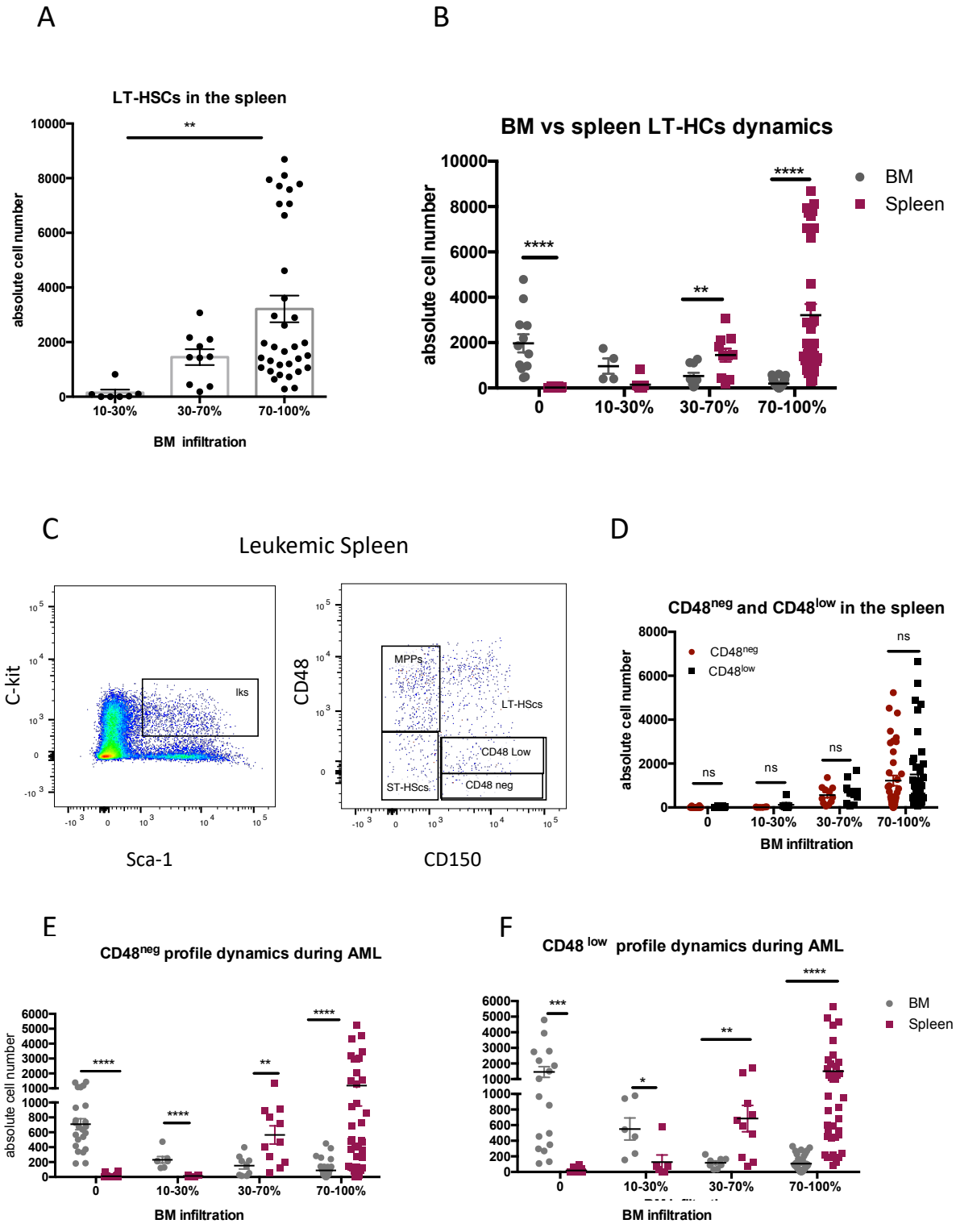

Figure 4.6-LT-HSCs compartment dynamic in the liver relative to AML growth in the BM

Data show the dynamic of LT-HSCs and the two sub-population included in it in liver, and in comparison, to the BM. (**A**) LT HSCs number significantly increases in liver over the course of AML progression while decreasing in the marrow (**B**).

(**C**) Representative FACS plots showing the gating strategy for the CD48^{low} and CD48^{neg} LT-HSCs in liver of AML burden mice. (**D**)Within the liver the number of CD48^{low} LT-HSCs is significantly higher than the CD48^{neg} at both medium and high BM infiltration. When compared to the BM, the number of both populations significantly increases in the liver while significantly decreasing in their natural niche (**E**) and (**F**)**.** Each dot represents a mouse, mice pooled from 7 independent experiments. Error bars represent the mean ±s.e.m. Statistical significance determined using Student's t-test (* represent $p<0.05$, ** represent $p<0.01$ *** represent $p<0.001$ ****represent $p<0.0001$)

4.6- The spleen as a pivotal site of EMH during AML

Following the HSPCs journey, I highlighted how these population migrate and colonise both the spleen and liver with a dynamic that correlates with BM infiltration. However, as cells can freely migrate, I next wondered whether both organs play the same role in this process or not. In order to elucidate this, I compared EMH in both liver and spleen from the same mice at different time points during leukaemia progression. Once again, I plotted the obtained cell numbers based on ranges of BM infiltration (10-30%, 30-70%, 70-100%). I started from the undifferentiated Lin$^-$ population containing the HSPCs (Figure 4.7 A). When plotted against each other, data showed that there is no difference between the detected number of Lin$^-$ cells between the spleen and liver at low (10-30%) and medium (30-70%) BM infiltration time points. However, the difference appears to be significant when the marrow is highly infiltrated, with the majority of Lin$^-$ cells detected in the spleen. I then carried out analysis of the HSPC compartments to try and elucidate where these cells mainly localise. Data analysis of LT-HSCs dynamics in these extramedullary sites revealed that there is no difference between these two organs in terms of LT-HSC number at low and medium-high infiltration, while there is a significant difference in their number when the BM exceeds 70% of infiltration (Figure 4.7 B). On the other hand, ST-HSCs and MPPs are equally distributed between the spleen and liver for each time point (Figure 4.7 C and Figure 4.7 D). The dynamics emerging from these data analysis propose a model in which LT-HSCs, once leaving their BM niche, preferentially migrate to the spleen, revealing how this organ may be a better place for sustaining EMH. In line with these data, the CD48^{neg} LT-HSCs appeared to be significantly higher in the spleen for each infiltration range, followed by the CD48^{low} that are equally distributed at low and medium

infiltration while predominantly in the spleen for a highly infiltrated marrow (Figure 4.7 E and Figure 4.7 F). Particularly, this data fit with what I previously showed regarding this population's dynamics in liver where the majority of LT-HSCs appear to be represented by the CD48^{low} sub-compartment.

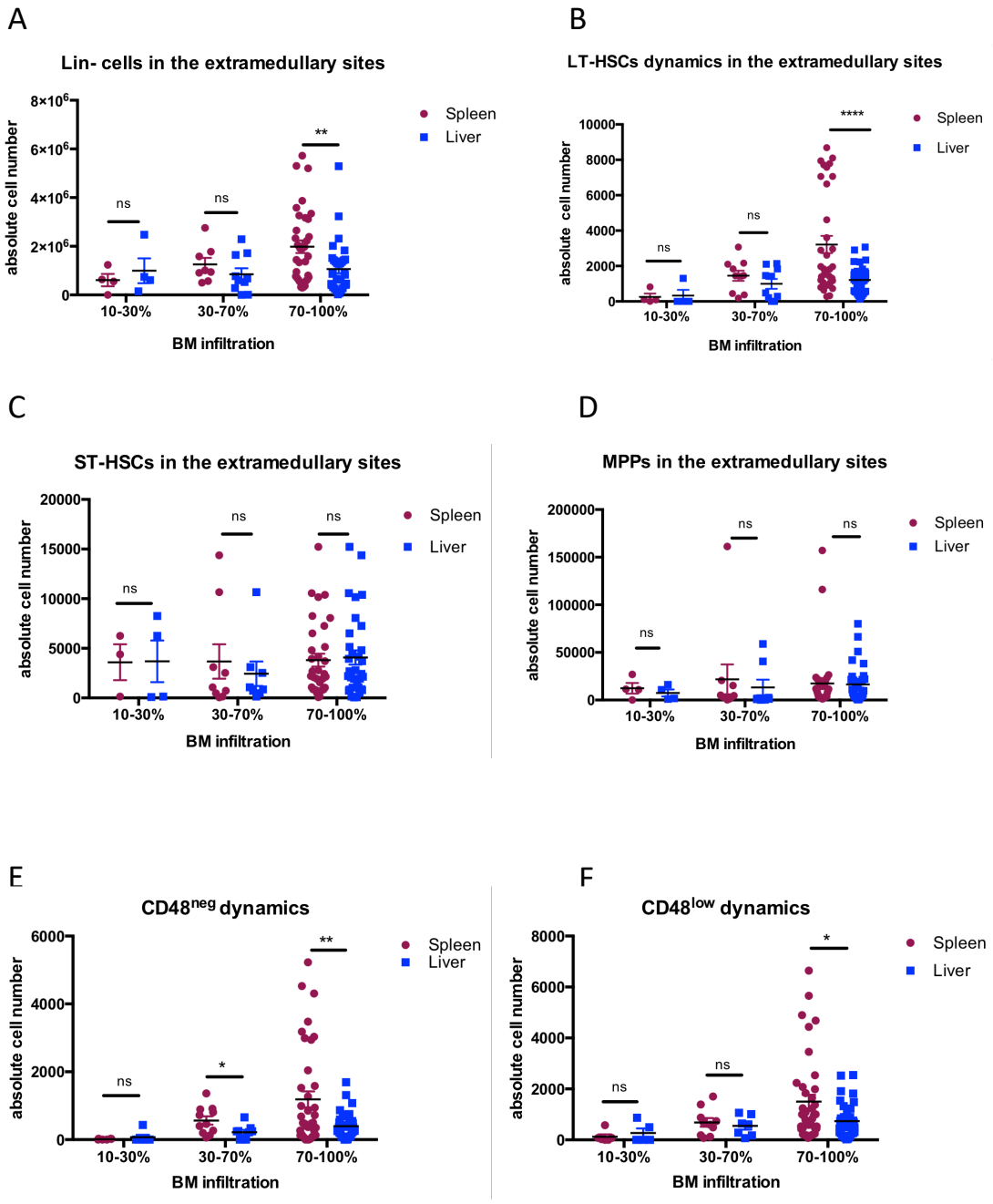

Figure 4.7-Comparison of HSPCs dynamics in the extramedullary sites

Direct comparison of HSPCs distribution in spleen and liver during AML progression. (**A**) At the late BM infiltration range the number of the Lineage$^-$ cells is significantly higher in the spleen than in the liver of AML burden mice**.** (**B**) Similar dynamics involve the LT-HSCs compartment, with cell numbers significantly higher in the spleen than in liver at late disease stages. On the other hand, the number of ST-HSCs (**C**) and MPPs (**D**) do not significantly differ between the two

sites. Analysis of the $CD48^{low/neg}$ LT-HSC revealed that the number of $CD48^{neg}$ cells is significantly higher in the spleen at both medium and high BM infiltration (**E**), while the number of $CD48^{low}$ cells is significantly different only when the marrow is fully infiltrated (**F**). Each dot represents a mouse. Data pooled from 7 independent experiments. Error bars represent the mean ± s.e.m. Statistical significance determined using Student's t-test (* represent $p<0.05$, ** represent $p<0.01$; ****represent $p<0.0001$)

4.7- The expansion of ECs and level of IL-6 differ between the splenic and the hepatic niche

Given that the HSC colonization of the spleen can be facilitated by an expansion of ECs followed by an over-production of cytokines such as CXCL12, SCF and IL-6, I next questioned if the difference in HSC distribution between these two organs could be the results of a differential EC expansion and cytokines expression. Therefore, I evaluated if AML progression induces an expansion of liver ECs and if I could detect any difference in the levels of CXCL12, SCF and IL-6. FACS analysis revealed that the absolute number of ECs was significantly increased in the liver of leukemic mice, where EMH was detected, in comparison to healthy control ones (Figure 4.8 A). Thus, even in the liver this cell population expands in response to AML. However, as HSPCs seem to migrate preferentially to the spleen, I compared the number of splenic ECs with the one observed within the liver at stages in which I could detect EMH. In line with the hypothesis that the spleen constitutes a better niche for HSPCs, the number of ECs in this organ was significantly higher than in liver (Figure 4.8 A).

I next questioned if the increase in ECs number was related to differential or greater production of cytokines in the spleen rather than in the liver. Despite the EC expansion, CXCL12 and SCF expression did not differ in the leukemic liver in comparison to control (Figure 4.8 B and Figure 4.8 C), while IL-6 seemed to be significantly higher in the liver of AML burdened mice (Figure 4.8 D). It should be noted that in liver IL-6 promotes hepatic regeneration by stimulating the production of acute phase proteins following liver injury (Gauldie, Richards & Baumann, 1992;

Cressman *et al.*, 1996; Selzner *et al.*, 2003; Taub, 2004; Michalopoulos, 2007). Therefore, considering the increased number of blasts in the liver, a boost in this cytokine expression can be interpreted as the liver's response to AML-induced damage and not only as a signal leading to HSPC expansion.

Next, I compared cytokines' level between liver and spleen and questioned whether I could detect any difference. With the exception of IL-6 (figure 4.8 D), data analysis revealed that the levels of CXCL12 and SCF were not different between the two extramedullary sites.

These data indicate that, based on the analysed components, the EC expansion represents the main difference between the spleen and liver. Despite both organs being able to accommodate HSPCs during AML development, the spleen seems to represent a site better supportive of EMH. However, the presence of HSPCs in the liver indicate that a certain level of EMH can also happen there, raising the hypothesis that this process is limited by the spleen's presence. Therefore, to truly understand the liver's ability in supporting EMH, I evaluated the HSPC distribution in splenectomised mice.

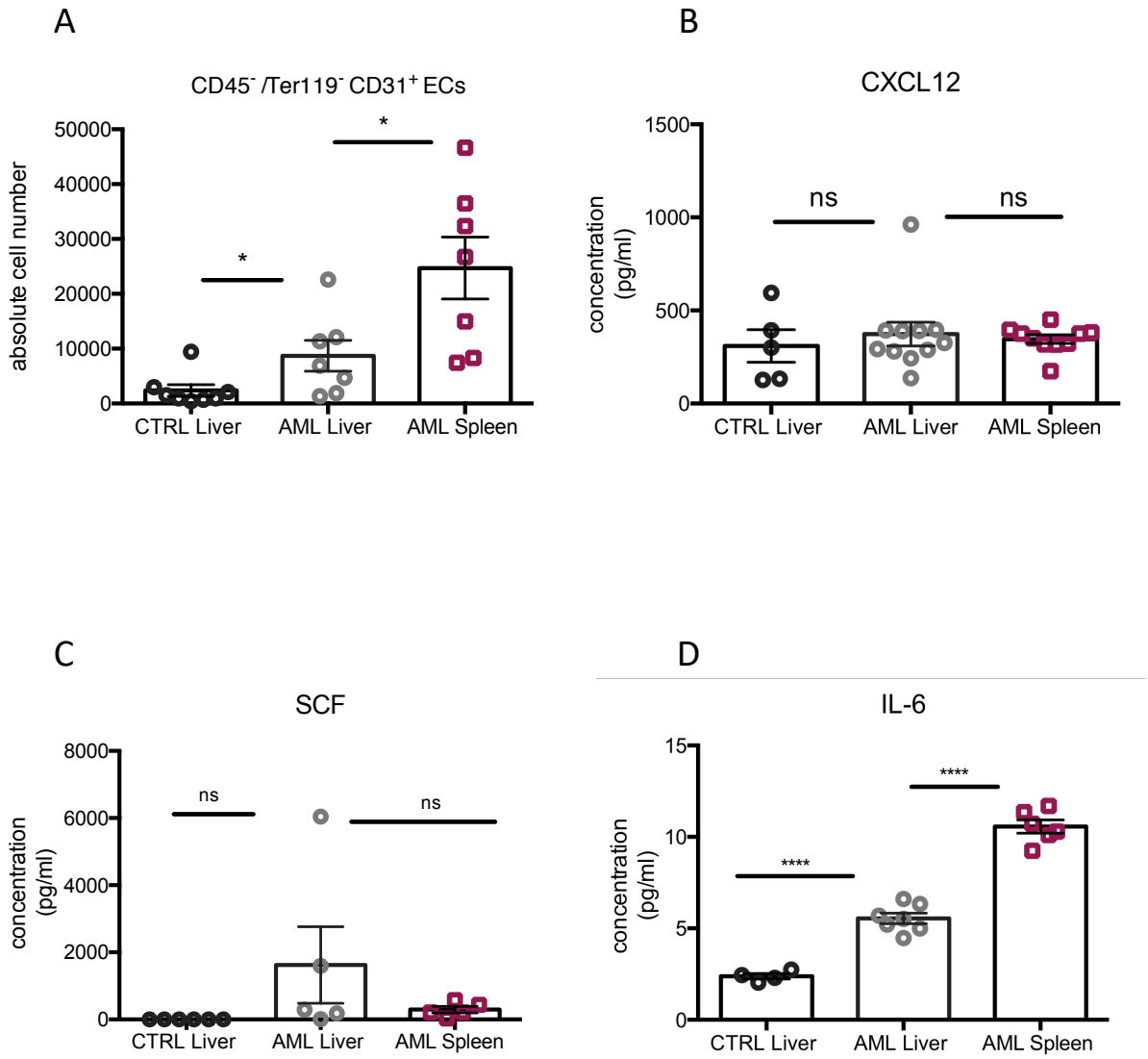

Figure 4.8- ECs expansion and cytokines' expression comparison between extramedullary sites

Data show how ECs and cytokines expression vary in the liver in comparison to the spleen during AML**.** (**A**) ECs significantly expands in liver of AML-burden mice in comparison with control animals. However, when compared with the spleen, the increase in ECs is significantly higher in the spleen than in liver. Analysis of cytokines levels show than there is no significant difference regarding the expression of (**B**) CXCL12 between control and leukemic liver and leukemic liver vs spleen. A similar profile is showed for SCF expression (**C**). On the other hand, IL-6 expression is significantly higher in leukemic liver compared to the control but its expression is higher in the spleen when compared to the liver (**D**) Each dot represents a mouse. n= at least 3 mice per group. Cytokines analysis was performed on liver and spleen supernatant. Error bars represent the mean ±s.e.m. Statistical significance determined using Student's t-test (* represent $p<0.05$; **** represent $p< 0.0001$)

4.8- Splenectomy slightly increases the hepatic niche capacity

The removal of the spleen, also known as splenectomy, is a surgical procedure in which all or part of the spleen is removed. This procedure is often performed in patients with both benign and cancerous condition such as Hodgkin's and non-Hodgkin's lymphoma, CLL and hairy cell leukaemia (Brodsky, Abcar & Styler, 1996; Cusack *et al.*, 1997; Xiros *et al.*, 2000; Sarid *et al.*, 2015; Petroianu, 2015). In the majority of cases, splenectomy is performed to achieve the removal of the bulk of disease, ameliorate hypersplenism and to improve the immune-mediated cytopenias (Cusack *et al.*, 1997; Xiros *et al.*, 2000). Numerous studies have reported a beneficial hematologic response to splenectomy (Adler *et al.*, 1975; Delpero *et al.*, 1990; Thiruvengadam *et al.*, 1990; Cusack *et al.*, 1997) including survival improvement in CLL. However, despite living a generally safe life, patients that undergo splenectomy have a major risk of infections together with cardiovascular complication in the long-term. Nevertheless, most of these risks seem to be due to other underlying conditions rather than the surgery itself (Weledji, 2014). In this context, the liver takes over part of the spleen's role in the immunological response. Several studies have also reported that splenectomy increases the liver's functionality and regeneration in various pathological conditions (Elsebae & Abu-Zekri, 2008; Murata *et al.*, 2008; Weledji, 2014).

As discussed above, in AML both spleen and liver are sites of EMH. However, the number of primitive HSCs and specifically the LT-HSCs, appear to be more localised in the spleen of leukemic mice when compared to their number in liver. Therefore, the spleen appeared to be the principal site where hematopoietic cells, forced to leave from the marrow by the growing blasts, relocate and start the EMH process. Although the spleen represents the preferred extramedullary niche, the fact that a small number of primitive hematopoietic cells could be

detected in the liver made me question whether the presence of the spleen was limiting the liver's ability to accommodate EMH.

To test this hypothesis, I decided to work with splenectomised mice in which the spleen is surgically removed at age ≥ 21 days. Splenectomised mice were allowed to recover for 6-7 weeks after the surgery and AML was injcetd only at this point. Following the dynamics with which HSPCs are dislodged from their niche and bearing in mind the previous data demonstrating the time-window in which these cells migrate and localise in the extramedullary sites, I decided to focus my analyses on the later time points of AML development. Thus, for the splenectomised mice, collected data relates to a BM infiltration range of 70-100%.

Alongside the splenectomised mice, I injected AML in an age-matched cohort of un-splenectomised animals in order to have a comparison between the two. I then started my analysis comparing the numbers of lineage$^-$ undifferentiated cells found in the livers of both groups at late infiltration. In the splenectomised group, lineage$^-$ cells show a trend toward increase although not significant in comparison to the un-splenectomised group (Figure 4.9 A). Next, I moved on to the analysis of HSPCs aiming to see if there was any improvement in their number in the liver due to the spleen's absence. LT-HSCs data analysis revealed a significant boost of these cells in the liver of splenectomised mice in comparison to the un-splenectomised animal (Figure 4.9 B). On the other hand, despite having a trend towards increase, the number of both ST-HSCs and MPPs were not significantly higher in splenectomised mice (Figure 4.9 C and Figure 4.9 D).

Considering the increase in LT-HSCs that migrated to the liver of splenectomised mice, I then determined which part of the two sub-populations was mostly represented in the LT-HSCs

compartment. Thus, I evaluated the dynamic of $CD48^{neg}$ and $CD48^{low}$ in this context. Again, despite their increasing trends, the $CD48^{neg}$ were not significantly higher in the liver of splenectomised mice (Figure 4.9 E). On the other hand, the $CD48^{low}$ number expanded significantly (Figure 4.9 F). These results are in line with what I have previously showed in the liver of non-splenectomised mice, where the majority of LT-HSCs that relocate there due to AML are represented by the $CD48^{low}$ sub-population.

Overall, the obtained data show how the absence of the spleen improves the liver's ability to retain HSPCs. Despite the increase being significantly higher only for the LT-HSC population, these cells have long-term reconstitution ability and therefore their presence in the liver indicate that this organ can potentially maintain haematopoiesis in the short-term. Despite the presence of HSPCs in extramedullary sites represent itself the EMH, the question remained open whether they were functional in either site.

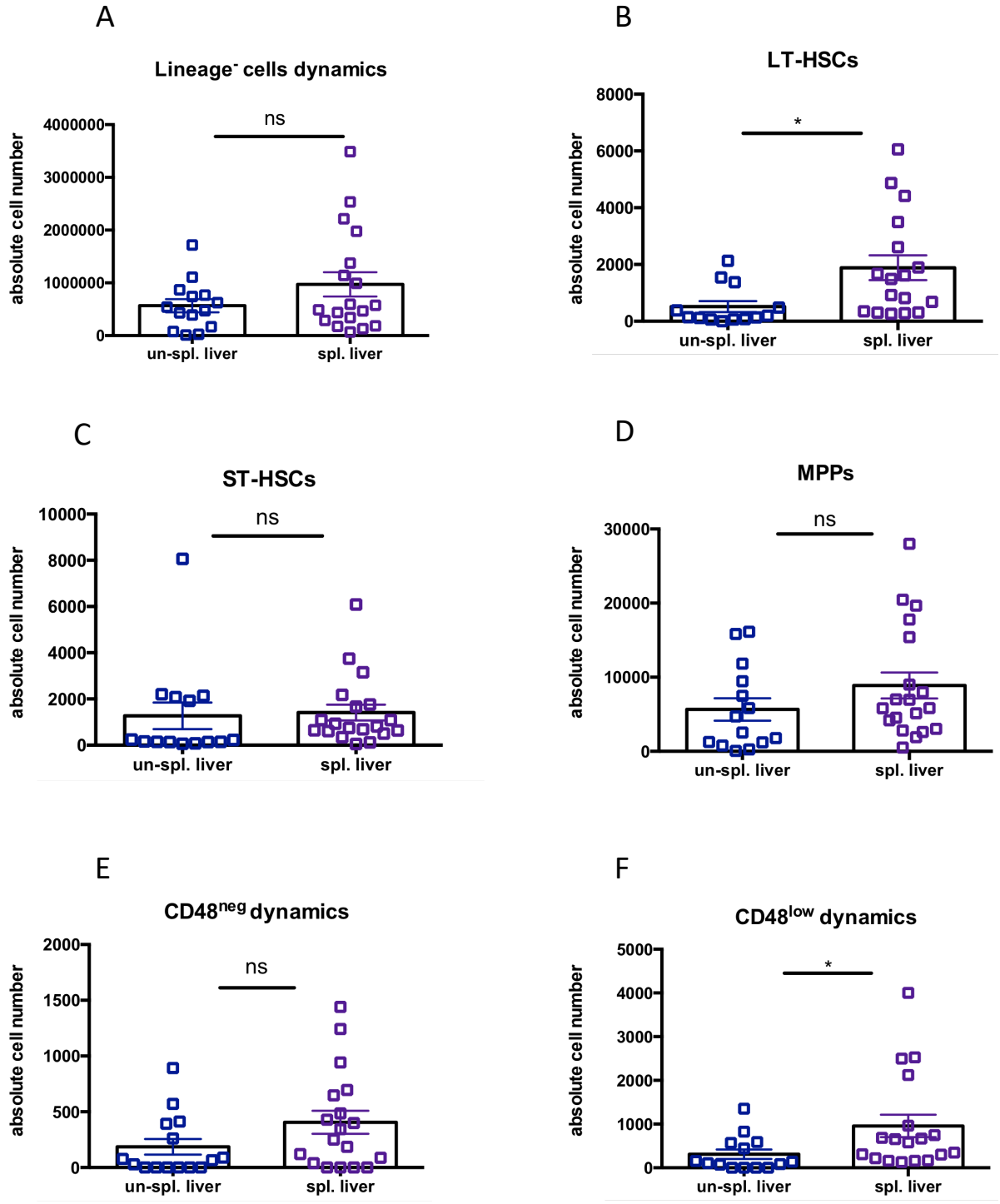

Figure 4.9-Splenectomy slightly enhances EMH in liver

Data show the effect of spleen removal on the dynamic of HSPCs in liver during AML progression. (**A**) despite not being significant, undifferentiated (Lineage$^-$) cells show a trend towards increasing in the liver of splenectomised animals when compared to un-splenectomised ones. (**B**) the absolute number of LT-HSCs significantly increases in the liver of splenectomised mice while no difference was detected for the (**C**) ST-HSCs and (**D**) MPPs numbers among the two groups. Analysis of the CD48$^{low/neg}$ LT-HSCs revealed that splenectomy does not increase significantly the number of (**E**) CD48^{neg} that localised in the liver, although it shows a trend towards increase. On the other hand, the CD48^{low} cells number has a significant boost following spleen removal (**F**)**.** Each dot represents a mouse, 32 mice in total from 3 independent experiments. Error bars represent the mean ± s.e.m. Statistical significance determined using Student's t-test (* represent $p<0.05$)

4.9- Transplantation assays reveal enhanced engraftment of cells from the splenic niche

The functional potential of HSPCs is typically assessed by transplanting them into lethally irradiated mice and measuring their engraftment capacity over time. Given the findings shown so far, I wanted to investigate whether HSCs found in spleen and liver maintain their functionality and are able to give rise to functional EMH. Answering this question will be important to delineate if the extramedullary sites can truly be considered as alternative niches once BM function is compromised.

Despite the enrichment of HSPCs at the extramedullary sites during later stage of AML, sorting the appropriate number of LT-HSCs from spleen and liver to obtain a statistically robust number of recipients per group was challenging. Therefore, to overcome this issue, I set up a transplantation assay in which I sorted the Lineage$^-$ c-kit$^+$ Sca-1$^+$ (LKS) population, which highly enriches for HSPCs and has been used as a robust readout of HSC function in previous work (Rashidi *et al.*, 2014). Lethally irradiated WT recipient mice each received 500 LKS cells from BM, Spleen or Liver of mT/mG donor mice that were previously injected with YFP AML. At the same time, LKS were also sorted from the BM of healthy control donors. In all cases, LKS cells were injected together with 2 x 10^5 WT whole BM competitor cells for haematopoietic support (Figure 4.10 A). Next, I measured the donor-derived reconstitution in the recipient's peripheral blood at 8, 12, 16, and 20 weeks post-transplant together with the proportion of donor-derived B, T and myeloid cells as a measure of multilineage reconstitution. Furthermore, reconstitution of the whole BM and HSPC populations in the BM were also analysed at week 21. Data analysis revealed a significant difference in the proportion of overall donor-derived tomato$^+$ cells when comparing peripheral blood reconstitution of BM LKS cells from control and leukemic mice (Figure 4.10 B). Analysis of the proportion of donor-derived

B, T and myeloid cells in peripheral blood demonstrated a significant difference in the myeloid compartment at week 12 (Figure 4.10 C). At this time point the LKS from control BM contributed to this compartment significantly more than the respective counterpart from leukemic marrow. However, by week 20 there was no significant difference in the lineage output between the two groups.

When I compared the reconstitution ability of LKS cells isolated from the spleens and livers of leukemic mice, data analysis revealed a significant difference at most time points in the proportion of overall donor-derived tomato^{+} cells (Figure 4.10 D). In particular, cells isolated from the spleen had significantly higher reconstitution potential than those isolated from the liver. Analysis of proportion of donor-derived B, T and myeloid cells showed a significant difference for B and T cells compartments at week 8 and 12 (Figure 410 E). In this case, the spleen LKS cells contribute significantly more to the B cell output at both weeks compared to the liver ones. On the other hand, the proportion of T cells produced by the LKS cells isolated from the liver is significantly higher (week 8 and 12). Also, despite not being statistically significant, transplantation of LKS isolated from the spleen resulted in higher levels of myeloid cell production. However, by week 20 there was no significant difference in multi-lineage output between transplanted spleen and liver LKS cells.

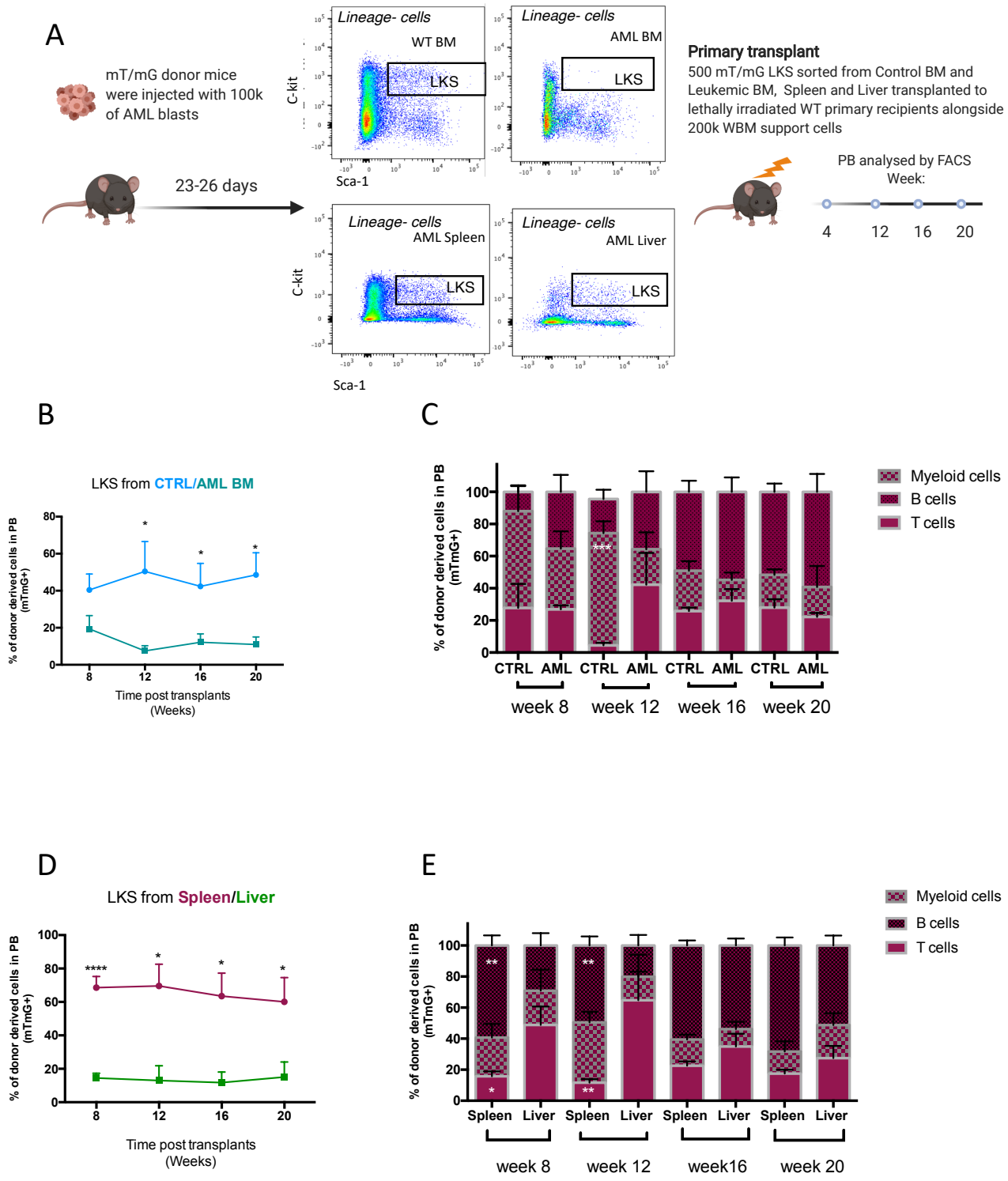

Figure 4.10-Comparison of reconstitution potential of LKS cells sort-purified from BM, spleen and liver

(**A**) mT/mG mice were injected with 100,000 YFP+ AML cells and sacrificed 23-26 days post injection when the marrow reached >90% of infiltration. LKS cells were sort-purified from BM, spleen and liver of AML burdened mice alongside control BM. At this point isolated cells were transplanted into lethally irradiated recipients and PB reconstitution was followed by flow cytometry over time. Comparison of **(B)** PB blood reconstitution and **(C)** the mean proportion of donor-derived B, T and myeloid cells between control and leukemic BM. Similarly, **(D)** PB blood reconstitution and **(E)** the mean proportion of donor-derived B, T and myeloid cells were analysed. n= 5 mice in each group. Error bars represent the mean ± s.e.m. Statistical significance determined using Student's t-test (* represent $p<0.05$, ** represent $p<0.01$; *** represent $p<0.001$)

Following the peripheral blood reconstitution and donor-recipient lineage restricted cells analysis, 21 weeks post-transplant I sacrificed the mice and investigated the donor-derived BM reconstitution and the presence of multiple HSPC populations. When compared altogether, the LKS sorted from control BM and leukemic spleen show higher engraftment of recipients' BM (Figure 4.11 A). Moreover, the BM engraftment capacity of the cells isolated from control BM was significantly higher than that of those sorted from leukemic BM, and liver respectively. At the same time, LKS cells from the spleen led to significantly higher engraftment than liver LKS cells. I also found no difference between LKS from the control BM and leukemic spleen in their engraftment capacity of recipients' BM.

Analysis of donor-derived HSPCs in the BM revealed a significant increase in the proportion of donor-derived LT-HSCs in recipients of LKS cells from the spleen compared to the liver while I detected no difference between the ones from the control BM and AML BM (Figure 4.11B). Similarly, I found a significant increase in the proportion of donor-derived ST-HSCs in recipients of LKS cells from the spleen compared to the liver ones while no difference was detected between those and the other groups. Finally, there was no difference in the proportion of donor-derived MPPs among the four groups.

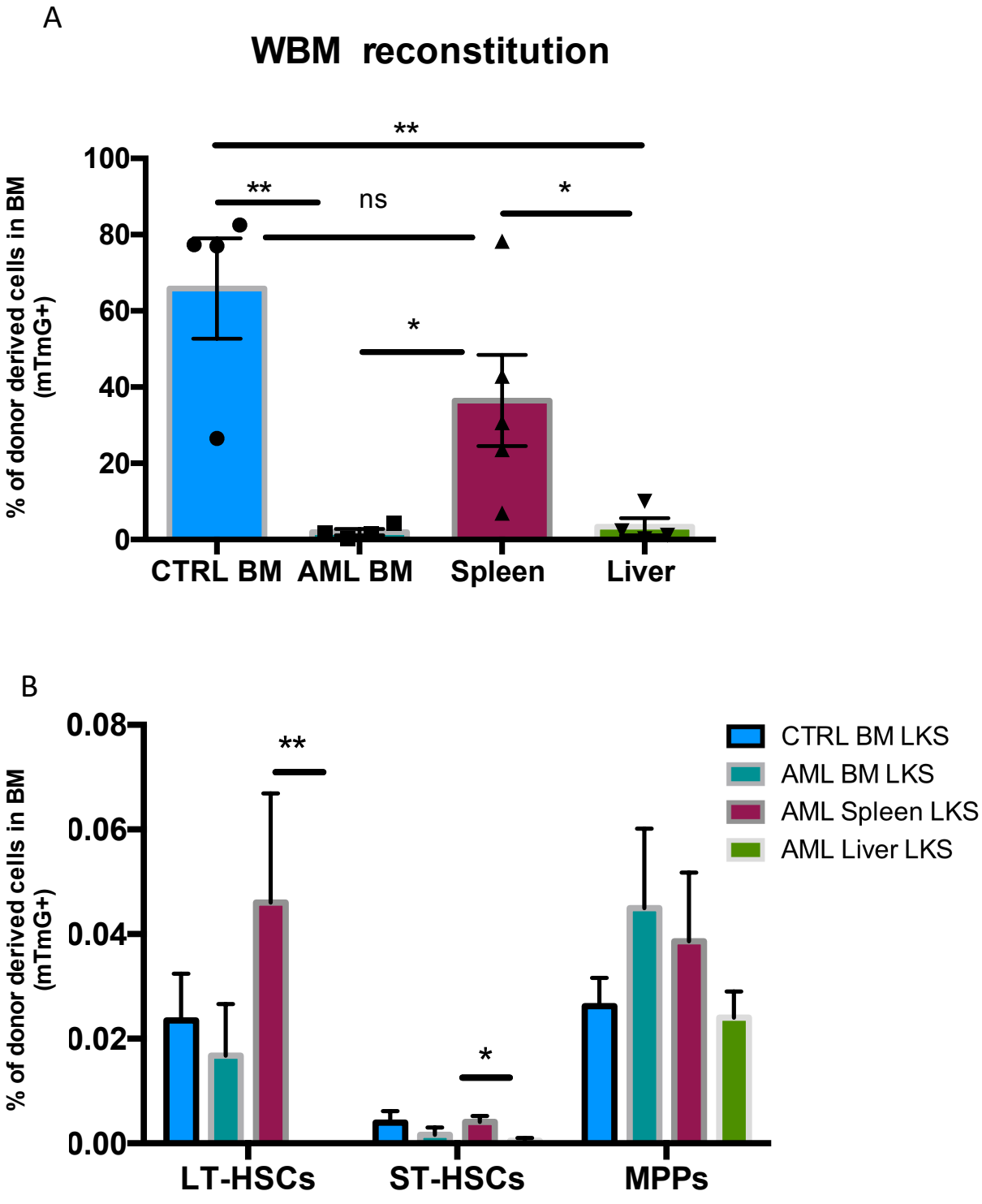

Figure 4.11-Contribution of BM and extramedullary LKS cells to BM haematopoiesis of irradiated recipients

LKS cells isolated from spleen and liver of AML burdened mice were compared with LKS isolated from control and leukemic BM for their ability to reconstitute the BM of lethally irradiated recipients**.** The total reconstitution in the BM of recipients was analysed 21 weeks post-transplant, when mice where sacrificed **(A)**. Analysis of donor-derived LT-HSCs, ST-HSCs and MPPs was also carried out at the end of the primary transplant **(B)**. n= 5 mice in each group. Error bars represent the mean ±. Statistical significance determined using Student's t-test or the Mann-Whitney test (* represent $p<0.05$; ** represent $p<0.01$; *** represent $p<0.001$; **** represents $p<0.0001$)

As the LKS isolated from the liver seemed to have the lowest reconstitution capacity in comparison to the spleen, I decided to look at the proliferative status of the LT-HSC in the extramedullary sites as it is known that proliferation affects engraftment ability (Akinduro *et al.*, 2018). Thus, I performed the 5-ethynyl-2'-deoxyuridine (EdU) assay in which EdU is administered to the mice and is subsequently incorporated within the DNA of dividing cells, allowing us to measure cell proliferation using flow cytometry. Data analysis revealed that the LT-HSCs detected in the liver are significantly more proliferative that those in the BM (Figure 4.12 A) while there was no significant difference in the proportion of proliferating ST-HSCs and MPPs among the three organs (Figure 4.12 B and Figure 4.12 C). Most importantly, the LT-HSCs found in the liver are significantly more proliferative of those in the spleen. Thus, these data show that the liver contains the most proliferative LT-HSCs, contributing to the explanation for their low engraftment capacity. Also, this finding fits with what I have previously shown about the proportion of $CD48^{low/neg}$ LT-HSCs being mobilised to the extramedullary sites during AML. Specifically, in the liver, the proportion of $CD48^{low}$ is significantly higher than the $CD48^{neg}$ LT-HSCs, and the latter have the lower proliferative rate and the greatest long-term engraftment ability (Akinduro *et al.*, 2018).

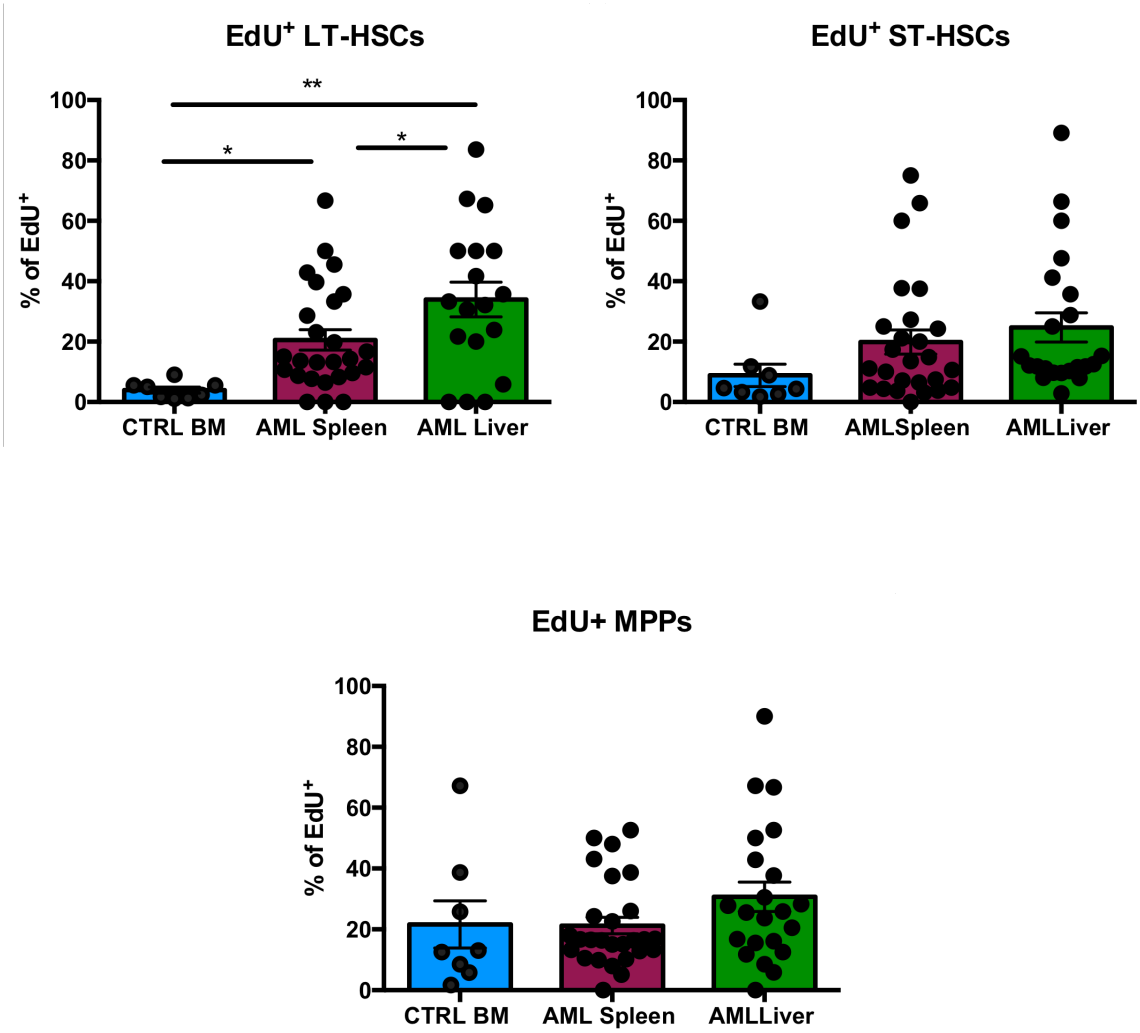

Figure 4.12-HSPC proliferation in BM and extramedullary sites

Quantification of the proliferation rate of HSPCs within different sites. (**A**) LT-HSCs are significantly more proliferative in the extramedullary sites when compared to those in the BM niche. Despite the liver showing a trend through higher proliferation, the EdU uptake for (**B**) ST-HSCs and (**C**) MPPs is not significant different among the tree sites. Each dot represents a mouse, mice pooled from 3 independent experiments. Error bars represent the mean± s.e.m Statistical significance determined using Student's t-test (* represent $p<0.05$, ** represent $p<0.01$)

Overall these data suggest that HSPC detected in extramedullary sites during AML maintain a certain level of functionality with the LKS isolated from leukemic spleen performing better than those from the liver in terms of engraftment and B, T and myeloid cell production potential. As HSCs are able to explicate its own function and give rise to the haematopoiesis process only when surrounded by a proper niche, these data suggest that the splenic

extramedullary site appears to function better than the hepatic ones in supporting the onset of functional EMH.

4.10- Splenectomy does not enhance the engraftment capacity of cells from the hepatic niche

As shown before, splenectomy induces a significant increase in HSPC numbers in the liver of AML-burdened mice in comparison with un-splenectomised animals. Therefore, I wanted to test if removal of the spleen would also enhance the capacity of AML-induced extramedullary cells seeding in the liver to engraft and reconstitute the BM of lethally-irradiated mice.

Once again, I set up a transplantation assay in which I sorted LKS from liver of donor C57BL/6 mice splenectomised at the age of 21 days. Alongside the splenectomised mice, I sorted LKS cells from the livers of an age-matched cohort of un-splenectomised animals in order to have a comparison between the two. Lethally irradiated mT/mG recipient mice received 500 LKS from liver of C57BL/6 splenectomised and un-splenectomised donor mice that were previously injected with YFP AML blasts. In both cases, LKS cells were injected together with 2 $x10^5$ mT/mG whole BM competitor cells for haematopoietic support.

Thus, I measured the donor-derived reconstitution in the recipient's peripheral blood at 8,12,16 and 20 weeks post-transplant together with the proportion of donor-derived B, T and myeloid cells as a measure of multilineage reconstitution. Together with that, I analysed the reconstitution of the whole BM and HSPCs population in the BM at week 21, when mice were sacrificed.

With the exception of week 8 post-transplant, data analysis revealed no difference in the proportion of overall donor-derived WT cells when comparing PB reconstitution of LKS cells from liver of splenectomised and un-splenectomised AML- burdened mice (Figure 4.13 A). At

the same time, analysis of the proportion of donor-derived B, T and myeloid cells in peripheral blood demonstrated a significant difference in the B and T cell compartments at week 8 between the two group (Figure 4.13 B). In fact, at 8 weeks post-transplant, LKS from the livers of un-splenectomised mice contribute to the B cell compartment significantly more than their respective counterparts from splenectomised animals. On another hand, LKS from livers of splenectomised mice produce significantly more T cells than the ones from the other group while no difference was detected for the myeloid compartment. Finally, by week 20 there was no significant difference in the lineage output between the two groups.

Following this analysis, 21 weeks post-transplant I sacrificed the mice and evaluated the donor-derived BM reconstitution and the presence of multiple HSPC populations. Consistently with the data from PB, data analysis revealed no difference in the BM engraftment capacity of the cells isolated from the liver between the two groups (Figure 4.13 C). Similarly, despite a trend for an increase in the proportion of donor-derived LT-HSCs, ST-HSC and MPPs in recipients of LKS from the liver of splenectomised mice, the difference was not statistically significant between the two groups (Figure 4.13 D).

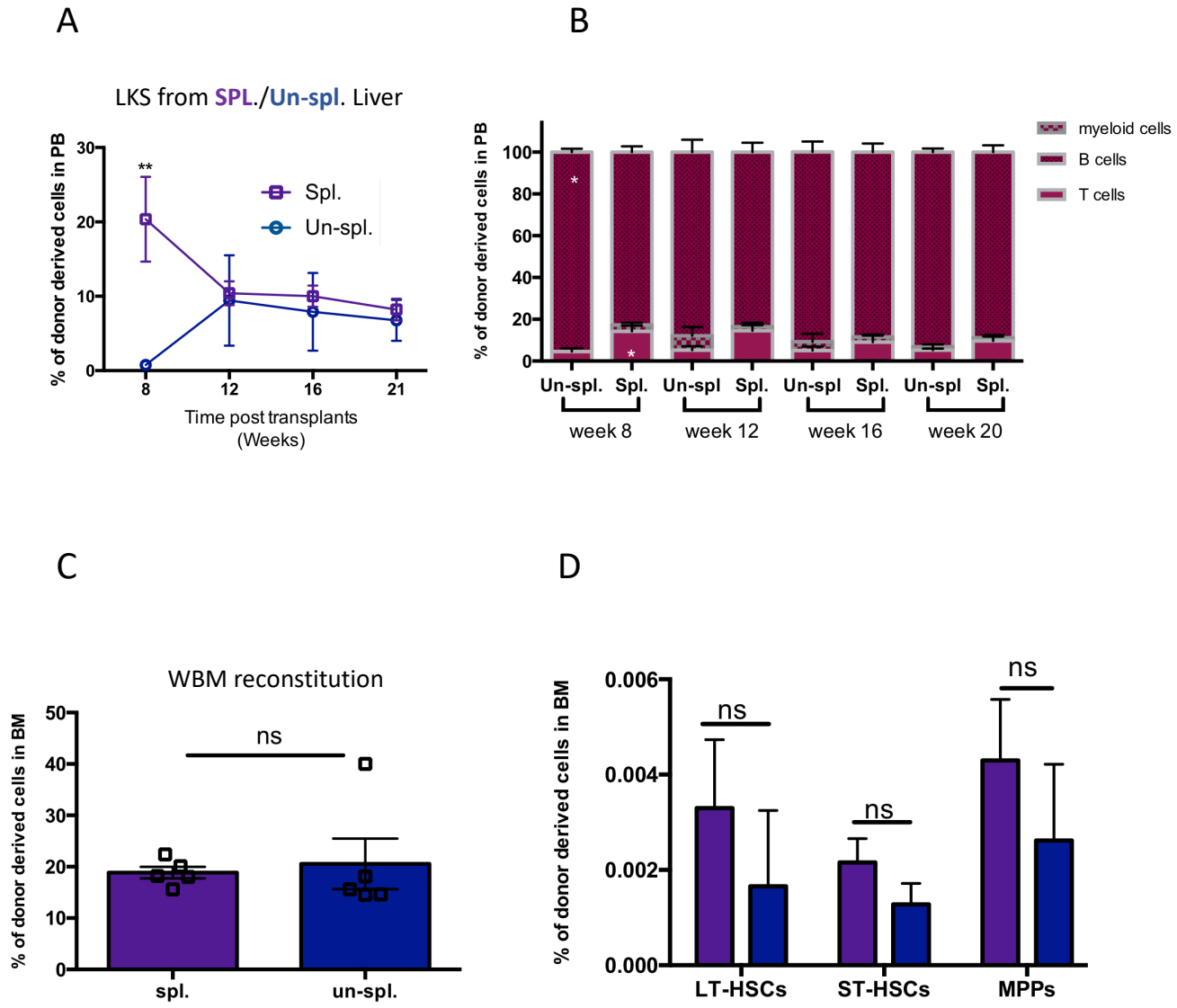

Figure 4.13-Comparison of reconstitution potential of LKS sort-purified from liver of splenectomised/ un-splenectomiced mice

C57BL/6 splenectomised and un-splenectomised mice were injected with 100k of YFP-AML and sacrificed 23-26 days post injection when the marrow reached >90% of infiltration. LKS cells where sort-purified from liver of splenectomised and un-splenectomised mice. At this point isolated cells were transplanted into lethally irradiated recipients and PB reconstitution was followed by flow cytometry over time. Comparison of (**A**) PB reconstitution and (**B**) the mean proportion of donor-derived B, T and myeloid cells between the LKS isolated from AML-burden liver of splenectomised and un-splenectomised mice. (**C**) At week 21, the total reconstitution in the BM of recipients was analysed. (**D**) analysis of donor-derived LT-HSC, ST-HSCs and MMPs was also carried out at the end of the primary transplant. n = 5 mice in each group. Error bars represent the mean ± sem. Statistical significance determined using Student's t-test or the Mann-Whitney test (* represent $p<0.05$)

Overall this data suggests that, although splenectomy improves the liver's ability to retain HSPCs, their engraftment capacity is not enhanced but remain similar to that of cells found in the livers of un-splenectomised animals. This data could partially be explained by the fact that,

as shown before, following splenectomy the increase in LT-HSC number in the liver is mostly due to a greater number of $CD48^{low}$ LT-HSCs seeding in this organ while the ones with better engraftment capacity preferentially migrate to the spleen.

4.11- Discussion

In this chapter, following the results obtained in chapter 3, I questioned the fate of circulating HSPCs, whether these populations were able to localize to other sites outside of the marrow and if these sites could maintain their functionality and drive an emergency haematopoiesis in the short-term.

In the first part, I used flow cytometry to analyse HSPCs throughout disease progression and highlighted how these cells, in correlation with the increasing BM infiltration, migrate to and localise in the spleen and liver. Considering that the presence of HSPCs in organs outside of the BM is defined as EMH, this first result indicated that AML is able to trigger the onset of this process. Furthermore, when comparing both sites for the HSPCs number, I showed that the majority of HSPCs migrate to the spleen with only a small number of cells reaching the liver.

Next, I dissected the LT-HSC compartment aiming to evaluate the proportions of $CD48^{low/neg}$ cells localising in both the extramedullary niches. In fact, we have previously shown that these two subpopulations are functionally different with the $CD48^{neg}$ cells proliferating less than $CD48^{low}$ and displaying significantly better long-term engraftment capacity (Akinduro *et al.*, 2018). Data analysis revealed that both populations significantly increase in the spleen throughout disease progression while their number decrease in the marrow. However, when I carried out a similar analysis for the liver, it emerged how the number of $CD48^{low}$ was

statistically higher compared to the $CD48^{neg}$ population. Thus, this finding revealed a different migratory dynamic for the $Cd48^{low/neg}$ in which the more quiescent HSCs seems to preferentially localise to the spleen.

Next, I questioned the factors that could have a role in attracting HSPCs to the spleen and liver making these environments good niches. Several studies have demonstrated that an expansion of ECs together with an upregulation of CXCL12 and SCF are factors implicated in EMH that happens in the splenic red pulp (Miwa *et al.*, 2013; Inra *et al.*, 2015; Yamamoto *et al.*, 2016). Therefore, I decided to evaluate if AML results in similar events in the spleen at the time of EMH. I found a significant difference in the expression for CXCL12, SCF and IL-6. In fact, their expression was significantly higher in the spleen of leukemic mice, characterised by EMH, in comparison to control mice. At the same time, for the hepatic niche, cytokine expression comparisons showed that only IL-6 significantly differs in its expression between the leukemic liver and the steady state one. Similar result emerged by comparing the leukemic liver and spleen, with the latter showing significant higher level of IL-6 comparing to the liver.

Furthermore, I detected a significant expansion of ECs in both the spleen and liver of AML-burden mice in comparison to their steady state counterpart. Most important, the ECs expansion in the leukemic spleen appeared significantly higher than the one in the liver. Whether the increased levels of CXCL12, SCF and IL-6 are produced by endothelial cell remains to be elucidated.

Altogether these findings lead to the hypothesis that the spleen has a pivotal role as an extramedullary niche during AML.

As I could still detect a number of LT-HSCs in the liver, I decided to evaluate EMH in splenectomised mice wondering if the presence of the spleen was the limiting factor for the ability of this organ in demonstrating proper function as a niche. In fact, in haematological malignancy the removal of the spleen is often carried out with the aim of reducing anaemia, neutropenia or thrombocytopaenia arising as a result of the hypersplenism (Delpero *et al.*, 1990; Weledji, 2014). In this context, the role of the spleen in various settings is then taken over by the liver and there are several studies that have shown how splenectomy improves the liver's regenerative capacity (Adler *et al.*, 1975; Elsebae & Abu-Zekri, 2008; Murata *et al.*, 2008). Data analysis showed that splenectomy significantly increases the number of LT-HSCs that localised in the liver compared to un-splenectomised mice. However, the absence of the spleen does not significantly increase the number of $CD48^{neg}$ LT-HSCs although they show a trend through an increase in comparison with un-splenectomised animals. Overall, these data fit with the role that has been described for the spleen in other pathological conditions in which EMH represents a secondary event (Inra *et al.*, 2015).

Finally, I performed transplantation assays to test the functionality of these LT-HSCs migrated to the extramedullary sites. Surprisingly and opposite to what has been previously shown, cells isolated from control BM had better engraftment than the leukemic ones (Miraki-Moud *et al.*, 2013; Cheng *et al.*, 2015). It should be considered that the disease stage at the time of cells sorting might influence their functionality explaining the difference in the result. In particular, in previous studies HSCs were sorted when the percentage of AML in the BM was around 60-80% (Miraki-Moud *et al.*, 2013; Cheng *et al.*, 2015). However, as I needed to isolate an appropriate number of cells from liver and spleen, I sacrificed mice when BM infiltration was over 90-95%. At this stage of disease, the stress which residual HSCs are exposed to is much higher than at lower infiltrations and could result in a reduction to their functionality.

Furthermore, at this stage of AML as we previously showed, the BM niche is mostly compromised, with vasculature and osteoblasts being loss (Duarte *et al.*, 2017). As both components are essential for the maintenance of HSCs quiescence and fitness, these could also lead to a further reduction in residual HSCs functionality.

When compared with donor-derived lineage restricted cells production, the control BM maintains higher myeloid cell production, followed by the spleen, with the liver being the worse in its ability to reconstitute this compartment. The ability to produce at least 1% of circulating myeloid cells for at least 16 weeks is what distinguishes LT-HSCs from ST-HSCs which have been defined by an inability to produce detectable granulocyte and myeloid progeny beyond 6-12 weeks (Morrison & Weissman, 1994; Adolfsson *et al.*, 2001; Christensen & Weissman, 2001; Yang *et al.*, 2005; Wagers & Weissman, 2006; Dykstra *et al.*, 2007).
This data also fits with those presented previously regarding the different proportion of $CD48^{low/neg}$ cells found in the two organs and strengthened by the HSPC proliferation data showing that within the liver the LT-HSCs, show significant higher levels of proliferation.

Although little is known about human EMH, this process has recently been elucidated in steady state conditions, highlighting the composition at the molecular level of the extramedullary HSPCs in BM, spleen and PB respectively (Mende *et al.*, 2020). At steady-state, a rare population of phenotypic HSCs has been shown to reside in the murine spleen (Morita et al 2011) however from the data above it appears that during AML the dynamics with which this population appear in this organ and decrease in the marrow delineate a migratory pathway for BM HSPCs more than an expansion of the splenic residual ones.

The results presented in this chapter raised a number of questions that will need further experiments to be answered. Here, I dissected the arising EMH by analysing cells based on the LKS SLAM family markers and then I looked at difference between more and less quiescent populations within the LT-HSC compartment using the $CD48^{low/neg}$ subdivision (Akinduro *et al.*, 2018) previously elucidated in the lab. Further analyses should include extra markers such as $CD34^{+/-}$ or $EPCR^{+}$ (Kent, Dykstra & Eaves, 2007) to evaluate other subpopulations of quiescent HSCs as it would be interesting to investigate if their migratory pathway among the spleen and liver is consistent with what I already observed. Even within the populations I identified, functional assays and proliferation analyses should be focussed on the $CD48^{neg}$ HSC subpopulation, as it is less heterogeneous and more primitive (Akinduro et al., 2018). Furthermore, future work should investigate the transcriptional states of cells found in extramedullary sites and make a comparison between them and those in the marrow and peripheral blood as these results may help to highlight a preferential migratory pathway that BM cells follow during the onset of EMH. Recently, it has been shown that in humans at steady-state, HSCs found in the spleen and peripheral blood are transcriptionally different from those found in the BM (Mende *et al.*, 2020) This difference is important as it elucidates a differential molecular structure for haematopoietic cells resulting in their varying abilities to rapidly produce lineage- primed cells in the case of emergency haematopoiesis (Mende *et al.*, 2020).

According to the data presented here, the onset of EMH in both spleen and liver correlates with a migration of HSPCs from the marrow once that it becomes fully infiltrated with blasts. However, it would be interesting to investigate whether during AML growth the small population of HSPCs that reside in both organs would contribute to EMH during the increased hematopoietic demand and whether these cells rely on a different molecular structure. Furthermore, a similar approach could highlight if cells derived from splenectomised mice

maintain the haematopoietic genes landscape or whether the absence of the spleen generate changes in the molecular signature. It is known that together with the spleen and liver, other organs can work as a site for EMH in emergency situations. Thus, in the future, it will be worth investigating this process in other sites such the thymus, lungs and lymph nodes.

Regarding the environment of these alternative niches, despite the fact that I have shown an expansion in the $CD45^{-}/Ter119^{-}$ $CD31^{+}$ ECs in both the leukemic spleen and liver, in order to lead this event to an increase in cytokines production it would be worth performing, an intracellular staining of ECs derived from both organs in leukaemia and steady state condition and analysing it by flow cytometry. This would be useful to evaluate the involvement in cytokines production of other cells within these sites and it will be interesting to use transgenic mice such as the Scf^{GFP} and the $Cxcl12^{DsRed}$ knock-in as they will allow us to analyse and quantify the differential expression of both cytokines within the spleen and liver (Ding *et al.*, 2012; Ding & Morrison, 2013; Inra *et al.*, 2015). Although CXCL12 and SCF have been linked to onset of EMH, to really prove that this is the case in AML it would important to carry out experiment aiming to block these cytokines, using specific blocking antibodies that will combine with the cytokines preventing them from binding to their receptor or using conditional knock-out mice such for example the $CLXL12^{fl/+}$ or the $Scf^{fl/+}$ as this will demonstrate if their role is truly pivotal for the colonization of extramedullary sites. Here, together with CXCL12 and SCF, I have focused on cytokines known to be important for HSC maintenance within the marrow and therefore are able to promote the EMH. However, in future it would be useful to perform a multiplex ELISA assay as this will allow to evaluate whether any other factors may contribute to the promotion and maintenance of EMH. Furthermore, it is opportune to consider whether the level of these cytokines change based on disease progression and if the number of HSPCs found in the two organs correlates to the changing levels. Although I performed IVM

of the mouse spleen, more imaging should be carried out and should include different disease stages. In fact, carrying out IVM of the spleen (and liver) would allow to directly visualise how the extramedullary HSPCs interact with the new environment, catching in real-time cell-cells dynamics that may promote EMH maintenance. For example, performing IVM on Scf^{GFP} and $Cxcl12^{DsRed}$ reporting mice, would allow to characterise how close to the SCF and CXCL12 expressing cells the extramedullary HSPCs seed and how they interact with them. Furthermore, in future, immunohistochemistry experiments together with histopathological analysis of the spleen and liver should be considered in order to better characterise the cell types and niche factors that support the functional EMH. At the same time, similar approaches should be applied for studying the liver of splenectomised mice.

After having shown the onset of EMH, I questioned the functionality of the HSCs found in these sites and therefore I set up transplant assays. In these experiments I sorted the LKS cells from the spleen and liver of leukemic mice as this population is highly enriched with HSPCs. However, future experiments could aim to sort and transplant LT-HSCs as this will allow direct comparisons of the functionality of cells that are known to be the ones with long-term reconstitution capacity and at the top of the hematopoietic hierarchy. Data analysis shows that the LKS isolated from the spleen had a better engraftment and reconstitution potential that those from the liver. This could be explained by the fact that the majority of the $CD48^{neg}$ LT-HSCs migrated to the spleen and these cells are the ones with the higher engraftment capacity(Akinduro *et al.*, 2018). Thus, further work could focus on isolating and transplanting the $CD48^{neg}$ LT-HSCs from both organs in order to assess whether these cells are functionality different. On the other hand, as the liver contains greater proportions of $CD48^{low}$ cells than $CD48^{neg}$ LT-HSCs, this could reflect a situation in which the LT-HSCs cells migrating to this organ, being more primed to give rise to progenitors and mature cells, could be the ones able

to respond quicker to a demand for alternatively sourced haematopoiesis. This idea is in part suggested by the data showing that the HSPCs in the liver are more proliferative than those in the spleen. These data were generated using EdU assays that give a qualitative indication of the proportion of proliferating HSPCs within these organs. However, in future, it may be worth performing the dual-pulse method using both EdU and BrdU previously developed by our research group (Akinduro *et al.*, 2018). This assay provides a more quantitative measure of proliferation and allows us to determine cells that are entering, within and exiting S-phase, thus allowing the calculation of proliferation rates. The use of this technique to analyse leukemic mice demonstrated that a constant proportion of AML cells enter S phase per hour with the only exception of blasts in PB that are less proliferative (Akinduro *et al.*, 2018). Therefore, it would be interesting to analyse in this context what happens to the proliferation rates of cells at extramedullary sites. In fact, the increase in HSPCs number and proliferation rate could both potentially result from relocation of these population in the extramedullary sites but also from an expansion of super rare pre-existing cells. Here, the steep curves related to the HSPCs cells number in PB and extramedullary sites suggested that the AML-induced EMH may results from a relocation process instead of an expansion of pre-existing cells. At the same time, data analysis showed that both the Lineage$^-$ and MPPs population accumulate faster in the spleen than in the PB, while LT-HSCs and ST-HSCs have a similar dynamic in the two sites. This observation suggest that the spleen may represent a better niche for progenitors than for HSCs and that the detected ECs expansion may have to occur first and before HSCs can seed in this alternative niche. However, to get a deeper insight into the migration and differentiation dynamics of haematopoietic compartments in each organ, would be important to generate a mathematical model. For example, a simple mathematical model could be obtained by evaluating the proliferation rate of HSPCs in spleen and liver and combining this data with the information related to the HSCPs absolute number. Then, the use of this model will allow to

compare the HSPCs migration and differentiation dynamics in steady-state and disease condition.

It should be noted that the transplantation assays in this chapter were performed by isolating and transplanting LKS cells from mice fully infiltrated with AML and that this may have determined a different outcome regarding the engraftment capacity of LKS isolated from AML BM. Thus, future work could also consider performing transplantation assays at earlier disease stages in which the EMH is still detectable. This will give more insight into how HSCs isolated from early stage of AML perform compared to the ones isolated at late disease timepoint. Despite the analysis of the WBM at week 21 suggesting that between the LKS isolated from the two extramedullary sites, only those from the spleen could sustain a secondary transplant, this experiment should be repeated in future in order to confirm whether these cells are able to reconstitute haematopoiesis in the long-term and whether they are able to self-renew.

Overall, I have shown that AML induces HSPC loss by dislodging them from their BM niche (Chapter 3) and that these cells then likely migrate to extramedullary sites from which, with varying capabilities, they maintain the hematopoietic process in the short-term. The discovery of alternative extramedullary niches able to sustain the haematopoietic process could have significant clinical relevance. In fact, from these sites, HSPCs not only may maintain haematopoiesis but most importantly, following chemotherapy treatment, these cells could potentially find their way back and replenish the BM. Future work should aim to evaluate the behaviour of the HSPCs in the extramedullary sites after chemotherapy treatment and whether an increase in the numbers of these cells repopulating the marrow correspond to a reduction of those located in the extramedullary sites. For example, a single cells RNAseq experiment

would highlight whether the HSCs that replenish the marrow after chemotherapy have the same signature of the ones founded in the extramedullary sites.

Despite understanding and improving EMH being undoubtedly useful, reducing the loss of healthy cells from the marrow would ultimately confer great improvement in AML treatment as it would circumvent an even temporary need for alternative niches. Therefore, in the next chapter I aim to find a potential therapeutic target for reducing HSPC loss and maintaining a greater number of residual HSPCs in the marrow throughout disease progression.

Chapter 5

Maintaining healthy haematopoietic cells in their niche: the effect of metalloproteinase inhibition

5.1- Introduction

In chapter 3, IVM data analysis of AML blasts interacting with healthy cells revealed how the latter are induced to egress from the marrow as large clusters, while in healthy control mice egressing cells are mostly single or in clusters of two. This observation led to the hypothesis that leukemic blasts may be able to gradually digest the ECM and this could contribute to healthy cells' dislodgment. Due to their role in matrix remodelling and their implication in cancer, I wondered if MMPs could play a role in promoting blast migration across the BM parenchyma – a process that ultimately leads to the elimination of entire clusters of healthy cells from the tissue. Most importantly, in Chapter 4, I highlighted how the majority of these dislodged cells were relocating in extramedullary sites where, thanks to the maintenance of some of their functionality, they could drive EMH. As the loss of healthy cells from their natural niche results in cytopenia, finding a mechanism to prevent this loss and promote HSPCs retention in the marrow will be fundamental for the development of new therapeutic approaches in AML.

Therefore, in this chapter, I investigate the effect of a specific MMPI on leukaemia progression and the haematopoietic system. Tumour growth is a multistep process that includes cell division and proliferation, alongside ECM digestion that promotes blasts migration through the basement membranes in order to reach the circulatory system and give rise to metastases

(Kleiner & Stetler-Stevenson, 1999; Lochter & Bissell, 1999; Hidalgo & Eckhardt, 2001). Nowadays, several evidences have linked MMPs dysregulation with cancer progression, invasion and metastasis. This is particularly the case for the gelatinases MMP-2 and MMP-9 for which changes in their expression lead to poor prognosis and aggressiveness of several cancers, including leukaemia (Li *et al.*, 2004; Stefanidakis & Koivunen, 2006; Gobin *et al.*, 2019).

Considering that, I first compare MMPs expression levels between AML blasts and healthy cells. Among the various overexpressed MMPs, I decided to focus on the MMP-9 as it has been mostly implicated in cancer progression and metastasis. For example, in chronic lymphocytic leukaemia (CLL), the elevated levels of MMP-9 have been shown to correlate with advanced disease stages and poor patient survival (Kamiguti *et al.*, 2004). Furthermore, recently, MMP-9 inhibition has been reported to decrease cell invasion and tumour burdened in B-ALL (Verma *et al.*, 2020). Therefore, I treated AML-burdened mice with prinomastat, an MMPI particularly active on MMP-9. I examined the direct effect of this inhibition on AML blasts migration, growth and apoptosis. I then used a combination of IVM and flow cytometry to investigate effects on BM vasculature as MMPs have been shown to have a role in vascular remodelling. Next, I questioned the effect of MMPI treatment on HSPC maintenance through AML growth and its role as a new therapeutic approach in combination with chemotherapy treatment. This study highlights a potential strategy to retain HSPCs in their natural niche and suggests a new therapeutic target to improve the overall response to AML treatment.

5.2- Metalloproteinase expression and targeting in AML

As mentioned early, MMPs are part of the proteolytic enzymes family implicated in ECM remodelling. MMPs are generally classified into 5 classes according to their primary structure and substrate specificity. Thus, it is possible to distinguish between the collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, MMP-11 and MMP-12), membrane type (MT)-MMPs (MT1-MMP, MT2-MMP, MT3-MMP and MT4-MMP) and finally the non-classified MMPs (Kleiner & Stetler-Stevenson, 1999; Hidalgo & Eckhardt, 2001). For each group, relative substrates could be found in Table 2. Normally, MMP expression is highly regulated at both the genetic and activation level. In fact, these enzymes are not constitutively expressed *in vivo* and their transcription is induced by various stimuli including cytokines, growth factors and cell/cell or cell/matrix interaction (Hidalgo & Eckhardt, 2001). Moreover, their function is tightly controlled and their proteolytic activity is inhibited by nonspecific protease inhibitors (α2microglobulin and α1antiprotease)and by specific tissue inhibitors of metalloproteinases (TIMPs) (Westermarck & Kähäri, 1999).

Group	Metalloproteinase	Substrates
Collagenases	MMP-1; MMP-8; MMP-13	Fibrillar collagen
Gelatinases	MMP-2; MMP-9	Gelatine, Collagen I, IV, V
Stromelysins	MMP-3; MMP-7; MMP-10; MMP-11, MMP-12	Laminin, fibronectin, Non-helical collagen, gelatinine
MT-MMPs	MMP-14; MMP-15; MMP-16; MMP-17	Collagen I,II,III , gelatine
Non-classified MMPs	MMP-19, MMP-20, MMP-21; MMP-22; MMP-23; MMP-27; MMP-28	Elastin, fibronectin, laimini, nidogen,tenascin, aggrecan, cartilage, oligomeric protein, amelogenin

Table 2 MMPs classification and substrates

Thus, I started by exploring the expression profile of MMPs in our AML model. I had access to raw RNA sequencing data, previously generated by Delfim Duarte (Duarte et al., 2018), which allowed me to analyse the gene expression profiles of AML blasts against granulocytes-monocyte progenitors (GMPs), the latter chosen because our AML model is based on the retroviral transduction of GMPs sorted from healthy donors and is propagated by leukaemia stem cells which resemble GMPs. I aimed to investigate whether or not leukemic blasts expressed MMP genes and if this expression varied comparing to that of GMPs. Surprisingly, the data analysis revealed that, among other genes, AML blasts highly expressed the collagenase MMP-8, the gelatinase MMP-9 (also known as gelatinase B) and the non-classified MMP-19 (also known as a RASI-1) (Figure 5.1 A). Out of these MMPs, I decided to focus on MMP-9 as its overexpression has been shown to promote cancer cell migration and invasion in several human tumours (Scorilas *et al.*, 2001; Li *et al.*, 2004; Paupert *et al.*, 2008)In B cell

acute lymphoblastic leukaemia (B-ALL), for example, MMP-9 overexpression together with MMP-2 were associated with tumour extramedullary infiltration and poor prognosis specifically related to MMP-9 expression (Pan *et al.*, 2014)

Thus, I decided to test the effect on AML of a selective MMPI called prinomastat (AG3340). Prinomastat shows high affinity for MMP-2 and MMP-9 and has low affinity for MMP-13 and MMP-14. This drug has been studied in the metastatic setting of ovarian, pancreatic, lung, breast and prostate carcinoma (Liu *et al.*, 2003; Hande *et al.*, 2004; Bissett *et al.*, 2005) and has been shown to reduce metastasis and enhance animal survival when combined with carboplatin in an orthotopic model of non-small cell lung cancer (Liu *et al.*, 2003). I planned prinomastat treatment in a way that allows the MMPI to properly explicate its mechanism of action, as it is known that the effect of these molecules relates to a specific time window of disease progression. Specifically, MMPIs are effective before the disease starts to spread and therefore at the early stages. Thus, mice injected with leukaemia started to receive daily intravenous injection of prinomastat 7 days post-AML injection for a total length of 15 days of treatment (Figure 5.1 B). Control mice were injected with AML at the same time, and received PBS as the vehicle control and following the same therapeutic scheme as for prinomastat. Disease progression and any other sign of distress were carefully monitored throughout the regimen. Peripheral blood (PB) was routinely analysed by flow cytometry to quantify infiltration as an indication of disease advancement. Data analysis revealed no difference in the percentage of blasts in peripheral blood between the two groups (Figure 5.1 C). I next carried out IVM to evaluate the presence of AML blasts in the calvaria of mice from both groups, as this would give an indication of the disease spread within the BM. Surprisingly, high-resolution images of the whole calvaria revealed that the BM of prinomastat treated mice was less infiltrated by leukemic blasts when compared to that of PBS treated ones, despite the similar PB infiltration

(Figure 5.1 D). This observation was promising as it suggested that prinomastat was having a specific effect on leukemic blasts within the marrow. Flow cytometry analysis revealed that, at time of sacrifice (PB >20% of blasts) the BM, spleen and liver of prinomastat treated mice were significantly less infiltrated that those from the PBS treated group (Figure 5.1 E, F and G). These first results strongly suggest that prinomastat treatment affects the ability of leukaemia to spread as it reduces blast invasion within the BM and in the extramedullary sites. Following these observations, I decided to analyse specific AML blast features with the aim of uncovering any changes induced by the treatment that could disrupt disease outcome.

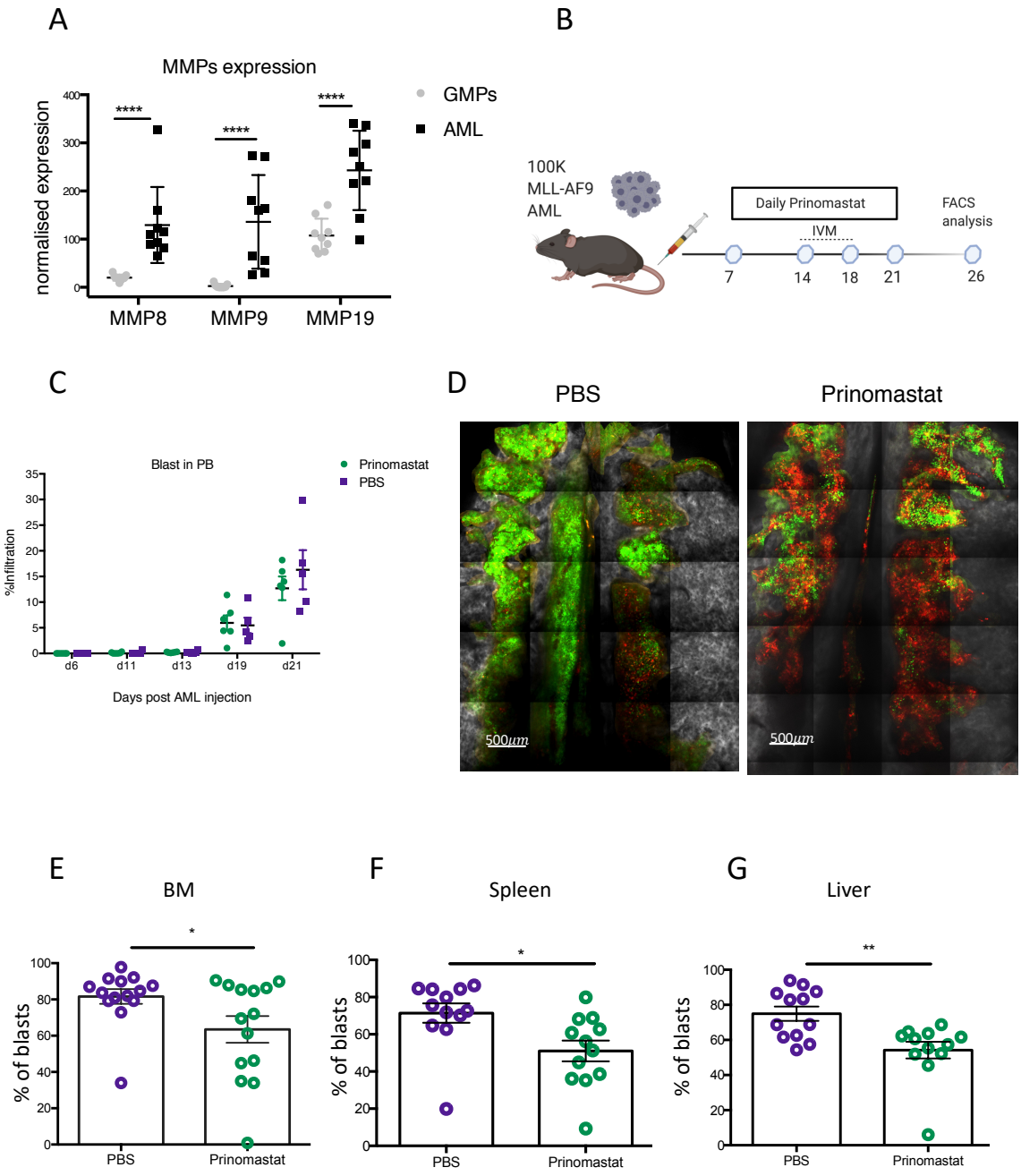

Figure 5.1-MMPs expression and prinomastat treatment of AML-burdened mice

(**A)** Differential expression of MMPs in found in MLL-AF9 AML blasts in comparison to GMPs. Each dot represents a mouse, 9 mice in total. Error bars represent the mean ± se.m. Statistical significance obtained using Student T-test (**** represent $p<0.0001$). (**B**) shows the treatment plan for the MMPI prinomastat. Mice injected with 100,000 AML blasts receive 13.5mg/Kg of prinomastat daily via IV injections for a total of 15 days. (**C**) PB infiltration shows no difference between the groups. (**D**) representative high-resolution tile-scans of prinomastat treated and PBS treated mice calvaria having similar PB infiltration; green is AML, red are healthy hematopoietic cells**.** (**E**)(**F**)(**G**) show the percentage of AML blasts in BM, Spleen and Liver respectively. Each dot represents a mouse, n= at least 3 mice per group from 2 independent experiments. Error bars represent mean ± s.e.m. Statistical significance determined using Student's t-test (* represent $p<0.05$)

5.3- Prinomastat affects blasts speed, proliferation and apoptosis

Analysis of the PB showed no difference in the percentage of circulating blasts among the two groups. However, when analysed by flow cytometry alongside IVM, the BM, spleen and liver of treated mice showed a lower percentage of blasts. I questioned whether a reason for the decrease in AML infiltration within these organs could be an impairment of parameters such as blast speed, proliferation and apoptotic rate. We previously showed that AML blasts are highly dynamic at early disease stages and that seeding AML cells migrate significantly faster than the seeding T-ALL blasts (Duarte *et al.*, 2018) Therefore, migration is a conserved trait of malignant cells and targeting it could impair the blasts ability to move through the microenvironment.

Thus, I performed IVM on PBS and prinomastat treated mice with the aim of discovering any change in leukemic blast migratory behaviour. Surprisingly, data analysis revealed that within the marrow, blasts in the prinomastat treated group were migrating with a significantly lower speed that those in the control group (Figure 5.2 A). However, as a reduction in speed does not entirely explain the lower percentage of blasts within the marrow and in extramedullary sites, I questioned whether MMP-9 inhibition could somehow impair the growth of AML. In fact, the observed reduction in blasts infiltration could be explained by an alteration of their proliferative and apoptotic rates. To test this hypothesis, I studied AML blasts in PBS and prinomastat treated leukemic mice using flow cytometry analysis of EdU uptake to measure proliferation and of Annexin V/7AAD labelling to quantify apoptosis of AML cells. I analysed AML blasts from the BM, spleen and liver. Analysis of leukaemia proliferation revealed that in the prinomastat treated group, AML cells proliferate significantly less in BM and liver in comparison to the PBS treated group (Figure 5.2 B and D). On the other hand, blast proliferation in the spleen was not significantly different between the two groups (Figure 5.2

C). In parallel, I could observe a significant increase in apoptosis of AML blasts in the BM and spleen of prinomastat treated mice (Figure 5.2E and F). However, no significant difference in apoptosis of AML cells was detected in the liver between the two groups (Figure 5.2G).

Altogether, these results suggest that AML growth is profoundly altered by prinomastat treatment, and that the combination of a decreased proliferation and increased apoptosis, together with a reduction in AML blast migratory capacity result in a lower number of leukemic cells within the analysed tissue.

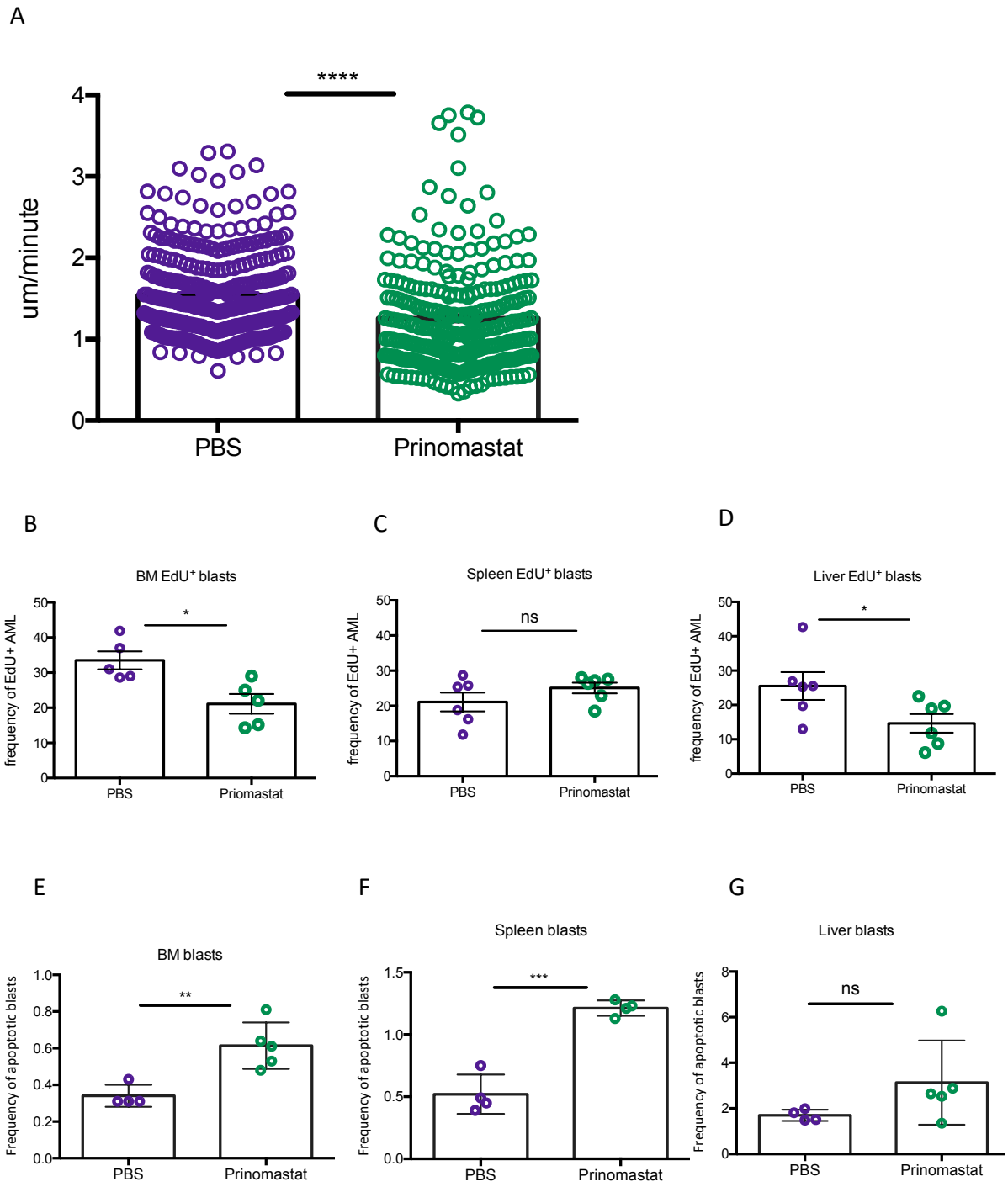

Figure 5.2-Prinomastat affects blasts speed, proliferation and death

Data show the effects of prinomastat treatment on leukemic blasts**.** (**A)** Mean speed of individual AML cells located in the BM parenchyma. Data obtained from 3 mice per condition, from two independent experiment. 338 AML cells for PBS and 293 for prinomastat treated. Error bars represent the mean ± s.e.m. Statistical significance identified using Student t test (****represent p<0.0001). Graph shows the comparison between prinomastat treated and PBS treated mice for the proportion of EdU+ blasts in the BM (**B)**, spleen (**C**) and liver (**D**) respectively. Each dot represents a mouse. n= at least 3 mice per condition from 2 independent experiments. (**E**)(**F**)(**G**) show the frequency of apoptotic blasts obtained from the Annexin V/7AAD analysis on blasts from BM, spleen and liver respectively. Each dot represents a mouse, n= at least 3 per group. Error bars represent mean ±s.e.m. Statistical significance identified using Student T test (** represent p<0.01; *** represent p<0.001)

5.4- The effects of prinomastat treatment on healthy cells egress from BM

As previously shown in Chapter 3, while leukaemia invades the BM, healthy haematopoietic cells egress from it. IVM data analysis have highlighted how, during AML, healthy cells are dislodged and enter into the circulation as clusters of different size which is in contrast to the steady-state situation where they extravasate as single cells or in clusters of two. The presence of large clusters in leukemic mice led to the hypothesis that AML blasts are able to carry out a certain level of matrix digestion, resulting in the healthy cells leaving the parenchyma in groups. As discussed above, the role of MMPs is to digest specific components of the matrix therefore facilitating cell migration in both steady-state and cancer. Considering this, I hypothesised that MMP-9 inhibition could reduce matrix digestion carried out by AML, resulting in less clusters of healthy cells seen in circulation. Thus, I performed IVM on PBS and prinomastat treated, leukemic, chimeric mice in which healthy hematopoietic cells are from mT/mG donor mice and can therefore be visualised as tomato^{+} cells (Figure 5.3 A and B). From previous experiments and data analysis, I was able to narrow down a specific window of disease progression, where mice displayed intermediate levels of infiltration, within which I could visualise healthy cells in circulation. IVM data analysis revealed that the circulating clusters of healthy cells in prinomastat treated mice are overall reduced (Figure 5.3 A and B). Despite not being significant, a greater number of single healthy cells were detected in the circulation of prinomastat treated mice, while in the pbs treated group circulating cells were mostly in clusters of two to eight cells (Figure 5.3 C).

All together these results suggest that MMP-9 inhibition through prinomastat reduces the impact of invading AML blasts within the BM parenchyma, however the question remained whether other niche components may be affected too and could, in turn, affect AML invasion.

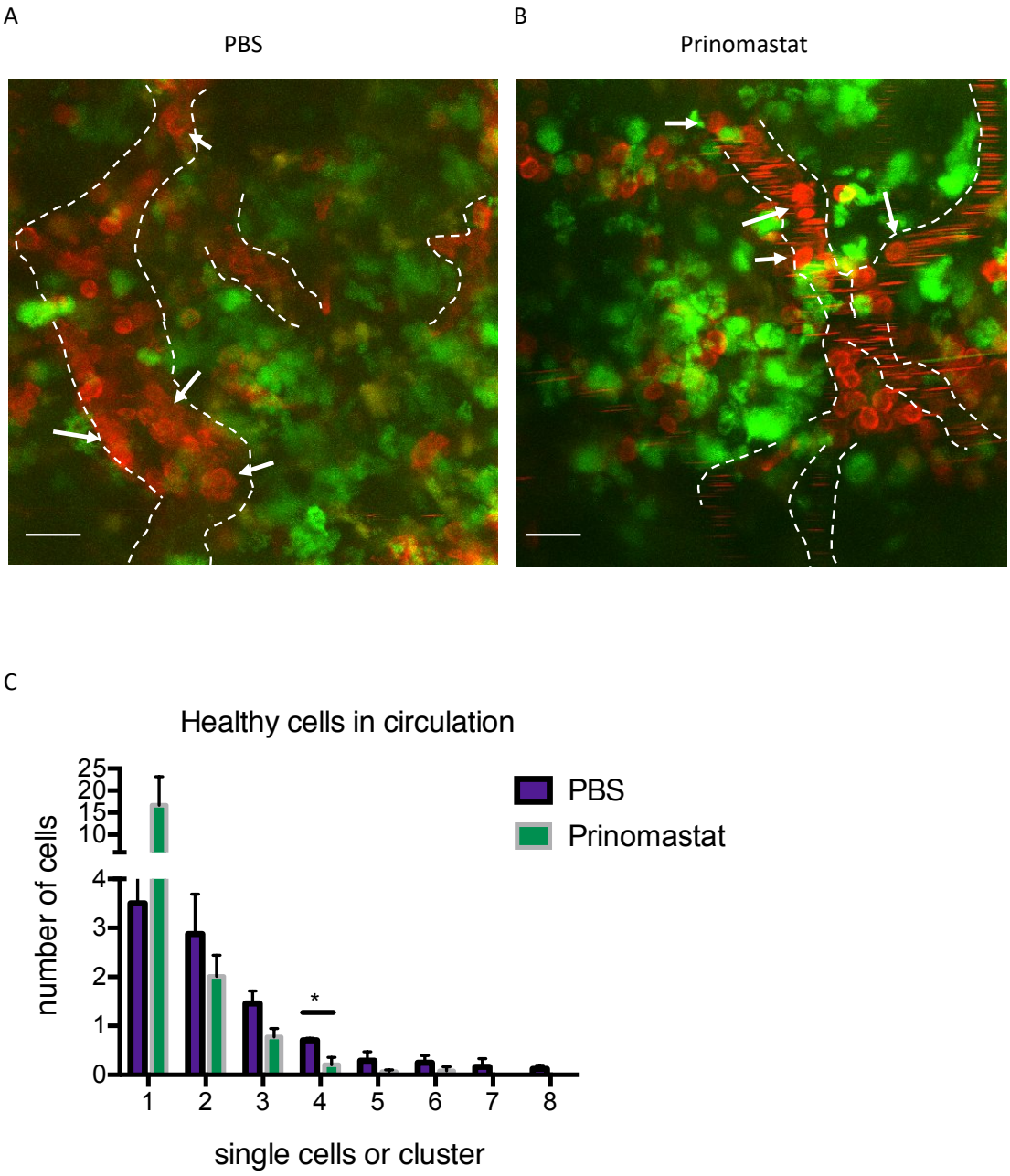

Figure 5.3-Prinomastat reduces the size of healthy cell clusters extravasating from the marrow

Data show the effect of MMP-9 inhibition on the size of healthy clusters extravasating due to AML. Maximum projection of representative time frames of time lapse datasets from PBS treated (**A**) and prinomastat treated (**B**) mice. Healthy cells are in red while leukaemia blasts are in green. Arrows point to cells clusters (**A**) or single cells (**B**) in the circulation. Scale bar represents 80µm. Blood vasculature is delineated by dashed lines. (**C**) Average of number of cells/clusters detected in circulation. 3 mice per condition. Error bars represent the mean ±s.e.m. Statistical significance identified using Student T test (* represent $p<0.05$)

5.5- Prinomastat improves vascular damage induced by AML

Previous studies have shown that MMPs are secreted by other niche components such as neutrophils and ECs (Magid, Murphy & Galis, 2003; Ong *et al.*, 2015). MMPs secretion by ECs has been suggested to be a mechanism for regulating the proteolytic activity that is fundamental during local remodelling (Galis & Khatri, 2002). In solid tumours, for example, ECs secrete MMP-2 and MMP-9 in response to angiogenic stimulation from vascular endothelial growth factor (VEGF), which contributes to increased vessel permeability (Sirois & Edelman, 1997; Taraboletti *et al.*, 2002; Weis & Cheresh, 2005; Mahecha & Wang, 2017). AML has been associated with the production of VEGF (Xu *et al.*, 2010; Duarte *et al.*, 2017; Passaro *et al.*, 2017) and alterations of vascular permeability resulting in increased leakiness (Passaro *et al.*, 2017). Reducing vascular leakiness could improve the retention of residual healthy cells within the BM. With this in mind, I questioned if prinomastat treatment could have a wider effect on the BM and could alter vascular permeability.

I carried out IVM on Flk1-GFP transgenic mice in which ECs express GFP and can be identified surrounding the blood vessels (Xu *et al.*, 2010). Therefore, I performed time lapse imaging of randomly selected calvarium BM areas of healthy control, PBS and prinomastat treated leukemic mice at early and late disease stages for a duration of 10-15 minutes following the injection of a low molecular weight TRITC-labelled dextran (Figure 5.4 A and Supplementary Video 3). Quantification of the fold change in TRITC-dextran intensity in the images over time revealed that PBS treated, leukemic mice had highly permeable vessels, demonstrated by the rapid diffusion of dextran throughout the BM parenchyma, in comparison to the prinomastat treated, leukemic mice at late disease stage (PB> 10%) (Figure 5.4 C). In fact, at this stage of disease, the difference in vascular permeability was statistically significant with the pbs treated group showing a 2-fold increase in leakiness in comparison with the

prinomastat treated, leukemic animals (Figure 5.4 C). Surprisingly, vascular leakiness could be also detected at early AML stages (PB<10%), when the number of seeding AML blasts within the marrow was still very low (Figure 5.4 B and Supplementary Video 4). Previous studies on changes in vasculature permeability have focused mostly on later disease stages, when seeding blasts in the marrow were more than 40% of total cells. Based on these data, it could be proposed that changes in vasculature permeability start at even earlier stages of AML - likely due to the inflammatory environment that characterizes AML (Duarte *et al.*, 2017; Craver *et al.*, 2018). Altogether, these results suggest that prinomastat treatment limits vascular damage in the context of AML.

As discussed above, AML is known to produce high levels of VEGF(Duarte *et al.*, 2017; Passaro *et al.*, 2017b) which has been associated with increased vascular permeability (Weis & Cheresh, 2005; Bates, 2010). At the same time, prinomastat treatment has been shown to reduce the VEGF levels in solid tumours (Woenne *et al.*, 2010). Therefore, I decided to investigate whether the prinomastat-driven limit in vascular damage could be as a result of reduced VEGF levels. Surprisingly, analysis of VEGF levels in BM supernatants from PBS and prinomastat treated leukemic mice by ELISA revealed no difference in the levels among the two groups (Figure 5.4 D).

Recently, it has been shown that AML causes an increase in the levels of reactive oxygen species (ROS) and nitric oxide (NO) in ECs through the NOX4-NOS3 axis (Passaro *et al.*, 2017). Interestingly, this pathway has also been proposed to be partially involved in AML-induced vascular leakiness (Thomas, Chen & Keaney, 2001; Craige *et al.*, 2011; Passaro *et al.*, 2017). Considering this, I questioned whether the reduced leakiness of vessels induced by prinomastat could be as a result of reduced EC ROS levels. Thus, I analysed the intracellular

ROS levels of ECs in prinomastat and PBS treated, leukemic mice at late disease stages. Flow cytometry analysis showed that the proportion of ROS-positive ECs was significantly lower in prinomastat treated mice compared to PBS treated (Figure 5.4 E). These data suggest that changes in the proportion of ROS^+ ECs in prinomastat treated mice could lead to reduced effects of AML on the vasculature, including reducing their permeability. Next, I evaluated if the MMPI treatment was impacting ECs in terms of their absolute numbers., I compared the absolute number of $CD31^+$ $Sca\text{-}1^+$ ECs and specifically the $Sca\text{-}1^{high}$ arteriolar ECs among the two groups, as arteriolar ECs have been shown to be highly involved in the maintenance of HSCs within the BM niche (Kunisaki *et al.*, 2013). Once again, data analysis revealed no difference in the number of ECs among the two groups (Figure 5.4 F and G).

Overall these data demonstrate that prinomastat treatment not only directly impacts leukemic blasts but can also target the vascular niche by reducing the vascular leakiness associated with AML.

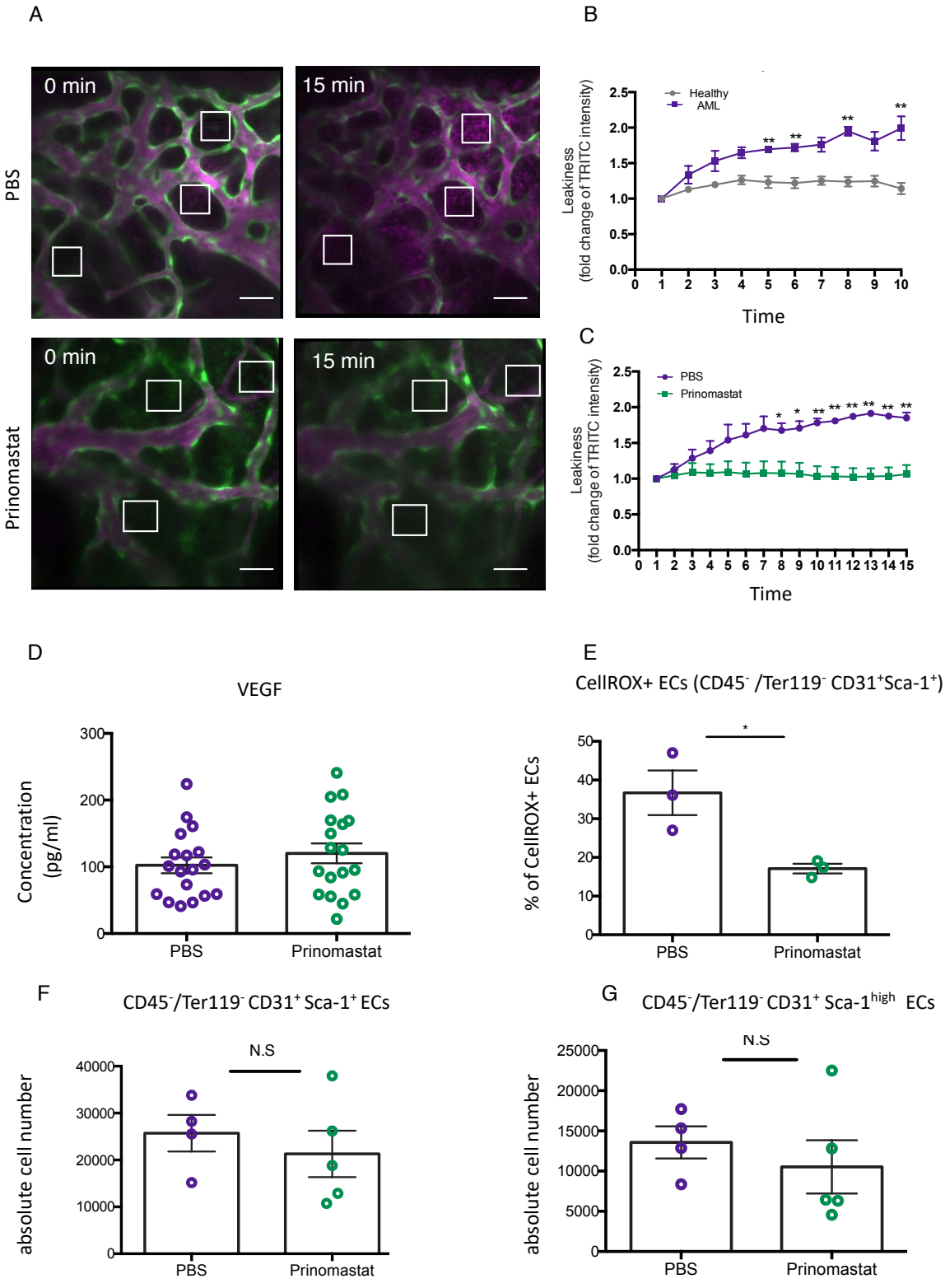

Figure 5.4-Prinomastat improves vascular permeability

Data show the effect of prinomastat treatment on vasculature during AML. **(A)** Vasculature leakiness was assessed by time lapse imaging of the calvarium for 15 minutes after injecting 3mg 65-85kDa TRITC-dextran i.v. Selected frames from representative time lapses data of PBS and prinomastat treated Flk1-GFP mice at late disease stage (PB>10% blasts). White boxes highlight regions of interest within the parenchyma were measurement were taken. Scale bar represents 50µm. Quantification of vascular leakiness in early AML (PB AML <10%) vs healthy mice **(B)** and in late stage AML, PBS vs prinomastat treated mice **(C)**. Analysis was done by measuring the fold change of TRITC-dextran intensity in 4 regions of interest per position (field of view). Data pooled from 3 mice per condition per time point with 4 positions acquired per

mouse. Error bars represent the mean ±s.e.m. Statistical significance obtained using Student's t-test with post-hoc Bonferroni correction for multiple comparisons (* represent $p<0.05$). **(D)** VEGF levels in BM supernatant of PBS and prinomastat treated mice. Each dot represents a mouse. 36 mice in total, 18 per condition. Data obtained from 2 independent experiments. Error bars represent mean± s.e.m. **(E)** Quantification of the proportion of ROS positive ($CellROX^+$) endothelial cells in both groups at late disease stage. Each dot represents a mouse, 3 mice per condition. Error bars represent mean±s.e.m. Statistical significance obtained using Student's t-test. (* represent $p<0.05$). **(F) (G)** Quantification of absolute cells number of $Ter119/CD45^-$ $Cd31^+$ $Sca\text{-}1^+$ endothelial cells and $CD31^+Sca\text{-}1^{high}$ arteriolar ECs. Each dot represents a mouse, 9 mice in total, 4 PBS and 5 prinomastat treated. Error bars represent the mean±s.e.m

5.6- Maintaining HSPCs in their niche: the effect of prinomastat on healthy cell dynamics

So far, the data presented in this chapter suggests that prinomastat can have a significant effect on leukaemia outcome. I hypothesised that the reduction in blast proliferation and their capacity to migrate and digest the ECM within the BM parenchyma, together with the reduction in vasculature permeability could promote healthy hematopoietic cell retention within their niche. To test this hypothesis, I compared PBS and prinomastat treated mice with equal amounts of blasts within the marrow. Specifically, 30-75% is medium infiltration and 75-100% is high infiltration respectively. Flow cytometry plots immediately highlighted a completely different dynamic for the hematopoietic cells among the two groups. For example, for a 90% of blast infiltration within the marrow, prinomastat treated mice still had clearly detectable number of LKS cells, reflecting a higher proportion of HSPCs (Figure 5.5 A). Data analysis confirmed the statistically significant difference for this population at both analysed time points (Figure 5.5 B). I then compared the number of undifferentiated lineage- cells as they contain the stem and progenitor haematopoietic cells. Even In this case, data analysis revealed a significant difference in the residual $lineage^-$ population among the two groups (Figure 5.5 C). Despite having a similar amount of blasts within their marrow, mice receiving prinomastat had a higher number of $lineage^-$ and LKS cells. Next, I focussed my analysis on the primitive HSPC compartments in order to confirm the hypothesis of a favourable effect of prinomastat on these populations. Flow cytometry data analysis revealed how, for a similar range of infiltration, the marrow of prinomastat treated mice contains a significantly higher number of LT-HSCs at both

medium and high infiltration points (Figure 5.5 D). Similarly, the number of both ST-HSCs and MPPs were higher in the group under MMPI treatment (Figure 5.5 E and F). Specifically, significantly more ST-HSCs were maintained within the marrow of prinomastat treated mice at medium AML stage while their amount appeared similar to the pbs control group at late disease (Figure 5.5 E). On the other hand, the MPP3-4 were following a similar dynamic to the LT-HSCs, and significantly differed at both disease stages (Figure 5.5 F).

These results were truly surprising and revealed, for the first time, that the use of a selective MMPI can significantly reduce the loss of HSPCs induced by AML. Therefore, by altering blast invasiveness, proliferation and apoptosis and by improving vasculature leakiness, prinomastat increases HSPC's ability to remain in their natural microenvironment.

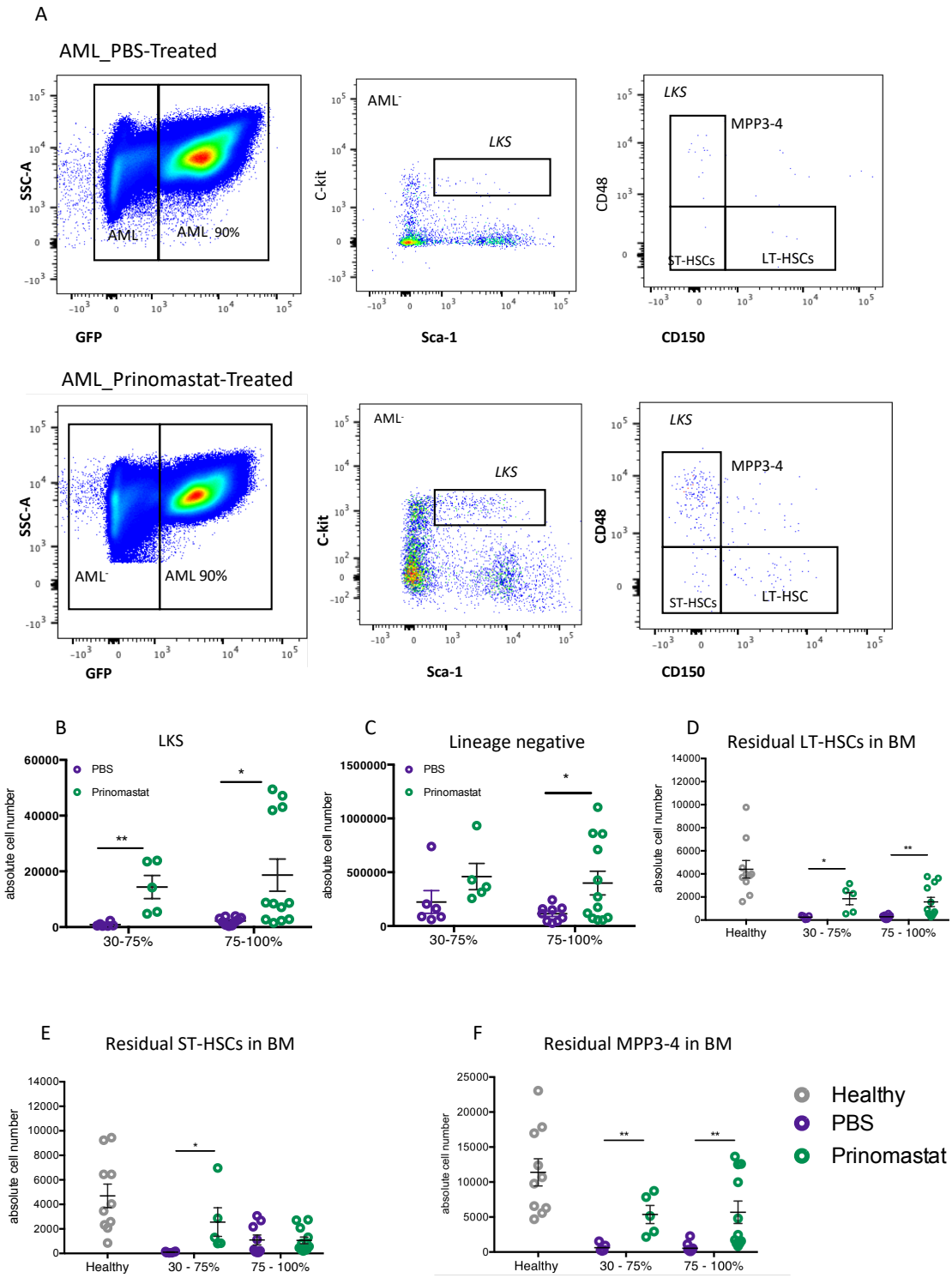

Figure 5.5-Prinomastat affects HSPCs dynamics within the marrow

Data show the impact of MMPI treatment on the haematopoietic stem and progenitor cells during the course of AML. (**A**) Representative flow cytometry plots showing the residual HSPCs in PBS and prinomastat treated mice. (**B**) Absolute number of residual LKS population in PBS and prinomastat treated mice. (**C**) Absolute number of residual lineage negative population in PBS and prinomastat treated mice. Each dot represents a mouse, 33 mice in total from 3 independent experiments Error bars represent the mean± s.e.m. The comparison of residual amount of LT-HSCs, ST-HSCs and MMP3-4 is shown in graph (**D**) (**E**) (**F**) respectively. Error bars represent the mean± s.e.m. each dot represents a mouse, n=10 healthy, n=15 PBS treated and n=17 prinomastat treated. Statistical significance obtained using Student's t test (*represent $p<0.05$, ** represent $p<0.01$)

Within the marrow, several molecular signals are known to be essential for functional haematopoiesis. Cytokines such as CXCL12 and SCF play a pivotal role in HSPC retention and maintenance in the niche and as I showed before, their upregulation in extramedullary sites is associated with HSPC migration and colonization there. As prinomastat treatment enhances the number of residual steady-state haematopoietic cells in the BM, I next questioned whether this effect could be explained by an increase in the level of CXCL12 and SCF in the marrow. Thus, I performed ELISA experiments aiming to evaluate the levels of both cytokines in the BM supernatant of PBS and prinomastat treated, leukemic mice. This analysis revealed no difference in the levels of either cytokine between the two groups (Figure 5.6 A and B). Therefore, from these data, I could conclude that prinomastat treatment does not induce HSPC retention within the marrow by increasing the levels of CXCL12 and SCF.

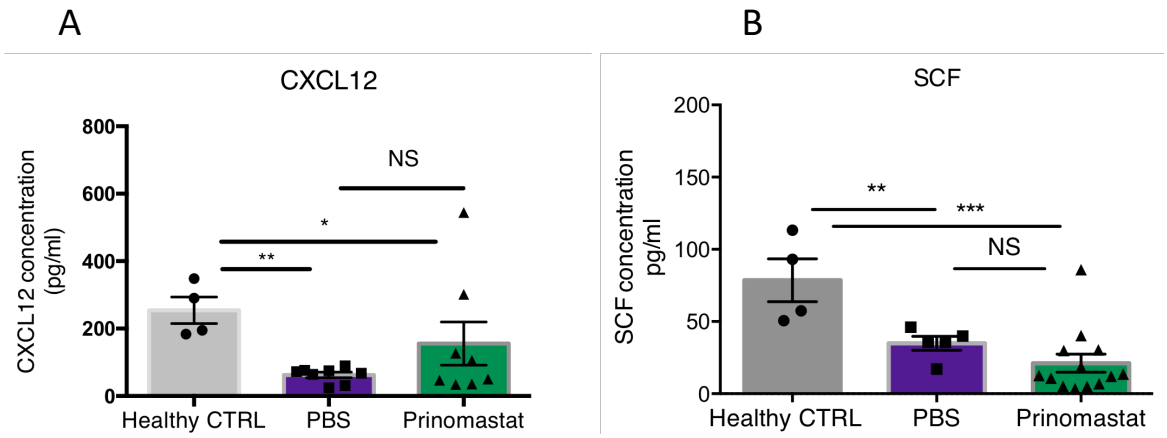

Figure 5.6-Prinomastat treatment does not change CXCL12 and SCF levels within the marrow

ELISA performed on the BM supernatant revealed no difference in the level of CXCL12 (**A)** and SCF (**B**) between the PBS and prinomastat treated group. Both CXCL12 and SCF levels significantly differ between the healthy control and both PBS and prinomastat treated mice. Error bars represent mean ± s.e.m. Each dot represents a mouse, 20 mice in total for (**A**) and 22 for (**B**). Statistical significance obtained using Student's t-test (* represent $p<0.05$, ** represent $p<0.01$, *** represent $p<0.001$)

5.7- MMPI treatment reduces the onset of extramedullary haematopoiesis

As shown above, despite AML progression, prinomastat treatment improves the ability of HSPCs to remain in their own niche. To further test the hypothesis that EMH is initiated by HSCPs displaced from the BM, I decided to investigate EMH in prinomastat treated, leukemic mice. In line with what I had demonstrated in chapter 4, I hypothesised that MMPI treatment should result in a reduction of EMH onset and reduced numbers of HSPCs in spleen and liver.

I started my analysis comparing the increase of HSPCs in the spleens of pbs and prinomastat treated, leukemic mice and then I subsequently analysed the liver. Again, mice were grouped based on their BM infiltration: healthy control, 30-75% medium and 75-100% high infiltrated. I first evaluated the absolute number of lineage$^-$ cells found in the spleen from both groups. This revealed a significant reduction in the absolute number of lineage$^-$ cells in prinomastat treated mice compared to PBS treated at both infiltration stages (Figure 5.7 A). These data hinted that there may be a reduction in EMH and this was reinforced by flow cytometry plots showing a decrease in LKS cells in the MMPI treated group (Figure 5.7 B). Further, quantification of this population revealed a statistically significant difference between the two groups at the late time point (Figure 5.7 C). When I analysed HSPC subpopulations, flow cytometry data revealed that all populations were lower in their number in the spleen of prinomastat treated mice when compared to PBS treated animals. Importantly, the number of LT-HSCs (Figure 5.7 D), ST-HSCs (Figure 5.7 E) and MPP3-4 (Figure 5.7 F) was significantly lower in the spleen of highly infiltrated, prinomastat treated mice. Overall, these data revealed that prinomastat treatment, by keeping the HSPCs in their niche, effectively reduces the onset of EMH in the spleen and confirmed that this phenomenon is due to the migration and colonization of this site by the cells egressing from the marrow.

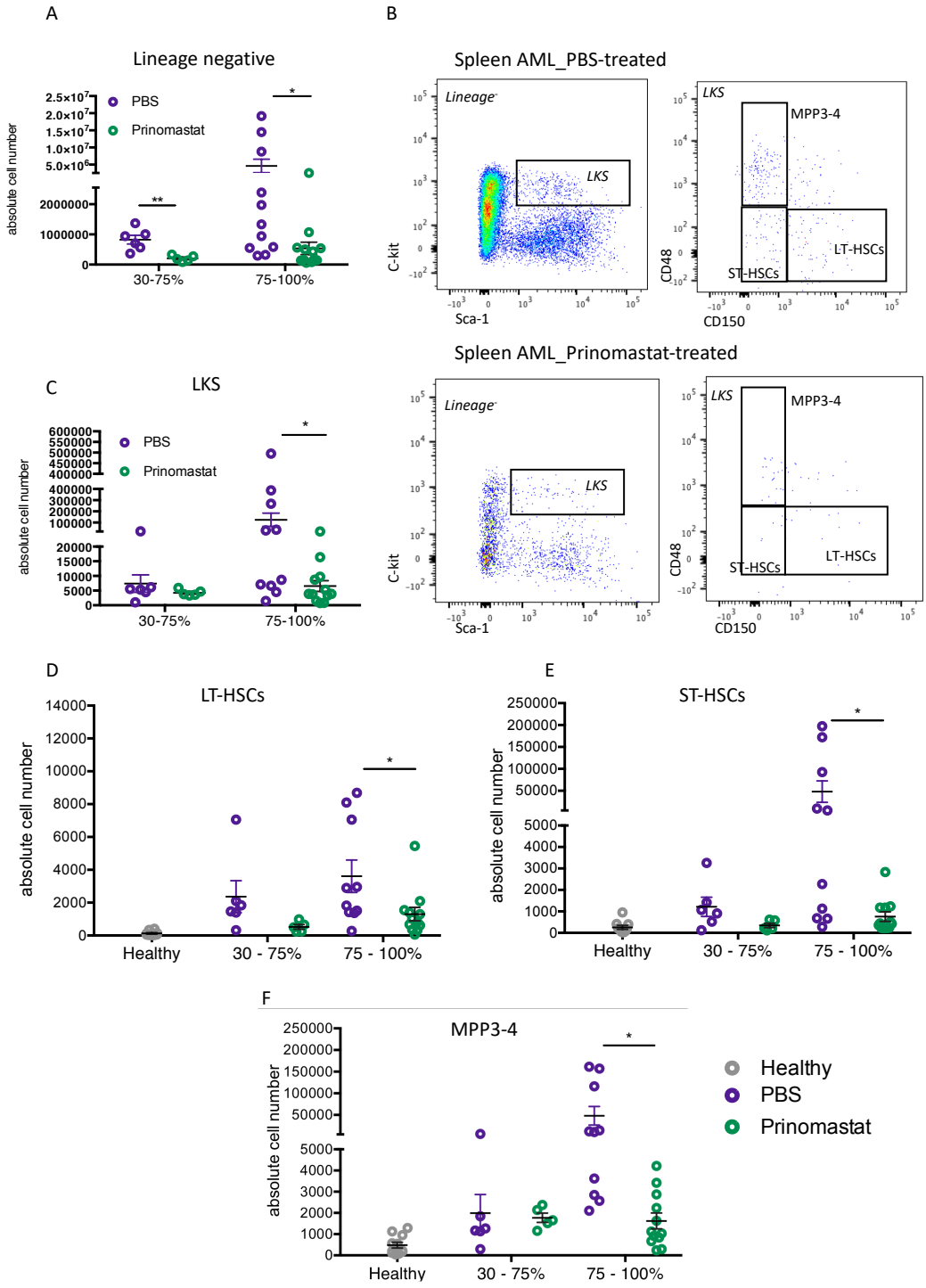

Figure 5.7-Splenic EMH is reduced by prinomastat treatment

(**A**) the Lineage- population is reduced in the spleen of prinomastat treated mice. Each dot represents a mouse, 33 mice in total from 3 independent experiments. (**B**) Representative flow cytometry plots showing the LKS and HSPCs population in the spleen of both groups. (**C**) shows the comparison between the absolute number of LKS cells among the PBS and prinomastat treated mice. Each dot represents a mouse, 33 mice in total from 3 independent experiments. (**D**) (**E**) (**F**) Comparison between the absolute number of LT-HSCs, ST-HSCs and MMPs found in the spleen of Prinomastat and PBS treated mice per each BM infiltration range. Each dot represents a mouse, n=10 healthy, n=16 PBS and n=17 prinomastat treated from 3 independent experiments. Error bars represent the mean±s.e.m. Statistical significance obtained using Student's T test (* represent $p<0.05$)

Having shown splenic EMH reduction, I next analysed the liver, wondering if the drug would have a similar effect on EMH arising at this other site. Once again, I started my analysis comparing the lineage$^-$ population among both groups of mice. Similar to the spleen, the absolute number of lineage$^-$ cells was significantly reduced in the liver of prinomastat treated mice in comparison to the PBS treated animals (Figure 5.8 A). As seen in the spleen, flow cytometry plots and data analysis revealed a trend towards a decrease in the LKS cells in the liver of prinomastat treated mice - which suggested a similar reduction in the number of the primitive stem and progenitor cells within this organ (Figure 5.B and C). Flow cytometry data analysis showed a significant reduction in the absolute number of LT-HSCs (Figure 5.8 D) located in the liver of mice under the MMPI regimen at both 30-75% and 75-100% BM infiltration range. Despite showing a trend towards a decrease, the number of ST-HSCs was not significantly different between the two groups (Figure 5.8 E). At the same time, the MPP number was not different among the two groups (Figure 5.8 F). Although the number of ST-HSCs and MMPs was not significantly different from the pbs treated mice, these data confirm that prinomastat affects EMH in the liver by significantly reducing the number of LT-HSCs that localise within it.

As shown in chapter 4, once dislodged from their niche, the majority of circulating HSPCs preferentially localise in the spleen, which becomes an important extramedullary site able to sustain functional EMH in the short-term. Therefore, even if the prinomastat treatment does not impact EMH in the liver as strongly as in the spleen, the major effect that it has on splenic onset represents an indirect proof of its ability to maintain HSPCs in the BM.

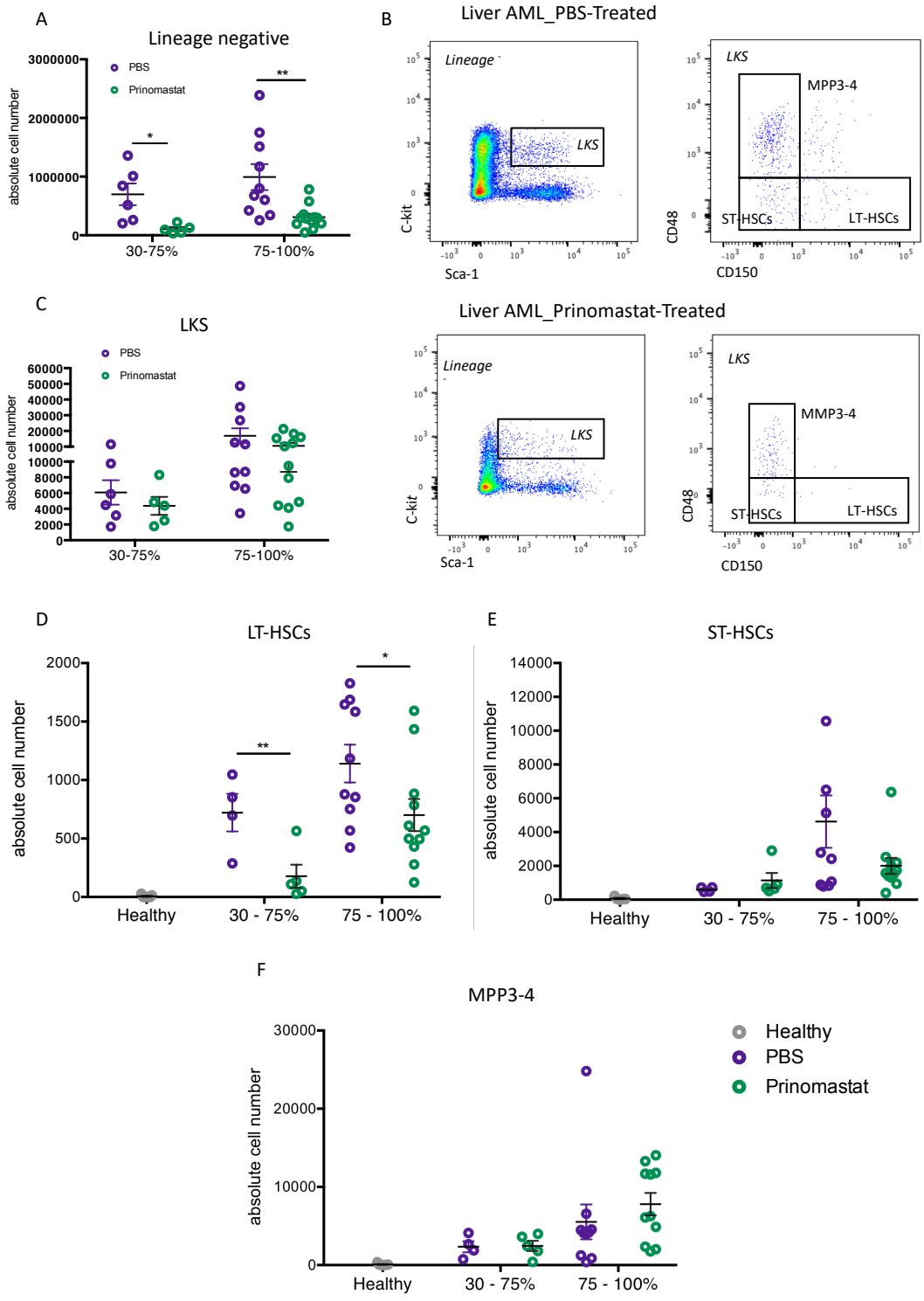

Figure 5.8-Prinomastat reduces the hepatic EMH

Data show the effect of the drug on liver EMH (**A**) shows the comparison between the absolute number of livers linege- cells among the PBS and prinomastat treated mice. (**B**) Representative flow cytometry plots showing the decrease in LKS and HSPCs populations in the liver of MMPI treated mice compared to the PBS ones. (**C**) shows the comparison between the absolute number of liver LKS cells among the PBS and prinomastat treated mice. Each dot represents a mouse, 33 mice in total from 3 independent experiments. Error bars represent the mean ±s.e.m. Statistical significance obtained by using the Student's T test (* represent $p<0.05$). (**D**)(**E**)(**F**) show the comparison between the number of LT-HSCs, ST-HSCs and MMP3-4s, respectively, founded in the liver of PBS and prinomastat treated mice. Each dot is a mouse, n=7 healthy, n=14 PBS and n=16 prinomastat treated from 3 independent experiments. Error bars represent the mean ±s.e.m. Statistical significance obtained using Student's t-test (* represent $p<0.05$)

5.8- Prinomastat enhances the effect of chemotherapy by extending survival

Current treatments for AML are based on chemotherapy and HSC transplantation. The established chemotherapy treatment is based on a first "induction" phase that aims to reduce the number of circulating blasts to an undetectable level and a "consolidation" phase aiming to eradicate the residual disease. Despite the advancement in treatments, AML patients still have a poor prognosis with a high percentage of relapse (Islam, 2017). Furthermore, patients who relapse become chemo-resistant and fail to undergo further remission (Islam, 2017). Therefore, there is an incredible need for new therapeutic approaches aiming to treat and improve AML-free survival.

The results obtained so far in this work lead me to conclude that prinomastat treatment affects AML's growth and its impact on the haematopoietic system. Thus, I hypothesized that AML burdened mice could also benefit from prolonged survival when treated with the MMPI prinomastat alone or in combination with chemotherapy.

In mice, chemotherapy treatment for AML is based on a regimen that mimics the clinical AML induction therapy (Zuber *et al.*, 2009). This established therapeutic model consists of intravenous (or intraperitoneal) administration of Ara-c (100mg/Kg) for a total of 5 days in conjunction with doxorubicin (Doxo) (3mg/kg) over the course of 3 days (Zuber *et al.*, 2009). Thus, in mice, this "5+3" chemotherapy regimen fully corresponds to the "7+3" one used in human patients. Therefore, I planned to test prinomastat alone or in combination with the above chemotherapy and evaluate the possibility of a positive effect on animal survival after AML.

To test my hypothesis, I followed disease progression and survival outcome in a cohort of 22 mice. Animals were injected with AML and divided into 4 groups as follows: 6 mice received

chemotherapy only, 6 mice received a combinatory treatment of prinomastat and chemotherapy, 5 were treated with prinomastat alone while the last 5 received only vehicle-control (PBS). For the groups receiving chemotherapy, treatment was started when the percentage of blasts in the PB reached 15%, usually 14-15 days after AML transplant. Prinomastat was given intravenously 7 days after AML transplant for a total length of 15 days of treatment. Thus, mice receiving both chemotherapy and prinomastat were already undergoing MMPI treatment for a period of time before ara-c and doxo were administered (Figure 5.9 A). In all cases, animals were culled before reaching the humane end point. As expected, mice receiving the vehicle-control were the first ones to develop visible leukaemia symptoms, with a median survival of 25 days. At the same time, despite reducing leukaemia infiltration, mice treated with the MMPI alone had a median survival of 27 days, showing that unfortunately, the use of prinomastat alone is not sufficient to prolong survival. This result was in line with what has been seen in another study, where the use of prinomastat as a single agent had no effect on mouse survival in an orthotopic model of lung cancer (Liu *et al.*, 2003).

On the other hand, chemotherapy alone significantly prolonged survival when compared to the vehicle-control. Finally, and surprisingly, the survival outcome for the group receiving both chemotherapy and prinomastat was significantly higher compared to all other treatment groups (Figure 5.8B) with mice surviving until 41 days after AML induction. This clearly indicates that the use of prinomastat alongside conventional therapy is able to improve overall survival in AML.

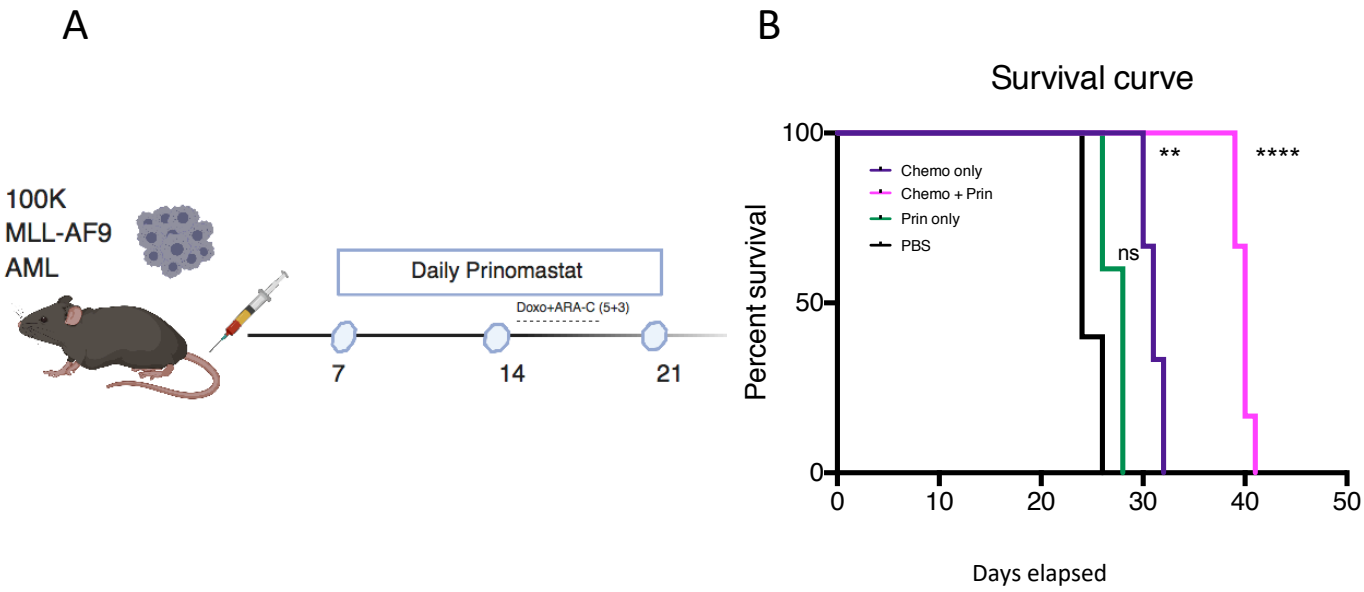

Figure 5.9-Prinomastat and chemotherapy combination enhances mice' survival

(**A**) Schematic representation of experimental set up. Mice receiving both prinomastat and vehicle-control were treated from day 7 post AML cells injection. Chemotherapy treatment was started around day 14-15 post AML injection, when PB reached at least 15% of infiltration, figure created with BioRender.com. (**B**) Kaplan-Meyer curve showing improved survival in mice receiving both prinomastat and conventional chemotherapy regimen. N=6 Chemo only, n=6 chemo + prinomastat, n=5 prinomastat only, n=5 vehicle-control only. Log-rank **** represent p<0.0001

Most importantly, considering the need of new therapeutic approaches in AML, this data strongly suggests that targeting MMPs could coordinate with conventional chemotherapy regiments to improve AML outcome.

5.9- Discussion

In this chapter, I investigate a possible role for MMPs in leukaemia progression and whether the use of an MMP inhibitor could affect disease outcome. In Chapter 3, IVM analysis of calvarium BM revealed that under blasts pressure, healthy haematopoietic cells egress from their niche as a cluster of two to eight cells. This observation led to the hypothesis that behind this process, leukaemic blasts may possess the ability to digest the ECM, forcing the healthy cells to leave in bulk. MMPs are proteolytic enzymes that, by digesting specific ECM components (e.g. collagens, gelatin, laminin), participate in its remodelling in both homeostatic and pathologic conditions (Daley, Peters & Larsen, 2008; Gattazzo, Urciuolo & Bonaldo, 2014; Jabłońska-Trypuć, Matejczyk & Rosochacki, 2016). In the past decades the dysregulated expression of MMPs has been associated with tumour invasiveness, metastasis formation and poor prognosis. Specifically, MMP-9 (gelatinase B) has been demonstrated to have a pivotal role in several cancers, including leukaemia, where its associated with tumour aggressiveness and poor patient survival (Fridman *et al.*, 2003; Paupert *et al.*, 2008; Pan *et al.*, 2014; Verma *et al.*, 2020). Therefore, MMPs have been considered as a therapeutic target in cancer research and numerous drugs have been developed in the past decades to reach this aim. However, little is known about the role of these molecules in AML and whether their inhibition could affect disease growth and progression.

Here, by analysing RNAseq data, previously generated in the lab on AML blast gene expression profiles, I was able to highlight how leukemic cells overexpress specific MMPs, including MMP-9, in comparison to GMPs used as a positive control. Therefore, considering its role in cancer, I decided to focus on MMP-9 and to investigate whether its inhibition could affect the outcome of AML. Thus, I chose the MMPI inhibitor prinomastat because it inhibits MMP-9 among others(Hande *et al.*, 2004) and it has been seen to be able to reduce tumour

growth and metastasis in a several models of solid cancer (Liu *et al.*, 2003; Hande *et al.*, 2004). Considering that MMPIs work optimally when administrated at the early stages of disease, I set up a treatment regimen in which the drug was given 7 days after AML transplant for a total of 15 days. Despite data on PB revealing no difference in the amount of circulating blasts, I showed a reduction in BM, spleen and liver infiltration due to prinomastat treatment. This result was specifically corroborated by IVM of calvarium BM, showing that, at equal PB infiltration, blasts were reduced within the marrow. This could be explained by a reduced ability of circulating AML cells to extravasate and re-enter the BM parenchyma and would be consistent with the observed reduced vasculature permeability and with reduced spleen infiltration, which in this model is driven by trafficking of cells from BM.

Further IVM analysis revealed an overall decrease in blasts speed that fits with previous observations linking MMP-9 expression with tumour migration and invasion (Fridman *et al.*, 2003; Mu, Song & Zhang, 2016). In fact, the production and release of this proteolytic enzyme by cancer cells has been shown to promote ECM degradation proteins resulting in enhanced tumour progression (Kessenbrock, Plaks & Werb, 2010). Therefore, the obtained data highlight how MMP inhibition could lead to a lower invasiveness capacity. Furthermore, proliferation and apoptosis of AML blasts were impacted by prinomastat, with blasts being less proliferative and showing more apoptosis. These data suggested that inhibiting MMP-9 affects leukaemia itself.

Moreover, quantification of IVM data revealed a reduction in the size of clusters leaving the marrow during the course of leukaemia, with the majority of cells now leaving as a single unit. The obtained result suggests that prinomastat treatment is beneficial for healthy cells, likely because the ECM surrounding them is preserved. Next, as MMP-9 has been shown to be

produced by ECs in response to VEGF, and AML cells are known to produce VEGF, especially in our model (Duarte et al., 2018) I questioned whether prinomastat could also have a general effect on that. This point is particularly important as in AML, the VEGF is known to increase vascular leakiness that may ultimately promote cells egression. Furthermore, increased vascular leakiness can lead to poor drug delivery favouring a microenvironment that will promote resistance against chemotherapy (Passaro *et al.*, 2017). Thus, improving vasculature permeability could help maintaining HSPCs in the marrow and improve therapy effectiveness. Here, I demonstrated that prinomastat reduces the vasculature leakiness associated with AML at late disease stage. ELISA and FACS analysis showed that this effect was not mediated by reduced levels of VEGF, however ROS levels in ECs were reduced. An increased level of ROS in ECs has been proposed as a mechanism contributing to vasculature leakiness (Thomas, Chen & Keaney, 2001; Craige *et al.*, 2011; Passaro *et al.*, 2017b). Also, recently, it has been proposed that highly permeable blood vessels, through a boost in ROS levels, augment HSPC migration and differentiation compromising their long-term repopulation and survival (Itkin *et al.*, 2016). Thus, vascular leakiness actively participates in the pathophysiology of AML.

My data suggest that the improved vasculature leakiness could result from prinomast acting at multiple levels on ECs: reducing ROS, reducing ECM degradation and reducing potential pressure from growing AML cells. Most importantly, I showed that prinomastat could reduce the impact that AML has on healthy haematopoietic cells and prolong mice survival once combined with chemotherapy. This was further evidence that inhibit MMPs could be beneficial in AML treatment.

Though these experiments provide an insight into the role of MMPs in AML and suggest that they are potential therapeutic targets, a number of questions arose from this work. Based on the gene expression profile, I observed that AML blasts overexpressed three different MMPs and specifically the MMP-8, MMP-9 and MMP-19. I decided to focus my interest on the MMP-9 as its dysregulation has been shown to be crucial in both CLL and B-ALL (Kamiguti *et al.*, 2004; Verma *et al.*, 2020)However, MMP-8 and MMP-19 have been also proposed to promote metastatic behaviour and tumour progression in solid tumour such as non-small cell lung cancer and hepatocellular carcinoma (Yu *et al.*, 2014; Qin *et al.*, 2016). Therefore, it would be worth using an inhibitor that targets these three enzymes together and evaluate whether stronger effects could be achieved. Also, together with the MMP-9, prinomastat inhibits MMP-2, MMP-13 and MMP-14, raising the possibility that other mechanisms contribute to the improvement in disease outcome observed here alongside MMP-9 inhibition. To control for this in future work, it could be useful to test one of the recent highly selective inhibitors for MMP-9 such as the AB0046 antibody or the MMP-9 Pex inhibitor, both of which have been shown to reduce tumour growth and metastasis (Levin *et al.*, 2017; Verma *et al.*, 2020). Furthermore, to confirm that behind the reduction in clusters of healthy cells leaving the marrow there is a decrease on matrix digestion, it would be necessary to perform immunostaining of long bones and quantify changes in ECM components due to AML and under prinomastat treatment.

To confirm that the observed effects are due to the MMP-9 inhibition on the AML blasts instead of a wider effect on the BM, future work could consider the generation of AML blasts by transducing GMPs from MMP-9 knockout mice or using the CRISPR-Cas9 technology to specifically delete this gene from leukemic cells. Both methodologies would allow to confirm if the observed changes in blasts behaviour are a consequence of the MMP-9 inhibition

specifically within AML cells. This would be especially useful to dissect whether the prinomastat effects on vascular leakiness are due to inhibition of EC-derived MMPs. Interestingly, a single cell RNA-seq study indicated no increased expression of MMPs in ECs in mice carrying AML (Baryawno *et al.*, 2019). Here, I showed a decrease in the level of ROS as one of possible mechanism behind this phenomenon, however it would be interesting investigate whether other pathways are also involved. For example, it could be worth it to evaluate if the MMPI treatment affect the level of inflammatory molecules such as IFNγ or TNF, both known to be able to affect vasculature permeability (Sprague & Khalil, 2009). Furthermore, in the future, it could be worth performing bulk RNA-seq of BM-derived ECs from PBS and prinomastat treated mice as this will allow to capture any transcriptomic changes in ECs due to the treatment, highlight genes that may be important molecular players in the rescue of vasculature permeability. At the same time, as ROS are also involved in HSC quiescence and mobilization (Ito *et al.*, 2006) and it would be interesting to evaluate if prinomastat treatment has also an effect on their level within the HSPCs population.

Flow cytometry data analysis revealed that prinomastat increased the number of residual HSPC within the marrow in comparison with PBS treated mice showing similar infiltration. It has been shown that residual HSCs from mice with leukaemia have better engraftment in comparison to those from healthy donors (Miraki-Moud *et al.*, 2013; Cheng *et al.*, 2015). However, in chapter 4, I obtained a different result, probably due to the high percentage of infiltration at the time of the sort, suggesting that HSCs function may decline at very late stage of leukaemia. Therefore, it would be interesting to investigate whether prinomastat, as well as increasing the number of residual HSCs, is able to preserve their functionality. Thus, future experiments could consider transplantation assays at various disease stages to compare HSCs fitness between the prinomastat and PBS treated group.

The data presented here show that treating mice with prinomastat alone failed to increase their survival, however when the treatment is given in combination with chemotherapy, mouse survival is statistically significantly prolonged. In these experiments, mice were treated with a dose of 13.5 mg/kg i.v. per day. It could be worth in the future to try a higher dose of prinomastat and evaluate if this could have a better impact on animals' survival. At the same time, given the positive results when both treatments were used, it could be interesting to investigate the impact on residual HSCs carrying out experiments to evaluate whether having both treatments would increase even more their number and so their change to remain in the marrow. It should be considered that, from a clinal point of view, it will be unfeasible to treat patients at early AML stages as those stages are usually undetected. Therefore, an optimal window for the prinomastat treatment could be represented by the stage that follows chemotherapy, when patients have a low blast infiltration. At the same time, it would be worth it to evaluate the disease relapse following chemotherapy alone or following the combinatory treatment as this will give information on whether prinomastat may affects the number and capacity to re-propagating of residual leukemic cells.

Also, it should be noted that all the above experiments were performed using a murine model of AML. Therefore, future works should consider to perform human AML-patients derived xenograft (PDX) experiments to test whether the MMPI inhibitor maintains its ability to impact AML outcome. Complimentary to that, *in vitro* experiments using AML-patient derived cells could be provide an indication for whether prinomastat affects blasts features.

Overall, the data presented here shows that MMPs have a role in leukaemia pathogenesis, suggesting that their inhibition could disrupt AML growth and promote the maintenance of steady state haematopoiesis over the course of disease. These findings could have a significant

implication for leukaemia research as represent the first evidence that MMPIs could be beneficially implemented in new therapeutic approaches in AML. Specifically, the data above suggests that the use of prinomastat alongside chemotherapy could be evaluated for patients in which MMP-9 results overexpressed. Recently, transcriptomics data analysis revealed that among the MLL rearrangement, the MMP-9 and MMP-14 have a trend through overexpression in patients in which AML is driven by the fusion protein MLLT3-KMT2A (Tyner *et al.*, 2018). As this particular AML subtype is the equivalent of our murine MLL-AF9 model and considering that prinomastat is able to inhibits both of those MMPs, it is reasonable to think that prinomastat treatment may improve AML outcome in these class of patients. Therefore, considering all the above results, the effects of prinomastat treatment alone or in combination with other agents should be evaluated in future clinical trials aiming to improve AML treatment at least in these patients.

Chapter 6

Summary, conclusion and future directions

Blood cell production is highly regulated and maintained throughout the human' lifespan via the process of haematopoiesis. This depends on the interaction of haematopoietic stem cells (HSCs) with a specific microenvironment or niche composed of several specialised components which orchestrate their functionality. In adulthood, the BM microenvironment represents the final niche for the HSCs and within it, osteoblasts, ECs and MSCs have a key role in regulating HSCs and healthy haematopoiesis.

Acute leukaemias, including both the T lymphoblastic (T-ALL) and myeloid (AML) are characterised by poor prognosis and high relapse rates. Despite some differences, both leukaemia types are known to remodel the BM niche at the expense of healthy HSCs and impairing steady-state haematopoiesis. In AML, the impact on healthy haematopoiesis is reflected by life-threating cytopenias that cause patients to suffer from bleeding and recurrent infections. This main symptom is caused by the loss of healthy haematopoietic stem and progenitor cells (HSPCs) that characterise the disease development and is exacerbated at late disease stages. Despite several mechanisms having been proposed to drive HSPC loss, including a differentiation block at the HSC-progenitor transition (Miraki-Moud *et al.*, 2013), a reduction in stem and progenitor cell production rate (Cheng *et al.*, 2015) and an increase in apoptosis or emigration (Akinduro *et al.*, 2018) the exact process by which leukaemia outcompetes healthy haematopoiesis remains poorly understood. Deciphering this mechanism will provide new therapeutic targets for AML.

In this thesis, I investigated how AML impacts steady-state HSPCs during its progression (Chapter 3), whether extramedullary sites are able to sustain haematopoiesis in the short term and if alternative HSPC niches are developed in response to malignancy (Chapter 4) and I explored new targets that could delay AML progression and maintain/restore healthy haematopoiesis within the BM (Chapter 5). To address these questions, I used a mouse model of AML and studied it using IVM of the calvarium BM and spleen, complemented by flow cytometry and ELISA assays. In this final chapter, I will summarise the findings and conclusions related to each of my research questions, propose follow up experiments and highlight the wider implications of this work.

What are the cellular dynamics of the competition between AML and healthy haematopoiesis?

In Chapter 3, I used a combination of IVM and flow cytometry to gain a deeper insight into how leukaemic cells interact with their healthy counterparts and, building on previous work from our research group, I explored the dynamics by which HSPCs are lost throughout disease progression. IVM data showed that while leukemic cells grow and invade the BM space, healthy haematopoietic cells are lost in parallel, suggesting that there is competition between these two populations for the BM niche. Furthermore, high-resolution time lapse imaging followed by cell track analysis revealed how leukaemic blasts appear heterogenous in their morphology and behaviour, with some of them exhibiting longer track lengths, higher total displacement and average speed compared to others, raising questions about the role of this blast sub-population in the process with which AML overtakes the healthy cells. At the same time, IVM experiments captured healthy haematopoietic cells egressing in bulk from the BM parenchyma via the circulation, suggesting that these cells may be displaced from their natural

niche by the growing AML. In line with this, I performed a detailed analysis of the circulating non-malignant cells in healthy and leukemic mice. These data revealed how differentially from the steady-state condition, in which they intravasate as single cells or in clusters of 2 cells, in the context of leukaemia the majority of healthy haematopoietic cells intravasate as clusters of up to 8 cells. These observations suggested that invading blasts may physically dislodge the healthy cells by affecting their anchorage with the extracellular matrix and pushing them through the endothelium and into the vasculature. I then hypothesized that if HSPCs were displaced from the marrow in an AML-dependent manner, we then should expect an increased number of HSPCs in the peripheral blood linked to their decrease in the BM. As hypothesized, flow cytometry analysis of the peripheral blood of AML-burdened mice at multiple stages of disease highlighted an increasing number of lineage negative haematopoietic cells, the number of which was directly proportional to the percentage of BM infiltration. At the same time, analysis of the BM during disease development revealed how the number of lineage negative population and the HSPCs included in it, progressively decreased during disease development. Particularly, while the ST-HSCs and MPPs follow similar kinetics with cells decreasing over time during AML, the LT-HSCs appeared to be the more resilient ones. A deeper analysis of the $CD48^{neg}$ and $CD48^{low}$ LTHSCs revealed how among these two sub-populations, the less proliferative $CD48^{neg}$ cells seemed able to better resist the perturbation caused by AML. This finding is in line with our previous study showing that AML ousts healthy cells with a kinetic that is independent from their cell cycle status with the exception of the $CD48^{neg}$ (Akinduro *et al.*, 2018). Furthermore, analysis of the $CD45^{-}$/$Ter119^{-}$ $CD31^{+}$ ECs at late disease stages revealed how AML leads to the overall reduction of ECs within the BM parenchyma. As in the BM the HSCs are maintained by a perivascular niche, the reduction of ECs could also enforce HSC mobilization from the BM parenchyma (Duarte *et al.*, 2017).

Recently, it has been shown that AML patients with lower HSC frequencies have lower disease-free survival (Wang *et al.*, 2017). Together with this, mathematical modelling suggests that the loss of HCS before AML relapse is due to their dislodgement from the niche (Wang *et al.*, 2017). Overall, the results presented in chapter 3 provide a unique insight into the dynamic interactions of AML with its steady-state counterpart as disease progresses, suggesting that instead of being lost, HSPCs are progressively displaced from their niche by competing blasts. Future studies should investigate further the nature and the role that the blasts sub-population, here called pioneer cells, may have in this process, as well as elucidate if the impact on the haematopoietic system caused by leukaemia is similar among different AML subtypes.

Does leukaemia induce extramedullary haematopoiesis and alternative niches for HSPCs?

Building on these findings (Chapter 3), in Chapter 4 I addressed how AML induces functional extramedullary haematopoiesis (EMH) using flow cytometry and ELISA combined with transplantation assays and demonstrated that, consistent with previous studies that analysed EMH in other pathological conditions (Inra *et al.*, 2015; Yamamoto *et al.*, 2016; Mohyuddin & Yacoub, 2016; Yang *et al.*, 2020), the spleen becomes the preferential site able to accommodate HSPCs that are displaced from the marrow by leukaemic blasts. I observed how the decrease in absolute number of HSPCs in the marrow lead to their increase in the spleen and liver of AML-burdened mice. Further analysis of the HSPC journey to extramedullary sites showed that HSPCs preferentially migrate to the spleen, where LT-HSCs could be found in greater numbers compared to the liver. It has been shown that the production of SCF and CXCL12 from perivascular cells promotes EMH in the splenic niche during myeloablation, blood loss or pregnancy(Inra *et al.*, 2015). Here, in line with that, analysis of spleen

supernatants revealed an increase in the levels of both cytokines in AML-burdened mice, which had developed EMH, compared with healthy animals, in which EMH was not detected. At the same time, I observed an increase in the number of $CD45^{-}/Ter119^{-}$ $CD31^{+}$ ECs in the spleens where the onset of EMH was detected. In the BM, HSCs have been found in close proximity to sinusoidal blood vessels leading to the hypothesis of a perivascular niche that is able to sustain them and similarly, when the EMH occurs in the spleen, HSCs have been found mainly around sinusoids in the red pulp (Kiel *et al.*, 2005; Kiel, Radice & Morrison, 2007; Kiel & Morrison, 2008; Inra *et al.*, 2015). Therefore, the observed expansion of ECs absolute number in the spleens of AML-burdened mice coincides with the establishment of EMH and with the increased number of HSPCs seeding in this site. I next questioned whether the HSPCs found in extramedullary sites maintained their functionality and as a consequence are able to maintain haematopoiesis in the short-term. Transplantation assays confirmed that HSPCs found in both sites maintain a certain level of functionality and highlighted how those isolated from leukaemic spleens had better engraftment and reconstitution potential compared to those isolated from leukaemic livers. This result is in line with the fact that when looking in detail at the dynamic with which the $CD48^{low/neg}$ LT-HSCs are found in both sites, it emerged that the spleen contained a significantly higher number of both $CD48^{neg/low}$ LT-HSCs than the liver. Overall these data suggested that while AML blasts overtake the BM space, the displaced HSPCs migrate in different proportions to the spleen and liver where the increased ECs and levels of regulatory cytokines make both sites, especially the spleen, alternative niches for HSCs. Furthermore, HSPCs maintain their functionality raising the possibility of a role for them in the haematopoiesis maintenance in the short-term. As explained before, AML induce a life-threatening cytopenia with patients often die due to the insufficient haematopoiesis and recurrent infections. Therefore, in this context, thinking of the EMH as a way to maintain the haematopoiesis in the short-term, get a deep insight into the nature of these alternative niches

and the EMH process could lead to the improvement of the haematopoietic process in AML patients. For example, by analysing the spleen environment in comparison to the liver one, it will be possible to highlight which factors make the spleen a better place for HSPCs to seed. Then, once that these signals have been recognised, it could be worth it to induce them in the liver as this will allow a wider tissue to become available for the EMH. At the same time, both extramedullary niches could be compared with the BM one with the aim to recapitulate some of the BM haematopoiesis features that will improve the extramedullary haematopoiesis in both sites.

MMPI treatment delays AML progression and maintains/restores healthy haematopoiesis

Despite the findings in Chapter 4 suggesting that the onset of EMH could maintain emergency haematopoiesis in the short-term, the impact on the HSPCs, resulting in life-threating cytopenias, remains the main problem in AML. Recently, healthy blood count recovery following chemotherapy has emerged as a the second most important predictor of disease-free survival after minimal residual disease burden (Wang *et al.*, 2017). Considering that blood cell recovery is dependent on healthy HSPCs and a healthy niche, the development of therapeutic interventions that can strengthen healthy haematopoiesis and protect HSPCs against leukaemia is necessary.

Building on the findings in Chapter 3 and 4, in Chapter 5 I addressed whether the use of a metalloproteinase inhibitor (MMPI) could reduce the effects of AML on the haematopoietic system resulting in a greater number of HSPCs being maintained in their niche. The finding in Chapter 3 regarding the intravasation process under AML led me to hypothesise that leukaemic

blasts could affect the healthy cells' anchorage to the extracellular matrix (ECM) resulting in the healthy cells intravasating in bulk. In this process, changes to vascular permeability associated with AML could promote cell egression from the BM parenchyma (Itkin *et al.*, 2016; Bixel *et al.*, 2017). As in other tissues, the ECM is an active component of the BM niche where, thanks to its fine regulation, it promotes tissue homeostasis by regulating cell/cell and cells/microenvironment interaction during steady state development (Lapidot, Dar & Kollet, 2005; Daley, Peters & Larsen, 2008; Gattazzo, Urciuolo & Bonaldo, 2014). However, it is recognised that alterations to the ECM and its regulation promote tumour progression and invasiveness (Kleiner & Stetler-Stevenson, 1999; Daley, Peters & Larsen, 2008; Lu *et al.*, 2011; Gaffney *et al.*, 2015). In this context, among the various enzymes taking part in ECM turnover, a specific role in cancer development has been associated to the dysregulation of the expression levels of metalloproteinases (MMPs) (Fridman *et al.*, 2003; Magid, Murphy & Galis, 2003; Gobin *et al.*, 2019; Pan *et al.*, 2014). Their involvement has been proven for the metastatic capacity of solid cancer and, recently, the overexpression of MMPs has been associated with progression and poor prognosis in myeloproliferative neoplasms and leukaemia (Lin *et al.*, 2002; Kamiguti *et al.*, 2004; Paupert *et al.*, 2008; Gobin *et al.*, 2019; Verma *et al.*, 2020). Here, considering the data obtained by IVM, I wondered about the expression of MMPs in our AML model and using RNAseq data previously generated in our lab, I demonstrated that AML blasts overexpress certain MMPs when compared to healthy GMPs. I then decided to test whether targeting these enzymes could impact AML outcome. I choose the metalloproteinase inhibitor prinomastat, which targets multiple MMPs including MMP-9, the latter strongly implicated in the invasiveness, progression and metastatic capacity of several tumours (Scatena, 2000; Liu *et al.*, 2003; Hande *et al.*, 2004; Bissett *et al.*, 2005; Verma *et al.*, 2020). Flow cytometry analysis of blasts during AML progression revealed that despite no differences detected in the percentage of circulating blasts, prinomastat treatment

reduced blast infiltration within the BM, spleen and liver. At the same time, I could observe a decrease in the blasts' speed in prinomastat treated, leukemic mice together with an increase in the proportion of apoptotic blasts in the marrow and spleen and a decrease in the proportion of proliferating blasts in BM and liver of AML burdened-mice. These results suggested that treating the mice with this MMPI directly affects leukaemic blasts. Furthermore, IVM data showed a reduction in the healthy cells intravasating as clusters, together with an increase in the number of single circulating cells. Considering that and the fact that changes in vasculature permeability promote cell egress (Itkin *et al.*, 2016; Bixel *et al.*, 2017), I questioned if the lower number of healthy clusters observed in circulation could be the effect of the treatment limiting vasculature leakiness associated with AML. In fact, it is known that ECs express MMPs during local remodelling induced by angiogenic stimuli such as VEGF that on the other hand, is associated with the increased vasculature leakiness detected in AML (Sun *et al.*, 2017; Mahecha & Wang, 2017; Passaro *et al.*, 2017; Duarte *et al.*, 2017). Surprisingly, prinomastat treatment induced a 2-fold decrease in vasculature permeability comparing to the pbs treated, leukemic mice. Given these data, I then questioned whether prinomastat treatment could affects the AML outcome on the healthy haematopoietic counterpart. Flow cytometry data analysis revealed that when compared by BM infiltration, prinomastat treated mice showed an increased number of residual HSPCs in the BM and a reduction in the EMH phenomenon. As I did not observe a change in the level of key cytokines in prinomastat treated mice, and I concluded that the observed retention of HSPCs in their natural niche is likely to be mediated by a combination of the effects on blast dynamics and vasculature permeability. Finally, I tested whether prinomastat treatment alongside a conventional chemotherapy could have an impact on survival. Unfortunately, despite reducing leukaemia infiltration, prinomastat treatment alone failed to prolong animal survival in comparison to the PBS treated and the chemotherapy only group. However, when given in combination with chemotherapy, I observed a significant

increase in animal survival. Given the need for new therapeutic strategies to improve AML patients' outcome, these findings represent the first evidence that the use of MMPIs could be beneficially implemented alongside conventional AML treatments to improve disease outcome. From this work it emerged that inhibiting MMP-9 improves disease outcome therefore highlighting how these proteases could be a target in leukaemia treatment. However, it will be beneficial to test whether the combinatorial inhibition of other MMPs would have a stronger effect leading to a higher impact on AML and the improvement of vasculature permeability. Specifically, it would be worth it to analyse how AML changes the overall BM ECM and specifically the HSC niche ECM. This analysis will highlight other components a part from the MMPs that may be used as therapeutic targets for AML treatment. Also, how shown here and from other studies, in order to be effective, the MMPI treatment has to start at early stage of AML as delays in starting the treatment lead to its effect to be inefficient. This last point, highlight the need of an early diagnose for AML which might become feasible with the current discovers that link clonal haematopoiesis with an increased risk of AML development. Alternatively, it may be worth considering prinomastat as a way to delay or reduce AML relapse following chemotherapy treatment.

Conclusion

In this thesis, I have carried out a longitudinal analysis of the effects of acute myeloid leukaemia on haematopoiesis and evaluated new approaches for its therapeutic treatment. Based on these studies, I have suggested a number of future directions for this work.

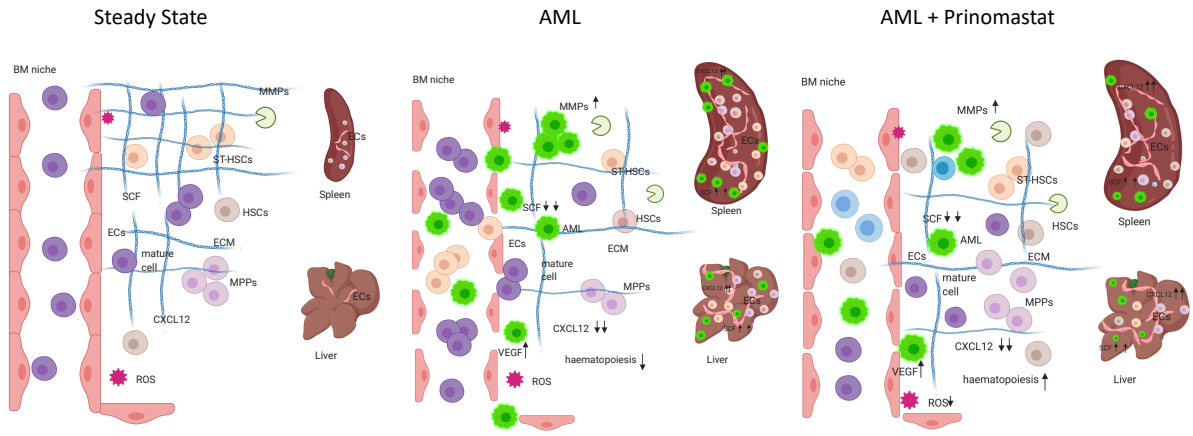

Figure 6.1-AML affects haematopoiesis and induces alternative niches

At steady state, HSPCs are localised in the BM where different cells types and signals provide for their maintenance. During AML progression the blasts that overexpress MMPs compete for the BM space while healthy cells are dislodged through the circulation. In this contest, the increase in vascular permeability induced by AML facilitates cells' egress and likely contributes to the loss of normal haematopoietic cells from the BM. At the same time, the dislodged HSPCs migrate and colonise the spleen and liver, which become sites of EMH in the short-term. Treating mice with prinomastat leads to a decrease in the percentage of blasts within the BM, spleen and liver together with a decrease in the vascular leakiness associated with AML. As a result, prinomastat treatment increases the number of residual HSPCs in the BM trough disease development and decrease the EMH associated with it

It will be of great importance to next elucidate if among the AML blasts, the pioneer cells have a specific signature that results in them having a pivotal role in the mechanism that leads to the loss of HSPCs during AML. In this case, the use of the photoconversion technique would allow to isolate few of these cells among other leukemic blasts. Thus, this approach will be useful

to overcame the lack of specific marker for the pioneer blasts, allowing to evaluate and compare genes signature between pioneer and no-pioneer leukemic cells. In fact, if difference in genes signature would be confirmed, targeting specifically these cells could represent a new strategy that aims to reduce AML impact on the steady-state counterpart. As shown, once HSPCs egress from the BM, EMH onset results in alternative sites from which these cells can respond and carry out emergency haematopoiesis in the short-term. From this work it emerged that ECs expansion and increasing levels of CXCL12 and SCF could maintain HSPCs in these alternative niches. However, here I analysed a limited number of niche components that form the new HSPC microenvironment and to gain a more systematic understanding of the changes taking part in extramedullary sites development, it will be important to analyse other elements, for example the perivascular stromal cells, using available reporter mice and by performing single cell RNAseq of the ECs and stroma population in both microenvironments similar to what has been done for the BM niche (Baryawno *et al.*, 2019). Furthermore, an exciting avenue to explore is whether HSPCs that localise in the extramedullary sites find their way back and actively participate to the BM regeneration following chemotherapy. For this aim, a detailed analysis of the genetic landscape of the HSPCs founded within different sites may help to decipher their contribution in BM and haematopoietic regeneration.

Finally, it will be essential to gain a deeper insight into the use of MMPIs as a possible new treatment in AML. As the use of prinomastat beneficially affects vasculature permeability it would be crucial to decipher the molecular mechanisms that allow a decrease in vascular leakiness and understand the contribution of MMP inhibition on the different components of the BM microenvironment. Ultimately, it would be extremely important to validate my current and further findings using patient-derived xenografts (PDX) to evaluate if it reflects the observations obtained using the murine AML. Clinically speaking it will be important to find

the optimal treatment-window through AML stages that will allow patients to get the best outcome from MMPI treatment. It should be considered that MMPs are enzymes that are widely implicated in a variety of process. Therefore, from a translational point of view, while they represent interesting targets in cancer treatment, some considerations are necessary regarding potential side effects due to their cross reactivity and the effects that inappropriate MMP inhibition could generate in patients. For example, among the early developed MMPIs, none survived clinical trials as a result of intolerable side effects like musculoskeletal syndrome including joint stiffening. Again, the observed side effects could be at least partially attributed to the cross reactivity of the inhibitors as well as a poor understanding of their complex biology (Turk, 2006). In fact, early inhibitors did not distinguish between individual MMPs but blocked the whole panel. This is of particular relevance as, for example, despite their involvement in tumour progression, it is now recognised that some members of the MMP family act to protect against tumours (Levin *et al.*, 2017). Considering that, a great challenge for pharmaceuticals targeting MMPs lies in the high redundancy within the MMP network. In fact, it is crucial to consider MMP activity not just on the basis of the individual activity of individual enzymes but as an interconnected and interdependent proteolytic system (Levin *et al.*, 2017). For example, if one MMP is selectively inhibited, other MMPs can compensate for the lack in function and at the same time, each MMP can hydrolyse multiple substrates with different substrates cleaved by various MMPs (Zucker, Cao & Chen, 2000). Taking everything into consideration, in order to get the best outcome from MMPI treatment, it would be of crucial importance to screen patients for MMP gene expression profiles before considering MMPI treatment, as this will help to identify the best inhibitor that suits their needs. Also, it should be considered that the timeframe of targeting MMPs often differ depending on the cancer stage, as the expression profile as well as the activity of MMPs will not be the same at early and late tumour stages (Gialeli, Theocharis & Karamanos, 2010). As explained before, considering that

the early stages of AML are usually undetected, the best option for starting prinomastat in patients may be represented by the stage that follows chemotherapy, when the blasts infiltration is low. In this context, MMPI treatment could be a way to delay AML relapse. Considering that disease relapse represents the main cause of the overall poor prognosis associated with AML, the use of prinomastat in this context may be truly beneficial for patient's outcome. On another note, from a clinical and translational point of view, it will be important also to consider the pharmacokinetics of these drugs in order to reach a satisfying and safe administration into patients. For example, one reason behind the failure of batimastat in Phase III of clinical trials, was linked to its poor solubility and as a consequence its poor bioavailability that made it mostly impossible for oral administration (Rasmussen & McCann, 1997). Considering that and thinking about the side effects linked to MMPI treatment, the use of systems allowing local drug delivery as a safe alternative to their systemic administration should be investigated. In fact, a system that will specifically carry the MMPI to desired sites of therapeutic action may bypass issues often linked to systemic drug delivery, such as poor bioavailability, high-dose requirements, adverse side effects and non-specific targeting. This may be more feasible for the treatment of solid cancers for which several innovative methods of drug delivery are being used (Senapati *et al.*, 2018). These includes a wide range of nanoscale compounds based on synthetic polymers, proteins, lipids together with organic and inorganic particles. Recently, Purcel *et al* have demonstrated the benefit of localised delivery of tissue inhibitors of MMPs in the context of ECM degradation (Senapati *et al.*, 2018). Recently, Purcel *et al* have demonstrated the benefit of localised delivery of tissue inhibitors of MMPs in the context of ECM degradation (Purcell *et al.*, 2014). Specifically, their work describes the synthesis of a polysaccharide-based hydrogel that can be locally injected into tissues and releases a recombinant tissue inhibitor of MMPs in response to MMP activity, demonstrating as on-demand MMP inhibition could be achievable using an injectable and bioresponsive hydrogel

(Purcell *et al.*, 2014). Therefore, it would be interesting to evaluate if something similar would be feasible for prinomastat and in leukaemia, where the main site for therapeutic drug delivery is represented by the bone marrow.

In summary, in this thesis I aimed to study the mechanisms leading to the loss of healthy haematopoiesis in AML and find new targets to strengthen and maintain the residual HSPCs in their niche. Using IVM, I was able to capture dynamic events leading to the loss of healthy cells and to analyse, with high-resolution, cellular interactions in 3D and in their natural *in vivo* microenvironment. This imaging strategy has the advantage of providing real time, tissue-specific context, which is not measurable by flow cytometry. This approach allowed us to gain a unique insight into the biological interactions and mechanisms of action operated by AML blasts to displace healthy haematopoietic cells from their natural microenvironment. At the same time, flow cytometry was applied to corroborate the IVM findings and extensively study the journey that haematopoietic cells are forced to undergo during AML development. Furthermore, given the need of new therapeutic approaches in AML, I highlight how targeting MMPs could benefit AML' patients as it affects leukaemia growth, improves vasculature leakiness and maintains HSPCs within the BM niche.

I believe the data presented in this thesis contribute greatly to our knowledge and understanding of the impact of AML on the haematopoietic dynamics and to evaluate new therapeutic approaches that can be combined with current chemotherapy treatments. The impact of this work will be maximised through follow up translational studies with the ultimate aim of improving the morbidity and mortality of AML patients.

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Appendix A

RNA sequencing and analysis

Kindly provided by Dr Delfim Duarte

C57BL/6 mice were transplanted with primary AML cells from 3 different donors and scarified at time when fully BM infiltration was reached. Tibias and femurs were then harvested and AML cells were isolated by crushing the metaphysis and sorting $DAPI^{-}$ $GFP^{+}mTomato^{+}$ cells. At the same time, the control population, GMPs, was sorted from whole BM of age- and sex-matched wild-type mice. The RNeasy® Mini kiy (Quiagen, Hilden, Geramany; Cat #74104) was used to extract the total RNA according to the manufacturer's instructions. The extracted RNA was then shipped to the Walter and Eliza Institute of Medical Research (WEHI, Melbourne) were it was processed. Briefly, the extracted RNA was analysed on the Agilent 4200 Tapestation prior to library preparation. High quality RNA with RIN values greater than 9 was used for downstream application. 3'mRNA-sequencing libraries were prepared from 100ng of total RNA using the QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen) according to the manufacturer's instructions and sequenced on a NextSeq 500 (Illumina). The single-end 75bp were demultiplexed using CASAVAv1.8.2 and Cutadapt (v1.9) was used for read trimming. The trimmed reads were subsequently mapped to the mouse genome (mm10) using HISAT2. FeatureCounts was used for read counting (Liao et al., 2014) after which differential gene expression analysis was performed using Voom- LIMMA packages (Law et al., 2014). GSEA2-2.2.2 was used for Gene set enrichment analysis (GSEA) (Liberzon et al., 2015; Subramanian et al., 2005).

Appendix B

Supplementary video legends

Supplementary Video 1: Representative maximum projection of 3D time lapses acquired every 3 minutes for 102 minutes (shown at 3 frame per second) of an area from a leukemic mouse (shown in Figure 3.7). Among leukemic blasts, white arrows highlight the explorative cells. Tomato^{+} healthy haematopoietic cells are shown in red; leukemic blasts are YFP^{+} and shown in green.

Supplementary Video 2: Representative maximum projection of 3D time lapse data acquired every 3 minutes for 24 minutes (shown at 3 frames per second) of an area from a leukemic Flk1-GFP mouse (shown in Figure 3.10). Healthy haematopoietic cells are shown in red; leukemic blasts are YFP^{+} and shown in dim green. Endothelial cells are GFP^{+} and shown in bright green. White arrows indicate the healthy haematopoietic cells intravasating into the circulation.

Supplementary Video 3: Representative maximum projection of 3D time lapse data (shown at 2 frames per second) of an area from a leukemic PBS treated (left) and leukemic prinomastat treated (right) Flk1-GFP mouse at late disease stage (blasts in PB >10%), injected with 3mg 65-80kDA TRITC-dextran (shown in Figure 5.4A). An image was recorded every minute for 15 minutes immediately after injecting the vascular dye in order to assess vascular leakiness. Flk1-GFP+ ECs are shown in green; TRITC-dextran is shown in magenta.

Supplementary Video 4: Representative maximum projection of 3D time lapse data (shown at 2 frames per second) of an area from a healthy (left) and a leukemic (right) Flk1-GFP mouse at early disease stage (blasts in PB <10%), injected with 3mg 65-80kDA TRITC-dextran (related to graph obtained in figure 5.4B). An image was recorded every minute for 10 minutes immediately after injecting the vascular dye in order to assess vascular leakiness. Flk1-GFP^{+} ECs are shown in green; TRITC-dextran is shown in magenta.