MONASH University

Mechanisms of differentiation therapy response and relapse in acute myeloid leukaemia

Steven Ngo

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Abstract

Acute myeloid leukaemia (AML) is an aggressive malignancy characterized by the accumulation of transformed immature myeloid blasts. In recent years, genome wide analyses of large cohorts of AML patients has identified AML as a genetically heterogenous disease. Despite the diversity in genetic lesions capable of driving the disease, AML genomes have considerably fewer mutations than other adult cancers. From such studies, a number of co-occurring genetic lesions were identified to drive AML. However, perhaps more remarkably was the striking mutual exclusivity between some of the most common mutations known to cause a differentiation block in AML. The mutual exclusivity is not only a testament to the genetic simplicity of AML, but also strongly suggests that these particular genetic lesions are likely to converge on and dysregulated a single common gene to enforce the differentiation block. The ETS family member transcription factor PU.1 is a master regulator of myeloid cell differentiation. Although point mutations to the transcription factor is rarely seen in AML patients, mouse models of AML have been generated by . Previously in the Dickins laboratory, a reversible AML model was also generated by toggling PU.1 expression in p53 -/- HSPCs, via the infection of a Tet-regulated shPU.1 hairpin (known as AML246). Consistent with the literature, loss of PU.1 expression in the presence of a p53 -/- mutation triggered the development of an aggressive AML. Conversely, restoring endogenous PU.1 expression in AML blasts triggered differentiation into mature granulocytes, thus showcasing the differentiation block that is caused by dysregulated PU.1 function. Remarkably, re-engagement of PU.1 suppression triggered differentiated leukemic cells to de-differentiate and return to a blast like state, showcasing the maturational plasticity of mature AML-derived cells. Interestingly, consistent with the literature, we found that the overexpression of a range of commonly occurring, oncogenic lesions such as AML1-ETO and PML-RARα, perturbed PU.1-mediated differentiation and apoptosis of our inducible AML model. Hence, dysregulation of PU.1 may be a common process by which mutually exclusive oncogenic mutations may drive AML.

Although standard cytotoxic chemotherapy routinely induces disease remission, most AML patients ultimately relapse with resistant disease. A notable exception is the AML subtype known as acute promyelocytic leukemia, where retinoic acid induces leukaemia maturation and transient remission as a single agent and is frequently curative in combination with arsenic trioxide. Recently agents including mutant IDH1/2 inhibitors and DHODH inhibitors have

been shown to induce maturation and regression of other AML subtypes, sparking renewed interest in AML differentiation therapy. The inducible PU.1 AML model described above (AML246) was transplanted in mice to mimic differentiation therapy in the clinic. Restoration of PU.1 in AML blasts triggered widespread differentiation and disease remission *in vivo,* however these mice also invariably succumb to disease relapse. Notably, time course studies of *in vivo* AML treatment reveal that one week after differentiation stimulus leukemic blasts mature into two myeloid lineages, the neutrophil-like and eosinophil-like populations. Whereas AML-derived neutrophils were cleared following differentiation AML-derived eosinophils persisted during disease remission in the bone marrow, as well as extramedullary organs such as the spleen, liver and kidneys. Hence, this data suggests that persistent AML-derived eosinophils were responsible for seeding relapse. Remarkably, CRISPR-Cas9-mediated KO of the key eosinophil transcription factors such as Gata1 and Xbp1 prevented AML blast differentiation along the eosinophil lineage following differentiation stimulus. Although experiments are still ongoing, prevention of AML eosinophil differentiation has thus far resulted in improved overall survival and the prevention of relapse in a significant number of mice.

Ultimately, these results demonstrate that AML differentiation therapy can produce long-lived sublineages of mature AML-derived cells from which relapse can originate. Hence, understanding the multilineage potential of AML blasts in individual patients may inform strategies that preclude or eradicate mature AML-derived cells to improve differentiation therapy outcomes.

Declaration

This is to certify that:

I) The thesis comprises of only my original work towards the degree of the PhD except where indicated in the Preface,

II) Due acknowledgement has been made in the text to all other material used,

III) The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

Steven Ngo
June 2019

Preface

Pursuant to the regulations governing the degree of Doctor of Philosophy at Monash University, I hereby submit that:

In Chapter 3, splenectomy of mice was performed by Dr. Peter Kanellakis. Fluorescence activated cell sorting was performed by AMREPflow core facility members. All analytical flow cytometry was performed by myself. MGG stains were performed by Alfred Hospital Histology group. The author's contribution to this chapter was 90%.

In Chapter 4, cloning of sgRNA into lentiviral vectors was performed by Max Garwood. PCR and sequence verification of potential CRISPR-Cas9 KO clones was also performed by Max Garwood. Splenectomy of mice was performed by Dr. Peter Kanellakis. The author's contribution to this chapter was 90%.

In Chapter 5, our AML model driven by inducible PU.1 knockdown (AML246) was originally generated by Dr. Mark McKenzie. Initial characterization of AML246, including assessment of differentiation in response to Dox (and de-differentiation upon removal of Dox) was performed by both Dr. Mark McKenzie and Dr. Margherita Ghisi. The author's contribution to this chapter was 85%.

Publications

McKenzie MD*, Ghisi M*, Oxley EP*, **Ngo S**, Cimmino L, Esnault C, Liu R, Salmon JM, Bell CC, Ahmed N, Erlichster M, Witkowski MT, Liu GJ, Chopin M, Dakic A, Simankowicz E, Pomilio G, Vu T, Krsmanovic P, Su S, Tian L, Baldwin TM, Zalcenstein DA, DiRago L, Wang S, Metcalf D, Johnstone RW, Croker BA, Lancaster GI, Murphy AJ, Naik SH, Nutt SL, Pospisil V, Schroeder T, Wall M, Dawson MA, Wei AH, De The H, Ritchie ME, Zuber J, Dickins RA. Interconversion between tumorigenic and differentiated states in acute myeloid leukemia. *Cell Stem Cell*, in press (2019).

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To my partner Nicole, words cannot describe how much your support has meant to me over the last three years. Thank you for always finding time to hear me vent about every minute setback, and for pulling me out of the spiral of negativity I often find myself in when things don't go to plan in the lab (with herself completing an intense medical degree and working a highly stressful job all the while!). You are truly brilliant.

My sisters Helen and Cathy, thank you for your constant encouragement and support throughout my life. It's been amazing to finally have all of us living in the same country once more, and to also watch both your families grow. To my beautiful nephews and nieces Ryan, Maddy, Maia and Oscar, thank you for making me laugh and helping me maintain perspective in life- there is nothing more I've enjoyed than spending my time with you all on the weekends.

Lastly, I'd like to thank my parents, Thanh-Chi and Thuc Trinh Ngo. 2019 marks the 40th year anniversary since my parents (and sister) took the life-threatening, fateful journey on a small boat from war-torn Vietnam to Australia. It goes without saying that none of my achievements would be possible without the sacrifices you have both made.

Abbreviations

AML	acute myeloid leukaemia
AS	arsenic
ATRA	all-*trans* retinoic acid
ATO	arsenic trioxide
BM	bone marrow
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CNS	central nervous system
CR	complete remission
crRNA	CRISPR RNA
CRT	calreticulin
CRISPR	clustered regularly interspaced palindromic repeats
CSC	cancer stem cell
Dox	Doxycycline
DS	differentiation syndrome
DSB	double stranded breaks
dsRNA	foreign double-stranded RNA
EM	extramedullary relapse
EoPs	eosinophil progenitors
ER	endoplasmic reticulum
FSC	forward scatter
GOI	gene of interest
GMPs	granulocyte monocyte progenitors
HDAC	histone deacetylases
HDR	homologous directed repair
HSCs	haematopoietic stem cells
HSCT	haematopoietic cell transplantation
HSPCs	haematopoietic stem/progenitor cells
IDH2	isocitrate dehydrogenase 2
IVIS	In Vivo Imaging System
LICs	leukemia initiating cells
LPS	lipopolysaccharide

LSC	leukemic stem cell
MBP	major basic protein
MegE	megakaryocyte/erythroid
MEPs	megakaryocyte/erythroid progenitors
microRNA	miRNA
mIDH2	mutant IDH2
NGS	next generation sequencing
NHEJ	non-homologous end joining
PAM	protospacer adjacent motif
PrCR	programmed cell removal
pri-miRNA	primary transcript micro RNA
RA	retinoic acid
RNAi	RNA interference
rrAML	relapse refractory AML
RT-PCR	reverse transcriptase PCR
rtTA	Tet-on transactivators
sgRNA	single guide RNA
shRNA	short hairpin RNA
SNVs	single-nucleotide variants
SSC	side scatter
tracrRNA	transactivation RNA
TRE	Tet-responsive promoter
tTA	Tet-off transactivators
UPR	unfolded protein response
VAF	variant allele frequency
WBC	white blood cells
WES	whole exome sequencing
WGS	whole genome sequencing

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Chapter 1: Literature Review

1. Epidemiology and clinical presentation of AML

Acute myeloid leukaemia (AML) is an aggressive haematological malignancy characterized by the accumulation of transformed immature myeloid blasts. It is a remarkably aggressive disease, with patients diagnosed with AML having a 5-year survival rate of 25% (De Kouchkovsky et al., 2016; Pulte et al., 2013). With many forms of cancer, the overall survival rate gradually decreases as patients get older, which is particularly concerning given the ever-aging population globally. Patients with AML not only suffer from the accumulation of transformed white blood cells (WBC), but also present with anaemia and very low platelet counts (De Kouchkovsky et al., 2016). Currently the most common method of treatment involves the use of cytotoxic chemotherapy agents such as Cytarabine, Fludarabine and Azacytidine (Dombret et al., 2016). The non-specific nature of these chemotherapeutic agents may contribute largely to the poor prognosis of AML patients, hence there is a growing need to find alternative methods of treating AML. The development of AML is triggered by the acquisition of oncogenic mutations that not only promote cell survival (traditionally known as class I mutations), but more importantly, mutations that block myeloid cell differentiation (class II mutations). The retention of AML blasts in an immature, proliferative state ultimately leads to the development of the disease. Recent advancements in genome and exome sequencing has identified a number of oncogenic mutations responsible for driving AML, however in order to understand how such oncogenic mutations trigger AML mechanistically, it is important to understand the normal process of myeloid cell production, otherwise known as myelopoiesis.

1.2 Myelopoiesis

1.2.1 PU.1 is a master regulator of myeloid cell production

Myelopoiesis involves a series of maturational steps that ultimately leads to the production of the myeloid compartment, encompassing neutrophils, basophils, mast cells, eosinophils and monocytes (Raskin, 1996). There are a number of transcription factors that play a role in the development of the myeloid compartment, however central to the production of mature myeloid cells is the transcription factor PU.1. PU.1 is encoded by the *Spi1* gene and is a member of the highly conserved ETS transcription factor family. PU.1 can autoregulate its own

expression, as the PU.1 protein is able to bind to its own promoter and drive further downstream transcription of the gene (Okuno et al., 2005). Although PU.1 is synonymous with myeloid cell differentiation, low levels of PU.1 also play an important role in B cell development (Torlakovic et al., 2001). In conjunction with other regulatory factors (which will be explored in further detail later), PU.1 is responsible for activating the expression of several differentiation cell surface markers such as CD11b, CD16, CD18 and CD64 (Fisher et al., 1998; Kastner et al., 2008). Furthermore, the expression of this transcription factor also facilitates the expression of cytokine receptors for G-CSFR and M-CSFR, both of which promote the differentiation of the myeloid progenitor into different myeloid subtypes (Gangenahalli et al., 2005). Consequently, PU.1 null mice die during late gestation due to the lack of fetal liver granulocytes, macrophages and B lymphocytes (Fisher et al., 1998; Kastner et al., 2008). PU.1^{gfp} reporter mice accurately display the level of PU.1 expression throughout the hematopoietic system during different stages of maturation. PU.1 levels are high in haematopoietic stem cells (HSCs), suggesting an important role for PU.1 during the early stages of haematopoietic development. PU.1 expression is still present in common lymphoid progenitors (CLPs), however as the progenitor cells matures into more committed lymphoid lineages, PU.1 expression levels gradually decrease, such that PU.1 expression is lower in mature B cells and absent in T lymphocytes (Nutt et al., 2005). Whereas PU.1 mRNA and protein levels gradually decrease along the lymphoid lineage, the expression levels of the PU.1 transcription factor varies in different myeloid cell subtypes. Like CLPs, common myeloid progenitors (CMPs) also express high levels of PU.1. Downregulation of PU.1 along the myeloid lineage is associated with the restriction of CMP differentiation into megakaryocytes and erythrocytes, however PU.1 expression levels are highest in neutrophils and macrophages (Chen et al., 1995; Nutt et al., 2005). Hence, there is a greater importance of the PU.1 transcription factor in myeloid lineage differentiation in comparison to the lymphoid lineage.

1.2.2 PU.1 /Gata1 cross-antagonism promotes erythropoiesis and megakaryopoiesis

Although PU.1 is considered the master regulator of myeloid cell differentiation, its interactions with other key transcription factors play an integral role in the commitment of haematopoietic stem/progenitor cells (HSPCs) along either the myeloid, lymphoid or megakaryocyte/erythroid (MegE) lineages. Most notable is the antagonistic relationship between PU.1 and Gata1. As the name suggests, Gata1 is part of the GATA family of transcription factors, characterized by their ability to bind to the 'GATA' DNA sequence

(Merika et al., 1993). GST pull-down assays and co-immunoprecipitation assays show that PU.1 physically binds to the C terminal binding domain of Gata1 (Nerlov et al., 2000). Much like PU.1, Gata1 is a pioneering transcription factor that directly binds to chromatin and recruits other transcription factors important for the initiation of cell differentiation (Kadauke et al., 2012). Their relationship is often cross antagonistic, however emerging evidence suggests that the nature of their interaction is often context dependent (Burda et al., 2010; Du et al., 2002). In the case of erythro-megakaryopoiesis, Gata1 functions to negatively regulate PU.1 to induce differentiation into megakaryocytes and erythroid cells (Fig 1.1) (P. Zhang et al., 1999). Murine erythroleukemia cells (MEL) co-express both the Gata1 and PU.1 transcription factors. Treatment of MELs with chemical inducers such as DMSO or HMBA results in the terminal differentiation of blast cells into the erythroid lineage. Interestingly, this corresponds to a gradual decrease in PU.1 transcription, indicating that low levels of PU.1 are required to produce erythrocytes and megakaryocytes (P. Zhang et al., 2000). Consistent with this observation, exogenous expression of Gata1 also promotes erythroid differentiation whereas overexpression of PU.1 inhibits Gata1 target genes and blocks Gata1 mediated differentiation, leading to erythroleukemia in MEL cells (Choe et al., 2003). Importantly, the expression of Gata1 alone does not trigger megakaryopoiesis or erythropoiesis, but rather prevents PU.1 from promoting HSPC maturation along the myeloid lineage (Cantor et al., 2002). Gata1 expression in conjunction with other transcription factors including Friends of Gata1 (FOG-1) are required to form complexes with Gata1 and activate several megakaryocytic and erythroid genes. Gene expression assays also outline the antagonistic relationship between PU.1 and Gata1 during erythro-megakaryopoiesis, with the activation of Gata1 corresponding to the downregulation of key target genes of PU.1 and myeloid cell differentiation such as Cebpα and Itgam (CD11b) (Burda et al., 2010; Du et al., 2002). In the case of Itgam, the c-Jun transcription factor functions as a coactivator of PU.1 for various myeloid promoters, including M-CSFR (Nerlov et al., 2000). Gata1 can repress the M-CSFR by displacing the c-Jun transcription factor from the PU.1 Ets domain, leading to the inhibition of PU.1 activity (P. Zhang et al., 1999). Therefore, mechanistically Gata1 can physically repress PU.1 target genes by competing for PU.1 DNA binding domains, ultimately promoting differentiation along the erythro-megakaryocyte lineage.

1.2.3 PU.1/Gata1 switch does not initiate lineage choice of HSPCs

Traditionally, it has been proposed that the decision of a HSPC to differentiate along the erythroid or myeloid lineage was determined by the cross-antagonism of both PU.1 and Gata1 in the CMP, the final common ancestor before bifurcation into the myeloid or Meg/E lineages (Figure 1.1). Although PU.1 and Gata1 undoubtedly play an important role in myeloid and Meg/E lineage differentiation respectively, traditional models of haematopoiesis suggests that lineage choice was determined by random fluctuations in the expression of both transcription factors, before one transcription factor 'conquers' the other and commits the CMP into a particular lineage (Graf et al., 2009). Therefore, in this model, PU.1/Gata1 expression in the CMP not only promotes lineage commitment, but also initiates the process (Arinobu et al., 2007). However, with the emergence of more sensitive flow cytometry and live imaging techniques, this model of lineage choice has been challenged in recent years. Consistent with previous studies, $PU.1^{YFP}$;$Gata1^{mCherry}$ reporter mice show upregulated expression of Gata1 and downregulation of PU.1 in megakaryocyte/erythroid progenitors (MEPs), and inverse expression in GMPs (high PU.1, negative for Gata1) (Hoppe et al., 2016). However, contrary to previous models, a PU.1, Gata1 double positive CMP population did not exist in this model. In fact, CMPs could already be distinctly separated into $PU.1^{+}$ $Gata1^{-}$, or $PU.1^{-}$ $Gata1^{+}$ populations. Hence, lineage choice into the myeloid or Meg/E lineages preceded the CMP stages of development. Furthermore, using live imaging of individual HSPCs from the $PU.1^{YFP}$;$Gata1^{mCherry}$ reporter mice was also able to show that in the context of myeloid vs Meg/E differentiation, Gata1 does not cause the downregulation of PU.1 during differentiation along the Meg/E lineage, as PU.1 levels are already undetectable long before Gata1 protein can be detected in the differentiating HSPCs (Hoppe et al., 2016). Therefore, although Gata1 and PU.1 can enforce lineage commitment, the random fluctuations in expression of these transcription factors does not initiate the lineage choice of the HSPC. This decision is likely to occur much earlier during haematopoiesis, however the mechanisms involved are currently unknown (Strasser et al., 2018).

1.2.4 Eosinophil development is regulated by co-operative Gata1/PU.1 interactions

Whereas in the context of Meg/E lineage differentiation Gata1 and PU.1 function antagonistically, both these transcription factors function co-operatively in the development of a sub population of myeloid cells known as the eosinophil. Eosinophils are a rare subpopulation of post-mitotic, pro-inflammatory granulocytes that combat parasites and promotes allergic reactions (Y. M. Park et al., 2010). The development of eosinophils begins with the lineage commitment of the granulocyte monocyte progenitors (GMPs) into eosinophil progenitors (EoPs). Eosinophil progenitors continue to mature in the bone marrow (BM), where extensive changes to the transcriptional profile takes place in the maturing eosinophil. This is also coupled with the upregulation of Ccr3 in terminally differentiated eosinophils, whereby these fully mature eosinophils will then migrate into the peripheral blood (Lamkhioued et al., 2003; Tiffany et al., 1998). The interplay between a number of transcription factors facilitates the maturation of eosinophils during different stages of eosinophil development. Both neutrophils and eosinophils are sub-populations of the granulocytic lineage. Unlike transcription factors such as Id2 which regulates the differentiation of both lineages (Buitenhuis et al., 2005; Uhm et al., 2012), Gata1 is uniquely important for eosinophil development and not the maturation of neutrophils (Hirasawa et al., 2002). Gene expression profiling of single cell murine progenitor cell demonstrates the importance of Gata1 to the eosinophil lineage, with the segregation of multipotent progenitor cells committing to the eosinophil lineage corresponding neatly to the expression of the Gata1 transcription factor (Olsson et al., 2016). Isolation of GMPs from Gata1^{GFP} reporter mice cultured *in vitro* shows that eosinophils are only found in the GFP fraction, further outlining the requirement of Gata1 for eosinophil development (Suzuki et al., 2009). Given this, deletion of a high affinity GATA site within the Gata1 promoter predictably leads to the selective loss of the eosinophil lineage *in vivo* (Yu et al., 2002). As mentioned previously, during the early hematopoietic stages, high Gata1 expression is inhibitory to eosinophil differentiation, as it prevents PU.1-mediated commitment of multipotent progenitors to the myeloid lineage. Conversely, high PU.1 levels not only commits HSPCs along the myeloid lineage, but it also essential for downregulating Gata1 expression in myeloid progenitors (myeloblasts) to an intermediate level that allows for eosinophil differentiation. Importantly, absence of Gata1 expression in the myeloid lineage leads to complete ablation of the eosinophil lineage without impact on neutrophils or eosinophils (Galloway et al., 2005). Hence PU.1-mediated downregulation of Gata1 (but not complete repression) is required to promote myeloid lineage differentiation, and to also bring Gata1

levels to an intermediate level that is suitable for eosinophil differentiation (Du et al., 2002). As an example of their co-operation in promoting eosinophil differentiation, both transcription factors are required for the expression of Major Basic Protein (MBP), a gene that is upregulated in EoPs to promote differentiation into mature eosinophils (Uhm et al., 2012). The P2 promoter region of MBP contains consensus binding sites for PU.1 and Gata1. In early eosinophil progenitors, Gata1 alone is able to transactivate the MBP promoter, however it is synergistically transactivated in the presence of low levels of PU.1 (Du et al., 2002). Interestingly, CEBPe functions as a repressor and blocks Gata1 interaction with PU.1 at the MBP promoter region, thereby blocking transcription of MBP and further development along the eosinophil lineage (Gombart et al., 2003). Hence, although Gata1 and PU.1 play important roles, eosinophil differentiation is a multi-factorial process involving the co-ordination of a number of key transcription factors.

1.2.5 Xbp1 is selectively required for eosinophil differentiation

Xbp1 is a transcription factor normally associated with the 'unfolded protein response' (UPR), a pathway activated to ameliorate cellular endoplasmic reticulum (ER) stress due to protein overload (Sha et al., 2009), but more recently implicated uniquely in the differentiation of the eosinophil lineage. Briefly, the UPR involves three transmembrane stress sensors, IRE1α, PERK and ATF6α. Activation of PERK leads to a block in protein translation and activation of downstream compensatory autophagy (Bravo et al., 2013; Lebeau et al., 2018), whereas involvement of the ATF6α branch of UPR leads to removal of misfolded proteins. The activation of IRE1α however, leads to the splicing of Xbp1, a potent transcriptional activator (Jiang et al., 2015). In the context of UPR, Xbp1 target genes play an important role in increasing protein folding capacity of the ER (Jiang et al., 2015). In myelopoiesis however, the expression of Xbp1 is specifically upregulated in GMPs and EoPs, before there is a gradual decrease in terminally differentiated eosinophils, presumably due to a significant drop in protein synthesis demand in a fully mature cell (Bettigole et al., 2015). More importantly, conditional knockout out of Xbp1 in the myeloid lineage leads to complete ablation of the eosinophil lineage, with no impact on total BM cellularity or other lymphoid and myeloid lineages (Bettigole et al., 2015; Shen et al., 2015). The loss of Xbp1 appears to impact the eosinophil lineage at the progenitor stage, with a significant increase in EoP cell death in Xbp1 KO mice (Bettigole et al., 2015). Interestingly, despite high frequency of Xbp1 mRNA splicing in GMPs, GMP frequency in Xbp1 deficient mice was comparable to normal, once again

outlining the maturation stage specific requirement for spliced Xbp1 in the development of eosinophils. Of the residual eosinophils that were present in Xbp1 deficient mice, these EoPs downregulated Gata1 as well as key eosinophil effectors Prg2 and Epx (Bettigole et al., 2015). Therefore, given the defects to the granule proteins (Prg2 and Epx), it is believed that the loss of the eosinophil lineage in Xbp1 deficient mice may be attributed to major reduction and misfolding of eosinophil granule proteins, such that these dramatic changes may induce ER stress and disrupt terminal differentiation (Bettigole et al., 2015).

1.2.6 Transcription factors that co-operate with PU.1 to trigger neutrophil and monocyte lineage differentiation

PU.1 is indispensable in the development of neutrophils and macrophages, as GMPs are unable to differentiate into either lineage in conditional PU.1 knockout mice (Chen et al., 1995). However, PU.1 alone cannot facilitate differentiation of the neutrophil and monocyte/macrophage lineages, but instead requires further activation of different transcription factors to complete myeloid cell maturation. Most notably, CCAAT/enhancer binding protein α (Cebpα) is a transcription factor that physically binds to the enhancer region of PU.1 to promote monocyte and granulocytic differentiation (D. Wang et al., 2006). Retroviral transduction of Cebpα in PU.1 competent and PU.1 haploinsufficient cells greatly increases monocyte production (Yeamans et al., 2007). Cebpα expression in early progenitors also promotes granulopoiesis, such that ablation of Cebpα results in impaired neutrophilic and eosinophilic differentiation (Ma et al., 2014). Interestingly, Cebpα null mice retain the monocytic lineage (D. E. Zhang et al., 1997) however, overexpression of Cebpα directly downstream of the GMP stage results in increased production of monocytes. Therefore, it is likely that Cebpα may be an important transcription factor for monocyte development, although its expression may not be essential. Notably, cell fate commitment into either the granulocytic or monocytic lineages upon Cebpα expression appears to be in part, dependent on PU.1 (Yeamans et al., 2007). Overexpression of Cebpα in PU.1^{low} cells differentiating into granulocytes, and overexpression in PU.1 competent cells resulting in monocytic differentiation (Yeamans et al., 2007). In addition to Cebpα, transcription factors such as Egr-2 and Nab-2 are induced upon PU.1 expression to promote monocyte/macrophage lineage commitment (Dahl et al., 2007). Both Egr-2 and Nab-2 bind the promoter region of PU.1, thereby repressing PU.1-mediated activation of Gfi-1, a key transcription factor for neutrophil fate determination (Dahl et al., 2007). Conversely, overexpression of the Gfi-1 transcription

factor blocks PU.1 induced macrophage differentiation by physically binding to PU.1 itself, and antagonizes the PU.1 transactivation of target genes that promote macrophage differentiation such as M-CSFR (Csf1r) and Mac-3 (Dahl et al., 2007). Gfi-1 also counter-represses Erg-2, further inhibiting the expression of key macrophage genes and thus promoting the differentiation of progenitor cells into neutrophils at the expense of macrophages (Dahl et al., 2007). Hence, in addition to the master regulator PU.1, the co-operative or antagonistic interaction between transcription factors mentioned above dictate the differentiation of progenitor cells along the neutrophilic or monocytic/macrophage lineage.

1.2.7 Extrinsic factors that promote myeloid lineage specific differentiation

Transcription factors play an integral role in the differentiation of progenitors into committed myeloid lineages. However, in addition to these intrinsic signals, extrinsic cues also play an essential role for dictating myeloid cell differentiation. PU.1 regulates the expression of a range of myeloid specific genes such as cytokine receptors granulocyte stimulating factor receptor (G-CSFR), macrophage-CSFR (M-CSFR) and granulocyte/macrophage CSFR (GM-CSFR) (Fisher et al., 1998). The cytokine G-CSF promotes the survival and differentiation of neutrophils (Roberts, 2005), as well as mobilization of HSPCs from the BM (Petit et al., 2002). Predictably, given its essential role in neutrophils, mice lacking G-CSF are severely neutropenic (Lieschke et al., 1994), however considerably lower numbers of mature neutrophils are still present in these mice suggesting that other factors also contribute to the differentiation of neutrophils (Lieschke et al., 1994). Interestingly, monocytes also express low levels of G-CSFR, and thus G-CSF null mice also see reduced numbers in monocytes and macrophages (Lieschke et al., 1994). Neutropenic, G-CSF lacking *Csf3*-deficient mice crossed with haploinsufficient PU.1 ($PU.1^{+/-}$) mice produce progeny with increased neutrophils in the BM (thereby rescuing the neutropenia), whereas mice with a PU.1 competent genetic background ($Csf3^{-/-}PU.1^{+/+}$), generate more macrophages than their haploinsufficient counterpart (Dahl et al., 2003).This is consistent with early studies showing that higher PU.1 expression is required for monocytes/macrophages than neutrophils (Dahl et al., 2003). Similarly, M-CSF null mice are severely monocytopenic, however they are not completely bereft of functioning monocytes and macrophages (Schonlau et al., 2003). Mice lacking all three major myeloid cytokines (G-CSF, M-CSF, GM-CSF) showed significantly reduced circulating number of neutrophils and monocytes as well as a shortened lifespan (Hibbs et al., 2007). However, these triple knockout mice still produced low numbers of both neutrophils

and monocytes, re-emphasizing the role of compensatory factors that contribute to the production of both lineages (Hibbs et al., 2007). Nevertheless, these cytokines evidently play a very important role in the development of neutrophils and monocytes. In the case of eosinophil development, IL-5 serves as the major maturation and differentiation factor (Kouro et al., 2009). Ectopic expression of IL-5 significantly increases eosinophil production *in vivo*, and administration of anti-IL-5 is highly effective in treating patients with hypereosinophilia (Rothenberg et al., 2008). IL-5 null mice also have significantly reduced, but not complete ablation of the eosinophil lineage (Matthaei et al., 1997). Together, the generation of cytokine deficient mice shows the importance of these external factors in the regulation of myeloid cell differentiation into specific lineages.

1.3. Acute myeloid leukaemia

As described above, the generation of the myeloid lineage is a tightly regulated process governed by the regulation of transcription factors as well as the presence of external cytokines. However, genetic mutations that compromise the function of these transcription factors not only cause a differentiation block in the progenitor cell population, but retention of progenitor cells in this immature and highly proliferative state may be the catalyst for myeloid cell transformation into a malignant state. Furthermore, AML can be categorized into many different subgroups based on morphology, encompassing leukaemias where blasts are more monocytic (Fenaux et al., 1990), as well as those that resemble eosinophils such as the case with some core binding factor (CBF) and inv(16) driven AMLs (Duployez et al., 2016; Xiao et al., 2018). Recent developments in single cell RNAseq has shown that HSC commitment into mature lineages is a continuous process (Eaves, 2015). Likewise, although AML is driven by mutations that prevent further myeloid cell maturation, the differentiation blocking mutation can be acquired during many stages of the myeloid cell differentiation continuum. Consequently, the cellular makeup of AML can be heterogenous, with some leukemic blasts transcriptionally resembling immature GMPs, whereas others demonstrate a more monocytic immunophenotype (van Galen et al., 2019) .To match the cellular heterogeneity of AML, there is also an array of genetic mutations that can cause AML (Ley et al., 2013). However, despite the genetic and cellular diversity of AML, it is a relatively simple genetic disorder, with an average of only 5 genetic driver mutations found within an AML patient (Ley et al., 2013). Notably, most of the mutations found in the AML genome are random events that precede the disease-initiating mutation (Welch et al., 2012). Therefore, the co-operation of only a few

(average of 5) mutations is required to generate the founding malignant clone, emphasizing the genetic simplicity of the disease.

1.3.1 The genetic landscape of AML

Recent advancements in genome sequencing technology allowing for a greater understanding of the molecular mechanisms that underpin the disease. For decades, cytogenetic analysis alone was used to determine the pathogenesis of AML (Grimwade et al., 2011). Despite complex cytogenetics (such as a monosomy karyotype) being a subtype with a poor survival outcome, over 50% of AML patients possess a normal karyotype (Ley et al., 2013). Hence the emergence of next generation sequencing (NGS) has revolutionized our understanding of AML as well as our approach in treating the disease. Whole genome or exome sequencing of large AML patient cohorts has been able to successfully map out the AML genome (Döhner et al., 2017; Ley et al., 2013). From such studies, it is evident that AML is a genetically heterogenous disease, with only twenty-three genes commonly mutated across all the AML samples that were sequenced (Döhner et al., 2017; Kihara et al., 2014; Ley et al., 2013; Papaemmanuil et al., 2016), thus re-iterating the genetic simplicity of the disease. As a result of such large scale AML genome studies, a number of commonly occurring mutations are used as key diagnostic and prognostic markers of AML in the clinical sphere (NPM1, FLT3 ,CEPBα,RUNX1, TP53 and ASXL1 mutations etc.) (Dohner et al., 2015) (Grossmann et al., 2012). Monitoring fusion oncogenes such as PML-RARα (Albano et al., 2015; Chendamarai et al., 2012), AML1-ETO (L. Zhang et al., 2014; L. Zhang et al., 2013) and CBFb-MYH11 (Ravandi et al., 2018) using polymerase chain reaction (PCR)-based assays during periods of minimal residual disease (MRD) is now common practice clinically, and is able to accurately predict the risk of relapse in patients harbouring these given mutations. Collectively, it is apparent that the application of NGS to uncover the genetic landscape of AML has shaped our understanding of the pathogenesis of the disease and subsequently, how patients can be effectively treated.

1.3.2 Categorization of genetic lesions in AML

Traditionally oncogenic driver mutations of AML were broadly categorized into two major groups: those that promote cell survival (class I) and those that block differentiation (class II) (Frohling et al., 2005; Kelly et al., 2002). The emergence of genome sequencing and the identification of the AML genome, has led to more sophisticated characterization of AML related mutations, separating recurrent mutations into nine distinct subgroups defined by

biological function (Ley et al., 2013). Although the previous 'class I' and 'class II' model has been superseded by the generation of these functionally defined subgroups, the conceptual framework of this model (which will be explained below) still provides a broad outline of the pathogenesis of AML. Furthermore, combining individual 'class I' and 'class II' genetic lesions *in vivo* is still commonly used to build accurate and informative animal models of various subtypes of AML disease (Zuber et al., 2009).

1.3.3 Co-operating mutations in AML

Prior to whole genome analysis of AML somatic mutations were grouped as lesions that either constitutively promote cell survival and proliferation (class I), or mutations that block haematopoietic cell differentiation (class II) (Kelly et al., 2002; Mazzarella et al., 2014)u3. More recently, epigenetics has been considered as a third class of mutations which also contribute to disease (Ley et al., 2013; Takahashi, 2013). Importantly, this model proposes that the co-operation of mutations from each category is required for the development of AML (Kelly et al., 2002). In support of this proposed model, genetic lesions found in AML very rarely cause disease in isolation. Co-operative mutations that promote proliferation in addition to blocking myeloid cell differentiation is often required for a founding clone to become malignant. The FMS-like tyrosine kinase 3 (FLT3) gene is among the most frequently occurring somatic mutations found in AML (25%-45% of all AML patients) (Ley et al., 2013), with the most common mutation to this gene being an intern tandem duplication (ITD) between exon 14 and 15. It is considered to be a class I mutation, and in transgenic mouse models FLT3-ITD mutations alone are unable to induce AML, but instead cause a myeloproliferative disease that resembles human chronic myelomonocytic leukaemia (B. H. Lee et al., 2007). However, when coupled with a differentiation blocking mixed lineage leukaemia fusion mutation (MLL-F) (class II), the myeloproliferative cells are transformed into an acute leukaemia (Ono et al., 2005). Similarly, expression of the MLL-F oncogene alone results in a myeloproliferative disorder with long disease latency in mice, but is unable to induce AML (Ono et al., 2005). Interestingly, the class I FLT3-ITD mutation most commonly co-occur with differentiation blocking, cytogenetically abnormal class II mutations such as t(8:21) (AML1-ETO), t(15:17) (PML-RARα) and variations of 11q23 (MLL-F), emphasizing the co-dependency between differentiation blocking and pro-survival mutations in inducing leukaemogenesis. Other co-operating oncogenes in AML include combinational mutations to AML1-ETO (differentiation blocking) and pro-survival such as Nras (Zuber et al., 2009) or c-kit (Y. Y. Wang et al., 2011),

where the co-expression of AML1-ETO (class II) with either Nras or c-kit mutation (class I) leads to a greatly accelerated AML (Zuber et al., 2009).

1.3.4 Mutually exclusive mutations in AML

Next generation sequencing reveals a wide range of co-operative mutations that drive AML. Interestingly, there is also clear mutual exclusivity within different subgroups of genetic lesions (Fig 1.2). Most strikingly, genetic lesions shown to block differentiation very rarely co-occur within a single patient (Kihara et al., 2014; Ley et al., 2013), suggesting that acquisition of a single mutation that perturbs transformed myeloid blast differentiation is sufficient in contributing to the process of AML. Furthermore, the mutual exclusivity also strongly suggests a common pathway in which these different oncogenes may converge to block differentiation. Hence, identifying a common downstream target that is shared by each of these differentiation blocking lesions may have significant therapeutic implications. Through a range of different studies, it has become increasingly evident that the inhibition of the transcription factor PU.1 is the common process that allows for these mutually exclusive oncogenes to block myeloid cell differentiation in AML.

1.3.5 Dysregulation of PU.1 results in the development of AML in mice and humans

Dysregulated PU.1 expression has been associated with a range of different malignancies (Okuno et al., 2012; Xu et al., 2012; Yuki et al., 2013), however more recently it has been proven to play an important role in the development of AML. Targeted deletion of enhancer regions of PU.1 which reduce PU.1 expression by 80% results in the accumulation of transformed immature myeloid cells (lacking expression of classic differentiation markers M-CSFR and G-CSFR) and the development of AML (Metcalf et al., 2006). Interestingly, the level of PU.1 reduction is crucial in the development of AML, as mice that carry heterozygous mutations to PU.1 did not develop leukemia (Rosenbauer et al., 2004). Mice harbouring a hypomorphic PU.1 allele enter a pre-cancerous state and develop AML after a few months; however the disease is greatly accelerated when the PU.1 mutation is present on a p53 null ($p53^{-/-}$) background. Despite being a potent tumour suppressor itself, a $p53^{-/-}$ mutation is unable to generate a leukaemia, hence in this context, it is PU.1 inhibition that effectively blocks myeloid differentiation and drives aggressive AML (Basova et al., 2014).

In the context of human AML, miR-155 regulates PU.1 on a post transcriptional level. Elevated levels of miR-155 are commonly found in AML as well as other myeloproliferative diseases (Huskova et al., 2015), hence emphasizing the important role PU.1 also plays as a tumour suppressor. Although there has been extensive research exploring the leukaemogenic potential of dysregulated PU.1 in different mouse models, point mutations to PU.1 itself are rarely found in AML patients (Lavallee et al., 2015; Ley et al., 2013; Mazzarella et al., 2014). However, despite the low rate of PU.1 mutations found in AML, many of the commonly occurring mutations indirectly compromise PU.1 function to drive disease.

1.3.6 PML-RARα fusion oncogene inhibits PU.1 expression and function

Acute Promyelocytic Leukaemia (APL) is a subset of AML characterized by the accumulation of immature promyelocytes. It accounts for 10%-15% of AMLs and is driven by the chromosomal translocation of retinoic acid receptor alpha (RARα) on chromosome 15 to the PML located on chromosome 15, yielding the PML-RARα fusion oncoprotein. The expression of PML-RARα results in the inhibition of many genes integral to myeloid development including PU.1. Early transgenic mouse models of PML-RARα driven AML show that penetrance of disease was greatly enhanced if the PML-RARα mutation was coupled with a deletion of one copy of the PU.1 gene (Walter et al., 2005). Furthermore, the presence of the fusion oncogene itself also repressed the residual PU.1 allele, and greatly accelerating the development of APL *in vivo* (Walter et al., 2005). ChIP-seq analysis also shows highly significant consensus binding sites between the two proteins, as well as the ability of PML-RARα to repress PU.1 mediated transactivation in myeloid precursor cells (K. Wang et al., 2010). This is also supported by gene expression analysis in APL patient samples, where increased expression of PML-RARα was coupled with decreased levels of PU.1 (Zhu et al., 2012). Collectively, there is strong evidence from both human and mouse models to suggest that PML-RARα directly binds and inhibits PU.1 to drive APL disease.

More recently, all-*trans* retinoic acid (ATRA) has been proven to be highly effective in treating patients with APL. ATRA degrades the PML-RARα protein and consequently alleviates the differentiation block caused by the fusion oncogene, resulting in the differentiation of transformed myeloid cells into neutrophil-like cells (Ablain et al., 2013). Interestingly, the ATRA-induced degradation of the fusion oncoprotein in PML-RARα leukemic cell lines is

associated with the restoration of PU.1, suggesting that the differentiation of leukemic cells is driven by PU.1 (Mueller et al., 2006).

1.3.7 RUNX1 mutation drives AML through the inhibition of PU.1

RUNX1 (otherwise known as AML1) is a DNA binding transcription factor that plays a pivotal role in haematopoiesis. The RUNX-PU.1 pathway is of major importance in HSC maintenance and differentiation (Growney et al., 2005) (Hu et al., 2011). Binding of RUNX1 to PU.1 results in the formation of a chromosomal loop at the PU.1 locus between the PU.1 enhancer and its proximal promoter, therefore facilitating PU.1 transcription. In addition to mediating PU.1 transcription, the binding of RUNX proteins to PU.1 also prevents interaction of PU.1 to its corepressors such as SIN3, ETO2 and HDAC2 (Gaidzik et al., 2016). Therefore, RUNX1 both directly and indirectly promotes the transcription of PU.1 and subsequent activation of myeloid differentiation genes such as MCSFR and GMCSFR. Given the important role RUNX1 plays in regulating PU.1 and myeloid cell differentiation, RUNX1 loss of-function mutations or translocations can lead to the development of AML (Gaidzik et al., 2016). Co-immunoprecipitation assays show that downregulation of PU.1 and transformation of myeloid cells into a pre-leukemic state can result from point mutations to the C-terminus of RUNX1. The highly conserved C-terminus of RUNX1 plays a pivotal role in excluding corepressor interaction with PU.1, such that RUNX proteins lacking the C-terminus are still able to bind to PU.1 but are unable to block interaction with corepressors such as ETO2 or SIN3A (Hu et al., 2011; Zhao et al., 2008). Therefore, access to the region where RUNX1 C-terminus normally binds to PU.1 allows corepressors to inhibit PU.1 expression, resulting in the downregulation of key myeloid differentiation genes, thereby functioning as a differentiation blocking genetic lesion (Gaidzik et al., 2016).

1.3.8 AML1-ETO fusion oncogene is a classic differentiation blocking mutation in AML

Although mutations to RUNX1 (otherwise known as AML1) in AML are rare, the gene encoding RUNX1 (AML1) is also a hot spot for chromosomal rearrangements that are among the most frequently identified mutations in AML (Miyoshi et al., 1991). In combination with point mutations to AML1, AML1-ETO mutations constitute 10%-15% of driver mutations found in AML, and is one of the earliest mutations in AML shown to inhibit PU.1 function (Vangala et al., 2003).Patients harbouring the AML1-ETO mutation have a comparatively lower PU.1 expression level than AML patients without the t8:21 translocation (Staber et al.,

2014). The AML1-ETO fusion protein results from the fusion of AML1 (RUNX1) on chromosome 21 to the ETO repressor protein located on chromosome 8. Exposure of the AML1 C- terminus allows for ETO binding, thus providing access for corepressors to PU.1. Genome wide analysis shows that both AML1 and AML1-ETO share and compete for identical binding sites, however unlike AML1, the fusion oncoprotein has a greater preference to binding to corepressors rather than coactivators (Ptasinska et al., 2014). In addition to increased corepressor binding, AML1-ETO also shares identical binding sites to the PU.1 coactivator c-Jun (Vangala et al., 2003). Therefore, the presence of AML1-ETO not only competes for binding sites with wildtype AML1, but also with c-Jun, thereby downregulating PU.1 transcription and the transactivation of PU.1 targets (Ptasinska et al., 2014). Hence, given that the AML1-ETO fusion oncoprotein provides greater opportunities for co-repressors to bind to PU.1 and inhibit its transcriptional activity, the development of AML can often hinge on the equilibrium between AML1-ETO and AML1 (Ptasinska et al., 2014). Predictably, human AML cell lines (Kasumi-1) expressing AML1-ETO also express low levels of PU.1, however differentiation of the leukemic cells was induced by the overexpression of PU.1 (Vangala et al., 2003).

1.3.9 MLL-F fusion oncoprotein interact with PU.1

RUNX1 expression is regulated through an autoregulatory positive feedback loop. In hematopoietic progenitors, high levels of RUNX1 expression promotes the recruitment of mixed lineage leukaemia (MLL), a methyltransferase which binds to the RUNX1 URE resulting in further transcription of RUNX1 (Huang et al., 2011).Therefore, mutations that impair RUNX1-MLL interactions also result in the development of AML (Huang et al., 2011). MLL can undergo fusion with several different partners, including AF9, ENL and AF10 (Zhou et al., 2014). Collectively, the translocation of MLL to its array of fusion partners are known as MLL-F. In isolation, MLL-F mutation alone is insufficient in driving AML, however MLL-F fusion oncogenes often co-occur with common pro-survival and proliferation oncogenes such as Flt3 mutations and mutations to Ras (Ley et al., 2013; Ono et al., 2005). When paired with such oncogenes, patients harbouring the MLL-F driven mutation represents a very aggressive subtype of AML. Interestingly, much like the case in AML1-ETO mouse models, further inhibition of PU.1 in MLL-F driven leukaemia (MLL-F expression in a hypomorphic PU.1 mouse model), also results in a delay in disease onset (Zhou et al., 2014). In this context, MLL-F drives the activation of the MEIS/HOXA9 pathway, which is essential for the initiation of

MLL driven leukaemia (Zhou et al., 2014). PU.1 directly binds to MEIS and many of its downstream targets, thereby sustaining the activation of the MEIS/HOXA9 pathway.

1.3.10 NPM1c: a differentiation blocking mutation in AML through the inhibition of PU.1

Nucleophosmin (NPM1) is a ubiquitously expressed protein that shuttles proteins between the cytoplasm and the nucleus. Genetic aberrations to NPM1 (known as NPM1c) are the most common mutations found in AML, with approximately 35% of all AML patients harbouring the mutation (Jain et al., 2014). However, despite being the most frequent *de novo* mutation found in AML, the oncogenic function of NPM1c remains unclear. Interestingly, genome-wide analysis of AML patients shows strong co-occurrence of NPM1c with FLT3 and DNMT3A, but also a clear mutual exclusivity with other well characterized differentiation blocking mutations such as AML1-ETO and PML-RARα (Ley et al., 2013). Therefore, from a genetic standpoint, mutations to NPM1 in AML appear to behave similarly to the other common differentiation blocking mutation. In wildtype cells, NPM1 is predominately located in the nucleolus and functions as a histone chaperone protein (Box et al., 2016). More recently, it was found that a mutation to NPM1 results the relocalization of NPM1 as well as key haematopoietic transcription factors into the cytoplasm, including PU.1. Notably, Cebpα and RUNX1 remain in the nucleus in NPM1c mutant cells (X. Gu et al., 2018). However, the disconnection of PU.1 and its coregulators leads to the repression (rather than activation) of hundreds of pro-granulocytic and monocytic differentiation genes (X. Gu et al., 2018). Interestingly, preventing the nuclear export of proteins using chemical inhibitors (KPT-330) retains nuclear localization of PU.1/NPM1, and induces monocyte differentiation of AML cell lines harbouring the NPM1 mutation (X. Gu et al., 2018). Therefore, the differentiation block observed in NPM1 mutated AMLs is also likely due to the loss of PU.1 transcription directly caused by the shuttling of PU.1 into the cytoplasm by NPM1c. Furthermore, treatment of mutated AML cells with the XPO1 inhibitor Selinexor retains NPM1c and PU.1 in the nucleus and triggers differentiation *in vitro* and *in vivo* (Xiaorong Gu et al., 2017).

Considering the recent results suggesting NPM1c compromises PU.1 function, it appears increasingly evident that central to most differentiation blocking mutations is the ability to inhibit PU.1 mediated differentiation of myeloid progenitor cells. In fact, having described the genetic lesions that have thus far been shown to dysregulate PU.1 function, it is possible that

>50% of AML case have compromised PU.1 function. Hence, despite the heterogeneity of genetic mutations that can drive AML, alleviating the differentiation block caused by the impediment of this single transcription factor could benefit a large proportion of patients suffering from AML.

1.4 Differentiation therapy of AML

Uncovering the genetic landscape of AML has greatly improved our understanding of the underlying mechanisms that cause AML. Although our understanding of the disease continues to grow, current treatment methods for AML patients still primarily involve the use of cytotoxic agents such as Cytarabine, Azacitidine and Fludarabine (Dombret et al., 2016). These chemotherapeutic agents kill both host and disease proliferating cells indiscriminately. Hence the dose-limiting toxicity of chemotherapeutic agents may also contribute to the poor prognosis of AML patients. Therefore, given the cytotoxicity of chemotherapeutic agents as well as its ineffectiveness in treating AML patients, finding more effective alternative therapies is of great importance.

In recent years, a more targeted approach known as differentiation therapy has yielded very promising results in certain subtypes of AML. Unlike chemotherapy which induces apoptosis, differentiation-based therapies look to re-engage normal myeloid cell differentiation of immature leukemic cells. Therefore, by driving differentiation of leukemic cells into 'normal' myeloid cells, the bulk of the tumour is subsequently cleared by homeostatic mechanisms that are responsible for clearing normal myeloid cells. The following sections will look to outline the efficacy as well as the shortfalls of differentiation-based therapy in AML.

1.4.1 The use of ATRA in treating APL

The hallmark example of the efficacy of differentiation therapy in the clinic is the of all-trans retinoic acid (ATRA) and arsenic (ATO) combinational therapy in APL. Prior to ATRA-based therapies, treatment of APL consisted of cytarabine and azacytidine chemotherapy. The use of such chemotherapeutic agents yielded complete remission (CR) in over 65% of APL patients (De Kouchkovsky et al., 2016). Of those that reached CR following initial chemotherapy, 50% of these patients relapsed shortly, and only one third of APL patients survived two years following treatment (J. Park et al., 2011). Therefore, prior to the development of ATRA-based therapies, patients with APL disease previously had the poorest survival rate amongst all AML

subtypes. However, landmark studies involving the use of combinational ATRA+ATO therapy has greatly improved APL patient outcome, with 95% of APL patients treated with the combinational therapy achieving CR (Abaza et al., 2017; Iland et al., 2012; Lo-Coco et al., 2013).

1.4.2 Mechanism of ATRA based therapies on APL

The majority of APL are characterized by the t(15:17) chromosomal translocation that encodes the PML-RARα fusion oncogene. PML-RARα is often the sole driver mutation for APL and has two primary functions: inhibit transcription of key differentiation genes and to disrupt the formation of PML nuclear bodies essential for p53 activation (Guo et al., 2000). Currently, the most common method of treatment is the combinational use of ATRA and arsenic trioxide (ATO). The binding of the PML to RARα leads to increased corepressor binding of RARα, resulting in direct repression of differentiation genes (K. Wang et al., 2010). The direct inhibition of PU.1 by the fusion protein blocks differentiation, and conditional overexpression of PU.1 can induce granulocytic differentiation of APL cells (Mueller et al., 2006). Retinoic acid (ATRA) is a master regulator of myeloid cell differentiation through the activation of RARα. In APL disease, ATRA has dual effect on the APL. Firstly, RA binds to the RARα portion of the PML-RARα fusion oncoprotein, thereby dissociating RARα from its co-repressors. This leads to the reactivation of suppressed differentiation genes including PU.1, resulting in granulocytic differentiation of APL cells (Mueller et al., 2006). Secondly, ATRA induced degradation of PML-RARα alleviates PML (which exerts growth suppressive properties) and allows for the reformation of PML nuclear bodies (Ablain et al., 2013). These nuclear bodies anchor many nuclear functions, including the activation of p53, which is essential for the loss of APL cell self-renewal and subsequent clearance of the disease (de The, 2018).

The RARα portion of PML-RARα was previously considered the main component that suppresses differentiation in APL. However, growing evidence suggests that targeting the PML rather than RARα may have greater effect in treating APL (X. W. Zhang et al., 2010). Unlike RA, ATO targets the PML portion of the fusion oncoprotein in APL. ATO is also able to break down PML-RARα, leading to transcription of differentiation genes, many of which are regulated by PU.1. However in addition to targeting the PML-RARα, ATO also targets wildtype PML protein, leading to increased formation of PML NBs and thus, more effective

clearance of differentiated APL cells (de The, 2018). As a result, increased formation of NBs, ATO has a more profound effect on treating APL than RA, such that the use of ATO as a single agent has been shown to cure 70% of patients with APL (Mathews et al., 2011; Zhou et al., 2014). Conversely, RA is relatively ineffective in treating APL as a single agent. However, the combinational therapy of ATO and ATRA have been shown to function synergistically, with many trials reporting 95% CR rates and is thus the gold standard therapy for APL disease (de The, 2018; Iland et al., 2012; Lo-Coco et al., 2013).

1.4.3 Multi-lineage potential of APL cell differentiation in response to ATRA-based therapies

ATRA-based therapies often lead to differentiation of AML blasts into neutrophils. In fact, ATRA treatment of APL patients can also lead to patients suffering from differentiation syndrome, where the *en masse* differentiation of AML blasts into neutrophils which is in itself life-threatening (Fathi et al., 2018; Montesinos et al., 2009). In addition to neutrophils however, AML blasts have also been shown to undergo maturation along other myeloid lineages. Most notably, ATRA based treatment can induce monocytic differentiation of AML blasts in patients (Naeem et al., 2006). Interestingly, *in vivo* and *in vitro* analysis of APL cells treated with ATRA shows a strong correlation between monocytic differentiation of AML blasts and the expression of M-CSFR (Riccioni et al., 2003). Patient samples consistently showed variable percentage of monocytic differentiation when treated with ATRA, and monocyte differentiation was greatly increased in cultured samples when APL cells were treated with a combination of ATRA and M-CSF (Riccioni et al., 2003). This is consistent with APL cell lines such as NB4 and HL-60 harbouring multilineage potential, including differentiation into monocytes when treated with ATRA and M-CSF (Khanna-Gupta et al., 1994; Ozeki et al., 2008). Furthermore, overexpression of MCSF-R in NB4 human cell lines also results in the presence of ATRA (Riccioni et al., 2003), outlining the potential for bidirectional differentiation in AML cells. This in turn depends on the stage of AML differentiation block, and whether the block is at a CMP-like or GMP-like stage of immaturity.

1.4.4 APL cell lines differentiate into eosinophils in the presence of IL-5

In addition to monocytic differentiation, AML blasts are also capable of differentiating along the eosinophilic lineage given the correct circumstances. Treatment of APL cell line HL-60 with ATRA in combination with IL-5 is able to skew differentiation from the neutrophil lineage

to the eosinophilic lineage *in vitro* (Ingley et al., 1991; Thivierge et al., 2000). A similar phenomenon is also observed in HT93 APL cell lines, where the same combination of ATRA and IL-5 can drive eosinophilic differentiation (Kishi et al., 1998). Although clinically understudied, these *in vitro* assays show the possibility of AML blast differentiation along particular mature myeloid lineages in the presence of given cytokines. Multilineage differentiation of AML blasts may already occur in patients following differentiation therapy, however historically has been difficult to detect.

1.4.5 ATRA and ATO combinational therapy on the NPM1c driven AML

Outside its use in treating patients with APL, the ATRA and ATO based combinational therapy has shown promise in treating AML patients harbouring the NPM1c driver mutation. Previous clinical trials involving the use of chemotherapy agents in conjunction with ATRA has shown conflicting evidence on the efficacy of ATRA in NPM1 mutant driven AMLs. Some studies have suggested it as an effective alternative for patients unfit for intensive chemotherapy (Forghieri et al., 2016), whilst others clinical trials have shown that the rate of CR is almost identical between chemotherapy patients treated with or without ATRA (Nazha et al., 2013). Despite the conflicting evidence with regards to the efficacy of ATRA in treating NPM1c driven AMLs in the clinic, there is also emerging evidence to suggest that the addition of ATO could have significant therapeutic benefits. NPM1 is a chaperone protein responsible for shuttling proteins between the nucleus and cytoplasm. Mutation to NPM1 leads to the improper distribution of proteins into the cytoplasm, including PML (El Hajj et al., 2015) (Martelli et al., 2015). Like PML-RARα , ATRA and ATO also drives proteasome degradation of the NPM1 mutant protein (Balusu et al., 2011). Hence the degradation of NPM1c also leads to the re-formation of PML bodies, resulting in the clearance of leukemic cells by the activation of p53 (El Hajj et al., 2015; Martelli et al., 2015). Furthermore, OCI-AML3 cells that harbour the NPM1 mutation are sensitive to ATRA induced granulocytic/monocytic differentiation and apoptosis. This effect is further accentuated in the presence of a hairpin targeting NPM1. Therefore, given the sensitivity to ATRA-induced differentiation and ATO-mediated formation of PML NBs, the use of ATRA and ATO differentiation therapy has strong potential in treating patient with NPM1 mutant driven AMLs.

1.4.6 HDAC inhibitors induce terminal myeloid differentiation in AML1-ETO driven leukaemias

ATRA based therapies have paved the way for the use of targeted differentiation therapy in AML. Although ATRA+ATO therapy has been widely effective in treating APL disease, this only constitutes 10-15% of patients with AML (Ley et al., 2013). Therefore, differentiation agents that target other subtypes of AML are still of high priority. HDAC inhibitors represent a group of therapeutic agents that aim to induce differentiation in AML. AML1-ETO is a common differentiation blocking driver mutation found in 10% of AML patients (Ley et al., 2013). AML1-ETO fusion protein recruits histone deacetylases (HDACs), which are responsible for altering the acetylation of several proteins, including those that regulate cell proliferation (Tabe et al., 2007). The HDAC inhibitor valproic acid (in combination with ATRA) has been used in the clinic on patients deemed unfit for intensive chemotherapy with some success, with 5% of these patients experiencing prolonged periods of disease remission (Fredly et al., 2013). Interestingly, terminal differentiation of AML blasts has not been observed with the use of Valporic acid. More recently, a more potent HDAC inhibitor in Panobinostat, has shown to not only trigger proteasome degradation of AML1-ETO, but also driving cell cycle arrest and the terminal differentiation of AML cells into granulocytes (along with upregulation of PU.1 and CEBPα) (Bots et al., 2014). In an AML1-ETO mouse model, the use of Panobinostat could prolong mouse survival and was also effective on AMLs with a p53 -/- background, suggesting that response to Panobinostat is independent of p53 activation (Bots et al., 2014). Given that Panobinostat functions independently of p53, combinational therapy of Panobinostat in conjunction with ATO (a p53 activator) has shown synergistic effects, resulting in prolonged disease remission in mice (Salmon et al., 2015). Therefore, given the development of more potent HDAC inhibitors and its efficacy in mice (in combination with ATO), the use of HDAC inhibitors in the clinic may not be too distant from the future.

1.4.7 $IDH2^{mut}$ inhibitors also trigger differentiation of immature AML blasts

In recent years, Enasidenib has emerged as a differentiation inducing therapeutic agent for AML patients that harbour isocitrate dehydrogenase 2 (IDH2) mutations. IDH2 is an enzyme involved in DNA methylation that is recurrently mutated in AML (in approximately 12% of patients) (Kihara et al., 2014; Ley et al., 2013). The results from phase 1 and 2 clinical trials as a single agent have been very promising given that over 40% $IDH2^{mut}$ refractory or relapse

AML (rrAML) over 40% IDH2^{mut} refractory or relapsed AML (rrAML) respond to treatment and achieve complete remission (CR) (Amatangelo et al., 2017). Treatment with Enasidenib in rrAMLs results in normalization of the HSPC compartment, due to a significant reduction in the percentage of immature leukemic blasts. Importantly, this is coupled with an increase in mature myeloid cells harbouring the mIDH2 allele (variant allele frequency (VAF) remains constant between pre-treatment and neutrophil samples collected at CR), suggesting that Enasidenib induces leukemic cell differentiation to achieve CR. Similar to other differentiation therapy agents, patients treated with Enasidenib often experience differentiation syndrome (DS) due to the on-mass differentiation of leukemic blasts into mature myeloid cells (Fathi et al., 2018). Interestingly, Enasidenib also induces bifurcated differentiation of AML blasts into predominately neutrophils and erythrocytes (Amatangelo et al., 2017). Although the mechanisms are unclear, bifurcated differentiation is likely driven by interactions with extrinsic cues such as the cytokine milieu during blast differentiation. Multilineage differentiation of AML cells following the release of the differentiation block is seen with a number of differentiation therapy agents, however its consequence is currently unknown.

1.5 AML relapse following differentiation therapy

The emergence of targeted differentiation therapy has greatly improved patient outcome in AML however disease relapse remains an ongoing issue with differentiation-based therapy. Both HDAC and IDH2^{mut} inhibitors have recently entered use in the clinic, hence data in relation to relapse following these treatments remains relatively unknown. ATRA-based therapies however, have existed in the clinic for decades, hence the prevalence of disease relapse in APL patients treated with ATRA is relatively well understood. ATRA as a single agent can only induce terminal differentiation in APL patients, however disease regression is only seen at high concentrations of ATRA (Muindi et al., 1992). Additionally, treatment of APL with drugs that inefficiently degrade the PML-RARα protein such as 13-cis-RA or etretinate is also able to differentiate *ex vivo*, but is unable to induce disease remission when used in the clinic (Ablain et al., 2013). Combining ATRA with chemotherapy saw improvements in dramatic improvements in preventing relapse compared to ATRA as a single agent reducing relapse rates from 50% to 20% (Asou et al., 2007; Sanz et al., 2009). Despite the improvements, it was not until ATRA was combined with ATO where APL became a highly curable disease, with >95% of APL patients achieving CR (Grossmann et al., 2012). Furthermore, combined ATRA + ATO therapy on rrAML patients has also increased CR

remission from 50% to 80% in these patients (Thirugnanam et al., 2009). The median time for the emergence of disease relapse is 17 months following differentiation therapy (Abaza et al., 2017; Thirugnanam et al., 2009), however remarkably relapse has also been seen in APL patients initially treated with ATRA and ATO following 17 years of continuous disease remission (Sakurai et al., 2018). Interestingly, induction of ATRA + ATO therapy leads to differentiation of APL blasts into mature granulocytes that retain the PML-RARα fusion oncogene (Ablain et al., 2013; Gocek et al., 2011). Of note, the $IDH2^{mut}$ inhibitor Enasidenib is also very efficient in driving terminal differentiation, however granulocytes harbouring the IDH^{mut} persist following treatment (Amatangelo et al., 2017; Takahashi, 2013). Therefore, although the source of relapse may come from a rare population of leukemic blasts that were insensitive to the initial treatment with differentiation therapy agents such as ATRA+AS (such as a quiescent leukemic stem cell (LSC), the presence of AML-derived granulocytes that retain expression of the fusion oncogene suggests that the origin of relapse may also be from the differentiated AML-derived cell. Furthermore, a recent publication from our group (McKenzie et al, 2019, in press) was able to show that the human APL cell line NB4 treated with ATRA not only differentiated, but could also undergo de-differentiation and revert back to an APL blast following the removal of ATRA. Hence, the plasticity of APL cell differentiation further supports the possibility that relapse in differentiation therapy may originate from a differentiated APL-derived cell. By extension, relapse resulting from other differentiation therapy agents such as (HDAC and $ID2^{mut}$ inhibitors) may also originate from differentiated AML cells that retain the leukaemia driving oncogene.

1.5.1 Extramedullary relapse in AML

Although the majority of AML relapse originates from the BM, over 5% of relapse cases in APL treated with ATRA originate from an extramedullary organ (organ that is not the BM) (de Botton et al., 2006). The most common location of extramedullary relapse is the central nervous system (CNS) (de Botton et al., 2006), however relapse can also originate from organs such as the breast (Bakst et al., 2011; Harris et al., 2013). The residency of dormant, mature, residual APL (or AML)-derived cells is particularly interesting when focusing on the cellular origin of relapse. The release of the differentiation block is often considered to result in granulocytic differentiation of AML blasts. However, it is plausible that an unappreciated number of AML cells also differentiate into other myeloid lineages. Multilineage differentiation of AML cells may have important clinical implications, given that there are a

number of inherent differences between mature myeloid cell subtypes. Most notably, neutrophils are short living cells; whereas tissue resident eosinophils and monocytes are significantly longer living (Hashimoto et al., 2013) (Patel et al., 2017) (Y. M. Park et al., 2010; Willebrand et al., 2017). Therefore, perhaps an underappreciated reason as to why differentiation therapy has been so effective is due to the differentiation of leukemic blasts predominately into the neutrophil-like lineage, a myeloid cell type with a particularly short lifespan. Conversely, causes of relapse (particularly extramedullary relapse) may be attributed to APL-derived cells that have differentiated into mature myeloid lineages with longer lifespans such as monocytes or eosinophils, that have the capacity to survive long term in particular organs and tissues. Interestingly, very late relapse (relapse occurring after 3 years of CR) is associated with patients that had AMLs featuring monocytic differentiation and normal cytogenetics (Watts et al., 2016). Leukaemia cutis (LC) is a common manifestation of extramedullary relapse and is also associated with monocytic differentiation of AML cells (Bakst et al., 2011; Luskin et al., 2015). Therefore, differentiation of AML blasts into non-neutrophilic lineages opens up the possibility that mature, AML-derived cells may contribute to extramedullary relapse following differentiation therapy.

1.6 Clearance and lifespan of mature myeloid cells

Given the possibility of differentiated AML-derived cells being the source of disease relapse, it is important to understand the mechanisms that normally regulate the clearance of mature myeloid cells during homeostasis. Furthermore, given that differentiation therapy looks to re-engage the normal process of myeloid cell differentiation, it is also likely that differentiated AML-derived cells are cleared through the same mechanisms that clear aging or dying mature myeloid cells.

1.6.1 Mature myeloid lineages have differing lifespans

As mentioned in greater detail in Chapter 3 (§3.9), along with functional differences between mature myeloid cells, there are also dramatic differences in the lifespan of different mature myeloid cell subtypes. Most notably, neutrophils are the most abundant leukocyte found in the body, with an estimated 10^{11} neutrophils produced daily (Furze et al., 2008) . Neutrophils also have a very short lifespan of less than 7 hours (Bratton et al., 2011) , although this has been contended in recent years (Pillay et al., 2010). Conversely, eosinophils, which are also a sublineage of granulocytes and are the rarest population of myeloid cells (Y. M. Park et al.,

2010), however (tissue resident) eosinophils have the capacity to survive for several weeks within tissues (Willebrand et al., 2017). Lastly, monocytes and macrophages have the longest lifespan amongst all myeloid lineages, with tissue resident monocytes/macrophages shown to be lifelong cell types. Although the aforementioned myeloid lineages vary greatly in their lifespan, it is likely that a single common homeostatic process encompasses the clearance all of aging or dying myeloid cells, although thus far the study of myeloid cell clearance has focused largely on the neutrophil population.

1.6.2 Clearance of normal aged or dying granulocytes is governed by tissue specific macrophages

The field of granulocyte clearance has grown in recent years, and may provide insight as to how AML-derived cells are cleared once they have matured. Neutrophils are the most abundantly available myeloid cell population, hence matching the enormous daily production rate of neutrophils with efficient clearance of senescent and aging neutrophils is of great importance. Granulocytes are primarily cleared in the spleen and liver, however there is growing evidence to suggest that clearance of aged neutrophils is also seen in the BM (Furze et al., 2008). In each of these sites of clearance, resident macrophages are responsible for phagocytosing mature granulocytes (Gordy et al., 2011).

Although little is about the mechanisms of mature myeloid cell clearance, a proposed model is the macrophage mediated programmed cell removal (PrCR) (Feng et al., 2018). Programmed cell removal is the process by which resident macrophages are able to clear aging or dying neutrophils in an immunologically silent manner. In short, PrCR relies on the ability of macrophages to recognise damaged or aged cells from the blood stream through the expression of different cell surface signals. Broadly, these signals can be categorized into two groups: the 'don't eat me signal' expressed on healthy, normal cells, and the 'eat me' signal that is expressed when a cell is no longer fit for survival. Although many of these signals have yet to be elucidated, one well defined 'don't eat me' signal important for the prevention of phagocytosis is the CD47/Sirpα interaction between healthy cells and circulating macrophages.

Sirpα is a receptor expressed primarily on macrophages, with evidence to suggest expression of this receptor on eosinophils also (Verjan Garcia et al., 2011). Whereas Sirpα is expressed predominately on macrophages, its ligand CD47 is expressed ubiquitously on healthy cells, and the interaction between these two proteins is essential for the prevention of macrophage

mediated phagocytosis of healthy cells. The relationship between CD47 and Sirpα was initially identified by tracking the interactions between HSCs and macrophages (Jaiswal et al., 2009). During periods of hematopoietic stress, HSCs often mobilize to specialised, ectopic niches (such as the spleen) to undergo extramedullary haematopoiesis (Kim, 2010). Circulating HSCs are particularly prone to phagocytosis, given that macrophages normally line the vascular sinusoids to remove foreign pathogens. Consequently, triggering a pro-inflammatory response via lipopolysaccharide (LPS) administration in xenograft mice saw not only the mobilization of the human HSCs, but also an increase in CD47 expression in both cord blood and BM HSCs, suggesting that the upregulation of CD47 was required to prevent engulfment by host macrophages (Jaiswal et al., 2009). Consistent with this, CD47 heterozygous HSCs had a significantly reduced fitness for reconstitution in recipient mice compared to wildtype HSCs due to phagocytosis by macrophages, however this difference was attenuated when recipient mice were first macrophage depleted via clodronate (Jaiswal et al., 2009). Therefore, the reduced fitness is most likely due to increased macrophage mediated phagocytosis of HSCs as a result of heterozygous expression of CD47 (Jaiswal et al., 2009).

1.6.3 Loss of 'don't eat me' signal alone is insufficient to trigger phagocytosis

Despite the expression of CD47 on healthy cells playing an important role in preventing macrophage-mediated phagocytosis, loss of the CD47 signal alone is unable to trigger engulfment by macrophages (Gardai et al., 2005). In addition to the loss of CD47, aging or dying cells also need to express signals that trigger macrophage engulfment, known as 'eat me' signals.

1.6.4 Calreticulin functions as a key 'eat me' signal on aging or dying cells

The 'eat me' signal that is perhaps best characterized is the cell surface expression of calreticulin (CRT) on aging or dying myeloid cells. CRT can be found in the ER, and functions as a chaperone protein (Heal et al., 1998). Although it is also normally expressed on healthy cells such as neutrophils, CRT is cleaved from the ER and cell surface expression of the ligand is significantly upregulated in response to stress (Heal et al., 1998). Initially, it was believed that neutrophil expression of cell surface CRT was self-mediated, however there is emerging evidence to suggest that macrophages actively secrete cell free CRT to 'label' aging or dying neutrophils (Feng et al., 2018). Isolation of neutrophils *in vitro* shows that CRT levels are only upregulated in neutrophils when co-cultured with macrophages, however low basal levels of

CRT is always present on neutrophils *in vivo* (Feng et al., 2018), hence neutrophil expression of CRT may be regulated by both intrinsic and extrinsic mechanisms. Importantly, neutrophils become more receptive to exogenous CRT over time whereas macrophages gradually lose affinity for CRT, suggesting that gain of CRT expression on neutrophils is important for their phagocytosis as they age (Feng et al., 2018). As neutrophils age, cell free CRT secreted by macrophages bind to asialoglycans that are expressed on the neutrophil cell surface. Furthermore, the expression of neutrophil asialoglycans is regulated by Neu4, such that suppression of Neu4 is able to significantly decrease the phagocytosis of an aging neutrophil (Feng et al., 2018). Following cumulative 'labelling' of neutrophils with CRT, significant redistribution of the ligand into distinct patches is observed on the neutrophil cell surface. Such reconfiguration of cell surface CRT is subsequently recognised by macrophages and triggers phagocytosis (Feng et al., 2018).

Additionally, the increase in cell surface CRT not only correlates with decreased CD47 expression, but CRT is redistributed away from CD47 on the cell surface of an apoptotic neutrophil (Gardai et al., 2005). Furthermore, CRT colocalizes with another well characterized 'eat me' signal phosphatidylserine (PS) on apoptotic cells, suggesting that the combined loss of the 'don't eat me' CD47 signal in conjunction with the gain of 'eat me' signals such as CRT or PS are both required for the effective phagocytosis of aging or dying cells (M. Liu et al., 2019).

1.6.5 Macrophage mediated PrCR is also responsible for the clearance of AML cells

The interplay between 'eat me' and 'don't eat me' signals on target cells such as CRT and CD47 respectively have been studied mostly in the context of clearance of aging and dying myeloid cells. However, the expression of these cell surface markers also has a significant impact in AML. Given the inhibitory role CD47 expression plays with regards to phagocytosis, LSCs in both human and mouse AMLs predictably express significantly high levels of CD47 in comparison to normal myeloid progenitor cells. Furthermore, high levels of CD47 expression correlates with both stronger tumorigenic potential of AML cells as well as the ability of AML cells to evade macrophages *in vivo* (Jaiswal et al., 2009). In human AML, separation of AML patients into $CD47^{hi}$ and $CD47^{lo}$ groups reveals a significantly poor clinical outcome and increased risk of death for patients with $CD47^{hi}$ AML. There were no significant differences in CD47 expression between different cytogenetic and molecular subgroups of AML, however a noticeable exception was the noticeably lower expression of CD47 in AML

patients harbouring AML1-ETO fusion oncogene mutation. The t(8:21) translocation patients are in a favourable risk group, but whether or not this is directly linked to low CD47 expression remains to be seen. Interestingly, the use of monoclonal antibodies directed against CD47 or Sirpα have been shown to be highly effective in increasing phagocytosis of human AML LSCs by both human and mouse macrophages. This effect is ameliorated when mice are first depleted of phagocytes with clodronate, suggesting that anti-CD47 (and anti-Sirpα) alone do not induce AML cell apoptosis, but rather their efficacy relies on triggering pro-phagocytic signals in the macrophages (Majeti et al., 2009).

Similarly, CRT exposure on malignant cells correlates with improved clinical outcome in AML patients, with CRT^{high} AML patients exhibiting improved relapse free survival (Fucikova et al., 2016). In the case of AML CRT expression however, the improved overall survival of CRT^{high} AML patients may rely more on the activation of the adaptive immune system, with high levels of surface CRT correlating with an increase in NK, CD4+ and CD8+ T cells. Notably, ectopic expression of CRT on leukemic blasts also inversely correlated with CD47 expression. Hence it is possible that macrophage mediated PrCR may also contribute to the favourable prognosis of CRT^{high} AML patients (Wemeau et al., 2010).

1.6.6 Metabolic changes in macrophages dictates the efficacy of tumour cell clearance

In addition to the recognition of 'don't eat me' or 'eat me' signals on tumour cells, metabolic rewiring of macrophages is also required for the effective clearance of tumour cells. In response to cytokines and the activation of certain pathogen recognition receptors, macrophages can be categorized as proinflammatory (M1) or anti-inflammatory (M2), both of which have different metabolic needs. In the context of cancer however, macrophages don't appear to conform to these classic M1 or M2 categories, but rather acquire a phenotype that encompasses both categories (M. Liu et al., 2019). Interestingly, phagocytosis of tumour cells is associated with dramatic changes in the metabolic requirements of the macrophage. Macrophages that were more adept to metabolizing fatty acids were more efficient in the clearance of tumour cells (M. Liu et al., 2019). Consequently, inhibition of fatty acid oxidation by macrophages via etomoxir blocked BM macrophage phagocytosis of tumour cells (M. Liu et al., 2019). Although it is unclear why fatty acid oxidation and metabolism in macrophages is required for tumour cell phagocytosis, a possible explanation is that fatty acid oxidation is required to fulfil the high

metabolic load involved in the process of phagocytosis. Alternatively, fatty acid oxidation may also enable macrophages to break down large lipid loads following tumour cell engulfment.

1.7 Genetic tools and the manipulation of gene function

This thesis describes a number of loss of function approaches that serve as powerful genetic tools to study the function of any given gene of interest. The most commonly used methods of genetic manipulation include the use of RNA interference (RNAi) as well as the more recent development of CRISPR-Cas9. Both of these techniques will be discussed below.

1.7.1 Interrogating gene function using RNA interference

RNAi is a conserved biological mechanism involved in the precise regulation of endogenous gene expression, however it also serves a defence mechanism against invading viruses through the response towards foreign double-stranded RNA (dsRNA) (Y. Lee et al., 2004). The major components of RNAi include the micro RNA (miRNA) and the RISC complex. Endogenous miRNA in the nucleus is initially transcribed by RNA polymerase II (Y. Lee et al., 2004) from a longer RNA-coding gene to form the primary transcript (pri-miRNA). The pri-mRNA molecule then undergoes significant post-transcriptional modification (such a poly-adenylation) to produce the characteristic stem-loop that is essential for further processing. Nuclear pri-mRNA is cleaved at the base of the dsRNA stem by Drosha to produce the pre-miRNA. The pre-miRNA is then transported out to the cytoplasm, where the enzyme Dicer cleaves dsRNA into shorter fragments that are then separated two single stranded RNA strands: the passenger RNA (which is subsequently degraded), and the guide RNA. The guide strand is then incorporated into the RISC complex, which is composed of Dicer-2, R2D2 and most importantly Argonaute 2 (Ago2). The single stranded guide RNA integrated to the RISC complex allows it to target complementary cytoplasmic messenger RNA (mRNA), where upon Ago2 cleaves the target mRNA, resulting in the post transcriptional silencing of the gene of interest.

1.7.2 Tet-regulated RNAi in mice

The ability of RNAi to suppress gene expression at the post-transcriptional level importantly leaves the genome intact. Consequently, this allows for gene silencing to be reversed when used in conjunction with Tetracycline (Tet)-regulated platforms. Such platforms rely on the

use of tetracycline, or its analogue Doxycycline (Dox) to regulate gene expression (Gossen et al., 1992; M. Gossen et al., 1995). This system comprises of two main components: the Tet-responsive promoter (TRE), and the Tet-transactivator protein. Tet-transactivators themselves come in two different classes. Tet-off transactivators (tTA) are active in the absence of Tetracycline or Doxycycline, allowing for binding and driving expression of the TRE promoter. Conversely, Tet-on transactivators (rtTA) are inactive, but upon treatment with Tet or Dox, and require the presence of these molecules to activate, thereby allowing for rtTA to bind and activate the TRE promoter. Incorporating RNAi technology to the Tet-regulated system therefore allows for the reversible silencing of genes of interest. Placing shRNA-miR30s or miREs directly downstream of a TRE promoter allows not only for the expression of the shRNA, but also allows for the expression to be reversible through the administration of Tetracycline or Doxycycline (Dickins et al., 2007). A further level of specification can be achieved by using tissue specific promoters to drive the Tet-transactivators (Dickins et al., 2007). Using these methods, it is possible to investigate the function of specific genes and also generate transgenic mouse models with reversible knockdown of a gene of interest.

1.7.3 CRISPR-Cas9 technology

In recent years, CRISPR-Cas9 systems have also emerged as a highly effective method investigating gene function. CRISPR (clustered regularly interspaced palindromic repeats) was initially found in bacteria, functioning as an innate anti-viral immune system (Marraffini et al., 2010). CRISPR are a family of DNA sequences found in the genome of prokaryotic organisms such as bacteria. These palindromic sequences are derived from invading viruses and serve as 'cellular' memory of non-self-viruses. In contrast to RNAi-mediated gene manipulation, an important distinction between the two technologies is that CRISPR-Cas9 introduces irreversible genome editing, whereas RNAi does not. CRISPR-Cas9 mediated knock out of a gene comprises of two major components: the single guide RNA (sgRNA) and the CRISPR endonuclease (such as Cas9). The sgRNA comprises the CRISPR RNA (crRNA) and the transactivation RNA (tracrRNA) components. The crRNA contains the target sequence and the tracrRNA is a significantly longer RNA sequence that forms the stem-loop structure required to bind to the Cas9 enzyme (Ran et al., 2013). The sgRNA directly binds the DNA target gene, where the Cas9 enzyme will form a double stranded break. Importantly, Cas9 can only cleave the genomic DNA in the presence of a protospacer adjacent motif (PAM) 2-6 bp directly downstream of the target DNA sequence (Fig 1.3) (Ran et al., 2013). Once the DNA has been

cleaved, the double stranded DNA break triggers endogenous DNA repair mechanisms, either through non-homologous end joining (NHEJ) or homologous directed repair (HDR). Given its error prone nature, NHEJ often leads to insertion/deletion (indel) mutations, resulting in frameshifts that cause loss of functional protein. HDR is a more accurate method of repair, however it requires the presence of a homologous repair template. This second repair approach can be exploited by the CRISPR knock-in approach, where a homologous artificial repair template is introduced containing base pair substitutions or gene of interest sequence. Due to recent advancements to CRISPR-Cas9 technology, it is now possible to lentivirally or retrovirally infect Cas9 complexes targeting the gene of interest (through synthetically produced sgRNAs) to permanently knock out (KO) genes of interest to ultimately interrogate gene function.

Project Rationale and Aims:

In recent years, differentiation therapy has revolutionized the treatment of AML patients with certain genetic aberrations. Despite the efficacy of differentiation therapy agents such as ATRA, the mechanisms of AML cell differentiation, clearance and causes of relapse remain largely unknown. Furthermore, given the development of new differentiation therapy agents that are entering the clinic (such as Enasidenib and HDAC inhibitors), understanding the nature of AML differentiation as well as the source of relapse is of great therapeutic importance. There are many oncogenic lesions that are capable of driving AML. Interestingly, there is increasing evidence to suggest that a set of recurrent but mutually exclusive oncogenic mutations all converge on the dysregulation of PU.1 to drive AML. AML246 is an inducible mouse model of AML previously generated in the Dickins laboratory (McKenzie et al, 2019, in press). In this model, the leukaemia is driven by the inducible knock down of the transcription factor PU.1. Importantly, restoring endogenous PU.1 in AML blasts in this model induces widespread leukemic cell differentiation (refer to Chapter 3.1). Therefore, using this genetic, PU.1-mediated mouse model of differentiation therapy, this thesis examines the following questions:

Chapter 3: Determining the kinetics and mechanisms of AML blast differentiation and clearance *in vivo*

Chapter 4: Identifying and preventing the source of relapse in AML following differentiation therapy

Chapter 5: Interrogating the PU.1 dependent and independent functions of commonly occurring AML oncogenes

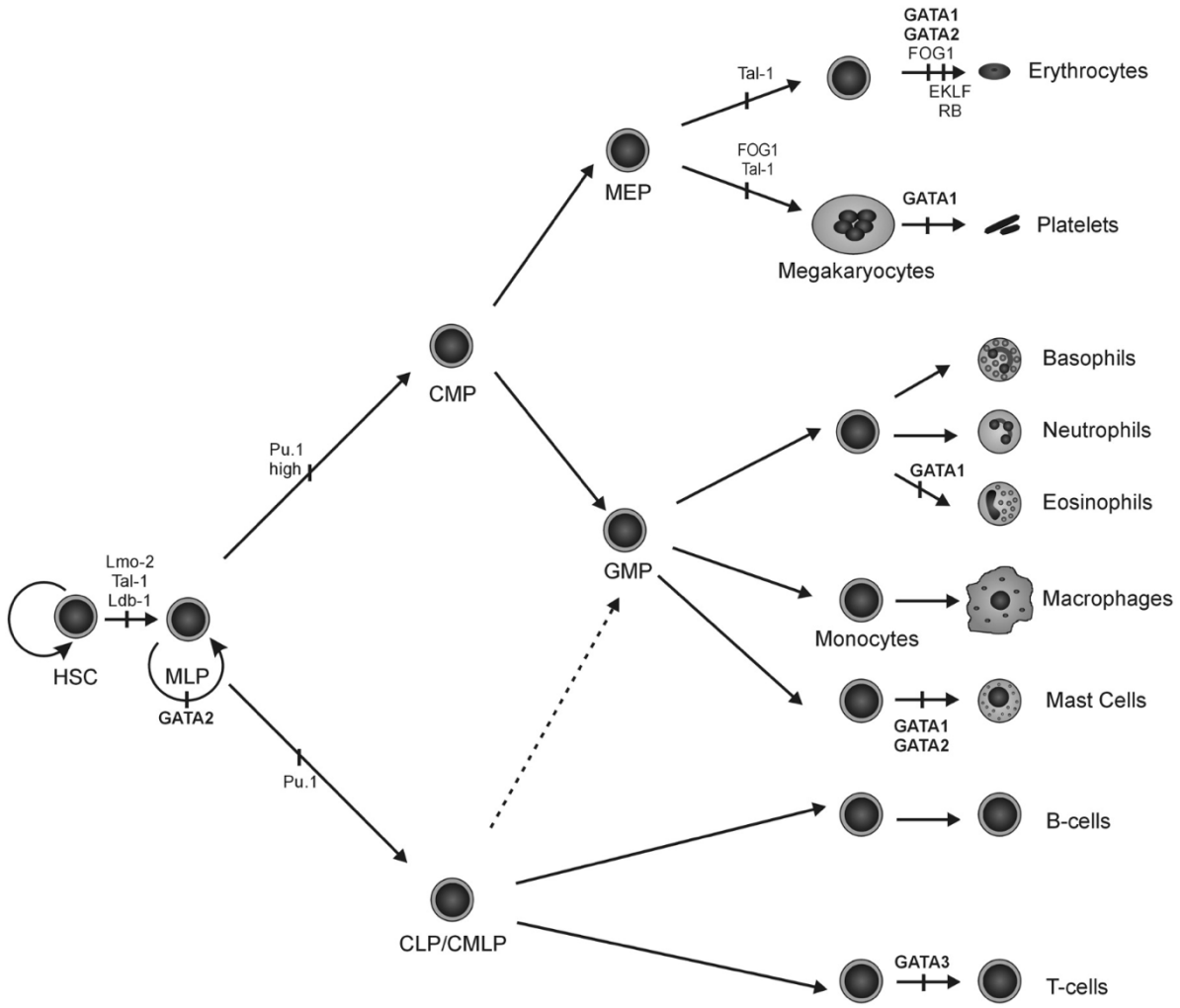


Figure 1.1 Involvement of PU.1 and Gata family transcription factors during haematopoiesis.

High PU.1 expression is required for the commitment of early progenitor cells to the myeloid lineage. Once commitment to the myeloid lineage, Gata1 plays an important role in promoting megakaryocyte, erythroid and eosinophil production. (Adapted from Ferreira et al. 2005)

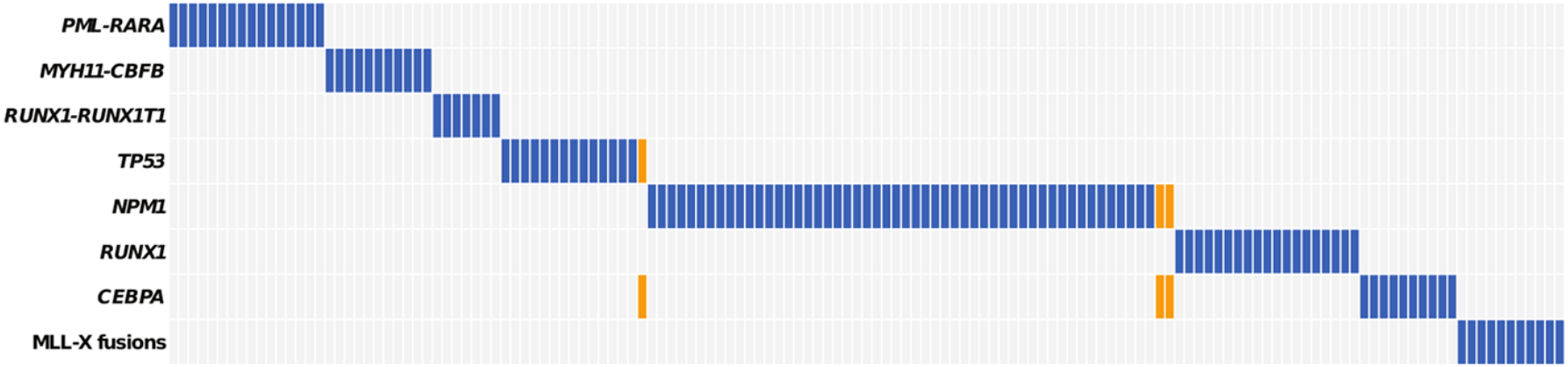


Figure 1.2 Commonly occurring oncogenic lesions identified though genome sequencing of AML patients

Over 200 AML patient samples were exome sequenced and the driver mutations identified for each patient. Exome sequencing data shows remarkable mutual exclusivity of oncogenic mutations shown to block differentiation between patients. (Adapted from Ley, T. J., et al. 2013)

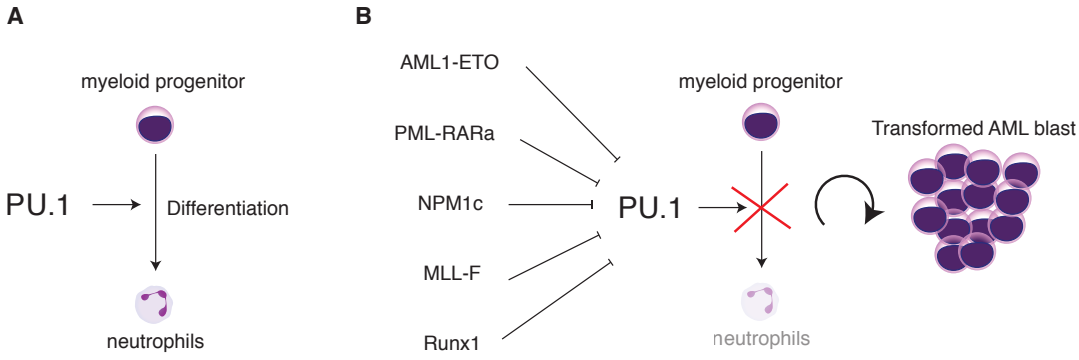


Figure 1.3 Dysregulation of PU.1-mediated myelopoiesis drives AML.

(A) Expression of master regulator PU.1 in myeloid progenitor cells drives differentiation into mature granulocytes. (B) Commonly occurring oncogenic mutations that directly inhibit PU.1 function to drive AML.

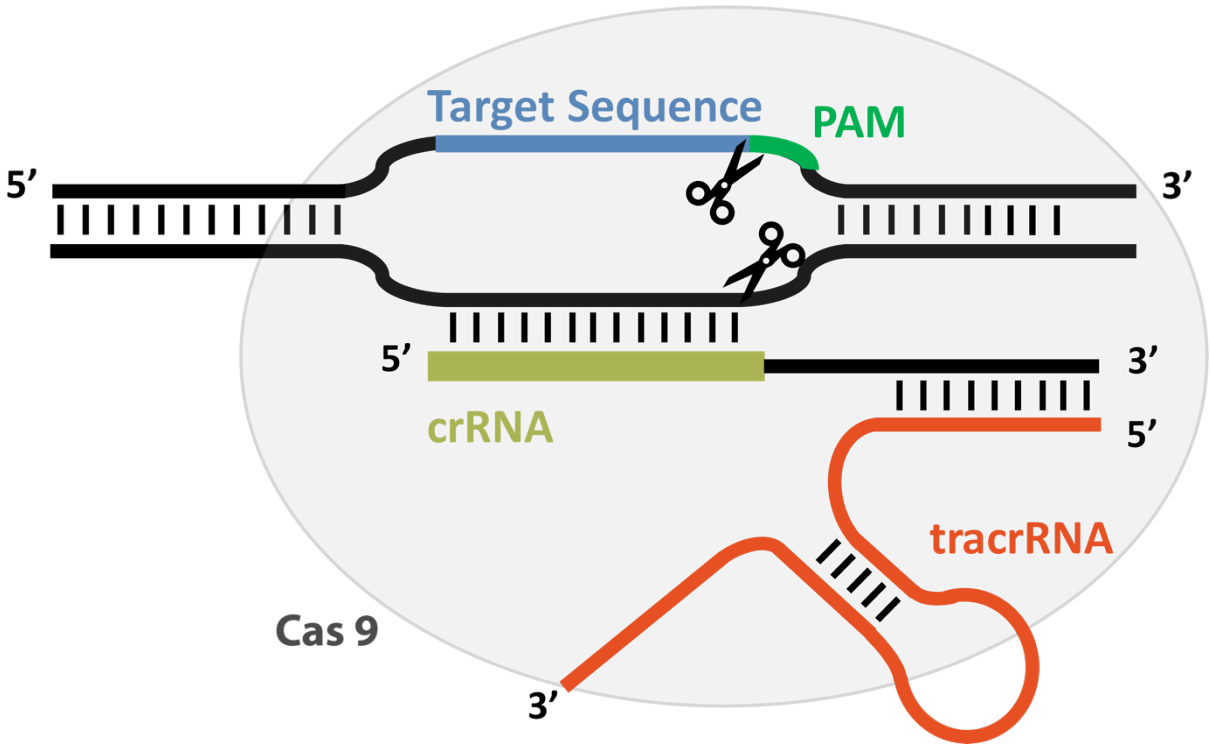


Figure 1.4 Schematic representation of CRISPR-Cas9-mediated cleavage of DNA.

crRNA component of the sgRNA binds to complementary DNA sequence whereas tracrRNA component of sgRNA forms the stem loop necessary for Cas9 mediated cleavage. Target site is directly upstream from PAM site. (Adapted from www.microsynth.ch)

Chapter 2: Materials and Methods

2.1 Cloning and sgRNA generation

2.1.2 Gibson Assembly cloning

Gibson Assembly cloning was used to generate mCherry labelled vectors that constitutively overexpress our oncogenes of interest. Restriction digest (RD) was performed to linearize the circular empty mCherry labelled vector backbone. Xho1 and Ecor1 restriction enzymes and Cutsmart Buffer were used for the RD reaction. 2.5µg of plasmid DNA was incubated at 37°C for 90 minutes with 2µL CutSmart Buffer 10x (NEB), 1µL EcoRI (NEB), 1µL XhoI (NEB), H_2O up to 20µL. To inactivate restriction enzymes, sample was incubated at 65°C for 20 minutes. In a separate reaction, oncogene of interest was PCR amplified using primers flanked with 16-20bp overhangs that are complementary to either the 5' or 3' end of the linearized vector backbone. For PCR amplification of DNA insert, Q5 High Fidelity DNA polymerase was used (NEB). 2.5 µL of the forward oligonucleotide (10uM) and 2.5 µL of the reverse oligonucleotide (10um) were added to mix containing 1ul of 10mM dNTPs, 10 µL 5x Q5 Reaction Buffer, 0.5 µL Q5 High-Fidelity DNA polymerase, 1ng of template DNA and Nuclease-Free water to make up final volume of 50ul reaction. The sample was then incubated at 98°C for 30 seconds, followed by 34x cycles of 60-65°C (depending on Tm of oligonucleotides) for 20 seconds and 72°C for 1 minute, followed by a further 2-minute incubation at 72°C. Samples were then placed on ice following PCR reaction. Both vector backbone and DNA insert are run on 2% agarose gels and bands containing the correct sized backbone/DNA insert are isolated using QIAquick Gel Extraction Kit (Qiagen) and eluted in 40ul buffer EB. To assemble the desired vector using Gibson Assembly, both DNA fragments (PCR amplified GOI insert and vector backbone) are added to 2x Gibson Assembly Master Mix (NEB) (10 µL). Volumes of PCR fragment and linearized vector added to the reaction vary depending on the concentration of gel isolated samples, however generally a 4:1 ratio of PCR fragment to vector backbone is used for the Gibson reaction. Total volume of Gibson reaction is 20 µL. Sample is incubated in a BioRad T100 Thermal cycler for 15 minutes at 50°C.

2.1.3 Cloning hairpin into LENC vector

For cloning hairpins into mCherry vector (as is the case for shRUNX1), Oligonucleotides were synthesised by Sigma-Aldrich with the forward and reverse strands of the 97mers flanked by XhoI and EcoRI restriction site overhangs. The forward and reverse single strands were phosphorylated and then annealed in the same reaction. 2.5µL of the forward oligonucleotide (40µM) and 2.5µL of the reverse oligonucleotide (40µM) were added to a mix containing 5µL Kinase Buffer 10x (NEB), 5µL ATP (10mM), 1µL of T4 polynucleotide Kinase (NEB), and 34µL of H_2O. The solution was incubated at 37°C for 30 minutes, then 96°C for 10 minutes on an AccuBlock Digital Dry Bath. Samples were cooled slowly to 80°C over 1 hour on the heat block, and then rapidly cooled on ice. To ligate the hairpin to the vector backbone, 3 µL of hairpin (isolated via gel electrophoresis and gel extraction) (2.7ng/ µL, 0.08pmol) and 7 µL of gel isolated vector backbone (13.2ng/µL, 0.02pmol) was added to 2 µL Ligase buffer (NEB) and 1 µL T4 Ligase (NEB). Ligation reaction was incubated overnight at 4°C.

2.1.4 sgRNA cloning for CRISPR-Cas9

sgRNA sequence of interests was cloned into a lentiviral vector (sgETN) containing a Thy1.1 reporter provided to us by Prof. Johannes Zuber. sgRNA sequences were ordered from Sigma Aldrich. To anneal sgRNA, 1 µL of both forward and reverse oligonucleotides (10 µM) was added to 1 µL of T4 Ligase buffer, 1 µL T4 PNK (NEB) and 6 µL nuclease free water to make a total volume of 10 µL. DNA mix is then vortexed and annealed in a thermocycler (BioRad) at 37°C for 30 minutes, followed by 5 minutes at 95°C. After 5 minutes, a 5°C/min ramping down of the temperature is performed until final temperature reaches 25°C. Annealed sgRNAs are diluted 1:250 for use in ligation reaction to vector backbone. Restriction digest of vector backbone (sgETN) was performed using BsmB1 (NEB) at 55°C for >3 hours. Linear backbone is then dephosphorylated with Calf Intestinal Alkaline Phosphatase CIP (NEB) for 30 minutes at 37°C. To ligate sgRNA to linear vector backbone, 1 µL of 1:250 sgRNA is added to 1 µL of T4 Ligase (NEB), µL T4 Ligase buffer (NEB) and 100ng of vector backbone (volume varies depending concentration of backbone purified from agarose gel). Reaction is performed at room temperature for 1 hour.

2.2 Bacterial Transformation

Plasmids were transformed into chemically competent bacteria. 1-5 µL (usually 10 pg - 100 ng) of the plasmid is added into 20-50 µL of competent cells and incubated on ice for 30 minutes. Bacteria then undergo heat shock transformation via incubation at 42°C. for 45 seconds and 4 minutes on ice. 1 mL of SOC media is added to the competent cells and incubated on a 37°C. shaker for 45 minutes. 100 µL of the competent cells in SOC media is then placed on LB ampicillin agarose gels (made in Dickins Lab). Plates are incubated at 37°C overnight.

2.3 Plasmid amplification

To amplify plasmids, transformed bacteria were picked from agarose plates and grown in LB with 100µg/mL Ampicillin overnight (37°C). Plasmid DNA was using Plasmid Miniprep or Maxiprep Kits (Qiagen) as per manufacturer's instructions. Plasmids were quantified by Nanodrop 1000 spectrophotometer analysis of 260/280nm wavelength absorption. Plasmid DNA was stored at -20°C.

2.4 Sequencing

All cDNA and DNA collected from experiments were sent to Micromon DNA Sequencing Facility and Monash University, where Sanger sequencing was performed. PCR products were sequenced using NCBI blast designed PCR primers. LENC vectors were sequenced using either MSCV 5' or IRES Forward or Reverse primers. CRISPR KO clones were also sent off were Sanger sequencing to determine genetic KO clones, using primers specified in Tables 2.1 and 2.5.

2.5 Lentiviral and retroviral production

To infect retroviral CMV vectors into AML246, calcium phosphate transfection was used for these experiments. $3.5x10^6$ 293T cells were plated in $100mm^2$ polystyrene plates and incubated overnight. Prior to transfection, media from 293T cells is replaced with fresh DMEM media. DNA plasmid mix was initially made to infect 293T cells. 4.8µg of the structural vector pMDL-GAG-POL, 2.4µg of the envelope vector ECO, and 14.4µg of the transgene expressing vector was added to 250µL of HEPES H_2O (Gibco), as well as 250µL of 0.5M $CaCl_2$. This mix is then added to 2x HBS (50mM HEPES, 10mM KCL, 12mM Dextrose, 180mM NaCl, 1.5mM Na_2hPO_4, ph 7.05), ensuring that each drop of DNA containing master mix is mixed well into

the 2x HBS. The solution is kept at room temperature for 20 minutes to allow for the formation of calcium phosphate precipitate, which is then subsequently added to the 293T cells in a dropwise manner. Media was then replaced with 6mL of fresh media following at least 8 hours of incubation (at 37°C, 10% CO_2). Cells are then left overnight, whereby virus is secreted into media by infected 293T cells.

Lentiviral packaging was used for infection of AML246/AML410 with Cas9 and sgRNA vectors given that they were infected into lentiviral vectors. The method of viral production is identical to that of retroviral production, however different packaging vectors were used. For lentiviral production, the DNA plasmid mix contained 5µg of the structural vector pMDL-GAG-POL, 3µg of the envelope vector (either ECO or VSV-G), 2.5µg of pRSV-Rev, and 10µg of the transgene expression vector.

2.5.1 Lentiviral and Retroviral Transduction

Viral containing supernatant was filtered through 0.45µm Minisart syringe filter unit to remove debris from the virus. In order to increase the viral titre, virus was concentrated by centrifugation via an Amicon Ultra-15 Centrifugal Filter Device at 3,000RPM for 5 minutes. Following concentration, 2mL of virus was added to 500,000 AML246/AML410 cells (suspended in 50uL of IMDM media) in a round bottom FALCON polystyrene tube. For infection, cells were centrifuged at 3,200RPM for 90 minutes at 37°C with virus added. Following centrifugation, cells were washed using PBS and resuspended in IMDM media. Cells were cultured at a concentration of $5x10^5$ cells/mL. After 72 hours, cells were washed twice again to eliminate remaining viral particles.

2.6 Molecular Biology

2.6.1 DNA extraction

Genomic DNA was extracted from $0.2x10^6$ to $5x10^6$ cells depending on the experiment. Cell pellets were lysed and DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) as per manufacturer's instructions. Quantification was assessed by Nanodrop 1000 spectrophotometer analysis of 260/280 nm wavelength absorption. DNA was stored at -20°C.

2.6.2 RNA extraction

RNA extracted from FACs sorted samples was performed using of the RNeasy Mini Kit (Qiagen) as per manufacturer's instructions. Generally, RNA extraction was performed on $0.2x10^6$ to $5x10^6$ cells and quantification of RNA was determined by Nanodrop 1000 spectrophotometer analysis of 260/280nm wavelength absorption. RNA was stored at -80°C.

2.6.3 cDNA synthesis

Superscript III first strand synthesis supermix kit (Invitrogen) was used to synthesize cDNA from RNA samples. On ice, up to 5 µg of total RNA is 1 µL random hexamer, 1 µL of annealing buffer and RNase-free water (to make total volume up to 8µL). This is incubated in a thermal cycler (BioRad) for 5 minutes at 65°C then immediately placed on ice for 1 minute. On ice, 10 µL of 2x First-Strand Reaction Mix (Invitrogen) is added to the sample, along with 2 µL of SuperScriptTM III/RNaseOUTTM Enzyme Mix (Invitrogen). Incubate at 25°C for 10 minutes, followed by 50 minutes at 50°C. Reaction is terminated at 85°C for 5 minutes and placed in -20°C.

2.7 Western Blot

Protein was extracted from $2x10^6$ cells of interest. Cells were centrifuged at 1,500 RPM for 5 minutes and washed in 1mL PBS (Sigma-Aldrich). Cells were pelleted again and supernatant was removed. Cell pellet was then resuspended in 200µL Laemmli solution. The lysate was vortexed and denatured for 5 minutes at 100°C on heat block. Protein lysate was quantified by Nanodrop 1000 spectrophotometer at 280nm wavelength, and stored at -20°C. For western blot experiments, 35µg of protein was loaded with 5µL of loading dye (40% Bromophenol Blue 0.1%, 10% β-mercaptoethanol, 50% ddH_2O) for each sample. All samples were made up to 30µL with Laemmli solution and vortexed before denaturation for 5 minutes at 100°C. Protein lysate is then loaded into Mini-protean pre-cast Gels 4-15% (BioRad). Electrophoresis was performed for 1 hour at 100V in running buffer (14.4g Glycine, 3g Tris and 1g SDS in 1L H_2O). To activate the PVDR membrane, (Immobilon-P), membrane was placed in methanol for 1 minute. Protein was transferred from the gel to the membrane for 1 hour at 120V in transfer buffer (14.4g Glycine and 3g Tris in 1L H_2O 20% methanol).

Membrane containing transferred protein was washed with 0.1% PBS-T (PBS-Tween20) (EMD Chemicals Inc.) on a rotator for 30 minutes (PBS-T was changed every 10 minutes). Following wash, membrane is blocked with 5% milk PBS-Tween20 overnight on a mechanical roller to minimize non-specific antibody binding at 4°C. Blocking solution was removed and washed twice with PBS, before membrane was incubated with primary antibody with rabbit polyclonal anti-PU.1 (T-21, SantaCruz Biotechnology) overnight at 4°C. Membranes were washed with PBS-Tween20 for 10 minutes 3 times, then incubated for 1 to 2 hours with secondary polyclonal swine anti-rabbit (Dako). To image the bands, 4mL of Luminata Forte Western HRP substrate was added onto the membranes and left to incubate for 1-2 minutes. A ChemiDoc Touch Imaging System (BioRad) was utilised to acquire both colorimetric and chemiluminescent signal readings. α-Tubulin was used as a loading control for western blot analysis. α-Tubulin was applied to the membrane following imaging of PU.1. Membrane was incubated in 1:10,000 α-Tubulin for 30 minutes at room temperature. Membrane was then washed using PBS-T three times and imaging protocol was repeated using Luminata Forte Western HRP substrate.

2.8 RT-PCR

RNA extraction and cDNA synthesis were performed on cells of interest as described in §2.6.1-2.6.3). Amount of cDNA synthesis was determined by the lowest concentration amongst all the samples for the same qPCR reaction. Following cDNA synthesis, 1 µL of cDNA from each sample (cDNA made from the same concentration of RNA for each sample) was added to 5 µL Promega Master mix 2x, 2 µL of primers (3 µM) and 2 µL of H_2O (for a final volume of 10 µL) in a 384 well plate. Primers were designed to span the exon-exon junction in order to avoid amplification of potential gDNA contamination. Primers were designed such that product size would be approximately100- 500bp in length. Primer sequences can be found in Table 2.3.

RT-PCR reaction was performed using a LightCycler 480 (Roche). Protocol involved 3-step PCR with the following programming: 95°C 3 minutes, (95°C 15 seconds, 60°C 30 seconds, 72°C 30 seconds) x 45

Relative transcript quantification was deduced from ΔΔCt method:

Average Ct (Target gene) – Average Ct (Rpl32) = ΔCt

ΔCt (test sample) – ΔCt (control sample) = $\Delta\Delta Ct$

$2^{-\Delta\Delta Ct}$ = Fold change gene expression

2.9 in vitro culture conditions

AML246 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) with 10% Fetal Calf Serum (FCS) (Sigma-Aldrich), 1% penicillin-streptomycin (Gibco) and 10ng/mL IL-3 (Peprotech). Cells were plated at an approximate concentration of $1x10^6$ cells per mL and incubated at 37°C, 10% CO_2. In some experiments, Doxycycline (Dox) (Sigma Aldrich) was added into the media in order to repress gene expression in our Tet-off system. For all experiments involving Dox treatment, Dox was diluted at 1µg/mL in IMDM. For time course assays, cells were resuspended in freshly made Dox IMDM media every 2-3 days. Old media was removed by centrifuging cell cultures at 1,500 RPM for 5 minutes and removal of supernatant. Generally, cells were split every 3 days unless stated otherwise.

293T cells were cultured in in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with 10% FCS (Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco). As 293T cells are adherent, cells were treated with Trypsin EDTA (Gibco) and incubated briefly at 37°C, 10% CO_2 to detach cells from flask. 293T cells were also normally incubated at 37°C, 10% CO_2.

2.10 Generation of sgRNA clones of AML246 mCherry

To generate CRISPR KO clones of AML246 mCherry cells, cells were lentivirally infected with pHRSIN pSFFV FLAG-NLS-Cas9-NLS pSV40 Blasticidin. Given Cas9 infected cells were also Blasticidin resistant, infected cells were selected for by treating AML246 cells with 10 µL of Blasticidin (Gibco) for 12 days to generate an AML246 Cas9 expressing cell line. Blasticidin was replenished in the IMDM media every 3 days. AML246 mCherry Cas9 infected cells were then lentivirally infected with vector encoding sgRNA sequence of interest. After 3 days post infection, Cas9 and sgRNA infected AML246 mCherry cells were single cell sorted using the BD influx based on Thy1.1 expression (expressed by cells infected by sgRNA vector). Single cells were cultured in IMDM media (10% Fetal Calf Serum (FCS) (Sigma-Aldrich), 1% penicillin-streptomycin (Gibco) and 10ng/mL IL-3 (Peprotech) and 10 µg/mL Blasticidin several weeks before >500,000 cells were harvested for gDNA synthesis. gDNA

was made as per §2.6.1 and was sent off for sequencing as described in §2.4. Oligonucleotides used to sequence AML246 mCherry CRISPR-Cas9 KO clones can be found in Table 2.5.

2.11 In vivo experimentation on AML

2.11.1 Generation of AML246 mCherry

To generate AML246 mCherry mouse model, AML246 cells were initially retrovirally infected with empty mCherry vector (MICR) as mentioned in §2.5.1. mCherry infected AML246 cells were then single cell sorted by FACs BD Influx and cultured in 96 well plates containing IMDM media. Single cell clones were then expanded in tissue culture for several weeks before transplant into Ly5.1 Rag mice via intravenous injections into the tail vein. 1.0 x 10^6 AML246 mCherry cells was resuspended into 200 µL of PBS for transplantation.

2.11.2 Leukemia Transplant

For subsequent transplants, frozen AML cells were rapidly thawed and washed with PBS. Cell viability was determined by Trypan Blue staining (Sigma Aldrich). 1.0 x 10^6 cells resuspended into 200 µL of PBD was transplanted into unirradiated Ly5.1 Rag or Rag1-/- recipient mice through intravenous tail vein injections.

2.11.3 Mouse monitoring

Experimental animals were monitored three times a week for overt and general signs of health and wellbeing. To monitor any signs of leukemia, examination of overt signs of sickness included dramatic weight loss, hind leg paralysis and breathing difficulties. After 3 weeks post transplantation, blood was collected from mandible or tail vein of mice to collect volumes for blood ranging from 20 µL - 100 µL on a weekly basis to track the progression of disease. Blood was collected into EDTA coated Microvette bleed tubes (Microvette). Following two weeks of Dox treatment. Mandible or tail vein bleeds became less frequent, with blood taken from animals every 2-3 weeks.

2.12 Tissue sample preparation and processing

2.12.1 Red Blood Cell Lysis

To monitor the presence of mCherry AML derived cells in the PB, 50µL of collected PB from the mandible bleeds is treated with 3mL of 1x RBC lysis buffer (4g NH_4Cl, 1g $KHCO_3$, 37mg EDTA IN 1L H_2O). Blood is mixed well via 1000µL pipette. Following 2 minutes of incubation at room temperature, sample is then centrifuged at 1,500RPM for 5 minutes. Supernatant is removed and sample is processed depending on the purpose of the experiment.

2.12.2 Tissue harvesting and processing

At given timepoints (specified in the figures), mice were culled via cervical dislocation or CO_2 gas asphyxiation. Different organs were harvested depending on the purpose of the experiment. For flow cytometry analysis of the BM, spleen and liver, organs were removed from the culled animal and filtered through a 40 µM nylon cell strainer (FALCON) using PBS and the blunt end of a plastic 3 mL syringe (FALCON). Filtered sample was then centrifuged at 1,500RPM for 5 minutes and supernatant was removed. 3 mL of 1x RBC lysis buffer is added to each sample to remove any RBCs and incubated at room temperature for 2 minutes. Sample in RBC lysis buffer is centrifuged once more and resuspended in FACs buffer for flow cytometry analysis on the LSRII or FACs sorting via the BD Influx.

For IVIS imaging, organs of interest were harvested from culled animals at given timepoints and kept in PBS. Liver, and spleen samples for IVIS were not processed and filtered through 40 µM cell strainer as would be the case for FACs analysis. Instead, these organs were also directly placed in PBS and on ice until organs were ready for imaging.

2.12.3 Dox treatment *in vivo*

To suppress shPU.1 hairpin expression of AML246 and AML410 cells in vivo, mice were fed Dox food pellets (600mg/kg) following the emergence of AML detected in the blood. Dox food pellets were purchased from Specialty Feeds via AMREP PAC.

2.12.4 Splenectomy

Splenectomies were performed by Dr. Peter Kanellakis. Briefly, mice are anaesthetized with xylazine/ketamine cocktail (administered via intraperitoneal injection at ketamine-65mg/kg and xylazine- 5mg/kg). Anesthetized mice were positioned on 37C heat pad (to provide warmth throughout surgical procedure) to provide clear view on shaved area. They were swabbed with alcohol followed by iodine and a small incision (10-15 mm) was made. The spleen was identified and carefully manipulated through the incision to sit outside the mouse on a moist gauze swab. After the splenic artery and vein are carefully cauterised, the spleen was removed. The abdominal muscle wall is then closed using a 4-0 synthetic absorbable suture. The skin incision is closed using silk sutures. Before suturing the abdominal muscle, about 100 µL of warm normal saline was added into the peritoneal cavity as a fluid replacement for blood loss during surgical procedure.

2.13 Flow Cytometry analysis

All flow cytometry-based analysis was performed on the BD LSRII at the AMREPflow core facility. Cells were initially pelleted by centrifugation at 1,500 RPM for 5 minutes. Following the removal of media supernatant, cells were resuspended in FACs buffer (10% FCS in PBS) containing 1µg/mL of viability marker SYTOX blue (ThermoFisher Scientific). In experiments involving the use of antibodies, cells were initially resuspended into 50 µL of Fc Block (1µg/mL) (WEHI) for 5 minutes to minimise non-specific binding of antibodies. Different combinations of mature myeloid markers listed in Table 2.6 was used depending on the given experiment. Cells were incubated in given antibody mix for 20 minutes at room temperature or 30 minutes on ice, before being washed using PBS. Cells were centrifuged and resuspended in FACs buffer.

2.13.1 Cell Cycle analysis

For cell cycle analysis, cells were initially washed twice in PBS. After the second wash, cells were centrifuged and supernatant removed. Cells were then stained with 1:1000 Fixable Viability Dye (ThermoFisher) for 30 minutes on ice. After 30 minutes, sample was washed twice with PBS to remove residual dye. Cells were then fixed and permeabilized with 250 µL of CytoFix/CytoPerm Buffer (BD Biosciences) for 30 minutes on ice. Following permeabilization, cells are then washed twice with 250 µL of CytoWash Buffer (BD

Biosciences). Following second wash, cells are centrifuged and supernatant is removed. Cells were then resuspended in 10% FCS containing 1:200 ki67 (BD Biosciences) and 1:500 DAPI (Sigma Aldrich) and incubated at room temperature in the dark for 30 minutes. Following incubation, cells are then washed with PBS and resuspended in FACs buffer (10% FCS in PBS) for analysis on the LSRII machine.

2.13.2 FACs based cell sorting

For cell sorting, cells in suspension were initially centrifuged (at 1,500RPM for 5 minutes) and washed twice with PBS. Cells in PBS are then filtered through a 70 μM mesh cap provided with a 3 mL polystyrene tube (FALCON) and centrifuged once more (same condition as above). For cell sorts based on the expression of given surface markers, antibody staining as mentioned in §2.13 was performed. Cells were then resuspended in FACs buffer (10% FCS in PBS) and resuspended in 3 mL polystyrene tube for fluorescence-activated cell sorting (FACS) on the BD Influx machine operated by AMREPflow core facility members. FACs sorted cells were collected in 15mL FALCON tubes (FALCON) containing 3 mL of IMDM media unless otherwise state. For single cell purification, cells were sorted on BD Influx (BD Biosciences) cell sorter into a 96-well plate with 100μL of IMDM media.

2.14 IVIS imaging

IVIS Spectrum In Vivo Imaging System was used to image mCherry fluorescence in organs harvested from mice. Software imaging wizard was used to adjust settings appropriate for mCherry fluorescence imaging. 580nm Excitation peak and 620nm Emission peak (and 587/610 Filter) was used to detect mCherry signal in organs. Exposure was set to Auto, Binning to Medium, Field of View set at 10cm. Adaptive FL background subtraction was utilized to remove background noise from images. Organs harvested from $Rag1^{-/-}$ or Ly5.1 Rag mice not transplanted with AML were used as a negative control for IVIS imaging.

2.15 Cytospin, MGG staining and histology imaging

FACs sorted cells in suspension were centrifuged at 1,500 rpm for 5 minutes. Cell pellet was resuspended in 150 μL of PBS. Cells suspended in PBS were then added to a slide chamber positioned on top of a microscope slide (Citoglas) and blot paper with a small hole (where the cells will be spun onto). The sample in the slide chamber was then spun down onto the slide

using a Cytocentrifuge (ThermoFisher, Cytospin 4) at 800 RPM for 5 minutes. Microscope slide containing spun down cells was then air dried at room temperature for 1 hour, followed by methanol fixation for 45 seconds. Fixed microscope slide was then sent down to Alfred Hospital pathology for May-Grünwald Giemsa (MGG) staining. Stained slides were then cover slipped using DPX (Merck) and 24mm x 40mm coverslips (Menzel-Glaser) and left overnight. Images of the histologically stained slights were performed on a Nikon Bright Field Microscope (OptiPhoto-2).

Table 2.1 Gibson and sequencing primers for cloning into MSCV-IRES-mCherry vector

Gene of interest	Primer sequence
NPM1c	Forward 5’ CTAGGCGCCGGAATTAGATCTCGCGACTCGAGCCACCATGCAAGCAAGTATAGAAAAAG 3’
	Reverse 5’ GTTAGGGGGGGGGCGGAATTGATCCCGCTCGAGAATTCTTAAAGAGACTTCCTCCAC 3’
PMLRARα	Forward5’ CTAGGCGCCGGAATTAGATCTCGCGACTCGAGCCACCATGGAGCCTGCACCCGCCCGATCTCCGAGGCCCCAG 3’
	Reverse 5’ GTTAGGGGGGGGGCGGAATTGATCCCGCTCGATCACGGGGAGTGGGTGGCCGGGCTGC 3’
MSCV 5’	5’ - CCCTGAACCTCCTCGTTCGA -3’
IRES F	5’ TCTAGGCCCCCCGAACCACGGGGAC 3’

Table 2.2 97mer oligonucleotide sequence

sgRNA	5' linker	97mer Forward Sequence	3' linker
shRUNX1	TGCTGTTGACAGTGAGCG	ACCATCCTGTTTGGCATCTATTTAGTGAAGCCACAGATGTAAATAGATGCCAAACAGGA TGGG	TGCCTACTGCCTCGG A

Table 2.3 Primers for qPCR

Gene of interest	F sequence	R sequence	Predicted product size
Gata1 1	GGGATCACCCTGAACTCGTC	GGTTGAACCTGGGCTTGTTG	70 bp
Siglec-F 1	GGTCTCACAGGTGAAGGTCC	GGCAAGATGGTTGCCTTTCG	73 bp
XBP1 1	CTGAGTCCGCAGCAGGTG	TTCCAGCTTGGCTGATGAGG	260 bp
Prg3 1	CCCTTGGGTAGTGAGTGCTG	GTCCAACGCAGCTTCTATGC	95 bp
EPX 1	CTGCTTAGCTGTAGTGGGGG	TGTGAGCACATCAGTGGCAT	390 bp
Ly6G 1	AGAGGAAGTTTTATCTGTGCAGCC	TCAGGTGGGACCCCAATACA	256 bp
Coro1a	5'- GGGCTGAGTCCCCCATTAAG -3'	5'- GAGACGCGCACATCCTCATA -3'	126 bp
PU.1	5'-CTGGAGCTCAGCTGGATGTTAC-3'	5'-GCCATCAGCTTCTCCATCAGAC-3'	477 bp

Table 2.4 sgRNA sequences for CRISPR-Cas9 KO

Gene name	sgRNA no:	Primer sequences
sg.Gata1	2	Forward: 5'caccGCTGGGCCTATGGCAAGA 3'
		Reverse: 5' aaacTCTTGCCATAGGCCCAGC 3'
sg.Gata1	3	Forward: 5' caccGGCCCTGGAAGACCAGGA
		Reverse: 5' aaacTCCTGGTCTTCCAGGGCC 3'
Sg.Csf1r	2	Forward: 5' caccGCGAGGGTTCATTATCCGCA 3'
		Reverse: 5' aaacTGCGGATAATGAACCCTCGC 3'
sg.Xbp1	1	Forward: 5'caccGGACACGCTGGATCCTGACG 3'
		Reverse: 5'aaacCGTCAGGATCCAGCGTGTCC 3'
sg.Ccr3	1	Forward: 5'caccGAGGCCGATGATGAACACCA 3'
		Reverse: 5'aaacTGGTGTTCATCATCGGCCTC 3'

Table 2.5 Primers used to sequence potential CRISPR-Cas9 KO

Gene name	sgRNA	Primer sequences	Product size
Gata1	2	Forward 5'CTCTCTACCCCTTCAACAGTCT 3'	426 bp
		Reverse 5'TCAAAAAGGGACCAGGAGGT 3'	
Gata1	3	Forward and reverse sequences same as above	426 bp
Csf1r	2	Forward 5'TCGGGCAGATGCTCAGGCCTTTG 3'	505 bp
		Reverse 5'CACAGCTGGTGTCATGGTCAG 3'	
Xbp1	1	Forward 5'CTCTGTCCCATTAGCCACCG 3'	499 bp
		Reverse 5'TGAATTTTCCCTGTTTCCTTGAACT 3'	
Ccr3	1	Forward 5'GATGACTTTGAGAGCTACAGAGC 3'	524 bp
		Reverse 5'GAGTGGGGTTTTGGCCACTA 3'	

Table 2.6 Antibodies and viability dyes used for Flow Cytometry

Antigen	Clone	Conjugate	Supplier	Dilutions
CD11b	M1/70	PE	BD Biosciences	1:400
		BV711		1:400
Gr-1	RB6-8C5	APC	BD Biosciences	1:400
Ly6G	1A8	APC	BD Biosciences	1:400
Siglec-F	E50-1440	PE	BD Biosciences	1:400
Ccr3	J073E5	APC	Biolegend	1:300
F4/80	BM8	PE-Cy7	Invitrogen	1:400
CD125	T21	BV421	BD Biosciences	1:400
CD115	AFS98	APC	BD Biosciences	1:400
Thy1.1	OX-7	APC	Biolegend	1:400
Ki67	SolA15	AF700	Invitrogen	1:100
CD45.1	A20	AF700	BD Biosciences	1:400
CD45.2	104	BV421	BD Biosciences	1:400
		PE-Cy7		1:400
Sytox Blue				1:1000
Fixable Live Dead Dye			BD Horizon	1:1000

Table 2.7 Antibodies for Western Blot analysis

Antigen	Clone	Supplier	Dilution
Anti-PU.1 (mouse)	C-3	Santa Cruz	1:1000
α -Tubulin	T9026	Sigma	1:5000

Chapter 3: Determining the kinetics and mechanisms of AML blast differentiation and clearance *in vivo*

3.1 Introduction

The transcription factor PU.1 is the master regulator of myeloid cell differentiation. Although PU.1 itself is rarely mutated in AML, PU.1 function is compromised in several AML subtypes. Therefore, to investigate global principles of the mechanisms of AML blast differentiation and clearance, a mouse model of AML driven by loss of PU.1 was previously generated in the Dickins laboratory. AML246 is a mouse AML model previously generated by Dr. Mark McKenzie (McKenzie et al, 2019, in press). It is driven by combined RNAi-mediated knockdown of PU.1 and p53 deficiency, however the model also contains an activating Kit mutation and a normal karyotype (McKenzie et al, 2019, in press). This AML model was generated by co-transducing p53-/-, fetal liver derived haematopoietic stem/progenitor cells with two retroviral vectors. The first vector contains a TRE3G promoter that controls the expression of a GFP reporter as well as a short hairpin RNA (shRNA) targeting PU.1. The second retroviral vector stably expresses the protein tTA. Together, these two vectors make a Tet-OFF regulated system where the expression of shRNA targeting PU.1 can be toggled in the absence or presence of Doxycycline (Dox). In the absence of Dox, tTA binds and transactivates the TRE3G promoter, leading to the expression of the hairpin targeting PU.1. PU.1 knockdown leads to the proliferation of the leukemic blasts, and recipient mice transplanted with primary AML246 develop an accelerated GFP^+ AML. Doxycycline (Dox) binds to tTA, thereby preventing tTA binding to the TRE3G promoter, leading to lost expression of genes downstream of the TRE3G promoter (Fig 3.1A). In this particular context, Dox administration for 10 days leads to reduced expression of the GFP reporter and the shRNA targeting PU.1. As such, the restoration of endogenous PU.1 drives leukemic blast differentiation *in vitro* and *in vivo* (Fig 3.1C) (McKenzie et al, 2019, in press). Hence, AML246 functions as a genetic mouse model of differentiation therapy. Importantly, when PU.1 suppression is re-engaged by the removal of Dox in culture, mature, AML-derived granulocytes remarkably de-differentiate and revert back to a blast like state (McKenzie et al, 2019, in press). These results not only question the uni-directional model of myeloid cell differentiation, but also raise the possibility that all AML-derived cells (regardless of maturity) can be leukaemia initiating. Furthermore, results from McKenzie et al. shows that despite uniform differentiation of leukemic blasts into mature, non-leukaemogenic cells *in vivo*, mice

kept on long term Dox ultimately succumb to disease relapse (Fig 3.1B). Thus, AML246 provides a unique model of AML that allows us to investigate the differentiation and clearance of AML blasts in an established disease.

Hence, the focus of this chapter is to use this mouse model of differentiation therapy to determine not only the mechanisms of AML differentiation and clearance *in vivo*, but to also investigate the cause of relapse following leukemic blast differentiation.

3.2 Generation of a trackable AML246 mouse model in vivo

Intravenous transplantation of AML246 via tail vein injection leads to the establishment of a GFP^+ AML following 4 weeks post-transplant. The administration of Dox (via Dox food pellets) to Rag -/- mice transplanted with AML246 leads to uniform AML blast differentiation and disease regression *in vivo*. However, a limitation to the original AML246 model is that the presence of Dox not only represses the expression of the shRNA targeting PU.1, but also the expression of the GFP reporter (McKenzie et al, 2019, in press). As such, it is difficult to track the AML-derived cells *in vivo* after mice have been kept on Dox long term. To address this, a retroviral vector expressing stable mCherry protein (MICR) was transduced into AML246 to generate AML246 mCherry (Fig 3.2A). Therefore, given that the mCherry reporter is driven by a promoter independent of the TRE3G promoter controlling GFP and shPU.1 expression, AML-derived cells remain mCherry positive in the absence or presence of Dox treatment. AML246 cells transduced with MICR were subsequently single cell cloned to give uniform mCherry signal and expanded *in vitro*. As such, mCherry expression can be used to track the presence of AML-derived cells following long term Dox *in vivo*.

3.3 Investigating the kinetics of AML engraftment, differentiation and regression in vivo

In order to characterize the behaviour of a chosen single cell clone of AML246 mCherry *in vivo*, 18 CD45.1 Rag mice (n=3 for each time point) were transplanted with 1.0 $x10^6$ mCherry leukemic cells. Weekly mandible bleeds were performed to monitor the development of an mCherry positive AML. Mice transplanted with AML246 mCherry were culled at given time points prior to and post PU.1 restoration to monitor disease burden in central organs such as the bone marrow (BM) and spleen. AML246 mCherry cells were first detected in both the BM and spleen two weeks following tail vein transplant. Following an extra two weeks, the mCherry leukemic cells continued to propagate and could eventually be detected in the

peripheral blood (PB). Of note, in an untreated mouse, the majority of the leukemic burden was localised to the spleen rather than the BM (Fig 3.2B). Once AML246 mCherry was be detected in the PB, the mice were then placed on Dox to trigger PU.1 restoration differentiation of the leukemic blasts. Following 7 days on Dox significant disease burden was detected in each of the PB, BM and spleen of the mice. Importantly, there was also uniform downregulation of the GFP reporter. Loss of GFP expression therefore indicated that the shPU.1 hairpin had been switched off and the cells were undergoing differentiation. This was supported by the restoration of PU.1 protein expression of the AML-derived cells following 8 days on Dox (Fig 3.2D, lane 2). From basic flow cytometry analysis, AML-derived cells could not be detected in the PB, BM or spleen after 14 days on Dox, therefore the disease was either cleared, or residual AML derived cells were still present at very low frequency that was not detected. Hence following PU.1 mediated differentiation of leukemic blasts, the AML-derived cells are cleared and the mice experience disease regression (Fig 3.2B).

3.4 Long term Dox treatment leads to a mCherry, GFP positive relapse

For the purposes of our experiments, mice were deemed to be 'in remission' once no AML-derived cells could be detected in the PB. Interestingly, mice transplanted with AML246 mCherry kept on long term Dox were in remission for several weeks before succumbing to disease relapse, as is indicated by the re-emergence of an AML-derived mCherry population (Fig 3.2B and C). Notably, the length of time on Dox before succumbing to relapse was highly variable, with mice falling to a mCherry positive relapse as early as 55 days post Dox, whereas others succumb to relapse after over 150 days on Dox (Fig 3.2C). Interestingly, with the exception of one mouse (out of 22 mice total), all disease relapses were also GFP positive, suggesting that the AML-derived cells had acquired a mutation that allowed for the shPU.1 hairpin to be switched on in the presence of Dox. This is supported by equally low PU.1 protein levels in relapse samples compared to PU.1 protein in the UT samples by western blot analysis (Fig 3.2D). mCherry/GFP positive relapse samples from the spleen were also sorted by flow cytometry (FACs) and subsequently cultured in the absence or presence of Dox. As expected, AML246 mCherry cells from an UT mouse lost GFP expression after 8 days on Dox *in vitro* (Fig 3.2E). Conversely, AML246 mCherry/GFP positive cells harvested from a relapse sample remained GFP positive in both the absence or presence of Dox, suggesting that the relapse cells are now insensitive to Dox treatment (Fig 3.2E). Therefore, these results suggest that the TRE3G promoter has been activated to re-engage PU.1 suppression in the presence of Dox.

Although it currently remains unclear, this is likely due to a mutation which has ultimately rendered the promoter insensitive to Dox. The unexpected development of Dox insensitivity proved to be serendipitous, as this failure to respond to Dox provided a unique opportunity to investigate the origins of disease relapse following differentiation therapy. This would not have been possible had the AML-derived cells remained sensitive to Dox as they likely would not have developed disease relapse.

3.5 Restoration of PU.1 in AML cells triggers bifurcated myeloid cell differentiation in vivo

From the initial time course assay, it was determined that endogenous PU.1 was restored in leukemic cells following 7-8 days of Dox. Flow cytometry analysis was used to determine the changes in immunophenotype of AML-derived cells as they underwent differentiation. As expected UT leukemic blasts were negative for the lineage specific myeloid markers Ly6G, F4/80 and Siglec-F, but were positive for CD11b (Fig 3.3A). The lack of lineage specific marker expression coupled with the expression of CD11b therefore suggests that the AML cells in an UT animal were immature, cells of the myeloid compartment. Morphologically, FACs sorted AML cells from a UT animal were large cells, with large undifferentiated nucleus characteristic of an immature myeloid blast (Fig 3.3A). Interestingly, despite the uniform restoration of endogenous PU.1 (as is indicated by downregulation of GFP), the AML-derived cells could be separated into two distinct populations based on their side scatter (SSC) profile after 8 days on Dox (Fig 3.3A).

Following 8 days on Dox, the majority of AML cells differentiated into SSC^{LOW} neutrophil-like cells, as is indicated by the expression of the pan mature myeloid marker CD11b as well as the neutrophil specific marker Ly6G (Fig 3.3A, bottom row). Conversely, a smaller proportion of AML-derived cells matured into a SSC^{HIGH} population that was negative for Ly6G. Instead, this subpopulation expressed the classic markers of the eosinophil lineage F4/80 and Siglec-F (Fig 3.3A, top row) (Hey et al., 2015). This is consistent with the histological stains from sorted SSC^{HIGH} and SSC^{LOW} AML-derived populations, where the SSC^{LOW} cells have the distinct multi-lobulated nucleus characteristic of neutrophils, and the SSC^{HIGH} cells possess a distinctly red, granular cytoplasm reminiscent of mature eosinophils (Fig 3.3A). In addition to the immunophenotypic and morphological differences between the SSC^{HIGH} and SSC^{LOW} AML-derived cells (Fig 3.3A bottom row), transcriptional differences were also observed between the two sub- populations. Initially, we had attempted to extract RNA from

AML-derived neutrophil-like and eosinophil-like cells following 8 days on Dox, however we were unable to extract good-quality RNA from AML-derived eosinophils, presumably due to the uniquely large RNase load of eosinophils (Domachowske et al., 1998). Fortunately, bifurcation of AML-derived cells was observed following 5 days on Dox (data not shown) hence SSC^{HIGH} and SSC^{LOW} cells were harvested from the BM at this timepoint for RT-qPCR analysis. As expected, the SSC^{HIGH} cells upregulated important eosinophil specific genes such as Gata1, Prg3, XBP1 and Siglec-F (Fig 3.3B). SSC^{LOW} cells however, only upregulated neutrophil specific genes such as Coro1a and Ly6G (Fig 3.3B). Interestingly, SSC^{LOW} cells also downregulated the eosinophil specific genes, suggesting that the process of neutrophilic differentiation may not rely on the expression of neutrophil specific genes, but may also require the active downregulation of other lineage specific genes, thereby preventing them from differentiating into another myeloid lineage (Bararia et al., 2016; Fiedler et al., 2012; Lawrence et al., 2018). To summarize, upon the restoration of PU.1 in AML blasts *in vivo*, AML blasts differentiate along two distinct myeloid lineages separated by their SSC profile. Further characterization shows that SSC^{HIGH} and SSC^{LOW} cells, immunophenotypically, morphologically and transcriptionally resemble eosinophils and neutrophils respectively.

3.5.1 Bifurcated leukemic cell differentiation observed in multiple PU.1 driven models of AML

To determine whether the bifurcation of AML-derived cells following differentiation was specific to the AML246 mCherry clone, the experiment was repeated using a parental AML246 clone that had not been previously transduced by the mCherry vector (AML246 clone 2). Similar to AML246 mCherry, intravenous injection of AML246 clone 2 resulted in establishment of disease following 4 weeks post-transplant. As expected, restoration of PU.1 following 10 days of Dox also resulted in bifurcation of AML-derived cells into SSC^{HIGH} eosinophilic and SSC^{LOW} neutrophilic lineages (Fig 3.4A and B).

In addition to AML246, McKenzie et al. also generated an independent inducible AML model known as AML410. Similarly to AML246, the generation of AML410 involved the co-infection of $p53^{-/-}$ FLCs with a vector encoding stable expression of tTA, as well as a second vector containing an independent shPU.1 hairpin (shPU.1 1293) driven by a TRE3G promoter. Importantly, the AML was generated in a mouse independent of the generation of AML246, hence the genetic background of AML410 also differs from AML246 (McKenzie et al, 2019, in press). To replicate our experiments from AML246 to this second inducible model, AML410

cells were transduced with a stable mCherry vector and mCherry infected cells were single cell cloned and characterized as previously described in §3.2. An AML410 mCherry clone was then transplanted into recipient CD45.1 Rag mice via intravenous injection and an mCherry, GFP+ disease was established after 3 weeks. After 15 days on Dox, AML410 cells also bifurcated, however AML-derived cells were distinctly separated by Siglec-F, IL5Rα expression, with SSC^{HIGH} cells being Siglec-F^{+}, IL5Rα^{-}, whereas the SSC^{LOW} cells were Siglec-F^{-}, IL5Rα^{+}. Given that both Siglec-F and IL5Rα (Wilson et al., 2011) are classic markers of eosinophils, AML cell bifurcation in this model may not be multilineage, but rather the differentiation into different maturational stages of the eosinophilic lineage. Nevertheless, collectively these data suggest that AML blasts from the AML246 model are capable of maturing into multiple subpopulations of differentiated non-leukaemogenic myeloid cells following the restoration of endogenous PU.1.

3.6 Transformation of AML cells correlates with changes in cell cycle status

In addition to immunophenotypical characterization of AML cells throughout the time course, we also determined the cell cycle profile of AML cells as they transition between immature and mature states during the course of Dox administration. In order to do this, PB, BM and spleen samples were harvested from mice that were untreated, treated with Dox for 7 days, or mice that had succumbed to relapse. AML-derived cells from each time point were then permeabilized and stained with 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent marker used to distinguish between cells that are in a quiescent phase (G0/G1), synthesis phase (S phase) or post replicative/ mitotic phase (G2/M) (Pozarowski et al., 2004). As expected, AML246 mCherry cells harvested from UT mice had approximately 15% of AML cells in the S/G2/M phase throughout the BM and spleen (Fig 3.5A and B). Noticeably, AML cells in the PB were less proliferative, with only 2.5% of PB AML cells in the S/G2/M phase. Following the restoration of PU.1 however (7 days Dox), the remaining AML-derived cells all remained arrested in G0/G1 phase (Fig 3.5A and B). Coupled with the immunophenotype data from the corresponding timepoint, the subpopulation of AML-derived cells that remain after 8 days on Dox appear to be post mitotic mature myeloid-like cells. Overall, differentiation of AML blasts correlates with loss of proliferative capacity, as was expected. Finally, much like the UT mCherry AML, the mCherry, GFP positive AML population that emerges following long term Dox treatment are also highly proliferative, thus confirming that these once differentiated AML cells have reverted back to a blast like state to propagate relapse (Figure 3.5A and B).

To summarize these results, the restoration of endogenous PU.1 *in vivo* via Dox treatment transforms the highly proliferative leukemic blasts into two distinct, non-proliferative mature myeloid cell populations, namely the SSC^{HIGH} eosinophils and the SSC^{LOW} neutrophils. Upon extended period of disease remission, AML-derived cells acquired a mutation that allows for the expression of shPU.1 in the presence of Dox, the AML-derived cells revert back to a disease that resembles the original UT AML that propagates relapse. This is consistent with observations made by McKenzie et al, whereby re-engagement of PU.1 suppression through the withdrawal of Dox from matured AML-derived cells saw reversion of the AML-derived neutrophil back to a blast like state. Although we are also likely to have observed de-differentiation of mature AML cells, an important distinction is that de-differentiation in our AML246 mCherry *in vivo* model has occurred in the presence of Dox, most likely attributed to a break in the TRE3G-tTA inducible system.

3.7 Clearance of differentiated AML cells is facilitated by splenic macrophages

The restoration of endogenous PU.1 results in differentiation of AML blast after a week on Dox, and after two weeks on Dox the AML-derived cells are cleared from the PB, BM and spleen. Therefore, rapid clearance of differentiated AML cells must be occurring during these time points to drive disease into regression and remission. Interestingly, following a week on Dox, in addition to the differentiation of AML blasts, a distinctive mCherry 'smear', GFP negative population appears uniquely in the spleen but not in the BM or PB (Fig 3.6A, also refer to 3.2B). Importantly, unlike the AML population that expressed uniform mCherry, the 'smear' signal encompasses a range of mCherry levels, with mCherry signal in a portion of 'smear' cells being noticeably higher than the AML-derived mCherry signal. As the AML246 mCherry cells were transplanted into Ly5.1 Rag mice, CD45.1 and CD45.2 staining was used to determine the origin of the mCherry 'smear' signal. Unlike the AML cells which were exclusively CD45.2 positive, the mCherry 'smear' signal is unanimously CD45.1 positive, suggesting that these cells are not AML-derived cells, but are host-derived cells. Flow cytometry analysis reveals that the host-derived mCherry 'smear' cells express mature macrophage markers CD11b and F4/80 (Fig 3.6A). This is also supported by morphological analysis, where FACs sorted mCherry 'smear' cells morphologically resemble macrophages (Fig 3.6A). Although there was also a low percentage of mCherry 'smear' cells present in the BM, this data strongly suggests that differentiated AML-derived cells are engulfed by host

derived macrophages specifically in the spleen. As a result, there is the emergence of a mCherry smear signal predominately in the spleen following a week on Dox.

3.8 Accelerated relapse following AML blast differentiation in the absence of a spleen

Our observations from mice treated with Dox for 8 days suggested that macrophages in the spleen play an important role in the clearance of differentiated AML cells. To test this hypothesis, the spleen was removed from a group of mice prior to leukemia transplant. Following two weeks post-surgery, both splenectomised and sham control mice were transplanted with AML246 mCherry. Length of time for the establishment of AML was similar between both splenectomised and sham control groups (emergence of $mCherry^+$ GFP^+ cells was delayed by 4 days in sham control group). To address this, disease burden at the time of Dox treatment was standardized between the two groups (mice were put on Dox when PB disease burden was at 10-20%). Once disease had been established, mice were placed on Dox to trigger differentiation and subsequent clearance (Fig 3.6B). Longitudinal analysis of disease clearance in the PB was performed on both groups of mice to determine whether the absence of a spleen impaired clearance of differentiated AML cells (Fig 3.6C). Surprisingly, following 15 days on Dox, splenectomised mice were able to clear the AML as effectively as our sham control. However, despite clearance of disease in the PB, splenectomised mice ultimately succumb to relapse faster than the sham controls, however the difference was not statistically significant ($p=0.05$, median survival for splenectomised = 45 days, median survival for sham control =57.5 days) (Fig 3.6D). Therefore, consistent with the characterization of the mCherry 'smear' cells, the macrophages in the spleen appear contribute to the clearance of AML-derived cells following differentiation *in vivo*, however it may not be the only organ that is responsible for doing so.

3.9 Original leukemic burden impacts the kinetics of differentiated AML cell clearance but not relapse

The emergence of a distinctive mCherry smear population following a week of Dox treatment signalled the clearance of AML cells following differentiation. Interestingly, a lower mCherry smear population correlated with a larger AML-derived burden following a week on Dox (Fig 3.7A). Hence to determine if the leukemic burden present prior to treatment impacts the kinetics of AML cell clearance following AML cell differentiation, groups of AML246 mCherry transplanted mice were placed on Dox at different degrees of leukemic burden. Mice

placed on Dox when PB AML burden was <10% were considered 'low disease' burden, whereas the 'high disease' burden group was comprised of mice that were placed on Dox when PB AML burden was >20%. Following 7 days on Dox, the BM and spleen was harvested from mice in each group and both host and AML-derived cells were analysed. Interestingly, a large population of host derived, mCherry cell engulfing macrophages were present in low burden mice following 7 days on Dox, however the emergence of this population was delayed in high disease burden mice (Fig 3.7A). Bifurcation of AML blasts into SSC^{LOW} neutrophil and SSC^{HIGH} lineages was observed in both groups of mice, however unexpectedly, the percentage of SSC^{HIGH} cells present in the high disease burden mice was considerably lower than those in the low disease burden group (Fig 3.7A and B). This was also coupled with a significantly higher percentage of SSC^{LOW} neutrophils in high disease burden mice compared to low disease burden mice (Fig 3.7B). Therefore, given the strong correlation between the reduction in SSC^{LOW} population and the presence of host derived macrophages (mCherry smear), this data strongly suggests that following differentiation, AML-derived neutrophils are cleared prior to the clearance of AML-derived eosinophils. Intriguingly, wildtype neutrophils have a notoriously short lifespan of less than 24 hours (Kotzin et al., 2016; Tak et al., 2013), whereas tissue resident eosinophils have the capacity to survive for several weeks (Y. M. Park et al., 2010; Uhm et al., 2012). Therefore, these differences in lifespan may be reflected in our data, where the AML-derived SSC^{LOW} neutrophils are cleared first, followed by the clearance of AML-derived SSC^{HIGH} eosinophils.

Given the delayed clearance of differentiated AML cells in high burden mice, we expected that the rate of relapse would be faster in mice with high disease burden compared to low burden mice, however surprisingly the difference was not statistically significant ($p=0.051$, median survival for <10% PB burden= 79.5 days, median survival for >20% burden =48.5 days) (Fig 3.7C). Although there is a positive trend, the lack of statistical significance implies that the impact of leukemic burden on relapse may be minimal relative to other factors, or that greater number of mice in each category need to be analysed to reveal the true impact of leukemic burden and rate of relapse. For example, differentiation of AML blasts into certain myeloid lineages may have a greater impact on relapse than the initial disease burden itself. Alternatively, although analysis of the PB provides an opportunity to monitor the disease longitudinally, the PB disease burden may not be a consistent reflection of the central disease burden. For example, an individual mouse with a low PB AML burden may relapse faster due

to a significantly higher disease burden in the spleen compared to a mouse with a high PB disease burden.

3.10 AML-derived SSC^{HIGH} eosinophils persist during disease remission and likely seed relapse

From the initial time course analysis, no AML-derived cells could be detected following 14 days on Dox, however mice still succumb to a mCherry AML relapse (Fig 3.2B). Given this, a rare population of residual AML-derived cells must be present during remission that was ultimately responsible for seeding relapse. To investigate this, recipient CD45.1 Rag mice transplanted with AML246 mCherry were treated with Dox for 16 and 18 days to drive the AML into remission. Importantly, CD45.1 mice were used for this experiment given that the emergence of an mCherry smear population following 8 days on Dox made it difficult to distinguish between AML-derived and host-derived mCherry^{+} cells (Fig 3.7A, spleen sample). Therefore, using CD45.1 mice, it was possible to clearly distinguish between AML-derived (CD45.2^{+}) and host-derived (CD45.1^{+}) cells. The BM and spleen were harvested at each of these time points and deep flow cytometry analysis performed to detect residual AML-derived cells that may be persisting in these organs. A combination of CD45.2^{+} and mCherry expression was used to assist in the identification of AML-derived cells in the BM and spleen at these later timepoints (Fig 3.8A and B). To begin with, the AML-derived cells present following 8 days on Dox include both SSC^{LOW} neutrophils and SSC^{HIGH} eosinophils. After deep flow cytometry analysis of organs harvested from mice treated with Dox for 16 days (over 1 million viable cells were recorded from each organ), >8,000 out of 3 million cells in total from the BM and spleen were AML-derived cells. The AML-derived cells were entirely GFP low, indicating that the persistent population that seeded disease relapse were cells that had switched the shPU.1 hairpin off. Therefore, the origin of relapse must come from a differentiated AML-derived cell that had once switched the shPU.1 hairpin off, as opposed to a rare population of AML-derived cells that was insensitive to Dox from the beginning. Surprisingly, further characterization revealed that the residual AML-derived cells present in both organs at remission were not only GFP^{LOW}, but also exclusively SSC^{HIGH} AML-derived cells (Figure 3.8A and B). These results were observed in multiple mice harvested at this timepoint (2 out of the 3 mice harvested at Dox day 16), and was also recapitulated in mice that were treated with Dox for 18 days (2 out of 2 mice at Dox day 18). Therefore, despite the vast majority of AML-derived cells differentiating into the SSC^{LOW} neutrophil-like population following the

endogenous restoration of PU.1, the only AML-derived cells that remain during remission are residual AML-derived SSC^{HIGH} eosinophil-like cells (Fig 3.8C) and thus, are likely responsible for seeding relapse.

3.11 Detection of residual AML-derived populations in extramedullary organs during remission

AML is a disease associated with a dysfunctional BM due to excess AML blasts, however it is also an aggressive, disseminated disease that can easily spread throughout the body. AML-derived cells were extensively analysed in the BM and spleen in the previous experiments mentioned above. To determine whether AML-derived cells were also present in organs independent of the BM and spleen, we investigated whether mCherry AML-derived cells could be found in other organs through the use of Spectrum In Vivo Imaging System (IVIS). IVIS is an imaging system used to detect the presence of fluorescent or bioluminescent labelled cells *in vivo*. mCherry allowed us to detect the presence of AML-derived cells in a range of harvested organs using IVIS imaging. As a negative control, organs of interest (spleen, stomach, brain liver, lung, uterus, heart and kidneys) were harvested from a gender and age matched mouse that was not transplanted with AML246 mCherry. As expected in an AML model, the mCherry AML appears to be widespread in an UT mouse, as was indicated by the presence of mCherry signal throughout all the organs harvested (except the heart) (Fig 3.8D). Following 16 days of Dox treatment, the leukemia was cleared from most organs harvested, however consistent with the flow cytometry data for the corresponding time point (16 days on Dox), a weak mCherry signal was also detected in the spleen (Fig 3.8D). Interestingly, in addition to the spleen, the presence of AML-derived cells could also be detected in the stomach, liver and kidneys at 16 days on Dox. The same result was also seen in organs harvested from mice following 18 days on Dox, however mCherry signal was no longer present in the stomach. Therefore, during remission, residual AML-derived cells are not only detected in the BM by flow cytometry (Fig 3.8A and B), but can also be found in extramedullary organs such as the spleen, kidney and liver via IVIS imaging. Persistence of AML-derived cells in extramedullary organs such as the kidney and liver may be particularly interesting, given that their clearance may not be subject to macrophage-mediated clearance mechanisms we observed in the spleen.

3.12 Discussion

3.12.1 Introduction

AML246 is a mouse model of differentiation therapy where it is possible to drive leukemic cell differentiation upon the restoration of endogenous PU.1, is a transcription factor functionally compromised in >60% of AML patients (Ley et al., 2013). The emergence of differentiation therapy has provided an exciting and effective alternative therapy for patients with certain subtypes of AML. ATRA-based therapies remain the gold standard for differentiation therapy in AML, however its efficacy is restricted to patients that harbour the PML-RARα oncogenic lesion. Given its targeted, non-genotoxic approach, there has been renewed interest in the development of therapeutic agents that also look to engage normal myeloid cell differentiation in different AML subtypes as mentioned in §1.4). As these therapeutic agents are developed, it is equally important to establish a strong understanding of the mechanisms of AML blast differentiation, and the potential unforeseen long-term implications these therapies may have. Using AML246, we were able to investigate a number of these questions *in vivo*, including the mechanisms of AML cell differentiation and regression, location of residual disease and ultimately, the source of relapse. This was made possible by infecting AML246 cells with a stable empty mCherry vector, thereby allowing us to track the AML cells prior to and during Dox treatment.

3.12.2 Bifurcated differentiation of AML may rely on external signals found in specialized niche

Dox induced restoration of PU.1 resulted in the differentiation of highly proliferative AML blasts into mature post mitotic myeloid-like cells. Remarkably, flow cytometry analysis was able to show that AML blasts differentiated into two distinct granulocytic sublineages (Iwasaki et al., 2005); the SSC^{LOW} neutrophils and the SSC^{HIGH} eosinophils (Fig 3.3). Flow cytometry analysis of differentiated AML cells was supported by RT-PCR analysis, confirming the expression of hallmark eosinophil and neutrophil genes in the SSC^{HIGH} and SSC^{LOW} populations respectively. Although the results from the RT-PCR analysis were able to provide important evidence of multilineage differentiation, in experiments beyond the scope of this PhD, we intend to conduct a more comprehensive transcriptional analysis of the AML-derived neutrophils and eosinophils via RNAseq to determine the stage of maturation each sublineage is able to reach. Alternatively, functional assays could also be used to determine the maturation

of the SSC^{HIGH} and SSC^{LOW} AML-derived lineages. CRISPR-mediated KO of key late stage eosinophil genes such as Cebpe (Bedi et al., 2009) or Notch (Kang et al., 2005) would provide strong indication of whether the AML-derived eosinophil-like cells are fully mature myeloid cells, or whether they are at an intermediate stage of maturation. More details to these experiments will be outlined in Chapter 4.

ATRA-based treatment of APL patients (harbouring the PML-RARα genetic lesion) is the most common example of differentiation therapy in AML. It is well documented that APL blasts mature into neutrophils in the presence of ATRA, however monocytic differentiation of AML blast upon ATRA treatment has also been documented (Naeem et al., 2006; Riccioni et al., 2003). APL mouse models (Liu et al., 2017), cell lines such as NB4 or freshly isolated APL cells cultured in the presence of ATRA and M-CSF are also able to undergo monocytic differentiation (Naeem et al., 2006; Riccioni et al., 2003). HL60, a human APL cell line can also undergo eosinophilic differentiation when cultured in ATRA and IL-5 (Ishihara et al., 2005; Thivierge et al., 2000; Tiffany et al., 1998). More recently, the use of $IDH2^{mut}$ inhibitors such as Enasidenib clinically has also been shown to induce both erythroid and neutrophilic differentiation of AML blasts (Amatangelo et al., 2017). Complete remission is achieved using Enasidenib in over 40% of relapse/refractory (rrAML) patients (Amatangelo et al., 2017). Interestingly, functional *mIDH2* positive neutrophils and erythroid cells following treatment (Quek et al., 2018), however the clearance of these mature *mIDH2* cells is currently not well understood. Therefore, these studies are able to independently show the multipotential nature of leukemic blasts differentiation in different subtypes of AML. Furthermore, these examples also suggest that the multilineage differentiation of AML blasts is dependent on external growth factors and cytokines. Importantly, in our AML246 mCherry mouse model, PU.1 expression was comparable between both differentiated SSC^{LOW} and SSC^{HIGH} populations via RT-PCR, suggesting that the commitment into different lineages not only requires the regulation of intrinsic factors (such as the expression/repression of certain transcription factors), but may also require extrinsic niche factors that promote differentiation of exposed AML blasts into their respective lineages. For example, the differentiation of myeloid progenitors to mature neutrophils requires the presence of cytokines such as G-CSF and GM-CSF (Demetri et al., 1991; Gasson, 1991), such that G-CSF null mice are chronically neutropenic (Lieschke et al., 1994). Conversely, eosinophils require the presence of IL-5 for their development, IL-5 null mice suffer from severe depletion in eosinophil numbers (Robertson et al., 2000). These cytokines and may play an essential role for the development

of neutrophils and eosinophils, however commitment to these lineages are likely to be dynamic and multifactorial. From previous studies in the Dickins lab, we found that culturing AML246 cells in the presence of M-CSF was able to drive monocytic differentiation of AML blasts *in vitro*, however we were unable to promote eosinophilic differentiation in the presence of IL-5 (data not shown). Using AML246 mCherry, we were unable to recapitulated the multilineage differentiation we see in our mouse model, with AML cells differentiating exclusively into the neutrophil lineage in culture *in vitro* (data not shown). This was likely due to the inability to replicate the specific milieu of cytokines and growth factors required to facilitate bifurcation of AML-derived cells *in vivo*, reaffirming the importance of a highly specialised BM or splenic niche to facilitate eosinophil differentiation of AML blasts. Although we were unable to show multilineage differentiation *in vitro*, these results suggest that AML246 mCherry blasts may preferentially differentiate into neutrophils by default, but sublineages can emerge given the right circumstances. Given the likely dependency on cytokines and growth factors for multilineage differentiation, it would be interesting to transplant AML246 mCherry into cytokine deficient mice (such as G-CSF or IL-5 null) mice to test if the leukemic cells are able to undergo neutrophilic or eosinophilic differentiation in the absence of these key factors *in vivo*.

Accessibility to the cytokine milieu may also impact the ratio of SSC^{HIGH} eosinophil-like and SSC^{LOW} neutrophil-like differentiation *in vivo*. We found that an increased leukemic burden resulted in a change in the ratio of AML blast differentiation along the eosinophil/neutrophilic lineages. High disease burden AML246 mCherry mice treated with Dox had a lower percentage of AML-derived SCC^{HIGH} eosinophils compared to mice treated with Dox at a lower disease burden. From these experiments, we concluded that the differences in percentage of AML-derived eosinophils was likely to be attributed to the delayed clearance of AML-derived neutrophils following differentiation in a high disease burden mouse. However, from these results we cannot rule out the possibility that a high disease burden may also disrupt the BM/splenic niche, such that accessibility to cytokines that promote eosinophil differentiation may be limited. Although the immunophenotype of SSC^{HIGH} eosinophils is similar between high and low disease burden mice (data not shown), the expression of surface markers alone may not be the best indication of disrupted myeloid cell differentiation. Therefore, RNAseq may be useful in determining the stage of maturation the AML-derived eosinophils reach in both contexts. Furthermore, comparison of our RNAseq data to public databases such as Haemosphere (Choi et al., 2018), where gene expression analysis has been broken down into

specific maturational stages of eosinophil development, can also provide a strong indication of what stage of maturation the AML-derived eosinophils ultimately reach. The implications of this will be further discussed in Chapter 4.

3.12.3 Implications for patient outcomes following AML cell multilineage differentiation

As mentioned above, multilineage differentiation of AML blasts has been clinically in some cases of APL following ATRA-based treatment. However, in this Chapter we were able to show for the first time the implications of bifurcated AML blast differentiation. The restoration of endogenous PU.1 drives leukemic cell differentiation predominately into the neutrophil lineage, with a minor subpopulation of cells differentiating into eosinophils. Notably, the differences in lifespan of the AML-derived granulocytes is consistent with the disparity in lifespan of normal neutrophils and eosinophils. Neutrophils are the most abundant myeloid population found in the human body, with over 10^{11} released in circulation on a daily basis (Dancey et al., 1976). However, they also have a very short lifespan of less than <24 hours (Tak et al., 2013). Conversely, although eosinophils are a very rare population of myeloid cells, they have a considerably longer lifespan than the neutrophil population, with evidence of tissue resident eosinophils living for several weeks following maturation (Y. M. Park et al., 2010). Therefore, similar to normal myeloid cell biology, the AML-derived neutrophils have a seemingly short lifespan and are cleared rapidly following differentiation, whereas AML-derived eosinophils were found in tissues such as the liver (data not shown for day 16-18, but liver data for day 21 is available in Fig 4.1) and spleen (as well as BM) following 16-18 days on Dox. Consequently, given that the eosinophils are able to survive longer than the neutrophils, following 16 days on Dox, they are the only AML-derived population present to acquire a genetic lesion that triggers de-differentiation and relapse.

Notably the relapse that emerged from long term Dox treatment of AML246 was $mCherry^{+}GFP^{+}$, suggesting that a mutation had rendered the Tet-regulated shPU.1 hairpin insensitive to Dox. Initially, we believed that a mutation to the tTA resulted into its conversion to an rtTA, causing the TRE promoter to become activated in the presence of Dox. This was our hypothesis given that tTA and rtTA only differ by 3 amino acids (Manfred Gossen et al., 1995). However, when relapse cells were cultured in the absence of Dox *in vitro* (which would normally inactivate the TRE-promoter in a Tet-ON regulated system), the cells remained GFP^{+}, suggesting that the TRE-promoter in the relapse cells continued to transcribe GFP as well as the shPU.1 hairpin (Fig 3.2E). Furthermore, sequencing of the tTA revealed that the Tet

transactivator was not mutated (data not shown), confirming that the system had not switched to a Tet-ON system. For future experiments we hope to perform 5' race experiments to identify the genetic mutation that ultimately allows for shPU.1 hairpin expression in the presence of Dox, leading to disease relapse.

In the clinical setting, the prevalence of multilineage AML blast differentiation following differentiation therapy is currently unknown. Differentiation of AML blasts is often considered neutrophilic, likely due to excessively high neutrophil counts in patients that suffer from differentiation syndrome, following differentiation therapy (Denu et al., 2016; Montesinos et al., 2009; Sanz et al., 2014). However, it is also very likely that AML blasts mature into a range of myeloid lineages following the release of the differentiation block, however the AML-derived myeloid cells may be morphologically indistinguishable from normal myeloid cells. Therefore, to understand whether this is truly the case clinically may prove to be very important, given that the source of relapse may be hiding in plain sight. In future experiments beyond the scope of this PhD, we hope to collect blood samples from AML patients treated with differentiation therapy agents (such as ATRA or Enasidenib) to determine the prevalence of multilineage differentiation clinically. For these experiments, we would rely on well characterized surface markers to separate out specific mature myeloid lineages from the patient samples using FACs-based sorting. Following this, we could then determine the percentage variant allele frequencies (VAF) of the mutation of interest in each myeloid cell lineage. From these experiments, we would therefore develop a stronger understanding of multilineage differentiation in the clinical setting, and also begin uncover the sup-population of AML-derived cells ultimately responsible for seeding disease relapse.

Regardless of whether eosinophilic differentiation of AML blasts exists clinically, we believe that the principles of our findings remain highly relevant. Ultimately, the lineage in which AML blasts differentiation into can greatly impact the likelihood of disease relapse, due to inherent differences in lifespan and clearance of independent mature myeloid lineages. As mentioned previously, monocytic differentiation of leukemic blasts has been shown in APL patients treated with ATRA based therapies. This could be particularly interesting, as monocytes and macrophages have a considerably longer lifespan than all other myeloid cell types, with some tissue resident macrophages estimated to be life-long (Shaw et al., 2018). Consistent with this, late APL relapse does exist (Sakurai et al., 2018) and they are often extramedullary (de Botton et al., 2006; Harris et al., 2013; Watts et al., 2016). Given this, it

would be interesting to identify whether or not relapse following differentiation therapy is associated with monocytic differentiation of AML blasts during ATRA treatment, or if monocytic differentiation is associated with late relapse.

3.12.4 Uncovering clearance mechanisms of AML cells following differentiation

In our AML model, we have shown the differentiation of AML blasts into particular myeloid lineages is likely to impact disease relapse. Highly related to this issue is also the mechanisms of clearance following AML cell differentiation, given that effective clearance of differentiated AML cells is what drives mice into remission, and thereby impacting the likelihood of relapse. In our experiments, we serendipitously saw the emergence of a distinctive mCherry 'smear' population specifically in the spleen following a week on Dox. The emergence of this population coincided with the differentiation of AML blasts into their respective myeloid lineages. Upon further characterization, it was established that the mCherry smear population were host derived macrophages that had engulfed AML-derived mCherry cells following their differentiation. Given that differentiation therapy looks to re-engage the normal process of myeloid cell maturation, we would predict the clearance of differentiated AML cells would involve the same mechanisms for clearing normal aging myeloid cells during homeostasis. The BM is considered to be a major site of aging neutrophil clearance (Furze et al., 2008; Gordy et al., 2011), however organs such as the liver and spleen also play an important role in the removal of both aging and dying cells (Furze et al., 2008). Removal of the spleen prior to leukemia transplant and differentiation resulted in accelerated relapse, however the difference was not statistically significant ($p=0.05$). Notably, these experiments were performed on a small cohort of mice ($n=4$ for each group), hence the experiment may lack statistical power. However given the promising results from the initial pilot study, it may be worthwhile repeating the experiment with larger cohorts to truly determine whether the removal of the spleen results in accelerated relapse due to impaired clearance of differentiated AML cells mediated by splenic macrophages.

Regardless of the limitations of our original splenectomy studies, we observed that despite the absence of a spleen, there was still effective clearance of AML-derived cells at least in the PB following 15 days on Dox, suggesting that other organs (such as the liver) may also facilitate the clearance of differentiated AML cells. Therefore, it is likely that multiple organs are responsible for the clearance of aging myeloid cells, with the spleen being a major contributor to this process. Consistent with this is the presence of a minor 'smear' population in the BM

during these periods of mass AML cell clearance. On a cellular level, splenic macrophages and macrophage in the liver (Kupffer cells) have been implicated in the clearance of aging neutrophils through a process known as programmed cell removal (Bugl et al., 2013; Davies et al., 2018; Gordy et al., 2011). Briefly, it is a macrophage mediated process responsible for facilitating an immunologically silent clearance of unwanted (aging, dying, dysfunctional) cells. It is a process that requires dynamic interactions between the macrophage and the target cell of interest. These signals can be broadly categorized into two types: the 'don't eat me' signal (such as CD47) (Matozaki et al., 2009) expressed on healthy cells as well as the 'eat me' signals (such as calreticulin and asialoglycans etc) (Feng et al., 2018) that are displayed on the aging or dying target cell. A combined loss of a 'don't eat me signal' and the expression of an 'eat me' signal by the target cell is required for the macrophage to recognise and engulf (Barrera et al., 2017; Jaiswal et al., 2009). Interestingly, the expression of CD47 has been implicated in AML, with increased CD47 expression on leukemic cells being associated with poor patient outcome (Majeti et al., 2009). With the exception of the examples mentioned above, the current understanding on normal or leukemic cell clearance remains largely unknown.

In future experiments, AML246 mCherry provides an ideal model to delineate novel mechanisms involved in this process, given that we can not only track the differentiation of a leukemic blast into a differentiated myeloid cell, but also track the emergence of the host macrophage population responsible for clearing the differentiated cells. Using the techniques described in Chapter 4, we intend to delete CD47 from our AML246 mCherry cells to determine whether the loss of this signal disrupts the normal kinetics of AML cell clearance following differentiation. However prior to these experiments, it may be firstly important to certify that clearance of differentiated AML-derived cells is indeed macrophage-dependent in our AML model. To address this, macrophage depletion assays could be performed to determine if the absence of macrophages negatively impacts the clearance of differentiated AML cells. Intraperitoneal injections of Clodronate-containing liposomes is a method commonly used to deplete macrophages in mice (Weisser et al., 2012). Therefore in future experiments, it would be interesting to deplete macrophages pharmaceutically using Clodronate and determine whether macrophage depletion prior to leukemia transplant impairs leukemia cell clearance following differentiation, thereby resulting in accelerated relapse due to improper clearance of AML-derived cells. Ultimately, the experiments described above

could provide invaluable insight towards the general mechanisms of AML cell clearance in response to differentiation therapy.

3.12.5 Measurable residual disease persists in extramedullary organs during remission

Deep flow cytometry analysis of the BM and spleen revealed a rare, residual population of AML-derived eosinophils in both organs at 16 days on Dox. Clinically, measurable residual disease (MRD) involves aspirates from the BM and refers to AML blast counts of $1:10^4$ to $1:10^6$ white blood cells, compared to the 1:20 that is considered an established AML (Schuurhuis et al., 2018). During periods of remission, we consistently found approximately 0.05-0.1% of total BM or spleen to be exclusively AML-derived eosinophils (no AML blasts were present during remission). Although the AML-derived cell to white blood cell ratio never reaches as low as $1:10^4$ to $1:10^6$ in our AML model, we are still able to detect a considerably rare population of AML-derived cells during a time period where disease is completely absent from the PB. Using IVIS imaging, we found that residual AML-derived cells were present not only in the BM and spleen, but also the kidney and liver during disease remission. These findings therefore open up the possibility of an extramedullary source of relapse in our AML model. In AML, relapse tends to originate from the BM, however there is evidence of relapse, particularly late stage relapse, originating from extramedullary organs (de Botton et al., 2006). Interestingly, late stage, extramedullary relapse is seen in APL patients treated with differentiation therapy agent ATRA (de Botton et al., 2006; Sakurai et al., 2018; Watts et al., 2016). Therefore, consistent with these clinical observations, in our AML246 mCherry model we have shown that differentiated AML-derived eosinophils are able to survive long term in extramedullary organs such as the spleen, hence there is every chance that relapse following differentiation therapy could also be extramedullary.

It is important to note that the findings described in this chapter were made in exclusively one mouse model of AML differentiation therapy (AML246). Bifurcation was also observed in different clones of AML246 (Fig 3.4), however alternative AML models are required to determine whether or not bifurcated AML cell differentiation and sublineage persistence is a common occurrence in AML differentiation therapy or if these results are unique to our mouse model. For example, xenograft models driven by APL cell lines such as NB4 or HT93 (both harbour the PML-RARα mutation) could provide a more clinically relevant model to

investigate the prevalence of multilineage differentiation following differentiation therapy using ATRA. In addition to xenograft models, it would also be interesting to look at blood samples of patients that have undergone differentiation based therapies such as ATRA+ATO or IDH1/2^{mut} inhibitors. Using digital droplet PCR (ddPCR) or Fluorescence in situ hybridization (FISH) methods, it may be possible to track the differentiation of leukemic cells into different myeloid lineages following differentiation therapy. Therefore using these models, it may be possible to determine the true clinical relevance of the findings we have made in our AML246 mCherry mouse model of differentiation therapy.

3.13 Conclusion

In conclusion, the restoration of endogenous PU.1 in AML246 mCherry drives bifurcated differentiation into two sublineages of granulocytes: the SSC^{LOW} neutrophils and the SSC^{HIGH} eosinophils. Intriguingly, AML-derived neutrophils are cleared following differentiation, however a minor subpopulation of AML-derived eosinophils persist and ultimately drive relapse. Therefore, for the first time, we have shown in our mouse model that differentiation of AML blasts into longer living myeloid lineages has serious implications for AML relapse, and by extension, potential implications in the clinic. Through rigorous interrogation of mice in remission, we show that residual AML-derived eosinophils persist not only in the BM, but in extramedullary organs such as the spleen, liver and kidneys. Finally, in addition to the characterization of AML blast differentiation, we were also able to identify a potential organ specific clearance mechanism responsible for the removal of differentiated AML cells.

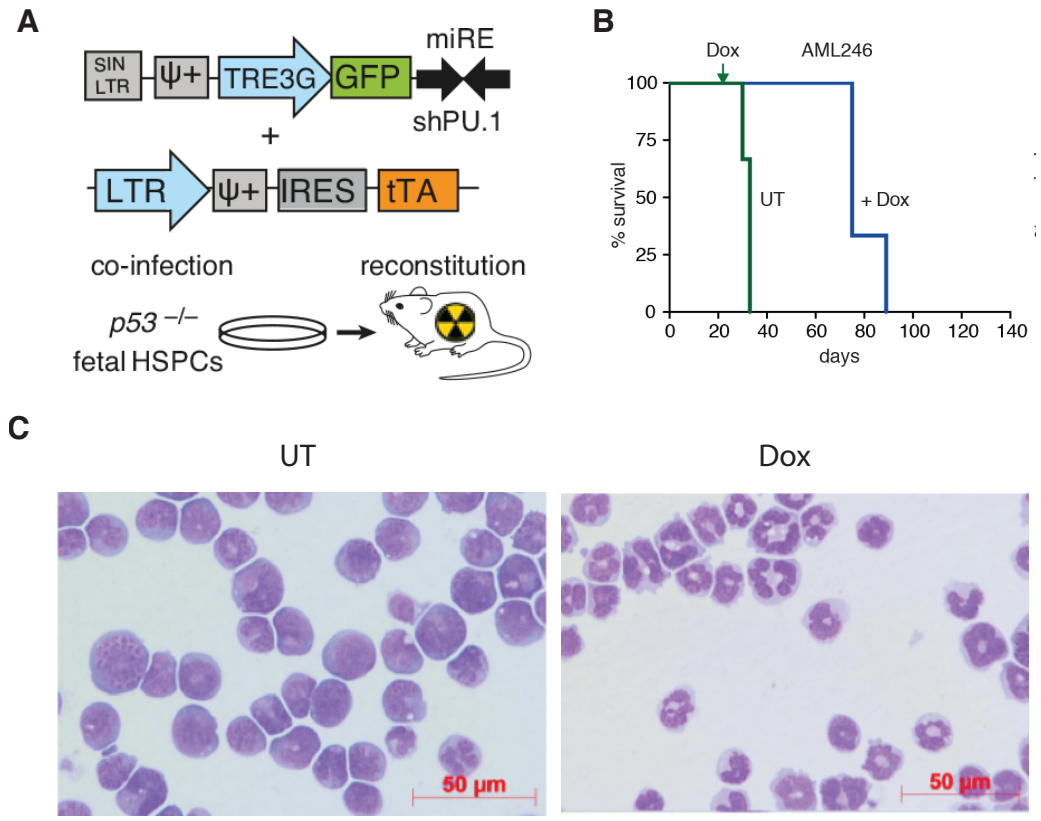


Figure 3.1 AML246 is an inducible mouse model of AML driven by suppression of PU.1

(A) Schematic of dual vectors infected into p53-/- fetal HSPCs to generate AML 246. (B) Kaplan-Meier survival analysis of mice transplanted with AML246 treated with Dox following disease establishment compared to those left untreated (UT). (C) Cytospins of AML246 cells harvested from UT mouse and mouse treated with Dox for 10 days. Data displayed in Fig 3.1 was taken from McKenzie et al., 2019, in press.

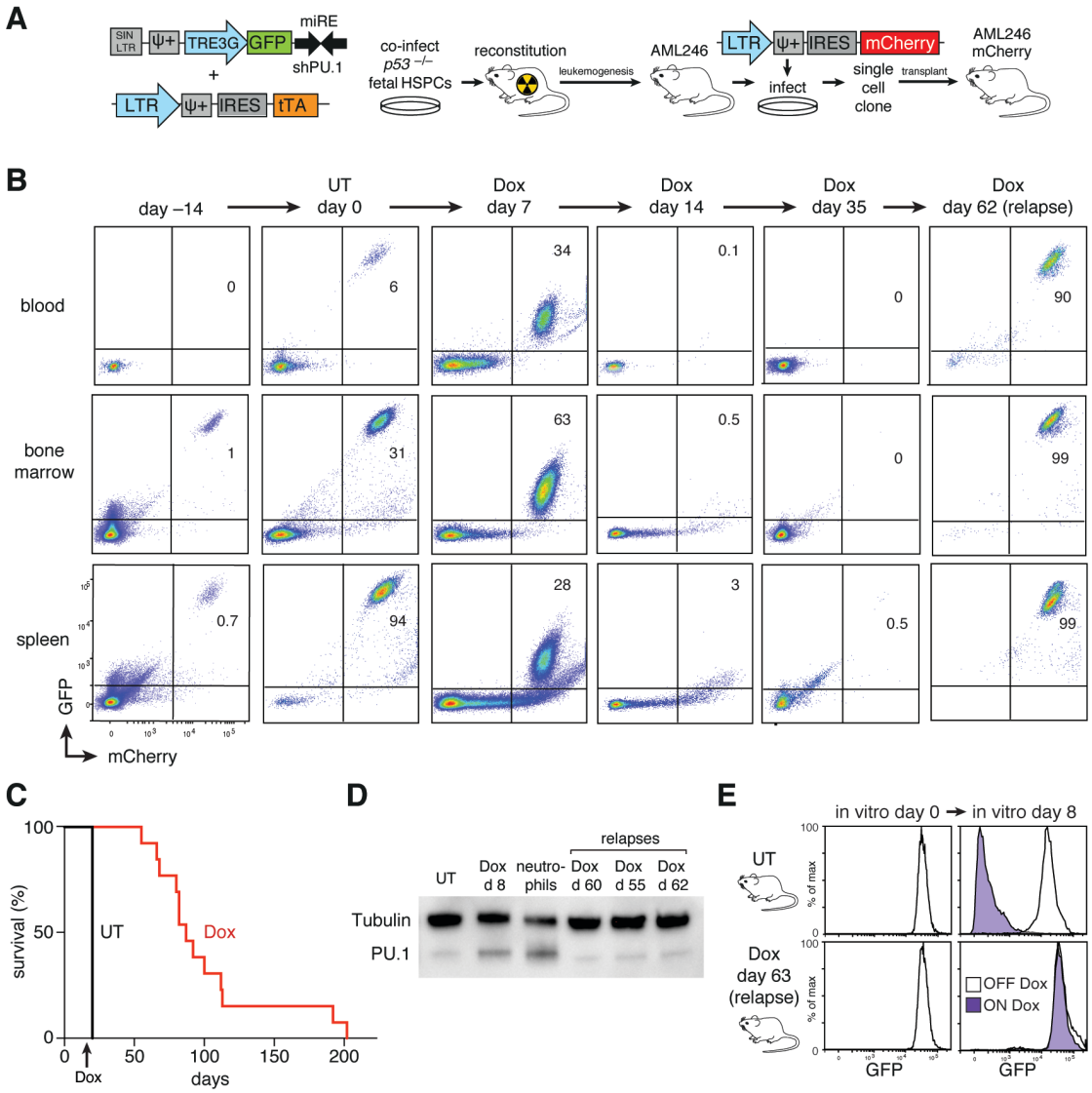


Figure 3.2 An mCherry positive p53-deficient AML model driven by reversible PU.1 knockdown.

(A) Schematic of the generation of AML246 mCherry mouse model. (B) Flow cytometry analysis tracking disease burden (mCherry positive cells) prior to and following Dox treatment in vivo. Mice transplanted with AML246 mCherry developed AML after 4 weeks and were put on Dox once disease burden reached 5-10% in the PB. n = 3 at each time point. (C) Kaplan-Meier survival analysis of mice transplanted with AML246 mCherry cells subsequently treated with Dox. n = 13 for mice maintained on Dox until relapse. (D) Western blot analysis of PU.1 protein expression in UT, Dox day 8 treated, and relapse samples compared to wildtype neutrophils. Wildtype neutrophils were sorted based on Ly6G and CD11b expression. (E) GFP flow cytometry analysis of relapse cells in vitro. mCherry, GFP positive cells present in the

bone marrow at relapse were FACs sorted and cultured in vitro either in the presence or absence of Dox for 8 days. AML246 mCherry cells from the bone marrow of an untreated mouse was also harvested and cultured in the presence or absence of Dox as a control.

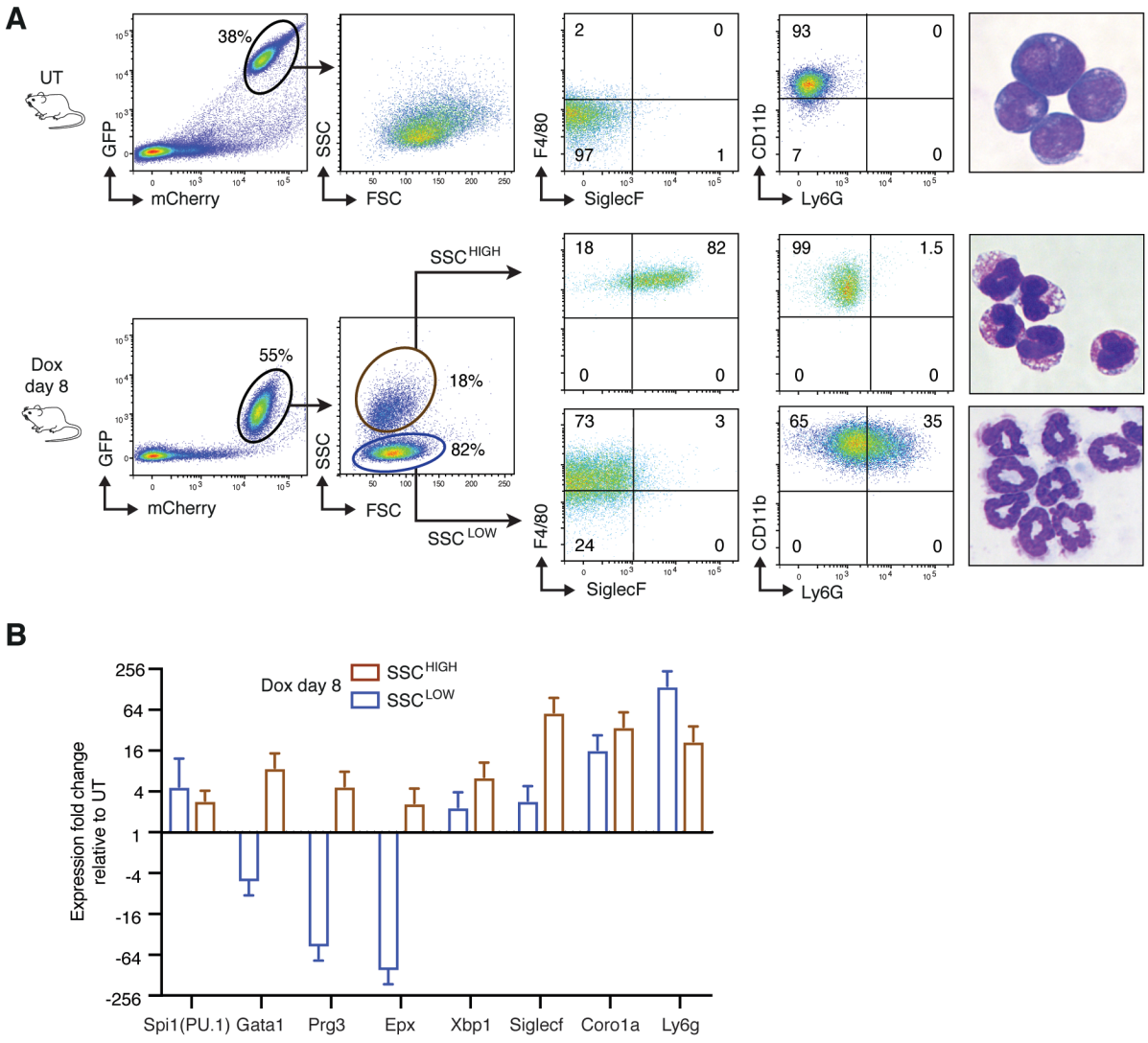


Figure 3.3 Bifurcation of AML derived cells following 8 days on Dox.

(A) Flow cytometry analysis of Rag 1 -/- mice transplanted with AML246 mCherry cells left untreated or treated with Dox for 8 days upon disease establishment. The immunophenotype of AML derived cells was determined by initially gating on mCherry positive cells, followed by analysis of FSC/SSC profile and expression of key myeloid markers (F4/80, Siglec-F, CD11b,Ly6G, Ccr3 and CD125). Cytospins of AML derived cells present in the bone marrow and spleen were sorted on the basis of mCherry expression. (C) Reverse transcriptase PCR performed on AML246 mCherry cells sorted from spleen following 5 days on Dox. The two AML derived populations were sorted on the basis of SSC, Siglec-F and Ly6G expression.

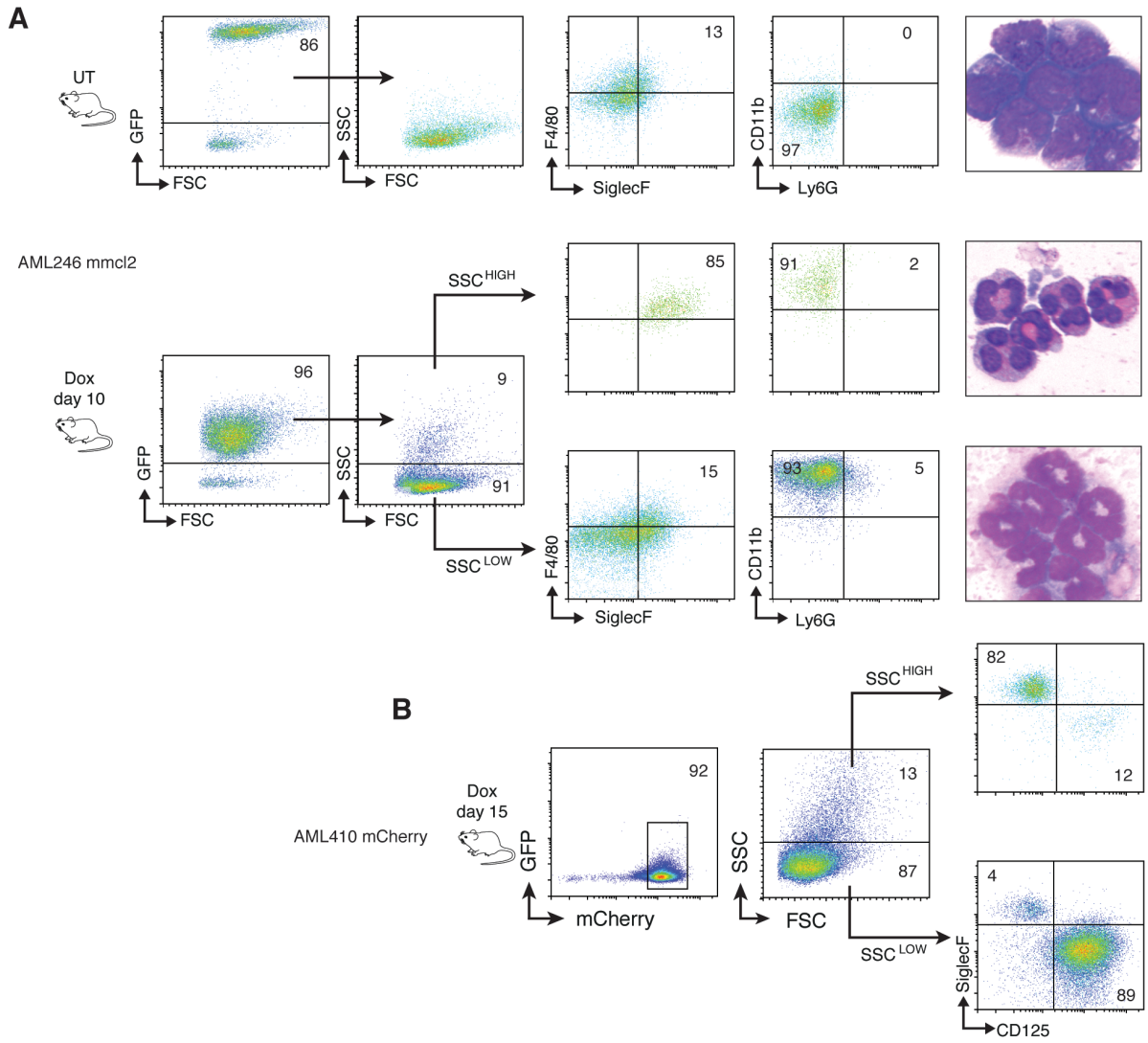


Figure 3.4 Bifurcated differentiation of AML blasts in independent reversible PU.1 AML models.

(A) Flow cytometry analysis of BM harvested from mice transplanted with AML246 mmcl2 following 10 days on Dox, or left untreated. BM samples were stained with lineage specific myeloid markers CD11b, F4/80, Ly6G and SiglecF to determine the immunophenotype of the AML derived cells. AML derived SSCHIGH and SSCLOW cells were FACs sorted and histologically stained via MGG (far right panel). (B) Immunophenotypical analysis of AML derived cells harvested from the BM of a representative Ly5.1 Rag mouse transplanted with AML410 mCherry using flow cytometry. BM samples were stained with Siglec-F and CD125.

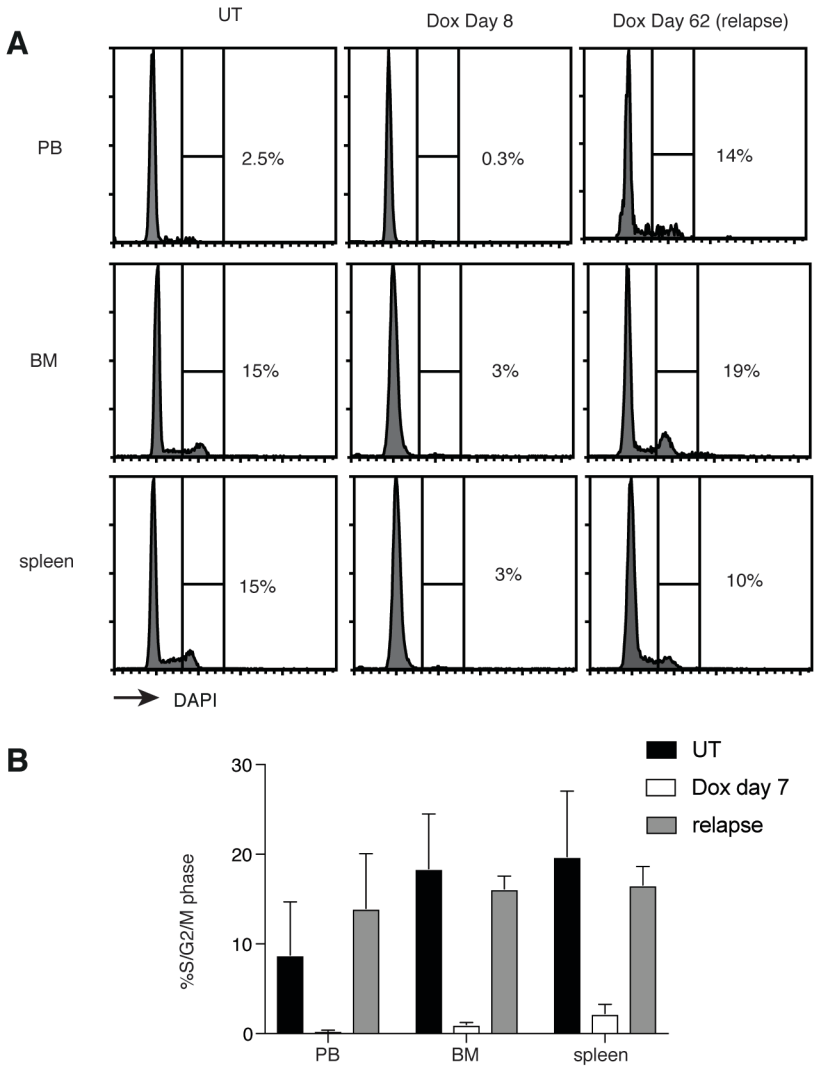
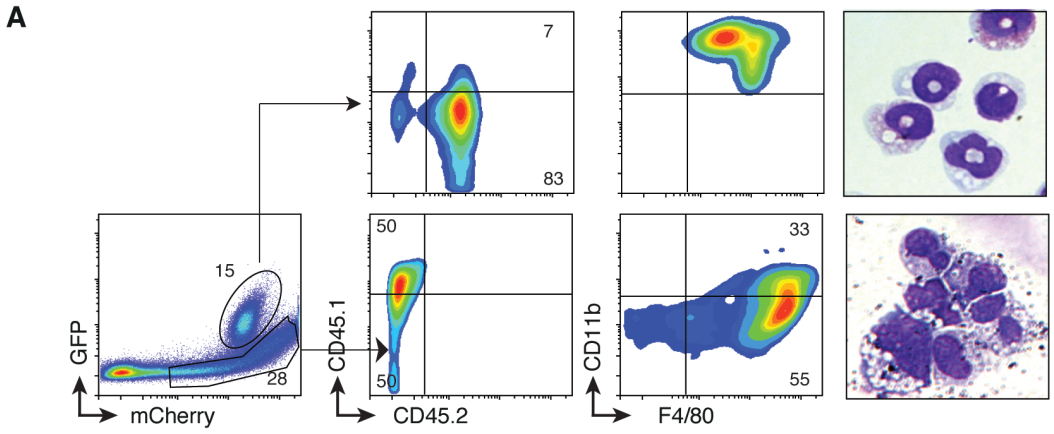
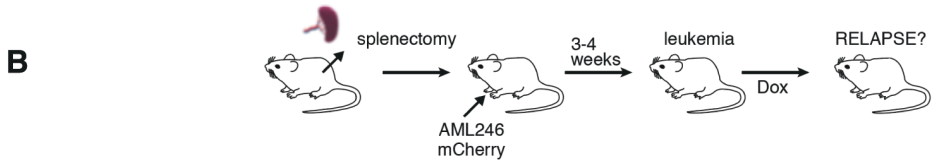
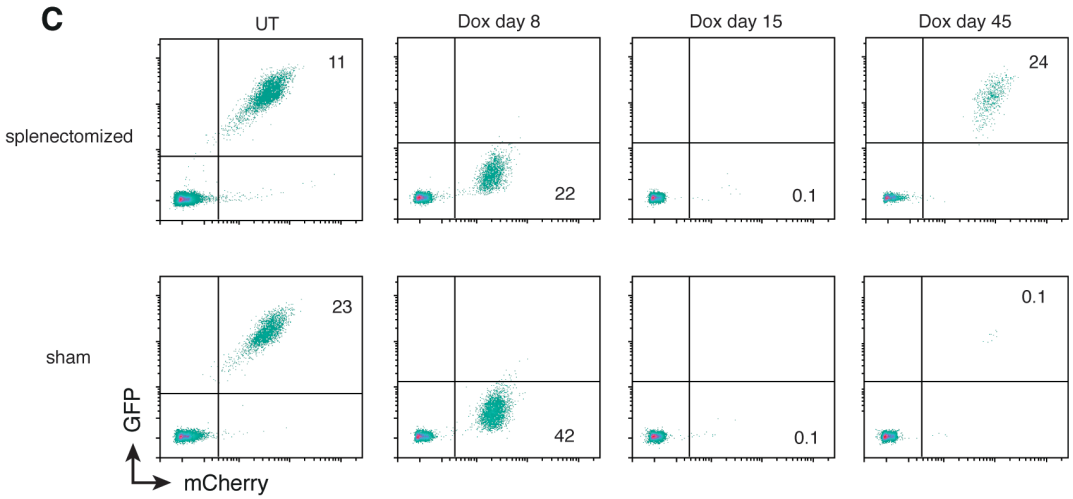


Figure 3.5 Differentiation of AML blasts is coupled with loss of proliferative capacity.

A) Representative DAPI cell cycle analysis of AML246 mCherry cells from the PB, BM and spleen in an untreated, Dox day 7 treated and relapse mouse. Percentage of mCherry cells that are in S/G2/M phase at each time point is shown. (B) Statistical analysis of percentage of AML derived mCherry cells in S/G2/M phase in each sample at given time points.


A
7
83
15
28
GFP
mCherry
50
50
CD45.1
CD45.2
33
55
CD11b
F4/80


B
splenectomy
AML246
mCherry
3-4
weeks
leukemia
Dox
RELAPSE?


C
UT
Dox day 8
Dox day 15
Dox day 45
splenectomized
sham
11
22
0.1
24
23
42
0.1
0.1
GFP
mCherry

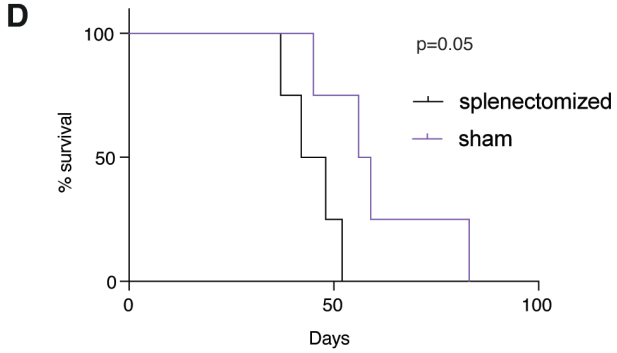

D
100
50
0
% survival
0
50
100
Days
p=0.05
splenectomized
sham

Figure 3.6 Macrophage in the spleen play in important role in the clearance of differentiated AML cells

A). Flow cytometry analysis of AML derived cells compared to mCherry 'smear' population that emerges specifically in the spleen following 8 days on Dox.CD45.1 and CD45.2 staining was used to determine the origin of different mCherry positive populations. (B) Experimental outline of detailing the splenectomies during remission. (C) Representative longitudinal flow cytometry analysis tracking AML246 mCherry cells of splenectomised and sham control mouse following Dox treatment. (D) Kaplan-Meier survival analysis of mice splenectomised (n=4) or sham control mice (n=4) that relapse with mCherry positive AML following long term Dox treatment. Mantel Cox analysis was performed to determine the statistical significance between both groups of mice.

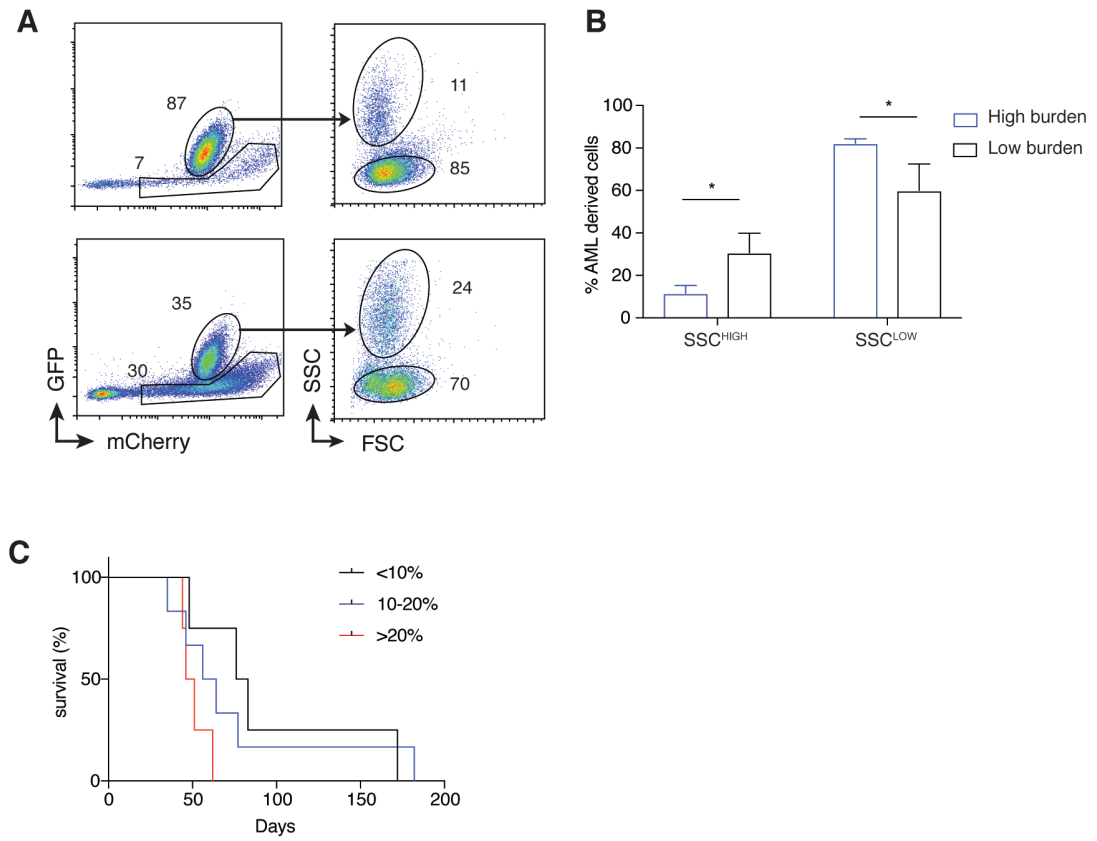


Figure 3.7 Leukemic burden impacts the ratio of SSC^{HIGH} and SSC^{LOW} cells present following 8 days on Dox.

A) Flow cytometry analysis of mCherry, GFP profile as well as emergence of mCherry 'smear' population in the spleen from AML246 mCherry Ly5.1 Rag mice with high disease burden (top row) compared to mice treated with Dox at low disease burden (bottom row) treated with Dox for 8 days. 'High' and 'low' disease burden was determined based on percentage of mCherry cells in the PB at Day 0 Dox treatment. (B) Statistical analysis of percentage of SSC^{HIGH} and SSC^{LOW} AML derived cells present in mice with high disease burden (blue) compared to low disease burden mice (black). Statistical significance was determined by unpaired t-test analysis. Histogram plot shows mean ± SEM, n=3 primary AML spleen samples harvested at 8 days Dox in each group $p<0.05$. (C) Kaplan-Meier survival analysis of mice transplanted with AML246 mCherry cells subsequently treated with Dox, separated by PB disease burden (<10% n=4, 10-20% n=6 , >20% n=4, n total= 14). Mantel Cox was performed to determine the statistical significance between each group of mice <10% vs 10-20% p=0.72, 10-20% vs >20% p =0.18, <10% vs >20% p= 0.051.

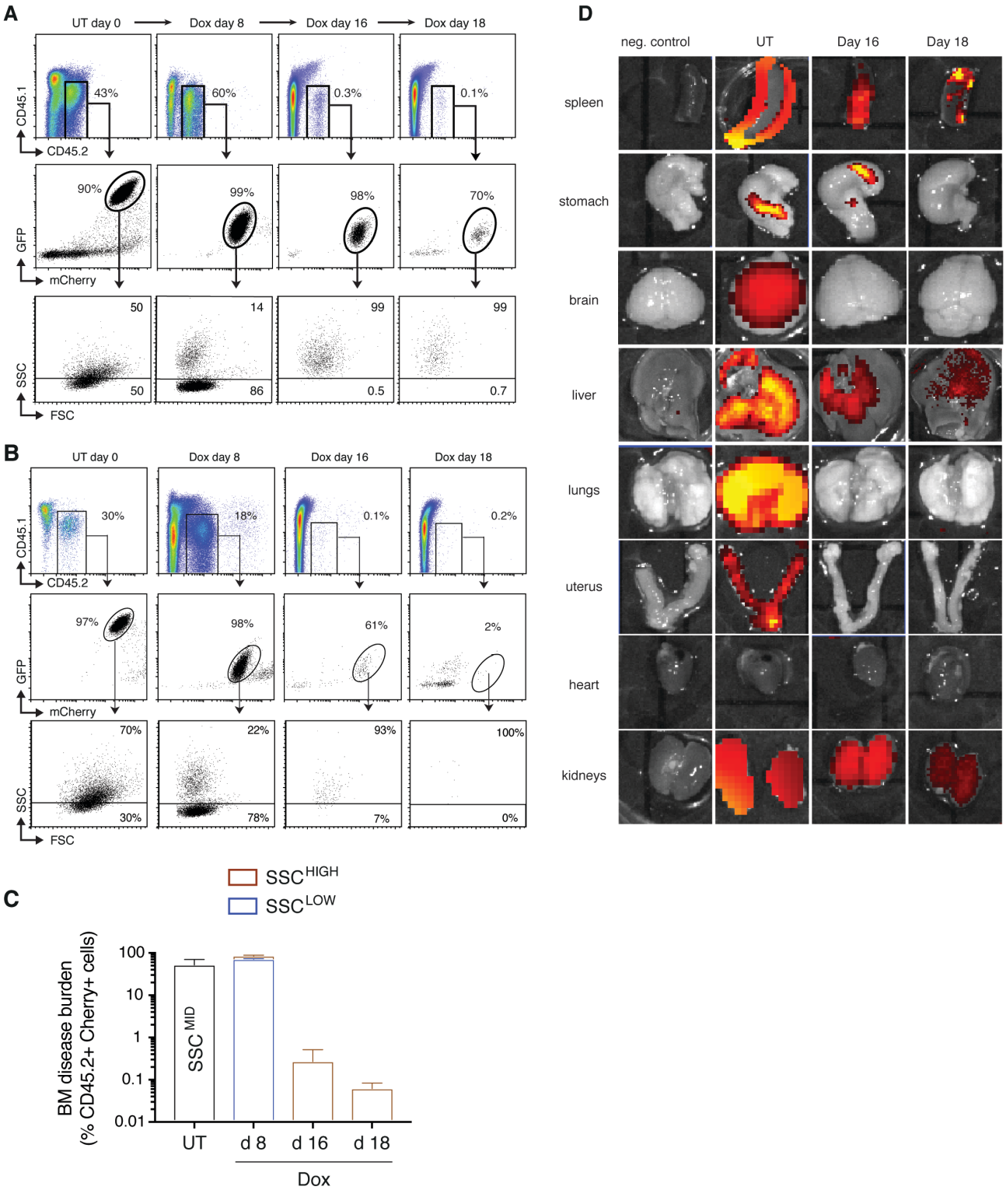

A
UT day 0
Dox day 8
Dox day 16
Dox day 18
CD45.1
CD45.2
43%
60%
0.3%
0.1%
GFP
mCherry
90%
99%
98%
70%
SSC
FSC
50
50
14
86
99
0.5
99
0.7
B
UT day 0
Dox day 8
Dox day 16
Dox day 18
CD45.1
CD45.2
30%
18%
0.1%
0.2%
GFP
mCherry
97%
98%
61%
2%
SSC
FSC
70%
30%
22%
78%
93%
7%
100%
0%
C
SSC HIGH
SSC LOW
BM disease burden
(% CD45.2+ Cherry+ cells)
100
10
1
0.1
0.01
SSC MID
UT
d 8
d 16
d 18
Dox
D
neg. control
UT
Day 16
Day 18
spleen
stomach
brain
liver
lungs
uterus
heart
kidneys

Figure 3.8 Persistence of AML-derived SSC^{HIGH} eosinophil-like cells during disease remission.

(A, B) Flow cytometry analysis of FSC/SSC profile of AML-derived cells harvested+/ from the bone marrow (A) and spleen (B) of representative AML246-Cherry leukemic mice during an 18 day Dox treatment time course, showing viable AML-derived cells identified based on surface CD45.2 and mCherry (UT n=3, day 8 n=3, day 16 n=3, day 18 n=2). (C) Abundance and proportion of SSC^{HIGH} and SSC^{LOW} AML-derived cells during the 18 day Dox treatment time course (mean +/– standard error, n=3 mice at Dox day 8 and day 16, n=2 mice at day 18). (D) mCherry imaging by IVIS of organs from AML246-Cherry leukemic mice, either untreated (representative mouse) or following 16-18 days Dox treatment (same mice from Figure 3A and 3B). Negative control organs are from a non-transplanted *Rag1*$^{-/-}$ mouse.

Chapter 4: Preventing AML relapse in AML246 mCherry

4.1 Introduction

The focus of the previous chapter was to characterize in detail the mechanisms of AML differentiation and clearance *in vivo* using our inducible PU.1 AML model, AML246. In doing so, we not only saw a fully penetrant relapse in our AML model, but also identified a potential cause of relapse following differentiation therapy. From previous studies in our lab, we were able to show that differentiated AML cells had the capacity to de-differentiate and revert to a blast-like state following the re-engagement of PU.1 suppression (McKenzie et al, 2019, in press). Consistent with this, in our AML246 mCherry *in vivo* model, we found a rare subpopulation of mature, AML-derived eosinophils that persisted during remission and likely responsible for seeding disease relapse. Although relapse rates in APL patients treated with combinational ATRA and ATO therapy is rare, de-differentiation of mature AML-derived cells (maintaining the PML-RARα mutation) could explain the cause of relapse for these rare cases. Furthermore, given the recent emergence of new leukemic cell differentiating therapeutic agents such as HDAC inhibitors and mIDH2 inhibitors, it is important to identify potential causes of relapse in a global mouse model of differentiation therapy. Hence using AML246 mCherry, this chapter describes our attempts to prevent disease relapse in our inducible AML model.

4.2 Identifying the origin of AML246 relapse

Using both deep flow cytometry analysis and IVIS imaging in Chapter 3, we found rare a subpopulation of AML-derived eosinophils in the BM, spleen and potentially liver and kidneys during remission. To determine the origin of relapse, the transplanted mice treated with Dox at later timepoints were harvested in order to detect where GFP positive AML-derived cells first emerge. At 21 days on Dox, AML-derived cells detected in the BM and liver remained $GFP^{LOW}SSC^{HIGH}$ eosinophils. Surprisingly, the AML-derived cells detected in the spleen of the same mice were uniformly GFP^{HIGH} (Fig 4.1A). Furthermore, the AML-derived cells in the spleen were no longer SSC^{HIGH} and had also lost the expression of the mature eosinophil lineage markers F4/80 and CD11b (Fig 4.1A). Hence, AML-derived cells had re-engaged the shPU.1 hairpin, apparently resulting in de-differentiation into an AML blast population. Interestingly, re-engagement of AML cell PU.1 suppression (switching shPU.1 hairpin and

GFP back on) initially in the spleen was observed in 2 out of 8 mice following 21 days of Dox treatment (Fig 4.1B). The remaining 6 out of 8 mice showed $mCherry^{+}GFP^{+}$ present in both the BM and spleen after 21 days on Dox, but importantly no Dox treated mice harvest at this timepoint showed the presence of $mCherry^{+}GFP^{+}$ relapse cells exclusively in the BM (Fig 4.1B). To determine whether or not the source of relapse could originate from another organ, IVIS imaging was also performed in order to detect AML-derived cells located in organs independent of the BM and spleen at 21 days on Dox. IVIS imaging is a highly sensitive technology often used in the detection of bio-labelled, measurable remission disease or rare cells in a range of different cancer models (Fujiki et al., 2008; Sasportas et al., 2014; Terziyska et al., 2013). From our previous experiments involving IVIS imaging of organs of mice in remission (Fig 3.8D), we found that mCherry signal was detected by IVIS in the spleen following 18 days on Dox. Flow cytometry analysis of the very same spleen (Fig 3.8C) showed that <0.1% of total spleen cells were $mCherry^{+}$ (10 out of 216,990 total spleen cells), thus giving an indication of the sensitivity of IVIS imaging in the detection of mCherry signal from AML-derived cells. Therefore, coupling IVIS imaging with flow cytometry data (where possible) of the organs was able to provide important information regarding the presence of residual AML-derived cells during disease remission. Consistent with the flow cytometry analysis at 21 days on Dox, IVIS imaging revealed the presence of mCherry cells in the liver and spleen (Fig 4.1C). Importantly, mCherry signal was also detected in the kidneys at this time point. Therefore, although flow cytometry data suggests that the mCherry cells in the liver are differentiated AML-derived eosinophils, IVIS data cannot rule out the possibility of relapse also emerging from the kidneys.

4.3 Splenectomising mice during remission does not prevent disease relapse

Given that both flow cytometry and IVIS analysis of AML246 mCherry mice in remission showed re-emergence of GFP^{HIGH} cells initially in the spleen, we postulated that removing the spleen during remission (before the AML-derived cells in the spleen have been able to switch the PU.1 hairpin back on) may prevent relapse in our model of AML. As mentioned previously, mice were considered to be in remission when AML-derived cells were undetectable in the PB following Dox treatment. Based on our initial time course studies, 16 days of Dox treatment reliably drove AML246 mCherry transplanted mice into remission. Following 16 days on Dox, mice that were in remission were either splenectomised or were used as a sham control (Fig 4.1D) (refer to §2. 12.4 for details of splenectomy). For sham controls, mice were anaesthetized

and a small incision was made, however the incision was immediately stitched together and the spleen was not removed. Splenectomized and sham mice were then kept on Dox following surgery and the emergence of disease relapse was detected by fortnightly mandible bleeds. Surprisingly, despite the detection of GFP^{HIGH} cells initially in the spleen, mice that underwent splenectomies during remission still succumb to relapse at a similar rate to the sham controls (Fig 4.1E). Hence, the removal of the spleen during remission has no impact on likelihood of relapse. This is plausible given that despite the emergence of GFP positive cells first seen in the spleen, AML-derived cells can also be detected in the BM and possibly the liver and kidneys at this timepoint. Hence, AML-derived cells detected in these organs may be equally capable of switching the PU.1 hairpin back on to de-differentiate and ultimately drive relapse, albeit in a slightly delayed response. Alternatively, AML-derived cells that have undergone de-differentiation in other organs may preferentially home to the spleen following reversion back to a blast-like state.

4.4 Generation of AML246 mCherry KO clones of various genes

From our previous experiments it was established that AML-derived eosinophils persisted following differentiation and seeded relapse. Therefore, we hypothesized that preventing AML cells from maturing along the eosinophil lineage could also lead to the prevention of relapse in our AML model. To do this, we opted to knock out genes in the AML blast that were essential for normal eosinophil maturation using CRISPR-Cas9. Briefly, the CRISPR-Cas9 system is a genome editing technique adapted from a naturally occurring gene editing system found in bacteria (Hsu et al., 2014). CRISPR-Cas9 gene editing involves two major components: the short guide RNA (sgRNA) that binds to the specific DNA target sequence of interest, as well as the Cas9 enzyme which is responsible for introducing the double stranded breaks (DSB) at the target sequence (Hsu et al., 2014). The formation of DSB leads to either non-homologous end joining (NHEJ) or homology-directed DNA repair (HDJ). NHEJ DNA repair is most common and results in the introduction of insertion or deletion mutations (indels). As a result of the indels, frameshift mutations are introduced, resulting in improper translation and loss of gene of interest expression.

To knock out genes of interest in our AML blast using CRISPR-Cas9, AML246 mCherry cells were retrovirally infected with two independent vectors: one vector encoding Cas9 (as well as blasticidin resistance), and the other encoding the sgRNA target sequence of interest (Fig 4.2A). Details of the methodology in regards to generating CRISPR KO clones of our AML246

mCherry cells are described in §2.10. Genes that were targeted were those that played an important role in eosinophil maturation during haematopoiesis. As previously mentioned in §1.2.4 the transcription factor Gata1 is the master regulator of eosinophil differentiation, whereby targeted deletion of the Gata1 promoter leads to ablation of the eosinophil lineage (Yu et al., 2002). Likewise, Xbp1 is also a transcription factor that is selectively required for eosinophil differentiation, such that a loss of Xbp1 leads to a significant reduction is eosinophil progenitor cells (Bettigole et al., 2015). Given their integral roles in eosinophil differentiation, both transcription factors were targeted in our CRISPR KO experiments. As mentioned in §2.10, following retroviral infection, single cell clones of dual infected cells were generated and Sanger sequencing was used to determine the genetic mutations that were introduced for each clone. Table 4.1 outlines the list of genes targeted as well as details of the genetic mutations of each individual KO clone generated for these experiments.

4.5 Loss of essential macrophage gene csf1r does not prevent AML cell differentiation into persistent SSC^{HIGH} lineage

Previous characterization of the AML-derived SSC^{HIGH} population showed strong evidence to suggest that they most resembled mature eosinophils. Both F4/80 and Siglec-F are classic surface markers of eosinophils, however there is also a specialized population of alveolar macrophages that express both markers (Misharin et al., 2013). Furthermore, like eosinophils, alveolar macrophages are highly granular and also have a SSC^{HIGH} profile. Therefore, to eliminate possibility that the AML-derived SSC^{HIGH} cells are in fact a subpopulation of macrophages, the essential macrophage gene csf1r (Dai et al., 2002), was knocked out in AML246 mCherry to determine whether or not this would impact leukemic cell differentiation into the SSC^{HIGH} lineage. A sequence verified AML246 mCherry csf1r KO clone (refer to Table 4.1) was transplanted into recipient Rag mice and treated with Dox for 8 days once disease was established. As a control for these experiments, AML246 mCherry cells infected with a scramble sgRNA vector (sgCON) were also single cell cloned, expanded and transplanted into mice in parallel with the csfr1 KO clone. Following the endogenous restoration of PU.1, sgCON clones were able to bifurcated into the SSC^{LOW} neutrophils and SSC^{HIGH} eosinophils (Fig 4.2B), suggesting that further single cell cloning of AML246 mCherry did not impair bi-lineage differentiation of AML blasts. AML246 mCherry csf1r KO clones were also able to bifurcate into both SSC^{LOW} and SSC^{HIGH} lineages, suggesting that csf1r was not required for the development of the persistent SSC^{HIGH} cells (Fig 4.2B).

Predictably, SSC^{HIGH} cells remained present during periods of disease remission in mice harbouring the AML246 mCherry csfr1 KO clone (Fig 4.2C), confirming that the loss of csf1r had no impact on the SSC^{HIGH} population. Given that csf1r is essential for macrophage differentiation, this data therefore indicates that the AML-derived Siglec-F^{+} $F4/80^{+}$ SSC^{HIGH} cells are not alveolar macrophages.

4.6 Gata1 KO prevents AML cell differentiation into eosinophil lineage following PU.1 restoration

Gata1 is a transcription factor that regulates megakaryocytic, erythrocytic and eosinophilic differentiation (Du et al., 2002; Galloway et al., 2005; Yu et al., 2002; P. Zhang et al., 2000). Given its essential role in eosinophil differentiation, Gata1 was knocked out in AML246 mCherry cells using CRISPR-Cas9. To minimize the effects of clonal variability resulting from single cell cloning (i.e. Selection for a clone particularly susceptible or resistant to relapse), three independent Gata1 KO clones (from two independent Gata1 sgRNAs) of AML246 mCherry were generated and analysed (details of genetic mutations can be found in Table 4.1). Notably, AML246 mCherry leukemic blasts lacking Gata1 expression behaved similarly to the original AML246 mCherry CRISPR-Cas9 control blasts. In a Dox-free *in vitro* setting, both Gata1 KO clones and control counterparts expressed mCherry and GFP at similar levels and shared a similar immunophenotype (Fig 4.3A). Hence, preliminary *in vitro* characterization of Gata1 KO AML246 mCherry suggests that not only was the loss of Gata1 tolerated in the untreated leukemic blast, but also does not appear to change the behaviour of the AML blast. Hence, this also suggests that the AML blasts were in an early, Gata1-independent stage of maturation. AML246 mCherry Gata1 KO clones were transplanted into recipient Ly5.1 Rag mice (in parallel with three independent AML246 mCherry sgCON clones) to determine if the AML cells were able to differentiate into the eosinophil lineage upon PU.1 restoration in the absence of Gata1 expression. Remarkably, whereas bifurcated differentiation was still seen in all mice transplanted with AML246 mCherry sgCON after 8 days on Dox, no AML-derived cells differentiated into the SSC^{HIGH} eosinophil lineage in either of the three Gata1 KO clones tested (Figure 4.3B) shows results from 1x sgCON clone and 2x Gata1 KO clones from 2 independent Gata1 sgRNAs). This was consistent with the absence of a Siglec-F^{+} $F4/80^{+}$ AML-derived cells following PU.1 restoration. Instead, all AML-derived cells were SSC^{LOW} and morphologically resembled neutrophils. The loss of the eosinophil population was also reflected in cytospins of FACs sorted AML-derived cells, where there were no cells that had

the classic red and granular cytoplasm characteristic of an eosinophil. Therefore, knocking out Gata1 in the AML246 mCherry blast successfully prevented the leukemic cells from differentiating into the SSC^{HIGH} eosinophil lineage. Furthermore, the absence of SSC^{HIGH} eosinophils in Gata1 KO clones provides genetic confirmation that the AML-derived eosinophils are Gata1-dependent, and along with flow cytometry and histological analysis, strongly suggests that the SSC^{HIGH} cells are indeed eosinophil-like.

4.7 Preventing eosinophil differentiation of AML blasts can lead to total clearance of leukemia and improved overall mouse survival in vivo

To determine whether the prevention of AML blast differentiation along the eosinophil lineage resulted in total clearance of the AML following differentiation, IVIS imaging of organs harvested from AML246 mCherry Gata1 KO mice treated with long term Dox was performed (45 days). Remarkably, whereas residual AML-derived cells were detected in the spleen and stomach of sgCON control mice, there was complete absence of mCherry AML-derived cells in all the organs harvested from Gata1 KO transplanted mice at the same time point (Fig 4.3C) . In support of this, deep flow cytometry analysis of the BM and spleen (> 1 million viable cells recorded from each organ) harvested from sgCON mice showed early $mCherry^{+}$ GFP^{+} relapse emerging predominately in the spleen ,whereas no mCherry, AML-derived cells were found in most Gata1 KO mice (Fig 4.3D). Furthermore, many of the sgCON mice treated with long term Dox (12 out of 21 pooled from three independent sgCON clones) ultimately died from disease relapse. Conversely, most of the mice transplanted with Gata1 KO AMLs have not yet succumb to disease relapse (pooled from three Gata1 KO clones derived from two independent Gata1 sgRNAs) . Importantly, some Gata1 KO mice also relapsed (4 out of 18), however the rate of relapse in Gata1 KO mice was significantly less than sgCON mice (Fig 4.3E). Similarly to relapse from control mice, Gata1 KO relapse was also GFP^{HIGH}, suggesting that residual AML-derived cells had switched the PU.1 hairpin back on in order to drive relapse. Furthermore, the relapse sample retained the Gata1indel that was observed in the original leukemia (data not shown), thus ruling out the possibility of escaper cells (where Gata1 was not knocked out) being the source of relapse in these mice. These experiments relating to long term Dox treatment and relapse are still ongoing, however we intend to end the experiment once mice have reached 200 days on Dox. Nevertheless, to date it appears that the prevention of AML cell differentiation into eosinophils following PU.1 restoration results in a significantly improved overall survival of mice ($p=0.02$).

4.8 Loss of Xbp1 prevents differentiation of AML blast into SSC^{HIGH} eosinophil lineage

Gata1 is the master regulator of eosinophil differentiation, however it is also important for differentiation into the megakaryocyte and erythroid lineages. Recently, Xbp1 has been identified as a key transcription factor for the differentiation of uniquely the eosinophil lineage (Bettigole et al., 2015; Shen et al., 2015). Therefore, given the specificity of Xbp1 requirement exclusively for eosinophilic differentiation, Xbp1 was also knocked out in AML246 mCherry to prevent differentiation into the persistent SSC^{HIGH} lineage. Interestingly, much like the loss of Gata1, knocking out Xbp1 also resulted in the inability of AML246 mCherry to differentiated into the eosinophil lineage, as indicated by the absence of SSC^{HIGH} AML-derived cells following 8 days of Dox treatment (Fig 4.4A). Instead, all AML-derived cells differentiated into the SSC^{LOW} neutrophil-like lineage. The same result was observed in two independent AML246 mCherry Xbp1 KO clones (Table 4.1), confirming that in the absence of Xbp1, AML blasts are unable to undergo eosinophilic differentiation in response to the restoration of PU.1. The impact this may have on overall survival is still unknown as the experiments are ongoing, however the KM curve to date shows a strong trend to suggest that prevention of eosinophil differentiation via Xbp1 KO may also result in improved overall survival for leukemic mice treated with Dox (Fig 4.4B).

4.9 C-C chemokine receptor3 (Ccr3) expression is not required for AML differentiation into eosinophil lineage

Ccr3 is a chemokine receptor readily expressed on mature eosinophils (Choi et al., 2018; Stirling et al., 2001; Tiffany et al., 1998). Its primary function is to regulate migration of eosinophil to sites of inflammation however there is also evidence to show its importance in eosinophil differentiation (Lamkhioued et al., 2003). AML246 mCherry Ccr3 KO clones were also generated to determine if this also prevented AML eosinophilic differentiation. AML246 mCherry Ccr3 KO clone (Table 4.1) was transplanted into recipient Rag mice and treated with Dox following disease establishment (Fig 4.5A). Unlike knocking out the transcription factors Gata1 and Xbp1, loss of Ccr3 in AML246 mCherry did not prevent leukemic cell differentiation into the SSC^{HIGH} eosinophil-like lineage (Fig 4.5A). These results were unexpected given the apparent involvement of Ccr3 plays in promoting eosinophil differentiation of CD34^{+} progenitor cells *in vitro* (Lamkhioued et al., 2003). Importantly, Ccr3-mediated differentiation of progenitor cells into eosinophils is eotaxin-dependent, a potent chemoattractant predominately found in the lung. Eotaxin is produced by epithelial and

endothelial cells in the airways, normally triggering eosinophil migration to the lungs in response to pathogens (Huaux et al., 2005). Hence, Ccr3-mediated differentiation of CD34+ progenitor cells may be context dependent, and may only occur in the presence of pathogens to stimulate increased eosinophil production. Whether or not it is required for eosinophil differentiation in the homeostatic context is unknown. Similarly to the Gata1 and Xbp1 KO clones, the impact of knocking out Ccr3 in AML246 mCherry with regards to relapse is yet to be determined given that the experiment is still ongoing (Fig 4.5B). Nevertheless, the ability of AML246 mCherry cells to undergo bifurcated differentiation in the absence of Ccr3 suggests that its expression is not required for AML eosinophilic differentiation.

4.10 Discussion

4.10.1 The origin of relapse following AML246 cell differentiation is extramedullary

In our inducible AML model, AML-derived eosinophils can be found in both haematopoietic and extramedullary organs during remission. Currently, it is unclear why rare subpopulations of AML-derived eosinophils are able to evade clearance and persist during remission. One possible explanation may be following differentiation, AML-derived eosinophils are able to extravasate from the vessels and into the tissue of the spleen, liver or kidneys. Notably, tissue resident eosinophils have a significantly longer lifespan than circulating eosinophils, suggesting that localization into the tissue may be important for eosinophil survival (Geslewitz et al., 2018; Y. M. Park et al., 2010). Exposure to pro-survival signals as well as limited accessibility of phagocytes to tissue resident eosinophils compared to those in circulation may attribute to the longer lifespan in tissue. It would be interesting to perform multiphoton microscopy imaging of the spleen following AML cell differentiation to determine whether extravasation of AML-derived eosinophils does in fact occur in our model. Notably, the spleen is highly vascular organ (Malinovsky et al., 1995), therefore circulation of AML-derived eosinophils and neutrophils may be occurring very often, and the chance to extravasate may be high. Eosinophils normally extravasate into tissues in response to the presence of foreign pathogens and allergens (Johansson, 2017; Knol et al., 1996). Chemokines and chemoattractants such as eotaxin and IL-5 are important for the chemotaxis of circulating eosinophils to different organs such as the lungs, liver, spleen (Geslewitz et al., 2018). Interestingly, a recent paper tracking real time migration of neutrophils and eosinophils in humans shows significant differences in migration kinetics between the two sub-granulocytic

populations (Lukawska et al., 2014). Radio-labelled eosinophils and neutrophils were re-infused into healthy volunteers and efflux of the two populations from the lungs to the spleen, liver and kidneys showed that eosinophils migrated significantly faster than neutrophils. Although speculative, the inherent ability of eosinophils to migrate into different tissues may assist in its evasion from circulating phagocytes, whereas the considerably slower neutrophils may be phagocytosed more efficiently due to prolonged time in circulation. Despite AML-derived cells localized in a number of organs during remission, IVIS imaging and deep flow cytometry analysis of mice on Dox after 21 days shows re-emergence of $mCherry^+$ GFP^+ relapse originally in the spleen on a remarkably consistent basis. This data therefore suggests two possible scenarios: either the AML-derived eosinophils localized in the spleen are particularly susceptible to mutations that re-engage PU.1 suppression, or cells that have already acquired mutations rendering them insensitive to Dox treatment preferentially home to the spleen once they have reverted to a blast-like state. To this point, from our initial time course assays, we consistently see the largest leukemic burden in the spleen rather than the BM in untreated mice (Fig 3.2B). The distribution of leukemic blasts prior to Dox treatment as well as the presence of GFP^+ AML cells in the spleen during early stage relapse suggest that the AML blasts have a particular affinity to the spleen. The spleen itself is typically involved in filtration and clearance of blood cells (Duez et al., 2015; Pivkin et al., 2016), however prior to the development of the BM (during fetal development), extramedullary haematopoiesis also occurs in this organ (Kim, 2010). Furthermore, during periods of BM stress (such as disruption to the BM niche), HSPCs are also able to migrate to the spleen and undergo extramedullary haematopoiesis (Inra et al., 2015; Kim, 2010; Oda et al., 2018; Short et al., 2019). Therefore, given that the splenic niche is conducive for stem cell and progenitor cell maintenance under given circumstances, the cytokine and growth factor milieu that is able to support their maintenance is also likely to support the survival of the transformed immature AML blasts. Hence, although AML is typically considered a disease of the BM, our data implicates the spleen as another important site of AML development and relapse.

4.10.2 Extramedullary relapse in AML is associated with monocytic differentiation

Extramedullary relapse (EM) has been seen in AML previously, often following allogeneic haematopoietic cell transplantation (HSCT). EM relapse typically involves a range of different organs such as the CNS, breast, cutaneous or soft tissue (Harris et al., 2013). Interestingly, monocytic differentiation of AMLs is often associated with EM relapse (Bakst et al., 2011),

perhaps relating to the long lifespan of tissue resident monocytes (Shaw et al., 2018). Although in our mouse model the differentiation of AML cells observed is eosinophilic (as well as neutrophilic), the considerably longer lifespan compared to neutrophils (Kotzin et al., 2016; Tak et al., 2013; Willebrand et al., 2017) as well as the ability to reside in tissues (Weller et al., 2017) (there is currently no evidence to suggests neutrophils reside in tissues), may shed light as to why AML-derived eosinophils are able to persist long term and ultimately drive EM relapse in our model.

4.10.3 The spleen contributes to differentiated AML cell clearance and is also organ where relapse is initially observed

In Chapter 3 the spleen was identified as a major site of differentiated AML cell clearance. However, despite the role it plays in the removal of AML cells, in this Chapter the spleen has also been implicated as the original site of relapse in our AML model. Although the spleen has a seemingly contradictory role in our model, it may be that the spleen is a major site of both AML cell clearance following differentiation as well as an organ where AML blasts preferentially reside. The spleen may initially facilitate AML blast proliferation, however once differentiation is triggered, splenic macrophages may then be responsible for clearing these differentiated cells. Residual AML-derived eosinophils (residing in the spleen or elsewhere) that revert back to an AML blast may preferentially home back to the spleen given that it appears to be a microenvironment that promotes AML blast survival and proliferation. Intriguingly, the spleen has also been implemented as the site of leukemia initiating activity in other models of AML (Krivtsov et al., 2006; Somervaille et al., 2006). Importantly, despite early $mCherry^{+}GFP^{+}$ AML relapse initially detected in the spleen, the removal of the spleen during remission was unable to prevent mice from succumbing to disease relapse. Therefore, although the spleen is where $mCherry^{+}GFP^{+}$ AML cells were initially observed, in its absence, AML-derived eosinophils in other organs (such as BM, liver or kidneys) may also re-engage PU.1 suppression and drive disease relapse. Transplantation of AML-derived cells harvested and sorted from each organ into secondary recipient mice would answer whether leukemia initiating potential can be narrowed down to a subpopulation of AML-derived cells localized in a specific extramedullary organ. Although this in theory could help identify the source of relapse in our AML model, our lab has previously described the difficulty in generating secondary AMLs via the transplantation of differentiated AML-derived cells into recipient mice (McKenzie et al., 2019, in press) Whereas previous literature has been able to identify

potential subpopulations of leukemia initiating cells through secondary transplant assays (Shlush et al., 2014; Somervaille et al., 2006; Taussig et al., 2010), these experiments often involve the transplantation of AML cells with blast-like features. Whereas an AML blast is designed to home to the BM and engraft in a secondary recipient, differentiated AML-derived cells presumably behave similarly to differentiated myeloid cells, and are thus not a cell type that would naturally home to the BM, but rather migrate out of the BM and into circulation. Hence due to the inherent differences in our hypothesized leukemia initiating cell population, we have thus far been unsuccessful in our attempts to generate leukemias when transplanting these cells into secondary recipient mice.

4.10.4 Loss of Gata1 and Xbp1 prevents AML blast differentiation along the eosinophil lineages

Gata1 is a master regulator of eosinophil differentiation during haematopoiesis (Gombart et al., 2003; Hirasawa et al., 2002; Yu et al., 2002). CRISPR-Cas9 mediated KO of Gata1 in AML blasts also prevented their differentiation into the eosinophilic lineage. Importantly, consistent with normal myeloid cell differentiation, Gata1 KO AMLs were still able to undergo neutrophilic differentiation, suggesting that its expression is not required for the development of this particular granulocytic lineage (Drissen et al., 2016; Hirasawa et al., 2002). As predicted, monolineage differentiation of AML blasts into the neutrophil lineage resulted in complete clearance of the AML in some mice following the induction of differentiation. This is likely due to the highly effective clearance mechanisms in place that are normally responsible for clearing billions of normal neutrophils on a daily basis (Furze et al., 2008; Gordy et al., 2011). Notably, despite the absence of a SSC^{HIGH} population in Gata1 KO mice, some of the Gata1 KO mice still suffered from a GFP^{HIGH} disease relapse (4 out of 18). This could possibly be due to AML-derived neutrophils in Gata1 KO clones acquiring relapse-causing mutations before the clearance of AML-derived neutrophils. To this point, following the administration of Dox, there was typically a 10 to 14-day window before the AML-derived cells differentiated into neutrophils and were cleared. Therefore, it is feasible that AML-derived neutrophils during this window acquire mutations that re-engage PU.1 suppression and thus seed relapse. Alternatively, relapse in the Gata1 KO mice may also be attributed to a pre-existing AML cell that was not responsive to the initial Dox therapy. Similarly to our experiments in Chapter 3, it would be important to investigate Gata1 KO mice during periods of disease regression and remission to ensure that all residual AML-derived cells at these time points (between 8-16 days

on Dox) are indeed GFP^{LOW} and have shut off the PU.1 hairpin. While currently our data does not directly eliminate the possibility or a pre-existing clone driving relapse, the fact that loss of Gata1 expression (and in turn the eosinophil AML lineage) resulted in a significant decrease in relapse following differentiation therapy suggests that relapse can also originate from a mature AML-derived lineage. Consequently, mice transplanted with Gata1 KO AMLs survive significantly longer than mice transplanted sgCON AMLs following Dox.

Similar to knocking out Gata1, the loss of Xbp1 in AML blasts also prevented AML246 mCherry differentiation along the eosinophil lineage. Xbp1-IREIα signalling has been implicated in the preservation of LSC self-renewal (L. Liu et al., 2019). However, the effect caused by the loss of Xbp1 expression in our model is most likely linked to the prevention of eosinophil differentiation rather than the perturbation of AML blasts, given that Xbp1 KO is also well tolerated in the AML246 blast. Furthermore, in normal eosinophil development, the loss of Xbp1 results in ablation of EoPs and an accumulation of GMPs during haematopoiesis (Bettigole et al., 2015). Therefore, consistent with our AML model, knocking out Xbp1 is likely to leave the AML blast in a GMP-like state, hence they are still able to differentiate along the neutrophil lineage following the restoration of PU.1. As the long term Dox experiments are currently ongoing, it is difficult to conclude whether the prevention of AML-eosinophil differentiation via the loss of Xbp1 translates to improved overall survival. Much like the Gata1 KO clones, mice transplanted with Xbp1 KO AMLs thus far appear to be less susceptible to relapse, however 1 out of the 6 transplanted mice has succumb to a $mCherry^{+}GFP^{+}$ relapse. We believe that this relapse is due to an AML-derived neutrophil acquiring a relapse causing event prior to the clearance of the differentiated AML cells.

4.10.5 Preventing relapse by removal of AML-derived eosinophils questions the LSC model

As it currently stands (3rd of June 2019), the results from the KM curves suggests that prevention of AML differentiation along the SSC^{HIGH} eosinophil lineage via Gata1 KO leads to improved overall survival in mice following long term Dox treatment. Although relapse still occurs in Gata1 KO mice, the rate of relapse is significantly less frequent than the sgCON counterpart. Hence, this data strongly suggests that following differentiation therapy in AML246, differentiated AML cells are the source of relapse. Consequently, these results also provide an interesting alternative source of relapse that may not stem from a dormant, non-

responsive cancer stem cell (CSC), but from a differentiated AML cell derived from the 'bulk' AML population. As described extensively in the literature, The CSC model hypothesizes that a rare, immature, stem cell like population of cancer cells is responsible for propagating the disease. Much like normal HSCs and progenitor cells, the leukemic stem cell (LSC) is at the apex of the cellular hierarchy and can partially differentiate to give rise to the bulk AML (Bonnet et al., 1997; Jordan, 2007). Importantly, in the CSC model, differentiation of an AML blast is unidirectional, and thus bulk AML cells are no longer leukemia initiating cells. Therefore, chemoresistance or disease relapse in this model can be explained by ineffective clearance of the LSC (Abdullah et al., 2013; Gentles et al., 2010). In our AML246 model however, we have previously shown AML blast differentiation is not unidirectional, and that mature AML cells have the capacity to de-differentiate (McKenzie et al, 2019, in press). Therefore, although relapse can originate from pre-existing LSCs, evidence of maturational plasticity coupled with a significant reduction in relapse when we prevent eosinophil maturation of AML246 *in* vivo suggests that relapse can also arise from differentiated AML cells. Hence, relapse initiating capacity may not be restricted to a rare population of dormant CSCs, but is rather present in potentially all AML-derived cells.

To our understanding, the results in this chapter have not yet been previously described clinically or in other models of AML. Although these results raise a novel source of relapse following differentiation therapy in AML, it is also important to note that an AML-derived eosinophil source of relapse has thus far only been made in our single inducible AML model system. Therefore to strengthen our hypothesis that relapse from differentiated AML cells is clinically relevant, it is important to see whether a similar phenomenon is observed in a second model of AML differentiation therapy. Encouragingly, evidence of eosinophilic differentiation of AML cells has recently been shown following the use of Enasidenib in an AML patient, however the implications of AML-derived eosinophils and relapse was not explored further (Galeotti et al., 2019). In future experiments it would be interesting to treat xenograft models of AML driven by HT93 or NB4 cells (harbouring the PML-RAR fusion oncogene mutation) with ATRA+ATO to determine if multilineage differentiation is observed in a more clinically relevant model of AML. Notably, HT93 cells have been shown to differentiate into eosinophils *in vitro* in the presence of IL-5, however this has yet to be documented *in vivo*, and thus it is unknown if these APL-derived eosinophils also persist and seed relapse in the xenograft model.

4.10.6 Are AML-derived eosinophils particularly susceptible to de-differentiation?

From our experiments we found that AML-derived eosinophils persist and often seed relapse. Although it is likely that AML-derived eosinophils drive relapse simply due to their comparably longer lifespan (and thus greater chance of acquiring mutations that re-engage PU.1 suppression), our results also bring into question whether AML-derived eosinophils are inherently more likely to de-differentiate than other mature myeloid cell subtypes such as neutrophils. Eosinophils are a cell type involved in significant protein production and secretion (Acharya et al., 2014). This is unsurprising given that their primary function is to secrete highly basic granule proteins in response to pathogen exposure. Eosinophilopoiesis requires the activation of physiological endoplasmic reticulum (ER) stress, and considerably high levels of protein synthesis are required before eosinophils terminally differentiate (Bettigole et al., 2015). Interestingly, transgenic mice with mutations to genes that are involved in the processing and release of secreted proteins are also deficient in eosinophils (Bettigole et al., 2015). Hence, the survival and differentiation of eosinophils is intertwined with tightly regulated protein production. Whereas normal eosinophils with defects in protein production rapidly undergo cell death, AML-derived eosinophils may be unique in that they are able to survive despite dysregulated protein synthesis due to their leukemic background (which is inherently pro-survival). Furthermore, whether the same differentiation program required for normal eosinophil differentiation is also activated in AML-derived eosinophil differentiation (following PU.1 restoration) remains to be seen. Hence, comparing the transcriptional profile of AML-derived eosinophils and normal eosinophils is of great importance. Nevertheless, the leukemic origin of the AML-derived eosinophils coupled with eosinophil maturation depending on tightly regulated protein production may not only render it unable to terminally differentiate, but in doing so, may also allow these transient eosinophils to de-differentiate given the right circumstances.

4.10.7 Disease relapse may be directly related to stage of AML-derived eosinophil maturation

Until recent work from our laboratory (McKenzie et al, 2019 in press), de-differentiation of AML-derived myeloid cells has not been previously described, therefore the potential and limitations of ‘mature’ AML cell de-differentiation is currently unknown. For example, in our model, the ability to de-differentiate and drive relapse may be dependent on the maturation stage of the AML-derived eosinophil originally produced by the differentiation stimulus. In

addition to RNAseq analysis of normal and AML-derived eosinophils, using CRISPR-Cas9 to KO genes known to be important for specific stages of eosinophilic differentiation may also indicate the stage of AML-derived eosinophil maturation. As shown in our data, knocking out the transcription factors Gata1 and Xbp1 both prevented the formation of AML-derived eosinophils. Gata1 expression is required during multiple stages of eosinophil development (refer to §1.2) , whereas Xbp1 is specifically required for differentiation into EoPs (Bettigole et al., 2015; Shen et al., 2015). Hence, the loss of Gata1 or Xbp1 expression is likely to have prevented AML cells from progressing past EoPs, leading to the absence of AML-derived eosinophils. Ccr3 is a gene that is expressed specifically on terminally differentiated eosinophils, with some evidence to suggest that it is also involved in eosinophil differentiation (Lamkhioued et al., 2003). However, in our AML model, the loss of Ccr3 did not prevent AML cell eosinophilic differentiation. As mentioned in §4.9, Ccr3-mediated differentiation may be context dependent, and rely on the presence of eotaxin to trigger differentiation of CD34+ progenitor cells in the presence of an allergen or pathogen. Hence, in these circumstances of AML cell differentiation, Ccr3 may not be essential for eosinophil differentiation. Overall, our data thus far suggests that the AML-derived eosinophils have matured at least beyond the point of EoPs. Knocking out other regulators of eosinophil development such as Id2 would be particularly interesting given that they are all required for late stage eosinophil differentiation (Bedi et al., 2009; Buitenhuis et al., 2005; Kang et al., 2005). Id2 is required for terminal differentiation of eosinophils, hence if the loss of Id2 expression also prevents the formation of eosinophils following AML blast differentiation, this would strongly suggest that these SSC^{HIGH} eosinophils are in fact, fully mature eosinophils. Consequently, this would provide strong evidence to suggest that 'terminally differentiated' AML-derived eosinophils still maintain the ability to revert and drive relapse following the re-engagement of PU.1 suppression. Conversely, AML cell differentiation into the eosinophil lineage in AML246 mCherry Id2 KO clones would indicate that the AML-derived eosinophils are not fully mature, and are likely to be at an intermediate stage of complete maturation.

Unlike Id2, Cebpe and Notch are both negative regulators of eosinophil terminal differentiation (Bedi et al., 2009; Kang et al., 2005). Therefore, knocking out either transcription factor may allow AML-derived eosinophils to fully mature. In this context, it would be interesting to see if mature AML eosinophils are still able to seed relapse, or whether promoting further maturation of AML-derived eosinophils leads to their clearance and the prevention of relapse. Nevertheless, in doing these genetic experiments we would be able to identify how far down

the differentiation pathway a ‘mature’ eosinophil can reach before it can no longer de-differentiate , or whether a ‘point of no return’ indeed exists in differentiated AML cells.

4.10.8 Identifying therapeutic methods of eliminating persistent AML-derived to influence relapse

Genetic approaches have proven to be highly effective in the prevention of AML cell differentiation into eosinophils in our mouse model of AML. Knocking out Gata1 and Xbp1 in particular has been effective in preventing eosinophil differentiation of AML cells, however transcription factors are difficult to target therapeutically. Therefore, moving forward it is also important to identify other, targetable regulators of eosinophil differentiation. In addition to transcriptional regulators, normal eosinophils also rely on the presence of external signals such as IL-5 for their development and survival (Matthaei et al., 1997; Takatsu et al., 2008). Intriguingly, previous attempts to knock out IL5Rα in AML246 mCherry were unsuccessful, as we were unable to generate viable KO clones with homozygote deletions (data not shown). This may attest to the potential eosinophilic bias of AML246 mCherry blasts, such that IL5Rα expression is essential for leukemic cell survival. Nevertheless, anti-IL5 (mepolizumab) is currently used clinically to treat patients with eosinophilia and is highly effective in reducing eosinophil numbers in patients (Farne et al., 2017). Co-treatment of differentiation therapy agents in conjunction with anti-IL-5 may prevent leukemic blast differentiation into the eosinophil lineage and improved clearance of AML-derived cells. Unfortunately, mepolizumab does not cross react with mouse IL-5, hence we are unable to test whether pharmaceutical depletion of IL-5 prevents leukemic cell differentiation into eosinophils in our AML246 mouse model.

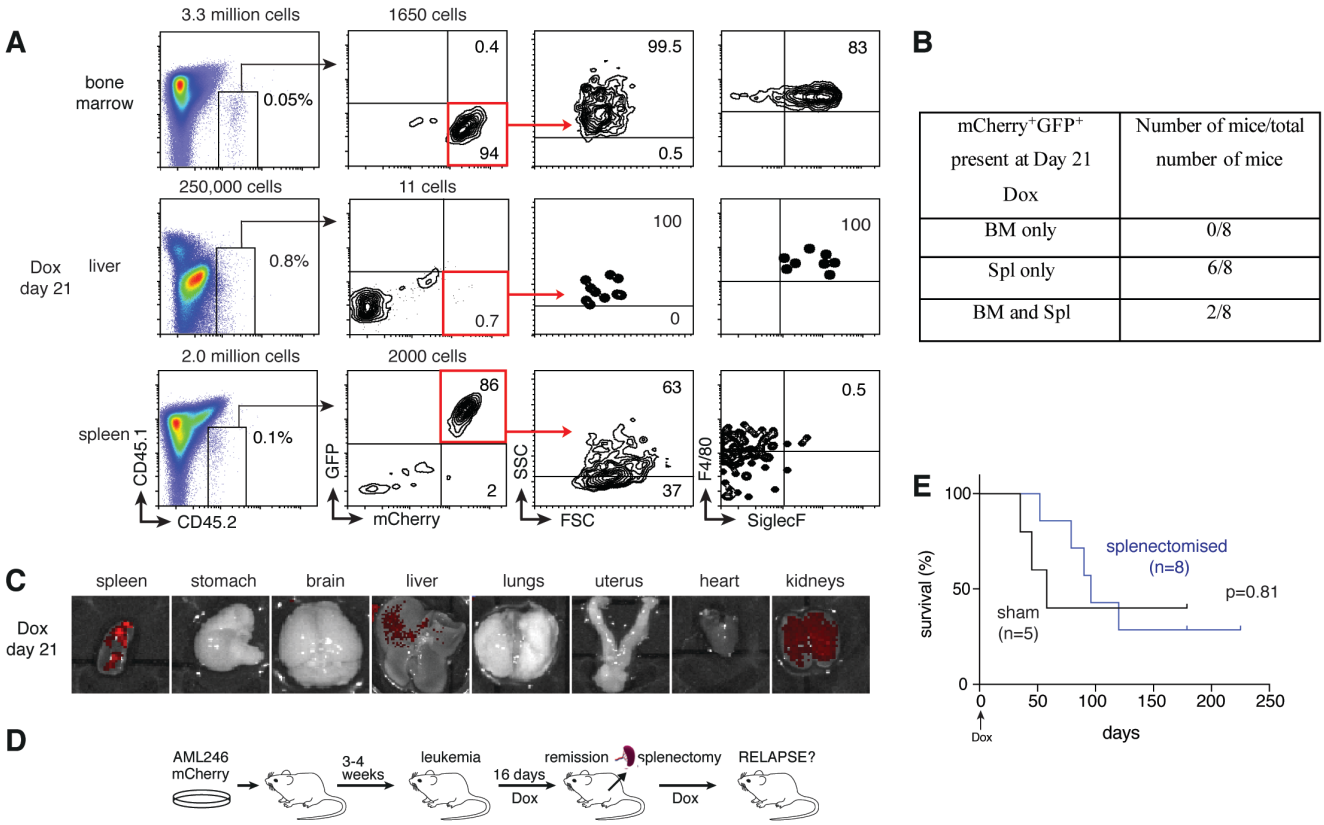
Alternatively, lineage skewing of AML-derived blasts exclusively into the rapidly cleared neutrophil population may also be a viable therapeutic option to prevent relapse. Whereas GM-CSF promoted both neutrophil an eosinophil production (Esnault et al., 2002; Fossati et al., 1998), G-CSF appears to be uniquely important for neutrophil differentiation (Basu et al., 2002; Queto et al., 2011; Roberts, 2005). Interestingly, in pulmonary allergy inflammation mouse models (ovalbumin-sensitive mice), pre-treatment of mice with G-CSF prevented BM eosinophil production whilst selectively increasing neutrophil colony formation in response to ovalbumin challenge (Queto et al., 2011). Therefore, the use of G-CSF in combination with differentiation therapy agents may be able to push AML blasts into the single neutrophil lineage

at the expense of eosinophil production. Importantly, in addition to being a pro-neutrophil growth factor, G-CSF may also trigger leukemic blast proliferation (Murayama et al., 1998), hence the timing of G-CSF induction would be critical in this scenario.

4.11 Conclusion

The aim of Chapter 3 was to characterize in detail the kinetics and mechanism of AML differentiation of our inducible PU.1 AML model *in vivo*. From these experiments, we found that following the establishment of AML, restoration of PU.1 triggered bifurcated differentiation of AML cells into AML-derived neutrophils, as well as a considerably rare population of AML-derived eosinophils (§3.5.1). Serendipitously, we found that differentiated AML-derived cells were predominately cleared through macrophages the spleen, and that the absence of a spleen resulted in ineffective clearance of AML-derived cells, leading to an accelerated relapse (§3.8). In addition to the preliminary data relating to potential AML cell clearance mechanisms, we were also able to identify the likely source of disease relapse following differentiation therapy. Whereas the AML-derived neutrophil population was efficiently cleared following differentiation, AML-derived eosinophils persisted during remission in the BM as well as extramedullary organs such as the spleen, liver and likely the kidneys (Fig 3.8D and Fig 4.1C). Therefore, given that the only AML-derived cells detected during disease remission (after 16 days on Dox when no AML cells could be found in the PB) were exclusively AML-derived eosinophils, we postulated that the source of relapse must come from this mature AML cell population. Furthermore, combined flow cytometry and IVIS imaging analysis showed that early $mCherry^{+}GFP^{+}$ relapse was likely to originate from the spleen (Fig 4.1A-C). Hence, these findings from Chapter 3 fuelled our investigation into preventing AML relapse following differentiation therapy in Chapter 4. Given our hypothesis, our aim was to prevent leukemic cell differentiation along the eosinophil lineage to prevent disease relapse. According to the literature, there are a number of genes that regulated specific stages of eosinophil maturation during eosinophilopoiesis (Uhm et al., 2012). Amongst these genes, the transcription factors Gata1 and Xbp1 appear to be uniquely required for eosinophil differentiation (Bettigole et al., 2015; Yu et al., 2002). Consistent with this, CRISPR-Cas9-mediated KO of Gata1 in AML246 blasts was able to successfully prevent their differentiation along the eosinophil lineage. Consequently, AML blasts underwent monolineage differentiation into neutrophil-like cells following PU.1 restoration, resulting in the clearance of AML-derived cells following long term Dox treatment in a number of mice (Fig 4.3D).

Experiments are still ongoing, however thus far the absence of an AML-derived eosinophil population in Gata1 KO mice resulted in improved overall survival in comparison to sgCON controls, however prevention of relapse was not observed in all mice. We speculate that the cases of relapse in Gata1 KO mice originated from AML-derived neutrophils that acquired relapse-causing mutations during early stages of differentiation therapy. Similar observations were also seen in mice transplanted with Xbp1 KO AML blasts, where the loss Xbp1 expression prevented AML246 cell differentiation into eosinophils (Fig 4.4A). Much like the Gata1 KO mice, these experiments are still ongoing, however we anticipate that the prevention of eosinophil differentiation in Xbp1 KO mice may also translate to a statistically significant minimization of relapse in these mice. Ultimately, our data suggests that differentiation therapy can trigger differentiation of AML blasts into multiple mature lineages. Therefore by promoting differentiation along a myeloid lineage that is effectively cleared, instead of lineages that have a long lifespan may have significant impact on patient outcomes and overall survival.



mCherry⁺GFP⁺ present at Day 21 Dox	Number of mice/total number of mice
BM only	0/8
Spl only	6/8
BM and Spl	2/8

Figure 4.1 Early relapse is first detected in the spleen

(A) Flow cytometry analysis of the bone marrow, liver and spleen of AML246 mCherry transplanted mice in remission. AML derived cells present in these organs was determined by the expression of CD45.2 and mCherry. (B) Location of mCherry, GFP positives in mice treated with Dox for 21 days. (C) IVIS imaging of a mouse in early relapse shows the presence of mCherry signal in the spleen, kidneys and liver. (D) Experimental outline detailing the removal of the spleen (splenectomy) in AML246 mCherry transplanted mice that are in remission following 16 days of Dox treatment. (E) Kaplan-Meier survival analysis of mice splenectomised during disease remission compared to sham controls. Mantel Cox analysis was used to determine statistical significance between both groups of mice (p=0.81).

Table 4.1 Genetic sequences for CRISPR KO clones verified by Sanger Sequencing

Gene	clone	alleles	Amino acid
Gata1	sgRNA3 clone 8	TTCCCAGGCCCTGGAAGACCAGGAAGGGAA wt TTCCCAGGCCCTGGAAGACCAAGGAAGGGAA a1 TTCCCAGGCCCTGGAAGACCAAGGAAGGGAA a2	SQALEDQEG SQALEDQGR SQALEDQGR
Gata1	sgRNA3 clone 5	TTCCCAGGCCCTGGAAGACCAGGAAGGGAA wt TTCCCAGGCCCTGGAAGA-CAGGAAGGGAA a1 TTCCCAGG----------GAAGGGAA a2	SQALEDQEG SQALEDRKG SQGREREEQ
Gata1	sgRNA2 clone 5	ATGCTAGCTGGGCCTATGGCAAGACGGCAC wt ATGCTAGCTGGGCCTATGGCAAAGACGGCAC a1 ATGCTAGCTGGGCCTATGGCAAAGACGGCAC a2	ASWAYGKTA ASWAYGKDG ASWAYGKDG
Ccr3	sgRNA1 clone 6	ACTCCCTGGTGTTCATCATCGGCCTCC wt ACTCCCTGGTTGTTCATCATCGGCCTC a1 ACTCCCTGGT--TCATCATCGGCCTCC a2	TPWCSSSAS TPWLFIIGL TPWFIIGLL
Xbp1	sgRNA1 clone 1	CAGGAGTTAAGAACACGCTTGGGAATGGA wt CA----------GGA a1 CA----------A a2	QELRTRLGM QEWTRWILT QMDTLDPDP
Xbp1	sgRNA1 clone 11	TTAAGAACACGCTTGGGAATGGACACG wt TTAAGA----------ACACGCG a1 TTAAGAACACGCTTGAGGAATGGACAC a2	LRTRLGMDT LRTRAGS**Stop** LRTRLRNGH
Csf1r	sgRNA 2 clone 4	AACGGTCTACTTCTTCTCGCCATGGCGAGGGTTCATTATCCGCAAGGCTAAAGTCCTTGACA wt AA----------GTCCTTGACA a1 AACGGTCTACTTCTTCTCGCCATGGCGAGGGTTCATT----------ACA a2	QNGLLLLAMARVHYP QNGLLLLAMARVHYS QKSLTAIPTCARPWS

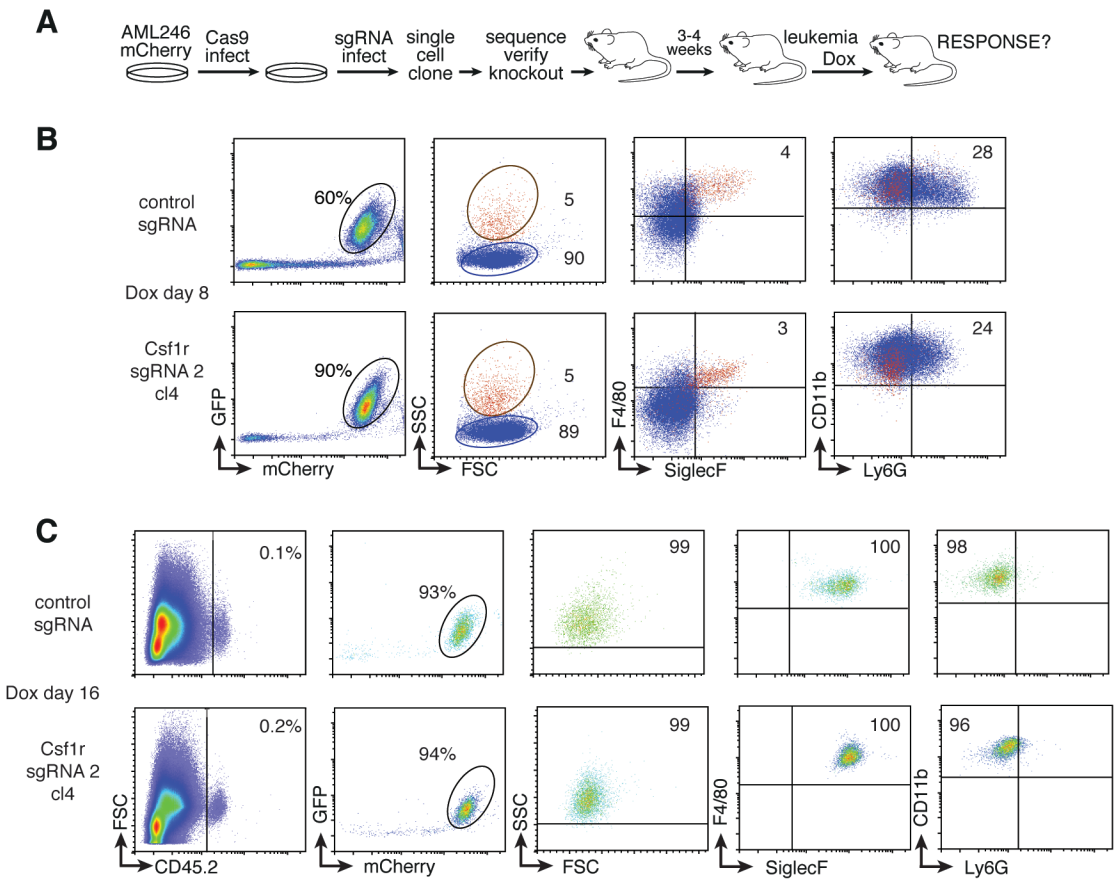


Figure 4.2 AML246 mCherry undergo bifurcated differentiation in the absence of Csf1r

(A) Schematic representation of workflow involved in the generation of CRISPR/Cas9 KO clones of genes of interest. (B) Flow cytometry analysis AML246 mCherry Csf1r KO clones. The immunophenotype of the AML derived population present after 8 days on Dox was determined by initially gating on mCherry positive cells, followed by analysis of their FSC/SSC profile along with the expression of myeloid markers F4/80, Siglec-F, CD11b and Ly6G. (C) Immunophenotype of AML derived cells present in Csf1r KO mice following 16 days on Dox compared to control sgRNA mice.

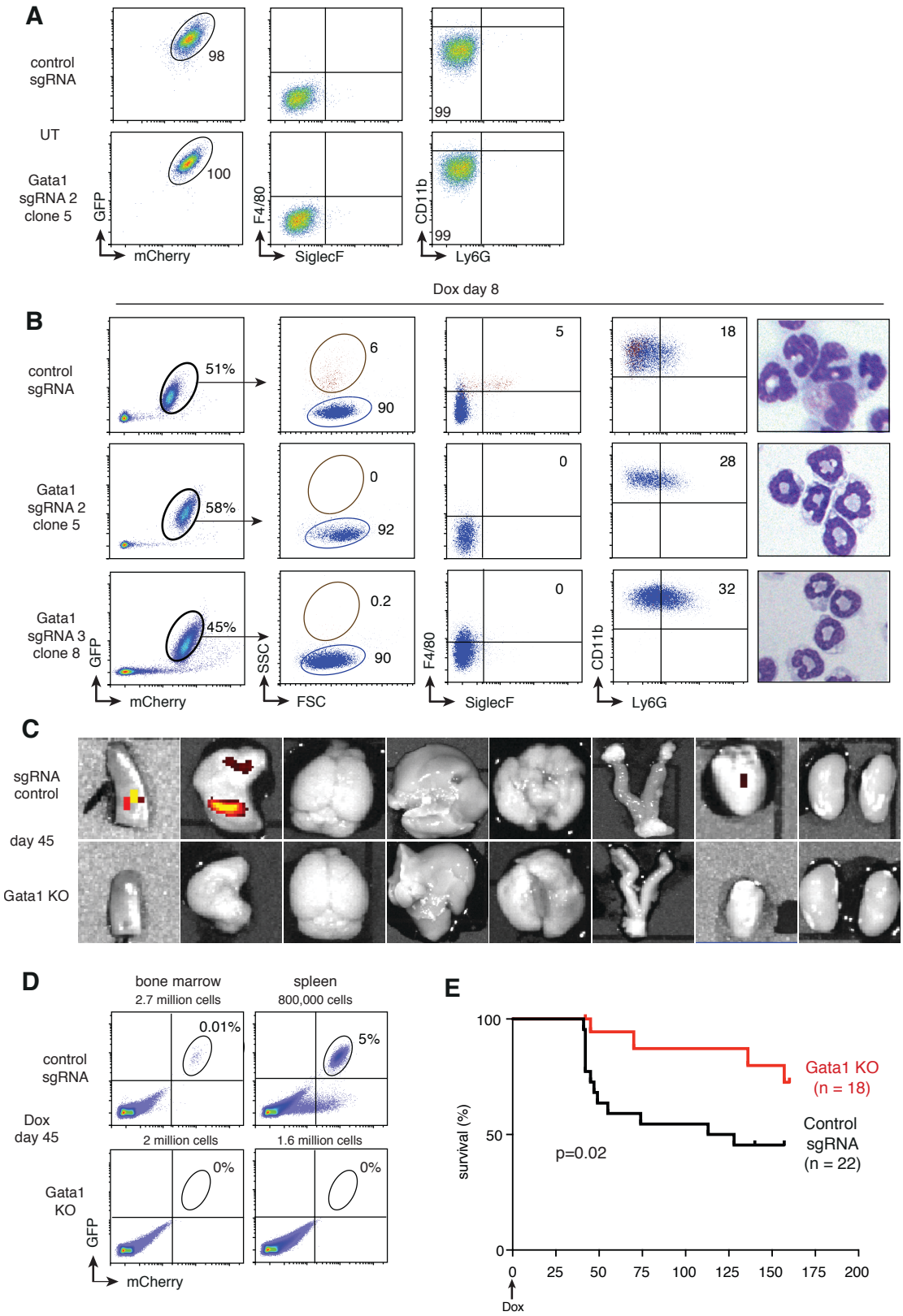

A
control sgRNA
UT
Gata1 sgRNA 2 clone 5
98
100
99
99
GFP
mCherry
F4/80
SiglecF
CD11b
Ly6G
Dox day 8
B
control sgRNA
Gata1 sgRNA 2 clone 5
Gata1 sgRNA 3 clone 8
51%
58%
45%
6
90
0
92
0.2
90
5
0
0
18
28
32
GFP
mCherry
SSC
FSC
F4/80
SiglecF
CD11b
Ly6G
C
sgRNA control
day 45
Gata1 KO
D
bone marrow
2.7 million cells
spleen
800,000 cells
control sgRNA
Dox day 45
Gata1 KO
0.01%
5%
2 million cells
1.6 million cells
0%
0%
GFP
mCherry
E
100
50
0
survival (%)
p=0.02
Gata1 KO (n = 18)
Control sgRNA (n = 22)
0
25
50
75
100
125
150
175
200
Dox

Figure 4.3 Loss of key eosinophil transcription factor Gata1 prevents differentiation of AML blasts into SSCHIGH population following Dox treatment

(A) Flow cytometry analysis comparing mCherry/GFP expression and immunophenotype of AML246 mCherry Gata1 KO clone vs sgCON clone in untreated media. (B) Flow cytometry analysis of two independent AML246 mCherry Gata1 (second and third row) KO clones transplanted into Rag 1 -/- mice that were treated with Dox for 8 days. The immunophenotype of the AML derived population present after 8 days on Dox was determined by initially gating on mCherry positive cells, followed by analysis of their FSC/SSC profile along with the expression of myeloid markers F4/80, Siglec-F, CD11b and Ly6G. Cytospins of the AML derived population residing in the BM was performed on FACs sorted mCherry positive cells (sytox blue was used as the viability marker). This data is from a single mouse representative of 3 mice per clone. (C) Comparison of the disease burden in Rag 1 -/- mice transplanted with either AML246 mCherry Gata1 KO or AML246 mCherry sgCON cells during disease remission (45 days on Dox). These flow cytometry plots are from a single mouse representative of 3 mice pulled from 2 independent Gata1 KO clones. sgCON flow cytometry plot is also a single mouse representative of 3 mice pulled from 2 independent sgCON clones (D) IVIS imaging of mCherry fluorescent signal in organs harvested from Gata1 KO and sgCON mice following 45 days of Dox treatment. (E) Kaplan Meier survival analysis of mice transplanted with either AML246 mCherry Gata1 KO or AML246 mCherry sgCON cells that were subsequently treated with Dox following disease establishment, as of Day 170 on Dox (3.5.19). Experiment will terminate once mice reach 200 days on Dox. Mantel-Cox analysis was performed to determine statistical significance ($p=0.02$)

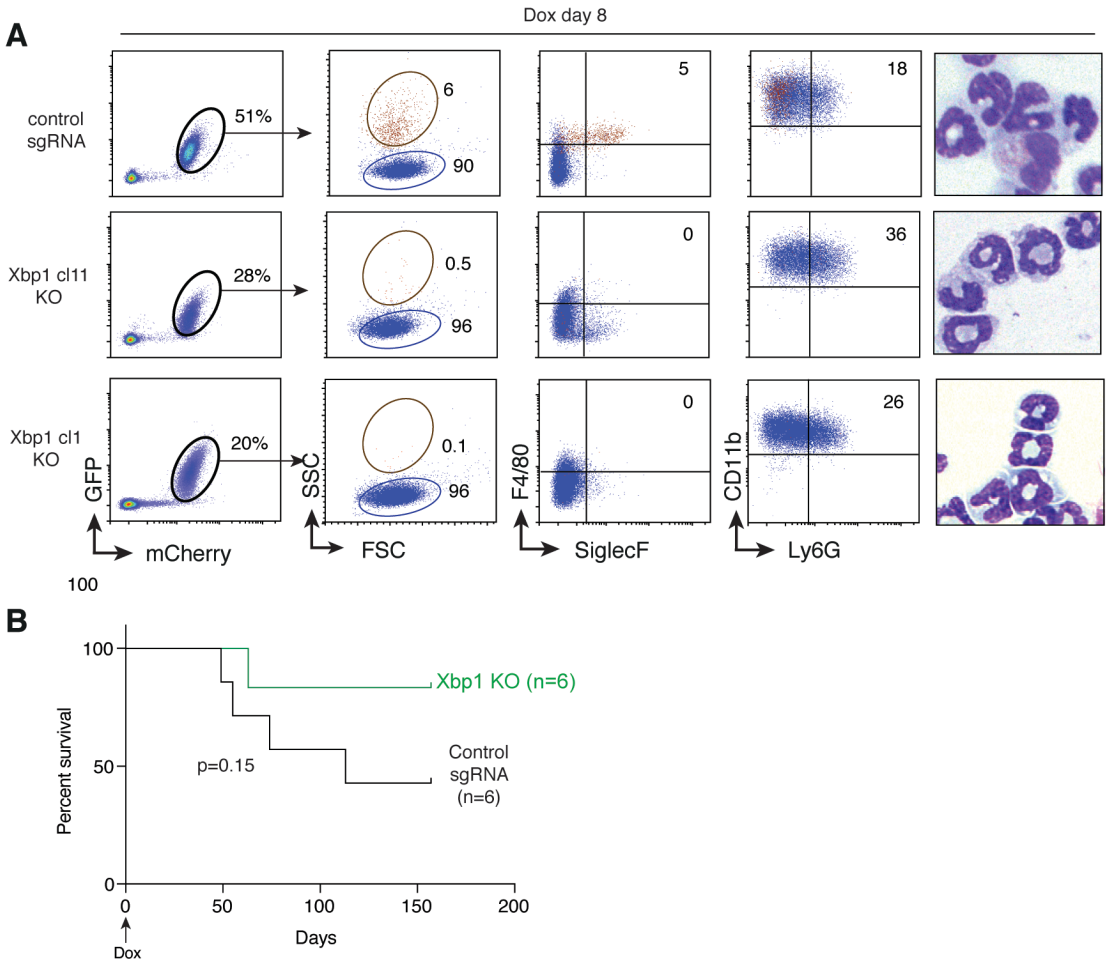


Figure 4.4 Loss of Xbp1 expression prevents eosinophil differentiation of AML246 mCherry

(A) Flow cytometry analysis of two independent AML246 mCherry Xbp1 KO clones (second and third row) transplanted into Rag 1 -/- mice that were treated with Dox for 8 days. Each row is a single representative mouse of 3 mice per clone. The immunophenotype of the AML derived population present after 8 days on Dox was determined by initially gating on mCherry positive cells, followed by analysis of their FSC/SSC profile along with the expression of myeloid markers F4/80, Siglec-F, CD11b and Ly6G. Cytospins of the AML derived population residing in the BM was performed on FACs sorted mCherry positive cells (sytox blue was used as the viability marker). (B) Kaplan Meier survival analysis of mice transplanted with either AML246 mCherry Xbp1 KO or AML246 mCherry sgCON cells that were subsequently treated with Dox following disease establishment, as of Day 150 on Dox (3.5.19). Experiment will terminate once mice reach 200 days on Dox. Mantel-Cox analysis was performed to determine statistical significance (p=0.15)

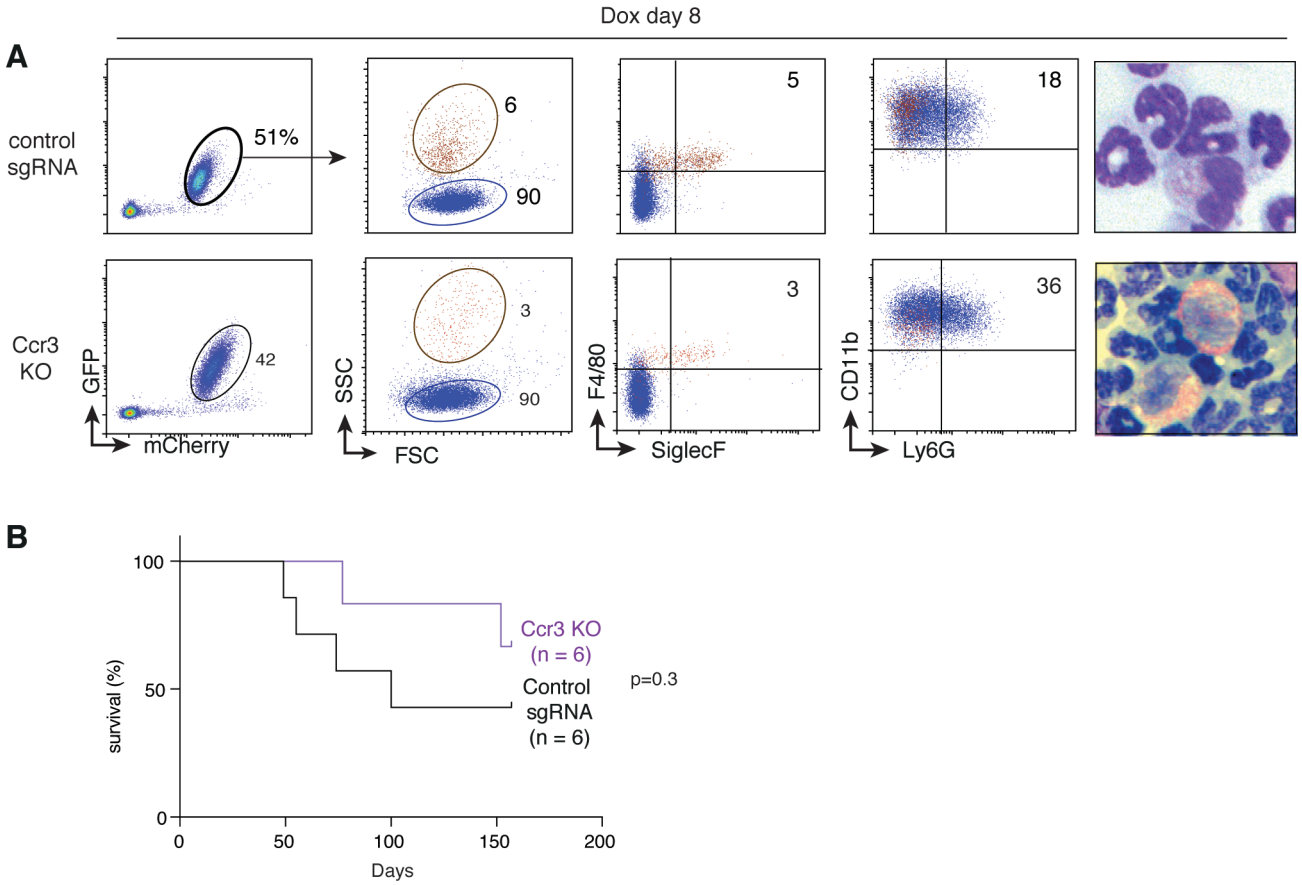


Figure 4.5 Ccr3 is not required for AML blast differentiation along eosinophil lineage

Flow cytometry analysis AML246 mCherry Ccr3 KO clone 6 transplanted into Rag 1 -/- mice that were treated with Dox for 8 days. The immunophenotype of the AML derived population present after 8 days on Dox was determined by initially gating on mCherry positive cells, followed by analysis of their FSC/SSC profile along with the expression of myeloid markers F4/80, Siglec-F, CD11b and Ly6G. Flow cytometry data is of a single representative mouse of 3 mice analysed from this Ccr3 KO clone. Cytospins of the AML derived population residing in the BM was performed on FACs sorted mCherry positive cells (sytox blue was used as the viability marker). (B) Kaplan Meier survival analysis of mice transplanted with either AML246 mCherry Ccr3 KO or AML246 mCherry sgCON cells that were subsequently treated with Dox following disease as of day 157 on Dox. Experiment will terminate once mice have reach 200 days on Dox. Mantel-Cox analysis was performed to determine statistical significance (p=0.3).

Chapter 5: Interrogating PU.1 dependent and independent functions of commonly occurring AML oncogenes.

5.1 Introduction

AML is a genetically heterogenous disease involving a number of driver mutations (as discussed in Chapter 1). A number of genetic lesions found in AML primarily act to block differentiation of the transformed myeloid blast. Previous literature has shown that a number of these differentiation blocking mutations dysregulate PU.1 function in order to drive AML. As discussed in Chapter 3, AML246 and AML410 leukemic models generated in the Dickins Lab are both mouse models of AML driven by the shRNA targeting PU.1. In these AML cells, the absence of Dox allows for the expression of the shPU.1 hairpin. As a result of PU.1 knockdown, AML cells proliferate uncontrollably *in vitro* and *in vivo*. Administration of Dox restores endogenous PU.1 levels, triggering leukemic cells to differentiate into predominately mature myeloid cells (neutrophils for AML246 and macrophages for AML410), followed by their cell death. Thus, using a cell line where it is possible to toggle endogenous PU.1 expression, this chapter looks to assess the ability of commonly occurring, mutually exclusive oncogenes to disrupt PU.1 function. These experiments may also provide a useful platform to determine the mechanisms of how given oncogenes are able to suppress PU.1 activity, as well as the PU.1 independent functions these oncogenes may have in order to drive AML.

5.2 Competition assay to determine oncogene inhibition of PU.1 mediated leukemic cell differentiation.

As mentioned above and throughout Chapters 3 and 4, AML246 is an AML cell line driven by a Dox-regulatable shPU.1 hairpin. Restoration of endogenous PU.1 (with Dox) drives leukemic cell differentiation. Therefore, overexpressing genetic lesions of interest in AML246 (or AML410) can be used to determine whether any given genetic lesion is able to block PU.1 mediated leukemic cell differentiation.

To determine this, a competition assay was established *in vitro*. AML246 and AML410 were infected with vectors where oncogene expression is linked to mCherry by an IRES element. Following retroviral transduction, the mixed population of infected (mCherry positive, oncogene expressing) and uninfected (mCherry negative) cells is treated with Dox to restore endogenous PU.1 and drive leukemic cell differentiation (Fig 5.1A). If the oncogene blocks

PU.1 induced differentiation, cells that express the oncogene would be less susceptible to differentiation, and thus survive and continue to proliferate in an immature state. Conversely, the uninfected AML246/AML410 cells will differentiate in response to PU.1 restoration, and thus undergo cell cycle arrest followed by cell death. Therefore, by assessing the percentage of mCherry positive cells that remain in the viable cell population over a given time course, we can determine if the expression of the class II oncogene can block PU.1 induced differentiation.

5.3 Cloning differentiation blocking oncogenes into stable vectors encoding mCherry reporter

To distinguish between AML246 cells that were either infected or not infected with the oncogene of interest, the oncogenes selected for these experiments were expressed in a common stable empty mCherry vector via Gibson Assembly. Briefly, Gibson Assembly is a novel technique that allows for the joining of multiple DNA fragments through a single isothermic reaction. This requires the presence of 20-40 base pair (bp) overlaps between adjacent DNA fragments. In the single isothermic reaction, exonuclease mediated digestion of 5' ends of the DNA fragments will yield 'sticky ends'. The complementary sticky ends of each DNA fragment will then be joined together with the assistance of DNA polymerase and ligase , resulting in the joining of multiple DNA fragments into one linearized or circular fragment. For the purposes of our experiments, a stable empty vector encoding an mCherry reporter (MICR) was used as the vector backbone for all oncogenes of interest. The MICR vector was linearized via Xho1 Eco1 restriction digest. PCR primers were designed to amplify individual oncogenes of interest, however importantly each PCR primer also contained overhangs that were complementary to the 5' or 3' ends of the vector backbone. Using this approach, all oncogenes of interest were successfully cloned into a stable mCherry vector. These mCherry retroviral vectors encoding the oncogene of interest were then infected into AML246 or AML410) cells *in vitro* for the competition assay described above.

5.4 Stable expression of independent shPU.1 hairpin provides survival advantage for AML246 clone 1 following endogenous PU.1 restoration in vitro

As mentioned in §1.3.4, genome sequencing of cohorts of AML patients revealed that many of the differentiation blocking mutations in AML rarely co-occur within the same patients (Ley et al., 2013; Papaemmanuil et al., 2016). Hence, these mutually exclusive mutations were selected for these experiments to determine if their differentiation blocking properties could

potentially be a result of their ability to dysregulate PU.1 function. To assess the ability of chosen oncogenes to perturb PU.1 function in our competition assay, both positive and negative controls were carefully chosen for these experiments. As a negative control AML246 and AML410 were infected with an empty mCherry vector (MICR) (Fig 5.1B). Overexpression of MICR provided no selective advantage for infected cells, resulting in a steady percentage of mCherry positive representation in Dox cultured media after 12 days (relative to day 0) (Fig 5.1C, black lines). As a positive control, AML246 clone 1 cells were infected with an independent mCherry labelled shPU.1 hairpin. Whereas the percentage of shPU.1 infected AML246 cells cultured in untreated media was relatively steady over the time course, AML246 cells infected with the shPU.1 hairpin had a significant competitive advantage over non-infected cells in Dox cultured media (Fig 5.1C, first graph). Hence, a stable expression of the second shPU.1 hairpin makes AML246 cells resistant to differentiation induced by shutdown of the Dox-regulatable hairpin as expected, resulting in increased representation of mCherry positive cells in Dox cultured media after 12 days.

5.5 Overexpression of commonly occurring oncogenes provides survival advantage for AML246 infected cells following PU.1 restoration.

Recent studies involving the genome sequencing of large cohorts of AML patients (described in §1.3.4) has also revealed remarkable mutual exclusivity between a number of commonly occurring mutations such as PML-RARα, AML1-ETO, MLL-AF9, and NPM1c. Each oncogene has independently been shown to disrupt PU.1 function in other models (X. Gu et al., 2018; Mueller et al., 2006; Vangala et al., 2003; Zhou et al., 2014). To determine whether these oncogenes also perturb PU.1 function in our inducible PU.1 AML model, AML246 cells were also infected with mCherry labelled vectors encoding the aforementioned oncogenes. Percentage of infection varied between the different vectors, with vectors encoding large fusion oncogenes (MLL-AF9, PML-RARα and AML1-ETO) typically having a lower efficiency (Fig 5.1B). Following infection, cells were then treated with Dox to determine whether AML246 oncogene cells had a selective advantage over non-infected cells upon triggering of PU.1 induced differentiation and apoptosis. AML246 cells overexpressing fusion oncogenes AML1-ETO, MLL-AF9 and PML-RARα all resulted in positive selection in the presence of Dox (compared to uninfected AML246 cells) (Fig 5.1C). The overexpression of NPM1c however did not provide a statistically significant selective advantage in the presence of Dox (Fig 5.1C). Interestingly, PML-RARα infection was not well tolerated by untreated cells, resulting in a

significant drop in mCherry positive representation in first 7 days of Dox treatment), but was remarkably selected for upon Dox induced differentiation (Fig 5.1C).

5.6 Knockdown of RUNX1 expression leads to positive selection of AML246 following endogenous restoration of PU.1

The RUNX1 transcription factor regulates corepressor activity of PU.1 during myeloid cell differentiation (Hu et al., 2011). Incidentally, RUNX1 mutations are also a common driver mutation of AML that does not co-occur with other differentiation blocking mutations (Ley et al., 2013; Papaemmanuil et al., 2016). Interestingly knockdown of RUNX1 in AML246 clone 1 was also positively selected for upon the restoration of PU.1 (Fig 5.1C), suggesting that much like the oncogenes tested above, loss of RUNX1 expression may directly dysregulate PU.1-mediated differentiation and apoptosis.

5.7 Overexpression of LIC marker Gpr56 does not provide selective advantage for AML246 infected cells.

Gpr56 is a stable marker of leukemia initiating cells (LICs) in AML. Although does not fall into the category of commonly occurring, mutually exclusive driver mutation of AML, previous RNAseq analysis of AML246 revealed Gpr56 as the most repressed gene following PU.1 restoration *in vitro* (McKenzie et al, 2019 in press). Therefore, to determine whether ectopic expression of Gpr56 also perturbed PU.1 function, a mCherry labelled Gpr56 vector was infected into AML246 (Fig 5.1B, last panel). Interestingly, overexpression of Gpr56 did not provide a selective advantage for infected AML246 cells (Fig 5.1 C), suggesting that it was unable to directly dysregulate PU.1 function.

5.8 Oncogene-mediated dysregulation of PU.1 activity was also observed in independent shPU.1 driven AML models

As further verification of our results, the same experiment was also performed in an independent AML246 clone (AML246 cl2). Consistent with the results using AML246 cl1 cells, the expression of genetic lesions AML1-ETO, PML-RARα, shRUNX1 and MLL-AF9 all provided the AML246 cl2 cells with a selective advantage in the presence of Dox (Fig 5.2B). Similar to AML246 cl1 however, overexpression of Gpr56 in AML246 cl2 did not provide a selective advantage for AML cells following PU.1 restoration (Fig 5.2B).

Importantly, whereas the selective advantage of NPM1c overexpression was not significant in AML246 cl1, ectopic expression of this oncogene provided a statistically competitive advantage when infected into AML246 cl2 cells (Fig 5.2B). Although ectopic expression of the oncogenes tested (with exception to Gpr56) provided selective advantage for AML246 cl2 cells in the presence of Dox, overall viability of Dox treated cells declined rapidly after 7 days on Dox (Fig 5.2C).

Lastly, the competition assay was also performed on an independent inducible PU.1 model, known as AML410. Briefly, similar to AML246, AML410 is also driven by the expression of a Tet-regulated shPU.1 hairpin on a p53-/- genetic background, however the shPU.1 hairpin used for AML410 (shPU.1 1293) differs from AML246 (shPU.1 200). Furthermore, the leukemia from the shPU.1 1293 hairpin arose from an independent mouse from AML246, and is therefore genetically distinct from AML246 (details of genetic background can be found in McKenzie et al, 2019, in press). Retroviral mCherry vectors encoding the oncogene of interest was infected into AML246 cells at various efficiencies (Fig 5.3A). Much like the results from both AML246 clones, overexpression of given oncogenes (except Gpr56) resulted in a selective advantage for these cells compared to un-infected cells in the presence of Dox (Fig 5.3B). With the exception of AML410 cells infected with shPU.1, AML1-ETO and PML-RARα, despite the selective advantage of oncogene expressing cells, cell viability continued to decline following 8-12 days of Dox treatment (Fig 5.3C). Therefore, although the ectopic expression of these oncogenes provides AML410 infected cells with an overall survival advantage, these are unlikely to remain blast-like in the presence of Dox, as infected cells still ultimately succumb to apoptosis.

5.9 Discussion

5.9.1 AML246 and AML410 are ideal AML models to determine oncogene impact on PU.1 function

In recent years, the use of next generation sequencing has uncovered the genetic and epigenetic landscape of AML (Döhner et al., 2017; Ley et al., 2013; Papaemmanuil et al., 2016). Through genome and exome sequencing of AML patient samples, it becomes apparent that many of the oncogenic mutations that block differentiation of immature, transformed myeloid blasts are mutually exclusive (Döhner et al., 2017; Ley et al., 2013). The lack of co-occurrence is likely due to these mutations affecting the same process to drive the differentiation block. PU.1 is a

master regulator of myeloid cell differentiation (Fisher et al., 1998). Furthermore, a number of oncogenes such as AML1-ETO, PML-RARα and NPM1c physically interact with PU.1 to inhibit its function (X. Gu et al., 2018; Vangala et al., 2003; K. Wang et al., 2010), suggesting that oncogenes that exhibit a differentiation blocking phenotype are likely to do so via the dysregulation of PU.1. Therefore, to test our hypothesis, commonly occurring, mutually exclusive oncogenic lesions (AML1-ETO, PML-RARα, MLL-AF9, NPM1c, and inhibition of RUNX1) were selected for our assay to determine if they could perturb PU.1 function in our PU.1 driven AML model. AML246 and AML410 are both leukemic cell lines regulated by PU.1 expression; such that differentiation of AML blasts is solely driven by restoration of endogenous PU.1. Therefore, ectopic expression of differentiation blocking oncogenes in this cell line provides an effective screening platform to determine if the expression of a given oncogene is sufficient to block PU.1 differentiation in our model.

For a future aim beyond the scope of this PhD, we hope to perform RNAseq analysis of oncogene infected AML246 cells in the presence and absence of Dox in comparison to uninfected cells to uncover the mechanism of PU.1 suppression, as well as oncogene functions that are independent of PU.1 dysregulation. Furthermore, although mCherry is a surrogate reporter for the expression of the oncogene of interest, it does not necessarily correlate to the degree of oncogene expression. Therefore for further validation, it would be worth considering performing western blot and RT-PCR analysis to gain a true measure of oncogene expression in the infected cells.

5.9.2 Oncogene infected AML246 and AML410 cells are negatively selected for in untreated media

Surprisingly, in each AML246 and AML410 cell line tested, cells infected different oncogenes such as AML1-ETO, NPM1, MLL-AF9 and PML-RARα were at a competitive disadvantage compared to non-infected AML246/AML410 cells when cultured in the absence of Dox (Fig 5.1B, Fig 5.2B and Fig 5.3B). This is indicated by the gradual decline of mCherry positive representation when mixed populations were cultured in Dox-free media. Hence, although inhibition of PU.1 in AML246 normally promotes continued proliferation due to a differentiation block, further downregulation of PU.1 by the oncogenes mentioned above may push PU.1 levels below the threshold required for cell survival. Thus, minimal levels of PU.1 expression may also be required for the survival of AML blasts. This is consistent with PU.1

function described in the literature, where PU.1 expression is indispensable for HSPC function (Fisher et al., 1998). Furthermore, in the context of AML, PU.1 is often downregulated in many subtypes of AML (including patients harbouring AML1-ETO, NPM1 and PML-RARα mutations), however it is rarely completely lost (Zhu et al., 2012). Interestingly, further downregulation of PU.1 in AML also leads to AML blast apoptosis (Antony-Debre et al., 2017). Interestingly, in the case of MLL-F driven leukemias, PU.1 is essential for the promotion of cell cycle progression and inhibition of AML cell apoptosis, partially via the MEIS/HOX pathway (Zhou et al., 2014). Therefore, low PU.1 levels in MLL-F expressing cells (such as MLL-AF9, AML246 cells) may be suboptimal for survival, resulting in gradual loss of representation when co-cultured with non-infected AML246 cells.

Oncogene expression resulted in selective disadvantage of leukemic blasts, however the degree of selective disadvantage varied between the different oncogenes tested (e.g. selective disadvantage is dramatic in PML-RARα infected cells compared to those infected with NPM1c or the hairpin targeting Runx.1). It is currently unclear as to why this may be the case, however this variation in selective disadvantage may attest the PU.1 related function of the given oncogenes themselves. For example, both PML-RARα and AML1-ETO are known to physically inhibit PU.1 function (Vangala et al., 2003; K. Wang et al., 2010). In both these cases the dramatic selective disadvantage AML246/AML410 cell lines have when infected with such oncogenes could be due to the inhibition of PU.1 being one of their primary functions in driving AML. Hence, further downregulation of PU.1 by these oncogenes in a cell line that already expresses low levels of PU.1 (in untreated media) not only results in a selective disadvantage, but may also showcase the potency of PU.1 inhibition by these given oncogenes in their ability to drive AML. This hypothesis is strengthened by the fact that the most dramatic selective advantage that is observed following the restoration of PU.1 (on Dox) is seen in both PML-RARα and AML1-ETO infected cells. Therefore this particular sensitivity to PU.1 toggling in PML-RARα and AML1-ETO infected cells may indicate how PU.1 perturbation may be the primary function of these given oncogenes in driving AML. Conversely, the oncogenic properties of other differentiation blocking mutations such as NPM1c overexpression may not solely rely on the downregulation of PU.1. Therefore, when NPM1c is infected into AML246/AML410, there is only a slight selective disadvantage in untreated media, as further PU.1 downregulation may not be so severe. Alternatively, the variation in the level of selective disadvantage could also be due to differences in retroviral integration and levels of oncogene expression in the infected cells. To determine whether or not this is the case,

it would be important to look at transcriptional levels or protein levels (via qPCR/western blot analysis) of not only PU.1 but the oncogene of interest in infected AML246/AML410 cells to determine the degree of PU.1 downregulation and oncogene expression.

5.9.3 Ectopic expression of PU.1 binding oncogenes AML1-ETO and PML-RARα provide selective advantage for AML246/AML410 cells following Dox treatment

Restoring endogenous PU.1 drives leukemic cell differentiation and subsequent apoptosis of AML246/AML410 cells. Both AML1-ETO and PML-RARα physically bind PU.1 to initiate the differentiation block (Vangala et al., 2003; K. Wang et al., 2010). In our AML246/AML410 models, the overexpression of commonly occurring fusion oncogenes AML1-ETO and PML-RARα perturbed PU.1 mediated differentiation of AML blast, resulting in the selective advantage of AML1-ETO/ PML-RARα infected AML246/AML410 cells. Whereas in previous studies, PU.1 has been overexpressed in AML1-ETO or PML-RARα expressing cell lines to overcome the differentiation block (and drive apoptosis) (Durual et al., 2007; K. Wang et al., 2010), the overexpression of both fusion oncogenes upon PU.1 restoration in our AML cell line resulted in a significant survival advantage for these cells. Despite the clear survival advantage of oncogene expressing cells following endogenous PU.1 restoration, some AML1-ETO and PML-RARα expressing AML246/AML410 cells were likely to have undergone differentiation and apoptosis, contributing to the large drop in cell viability of Dox treated cultures. However, this is likely due to technical issues inherent to retroviral infection of cells. The number of integrations to the AML246/AML410 genome may vary greatly between infected cells, and thus the expression levels of the oncogenes are likely to vary also. Therefore it is difficult to determine the degree of oncogene expression required to properly disable PU.1 restoration. Consequently, AML246/AML410 cells with suboptimal AML1-ETO or PML-RARα overexpression may still undergo differentiation and apoptosis upon Dox treatment. However, given the gradual increase in oncogene expressing AML246/AML410 cells (AML1-ETO, PML-RARα, NPM1c, MLL-AF9 and shRUNX1) following Dox treatment, it is likely that optimal levels of oncogene expression is capable of blocking PU.1 induced differentiation of AML246/AML410 *in vitro*. For future experiments, it will be important to determine whether the selective advantage of mCherry oncogene expressing cells treated with Dox is coupled with the absence of mature myeloid marker induction (such as CD11b or Gr-1) to confirm that the positive selection is due to a lack of leukemic cell differentiation.

5.9.4 Suppression of coregulator RUNX1 (AML1) disrupts PU.1 function in AML246 and AML410

Point mutations to the non-truncated form of AML1 (otherwise known as RUNX1) are often associated with myelodysplastic syndrome (MDS) (Steensma et al., 2015), or preleukemic mutations that initiate clonal haematopoiesis (Steensma et al., 2015), however it is also a commonly occurring driver mutation found in AML (Gaidzik et al., 2016). RUNX1 regulates co-repressors of PU.1 such that RUNX1 haploinsufficiency increased coimmunoprecipitation of corepressors such as Eto2 and Hdac2 to PU.1 (Hu et al., 2011; Huang et al., 2008). Therefore, predictably in our model, hairpin mediated knock down of RUNX1 in AML246 resulted in a positive selection for shRUNX1 infected AML cells upon Dox treatment, likely due to the sustained activation of PU.1 co-repressors despite endogenous restoration of PU.1. As such, PU.1 levels remain low in the presence of Dox, preventing these cells from differentiating and undergoing apoptosis. To confirm this, we intend to determine whether this is the case via RT-PCR and Western Blot analysis to determine PU.1 levels in the presence of shRUNX1.

5.9.5 NPM1c overexpression dysregulates PU.1 mediated differentiation

Whereas the interactions between PU.1 and a number of oncogenes (such as AML1-ETO and PML-RARα) have previously been identified, the link between other common differentiation blocking AML mutations have been less clear. NPM1c is a chaperone protein that is also among the most commonly mutated driver mutations found in AML (Ley et al., 2013). Intriguingly, the NPM1c mutation also rarely co-occurs with fusion oncogenes such as AML1-ETO and PML-RARα AML patients, suggesting functional redundancy between NPM1c and these differentiation blocking mutations (Ley et al., 2013). Until recently, the leukemogenic properties of NPM1c have been attributed to the mutant oncoproteins misplacement of PU.1 protein into the cytoplasm (X. Gu et al., 2018). Given that PU.1 regulates itself through and autoregulatory loop (Chen et al., 1995), the accumulation of cytoplasmic PU.1 results in downregulated expression of the transcription factor, thus causing a differentiation block in the cell. Consistent with these recent findings, our competition assay also shows a significant competitive advantage for NPM1c expressing AML246/AML410 cells in the presence of Dox, suggesting that the expression of the oncogene is protective of PU.1 induced differentiation and apoptosis of leukemic blasts. For future experiments, it would be interesting to perform

immunofluorescence microscopy analysis to determine and compare localization of PU.1 protein in AML246/AML410 NPM1c infected and non-infected cells. Given the description of NPM1c function in the literature (X. Gu et al., 2018), we would anticipate observing localization of PU.1 protein in the cytoplasm of NPM1c expressing cells following Dox treatment, whereas Dox treatment on non-infected cells should result in localization of PU.1 protein in the nucleus. Nuclear and cytoplasmic fractionation and subsequent western blot analysis for PU.1 protein in each fraction may also be used to determine whether overexpression of NPM1c does misplace PU.1 protein in the cytoplasm of AML246/AML410 cells.

5.9.6 MLL-AF9 overexpression provides selective advantage in the presence of Dox

Consistent with the literature, ectopic expression of oncogenes such as NPM1c, AML1-ETO, PML-RARα and RUNX1 knockdown lead to disrupted PU.1 mediated differentiation of AML blasts *in vitro*. MLL-fusion (F) translocations are relatively uncommon mutations in AML (<5%) (Zhou et al., 2014). However much like the oncogenes mentioned previously, it also do not co-occur with differentiation blocking mutations (Ley et al., 2013). As mentioned previously, PU.1 is required for MLL leukemic cell growth and prevention of apoptosis, thus explaining the selective disadvantage of MLL-AF9 infected AML246/AML410 cells in untreated media (Fig 5.2B, Fig 5.3B). Interestingly, much like the other oncogenes tested in this competition assay, overexpression of the fusion oncogene MLL-AF9 also provided selective advantage for AML cells following restoration of endogenous PU.1. Therefore, in this context, Dox treatment may restore PU.1 to a level optimal for MLL-AF9 expressing AML246/AML410 cells, such that its selective advantage is a result of promoting leukemic cell growth rather than a block in differentiation.

5.9.7 The LSC marker Gpr56 is unable to block PU.1 mediated differentiation

Lastly, Gpr56 is an adhesion molecule that functions as a robust marker of LICs (C. Pabst et al., 2016). Although the function of Gpr56 remains unclear, Gpr56 expression levels correlates with poor survival in AML patients (Caroline Pabst et al., 2016). Interestingly, RNAseq data analysis described in McKenzie et al. 2019 revealed that Gpr56 was the most downregulated gene following PU.1 restoration in AML246 cells. Hence, we speculated that a primary function of Gpr56 was to block PU.1 induced differentiation of transformed myeloid blasts to drive AML. Despite this, from the competition assays described in this chapter, we found that

overexpression of Gpr56 was unable to perturb PU.1 restoration, given that there was no change in Gpr56 AML246/AML410 cell representation following 7-12 days of Dox treatment. Therefore it is unlikely that the function of Gpr56 is to block PU.1 induced differentiation. More recently, it has been shown that suppression of Gpr56 in AML cells induces apoptosis, suggesting that expression of Gpr56 may have a pro-survival role, rather than a differentiation blocking role for AML cells (Saha et al., 2018).

5.10 Conclusion

To summarize, AML is a genetically heterogenous disease with a range of differentiation blocking driver mutations. Next generation sequencing (NGS) of AML patient samples reveals a striking mutual exclusivity between a number of commonly occurring oncogenes (Döhner et al., 2017; Ley et al., 2013), many of which have previously been identified to directly dysregulate PU.1 function (X. Gu et al., 2018; Vangala et al., 2003; K. Wang et al., 2010; Zhou et al., 2014). Therefore, this genetic data strongly suggests that many of these mutually exclusive oncogenes may converge on PU.1 dysregulation in order to induce a differentiation block and drive disease. AML246 and AML410 are leukemic cell lines that undergo differentiation upon the restoration of endogenous PU.1 (via Dox treatment). Using these cell lines, we found that ectopic expression of a number of these common oncogenes such as NPM1c, AML1-ETO, PML-RARα and RUNX1 knockdown led to a dysregulation of PU.1 mediated differentiation and apoptosis. As such these cells were positively selected for and eventually dominated the previously mixed culture of oncogene infected and non-infected cells. Although further analysis may be required for these assays (such as flow cytometry analysis of mature myeloid markers and PU.1 gene expression analysis of oncogene infected cells via RT-PCR), the competition assays provide sound evidence to suggest that many of these mutually exclusive oncogenes in AML block differentiation via the perturbation of PU.1. Regardless, identifying the expression of differentiation surface markers through flow cytometry (such as upregulation of myeloid lineage markers such as CD11b of Ly6G) or performing cytopsins to determine the morphology of oncogene expressing cells following PU.1 restoration would provide significant insight regarding the differentiation status of the oncogene expressing cells that have a competitive advantage. Through these experiments, it can be determined whether the ectopic expression of a given oncogene truly prevents differentiation upon the restoration of PU.1, or whether differentiation still occurs, but cell death is delayed due to PU.1 independent functions of the given oncogene.

To this point, the competition assay established also provides an ideal platform to investigate PU.1 dependent and PU.1 independent functions of any oncogene of interest. Infection with vectors expressing mCherry reporter allows for the distinction between oncogene expressing and oncogene non-expressing AML246 cells. Furthermore tracking GFP expression allows us to determine whether PU.1 has been knocked down (GFP^{high} = $PU.1^{low}$, GFP^{low} = $PU.1^{ON}$). Therefore using these parameters we are able to sort distinct populations of cells using flow cytometry (FACs) for RNAseq analysis to compare the transcriptional profiles of each population. For example, comparing the transcriptional changes in UT and Dox treated cells in the absence of oncogene to the changes from UT and Dox treated cells in the presence of the oncogene may be able to determine the PU.1 dependent function of the oncogene of interest. Conversely, comparing the RNAseq data from Dox treated cells in the absence or presence of the oncogene may allow us to determine the other functions of the given oncogene independent of PU.1 inhibition. Ultimately, these experiments are beyond the scope of the PhD, however establishing this platform will allow us to uncover novel mechanisms of commonly occurring oncogenes in our AML model.

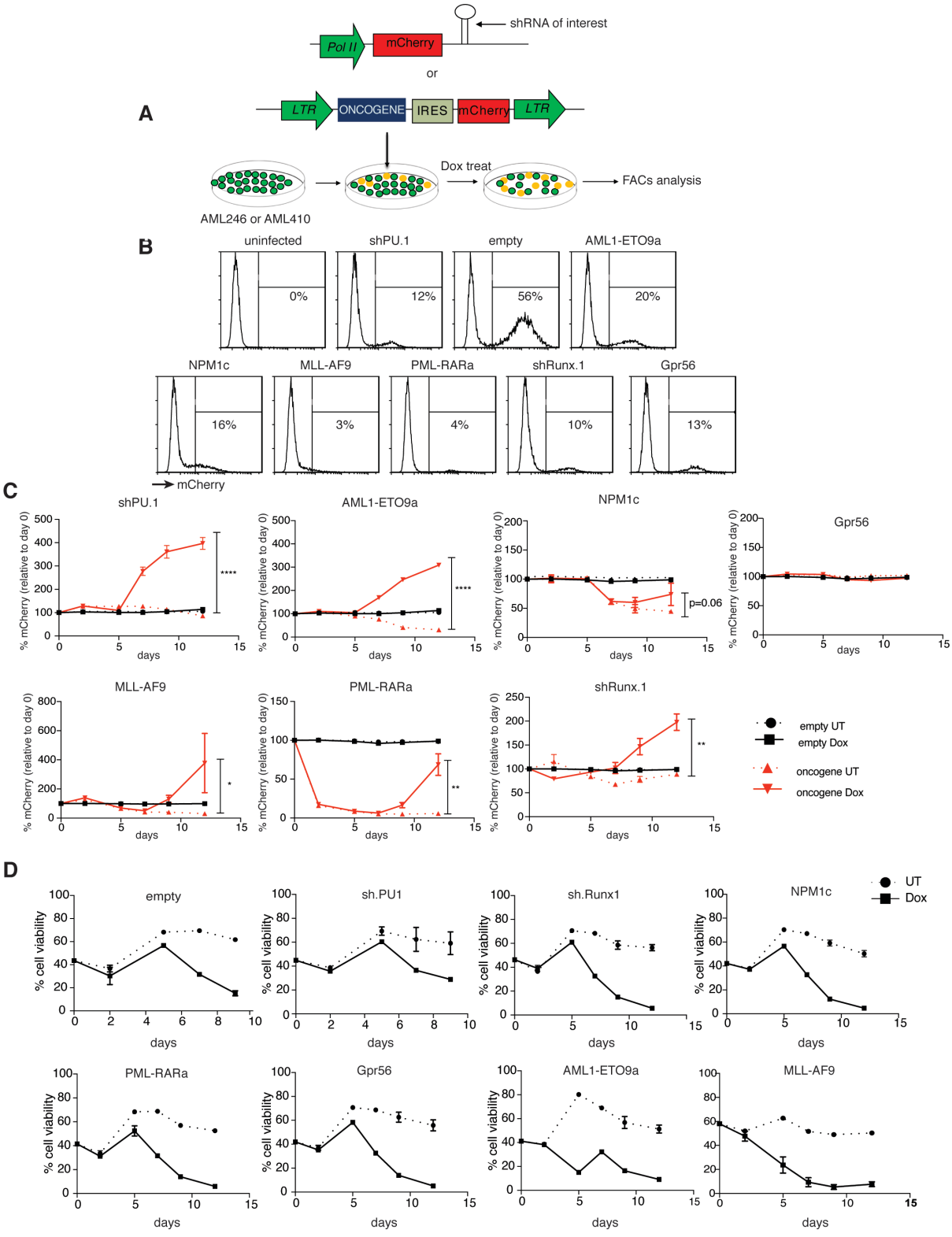

A
shRNA of interest
Pol II
mCherry
or
LTR
ONCOGENE
IRES
mCherry
LTR
Dox treat
FACs analysis
AML246 or AML410
B
uninfected
shPU.1
empty
AML1-ETO9a
0%
12%
56%
20%
NPM1c
MLL-AF9
PML-RARa
shRunx.1
Gpr56
16%
3%
4%
10%
13%
mCherry
C
shPU.1
AML1-ETO9a
NPM1c
Gpr56
MLL-AF9
PML-RARa
shRunx.1
% mCherry (relative to day 0)
days
****
p=0.06
*
**
empty UT
empty Dox
oncogene UT
oncogene Dox
D
empty
sh.PU1
sh.Runx1
NPM1c
PML-RARa
Gpr56
AML1-ETO9a
MLL-AF9
% cell viability
days
UT
Dox

Figure 5.1 Selective advantage for oncogene expressing AML246 clone 1 cells following restoration of endogenous PU.1

(A) Experimental outline of competition assay. (B) Percentage of infection of AML246 cl1 cells with mCherry labelled, oncogene expressing vectors. (C) Competition assay comparing percentage of mCherry positive cells in untreated (dotted lines) and Dox treated media (bold lines) following a 12 day Dox treatment time course. Unpaired student t-test performed to determine statistical significance of percentage of mCherry positive cells at day 12 on Dox. **** = $p<0.0001$, *** = $p<0.001$, ** = $p<0.01$, * = p.0.05 (D) % viability of untreated (UT) and Dox treated cultures bulk infected with mCherry labelled oncogene vectors over a 10-12 day time course .

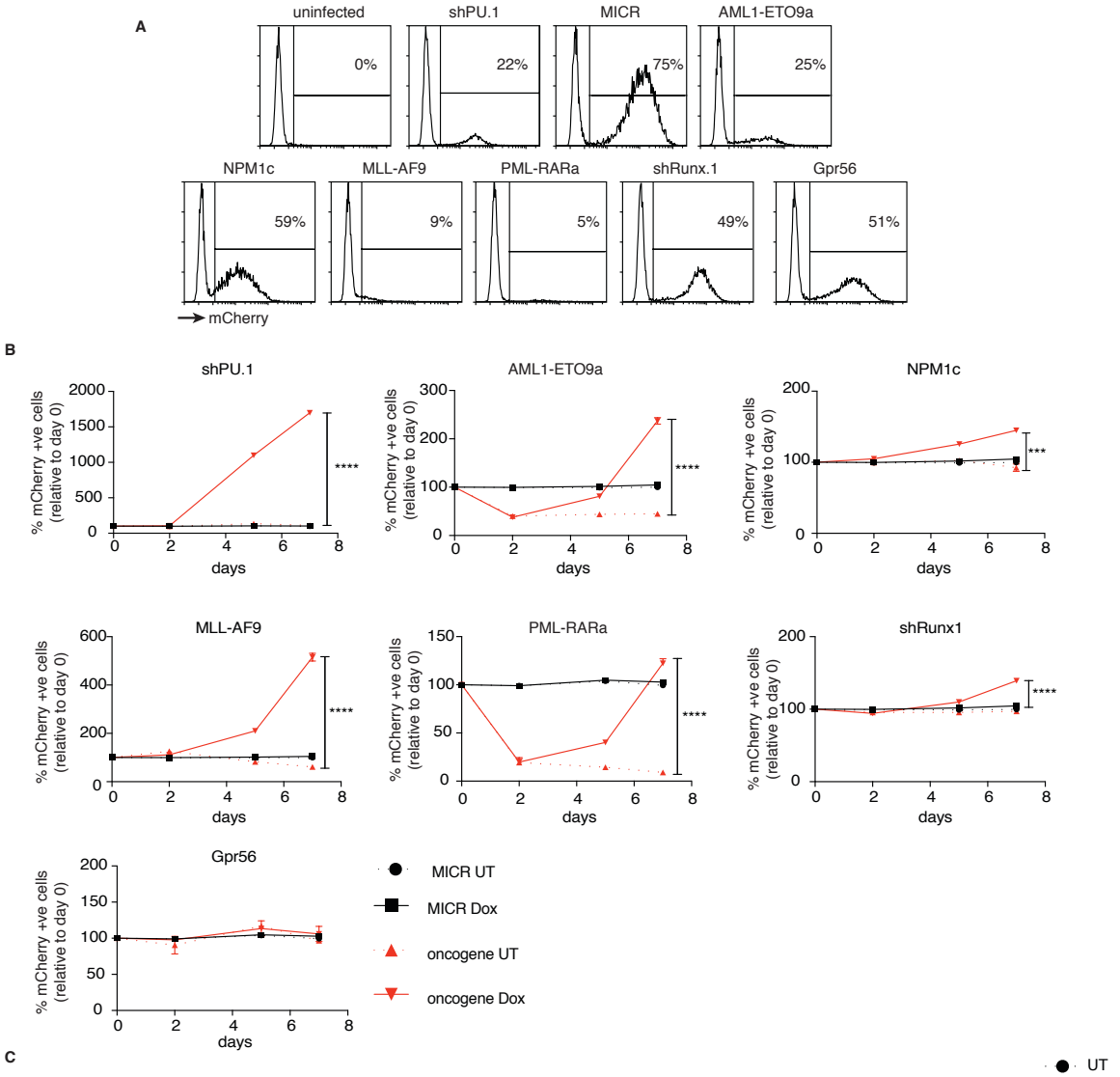

A
uninfected
shPU.1
MICR
AML1-ETO9a
0%
22%
75%
25%
NPM1c
MLL-AF9
PML-RARa
shRunx.1
Gpr56
59%
9%
5%
49%
51%
mCherry
B
shPU.1
AML1-ETO9a
NPM1c
MLL-AF9
PML-RARa
shRunx1
Gpr56
% mCherry +ve cells (relative to day 0)
days
****
***
MICR UT
MICR Dox
oncogene UT
oncogene Dox
C
MICR
sh.PU1
sh.Runx1
NPM1c
UT
Dox
PML-RARa
Gpr56
AML1-ETO9a
MLL-AF9
% cell viability
days

Figure 5.2 Selective advantage for oncogene expressing AML246 clone 2 cells following restoration of endogenous PU.1

(A) Percentage of infection of AML246 cl1 cells with mCherry labelled, oncogene expressing vectors. (B) Competition assay comparing percentage of mCherry positive cells in untreated (dotted lines) and Dox treated media (bold lines) following an 8 day Dox treatment time course. Unpaired student t-test performed to determine statistical significance of percentage of mCherry positive cells at day 8 on Dox. **** = $p<0.0001$, *** = $p<0.001$, ** = $p<0.01$, * = p.0.05 (C) % viability of untreated (UT) and Dox treated cultures bulk infected with mCherry labelled oncogene vectors over an 8-day time course.

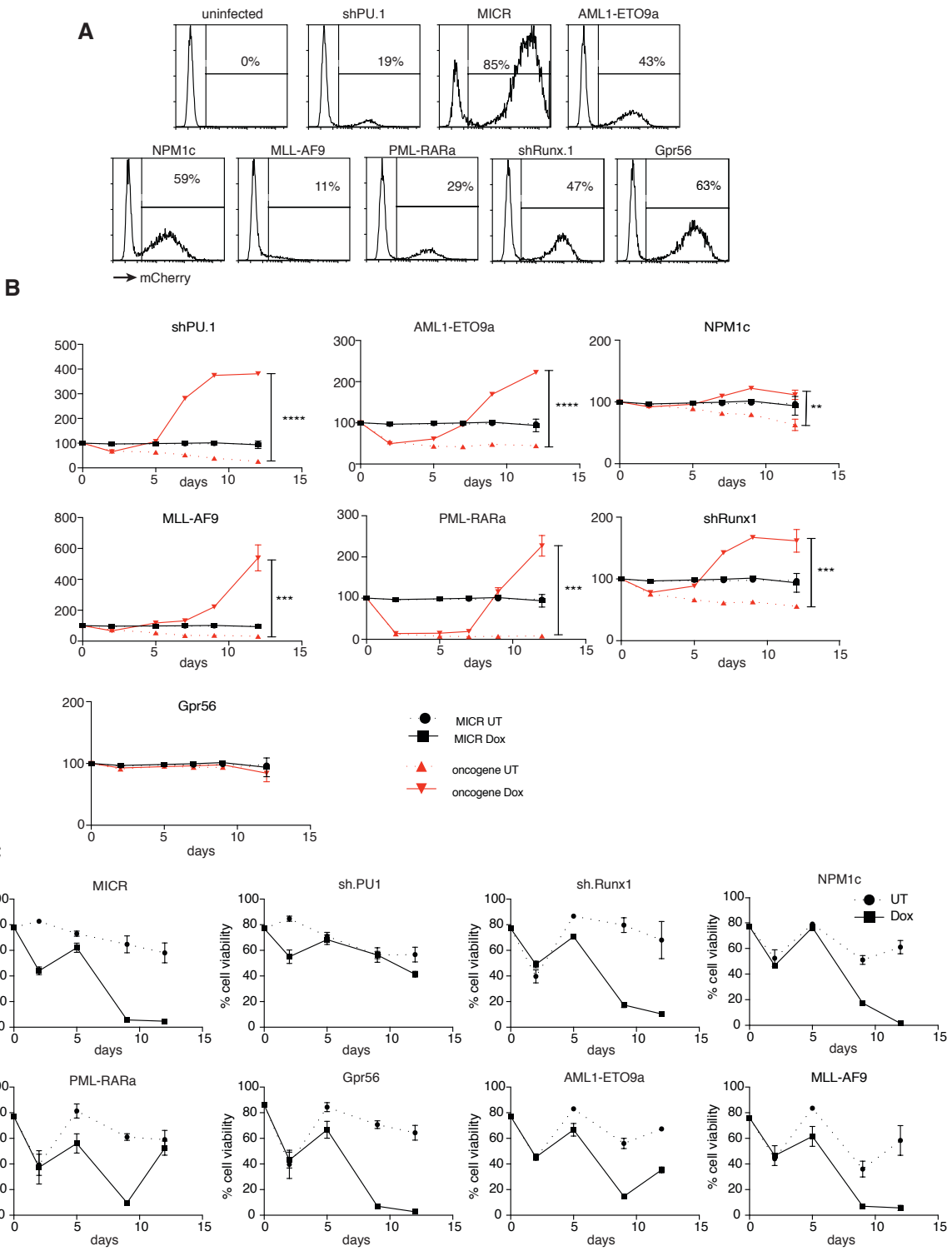

A
uninfected
shPU.1
MICR
AML1-ETO9a
0%
19%
85%
43%
NPM1c
MLL-AF9
PML-RARa
shRunx.1
Gpr56
59%
11%
29%
47%
63%
mCherry
B
shPU.1
AML1-ETO9a
NPM1c
MLL-AF9
PML-RARa
shRunx1
Gpr56
days
MICR UT
MICR Dox
oncogene UT
oncogene Dox
C
MICR
sh.PU1
sh.Runx1
NPM1c
UT
Dox
PML-RARa
Gpr56
AML1-ETO9a
MLL-AF9
% cell viability
days

Figure 5.3 Selective advantage for oncogene expressing AML410 cells following restoration of endogenous PU.1

(A) Percentage of infection of AML246 cells with mCherry labelled, oncogene expressing vectors. (B) Competition assay comparing percentage of mCherry positive cells in untreated (dotted lines) and Dox treated media (bold lines) following a 12 day Dox treatment time course. Unpaired student t-test performed to determine statistical significance of percentage of mCherry positive cells at day 12 on Dox. **** = $p<0.0001$, *** = $p<0.001$, ** = $p<0.01$, * = p.0.05 . (C) % viability of untreated (UT) and Dox treated cultures bulk infected with mCherry labelled oncogene vectors over a 10-12-day time course .

Chapter 6: Perspectives and Future Directions

Currently, chemotherapy remains the gold standard method of treatment of AML patients clinically. However given the genotoxic nature of chemotherapeutic agents as well as the relatively poor success rate of chemotherapy in a wide range of AML subtypes, it has become increasingly necessary for the use of more targeted approaches in the treatment of AML. Famously, ATRA+ATO differentiation therapy targeting the PML-RARα oncogenic mutation has turned a once largely incurable subtype of AML, to an AML subtype with the best prognosis (Abaza et al., 2017). Therefore given the success of differentiation therapy, new differentiation therapy agents have recently emerged (such as HDAC and IDH mutant inhibitors) in the hope of mimicking the success of ATRA based therapies in other subtypes of AML (Amatangelo et al., 2017; Fredly et al., 2013; Quek et al., 2018; Tabe et al., 2007). Whilst the emergence of such targeted therapies is promising, AML patients treated with such differentiation therapy agents still succumb to disease relapse following periods of disease remission (Galeotti et al., 2019). Therefore, although differentiation therapy has been a promising method of treatment in certain subtypes of AML, there remains a significant lack of understanding regarding the biology of AML cell differentiation and clearance that may potentially hinder the efficacy of such therapies.

To investigate the causes of relapse, this theses relied on the use of a mouse model of AML driven by the Tet-regulated, inducible knockdown of the myeloid transcription factor PU.1. On a p53 -/- genetic background carrying an activating Kit mutation and a normal karyotype, the shRNA mediated knockdown of PU.1 resulted in the proliferation of AML blasts. However restoration of endogenous PU.1 (via treatment of Dox) triggered widespread differentiation and clearance of differentiated AML cells *in vivo*. Mice that were treated with Dox were in disease remission for several weeks before ultimately succumbing to disease relapse caused by mutations that allowed for the re-engagement of PU.1 inhibition of AML-derived cells. Intriguingly, during the throngs of AML cell differentiation, AML-derived cells in our model bifurcated into two distinct mature myeloid cell populations. Consistent with the observations made clinically, triggering leukemic cell differentiation largely resulted in the differentiation of AML blasts into neutrophils. Surprisingly, a small population of AML-derived cells also differentiated into mature myeloid cells that greatly resembled eosinophils. Importantly, during periods of disease remission (where AML-derived cells were not detectable in the PB), no AML blasts could be detected, and the only AML-derived cells that could be detected were

exclusively differentiated eosinophils. Therefore, consistent with normal mature myeloid cells, AML-derived neutrophils are rapidly cleared following differentiation (due to their short lifespan), whereas AML-derived eosinophils were able to persist due to their inherently longer lifespan (Uhm et al., 2012). As such, there is a greater chance for relapse causing mutations to occur in AML-derived eosinophils given that they persist for a longer period of time. From this it was hypothesized that the source of relapse in our AML model may come from differentiated AML-derived eosinophils. To investigate this, CRISPR-Cas9 technology was used to prevent AML cell differentiation into the eosinophilic lineage by knocking out genes instrumental in the differentiation of eosinophils, namely Gata1 and Xbp1. Remarkably, prevention of AML cell differentiation into the eosinophil lineage (by either knocking out Gata1 or Xbp1) resulted in total clearance of AML-derived cells in the majority of mice, leading to a significant reduction in the rate of relapse compared to leukemias that were able to bifurcate into the persistent eosinophil lineage.

6.1 Source of relapse may originate from both LSCs and mature AML-derived cells

Historically, relapse in AML (treated either with chemotherapy or differentiation therapy) was believed to originate from a rare population of LSCs that were not responsive to the initial therapy. Bulk leukemic cells undergo therapy induced apoptosis or clearance, whereas a dormant population of stem cell-like leukemic cells are insensitive to treatment and consequently propagate the relapse (Shlush et al., 2017). Interestingly, in our mouse model of AML described, we find that only differentiated AML-derived cells remain during disease remission, and by preventing leukemic cell differentiation in the long living eosinophil lineage, it is possible to greatly reduced the rate of relapse in these mice. Relapse still occurs in a small percentage of mice transplanted with Gata1 KO or Xbp1 KO AMLs. Our explanation for this is that relapse causing events could also be occurring in the AML-derived neutrophil population during the short period of time they are alive, hence relapse can still occur in the absence of an AML-derived eosinophil population. However despite the uniform differentiation of AML cells following the restoration of PU.1, as well as the lack of evidence of rare, persistent blast cells during disease remission, our data does not discount the possibility of LSCs being the source of relapse in some cases. It is possible that the prevalence of non-responsive LSCs is beyond our detection limit, hence relapse from these eosinophil-less AMLs could be caused by LSCs. Despite this, the fact that prevention of eosinophilic differentiation in our Gata1 KO or Xbp1 KO experiments can greatly reduce relapse suggests that relapse can

also come from 'bulk' AML-derived cells, a concept that has previously been unappreciated. This of course, has significant therapeutic implications, and suggests that all AML-derived cells must be targeted to ensure the absence of relapse following differentiation therapy.

The capacity of differentiated AML-derived cells to seed relapse has not been reported previously. Our findings from this thesis also emphasizes the importance of lineage specific differentiation of AML blasts and how this may potentially impact the likelihood of relapse. As mentioned previously, normal mature myeloid cells possess varying half-lives. Neutrophils have a notoriously short lifespan, whereas monocytes and eosinophils can survive for long periods of time. Therefore, given the inherently short lifespan and effective clearance of neutrophils, skewing AML cell differentiation away from longer living myeloid lineages (such as monocytes and eosinophils) and towards a rapidly cleared myeloid lineage (such as neutrophils) could potentially greatly impact the likelihood of relapse in patients that are undergoing differentiation therapy. Further investigation to this in other models of AML may be required to determine if this is truly the case.

6.2 Clinical implications and the importance of patient sampling

As mentioned previously in Chapter 5, PU.1 itself is rarely mutated in AML, however many of the commonly occurring oncogenes that drive AML have all been shown to directly inhibit PU.1 function (more details in Chapter 5). Hence, despite AML246/410 being a single mouse model of AML and differentiation therapy, given that PU.1 is functionally compromised in a large percentage of AML patients, findings from this model are likely to be widely applicable to other models of AML as well as what may be occurring clinically. In addition to xenograft models (briefly outlined in §4.10.5), in future experiments we would like to determine the clinical relevance of our findings in AML246, and address the prevalence of multilineage differentiation following differentiation therapy treatment in AML patients. Differentiation of AML cells often seen as neutrophilic clinically, however there has also been some evidence of monocytic differentiation of AML blasts following ATRA treatment of APL patients (Gocek et al., 2011; Naeem et al., 2006). Furthermore, treating patients with IDH2 mutations with Enasidenib has also resulted in erythroid and eosinophilic differentiation (Galeotti et al., 2019; Yen et al., 2017). Although there has been sporadic evidence of AML cells differentiating into non-neutrophilic myeloid lineages, there has yet to be a systematic approach addressing its true prevalence clinically, as well as the potential implications this may have on patient relapse. It

would be useful to use blood records that are available on AML patients that have undergone differentiation therapy, and important thing to consider is the preparation of blood samples prior to analysis. For example, patient samples often undergo Ficoll separation in order to enrich for populations of interest. Therefore, it is possible that through the use of Ficoll centrifugation, AML-derived eosinophils or monocytes may be removed from further analysis, and thus there may be a significant underrepresentation of AML-derived cells documented in the patient's sample. Hence, although our evidence of multilineage differentiation comes from a single AML mouse model, this concept of bi or multilineage differentiation of AML blasts may also be an important thing to consider in how patient samples are processed clinically, particularly during times of disease remission, where clinicians may only be looking for the presence of AML blasts.

In addition to the processing of patient samples, data from our mouse model also suggests that where patient samples are collected from may play an integral role in the early detection of relapse in patients. In our model of differentiation therapy, we found that during periods of disease remission, AML-derived cells were not only detected in the bone marrow, but also in other organs such as the spleen, liver and kidneys. Furthermore, relapse cells consistently re-emerged initially in the spleen rather than the bone marrow. Hence, the source of relapse in our model is likely to be extramedullary. Extramedullary sources of relapse have been documented in AMLs treated with differentiation therapy previously (Bakst et al., 2011; de Botton et al., 2006; Harris et al., 2013), therefore bone marrow aspirates of these patients during remission may not be an informative method in the detection of early relapse. Interestingly, in our mouse model the removal of the spleen during disease remission was unable to prevent relapse in these mice, suggesting that AML-derived cells in other organs are equally capable of driving relapse. Nonetheless, non-bone marrow origin of relapse may also be occurring at an under-appreciated rate clinically and may be a topic of interest in address further.

6.3 Future directions in the clinical sphere

Although AML246 mCherry is a mouse model of differentiation therapy, it would be interesting to investigate the response of the AML to chemotherapy. Chemotherapy may result in the eradication of AML cells in our mouse model, however relapse may also emerge from the spleen. Although chemotherapy looks to induce apoptosis via cell cycle arrest, a secondary effect of cell cycle arrest may also be differentiation of the immature cell (Myster et al., 2000;

Wahba et al., 2018). Consequently, differentiated, post-mitotic AML cells may also be the origin of relapse in chemotherapy patients, given that chemotherapeutic agents specifically target actively dividing cells. It would be interesting to determine in our mouse model if chemotherapy alone can induce differentiation of AML blasts, and if relapse can be eradicated by eliminating the differentiated AML cell.

Results from this thesis provide strong scientific evidence to warrant further investigation in the clinical sphere. In future experiments, we hope to collect samples from AML patients treated with differentiation therapy agents such as ATRA or Enasidenib and perform flow based assays to determine the percentage of AML-derived in each lineage, by tracking the variant allele frequency (VAF) of the mutation of interest in each myeloid lineage. Alternatively, ddPCR could also be used during periods of remission to detect measurable residual disease in the myeloid lineages of interest. With this approach, we would be able to gauge not only the prevalence of multilineage differentiation, but also the variability in multilineage differentiation that may be present between patients treated with such therapeutic agents. In doing so, we may also determine whether certain genetic subtypes of AML are more or less pre-disposed to multilineage differentiation, and if differentiation into given lineages may impact the likelihood of relapse in these patients. Consequently, by developing a stronger understanding of the nature of AML cell differentiation in patients, we can begin to implement new strategies in our treatment methods in the hope of reducing the rate of relapse in these patients. If it can be shown systematically that multilineage differentiation of AML blasts is prevalent, and that relapse can originate from mature AML-derived cells, the addition of new lineage depletion antibodies to current differentiation therapeutic agents in the treatment of certain subtypes of AML patients may greatly improve the overall survival of patients.

Ultimately, we hope that the novel concepts that have been raised in this thesis including multilineage AML cell differentiation, relapse from mature AML-derived cells and the potential of extramedullary relapse may contribute to the improvement and hopefully cure of AML patients that are treated with differentiation therapy.

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