Bloodlines Matter: leukemia associated mutation FLT3-ITD disrupts dendritic cell homeostasis and leads to altered T cell phenotypes

By

Patrick A. Flynn

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CERTIFICATE OF APPROVAL

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This is to certify that the PhD dissertation [or] Master's thesis of

Patrick A. Flynn

has been approved

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Mentor/Advisor

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Table of Contents

List of Figures

List of Abbreviations

ALL	Acute lymphocytic leukemia
AML	Acute Myeloid Leukmeia
APC	Antigen prosenting cell
ATP	Adenosine triphosphate
BATF	Basic leucine zipper ATF-like
BMDC	Bone marrow derived DC
cDC	Conventional Dendritic Cell
CDP	Common DC progenitor
CITE-seq	Combinatorial Indexing of Transcriptomes and Epitopes
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitor
CLR	C-type lectin receptor
CML	Chronic myeloid leukemia
CMML	Chronic myelomonocytic leukemia
CMP	Common myeloid progenitor
CR	Clinical response
Cre	Cre-recombinase
DAMP	Damage associated molecular pattern
DC	Dendritic Cell
DTR	Diptheria toxin receptor
ECD	Extracellular domain
ER	Endoplasmic Reticulum
ERAP	ER Aminopeptidases
ETS	Erythroblast transformation specific
FDA	Food and Drug Administration
FLT3	Fms-like tyrosine kinase 3
FLT3-ITD	FLT3-Internal Tandem Duplication
FLT3L	FLT3 Ligand
GEMM	Genetically engineered mouse model
HD	Healthy donor
HSC	Hematopoietic stem cell
JMP	Juxtamembrane domain
KO	Knockout
LysM	Lysozyme M
MDP	Macrophage/DC progenitor
MHC-I	Major histocompatibility complex I
MHC-II	Major histocompatibility complex II
MLR	Mixed Lymphocyte Reaction
MPN	Myeloproliferative neoplasms
MPP	Multi-potent progenitor

NCI SEER	National Cancer Institute Surveillance, Epidemiology, and End Results
NLR	NOD-like receptor
OVA	Ovalbumin
P2C	Phagosomal to cytosol
PAMP	Pathogen associated molecular pattern
pDC	Plasmacytoid Dendritic Cell
PRR	Pattern recognition receptor
R/R	Relapsed Refractory
RAS-GTP	Rat sarcoma virus guanine triphosphate
RLR	RIG-I-like receptor
RTK	Receptor tyrosine kinase
scRNA-seq	Single cell RNA sequencing
STAT3	Signal transducer and activator of transcription 3
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T-helper cell
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptor
Vav-Cre	Human vav 1 oncogene-Cre recombinase
WGS	Whole genome sequencing
Zbtb46	Zinc finger and BTB domain containing 46

Acknowledgements

To begin, I would like to give my most sincere thank you to my mentor Evan Lind, PhD. I have the great honor to be Evan's first graduate student that was solely under his wing and I will always remember this special position I find myself in. Because Evan co-mentored Kyle Romine with Jeffrey Tyner, PhD, I will always consider myself to be Evan's "first." From the first time I met Evan on the 5th floor of RJH at the Flow Core running samples on the CyTOF (1st gen by the way), I immediately felt a comradeship with him and was excited to find out that he also loves dendritic cells. It was also a welcome sign to me that having visible tattoos does not prevent you from achieving the lofty goal of pursuing a PhD. I am so grateful for all of the patience and support Evan has had for me while during my time as a graduate student. Due to my personal life, I had many challenges come up during my PhD training that I don't know if many other mentors would be willing to understand and listen to. The boundless enthusiasm and positivity that Evan has is so infectious, that even in my darkest moments he was able to keep a light on at the end of the tunnel for me. Our journey together through a pandemic and changing thesis projects has made me incredibly proud of what we were able to accomplish. His confidence in my ability to drive a novel project in the lab in such a short time is humbling. I promise to carry forward the intellectual fortitude and excitement for all things immune related for the rest of my career.

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and now moved on to medical schools. Jessica Mulkey who is currently in the lab, now carries the torch of being a great technician for the lab. Our Second Year graduate student Ravina Pandita, who is so sharp and has great things ahead of her. Your work on T cells in AML will bring you much success in the future! It is a great feeling to know that I can take the lessons I have learned and use those to help mentor and guide you when you ask for it. I have to save Yoko Kosaka, PhD for last. Yoko is the glue that holds us together and has become more than a mentor but also a friend to me. Yoko has been so helpful for improving my scientific writing skills but also filling the gaps when something goes wrong or an emergency comes up. Your hard work has been an inspiration to me and I am excited to stay in touch as time goes on. A sincere thank you to all of you.

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Abstract

Dendritic cells (DC) are innate myeloid cells that specialize in the priming and activation of adaptive immune cells. DC development is determined by signaling through the receptor tyrosine kinase Fms-like tyrosine kinase 3 (FLT3) in bone marrow myeloid progenitors. Recently the naming conventions for DC phenotypes have been updated to distinguish between "Conventional" DCs (cDCs) and plasmacytoid DCs (pDCs). Activating mutations of FLT3, including Internal Tandem Duplication (FLT3-ITD), are associated with poor prognosis for leukemia patients. To date, there is little information on the effects of FLT3-ITD in DC biology. We examined the cDC phenotype and frequency in bone marrow aspirates from patients with acute myeloid leukemia (AML) to understand the changes to cDCs associated with FLT3-ITD. When compared to healthy donor (HD) we found that a subset of FLT3-ITD+ AML patient samples have overrepresented populations of cDCs and disrupted phenotypes. Using a mouse model of FLT3-ITD+ AML, we found that cDCs were increased in percentage and number compared to control wild-type (WT) mice. Single cell RNA-seq identified FLT3-ITD+ cDCs as skewed towards a cDC2 T-bet^{-} phenotype, previously shown to promote Th17 T cells. We assessed the phenotypes of CD4+ T cells in the AML mice and found significant enrichment of both Treg and Th17 CD4+ T cells. Adoptive transfer of naïve CD4+ T cells into AML hosts showed increased retention and activation when compared to WT hosts. When stimulated *ex vivo* CD4+ T cells from AML mice secreted more IL-17A than WT derived CD4+ T cells. Moreover, co-culture of AML mouse-derived DCs and naïve OT-II cells preferentially skewed T cells into a Th17 phenotype. Together, our data suggests that FLT3-ITD+ leukemia-associated cDCs polarize CD4+ T cells into Th17 subsets, a

population that has been shown to be negatively associated with survival in solid tumor contexts. This illustrates the complex tumor microenvironment of AML and highlights the need for further investigation into the effects of FLT3-ITD mutations on DC phenotypes and their downstream effects on T cells.

Chapter I: Introduction

1.1 A short history of dendritic cells

Dendritic cells (DCs) are sentinel leukocytes that have been found among all mammalian species. They perform functions in both arms of the immune system: innate and adaptive. DCs have critical roles initiating and regulating immune responses at both the early stage (innate) and the late stage (adaptive) immune response. DCs were first described by Steinman and Cohn in 1973, then characterized in more detail through 1979 [1-3]. Steinman and colleagues discovered myeloid cells that were distinct from macrophages, both morphologically (tree like processes) and cytologically (fewer lysosomal granules), showed low-adherence to surfaces *in vitro*, and represented a small fraction (~1-percent) of splenocytes [4]. The discovery of DCs officially united the ideas of Metchnikov and Ehrlich (regarded as the "fathers" of immunology), for their pioneering experiments identifying phagocytosis (innate) and anti-sera (adaptive) respectively [5], which lead to decades of research investigating the specialized functions of DCs and their distinction from other similar cell-types (*i.e.* Langerhan's cells or monocyte-derived cells).

During early characterization of DCs it was proposed by the Steinman group that DCs are the best activators of T-cells in 1983 [3]. To prove this specialized function, Steinman and colleagues used Mixed Lymphocyte Reactions (MLR) to show that when DCs are removed from the reaction, the stimulatory capacity of the reaction was reduced 90 percent [3]. Despite being a small fraction of the immune cells of the spleen, DCs have the potent ability to activate lymphocytes. The idea that DCs are the special "accessory cells" by

which adaptive immune responses are started with had already been tested by Nussenzweig and colleagues in 1980 [6] and further tested by Hamilos and colleagues in 1989 [7]. Considered canon in today's modern era, the work on DCs by Steinman and his peers was highly controversial through the 1980's regarding their ability to be the most efficient stimulators of T cells. Many years after the discovery of DCs, Ralph Steinman was awarded the Nobel Prize posthumously in 2011 for his pioneering works [8]. Flash forward to 2023 and the field of DC biology has significantly advanced our understanding of DC ontogeny, phenotype, and function (Figure 1.1) via technological advances in genetics and assays which will be covered in the following sections.

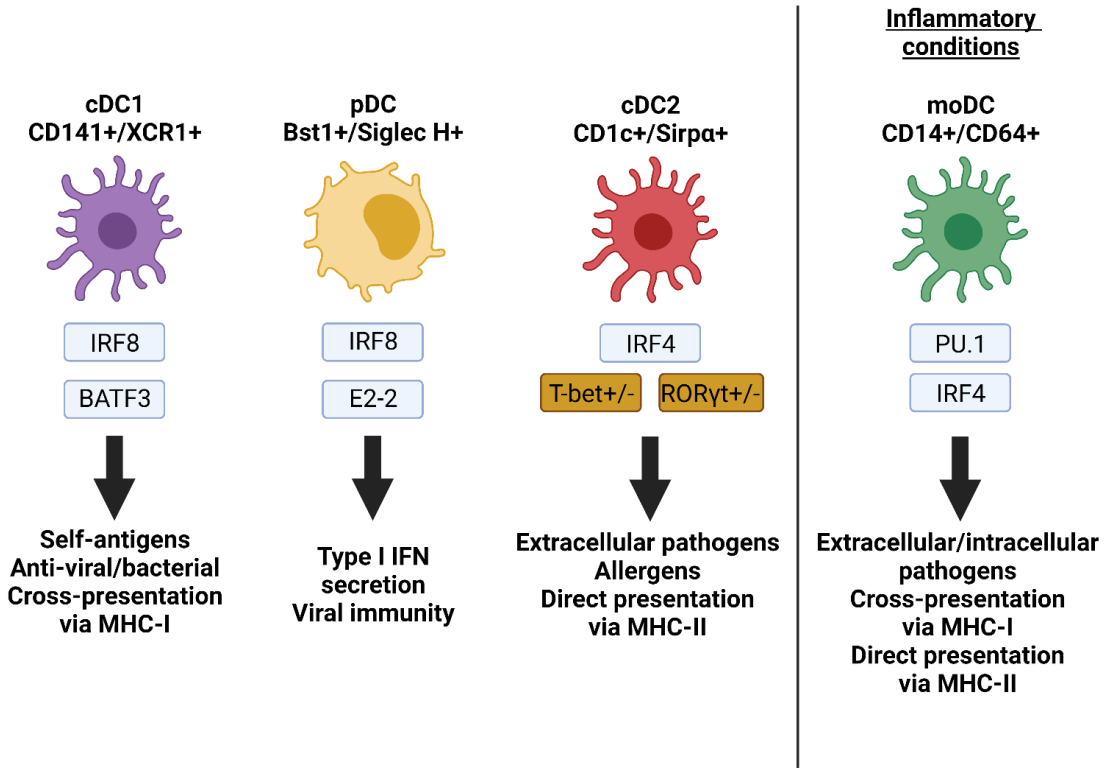


Figure 1.1 Mammalian classes of dendritic cells

Figure 1.1. Mammalian classes of dendritic cells. Due to the recent advances in genetics the nomenclature for dendritic cells has changed to divide them into two major categories during steady state, Conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Related to DCs and separated by a vertical line, are so called monocyte-derived DCs (moDCs). These cells arise during acute inflammatory conditions and perform antigen presenting cell (APC) functions similar to cDCs, but are not considered to be of the same lineage. DCs are labeled with their human (e.g. CD141) and murine (e.g. XCR1) surface proteins used to discriminate them from other DC subsets in flow cytometry. The major lineage determining transcription factors are labeled beneath each DC subtype (i.e. IRF8 and BATF3 are required for cDC1). Canonical functions for each lineage is listed at the bottom.

1.2 Dendritic cells depend upon the receptor FLT3 for development

During the 1990's and the 2000's there was great interest in understanding how DCs grow during hematopoiesis. Like all other immune cells, DCs are descendants of hematopoietic stem cells (HSCs) from the bone marrow that become fate-determined by the expression of surface receptors and environment cytokines [9-11]. DC hematopoiesis became distinct from other myeloid lineage cells when it was discovered that DCs required access to fms-like tyrosine kinase receptor 3 a.k.a. fetal liver kinase 2 (FLT3 or Flk2 or CD135) signaling to develop in the 1990s [12] (Figure 1.2).

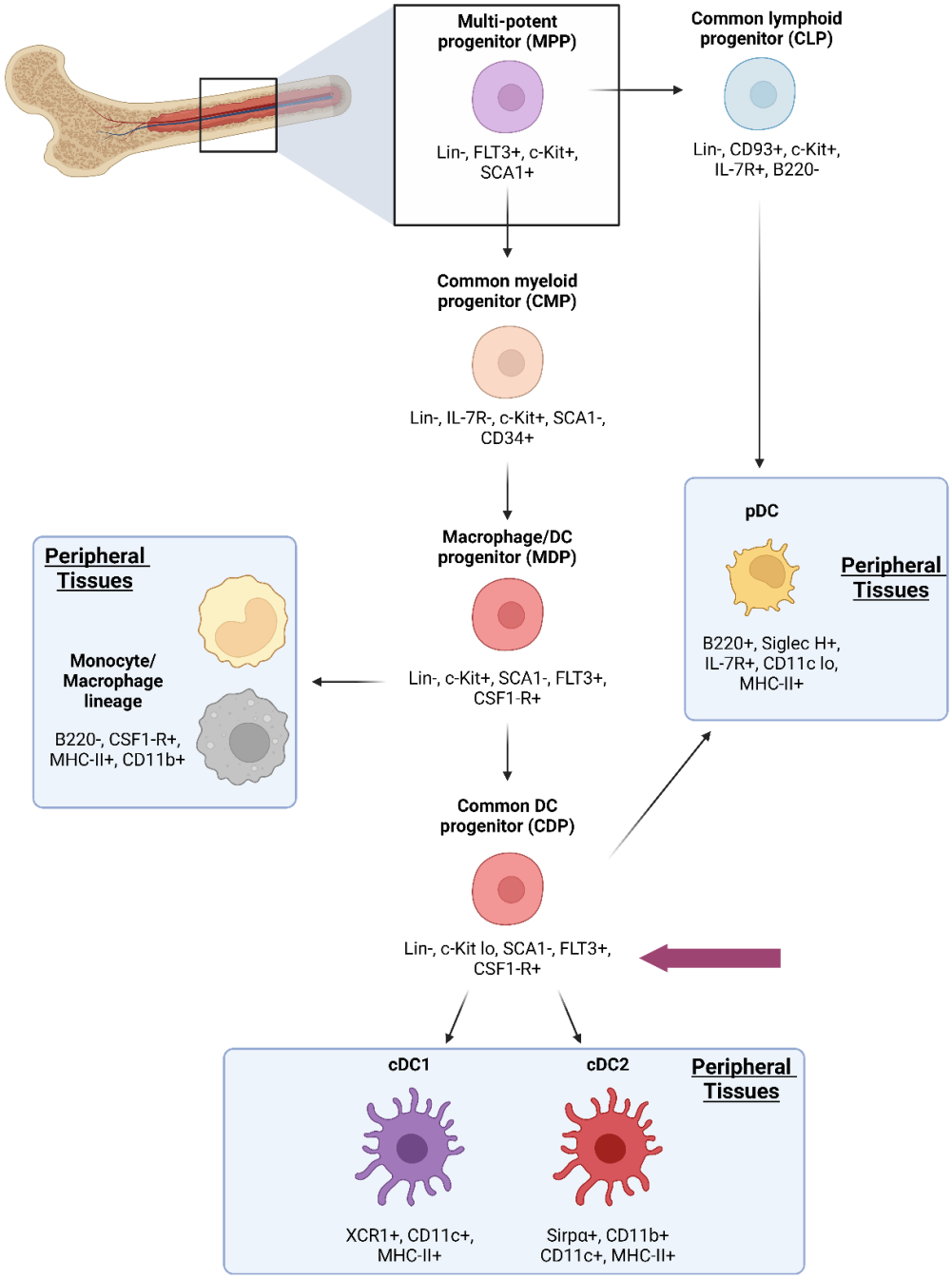


Figure 1.2 DC hematopoiesis

Figure 1.2. DC hematopoiesis. MPPs in the bone marrow differentiate into lymphoid and myeloid cell fate lineages early during hematopoiesis. Myeloid cell progenitors that express that express high levels of FLT3 commit to DC cell fate and differentiation (CDP with purple arrow). Notably, the origins of pDCs can come from both lymphoid and myeloid progenitors and is still under investigation how the two branches result in pDCs. After extravasation from the bone marrow into the blood to peripheral tissues then completes the development of DCs and the related myeloid cells monocytes and macrophages.

FLT3 was initially discovered and characterized as a surface protein on multipotent HSCs by Matthews et. al. in 1991 [13]. The expression of FLT3 (Flk2) by mRNA was restricted to primitive/immature/progenitor cell populations of the bone marrow, liver, brain, and thymus [13]. Further sequence alignment showed homology with the sibling Type III receptor tyrosine kinases (RTKs); c-kit, c-fms, and Pdgfra/b proteins [13]. Shortly after the published work of Matthews et. al., was the work from Stewart Lyman and colleagues at Immunex in Seattle, WA where they cloned the ligand for the receptor FLT3, so called FLT3L [14] and showed *in vivo* that mouse DC numbers are increased after injection of exogenous FLT3L [15] and a reduction of global DC numbers when FLT3L is deficient [16]. This suggested that despite the expression of FLT3 broadly being expressed on on HSCs that could lend functionality to any number of to non-DC myeloid cells and lymphocytes, the sensing of FLT3L is more important to DCs development (differentiation) than other immune cell subsets.

Similarly to other Type III RTKs, FLT3 signal transduction is involved in many biochemical pathways that promote proliferation and survival of hematopoietic cells [17, 18] (Figure 1.3). RAS-GTP was confirmed to be downstream of FLT3 activation by transfection of NIH 3T3 and Ba/F3 cell lines with a chimeric receptor (FF3) consisting of extracellular human FMS and the transmembrane domain of FLT3 [19]. Around the same time PI3K was also confirmed to be activated downstream of FLT3 using the same method [20, 21]. Through the 1990's work was done to characterize the signal transduction pathways downstream of FLT3, but there was not much known about the transcription factors important to DCs in response to FLT3 signaling. In 2003 Yasmina Laouar et. al.

published an important finding, where mice that completely lacked expression of STAT3 had significantly ablated DC frequency in number across lymphoid tissues [22].

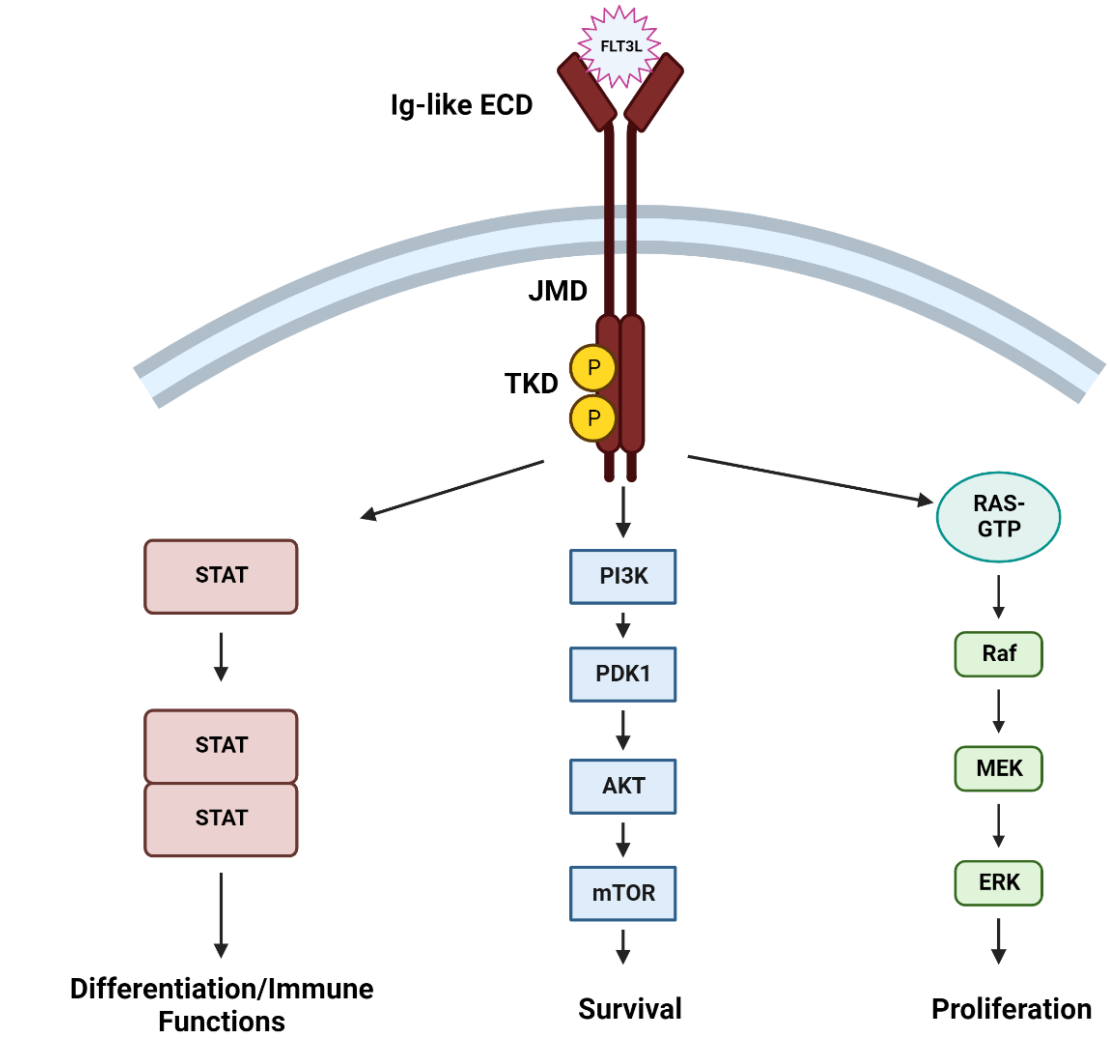


Figure 1.3 FLT3 receptor structure and signaling

Figure 1.3. FLT3 receptor structure and signaling. Upon engagement with FLT3L, the FLT3 receptor homodimerizes and phosphorylates the tyrosine kinase domain (TKD) of FLT3. Linker proteins (e.g. GRB2 – not shown) provide scaffolds to allow signal transduction through the three canonical branches: STAT, PI3K, and RAS. The activity of these pathways lead to activation of differentiation, immune functions, survival, and proliferation. Extra-cellular domain (ECD), juxtamembrane domain (JMD), tyrosine kinase domain (TKD).

Furthermore, they also showed that the critical transition step from DC-progenitor cells at the common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) is where STAT3 expression is required for the commitment to DC lineage. To illustrate this, they treated STAT3KO mice with FLT3L (10 μg) for nine consecutive days and observed comparable amounts of HSCs and CMP/CLP populations between STAT3KO and controls, but STAT3KO mice lacked fully committed DCs in the periphery [22]. They conclude that STAT3 expression is required at the CMP/CLP to DC precursor differentiation during hematopoiesis and that STAT3 is activated downstream of FLT3 signaling.

1.3 Insights into transcriptional regulation of DC development

Simultaneous to the efforts to understand cytokines and identity defining proteins related to DCs, was the endeavor to investigate transcriptional regulation of DCs. The identification of STAT3 requirement for DC development was an important step, but there are additional key transcription factors that are necessary for DC cell fate. Important to all DC subsets is the activity of the erythroblast transformation specific (ETS) family transcription factor PU.1 (endcoded by *Spi1* gene) [23, 24]. Furthermore, PU.1 activity was shown to be critical for FLT3 expression and responsiveness to FLT3L cultured BMDCs [25]. Important to note, complete knockout of PU.1 in mice results in fetal lethality [25], highlighting the importance of PU.1 expression throughout hematopoiesis.

Another transcription factor that is involved in DC development is the transcription factor zinc finger and BTB domain containing 46 (Zbtb46) [26]. Utilizing a diphtheria toxin receptor (DTR) knock-in model, it was shown using bone marrow chimeras in mice, that injection of diphtheria toxin (DT) was able to specifically deplete cDCs. Until this time, a CD11c-DTR model was previously used [27] to study DC depletion but had major caveats, due to the fact that CD11c is also expressed on macrophages, monocytes, subpopulations of activated T cells, and cardiac endothelium [28, 29].

Also critical to the generation of DCs is the transcription factor interferon regulatory factor 8 (IRF8). In mice that are lacking IRF8 the cDC1 population is absent and their pDC phenotype is altered, such that the pDCs produce less Type I Interferon and exhibit improved T cell stimulatory ability [30, 31]. Coordinating with IRF8 is the basic leucine zipper ATF-like (BATF) family member BATF3 transcription factor, where the loss of

BATF3 prevents the generation of cDC1 cells and instead skews them towards cDC2 phenotype [32].

Specification of CDPs towards cDC2 phenotype has been shown to rely on the expression of IRF4 at the expense of IRF8 [33, 34], although this area of DC biology is still actively under study. Using a CD11c-Cre model to knockout IRF4 expression in all CD11c expressing cells showed that the frequency of CD11b+ Sirpα+ cDC2s was reduced in the lungs and spleen, without affecting the macrophage population in those tissues [35]. The competition between IRF4 and IRF8 *in vivo* using the CD11c-Cre system nicely showed that cDC1 cells are more reliant on IRF8 (heterozygous expression still results in cDC1 depletion) and cDC2s can still develop *in vivo* despite heterozygous IRF4 expression [35, 36]. Historically the classification of DCs were restricted to three types, but the improvements to DC-ontogeny has left the field in a more complex model than originally anticipated. When compared to other immune cells such as macrophages, B Cell, or T cells, DCs have a surprisingly complex development program that has made it difficult to generate DC-specific knockouts in mice, due to shared myeloid lineage markers and transcription factors [29].

1.4 Fundamentals of antigen processing and presentation

As professional antigen presenting cells, DCs are defined by their efficient ability to survey their environment and collect molecules for presentation of short-length peptides on MHC-I and MHC-II molecules to T cells. The pathways that drive their antigen presentation have been characterized in great detail and has been reviewed extensively [37-39]. There are some similarities to MHC-I and MHC-II antigen presentation, but there are fundamental differences to how they work and are specialized to provide breadth of protection against pathogens both intracellular and extracellular. As a general rule, the steps for antigen processing and presentation are as follows: acquisition of antigen, proteolysis of protein, transfer and loading to MHC, and surface display on the cell as a peptide:MHC complex [40]. This is referred to as Classical Antigen Presentation which will be discussed more in depth below. It is worth noting that there are Non-Classical MHC-I "like" molecules that can present lipids using CD1 on their surface which also play a role in immunity to pathogens as well, but will not be discussed here [41].

Starting with the context of MHC-I, the acquisition of antigen occurs within the cytosol of the cell where both self-proteins and foreign proteins can be identified by ubiquitinating proteins for degradation by the proteasome; if this fails then the antigen cannot be presented in complex by MHC-I [42]. After proteasomal degradation the resulting oligopeptides are shuttled by the Transporter Associated with Antigen Processing (TAP) into the Endoplasmic Reticulum (ER) [43, 44]. Further editing by ER aminopeptidases (ERAP) will reduce the size of the oligopeptides into shorter length peptides of 8-11 amino acids in length where the peptide can be loaded onto nascent MHC-I molecules in the lumen of the ER [45, 46]. Now a peptide:MHC-I (pMHC-I) complex, it can now translocate from the

ER to the Golgi secretory pathway and be presented on the cell surface for recognition by a T cell [45, 46]. In most contexts MHC-I is thought of as an intracellular signaling pathway, meaning peptides from self or intracellular pathogens can be loaded onto MHC-I. However this theory was altered during the characterization of MHC molecules. The discovery of an alternative pathway referred to as Cross Presentation by MHC-I changed our understanding of MHC-I activity [47]. Cross presentation is the idea that exogenous molecules from the extracellular environment can be acquired and loaded onto endosomal MHC-I molecules without the need to be processed by the proteasome or brought to the ER [48]. The pathways that contribute to cross-presentation are still being studied to this day, but there is a body of evidence partially explaining how this works. One of the well characterized pathways is the phagosomal to cytosol (P2C) pathway. This is where the APC can internalize exogenous materials either by micropinocytosis or receptor-mediated endocytosis where 1) peptidases/cathepsins within the lumen of the endosome can digest the molecules into peptides and directly loaded onto MHC-I molecules or 2) the endocytosed molecules are released into the cytosol for ubiquitination and proteosomal degradation and subsequent pMHC complexing via the ER-Golgi secretory pathway [40, 49, 50].

For MHC-II presentation, the dogma is that this antigen presentation molecule specifically presents to CD4+ T cells, but it shares many of the same pathways that are characterized for MHC-I. MHC-II antigen acquisition is driven by extracellular molecule internalization via endocytosis, phagocytosis, and micropinocytosis wherein the vesicles containing exogenous materials can be degraded by cathepsins and proteases [51]. Rather than going through the TAP and into the ER-Golgi, the loading of exogenous peptides onto

MHC-II molecules is primarily performed in the lumen of recycling endosomes that contain MHC-II molecules that are internalized from the cell surface into endosomal compartments [52, 53]. Regardless of how the complex is achieved, the presentation of peptide either in the MHCI or MHCII molecules form the basis of cognate T cell/DC interactions. These interactions of MHC-I and MHC-II on the surface of DCs are critical for the priming and activation of cognate antigen specific CD8+ and CD4+ T cells respectively, which will be discussed below.

1.5 DCs are important influencers of T cells

Concurrent with the investigations into DC-intrinsic biology was research studying the effects DCs have on other immune cells. There has been great interest to study the relationship between DCs and αβ T cells due to their clinical relevance for infectious diseases, autoimmunity, and oncology. Among the various antigen presenting cells (APCs) in mammals (macrophage, B cell, epi/endo-thelial cells, and DC), it has been established that DCs are the most efficient APCs for priming and activating naïve, antigen-specific T cell responses [3, 6, 54, 55] (Figure 1.4). How these two cells interact and influence each other is discussed in detail below.

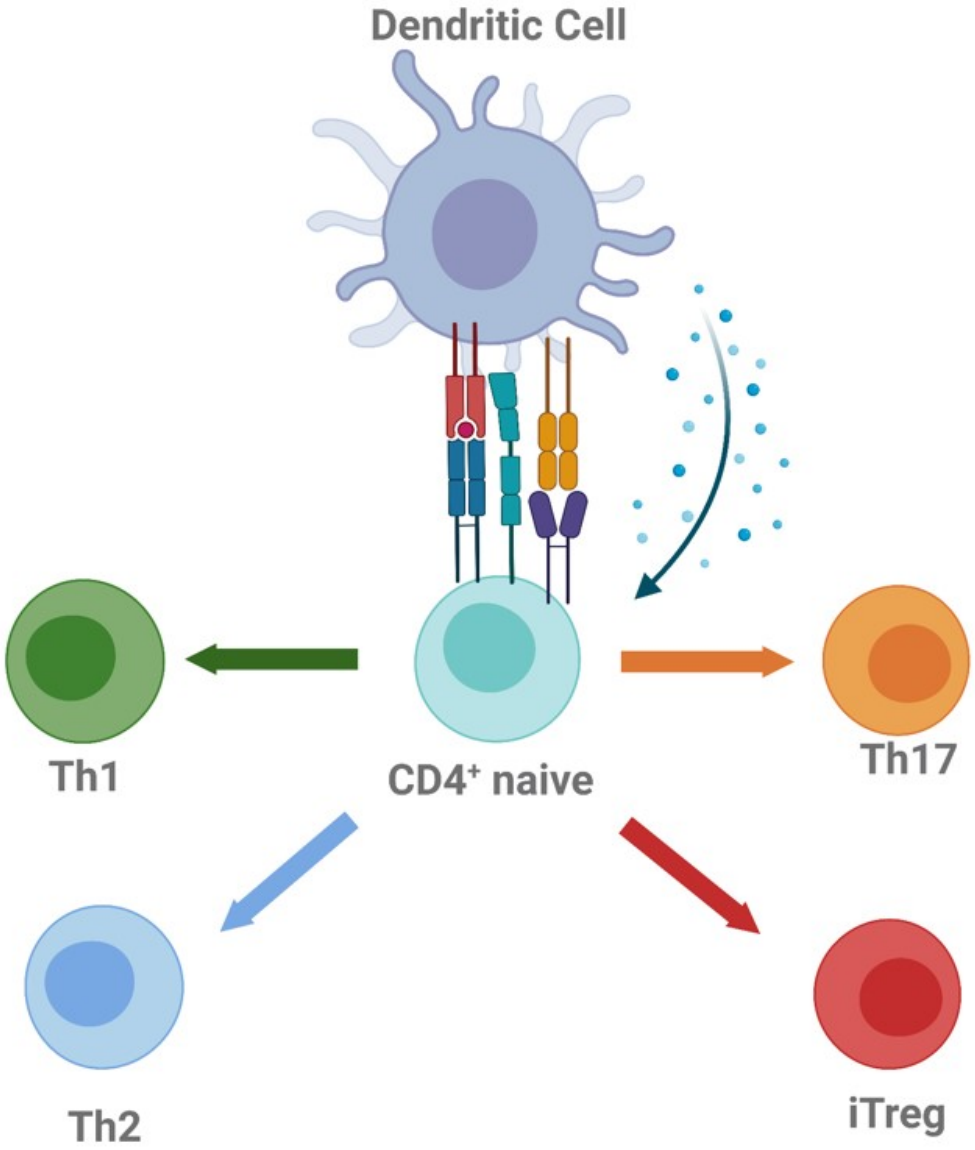


Figure 1.4 DCs prime naive T cells for antigen specific responses

Figure 1.4. DCs prime naïve T cells for antigen specific responses. A DC interacting with a naïve CD4+ T cell that can be polarized into various Th-subsets.

The mechanisms of DC-induced polarization of naïve T cells can be applied to all cDC subsets, with slight variations for non-lymphoid tissues such as mucosal barriers (i.e. CD103+ gut-DCs) or the epidermis of the skin (i.e. Langerhan's cells) [56, 57]. To start, cDCs at steady-state are in a so-called "sentinel" mode where they constantly sample molecules in lymphoid and non-lymphoid tissues until a Pattern Recognition Receptor (PRR) is stimulated [58, 59]. PRRs are innate immune receptors that recognize evolutionary conserved bacterial and viral molecules that are not found in mammalian hosts [60]. These microbial molecules are termed Pathogen Associated Molecular Patterns (PAMPs) and act as ligands for PRRs. PRRs are a large family of receptors and sensors that can detect the presence of pathogens externally and internally. The first PRRs identified are the Toll-like Receptors (TLRs) that are present on the cell surface and in the lumen of endosomes [60]. Similar to TLRs are the C-type Lectin Receptors (CLRs) that are found on the cell surface and can modulate TLR expression after pathogen encounter [61]. Additionally, DCs have the ability to detect pathogens that access the cytoplasm free from endosomal compartments [62]. The three major classes of PRRs within the cytosol are NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and cGAS-STING [63, 64]. Originally it was thought that PRRs recognized foreign ligands but it was discovered that self-ligands from damage can also trigger responses by PRRs, so called Damage Associated Molecular Patterns (DAMPs) [65].

Upon ligand interaction with any of the aforementioned PRRs the DC switches from a sentinel-like state to now an activated state whereby it can now communicate signals to other immune cells through the secretion of cytokines and other inflammatory factors [66]. Furthermore, this will allow the DC to induce antigen-specific adaptive immune responses

by the T and B lymphocytes. To communicate pathogen information to lymphocytes, dendritic cells process internalized pathogen molecules and break them down into small peptide fragments that can fit on either major histocompatibility complex—I (MHC-I; for CD8+ T cells) or MHC-II (for CD4+ T cells) [67-69]. The peptide-MHC (pMHC) complex will be recognized by naïve cognate T cells with the appropriate T cell receptor (TCR) and stabilizes their connection [70]. This step of DC-T cell interaction has been termed "Signal 1" (Figure 1.5). This now becomes a two-way communication stream, where surface ligands on the T cell can interact with additional receptors on the DC which causes them to be stimulated even more, such as CD40 (expressed on DC)+CD40L (expressed on T cell) [71]. This activity is termed "licensing" and induces strong upregulation of costimulatory molecules on the surface of the DC, most classically CD80/86 (a.k.a. B7.1/2) [72, 73]. The DC can then stimulate the T cell through CD28 on its surface using these molecules which is called Co-stimulation or "Signal 2." This will induce activation and nuclear translocation of transcription factors (TF) that determine the acquisition of T specific T cell fates post-translocation [74-77]. Finally, based on the pathogen context and these signals between DC and T cell, the secreted inflammatory cytokines from the DC received by the T cell in conjunction with the TFs cause phenotype switching, from a naïve T cell to now an effector T cell that can help eradicate the pathogen [78, 79] (Figure 1.5). The sensing of cytokines from DCs to T cells is referred to as "Signal 3" (Figure 1.4). The context-dependent signals that the DCs receive and translate to naïve T cells produces what is now called T-helper subsets (Th), defined by their canonical TFs and the primary cytokines the T cells secrete. Th1 (T-bet and IFNγ), Th2 (GATA3 and IL-4), Th17 (RORγt

and IL-17), Tfh (Bcl-6 and IL-21), and Treg (FOXP3 and IL-10) are the various subtypes of functional T cells described in the field [78, 79].

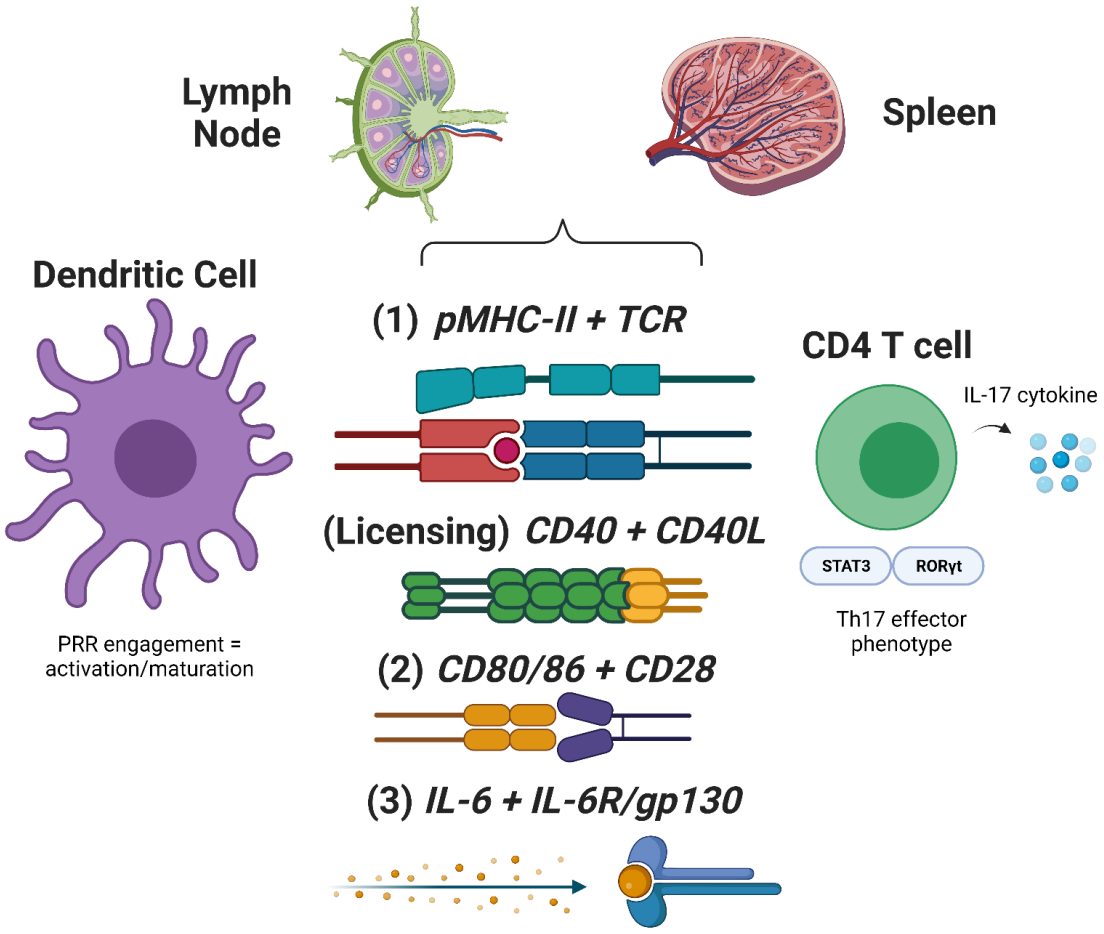


Figure 1.5 DC T cell interactions lead to T cell polarization in lymphoid tissues

Figure 1.5. DC-T cell interactions lead to T cell polarization in lymphoid tissues. Dendritic cells (DCs) sense pathogens with pattern recognition receptors (PRR) (e.g. TLR4) where engagement of PRR activates the DC, resulting in internalization of pathogen into endosomal compartments for digestion by enzymes into small protein fragments called peptides, which can then be loaded onto MHC-II molecules that are also within endosomes. Expression of the combined peptide-MHC-II (pMHC-II) can then engage with a naïve CD4+ T cell and the TCR (Signal 1). CD40 on the surface of the DC will engage with the CD40L on the cognate CD4+ T cell (Licensing), promoting the increased expression of CD80/86 proteins (Signal 2) on the DC surface that potently stimulate the CD4+ T cell. This also results in the production of IL-6 cytokine (TGF-β is also important) (Signal 3) from the DC which is received by the surface IL-6R/gp130 heterodimer on the CD4+ T cell. The combination of the three signals will initiate priming, activation, and polarization of the naïve CD4+ T cell to upregulate STAT3 and RORγt transcription factors, therefore becoming a T helper 17 (Th17) as defined by the secretion of IL-17 cytokine by the T cell.

1.6 Acute Myeloid Leukemia is a hematopoietic cancer

Leukemia is a diverse group of lymphoid and myeloid neoplasms that contain heterogeneous mutations and defined as a clonal disease of immature white blood cells in the bone marrow that rapidly expand. This cancer has been medically recognized for about 200 years, where the earliest cases reported in Europe described patients with "milky blood" or blood appeared to be "pus filled" during autopsies of patients [80]. Once microscopes became more widely available closer to the 1840's, doctors were able to more accurately describe the pathology of the disease and recognized that white blood cells were the primary source of pathology in the patients. Most of the credit for originally describing leukemia goes to four European doctors: Alfred Velpeau, Alfred Donné, John Bennett, and Rudolf Virchow [80-82]. It is certain that outside of Europe there were cases of leukemia although there is no record of them in the literature earlier than those recorded in Europe in the 18^{th} or early 19^{th} centuries.

The focus of this thesis is the pathology of acute myeloid leukemia (AML) but the leukemia umbrella contains many variations of this disease, splitting between Acute and Chronic phenotypes and includes: Chronic Myeloid Leukemia (CML), Chronic Myelomonocytic Leukemia (CMML), Myeloproliferative Neoplasms (MPN), Acute Lymphocytic Leukemia (ALL), and Chronic Lymphocytic Leukemia (CLL) [83]. The National Cancer Institute SEER program estimates about 30,000 adults and children are diagnosed with a leukemia in the United States each year. Regardless of what type of leukemia a patient is diagnosed with, many types have poor prognoses to this day, particularly in older individuals who cannot withstand standard chemotherapy regimens that often relapse after treatment [84-86]. The 5-Year Relative Survival rate for patients is

~32% (Figure 1.6). This has led the field to characterize the mutations that drive this cancer and those associated with AML specifically are discussed below.

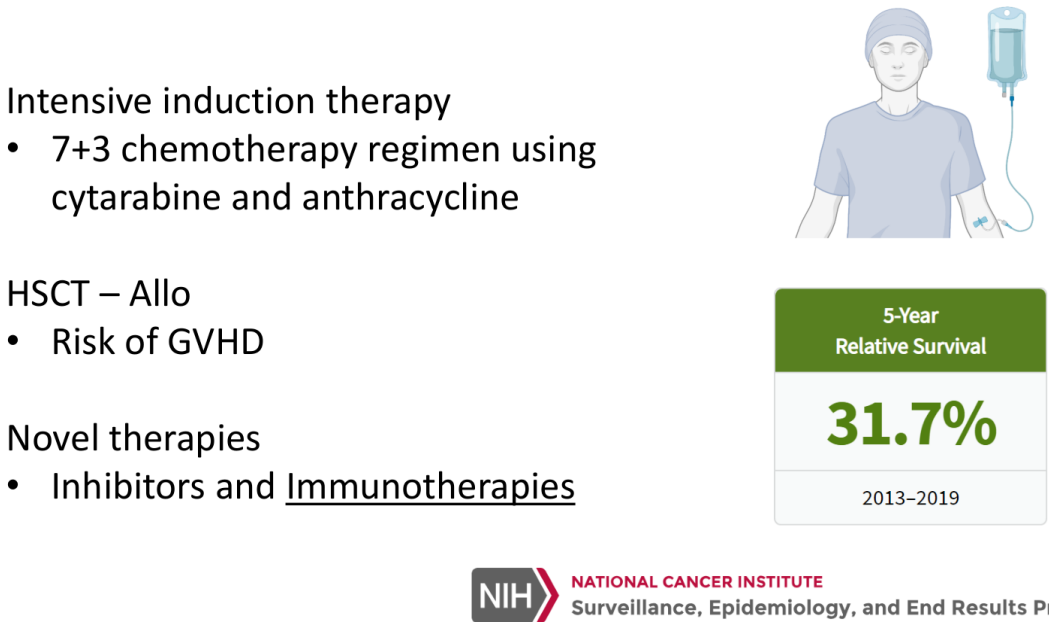


Figure 1.6 NCI SEER 5-year relative survival and AML patient treatments

Figure 1.6. NCI SEER 5-Year Relative Survival and AML patient treatments. Cartoon schematic of three major categories of clinical treatment for AML patients that includes the NCI-SEER 5-year survival data.

The tumor cells in AML are referred to as “blasts” due to their large size and heterogeneous phenotype as viewed by the microscope and measured with flow cytometry (Figure 1.7). There is no single unifying mutation that causes AML, but rather a combination of mutations that affect proliferation, differentiation, and the epigenetics of myeloid precursors that results in blast formation and tumor outgrowth [84, 87-90] (Figure 1.8). In a case study of 200 AML patients, whole genome sequencing (WGS) was performed on matched primary tumor tissue and normal skin tissues, revealing that the top three mutated genes are FLT3 (proliferation/differentiation), NPM1 (proliferation/differentiation), and DNM3TA (epigenetics) [84, 87, 91] (Figure 1.9). Furthermore, mutations in TP53 (tumor suppressor), TET2 (DNA methylation/epigenetics), NRAS (proliferation/differentiation), CEBPA (myeloid differentiation), RUNX1 (proliferation/differentiation), and IDH1/2 (epigenetics) are also common in AML patients [84, 87, 91]. Patients that harbor combinations of mutations with FLT3-ITD (*e.g.* FLT3-ITD+TET2) show even worse survival [92] (Figure 1.10, 1.11). Genetically engineered mouse models (GEMM) to study AML have been difficult to produce based on the requirement for a minimum of two or three gene modifications to induce disease, but they do cover both inducible and constitutive expression models with varying degrees of disease induction allowing for mechanistic experiments that study the tumor blasts and drug responses [93].

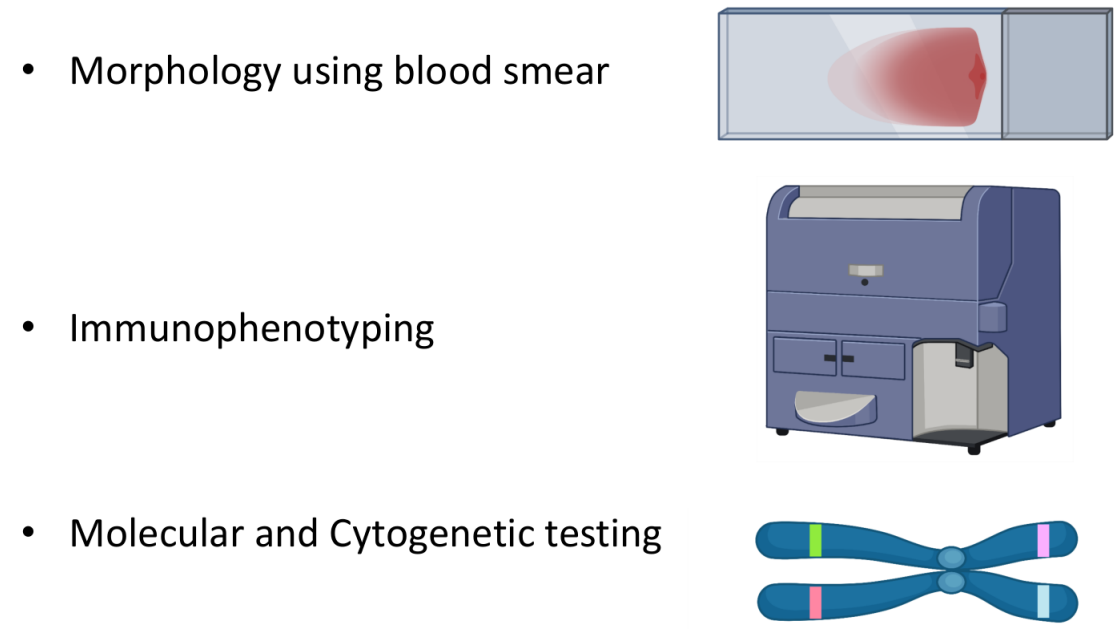


Figure 1.7 Techniques used to diagnose AML patients

Figure 1.7. Techniques used to diagnose AML patients. Cartoon schematic of three techniques used in the clinic to diagnose AML patients.

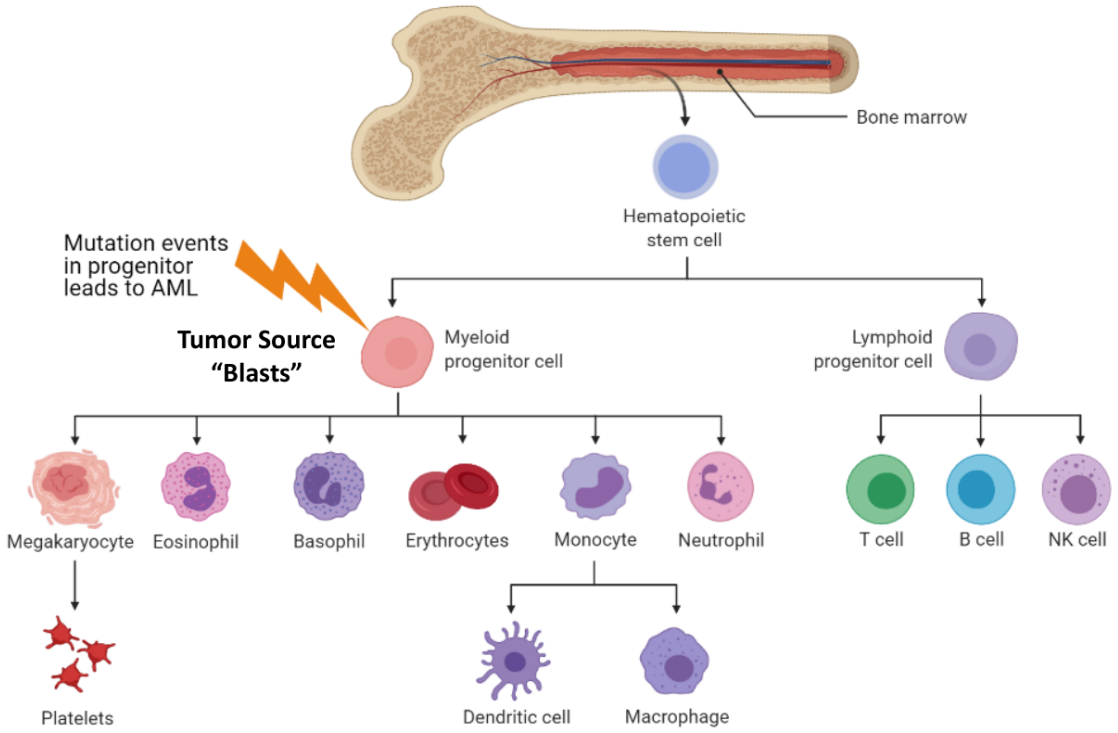


Figure 1.8 Immature myeloid progenitor cells are the source of AML tumor blasts

Figure 1.8. Immature myeloid progenitor cells are the source of AML tumor blasts. Cartoon schematic of normal hematopoiesis that highlights myeloid progenitor cells that acquire mutational events that can lead to a tumor clone becoming an AML tumor Blast. The AML Blast will expand and cause imbalance of downstream myeloid lineage cell populations.

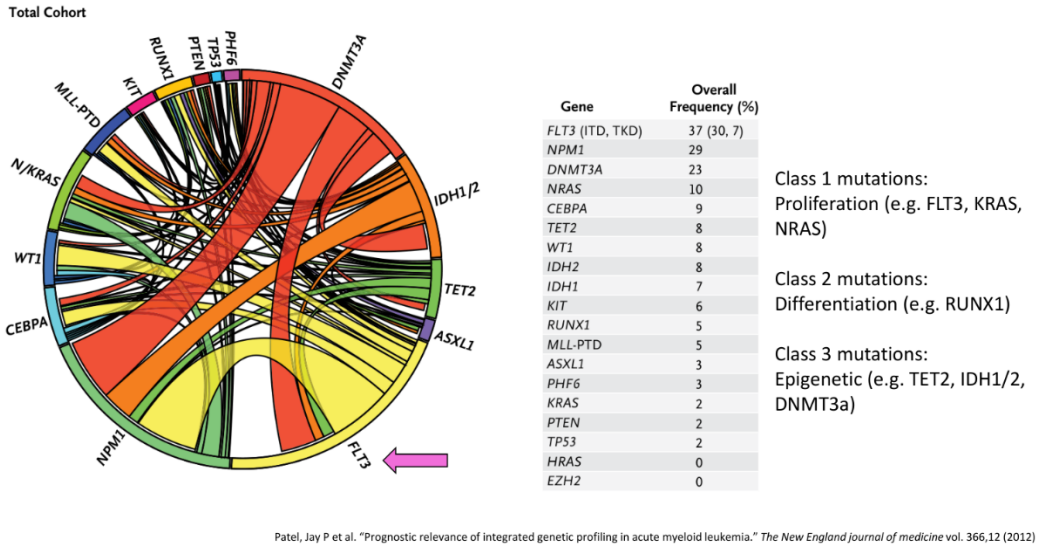


Figure 1.9 Mutated genes that are found in AML patients

Figure 1.9. Mutated genes that are found in AML patients. Circus diagram showing the relative frequency and co-occurrence of mutations in patients diagnosed with AML. Pink arrow highlights region denoting FLT3 mutations. Adapted with permission from *Patel et. al.* [98].

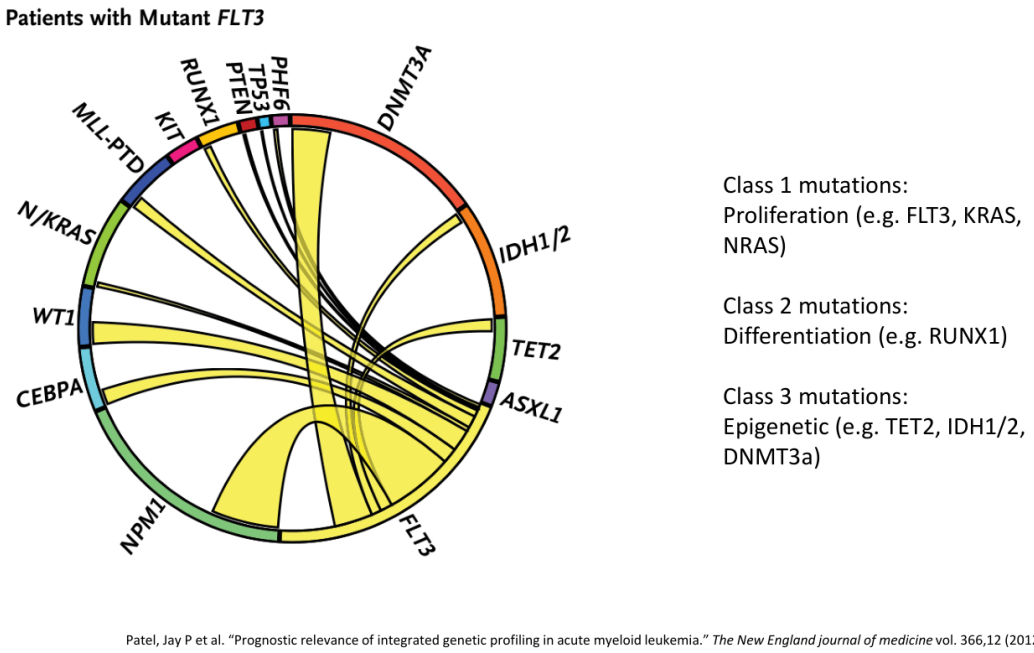
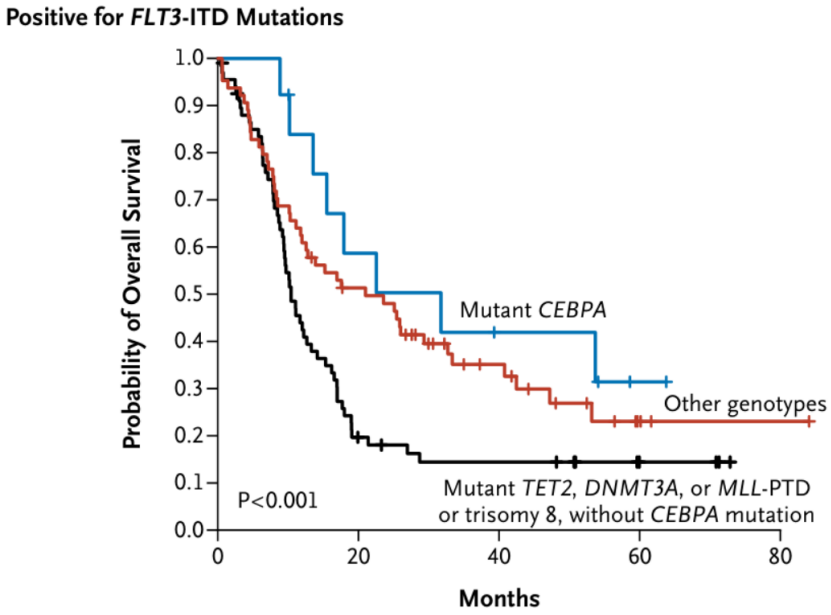


Figure 1.10 Mutated genes that associate with FLT3 in AML patients

Figure 1.10. Mutated genes that associate with FLT3 in AML patients. Circus diagram showing the relative frequency and co-occurrence of mutations combined with FLT3 in patients diagnosed with AML. Adapted with permission from *Patel et. al.* [98].



Patel, Jay P et al. "Prognostic relevance of integrated genetic profiling in acute myeloid leukemia." *The New England journal of medicine* vol. 366,12 (2012)

Figure 1.11 Multivariate risk classification of patients with FLT3-ITD+ AML

Figure 1.11. Multivariate Risk Classification of Patients with FLT3-ITD+ AML. Kaplan–Meier estimates of overall survival are shown for the risk stratification of patients with intermediate-risk AML that are positive for FLT3-ITD mutation. Adapted with permission from *Patel et. al.* [98].

1.7 Mutations of FLT3 in the context of AML

For 30 years the field has known that high expression of FLT3 is present in leukemias in both CD34+ and CD34- hematopoietic cells [18, 88, 94-97]. Consequently, activating mutations of FLT3 are one of the most common mutation found in AML patients, up to 30%, caused by internal tandem duplications (FLT3-ITD) within the cytoplasmic domain of FLT3 [87, 98-102] resulting in ligand independent signaling [98, 103] (Figure 1.12). This has led to development of tyrosine kinase inhibitors (TKIs) that abrogate FLT3-ITD signaling to treat leukemias by competing for the adenosine triphosphate (ATP) binding site of FLT3, therefore reducing activity of FLT3-ITD signaling and its downstream targets [104]. The off-target affects are broad given the homology of tyrosine kinase structures This is why there are two generations of FLT3 inhibitors developed to treat FLT3-mutated leukemias, where the second generation of drugs were designed to be more specific than the first generation [85]. For example, the first FDA approved TKI for treating FLT3-mutated leukemia called Midostaurin, was originally developed as a TKI for Protein Kinase C (PKC) activating mutations that are found in various solid tumor diseases (e.g. Colon, Breast, and Melanoma) where it showed synergy with chemotherapy and radiotherapy for patient treatment [105]. The anti-proliferative and RTK inhibition properties of Midostaurin seen in solid cancers led to the use of Midostaurin for treating FLT3 mutated leukemias in multiple clinical trials. It demonstrated survival benefits and reduced tumor burden in both newly diagnosed leukemia patients and Relapsed/Refractory (R/R) patients when combined with chemotherapy [106-108]. Midostaurin is considered to be a 1st-Generation TKI due to lacking specificity to FLT3 and this drug among others drove the development of 2nd-

Generation TKIs that are more specific and have reduced off-target effects [85]. One of the most prominent of these 2nd-Generation TKIs for treating FLT3-mutated AML is Gilteritinib. Gilteritinib is not FLT3 specific because it also has been shown to inhibit signaling of the related tyrosine kinase AXL [104, 109], however Gilteritinib has potent anti-phosphorylation effects in cell lines by

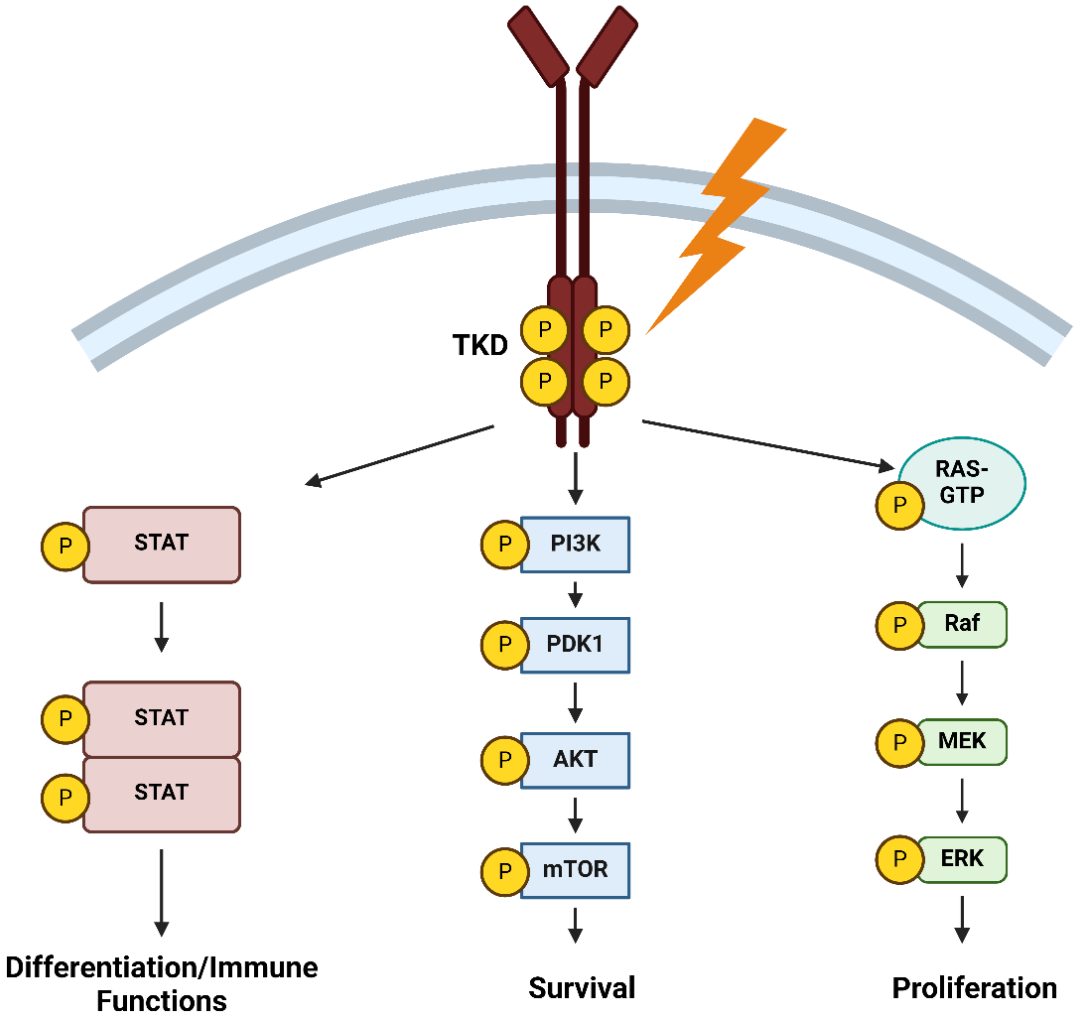


Figure 1.12 FLT3-ITD mutation casues ligand independent signaling

Figure 1.12. FLT3-ITD mutation causes ligand independent signaling. Cartoon schematic showing the hyperphosphorylated region of the TKD of FLT3. Pathways downstream of FLT3 are also activated and denoted with increased phosphorylation.

inhibiting proteins downstream of FLT3 such as: pFLT3, pERK, pSTAT5, and pAKT [104]. Additionally the drug was able to reduce tumor burden and prolong survival in mouse AML xenograft models [104], paving the way for eventual clinical evaluation in AML patients. In this trial, R/R AML patients treated with Gilteritinib showed inhibition of pFLT3 as measured by Western Blot and achieved clinical responses (CR) for a majority of patients that were FLT3-mutant when compared to FLT3-WT [110]. Because the drug was well tolerated and the general safety profile was within guidelines the U.S. FDA officially approved the use of Gilteritinib to treat AML patients in November 2018 [111]. This was a significant finding for the AML field. Many AML patients who are resistant to standard of care chemotherapies or relapse after care were able to benefit from administration of Gilteritinib when no other drugs are available, providing an overall survival benefit of four months compared to salvage chemotherapy [110].

1.8 A novel study of FLT3-ITD+ DCs

Despite the presence of FLT3 mutations in AML and the requirement for FLT3 in normal DC-development, there is very little understood about the effects of this mutation on DC populations in FLT3-IDT patients. If FLT3-ITD is a key mutation for AML, why is AML not a disease of dendritic cells? There are very few reports characterizing blood DC-subsets in any human leukemias or myelodysplastic syndromes and sample sizes are limited [112, 113]. Recently it has been reported that some AML tumor cells may express CD11c, a key marker of DC identification *in vivo* [114]. Because of the genetic variability of the disease, it has been difficult to distinguish *bona fide* DCs from tumor cells with aberrant expression of DC-related proteins. Mouse models to study AML have diverse mutation targets and cover both inducible and constitutive expression models with varying degrees of disease induction [93]. Despite the variety of models available, there is a paucity of DC-specific reports. It was shown in a non-leukemia model that the FLT3-ITD mutation produces functional DCs without pathogenic side effects but does result in increased DC abundance and moderate effects on CD4+ T cell phenotype [115]. There remains a large gap in knowledge regarding the role DCs play in FLT3-ITD+ leukemias. Therefore, I sought to interrogate the relationship between mutated FLT3 and DCs, in both primary patient samples and a murine model of FLT3-ITD AML. The central hypothesis that started my investigations into this complex system of FTL3-ITD, DCs, T cells, and AML is: AML driving mutation FLT3-ITD expands the DC compartment and alters their phenotype and function that supports the tumor microenvironment of AML. I started with two major questions to test my hypothesis: how does FLT3-ITD alter DC homeostasis?; Are FLT3-ITD+ DCs different from WT DCs by phenotype or function? (Figure 1.13).

To provide insight into the phenotypic and molecular changes in DCs during AML, I investigated samples isolated from patients with AML and a genetically-engineered mouse model (GEMM) of AML that spontaneously develops disease. I first identified a disruption in the frequency

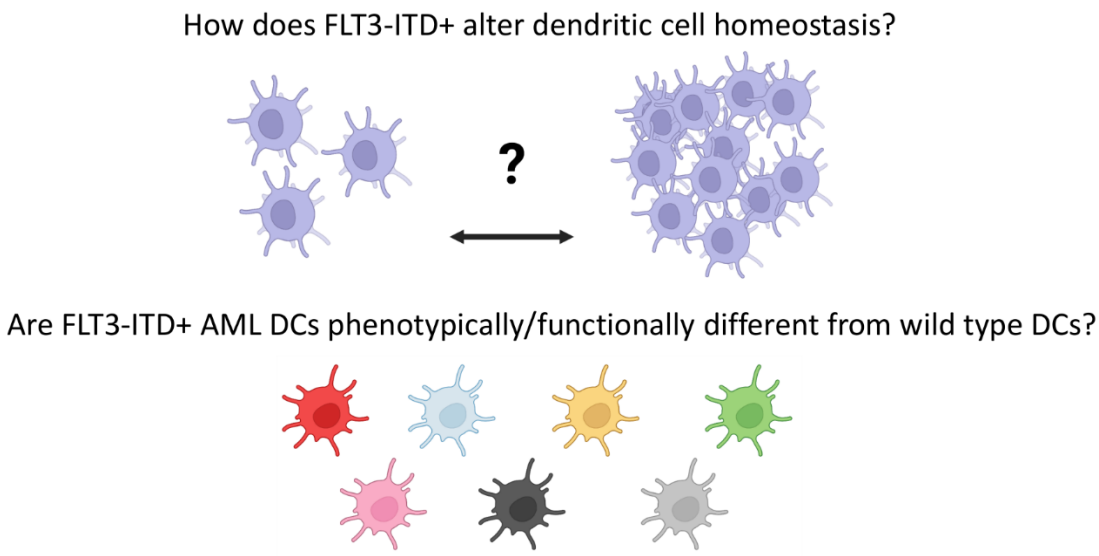


Figure 1.13 Unresolved questions regarding FLT3-ITD, AML, and DCs

Figure 1.13. Unresolved questions regarding FLT3-ITD, AML, and DCs. Upper panel posing the question of whether DC homeostasis is changed under FLT3-ITD+ AML context. Lower panel posing the question of FLT3-ITD+ AML altering the phenotype and function of DCs in the context of disease.

of cDCs in the bone marrow of patients with AML segregated by their FLT3-ITD status, suggesting that some AML patients have increased output of DCs compared to healthy donors. I then utilized our GEMM AML mice and confirmed that the FLT3-ITD mutation causes significant expansion of cDCs both in the bone marrow and spleens of AML mice compared to FLT3-ITD- controls and healthy mice. Based on that finding I used single cell RNA-sequencing (scRNA-seq) to interrogate changes to AML cDCs at the transcript level and correlated the expression of surface proteins using antibody-derived tags (ADTs), a technology that uses nucleic acid barcodes attached antibodies. I was able to identify bona fide cDCs transcriptionally and found an enrichment of the recently described T-bet- cDC2 that are efficient at polarizing naïve CD4+ T cells into Th17 cells. In the mice with AML, I observed elevated levels of blood serum cytokines that are permissive to Th17 polarization of CD4+ T cells. There is a significant enrichment of both Treg and Th17 phenotypes in the blood of AML mice, suggesting that AML DCs may be driving their polarization. To measure the impact of DCs on T cell skewing I used adoptive transfer studies to compare AML and WT healthy mice. I did not find Treg or Th17 polarization; however, AML hosts expanded and retained significantly more transferred OT-II cells compared to WT, suggesting that the increased DC frequency supports naïve OT-II T cell survival. Finally, I found that co-culturing naïve OT-II cells with AML DCs and whole-OVA protein results in increased IL-17A production, suggesting that AML DCs play a role in polarizing Th17 cells in the presence of cognate antigen. The results of my work result in the hypothesis: **FLT3-ITD+ cDCs in AML polarize naïve CD4+ T cells into Th17 by the secretion of IL-27, TGF-β, and IL-10, thereby supporting AML tumor survival**.

The following chapter will describe in detail how my experiments tested and supported this hypothesis.

Materials and Methods

Ethics statement. Studies involving patients was collected under OHSU IRB 4422 Marc Loriaux, PI. in accordance with all local and institutional requirements. Animal studies were approved and conducted under OHSU IACUC protocol "Immune-based therapeutic approaches for acute myeloid leukemia" IP00000907 Evan Lind, PI.

Human samples. Flow cytometry analisis was performed on 22 samples, 12 FLT3 WT and 10 FLT3-ITD+. Sample characteristics including mutational status, surface phenotype and karyotype are displayed in Supplemental Table 1. Bone marrow aspirates were separated by Ficoll density gradient centrifugation and frozen using a cocktail of 90%FBS+10%DMSO for cryogenic storage in liquid nitrogen. All human flow cytometry experiments were performed using liquid nitrogen stored samples. Samples were carefully thawed using a 37°C water bath before immediate slow transfer into warm 10%FBS 1XDMEM media and centrifugated at 300xg for 10 minutes. DNAse-I was added to pelleted cells for a final concentration of 50 µg/mL and 10 mL of warm media to resuspend cell pellet. Any bone fragments remaining were filtered using 70 µm mesh filters before antibody staining. Antibody clones and source are listed in Table 1. Viability was determined by Zombie Aqua staining and doublets were gated out of analysis by FSC-A vs. FSC-H. Flow cytometry data were acquired on a BD LSRFortessa or Cytek Aurora and analyzed using FlowJo v10 software. All human sample experiments are approved under IRB protocol #00004422, "Pathogenesis of Acute Leukemia, Lymphoproliferative Disorder and Myeloproliferative Disorders" (PI: Marc Loriaux, MD, PhD). Informed consent was obtained from all patients.

DC Cell-Type Scores. FLT3-ITD+ and FLT3-WT AML patient samples were identified using Weighted Gene Co-expression Network Analysis (WGCNA) from BEAT AML Bottomly et. al. 2022 https://doi.org/10.1016/j.ccell.2022.07.002. BEAT AML cohort RNAseq samples were divided by bone marrow or peripheral blood/leukapheresis before being centered and scaled separately. Using the top 30 genes for each of six single-cell AML tumor-derived signatures gene set scores were computed. Scores were based on the first principal component and aligned with average expression.

AML Murine Model. Mice expressing FLT3-ITD under the endogenous FLT3 promoter (strain B6.129-Flt3tm1Dgg/J, The Jackson Laboratory, stock no. 011112) [116] were crossed to mice with the TET2 gene flanked by LoxP sites (strain B6;129STet2tm1.1Iaai/J, The Jackson Laboratory, stock no. 017573) [117]. The Flt3ITD/Tet2 flox mice were then crossed to mice expressing Cre recombinase under the Lysm promoter (strain B6.129P2-Lyz2tm1(cre)Ifo/J, The Jackson Laboratory, stock no. 004781). The Flt3ITD/Tet2/LysMCre mice were bred to mice with the TP53 gene flanked by LoxP sites (strain B6.129P2-Trp53tm1Brn/J, The Jackson Laboratory, stock no. 008462). All breeding animals were purchased from The Jackson Laboratory. All mice used in these experiments were bred as heterozygous for FLT3-ITD and LysCre but homozygous for TET2 and TP53. All mouse experiments were performed in accordance with the OHSU Institutional Animal Care and Use Committee protocol IP00000907. No inclusion or exclusion criteria were used on the animals with correct genotype. Mice were selected and assigned to groups randomly while maintaining a 50% male 50% female ratio per experiment. No blinding was performed. Average age of mice used for in vivo studies was 30 weeks.

***Ex vivo* cell preparation**. Spleens were harvested from mice and mechanically dissociated using frosted microscope slides then rinsed with 1x PBS. Single cell suspensions were passed through 70-µm cell strainers and red blood cells were then lysed with ammonium chloride-potassium (ACK) lysis buffer. Cells were counted with hemacytometer and 3–5 × 10^6 cells were used per antibody-staining reaction. For experiments requiring enrichment of splenic cDCs, cells were enriched from total spleen cells with MojoSort™ Mouse CD11c Nanobeads (BioLegend Cat: 480078) according to manufacturer's protocol. After positive selection cells were counted via hemacytometer to assess viability and cell count with purity assessed via flow cytometry. For adoptive transfer and co-culture experiments naïve OT-II cells were magnetically enriched with MojoSort™ Mouse CD4 Naïve T Cell Isolation Kit (BioLegend Cat: 480040) according to manufacturer's protocol. After positive selection cells were counted via hemacytometer to assess viability and cell count with purity assessed via flow cytometry.

Flow cytometry staining. Bone marrow, blood, or splenocytes were processed and subjected to red blood cell lysis by ACK before counting via hemacytometer. Cells were resuspended in PBS and stained at 4°C with 100 µL 1:500 Zombie Aqua viability dye (BioLegend, Cat# 423102) and 1:200 mouse FC block (TruStain FcX, BioLegend Cat# 101320) for 15 min, covered from light. Cells were pelleted for 5 minutes at 300xg. 100 µL of cell surface staining antibody cocktail was added directly on top of the cells and stained on ice for 30 min in FACS buffer (1X PBS, 1.5% calf serum, 0.02% sodium azide, 2mM EDTA). For intracellular staining, the cells were then washed with FACS buffer, permeabilized, and stained for intracellular targets according to the manufacturer's protocol (eBioscience FOXP3 Transcription Factor Staining Buffer Set, Cat# 00-5523-00),

then resuspended in FACS buffer before analyzing on either a BD Fortessa or Cytek Aurora flow cytometer. Data were analyzed using FlowJo software.

Adoptive transfer experiments. Spleens from CD45.2$^+$ OT-II mice [118] were harvested from mice and prepared for naïve OT-II cell transfer as described above before transfer to AML CD45.1$^+$ or WT CD45.1$^+$ recipient mice. On Day 0 200,000 naïve OT-II cells were intravenously injected into recipient mice. On day 1 soluble OVA protein (200 µg) was injected into recipient mice. On Day 11 spleens from recipient mice were harvested and splenocytes were prepared for flow cytometry staining to asses OT-II populations for cell number and frequency as described above.

Ex vivo stimulation of splenocytes for cytokine secretion. Splenocytes from WT and AML mice were processed and subjected to red blood cell lysis by ACK before counting via hemacytometer. 500,000 cells were placed into tissue-culture treated 96-well plates that were treated with anti-mouse CD3ε IgG antibody (BioLegend Cat: 100340; Clone: 145-2C11) at a final concentration of 2.5 µg/mL plus anti-mouse CD28 (BioXcell Cat: BE0015-1; Clone: 37.51) at a final concentration of 20 µg/mL or control Armenian Hamster IgG antibody (BioLeged Cat: 400959; Clone: HTK888) at a final concentration of 2.5 µg/mL for Unstimulated control group and 1X Cell Activation Cocktail with Brefeldin A (CAC) (BioLegend Cat: 423303). Cells were then incubated for 6-hours in 1X Culture Media (10% FBS, 1% Pen/Strep, 1X MEM, 1 µM HEPES, 50 µM 2-ME). Cells were harvested after incubation, washed 2x with 1X PBS before processing for intracellular flow cytometry.

Antibodies.

Table 1 Human antibody list:

Antigen	Fluor	Clone	Catalogue	Vendor
AXL	Alexa Fluor 350	108724	FAB154U-100G	BD Bio
XCR1	BV421	S15046E	372610	BioLegend
CD11b	Pacific Blue	ICRF44	558123	BD Bio
Zombie Aqua			423102	BioLegend
CD45	BV605	HI30	304042	BioLegend
CD123	BV650	6H6	306020	BioLegend
CD33	BV711	WM53	303424	BioLegend
CD19	BV786	HIB19	302240	BioLegend
CD11c	Alexa Fluor 488	Bu15	337236	BioLegend
CD3e	PerCP	OKT3	317338	BioLegend
CD34	PerCP-Cy5.5	561	343612	BioLegend
CLEC10A	PE	H037G3	354704	BioLegend
CD56	PE Dazzle 594	HCD56	318348	BioLegend
HLA-DR	PE-Cy5	L243	307608	BioLegend
CD1c	PE-Cy7	L161	331516	BioLegend
CD38	Alexa Fluor 647	HH2	303514	BioLegend
CD14	Alexa Fluor 700	HCD14	325614	BioLegend

Table 2 Mouse antibody list

Antigen	Fluor	Clone	Catalogue	Vendor
CD45	BUV496	30-F11	749889	BD Bio
CD86	BUV737	GL1	741737	BD Bio
I/A-I/E	BV421	M5/114.15.2	107631	BioLegend
CD80	BV650	16-10A1	104732	BioLegend
CD317/BST2/PDCA-1	BV711	927	104732	BioLegend
XCR1	BV785	ZET	148225	BioLegend
CD103	Alexa Fluor 488	2_E7	121408	BioLegend
CD135	PE	A2F10	135306	BioLegend
CD11b	PE-Cy7	M1/70	101216	BioLegend
F4/80	APC	BM8	123116	BioLegend
CD25	PE-Cy7	PC61	102026	BioLegend
CD45.1	BV711	A20	110739	BioLegend
CD11c	Alexa Fluor 488	N418	117311	BioLegend
Ly6G	PerCP-Cy5.5	1A8	127616	BioLegend
CD3e	PE	145-2C11	100308	BioLegend
SIRPα	PE-Dazzle 594	P84	144016	BioLegend
CD19	PE-Dazzle 594	6D5	115554	BioLegend
GR-1	BV605	RB6-8C5	108439	BioLegend
CD3e	BV421	145-2C11	100336	BioLegend
CD317/BST2/PDCA-1	BV711	927	127039	BioLegend
CD11b	APC	M1/70	101212	BioLegend
Ly6C	Alexa Fluor 700	HK1.4	128024	BioLegend
CD62L	APC-Cy7	MEL-14	104428	BioLegend
CD86	PE-Cy7	PO3	105116	BioLegend
CD86	BV650	GL-1	105035	BioLegend
XCR1	BV650	ZET	148220	BioLegend
CD86	APC-Cy7	GL-1	105030	BioLegend
B220	PerCP-Cy5.5	RA3-6B2	103236	BioLegend
I/A-I/E	PE	M5/114.15.2	107608	BioLegend
IFNγ	Pacific Blue	XMG1.2	505818	BioLegend
IL-4	BV605	11B11	504126	BioLegend
I/A-I/E	BV650	M5/114.15.2	107641	BioLegend
IL-17A	BV711	TCH11-18H10.1	506941	BioLegend
FOXP3	PE-CY7	FJK.16S	25-5773-82	eBioscience
GATA3	APC	16E10A23	653805	BioLegend
T-bet	BV786	O4-46	564141	BioLegend
RORγt	PE	B2D	12-6981-82	eBioscience
CD25	APC-R700	PC61	565134	BD Bio
CD45.2	APC-Cy7	104	109824	BioLegend
CD11c	BV421	N418	117330	BioLegend
CD8a	Pacific Blue	53-6.7	100725	BioLegend
CD44	BV605	IM7	103407	BioLegend
NK1.1	BV650	PK136	108736	BioLegend
CD19	BV711	6D5	115555	BioLegend
CD3e	Alexa Fluor 488	145-2C11	100321	BioLegend
I/A-I/E	PerCP	M5/114.15.2	107624	BioLegend
CD4	PerCP-Cy5.5	RM4-4	116012	BioLegend
Zombie Aqua			423102	BioLegend

Table 3 ADT list:

Antigen	Clone	Catalogue	Vendor
TotalSeq™-A0002 anti-mouse CD8a	53-6.7	100773	BioLegend
TotalSeq™-A0103 anti-mouse/human CD45R/B220 Antibody	RA3-6B2	103263	BioLegend
TotalSeq™-A0106 anti-mouse CD11c Antibody	N418	117355	BioLegend
TotalSeq™-A0014 anti-mouse/human CD11b Antibody	M1/70	101265	BioLegend
TotalSeq™-A0117 anti-mouse I-A/I-E Antibody	M5/114.15.2	107653	BioLegend
TotalSeq™-A0200 anti-mouse CD86 Antibody	GL-1	105047	BioLegend
TotalSeq™-A0201 anti-mouse CD103 Antibody	2E_7	121437	BioLegend
TotalSeq™-A0212 anti-mouse CD24 Antibody	M1/69	101841	BioLegend
TotalSeq™-A0422 anti-mouse CD172a (SIRPα) Antibody	P84	144033	BioLegend
TotalSeq™-A0563 anti-mouse CX3CR1 Antibody	SA011F11	149041	BioLegend
TotalSeq™-A0568 anti-mouse/rat XCR1 Antibody	ZET	148227	BioLegend
TotalSeq™-A0811 anti-mouse CD317 (BST2, PDCA-1) Antibody	927	127027	BioLegend
TotalSeq™-A0849 anti-mouse CD80 Antibody	16-10A1	104745	BioLegend
TotalSeq™-A0903 anti-mouse CD40 Antibody	3/23	124633	BioLegend
TotalSeq™-A1010 anti-mouse CD205 (DEC-205) Antibody	NLDC-145	138221	BioLegend
TotalSeq™-A0093 anti-mouse CD19 Antibody Antibody	6D5	115559	BioLegend
TotalSeq™-A0981 anti-mouse TCR Vα2 Antibody	B20.1	127831	BioLegend
TotalSeq™-A0013 anti-mouse Ly-6C Antibody	HK1.4	128047	BioLegend
TotalSeq™-A0015 anti-mouse Ly-6G Antibody	1A8	127655	BioLegend
TotalSeq™-A0098 anti-mouse CD135 Antibody	A2F10	135316	BioLegend
TotalSeq™-A0105 anti-mouse CD115 (CSF-1R) Antibody	AFS98	135533	BioLegend

LegendPlex Blood Serum Assay. Peripheral blood from WT and AML was collected via retro-orbital sources. Blood was collected in Sarstedt Microvette® 500 Serum Gel tubes and centrifugated at 10,000 x G for 5 minutes at room temperature to remove cells from the serum. Aliquots of the remaining serum were stored at -80°C before assayed. On day of being assayed aliquots were thawed slowly on ice before being tested using the flow cytometry based LEGENDplex™ Mouse Inflammation Panel (BioLegend Cat: 740446) according to the manufacturer's instructions for sample processing. Samples were measured on a BD Fortessa according to the manufacturer's instructions for calibration and data collection. Data was analyzed using the online resource from BioLegend (https://legendplex.qognit.com).

ADTs and Single cell RNA-seq. Spleens from WT and AML mice were harvested and processed into filtered single cell suspensions as described above. After counting, cells were enriched for DC populations using negative selection EasySep™ Mouse Pan-DC Enrichment Kit II (StemCell Cat: 19863) according to the manufacturer's protocol. After magnetic enrichment unlabeled cells were counted and aliquoted into $1x10^6$ total cells for TotalSeq™-A (BioLegend, custom panel) ADT staining. Cells were incubated with 1:200 TruStain FcX™ PLUS (BioLegend Cat: 156604) in a final volume of 50 µL 1X FACS buffer for 10 minutes at 4°C. ADT 2X cocktail master mix was prepared for a final dilution of approximately 1 µg per ADT as follows: 1.8 µL per ADT for a total volume of 37.8 µL of just ADTs. 412.2 µL of 1X FACS buffer added to the ADTs for a final volume of 450 µL 2X ADT master mix cocktail. Add 50 µL of the master mix to each sample for a final volume of 100 µL. Incubate samples for 30 minutes at 4°C. Add 100 µL 1X FACS buffer and pellet cells for 5 minutes at 300xg. Wash two more times to remove any unbound

ADTs. Resuspend cell pellets at 1,000 cells per µL with 1X PBS 0.04% BSA. Samples were transferred to the OHSU Massively Parallel Sequencing Shared Resource (MPSSR) for 10X Genomics Chromium CITE-seq library and scRNA-seq cDNA library preparation according to 10X Genomics protocols. Each sample was sequenced on its own lane on the chip with no multiplexing. Each sample was sequenced with a target number of 10,000 cells per sample at a reads of 20,000 per cell for an approximate read depth of 200 million reads.

CITE-seq Analysis Performed in collaboration with Mark D Long PhD. Raw sequence data demultiplexing, barcode processing, alignment (mm10) and filtering for true cells were performed using the Cell Ranger Single-Cell Software Suite (v6.0.2), yielding 89,537 cells (WT: 11,326 cells per sample, AML: 11,059 cells per sample) with a mean of 23,788 reads/cell (91.38% mapping rate), median of 1,676 genes/cell, 20,037 total unique detectable genes, and 5,204 median UMI counts/cell. Subsequent filtering for high quality cells, and downstream analyses were performed using Seurat (v4) [119]. Genes expressed in less than 3 cells and cells that express less than 300 genes were excluded from further analyses. Additional filtering of cells was determined based on the overall distributions of total RNA counts (< 80,000) and the proportion of mitochondrial genes (< 10%) detected to eliminate potential doublets and dying cells, respectively. Additional detection of doublets was performed using Scrublet [120] using a cut-off threshold based on total distribution of doublet scores (doublet score < 0.2). Quantification of mitochondrial and ribosomal gene expression was calculated using the PercentageFeatureSet function, using gene sets compiled from the HUGO Gene Nomenclature Committee database. Cell cycle phase scoring was accomplished against normalized expression via the CellCycleScoring

function using mouse genes orthologous to known cell cycle phase marker genes. Ultimately, 74,198 high quality cells (WT: 37,033 cells, AML: 37,165 cells) across samples were included in downstream analyses. Ambient RNA correction was performed using SoupX [121]. Normalization and variance stabilization of gene expression data were conducted using regularized negative binomial regression (sctransform) implemented with Seurat. Normalization of ADT abundances was accomplished via the centered log-ratio method. Principal component analysis (PCA) was performed on normalized data and optimal dimensionality of the dataset was decided by examination of the Elbow plot, as the total number of PCs where gain in cumulative variation explained was greater than 0.1% (PCs = 41). Unsupervised cluster determination was performed against a constructed SNN graph via the Louvain approach using a resolution of 0.08. UMAP was applied for non-linear dimensionality reduction to obtain a low dimensional representation visualization of cellular states. Differential expression between clusters or samples was determined using the MAST method [122] via the FindMarkers function, using a minimum expression proportion of 25% and a minimum log fold change of 0.25. Mean expression of markers found within each cluster or cell annotation were used for subsequent analyses including dotplot visualization. Major cell lineages were determined using SingleR [123] using gene sets derived from the ImmGen database. Reference based mapping of the DC compartment was performed against a previously published dataset characterizing DC heterogeneity in mouse spleen (Brown et. al. [124]). Raw counts were obtained from the Gene Expression Omnibus (GSE137710) and re-processed utilizing a Seurat based pipeline described above. Reference based mapping and label transfer of previously annotated cells was performed using the FindTransferAnchors and MapQuery functions implemented within Seurat. DC-

specific annotations were further refined using curated marker assessment. Unbiased T-cell phenotype annotation was performed using ProjecTILs [125] and refined using knowledge-based assessment of canonical gene markers.

Statistics. A 2-sample Student t test with unequal variances was used to test for a statistical difference between 2 groups or a 1-sample Student t test was used to test for a statistical difference in the percent change from 0% where indicated. Unless otherwise indicated, all hypothesis tests were 2-sided, and a significance level of 0.05 was used. Ordinary One-Way ANOVA used to test-for statistical differences between 3 groups. Statistics and Graphs were generated by using Prism 9 software (GraphPad Software https://www.graphpad.com/).

Chapter II: Leukemic mutation FLT3-ITD is retained in dendritic cells and disrupts their homeostasis leading to expanded Th17 frequency

Adapted from manuscript Flynn *et. al*., accepted for publication at Frontiers in Immunology:

Flynn PA & Long MD, Kosaka Y, Mulkey JS, Coy JL, Long N, Agarwal A, Lind EF. Leukemic mutation FLT3-ITD is retained in dendritic cells and disrupts their homeostasis leading to expanded Th17 frequency. *Frontiers in Immunology 2024.*

Contributions: P.A.F conceived the study and designed all experiments with E.F.L. P.A.F. performed all the experiments, analyzed, and interpreted the data. M.D.L. analyzed single-cell data and generated single-cell figures in collaboration with P.A.F. and share first authorship. P.A.F., M.D.L., and E.F.L. wrote the manuscript. Y.K. provided manuscript editing. J.S.M and J.L.C. helped with sample processing. N.L. provided AML patient clinical data. A.A. provided AML patient serum data.

2.1 Bone marrow samples from patients with AML exhibit disrupted development of cDCs

To begin characterizing changes to the DC compartment of AML patients we identified frozen bone marrow aspirates that had been collected during the development of the multi-site BEAT AML Trial [87]. From this dataset we identified 12 WT-FLT3 status AML patients and 10 FLT3-ITD+ status AML patients and nine healthy donors so that we can compare between healthy tissue, disease, and disease present with FLT3-ITD mutation to determine the impact of FLT3-ITD on patient cDCs. The frozen bone marrow aspirates were processed into single cell suspensions by carefully thawing cells and treating them with low concentration DNAse I to prevent cell clumping after thaw. After washing the cells with PBS and filtered for bone fragements, single cell suspensions were stained with antibodies for known DC markers and analyzed by flow cytometry to measure frequencies of cDCs based on their expression of high levels of CD11c and HLA-DR on their surface after gating out non-DC immune cells (Figure 2.1, Figure 2.3A). We investigated AML

Blast gating on our patient samples to identify any significant enrichment of CD11c+ HLA-DR+ cells and did not find that our DC populations are within the Blast compartment (Figure 2.2).

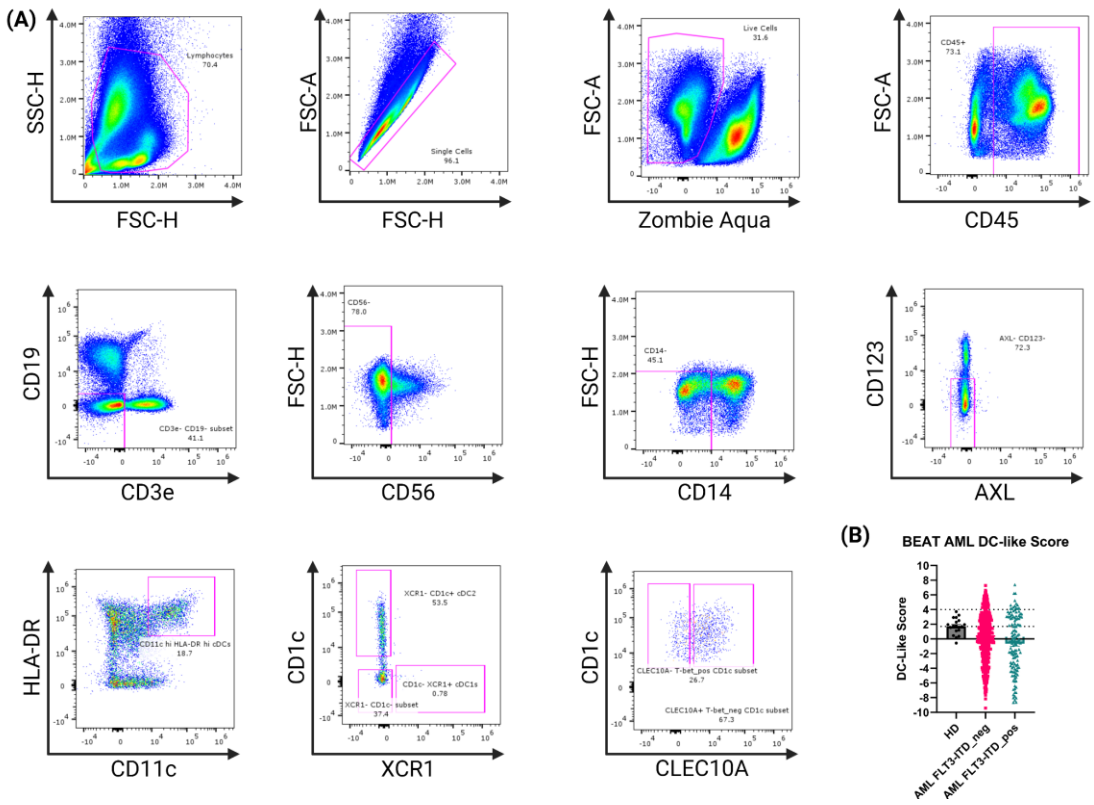


Figure 2.1 Gating scheme for human bone marrow flow cytometry and DC-like score

Figure 2.1 Gating scheme for human bone marrow flow cytometry and DC-like score. A. Frozen stocks from AML patient bone marrow aspirates were thawed and processed into single-cell suspensions for phenotyping by flow cytometry. cDC populations were identified by their high expression of surface molecules HLA-DR and CD11c after sequentially removing non-DC populations from the gating hierarchy. To identify cDC1 and cDC2 frequencies the surface molecules XCR1 and CD1c were used respectively. Based on the surface expression of CLEC10A the identification of cDC2 subsets were identified as CLEC10A positive or negative. B. Dot plots showing DC Cell-Type scores from AML patient samples.

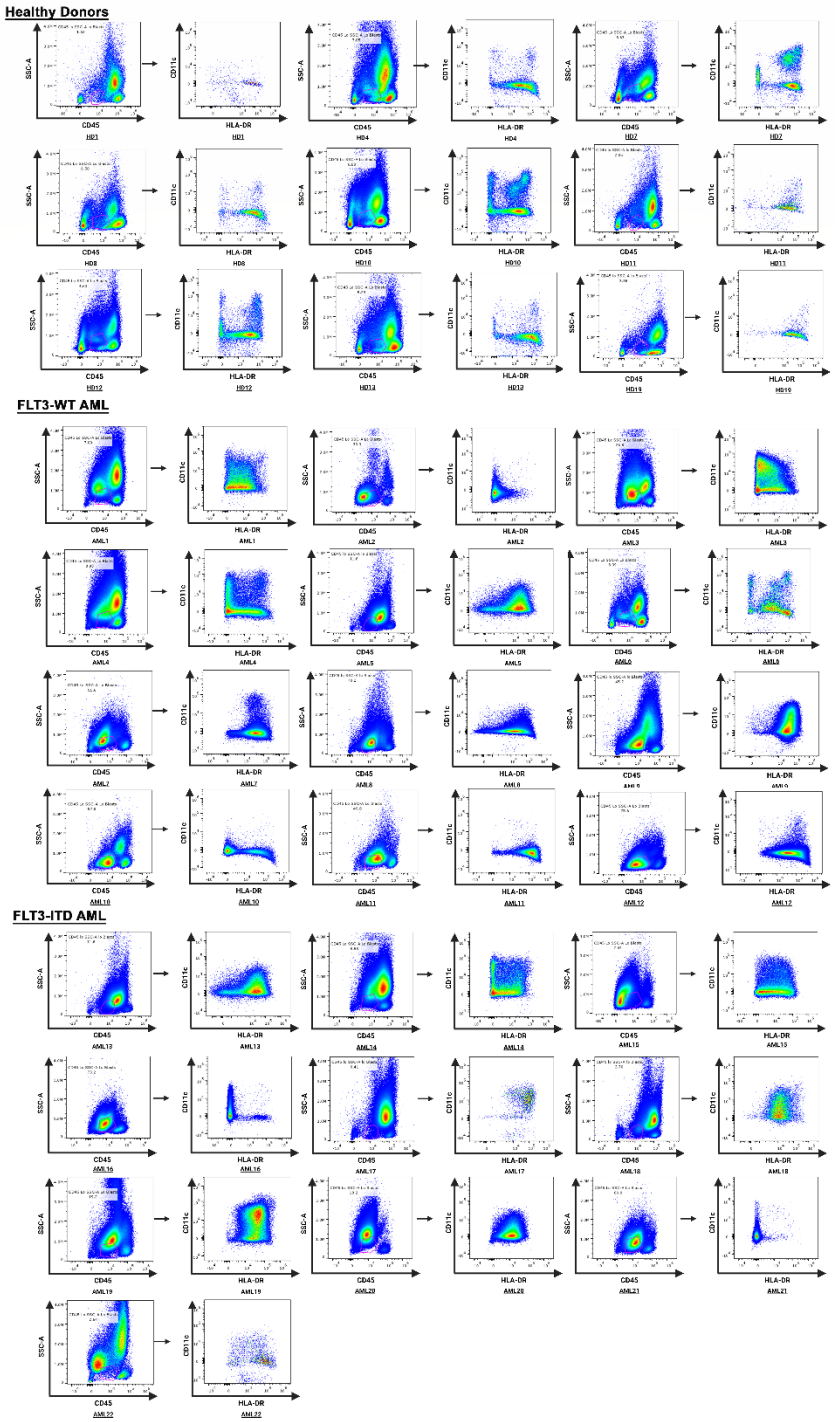


Figure 2.2 AML blast cytometry gating scheme of the bone marrow

Figure 2.2 AML Blast expression of CD11c and HLA-DR surface proteins. Blast populations identified by expression of SSC-A and CD45 low. Within this gate the expression of CD11c and HLA-DR are shown for each HD (n=9), FLT3-ITD+ (n=10), and FLT3-WT (n=12) AML samples.

We found that DCs from patients with AML showed a heterogeneous phenotype compared to healthy donors (HD). For a subset of patients, the frequency of cDCs as a proportion of CD45+ cells was increased compared to HD and this trend was even more pronounced in the samples from FLT3-ITD+ patients (Figure 2.3B). We then sought to investigate if there were any changes to the major cDC subtypes. We therefore assessed the expression of CD1c and XCR1 to identify cDC2 and cDC1, respectively (Figure 2.3C). The frequency of XCR1+ cDC1s as a proportion of all cDCs was significantly lower in both AML patient groups compared to HD (Figure 2.3D). The frequency of CD1c+ cDC2 was significantly lower in FLT3-ITD+ patient samples but there was no difference between HD and FLT3-WT AML (Figure 2.3E). Unexpectedly the CD1c XCR1 double-negative population was significantly higher in the FLT3-ITD+ AML group compared to HD and FLT3-WT (Figure 2.3F). It has been recently appreciated that cDC2 can be further subtyped by their expression of cell surface CLEC10A as an alternative to intracellular T-bet staining [124] and therefore CLEC10A expression was assessed on CD1c+ cDC2s (Figure 2.3G). We observed that the frequency of CLEC10A+ CD1c+ cells was significantly lower in the FLT3-ITD+ AML group compared to HD and FLT3-WT AML (Figure 2.3H) and the same was true for the CLEC10A- cDC2s (Figure 2.3I). Taken together, these data suggest that in some patients with FLT3-ITD+ AML, the homeostasis of bone marrow cDCs is disrupted and is characterized by a previously unreported expansion of double-negative XCR1-cDC1- poorly-differentiated cDCs.

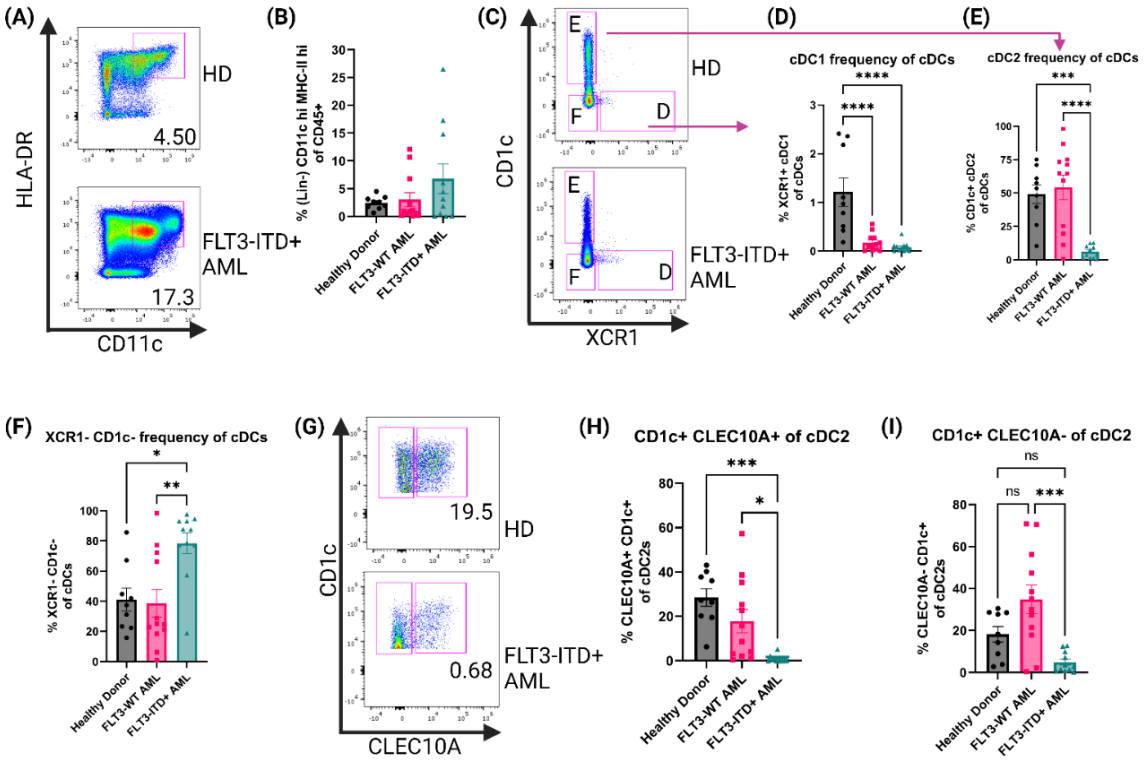


Figure 2.3 Identification of cDCs and their subsets in AML bone marrow

Figure 2.3 Identification of cDCs and their subsets in AML bone marrow. Frozen stocks from AML patient bone marrow aspirates were thawed and processed into single-cell suspensions for phenotyping by multi-parameter flow cytometry. A. Representative dot plots showing human bone marrow cDCs. cDCs defined as Lin(CD3e, CD19, CD56, CD14, AXL, CD123)-CD45+CD11c+HLA-DR+. B. Summary graph for (A). Each symbol is one human sample. HD n=9, FLT3-WT AML n=12, FLT3-ITD+ AML n=11. C. Representative dot plots showing cDC1 and cDC2 subsets of the cDCs identified in (A). cDC1 defined as CD1c-XCR1+ and cDC2 defined as XCR1-CD1c+. (D-F) Summary graphs for (C). Each symbol is one human sample. HD n=9, FLT3-WT AML n=12, FLT3-ITD+ AML n=11. (G) Representative dot plots showing CLEC10A expression of cDC2s. (H-I) Summary dot plots of CLEC10a+ and CLEC10a- cDC2s as a frequency of total cDCs (A).

2.2 Changes in cDCs in a FLT3-ITD+ mouse mode of AML

To better understand how the FLT3-ITD mutation in AML affects cDCs, we characterized cDCs in a mouse model of AML. Our lab has previously published work using AML mice that harbor the FLT3-ITD mutation and spontaneously develop AML to interrogate T cell dysfunction [126, 127]. Here we utilized mice that express one copy of FLT3-ITD under the endogenous FLT3 promoter, have a homozygous loss of TET2 and a homozygous loss of p53 using Lysozyme M (LysM) Cre-mediated deletion of LoxP-flanked alleles (AML mice) [103, 117, 128, 129]. This model is similar to others who reported combining FLT3-ITD with loss of TET2 [92]. In our system, TET2 and p53 mutations are limited to the myeloid compartment by use of LysM-Cre rather than all hematopoietic cells (*e.g.* Vav-Cre), thereby retaining a wild-type lymphoid compartment. This combination of mutations produces spontaneous AML, as evidenced by splenomegaly (Figure 2.4 A&B) and increased CD11b+ cell frequency in both the spleen and the blood (Figure 2.4 C&D). Furthermore, AML mice succumb to disease and die prematurely (Figure 2.4 E). We then measured populations of DCs using the canonical surface markers CD11c and MHC-II (Figure 2.4 F) which are expressed at high levels by cDCs compared to other myeloid subsets.

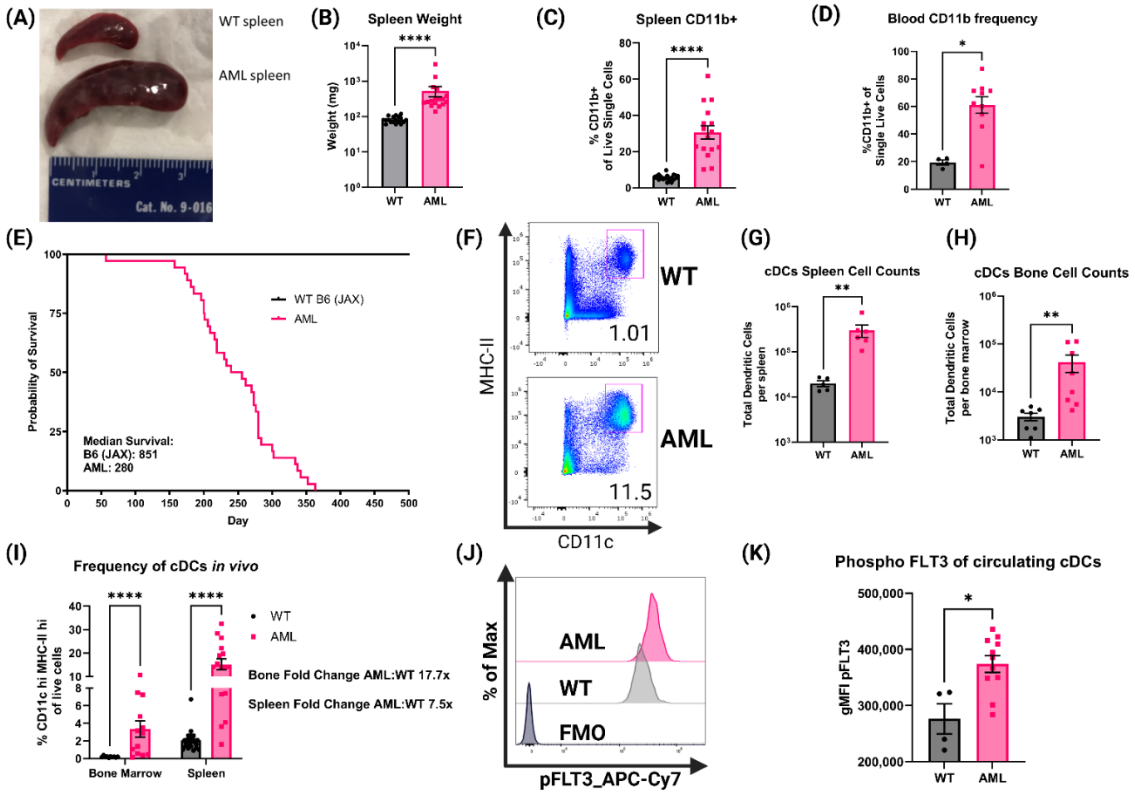


Figure 2.4 AML mice have increased frequencies of myeloid cells and cDCs

Figure 2.4 AML mice have increased frequencies of myeloid cells and cDCs. A. Comparison of dissected spleens from healthy WT mice and diseased AML mice. Ruler for scale (cm). B. Summary graph of spleen wet weight for WT and AML mice. Each symbol is one mouse. WT n=18 AML n=17. C. RBC lysed single-cell suspensions of WT and AML mice spleens were stained with monoclonal antibodies for known immune cell markers. Summary graph for frequency of CD19-CD3e-CD11b+ splenocytes. Each symbol is one mouse. WT n=17 AML n=16. D. RBC lysed single-cell suspensions of WT and AML PBMCs were stained with monoclonal antibodies for known immune cell markers. Summary graph for frequency of CD19-CD3e-CD11b+ PBMCs. Each symbol is one mouse. WT n=4 AML n=10. E. Survival curves of reference C57BL/6J mice from Jackson Laboratories (B6) and our mouse model AML mice. F. Representative dot plots showing mouse splenic cDCs. cDCs defined as Lin(Ly6C, Ly6G, F4/80, CD3e, CD19, NK1.1)-CD45+CD11chiMHC-IIhi. G. Summary graph of cDC cell counts per spleen of WT and AML mice. Each symbol is one mouse. WT n=5 AML n=6. H. Summary graph of cDC cell counts per bone marrow of WT and AML mice. Each symbol is one mouse. WT n=5 AML n=6. I. Summary graph of (F). Each symbol is one mouse. WT bone marrow n=10 WT spleens n=23. AML bone marrow n=13 AML spleens n=16. J. Representative histograms of phospho-FLT3 (pFLT3) staining in cDCs as measured by flow cytometry. RBC lysed single-cell suspensions of WT and AML PBMCs were stained with monoclonal antibodies for known immune cell markers at the surface and were stained for pFLT3 in the cytoplasm. K. Summary graph showing geometric mean fluorescent intensity (gMFI) pFLT3 staining of blood circulating cDCs. Each symbol is one mouse. WT n=4 AML n=11.

We found that in both compartments, AML mice exhibited significantly increased cDCs (Figure 2.4G&H). When comparing the frequency of cDCs in the bone marrow and spleen to healthy WT controls we found that cDCs were significantly more abundant in AML mice (Figure 2.4I). The phenotype observed in AML mice was not observed in littermates that have wild-type FLT3 but still harbored loss of both TET2 and p53 (Figure 2.5B), supporting our hypothesis that FLT3-ITD would lead to increased abundance of cDCs *in vivo*. To confirm that FLT3-ITD has constitutive signaling in cDCs, we performed intracellular flow cytometry to measure phosphorylated FLT3 (pFLT3) using an antibody that recognizes Y591 of the intracellular domain of FLT3 (Figure 2.4J) [18, 96]. When comparing the geometric mean fluorescent intensity (gMFI) between WT and AML we found that circulating cDCs in AML blood have more pFLT3, as expected in mice with a FLT3-ITD mutation (Figure 2.4K). In agreement with our gating approach used with human patient samples, we evaluated the GEMM tumor phenotype within bone marrow and spleen to which we did not observe an enrichment of DC-phenotype markers (Figure 2.6), confirming that our gating criteria are selective for differentiated cDCs in our GEMM data. Taken together, our AML mouse model displays significant expansion of cDCs as a result of FLT3-ITD.

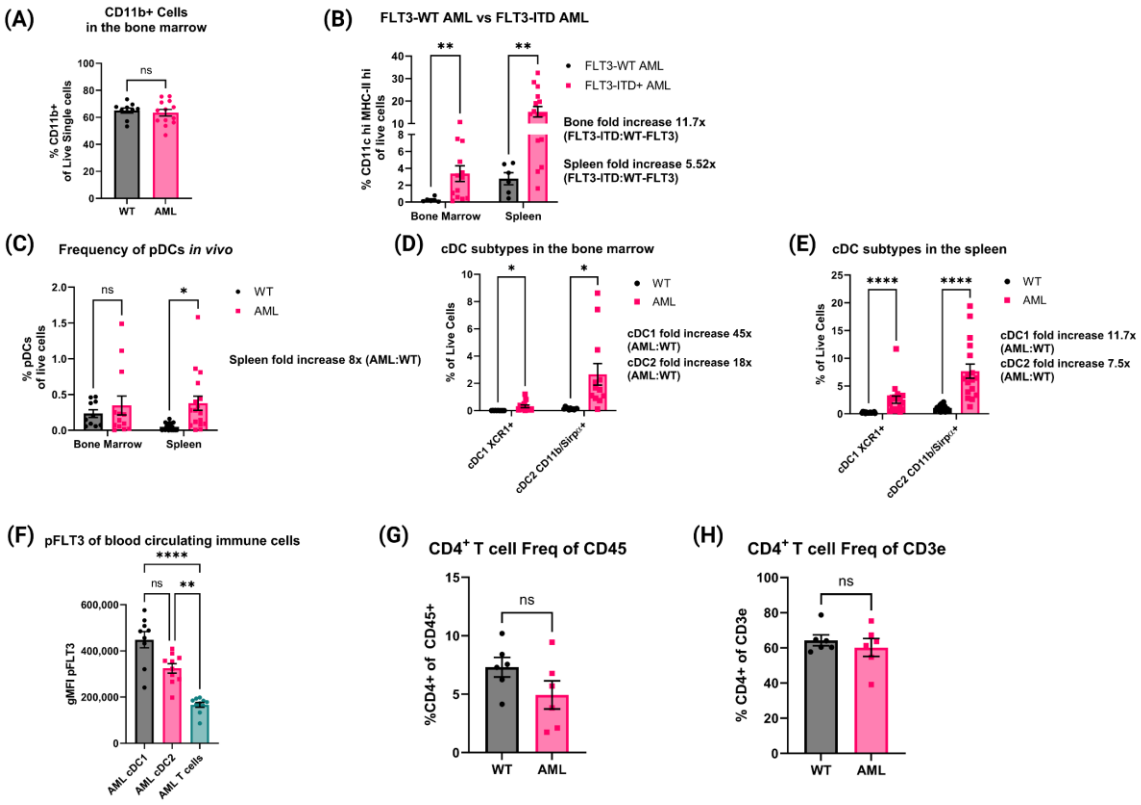


Figure 2.5 AML mice have increased frequencies of myeloid cells and cDCs

Figure 2.5 AML mice have increased frequencies of myeloid cells and cDCs. A. Summary bar chart of CD11b+ cell frequency in the bone marrow of WT (n=10) and AML (n=13) mice. B. Summary bar chart of cDC frequency in the bone marrow and spleen compartments of WT-FLT3 LysM-Cre+ TET2loxp/loxp mice (n=6) and AML mice (n= bone marrow n=13 AML spleens n=16). C. Summary bar chart of pDC frequency in bone marrow and spleen. Each symbol is one mouse. WT bone marrow n=10 WT spleens n=23. AML bone marrow n=13 AML spleens n=16. D. Summary bar chart of cDC1 and cDC2 frequency in bone marrow. Each symbol is one mouse. WT bone marrow n=10. AML bone marrow n=13. E. Summary bar chart of cDC1 and cDC2 frequency in spleens. Each symbol is one mouse. WT bone marrow n=23. AML bone marrow n=16. F. Summary bar chart of blood circulating AML cDC1 and AML cDC2 and AML T cells gMFI pFLT3. N=10. G. Summary bar chart of blood circulating CD4+ T cells as a proportion of CD45+ cells. WT n=6 and AML n=6. H. Summary bar chart of blood circulating CD4+ cells as a proportion of total CD3ε+ T cells. WT n=6 and AML n=6.

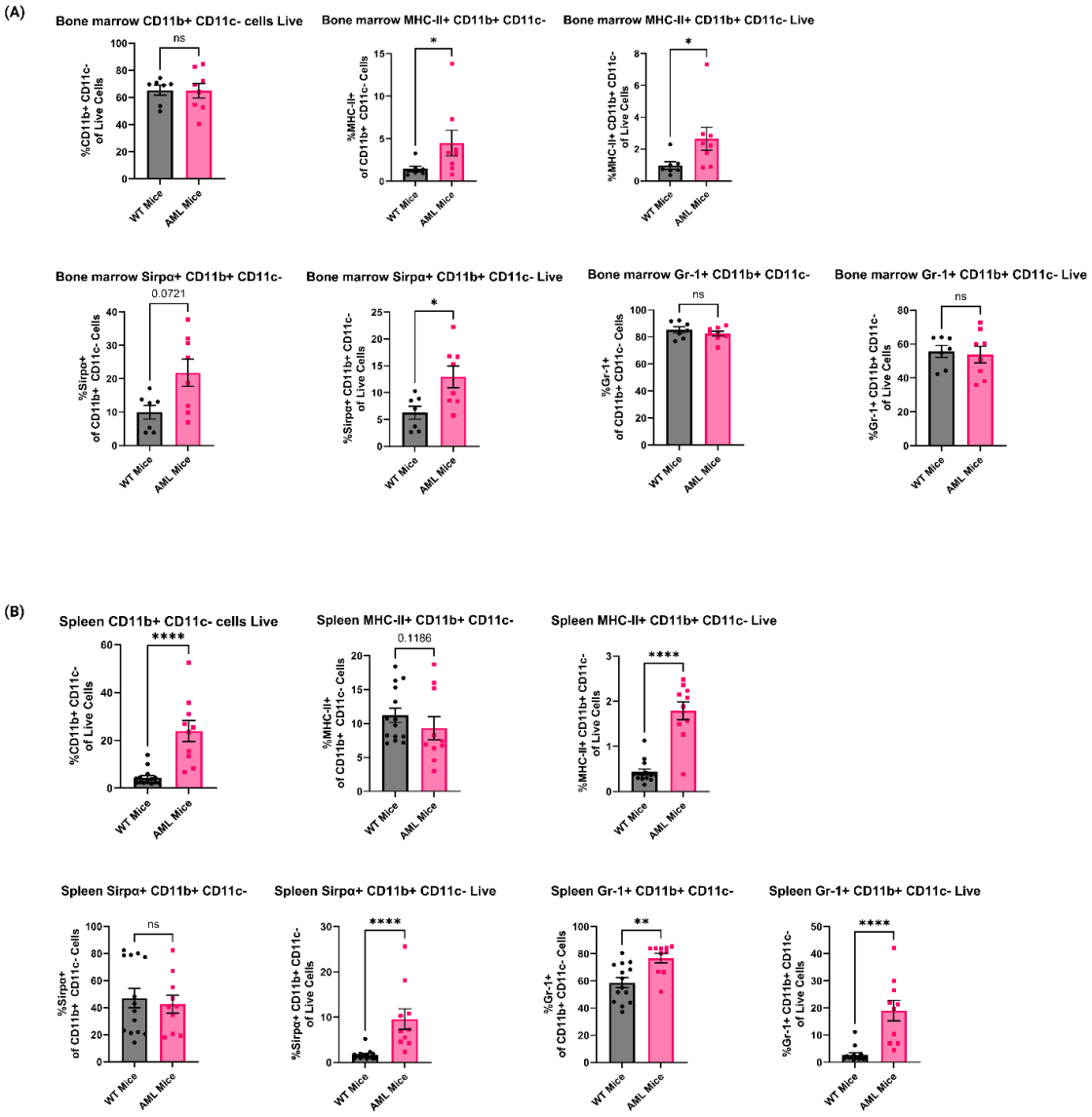


Figure 2.6 GEMM tumor phenotype in the bone marrow and spleen

Figure 2.6 GEMM tumor phenotype in the bone marrow and spleen. A. Summary bar charts of myeloid phenotyping GEMM tumor cells (CD11b+ CD11c-) frequency in the bone marrow of WT (n=7) and AML (n=8) mice. B. Summary bar charts of of myeloid phenotyping GEMM tumor cells (CD11b+ CD11c-) frequency in the spleen of WT (n=14) and AML (n=10).

2.3 Single-cell profiling of splenic cDCs in the context of FLT3-ITD AML

After confirming the phenotype of DCs in our AML mice, we interrogated how DCs are altered in the FLT3-ITD AML environment at the single-cell level using paired transcriptome and cell surface protein profiling (CITE-seq). In total, 74,198 high-quality cells were profiled across samples (WT: n = 4, AML: n = 4) after quality assessment and filtering procedures (Figure 2.7 A-B). Unsupervised cell clusters were annotated to major cell lineages using a combination of supervised and knowledge-based cell annotation procedures, and allowed for the identification of bona fide cDCs from other immune cell subsets as well as any possible AML-tumor related myeloid cells from spleen tissue (Figure 2.7 3C-D). As expected, splenic cells captured from AML mice had a significantly higher proportion of DCs relative to WT mice (Figure 2.7 D&E). Cell surface protein abundances measured by Antibody Derived Tags (ADTs) confirmed major cell lineages determined by transcriptome-based annotations (Figure 2.8 A&B; Figure 2.9), and strongly correlated with corresponding transcripts across cells (Figure 2.8 C).

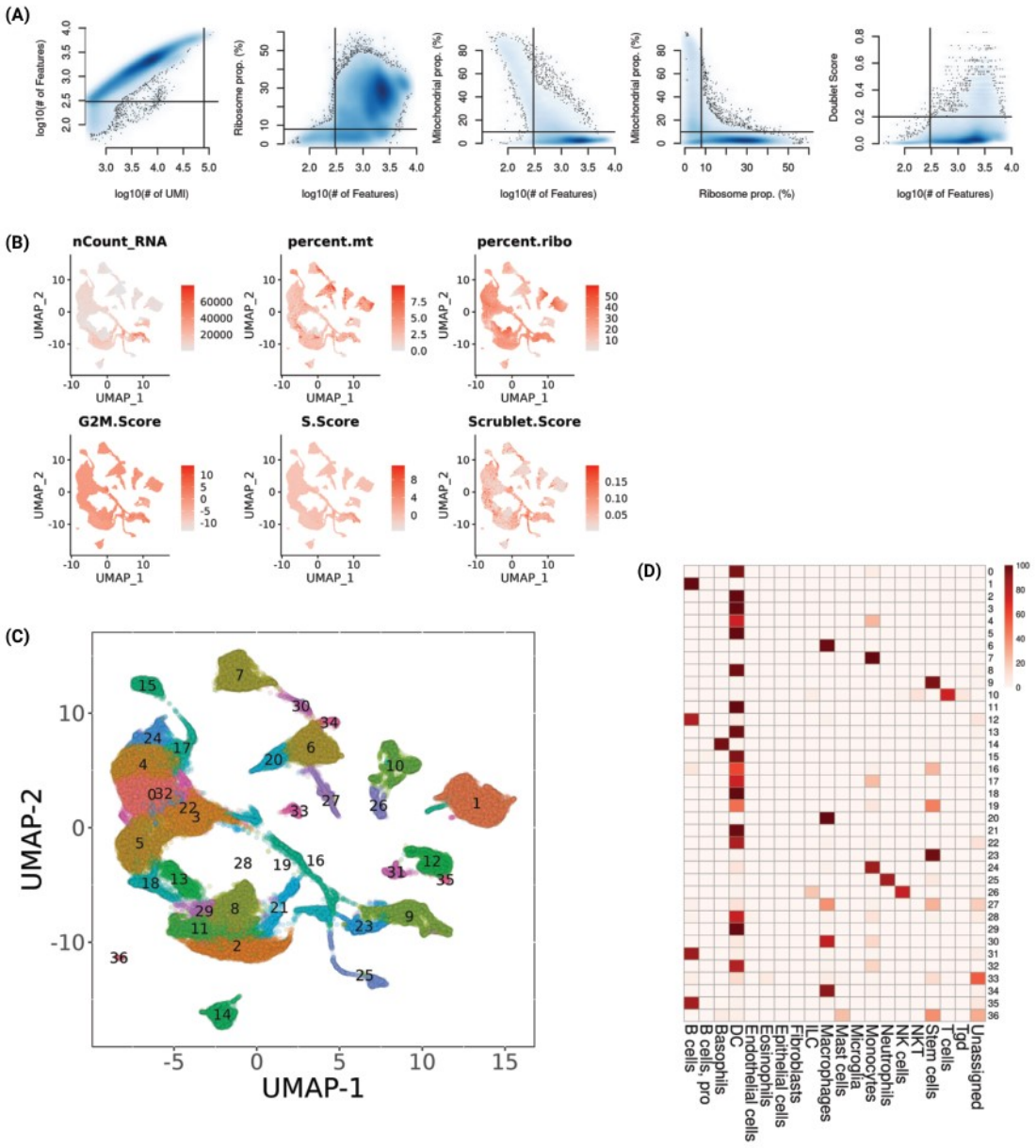


Figure 2.7 Mouse scRNA-seq QC and processing metrics

Figure 2.7 Mouse scRNA-seq QC and processing metrics. A. Filtering criteria used to isolate high-quality cells for downstream analysis, including number of detectible features/UMI, ribosomal and mitochondrial gene proportions, and Doublet Score. B. QC metrics and cell-cycle scoring shown for all cells. C. UMAP representation of all high-quality cells. Colors represent final clusters determined by unsupervised clustering analysis. D. Proportion of each cluster annotating to major lineages determined by SingleR supervised classification against the Immgen Database. Figures generated by Mark D. Long with input from PAF.

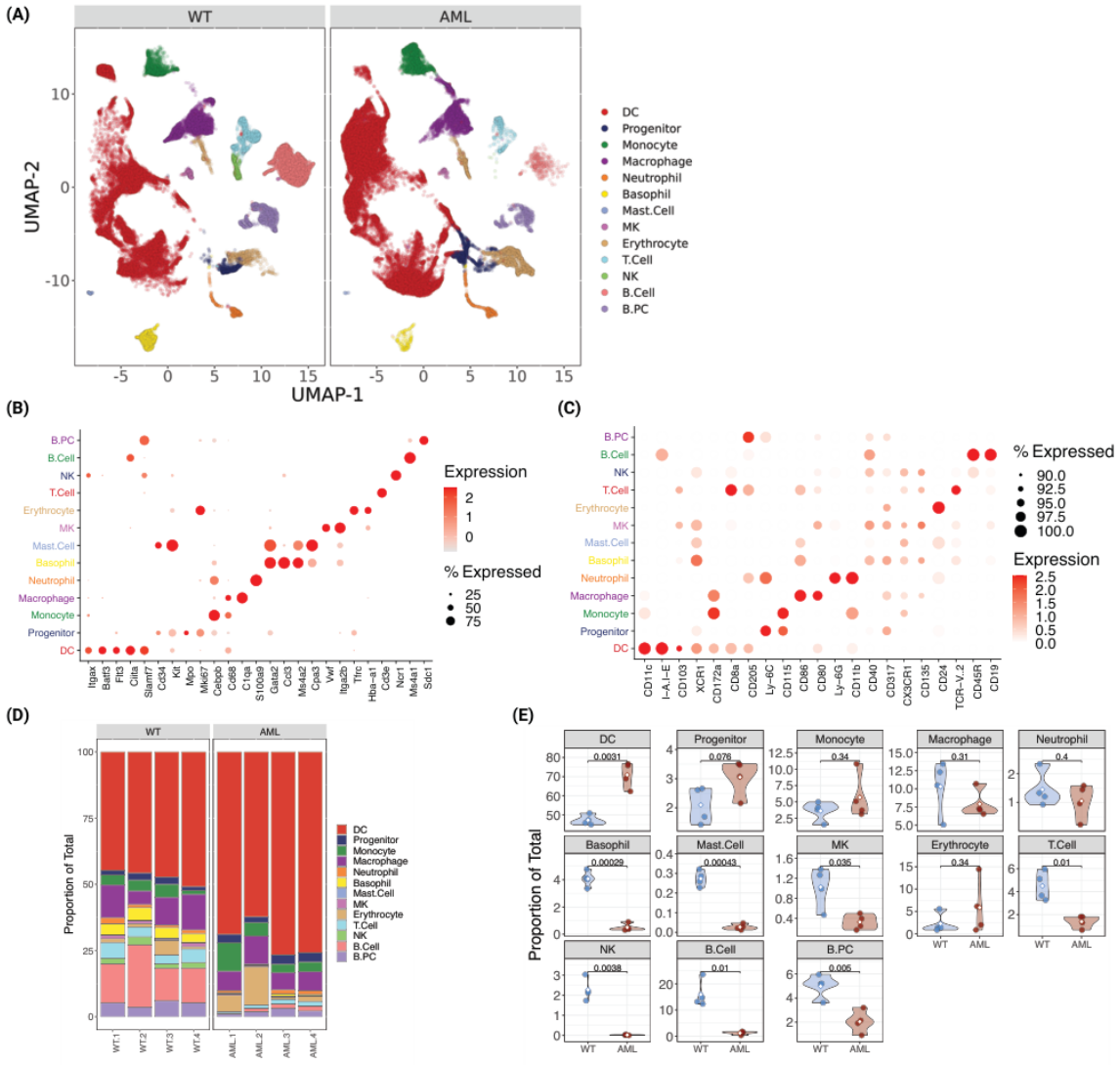


Figure 2.8 Single cell RNA-seq profiling of AML and WT mouse spleens

Figure 2.8 Single cell RNA-seq profiling of AML and WT mouse spleens. A. UMAP plot of total cells derived from WT and AML mice after magnetic bead enrichment for DCs. B. Mean expression of various linage markers across annotated cell types. C. Mean abundance of protein markers across annotated cell types. D. Cell type proportions across samples AML (n=4) and WT (n=4). E. Violin plots of cell type proportions compared between WT and AML groups. Differences in means were determined using Student's t-test. Figures generated by Mark D. Long with input from PAF.

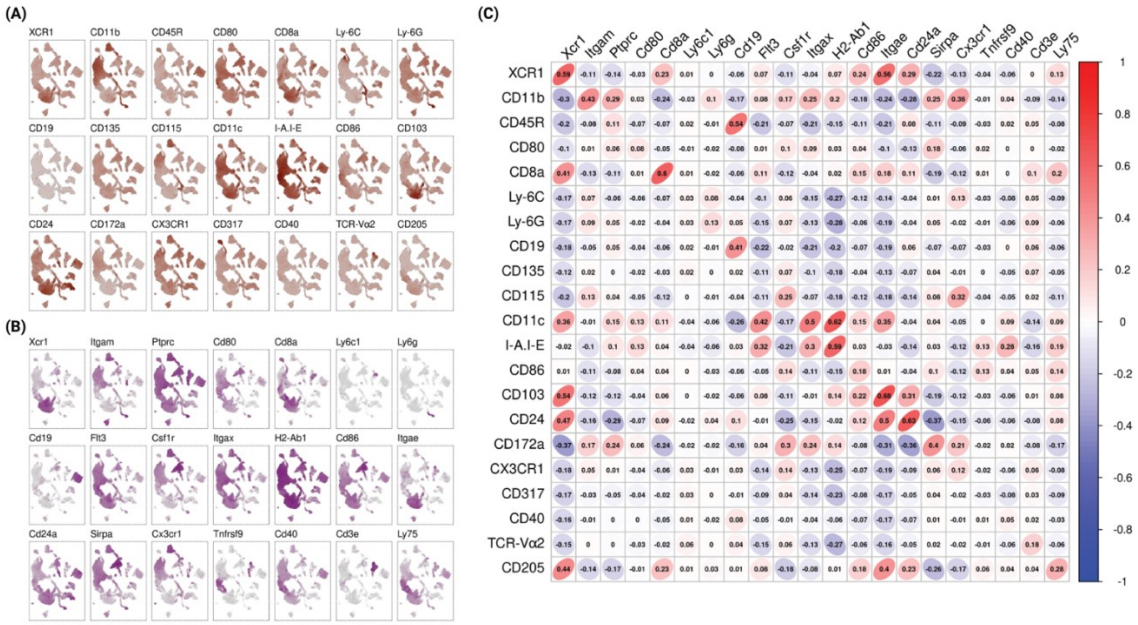


Figure 2.9 Comparison of GEX and Protein ADT profiles across single cells

Figure 2.9 Comparison of GEX and Protein ADT profiles across single-cells. A. UMAP representation of for protein ADT abundances. B. UMAP representation of gene expression markers associated with proteins profiled in (A). C. Correlation matrix for surface proteins and corresponding transcripts across all cells. Figures generated by Mark D. Long with input from PAF.

Having isolated the DC compartment from other immune cells in the WT and AML splenic environment, we next sought to further define DC phenotype heterogeneity and how it is altered in AML. Recent single-cell profiling of DCs has established further heterogeneity than previously thought, for instance that cDC2 subsets can be segregated by their expression of T-bet [124]. To identify DC subtypes within our mouse splenic cells, we implemented a referenced based classification approach utilizing single-cell data derived from mouse splenocytes reported by Brown *et. al.* [124] (Figure 2.10) to help in delineating DC phenotypes in our data. Using this approach paired with subsequent marker based assessment, we detected 9 distinct DC subsets including cDC1, T-bet- and T-bet+ cDC2, migratory *Ccr7* expressing DCs (CCR7+ DC), plasmacytoid DCs (pDC) and Siglec-H expressing pDC precursors (Siglec-H DC), monocyte-like DCs, as well as proliferative DC subsets which were strongly supported by canonical marker assessment (Figure 2.11 A-C).

Notably, upon examination of DC subtype proportions between WT and AML splenocytes, we observed a strong and significant shift in cDC2 subsets with AML splenocyte cDC2s being dominated by a T-bet- phenotype, while WT cDC2s maintained a T-bet+ phenotype (Figure 2.11 D-E). The expression of *Clec10a* and *Cd209a* were highly expressed on T-bet- cDC2s (Figure 2.11 B) in accordance with data published [124]. Surface protein detection by ADT confirmed that T-bet- cDC2s had low expression of CD80 but high expression of CD172a and CD11b as expected (Figure 2.11 C). Among other DC subtypes, there was no observed difference in the proportion of cDC1s between AML and WT, but significant decreases in pDCs and CCR7+ DCs, and increase in monocyte-like DCs were also observed in AML splenocytes, although to a lesser extent

than the shift in Tbet-/+ cDC2s. T-bet- cDC2s were previously reported to preferentially skew naïve CD4+ T cells into Th17s *in vitro* [124]. Th17 cells have been shown to be elevated in AML patients and are associated with negative prognosis in AML [130]. IL-17A promoted proliferation of AML cells and increased IL-17R expression on the surface of tumor cells and the presence of IL-17A reduced the frequency of Th1 cells, suggesting that for hematological diseases IL-17A promotes tumor survival [130]. In summary, our data shows that *bona fide* DCs are present in AML and they are skewed towards T-bet- cDC2 phenotype that may contribute to Th17 skewing *in vivo*.

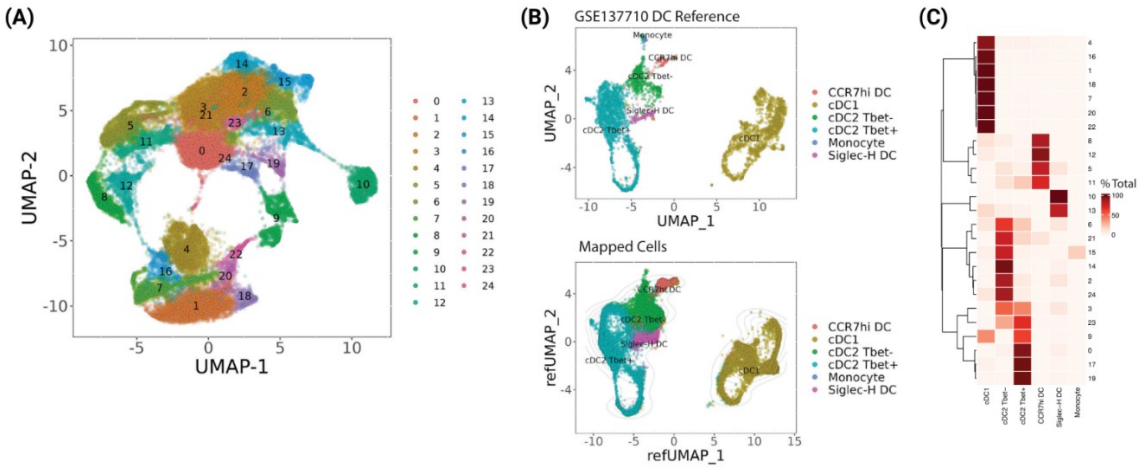


Figure 2.10 Reference based mapping delineates DC heterogeneity in WT and AML mouse spleens

Figure 2.10 Reference based mapping delineates DC heterogeneity in WT and AML mouse spleens. A. UMAP visualization of the identified DC compartment. B. Upper panel, GSE137710 DC Reference UMAP from re-analysis of data derived from Brown et. al. (GSE137710). Bottom panel, AML and WT mouse splenocytes mapped to Brown et. al. reference. C. Proportion of cells within each cluster annotating to various DC subtypes after classification from reference based mapping. Figures generated by Mark D. Long with input from PAF.

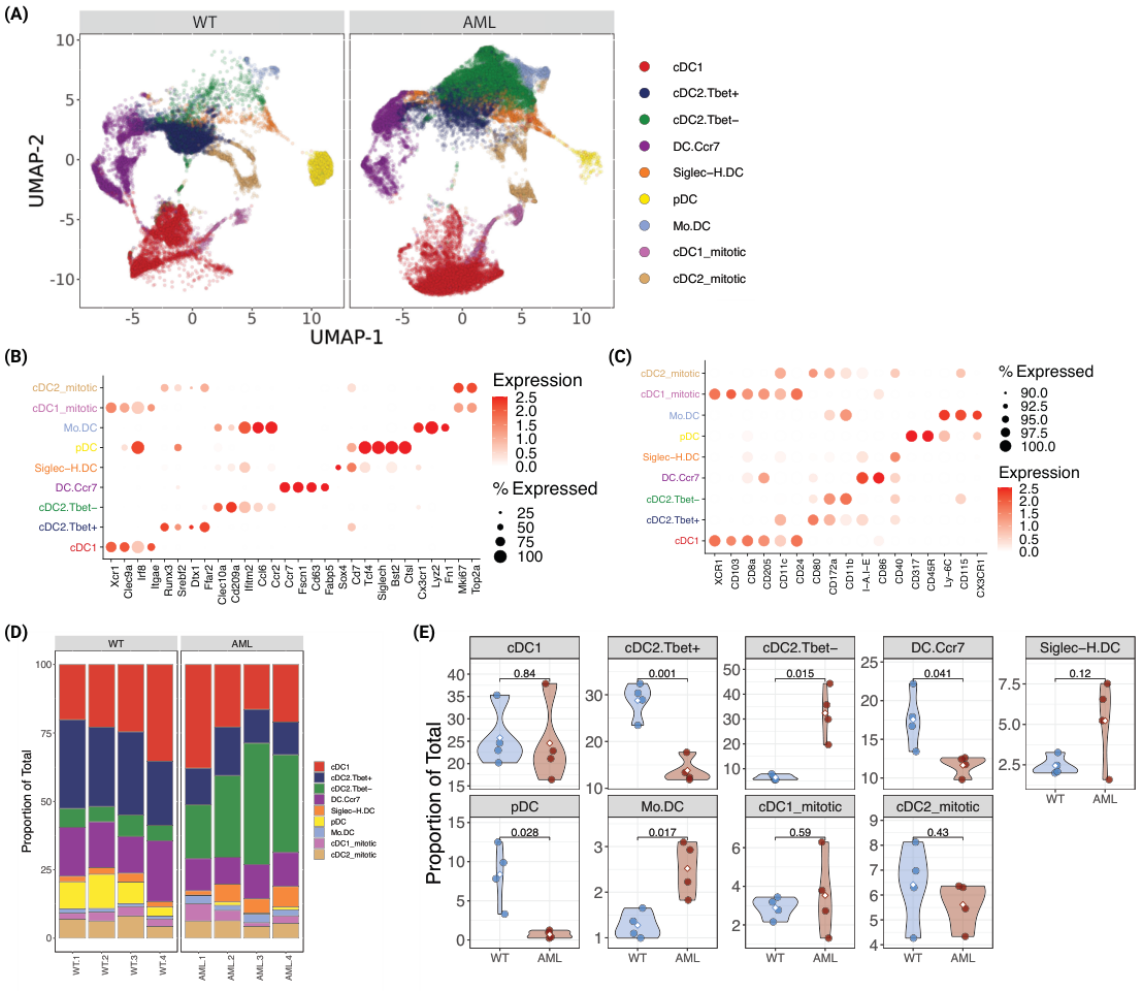


Figure 2.11 Single cell RNA-seq analysis of AML and WT mouse spleens identify changes in DC populations

Figure 2.11 Single cell RNA-seq analysis of AML and WT mouse spleens identify changes in DC populations. A. UMAP plot of DC subsets identified from WT and AML mice after magnetic bead enrichment for DCs. B. Mean expression of various DC gene markers across annotated DC subtypes. C. Mean abundance of protein markers across annotated DC subtypes. D. DC subtype proportions across samples AML (n=4) and WT (n=4). E. Violin plots of DC subtype proportions compared between WT and AML groups. Differences in means were determined using Student's t-test. Figures generated by Mark D. Long with input from PAF.

2.4 AML mice have disrupted CD4+ T cell phenotype and cytokines

It has been shown that mature DCs tune adaptive T cell responses into T-helper subsets (e.g. Th1, Th2, Th17, and Treg) [131-133] and given that the AML mice contain significantly increased levels of cDCs *in vivo* we hypothesized that CD4+ T cell populations would be altered systemically in the context of AML. We sampled peripheral blood from AML and WT healthy mice to measure the frequency of CD4+ T cells (Figure 2.12). The frequency of circulating CD4+ T cells was not statistically different but when we compared the canonical T-helper transcription factors T-bet, GATA3, FOXP3, and RORγt we found that in AML mice they had increased Treg and Th17 populations (Figure 2.13 B). Examination of the T-cell compartment identified from single-cell profiling of WT and AML splenocytes supported a strong shift away from naïve CD4+ T cells and an expansion of Tregs in AML spleen (Figure 2.14). Th17 cells could not be identified given the relatively low number of total T cells available for analysis (Figure 2.14). We further confirmed this T helper phenotype in the AML mice by analyzing the putative T helper transcription factors in the bone marrow and spleen (Figure 2.15). We consistently see that in all three tissues (blood, bone, and spleen) that CD4+ T cells are skewed towards Treg and Th17 phenotypes.

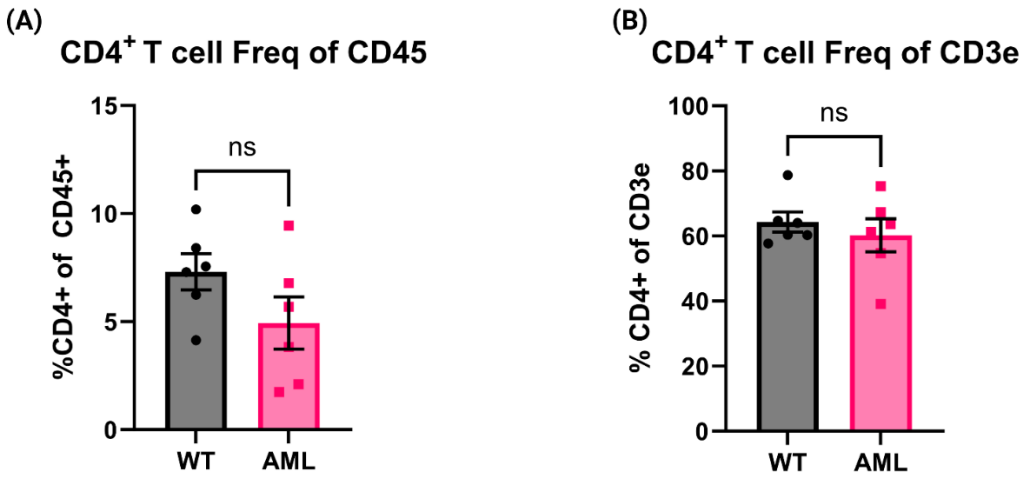


Figure 2.12 CD+ T cell frequencies are not altered in AML mice

Figure 2.12 CD4+ T cell frequencies are not altered in AML mice. A&B. Summary bar chart of splenic CD4+ T cell frequencies. WT (n=6) AML (n=6).

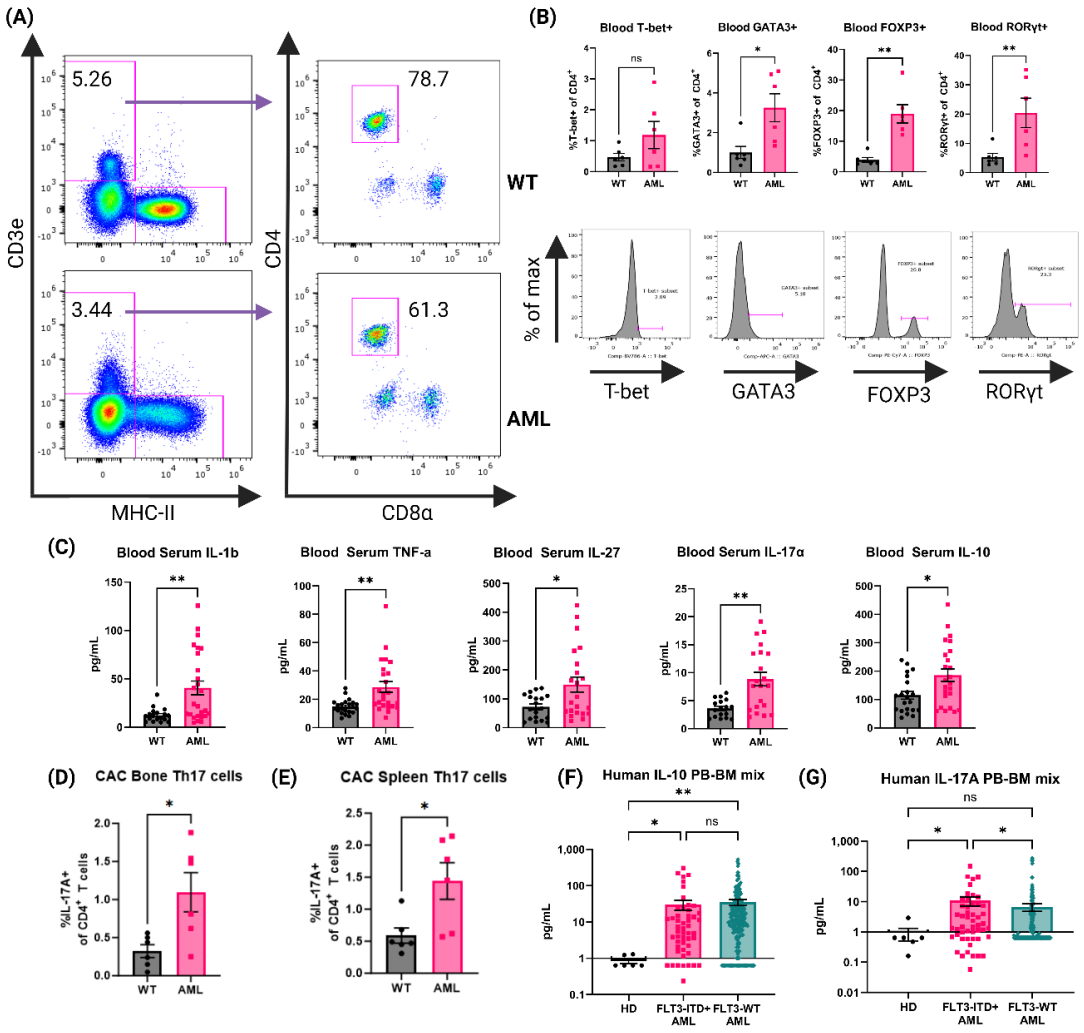


Figure 2.13 AML mouse blood exhibits a Th17 inflammatory phenotype

Figure 2.13 AML mouse blood exhibits a Th17 inflammatory phenotype. A. Representative dot plots for gating on mouse blood CD4+ T cells by flow cytometry. Top row is WT and bottom row is AML. B. Transcription factor expression of CD4+ T cells. Top row is summary bar charts of transcription factor expression in CD4+ T cells from (A). Each symbol is an individual mouse. WT (n=6) and AML (n=6). Bottom row is representative histograms of each transcription factor. C. Blood serum cytokines in WT (n=21) and AML (24) mice. Each symbol is an individual mouse. Summary bar charts for selected inflammatory cytokines measured. D. Summary bar chart for bone marrow IL-17A+ CD4+ T cells after stimulation with Cell Activation Cocktail. WT n=6 and AML n=6. E. Summary bar chart for spleen IL-17A+ CD4+ T cells after stimulation with Cell Activation Cocktail. WT n=6 and AML n=6. F. Summary bar charts for Luminex measurement of human IL-10 cytokine detected in HD and AML patient samples of mixed peripheral blood (PB) and bone marrow (BM). HD (n=6) and FLT3-ITD+ AML (n=50) and FLT3-WT AML (n=251). G. Summary bar charts for Luminex measurement of human IL-17 cytokine detected in HD and AML patient samples of mixed peripheral blood (PB) and bone marrow (BM). HD (n=6) and FLT3-ITD+ AML (n=50) and FLT3-WT AML (n=251).

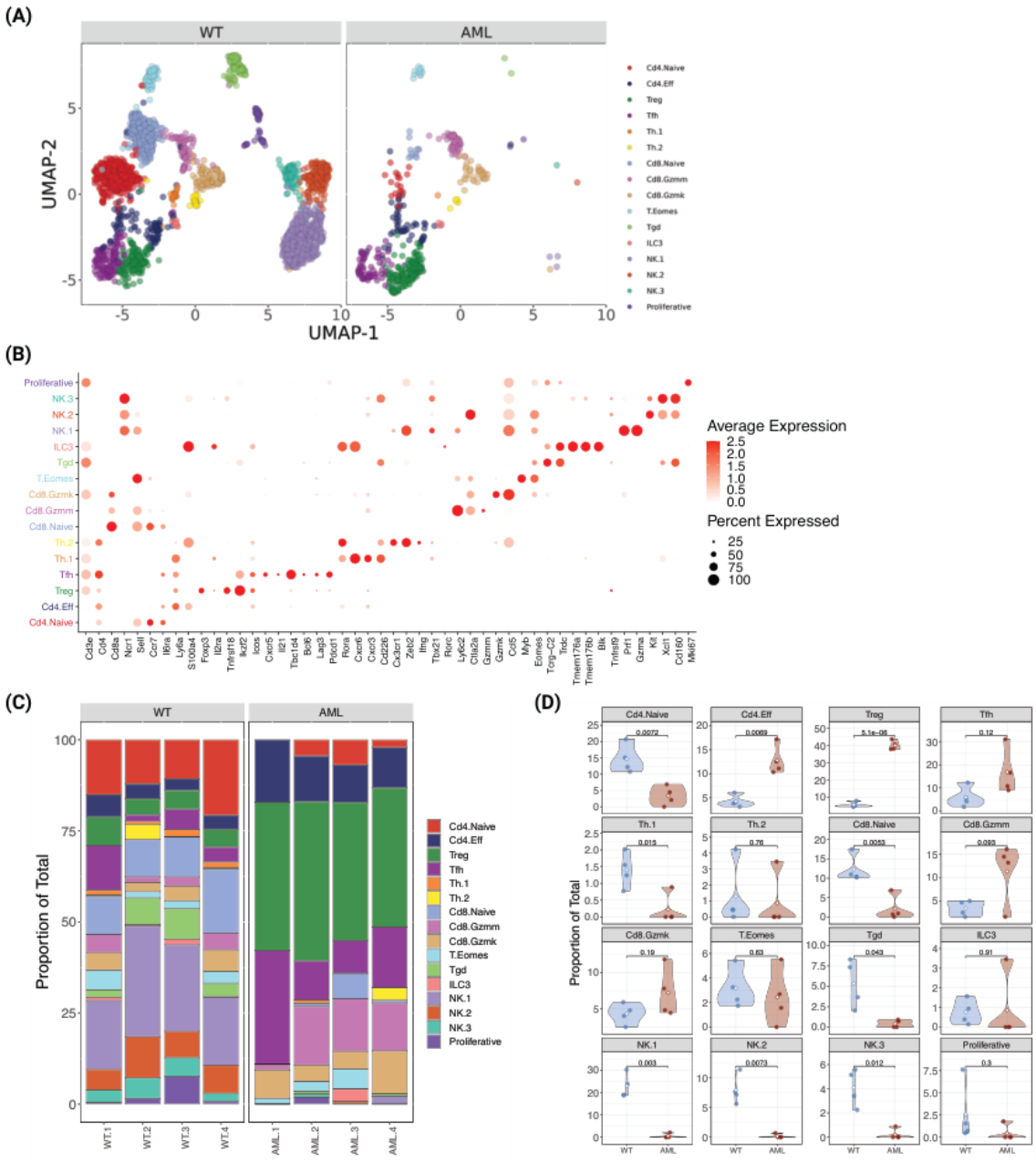


Figure 2.14 Single cell RNA-seq analysis of AML and WT mouse spleens identify changes in T cell phenotypes

Figure 2.14 Single cell RNA-seq analysis of AML and WT mouse spleens identify changes in T-cell phenotypes. A. UMAP plot of T-cell and NK subsets identified from WT and AML mice. B. Mean expression of various T and NK cell gene markers across annotated T-cell subtypes. C. T and NK cell subtype proportions across samples AML (n=4) and WT (n=4). D. Violin plots of T and NK cell subtype proportions compared between WT and AML groups. Differences in means were determined using Student's t-test.

The increased abundance of cDCs and the altered T-helper compartment led us to hypothesize that the cytokine profile in AML mice would also be altered. Serum samples from AML and WT healthy mice were analyzed by LegendPlex Mouse Inflammation Panel for inflammation associated cytokines (Figure 2.13C). We found that AML mice had increased levels of IL-1β and TNF-α, consistent with a chronic disease phenotype. We also observed increased levels of IL-27 which is a potent DC-secreted cytokine that can influence T cell responses after activation [49, 50] as well as the Treg and Th17 associated cytokines IL-10 and IL-17A (Figure 2.13C). To confirm the source of IL-17A in our GEMM, we performed ex vivo stimulation of bone marrow and splenocytes from WT and AML mice. We stimulated cells with either Cell Activation Cocktail containing PMA+ Ionomycin and Brefeldin A (CAC) or anti-CD3ε+anti-CD28 and performed intracellular cytokine staining and flow cytometry analysis. In both bone marrow and spleen tissue we see increased IL-17A in the AML samples when compared to WT mice. We identified CD4+ T cells as the source of IL-17A (Figure 2.13D,E; Figure 2.16A). Furthermore, we analyzed the non-T cell compartment by analyzing CD3ε- cells in our assay and we do not find a detectable presence of IL-17A in non-T cells (Figure 2.16B). Based on these finding we analyzed serum from bone marrow and peripheral blood of HD, FLT3-ITD+ AML, and FLT3-WT patient samples by Luminex. We saw significantly increased levels of IL-10 in both AML groups compared to HD, but we do not see a difference between AML groups (Figure 2.13F). There is a significant difference of IL-17A detected in patients in the FLT3-ITD+ AML group when compared to both HD and FLT3-WT AML (Figure 2.13G). Together, these data suggest that in the context of AML, naïve CD4+ T cells are directed towards Th17 and Treg phenotypes by cDCs.

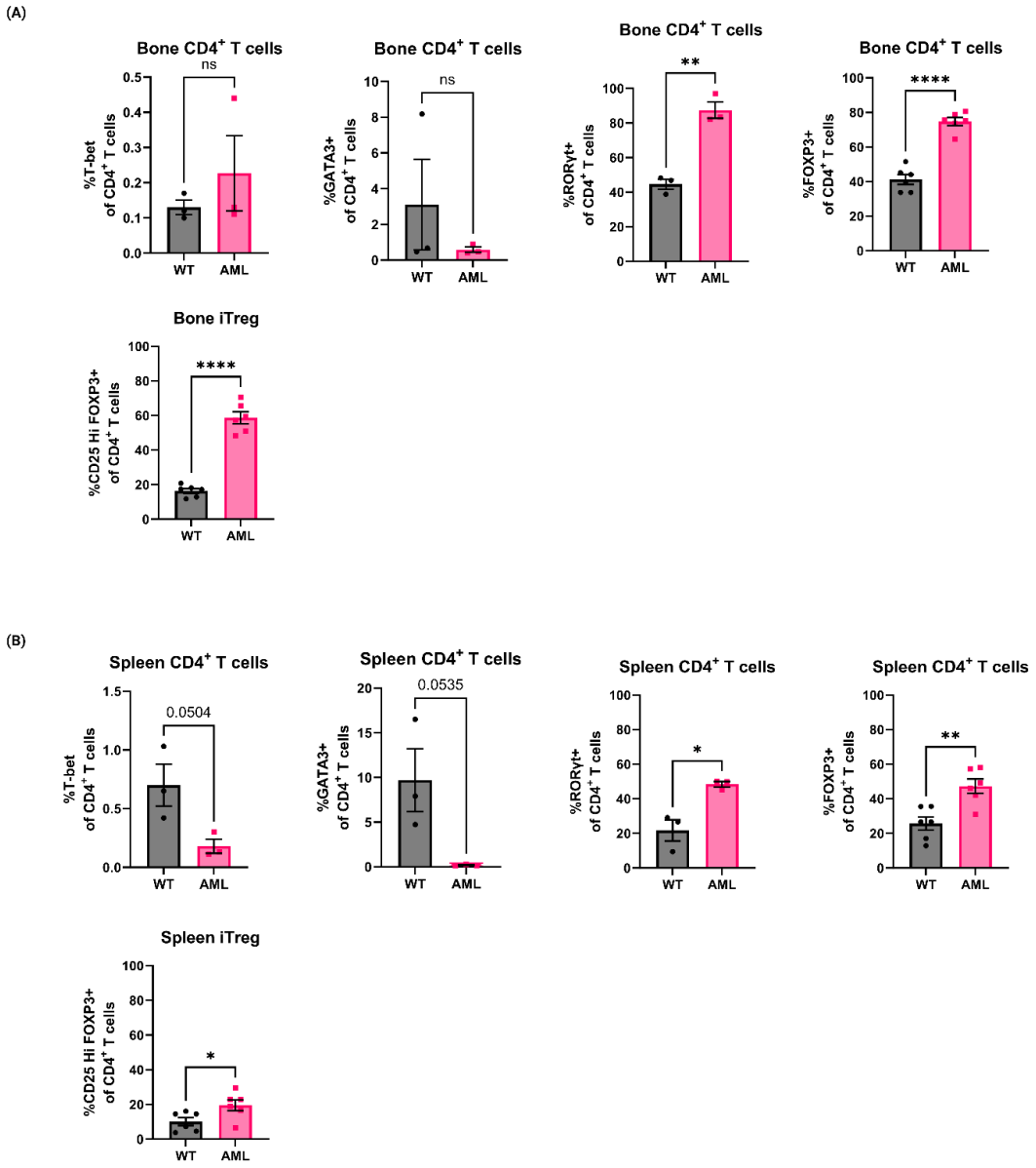


Figure 2.15 GEMM CD4+ T cell transcription factor phenotyping in the bone marrow and spleen

Figure 2.15 GEMM CD4+ T cell transcription factor phenotyping in the bone marrow and spleen. A. Summary bar charts of phenotyping GEMM Thelper transcription factors in the bone marrow of WT (n=3) and AML (n=3) mice. FOXP3+ and CD25+ T cell n=6 for both genotypes. B. Summary bar charts of phenotyping GEMM Thelper transcription factors in the spleen of WT (n=3) and AML (n=3). FOXP3+ and CD25+ T cell n=6 for both genotypes.

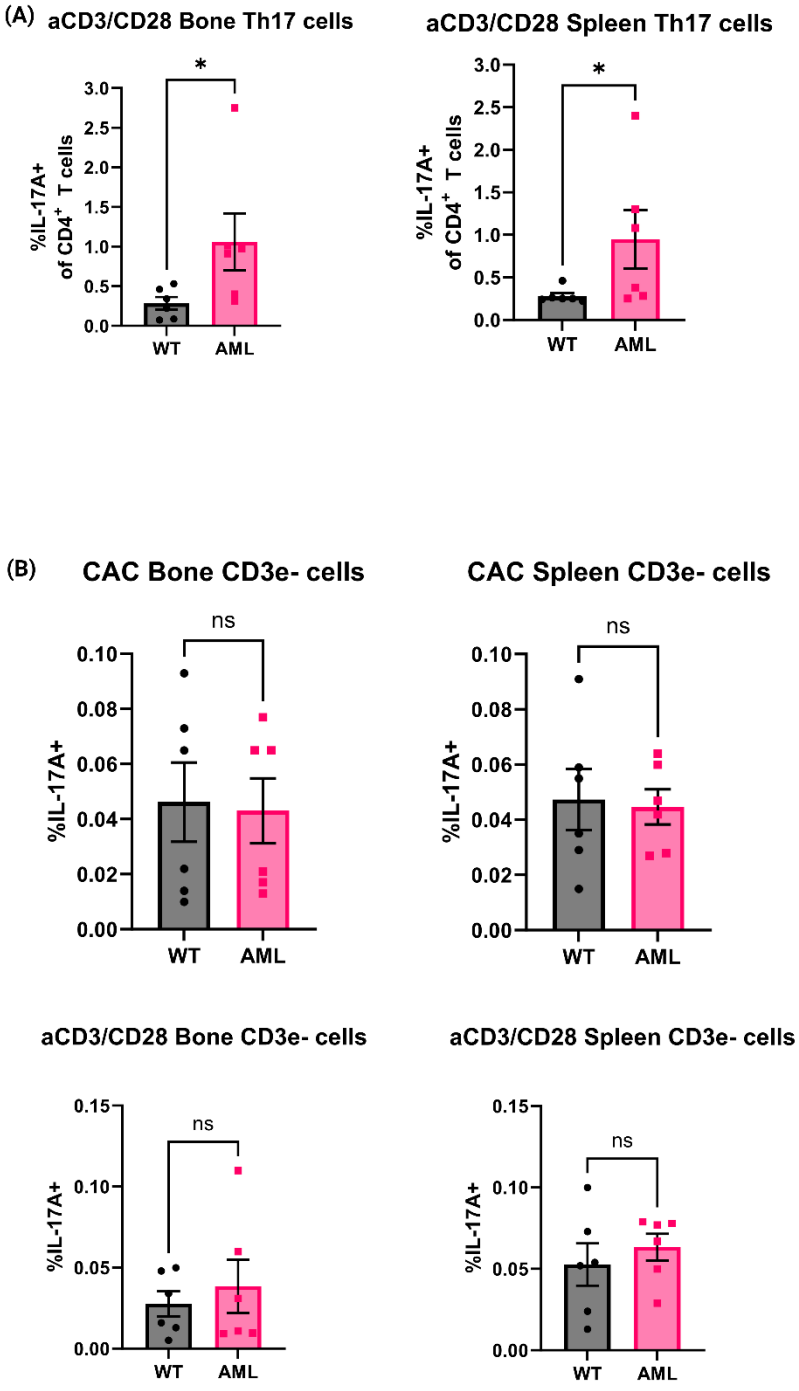


Figure 2.16 IL-17A cytokine detection after ex vivo stimulation

Figure 2.16 IL-17A cytokine detection after ex vivo stimulation. A. Summary bar charts of IL-17A+ CD4+ T cells from the bone marrow or spleens of WT (n=6) and AML (n=6) mice after stimulation with anti-CD3ε anti-CD28 in vitro for six hours. B. Summary bar charts of IL-17A+ events in the CD3ε- compartment of the bone marrow or spleen of WT (n=6) and AML (n=6) mice after stimulation with Cell Activation Cocktail or anti-CD3ε anti-CD28 in vitro for six hours.

2.5 AML mice support expansion of OT-II cells *in vivo* and AML DCs promote Th17 skewing *in vitro*

Given that we found the increase in T-bet- cDC2s and Th17 cells in AML mice, there was reason to investigate whether the CD4+ T cell phenotype was the result of interactions with DCs. We performed adoptive cell transfer (ACT) using naïve T Cell Receptor (TCR) transgenic CD4+ OT-II cells that are specific to OVA peptides [118, 134, 135], as a mechanistic tool to study antigen specific activation of CD4+ T cells. Age-matched healthy WT or AML mice received equal numbers of naïve OT-II cells and one day following received an equal dose of 200 μg whole-OVA protein. Spleens were harvested for flow cytometric analysis 10 days after OVA injection (Figure 2.17A). Transferred cells were identified using congenic markers CD45.1 and CD45.2 (Figure 2.17B). When probed for transcription factors FOXP3 and RORγt we did not see expression of either transcription factor. We did observe significantly more CD44+ OT-II cells and with increased frequency in AML host mice at Day 11 suggesting that AML are more supportive of CD4+ T cell activation and retention after transfer, suggesting DC-induced priming of naïve OT-II cells (Figure 2.17C). To confirm our findings we also analyzed a cohort of mice that were injected with irrelevant antigen whole-BSA protein after adoptive transfer, where we do not observe an activation phenotype (Figure 2.18). Since we did not see evidence of specific Th skewing *in vivo* we next tested an *in vitro* approach where magnetically isolated splenic cDCs and naïve OT-II T cells were co-cultured with OVA_{323} peptide (Figure 2.17D). After five days of culture we analyzed secretion of IL-17A by flow cytometry (Figure 2.17E). In the groups that had the addition of the OT-II dominant antigen $OVA_{323,}$ we saw an increased trend of IL-17A secretion in

the AML cDC co-cultures (Figure 2.17F). Taken together our data suggests that AML mice have a DC phenotype that supports CD4+ T cell retention and polarization of naïve CD4+ T cells into a Th17 phenotype. In summary, we find that in the context of our AML mouse model, DCs inherit the FLT3-ITD mutation and DC homeostasis is disrupted. We also report that CD4+ T cells are skewed towards Treg and Th17 phenotypes *in vivo* and adoptively transferred OT-II cells are retained in higher numbers in AML recipients. *In vitro* co-cultures of OT-II cells and DCs resulted in more IL-17A secretion in the AML samples. All of these data together suggest that the increased DC phenotype observed also directly impacts the CD4+ T cell compartment, which results in a microenvironment that is tumor supportive.

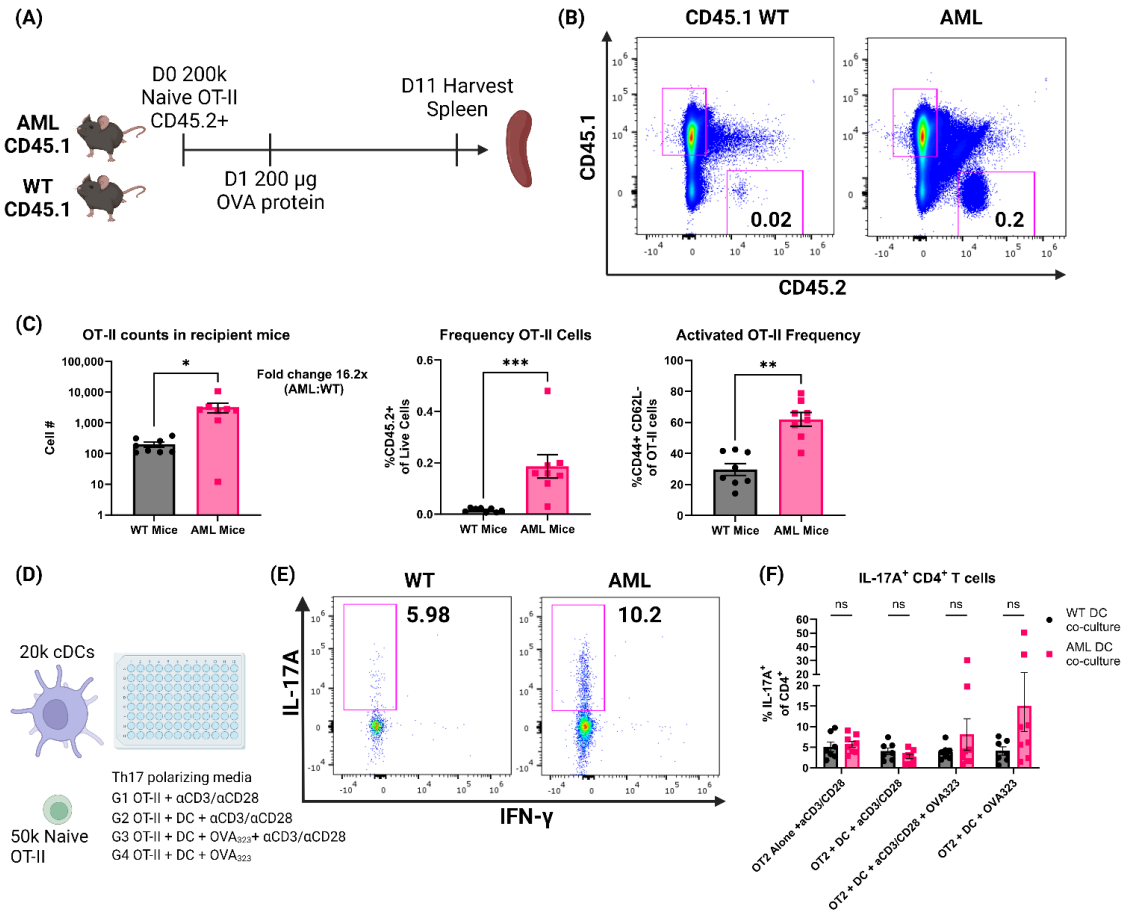


Figure 2.17 AML mice retain more OT-II cells and AML DCs promote IL-17A production

Figure 2.17 AML mice retain more OT-II cells and AML DCs promote IL-17A production. A. Experimental outline of OT-II adoptive cell transfer. B. Representative dot plots of spleen resident CD45.2+ OT-II cells by flow cytometry. Frequency of live single cells noted in the plots. C. Summary bar charts of OT-II cell counts (left panel) and frequency of live single cells (right panel). Each symbol is an individual mouse and summary data collected from two experiments. AML (n=8) and WT (n=8). D. Experimental outline of in vitro co-cultures of OT-II cells and cDCs. E. Representative dot plots of IL-17A+ OT-II cells by flow cytometry. Frequency of CD4+ cells noted in the plots. F. Summary bar charts of IL-17A+ CD4+ T cells from (E). Each symbol is an individual sample and summary data collected from four experiments. AML (n=8) and WT (n=7).

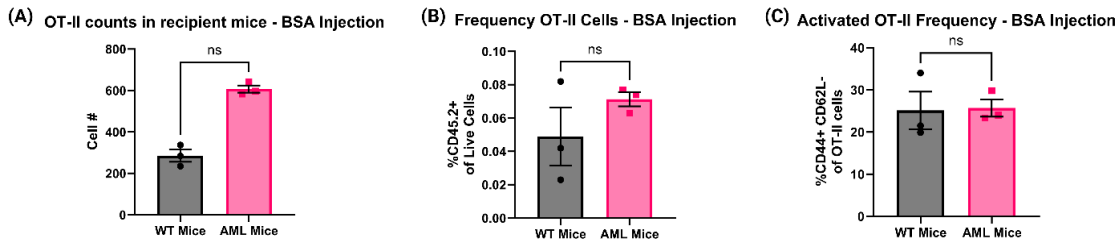


Figure 2.18 OT-II cells are antigen specific and do not respond to irrelevant BSA protein in vivo

Figure 2.18 OT-II cells are antigen specific and do not respond to irrelevant BSA protein in vivo. A. Summary bar chart of detectable CD45.2+ OT-II cells in the spleens of WT (n=3) and AML (n=3) mice. B. Summary bar chart frequency of CD45.2+ OT-II cells in the spleens of WT (n=3) and AML (n=3) mice. C. Summary bar chart frequency of CD44+, CD62L- OT-II cells in the spleens of WT (n=3) and AML (n=3) mice.

2.6 Discussion

FLT3-ITD is one of the most common mutations found in AML patients and research focus has been placed mainly on the AML blasts. DCs are the most responsive cell type to FLT3 signaling and they rely on this pathway for development [136, 137]. The connection between FLT3-ITD AML and DCs remains to be understood, given that AML is not considered to be a disease of DCs. We found that in bone marrow from FLT3-ITD+ AML patients, the frequency of cDCs was heterogeneous and patients could be stratified into high/medium/low frequencies. FLT3-ITD- bone marrow samples also had elevated levels of cDCs in the bone marrow compared to HD, but the highest observed frequencies were in the FLT3-ITD+ AML, suggesting that there are cDC precursors that are not tumor blasts that retain this mutation and expand. Our data shows that in AML the cDC1 and cDC2 subsets are disrupted and the frequency of XCR1/cDC1 double-negative cDCs was significantly higher in FLT3-ITD+ samples. This may suggest that patients with FLT3-ITD+ AML may retain more precursors than fully committed DC cells. Reports on bone marrow frequency of cDCs in the literature are sparse [138] but what we observe is consistent with the data reported, where a subset of AML patients have increased levels of cDCs in their bone marrow. However, that study did not have FLT3-ITD mutation status for patients. The majority of AML-DC reports focus on measuring circulating pDCs and cDCs [113, 139, 140] support the idea that FLT3-ITD+ AML patients have increased DC frequency in the peripheral blood. Immunophenotyping more FLT3-ITD+ AML patients to understand the changes to DC subsets in the bone marrow and the peripheral lymphoid tissues can help predict DC-induced polarization of CD4+ T cells. This has important

implications for the efficacy of current and future immunotherapies where T cell modulation is the goal.

We found that our spontaneous AML mice had a significant DC phenotype. It has been reported previously in a non-AML context that the FLT3-ITD mutation led to cDC expansion systemically [115]. We see that in the context of AML the expansion of cDCs is even more profound and was a result of FLT3-ITD *in vivo*. This suggests that in our model, the FLT3-ITD mutation drives a proportion of DC-precursors into mature DCs rather than malignant cells. The molecular understanding of DCs has increased significantly due to advances in sequencing technologies [31, 141, 142]. We used the models from Brown et. al. to inform our scRNA-seq analysis [124]. To the best of our knowledge, our report is the first to provide scRNA-seq data on DCs in the context of a model of FLT3-ITD+ AML. In combination with our ADT data, we can confidently identify *bona fide* DCs in the spleen. This has allowed us to more accurately describe changes to the DC population without contamination from other myeloid subsets and tumor blasts that have a shared lineage and could confound our DC findings. Our AML mice had more T-bet$^-$ cDC2s, a population of cells that have been shown to promote polarization of naïve CD4+ T cells into Th17 phenotypes [124]. This finding was unexpected to us because reports suggest that strong FLT3 signaling *in vivo* drives all DC subsets to expand [22, 115, 143]. This T-bet$^-$ cDC2 cell type is novel and will be the subject of future studies to understand how it is functionally different from other DCs in AML. Our data is concordant with what was reported by Brown et. al., which could have clinical implications for AML patients. The role of Th17 cells in cancer is controversial but in AML they have been found to be deleterious for patient survival [130, 144-146]. Our mouse model of AML

recapitulates findings from humans where the inflammatory cytokine milieu is permissive to Th17 polarization [147, 148].

We recognize that the DC phenotype we describe in our human samples is different from the mouse DC phenotype. This was not unexpected as AML is a very genetically complicated cancer [87, 149]. Our mouse model has a select number of mutations (FLT3-ITD, TET2 KO, and TP53 KO) that leads to AML-like disease but is a significantly smaller list of mutations compared to human AML patients [87]. It is likely that the increased complexity of mutations in the AML patient samples is impacting DC subsets in a way that we cannot replicate in the genetically engineered mouse model (GEMM) that we use. What may address this discrepancy is the use of ATACseq and scRNA-seq on both mouse and human AML DCs to compare their chromatin accessibility and transcriptomes. It will be important to compare the tissues of the AML microenvironment (blood, bone marrow, and spleen) between species to interpret future findings. Such data would shed light on what differences and parallels exists between species regarding the active sites of transcription and gene expression as a result of inherited leukemia mutations and therefore enabling future mechanistic studies.

It is important in our view to consider the contribution of DCs on the T cell compartment as DCs are continuously supplied to the periphery and difficult to ablate compared to lymphocytes. In summary, we have shown that in some subsets of FLT3-ITD+ AML patients the frequency of bone marrow DCs is increased. Using a mouse model of AML we characterized this increase in DC abundance as a result of FLT3-ITD and that they are skewed towards a T-bet$^-$ cDC2 phenotype. These cells are adept at polarizing naïve CD4+ T cells into the Th17 subset. This work provides novel scRNA-seq data on DCs and

further supports rationale for the need to improve the characterization and function of non-tumor myeloid cells and their impact on anti-tumor responses. This is particularly important with the growing interest in developing immunotherapies for AML [126, 127, 150, 151].

Chapter III: Summary and Future Directions

3.1 Summarizing data from the FLT3-ITD project

Based on my findings**, I propose that FLT3-ITD+ cDCs in AML polarize naïve CD4+ T cells into Th17 by the secretion of IL-27, TGF-β and IL-10, thereby contributing to the immune suppressive microenvironment of AML** (Figure 3.1). Until now there has been little to no published findings on the role of DCs in AML. Here we demonstrate with human and mouse data in the context of AML, DC populations are disrupted and this can have impacts on the CD4+ T cell compartment. First, we showed in AML patient bone marrow samples the phenotype for cDCs was heterogeneous and altered in FLT3-ITD+ AML. The cDC phenotype in FLT3-ITD+ AML lacked expression of the canonical subtype proteins XCR1 and CD1c. When I probed these Double-negative DC cells for the surface antigens CD33, CD34, and CD38 for stemness, I observed an overall positive expression of CD38 and mixed positive expression of CD33 and CD34 (Figure 3.2). Furthermore, I inspected the Double-negative DC cells for expression of IL-3 Receptor (IL-3R a.k.a CD123) which is expressed at high levels on AML blasts [152] but do not see a positive signal for this surface marker, suggesting that our phenotyping data is not being swayed by contaminating AML tumor blasts (Figure 2.1). Moreover, I compared the expression of CD11c and HLA-DR on AML blast cells and did not see a consistent result across patient samples (Figure 2.2). We are also publishing the details on the OHSU Clinical Flow Core panel that was used for typing the patients that are in our manuscript, adding to the analysis from our work. Due to the complex combination of mutations and the effect on hematopoiesis, it is not surprising that from primary AML

patient bone marrow samples the characterization of specialized immune cells such as DCs will have some hurdles to overcome. Despite the caveats to our flow cytometry data, there is evidence from the literature to support our argument that DCs are not tumor blasts in AML, and that true AML is determined by expression of of CD33+/CD38-/CD123+ immature stem cells detected in the blood and bone marrow [152-154]. This dogma is subject to change as assays and methodologies improve over time.

The experiments performed using the GEMM to study the biology of FLT3-ITD+ AML have shown a novel phenotype that has not been previously described in the literature of AML. The recently appreciated discovery that DCs can express T-bet and the expression of this transcription factor modulates their function is in the early stages of understanding [124]. The data from my manuscript concurs with the data published by Brown et. al., suggesting that T-bet expression in DCs controls an expression network that promotes Th17 polarization of naïve CD4+ T cells. It remains consistent that FLT3 signaling is a key factor for DC hematopoiesis and the inclusion of FLT3-ITD mutation in DCs drives expansion much like what is seen by administration of exogenous FLT3L [15, 16, 22, 115, 143]. There is a simultaneous expansion of both the tumor cells (CD11b+ myeloid cells) and DCs in FLT3-ITD+ GEMM, suggesting that leukemia mutations can exist in DCs but they do not become the tumor, based on flow cytometry data. The dramatic expansion of DCs in the GEMM result in skewing the CD4+ T cells into Th17 and iTreg phenotypes, suggesting a role in the immunosuppressive environment seen in AML. The use of CITE-seq allowed us to identify at the transcriptional level what was observed at the protein level by flow cytometry, for both DCs and T cells respectively. The generation of a robust transcriptional dataset for an AML GEMM is novel and will hopefully serve the

community to drive new areas of research. There are several ways to further investigate the findings from my research which will be outlined below.

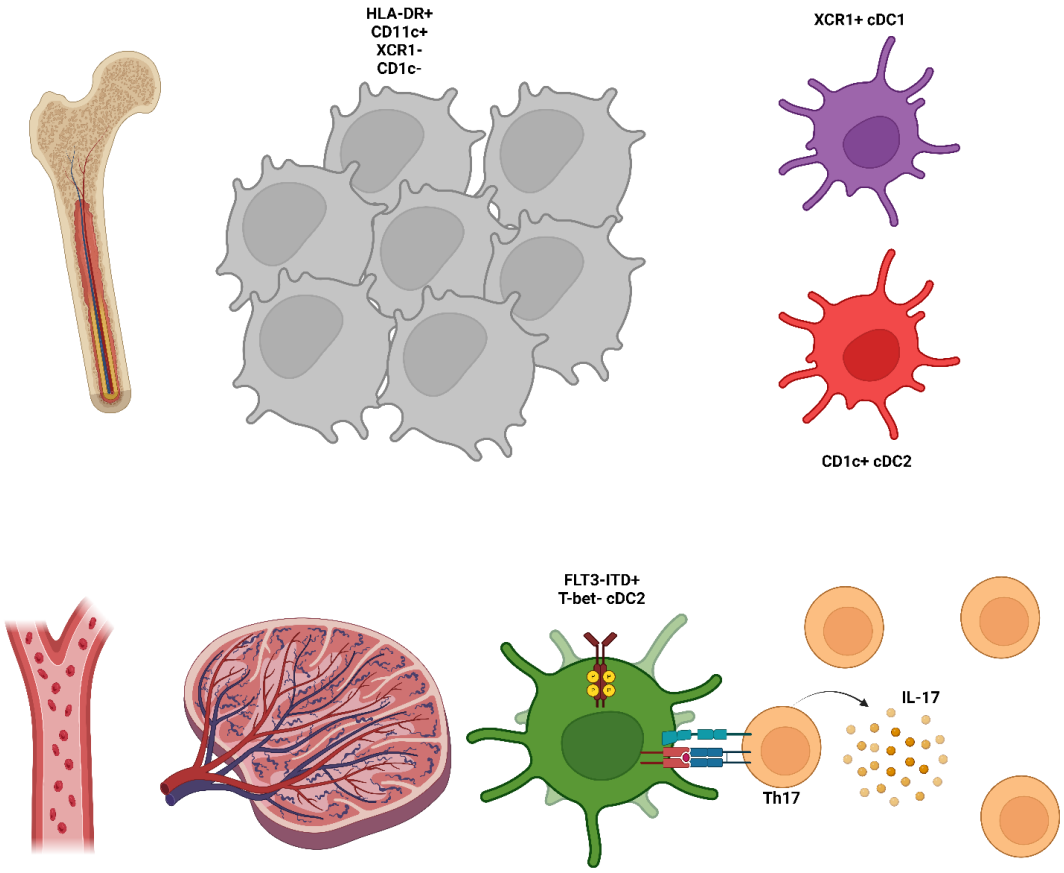


Figure 3.1 Model of FLT3-ITD+ AML DCs and Th17 phenotype

Figure 3.1 Model of FLT3-ITD+ AML DCs and Th17 phenotype. Cartoon schematic of FLT3-ITD+ AML where patients exhibit a phenotype of disrupted DC development that results in a reduction of committed cDC cells and an expansion of uncommitted DC-precursors (XCR1-, CD1c-, HLA-DR+, CD11c+) in the bone marrow. In the blood and lymphoid tissues T-bet- cDC2s are enriched in FLT3-ITD+ AML and polarize naïve $CD4^+$ T cells into the Th17 subset.

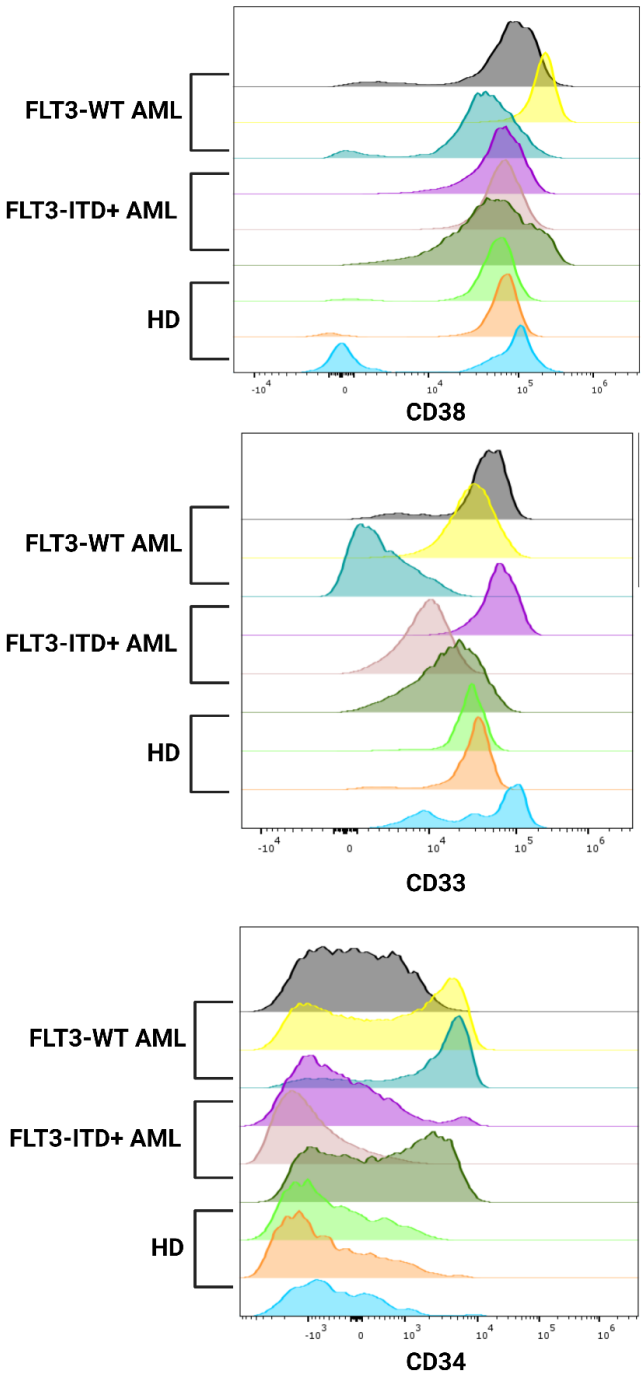


Figure 3.2 Human AML cDC expression of hematopoietic markers

Figure 3.2 Human AML cDC expression of hematopoietic markers. Histograms of representative human bone marrow samples showing cDC expression of CD38, CD33, and CD34 surface proteins associated with hematopoiesis. N=3 for each group.

3.2 Future experiments investigating human FLT3-ITD+ DCs

Future opportunities to delve deeper into this project I propose are to expand the characterization of the patient bone marrow samples to also include intracellular staining for transcription factors and other cytoplasmic proteins. It has been shown previously in 2009 that T-bet overexpressing human DCs can promote Th1 skewing of naïve CD4+ T cells *in vitro*, suggesting a functional role of T-bet in DCs [155]. Recent work does not show a difference in Th1 skewing capability but rather a difference in Th17 skewing based on T-bet expression in DCs [124]. Directly probing for T-bet and TGFβ would be helpful for identifying cDC2 subsets if present in the bone marrow of AML patients. Matched AML patient PBMCs would also be informative for identifying cDCs if they are present in the blood of FLT3-ITD+ patients. This may require a very large cohort of samples (100+) to thoroughly investigate the phenotype of their DCs, which could take years to accomplish given the incidence rate of FLT3-ITD being ~30%. If possible, analysis of splenic tissue and peripheral blood from AML patients would be allow us to further compare our data from the GEMM, where we were able to characterize bone marrow, blood, and spleen tissues. Published findings in the literature characterizing DCs in AML patients is generally confounded by the shared lineage problem but modern multi-parameter flow cytometry technologies are tackling this problem [113, 138-140]. In our hands we can generate a panel of 15+ colors for flow cytometry and the promise of spectral-based cytometers such as the CyTEK Aurora conceivably can be 30 or even 40 colors. Alternatively, the use of mass-cytometry is attractive as well, where a 50 marker panel is routinely used to characterize immune cells. The biggest downsides to this technology are price and availability of antigens to conjugate to metals. Regardless of approach, I argue that the panels of increased

complexity will be critically important moving forward to address the problem of *bone fide* DCs from AML patients.

The power of sequencing technologies have revolutionized traditional immunology and cancer immunology. I argue that leukemias in particular will benefit from these technologies to allow researchers to interrogate the complex microenvironment(s) that this disease imparts on patients. AML is a disease defined by a tumor where immature myeloid precursor cells that cause disease, which has to be carefully detected and separated from downstream differentiated myeloid cells. The BEAT AML study [84, 87] has paved the way for studies that leverage single-cell RNA sequencing (sc-RNAseq) on white blood cells and in leukemia [114, 156, 157]. The BEAT AML study used Bulk-RNA sequencing analysis of their cohorts, which reduced our ability to test specific hypotheses regarding DCs and the impact of FLT3-ITD mutation in patient samples. Despite that limitation we were given DC-like scores from that team and it does provide the basis to propose further studies using scRNA-seq where we can identify patient samples that have expanded DCs and compare them transcriptionally to FLT3-WT AML and HDs. The advantages here is that we will have the power to computationally identify *bone fide* DCs from other myeloid cells and from AML tumor blasts, with literature references to support identification of immune cells with this technology [156, 157]. The literature resource we used to inform our computational approach to identifying DCs used healthy human spleen tissue and melanoma patient FFPE tissues, where the identification of DCs is clearer and more defined in the tumor microenvironment [124]. Furthermore, the treatment status of AML patients is important to consider as well when moving forward with experiments since the inclusion of chemotherapeutics can affect the interpretation of the data. I think the more

holistic approach where you would include *de novo* and treatment relapsed patient samples for testing hypotheses would be best because patients who "fail" at the clinic can teach us why the drug does not work for all and how can we improve engagement with the immune system for those that are resistant to current biochemical therapies.

I would approach this scRNA-seq experiment in two ways: unbiased and DC-enriched. The first approach is the unbiased analysis of AML patients with confirmed FLT3-WT and FLT3-ITD mutation status and HD controls. I would analyze matched bone marrow and PBMCs with spleen tissue or lymph nodes if possible. With these samples available I would process them for CITE-seq analysis so I can capture the proteins in addition to transcripts. I would use the same computational approach used with my manuscript (*e.g.* 10X Chromium, Seurat 4.0, etc.) and identify changes to the DC-compartment. The advantage of this first approach is that with matched tissue samples across patients we can find similarities and differences in the distribution of DC and DC subsets between the primary sites of disease (bone marrow and blood) and lymph node/spleen (sites typically not evaluated for diagnosis). I would test several questions using this dataset: 1) Do we see an increased abundance of T-bet- cDC2s in FLT3-ITD+ AML compared to HD and FLT3-WT AML? 2) Is there an increased signature of actively dividing Mitotic-DCs (DCs that are actively engaged in cell division) that is dependent on FLT3 mutation status? 3) Are there increased signatures for DC:T cell activation in the lymph node/spleen compared to blood/bone marrow (*e.g.* CD40, MHC-II, Co-Stim, etc.)? 4) Does FLT3 mutation status correlate with Th17 phenotypes? I would expect for Question 1, the abundance of T-bet- cDC2s in FLT3-ITD+ AML would be higher in lymphoid tissues compared to HD based on our findings from the GEMM data. In the bone

marrow and blood it is expected to follow suit with my flow cytometry data, where in FLT3-ITD+ patients their cDCs were significantly ablated in favor of double-negative XCR1-CD1c- cells. For Question 2, I expect the results to be more dependent on HD versus AML being the strongest difference, while the differences between FLT3-WT AML and FLT3-ITD+ AML may be less clear given the complex mutation status of patients. Question 3 I would expect to see a similar phenotype that was reported in the GEMM, where the increased abundance of DCs in FLT3-ITD+ patients in the peripheral lymphoid tissues will also be of a T-bet- cDC2 dominant phenotype. Quantification of cell-types for Question 4 will quickly answer this question. The cytokine data for IL-17A in both FLT3-ITD+ patients and mice were increased over healthy controls, therefore I would expect the FLT3 mutation status and Th17 phenotype to be consistent and Th17 abundance will be increased in FLT3-ITD+ AML compared to HD and FLT3-WT AML. The IL-17A cytokine signature we found in our samples has been previously reported by another group, although the AML mutation status was not disclosed [130]. Their data suggests that in the AML environment, IL-17A cytokine promotes AML tumor growth through the activity of IL-17R on the tumor cells [130]. I would investigate this phenotype as well in future FLT3-ITD+ AML patient samples, where I can test cultures of patient AML blasts in the presence of IL-17A with and without IL-17R blocking antibody. This will answer whether or not IL-17A promotes tumor growth and survival. I would expect in the FLT3-ITD+ AML cultures the addition of IL-17A will promote tumor growth through IL-17R. It is important to also translate the *in vitro* co-culture experiments using DCs and naïve CD4+ T cells from human sources. If bone marrow isolation of DCs from FLT3-ITD+ and FLT3-WT AML does not supply enough cells for the assay, then sourcing DC from peripheral blood may

be a secondary source of cells. It has been shown in the literature that strategies to generate DCs from bone marrow precursors and peripheral blood precursors can generate sufficient numbers of cells to perform these experiments [158-160].

A caveat to this first approach to using single-cell sequencing of AML patient samples is that the abundance of AML tumor blasts can reduce the clarity of the data. From our hands-on experience of working with primary patient bone marrow aspirates is that they are comprised of primarily tumor cells. Therefore, the second approach I would propose is to enrich for DCs that was used for the GEMM experiment. The negative-selection kit from StemCell offers a good balance of improving DC-yields while retaining Lineage+ cell types that are critical to the computational analysis steps of these kinds of data. From this approach we improve our resolution of DC-specific questions and answers, but again we risk the loss of information regarding the T and B lymphocytes similarly to the GEMM data. Given unlimited resources and patient samples to choose from, I would proceed on both experimental designs so that I get the advantages of both without the worry of their caveats taking away from the data. These two datasets combined would be a significant immune atlas of AML that to my knowledge does not yet exist and would complement the existing BEAT AML database that is an excellent resource for drug discovery, predicting patient drug resistances, and tumor-intrinsic biology [84, 87]. From the transcriptional data that would result from these experiments I would complement them with DNA methylation sequencing experiments so that I could begin mapping epigenetic changes in FLT3-mutated patient samples as they often have co-mutations with TET2 and DNMT3A proteins resulting in dysregulated DNA methylation [98], to begin to understand the effect of AML mutations on DC regulatory networks and changes to promoter regions.

I would expect that in both FLT3-WT and FLT3-ITD+ AML samples compared to HD will have significantly altered epigenetics.

Finally, I would want to pursue research outside of single-cell technologies where I would also drive an effort to study the functional differences in AML-grown human DCs. To this end I would flow-sort DCs from AML patient samples and HD samples to use in classic *in vitro* assays to study their response to TLR stimulation, where it has been shown that TLR signaling activates DCs for T cell priming [58, 161]. It has been published previously that anti-AML T cell responses are driven by DCs, suggesting that DC dysregulation would result in poor T cell responses [162]. I would design the experiment to have unstimulated and stimulated groups split into cDC1, T-bet+ cDC2, and T-bet- cDC2 then group them with TLR agonists for TLR3 (dsRNA; Poly:IC), TLR4 (bacterial cell wall; LPS) TLR7 (ssRNA; R848), and TLR9 (CpG DNA; ODN2216). With these kinds of experiments I would characterize surface and intracellular proteins related to DC-maturation (*e.g.* CD80/86, MHC-II, CD40). Additionally I would measure cytokines secreted into the serum and identify canonical Th-skewing cytokines (*e.g.* IL-12, IL-4, TGFβ, IL-23, etc.). Based on the findings from our mouse data I would expect similar levels of maturation between HD and AML samples, but in terms of cytokines released I hypothesize a more Th17/iTreg polarizing phenotype in AML samples compared to HD. Using the results of these *in vitro* experiments then I would move on to DC-T cell co-culture experiments where I would study AML-DC impacts on T cell proliferation and polarization *in vitro*. Again, I would expect FLT3-ITD+ DCs to drive Th17/iTreg polarizations compared to HD and FLT3-WT AML.

3.3 Future experiments investigating GEMM AML DCs

Using a novel GEMM system of AML I was able to begin characterizing the molecular and phenotypic changes to cDCs *in vivo*. The data from these mouse experiments also informed my thinking on how to pursue future human experiments as well. Mouse models of AML are highly heterogeneous and primarily focus on studying drug resistance and tumor intrinsic biology [93] and only more recently has GEMM systems been used to characterize immune cells, in particular the CD8 T cell compartment [127]. This has left a space open to study DCs in AML GEMM systems. Therefore the field has primarily focused on two questions based on historical precedent: how can we treat the cancer with drugs and how can we enable anti-AML responses in T cells? To date the current standard of care remains chemotherapy and small molecule inhibitors to target the AML tumor blasts [84-87, 97, 106-109, 149, 163, 164]. There is growing rationale to explore immunotherapies in myeloid leukemia and immunological mechanisms of disease resistance to therapy [126, 127, 150, 151, 165-172]. In the research presented here, I was able to add to this discussion about immunity in AML through the lens of the DC. I was able to show in a spontaneous GEMM of AML that cDCs are significantly expanded both in frequency and number for both spleen and bone marrow tissue. This was dependent on FLT3-ITD presence, confirmed by the staining for phosphorylated FLT3 and comparison to FLT3-WT AML control littermates (TET2 KO, TP53 KO, LysM-Cre+) who had comparable frequency to WT healthy controls. FLT3-ITD+ AML mice also supported retention of adoptively transferred transgenic OT-II cells and the CD44 expression on the OT-II cells after 11 days was significantly higher compared to healthy WT hosts.

The use of DC-ablating technologies will be critical for testing the hypothesis given. There are multiple options of mouse models to deplete DCs *in vivo* [28, 29]. It would be useful to cross the AML GEMM used in our lab with the CD11c-DTR expressing mice [173], allowing us to observe any phenotype changes when all DC-subsets are depleted *in vivo*. Crossing AML mice to the more DC-specific Zbtb46-DTR system, we can limit the ablation effects to only the cDCs and not impacting pDCs and non-DC myeloid and lymphoid cells [26]. From either system I would evaluate AML tumor burden by measuring CD11b+ myeloid cells, stem cell populations in the bone marrow and spleen compartments, and CD4+ T cell phenotypes before and after depletion. If the DC phenotype we reported is supporting the tumor, then I would expect the CD11b+ tumor burden to be decreased after DC-ablation and stem cell populations to be reduced. Moreover, the Th17 and Treg phenotypes seen in AML mice would be reduced as well. If the DCs do not play a role proposed in the hypothesis, then I would not expect to see differences in CD11b+ cells, stem cells, and Th17/Treg phenotypes. In both DTR models, it was shown that priming of both CD4+ and CD8+ T cells is abrogated [26, 173], suggesting that DC-depletion in AML mice will result in less activation and retention of adoptively transferred cells.

We published robust scRNA-seq data on FLT3-ITD+ mice where we discovered a shift in DC-subsets towards a T-bet- cDC2 phenotype that had been shown to polarize naïve CD4+ T cells towards a Th17 phenotype [124]. Thus I characterized the CD4+ T cell compartment in our GEMM system where I found significant frequencies of both Treg and Th17 cells *in vivo*, which was supported by the elevation of inflammatory cytokines such as IL-1β and TNF-α but also IL-27 and IL-17A and IL-10, suggesting a DC:T cell axis in

these mice. These cytokines are also identified in our human data as well as in the literature [130, 140, 147, 148, 174]. We used *in vitro* co-culture experiments to test the hypothesis that AML DCs will polarize more naïve CD4+ T cells into Th17 phenotype compared to WT DC controls. We were not able to show a statistical significance with our set of data, however the trend showed that in AML DC co-cultures the polarization was stronger in AML-DC co-cultures compared to WT-DC co-cultures. Overall, our data supports the idea that FLT3-ITD can induce significant expansion of cDCs *in vivo* and in combination with TET2 and TP53 mutations there is a resulting skew towards T-bet- cDC2 phenotype that promotes increased frequency of Th17 cells.

The important findings from my research has revealed a previously unexplored impact of the AML associated mutation FLT3-ITD when expressed in DCs. The data from our GEMM suggest that DCs play a role in the immune response to AML by redirecting naïve CD4+ T cells towards Th17 phenotype. More functional experiments will be important to elucidate the individual contributions from cDC1s and cDC2s in our model. The relationship between DCs and T cells has long been studied, where increased cDC abundance will coincide with changes to the T cell compartment, particularly iTregs in naïve mice [115, 175]. In our model, there is evidence of DC induced expansion and retention of adoptively transferred OT-II cells, but unfortunately those cells did not exhibit detectable expression of FOXP3 or RORγt transcription factors. Nonetheless, the adoptive transfer experiment and the co-culture experiments both point in the direction that these AML-DCs also exhibit functional differences in the presence of cognate antigen; in the case of whole OVA protein in the adoptive transfer context or with peptide when using OVA_{323} *in vitro*. I argue that these data provide the rationale to further test the antigen

processing and presentation abilities of the cDC1 and cDC2 subsets to naïve OT-II cells. I would propose experiments to isolate cDC1 and cDC2 subsets from FLT3-ITD and FLT3-WT AML mice and WT healthy controls via FACS, then co-culture them with naïve OT-II cells under Th1, Th2, iTreg, and Th17 polarizing conditions. This would help identify the individual contributions to the *in vivo* phenotype I describe in our manuscript. I would expect that in FLT3-ITD+ T-bet- cDC2 cultures the polarization of Th17 cells will be higher compared to FLT3-WT and WT T-bet- cDC2 controls. I hypothesize that FLT3-ITD+ cDC1s will polarize more iTregs when compared to FLT3-WT and WT cDC1 controls based on our *in vivo* data. Although it was not a focus of my research, I would like to examine the effects FLT3-ITD+ cDCs have on naïve OT-I cells in the same fashion as the OT-II cells, as it has been published that CD8+ T cells that are activated by DCs are important for anti-tumor immunity [176-183]. The adoptive transfer experiment showed that the transferred OT-II cells had more CD44+ compared to WT hosts and I hypothesize that adoptively transferred naïve OT-I cells will also have CD44+ cells in FLT3-ITD+ hosts compared to WT hosts. The inclusion of additional markers of effector phenotype such as KLRG1 and CD127 are important to identify effector CD8+ T cells [184].

In addition to the co-culture experiments to test the influence of cDCs on naïve T cells, I would perform DC maturation/activation experiments *in vitro* using FACS to isolate cDC subsets using TLR stimulation in the same manner as I propose for the human sourced DCs. These experiments would allow me to characterize canonical surface and intracellular proteins of maturation/activation (*e.g.* CD40, MHC-II, Co-Stim, NF-κB, IRF3, AP-1). I hypothesize that in FLT3-ITD+ cDCs they will have a more tolerizing phenotype compared to FLT3-WT and WT cDCs. Moreover, I hypothesize tolerizing cytokine concentrations

will be increased as well (*e.g.* TGF-β, IL-10). These experiments will also help determine which cDC from our GEMM are the primary source of the increased IL-27 and TGF-β observed in the blood serum.

The role of IL-27 has become appreciated across a spectrum of contexts, from vaccine design to graft versus host disease to cancer [185-187] for eliciting effector T cell responses and speaks to the pleiotropic nature of IL-27 signaling in T cells. The literature for IL-27 and AML/leukemia is sparse but it was shown in four human AML cell lines that IL-27 can promote tumor proliferation *in vitro*, suggesting a controversial role of IL-27 in the context of AML [174], but in the context of chronic lymphocytic leukemia (CLL) IL-27 was shown to be associated with a protective phenotype that was CD8+ T cell dependent [188]. Therefore the activity of IL-27 must be inspected in a disease context manner. Based on our GEMM scRNA-seq data I hypothesize that the primary source of IL-27 *in vivo* would be the T-bet- cDC2s and this would be reflected by higher IL-27 cytokine from the *in vitro* culture experiments. This may explain the dual-expansion of iTregs and Th17 cells in our GEMM as it has been shown that IL-27 supports the function and presence of iTregs in the GVHD model [186].

One of the unexpected findings from our GEMM is the absence of pDCs in FLT3-ITD+ mice in our scRNA-seq dataset. There was not a significant difference in the frequency of pDCs in the bone marrow tissue, but there was an increase seen in the spleen *in vivo*, suggesting that in FLT3-ITD+ mice that peripheral tissue pDCs are favored over bone marrow tissue pDCs which could be related to cues from the stromal environment differences. The frequency of pDCs as a fraction of live cells is very low however, ~0.5%, so this may be due to the comparison of naïve healthy mice to inflamed AML mice or the

magnetic bead enrichment process I used to prepare the splenocytes for sequencing. This was unexpected as pDCs are reliant on FLT3 signaling much like cDC1s are [189]. Canonically the activity of pDCs is related to viral infections as they are important influencers of Type I interferons during acute infection and so it could be that the inflammatory cytokine milieu of AML may be repressing the expansion of pDCs in favor of cDC cell fate. Moreover, the ontogeny of pDCs are shared between myeloid and lymphoid precursors, adding a layer of complexity that would require careful experimental designs in the context of AML to tease apart [190]. Our scRNA-seq dataset can be used to look for differences in the transcripts for IRF8, E2-2, RUNX2, and SPI-B to provide evidence of cell-fate disruption. Based on the data I currently have, I hypothesize that IRF8 and E2-2 are downregulated in pDC-precursors in FLT3-ITD+ cells even though they have increased signaling through FLT3.

The novel scRNA-seq dataset that we have published in FLT3-ITD+ mice is the first of its kind for the field of AML. We were able to achieve robust profiling of splenic cells and in particular DCs. One of the unfortunate side-effects of the DC-enrichment that I used during sample preparation was the loss of T cells in both groups. We were able to reliably identify more prominent T cell populations such as naïve CD4+ T cells and effector CD4+ T cells for example, but critically we were unable to identify Th17 cells with the transcriptional data even though we could resolve Tregs. Despite the data for the T cells, the data for the DC compartment was clear. One of the quality control measures I wanted to include with our transcripts were the ADTs. Regardless of genotype, the correlation matrix for transcript and protein was a great addition to our analysis pipeline. Where possible I recommend the addition of ADTs to any RNA-seq experiment and can serve as

a resource for future DC-seq experiments for any researcher since we also have these data for WT naïve C57BL/6 mice. Similarly to the future plans I have for human AML DCs, I would also want to interrogate the changes in transcription factor activity in these FLT3-ITD+ mice and it would be informative to perform experiments to sequence the DNA methylation patterns DCs from FACS. Having a TET2 KO in the DCs from AML mice will certainly have an effect on their epigenomes compared to WT DCs and I hypothesize that FLT3-ITD will show differences compared to FLT3-WT samples as well. This will additionally provide insight into the gene regulation differences that are driving this expansion of T-bet- cDC2s and the reduction of pDCs observed *in vivo*.

3.4 Where the research fits in the leukemia field now and where it leads in the future

The largest impacts from my research of FLT3-ITD+ AML are: 1) the disruption of the DC-compartment that leads to the skewing of T-bet- cDC2 phenotype which in turn can 2) promote the polarization of naïve CD4+ T cells into Th17 phenotype and 3) a robust CITE-seq dataset of both healthy and FLT3-ITD+ AML DCs. These findings provide a rationale to further study the biology of DCs in leukemia. To the best of my knowledge, there are no reports of specifically characterizing cDCs in FLT3-ITD+ patient bone marrow samples and the reports available that specifically measure DCs in AML lacked more modern staining methodologies to identify cDCs or used GM-CSF+IL-4 *in vitro* culture methods which produces heterogeneous cell types [112, 191, 192]. Regardless, there is historical evidence for dysregulation of DC populations in AML patients, suggesting a role of DCs in this complex disease [139, 140]. Moreover, the heterogeneity of AML is likely the reason for why FLT3-ITD+ AML is not a disease of DCs, even though it is thought that FLT3 signaling expands DCs [15, 16, 22, 115, 136, 137, 192-197]. For the future of the field it will be important to have translational research scientists continue to collaborate with clinicians for access to primary AML patient tissues and continue to analyze immune cells, particularly DCs since their impact on T cell responses are important in solid tumors [158, 177-179, 182, 198-203]. There is also some clinical evidence in AML where DCs can provide therapeutic benefit [165]. The idea for DC-based therapies came from the observation that for some AML patients who undergo allogeneic HSCT they exhibit a so-called "Graft versus Leukemia" response driven by host immune cells [204, 205]. However this treatment can develop graft versus host disease (GVHD), where transplanted donor

cells attack host tissues. This reaction has killed many patients in clinical trials and requires immunosuppression drugs after HSCT, where some patients relapse after HSCT [206-208]. Thus clinicians and researchers opted to deliver patient derived DCs that have been modulated *ex vivo* where the natural priming abilities of DCs can induce anti-AML responses by CD8+ T cells. In clinical trials it was primarily the use of autologous use of extracted DCs or CD34+ cells that DCs are generated from *ex vivo* culture before adoptive transfer back into patients [165]. Multiple approaches have been tried on patients who are refractory to available treatments: AML-DCs are peptide pulsed with Wilms' tumor 1 (WT-1) peptides [209], cultured with irradiated tumor [210], autologous tumor/DC hybridization (electrically fusing cells together) [211, 212], and mRNA treatment of DCs [172] (Figure 3.3). This is still a highly controversial area of medicine and will require many years of development, but even in small case studies of one patient, these small successes will eventually fulfill the goal of the DC-field to utilize DCs in immunotherapy. In the future it may be possible where an AML patient can have their own purified DCs extracted, loaded with AML tumor antigens and activation stimuli, adoptively transferred back into them, and generate durable CD8+ T cell responses to control their disease. The potential upside to this cell-based therapy is that you avoid the problems associated with CAR T technology [213]. DCs do not form memory cells that persist for the life of the host as T cells do, where the lifespan of a DC is less than one week and therefore avoiding long-term activation pathology [4, 214].

- Improvements to DC-vaccines (DCs pulsed with tumor antigens, DCs cultured with irradiated tumor cells, etc.)

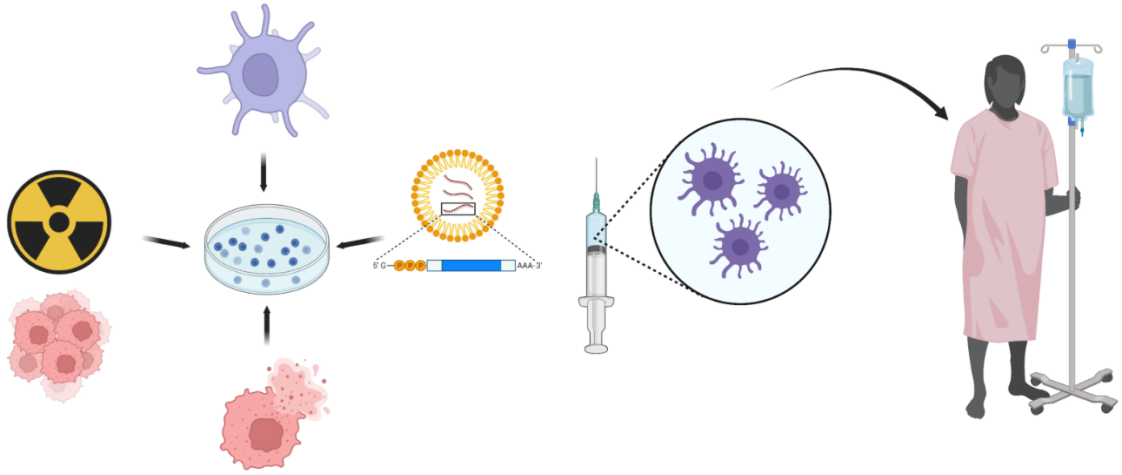


Figure 3.3 DC-vaccines for immunotherapy

Figure 3.3 DC-vaccines for immunotherapy. Cartoon schematic of methods to utilize DC-vaccines as a tool for immunotherapy.

The further characterization of DC-subtypes in AML patients will be important for the application of DC-ablation technologies. Currently the DC-field of immunology is undergoing a shift in our understanding of how complex DC-poiesis is (*e.g.* myeloid and lymphoid derived DCs) [190]. Molecular tools to ablate DCs in mice are still being developed and does not have the specificity that other lineages have (*i.e.* T cells) where off-target affects and incomplete depletion is still the case [29]. This can change dramatically in the next 10-20 years however, as the rise of CRISPR/Cas9 technology is providing a new molecular revolution to basic science [215] and in the clinic (Breyanzi from BMS) [216]. It is likely that in the context of FLT3-ITD+ AML, those patients have skewed cDCs such as T-bet- cDC2s that are counter-productive to producing Th1 and cytotoxic CD8+ T cells. Conceivably with improvements to DC-specific ablation a patient could receive a regimen that can temporarily reduce the frequency of "bad" T-bet- cDC2s, thus allowing expansion of anti-tumor Th1 cells or reduction in the frequency of Th17 subset. I would imagine something like an antibody drug conjugate (ADC) that can specifically engage the cDC of choice and then internalize it to induce cell death program of apoptosis via Caspase-3 and Bax (Figure 3.3). So far the role of Th17 cells in leukemia is a negative correlation, where increased IL-17A cytokine detected is associated with worse prognosis [130, 146, 147]. The role of IL-17 cytokines are still being studied as it has a complex role in solid tumors but is associated with pro-tumor properties such as myeloid cell recruitment and would healing programs [145, 217-219]. My data suggests that this axis is at play in AML, therefore it would be rationale to explore IL-17 depletion. IL-17 depletion has been successful in the treatment of autoimmune diseases such as psoriasis and rheumatoid arthritis [220]. This drug would be useful to test in our AML

GEMM and with human AML co-cultures *in vitro*. I would hypothesize that the Th17 phenotype in AML would be reduced after injection of secukinumab treatment and reduced myeloid inflammamatory profile (*e.g* IL-1α/β and TNF-α).

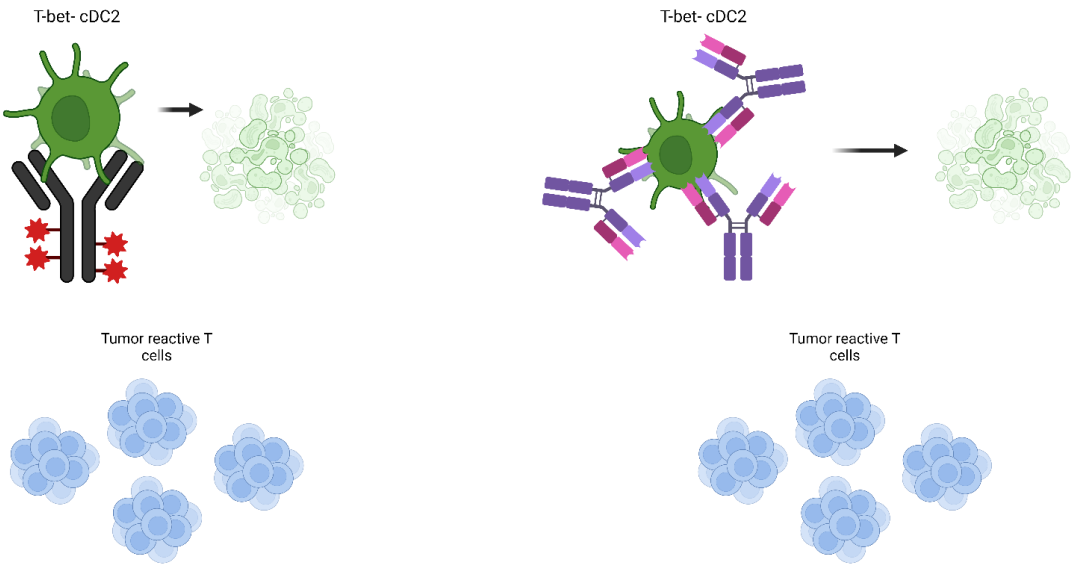


Figure 3.4 DC-targeting antibodies to expand tumor reactive T cells

Figure 3.4 DC-targeting antibodies to expand tumor reactive T cells. Cartoon schematic of methods to utilize DC-targeting antibodies inducing apoptosis to allow expansion of tumor reactive T cells *in vivo*.

Finally, I imagine a future where our CITE-seq data becomes the stepping stone to an AML Atlas of the immune system, where each type of DC is characterized at the protein, transcript, and epigenetic levels (Figure 3.5). There is a growing body of research that is characterizing DCs with these methods [124, 143, 221, 222], identifying transcriptional activity that was previously undetected. These types of analyses can be used to identify pathway modulation from drug screens [84, 87, 223]. The use of small molecule inhibitors to target mutated pathways in tumor cells have been used for about 20 years, with varying degrees of success [224]. For example, it has been shown that repurposing drugs that were designed to kill leukemias can also modulate immune function [126, 127]. It is therefore a rationale to perform these experiments on non-tumor DCs to identify pathways that can be modulated for a desired clinical benefit. By collaborating with expert chemists, these datasets can lead the way towards small molecule drugs that can possibly modulate DCs. It is possible that there are cDC unique-proteins or signaling cascades that if given the correct small molecule could enhance their survival so that the success rate of engagement with cognate T cells to provide the immunological benefit desired. The lessons learned from studying AML DCs and FLT3-ITD+ DCs may eventually lead to new tools that scientists can leverage not only for leukemia but other disease contexts as well.

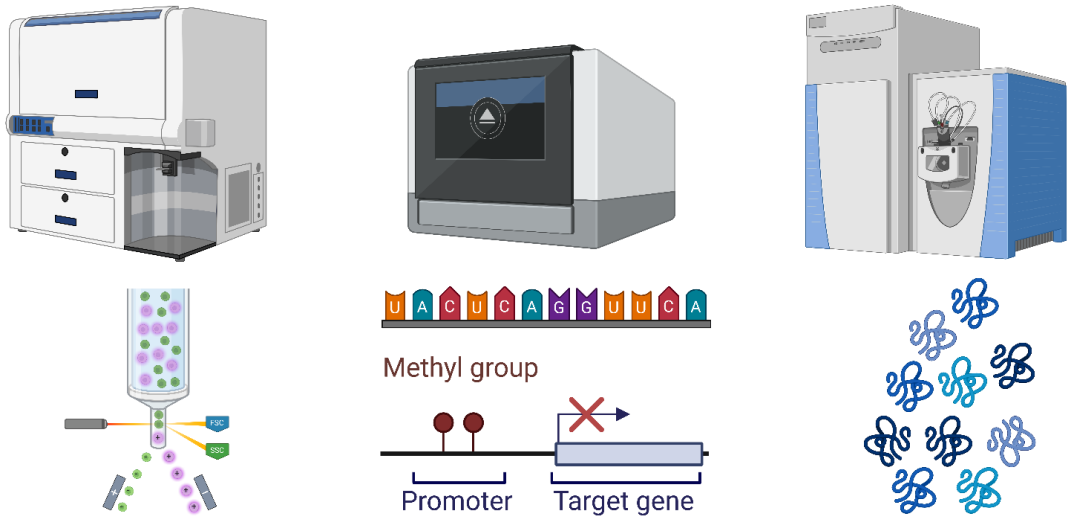


Figure 3.5 Technologies to develop an immune-atlas of AML

Figure 3.5 Technologies to develop an immune-atlas of AML. Cartoon schematic of technologies (flow cytometry, RNA/DNA sequencing, and proteomics) that can be used to build a thorough immune-atlas of AML.

Chapter IV: Loss of miR-146a expression in dendritic cells enhances responsiveness to TLR stimulation and T cell priming

4.1 microRNA biology and the canonical microRNA pathway

The first discovery of microRNA (miR) was in the model system *C. elegans* by two research groups in 1993 [225, 226]. It was suggested that translation of the mRNA *lin-14* is controlled by short anti-sense nucleotide sequences that are complementary to nucleotides in the 3' untranslated region (UTR) of *lin-14*, therefore causing an RNA-RNA complex that reduces translation [225, 226]. Studies in the model system *Drosophila* provided more mechanistic insight into miR suppression of mRNA translation that is mediated by interactions at the 3'-UTR of their targets [227, 228]. Soon after these works it was shown that miRs are conserved across species, indicating that expression of miRs is an important biological process for life [229]. Today, much of the pathway to generate functional miRs has been described (Figure 4.1) [230, 231]. This process is as follows: a primary miR (pri-miR) is transcribed in the nucleus by RNA polymerase II (Pol II) in a hairpin shape. The pri-miR is edited by two enzymes, Drosha and DGCR8, into a cropped precursor miR (pre-miR) as a hairpin shape. This pre-miR is transported from the nucleus to the cytoplasm by nuclear transport receptors (*e.g.* Exportin 5). The cytoplasmic pre-miR is recognized as a substrate for the enzyme Dicer to remove the hairpin terminal loop sequence and retaining the double-strand motif. The "diced" pre-miR duplex can now be a substrate for an Argonaute (Ago) protein for conversion to the functional single-strand miR sequence. The miR-Ago complex (RNA-induced silencing complex, a.k.a. RISC) can now repress target mRNA sequences that join the RISC. It has become appreciated that the

actions of miRs play a role in many aspects of cell biology, from development to host defense against infections [225, 226, 229, 231-235].

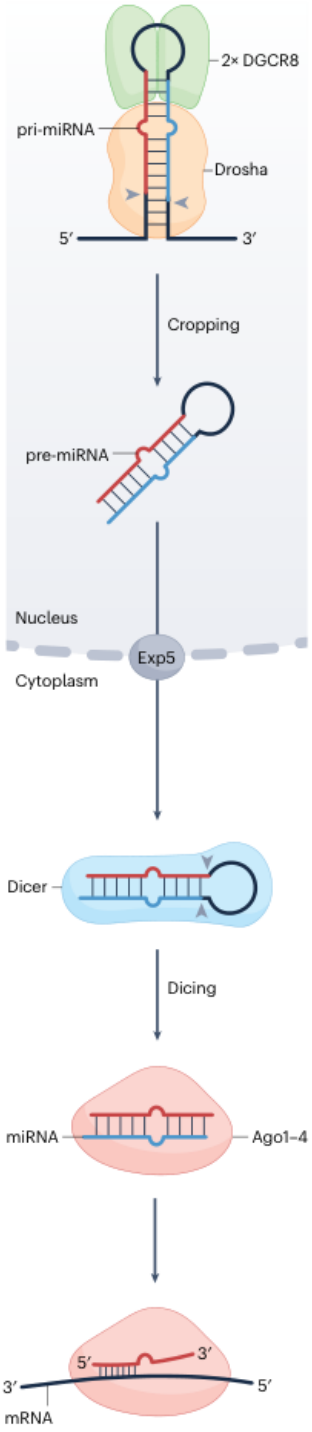


Figure 4.1 Canonical pathway of miRNA production

Figure 4.1 Canonical pathway of miRNA production. Cartoon schematic of miRNA biogenesis with permission from Shang *et. al.* [231].

4.2 miR-146a expression affects DC frequency and T cell activation

Full pathogen clearance often requires the response of activated adaptive immunity mediated by DCs [236-238]. Dendritic cells become activated and mature upon stimulation through their pattern recognition receptors (PRRs) by pathogen associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) [239, 240]. Pathogens have evolved many tools to escape destruction and manipulate host immune cells for their survival [241]. One of these mechanisms is the suppression of inflammatory signaling. This can be mediated by regulating protein activity or modulating RNA abundance in immune signaling pathways. A key component of cell-intrinsic regulation is the expression of microRNAs [232, 242], short non-coding RNAs of 19-25 nucleotide length that post-transcriptionally suppress mRNA translation [242]. Upon activation, DCs upregulate miRs that enhance or suppress their function [243-247]. One miR expressed at high levels in DCs upon activation is miR-146a. miR-146a is thought to function as a negative feedback mechanism important in preventing autoimmunity [248, 249]. This miR has been shown to target transcripts for two proteins involved in NFκB signaling, IRAK1 and TRAF6, that abrogate inflammatory responses [250-252]. Bone marrow derived DCs stimulated Toll-like receptor (TLR) ligands have increased miR-146a expression, suggesting that miR-146a may play an important role in DC responses to infection by both bacteria and viruses. (Fig. 4.2).

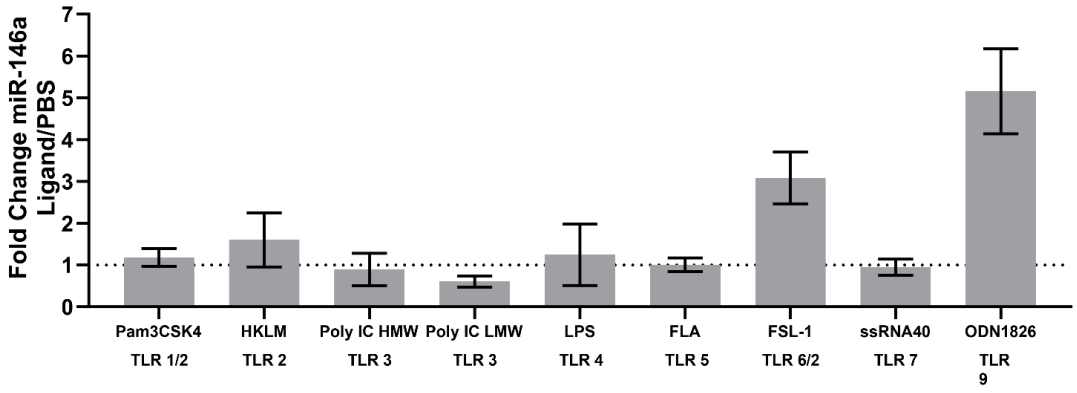


Figure 4.2 miR-146a expression in DCs after TLR stimulation

Figure 4.2 miR-146a expression in DCs after TLR stimulation. Bone marrow derived DCs were incubated with TLR ligands overnight. MicroRNA fold change normalized to the internal standard sno-202 RNA.

Multiple pathogens have been shown to manipulate host immune cells and microRNAs during infections for their survival benefit. Mycobacteria such as *M. bovis* can manipulate miR-146a levels in macrophages for improved survival by reducing iNOS production in macrophages [252]. Similar strategies are used also by gram-negative bacteria such as *H. pylori* and *S. enterica* [233, 234]. Subversion of the host miRs is also a tactic of many virus families [253-255]. Much of this work studying pathogen-microRNA relationships has only covered macrophages or non-immune cells. What remains to be studied in detail is how pathogens target miRs in DCs and if that benefits their survival. Determining the role of miR-146a in the context of pathogen infection of DCs may reveal new therapeutic targets that take advantage of immune responses. Previous work on mouse models to investigate the role of miR-146a relied on whole knockout (miR-146a WKO) mice, which can mask the specific contribution of dendritic cells in response to stimuli [249, 250]. Therefore, our lab sought to employ a mouse model with a DC-specific deletion of miR-146a. Utilizing the Cre/loxP system, we have generated a conditional DC-specific miR-146a KO (cKO) mouse model by expressing Cre under the endogenous CD11c promoter with loxP-sites flanking the miR-146a sequence.

This mouse has allowed us to evaluate endogenous changes in the DC and T cell populations in the spleens of naïve mice. We found that miR-146a cKO mice had increased CD8a+ DCs and CD103+CD8α+ DCs compared to WT (Figure 4.3). This cell population is critical in priming CD8+ T cells, which would then be able to attack pathogen infected cells [256-258]. Furthermore, miR-146a cKO mice have increased CD11b+ cDC2 cells compared to WT (Figure 4.4). These data suggest that miR-146a plays a role in DC growth and function.

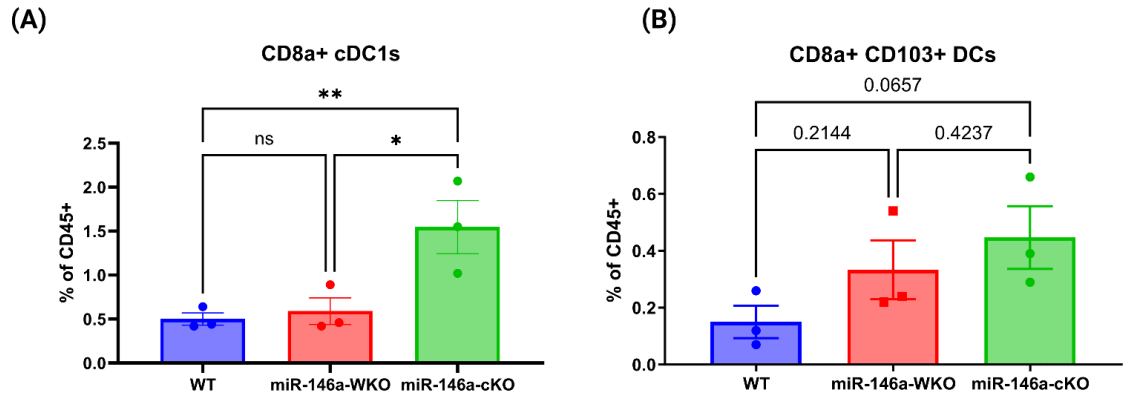


Figure 4.3 cDC1 frequency in miR-146a mice

Figure 4.3 cDC1 frequency in miR-146a conditionally deficient (CD11c-CRE) mice. A. Summary bar chart of CD8α+ cDC1 cells in the spleens of naïve mice. N=3 for all three genotypes. B. Summary bar chart of CD8α+CD103+ cDC1 cells in the spleens of naïve mice. N=3 for all three genotypes. Each dot represents an individual animal.

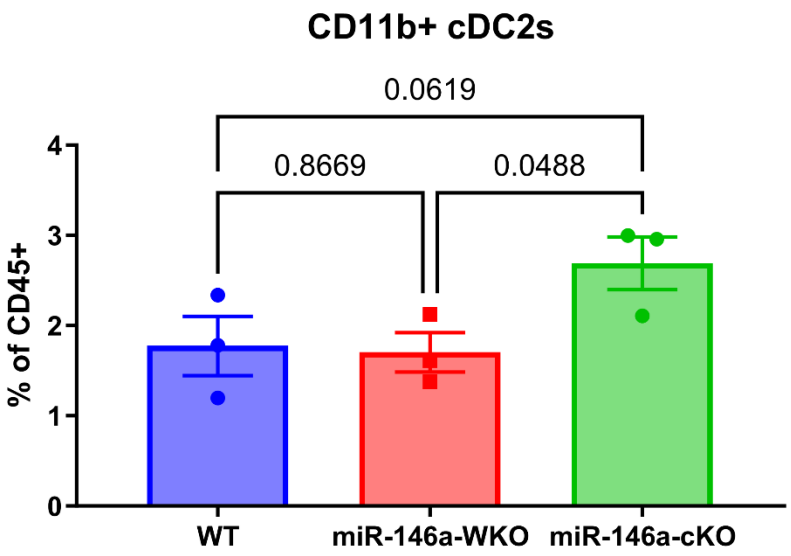


Figure 4.4 cDC2 frequency in miR-146a mice

Figure 4.4 cDC2 frequency in miR-146a conditionally deficient (CD11c-CRE) mice. A. Summary bar chart of CD11b+ cDC2 cells in the spleens of naïve mice. N=3 for all three genotypes. Each dot represents an individual animal.

Because the DC populations were changed in miR-146a cKO mice, we then hypothesized that T cell populations may also be altered in the spleen. We found that the miR-146a cKO mice had slightly increased proportion of CD3ε+ T cells compared to WT mice and miR-146a WKO mice (Figure 4.5A). Next we measured the frequencies of CD4+ T cells and CD8+ T cells, where in miR-146a cKO mice the CD4+ T cells were increased compared to WT and miR-146a WKO mice (Figure 4.5B). The CD8+ T cell frequency was not changed between genotypes (Figure 4.5C). We did not observe a change in CD4+ T cell or CD8+ T cells as a proportion of total CD3ε+ (data not shown). These data suggest that loss of miR-146a in DCs alters T cell homeostasis.

We reasoned that with both DC and T cell homeostasis altered, T cell phenotypes would also be affected in miR-146a cKO mice. We measured two markers of T cell activation, CD44 and CD69, to test whether miR-146a cKO mice have a stimulated signature compared to WT mice. CD8+ T cells showed increased CD44+ frequency in miR-146a mice compared to WT and miR-146a WKO mice Figure (4.6A). CD4+ T cells had increased CD44+ frequency in both miR-146a genotypes compared to WT (4.6B). When probed for CD69+ frequency, CD8+ T cells did not show a difference between genotypes (Figure 4.6C), however CD4+ T cells had increased CD69+ frequency in both miR-146a genotypes (Figure 4.6D). These data suggest that when miR-146a is deficient in DCs they are promiscuously activating T cells in naïve mice.

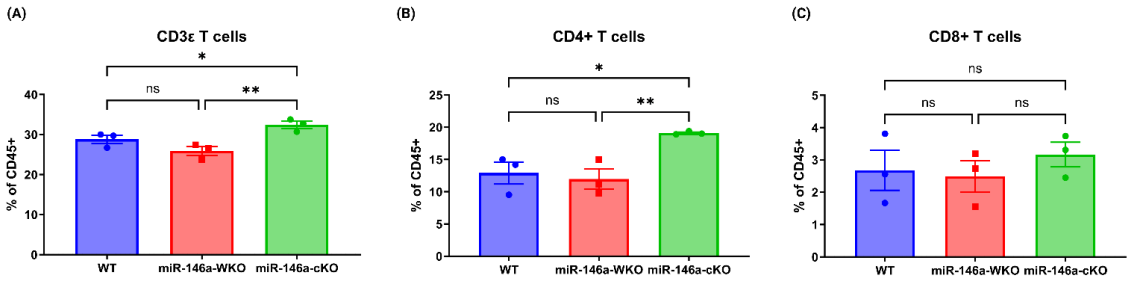


Figure 4.5 T cell frequency in miR-146a mice

Figure 4.5 T cell frequency in miR-146a conditionally deficient (CD11c-CRE) mice. A. Summary bar chart of CD3ε+ T cells in the spleens of naïve mice. N=3 for all three genotypes. B. Summary bar chart of CD4+ T cells as a proportion of CD45+ cells in the spleen. N=3 for all three genotypes. C. Summary bar chart of CD8+ T cells as a proportion of CD45+ cells in the spleen. N=3 for all three genotypes. Each dot represents an individual animal.

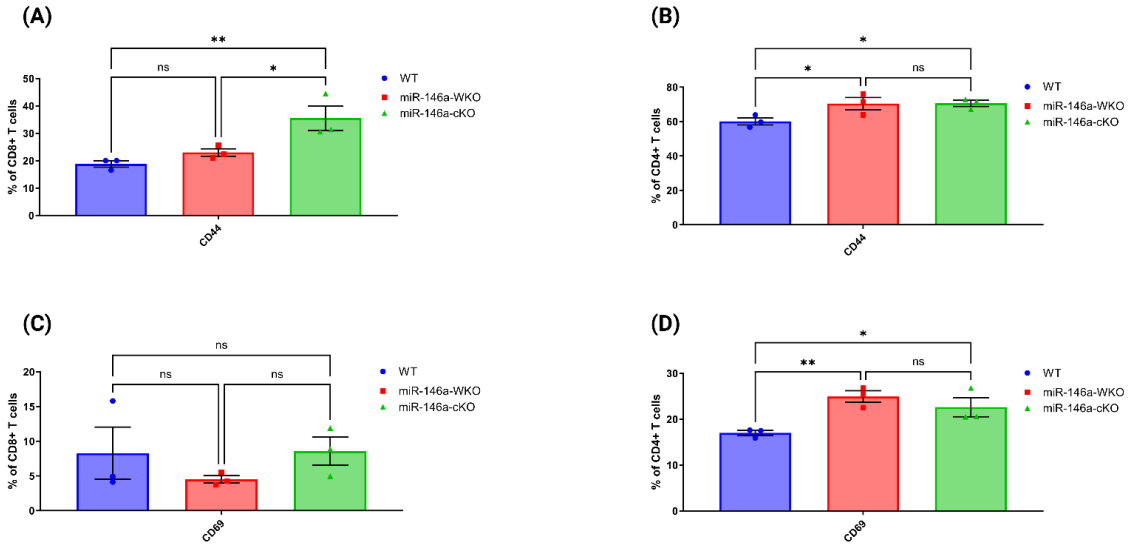


Figure 4.6 CD8+ and CD4+ T cells have increased activation markers in miR-146a deficient mice

Figure 4.6 CD8+ and CD4+ T cells have increased activation markers in miR-146a conditionally deficient (CD11c-CRE) mice. A. Summary bar chart of CD44 frequency in CD8+ T cells in the spleens of naïve mice. N=3 for all three genotypes. B. Summary bar chart of CD44 frequency in CD4+ T cells in the spleens of naïve mice. N=3 for all three genotypes. C. Summary bar chart of CD69 frequency in CD8+ T cells in the spleens of naïve mice. N=3 for all three genotypes. D. Summary bar chart of CD69 frequency in CD4+ T cells in the spleens of naïve mice. N=3 for all three genotypes. Each dot represents an individual animal.

4.3 *In vitro* analysis of miR-146a KO BMDC

The phenotyping data from the miR-146a WKO and miR-146a cKO mice suggested that loss of miR-146a causes DCs to increase in frequency and activate T cells *in vivo*. Therefore we sought to characterize DC responses to TLR stimulation *in vitro* to test their responsiveness to PRR engagement. Freshly isolated bone marrow cells from WT and miR-146a WKO mice were seeded into non-tissue culture treated petri dishes at 2e6 cells/dish in complete media (RPMI + 10%FCS + 1% Pen/Strep + 50 μM 2-ME) + 40 ng/mL GM-CSF on Day 0. On Day 3 fresh complete media + 40 ng/mL GM-CSF was added to each plate. On Days 6&8 half of the culture media + GM-CSF was removed and replaced with fresh complete media + 40 ng/mL GM-CSF. On Day 9 non-adherent cells were collected from the dishes and counted to 2e6 cells/mL concentration. Harvested cells were seeded into tissue culture treated 24-well plates at 2e6 cells/mL in complete media and treated with either LPS at 10 ng/mL or CpG (ODN1826) overnight. On Day 10 cultured cells were harvested and washed 3X before flow cytometry analysis.

When probed for the co-stimulatory molecules CD80 and CD86 on their surface unstimulated BMDCs from WT and miR-146a WKO mice did not show a difference in gMFI. However after TLR stimulation with both LPS (TLR4) and CpG (TLR9) miR-146a KO BMDCs had increased gMFI of CD80 and CD86 on their surface, suggesting increased sensitivity to TLR4 and TLR9 stimulation and ability to activate naïve T cells (Figure 4.7A,B). Downstream proteins IRAK1 and TRAF6 are reported targets of miR-146a [250, 255, 259], therefore IRAK1 and TRAF6 proteins may be increased in miR-146a KO BMDCs. We next wanted to test for any changes in cytokine secretion after TLR4 stimulation of BMDCs. After generating BMDCs as described above, WT and miR-146a

KO BMDCs were incubated overnight with 10 ng/mL LPS. The following day supernatants were collected and measured for myeloid inflammatory cytokines using the BioLegend LegendPlex assay.

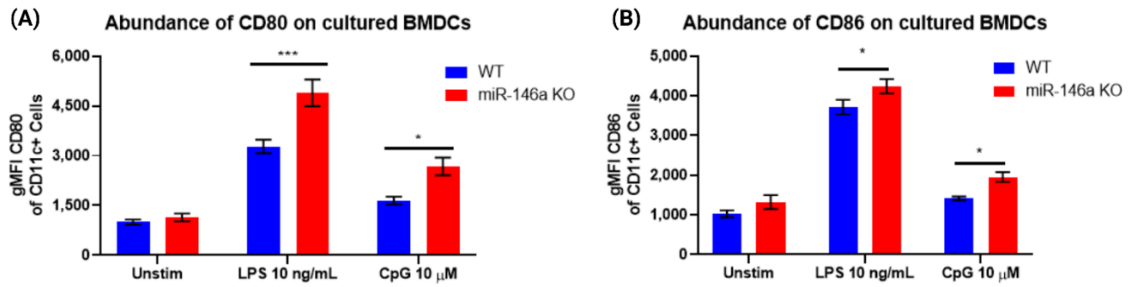


Figure 4.7 TLR stimulation of BMDCs increased CD80 and CD86 surface abundance

Figure 4.7 TLR stimulation of BMDCs increased CD80 and CD86 surface abundance. A. Summary bar chart of CD80 gMFI on BMDCs unstimulated or stimulated with LPS (10 ng/mL) or CpG (10 μM) overnight. N=3 for all three groups. B. Summary bar chart of CD86 gMFI on BMDCs unstimulated or stimulated with LPS (10 ng/mL) or CpG (10 μM) overnight. N=3 for all three groups.

At resting state BMDCs from WT and miR-146a samples produced low levels of IL-1α, IL-1β, and IL-12p40 (Figure 4.8A). After overnight stimulation with LPS the range of detectable cytokines from the supernatant increased to include IL-3, IFN-β, GM-CSF, and IL-27 (Figure 4.8B). The levels of IL-1α/β and IL-12p40 increased after TL4 stimulation, but there was no difference in concentrations of cytokines. Of the detectable cytokines, IL-27 had a trending decrease in concentration when comparing miR-146a KO to WT. These data suggest that loss of miR-146a in BMDCs does not affect their ability to secret cytokines after TLR4 stimulation with LPS.

To further evaluate differences in DC function, we opted to incubate immature and mature BMDCs with the self-quenching fluor DQ-OVA (ThermoFisher; Molecular Probes) [260] to test changes in antigen uptake and processing. BMDCs from WT, miR-146a WKO, and miR-146a cKO mice were incubated in the presence of DQ-OVA (1 mg/mL) for 10 minutes or one hour, where upon exposure to low pH, the DQ-OVA can fluoresce and the signal can be measured by flow cytometry (Figure 4.9A). After 10 minutes of incubation there was no difference in the percent positive BMDCs between WT and miR-146a WKO samples, but miR-146a cKO BMDCs had increased DQ-OVA signal (Figure 4.9A). After one hour of incubation WT and miR-146a WKO cells had no difference in frequency, but the percent of miR-146a cKO cells positive for DQ-OVA were lower than both groups (Figure 4.9A). These data suggest that loss of miR-146a under the control of the CD11c promoter increases antigen uptake at early exposure, but reduces long-term accumulation of antigen. We also evaluated BMDCs that were LPS stimulated overnight (16 hours) and measured percent of DQ-OVA positive BMDCs (Figure 4.9B). Again we find that miR-146a cKO cells have higher signal for DQ-OVA at 10 minutes of

incubation, but is the lowest for percent positive DQ-OVA at one hour with miR-146a WKO cells also showing reduced signal compared to WT (Figure 4.9B). These results suggest that after TLR4 stimulation loss of miR-146a increased early uptake of antigen but has the opposite effect for long exposure to antigen.

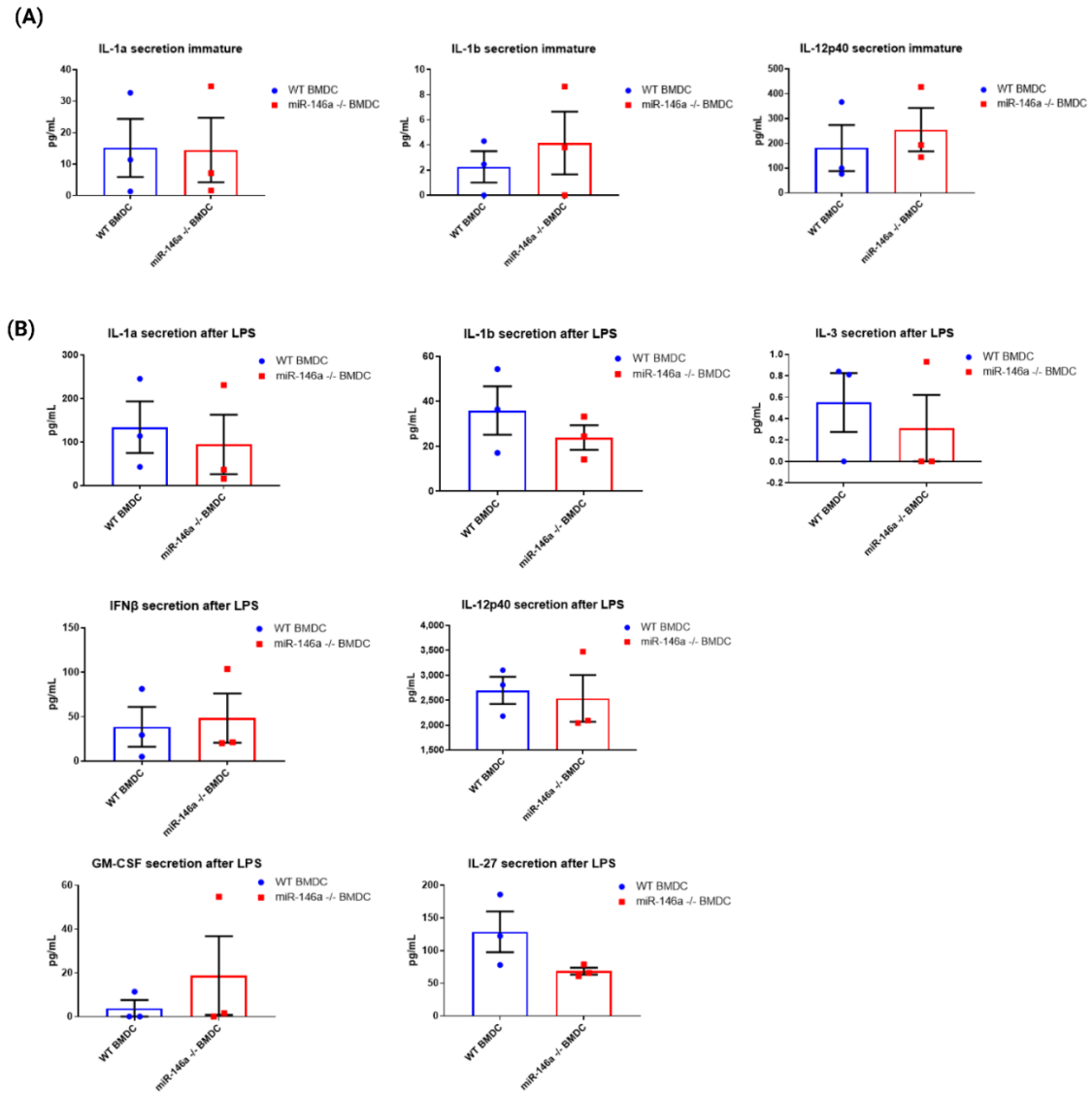


Figure 4.8 Cytokine secretion of WT and miR-146a BMDCs

Figure 4.8 Cytokine secretion of WT and miR-146a BMDCs. A. IL-1α, IL-1β, and IL-12p40 cytokines secreted by unstimulated WT and miR-146a KO BMDCs. N=3 for both genotypes. B. IL-1α, IL-1β, IL-3, IFN-β IL-12p40, GM-CSF, and IL-27 cytokines secreted by WT and miR-146a BMDCs after overnight stimulation with LPS. N=3 for both genotypes. Each dot represents an individual animal.

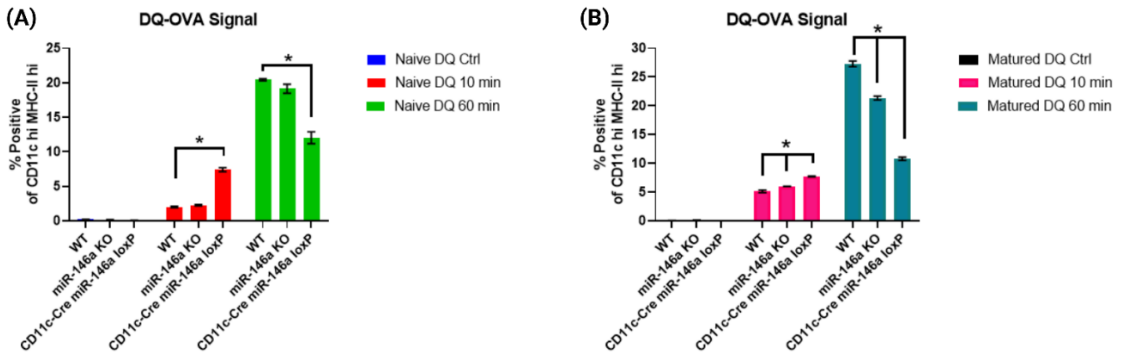


Figure 4.9 DQ-OVA uptake by WT and miR-146a deficient BMDCs

Figure 4.9 DQ-OVA uptake by WT and miR-146a deficient BMDCs. A. BMDCs from WT and miR-146a WKO and miR-146a cKO were incubated with DQ-OVA for 10 minutes or 60 minutes before measuring DQ-OVA signal by flow cytometry. B. LPS stimulated BMDCs from WT and miR-146a WKO and miR-146a cKO were incubated with DQ-OVA for 10 minutes or 60 minutes before measuring DQ-OVA signal by flow cytometry.

4.4 LCMV infection of miR-146a cKO mice

Co-stimulation and early antigen uptake is increased in miR-146a deficient BMDCs after TLR stimulation, suggesting improved ability to prime naïve T cells. Given the data that was observed from the *in vitro* studies, we opted to test antigen-specific T cell activation by DCs using a mouse model of infection using lymphocytic choriomeningitis virus (LCMV) in miR-146a cKO mice. LCMV infection is a model system that can be used to track antigen-specific T cells *in vivo* [261]. We infected 4 WT and 4 miR-146a cKO mice two days after collecting blood aliquots from all mice for a baseline (Figure 4.10A). Mice were injected with 2,000 PFUs of LCMV-Armstrong strain to produce an acute infection so we can measure the expansion and contraction of antigen-specific CD8+ T cells over time up to 43 days after infection (Figure 4.10A). By using a tetramer during flow cytometry staining we can identify CD8+ T cells that recognize one of the viral epitopes gp33 (MBL H-2Db LCMV gp33 [C9M] Tetramer-KAVYNFATM-PE) We found that in miR-146a cKO hosts the frequency of LCMV specific CD8+ T cells circulating in the blood was lower than WT hosts starting at day 14 (Figure 4.10B). Once the mice were sacrificed at day 43 after infection, we analyzed their spleens for tetramer positive cells. We find that the spleens of WT and miR-146a cKO mice have frequencies of tetramer positive cells that reflect what was seen in the blood (Figure 4.10C). Again we find that at the terminal endpoint miR-146a cKO mice have fewer tetramer positive cells. Moreover, when we measured estimated numbers of tetramer positive cells in the spleen, we find that miR-146a cKO mice are lower compared to WT (Figure 4.10D). Finally, we measured surface markers CCR7, CD62L, CD69, and CD103 to see if tetramer positive cells showed differences in phenotype (Figure 4.10E). We did not find any signal for CCR7

or CD62L for either genotype. We did find that more tetramer positive cells were positive for CD69 in miR-146a cKO hosts and a trending increase of CD103 in miR-146a cKO hosts, but the frequencies are low (Figure 4.10E). Taken together, miR-146a cKO mice have less capacity to generate antigen-specific CD8+ T cell responses after acute viral challenge.

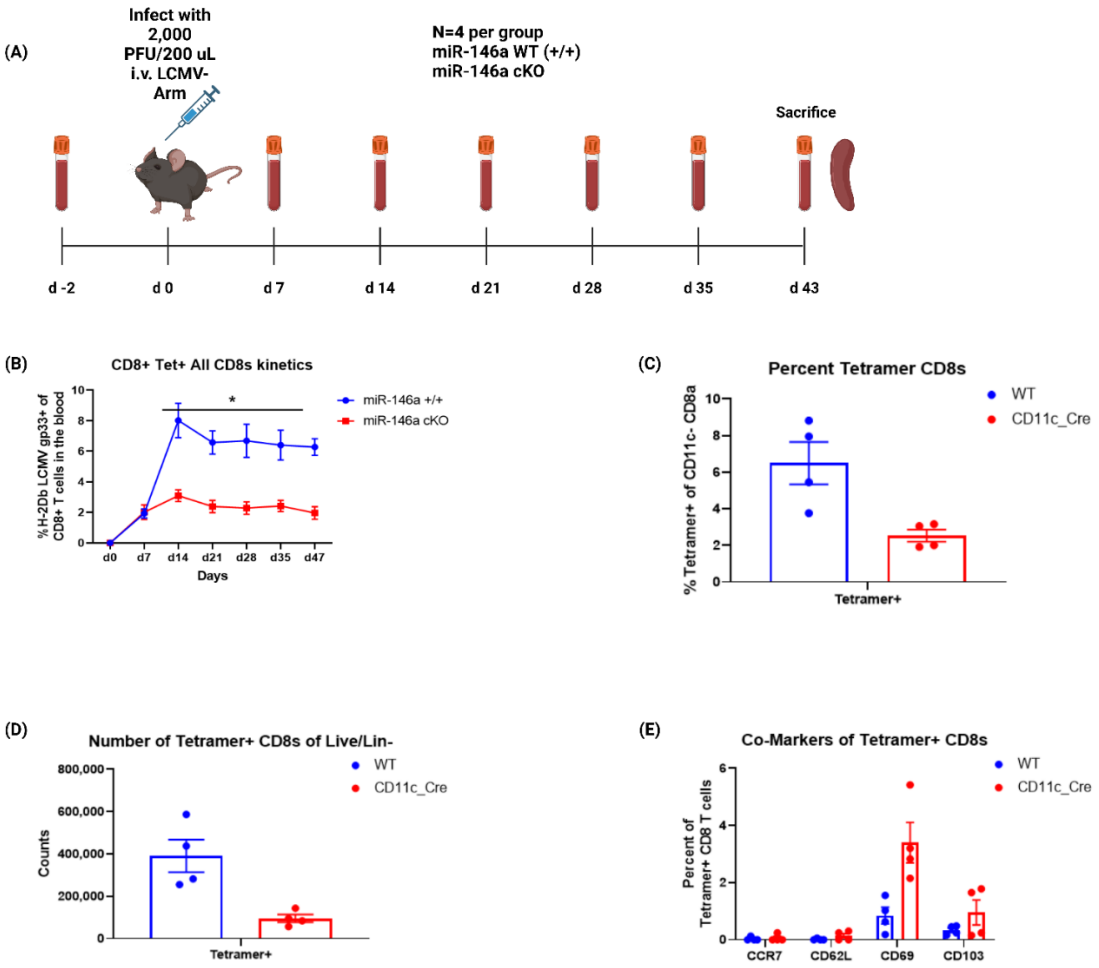


Figure 4.10 LCMV infection of WT and miR-146a cKO mice

Figure 4.10 LCMV infection of WT and miR-146a cKO mice. A. Experimental layout of sample collection timepoints, infection, and sacrifice endpoint. B. Timepoint kinetics of antigen-specific CD8+ T cells detected by gp33-tetramer staining in the blood. C. Summary bar charts of the frequency of gp33-tetramer positive CD8+ T cells in the spleen of WT and miR-146a cKO mice at day 47. D. Estimated cell counts of gp33-tetramer positive cells in the spleen of WT and miR-146a cKO mice at day 47. E. Frequency of activation markers CCR7, CD62L, CD69, and CD103 on tetramer positive cells.

4.5 Summary and Future Directions

The characterization of miR-146a deficient mice in our lab has given some conflicting results. The *in vivo* phenotyping from miR-146a spleens indicated heightened immune activity and the *in vitro* testing of BMDCs indicated DCs that have increased response to TLR stimulation and antigen. However when miR-146a cKO mice were evaluated for antigen-specific T cell responses after viral infection, they showed fewer viral specific T cells. These data taken as a whole can be interpreted as a complex role for miR-146a activity in DCs specifically that warrant further studies. The hypothesis to test experimentally is: **loss of miR-146a in DCs hinders T cell responses to viral infection.**

Upregulation of miR-146a in response to TLR signaling is supported in the literature and by experimental data from our lab [249, 250, 253, 255, 262] (Figure 4.2). While IRAK1 and TRAF6 have been characterized as targets of miR-146a, it is likely that additional targets exist for miR-146a repression. Indeed it was published that IRAK2 is a *bona fide* target of miR-146a using VSV infection of macrophages [255]. Therefore I would screen DCs in miR-146a overexpression and knockout experiments to identify previously unidentified targets. I would FACS purify splenic DCs from WT mice and seed them into tissue culture plates where I can transfect DCs with miR-146a mimics using double-stranded RNA oligonucleotides to overexpress miR-146a before incubation overnight with TLR ligands. I would then harvest the DCs for bulk RNA sequencing for unbiased detection of TLR pathway changes. The experiment would be repeated with miR-146a cKO sourced splenic DCs to avoid non-specific impacts on the development of DCs in a whole-KO before bulk RNA sequencing. Target prediction from *in silico* analyses such as Targetscan.org or Mirbase.org would supplement the RNA sequencing analysis. I would

expect that novel miR-146a targets will be identified that are part of TLR signaling as well as TLR-independent pathways. Any identified targets from sequencing will be confirmed using 3'UTR luciferase reporter assays as they provide a reliable *in vitro* method to evaluate miR targets [255].

The data from these sequencing experiments can also provide new insights to the findings that loss of miR-146a increases cDC frequency *in vivo* (Figures 4.3,4.4). The samples that do not receive TLR stimulation will provide information on changes to pathways associated with cell cycle or apoptosis programs. It was published that loss of miR-146a expression in BMDCs led to an increase in the anti-apoptosis protein Bcl-2, suggesting that loss of miR-146a in DCs will increase their survival and retention [251]. I would expect from RNA sequencing data anti-apoptosis pathways will be enriched in miR-146a KO DC populations. Moreover, I would expect transcripts for CD80 and CD86 to be increased in miR-146a KO DCs based on the *in vitro* data generated in our lab (Figure 4.7).

The DQ-OVA experiments in miR-146a deficient BMDCs indicate changes to antigen uptake or processing. It is not known if miR-146a modulates phagocytosis proteins (e.g. mannose receptor) or antigen processing proteins (*e.g.* TAP). Therefore, I would perform DC-T cell co-culture experiments to evaluate changes in T cell activation. Using FACS to purify splenic DCs I would incubate them with whole-OVA protein and stimulate with LPS or CpG for 3 hours before co-culture with transgenic OT-I cells. I would probe for proliferation of OT-I cells using carboxyfluorescein scuccinimidyl ester (CFSE) dye dilution after culture with miR-146a competent and deficient DCs at 24, 48, and 72 hours. Engagement with DCs has been reported to take as long as 72 hours to provide optimal T cell activation [263] but in miR-146a deficient DC culture this process may be shorter.

Furthermore, intracellular cytokine staining for IL-2, TNF-α, and IFN-γ in OT-I cells would be assessed for functional ability. Based on our *in vivo* infection experiment I would expect OT-I cells that are cultured with miR-146a deficient DCs to have less proliferation and functional cytokines by flow cytometry analysis.

I would repeat the LCMV-Armstrong infection experiment and incorporate some additional metrics that were not recorded. Although the tetramer positive OT-I cells were lower in miR-146a cKO mice, we do not know if viral titers were affected in those mice. Moreover, we do not know if class-switching of antibodies was different between miR-146a cKO hosts and WT hosts. It is possible that a humoral response that was undetected played a role in addition to the CD8+ T cell response to the infection. Therefore I would include at the early timepoints after infection collection of sera for viral titer analysis using plaque assays from WT and miR-146a cKO infected hosts. Days 1, 3, 5, and 7 would provide helpful kinetics information on viral burden. I would also include a LCMV-specific IgG ELISA assay for detection of differences in LCMV specific antibody responses [264]. Finally I would include intracellular cytokine staining for IL-2, TNF-α, and IFN-γ to asses functional ability of OT-I cells that respond to infection. I would expect that in OT-I cells from miR-146a cKO hosts will exhibit lower cytokines by flow cytometry.

4.6 Materials and Methods

Ethics statement. Animal studies were approved and conducted under OHSU IACUC protocol "Micro-RNA (miRNA) in adaptive and innate immune responses" IP00000915 Evan Lind, PI.

miR-146a KO Murine Model. Mice that are complete knockout for miR-146a were purchased from Jackson Labs [249] strain#016239 B6.Cg-Mir146tm1.1Bal/J. miR-146a cKO mice CD11c-Cre miR-146a loxP were a generous gift from Li-Fan Liu, PhD. All breeding animals were purchased from The Jackson Laboratory. All mice used in these experiments were bred as homozygous. All mouse experiments were performed in accordance with the OHSU Institutional Animal Care and Use Committee protocol IP00000915. No inclusion or exclusion criteria were used on the animals with correct genotype. Mice were selected and assigned to groups randomly while maintaining a 50% male 50% female ratio per experiment. No blinding was performed. Average age of mice used for *in vivo* studies was 10 weeks.

***Ex vivo* cell preparation**. Spleens were harvested from mice and mechanically dissociated using frosted microscope slides then rinsed with 1x PBS. Single cell suspensions were passed through 70-µm cell strainers and red blood cells were then lysed with ammonium chloride-potassium (ACK) lysis buffer. Cells were counted with hemacytometer and 3–5 × 10^6 cells were used per antibody-staining reaction.

Flow cytometry staining. Bone marrow, blood, or splenocytes were processed and subjected to red blood cell lysis by ACK before counting via hemacytometer. Cells were resuspended in PBS and stained at 4°C with 100 µL 1:500 Zombie Aqua viability dye

(BioLegend, Cat# 423102) and 1:200 mouse FC block (TruStain FcX, BioLegend Cat# 101320) for 15 min, covered from light. Cells were pelleted for 5 minutes at 300xg. 100 µL of cell surface staining antibody cocktail was added directly on top of the cells and stained on ice for 30 min in FACS buffer (1X PBS, 1.5% calf serum, 0.02% sodium azide, 2mM EDTA). Cells were washed 2X with 1X FACS buffer, then resuspended in 1X FACS buffer before analyzing on a BD Fortessa flow cytometer. Data were analyzed using FlowJo software.

Generation of BMDCs and TLR Simulation. Protocol adapted from Lutz et. al. [265]. Femurs and tibia are collected from mice and bone marrow cells flushed using a 25G needle. Cells are pelleted and resuspended in complete DC media (10%FCS + 1% Pen/Strep + 50µM 2-ME). Bone marrow cells are seeded inot non-tissue culture treated 100 mm petri dishes at 2e6 cells/10 mL. 40 ng/mL GM-CSF (Peprotech catalogue: 315-03) cytokine added to culture. On Day 3 10 mL of complete media containing 40 ng/mL GM-CSF is added to cultures. Day 6 10 mL supernatant is removed from cultures and replaced with fresh 10 mL complete media containing 40 ng/mL GM-CSF. Day 8 repeat 10 mL media replacement. Day 9 harvest loosely adherent cells and pellet them for resuspension at 2e6 cells/mL. Seed wells in 24-well plate to stimulate with TLR ligands 10µM CpG (ODN1826 – Invivogen) and 10 ng/mL or 1 mg/mL LPS (Sigma-Aldrich – 026:B6). Stimulate DCs overnight (16 hours) and harvest Day 10 cells for analysis. DQ-OVA (ThermoFisher; Molecular Probes) [260] was incubated with BMDCs at a concentration of 1 mg/mL for 10 minutes or 60 minutes at 37°C.

Viral infection using LCMV-Armstrong. Frozen stock of LCMV-Armstrong ($8x10^7$ pfu/mL) was a generous gift from Lund Lab. On day of infection, frozen viral

aliquot was carefully thawed and diluted with 1X PBS for a final concentration of 10 pfu/µL for an injection dose of 2,000 pfu/200 µL. On Day 0 2,000 pfu/200 µL LCMV-Armstrong was intravenously injected into recipient mice. On days -2, 7, 14, 21, 28, 35, and 43 peripheral blood aliquots were collected for tetramer kinetics. On Day 43 spleens from recipient mice were harvested and splenocytes were prepared for flow cytometry staining to asses CD8+ T cells for tetramer staining. MBL H-2Db LCMV gp33 [C9M] Tetramer-KAVYNFATM-PE https://products.mblintl.com/products/tb-m512-2/.

Antibodies.

Table 1 Mouse antibody list

Antigen	Fluor	Clone	Catalogue	Vendor
I/A-I/E	BV421	M5/114.15.2	107631	BioLegend
CD80	BV650	16-10A1	104732	BioLegend
CD103	Alexa Fluor 488	2_E7	121408	BioLegend
CD135	PE	A2F10	135306	BioLegend
CD11b	PE-Cy7	M1/70	101216	BioLegend
F4/80	APC	BM8	123116	BioLegend
CD11c	Alexa Fluor 488	N418	117311	BioLegend
Ly6G	PerCP-Cy5.5	1A8	127616	BioLegend
CD3e	PE	145-2C11	100308	BioLegend
CD19	PE-Dazzle 594	6D5	115554	BioLegend
GR-1	BV605	RB6-8C5	108439	BioLegend
CD3e	BV421	145-2C11	100336	BioLegend
CD11b	APC	M1/70	101212	BioLegend
Ly6C	Alexa Fluor 700	HK1.4	128024	BioLegend
CD62L	APC-Cy7	MEL-14	104428	BioLegend
CD86	PE-Cy7	PO3	105116	BioLegend
CD86	BV650	GL-1	105035	BioLegend
CD86	APC-Cy7	GL-1	105030	BioLegend
B220	PerCP-Cy5.5	RA3-6B2	103236	BioLegend
I/A-I/E	PE	M5/114.15.2	107608	BioLegend
I/A-I/E	BV650	M5/114.15.2	107641	BioLegend
CD11c	BV421	N418	117330	BioLegend
CD8a	Pacific Blue	53-6.7	100725	BioLegend
CD44	BV605	IM7	103407	BioLegend
NK1.1	BV650	PK136	108736	BioLegend
CD19	BV711	6D5	115555	BioLegend
CD3e	Alexa Fluor 488	145-2C11	100321	BioLegend
I/A-I/E	PerCP	M5/114.15.2	107624	BioLegend
CD4	PerCP-Cy5.5	RM4-4	116012	BioLegend
Zombie Aqua			423102	BioLegend
H-2Db LCMV gp33	PE	C9M	TB-M512-1	MBL

LegendPlex Blood Serum Assay. Peripheral blood from WT and miR-146a cKO mice was collected via retro-orbital sources. Blood was collected in Sarstedt Microvette® 500 Serum Gel tubes and centrifugated at 10,000 x G for 5 minutes at room temperature to remove cells from the serum. Aliquots of the remaining serum were stored at -80°C before assayed. On day of being assayed aliquots were thawed slowly on ice before being tested using the flow cytometry based LEGENDplex™ Mouse Inflammation Panel (BioLegend Cat: 740446) according to the manufacturer's instructions for sample processing. Samples were measured on a BD Fortessa according to the manufacturer's instructions for calibration and data collection. Data was analyzed using the online resource from BioLegend (https://legendplex.qognit.com).

Statistics. A 2-sample Student t test with unequal variances was used to test for a statistical difference between 2 groups or a 1-sample Student t test was used to test for a statistical difference in the percent change from 0% where indicated. Unless otherwise indicated, all hypothesis tests were 2-sided, and a significance level of 0.05 was used. Ordinary One-Way ANOVA used to test-for statistical differences between 3 groups. Statistics and Graphs were generated by using Prism 9 software (GraphPad Software https://www.graphpad.com/).

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