Investigating the role of cytokines in clonal hematopoiesis

Tania Fabo
Balliol College

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COVID-19 Statement

Due to the COVID-19 pandemic, I was unable to complete my proposed research plan to the extent that I had originally intended. When the pandemic began in March, I flew home to the United States to be with my family and help take care of my siblings, given that my mother, a nurse, was putting herself at risk at the frontlines of the pandemic. As a result, I was not able to complete the lab work I had planned to do and was not able to return to the university once labs began to open again. As I have mentioned throughout this thesis, there were several experiments I had planned which I was no longer able to do.

The first of these was the Luminex cytokine analysis I had planned to do on the bone marrow samples that had been collected from CHIP and control patients. Although the Luminex analysis of peripheral blood revealed interesting information on which cytokines may be dysregulated in CHIP, analysis of the bone marrow samples would have given greater insights into how CHIP mutant clones might modify cell signaling in the microenvironment to benefit their own expansion. I had also planned to repeat the BCA protein quantification experiments which I had inaccurately performed to be able to normalize cytokine expression to total protein. Following these experiments, I had planned to map this information onto the TARGET-Seq that my graduate student collaborator, Asger Jakobsen, had performed in HSCs from CHIP patients. Given that TARGET-Seq allows for simultaneous mutational analysis and RNA-Seq, this would have helped me determine whether HSPC receptors and signaling pathways for cytokines of interest are differentially expressed in HSCs carrying CHIP mutations compared to those that are not. The next step would have been to establish causality, which I planned to do through replating assays to determine how certain cytokines affect proliferation and self-renewal of CHIP

clones. Finally, and perhaps most ambitiously given my timeframe, I had hoped to sort bone marrow from CHIP and control patients for cells shown to be dysregulated in MDS, a common precursor to AML, and perform RNA-Seq to study how these niche cells might become dysregulated in CHIP.

Abstract

Clonal hematopoiesis of indeterminate potential (CHIP) is a hematologic disorder in which individual hematopoietic stem cells acquire a mutation which allows them to clonally expand (Genovese et al., 2014; Jaiswal et al. 2014). CHIP is known to occur disproportionately in older adults. While CHIP itself usually remains clinically benign, it has been shown that CHIP may serve as a precursor for acute myeloid leukemia (AML), a hematologic cancer which, like clonal hematopoiesis, increases in risk and mortality with age (Deschler and Lübbert, 2006). However, it is not fully known what drives CHIP mutant clones to expand, nor what causes CHIP to develop into advanced hematologic malignancies such as AML. To date, a great deal of research has focused on understanding how cell intrinsic factors, like the gene carrying the CHIP mutation and further mutations acquired during AML development, may provide insight into CHIP development and progression. However, investigating cell extrinsic factors, such as cytokine signaling, in the context of CHIP may provide additional important clues into how CHIP mutants are able to gain the clonal advantage and ultimately develop into AML. In this thesis, I undertook a large-scale panel analyzing how expression of 25 cytokines may be dysregulated in the peripheral blood of CHIP patients. I identified 5 cytokines, TNFα, IL-8, CCL7, IFNγ and FGF-basic, which had significantly dysregulated expression between CHIP patients and controls. Although these results were not significant after Bonferroni multiple testing correction, they warrant further investigation into how they may impact HSC mutant clone expansion. Additionally, I analyzed RNA-Seq data to determine how these and a few other cytokines and their receptors are differentially expressed amongst blood progenitors, both in healthy patients and AML patients. Findings from the CHIP cytokine panel and my analysis of

the RNA-Seq data suggest that inflammatory signaling may play a key role in both driving HSC mutant clone expansion in CHIP and promoting the eventual development of AML.

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List of Abbreviations

AML	Acute myeloid leukemia
ANGPT1	Angiopoietin-1
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BM	Bone marrow
CCL11	Chemokine ligand 11
CCL3	Chemokine ligand 3
CCL7	Chemokine ligand 7
CHIP	Clonal hematopoiesis of indeterminate potential
CMP	Common myeloid progenitor
CXCL12	C-X-C motif chemokine ligand 12
FGF-basic	Basic fibroblast growth factor
Flt3 (ligand)	Fms-like tyrosine kinase 3 (ligand)
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte-macrophage progenitor
HSC	Hematopoietic stem cell
IFNγ	Interferon gamma
IL-10	Interleukin 10
IL-11	Interleukin 11
IL-17	Interleukin 17

IL-18	Interleukin 18
IL-1β	Interleukin 1 beta
IL-3	Interleukin 3
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-8	Interleukin 8
LIF	Leukemia inhibitory factor
LMPP	Lymphoid-primed progenitor
LSC	Leukemic stem cell
M-CSF	Macrophage colony-stimulating factor
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte erythroid progenitor
MLP	Multi-lymphoid progenitor
MPP	Multipotent progenitor
MSC	Mesenchymal stromal cells
PB	Peripheral blood
PBS	Phosphate-buffered saline
RDW	Red blood cell distribution width
SCF	Stem cell factor
TNFα	Tumor necrosis factor
TPO	Thrombopoietin
VAF	Variant allele frequency

VEGF	Vascular endothelial growth factor

1) Introduction

Humans have between 50,000 and 200,000 stem cells residing in the bone marrow that contribute daily to blood production, or hematopoiesis. These stem cells, called hematopoietic stem cells (HSCs), are multipotent self-renewing cells that play a crucial function in the long-term maintenance and production of all mature blood cell lineages during a person's lifespan (Dzierzak and Bigas, 2018). This blood production is polyclonal, whereby each HSC in a person's bone marrow contributes relatively equally to blood production. Key historical experiments have validated the polyclonal nature of hematopoiesis. Till and McCulloch (1961) found that when irradiated mice were injected with bone marrow cells, the number of cells injected was directly proportional to the number of colonies in the spleen (an important site of hematopoiesis). Becker, McCulloch and Till (1963) built upon this work by showing direct evidence of the unicellular origin of these colonies, using radiation to generate unique abnormal karyotypes in HSCs that could be traced in colony formation. Taken together, this work provided the earliest evidence for HSCs and the polyclonal nature of blood production.

HSCs can both self-renew, meaning that they give rise to another HSC during cell division, and differentiate, meaning they progress further down the blood lineages and away from multipotency (Till and McCulloch, 1961; Becker et al., 1963; Siminovitch et al., 1963; Wu et al., 1968; Seita and Weissman, 2010). As HSCs are dividing or self-renewing, normal somatic mutations can occur from errors in DNA replication as they do for all dividing cells. Somatic mutations accumulate as a person gets older (Kenyon, 2010; Risques and Kennedy, 2018). If these mutations occur in genes that give a blood stem cell an advantage over other stem cells, that stem cell will go on to disproportionately contribute to blood cell production compared to

other blood stem cells and have a larger clone size. This process is known as clonal hematopoiesis.

Age-related clonal hematopoiesis was first demonstrated in studies that showed that 38% of healthy women over the age of 60 had nonrandom X chromosome inactivation in peripheral blood cells, suggesting preferential clonal selection in an early precursor (Busque et al., 1996; Champion et al., 1997).

Further large retrospective cohort studies have found that clonal hematopoiesis of somatic variants in the peripheral blood cells increases with age, from less than 1% in persons younger than 50 to 9.5% in persons aged 70-90, 11.7% in persons aged 80-89, and 18.4% in persons aged 90-108 (Genovese et al., 2014; Jaiswal et al. 2014). These somatic variants most frequently occurred in the genes DNMT3A, TET2, and ASXL1. Importantly, these age-related clonal hematopoiesis mutations are associated with at least 11-fold increased risk of hematologic cancers, as well as increased risk of coronary heart disease and ischemic stroke. Genovese et al. (2014) found that 42% of hematologic cancers in their cohort arose in patients who had clonal hematopoiesis at the time of sequencing. These findings suggest that clonal hematopoiesis of HSCs carrying these somatic mutations is the first step in developing hematologic cancers and could be used to screen for future risk.

To understand how clonal hematopoiesis might confer greater risk for hematologic cancers, it is important to understand how cancer develops. Cancer is thought to arise from a series of stepwise genetic and epigenetic changes and mutations (Nowell, 1976). These changes will transform a normal cell into a premalignant and ultimately malignant cell. In hematologic cancers, sequential acquired mutations which select for clonal expansion in the blood will give

rise to cancerous clones which can end up dominating a person's blood. A precursor to this process may be clonal hematopoiesis, whereby an HSC clone has begun to expand due to a beneficial mutation but has not yet transformed into a malignant phenotype.

Clonal hematopoiesis may specifically serve as a precursor for acute myeloid leukemia (AML), a hematologic cancer which, like clonal hematopoiesis, increases in risk and mortality with age (Deschler and Lübbert, 2006). The median age for new AML diagnosis is 65 and the disease is rarely diagnosed before the age of 40. 5-year survival for AML is also stratified by age, from 48.5% survival for patients younger than 45, to 4.3% survival for patients older than 65. Progression to AML has been connected to preleukemic HSCs and founder clones that harbor some of the mutations found in leukemic cells (Jan et al., 2012; Welch et al., 2012). These preleukemic mutations are in genes that are also commonly mutated in clonal hematopoiesis, including *DNMT3A*, *TET2*, and *ASXL1* (Corces-Zimmerman et al., 2014; Genovese et al., 2014; Jaiswal et al., 2014). These frequently mutated genes tend to be "landscaping" genes that are involved in chromatin remodeling and epigenetic modifications (Challen et al., 2012; Moran-Crusio et al., 2011).

Mutations in these genes are prevalent in AML patients and associated with poorer survival. Over 20% of patients with acute myeloid leukemia carry *DNMT3A* mutations (Ley et al., 2010; Yan et al., 2011). Median survival for patients with *DNMT3A*-mutant leukemia is significantly shorter (12.3 months) than among patients without this mutation (41.1 months) (Ley et al., 2010). Similarly, *TET2* mutations occur in approximately 25% of acute myeloid leukemia patients and are associated with older patients (Chou et al., 2011; Metzeler et al., 2011; Weissman et al., 2012). Median survival for patients with *TET2* mutations is also significantly

lower (6.7 months) compared to patients without these mutations (18.7 months) (Metzeler et al., 2011; Weissman et al., 2012). Furthermore, founder clones carrying common clonal hematopoiesis mutations like DNMT3A can also be a source of subclones for disease relapse (Ding et al., 2012; Jan et al. 2012; Welch et al., 2012; Corces-Zimmerman et al., 2014). These findings highlight the connection between mutations commonly found in clonal hematopoiesis and aggressive AML.

Clonal hematopoiesis is also linked to higher risk for myelodysplastic syndromes (MDSs), a common precursor to AML. MDSs are a heterogenous group of clonal hematopoietic disorders characterized by ineffective hematopoiesis and cytopenia, or reduction in the number of mature blood cells (NCI, 2019). Approximately 30% of patients with MDS will go on to develop AML (Klepin, 2016; Walter et al., 2011). *DNMT3A* mutations, commonly found in clonal hematopoiesis, are detected in approximately 8% of MDS, and these mutations are associated with poorer survival and more rapid progression to AML (Walter et al., 2011). Mutations in other genes commonly mutated in clonal hematopoiesis, such as *TET2, SF3B1,* and *ASXL1*, are also frequently mutated in MDS (Haferlach et al., 2013).

However, it is important to note that while clonal hematopoiesis confers increased risk for MDS and AML, most individuals who develop clonal hematopoiesis during aging will never go on to develop MDS or AML (Steensma et al., 2015). This is why age-related clonal hematopoiesis is commonly referred to as clonal hematopoiesis of indeterminate potential (CHIP), because potential for these expanded HSC clones to develop into disease has not yet been clearly defined. While CHIP mutations can serve as an important prognostic marker for risk of hematologic diseases like MDS and AML, it is important to understand what drives HSC

clones carrying these mutations to expand and develop into disease. This could be the key to targeting these expanded clones before they develop into hematologic cancers and targeting the founder clones that are responsible for relapse.

Because cells in the body exist in dynamic crosstalk with other cells, one potential strategy is to look at how the microenvironment where mutant HSC clones reside contributes to their expansion. Cells and signaling in the bone marrow niche, where most HSCs are located, play a key role in regulating HSC maintenance and function. The normal bone marrow niche is composed of diverse cell types that send signals and provide physical interactions to HSCs that are crucial for HSC maintenance and the regulation of hematopoiesis (Schepers et al., 2015). Important cell types in the niche include vascular endothelial cells, perivascular mesenchymal stromal cells (MSCs), and megakaryocytes (Kiel et al., 2005; Bruns et al., 2014; Day and Link, 2014; Morrison and Scadden, 2014; Ramalingam et al., 2017). These cells provide important regulatory signals in close proximity to HSCs. Other cells found in the bone marrow niche include osteoblasts, specialized macrophages and nerve cells.

There are several signaling molecules in the bone marrow, most of which are cytokines, that play key roles in controlling normal HSC function. Stem cell factor (SCF), transforming growth factor beta-1 (TGF-β1), platelet factor 4 (PF4 or CXCL4), angiopoietin 1 (ANGPT1), and thrombopoietin (TPO) are all critical enforcers of HSC quiescence, or dormancy (Zhang and Lodish, 2008; Mirantes et al., 2015; Schepers et al., 2015). Chemokine stromal-derived factor 1 (SDF1α or CXCL12) and its receptor C-X-C chemokine receptor type 4 (CXCR4), vascular adhesion protein 1 (VCAM-1), and various selectins and extracellular matrix (ECM) proteins like fibronectin are all essential regulators of HSC homing and anchoring in the niche. Notch

ligands and other cell-bound molecules, as well as locally secreted cytokines like interleukin 7 (IL-7) and erythropoietin (EPO) help control HSC proliferation and differentiation activity (Robb, 2007). As a group, these signaling molecules help regulate when HSCs self-renew and when they differentiate. Given that CHIP, and the diseases that can arise from it, are disorders of dysregulated HSC function, it may be that signaling in the bone marrow is also disrupted to help drive clonal expansion and disease.

Research has shown that the niche can help hematologic diseases progress and even remodel itself as disorders develop (Ghobrial et al. 2018). Altered expression of proinflammatory and regulatory cytokines, such as TNFα, IL-6, TGF-β, and CXCL12, in mesenchymal stromal cells (MSCs) has been shown to enhance adhesion, proliferation, and drug resistance of MDS and leukemia cells (Wang and Xiao, 2014; Shastri et al., 2017; Ghobrial et al., 2018). MSCs from patients with MDS have also been shown to overproduce the microenvironmental factors N-Cadherin, IGGB2, VEGFA, and LIF to enhance MDS cell expansion in patient xenograft experiments (Medyouf et al., 2014). Stromal fibroblasts and macrophages in the bone marrow of MDS patients also have altered expression of genes encoding proinflammatory cytokines and other factors, including TNFα, IFN-γ, IL-6, TGF-β, and CCL3 (Kitagawa et al., 1997; Allampallam et al., 2002; Feng et al., 2011). Additionally, MSCs in the bone marrow have been shown to subvert and regulate innate and adaptive immunity by modulating secretion of cytokines like TGF-β, IFN-γ, IL-10, TNFα (Ghannam et al., 2010a; Ghannam et al., 2010b). MSCs may be co-opted in the progression of hematologic disease to help suppress the immune response and promote clonal expansion.

The relationship between CHIP and the microenvironment and cytokine signaling has been less explored. Using aggregate whole-genome sequencing data from peripheral blood samples, Bick et al. (2020) found that CHIP is associated with higher levels of IL-6 and IL-1β. They also found important mutation-specific effects, where TET2-mutant individuals with CHIP had significantly increased IL-1β and JAK2- and SF3B1-mutant CHIP carriers had significantly increased IL-18. Jaiswal et al. (2017) found that plasma IL-8 levels are significantly elevated in TET2-mutant CHIP compared to controls. Other studies have uncovered additional CHIP-wide and mutant-specific cytokine expression differences, including higher levels of serum IL-6 in TET2-mutant CHIP, higher levels of CCL11 and TNFα in DNMT3A-mutant CHIP, and higher IL-6 and TNFα in all CHIP carriers (Cook et al., 2017; Cook et al., 2019). Additionally, studies in mice have shown that elevated TNFα is advantageous for proliferation of TET2-mutant HSCs (Abegunde et al., 2018). In addition to altered cytokine levels, researchers have found a significant association between red blood cell distribution width (RDW) in CHIP patients and risk of progression to AML (Abelson et al., 2018; Bick et al., 2020). Importantly, raised RDW is correlated with inflammation, suggesting that intercellular inflammatory signaling and interactions may play a role in determining which CHIP clones progress to AML.

While this prior work has taken important steps to uncovering how the microenvironment can impact CHIP clones, it is clear that a more comprehensive look at how expression of regulatory and inflammatory cytokines varies for CHIP patients in both the peripheral blood and the bone marrow is needed. In this thesis, I investigated how expression of a large panel of cytokines varies between CHIP and control patients. For this work, I used samples that had been previously collected by Asger Jakobsen, a graduate student in the Vyas Lab. Prior to my work on

this project, Asger set up a study to collect peripheral blood plasma and bone marrow from 200 older adults with no history of hematologic cancers. These samples were collected during hip replacements, a procedure done predominantly on older adults, and were genotyped using DNA probes complimentary to the genomic regions of interest. The samples were collected as part of a larger project aimed at understanding the mechanisms for how HSCs with CHIP mutations are able to gain a clonal advantage over non-mutant cells.

My work for this thesis focused specifically on the role that the microenvironment may play in promoting HSC clone expansion. In this thesis, I undertook several lines of investigation to explore this question. I began by using a Luminex panel to compare overall cytokine expression levels between patients with and without CHIP. I then looked at whether there were any specific mutation, number of mutations, or allele frequency effects in cytokine expression levels. Finally, I used RNA-Seq data from the Vyas Lab to look at which cells normally express the dysregulated cytokines and their receptors and identify hematopoietic cell types that might be in crosstalk with CHIP HSC clones and promote their expansion.

These experiments and analyses work to improve our understanding of what extracellular factors and cells are interacting with HSCs carrying CHIP mutations and how they might play a role in clonal hematopoiesis. By better characterizing how CHIP clones gain the clonal advantage, this research can inform future work on what causes a CHIP clone to progress to further hematologic disease, like MDS and AML. This may lead to better prognostic and early interventions to prevent patients from developing these life-altering diseases.

2) Materials and Methods

2.1 Patient samples

Patient samples were collected with informed consent under the Mechanisms of Age-Related Clonal Haemopoiesis (MARCH) Study (REC Ref: 17/YH/0382). Participants were recruited from individuals due to undergo elective total hip replacement surgery at the Nuffield Orthopaedic Centre, Oxford.

At the time of surgery, bone marrow aspirates were obtained from the femoral canal and collected in anticoagulated buffer containing acid-citrate-dextrose, heparin sodium and DNase. Samples of peripheral blood were collected in EDTA vacutainers.

All samples were processed within 24 hours after collection. Peripheral blood samples were diluted 1:2 in RPMI and filtered through a 70μm cell strainer. Bone marrow aspirates were filtered through a 70μm cell strainer to obtain a single cell suspension. Peripheral blood and bone marrow mononuclear cells (MNCs) were then isolated by Ficoll density gradient centrifugation. Bone marrow CD34+ cells were purified using a CD34 MicroBead kit and MACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Unseparated MNCs, CD34+ and CD34-deplete fractions were frozen in 90% FBS/10% DMSO, stored in liquid nitrogen and subsequently thawed on the day of the experiment.

Peripheral blood granulocytes isolated by Ficoll density gradient centrifugation were frozen for analysis of mutant allele frequency in peripheral blood.

Peripheral blood and bone marrow plasma samples were obtained by collecting the supernatants after Ficoll density gradient centrifugation, further centrifuged at 500g for 5 mins to remove cells and frozen at -80°C.

2.2 Targeted sequencing

Genomic DNA was extracted from bone marrow MNCs using a DNeasy Blood & Tissue Kit (Qiagen). Pre-capture DNA libraries were prepared using the KAPA HyperPlus protocol (KK8514, Roche).

Targeted capture was performed using a custom pool of biotinylated capture probes (SeqCap EZ Prime Choice, Roche) targeting 97 genes recurrently mutated in clonal haematopoiesis and myeloid malignancies.

Post-capture amplified DNA libraries were quantified by Qubit (Life Technologies) and size distribution and quality analysed using a Bioanalyser chip (Agilent Technologies) or Tapestation (Agilent Technologies). Libraries were pooled to a final concentration of 4 nM and were sequenced on an Illumina NextSeq 500 using paired-end reads.

2.3 Variant calling

Sequencing data were analysed in a custom pipeline based on the GATK Best Practices (GATK v4.0.5.1 and Picard v2.18.7). Raw sequencing reads were converted to an unmapped BAM file

and trimmed of adapter sequences using Picard MarkIlluminaAdapters. Somatic variant calling was performed using VarDictJava and Mutect2. For VarDict, variants were called with a minimum variant allele frequency of 0.01, minimum base quality score of 25 and minimum supporting reads of 2. For Mutect2, a minimum tumour LOD of 1 was used and variants were filtered for sequence context-dependent artefacts using FilterByOrientationBias.

The following post-processing filters were applied to exclude likely sequencing artefacts: (1) Minimum of 5 variant reads for SNVs (with at least 2 reads in forward and reverse directions), or minimum of 10 variant reads for indels (with at least 4 reads in forward and reverse directions). (2) Minimum base quality score 30. (3) Minimum mapping quality score 40. (3) No strand bias. (4) No position bias towards beginning or end of reads.

2.4 Thermo Scientific Bicinchoninic Acid (BCA) Protein Assay

BCA protein quantification assay was performed on processed bone marrow and peripheral blood plasma samples using the Pierce™ BCA Protein Assay Kit. Samples were thawed on ice to prevent protein degradation. Nine standard dilutions were prepared in accordance with kit instructions. Each sample was first diluted 1:25 by pipetting 2 µL of sample into 48 µL PBS. 1:25 dilutions were subsequently diluted 1:10 by pipetting 5 µL of diluted sample into 45 µL PBS. Final assay sample dilutions were stock, 1:25, and 1:250. Standards were run in duplicates. Dilutions were not run in replicates. The assays were run in standard, flat-bottom 96-well plates. After mixing with working buffer, assay plates were placed on a shaker for 30 seconds and incubated at 37ºC for 30 minutes. Plates were then set out to sit at room temperature for 5

minutes. Following incubation, plate absorbance was measured at 562 nm using the ClarioStar plate reader. Sample concentrations were determined from standard concentrations.

2.5 Magnetic Luminex Performance Assay

Luminex assays were performed on peripheral blood samples using R&D Magnetic Luminex® Performance Assay kits. One 24-plex assay kit and a single-plex assay kit for CXCL12 was used. Samples and assay reagents were prepared according to kit instructions. Peripheral blood samples were not diluted due to being diluted 1:2 during initial processing. Standard cocktails were prepared and diluted according to kit instructions. Samples were diluted with microparticle cocktail in 96-well plate provided by the kit and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 800 ± 50 rpm. The plate was washed using a magnetic washer. Biotin-antibody cocktail and Streptavidin-PE were added separately and washed using the magnetic washer. Microparticles containing bound sample were resuspended in wash buffer and read within 90 minutes on the Bio-Rad Bio-Plex 200 analyzer. If bead aggregation issues were encountered, the sample plate was set to shake for an additional 2 minutes or the beads were resuspended by manual pipetting.

2.6 RNA-Seq data analysis

RNA-Seq was performed on a range of FACS-sorted blood progenitor cell populations from 3 normal donors and 17 leukemia patients. Progenitor cells obtained from normal donors were HSCs, MPPs, LMPPs, MLPs, CMPs, GMPs, and MEPs. Progenitor cells obtained from leukemia donors were GMPs, LMPPs, and MLPs. MLPs were excluded from analysis because these cells

were only obtained and sequenced for one patient. RNA-Seq file containing gene expression information for all cell types and patient replicates containing expression information based on Ensembl IDs was loaded into RStudio and merged with file containing Ensembl ID and gene names. One-way repeated measures ANOVA was first performed on healthy patient donors. Each 'repeated measure' was the replicate number assigned to different cell type sample (3 total replicates). If one-way ANOVA was significant, post-hoc testing was conducted to determine which cell pairings had significantly different expression. Two-way repeated measures ANOVA, or mixed ANOVA, was performed to compare leukemic GMPs and LMPPs to healthy GMPs and LMPPs. 16 matching replicates were sequenced from leukemic GMP and LMPP donors (one LMPP replicate had no sequencing information for GMPs). If mixed ANOVA showed significant leukemic effect or interaction effect, post-hoc testing was conducted to reveal the nature of the effect.

2.7 Data processing and statistical analysis

Data from the Luminex assays was processed using the tidyverse and dplyr packages in RStudio. These packages can be installed using the `install.packages("tidyverse")` and `install.packages("dplyr")` function in RStudio, followed by `library(tidyverse)` and `library(dplyr)`. Figures were generated using these functions in RStudio. Statistical analyses were conducted in Prism 8. CHIP, control, and subgroup sample were first tested for normality using the Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests in Prism. Almost all groups of samples

did not have normal distribution. Therefore, parametric tests, such as t-tests, were not valid and the Mann-Whitney U test (a type of non-parametric test) was performed instead.

RNA-Seq data was processed and analyzed using tidyverse, ggpubr, and rstatix packages in RStudio. Packages can be installed using the `install.packages` function for each package and loaded using the `library` function, as noted above. ANOVA tests, post-hoc tests and figure generation were performed in RStudio.

3) Results

3.1 Overview of cytokine analysis experiments

The goal of this thesis was to investigate how the bone marrow microenvironment may contribute to the expansion of mutant hematopoietic stem cell (HSC) clones in patients with CHIP. To answer this question, I carried out Luminex cytokine assays to measure the fluorescence and concentration of a set of cytokines in the peripheral blood plasma samples of healthy and CHIP patients. CHIP and control patient pools were matched based on age, sex, and past medical history to control for any potential physiological confounders. BCA assays were carried out to measure the total concentration of protein in the samples prior to performing the Luminex assays. Following the Luminex assays, a list of the most interesting cytokines whose expression may be dysregulated in CHIP patients was compiled. Using this list, I analyzed data generated from a previous RNA-Seq experiment in the lab to determine how expression of these cytokines and their receptors varies among different blood progenitor cells, both in healthy patients and in patients with acute myeloid leukemia. In this section, I will explain the rationale and findings for each of the aforementioned experiments. I will then move on to the discussion section where I will discuss and analyze the results, discuss limitations in interpreting the findings, and propose future experiments to validate my findings and explore the theories posed in this thesis.

3.2 Patient data and matching controls

Prior to my work on this project, Asger Jakobsen, a graduate student in the Vyas Lab, set up a study to collect peripheral blood plasma and bone marrow samples from 200 older adults

with no prior history of hematologic cancers or malignancies. These samples were collected during total hip arthroplasty, or hip replacement, a procedure which is typically performed on older adults. Because CHIP is also disproportionately seen in older adults, the likelihood of identifying individuals with CHIP in this patient population was high. Following collection of bone marrow and peripheral blood, the samples were processed and genotyped using DNA probes complimentary to the genomic regions of interest. Information on patients' past medical history was also collected from clinicians. A total of 170 patient samples were collected and genotyped by the time I began my project in October 2019.

Out of the 170 patients whose peripheral blood and bone marrow samples were collected, 66 patients (38.8%) carried a clonally expanded mutation in genes commonly associated with CHIP. This rate of occurrence is higher than what has been observed in prior CHIP studies, which report an occurrence rate of up to approximately 20% in their oldest patients (Genovese et al., 2014; Jaiswal et al. 2014). This is because, while prior studies classified CHIP as any expanded HSC clone with variant allele frequency (VAF) greater than 0.02 (that is, the expanded mutant CHIP clone makes up 2% of the total HSC population), the sensitivity of the genotyping method used on our samples allowed VAFs as low as 0.01 to be detected and classified as CHIP.

In preparation for Luminex cytokine analysis, I created two patient groups: 41 CHIP patients and 41 control patients. Patients in the CHIP group were prioritized for selection if they carried one of the two most commonly occurring CHIP mutations, DNMT3A and TET2. This would strengthen the power of mutation-specific analyses I planned to conduct on my Luminex data. Samples across the two groups were matched as best as possible based on age range (40-49, 50-59, 60-69, 70-79, 80-89), sex (male or female), and histories of heart disease (none, moderate,

or severe [ischemic heart disease or coronary artery bypass grafting]), hypertension (yes or no), diabetes (none, pre-diabetes, or full diabetes), stroke (none or transient ischemic attack), and smoking (none, former smoker, or current smoker) (Table 1). CHIP and control cases were matched to eliminate potential non-CHIP physiological confounders for any observed cytokine dysregulation.

Characteristic	CHIP	Control
N. of patients	41	41
Age range, n. of patients		
40-49	1	0
50-59	5	3
60-69	8	13
70-79	14	19
80-89	13	6
Sex, n. of patients		
Male	19	18
Female	22	23
Heart disease, n. of patients		
None	36	36
Moderate	2	2
Severe	3	3
Hypertension		
None	25	24
Yes	16	17

Diabetes			
	None	35	36
	Pre-diabetes	1	1
	Diabetes	5	4
Stroke			
	None	40	40
	TIA	1	1
Smoking			
	No	20	18
	Former	19	17
	Current	2	6

Table 1. CHIP samples and controls were matched as best as possible for Luminex analysis based on sex, age range, and history of heart disease, hypertension, diabetes, stroke, and smoking.

3.3 Protein quantification of peripheral blood and bone marrow samples

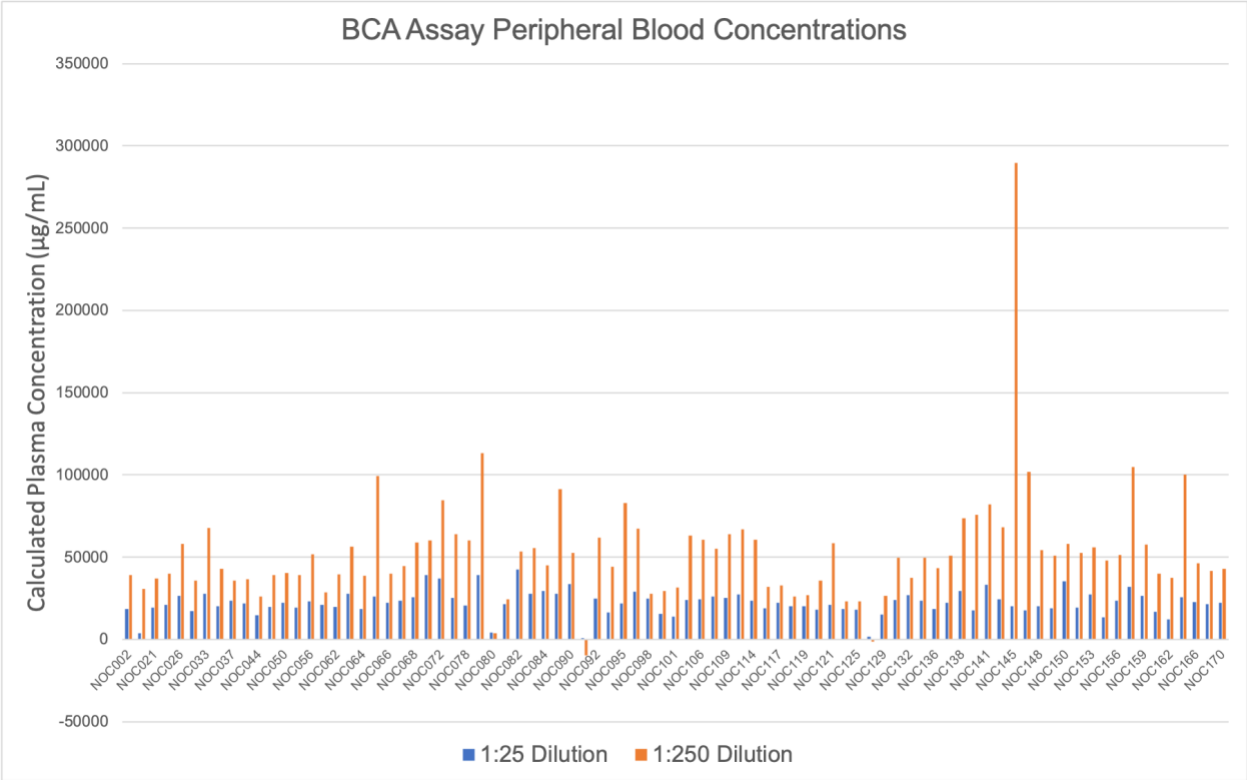
After finalizing a list of patients for the Luminex experiments, I performed bicinchoninic acid (BCA) protein assays on the peripheral blood and bone marrow samples from these patients to calculate total protein and confirm that there were no aberrant levels of total protein in any of the samples which would cause errors when interpreting Luminex assay results. The goal was to normalize observed cytokine expression levels from the Luminex essay to total protein levels in the same sample.

The upper limit of detection for the BCA assay is 2000 μg/mL while the lower limit is 20 μg/mL. The average total concentration of protein in blood plasma is 80,000 μg/mL. At the time

of collection and processing, blood plasma samples were diluted 1:2, such that the starting concentration of our samples was 40,000 μg/mL. I was unable to determine the starting concentration of the bone marrow samples because, unlike for the blood plasma, the dilution of bone marrow samples during processing was not standardized. I decided to dilute peripheral blood and bone marrow samples by the same factors and repeat the experiment for the bone marrow samples if their concentrations were out of range.

Each sample was diluted twice, first 1:25 (to achieve approximate concentration of 1600 μg/mL) and then 1:10 (approx.160 μg/mL). As can be seen in Figure 1, there is great deal of variation in the calculated protein concentrations from the BCA assay. While the expected concentration of the peripheral blood samples was ~40,000 μg/mL, almost none of the samples reached this concentration. Furthermore, there was a great deal of variation in concentrations, both across samples and between the 1:25 and 1:250 dilution of the same sample. A key unanticipated limitation to the design of this experiment which may explain these aberrant results was that very small volumes were pipetted to achieve the aforementioned dilutions. To get the 1:25 dilution, I pipetted 2 μL of sample into 48 mL of PBS. For the 1:10 dilution, I pipetted 5 μL of the 1:25 dilution into 45 μL of PBS. These small volumes, which are difficult to pipette accurately, likely led to a great deal of variation in concentrations. Given this error, I decided to move forward with performing the Luminex cytokine assays and repeat the BCA assays at a later time. However, due to the COVID-19 pandemic and resulting shelter-in-place orders and travel restrictions, I was unable to return to the lab to repeat the BCA assay experiments.

A)


BCA Assay Peripheral Blood Concentrations
Calculated Plasma Concentration (µg/mL)
350000
300000
250000
200000
150000
100000
50000
0
-50000
1:25 Dilution
1:250 Dilution

B)

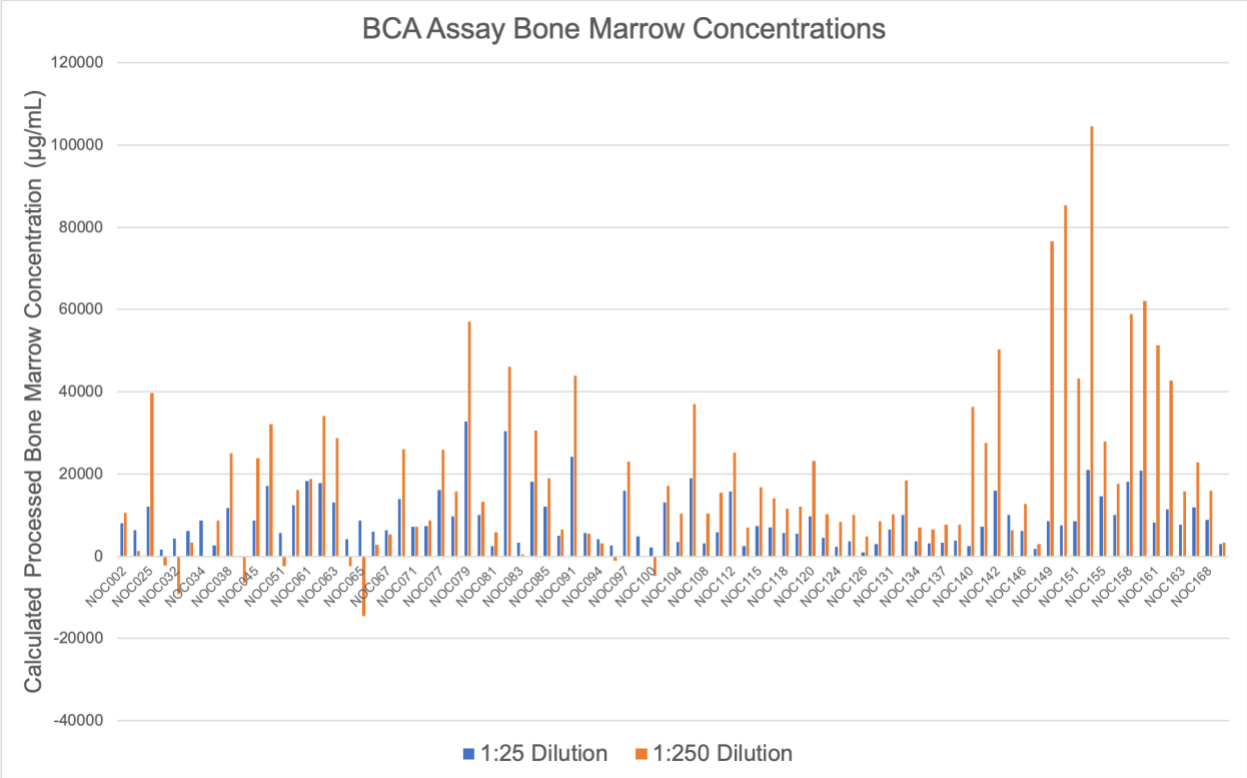

BCA Assay Bone Marrow Concentrations
Calculated Processed Bone Marrow Concentration (µg/mL)
120000
100000
80000
60000
40000
20000
0
-20000
-40000
1:25 Dilution
1:250 Dilution

Figure 1. Protein concentrations calculated based on BCA assay standards showed a great deal of variation both across samples and between samples of different dilution, likely due to pipetting errors. a) Peripheral blood sample concentrations based on the BCA assay showed a great deal of variation. **b)** Bone marrow sample concentrations showed even more variation, with several samples appearing to have negative concentrations.

3.4 Luminex cytokine analysis of peripheral blood samples

The Luminex cytokine assay was performed on a panel of 25 cytokines (24 multiplexed and 1 single panel): Angiopoietin-1, CCL3, CCL7, CCL11, CXCL12, FGF basic, Flt3 ligand, G-CSF, GM-CSF, IFNγ, IL-1β, IL-3, IL-6, IL-7, IL-8, IL-10, IL-11, IL-17, IL-18, LIF, M-CSF, SCF, Thrombopoietin, TNFα, and VEGF (Table 2). These cytokines were selected based on their known roles in HSC quiescence, regulation, and expansion, as well as on any previous studies showing that their expression may be dysregulated in CHIP, MDS, and AML.

Cytokine	Function
Angiopoietin-1	Critical enforcer of HSC quiescence (Arai et al., 2004). Overexpression of Ang1 in subset of AMLs may promote cell quiescence and drug resistance (Ichihara et al., 2011).
CCL3	Promotes myeloid differentiation and regulates size of HSC pool (Staversky et al., 2018). Stromal fibroblasts and macrophages in bone marrow of MDS patients have altered expression of CCL3 (Feng et al. 2011)
CCL7	Chemokine that attracts monocytes and regulates macrophage function during inflammation (Liu et al., 2018).
CCL11	Eosinophil chemoattractant that stimulates recruitment of eosinophils from blood to sites of allergic inflammation (Palframan et al., 1998). DNMT3A-mutant CHIP associated with elevated serum Eotaxin-1/CCL11 (Cook et al., 2017).
CXCL12	Essential regulator of HSC homing and anchoring in the niche

	(Schepers et al., 2015). Expression of CXCL12 is dysregulated in leukemia (Schepers et al., 2015; Ghobrial et al., 2018).
FGF basic	Growth and signaling factor which is expressed in the bone marrow and helps positively regulate hematopoiesis (Allouche and Bikfalvi, 1995). May mediate early therapeutic resistance in AML and assist in stromal protection of leukemia cells (Traer et al., 2016; Javidi-Sharifi et al., 2019)
Flt3 ligand	Regulates hematopoiesis by stimulating proliferation and differentiation of various blood cell progenitors (Tsapogas et al., 2017). Its receptor, Flt3, frequently carries activating mutations in AML (Tsapogas et al., 2017).
G-CSF	Induces HSC mobilization from bone marrow into bloodstream and initiates proliferation and differentiation of granulocytes (Basu et al., 2002). G-CSF has been used in the treatment of AML (Nomdedeu et al., 2015).
GM-CSF	Growth factor that stimulates stem cells to produce granulocytes and monocytes (Hercus et al., 2009). GM-CSF expression shown to be lower in AML and MDS patients than healthy controls (Kassem et al., 2018).
IFNγ	Pro-inflammatory cytokine critical for innate and adaptive immunity (Tau and Rothman, 1999). Shown to have dysregulated expression in bone marrow of MDS and leukemia patients (Kitagawa et al., 1997; Ghobrial et al., 2018). Zhang et al. (2019) showed in UC patients that serum IFNγ is significantly higher in DNMT3A+ CHIP patients.
IL-1β	Important mediator of inflammatory response, involved in cell proliferation, differentiation, and apoptosis (Ren and Torres, 2009). Shown to have altered expression in MSPCs of MDS and leukemia patients (Ghobrial et al., 2018). Aggregate data from Bick et al. (2020) found that CHIP is associated with increased IL-1β and TET2 CHIP carriers had more significantly increased IL-1β.
IL-3	Stimulates the differentiation of HSCs into myeloid and lymphoid progenitor cells and stimulates proliferation of cells in myeloid lineage (Hercus et al., 2013). Its receptor, IL-3R, is shown to be overexpressed in AML (Testa et al., 2004).
IL-6	Acts as both a pro-inflammatory and anti-inflammatory cytokine (Tanaka et al., 2014). Shown to have altered expression in MSPCs of MDS and leukemia patients (Ghobrial et al., 2018). TET2-mutant

	CHIP is associated with overproduction of IL-6 (Cook et al., 2017; Cai et al., 2018) which promotes mutant HSC expansion and accelerates atherosclerosis
IL-7	Stimulates differentiation of HSCs into lymphoid progenitor cells (ElKassar and Gress, 2010). Known to promote hematological malignancies like acute lymphoblastic leukemia and T cell lymphoma (Or et al., 1998).
IL-8	Important mediator of immune reaction in innate immune system response by inducing target cells to migrate towards site of infection (Bickel, 1993). High expression of IL-8 correlated with poor prognosis in certain subsets of AML (Kuett et al., 2015). Jaiswal et al. (2017) showed that plasma IL-8 levels are significantly elevated in patients with TET2-mutant CHIP than in controls.
IL-10	Anti-inflammatory cytokine involved in immunoregulation and inflammation (Iyer and Cheng, 2012). Secreted by dysregulated stromal cells in the bone marrow tumor microenvironment to regulate innate and adaptive immune responses (Ghobrial et al., 2018).
IL-11	Can act as both an anti-inflammatory and pro-inflammatory cytokine and is associated with adipogenesis, osteoclastogenesis, neurogenesis and platelet maturation (Xu et al., 2016). Increased IL-11 signaling may also play a key role in various cancers.
IL-17	Cytokine which induces and mediates pro-inflammatory response (Zenobia and Hajishengallis, 2000). Expression dysregulated in many autoimmune and inflammatory diseases.
IL-18	Pro-inflammatory cytokine that facilitates adaptive immune response and is able to induce severe inflammatory reactions (Dinarello et al., 2013). Overexpression of IL-18 may mark poor prognosis in AML (Zhang et al., 2002). Bick et al. (2020) found that carriers of JAK2 and SF3B1 CHIP mutations had significantly increased IL-18.
LIF	Plays important role in leukemia cell differentiation, inflammatory response, stem cell self-renewal, and other biological processes (Yue et al., 2015).
M-CSF	Induces differentiation of HSCs into macrophages (Hamilton and Achuthan, 2013). M-CSF shown to have high expression in AML patients (Wang et al., 2018).
SCF	Plays a critical role in HSC quiescence and maintenance (Schepers et

	al., 2015). Decreased expression of SCF and other HSC-supportive factors by stromal cells may promote leukemia.
Thrombopoietin	Enforcer of HSC quiescence (Schepers et al., 2015).
TNFα	Important for HSC survival and myeloid regeneration (Idriss and Naismith, 2000). Analyses of young/aged mouse HSCs and human MDS and AML samples showed enrichment for pro-survival HSC-specific TNFα signature genes (Yamashita and Passegué, 2019). Both TET2- and DNMT3A-mutant CHIP are linked to elevated serum TNFα (Cook et al., 2017).
VEGF	Pro-angiogenic factor promoting growth of new blood vessels (Shibuya, 2011). Production of VEGF is altered in MSPCs of MDS and leukemia patients (Ghobrial et al., 2018).

Table 2. 25 cytokines were selected for Luminex panels based on their roles in HSC regulation and inflammatory signaling, as well as evidence of dysregulation in prior CHIP, MDS, and AML studies.

I began by performing the Luminex assays on the 82 peripheral blood samples I had selected. The Luminex analyzer measured the fluorescence levels for each unique tagged cytokine and calculated cytokine concentration based on diluted standards. Because many of these expression values were out of range based on the standards, I opted to analyze the Luminex assay data by using fluorescence levels as representative of relative expression of each cytokine.

I first compared expression level differences between all CHIP and control samples for each cytokine by Mann-Whitney test. I used this non-parametric test because CHIP and control sample groups were not normally distributed (normality was tested for using the Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests). I found that TNFα ($p=0.0297$), CCL7 ($p=0.0381$), and IL-8 ($p=0.0100$) were all significantly overexpressed in CHIP samples compared to controls, while FGF-basic was significantly downregulated ($p=0.0375$) (Figure 2). However, it is important to note that these differences in expression were

no longer significant after Bonferroni multiple-test correction, where p must be less than 0.002 because n=25 (25 cytokines tested and analyzed).

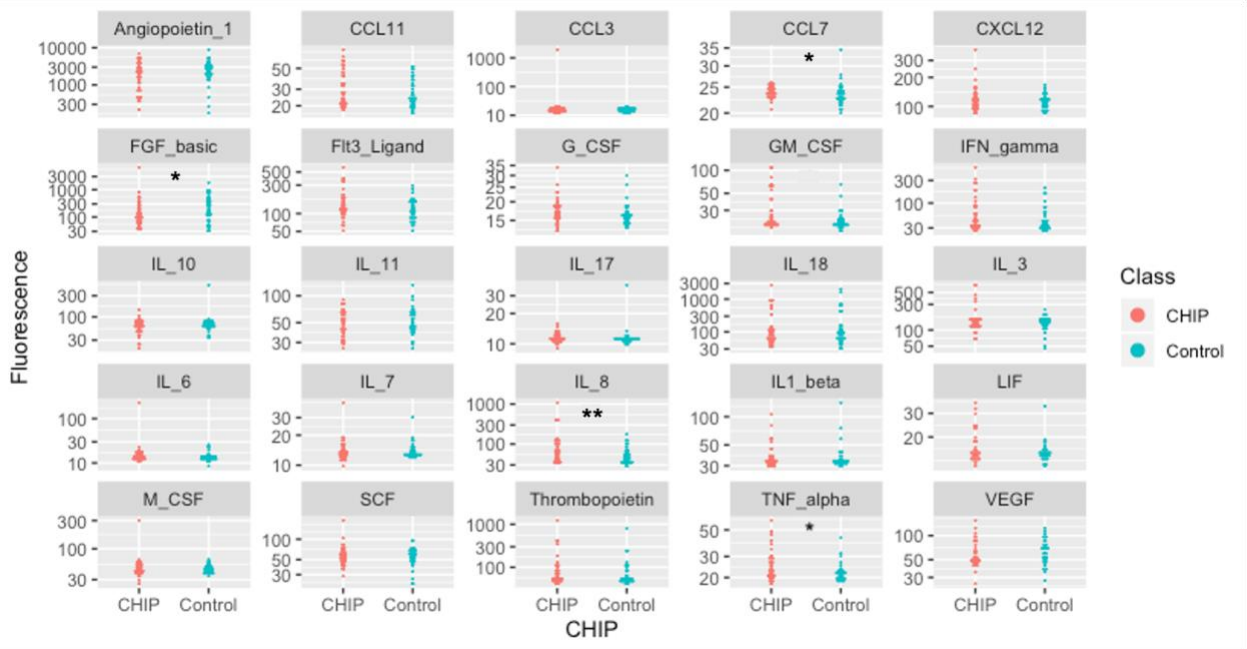


Figure 2. Luminex assay on panel of 25 cytokines showed that TNFα, CCL7, and IL-8 were significantly elevated in CHIP samples compared to controls, while FGF-basic was significantly downregulated. These differences were not significant after Bonferroni multiple test correction.

In addition to comparing all CHIP samples to controls, I also analyzed several subcategories of CHIP samples in comparison to controls to determine whether any characteristics of CHIP mutant clones resulted in uniquely dysregulated cytokine expression. I compared expression levels of DNMT3A- and TET2-mutant CHIP samples against controls to test for mutation-specific cytokine effects using the Mann-Whitney test (Figure 3). I found that TNFα was significantly upregulated in TET2-mutant CHIP samples compared to controls (p=0.0363). However, this difference was not significant after Bonferroni multiple-test

correction. No cytokines were significantly differentially expressed in DNMT3A-mutant CHIP samples compared to controls (Supplementary Figure 1).

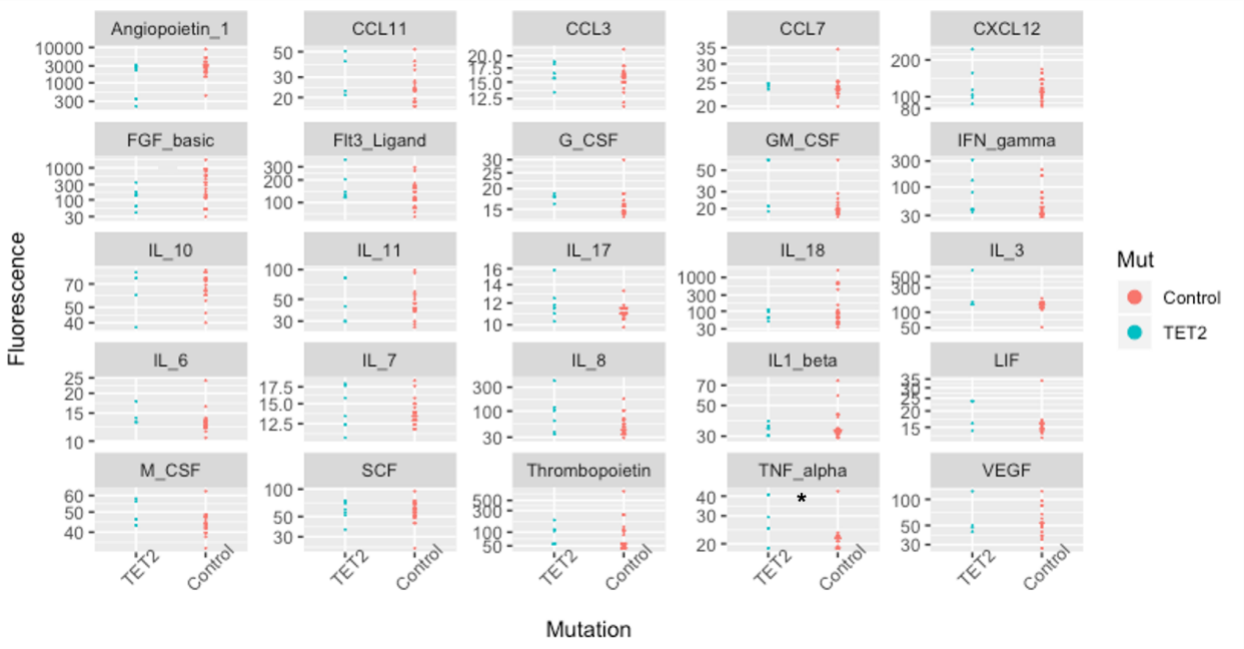


Figure 3. Luminex assay analysis showed that TNFα was significantly elevated in CHIP samples carrying TET2 mutations compared to controls. This difference was not significant after Bonferroni multiple test correction.

I also looked for mutation number effects by comparing cytokine expression levels of CHIP samples with either 1 mutation or >1 mutation against controls using the Mann-Whitney test (Figure 4). I found that TNFα (p=0.0295), CCL7 (p=0.0225), IL-8 (p=0.0462) and IFNγ (p=0.0379) were significantly upregulated in CHIP samples with multiple mutations compared to controls. However, this was also not significant after Bonferroni multiple-test correction.

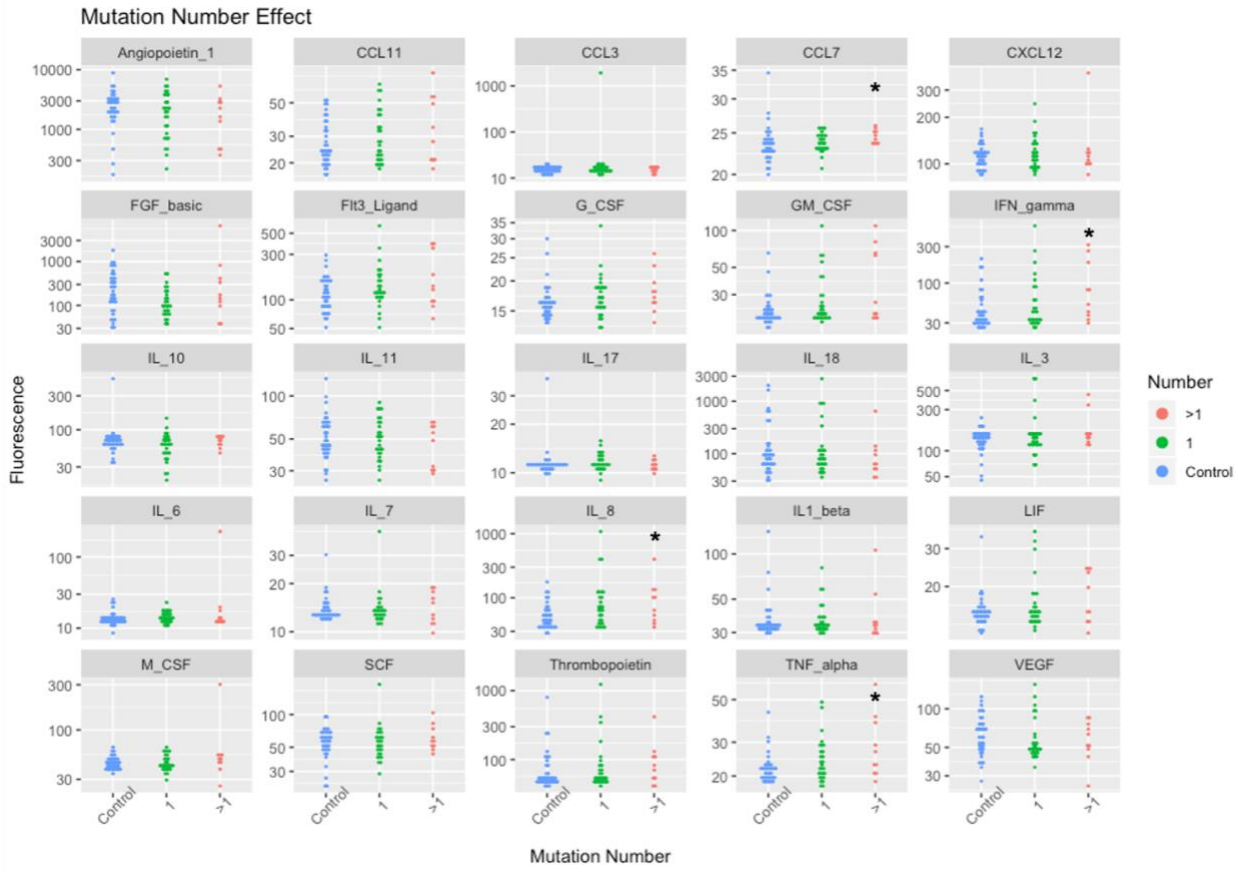
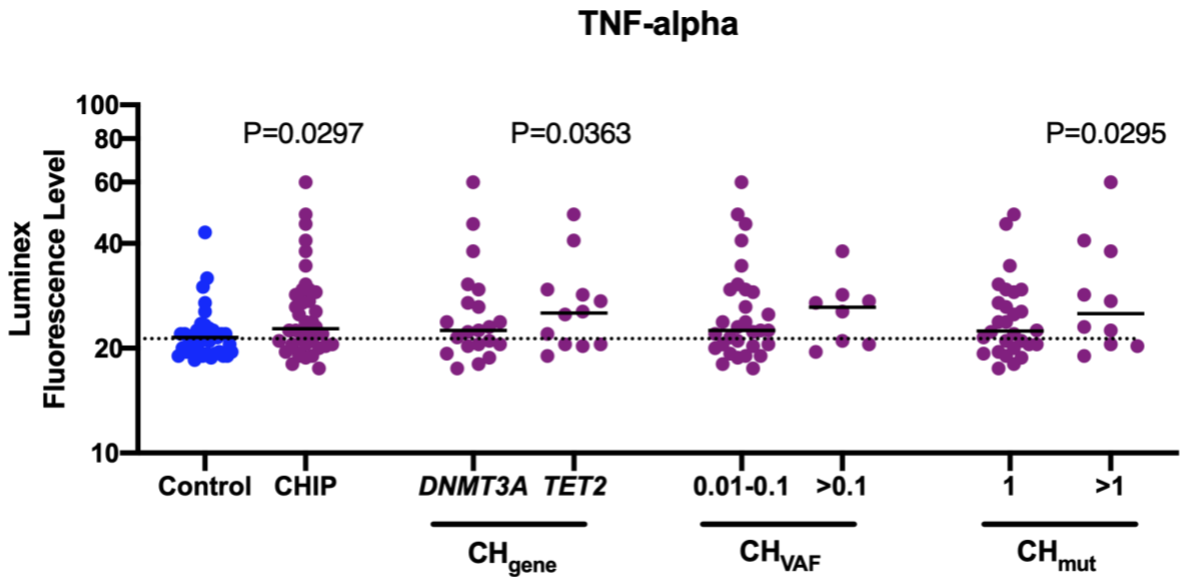


Figure 4. Luminex assay analysis showed that TNFα, IFNγ, IL-8 and CCL7 were significantly elevated in CHIP samples carrying multiple mutations. These differences were not significant after Bonferroni multiple test correction.

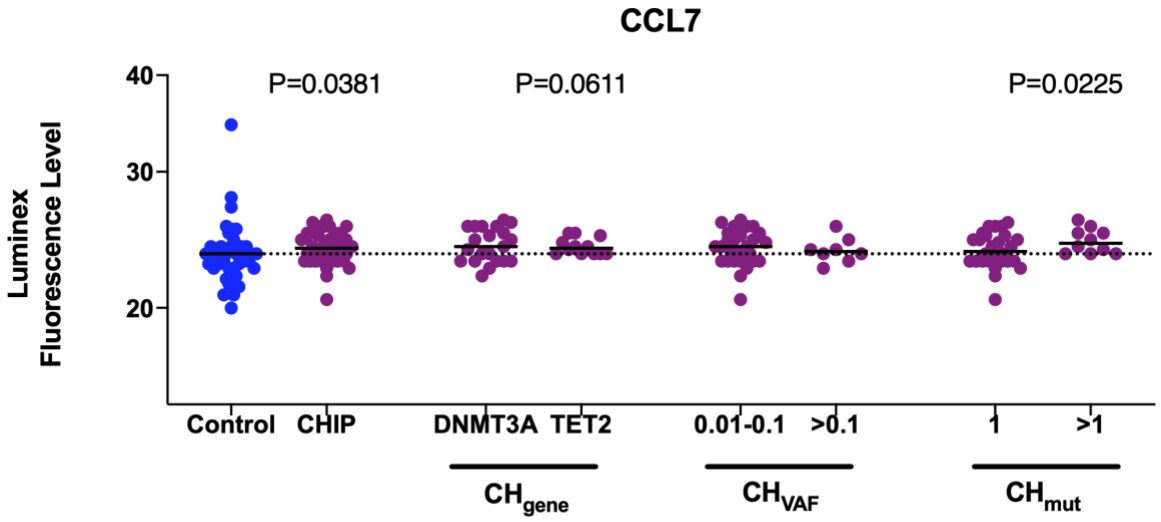
Finally, I looked for VAF size effects by splitting CHIP samples up based on VAF ≥0.1 (large) and VAF 0.01-0.1 (small) and compared cytokine expression levels of these grouped samples against controls using the Mann-Whitney test. No cytokines were significantly differentially expressed between controls and samples with either large or small VAF (Supplementary Figure 2).

More detailed figures for each of these most significantly dysregulated cytokines and how their expression varies across patients with CHIP and subcategories of CHIP samples can be found below (Figure 5a-e).

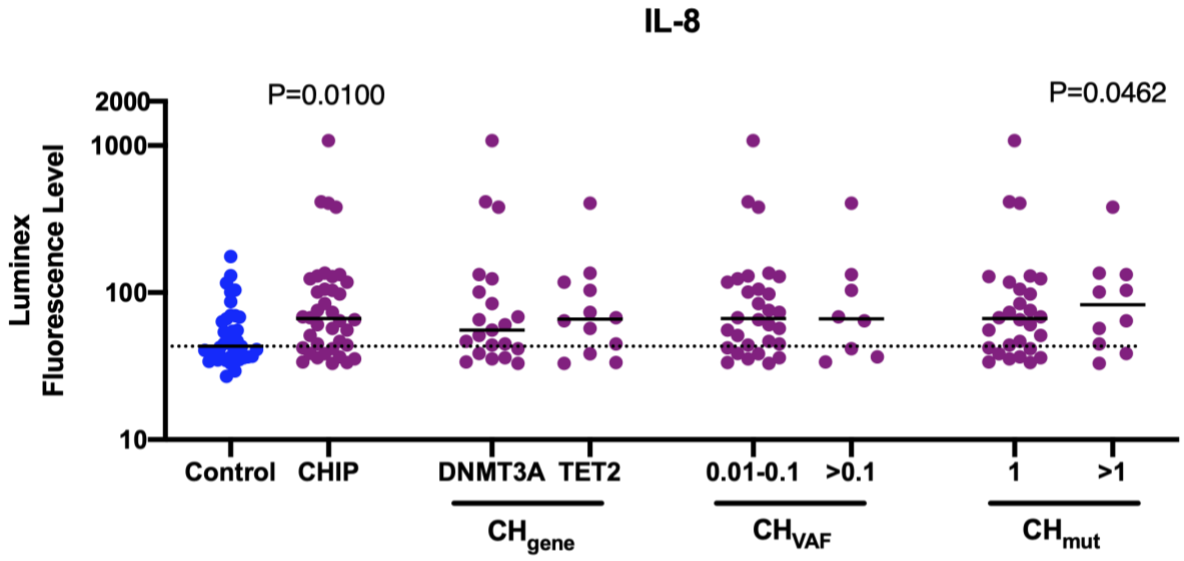
A)



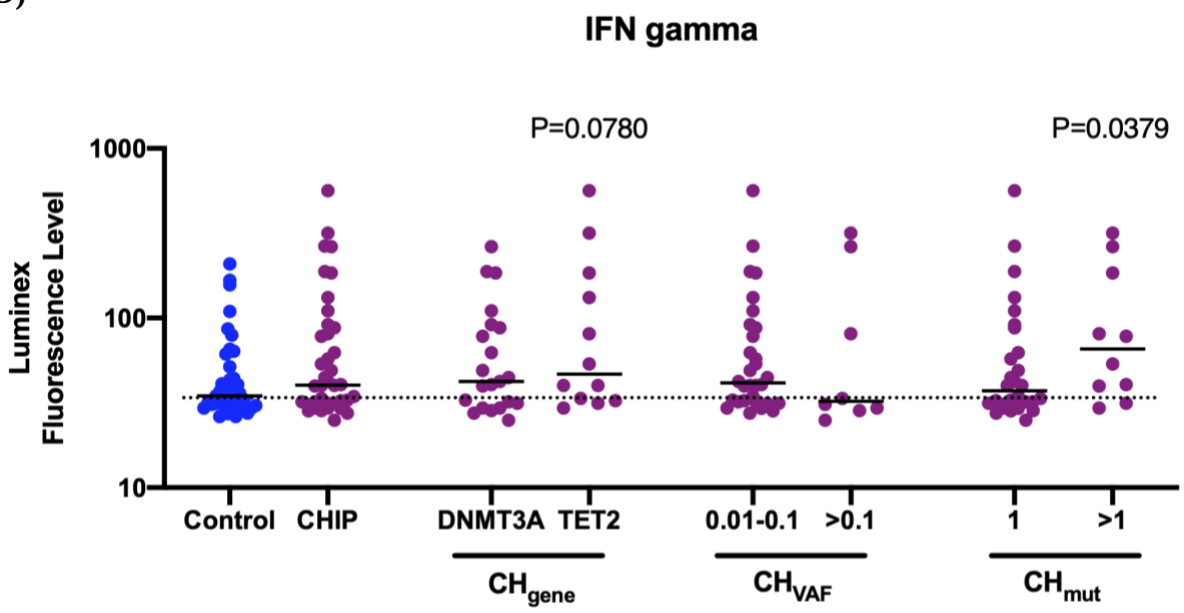
B)



C)


IL-8
P=0.0100
P=0.0462
2000
1000
100
10
Luminex Fluorescence Level
Control
CHIP
DNMT3A
TET2
0.01-0.1
>0.1
1
>1
CHgene
CHVAF
CHmut

D)


IFN gamma
P=0.0780
P=0.0379
1000
100
10
Luminex Fluorescence Level
Control
CHIP
DNMT3A
TET2
0.01-0.1
>0.1
1
>1
CHgene
CHVAF
CHmut

E)

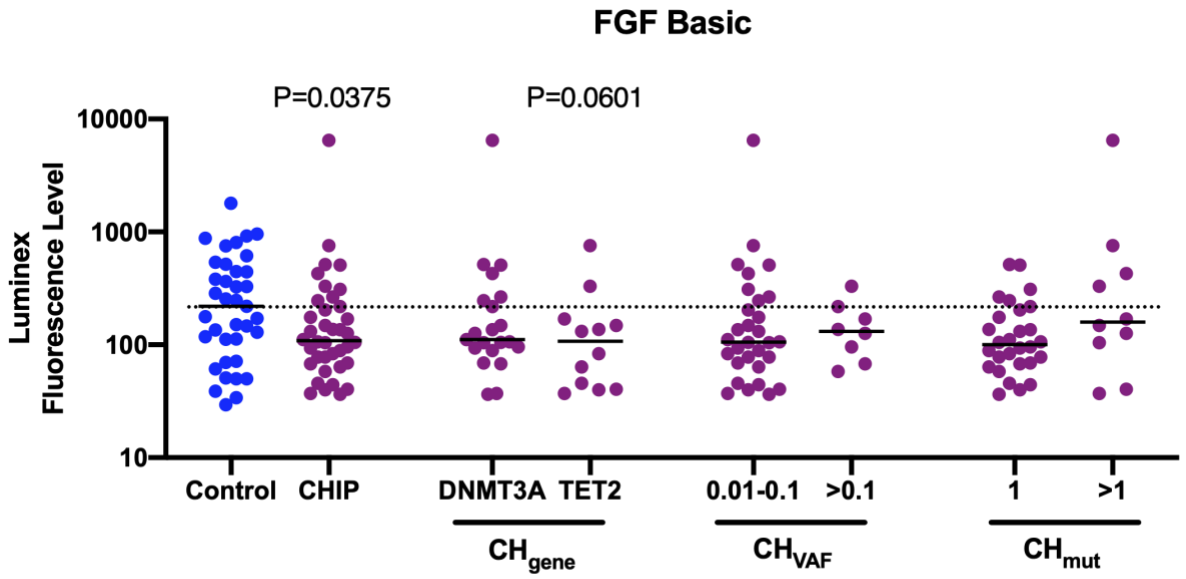


Figure 5. More detailed view of how TNFα, CCL7, IL-8, IFNγ, and FGF-basic are dysregulated in CHIP. a) TNFα is upregulated across CHIP and CHIP subcategories compared to controls, with the most significant difference being between overall CHIP and controls, TET2 CHIP compared to controls, and patients carrying multiple CHIP mutations (mut >1) compared to controls. **b)** CCL7 is most significantly upregulated in CHIP samples compared to controls, as well as mut >1 samples compared to controls. **c)** IL-8 is upregulated across CHIP and CHIP subcategories compared to controls, with the most significant difference being between overall CHIP and controls, and mut >1 samples vs controls. **d)** IFNγ is upregulated across CHIP and CHIP subcategories compared to controls, with the most significant difference being between mut >1 samples vs controls. **e)** FGF-basic is downregulated across CHIP and CHIP subcategories compared to controls, with the most significant difference being between overall CHIP and controls.

3.5 RNA-Seq analysis of cytokine and receptor expression in healthy and leukemic patients

Following analysis of the Luminex cytokine assay, I was interested in connecting my findings to RNA-Seq data to determine which cell types express or have receptors for these cytokines and whether this differs for patients with hematologic malignancies. Doing so might provide insight into how the body and bone marrow microenvironment either respond to or

support the aberrant growth of mutant HSC clones in CHIP and advanced hematologic malignancies. To do this, I used RNA-Seq data previously collected by Zahra Aboukhalil, a former graduate student in the Vyas Lab. Zahra had performed RNA-Seq on peripheral blood samples collected from 3 healthy patients and 17 patients with AML. In the healthy patients, Zahra had sorted and sequenced the RNA of different blood progenitors: hematopoietic stem cells (HSCs), multipotent progenitors (MPPs), lymphoid-primed multipotent progenitors (LMPPs), multi-lymphoid progenitors (MLPs), common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMP), megakaryocyte/erythrocyte progenitors (MEPs) (Figure 11). In the leukemic patients, Zahra had sorted and sequenced LMPPs and GMPs, progenitors which have been shown to make up the leukemic stem cell population in AML (Goardon et al., 2011). In deciding on which cytokines and their receptors to analyze the expression of in the RNA-Seq data, I included all cytokines with $p<0.05$ for overall CHIP vs Control comparison, as well as for subgroup comparisons. These cytokines were TNFα (overexpressed in overall CHIP, p=0.0297; overexpressed in TET2 CHIP, p=0.0363; overexpressed in CHIP with multiple mutations, p=0.0295), IL-8 (overexpressed in CHIP, p=0.0100), CCL7 (overexpressed in overall CHIP, p=0.0381; overexpressed in CHIP with multiple mutations, p=0.0225), FGF-basic (downregulated in CHIP, p=0.0375) and IFNγ (overexpressed in CHIP with multiple mutations, p=0.0379). Although none of these cytokines were significant after Bonferroni multiple test correction, I treated these RNA-Seq analyses as exploratory to establish grounds for future cytokine investigations in CHIP.

To broaden the scope of analysis, I also included all cytokines for which the difference of expression levels was $p<0.1$ for either broad CHIP or subgroup analysis (Supplementary Figure

3). GM-CSF (p=0.0521) was all overexpressed in CHIP compared to control samples (not significant). IFNγ (p=0.0780), LIF (p=0.0774), and CCL7 (p=0.0611) were overexpressed in TET2 CHIP samples compared to controls, while FGF-basic was downregulated in TET2 CHIP samples (p=0.0601) (not significant). GM-CSF (p=0.0647) was overexpressed in CHIP samples with more than 1 mutation relative to controls (not significant).

In addition to investigating the expression of these cytokines in this RNA-Seq dataset of blood progenitors, I also aimed to look at expression of their receptors to see how their dysregulated expression might affect progenitor function and proliferation in healthy and leukemic patients (Table 3). Cytokines GM-CSF and CCL7, and receptor CXCR1 were not present in the RNA-Seq data, so analyses were performed on the mRNA of a total of 16 cytokines and receptors.

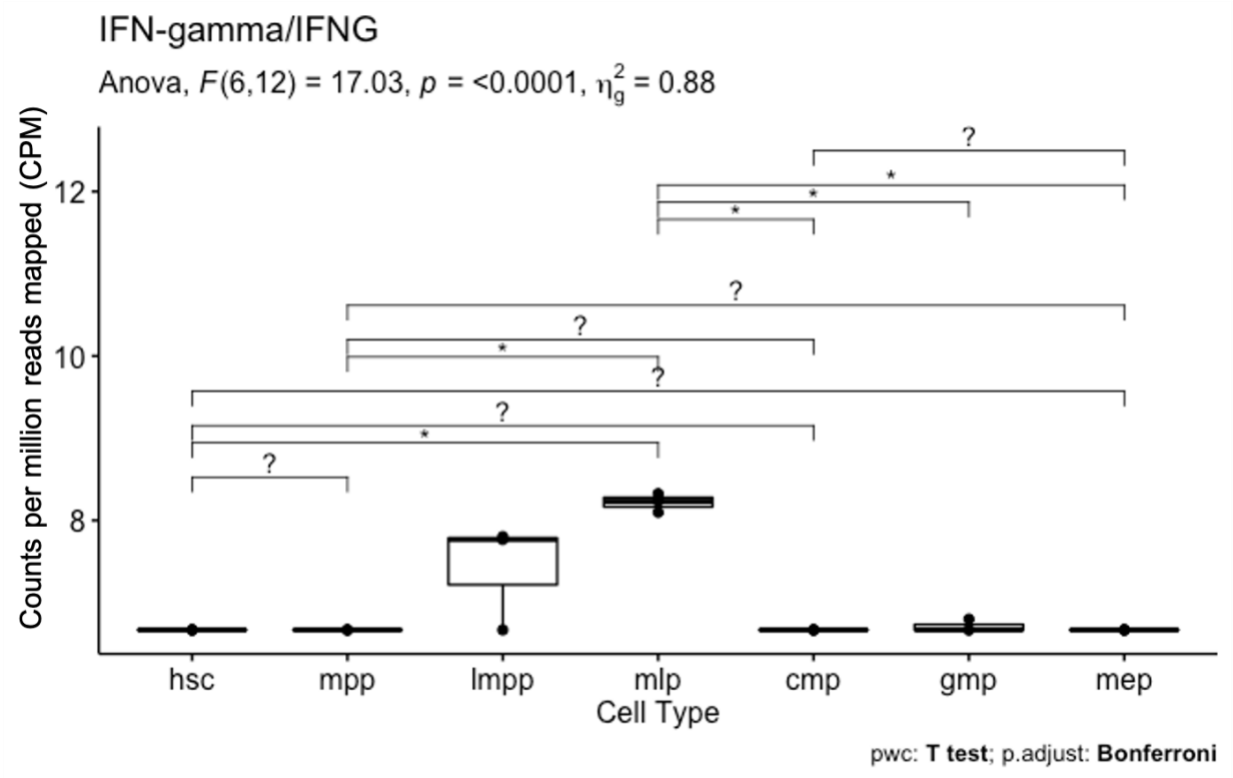
Cytokines of Interest	Receptor(s)
CCL7	CCR1, CCR2, and CCR3 (Liu et al., 2018)
FGF basic	FGFR1 (Ornitz and Itoh, 2015)
GM-CSF/CSF2	GM-CSF receptor/CD116 (Hercus et al., 2009)
IFNγ	IFNGR1 and IFNGR2 (Tau and Rothman, 1999)
IL-8	CXCR1 and CXCR2 (Brat et al., 2005)
LIF	LIFR (Cullinan et al., 1996)
TNFα	TNFR-1 and TNFR-2 (Idriss and Naismith, 2000)

Table 3. mRNAs of cytokines (and their receptors) were selected for RNA-Seq analysis if the p-value of the Mann-Whitney test measuring their dysregulation in CHIP was <0.1. CCL7, GM-CSF, and CXCR1 were not included in analysis because they were missing from the RNA-Seq data.

I began by performing one-way repeated measures ANOVAs on the sequencing data of blood progenitor cells from normal, non-leukemic patients. Repeated measures ANOVA was important to track differences in expression across the same patient for a more accurate assessment of expression differences. Because expression of 16 mRNAs for cytokines and receptors was analyzed, ANOVAs would need to have $p<0.003$ to be significant after Bonferroni multiple test correction. For each ANOVA test which indicated significance variance (either before or after Bonferroni correction), I conducted post-hoc tests to determine which cell types have expression levels that are significantly different from other cell types. In the R-generated plots, post-hoc comparisons were automatically only labeled as significant if they remained significant after Bonferroni correction.

ANOVA testing showed that IFNγ expression was significantly different among healthy progenitor cells ($p<0.0001$, remains significant after Bonferroni correction) and post-hoc tests showed that MLPs have significantly higher expression of IFNγ than all other progenitor cell types except LMPPs (Figure 6a). These post-hoc differences were significant after Bonferroni correction. TNFR-2 (receptor for TNFα) also showed significant difference in expression among progenitors in ANOVA testing ($p=0.011$), although this ANOVA difference is not significant after Bonferroni correction (Figure 6b). Post-hoc tests on TNFR-2 expression showed that MLPs significantly overexpress TNFR-2 compared to CMPs and MEPs.

A)


IFN-gamma/IFNG
Anova, F(6,12) = 17.03, p = <0.0001, η²g = 0.88
Counts per million reads mapped (CPM)
12
10
8
hsc
mpp
lmpp
mlp
cmp
gmp
mep
Cell Type
pwc: T test; p.adjust: Bonferroni

B)

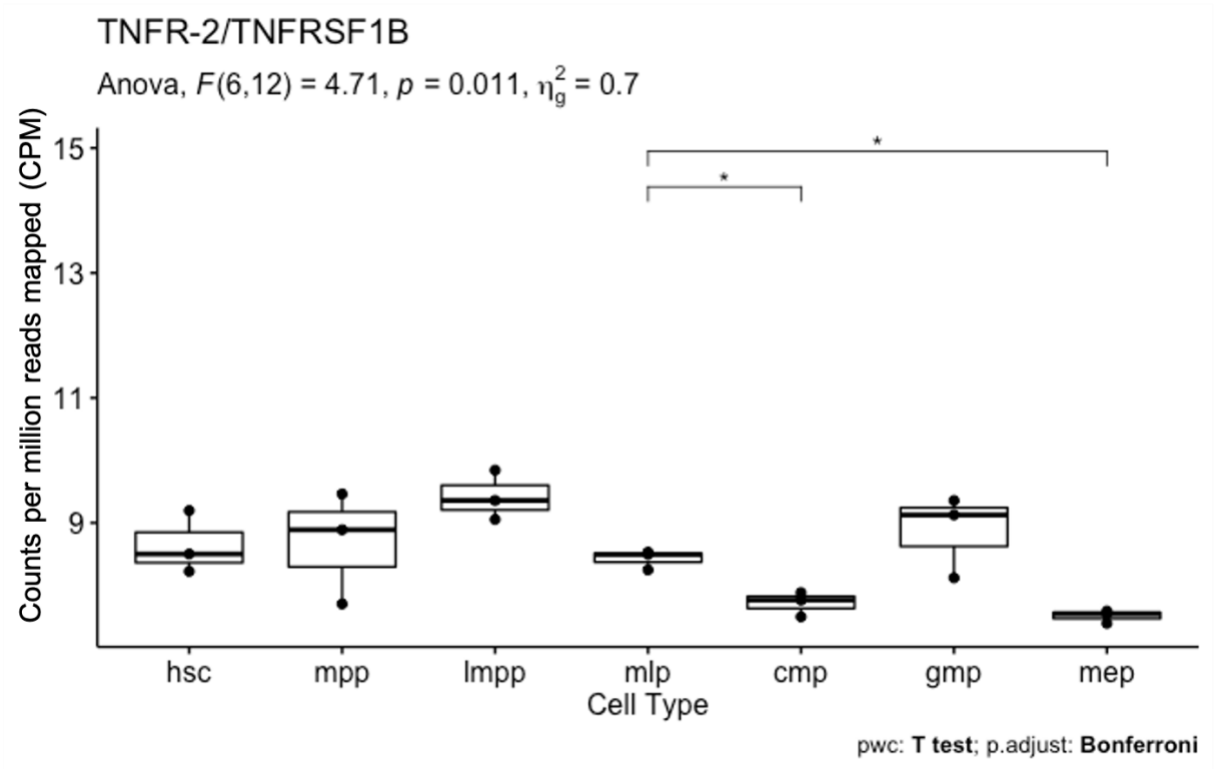
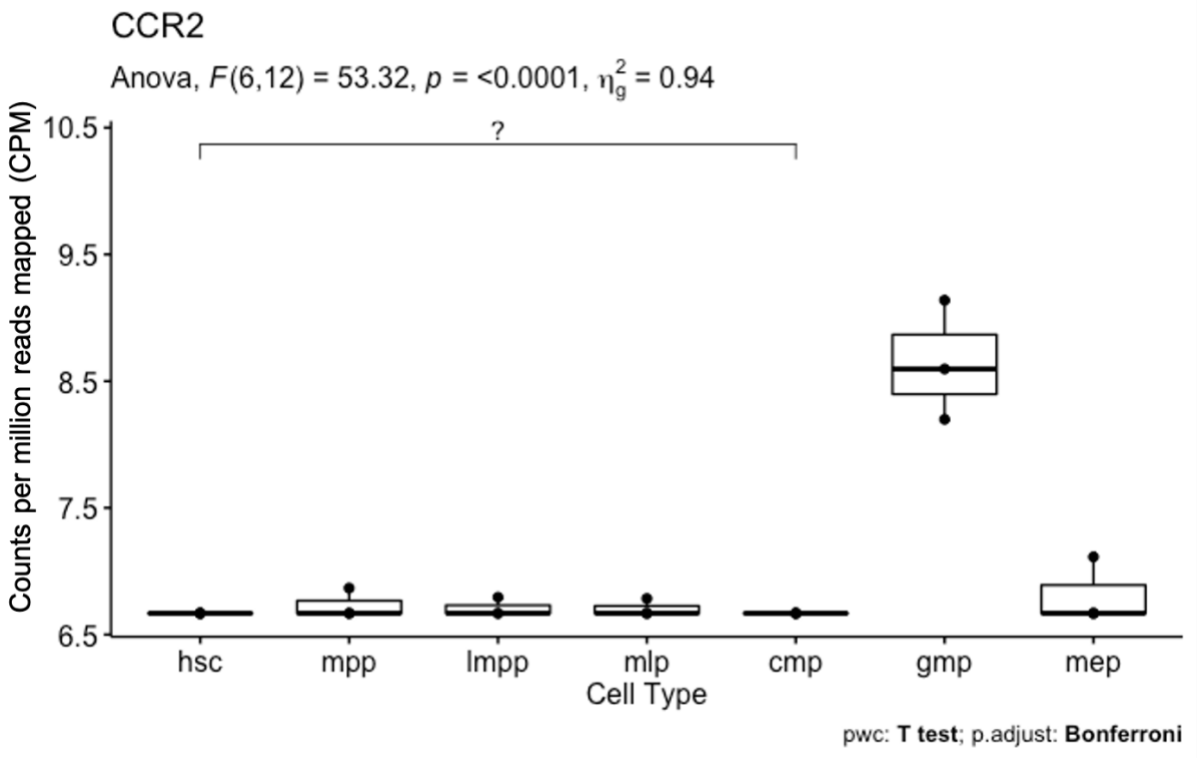

TNFR-2/TNFRSF1B
Anova, F(6,12) = 4.71, p = 0.011, η²g = 0.7
Counts per million reads mapped (CPM)
15
13
11
9
hsc
mpp
lmpp
mlp
cmp
gmp
mep
Cell Type
pwc: T test; p.adjust: Bonferroni

Figure 6. IFNγ and TNFR-2 were significantly differentially expressed between at least two blood progenitor types. a) IFNγ had a significant ANOVA test, even after Bonferroni correction. Post-hoc testing showed that MLPs significantly overexpressed IFNγ compared to every cell type except LMPPs. Question marks in graph are because R was unable to run statistical test on cell types that had the same expression value resulting from a cut-off imposed during initial analysis. **b)** TNFR-2 had a significant ANOVA test, although this was not significant after Bonferroni correction. Post-hoc testing showed that MLPs significantly overexpressed TNFR-2 compared to CMPs and MEPs.

In addition, CCR2 (receptor for CCL7) ($p<0.0001$) and TNFR-1 (receptor for TNFα) ($p=0.002$) had significant ANOVAs but their post-hoc tests were not significant after Bonferroni correction (Figures 7a-b). However, the ANOVA plot for CCR2 expression across cell types suggests that CCR2 is more expressed in GMPs than other cell types, albeit not significantly (Figure 7a).

A)



B)

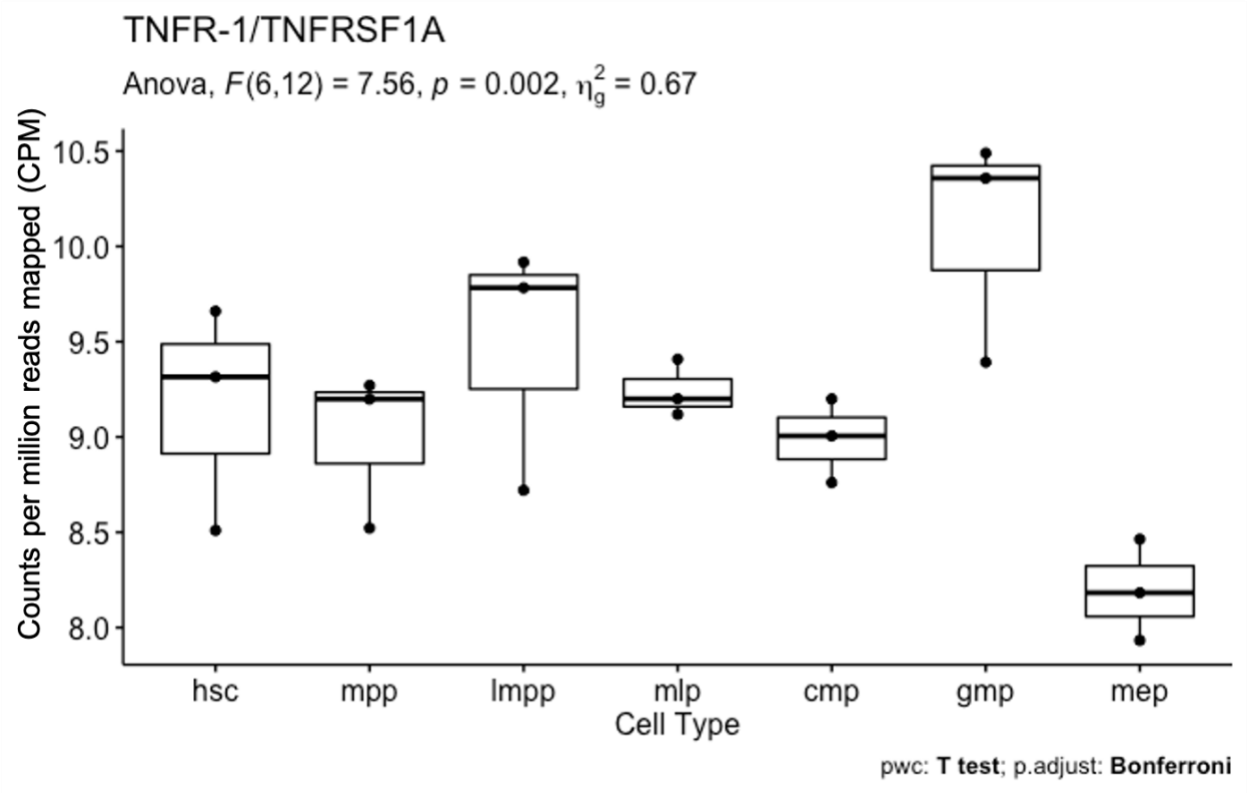


Figure 7. CCR2 and TNFR-1 were significantly differentially expressed based on ANOVA, even after Bonferroni correction, but post-hoc tests were not significant. a) Plot suggests that GMPs overexpress CCR2 relative to all other cell types, although this is not significant. Question mark in graph is because R was unable to run statistical test on cell types that had the same expression value resulting from a cut-off imposed during initial analysis. **b)** Plot shows similar pattern of expression as TNFR-2, with highest expression in LMPPs, MLPs, and GMPs.

Finally, there were several cytokines for which the ANOVA tests were in the significant range but not after Bonferroni correction, and whose post-hoc tests were also not significant after Bonferroni correction. These included FGFR1 (p=0.023), IFNGR1 (p=0.014), and LIFR (p=0.013) (Supplementary Figure 4).

In addition to one-way repeated measures ANOVAs on healthy progenitors, I also performed mixed (repeated measures two-way) ANOVAs comparing gene expression in GMPs and LMPPs between normal patients and patients with AML. Similar to the one-way ANOVA, I treated expression values from each patient replicate as paired values (patient 14 was removed because they had no GMP expression values). Through mixed ANOVAs, I was able to determine how expression varied between normal and leukemic GMPs and LMPPs. Importantly, mixed ANOVAs allowed me to determine the interaction effect between leukemia and cell type—that is, how having AML might uniquely affect cytokine expression depending on cell type. For each cytokine, if the mixed ANOVA indicated a significant interaction effect, I performed a simple main effect test, which is a one-way model to investigate the effect of malignancy on expression for each cell type. If the simple main effect was significant, I performed post-hoc tests to determine how cytokine or receptor expression varied based on malignancy status and cell type.

CCR2 was the only protein for which the mixed ANOVA showed significant interaction between cell type and malignancy, even after Bonferroni adjustment ($p<0.0001$) (Figure 8). After finding that the simple main effect was significant, post-hoc testing showed that normal GMPs significantly overexpress CCR2 compared to leukemic GMPs, while there is no difference between normal and leukemic LMPPs. This suggests that expression of CCR2, the receptor to CCL7, is unique to GMPs in healthy patients and that this expression is lost in leukemia.

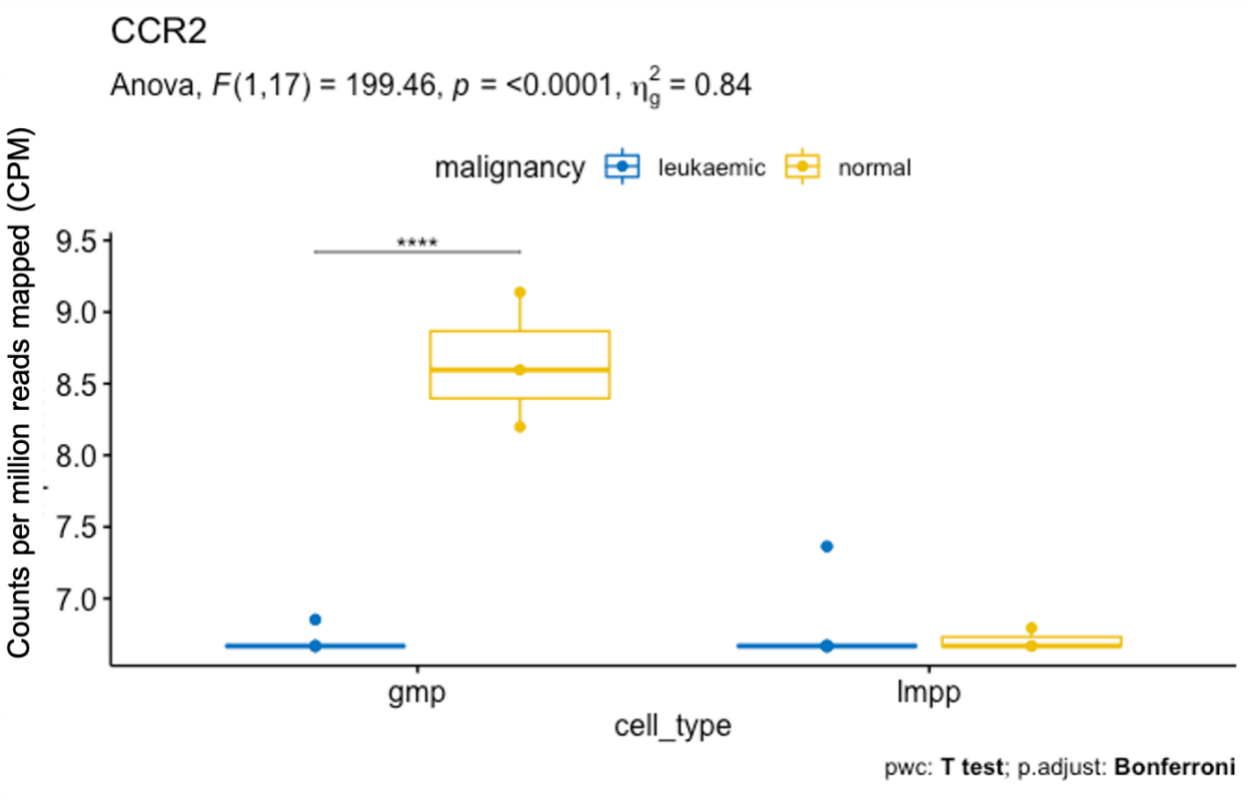


Figure 8. CCR2 expression showed significant interaction between malignancy status and cell type, even after Bonferroni correction. Post-hoc testing showed that normal GMPs significantly overexpress CCR2 compared to leukemic GMPs, while normal and leukemic LMPPs have similar CCR2 expression.

IFNγ (p=0.007) showed significant interaction between cell type and malignancy, although this was not significant after Bonferroni correction (Figure 9). However, I proceeded with analysis and found that the simple main effect was significant for both proteins. Post-hoc testing showed that while normal LMPPs overexpress IFNγ compared to leukemic LMPPs, there is no such difference between normal and leukemic GMPs. This suggests that expression of IFNγ is unique to LMPPs in healthy patients and that this is lost in leukemia.

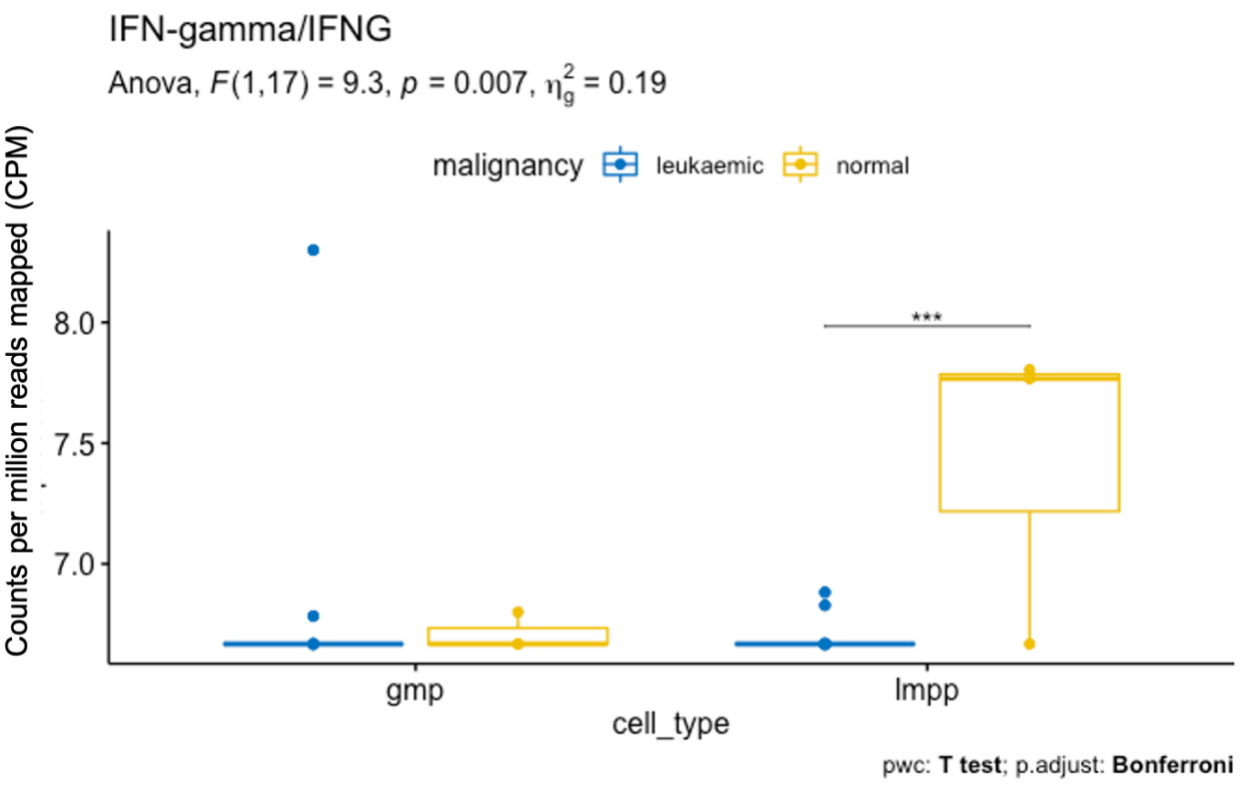


Figure 9. IFNγ expression showed significant interaction between malignancy status and cell type, although this was no longer significant after Bonferroni correction. Post-hoc testing showed that normal LMPPs significantly overexpress IFNγ compared to leukemic LMPPs, while normal and leukemic GMPs have similar IFNγ expression.

TNFR-2 (receptor to TNFα) was overall more expressed in normal cells than leukemic cells, though this was not significant after Bonferroni adjustment (Figure 10).

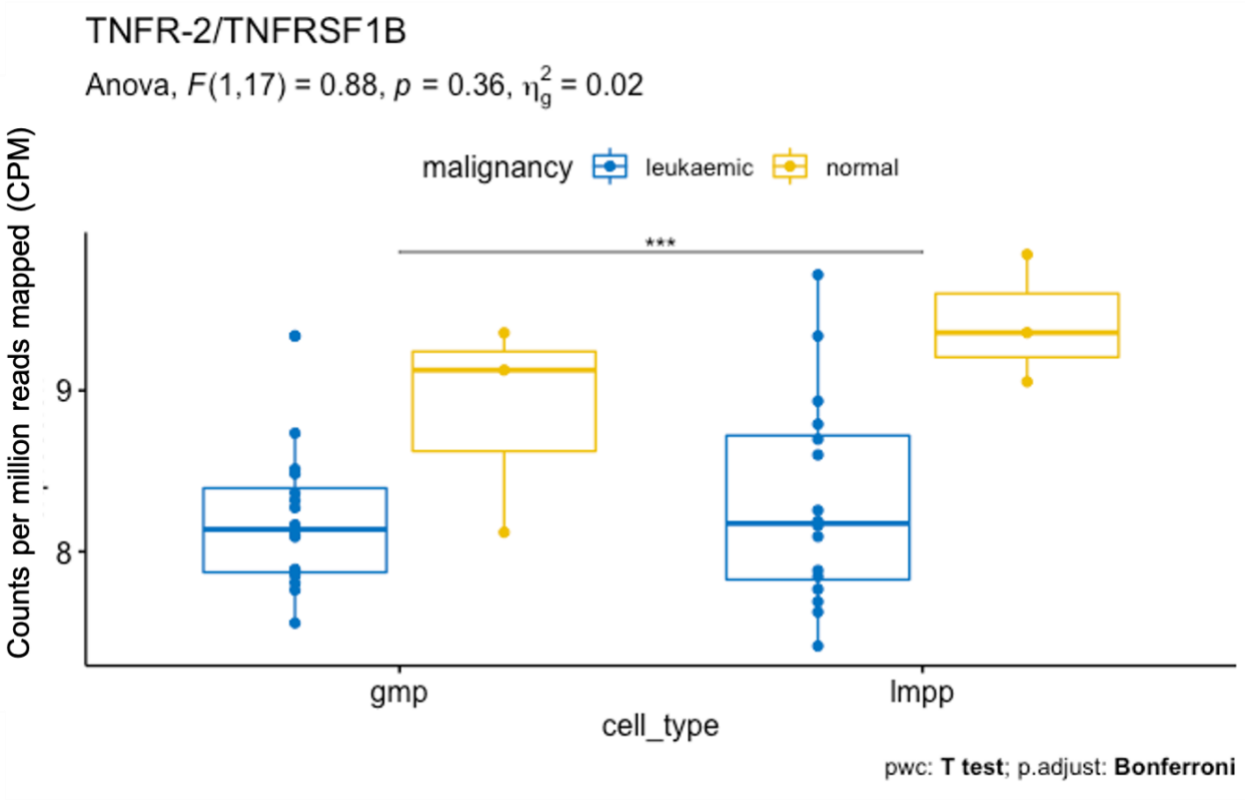


Figure 10. TNFR-2 expression showed a significant leukemic effect. Normal GMPs and LMPPs overall express TNFR-2 relative to their leukemic counterparts overall.

4) Discussion and Future Directions

In this thesis, I aimed to improve our understanding of how signaling in the bone marrow microenvironment could contribute to the expansion of hematopoietic stem cell (HSC) mutant CHIP clones. I performed Luminex cytokine assays on a panel of 25 cytokines to determine how their expression is dysregulated in patients with CHIP. In my analyses, I focused on how expression differed between CHIP patients and controls. I also further split CHIP samples in several subcategories (mutated gene, VAF size, and number of mutations) to determine if there were any unique CHIP subcategory effects. Through these analyses, I found five cytokines to be differentially expressed in CHIP, although this was not significant after Bonferroni multiple test correction. After identifying several cytokines of interest based on the Luminex experiments, I analyzed previously collected RNA-Seq data on normal and leukemic blood progenitor cells to determine how expression of these cytokines and their receptors varies among progenitors. I was able to identify a few cytokines and receptors that were differentially expressed in certain blood progenitor cells, as well as a few whose expression varied between normal and leukemic patient progenitors.

4.1 Luminex data

The first stage of this project involved performing Luminex cytokine assays to see how expression levels of different cytokines vary between patients with CHIP and healthy controls. By determining the degree of cytokine dysregulation in CHIP samples, I hoped to gain insight on how the bone microenvironment is modified in patients with CHIP, both in reaction to the

aberrant expansion of mutant clones and also to support the expansion of these clonal populations.

Prior to conducting the Luminex assays, I performed BCA protein quantification assays to calculate the concentration of total protein in the 82 samples selected for Luminex analysis. However, as I noted in the results section, due to the small volumes which were pipetted to dilute the samples into the BCA assay range of detection, the concentrations observed were highly variable and most likely inaccurate. I proceeded to carry out the Luminex experiments with the goal of later repeating the BCA assays with more accurate dilutions. However, due to the COVID-19 pandemic and the travel restrictions and shelter-in-place orders that resulted from the pandemic, I was unable to return to the lab to repeat the BCA assay experiment. Thus, it should be noted that an important limitation to interpreting the results of the Luminex assays is that none of the expression levels have been normalized to total protein concentrations. Furthermore, while I had originally intended to carry out Luminex experiments on both peripheral blood and bone marrow samples, I was unable to perform the bone marrow experiments due to the COVID-19 pandemic. Therefore, my Luminex analyses will focus solely on peripheral blood data. As previously noted, the peripheral blood samples collected were blood plasma, rather than serum. Plasma and serum are two types of blood samples that can be obtained via different methods of processing blood. Importantly, plasma and serum can yield different cytokine levels due to these distinct methods. It has not been definitively shown that either serum or plasma is better for measuring cytokine levels, and this may in fact differ based on the cytokine being measured (Guo et al., 2013; Gruen et al., 2016). Furthermore, some prior studies of dysregulated cytokine expression in CHIP have looked at serum samples rather than plasma (Cook et al., 2017; Zhang

et al., 2019). Thus, the results from the Luminex experiment should be interpreted with the caveat that cytokine levels in the samples may not reflect physiological levels due to the method of blood processing.

In my Luminex analysis, I found that three cytokines were significantly overexpressed in patients with CHIP compared to controls: TNFα, CCL7, and IL-8. I also found that TNFα was uniquely overexpressed in patients with TET2 CHIP mutations, and that TNFα, CCL7, IFNγ, and IL-8 were overexpressed in patients carrying multiple CHIP mutations compared to controls However, these results were not significant after correcting for the number of tests performed. This may be due to low power of the study as a result of limited case numbers. Future work with a larger cohort should focus on validating whether expression of these cytokines is in fact dysregulated in patients with CHIP. If these cytokines are in fact differentially expressed in CHIP, it could reveal interesting clues as to how increased microenvironmental cytokine signaling might promote the expansion of CHIP clones.

TNFα is a cytokine that is important for HSC survival and myeloid regeneration. If it is overexpressed in CHIP patients, it could be acting as a CHIP-promoting cytokine by promoting survival and expansion of the mutant clones. It also makes sense that a cytokine involved in myeloid regeneration might be upregulated, given that CHIP appears to predispose patients to MDS and AML. Furthermore, previous research has shown that an inflammatory *in vitro* environment containing TNFα favors clonal hematopoiesis of Tet2-knockout murine and TET2-mutant human HSPCs (Abegunde et al. 2018). Our finding that TNFα is elevated specifically in patients with TET2 CHIP mutations supports these findings. Further studies should investigate whether CHIP-mutant HSC clones are particularly sensitive to TNFα

signaling and which cells in the bone marrow might have upregulated TNFα signaling in CHIP patients. Studies should also investigate the mechanism by which TET2-mutant CHIP clones may uniquely take advantage of elevated TNFα in the microenvironment to disproportionately expand.

IL-8 is a cytokine which plays an inflammatory role by recruiting neutrophils to the site of infection. Prior studies have also shown that IL-8 is elevated in CHIP, particularly in TET2-mutant CHIP, and that overexpression of IL-8 is correlated with poor prognosis in certain AML subsets (Jaiswal et al., 2017; Kuett et al., 2015). Given the paradoxical role that inflammation has been shown to play in cancer initiation and progression, overexpression of IL-8 may induce HSC exhaustion and promote expansion of mutant clones that are resistant to the inflammatory microenvironment, thereby allowing for initiation and progression of myeloid malignancies (Schuettpelz and Link, 2013). Further inflammatory signaling during cancer (as seen with high levels of IL-8 expressed in AML) may be co-opted by cancers to promote angiogenesis and a pro-tumorigenic microenvironment (Coussens and Werb, 2002). Further work must be done to explore how normal and mutant HSCs in CHIP patients respond to proinflammatory IL-8 signaling to determine the role that elevated IL-8 expression may play in promoting expansion of CHIP mutant clones.

CCL7 is a chemokine which attracts and regulates macrophage function during inflammation. Given that CHIP manifests as the dysregulated proliferation of HSC clones, the elevated CCL7 we observed in CHIP patients during Luminex analysis could be a result of macrophages being recruited in CHIP to mount an inflammatory response against aberrant clonal expansion. However, previous research has also shown that *Tet2*$^{-/-}$ murine macrophages

upregulate expression of several inflammatory genes, such as *Ccl7* (Cull et al. 2017). While our Luminex experiments did not find significant elevation of CCL7 in TET2 CHIP patients specifically, this prior research still suggests that the elevated CCL7 we found in CHIP patients overall could result from the dysregulated gene expression of macrophages that are derived from these CHIP HSCs. Further mechanistic work must be done to determine whether this elevated CCL7 is only a result of dysregulated expression by CHIP-derived macrophages or if CCL7 signaling has an effect on the HSCs occupying the bone marrow niche.

IFNγ is a proinflammatory cytokine that is critical for innate and adaptive immunity and appears to play a critical role in antitumor and antiproliferative effects. Based on my Luminex analysis, IFNγ may be more expressed in patients with multiple CHIP patients. Other studies have shown that IFNγ is more significantly expressed in DNMT3A-mutant CHIP patients, as well in MDS and subsets of AML (Kitagawa et al., 1997; Ciciarello et al., 2019). However, as with many CHIP studies, these findings are only correlative and do not prove causation. It may be that elevated IFNγ helps promote the growth of mutant HSCs, or it could be that mature blood cells derived from mutant CHIP simply demonstrate dysregulated expression of inflammatory cytokines like IFNγ. Further mechanistic work must be done to elucidate the effect of elevated IFNγ on HSCs.

In addition to these four cytokines, I also found that FGF-basic was downregulated in patients with CHIP, although this finding was not significant after Bonferroni correction. FGF-basic is a growth and signaling factor which is expressed in the bone marrow and helps positively regulate hematopoiesis. Interestingly, my finding that FGF-basic may be downregulated in CHIP appears to contradict with other studies which have shown that FGF-

basic is elevated in the bone marrow of patients with myeloid disorders like AML (Bieker et al., 2003). Future work might elucidate whether FGF-basic is in fact downregulated in CHIP and, if so, how the role of FGF-basic might differ between CHIP, where it is downregulated, and AML, where it is overexpressed.

As I have noted, given that no cytokines were significantly dysregulated after Bonferroni correction, future studies will need to confirm whether or not the aforementioned cytokines are in fact differentially expressed in CHIP patients. It will be further important to elucidate whether there is high expression because mutant cells and the blood cells derived from them secrete more cytokines or because these cytokines are critical for promoting mutant cell expansion.

Furthermore, given that these Luminex experiments were only performed with peripheral blood samples, it is not known whether any observed changes reflect differential cytokine expression within the bone marrow itself, which is the site of HSC mutant clone expansion. Thus, future work on this project will require performing Luminex experiments on the collected bone marrow samples to determine how cytokine expression within the bone marrow microenvironment differs between CHIP and control patients.

4.2 RNA-seq data

Following Luminex analysis, I analyzed existing RNA-Seq data on genes expressed by blood progenitors in healthy and leukemic patients. My goal was to determine if any cytokines of interest (identified based on the Luminex assay panel) and their receptors were differentially expressed by blood progenitors. Understanding how expression differs in healthy patients could help us understand how dysregulated cytokine signaling in early stages of CHIP could

potentially assist or hinder expansion of mutant clones. Additionally, understanding how expression differs in leukemic patients could help us understand how certain patterns of dysregulation might drive CHIP mutant clones towards future myeloid malignancies.

To expand the scope of analysis, I included the cytokines mentioned above as well as any cytokine for which $p<0.1$ in CHIP Luminex analysis. While expression differences for many of these cytokines are above the threshold of significance, both before and after Bonferroni correction, I approached the RNA-Seq analysis as an exploratory project that could serve as grounds for future work to validate any findings.

The blood progenitors which had been sequenced were hematopoietic stem cells (HSCs), multipotent progenitors (MPPs), lymphoid-primed multipotent progenitors (LMPPs), multi-lymphoid progenitors (MLPs), common myeloid progenitors (CMPs), granulocyte/macrophage progenitor (GMPs), and megakaryocyte/erythroid progenitor (MEPs) (Figure 11). HSCs are the least differentiated of the progenitors, while MPPs are the second least differentiated. LMPPs are progenitors that are primed to proceed down the lymphoid differentiation pathway. However, LMPPs are also thought to be capable of differentiating into GMPs, a myeloid progenitor. MLPs are committed to the lymphoid lineage. CMPs are the least differentiated myeloid progenitor. GMPs are more differentiated than CMPs and serve as the precursor to monoblasts and myeloblasts, progenitors which go on to develop into macrophages and granulocytes. MEPs are progenitors which give rise to megakaryocytes and erythrocytes. Given the variation in progenitor lineages and stages of differentiation in this RNA-Seq dataset, analysis for cytokine expression could give important insight into how dysregulated signaling in CHIP might affect proliferation and differentiation.

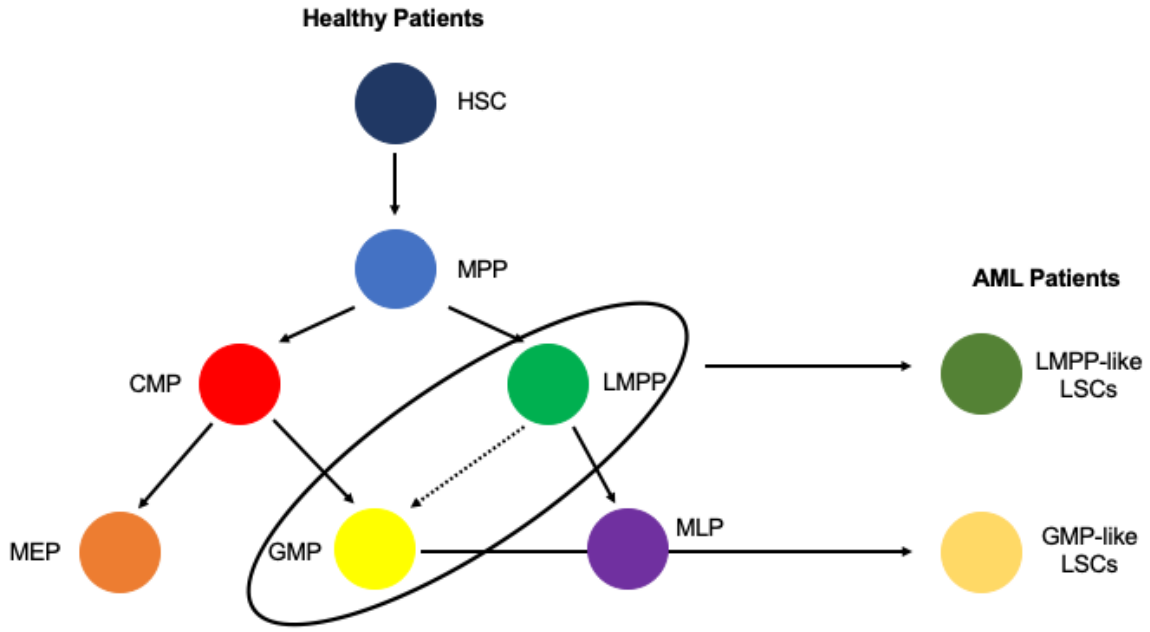


Figure 11. Blood progenitors sequenced from healthy patients represented different stages of differentiation and blood lineages, while leukemic samples had sequencing data from LMPP- and GMP-like LSCs. Based on graphical abstract from Goardon, et al. (2011).

I first performed one-way repeated measures ANOVA on the RNA-Seq data of healthy patient cells and found IFNγ to be the only cytokine or receptor whose differential expression was significant after Bonferroni correction and whose post hoc tests also showed significant difference. IFNγ was significantly more expressed by MLPs than any other progenitor except for LMPPs, suggesting that lymphoid progenitors overexpress IFNγ relative to myeloid progenitors. As I previously mentioned in discussion of my Luminex findings, IFNγ is a proinflammatory cytokine that is critical for innate and adaptive immunity and appears to play a critical role in antitumor and antiproliferative effects. Interestingly, it has been shown that IFNγ is particularly effective at suppressing lymphomagenesis, perhaps due to its elevated signaling among cells of

the lymphoid lineage as I have shown (Street et al., 2002). Based on my RNA-Seq findings, IFNγ may play a paradoxical inflammatory role in myeloid malignancies, whereby it is more expressed in CHIP patients as the body mounts an inflammatory response to suppress dysregulated HSC clonal expansion. Later, it may become co-opted by advanced myeloid malignancies like MDS and AML to promote proliferation, expansion, and spreading as has been demonstrated with chronic inflammation in cancers (Coussens and Werb, 2002). However, more work must be done to understand the role that elevated IFNγ expression might play in CHIP, which cells in the bone marrow have dysregulated IFNγ expression in CHIP, and how the role of IFNγ changes across myeloid malignancies.

TNFR-2, one of the receptors to TNFα, is another receptor for which ANOVA testing was significant, although not significant after Bonferroni correction. However, post-hoc tests for TNFR-2 were significant after Bonferroni correction. Post-hoc tests showed that TNFR-2 was more significantly expressed in MLPs compared to CMPs and MEPs. Figure 6b showed that LMPP expression of TNFR-2 is elevated and GMPs overexpress TNFR-2 compared to other cells of the myeloid lineage, although this was not significant. Expression also appears to be elevated in HSCs and MPPs relative to CMPs and MEPs, although this is also not significant. In addition, TNFR-1, the other receptor to TNFα, had a significant ANOVA after Bonferroni correction. Although post-hoc testing was not significant, TNFR-1 showed a similar pattern of expression as TNFR-2 where LMPPs and GMPs appear to have relatively high levels of expression compared to other cell types. Furthermore, my Luminex analysis showed that TNFα is overexpressed in CHIP patients compared to controls, although this was not significant after Bonferroni correction. Other work has shown that DNMT3A- and TET2-mutant CHIP have

elevated TNFα expression (Cook et al., 2017). Thus, it is important to consider what role elevated TNFα may play in CHIP and progression to myeloid malignancies, given what the RNA-Seq data has revealed about which cells might be particularly susceptible to increased TNFα signaling. TNFα is tied to cancer-related inflammation and is known to play a rather paradoxical role in cancer, both abating and promoting cancer progression. If its role is to abate CHIP, it may be initiating an inflammatory response to dysregulated mutant clone proliferation, to which HSCs would appear to be susceptible due to their relatively high levels of TNFR-2. If TNFα is playing a supportive role, high expression of its receptor particularly in LMPPs and GMPs may play a critical role in promoting the development of leukemic stem cell populations in CHIP patients. Future studies must work to understand how TNFα signaling becomes dysregulated in CHIP and elucidate the mechanistic role that elevated TNFα plays in expansion of CHIP clones and progression to MDS and AML.

There were additional cytokines whose ANOVAs were significant after Bonferroni correction, but whose post-hoc tests were not. One of these was CCR2, which is a receptor to CCL7, a chemokine which, as previously discussed, attracts and regulates macrophage function during inflammation. While post-hoc testing was not significant, Figure 7a shows that CCR2 is most expressed in GMPs compared to other cytokines, which makes sense given that GMPs are the progenitors which ultimately differentiate into macrophages. Luminex analysis showed that CCL7 is more expressed in CHIP patients overall and those with multiple mutations compared to controls. However, if CCL7 is indeed more expressed, this may suggest that macrophages are recruited in CHIP to mount an inflammatory response to the dysregulated proliferation of multiple HSC clones.

Two-way repeated measures (mixed) ANOVA added another dimension of analysis by allowing me to compare expression of cytokines and their receptors between leukemic stem cells and healthy blood progenitors. The two cell populations analyzed were GMPs and LMPPs which make up the LSC population in AML. The most significantly differentially expressed protein was CCR2, which was shown to be overexpressed in normal GMPs compared to leukemic GMPs but with no such difference between normal and leukemic LMPPs. These findings suggest that expression of CCR2, and the associated biological effects, are unique to GMPs in healthy patients. As previously mentioned, CCR2 is the receptor to CCL7, a cytokine which recruits macrophages during inflammation. If leukemic GMPs lose CCR2 expression, they become less susceptible to inflammatory CCL7 signaling. Loss of CCR2 may be an important counter-inflammatory mutation gained in AML due to chronic inflammation. Thus, if CCL7 is indeed overexpressed in CHIP patients, it may play an important role in mounting an inflammatory response to the expansion of mutant clones. Acquired CCR2 mutations in GMPs may therefore be an important later step in progression from CHIP to AML. Future work must validate whether or not CCL7 is in fact overexpressed in CHIP to determine how resulting chronic inflammation may drive acquired resistance and disease progression.

IFNγ had a significant mixed ANOVA, though not after Bonferroni correction. However, IFNγ did show significant interaction in post-hoc tests. IFNγ was shown to be overexpressed in normal LMPPs relative to leukemic LMPPs, with no such difference between normal and leukemic GMPs, suggesting low IFNγ expression in LSCs. Furthermore, recall that in healthy blood progenitors, IFNγ is most expressed in LMPPs and MLPs, two lymphoid progenitors. As previously noted, IFNγ is known to particularly inhibit lymphomagenesis, perhaps due to inter-

and intracellular IFNγ amongst these cell types. Thus, it would make sense that IFNγ expression is reduced in leukemic stem cells. However, interestingly, as I also noted, IFNγ is overexpressed overall in CHIP, MDS, and AML. This points to a rather paradoxical role for IFNγ expression, further complicated by the fact that expression is lost by this LMPP-like LSC population in AML patients. It may be that, while LMPP expression of IFNγ is lost, other cells in the bone marrow express IFNγ and that AML and other late-stage myeloid malignancies have co-opted IFNγ expression for its protumorigenic effects. However, the question of IFNγ's role in CHIP and AML, how the function of IFNγ signaling varies depending on the stage of myeloid malignancy, and which cells in the bone marrow express IFNγ will need to be investigated further.

It is important to note that there were several key findings from other papers on peripheral blood cytokine differences in CHIP patients that were not reproduced by our Luminex assay. For example, Bick et al. (2020), Fuster et al. (2017), and Jaiswal et al. (2017) all found an association between elevated IL-1β and *TET2* mutations which we did not find. Similarly, Bick et al. (2020), Jaiswal et al. (2017), and Meisel et al. (2018) found an association between elevated IL-6 and *TET2* mutations that our assay was not able to reproduce. However, these differences may at least in part be due to variability in assays designed to measure cytokine expression. For example, Jaiswal et al. (2017) performed RNA-Seq on *Tet2* knockout macrophages to measure differences in cytokine expression in CHIP mutant HSC-derived myeloid cells rather than directly measuring cytokine protein levels in their blood samples. Bick et al. (2020) used data on inflammatory markers measured in other studies. Fuster et al. (2017) assessed elevated expression of IL-1β in *Tet2*$^{-/-}$ mouse-derived macrophages using qtPCR. Future

work should test differences in cytokine expression by using assays that look at both gene expression and protein levels to account for variability in assays that measure cytokine levels.

In addition, while my analysis of results has focused primarily on the effect that dysregulated cytokine expression in CHIP could have on mutant HSCs, it is important to note that clonal expansion resulting from dysregulated cytokine levels in blood could be due to either their effect on mutant HSCs or normal HSCs. As I have discussed thus far, the cytokines of interest could be positively regulating the growth of mutant HSCs, promoting clonal expansion due to the greater self-renewal capacity of mutant HSCs when targeted by these cytokines (i.e., the CHIP mutation and extracellular signaling together help the HSC clone expand). However, clonal expansion could also stem from negative regulation of normal HSCs, resulting in impeded self-renewal capacity that therefore allows the mutant HSCs which are immune to this signaling to expand faster. This is one way to reconcile the at times paradoxical role that these cytokines appear to be playing, with antiproliferative cytokines like IFNγ being potentially overexpressed in CHIP. This mechanistic difference in clonal expansion cannot be elucidated by looking at cytokine levels in blood. Future work must study the downstream effects of elevated cytokine levels, looking particularly at pathways that might be dysregulated in mutant HSCs and normal HSCs in the presence of certain cytokines to determine how these cytokines are impacting clonal expansion in CHIP patients.

Overall, findings from analysis of the Luminex and RNA-Seq data suggest that inflammation plays an important and potentially paradoxical role in CHIP and myeloid malignancies. Several pro-inflammatory cytokines, including IFNγ, CCL7, IL-8 and TNFα may be overexpressed in CHIP patients to mount a paradoxical inflammatory response that both

abates and promotes the dysregulated proliferation seen in CHIP. Other cytokines, such as FGF-basic, might be downregulated as the microenvironment remodels to support CHIP. Furthermore, my findings show that there may be a difference between the role of cytokines in CHIP versus MDS and AML. For example, while IFNγ may play an inhibitory role in CHIP, IFNγ signaling could be used by AML to support its growth and proliferation. Furthermore, chronic inflammatory signaling in CHIP may promote the development of resistant leukemic clones, as can be seen with CCR2, the receptor to CCL7, being lowly expressed in leukemic cells compared to normal progenitor cells. It is clear that further work must be done to determine which cytokines are playing a promoting and inhibitory inflammatory role in CHIP, how this inflammatory signaling might be co-opted by AML, and what mutations are acquired to resist the effects of inflammatory signaling. Doing so will give us better insight into how the bone marrow microenvironment contributes to the expansion of CHIP clones and whether signaling in the microenvironment can predict which patients will go on to develop AML.

4.3 Limitations

There are several limitations to interpreting these findings which I have noted throughout this thesis. As I have previously noted, I was not able to normalize expression levels from Luminex data to total protein given the accuracy issues I encountered in the BCA assay and the fact that I was unable to repeat the assay due to the COVID-19 pandemic. Thus, it may be that certain observed differences in cytokine expression between CHIP and control patients are due to differences in total protein levels between patients. In addition, only a few cytokines mentioned were significantly expressed in Luminex analysis based on $p<0.05$, and none were significant

after Bonferroni correction. Furthermore, a few cytokines and receptors selected for RNA-Seq analysis were based on a broadened list that included cytokines with $p<0.1$. Thus, the results outlined in this thesis should be interpreted with caution and future work must validate whether these cytokines are in fact differentially expressed in CHIP and to mechanistically understand the effect that they have on CHIP and AML.

Another important limitation to interpreting these findings is that the Luminex analysis was only performed on peripheral blood samples and not bone marrow samples. While the original goal of the project had been to analyze both peripheral blood and bone marrow samples, the timing of the COVID-19 pandemic did not allow this to happen. Thus, while any observed dysregulation of cytokine expression may reflect changes in expression in the bone marrow where HSCs reside, this cannot be confirmed without also looking at how cytokine expression is dysregulated in the bone marrow of CHIP patients. Future research must look at how cytokine expression within the bone marrow is dysregulated.

4.4 Future directions

Given these limitations and the gaps in knowledge outlined in this discussion, there are several important lines of future work that should be undertaken to better understand how cytokine dysregulation in CHIP affects HSC mutant clone expansion and potential progression to advanced myeloid malignancies. Many of these were experiments I had hoped to perform if not for the COVID-19 pandemic. As previously mentioned, it will be important to perform Luminex cytokine analysis experiments on bone marrow samples to see if there are any differences in the cytokines that are dysregulated in the bone marrow microenvironment of CHIP patients. Doing

so will improve our understanding of how signaling in the stem cell niche might affect CHIP mutant clonal expansion. However, it is important to note that, unlike peripheral blood collection, bone marrow sampling is prone to erroneous sampling, including contamination with peripheral blood. This can be seen in the BCA assay experiments I conducted which, although imperfect, still showed much greater variability in the bone marrow sample protein concentrations compared to peripheral blood (Figure 1). This makes it not only difficult to obtain a pure bone marrow sample, but also difficult to sample consistently across patients. Thus, if future cytokine work is to proceed with the collected bone marrow samples, steps must be taken to normalize to proteins found predominantly in the bone marrow or other sample characteristics that would be exclusive to bone marrow.

Additionally, while the RNA-Seq data was important for understanding how cytokine dysregulation might affect healthy and leukemic blood progenitors, it is still not known how expression of cytokines and their receptors differs in progenitor cells within CHIP patients. Thus, RNA-Seq experiments studying expression in CHIP blood progenitors is an important next step. Asger Jakobsen, the graduate student in the Vyas Lab who was my collaborator on this project, is currently carrying out TARGET-Seq experiments (a technique which allows for parallel mutational analysis and RNA sequencing) to study gene expression of HSCs from CHIP patients. Extending this work to other blood progenitors could help us understand how cytokine and receptor expression varies from healthy to CHIP to AML patients. Furthermore, to understand the role of bone marrow microenvironment signaling, it will also be important to perform RNA-Seq experiments to study how non-blood progenitor cells in the bone marrow dysregulate cytokine expression in CHIP to see how they may assist or abate the expansion of CHIP clones

and, later, AML. To establish causality, experiments such as replating assays must be carried out to determine how cytokines of interest affect proliferation and self-renewal of HSCs with CHIP mutations. Carrying out these lines of investigation will allow us to gain a better understanding of how CHIP mutant clones drive dysregulated cytokine expression and the role that this dysregulated expression plays in progression to AML.

5) Conclusion

Through my thesis work, I advanced our understanding of how dysregulated cytokine signaling in patients with clonal hematopoiesis may affect the expansion of HSC mutant clones. These findings give more insight into how signaling of cells in the bone marrow microenvironment might change in response to CHIP. In my analysis, I focused particularly on dysregulated proinflammatory cytokines and the paradoxical role they may play in CHIP and myeloid malignancies. I discussed how proinflammatory cytokines may initially have an inhibitory effect and later be co-opted to promote the advancement of myeloid malignancies and leukemia. While many of the cytokine dysregulations I found were not significant, particularly after Bonferroni correction, my findings should establish grounds for future exploratory and validating work.

Findings from my analysis of the RNA-Seq data furthered our understanding of how dysregulated cytokine signaling in CHIP may affect blood progenitors based on how these cytokines and their receptors are expressed in healthy blood progenitors and leukemic stem cells. My analysis revealed how differential cytokine and receptor expression in different progenitor cell types can leave certain cell types susceptible to signaling that might drive myeloid malignancies. Importantly, these findings showed how dysregulated expression of such cytokines during CHIP may play a crucial role in transforming blood progenitors from the healthy to the leukemic state.

Future work will be important to further improve our understanding of how cytokine signaling in CHIP patients, particularly in the bone marrow microenvironment, affects proliferation of blood progenitors. It will be important to look particularly at changes in the

proliferation of HSCs and known precursors to leukemic stem cells in response to dysregulated cytokine expression to understand how this signaling may promote the development of AML.

Given the current gaps in understanding in how CHIP clones gain the clonal advantage and what causes them to progress to more advanced myeloid malignancies like MDS and AML, insights into extracellular signaling may provide important clues to answer these questions. The work in this thesis provides evidence that cytokine dysregulation may play an important role in affecting CHIP clone expansion and in promoting the development of further myeloid malignancies. Additional work exploring the ideas posed in this thesis will be important for furthering our understanding of how cytokine signaling affects CHIP. Doing so may improve our ability to intervene early and accurately for patients with CHIP to prevent the development of advanced, life-altering hematologic malignancies.

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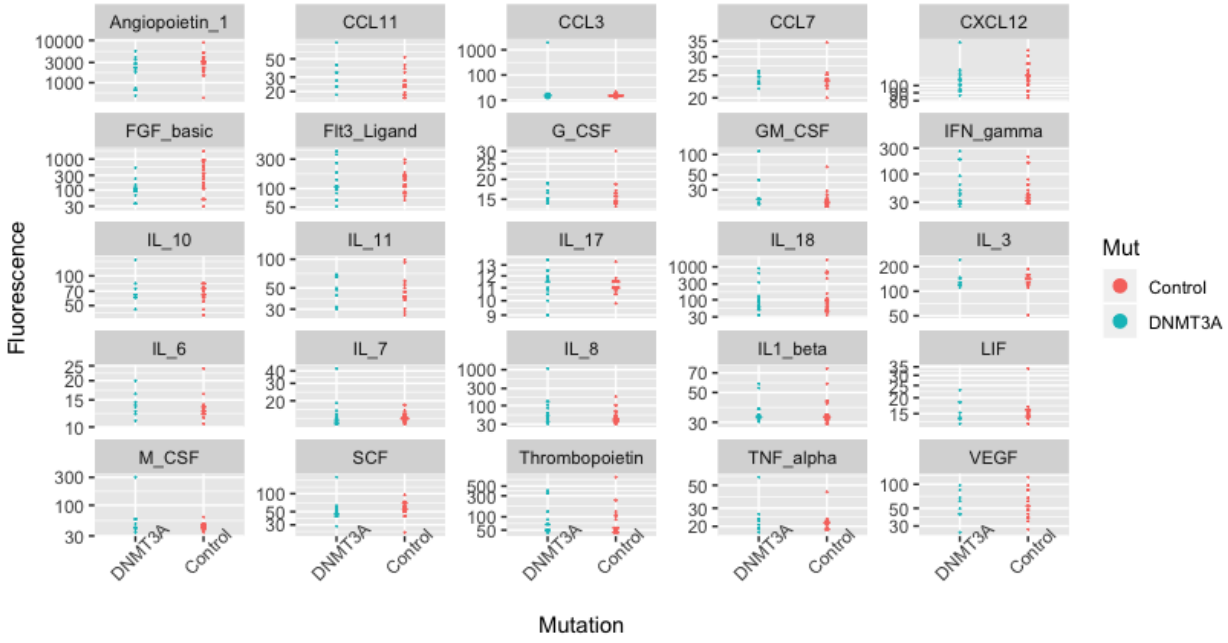
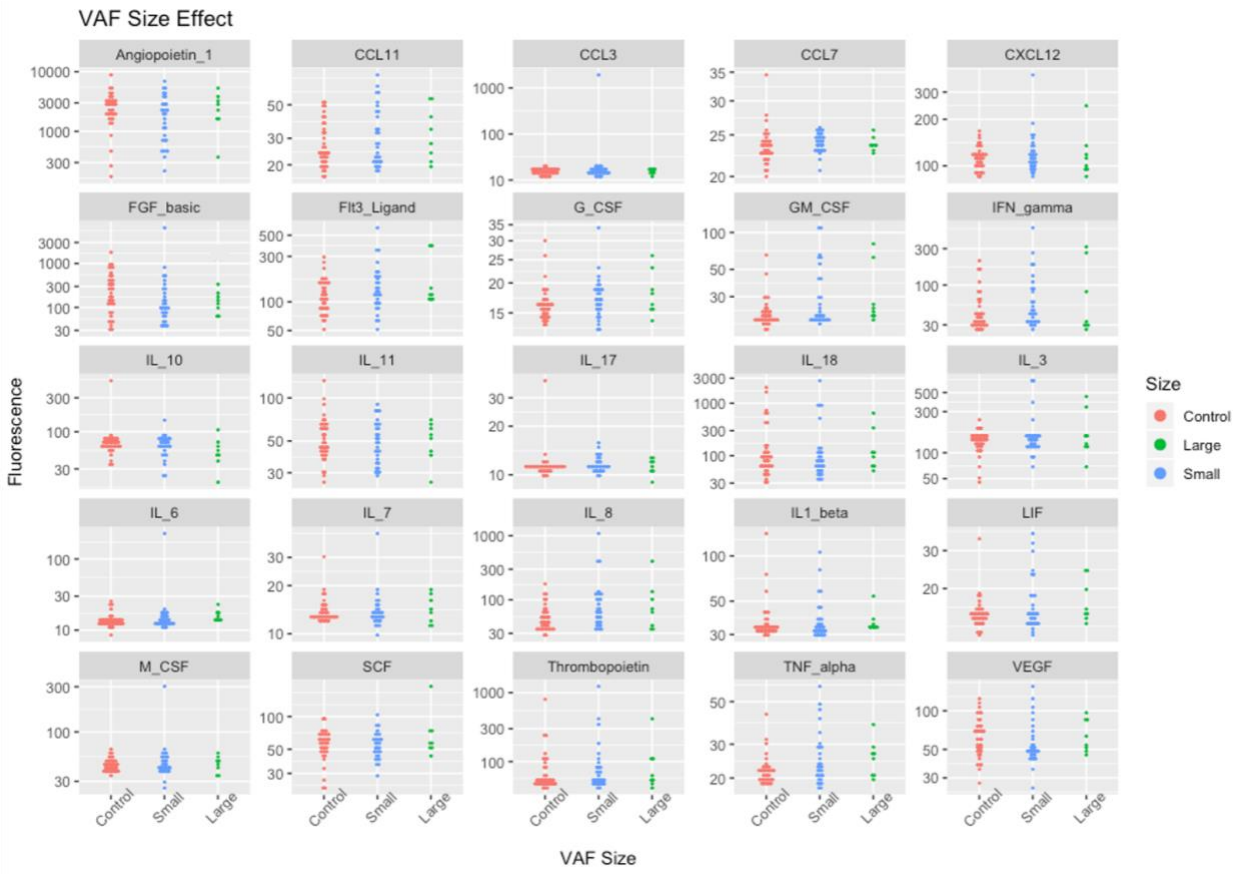
Supplementary Figures

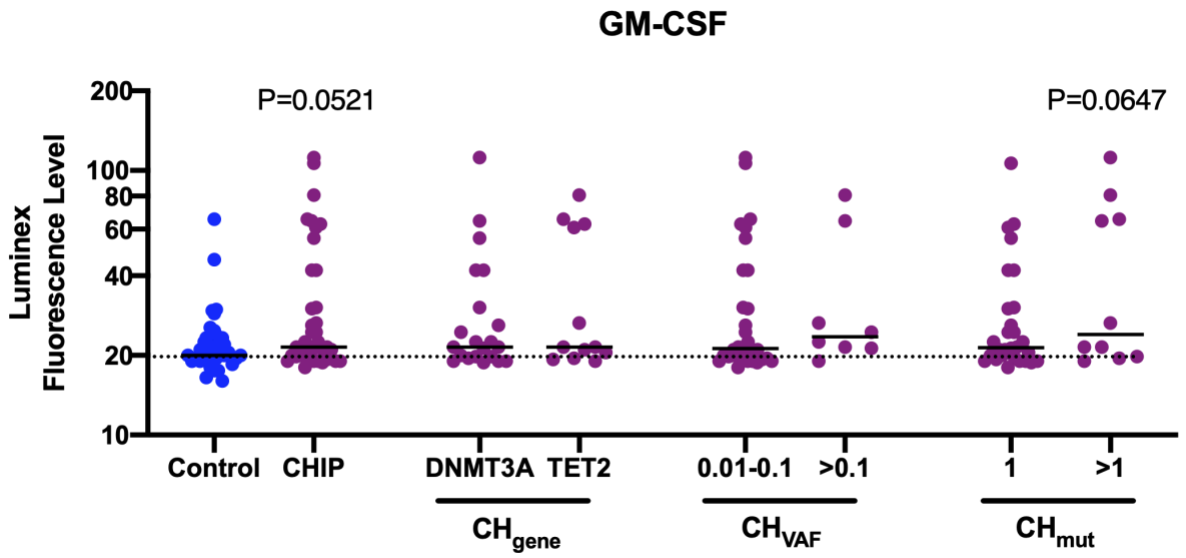


Supplementary Figure 1. Expression levels between DNMT3A-mutant CHIP and control samples.

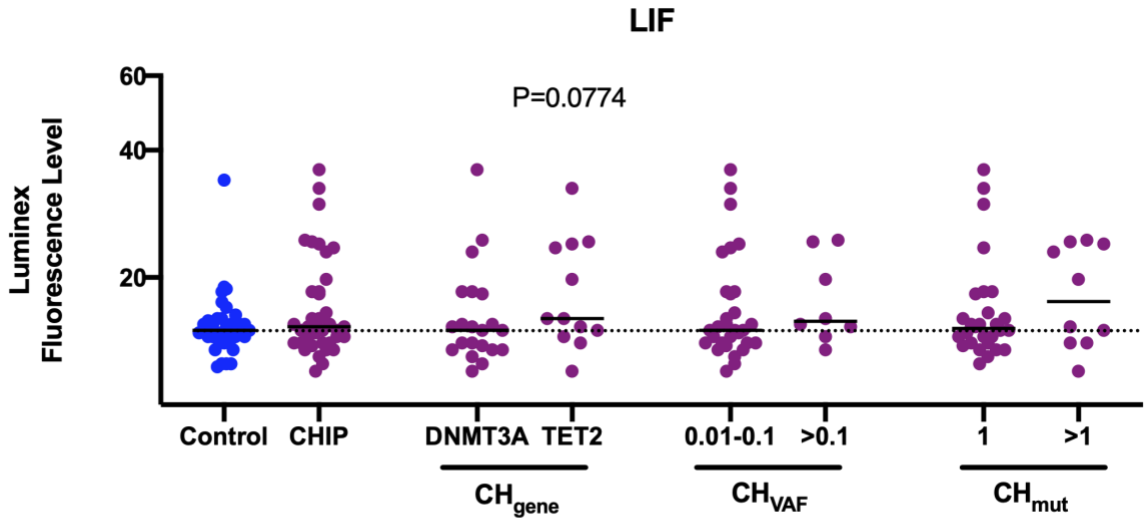


Supplementary Figure 2. Expression levels between CHIP samples with VAF >0.1, CHIP samples with VAF 0.01-0.1, and control samples.

A)

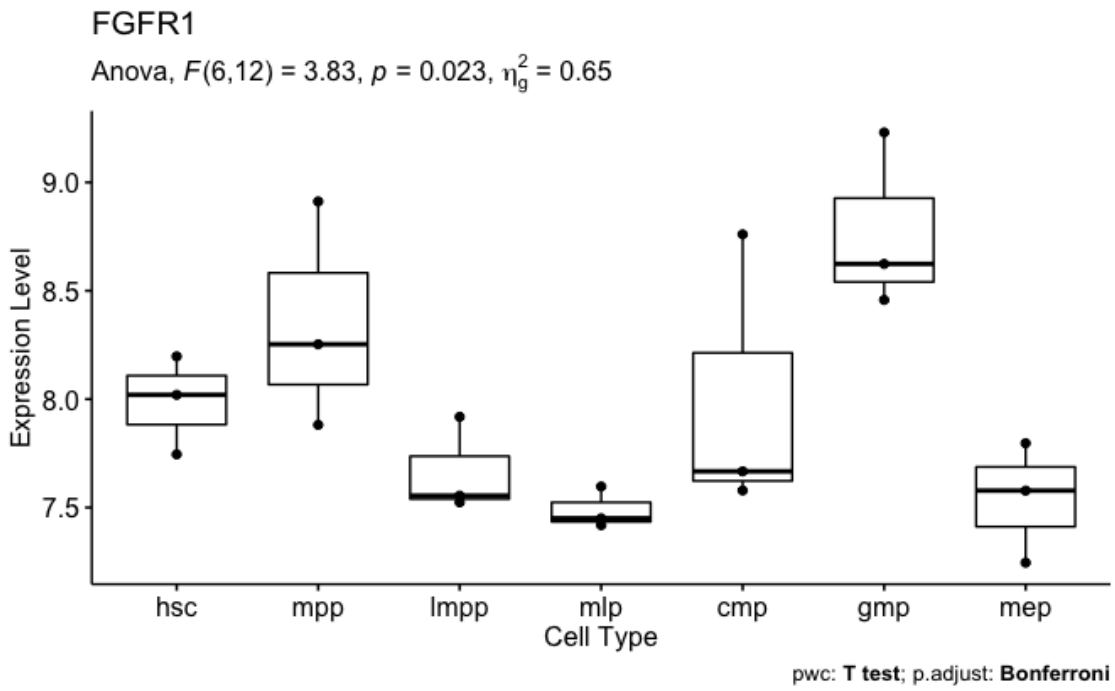


B)

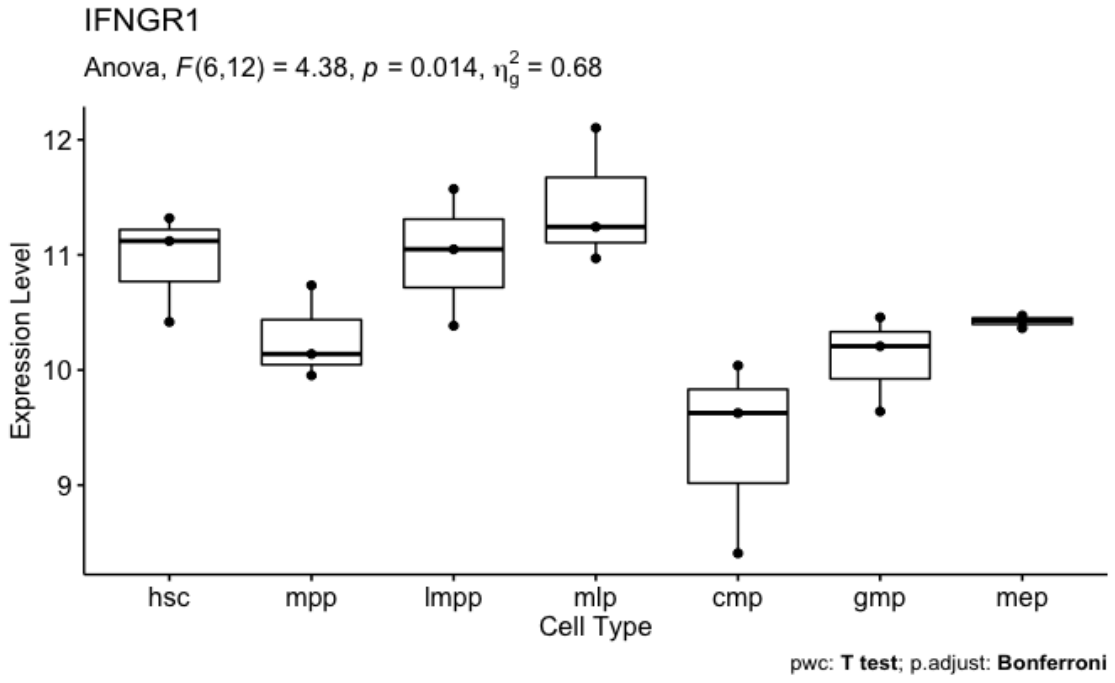


Supplementary Figure 3. Cytokines which were included for RNA-Seq analysis along with their receptors. These cytokines had some dysregulated expression in CHIP where p<0.1. **a)** GM-CSF was differentially expressed between total CHIP and control samples, as well as between CHIP samples carrying multiple mutations and controls. **b)** LIF was differentially expressed between TET2-mutant CHIP and control samples.

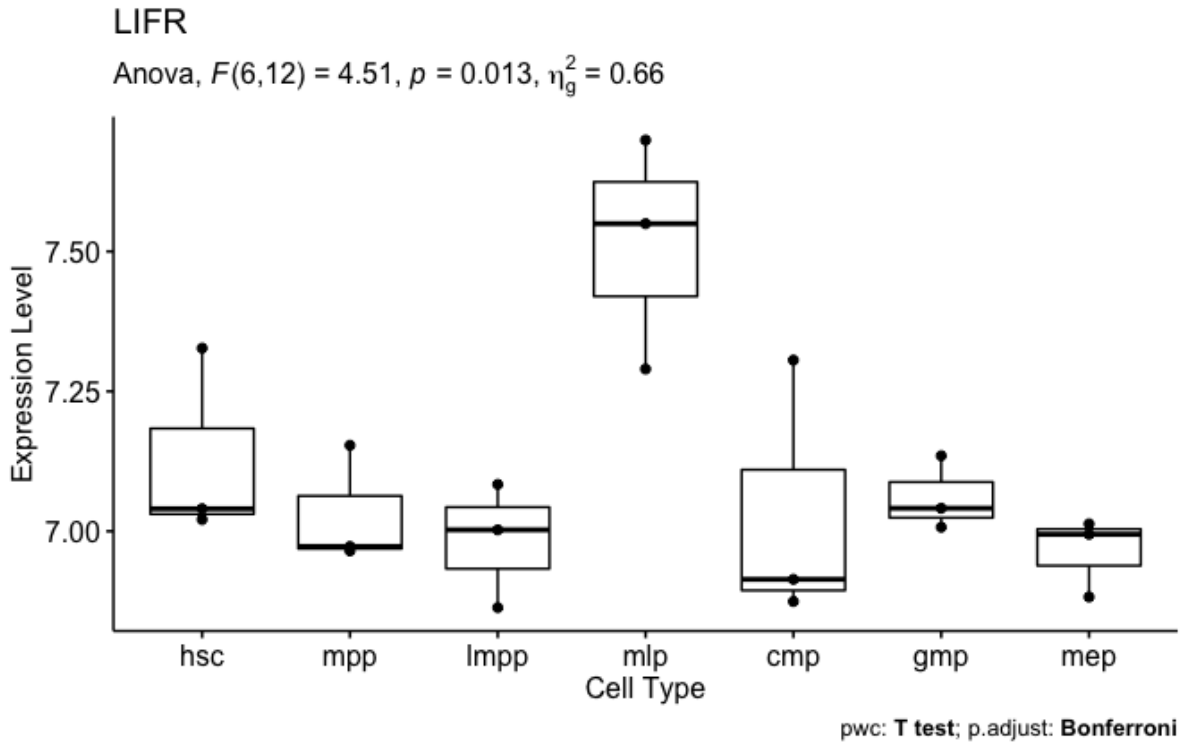
A)


FGFR1
Anova, F(6,12) = 3.83, p = 0.023, η²g = 0.65
Expression Level
9.0
8.5
8.0
7.5
hsc
mpp
lmpp
mlp
cmp
gmp
mep
Cell Type
pwc: T test; p.adjust: Bonferroni

B)


IFNGR1
Anova, F(6,12) = 4.38, p = 0.014, η²g = 0.68
Expression Level
12
11
10
9
hsc
mpp
lmpp
mlp
cmp
gmp
mep
Cell Type
pwc: T test; p.adjust: Bonferroni

C)



Supplementary Figure 4. Cytokines/receptors which were had significant ANOVA indicating differential expression between blood progenitors, although not significant after Bonferroni correction. a) FGFR1; **b)** IFNGR1; **c)** LIFR. All expression levels are counts per million reads mapped (CPM).