TRIBBLES HOMOLOGUE 1 CONTROLS GRANULOCYTE PROGENITOR COMMITMENT AND TERMINAL CELL IDENTITY AND FUNCTION

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DEDICATION

This dissertation is dedicated to my grandmothers, Judy Greenfeld and Olga Mack, both of whom I lost to lung cancer during my time in graduate school. These strong and amazing women were two of my staunchest and most vocal supporters. I hope that this work and the work I do going forward in some small way advances our knowledge to the point that deaths like theirs could be preventable.

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This body of work is the result of a truly collaborative effort. Without the contributions of the people mentioned below, as well as countless others, it is highly unlikely that I would have completed this endeavor. They supported me when I was struggling with the pressures of graduate school and celebrated me on the rarer occasions when things went my way.

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ABSTRACT

TRIBBLES HOMOLOGUE 1 CONTROLS GRANULOCYTE PROGENITOR COMMITMENT AND TERMINAL CELL IDENTITY AND FUNCTION

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Eosinophils and neutrophils are critical for host defense, yet gaps in understanding how granulocytes differentiate from HSCs into mature effectors remain. The pseudokinase Trib1 is an important regulator of granulocytes; knockout mice lack eosinophils and have increased neutrophils. However, how Trib1 regulates cellular identity during eosinophilopoiesis and cellular function of mature eosinophils and neutrophils is not understood. *Trib1* expression markedly increases with eosinophil-lineage commitment in eosinophil progenitors (EoPs), downstream of the GMP. Using hematopoietic- and eosinophil-lineage-specific *Trib1* deletion, we found that Trib1 regulates both granulocyte precursor lineage commitment and mature eosinophil identity. Conditional Trib1 deletion in HSCs reduced the size of the EoP pool and increased neutrophils, whereas deletion following eosinophil lineage commitment blunted the decrease in EoPs without increasing neutrophils. In both modes of deletion, Trib1-deficient mice expanded a stable population of $Ly6G^{+}$ eosinophils that retained neutrophilic characteristics and functions, and had increased C/EBPα p42. Using an *ex vivo* differentiation assay, we identified a previously uncharacterized role for IL-5 in supporting both eosinophil and neutrophil production from the GMP; Trib1 suppressed the neutrophil gene program in lineage-committed eosinophil precursors in response to IL-5 signaling. Furthermore, we demonstrated that Trib1 loss blunted eosinophil migration and altered chemokine receptor expression, both *in vivo* and *ex vivo*. We showed that Trib1 controls eosinophil identity by modulating C/EBPα. Trib1 also controls neutrophil inflammatory function by modulating activation of the AKT, MAPK, and NF-κB pathways Together, our findings provide new insights into early events in myelopoiesis, whereby Trib1 functions at multiple distinct stages. Trib1 guides eosinophil lineage commitment from the GMP, suppresses the neutrophil program, and limits neutrophil inflammatory function, together promoting granulocyte terminal identity, lineage fidelity, and homeostasis.

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PREFACE

Every day, billions of blood cells are generated in the bone marrow (BM) and this process continually produces new cells to meet the demands of the host organism, both at steady state, and under settings of stress[1]. Hematopoiesis, the process of generating new blood cells, can broadly be divided into the production of the three main lineages of blood products: red blood cells (erythrocytes), white blood cells (leukocytes), and platelets. Full tri-lineage hematopoiesis is required to sustain life and failure of any one arm can lead to illness or death. Any alterations in this, from underproduction, overproduction, to the development of malignancy, is detrimental to the host. In addition, once a cell adopts an individual identity, it must actively maintain that identity during its lifetime and not transition either backwards into an undifferentiated state or to another mature lineage. Thus, hematopoiesis is tightly controlled and cellular identity is rigidly set. In this work, I investigate how different hematopoietic cell populations with highly divergent functions arise from common progenitors and how those cells maintain their distinct characteristics. For these studies, I used mice to model the dynamics of hematopoiesis in a tractable system. Ultimately, the goal of this work is to understand how cellular identity is shaped and maintained, sustaining normal hematopoietic production and organismal homeostasis.

CHAPTER 1: INTRODUCTION

The immune system is a complex network of cells, signaling molecules, and soluble proteins that silently functions to guard the body against external assaults and dangers from within. Leukocytes continually patrol peripheral tissues and have a range of phenotypes and functions, specialized to serve unique purposes. Leukocytes can be divided into two branches: innate and adaptive. Innate leukocytes, including granulocytes, macrophages, dendritic cells (DCs), and innate lymphocytes, are the first responders to sites of infection and serve to alert and prime other populations to amplify danger signals. These cells also possess a set of fixed, germline-encoded receptors that allow them to recognize and respond to a broad, yet defined, array of targets. In contrast, adaptive immune cell populations, consisting of T and B lymphocytes, are able to tune their responses to specific threats and improve their functions upon secondary challenge. These two arms of the immune system, while consisting of separate populations, are fundamentally linked and are both required for survival.

All hematopoietic cells originate from a common progenitor, the hematopoietic stem cell (HSC)[2]. These cells possess the ability to both self-renew and give rise to all terminally differentiated blood cells. HSCs and their downstream progeny face multiple decision points as they pass from the undifferentiated state to mature effector cells. These decision points, both stochastic and influenced by external signals, determine which cells are made. Governed by the interplay of signaling molecules, receptors, and transcription factors, cellular fate decisions are some of the most complex. Once a progenitor specifies a particular lineage, the identity of that cell must be actively maintained and other programs repressed in order to preserve a particular cell state.

Elegant work in T cells, for example, demonstrated that cells must both turn on their chosen program, or specify that lineage, as well as repress alternative lineage programs, or commit to that lineage[3]. While these questions have been extensively studied in other lineages, similar studies are only beginning to be undertaken in some innate populations. In particular in this study, we will focus on how two populations of innate immune cells, eosinophils and neutrophils, diverge from a common progenitor to establish unique and fixed identities and how their terminal functions are controlled.

Granulocytes

The innate immune system is characterized by fast response times, lack of germline re-arrangeable antigen receptors, and the ability to respond to a broad range of threats. While a traditional hallmark of the innate immune system is lack of memory development, recent work has challenged this paradigm, showing improvement on re-challenge in NK cells[4], macrophages[5], and group 2 innate lymphocytes (ILC2)[6]. Further subdividing the cellular innate immune system, this branch can be broken down into myeloid and lymphoid innate cells. The innate myeloid compartment, including granulocytes, macrophages/monocytes and dendritic cells, contains some of the most abundant cells of the hematopoietic tree. These populations are critical for defense against invading pathogens and are continually patrolling barrier surfaces, including the lung, gut, and skin. Furthermore, these cells are critical bridges between the innate and adaptive systems. One of the first cell types to respond to pathogenic insults are granulocytes. This family of cells is known for powerful effector mechanisms and short half-lives. Named for the characteristic granules present in the cytoplasm, granulocytes include neutrophils, eosinophils, and basophils. Of particular focus to this study are neutrophils and eosinophils.

Neutrophils: First line defense

Neutrophils, sometimes called polymorphonuclear leukocytes (PMN) in reference to their highly segmented nucleus, are the true first responders of the immune system[7]. Neutrophils are rapidly recruited to sites of infection or tissue injury. They use multiple mechanisms to both eliminate pathogens, in particular bacteria, and amplify the danger signals to recruit other innate and adaptive populations. Neutrophils are phagocytic and use reactive oxygen and nitrogen species (ROS/RNS) to destroy phagocytosed bacteria[8]. Furthermore, neutrophils release proteases and anti-microbial peptides from pre-formed granules[9]. This can be beneficial to the host by supporting bacterial clearance, but can also lead to immunopathology in a range of settings from acute lung injury[10] and sepsis[11] to autoimmune disease[12] and cancer[13]. Because many of these functions are tissue destructive, identifying ways to control neutrophil activation is critically important.

In addition, neutrophils are able to release large clouds of DNA and other cellular contents in structures known as neutrophil extracellular traps (NETs). NETs trap bacteria and increase local concentrations of cytokines, chemokines, and other effector molecules[14-17]. Furthermore, they act as danger signals to attract other populations. NET formation has also been implicated the development of autoimmune diseases such as lupus[18,19].

Defects in neutrophil populations, either due to decreased production or lack of effector functions leads to severe immunodeficiency manifested by recurrent opportunistic infections[20]. For example, lack of neutrophil ROS generation is seen in chronic granulomatous disease (CGD), and is characterized by the formation of large granulomas of neutrophils that are unable to eliminate bacteria[21]. As neutrophils are

powerful effector cells, their function must be tightly controlled. Prolonged or inappropriate neutrophil activation can be tissue destructive and cause immunopathology. As noted above, neutrophils are implicated in the pathogenesis of multiple diseases including autoimmune arthritis[12], cancer[13,22], and type 2 diabetes[23].

Eosinophils: protective and problematic

In contrast to neutrophils, which are primarily anti-bacterial effector cells, eosinophils, another population of granulocytes, are classically thought to be important for defense against parasites[24,25]. However, there is significant debate about the direct anti-parasitic functions of eosinophils[26,27]. In fact, in certain settings, eosinophils may contribute to parasite persistence by propagating a Th2-biased immune response [28]. Much less abundant than neutrophils, eosinophils are characterized by a highly granular cytoplasm, rich in destructive cationic proteins such as major basic protein (MBP) and eosinophil peroxidase (EPX)[29]. These granules also contain pre-formed cytokines, such as IL-4, IL-5, and TNFα, and chemokines, such as RANTES, for rapid release upon activation[30-35]. Studies of mice and humans genetically lacking eosinophils as well as humans treated with anti-IL-5 therapy[36,37] raise the question of the true importance of eosinophils to human health, as individuals lacking eosinophils appear to suffer no ill consequences[38].

Eosinophils also expand under allergic or atopic conditions and participate in Th2-polarized immune responses. Eosinophil numbers in the blood and lung correlate with asthma severity[39] and control[40]. Furthermore, anti-IL-5 therapy to limit eosinophilia in atopic patients has shown promise in patients with eosinophil-dominant asthma[36,37]. This relationship has been further dissected using mouse models. In particular, using eosinophil-deficient strains, these cells were shown to play a causative role in the

development of atopic lung pathology[41]. Specifically, eosinophils are required to recruit $CD4^+$ T cells to the lung[42], as well as modulate DC activation and migration in the small intestine following allergen challenge[43,44].

As part of the larger Th2 program, eosinophils support macrophage polarization to a more anti-inflammatory/"M2-like" state, through the production of IL-4 and IL-13[45]. This was shown to be critical to the maintenance of metabolic homeostasis in adipose tissue to prevent glucose intolerance. Eosinophils can also play roles in traditionally Th1-biased diseases such as respiratory viral infection[46]. Finally, while eosinophils were suggested to be required for BM plasma cell survival[47], recent reports dispute this[48]. Together, these reports provide insight into the important role that eosinophils play at steady state and under settings of stress and highlight the critical impotence of understanding their origin and identity.

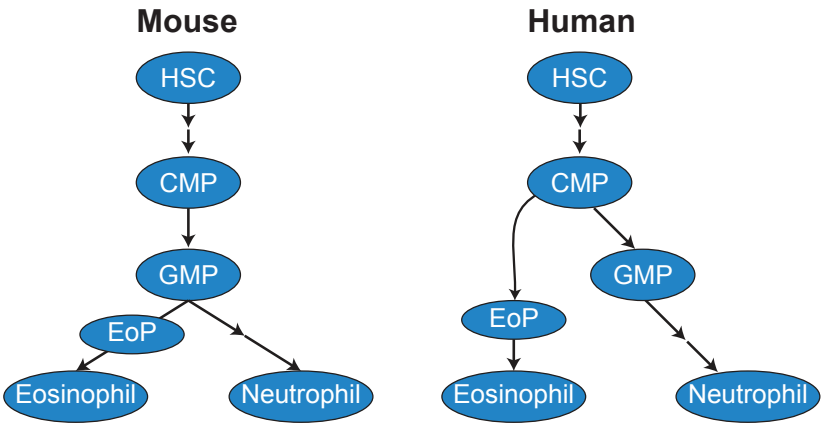

Fig. 1.1: Model of granulopoiesis. Model of mouse (left) and human (right) granulopoiesis[50, 51].

Myeloid lineage commitment

Granulocytes cells pass through several well-described populations as they differentiate from the HSC. These populations were characterized using expression various cell surface antigens. In particular, the common myeloid progenitor (CMP) and the granulocyte/macrophage progenitor (GMP) were shown through transplant studies to give rise to all granulocyte lineages[49]. These studies were done transplanting by bulk sorted cells and as such, the heterogeneity of these populations was not assessed. The issue of progenitor heterogeneity will be discussed further below.

IL-5 regulates eosinophil developmental intermediates

Eosinophils pass through a lineage-committed progenitor stage, termed the eosinophil progenitor (EoP), which is derived from the GMP in mice[50]. In humans, the EoP branches off directly from the upstream CMP[51] **(Fig. 1.1)**. The EoP is characterized by expression of the high affinity alpha chain of the IL-5 receptor (IL-5Rα)[50]. Recently, a $CD11b^{+}$ $SiglecF^{+}$ $IL\text{-}5Ra^{-}$ population was described in the BM as being a GMP-like population that selectively gives rise to neutrophils[52]. However, the functional significance of this population has not been evaluated. The cytokine IL-5 drives eosinophil development and proliferation *in vivo*[53] and *ex vivo*[54]. Furthermore, massive eosinophilia is observed in IL-5 transgenic mice whose T cells constitutively express IL-5 (ref. [55]). Despite this, mice deficient in IL-5 retain homeostatic levels of eosinophils[56,57], suggesting that other factors either can compensate for lack of IL-5 or normally act to support steady-state eosinophil production. IL-33, one of these additional factors, will be discussed below.

Recent work demonstrated that IL-5 promotes a network of factors that shape eosinophil development, including IL-4 and CCL3[58]. This report highlighted that IL-4/IL-

4Rα expression was induced in developing eosinophils following IL-5 stimulation and this interaction further drove eosinophil expansion. Interestingly, in the absence of IL-5, IL-4 inhibited eosinophil survival *ex vivo*. In contrast, CCL3 produced by immature eosinophils acted in an autocrine fashion to stimulate eosinophil maturation in the absence of IL-5. Finally, while IL-5 plays a critical role in eosinophil development, it is unclear what drives upregulation of IL-5Rα or even if it is a driver or a consequence of lineage commitment itself.

The role of IL-33 in eosinophil development

The cytokine IL-33 is also implicated in eosinophil lineage commitment and development. IL-33 was initially characterized as an IL-1 family member that induced a Th2-polarized response and generated eosinophilia when administered to mice[59]. Further work revealed that it functions as an alarmin, or danger signal, released by damaged epithelium at barrier surfaces to initiate innate responses[60]. Critically, to induce eosinophilia following allergen or parasite exposure, IL-33 activates Th2 cells and ILC2s to produce IL-5 and IL-13[61-64]. Eosinophils express the IL-33 receptor (ST2) and upregulate it upon recruitment to the airway after allergen challenge[65]. IL-33 activates eosinophils[66] and promotes cell survival in conjunction with GM-CSF[67].

These reports highlight the role of IL-33 in the development of eosinophilia following challenge, yet these functions are largely dependent on IL-33-induced IL-5. Studies attempting to delineate an IL-5-independent role for IL-33 in eosinophils development struggled to separate the two and there are conflicting reports on the ability of IL-33 to promote eosinophil development *ex vivo*. One study reported that IL-33 promoted eosinophil production from c-Kit$^+$ BM progenitors *ex vivo*[65]. In contrast, another report found that IL-33 did not support eosinophil production *ex vivo* and, in fact,

antagonized IL-5-driven eosinophil production[68]. Eosinophil development has also been analyzed in IL-33 or ST2 knockout mice and steady-state eosinophil levels were reduced[69].

Interestingly, while IL-33 required IL-5 to promote eosinophil production *in vivo*, the inverse was also partly true. IL-5 transgenic mice that lacked ST2 had a partial decrease in eosinophilia, albeit not down to WT levels[69]. Of note, ST2 knockout mice also showed reduced neutrophils, suggesting that IL-33 plays a role in neutrophil development as well. Taken together, IL-33 and IL-5 are strongly interconnected and interdependent in their roles in eosinophil development, and more work is required to truly delineate an independent role for IL-33 in eosinophilopoiesis. Further, the true signals that drive eosinophil lineage specification or commitment are unknown.

Neutrophil developmental intermediates

While the histologic differentiation of neutrophils is established, stable intermediates of neutrophil differentiation analogous to the EoP are now just beginning to be characterized[70-73]. These studies identified sortable intermediates that derive from the GMP and are restricted to the neutrophil lineage. The strongest candidate for this restricted neutrophil progenitor in mice is the preNeu[72]. This population was identified as Lineage$^-$ (CD90, NK1.1, B220), CD115$^-$, SiglecF$^-$, Gr1$^+$ CD11b$^+$ c-Kit^{int} CXCR4$^+$ and when transplanted into WT recipients, only produced neutrophils, with a notable lack of eosinophils. Despite this, further work is required to determine the precise lineage path in neutrophil differentiation.

Transcription factors in granulocyte development

Multiple TFs are required for eosinophil and neutrophil development, including C/EBPα, C/EBPε, PU.1, Gfi1, GATA-1, and GATA-2. C/EBPα$^{-/-}$ mice lack neutrophils and eosinophils, due to a block in the CMP to GMP transition[74,75]. C/EBPα is also required to balance neutrophil and monocyte development, with high C/EBPα favoring neutrophils and intermediate levels favoring monocytes[76,77]. Critically, there are two C/EBPα isoforms, p42 and p30[78]. C/EBPα p42 is required for terminal myeloid differentiation and is anti-proliferative via suppression of E2F[79]. Conversely, C/EBPα p30 supports GMP formation, yet cannot induce subsequent myeloid differentiation. This, together with the inability of C/EBPα p30 to interact with E2F, induces acute myeloid leukemia (AML) if p30 is present alone[80]. In studying the role of C/EBP proteins in eosinophil development, Nerlov, Graf, and colleagues observed that different functions were required to regulate eosinophil development. They observed that both C/EPBα and C/EPBβ could induce eosinophil lineage commitment, but maturation required an intact transactivation domain, at least in the case of C/EPBβ[81].

In human hematopoietic progenitors, enforced C/EBPα expression promotes neutrophil and eosinophil development[82], whereas over-expression of GATA-1 or GATA-2 promotes only eosinophil differentiation[83]. More specifically, using a transformed chicken multipotent progenitor system, intermediate levels of GATA-1 expression, in conjunction with C/EBPβ expression, generated eosinophils whereas high levels of GATA-1 failed to induce differentiation[84,85]. This was correlated with decreasing levels of the GATA co-factor, FOG, as cells differentiated into eosinophils. Conversely, forced expression of FOG was able to block GATA-1-mediated eosinophil differentiation. Interestingly, this process was antagonized by PU.1, which was able to mediate down-

regulation of GATA-1 in multipotent progenitors, leading to the generation of myeloblasts.

Eosinophils also require GATA-1 for development, as mice with a mutation in the *Gata1* promoter lack eosinophils[86]. GATA-1 also interacts with PU.1 and C/EBPε to modulate eosinophil granule protein production[87]. PU.1 is also required at earlier stages for eosinophil, neutrophil, and macrophage differentiation[87-89]. Finally, Gfi1 is required to regulate neutrophil versus monocyte lineage choice[90]. Gfi1-deficient mice develop an aberrant population of neutrophil-lineage committed cells with monocytic characteristics in place of mature neutrophils. These examples illustrate the precise timing and levels of TF expression required for optimal neutrophil and eosinophil development.

While the above data suggests that C/EBPβ may functionally overlap with C/EBPα, C/EBPε appears to have distinct functions. C/EBPε$^{-/-}$ mice lack eosinophils and present with atypical and defective peripheral neutrophils as well as myelodysplasia[91]. These mice died prematurely from opportunistic infections. C/EBPε was later shown to be required for terminal granule maturation in both eosinophils and neutrophils[92,93]. This would suggest that C/EBPε plays a key role separate from C/EBPα after differentiation from the GMP toward both neutrophils and eosinophils. Interestingly, in humans, C/EBPε has 4 different isoforms (ε^{32}, ε^{30}, ε^{27}, and ε^{14}) and these isoforms have differential abilities to promote eosinophil or neutrophil development[94]. Using CD34^{+} human BM progenitors, expression of only C/EBPε$^{32/30}$ yielded eosinophils (independent of IL-5), whereas expression of C/EBPε^{27} or C/EBPε^{14} strongly inhibited it, even in the presence of IL-5[95]. It should be noted that mice only possess two isoforms that have reported to have similar functions (C/EBPε^{36} and ε^{34})[96].

Finally, while cooperative networks of transcription factors can act to reinforce or oppose cell-type specific gene expression, an elegant set of *in vitro* experiments

demonstrated that there are graded differences even in co-expression of two factors. The authors used overexpression of either C/EBPα and GATA-2 in purified common lymphoid progenitors (CLPs) and temporally regulated their expression[97]. Their results showed that if C/EBPα was expressed prior to GATA-2, eosinophils were generated. In contrast, if GATA-2 was expressed prior to C/EBPα, basophils were the main cell type produced. Recent work examining the factors that mediate lineage choice identified that GATA-1 expression delineates progenitors capable of generating neutrophils, monocytes, and lymphocytes and those that produce eosinophils, and erythroid/megakaryocytic lineage cells[98]. In this study, they identified that GATA-1-expressing progenitors were biased toward eosinophils. Taken together, these data suggest that cell fate choice is a dynamic process that is initiated early during development through the action of transcription factor or enhancer activity.

Generation of eosinophil-deficient mice

Various key factors were identified through knockout studies as required for eosinophil development. These broadly fall two several categories: transcription factors or granule proteins. As noted above, mice lacking C/EPBα lack all granulocytes[74,75] and mice lacking C/EBPε cannot produce eosinophils or neutrophils due to defective granule production[91-93]. Deletion of a double binding site for GATA-1 in the *Gata1* promoter ablates eosinophils and basophils[86,99]. Granule protein morphogenesis is also critical for eosinophil development. Mice lacking XBP1, a regulator of the unfolded protein response, cannot produce eosinophils due to the accumulation of misfolded granule proteins and overwhelming cellular stress[100]. Along these lines, deletion of two of the major eosinophil granule proteins in tandem, MBP and EPX, blocks granule morphogenesis and eosinophil production[101]. Together, these studies indicate that there

are two critical steps in eosinophil production: lineage commitment and terminal maturation through granule morphogenesis.

Cell lineage choice is set early and correlates with enhancer landscapes

To understand how fate choices are made, it must first be understood when these choices occur. Recent work at the single cell level demonstrated that myeloid progenitor populations are highly heterogenous. Furthermore, they found that many of these cells are already primed or pre-committed to a particular lineage[98,102-104]. These studies highlight that transcription factor networks orchestrate set gene programs and these are often either primed for expression or expressed in these progenitor stages. Interestingly, one study found that within the CMP and GMP populations, there is a subset of cells that expresses elements of two or more terminal cell programs, termed "multi-lineage primed"[103]. They further found that this population can expand or contract based on the balance of the transcription factors Irf8 and Gfi1. In addition, work investigating the heterogeneity of MPPs, directly downstream of the HSC, showed that there are multiple subsets within this population that can be fractionated using cell sorting that are biased toward different lineages[105]. Together, these studies suggest that while the potential of differentiating progenitors is specified earlier than previously thought, the precise balance of transcription factor levels determines the ultimate cellular output.

Further supporting the idea that cellular fate is determined before true phenotypic differentiation, work utilizing inducible differentiation in long-term cultures of hematopoietic progenitors demonstrated that the enhancer landscapes in multi-potent progenitors (MPPs) resembles more differentiated lymphoid or myeloid cells[106]. This group examined enhancer patterns in MPPs and found that enhancers for genes

associated with multiple lineages (e.g. *Cebpa, Ebf1, Vpreb3, Thy1*) displayed H3K4 mono-methylation (me1) marks, suggesting they are poised for activation. Subsequent work from another group examined genome-wide H3K4me1 marks in progenitors and mature cells and found that progenitors for the myeloid and erythroid lineages clustered with their respective mature progeny[107]. However, when using RNA-based clustering, they found that these progenitors clustered with HSCs and MPPs. Taken together, these studies indicate that true lineage specification takes place earlier than previously thought.

Tribbles

Initially identified in Drosophila, Tribbles proteins play diverse roles and can function as both oncogenes and tumor suppressors in cancer[108-110]. There are three mammalian Tribbles homologues, including Trib1, primarily expressed by mature myeloid cells, and Trib2, largely confined to the lymphoid lineage[111,112]. Trib3 is expressed mainly in non-hematopoietic tissues. Tribbles homologues function as scaffold proteins and act mainly to promote protein degradation and sequestration. Known Tribbles protein functions can be broadly classified as being C/EBPα dependent or independent. Both are discussed below.

Tribbles proteins regulate normal and malignant hematopoiesis

Many of the known roles of Tribbles proteins in hematopoiesis depend on their interaction with C/EBPα **(Fig. 1.2)**. Ectopic expression of either *Trib1* or *Trib2* in murine hematopoietic progenitors induced acute myeloid leukemia (AML) through enhanced C/EBPα degradation, mediated by interaction with the E3-ubiquitin ligase, COP1[113-116]. Prior to developing AML, *Trib2* over-expressing mice showed skewed myelopoiesis with

decreased granulocytic and enhanced monocytic differentiation[113]. Trib2-induced AML cells demonstrated decreased C/EBPα p42 protein[113]. Trib1 directly interacts with both COP1 and C/EBPα, allowing for C/EBPα ubiquitination and degradation. The importance of the Trib1:C/EBPα interaction will be discussed in **CHAPTER 4**.

In a similar manner, loss of Trib2 in a Notch-driven model of T cell acute lymphoblastic leukemia (T-ALL), resulted in increased penetrance and decreased latency of disease[117]. Trib2-deficient leukemic blasts from these mice had increased expression of C/EBPα p42, but did not show changes in ERK or AKT pathway activation. These studies underscore the critical role exerted by Tribbles proteins in regulating cellular identity and differentiation.

Recent work demonstrates that Trib1 regulates steady state myeloid

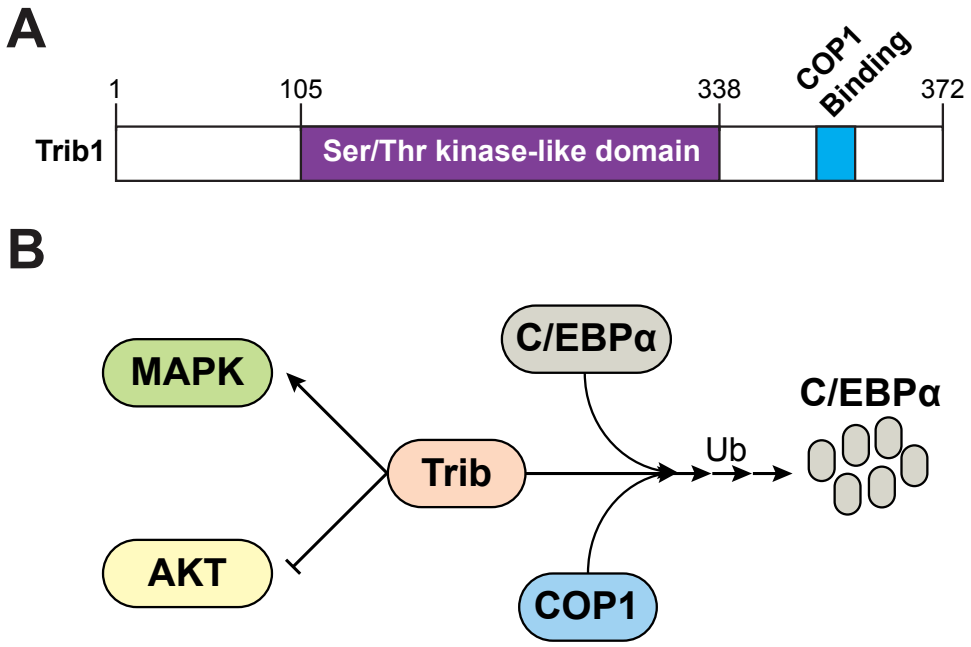

Fig. 1.2: Overview of Trib1 structure and Tribbles protein function. A) Model of Trib1 structure, highlighting the kinase-like domain and the COP1 binding site. B) Model of known protein/pathway interactions with all Trib homologues. Adapted from ref. 112.

development; Trib1$^{-/-}$ mice lack M2 macrophages and eosinophils, and have more neutrophils[118]. An increase in C/EBPα protein levels was observed, and *Cebpa* knockdown partially rescued *ex vivo* myeloid differentiation[118]. In an analogous manner, Trib1 regulates hepatic lipid metabolism by facilitating the degradation of C/EBPα[119]. This work also demonstrated that C/EBPα regulates *Trib1* expression itself in the liver through a negative feedback loop. It is unknown, however, how *Trib1* expression is regulated during myeloid development. Furthermore, despite these data, it is unclear at what developmental stage Trib1 modulates the balance between eosinophil and neutrophil cell identity.

Tribbles homologues regulate cell function

As noted above, loss of Trib2 in a Notch-driven T-ALL model did not alter ERK or AKT pathway activation[117]. These data are in contrast to reports showing that Tribbles proteins can modulate activation of these signaling pathways. Tribbles proteins have been reported to interact with the MAPK pathway. In particular, human TRIB1 and TRIB3 suppressed MAPK activation in HeLa cells[120] and human TRIB2[121] inhibited ERK and JNK activation in human monocytes. In addition, both mouse Trib2 and Trib3 were shown to inhibit AKT activation during adipocyte differentiation[122,123]. Together, through a variety of pathways, Tribbles proteins impact cellular differentiation and function. Despite these data, much remains to be learned regarding how Tribbles proteins function. The role of Trib1 in modulating these pathways in neutrophils will be discussed in **CHAPTER 5**.

Tools to study Trib1 function in granulopoiesis

To evaluate the role of Trib1 in granulocyte development and identity, I took several approaches **(Fig. 1.3)**. The bulk of the work I discuss here centers on the generation and testing of new conditional deletion models to selectively ablate *Trib1* expression. Our group and others found that global deletion of Trib1 impairs embryonic viability **(data not shown and ref.[118])**. To circumvent this, prior to my studies, the lab took advantage of a mouse with a *Trib1* allele flanked by LoxP sites to allow for Cre-mediated deletion[119]. Using this mouse, we generated a mouse strain lacking *Trib1* in all hematopoietic cells using VavCre[124]. These mice, termed Trib1$^{\Delta HSC}$, will show the role of Trib1 both early and late during granulopoiesis and result in both terminal eosinophils and neutrophils lacking *Trib1*. Prior studies demonstrated that Trib1 impacts multiple lineages. To specifically delete Trib1 in eosinophils and to separate out effects on terminal cell identity from lineage commitment, I generated a conditional Trib1 deletion strain using EoCre[125]. This allows for selective ablation of *Trib1* only in eosinophil lineage cells. To have more temporal control over when Trib1 deletion occurs, we generated mice with the conditional *Trib1* allele crossed to a tamoxifen-inducible *Cre*, ER^{T2}-Cre[126]. These three mouse strains will allow me to examine the dynamics of Trib1-mediated regulation of granulocyte development and to answer more fundamental questions of how the granulocyte lineage is both established and maintained.

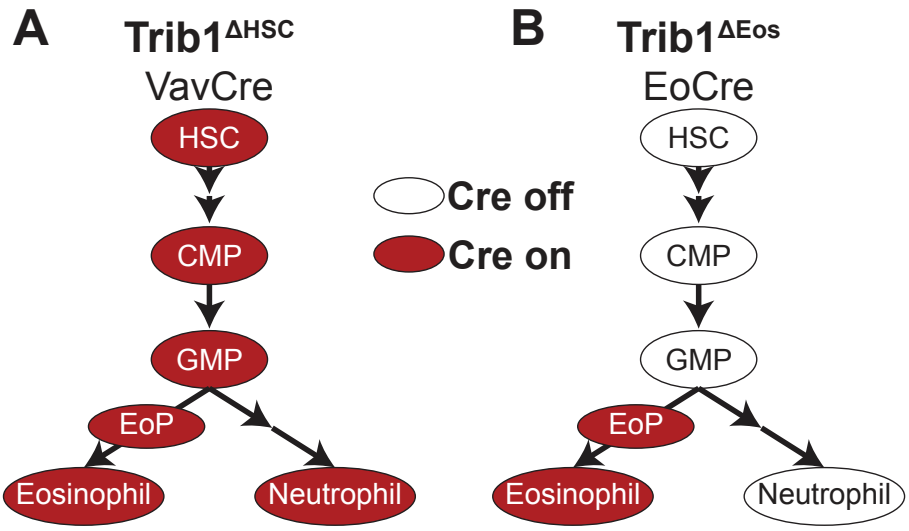

Fig. 1.3: Models of Trib1 deletion strategies. Deletion of Trib1 in mouse granulocyte development using VavCre (A) to delete in all hematopoietic cells or EoCre (B) to delete in only eosinophil lineage cells following lineage commitment.

CHAPTER 2: TRIB1 REGULATES GRANULOCYTE PROGENITOR LINEAGE PROGRAMMING[1]

INTRODUCTION

Hematopoietic cells depend on a finely balanced network of signaling pathways to progress from multipotent progenitors to terminal effectors and maintain cellular identity. Eosinophils and neutrophils are vital for host defense yet contribute to the pathogenesis of many atopic and inflammatory conditions[7,127]. These subsets develop from the granulocyte/macrophage progenitor (GMP)[49], and eosinophils subsequently arise from a downstream committed eosinophil progenitor (EoP) in the bone marrow (BM)[50]. However, how eosinophil lineage commitment is regulated is not well understood.

Cytokine signals influence cell fate decisions

During hematopoietic development, cellular output must meet the needs of the organism, whether that is enhanced erythropoiesis to cope with blood loss or enhanced granulopoiesis to support bacterial clearance[128]. To that end, progenitors must adapt to changing conditions in the BM and alter cellular output. It was first reported by Weismann and colleagues that forced expression of the IL-2 and GM-CSF receptors on common lymphoid progenitors (CLPs) could convert them myeloid lineage cells[129]. The authors further demonstrated that HSCs express low levels of myeloid-associated cytokine receptors and that these are lost upon differentiation to CLPs

[1]Portions of this chapter are adapted from "Trib1 regulates eosinophil lineage commitment and identity by restraining the neutrophil program", Ethan A. Mack, Sarah J. Stein, Kelly S. Rome, Lanwei Xu, Gerald B. Wertheim, Rossana C.N. Melo, and Warren S. Pear, *Blood*, in revision.

Subsequently, multiple reports identified that cytokines act directly on HSCs and precursor cells to alter their developmental potential. Both IFN-α[130] and IFN-γ[131] activate quiescent HSCs to proliferate. Further work illustrated that inflammatory signals direct hematopoietic progenitors toward the myeloid fate. When stimulated with toll-like receptor (TLR) ligands, HSCs are activated and differentiate into myeloid lineage cells[132,133]. It was additionally demonstrated that HSCs and progenitors directly sense inflammatory stimuli and respond with increased proliferation, elevated cytokine production, and altered differentiation potential[134-137]. Together, this suggests that there are multiple extrinsic signals that can modulate development of HSCs and progenitors.

This work raises the question of if these cytokine signals act in an instructive manner to alter cellular development or if they provide survival signals that allow for pre-scripted programs to be carried out. By using a Bcl-2 transgene, one group demonstrated that cytokine signaling did not rescue alternatively primed precursors from apoptosis[129]. Taken together, the above reports suggest that cytokine signals act in concert with transcription factors to alter BM development, indicating that progenitor potential is malleable and able to respond to host requirements.

Trib1 in myeloid development

Recent work implicates the Tribbles pseudokinase family in myelopoiesis. Tribbles proteins primarily act as adaptors to promote protein degradation and/or sequestration[108-110]. There are three mammalian tribbles homologues (Trib1-3) that are defined by a central serine/threonine kinase-like domain and C-terminal sequences that bind the E3 ubiquitin ligase COP1[113-116]. Mice with a germline deletion of Trib1 lack “M2” macrophages and eosinophils, and have more neutrophils[118], a phenotype that is influenced by the failure of Trib1 to promote CCAAT/Enhancer Binding Protein Alpha

(C/EBPα) protein degradation[118]. C/EBPα plays a critical role in myeloid development and will be discussed separately in **CHAPTER 4**. Myelopoiesis is unaffected in mice lacking Trib2 or Trib3[117,118].

While previous work revealed alterations in myeloid populations with Trib1 loss, the identity of the factors involved in lineage priming during granulopoiesis and terminal granulocyte identity are not well established. Using hematopoietic *Trib1* deletion, we found that Trib1 regulates eosinophil precursor lineage commitment. Conditional Trib1 deletion in HSCs reduced the size of the EoP pool. We further demonstrate that IL-5 drives eosinophil differentiation from the GMP, and Trib1 acts to suppress the neutrophil program in lineage-committed eosinophils precursors. Furthermore, we show that Trib1 normally suppresses neutrophil numbers at the end of a short course IL-5 culture. Together, our findings provide new insights into early steps in granulocyte development, where Trib1 acts to control eosinophil lineage commitment from the GMP and suppress the neutrophil program in response to IL-5, promoting eosinophil terminal identity and lineage fidelity.

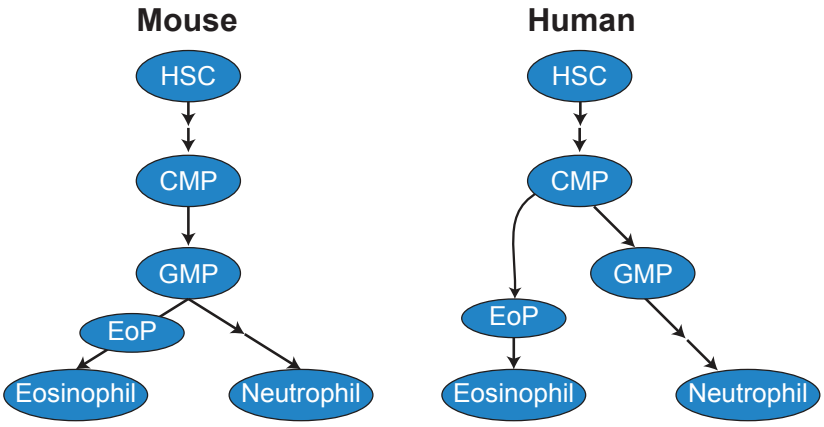

Fig. 2.1: Model of granulopoiesis. Model of mouse (left) and human (right) granulopoiesis[49, 51].

RESULTS

Trib1 regulates eosinophil lineage commitment from the GMP

To understand Trib1 function in eosinophil commitment, we determined its expression in myeloid progenitors. During murine eosinophilopoiesis, EoPs arise from GMPs[50] **(Fig. 2.1)**. We measured *Trib1* mRNA expression in CMP, GMPs, and EoPs and found that *Trib1* is highly expressed in EoPs compared to CMPs or GMPs **(Fig. 2.2A)**. *TRIB1* is also upregulated during human eosinophil differentiation[138], in which eosinophils arise from the CMP[51] **(Fig. 2.2B)**. Subsequently, we measured expression of *Trib1* and found high expression in neutrophils with lower, but detectable expression in eosinophils **(Fig. 2.2C)**. To investigate how Trib1 modulates granulocyte development, we generated mice expressing VavCre[124] and a conditional *Trib1* allele[119] to delete *Trib1* in all post-embryonic hematopoietic cells ($Trib1^{\Delta HSC}$). *Trib1* deletion was validated by qPCR in the BM compared to mice expressing VavCre alone ($Trib1^{+/+}$) **(Fig. 2.2D)**. Similar to Trib1 germline knockout mice[118], $Trib1^{\Delta HSC}$ mice had increased neutrophils and markedly decreased eosinophils in the spleen, blood, lung, and colon **(Fig. 2.3 and data not shown)**. Of note, $Trib1^{\Delta HSC}$ mice had significantly larger spleens compared to $Trib1^{+/+}$ **(Fig. 2.3J)**, reflecting the expansion of neutrophils.

Our conditional Trib1 deletion model provided the opportunity to investigate the effect of Trib1 deletion on the dynamics of eosinophil differentiation and identity, which were not previously explored. Analysis of different myeloid developmental subsets in Trib1$^{\Delta HSC}$ mice showed that the CMP and GMP populations were unperturbed **(Fig. 2.4)**; whereas the EoP population was significantly decreased **(Fig. 2.5)**. These data not only show that *Trib1* expression is induced following GMP differentiation, but that this increased expression functionally impacts eosinophil commitment in an early progenitor.

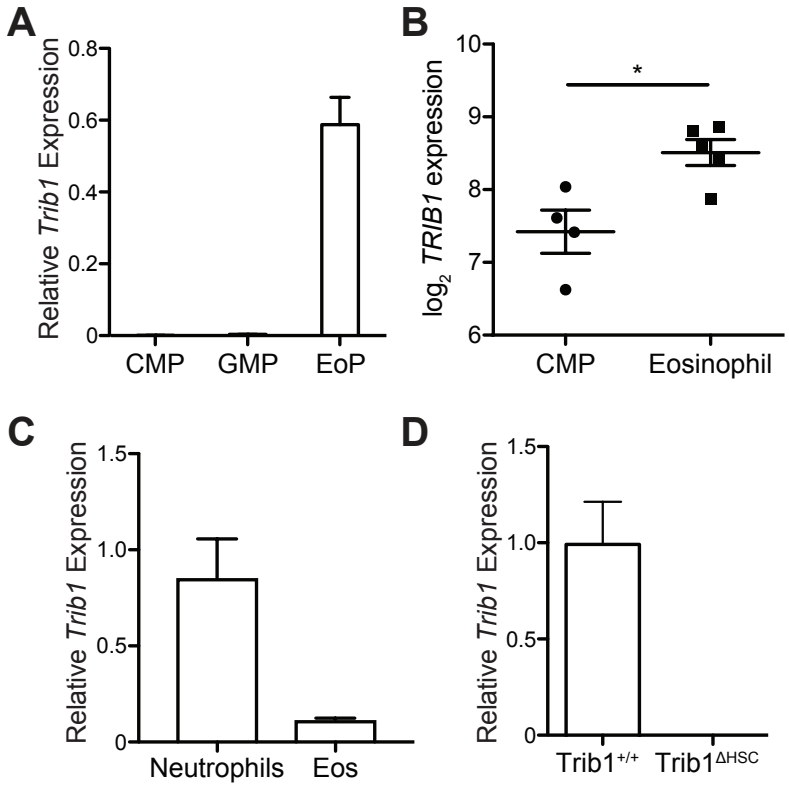

Fig. 2.2: *Trib1 expression increases with eosinophil commitment*. A) *Trib1* expression in sorted CMP, GMP, and EoP from WT C57BL/6 mice relative to *18s*, normalized to Trib1$^{+/+}$ neutrophils, representative of 3 experiments. B) Relative expression of *TRIB1* in human CMP and eosinophils obtained from the DMAP dataset[138] using BloodSpot[271]. C) *Trib1* expression in sorted BM neutrophils and eosinophils from Trib1$^{+/+}$ mice, relative to *18s*, normalized to Trib1$^{+/+}$ neutrophils, n=3 mice/group, representative of 3 experiments. D) qPCR of Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ whole BM for *Trib1* expression relative to *18s*, normalized to Trib1$^{+/+}$ BM, representative of 3 experiments. *p=0.0131, unpaired student's *t* test.

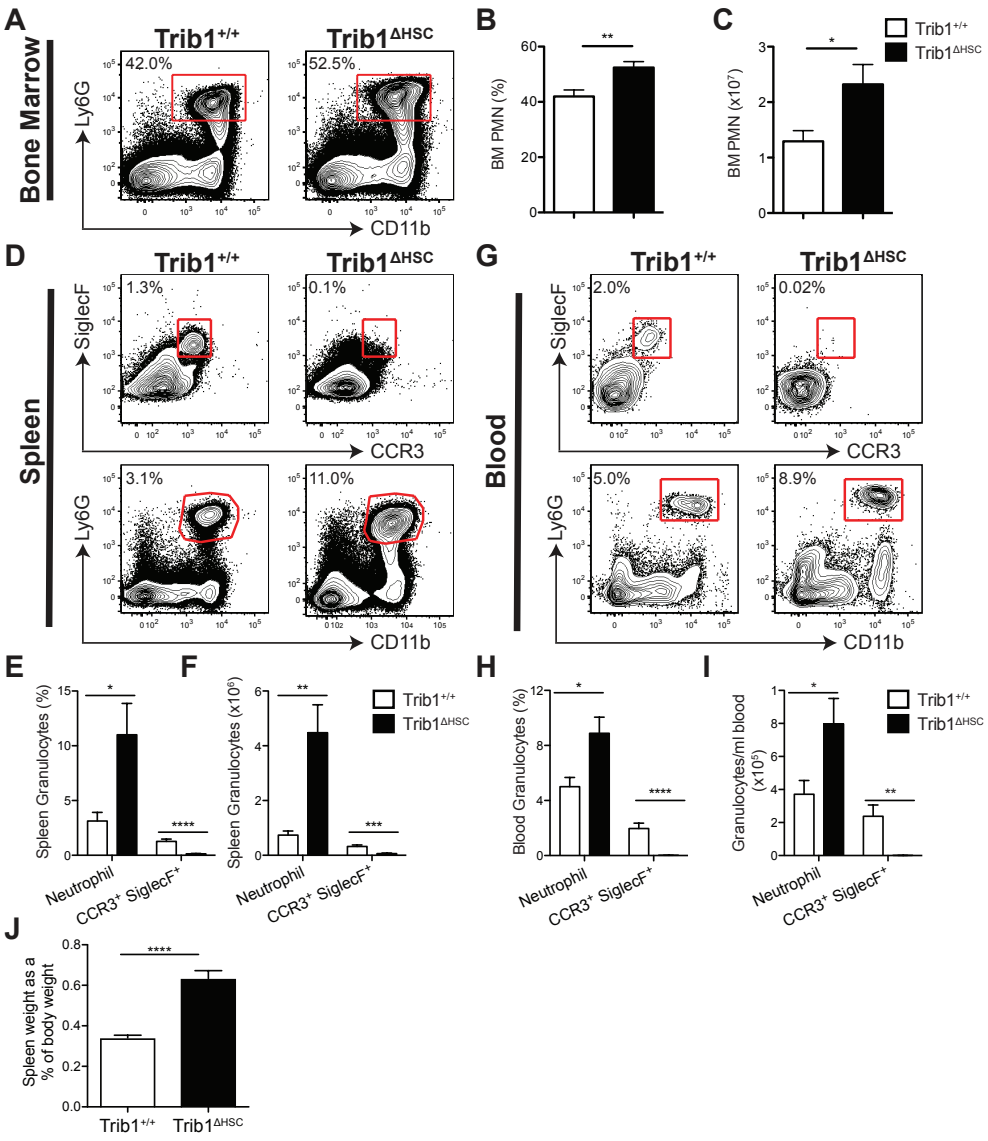

Fig. 2.3: Early Trib1 deletion increases neutrophils and diminishes peripheral eosinophils. A) Representative plots of BM neutrophils from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice. B) Frequency of live cells and C) absolute numbers of BM neutrophils, n=11-12 mice/group from 4 expts. D) Representative plots of splenic eosinophils (upper) and neutrophils (lower) from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice. E) Frequency of live cells and F) absolute numbers of splenic granulocytes, n=11-12 mice/group from 4 expts. G) Representative plots of blood eosinophils (upper) and neutrophils (lower) from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice. H) Frequency of live cells and I) absolute numbers of blood granulocytes. Eosinophil plots gated on live, CD11b^{+} cells, neutrophil plots gated on live, SiglecF^{-} cells. Frequency n=12-13 mice/group from 3 expts, absolute numbers n=7 mice/group, representative of 2 expts. J) Spleen weight as a fraction of body weight n=23-24 mice/group, pooled from 8 experiments. *p<0.0230, **p<0.0047, ***p=0.0001, ****p<0.0001, unpaired student's *t* test. Frequencies and error bars are mean±SEM of live cells.

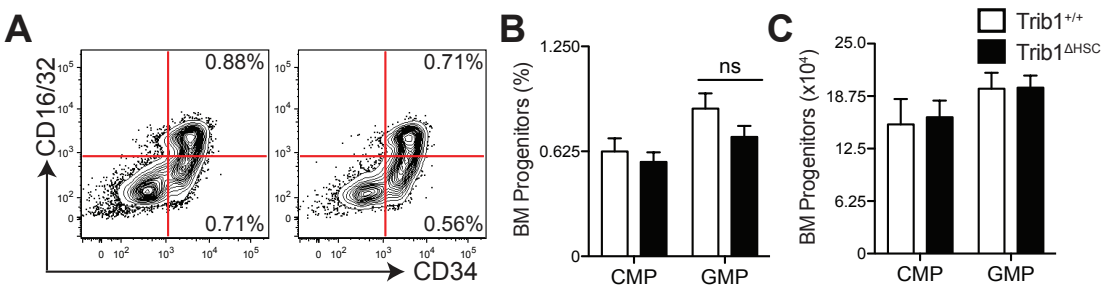

Fig. 2.4: No change in CMP and GMP populations with hematopoietic Trib1 deletion A) Representative plots of BM myeloid progenitors from Trib1$^{+/+}$ or Trib1$^{\Delta HSC}$ mice, gated on live, lineage^{-} c-Kit^{+} cells. B) Frequency of live cells and C) absolute number of CMP and GMP, n=10/group pooled from 4 experiments. ns=not significant, unpaired student's *t* test. Frequencies and error bars are mean±SEM of live cells.

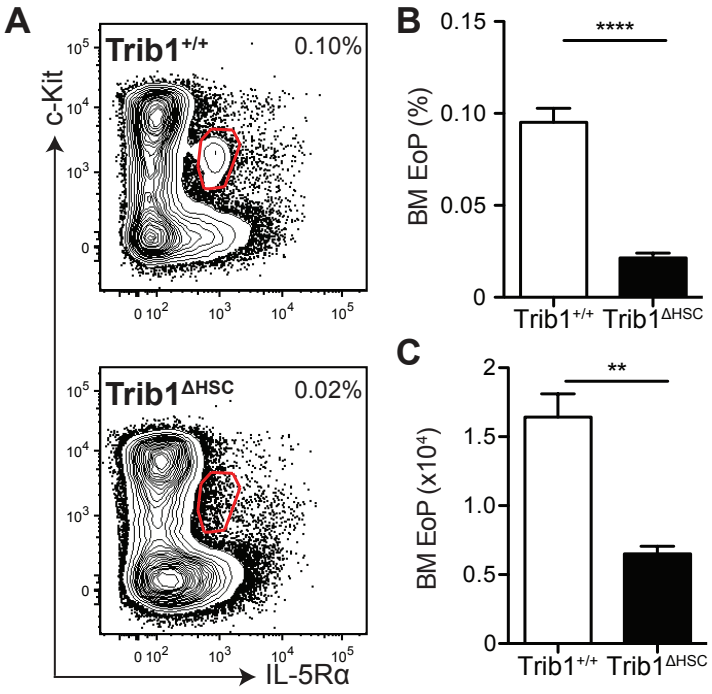

Fig. 2.5: Trib1 is required for EoP homeostasis. A) qPCR of Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ whole BM for *Trib1* expression relative to *18s*, normalized to Trib1$^{+/+}$ BM, representative of 3 experiments. B) Representative plots of BM EoP, gated on live, lineage^{-} CD34^{+} cells. D) Frequency of live cells and D) absolute number of BM EoP, n=4 mice/group, representative of 2 experiments. **p=0.0013, ****p<0.0001, unpaired student's *t* test. Frequencies and error bars are mean±SEM of live cells.

Trib1 represses the IL-5 driven neutrophil program in developing eosinophils ex vivo

The cytokine IL-5 is sufficient to drive eosinophil development *ex vivo*[58,139]. In order to assess the ability of exogenous IL-5 to restore normal eosinophil development in Trib1-null progenitors, we used a previously described *ex vivo* culture system[54] **(Fig. 2.6A)**. After culture with IL-5, Trib1$^{+/+}$ whole BM yielded a large population of eosinophils, while eosinophil output by Trib1$^{\Delta HSC}$ BM was decreased **(Figs. 2.6B-D)**. Unexpectedly, neutrophils were expanded in IL-5 culture of Trib1$^{\Delta HSC}$ BM. In **CHAPTER 3**, we will functionally characterize these cells. This neutrophil bias is similar to what was observed *in vivo* in Trib1$^{\Delta HSC}$ mice, with a neutrophil expansion and a loss of peripheral eosinophils **(Fig. 2.3)**. Together, this suggests that IL-5 alone is not sufficient to restore normal

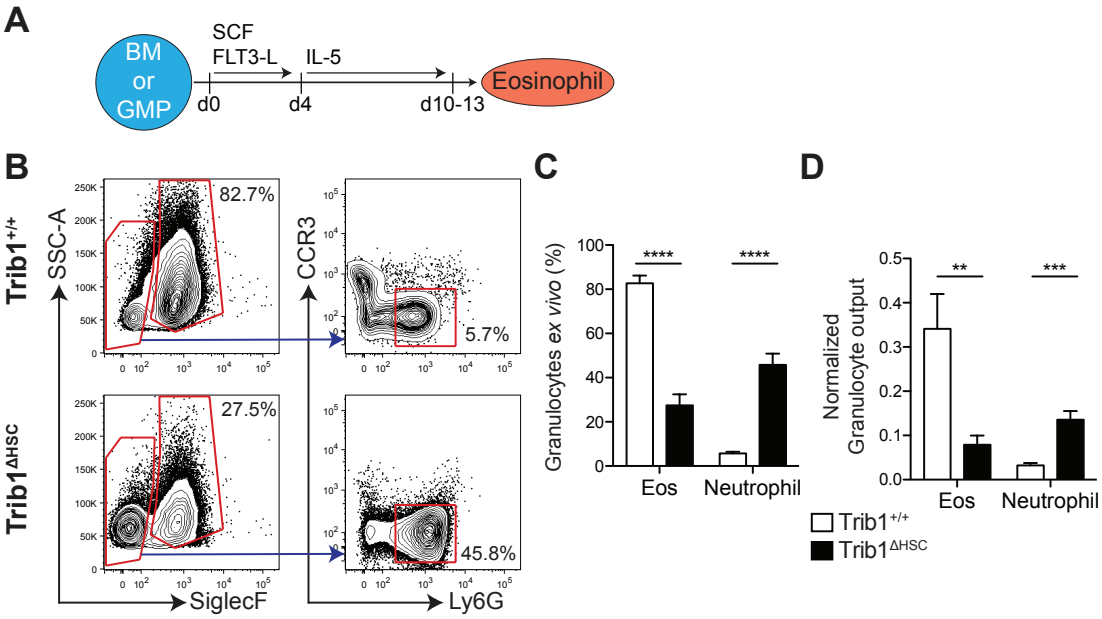

Fig. 2.6: Trib1 represses neutrophil differentiation ex vivo in response to IL-5. A) Schematic of *ex vivo* eosinophil differentiation assay. B) Representative plots of d10 IL-5 cultures Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ whole BM gated on live cells, representative of 4 experiments. C) Frequency of live cells and D) d10 cell output normalized to d4 cell counts assay using Trib1$^{+/+}$ or Trib1$^{\Delta HSC}$ BM. Frequency n=5 mice/group pooled from 3 experiments; cell output n=6 mice/group pooled from 4 experiments. Eosinophils gated on SiglecF^{+} CCR3^{+}; neutrophils gated on Ly6G^{+} SiglecF^{-}. Frequencies and error bars are mean±SEM of live cells.

granulocyte output from Trib1-deficient BM. Furthermore, this indicates that Trib1 normally functions to restrain neutrophil differentiation, survival, and/or proliferation.

To determine how Trib1 influences myeloid progenitor potential, we sorted and cultured GMP from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice. After 10 days of IL-5 culture, Trib1$^{+/+}$ GMPs generated predominantly eosinophils, while eosinophil output from Trib1$^{\Delta HSC}$ GMPs was significantly decreased **(Figs. 2.7A-B)**. Additionally, Trib1$^{\Delta HSC}$ GMPs produced more neutrophils, similar to data obtained using whole Trib1$^{\Delta HSC}$ BM **(Figs. 2.6B-D)**. We next measured *Trib1* in cells derived from GMPs cultured in IL-5 to evaluate its expression kinetics. *Trib1* expression increased from undetectable levels to detectable levels by d4 of culture, and continued to increase post-IL-5 addition **(Fig. 2.7C)**. *Epx* expression was blunted in cultured Trib1$^{\Delta HSC}$ GMPs compared to Trib1$^{+/+}$ cultures **(Fig. 2.7D)**. Conversely, *Ltf* expression was markedly increased in Trib1$^{\Delta HSC}$ GMP cultures during the 10-day culture period, whereas expression in Trib1$^{+/+}$ cells decreased after d6 **(Fig. 2.7E)**. These data suggest that Trib1 normally prevents neutrophil gene expression and that Trib1 works together with IL-5 to guide normal eosinophil eosinophil development. In the absence of Trib1, developing progenitors are unable to extinguish or are biased toward the neutrophil program, beginning at or prior to the GMP stage.

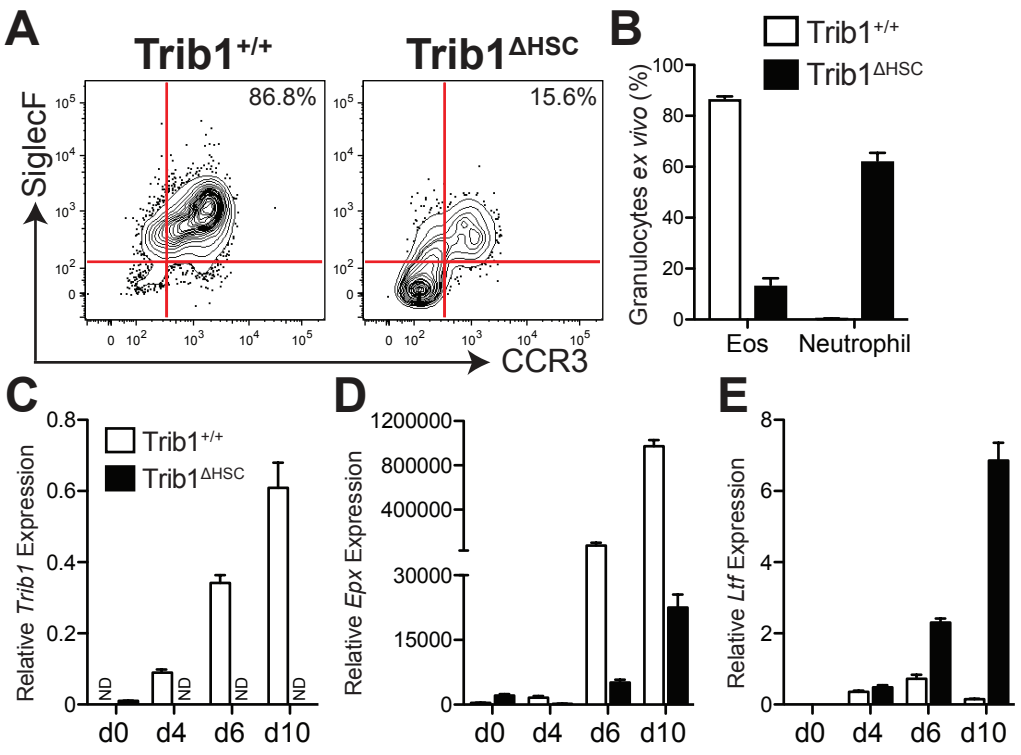

Fig. 2.7: Trib1 loss skews GMP potential toward neutrophils A) Representative plots of d10 IL-5 cultures of sorted GMPs from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice, gated on live, CD11b^{+} cells. B) Quantification of granulocyte output at d10 IL-5 culture of sorted GMPs, n=3-5 wells/genotype. Representative of 2 experiments. C) *Trib1*, D) *Epx*, and E) *Ltf* expression during eosinophil *ex vivo* culture of sorted Trib1$^{+/+}$ or Trib1$^{\Delta HSC}$ GMPs relative to *18s*, normalized to Trib1$^{+/+}$ neutrophils, representative of 3 experiments. ND=not detected. Cells were cultured as in **Fig. 2.6A**.

To further characterize the kinetics of Trib1-mediated regulation of granulocyte output from BM progenitors, we generated an inducible Trib1 knockout mouse using a tamoxifen-inducible *Cre* recombinase (ER^{T2}-Cre)[126] crossed to a mouse with the conditional Trib1 allele[119]. When treated with tamoxifen, these mice will delete *Trib1* in all cells (control ER^{T2}-Cre^{+} $Trib1^{+/+}$; knockout ER^{T2}-Cre^{+} $Trib1^{Fl/Fl}$). Using the above culture system **(Fig. 2.6A)**, we cultured lineage-depleted BM from either ER^{T2}-Cre^{+} $Trib1^{+/+}$ or ER^{T2}-Cre^{+} $Trib1^{Fl/Fl}$ mice. In parallel cultures, we added tamoxifen either on d0, d4, or d8, and analyzed culture output on d12 **(Fig. 2.8)**. Tamoxifen addition at d0 mirrored the results seen with $Trib1^{\Delta HSC}$ mice. However, later tamoxifen addition to ER^{T2}-Cre^{+} $Trib1^{Fl/Fl}$ cultures at d4 and more so at d8, both reduced the neutrophil output frequency and increased the number of eosinophils generated. These data, together with what we observed above, indicates that Trib1 regulates the switch between neutrophil and eosinophil production in response to IL-5 signaling and this switch occurs early in development, likely at the GMP or immediately preceding lineage commitment. We will

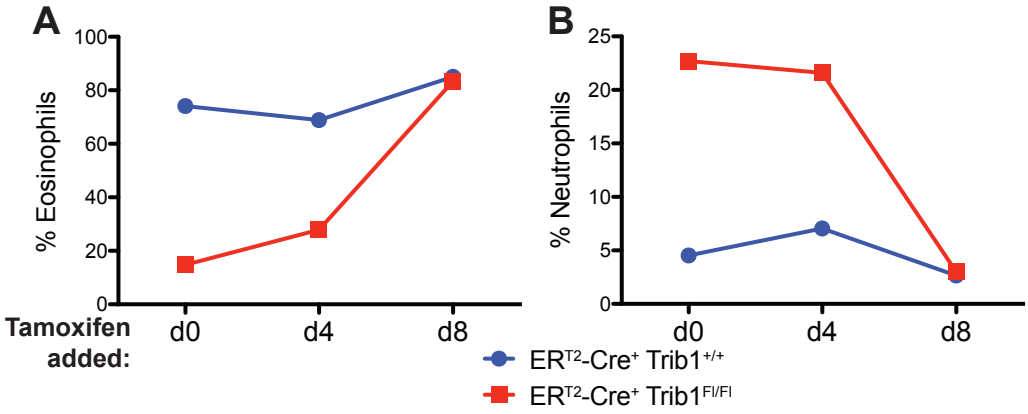

Fig. 2.8: Trib1 is required early following IL-5 stimulation to control eosinophil lineage commitment and to suppress the neutrophil program. Quantification of A) eosinophil and B) neutrophil output assessed at d12 IL-5 culture of lineage negative BM from ER^{T2}-Cre^{+} $Trib1^{+/+}$ mice (blue) or ER^{T2}-Cre^{+} $Trib1^{Fl/Fl}$ mice (red), n=1 well/timepoint, representative of 2 experiments. 500nM 4-OH-tamoxifen added on the days indicated.

continue to explore this idea in **CHAPTER 3**.

While it appears that Trib1 loss supports neutrophils following IL-5 signaling, it is unclear how Trib1 shapes the response to differentiation in response to G-SCF, one of the main cytokines normally driving neutrophil differentiation[140]. To address this, we cultured Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ BM in G-CSF. Cells were cultured for 4 days in Flt3L and SCF followed by 4 days in G-CSF. The frequency and absolute number of neutrophils generated were increased in the absence of Trib1 **(Fig. 2.8)**. This indicates that in the absence of Trib1, developing granulocytes are better able to generate neutrophils in response to neutrophil-promoting conditions. This likely correlates with the increase in neutrophils seen *in vivo* in Trib1$^{\Delta HSC}$ mice **(Fig. 2.3)**.

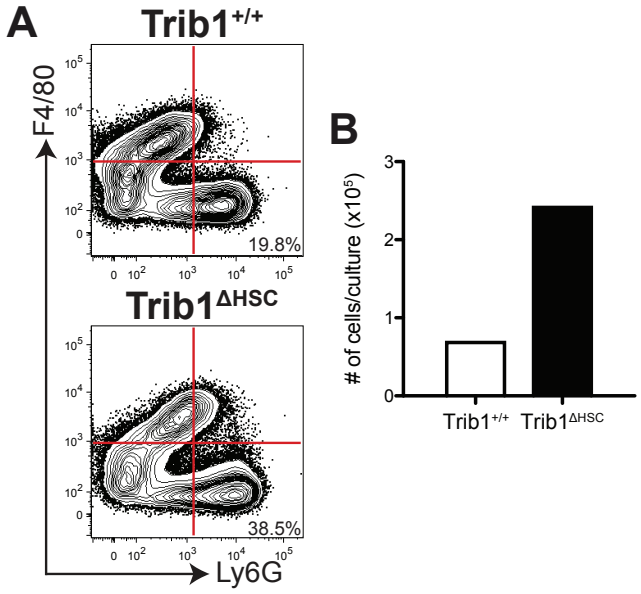

Fig. 2.9: Trib1 loss increases G-CSF-driven neutrophil production *ex vivo*. A) Representative plots of cultured BM at d8 after 4 days of 100ng/ml SCF and 100ng/ml Flt3L, followed by 4 days of 10ng/ml G-CSF from Trib1$^{+/+}$ or Trib1$^{\Delta HSC}$ mice. Plots gated on live, CD11b^{+} cells. Frequency of live cells shown. B) Absolute number of neutrophils generated during G-CSF culture at d8. n=1 mouse/group, representative of 1 experiment.

DISCUSSION

Differentiation of hematopoietic cells from multipotent progenitors to terminally differentiated cells requires early progenitors to establish a unique cellular identity through activating and repressing specific gene sets. Previous work using germline Trib1 knockout mice demonstrated that eosinophil numbers were suppressed and neutrophil numbers were enhanced in the absence of Trib1[118], yet at what developmental stage Trib1 functioned was unknown. We found that *Trib1* is expressed in EoPs, but is undetectable in CMPs and GMPs, with similar dynamics in human eosinophil development, suggesting a role for Trib1 at the earliest stages of eosinophil development. We show that Trib1 acts at to modulate the earliest stages of granulocyte development. Trib1 loss in HSCs leads to a selective decrease in EoPs and a concomitant increase in neutrophils, suggesting that blocking early eosinophil commitment shunts myeloid progenitors toward neutrophils.

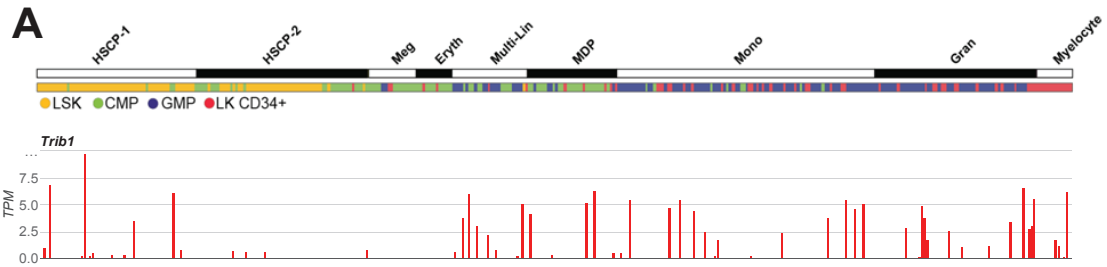

Fig. 2.10: Select single GMPs express *Trib1*. A) Single-cell RNAseq data from ref. 103 analyzed for *Trib1* expression. Upper black and white portion represents the putative clusters derived from transcriptional profiling. The colored lines are the surface phenotypes of the cells sorted for sequencing.

Our data show that GMPs lacking Trib1 produce both eosinophils and neutrophils when cultured with IL-5, while WT GMPs primarily generate eosinophils **(Figs. 2.7A-B)**, suggesting that Trib1 modulates lineage priming of myeloid progenitors. Additionally,

Trib1$^{\Delta HSC}$ progenitors cultured with G-CSF were better able to make neutrophils than their Trib1$^{+/+}$ counterparts **(Fig. 2.9)**. This suggests that either in the absence of Trib1, the progenitor pool is enriched for neutrophil-primed cells, or Trib1 loss enhances the ability of G-CSF to signal for neutrophil differentiation or survival. While we do not observe *Trib1* expression in the bulk GMP population, recent work identified *Trib1* expression in individual CMP and GMP cells[103] **(Fig. 2.10)**. Furthermore, these reports show that individual myeloid progenitors may be "pre-committed" to a specific lineage[98,102,103]. Future studies are required at the single-cell level to determine if Trib1 influences eosinophil lineage programming in CMPs or GMPs, prior to the EoP stage, and to delineate the precise origin of these cells.

Culture in IL-5 alone is sufficient to expand neutrophils from Trib1-deficient BM. Using temporally controlled deletion with ER^{T2}-Cre, we demonstrate that early in development, there is a high degree of lineage plasticity. Specifically, early deletion with tamoxifen treatment at d0 or d4 of culture, prior to IL-5 addition, showed cells that were permissive for neutrophil development. In contrast, deletion at d8, 4 days after the cells first saw IL-5, demonstrated that the eosinophil program was fixed. Together, these data support the idea that IL-5 can support both the eosinophil and neutrophil programs, and that Trib1 modulates the response to this cytokine.

We observe that Trib1-deficient progenitors yielded more neutrophils when cultured in IL-5 compared to WT progenitors. These data, together with the *in vivo* neutrophil expansion that we and others observe with Trib1 loss,[118] suggest that Trib1 expression in early myeloid progenitors restricts neutrophil development. However, the precise stage when Trib1 modulates neutrophil development and function is not known. Furthermore, in the IL-5 culture, it is unclear if Trib1 regulates neutrophil development itself. Our data cannot distinguish this from Trib1 regulation of survival or proliferation.

Alternatively, it is possible that this increase in neutrophils is independent of IL-5 and simply an intrinsic result of Trib1 loss. Recent work has begun to characterize stable intermediates of neutrophil differentiation[70-73]. Thus, further work is required to determine the precise role of Trib1 in neutrophil differentiation and function and will be discussed in **CHAPTER 5**.

In summary, our studies reveal Trib1 as a key regulator of eosinophil development and homeostasis. We find that Trib1 influences at the earliest stages of eosinophil commitment, by modulating the response to cytokine signals. These findings clarify long-standing questions in granulopoiesis regarding regulation of cell lineage choice, and provide a path forward in the study of eosinophil and neutrophil development.

CHAPTER 3: TRIB1 REPRESSES THE NEUTROPHIL PROGRAM TO CONTROL EOSINOPHIL IDENTITY[2]

INTRODUCTION

Cellular differentiation into mature cells requires cells to activate certain gene sets and repress others. Yet this begs the question: how fixed are these cellular identities once they are adopted? Work over the last several years demonstrated that ectopic expression of transcription factors is able to dramatically alter cellular phenotype and identity. The subsequent work suggests that while mature cell identity is stable, external stimuli or internal perturbations that alter the transcriptional landscape of the cell could have profound consequences on lineage.

Demonstrating the importance of transcription factors in controlling cellular identity, two studies used forced expression of the myeloid transcription factor C/EBPα to transform lymphocytes into myeloid cells. Firstly, in $CD19^+$ B cell precursors isolated from the BM, overexpression of C/EBPα converted these to $CD11b^+$ myeloid cells[141,142]. The authors showed that this same strategy works with mature splenic B cells, notably with a decrease in transformation efficiency. Subsequently, the same group used a similar approach, but took pre-T cells and used C/EBPα or PU.1 to transform them into macrophages or dendritic cells, respectively[143]. This second report did not attempt to transform peripheral T cells. It should be noted that in these studies, there was a requirement for cell division or proliferation to allow for the transdifferentiation. Finally, it is interesting to place this in the context of work demonstrating that a critical step in T cell development is the suppression of C/EBPα by the Notch-target Hes1[144], suggesting

[2]Portions of this chapter are adapted from "Trib1 regulates eosinophil lineage commitment and identity by restraining the neutrophil program", Ethan A. Mack, Sarah J. Stein, Kelly S. Rome, Lanwei Xu, Gerald B. Wertheim, Rossana C.N. Melo, and Warren S. Pear, *Blood*, in revision.

that active suppression of alternative cell fates is required for lineage determination. This occurs in granulocytes as well, with C/EPBα inducing a microRNA that suppresses Notch1[145]. This indicates that exclusion of alternative fates is critical for proper lineage programming.

When assessing the ease of altering cell identity, the above studies suggest that more terminally differentiated cells are more difficult to transform. In a study examining the myeloid conversion of pro-T cells through ectopic IL-2Rβ expression, only pro-T1 and pro-T2 cells were malleable, whereas more mature pro-T3 cells could not be converted to the myeloid fate[146]. Further supporting this hypothesis, when a separate group used the Yamanaka factors (Oct4, Sox2, Klf4 and c-Myc) to reprogram B cells to induced pluripotent stem (iPS) cells, they found that while these factors were sufficient to reprogram pro- and pre-B cells, they needed to either ablate Pax5 or add C/EBPα to reprogram mature B cells[147]. This indicates that more mature cells present roadblocks to alterations in identity that are not present in less differentiated cells. This further suggests cell intrinsic differences that make cell identity more fixed in terminally differentiated cells as compared to their more immature precursors.

In **CHAPTER 2**, I discussed the role of Trib1 in modulating granulocyte precursor potential and eosinophil lineage commitment. Prior to this work, little was known about at what developmental stage Trib1 acted to influence the balance between eosinophils and neutrophils. I showed in **CHAPTER 2** that Trib1 loss in HSCs using VavCre reduced the EoP population. Furthermore, Trib1$^{\Delta HSC}$ progenitors preferentially yielded neutrophils on exposure to IL-5. These data show that Trib1 is able to modulate early progenitor potential and tune the response to IL-5 signaling. However, this work does not shed light on if or how Trib1 acts to modulate the identity of mature granulocytes. In this chapter, I will explore how Trib1 controls mature eosinophil identity. For the studies below, I

generated a new mouse strain to specifically isolate the impact of Trib1 loss to the eosinophil lineage. By crossing mice with a conditional Trib1 allele (cTrib1) [119] to mice expressing *Cre* recombinase under the control of the *Eosinophil peroxidase* (*Epx*) promoter[125], I am able to selectively ablate *Trib1* in eosinophil lineage cells, starting at the EoP. In this chapter, I will refer to these mice as Trib1$^{\Delta Eos}$. Using these conditional deletion strains, I further characterize the role of Trib1 in granulocyte development. I demonstrate that Trib1 controls both eosinophil lineage commitment and terminal identity.

RESULTS

Trib1$^{\Delta HSC}$ mice develop a population of Ly6G^{+} eosinophils in the BM

Given that the EoP defect occurs with Trib1 loss **(Fig. 2.5)**, we next examined terminal eosinophil maturation. As eosinophils mature in the BM, they gain expression of CCR3[148]. While previous reports demonstrated an absence of BM eosinophils with global Trib1 loss[118], we observed a preservation of eosinophils in Trib1$^{\Delta HSC}$ BM. Both CCR3^{-} and CCR3^{+} eosinophils were present in the BM of Trib1$^{\Delta HSC}$ mice **(Figs. 3.1A-C),** and the number of CCR3^{+} eosinophils was increased in Trib1$^{\Delta HSC}$ BM. This was accompanied by a decrease in the frequency and number of CCR3^{-} eosinophils **(Figs. 3.1A-C)**. Strikingly, nearly all CCR3^{+} eosinophils in Trib1$^{\Delta HSC}$ BM expressed surface Ly6G, a neutrophil-specific antigen[149] **(Fig. 3.1D)**. Ly6G expression increased as the cells gained CCR3 expression **(Figs. 3.1D-E)**. The Ly6G^{+} SiglecF^{+} CCR3^{+} cells found in Trib1$^{\Delta HSC}$ mice were similar in number to Ly6G^{-} eosinophils in Trib1$^{+/+}$ BM **(Fig. 3.1F)**. Trib1$^{\Delta HSC}$ Ly6G^{+} eosinophils had reduced expression of the eosinophil granule protein gene *Eosinophil peroxidase* (*Epx)* **(Fig. 3.2A)**, but maintained high side-scatter, as well as surface expression of Ly6C, F4/80, and IL-5Rα (CD125) **(Figs. 3.3A-B)**. In line with

our observation of increased Ly6G expression, we observed increased SiglecE surface expression on Trib1$^{\Delta HSC}$ eosinophils compared to Trib1$^{+/+}$ **(Fig. 3.3C)**. SiglecE, while not as neutrophil-restricted as Ly6G, is highly expressed on neutrophils, and absent from WT eosinophils[150]. In contrast to Trib1$^{+/+}$ CCR3^{+} eosinophils, Trib1$^{\Delta HSC}$ eosinophils trended towards increased expression of the neutrophil secondary granule protein gene *Lactoferrin (Ltf)* **(Fig. 3.2B)**, and had hypersegmented nuclear architecture **(Fig. 3.4)**. These cells were also present in chimeras engrafted with Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ BM **(Fig. 3.5)**, establishing the cell-intrinsic nature of this population.

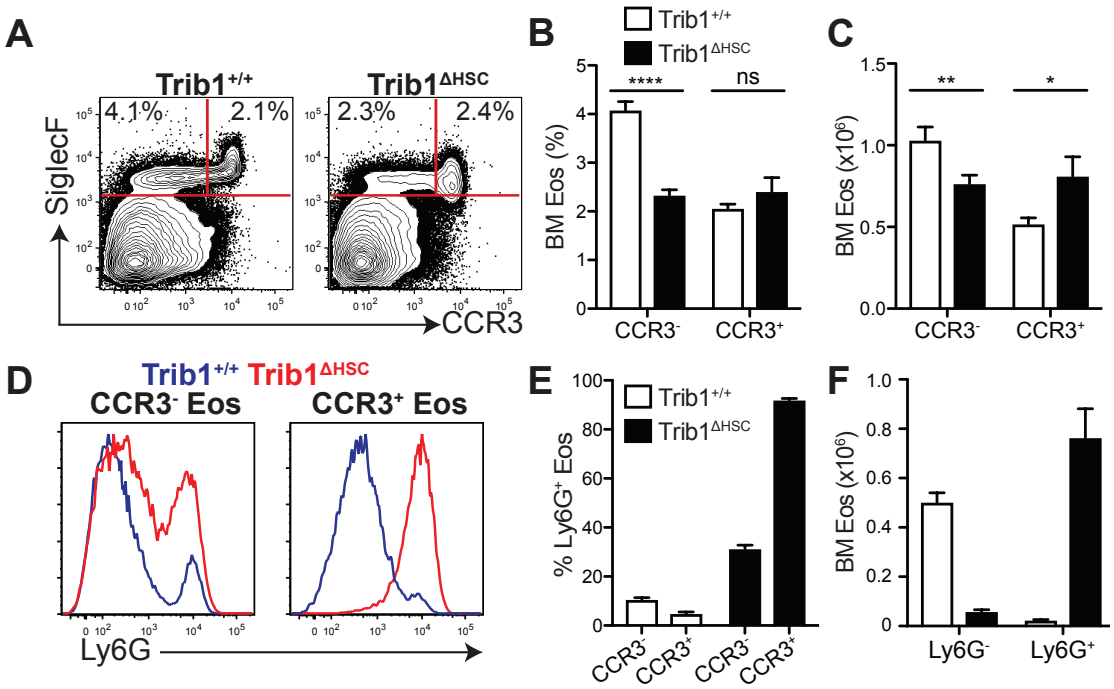

Fig. 3.1: Ly6G$^+$ eosinophils are expanded in Trib1-deficient bone marrow A) Representative plots of eosinophils in the BM from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice, gated on live, CD11b$^+$ cells. B) Frequency of live cells and C) absolute number of mature eosinophils in the BM from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice, n=24 mice/group pooled from 7 experiments. D) Representative histogram of Ly6G expression by immature SiglecF$^+$ CCR3$^-$ (left) and mature SiglecF$^+$ CCR3$^+$ (right) eosinophils, Trib1$^{+/+}$ (blue), Trib1$^{\Delta HSC}$ (red). E) Percent of immature and mature eosinophils expressing Ly6G. F) Absolute number of BM mature eosinophils by Ly6G expression, n=11-12 mice/group pooled from 4 experiments. ns=not significant, *p<0.0232, **p<0.0084, ****p<0.0001, unpaired student's *t* test. Frequencies and error bars are mean±SEM of live cells.

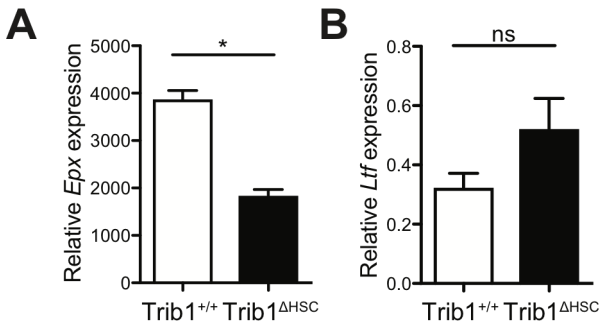

Fig. 3.2: Trib1-deficient eosinophils show altered gene expression A) *Epx* and B) *Ltf* expression in sorted mature BM eosinophils (SiglecF$^+$ CCR3$^+$ F4/80$^+$ CD11b$^+$) relative to *18s* and normalized to Trib1$^{+/+}$ neutrophils, n=2 mice/group, representative of 4 experiments. ns=not significant, *p<0.0232

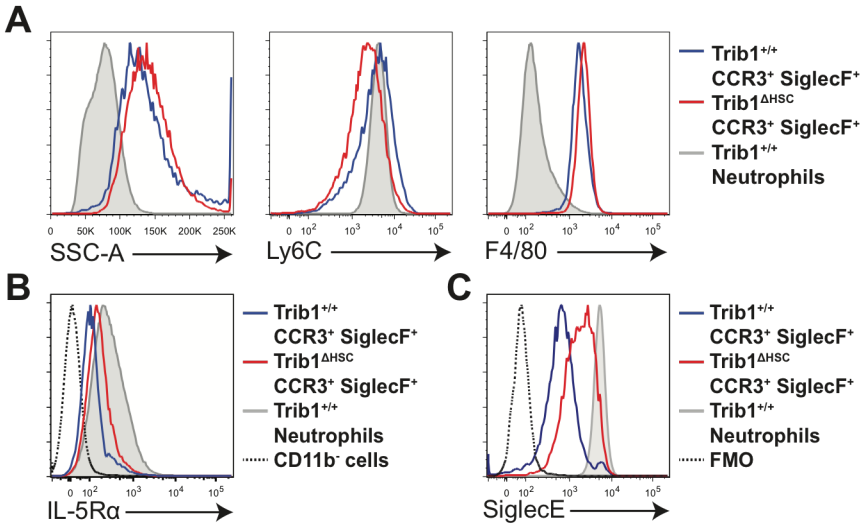

Fig. 3.3: Trib1-deficient Ly6G$^+$ eosinophils express eosinophil surface markers and upregulate SiglecE A) Representative histograms of BM eosinophils from Trib1$^{+/+}$ (blue) and Trib1$^{\Delta HSC}$ (red) mice with Trib1$^{+/+}$ neutrophils (gray solid) as a control. SSC-A: side-scatter area, representative of 4 expts. B) Representative histogram of IL-5Rα (CD125) expression on BM eosinophils from Trib1$^{+/+}$ (blue) and Trib1$^{\Delta HSC}$ (red) mice with Trib1$^{+/+}$ neutrophils (grey filled) and FMO (black dashed) as controls, representative of 2 experiments. C) SiglecE expression on BM eosinophils from Trib1$^{+/+}$ (blue) and Trib1$^{\Delta HSC}$ (red) mice with Trib1$^{+/+}$ neutrophils (gray solid) and FMO (black dashed) as controls, representative of 1 expt. For all histograms, eosinophils gated on live, CD11b$^+$, SiglecF$^+$, CCR3$^+$ cells, neutrophils gated live, CD11b$^+$ Ly6G$^+$ SiglecF$^-$.

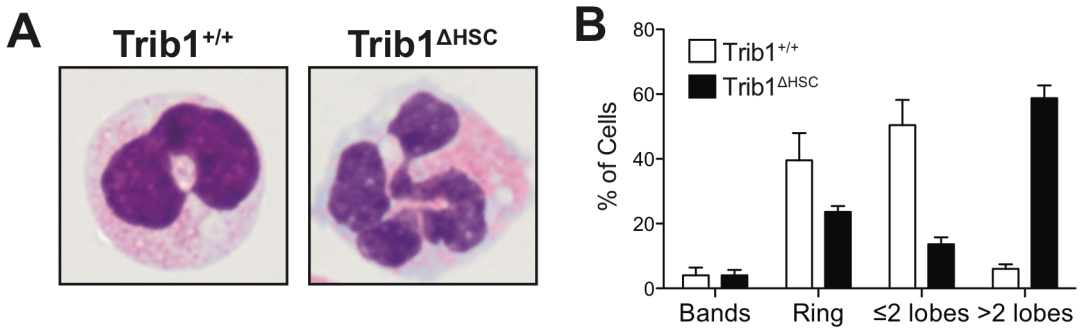

Fig. 3.4: **Trib1 loss yields hypersegmented Ly6G$^+$ eosinophils.** A) Representative cytospins of sorted eosinophils (CD11b$^+$ SiglecF$^+$ CCR3$^+$ F4/80$^+$) at 100x magnification with Diff-quik stain, representative of 3 expts. B) Scoring of cytospins of sorted mature eosinophils for nuclear lobation, n=5 mice/group from 3 expts.

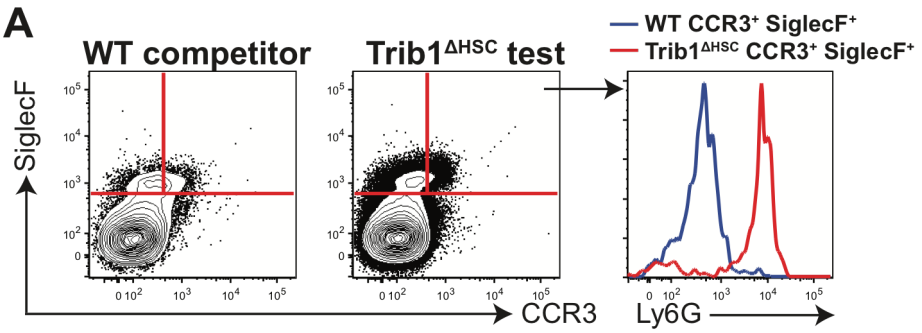

Fig. 3.5 The Trib1-deficient eosinophil phenotype is cell intrinsic. A) Analysis of mixed BM chimeras, WT competitor BM (CD45.1$^+$, left) and Trib1$^{\Delta HSC}$ BM (CD45.2$^+$, right). Representative histogram of mature eosinophils showing Ly6G expression (WT CD45.1$^+$, blue; Trib1$^{\Delta HSC}$ CD45.2$^+$, red). Mice were analyzed at 12-20 weeks post-transplant. Representative of 3 experiments.

For a detailed morphological characterization of Trib1$^{\Delta HSC}$ eosinophils, cells were prepared for conventional transmission electron microscopy (TEM)[30]. Both Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ eosinophils showed a typical cytoplasmic population of large, specific granules with a crystalloid electron-dense core and an outer electron-lucent matrix, delimited by a membrane **(Figs. 3.6A-C)**. This unique granule morphology is specific to eosinophils[151]. The cytoplasm of Trib1$^{\Delta HSC}$ eosinophils also showed increased amount of endomembranes compared to Trib1$^{+/+}$ eosinophils and numerous smaller, less dense, round or elongated structures, which resembled neutrophil specific granules **(Fig. 3.6C)**; thus Ly6G^{+} eosinophils from Trib1$^{\Delta HSC}$ mice contain granules typical of both eosinophils and neutrophils. The nuclei of Trib1$^{+/+}$ eosinophils appeared as ring-like structures, characteristic of mouse eosinophils or multiple nuclear compartments, indicative of the ring-like nucleus crossing in and out of the section's plane[152] while Trib1$^{\Delta HSC}$ eosinophils exhibited a more segmented nucleus **(Figs. 3.6A-B)**. Consistent with these neutrophil-like characteristics, Trib1$^{\Delta HSC}$ eosinophils were more phagocytic than their WT counterparts **(Figs. 3.6D-E)** and produced more ROS when stimulated *ex vivo* with phorbol 12-myristate 13-acetate (PMA) **(Fig. 3.6F)**. Together, these data suggest that Trib1 controls mature eosinophil identity by repressing the neutrophil program.

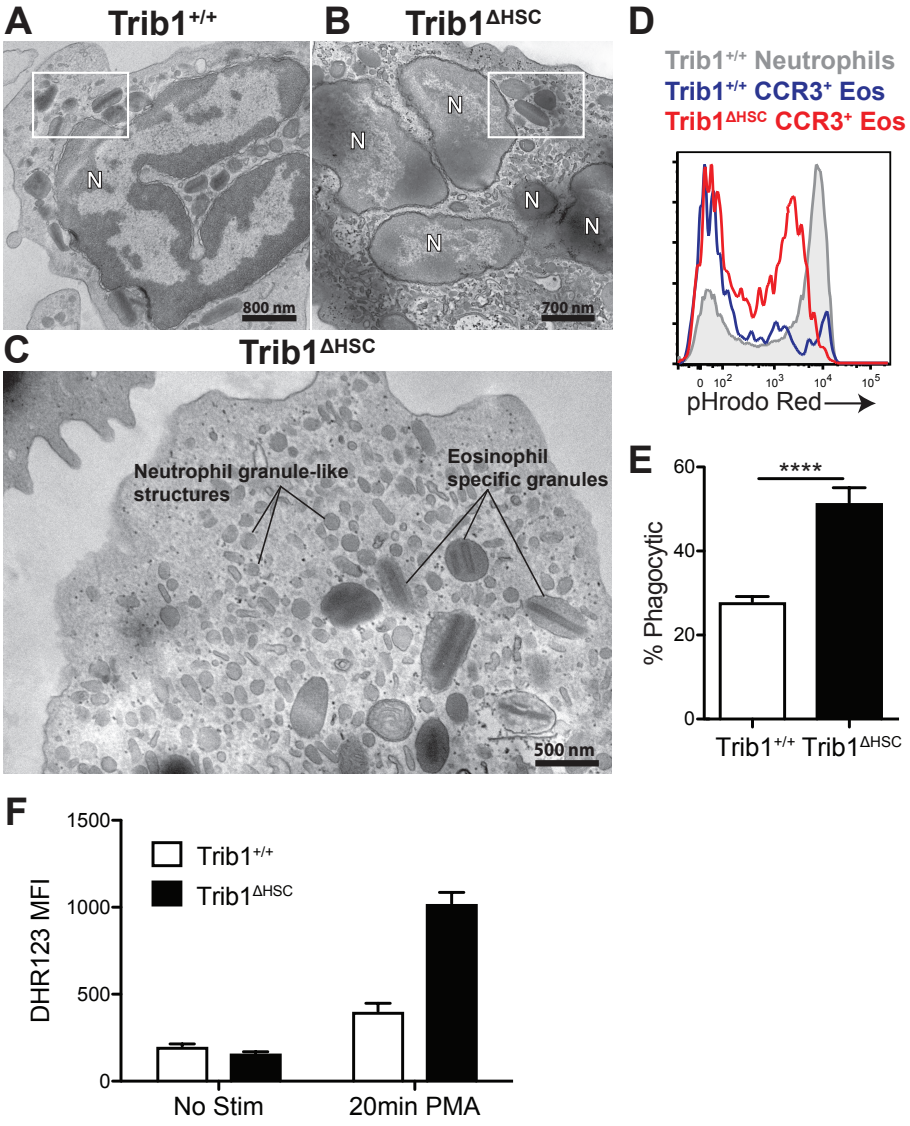

Fig. 3.6: Trib1 deficient eosinophils have neutrophil granules and functions. A-B) Representative electron micrographs show typical eosinophil specific granules (boxed areas in A and B) with a centrally located crystalloid electron dense core in both Trib1$^{+/+}$ (A) and Trib1$^{\Delta HSC}$ (B) eosinophils. C) The cytoplasm of Trib1-deficient eosinophils also contains numerous round and smaller structures similar to neutrophil specific granules. Representative of two experiments with a total of 75 electron micrographs evaluated. N, nucleus. D) Phagocytosis assay using pHrodo red-labeled *E. coli* bioparticles with whole BM gated on Trib1$^{+/+}$ neutrophils (filled gray), Trib1$^{+/+}$ CCR3^{+} eosinophils (blue), and Trib1$^{\Delta HSC}$ CCR3^{+} eosinophils (red). M) Quantification of pHrodo red expression by CCR3^{+} BM eosinophils, n=7 mice/group, pooled from 3 experiments. F) Quantification of DHR123 MFI as a measure of ROS production by BM eosinophils (live, CD11b^{+} SiglecF^{+}) following 20min PMA (10ng/ml) stimulation, n=2 mice/group, representative of 2 experiments. ****p<0.0001, unpaired student's *t* test. Frequencies and error bars are mean±SEM of live cells.

Deletion of Trib1 following eosinophil lineage commitment results in a stable population of BM eosinophils that is restricted to the eosinophil lineage

We observed Ly6G^{+} eosinophils with characteristics of both eosinophils and neutrophils in the BM of Trib1$^{\Delta HSC}$ mice, suggesting that these cells are a granulocyte lineage intermediate that is capable of transitioning to either the neutrophil or eosinophil lineage. To test their developmental potential, we used a fate tracking strategy to follow the effects of Trib1 loss after eosinophil lineage commitment. Using mice expressing *Cre* under the control of the *Epx* promoter (EoCre)[125], we generated "Trib1$^{\Delta Eos}$" mice, lacking Trib1 in EoP and eosinophils. We verified *Trib1* deletion in BM-derived eosinophils from Trib1$^{\Delta Eos}$ mice compared to mice expressing EoCre alone (Trib1$^{+/+}$) **(Fig. 3.7A).** We next crossed Trib1$^{\Delta Eos}$ mice to a YFP reporter mouse[153], termed "Trib1$^{\Delta Eos\text{-}YFP}$" **(Fig. 3.7B)**, resulting in YFP expression in EoCre-expressing cells. Nearly all YFP-expressing Trib1$^{\Delta Eos\text{-}YFP}$ BM cells were SiglecF^{+} eosinophils, the majority of which expressed Ly6G, while YFP^{+} neutrophils (SiglecF^{-} Ly6G^{+}) or other cells were absent **(Fig. 3.8)**. Some eosinophils from Trib1$^{\Delta Eos\text{-}YFP}$ BM had lower SiglecF expression, but resembled eosinophils by side scatter and F4/80 expression **(data not shown)**. These data confirm

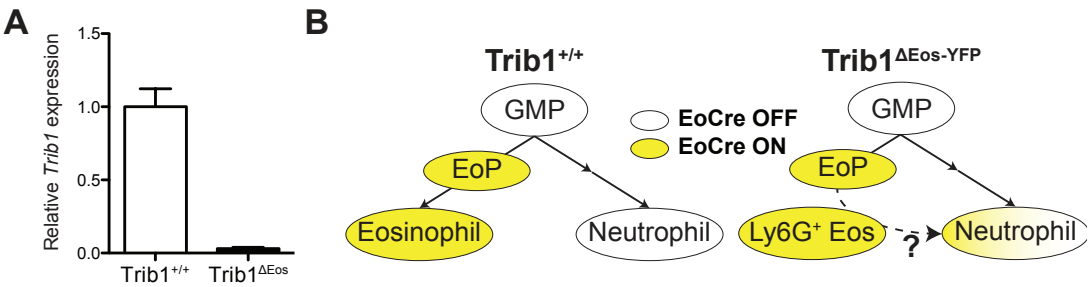

Fig. 3.7: Model of eosinophil-specific Trib1 deletion. A) qPCR of Trib1$^{+/+}$ and Trib1$^{\Delta Eos}$ d13 cultured eosinophils for *Trib1* expression relative to *18s*, normalized to Trib1$^{+/+}$ eosinophils, representative of 2 experiments. B) Model of fate tracking EoCre-mediated deletion of Trib1, and hypothesis of a full transition of Ly6G^{+} eosinophils from Trib1$^{\Delta Eos}$ BM to neutrophils.

that Trib1-deficient Ly6G^{+} eosinophils develop from eosinophil-committed cells and do not transition to other lineages.

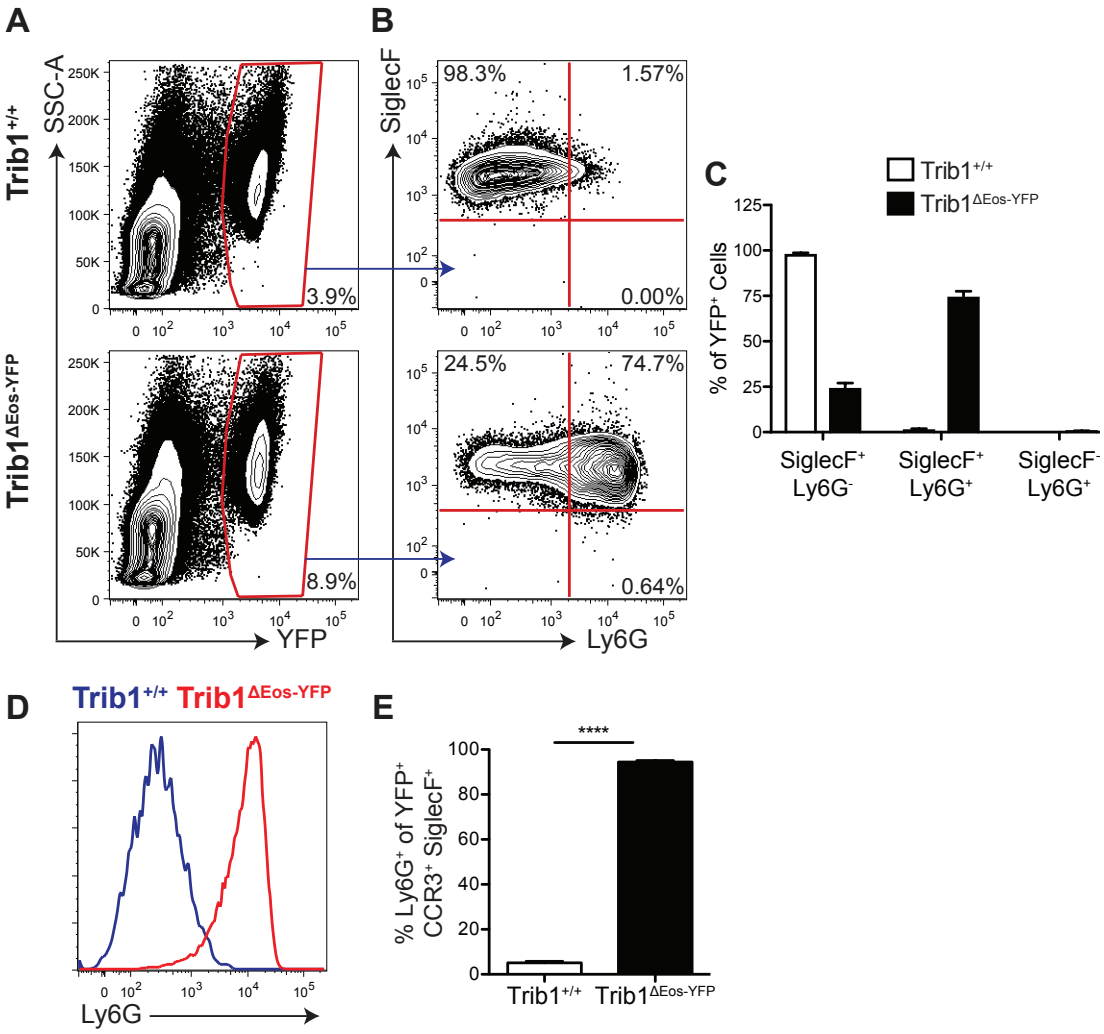

Fig. 3.8: Trib1-deficient Ly6G^{+} eosinophils are a stable population that does not transition to other lineages. A) Representative plots of BM from Trib1$^{+/+}$ and or Trib1$^{\Delta Eos\text{-}YFP}$ mice, gated on live cells. B) Representative plots of YFP^{+} BM cells gated on live, YFP^{+} cells. C) Distribution of YFP^{+} cells by SiglecF and Ly6F expression, as a fraction of YFP^{+} BM cells, n=3 mice/group, representative of 4 experiments. D) Representative histogram of Ly6G expression by YFP^{+} SiglecF^{+} CCR3^{+} cells, Trib1$^{+/+}$ (blue), Trib1$^{\Delta Eos\text{-}YFP}$ (red). E) Frequency of Ly6G expression by YFP^{+} SiglecF^{+} CCR3^{+} cells. n=11 mice/group pooled from 4 experiments. ****p<0.0001, unpaired student's *t* test. Frequencies and error bars are mean±SEM of live cells.

To determine when in development Trib1 modulates eosinophil identity, we further characterized the Trib1$^{\Delta Eos}$ mice. In contrast to Trib1$^{\Delta HSC}$ mice, we observed a much smaller reduction in EoP numbers in Trib1$^{\Delta Eos}$ BM **(Fig. 3.9)**. Despite this, the majority of BM eosinophils expressed Ly6G **(Fig. 3.10)**, and few eosinophils were detected in the periphery **(Figs. 3.11A-E)**, similar to Trib1$^{\Delta HSC}$ mice. In a notable difference from the Trib1$^{\Delta HSC}$ mice, which show a neutrophil expansion **(Figs. 2.3)**, the frequency and number of neutrophils were unchanged in the spleen, blood, and BM of the Trib1$^{\Delta Eos}$ mice **(Fig. 3.11)**. Together, these findings reveal that Trib1 regulates two distinct stages of eosinophil development: lineage commitment and terminal differentiation.

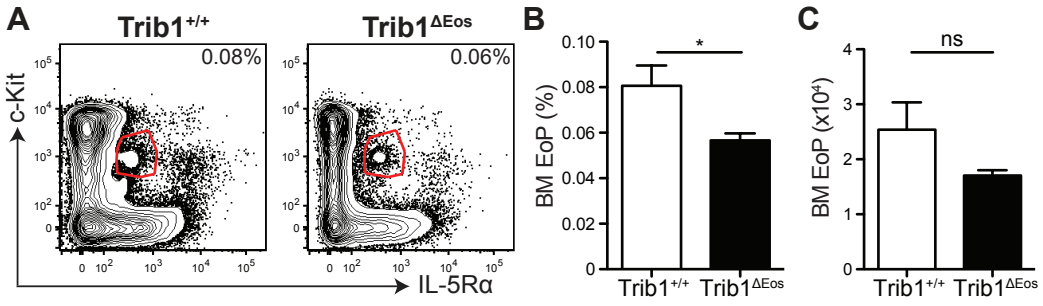

Fig. 3.9: Eosinophil-specific deletion of Trib1 results in improved preservation of EoPs. A) Representative plots of BM EoP, gated on live, lineage$^-$ CD34$^+$ cells from Trib1$^{+/+}$ and Trib1$^{\Delta Eos}$ mice. B) Frequency of live cells and C) absolute numbers of BM EoP from Trib1$^{+/+}$ and Trib1$^{\Delta Eos}$ mice, n=6 mice/group pooled from 2 experiments. ****p<0.0001, unpaired student's *t* test. Frequencies and error bars are mean±SEM of live cells.

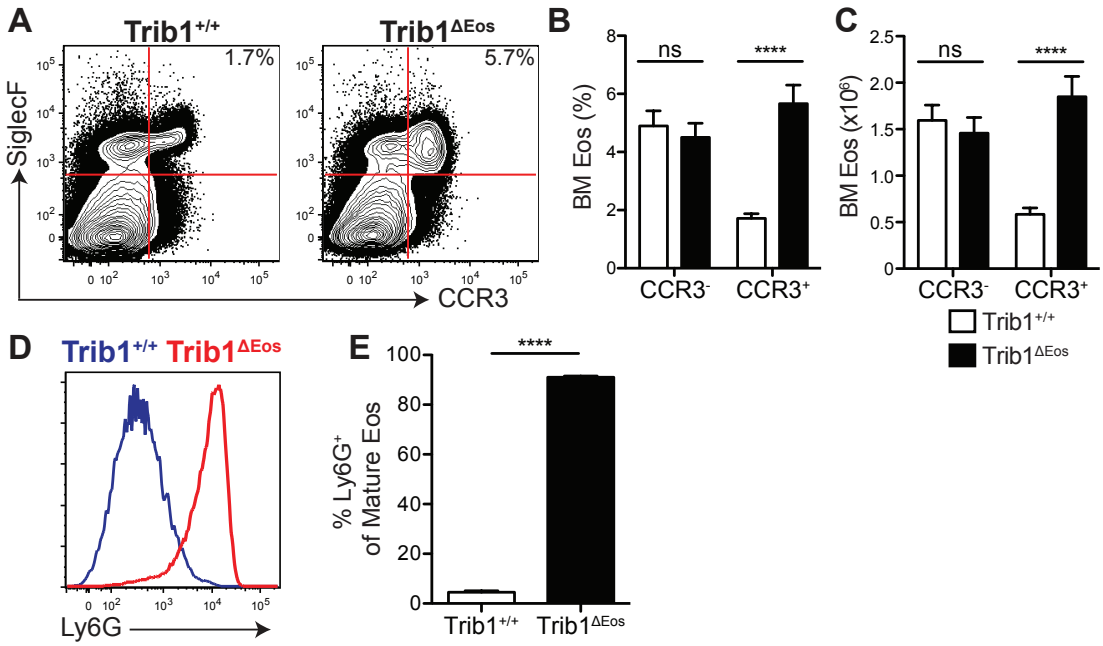

Fig. 3.10: Eosinophil-specific deletion of Trib1 results in Ly6G$^+$ eosinophil development. A) Representative plots of BM eosinophils from Trib1$^{+/+}$ and Trib1$^{\Delta Eos}$ mice gated on live, CD11b$^+$ cells. B) Frequency of live cells and C) absolute numbers of BM eosinophils by CCR3 expression. D) Representative histogram of Ly6G expression by SiglecF$^+$ CCR3$^+$ cells, Trib1$^{+/+}$ (blue), Trib1$^{\Delta HSC}$ (red). E) Frequency of SiglecF$^+$ CCR3$^+$ cells expressing Ly6G, n=11 mice/group pooled from 4 experiments. ****p<0.0001, unpaired student's *t* test. Frequencies and error bars are mean±SEM of live cells.

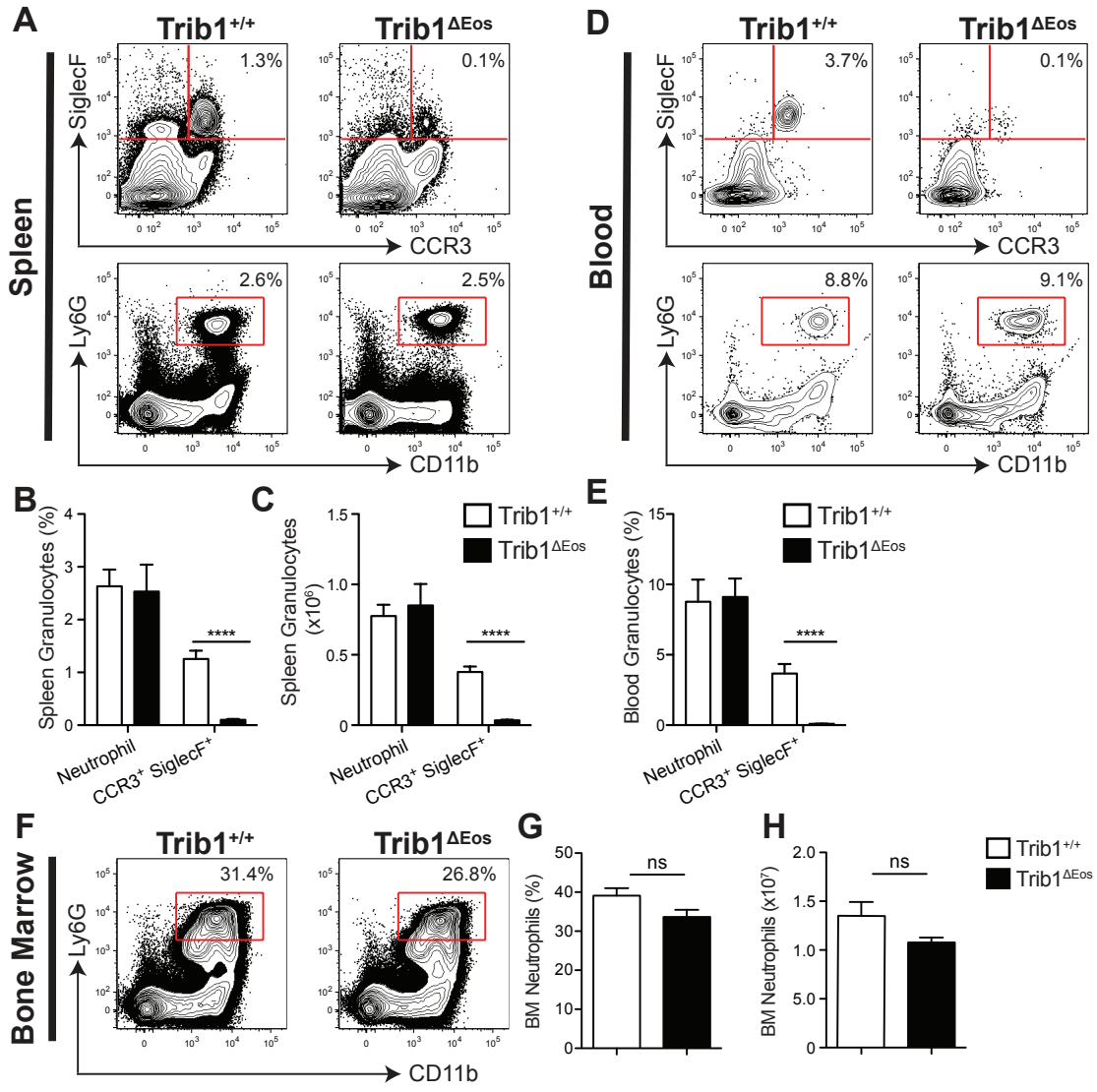

Fig. 3.11: Reduced eosinophils without a concomitant increase in neutrophils in the spleen and blood of Trib1$^{\Delta Eos}$ mice. A) Representative plots of splenic eosinophils (upper) and neutrophils (lower) from Trib1$^{+/+}$ and Trib1$^{\Delta Eos}$ mice. B) Frequency of live cells and C) absolute numbers of splenic granulocytes, n=11 mice/group pooled from 4 experiments. D) Representative plots of blood eosinophils (upper) and neutrophils (lower) from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice. E) Frequency of blood granulocytes, n=9 mice/group pooled from 3 experiments. F) Representative plots of BM neutrophils from Trib1$^{+/+}$ and Trib1$^{\Delta Eos}$ mice, gated on live, SiglecF^{-} cells. G) Frequency of live cells and H) absolute numbers of BM neutrophils, n=11 mice/group pooled from 4 experiments. Eosinophil plots gated on live, CD11b^{+} cells; neutrophil plots gated on live, SiglecF^{-} cells. ns=not significant, ****p<0.0001, unpaired student's *t* test. Frequencies and error bars are mean±SEM.

IL-5 culture promotes development of Ly6G$^+$ eosinophils from Trib1-deficient BM

As noted above, the cytokine IL-5 supports eosinophil development *in vivo* and is required for eosinophilia under a number of settings[53,56,57]. Furthermore, IL-5 alone can drive eosinophil development *ex vivo*[54,58]. As shown in **CHAPTER 2**, Trib1$^{\Delta HSC}$ BM or GMPs in cultured in IL-5 showed reduced eosinophil potential and increased neutrophil output compared to Trib1$^{+/+}$ cells **(Figs. 2.6, 2.7, 2.8)**. While these data show that Trib1 influences early lineage priming/potential of progenitors, it is unclear how Trib1 loss impacts more terminal differentiation steps. Importantly, it is unknown if the Ly6G$^+$ eosinophils we observe *in vivo* in Trib1$^{\Delta HSC}$ BM **(Fig. 3.1)** can be supported by IL-5 alone. When cultured in IL-5, Trib1$^{\Delta HSC}$ BM yielded fewer eosinophils than Trib1$^{+/+}$ BM **(Fig. 3.12A-C)**; yet the majority of the Trib1-defcient eosinophils generated were Ly6G$^+$ **(Fig. 3.12D)**, similar to what was observed *in vivo* **(Fig. 3.1)**. Interestingly, compared to *in vivo*, there was an expansion of Ly6G$^+$ eosinophils from Trib1$^{+/+}$ BM. Together, this indicates that IL-5 alone is sufficient to induce the generation and/or expansion of the Ly6G$^+$ eosinophil population.

Subsequently, to determine if IL-5 could rescue normal eosinophil development following eosinophil lineage commitment, we cultured Trib1$^{\Delta Eos}$ BM in IL-5. In contrast to what was observed with Trib1$^{\Delta HSC}$ BM, we did not observe a neutrophil expansion in Trib1$^{\Delta Eos}$ cultures **(Figs 3.13A-C)**. Trib1$^{\Delta Eos}$ produced similar numbers of eosinophils compared to Trib1$^{+/+}$ BM. Finally, similar to Trib1$^{\Delta HSC}$ BM cultures, Trib1$^{\Delta Eos}$ BM yielded predominantly Ly6G$^+$ eosinophils **(Fig. 3.13D)**. Together, these data further reinforce the dual role of Trib1 in granulocyte development. Trib1 loss prior to eosinophil lineage commitment results in reduced eosinophilic potential, while Trib1 loss following lineage commitment promotes the development of Ly6G$^+$ eosinophils.

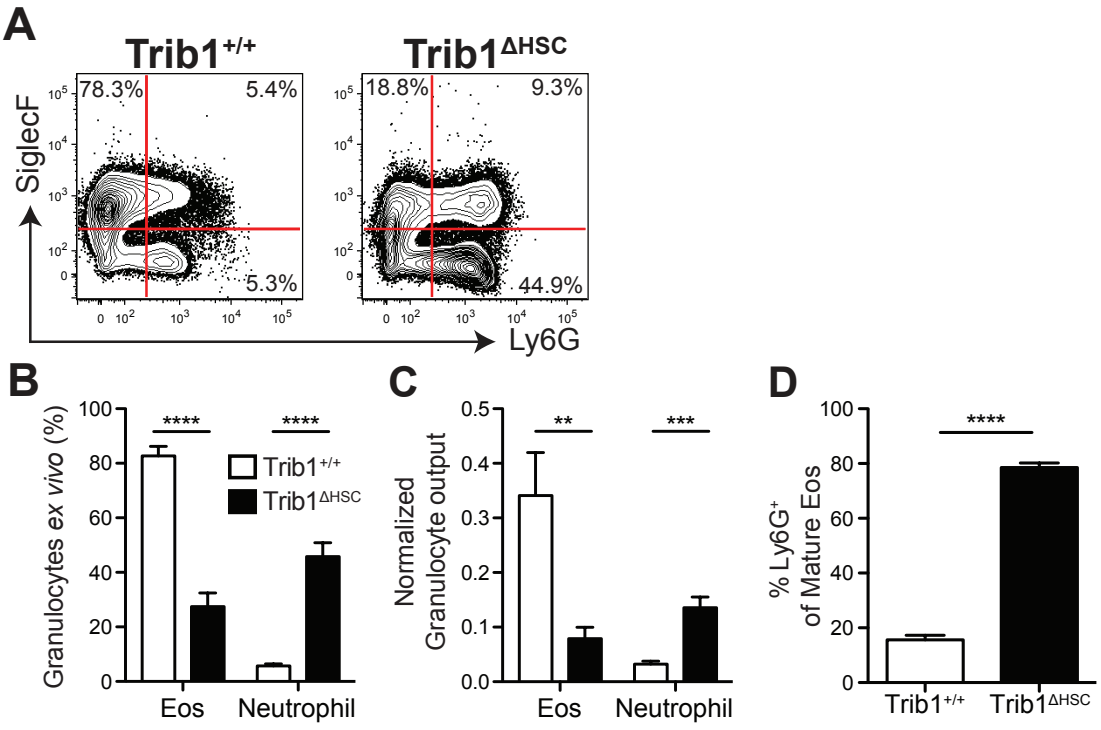

Fig. 3.12: Hematopoietic Trib1 loss results in neutrophil and Ly6G$^+$ eosinophil production *ex vivo*. A) Representative plots of d10 IL-5 cultures Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ whole BM gated on live cells. B) Frequency of live cells and C) d10 cell output normalized to d4 cell counts assay using Trib1$^{+/+}$ or Trib1$^{\Delta HSC}$ BM. Frequency n=5 mice/group pooled from 3 experiments; cell output n=6 mice/group pooled from 4 experiments. D) Frequency of Ly6G expression on SiglecF$^+$ CCR3$^+$ cells at d10, n=3 mice/group, representative of 4 experiments. Eosinophils gated on SiglecF$^+$ CCR3$^+$; neutrophils gated on Ly6G$^+$ SiglecF$^-$. **p<0.0063, ***p=0.0002, ****p< 0.0001, unpaired student's *t* test. Frequencies and error bars are mean±SEM of live cells.

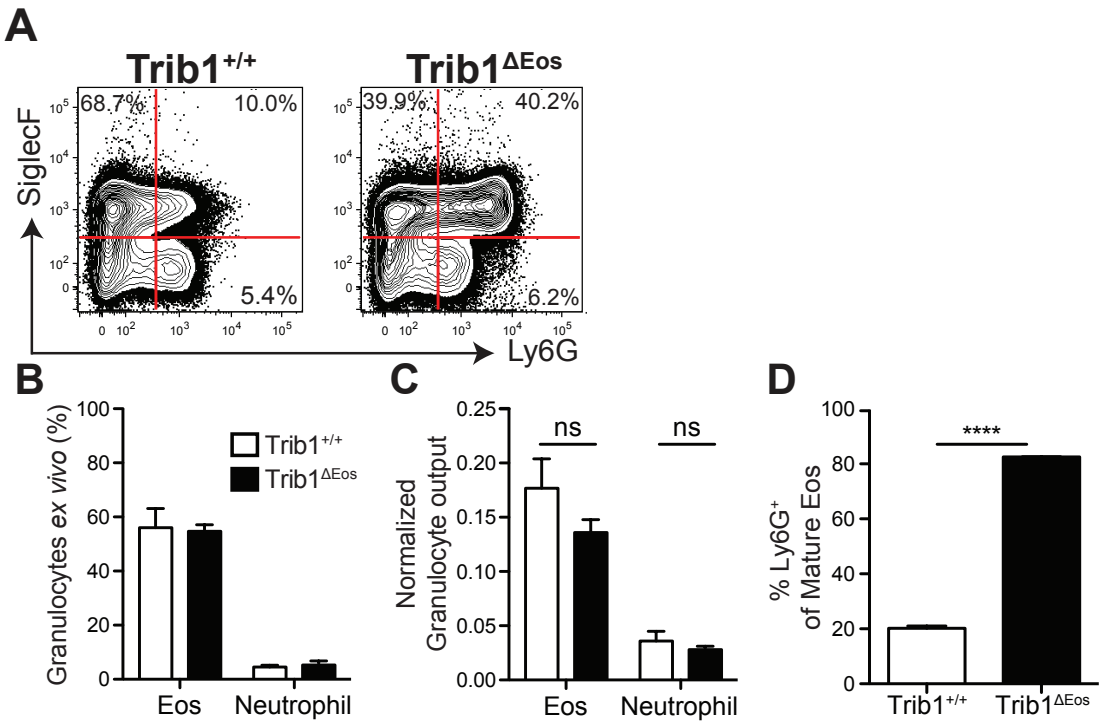

Fig. 3.13: Trib1$^{\Delta Eos}$ BM yield Ly6G^{+} eosinophil *ex vivo* with no increase in neutrophil production. A) Representative plots of d10 IL-5 cultures Trib1$^{+/+}$ and Trib1$^{\Delta Eos}$ whole BM gated on live cells. E) Frequency of live cells and F) d10 cell output normalized to d4 cell counts using Trib1$^{+/+}$ or Trib1$^{\Delta Eos}$ BM. Frequency n=2-3 mice/group, representative of 6 experiments; cell output n=7 mice/group pooled from 5 experiments. G) Frequency of Ly6G expression on SiglecF^{+} CCR3^{+} cells, n=2-3 mice/group, representative of 6 experiments. Eosinophils gated on SiglecF^{+} CCR3^{+}; neutrophils gated on Ly6G^{+} SiglecF^{-}. ****p< 0.0001, unpaired student's *t* test. Frequencies and error bars are mean±SEM of live cells.

We next sorted eosinophils and neutrophils from d10 IL-5 cultures of Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ BM to measure changes in gene expression. As expected, *Trib1* was highest in neutrophils and absent in Trib1$^{\Delta HSC}$ cells **(Fig. 3.14A)**. We found that *Epx* expression was highest in Ly6G^{-} eosinophils derived from either Trib1$^{+/+}$ or Trib1$^{\Delta HSC}$ BM and lower in Trib1$^{+/+}$ Ly6G^{+} eosinophils **(Fig. 3.14B)**. *Ltf* was expressed in neutrophils but not eosinophils (Ly6G^{+} or Ly6G^{-}) from Trib1$^{+/+}$ BM. In contrast, in cells derived from Trib1$^{\Delta HSC}$ BM, *Ltf* was expressed in both neutrophils and Ly6G^{+} eosinophils **(Fig. 3.14C)**, similar to our *in vivo* data **(Fig. 3.2B)**.

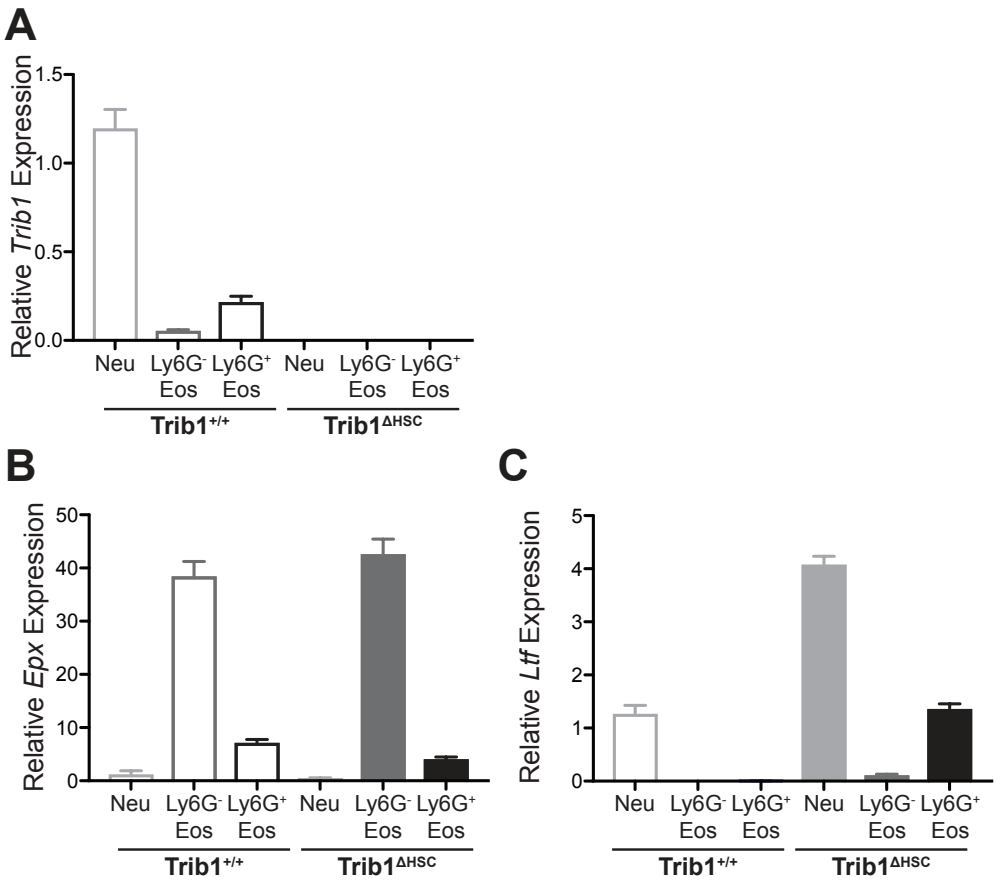

Fig. 3.14: Trib1 loss alters granulocyte gene expression *ex vivo*. qPCR analysis of sorted neutrophils (CD11b^{+} Ly6G^{+} SiglecF^{-}), Ly6G^{-} and Ly6G^{+} eosinophils (CD11b^{+} SiglecF^{+} CCR3^{+}) from d10 IL-5 culture of Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ BM for A) *Trib1*, B) *Epx*, C) *Ltf*, relative to *18s*, normalized to Trib1$^{+/+}$ neutrophils, n=3, representative of 2 experiments.

A key function of eosinophils is cytokine production, many of which are stored pre-formed in cytoplasmic granules. IL-4 is one of these eosinophil-associated cytokines[31], and we measured release of IL-4 by IL-5-cultured eosinophils. IL-5 alone is sufficient to stimulate eosinophil activation and granule release[154]. Both Trib1$^{+/+}$ and Trib1$^{\Delta Eos}$ eosinophils released similar levels of IL-4 into the culture supernatant at d12 of IL-5 culture **(Fig. 3.15A)**. We did not detect G-CSF in the culture supernatant and there was no change in the level of GM-CSF produced **(Fig. 3.15B and data not shown)**. Of note, we also observed that Trib1$^{\Delta Eos}$ cultures produced more MCP-1 compared to Trib1$^{+/+}$ cultures **(Fig. 3.15C)**. MCP-1 (CCL2) is a chemoattractant that recruits monocytes as well as acting on other populations, including T cells to augment Th2 responses[155-158]. It can be produced by eosinophils in response to the complement split product C5a, with IL-5 alone inducing minimal MCP-1 production[159]. This further conforms that Trib1-deficient eosinophils have an altered response to IL-5, in this case, by producing MCP-1.

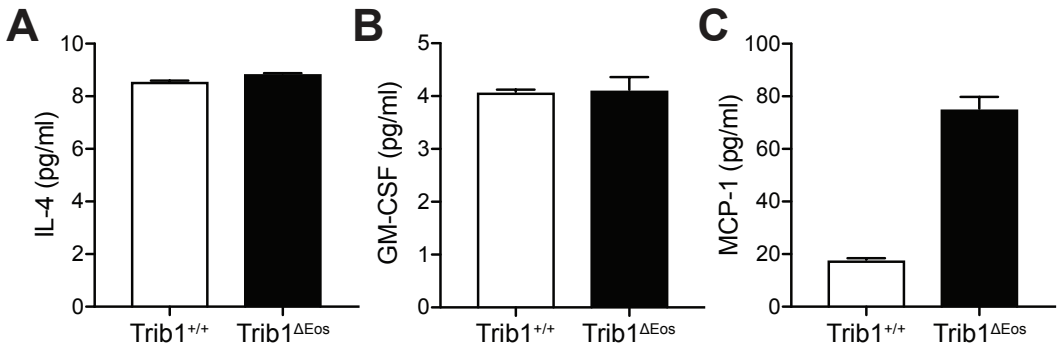

Fig. 3.15: Trib1-deficient eosinophils produce increased MCP-1 *ex vivo*.
Quantification of A) IL-4 B) GM-CSF and C) MCP-1 levels in the supernatant of IL-5 cultures at d12 of Trib1$^{+/+}$ and Trib1$^{\Delta Eos}$ BM by cytokine bead array. n=2 wells/group, representative of 4 experiments. Error bars are mean±SEM. *Cytokine bead array performed by D. Bellissimo*.

To assay the functional capability of cells derived from the IL-5 culture system, we performed transwell assays to measure cell migration to eotaxin (CCL11) or KC (CXCL1), which attract eosinophils[160,161] or neutrophils[162,163] respectively **(Figs. 3.16A-B)**. Eosinophils derived from cultured $Trib1^{+/+}$ BM migrated to eotaxin with greater efficiency than to KC, as expected **(Figs. 3.16C-D)**. $Ly6G^{+}$ eosinophils derived from $Trib1^{\Delta HSC}$ and $Trib1^{\Delta Eos}$ cultured cells also migrated to eotaxin, albeit with reduced efficiency, and minimally migrated to KC **(Figs. 3.16C-D)**. Lastly, $Trib1^{\Delta HSC}$ neutrophils migrated to KC **(Fig. 3.16E)**, indicating that these cells functionally resemble *bona fide* neutrophils. As few neutrophils were produced from $Trib1^{+/+}$ cultures, we were unable to analyze their migration. Thus, both the eosinophils and neutrophils derived from Trib1-deficient cultures migrated to their respective attractants/chemokines, indicating that these are functionally competent granulocytes.

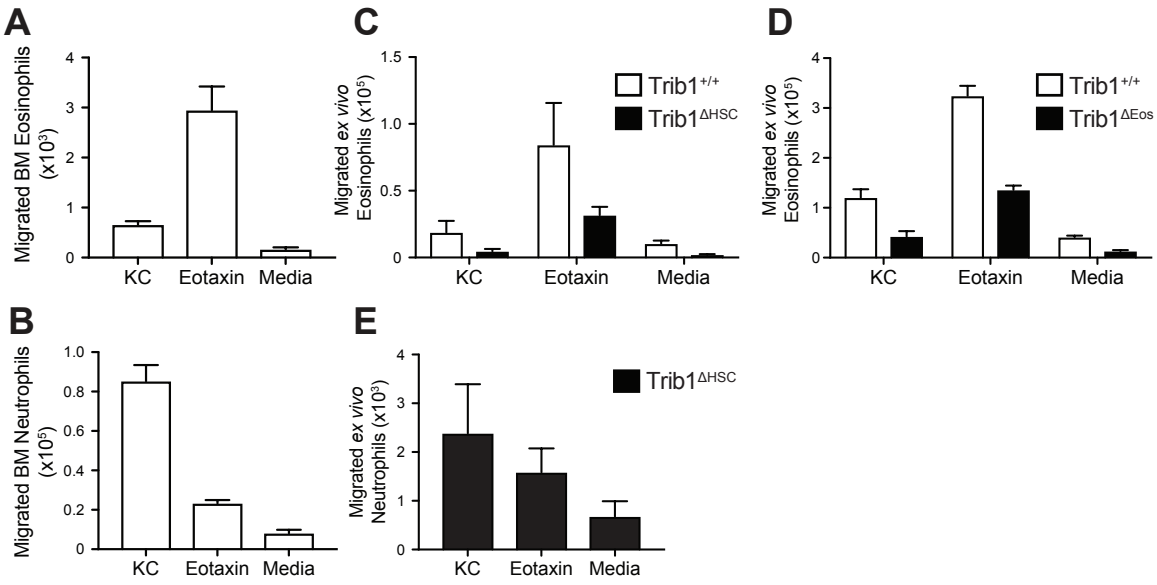

Fig. 3.16: IL-5-derived granulocytes maintain lineage-specific migration patterns with Trib1 loss. A) Quantification of neutrophil or B) eosinophil migration using freshly isolated WT BM gated on neutrophils or eosinophils, respectively. n=3/group, representative of 3 experiments. C) Quantification of eosinophil migration after 2 hours in a transwell chemotaxis assay starting with $1x10^6$ d12 IL-5 cultured BM from $Trib1^{+/+}$ or $Trib1^{\Delta HSC}$ mice to either KC/CXCL1 (50ng/ml) or eotaxin/CCL11 (500ng/ml). n=3, representative of 3 experiments. D) Quantification of eosinophil or E) $Trib1^{\Delta Eos}$ neutrophil migration after 2 hours in a transwell chemotaxis assay starting with $1x10^6$ d10 IL-5 cultured BM from $Trib1^{+/+}$ or $Trib1^{\Delta HSC}$ mice to either KC/CXCL1 (50ng/ml) or eotaxin/CCL11 (500ng/ml). n=3/group, representative of 3 experiments. In all panels, eosinophils gated $CD11b^+$ $SiglecF^+$ $CCR3^+$, neutrophils gated $CD11b^+$ $Ly6G^+$ $SiglecF^-$. Frequencies and error bars are mean±SEM of live cells.

Trib1-deficient eosinophils show impaired mobilization in response to lung inflammation

The Ly6G^{+} eosinophils present in Trib1$^{\Delta HSC}$ and Trib1$^{\Delta Eos}$ mice are predominantly restricted to the BM and do not transition to other lineages **(Figs. 2.3 and 3.11)**. To determine whether these cells can be mobilized, Trib1$^{+/+}$ and Trib1$^{\Delta Eos}$ mice were treated intranasally with papain, a protease allergen that stimulates eosinophil recruitment to the lung[60,164,165]. Papain-treated Trib1$^{\Delta Eos}$ mice mobilized eosinophils to the lung; however, the magnitude of mobilization to the lung was decreased **(Figs. 3.17A-C)**. Nearly all of the eosinophils recruited to the lungs of Trib1$^{\Delta Eos}$ mice post-papain treatment were Ly6G^{+} **(Figs. 3.17D-E)**. In addition, mobilization of Trib1$^{+/+}$ eosinophils following papain was seen systemically, while there was a decrease in Trib1$^{\Delta Eos}$ mobilization to the blood and spleen **(Fig. 3.18)**. These data show that the Ly6G^{+} eosinophils generated by Trib1-deficient BM are *bona fide* eosinophils, as they are mobilized in response to type 2 inflammation; however, Trib1 is required for optimal migration.

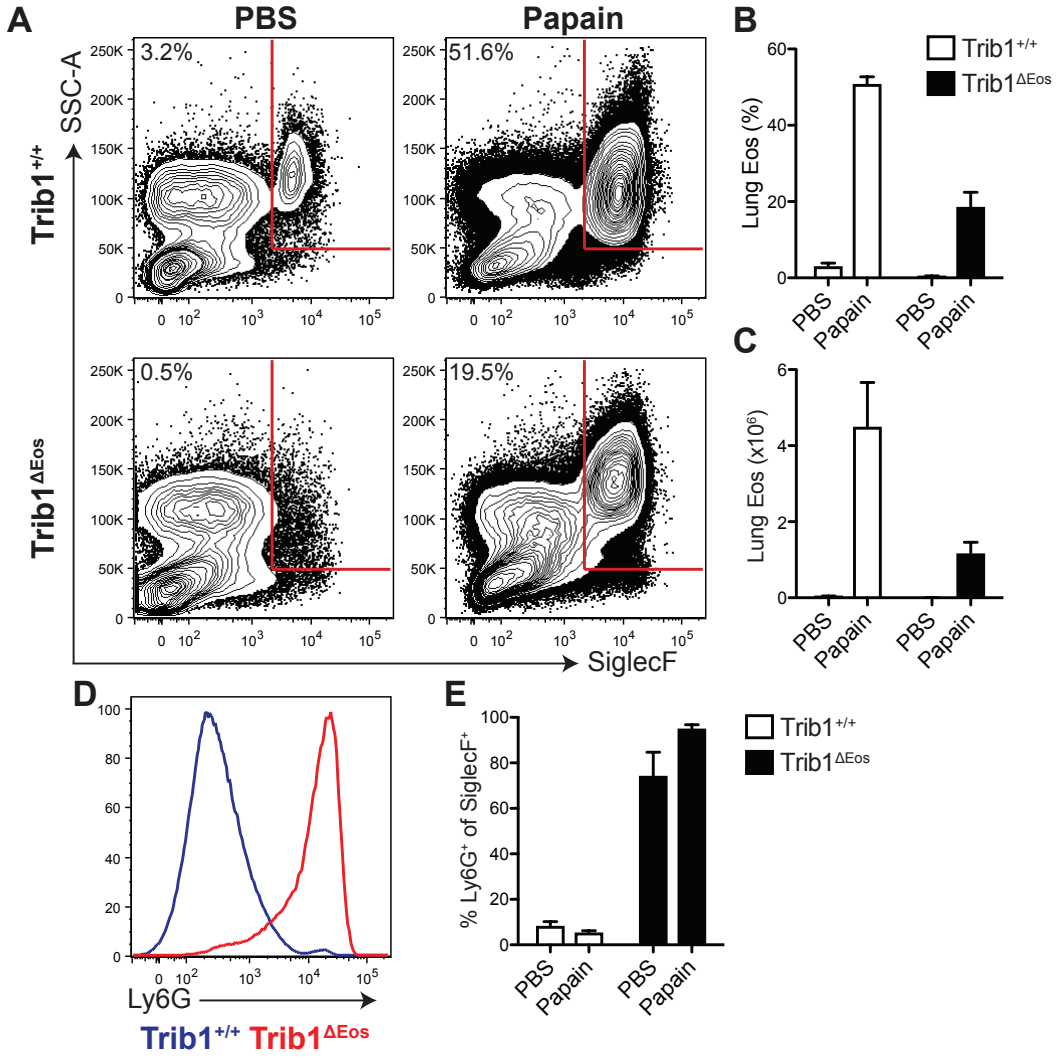

Fig. 3.17: Partial rescue of Trib1-deficient eosinophil mobilization in response to type 2 lung inflammation. Mice were treated for 5 days with intranasal PBS or 30µg papain. A) Representative plots of lung leukocytes gated on live, CD45^{+} CD11b^{hi}. B) Frequency of live cells and C) absolute number of lung eosinophils gated on live, CD45^{+} CD11b^{hi} CD11c^{-} SiglecF^{+} cells. D) Representative histogram of Ly6G expression by lung eosinophils gated live, CD45^{+} CD11b^{hi} CD11c^{-} SiglecF^{+}, Trib1$^{+/+}$ (blue), Trib1$^{\Delta Eos}$ (red). E) Fraction of lung eosinophils expressing Ly6G. n=3 mice/group, representative of 2 experiments. Frequencies and error bars are mean±SEM of live cells.

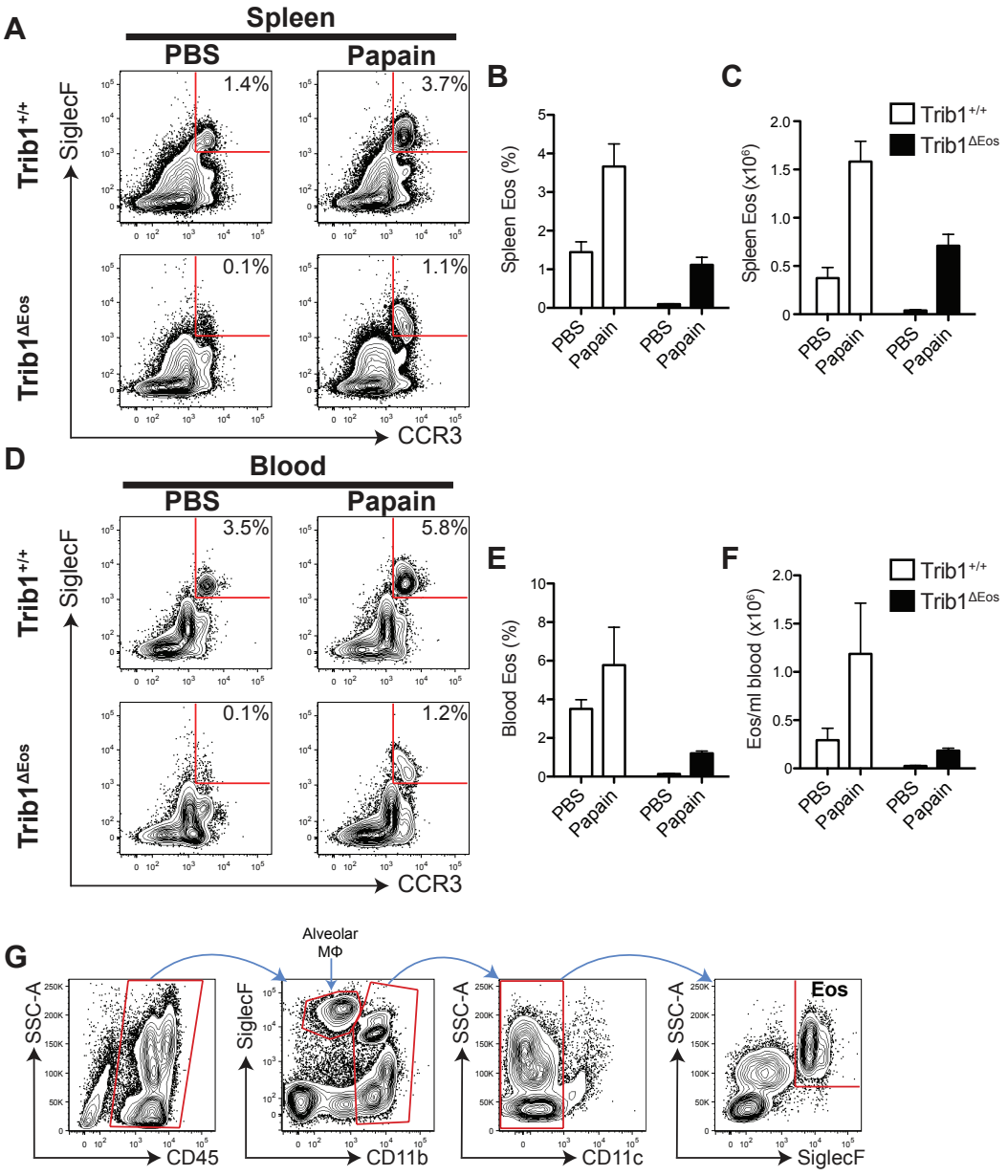

Fig. 3.18: Peripheral mobilization of Trib1-deficient eosinophils in response to papain. Analysis of splenic eosinophils following papain treatment in Trib1$^{\Delta Eos}$ mice. A) Representative plots of splenic eosinophils gated live, CD11b+ cells. B) Frequency of live cells and C) absolute number of CCR3^{+} SiglecF^{+} cells in the spleen. D) Representative plots of blood eosinophils gated live, CD11b^{+} cells. E) Frequency of live cells and F) cells/ml of blood of CCR3^{+} SiglecF^{+} cells. n=3/group, representative of 2 experiments. Frequencies and error bars are mean±SEM of live cells. G) Representative gating strategy for identifying lung eosinophils. First left plot gated on singlet, live cells. Alveolar macrophages (AMΦ) identified as CD11b^{lo} SiglecF^{+}. After excluding AMΦ, eosinophils were identified as CD11b^{+} CD11c^{-} SiglecF^{+} SSC^{hi}.

We observe that Trib1-deficient eosinophils are restricted to the BM under steady state conditions and are mobilized with reduced efficiency following papain treatment **(Fig. 3.17)**. As dynamic expression of chemokine receptors influences retention of neutrophils and other cell populations in the BM[166], we investigated expression of various key chemokine receptors on Trib1-deficent eosinophils. We observed that CCR3 and CXCR2 expression were unchanged **(Fig. 3.19A)**, while CXCR4 expression was increased **(Figs. 3.19A-B)**. During development in the BM, we observe that eosinophils normally downregulate CXCR4 as the acquire CCR3 **(Figs. 3.19C-D)**. This downregulation did not occur in the absence of Trib1 and $CCR3^{+}$ eosinophils maintained high CXCR4 expression.

Thus, we hypothesize that increased CXCR4 expression increases BM eosinophil retention in the absence of Trib1. To test this, we used a specific inhibitor of CXCR4, AMD3100[167]. CXCR4 expression normally increases cell retention in the BM and AMD3100 rapidly mobilizes BM progenitors and neutrophils into circulation[166-169]. 2 hours following AMD3100 injection, eosinophils were robustly mobilized into the circulation of $Trib1^{\Delta Eos}$ mice **(Fig. 3.20A-C)**. We also observed an increase in the number of circulating eosinophils in $Trib1^{+/+}$ mice, as well as an increase in blood neutrophils in both $Trib1^{+/+}$ and $Trib1^{\Delta Eos}$ mice **(Fig. 3.20D-F)**. We next measured the expression of surface CXCR4 to determine if AMD3100 impacted its expression, as we hypothesize that antagonism of CXCR4 will lead to its upregulation. We observed that the few eosinophils present in the circulation of PBS-treated $Trib1^{\Delta Eos}$ mice had increased CXCR4 expression **(Fig. 3.21A)**. Both $Trib1^{+/+}$ and $Trib1^{\Delta Eos}$ eosinophils saw increased CXCR4 expression following AMD3100 treatment, with $Trib1^{\Delta Eos}$ cells showing the largest increase. $Trib1^{\Delta Eos}$ neutrophils had similar CXCR4 expression compared to

Trib1$^{+/+}$ neutrophils and both genotypes saw similar increases in CXCR4 following AMD3100 treatment **(Fig. 3.21B)**. Together, this suggests that increased CXCR4 expression on Trib1-deficient eosinophils contributes to their BM retention, and CXCR4 antagonism can relieve this block in egress.

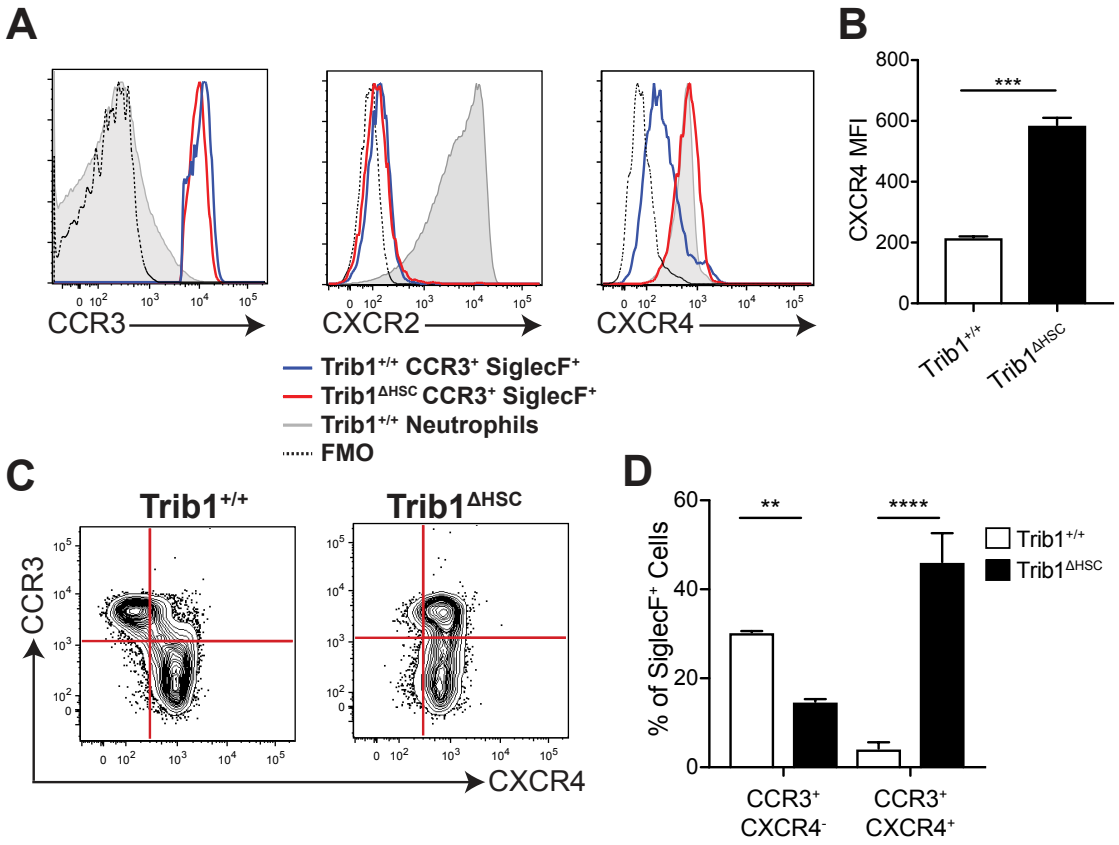

Fig. 3.19: Increased CXCR4 expression on Trib1-deficient BM eosinophils. A) Representative histograms of CCR3, CXCR2, and CXCR4 staining on BM eosinophils from Trib1$^{+/+}$ (blue) or Trib1$^{\Delta HSC}$ (red) mice with Trib1$^{+/+}$ neutrophils (gray solid) and CXCR4 FMO (dashed black) shown as controls, representative of 1 (CXCR2) or 3 (CCR3, CXCR4) expts. B) Quantification of CXCR4 MFI on CD11b^{+} CCR3^{+} SiglecF^{+} eosinophils, n=3 mice/group, representative of 3 expts, ***p=0.0001, unpaired student's t test. C) Representative plots of BM eosinophils gated on live, CD11b^{+} SiglecF^{+} cells. D) Quantification of CCR3 vs CXCR4 expression on CD11b^{+} SiglecF^{+} BM eosinophils. n=3 mice/group, representative of 3 experiments. **p=0.0027, ****p<0.0001, 2-way ANOVA with multiple comparisons.

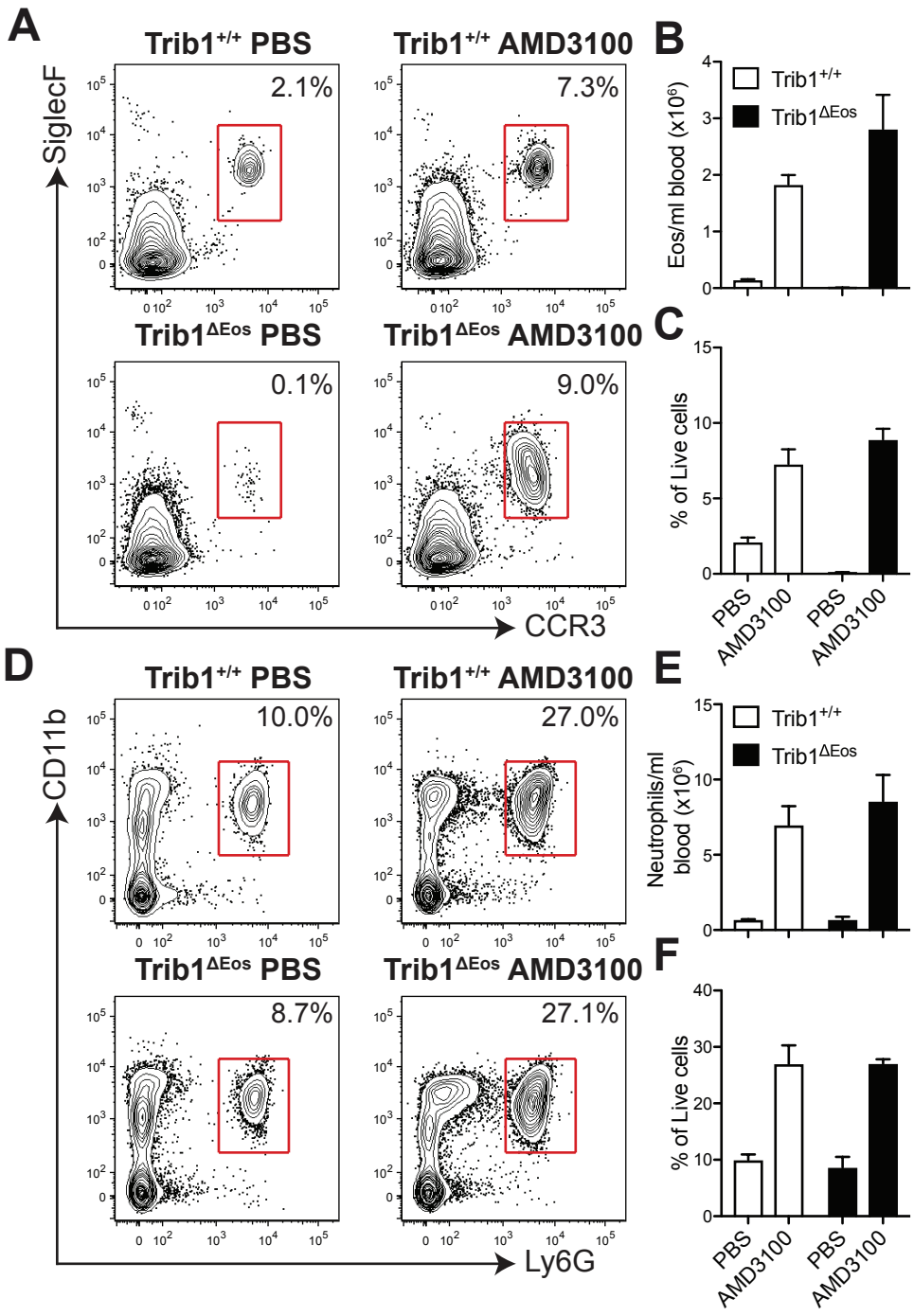

Fig. 3.20: CXCR4 antagonism mobilizes Trib1-deficient BM eosinophils into the blood. 2hrs following PBS or 5mg/kg AMD3100 subcutaneous injection. A) Representative plots of blood eosinophils gated live, CD11b^{+}. B) absolute number/ml and C) frequency of live cells of blood eosinophils gated live, CD11b+ SiglecF^{+} CCR3^{+}. D) Representative plots of blood neutrophils gated live, SiglecF^{-}. E) Absolute number/ml and F) frequency of live cells of blood neutrophils gated live, CD11b^{+} SiglecF^{-} Ly6G^{+}. n=3 mice/group, representative of 1experiment. ns=not significant, *p=0.02, **p<0.005, unpaired student's *t* test. Frequencies and error bars are mean±SEM of live cells.

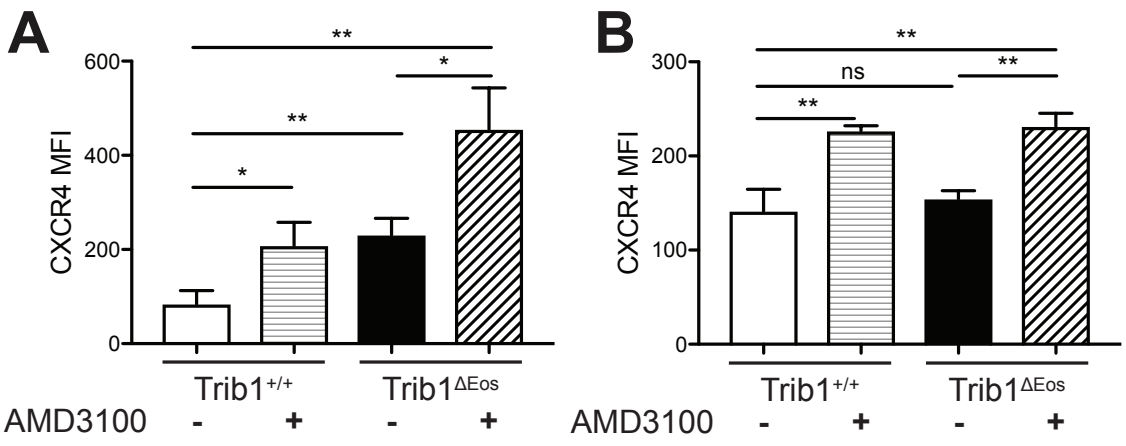

Fig. 3.21: CXCR4 antagonism increases granulocyte CXCR4 expression. Quantification of CXCR4 MFI on A) blood eosinophils gated live, $CD11b^+$ $SiglecF^-$ $CCR3^+$ and B) blood neutrophils gated live, $CD11b^+$ $SiglecF^-$ $Ly6G^+$ 2hrs following PBS or 5mg/kg AMD3100 subcutaneous injection. n=3 mice/group, representative of 1experiment. ns=not significant, *p=0.02, **p<0.005, unpaired student's *t* test. Frequencies and error bars are mean±SEM.

DISCUSSION

We show that Trib1 acts at two distinct points during eosinophil development, as revealed through stage-specific deletion studies. Trib1 loss in HSCs leads to a selective decrease in EoPs and a concomitant increase in neutrophils, suggesting that blocking early eosinophil commitment directs myeloid progenitors toward neutrophils. In contrast, Trib1 loss in EoPs using EoCre does not impact eosinophil fidelity. However, the resulting BM eosinophils express aspects of the neutrophil program; this phenotype is also observed in eosinophils generated from $Trib1^{\Delta HSC}$ progenitors. These data suggest that Trib1 is required to both specify the eosinophil lineage and guide its proper differentiation.

We observe a marked expansion of a $Ly6G^+$ eosinophil population in Trib1-deficient BM, in contrast to WT mice, where Ly6G is primarily restricted to neutrophils[149]. $Ly6G^+$ eosinophils are also present in WT BM, albeit at a much lower frequency, and are

predominantly found among CCR3$^-$ eosinophils **(Figs. 2D-F)**. Additionally, a small population of Ly6G$^+$ eosinophils was identified in WT murine lungs following fungal allergen challenge, as well as in *ex vivo* eosinophil cultures of WT BM[170]. Our studies provide new insights into the characteristics and maintenance of this population by Trib1. Furthermore, without Trib1, aspects of the neutrophil gene program are either activated or remain active, which stabilizes the existing Ly6G$^+$ CCR3$^-$ and/or CCR3$^+$ eosinophil populations. Neither the previously published work nor our studies determined the true function of these cells at homeostasis. It is likely that in WT mice, the Ly6G$^+$ eosinophils are a differentiation intermediate. As noted in the discussion of **CHAPTER 2**, recent work identified a population of GMPs that express elements of multiple cell lineage programs[103]. We hypothesize that Ly6G$^+$ eosinophils in WT mice may be an extension of this multi-lineage primed population. Alternatively, it is possible that these cells differentiate into mature cells yet re-express elements of alternative programs. The Trib1-deficient Ly6G$^+$ eosinophils phenotypically and functionally resemble eosinophils, yet also retain neutrophilic characteristics, including expression of Ly6G and SiglecE, increased phagocytosis and ROS production, and neutrophil-type granules. These findings further implicate Trib1 as an important regulator of granulocyte identity.

These findings show that Trib1 tunes the response to IL-5 to maintain eosinophil lineage fidelity, with Trib1 functioning to repress neutrophil gene expression following IL-5 stimulation. The above IL-5 data suggest that IL-5 responsiveness is fundamentally altered. Potential causes of the change in IL-5 responsiveness are discussed in **CHAPTER 6**. Furthermore, we observe that Trib1-deficient eosinophils increase their production of MCP-1 when in IL-5 culture **(Fig. 3.15C)**, a cytokine that normally is not induced in eosinophils by IL-5[159]. While it is unknown if IL-5 drives this phenotype *in vivo* as well, future work will focus on the *in vivo* consequences of Trib1 loss in eosinophils.

However, given the defective mobilization of eosinophils from the BM, it will may be challenging to delineate the functional consequences of altered cell function from differences in recruitment kinetics.

Although our papain challenge and transwell migration studies indicate that functional eosinophils are present in Trib1-deficient mice, eosinophil migration was altered **(Figs. 3.16-3.18)**. Of particular interest is our finding that CXCR4 is increased on Trib1-deficient BM eosinophils **(Figs. 3.19D-E)**. We hypothesize that increased CXCR4 expression on Trib1-deficient BM eosinophils limits egress of these cells from the BM.[168,169] This expands BM eosinophils **(Figs. 3.1A-C, 3.10A-C),** reduces peripheral eosinophil numbers **(Figs. 2.3D-I)** and decreases migration **(Figs. 3.16-3.18)**. Our studies using AMD3100 demonstrated that CXCR4 antagonism could mobilize eosinophils into the blood **(Figs. 3.20-3.21)**. This further suggests that increased CXCR4 expression on Trib1-deficient eosinophils contributes to their BM retention and diminished migration in response to papain. Of note, $Trib1^{+/+}$ eosinophils were also mobilized in response to AMD3100 treatment. This implicates CXCR4 in WT eosinophils as well as in the absence of Trib1. However, future studies are required to evaluate the true importance of CXCR4 to eosinophil chemotaxis and migration.

As noted above, Trib1-deficient eosinophils have some functions characteristic of neutrophils. Despite this, it is unclear how the function of neutrophils is altered with Trib1 loss. This question will be explored in **CHAPTER 5**. Previous studies in mutant mice defined defects in eosinophil production due to alterations in transcription factors or granule proteins, or changes in eosinophil recruitment due to loss of CCR3 or eotaxin[86,100,101,171-175]. Our current study shows that Trib1 also influences eosinophil production and recruitment. How Trib1 signals integrate with these other pathways remains to be defined.

The above data indicate that Trib1 loss instructs granulocyte differentiation by modulating IL-5 signaling at two points, both early in differentiation at lineage commitment, as well as in the control of terminal identity. How this is controlled or the mechanism through which Trib1 acts remains unknown, and will be studied further in **CHAPTER 4**.

CHAPTER 4: TRIB1 REGULATES C/EBPA LEVELS TO CONTROL EOSINOPHIL IDENTITY[3]

INTRODUCTION

As cells differentiate, cellular identity is intimately connected with the transcriptional landscape of that cell, largely controlled by a set of key transcription factors. These transcription factors, often called master regulators, play a central role in determining cellular identity, as they control genes correlating with lineage-specific programs. They also modulate the levels of other transcription factors either associated with or opposed to a certain cell fate. During the process of differentiation, there are multiple possibilities for how progenitors determine cell identity, including through external instructive signals, such as cytokines, or through a stochastic process mediated by the balance of transcription factors in a particular cell. These processes are not necessarily mutually exclusive and likely operate in tandem.

Several studies highlighted the transcriptional networks that mediate lineage specification. One report focused on the interplay between two critical transcription factors required for lymphoid and myeloid development, Gfi1 and PU.1. Work studying their opposing functions demonstrated that these two factors are able to both directly and indirectly repress transcription of the other[176,177]. This was especially clear in the case of Gfi1, which binds directly to the PU.1 (*Spi1*) promoter and suppress its transcription. Furthermore, this group demonstrated that Gfi1 and PU.1 have overlapping recognition sequences, suggesting they compete for DNA binding sites. This indicates that lineage-promoting transcription factors maintain cell fate choices in part by

[3]Portions of this chapter are adapted from "Trib1 regulates eosinophil lineage commitment and identity by restraining the neutrophil program", Ethan A. Mack, Sarah J. Stein, Kelly S. Rome, Lanwei Xu, Gerald B. Wertheim, Rossana C.N. Melo, and Warren S. Pear, *Blood*, in revision.

repressing opposing factors. While PU.1 and Gfi1 oppose each other, a system was reported in B cells involving E2A, EBF1, and Foxo1, which form a network to direct B lineage fate[178]. This group demonstrated that coordinated binding of these factors enhanced B cell-specific gene expression, suggesting that these factors cooperatively function to facilitate lineage specification. Finally, it was demonstrated that transcription factors actively work to promote epigenetic alterations in enhancers and promoters[179]. Taken together, these data suggest that cell fate choice is a dynamic process, initiated early during development through transcription factors, enhancer activity, or both.

As noted in **CHAPTER 2**, cytokine signals influence hematopoietic progenitor priming. These demand-associated alterations in cell output are not only dependent on cytokine signals, but activate alternative transcription factors or transcriptional programs to direct cell production. Emergency granulopoiesis, the process of increasing neutrophil production during bacterial infection, is mediated by G-CSF signals through STAT3[180-182]. Importantly, while C/EPBα is normally a key regulator of neutrophil differentiation, C/EPBβ is required for emergency granulopoiesis[183]. Without C/EPBβ, neutrophil production cannot respond to these demand signals. In fact, C/EPBβ is a direct STAT3 target in response to G-CSF and together, they co-induce c-Myc expression to support increased proliferation during emergency granulopoiesis[181].

As noted in **CHAPTER 1**, there are several transcription factors that are key for eosinophil lineage commitment and terminal differentiation. Central among them are C/EPBα, C/EBPε, GATA-1, and GATA-2. The level of expression of these factors is critical. Different expression levels of C/EPBα separated neutrophil and monocyte differentiation[77], and intermediate levels of GATA-1 corresponded with eosinophil instead of thromboblast-type cells, which favored higher levels[84,85]. In addition to the simple expression of these transcription factors, the order in which they are expressed is

critical for proper development. C/EPBα expression prior to GATA-2 yielded eosinophils, whereas basophils were generated if the order was reversed[97]. Finally, in studying the different functions of C/EBP proteins in eosinophils differentiation, lineage commitment and terminal maturation were found to have differential requirements for the C/EBP transactivation domain[81].

One of the first described functions of *Tribbles* in drosophila was to facilitate the degradation of *Slbo*, the drosophila homologue of the C/EBP transcription factors[110]. Furthermore, in mammalian systems, C/EBPα was shown to be key to Tribbles-protein mediated phenotypes seen in leukemic and knockout settings[113,118]. A direct interaction between Trib1 and C/EBPα was also demonstrated[184] and C/EBPα binding to Trib1 was shown to drive a conformational change in Trib1, exposing other binding sites, possibly for the E3-ubiquitin ligase COP1[185]. Of note, Trib1 selectively interacts with C/EPBα p42 and not p30[184]. Despite these data, it was unknown if and how C/EBPα contributed to the phenotypes seen in Trib1-deficient mice. We observe that Trib1 normally functions to restrain C/EPBα p42 protein levels and that C/EPBα partly controls eosinophil phenotype. Finally, we observe that Trib1 can both repress the neutrophil program while simultaneously inducing the eosinophil lineage program.

RESULTS

Trib1 modulates granulocyte identity in part through regulation of C/EBPα

C/EBPα is a key regulator of granulocyte development[74-77] and is a target of Trib1-mediated protein degradation[114,118,184]. We hypothesized that Trib1 modulates C/EBPα protein levels in order to regulate granulocyte development. We measured the expression of C/EBPα protein in sorted BM neutrophils and $CCR3^-$ and $CCR3^+$ eosinophils from $Trib1^{+/+}$ and $Trib1^{\Delta HSC}$ mice. Strikingly, C/EBPα p42 protein expression

was increased in all three populations with minimal change in C/EBPα p30 **(Fig. 4.1A)**. We also measured expression of GATA1 and C/EBPε, two key transcription factors in eosinophil development[83,84,94,186], in eosinophils from Trib1$^{+/+}$ or Trib1$^{\Delta HSC}$ mice. GATA-1 expression was decreased in CCR3^{-} eosinophils lacking Trib1, but unaffected in CCR3^{+} Trib1$^{\Delta HSC}$ eosinophils, while C/EBPε, a target of C/EPBα during granulopoiesis[77], was increased in the absence of Trib1 **(Fig. 4.1B)**. GATA1 was proposed as a marker to

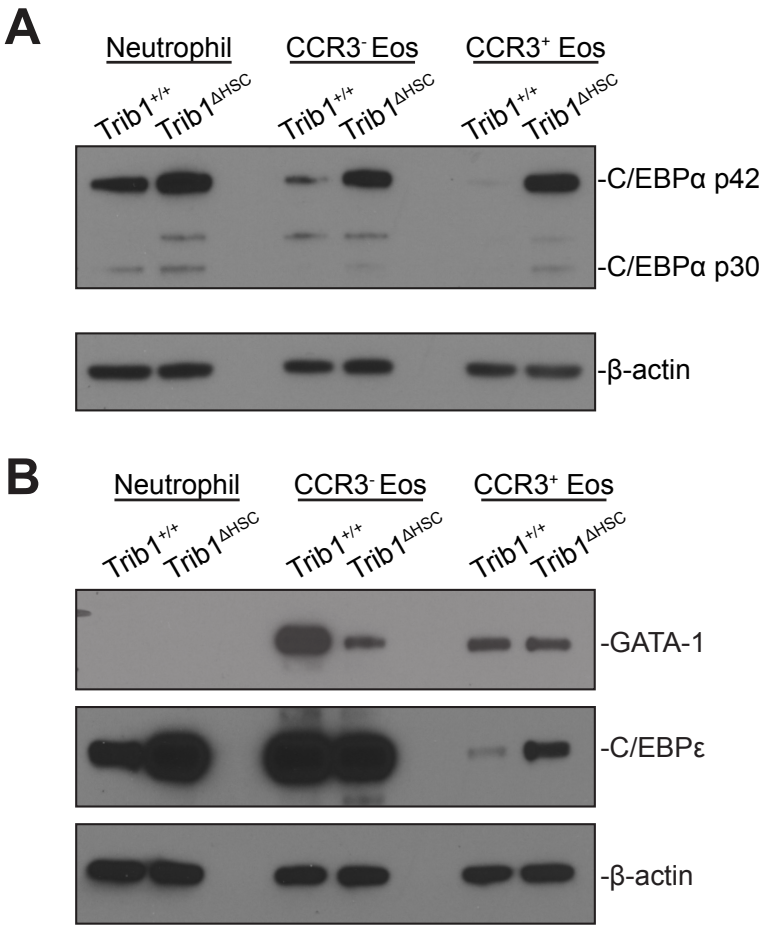

Fig. 4.1: Trib1 loss increases granulocyte C/EBPα, GATAT-1, and C/EBPε expression. A) C/EBPα immunoblot analysis of sorted neutrophils (CD11b^{+} Ly6G^{+} SiglecF^{-} F4/80^{-}), and CCR3^{-} and CCR3^{+} eosinophils (CD11b^{+} SiglecF^{+} F4/80^{+}) from the BM of Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice, β-actin is the loading control. Representative of 2 experiments. B) Immunoblot of sorted CCR3^{+} eosinophils (CCR3^{+} SiglecF^{+} F4/80^{+} CD11b^{+}) probed for GATA-1 and C/EBPε, with β-actin as a loading control. Representative of 3 experiments.

distinguish between progenitors with neutrophil versus eosinophil potential[98] and our data suggest that Trib1 modulates GATA-1 levels, possibly altering granulocyte cell identity.

To determine if C/EBPα upregulation was responsible for altered granulocyte development in the absence of Trib1, we knocked down C/EBPα in hematopoietic progenitors from $Trib1^{+/+}$ or $Trib1^{\Delta HSC}$ mice using two previously validated shRNAs[77], transplanted the cells into irradiated recipients, and assessed BM eosinophils by flow cytometry after 9 weeks **(Fig. 4.2)**. We also sorted GFP^+ BM cells to assess knockdown efficiency **(Fig. 4.2A)**. We observed decreased Ly6G expression on $SiglecF^+$ eosinophils in the BM of recipients reconstituted with $Trib1^{\Delta HSC}$ progenitors transduced with C/EBPα shRNAs B9 and B11 **(Fig. 4.2C)**. Together, these findings indicate that Trib1 modulates C/EBPα levels to promote eosinophil cell identity.

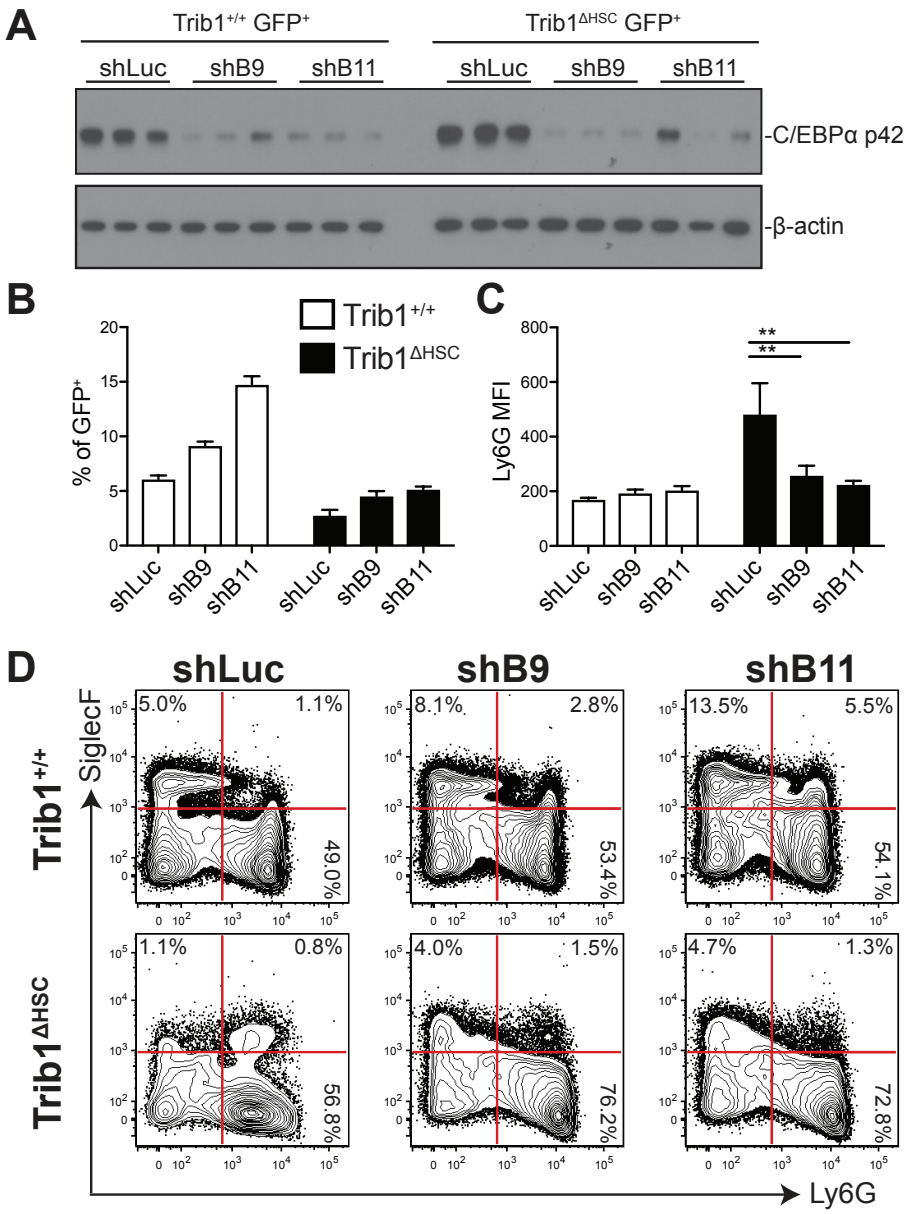

Fig. 4.2: C/EBPα knockdown partly restores normal eosinophil production from Trib1-deficient BM. A) C/EBPα immunoblot analysis of sorted GFP$^+$ BM cells from mice transplanted with shRNA-transduced BM cells, 9 weeks after transplantation with β-actin as a loading control. shLuc control targets firefly luciferase, shB9 and shB11 target *Cebpa.* Sorted GFP$^+$ cells from 3 representative mice are shown. Representative of 2 experiments. B) Percentage of GFP+ cells expressing control or C/EBPα shRNAs B9 or B11 cells expressing SiglecF with surface expression of SiglecF. Representative of 2 experiments. D) MFI of Ly6G expression on BM eosinophils (GFP$^+$ CD11b$^+$ SiglecF$^+$) expressing either control or C/EBPα shRNAs B9 or B11. n=4-5 mice/group, representative of 2 experiments. **p<0.0052, 2-way ANOVA with multiple comparisons. Frequencies and error bars are mean±SEM of GFP$^+$ cells

As an alternative approach to studying the role of C/EBPα in eosinophil identity, we used a stepwise genetic approach. We bred Trib1$^{\Delta HSC}$ with mice bearing floxed *Cebpa* alleles[75]. This allowed deletion of both Trib1 and C/EBPα in all hematopoietic cells. As noted above, loss of C/EBPα prior to the GMP creates a block in differentiation at the CMP to GMP transition[74,75]. An advantage of our approach, however, is that we were able to generate full Trib1 knockouts while only deleting one *Cebpa* allele. These VavCre^{+} Trib1$^{Fl/Fl}$ C/EBPα$^{Fl/+}$ mice, termed 'Trib1$^{\Delta HSC}$ C/EBPα$^{\Delta HSC/+}$", facilitated a 50% reduction in C/EBPα gene dosage.

To circumvent the issue of blocking the CMP to GMP transition with full C/EBPα knockout, we generated a second conditional *Cebpa* deletion strain using the Trib1$^{\Delta Eos}$ mice. By crossing Trib1$^{\Delta Eos}$ mice to C/EBPα$^{Fl/Fl}$ mice, we were able to generate mice lacking both *Trib1* and either one or both copies of *Cebpa*. These mice, termed 'Trib1$^{\Delta Eos}$ C/EBPα$^{\Delta Eos/+}$', allowed us to reduce *Cebpa* levels by half. Importantly, as EoCre expression begins following eosinophil lineage commitment from the GMP and C/EBPα expression is high in the GMP, it is likely that genetic deletion of one allele of *Cebpa* will not be sufficient to reduce C/EBPα protein. This may be further compounded by the lack of Trib1 in these mice. As Trib1 is a post-translational regulator of C/EBPα levels, absence of Trib1 may further stabilize C/EBPα even after the *Cebpa* allele is excised. In both models, we observed a reduction in Ly6G expression on Trib1-deficient CCR3^{+} BM eosinophils when one copy of *Cebpa* was removed (**Figs 4.3-4.4)**, further supporting our hypothesis that Trib1 modulates C/EBPα levels to influence eosinophil identity.

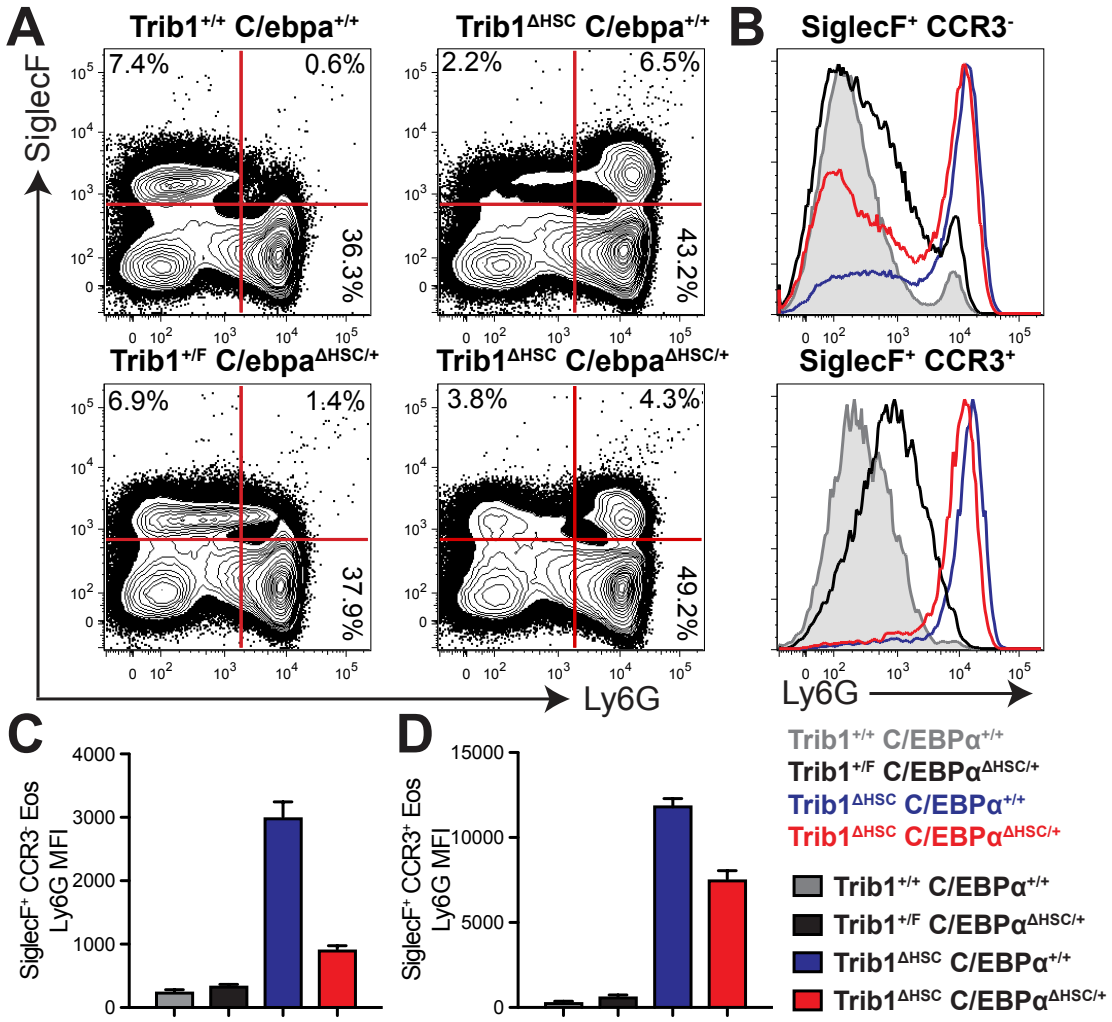

Fig. 4.3: Genetic reduction in C/EBPα reduces Ly6G expression on Trib1$^{\Delta HSC}$ eosinophils. A) Representative plots of BM granulocytes gated on live, CD11b^{+} cells B) Representative histograms of Ly6G expression by CCR3^{-} (top) and CCR3^{+} (bottom) eosinophils. Quantification of Ly6G MFI on CCR3^{-} (C) and CCR3^{+} (D) BM eosinophils. n=3 mice/group, representative of 1 experiment. Frequencies and error bars are mean±SEM. Frequencies and error bars are mean±SEM of live cells.

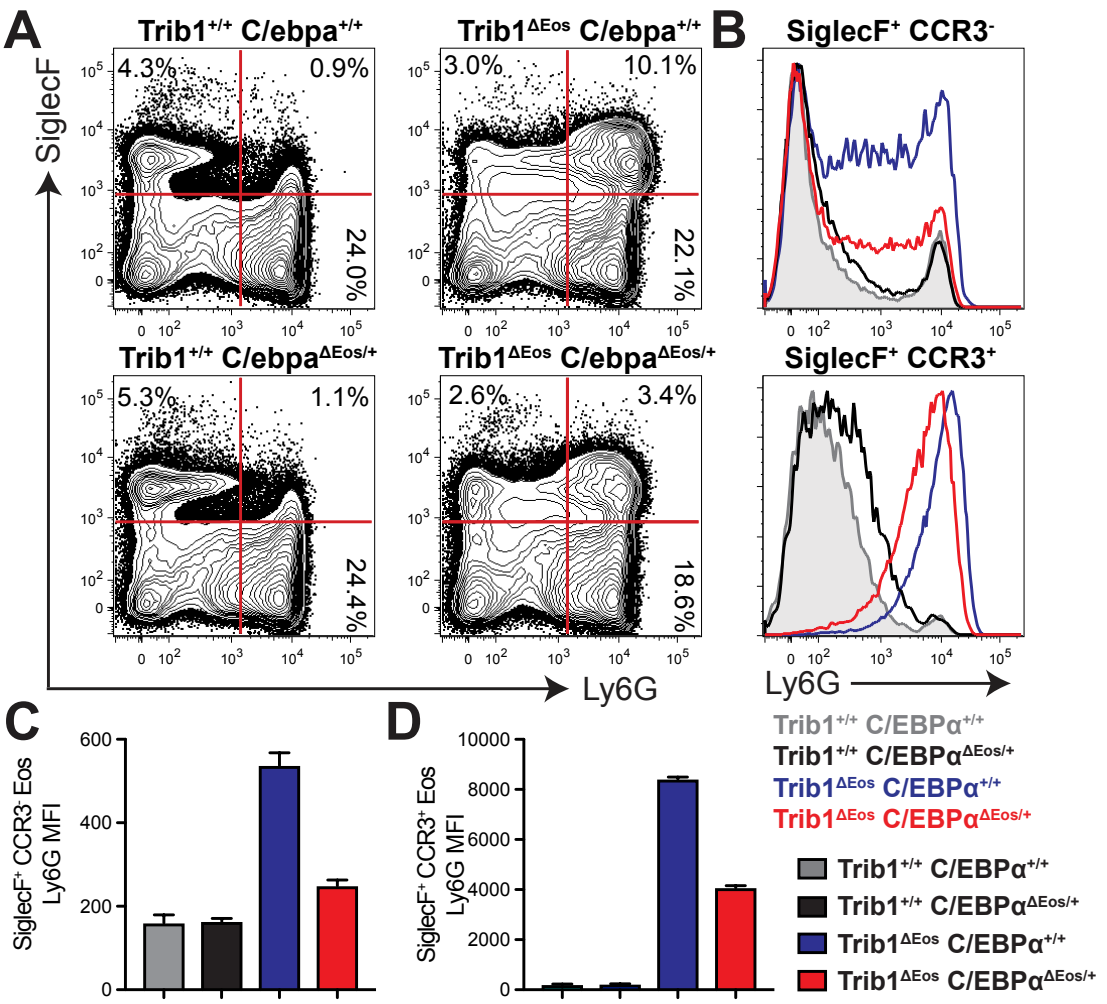

Fig. 4.4: Genetic reduction in C/EBPα reduces Ly6G expression on Trib1$^{\Delta Eos}$ eosinophils. A) Representative plots of BM granulocytes gated on live, CD11b^{+} cells B) Representative histograms of Ly6G expression by CCR3^{-} (top) and CCR3^{+} (bottom) eosinophils. Quantification of Ly6G MFI on CCR3^{-} (C) and CCR3^{+} (D) BM eosinophils. n=2-3 mice/group, representative of 1 experiment. Frequencies and error bars are mean±SEM.

Trib1 can enforce the eosinophil program

While the data in the preceding chapters suggest that Trib1 promotes eosinophil lineage commitment from the GMP and represses the neutrophil program in response to IL-5 signaling, it is unclear if Trib1 truly enforces the eosinophil program. To determine if there is an instructive function for Trib1 in eosinophil lineage specification, we turned to an overexpression system. Previously, our lab demonstrated that overexpression of either Trib1 or Trib2 lead to the development of AML[113,114]. In both settings, there was increased C/EBPα p42 degradation. In the case of Trib2, it was shown that Trib2 overexpression promoted a change in the ratio of C/EBPα p42 and p30, with a loss of p42 and an increase in p30. Prior to AML development, mice with BM overexpressing Trib2 lost a population of $CD11b^+$ $Gr1^+$ cells and expanded a population of $CD11b^+$ $F4/80^+$ cells[113]. This switch from granulocytic to monocytic/macrophage development, suggested that Trib2 modulated both leukemogenesis, as well as normal development.

To evaluate the role of Trib1 in enforcing the eosinophil lineage program, we retrovirally overexpressed mouse Trib1 in WT hematopoietic progenitors. We then evaluated the BM at 8.5 weeks following transplantation. In mice transplanted with cells overexpressing Trib1, we observed the formation of $SiglecF^+$ blasts in the BM and circulation **(Figs. 4.5-4.6)**. These cells had nearly no C/EBPα p42 with a dramatic increase in C/EBPα p30 **(Fig. 4.5A)**. Phenotypically, they resembled immature eosinophils cells with expression of Ly6C, CD11b, F4/80, but lacked CCR3 and had low side-scatter **(Figs. 4.5B-C)**. As seen previously, the white blood cell count (WBC) reflected leukemia development **(Fig. 4.5D)**. We confirmed their blast-like morphology by cytospin **(Figs. 4.6A-B)**. To determine if Trib1 overexpression altered eosinophil versus neutrophil gene expression, we measured *Epx* and *Ltf* expression in sorted GFP^+ cells from the BM of these mice. We observed an increase in *Epx* and a concomitant

decrease in *Ltf* in cells overexpressing Trib1 **(Fig. 4.6C-E)**. Together with the surface SiglecF expression, this suggests that Trib1 can play an instructive role in the eosinophil lineage program in addition to repressing the neutrophil program.

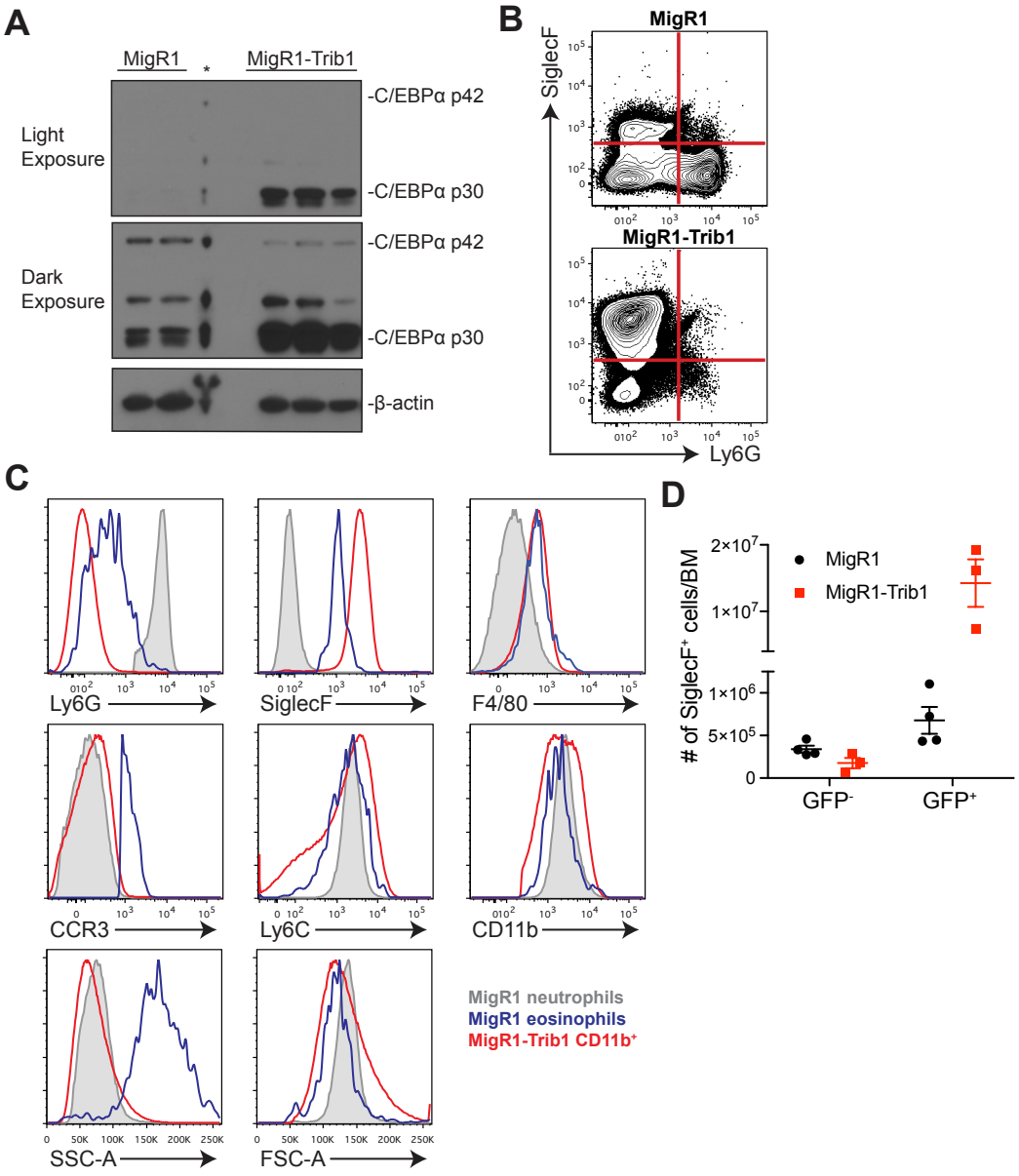

Fig. 4.5: Trib1 overexpression results in blasts with an altered C/EBPα p42:p30 ratio and eosinophilic surface features. A) C/EBPα immunoblot of sorted GFP$^+$ cells from mice transplanted with BM transduced with either MigR1 empty vector or MigR1-mTrib1. *=poor lysis sample. Each lane is an individual mouse. B) Representative plots of BM granulocytes gated on live, GFP$^+$ CD11b$^+$ cells. C) Representative histograms characterizing GFP$^+$ CD11b+ blasts from Trib1-overexpressing BM. MigR1-EV neutrophils (gray) and eosinophils (blue) shown as a control. D) Absolute number of GFP$^+$ CD11b$^+$ SiglecF$^+$ cells in the BM. n=3-4 mice/group, representative of 1 experiment. Mice analyzed at 8.5 weeks post-transplant. Frequencies and error bars are mean±SEM.

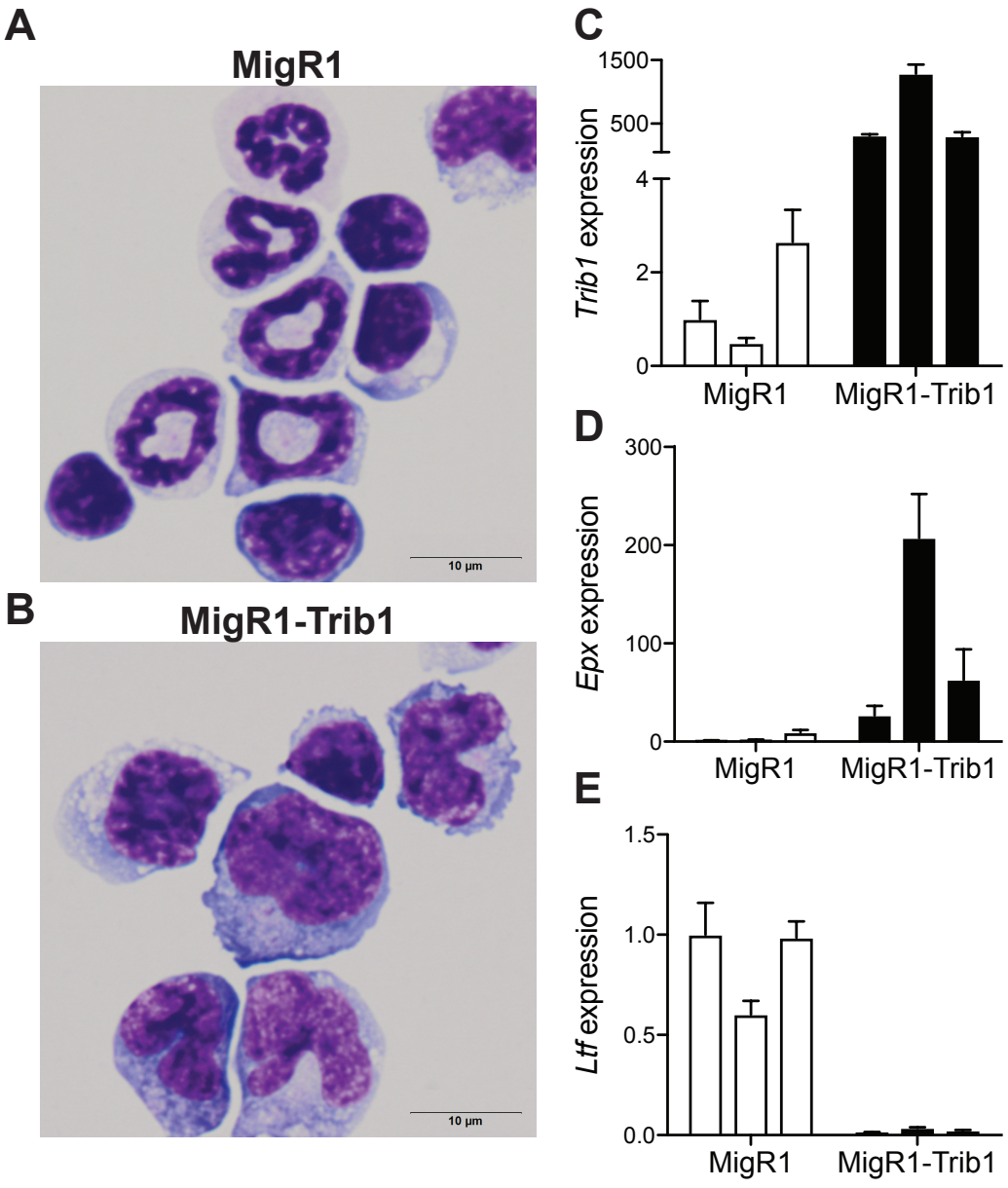

Fig. 4.6: Cells overexpressing Trib1 show eosinophil-biased gene expression. A-B) Representative micrographs of cytospins of sorted BM GFP$^+$ cells from mice transplanted with BM transduced with either MigR1 empty vector or MigR1-mTrib1 at 100x magnification. qPCR analysis of sorted BM GFP$^+$ cells for C) *Trib1*, D) *Epx*, and E) *Ltf*, relative to *18s*, normalized to MigR1-EV #1. n=3 mice/group, representative of 1 experiment. Mice analyzed at 8.5 weeks post-transplant. Frequencies and error bars are mean±SEM.

DISCUSSION

As reported, cells lacking Trib1 have increased amounts of C/EBPα[118] **(Fig. 4.1A)**. The precise regulation of C/EBPα is critical in myeloid development[74-77,82,97]; and neutrophils express higher levels of C/EBPα than eosinophils **(Fig. 4.1A)**. Furthermore, knockdown of C/EBPα partly normalized eosinophil differentiation in the absence of Trib1 **(Fig. 4.2C)**, suggesting that Trib1 controls eosinophil identity by regulating C/EPBα levels. Our observation of increased C/EBPε expression in Trib1-deficient eosinophils **(Fig. 4.1B)** is consistent with the ability of C/EPBα to upregulate C/EBPε during granulopoiesis, possibly altering granule development[77]. Finally, we do observe a decrease in GATA-1 levels in CCR3- eosinophils lacking Trib1, suggesting that Trib1 may play a role in recent reports noting that GATA-1 expression segregates eosinophil versus neutrophil fate[98]. These findings suggest that Trib1 normally represses the neutrophil gene program in developing eosinophils, partly by decreasing C/EBPα protein expression, in order to promote eosinophil development. Critically, our data suggest that the precise regulation of C/EPBα is required for proper granulocyte development.

C/EBPα has long been known to be critical for myeloid cell development and centrally, it is required for the CMP to GMP transition[75]. Among the lineages differentiating from the GMP, including macrophages/monocytes and neutrophils, it is clear that the level of C/EBPα following the GMP is critical for lineage specification. In particular, reduced levels of C/EBPα subsequent to the GMP favor monocyte output over neutrophils[76,77]. Importantly, C/EBPα knockdown rescued the *ex vivo* changes in colony formation from Trib1$^{-/-}$ BM[118]. Previous work suggests that the degree of C/EBPα homodimerization versus heterodimerization with transcription factors like AP-1 family members may alter the balance between neutrophil and monocyte identity[187]. These studies did not examine the role of C/EBPα levels in eosinophil development. As such,

we hypothesize that higher levels of C/EBPα and subsequent formation of C/EBPα homodimers drives neutrophil-specific gene expression, whereas eosinophil-specific gene expression is predominantly driven by C/EBPα heterodimers with other transcription factors. Future studies will focus on the specific interactions between C/EBPα and other transcription factors and mechanistically how C/EBPα levels impact identity. This will be further discussed in **CHAPTER 6**.

C/EBPα is post-translationally processed into 2 isoforms, p42 and p30[78]. Intriguingly, Trib1 appears to disproportionally impact p42 levels while sparing p30 **(Fig. 4.1A)**. This effect is in line with the previously reported selectivity for the interaction with Trib1 and C/EBPα p42[184]. We previously observed that overexpression of Trib1 or Trib2 induced AML[113,114]. Our data here replicate this phenotype and we further characterize the blasts seen with Trib1 overexpression. The AML effect seen in **Fig. 4.5** is likely due to an imbalance in the levels of C/EBPα p42 and p30. Furthermore, as noted above, different C/EBP functions are required for eosinophil lineage commitment versus terminal differentiation[81]. As C/EPBα p30 lacks one of the two transactivation domains present in p42[78], this may be a possible mechanism for altered gene expression or differentiation lineage programming seen in the absence of Trib1, with a shift in the ratio of p42 to p30.

While we hypothesize that Trib1 selectively degrades p42 without interacting with p30, it is unclear what drives the increase seen in p30 with Trib1 overexpression. There are several possibilities: 1) Trib1 facilitates processing of C/EBPα p42 into p30, 2) Trib1 directly drives increased production of p30, 3) Trib1 indirectly drives increased production of p30. Of these three possibilities, the first and third and the most likely, but further work is needed to determine how Trib1 modulates p30 levels. For this latter possibility, Trib1-mediated degradation of C/EBPα p42 could lead to a feedback

mechanism whereby the cell would increase *Cebpa* transcription. While this would initially increase both p42 and p30, Trib1 would continue to selectively degrade p42, sparing p30. The net result is an isolated increase in C/EBPα p30. Interestingly, recent work demonstrates that C/EBPα p42 is required for Trib2-mediated leukemia initiation[188]. It remains unknown, however, if this reflects that p42 is needed to drive differentiation far enough for p30 to take over, or if p42 in some way cooperates in inducing the leukemic program itself.

As noted above, p30 alone is leukemogenic[80] and we see a true AML phenotype with Trib1 overexpression. While it is difficult to draw conclusions on how Trib1 acts normally from the AML context, we do observe elements of the eosinophil program active in these blasts. As seen in **Figs. 4.5B-C** and **Figs. 4.6D-E**, cells overexpressing Trib1 expressed SiglecF, F4/80, and Ly6C on their surface and slightly downregulated Ly6G. By qPCR analysis, we observed increased *Epx* coupled with a near complete suppression of *Ltf* transcripts. Together, these data suggest that Trib1 can both activate the eosinophil program, but also repress the neutrophil program when overexpressed. Despite this, they also had low side-scatter and no granularity or eosinophilic staining visible on cytospin **(Figs. 4.6A-B)**, indicating that the leukemogenic effects of p30 may overwhelm any differentiation programs operating in the background.

C/EBPα is a dynamic transcription factor and has multiple points of regulation and tuning to alter its function and activity. In this chapter, I demonstrate that one of those methods, post-translational control of protein stability, fundamentally impacts eosinophil identity. While the C/EBPα shRNA knockdown and genetic deletion studies show that C/EBPα directly impacts terminal identity **(Fig. 4.2-4.4)**, it remains unknown if or how the changes in C/EBPα seen with Trib1 loss alter granulocyte progenitor heterogeneity, eosinophil lineage commitment, neutrophil development or cellular

function. Furthermore, our genetic knockout studies using the conditional *Cebpa* allele demonstrate that by partly reducing the C/EBPα gene dosage, we can to some degree rescue the neutrophilic phenotype seen on Trib1-defcient eosinophils. However, it is difficult to interpret these data in the absence of C/EBPα western blots confirming a reduction in C/EBPα levels. These issues and directions for future work will be discussed in **CHAPTER 6**.

CHAPTER 5: TRIB1 CONTROLS NEUTROPHIL FUNCTION

INTRODUCTION

As noted in **CHAPTER 1**, neutrophils are powerful effector cells that are critical effectors against bacterial infection, and can also be damaging and immunopathogenic. This duality in their function serves to highlight the importance of tight regulation of neutrophil function. While the control of neutrophil production is one of the central regulators of overall neutrophil function, I will not focus on the factors controlling neutrophil development here, as granulopoiesis is reviewed in **CHAPTERS 1-4**. In this chapter, I will focus on regulators of terminal neutrophil function and highlight possibilities for how Trib1 modulates these pathways. In reviewing the many pathways that transduce signals in neutrophils, I will discuss those we examined in detail experimentally, including the AKT/mTOR, MAPK, and NF-κB pathways.

Signaling cascades in neutrophils

The AKT/mTOR pathway is a conserved signaling cascade that regulates cellular anabolic pathways[189]. This pathway is critically important for supporting proliferation and survival, as well as the development and function of immune cells[190]. In neutrophils, this pathway is required for chemotaxis[191-193] and the generation of reactive oxygen species (ROS)[194,195], critical elements of neutrophil inflammatory function. Negative regulation of this pathway decreases neutrophil activity[196,197], and increased AKT activation correlates with worse patient outcomes in acute lung injury[198]. This is thought, in part, to be neutrophil-dependent[199]. The AKT pathway through mTOR can also regulate NET formation[200,201]. In neutrophils, AKT signaling is often activated by G-protein-coupled receptors (GPCRs), such as complement, chemokine, or formyl peptide receptors[195,202].

There are two readouts that I will use to measure activation of the AKT/mTOR pathway. The first is directly looking at the activation of AKT itself, reading out phosphorylation at serine 473. The second is a more downstream target of the AKT/mTOR pathway, S6. S6, a ribosomal protein involved in ribosome biogenesis and protein synthesis, is activated by S6 kinase (S6K), an mTOR target[203-205]. I use it here as a readout for AKT/mTOR pathway activity.

The NF-κB pathway is also involved in neutrophil activation and function[206]. NF-κB is a family of transcription factors that is inducibly regulated by multiple external and internal signals including toll-like receptor agonism, cytokine stimulation, and internal reactive oxygen and nitrogen intermediates[207-212]. NF-κB subunits are rapidly activated following stimulation of neutrophils with LPS, TNFα, or the formyl peptide fMLP. NF-κB regulates multiple cellular processes in neutrophils including cytokine production[213] and survival[214,215]. While classically thought of as a pro-inflammatory pathway, NF-κB activation can trigger both pro- and anti-inflammatory gene expression[213,216]. NF-κB activation is regulated at many steps, given the potent pro-survival and often pro-inflammatory functions of this pathway. One of the key regulatory steps that I will examine below is the degradation of the negative regulator, IκBα. IκBα normally functions to sequester NF-κB subunits in the cytosol, preventing nuclear translocation. Upon pathway activation, IκBα is phosphorylated by IKK and targeted for degradation, freeing NF-κB subunits to enter the nucleus and activate transcription[217].

In addition to the above-mentioned pathways, the mitogen-activated protein kinase (MAPK) pathway also plays a major role in neutrophil function. The MAPK pathway consists of signaling cascades that transduce extracellular signals downstream of multiple receptors including GPCRs, cytokine receptors, and TLRs[218]. This cascade can also modulate neutrophil apoptosis[219], as well as supporting neutrophil ROS

production[220]. One of the many members of the MAPK family are the extracellular signal-regulated kinase 1 and 2 (ERK1/2)[221]. These two highly homologous kinases transduce activation signals from growth factors, GPCRs, and other mitogens. They are directly activated by Ras and Raf proteins, which activates MEK1/2. MEK1/2 phosphorylate ERK1/2, which can then phosphorylate a variety of substrates including transcription factors such as NFAT[222], STAT5a[223], and c-Fos[224,225].

One important shared function is the regulation of cell survival. Stimulation of human neutrophils with different toll-like receptor agonists activated both AKT and NF-κB pathways, promoting cell survival[226]. All three of these pathways can regulate cell survival. AKT/mTOR controls caspase activation[227] and AKT blockade increased neutrophil apoptosis[228]. In contrast, activation of the MAPK member, JNK downstream of TNFα signaling induced apoptosis, which was negatively regulated by NF-κB[229]. Of note, TNFα-induced ROS production was suppressed by NF-κB, promoting cell survival[230,231].

Finally, these pathways are often co-activated following activation through different cell surface receptors[233]. For example, downstream of G-CSF signaling, multiple pathways are activated, including STAT signaling[180], MAPK[234], and AKT[235,236]. While other signaling and transcriptional cascades contribute to regulating terminal neutrophil functions, these three constitute the bulk of the regulatory potential, and importantly for this study, show the potential to interact with Tribbles proteins.

Tribbles proteins in cell function

The data presented in the preceding chapters illustrate that Trib1 fundamentally influences granulocyte development and identity, integrating signals from IL-5 and C/EPBα. Despite these data, it is unclear if Trib1 also regulates cellular function, independently of differentiation. In **CHAPTER 3**, I demonstrate that Trib1-deficient

eosinophils have reduced ability to migrate to sites of inflammation *in vivo* and to chemoattractants *ex vivo* **(Figs. 3.16-3.18)**, possibly correlated with their increased expression of CXCR4 **(Figs. 3.19-3.20)**. In addition, Trib1$^{\Delta Eos}$ eosinophils show altered chemokine expression following IL-5 stimulation, producing increased amounts of MCP-1 **(Fig. 3.15C)**. In light of these data, it is possible that aspects of neutrophil function are also impacted by Trib1 loss. While Trib1 is known more for its ability to modulate differentiation, other tribbles homologues are implicated in the regulation of activation. For example, mouse Trib2 regulates monocyte IL-8 production via the MAPK pathway[121] and human TRIB2 modulates MAPK signaling[120]. Elevated human TRIB1 expression was found in a study of hepatocellular carcinoma samples and corelated with activated ERK signaling[237]. Both Trib2 and Trib3 suppress AKT pathway activation, Trib3 through protein sequestration[122,123]. Finally, the report characterizing terminal myeloid alterations in Trib1$^{-/-}$ mice noted a defect in M2 macrophages in the absence of Trib1[118]. Macrophage polarization can reflect both functional or developmental changes, and it is unclear how Trib1 controls this phenotype. While these reports highlight the function of Tribbles homologues in regulating cell function or phenotype, it is unknown if Trib1 mediates similar effects in neutrophils.

RESULTS

Trib1 regulates neutrophil size and segmentation

As noted above, multiple Tribbles homologues impact cellular function. To asses changes in neutrophil function in the absence of Trib1, I measured cell size, as this can corelate with cellular activation. Cellular size is regulated by a number of factors including the metabolic state of the cell, and in particular, activation of the AKT pathway[238,239]. Neutrophil size may also correlate with cell function[240]. I found that Trib1-deficient BM neutrophils were larger as measured by forward scatter **(Fig. 5.1)**. In addition to their increased size, these cells had a hypersegmented nuclear architecture **(Figs. 5.2A-B)**. Recent work characterizing heterogeneity in peripheral neutrophil populations indicates that as neutrophils age in the circulation, their nuclei become increasingly segmented[241]. Aged neutrophils also express lower levels of CD62L, and I observed a slight reduction in CD62L expression on BM neutrophils from Trib1$^{\Delta HSC}$ mice

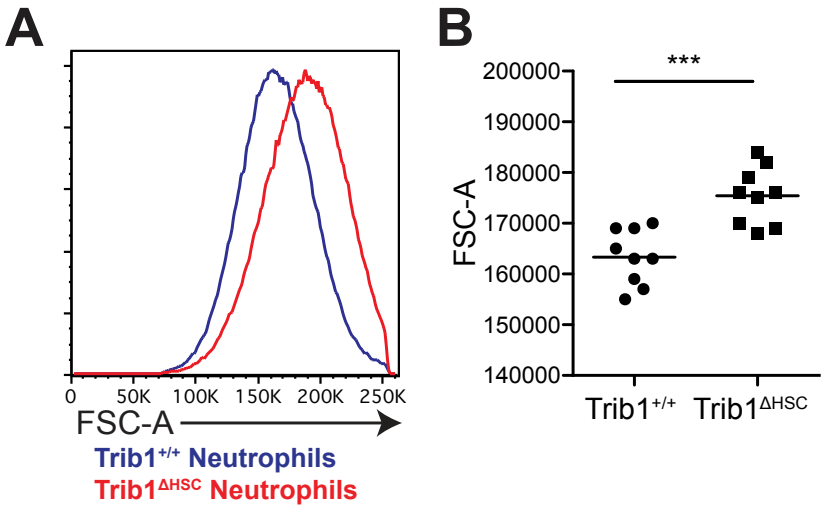

Fig. 5.1: Trib1 loss increases neutrophil cell size. A) Representative plots of BM neutrophils from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice, gated on live, CD11b^{+} SiglecF^{-} Ly6G^{+} cells. B) Geometric mean of the FSC-A for BM neutrophils, n=9 mice/group pooled from 3 experiments. ***p=0.0003, unpaired student's *t* test.

(Figs. 5.2C-D). While this reduction is not as significant as what is seen in aged blood neutrophils, there may be a correlation with the changes in nuclear segmentation.

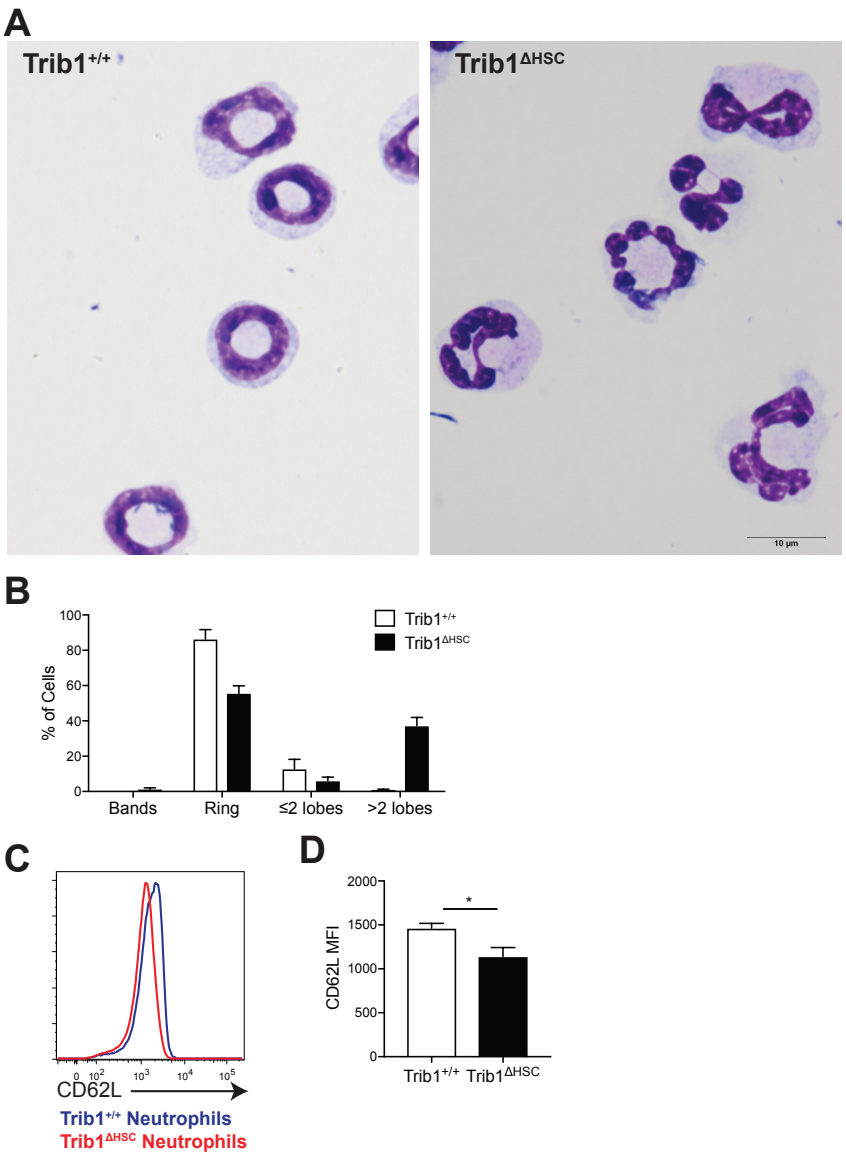

Fig. 5.2: Trib1-deficient neutrophils are hypersegmented. A) Representative micrographs of cytospins of sorted BM neutrophils from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice at 100x magnification stained with Diff-quik stain. Cells sorted on live, CD11b^{+} Ly6G^{+} SiglecF^{-} F4/80^{-}. Scale bar shown in right panel applies to both images. Representative of 3 experiments B) Quantification of nuclear lobation in sorted BM neutrophils, n=5 mice/group from 3 experiments. C) Representative histogram of BM neutrophils. Cells gated on live, CD11b^{+} Ly6G^{+} SiglecF^{-}. D) Quantification of CD62L MFI on BM neutrophils. n=3 mice/group, representative of 2 experiments. *p=0.05, unpaired students *t* test. Frequencies and error bars are mean±SEM. *Cytospin scoring done by G. Wertheim.*

Trib1-deficient neutrophils are hyperactivated

These data suggest that Trib1-deficient neutrophils may show altered AKT activation. To investigate this finding, I stimulated whole BM from $Trib1^{+/+}$ and $Trib1^{\Delta HSC}$ mice and examined AKT activation. I found that Trib1-deficient neutrophils have increased phosphorylation of AKT at Ser473 both at baseline and following LPS stimulation **(Fig. 5.3)**. To measure downstream consequences of elevated AKT activation, I repeated this stimulation on sorted BM neutrophils and measured phosphorylation of S6, a downstream target of AKT and mTOR[242]. $Trib1^{\Delta HSC}$ neutrophils showed increased phosphorylation of S6 at Ser235/236 both at rest and with short LPS stimulation **(Fig. 5.4)**. As LPS activates other pathways downstream of TLR4 aside from AKT, I examined both MAPK and NF-κB pathway activation in this stimulation of sorted neutrophils. $Trib1^{\Delta HSC}$ neutrophils had increased phosphorylation of ERK1/2 at Thr202/Tyr204 **(Fig. 5.4)**. Lastly, I examined degradation of IκBα, a negative regulator of canonical NF-κB signaling[217]. IκBα is degraded following stimulation and Trib1 loss did not appear to alter the kinetics of IκBα degradation. Together these data suggest that Trib1 may impact AKT and MAPK pathway activation while sparing NF-κB.

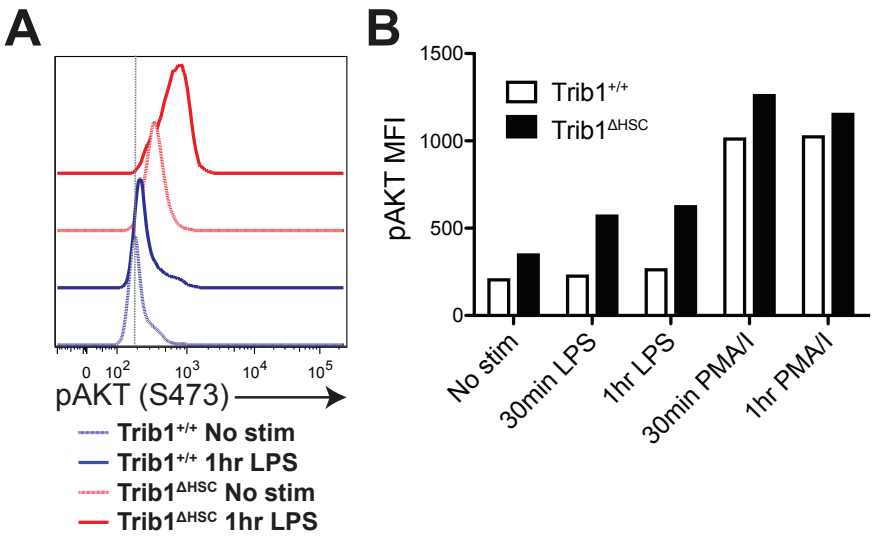

Fig. 5.3: Increased AKT phosphorylation in Trib1-deficient neutrophils after LPs stimulation. A) Representative histogram of BM neutrophils at rest or stimulated with 100ng/ml of LPS for 1hr. Cells gated on live, CD11b^{+} Ly6G^{+} SiglecF^{-}. Representative of 2 experiments B) Quantification of pAKT MFI following stimulation with LPS or PMA/ionomycin (2ng/ml PMA, 20ng/ml ionomycin), n=1 mouse/group representative of 2 experiments.

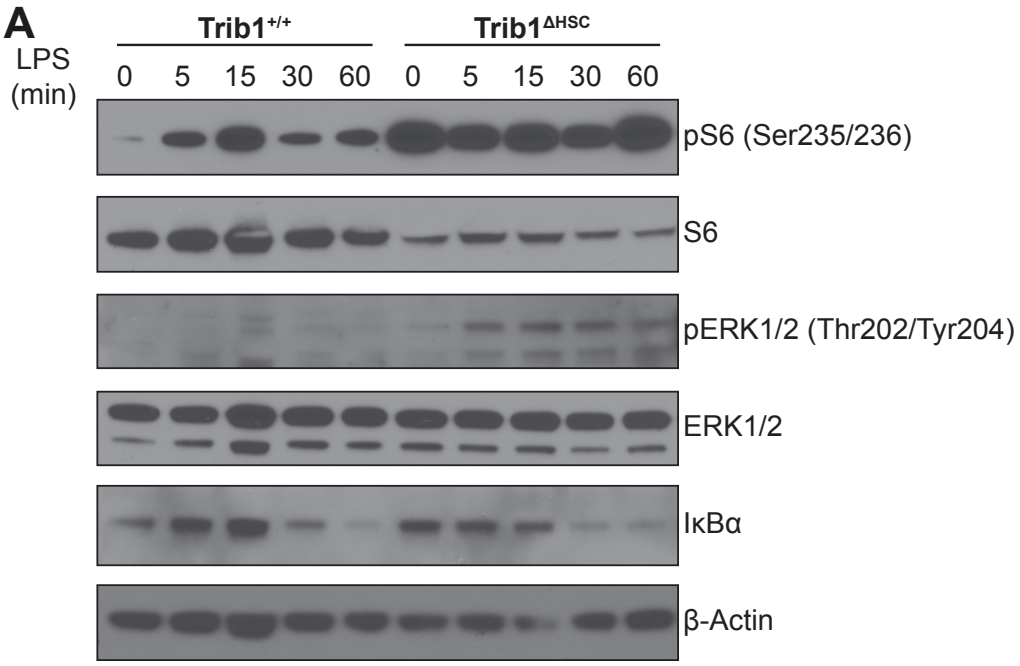

Fig. 5.4: Increased neutrophil S6 and ERK phosphorylation with Trib1 loss. A) Immunoblot of a time-course stimulation of sorted BM neutrophils. Cells sorted on live, CD11b^{+} Ly6G^{+} SiglecF^{-} F4/80^{-}. Representative of 2 experiments for pS6, 1 experiment for pERK, and IκBα.

The above data suggest that Trib1 alters intracellular signaling in neutrophils. To measure the impact of these changes on cell function, I measured neutrophil cytokine and ROS production. TNFα is a direct NF-κB target[243], and neutrophils can produce TNFα when activated. In particular, neutrophil TNFα production is associated with a pro-inflammatory neutrophil phenotype, thought to have anti-tumor effects[13]. While I did not observe changes in IκBα degradation following brief LPS stimulation **(Fig. 5.4)**, it is possible that other elements of the NF-κB pathway are altered or a longer stimulation is required to see differences. To investigate this, I stimulated whole BM from $Trib1^{+/+}$ or $Trib1^{\Delta HSC}$ mice with LPS for 2 or 4hrs and measured intracellular accumulation of TNFα. $Trib1^{\Delta HSC}$ neutrophils stimulated with LPS for both 2 and 4hrs showed increased TNFα production **(Fig. 5.5)**. Interestingly, the entire population of $Trib1^{\Delta HSC}$ neutrophils shifted in their production of TNFα, whereas in $Trib1^{+/+}$ BM, only a small fraction of neutrophils increased TNFα production.

To further probe neutrophil function in the absence of Trib1, we examined neutrophil ROS production. ROS production is one of the central methods neutrophils use to eliminate bacteria. Importantly, ROS production downstream of activators such as fMLP or C5a is regulated by inputs from AKT[244] and ERK[220]. To broadly evaluate the ability of neutrophils to produce ROS in the absence of Trib1, I stimulated whole BM from $Trib1^{+/+}$ and $Trib1^{\Delta HSC}$ mice with PMA. When stimulated with PMA for 20 minutes, $Trib1^{\Delta HSC}$ neutrophils produced more ROS compared to $Trib1^{+/+}$ neutrophils **(Fig. 5.6)**. Of note, there was no change in ROS production without stimulation, suggesting that the at rest changes seen in pS6 and cell size may be confined to anabolic pathways and not cellular effector functions.

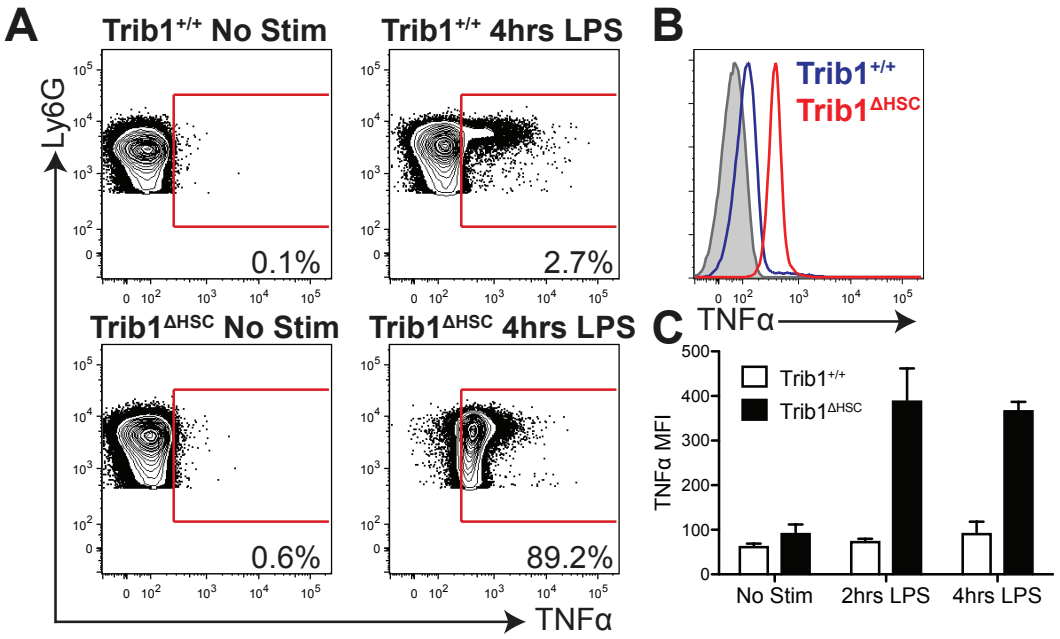

Fig. 5.5: Trib1-deficient neutrophils produce more TNFα following LPS stimulation. A) Representative plots of stimulated whole BM gated on neutrophils (live, CD11b$^+$ SiglecF$^-$ Ly6G$^+$) from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice. Cells with media alone or stimulated for 4hrs with 100ng/ml LPS with brefeldin A (2ug/ml). B) Representative histogram of intracellular TNFα gated on live, CD11b$^+$ SiglecF$^-$ Ly6G$^+$. C) Quantification of neutrophil TNFα MFI at 2 and 4hrs of LPS stimulation, n=2 mice/group, representative of 2 experiments. Frequencies and error bars are mean±SEM of neutrophils.

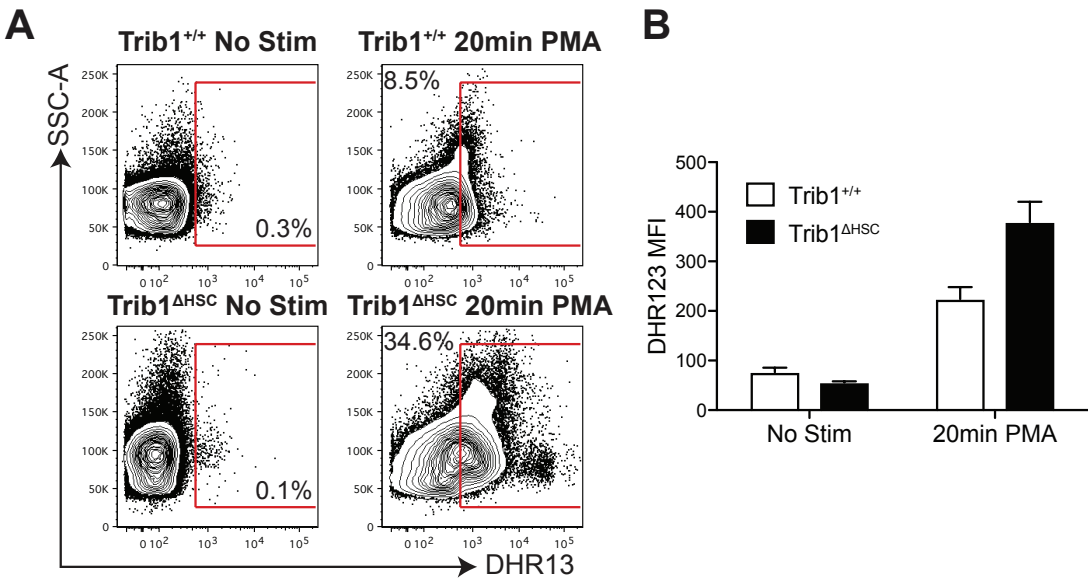

Fig. 5.6: Trib1-deficient neutrophils produce more ROS following PMA stimulation. A) Representative plots of stimulated whole BM gated on neutrophils (live, CD11b^{+} SiglecF^{-} Ly6G^{+}) from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice. Cells with media alone or stimulated for 20min with 10ng/ml PMN with 100ng/ml DHR123 to detect ROS production. B) Quantification of neutrophil DHR123 MFI after 20min PMA stimulation, n=2 mice/group, representative of 2 experiments. Frequencies and error bars are mean±SEM of neutrophils.

The above data suggest that Trib1$^{\Delta HSC}$ neutrophils are more activated both at rest and with stimulation. I hypothesized that hyperactive neutrophils would lead to the development of systemic immunopathology or autoimmunity. To probe this, I aged both Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice to examine the development of tissue damage or a change in phenotype. Trib1$^{\Delta HSC}$ mice were taken out as far as 433 days and no signs of immunopathology were observed. In addition, I did not observe a change in the frequency of neutrophils or eosinophils in the spleen or BM of aged mice compared to younger mice in the absence of Trib1 **(data not shown)**. Furthermore, there was no correlation between spleen weight and age **(Fig. 5.7)**, Similar to what was seen in **Fig. 2.3J**, Trib1$^{\Delta HSC}$ mice consistently had larger spleens.

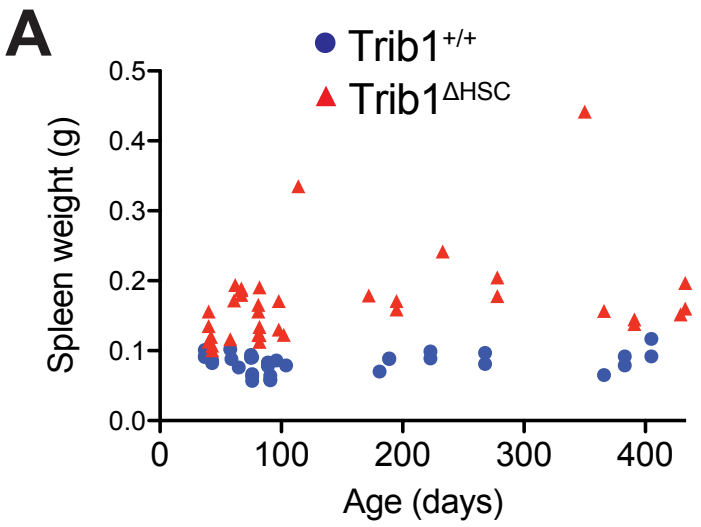

Fig. 5.7: No correlation between spleen size and age with Trib1 loss. A) Correlation between spleen weight and mouse age. n=35=37 mice, pooled from 9 experiments.

DISCUSSION

The preceding chapters focused on how Trib1 impacts cellular development and cellular identity. While some work was done characterizing the function of Trib1-deficient eosinophils, our goal in those studies was to determine where on the continuum of eosinophils-to-neutrophils Trib1-deficient $Ly6G^+$ eosinophils were located. In other words, were they more similar functionally to a WT eosinophil or to a WT neutrophil. One of the questions that remained unaddressed in the preceding chapters was how Trib1 controlled the function of neutrophils. In this chapter, I began to address that question and my preliminary data suggest that Trib1-deficient neutrophils are functionally altered. I observe that $Trib1^{\Delta HSC}$ BM neutrophils are larger at steady state **(Fig. 5.1)** and this corelates with increased AKT/mTOR pathway activation both at rest and with LPS stimulation **(Figs. 5.3-5.4)**. Trib1-deficent neutrophils also show increased ERK1/2 phosphorylation with LPS stimulation, but no change in the rate of degradation of IκBα. Finally, $Trib1^{\Delta HSC}$ neutrophils are better able to produce TNFα and ROS following stimulation **(Figs 5.5-5.6)**.

While I used PMA to induce neutrophil respiratory burst, this represents a non-physiologic stimulation. *In vivo* inducers of ROS, such as bacterial phagocytosis, formyl peptides, and C5a, among others, are better model stimuli and future studies will use these activators to probe the role of Trib1 in ROS production. As noted above, multiple pathways can regulate ROS production, yet PMA bypasses most of proximal steps in these regulatory pathways. Thus, it is difficult to determine how Trib1 loss impacts ROS production in the absence of a more physiologic stimuli.

$Trib1^{\Delta HSC}$ neutrophils have increased resting cell size **(Fig. 5.1)**. As noted above, the AKT pathway can directly contribute to this anabolic phenotype. Supporting this, we see increased phosphorylation of S6 at rest. This suggests that there is an increase in

tonic AKT/mTOR activation. How Trib1 regulates this is still an open question. Previous work showing a suppressive interaction between Trib2/Trib3 and AKT postulated that Trib3 sequestered AKT, preventing it from activating its downstream effectors[122,123]. While the physical interaction between Trib1 and C/EBPα is well documented[184,185], it is unclear if there is a physical interaction between Trib1 and AKT itself.

Given the significant neutrophil expansion as well as the neutrophil activation changes seen in Trib1$^{\Delta HSC}$ mice, I hypothesized that with age, Trib1-deficient mice would show signs of immunopathology or myeloproliferative disease (MPD). Surprisingly, Trib1$^{\Delta HSC}$ mice show no signs of autoimmunity or immunopathology with age. Furthermore, while Trib1$^{\Delta HSC}$ mice show increased spleen size due to a larger neutrophil population **(Fig. 2.3J)**, there was no correlation between age and spleen size **(Fig. 5.7)**, suggesting that no MPD developed. It is possible that the lack of tissue-destructive effects see in Trib1$^{\Delta HSC}$ mice is due to the absence of external stimuli. I hypothesize that with some form of inflammatory challenge, we would observe increased pathology in Trib1$^{\Delta HSC}$ mice. Experiments probing this will be discussed in **CHAPTER 6**.

Trib1$^{\Delta HSC}$ neutrophils from the BM have increased nuclear segmentation compared to Trib1$^{+/+}$ BM neutrophils, which are predominantly ring forms **(Fig. 5.2)**. While little is known about how changes in neutrophil nuclear architecture are either a readout for or impact cell function, this change in phenotype suggests of larger changes in cell state. Clinically, neutrophil nuclear hypersegmentation is seen with vitamin B12 or folate deficiency[245-247]. Neutrophil segmentation is largely be controlled by the interplay of various nuclear lamin receptors, in particular lamin B[248-250]. Interestingly, in human neutrophils lacking segmentation, there appear to be no functional consequences[251].

As noted above, recent work characterizing the temporal dynamics of neutrophil ageing in circulation demonstrated that neutrophil nuclei become increasingly

segmented with age[241]. The authors of this study however, do not comment on why aged neutrophils are hypersegmented. Another group identified a population of hypersegmented neutrophils in humans following LPS challenge [252]. These cells were also found to be immunosuppressive, a phenotype that required neutrophil CD11b expression. This is in line with reports from another group identifying a tumor-associated neutrophil phenotype with hypersegmentation that supported tumor growth[13]. These reports contrast with the above observations that Trib1-deficient neutrophils are hyperactive as measured by TNFα and ROS production. Despite this, there still may be a correlation, as so called "myeloid-derived suppressor cells" can suppress T cell activation partly through ROS production[253-255].

It is important to place these data in the context of what I observe in the preceding chapters regarding eosinophil development. These pathways mentioned above also play important roles in eosinophils and much remains to be studied as to how Trib1 modulates cellular signaling activation in eosinophils. Of note a recent report highlighted that loss of the NF-κB negative regulator, IκBα, and subsequent NF-κB activation increased eosinophil survival[256]. This was shown to act through increased expression of Bcl-x_L. As noted above, NF-κB is critical for countering MAPK-induced apoptosis signals[229-231]. It is possible that similar processes are active in eosinophils, however, future studies are required to investigate this. Of note, I observe increased phosphorylation of S6 at rest as well as with stimulation. S6 was shown to regulate cell size, and cells unable to phosphorylate S6 were observed to be smaller[257]. As I observe an increase in resting neutrophil size with Trib1 loss, I hypothesize that normally Trib1, either directly or indirectly, suppresses S6 activation to decrease cell size. Furthermore, as the pathways discussed in this chapter regulate cell survival, it is possible that

alterations in any one of these will lead to changes in apoptosis. Future studies are needed to address this question and will be discussed in **CHAPTER 6**.

Together, in the absence of *in vivo* functional assays for neutrophil activity, it is difficult to determine directly how Trib1 modulates neutrophil function. The above data suggest both baseline and stimuli-dependent increases in activation of key signaling pathways. Yet in the context of the current literature, it is unclear if this will result in measurable impacts *in vivo.* Ongoing and future studies will investigate the role of Trib1 in neutrophils *in vivo* and will be discussed in **CHAPTER 6**.

CHAPTER 6: DISCUSSION AND FUTURE DIRECTIONS

In the preceding 4 chapters of data, I present an analysis of the multiple functions of Trib1 in granulocytes. With temporally-controlled genetic deletion studies constituting the bulk of the work, I determined that Trib1 serves dual roles during eosinophil differentiation. During the critical early steps of differentiation from the GMP, Trib1 functions to support eosinophil lineage commitment at the GMP to EoP transition. Subsequently, Trib1 suppresses the neutrophil gene program in response to IL-5 signals, allowing for proper eosinophil differentiation. In the absence of Trib1, I observe the expansion of a small pre-existing lineage intermediate population of $Ly6G^+$ eosinophils. These cells fail to repress neutrophilic features such as neutrophil type-granules and increased phagocytosis, ROS production, and CXCR4 receptor expression. I demonstrate that there is increased C/EBPα p42 protein expression in Trib1-deficient granulocytes and that this increase in C/EBPα contributes to the expansion of $Ly6G^+$ eosinophils seen in the absence of Trib1. Finally, in examining the functional consequences of Trib1 loss on neutrophils, I observe that Trib1-deficient neutrophils showed signs of increased baseline and stimulation-dependent activation.

Overall, I present a model showing a proposed mechanism for how Trib1 regulates the eosinophil and neutrophil lineages **(Figs. 6.1-6.3)**. In this model, Trib1 functions as a dam to prevent alternative program expression. Normally, there is some crossover, in the form of the small population of $Ly6G^+$ eosinophils present in WT mice. In addition, this separation is in part driven by a gradient of C/EPBα expression. At steady state, Trib1 functions to prevent a breakdown of lineage choice by tuning C/EPBα activity and altering responsiveness to IL-5. Using stage-specific knockouts, I demonstrated that normally Trib1 controls lineage commitment and terminal identity independently of the other.

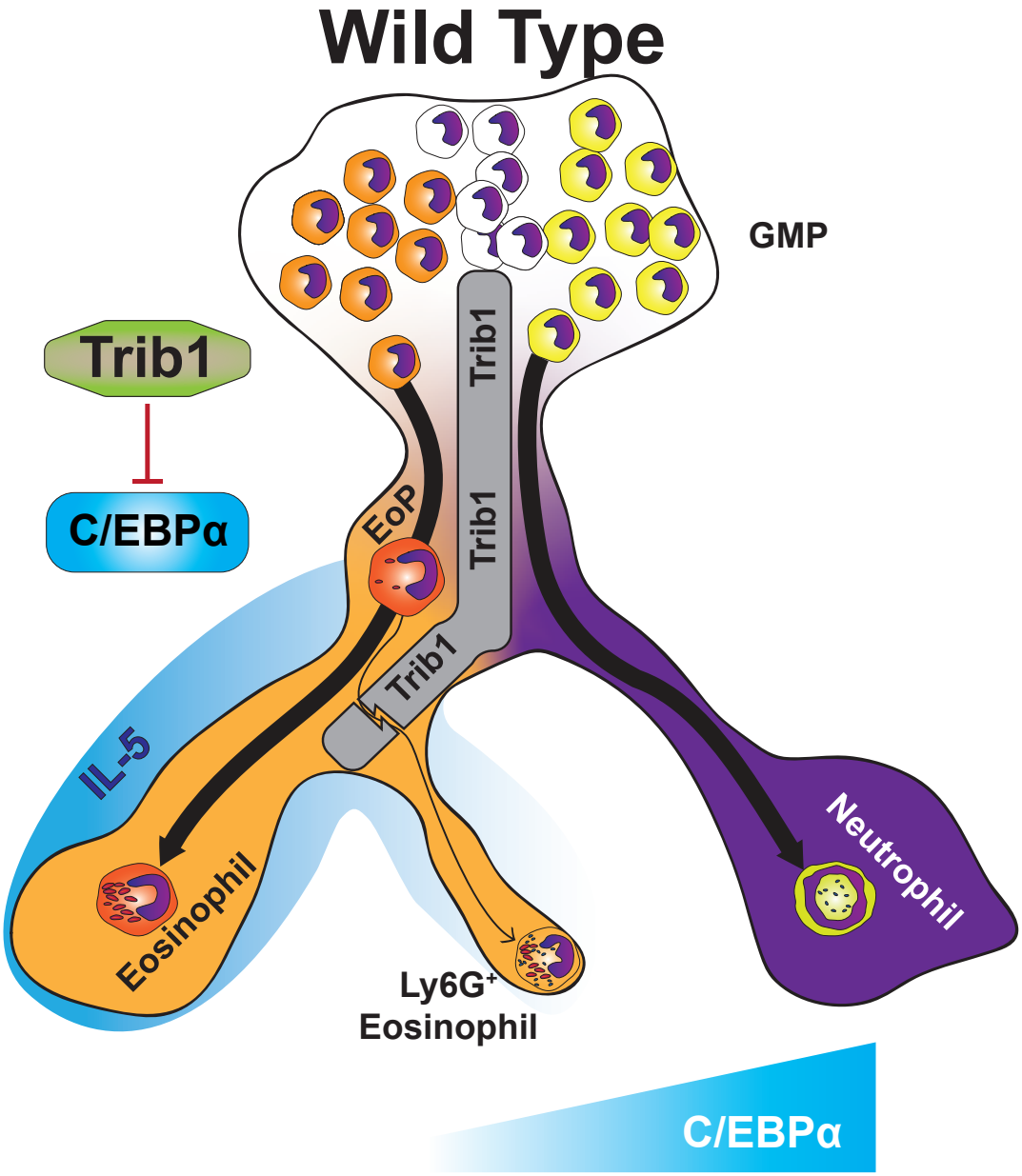

Fig. 6.1: Model of Trib1 function in wild type mice.

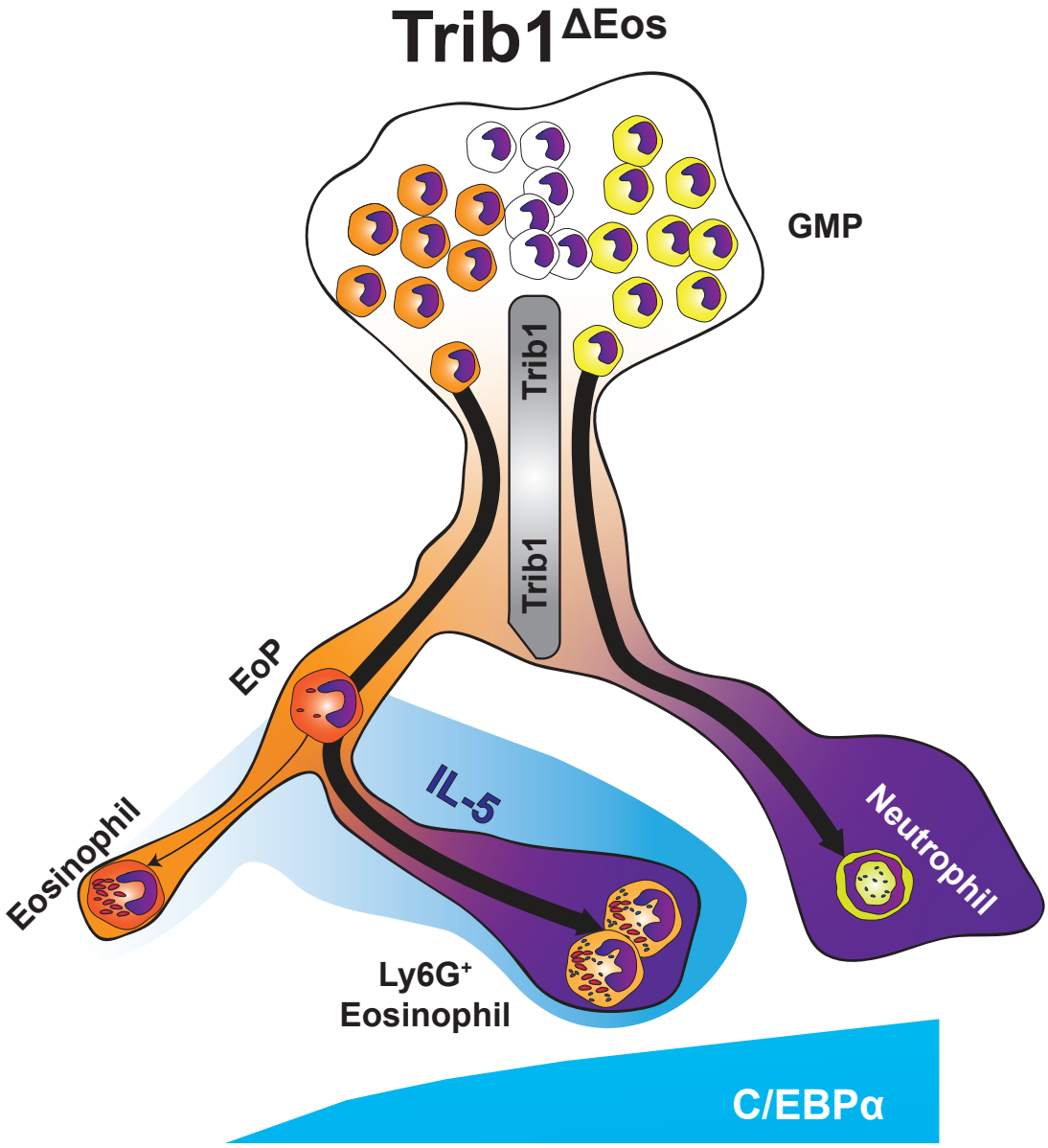

Fig. 6.2: Model of Trib1 function in Trib1$^{\Delta Eos}$ mice.

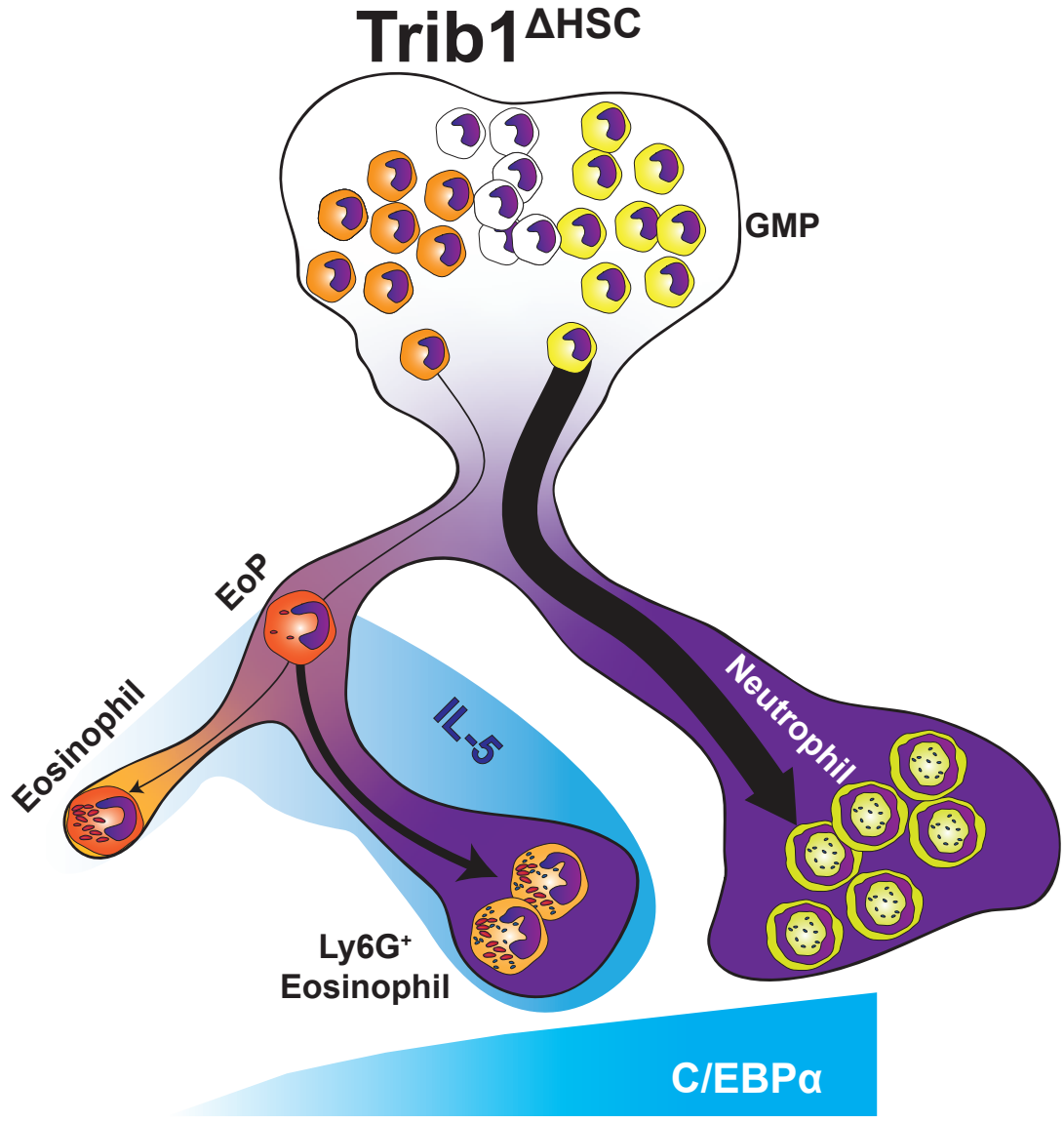

Fig. 6.3: Model of Trib1 function in Trib1$^{\Delta HSC}$ mice.

When I began this project, little was known regarding the mechanism of action of how Trib1 controlled granulocyte development and identity. In previous work from our lab, Trib1 or Trib2 overexpression induced AML development, associated with an alteration in the ratio of C/EBPα p42 to p30[113,114,258]. Early in the disease course of Trib2-induced AML, there appeared to be a shift away from granulocytic development toward the production of F4/80$^+$ monocytes/macrophages[113]; yet it was unclear if this represented a true alteration in myelopoiesis or early blast development. Furthermore, while this provided insight into how Trib proteins functioned, due to the cellular transformation induced largely by the increase of C/EBPα p30, it was difficult to study if or how Tribbles proteins modulated normal hematopoiesis. Subsequently, global loss of Trib1 was shown to expand mature neutrophils and ablate mature eosinophils[118]. Yet at what developmental stage Trib1 acted was unknown. Furthermore, while the authors showed that C/EBPα knockdown rescued eosinophil colony formation *ex vivo*, they failed to demonstrate this effect *in vivo* in a physiologic setting, with IL-5 present.

Taking these studies together, many outstanding questions remain regarding how Trib1 functioned to control normal granulopoiesis.

Regulation of Trib1 expression

Fundamentally, the kinetics of *Trib1* expression were unknown prior to this work. I observed that *Trib1* expression is induced following eosinophil lineage commitment from the GMP with expression detectable in the EoP and not in the CMP or GMP **(Fig. 2.2A)**. This raises the question of what regulates *Trib1* expression itself. Recent single-cell analysis of hematopoietic progenitors illustrated that select progenitors, including some GMP, express *Trib1* **(ref.[103] and Fig. 2.10)**. From those data, it appears that cells primed/pre-committed to different lineages expressed *Trib1*, including cells destined to

be granulocytes. Of note, this work did not distinguish between the neutrophil and eosinophil programs. Furthermore, there was also *Trib1* expression in cells in the multi-lineage primed population, suggesting that Trib1 can contribute to regulating lineage choice in cells stably expressing multiple transcriptional programs. As I note above, I hypothesize that the small population of $Ly6G^{+}$ eosinophils in WT mice represents a continuation of this multi-lineage primed population.

Given these data, along with my own, there are two possibilities for what controls *Trib1* expression. Trib1 may be a part of cell-type specific transcriptional programs, induced as soon as a progenitor specifies a particular lineage. Alternatively, progenitors may upregulate *Trib1* before true lineage specification. Work from other cell populations may offer insight into this process. In hepatocytes, *Trib1* expression is positively regulated by C/EBPα, which then forms a negative feedback loop to suppress C/EBPα levels in the liver[119]. Interestingly, in C/EBPα ChIP-seq data from GMP and

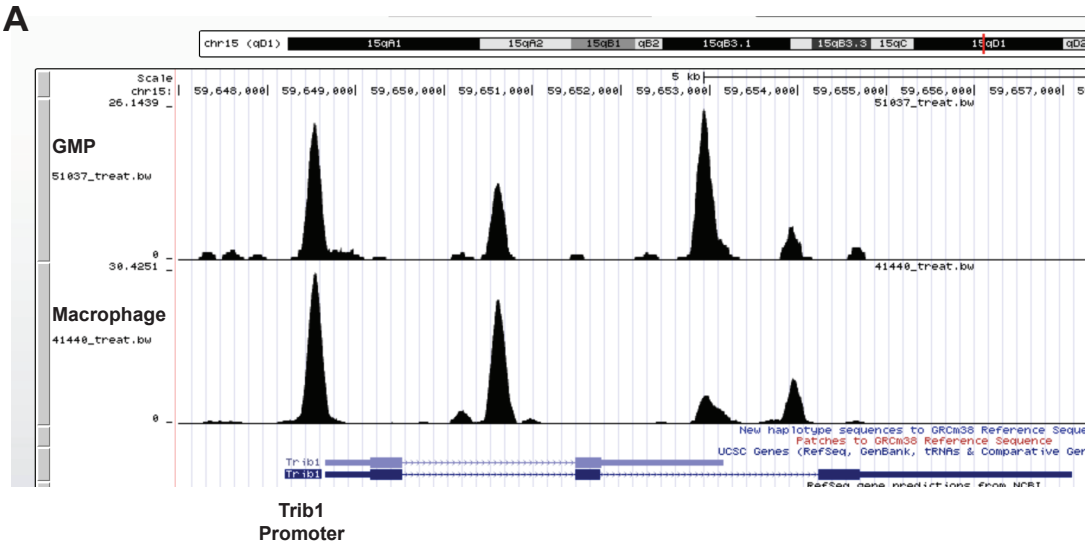

Fig. 6.4: C/EBPα binds around the *Trib1* locus in GMPs and macrophages. A) ChIP-seq tracks for C/EBPα from GMP **(ref. 259)** and macrophages **(ref. 260)**.

macrophages, there is C/EBPα binding in and around *Trib1*[259,260] **(Fig. 6.4)**. Given that there is low to undetectable *Trib1* expression in GMP, it is difficult to conclude how C/EPBα binding at the *Trib1* locus impacts its expression in these cells. It is possible that by binding around *Trib1,* its expression is primed, and on further differentiation, other transcription factors or transcriptional activators are recruited to initiate *Trib1* transcription. It is clearer in macrophages, as there is active *Trib1* expression in these cells. C/EPBα binding around *Trib1* in macrophages may direct its expression in those cells. As there are no published data sets of C/EPBα ChIP-seq in eosinophils, future work will focus on the role of C/EBPα in these cells.

Recent work examined the epigenetic state of GMPs and mature granulocytes using a combination of H3K4me1 and H3K27Ac marks to delineate primed chromatin[261]. In these data sets, there was robust H3K3me1 deposition in and around Trib1 in GMPs,

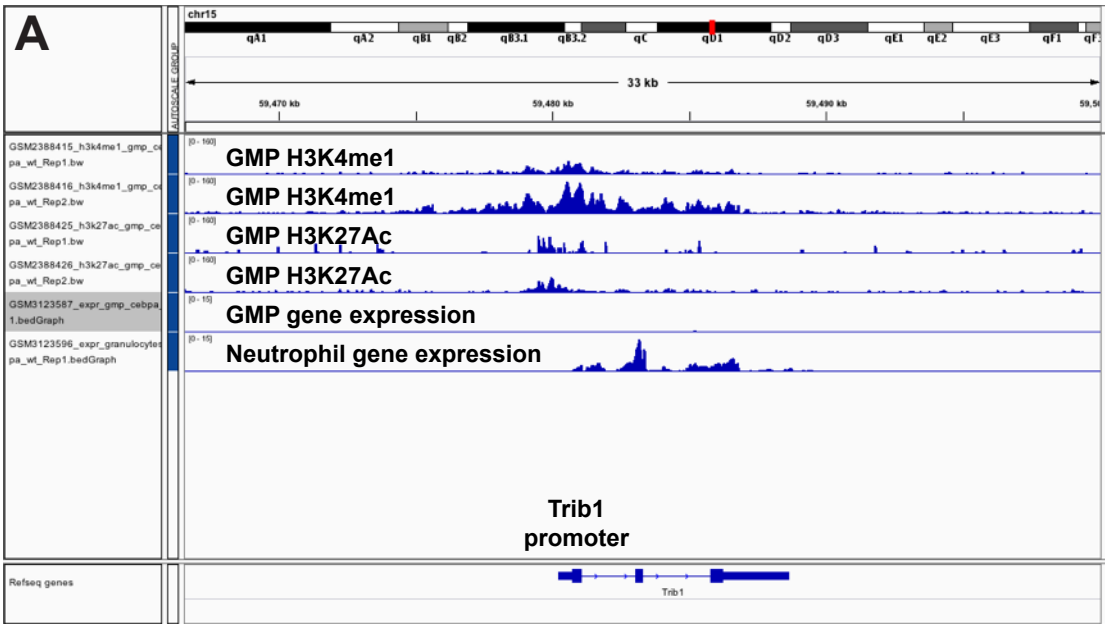

Fig. 6.5: The *Trib1* locus is primed in GMPs. A) Duplicate H3K4me1 and H3K27Ac tracks in GMPs with gene expression in GMPs and granulocytes from **ref. 261.**

corelated with H3K27Ac marks **(Fig. 6.5)**. In addition, there was minimal *Trib1* expression at the transcript level in bulk GMPs, indicating that this locus is primed, but not active. In contrast, there is high *Trib1* expression in mature granulocytes, suggesting that this locus becomes transcriptionally active following lineage commitment, yet is primed for activation in the GMP. This then raises the question as to what primes *Trib1* expression. As noted in **CHAPTER 1**, cytokine signals can influence the fate of early progenitors. It is possible that these signals, in concert with early pioneering myeloid transcription factors like C/EPBα, set up the *Trib1* locus for expression later in differentiation. What then remains unknown, is under settings of stress, such as infection, when different populations are needed from the BM, how Trib1 contributes to this process.

It is interesting to observe that the data in **Fig. 6.5** corroborate what I see in **Fig. 2.2A**, with minimal Trib1 expression in bulk GMPs. The single cell data in **Fig. 2.10** taken together with the bulk qPCR and ChIP-seq in **Figs. 2.10 and 6.5**, indicates that there is globally limited *Trib1* expression at the GMP stage, with select cells expressing it. As I noted in the discussion for **CHAPTER 2**, the recently reported heterogeneity within the GMP population may contribute to this disparity. Further studies are needed to dissect the unique characteristics of the individual GMPs that do express *Trib1*. Together, additional work is needed to investigate how *Trib1* expression is induced and regulated. This will be discussed in more detail below.

Trib1 in gene regulation: inductive or repressive

The majority of the data presented here demonstrate that Trib1 plays a repressive role in silencing neutrophil gene expression in developing eosinophils. I observe that in the absence of Trib1, eosinophils take on neutrophil characteristics

(Figs. 3.1-3.6, 3.8, 3.10, 3.12-3.14, 4.1A). In addition, I observe a decrease in *Ltf* expression with Trib1 overexpression **(Figs. 4.5-4.6)**. However, there are some indications that Trib1 may also play an inductive role in supporting the eosinophil program. My initial observation that Trib1 supports eosinophil lineage commitment from the GMP indicates to some extent that Trib1 can facilitate eosinophil gene program initiation or maintenance **(Fig. 2.5)**. Subsequently, I observed a decrease in *Epx* expression with Trib1 loss in BM CCR3^{+} eosinophils **(Fig. 3.2A)**. Finally, with Trib1 overexpression, I observed an increase in *Epx* expression coupled with surface SiglecF expression **(Figs. 4.5-4.6)**. These three observations together indicate an active and inductive role for Trib1 in eosinophil gene expression. Together, this suggests that Trib1 plays both inductive and repressive roles in supporting eosinophil lineage specification and commitment.

This then raises the question of how Trib1 mediates its function to alter gene expression. The majority of my data, together with previous work, points to C/EBPα as well as modulation of IL-5 signaling. Both will be discussed below.

IL-5 signaling and Trib1

In **CHAPTER 3**, I describe that Ly6G^{+} eosinophils fail to repress neutrophilic features. What is unknown from my data, however, is what the inductive signals are for the set of genes that drives this phenotype. As I see these Ly6G^{+} eosinophils arise in culture with only IL-5, I hypothesize that IL-5 directly drives this program. Alternatively, it is possible that during their development, these cells gain the ability to produce another cytokine that acts in an autocrine manner to induce neutrophil-specific genes. I examined the most likely candidates, G-CSF and GM-CSF. I did not detect G-CSF and saw no change in GM-CSF levels in supernatants taken at the end of culture of Trib1$^{\Delta Eos}$

BM **(Fig. 3.15B and data not shown)**. While I did observe increased MCP-1 expression **(Fig. 3.15B)**, MCP-1 has not been reported to alter progenitor differentiation.

These data, together with the data shown in **CHAPTER 2**, indicate that IL-5 participates in both stages of Trib1-mediated regulation of granulocyte development. IL-5 drives both the shift toward neutrophils seen in the absence of Trib1 as well as the production of $Ly6G^{+}$ eosinophils from Trib1-deficient BM. What remains unanswered then is how IL-5 signals could alternatively drive 2 different programs and if this occurs in the same cell. Furthermore, it is unclear if IL-5 alone directly supports neutrophil differentiation. While I observe a neutrophil expansion from $Trib1^{\Delta HSC}$ BM and GMP *ex vivo* in IL-5 culture, these data cannot clarify if Trib1 normally functions to restrain neutrophil differentiation or if it regulates neutrophil survival and/or proliferation. IL-5 preferentially signals through STAT5 to drive gene expression[262]. I hypothesize that Trib1 loss may alter the selectivity of IL-5 for STAT5 with STAT3 substituting to drive an alternative, more neutrophil-biased program. There are reports that IL-5 can signal through STAT3 in some settings[263]. Furthermore, G-CSF, which drives neutrophil development, signals predominantly through STAT3[180]. Thus, one mechanism for IL-5 driving dual programs in the same cell could be either a switch from STAT5 to STAT3 downstream of the IL-5 receptor or a reliance on both.

Finally, the effects on neutrophils could be IL-5 independent and solely dependent on Trib1. This possibility is further strengthened by our observation that while the IL-5Rα is detectable on the surface of both eosinophils and neutrophils from wild-type and Trib1-deficient mice, the mRNA expression of *Cd125* (the transcript for the IL-5Rα) was undetectable in neutrophils sorted from both $Trib1^{+/+}$ and $Trib1^{\Delta HSC}$ BM and IL-5 cultures **(Fig 6.6)**. Future work is required to determine the interplay between Trib1 and IL-5 and what other roles Trib1 plays in shaping granulocyte output.

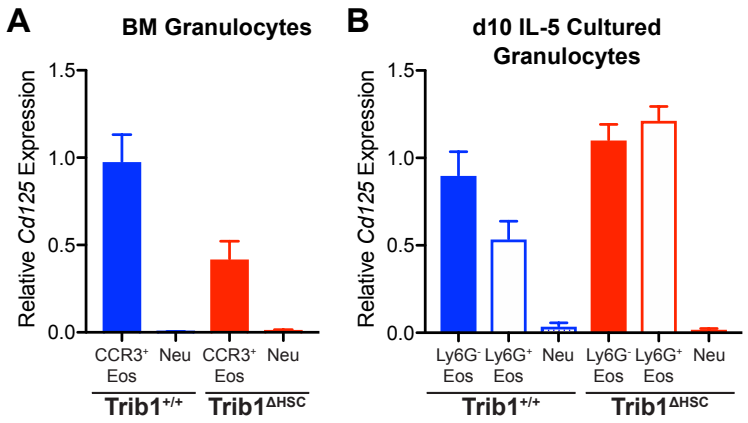

Fig. 6.6: Neutrophils do not express *Cd125* mRNA. A) qPCR analysis of *Cd125* expression by sorted BM CCR3^{+} eosinophils and neutrophils from Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ mice. Relative to *18s*, normalized to Trib1$^{+/+}$ eosinophils. B) *Cd125* qPCR analysis of sorted eosinophils based on Ly6G expression and neutrophils from d10 IL-5 culture of Trib1$^{+/+}$ and Trib1$^{\Delta HSC}$ BM. Relative to *18s*, normalized to Trib1$^{+/+}$ Ly6G^{-} eosinophils. n=3 mice/group. Representative of 1 experiment. Neu= neutrophil. Error bars are mean± SEM.

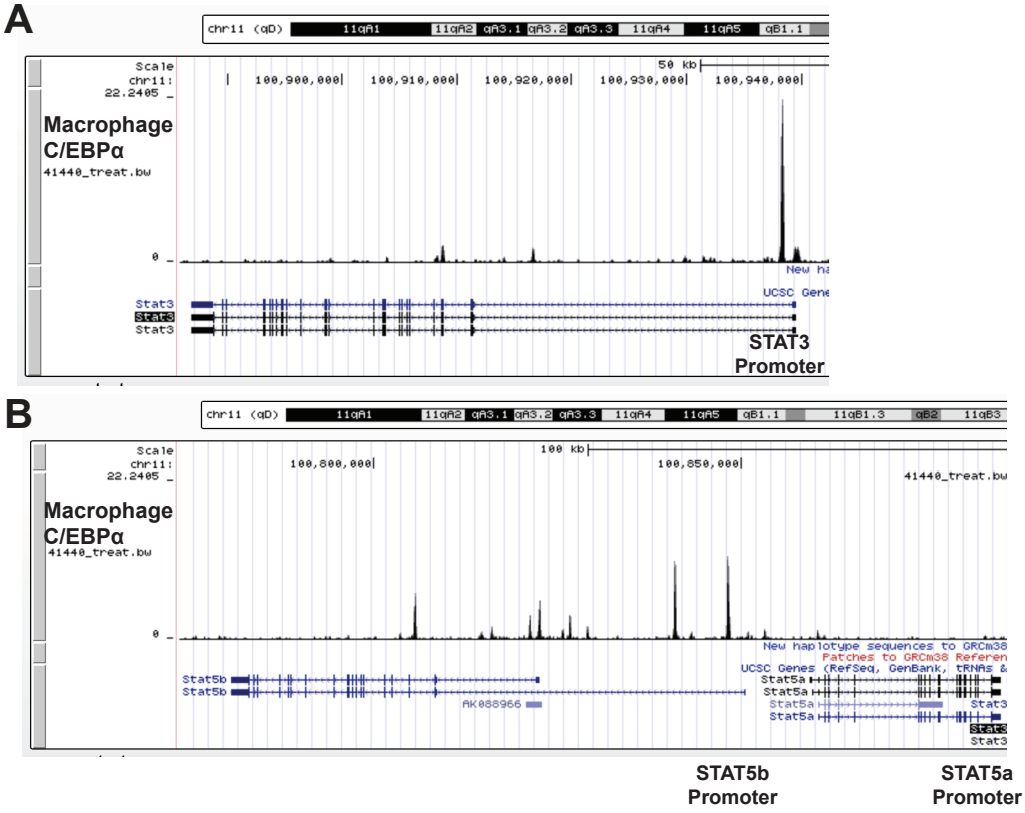

Fig. 6.7: C/EBPα binds around *Stat3* and *Stat5b* in macrophages. ChIP-seq tracks for C/EBPα from macrophages **(ref. 260)** at the A) *Stat3* locus and B) *Stat5a* and *Stat5b* loci. Each track is on the same scale.

In examining macrophage C/EBPα ChIP-Seq data[260], I observed C/EBPα binding at the *Stat3* promoter **(Fig. 6.7A)**, suggesting that C/EBPα contributes to STAT3 regulation. Moreover, increased C/EBPα may drive elevated STAT3, switching the balance of eosinophil-lineage targets downstream of STAT5 to neutrophil-lineage targets downstream of STAT3. C/EBPα also binds to the *Stat5b* locus **(Fig. 6.7B)**. What remains unknow, is how C/EBPα binds to these targets. This will be further explored below.

C/EBPα levels impact activity and dimerization

In **CHAPTER 4**, I introduce the idea that Trib1 modulates C/EBPα levels and this partly controls eosinophil terminal identity. While it was clear from previous studies that Trib1 both interacted with and regulated the protein expression of C/EBPα[118], what was unknown was when this regulation occurred and how it actually impacted granulocyte development. As noted earlier, C/EBPα is required for the CMP to GMP transition[75]. After that step, C/EBPα is still required to direct the differentiation of multiple myeloid lineages where graded C/EBPα expression is seen across different lineages. Neutrophils require the highest level of C/EBPα for their differentiation whereas monocytes/macrophages require less[77]. It was unknown, however, what level of C/EBPα is required for eosinophil differentiation. My data demonstrate that eosinophils require lower levels of C/EBPα compared to neutrophils as increased C/EBPα causes the cells to have a more neutrophilic identity. What remains unresolved from my data is how Trib1 and C/EBPα together impact the earlier stages in eosinophil and neutrophil development. I was unable to conclusively demonstrate that knockdown of C/EBPα increased EoP numbers or reduced the neutrophil output *ex vivo* from Trib1-deficient BM. Furthermore, while my data show that mature granulocytes lacking Trib1 have

elevated C/EBPα p42 **(Fig. 4.1A)**, it is unknown what the levels of C/EBPα are in granulocyte precursors. Future studies described below will begin to address this.

Increased levels of C/EBPα cause increased C/EBPα activity, but may also impact C/EBPα binding partners. C/EBPα contains a bZIP domain with a leucine zipper (LZ) that facilitates interaction with other LZ-containing proteins, such as c-Jun, c-Fos, JunB, and ATF-2[187,264]. C/EBPα can interact with DNA in multiple ways, either as a homodimer with itself, or as a heterodimer with the above factors. Studies from the Friedman group examined the signaling pathways downstream of the G-CSF and M-CSF receptors[265]. They observed that G-CSF preferentially induced STAT3 and SHP2 phosphorylation, whereas M-CSF stimulation resulted in ERK activation with increased c-Fos. The authors speculated that the increase in c-Fos following M-CSF stimulation lead to the formation of C/EBPα:c-Fos heterodimers. Previous work from this group demonstrated that C/EBPα:c-Fos heterodimers bound to the PU.1 (*Spi1*) promoter and a C/EBPα:c-Jun heterodimer activated *Spi1* transcription, which favors monocytic differentiation[266]. After G-CSF signaling, they speculate that the above signaling drives high levels of C/EBPα, leading to the formation of C/EBPα homodimers.

Without knowing the flanking sequences around these C/EBPα binding sites, it is difficult to conclude if differential C/EBPα binding partners influence STAT levels. I hypothesize that IL-5 signaling in the absence of Trib1 mimics G-CSF signaling by activating STAT3. This, either directly or through Trib1-mediated effects, would increase the frequency of C/EBPα homodimers. In contrast, when Trib1 is present, IL-5 would be restricted to activating STAT5, directing eosinophil-lineage differentiation, possibly through C/EBPα heterodimers with another factor. C/EBPα levels would also be lower, with Trib1 acting to facilitate C/EBPα degradation. In both of these situations, the level of C/EBPα itself may set this pathway in motion, given the above data showing C/EBPα

binding to both *Stat3* and *Stat5b* loci. However, I cannot rule out a direct interaction between Trib1 and elements of the IL-5 or STAT signaling pathway. Studies examining the role of differential STAT regulation will be explored below in *Future Directions*.

IL-5 can also activate NF-κB[267] as well as the MAPK pathway[268]. As I observe alterations in neutrophils in ERK signaling **(Fig. 5.4)** and the NF-κB target, TNFα **(Fig. 5.6)**, it is possible that non-STAT signaling events downstream of IL-5 contribute to the altered lineage programming. Moreover, from the above studies, treatment of lineage$^-$ BM cells with a MEK inhibitor reduced monocytic colony formation whereas SHP2 inhibition reduced the frequency of granulocytic colonies[265]. This would suggest that the signaling alterations I observe in neutrophils may also impact cell differentiation. As these pathways also intersect with the STAT pathway, alterations in STAT signaling could have broad impacts. Future studies are described below to evaluate this.

Neutrophils: rationalizing high Trib1 with high C/EBPα

In the above discussion, based on my data, I speculate that C/EBPα, possibly in conjunction with dysregulated signaling in response to IL-5, drives the alterations seen with Trib1 loss. While this mechanism makes conceptual sense in eosinophils, it is more difficult to understand Trib1 function in neutrophils, where *Trib1* is very highly expressed. It is difficult therefore to rationalize this expression data with the proposed mechanism for how Trib1 modulates granulocyte development. Neutrophils express higher levels of *Trib1* compared to eosinophils **(Fig. 2.2C)**, yet also have much higher levels of C/EBPα **(Fig. 4.1A)**. If the impact of Trib1 is solely to degrade C/EBPα, I would expect cells with high *Trib1* expression to have lower levels of C/EBPα. I hypothesize that as the levels of C/EBPα are much higher in neutrophils, the C/EBPα-lowering effect of Trib1 does not significantly impact total protein levels. Alternatively, the physical localization of Trib1

may be altered in neutrophils compared to other cell types. As there is no antibody we believe specific for Trib1 protein, I have been unable to perform these types of studies. Our lab is currently working to generate a mouse with a knock-in tagged Trib1 that will facilitate more proteomics and localization type experiments. These studies will be discussed below.

The neutrophil expansion observed in the Trib1$^{\Delta HSC}$ mice both *in vivo* and *ex vivo* in IL-5 and G-CSF cultures, suggests either increased commitment to the neutrophil lineage, increased proliferation of neutrophil intermediates, or increased survival. As noted in **CHAPTER 2**, stable, flow-sortable, neutrophil-lineage restricted progenitors have only recently been characterized in mice and humans[70-73]. These studies describe

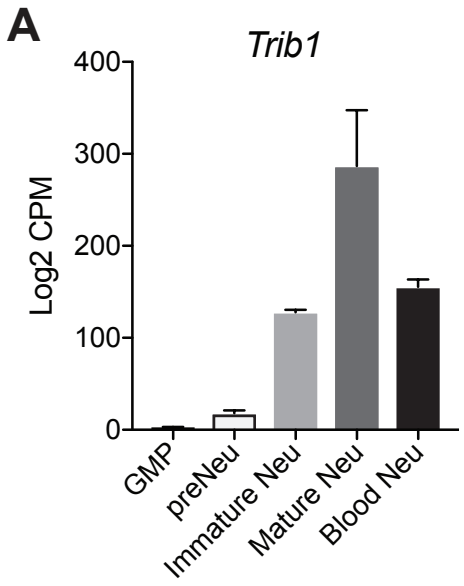

Fig. 6.8: *Trib1* expression increases with neutrophil differentiation. A) RNA-seq of BM neutrophil differentiation intermediates and blood neutrophils. GMP sorted as Lineage$^-$ (CD90, NK1.1, B220), CD11b$^-$ c-Kit^{hi}, CD34$^+$ FcγII/IIIR$^+$. PreNeu sorted as Lineage$^-$ (CD90, NK1.1, B220), CD115$^-$, SiglecF$^-$, Gr1$^+$ CD11b$^+$ c-Kit^{int} CXCR4$^+$. Immature neutrophils sorted as Lineage$^-$ (CD90, NK1.1, B220), Gr1$^+$ CD11b$^+$ c-Kit$^-$ CXCR4$^-$, Ly6G$^{lo\ to\ +}$, CXCR2$^-$. Mature and blood neutrophils sorted as Lineage$^-$ (CD90, NK1.1, B220), Gr1$^+$ CD11b$^+$ c-Kit$^-$ CXCR4$^-$, Ly6G$^+$, CXCR2$^+$. From **ref. 72**. *Data courtesy of Lai Guan Ng*.

cells derived from the GMP that are only able to produce neutrophils, analogous to the EoP. Yet, it was unknown if, as during eosinophil development, *Trib1* expression increases during neutrophil differentiation from the GMP. In analyzing data from one of these recent studies, I found that *Trib1* increases in a stepwise manner as neutrophils pass from the GMP through two intermediate stages in the BM[72] **(Fig. 6.8)** This group identifies the preNeu as the first neutrophil-lineage restricted population that lacks eosinophil potential *in vivo*. Interestingly, blood neutrophils showed reduced *Trib1* expression, possibly reflecting changes due to the physical location of the cell or the age of the cell. Together, this suggests that the preNeu, which expresses *Trib1*, is a potential site of Trib1-mediated regulation of the neutrophil lineage. Future studies discussed below will focus on this population.

Future Directions

While to a small extent, this work addresses the questions of how granulocyte lineage choice and identity are controlled and how Trib1 modulates this process, much remains unanswered. In the preceding discussion, I raise several questions that future experiments in the lab will focus on. One of the most intriguing questions and one with the broadest impact, is based on the observation that IL-5 alone can support both eosinophil and neutrophil production in the absence of Trib1. To evaluate this, we plan to do single-cell RNA-seq (scRNA-seq) on Trib1-deficent cells following exposure to IL-5. Using the IL-5 culture system **(Fig. 2.6A)**, I will start with sorted $Trib1^{+/+}$ and $Trib1^{\Delta HSC}$ GMPs and preform the scRNA-seq following 4 days of SCF and Flt3L exposure and following 2 subsequent days of IL-5 treatment. The single cell approach is necessary to determine if, in the absence of Trib1, both eosinophil and neutrophil lineage programs are induced in one cell simultaneously, or if each cell adopts a particular lineage early

on. I hypothesize that in the Trib1$^{\Delta HSC}$ cultures, both programs will be initiated in individual cells. If we were to profile later during the culture, I predict we would eventually see the neutrophil program predominate in those cells destined to be neutrophils. In contrast, the eosinophil program would likely dominate in the Ly6G^{+} eosinophils generated but elements of the neutrophil program would remain active. This experiment will shed light on how IL-5 signaling is altered in the absence of Trib1 and how this impacts both lineage specification and commitment.

In addition, while we will be examining GMPs after 4 days of SCF and Flt3L treatment, this will give us some idea of the changes in GMP heterogeneity in the absence of Trib1. As we know that select GMP express *Trib1*, we will see if these select GMP have a particular lineage bias and how that bias is altered when Trib1 is deleted. As the SCF/Flt3L pre-treatment may alter GMP pool composition, we can also perform this sequencing on cells directly from the BM. While it is possible that we may not see any changes in the GMP compartment with Trib1 loss, it will set up a baseline for future studies, especially of examining changes in EoP gene expression in the absence of Trib1.

Finally, as a parallel to this experiment, we will perform similar analyses with G-CSF cultures to determine how signaling downstream of G-CSF augments neutrophil development. As I hypothesize that G-CSF-induced differentiation, unlike IL-5, only drives the neutrophil program in the absence of Trib1, bulk transcriptome analysis will be sufficient to determine differences. Unlike with IL-5-induced eosinophil differentiation, I do not expect to see multiple programs active in one cell. Instead, I hypothesize that there will either be stronger induction of the neutrophil program itself or increased proliferation of developing cells.

To address the role of altered STAT signaling in the absence of Trib1, we will examine the kinetics of STAT5a/b and STAT3 phosphorylation following IL-5 after Trib1 loss. I hypothesize that there is a switch from STAT5 to STAT3 downstream of IL-5 in developing Trib1-deficient eosinophils. To evaluate this, we will harvest cells at various points during IL-5 culture and measure phosphorylation of STAT5 and STAT3. If this reveals differences, we can perform STAT3 and STAT5 ChIP-seq to determine the specific targets influenced by changes in STAT activation. It is unknown, however, if a change in STAT dynamics would be a direct result of Trib1 loss or if it is mediated by another factor, such as C/EBPα.

To address this and to more broadly investigate direct Trib1 targets in eosinophil and neutrophils, our lab is generating a Trib1 knock-in tagged mouse, where the endogenous *Trib1* locus is replaced with a *Trib1* construct with flag and HA tags. This mouse will allow for cell localization studies to determine where Trib1 is actually located within the cell. Importantly, we will also be able to determine Trib1 interacting partners through a combination of targeted immunoprecipitation and mass spectrometry. Previous proteomic screens which identified COP-1 as a Trib1 interactor were done in HeLa cells with Trib1 overexpression. As such, performing these studies in a more relevant cell type with physiologic levels of Trib1 will be key to discovering true Trib1 targets.

While my data suggest that $Ly6G^+$ eosinophils expand in the absence of Trib1, it is unclear what the function is of this population in WT mice. We and others have observed this population in WT mice[170], and I present data showing that in Trib1-deficient mice, they have a mixed phenotype and functional capabilities. Finally, I demonstrated that these cells are restricted to the eosinophil lineage. Despite this, I cannot determine from my data if in WT mice they maintain their expression of Ly6G or

transition to phenotypically 'normal' Ly6G^{-} eosinophils. To investigate this, I will sort Ly6G^{+} eosinophils from WT mice with the YFP reporter and adoptively transfer them into congenic recipients. I will subsequently observe them over time to see if they maintain or downregulate their expression of Ly6G. If they do downregulate Ly6G, it would suggest that these cells normally transition to true eosinophils and are an eosinophil lineage intermediate. If they fail to downregulate Ly6G, I would conclude that they are more similar to a stable population rather than a lineage intermediate. Regardless, my data suggest that Trib1 expands and/or stabilizes this population.

To evaluate how C/EBPα levels influence its activity, we will endeavor to determine changes in C/EBPα binding partners in the absence of Trib1. We will use C/EBPα ChIP-seq in WT and Trib1-deficent culture-derived eosinophils. We will examine changes in where C/EBPα binds and in the flanking sequences of these C/EBPα binding sites. I hypothesize that we will see more occupied tandem C/EBPα binding sites in Trib1-deficent eosinophils. In contrast, in Trib1$^{+/+}$ eosinophils, C/EBPα peaks will be adjacent to binding sites for other factors such as c-Fos or c-Jun. Should cell numbers be limiting or if we want to perform this analysis on smaller progenitor populations, such as the EoP, ATAC-seq to determine open chromatin regions, followed by motif analysis will provide similar information.

In **CHAPTER 3**, I suggest that increased CXCR4 expression on Trib1-deficient Ly6G^{+} eosinophils is partly responsible for the absence of these cells in the periphery. While the study using AMD3100 to antagonize CXCR4 mobilized these cells, it also mobilized other cell populations. To more specifically test the role of CXCR4 in eosinophil biology, I will cross the Trib1$^{\Delta Eos}$ mice with a mouse bearing a conditional *Cxcr4* allele. This strategy will allow me to isolate the impact of CXCR4 solely to the

eosinophil lineage and I hypothesize that loss of CXCR4 will allow for both WT and, to a greater extent, Trib1-deficient eosinophils to egress from the BM.

The *ex vivo* studies with G-CSF will parallel *in vivo* analysis of neutrophil development. As noted earlier, the neutrophil progenitor/preNeu population expresses *Trib1* **(Fig. 6.8)** and may be a site of Trib1-mediated regulation. We will first use the Trib1$^{\Delta HSC}$ mice to determine if Trib1 loss before neutrophil lineage commitment alters the size of the preNeu population, similar to the EoP; with the hypothesis that it will be expanded. Subsequently, we will use a combination of BrdU incorporation assays with Ki-67 and AnnexinV staining to measure changes in cell turnover, proliferation, and apoptosis. Together with increased commitment to the neutrophil lineage, changes in any of the three above parameters could account for the neutrophil expansion seen in the Trib1$^{\Delta HSC}$ mice. Given the changes seen with eosinophil differentiation, it is likely at the level of lineage commitment itself, however, the other factors may contribute as well.

Along these lines, to more specifically study the role of Trib1 in neutrophil function with limited impact on other lineages, we will use *Cre* under the control of the *Mrp8* (S100A8) promoter, which is highly specific for neutrophils[269,270]. In a report using a YFP reporter to track expression of different *Cre* drivers across various myeloid lineages, MRP8Cre was found to be one of the most specific for neutrophils, with some expression in peripheral monocytes/macrophages but no expression in eosinophils[270]. Using these mice, we will functionally assay Trib1-deficent neutrophils.

Based on my preliminary data shown in **CHAPTER 5**, I hypothesize that Trib1-deficent neutrophils are more activated both at steady state as well as with stimulation. To determine if this translates to altered activation *in vivo*, I will challenge the MRP8Cre-cTrib1 mice (Trib1$^{\Delta PMN}$) mice with bacterial pneumonia. I hypothesize that these mice will clear the infection more rapidly, albeit with an increase in tissue damage due to

increased neutrophil infiltration/activity. A possible confounding issue in the *in vivo* studies will be how to separate protective/pathologic effects of a developmental neutrophil expansion from cell intrinsic alterations in function. Thus, it will be of critical importance to validate our *in vivo* findings with *ex vivo* assays as well as with neutrophil adoptive transfer strategies.

If we observe changes with stress, we will transcriptionally profile BM neutrophils from Trib1$^{\Delta PMN}$ mice both at steady state and with stimulation. We will combine this with a more targeted analysis of signaling intermediates of the NF-κB, AKT/mTOR, and MAPK pathways following stimulation. As my preliminary data show alteration in the activation/activity of these pathways, I hypothesize that we will observe similar differences in the Trib1$^{\Delta PMN}$ neutrophils. By combining global profiling with a more targeted signaling analysis, we will get a fuller picture of how Trib1 loss impacts neutrophil function. We will perform *ex vivo* assays of neutrophil function in the absence of Trib1 including measuring cytokine and ROS production, phagocytosis, and NET formation with the hypothesis that these pro-inflammatory functions will be increased.

As the pathways studied in **CHAPTER 5** regulate cell survival, I will measure the lifespan and rate of apoptosis of Trib1-deficient neutrophils. As AKT and NF-κB are pro-survival[227-231], I hypothesize that there will be increased neutrophil survival in the absence of Trib1, possibly contributing to the increase in neutrophil numbers in Trib1-deficient mice.

To determine the mechanism of action of Trib1-mediated regulation of neutrophil function, we can take advantage of the Trib1 knock-in tagged mouse discussed above. This will allow us to perform both targeted immunoprecipitation studies as well as more global analyses for Trib1 interacting partners. Furthermore, as the proposed mechanism

for the suppressive effect of Trib3 on AKT activation was due to sequestration[123], we can perform co-localization microscopy studies to further validate this with Trib1.

Concluding remarks

The regulation of transcription factors and cell signaling is critical for both the establishment and maintenance of cellular identity. Here, I identify a previously unknown role for Trib1 in modulating both granulocyte lineage commitment and terminal cell identity and function, integrating C/EBPα and IL-5 signals. While I endeavored to move this study away from one purely focused on the action of Trib1 and more to one that studies the factors involved in establishing and maintaining the eosinophil and neutrophil lineages, significant gaps remain. Some of those have been discussed above. Yet the question still remains: why have a factor such as Trib1? I propose that Trib1 functions to tune both the levels and the activity of C/EBPα as well as to alter the response to cytokine signaling, shifting differentiation pathways based on the needs of the host. This allows for greater flexibility in cellular responses.

Furthermore, the true factors controlling lineage commitment remain unresolved. Given the recent work showing pre-commitment in progenitor populations, this likely happens earlier than previously thought through a combination of stochastic events and instructive signals. This is especially true with the eosinophil lineage as the signals that direct the earliest steps in eosinophil lineage commitment are unknown. While both IL-5 and IL-33 were proposed to support this initial step, no globally accepted model has prevailed. By regulating both transcription factor levels and cytokine responsiveness, Trib1 may control this process by facilitating exclusion of alternative programs. Again, if Trib1 truly participates in eosinophil program, specification is unknown.

Overall, in this study, I use Trib1 as a tool to dissect questions of cell identity and programming. While much remains to be learned regarding how granulocyte lineage programs are induced, maintained, and regulated, this study provides insight into this process, opening up new avenues for future work.

CHAPTER 7: MATERIALS AND METHODS

Mice

Conditional Trib1 mice (cTrib1; C57BL/6-*Trib1*$^{tm1.\ mrl}$, Taconic #10265) [119] were crossed to VavCre^{+} (Tg$^{(Vav1\text{-}cre)1Graf}$) mice[124] to generate Trib1$^{\Delta HSC}$ mice. EoCre^{+} mice (Epx$^{tm1.1(cre)Jlee}$)[125], a gift from James Lee, were crossed to cTrib1 mice to generate Trib1$^{\Delta Eos}$ mice, and then to Rosa26-YFP reporter mice (*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$; Jackson Labs #006148) to generate Trib1$^{\Delta Eos\text{-}YFP}$ mice. ER^{T2}-Cre mice[126] (*Gt(ROSA)26Sor*$^{tm1(cre/ESR1)Tyj/J}$; Jackson Labs #008463) were crossed to cTrib1 mice to generate the tamoxifen-inducible deletion strain. To generate conditional C/EBPα deletion mice, Trib1$^{\Delta HSC}$ or Trib1$^{\Delta Eos}$ mice were bred to mice with a conditional C/EBPα allele (Cebpa$^{tm1Dgt/J}$; Jackson Laboratory # 006447) [75]. Throughout, all Trib1$^{+/+}$ mice used were Cre^{+}, either VavCre^{+}, EoCre^{+}, or ER^{T2}-Cre^{+} as appropriate, with the WT cTrib1 allele. All mice were on the C57BL/6 background and were analyzed between 5-12 weeks of age. Animals were housed in a specific pathogen-free facility at the University of Pennsylvania. Experiments were performed according to the guidelines from the National Institutes of Health with approved protocols from the University of Pennsylvania Animal Care and Use Committee.

Flow cytometry and cell sorting

A complete list of antibodies is provided in in **Table 1** below. BM, spleens and blood were collected and processed using cold PBS with 2% heat-inactivated FBS (Gibco). Red blood cells were lysed using ACK lysis buffer (Lonza) for 5 minutes at room temperature. Cells were counted and stained at 20x10^{6} cells/ml in the presence of 5% 2.4G2 Fc blocking antibody. Zombie Violet (BioLegend) or 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Sigma) was used for live/dead discrimination. For

all analysis and sorting, doublets were excluded. For mature cell sorting, cells were processed as above. For progenitor cell sorting, cells were processed as above, lineage depleted with biotinylated antibodies and streptavidin MACS beads (Miltenyi), and then surface stained without Fc blockade. For GMPs and CMPs, the lineage panel included Sca1, CD3ε, CD19, B220, NK1.1, Ter-119, CD127, CD11b, and Gr-1. The EoP lineage panel mirrored that for GMP/CMP sorting, except CD11b was excluded and Sca1 was gated out separately. Cells were analyzed on an LSR II or LSR Fortessa flow cytometer (BD) and data were analyzed with FlowJo software v.9.7 (TreeStar). Cells were sorted on a FACSAria II (BD) using a 70μm nozzle at 70psi. Gating strategies for mature cells **(Figs. 1.3A-B)** and progenitors **(Figs. 1.3C-D)** are included below.

Ex vivo eosinophil culture

Eosinophils were generated *ex vivo* as previously described[54]. Briefly, either whole BM or sorted GMPs were seeded at $1x10^6$ cells/ml in RPMI media (Corning) supplemented with 15% fetal bovine serum (FBS) (Hyclone), 1% penicillin/streptomycin (Gibco), 2mM L-glutamine (Gibco), 1mM sodium pyruvate (Gibco), 10mM HEPES (Gibco), and 50μM 2-mercaptoethanol (Sigma), in the presence of 100ng/ml rhFlt3-L (Peprotech) and 100ng/ml rmSCF (Peprotech) and cultured for 4 days. On day 4, non-adherent cells were counted and resuspended at $1x10^6$ cells/ml in the above RPMI media supplemented with 10ng/ml rmIL-5 (Peprotech). The media was changed on day 8, and every two days thereafter, and cells were resuspended at $1x10^6$ cells/ml with fresh IL-5. On day 10-13, cells were counted and processed for RNA or flow cytometry.

Ex vivo neutrophil culture

For neutrophil differentiation, cells were processed and cultured as above for eosinophil differentiation. Briefly, $1x10^6$ cells/ml were plated in the above media in presence of 100ng/ml rhFlt3-L (Peprotech) and 100ng/ml rmSCF (Peprotech) and cultured for 4 days. On day 4, non-adherent cells were counted and resuspended at $1x10^6$ cells/ml in the above RPMI media supplemented with 60ng/ml rhG-CSF (Peprotech) for 4 days. Cells were counted and analyzed on day 8.

Phagocytosis assay

Whole BM was isolated as described above and resuspended in Live Cell Imaging Solution (Invitrogen). Cells were incubated with pHrodo Red *E. coli* BioParticles (Invitrogen) for 2hrs at 37°C. Uptake was analyzed by flow cytometry as above.

Transwell chemotaxis assay

Cultured eosinophils or whole BM were isolated and resuspended in RPMI 1640 Medium with no phenol red (Gibco), supplemented with 0.5% low endotoxin BSA (Sigma). $1x10^6$ cells were loaded in the upper chamber of a 6.5mm transwell insert with a 5.0μm pore polycarbonate membrane (Corning). The lower chamber contained media without (control) or with KC/CXCL1 (50ng/ml) (Peprotech) or eotaxin/CCL11 (500ng/ml) (BioLegend). Cells were allowed to migrate for 2hrs at 37°C, and then collected, counted, and analyzed by flow cytometry. Whole BM was used as a control for cell migration.

pAKT, TNFα, and ROS production assays

For pAKT, whole BM was processed as above and rested for 30 minutes at 37°C. Cells were then stimulated for 30min to 1hr with 100ng/ml of ultrapure LPS B5 (Invivogen) or 2ng/ml PMA with 20ng/ml ionomycin (Sigma). Cells were then added directly to 10 volumes of pre-warmed Lyse/Fix buffer (BD) and incubated for 10min at 37°C. Cells were then washed and surface stained followed by permeabilization with Perm/Wash buffer (BD). Intracellular pAKT staining (BD) was done in Perm/Wash buffer for 30 minutes. Cells were then washed and analyzed as above. For TNFα production, whole BM was processed as above and rested for 30 minutes at 37°C. Cells were stimulated with 100ng/ml ultrapure LPS B5 (Invivogen) for 4hrs at 37°C in the above eosinophil culture media, containing 2ug/ml brefeldin A (Sigma). Cells were then washed and surfaced stained as above, followed by overnight fixation with Cytofix/Cytoperm (BD). Cells were then intracellularly stained with anti-TNFα (BD) and analyzed as above. For ROS production, whole BM was processed and surface stained as above. Cells were then rested for 15min at 37°C in PBS. 10ng/ml PMA (Sigma), 100ng/ml dihydrorhodamine 123 (DHR123) (Sigma), and Zombie Aqua (BioLegend) was then added for 15min at 37°C. Cells were washed and analyzed as above.

Cytospins and light and electron microscopy

$5x10^4$ cells were resuspended in PBS and spun onto microscopy slides using a Cytospin 3 Cytocentrifuge (Shandon). Slides were air-dried and stained with Diff-Quik (Dade Behring). Images were acquired using a BX41 microscope (Olympus). For electron microscopy, eosinophils were sorted as above and fixed with 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1M sodium cacodylate buffer, pH7.4, for 1hr at room

temperature. After subsequent buffer washes, cells were resuspended in molten 2% agar in 0.1M sodium cacodylate buffer, pH 7.4, and recentrifuged. The samples were then post-fixed in 2.0% osmium tetroxide for 1hr at room temperature. After dehydration through a graded ethanol series, the tissue was infiltrated and embedded in EMbed-812 (Electron Microscopy Sciences). Thin sections were stained with lead citrate and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage image capture software. A total of 75 electron micrographs from two experiments were analyzed.

Bone marrow transplantation and C/EPBα shRNA knockdown

For mixed chimeras, CD45.1^{+} BM was mixed 1:1 with CD45.2^{+} Trib1$^{+/+}$ or Trib1$^{\Delta HSC}$ BM and 2x10^{6} cells were injected via the tail vein into lethally irradiated C57BL/6.SJL mice. Mice were analyzed at 12-20 weeks post-transplant. For C/EPBα shRNA knockdown, cells were processed and transduced as described previously[117]. Briefly, BM was collected from Trib1$^{+/+}$ or Trib1$^{\Delta HSC}$ 4 days after intravenous administration of 5-fluorouracil (5-FU) (250mg/kg). Cells were transduced with lentivirus and 1x10^{6} cells were injected intravenously into lethally irradiated recipients. Mice were analyzed at 9 weeks post-transplant. For Trib1 overexpression, BM was collected from WT mice 4 days after intravenous administration of 5-FU (250mg/kg). Cells were transduced with retrovirus and 1x10^{6} cells were injected intravenously into lethally irradiated recipients. Mice were analyzed at 8.5 weeks post-transplant.

Constructs and viruses

Production of high-titer virus was performed as described previously[117]. Briefly, lentiviral pLKO.1 shRNA constructs[77] were co-transfected into 293T cells (ATCC, CRL-3216) with

pMDL (gag-pol), pRSV-Rev and pHIT123 (envelope). Retroviral constructs were co-transfected into 293T cells with pCGP (gag-pol) and pHIT123 (envelope). Viral titers were determined using 3T3 fibroblasts. Viral supernatants were stored at -80°C.

Papain treatment

Mice anesthetized with isoflurane (Phoenix) received 30µl intranasal PBS or 30µg papain (Millipore) daily for 5 days and were euthanized 24hrs later. Lungs were harvested following retrograde flushing through the heart with PBS, and were digested with collagenase D (Roche) and DNaseI (Sigma) for 45min at 37°C. Lung digests were passed through a 70-micron filter and processed for flow cytometry.

Immunoblotting

Cells were directly lysed in 2x SDS sample buffer (10% SDS) (BioRad) and boiled for 10min. Cell lysates were clarified, separated by SDS-PAGE and transferred to PVDF (Millipore). Antibodies used were anti-C/EBPα (8178), anti-GATA1 (3535), anti-pS6 (Ser235/236) (4856), anti-S6 (2317), anti-p-ERK1/2 (p44/42 MAPK) (Thr202/Tyr204) (9101), anti-ERK1/2 (p44/42 MAPK) (9012), anti-IκBα (4812) (all from Cell Signaling Technology), anti-C/EBPε (C-10, Santa Cruz Biotechnology), anti-β-actin (A5316, Sigma), anti-rabbit-HRP (NA934V, GE Healthcare), and anti-mouse-HRP (NA931V, GE Healthcare).

Quantitative PCR (qPCR)

RNA was extracted using Trizol (Ambion), followed by cDNA synthesis (SuperScript III kit; Invitrogen). qPCR was performed using TaqMan PCR master mix (Applied Biosystems) on a ViiA 7 system (ABI) and mRNA quantities were normalized to *18s*.

Primer/probe sets were *18s* (4319413E), *Trib1* (Mm00454875_m1), *Ltf* (Mm00434787_m1), *Epx* (Mm00514768_m1) and were purchased from Life Technologies.

ChIP-seq and RNA-seq data analysis

Published data sets as .Bigwig files were downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). Tracks were then analyzed in IGV 2.4 (Broad Institute)

Statistical analysis

Statistical analyses were performed using GraphPad Prism v.7.

Table 7.1

Antibody	Color	Clone	Manufacturer	Catalogue number
SiglecF	PE	E50-2440	BD Bioscience	552126
	BV421			562681
Ly6G	PE-Cy7	1A8	BioLegend	127617
CCR3 (CD193)	APC	J073E5	BioLegend	144512
CD11b	PerCP-Cy5.5	M1/70	BioLegend	101228
F4/80	FITC	BM8	eBioscience	11-4801-82
	APC-Fire/750	BM8	BioLegend	123152
CD11c	APC-Cy7	N418	BioLegend	117324
Ly6C	BV711	HK1.4	BioLegend	128037
SiglecE	PE	M1304A01	BioLegend	677103
CD62L	APC-Cy7	MEL-14	BioLegend	104427

CXCR4	Biotin	2B11	ThermoFisher	13-9991-82
CXCR2	PE	SA044G4	BioLegend	149303
CD34	eFluor 660	Ram34	eBioscience	50-0341-82
Ckit	PE-Cy7	2B8	BioLegend	105814
FcγII/IIIR (CD16/32)	APC-Cy7	93	BioLegend	101328
IL-5Rα (CD125)	PE	T21	BD Bioscience	558488
CD3ε	Biotin	145-2c11	BioLegend	100304
CD19	Biotin	6D5	BioLegend	115504
B220	Biotin	RA3-6B2	BioLegend	103204
NK1.1	Biotin	PK136	BioLegend	108704
Gr-1	Biotin	RB6-8C5	BioLegend	108404
Ter-119	Biotin	TER-119	BioLegend	116204
CD127	Biotin	A7R34	BioLegend	135006
Sca1	Biotin	D7	BioLegend	108104
CD11b	Biotin	M1/70	BioLegend	101204
TNFα	AF700	MP6-XT22	BD Bioscience	558000
pAKT (S473)	PE	M89-61	BD Bioscience	560378
Streptavidin	BV605	-	BioLegend	405229

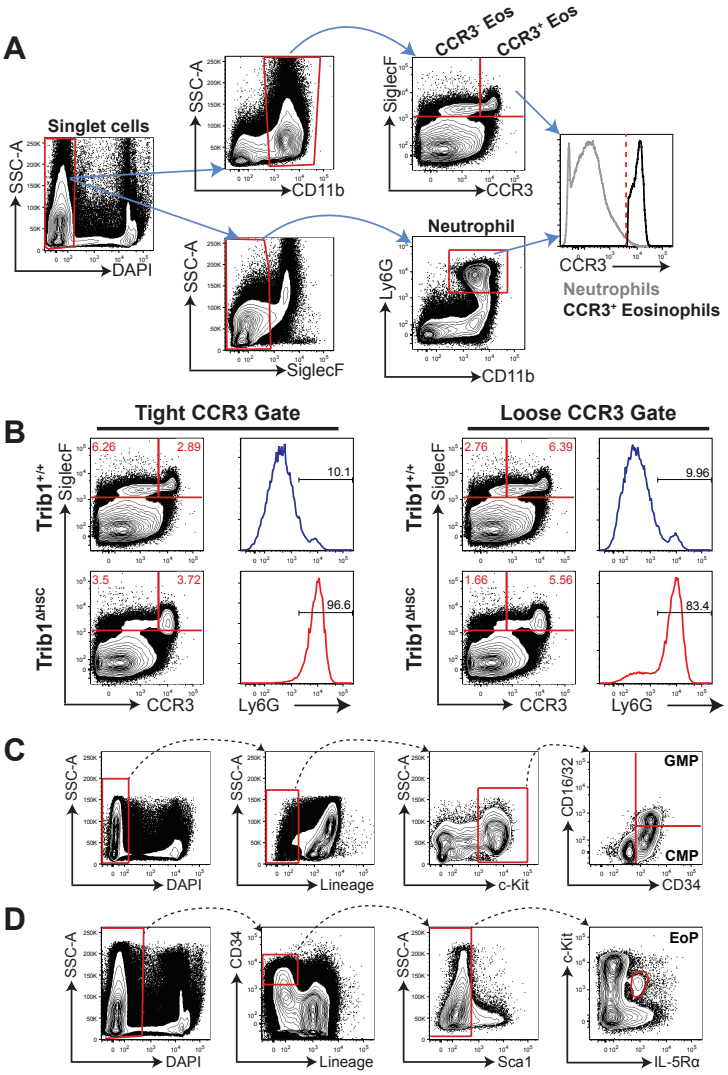

Fig. 7.1: Gaiting strategies. A) Representative gating strategy for BM eosinophils and neutrophils. First panel gated on singlet cells. Live cells are identified using DAPI exclusion. Eosinophils are subsequently gated as CD11b^{+} then SiglecF^{+} and CCR3$^{+/-}$ (upper panels). Neutrophils are gated as SiglecF^{-} then Ly6G^{+} CD11b^{+} (lower panels). The far-right histogram depicts the strategy for setting the CCR3 gate where neutrophils, which do not express CCR3, are used as a negative staining control. B) Comparison of a tight versus loose CCR3 gate for identifying eosinophils. Dot plots gated on singlet, live, CD11b^{+} cells and histograms are subsequently gated as SiglecF^{+} CCR3^{+}. The left panels are set with a tight CCR3 gate using neutrophils as a negative control as in (A). The right panels are set with a loose CCR3 gate. C) Representative gating/sorting strategy for isolating BM GMP and CMP. Left panel gated on singlet cells. CMP/GMP lineage cocktail includes Sca1, CD3ε, CD19, B220, NK1.1, Ter-119, CD127, CD11b, and Gr-1. D) Representative gating/sorting strategy for isolating BM EoP. Left panel gated on singlet cells. EoP Lineage cocktail includes CD3ε, CD19, B220, NK1.1, Ter-119, CD127, and Gr-1.

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