UAB LIBRARIES
The University of Alabama at Birmingham

University of Alabama at Birmingham
UAB Digital Commons

All ETDs from UAB

UAB Theses & Dissertations

2020

Distinct Tissue Specific Functions Of Bone Marrow Regulatory T Cells, And Their Therapeudic Applications In Myeloid Neoplasms

Virginia Camacho
University of Alabama at Birmingham

Follow this and additional works at: https://digitalcommons.library.uab.edu/etd-collection

 Part of the Medical Sciences Commons

Recommended Citation

Camacho, Virginia, "Distinct Tissue Specific Functions Of Bone Marrow Regulatory T Cells, And Their Therapeudic Applications In Myeloid Neoplasms" (2020). *All ETDs from UAB*. 755.
https://digitalcommons.library.uab.edu/etd-collection/755

This content has been accepted for inclusion by an authorized administrator of the UAB Digital Commons, and is provided as a free open access item. All inquiries regarding this item or the UAB Digital Commons should be directed to the UAB Libraries Office of Scholarly Communication.

DISTINCT TISSUE SPECIFIC FUNCTIONS OF BONE MARROW REGULATORY T CELLS, AND THEIR THERAPEUDIC APPLICATIONS IN MYELOID NEOPLASMS

by

VIRGINIA CAMACHO

ROBERT WELNER, CHAIR
CHRIS KLUG
CHANDER RAMAN
DAVID SCHNEIDER
ALLAN ZAJAC

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2020

Copyright by
VIRGINIA CAMACHO
2020

DISTINCT TISSUE SPECIFIC FUNCTIONS OF BONE MARROW TREGS AND THEIR THERAPUEDIC IMPLICATIONS IN MYELOID NEOPLASMS

Virginia Camacho

IMMUNOLOGY

ABSTRACT

In the bone marrow, Regulatory T cells (Tregs) constitute nearly 30% of CD4+ T cells, a significantly higher ratio than other tissues. Although a few of the mechanisms by which Tregs regulate normal hematopoiesis have been elucidated, our understanding of this process during leukemogenesis is extremely limited. In this work, we define how Tregs maintain and support the bone marrow microenvironment. We propose that the role of Tregs extends beyond their canonical immuno-suppressive function and that these cells are required to maintain healthy hematopoiesis. We have defined tissue-specific roles for Tregs in the bone marrow including the maintenance of hematopoietic stem cells (HSCs) as well as crosstalk with mesenchymal stromal cells. We observed that depletion of Tregs has widespread effects across several bone marrow populations. Loss of Tregs induces proliferation and cycling of HSCs, and results in accumulation of myeloid cells, all hallmark traits of neoplasia. Moreover, the supportive capacity of stromal niche populations is negatively altered by Treg depletion suggesting a remodeling of the marrow microenvironment. Specifically, we underscore the importance of Treg IL-10 in maintaining stromal cell function and supporting hematopoiesis.

We expand on these findings to show how that Tregs are altered in a leukemic setting and utilize immunotherapeutic agents to restore Treg function and mitigate leukemic transformation. These projects further examine how loss of Treg function and IL-10 regulatory mechanisms promote disease progression in the context of chronic

myeloid leukemia. The findings presented here provide a foundation to devise more comprehensive immune therapies in myeloid malignancies. Defining how and why Treg dysregulation occurs, as well as determining specific mechanisms that are lost during leukemic transformation are the focus of these studies with the goal of improving our understanding of the immune landscape in hematological disease. The conclusions from this work provide a direction for manipulating the trajectory of the immune response in myeloid neoplasms.

Keywords: Regulatory T cells, Stromal cell, Hematopoietic Stem Cell, Interleukin 10

DEDICATION

This work is dedicated to parents, for their unconditional love and support. To my mom for her unwavering care, her laughter, her love, and for instilling in me the resilience to always get up and try again. Thank you for letting me fill our house with books and for letting me bring science textbooks on vacation. To my brothers, for making me laugh through a lifetime of failures. Finally, this is dedicated to Rob, thank you for your leadership, your mentorship, and example. Thank you for fighting always fighting for us and for demonstrating that is it possible to lead with scientific joy, with integrity, and without negativity. Most of all thank you never letting me lose sight of the things in life that matter most, and to never put science above people.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor Rob Welner for giving the PhD of my dreams, for letting me pursue all the crazy ideas, and for supporting me at every turn. Your patience, creativity and ability to always look on the bright side led my projects to the most exciting directions. I am so thankful for the opportunity to have tried every crazy experiment and idea. I am grateful for your eternal patience and for 'the process'. I can only hope to have my own lab one day so that I can train the next generation with the same level of dedication, love and support.

I would like to also thank my thesis committee Dr. Schneider, Dr. Raman, Dr. Klug, and Dr. Allan Zajac for their guidance and collaboration. I feel fortunate to have had a committee that supported me and challenged me. This work would not have been possible without my wonderful lab mates. In particular, Sweta Patel and Victoria Matkins. Thank you for the incredible intriguing discussions, thoughtful advice, technical expertise, feedback, and bringing lots of laughs to my science. Last but not least, I would like to thank Sam Lederman for moving down to Alabama to support me on this adventure, for his endless patience with my lab hours and his unconditional support, and love.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Modulating IL-10 production by Regulatory T cells for therapeutic intervention in Chronic Myeloid Leukemia

Discussion

INTRODUCTION

The human immune system is classically divided into two compartments: an innate and adaptive arm, bifurcated according to functional diversities(1). T cell lymphocytes (T-cells) are critical actors within the adaptive arm. They are involved in the regulation of immune reactions, the establishment of immunological memory, host-pathogen interactions, and barrier tissue-immunity(2). T cells are antigen-specific cells. Upon activation and exposure to their cognate antigen, they undergo maturation and clonal expansion, or can become long-lived memory cells poised for future recall responses(3, 4).

In addition to their immunological roles, T cells function is also compartmentalized across various organs and tissues interfaces. T-cell function is highly heterogenous, responsive, and context dependent(5). At the basic level it involves dynamic interactions with other cellular (immune and non-immune) components and often relies on the secretion of chemokines, cytokines, and effector molecules. Characteristically, the role of T cells is that of immune protection, surveillance and clearance. However, it is becoming clear that T cell identity is highly intertwined with anatomical location and it has been demonstrated that these adaptive immune cells reciprocally influence tissue homeostasis. The diverse physiological requirements of cellular niches are proportionally reflected by the immense diversity of T cell subsets localized to different areas of the body(5). Recent investigations demonstrate that the demands of individual microenvironments dynamically influence the developmental trajectory and identity of resident T cells populations. In numerous contexts, the function of T cells occupying a tissue niche is often adapted to optimize for integration and regulation of their corresponding site of residence(6).

The bone marrow (BM) is considered a ‘primary lymphoid’ organ, indicating its capacity to support lymphopoiesis in an antigen independent fashion(7, 8). In addition to providing the framework for the differentiation and maturation of progenitor cells into the lymphoid lineage, the bone marrow also serves as a critical immunological transit point and an active site of immune regulation(9, 10). It is increasingly recognized that the marrow is a reservoir for multiple T-cell subsets (11). Prominent actors include cytotoxic CD8+ cells, effector CD4+ cells, memory T- cells, and Foxp3+ Regulatory T cells (Tregs). Notably, the bone marrow can also serve as a secondary lymphoid organ. It provides the architecture and cellular contacts to support proliferation, self-renewal, and migration of multiple T-cell populations. It also confers the appropriate environmental signals for T cell survival and T cell priming, (8, 12) Thus, the marrow not only provides a dedicated niche for circulating antigen experienced cells to survive and proliferate, but also serves a hub for dormant resident memory T cell populations(13-15). After executing their immune function, memory T cells migrate to the marrow for homeostatic maintenance.(16) Here, the stromal compartment is of eminent importance for their maintenance. T cell survival niches are organized by stromal cells, and bear resemblance to niches responsible for maintaining memory plasma cells. This includes the presence CXCL12-expressing mesenchymal cells, VCAM1+ stromal cells. The stromal architecture also provides critical cell contacts and regulates T cells through the secretion of chemokines and cytokines, such as IL-7 and IL-15. (9, 17-19).

Thus far, significant contributions been made showing how the bone marrow microenvironment contributes to the organization and regulation of effector T cell immunity, including long-term memory responses, secondary immune reactions, and surveillance(10). Despite these advances, information is currently lacking on how the stromal niches interact and crosstalk with the regulatory T cell compartment. Similarly,

much less in known about how identity of stromal populations may be reciprocally imprinted or influenced by the immunological cells which they sustain.

REGULATORY T CELLS

The notion of an immunomodulatory T cell population was first proposed in 1970 by Gershon and Kondo, who introduced the concept of a thymically derived ‘suppressor’ T cell (20). For many frustrating decades following the 1970 proposal of a ‘suppressor’ T cell, numerous groups searched for a marker that would cleanly identify this population. Thus, the next decisive turning point for the field came in 1995 with Shimon Sakaguchi’s seminal paper outlining that CD25 (IL-2Rα) reliably marked a thymically derived $CD4^+$ T cell subset that was able to exert suppressive functions and protect thymectomized mice from autoimmunity(21). The ability of these T cells to regulate the immune response, led to their eponym as Regulatory T cells. Since then, the Treg biology field has undergone an enthusing and exponential boom.

Tregs are critical for the preservation of immunological homeostasis. They comprise the regulatory arm of the immune system, and function by curtailing inflammatory responses, and preventing over-activation. These cells have a complex repertoire of biological capacities and rely on different mechanisms to temper the immune response. Principally they suppress the proliferation and cytokine production of effector T cells(22). This can be contact-dependent, or through Treg-associated suppressor molecules and soluble factors including IL-10, TGF-β, GITR, CTLA-4, IFN-γ, IL-9, IL-35 (23, 24).

Treg generation *in-vivo* occurs through two distinct pathways: the thymus (tTregs) or the periphery (pTreg). As was evident early on in their identification, a sizeable proportion of Tregs are generated in the thymus. Here Tregs encounter self-antigens presented by thymic epithelial cells and undergo a concerted process of

positive selection and clonal deletion(25). The precise mechanisms that underlie thymic Treg regeneration are under resolution, but of importance are high-affinity T cell receptor (TCR) interactions with self-peptides, modulation of signal strength through a variety of costimulatory molecules, and the integration of cytokine signals, particularly the common γ-chain cytokines IL-2-, IL-7, IL-15 as well as transforming growth factor β (TGF-β). Conversely, peripherally derived Tregs (pTregs) arise from naïve T cells in secondary lymphoid tissues. These pTregs subsequently go out into circulation where they encounter tissue-specific antigens and contribute to the suppression of effector responses and the establishment of tolerance at these peripheral organ and tissues sites(26, 27). The tolerogenic functions of pTregs are particularly relevant in organs that interface the external microenvironment or depend on micro-organismal colonization, such as the skin or intestine, where it is imperative to avoid adverse or overactive immune reactions.

Although Tregs are diverse, plastic, and heterogeneous pool, they share certain uniting features. The specialized functions of Tregs cells are imprinted by their lineage defining transcription factor, forkhead box protein 3 (Foxp3) which plays a critical role in reinforcing fidelity to the Treg identity(28, 29). Another key Treg characteristic is the constitutive expression of CD25, the IL-2 receptor alpha chain, the which allows them to scavenge IL-2 signals necessary for their survival, suppressor function, and lineage stability(30, 31).

The link between Foxp3 and Treg identity was first established in murine models. Mutational inactivation of the *Foxp3* gene in this mutant mouse, referred to as the Scurfy mouse, resulted in a lethal phenotype marked by aggressive lymphoproliferation, spontaneous inflammation and multi-organ autoimmunity. The translational relevance of *Foxp3* was cemented by the discovery of a corresponding human phenotype. The lethal autoimmune manifestations in patients suffering from immunodysregulation poly-

endocrionopathy, X linked (IPEX), were found to arise from mutations in *Foxp3(32)*. Follow up studies have further revealed the importance of *Foxp3* in Treg development, maintenance, and the acquisition of immunosuppressive properties.

PHENOTYPIC TRAITS OF TISSUE TREGS

Until recently, our conceptual vision of Regulatory T cell function was guided by experimental systems that focused dogmatically on their immunosuppressive functions in the context of autoimmunity, cancer, infection of inflammation. Likewise, our understanding of Regulatory T cell identity was principally informed by characterization of circulating Tregs or those in lymphoid tissues. More recently however, the recognition that Tregs occupy barrier tissue niches and non-hematopoietic organs has shifted the conceptual paradigm of Tregs biology. There is increasing evident Tregs are able to shape their corresponding niches and create a favorable environment to support tissue homeostasis.

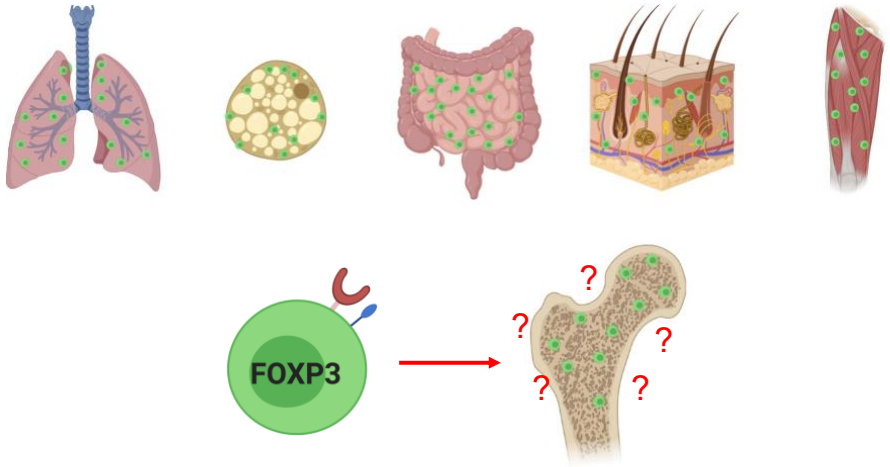


Figure 1: Tissue Tregs
Tregs are a specialized lineage of suppressive CD4 T cells that act as critical negative regulators of immune in various biological contexts. They have well described roles in immune regulation including by-stander suppression, the induction of tolerance, and the resolution of inflammation. Recent work has shown that Tregs also reside within specific tissues and contribute to non-immune regulation of their local microenvironments. Distinct tissue populations have been described in the Lung, Adipose (VAT), Gut, Skin and Muscle that perform non-immune functions. This includes promoting stem cell quiescence, tissue regeneration, and metabolic processing. The focus of this project is to dissect the phenotype and function of bone marrow Tregs, with focus on their key features and their contributions to the regulation of hematopoiesis.

Within parenchymal tissues, Tregs have specialized non-immune roles indicating that there is functional and molecular heterogeneity within this population. These 'tissue' Tregs are enriched in various organs including visceral adipose tissue (VAT), skeletal muscle, epithelium, and lamina propria. They differ from peripheral Tregs in several key characteristics including, cytokine production, transcriptional profile, and surface phenotype (33). A uniting feature of these non-conventional Tregs is that their profile is indicative of tissue adaptation. Different tissue Treg subsets express features that are not typically observed in peripheral Tregs, and instead mirror their tissue of residence. Tissue Tregs are also distinguished by their regenerative capacities and their important crosstalk with stem cell populations in different niches(34).

VISCERAL ADIPOSE TREGS

The observation that Tregs are highly accumulated in adipose tissue (~50% of $CD4^+$ T cells) was critical for launching the 'tissue Treg' paradigm(35, 36). Visceral adipose tissue (VAT) refers to the white fatty tissue lining various internal organs. It primarily acts as a reservoir for energy storage but also possesses unique immunological and metabolic properties. Importantly, it has been implicated in human disease including metabolic disorders, insulin resistance, and type 2 diabetes. Within the adipose tissue, VAT Tregs display unique tissue-specific phenotypes and functional characteristics. Broadly, VAT Tregs suppress adipose tissue inflammation and also promote metabolic homeostasis. This is partly done by decreasing the release of pro-inflammatory cytokines including IFN-y, TNF-*α*, IL-6, and RANTES within VAT and preventing the accumulation of pro-inflammatory macrophages and monocytes.

The tissue adaptation of VAT Tregs is underscored by their dependency on the nuclear receptor family member peroxisome proliferator-activated receptor gamma (PPAR-*γ*). This ligand-inducible transcription factor is the master regulator of adipocyte differentiation and is also involved in fatty acid processing, and glucose metabolism(37, 38). Surprisingly, VAT Tregs express PPAR*γ* at high levels, and their unique phenotype critically depends its expression(39). Moreover, VAT Treg function can be specifically stimulated by administration of PPAR*γ* agonists. Boosting VAT Tregs through this mechanism helps to tempter insulin resistance and lower circulating glucose levels. Conversely, depletion of the VAT Treg pool via conditional knockout of *PPARγ* in Foxp3+ cells results in a dramatic contraction of the VAT Treg pool and decreased metabolic function.

SKELETAL MUSCLE TREGS

Skeletal muscle Tregs mediate muscle repair after injury via production of amphiregulin (AREG), a low-affinity EGFR ligand and IL-10. The power of their regenerative function has been neatly illustrated using models of the mdx mouse model of Duchenne muscular dystrophy (DMD)(40). Although Tregs are rare in healthy skeletal muscle, Tregs are recruited in response to injury and their numbers increase dramatically in proportion to disease progression. Specifically, muscle Tregs occupy necrotic lesions and secrete increased amounts of IL-10 and AREG in response to muscle inflammation(41, 42). In the context of DMD, depletion of Tregs was shown to accelerate disease progression, tissue inflammation, and muscular dystrophy. Conversely, boosting Treg numbers with IL-2/anti-IL-2 complexes had a therapeutic effect that resulted in decreased myofiber injury. Similar mechanisms of regeneration have been observed in the context of inflammation induced damage to skeletal muscle(43).

Within the muscle, a direct crosstalk has been outlined between Tregs and specialized skeletal muscle stem cells, referred to as satellite cells. This interaction was shown to be critical to muscle regeneration, thus highlighting the instrumental role that Tregs play in regeneration of muscular tissue. In the context of acute inflammation, Tregs were found to co-localize in close proximity to satellite cells, at sites of tissue lesion, where they were shown to promote myogenesis. At the site of injury, skeletal muscle Tregs promoted the differentiation and expansion of resident satellite stem cells(44). Interestingly, culture of skeletal muscle Tregs with satellite cells resulted in Treg activation, the upregulation of effector molecules highlighting an important crosstalk between Tregs and muscle stem cells in during the course of muscle regeneration.

SKIN TREGS

The association between Tregs and hair follicle stem cells (HFSC) further contributes the growing paradigm of Tregs influencing the differentiation and maturation of tissue stem cells. Within the epithelial barrier, unique stem cell populations participate in the regeneration of hair follicles throughout the lifespan of an organism(45). Histological analysis has revealed that Tregs enriched in the follicular epithelium. They are localized to the hair follicle (HF) in the vicinity of the bulge region(46). This bulge region is a preferred niche for hair follicle stem cells that are responsible for the hair follicle to regeneration. Within the epithelium, skin Tregs participate in the regulation of the hair follicle cycle and specifically facilitate anagen (growth phase) induction. The interactions between Treg and HFSC within the hair bulge region was shown to be mediated by Notch signaling. Tregs expression of Jag1was shown to interact with Notch receptors on HFSC and Treg specific deletion of Jag1 resulted in a significant downregulation of critical epidermal differentiation genes. Although other ligand-receptor interactions may contribute to Treg HF regeneration, this regenerative function is at least partly mediated via Jag.

INTESTINAL TREGS

Within the intestine there is significant phenotypic heterogeneity among the Tregs pool, with different intestinal Treg subsets co-expressing various lineage defining transcription factors such as *GATA3*, *Helios* and *Rorc,* based on anatomical location. Tregs are most abundant in the colon comprising (35-40%) of residing $CD4^+$ T cells. Akin to the *PPARγ* expression in VAT Tregs, many colonic Tregs express *Rorc* which encodes retinoic acid receptor-related orphan receptor γt (RoRγt) in conjunction with *Foxp3*. Of the tissue populations, colonic Tregs are the most sensitive to commensal microbiota colonization and depend on microbial antigens for their maintenance. Thus, a sizeable portion of the intestinal Treg pool is not thymically derived but instead is

generated in the periphery (pTreg) through exposure to foreign peptides. This has been elegantly teased apart using gnotobiotic murine models, where the intestinal Treg pool has been characterized in mice that have had limited of exposure to external microorganisms. In germ-free mice, raised in sterile conditions that prevent exposure to all external microorganisms, the intestinal Treg pool contracts dramatically highlighting a dependency to foreign antigens. Similar reductions are observed under conditions of prolonged antibiotic treatment thus underscoring symbiotic role of the microbiome in establishing the colonic Treg pool.

A critical function of colonic Tregs is to suppress gastrointestinal inflammation and aid in the maintenance of oral tolerance. During intestinal inflammation Tregs play specialized roles as immunosuppressors. The transfer of effector T cell populations ($CD45RB^{high}$ $CD4^{+}$ T cells) in the absence of Tregs results in the induction of colitis and exacerbated intestinal pathology. To execute these functions intestinal Tregs rely on different transcription factors and cytokine receptors. The transcription factors GATA3 and Helios are functionally necessary for Treg to limit tissue inflammation and Treg specific deletion of GATA3 and Helios results in increased colitis severity and more pronounced colonic inflammation. Intestinal Tregs also regulate ongoing inflammation via specific cytokine signaling axes. Two cytokines ones are TGF-*β* and IL-10 and both TGF-β and IL10 is also essential for colonic Treg function. Deletion of these cytokine receptors (TGF-*β*RI or IL10Ra) results in increases in monocyte and neutrophil infiltration to the colon as well and mice fail to resolve colitis.

In addition to facilitating tolerance and suppression intestinal Tregs also have regenerative roles. It has been described that intestinal Tregs are also able to mediate tissue regeneration and repair, akin to Treg populations in the epithelium and skeletal muscle. As with satellite stem cells, Treg repair functions on the intestinal epithelium appear to be partially mediated by Amphiregulin, and it has been demonstrated that

AREG highly expressed in intestinal Tregs. Upon injury intestinal epithelial cells secrete IL-33 which acts on IL-33R and drives increased AREG production(47). Interactions between Tregs and intestinal stem cells have also been visualized and dissected transcriptionally at the single-cell level but the precise mechanisms underlying this regulation are yet to be elucidated(48).

BONE MARROW TREGS

In the bone marrow, Tregs constitute nearly 30% of CD4+ T cells, a significantly higher ratio than other hematopoietic tissues (49). In vivo imaging has demonstrated that Tregs cluster proximal to HSCs, where they are speculated to create an immune-privileged niche (50). Specifically, it has been shown that adenosine generated by CD39 of BM Tregs helps maintain HSC quiescence and can promote allogeneic-HSC engraftment(51). Recent studies have also underscored a role for Tregs in bone homeostasis via interactions with osteoclasts; others have shown that Treg depletion negatively affects B-cell lymphopoiesis via dysfunction of perivascular stromal cells (52-54). These observations suggest that BM Tregs regulate HSCs and the hematopoietic niche during steady-state and disease and allude to a potential function for Tregs in regulating the marrow tissue niche. The present work expands on these investigations of Tregs in the bone marrow. It outlines the origin, phenotype and function of bone marrow Treg cells defining them as a specialized tissue population. Importantly the subsequent studies highlight a major functional role for Treg derived IL-10 in maintaining stromal cell function and supporting long-term hematopoiesis both in the steady state and in the course of leukemic transformation.

INTERLEUKIN 10

The cytokine interleukin-10 (IL-10) was first described in 1989(55). Since then, years of investigation have outlined the diverse roles of IL-10 in the context of inflammation, regeneration, autoimmunity, tumor responses, and cellular communication. The ubiquity of IL-10 expression and the conservation of its regulatory functions across vertebrate species highlight the evolutionary importance and multifunctional nature of this cytokine (56). The ability to integrate IL-10 signals is found across the majority of hematopoietic and non-hematopoietic cells underscoring its biological relevance(55, 57). With regards to the immune system, IL-10 is indispensable and has been implicated in numerous disease models developmental processes(58-60). Nearly all innate and adaptive immune cells are able to either produce IL-10 or respond to IL-10 signals through the corresponding receptors (IL-10R1) and IL-10R2(61, 62). IL-10 is a 'Type II cytokine' branded by the original studies on IL-10 in shaping the humoral immune response, and its association as an inhibitory T-helper 2 (Th-2) cytokine(63).

At the basic level, IL-10 signals through the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling pathways. Downstream integration of this signal is both context-dependent and cell type-specific involving layers of genomic, epigenetic and transcriptional regulation(64-66). While the functions of IL-10 are vast, it is principally recognized as an inhibitory soluble mediator that with regulatory functions related to the suppression of cellular activation and downregulation of inflammatory responses. Moreover, IL-10 is necessary for the formation of immune tolerance and prevention of excessive immune reactions at barrier tissue interfaces which rely on symbiotic relationships with microbes. IL-10 signaling is essential for the resolution phase of an immune response as it helps to limit excessive collateral damage in host tissues. One of its common functions is to limit the presentation of peptides by antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages, monocytes

and myeloid cells. In response to IL-10 signals, these cells downregulate the expression of MHC II and associated co-stimulatory and adhesion molecules thereby limiting T cell activation. IL-10 signals also constrain myeloid cell maturation. and block the secretion of inflammatory cytokine. In the context of injury, IL-10 can also promote macrophages polarization from pro-inflammatory (M1) to an anti-inflammatory and wound healing state (M2) (67-70).

IL-10 is a broadly expressed cytokine. It is secreted by various innate immune cells including DCs, macrophages, mast cells, natural killer (NK) cells, neutrophils, and monocytes. It can also be produced by T-cells and B cells(71-73) and can directly inhibit T cells, particularly Th17 and Th2 cells. Although most T cells, both CD8 T cells and CD4 T helper cells have the capacity to secrete IL-10, the dominant sources are Th2 cells, Type 1 regulatory cells (Tr1) and Tregs(74). Of the $CD4^+$ T subsets Tregs are the universal IL-10-producers and Treg regulation of immunity via IL-10 has been the subject of intense investigation(75-77).

While waves of IL-10 secretion can be seen in response to injury, infection, or inflammation Treg secretion of IL-10 is highly dynamic, tightly regulated, and tissue specific (78-80).The gut, has a particular dependency on IL-10 and production of this anti-inflammatory cytokine by intestinal Tregs in required for the maintenance of immunological tolerance. Within the human system, genomic investigations have revealed that polymorphisms in the *IL10* and *IL10RA genes* are clinically associated with intestinal inflammation and autoimmunity in the form of intestinal bowel disease IBD, Crohn's disease (CD) and Ulcerative Colitis (UC
(80). Thus, of the various Treg subsets, intestinal Tregs are recognized for their capacity to produce high levels of IL-10(81-88). The ability of Tregs to integrate IL-10 signals is also critical for their regulatory functions. The expression of IL10R is necessary for Treg suppression of Th17 cell-mediated inflammation, and Treg-specific deletion of IL10R

results in spontaneous Th17 cell driven colitis(89, 90). Furthermore, it has recently been shown that IL-10 is involved in modulating non-hematopoietic cells intestinal epithelial cells(91, 92).

HEMATOPOIESIS

Hematopoiesis refers to the continuous process of blood cells production that is active for an organisms' lifetime. Throughout life, the hematopoietic system carefully balances blood cell quiescence, self-renewal, differentiation, regeneration in accordance with extrinsic and organismal demands. Hematopoiesis occurs in a series of waves, with each successive wave producing groups of multi-potent cells that tend to restrict their multilineage-potential as development advances. The first wave occurs during embryonic development. During this wave there hematopoietic stem and progenitor cells (HSPCs) are seeded into the aortic endothelium or intraembryonic aorta region in the yolk sac, a process that is relatively conserved across different animal models (93). As waves of hematopoiesis progress, primitive progenitor cells and HSCs migrate to colonize the fetal liver and as adult mammals mature, the process shifts from the liver to the bone marrow(94-96). After birth the bone marrow becomes the principal site of hematopoiesis, and of HSC (hematopoietic stem cell) maintenance throughout adulthood. Hematopoietic stem cells sit at the apex of a well-orchestrated and tightly regulated the hematopoietic hierarchy.

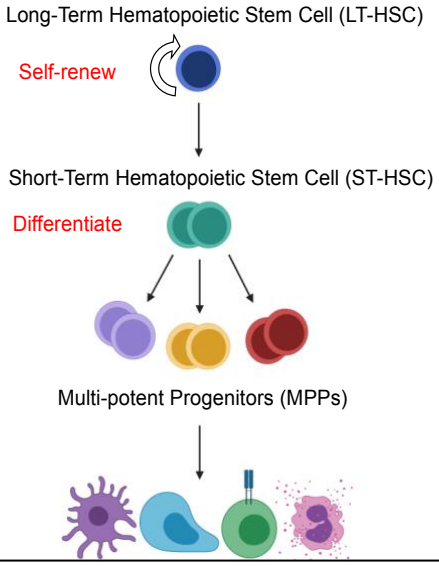


Figure 2: Hematopoietic stem cell hierarchy

Hematopoiesis is the process by which the cellular components of blood are replenished, a process which occur throughout lifetime. The continuous production of our blood cell requires a highly regulated, and highly responsive, system. Within this system hematopoietic stem cells sit at the top of the hierarchy. In the adult they are primarily found in the bone marrow where they are characterized by their ability to self renew and differentiate into mature blood lineages. In the steady state, most HSCs are quiescent, which protects them from genotoxic insults and the bulk of hematopoiesis is ensured by downstream progenitors.

At the basic level, HSC's are defined experimentally by their capacity to self-renew when serially transplanted, and to produce all mature blood cell lineages including lymphoid, myeloid, erythroid, and platelet subsets. A defining property of most HSCs is their relative state of dormancy. Most adult HSCs maintain a state of cellular quiescence. This is done to prevent their exhaustion, ensure their long-term repopulating ability, protect from genotoxic stress, and limit the acquisition of heritable and malignant mutations to downstream progeny(97-100). In steady state conditions, a dynamic and continuous stream of intrinsic and extrinsic cellular signals dictate the balance between self-renewal and lineage commitment. HSC and more differentiated multi-potent progenitors (MPPs) begin the process of lineage differentiation and expansion in response to environmental demands which require the amplification of specific mature blood lineages. This can include conditions of stress, infection, regeneration, or disease. The markers defining HSCs have evolved dramatically in recent years, and numerous functional and transcriptomic studies have revealed an immense degree of heterogeneity and functional variability within the HSC compartment. This includes diversity in lineage output (myeloid vs lymphoid biases) as well as differences in kinetics

of cell division, differentiation status, and cellular quiescence (100-106). Recent lineage fate mapping models in combination with single cell transcriptomic approaches have likewise revealed a division of labor among the hematopoietic hierarchy with multi-potent progenitor populations contributing significantly more to blood cell generation than was previously appreciated(107-109).The question of how much of a specific HSC program is predetermined intrinsically during development and how much heterogeneity is acquired by extrinsic signals encountered during development and colonization of respective niches remains an active and evolving topic of investigation.

THE STROMAL CELL NICHE

It is widely recognized that the local bone marrow (BM) microenvironment, referred to as the "niche," is a critical regulator of HSC function. Nearly all aspects of hematopoietic development involve a synchronized orchestration and integration of temporally and spatially dynamic extrinsic signals(110, 111). The hypothesis of a specialized niche was proposed in 1978 by Ray Schofield who put forth the notion that processes related to HSC lineage output, HSC quiescence, and stability of the stem cell gene expression program were influenced by and dependent on interactions with the surrounding stromal microenvironment(112, 113). It is now well appreciated that the organization and function of the stromal microenvironment plays a crucial role in regulating HSC behavior. The non-hematopoietic cells that provide the supporting architecture and cellular backdrop for hematopoiesis are loosely referred to as the 'stroma'. The stromal niche is comprised of a myriad of cell types which regulate HSC survival, self-renewal, migration, quiescence, and differentiation via different combination of secreted proteins, extracellular matrix interactions and cell-cell contacts. This includes mesenchymal stromal cells (MSCs), osteoblasts, endothelial cells, non-myelinating Schwann cells, and sympathetic nerves. Akin to stem cells, MSCs are a

long-term self-renewing cell with the capacity to give rise to multiple diverse progenies of different linages, including fat, bone and cartilage(11, 114, 115).

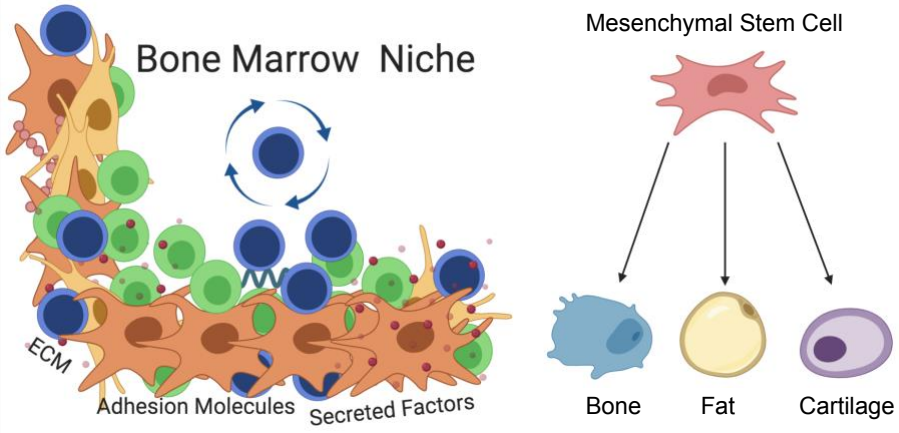


Figure 3: Stromal Cell Niche
(HSCs) are maintained by a dynamic interactions within a specialized microenvironment known as the bone marrow niche. This niche is made up by a complex network of non-hematopoietic stromal cells *stroma*. Stromal cells are known to regulate important stem cell properties including self-renewal, differentiation, and quiescence via release of soluble factors as well as cell-cell interactions . Akin to the hematopoietic tree, there is also developmental hierarchy among stromal populations. At the top of the hierarchy are mesenchymal stromal cells (MSCs). (MSCs), a stromal cell with multi-lineage differentiation capacity to bone, fat , and cartilage.

MSCs are distributed throughout the bone marrow and can be distinguished by an evolving panel immunophenotypic markers. Numerous genetic lineage–tracing studies have defined markers including Nestin (Nes^+), Osterix (Osx^+), Paired-related homeobox 1($Prx1^+$), and Leptin Receptor ($Lepr^+$) that enrich for mesenchymal progenitors that regulate different HSC properties in both physiological and pathological conditions(116). However, while several subpopulations have been shown to be contribute to HSC maintenance, key secreted such as factors the chemokine C-X-C motif ligand 12 (CXCL-12) and stem cell factor (SCF), are produced my multiple stromal subsets highlighting the functional overlap among stromal subsets. The niche factor CXCL12 is indispensable for migration and retention of HSCs to the bone marrow via binding to

CXCR4. In line with this, numerous investigations have visualized the colocalization of HSCs with CXCL12-abundant reticular (CAR) cells which express high amounts of CXCL12. Similarly, it has also been demonstrated that CXCL12, in coordination with other extrinsic cues, also influences the long-term-repopulating activity of HSC (117-120). Another secreted niche factor of critical importance is stem cell factor (SCF; also known as KITL). SCF plays important roles in the early organization of the developing HSCs and more homeostatic functions that enhance HSC maintenance and promote HSC recovery after myeloablation(79, 118, 121-127).

Distinct stromal niches have been described based on their anatomical location and their contributions to HSC regulation. This includes an endosteal niche proximal to the outer edge of the bone that is enriched in osteoblasts, bone forming cells, that provide support to HSC *in vivo* through a number of secreted factors(128-131). The perivascular niche, which is closer to the inner core of the bone marrow, also supports HSC function. This niche can be further subdivided into arteriolar and sinusoidal compartments. The arteriolar niche is composed of arteriolar pericytes, endothelial cells, which secrete SCF, CXCL12, and E-selectin, as well as sympathetic nerves which regulate CXCL12 expression in the stroma, and non-myelinating Schwann cells, which secrete TGF-β. Collectively these signals contribute to HSC migration, and also maintain HSC health and homeostasis. TGF-β1, specifically maintains hemopoietic stem and progenitor cells (HSPCs) in quiescence and modulates expression of mucin-like protein CD34, a key marker of immature HSPCs,(121, 132-136). A sinusoidal niche has comparably been described that is enriched with CAR cells and megakaryocyte progenitors. Within this niche, the medullary vascular sinuses are lined with endothelial cells, CAR cells, and MSCs which form a reticular network that supports HSCs(120, 137). Chemokine C-X-C motif ligand 4 (CXCL4) released by megakaryocytes regulates

HSC cell cycle activity. Megakaryocytes also contribute to HSC quiescence during homeostasis promoting HSC regeneration after chemotherapeutic stress.(136, 138)

Recent advances in high resolution microcopy have revealed a previously unappreciated volume and diversity to the stromal compartment. They have also shed light on the physiological association of HSC with different stromal cells and endothelial compartments and revealed a significant degree of redundancy and overlap among these stromal niches. (139-144). Together, it is evident that the bone marrow niche is highly heterogenous defined by multiple cellular signals and interactions. Concurrently the stromal compartment is equally diverse. It is composed of different subsets and the effect on hematopoiesis is both cell-type and location dependent. Thus, the functional properties of different stromal cell subsets and their stem- cell regulatory abilities remain an open area of investigation.

MEYLOPROLIFERATIVE NEOPLASMS

Myelodysplastic and myeloproliferative neoplasms (MDS/MPNs) are highly heterogeneous clonal blood disorders that vary in cellular composition, genetic pathology, and disease course. Despite their structural and molecular differences, these myeloid malignancies share several commonalities in biology. Their underlying cellular origin is typically transformed hematopoietic stem or progenitor cells which outcompete the healthy counterparts. The clonal nature of these disorders arises from an uncontrolled expansion of differentiation-blocked cells which are unable to progress to mature lineages. This skewing creates to defects in hematopoietic output and eventually leads to clonal hematopoiesis and leukemic progression. Moreover, normal hematopoietic cells are forced into competition with mutant leukemic cells as the two populations occupy the same microenvironment. There is also is increasing evidence that the niche remodeling that accompanies transformation results creates an aberrant microenvironment, unfavorable to healthy cells and that the bone marrow niche can be a

driver for malignancy in itself (145-151). The causal relationship between both of these hypothesis remains to be defined, and it is likely that both of these scenarios occur concurrently or with some degree of overlap. Other hallmark phenotypes include the uncontrolled production of myeloid cells, excessive inflammatory signaling, and immune dysregulation. Recently, it is become well recognized that the immune microenvironment plays a critical role in the leukemic growth and survival.

CHRONIC MYELOID LEUKEMIA

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell disorder that results from the reciprocal translocation t (9;22) (q34; q11). The chromosomal translocation is commonly referred to as the 'Philadelphia chromosome' named after its original identification in 1960, in a patient with CML(152).The translocation of the tyrosine-protein kinase (*ABL1*) on chromosome 9 to the breakpoint cluster region (*BCR*) gene on chromosome 22 creates a oncogenic fusion gene (BCR-ABL1) with persistently enhanced tyrosine kinase activity(153). In the recent years tyrosine kinase inhibitors (TKIs) have dramatically improved quality of life and survival outcome with many patients achieving deep molecular response (DMR) and molecular remission even after TKIs discontinuation(154, 155). Of note, it is becoming appreciated that immunological function may play a role in achievement of DMR in CML patients on TKI therapy. Similarly, a number of immune phenotypes are beginning to emerge as prognostic indicators of disease outcome(156). Some of the key cellular actors that have been functionally implicated in CML are Natural killer (NK) cells, effector T cells, Tregs, and Myeloid-derived suppressor cells (MDSCs). Dormant or resistant BCR–ABL+ leukemic cells are often a cause of disease relapse after therapy. These residual cells and can be detected even in patients that achieve molecular remission after TKI discontinuation. Thus, strategies for eliminating residual leukemic cells are of great interests. NK cells

are innate immune cells equipped for enhanced immunosurveillance and cytotoxicity. These properties make them an attractive candidate for prospective immunotherapy in CML.

An alternative to pharmacological intervention in CML is allogeneic hematopoietic stem cell transplantation. This therapy has shown potent positive responses even in patients relapsed disease. The strong anti-leukemic effect is attributed to the robust graft-versus-leukemia reactions which are able to target and eliminate oncogenic cells. It has been demonstrated that autologous NK cells can be activated to exhibit a cytolytic activity against malignant progenitors in vitro (157). Clinically it has also been observed that higher counts of peripheral blood NK cells correlate with reduced risk of relapse, suggesting a role in tumor surveillance(158, 159). Importantly, specific TKI BCR/ABL-inhibitors, which inhibit T cell function do not seem to alter NK cell cytotoxicity and cytokine secretion(160). The majority of these studies have looked at NK immunosurveillance and cytotoxity in the context of residual leukemia following sustained TKI treatment. Thus, a necessary question that needs to be answered is whether NK cells are functional in untreated patients with active CML. Nonetheless, these studies provide insight into how to create future combinatory immunotherapeutic approaches and highlight the important role of immunological function in myeloid malignancies.

Immune altering effects of TKI-therapy on the T cell compartment have also been reported. Some groups report treatment with the TKI Imatinib inhibits T cell proliferation and activation(161). Other investigations have demonstrated that various immune phenotypes manifest concurrently and vary depending on disease severity, length of TKI treatment and depth of remission(162). In some studies, leukemic burden was correlated with a decrease in numbers of Tregs, and MDSCs, as well as increased NK cell numbers. It has also been reported that patients with high levels of BCR-ABL

transcripts have higher frequencies of T-reg cells(163). Conversely, others have shown that frequencies of Tregs are significantly elevated in CML patients at the time of diagnosis but decreased dramatically in patients with chronic phase CML(164). These results suggest that different immunological mechanistic may be pertinent at different phases of CML and there is currently a deficit in the available clinical data related to Treg function in different patient populations, at different stages of disease, and in response to different TKI treatments. It has also been reported that treatment with different TIK, Dasatinib, can trigger clonal T cell expansion, indicative of leukemic antigen recognition and anti-CML cytotoxicity (165). The Programmed cell death protein 1 (PD1)- PD-L1 pathway has also been implicated in CML pathology. In T cells PD-1, is a co-inhibitory receptor, upregulated by activated T cells and its ligation with PD-L1 is critical for immune tolerance and downregulation of immune responses. Dysregulated PD-1-PD-L1 signaling has been detected in T cells from CML patients with CML along increased expression of other exhaustion markers, including, T cell immunoglobulin and mucin domain 3 (TIM3), and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) indicative of potential T cell exhaustion(166, 167). Ongoing research has focused on identifying leukemia-associated antigens expressed on mutant cells with the goal of using these antigens for the development of functional immunotherapy in CML patients. However, functional targets that able to elicit a robust repose are still lacking, and the effect of the inflammatory cytokine environment on T cell activation presents another obstacle to these therapies (168).

Overall complex immune interactions, both immunosuppressive and activating, have been demonstrated in the context of CML and in response to TKI therapy. These observations raise questions on how the precise immune-escape mechanisms of leukemic blasts and how the inflammatory progression of disease affects the immune landscape during leukemogenesis.

OVERALL RESEARCH GOAL

Recently, T cell regulation of bone marrow has garnered a lot of attention, fueled in part by an explosion in the prevalence of immunotherapy and the dual recognition that immune dysregulation is both a dominant and accompanying mechanism of leukemic transformation. Importantly, it is now recognized that the non-hematopoietic stromal niche contributes to the organization and regulation of T cell homeostasis by directing cellular plasticity, differentiation, and development. Less however is known about the feedback mechanisms through which T cells crosstalk with the marrow stroma. This project focuses on the role of CD4+ T cells in maintaining the integrity of the bone marrow microenvironment and their role in regulating hematopoiesis in the steady state and during disease. Specifically, it introduces recent advances in the roles played by Foxp3+ Regulatory T cells (Tregs) in the maintenance and conditioning of the bone marrow microenvironment both in steady state and disease. It further dissects how the development of leukemia overlays with a stepwise loss of T cell function and identity.

Finally, it examines the role that dysfunctional T cells play in the evolution of hematological malignancy with a focus on how immune-modulatory drugs and interventions can restore T cell balance and delay disease progression. While the influence of Regulatory T cells (Tregs) has been explored in numerous contexts, their role with regards to stromal maintenance has not been elucidated, and their significance in within the context of hematopoietic regulation remains relatively unexplored. A comprehensive characterization of Regulatory T cells in the maintenance and regulation of the bone marrow niche, both in steady state and in the context of hematological malignancy is the focus of this dissertation and the present work.

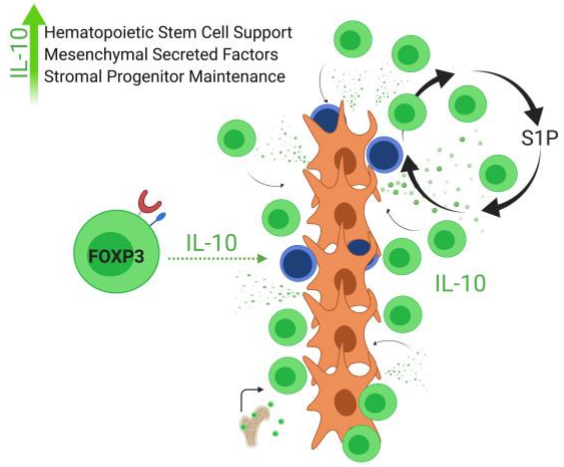
Bone marrow Regulatory T cells mediate stromal cell function and support hematopoiesis via IL-10

by

Virginia Camacho, Victoria R. Matkins, Sweta B Patel, Jeremie M Lever, Zhengqin Yang, Li Yang, Ashley E. Landyut, Emma C Dean, James F. George, Henry Yang, Paul Brent Ferrell, Craig L. Maynard, Casey T. Weaver, Heth R. Turnquist, Robert S. Welner

JCI Insight. 2020;5(22):e135681. https://doi.org/10.1172/jci. insight.135681.
Copyright
2020
by
Camacho et al.
Used by permission

Format adapted [and errata corrected] for [thesis or] dissertation



In Brief

Regulatory T cells within the bone marrow (BM Tregs) represent a specialized population whose functions are incompletely understood. In this study, we provide a novel mechanism by which marrow Tregs support the maintenance of stromal cells to direct hematopoiesis. We found that marrow Tregs are high producers of IL-10, an immunosuppressive cytokine that limits inflammation. In this study we describe how bone marrow Tregs condition the stromal cell niche and enhance their ability to support stem cell function and hematopoiesis via IL-10. Collectively, our observations suggest that Tregs residing within marrow are master regulators of hematopoiesis and that production of IL-10 accounts for at least some of their functions. This and other properties have the potential to be exploited in the context of transplantation or hematopoietic disease.

Highlights

- **Tregs in the marrow are unique**
  I. Highly Circulation
  II. Unique homing capacity Marrow
  III. Enhanced IL-10 secretion

- **Tregs support hematopoiesis via IL-10**
  i. IL-10 has a conditioning role for stroma
  ii. IL-10 is required for stromal cell maintenance and HSC-supporting activity

JCI Insight
Copyright
2020
by Camacho et. al. 2020 Used by permission
Format adapted for dissertation

Bone marrow Regulatory T cells mediate stromal cell function and support hematopoiesis via IL-10

Authors

Virginia Camacho[1], Victoria R. Matkins[1], Sweta B. Patel[1], Jeremie M. Lever[2], Zhengqin Yang[3], Li Ying[4], Ashley E. Landuyt[5], Emma C. Dean[5], James F. George[3], Henry Yang[4], Paul Brent Ferrell[6], Craig L. Maynard[5], Casey T. Weaver[5], Heth R. Turnquist[7] and Robert S. Welner[1*]

[1] Division of Hematology-Oncology, Department of Medicine, The University of Alabama at Birmingham, Birmingham, AL, 35294, USA. vcamacho@uab.edu 205-975-3960
[1] Division of Hematology-Oncology, Department of Medicine, The University of Alabama at Birmingham, Birmingham, AL, 35294, USA. vrmcclea@uab.edu 205-975-3960
[1] Division of Hematology-Oncology, Department of Medicine, The University of Alabama at Birmingham, Birmingham, AL, 35294, USA. patel791@uab.edu 205-975-3960
[2] Nephrology Research and Training Center, Division of Nephrology, Department of Medicine, The University of Alabama at Birmingham, Birmingham, AL, 35294, USA. jlever@uab.edu 205-934-4261
[3] Division of Cardiothoracic Surgery, Department of Surgery, The University of Alabama at Birmingham, Birmingham, AL. zhengqinyang@uabmc.edu 205-934-4261
[4] Cancer Science Institute of Singapore & Department of Biochemistry, National University of Singapore. csiliyi@nus.edu.sg +65 9658 7920
[5] Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA. ashland@uab.edu 205-996-0136
[5] Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA. emmadean@uab.edu 205-934-4092
[2] Nephrology Research and Training Center, Division of Nephrology, Department of Medicine, The University of Alabama at Birmingham, Birmingham, AL, 35294, USA. jfgeorge@uabmc.edu 205-934-4261
[4] Cancer Science Institute of Singapore & Department of Biochemistry, National University of Singapore. csiyangh@nus.edu.sg +65 9658 7920
[6] Division of Hematology/Oncology, Vanderbilt University Medical Center, Nashville, TN, USA. brent.ferrell@vanderbilt.edu 615 875-8619
[5] Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA. clmaynard@uab.edu 205 996-0136
[5] Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA. cweaver@uab.edu (205) 975-5537
[7] Department of Surgery, University of Pittsburgh, Pittsburgh, PA; Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA. het5@pitt.edu 412-624-6695
[1] Division of Hematology-Oncology, Department of Medicine, The University of Alabama at Birmingham, Birmingham, AL, 35294, USA. rwelner@uab 205-975-3960

* Corresponding author email – rwelner@uab.edu

Abstract

The non-immune roles of Regulatory T cells (Tregs) have been described in various tissues, including the bone marrow. In this study, we comprehensively phenotyped marrow Tregs, elucidating their key features and tissue specific functions. We show that marrow Tregs are migratory, and home back to the marrow. For trafficking, marrow Tregs utilize S1P gradients, and disruption of this axis allows for specific targeting of the marrow Treg pool. Following Treg depletion, the function and phenotype of both mesenchymal stromal cells (MSCs) and hematopoietic stem cells (HSCs) was impaired. Transplantation also revealed that a Treg-depleted niche has a reduced capacity to support hematopoiesis. Finally, we found that marrow Tregs are high producers of IL-10 and that Treg-secreted IL-10 has direct effects on MSC function. This is the first report revealing that Treg secreted IL-10 is necessary for stromal cell maintenance and our work outlines an alternative mechanism by which this cytokine regulates hematopoiesis.

Introduction

Regulatory T lymphocytes (Tregs) are required for the preservation of immunological homeostasis. Recent work has shown that Tregs also reside within specific tissues and contribute to non-immune regulation of their local microenvironments (1-6). While the crosstalk between Tregs and other immune cells has long been an active area of investigation, there is a critical gap in knowledge defining how Tregs interact with non-hematopoietic cells and how these Treg properties may be exploited to target non-hematopoietic populations within tissue microenvironments.

Tregs have specialized roles in tissue regeneration, oral tolerance, and metabolic responses reflecting the functional and molecular heterogeneity of these cells (1, 7). These 'tissue-Tregs' are enriched at various sites including skeletal muscle, epithelium, lamina propria, and adipose tissue (3, 4, 6, 8-11). Importantly, they differ from peripheral Tregs with respect to cytokine production, transcriptome profile, and metabolic capacities. In the bone marrow, Tregs are found at a significantly higher ratio than in other lymphoid tissues (30-40% of $CD4^+$ T cells) (12, 13). *In vivo* imaging of bone marrow has demonstrated that Tregs cluster proximal to hematopoietic stem cells (HSCs) and that adenosine generated by Tregs maintains HSC quiescence and can promote allo-HSC engraftment (14, 15). Additionally, Tregs have been shown to play a critical role in improving Graft-Versus-Host-Disease (GVHD) and recovery following bone marrow transplantation (16-18). These observations indicate that Tregs are active regulators of homeostasis within the bone marrow.

Non-hematopoietic components of the bone marrow and the mechanisms by which they maintain hematopoiesis have been extensively studied (19-23). Hematopoietic stem cells (HSCs) are kept in balance by a dynamic milieu of cellular interactions within specialized microenvironments known as niches (24-31). This network of non-hematopoietic cells is loosely referred to as the *stroma*, and it encompasses a complex system of specialized cells. Among these are mesenchymal stromal cells (MSCs), a stromal cell with multi-lineage differentiation capacity to bone, fat, and cartilage (27, 30, 32-34). Many studies have underscored a role for stromal regulation of HSCs. However, little is known about how T cells regulate the stromal niche and facilitate these functions. This study examines Treg regulation of the bone marrow microenvironment, with a direct focus on maintenance of mesenchymal stromal cells.

Here, we outline the characteristics that define bone marrow Tregs. We propose that marrow Tregs represent a specialized 'tissue-Treg' that actively regulates and conditions the stromal niche. In this study, we utilized multiple clinically validated immunomodulatory agents to manipulate the marrow Treg pool and assessed these effects on the bone marrow microenvironment. Our results provide mechanistic insight into Treg regulation of mesenchymal stromal cells, and we identify IL-10 signaling as highly relevant to this process. Importantly, our work provides a foundation for the further investigations into cell-based Treg therapy, specifically targeted at enhancing stromal cell function.

Results

Phenotypic markers distinguish peripheral and bone marrow Regulatory T cells

To evaluate the tissue-specific functions of bone marrow Regulatory T cells (marrow Tregs), we compared their distribution to Tregs in other tissues: spleen and inguinal

lymph node (LN). Consistent with the published literature, we found that the marrow was enriched in $CD3^+CD4^+$ $Foxp3^+$ cells. Notably, the frequency of Tregs is approximately three times greater in the marrow than other lymphoid organs (5, 13, 15, 35). Marrow Tregs comprise ~40% of total $CD3^+CD4^+$cells (Figure 1A-B) and about 0.005-0.01% of total bone marrow cells suggesting that they have important functions in this site.

To further profile marrow Tregs, we examined the expression of informative surface receptors (36) (Figure 1C) and S1A). We observed minimal differences in activation markers: CD103, CTLA-4, CD69, CD73, with the exception of increased PD-1. Though notable differences included increased levels of KLRG-1 (Killer Cell Lectin-Like Receptor Subfamily G, Member 1), as well as decreased expression of CD25 (IL2Rα) and ICOS (inducible co-stimulator) (Figure 1C). Of note, KLRG1 is a marker of terminal differentiation and tissue specialization suggesting that marrow Tregs represent a mature effector population (37-39). Intriguingly, we also observed high expression of CD127/IL7Rα (Figure 1D-E). Because the functions of Tregs are considered to be independent of IL-7, and Tregs are characterized by low CD127 expression (40, 41), we sought to determine if increased CD127 expression denoted differences in reactivity and signal transduction. Following IL-7 stimulation, we observed an increase in phospho-STAT5 indicating that marrow Tregs are indeed responsive to this cytokine. Our results also demonstrate that CD127 is an intrinsic characteristic of marrow Tregs, and that IL-7 signaling may represent an adaptive mechanism in a context where IL-7 is the prevalent cytokine.

Our analysis also revealed differences in proliferation and apoptosis. Marrow Tregs had similar rates of proliferation and cell death as Tregs in the spleen (Figure 1F), but than those in the LN. Similar to other 'tissue-Treg' populations (adipose, lung, and intestine) marrow Tregs highly expressed the IL-33 receptor, ST2 (Figure S1B). Marrow Tregs also highly expressed Helios and Neuropilin-1, indicating thymic origin (Figure 1G). To validate this, we transplanted $CD3^+$ DN ($CD4^-$ $CD8^-$) thymocytes from $Foxp3^+$ (CD45.1) mice and assessed Treg development in recipient tissues (CD45.2, non-GFP hosts) 8 weeks after transplantation. Our analysis revealed that a significant portion of Tregs in the marrow, as well as the spleen, were derived from transplanted thymocytes. $Foxp3^+$ cells were detected in the LN, but at significantly reduced proportions (Figure 1H). In conclusion, we ascertain that marrow Tregs harbor markers of tissue specialization and differ from those in the periphery with regards to their phenotype and signaling capacity.

Regulatory T cells in the bone marrow preferentially localize back to this site

To dissect transcriptional differences, we performed RNA sequencing analysis on Tregs isolated from the bone marrow, lymph node, and spleen (GSE138095). We observed that marrow Tregs possessed a distinct transcriptome and were enriched for signatures related to homing, migration, secreted factors, and activation. We identified 347 differentially expressed genes (181 downregulated DEGs, 166 upregulated DEGs) in marrow Tregs. GSEA indicated an enrichment for genes associated with chemotaxis and enhanced cytokine secretion. Of note, the upper majority of differentially expressed genes were those encoding chemokine and cytokine receptors (Figure 2A-B, S2A). To compare marrow Tregs to Tregs in other tissues, we overlaid our bulk transcriptomic data with recently published single-cell RNA-seq datasets (42). Pathways analysis and DEG signatures neatly distinguished marrow Tregs from other subsets according to their tissue-specific gene expression. Furthermore, unsupervised transcript clustering and t-SNE (t-distributed Stochastic Neighbor Embedding) visualization revealed marrow Tregs to have a distinct transcriptome. Marrow Tregs converged to some degree with Tregs in

VAT (visceral adipose tissue) as well as lymph node and peripheral blood, a finding that concurs with previous studies (9, 11, 43) and suggests that marrow Tregs might exchange with the circulating pool (Figure 2C, S2B-C). We further analyzed the expression of CXCR4 and CD150 using transcriptomic data in combination with single-cell datasets of 'tissue-Tregs'. No striking transcriptional differences were detected, and we observed that CD150 and CXCR4 are are expressed by Tregs in all tissues and not exclusive to marrow Tregs contrary to other studies. (Figure S2B-C). By surface analysis, we found equivalent CXCR4 expression across Treg sub-populations but did note an increase in CD150 in marrow Tregs, as has been reported (15, 43).

Across multiple tissues, the division of labor among Treg populations is maintained through synchronized homing patterns which position Tregs at sites where regulation is required (11, 12). Thus, we evaluated the expression of various chemokine and homing receptors in marrow Tregs with respect to those from spleen and LN. We observed decreased expression of CD62L (L-selectin), important for egress from lymphoid tissues and a marker for T-cell development (44). We also noted increased expression of CD49b (integrin alpha$_2$) relative to splenic cells, which delineates Treg specialization and correlates with enhanced effector function and migratory capacity (45) (Figure S2C). Next, we tested if there was selective recruitment of Tregs to the bone marrow using functional homing assays as have been used to study HSCs (46). We transplanted Tregs isolated from different tissues and compared Treg migration to different lymphoid organs. Foxp3^{GFP} cells were injected into congenic non-irradiated mice and homing to recipient tissues was assessed 24 hours post-transplant. Remarkably, marrow Tregs had an enhanced ability to return to the bone marrow, a characteristic resonant with stem cell function. Recipient marrows contained significantly higher numbers of marrow isolated Tregs implying that unique signaling cues concentrate Tregs back to this microenvironment. Interestingly, splenic Tregs did not preferentially home well to any tissue. We also assessed retention at extended timepoints and found that Tregs persisted in the marrow 8 weeks after transplant (Figure 2D). This demonstrates that marrow Tregs possess a distinctive ability to return to and persist in their tissue of origin.

To assess the kinetics of Treg circulation, we generated parabiotic pairs of Foxp3^{GFP} mice (CD45.1) and non-GFP partners (CD45.2). Parabiosis generates a shared circulation system and is a well-validated method for studying T-cell origin and trafficking (47, 48). After 28 days, parabionts and non-connected controls were sacrificed, and cellular exchange was examined. Analysis revealed that marrow Tregs were highly migratory non-sessile cells (Figure 2E). At the tissue level, the marrow had lower cellular exchange than the spleen and LN (Figure S2D). We also observed significantly decreased CD4$^+$ chimerism (~25.6% exchange), whereas CD8$^+$ exchange (~50.0%) was consistent across tissues. Although CD4$^+$ T cell chimerism was decreased in the marrow, Treg exchange (~20.4%) was much higher than the LN (~5.5%) and spleen (~5.4%). Marrow Tregs were exchanged significantly more than other T cells (Foxp3$^-$ CD4$^+$ cells and CD8$^+$ cells) (Figure S2E-F). This demonstrates increased affinity for the marrow environment and suggests there may be targeted recruitment of marrow Tregs to this tissue. Collectively, our parabiotic analysis indicates that marrow Tregs are highly specific to the marrow and suggests these cells may not need spleen or lymph node for instruction.

Regulatory T cell depletion does not result in bone marrow T cell activation or inflammation

To test the physiological relevance of marrow Tregs, we assessed how marrow populations responded to their manipulation. Tregs were depleted via administration of anti-CD25 antibody (aCD25). Using a low dose regimen, we were able to specifically target Tregs, avoid depletion of other T cells, and circumvent the confounds of autoimmunity. aCD25-mediated Treg depletion led to a significant (<50%) reduction in $Foxp3^+$ cells (Figure 3A-B), as has been previously reported (49-51). Following depletion, we found no changes to tissue cellularity (Figure S3A) and no significant expansion or increased proliferation of $Foxp3^-CD4^+$, $CD8^+$ or NK cells in the marrow demonstrating that the primary function of marrow Tregs is not immune-suppression (Figure 3C-D, S3B-C). This was surprising given that the canonical function of Tregs is to limit effector T cell expansion and proliferation(52). While we observed a reduction of $CD4^+$ cells numbers and increased $CD4^+$ apoptosis, this can be specifically ascribed to the loss of $CD4^+$ Tregs that are undergo cell death as a consequence of aCD25 (Figure 3E, S3C). Overall, we observed no indication of overt inflammation among other mature populations in spleen or marrow with the exception of a slight myeloid expansion (Figure S3D-E). Finally, to assess local and systemic effects of aCD25, we evaluated over 200 chemokine and cytokine targets in the serum and bone marrow fluid of Treg depleted mice. We detected negligible increase of inflammatory markers (including IL-1, IL-17, IL-22 and TNF-alpha, and IFN-gamma) in the serum (systemic) or the bone marrow fluid (local) with most targets relatively unchanged (Figure 3F, S3F). As a reference, we compared aCD25 depletion with a low dose TLR mimic lipopolysaccharide (LPS) which produced <100-fold change in expression. Only Artemis and IL2Ra had <2-fold change at 2.13 and 2.96 respectively. Thus, we determined that there was no inflammation using this treatment. Altogether, this suggests that inhibition of T cell activation and cytokine-mediated inflammation is not the principal function of marrow Tregs.

Bone marrow Regulatory T cells are required for stromal cell maintenance

Given the crosstalk that exists between Tregs and their local tissue environments (4), we hypothesized that Tregs might regulate the function of non-hematopoietic cells within the marrow. Indeed, we observed an expansion and increased proliferation of various stromal populations following Treg depletion, including $CD31^-CD51^+Sca1^+$ phenotypic mesenchymal stromal cells (MSCs) and $CD31^-CD51^+Sca1^-$ (bone precursors). A similar but not statistically significant trend was observed for $Sca1^+CD140\alpha^+$ (PDGF Receptor α) cells and no significant changes were noted for endothelial cells ($CD31^+$) (Figure 4A, S4A) (53-55). To validate this, we utilized a stromal-associated strain, Prrx1-Cre crossed to a ROSA/tdTomato reporter, in which $tdTOM^+$ labeling corresponds to stroma. Following aCD25, the numbers of $tdTOM^+$ cells were significantly increased, corroborating our initial findings (Figure 4B).

We then tested if the stromal phenotypes seen after Treg depletion could be rescued. We tracked the recovery of hematopoietic and stromal populations after stopping aCD25 treatment. We evaluated the stabilization of populations to baseline conditions (no treatment) during the course of short-term (4-8 weeks after discontinuing aCD25) and long-term (25 weeks) recovery. We observed that the normalization of the stromal compartment occurred proportionate to the restoration of Tregs (Figure 4C). This suggests that bone marrow stromal cells are finely tuned to the presence of Tregs.

Our in-vivo data suggested a meaningful role for Tregs with regards to stromal regulation. To validate this, we looked for methods that would specifically target the marrow Treg pool. One marker, Sphingosine-1-Phosphate Receptor 1 (S1PR1), was highly expressed on marrow Tregs (Figure 4D). Since others have shown that both T

cells and stem cells use gradients S1P to home back to the bone marrow (56, 57), we investigated if the S1P/S1PR1 axis was a potential mechanism for Treg trafficking. Mice were treated with S1P receptor modulator Phospho-Fingolimod (FTY720), which interferes with S1PR signaling and abrogates the S1P/S1P1-dependent egress of lymphocytes from lymphoid tissues. Because S1P trafficking is highly tuned to the surface expression of different S1P receptors (S1PR1-5), the time-course and dose of FTY720 was chosen to limit off-target effects to other S1PR1 expressing cells, including lymphocytes and HSCs. Targeting S1PR1-mediated recirculation allowed us to prevent the influx of Tregs to the marrow and assess the cellular response (57). After 7 Days of FTY720 we observed a significant decrease of Tregs in the marrow suggesting that that S1P/S1P1 gradients are involved in marrow Treg trafficking (Figure 4E-F, S4B). We specifically noted an accumulation of Tregs in the spleen, consistent with other reports (58). Importantly, this treatment did not alter the Treg numbers in the other peripheral tissues, allowing for more precise manipulation of marrow Tregs. With FTY720, we observed an expansion and increased proliferation of PDGFRα^{+} cells and MSCs (Figure 4G), similar to aCD25. No significant changes were observed for endothelial cells or bone progenitors. This substantiates our observations that stromal populations acutely respond to disruptions in the Treg pool.

Bone marrow Regulatory T cells enhance niche-dependent support of hematopoietic stem cells

Stromal cells regulate important hematopoietic stem and progenitor cells (HSPCs), properties including self-renewal, differentiation, and quiescence via release of soluble factors and cell-cell interactions (20). The changes to the stromal compartments led us to hypothesize that Tregs may regulate important stromal cell functions including HSC support. We focused on HSC support because blood stem cell regulation is a defining feature of the niche with immense clinical implications (59-62). To test the effect of Tregs on the HSC-stromal relationship, we assayed the ability of the Treg-depleted niche to support HSCs using non-genotoxic conditioning and transplantation. In order to maintain the existing niche architecture and circumvent the need for irradiation, recipients were cleared with ACK2, an antibody clone blocking cKit function (63). LT-HSCs were then transplanted into either WT or Treg depleted mice (aCD25). We observed appreciably reduced peripheral blood engraftment at 16-weeks post-transplant in the Treg depleted mice (Figure 5A). These findings indicate that the capacity of the stromal niche to support LT-HSCs is impaired in the absence of Tregs. Importantly, it demonstrates that Tregs influence extrinsic, niche-dependent stem cell regulation.

Others have shown that HSPCs are sensitive to environmental changes and are influenced by stromal alterations (64-66). Consistent with this, we noted an expansion of HSPCs after aCD25-mediated Treg depletion (Figure 5B). Specifically, an increase in proliferation of long-term (LT) HSCs (Lineage^{-}Sca1^{+}cKit^{+} CD150^{+} CD48^{-}; LT-HSCs) was observed (Figure 5B) with no significant changes in cell death (Figure 5C) (67, 68). We also observed the presence of extramedullary hematopoiesis as evidence by the increase of LT-HSC to the spleen (Figure S5C). To transcriptionally dissect this phenotype, we performed RNA sequencing on LT-HSCs. We identified 179 upregulated DEGs from aCD25 treated mice. GSEA indicated a decreased in classical stem cell signatures as well as increased proliferation (Figure 5D, S5A). We observed negligible alterations to more differentiated progenitor populations (Figure S5B). As HSCs are typically a quiescent, population sensitive to environmental cues (69-71), the phenotypic and transcriptional profiles following Treg depletion suggested functional decline. To test this, we assayed the effects of Treg loss on intrinsic properties of LT-HSC. This was

tested through competitive repopulation assays using different competitor donor ratios. At 16-weeks post-transplant, we observed lower total peripheral blood chimerism in mice transplanted with cells from aCD25 treated mice (Figure 5E, S5D). This reduced repopulating capacity indicates a loss of functional potential. Together, these results indicate that stem cells from a Treg depleted environment are phenotypically expanded and impaired even when placed in a healthy microenvironment.

Because our in-vivo treatments involved complex cellular interactions, we sought to dissect the effect of Tregs more directly through co-cultures. To do this, we generated an ex-vivo hematopoietic support assay in which HSC numbers act a proxy for stem cell maintenance. In our assay, HSC numbers indicate stem cell survival and act as a surrogate measure of stromal cell function (HSC-support). Although co-cultures do not entirely replicate the complexity of the marrow microenvironment, they are among the better ex-vivo assays available (72). We cultured HSCs in the presence or absence of stroma and assessed how Tregs altered this system (Figure 5F). In line without in-vivo observations, we found enhanced HSC support in conditions where stroma and Tregs were present. Marrow Tregs enhanced the ability of stromal cells to both maintain HSCs and promote maturation to phenotypic multipotent progenitors (Figure 5G). Importantly, this was exclusive to marrow Tregs, as splenic Tregs had no effect. Together with the transplant studies this suggest that marrow Tregs enhance the capacity of stromal cells to support hematopoiesis and maintain stem cells.

Regulatory T cell secreted IL-10 alters stromal cell proliferation, differentiation and hematopoietic stem cell support

A key cytokine associated with Treg function is IL-10. This pleiotropic anti-inflammatory cytokine allows Tregs to temper immune responses at various tissue interfaces (73-75). We utilized a previously established IL-10 reporter system (in which IL-10 expression is linked to Thy1.1) to analyze IL10 production in-vivo. (76, 77) We identified that marrow Tregs are high producers of IL-10 and generate significantly more IL-10 transcripts than other Tregs (Figure 6A-B). We also uncovered that Tregs are a principal source of IL-10 in bone marrow (Figure S6A). To resolve potential targets of Treg secreted IL-10, we analyzed our previously generated single-cell RNA sequencing datasets for the expression of IL-10 receptor alpha (IL-10Ra) across mature, progenitor and stromal bone marrow populations. Intriguingly, MSCs highly expressed the IL-10Ra. They also had the highest IL-10Ra expression across stromal populations (Figure 6C and S6B) (78). We detected no IL-10Ra expression in HSPCs. To probe this mechanism, we stimulated stroma with IL-10 and evaluated downstream signaling targets (Figure 6D). Indeed, IL-10 stimulation triggered robust STAT3 phosphorylation (40-fold increase in pSTAT3) indicating that stroma actively respond to IL-10 signaling.

To determine if the stromal response was brought on by disruptions to IL-10, we performed in-vivo neutralization of IL-10 signaling by blocking the IL-10 receptor (via anti-IL-10R). We observed that IL-10R blockade reproduced the stromal phenotypes seen with Treg depletion via aCD25, (Figure 4A, S4A) and Treg sequestration via FTY720 (Figure 4G). This resulted in expansion of MSCs and bone forming progenitors (Figure 6E). No significant changes were observed for endothelial or PDGFRα^{+} cells. We observed no significant changes to mature myeloid or T cell populations, but we did recapitulate the expanded LT-HSC phenotype (Figure S6C-D). These results concur with our other transient drug treatments and indicate that IL-10 selectively and specifically regulates both stromal cells and HSCs.

The robust stromal response to IL-10 led us to hypothesize that Treg secreted IL-10 may have a conditioning role in shaping the bone marrow microenvironment. To assess its long-term influence, we analyzed mice with selective knockout of IL-10 in Foxp3-expressing cells (Foxp3-Cre × IL-$10^{fl/fl}$). Intriguingly, life-long genetic deletion of Treg-IL-10 resulted in a reduction of MSCs, bone forming progenitors, and $PDGFRA^{+}$ cells (Figure S6E). Endothelial cells remained unchanged. This blunted phenotype suggests that Treg-secreted IL-10 is necessary for normal stromal development. We also observed an expansion of myeloid populations and no significant changes to T cells. In contrast to our transient treatments, no major changes were observed in HSPCs. (Figure S6F-G). To further dissect the developmental influence of IL-10 on stromal cells, we generated Prrx1-Cre×IL10R-$alpha^{fl/fl}$ mice in which IL10Ra was deleted from Prrx1 expressing stromal populations. With this additional life-long genetic deletion model, we recapitulated the blunted stromal phenotype. We observed a significant reduction in stromal populations similar to Foxp3-Cre×IL-$10^{fl/fl}$ mice (Figure S6H). These findings highlight the differential effects between transient and prolonged IL-10 perturbations on stroma. Our observations suggest that acute perturbations of Tregs/IL-10, as seen with aCD25, IL10R blockage and FTY720, lead to increased stromal proliferation consistent with a stress response (79). Conversely, life-long loss of IL-10 signaling, as seen with genetic deletions, appears to hamper stromal cell maturation resulting in decreased numbers. These findings suggest that Treg-derived IL-10 may have additional importance for shaping the developmental trajectories of stromal cells.

To gain a better understanding for the role of IL-10 on stromal differentiation and lineage commitment, we used the colony-forming unit (CFU) assay to test if IL-10 influenced the clonogenic potential of stromal progenitors. Stroma stimulated with IL-10 showed ~10% reduction in CFU activity, indicating that IL-10 limits colony formation (Figure 6F). Additionally, we tested how IL-10 influenced differentiation into bone and fat lineages. We utilized two additional stromal-associated strains: Osx-Cre (early-bone forming cells), and AdipoQ-Cre (adipocyte cells) crossed to ROSA/tdTomato reporters(80, 81). We observed fewer $tdTOM^{+}$ cells in Osx cultures indicating that IL-10 alters bone lineage commitment. No consistent differences were observed regarding adipocyte differentiation (Figure 6G). We also evaluated the expression of stromal-associated factors upon IL-10 stimulation. Upon stimulation, we detected increased levels of stromal associated transcripts *Runx2*, *LepR*, and *PDGFRa*, and *KITLG*, which have been associated with enhanced hematopoietic support (22, 82, 83). Conversely, transcripts associated with lineage commitment including *Col2A1*, *Spp1*, *Ppry*, and *Fabp4* were expressed at lower levels (33, 54, 84, 85). Interestingly, the receptor for IL-10, *IL-10ra* was also upregulated, as was IL-7, implying that a feedback loop might exists between Tregs and stroma (Figure 6H). These findings demonstrate that IL-10 is an important mediator of stromal secreted factors and suggest that IL-10 signals may serve checkpoint for stromal precursors to advance cell lineage decisions.

Finally, we sought to determine if stromal cell function (specifically HSC maintenance) was directly affected by Treg-secreted IL-10. To test this, we evaluated support of HSC in the presence of Tregs using our co-culture model, but this time introduced a neutralizing antibody to IL-10. We again tested phenotypic HSC maintenance ex-vivo because it provides a short-term read-out of one of the most clinically relevant stromal cell functions. Notably, we observed that neutralization of IL-10 significantly abrogated the capacity of stromal cells to maintain phenotypic HSCs (even in presence of Tregs). This indicates that IL-10 is a critical actor in Treg regulation of stromal cells (Figure 6I).

To validate the phenotypic HSC observations from the ex-vivo cultures, we tested the functional capacity of HSCs after 96 hours of co-culture with Tregs, stroma, and IL-10 neutralizing antibody. This was done by transplanting HSCs from individual wells and evaluating engraftment and long-term lineage-reconstitution (Figure 6I, S6I). Analysis of donor chimerism revealed that even short-term ex-vivo exposure to marrow Tregs was sufficient to enhance HSC support and improve engraftment. Critically, the Treg effect on engraftment was abrogated in cohorts where IL-10 was neutralized. In absence of other cellular actors, this suggests that Treg-derived IL-10 directly augments the ability of stromal cells to support HSCs. These findings expand on the conclusions from our co-culture analysis as HSCs demonstrating that stem cells are not just phenotypically maintained, but also functionally improved by presence of marrow Tregs and stroma. We also propose that this ex-vivo culture system is a useful model to functionally evaluate the effects of Treg IL-10 on stromal cell function in absence of other cellular variables.

In line with the previous profiling, we also observed decreased HSC maintenance using stroma derived from Foxp3-Cre×IL-10$^{fl/fl}$ mice (Figure S6J). This suggests that stroma with functional IL-10 signaling are more efficient at ex-vivo HSC support. Together with the transplant studies, this suggest that marrow Tregs enhance the capacity of stromal cells to maintain stem cells via IL-10. The data further demonstrate that IL-10 has a dual role: it regulates the differentiation trajectory of MSCs and enhances their function with regards to secreted factors and stem cell maintenance.

Discussion

Our study sheds light on the defining properties of marrow Tregs and outlines an uncovered role for Treg secreted IL-10 in regulating stromal cell function and development. We found that Tregs are enriched within the marrow and are specialized for the maintenance of this tissue. Furthermore, transcriptional and functional characterization of marrow Tregs identified a unique profile of chemokine receptors, surface markers, and circulation patterns. Notably, depletion of Tregs showed no increases in T cell proliferation pro-inflammatory cytokines suggesting that their role in the marrow is not immune suppression. In contrast, stem cells and mesenchymal stromal cells were severely altered Treg depletion. Importantly, the effects of Treg depletion were replicated with different methods of manipulation including Treg sequestration and blockade of IL-10 signaling underscoring the importance of T IL-10 as a key mediator of these phenotypes.

Our work provides a comprehensive evaluation of bone marrow Tregs in relation to those in the periphery as well as tissue-specific subsets. We conclude that bone marrow Tregs are tissue-specialized. Marrow Tregs express genes associated with activation and terminal differentiation and they harbor a unique profile of chemokine receptors. Another distinguishing property of marrow Tregs was high expression of CD127. In the context of Treg biology, this could be an environmental adaptation the low IL-2 levels in the marrow, and it is likely that Tregs in rely on IL-7, provided by the surrounding stroma (86). Our observations are in line with studies showing co-localization of bone marrow memory CD8 cells T cells in the proximity of IL-7 producing stromal cells (87). In fact, the decrease in proliferation of CD8 cells we observed upon Treg depletion may itself be a result of stromal alterations. Given that the marrow serves a memory T cell niche, our CD127 phenotype supports the notion that Tregs in different environmental contexts can be sustained through IL-7 and can signal through CD25-independent mechanisms.

We have also identified a clinically relevant mechanism to disrupt Treg trafficking to the bone marrow. We provide evidence that marrow Tregs depend on sphingosine-1-phosphate (S1P) gradients for migration and that disruption of this axis allows for sequestration of Tregs out of the marrow. More detailed investigations into S1P mediated Treg trafficking are required to further develop this system for Treg manipulation. Remarkably marrow Tregs are able to home back to the marrow and they persist in this tissue in a similar fashion as HSCs (56). We also demonstrate that marrow Tregs are highly migratory and not tissue resident. This circulatory profile was corroborated by parabiosis. We propose that the homing capacity of marrow Tregs makes them an attractive candidate for targeted drug delivery. Importantly, it raises the possibility that stem cell therapy, GVHD, and transplantation could be enhanced via marrow Tregs because these cells equipped to provide site-specific regulation and can directly target mesenchymal components of the bone marrow.

In other studies, localization of Tregs to the marrow was attributed to increased expression of CXCR4 (15). We evaluated CXCR4 expression at the protein, bulk transcript and single cell levels and observed no differences. Because marrow Tregs are highly circulatory, a Foxp3-specific deletion of CXCR4 likely prevents Treg re-entry to the marrow, resulting in diminished numbers, but also targets peripheral Tregs nonspecifically. Furthermore, manipulation of CXCR4 negatively alters Treg function, as CXCR4 antagonists have shown to inhibit Tregs' suppressive activity (88). Given the multiple roles that CXCR4 has in cellular trafficking to the marrow and T cell trafficking specifically, disruption of CXCR4 signaling does not represent a translationally feasible mechanism by which to target the marrow Tregs pool.

Upon Treg depletion, a significant expansion of HSPCs and stromal cells was observed, as well as increased proliferation of HSCs. We propose this is a direct consequence of the alterations imparted on the marrow microenvironment. Moreover, the absence of Treg cells compromised the capacity of stromal cells to support HSCs both in-vivo and in co-culture. Using three different methods: Treg depletion, Treg sequestration and IL-10R blockade, we have shown that loss of marrow Tregs function has a critical effect on HSCs in a stromal cell dependent manner. This indicates that marrow Tregs directly regulate the mesenchymal components responsible for maintaining HSCs and our work supports the notion that Tregs act on HSCs both directing and indirectly via modulation of the stroma niche.

In the process of selecting a method for Treg depletion, we also evaluated a Foxp3-DTR (Diptheria Toxin receptor) model where Foxp3$^+$ cells are ablated following Diptheria Toxin administration (89). While this is a widely accepted immunological model, DT regiments produce unwanted side effects (90, 91). In Foxp3-DTR mice, we observed accelerated deterioration and rapid onset of autoimmunity including increased cell death. Furthermore, multiple populations directly respond to inflammatory stimuli and these represent off-target effects incompatible with our studies. Given the therapeutic potential of Tregs, we instead aimed to utilize models with translational applicability. Thus, a significant advantage of aCD25 administration, is that the humanized anti-hCD25 antibody (daclizumab) has been clinically validated (51, 92).

Other studies have demonstrated that MSCs enhance the function of various immune cells, and of Tregs specifically (93-100). However, few have addressed if the reverse also occurs. Similarly, while it is known that IL-10 is critical for immune-suppression (74,

75, 101), a role for IL-10 in hematopoiesis and in stromal cell conditioning was previously unexplored. Here, we identified a unique mechanism for Treg-derived IL-10 in shaping a tissue niche. We demonstrate that IL-10 influences stromal capacity to support HSCs, consistent with studies in other tissues, for example, of IL-10 acting on intestinal epithelial cells (102). While this does not obscure the potential for Tregs to regulate stromal cells via other cell-cell contact mechanisms or secreted factors, it offers a viable target for modulating the stromal environment. We observed changes to stroma when we manipulated IL-10 signaling in an acute manner (via loss of Tregs or blocking IL10 signaling) and systemically using a lifelong elimination of Treg secreted IL-10 (Foxp3-Cre×IL-10$^{fl/fl}$) or IL-10 stromal cell signaling (Prrx1-Cre×IL-10R$^{fl/fl}$) mice. We attribute the disparity in some phenotypes to the differences in treatment length (acute versus long-term) and animal models: transient treatments versus genetic deletions (life-long IL-10 perturbations). With the transient treatments of aCD25, IL10R blockage and FTY720, the loss of IL10 signaling is temporary, resulting in increased proliferation, and consistent with a stress response expansion (79). Conversely, we propose that IL-10 is critical for MSCs. Thus, having limited access to a major source of IL-10 from birth likely blunts stromal cell development resulting in decreased numbers. Collectively, both observations support a model in which the stromal alterations are precipitated by the absence of IL-10 producing Tregs. Our transplantation and ex-vivo assays demonstrate that Tregs render the bone marrow niche more supportive and functional. Translationally, it may even be possible to improve strategies for ex-vivo HSC expansion in culture by advancing our understanding of how immune cells influence the niche-dependent mechanisms that promote HSC maintenance. We propose Treg derived IL-10 directly contributes to HSC support in a stromal cell dependent manner. As Treg dysregulation has been implicated in numerous diseases, future studies into how IL-10 signaling is dysregulated in the context of inflammation or hematological malignancy represent promising areas of investigation.

Overall, this work sheds light on how mature immune cells actively regulate hematopoiesis via the local microenvironment. We have discovered a unique Treg-to-stromal cell interaction pathway which reconciles previous investigations on marrow Tregs and expands our understanding of their tissue specialization. These studies provide a foundation for the development of therapies aimed to manipulate stromal populations and enhance their function. This has broad implications for the treatment of hematological malignancies, transplantation and the emerging fields of Treg and mesenchymal cell therapy.

Methods

Anti-CD25
(0.5mg/ mouse) of InVivoMAb anti-mouse CD25 (PC-61.5.3) or InVivoMAb, or rat IgG1 isotype control (anti-horseradish peroxidase was administered intraperitoneally weekly for 4 weeks. before being euthanized for analysis.

Blockade of IL-10R
100µg InVivoMAb anti-mouse IL10R (1B1.3a; BioXCell) or vehicle (PBS) was administered 4 times at 5-day intervals. Mice were euthanized for analysis 10 days after last injection.

EdU
(1mg/mouse) of EdU in 200 µl PBS was administered intraperitoneally 16h before being euthanized for analysis. Cells were analyzed 16 hours post-injection and EdU incorporation assessed according to the manufacturer's protocol.

BrdU
(1mg/ mouse) of BrdU in 200 µl PBS was administered intraperitoneally 16h before being euthanized for analysis. Cells were analyzed 16 hours post-injection and BrdU incorporation assessed according to the manufacturer's protocol.

AnnexinV
Analysis of apoptotic cells were performed using APC-conjugated Annexin V according to the manufacturer's protocol (Biolegend, San Diego, CA.)

STAT5
Cells were isolated from of 6-8-week-old *Foxp3*^{GFP} mice and incubated for 15 min at 37 C with or without recombinant murine IL-7 or IL-10 (Peprotech). Cells were processed according to manufacturer's protocol (BD Phosflow™ Perm Buffer III). Cells were then stained with primary Anti-Stat5 (pY694) or isotype control (BD Biosciences).

FTY720
FTY720 (reconstituted to 1 mg/ml in 1% DMSO and PBS). (1 mg/kg) FTY720 or PBS was administered intraperitoneally weekly for 7 days before being euthanized for analysis.

Bulk RNA sequencing
GEO accession number: GSE138095. Bone marrow, spleen and lymph node Tregs; Control and anti-CD25 long-term hematopoietic stem cells.

Treg cells from lymphoid tissues
Live $CD4^+$ $CD8\alpha^-$ $Foxp3^+$ $NK\text{-}1.1^-$ (Tregs) were sorted from these tissues (bone marrow, inguinal lymph node, and spleen) of 6-8-week-old *Foxp3*^{GFP} mice and resuspended in Buffer RLT. RNA was extracted using RNAeasy micro kit (QIAGEN, Valencia, CA), with 3 biological replicates per tissue.

Hematopoietic Stem Cells
Live $Lin^-Sca\text{-}1^+c\text{-}Kit^+Flt3^-CD150^+CD48^-$ (long-term hematopoietic stem cells) were sorted from 10-12-week-old B6 mice treated with anti-CD25 or IgG1 and resuspended in

Buffer RLT. RNA was extracted using RNAeasy micro kit (QIAGEN, Valencia, CA), with 4 biological replicates per treatment condition.

Library Amplification and Analysis
Libraries were prepared using the TruSeq RNA Library Preparation Kit v2 (catalog RS-122–2001, Ilina) according to the manufacturer's protocol. Sequencing was performed on the Illumina HiSeq2500. Reads of RNA-seq data were mapped to mouse mm10 genome using STAR aligner. Mapped reads number of each gene were used to identify gene expression by feature Counts. Gene expression counts were further normalized among samples based on the total numbers of all mapped reads, and subsequently log2-tranformed. Normalized data were further employed to identify differentially expressed genes with the cutoff values: 2 for the average fold change between the comparing two groups and 0.05 for the p value of t-test cross the replicated samples. DAVID (https://david.ncifcrf.gov/) was used for GO pathway analysis of differentially expressed genes. Hierarchical clustering with average linkage was performed for the normalized expression data cross different tissues, and subsequently, the heatmaps were generated from the clustering results. Gene set enrichment analysis (GSEA) was performed based on the normalized data using GSEA v2.0 tool (http://www.broad.mit.edu/gsea/).

Stromal Cell isolation
Non-hematopoietic stromal cells were processed as follows: residual bone fragments were crushed and digested in PBS and collagenase/dispase (1 mg/mL; Cat: 11097113001; Sigma-Aldrich), for 45 minutes at 37°C, and then combined with the single cell bone marrow suspension.

Treg homing assay
FACS sorted $Foxp3^+$ cells were isolated from bone marrow or spleen of CD45.2+ mice were transplanted retro-orbitally into CD45.1 non-GFP recipients (20,000 cells per mouse). Recipient groups received either bone marrow or spleen $Foxp3^+$ cells. Mice were euthanized 24hrs post-transplantation, and homing/distribution of $Foxp3^+$ cells to bone marrow, lymph node, and spleen of CD45.1 non-GFP recipients was assessed.

Thymocyte Transplant assay
FACS sorted $CD3^+$ DN ($CD4^-$ $CD8^-$ $Foxp3^{-)}$ thymocytes from $Foxp3^+$(CD45.1) mice were transplanted retro-orbitally into (CD45.2) non-GFP hosts (50,000 cells per mouse). Mice were euthanized 8-weeks post transplantation, and generation of $Foxp3^+$ cells in bone marrow, lymph node, and spleen of CD45.2 non-GFP recipients was assessed.

ACK2 Administration
Antibody conditioning and transplantation was done in two rounds. 500µg of purified anti-mouse CD117 (c-kit) antibody [clone ACK2] was administered retro-orbitally into CD45.2 mice. Seven days after ACK2, 500 sorted CD45.1+ HSCs (Lin^- $c\text{-}kit^+$ $Sca1^+$ $CD150^+$ $CD48^-$) were transplanted. Mice were allowed to recover for seven days. Post recovery they were reconditioned with another, 500µg of ACK2 and transplanted an additional 500 CD45.1+ HSCs 7 days later.

Quantitative reverse transcription PCR
Passage 1 stroma were cultured in the presence or absence of IL-10 (10ng/mL) for 7 days, RNA was extracted using RNAeasy micro kit (QIAGEN, Valencia, CA), and cDNA synthesized using the Superscript III First-Strand Kit (Invitrogen, Grand Island, NY). q-

PCR was performed in triplicate using Invitrogen™ SuperScript™ IV Reverse Transcriptase kit. Power SYBR Green Master Mix gene-expression assays were performed for murine Runx2, Twist1, LepR, PDGFR, Gli1, kitL, Angpt2, IL10ra, IL1-rb, col2A1, Spp1, Fabp4, Ppry, LPL, IL7 and were normalized to the level of TBP1 mRNA.

Flow cytometry and cell sorting

Single cell suspensions from bone marrow, inguinal lymph node, thoracic duct, spleen, and peripheral blood were analyzed using a BD Fortessa X-20 flow cytometer or BD LSRII (BD Biosciences, San Jose, CA) sorted using a FACSAria (BD Biosciences, San Jose, CA). Diva software (BD) and FlowJo (Tree Star) was used for data acquisition and analysis, respectively. Surface Markers: KLRG1, ICOS, CD25, CD103, CD44, CD62L, CD49b, CD127, CTLA-4, PD-1, CD73, CD69, CXCR4, CD150, S1PR1, ST2.

Mature populations were defined as follows: CD4 T cell ($CD3^+$ $CD4^+$ $NK1.1^-$), CD8 T cell ($CD3^+$ $CD8^+$ $NK1.1^-$) Treg ($CD3^+$ $CD4^+$ $Foxp3^+$ $NK1.1^-$), Natural Killer T cells (NKT; $CD3^+$ $NK1.1^+$), Neutrophils ($Gr\text{-}1^+$,$CD115^-$), Macrophages ($Gr\text{-}1^-$,$F4/80^+$). B cells ($CD19^+$ $B220^+$) Immature-B cells ($CD43^+$ $CD19^+$ $B220^+$).

Hematopoietic stem and progenitor cells were defined as follows: granulocyte-macrophage progenitors (GMP; $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+Fc\gamma R^+$), megakaryocyte-erythroid progenitors (MEP; $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^-Fc\gamma R^-$), long-term hematopoietic stem cells (LT-HSC; $Lin^-Sca\text{-}1^+c\text{-}Kit{+}Flt3^-CD150^+CD48^-$), short-term hematopoietic stem cells (ST-HSC; $Lin^-Sca\text{-}1^+c\text{-}Kit^+Flt3^-CD150^-CD48^-$), multipotent progenitors (MPP; $Lin^-Sca\text{-}1^+c\text{-}Kit^+Flt3^-CD48^+$).

Non-hematopoietic stromal cells were defined as follows: non-hematopoietic, non-endothelial cells ($CD45^-$/$Ter119^-$/$CD31^-$), endothelial cells ($CD45^-$/$Ter119^-CD31^+$), mesenchymal stromal cells ($CD45^-$/$Ter119^-$/$CD31^-$ $CD51^+Sca\text{-}1^+$), osteoblasts ($CD45^-$/$Ter119^-$/$CD31^-CD51^+Sca\text{-}1^-$), $PDGFRA^+$ cells ($CD45^-$/$Ter119^-$/$CD31^-CD140\alpha^+Sca\text{-}1^+$).

Donor cell engraftment and lineage contribution was determined via analysis of the peripheral blood using anti-CD45.1 and CD45.2 antibodies and lineage markers: CD3e for T-Cell lineage, B220 for B-Cell lineage, and CD11b for Myeloid lineage

For complete antibody please refer to Supplementary Table 1.

Stromal Cell Culture

All cultures with stromal cells were carried out with murine stromal cells from harvested from femurs and tibias and cultured through one passage (P1) and supplemented with Gibco™ MEM α, Nucleosides, No Phenol Red Media supplemented with 100 U/mL penicillin-streptocycin, 100 U/mL Antibiotic Antimycotic Solution and 20% FBS. Media and cytokines were replaced every 3 days.

Stromal Treg and HSC Co-culture

LT-HSC ($Lin^-Sca\text{-}1^+c\text{-}Kit^+Flt3^-CD150^+CD48^-$) were cultured with Tregs sorted from bone marrow or spleen ($CD3^+CD4^+Foxp3^+$) at a 2:1 ratio with or without stromal cells (200 sorted $CD45^-$/$Ter119^-$/$CD31^-$ cells) in Lonza™ BioWhittaker™ X-VIVO™ 15 Hematopoietic Serum-Free Culture Media supplemented with 100 U/mL penicillin-streptocycin, 100 U/mL Antibiotic Antimycotic Solution; murine SCF 20ng/mL, Flt3 50ng/mL, IL-3 10ng/ML, IL-2 50ng/mL, IL-7 50ng/mL. Cultures were supplemented with

10ng/mL of IL-10 neutralizing antibody at a neutralization Dose (ND50) of 0.015 µg/mL) as indicated. Cultures were carried out for 96 hours in a 96 well flat bottom plate.

MSC Colony forming Unit-Fibroblast (CFU-F) assay

10,000 stromal cells were cultured in a 24 well plate for 7 days supplemented with Gibco™ MEM α, Nucleosides, No Phenol Red Media supplemented with 100 U/mL penicillin-streptocycin, 100 U/mL Antibiotic Antimycotic Solution and 20% FBS in the presence or absence of IL-10 (10ng/mL). Media and cytokines were replaced every 3 days. Cells were rinsed with PBS, fixed with methanol and stained with Giemsa staining solution. Cell clusters with more than 20 cells were counted as a colony.

Western Blot and Protein Quantification

The cells were lysed using 50µL of RIPA lysis buffer per 1 million cells. 10% SDS-PAGE gels were then loaded with 30µg of protein for separation and then transferred to a nitrocellulose membrane using a TransTurbo (Bio-Rad). The membrane was blocked in TBST with 5% non-fat skimmed milk for 1 hour at RT. Blots were probed for pSTAT3-Y705 (D3A7 XP, Cell Signaling) and re-probed for tSTAT3 (F-2, Santa Cruz). The images were taken using a GBox imager. Densitometry analysis of the blots was carried using ImageJ normalized to the housekeeping gene B-Actin (C4, Santa Cruz).

Competitive Repopulation

CD45.2 bone marrow cells from control or aCD25 treated mice (competitors) were mixed with wild-type bone marrow cells (donors) and a total of $5x10^6$ cells was injected into lethally irradiated $CD45.1^+$ recipients. Donor competitor ratios of 1:1 (1 competitor: 1 donor) 1:3 (1 competitor: 3 donor) 3:1 (3 competitor:1 controls) were used.

Ex-Vivo HSC Transplantation Assay

$CD45.2^+$ HSCs were collected from individual co-culture wells after 96 hours of exposure to Tregs, stroma, and/or IL-10 neutralizing antibody. The contents of individual wells were normalized to ensure that equal HSC numbers were transplanted. HSCs were retro-orbitally injected into cohorts of sub-lethally irradiated $CD45.1^+$ recipients (550 rads).

Single cell RNA sequencing

scRNAseq data (42) was filtered and visualized by graph-based clustering and tSNE visualization followed by differentially expressed gene and pathway analysis using Partek Flow, version 8.0, Partek Inc., St. Louis, MO, USA.

Statistical Analysis

Statistical analyses were reported as means ± standard deviation (SD) or standard errors of means (SEM). A Shapiro-Wilk test was used to determine normal versus abnormal distributions, and all continuous variables were tested for mean differences. Depending on the spread of variable both nonparametric: Mann–Whitney U test, ANOVA Kruskal-Wallis test, Wilcoxon test, and parametric: Student's t-test and ANOVA were used. For ANOVA, Tukey's post-test was used to compare individual groups (GraphPad Prism version 6.0, La Jolla, CA). A-priori sample size calculation was determined based on estimates from preliminary data in order to provide power of >80% to detect a 30% difference with an alpha error of 0.05. p values < 0.05 were considered statistically significant. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$; ns = not significant.

Study Approval

Murine Strains: The Jackson Laboratory

C57BL6 mice (stock no.000664) were obtained from The Jackson Laboratory
Foxp3/GFP mice (Stock No: 023800) were obtained from The Jackson Laboratory
Foxp3/DTR mice (Stock No: 016958) were obtained from The Jackson Laboratory
TdTomato mice (Stock No: 007909) were obtained from The Jackson Laboratory
Ocn-Cre (Stock No: 019509) were obtained from The Jackson Laboratory
Osx-Cre (Stock No: 006361) were obtained from The Jackson Laboratory
AdipoQ-Cre: (Stock No: 010803) were obtained from The Jackson Laboratory
Prrx1-Cre: (Stock No:005584) were obtained from The Jackson Laboratory

Murine Strains: Donated

10BiT.Foxp3^{gfp} (CD45.1 and CD45.2); immunocompetent, donated by Dr. Casey Weaver. Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA. cweaver@uab.edu (205) 975-5537
Foxp3-Cre/YFP-IL10flox (CD45.2); immunocompetent, donated by Dr. Heth Turnquist. Department of Surgery, University of Pittsburgh, Pittsburgh, PA; Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA. het5@pitt.edu 412-624-6695

All murine experiments were conducted using 6-12 weeks old male and female mice. Mice were separated by sex and housed with 5-7 mice per cage. Littermates of the same sex were randomly assigned to experimental groups. Mice for use in these studies were maintained in a specific pathogen-free state in micro-isolator caging and received autoclaved water and standard rodent chow diet. Mice were tested quarterly (maximum interval) for all murine pathogens by culture, histopathology and serology. All mice were maintained under the guidance of the UAB Animal Resources Program. The Department of Comparative Medicine at the University of Alabama at Birmingham provided veterinary care. UAB complies with the current NIH policy on animal welfare, the Animal Welfare Act, and all applicable federal, state, and local laws. The present study was approved by the Institutional Review Board at UAB in accordance with assurances filed with the Department of Health and Human Services and met all requirements of the Declaration of Helsinki. UAB Office of Research, 720 Administration Building, 701 20th Street South, Birmingham, AL 35233. Corresponding IRB-160420008.

Parabiosis Surgery

Parabiotic surgeries were conducted as previously published by Lever and colleagues(47, 103).

Acknowledgments

We would like to thank Drs. Paul Kincade and Allan J. Zajac for their feedback. This project was supported by the following grants: HL131477 (NIH), ASH Bridge Funding 2018, National Institutes of Health (NIH) Immunology T32 Training Grant 2T32AI007051 and funds from the Division of Hematology/Oncology at UAB.

Author contributions

V.C. designed, planned, and performed experiments, analyzed data and wrote the manuscript; J.M.L., Z.Y., V.R.M, S.B.P, A.E.L, and P.B.F performed experiments; L.Y.H.Y. analyzed data and edited the manuscript; HRT and C.L.M assisted with data interpretation and experimental design, and edited the manuscript. J.F.G, E.C.D, and

C.T.W. supervised the study and edited the manuscript; R.S.W supervised the study and assisted with data interpretation and manuscript writing. All authors declared no conflict of interest.

References:

1. Vasanthakumar A, Moro K, Xin A, Liao Y, Gloury R, Kawamoto S, et al. The transcriptional regulators IRF4, BATF and IL-33 orchestrate development and maintenance of adipose tissue-resident regulatory T cells. *Nature immunology.* 2015;16(3):276-85.
2. Mackay LK, and Kallies A. Transcriptional Regulation of Tissue-Resident Lymphocytes. *Trends in immunology.* 2017;38(2):94-103.
3. Panduro M, Benoist C, and Mathis D. Tissue Tregs. *Annu Rev Immunol.* 2016;34:609-33.
4. Zhou X, Tang J, Cao H, Fan H, and Li B. Tissue resident regulatory T cells: novel therapeutic targets for human disease. *Cellular & molecular immunology.* 2015;12(5):543-52.
5. Glatman Zaretsky A, Konradt C, Depis F, Wing JB, Goenka R, Atria DG, et al. T Regulatory Cells Support Plasma Cell Populations in the Bone Marrow. *Cell Rep.* 2017;18(8):1906-16.
6. Ali N, Zirak B, Rodriguez RS, Pauli ML, Truong HA, Lai K, et al. Regulatory T Cells in Skin Facilitate Epithelial Stem Cell Differentiation. *Cell.* 2017;169(6):1119-29.e11.
7. Kalin S, Becker M, Ott VB, Serr I, Hosp F, Mollah MMH, et al. A Stat6/Pten Axis Links Regulatory T Cells with Adipose Tissue Function. *Cell metabolism.* 2017;26(3):475-92.e7.
8. Ali N, and Rosenblum MD. Regulatory T cells in skin. *Immunology.* 2017;152(3):372-81.
9. Cipolletta D, Feuerer M, Li A, Kamei N, Lee J, Shoelson SE, et al. PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature.* 2012;486(7404):549-53.
10. Cipolletta D, Cohen P, Spiegelman BM, Benoist C, and Mathis D. Appearance and disappearance of the mRNA signature characteristic of Treg cells in visceral adipose tissue: age, diet, and PPARgamma effects. *Proceedings of the National Academy of Sciences of the United States of America.* 2015;112(2):482-7.
11. Li C, DiSpirito JR, Zemmour D, Spallanzani RG, Kuswanto W, Benoist C, et al. TCR Transgenic Mice Reveal Stepwise, Multi-site Acquisition of the Distinctive Fat-Treg Phenotype. *Cell.* 2018;174(2):285-99.e12.
12. Wei S, Kryczek I, and Zou W. Regulatory T-cell compartmentalization and trafficking. *Blood.* 2006;108(2):426-31.
13. Zou L, Barnett B, Safah H, Larussa VF, Evdemon-Hogan M, Mottram P, et al. Bone marrow is a reservoir for CD4+CD25+ regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer research.* 2004;64(22):8451-5.
14. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R, et al. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature.* 2011;474(7350):216-9.
15. Hirata Y, Furuhashi K, Ishii H, Li HW, Pinho S, Ding L, et al. CD150(high) Bone Marrow Tregs Maintain Hematopoietic Stem Cell Quiescence and Immune Privilege via Adenosine. *Cell stem cell.* 2018;22(3):445-53.e5.
16. McDonald-Hyman C, Muller JT, Loschi M, Thangavelu G, Saha A, Kumari S, et al. The vimentin intermediate filament network restrains regulatory T cell suppression of graft-versus-host disease. *The Journal of clinical investigation.* 2018;128(10):4604-21.

17. Thangavelu G, and Blazar BR. Achievement of Tolerance Induction to Prevent Acute Graft-vs.-Host Disease. *Frontiers in immunology.* 2019;10:309.
18. Riley JS, McClain LE, Stratigis JD, Coons BE, Ahn NJ, Li H, et al. Regulatory T cells promote alloengraftment in a model of late-gestation in utero hematopoietic cell transplantation. *Blood advances.* 2020;4(6):1102-14.
19. Tay J, Levesque JP, and Winkler IG. Cellular players of hematopoietic stem cell mobilization in the bone marrow niche. *International journal of hematology.* 2016.
20. Cordeiro Gomes A, Hara T, Lim VY, Herndler-Brandstetter D, Nevius E, Sugiyama T, et al. Hematopoietic Stem Cell Niches Produce Lineage-Instructive Signals to Control Multipotent Progenitor Differentiation. *Immunity.* 2016;45(6):1219-31.
21. Li L, and Neaves WB. Normal stem cells and cancer stem cells: the niche matters. *Cancer Res.* 2006;66(9):4553-7.
22. Puverel S, Kiris E, Singh S, Klarmann KD, Coppola V, Keller JR, et al. RanBPM (RanBP9) regulates mouse c-Kit receptor level and is essential for normal development of bone marrow progenitor cells. *Oncotarget.* 2016.
23. Camacho V, McClearn V, Patel S, and Welner RS. Regulation of normal and leukemic stem cells through cytokine signaling and the microenvironment. *International journal of hematology.* 2017;105(5):566-77.
24. Morrison SJ, and Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature.* 2014;505(7483):327-34.
25. Itkin T, Gur-Cohen S, Spencer JA, Schajnovitz A, Ramasamy SK, Kusumbe AP, et al. Distinct bone marrow blood vessels differentially regulate haematopoiesis. *Nature.* 2016;532(7599):323-8.
26. Baryawno N, Severe N, and Scadden DT. Hematopoiesis: Reconciling Historic Controversies about the Niche. *Cell Stem Cell.* 2017;20(5):590-2.
27. Yu VW, and Scadden DT. Hematopoietic Stem Cell and Its Bone Marrow Niche. *Curr Top Dev Biol.* 2016;118:21-44.
28. Hoggatt J, Kfoury Y, and Scadden DT. Hematopoietic Stem Cell Niche in Health and Disease. *Annual review of pathology.* 2016;11:555-81.
29. Yu VW, and Scadden DT. Heterogeneity of the bone marrow niche. *Current opinion in hematology.* 2016;23(4):331-8.
30. Kfoury Y, and Scadden DT. Mesenchymal cell contributions to the stem cell niche. *Cell Stem Cell.* 2015;16(3):239-53.
31. Scadden DT. The stem-cell niche as an entity of action. *Nature.* 2006;441(7097):1075-9.
32. Mishra PJ, and Banerjee D. Activation and Differentiation of Mesenchymal Stem Cells. *Methods in molecular biology (Clifton, NJ).* 2017;1554:201-9.
33. Almalki SG, and Agrawal DK. Key transcription factors in the differentiation of mesenchymal stem cells. *Differentiation; research in biological diversity.* 2016;92(1-2):41-51.
34. Liu H, Xia X, and Li B. Mesenchymal stem cell aging: Mechanisms and influences on skeletal and non-skeletal tissues. *Experimental biology and medicine (Maywood, NJ).* 2015;240(8):1099-106.
35. Pierini A, Nishikii H, Baker J, Kimura T, Kwon HS, Pan Y, et al. Foxp3(+) regulatory T cells maintain the bone marrow microenvironment for B cell lymphopoiesis. *Nature communications.* 2017;8:15068.
36. Larbi A, and Fulop T. From "truly naive" to "exhausted senescent" T cells: when markers predict functionality. *Cytometry Part A : the journal of the International Society for Analytical Cytology.* 2014;85(1):25-35.

37. Beyersdorf N, Ding X, Tietze JK, and Hanke T. Characterization of mouse CD4 T cell subsets defined by expression of KLRG1. *European journal of immunology.* 2007;37(12):3445-54.
38. Cheng G, Yuan X, Tsai MS, Podack ER, Yu A, and Malek TR. IL-2 receptor signaling is essential for the development of Klrg1+ terminally differentiated T regulatory cells. *Journal of immunology (Baltimore, Md : 1950).* 2012;189(4):1780-91.
39. Kornete M, Mason E, Istomine R, and Piccirillo CA. KLRG1 expression identifies short-lived Foxp3(+) Treg effector cells with functional plasticity in islets of NOD mice. *Autoimmunity.* 2017;50(6):354-62.
40. Rodriguez-Perea AL, Arcia ED, Rueda CM, and Velilla PA. Phenotypical characterization of regulatory T cells in humans and rodents. *Clinical and experimental immunology.* 2016;185(3):281-91.
41. Yu N, Li X, Song W, Li D, Yu D, Zeng X, et al. CD4(+)CD25 (+)CD127 (low/-) T cells: a more specific Treg population in human peripheral blood. *Inflammation.* 2012;35(6):1773-80.
42. Delacher M, Imbusch CD, Hotz-Wagenblatt A, Mallm JP, Bauer K, Simon M, et al. Precursors for Nonlymphoid-Tissue Treg Cells Reside in Secondary Lymphoid Organs and Are Programmed by the Transcription Factor BATF. *Immunity.* 2020;52(2):295-312.e11.
43. Zemmour D, Zilionis R, Kiner E, Klein AM, Mathis D, and Benoist C. Single-cell gene expression reveals a landscape of regulatory T cell phenotypes shaped by the TCR. *Nature immunology.* 2018.
44. Wedepohl S, Beceren-Braun F, Riese S, Buscher K, Enders S, Bernhard G, et al. L-selectin--a dynamic regulator of leukocyte migration. *European journal of cell biology.* 2012;91(4):257-64.
45. Fan X, Moltedo B, Mendoza A, Davydov AN, Faire MB, Mazutis L, et al. CD49b defines functionally mature Treg cells that survey skin and vascular tissues. *J Exp Med.* 2018;215(11):2796-814.
46. Lapidot T, Dar A, and Kollet O. How do stem cells find their way home? *Blood.* 2005;106(6):1901-10.
47. Lever JM, Yang Z, Boddu R, Adedoyin OO, Guo L, Joseph R, et al. Parabiosis reveals leukocyte dynamics in the kidney. *Lab Invest.* 2018;98(3):391-402.
48. Steinert EM, Schenkel JM, Fraser KA, Beura LK, Manlove LS, Igyarto BZ, et al. Quantifying Memory CD8 T Cells Reveals Regionalization of Immunosurveillance. *Cell.* 2015;161(4):737-49.
49. Huss DJ, Pellerin AF, Collette BP, Kannan AK, Peng L, Datta A, et al. Anti-CD25 monoclonal antibody Fc variants differentially impact regulatory T cells and immune homeostasis. *Immunology.* 2016;148(3):276-86.
50. Chinen T, Kannan AK, Levine AG, Fan X, Klein U, Zheng Y, et al. An essential role for the IL-2 receptor in Treg cell function. *Nature immunology.* 2016;17(11):1322-33.
51. Huss DJ, Mehta DS, Sharma A, You X, Riester KA, Sheridan JP, et al. In vivo maintenance of human regulatory T cells during CD25 blockade. *J Immunol.* 2015;194(1):84-92.
52. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, and Yamaguchi T. Regulatory T cells: how do they suppress immune responses? *International immunology.* 2009;21(10):1105-11.
53. Mendez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature.* 2010;466(7308):829-34.

54. Li H, Ghazanfari R, Zacharaki D, Ditzel N, Isern J, Ekblom M, et al. Low/negative expression of PDGFR-alpha identifies the candidate primary mesenchymal stromal cells in adult human bone marrow. *Stem cell reports.* 2014;3(6):965-74.
55. Beerman I, Luis TC, Singbrant S, Lo Celso C, and Mendez-Ferrer S. The evolving view of the hematopoietic stem cell niche. *Experimental hematology.* 2017;50:22-6.
56. Massberg S, and von Andrian UH. Novel trafficking routes for hematopoietic stem and progenitor cells. *Annals of the New York Academy of Sciences.* 2009;1176:87-93.
57. Halin C, Scimone ML, Bonasio R, Gauguet JM, Mempel TR, Quackenbush E, et al. The S1P-analog FTY720 differentially modulates T-cell homing via HEV: T-cell-expressed S1P1 amplifies integrin activation in peripheral lymph nodes but not in Peyer patches. *Blood.* 2005;106(4):1314-22.
58. Eken A, Duhen R, Singh AK, Fry M, Buckner JH, Kita M, et al. S1P1 deletion differentially affects TH17 and Regulatory T cells. *Scientific reports.* 2017;7(1):12905.
59. Janczewska S, Ziolkowska A, Durlik M, Cybulska E, Olszewski WL, and Lukomska B. Requirement of stromal cells in the bone marrow transplant for rapid lymphoid replenishment. *Transplantation proceedings.* 1999;31(1-2):696-9.
60. Squillaro T, Peluso G, and Galderisi U. Clinical Trials With Mesenchymal Stem Cells: An Update. *Cell transplantation.* 2016;25(5):829-48.
61. Abbuehl JP, Tatarova Z, Held W, and Huelsken J. Long-Term Engraftment of Primary Bone Marrow Stromal Cells Repairs Niche Damage and Improves Hematopoietic Stem Cell Transplantation. *Cell Stem Cell.* 2017;21(2):241-55 e6.
62. Crippa S, and Bernardo ME. Mesenchymal Stromal Cells: Role in the BM Niche and in the Support of Hematopoietic Stem Cell Transplantation. *HemaSphere.* 2018;2(6):e151.
63. Czechowicz A, Kraft D, Weissman IL, and Bhattacharya D. Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches. *Science.* 2007;318(5854):1296-9.
64. Si S, Nakajima-Takagi Y, Iga T, Tsuji M, Hou L, Oshima M, et al. Hematopoietic insults damage bone marrow niche by activating p53 in vascular endothelial cells. *Experimental hematology.* 2018;63:41-51.e1.
65. Asada N, Takeishi S, and Frenette PS. Complexity of bone marrow hematopoietic stem cell niche. *International journal of hematology.* 2017;106(1):45-54.
66. Ludin A, Gur-Cohen S, Golan K, Kaufmann KB, Itkin T, Medaglia C, et al. Reactive oxygen species regulate hematopoietic stem cell self-renewal, migration and development, as well as their bone marrow microenvironment. *Antioxidants & redox signaling.* 2014;21(11):1605-19.
67. Pietras EM, Reynaud D, Kang YA, Carlin D, Calero-Nieto FJ, Leavitt AD, et al. Functionally Distinct Subsets of Lineage-Biased Multipotent Progenitors Control Blood Production in Normal and Regenerative Conditions. *Cell Stem Cell.* 2015;17(1):35-46.
68. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, and Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell.* 2005;121(7):1109-21.
69. Lin KK, Rossi L, Boles NC, Hall BE, George TC, and Goodell MA. CD81 is essential for the re-entry of hematopoietic stem cells to quiescence following stress-induced proliferation via deactivation of the Akt pathway. *PLoS biology.* 2011;9(9):e1001148.

70. Chambers SM, Shaw CA, Gatza C, Fisk CJ, Donehower LA, and Goodell MA. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. *PLoS biology.* 2007;5(8):e201.
71. Sawen P, Lang S, Mandal P, Rossi DJ, Soneji S, and Bryder D. Mitotic History Reveals Distinct Stem Cell Populations and Their Contributions to Hematopoiesis. *Cell reports.* 2016;14(12):2809-18.
72. Wohrer S, Knapp DJ, Copley MR, Benz C, Kent DG, Rowe K, et al. Distinct stromal cell factor combinations can separately control hematopoietic stem cell survival, proliferation, and self-renewal. *Cell Rep.* 2014;7(6):1956-67.
73. Piazzon MC, Lutfalla G, and Forlenza M. IL10, A Tale of an Evolutionarily Conserved Cytokine across Vertebrates. *Crit Rev Immunol.* 2016;36(2):99-129.
74. Trifunovic J, Miller L, Debeljak Z, and Horvat V. Pathologic patterns of interleukin 10 expression--a review. *Biochemia medica.* 2015;25(1):36-48.
75. Gabryšová L, Howes A, Saraiva M, and O'Garra A. The regulation of IL-10 expression. *Curr Top Microbiol Immunol.* 2014;380:157-90.
76. Landuyt AE, Klocke BJ, Colvin TB, Schoeb TR, and Maynard CL. Cutting Edge: ICOS-Deficient Regulatory T Cells Display Normal Induction of Il10 but Readily Downregulate Expression of Foxp3. *J Immunol.* 2019;202(4):1039-44.
77. Maynard CL, Harrington LE, Janowski KM, Oliver JR, Zindl CL, Rudensky AY, et al. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nature immunology.* 2007;8(9):931-41.
78. Wolock SL, Krishnan I, Tenen DE, Matkins V, Camacho V, Patel S, et al. Mapping distinct bone marrow niche populations and their differentiation paths. *Cell reports.* 2019.
79. Helbling PM, Pineiro-Yanez E, Gerosa R, Boettcher S, Al-Shahrour F, Manz MG, et al. Global Transcriptomic Profiling of the Bone Marrow Stromal Microenvironment during Postnatal Development, Aging, and Inflammation. *Cell Rep.* 2019;29(10):3313-30 e4.
80. Rodda SJ, and McMahon AP. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development.* 2006;133(16):3231-44.
81. Eguchi J, Wang X, Yu S, Kershaw EE, Chiu PC, Dushay J, et al. Transcriptional control of adipose lipid handling by IRF4. *Cell Metab.* 2011;13(3):249-59.
82. Arthur A, Cakouros D, Cooper L, Nguyen T, Isenmann S, Zannettino AC, et al. Twist-1 Enhances Bone Marrow Mesenchymal Stromal Cell Support of Hematopoiesis by Modulating CXCL12 Expression. *Stem cells (Dayton, Ohio).* 2016;34(2):504-9.
83. Anthony BA, and Link DC. Regulation of hematopoietic stem cells by bone marrow stromal cells. *Trends in immunology.* 2014;35(1):32-7.
84. Zhou BO, Yue R, Murphy MM, Peyer JG, and Morrison SJ. Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell.* 2014;15(2):154-68.
85. Yue R, Zhou BO, Shimada IS, Zhao Z, and Morrison SJ. Leptin Receptor Promotes Adipogenesis and Reduces Osteogenesis by Regulating Mesenchymal Stromal Cells in Adult Bone Marrow. *Cell stem cell.* 2016;18(6):782-96.
86. Schmaler M, Broggi MA, Lagarde N, Stocklin BF, King CG, Finke D, et al. IL-7R signaling in regulatory T cells maintains peripheral and allograft tolerance in mice. *Proceedings of the National Academy of Sciences of the United States of America.* 2015;112(43):13330-5.

87. Bradley LM, Haynes L, and Swain SL. IL-7: maintaining T-cell memory and achieving homeostasis. *Trends in immunology.* 2005;26(3):172-6.
88. Santagata S, Napolitano M, D'Alterio C, Desicato S, Maro SD, Marinelli L, et al. Targeting CXCR4 reverts the suppressive activity of T-regulatory cells in renal cancer. *Oncotarget.* 2017;8(44):77110-20.
89. Kim J, Lahl K, Hori S, Loddenkemper C, Chaudhry A, deRoos P, et al. Cutting edge: depletion of Foxp3+ cells leads to induction of autoimmunity by specific ablation of regulatory T cells in genetically targeted mice. *J Immunol.* 2009;183(12):7631-4.
90. Christiaansen AF, Boggiatto PM, and Varga SM. Limitations of Foxp3(+) Treg depletion following viral infection in DEREG mice. *Journal of immunological methods.* 2014;406:58-65.
91. Nystrom SN, Bourges D, Garry S, Ross EM, van Driel IR, and Gleeson PA. Transient Treg-cell depletion in adult mice results in persistent self-reactive CD4(+) T-cell responses. *European journal of immunology.* 2014;44(12):3621-31.
92. Gillard GO, Saenz SA, Huss DJ, and Fontenot JD. Circulating innate lymphoid cells are unchanged in response to DAC HYP therapy. *Journal of neuroimmunology.* 2016;294:41-5.
93. Zhang Q, Fu L, Liang Y, Guo Z, Wang L, Ma C, et al. Exosomes originating from MSCs stimulated with TGF-beta and IFN-gamma promote Treg differentiation. *Journal of cellular physiology.* 2018;233(9):6832-40.
94. Lee HJ, Kim SN, Jeon MS, Yi T, and Song SU. ICOSL expression in human bone marrow-derived mesenchymal stem cells promotes induction of regulatory T cells. *Scientific reports.* 2017;7:44486.
95. De Miguel MP, Fuentes-Julian S, Blazquez-Martinez A, Pascual CY, Aller MA, Arias J, et al. Immunosuppressive properties of mesenchymal stem cells: advances and applications. *Current molecular medicine.* 2012;12(5):574-91.
96. Khosravi M, Bidmeshkipour A, Moravej A, Hojjat-Assari S, Naserian S, and Karimi MH. Induction of CD4(+)CD25(+)Foxp3(+) regulatory T cells by mesenchymal stem cells is associated with RUNX complex factors. *Immunologic research.* 2017.
97. Chen C, Liang J, Yao G, Chen H, Shi B, Zhang Z, et al. Mesenchymal stem cells upregulate Treg cells via sHLA-G in SLE patients. *International immunopharmacology.* 2017;44:234-41.
98. Tang RJ, Shen SN, Zhao XY, Nie YZ, Xu YJ, Ren J, et al. Mesenchymal stem cells-regulated Treg cells suppress colitis-associated colorectal cancer. *Stem cell research & therapy.* 2015;6:71.
99. Wang D, Huang S, Yuan X, Liang J, Xu R, Yao G, et al. The regulation of the Treg/Th17 balance by mesenchymal stem cells in human systemic lupus erythematosus. *Cellular & molecular immunology.* 2017;14(5):423-31.
100. Rashedi I, Gomez-Aristizabal A, Wang XH, Viswanathan S, and Keating A. TLR3 or TLR4 Activation Enhances Mesenchymal Stromal Cell-Mediated Treg Induction via Notch Signaling. *Stem cells (Dayton, Ohio).* 2017;35(1):265-75.
101. Rutz S, and Ouyang W. Regulation of Interleukin-10 Expression. *Advances in experimental medicine and biology.* 2016;941:89-116.
102. Wang Y, Chen Q, Zhang Z, Jiang F, Meng X, and Yan H. Interleukin-10 overexpression improves the function of endothelial progenitor cells stimulated with TNF-alpha through the activation of the STAT3 signaling pathway. *Int J Mol Med.* 2015;35(2):471-7.

103. George JF, Lever JM, and Agarwal A. Mononuclear phagocyte subpopulations in the mouse kidney. *American journal of physiology Renal physiology.* 2017;312(4):F640-f6.

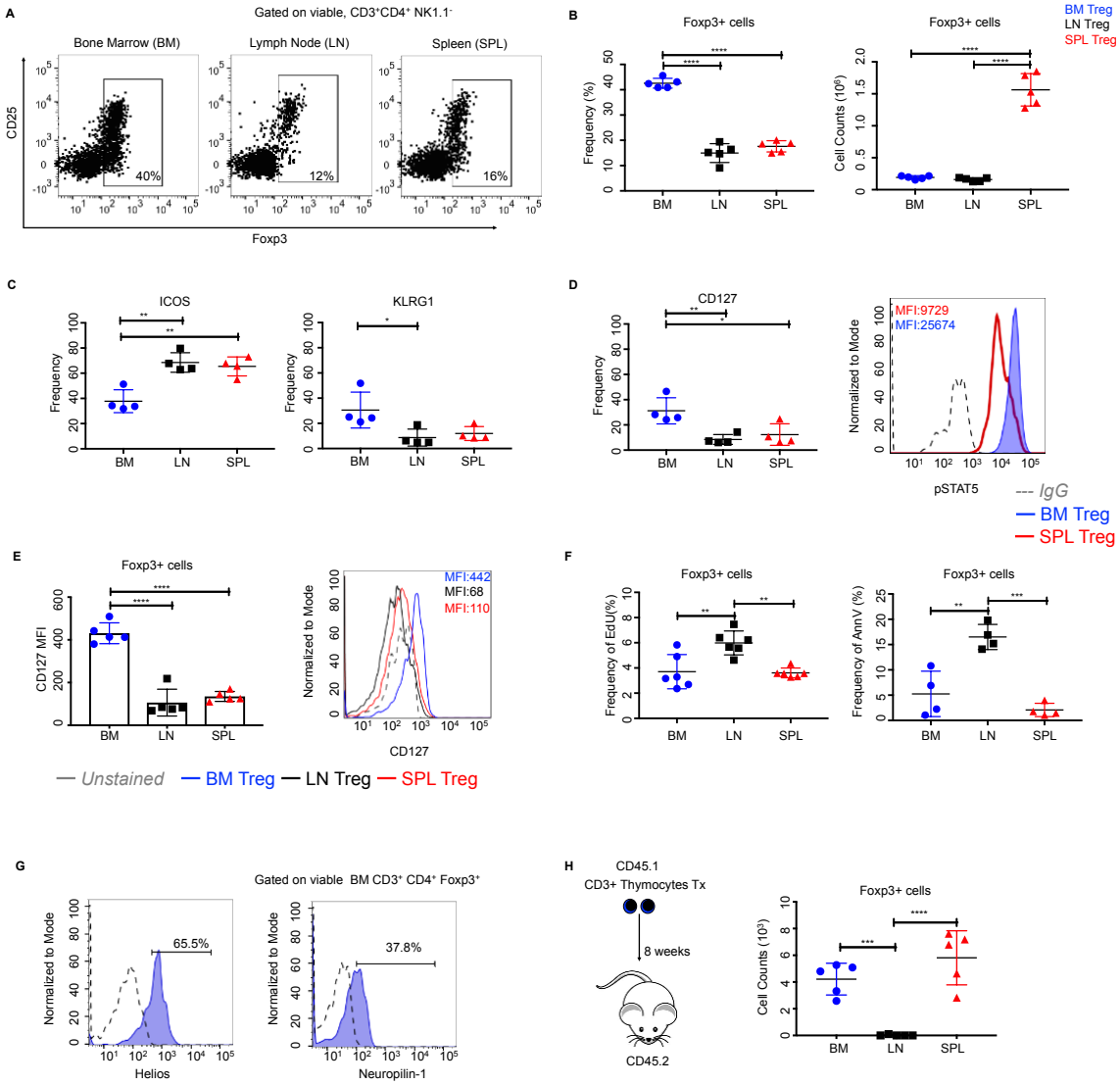

A
Gated on viable, CD3+CD4+ NK1.1-
Bone Marrow (BM)
Lymph Node (LN)
Spleen (SPL)
CD25
Foxp3
40%
12%
16%
B
Foxp3+ cells
Frequency (%)
Cell Counts (10^6)
BM Treg
LN Treg
SPL Treg
C
ICOS
KLRG1
Frequency
D
CD127
Normalized to Mode
MFI:9729
MFI:25674
pSTAT5
--- IgG
BM Treg
SPL Treg
E
Foxp3+ cells
CD127 MFI
MFI:442
MFI:68
MFI:110
CD127
Unstained
BM Treg
LN Treg
SPL Treg
F
Foxp3+ cells
Frequency of EdU(%)
Frequency of AnnV (%)
G
Gated on viable BM CD3+ CD4+ Foxp3+
65.5%
37.8%
Helios
Neuropilin-1
H
CD45.1
CD3+ Thymocytes Tx
8 weeks
CD45.2
Foxp3+ cells
Cell Counts (10^3)
BM
LN
SPL

Figure 1: Profiling of bone marrow Tregs

A: Representative plots of Foxp3$^+$ cells.
B. Frequencies and absolute counts of Foxp3$^+$cells; n=5 animals.
C. Frequencies of ICOS$^+$, KLRG1$^+$, CD127$^+$, CD25$^+$; n=5 animals.
D. Frequency of CD127; MFI of STAT5 phosphorylation; n=5 animals.
E. Histogram and MFI of CD127 in; n=5 animals.
F. Frequency of EdU$^+$ cells; n=6 and AnnexinV$^+$ cells; n=4 animals.
G. Histograms of Helios and Neuropilin-1.
H. Thymocyte transplant assay. Counts of Foxp3$^+$cells 8-weeks post transplantation; n=5 animals.
Data are shown as mean ± SD; graphs represent data from at least three independent experiments. Statistics performed with one-way ANOVA with Tukey's multiple comparisons test at 95.00% CI of diff; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

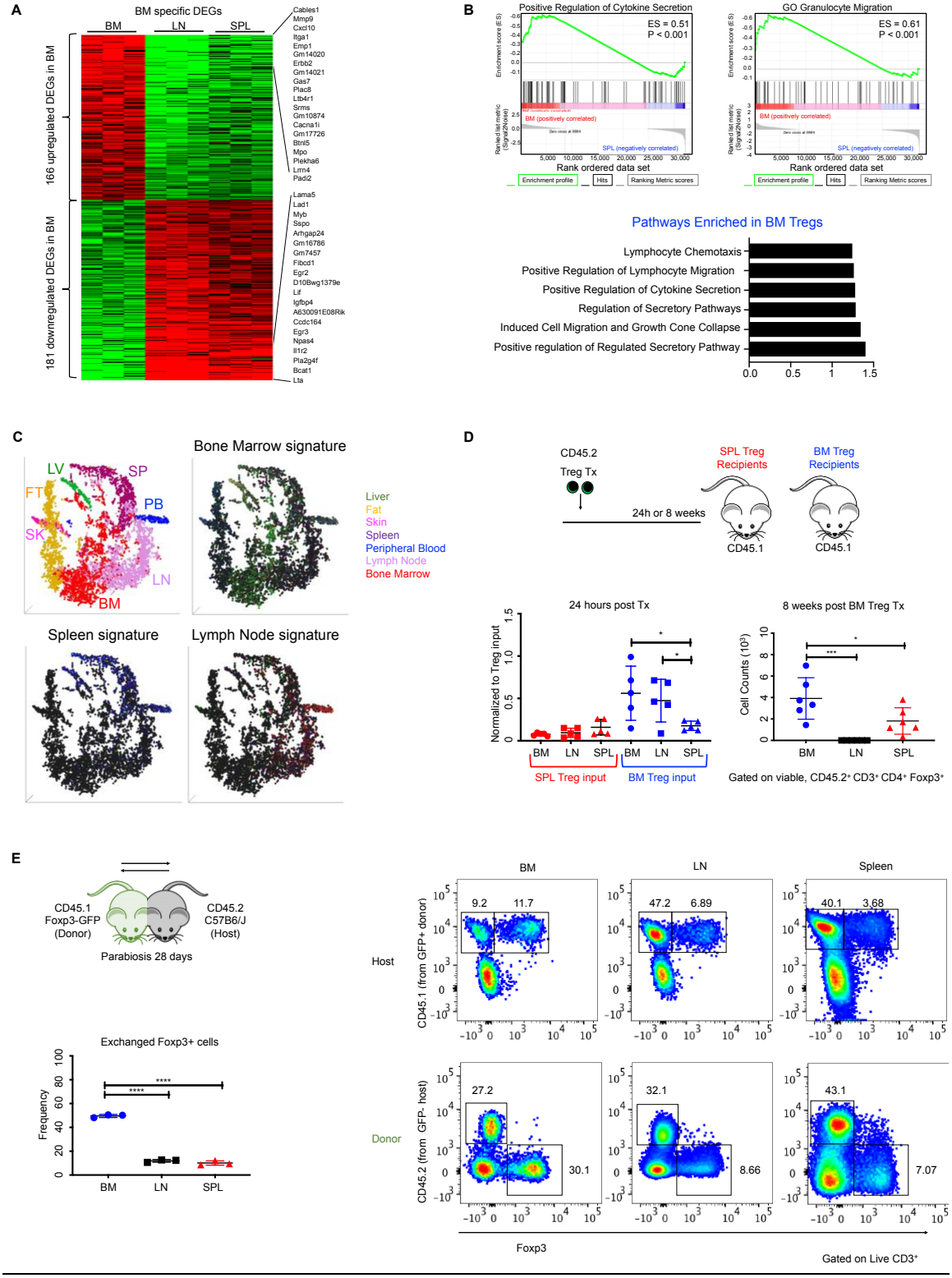

A
BM specific DEGs
BM
LN
SPL
166 upregulated DEGs in BM
181 downregulated DEGs in BM
Cables1
Mmp9
Cxcl10
Itga1
Emp1
Gm14020
Erbb2
Gm14021
Gas7
Plac8
Ltb4r1
Srms
Gm10874
Cacna1i
Gm17726
Btnl5
Mpo
Plekha6
Lrrn4
Padi2
Lama5
Lad1
Myb
Sspo
Arhgap24
Gm16786
Gm7457
Fibcd1
Egr2
D10Bwg1379e
Lif
Igfbp4
A630091E08Rik
Ccdc164
Egr3
Npas4
Il1r2
Pla2g4f
Bcat1
Lta
B
Positive Regulation of Cytokine Secretion
ES = 0.51
P < 0.001
GO Granulocyte Migration
ES = 0.61
P < 0.001
BM (positively correlated)
SPL (negatively correlated)
Rank ordered data set
Enrichment profile
Hits
Ranking Metric scores
Pathways Enriched in BM Tregs
Lymphocyte Chemotaxis
Positive Regulation of Lymphocyte Migration
Positive Regulation of Cytokine Secretion
Regulation of Secretory Pathways
Induced Cell Migration and Growth Cone Collapse
Positive regulation of Regulated Secretory Pathway
C
Bone Marrow signature
LV
SP
FT
PB
SK
LN
BM
Spleen signature
Lymph Node signature
Liver
Fat
Skin
Spleen
Peripheral Blood
Lymph Node
Bone Marrow
D
CD45.2
Treg Tx
24h or 8 weeks
SPL Treg Recipients
BM Treg Recipients
CD45.1
CD45.1
24 hours post Tx
8 weeks post BM Treg Tx
Normalized to Treg input
Cell Counts (10³)
BM
LN
SPL
BM
LN
SPL
SPL Treg input
BM Treg input
Gated on viable, CD45.2⁺ CD3⁺ CD4⁺ Foxp3⁺
E
CD45.1
Foxp3-GFP
(Donor)
CD45.2
C57B6/J
(Host)
Parabiosis 28 days
Exchanged Foxp3+ cells
Frequency
BM
LN
SPL
BM
LN
Spleen
Host
Donor
CD45.1 (from GFP+ donor)
CD45.2 (from GFP- host)
9.2
11.7
47.2
6.89
40.1
3.68
27.2
30.1
32.1
8.66
43.1
7.07
Foxp3
Gated on Live CD3⁺

Figure 2: Marrow Tregs localize to their site of origin

A: Heat map showing DEGs in Foxp3$^+$cells; n=3 replicates per tissue. DEG in marrow Foxp3$^+$cells. Genes with FC< 1/2 or >2, and adjusted p-value <0.05 are shown.

B. GSEA plot of marrow Foxp3$^+$cells (top), pathway analysis (bottom); n=3 replicates per sample.

C. An overlay of RNAseq DEGs into tSNE plots highlighting Tregs expressing signatures for bone marrow (green) spleen (blue) and lymph node (red).

D. Homing measure 24 hours (left) and 8 weeks post transplantation (right). Data expressed as fold-change, normalized to input (24 hours); n=5 or counts (8 weeks); n=6 recipients.

E. Frequency of exchanged Foxp3$^+$cells (left). Plots of exchanged CD3$^+$ populations (right); n=3 parabionts.

Data are shown as mean ± SD; graphs represent data from at least three independent experiments (2A-D); two independent experiments (2E). Statistics performed with two-way ANOVA (2D-left), one-way ANOVA (2D-right,2E) with Tukey's multiple comparisons test at 95.00% CI of diff; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

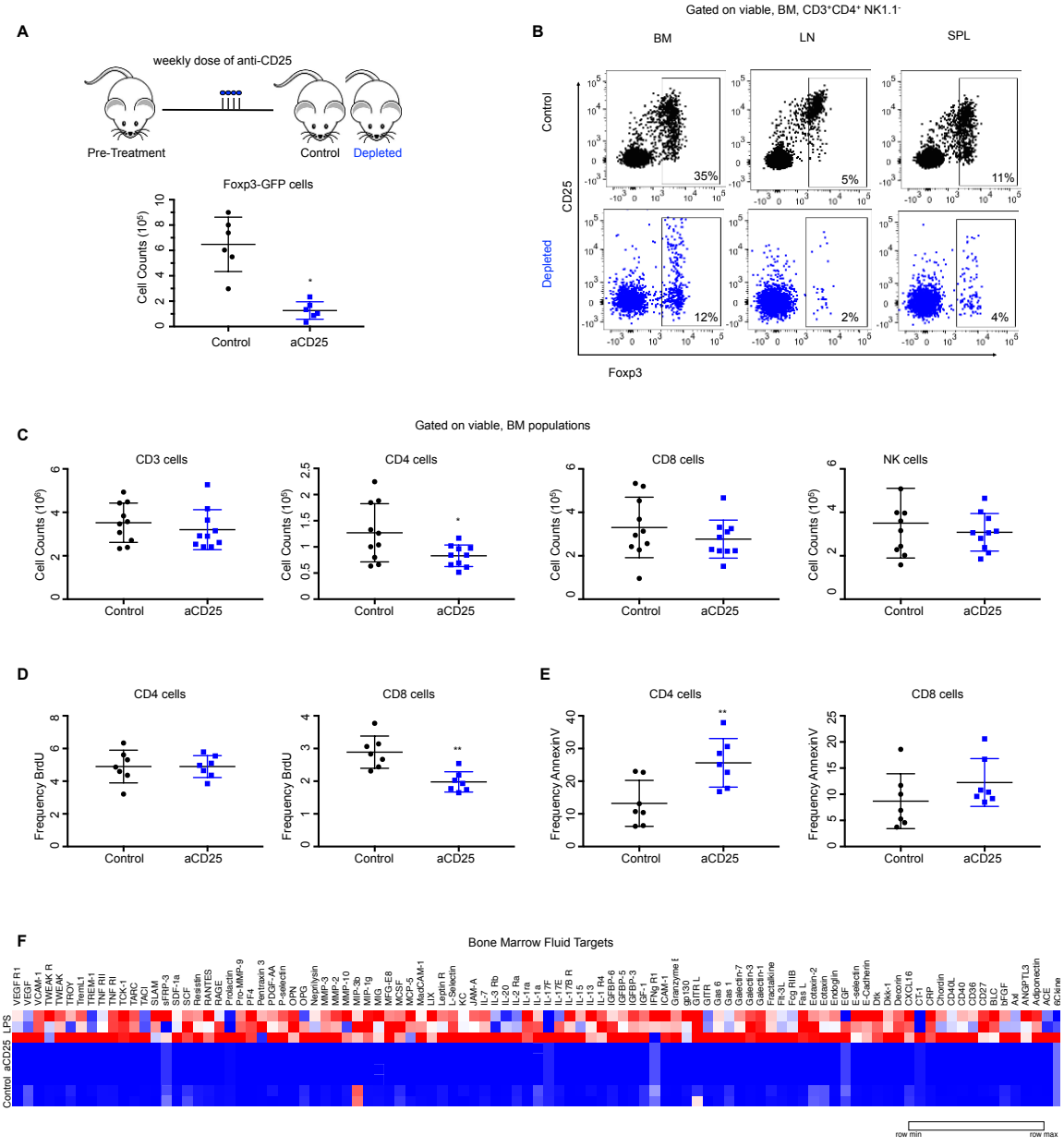

A
weekly dose of anti-CD25
Pre-Treatment
Control
Depleted
Foxp3-GFP cells
Cell Counts (10⁵)
Control
aCD25
B
Gated on viable, BM, CD3⁺CD4⁺ NK1.1⁻
BM
LN
SPL
Control
Depleted
CD25
Foxp3
35%
5%
11%
12%
2%
4%
C
Gated on viable, BM populations
CD3 cells
CD4 cells
CD8 cells
NK cells
Cell Counts (10⁶)
Cell Counts (10⁵)
Control
aCD25
D
CD4 cells
CD8 cells
Frequency BrdU
E
CD4 cells
CD8 cells
Frequency Annexin V
F
Bone Marrow Fluid Targets
LPS
aCD25
Control
row min
row max

Figure 3: Treg depletion does not result in inflammation

A. Counts of Foxp3$^+$cells; n=6 animals per group.
B. Plot of Foxp3$^+$cells following anti-CD25.
C. Counts of marrow CD3$^+$, CD4$^+$, NK1.1$^+$ and CD8$^+$ cells; n=10 animals per group.
D. Frequency of BrdU$^+$ CD4$^+$ and CD8$^+$ cells; n=7 animals per group.
E. Frequency of AnnexinV$^+$ CD4$^+$ and CD8$^+$ cells; n=7 animals per group.
F. Heatmap of top differentially expressed cytokines in marrow fluid; n=3 replicates per condition. Differentially expressed cytokines defined with FC< .5 or >.5 and adjusted p-value <0.05.
Data are shown as mean ± SD; graphs represent data from at least three independent experiments. Statistics performed with unpaired two-tailed Student *t* test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

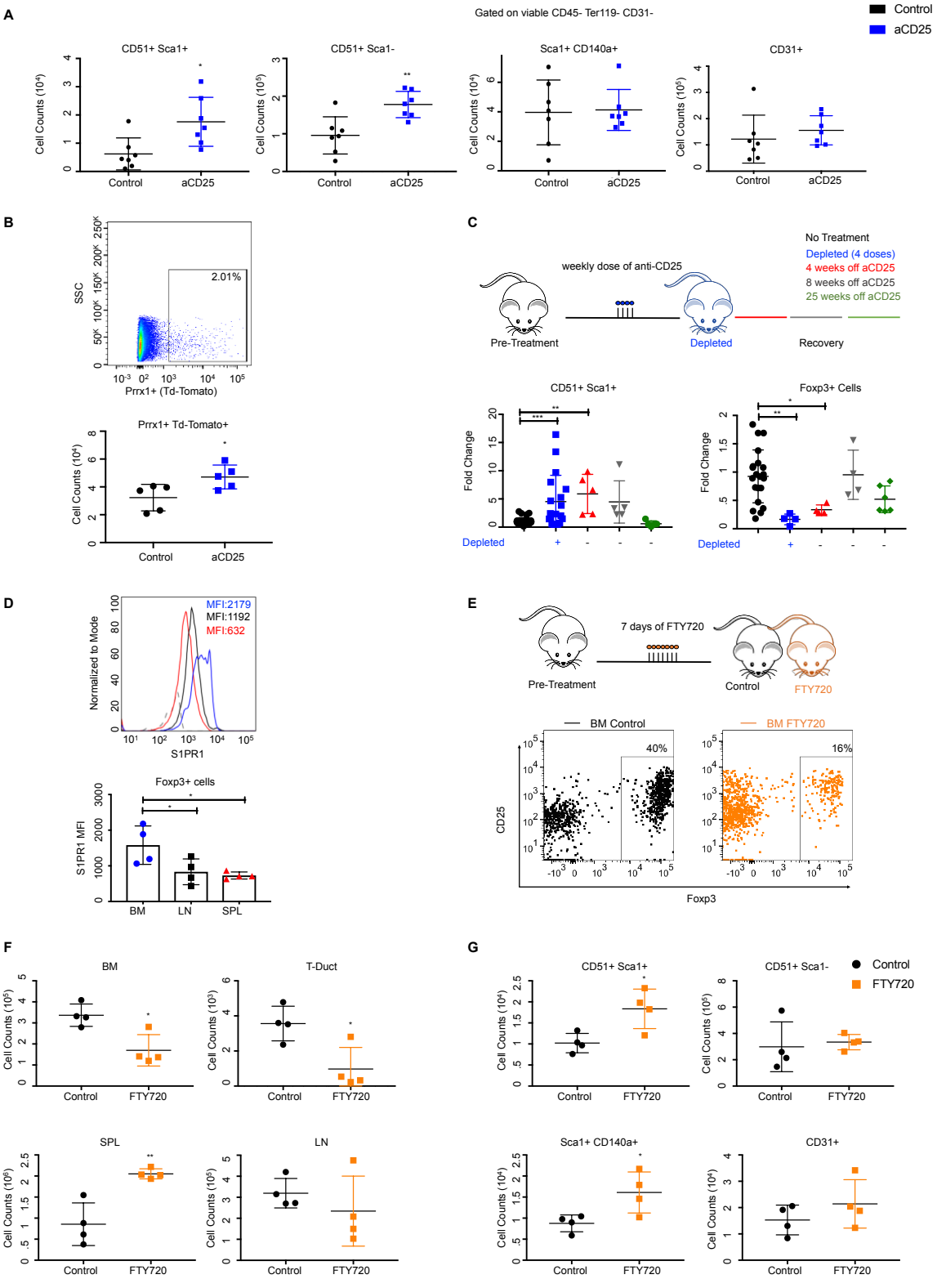

A
Gated on viable CD45- Ter119- CD31-
Control
aCD25
CD51+ Sca1+
CD51+ Sca1-
Sca1+ CD140a+
CD31+
Cell Counts (10⁴)
Cell Counts (10⁵)
Control
aCD25
B
SSC
2.01%
Prrx1+ (Td-Tomato)
Prrx1+ Td-Tomato+
C
No Treatment
Depleted (4 doses)
4 weeks off aCD25
8 weeks off aCD25
25 weeks off aCD25
weekly dose of anti-CD25
Pre-Treatment
Depleted
Recovery
CD51+ Sca1+
Foxp3+ Cells
Fold Change
D
MFI:2179
MFI:1192
MFI:632
Normalized to Mode
S1PR1
Foxp3+ cells
S1PR1 MFI
BM
LN
SPL
E
7 days of FTY720
Pre-Treatment
Control
FTY720
BM Control
BM FTY720
40%
16%
CD25
Foxp3
F
BM
T-Duct
SPL
LN
Control
FTY720
G
CD51+ Sca1+
CD51+ Sca1-
Sca1+ CD140a+
CD31+
Control
FTY720

Figure 4: Tregs maintain stromal cell.
A: Counts of stromal populations following anti-CD25; n=7 animals.
B: Plot and counts of TdTomato$^+$ stromal cells; n=5 animals.
C: Recovery post anti-CD25. Quantification of MSCs and Foxp3$^+$cells at 4, 8, and 25 weeks post anti-CD25. Data expressed as fold-change relative to untreated.
D. Histogram and quantification of S1PR1 MFI in Foxp3$^+$cells; n=4 animals.
E: Representative plot of Foxp3$^+$cells post FTY720.
F. Counts of Foxp3$^+$ cells following FTY720; n=4 animals per group.
G. Counts of stromal populations following FTY720; n=4 animals.
Data are shown as mean ± SD; graphs represent data from at least three independent experiments. Statistics performed with unpaired two-tailed Student *t* test (4A-B, F-G) and one-way ANOVA with Tukey' multiple comparisons test at 95.00% CI of diff (4C-D); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

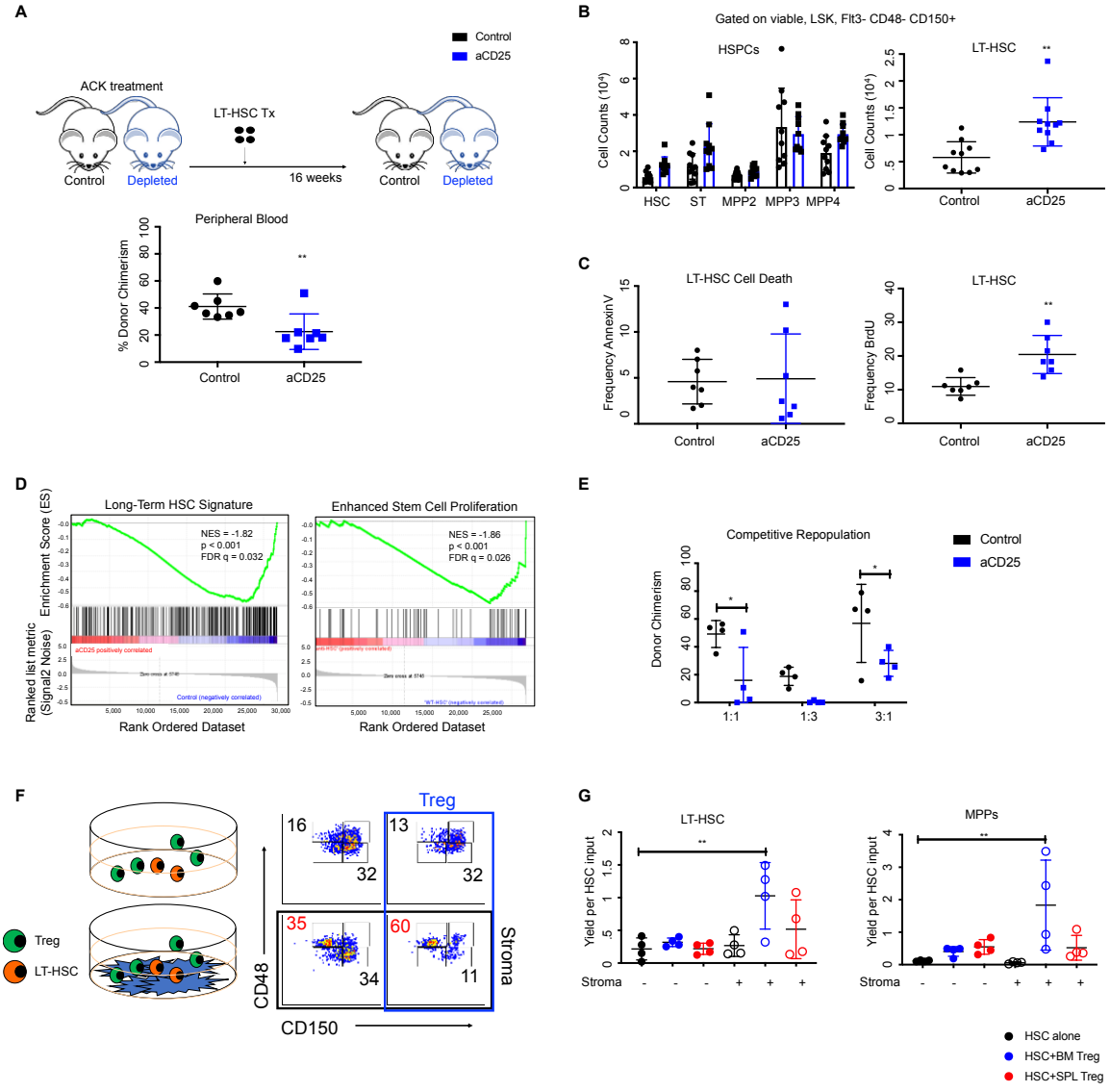

A
Control
aCD25
ACK treatment
LT-HSC Tx
16 weeks
Control
Depleted
Control
Depleted
Peripheral Blood
% Donor Chimerism
Control
aCD25
B
Gated on viable, LSK, Flt3- CD48- CD150+
HSPCs
Cell Counts (10⁴)
HSC
ST
MPP2
MPP3
MPP4
LT-HSC
Cell Counts (10⁴)
Control
aCD25
C
LT-HSC Cell Death
Frequency AnnexinV
Control
aCD25
LT-HSC
Frequency BrdU
Control
aCD25
D
Long-Term HSC Signature
NES = -1.82
p < 0.001
FDR q = 0.032
Enhanced Stem Cell Proliferation
NES = -1.86
p < 0.001
FDR q = 0.026
Enrichment Score (ES)
Ranked list metric (Signal2 Noise)
Rank Ordered Dataset
Rank Ordered Dataset
E
Competitive Repopulation
Control
aCD25
Donor Chimerism
1:1
1:3
3:1
F
Treg
LT-HSC
Treg
16
13
32
32
35
60
34
11
Stroma
CD48
CD150
G
LT-HSC
Yield per HSC input
Stroma
MPPs
Yield per HSC input
Stroma
HSC alone
HSC+BM Treg
HSC+SPL Treg

Figure 5: Marrow Tregs enhance HSC-supportive activity of stromal cells

A: Clearance via ACK2 treatment and peripheral blood chimerism; n=10 animals.
B: Counts of HSPCs following anti-CD25; n=10 animals.
C. Frequency of AnnexinV$^+$ and BrdU$^+$ LT-HSC following anti-CD25; n=8 animals.
D: GSEA plot for aCD25 LT-HSCs; n=4 replicates.
E. Competitive Repopulation Assay and peripheral blood chimerism; n=4 recipients.
F: Co-culture experiment and representative flow plot of HSPC, Tregs and stromal cells.
G: Quantification of HSCs and MPPs. Data expressed as fold-change and normalized to input of HSCs; n=4 wells per condition.
Data are shown as mean ± SD; graphs represent data from at least three independent experiments. Statistics performed with unpaired two-tailed Student *t* test (5A-E); and one-way ANOVA with Tukey' multiple comparisons test at 95.00% CI of diff (5G) *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

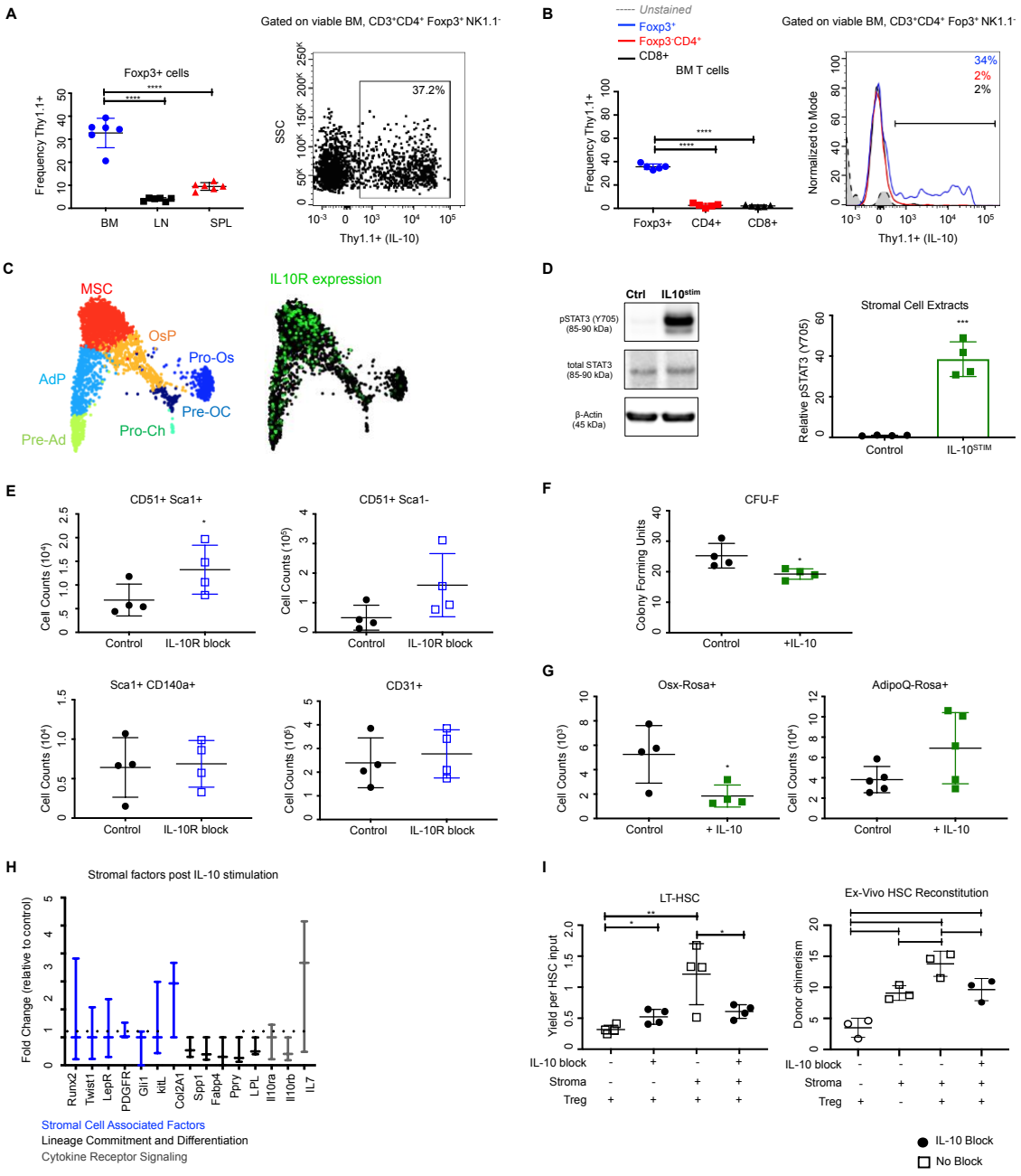

A
Foxp3+ cells
Frequency Thy1.1+
BM
LN
SPL
Gated on viable BM, CD3+CD4+ Foxp3+ NK1.1-
37.2%
SSC
Thy1.1+ (IL-10)
B
Unstained
Foxp3+
Foxp3-CD4+
CD8+
BM T cells
Foxp3+
CD4+
CD8+
Gated on viable BM, CD3+CD4+ Fop3+ NK1.1-
34%
2%
2%
Normalized to Mode
Thy1.1+ (IL-10)
C
MSC
OsP
Pro-Os
AdP
Pre-OC
Pre-Ad
Pro-Ch
IL10R expression
D
Ctrl
IL10stim
pSTAT3 (Y705) (85-90 kDa)
total STAT3 (85-90 kDa)
β-Actin (45 kDa)
Stromal Cell Extracts
Relative pSTAT3 (Y705)
Control
IL-10STIM
E
CD51+ Sca1+
CD51+ Sca1-
Cell Counts (10⁴)
Cell Counts (10⁵)
Control
IL-10R block
Sca1+ CD140a+
CD31+
F
CFU-F
Colony Forming Units
Control
+IL-10
G
Osx-Rosa+
AdipoQ-Rosa+
Cell Counts (10³)
Control
+ IL-10
H
Stromal factors post IL-10 stimulation
Fold Change (relative to control)
Runx2
Twist1
LepR
PDGFR
Gli1
kitL
Col2A1
Spp1
Fabp4
Pprγ
LPL
Il10ra
Il10rb
IL7
Stromal Cell Associated Factors
Lineage Commitment and Differentiation
Cytokine Receptor Signaling
I
LT-HSC
Yield per HSC input
IL-10 block
Stroma
Treg
Ex-Vivo HSC Reconstitution
Donor chimerism
IL-10 Block
No Block

Figure 6: Treg IL-10 restricts stromal cell proliferation and differentiation

A: Frequencies and plot of Thy1.1^{+}Foxp3^{+}cells in bone marrow (right); n=6 animals per group.

B. Frequencies and histogram of Thy1.1^{+}cells: CD4^{+} Foxp3^{+}(blue), CD3^{+} CD4^{+} Foxp3^{-}(red), CD3^{+} CD8^{+}(black); n=5 animals.

C: SPRING plots of stromal cells transcriptomes. Pre-adipocyte (pre-AD), Adipocyte progenitor (AdP), Mesenchymal stromal cell (MSC), Osteoblast/chondrocyte progenitor (OsP), Pre-osteoblast/chondrocyte (Pre-OC), Pro-osteoblast (Pro-Os), and Pro-chondrocyte (Pro-Ch). Relative abundance of IL-10Ra (green).

D. Western blot of STAT3 phosphorylation (Tyr 705) and densitometric protein analysis; n=4 biological replicates.

E. Counts of stromal populations in IL-10R^{BLOCK} mice; n=4 animals per group.

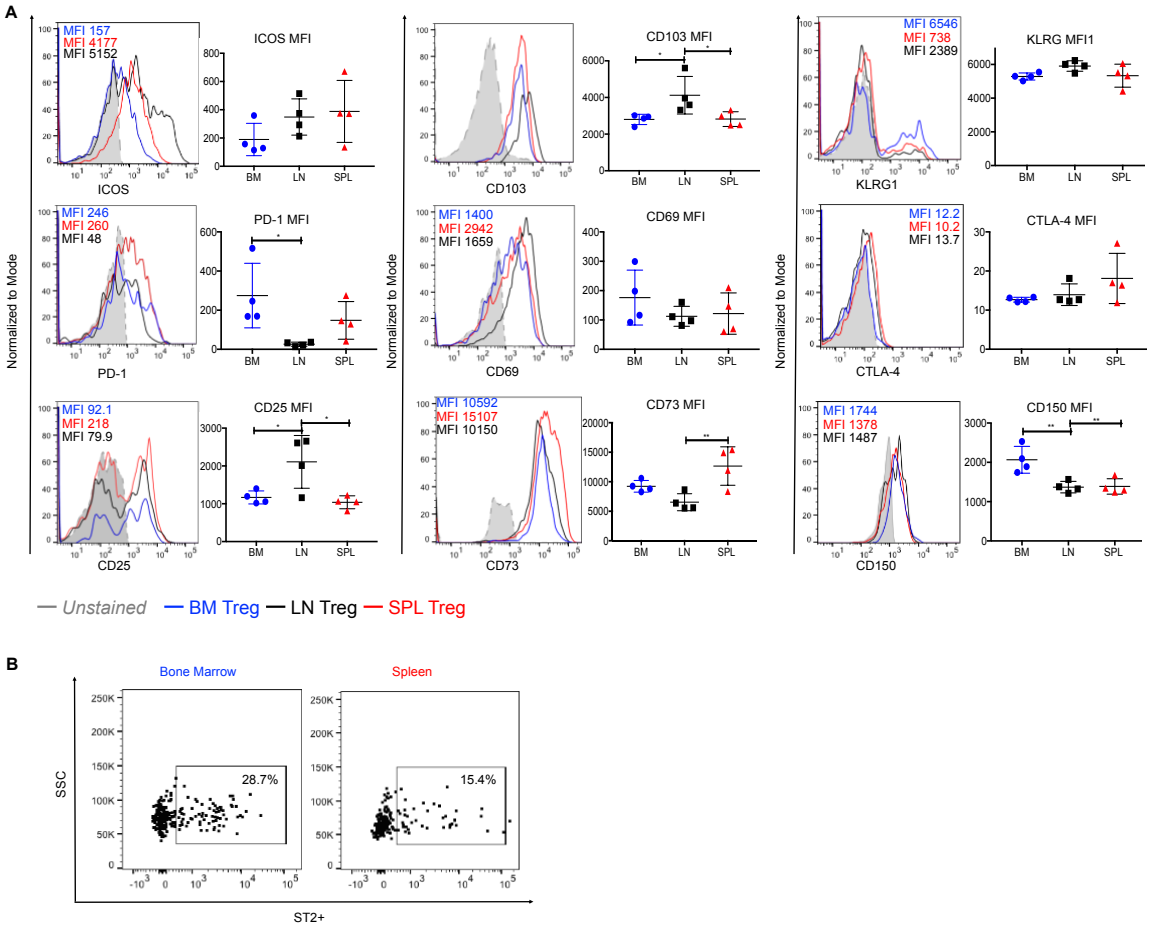
F. CFU-Fs of stromal cells following 7 days of IL-10 stimulation; n=4 wells per condition.

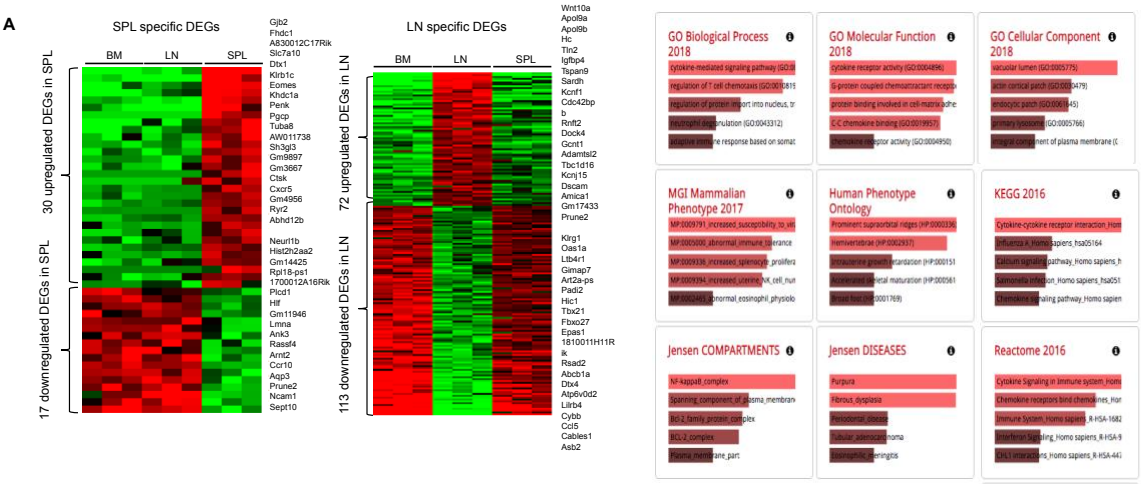
G. Counts of TdTomato+ cells from Osx-Cre and AdipoQ-Cre mice following 7 days of IL-10 stimulation; n=4 wells per condition.

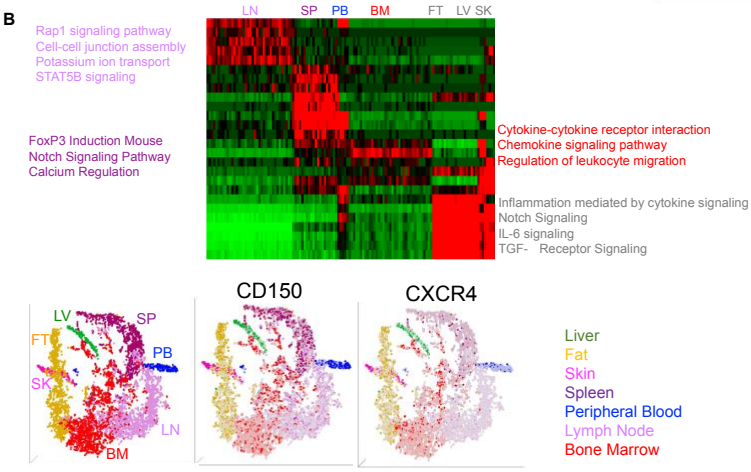
H. qRT-PCR analysis of transcripts following 7 days of IL-10 stimulation. Genes presented as fold-change (relative to control); n=3 biological replicates, each point was done in triplicate (normalized to TBP1).

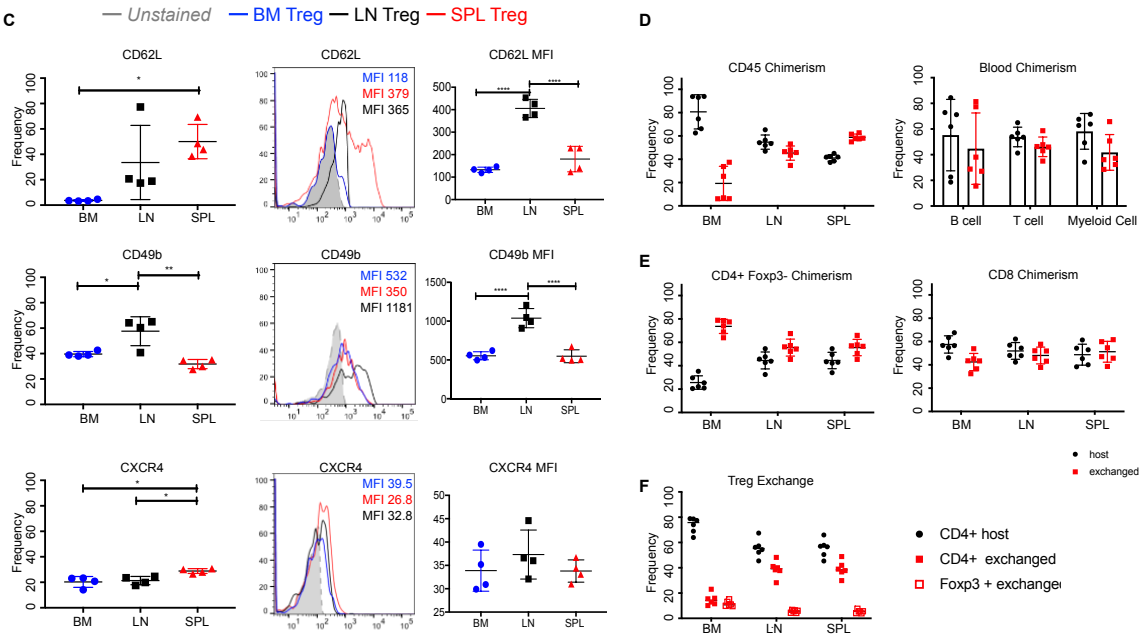
I. Co-culture of LT-HSCs, Foxp3^{+} cells, stromal cells with IL-10 blocking antibody for 96 hours; n=4 wells per condition (left). Transplantation assay of *ex-vivo* HSCs collected from individual co-culture wells after 96 hours of exposure. Peripheral blood chimerism of donor cells; n=3 recipients per group (right).

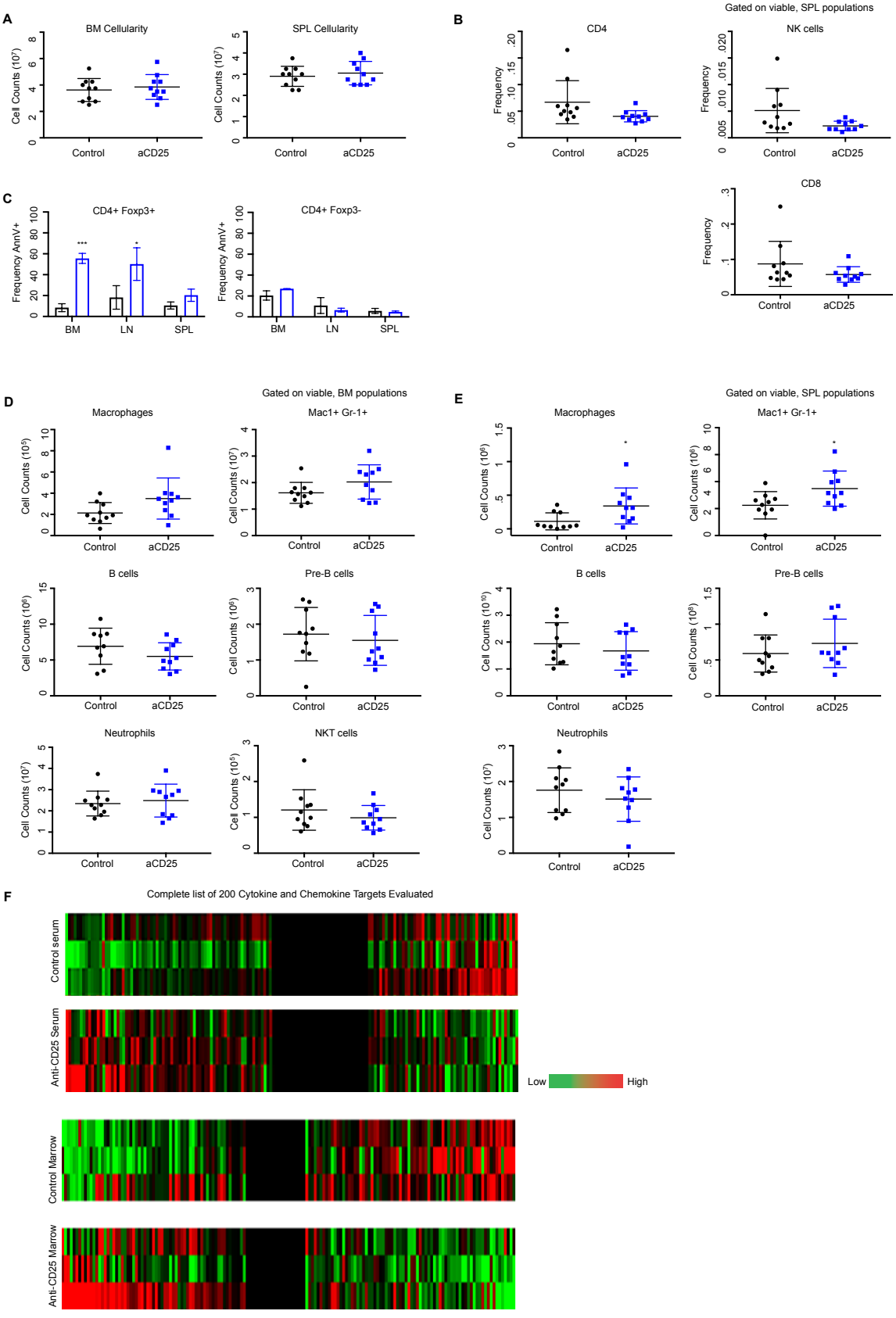
Data are shown as mean ± SD (6A-B, E-G, I) or SEM (6H); graphs represent data from at least three independent experiments Statistics performed with unpaired two-tailed Student *t* test (6E-G) and one-way ANOVA with Tukey' multiple comparisons test at 95.00% CI of diff (6A-B, I); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

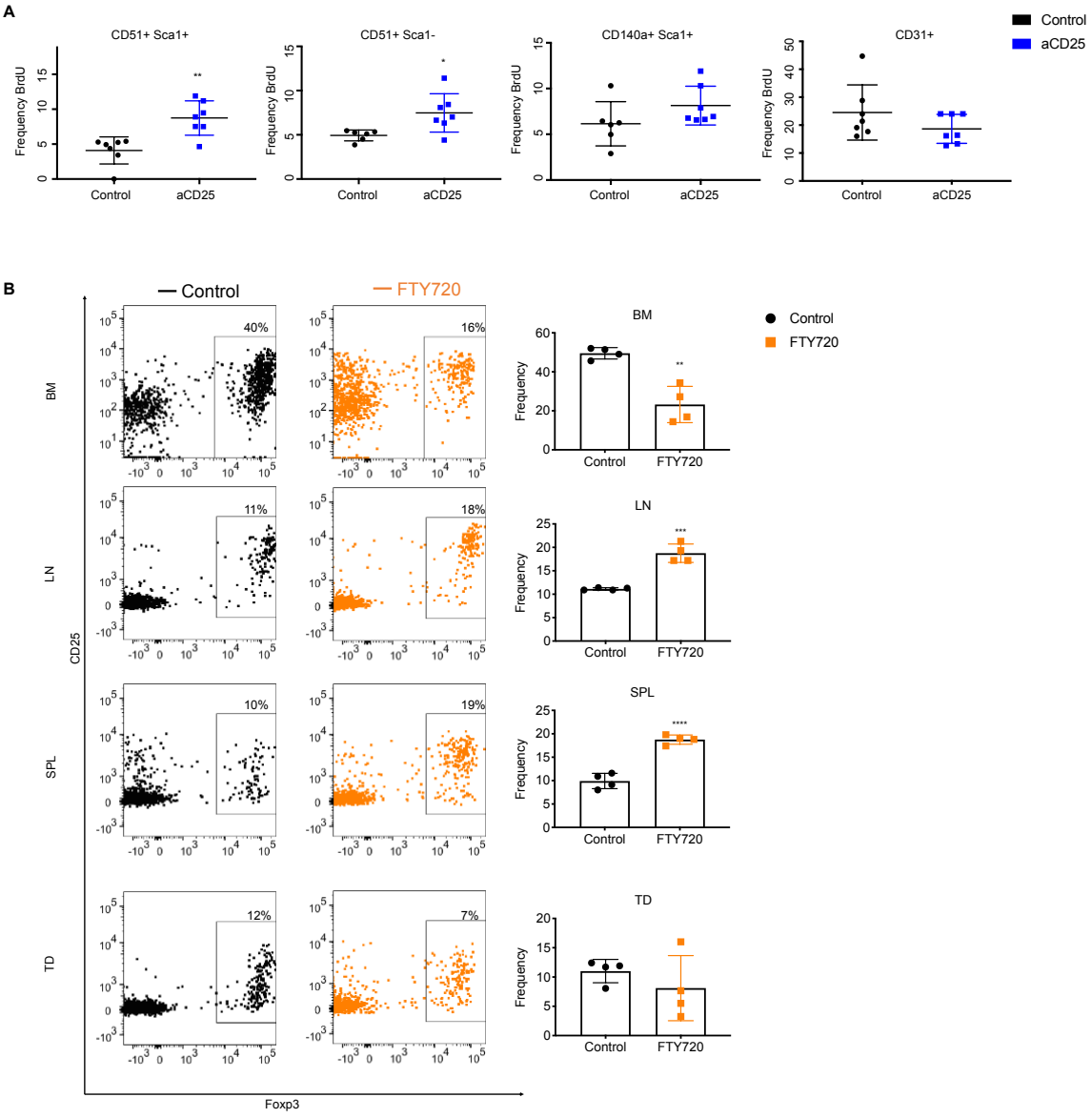

A
Normalized to Mode
MFI 157
MFI 4177
MFI 5152
ICOS
ICOS MFI
MFI 246
MFI 260
MFI 48
PD-1
PD-1 MFI
MFI 92.1
MFI 218
MFI 79.9
CD25
CD25 MFI
MFI 1400
MFI 2942
MFI 1659
CD103
CD103 MFI
CD69
CD69 MFI
MFI 10592
MFI 15107
MFI 10150
CD73
CD73 MFI
MFI 6546
MFI 738
MFI 2389
KLRG1
KLRG MFI1
MFI 12.2
MFI 10.2
MFI 13.7
CTLA-4
CTLA-4 MFI
MFI 1744
MFI 1378
MFI 1487
CD150
CD150 MFI
BM
LN
SPL
Unstained
BM Treg
LN Treg
SPL Treg
B
Bone Marrow
Spleen
SSC
ST2+
28.7%
15.4%

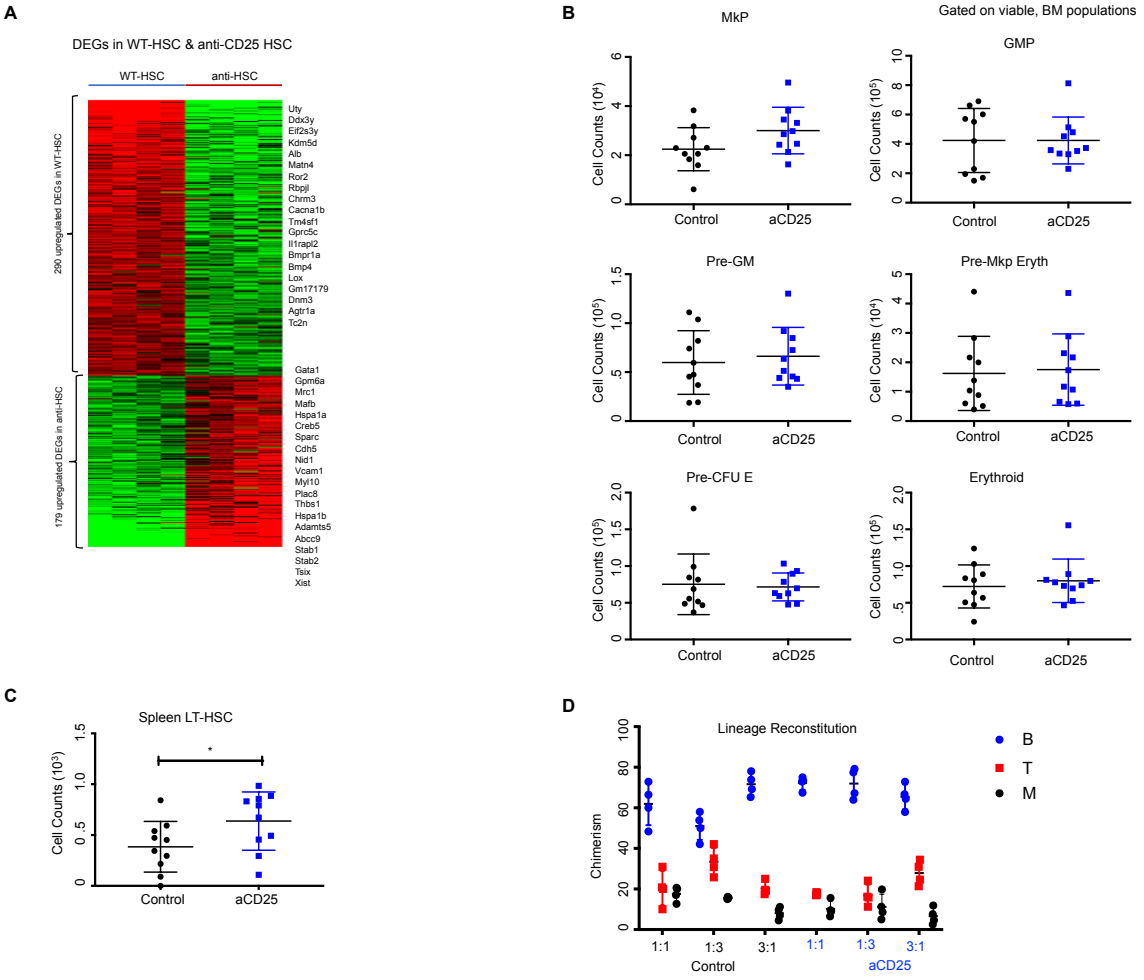

A
SPL specific DEGs
LN specific DEGs
BM
LN
SPL
30 upregulated DEGs in SPL
17 downregulated DEGs in SPL
72 upregulated DEGs in LN
113 downregulated DEGs in LN
GO Biological Process 2018
GO Molecular Function 2018
GO Cellular Component 2018
MGI Mammalian Phenotype 2017
Human Phenotype Ontology
KEGG 2016
Jensen COMPARTMENTS
Jensen DISEASES
Reactome 2016

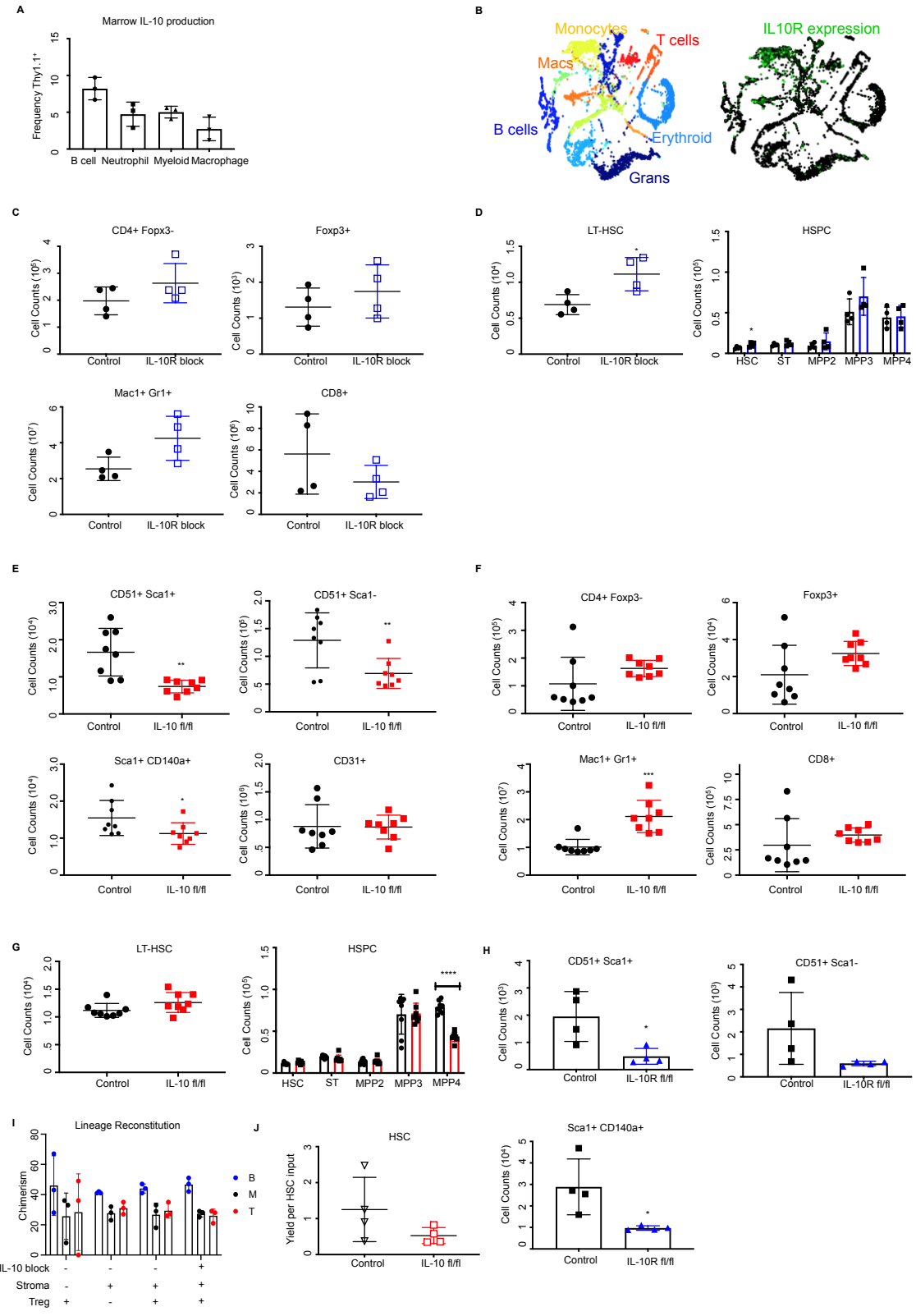

B
LN SP PB BM FT LV SK
Rap1 signaling pathway
Cell-cell junction assembly
Potassium ion transport
STAT5B signaling
FoxP3 Induction Mouse
Notch Signaling Pathway
Calcium Regulation
Cytokine-cytokine receptor interaction
Chemokine signaling pathway
Regulation of leukocyte migration
Inflammation mediated by cytokine signaling
Notch Signaling
IL-6 signaling
TGF- Receptor Signaling
CD150
CXCR4
Liver
Fat
Skin
Spleen
Peripheral Blood
Lymph Node
Bone Marrow


C
Unstained
BM Treg
LN Treg
SPL Treg
CD62L
CD62L MFI
MFI 118
MFI 379
MFI 365
CD49b
CD49b MFI
MFI 532
MFI 350
MFI 1181
CXCR4
CXCR4 MFI
MFI 39.5
MFI 26.8
MFI 32.8
Frequency
D
CD45 Chimerism
Blood Chimerism
B cell
T cell
Myeloid Cell
E
CD4+ Foxp3- Chimerism
CD8 Chimerism
host
exchanged
F
Treg Exchange
CD4+ host
CD4+ exchanged
Foxp3 + exchange


A
BM Cellularity
Cell Counts (10⁷)
Control
aCD25
SPL Cellularity
Cell Counts (10⁷)
Control
aCD25
B
CD4
Frequency
Control
aCD25
Gated on viable, SPL populations
NK cells
Frequency
Control
aCD25
CD8
Frequency
Control
aCD25
C
CD4+ Foxp3+
Frequency AnnV+
***
*
BM
LN
SPL
CD4+ Foxp3-
Frequency AnnV+
BM
LN
SPL
D
Gated on viable, BM populations
Macrophages
Cell Counts (10⁵)
Control
aCD25
Mac1+ Gr-1+
Cell Counts (10⁷)
Control
aCD25
B cells
Cell Counts (10⁶)
Control
aCD25
Pre-B cells
Cell Counts (10⁶)
Control
aCD25
Neutrophils
Cell Counts (10⁷)
Control
aCD25
NKT cells
Cell Counts (10⁵)
Control
aCD25
E
Gated on viable, SPL populations
Macrophages
Cell Counts (10⁶)
*
Control
aCD25
Mac1+ Gr-1+
Cell Counts (10⁶)
*
Control
aCD25
B cells
Cell Counts (10¹⁰)
Control
aCD25
Pre-B cells
Cell Counts (10⁸)
Control
aCD25
Neutrophils
Cell Counts (10⁷)
Control
aCD25
F
Complete list of 200 Cytokine and Chemokine Targets Evaluated
Control serum
Anti-CD25 Serum
Low
High
Control Marrow
Anti-CD25 Marrow


A
CD51+ Sca1+
CD51+ Sca1-
CD140a+ Sca1+
CD31+
Frequency BrdU
Control
aCD25
B
Control
FTY720
BM
LN
SPL
TD
CD25
Foxp3
40%
16%
11%
18%
10%
19%
12%
7%
Frequency


A
DEGs in WT-HSC & anti-CD25 HSC
WT-HSC
anti-HSC
290 upregulated DEGs in WT-HSC
179 upregulated DEGs in anti-HSC
Uty
Ddx3y
Eif2s3y
Kdm5d
Alb
Matn4
Ror2
Rbpjl
Chrm3
Cacna1b
Tm4sf1
Gprc5c
Il1rapl2
Bmpr1a
Bmp4
Lox
Gm17179
Dnm3
Agtr1a
Tc2n
Gata1
Gpm6a
Mrc1
Mafb
Hspa1a
Creb5
Sparc
Cdh5
Nid1
Vcam1
Myl10
Plac8
Thbs1
Hspa1b
Adamts5
Abcc9
Stab1
Stab2
Tsix
Xist
B
MkP
Gated on viable, BM populations
GMP
Pre-GM
Pre-Mkp Eryth
Pre-CFU E
Erythroid
Cell Counts (10⁴)
Cell Counts (10⁵)
Cell Counts (10⁶)
Control
aCD25
C
Spleen LT-HSC
Cell Counts (10³)
*
D
Lineage Reconstitution
Chimerism
B
T
M
1:1
1:3
3:1
Control
aCD25


A
Marrow IL-10 production
Frequency Thy1.1+
B cell
Neutrophil
Myeloid
Macrophage
B
Monocytes
T cells
Macs
B cells
Erythroid
Grans
IL10R expression
C
CD4+ Fopx3-
Foxp3+
Mac1+ Gr1+
CD8+
Cell Counts
Control
IL-10R block
D
LT-HSC
HSPC
HSC
ST
MPP2
MPP3
MPP4
E
CD51+ Sca1+
CD51+ Sca1-
Sca1+ CD140a+
CD31+
IL-10 fl/fl
F
CD4+ Foxp3-
Foxp3+
Mac1+ Gr1+
CD8+
G
LT-HSC
HSPC
H
CD51+ Sca1+
CD51+ Sca1-
IL-10R fl/fl
I
Lineage Reconstitution
Chimerism
B
M
T
IL-10 block
Stroma
Treg
J
HSC
Yield per HSC input
Sca1+ CD140a+

Supplement Figures

Supplementary Figure 1: Profiling of bone marrow Tregs
S1A. Histograms and quantification of MFI of ICOS, CD103, KLRG1, PD-1, CD69, CTLA-4, CD25, CD73 and CD150; n=4 animals.
S1B. Representative dot plots of $ST2^+$ cells in indicated tissues.
Data are shown as mean ± SD; graphs shown are representative of at least three independent experiments. Statistical analysis performed with one-way ANOVA with Tukey' multiple comparisons test at 95.00% CI of diff (S1C); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplementary Figure 2: Marrow Tregs localize to their site of origin.
S2A. Heatmap of selected top differentially expressed genes in $Foxp3^+$ cells from spleen and lymph node; n=3 biological replicates per tissue sample. Genes with FC with FC< 2 or >2, and adjusted p-value <0.05 are shown (right). GO analysis of enriched pathways in bone marrow $Foxp3^+$ cells; n=3 biological replicates (left).
S2B. Heatmap of top differentially expressed pathways from single-cell transcriptomes of tissue Treg populations: lymph (LN), spleen (SP), peripheral blood (PB), bone marrow (BM), fat (FT), and skin (SK) Tregs (right). Relative abundance of CD150 and CXCR4 expression among tissue Treg populations is shown (bottom).
S2C. Frequencies and representative MFI of $Foxp3^+$cells showing frequencies and representative MFI histograms of CD62L, CD49b, and CXCR4 from in indicated tissues and unstained controls.
S2D: Total chimerism in bone marrow, lymph node, and spleen of parabionts (left). Total chimerism of B cell, T cell, and Myeloid cell lineages in peripheral blood (right).
S2E: Total chimerism of $CD4^+$ (left) and $CD8^+$ cells (right).
S2F: Frequency of Treg exchange among $CD4^+$ cells.
Data are shown as mean ± SD; graphs shown are representative of at least three independent experiments. Statistical analysis performed with one-way ANOVA with Tukey' multiple comparisons test at 95.00% CI of diff (S2C); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplementary Figure 3: Treg depletion does not result in inflammation
S3A: Counts of bone marrow and spleen cellularity following anti-CD25; n=10 animals per group for each tissue.
S3B: Frequencies of $CD4^+$, $CD8^+$ and $NK1.1^+$ cells following anti-CD25; n=10 animals.
S3C. Counts of $Foxp3^+CD4^+$ (grey) and $Foxp3^-CD4^+$ (black) following anti-CD25 (right). Frequency of $AnnexinV^+$ cells in $Foxp3^+CD4^+$ and $Foxp3^-CD4^+$ cells following anti-CD25; n=4 animals.
S3D. Counts of bone marrow populations following anti-CD25: Macrophages, Myeloid cells, B cells, Neutrophils, and NKT cells; n=10 animals.
S3E. Counts of splenic populations following anti-CD25: Macrophages, Myeloid cells, B cells, Neutrophils; n=10 animals.
F. Complete target list of serum and bone marrow fluid cytokines and chemokines following anti-CD25 depletion (ordered based on fold change).
Data are shown as mean ± SD; graphs shown are representative of at least three independent experiments. Statistical analysis performed with unpaired two-tailed Student *t* test S3A-E). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplementary Figure 4: Tregs are required for stromal cell maintenance
S4A. Frequency of $BrdU^+$ cells: MSCs, bone precursors, $PDGFR\alpha^+$ cells, endothelial cells following anti-CD25;; n=7 animals per group.
S4B: Representative plot of $Foxp3^+$ cells in bone marrow, lymph node, spleen and thoracic duct (right); frequencies of $Foxp3^+$ cells (left); n=4 animals per group. Data are shown as mean ± SD; graphs shown are representative of at least three independent experiments. Statistical analysis performed with unpaired two-tailed Student *t* test (S4A-B)*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplementary Figure 5: Bone marrow Tregs enhance HSC-supportive activity of stromal cells
S5A. Heatmap of top differentially expressed genes in LT-HSC's from control and anti-CD25 mice; n=4 biological replicates per treatment condition. Genes with FC< 1/2 or >2, and adjusted p-value <0.05 are shown.
S5B. Counts of bone marrow MkP, GMP, pre-GM, Pre Mkp Eryth, Pre-CFU-E, Erythroid following anti-CD25; n=10 animals per group.
S5C. Counts of splenic LT-HSC following anti-CD25; n=10 animals.
S5D. Multi-lineage engraftment of B cell (blue); T cell (red) and Myeloid cell (black) lineages; n=4 animals.
Data are shown as mean ± SD; graphs shown are representative of at least three independent experiments. Statistical analysis performed with unpaired two-tailed Student *t* test (4A-C) *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplementary Figure 6: Treg IL-10 restricts stromal cell proliferation and differentiation
S6A: Quantification of IL-10-expressing ($Thy1.1^+$) cells in B cell, Neutrophil, Myeloid cells, Macrophages; n=3 animals.
S6B: SPRING plots of single-cell transcriptomes of mature bone marrow populations: B cell, Neutrophil, Myeloid cells, Macrophages (right). Relative abundance of IL-10Ra expression shown in green (left).
S6C. Counts of $Foxp3^-$ $CD4^+$, $Foxp3^+CD4^+$, $CD8^+$, and $Mac1^+Gr1^+$ cells in $IL\text{-}10R^{BLOCK}$ mice; n=4.
S6D. Counts of HSPCs in $IL\text{-}10R^{BLOCK}$ mice; n=4 animals per group.
S6E. Counts of $CD51^+$ $Sca1^+$cells (MSCs), $CD51^+Sca1^-$(bone precursors), and $CD140\alpha^+Sca\text{-}1^+$ ($PDGFR\alpha^+$ cells), endothelial cells ($CD45^-/Ter119^-CD31^+$) in Foxp3-Cre×$IL\text{-}10^{fl/fl}$ mice; n=8 animals.
S6F. Counts of $Foxp3^-$ $CD4^+$, $Foxp3^+CD4^+$, $CD8^+$, and $Mac1^+Gr1^+$ cells in Foxp3-Cre×$IL\text{-}10^{fl/fl}$ mice; n=8 animals.
S6G. Counts of HSPCs in Foxp3-Cre×$IL\text{-}10^{fl/fl}$ mice; n=8 animals.
S6H. Counts of $CD51^+Sca1^+$cells (MSCs), and $CD31^-CD51^+$ $Sca1^-$ bone precursors, and $CD140\alpha^+Sca\text{-}1^+$ ($PDGFR\alpha^+$), in Prrx1-Cre×$IL10R\alpha^{fl/fl}$ mice; n=4 animals.

S6I. Multi-lineage engraftment of *ex-vivo* transplanted HSC; B cell (blue); T cell (red) and Myeloid cell (black); n=3 animals per group.
S6J. Co-culture for calculating yields of LT-HSCs maintained on WT stroma (black) or Foxp3-Cre×IL-10$^{fl/fl}$ stroma (red) for 96 hours; n=4 wells per condition. Data are shown as mean ± SD; graphs shown are representative of at least three independent experiments. Statistical analysis performed with unpaired two-tailed Student *t* test (6C-G). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplemental Table 1: Complete Antibody Information

Antibody	Clones	Catalog Number	Source
KLRG1	14C2A07	138411	Biolegend
ICOS	15F9	117405	Biolegend
CD25	PC67	102049	Biolegend
CD103	2E7	121423	Biolegend
CD44	IM7	103028	Biolegend
CD62L	MEL-14	104410	Biolegend
CD49b	DX5	108909	Biolegend
CD127	A7R34	135010	Biolegend
CTLA-4	UC10-4B9	17-1522-82	eBioscience
PD-1	RMP1-14	114117	Biolegend
CD73	AD2, TY/11.8	563198, 127210	Fisher Scientific, Biolegend
CD69	FN50	310911	Biolegend
CXCR4	12G5	306509	Biolegend
CD150	TC15	115941	Biolegend
S1PR1	SW4GYPP	50-3639-41	ThermoFisher Scientific
ST2	U29-93	U29-93	BD Biosciences
CD3	145-2C11, 17A2	100351, 100222	Biolegend
CD4	GK1.5	100426	Biolegend
CD8	53-6.7	100714	Biolegend
NK-1.1	PK136	108719	Biolegend
Ly-6G/Ly-6C (Gr-1)	RB6	108410	Biolegend
CD115	AFS98	135510	Biolegend
CD11b	M1/70	101204	Biolegend
F4/80	BM8	123118	Biolegend
CD45R/B220	RA3-6B2	103206	Biolegend
CD19	6D5	115510	Biolegend
CD43	S11	143204	Biolegend
Ter119	Ly-76	116233	Biolegend
Flk-2, Flt3	A2F10	135315	Biolegend
CD34	RAM34	11-0341-82	Biolegend
CD48	HM48-1	103439	Biolegend
CD45	30-F11	103110	Biolegend
Ly-6A/E (Sca-1)	D7	108134	Biolegend
CD31	390	102422	Biolegend
c-kit (CD117)	2B8	105826	Biolegend
CD140a	APA5	25-1401-80	eBioscience
CD51	RMV-7	104106	Biolegend
CD45.2	104	109806	Biolegend
CD45.1	A20	110741	Biolegend
CD41	MWReg30	133904	Biolegend

Table 1: Summary of Treg Treatments

Hypothesis	Model	Duration	Rationale	Variables	Readout	Results	Strengths	Limitations	Rigor/ Reproducibility
Bone marrow Tregs have tissue specific (non-immune) functions in the bone marrow If marrow Tregs are relevant to hematopoiesis we should see changes to HSPCs and stromal cells	aCD25 *Mechanism:* blocking IL-2 signal *Result:* Treg lose IL-2 signal and are depleted	4 doses of aCD25 28 days Long-term	Total ablation of Tregs results in autoimmunity Reduction of Tregs to measure HSC and stromal response	*Independent variable:* Number of Tregs *Dependent variable:* Changes to stroma & HSPCs	- Flow Cytometry profiling - Cell Counts - Proliferation - Cell Death - RNA sequencing of HSCs - Transplant assays using genotoxic and non-genotoxic conditioning	Expansion of stromal cell populations Expansion of LT-HSC Altered transcriptional of LT-HSC Decreased engraftment	Circumvents autoimmune phenotypes Clinically validated for human use and FDA approved	Depletes Tregs across tissues	Foxp3 DTR model was evaluated and incompatible with these studies aCD25 dosing optimized to deplete Tregs and avoid inflammation and based on relevant publications
Tregs regulate the function stromal cells The stromal cell expansion is due to loss of marrow Tregs not because of changes to ALL Tregs	FTY720 *Mechanism:* Causes down regulation of S1PR1 *Result:* inhibits Treg circulation from lymphoid tissues and re-entry into marrow	7 doses of FTY720 7 days Short-term	Marrow Tregs express more S1PR1 Maintains Tregs in other tissues Selectively reduces marrow Tregs numbers	*Independent variable:* Number of Tregs in the marrow *Dependent variable:* Changes to stroma & HSPCs	- Flow Cytometry profiling Cell Counts	Expansion of stromal cell populations No changes to LT-HSC	Does not deplete peripheral Tregs Selectively reduces marrow Tregs Clinically validated for human use and FDA approved	Targets S1PR1 in many cells Did not see an increase in HSC pool Short term treatment duration	FTY720 dosing optimized to preferentially target marrow Tregs due to higher S1PR1 expression than other Tregs
The stromal expansion seen after aCD25 and FTY720 are driven by disruption to IL-10	Anti-IL10 Receptor Mechanism: blocks IL-10 binding Result: Cells Lose IL-10 signal	5 doses 21 days Long-term	MSC's highly express IL10 Receptor	*Independent variable:* IL-10 signaling *Dependent variable:* Changes to stroma & HSPCs	- Flow Cytometry profiling Cell Counts	Expansion of stromal cell populations Expansion of LT-HSC	Long-term treatment duration (similar to aCD25 in length) Does not deplete Tregs Specific to IL10 signaling Circumvents autoimmune phenotypes	Targets IL10 receptor in all cells	IL10R depletion dosing optimized to avoid inflammation caused by loss of IL10

Table 1: The Tregs treatments were all done on healthy, young C57BL/6 mice. All of three treatments represent transient perturbations of Tregs or IL-10 signaling using different drugs. We feel it is appropriate to compare these approaches as mice developed normally and were controlled for age and sex. The length of treatment and the drug target represent the biggest variables. Mice were subjected to either a short-term treatment (FTY720) or long-term treatments (aCD25, IL10R block).

Table 2: Summary of Stromal phenotypes

Hypothesis	Model	Duration	Rationale	Variables	Readout	Results	Strengths	Limitations	Rigor/ Reproducibility
Treg secreted IL-10 supports stromal cell function	Foxp3 Cre/YFP-IL-10 flox *Mechanism:* Deletion of IL10 production in Tregs *Result:* loss of Treg IL10	Loss of Treg IL10 from birth	Transient IL10 block perturbs stroma Stromal alterations are expected if If Treg-derived IL10 is important	*Independent variable:* Treg derived IL-10 *Dependent variable:* Stromal cells HSPCS	Flow Cytometry profiling Cell Counts	Decrease in stromal cell populations No change to LT-HSC	Maintains Tregs Specific to the Treg derived IL-10	Other populations rely on Treg IL-10 Not transient manipulation Loss of Treg-derived IL-10 from birth	Opposite phenotype as transient perturbations Similar stromal effect as blocking IL-10 signal in Prrx
Treg secreted IL-10 supports stromal cell function	Prx1-Cre/IL10Receptor flox *Mechanism:* Deletion of IL10 receptor in stroma *Result:* loss of IL10 signaling in stroma	Loss of IL10 signaling in Prrx+ cells from birth	Foxp3 Cre/YFP-IL-10 flox mice have a contracted stromal pool	*Independent variable:* IL10 Receptor in Prrx cells *Dependent variable:* Stromal cells HSPCs	Flow Cytometry profiling Cell Counts	Decrease in stromal cell populations Decrease in LT-HSC	Maintains Tregs Maintains Treg derived IL-10 Limits effect of Prrx Stromal populations	Targets Prrx expressing cells in other tissues Not transient manipulation Loss of IL-10 signaling from birth	Opposite phenotype as transient perturbations Similar stromal effect as removing Treg derived IL10 from birth

Table 2: These experiments evaluate the effects of IL-10 signaling. Both of these mouse models provide a scenario where IL-10 signaling has been abrogated from birth. Transient manipulation of Tregs or IL10 signal suggest a stromal phenotype. We observed (via qPCR) the upregulation of different stromal transcripts and observed (via western blot) phosphorylation of STAT3 upon IL-10 stimulation. Because IL-10 signal has been disrupted from birth we feel it is more equitable to compare these two models to each other than to normal mice. Given the importance of IL-10 in many cell types, its pleiotropic effects, and the role of IL-10 in development we feel there are many confounds in comparing mice that experienced transient Treg/IL10 perturbations (as was done with aCD25, FTY720 and IL10R block) to mice where IL-10 signaling has been compromised from ontogeny.

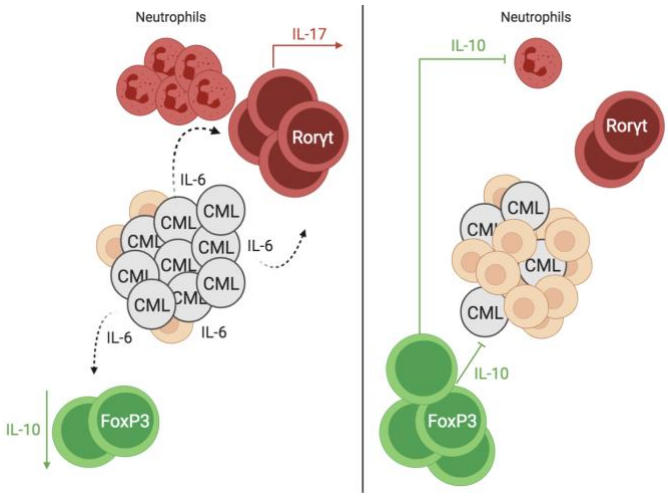
Modulating IL-10 production by Regulatory T cells for therapeutic intervention in Chronic Myeloid Leukemia

by

Virginia Camacho1, Sweta B. Patel, Victoria R. Matkins, Valeriya Kutznetzova, Ashlyn Anderson, Laurie E. Harrington, Robert S. Welner

In preparation for *Science Immunology*

Format adapted for [thesis or] dissertation



In Brief

The non-immune roles of Regulatory T cells (Tregs) have been described various tissues, including the bone marrow, but their role in leukemogenesis remains elusive. In this study, we uncovered that the Chronic Myeloid Leukemia (CML) environment effectively deteriorates Treg function and stability. We also observed that Treg/Th17 ratios are disrupted and influence disease progression. We demonstrate that restoring Treg function and expanding their IL-10 production can be used therapeutically to delay disease progression and protect the bone marrow niche. These findings highlight how the Treg/Th17 balance regulates disease progression in the context of leukemic transformation. This is the first report revealing that Treg secreted IL-10 can be used therapeutically in the context of chronic myeloid leukemia.

Highlights

- **Tregs are altered in CML**
  I. Highly Activated
  II. Loss of Foxp3
  III. Decreased IL-10 secretion

- **Expanding Tregs can delay leukemic progression**
  i. Increasing IL-10 production curbs myeloid cell expansion
  ii. IL-10 directly influences myeloid and neutrophil differentiation of leukemic progenitors

In Preparation for submission
Format adapted for dissertation

Modulating IL-10 production by Regulatory T cells for therapeutic intervention in Chronic Myeloid Leukemia

Virginia Camacho[1], Sweta B. Patel[1], Victoria R. Matkins[1], Valeriya Kutznetzova[1], Ashlyn Anderson[2], Laurie E. Harrington[2], Robert S. Welner[1]

[1] Division of Hematology-Oncology, Department of Medicine, The University of Alabama at Birmingham, Birmingham, AL, 35294, USA. vcamacho@uab.edu 205-975-3960
[2]Department of Cell, Developmental, and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL.

* Corresponding author email – rwelner@uab.edu

The non-immune roles of Regulatory T cells (Tregs) have been described across various tissues, including recent work on bone marrow Tregs in stromal cell maintenance and steady-state hematopoietic support. In CML, there is an increase in pro-inflammatory cytokines and inflammation that promotes the pathogenic skewing of regulatory and effector T cells. In the leukemic marrow, we found that Tregs are unstable and dysregulated. This phenotype was also strongly associated with markers of immune activation and loss of IL-10 production. Specifically, our findings uncovered that Treg/Th17 ratios are disrupted in the context of leukemia and influence disease progression. Moreover, we found that Treg-secreted IL-10 has direct effects on myeloid cell production by leukemic progenitors. We demonstrate that restoring Treg function and expanding their IL-10 production can be used therapeutically to mitigate disease progression. Our results support the hypothesis that Tregs are compromised during leukemic progression as a consequence of pro-inflammatory cytokines from the myeloid associated disease. This is the first report revealing that Treg secreted IL-10 is can be used as a therapeutic intervention in the context of CML and our work outlines an alternative mechanism by which this cytokine regulates hematopoiesis both in steady-state and disease.

INTRODUCTION

Chronic Myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder that arises from the genetic translocation (t9;22) (q34; q11.2) of the *BCR* and *ABL* genes. This generates an oncogenic fusion protein that results in a constitutively active *ABL1* kinase (1-3). CML initiates within the hematopoietic stem cell (HSC) compartment where dysregulated kinase activity leads to progressive and chronic expansion of neoplastic cells. As a myeloproliferative neoplasm (MPN), CML is categorized by the uncontrolled production of myeloid cells, neutrophil expansion, excessive inflammatory signaling, immune dysregulation and ineffective hematopoiesis(4, 5).

In CML, inflammatory cytokines contribute to the maintenance of oncogenic blasts in both an autocrine and paracrine manner exacerbating disease progression. The overproduction of inflammatory mediators such as interleukin 6 (IL-6), and interleukin (IL-1) play vital roles in the development of CML(6). Along with persistent inflammation, immune dysregulation is emerging as a contributing factor in the development of CML (7-12). However, the cellular actors involved, and their specific contributions remain ill-defined. In particular, there is limited information about the role of CD4+ T helper (Th) subsets in CML pathophysiology. T helper cells play important roles in the development and progression of infection, inflammation, autoimmunity, and tumors and have received increasing attention in in the context of hematological malignancy (13-16). In particular, Regulatory T cells (Tregs) are thought to have clinical relevance in predicting therapeutic responses and disease severity (11). They have also been demonstrated to have important role in supporting hematopoiesis (17). In this regard, we recently showed that bone marrow Treg are a tissue specific subset and have key functions related to the maintenance of stromal cells and stem cells via IL-10 secretion.

Regulatory T cells (Tregs) are a specialized CD4+ T cell characterized by the expression of their lineage defining transcription factor Foxp3 (18). Tregs are involved in a plethora of biological activities spanning the instruction of immune tolerance, oncogenesis, autoimmunity and inflammation(19-26). In acute settings, Tregs are highly equipped to exert their immunosuppressive functions, and remain stable when entering inflammatory settings. It has been demonstrated that during injury and acute infection Tregs directly participate in tissue repair, the tampering of the immune response, and the resolution of inflammation. However, in conditions of chronicity, such as oncogenesis or autoimmunity, Tregs can become unstable (20, 27-30). The ability to produce anti-inflammatory cytokines and control inflammation is often lost upon sustained exposure to persistent inflammatory environments. The differentiation trajectory Tregs is closely tied with the differentiation of T-helper 17 (Th17) cells and these two subsets often play contradictory roles in the context of inflammatory responses (22, 23, 31-33). Th17 are defined by the transcription factor retinoic acid-related orphan receptor (RORγt) and the production of IL-17A among other chemokines and cytokines. Th17 immunity is conventionally associated with autoimmune conditions, where they have been implicated in pathogenesis as well as amplification of inflammatory responses. Specifically, Th17 cells have well defined roles in the recruitment and activation of myeloid cells and have been shown to promote granulopoiesis, neutrophil recruitment (22, 34, 35).

Given the function of Th17 cells as inflammatory mediators, they are typically regarded as the counterpart to Tregs and immunological homeostasis depends on their appropriate counterbalance (15, 21, 34, 36-39). Nevertheless, Tregs and Th17 cells often play reciprocal roles and the two lineages are closely linked developmentally. The

maintenance and differentiation of Th17 cells requires the presence of IL-1, IL-6, and transforming-growth factor beta 1 (TGF-β1) and the STAT3 pathway (21). Interestingly these signals are detrimental to Treg stability and antagonize Foxp3 mediated differentiation. A compelling body of work now supports the notion that Foxp3$^+$ Tregs are highly plastic and acquire Th17 effector functions in the context of inflammation (15, 22, 32). Across many models, the dual expression of Th17 and Treg signature genes has been reported in the same cells, and it has been shown that Tregs can differentiate to IL-17 producing Foxp3$^+$ Treg cells. Moreover, Tregs can also acquire the ability to secrete IL-17 and have been shown to co-express both RORγt and Foxp3 (40-43).

In this work we also characterized a dysregulated balance of Treg and Th17 and in the context of CML. We observed that the CML cytokine environment is able to promote Th17 generation and increased production of IL-17A both in culture and in vivo. This emergent phenotype is likely mediated by increased inflammatory signals, specifically IL-6. Our data suggests that the increased levels of IL-6 seen in CML negatively impact Treg development and biases the Treg/Th17 balance in favor of inflammatory Th17 cells. We show that in CML Tregs become unstable, lose Foxp3 expression, and acquire an activated STAT3 signature. Furthermore, our profiling indicates that Tregs in CML lose the ability to secrete high levels of IL-10 in response to the inflammatory CML environment.

In the context of CML, Treg profiling has done in the context of residual leukemia following a prolonged TKI regimen or at advanced stages of disease. Thus, mechanistic roles for Tregs in the development of CML has not been systematically addressed. Thus, in the present work, we investigated whether boosting Treg function could alter disease progression. We observed that expanding Tregs prior to disease initiation was able to reduce leukemic burden and curb myeloid expansion. We investigated the effect of IL-10 on myeloid differentiation and found that in the presence of IL-10 cells, granulocyte-monocyte progenitors had reduced differentiation into neutrophil and myeloid lineages. Importantly the effect of IL-10 was dose dependent and was more prominent in leukemic cells. These results provided compelling evidence that the Treg-IL-10 axis exerts pleiotropic effects on CML progression. The data also highlight a previously uncovered for the CML microenvironment in promoting Th17 differentiation. Thus, manipulating Treg function and enhancing IL-10 may represent a targetable therapeutic modality for CML patients.

RESUTS

Altered Treg populations in Chronic Myeloid Leukemia.

To interrogate the role of Tregs during the course of leukemic progression, we utilized a well-established inducible transgenic murine model (SCL-tTA×BCR-ABL) of Chronic Myeloid Leukemia (CML) CML is characterized by the genetic translocation (t(9;22)(q34;q11.2) of BCR and ABL genes to generate an oncogenic fusion protein that results in a constitutively active *ABL1* kinase (citations). In our inducible model, tetracycline (TET) withdrawal results in the development of a myeloproliferative neoplasm that closely recapitulates the progression seen in patients. In order to track Treg populations through the course of disease development, we bred CML mice to Foxp3-GFP reporter mice. However, because CML is driven by over-active STAT5 signals from the kinase, and there is a meaningful role for STAT5 in Treg biology, we elected to utilize chimeric mice which would allow us to study non-transformed Treg

populations in the context of a leukemic environment. Chimeric mice were generated by transplanting transformed cells (expressing the BCR/ABL fusion protein) into wild-type Foxp3-GFP mice (Fig 1A). The use of congenic markers allowed us to differentiate between leukemic exposed (CD45.1) Tregs and BCR-ABL+ (CD45.2) cells. In order to recapitulate the human biology, we chose to focus our studies on the non-transformed (CD45.1) cells within the leukemic environment. Our chimeric mice recapitulated the established CML disease phenotypes; we observed neutrophilic leukocytosis, splenomegaly, accumulation of myeloid progenitors, and expansion of granulocytes and granulocyte-monocyte progenitors (GMP) (S1A-D). Through the use of congenic markers, we were able to monitor the effects of leukemic exposure on Treg populations.

We observed a reduction of Treg populations in proportion to increased leukemic burden underscoring a possible vulnerably to the CML microenvironment (Fig 1B). In accordance with this, Tregs from CML mice had an altered phenotype as assessed by upregulation of various activation receptors (ICOS, GITR, KLRG1, CD44, CD103) (Fig 1C). We also observed a downregulation of markers associated with Treg stability including PD-1 and CD25. In the context of Treg biology, constitutive CD25 expression is of their identifying phenotypes contingent on Tregs' unique dependency on IL-2. Significant work has defined IL2R signaling as essential for the development, homeostasis, and function of Tregs. It has also been shown that IL-2 is the primary cytokine contributing to the homeostasis of peripheral Treg cells. Functionally, constitutive expression of CD25 is necessary for Tregs scavenge the paracrine IL-2 and to promote IL-2-mediated Treg functions including immune tolerance and anti-inflammation (44). Notably, the loss of CD25 expression marker has been associated with Treg dysfunction across various disease models. We observed downregulation of CD25 across Treg populations from leukemic mice (Fig S1E). Surprisingly, we observed the loss of CD25 in BCR-ABL+ Treg populations, despite the constitutively STAT5 inherent to the oncogene, which should reinforce the STAT-5 mediated Treg phenotype. Collectively this suggest that environmental cues from the CML environment impose significant pressure to critical Treg pathways.

Inflammatory cytokines direct development of Th17 cells in CML

High levels of IL-6 are hallmarks of CML and it is well established that IL-6 is critical to disease maintenance and propagation. In the context of T cell biology, IL-6 acts as a potent pro-inflammatory cytokine that promotes of Th17 differentiation and inhibits Treg differentiation. To understand the function of leukemic cytokines in the context of Treg differentiation, we isolated naïve CD4+ T cells from CML mice at the peak of disease and tested the ability of these cells to generate Tregs when removed from a hostile inflammatory setting. We observed a gross defect in the generation of Foxp3+ cells when utilizing naïve CD4 cells from CML mice at the peak of disease (Fig 2A).

In our CML model, we observed reduced Treg numbers indicating potential instability. The acquisition of Treg fate, and differentiation to the Treg lineage is developmentally linked with the expression of *Foxp3*, which reinforces a major component of the Treg transcriptional and functional landscape. Importantly it also sustains high expression key Treg signatures and is involved in the necessary repression of Th1, Th2, and Th17 effector functions (45). Although Tregs are typically stable in healthy homeostatic settings, chronic inflammatory scenarios are associated with loss of *Foxp3* and the generation of 'ex-Tregs'. These ex-Tregs often acquire the potential to differentiate into effector T cells and have been shown to secrete inflammatory cytokines(46-49). To trace

the history of Foxp3^{+} Tregs in the context of CML, we generated a lineage-tracing model which would allow us to test how the inflammatory pressures of CML environment affected *Foxp3* expression and the stability of Tregs. To do this, we crossed mice carrying *a* YFP-Cre fusion protein knocked into the 3' UTR of the *Foxp3* gene with a Rosa-LSL-Tdtomato reporter mice containing a transgene encoding an enhanced tandem dimer tomato red fluorescent protein (tdTomato Red) in the *ROSA26* locus. In this model, even if Tregs lose Foxp3, these 'YFP^{-}TdTom^{+} 'exTregs' will be pre-labeled with TdTom, allowing us to trace the history of Foxp3 expression and differentiate Tregs that have lost Foxp3 from those that never expressed it. To probe the issue of Foxp3 stability and the subsequent outcome for Tregs in CML, we generated chimeric mice using (CD45.1/2) BCR+SCL+ leukemic cells as described and traced Foxp3 expression during disease progression. In our chimeric mice, we identified a sizeable fraction of ex-Tregs (10–15%) even under homeostatic conditions. We attribute the proportion of ex-Tregs to the exposure to sub-lethal dose of X-ray irradiation, as expansion of the ex-Treg pool has been reported in lymphopenic or transplant conditions. Remarkably we noted a significant increase in YFP^{-}TdTom+ cells corresponding to an increase in ex-Treg cells in CML (Fig S2B). Previous studies have shown that loss of Treg identity and acquisition of Th17 function are regulated in part by STAT3 signaling (36). To explore this, we performed expression analysis of selected Th17 associated genes on CD4+ Foxp3+ cells. We detected increased levels of STAT3 transcripts in Foxp3+ Tregs from CML mice relative to WT Tregs. Interestingly, we also observed an increase in AHR, a transcription factor expressed in high levels by Th17 cells and has been implicated in Treg/Th17 conversion (Fig 2B) (50, 51). In combination with our fate-mapping experiments, this indicates that Tregs is less stable in the context of CML, and that this leukemic environment prompts Tregs to downregulate Foxp3 and activate STAT3.

Increased levels of IL-6 are known to induce Th17 cells (52-57). Therefore, we hypothesized that cytokine profile of CML might favor the generation of Th17 cells. To investigate if the inflammatory signals from the CML environment facilitated the initiation of Th17 cells, we isolated naïve CD4+ T cells and polarized them using conditioned media from leukemic mice. In our cultures, we observed that CML conditioned media let to robust generation of RORγt+, IL17+ cells, almost identical to Th17 polarizing conditions (Fig 2C, S2A). To validate these observations, we made use of IL17-GFP reporter, which faithfully labels IL17 producing cells. Indeed, analyses of naïve CD4+ cells polarized with CML conditioned media revealed expansion of IL17+ GFP cells (Fig 2D). We next sought to determine if these findings applied to Th17 cells *in vivo*, in the context of leukemic transformation. To enable the study of non-transformed Th17 cells in the context of CML inflammation, we generated chimeric mice using (CD45.2) IL17-GFP+ cells, (CD45.1/2) BCR+SCL+ leukemic cells and examined GFP-reporter expression following disease onset. Remarkably, there was a significant expansion of total CD4+ IL17+ cells in the bone marrow of CML mice (Fig 2E, S2C). This provides evidence that that CML cytokines play a role in inducing IL-17A production and that these leukemic conditions drive the generation of Th17 cells.

DR3 Treg expansion ameliorates leukemic disease in CML mice

We previously outlined a critical role for bone marrow Tregs in the maintenance of hematopoiesis and hematopoietic stem function (HSC) (citation). We have shown that depletion of Tregs has deleterious effects on stem function including loss of repopulation capacity, and acquisition of myeloid bias. Loss of Tregs also impacts the stromal niche, in particular mesenchymal stem cells (MSCs) reducing their capacity to support HSCs.

Importantly Treg support MSC is mediated via secreted IL-10. These critical roles of Treg secreted IL-10 in the marrow led us to hypothesize that Tregs may represent a target by which to alter the disease trajectory. Given the importance of Treg-secreted IL-10 in the maintenance of hematopoiesis, we aimed to select a method of Treg expansion that also enhanced IL-10 secretion. A method of Treg expansion that has shown tremendous efficacy is the targeting of Death receptor 3 (DR3), a tumor necrosis factor receptor superfamily member (TNFRSF25). TNFRSF25 (DR3) is a type I transmembrane protein that can be induced on activated T cells and is expressed at low levels of various immune populations (58-60). However, it is constitutively expressed at high levels on Foxp3$^+$ Tregs. Others have demonstrated that the administration of an agonistic antibody (4C12) not only expands Tregs increases but specifically boots their effector functions and enhances IL-10 secretion (61-63). Thus, we elected to expand Tregs through DR3 activation in our CML model. Consistent with other studies, we observed that administration 4C12 significantly expanded Foxp3+ Treg cell and also enhanced their IL-10 production (Fig 3A, S3A).

To analyze Treg IL-10 production in in CML, we utilized a previously established IL-10 reporter system, the 10BiT mice, in which IL-10 expression is linked to Thy1.1 (64, 65). By generating chimeric mice with this IL-10 transcriptional reporter model, we were able to monitor changes to IL-10 production in vivo in the context of CML. We observed significantly fewer IL-10 transcripts in Tregs exposed to the CML environment, consistent with their altered profile (S3B). Given these alterations, we hypothesized that alterations to Tregs and loss of IL-10 signals might contribute the inflammatory profile in CML. To examine the effects of Treg manipulation on disease progression, we treated control and CML mice with Treg-modulating agents; either 4C12 (to expand) or anti-CD25 (to deplete). Our previous work showed the detrimental effects of Treg depletion on hematopoiesis. Consistent with this, mice receiving CD25 progressed more rapidly and succumbed to leukemia beginning 6–8 weeks after treatment. Remarkably, expanding Tregs via DR3 prior to disease onset delayed leukemic burden and significantly curbed myeloid cell expansion. Boosting Treg function via 4C12 significantly protected from CML mice from death with a median disease onset of 10 weeks. Importantly, the DR3-treated mice had appreciably reduced splenomegaly and myeloid expansion (Fig 3B, S3C-E). To rule out any direct effects of DR3 on leukemic cells, we treated K562 cells with increasing doses of DR3 and found no increased cell death or toxicity (S3F). We also evaluated the effects of DR3 stimulation on colony forming potential of mutant and non-mutant lineage negative, Sca-1$^+$ c-kit$^+$ cells (LSK) (S3G). We observed no changes to colony forming capacity suggesting that DR3 is mediating anti-leukemic via indirect mechanisms. This indicates that enhancing Treg functions, specifically IL-10 production has therapeutic effects on CML disease development.

Direct effects of IL-10 on Leukemic Progenitors

To investigate the mechanism by which DR3 prolonged the survival of CML mice, we analyzed the effects of treatment on the growth of leukemic cells in the bone marrow. Activation of Tregs via DR3 significantly suppressed the frequency of BCR-ABL-transformed cells and we observed a dramatic reduction in leukemic granulocyte-monocyte progenitors (GMP), (Gr1+ Mac1+) myeloid cells, and neutrophils (Fig 3C). To understand the mechanisms by which increased IL-10 production altered leukemic progression, we evaluated potential cellular targets of IL-10. We have previously assessed the expression of the IL-10 receptor (IL10Ra) among the hematopoietic stem and progenitor cells (HSPCs) in the bone marrow and found no expression. However,

given the importance of IL-10 for myeloid cell differentiation and function, we examined previously generated datasets for cellular targets of IL-10 (66). We confirmed that IL-10Ra was expressed on GMP populations (Fig 4A). To measure the effects of IL-10 in the context of leukemic expansion, we differentiated GMP into various myeloid subsets and added increasing doses of IL-10. Remarkably we found that IL-10 significantly decreased myeloid cell output and this effect was selective for cells carrying the BCR-ABL oncogene. We observed a dose-dependent response in response to IL-10 noting the decrease in the differentiation to neutrophil cells (Fig 4B). Collectively, these findings suggest that Treg secreted IL-10 may be able to counter the pathogenic inflammatory signals and delay CML progression by acting directly on leukemic myeloid progenitors (Fig 4C).

Discussion

In summary, we describe a therapeutic function for Tregs and IL-10 in the context of chronic myeloid leukemia. The expansion of Tregs has been a topic of debate in the context of tumorigenesis, where excessive Treg function is typically considered detrimental and pathogenic. Contrary to this, we observed a significant acceleration of disease pathology when Tregs were depleted, suggesting a constructive function for Tregs in limiting inflammatory disease. In line with this notion, the expansion of Tregs, and enhancement of their IL-10 production was able to curb myeloid cell production and reduce leukemic burden. The results presented here feature a new theme, were boosting Tregs might be exploited as a means of improving the immune response in the context of leukemic transformation.

Tregs numbers have previously been profiled following TKI intervention in CML but their contributions to disease or mechanisms of action remain unclear (67). In our model, we found altered Treg profiles including decreased numbers, upregulation of key activation markers, and loss of IL-10 production. Collectively, these results suggest that chronic exposure to the leukemic environment may be detrimental to Treg stability. CML is associated with elevated levels of IL-6, and this cytokine is critical for disease propagation. In patients, IL-6 levels have been valuable prognostic indicators of disease outcome with the highest levels of IL-6 are typically found in the blast crisis phase (6, 68-73). Accordingly, IL-6 is associated with accelerated transformation and pathology in CML. Consistent with this, blockade of IL-6 signaling has been demonstrated be an effective treatment strategy for therapeutic intervention (74). With regards to T cell biology IL-6 is critical actor that synchronizes the balance between Tregs and Th17 cells (28). IL-6 is a known inducer of RORγt and the activation of this transcription factor via STAT3 has been shown to support acquisition of Th17 fate (22, 31, 32, 35).

A unique finding described in this work is the presence of Th17 cells in CML. We observed that the CML microenvironment was able to promote the development and differentiation of Th17 cells both in culture and in vivo. These results suggest a direct pathway for induction of Th17 cell fate in leukemia likely arising from the greater availability of IL-6 and other inflammatory cytokines(21, 52, 75). Further elucidation of the role of Th17 cells in modulating CML progression could provide a new perspective on pathogenic immunological mechanisms that underlie this disease and could expand options for clinical cellular therapy. In the context of both autoimmunity and solid tumors increased levels of IL-17 and presence of Th17 cells in inflamed tissues have been hallmarks of pathology. Interestingly, there is specific role of IL-17 in myeloid cell activation (38, 76-78). We can speculate that increased levels of IL-17 in inflamed

tissues may act as a positive signal that amplifies myeloid recruitment, and specifically neutrophil activation and differentiation in CML.

Our results expanding Tregs in CML are consistent prior literature related to the use of Death receptor 3 (DR3) (TNFRSF25) to target Treg populations. We show that DR3 is a targetable receptor that permits the preferential expansion of Tregs in the context of CML. Using this method, we were able to expand Tregs and enhance their IL-10 producing capacity to limit inflammatory disease. We also observed a direct effect of IL-10 on leukemic GMP progenitors. In a dose dependent manner, IL-10 signals inhibited the differentiation of leukemic progenitors into myeloid and neutrophil lineages. We propose that IL-10 may play an important role in mediating the balance between inflammatory and anti-inflammatory responses in hematological malignancy more broadly.

In conclusion, we observed that expansion of Tregs via DR3 stimulation in CML mice reduced the severity of disease. We specifically observed prolonged survival of DR3 treated mice, reduced myeloid expansion, reduced splenomegaly and reduced neutrophilia. We show that this is due, at least in part, to increased IL-10 production. We speculate that other Treg secreted molecules, such as Amphiregulin may be involved in this regulation and this represents an exciting area for future studies. The functional role of IL-10 and in other myeloid neoplasms also warrants further investigation. Future studies into the Treg/Th17 balance may have clinical potential for decelerating the oncogenic effects of inflammation in the context of leukemogenesis. Collectively, our data further underscore the importance of adaptive immune cells in modulation of pathology of myeloid leukemia and provide a new perspective on the immune mechanisms that underlie leukemic transformation.

Methods

Murine Strains: The Jackson Laboratory

C57BL/6 Stock No: 000664; immunocompetent
Foxp3/GFP (CD45.2): C57BL/6-Tg: 90Pkraj/J Stock No: 023800; immunocompetent
BCR-ABL/SCL-tTA mice; immunocompetent
IL17A-IRES-GFP-KI (CD45.2): C57BL/6-*Il17a*^{tm1Bcgen}/J Stock No: 018472
Foxp3$^{YFP\text{-}cre}$(CD45.2): B6.129(Cg)-*Foxp3*$^{tm4(YFP/icre)Ayr}$/J Stock No: 016959
tdTomato: (CD45.2): B6. Cg-Gt (ROSA)26Sortm9(CAG-tdTomato) Hze/J Stock No: 007909; immunocompetent

Transgenic mice

Foxp3GFP-CML mice were generated by breeding BCR-ABL/SCL-tTA mice to Foxp3/GFPmice
Treg fate-mapper mice were generated by breeding Foxp3$^{YFP\text{-}cre}$ to CAG-tdTomato mice

Murine Strains: Donated

10BiT.Foxp3^{gfp} (CD45.1 and CD45.2); immunocompetent, donated by Dr. Casey Weaver.
BCR-ABL/SCL-tTA mice (CD45.1/2); immunocompetent, donated by Dr. Ravi Bhatia
IL17A-IRES-GFP-KI: C57BL/6-*Il17a*^{tm1Bcgen}/J Stock No: 018472, donated by Laurie Harrington

All murine experiments were conducted using 6-12 weeks old male and female mice. Experimental mice were separated by sex and housed with 5-7 mice per cage. Littermates of the same sex were randomly assigned to experimental groups. Mice for use in these studies were maintained in a specific pathogen-free state in micro-isolator caging and received autoclaved water and standard rodent chow diet. Mice for leukemic profiling experiments were monitored and sacrificed at ~60% Gr1+ Mac1+ cells in the peripheral blood, and elevated neutrophil counts as assayed by HemaVat. All mice were maintained under the guidance of the UAB Animal Resources Program under the direction of full-time veterinarians fully accredited by the American Association for Accreditation of Laboratory Animal Care. The Department of Comparative Medicine at the University of Alabama at Birmingham provided veterinary care. UAB complies with the current NIH policy on animal welfare, the Animal Welfare Act, and all applicable federal, state, and local laws.

Anti-CD25

(0.5mg/ mouse) of *InVivo*MAb anti-mouse CD25 (PC-61.5.3) or *InVivo*MAb, or rat IgG1 isotype control (anti-horseradish peroxidase) was administered intraperitoneally weekly (7D) before being euthanized for analysis.

anti-mouse DR3

(10ug/mouse) of Ultra-LEAF™ Purified anti-mouse DR3 (TNFRSF25) antibody, or rat IgG1 isotype control (anti-horseradish peroxidase) was administered intraperitoneally bi-weekly (14D) before being euthanized for analysis.

BrdU

(1mg/ mouse) of BrdU in 200 µl PBS was administered intraperitoneally 16h before being euthanized for analysis. Cells were analyzed 16 hours post-injection and BrdU incorporation assessed according to the manufacturer's protocol.

AnnexinV

Analysis of apoptotic cells were performed using APC-conjugated Annexin V according to the manufacturer's protocol (Biolegend, San Diego, CA.)

Flow cytometry and cell sorting

Single cell suspensions from bone marrow, inguinal lymph node, thoracic duct, spleen, and peripheral blood were analyzed using a BD Fortessa X-20 flow cytometer or BD LSRII (BD Biosciences, San Jose, CA) sorted using a FACSAria (BD Biosciences, San Jose, CA). Diva software (BD) and FlowJo (Tree Star) was used for data acquisition and analysis, respectively. Surface Markers: KLRG1, ICOS, CD25, CD103, CD44, CD62L, CD127, PD-1, CD69, GITR.

Mature populations were defined as follows: CD4 T cell ($CD3^+$ $CD4^+$ $NK1.1^-$), CD8 T cell ($CD3^+$ $CD8^+$ $NK1.1^-$) Treg ($CD3^+$ $CD4^+$ $Foxp3^+$ $NK1.1^-$), Natural Killer T cells (NKT; $CD3^+$ $NK1.1^+$), Neutrophils ($Gr\text{-}1^+$,$CD115^-$), Macrophages ($Gr\text{-}1^-$,$F4/80^+$). B cells ($CD19^+$ $B220^+$) Immature-B cells ($CD43^+$ $CD19^+$ $B220^+$).

Hematopoietic stem and progenitor cells were defined as follows: granulocyte-macrophage progenitors (GMP; $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+Fc\gamma R^+$), megakaryocyte-erythroid progenitors (MEP; $Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^-Fc\gamma R^-$), long-term hematopoietic stem cells (LT-HSC; $Lin^-Sca\text{-}1^+c\text{-}Kit{+}Flt3^-CD150^+CD48^-$), short-term hematopoietic stem cells (ST-HSC; $Lin^-Sca\text{-}1^+c\text{-}Kit^+Flt3^-CD150^-CD48^-$), multipotent progenitors (MPP; $Lin^-Sca\text{-}1^+c\text{-}Kit^+Flt3^-CD48^+$).

Non-hematopoietic stromal cells were defined as follows: non-hematopoietic, non-endothelial cells ($CD45^-$/$Ter119^-$/$CD31^-$), endothelial cells ($CD45^-$/$Ter119^-CD31^+$), mesenchymal stem cells ($CD45^-$/$Ter119^-$/$CD31^-$ $CD51^+Sca\text{-}1^+$), osteoblasts ($CD45^-$/$Ter119^-$/$CD31^-CD51^+Sca\text{-}1^-$), $PDGFRA^+$ cells ($CD45^-$/$Ter119^-$/$CD31^-CD140\alpha^+Sca\text{-}1^+$).

<u>CD4 T-helper polarization</u>

Naïve CD4 T cells were isolated by magnetic bead enrichment using the mouse naive CD4 isolation kit (Stem Cell Technology) according to the manufacturer's protocol and cultured in RPMI 1640 with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 1× nonessential amino acids, 1 µM sodium pyruvate, and 50 µM β-mercaptoethanol) with plate-bound 10 µg/ml anti-CD3ε, and soluble 1 µg/ml anti-CD28. Th17: 20 ng/ml rmIL-6, 2.5 ng/ml rhTGFβ1 10 ng/ml rmIL-23, 10 µg/ml anti-IL-4, and 10 µg/ml anti-IFNγ. Treg: 1 ng/mL TGF-β, and 50ng/ml IL-2, 10 µg/ml anti-IL-4, and 10 µg/ml anti-IFNγ in 96-well flat-bottom plates.

Intracellular staining

intracellular staining was performed following stimulation with Leukocyte Activation Cocktail, with BD GolgiPlug™ (BD Pharmingen™). Cell viability was determined by Fixable Aqua Dead Cell Stain Kit (Invitrogen L34957) Intracellular staining for IL-17A, RORγt, and Foxp3, and was performed using the Foxp3 Permeabilization/Fixation kit (eBioscience).

Stromal Cell Culture

All cultures with stromal cells were carried out with murine stromal cells from harvested from femurs and tibias and cultured through one passage (P1) and supplemented with Gibco™ MEM α, Nucleosides, No Phenol Red Media supplemented with 100 U/mL penicillin-streptocycin, 100 U/mL Antibiotic Antimycotic Solution and 20% FBS. Media and cytokines were replaced every 3 days.

Myeloid Differentiation
Granulocyte-Monocyte progenitors (control or BCR-ABL1+) were sorted from bone marrow and cultured with) in Lonza™ BioWhittaker™ X-VIVO™ 15 Hematopoietic Serum-Free Culture Media supplemented with 100 U/mL penicillin-streptocycin, 100 U/mL Antibiotic Antimycotic Solution; murine SCF 10ng/mL, Flt3 50ng/mL, IL-3 10ng/mL, GM-CSF 10ng/mL, M-CSF 10ng/mL, Cultures were supplemented with murine IL-10. Cultures were carried out for 7-10 wand cytokines replaced every 3 days in a 96 flat bottom plate.

Colony forming Unit (CFU) assay
20,000Lin-cKit+Sca1+ cells were sorted from WT or CML mice and were cultured for 10 days in MethoCult GF M3434 media (StemCell Technologies Inc, Vancouver, BC, Canada) supplemented with 10ug/mL of DR3. Media and cytokines were replaced every 3 days. Colonies were counted and analyzed according to the technical manual.

Quantification and Statistical Analysis
Statistical analyses were performed depending on the spread of the variable as specified and were reported as means ± standard deviation (SD) or standard errors of means (SEM). A Shapiro-Wilk test was used to determine normal versus abnormal distributions, and all continuous variables were tested for mean differences. Depending on the spread of variable both nonparametric: Mann–Whitney U test, ANOVA Kruskal-Wallis test, Wilcoxon test, and parametric: Student's t-test and ANOVA were used. For ANOVA, Tukey's post-test was used to compare individual groups (GraphPad Prism version 6.0, La Jolla, CA). A-priori sample size calculation was determined based on estimates from preliminary data in order to provide power of >80% to detect a 30% difference with an alpha error of 0.05. p values < 0.05 were considered statistically significant. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$; ns = not significant.

Acknowledgments

We would like to thank Drs. Paul Kincade and Allan J. Zajac for their feedback. This project was supported by the following grants: HL131477 (NIH), ASH Bridge Funding 2018, National Institutes of Health (NIH) Immunology T32 Training Grant 2T32AI007051 and funds from the Division of Hematology/Oncology at UAB.

Author contributions

V.C. designed, planned, and performed experiments, analyzed data and wrote the manuscript; V.R.M, S.B.P, performed experiments; A.A. and L.E.H. supervised the study; R.S.W supervised the study and assisted with data interpretation and manuscript writing. All authors declared no conflict of interest.

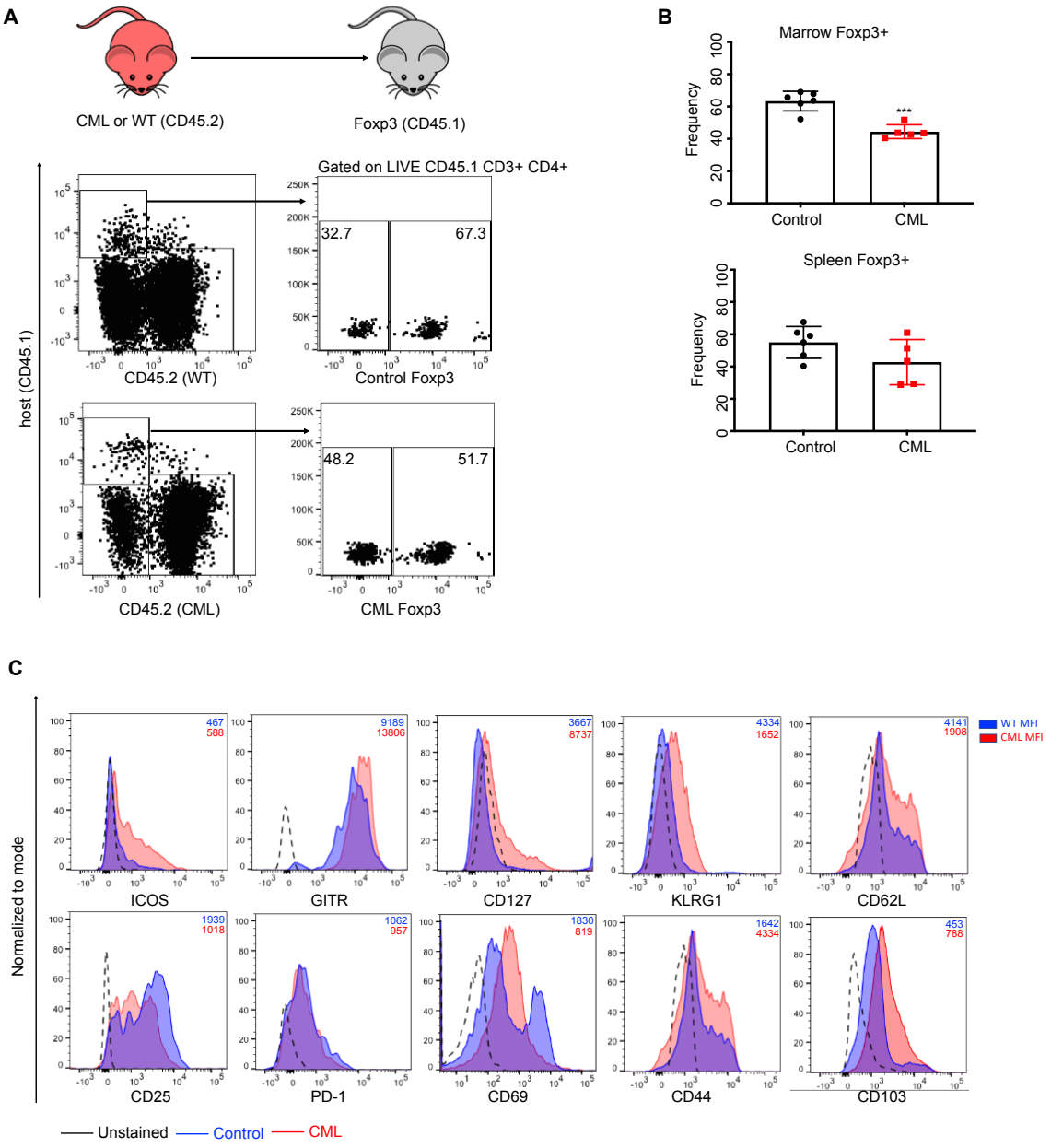

A
CML or WT (CD45.2)
Foxp3 (CD45.1)
Gated on LIVE CD45.1 CD3+ CD4+
host (CD45.1)
CD45.2 (WT)
Control Foxp3
32.7
67.3
CD45.2 (CML)
CML Foxp3
48.2
51.7
B
Marrow Foxp3+
Frequency
Control
CML
***
Spleen Foxp3+
C
Normalized to mode
ICOS
GITR
CD127
KLRG1
CD62L
CD25
PD-1
CD69
CD44
CD103
WT MFI
CML MFI
Unstained
Control
CML

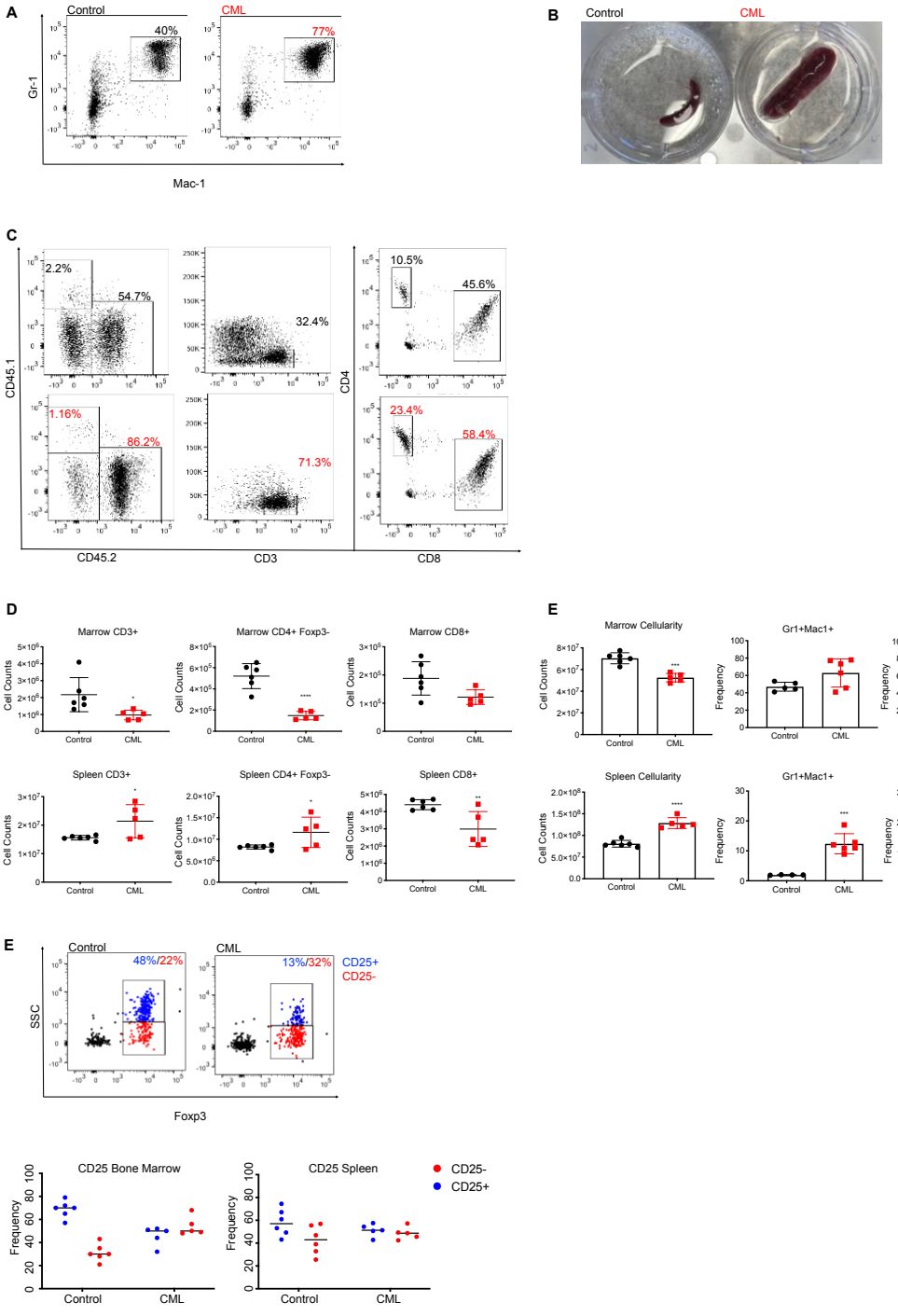
Figure 1: Altered Treg populations in Chronic Myeloid Leukemia.

1A: Schematic of CML model (top). Representative plots of $Foxp3^+$ cells in control and CML chimeric mice.

1B: Frequency of $Foxp3^+$ cells in control (black) and CML (red) in bone marrow and spleen; n=6 mice per group.

1C: Representative histogram and MFI of ICOS, GITR, CD127, KLRG1, CD44, CD62L, CD25, PD-1, CD103, CD69 in indicated tissue control (blue), CML (red), unstained (dotted).

Data are shown as mean ± SD; graphs represent pooled data from at least three independent experiments. Statistical analysis performed with Statistical analysis performed with unpaired two-tailed Student t test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.


A
Control
CML
40%
77%
Gr-1
Mac-1
B
Control
CML
C
2.2%
54.7%
32.4%
10.5%
45.6%
1.16%
86.2%
71.3%
23.4%
58.4%
CD45.1
CD4
CD45.2
CD3
CD8
D
Marrow CD3+
Marrow CD4+ Foxp3-
Marrow CD8+
Spleen CD3+
Spleen CD4+ Foxp3-
Spleen CD8+
Cell Counts
Control
CML
E
Marrow Cellularity
Gr1+Mac1+
Neutrophils
Spleen Cellularity
Frequency
E
Control
CML
48%/22%
13%/32%
CD25+
CD25-
SSC
Foxp3
CD25 Bone Marrow
CD25 Spleen
Frequency

Supplementary Figure 1: Altered Treg populations in Chronic Myeloid Leukemia.

1A: Representative plots of $Mac1^+Gr1^+$ cells in control (black) and CML (red) mice.

1B: Representative image of in control and CML spleen size.

1C: Gating strategy for chimeric CML mice.

1D: Absolute counts of $CD3^+$, $CD4^+Foxp3^-$, $CD4^+Foxp3^+$ and $CD8^+$ cells in control (black) and CML (red) mice; n=6 mice per group in bone marrow (top) and spleen (bottom).

1E: Absolute counts of spleen and bone marrow cellularity, $Mac1^+Gr1^+$, and Neutrophils in control (black) and CML (red) mice; n=6 mice per group in bone marrow (top) and spleen (bottom).

1E: Representative plot of CD25 expression in $Foxp3^+$ in control and CML mice (top). Absolute counts of CD25+ (blue) and CD25- (red) bone marrow cells; n=6 mice per group.

Data are shown as mean ± SD; graphs represent pooled data from at least three independent experiments. Statistical analysis performed with unpaired two-tailed Student *t* test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

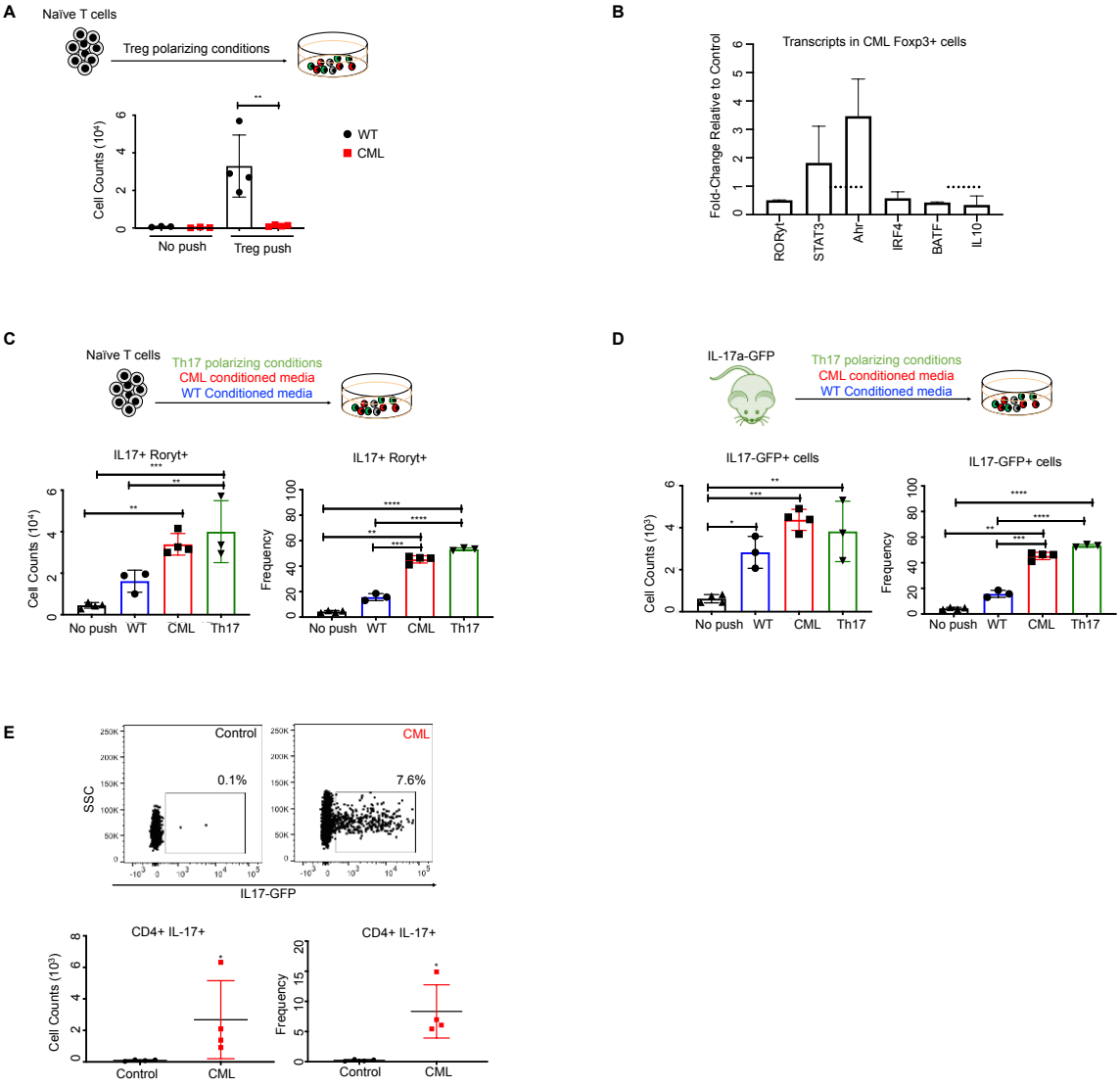
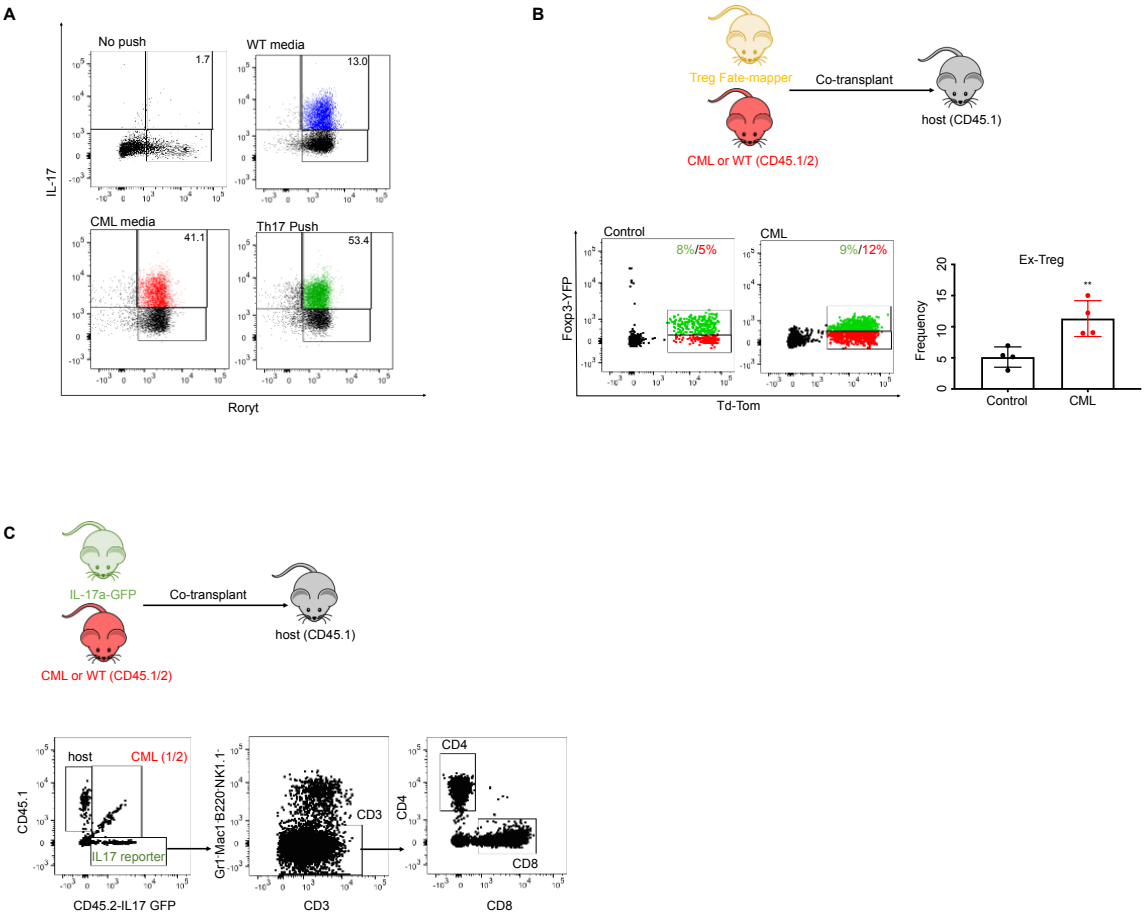

A
Naïve T cells
Treg polarizing conditions
Cell Counts (10⁴)
WT
CML
No push
Treg push
B
Transcripts in CML Foxp3+ cells
Fold-Change Relative to Control
RORγt
STAT3
Ahr
IRF4
BATF
IL 10
C
Naïve T cells
Th17 polarizing conditions
CML conditioned media
WT Conditioned media
IL17+ Roryt+
Cell Counts (10⁴)
Frequency
No push
WT
CML
Th17
D
IL-17a-GFP
Th17 polarizing conditions
CML conditioned media
WT Conditioned media
IL17-GFP+ cells
Cell Counts (10³)
Frequency
E
Control
CML
0.1%
7.6%
SSC
IL17-GFP
CD4+ IL-17+
Cell Counts (10³)
Frequency

Figure 2 Inflammatory cytokines direct development of Th17 cells in CML.
2A: Absolute counts of Foxp3$^+$ following polarization of naïve cells to Tregs in control (black)and CML (red) mice. n=4 mice per group.
2B: qRT-PCR analysis of T cell transcripts comparing Foxp3+ cells from CML and control. Target genes are presented as fold-change (relative to control) for each transcript; n=3 biological replicates per data point, each point was done in triplicate (normalized to TBP1).
2C: Schematic of T cell polarization (top). Differentiation of Th0 cells into Th17 using Th17 polarizing cytokines (green), WT-conditioned media (blue), and CML- conditioned media(red). Absolute counts and frequencies of IL17$^+$ Roryt$^+$ cells (bottom).
2D: Differentiation of Th0 from IL17a-GFP mice cells into Th17 using Th17 polarizing cytokines (green), WT-conditioned media (blue), and CML- conditioned media(red). Absolute counts and frequencies of IL17a-GFP+ cells.
2E: Representative plots of IL17a-GFP$^+$ cells in control and CML mice (top). Absolute counts and frequencies of GFP$^+$ cells in control (black) and CML (red) mice; n=4 mice per group.
Data are shown as mean ± SD (2A,C-F) or SEM (2B); graphs represent data from at least three independent experiments Statistics performed with unpaired two-tailed Student *t* test and one-way ANOVA with Tukey' multiple comparisons test at 95.00% CI of diff; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.


A
No push
WT media
CML media
Th17 Push
1.7
13.0
41.1
53.4
IL-17
Roryt
B
Treg Fate-mapper
Co-transplant
host (CD45.1)
CML or WT (CD45.1/2)
Control
CML
8%/5%
9%/12%
Foxp3-YFP
Td-Tom
Ex-Treg
Frequency
Control
CML
C
IL-17a-GFP
Co-transplant
host (CD45.1)
CML or WT (CD45.1/2)
host
CML (1/2)
IL17 reporter
CD45.1
CD45.2-IL17 GFP
Gr1+Mac1+B220+NK1.1+
CD3
CD4
CD8

Supplementary Figure 2: Inflammatory cytokines direct development of Th17 cells in CML.

S2A: Representative plots of Th0 differentiation into Th17 cells using Th17 polarizing cytokines (green), WT-conditioned media (blue), and CML- conditioned media(red).
S2C: Schematic of Foxp3-Fate mapper model (top). Representative plots of $YFP^{+}TdTom^{+}$ cells (green) and $TdTom^{+}$ cells (red) in control and CML mice. Frequencies of Ex Treg $TdTom^{+}$ cells (red) in control (black) and CML (red) mice; n=4 mice per group.
S2C: Schematic of CML and IL17a-GFP+ chimeric mice and gating strategy
Data are shown as mean ± SD; graphs represent pooled data from at least three independent experiments. Statistical analysis performed with unpaired two-tailed Student *t* test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

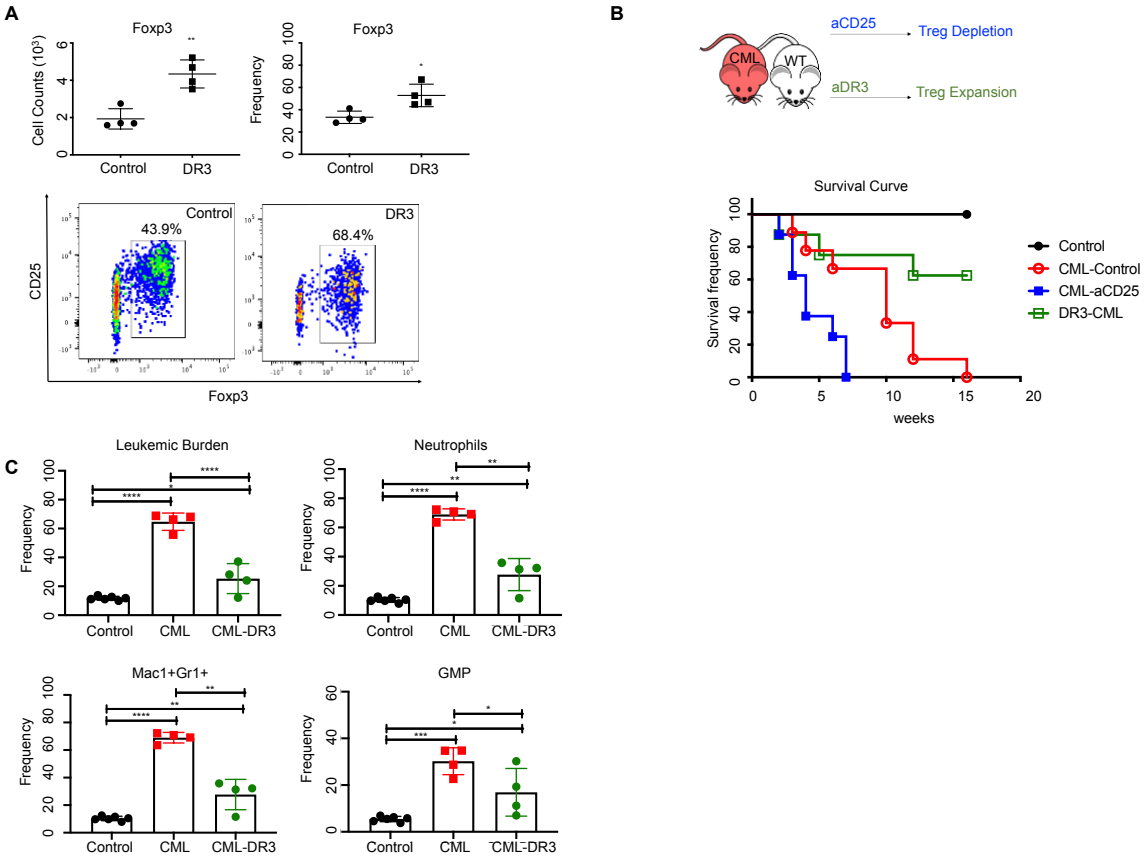

A
Foxp3
Cell Counts (10^5)
Control
DR3
Foxp3
Frequency
Control
DR3
Control
43.9%
DR3
68.4%
CD25
Foxp3
B
CML
WT
aCD25
Treg Depletion
aDR3
Treg Expansion
Survival Curve
Survival frequency
weeks
Control
CML-Control
CML-aCD25
DR3-CML
C
Leukemic Burden
Neutrophils
Mac1+Gr1+
GMP
Frequency
Control
CML
CML-DR3

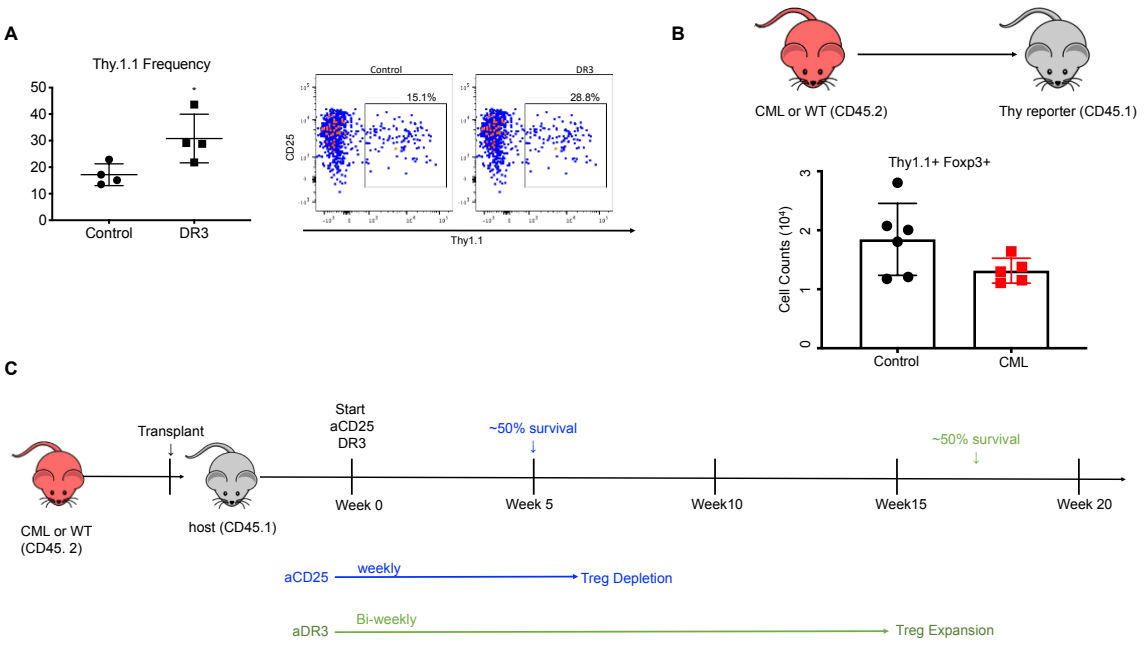
Figure 3:DR3 Treg expansion ameliorates leukemic disease in CML.

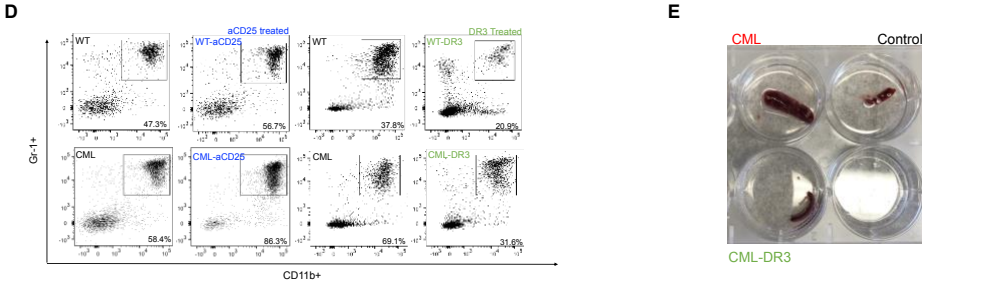
3A: Representative plots, absolute counts, and frequencies of $Foxp3^+$ cells following DR3 stimulation; n=4 per group.

3B: Schematic of DR3 and aCD25 treatment (top). 16-week survival curve of CML, control and Treg treatment groups (bottom). Representative plots of $Mac1^+Gr1^+$ cells in CML (red) and DR3 treated (green) mice.

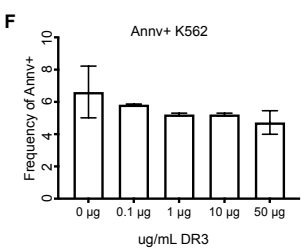
3C: Schematic of control or BCR-ABL+ GMP differentiation to myeloid lineages. Absolute counts of $Mac1^+Gr1^+$ cells, neutrophils in with increasing doses of IL-10; n=3 wells per condition.

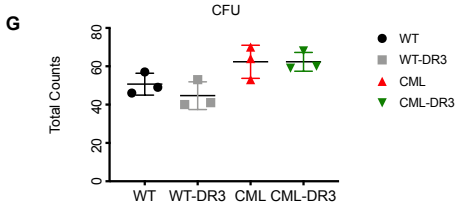
Data are shown as mean ± SD; graphs represent data from at least three independent experiments Statistics performed with unpaired two-tailed Student *t* test and one-way ANOVA with Tukey' multiple comparisons test at 95.00% CI of diff ; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.


A
Thy.1.1 Frequency
Control
DR3
CD25
Thy1.1
B
CML or WT (CD45.2)
Thy reporter (CD45.1)
Thy1.1+ Foxp3+
Cell Counts (10⁴)
Control
CML
C
Transplant
Start aCD25 DR3
~50% survival
~50% survival
CML or WT (CD45. 2)
host (CD45.1)
Week 0
Week 5
Week10
Week15
Week 20
aCD25
weekly
Treg Depletion
aDR3
Bi-weekly
Treg Expansion


D
aCD25 treated
DR3 Treated
WT
WT-aCD25
WT-DR3
CML
CML-aCD25
CML-DR3
Gr-1+
CD11b+

E
CML
Control
CML-DR3


F
Annv+ K562
Frequency of Annv+
0 µg
0.1 µg
1 µg
10 µg
50 µg
ug/mL DR3


G
CFU
Total Counts
WT
WT-DR3
CML
CML-DR3

Supplementary Figure 3 DR3 Treg expansion ameliorates leukemic disease in CML.

S3A: Representative plots, absolute counts, and frequencies of $Thy1.1^+$ cells following DR3 stimulation; n=4 per group.

S3B: Absolute counts, and frequencies of $Thy1.1^+$ cells from control (black) and CML (red) mice; n=6 mice per group.

S3C: Schematic timeline of survival curve and Treg treatments

S3D: Representative plots of $Mac1^+Gr1^+$ cells in aCD25 (blue) and DR3 (green) treated mice.

S3E: Spleen images of control, CML and DR3-treated CML mice at time of sacrifice

S3F. Dose response curve showing frequency of Annexin V^+ K562 cells with increasing doses of DR3

S3G: Colony forming assay of $Lin^-cKit^+Sca1^+$ cells were sorted from control and CML mice following DR3 treatment. Total CFUs after 10 days of culture.

Data are shown as mean ± SD; graphs represent pooled data from at least three independent experiments. Statistical analysis performed with unpaired two-tailed Student *t* test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

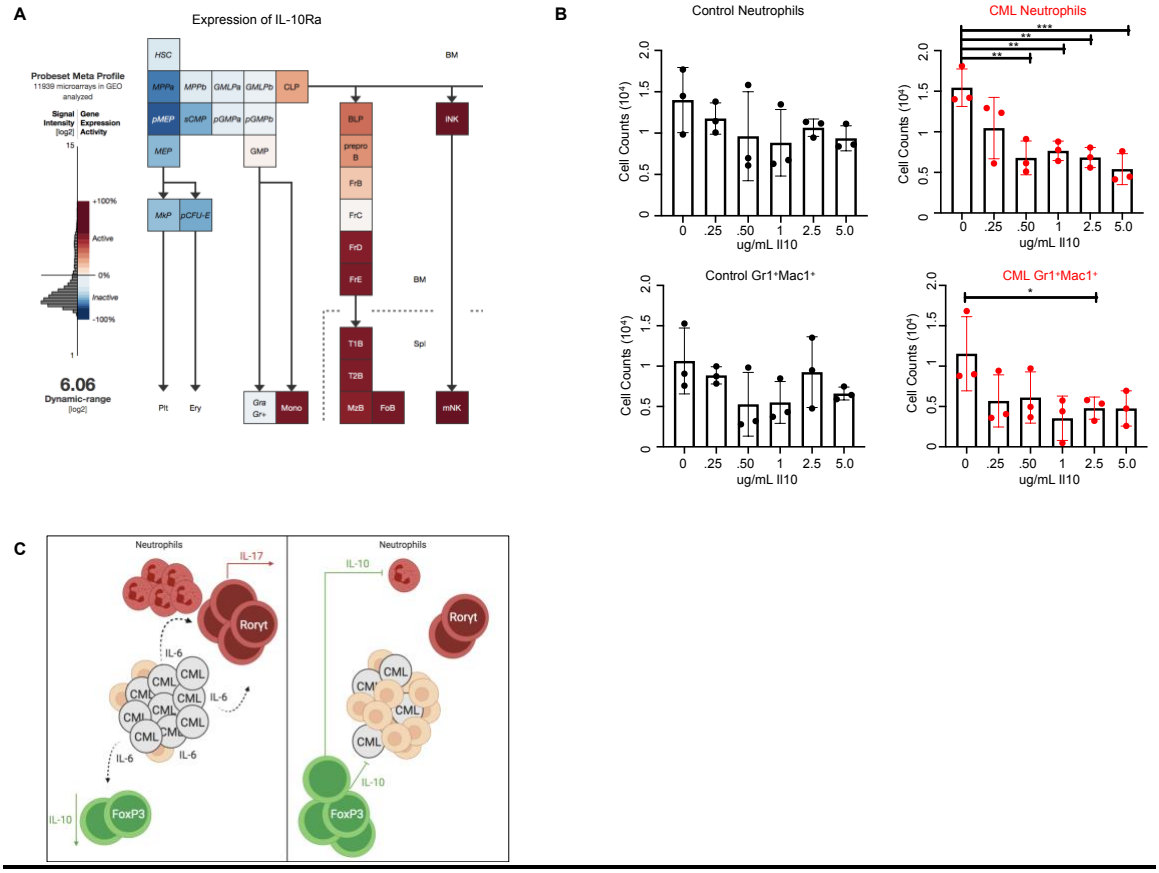

A
Expression of IL-10Ra
Probeset Meta Profile
11939 microarrays in GEO analyzed
Signal Intensity [log2]
Gene Expression Activity
15
+100%
Active
0%
Inactive
-100%
1
6.06
Dynamic-range [log2]
HSC
MPPa
MPPb
GMLPa
GMLPb
CLP
pMEP
sCMP
pGMPa
pGMPb
MEP
GMP
MkP
pCFU-E
Plt
Ery
Gra Gr+
Mono
BM
BLP
prepro B
FrB
FrC
FrD
FrE
T1B
T2B
MzB
FoB
Spl
iNK
mNK
B
Control Neutrophils
CML Neutrophils
Control Gr1+Mac1+
CML Gr1+Mac1+
Cell Counts (10⁴)
0
0.5
1
1.5
2.0
0
.25
.50
1
2.5
5.0
ug/mL Il10
**
***
*
C
Neutrophils
IL-17
Roryt
IL-6
CML
IL-10
FoxP3

Figure 4 Direct effects of IL-10 on leukemic progenitors.

4A: IL10Ra expression data of hematopoietic bone marrow cells from Gene Expression Commons

4B: Absolute counts of Neutrophils and $Mac1^{+}Gr1^{+}$ cells following 10 days of differentiation. Control and BCR-ABL+ GMP progenitors were differentiated with increasing doses of IL-10

4C: Schematic graphical summary of study findings

Data are shown as mean ± SD; graphs represent data from at least three independent experiments Statistics performed with one-way ANOVA with Tukey' multiple comparisons test at 95.00% CI of diff; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

References

1. Brück O, Blom S, Dufva O, Turkki R, Chheda H, Ribeiro A, et al. Immune cell contexture in the bone marrow tumor microenvironment impacts therapy response in CML. *Leukemia.* 2018;32(7):1643-56.
2. Zhou T, Medeiros LJ, and Hu S. Chronic Myeloid Leukemia: Beyond BCR-ABL1. *Curr Hematol Malig Rep.* 2018;13(6):435-45.
3. Houshmand M, Simonetti G, Circosta P, Gaidano V, Cignetti A, Martinelli G, et al. Chronic myeloid leukemia stem cells. *Leukemia.* 2019;33(7):1543-56.
4. Ureshino H, Shindo T, and Kimura S. Role of cancer immunology in chronic myelogenous leukemia. *Leukemia research.* 2020;88:106273.
5. Kang ZJ, Liu YF, Xu LZ, Long ZJ, Huang D, Yang Y, et al. The Philadelphia chromosome in leukemogenesis. *Chin J Cancer.* 2016;35:48.
6. Reynaud D, Pietras E, Barry-Holson K, Mir A, Binnewies M, Jeanne M, et al. IL-6 controls leukemic multipotent progenitor cell fate and contributes to chronic myelogenous leukemia development. *Cancer cell.* 2011;20(5):661-73.
7. Mumprecht S, Schürch C, Schwaller J, Solenthaler M, and Ochsenbein AF. Programmed death 1 signaling on chronic myeloid leukemia-specific T cells results in T-cell exhaustion and disease progression. *Blood.* 2009;114(8):1528-36.
8. Salih J, Hilpert J, Placke T, Grünebach F, Steinle A, Salih HR, et al. The BCR/ABL-inhibitors imatinib, nilotinib and dasatinib differentially affect NK cell reactivity. *Int J Cancer.* 2010;127(9):2119-28.
9. Kreutzman A, Juvonen V, Kairisto V, Ekblom M, Stenke L, Seggewiss R, et al. Mono/oligoclonal T and NK cells are common in chronic myeloid leukemia patients at diagnosis and expand during dasatinib therapy. *Blood.* 2010;116(5):772-82.
10. Rojas JM, Wang L, Owen S, Knight K, Watmough SJ, and Clark RE. Naturally occurring CD4+ CD25+ FOXP3+ T-regulatory cells are increased in chronic myeloid leukemia patients not in complete cytogenetic remission and can be immunosuppressive. *Experimental hematology.* 2010;38(12):1209-18.
11. Zahran AM, Badrawy H, and Ibrahim A. Prognostic value of regulatory T cells in newly diagnosed chronic myeloid leukemia patients. *Int J Clin Oncol.* 2014;19(4):753-60.
12. Hughes A, Clarson J, Tang C, Vidovic L, White DL, Hughes TP, et al. CML patients with deep molecular responses to TKI have restored immune effectors and decreased PD-1 and immune suppressors. *Blood.* 2017;129(9):1166-76.
13. O'Shea JJ, and Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science (New York, NY).* 2010;327(5969):1098-102.
14. Pepper M, and Jenkins MK. Origins of CD4(+) effector and central memory T cells. *Nature immunology.* 2011;12(6):467-71.
15. Zhou L, Chong MM, and Littman DR. Plasticity of CD4+ T cell lineage differentiation. *Immunity.* 2009;30(5):646-55.
16. Zhu J, and Paul WE. CD4 T cells: fates, functions, and faults. *Blood.* 2008;112(5):1557-69.
17. Hirata Y, Furuhashi K, Ishii H, Li HW, Pinho S, Ding L, et al. CD150(high) Bone Marrow Tregs Maintain Hematopoietic Stem Cell Quiescence and Immune Privilege via Adenosine. *Cell stem cell.* 2018;22(3):445-53.e5.

18. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, and Yamaguchi T. Regulatory T cells: how do they suppress immune responses? *International immunology.* 2009;21(10):1105-11.
19. Richards DM, Delacher M, Goldfarb Y, Kägebein D, Hofer AC, Abramson J, et al. Treg Cell Differentiation: From Thymus to Peripheral Tissue. *Prog Mol Biol Transl Sci.* 2015;136:175-205.
20. Barbi J, Pardoll D, and Pan F. Treg functional stability and its responsiveness to the microenvironment. *Immunol Rev.* 2014;259(1):115-39.
21. Kimura A, and Kishimoto T. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol.* 2010;40(7):1830-5.
22. Lee GR. The Balance of Th17 versus Treg Cells in Autoimmunity. *Int J Mol Sci.* 2018;19(3).
23. Naufel AO, Aguiar MCF, Madeira FM, and Abreu LG. Treg and Th17 cells in inflammatory periapical disease: a systematic review. *Braz Oral Res.* 2017;31:e103.
24. Ohue Y, and Nishikawa H. Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? *Cancer Sci.* 2019;110(7):2080-9.
25. Tanaka A, and Sakaguchi S. Targeting Treg cells in cancer immunotherapy. *Eur J Immunol.* 2019;49(8):1140-6.
26. Wolf D, Sopper S, Pircher A, Gastl G, and Wolf AM. Treg(s) in Cancer: Friends or Foe? *J Cell Physiol.* 2015;230(11):2598-605.
27. Dominguez-Villar M, and Hafler DA. Regulatory T cells in autoimmune disease. *Nature immunology.* 2018;19(7):665-73.
28. Munn DH, Sharma MD, and Johnson TS. Treg Destabilization and Reprogramming: Implications for Cancer Immunotherapy. *Cancer research.* 2018;78(18):5191-9.
29. Overacre-Delgoffe AE, and Vignali DAA. Treg Fragility: A Prerequisite for Effective Antitumor Immunity? *Cancer Immunol Res.* 2018;6(8):882-7.
30. Zhang Z, and Zhou X. Foxp3 Instability Helps tTregs Distinguish Self and Non-self. *Frontiers in immunology.* 2019;10:2226.
31. Fasching P, Stradner M, Graninger W, Dejaco C, and Fessler J. Therapeutic Potential of Targeting the Th17/Treg Axis in Autoimmune Disorders. *Molecules.* 2017;22(1).
32. Knochelmann HM, Dwyer CJ, Bailey SR, Amaya SM, Elston DM, Mazza-McCrann JM, et al. When worlds collide: Th17 and Treg cells in cancer and autoimmunity. *Cellular & molecular immunology.* 2018;15(5):458-69.
33. Shao S, Yu X, and Shen L. Autoimmune thyroid diseases and Th17/Treg lymphocytes. *Life Sci.* 2018;192:160-5.
34. Bedoya SK, Lam B, Lau K, and Larkin J, 3rd. Th17 cells in immunity and autoimmunity. *Clin Dev Immunol.* 2013;2013:986789.
35. Yasuda K, Takeuchi Y, and Hirota K. The pathogenicity of Th17 cells in autoimmune diseases. *Semin Immunopathol.* 2019;41(3):283-97.
36. Durant L, Watford WT, Ramos HL, Laurence A, Vahedi G, Wei L, et al. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity.* 2010;32(5):605-15.
37. Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM, et al. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity.* 2011;34(4):566-78.
38. Jadidi-Niaragh F, and Mirshafiey A. Th17 cell, the new player of neuroinflammatory process in multiple sclerosis. *Scand J Immunol.* 2011;74(1):1-13.

39. Stewart CA, Metheny H, Iida N, Smith L, Hanson M, Steinhagen F, et al. Interferon-dependent IL-10 production by Tregs limits tumor Th17 inflammation. *The Journal of clinical investigation.* 2013;123(11):4859-74.
40. Barilla RM, Diskin B, Caso RC, Lee KB, Mohan N, Buttar C, et al. Specialized dendritic cells induce tumor-promoting IL-10(+)IL-17(+) FoxP3(neg) regulatory CD4(+) T cells in pancreatic carcinoma. *Nature communications.* 2019;10(1):1424.
41. Korn T, Bettelli E, Oukka M, and Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol.* 2009;27:485-517.
42. Park SH, Jung HJ, and Kim TS. IL-33 changes CD25(hi) Tregs to Th17 cells through a dendritic cell-mediated pathway. *Immunol Lett.* 2020;218:5-10.
43. Downs-Canner S, Berkey S, Delgoffe GM, Edwards RP, Curiel T, Odunsi K, et al. Suppressive IL-17A(+)Foxp3(+) and ex-Th17 IL-17A(neg)Foxp3(+) T(reg) cells are a source of tumour-associated T(reg) cells. *Nature communications.* 2017;8:14649.
44. Turka LA, and Walsh PT. IL-2 signaling and CD4+ CD25+ Foxp3+ regulatory T cells. *Front Biosci.* 2008;13:1440-6.
45. Ohkura N, Hamaguchi M, Morikawa H, Sugimura K, Tanaka A, Ito Y, et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity.* 2012;37(5):785-99.
46. Sawant DV, and Vignali DA. Once a Treg, always a Treg? *Immunol Rev.* 2014;259(1):173-91.
47. Sawant DV, Yano H, Chikina M, Zhang Q, Liao M, Liu C, et al. Adaptive plasticity of IL-10(+) and IL-35(+) T(reg) cells cooperatively promotes tumor T cell exhaustion. *Nature immunology.* 2019;20(6):724-35.
48. Nakatsukasa H, Oda M, Yin J, Chikuma S, Ito M, Koga-Iizuka M, et al. Loss of TET proteins in regulatory T cells promotes abnormal proliferation, Foxp3 destabilization, and IL-17 expression. *International immunology.* 2019.
49. Yue X, Lio CJ, Samaniego-Castruita D, Li X, and Rao A. Loss of TET2 and TET3 in regulatory T cells unleashes effector function. *Nature communications.* 2019;10(1):2011.
50. Gagliani N, Amezcua Vesely MC, Iseppon A, Brockmann L, Xu H, Palm NW, et al. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. *Nature.* 2015;523(7559):221-5.
51. Liu X, Hu H, Fan H, Zuo D, Shou Z, Liao Y, et al. The role of STAT3 and AhR in the differentiation of CD4+ T cells into Th17 and Treg cells. *Medicine (Baltimore).* 2017;96(17):e6615.
52. Camporeale A, and Poli V. IL-6, IL-17 and STAT3: a holy trinity in auto-immunity? *Front Biosci (Landmark Ed).* 2012;17:2306-26.
53. Chang H, Zhao F, Xie X, Liao Y, Song Y, Liu C, et al. PPARα suppresses Th17 cell differentiation through IL-6/STAT3/RORγt pathway in experimental autoimmune myocarditis. *Exp Cell Res.* 2019;375(1):22-30.
54. Park SH, Kim MS, Lim HX, Cho D, and Kim TS. IL-33-matured dendritic cells promote Th17 cell responses via IL-1β and IL-6. *Cytokine.* 2017;99:106-13.
55. Subbanna M, Shivakumar V, Talukdar PM, Narayanaswamy JC, Venugopal D, Berk M, et al. Role of IL-6/RORC/IL-22 axis in driving Th17 pathway mediated immunopathogenesis of schizophrenia. *Cytokine.* 2018;111:112-8.
56. Wang B, Tang Y, Sun X, Ouyang X, Li H, Wei J, et al. Increased IL-6 expression on THP-1 by IL-34 stimulation up-regulated rheumatoid arthritis Th17 cells. *Clin Rheumatol.* 2018;37(1):127-37.

57. Zhao M, Tan Y, Peng Q, Huang C, Guo Y, Liang G, et al. IL-6/STAT3 pathway induced deficiency of RFX1 contributes to Th17-dependent autoimmune diseases via epigenetic regulation. *Nature communications.* 2018;9(1):583.
58. Li J, Shi W, Sun H, Ji Y, Chen Y, Guo X, et al. Activation of DR3 signaling causes loss of ILC3s and exacerbates intestinal inflammation. *Nature communications.* 2019;10(1):3371.
59. Siakavellas SI, Sfikakis PP, and Bamias G. The TL1A/DR3/DcR3 pathway in autoimmune rheumatic diseases. *Semin Arthritis Rheum.* 2015;45(1):1-8.
60. Valatas V, Kolios G, and Bamias G. TL1A (TNFSF15) and DR3 (TNFRSF25): A Co-stimulatory System of Cytokines With Diverse Functions in Gut Mucosal Immunity. *Frontiers in immunology.* 2019;10:583.
61. Wolf D, Schreiber TH, Tryphonopoulos P, Li S, Tzakis AG, Ruiz P, et al. Tregs expanded in vivo by TNFRSF25 agonists promote cardiac allograft survival. *Transplantation.* 2012;94(6):569-74.
62. Schreiber TH, Wolf D, Tsai MS, Chirinos J, Deyev VV, Gonzalez L, et al. Therapeutic Treg expansion in mice by TNFRSF25 prevents allergic lung inflammation. *The Journal of clinical investigation.* 2010;120(10):3629-40.
63. Madireddi S, Eun SY, Mehta AK, Birta A, Zajonc DM, Niki T, et al. Regulatory T Cell-Mediated Suppression of Inflammation Induced by DR3 Signaling Is Dependent on Galectin-9. *J Immunol.* 2017;199(8):2721-8.
64. Maynard CL, Harrington LE, Janowski KM, Oliver JR, Zindl CL, Rudensky AY, et al. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nature immunology.* 2007;8(9):931-41.
65. Landuyt AE, Klocke BJ, Colvin TB, Schoeb TR, and Maynard CL. Cutting Edge: ICOS-Deficient Regulatory T Cells Display Normal Induction of Il10 but Readily Downregulate Expression of Foxp3. *J Immunol.* 2019;202(4):1039-44.
66. Seita J, Sahoo D, Rossi DJ, Bhattacharya D, Serwold T, Inlay MA, et al. Gene Expression Commons: an open platform for absolute gene expression profiling. *PloS one.* 2012;7(7):e40321.
67. Chen P, Wang M, Li D, Jia Y, He N, Li W, et al. The alteration and clinical significance of Th22/Th17/Th1 cells in patients with chronic myeloid leukemia. *Journal of immunology research.* 2015;2015:416123.
68. Panteli KE, Hatzimichael EC, Bouranta PK, Katsaraki A, Seferiadis K, Stebbing J, et al. Serum interleukin (IL)-1, IL-2, sIL-2Ra, IL-6 and thrombopoietin levels in patients with chronic myeloproliferative diseases. *Br J Haematol.* 2005;130(5):709-15.
69. Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, et al. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell.* 2008;133(6):1019-31.
70. Ciarcia R, Vitiello MT, Galdiero M, Pacilio C, Iovane V, d'Angelo D, et al. Imatinib treatment inhibit IL-6, IL-8, NF-KB and AP-1 production and modulate intracellular calcium in CML patients. *J Cell Physiol.* 2012;227(6):2798-803.
71. Sanchez-Correa B, Bergua JM, Campos C, Gayoso I, Arcos MJ, Bañas H, et al. Cytokine profiles in acute myeloid leukemia patients at diagnosis: survival is inversely correlated with IL-6 and directly correlated with IL-10 levels. *Cytokine.* 2013;61(3):885-91.
72. Nievergall E, Reynolds J, Kok CH, Watkins DB, Biondo M, Busfield SJ, et al. TGF-α and IL-6 plasma levels selectively identify CML patients who fail to achieve an early molecular response or progress in the first year of therapy. *Leukemia.* 2016;30(6):1263-72.

73. Kuepper MK, Bütow M, Herrmann O, Ziemons J, Chatain N, Maurer A, et al. Stem cell persistence in CML is mediated by extrinsically activated JAK1-STAT3 signaling. *Leukemia.* 2019;33(8):1964-77.
74. Welner RS, Amabile G, Bararia D, Czibere A, Yang H, Zhang H, et al. Treatment of chronic myelogenous leukemia by blocking cytokine alterations found in normal stem and progenitor cells. *Cancer cell.* 2015;27(5):671-81.
75. Abousamra NK, Salah El-Din M, and Helal R. Prognostic value of Th17 cells in acute leukemia. *Med Oncol.* 2013;30(4):732.
76. Holz K, Prinz M, Brendecke SM, Hölscher A, Deng F, Mitrücker HW, et al. Differing Outcome of Experimental Autoimmune Encephalitis in Macrophage/Neutrophil- and T Cell-Specific gp130-Deficient Mice. *Frontiers in immunology.* 2018;9:836.
77. McGinley AM, Sutton CE, Edwards SC, Leane CM, DeCourcey J, Teijeiro A, et al. Interleukin-17A Serves a Priming Role in Autoimmunity by Recruiting IL-1β-Producing Myeloid Cells that Promote Pathogenic T Cells. *Immunity.* 2020;52(2):342-56.e6.
78. Wojkowska DW, Szpakowski P, Ksiazek-Winiarek D, Leszczynski M, and Glabinski A. Interactions between neutrophils, Th17 cells, and chemokines during the initiation of experimental model of multiple sclerosis. *Mediators Inflamm.* 2014;2014:590409.

Regulation of normal and leukemic stem cells through cytokine signaling and the microenvironment

by

Virginia Camacho, Victoria McClearn, Sweta Patel, Robert S Welner

Camacho V, McClearn V, Patel S, Welner RS. Regulation of normal and leukemic stem cells through cytokine signaling and the microenvironment. Int J Hematol. 2017 May;105(5):566-577. doi: 10.1007/s12185-017-2184-6. Epub 2017 Feb 7. PMID: 28176225.

Copyright
2017
by
Camacho et al
Used by permission

Format adapted [and errata corrected] for [thesis or] dissertation

Internation Journal of Hematology

"Progress in Hematology"

Regulation of normal and leukemic stem cells through cytokine signaling and the microenvironment

Virginia Camacho*, Victoria McClearn*, Sweta Patel*, and Robert S. Welner

* Co-first authors

Division of Hematology and Oncology, The University of Alabama at Birmingham, Birmingham, AL, USA

Corresponding Author:

Robert S. Welner

Comprehensive Cancer Center

1824 6th Ave S, WTI 510D

Birmingham, AL, USA 35294

Ph#: +1 205-975-3960

Email: rwelner@uab.edu

Summary: Leukemias depend on transformed stem cells for their growth and thus these cells represent important therapeutic targets. However, leukemic stem cells resemble normal hematopoietic stem cells (HSCs) with respect to most surface markers, gene expression patterns, and ability to be transplanted. Furthermore, the microenvironment that supports healthy HSCs non-hematopoietic populations, and immune cells together with the cytokines and adhesion molecules these cells produce is perturbed during leukemogenesis. This altered environment promotes leukemic growth through pro-inflammatory cytokines. Here, we characterize normal and leukemic signaling, as well as the instructive cues from the neighboring hematopoietic cells and the microenvironment, that promote stem cell self-renewal and differentiation.

All mature blood cells are derived from rare hematopoietic stem cells (HSCs) that reside in the adult bone marrow[1]. Evidence shows that proper gene expression and signaling transduction in HSCs is essential for homeostasis. Several transcription factors and signaling pathways are critical for the formation and function of HSCs, and for the differentiation to specific blood lineages. However, malfunctioning HSCs through dysregulated transcription factor expression can result in malignancies. Furthermore, following hematologic injuries or leukemia, alterations in non-transformed HSC result in life threatening complications from infection, bleeding, and anemia. In this review, we focus on work that strives to understand the cells and signaling pathways that regulate, maintain, and interact with HSCs and leukemic stem cells.

Cytokines and Immune Regulation of HSCs and LSCs

The hematopietic stem cells (HSC) rely on mature myeloid and lymphoid lineage cells to help regulate their differentiation and self-renewal. One function is to protect stem cells and allow them to undergo adequate maturation; another is to eliminate HSCs that have become malignant[2]. The transition from normal to aberrant hematopoiesis is accompanied by a spectrum of immunological alterations (**FIGURE 1**). During leukemogenesis vital cell functions such as proliferation, differentiation, and signal transduction become dysregulated[3,4]. Furthermore, the cytokines that regulate normal hematopoiesis are also perturbed. The interchange of these molecules is needed to maintain the hematopoietic microenvironment and immune homeostasis[5]. While cytokine networks mediate communication across numerous cell types, one of their principal functions is to serve as immunomodulating agents; they are specifically important for coordinating the class and magnitude of the immune response[6]. Downstream effects can be antagonistic, additive, or synergistic. Currently, cytokine immunotherapy is being applied to combat leukemic stem cell (LSC) pathophysiology[7,8]. However, the pleiotropic nature of cytokine signaling

makes it difficult for treatments to be specific and have a high efficacy; off target effects are a major obstacle. To move forward, a more detailed and precise mapping of the cytokine landscape is necessary.

LSCs utilize specific mechanisms to escape immune surveillance; they secrete various co-stimulatory molecules and suppressive cytokines[9]. This hinders effector immune cell function, and deleteriously reconstitutes the hematopoietic microenvironment. At the molecular level LSCs and their differentiated progeny exhibit unique genetic signatures and expression profiles[10,11] that allow them to express different antigens[12]. LSCs also target metabolic pathways[13], and create an anti-apoptotic environment which facilitates their own proliferation while distorting the recycling of immune cells[14]. Additionally, several surface proteins common to immune cells, including T-cell immunoglobulin mucin-3 (TIM-3) have been associated with LSC self-renewal and leukemic transformation[15].

The bone marrow is an important immunological transit point, as previously reviewed[16]. In addition to serving as a primary organ for hematopoiesis the bone marrow is also an integral site for lymphocyte circulation[17]. The cytokine networks that run through the bone marrow are important for HSC maturation, pluripotency, and contact with immune cells. Alterations of this milieu within the bone marrow have been implicated in clonal stem cell disorders as well as expansion LSCs[7]. For both LSCs and immune cells, the bone marrow serves as a supportive apparatus for growth and development. Correspondingly, it is the principal site for crosstalk between these cells. Thus, it is necessary to tease out the cell-cell contact mechanisms and soluble factors that are exchanged between LSC and immune cells within the bone marrow microenvironment. The trafficking of various immune cells within this microenvironment has been observed. These immune cell populations include, conventional T cells, regulatory T cells (T-regs), B cells, Natural Killer cells

(NKs), Neutrophils, Dendritic Cells (DCs), and myeloid derived suppressor cells (MDSCs)[18]. Not only does the bone marrow act as a reservoir for immune cells, it also serves as a differentiation regulatory organ. In thymectomized animals, the bone marrow provides necessary architectural elements to support T cell maturation[19,20]. In addition to modulating HSC compartment, the immune cell trafficked through the bone marrow also cross-talk with other resident cells. Therefore, it can be expected that, the dysregulation of immune networks within the bone marrow has sweeping effects on the niche populations.

During hematopoiesis, immune cells play a requisite modulatory role. Resident cells maintain HSC homeostasis through various mechanisms; they eradicate aberrant clones, balance cell proliferation, and protect stem cells from apoptosis[21]. T-regs are key mediators in this process. Notably, T-reg frequency in the bone marrow is higher than the spleen and lymph nodes; they have been shown to comprise as much as 30% of CD4+ T cells in the bone marrow[21]. T-regs are a dynamic class of cells that temper the immune response throughout the body. T-regs shield the stem cell compartment from autoimmunity, excessive inflammation, and apoptosis. Thus, the bone marrow serves as a site of immune 'privilege' where regulatory cells establish a gradient of immune suppression and regulation[22]. T-regs suppress CD4+ and CD8+ T cells cytokine production, prominently IL-2 [23]. They also secrete various inhibitory cytokines, such as IL-35 to regulate T cell activity and apoptosis[24,25]. Consequently, a growing body of evidence suggests that T-regs are hijacked by LSCs for protection and survival. It is likely that LSCs recruit T-regs and exploit their modulatory capacities to escape detection[26]. The mechanism by which LSC target T-regs remains to be elucidated; they may act on them directly, or employ signals from the periphery.

In particular, myelodysplastic syndrome (MDS) is a useful model for characterizing the immune regulation of LSCs. MDS is marked by various defects which encumber normal hematopoiesis; this includes autoimmunity, excess inflammation, dysregulated cell death, impaired signaling. Accumulation of these events leads to aggressive clonal proliferation within the bone marrow that goes undetected by the immune system. Given the intersectionality between MDS and leukemia, as well as the propensity to proceed from one to the other, it is worth noting that there are common pathologies associated with both diseases. Two key features are the dysregulation of innate immunity, and an increase in inflammation[27]. In leukemia, miR-29b has been shown to alter the capacities of NK cells, which disrupts the innate immune response and allows LSCs to escape surveillance[28]. Correspondingly, reduced NK function is seen in MDS patients as the disease progresses[29], and synthetic reactivation of NK cell activity has been explored as a potentially therapeutic treatment[30]. With respect to signaling, cytokine stimulation and differentiation of NK cell populations has been shown to enhance NK cell response and functionality[31]. It has also been shown that LSCs employ the CD160 signaling axis, a pathway common to NK cells and CD8 T cells, to facilitate their expansion[32].

Mast cells, which serve as activators of inflammation, have also been implicated. Mast cell growth factor, a ligand for c-kit, has been shown to promote hematopoiesis and proliferation of leukemic precursors[33,34]. Dysregulation of this receptor tyrosine kinase, accompanies the corrosion of the stem cell niche and the hematopoietic system in MDS and other stem cell malignancies. Likewise the regulation of c-kit receptor by Ran-binding protein microtubule-organizing center appears to function in the development of bone marrow progenitors[35]. Leukemia-initiating-cells have also been associated with a mast cell signature, and the IL2/CD25 axis may serve as a key regulator in leukemic cell activation[36]. As with leukemia, T-reg functionality is distorted in MDS; T-reg receptor profiles as well as migratory and homing capacities

to the bone marrow are altered to favor LSC development[37]. Both MDS and leukemia are marked by unique cytokine and chemokine expression patterns[9] and it is likely that this alters the communication landscape between immune cells, stem cells, and stromal cells of the hematopoietic system. Similarly, disequilibrium of the signal transduction pathway and malignant cell survival has also been observed to be correlated[38]. A notable feature of leukemia and MDS development is the deregulation of Toll-like Receptors (TLRs); these receptors are critical for innate immune signaling[39] and they are important for the cooperative regulation of pro-inflammatory cytokines and chemokines. In MDS, a significant upregulation of TLR1, 2 and 6 has been observed; specifically, the TLR2 signaling axis has been shown to mediate inflammation via the activation of activate IL-8 and histone demethylase JMJD3. Likewise, levels of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), transforming growth factor beta (TGF-β), and interleukins IL-6 and IL-8 are also elevated[40-42]. Moreover, TLR dysfunction has been implicated in the progression to leukemia where signal transduction of important proliferative and apoptotic pathways is not properly activated[43]. The cytokine imbalances and dysregulated activation of TLRs observed in MDS stifles immune cell function, increases inflammation, and disrupts cellular communication. Collectively, these changes contribute to the formation of the leukemic stem cell niche.

Many hematological disorders, such as MDS and leukemia are pathologies concomitant with aging. This increase in age causes significant remodeling of the bone marrow microenvironment. One feature, is the gradual replacement of the bone marrow compartment with adipose tissue[44]. Another component of aging is immunosenescence. The decline of immune function that results from aging has various consequences on the stem cell lineages; it hinders the bone marrow's ability to nurture precursors, and leads to dysplastic changes in resident cells, defective apoptosis, and cytopenia[45]. Loss of functionality disrupts cytokine and hormone

networks necessary to maintain the various hematopoietic lineages[46,47]. Because HSCs depend on adequate signaling from the bone marrow for proper quiescence and self-renewal, the age-related decline of immune function is often a trigger for leukemic transformation[48]. Cellular senescence increases chronic inflammation through the senescence-associated secretory phenotype (SASP). This phenotype engages cell cycle and metabolism related genes, soluble factors (chemokines, growth factors, and interleukins), secreted proteases and ECM components[49-51]. IL-1, IL-6, and IL-8 are some of the most significantly upregulated interleukins[52]. The insulin-like growth factor (IGF)/IGF receptor signaling axis also contributes to SASP profile and an overall inflammatory phenotype[53]. Senescent cells also amplify the CXCR2 signaling networks via increased secretion of CXCR-2 (IL-8RB)-binding chemokines[54]. The aggregate effects of disrupted cellular signaling in combination with the loss of immunological capacity create a climate permissive of LSC expansion. Aging and cellular senescence introduces changes to the bone marrow niche, and resident populations which alter the landscape of soluble and insoluble factors in the microenvironment. These changes syngergisically impeding proper hematopoietic maturation.

Under normal conditions HSCs move through a series of steps as they evolve into specialized cells. The burden of regulating these transitions falls heavily on the immune system. Proper clonal expansion and trimming of malignant cells is carried out under the directive of immune cells. In hematopoietic pathologies, such as leukemia, the transition from HSCs to LSCs is accompanied by a loss of immune function. When immune regulation fails, HSCs lose the ability to balance self-renewal and lineage commitment; hallmarks of LSC development include abnormal expansion and dysregulated proliferation. Given the dependency between these cell types, an investigation of immune components is required to determine the regulatory checkpoints that go awry during LSC biogenesis. The balance between immune

surveillance and immune evasion is a critical topic of exploration regarding LSC development and disease survival.

LSCs Crosstalk with the Microenvironment

The bone marrow niche is a complex arrangement of cells such as osteoblasts, endothelial cells, mesenchymal stem cells, stromal cells, and the above-mentioned immune cells. Within the bone marrow (BM) two distinct niches have been observed, the endosteum and vascular niche. The majority of HSCs reside in the endosteum, inner surface of the BM, which is lined with cells such as osteoblasts and osteoclasts. The sinusoidal (vascular) niche is comprised of thin vessels along with reticular cells, stromal cells, mesenchymal stromal cells (MSCs), and neurons, all of which can regulate and sustain HSCs and allow for communication with the rest of the body. Similar to immune cells within the microenvironment, niche cells communicate with HSCs other through cytokines, chemokines, and adhesion molecules that regulate self-renewal, quiescence, and mobilization.

In order to define the cells that constitute the niche, fate-mapping models have shown that stromal tissue, bone, and cartilage are derived from increasing lineage-restricted stem and progenitor cells[55]. Growth plate studies reveal a high frequency of non-hematopoietic, non-endothelial cells that contain 8 distinct subpopulations with differential expression of CD105, Thy, 6C3, and CD200. The populations resolved from this staining are: skeletal stem cells (SSC), bone, cartilage, and stromal progenitor (BCSP), pre-BCSP, B-lymphocyte stromal progenitors (BLSP) 6C3 (as defined by expression of Ly-51), hepatic leukemia factor expressing cells (HEC), Thy (based on CD90/Thy1 expression), and pro-chondrogenic progenitor (PCP). SSC, pre-BCSP, and BCSP had the potential to give rise to bone, cartilage, and marrow, while BLSP, 6C3, HEC, and Thy made primarily bone. As suggested by its name, PCP gave rise to cartilage[55]. Similar to the hematopoietic system of differentiation,

the skeletal system has a hierarchy of progenitor-precursor relationships that are just now being discovered.

Several factors known to support hematopoietic stem cells have been used to resolve subsets of cells within the Bone Marrow Microenvironment (BMM). These include CXCL-12, angiopoietin-1, stem cell factor (SCF), jagged-1, and thrombopoietin[56]. Recent work has taken this a step further by using single cell analysis to define populations in close proximity to HSCs[57]. Cells within short distances of HSPCs had upregulation of cell surface proteins Vcam1, Adam9, and Amot as well as immune response genes Map3K14, CXCL12, and IL-18. Additionally, these proximal cells were found to be lineage committed but still immature. Conditional knockout-models have showed that mature osteoblastic cell derived RNase angiogenin (ANG) regulates lymphoid progenitors, while mesenchymal and osteolineage progenitor cell derived ANG aids in HSC quiescence and repopulation. Furthermore, it was shown that proximal cells secrete IL-18 and employ this cytokine to regulate quiescence of short-term progenitors. Lastly, it has been demonstrated that antibody inhibition of cell adhesion molecule, such as Embigin, promotes an increase in the frequency of HSCs, progenitors, and colony-forming cells. Taken together these results establish that the BMM is a highly organized unit of cells, and that these populations cross talk with HSCs to regulate hematopoiesis.

The study of the BMM on the pathogenesis of hematopoietic malignancies is not well understood. Current literature points to an interaction between LSC and MSCs which may impact both populations, perhaps modulating the molecular pathways that regulate normal HSCs. Understanding the niche in leukemia will provide insight on how to alter the microenvironment to target malignant cells. Disruption of the niche generates an array of problems, and has been shown to help LSCs evade

therapeutic agents[58]. In xenograft models, LSCs disrupt the microenvironment and normal HSCs behavior through chemokine signaling. Stromal Cell Derived Factor 1 (SDF-1), also known as CXCL12, is a chemokine secreted by the microenvironment to maintain HSCs and aid in their homing; it is upregulated during times of hypoxia and inflammation. However, upon serial transplantation, acute lymphoblastic leukemia LSCs were found to home to SDF-1 negative areas. Through cytokine signaling these altered niches are able to dysregulate the microenvironment to exclude HSCs[59]. Confocal microscopy studies reveal that, healthy HSCs compete with acute myeloid leukemia (AML) LSCs for physical niche occupancy in competitive transplantation models[60]. Mice receiving higher doses of either whole BM or HSCs were able to survive longer with LSCs due to either competition for occupancy or resources. However, the two cells types were not seen to inhabit the same environment. Together these studies demonstrate that the niche is altered during leukemia and suggests that LSCs physically outcompete HSCs.

The specific ways in which leukemic cells alter the microenvironment to disrupt normal hematopoiesis is not well defined. As mentioned previously, CXCL12 is decreased in some leukemia[59]; however, the role CXCL12 plays in LSCs maintenance is still unknown. Using high resolution imaging of fate mapping mouse models, it has been shown that LSCs home to the endothelial region of the BMM where CXCL12 production was stable[61]. Additionally, the burden of LSCs decreases upon the deletion of the CXCL12 gene from endothelial cells. Moreover, deletion or inhibition of CXCR4 from LSCs decreases the leukemic burden. To demonstrate therapeutic potential, a small molecule, AMD3465, has been used as an antagonist to the receptor in both murine and human leukemic xenograft models. In both models, leukemic burden decreased upon addition of this small molecule therapy[61]. Similarly, AML mouse models have shown that the LSCs induce transcriptional changes in normal MSCs. BM stromal cells from AML patients show a shift in

differentiation with more osteoblastic cells and less undifferentiated cells[62]. Additionally, the AML BM was also unable to form colonies and proliferate as readily as healthy BM. They also showed that cell-signaling molecules such as Jagged-1 and CXCL12 were altered in the BM microenvironment. Jagged-1 percentage was decreased in leukemic MSCs compared to healthy MSCs when co-cultured with normal HSCs. Moreover, there was a significant suppression of Hes-1 and Hes-5, downstream targets of notch signals. A similar trend was seen with CXCL12 expressing cells. Besides, healthy HSCs are unable to expand when cultured on leukemic MSCs[62].

Notch is a plasma membrane protein that functions in cell signaling by having it's extracellular domain and intracellular domain cleaved. There are four types of Notch and majority of acute lymphoid leukemia patients test positive for a gain of function mutation in the intracellular domain of Notch1. A mouse model of T-ALL was used to determine the mechanism behind the leukemic expansion in the BM[58]. As with other leukemia, these leukemic cells were able to displace normal cells for space within the BMM. Additionally, there was a reduction of osteoblasts in the marrow. This was correlated with decreased expression of osteoblast transcription factors such as Runx2, osterix, osteocalcin, and ostegrin, as well as CXCL12. Meanwhile, stromal cells showed an increased expression of IL-6, SCF, HIF1a, VEGF, and Jag1. Furthermore, Notch1 was found to be able to bind to the promoter of the CXCL12 gene and affect expression[58]. Notch1 activation suppresses osteoblasts by decreasing CXCL12 that results in inhibition of normal hematopoiesis. This allows for a niche-targeted approach to regain osteoblast function and restore normal hematopoiesis.

LSCs use different niche environments to aid in chemo-resistance. During treatment, LSCs 'home' to adipose tissue in order to escape detection[63]. The adipose

niche induces a pro-inflammatory response from the LSCs. This causes the cells to secrete cytokines to increase lipolysis and release of fatty acids. LSCs then take up these the fatty acids to increase fatty acid oxidation (FAO). The expression of CD36 on LSCs seems to confer an advantage for the uptake of fatty acids. Additionally, the $CD36^+$ LSCs have lower ATP levels than the $CD36^-$ cells even with comparable mitochondrial mass. With the high FAO, low ATP levels, and their higher dependence on glycolysis, CD36+ LSCs resemble quiescent HSCs. Since chemotherapy targets cycling cells, $CD36^+$ cells demonstrate a higher resistance to chemotherapeutics[63]. Taken together, these illustrate the role the leukemic microenvironment plays in LSCs maintenance and drug resistance.

The majority of leukemic research has focused on genetic alterations that increase the risk of developing leukemia or that drive the leukemogenesis. Another recent emphasis is determining mutations that lead to a stressed environment for the HSCs. In Shwachman-Diamond Syndrome (SDS) and Myelodyplastic Syndrome (MDS), mesenchymal inflammation has been shown to stress HSCs and increase the risk of leukemic[64]. Furthermore, a conditional knockout specifically in mesenchymal cells for the *Sbds* gene results in similar bone formation as in human SDS. Additionally, these targeted mice have an increase in mitochondrial dysfunction and increased DNA damage in the HSCs. However, in the Sbds KO mice, unlike ROS induced damage in healthy controls, the pathways for the DNA damage, such as base excision repair and ataxia telangiectasia Rad3, are transcriptionally altered. p53, a tumor suppressor gene, is overexpressed in the mutant niche cells as a potential mechanism for this genotoxic stress[64]. This upregulation of p53 causes an increase in secretion of S100A8/9 heterodimer, which is a member of the S100 family and involved in cellular processes such as differentiation. S100A8/9 in turn binds to Toll-Like Receptor 4 (TLR4) to cause the increase in ROS and DNA damage of HSCs. Similar analysis was done with Noonan Syndrome (NS) to study the basis for

an increased risk of developing leukemia[65]. In these patients, the gene PTPN11 encodes SHP2, tyrosine phosphatase, a positive regulator of the RAS signaling pathway, is mutated in 50% of the patients. Using niche specific expression of a mutant *Ptpn11$^{E76K/+}$ gene* showed that Erk, Akt, NF-κB intracellular signaling pathways were highly upregulated in the HSCs of the mutant mice. Along with increasing intracellular signaling, the BM plasma show elevated levels of pro-inflammatory cytokines such as IL-1β, TREM-1, CCL3, CCL12, CCL4, and TIMP-1, but a decrease in CXCL12. Moreover, the anti-inflammatory cytokine IL-1ra was also increased. Patients with elevated CCL3 show an increase in complications from NS as compared to healthy patients. However, when looking at the HSCs, they found that IL-1β causes the HSCs to differentiate towards myeloid cells and monocytes, while CCL3, CCL4, and CCL12 appear to show no differentiation effect. Meanwhile, the impact of these factors on the MSCs results an increase in proliferation. Also, these perturbed MSCs recruit monocytes that produce pro-inflammatory cytokines. This causes the HSCs to be hyper-activated and displaced from the niche causing myeloproliferative neoplasm (MPN). These studies demonstrate the role of the mutant niche in causing disruption of the hematopoietic system. The combined effects of high stress and inflammation cause HSCs to be displaced and acquire mutations leading to leukemic disease.

As depicted in **FIGURE 2** many molecules play a role in the complex network of communication between the BMM and HSCs or LSCs. The microenvironment secretes signals that maintain and regulate HSCs. LSCs hijack the HSC niche to fulfill their demands, and promote their own survival. Changes within the leukemic microenvironment makes LSCs survive and resistant to chemotherapy. They remain more quiescent in the altered niche making targeted therapies problematic. Therefore, therapeutic treatment for leukemia needs to evaluate niche environment

holistically. Understanding how LSCs alter the microenvironment will aid in finding new opportunities to treat the illness.

Dysregulated signaling pathways contributing to LSCs

As mentioned above, recent studies have focused on genetic modifications that increase the risk of developing leukemia or promote transformation of normal HSCs. A combination of genetic and epigenetic alterations, signaling pathway disturbances, and changes to the bone marrow microenvironment permit the survival of LSCs over HSCs within the leukemic niche[66]. Futhermore, these pathways modify signaling pathways from upstream cytokines receptors to determine self-renewal and differentiation. Both stem cells have been defined by the ability to reconstitute normal hematopoiesis or leukemia following transplantation. This requires differentiation and self-renewal of the stem cells. The signaling pathways controlling self-renewal have been characterized and defined using mouse models. These include hedgehog ligands that promote HSC proliferation[67]. Another pathway is Wnt signaling. Wnt ligands bind to their cell-surface receptors to stabilize β-catenin and this leads to HSC expansion[68]. Furthermore, similar to expression within the BMM, expression of the intracellular domain of Notch (ICN) within hematopoietic cells leads to HSC expansion[69]. Therefore, several signaling pathways are known to regulate the self-renewal and maintenance of HSCs and LSCs (**FIGURE 3**). Mutations in any of these pathways or those that dysregulate these signal pathways result in transformation of HSCs to malignant cells.

Proper regulation of Wnt signaling is required to maintain a balance between the quiescent and active form of HSCs by regulating self-renewal and differentiation. Recently, WNT5A, a main stimulator of Wnt signaling, was found within the inflammatory niche[70]. Moreover, haploinsufficiency of WNT5A led to impaired actin polarization due to dysregulation of the *zeb1* transcription factor. This affects the

homing, migration and adhesion of LSCs[70], suggesting that alterations of this signaling pathway contribute to the recurrence of disease. Furthermore, Ctnnb1 and Wnt receptors frizzled 4/6 and the target gene, cyclin D2, were upregulated in LSC populations[71]. While the knockdown in expression of β-catenin results in the reduced growth of these cells; ablation or conditional deletion elminates the oncogenic potential of the MLL-transformed cells[66]. Consistent with this idea, the expression of β-catenin can be down-regulated by p53, a commonly dysregulated pathway in various types of leukemia. Based on these findings, a protein that negatively regulates the self-renewal of stem cells is Aspp1 (apoptosis-stimulating protein of p53), a co-factor of p53 that enhances its pro-apoptotic effects was recently discovered[72]. In fact, HSCs deficient in both Aspp1 and p53 accumulate DNA damage and develop haematological malignancies. However, Aspp1 can regulate the pool of HSC via p53 dependent and p53 independent mechanisms. Also, downregulation of Aspp1 by promotor hypermethylation has been observed in leukemic cells[72]. Additional studies that support these findings show that dual targeting of p53 and c-MYC simultaneously improves the selectivity for targeting LSCs in CML as compared to tyrosine kinase inhibitors alone that are unable to eradicate the LSCs responsible for relapse[73]. Moreoever, resistance to BET inhibitors (Bromodomain and extra terminal protein that bind acetylated chromatin marks) as seen in leukemic cells, is partially due to activation of Wnt/β-catenin signaling, and targeting this pathway restored the sensitivity of cells towards these inhibitors[74].

Interestingly, the Notch signaling pathway activity is also regulated by β-catenin protein (Ctnnb1)[66]. Within this pathway, the complex γ-secretase and the target genes of Hes family, c-Myc, cyclins D1 and D3, Notch1 and 3 have all been proven to be important in HSCs in quiescence, while Notch1 has tumor suppressor activity in AML[66,75]. *Hes1*, a target gene for Notch as well as Transforming Growth Factor (TGF-β) signaling are transcriptionally regulated by the factor JunB to control the

proliferation of HSCs for production of myeloid progenitors[75]. Meanwhile, HSCs deficient in *junB* have reduced activity of many downstream effectors for this pathway, like *Smad7* and *p57*. Supporting this idea, reduction in *Hes1* causes the loss of quiescence and hyperproliferation of the LSCs[75]. These data confirm that Wnt/β-catenin and Notch signaling are critical regulators of HSCs and LSCs.

Not surprising, that GSK-3β has been considered a key therapeutic target for leukemia because of the variety of pathways with which it interacts, including Wnt/β-catenin, Notch, PI3K/PTEN/Akt, and Hedgehog (Hh)[76-78]. Indeed, miR-126 regulates LSCs quiescence by targeting the components of this PI3K/AKT/GSK3β signaling axis in leukemia resulting in disease relapse and treatment resistance[79]. However, others have shown that the inhibition of miR-126 leads to LSC differentiation and cell cycle activation improving the sensitivity of anti-proliferative drugs[79]. Additionally, the negative regulator of PI3K pathway, *Pten* is also important to maintain the quiescent state of the HSCs[80]. That is, cells deficient in *Pten* are sensitized to IFNα and G-CSF. This results in hyper-activation of PI3K pathway, increase of protein synthesis and tumour suppression expression and depletion of HSCs[80].

Providing further evidence to the interplay between these signaling pathways, *Pten* deficient B cells activate the hedgehog (HH) pathway resulting in an expansion of B lineage cells but do not display a CLL-like phenotype[81]. In this model, activation of the Hh/Gli1 along with PI3K/AKT signaling was unable to cause expansion of the B lineage cell. This proves that his pathway does not play a role in the onset of CLL, but is rather required for their survivalas proven by both stroma-derived HH signaling and PI3K/AKT pathway[81]. In another model of lymphoid leukemia, using a JAK3 mutant mouse model[82], an increase in the expression of stimulatory ligands, *Dll4*, *Il7* and *Vegf*, that promote proliferation and cell survival was observed. Similar to CLL-like model, this was due to the upregulation in expression of the GLI1 transcription

factor and ectopic expression of Sonic Hedgehog (SHH) and Indian Hedgehog (IHH), ligands of the hedgehog pathway in the T-cell progenitors. These JAK3 mutant mice were also shown to be sensitive to treatment of the Shh pathway include smoothened (SMO) and glioma-associated oncogene homolog (GLI) inhibitors[82]. Futhermore, cases of AML having FMS-like tyrosine kinase (FLT3) internal tandem duplication (ITD) mutation had an increased expression of GLI2, an effector of the HH pathway, which regulates the transcription of CyclinD1 and N-Myc. The constitutive activation of Hh/Gli led to enhancement of STAT5 and hence proliferation of myeloid progenitors. And, the combined use of SMO and FLT3 antagonists inhibited the growth of AML cells *in vitro* as well as *in vivo*[83]. Targeting of the these pathways, including Wnt/β-catenin and Hedgehog (Hh), have proven difficult, as these self-renewal and stem cell pathways are essential in other tissues throughout the body.

Apart from these signaling pathways described above, the JAK/STAT pathway plays a mechanistic role in the development of leukemia due to the interplay of the pathway with the cytokines and the inflammatory niche. The constitutive activation of the JAK/STAT pathway drives leukemogenic potential as observed in BCR/ABL$^+$ CML, BCR/ABL$^-$ myeloproliferative neoplasms and is even associated with poor prognosis of AML LSCs[84-86]. Janus kinases (JAKs), cytoplasmically associated with cytokine receptors are phosphorylated at their tyrosine residues and are activated when cytokines and growth factors bind to their receptors. Mutated and constitutively active JAK, cause the phosphorylation and dimerization of STATs that are transcription factors regulating genes important for cell survival and proliferation. There has been significant advances in targeting these pathways in leuekmia and solid tumors[84,87]. Interestingly, tyrosine-unphosphorylated STAT5 (uSTAT5) represses megakaryocytic transcriptional program in the absence of thrombopoietin

(THPO) and restricted the access of ERG, a transcriptional factor to its target genes, proving the role of cytokine in megakaryocytic differentiation[87]. Therefore, although the focus has been on active or phosphorylated STAT protein, the inactive form also regulates gene expression and cell fate. Additionally, downregulating the expression of THPO receptor MPL reduced the THPO-stimulated STAT3/5 activation in turn decreasing the expression of STAT target genes *Bcl*-XL, *Bcl2*, *Hif2a*, *Myc*, *Osm* and *Pim1* responsible for cell cycle progression and inhibition of apoptosis[85]. Moreoever, UT2, an endogenous transmembrane protein upstream-of-mTORC2 that negatively regulates STAT3, inhibiting the phosphorylation of STAT3 at tyrosine 705 and reducing the cytokine IL-6 signaling, was decreased in multiple myeloma[88]. This suggests that phosphorylated STAT in the JAK/STAT pathway leads to activation of genes related to progression of cell cycle and inhibition of apoptosis aiding in the development and survival of LSCs, and hence can be therpeutically targeted for treatment.

Proinflammatory cytokine are typically elevated during myeloid malignancies. A few of these elevated factors include CCL2, CCL3, CCL5, CSF2, IFN-γ, IL-1, IL-3, IL-6, IL-8, IL-10, IL-12, IL-13, THPO, TNFα and stem cell factor[86,89-91]. Increased levels of these factors, as well as increased cell proliferation is observed in leukemic patients and people suffering from other myeloproliferative disorders[9,86]. IL-1, a proinflammatory cytokine triggers the phosphorylation and ubiquitination of downstream transcriptional targets of NF-κB, p38, JNK, MAPK and AP-1 signaling pathways. The target genes of this activation being the IL-1α, IL-1β, IL-6, IL-8, MCP-1, MKP-1 and TNFα, stimulating cell division and myeloid cell differentiation of HSCs while inhibiting lymphopoiesis and eryhtropoiesis[91,92]. Furthermore, IL-1β and CXCL2 were upregulated in AML due to a positive feedback mechanism phosphorylating GATA-2 via activation of the p38/ERK signaling promoting transcriptional activation and hence cell proliferation[93]. Tumour suppressive action of RIPK3, a target of the

TNF receptor signaling pathway, promotes cell-death of LSCs in AML from inflammasome-mediated release of IL-1β that restricts myeloid leukemogenesis[94]. Additionally, chronic exposure to IL-1 reversibly impaired self-renewal ability of HSC similar to long term exposure of IFNα while its acute production led to myeloid differentiation for blood regeneration observed during a physiological emergency state[92]. Meanwhile, an enhanced sensitivity to IL-1 induced NF-κB signaling and an upregulation IL-1 receptor Type 1 (IL-1-R1) and IL-1 receptor accessory protein (IL-1-RAP) was observed in CML LSCs[91]. A significant inhibition of the NF-κB signaling was observed after IL1-RA (IL1 receptor antagonist) on treatment with tyrosine kinase inhibitors in CML aiming to maintain treatment-free remissions by targeting the LSCs[91]. Apart from IL-1, the role of IL-6 in CML has been shown to be crucial for leukemic maintenance[89,90]. Non-transformed progenitors and normal HSCs were restored with anti-IL-6 treatment of chimeric CML mice. Additionally, changes in cytokine levels from the leukemia in this chimeric model affect the normal stem and progenitor cell function to transformation of the neighbouring cells[89]. Futhermore, BCR-ABL translocation regulated the expression of IL-6 through BCL6, a transcription regulator of IL-6 gene via the paracrine loop, reprogramming differentiation towards the myeloid lineage proving its importance in CML development. This finding was confirmed by the restoration of the normal low IL-6 levels on treatment with tyrosine kinase inhibitors. Disruption of the paracrine loop led to delays in the onset of CML with persistent BCR-ABL activity, proving the role of cytokines in the sustenance of LSCs[90].

It can be concluded that for the development, survival, and self-renewal of LSCs several signaling pathways, transcription factors and the cytokines play crucial roles. Numerous transcription factors known to regulate the genes required for cell cycle activation and inhibition of apoptosis for cell proliferation are activated in leukemia,

either by the oncogene or by changes to the microenvironment. Recent studies have revolved around the pro-inflammatory cytokines important to the development of LSCs and maintenance of the leukemic environment.

As discussed here, hematopoietic stem cells rely on cytokines from the bone marrow niche and neighboring hematopoietic cells to maintain their dormancy as well as differentiate to mature blood lineages to meet the needs of the individual. However, during malignancies, alteration in secreted factors and the niche contribute to maintenance of leukemic stem cells and disease progression. Here, we described the alterations in immune regulation, pro-inflammatory cytokines, self-renewal pathways and the microenvironment from leukemia. Findings in the past few years suggest that the future of treating leukemia will likely involve restoring the diseased microenvironment and inhibition of cytokines and secreted factors that promote leukemic maintenance.

References

1. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132:631-644, 2008.
2. Dadi S, Chhangawala S, Whitlock BM, Franklin RA, Luo CT, Oh SA, Toure A, Pritykin Y, Huse M, Leslie CS, Li MO. Cancer Immunosurveillance by Tissue-Resident Innate Lymphoid Cells and Innate-like T Cells. Cell 164:365-377, 2016.
3. Buggins AG, Milojkovic D, Arno MJ, Lea NC, Mufti GJ, Thomas NS, Hirst WJ. Microenvironment produced by acute myeloid leukemia cells prevents T cell activation and proliferation by inhibition of NF-kappaB, c-Myc, and pRb pathways. J Immunol 167:6021-6030, 2001.
4. Kitagawa Y, Wing JB, Sakaguchi S. Transcriptional and Epigenetic Control of Regulatory T Cell Development. Prog Mol Biol Transl Sci 136:1-33, 2015.
5. Li L, Neaves WB. Normal stem cells and cancer stem cells: the niche matters. Cancer Res 66:4553-4557, 2006.
6. Croft M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? Nat Rev Immunol 3:609-620, 2003.
7. Moudra A, Hubackova S, Machalova V, Vancurova M, Bartek J, Reinis M, Hodny Z, Jonasova A. Dynamic alterations of bone marrow cytokine landscape of myelodysplastic syndromes patients treated with 5-azacytidine. Oncoimmunology 5:e1183860, 2016.
8. Arellano M, Waller EK. GM-CSF + INF-alpha induce a graft-versus-leukemia effect in BMT patients with relapsed AML and ALL. J Clin Oncol 22:6634, 2004.
9. Kornblau SM, McCue D, Singh N, Chen W, Estrov Z, Coombes KR. Recurrent expression signatures of cytokines and chemokines are present and are independently prognostic in acute myelogenous leukemia and myelodysplasia. Blood 116:4251-4261, 2010.
10. Gentles AJ, Plevritis SK, Majeti R, Alizadeh AA. Association of a leukemic stem cell gene expression signature with clinical outcomes in acute myeloid leukemia. Jama 304:2706-2715, 2010.
11. Jung N, Dai B, Gentles AJ, Majeti R, Feinberg AP. An LSC epigenetic signature is largely mutation independent and implicates the HOXA cluster in AML pathogenesis. Nat Commun 6:8489, 2015.
12. Korsmeyer SJ, Arnold A, Bakhshi A, Ravetch JV, Siebenlist U, Hieter PA, Sharrow SO, LeBien TW, Kersey JH, Poplack DG, Leder P, Waldmann TA. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. J Clin Invest 71:301-313, 1983.
13. Curti A, Pandolfi S, Valzasina B, Aluigi M, Isidori A, Ferri E, Salvestrini V, Bonanno G, Rutella S, Durelli I, Horenstein AL, Fiore F, Massaia M, Colombo MP, Baccarani M, Lemoli RM. Modulation of tryptophan catabolism by human leukemic cells results in the conversion of CD25- into CD25+ T regulatory cells. Blood 109:2871-2877, 2007.
14. Milojkovic D, Devereux S, Westwood NB, Mufti GJ, Thomas NS, Buggins AG. Antiapoptotic microenvironment of acute myeloid leukemia. J Immunol 173:6745-6752, 2004.
15. Kikushige Y, Miyamoto T, Yuda J, Jabbarzadeh-Tabrizi S, Shima T, Takayanagi S, Niiro H, Yurino A, Miyawaki K, Takenaka K, Iwasaki H, Akashi K. A TIM-3/Gal-9 Autocrine Stimulatory Loop Drives Self-Renewal of Human Myeloid Leukemia Stem Cells and Leukemic Progression. Cell Stem Cell 17:341-352, 2015.
16. Zhao E, Xu H, Wang L, Kryczek I, Wu K, Hu Y, Wang G, Zou W. Bone marrow and the control of immunity. Cell Mol Immunol 9:11-19, 2012.

17. Massberg S, Schaerli P, Knezevic-Maramica I, Kollnberger M, Tubo N, Moseman EA, Huff IV, Junt T, Wagers AJ, Mazo IB, von Andrian UH. Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. Cell 131:994-1008, 2007.
18. Riether C, Schurch CM, Ochsenbein AF. Regulation of hematopoietic and leukemic stem cells by the immune system. Cell Death Differ 22:187-198, 2015.
19. Garcia-Ojeda ME, Dejbakhsh-Jones S, Weissman IL, Strober S. An alternate pathway for T cell development supported by the bone marrow microenvironment: recapitulation of thymic maturation. J Exp Med 187:1813-1823, 1998.
20. Dejbakhsh-Jones S, Jerabek L, Weissman IL, Strober S. Extrathymic maturation of alpha beta T cells from hemopoietic stem cells. J Immunol 155:3338-3344, 1995.
21. Zou L, Barnett B, Safah H, Larussa VF, Evdemon-Hogan M, Mottram P, Wei S, David O, Curiel TJ, Zou W. Bone marrow is a reservoir for CD4+CD25+ regulatory T cells that traffic through CXCL12/CXCR4 signals. Cancer Res 64:8451-8455, 2004.
22. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R, Gao W, Saito TI, Lo Celso C, Tsuyuzaki H, Sato T, Cote D, Sykes M, Strom TB, Scadden DT, Lin CP. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. Nature 474:216-219, 2011.
23. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. Regulatory T cells: how do they suppress immune responses? Int Immunol 21:1105-1111, 2009.
24. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 450:566-569, 2007.
25. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. Nat Immunol 8:1353-1362, 2007.
26. Ustun C, Miller JS, Munn DH, Weisdorf DJ, Blazar BR. Regulatory T cells in acute myelogenous leukemia: is it time for immunomodulation? Blood 118:5084-5095, 2011.
27. Albitar M, Manshouri T, Shen Y, Liu D, Beran M, Kantarjian HM, Rogers A, Jilani I, Lin CW, Pierce S, Freireich EJ, Estey EH. Myelodysplastic syndrome is not merely "preleukemia". Blood 100:791-798, 2002.
28. Mundy-Bosse BL, Scoville SD, Chen L, McConnell K, Mao HC, Ahmed EH, Zorko N, Harvey S, Cole J, Zhang X, Costinean S, Croce CM, Larkin K, Byrd JC, Vasu S, Blum W, Yu J, Freud AG, Caligiuri MA. MicroRNA-29b mediates altered innate immune development in acute leukemia. J Clin Invest, 2016.
29. Epling-Burnette PK, Bai F, Painter JS, Rollison DE, Salih HR, Krusch M, Zou J, Ku E, Zhong B, Boulware D, Moscinski L, Wei S, Djeu JY, List AF. Reduced natural killer (NK) function associated with high-risk myelodysplastic syndrome (MDS) and reduced expression of activating NK receptors. Blood 109:4816-4824, 2007.
30. Gleason MK, Ross JA, Warlick ED, Lund TC, Verneris MR, Wiernik A, Spellman S, Haagenson MD, Lenvik AJ, Litzow MR, Epling-Burnette PK, Blazar BR, Weiner LM, Weisdorf DJ, Vallera DA, Miller JS. CD16xCD33 bispecific killer cell engager (BiKE) activates NK cells against primary MDS and MDSC CD33+ targets. Blood 123:3016-3026, 2014.
31. Wagner JA, Berrien-Elliott MM, Rosario M, Leong JW, Jewell BA, Schappe T, Abdel-Latif S, Fehniger TA. Cytokine-Induced Memory-Like Differentiation Enhances Unlicensed Natural Killer Cell Antileukemia and FcgammaRIIIa-Triggered Responses. Biol Blood Marrow Transplant, 2016.
32. Liu FT, Giustiniani J, Farren T, Jia L, Bensussan A, Gribben JG, Agrawal SG. CD160 signaling mediates PI3K-dependent survival and growth signals in chronic lymphocytic leukemia. Blood 115:3079-3088, 2010.

33. Goselink HM, Williams DE, Fibbe WE, Wessels HW, Beverstock GC, Willemze R, Falkenburg JH. Effect of mast cell growth factor (c-kit ligand) on clonogenic leukemic precursor cells. Blood 80:750-757, 1992.
34. Valent P, Spanblochl E, Bankl HC, Sperr WR, Marosi C, Pirc-Danoewinata H, Virgolini I, Eichler HG, Agis H, Sillaber C, et al. Kit ligand/mast cell growth factor-independent differentiation of mast cells in myelodysplasia and chronic myeloid leukemic blast crisis. Blood 84:4322-4332, 1994.
35. Puverel S, Kiris E, Singh S, Klarmann KD, Coppola V, Keller JR, Tessarollo L. RanBPM (RanBP9) regulates mouse c-Kit receptor level and is essential for normal development of bone marrow progenitor cells. Oncotarget, 2016.
36. Kobayashi CI, Takubo K, Kobayashi H, Nakamura-Ishizu A, Honda H, Kataoka K, Kumano K, Akiyama H, Sudo T, Kurokawa M, Suda T. The IL-2/CD25 axis maintains distinct subsets of chronic myeloid leukemia-initiating cells. Blood 123:2540-2549, 2014.
37. Kotsianidis I, Bouchliou I, Nakou E, Spanoudakis E, Margaritis D, Christophoridou AV, Anastasiades A, Tsigalou C, Bourikas G, Karadimitris A, Tsatalas C. Kinetics, function and bone marrow trafficking of CD4+CD25+FOXP3+ regulatory T cells in myelodysplastic syndromes (MDS). Leukemia 23:510-518, 2009.
38. Nguyen AH, Berim IG, Agrawal DK. Chronic inflammation and cancer: emerging roles of triggering receptors expressed on myeloid cells. Expert Rev Clin Immunol 11:849-857, 2015.
39. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 140:805-820, 2010.
40. Kitagawa M, Saito I, Kuwata T, Yoshida S, Yamaguchi S, Takahashi M, Tanizawa T, Kamiyama R, Hirokawa K. Overexpression of tumor necrosis factor (TNF)-alpha and interferon (IFN)-gamma by bone marrow cells from patients with myelodysplastic syndromes. Leukemia 11:2049-2054, 1997.
41. Sawanobori M, Yamaguchi S, Hasegawa M, Inoue M, Suzuki K, Kamiyama R, Hirokawa K, Kitagawa M. Expression of TNF receptors and related signaling molecules in the bone marrow from patients with myelodysplastic syndromes. Leuk Res 27:583-591, 2003.
42. Wei Y, Dimicoli S, Bueso-Ramos C, Chen R, Yang H, Neuberg D, Pierce S, Jia Y, Zheng H, Wang H, Wang X, Nguyen M, Wang SA, Ebert B, Bejar R, Levine R, Abdel-Wahab O, Kleppe M, Ganan-Gomez I, Kantarjian H, Garcia-Manero G. Toll-like receptor alterations in myelodysplastic syndrome. Leukemia 27:1832-1840, 2013.
43. Kuninaka N, Kurata M, Yamamoto K, Suzuki S, Umeda S, Kirimura S, Arai A, Nakagawa Y, Suzuki K, Kitagawa M. Expression of Toll-like receptor 9 in bone marrow cells of myelodysplastic syndromes is down-regulated during transformation to overt leukemia. Exp Mol Pathol 88:293-298, 2010.
44. Takeshita S, Fumoto T, Naoe Y, Ikeda K. Age-related marrow adipogenesis is linked to increased expression of RANKL. J Biol Chem 289:16699-16710, 2014.
45. Lambert C, Wu Y, Aanei C. Bone Marrow Immunity and Myelodysplasia. Front Oncol 6:172, 2016.
46. Tsuboi I, Morimoto K, Hirabayashi Y, Li GX, Aizawa S, Mori KJ, Kanno J, Inoue T. Senescent B lymphopoiesis is balanced in suppressive homeostasis: decrease in interleukin-7 and transforming growth factor-beta levels in stromal cells of senescence-accelerated mice. Exp Biol Med (Maywood) 229:494-502, 2004.
47. Wang CQ, Udupa KB, Xiao H, Lipschitz DA. Effect of age on marrow macrophage number and function. Aging (Milano) 7:379-384, 1995.
48. Braig M, Pallmann N, Preukschas M, Steinemann D, Hofmann W, Gompf A, Streichert T, Braunschweig T, Copland M, Rudolph KL, Bokemeyer C, Koschmieder S, Schuppert A, Balabanov S, Brummendorf TH. A 'telomere-associated secretory

phenotype' cooperates with BCR-ABL to drive malignant proliferation of leukemic cells. Leukemia 28:2028-2039, 2014.
49. Cahu J, Bustany S, Sola B. Senescence-associated secretory phenotype favors the emergence of cancer stem-like cells. Cell Death Dis 3:e446, 2012.
50. Tchkonia T, Zhu Y, van Deursen J, Campisi J, Kirkland JL. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. J Clin Invest 123:966-972, 2013.
51. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 8:729-740, 2007.
52. Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, Aarden LA, Mooi WJ, Peeper DS. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell 133:1019-1031, 2008.
53. Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol 5:99-118, 2010.
54. Acosta JC, O'Loghlen A, Banito A, Guijarro MV, Augert A, Raguz S, Fumagalli M, Da Costa M, Brown C, Popov N, Takatsu Y, Melamed J, d'Adda di Fagagna F, Bernard D, Hernando E, Gil J. Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell 133:1006-1018, 2008.
55. Chan CK, Seo EY, Chen JY, Lo D, McArdle A, Sinha R, Tevlin R, Seita J, Vincent-Tompkins J, Wearda T, Lu WJ, Senarath-Yapa K, Chung MT, Marecic O, Tran M, Yan KS, Upton R, Walmsley GG, Lee AS, Sahoo D, Kuo CJ, Weissman IL, Longaker MT. Identification and specification of the mouse skeletal stem cell. Cell 160:285-298, 2015.
56. Nwajei F, Konopleva M. The bone marrow microenvironment as niche retreats for hematopoietic and leukemic stem cells. Adv Hematol 2013:953982, 2013.
57. Silberstein L, Goncalves KA, Kharchenko PV, Turcotte R, Kfoury Y, Mercier F, Baryawno N, Severe N, Bachand J, Spencer JA, Papazian A, Lee D, Chitteti BR, Srour EF, Hoggatt J, Tate T, Lo Celso C, Ono N, Nutt S, Heino J, Sipila K, Shioda T, Osawa M, Lin CP, Hu GF, Scadden DT. Proximity-Based Differential Single-Cell Analysis of the Niche to Identify Stem/Progenitor Cell Regulators. Cell Stem Cell 19:530-543, 2016.
58. Zhou HS, Carter BZ, Andreeff M. Bone marrow niche-mediated survival of leukemia stem cells in acute myeloid leukemia: Yin and Yang. Cancer Biol Med 13:248-259, 2016.
59. Colmone A, Amorim M, Pontier AL, Wang S, Jablonski E, Sipkins DA. Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. Science 322:1861-1865, 2008.
60. Glait-Santar C, Desmond R, Feng X, Bat T, Chen J, Heuston E, Mizukawa B, Mulloy JC, Bodine DM, Larochelle A, Dunbar CE. Functional Niche Competition Between Normal Hematopoietic Stem and Progenitor Cells and Myeloid Leukemia Cells. Stem Cells 33:3635-3642, 2015.
61. Pitt LA, Tikhonova AN, Hu H, Trimarchi T, King B, Gong Y, Sanchez-Martin M, Tsirigos A, Littman DR, Ferrando AA, Morrison SJ, Fooksman DR, Aifantis I, Schwab SR. CXCL12-Producing Vascular Endothelial Niches Control Acute T Cell Leukemia Maintenance. Cancer Cell 27:755-768, 2015.
62. Kim JA, Shim JS, Lee GY, Yim HW, Kim TM, Kim M, Leem SH, Lee JW, Min CK, Oh IH. Microenvironmental remodeling as a parameter and prognostic factor of heterogeneous leukemogenesis in acute myelogenous leukemia. Cancer Res 75:2222-2231, 2015.
63. Ye H, Adane B, Khan N, Sullivan T, Minhajuddin M, Gasparetto M, Stevens B, Pei S, Balys M, Ashton JM, Klemm DJ, Woolthuis CM, Stranahan AW, Park CY, Jordan

CT. Leukemic Stem Cells Evade Chemotherapy by Metabolic Adaptation to an Adipose Tissue Niche. Cell Stem Cell 19:23-37, 2016.
64. Zambetti NA, Ping Z, Chen S, Kenswil KJ, Mylona MA, Sanders MA, Hoogenboezem RM, Bindels EM, Adisty MN, Van Strien PM, van der Leije CS, Westers TM, Cremers EM, Milanese C, Mastroberardino PG, van Leeuwen JP, van der Eerden BC, Touw IP, Kuijpers TW, Kanaar R, van de Loosdrecht AA, Vogl T, Raaijmakers MH. Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia. Cell Stem Cell 19:613-627, 2016.
65. Dong L, Yu WM, Zheng H, Loh ML, Bunting ST, Pauly M, Huang G, Zhou M, Broxmeyer HE, Scadden DT, Qu CK. Leukaemogenic effects of Ptpn11 activating mutations in the stem cell microenvironment. Nature 539:304-308, 2016.
66. Heidel FH, Arreba-Tutusaus P, Armstrong SA, Fischer T. Evolutionarily conserved signaling pathways: acting in the shadows of acute myelogenous leukemia's genetic diversity. Clin Cancer Res 21:240-248, 2015.
67. Trowbridge JJ, Scott MP, Bhatia M. Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. Proc Natl Acad Sci U S A 103:14134-14139, 2006.
68. Reya T, Duncan AW, Ailles L, Domen J, Scherer DC, Willert K, Hintz L, Nusse R, Weissman IL. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 423:409-414, 2003.
69. Karanu FN, Murdoch B, Gallacher L, Wu DM, Koremoto M, Sakano S, Bhatia M. The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. J Exp Med 192:1365-1372, 2000.
70. Schreck C, Istvanffy R, Ziegenhain C, Sippenauer T, Ruf F, Henkel L, Gartner F, Vieth B, Florian MC, Mende N, Taubenberger A, Prendergast A, Wagner A, Pagel C, Grziwok S, Gotze KS, Guck J, Dean DC, Massberg S, Essers M, Waskow C, Geiger H, Schiemann M, Peschel C, Enard W, Oostendorp RA. Niche WNT5A regulates the actin cytoskeleton during regeneration of hematopoietic stem cells. J Exp Med 214:165-181, 2017.
71. Yeung J, Esposito MT, Gandillet A, Zeisig BB, Griessinger E, Bonnet D, So CW. beta-Catenin mediates the establishment and drug resistance of MLL leukemic stem cells. Cancer Cell 18:606-618, 2010.
72. Yamashita M, Nitta E, Suda T. Aspp1 Preserves Hematopoietic Stem Cell Pool Integrity and Prevents Malignant Transformation. Cell Stem Cell 17:23-34, 2015.
73. Abraham SA, Hopcroft LE, Carrick E, Drotar ME, Dunn K, Williamson AJ, Korfi K, Baquero P, Park LE, Scott MT, Pellicano F, Pierce A, Copland M, Nourse C, Grimmond SM, Vetrie D, Whetton AD, Holyoake TL. Dual targeting of p53 and c-MYC selectively eliminates leukaemic stem cells. Nature 534:341-346, 2016.
74. Fong CY, Gilan O, Lam EY, Rubin AF, Ftouni S, Tyler D, Stanley K, Sinha D, Yeh P, Morison J, Giotopoulos G, Lugo D, Jeffrey P, Lee SC, Carpenter C, Gregory R, Ramsay RG, Lane SW, Abdel-Wahab O, Kouzarides T, Johnstone RW, Dawson SJ, Huntly BJ, Prinjha RK, Papenfuss AT, Dawson MA. BET inhibitor resistance emerges from leukaemia stem cells. Nature 525:538-542, 2015.
75. Santaguida M, Schepers K, King B, Sabnis AJ, Forsberg EC, Attema JL, Braun BS, Passegue E. JunB protects against myeloid malignancies by limiting hematopoietic stem cell proliferation and differentiation without affecting self-renewal. Cancer Cell 15:341-352, 2009.
76. Welham MJ, Kingham E, Sanchez-Ripoll Y, Kumpfmueller B, Storm M, Bone H. Controlling embryonic stem cell proliferation and pluripotency: the role of PI3K- and GSK-3-dependent signalling. Biochem Soc Trans 39:674-678, 2011.

77. Gandillet A, Park S, Lassailly F, Griessinger E, Vargaftig J, Filby A, Lister TA, Bonnet D. Heterogeneous sensitivity of human acute myeloid leukemia to beta-catenin down-modulation. Leukemia 25:770-780, 2011.
78. Wickremasinghe RG, Prentice AG, Steele AJ. p53 and Notch signaling in chronic lymphocytic leukemia: clues to identifying novel therapeutic strategies. Leukemia 25:1400-1407, 2011.
79. Lechman ER, Gentner B, Ng SW, Schoof EM, van Galen P, Kennedy JA, Nucera S, Ciceri F, Kaufmann KB, Takayama N, Dobson SM, Trotman-Grant A, Krivdova G, Elzinga J, Mitchell A, Nilsson B, Hermans KG, Eppert K, Marke R, Isserlin R, Voisin V, Bader GD, Zandstra PW, Golub TR, Ebert BL, Lu J, Minden M, Wang JC, Naldini L, Dick JE. miR-126 Regulates Distinct Self-Renewal Outcomes in Normal and Malignant Hematopoietic Stem Cells. Cancer Cell 29:602-606, 2016.
80. Porter SN, Cluster AS, Signer RA, Voigtmann J, Monlish DA, Schuettpelz LG, Magee JA. Pten Cell Autonomously Modulates the Hematopoietic Stem Cell Response to Inflammatory Cytokines. Stem Cell Reports 6:806-814, 2016.
81. Kern D, Regl G, Hofbauer SW, Altenhofer P, Achatz G, Dlugosz A, Schnidar H, Greil R, Hartmann TN, Aberger F. Hedgehog/GLI and PI3K signaling in the initiation and maintenance of chronic lymphocytic leukemia. Oncogene 34:5341-5351, 2015.
82. Dagklis A, Demeyer S, De Bie J, Radaelli E, Pauwels D, Degryse S, Gielen O, Vicente C, Vandepoel R, Geerdens E, Uyttebroeck A, Boeckx N, de Bock CE, Cools J. Hedgehog pathway activation in T-cell acute lymphoblastic leukemia predicts response to SMO and GLI1 inhibitors. Blood 128:2642-2654, 2016.
83. Lim Y, Gondek L, Li L, Wang Q, Ma H, Chang E, Huso DL, Foerster S, Marchionni L, McGovern K, Watkins DN, Peacock CD, Levis M, Smith BD, Merchant AA, Small D, Matsui W. Integration of Hedgehog and mutant FLT3 signaling in myeloid leukemia. Sci Transl Med 7:291ra296, 2015.
84. de Freitas RM, da Costa Maranduba CM. Myeloproliferative neoplasms and the JAK/STAT signaling pathway: an overview. Rev Bras Hematol Hemoter 37:348-353, 2015.
85. Zhang B, Li L, Ho Y, Li M, Marcucci G, Tong W, Bhatia R. Heterogeneity of leukemia-initiating capacity of chronic myelogenous leukemia stem cells. J Clin Invest 126:975-991, 2016.
86. Kleppe M, Kwak M, Koppikar P, Riester M, Keller M, Bastian L, Hricik T, Bhagwat N, McKenney AS, Papalexi E, Abdel-Wahab O, Rampal R, Marubayashi S, Chen JJ, Romanet V, Fridman JS, Bromberg J, Teruya-Feldstein J, Murakami M, Radimerski T, Michor F, Fan R, Levine RL. JAK-STAT pathway activation in malignant and nonmalignant cells contributes to MPN pathogenesis and therapeutic response. Cancer Discov 5:316-331, 2015.
87. Park HJ, Li J, Hannah R, Biddie S, Leal-Cervantes AI, Kirschner K, Flores Santa Cruz D, Sexl V, Gottgens B, Green AR. Cytokine-induced megakaryocytic differentiation is regulated by genome-wide loss of a uSTAT transcriptional program. EMBO J 35:580-594, 2016.
88. Lee D, Wang YH, Kalaitzidis D, Ramachandran J, Eda H, Sykes DB, Raje N, Scadden DT. Endogenous transmembrane protein UT2 inhibits pSTAT3 and suppresses hematological malignancy. J Clin Invest 126:1300-1310, 2016.
89. Welner RS, Amabile G, Bararia D, Czibere A, Yang H, Zhang H, Pontes LL, Ye M, Levantini E, Di Ruscio A, Martinelli G, Tenen DG. Treatment of chronic myelogenous leukemia by blocking cytokine alterations found in normal stem and progenitor cells. Cancer Cell 27:671-681, 2015.
90. Reynaud D, Pietras E, Barry-Holson K, Mir A, Binnewies M, Jeanne M, Sala-Torra O, Radich JP, Passegue E. IL-6 controls leukemic multipotent progenitor cell fate and contributes to chronic myelogenous leukemia development. Cancer Cell 20:661-673, 2011.

91. Zhang B, Chu S, Agarwal P, Campbell VL, Hopcroft L, Jorgensen HG, Lin A, Gaal K, Holyoake TL, Bhatia R. Inhibition of interleukin-1 signaling enhances elimination of tyrosine kinase inhibitor treated CML stem cells. Blood, 2016.
92. Pietras EM, Mirantes-Barbeito C, Fong S, Loeffler D, Kovtonyuk LV, Zhang S, Lakshminarasimhan R, Chin CP, Techner JM, Will B, Nerlov C, Steidl U, Manz MG, Schroeder T, Passegue E. Chronic interleukin-1 exposure drives haematopoietic stem cells towards precocious myeloid differentiation at the expense of self-renewal. Nat Cell Biol 18:607-618, 2016.
93. Katsumura KR, Ong IM, DeVilbiss AW, Sanalkumar R, Bresnick EH. GATA Factor-Dependent Positive-Feedback Circuit in Acute Myeloid Leukemia Cells. Cell Rep 16:2428-2441, 2016.
94. Hockendorf U, Yabal M, Herold T, Munkhbaatar E, Rott S, Jilg S, Kauschinger J, Magnani G, Reisinger F, Heuser M, Kreipe H, Sotlar K, Engleitner T, Rad R, Weichert W, Peschel C, Ruland J, Heikenwalder M, Spiekermann K, Slotta-Huspenina J, Gross O, Jost PJ. RIPK3 Restricts Myeloid Leukemogenesis by Promoting Cell Death and Differentiation of Leukemia Initiating Cells. Cancer Cell 30:75-91, 2016.

DISCUSSION

Tregs comprise the regulatory arm of the immune system, and function by curtailing inflammatory responses, and preventing over-activation. While the impact of Tregs has been readily explored in the context of autoimmunity and solid tumors, their contribution to hematological malignancies has not. Moreover, while it is known that immune abnormalities accompany leukemic transformation the immune mechanisms that underlie loss of hematopoietic function lack resolution. This is particularly true of MPNs, where an association has been established between immune dysregulation and disease onset, but the pathogenic link is unknown(1, 2). Specifically, clinical investigations have defined a correlation between MPNs and autoimmune diseases, suggesting a potential defect in Treg function (3).

It is now recognized that the roles of Tregs span beyond traditional roles of damping of immune response to more specialized functions. A growing body of evidence now supports the critical role for mediated distinct “tissue Treg” subpopulations in tissue homeostasis (4). Within the bone marrow, Tregs have been shown to have unique supportive functions related to hematopoietic stem cell maintenance(5-8). The work presented here expands on these initial observations and highlights a cooperative relationship with and dependency on the stromal compartment for hematopoietic regulation. We demonstrate that bone marrow Treg are a tissue specific subset and have outlined their key features related to the maintenance of stromal cells and the support of hematopoiesis via enhanced IL-10 secretion.

DISTINCT TISSUE SPECIFIC FUNCTIONS OF BONE MARROW TREGS

In our characterization of bone marrow Tregs we identified distinctive characteristics and phenotypes. These differences in frequency, homing potential, and

cytokine output suggest a specialized biological function for marrow Tregs, which we propose is to sustain healthy hematopoiesis. Of particular interest is the migratory capacity and ability to home back to the bone marrow microenvironment. In functional homing assays using Tregs from the marrow, spleen, and inguinal lymph nodes we have observed that marrow Tregs preferentially traffic back to their tissue or origin with much higher efficiency than other Tregs. This unique property makes them an attractive candidate for cellular therapy as it opens up the possibility to use bone marrow Tregs for targeted drug delivery or to improve hematopoietic stem cell transplantation via conditioning of the stromal niche. In our work, we identified the S1P/S1PR1 as one of the relevant signaling axes involved in Treg trafficking, and we exploited the differential expression of this receptor to prevent bone marrow re-entry. Our sequencing analysis reveals a complex profile of chemokines and cytokines. Thus, it is likely that multiple signaling pathways are operative in the marrow Treg pool. The role of other migratory stimuli in directing bone marrow Treg trafficking or promoting retention of Treg cells in the marrow remains to be determined. It is well established that Treg localization to inflammatory sites is essential to their homeostatic function(9). Therefore, it will be important to ascertain how Treg homing to the marrow might be altered in non-homeostatic conditions of disease or malignancy. If this is concept is to be expanded up therapeutically, the profile of the patient particularly inflammatory status will be critical.

Expanding on previous studies of Tregs colocalizing with hematopoietic stem cells (HSC) we observed that depletion of Tregs via administration of anti-CD25 has widespread effects across several stem and progenitor populations, with a particularly severe effect on long-term hematopoietic stem cells, (LT-HSC). In our depletion mode, loss of Tegs resulted in increased HSC cycling and proliferation, while fostering myeloid skewed differentiation. Key stem cell features including repopulation capacity and dormancy are also disrupted. Expanding on these observations, it will be of interest to

examine how HSC function and differentiation are altered in chronic autoimmune conditions or other persistent inflammatory states where Treg dysregulation has been extensively described. This is a noteworthy area of investigation given the clinical association between antecedent autoimmune conditions, and the development of clonal hematopoiesis and specific MPNS(1, 10). In particular, it will be of interest to determine how the duration of Treg dysfunction influences stem cell self-renewal and differentiation. Along these lines, it is similarly worth considering how various Treg and T cell modulating agents might be having permanent indirect effects on the delicate process of hematopoiesis.

In addition to observing differences at the stem cell level, we also noted expansion of other multipotent progenitor populations. Across our different treatments (aCD25, FTY720, blockage of the IL-10 receptor) we observed notable differences in progenitor profiles. The differences in multi-potent progenitor (MPP) reconstitution represent an interesting observation. It is possible Tregs influence the niche-dependent mechanisms regulating MPP biases and differentiation or that Tregs target MPPs directly. Interestingly, across some treatments we consistently observed an expansion of the MPP4 compartment. One possibility is that following aCD25 the marrow is responding to the sustained tissue-wide absence of Tregs in a compensatory fashion and favoring lymphoid output in order to restore Treg numbers. Others have shown that MPP4 is lymphoid-biased (11). The pressure to generate more Tregs is less following FTY720 due to the short length of treatment (7 days vs 28 days) and because Tregs are not depleted at other sites. When we performed IL10 Receptor blockade we did not see increase in MPP4, possibly because there was no pressure to generate more Tregs. Elucidating these interactions will requires further studies and different models but could further expand the role of Treg and IL-10 in progenitor lineage commitment. It will be of interest to evaluate the duration and distribution of Treg interactions with other bone

marrow populations progenitor how these interactions are shaped by other environmental and extrinsic factors.

Bone marrow Treg regulation of HSPCs align the role of Tregs in maintaining specialized stem cell populations in other tissues including the skin and muscle. An open question that remains is whether other Treg factors, in particular those whose regenerative potential have described, also contribute to hematopoietic regulation. A secreted Treg factor of interest is Amphiregulin (AREG) which has been demonstrated to facilitate skeletal muscle regeneration, as well as the repair of infection-induced tissue damage in the lung. In other tissues, the role of AREG in stem cell regeneration has been well characterized. With regards to AREG production it has been demonstrated that ST2 (IL1RL1) Tregs are high producers of this factor(12-16). ST2 is activated by the cytokine IL-33, which has pleiotropic activity as a damage-associated molecular pattern (DAMP), serving as a regulator of cellular activation and recruitment. Among the various markers highly expressed we detected significant expression of ST2 on bone marrow Tregs as has been reported in other tissue subsets(12-16). This suggests that $ST2^+$ Tregs may provide a microenvironmental source of AREG in bone marrow niche. More work is needed to evaluate how other Treg secreted factors may cooperate with IL-10 for hematopoietic regulation.

TREG REGULATION OF THE STROMAL NICHE

Given the crosstalk that exists between Tregs and their local tissue environments, we hypothesized that Tregs may regulate the function of non-hematopoietic cells within the marrow. Using various methods of Treg manipulation we observed that supportive bone stromal populations were responsive to Treg alterations. Consistently across treatments there was increased cycling and proliferation of mesenchymal stromal populations. In addition to phenotypic changes, there were also

functional consequences associated with the expanded stromal compartment. Non-genotoxic conditioning and transplantation, revealed that the Treg depleted microenvironment is less supportive of HSCs, thus establishing a novel role for Tregs in maintaining niche function. Remarkably, the effects of Treg depletion were found to be long- lasting. In the absence of continued treatment, cohorts sustained a myeloid bias, as well as increased numbers of HSCs and stromal cells. These observations support the conclusion that marrow Tregs represent a unique subset that maintains stromal populations and have a
specialized function within this tissue.

In the context of hematopoiesis, the function of stromal cells has been primarily centered on the regulation that these cells provide to HSCs. We hypothesized that the stromal expansion is a stress response to the lack of Tregs, as has been previously documented. Our investigations support the notion that *intrinsic* HSC functions can be influenced by *extrinsic* niche-dependent regulation. To test this hypothesis, we evaluated both sides of this relationship through transplantation. Because HSCs are extremely sensitive their environmental cues, we functionally assayed how transient Treg loss impacted HSC-*intrinsic* properties. We noted how Treg depletion impaired the ability of HSCs to efficiently reconstitute in a competitive transplantation assay. We observed that HSC isolated from Treg depleted mice had reduced repopulation capacity when both groups were placed in equivalent wild-type stromal environments. We were also able to characterize the effects of *extrinsic*-niche-dependent HSC regulation. To test how HSC functions are affected by the external environment we transplanted healthy WT HSCs into either a WT niche or a Treg depleted niche (aCD25). This allowed us to determine if Treg depletion impaired the ability of stromal cells to efficiently support healthy stem cells. We observed that aCD25 stroma had reduced supportive capacity when engrafted with wild-type HSCs. While others have shown that Tregs may interact with HSCs and

regulate them directly, the focus of these studies was on the crosstalk of Tregs and stromal cells. It is possible to speculate that the stromal phenotypes precede the HSC phenotypes as stromal cells regulate stem cell quiescence and differentiation however this would require further investigation.

Collectively, there has been elegant work on how stromal cells regulate HSC health but there is far less information on how stromal cells themselves are regulated. Our investigations provide critical insight into immune support of stromal cells, and IL-10 provides a mechanistic explanation for this regulation. Many questions remain open with regards to how stromal cells integrate Treg and IL-10 signals and translate them to functional consequences. Our data suggest that Treg IL-10 influences stromal cell decision to be quiescent, self-renew, as evidenced by the lack of colony forming potential following IL-10 stimulation and the reduced differentiation into bone and fat linages. As was considered with HSC biology, it is also possible that other Treg factors are involved in this stromal crosstalk.

Our observations using various stromal cell models suggest developmental roles for Tregs and IL-10 in the preservation and regulation of the mesenchymal network. This is supported by two models where IL-10 signaling was deleted; a Foxp3Cre-IL-10 floxed mice (IL-10 production is ablated in Tregs from birth) and a Prrx-IL10R floxed model (where the IL-10 receptor is deleted in all stromal populations from birth). In these two systems we observed a blunted stromal compartment and reduced numbers of multiple populations suggesting that IL-10 may be important developmentally. Future work with these two stromal murine models has enthusing implications for dissecting the stromal contributions to hematological malignancy. An outstanding question with tremendous therapeutic implications is whether we can take advantage of signaling differences in normal vs leukemic stroma to alter disease progression. At the more fundamental level,

how is the stem cell compartment affected by this altered stroma and what are the implications for the process of hematopoiesis more broadly?

TREGS AND TH17 CELLS IN MYELOID LEUKEMIA

Numerous investigations have demonstrated that aberrant expression of inflammatory cytokines accompany CML. In particular, the focus has been on the role of inflammatory cues in propagating the survival of leukemic stem cells and how these signals alter the stroma to favor leukemic expansion. More recently however, it has been appreciated that leukemic transformation is also characterized by a dysregulated immune landscape. However, in comparison to solid tumors and autoimmune conditions, a comprehensive profiling of the $CD4^+$ T cell compartment in myeloproliferative neoplasms such as chronic myeloid leukemia (CML) is significantly lacking. Given critical functions for bone marrow Tregs in hematopoietic stem cell maintenance and stromal regulation, a logical extension of our work was was to assess the role of Tregs in hematological malignancy.

Broadly, the contribution of Tregs to the process of oncogenesis has been regarded as deleterious. In fact, it is well documented that accumulation of immunosuppressive Tregs contributes to immune evasion and decreased immunosurveillance in a wide spectrum of cancers effectively suppressing effective anti-tumor immunity. (17-19). In myeloid malignancies, Tregs are similarly associated with more aggressive disease, or with increased risk of relapse or drug resistance. However, most of these studies have involved analysis of Tregs at later stages of disease, or after significant therapeutic intervention. Thus, there is limited information on how the Treg profiles change in proportion to disease progression. A basic question that needs to be addresses is whether Tregs in CML are functional in patients with active or early disease. Nonetheless, a few reports have emerged detailing a beneficial prognosis, for

patients with higher Treg numbers(20-22). In our CML mode, we tested how prolonged exposure to the leukemic environment impacted Treg fate and function. Our data indicate that leukemic cytokines induce reprogramming of Treg cells that makes them unstable, a phenotype which is characterized by loss of Foxp3 and reduced IL-10 production. We attribute this to the elevated levels of inflammatory cytokines in CML, particularly IL-6. Increased levels of IL-6 are known to be deleterious to Treg development and the acquisition of inflammatory properties. IL-6 is also a potent inducer of Th17 cells.

Th17 cells have critical roles in activating host responses to extracellular pathogens, in particular with the activation neutrophils at barrier tissues. In addition to this, Th17 cells have emerged as notable inflammatory mediators in autoimmune diseases, and solid tumors. Across different models, Th17 related cytokines such as IL-17, IL-21, and GMCSF, have been demonstrated to act as potent immune activators that mediate chemokine production, leukocyte infiltration, and neutrophil recruitment(23-25). Recent studies suggest a potential prognostic value of Th17 cells in hematological malignancies but relatively little is known about their contribution to disease and it remains controversial whether these cells accelerate oncogenic inflammation, or regulate anti-tumor responses (26-33). In our model, we observed robust induction of Th17 cells and IL-17 production both in vivo and in culture. Remarkably, the presence of Th17 cells was detected in various tissues including the spleen and bone marrow. In solid tumors and autoimmune diseases, Th17 populations are typically isolated from the site of inflammation and their numbers are relatively scarce outside of mucosal or barrier tissues. Consistent with this, we did not detect IL-17 in steady state conditions.

The ubiquitous nature of leukemia in the body imposes unique immunological pressures and exciting challenges for evaluating the impact oncogenic transformation on the host immune system. Unlike solid tumors, leukemias are widely disseminated

throughout the body. This creates a system where immune cells, both in circulation and across tissues, are in close proximity to neoplastic cells and exposed to consistent inflammatory cues. We propose the pervasive nature of this inflammation fuels the robust induction of Th17 cells. Given that particular inflammatory mediators such as IL-1 and IL-6 are conserved across leukemic models and it is likely that Th17 cells are present in other myeloid neoplasms and exert direct or indirect effects on immune activation in these contexts. The bias in differentiation to Th17 cells at the expense of Tregs suggests that prolonged exposure to these environments may induce cell intrinsic biases in the developmental trajectory of naïve T cell populations.

Another area for further investigation relates to the specific leukemia signals affect T cell differentiation. Our work suggests that the leukemic environment co-opts the T cell compartment to favor its survival and propagation. In addition to Tregs and Th17 cells it is likely that naïve T cells may have defects or biases to other T helper fates. Answers to these inquiries this will require more comprehensive profiling of the key cytokines and chemokines in specific leukemic microenvironments as well as rigorous definitions of what constitutes short-term and long-term adaptation relative to inflammatory responses.

In allogeneic hematopoietic cell transplantation (HCT), augmenting the immune-modulatory function of Tregs has proven to be a promising therapeutic strategy to reduce the morbidities associated with Graft vs. Host Disease (GVHD). Numerous approaches for expanding Tregs have shown varying degrees of effectiveness at reducing an allogeneic reaction of donor T cells and damaged recipient tissues. Of the different strategies, the targeting of Death receptor 3 (DR3), a tumor necrosis factor receptor superfamily member (TNFRSF25) has been demonstrated to potently expand Tregs both in humans and in mice. TNFRSF25 (DR3) is a type I transmembrane protein

that can be induced on activated T cells and is expressed at low levels of various immune populations (34-36). However, it is constitutively expressed at high levels on Foxp3^{+} Tregs. In the context of HCT, the use of DR3 to expand Tregs has had significant success at reducing gross inflammatory responses (37-39). In light of this, we investigated whether treatment with the DR3 agonistic antibody, 4C12, could alter disease progression. We observed that expanding Tregs prior to disease initiation was able to mitigate leukemic progression with targeted effects on myeloid cells. Importantly, DR3 stimulation was able to induce potent IL-10 production in Tregs. To determine the function of Treg IL-10 on myeloid expansion we directly assessed the effects of IL-10 on myeloid cells. Interestingly, we noted a robust and dose dependent effect of IL-10 on myeloid and neutrophil differentiation. Granulocyte-monocyte progenitors carrying the BCR-ABL1 oncogene were acutely sensitive to IL-10 and had reduced differentiation into neutrophil and myeloid lineages.

Future studies should address whether how the competition of inflammatory and anti-inflammatory signals, such as IL-6 and IL-10 are integrated by leukemic stem and progenitor populations. It is likely, that there is significant competition between specific cytokines, and exploiting these differences in signal transduction could be used to delay the development of myeloid malignancies, extend the window of clinical intervention or improve combinatorial drug therapies.

FUTURE DIRECTIONS: TREGS IN MDS

Myelodysplastic syndromes (MDS), represent a heterogeneous group of hematological malignancies. While there are numerous contributions to MDS pathology, epigenetic alterations, cytopenia, cellular dysplasia, and acute risk of leukemic transformation are hallmark traits of the disease(40). Recent investigations have shown

that autoimmune diseases and inflammatory conditions are closely associated with the initiation of MDS and predict transformation to acute myeloid leukemias (AML). Despite these findings, current risk assessment and management of MDS is primarily focused on the cytogenetic constituents of the disease, overlooking important immunological components. One population which is significantly altered in MDS patients, is Tregs(41-44).

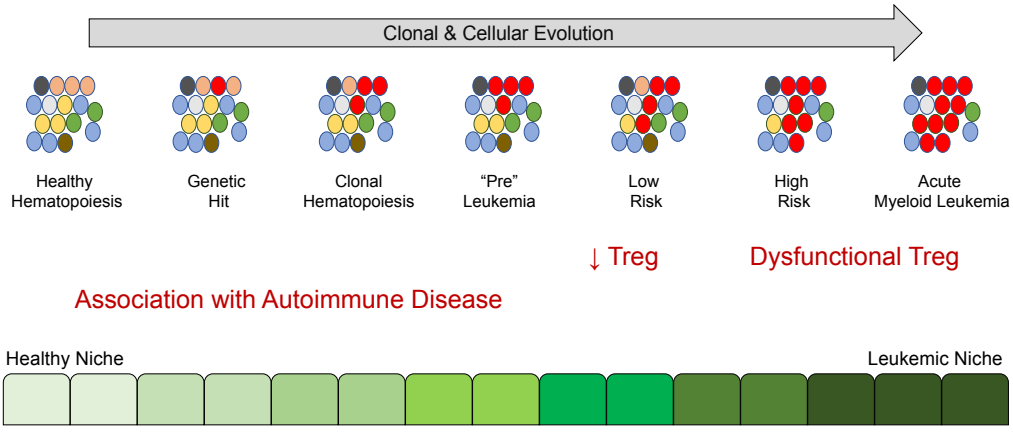


Figure 4: Tregs in Myelodysplastic syndrome
Myelodysplastic syndrome (MDS) is clonal disorder characterized by ineffective hematopoiesis and a tendency to evolve into acute myeloid leukemia (AML). Notably there is an increased risk of MDS development among patients with autoimmune diseases. Along these lines immune dysregulation and altered Tregs have been clinically implicated in the pathogenesis of disease and and have been utilized as prognostic indicators of disease progression and response to treatment. Specifically, Treg expansion is associated of high-risk MDS and progression to aggressive subtypes of the disease.

The onset of MDS has been linked to various molecular abnormalities including somatic mutations and cytogenetic lesions. This is substantiated by candidate-gene screens and Genome Wide Association Studies, which have shown that enzymes regulating the modification and methylation of DNA are frequently mutated across MDS patients(45, 46). Given the extreme heterogeneity of MDS and the vague of rubric for diagnosing patients compared to other myeloid malignancies, integrating an immune component to the existing prognostic models would greatly improve diagnosis as well and help inform treatment. The work presented here has focused on detailing the

function of Tregs in steady state hematopoietic regulation, and their therapeutic implications for myeloid leukemias. In a disease model such as MDS where clinical studies have already underscored a distinct Treg phenotype, is it likely that Treg alterations contribute to leukemogenesis. In MDS, assessing how Treg characteristics are altered in a leukemic setting and testing if alterations to Tregs alone are sufficient to drive the development of MDS represent exciting areas of investigation. Dissecting the contributions of Tregs to MDS pathogenesis has significant implications with regards to immunotherapy, and as well as diagnostic value.

References

1. Yang L, Qian Y, Eksioglu E, Epling-Burnette PK, and Wei S. The inflammatory microenvironment in MDS. *Cellular and molecular life sciences : CMLS.* 2015;72(10):1959-66.
2. Wei Y, Dimicoli S, Bueso-Ramos C, Chen R, Yang H, Neuberg D, et al. Toll-like receptor alterations in myelodysplastic syndrome. *Leukemia.* 2013;27(9):1832-40.
3. Fozza C. The burden of autoimmunity in myelodysplastic syndromes. *Hematological oncology.* 2018;36(1):15-23.
4. Zhou X, Tang J, Cao H, Fan H, and Li B. Tissue resident regulatory T cells: novel therapeutic targets for human disease. *Cellular & molecular immunology.* 2015;12(5):543-52.
5. Zou L, Barnett B, Safah H, Larussa VF, Evdemon-Hogan M, Mottram P, et al. Bone marrow is a reservoir for CD4+CD25+ regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer research.* 2004;64(22):8451-5.
6. Pierini A, Nishikii H, Baker J, Kimura T, Kwon HS, Pan Y, et al. Foxp3(+) regulatory T cells maintain the bone marrow microenvironment for B cell lymphopoiesis. *Nature communications.* 2017;8:15068.
7. Glatman Zaretsky A, Konradt C, Depis F, Wing JB, Goenka R, Atria DG, et al. T Regulatory Cells Support Plasma Cell Populations in the Bone Marrow. *Cell reports.* 2017;18(8):1906-16.
8. Hirata Y, Furuhashi K, Ishii H, Li HW, Pinho S, Ding L, et al. CD150(high) Bone Marrow Tregs Maintain Hematopoietic Stem Cell Quiescence and Immune Privilege via Adenosine. *Cell stem cell.* 2018;22(3):445-53.e5.
9. Kishore M, Cheung KCP, Fu H, Bonacina F, Wang G, Coe D, et al. Regulatory T Cell Migration Is Dependent on Glucokinase-Mediated Glycolysis. *Immunity.* 2017;47(5):875-89.e10.
10. Zhang CRC, Nix D, Gregory M, Ciorba MA, Ostrander EL, Newberry RD, et al. Inflammatory cytokines promote clonal hematopoiesis with specific mutations in ulcerative colitis patients. *Experimental hematology.* 2019;80:36-41.e3.
11. Pietras EM, Reynaud D, Kang YA, Carlin D, Calero-Nieto FJ, Leavitt AD, et al. Functionally Distinct Subsets of Lineage-Biased Multipotent Progenitors Control Blood Production in Normal and Regenerative Conditions. *Cell stem cell.* 2015;17(1):35-46.
12. Chen S, Chen B, Wen Z, Huang Z, and Ye L. IL-33/ST2-mediated inflammation in macrophages is directly abrogated by IL-10 during rheumatoid arthritis. *Oncotarget.* 2017;8(20):32407-18.
13. Takatori H, Makita S, Ito T, Matsuki A, and Nakajima H. Regulatory Mechanisms of IL-33-ST2-Mediated Allergic Inflammation. *Frontiers in immunology.* 2018;9:2004.
14. Yang J, Ramadan A, Reichenbach DK, Loschi M, Zhang J, Griesenauer B, et al. Rorc restrains the potency of ST2+ regulatory T cells in ameliorating intestinal graft-versus-host disease. *JCI insight.* 2019;4(5).
15. Halvorsen EC, Franks SE, Wadsworth BJ, Harbourne BT, Cederberg RA, Steer CA, et al. IL-33 increases ST2(+) Tregs and promotes metastatic tumour growth in the lungs in an amphiregulin-dependent manner. *Oncoimmunology.* 2019;8(2):e1527497.

16. Liu Q, Dwyer GK, Zhao Y, Li H, Mathews LR, Chakka AB, et al. IL-33-mediated IL-13 secretion by ST2+ Tregs controls inflammation after lung injury. *JCI insight.* 2019;4(6).
17. Ohue Y, and Nishikawa H. Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? *Cancer Sci.* 2019;110(7):2080-9.
18. Tanaka A, and Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Cell Res.* 2017;27(1):109-18.
19. Togashi Y, Shitara K, and Nishikawa H. Regulatory T cells in cancer immunosuppression - implications for anticancer therapy. *Nat Rev Clin Oncol.* 2019;16(6):356-71.
20. Wang J, and Ke XY. The four types of Tregs in malignant lymphomas. *J Hematol Oncol.* 2011;4:50.
21. Farinha P, Al-Tourah A, Gill K, Klasa R, Connors JM, and Gascoyne RD. The architectural pattern of FOXP3-positive T cells in follicular lymphoma is an independent predictor of survival and histologic transformation. *Blood.* 2010;115(2):289-95.
22. Droeser R, Zlobec I, Kilic E, Güth U, Heberer M, Spagnoli G, et al. Differential pattern and prognostic significance of CD4+, FOXP3+ and IL-17+ tumor infiltrating lymphocytes in ductal and lobular breast cancers. *BMC Cancer.* 2012;12:134.
23. Jadidi-Niaragh F, and Mirshafiey A. Th17 cell, the new player of neuroinflammatory process in multiple sclerosis. *Scand J Immunol.* 2011;74(1):1-13.
24. Wojkowska DW, Szpakowski P, Ksiazek-Winiarek D, Leszczynski M, and Glabinski A. Interactions between neutrophils, Th17 cells, and chemokines during the initiation of experimental model of multiple sclerosis. *Mediators Inflamm.* 2014;2014:590409.
25. McGinley AM, Sutton CE, Edwards SC, Leane CM, DeCourcey J, Teijeiro A, et al. Interleukin-17A Serves a Priming Role in Autoimmunity by Recruiting IL-1β-Producing Myeloid Cells that Promote Pathogenic T Cells. *Immunity.* 2020;52(2):342-56.e6.
26. Abousamra NK, Salah El-Din M, and Helal R. Prognostic value of Th17 cells in acute leukemia. *Med Oncol.* 2013;30(4):732.
27. Bi L, Wu J, Ye A, Wu J, Yu K, Zhang S, et al. Increased Th17 cells and IL-17A exist in patients with B cell acute lymphoblastic leukemia and promote proliferation and resistance to daunorubicin through activation of Akt signaling. *J Transl Med.* 2016;14(1):132.
28. Gaublomme JT, Yosef N, Lee Y, Gertner RS, Yang LV, Wu C, et al. Single-Cell Genomics Unveils Critical Regulators of Th17 Cell Pathogenicity. *Cell.* 2015;163(6):1400-12.
29. Jain P, Javdan M, Feger FK, Chiu PY, Sison C, Damle RN, et al. Th17 and non-Th17 interleukin-17-expressing cells in chronic lymphocytic leukemia: delineation, distribution, and clinical relevance. *Haematologica.* 2012;97(4):599-607.
30. Li P, Ji M, Park J, Bunting KD, Ji C, and Tse W. Th17 related cytokines in acute myeloid leukemia. *Front Biosci (Landmark Ed).* 2012;17:2284-94.
31. Shahid A, and Bharadwaj M. The connection between the Th17 cell related cytokines and cancer stem cells in cancer: Novel therapeutic targets. *Immunol Lett.* 2019;213:9-20.

32. Xiang M, Guo L, Ma Y, and Li Y. Expression of Th17 and CD4+ CD25+ T regulatory cells in peripheral blood of acute leukemia patients and their prognostic significance. *Pak J Pharm Sci.* 2017;30(2(Suppl.)):619-24.
33. Yousefi M, Movassaghpour AA, Shamsasenjan K, Ghalamfarsa G, Sadreddini S, Jadidi-Niaragh F, et al. The skewed balance between Tregs and Th17 in chronic lymphocytic leukemia. *Future Oncol.* 2015;11(10):1567-82.
34. Li J, Shi W, Sun H, Ji Y, Chen Y, Guo X, et al. Activation of DR3 signaling causes loss of ILC3s and exacerbates intestinal inflammation. *Nature communications.* 2019;10(1):3371.
35. Siakavellas SI, Sfikakis PP, and Bamias G. The TL1A/DR3/DcR3 pathway in autoimmune rheumatic diseases. *Semin Arthritis Rheum.* 2015;45(1):1-8.
36. Valatas V, Kolios G, and Bamias G. TL1A (TNFSF15) and DR3 (TNFRSF25): A Co-stimulatory System of Cytokines With Diverse Functions in Gut Mucosal Immunity. *Frontiers in immunology.* 2019;10:583.
37. Kim BS, Nishikii H, Baker J, Pierini A, Schneidawind D, Pan Y, et al. Treatment with agonistic DR3 antibody results in expansion of donor Tregs and reduced graft-versus-host disease. *Blood.* 2015;126(4):546-57.
38. Nishikii H, Kim BS, Yokoyama Y, Chen Y, Baker J, Pierini A, et al. DR3 signaling modulates the function of Foxp3+ regulatory T cells and the severity of acute graft-versus-host disease. *Blood.* 2016;128(24):2846-58.
39. Mavers M, Simonetta F, Nishikii H, Ribado JV, Maas-Bauer K, Alvarez M, et al. Activation of the DR3-TL1A Axis in Donor Mice Leads to Regulatory T Cell Expansion and Activation With Reduction in Graft-Versus-Host Disease. *Frontiers in immunology.* 2019;10:1624.
40. Sperling AS, Gibson CJ, and Ebert BL. The genetics of myelodysplastic syndrome: from clonal haematopoiesis to secondary leukaemia. *Nature reviews Cancer.* 2017;17(1):5-19.
41. Kordasti S, Marsh J, Al-Khan S, et al. Functional characterization of CD4+ T cells in aplastic anemia. Blood. 2012;119(9):2033-2043. *Blood.* 2020;136(9):1114.
42. Komrokji RS, Kulasekararaj A, Al Ali NH, Kordasti S, Bart-Smith E, Craig BM, et al. Autoimmune diseases and myelodysplastic syndromes. *Am J Hematol.* 2016;91(5):E280-3.
43. Kordasti S, Costantini B, Seidl T, Perez Abellan P, Martinez Llordella M, McLornan D, et al. Deep phenotyping of Tregs identifies an immune signature for idiopathic aplastic anemia and predicts response to treatment. *Blood.* 2016;128(9):1193-205.
44. Povoleri GAM, Nova-Lamperti E, Scottà C, Fanelli G, Chen YC, Becker PD, et al. Human retinoic acid-regulated CD161(+) regulatory T cells support wound repair in intestinal mucosa. *Nature immunology.* 2018;19(12):1403-14.
45. Chung YR, Schatoff E, and Abdel-Wahab O. Epigenetic alterations in hematopoietic malignancies. *International journal of hematology.* 2012;96(4):413-27.
46. Steensma DP, Bejar R, Jaiswal S, Lindsley RC, Sekeres MA, Hasserjian RP, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood.* 2015;126(1):9-16.

APPENDIX A

Institutional Animal Care Approval Form

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 28-May-2019

TO: Welner, Robert Samuel

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 28-May-2019.

Protocol PI:	Welner, Robert Samuel
Title:	Impact of leukemic cells on hematopoietic niche
Sponsor:	UAB DEPARTMENT
Animal Project Number (APN):	IACUC-20430

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 27-May-2022.

Institutional Animal Care and Use Committee (IACUC)

403 Community Health on 19th | 933 19th Street South

Mailing Address:
CH19 403 | 1720 2nd Ave South | Birmingham AL 35294-2041

phone: 205.934.7692 | fax: 205.934.1188
www.uab.edu/iacuc | iacuc@uab.edu