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The Ins and the Outs of Hematopoiesis: Intrinsic and extrinsic regulators of hematopoietic stem cell fate choices

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by

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Abstract

The Ins and the Outs of Hematopoiesis: cell intrinsic and extrinsic regulators of hematopoietic stem cell fate choices

Taylor S. Cool

Hematopoiesis, the process of generating all blood and immune cells from hematopoietic stem cells (HSCs), is a tightly regulated process orchestrated by cell-intrinsic and cell-extrinsic cues. This process occurs in "waves" during fetal development, with windows of distinct and transient hematopoietic stem and progenitor cells (HSPCs) giving rise to "non-traditional" mature cell types. These cells are referred to as non-traditional because they arise during fetal development from these unique HSPCs, and then self-maintain for the lifespan without contribution from the adult (or life-long definitive) HSC pool. In contrast, adult hematopoiesis occurs in a very well-characterized hierarchy, where HSCs differentiate in a more lineage-restricted progression into more lineage restricted progenitor cells before terminally differentiating into "traditional" mature cell types. These cell types are referred to as traditional mature blood and immune cells because they are constantly replenished from the life-long definitive HSCs for the remainder of life. This body of work identifies cell-intrinsic and cell-extrinsic regulators of this process both during steady state health development and maintenance, as well as how dysregulation of this process alters hematopoietic output and function for life.

Dedication

To my son, Patrick.

I hope you find this work an inspiration to always follow your dreams and to take every challenge head on.

I love you so much.

Also, to my grandmother, Gerry.

Who pushed me and inspired me to be the woman that I am today.

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He was there to lean on during my hardest of times. I could not have done this without him.

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Introduction

[This section is adapted from a review, Chasing Mavericks: The quest for defining developmental waves of hematopoiesis, published in Current Topics in Developmental Biology in 2019]

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Abstract

Hematopoiesis is the process by which mature blood and immune cells are produced from hematopoietic stem and progenitor cells (HSCs and HSPCs). The last several decades of research have shed light on the origin of HSCs, as well as the heterogeneous pools of fetal progenitors that contribute to lifelong hematopoiesis. The overarching concept that hematopoiesis occurs in dynamic, overlapping waves throughout development, with each wave contributing to both continuous and developmentally limited cell types, has been solidified over the years. However, recent advances in our ability to track the production of hematopoietic cells in vivo have challenged several long-held dogmas on the origin and persistence of distinct hematopoietic cell types. In this review, we highlight emerging concepts in hematopoietic development and identify unanswered questions.

Introduction

Hematopoietic stem cells (HSCs) are blood stem cells capable of self-renewal and differentiation into lineage-restricted progenitor cells that ultimately make mature cell types with highly specialized functions. HSCs were first described in the 1960s as cells capable of giving rise to spleen colonies containing clonally multipotent cells (Becker et al., 1963; A. M. Wu et al., 1968). These studies laid the groundwork for our initial understanding of blood stem cells and defined them as cells with combined self-renewal and multilineage hematopoietic differentiation capability upon transplantation into a suitable host. Subsequently, Spangrude et al. demonstrated that 30 HSCs were sufficient to rescue half a population of lethally irradiated mice, indicating the potent reconstitution capacity of these cells (Spangrude et al., 1988a). Since then, the field of molecular and cellular biology has advanced rapidly, allowing for extremely sensitive detection of donor-derived cells to assess the clonal capacity of distinct subsets of hematopoietic stem and progenitor cells (HSPCs).

While transplantation of HSPCs into conditioned hosts has long been the "gold standard" in the field, in situ fate mapping, barcoding, and RNA sequencing (RNA-seq) have become increasingly common to interrogate HSC identity and function. There are pros and cons to each of these techniques. Transplantation assays test the inherent potential of stem and progenitor cells to produce different cell types. However, the conditioning regimens typically used to ensure robust reconstitution may affect the

lineage capacity and/or preference of the incoming cells as well as the clearance of resident cell types, thus altering the perceived potential of the transplanted cell. Although reconstitution assays remain an essential tool to assess HSPC in vivo lineage potential and the therapeutic potential of cell subpopulations for regenerative medicine, HSPC transplantation might not reflect the cell ontogeny of normal hematopoiesis. Fate mapping using permanent genetic marking allows for tracking of HSPC differentiation in situ without disrupting normal hematopoiesis. Yet, it is difficult to reliably pinpoint the initiation of labeling to only HSCs, since different progenitors may transiently express the gene responsible for the labeling. In situ barcoding assays allow us to determine contributions of single clones to the hematopoietic system (Busch et al., 2015; Pei et al., 2017; Sun et al., 2014a) (reviewed in Dharampuriya et al., 2017). However, interpretation of the outcomes is complex and relies heavily on mathematical modeling, and the conclusions have been challenged (Sawai et al., 2016). In addition to these functional assays, RNA-seq of bulk populations or single cells is heavily utilized. This is an extremely useful, yet relatively simple, tool to identify differences between cells. Although the data collected from RNA-seq are descriptive and not without caveats (Arzalluz-Luque et al., 2017; Griffiths et al., 2018; Herring et al., 2018), they provide a valuable database of genes to further investigate. Regardless of caveats, these assays have collectively proven extremely beneficial in furthering our understanding of hematopoiesis.

Increasingly sophisticated versions of these techniques have led to reconsideration of some long-held dogmas of hematopoiesis. Vast heterogeneity in stem and progenitor populations throughout development and in adulthood has been revealed, and the idea that the adult HSC pool is derived from one small pool of cells, as previously believed, has been challenged (Ganuza et al., 2017; Pei et al., 2017; Yu et al., 2016). Accumulating evidence suggests that embryonic progenitors that precede lifelong, engraftable HSCs contribute to distinct cell types that persist with little replenishment from the adult HSC pool (Hoeffel et al., 2015; Kierdorf et al., 2015). The extent to which different HSCs, and their fetal precursors, contribute to hematopoiesis during development and over the lifespan is under debate (Sawai, 2016). Even the concept that definitive, repopulation-capable HSCs persist for life has been challenged (Beaudin et al., 2016). In order to solidify emerging views and continue to gain deeper insights into the biology of HSCs, many labs focus their attention to the developmental origin of HSCs and the lifelong contribution of discrete waves of hematopoiesis. Important goals include gaining an actionable understanding of the biology of hematopoietic disease, and how to generate and culture HSCs in vitro for therapeutic use. In this review, with emphasis on lessons learned from the mouse, we aim to summarize the current perspective on the contributions of developmental waves of HSPCs to the specification and maintenance of lifelong hematopoiesis, and to pinpoint questions that remain unanswered.

HSC emergence: Where, when, and how?

Cellular origins of HSCs: Hemangioblast versus hemogenic endothelium

The mechanisms of HSC emergence have been under debate for decades.One hypothesis posited that hematopoietic and endothelial lineages arise through a specialized bi-potent progenitor known as the hemangioblast (Fig. 1.1). This term was initially used to describe a group of mesodermal cells from the primitive streak that contained endothelial and hematopoietic cells (Murray, 1932). One study demonstrated that a subset of embryonic stem cell (ESC)-derived progenitors were capable of producing both endothelial and hematopoietic lineages (Lacaud et al., 2006). The authors concluded that this progenitor population was an in vitro equivalent of the hemangioblast. Using a brachyury-GFP transgene, Huber and colleagues identified a subset of Flk1+brachyury+ cells that modeled the mesodermal-to-hemangioblast transition (Huber et al., 2004) providing further ex vivo support for the existence of a hemangioblast. Ueno and colleagues tested the hemangioblast concept in vivo by making tetrachimeric mouse embryos that clonally expressed different fluorescent proteins (Ueno & Weissman, 2006). The dissimilar proportions of colored endothelial and hematopoietic cells within individual blood islands argued against a shared bi-potent progenitor that contributes equally to both lineages. Another group used a temporally-restricted fate mapping model, VE-cadherin-CreERT2, to selectively label endothelial cells and thereby distinguish between HSC emergence from the endothelium or underlying mesenchyme (Zovein et al., 2008). By injecting pregnant mothers at E9.5 with tamoxifen, they aimed to label aorta-gonad-mesonephros (AGM)-derived HSCs, while evading the earlier VE-cadherin expression of the yolk sac (YS).

By surveying additional models (SM22α-Cre and Myocardin-Cre) that labeled mesenchymal cells and their progeny, they ruled out a direct mesenchyme-to-HSC transition and concluded that only the endothelial lineage was capable of generating HSCs. Similarly supporting an endothelial ancestry, Oberlin et al. demonstrated that the majority of adult BM HSCs are derived from a VE-cadherin positive precursor (Oberlin et al., 2010). However, VE-cadherin may not be entirely restricted to the endothelium. One study demonstrated that fetal HSCs express VE-cadherin at E13.5, but lose expression by E16.5 (Kim et al., 2005); thus, the observed labeling of hematopoietic cells in this model may not signify an endothelial origin.

An alternative hypothesis to the bi-potent hemangioblast proposed that mesoderm first gives rise to endothelial cells, some of which possess the capacity to generate HSCs (Figure 1.1). In support of HSC emergence after endothelial specification, Lancrin and colleagues identified a hemogenic endothelial precursor intermediate that was capable of giving rise to the first hematopoietic cells (Lancrin et al., 2009). Another group, using in vitro time-lapse imaging, concluded that both ESC-derived mesodermal cells and mesodermal cells isolated from E7.5 embryos were able to give rise to hematopoietic cells through an endothelial intermediate, supporting the endothelial origin of HSCs (Eilken et al., 2009). Boisset and colleagues used in vivo time-lapse imaging to visualize HSC emergence from endothelium in the AGM region (Boisset et al., 2010). Similar findings were made simultaneously in zebrafish: two groups concluded that HSCs emerge from ventral aortic hemogenic endothelial cells prior to

the onset of blood circulation (Bertrand et al., 2010; Kissa & Herbomel, 2010). Thus, HSC emergence appears similar in zebrafish and mouse, although de Brujin et al. concluded that HSC activity was limited to a few cells within the endothelial layer of the dorsal aorta in mouse, as opposed to the ventral aorta in fish (M. F. T. R. de Bruijn et al., 2002).

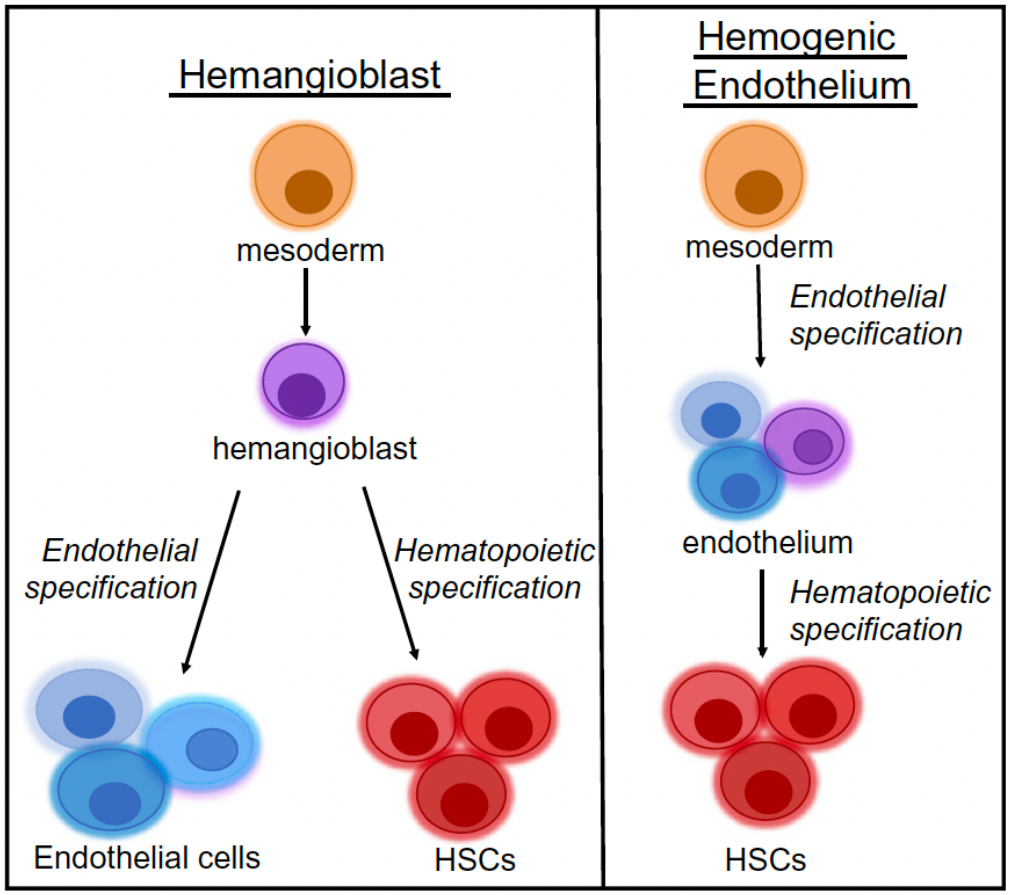

Figure .1. Hemangioblast versus hemogenic endothelium schematic. Two proposed models of HSC emergence in the embryo. One model proposes that a specialized bi-potent precursor named the hemangioblast gives rise to both endothelial cells and HSCs. An alternative model proposes that mesoderm gives rise first to endothelium,a small proportion of which can give rise to HSCs. In this schematic, the purple cell represents the subset of hemogenic endothelial cells. The different shades represent heterogeneity within both lineages.

More recently, a group used single cell isolation and RNA-seq to identify molecular signatures of pre-HSCs (F. Zhou et al., 2016). They concluded that Rictor, a component of the mTORC pathway, is required for the emergence of HSCs from endothelial cells, a finding that supports the notion that HSCs arise from endothelial cells (Figures 1.1 and 1.2). Ganuza and colleagues performed ex vivo cultures of murine cells from the midgestation aorta, vitelline artery, and umbilical artery and found that only a very small percentage of the cells had in vitro functional properties of hemogenic endothelium (Ganuza et al., 2018). Further, they suggested that heterogeneity within the hemogenic endothelial population might contribute to distinct populations with differential capacities to specify HSCs (Figure 2). This finding suggests that several types of precursors contribute to HSPCs. This idea is novel and striking given that some labs have believed for decades that one specific type of precursor contributes to the first HSCs. While no definitive conclusion has been made regarding the exact properties of the in vivo precursors to HSCs, it is clear that endothelial and hematopoietic lineages are closely linked (Figures 1.1 and 1.2). The concepts of the hemangioblast versus the endothelial-to-hematopoietic transition are quite similar and may not be mutually exclusive: in both cases, mesoderm cells give rise to endothelial and hematopoietic lineages (Figures 1.1 and 1.2). Several reviews discuss the hemangioblast versus hemogenic endothelial origin of HSCs in greater detail (Boisset et al., 2010; M. de Bruijn, 2014; Lacaud & Kouskoff, 2017; Nishikawa, 2012).

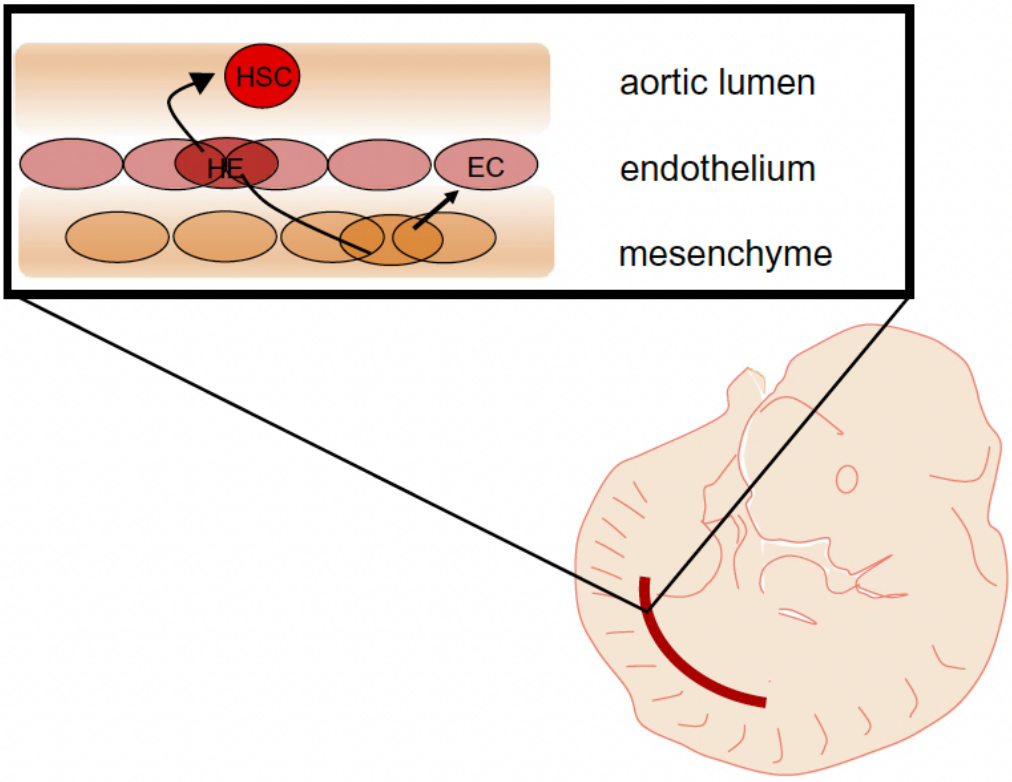

Figure 1.2 HSC emergence. It is currently believed that definitive HSCs emerge in the AGM region of E10.5 murine embryos. There has been substantial evidence supporting the notion that a subset of mesenchyme-derived endothelial cells (ECs), called hemogenic endothelium (HE), has the potential to give rise to HSCs. Such de novo HSC specification in the AGM may contribute to lifelong hematopoiesis in parallel with YS-derived hematopoietic cells.

Are HSCs specified multiple times in distinct locations?

Tracking the ontogeny of HSCs has proven challenging due to the different temporal and spatial emergence of blood cells. It has been proposed that HSCs emerge de novo in either the YS, placenta, and/or AGM, and eventually seed the fetal liver (FL) and adult bone marrow (BM). Early in vitro and in vivo colony forming assays (Huang & Auerbach, 1993; Liu & Auerbach, n.d.; Moore & Metcalf, 1970; Perah & Feldman, 1977; Toles et al., 1989) suggested that HSCs emerge in the YS, migrate to the FL, and

finally to the BM, where they reside in adulthood and contribute to lifelong hematopoiesis. In support of this model, Samokhvalov et al. demonstrated thatRunx1+ cells, labeled with a single tamoxifen injection at E7.5 in aMER-cre-MERmodel, were able to develop into fetal myeloid and lymphoid progenitors and contribute to adult hematopoiesis (Samokhvalov et al., 2007). They concluded that these cells were able to colonize the umbilical cord, AGM, and ultimately the FL, suggesting that at least some definitive HSCs might be descendants of hematopoietic cells first specified in the YS.More recent data from their group substantiate these findings by providing evidence that a subset of extraembryonic Runx1+Gata1– cells in the E7.5 embryo migrate to intraembryonic sites prior to the onset of circulation (initiation of a heartbeat) (Tanaka et al., 2014). Similar findings were made using a Lyve1-cre mouse model (Lee et al., 2016). These results are quite striking as they suggest that systemic circulation is not required for HSC migration through the embryo. However, it has also been suggested that HSCs are generated de novo in the AGM, challenging an exclusive YS origin of definitive HSCs (Medvinsky & Dzierzak, 1996a). Using an in vitro organ culture system to tease apart the hematopoietic potential of cells residing in the YS and AGM region, the authors concluded that the AGM is the source of definitive HSCs that ultimately seed the adult BM. Taoudi and colleagues reported that VEcadherin+CD45+ pre-definitive HSCs in the AGM region give rise to the first definitive HSC (Taoudi et al., 2008). This finding contradicted the YS origin of HSCs and suggested that HSCs are generated de novo in the AGM rather than migrating from the YS. Several other studies have also reported de novo generation of HSCs in the AGM region (M. F. T. R.

de Bruijn et al., 2000; Durand & Dzierzak, 2005; Peeters et al., 2009) or in the placenta, independent of circulation (Gekas et al., 2010; Rhodes et al., 2008).

Many labs have utilized Cre lineage tracing to directly assess the emergence of HSCs. Using an inducible Stem Cell Leukemia (SCL)-specific Cre line, one group addressed whether HSCs are generated *de novo* at each site during fetal development (Gothert et al., 2005). They injected tamoxifen into pregnant mothers at E10.5 in order to label AGM HSCs and analyzed the BM of adult progeny for labeled HSCs. As ~10% of the adult BM HSCs were labeled, at least some of the fetal HSCs appear to seed the adult BM. However, this result also revealed either inefficient HSC labeling (at this time point), or that additional HSCs may be specified after this time point. To address the possibility of de novo generation of HSCs at later timepoints, they compared the percent EYFP+ cells in the adult BM of mice dosed at E10.5 and E11.5 with the percent EYFP+ cells in the BM of mice transplanted with previously marked E14.5 FL cells. The authors reasoned that de novo generation of HSCs would have led to a decrease in EYFP+ cells in the BM of the transplanted mice. They found that mice transplanted with E14.5 FL HSCs had a similar percentage of labeled HSCs in their adult BM compared to the embryos treated with tamoxifen at E10.5, thus arguing against de novo generation beyond E10.5. However, it is important to note that the labeling efficiency of CreER models during embryonic development is frequently incomplete, and that the origins of the remaining 90% of unlabeled cells in this model remain unknown.

The lack of consensus on the anatomical location(s) of the emergence of the first HSC(s) is likely, in part, due to models and methods being used. One issue is the length of time tamoxifen persists in the system. For example, Nishikawa's group suggested that YS cells migrate to colonize the embryo; however, labeling of intraembryonic cells could potentially be explained by tamoxifen remaining in the system and labeling cells within the embryo that express Runx1 at a later time point. In addition, most groups use different Cre models, independently, that undoubtedly label divergent cell types. A thorough side by side comparison of the Cre lines that are specific to early hematopoietic development may help in our data analysis and interpretations, and ultimately better define hematopoietic development (Abram et al., 2014). Additionally, transplantation assays may not be sufficient to define which "HSCs" persist in situ (Figure 1.3). It has been demonstrated that embryonic HSCs engraft better in neonates compared to adults (Arora et al., 2014; Yoder et al., 1997). Additionally, evidence is accumulating that cells that do not engraft long term upon transplantation can persist for long periods of time in situ (Busch et al., 2015; Pei et al., 2017; Schoedel et al., 2016; Sun et al., 2014b). Models that do not rely on niche perturbations or culturing of cells may provide us with better insights to the development of the blood system in situ (Ganuza et al., 2018).

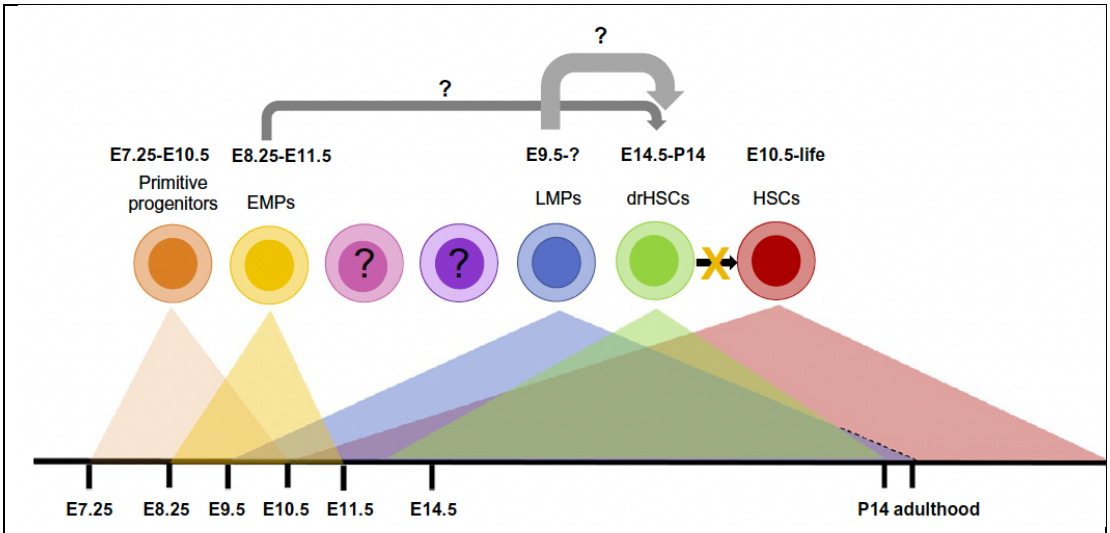

Figure 1.1. Fetal hematopoiesis occurs in waves. Several distinct progenitors exist at different developmental stages in situ. Primitive erythroid precursors can be isolated from E7.25 until E10.5. Erythro-myeloid progenitors (EMPs) exist from E8.25 until E11.5. Lymphomyeloid progenitors (LMPs) appear around E9.5. Engraftable, developmentally restricted (dr)HSCs can be detected from E12.5 until postnatal day 14 (P14). Definitive HSCs that contribute to lifelong hematopoiesis are identifiable from E10.5 onward. It remains to be determined whether the drHSC is derived from EMPs, LMPs, or an independent precursor. It is clear that drHSCs do not give rise to definitive HSCs. Whether other previously unidentified HSPCs exist during this timeframe remains to be determined.

Molecular regulation of HSC specification

What are the molecular drivers of HSC specification and maintenance?

The molecular drivers of hematopoiesis change several times from a developing embryo to a fully formed vertebrate (Table 1). Efforts focused on understanding the different genetic programs that regulate HSC specification, expansion, and maintenance have identified distinct transcription factors involved in primitive erythropoiesis. Several studies have demonstrated that SCL/TAL1 is required for primitive hematopoiesis and specification of HSCs (Robb et al., 1995; Shivdasani et al., 1995; Visvader et al., 1998) and divergence of cardiac and hematopoietic lineages during development (Org et al., 2015). SCL is required for the initial specification of

the hematopoietic system, but not for long-term HSC colonization in the FL (Schlaeger et al., 2005), and conditional deletion of SCL in adult mice showed that SCL is dispensable for adult HSC maintenance (Mikkola et al., 2003). Similarly, the transcriptional regulators Rbtn2 (LMO2) (Warren et al., 1994) and CBP (Tanaka et al., 2000) appear to be required for primitive hematopoiesis and specification of HSCs, but not for HSC maintenance.

Like SCL, Runx1 is a transcription factor and an essential regulator of hematopoietic specification. Okuda et al. showed that Runx1 is required for FL hematopoiesis (Okuda et al., 1996). Mice with a homozygous mutation of Runx1 exhibited normal YS erythropoiesis, but eventually died around E12.5 due to failed FL hematopoiesis. Importantly, endothelial-specific loss of Runx1 precluded HSC formation (M. J. Chen et al., 2009), whereas hematopoietic deletion of Runx1 allowed normal EMP and HSC formation (Ichikawa et al., 2004). This supports HSC derivation from endothelial cells in a Runx1-dependent process. To further define the window of Runx1 dependency, Tober *et al.* performed timed deletions of Runx1 from E7.5 until E13.5 (Tober et al., 2013). When Runx1 was deleted between E9.5 and E11.5, recipients had no engraftment, while deletion after E11.5 led to high levels of engraftment. The authors concluded that Runx1 is required for EMP and HSC formation up until E10.5 and E11.5, respectively. In addition to Runx1, ATF4 (Masuoka & Townes, 2002; Zhao et al., 2015), Cbfb (Bresciani et al., 2014; Sasaki et al., 1996), Gata2 (Ling et al., 2004; Mehta et al., 2017; N. P. Rodrigues et al., 2005; Tsai et al., 1994), Sox17 (Clarke et al.,

2013; Kim et al., 2007), and C-Myb (Emambokus et al., 2003; Lieu & Reddy, 2009; Mucenski et al., 1991; Zhang et al., 2011) have also been shown to be indispensable for normal mouse FL hematopoiesis (Table 1). Some of these factors also regulate adult HSC maintenance and function, while some are dispensable for adult hematopoiesis (Table 1). Likewise, a unique set of genes are required for the maintenance of adult HSCs (Hock et al., 2004; Semerad et al., 2009; van der Lugt et al., 1994; Ye et al., 2013; Zhuang et al., 1994). Interestingly, Sox17 and Lin28 are highly expressed in FL, but not adult, HSCs, and can be used to reprogram adult HSCs into cells that have regained at least some of the properties of fetal HSCs (He et al., 2011; Lang et al., 2013; Yuan et al., 2012). These studies have identified several key regulators of primitive and definitive hematopoiesis and have given us insight into the development and maintenance of distinct stages of HSCs (Table 1). However, the intricate network of genes being regulated by these factors remains under investigation, with the hopes of understanding the coordinated actions of key hematopoietic pathways that contribute to lifelong hematopoiesis.

Table 1. Molecular regulators of HSC specification

Stage	Molecular Regulator	Deficit when knocked out	Required for primitive hematopoiesis?	Required for definitive hematopoiesis?	References
Primitive hematopoiesis	Scl	No YS hematopoiesis. Disrupted capillary formation.	Yes	No	Visvader,Fujiwara, & Orkin, 1998; Robb et al., 1995; Shivdasani,Mayer, & Orkin, 1995; Y Tanaka et al., 2000; Mikkola et al., 2003; Schlaeger, Mikkola, Gekas, Helgadottir, & Orkin, 2005; Org et al., 2015
Primitive hematopoiesis	Cpb	Defective blood vessel formation.	Yes	?	Tanaka et al., 2000
Primitive hematopoiesis	Runx1	Absence of fetal liver hematopoiesis.	Yes	No	Okuda, van Deursen, Hiebert, Grosveld, & Downing, 1996; Chen, Yokomizo, Zeigler, Dzierzak, & Speck, 2009; Tober, Yzaguirre, Piwarzyk, & Speck, 2013
Primitive	Sox17	Reduced hemogenic endothelium and HSCs in AGM region.	Yes	No	Clarke et al., 2013; Injune Kim, Saunders, & Morrison, 2007
Primitive to definitive transition	ATF4	FL hematopoiesis impaired. HSC self-renewal impaired.	Yes	Yes?	Masuoka & Townes, 2002; Zhao et al., 2015
Primitive to definitive transition	Cbfb	Nascent HSCs stuck in AGM (cannot enter circulation)	No	Yes	Bresciani et al., 2014; Sasaki et al., 1996
Primitive to definitive transition	Gata-2	Blocked HSC formation in AGM. Adult HSCs have impaired proliferation.	No	Yes	Ling et al., 2004; Mehta et al., 2017; Rodrigues et al., 2005; Tsai et al., 1994
Primitive to definitive transition	C-myb	Blocked migration from AGM. Conditional deletion in adults leads to impaired self-renewal.	Yes	Yes	Emambokus et al., 2003; Lieu & Reddy, 2009; Mucenski et al., 1991; Zhang, Jin, Li, Qin, & Wen, 2011
Primitive to definitive transition	C/EBPa	Impaired granulopoiesis. Enhanced repopulation activity of FL HSCs. Conditional deletion in adults leads to increased proliferation of HSCs.	Yes	Yes	Ye et al., 2013
Definitive hematopoiesis	Tet/Etv6	Impaired megakrypoiesis	No	Yes	Hock et al., 2004
Definitive hematopoiesis	E2A	Blocked B cell development leading to postnatal death. HSC pool maintenance perturbed. Impaired myelolymphoid/myloerythroid progenitor maturation.	No	Yes	Semerad, Mercer, Inlay, Weissman, & Murre, 2009; Zhuang, Soriano, & Weintraub, 1994
Definitive hematopoiesis	Bmi-1	Impaired hematopoiesis postnatally. Reduced hematopoietic numbers. Increased adipocytes in bone marrow.	No	Yes	van der Lugt et al., 1994

Phenotypic and functional heterogeneity within the HSPC pool

Extensive transcriptome heterogeneity of cell populations

For several decades, the field has believed that one small pool of HSCs gives rise to a relatively homogeneous HSC pool and ultimately the blood system (Kumaravelu et al., 2002; Medvinsky et al., 2011; Medvinsky & Dzierzak, 1996b; Morrison et al., 1995; Spangrude et al., 1988b). Numerous studies have identified subsets of hematopoietic precursors and HSPCs using single-cell (sc)RNA-seq (Guo et al., 2013; Kowalczyk et al., 2015; Macaulay et al., 2016; Moignard et al., 2013; Wilson et al., 2015). In an impressive analyses of early mesoderm specification, one group assayed over 3,900 cells from developing embryos spanning E7.0 and E8.5 (Moignard et al., 2015). This revealed three cell clusters within the embryo: primitive streak/neural plate associated cells, a mixed group containing primitive streak/neural plate and hemogenic endothelial associated cells, and a final group consisting of hematopoietic associated cells. Computational analysis identified Sox7 as a potential regulator of early blood divergence. An inducible Sox7 overexpression model validated this prediction. Embryos overexpressing Sox7 displayed a clear reduction in primitive erythroid cells, indicating that downregulation of Sox7 is required for early blood cell commitment. Similarly, Scialdone *et al.* used bulk and scRNA-seq to interrogate the endothelial and hematopoietic specification from E6.5-E8.5 embryos (Scialdone et al., 2016). Gene expression data from wild type embryos and SCL mutants indicated that SCL regulates several genes associated with early blood specification, including Sox7.

In parallel to these embryonic studies, several groups have addressed heterogeneity within the adult HSPC pool using RNA-seq (Kowalczyk et al., 2015; Wilson et al., 2015). One group performed scRNA-seq of adult HSCs and their direct descendants, MPP1 cells, and identified subsets of quiescent and "active" HSCs, based on differential expression of cell cycle genes, DNA replication genes, and myeloid associated genes (Yang et al., 2016). The vast heterogeneity identified by these studies has prompted investigation by our lab and others as to whether other distinct stem and progenitor cells exist throughout the lifespan. Analogous scRNA-seq analysis of fetal stem and progenitor populations may shed light on previously unidentified populations in the developing embryo.

Functional heterogeneity of hematopoietic progenitor populations

To address the functional consequences of the revealed heterogeneity, many labs have utilized transplantation, barcoding, and lineage tracing with complex reporter systems. Interestingly, experiments that investigated the capacity of HSCs isolated from different developmental time points and tissues revealed extensive heterogeneity that changes throughout development and the lifespan (Benz and Eaves, 2012). This revelation suggested the existence of several distinct clones of HSCs and that pools are clonally selected based on supporting niches and the needs of the developing mouse. It also prompted the creation of more complex systems to address the clonal capacity of individual HSCs. Ganuza *et al.* utilized the Confetti system with several Cre lines to conclude, with the help of mathematical modeling, that several hundred developmental

precursors contribute to long-term hematopoiesis (Ganuza et al., 2017). Unlike previous estimations of HSC numbers in the developing embryo, this model did not rely on ex vivo culture or transplantation of embryonic cells. Also using a multicolor reporter strategy, Yu *et al.* reported that the murine hematopoietic system is composed of persistent and non-persistent clones, with a small population of "dominant" clones that persist and give rise to most of the blood system while others completely disappear (J. Wu et al., 2016). Such a non-persistent, developmentally restricted (dr)HSC that retain serial, multilineage reconstitution capability was recently identified in FL (Beaudin et al., 2016). Pei et al, using an artificial DNA recombination locus, "polylox", were able to tag thousands of single progenitor cells in the embryo and attribute the cell production capacity of individual clones (Pei et al., 2017). Like Ganuza and Yu, they concluded that the embryo is made up of many clones of HSCs and that some clones are more productive than others. Similar results have been made in the zebrafish (Henninger et al., 2017). While the extent of heterogeneity within the embryonic and adult HSC pools is still unclear, there is mounting consensus that many more clones than previously believed contribute to hematopoiesis during development and throughout the lifespan.

Do all HSCs persist?

There is conflicting evidence regarding the persistence of HSCs. One hypothesis is that all HSCs, endowed with self-renewal capability by definition, persist throughout the lifespan. As discussed above, the Nishikawa and Inlay/Mikkola groups have provided

evidence that hematopoietic cells specified in the YS can persist for life (Lee et al., 2016; Samokhvalov et al., 2007). However, there is also accumulating evidence for the converse model, with some "HSC" populations extinguishing over time (Beaudin et al., 2016; J. Wu et al., 2016). Recently, our lab identified a developmentally-restricted HSC (drHSC) that does not persist into adulthood in unperturbed mice, supporting the notion that not all pools of HSCs endure for life (Beaudin et al., 2016). We identified this drHSC via use of the $Flk2^{Cre}/Rosa^{mTmG}$, also known as "FlkSwitch", mice (Beaudin et al., 2016; Boyer et al., 2011, 2012). Although functional HSCs by serial transplantation assays, the drHSCs only exist perinatally (Figures 1.3 and 1.4). The irreversible, Cre-mediated genetic deletion that marks the drHSCs (but not adult HSCs) means that drHSCs cannot be precursors of adult HSCs, thereby questioning the idea that hematopoiesis is one linear progression through development. The temporal restriction of drHSCs to a perinatal window also defies the principle that engraftable HSCs persist and repopulate the entire blood system throughout the lifespan. The drHSCs efficiently gave rise to unique subsets of cells, including innate-like B and T lymphocytes, supporting the concept of developmental waves that comprise both unique lineage capacities and varying degrees of overlap in lineage potential (Figure 1.3). The ability to prospectively isolate drHSCs will enable new insights to the mechanisms regulating HSC persistence and lineage potential and extend recent work in the field that has highlighted the vast heterogeneity of hematopoietic progenitors (Table 2) (Eaves, 2015).

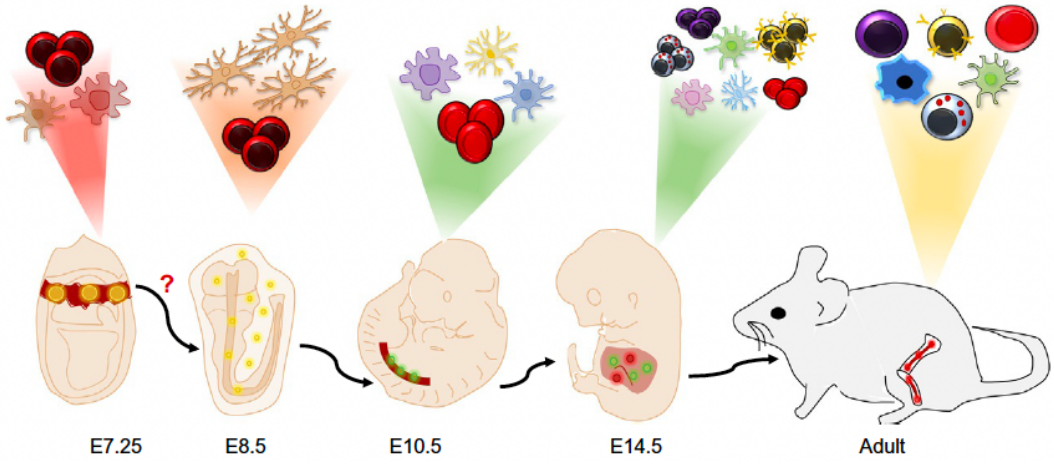

Figure 1.4 Progenitors at different time points give rise to both overlapping and distinct subsets of cells. A simplified model depicting key features of developmental hematopoiesis. Around E7, cells of the yolk sac blood islands give rise to primitive macrophages and to primitive erythrocytes that are nucleated and express fetal globins. At E8.5, EMPs contribute to the first wave of tissue resident macrophages, including microglia in the developing brain. By E10.5, the first engraftable HSCs emerge in the AGM region of the embryo proper and initiate production of traditional mature blood cells (erythrocytes, platelets, granulocytes, monocytes/macrophages, B cells, T cells, and NK cells. At E14.5, developmentally restricted (dr)HSCs and persisting HSCs coexist in the fetal liver; drHSCs efficiently produce innate-like and traditional lymphoid cells. After birth, definitive HSCs primarily reside in the bone marrow and contribute to "traditional" mature blood cells for the lifespan of the animal.

Table 2. Summary of different hematopoietic stem and progenitor cells (HSPCs) that contribute to development of the blood system.

Progenitor name	Stage	Persists throughout life?	Lineage biases	References
Primitive progenitors	E7.25-E10.5	No	Myeloid (primitive red blood cells)	Haar & Ackerman. 197
Erythromyeloid progenitors (EMPs)	E8.25-E11.5	No	Myeloid (erythrocytes, megakaryocytes, macrophages)	Palis et al. 1999
Lymphomyeloid progenitors (LMPs)	E9.5-life	Yes	Lymphoid, GM	Boiers et al., 2013
Developmentally-restricted HSCs (drHSCs)	E12.5-P14	No	Lymphoid (innate-like lymphocytes)	Beaudin et al., 2016
Definitive HSCs	E10.5-life	Yes	Clone dependent?	**Not sure who to put for this one since so many labs have described**

Purpose of developmental waves

Immune system layering

The very first hematopoietic cells produced during embryogenesis, prior to the emergence of reconstituting HSCs, are nucleated erythrocytes and macrophages (Table 2, Figure 1.3 and 1.4). Erythrocytes are responsible for carrying oxygen throughout the developing embryo, and macrophages play important roles in tissue development and homeostasis (Blander, 2017; Gomez Perdiguero & Geissmann, 2013; Kierdorf et al., 2015; Nicolás-Ávila et al., 2018). The specification of these cells is intriguing since they do not appear to derive from the classical hierarchy that defines later stages of hematopoiesis. Similarly, tissue resident macrophages (TrMacs) have been the topic of substantial interest in recent years due to their reported pre-HSC origin (Lichanska & Hume, 2000; Mizoguchi et al., 1992; Sorokin et al., 1992). TrMacs have been widely implicated in disease, from neurodegeneration and autoimmune disorders to cancer (Honold & Nahrendorf, 2018; Nicolás-Ávila et al., 2018; Sevenich, 2018; Yin et al., 2017; Z. Zhou et al., 2016). Understanding how TrMacs develop and how they are maintained long-term will provide a better understanding of several diseases and may uncover potential therapeutic targets.

In the last decade, several groups have utilized fate mapping to identify distinct subsets of TrMacs that arise from YS progenitors and "self-renew" in their respective tissues (Epelman et al., 2014a; Ghigo et al., 2013; Ginhoux et al., 2010; Hashimoto et al., 2013a; Hoeffel et al., 2012, 2015; Kierdorf et al., 2013; C. Schulz et al., 2012; S. Yona

et al., 2013)reviewed in (Davies et al., 2013; Hoeffel & Ginhoux, 2015a, 2018; Lavin et al., 2015a; Lavin & Merad, 2013). Neither resident brain macrophages (microglia) nor skin macrophages (Langerhans cells) appear to be maintained by BM-derived progenitors (Ajami et al., 2007b; Merad et al., 2002a). Instead, microglia and Langerhans cells are likely of fetal origin, without significant contribution from adult HSCs at steady state (Figure 1.4). Several groups have since reported BM-independent maintenance of additional TrMacs, including Kupffer cells of the liver, alveolar macrophages of the lung, cardiac macrophages of the heart, and red pulp macrophages of the spleen (Figure 1.4) (Epelman et al., 2014a; Hashimoto et al., 2013a; S. Yona et al., 2013). While most groups agree that many TrMacs have a fetal origin, the mechanisms that regulate seeding of these cells in distinct developmental waves and tissues requires further investigation. Surprisingly, we have found that tissue monocytes express IL7r$\boldsymbol{\alpha}$ and contribute to TrMacs in several tissues (Leung et al., 2019). This finding was unexpected, as IL7r$\boldsymbol{\alpha}$ is restricted to the lymphoid lineage in canonical hematopoiesis (Schlenner, 2010). Additional unanticipated regulators of tissue resident immune cells will likely be uncovered in the future.

Mast cells have also been suggested to have fetal origins. In 1983, one group injected YS- or FL-derived mast precursor cells into adult mice that lack mast cells to determine the site of precursor activity (C. Hayashi et al., 1983). They demonstrated that the precursor activity was limited to the YS, arguing that these likely do not originate from definitive HSCs. Very recently, Gentek *et al.* used a VEcadherin fate mapping model

to assess the ontogeny of mast cells (Gentek et al., 2018). They concluded that mast cells largely originate from YS progenitors, but that adult definitive HSCs also contribute. They also demonstrated that YS-derived mast cells are transcriptionally distinct from their adult-derived counterparts. This finding is supportive of multiple waves of HSPCs contributing to tissue resident myeloid cells.

In addition to TrMacs and mast cells, several types of innate-like lymphocytes have been shown to have primarily fetal origins. One such cell type, B1a cells that predominantly exist in serous cavities, has become highly scrutinized (Beaudin & Forsberg, 2016a). The Herzenberg group showed that fetal progenitor cells have a unique capability of generating Ly-1+ B cells (B1 cells)(Hayakawa et al., 1985). They concluded that only neonatal, not adult, cells had the capacity to generate B1a cells upon transplantation into irradiated recipients. This finding was further corroborated by additional studies (Barber et al., 2011; Ghosn et al., 2012). However, the conclusion that FL HSCs are incapable of contributing to B1a cells in an irradiated adult host (Ghosn et al., 2016) has been convincingly contested(Beaudin et al., 2016). The source of the divergent conclusions likely stem from strict phenotypic (Ghosn) versus functional (Beaudin and Kristensen) definitions of fetal HSCs. Several recent reviews provide more in depth discussion (Beaudin & Forsberg, 2016b; Hadland & Yoshimoto, 2018; Hardy & Hayakawa, 2015a; Herzenberg, 2015; Yoshimoto, 2015a). While the cellular precursors of B1a cells remain under debate, there is general consensus on a primarily fetal origin of the B1a lineage.

Although the exact contribution of each wave to the developing blood system remains under investigation, there is clearly substantial overlap in hematopoietic cell production between waves. It appears that the earlier waves contribute to innate immune cells that provide basic immunity and aid in tissue formation and organization, while later perinatal waves contribute to innate and adaptive immune cells that prepare the fetus for exposure to environmental pathogens after birth. How early life exposures affect the emergence and expansion of different immune cells, and how that influence immune cell function in later life, is an important avenue of investigation.

Conclusion and Future directions

As highlighted in this review, hematopoiesis undergoes continual molecular and functional changes throughout development and the lifespan. It seems likely that several clones of HSCs arise independently, in temporally and spatially regulated waves (Figure 1.4, Table 2). Exactly how many HSCs emerge and persist in the embryo, and their relative lifelong contribution to the blood system remains under debate. Cre-lox, barcoding, and transplantation tracking systems have, together with RNA-seq, improved our understanding of blood development, yet some questions remain difficult to address. It is unclear which waves are essential and how much functional overlap exists between them. Rigorous testing would require specifically deleting one lineage without perturbing the others, but that will depend on development of more precise and efficient tools. The novel genetic regulators identified by RNA-

seq, in conjunction with reconstitution assays, fate mapping models, and innovative microscopy techniques, promise to provide an increasingly comprehensive understanding of blood system development. Quantitative assessment of individual HSC output, in situ and upon transplantation, is also needed to provide deeper insight into the lifespan and differentiation capacity of HSCs. Additionally, understanding the amazing epigenetic memory of cells, such as the drHSCs that maintain lineage biased output upon serial transplantation, may allow us to better utilize, and potentially engineer, HSCs for therapeutic use, and will likely also shed light on the drivers of blood and immune disease. While our understanding of hematopoiesis has expanded immensely, the spatial and temporal regulation of hematopoietic development warrant continued investigation.

Chapter 1: intrinsic regulators of tissue resident immune cell establishment from fetal progenitors

[This section is adapted from a publication, The lymphoid-associated interleukin 7 receptor (IL7R) regulates tissue-resident macrophage development, Accepted at Development]

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Abstract

The discovery of a fetal origin for tissue-resident macrophages (trMacs) has inspired an intense search for the mechanisms underlying their development. Here, we performed *in vivo* lineage tracing of cells with an expression history of IL7Rα, a marker exclusively associated with the lymphoid lineage in adult hematopoiesis. Surprisingly, we found that Il7r-Cre labeled fetal-derived, adult trMacs. Labeling was almost complete in some tissues and partial in others. The putative progenitors of trMacs, yolk sac (YS) erythromyeloid progenitors, did not express IL7R, and YS hematopoiesis was unperturbed in IL7R-deficient mice. In contrast, tracking of IL7Rα message levels, surface expression, and Il7r-Cre-mediated labeling across fetal development revealed dynamic regulation of *Il7r*mRNA expression and rapid upregulation of IL7Rα surface protein upon transition from monocyte to macrophage within fetal tissues. Fetal monocyte differentiation *in vitro*produced IL7R^{+} macrophages, supporting a direct progenitor-progeny relationship. Additionally, blockade of IL7R function during late gestation specifically impaired the establishment of fetal-derived trMacs *in vivo*. These data provide evidence for a distinct function of IL7Rα in fetal myelopoiesis and identify IL7R as a novel regulator of trMac development.

Introduction

Hematopoietic stem cells (HSCs) are responsible for sustaining blood and immune cell production across the lifespan of the animal, under steady-state conditions, during infection, and following transplantation. However, recent findings have revealed that many tissue-resident immune cells are poorly generated or regenerated from adult HSCs, including subsets of tissue-resident macrophages (trMacs), such as microglia, epidermal Langerhans cells, liver Kupffer cells, and alveolar macrophages (Beaudin & Forsberg, 2016a; Cool & Forsberg, 2019; Ginhoux et al., 2010; Hardy & Hayakawa, 2015b; Hashimoto et al., 2013b; Herzenberg, 2015; Hoeffel et al., 2015; Kierdorf et al., 2015; Sawai, 2016; S. K. Yona Ki-Wook; Wolf, Yochai; Mildner, Alexander; Varol, Diana; Breker, Michal; Strauss-Ayali, Dalit; Viukov, Sergey; Guilliams, Martin; Misharin, Alexander V. ;. Hume, David A. ;. Perlman, Harris; Malissen, Bernard; Zelzer, Elazar; Jung, Steffen, 2012, 2012; Yoshimoto, 2015b). These trMacs are specialized macrophages that reside within tissues and have specific functions in tissue and immune homeostasis (Epelman et al., 2014b; Ginhoux et al., 2010; Hoeffel et al., 2012; Kierdorf et al., 2015; S. K. Yona Ki-Wook; Wolf, Yochai; Mildner, Alexander; Varol, Diana; Breker, Michal; Strauss-Ayali, Dalit; Viukov, Sergey; Guilliams, Martin; Misharin, Alexander V. ;. Hume, David A. ;. Perlman, Harris; Malissen, Bernard; Zelzer, Elazar; Jung, Steffen, 2012). Unlike classical adult monocyte-derived macrophages, which are recruited from circulation upon inflammation and have high turnover rates, trMacs are locally maintained by proliferation, independently of adult hematopoiesis (Ajami et al., 2007a; Hashimoto et al., 2013b; Hulsmans et al., 2017;

Merad et al., 2002b). Recent fate-mapping studies have revealed a fetal origin of specific trMacs (Epelman et al., 2014; Gomez Perdiguero et al., 2015; Hoeffel et al., 2015; Schulz et al., 2012; Yona et al., 2013), but the cellular and molecular mechanisms driving trMac establishment and expansion within fetal tissues are poorly understood.

Whether trMacs are seeded once from a common progenitor or in reiterative waves throughout development is under intense investigation. Accumulating evidence points towards extra-embryonic yolk sac (YS)-derived erythromyeloid progenitors (EMPs) as the initial cell of origin for macrophages that seed developing tissues, and the primary source of brain microglia (Gomez Perdiguero et al., 2015; Kierdorf et al., 2013). Microglia and other trMacs can differentiate directly from EMPs through either Myb-dependent or -independent pathways, likely through an EMP-derived intermediate (Hoeffel et al., 2015; Schulz et al., 2012). Macrophages derived from fetal liver (FL) precursors may replace or replenish certain fetal-derived trMac populations initially seeded by YS-derived cells, including lung alveolar macrophages, epidermal Langerhans cells and liver Kupffer cells (Guilliams et al., 2013; Hoeffel et al., 2015, 2012; Yona et al., 2013). However, both the precise cell of origin for FL precursors and their specific contribution to adult trMac compartments remain controversial, partly because of incomplete progenitor labeling and the difficulties in accurately tracking progeny *in situ*. Despite intense investigation of the mechanisms

regulating macrophage differentiation from YS progenitors, including gene expression programs (Mass et al., 2016) and mechanisms of tissue seeding (Stremmel et al., 2018), comparatively less is known about the developmental mechanisms that regulate the establishment of tissue macrophages from later waves of hematopoietic cell production.

Here, our investigation of hematopoietic development using the Il7r-Cre lineage-tracing model (Schlenner et al., 2010) revealed unexpected and robust labeling of adult trMacs in multiple tissues. Examination of fetal myeloid development revealed a transient wave of IL7Rα expression in developing fetal macrophages that was dynamically regulated as macrophages differentiated within developing resident tissues. Both germline deletion and specific blockade of IL7R during the developmental window in which trMacs express IL7R confirmed the functional requirement for IL7R signaling during fetal trMac development. In contrast, IL7Rα was not expressed by YS EMPs and YS hematopoiesis was unperturbed in *Il7r*$^{-/-}$ embryos. Together, these experiments reveal IL7R as a novel regulator of fetal macrophage development during late gestation.

RESULTS

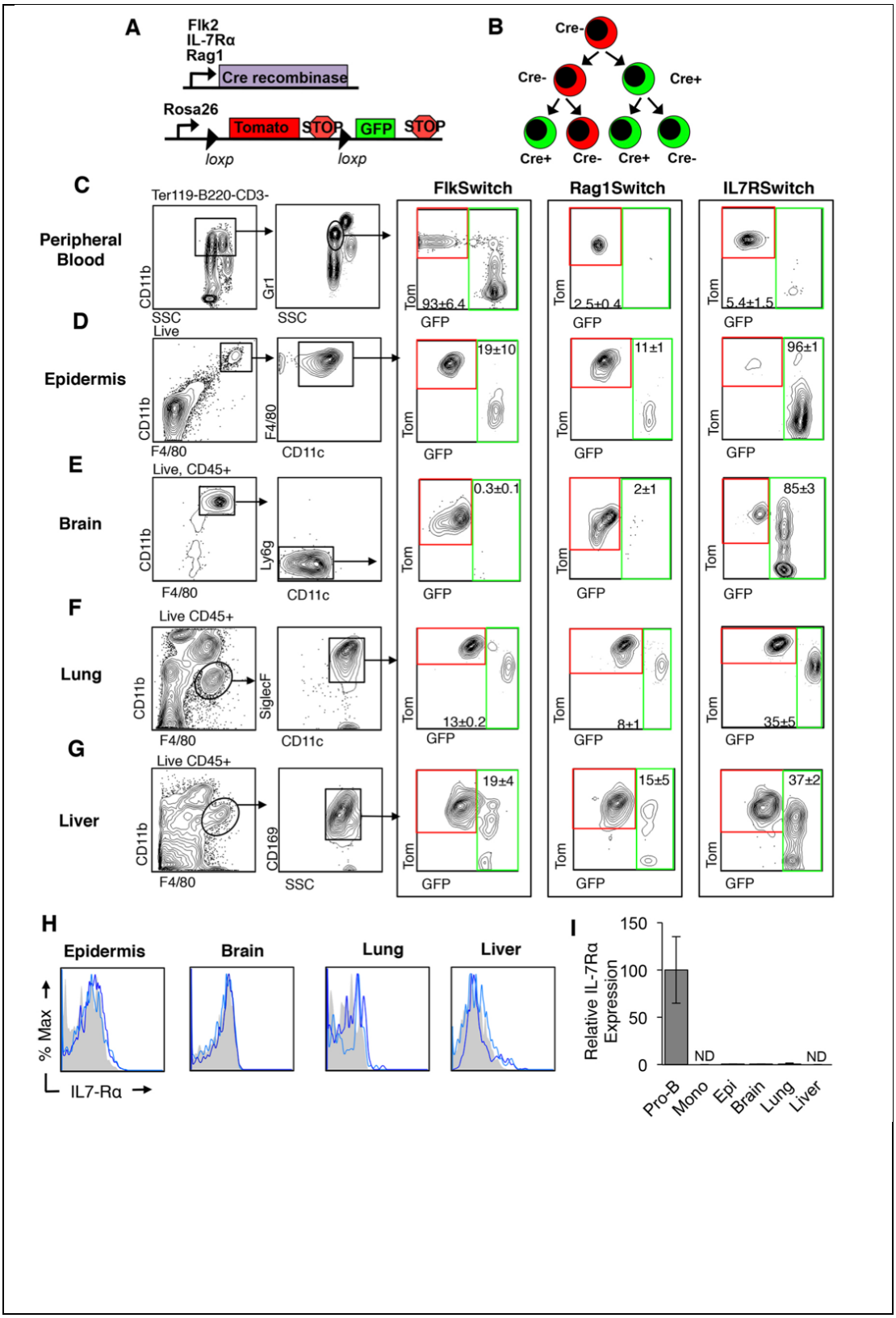
Il7r-Cre specifically labels adult tissue-resident macrophages

The lymphoid-associated genes *Flk2* (*Flt3*), *Il7r*, and *Rag1* label cells with increasingly restricted lymphoid potential in adult hematopoiesis (Adolfsson et al., 2005; Forsberg et al., 2006; Igarashi et al., 2002; Kondo et al., 1997). In contrast, during fetal development all three markers identify oligopotent hematopoietic progenitors with both myeloid and lymphoid potential (; Boiers et al., 2013). Although we and others have used the Flk2-Cre and Rag1-Cre models to track the contribution of fetal progenitors to trMac populations (Boiers et al., 2013; Epelman et al., 2014; Gomez Perdiguero et al., 2015; Hashimoto et al., 2013; Hoeffel et al., 2015), the contribution of IL7R-marked progenitors to the same populations has not been previously examined. To decipher the contribution of specific transient hematopoietic progenitors to adult trMacs, we compared adult trMac labeling across three lineage-tracing models: Flk2-Cre, Il7r-Cre and Rag1-Cre. We crossed mice expressing Rag1-Cre (Welner et al., 2009) or Il7r-Cre (Schlenner, 2010) to mTmG mice expressing a dual-color fluorescent reporter (Muzumdar et al., 2007), thereby creating 'Rag1Switch' and 'IL7RSwitch' models (Fig. 2.1A) analogous to the previously described FlkSwitch mouse (Beaudin et al., 2016; Boyer et al., 2011, 2012). In all models, all cells express Tomato (Tom) until Cre-mediated recombination results in the irreversible switch to GFP expression by that cell and all of its progeny (Fig. 2.1B).

We compared reporter expression in fetal-derived trMacs of all three models to reporter expression in adult bone marrow (BM)-derived circulating peripheral blood (PB) monocytes ($CD11b^{hi}Gr1^{lo}SSc^{lo}$; CD11b also known as ITGAM, Gr1 as Ly6G; SSc, side scatter parameter), the precursors of adult HSC-derived macrophages. As expected, Cre-driven GFP labeling of monocytes in IL7RSwitch and Rag1Switch mice was less than 5% (Fig. 2.1C) and paralleled the nominal labeling observed in adult HSCs and myeloid progenitors (Fig. S1.1A,B), as previously reported (Schlenner et al., 2010; Welner et al., 2009). Adult FlkSwitch mice exhibited high GFP labeling in PB monocytes (Fig. 2.1C), as we previously reported (Boyer et al., 2011). Intriguingly, examination of Cre-driven reporter switching in adult trMacs revealed distinct GFP labeling patterns across all three lineage-tracing models and across tissues. Within Langerhans cells (LCs) of the epidermis, around 20% of cells were labeled by GFP in the FlkSwitch model (Fig. 2.1D), consistent with previous reports (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015). Low GFP labeling was observed in LCs of Rag1Switch mice (Fig. 2.1D), as described previously for skin and other tissue macrophages during fetal development (Boiers et al., 2013). In sharp contrast, up to 96% of LCs expressed GFP in IL7RSwitch mice (Fig. 2.1D). A similar pattern was observed for microglia; consistent with a pre-HSC origin; virtually no reporter switching was observed in adult microglia of FlkSwitch mice (Fig. 2.1E) and minimal microglia labeling was observed in Rag1Switch mice. Remarkably, however, GFP labeling of microglia was over 85% in IL7RSwitch mice (Fig. 2.1E).

Examination of reporter expression across lineage-tracing models in trMacs of the lung (alveolar macrophages, AMs) and the liver (Kupffer cells, KCs) yielded a different labeling pattern compared with the LCs and microglia (Fig. 2.1F,G). In FlkSwitch and Rag1Switch mice, GFP labeling in AMs and KCs was low and roughly comparable to LCs (~8-19% GFP^+ cells; Fig. 2.1D,F,G). Surprisingly, in IL7RSwitch mice, GFP labeling in AM and KC populations was substantially higher, ~40%, compared with both FlkSwitch and Rag1Switch models (Fig. 2.1F,G). Although labeling in these two populations was considerably less than that of LCs or microglia in the same mice (~85-96%), it was substantially higher than labeling of adult BM-derived myeloid populations ($F4/80^{lo}CD11b^+$; F4/80 also known as ADGRE1) within the same tissues (<5%) (Fig. S1.1C-E). Comparison of Cre-mediated labeling between circulating BM-derived monocytes (<4%; Fig. 2.1C) and trMacs (40-90%; Fig. 2.1D-G) in adult IL7RSwitch mice also revealed stark differences in labeling, supporting previous reports that adult BM-derived monocytes did not substantially contribute (at steady-state) to the trMac populations investigated, including microglia, LCs, KCs, and AMs (Gomez Perdiguero et al., 2015; Hashimoto et al., 2013; Hoeffel et al., 2012; Sheng et al., 2015). Similarly, other myeloid cells in the tissues, such as neutrophils, were also minimally labeled (Fig. S1.1F,G), consistent with a previous report (Schlenner, 2010). Despite substantial Il7r-Cre-mediated labeling, IL7Rα surface expression was undetectable in any adult trMacs surveyed (Fig. 2.1H). *Il7r* was also virtually undetectable in trMacs compared with BM Pro-B-cells (Fig. 2.1I). The robust labeling of adult trMacs in the IL7RSwitch model in the absence of message or surface

expression therefore suggested that IL7Rα was expressed during an earlier window of macrophage development.


A
Flk2
IL-7Rα
Rag1
Cre recombinase
Rosa26
Tomato
STOP
GFP
STOP
loxp
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B
Cre-
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Cre+
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Cre-
Cre+
Cre-
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D
E
F
G
Peripheral Blood
Epidermis
Brain
Lung
Liver
FlkSwitch
Rag1Switch
IL7RSwitch
Ter119-B220-CD3-
CD11b
SSC
Gr1
SSC
Live
F4/80
CD11c
Live, CD45+
Ly6g
Live CD45+
SiglecF
CD169
Tom
GFP
93±6.4
2.5±0.4
5.4±1.5
19±10
11±1
96±1
0.3±0.1
2±1
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13±0.2
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H
Epidermis
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I
Relative IL-7Rα Expression
150
100
50
0
ND
ND
Pro-B
Mono
Epi
Brain
Lung
Liver

Figure 2.1 Il7r-Cre specifically labels adult tissue-resident macrophage populations. Representative flow cytometric analysis of reporter expression across different monocyte and macrophage populations in adult mice. Tomato (Tom) and GFP expression is highlighted by red and green boxes, respectively, in FlkSwitch, Rag1Switch and IL7Rswitch models. Values indicate mean frequencies±s.e.m. of gated Il7r-Cre marked GFP+ populations. Plots and values are representative of four or five mice each representing three independent experiments. (A) Schematic of the 'Switch' models. Cre recombinase expression was controlled by either Flk2, IL7Rα or Rag1 regulatory elements, respectively. Cre-driver mice were crossed to mice expressing a dual-color reporter expressing either Tom or GFP, under control of the Rosa26 locus. Expression of Cre results in an irreversible genetic deletion event that causes a switch in reporter expression from Tom to GFP. (B) Schematic of Cre-mediated reporter switching in the 'switch' models. All cells initially express Tom. Expression of Cre results in an irreversible switch from Tomato to GFP expression. Once a cell expresses GFP, it can only give rise to GFP-expressing progeny. (C) Representative flow cytometric analysis of reporter expression in circulating CD11bhiGrmid monocytes in the peripheral blood of adult FlkSwitch, Rag1Switch and IL7RSwitch mice. (D) Representative flow cytometric analysis of reporter expression in LCs (F4/80+CD11b+CD11cmid) in the epidermis of adult FlkSwitch, Rag1Switch and IL7RSwitch mice. (E) Representative flow cytometric analysis of reporter expression in microglia (CD45+F4/80hiCD11bhiLy6g−CD11c−) in the brains of FlkSwitch, Rag1Switch and IL7RSwitch adult mice. For additional gating see Fig. S1H. (F) Representative flow cytometric analysis of reporter expression in lung AMs (CD45+F4/80hiCD11bmid SiglecF+CD11c+) of FlkSwitch, Rag1Switch, and IL7RSwitch adult mice. G, Representative flow cytometric analysis of reporter expression in liver KCs (CD45+F4/ 80hiCD11bmidCD169+) in adult FlkSwitch, Rag1Switch and IL7RSwitch mice. (H) Lack of IL7Rα surface expression in the LCs of the epidermis, brain microglia, lung AMs, and liver KCs. For each tissue, IL7Rα surface expression of gated population is shown for two representative mice in blue. Gray shaded area represents fluorescence-minus-one (FMO) control. (I) Quantitative RT-PCR analysis of Il7r expression in sorted bone marrow- derived B220+CD43+ Pro-B cells and CD11b+Grmid monocytes, and LCs of the epidermis, brain microglia, lung AMs, and liver KCs isolated from WT adult mice. Data represent mean±s.e.m. for three independent experiments. Values are normalized to Pro-B cells, set to 100. ND, not detected. Additional analyses of adult tissue myeloid populations can be found in Fig. S1.

YS hematopoiesis does not depend on IL7R

YS EMPs have been proposed as a cell of origin for fetal trMac development, either through direct differentiation or through the Myb-dependent generation of intermediate progenitors that seed the fetal liver (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015, 2012; Mass et al., 2016; Schulz et al., 2012). To determine whether Il7r-Cre labeling of adult trMac populations initiated in YS EMPs during embryonic development, we examined Il7r-Cre-driven GFP labeling, IL7Rα surface expression and *Il7r* in YS EMPs and YS macrophages. YS EMPs (Ter119^{-}Kit^{+}CD41^{+}; Ter119 also known as LY67; CD41 also known as ITGA2B) at embryonic day (E)9.5 were minimally labeled by Il7r-Cre, consistent with a complete absence of IL7Rα surface

expression (Fig. 2.2A,A′). There was virtually no Il7r-Cre-induced reporter expression within the CD45^{+} (CD45 also known as Ptprc) fraction of the YS, which included myeloid cells expressing CD11b or F4/80 (Fig. 2.2B), and there was similarly no IL7Rα surface expression observed (Fig. 2.2B′). *Il7r* in E9.5 EMPs was detectable at very high Ct values (Fig. 2.2C,C′), and consequently we observed slightly more labeling in YS EMPs by E10.5 (Fig. 2.2A). However, increased reporter expression was not accompanied by surface expression (Fig. 2.2A′). Overall, labeling of YS EMPs was comparable to background labeling observed in adult hematopoiesis (Schlenner, 2010). To clarify whether IL7R expression regulated YS hematopoiesis, we examined the frequency of YS EMPs and YS macrophages in *Il7r*$^{-/-}$ embryos, which have a germline deletion of IL7Rα (Peschon et al., 1994). Neither YS EMPs nor YS macrophage frequency was significantly different between wild-type (WT) and *Il7r*$^{-/-}$mice (Fig. 2.2D,E). Together, these data indicate that IL7R is not required for the generation of YS progenitors or YS macrophages, and suggest that the robust labeling of F4/80^{hi}trMacs by Il7r-Cre occurs later in trMac ontogeny.

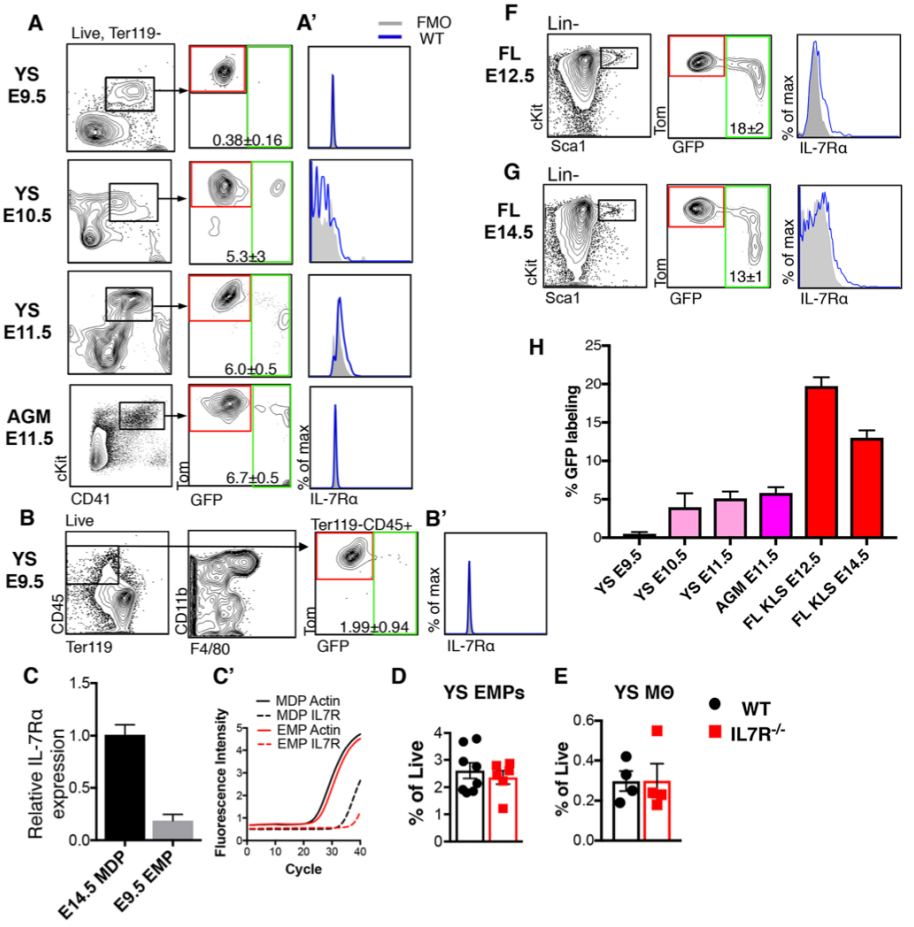

Figure 2.2 IL7Rα is dispensable for YS hematopoiesis. (A,A′) Representative FACS plots showing gating strategy, Il7r-Cre-driven GFP labeling, and IL7Rα surface expression in Ter119−Kit+CD41+ EMPs in the YS at E9.5, E10.5, E11.5 and in the AGM at E11.5. Red and green boxes denote gates for Tom+ and GFP+ progenitors, respectively. Gray shaded areas in histograms indicate FMO control. Plots and values indicate mean frequencies±s.e.m. of gated Il7r-Cre-marked GFP+ populations for four to seven mice from at least two independent experiments. (B) Representative FACS plots showing gating, Il7r-Cre-driven GFP labeling, and IL7Rα surface expression in CD45+Ter119− cells in the YS at E9.5. Adjacent plot shows CD11b and F4/80 expression within the CD45+Ter119− compartment. Red and green boxes denote gates for Tom and GFP expression of CD45+Ter119− cells, respectively. (C,C′) Quantitative RT-PCR analysis of Il7r mRNA in YS EMPs from E9.5. (C) ΔΔCT values normalized to beta-actin compared with Il7r expression levels in monocyte dendritic progenitors (MDPs; CD115+Kit+Flk2+Ly6c−) isolated from E14.5 FL. (C′) Representative amplification plot of beta-actin (solid lines) and Il7r (dashed lines) for quantitative RT-PCR of E14.5 MDPs and EMPs as described in C. Data show mean±s.e.m. from three independent experiments. (D,E) Quantification of the frequency of EMPs (D) and F4/80+ macrophages (E) in YS of Il7r−/− and WT embryos at E9.5. Data show mean frequency±s.e.m. representing three independent experiments. (F,G) Representative FACS plots showing gating, Il7r-Cre-driven GFP labeling, and IL7Rα surface expression in cKit+Lin−Sca1+ stem and progenitor cells at E12.5 (F) and E14.5 (G). (H) Quantification across development of the percentage (±s.e.m.) of Il7r-Cre-driven reporter labeling (GFP+) in the phenotypically defined stem and progenitor populations described in A-G. Additional analysis of Il7r-Cre-driven reporter labeling in fetal hematopoietic populations can be found in Fig. S2.

Il7r-Cre labels non-HSC progenitors in the fetal liver

As early YS progenitors and macrophages were minimally labeled by Il7r-Cre, we investigated labeling in putative progenitors slightly later in development. Labeling remained comparably low in both YS and aorta-gonad-mesonephros (AGM) Kit^+CD41^+EMPs at E11.5, and IL7R surface expression was still undetectable in the EMPs within the FL (Fig. 2.2A,A′; Fig. S1.2A). Higher Il7r-Cre-driven labeling was observed in FL Lin-Kit^+Sca1^+ (KLS) progenitors by E12.5 (18%), but surface expression was still low (Fig. 2.2F,H). Labeling subsequently declined in E14.5 progenitors (Fig. 2.2G,H; $P<0.001$), and all labeling in E14.5 progenitors was confined to the CD150 (SLAMF1)-multipotent progenitor compartment (Fig. S1.2B). Consistent with labeling of upstream multipotent cells in the FL, comparable Il7r-Cre labeling was observed in all downstream progenitor and mature compartments (Fig. S1.2C-G), with the exception of significant labeling in committed lymphoid progenitors (Fig. S1.2D) and mature lymphoid cells (Fig. S1.2E). Labeled progenitors at E14.5 did not possess long-term multilineage reconstitution, confirming that they were not definitive HSCs (Fig. S1.2H). These data suggest that IL7R reporter expression in late (E10.5) YS and AGM partially labels a transient progenitor that contributes to FL hematopoiesis but is not a definitive HSC.

Il7r-Cre labeling of myeloid cells is tissue and developmental stage specific

Despite low levels of labeling in YS and fetal liver progenitors, the Il7r-Cre labeling in adult trMacs (Fig. 2.1) was considerably higher compared with any progenitor we profiled across fetal development (Fig. 2.2H; Fig. S1.2). We reasoned that progenitor labeling alone could not entirely explain the labeling observed in those compartments. To resolve this discrepancy, we next evaluated Il7r-Cre-driven labeling and IL7Rα expression in myeloid-restricted precursors within peripheral tissues during FL stage development. We initiated our investigation at E12.5, the stage at which significant progenitor labeling by Il7r-Cre was initially observed (Fig. 2.2F,H). At this stage of development, macrophages derived from the extra-embryonic YS (F4/80^{hi}CD11b^{lo}) are already present in the tissues, but may be replaced by incoming FL-derived macrophage precursors or monocytes (F4/80^{lo}CD11b$^{mid/hi}$; Fig. 2.3A; Fig. S1.3) (Hoeffel et al., 2015). F4/80^{lo}CD11b$^{mid/hi}$macrophage precursors in fetal peripheral tissues are heterogeneous or low for Ly6c expression and express the fetal macrophage marker CD64 (FCGR1) (Fig. S1.4A-C; (Hoeffel & Ginhoux, 2015b), suggesting their propensity to differentiate into macrophages.

Analysis of Il7r-Cre-driven reporter expression revealed two very clear patterns: first, GFP labeling at E12.5 was significantly higher in all F4/80^{lo} monocytes compared with F4/80^{hi}macrophages across all tissues (Fig. 2.3B, E12.5). Second, cross-tissue comparison revealed higher GFP labeling in F4/80^{lo} monocytes in brain, lung and epidermis compared with liver (Fig. 2.3B, E12.5). Across all F4/80^{hi} macrophages, GFP labeling was highest in the skin (25%; $P<0.05$ compared with liver F4/80^{hi} macrophages). A very comparable pattern emerged at E14.5; however, between

E12.5 and E14.5, GFP labeling increased slightly across all F4/80^{hi} macrophage populations, but only significantly for the skin (Fig. 2.3C, Skin/Epi).

From late gestation into early neonatal development, the patterns of Il7r-Cre-mediated GFP labeling among F4/80^{hi} macrophages and F4/80^{lo} monocytes were strikingly different, both between cell types and between tissues (Fig. 2.3B,C). Beyond E17.5, GFP labeling in liver and lung was higher in F4/80^{hi} macrophages compared with F4/80^{lo}monocytes. This 'switch' reflected a significant increase in GFP labeling of F4/80^{hi}macrophages between E14.5 and postnatal day (P)0 and a concomitant decrease in F4/80^{lo}monocyte labeling (Fig. 2.3C), coincident with the HSC-dependent contribution to fetal monocytes later in gestation (Hoeffel & Ginhoux, 2015b; S. K. Yona Ki-Wook; Wolf, Yochai; Mildner, Alexander; Varol, Diana; Breker, Michal; Strauss-Ayali, Dalit; Viukov, Sergey; Guilliams, Martin; Misharin, Alexander V. ;. Hume, David A. ;. Perlman, Harris; Malissen, Bernard; Zelzer, Elazar; Jung, Steffen, 2012). GFP labeling in lung and liver macrophages reached adult levels by P0 (Fig. 2.3B, Fig. 2.1F,G; P>0.5). The increase in labeling of F4/80^{hi} macrophages across fetal development suggested either that Il7r-Cre labeling was occurring cell-autonomously in F4/80^{hi} macrophages, or that Il7r-labeled fetal monocytes were gradually replacing or contributing to tissue macrophage populations in the lung and liver during fetal hematopoiesis.

In contrast to lung and liver, progression of GFP labeling within the skin and brain across ontogeny relayed a different pattern. F4/80^{lo} monocytes were barely detectable

in the epidermis and brain after E14.5 (Fig. 2.3A, bottom right panels), as previously described (Hoeffel & Ginhoux, 2015b). GFP labeling of skin macrophages increased robustly from E17.5 to P0 (Fig. 2.3C, Skin/Epi), and continued to increase postnatally (Fig. 2.3B,C). Similarly, microglia labeling increased steadily across development, but had still not reached adult levels by P14 (Fig. 2.3B,C). The disparate GFP labeling that we observed across different tissues suggested a differential involvement of IL7R in the development of different trMac populations across ontogeny.

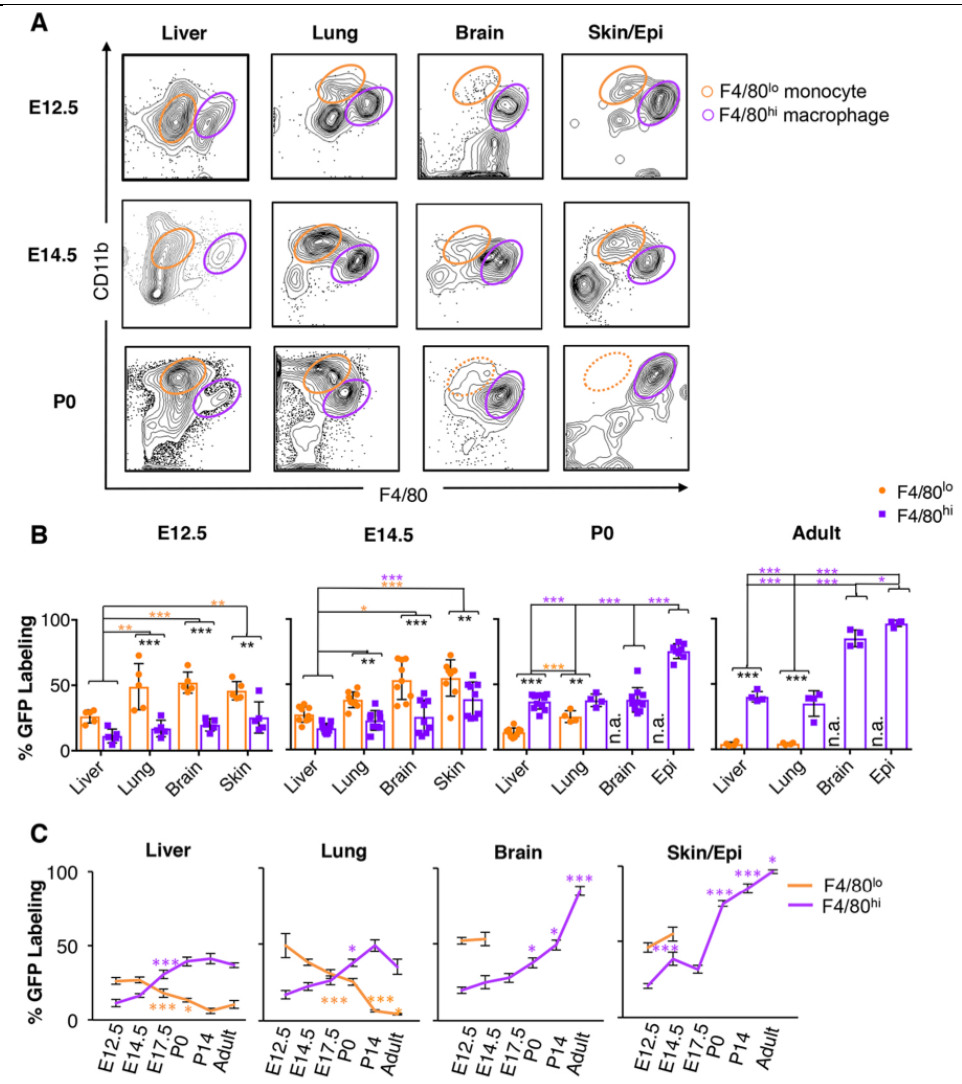

Figure 2.3 Il7r-Cre dynamically labels myeloid cells during tissue-resident macrophage development. (A) Representative flow cytometric analysis indicating gating for FL-derived F4/80lo monocytes (live Ter119−CD45+F4/80loCD11bmid/hi; orange gates) and previously seeded F4/80hi macrophages (live Ter119−CD45+F4/ 80hiCD11bmid; purple gates) in different tissues at E12.5, E14.5 and P0. Pre-gates are shown in Fig. S3. (B) Quantification of Il7r-Cre-driven GFP labeling within gated population indicated in A across different tissues at E12.5, E14.5, P0 and Adult (8-12 weeks). Orange asterisks denote cross-tissue differences in GFP labeling between F4/80lo monocytes and purple asterisks denote cross-tissue differences in GFP labeling between F4/80hi macrophages. Black asterisks denote differences in GFP labeling between F4/80lo monocytes and F4/80hi macrophages within each tissue. n=4-11 representing at least three independent experiments. n.a., not analyzed. (C) Time course of Il7r-Cre-driven GFP labeling in F4/80hi macrophages and F4/80lo monocytes shown in B across ontogeny. GFP labeling was quantified in adult tissue macrophages as phenotypically defined in Fig. 1. Asterisks indicate statistically significant differences between the percentage of GFP labeling at the previously measured time point, with purple asterisks denoting differences between the indicated F4/80hi macrophages and orange asterisks denoting differences between F4/80lo monocyte populations. Representative pre-gating for cell populations in Fig. 3A are shown in Fig. S3. *P<0.05, **P<0.005, ***P<0.0005. Error bars represent s.e.m.

IL7Rα expression is dynamically regulated during fetal tissue macrophage development

To gain insight into what was driving the observed pattern of Il7r-Cre-mediated GFP labeling within F4/80^{lo} monocyte and F4/80^{hi} macrophage populations across development, we probed *Il7r* levels beginning at E14.5 (Fig. 2.4A). Across tissues, F4/80^{lo}monocytes expressed low but consistently detectable levels of *Il7r* relative to fetal liver monocyte dendritic progenitors (MDPs), which are known to express *Il7r (Hoeffel & Ginhoux, 2015b; Fig 2.4A)*. Circulating peripheral blood Ly6c^{hi} monocytes also expressed detectable *Il7r* and were labeled by Il7r-Cre to a similar degree as liver F4/80^{lo} monocytes (Fig. S1.4C,D). Increased *Il7r* mRNA in peripheral tissue F4/80^{lo} monocytes corresponded with higher Cre-mediated GFP expression (Fig. 2.4A), as GFP labeling increased in monocytes once they had exited the liver at E14.5 (Fig. 2.3B). Lung and skin monocytes with the highest GFP labeling also expressed the highest levels of *Il7r* mRNA. In comparison, and consistent with significantly lower Cre-mediated GFP labeling, *Il7r* was virtually undetectable in F4/80^{hi} macrophages (Fig. 2.4A) at E14.5. Postnatally, at P14, *Il7r*levels continued to be negligible in macrophages of the lung and liver, but microglia and LCs expressed detectable *Il7r* (Fig. 2.4B), driving continued postnatal Cre-mediated recombination in those tissues. These data confirmed the fidelity of the Il7r-Cre model and revealed *Il7r* expression by fetal myeloid cells during development.

To ascertain the relationship between Il7r-Cre-mediated GFP labeling and IL7Rα surface expression in developing macrophages during fetal development, we profiled surface expression from E12.5 to P14. Considering the high degree of GFP labeling and expression of *Il7r* mRNA by F4/80^{lo} monocytes during fetal development, we expected to observe monocytes displaying surface IL7R. Unexpectedly, F4/80^{lo} monocytes never displayed detectable IL7Rα surface protein at any of the time points examined (Fig. 2.4C). Surprisingly, robust IL7Rα surface protein was instead observed on prenatal F4/80^{hi}macrophages in peripheral tissues (lung, brain and skin), whereas minimal surface expression was observed on F4/80^{hi} macrophages in the liver (Fig. 2.4D). IL7Rα surface protein on peripheral F4/80^{hi} trMacs was observed beginning at E12.5, and peaked at E17.5 (Fig. 2.4D, middle panel), despite significantly lower GFP labeling compared with F4/80^{lo} monocytes (Fig. 2.3B,C). The vast majority (>90%) of IL7Rα-expressing macrophages in the brain, lung and skin also co-expressed the common γ chain (CD132; also known as IL2RG; Fig. S1.5A), indicating expression of a functional IL7 receptor. By P0, macrophages in the liver and lung ceased to express IL7Rα, concomitant with a plateau in GFP labeling by birth (Fig. 2.3C). In contrast, some proportion of macrophages in the brain and epidermis continued to express IL7Rα protein at the surface postnatally, albeit at lower levels, and IL7Rα surface expression was detectable on epidermal macrophages as late as P14. Continued IL7Rα expression in these particular tissues was consistent with continued postnatal labeling by Il7r-Cre (Fig. 2.3C) and *Il7r* mRNA expression (Fig. 2.4B).

These data therefore revealed tightly regulated and highly coordinated expression of IL7Rα in developing macrophages across different tissues.

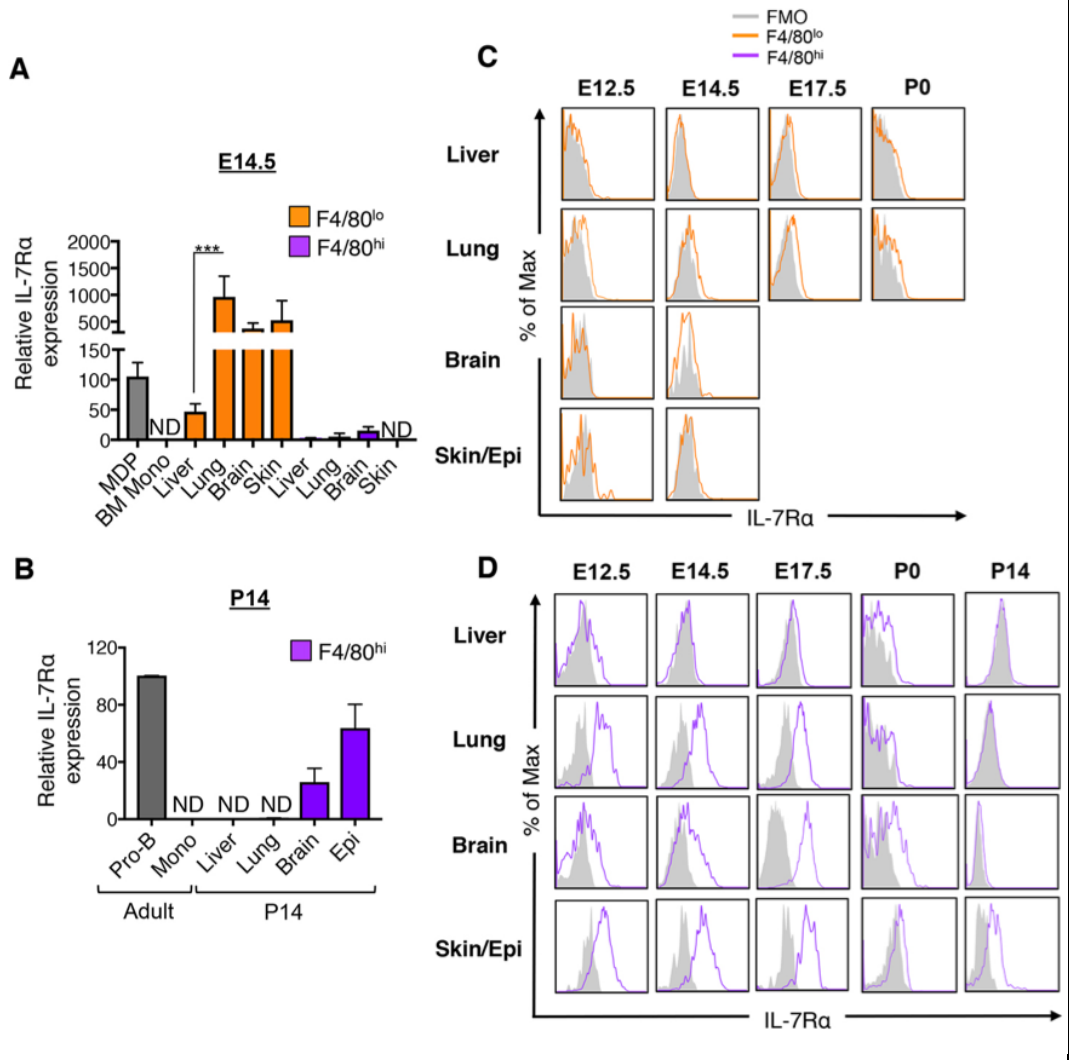

Figure 2.4 L7Rα message and surface expression are dynamically regulated during macrophage development. (A) Quantitative RT-PCR analysis of Il7r mRNA in monocytes (F4/80loCD11bhi) and macrophages (F4/80hiCD11blo) isolated from liver, lung, brain and skin of E14.5 fetuses. Fetal liver macrophage dendritic cell precursors (MDP; CD115+Kit+Flk2–Ly6c−CD11b−) and adult BM monocytes (CD11b+Gr1mid) were used as positive and negative controls, respectively. Data shown are mean±s.e.m. of ΔΔCT values calculated for Il7r and β-actin and normalized to MDPs (set to 100) across three independent experiments. (B) Quantitative RT-PCR analysis of Il7r mRNA in monocytes (F4/80loCD11bhi) and macrophages (F4/80hiCD11blo) isolated from liver, lung, brain and epidermis (Epi) of neonatal mice at P14. Adult BM Pro-B cells (B220+CD43+) and BM monocytes (CD11b+Gr1+) were used as positive and negative controls, respectively. Data shown are mean±s.e.m. of ΔΔCT values calculated for Il7r and beta-actin and normalized to BM Pro-B cells (set to 100) across three independent experiments. ND, not detected. (C,D) Representative flow cytometric analysis of IL7Rα surface expression in F4/80lo monocytes (CD45+F4/80loCD11bhi; C) and F4/80hi macrophages (CD45+F4/80hiCD11bmid; D) in different tissues at E12.5, E14.5, E17.5, P0 and P14. Gray shaded area indicates FMO control shown for each cell type, respectively. Plots are representative of analysis in five or six mice from three independent experiments. Analysis of common gamma chain expression in fetal tissue macrophages can be found in Fig. S4.

IL7Rα-expressing monocytes give rise to IL7R^{+} macrophages ex vivo

We hypothesized that F4/80^{lo} monocytes upregulate *Il7r* as they exit the liver and migrate to and enter fetal tissues, where they differentiate into F4/80^{hi} macrophages. IL7Rα surface expression is then rapidly switched on as F4/80^{lo} monocytes differentiate into F4/80^{hi} macrophages. To test our hypothesis, we isolated F4/80^{lo} monocytes from the fetal liver at E14.5 and differentiated them into F4/80^{hi} macrophages *ex vivo* in the presence of macrophage colony-stimulating factor (M-CSF). F4/80^{lo}CD11b^{mid} cells were sorted from FL based on Ly6c expression (Fig. 2.5A) and cultured with 20 ng M-CSF/ml for 5 days. FL monocytes expressed detectable *Il7r* (Fig. 2.4A), but IL7R surface expression was not observed (Fig. 2.5B-C′). M-CSF induced the differentiation of Ly6c^{hi}F4/80^{lo}CD11b^{hi} to F4/80^{hi} CD11b^{mid} macrophages that expressed CD64 after only 1 day in culture, and macrophage differentiation plateaued at 3 days (Fig. 2.5C,E,E′). Differentiated F4/80^{hi}macrophages could also be differentiated from F4/80^{lo} monocytes by their larger size and higher cellularity complexity, as defined by forward and side scatter (Fig. S1.5C′). Remarkably, differentiated F4/80^{hi} cells also upregulated IL7Rα expression on the cell surface (Fig. 2.5C,C′; also see Fig. S1.5D). IL7Rα was co-expressed with the common gamma chain (Fig. 2.5D,D′; Fig. S1.5D). In contrast to Ly6c^{hi} cells, Ly6c^{lo}CD11b^{hi} FL monocytes displayed limited differentiation into F4/80^{hi} macrophages, even after 5 days in culture with M-CSF (Fig. S1.5B). However, additional refinement of these populations by sorting for CD115-expressing cells enhanced macrophage differentiation from Ly6c^{hi}F4/80^{hi}CD11b^{mid} monocytes, and

also resulted in macrophage differentiation from Ly6c^{lo}CD115^{+}F4/80^{hi}CD11b^{mid} cells (Fig. S1.5C). Both MDP and Ly6c^{lo} monocytes displayed significantly higher Cre-mediated labeling compared with Ly6c^{hi} monocytes (Fig. S1.4D), suggesting distinct developmental pathways. These data reveal that Ly6c^{hi}CD11b^{hi} cells in the fetal liver that express *Il7r* (Fig. 2.4A; Fig. S1.4E) but not surface IL7R surface protein (Fig. S1.4F) have the capacity to differentiate into F4/80^{hi}macrophages, and that this differentiation is accompanied by upregulation of surface IL7R expression.

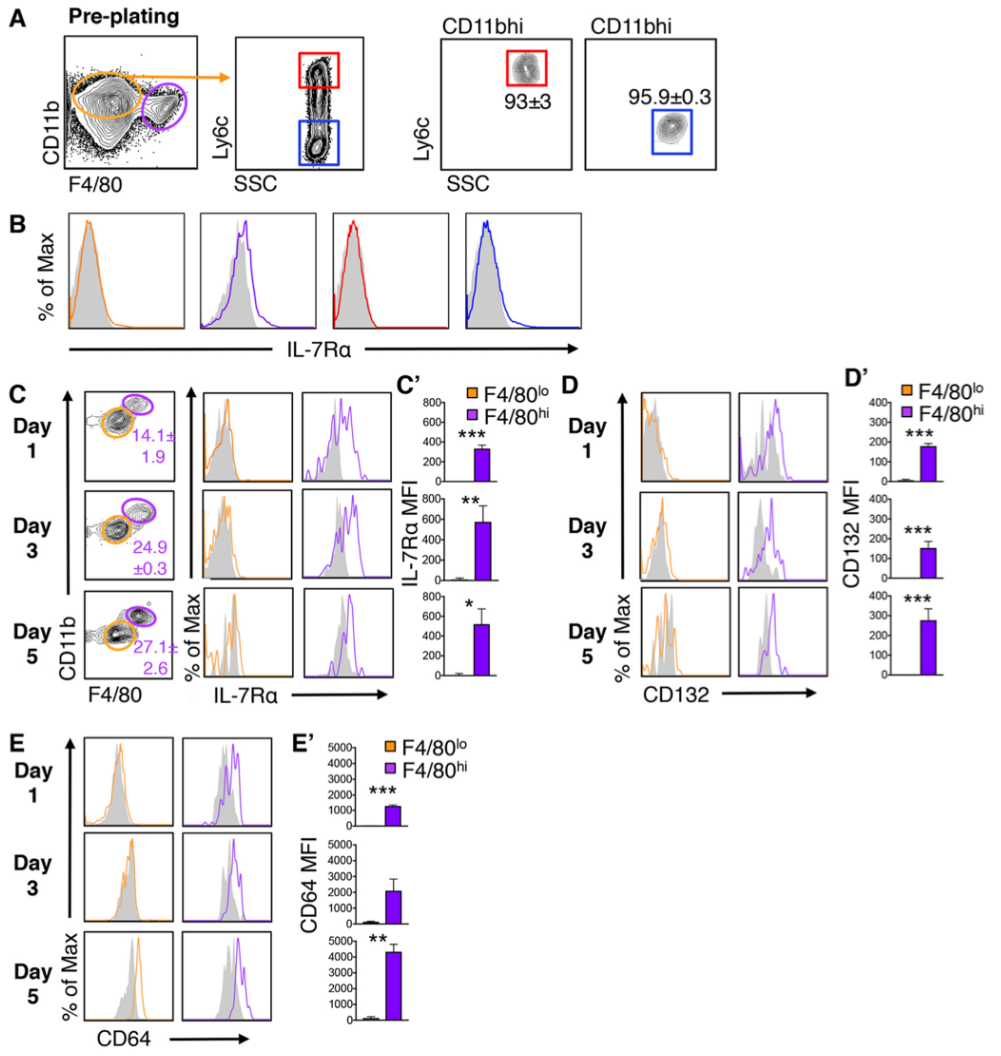

Figure 2.5 Fetal liver monocytes differentiate into IL7R-expressing macrophages ex vivo. (A) Representative gating of F4/80loCD11bhimonocytes (orange gates) from CD11b-enriched FL at E14.5, gated on Ly6c expression (Ly6chi, red; Ly6clo, blue). Adjacent plots represent post-sort purity analysis of FL CD11bhiF4/80lo Ly6chi and Ly6clo monocytes with mean±s.e.m. frequency of sorted populations in triplicate from three independent experiments. (B) Representative FACS plots show surface IL7Rα expression on macrophage (F4/80hi) and monocyte (F4/80loCD11bhi) populations as a function of Ly6c expression, color-coded as gated in A. (C-E′) Representative FACS plots showing gating of monocyte (F4/80loCD11b+) and macrophage (F4/80hiCD11b+) populations and expression of IL7Rα (C), CD132 (D) and CD64 (E) on gated populations following 1, 3 or 5 days of culture of sorted F4/80loCD11bhiLy6chi monocytes with 20 ng m-CSF. Mean fluorescence intensity (MFI; C′,D′,E′) was calculated as the geometric mean minus the FMO for each sample. Gray shaded area indicates FMO control. Values indicate mean frequencies±s.e.m. of gated populations for six to eight different samples in triplicate from three independent experiments. *P<0.05, **P<0.01, ***P<0.001; Student's t-test.

IL7Rα regulates tissue-resident macrophage development

The robust and dynamic expression of IL7R by monocyte precursors and developing tissue macrophages suggested that IL7R regulates fetal trMac development from precursors within fetal tissues. To test this hypothesis, we injected the highly specific IL7Rα monoclonal blocking antibody A7R34, or an IgG2A control, into pregnant mice during fetal development (Fig. 2.6A). Injection of A7R34 during pregnancy completely blocks IL7R signaling, as evidenced by deletion of Peyer's patches in developing embryos following a single injection (Hashizume, 2007; Yoshida, 1999). We injected pregnant WT mice with A7R34 or IgG2A control at E13.5 and E15.5, coincident with robust IL7Rα expression on fetal macrophages (Fig. 2.4B), and examined cellularity of macrophages in the epidermis, lung, liver and brain in neonates 9 days later (Fig. 2.6A). Macrophage cellularity in the epidermis, liver and lung was significantly reduced following this temporally limited blockade of IL7Rα signaling, whereas microglia were unchanged (Fig. 2.6B).

We further investigated trMac cellularity in *Il7r*$^{-/-}$ mice. Although *Il7r* deletion did not affect YS hematopoiesis (Fig. 2.2), examination at P19 revealed that *Il7r* deletion drastically reduced cellularity of trMacs in all tissues (Fig. 2.6C, Fig. S1.6A). *Il7r* deletion did not affect cellularity of other tissue myeloid populations, such as neutrophils (Fig. S1.6B). By adulthood, differences in macrophage cellularity had normalized (Fig. S1.6C), despite sustained impairments in lymphocyte development (Fig. S1.6C′). Cellularity of trMacs was similarly normalized

by adulthood following developmental IL7R blockade (Fig. S1.6D), as were B cells (Fig. S1.6D′). As trMacs are capable of self-maintenance across adulthood, these data suggest that IL7R plays a unique role in the establishment of these populations, but is not required for their homeostatic maintenance in adulthood. Together with the IL7R blocking experiments, these data indicate that IL7R plays a unique role in the establishment of trMacs during perinatal development.

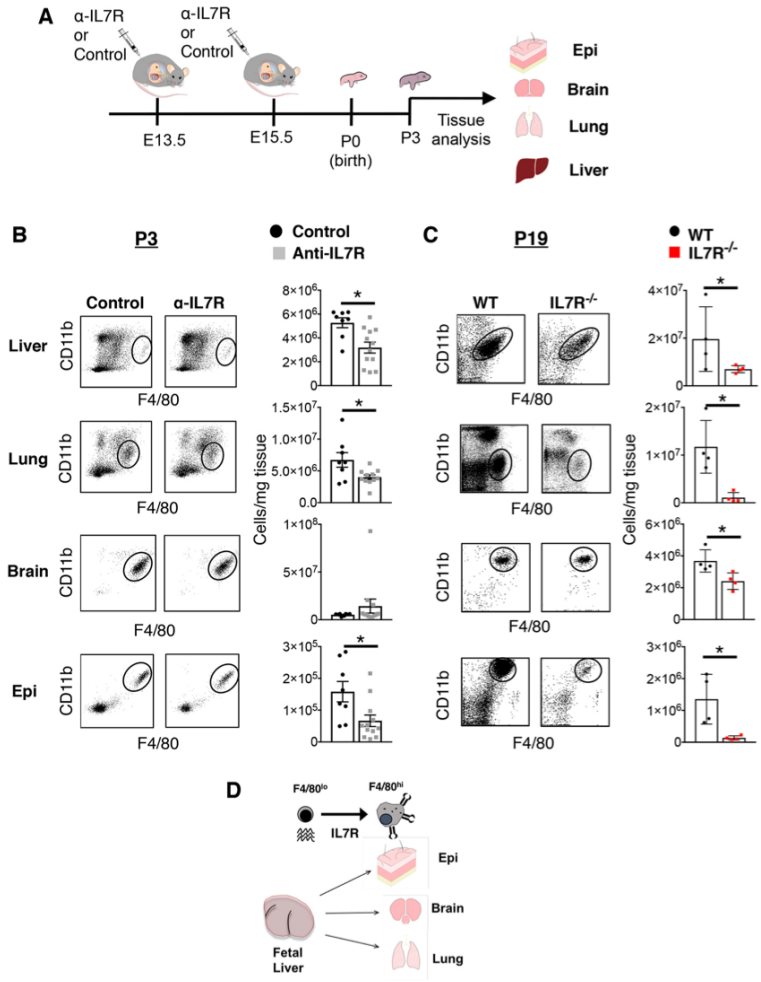

Figure 2.6 L7R regulates tissue-resident macrophage development. (A) Schematic illustrating the timeline for injection and analysis of the IL7R blocking antibody, A7R43, and IgG2A control. Timed-mated WT mice were injected at E13.5 and E15.5 with 600 mg of the IL7Rα blocking antibody or equivalent control injection. Pups were analyzed 9 days after final injection and cellularity of macrophages in the liver, lung, brain and epidermis (Epi) were determined. (B) Representative FACS plots and associated quantification of cells/mg of tissue macrophages (live CD45+F4/80hiCD11blo) in the liver, lung, brain and epidermis of neonates examined 9 days following maternal injections of the A7R43 IL7Rα blocking antibody or IgG2A control administered at E13.5 and E15.5. n=8-11 representing three independent experiments. *P<0.05. (C) Representative FACS plots and associated quantification of cells/mg of tissue macrophages (live CD45+F4/80hiCD11blo) in the liver, lung, brain and epidermis of Il7r−/− and WT neonates at P19. n=4 representing four independent experiments. *P<0.05. (D) A model for dynamic IL7Rα expression during fetal macrophage development. Fetal liver F4/80lo monocytes express Il7r message, and mRNA expression is upregulated as F4/80lo monocytes exit the liver and transit to peripheral tissues. However, upregulation of message is not associated with measurable surface expression in F4/80lo monocytes. IL7Rα surface expression only occurs upon differentiation of monocytes into F4/80hi macrophages in the tissues, at which point message levels dissipate. Continued message and surface expression postnatally within microglia and LCs account for higher levels of GFP labeling in those tissues, whereas GFP labeling is equilibrated by birth in the AMs of the lung and KCs of the liver.

Discussion

Our investigation has revealed a completely novel role for IL7R in fetal myeloid development, and specifically in the generation of fetal-specified trMacs. Tracing the progeny of cells mapped by the lymphoid marker IL7Rα revealed that trMacs derived from fetal hematopoiesis were distinctly marked by IL7Rα expression history (Fig. 2.1). Our comprehensive analysis of Il7r-Cre labeling, *Il7r* mRNA levels, and surface expression concluded that trMac labeling was not due solely to derivation from an IL7Rα-labeled bipotent progenitor, nor was labeling acquired in adulthood. Instead, our developmental analysis revealed that labeling of trMacs by Il7r-Cre occurred as a result of dynamic stage- and tissue-specific expression of IL7R during macrophage development within tissues during fetal development (Figs 2.3 and and 2.4). We showed that the CD11b^{hi}Ly6c^{hi} fetal liver monocytes rapidly upregulated surface IL7R as they differentiated into F4/80^{hi}macrophages *ex vivo* (Fig. 2.5), and observed robust IL7R surface expression on developing macrophages during a limited window of fetal development. Using two different loss-of-function approaches, we further demonstrated that blocking IL7R signaling during the window of robust IL7R expression by developing trMacs impairs their establishment (Fig. 2.6). In adult BM hematopoiesis, IL7Rα expression exclusively marks lymphoid cells (Schlenner, 2010), and the many functions of IL7R signaling in lymphopoiesis are well-delineated (Fry, 2005) , including proliferation, differentiation and survival. IL7R may therefore

regulate similar processes in developing macrophages, but the mechanisms and signaling pathways for IL7R in myeloid cells remain to be established. By revealing a role for IL7R in trMac development, our data identify a function of IL7R during fetal development that extends beyond regulation of the lymphoid lineage, contributing to accumulating evidence that fetal hematopoietic lineage specification is considerably less constrained than adult hematopoiesis (Beaudin et al., 2016; Mebius, 2001; Notta, 2015).

Common signaling factors, including dependency on the transcription factor PU.1 (also known as SPI1)(C. P. Schulz Elisa Gomez; Chorro, Laurent; Szabo-Rogers, Heather L. ;. Cagnard, Nicolas; Kierdorf, Katrin; Prinz, Marco; Wu, Bishan; Jacobsen, Sten Eirik W. ;. Pollard, Jeffrey W. ;. Frampton, Jon; Liu, Karen J. ;. Geissmann, Frederic, 2012) and signaling downstream of the CSF-1 receptor (Chitu, 2016), regulate macrophage development across multiple tissues, whereas other signals regulate and maintain tissue-specific identity and function (Guilliams, 2013; Mass, 2016; Scott, 2018). IL7R appears to function as a regulatory factor across different tissues, but may also have distinct roles in different tissues across ontogeny. Blocking IL7Rα function during a narrow fetal window (E13.5-E15.5) negatively affected cellularity of liver, lung and epidermal macrophages, with no effect on microglia (Fig. 2.6B). In contrast, germline deletion of IL7Rα in the *Il7r*$^{-/-}$ mouse significantly impaired trMac cellularity across all tissues, including microglia, examined at P19 (Fig. 2.6C). The different effect of total knockout and transient blockade of IL7Rα on trMac development suggests that the window of dependency on IL7R signaling differs between different tissues. The

requirement for IL7Rα during postnatal microglia development fits with recent reports of rapid changes in proliferation and apoptosis occurring postnatally in microglia (Askew, 2017; Nikodemova, 2014), and suggests the possibility that IL7Rα contributes to proliferation or survival of microglia in the transition between the postnatal period and adulthood. Although IL7Rα is not expressed by adult myeloid cells at homeostasis (Schlenner, 2010), there have been a handful of reports suggesting that IL7 can promote monocyte activation in the context of autoimmunity and infection (Z. K. Chen Seung Jae; Chamberlain, Nathan D. ;. Pickens, Sarah R. ;. Volin, Michael V. ;. Volkov, Suncica; Arami, Shiva; Christman, John W. ;. Prabhakar, Bellur S. ;. Swedler, William I. ;. Mehta, Anjali; Sweiss, Nadera J. ;. Shahrara, Shiva, 2013; Gessner, 1993), and a bipotent IL7R^{+}lympho-myeloid progenitor has been identified that emerges in the context of malaria infection (Belyaev, 2010). Whether autoimmunity or other inflammatory conditions can promote the usage of fetal hematopoietic differentiation pathways to confer distinct functions on myeloid cells during disease states is an interesting hypothesis that remains be tested experimentally.

We used three different lineage-tracing models based on lymphoid markers associated with both increased lymphoid potential (Flk2, Rag1, IL7Rα; Fig. 1) (Boyer et al., 2019; Forsberg et al., 2006; Igarashi, 2002; Kondo et al., 1997) and overlapping expression in FL progenitors (Beaudin et al., 2016; Böiers, 2013) to trace trMac origin. The stark contrast in labeling between these models allows us to exclude the possibility that labeling of adult trMacs by Il7r-Cre reflected inheritance from an earlier lymphomyeloid progenitor. A clear example is epidermal LCs: if labeling of LCs was

due to derivation from lymphomyeloid progenitors, LCs would be robustly labeled not only by Il7r-Cre, but also by Flk2-Cre and Rag1-Cre, as those genes are expressed in IL7R^{+} FL progenitors (Boiers et al., 2013). The high degree of Il7r-Cre labeling of trMacs was similarly not reflected in YS EMPs or YS macrophages (Fig. 2.2, Fig. S1.2) and IL7R deletion had no effect on YS EMP cellularity or the development of YS macrophages during embryonic hematopoiesis (Fig. 2.2D,E), but resulted in significant impairments later in trMac development (Fig. 2.6C). Together, these data suggest that although IL7R expression labels a multipotent fetal liver progenitor, expression of IL7R on developing tissues macrophages later in gestation regulates their development during later waves of macrophage seeding (De et al., 2018; Ferrero et al., 2018; Tan and Krasnow, 2016).

Our analysis of the IL7R knockout mouse and in response to physiological blockade of IL7R reveal IL7R as a novel regulator of tissue macrophage development. Functional inhibition of IL7R also affected lymphocyte cellularity (Fig. S1.6C′), as previously reported (Hashizume et al., 2008; Peschon et al., 1994; Yoshida et al., 1999). One caveat is that the impairments in trMac development may be secondary to impaired lymphocyte development. However, despite sustained impairments in lymphocyte development in the IL7R knockout (Fig. S1.6C′), trMac cellularity recovered by adulthood (Fig. S1.6C), arguing that lymphocyte impairment alone is not responsible for impaired trMac development. Deletion of IL7R had no effect on YS EMPs or YS macrophage generation, affirming that IL7R plays a more crucial role in fetal macrophage generation at later stages of hematopoiesis.

Fetal F4/80^{hi} macrophages expressed negligible *Il7r* message levels (Fig. 2.4A) yet expressed robust surface IL7R protein (Fig. 2.4D) and acquired Il7r-Cre labeling across fetal development (Fig. 2.3C). In lung and liver, for example, increased GFP labeling in F4/80^{hi}trMacs across fetal development did not reflect Cre-driven reporter expression, as trMacs did not express *Il7r* during fetal development (Fig. 2.4A). The most parsimonious explanation for this surprising discrepancy is the initiation of Cre recombination in IL7R-marked F4/80^{lo} macrophage precursors with subsequent inheritance of the floxed allele by F4/80^{hi} trMacs upon differentiation. F4/80^{hi} trMac differentiation is then coincident with the rapid translation of *Il7r* message into IL7Rα protein and surface display (Fig. 2.6D). Indeed, our *ex vivo* differentiation assay revealed that Ly6c^{+} FL monocytes expressing *Il7r* mRNA (Fig. 2.4C; Fig. S1.4E) differentiate into F4/80^{hi} macrophages that upregulate surface IL7R protein *ex vivo* (Fig. 2.5). *In vivo*, the rapid and dynamic regulation of IL7R protein surface expression may reflect a response to ligand in tissues, as surface expression of IL7R is regulated by IL7 availability (Clark et al., 2014; Wei et al., 2000). The lower surface expression of IL7R on fetal liver F4/80^{hi} macrophages compared with peripheral tissues (Fig. 2.4D) *in vivo* may also reflect surface regulation by immediate sources of IL7 ligand in the fetal liver (Namen et al., 1988).

By tracking GFP expression within fetal myeloid cells in the Il7r-Cre model, we observed the contribution or replacement of GFP-labeled F4/80^{lo} precursors to F4/80^{hi} trMacs in the lung and liver, as evidenced by increased labeling in F4/80^{hi} macrophages across ontogeny (Fig. 2.3C). Whereas trMacs of the lung and

liver exhibited robust labeling (~40%) that had plateaued at birth (Fig. 2.3A-C), *Il7r* mRNA expression (Fig. 2.4B,D; Mass et al., 2016) and Cre-mediated labeling of LC and microglia continued postnatally, leading to almost complete labeling by Il7r-Cre in adulthood. Although monocytes entering the epidermis and brain expressed both the highest levels of *Il7r* mRNA and had the greatest degree of Cre-mediated labeling, there was very little monocyte infiltration from E14.5 and beyond (Fig. 2.4A). Instead, the higher degree of labeling in the epidermis and brain could be attributed to higher and persistent expression of IL7Rα within trMacs peri- and postnatally, leading to increased GFP labeling (Fig. 2.3). Together, our analysis provides additional support for the contribution of FL F4/80^{lo} macrophage precursors to specific trMac compartments during late gestation (Guilliams et al., 2013; Hoeffel et al., 2015; Rantakari et al., 2016; Tan and Krasnow, 2016), and reveals how IL7R expression during macrophage differentiation regulates establishment of tissue macrophage compartments.

Materials & Methods

Mice

All animals were housed and bred in the AALAC accredited vivaria at UC Santa Cruz or UC Merced and group housed in ventilated cages on a standard 12:12 light cycle. All procedures were approved by the UCSC or the UC Merced Institutional Animal Care and Use (IACUC) committees. IL7Rα-Cre (Schlenner et al., 2010), Rag1-Cre (Welner et al., 2009) and Flk2-Cre (Benz et al., 2008) mice, obtained under fully executed Material Transfer Agreements, were crossed to homozygous Rosa26^{mTmG} females (Muzumdar et al., 2007) to generate 'switch' lines, all on the C57Bl/6 background. WT C56Bl/6 mice were used for controls and for all expression experiments. Adult male and female mice were used randomly and indiscriminately, with the exception of the FlkSwitch line, for which only males were used because of their high and uniform floxing efficiency. Similarly, mice for developmental analysis were used indiscriminately without knowledge of gender.

Tissue and cell isolation

Mice were sacrificed by CO_2 inhalation. Gravid uteri removed, and individual embryos dissected. Adult liver, lung, brain and skin were isolated and treated with 1× PBS (+/+) with 2% serum, 0.2-1 mg/ml collagenase IV (Gibco) with or without 100 U/ml DNase1 for 20 min to 2 h. For adult epidermis isolation, ears were first incubated with 1× PBS (+/+) containing 2.4 mg/ml dispase (Gibco) to separate the epidermis, followed by 2 h

incubation of the epidermis with 1/mg/ml collagenase. Following incubation, all tissues were passaged through a 16 g needle or a 19 g needle ten times, and then filtered through a 70 μm filter.

Flow cytometry

Cell labeling was performed on ice in 1× PBS with 5 mM EDTA and 2% serum. Antibodies used are listed in Table S1. Analysis was performed on BD FACS Aria II at University of California-Santa Cruz, and BD FACS Aria III and the University of California-Merced and analyzed using FlowJo.

Transplantation assays

Transplantation assays were performed as previously described (Beaudin et al., 2014; Beaudin et al., 2016; Smith-Berdan et al., 2015; Ugarte et al., 2015; Boyer et al., 2019). Briefly, sorted Tom^+ or GFP+ KLS cells were isolated from IL7RαSwitch or Rag1Switch E14.5 fetal liver donors. WT recipient mice aged 8-12 weeks were sublethally irradiated (750 rad, single dose). Under isofluorane-induced general anesthesia, sorted cells were transplanted intravenously. Recipient mice were bled at 4, 8, 12 and 16 weeks post-transplantation via the tail vein and peripheral blood was analyzed for donor chimerism by means of fluorescence profiles and antibodies to lineage markers. Long-term multilineage reconstitution was defined as chimerism in both the lymphoid and myeloid lineages of >0.1% at 16 weeks post-transplantation.

***Ex vivo* macrophage culture**

E14.5 fetal livers from WT embryos were harvested and homogenized via trituration. $CD11b^+$ cells were enriched by positive selection with CD11b biotin-conjugated antibodies and streptavidin microbeads, using LS columns (Miltenyi). $F4/80^{lo}$ $CD11b^{mid}$cells were sorted based on Ly6c expression or CD115 expression and cultured in a 96-well plate (DMEM, 20% FBS, 1 mM sodium pyruvate, 10 mM HEPES, 0.1 mM 2-mercaptoethanol and 50 mg/ml primocin, Life Technologies). Cells were cultured in triplicate in the presence of 20 ng/ml M-CSF and analyzed at three different time points (days 1, 3 and 5) for the presence of $F4/80^{hi}$ $CD11b^{mid}$ macrophages and for surface expression of IL7R, common gamma chain (CD132) and CD64.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

RNA isolation from sorted cells was accomplished using the RNeasy mini kit (Qiagen), and cDNA was reverse transcribed from RNA (High capacity cDNA reverse transcription kit, ThermoFisher Scientific). TaqMan probes (TaqMan Gene Expression Assay, ThermoFisher Scientific) were used for qRT-PCR analysis on a StepOnePlus Real-Time PCR System (ThermoFisher Scientific) in comparative CT mode. Samples were run in triplicate and were run with positive ($CD43^+$ $B220^+$ Pro B-cells), negative ($Gr1^+$ $CD11b^+$BM monocytes), and no cDNA controls.

Quantification and statistical analysis

Number of experiments, *n*, and what *n* represents can be found in the legend for each figure. Statistical significance was determined by two-tailed unpaired Student's *t*-test. All data are shown as mean±s.e.m. representing at least three independent experiments.

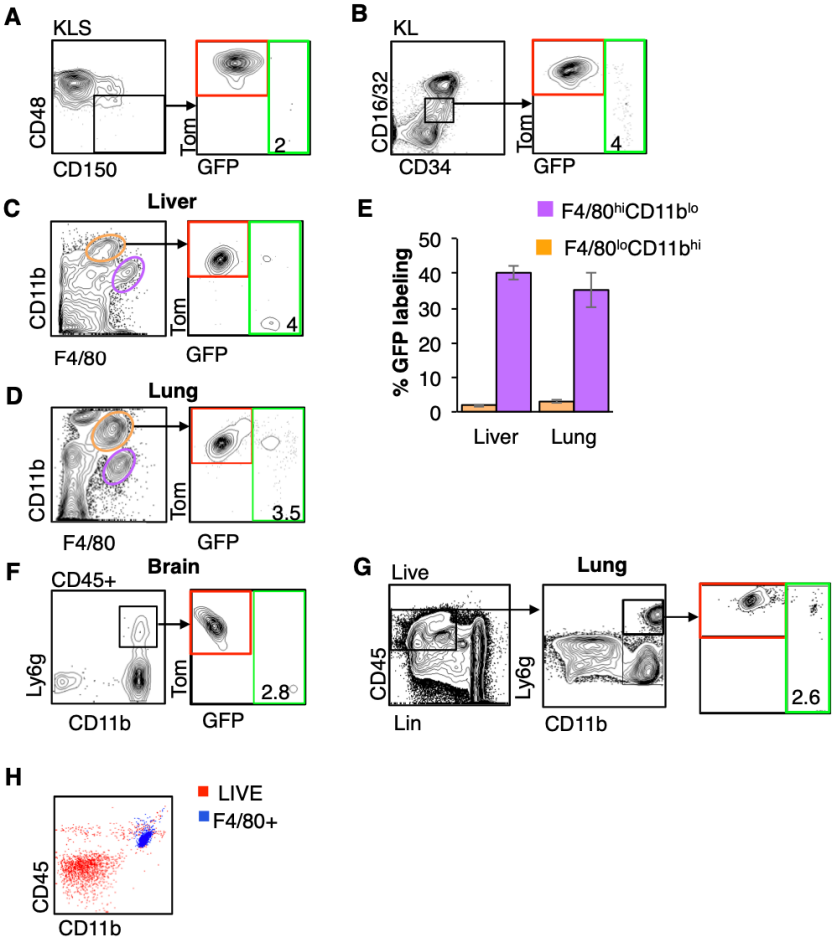

Figure S1.1 Analysis of Il7r-Cre driven labeling in additional adult populations. **A, B,** Representative FACS plots displaying gating of HSCs (Lin-cKit+Sca1+CD48-CD150+, A), and common myeloid progenitors, (Lin-cKit+Sca1-CD34midFcgramid, B) and corresponding representative flow plots showing the percentage of Il7r-Cre driver reporter labeling (GFP)+ in gates populations. Data are representative of three mice. **C, D,** Representative FACS plots displaying the gating of F4/80hi macrophages and F4/80lo Cd11bhi monocyte populations in the liver (**C**) and lung (**D**). Corresponding representative flow plots show the percentage of Il7r-Cre driver reporter labeling (GFP)+ in F4/80lo Cd11bhi monocytes. Numbers represent mean frequencies ± SEM of gated populations for the same mice described in Figure 1. **E,** Quantification of the percent of Il7r-Cre driven reporter labeling (GFP+) in the two populations shown in (**C,D**). Whereas fetal-derived Kupffer cells in the liver (F4/80hiCd11bloCD169+) and alveolar macrophages in the lung (F4/80hiCD11bloCD11c+ SiglecF+) exhibited high levels of reporter expression (Fig 1F, G), F4/80loCD11bhi cells in both tissues exhibited nominal Cre-mediated labeling, reflecting their derivation from adult BM hematopoiesis. **F,** Representative FACS plots displaying of Il7r-Cre driven reporter labeling (GFP+) in CD45+Ly6g+CD11b+ neutrophils in brain. Numbers show representative frequency of GFP+ cells in gated population. **G,** Representative FACS plots displaying of Il7r-Cre driven reporter labeling (GFP+) in CD45+Ly6g+CD11b+ neutrophils in the lung. Numbers show representative frequency of GFP+ cells in gated population of two independent experiments. **H,** Representative FACS plots displaying CD45 and CD11b expression by F4/80+ cells (Blue) overlayed on Live adult brain cells. Red dots depict all Live cells and Blue dots depict cells that are positive for F4/80 staining.

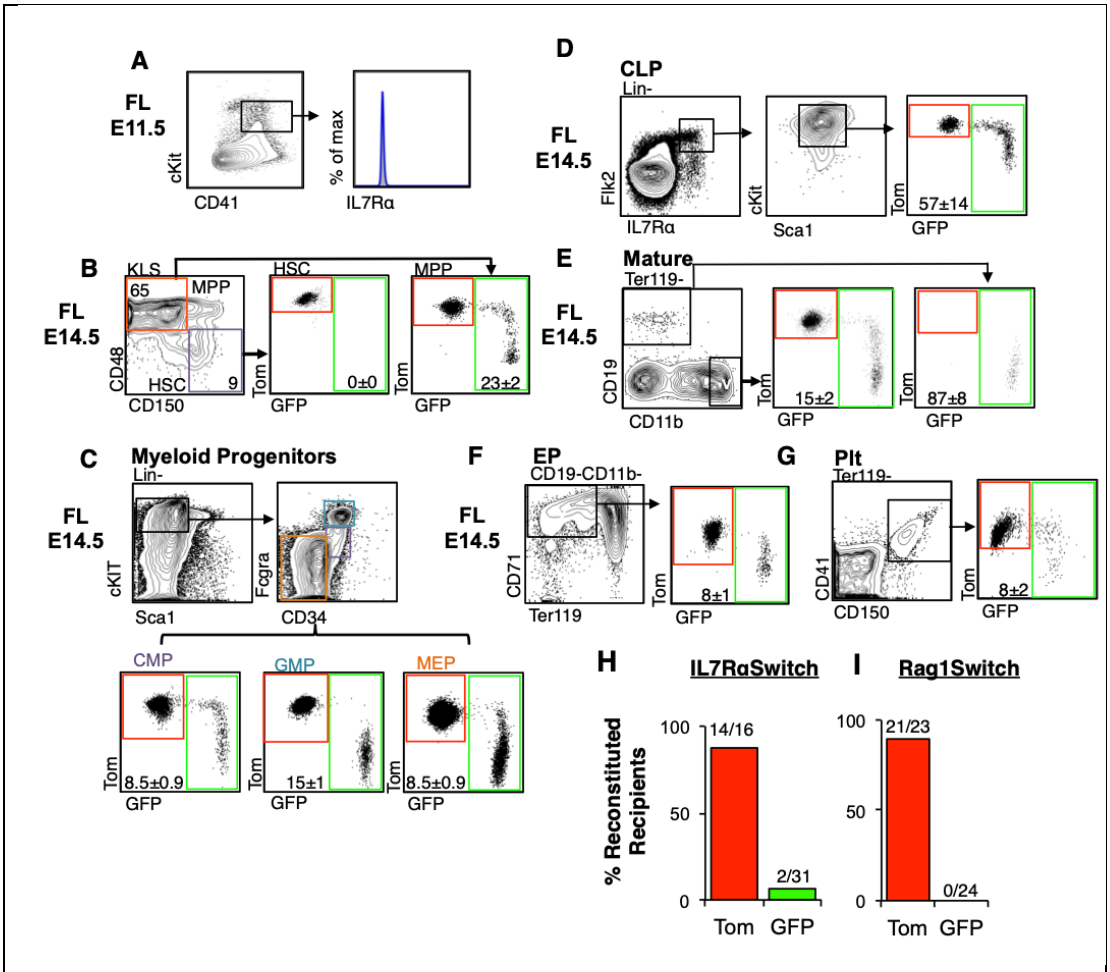

Figure S1.2 Analysis of additional Il7r-Cre-labeled populations across fetal development. A, Representative FACS plots of cKit+CD41+ erythromyeloid progenitors (EMPs) in the fetal liver (FL) at E11.5 and accompanying IL7Rα surface expression. Data are representative of 3-7 mice from at least 2 independent experiments. Gray shaded area indicates FMO control. **B**, Representative FACS plots showing gating and Il7r-Cre-driven GFP labeling within fractions of the cKit+Lin-Sca1+ (KLS) compartment of the E14.5 FL as defined by CD150 and CD48. Red and green boxes denote gated Tom+ and GFP+ fractions, respectively. Values indicate means frequencies ± SEM of gated Il7r-Cre marked GFP+ populations for 5 mice from three independent experiments. **C-G**, Representative FACS plots showing gating and IL7R- Cre-mediated GFP labeling within **C**, myeloid progenitors including CMP (Lin-cKit+ FcgramidCD34mid), GMP (Lin-cKit+ FcgrahiCD34+), and MEP (Lin-cKit+ Fcgra-CD34-); **D,** Cre-mediated GFP labeling within the CLP compartment (Lin-cKitmidScamidIL7Rα+Flk2+); **E,** Cre-driven GFP labeling within mature myeloid (CD11b)+ and lymphoid (CD19)+ cells; **F,** Cre-driven GFP labeling in erythroid progenitors (EP, CD19-CD11b-Ter119+CD71+) and **G,** Cre-driven GFP labeling of platelets (Ter119-CD41+CD150+). Red and green boxes denote gated Tom+ and GFP+ fractions. Values indicate mean frequencies ± SEM of gated Il7r-Cre marked GFP+ populations for 5 mice from three independent experiments. **H, I,** Proportion of sublethally irradiated adult recipients demonstrating long-term multilineage reconstitution (see methods for definition) following transplantation of 1000 Tom+ or 1000 GFP+ FL KLS cells isolated from IL7R-Switch (**H**) or Rag1-Switch (**I**) embryos, respectively.

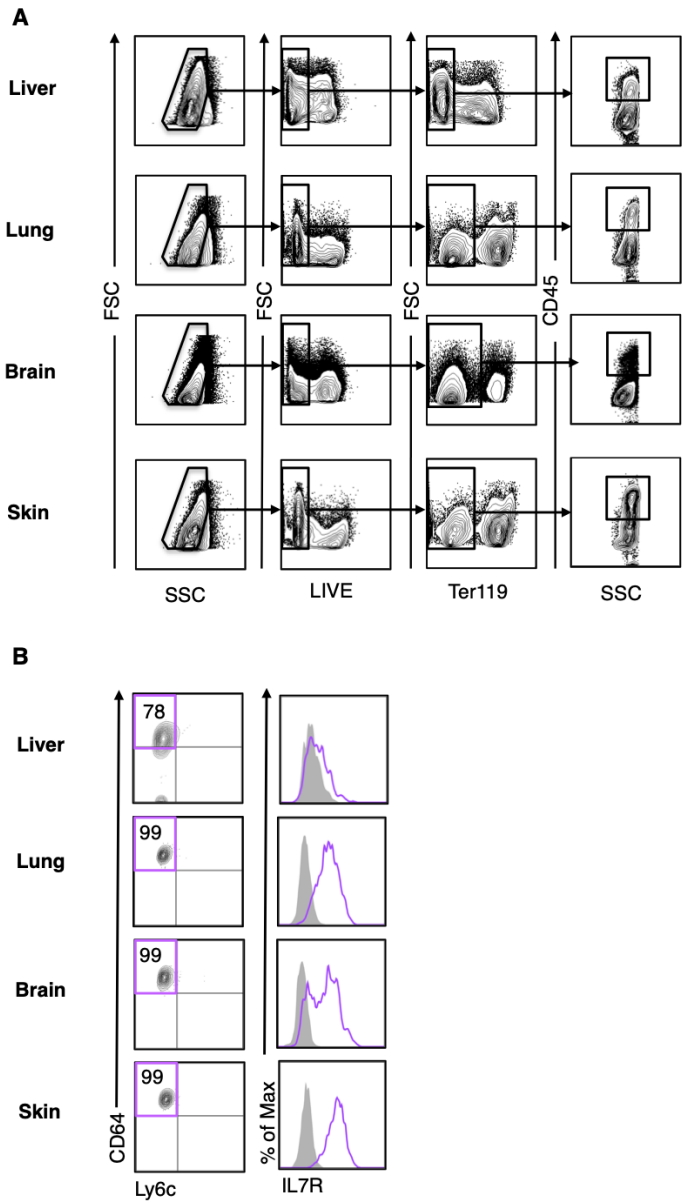

Figure S1.3 Additional gating of the fetal myeloid cells shown in Figure 3. **A,** Representative FACS plots show pre-gating analysis for the F4/80hi and F4/80lo populations shown in Figure 3 at E14.5. **B,** Additional gating of E14.5 F4/80hi macrophages (pregated on Live Ter119-CD45+F4/80hiCD11bmid, as in Fig. 3 and Fig S3A) using CD64 and Ly6c revealed comparable patterns of IL7R surface expression as macrophages not gated by CD64 and Ly6c. Purple squares indicate gated CD64+Ly6c-F4/80hiCD11blo macrophages. Numbers indicate representative frequencies of gated populations. Accompanying histograms depict IL7R expression on gated population (purple) as compared to FMO (solid gray).

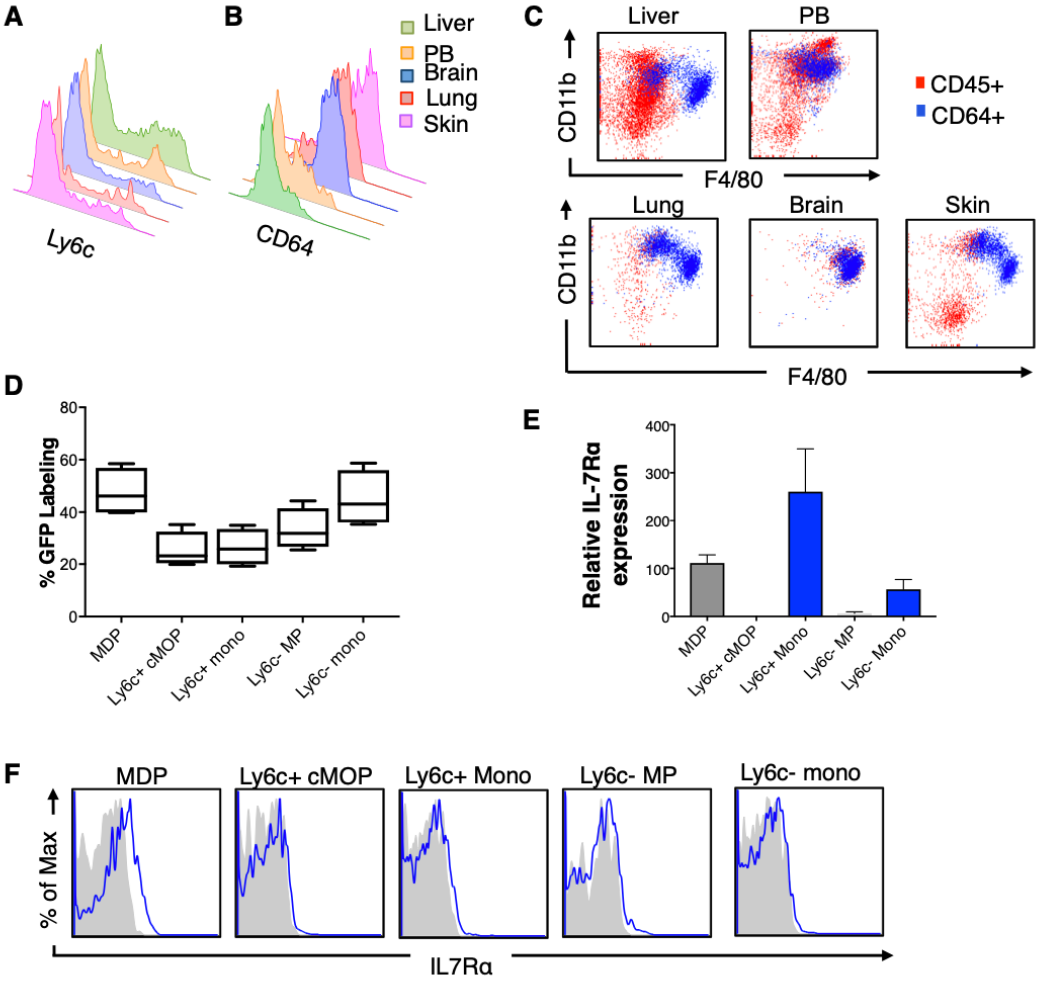

Figure S1.4 Heterogeneity among fetal myeloid cells. **A, B,** Representative FACS plots comparing expression of Ly6c (A) and CD64 (B) among F4/80loCD11b+ cells in the fetal liver, peripheral blood (PB), and peripheral tissues at E14.5. Ly6c is more highly expressed by fetal liver populations, whereas CD64 is more highly expressed by populations in peripheral tissues. **C,** Overlay of CD64 expression on CD45+ populations defined by CD11b and F4/80 across multiple tissues at E14.5. **D**, GFP labeling among E14.5 fetal liver macrophage-dendritic cell progenitors (MDPs; CD115+cKit+Flk2+Ly6c-CD11b-), common monocyte progenitor (cMOP; CD115+cKit+Flk2- Ly6c+), myeloid progenitors (MP; CD115+cKit+Flk2-Ly6c-) and monocytes (CD115+cKit- Flk2-CD11b+) sorted based on Ly6c expression, as previously described by Hoeffel et al, 2015. MDPs display the highest levels of IL7ra-Cre mediated labeling, consistent with previously reported IL7R expression (Hoeffel et al, 2015). Downstream populations show intermediate labeling, and Ly6c- populations shows higher labeling as compared to Ly6c+ populations. **E**, Quantitative qPCR of IL7Rα message in the same populations as defined in (**C**). ΔΔCT values were normalized to beta-actin and compared to IL7Rα expression levels in MDPs. Data represent three independent experiments. **F**, Representative FACS plots of three independent experiments showing IL7Ra surface expression among the same populations described in (C) in the E14.5 FL. Gray shaded area indicates FMO control.

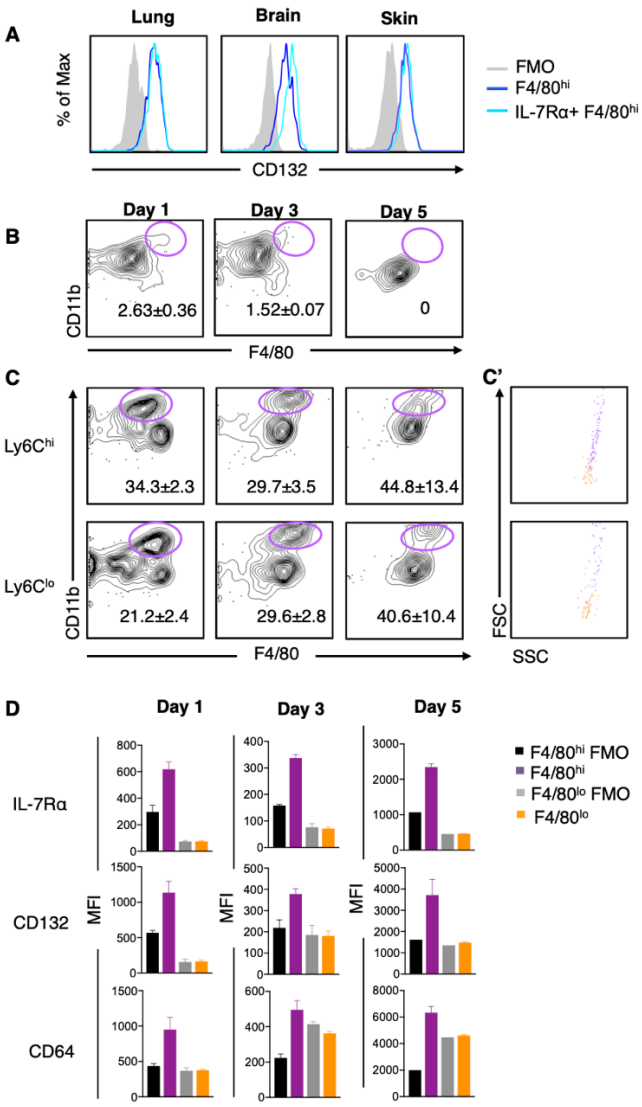

Figure S1.5 Properties of F4/80hi macrophages in vivo and ex vivo. **A,** Representative FACS plots showing expression of the common gamma chain (CD132) on F4/80hi macrophages and IL7Rα-expressing F4/80hi macrophages in the tissues indicated at E14.5. Gray shaded area indicates FMO control. Data are representative of three independent experiments. **B,** Representative FACS plots showing differentiation of Ly6cloCD11bhiF4/80lo fetal liver monocytes cultured with 20 ng M-CSF for 5 days. Values represent mean ± SEM frequencies of differentiated F4/80hi macrophages, gated in purple. Data are representative of three independent experiments performed in triplicate. **C, C',** Representative FACS plots showing differentiation of Ly6clo or Ly6chi CD115+ CD11bhiF4/80lo fetal liver monocytes sorted and cultured with 20 ng M-CSF for 5 days. Values represent mean ± SEM frequencies of differentiated F4/80hi macrophages, gated in purple. Data are representative of two independent experiments performed in triplicate. **C',** Representative FACS plots depicting size (FSC) and complexity (SSC) or F4/80lo monocytes, indicated in orange, and differentiated F4/80hi macrophages, indicated in purple, at day 5 for the same experiments described in C. **D,** Raw mean fluorescence intensity (MFI) analysis of the same data presented in **Fig 5C-E**.

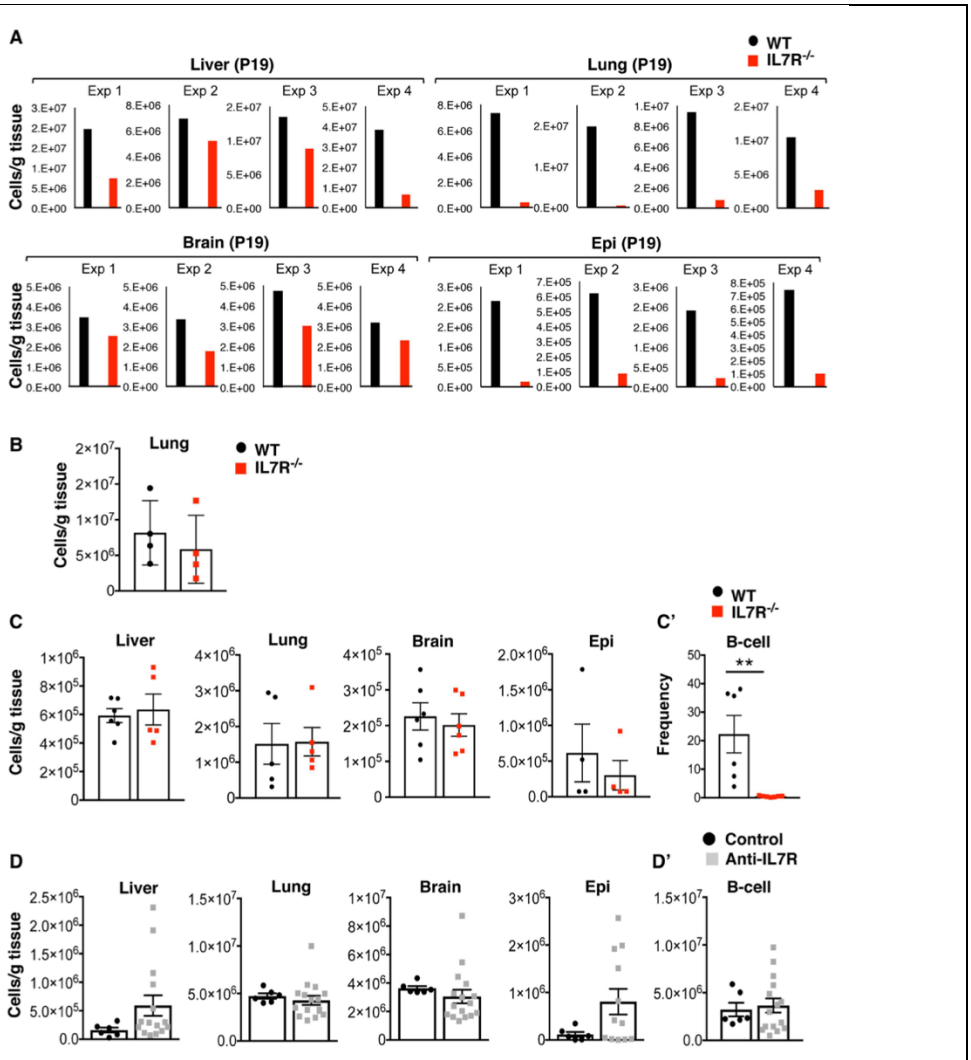

Figure S1.6 Impairment in tissue macrophage development is normalized by adulthood. **A,** Cellularity of trMacs in the four individual experiments that are shown as aggregate data in Fig. 6C. The decrease in trMacs in liver, lung, brain, and epidermis in IL7R-/- mice is reproducible across experiments, despite some inter-experiment variability in cell recovery. **B,** Cellularity of lung neutrophils (CD45+CD11bhiSSChi) in WT and IL7R-/- neonates at postnatal day (P)19, for the same experiments described in Fig. 6C. N = 4 representing four independent experiments. **C, C',** Cellularity of adult tissue macrophage compartments (**C**) and frequency of peripheral blood B-cells (Live,Ter119-B220+; **C'**) in adult WT and IL7R-/- mice. Data shown represent mean ± SEM from three independent experiments. Although tissue macrophage development in the liver, lung, and epidermis is impaired in neonates, these differences are normalized by adulthood. Data shown represent mean ± SEM from 4-6 mice in three independent experiments. **D, D',** Cellularity of adult tissue macrophages (F4/80hiCD11blo) and liver B-cells (Ter119- CD19+; **D'**) following prenatal injections of the A7R43 IL7R blocking antibody at E13.5 and E15.5, respectively. Data shown represent 4-6 mice from 3 independent experiments.

Chapter 2: extrinsic regulators of tissue resident immune cell establishment and homeostasis

[This section is adapted from a publication, Interleukin 7 receptor is required for myeloid cell homeostasis, Accepted at Experimental Hematology]

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Abstract

Respiratory diseases are a leading cause of death worldwide, with vulnerability to disease varying greatly between individuals. The reasons underlying disease susceptibility are unknown, but there is often a variable immune response in lungs often. Recently, we identified a surprising novel role for the interleukin 7 receptor (IL7R), a primarily lymphoid-associated regulator, in fetal-specified, lung-resident macrophage development. Here, we report that traditional, hematopoietic stem cell-derived myeloid cells in the adult lung, peripheral blood, and bone marrow also depend on IL7R expression. Using single- and double-germline knockout models, we found that eosinophil numbers were reduced on deletion of IL7Ra. We then employed two Cre recombinase models in lineage tracing experiments to test whether these cells developed through an IL7Ra+ pathway. Despite the impact of IL7Ra deletion, IL7R-Cre labeled only a minimal fraction of eosinophils. We therefore examined the intrinsic versus extrinsic requirement for IL7R in the production of eosinophils using reciprocal hematopoietic stem cell transplantation assays. These assays revealed that extrinsic, but not eosinophil-intrinsic, IL7R is required for eosinophil reconstitution by HSCs in the adult lung. To determine which external factors may be influencing eosinophil development and survival, we performed a cytokine array analysis between wild-type and IL7Ra-deficient mice and found several differentially regulated proteins. These findings expand on our previous report that IL7R is required not only for proper lymphoid cell development and homeostasis, but also for myeloid cell homeostasis in tissues.

Introduction

Hematopoiesis is the process of mature blood and immune cell production and maintenance from hematopoietic stem and progenitor cells (HSPCs). The majority of "traditional" blood and immune cells, including eosinophils, have short half-lives and are continually replaced by HSPCs (McRae et al., 2019). In contrast, fetally-derived tissue resident macrophage (trMacs) self-maintain in their respective tissues for life, without contribution from adult HSCs, thereby leading us to consider them "non-traditional" mature immune cells (Boyer et al., 2019; S. B. Smith-Berdan Alyssa; Rajendiran, Smrithi; Forsberg, E. Camilla, 2019; S. N. Smith-Berdan Andrew; Hong, Matthew A. ;. Forsberg, E. Camilla, 2015; Ugarte, 2015). Recently, our lab uncovered a novel role for interleukin 7 receptor (IL7R) in trMac specification during development (Rajendiran, 2020). Though IL7R had originally been reported to be essential exclusively for lymphocyte development and survival, a few recent studies, including ours, have found that it also plays roles in myeloid cell development(Cool & Forsberg, 2019; Lavin et al., 2015b; Rajendiran, 2020). Interestingly, in addition to our finding that IL7R regulates trMac specification during mouse fetal development4, studies in humans have reported that other myeloid cell types, including monocytes and eosinophils, may express IL7R(Cool & Forsberg, 2019; Sawai, 2016). Furthermore, these cells can upregulate IL7R mRNA and surface protein upon stimulation with lipopolysaccharide (LPS), a known activator of eosinophils. Here, we used germline knockout mice, lineage tracing, and transplantation assays to determine whether IL7R

plays a role in adult-derived "traditional" myeloid cell types in the lung, peripheral blood (PB), and bone marrow (BM).

Results and Discussion

Adult neutrophils and eosinophils are differentially affected by deletion of IL7R$\boldsymbol{\alpha}$

During our previous studies where we found that IL7R unexpectedly regulates trMac development, we also noted alterations in other cells types in the lungs of IL7R$\boldsymbol{\alpha}$-/- mice. We therefore employed Flk2-/- and IL7R$\boldsymbol{\alpha}$-/- mice and crossed the two strains to also create homozygous double mutants (referred to as FIDKO mice here) to investigate lung cellularity in three mutant cohorts. Flk2 is a tyrosine kinase receptor that regulates hematopoietic development10, including robust numbers of common myeloid progenitor cells (CMPs)(Beaudin & Forsberg, 2016b; Leung et al., 2019), the presumed precursors of eosinophils. Previously, our lab established that all adult-derived "traditional" mature blood and immune cells, but not trMacs (Kelly et al., 2009; Rajendiran, 2020), develop through a Flk2+ pathway (Al-Mossawi et al., 2019; Cook et al., 2012; Leung et al., 2019; Rajendiran, 2020). As expected based on previous reports of circulating lymphocytes15, B lymphocytes in the lungs were decreased in all three mutant strains relative to the WT controls,

with drastic reductions in the IL7R$\boldsymbol{\alpha}$-/- and FIDKO mice (Fig. 3.1A), while neutrophil numbers(Boyer et al., 2019) were normal in all three genetic models (Fig. 3.1B). Surprisingly, deletion of IL7R$\boldsymbol{\alpha}$ also led to a significant reduction in numbers of

eosinophils in the lungs, with a further significant reduction in the FIDKO mice (Fig. 3.1C). To determine whether this was a tissue-specific phenotype, we also analyzed the PB and BM of WT and IL7R-/- mice. We observed that eosinophils were similarly affected in these tissues (Fig. S2.1B and D). Interestingly, there was no significant difference in neutrophils numbers in the BM of these mice, but there were significantly more circulating neutrophils in the IL7Rα-/- mice (Fig. S2.1A and C). These data indicate that IL7R is differentially required for eosinophil and neutrophil homeostasis across tissues.

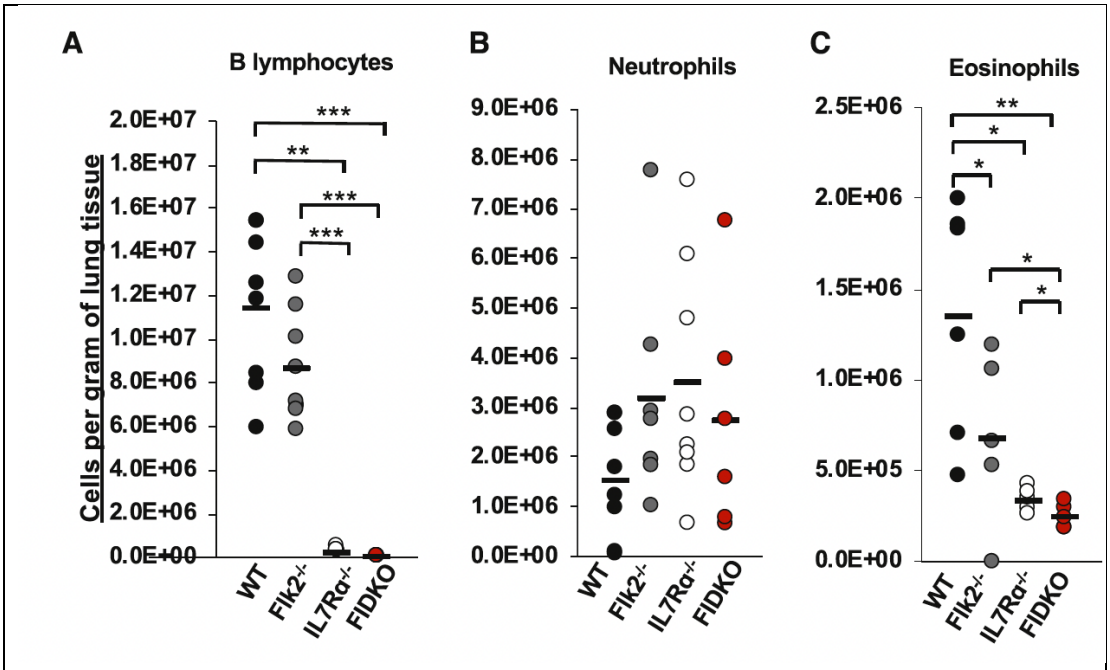

Figure 3.1 Deletion of Flk2 and/or IL7Rα differentially affects numbers of both lymphoid and myeloid cells in the lungs of adult mice. Cellularity was calculated by the total numbers of cells collected and dividing by the weight of the tissue. Dots represent individual mice. Black bars represent the average. *P<0.05, **P<0.005, ***P<0.001. **A,** B lymphocytes were significantly reduced in Il7Rα$^{-/-}$ and FIDKO, but not Flk2$^{-/-}$, lungs. Quantification of lymphocytes (Live, Ter119-Mac1-Gr1-CD19+) per gram of tissue in the lungs of wild type (WT) (black), Flk2$^{-/-}$ (gray), IL7Rα$^{-/-}$ (white), or FIDKO (red) adult mouse lungs. WT n=8, Flk2$^{-/-}$ n=8, IL7R$^{-/-}$ n=8, and FIDKO n=5 representing four independent experiments. **B,** Neutrophils were not affected by loss of Flk2 or IL7Rα. Quantification of neutrophils (Live, CD3-CD4-CD5-CD8-B220-Ter119-CD45+Ly6g+CD11b+) per gram of tissue in the lungs of wild type (WT) (black), Flk2$^{-/-}$ (gray), IL7Rα$^{-/-}$ (white), or FIDKO (red) adult mouse lungs. WT n=8, Flk2$^{-/-}$n=7, IL7R$^{-/-}$ n=8, and FIDKO n=6 representing four independent experiments.**C,** Eosinophils were significantly reduced in Flk2$^{-/-}$, IL7Rα$^{-/-}$, and FIDKO mouse lungs. Quantification of eosinophils (Live,CD3-CD4-CD5-CD8-B220-Ter119-CD45+Ly6g-CD11b+SiglecF^{mid}CD11c-) per gram of tissue in the lungs of wild type (WT) (black), Flk2$^{-/-}$ (gray), IL7Rα$^{-/-}$ (white), or Flk2$^{-/-}$IL7Rα$^{-/-}$ (FIDKO) (red) adult mouse lungs. WT n=6, Flk2$^{-/-}$ n=5, IL7R$^{-/-}$ n=5, and FIDKO n=5 representing four independent experiments.

IL7R-Cre does not label adult neutrophils or eosinophils

Recently, we established that IL7R-Cre, but not Flk2-Cre, robustly labels trMacs across several tissues due to transient, developmental expression of IL7R$\boldsymbol{\alpha}$ in the monocyte-macrophage lineage (Gabriel A. Leung1† Taylor Cool & 1, 2019). To interrogate whether eosinophils have a history of IL7R expression, we crossed mice expressing IL7R-Cre16 to mTmG mice containing a dual color fluorescent reporter, thereby

creating the “IL7RαSwitch” model analogous to the previously described FlkSwitch mouse (Fig.3.2A-3.2B) (Boyer et al., 2011). In both models, all cells express Tomato (Tom) unless Cre-mediated recombination cause an irreversible switch to GFP expression by Cre-expressing cells and all of their progeny (Fig. 3.2A-3.2B). Labeling of lung B lymphocytes was high (>95%) in both models (Fig. 3.2C), as has previously been reported for circulatory lymphocytes(Beaudin et al., 2016; Boyer et al., 2011; Schlenner, 2010). Similar to myeloid cells in the peripheral blood (PB), labeling of lung neutrophils and eosinophils was high (>95%) in the FlkSwitch mice (Fig. 3.2D-2E). In contrast, in the IL7R$\boldsymbol{\alpha}$Switch mice labeling of lung neutrophils was nominal (<3%) (Fig. 3.2D), similar to previously reported PB neutrophils(Beaudin et al., 2016; Boyer et al., 2011). Despite the significant reduction in eosinophil numbers in IL7R$\boldsymbol{\alpha}$-/- mice (Fig. 3.1C), labeling of eosinophils was also nominal (<3%) in the IL7RαSwitch mice (Fig. 3.2E). These data show that most lung eosinophils do not develop through an IL7R$\boldsymbol{\alpha}$+ pathway yet depend on IL7R for

maintenance of normal cell numbers.

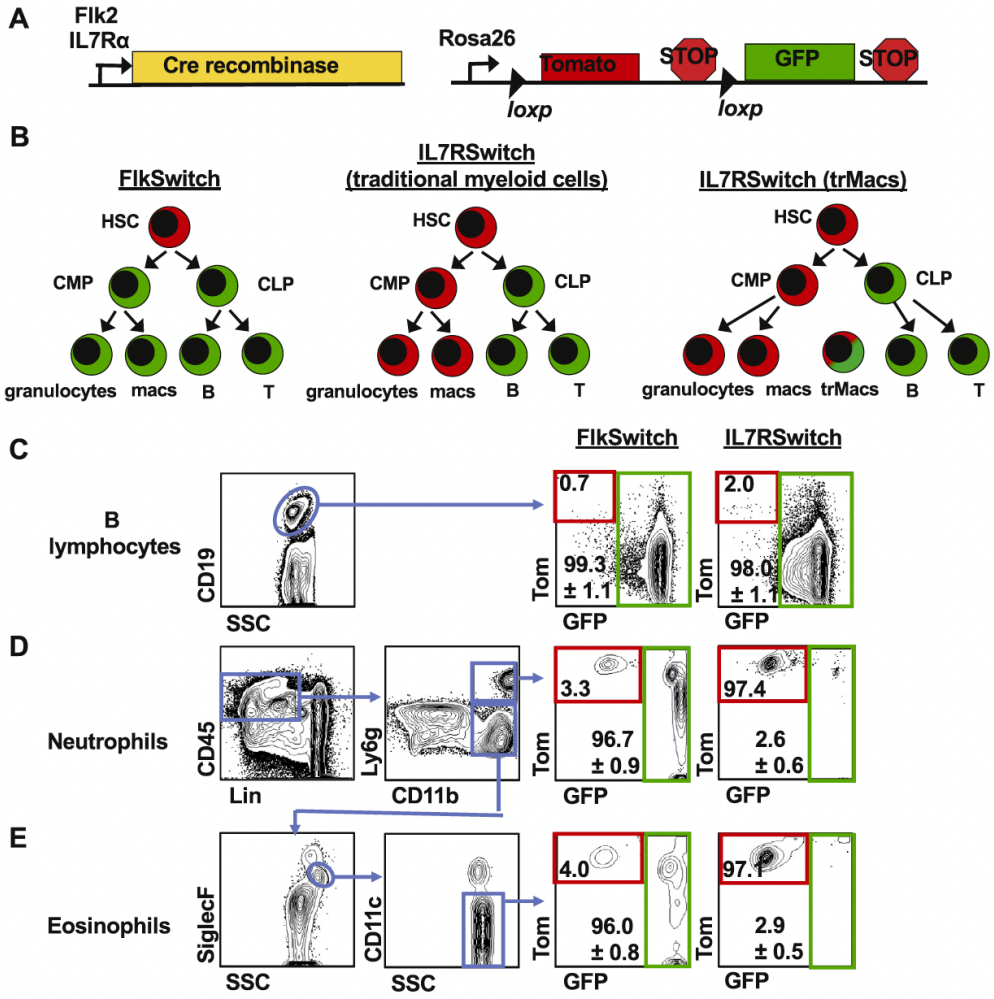

Figure 3.2 IL7R-Cre efficiently labels lung B cells but does not label adult lung neutrophils or eosinophil. **A,** Schematic of the 'Switch' models. Cre recombinase expression was controlled by either Flk2 or IL7Rα regulatory elements. Cre-driver mice were crossed to the mTmG mice expressing a dual-color reporter expressing either Tom or GFP, under control of the Rosa26 locus. Expression of Cre results in an irreversible genetic deletion event that causes a switch in reporter expression from Tom to GFP. **B,** Schematic of Cre-mediated reporter switching in the 'switch' models. All cells express Tom unless expression of Cre resulted in an irreversible switch from Tomato to GFP expression. Once a cell expresses GFP, it can only give rise to GFP-expressing progeny. **C,** Lung B cells were highly labeled by both Flk2-Cre and IL7R-Cre lineage tracing. Representative flow cytometric analysis of reporter expression in CD19+ lymphocytes in the lungs of adult FlkSwitch and IL7RSwitch mice. Tomato (Tom) and GFP expression is highlighted by red and green boxes, respectively, in FlkSwitch and IL7Rswitch models. **D,** Lung neutrophils were highly labeled by Flk2-Cre, but not IL7R-Cre, lineage tracing. Representative flow cytometric analysis of reporter expression in CD45+Lin-Ly6g^{hi} neutrophils in the lungs of adult FlkSwitch and IL7Rswitch mice. Tom/GFP gating strategies as in panel C. **E,** Lung eosinophils were highly labeled by Flk2-Cre, but not IL7R-Cre, lineage tracing. Representative flow cytometric analysis of reporter expression in CD45+Lin-Ly6g^{lo}SiglecF^{mid}CD11c^{-} eosinophils in the lungs of adult FlkSwitch and IL7Rswitch mice. Plots and values are representative of at least three or four mice per cohort from four independent experiments. Values indicate mean frequencies±S.E.M. of gated GFP+ populations.

IL7Rα-/- HSCs efficiently generate lung eosinophils upon transplantation

To determine whether IL7R-deficient progenitors lack the intrinsic potential to make eosinophils, we transplanted IL7Rα-/- or wild type (WT) HSCs into sublethally irradiated WT recipients and analyzed immune cell reconstitution in the blood and lung >4 months post-transplantation (Fig.3.3A). As expected, the IL7Rα-/- HSCs showed diminished ability to produce circulating lymphoid, but not "traditional" myeloid, cells in the blood of the same mice (Fig. S2.1A,B). In contrast, lung neutrophils and eosinophils were generated with equal efficiency by WT and IL7Rα-/- HSCs (Fig. 3B). The strong reconstitution potential of eosinophils and neutrophils of the lung, comparable to the GM donor chimerism in the periphery, further supports that these cells are "traditional", adult HSC-derived myeloid cell types and that they do not intrinsically require IL7R for their development.

Wild type HSCs fail to fully reconstitute eosinophils in IL7Rα-/- recipients

To determine whether the requirement for IL7R was eosinophil-extrinsic, WT HSCs were transplanted into either WT or IL7Rα-/- recipients (Fig. 3.3C). WT HSCs efficiently contributed to circulating GMs, Bs, and Ts (Fig. S2.1C,D), but, intriguingly, displayed significantly impaired reconstitution of neutrophils and eosinophils in the lungs of IL7Rα-/- recipients (Fig. 3.3D). Taken together, these data suggest that extrinsic IL7R is required for homeostasis of neutrophils and eosinophils in the lung, but not for other "traditional" myeloid cells which circulate in the periphery.

IL7R deletion caused alterations in cytokines involved in myeloid cell homeostasis. Previous literature has reported several factors implicated in eosinophil development, homing, and survival, including Eotaxin(Rose, 2010; Saito, 2016), IL-5 (Foster et al., 1996; Walker et al., 2020; Wiesner, 2017), and others(Cook et al., 2012; Kelly et al., 2009; Yi, 2018). To determine which factors may be influencing eosinophil development and survival in the IL7R mutant mice, we collected serum from WT and mutant mice and compared the relative concentration of several cytokines. Because IL-7 has previously been reported to be elevated in IL7Rα-/- mice(Kelly et al., 2009), we first tested IL-7 levels as our positive control. Consistent with previous data, we observed that IL-7 was upregulated several fold in IL7Rα-/- mice, although borderline statistically significant here (~10- fold, $p<0.1$; Fig. 3.3E). Importantly, we also observed that eosinophil-promoting Eotaxin was significantly downregulated in the IL7Rα-/- mice (Fig. 3.3E). Additionally, IL-5 was decreased 3-fold in the mutant mice, near statistical significance ($p<0.1$; Fig. 3.3E). Taken together, these data indicate that downregulation of Eotaxin and IL-5, and possibly additional factors, may play important roles in eosinophil development and survival in the IL7R mutant mice.

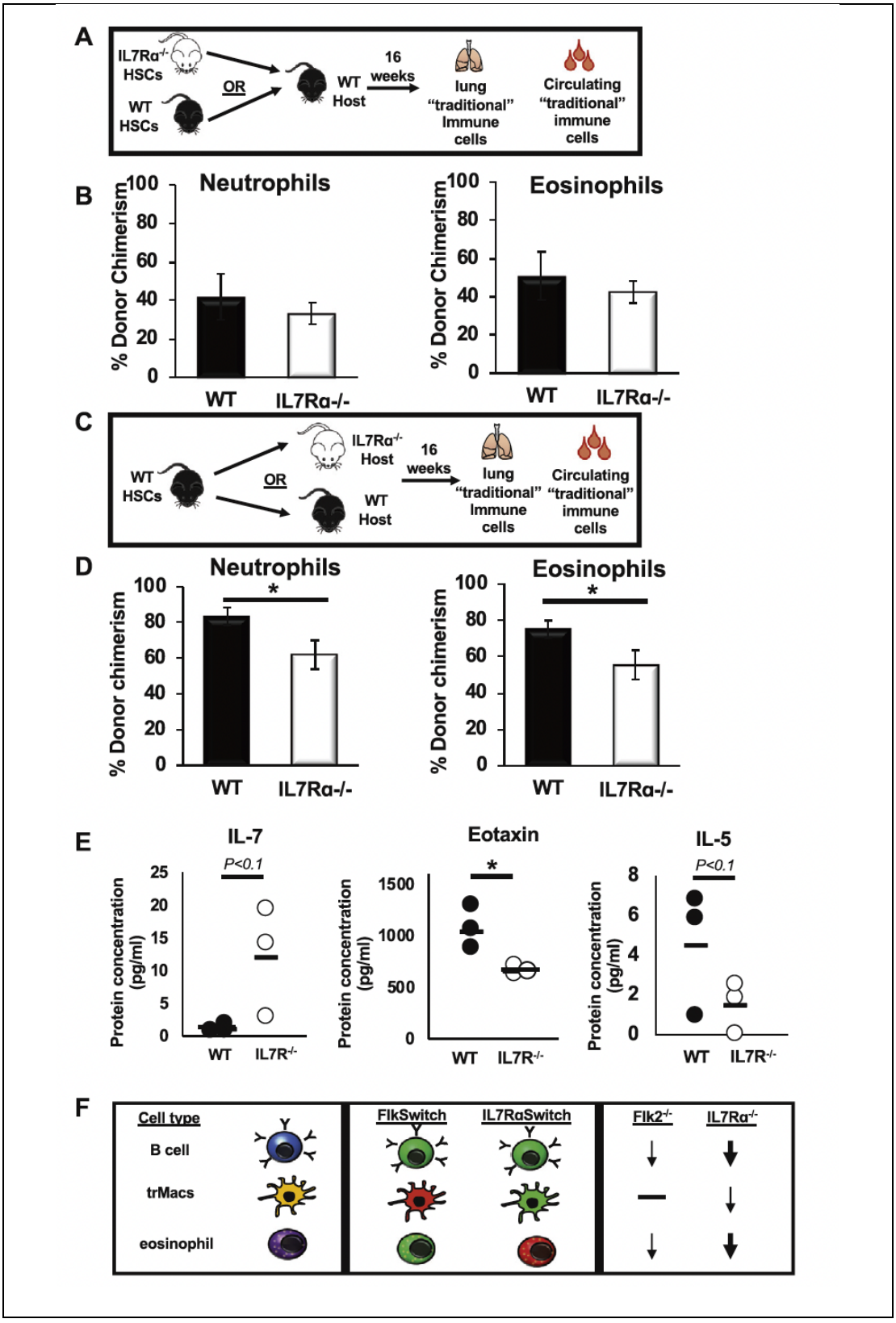
A
IL7Rα-/- HSCs
OR
WT HSCs
WT Host
16 weeks
lung "traditional" Immune cells
Circulating "traditional" immune cells
B
Neutrophils
% Donor Chimerism
100
80
60
40
20
0
WT
IL7Rα-/-
Eosinophils
% Donor Chimerism
WT
IL7Rα-/-
C
WT HSCs
OR
IL7Rα-/- Host
WT Host
16 weeks
lung "traditional" Immune cells
Circulating "traditional" immune cells
D
Neutrophils
*
% Donor chimerism
WT
IL7Rα-/-
Eosinophils
*
% Donor chimerism
WT
IL7Rα-/-
E
IL-7
P<0.1
Protein concentration (pg/ml)
25
20
15
10
5
0
WT
IL7R-/-
Eotaxin
*
Protein concentration (pg/ml)
1500
1000
500
0
WT
IL7R-/-
IL-5
P<0.1
Protein concentration (pg/ml)
8
6
4
2
0
WT
IL7R-/-
F
Cell type
B cell
trMacs
eosinophil
FlkSwitch
IL7RαSwitch
Flk2-/-
IL7Rα-/-

Figure 3.3 IL7Rα is extrinsically required for eosinophil homeostasis in the lung. A, Schematic depicting the transplantation experimental setup used to determine whether IL7R regulates eosinophil development by cell intrinsic mechanisms. 500 WT or IL7Rα$^{-/-}$ HSCs were transplanted into a ¾ sublethally irradiated wild type (WT) GFP recipient. After 16 weeks post transplant, the lungs were harvested and mature immune cells were analyzed via flow cytometry for donor chimerism. **B,** IL7R deletion did not alter the ability of HSCs to reconstitute lung eosinophils. Percent donor chimerism of neutrophils and eosinophils in the lungs of transplanted mice. Error bars are SEM, WT n = 4, IL7Rα$^{-/-}$ n=6 from 2 independent experiments. **C,** Schematic depicting the transplantation experimental setup used to determine whether cell extrinsic IL7R expression is necessary for eosinophil development and homeostasis. **D,** WT HSCs were less efficient at generating "traditional" myeloid cells in an IL7Rα$^{-/-}$ host. Percent donor chimerism of neutrophils and eosinophils in the lungs of transplanted mice. Error bars are SEM, WT n = 5, IL7Rα$^{-/-}$ n=13 from 4 independent experiments. *P<0.05, **P<0.005, ***P<0.001. **E,** Loss of IL7R results in altered cytokine profile in adult mice. Dot plots representing the protein concentration (in pg/ml) of WT (black dots) or IL7Rα$^{-/-}$ (white dots) in the serum of adult mice. Black bars represent the average. WT n=3, IL7Rα$^{-/-}$ n=3 from 3 independent mice; this was sufficient to reach a statistical power of 80% and * P<0.05. **F,** Flk2 and IL7R are differentially involved in the development of multiple hematopoietic cell types. Schematic depicting the differential expression and functional requirement for IL7R and Flk2 in traditional lymphocytes (B cells; top), tissue-resident macrophages (trMacs; middle), and adult lung myeloid cells (eosinophils; bottom). Lymphocytes are highly labeled by both Flk2-Cre (FlkSwitch) and IL7R-Cre (IL7RαSwitch), and functionally dependent on both receptors, although more drastic reductions in cell numbers were observed in IL7Rα$^{-/-}$ (thick arrow) than in Flk2$^{-/-}$mice (thin arrow). In contrast, trMacs are highly labeled by IL7R-Cre, but not Flk2-Cre, and functionally rely on IL7R (thin arrow), but not Flk2 (black line), for efficient development. Here, we report that lung eosinophils are highly labeled by Flk2-Cre, but not IL7R-Cre, yet depend on IL7R (thick arrow), but not Flk2 (black line), for efficient maintenance and reconstitution by HSCs.

Our data presented here reveal that IL7R is required for specific myeloid cell homeostasis in the lungs, PB, and BM of adult mice. Other groups have reported that human eosinophils have detectable levels of IL7R$\boldsymbol{\alpha}$ mRNA and surface protein (Cook et al., 2012; Kelly et al., 2009), and that IL7R mRNA can be induced in human monocytes with LPS stimulation(Al-Mossawi et al., 2019). Similarly, we previously found that trMacs in the lung and other tissues transiently express IL7Rα during development(Leung et al., 2019). However, our IL7R-Cre lineage tracing data of traditional lung myeloid cells reported here show that only a small proportion of eosinophils and neutrophils are labeled at steady state. This strongly argues against a requirement for expression by eosinophils or their precursors. Instead, we favor a model of cell-extrinsic requirement for IL7Rα in eosinophil development. This notion

is supported by the reciprocal transplantation assays, where we found that WT and IL7Rα-/- HSCs are equally capable of contributing to eosinophils and neutrophils in WT hosts (Fig. 3.3B). Conversely, when we transplanted WT HSCs into an IL7R null background, we observed that the donor chimerism of eosinophils and neutrophils in the lungs were significantly impaired (Fig. 3.3D), but that circulating "traditional" myeloid cells were efficiently generated (Fig. S2.1B). Interestingly, it has been reported that eosinophil homeostasis relies on lymphocyte- and stromal-secreted survival factors(Foster et al., 1996; Rose, 2010; Walker et al., 2020; Wiesner, 2017). We found that two known eosinophil regulators, Eotaxin and IL-5, were reduced in the IL7Rα-/- mice. Both of these cytokines are known to be secreted by lymphoid cells to promote eosinophil development, homing, and survival(Abdala-Valencia, 2018; Foster et al., 1996; Hartnell et al., 1993; Hogan et al., 2003; Rochman, 2008; Rose, 2010; Saito, 2016; Takatsu, 2011; Walker et al., 2020; Yi, 2018). Additionally, as previously reported7, we observed that IL-7 was upregulated in the IL7R-/- mice, likely due to unbound excess IL-7 in the absence of lymphoid cells. These data could potentially explain the mechanisms behind the eosinophil decrease in IL7Rα-/- mice (Fig. 3.1C, S2.1B,D, 3D), which have drastically reduced numbers of lymphocytes(Boyer et al., 2012) (Fig. 3.1A, S2.1B,D). The lack of lymphoid cells results in less secretion of Eotaxin and IL-5, resulting in poor eosinophil development and survival. Taken together, this suggests an extrinsic requirement for IL7R for eosinophil and neutrophil homeostasis. The data reported here represent a novel role of IL7R in "traditional" myeloid cell homeostasis, in a cell type-specific manner. We interpret our findings to

suggest a cell-intrinsic role for IL7R in lymphoid cell development and survival, which then, in turn, support traditional myeloid cells in the lungs of adult mice. The results presented here add to the body of knowledge of how complex and dynamic Flk2 and IL7R expression and function cooperate in the regulation of a variety of immune cells, including traditional myeloid cells in the adult lung (Fig. 3F). These findings are significant for lung health, because understanding hematopoietic homeostasis in the lung provides insight on susceptibility to respiratory disease.

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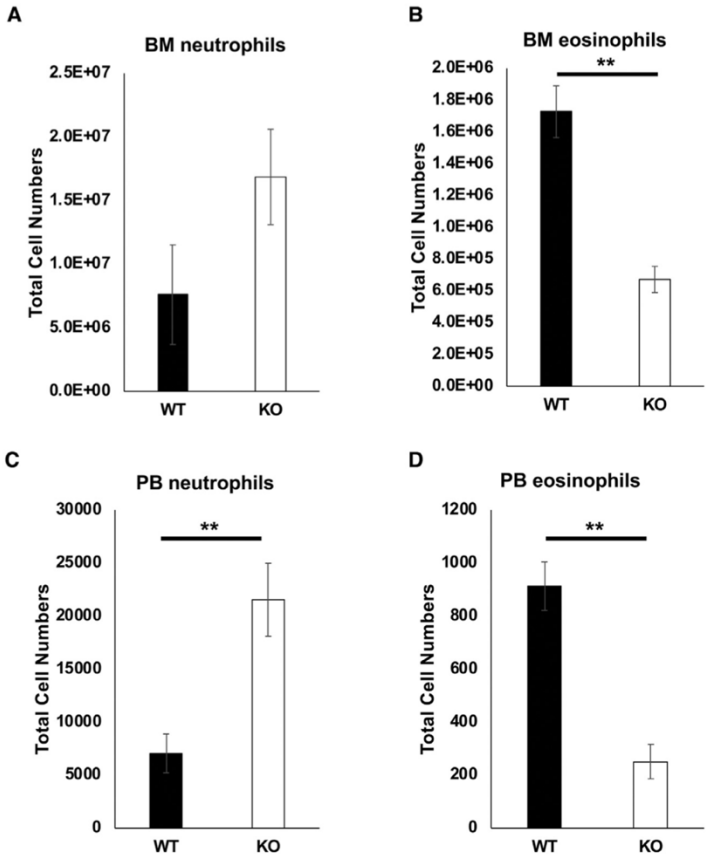

Figure S2.1 Neutrophil and eosinophil homeostasis are altered in the bone marrow (BM) and peripheral blood (PB) of IL7Ra/ mice. Quantification of total cells per 25ul of PB or 1 leg (for BM) of WT (black) or IL7Ra/ (white) adult mice. Error bars are SEM, WT n=3 and IL7Ra/ n=4 representing two independent experiments. *P<0.05, **P<0.005, ***P<0.001. A, Neutrophil numbers were not signifi- cantly different in the BM of Il7Ra/ mice. Quantification of neutrophils (Live, CD3-CD4-CD5-CD8-B220-Ter119-CD45+Ly6g+CD11b+) per 1 leg of wild type (WT) (black) or IL7Ra/- (white) adult mice. B, Eosinophil numbers were significantly reduced in the BM IL7Ra/ mice. Quantification of eosinophils (Live, CD3-CD4-CD5-CD8-B220-Ter119-CD45+Ly6g-CD11b+SiglecFmidCD11c-) per 1 leg of wild type (WT) (black) or IL7Ra/- (white) adult mice. C, Neutrophil numbers were significantly increased in the PB of IL7Ra/ mice. Quantification of neutrophils (Live, CD3-CD4-CD5-CD8-B220-Ter119-CD45+Ly6g+CD11b+) per 25ul of wild type (WT) (black) or IL7Ra/- (white) adult blood. D, Eosinophil numbers were significantly reduced in the PB of IL7Ra/ mice. Quantification of eosinophils (Live,CD3-CD4-CD5-CD8-B220- Ter119-CD45+Ly6g-CD11b+SiglecFmidCD11c-) per 25ul of wild type (WT) (black) or IL7Ra/- (white) adult blood.

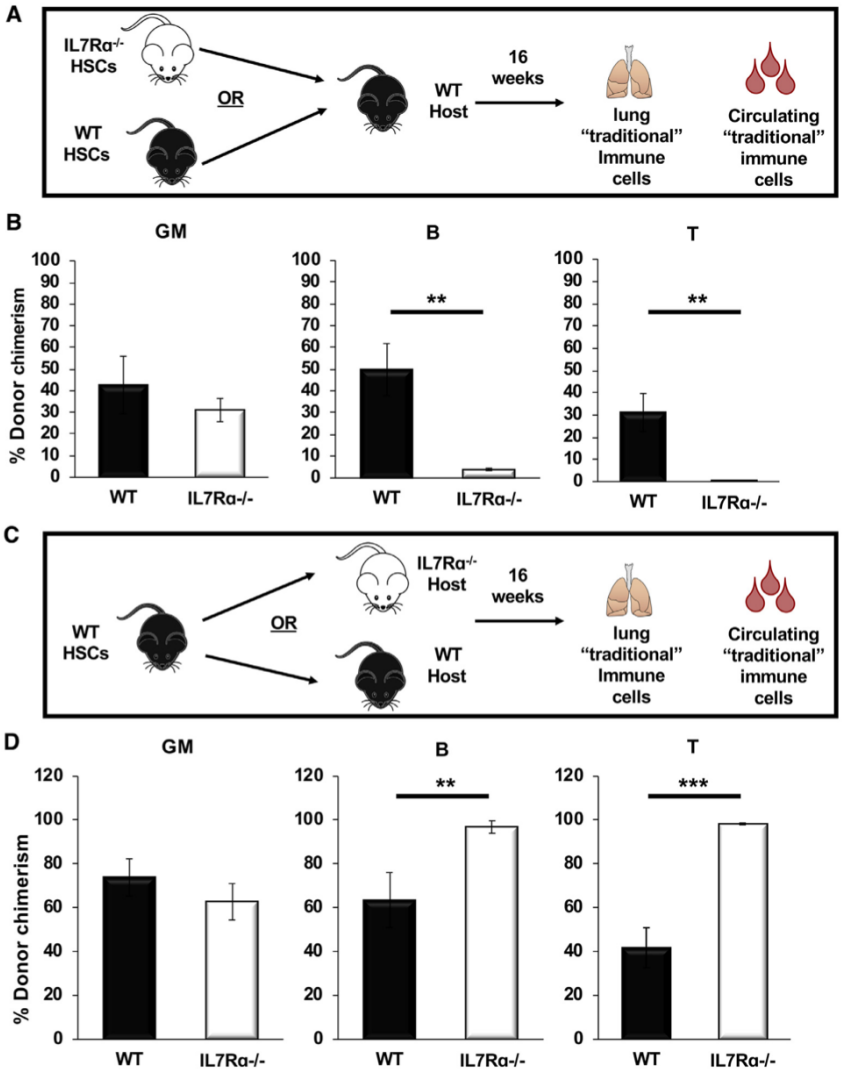

Figure S2.2 Donor chimerism of traditional mature cells in the peripheral blood of reciprocal transplants. A, Schematic depicting the transplantation experimental setup used to determine cell extrinsic mechanisms regulating "traditional" mature myeloid and lymphoid cell development. 500 WT or IL7Rα/ HSCs were transplanted into a 3/4 sublethally irradiated wild type (WT) GFP recipient. After 16 weeks post transplant, the peripheral blood was harvested and mature immune cells were analyzed via flow cytometry for donor chimerism. B, IL7R deletion did not alter the ability of HSCs to reconstitute "traditional" circulating myeloid cells, but did display reduced capacity to generate B and T lymphocytes. Percent donor chimerism of granulocyte/macrophage (GM) (Gr1+CD11b+), B lym- phocytes (B220+), and T lymphocytes (CD3+) in the peripheral blood of the same transplanted mice as in Figure 3B. Error bars are SEM, WT n = 5 IL7Rα/ n=6 from 2 independent experiments. ** = p<0.05, **P<0.005, ***P<0.001. C, A schematic depicting the transplantation experi- mental setup used to determine cell extrinsic mechanisms regulating "traditional" circulating myeloid and lymphoid cells. D, WT HSCs robustly reconstituted "traditional" circulating myeloid and lymphoid cells in an IL7Ra/ recipient. Percent donor chimerism of granulocyte/macrophage (GM) (Gr1+CD11b+), B lymphocytes (B220+), and T lymphocytes (CD3+) in the peripheral blood of the same transplanted mice as in Figure 3D. Error bars are SEM, WT n = 5, IL7Rα/ n=13 from 4 independent experiments. * = p<0.05, **P<0.005, ***P<0.001.

Chapter 3: effects of inflammation on fetal hematopoiesis and immune composition and function

I. Clearing the Haze: How Does Nicotine Affect Hematopoiesis before and after Birth?

[This section is adapted from a publication, Clearing the Haze: How Does Nicotine Affect Hematopoiesis before and after Birth?, Accepted at Cancers]

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Abstract

Hematopoiesis is a tightly regulated process orchestrated by cell-intrinsic and cell-extrinsic cues. Over the past several decades, much effort has been focused on understanding how these cues regulate hematopoietic stem cell (HSC) function. Many endogenous key regulators of hematopoiesis have been identified and extensively characterized. Less is known about the mechanisms of long-term effects of environmental toxic compounds on hematopoietic stem and progenitor cells (HSPCs) and their mature immune cell progeny. Research over the past several decades has demonstrated that tobacco products are extremely toxic and pose huge risks to human health by causing diseases like cancer, respiratory illnesses, strokes, and more. Recently, electronic cigarettes have been promoted as a safer alternative to traditional tobacco products and have become increasingly popular among younger generations. Nicotine, the highly toxic compound found in many traditional tobacco products, is also found in most electronic cigarettes, calling into question their purported “safety”. Although it is known that nicotine is toxic, the pathophysiology of disease in exposed people remains under investigation. One plausible contributor to altered disease susceptibility is altered hematopoiesis and associated immune dysfunction. In this review, we focus on research that has addressed how HSCs and mature blood cells respond to nicotine, as well as identify remaining questions.

Introduction

An environmental exposure that has become increasingly important in the research field is exposure to tobacco products. According to the National Institute of Health (NIH), one-fourth of the U.S. population uses tobacco products. An abundance of evidence has demonstrated that smoking is highly toxic to human health. Societal use of e-cigarettes has emerged recently and been marketed as a "safer alternative" to traditional tobacco products. Currently, 10 million adults and over 5 million middle and high school students use e-cigarettes in the US (Ramanathan, 2020). Over the last several decades, researchers have demonstrated that tobacco exposure is not only dangerous to the person using the products, but also to people who are exposed second- or even third-hand (Ferrante et al., 2013; Flouris et al., 2010). Tobacco products are highly complex and made up of several extremely toxic compounds, including hydrogen cyanide, formaldehyde, benzene, nicotine, and more (Mishra et al., 2015). One of the toxic compounds found in almost all present-day tobacco products is nicotine, a stimulant known for its addictive properties. The oral lethal dose (LD50) of nicotine in humans is ~0.8–1 mg/kg (Belkoniene, 2019; Mayer, 2013; Sommerfeld, 2016; Thornton, 2014). Nicotine is still highly prevalent in these so-called safer alternatives, presumably to promote addiction to the products. Nicotine has been associated with many deleterious health consequences, including cancer, pulmonary disease, and increased risk of infections (Mishra et al., 2015). How nicotine influences immunity in first, second, or third-hand exposed people remains an open area of investigation. Interestingly, nicotine has been shown to increase inflammation, as well

as white blood cell (WBC) counts (Chang et al., 2010; Fernández, 2012; Friedman, 1973; Roethig, 2010; Smith, 2003; Zalokar, 1981). WBCs are mature, terminally differentiated cells that come from hematopoietic stem cells (HSCs), in a process known as hematopoiesis. WBCs are immune cells with specialized functions, fighting infections and recognizing tumor and diseased cells. Thus, nicotine-induced systemic inflammation and increased WBC count increases the risk for and exacerbates a wide number of clinical conditions, including cancer, cardiovascular disorders, atherosclerosis, autoimmune syndromes, allergy, asthma, and pulmonary disease. The effects that nicotine has on the number and activity of each of these WBC types remains under investigation. This review focuses on the question: what lasting impacts does nicotine have on hematopoiesis and life-long immunity?

The Hematopoietic Hierarchy

The hematopoietic compartment consists of hematopoietic stem and progenitor cells (HSPCs) and their mature, terminally differentiated progeny (**Figure 4.1**a)(Rodriguez y Baena, 2021; Till, 1961). Hematopoietic stem cells (HSCs) are fascinating because they possess the ability to self-renew as well as differentiate into all mature blood and immune cells (**Figure 4.1**a). The hematopoietic process occurs in spatially and temporally distinct waves throughout development, and ultimately gives rise to the complex immune system that patrol all tissues. During fetal development, these distinct waves of HSPCs differentially contribute to mature blood and immune cells across tissues (Cool & Forsberg, 2019; Dzierzak, 2018; Klaus & Robin, 2017; Wittamer,

2020). This complex orchestra of immune layering consists of (1) early developmental, non-self-renewing progenitors that give rise to self-renewing progeny that persist throughout life with little to no contribution from adult HSCs, and (2) subsequently developed self-renewing progenitors (HSCs) that give rise to non-self-renewing progeny that are stably replenished throughout life (**Figure 4.1**b). At the interphase of these two well-established paradigms of hematopoietic development exist at least one population of developmentally restricted HSCs (drHSCs) that do not normally self-renew, but can be induced to do so upon transplantation into irradiated hosts (Beaudin et al., 2016). Based on their ability to give rise to robust numbers of traditional as well as atypical lymphoid cells, the physiological role of the drHSCs may be to boost production of lymphoid-mediated immunity that is needed after birth. It is also possible that drHSCs—or other normally transient progenitor cells—are induced to persist for longer time periods upon inflammatory stimulus like nicotine exposure, similar to their induced persistence in an irradiated environment (Beaudin et al., 2016; Beaudin & Forsberg, 2016a). This critical window of perinatal hematopoietic development, with distinct non-persisting stem and progenitor populations contributing alongside "true", life-long HSCs to a rapidly developing and dynamic immune system, poses as a vulnerable point of interrogation for long-term effects of altered hematopoiesis and immunity in nicotine-exposed individuals. Persisting alterations in cell function can occur in the absence of genetic mutations (de Laval et al., 2020); the likelihood of lasting physiological responses, however, is greater if cellular changes occur in cells with long half-lives or with self-renewal capacity than in cells with high turnover rates.

Potentially, nicotine alters HSCs (or other fetal progenitors) at the epigenetic level, leading to altered HSC function and/or lineage output for life. Alternatively, nicotine alters the numbers and/or epigenetics of mature blood cells, leading to permanently altered immune function (Figure 4.1b). Whether and how nicotine exposure alters HSCs or their progeny, or both, to cause long-term changes in disease susceptibility remains to be determined.

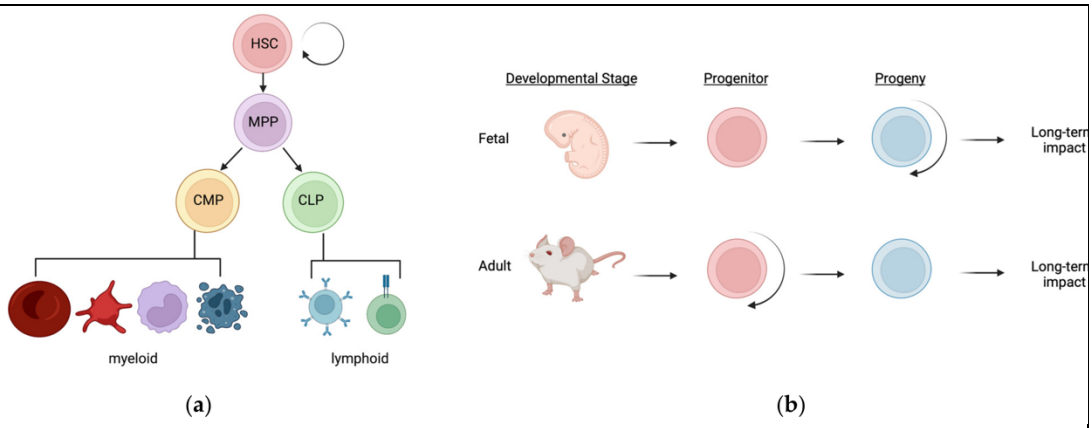

Figure 4.1 The hematopoietic hierarchy and sources of potential long-term impacts following nicotine exposure: (**a**) Hematopoiesis is the process of generating all mature blood and immune cells from hematopoietic stem cells (HSCs). This process occurs in a well-defined hierarchy during adult steady-state hematopoiesis, as depicted in this simplified tree structure. HSCs differentiate into multipotent progenitors, and then either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs), before terminally differentiating into mature blood and immune cells of either myeloid or lymphoid classification. HSCs are unique in their ability to both self-renew as well as differentiate into all of these progenitors and mature cells; (**b**) During fetal hematopoiesis, distinct waves of hematopoietic stem and progenitors (HSPCs) exist throughout development and adulthood. Many of the progenitors that exist during early fetal development are non-self-renewing but can give rise to self-renewing progeny such as "non-traditional" tissue-resident immune cells. Subsequently and during adult steady-state, hematopoiesis is sustained by self-renewing progenitors (HSCs) that give rise to non-self-renewing, short-lived progeny such as "traditional" circulating RBCs and WBCs. Nicotine exposure may influence life-long immunity by two potential mechanisms: (1) nicotine causes changes or persistence in HSPCs which results in altered hematopoietic output for life, or (2) nicotine causes a change in the long-lived immune cells during their establishment which alters immunity later in life. These mechanisms are not mutually exclusive and a combination of both could lead to altered hematopoiesis and altered immunity for life. Adapted from Cool and Forsberg, 2019.

Regulation of Hematopoietic Homeostasis

The development of this complex immune system relies on intrinsic and extrinsic cues (C. P. S. Rodrigues Maria; Akhtar, Asifa, 2020). Several key regulators of hematopoiesis have been identified and characterized in detail. Transcription factors such as Runx1, SCL, Gata-2, and C-myb play key roles in the intrinsic regulation of HSC potential during development (Cool & Forsberg, 2019; Gao et al., 2020; Wang et al., 2021). Additionally, inflammatory regulators like interferons, interleukin (IL)-1, IL-6, and TNFα provide HSC-extrinsic cues to regulate HSC fate choice and function (Baldridge et al., 2011; Collins et al., 2021; Pietras, 2017; Schuettpelz, 2013). Although we have relatively clear and convincing research describing how specific intrinsic and extrinsic regulators influence cell fate choice, many environmental factors that can also significantly impact HSC function and potential have not been thoroughly investigated. Importantly, the timing and duration of these exposures can impact hematopoietic development and function either transiently or for life. While acute exposures can have a transient effect on hematopoiesis, in this review, we focus on the potential mechanisms of altered hematopoiesis and HSC function after chronic challenge. As alluded to above, nicotine may alter long-term immunity via two potential mechanisms: (1) persistent changes in HSCs that then have lasting impacts on hematopoiesis (and immunity) for life, and/or (2) persistent changes in mature immune cells (Figure 4.1b). In this review, we focus on several studies that have investigated the effects of nicotine on HSCs and WBCs, as well as how nicotine alters inflammatory mediators. We

highlight potential mechanisms of altered hematopoiesis and immunity and identify experiments that could help untangle whether these effects are due to changes in HSCs or changes in their mature progeny.

Does Nicotine Alter Hematopoiesis by Direct Action on HSCs?

Tobacco product use is associated with increased WBC counts in peripheral blood which is a sign of increased systemic inflammation (Chmielewski, 2017; Fernández, 2012; Flouris et al., 2010; Friedman, 1973; Pedersen, 2019; Roethig, 2010; Smith, 2003; Zalokar, 1981). Specifically, it is known that nicotine in tobacco products can alter WBC counts in the long-term (Chang et al., 2010). However, the mechanism underlying these changes remains unclear. Additionally, whether tobacco product use alters counts of non-traditional immune cells in different tissues has not been investigated. Potentially, there are two plausible mechanisms of altered WBC counts: (1) nicotine directly affects hematopoietic cells by direct binding to nicotinic receptors expressed by HSCs and/or their progeny (**Figure 4.2**a), or (2) nicotine indirectly affects the hematopoietic compartment by triggering release of inflammatory-mediating cytokines from non-hematopoietic cells that then act on HSCs and/or their progeny (**Figure 4.2**b). It is important to note that these potential mechanisms are not mutually exclusive, and that the effects of nicotine could be a combination of both mechanisms (**Figure 4.2**a,b). In this section, we discuss the evidence for nicotine acting as a nicotinic cholinergic receptor agonist and directly affecting hematopoiesis via binding to nicotinic acetylcholine receptor (nAChRs) on hematopoietic cell types.

Do HSCs Express Nicotinic Acetylcholine Receptors (nAChRs)?

One potential mechanism of altered WBCs after nicotine exposure is that nicotine directly affects HSCs via nAChRs expressed on their cell membrane. nAChRs are a family of ligand-gated ion channels. There are 16 homologous subunits identified in mammals, and these subunits combine to form many different nAChR subtypes. Interestingly, these subtypes have various expression patterns across tissues, diverse functional properties and pharmacological characteristics (Dani, 2015; J. L. Wu Ronald J., 2011). A few groups have provided evidence that HSCs, and some of their mature immune cell progeny, express several subunits of the nAChRs at steady state, and that the expression of other subunits can be induced after exposure to nicotine (Chang et al., 2010; St-Pierre, 2016). Chang et al. reported an increase in both HSC and WBC numbers in nicotine-exposed mice, and expression of the nAChR alpha 7 subunit (nAChRα7) in total bone marrow, as well as on isolated HSCs (Chang et al., 2010). They used a combination of flow cytometry and immunofluorescence imaging to assess nAChRα7 expression on whole bone marrow (WBM) cells which contain HSPCs and mature cells, as well as on purified HSCs. In this experiment they used the nAChRα7 ligand Alpha Bungarotoxin (abgt) conjugated to a FITC fluorophore to determine whether WBM cells or HSCs expressed the receptor on the cell membrane. Abgt is known to bind with high affinity to the nAChRα7 subunit. Using this method, they determined that some cells within the WBM fraction and purified HSCs both displayed the receptor for abgt ligand. Although they demonstrated that WBM and HSCs had

positive staining for the abgt ligand (presumably binding to the nAChRα7 subunit), it should be noted that this assay did not directly determine the levels of nAChRα7 in these cells nor did they include a strong positive control (such as brain homogenate) (St-Pierre, 2016) or negative control (tissue known to not express nAChRα7) to rigorously decipher the relative expression of nAChRα7. An interesting experiment that would have strengthened their findings would be to perform the same experiment with HSCs from wild type (WT) mice and mice lacking nAChRα7. If they had observed that WT HSCs had positive staining for the FITC-abgt, but the mutant HSCs did not, this would more unequivocally have supported their conclusion that HSCs express nAChRα7.

Do other Hematopoietic Cells Express nAChRs?

In a separate study, St-Pierre et al. took a different approach to address this same question: Do hematopoietic cells express nAChRs (St-Pierre, 2016)? They used freshly isolated murine WBM cells and brain tissue (as a positive control) to perform RT-PCR for the several different subunits of nAChRs. Using this method, they determined that nAChRα9 and nAChRβ2 mRNAs were expressed by nearly all bone marrow cells, while nAChRα7 was expressed only in CD34+ progenitors, monocytes, and B cells. They concluded that long-term HSCs do not actually express the α7 or α9 nAChR subunits, but progenitors and some mature blood cells do. However, one important thing to note about these findings is that they were unable to detect these subunits using qRT-PCR as the detection levels were below threshold at 35 cycles. For this reason,

they performed nested RT-PCR instead and observed that nAChR mRNA expression was highly variable across hematopoietic populations. Since mRNA expression does not always result in protein expression, they also investigated whether nicotine could modulate bone marrow-derived myeloid cell numbers via nAChRα7 and nAChRα9 by performing in vitro and in vivo experiments using WT, nAChRα7 knockout (KO), and nAChRα9 KO mice. Interestingly, their in vitro and in vivo experiments provided contradictory results. In vitro, nicotine reduced total numbers of bone marrow-derived monocytes (BMDMs) in WT mice, but not in the two mutant mice. However, in their in vivo model, nicotine had a protective effect on BMDMs. It is important to note that these in vitro and in vivo experiments lacked a nicotine-only control group. A more convincing and straightforward experiment would have been to do a systematic side-by-side comparison of the effects on nicotine on BMDMs in vitro and in vivo in all 3 models (WT, nAChRα7 KO, and nAChRα9 KO) with nicotine only versus control.

Overall, the evidence for robust and functional cell surface expression of nAChR subunits on HSCs and other hematopoietic subsets is not unequivocally convincing. A more direct and definitive approach to determine this would be to purify various hematopoietic cell populations from the murine bone marrow, and test expression by flow cytometry or immunohistochemistry using antibodies specific to each nAChR subunit, with the corresponding cells from gene deletion models serving as controls. If HSCs and mature immune cells do in fact express nAChR subunits on the membrane, it could be assumed that they would *directly* respond to nicotine, at least in part, via

binding of the nAChRs expressed. Additionally, in vitro exposure of HSCs and/or their mature immune cell progeny to nicotine-containing media may provide more concrete evidence as to whether nicotine can directly affect these cells, and specifically which ones. At present, a more thorough investigation of the direct effects of nicotine on hematopoietic cell types is needed.

Does Nicotine Affect Hematopoiesis via an Altered Inflammatory State?

As an alternative to nicotine affecting hematopoiesis through binding nAChRs on hematopoietic cells and/or their progeny, nicotine may potentially affect hematopoiesis indirectly via inflammatory cues (**Figure 4.2**). Nicotine is known to induce the release of several inflammatory-mediating cytokines including TNFα, IL-6, IL-1, and others which are also known to play important roles in hematopoietic cell development and homeostasis (Collins et al., 2021; Hosseinzadeh, 2016; Mohsenzadeh et al., 2014; Osgoei, 2018; Strzelak, 2018; Von Chamier et al., 2017). While hematopoietic cells are known to be the source for some of these inflammatory mediators, other cell types may also contribute to altered inflammation, including epithelial cells (Strzelak, 2018). In this scenario, one might hypothesize that nicotine acts on non-hematopoietic cells that do express nAChRs, and once the nAChR signaling cascade is initiated in these cell types, the cells undergo molecular changes to respond to the stimulus and can send informative cues (cytokines) to neighboring cell types or into circulation to reach distant tissues and elicit cellular responses (Hajiasgharzadeh et al., 2019). There are several cell types that are known to respond directly to nicotine, including muscle cells

and neurons. The vagus nerve is a complex network of neurons that connects the brain with the rest of the organs in the body. It has been recently demonstrated that the vagus nerve, in addition to controlling heart rate, stress, and hormone secretion (Breit et al., 2018), also acts as an immunomodulator (Johnston & Webster, 2009). Coincidentally, acetylcholine is the main neurotransmitter of the vagus nerve, and controls immune cell function via the nAChRα7 subunit. Nicotine, which has a similar structure to acetylcholine, potentially binds to nAChRs to activate the release of inflammatory-mediating cytokines as a form of communication with surrounding organs (**Figure 4.2**). Hematopoietic cells (both HSCs and mature immune cells) are known to express receptors for many cytokines (Pietras, 2017) and, although they may not be able to directly respond to nicotine, they can therefore undoubtedly respond to many inflammatory cues.

Nicotine leading to an altered inflammatory state has been supported by several studies (H. L. Chen Gerard; Chan, Yik Lung; Chapman, David G. ;. Sukjamnong, Suporn; Nguyen, Tara; Annissa, Tiara F. ;. McGrath, Kristine C. Y. ;. Sharma, Pawan K. ;. Oliver, Brian G., 2018; Flouris et al., 2012; Gracia, 2005; Hajiasgharzadeh et al., 2019; St-Pierre, 2016; Wong, 2015). In rodents, it has been demonstrated that nicotine exposure increases the release of pro-inflammatory cytokines. Since ~10% of pregnant women continue smoking during gestation (Azagba, 2020), many studies have focused on understanding the effects of in utero nicotine exposure. Similar to data from adults, nicotine is also able to induce inflammation in a developing rodent fetus. Mohsenzadeh

et al. exposed pregnant rats to nicotine and measured the serum of their pups after birth to determine concentrations of several inflammatory-mediating cytokines (Mohsenzadeh et al., 2014). They found that hs-CRP, IL-6, and TNFα were all elevated in the nicotine-exposed pups compared to their control counterparts. They concluded that in utero nicotine exposure induces a dose-dependent increase in inflammatory-mediating cytokines. Similarly, Orellana et al. observed significantly elevated serum levels of IL-1β and TNFα in the offspring of nicotine-exposed mice (Orellana, 2014). Additionally, day 8.5 mouse embryos exposed to nicotine developed abnormalities and showed increased inflammation by elevated expression of TNFα, IL-1β, and caspase 3 (Lin, 2014). Another study aimed to determine the effects of in utero nicotine exposure found an increased risk for intrauterine infection and altered inflammatory profile of fetal tissues in rodents (Von Chamier et al., 2017). In this study, they measured cytokines from placental tissue and amniotic fluid from nicotine-exposed pregnant dams. Interestingly, they observed that nicotine exposure did not significantly increase TNFα in either tissue. IFNγ was significantly elevated in placental tissue, but significantly decreased in the amniotic fluid of nicotine-exposed fetuses. IL-6, which was unchanged in the nicotine-exposed placental tissues, was significantly increased in amniotic fluid. They concluded that these altered inflammatory mediators were the cause of increased infection susceptibility of the fetus of nicotine-exposed dams. It should be noted that the different outcomes of these studies could be attributed to several factors, including the mode and amount of exposure to nicotine, analysis of different tissues (fetal blood versus placenta/amniotic fluid), and time of tissue

collection (newborn rats versus gestational day 18). These studies also only probed a few cytokines and did not investigate the underlying mechanism of altered inflammation.

Overall, these studies indicate that the exposure of pregnant females to nicotine can lead to an altered inflammatory environment during gestation, which may have important consequences on fetal hematopoiesis (Apostol, 2020; Y. S. Hayashi Maiko; Takizawa, Hitoshi, 2019; Pietras, 2017). Moreover, it is yet unknown whether this is caused directly by nicotine in the fetal environment or by passage of maternal nicotine-induced inflammatory cytokines to the fetus during development. To understand the mechanism of altered inflammation in nicotine-exposed pups, it would be necessary to identify the cells to which nicotine is binding and initiating signaling, and then determining if those cells are the sole source of cytokines or if they work in concert with other cells to elicit an immune response. Although we know that nicotine can cross the placenta and accumulates in the fetal blood and in the breast milk (Bruin et al., 2010; Dahlström, 1990; Kang, 2017; Kierdorf et al., 2013; Qu, 2018), it remains unclear whether these significant fetal and neonatal exposures lead to direct changes in the fetal hematopoietic compartment. It is also unknown whether nicotine leads to transient or persisting alterations in developmental hematopoiesis.

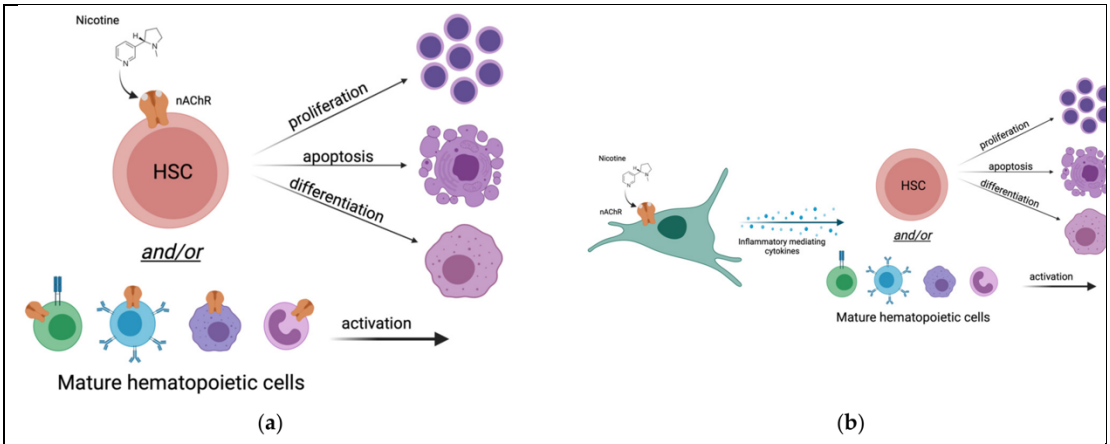

Figure 4.2 Potential mechanisms of altered hematopoiesis and immunity for life following nicotine exposure: (**a**) Nicotine directly influences the hematopoietic compartment. Nicotine binds to nicotinic-acetyl choline receptors (nAChRs) expressed by hematopoietic stem cells or other mature hematopoietic cell types. These cells then undergo molecular changes which lead to proliferation, apoptosis, differentiation, and/or activation; (**b**) Nicotine indirectly influences the hematopoietic compartment. Nicotine binds to nicotinic-acetyl choline receptors (nAChRs) expressed by non-hematopoietic cells. These cells undergo molecular changes that then lead to release of inflammatory-mediating cytokines. Cytokines released by the non-hematopoietic cells bind to receptors on hematopoietic cell types. Hematopoietic cells then undergo molecular changes in response to the cytokines (directly) and nicotine (indirectly) which leads to proliferation, apoptosis, differentiation, and/or activation.

Conclusions

Persisting alterations to hematopoiesis may serve as a mechanism of disease susceptibility later in life. Although it is widely agreed that nicotine is extremely toxic, the mechanism of altered immunity of nicotine-exposed individuals remains a topic of intense investigation. The emergence of e-cigarettes as alternatives to traditional tobacco products has prompted a new wave of health concerns and a need for scientific research into the effects of their toxic components on human health. Nicotine is highly addictive and found in almost all present-day tobacco products, new and old, and therefore serves as a logical starting point of investigation of the toxic effects of tobacco product use on human health. Decades of research have demonstrated that children of smoking mothers have diminished health (Bruin et al., 2010; Hofhuis et al., 2003; Wen,

2012), yet the mechanism of altered disease susceptibility remains unclear. As the hematopoietic system is the focal point of immunity and health, the effects of nicotine on hematopoietic cell types warrant further investigation. Nicotine potentially *directly* affects hematopoietic cells, including HSCs, via binding their nAChRs. Direct action on HSCs is consistent with the increase in HSC numbers and detection of nAChRα7 expression on the surface of HSCs (Chang et al., 2010). Alternatively, nicotine may *indirectly*affect hematopoietic cell types, by binding nAChRs on other cell types (likely epithelial, neuronal, muscular cells) that then secrete cytokines to induce inflammation. A third possibility is that nicotine affects hematopoiesis both directly and indirectly, leading to feedback loops that perpetuate inflammation. In order to advance our understanding of the effects of nicotine on hematopoietic cell types and immunity, thorough investigation into these possible mechanisms is needed. Experiments with genetic deletion or gain-of-function models, possibly facilitated by the many rapidly emerging CRISPR technologies (González-Romero, 2019; Nidhi, 2021), should enable unequivocal new results. Exciting approaches to move the field forward are increasingly feasible. For example, the effect of nicotine on mature immune cell subsets could be assessed at high resolution by single-cell RNA sequencing, possibly revealing alterations of activation genes in T cells. Analogously, ATAC-seq, of bulk or single cells (J. D. ; G. Buenrostro Paul G. ;. Zaba, Lisa C. ;. Chang, Howard Y. ;. Greenleaf, William J., 2013; J. D. ; W. Buenrostro Beijing; Chang, Howard Y. ;. Greenleaf, William J., 2015), could be implemented to test the hypothesis that fetal and/or adult HSCs have altered functional output in

response to nicotine due to lasting epigenetic changes. The discovery of drHSCs (Beaudin et al., 2016) and improved characterization of non-traditional immune cells (Cool et al., n.d.; Gabriel A. Leung1† Taylor Cool & 1, 2019; Merad et al., 2002b; Worthington et al., 2022) opens new and intriguing avenues of exploration. Together with a systematic investigation of the direct and indirect effects of nicotine on hematopoiesis, these strategies will provide insights needed to understand and mitigate damage to its exposure.

Author Contributions

Writing—original draft preparation, T.C.; writing—review and editing, T.C., A.R.y.B. and E.C.F.; visualization, T.C. and E.C.F.; funding acquisition, T.C., A.R.y.B. and E.C.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

II. In Utero Nicotine Exposure Leads to Persistent Changes in Hematopoietic Function and Maintenance

[This section is adapted from a manuscript in preparation for publication, In Utero Nicotine Exposure Leads to Persistent Changes in Hematopoietic Function and Maintenance. In preparation.]

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Abstract

Tobacco use during pregnancy has many deleterious health consequences on not only the smoking mother, but on the unborn fetus. Children of smoking mothers are known to have increased susceptibility to respiratory diseases later in life. The mechanisms driving this increased susceptibility are not clearly understood. On potential source of altered disease susceptibility is the immune system, which is derived from Hematopoietic Stem and Progenitor Cells (HSPCs). Here, we report that nicotine, one of the main toxic compounds found in many traditional and new tobacco products, has a deleterious effect on hematopoietic output and function. In utero nicotine exposure results in a transient decrease in numbers of HSCs, but persistent altered hematopoietic output. In utero nicotine exposure did not alter the functionality of HSCs by the gold standard transplantation assay. However, in utero nicotine exposure did result in altered hematopoietic function in a secondary insult model. These finding suggest a persistent change within HSCs that drive altered hematopoietic output.

Introduction

An emerging market of products that have become a focal point of research efforts is electronic cigarettes (e-cigs). These vape pens have been marketed as a safer alternative to traditional tobacco products, and often contain "exciting" flavor additives making them appealing to younger consumers. Although they are marketed as a safer alternative to traditional cigarettes, they still contain many toxic compounds with known deleterious health consequences (Cool et al., 2022). One of the many toxic compounds found in these products is nicotine. Nicotine binds to nicotinic acetyl choline receptors (nAChRs) and triggers cellular responses such as proliferation, apoptosis, and differentiation (Cool et al., 2022). Additionally, nicotine is known to cause inflammation (Gracia, 2005; Mohsenzadeh et al., 2014). The cellular and molecular mechanisms of altered health in nicotine-exposed children remains under investigation. Children of smoking mothers are known to have increased susceptibility to many diseases, especially diseases of the airways and lungs, including asthma, COPD, and increased respiratory infections later in life(Pattenden et al., 2006). However, what causes this increased disease susceptibility is poorly understood. From one perspective, in utero exposure to toxic compounds, such as nicotine, potentially alters health via affecting immune cell establishment and function.

Developmental immune layering is a complex orchestration of waves of unique Hematopoietic Stem and Progenitor Cells (HSPCs) give rise to developmentally distinct subsets of immune cells (Cool & Forsberg, 2019). This process is comprised

of non-self-renewing progenitors that give rise to long-lived self-renewing mature cells, such as tissue resident cells. The extent to which these fetal-derived immune cells persist and contribute to adult immunity, and whether these cells or their progenitor source may alter disease susceptibility for life, remains unclear. Additionally, there are self-renewing progenitors (such as traditional HSCs) that give rise to non-self-renewing progeny. Several perinatally-established subsets of white blood cells (WBCs), including trMacs and innate-like lymphoid cells, have been implicated as modulators of inflammation across tissues and thus pose as interesting points of interrogation for the link between early life exposures and elevated inflammation. In utero exposure to pathogens and toxicants coincides with developmental waves that generate long-lasting immune cells, which is one potential mechanism of altered life-long immunity in the children of smoking mothers.

Here, our examination of how in utero nicotine exposure alters hematopoietic establishment and function revealed that perinatal nicotine exposure (PNE) results in altered hematopoietic establishment and function for life. PNE transiently altered HSC numbers but lead to persistent differences in fetally-derived non-traditional immune cells in the lungs of mice. Additionally, although PNE did not alter HSC function by the gold standard transplantation assay, it led to persistent altered hematopoietic function in a secondary insult model. Together, these experiments demonstrate that in utero nicotine exposure has deleterious consequences on hematopoietic establishment and function for life.

Results

Perinatal nicotine exposure transiently alters HSC numbers

To determine how PNE affects hematopoiesis, we exposed pregnant dams to nicotine water at a level of exposure comparable to a moderate smoker and examined the offspring of these mice at different developmental stages representative of early postnatal life, childhood, or adulthood. We compared Hematopoietic Stem and Progenitor Cell (HSPC) numbers between nicotine-exposed animals and control animals from the main hematopoietic organ for the respective developmental timepoint (liver and bone marrow for P0 and bone marrow for P14 and adult mice). Interestingly, PNE resulted in a transient increase in bone marrow HSC numbers, but a decrease in liver HSC numbers at P0 (Figure 5.1A). By P14, there was a significant decrease in bone marrow HSC numbers (Figure 5.1B). This significant decrease was not detected in the adult mice, however there were persistently significantly fewer progenitor cell numbers in the adults, suggesting persistent altered hematopoietic output (Figure 5.1 C).

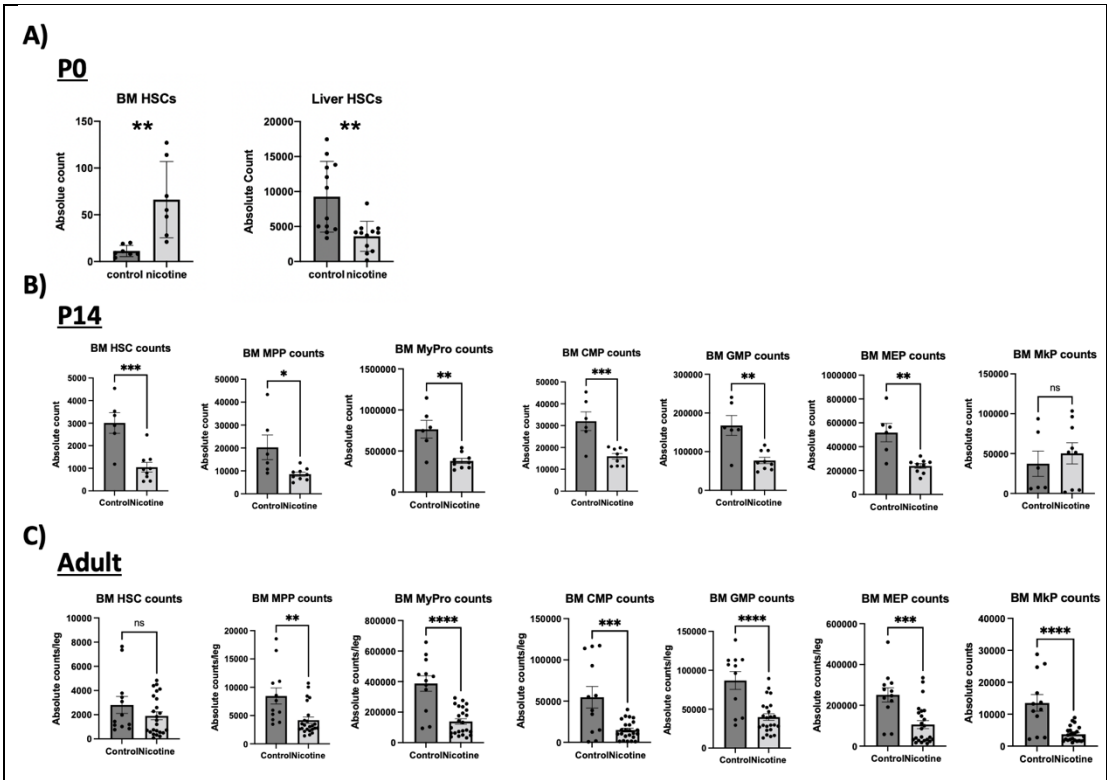

Figure 5.1 Perinatal nicotine exposure transiently alters HSC number. Quantification of total numbers of HSCs per whole liver or 2 legs (for P0), 1 leg (for P14 or adult) of control (dark bars)l or nicotine-exposed (light gray bars) mice. Error bars are SEM. A) Total numbers of HSCs (Live, CD3-CD4-CD5-CD8-B220-Gr1-Ter119-cKit+Sca1+Flk2-SLAM+) from P0 control or nicotine-exposed pups. Control n=7 (BM) and nicotine n=7 (BM) representing 2 independent experiments. B) Total numbers of HSCs (Live, CD3-CD4-CD5-CD8-B220-Gr1-Mac1-Ter119-cKit+Sca1+Flk2-SLAM+) from bone marrow of P14 control or nicotine-exposed pups. Control n=6 and nicotine n=9 representing 3 independent experiments. B) Total numbers of HSCs (Live, CD3-CD4-CD5-CD8-B220-Gr1-Mac1-Ter119-cKit+Sca1+Flk2-SLAM+) from bone marrow in adult control or nicotine-exposed mice. Control n=12 and nicotine n=24 representing 3 independent experiments. *P<0.05, **P<0.005, ***P<0.001.

Perinatal nicotine exposure does not affect traditional mature cell numbers

To determine if the altered HSPC numbers observed in the main hematopoietic compartments also result in decreased mature cell production, we next examined the mature cell numbers in the peripheral blood of these animals. Interestingly, although PNE lead to altered HSPC numbers, there was no significant differences in traditional

mature cells being produced in the periphery of these animals, suggesting HSC exhaustion (Figure 5.2).

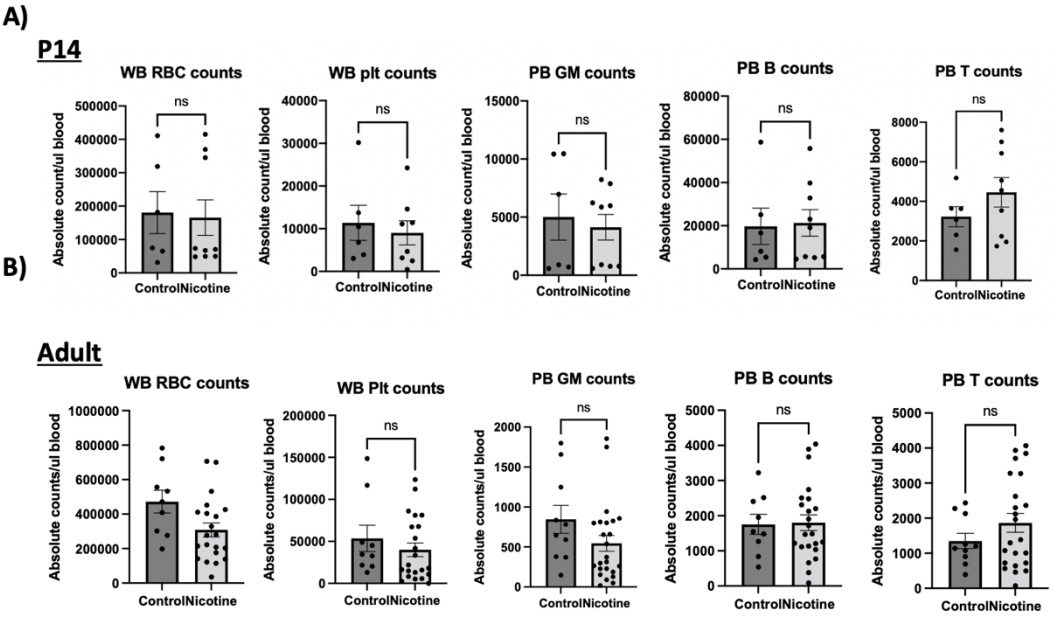

Figure 5.2 Perinatal nicotine exposure does not affect traditional mature cell production Quantification of total numbers of "traditional" mature cells in the peripheral blood (per 25ul) of control (dark bars) or nicotine-exposed (light gray bars) mice. Error bars are SEM. A) Total numbers of Red Blood Cells (RBC)((Live, Ter119+), Platelets (Plt)(Live, Ter119-CD61+), GMs(Live, Ter119-Mac1+GR1+), B (Live, Ter119-, B220+), and T cells (Live, Ter119-, CD3+) from P14 pups. Control n=6 and nicotine n=9 representing 3 independent experiments. B) Total numbers of Red Blood Cells (RBC)((Live, Ter119+), Platelets (Plt)(Live, Ter119-CD61+), GMs(Live, Ter119-Mac1+GR1+), B (Live, Ter119-, B220+), and T cells (Live, Ter119-, CD3+) from adult mice (8-12weeks). Control n=9 and nicotine n=23 representing 3 independent experiments. *P<0.05, **P<0.005, ***P<0.001.

PNE leads to persistent decrease in non-traditional immune cell numbers in the lungs of exposed mice

To determine if PNE resulted in persistent changes in establishment of non-traditional, or fetally-derived, immune cell populations, we next compared immune cell numbers in the lungs of exposed mice. By examining the lungs, we are also able to compare

traditional and non-traditional immune cell composition. Surprisingly, although we observed a transient trend towards a burst in the generation of T cells in P14 animals (Figure 5.3 A), there was a significant decrease in total number of CD3+ T cells by adulthood (Figure 5.3B). Furthermore, there was a significant decrease in the number of gamma delta T cells (TCRGD+), a subset of T cells that are considered fetally-derived (Figure 5.3B).

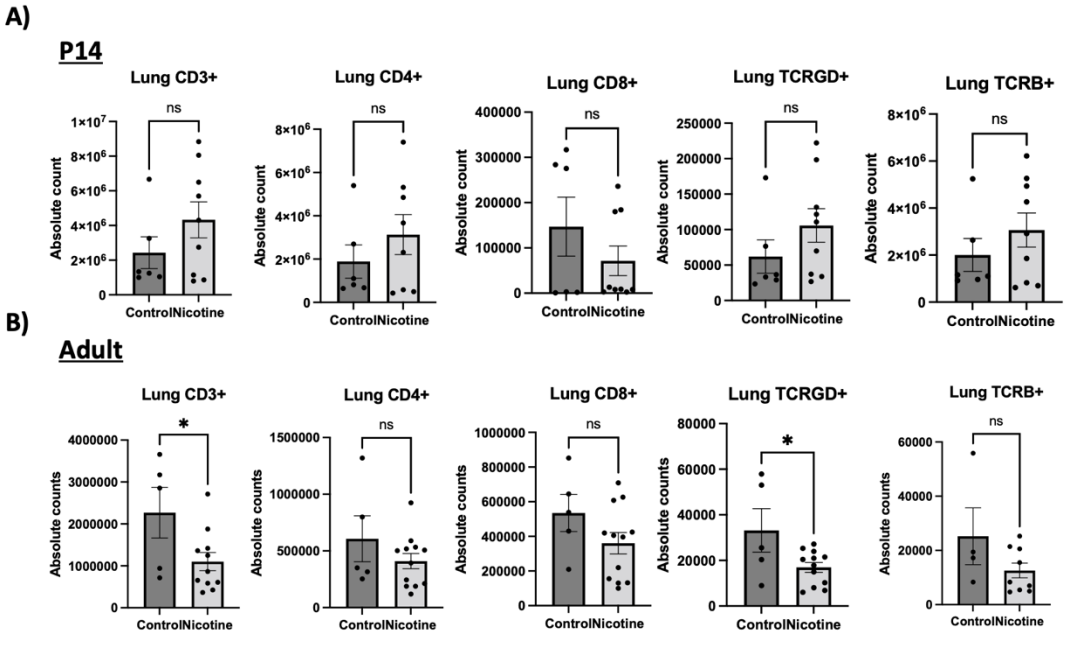

Figure 5.3 Perinatal nicotine exposure results in decreased non-traditional immune cells in the lungs. Quantification of total numbers of "traditional" and "non-traditional" mature immune cells in the lungs of control (dark bars) or nicotine-exposed (light gray bars) mice. Error bars are SEM. A) Total numbers of different T cell populations at P14. Control n=6 and nicotine n=9 representing 3 independent experiments. B) Total numbers of different T cell populations in adult mice (8-12weeks). Control n=5 and nicotine n=11 representing 2 independent experiments. *P<0.05, **P<0.005, ***P<0.001.

PNE does not alter HSC function

We next wanted to determine if PNE altered the functionality of HSCs. To test this, we isolated HSCs from the liver or bone marrow of P0 animals and transplanted them into sublethally-irradiated wild-type recipients (Figure 5.4). Surprisingly, PNE had no effect on the ability of HSCs to reconstitute the HSPC compartment in the recipients (Figure 5.4A). Additionally, nicotine-exposed HSCs retained multilineage potential and reconstituted all mature cells without any deficit (Figure 5.4B). Taken together, these data indicate that nicotine exposure does not affect HSC function or phenotype. The decrease in phenotypic HSCs observed in Figure 5.1, taken together with the data here, suggests a decrease in the number of functional HSCs.

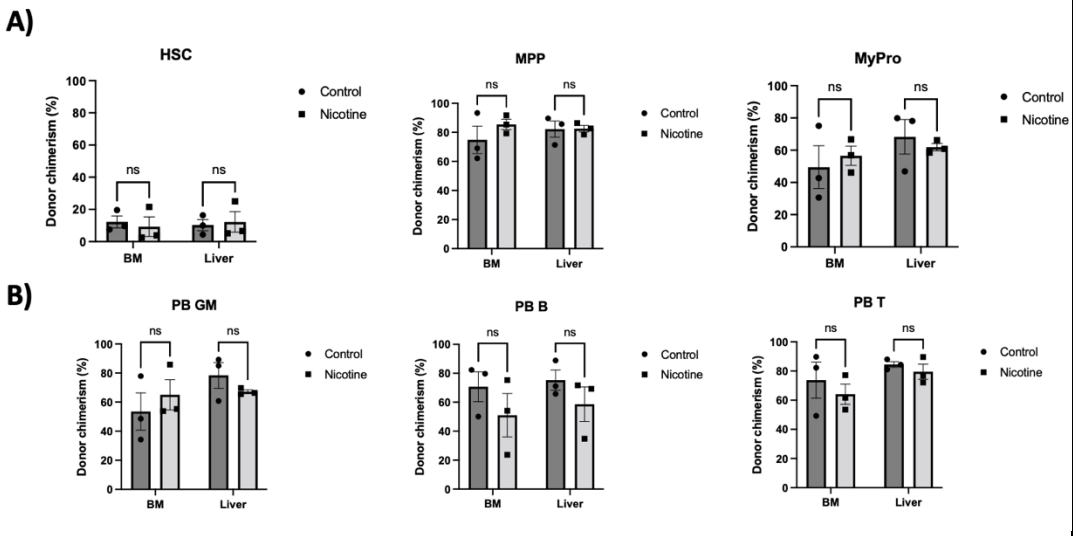

Figure 5.4 Perinatal nicotine exposure does not affect HSC function. Whole liver or bone marrow was processed to single cell suspension, and the equivalent of 100 HSCs from each tissue were transplanted into ¾ sublethally irradiated wild type (WT) recipients. After 16 weeks post transplant, the mice were sacrificed and analyzed via flow cytometry for donor chimerism in HSPC compartments and mature cells. A) Donor chimerism of HSCs (Live, CD3-CD4-CD5-CD8-B220-Gr1-Mac1-Ter119-cKit+Sca1+Flk2-SLAM+), MPPs (Live, CD3-CD4-CD5-CD8-B220-Gr1-Mac1-Ter119-cKit+Sca1+Flk2+SLAM-), and Myeloid Progenitors (MyPros) (Live, CD3-CD4-CD5-CD8-B220-Gr1-Mac1-Ter119-cKit+Sca1-) in the bone marrow of recipient mice transplanted with either control (dark gray bars) or nicotine-exposed (light gray bars) whole liver/whole bone marrow. B) Donor chimerism of peripheral blood GMs (Liver, Ter119-Gr1+Mac1+), B (Live, Ter119-B220+), and T (Live,Ter119-CD3+) cells of recipient mice transplanted with either control (dark gray bars) or nicotine-exposed (light gray bars) whole liver/whole bone marrow. Error bars are SEM, Control n = 3, nicotine n=3 from 1 independent experiment.

PNE alters immune response with secondary insult

Having demonstrated that PNE transiently decreased the number of functional HSCs, we next wanted to determine if PNE affected hematopoietic response in a secondary insult model. To test this, mice that had been exposed to nicotine perinatally were exposed to a single 35ug dose of Lipopolysaccharide (LPS) in adulthood and analyzed for their hematopoietic compartments across tissues. In this experiment, we expect there to be emergency myeloipoiesis (generation of myeloid progenitors and mature

cells such as GMs) in response to LPS stimulation. We observed a significant increase in HSCs and mature GMs in response to LPS alone (Figure 5.5A,C), and a trending increase in MPPs (Figure 5.5B). PNE plus secondary insult had no effect on HSC numbers (Figure 5.5A). Interestingly, PNE did result in a significant increase in MPPs (Figure 5.5B) as well as an increase (although not statistically significant) in GM production in the periphery (Figure 5.5C). Taken together, these data indicate that PNE exacerbates emergency myelopoiesis in response to LPS.

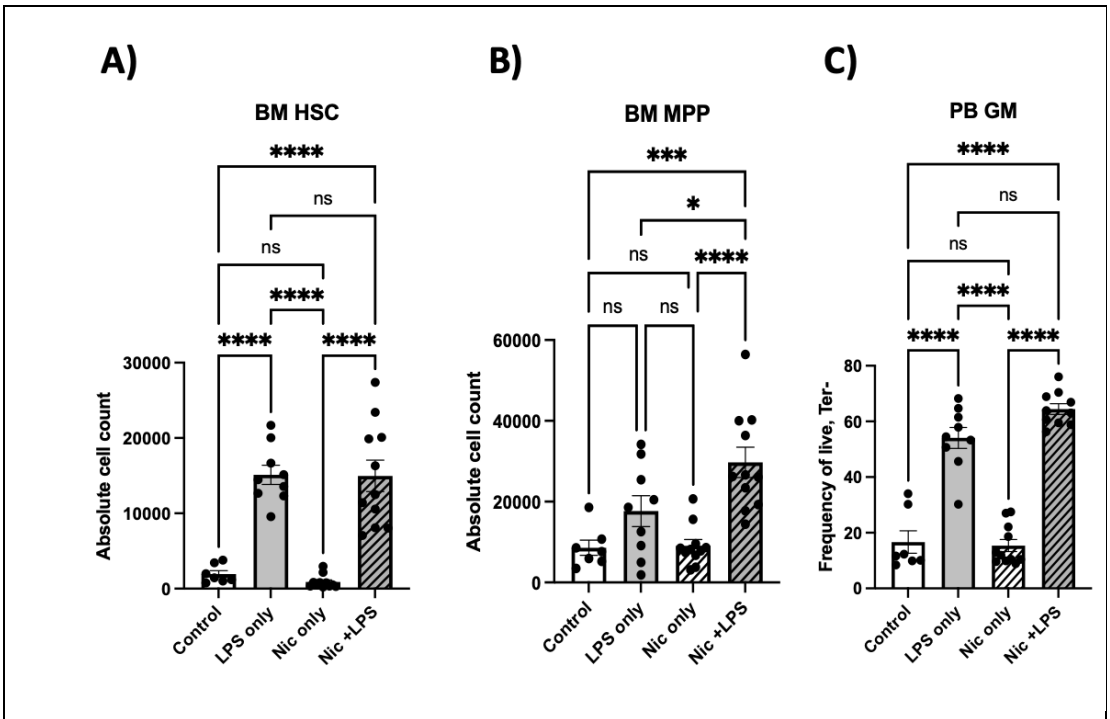

Figure 5.5 Perinatal nicotine exposure exacerbates secondary immune insult with LPS. Quantification of absolute cell numbers from control (white bars), LPS only (gray bars), nicotine only (striped white bars), or nicotine + LPS (gray striped bars) exposed animals. A) Total numbers of HSCs (Live, CD3-CD4-CD5-CD8-B220-Gr1-Mac1-Ter119-cKit+Sca1+Flk2-SLAM+) per 2 legs. B) Total numbers of MPPs (Live, CD3-CD4-CD5-CD8-B220-Gr1-Mac1-Ter119-cKit+Sca1+Flk2+SLAM-) per 2 legs. C) Total numbers of GMs (Live, Ter119-Gr1+Mac1+) per 25ul of blood. Error bars are SEM, Control n = 7, LPS only n= 9, nicotine only n=11, and nicotine + LPS n=11 from 2 independent experiments. *P<0.05, **P<0.005, ***P<0.001.

Discussion

Our investigation here revealed that PNE dynamically affects hematopoietic establishment and function for life. PNE resulted in a significant, but transient, decrease in total HSC numbers post birth. This deficit was recovered by adulthood, however there were persistent decreases in almost all downstream progenitors, indicating persistent altered hematopoietic output. Surprisingly, although we saw persistent altered HSPC numbers, there was no difference in traditional mature cell production in the peripheral blood of these animals., suggesting that PNE may lead to HSC exhaustion. Examining the lungs of the PNE mice revealed that there was a burst in the generation of T cells, but that by adulthood there were significantly fewer T cells overall, and specifically, fewer gamma delta T cells. This was interesting, because it demonstrates a dynamic situation where there is a transient burst in the generation of immune cells postnatally, but by adulthood there seems to be an exhaustion of the T cell compartment, and specifically the fetally-derived subset of T cells. Our examination of HSC functionality following PNE revealed that nicotine did not affect HSC function by gold standard definition of HSCs capability of long-term multilineage reconstitution (LTMR) and self-renewal capacity. However, there was altered hematopoietic response to secondary insult with PNE, indicating altered hematopoietic function in situ. We observed exacerbated emergency myelopoiesis in response to LPS following PNE. Taken together, these data suggest that PNE has permanent effects on hematopoietic establishment and function. Specifically, PNE seems to have an effect

on the establishment of non-traditional fetally-derived immune cells of the lungs, which is a plausible cellular mechanism of altered life-long immunity in children of smoking mothers.

Methods

Mice

All animals were housed and bred in the AALAC accredited vivaria at UC Santa Cruz and group housed in ventilated cages on a standard 12:12 light cycle. All procedures were approved by the UCSC or the UC Merced Institutional Animal Care and Use (IACUC) committees. WT C56Bl/6 mice were used for controls and for all expression experiments. Male and female mice were used equally and without sex discrimination for all experiments. Mice were fed normal chow diet and given nicotine solution (100ug/ml nicotine and 5% sucrose) *ad libitum*. Mice were sacrificed at P0, P14, or adulthood. Recipients for transplantation assays were adult mice (8-12 weeks of age). For LPS exposure, adult mice (8-12 weeks) were given a single intraperitoneal injection of 35ug of LPS. They were sacrificed for analysis ~16 hours post injection.

Tissue and cell isolation

Mice were sacrificed by CO2 inhalation. Lungs were harvested and treated with 1X PBS (+/+) with 2% serum and 2mg/ml Collagenase IV (Gibco) and 100U/ml DNaseI for 1 hour at 37C. Following incubation, tissue was passed through a 16g needle followed by 9g needle (approximately 10 times each) and then filtered through a 70um filter. Long bones were taken to isolate HSCs as well as to analyze bone marrow HSPCs. Peripheral blood was analyzed by tail bleeds for transplantation experiments, and then taken from the femoral artery at sacrifice for terminal analysis.

Flow cytometry

Cell labeling was performed on ice in 1× PBS with 5 mM EDTA and 2% serum. Analysis was performed on BD FACS Aria II or FACS LSRII at University of California-Santa Cruz.

Transplantation assays

Transplantation assays were performed as previously described (Beaudin et al., 2014; Beaudin et al., 2016; Smith-Berdan et al., 2015; Ugarte et al., 2015; Boyer et al., 2019). Briefly, P0 liver or bone marrow was harvested and processed into single cell suspensions for donors (Primary recipients of nicotine exposed HSCs). Cells were counted and the equivalent of 100 HSCs was transplanted into WT recipient mice aged 8-12 weeks were sublethally irradiated (750 rad, single dose). For adult HSC transplantation from nicotine + LPS mice, 10 million or 40 million whole bone marrow cells were transplanted. Under isofluorane-induced general anesthesia, cells were transplanted retro-orbitally. Recipient mice were bled at 4, 8, 12 and 16 weeks post-transplantation via the tail vein and peripheral blood was analyzed for donor chimerism by means of fluorescence profiles and antibodies to lineage markers. Long-term multilineage reconstitution was defined as chimerism in both the lymphoid and myeloid lineages of >0.1% at 16 weeks post-transplantation.

Quantification and statistical analysis

Number of experiments, *n*, and what *n* represents can be found in the legend for each figure. Statistical significance was determined by two-tailed unpaired Student's *t*-test or Chi. All data are shown as mean±s.e.m. representing at least three independent experiments.

Conclusion

The work I have presented here provide several novel findings on the role of interleukin 7 receptor (IL7R) in myeloid cell establishment and homeostasis, as well as novel findings on the effects of nicotine on hematopoietic establishment and function. For decades, IL7R has been studied with respect to lymphoid cell development and maintenance, but in chapters 1 and 2 here, I demonstrate and discuss the importance of this gene in the establishment and maintenance of both non-traditional and traditional myeloid cells. In chapter 1, I describe a previously uncharacterized wave of tissue resident macrophage (trMac) establishment which relies on IL7R signaling. Using lineage tracing models, examining gene and protein expression, and blocking signaling revealed that several subsets of trMacs develop through an IL7R+ wave during fetal development. In the germline deletion model (IL7R-/- mice), adults had no significant differences in total numbers of these cells, although we saw a transient decrease during development. In the future, it would be interesting to examine the compensatory mechanisms of development of these cell types. This indicates that these cells are able to compensate for loss of IL7R somehow. Additionally, a thorough examination of this IL7R/IL-7 signaling requirement across tissues would be very interesting. Understanding which tissues and which subsets of cells rely on IL7R/IL-7 signaling would be helpful in understanding tissue-specific requirements and environments required for generation of these cell types.

In the second chapter, I discuss a novel role of IL7R in the establishment and maintenance of adult-derived or "traditional" myeloid cells in the lungs. Using lineage tracing, knockout models, and reciprocal transplantation assays, I found that eosinophils in the lung require cell-extrinsic IL7R for their development and maintenance. Specifically, they require supportive cytokines from lymphoid cells, such as Eotaxin and IL-5 for their survival and maintenance. Interestingly, this was also a tissue specific, and cell type specific, phenotype. Adult-derived "traditional" eosinophils in the lung required IL7R in a cell-extrinsic manner, but similar "traditional" myeloid cells in the blood and bone marrow were not affected by the loss of IL7R, the lymphoid cells, or the changed cytokine environment. Here is another situation where a future intensive investigation into the tissue specificity of this signaling and cellular requirement of IL7R may be very interesting. It is peculiar that eosinophils in the lung were affected in these experiments. What specific cell types in the lung support eosinophil development and maintenance? Is it one specific cell type, or several that work together? Additionally, is Eotaxin or IL-5 alone enough to maintain eosinophils? Or similarly, are several cytokines required?

In the final chapter, I examined how exogenous factors, specifically nicotine, influence hematopoietic establishment and function for life. Our primary goal was to understand how in utero exposure to nicotine influenced the establishment of both HSCs as well as "traditional" and "non-traditional" immune cells across tissues, and specifically the lungs. Surprisingly, in utero nicotine exposure had transient effects on

numbers of HSCs, but resulted in a persistent deficit in “non-traditional” immune cells in the lungs (gamma delta T cells). Additionally, although in utero nicotine exposure did not affect HSC function by the gold standard transplantation assay in our field, there were differences in hematopoietic function in our secondary immune insult model with LPS. Taken together, these data demonstrated persisting effects on HSCs in situ. To understand these persisting effects on HSCs, we have a few future experiments set up that are not discussed here. To determine the molecular drivers of altered hematopoiesis in the nicotine exposed animals, we want to look at persisting epigenetic changes in HSCs. We hypothesize that genes implicated in immune response may be differentially regulated in our analyses here. To address persistent epigenetic changes, we will use ATACseq and RNAseq of HSCs from adult mice that were exposed to nicotine perinatally. By looking at adult HSCs, we will be able to examine persisting changes in these cells. Additionally, to understand whether HSCs are responding directly to nicotine, we are performing qRT-PCR for nicotinic acetyl choline receptors in bone marrow and on isolated HSCs. Alternatively, to determine if HSCs are responding to inflammation caused by nicotine exposure, we are going to analyze cytokines in the serum and bone marrow of nicotine exposed animals. This work will shed light on the molecular drivers of altered immunity in nicotine (and tobacco) exposed children.

Taken together, the work here describes several novel aspects of how endogenous and exogenous factors influence hematopoietic establishment, maintenance, and function.

The work presented here identifies novel roles for IL7R in myeloid cell development and maintenance, as well as examines how toxicants (nicotine) influence hematopoiesis for life. There are several exciting avenues of further exploration for these projects, and I am excited to see how these findings influence future research in this field.

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