



Erythroid Lineage Cells in the Liver: Novel Immune Regulators and Beyond

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Abstract

The lineage of the erythroid cell has been revisited in recent years. Instead of being classified as simply inert oxygen carriers, emerging evidence has shown that they are a tightly regulated in immune potent population with potential developmental plasticity for lineage crossing. Erythroid cells have been reported to exert immune regulatory function through secreted cytokines, or cell-cell contact, depending on the conditions of the microenvironment and disease models. In this review, we explain the natural history of erythroid cells in the liver through a developmental lens, as it offers perspectives into newly recognized roles of this lineage in liver biology. Here, we review the known immune roles of erythroid cells and discuss the mechanisms in the context of disease models and stages. Then, we explore the capability of erythroid lineage as a cell source for regenerative medicine. We propose that the versatile lineage of erythroid cells provides an underappreciated and potentially promising area for basic and translational research in the field of liver disease.

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Introduction

The human body relies mainly on the bone marrow for steady state erythropoiesis. Under erythroid stress conditions, such as chronic inflammation, spleen and liver are used to expand the erythropoietic capacity because suppressed erythropoiesis is induced in bone marrow due to inflammatory pathways. But whether erythropoietic cells directly participated in inflammatory processes was rarely studied, until very recently. Besides being inert oxygen carriers, erythroid lineage cells emerge to be a modulator of innate and adaptive immune response.¹ This review summarizes the most recent advances in newly found features of erythroid lineage cells, largely focusing on their immune modulatory functions, especially in neonatal immunity, as

evidenced by both *in vivo* and *in vitro* studies in mouse and human. In addition, we also shed some light on the emerging trends of erythroid cells in the fields of microbiome study and regenerative medicine.

Erythroid lineage cells: Natural history in the liver

Cellular markers for staging of erythroid cells

There are different stages during erythropoiesis. The cells of interest for this review, referred as "erythroid lineage cells" or "CD71+ erythroid cells", represent a mix of erythroblasts, including basophilic, polychromatic, and orthochromatic erythroblasts. A widely used assay relies on the cell-surface markers CD71 and Ter119, and on the flow-cytometric 'forward-scatter' parameter, which is a function of cell size.² However, because CD71 is expressed on all proliferating cells,³ the adhesion molecule CD44 has been used in some studies to distinguish between erythroblasts at successive developmental stages.⁴ It is well established that during murine erythropoiesis *in vivo*, one proerythroblast undergoes three mitoses to generate (sequentially) two basophilic, four polychromatic, and eight orthochromatic erythroblasts.⁵ Using this set of cell surface markers, along with the features of cell size and presence or absence of nucleus, erythroblasts can be easily distinguished from proerythroblasts (Ter119-) and successive reticulocytes (without nucleus) (Fig. 1).

Emergence of -omics approaches and the optimization of *ex vivo* erythroid cultures in this field will greatly enable us to investigate the continuous yet hierarchical structure of hematopoietic network, and uncover novel growth factor receptor regulators of the erythroid trajectory.^{6,7}

Erythroid cell origins and dynamics in developmental liver

Erythropoiesis occurs mainly in the bone marrow; but, that is true only for the adult stage. In fact, erythropoiesis involves many tissue origins and shifts locations during the early development stage. Therefore, to understand erythroid cell origins and dynamics in developing liver is key for us to understand their various biological roles. Differentiation and proliferation of erythroid lineage cells have been extensively studied over the years. Hematopoiesis, defined as the formation of cellular components in blood, occurs during embryonic development and throughout adulthood to replenish the blood system. Specifically, erythropoiesis, which refers to the

Keywords: Erythroblast; Immune suppression; Liver inflammation; Hepatogenesis.
Abbreviations: EMP, erythroblasts-macrophage protein; EPO, Erythropoietin; HSC, hematopoietic stem cells; Tregs, regulatory T cells.

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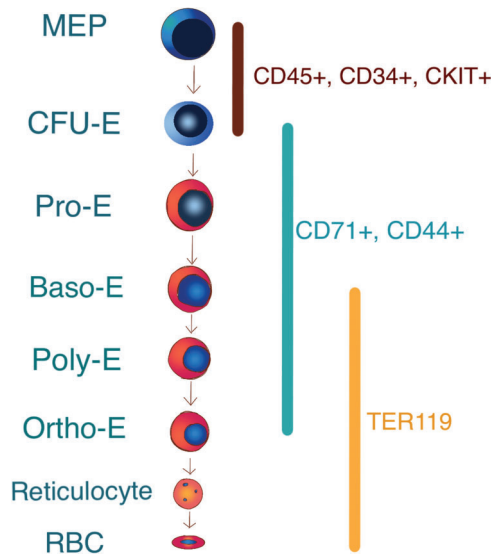


Fig. 1. Cellular markers for staging of erythroid cells. Representative cell surface markers for different stages of erythroid lineage cells. Most of the progenitors share CD45+, CD34+ and CKIT+. As they mature, they lose surface marker of CD71 and gain the marker of TER119. Eventually, reticulocyte and RBC lose their nucleus before being released into system circulation.

Abbreviations: MEP, megakaryocyte/erythroid progenitor; CFU-E, Colony Forming Unit-Erythroid cell; Pro-E, proerythroblast; Baso-E, basophilic erythroblast; Poly-E, polychromatic erythroblast; Ortho-E, orthochromatic erythroblast; RBC, red blood cell.

expansion and maturation of erythroid lineage cells, and is the earliest and largest population of cells in hematopoiesis.

We have learned from mouse models that there are two waves of hematopoiesis that occur during embryo development. The initial wave, called primitive hematopoiesis, starts at E7.5 in the extraembryonic yolk sac. The successive wave, called definitive hematopoiesis, starts at E9.5 in both the yolk sac and the intra-embryonic aorta-gonadomesonephros region.⁸ Later, those hematopoietic progenitors migrate and seed the fetal liver, as the yolk sac microenvironment does not support terminal differentiation into definitive blood cell lineages; it is, thus, here that they can efficiently generate blood cells for the fast-growing embryo.⁹ In detail, at E9.5-10.5, the liver rudiment is colonized by myeloerythroid progenitors. At E11.5, hematopoietic stem cells (HSCs) appear in the fetal liver, a time slightly later than that of the myeloerythroid progenitors.⁸ Notably, the early fetal liver does not produce HSC *de novo* but is believed to be the main site of HSC expansion and differentiation.

The early fetal liver is rich in colony-forming unit-erythroid and proerythroblasts, reflecting an active erythropoiesis state early on, whereas myeloid and lymphoid progenitors accumulate later in life. In mouse models, HSCs plateau at E15.5-16.5 and start to decline in fetal liver, where the microenvironment can no longer meet the changing needs of lineage differentiation and HSC expansion.⁹ The spleen starts to produce blood cells at E14 and continues to be a site of hematopoiesis after birth, at time of stress. At E18, the soft embryo starts to have solid bony structures, and bone marrow provides the suitable environment for the HSC and hematopoiesis throughout adulthood.¹⁰

The dynamics of erythropoiesis in developmental liver remains much less defined in humans. A recent study by Fanni *et al.*¹¹ shed some light on simplifying the time span into four stages, as follows: stage I lasts for the first 9 weeks (free of any clear sign of hematopoiesis); stage II from 10 weeks to 12 weeks (small and irregular erythrocytic foci); stage III from 13 weeks to 22 weeks (bigger foci in hepatic parenchyma); and, stage IV from 23 until 39 weeks (few round and isolated foci remains).

Immune regulating potency of erythroid cells

The immune regulatory capability of erythroid cells has been researched much less in previous years. However, this new emerging topic is the focus of this review. In the following sections, we will provide the updated evidence showing the interplay of erythroid cells with other immune cells and discuss which disease models have already been tested for their suppressive functions, with additional details given about the controversies of hypotheses in the biology of their immune potency (Fig. 2). Also, we will discuss the recent studies related to the microbiome and how it can be regulated by erythroid lineage cells. Finally, we will summarize the potential regulators of immunosuppressive erythroid cells that have been proposed in this field.

Interplay of erythroid cells with other immune cells

Erythropoiesis has been isolated and studied independently from other hematopoietic immune-related lineage cells, for decades. From the developmental perspective, the generation of lymphoid progenitors concurrently occurs with the development of myelo-erythroid progenitors, implying that the crosstalk of myeloid lineage and erythroid lineage is possibly quite common during development.¹²

Various cell types have been shown to interact with and be modulated by erythroid cells, including both lymphoid immune cells and myeloid immune cells. It is shown that nucleated erythroid cells exert a potent natural suppressor activity for both B and T cell-mediated immune processes.¹³ Also, the erythroid cells from neonate spleen had the capacity to modulate the differentiation of CD4+ T cells into effector cells and provide a bias towards a Th2 type instead of Th1 type by producing IL-6.¹⁴ CD71+ erythroid cells can also directly interact with immune regulatory T cells (known as Tregs), promoting the development and function of Treg cells through TGF-beta.¹⁵

The ability of erythroid cells to interact with myeloid immune cells has also been investigated in recent years. For example, it was found that the interaction between macrophage and erythroid cells happened throughout normal, stressed and pathological conditions, mediated by the adhesive molecule, erythroblast-macrophage protein, expressed on both cells.¹⁶ Another report provided data to show that nucleated red blood cells could also induce IL-10/IL-19 production by monocytes, even without cell-to-cell contact, to suppress a vigorous harmful innate immune reaction in fetuses.¹⁷

Disease models involving suppressive erythroid cells

The immune suppressive function of erythroid lineage cells has been shown in different disease models in recent studies. Dunsmore *et al.*¹⁸ reported that CD71+ cells compromise

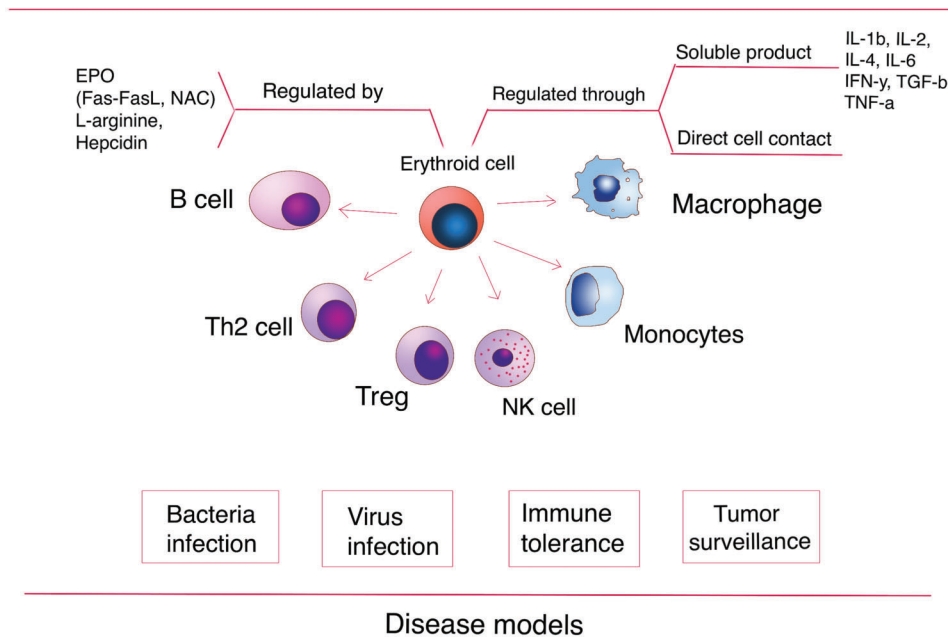


Fig. 2. Mechanism of immune regulation of erythroid cells. Erythroid cells can regulate on different cell types including both lymphoid cells (B cell, Th2 cell, Treg, NK cells and myeloid cells (macrophage, monocyte), through either soluble cytokines, or direct cell contacts. The upper stream regulators of erythroid cell include EPO (through Fas-FasL and NAC), L-arginine and Heparidin. The immune regulatory roles of erythroid cells have been evidenced across disease models, including bacteria and virus infection, as well as immune tolerance and tumor surveillance.

Abbreviations: EPO, erythropoietin; Fas-FasL, Fas-Fas ligand; NAC, N-acetylcysteine; NK cell, natural killer cell; Treg, regulatory T cell; TNF-a, tumor necrosis factor alpha; TGF-b, transforming growth factor beta; IFN-g, Interferon gamma.

innate immune responses against *Bordetella pertussis* infection in the lung. Apart from bacterial infection, two studies have shown that CD235a + CD71+ erythroid cells also modulate immune response against virus infection, including the role of erythroid cells in peripheral blood in human immunodeficiency virus-infected people,¹⁹ and in a biliary atresia model induced by rhesus rotavirus.²⁰

Besides immunity against pathogens, erythroid lineage cells also participate actively in immune tolerance and surveillance. Umbilical cord CD71+ erythroid cells have been shown to play a role in spontaneous preterm labor and maternal-fetal tolerance.^{21,22} In the enlarged spleen of hosts bearing advanced tumors, CD71+ erythroid cells were also found to be enriched and to facilitate tumor progression by secreting the neurotrophic factor artemin into the blood.²³ In both patients with advanced cancer and treatment-naïve mice bearing large tumors, CD71+ erythroid cells contributed to the impaired T cell responses, especially that of the CD8+ T cells.²⁴

Controversies: modulation or suppression, direct or indirect

In the field of research into the function of erythroid lineage cells, controversial results have been observed in a few studies, leading to debate over whether they are immune suppressive or modulatory and whether the interaction is direct or indirect. In a mouse sepsis model induced by endotoxin or polymicrobial challenge, neonatal CD71+ erythroid cells failed to modify sepsis mortality.¹⁹ Another study in the pathogenesis of preterm labor showed neonatal CD71+

erythroid cells to be immunomodulatory, rather than immunosuppressive.²⁵

The mechanism of erythroid cells' immune suppression activities, whether through direct cell contact or soluble products, is also an ongoing matter of debate. One human study showed direct contact, instead of soluble products, between neonatal CD71+ erythroid cells and maternal mononuclear immune cells and characterized it as the key step to release of pro-inflammatory cytokines and decrease of the anti-inflammatory cytokine TGF-beta.²⁵ Another study showed the opposite result; the investigators successfully used erythrocyte-derived conditioned media to induce a type-1 interferon response in macrophages, supporting an integrative role for soluble products in the immune response.²⁶ The spectrum of cytokines produced by erythroid cells is, surprisingly, quite widespread and includes IL-1beta, IL-2, IL-4, IL-6, IFN-gamma, TGF-beta1 and TNF-alpha.^{27,28}

Microbiome and erythroid lineage cells

It was Elahi *et al.*²⁹ who first reported that CD71+ erythroid cells suppress the exaggerated inflammatory process and establish immune tolerance towards colonized commensal microorganisms after birth, which in turn compromised host defense against pathogens. They also showed that arginase II from CD71+ erythroblasts is essential for neonatal innate-immune suppression, which was further validated by another group, demonstrating a key role of arginine in mucosal immunity, especially of susceptibility to gut-derived pathogens.³⁰ In a more recent human study about inflammatory bowel disease, the role of erythroid cells in regulating the gut microbiome was further investigated. Data showed that

reduced frequency and/or impaired functionality of CD71+ erythroid cells during pregnancy may predispose inflammatory bowel disease patients to a more pro-inflammatory milieu in their gastrointestinal tract and induce dysbiosis.³¹ Other recent mechanistic research has suggested heat-stable microbial products which circulate in the bloodstream might link to inflammatory signaling modulated by hematological compartments.³²

Potential regulators of immunosuppressive erythroid cell

Erythropoiesis is a highly regulated process of erythrocyte production. However, limited studies were done to investigate potential regulators for the immune suppressive features of those erythroid cells. Here, we summarize some of those proposed potential regulators, which could be promising for future therapeutic applications.

L-arginine which overrides immunosuppression of neonatal CD71+ cells that express the enzyme arginase-2 could also be a potential regulator of the immune response of erythroid cells.²⁹ Hcpidin expression was shown to be mediated by the transferrin receptor 1 TfR1 (also known as CD71) expression on erythroid precursors, which might be a potential regulator of immunosuppressive erythroid cells.³³

The erythropoietin (EPO) receptor is expressed abundantly on proerythroblasts and early-stage erythroblasts, indicating that EPO can be a potential regulator of the immune suppressive function of erythroid lineage cells. Several lines of evidence have demonstrated that liver is the predominant production site for EPO during development and is the major cellular sites of EPO gene expression.⁽³³⁾ Erythroid lineage progenitors in fetal liver are shown to be more sensitive to this effect of EPO than are those of adults,³⁴ implying an active regulatory role of EPO in the hepatic milieu. EPO has been proven to modulate the immune responses and dynamics of oxidative status in various studies both *in vivo* and *in vitro*,³⁵ through either the Fas and FasL pathway, modulation of N-acetyl-cysteine, a reactive oxygen species scavenger,³⁶ or by directly reducing production of neutrophils, accompanying accelerated erythropoiesis.³⁴ EPO can also signal through macrophages to promote apoptotic cell clearance and immune tolerance.³⁷ Targeting EPO and EPO-receptor have been shown to have great potential in regulating immune injury of various liver diseases, suggesting promising future clinical applications.³⁸⁻⁴¹

Erythroid lineage cells and neonatal immunity

The accumulation of erythroid lineage cells in the liver perinatally suggests they are more related to neonatal immunity than any other developmental stage later in life. Unsurprisingly, high frequencies of erythroid cells were found (median: 31%) in cord blood samples from term and preterm neonates. These erythroid cells disappear rapidly by 1 week of age.⁴² In neonates, the frequent onset of infection might not be attributed to an inherent immaturity of neonatal immune cells but rather to the immune suppression by CD71+ erythroid cells, which leave newborns vulnerable to infection.⁴³ Furthermore, the selective accumulation of erythroid cells in the spleen during development may explain differences of immune responses generated in infants and neonates.¹⁴

Immune potency of these CD71+ erythroid cells was also observed in neonatal salmonella infection but it seemed to have both positive and negative consequences for host immunity.⁴⁴ Immunosuppression mediated by CD71+ erythroid cells is also crucial for homeostasis in the perinatal period, as it has been shown to bring down TNF-alpha and IFN-gamma production through arginase-2 activity and PD-1/programmed death ligand-1 (PDL-1), contributing to fetomaternal tolerance.²² The critical window of erythropoiesis in the neonatal period suggests erythroid lineage cells may play an important role in neonatal immunity.

Lineage crossing and regenerative medicine

Erythroid progenitors replenish other cell types

Neonate liver does not have a quiescent state, as adult livers do; they are continually undergoing massive transitional changes, even when not confronted by any external stimulus.⁴⁵ Thus, any single gene in the liver may serve distinct functions in different stages. One of the emerging themes is that many of the same signaling pathways, transcription factors and even cell types are used reiteratively.^{46,47} Erythroid progenitors possess such versatility, making them capable of replenishing other cell types developmentally, as illustrated by the following examples. The non-hematopoietic cell fraction of the bone marrow, which contains heterogeneous stromal cell populations, has been shown to be generated from a hematopoietic rather than mesenchymal origin.⁴⁸ Adult connective tissue-resident mast cells, which are associated with various inflammatory processes, have been shown to originate from late erythro-myeloid progenitors.⁴⁹ Erythro-myeloid progenitors also constitute a source of endothelial cells.⁵⁰ The blood islands formed thereafter contain not only red blood cells but also endothelial cells.⁵¹ The ability of erythroid progenitors to replenish other cell types suggest its potential to be used in regenerative medicine.

Erythroid cells crosstalk with hepatogenesis

In the past, erythrocyte-related genes and erythrocytes were frequently excluded from research analysis, as presumably the cells only carry oxygen and do not interact with other cells or the environment.⁵² Specific retrieval and isolation protocols were constantly used to eliminate the majority of circulating erythroid cells to increase purity for "cells of interest".⁵³ For those reasons, how erythroid cells participate specifically in hepatogenesis is largely unknown.⁵⁴ As demonstrated in the previous paragraph, there are continuous lines of evidence showing that erythroid progenitors can replenish other cell types; therefore, their involvement in hepatogenesis needs to be further examined.

The biological process of erythropoiesis is weaved into hepatogenesis chronologically and spatially during liver development.⁵⁵ Hepatoblasts (hepatic endoderm cells) delaminate from epithelium and invade the adjacent septum transversum mesenchyme to form the liver bud at E9.5-E10.5.⁵⁶ After that, the liver bud undergoes a period of accelerated growth, as it is vascularized and colonized by hematopoietic cells.⁵⁷ The encounter of hepatoblasts and hematopoietic cells in the liver bud raises the possibility of cell-crosstalk during this developmental milestone of hepatogenesis. There are a handful of lines of evidence showing the

existence of such interactions between erythropoiesis and hepatogenesis in the early developmental stage.

An earlier study on the joining of the bile ducts during hepatogenesis of the mouse embryo has demonstrated that reciprocal cell interactions can possibly occur between the biliary epithelial cells from embryonic endoderm and erythroid cells from nearby mesoderm.⁵⁸ Using dynamic transcriptomic and proteomic profiling, relationships and extensive crosstalk between hematopoiesis and hepatogenesis in the mid-trimester fetal liver was characterized.⁵⁹ As we would expect, one possible means of crosstalk mediation can be cytokine secretion. Indeed, it has been shown that hematopoietic cells in the liver secrete cytokine oncostatin M, which in combination with the glucocorticoid hormones human growth factor and WNT, promotes hepatocyte differentiation and maturation.⁶⁰

Compared to crosstalk between erythropoiesis and hepatogenesis during the early developmental stage, the interaction between erythroid cells and parenchymal cells during liver regeneration in the mature liver is more debatable. It is, however, well known that mature liver still maintains great self-regeneration capability. As discussed above, in the liver bud, hematopoietic cells are in direct contact with hepatoblasts, the common precursors of hepatocytes and cholangiocytes. However, in the mature liver, erythroblasts reside in the sinusoid and lose direct contact with cholangiocytes; blood from the portal vein enters the sinusoid space and comes into direct contact with the basal surface of the hepatocyte,^{61,62} while bile is secreted from the apical surface of adjoining hepatocytes into the bile canaliculi (grooves in the cell surface).⁶³ The interaction between erythroid cells and regenerative parenchymal cells, if there is any, remains largely undefined.

Recent advances in single-cell RNA sequencing have provided new evidence that erythroblasts can cross lineage and participate in hepatogenesis. In fetal liver, two distinct ALB+ expressing populations and several nonhepatic populations, resembling erythroblast cell transcriptionally, types were found.⁶⁴ One of the biggest challenges and concerns of using induced pluripotent stem cells for regenerative medicine is the carcinogenesis potential of uncontrolled development of the seeding stem cells.^{65,66} As such, discoveries of safer progenitor cells for regeneration purposes in many organ systems have shifted focus to *in situ* precursors.^{67–70} Based on their versatility, hepatic erythroid cells can be used as an integral component of regenerative modeling in future studies (Fig. 3).

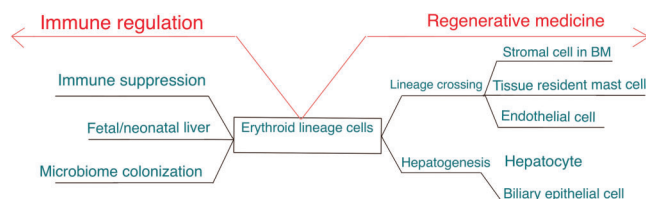


Fig. 3. Emerging roles of erythroid lineage cells in the liver. Erythroid lineage cells have shown potential of immune regulation and regenerative medicine. Future investigations may highlight their role in immune suppression, fetal/perinatal liver disease and microbiota-host interactions. On the arm of regenerative medicine, their role to participate in lineage crossing and hepatogenesis will be further investigated.

Conclusions

There are a few unique advantages of erythroid lineage cells. They are not permanently anchored and can be easily mobilized from sinusoid, having good potential for self-renewal, and being sensitive to external hazards and immune potent. They may function proactively through the developmental stage, the maturation of the immune and non-immune cells, and the different progressive stages of the disease. Those cells hold promise in research to highlight their utility in immune-related diseases and can be innovative targets for therapeutic options.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived and designed the review, and designed the figures (LY), co-wrote the manuscript (KL, LY).

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