Mechanistic and Therapeutic Studies Related to Anemia of Chronic Disease and Inflammation

Chanté Larelle Richardson
Oxon Hill, Maryland

Master of Business Administration, Ashford University, 2010
Master of Science, University of Virginia, 2009
Bachelor of Science, University of Pittsburgh, 2006

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Abstract

The erythroid iron restriction response (EIRR) results from lineage-selective inactivation of aconitase enzymes, causing diminished erythropoietin (Epo) responsiveness in early erythroid progenitors. Provision of exogenous isocitrate in either cell culture or murine models of iron deficiency restores Epo responsiveness and abrogates the erythropoietic block characteristic of the iron deprivation response. Although isocitrate administration can restore erythropoiesis in iron deficient mice, the response is transient. However, if inappropriate activation of the EIRR also contributes to anemias not actually caused by decreased body iron stores, isocitrate may provide a therapeutic benefit in those clinical settings. A major area of clinical controversy is the degree to which erythroid iron restriction contributes to anemia of chronic disease and inflammation (ACDI).

Numerous patients with chronic diseases such as kidney failure, cancer, and autoimmunity develop clinically significant anemias, collectively designated anemia of chronic disease and inflammation. ACDI arises from the diminished production of red cells by the bone marrow. In many patients, treatment with erythropoietin injections lessens the anemia and improves symptoms. However, erythropoietin treatment is expensive, places patients at risk for adverse side effects, and in many cases eventually loses its effectiveness. Two major abnormalities in ACDI underlie the defective marrow function and poor responsiveness to erythropoietin treatment. Firstly, defects in iron transport cause retention in storage pools and diminished delivery to the marrow red cell precursors, a situation known as iron restriction. Secondly, cells in the immune system

secrete inflammatory mediators, most notably interferon γ (IFNγ) and tumor necrosis factor α (TNFα), which directly bind to marrow red cell precursors and inhibit their development into red cells.

This study shows that iron restriction causes the red cell precursors to become extremely sensitive to the inhibitory effects of inflammatory mediators. Providing the compound isocitrate blocks all inhibition by inflammatory mediators in cell culture experiments and eliminates anemia in a rat arthritis model of ACDI. Additionally, this study dissects the ability of iron restriction to alter the response of the red cell precursors to the key inflammatory mediator IFNγ. In particular, iron restriction specifically changes the patterns of signaling within the cell as it responds to the mediator in the environment. We define a pathway in which iron restriction and IFNγ act in a cooperative manner on early erythroid progenitors to increase PU.1 expression and interfere with its normal downregulation. Iron restriction and isocitrate exert their influences, at least in part, through alteration of PKC activation. Thus, we propose a model of ACDI in which iron restriction and inflammatory signaling are both required to attain a critical threshold of erythroid PU.1, which may then interfere with early stages of lineage commitment. Through its reversal of PKC activation by iron restriction, isocitrate may act to keep PU.1 levels below this critical threshold. The ability of isocitrate to reverse these signaling abnormalities provides new evidence for how it exerts its beneficial effects in ACDI.

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ACD/ACI	Anemia of Chronic Disease/Anemia of Chronic Inflammation
ACDI	Anemia of Chronic Disease and Inflammation
ACO	Aconitase
AI	Anemia of Acute Inflammation
AKT	Protein Kinase B
ANOVA	Analysis of Variance
AOD	Autoimmune Ovarian Disease
BFU-e	Burst Forming Unit-Erythroid
BIM	Bisindolylmaleimide
BLAST	Basic Local Alignment Search Tool
C/EBP	CCAAT/enhancer binding protein
CaMK	Calmodulin-associated Kinase
CBC	Complete Blood Count
CFU	Colony Forming Unit
CFU-e	Colony Forming Unit-Erythroid
CFU-GEMM	Colony Forming Unit-Granulocyte, Erythrocyte, Monocyte, Megakaryocyte
CKD	Chronic Kidney Disease
CNS	Central Nervous System
DAPK	Death-Associated Protein Kinase
EAO	Experimental Autoimmune Orchitis
EIRR	Erythroid Iron Restriction Response
Epo	Erythropoietin
EpoR	Erythropoietin Receptor
ERK	Extracellular-Regulated Kinase
ESA	Erythropoietin Stimulating Agent
Fe	Iron
FSC	Forward Scatter
GAS	Gamma Activated Sequence

GATE	Gamma-Activated Transcriptional Element
GPA	Glycophorin A
HAT	Histone Acetyl Transferase
HB/Hb	Hemoglobin
HBKA	Heat-Killed *Brucella abortus*
HCT/Hct	Hematocrit
HIF	Hypoxia-inducible factor
IC	Isocitrate
IDA	Iron Deficiency Anemia
IFN	Interferon
IL	Interleukin
IMDM	Iscove's Modified Dulbecco Medium
IP	Intraperitoneal
IRE	Iron Response Element
IRF	Iron Response Factor
IRIDA	Iron-Refractory Iron Deficiency Anemia
IRP	Iron Response Protein
IscU	Iron Sulfur Cluster
ISGF	Interferon-stimulated gene factor
ISRE	Interferon-stimulated response element
IV	Intravenous
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
KSL	Kit^+ Sca^+ Lin^-
LDH	lactate dehydrogenase
L-NAME	L-NG-Nitroarginine Methyl Ester
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MCV	Mean Corpuscular Value
MEK	MAPK/ERK kinase
MONO	Monocyte

mRNA	messenger RNA
n.s.	Not significant
NAC	N-acetyl cysteine
NCBI	National Center for Biotechnology Information
NEU/ NE	Neutrophil
NF –κB	Nuclear Factor-Kappa B
Nfs	Cysteine desulferase
NO(S)	Nitric Oxide (Species)
PARP	Poly ADP-ribose polymerase
PBS	Phosphate Buffered Saline
PDTC	Pyrrolidine dithiocarbamate
PGPS	Peptidoglycan-polysaccharide
PHSC/HSC	Pluripotent Hematopoietic Stem Cell/Hematopoietic Stem Cell
PI	Propidium Iodide
PKC	Protein Kinase C
PLC	Phospholipase C
PLT	Platelet
pS	phospho serine
pY	phospho tyrosine
qRT-PCR	quantitative Real Time-Polymerase Chain Reaction
RA	Rheumatoid arthritis
RBC	Red Blood Cell
ROS	Reactive Oxygen Species
s.e.m.	Standard Error of the Mean
SCF	Stem Cell Factor
shRNA	short hairpin RNA
siRNA	short interfering RNA
SSC	Side Scatter
STAT	Signal Transducer and Activator of Transcription
TfR	Transferrin Receptor
TMPRSS6	Transmembrane protease, serine 6

TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TSAT	Transferrin Saturation
Tub	Tubulin
TWEAK	TNF-like weak inducer of apoptosis
Tx	Treatment
UV	Ultra-Violet
WBC	White Blood Cell

ACKNOWLEDGEMENTS & DEDICATION

First, I want to acknowledge my heavenly father, for without him none of this would have been possible. I dedicate the work presented in my dissertation to my family and many friends. I extend much gratefulness to my loving mother, Katrina Richardson, whose words of encouragement and undying support helped to sustain me throughout this entire process. My brother, Yardell Richardson and sister, Shanikka Richardson, who always remind me of the how much they see me as a role model and thus, failure or giving up was never an option—"Every set back, is a set-up, for a comeback!". To all the family members and friends whose names who are not singled out, I appreciate you as well as you celebrated with me in all my successes and prayed with me through my shortcomings. To a few special friends, (Chinedu Onwudiwe, Tara Hickman, Sarah Ellis, & Ashley Pope) "Thank you!" You all always showered me with praise and encouragement. To Kendra Simpson for always reminding me, that I am not alone, and that, I too, can get through this!

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Chapter 1:
General Introduction

1.1 Overview of hematopoiesis

Hematopoiesis is the process of production, multiplication, and specialization of blood cells in the bone marrow. It begins with the most basic blood cell, the pluripotent hematopoietic stem cell/hematopoietic stem cell (PHSC/HSC) as depicted in Figure 1.1. PHSCs self-renew and give rise to all hematopoietic lineages: white blood cells, which protect our bodies from infection; mature red blood cells that carry oxygen to the cells and tissues in our bodies; and platelets, which assist in clot formation during injury [1].

In mammals, the sites of hematopoiesis shift depending on the stage of development. As an embryo, initially hematopoiesis is carried out in the yolk sac, but shifts to the liver --a process known as primitive hematopoiesis. From the second trimester of development and beyond, the majority of hematopoiesis occurs in the marrow and spleen. Liver hematopoiesis only occurs in times of crisis at this developmental point. During adulthood, hematopoietic sites are limited to the bone marrow of skull, ribs, sternum, vertebral column, pelvis and proximal ends of the femurs--a process known as definitive hematopoiesis. This occurs in the extravascular compartment of the bone marrow, which has daily output of mature blood cells of approximately 2.5 billion erythrocytes, 2.5 billion platelets, and 50-100 billion granulocytes.

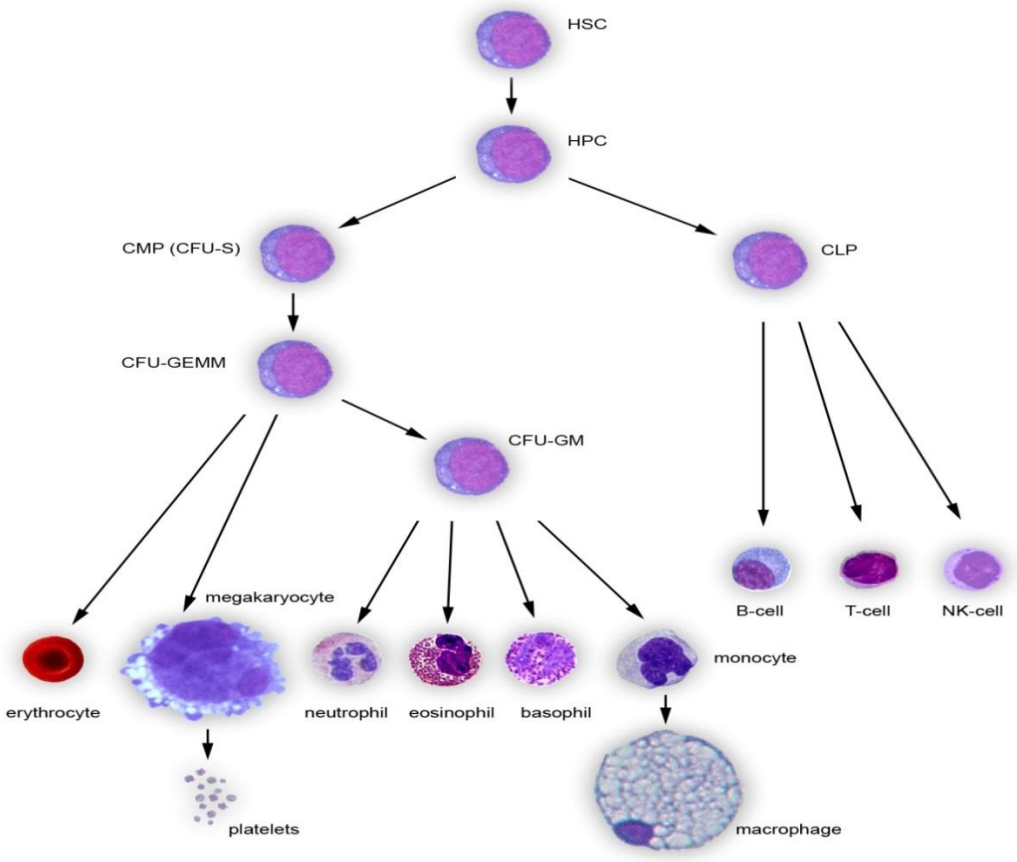

Figure 1.1 Hematopoiesis

Diagram was retrieved from the George Daley Laboratory website at HHMI/Children's Hospital Boston/Harvard Medical School, submitted by Dr. William Lensch

https://daley.med.harvard.edu/assets/Willy/willy.htm

1.2 Erythropoiesis: development and regulation

Erythropoiesis is a tightly regulated, complex physiologic process that leads to the formation of erythrocytes from PHSCs. The erythrocytes are formed during primitive hematopoiesis to facilitate tissue oxygenation as the embryo undergoes rapid growth [1]. PHSCs in the bone marrow differentiate into all the cellular elements of blood under the influence of various cytokines and growth factors. There are several transcription factors that influence erythroid cell fate, including the master regulators of erythroid versus myeloid lineage choice in the hematopoietic system, GATA-1 and PU.1. The C-finger region of GATA-1 represses PU.1 activity by functionally interfering with the activity of the ETS domain of PU.1, which allows erythroid differentiation [2]. PU.1 can bind to itself also, resulting in a block in GATA-1 binding, and promoting myeloid differentiation [3]. This cross-antagonistic mechanism between GATA-1 and PU.1 shown in Figure 1.2A highlights the lineage fate decisions that occur in the development of hematopoietic progenitors [4].

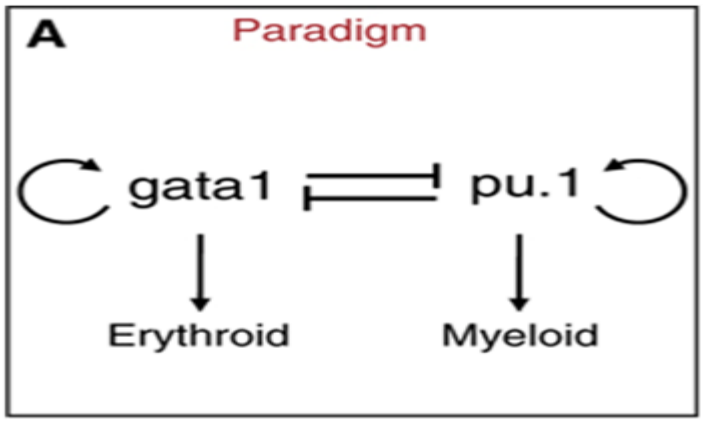

Figure 1.2 Schematic representation of the classical gata1/pu.1 cross-antagonistic paradigm.

Diagram was retrieved from Monteiro et al, *EMBO Journal*, 2011, Volume 30 Issue 6

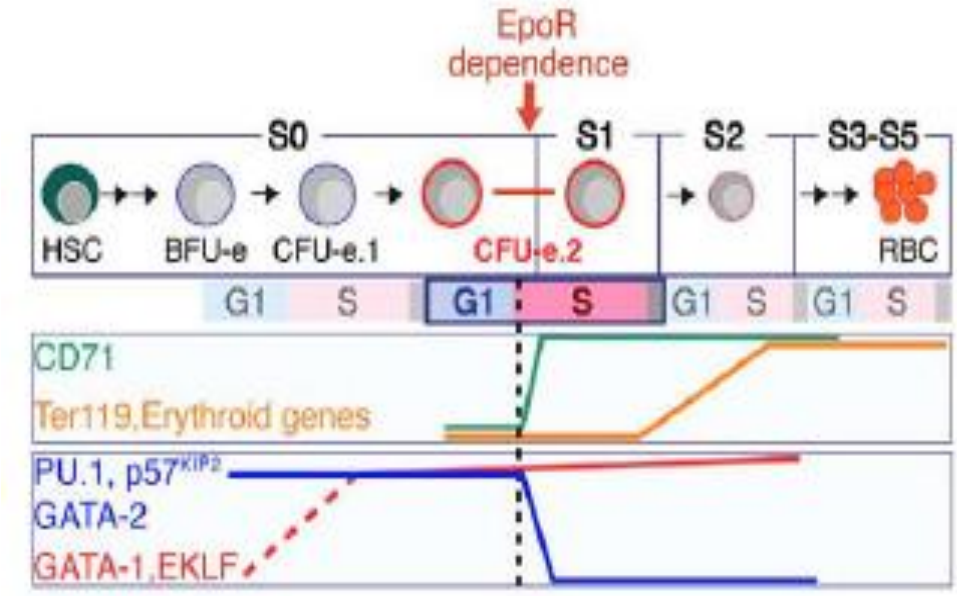

Figure 1.3 Representation of erythroid gene expression as cells undergo maturation.

Diagram was retrieved from Pop et al, *PloS Biology*, 2010; Volume 8, Issue 9.

Early hematopoietic progenitors committed to differentiation are called colony forming units (CFUs). A key intermediate in the differentiation of granulocytes, erythrocytes, monocytes and megakaryocytes is the CFU-GEMM shown in Figure 1.1. RBC differentiation from the CFU-GEMM requires erythropoietin (Epo), a major cytokine produced in the kidneys. The maturation stages of red cells and the specific cell surface antigen they express during different maturation stages is diagrammed in Figures 1.3 and 1.4. CFU-e through orthochromatic erythroblasts stages are mediated by Epo signaling.

As erythrocytes mature, they exit the bone marrow and lose their nuclei and cytoplasmic organelles. Epo exerts its effects by binding to Epo receptors (EpoR) expressed on target erythroid progenitors. It acts on these progenitors to promote proliferation, differentiation and survival. Epo binding to its receptor (EpoR) activates Janus Kinase 2 (JAK2), which subsequently phosphorylates and activates signal transducer and activator of transcription (STAT5). Figure 1.5 shows several signaling pathways are activated in response to Epo including the PLCγ, MAPK, and JNK cascades [5]. Dysregulation of signals downstream of the EpoR can promote or inhibit erythropoiesis leading to polycythemias or anemias, respectively. The selective ability of Epo to drive erythropoiesis has led to the development of recombinant forms of Epo to treat patients with anemia.

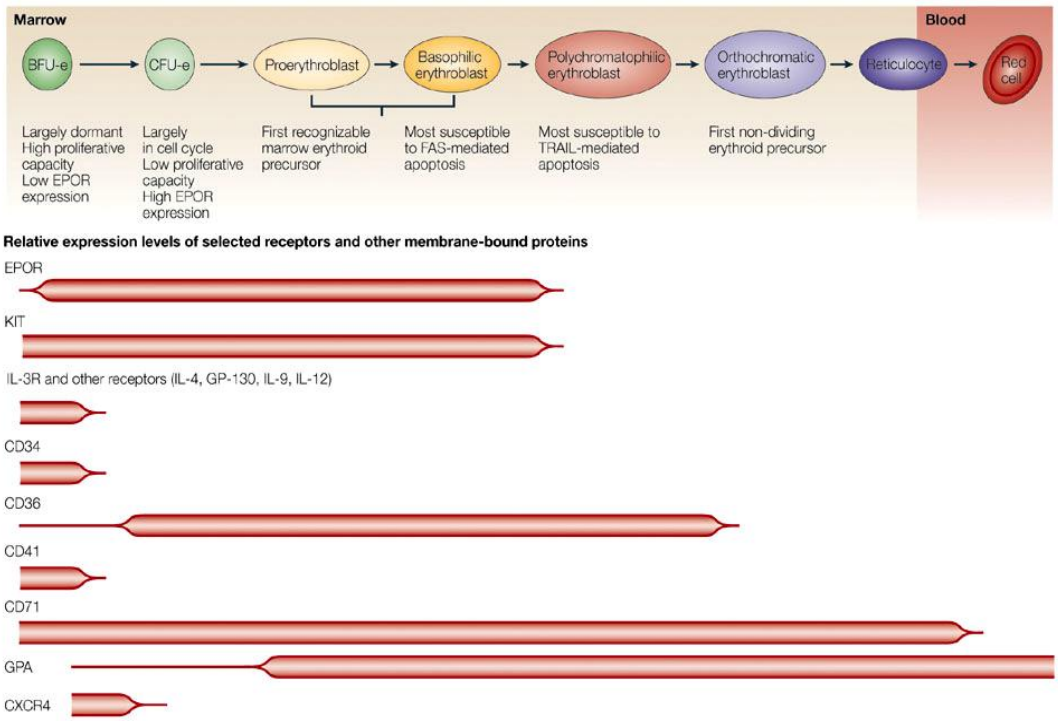

Figure 1.4 Erythroid Maturation Stages and the Relative Expression Levels of Surface Receptors and Membrane bound proteins

Diagram was retrieved from Spivak, *Nature Reviews Cancer*, 2005; Volume 5, 543-555.

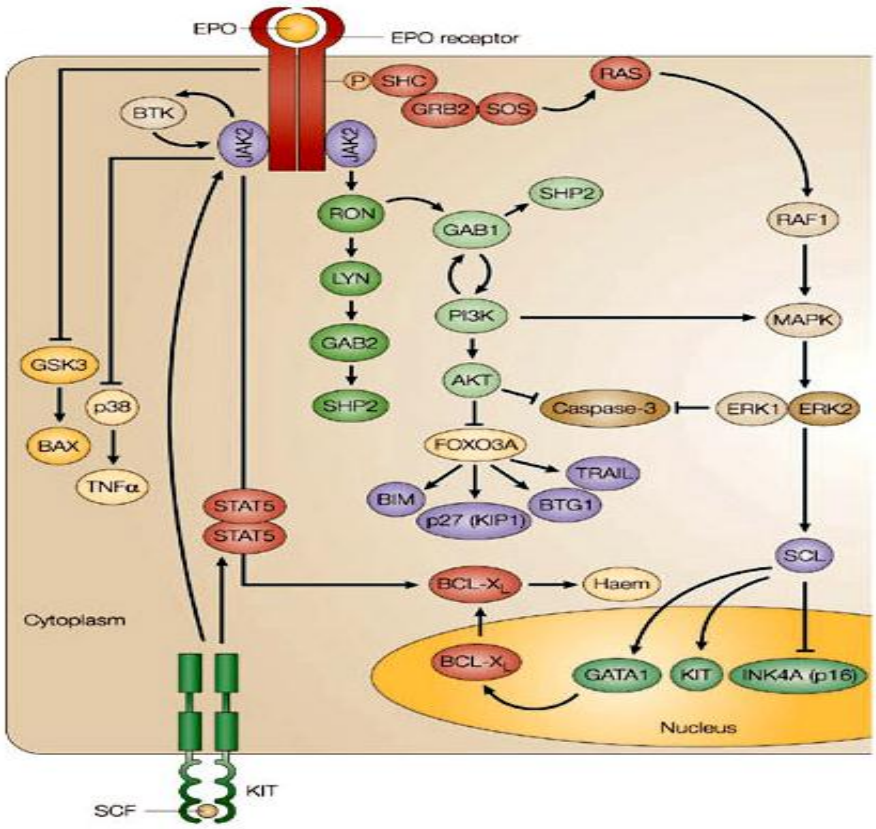

Figure 1.5: EPO Signaling Cascade

Diagram was retrieved from Spivak, *Nature Reviews Cancer*, 2005; Volume 5, 543-555.

1.3 Anemia of Chronic Disease and Inflammation

1.3.1 Clinical definition/description

More than 145 million Americans live with chronic conditions and projecting trends indicate by 2030 that number will increase by 18 percent [6]. Anemia is a common complication of malignancies, chronic inflammatory diseases, and rheumatologic diseases. The anemia that develops in these chronic diseases, such as chronic kidney disease (CKD), cancer, autoimmunity and chronic infections [7] is collectively called anemia of chronic disease and inflammation (ACDI). ACDI is a disorder of iron homeostasis in which iron becomes unavailable for heme synthesis. It is the most frequent anemia in hospitalized patients and when severe, this anemia can produce debilitating symptoms of fatigue, precluding many of the normal activities of daily life. Its adverse effects as well as an increased mortality rate [8-10] constitute a major public health problem.

Unlike hemochromatosis and iron deficiency anemia (IDA), the serum studies for ACDI patients show low serum iron, low transferrin, low total iron binding capacity, elevated serum ferritin, elevated hepcidin levels, normal transferrin receptor levels and a normocytic, and normochromic anemia. However, occasionally, the anemia can be microcytic and hypochromic. In both IDA and ACDI, anemia arises mainly due to suppression of erythropoiesis in a phenomenon known as iron restriction [11]. Iron restriction exerts its effects selectively on the erythroid lineage and impairs the responsiveness of early erythroid progenitors to Epo, the principal erythroid cytokine [11]. While ACDI and IDA are both results of impairments in iron metabolism, they do

so by two different mechanisms. IDA results from diminished total body iron stores, whereas ACDI results from hepcidin-mediated iron sequestration in inaccessible storage pools [12]. IDA is successfully managed by administration of iron and addressing the underlying cause of iron deficiency; however, ACDI is managed using a myriad of therapies, many of which are inadequate.

ACDI has been associated with red cell defects that include diminished production and shortened half-life [13]. The diminished red cell production, or impaired erythropoiesis, has been ascribed to: iron restriction, inflammatory cytokines, and diminished Epo production [13]. Many patients with ACDI also present with an inadequate formation of endogenous Epo; however, the contribution of Epo deficiency is ambiguous. In one major clinical study, ACDI patients had Epo levels no different from non-anemic controls [14]. In a second major clinical study, ACDI patients had significantly elevated Epo levels compared with non-anemic controls [15]. These studies suggest that Epo levels vary considerably within the ACDI population and Epo deficiency probably does not initiate the anemia in most cases of ACDI.

Table 1.1 The National Anemia Action Council's anemia statistics for U.S. patient populations.
http://anemia.org/resources/education-kit/anemia-statistics.php
(last updated 10/4/2010)

Chronic Disease or Condition	Cases (millions)	Anemia Prevalence
Chronic kidney disease	20	28%, in mild disease / 87% in severe disease
Chronic hepatitis C	3.2	67%, treatment related-anemia
Rheumatoid arthritis	2.1	30-60%
Inflammatory bowel disease	>1	10-72%, Crohn's / 8-74% ulcerative colitis / 17-41%, other
Chemotherapy patients		88% severe anemia (550,000 cancer deaths/yr)
HIV/AIDS		30%, HIV (22% treatment-related anemia) 75-80% AIDS

1.3.2 Role of Hepcidin

Multiple pathophysiologic mechanisms have been implicated in the development of ACDI, including elevated production of hepcidin and inflammatory cytokines, including IFNγ, TRAIL, Interleukins-1β, 6, 10, and 15, and TNFα shown in Figure 1.6 [13]. Hepcidin, an iron regulatory peptide produced by hepatocytes, contributes to anemia by causing iron retention in macrophages, which prevents iron delivery to developing erythroid progenitors. However, the mechanism that proinflammatory cytokines employ to directly inhibit erythroid differentiation is unknown. Elevated levels of hepcidin downregulate ferroportin on marrow histiocytes. Ferroportin is responsible for the export of iron from marrow histiocytes into erythroid progenitor cells and its downregulation leads to the retention of iron in the marrow during ACDI [14]. Iron refractory iron deficiency anemia (IRIDA) also shows high hepcidin expression suggesting that it shares common clincal and molecular features with ACDI [16]. Many studies have implicated IL-6 as the inflammatory mediator driving hepatocytes to increase production of hepcidin [17].

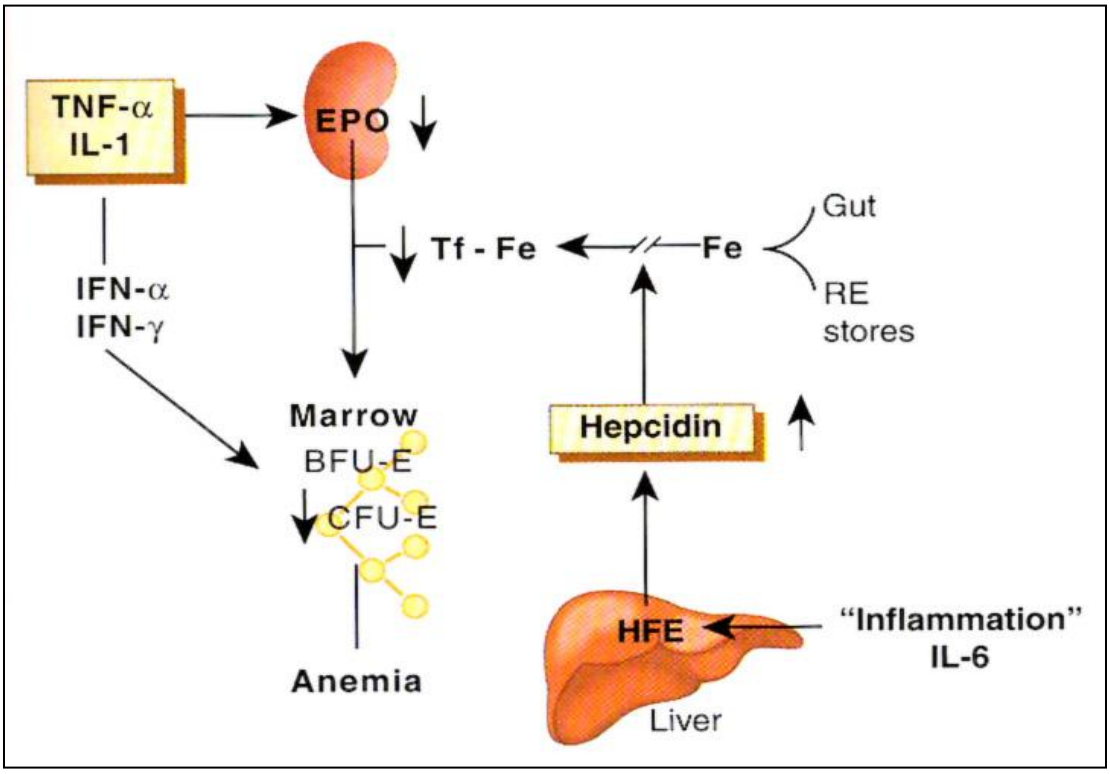

Figure 1.6 Factors contributing to ACI

Diagram was retrieved from Hematology in Clinical Practice by Hillman, Ault, Leporrier, Rinder (McGraw Hill Lange)

1.3.3 Implicated Inflammatory Activators

Underlying diseases, such as cancer, human immunodeficiency virus, or rheumatologic diseases lead to the sustained production of inflammatory cytokines, which exert cytotoxic effects on erythroid progenitor cells and disrupt iron regulation. Figure 1.6 details the multiple pathophysiological mechanisms that are involved in ACDI. Inflammatory cytokines exert cytotoxic effects on erythroid cells by increasing the production of free radicals, leading to cell damage and apoptosis. Increased uptake and retention of iron within monocytes under inflammatory conditions has been proposed as a central mechanism underlying ACDI. Ludwiczel et al propose that ACDI may be due to increased iron uptake by moncytes induced by a combined action of pro- and anti-inflammatory cytokines on transferrin receptor (TfR) mediated and non-TfR mediated iron uptake. The induced cytokines, specifically IFNγ, TNFα and IL-1 act directly on the kidney to inhibit the production of the Epo. Ludwiczek et al also demonstrated synergy between IFNγ and LPS in the down regulation of ferroportin expression results in increased storage and retention of iron [18]. Neither IFNγ nor LPS had an affect alone.

Several cytokines such as IFNγ and TNFα have been implicated to directly and indirectly suppress the differentiation of erythroid progenitor cells. Both, IFNγ and TNFα can activate the JAK/STAT, MAPK and AKT pathways and often collaborate to impair erythropoiesis. IFNγ is a heterodimer composed of binding (R1) and signaling (R2) peptides. The IFNγ receptor has an intracellular domain that serves as a docking site for JAKs. JAKs are phosphorylated when IFNγ binds to its receptor and JAKs further activate STATs. After phosphorylation and dimerization STATs are translocated to the nucleus and induce apoptotic and antiproliferative genes.

IFNγ is also involved in iron metabolism, as it has been linked to iron mobilization and direct suppression of CFU-E differentiation [19]. IFNγ induces short lived radicals and superoxide anions, which act on iron homeostasis by modulating ferritin translation, the mRNA stability of transferrin receptor and the down regulation of ferroportin transcription [14]. Reactive oxygen species (ROS) and nitric oxide (NO) damage iron sulfur cluster proteins. IFNγ can also induce ceramide production, EpoR downregulation, decreased Epo production and activity, and reduced expression of other pro-hematopoietic cytokines, such as stem-cell factor (SCF). However, none of this is clearly understood or well defined in the realm of ACDI.

TNFα independently activates NF-kB, JNK, and caspase signaling pathways. It is also a potent inhibitor of erythroid differentiation that has been implicated in Fanconi anemia, aplastic anemia and anemia of chronic disease (ACD) [20,21]. TNFα is known to cause ROS accumulation, and induces a caspase-independent, necrosis like cell death [22]. Cross talk between IFNγ and TNFα family members, such as TWEAK, has been shown by Felli et al, to upregulate Fas-L expression and increase caspase activation that subsequently suppresses erythropoiesis [23]. Mice injected with TNFα develop hypoferremia and show macrophage iron accumulation [24]. Rheumatoid arthritis (RA) patients with elevated serum TNFα and ACDI display increased apoptosis in bone marrow progentiors as opposed to normal patients.Treating these RA-ACD patients with anti-TNFα alleviated the anemia and decreased apoptosis [25].

1.3.4 Erythroid Iron Restriction Response

The erythroid iron restriction response (EIRR), involving lineage selective inhibition of erythropoiesis by diminished iron availability, functions to triage iron utilization under conditions of critical shortage. Mechanisms underlying this response comprise modulation of Epo signaling by transferrin receptors and by aconitase enzymes [26-28]. In two recent publications, we have shown that a key sensor in the EIRR consists of aconitases, which are iron-sulfur cluster-containing enzymes that convert citrate to isocitrate. The activity is dependent on an intact 4Fe-4S cluster.[29] Two human genes, *ACO1* and *ACO2,* encode two aconitase iso-enzymes. *ACO1* encodes the cytosolic aconitase 1/IRP1 and *ACO2* encodes mitochondrial aconitase. IRP1 binds to mRNAs that contain the iron response element (IRE) and represses translation. Mitochondrial aconitase is a component of the Krebs cycle and thus indirectly provides succinyl CoA for heme biosynthesis.

In response to iron deprivation, erythroid progenitors underwent lineage-specific inactivation of both mitochondrial (ACO2) and cytosolic (ACO1) aconitase isoforms. Furthermore, we identified through three lines of evidence that mitochondrial aconitase appears to function as a critical sensor of iron levels within developing erythroblasts, and its inactivation by iron deprivation triggers the iron restriction response: Firstly, knockout mice lacking cytosolic aconitase (*IRP*-/-) showed no anemia at steady state and no defects in their stress erythropoiesis response [27]. Secondly, fluoracetate, which produces fluorocitrate within mitochondria, inhibited erythropoiesis much more efficiently than fluorocitrate, which accumulates primarily in the cytosol [27]. Thirdly, shRNA

knockdown of mitochondrial aconitase, but not of cytosolic aconitase caused inhibition of erythroid differentiation [28].

High levels of aconitase activity are specifically required for erythropoiesis such that in vitro enzymatic inhibition blunts cellular responsiveness to Epo, and in vivo inhibition causes anemia [27,28]. Figure 1.7 depicts the pathway by which erythropoiesis is regulated by iron, aconitase and isocitrate. Under normal iron conditions, aconitase enzymes possess intact iron-sulfur clusters and the conversion of citrate to isocitrate can occur. Under conditions of iron restriction, these iron-sulfur clusters are destabilized, thus resulting in aconitase inactivation. Aconitase inactivation promotes protein kinase C (PKCα/β) hyperactivation, which in turn contributes to impaired viability and differentiation [28]. Providing exogenous isocitrate may prevent assembly of a repressive signal by stabilizing aconitase iron-sulfur clusters. We have shown that isocitrate in either cell culture or a murine model of iron deficiency abrogates the erythropoietic block characteristic of the erythroid iron restriction response and prevents PKCα/β hyperactivation [28]. Therefore, understanding the mechanisms underlying the erythroid iron restriction response and developing therapies to target this response are critically important.

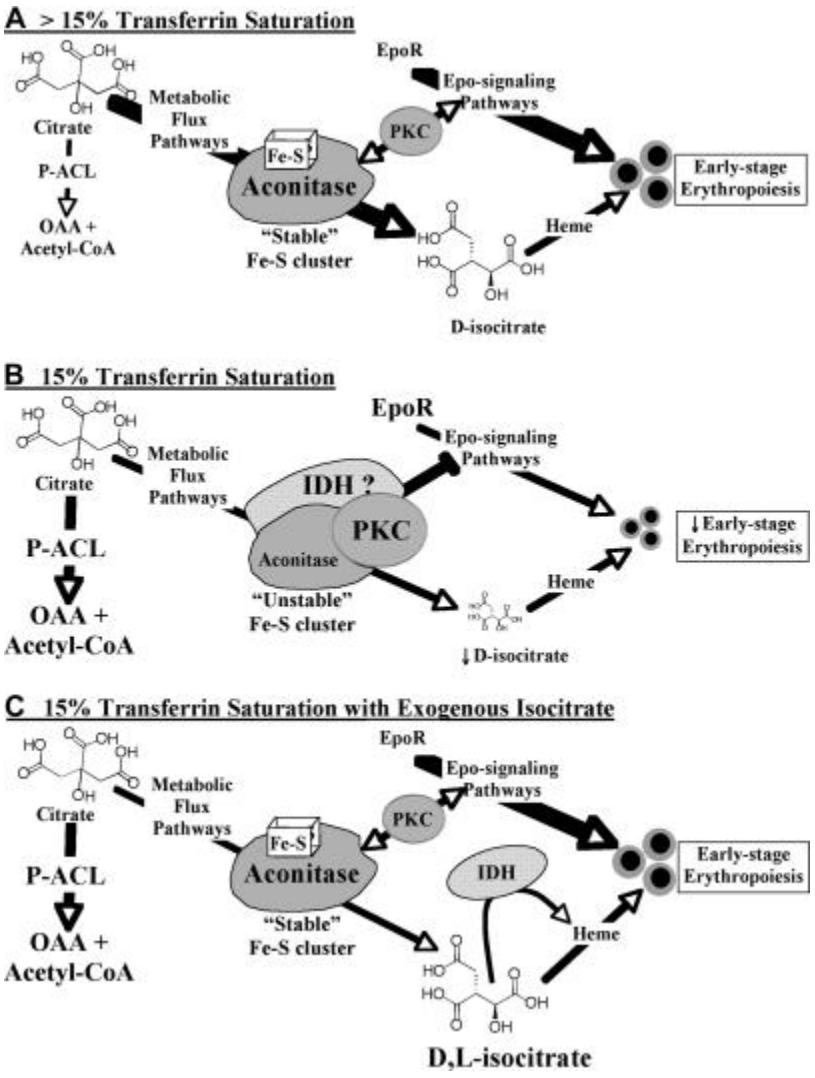

Figure 1.7 Model for erythropoietic regulation by an iron-aconitase-isocitrate pathway

Diagram retrieved from Bullock et al. *Blood*, 2010; Volume 116:97-108.

A) In the absence of iron restriction (>15%Transferrin Saturation) aconitase enzymes possess intact iron-sulfur clusters and function mainly in the conversion of citrate

to isocitrate via the “Metabolic Flux Pathway”. **B)** In the presence of iron restriction (15% Transferrin Saturation), destabilization of the aconitase iron-sulfur clusters induces assembly of a repressive signalosome which may act in part through protein kinase C (PKC) hyperactivation. In addition, diminished metabolic flux may compromise heme production and lead to shunting of citrate via activated ATP-citrate lyase (P-ACL) to oxaloacetate (OAA) and acetyl-CoA. Isocitrate may prevent assembly of a repressive signalosome via stabilization of aconitase iron-sulfur clusters and via binding to isocitrate dehydrogenase (IDH) enzymes. **C)** Isocitrate rescue (15% Transferrin Saturation with Exogenous Isocitrate) may prevent assembly of a repressive signalosome by stabilization of aconitase iron-sulfur clusters and by binding to isocitrate dehydrogenase (IDH) enzymes. In addition, exogenous D- but not L-isocitrate may support heme biosynthesis. However, exogenous isocitrate does not prevent shunting of citrate by activated ATP-citrate-lyase.

1.3.5 Current Therapies

The ideal treatment for ACDI is to treat the underlying chronic disease/inflammation successfully. However, most causes of ACDI are not curable, thus many patients with ACDI simply live with the symptoms and side effects. The adverse consequences of anemia in these patients range from activity-limiting fatigue to shortened life expectancy [8-10]. In treating these anemias, several nations spend billions of dollars per year on erythropoiesis stimulating agents (ESAs) [30,31], which consist of injectable recombinant human erythropoietin (Epo) and intravenous (IV) iron [32]. While these treatments often show efficacy in ameliorating anemia, they are expensive and pose health risks.

Epo treatment is associated with cardiovascular complications and enhancement of tumor growth in cancer patients [32,33]. A high proportion of patients show suboptimal responses to Epo as well as the development of resistance to erythropoietin [34]. IV iron poses risks for septicemia, iron overload, endothelial damage, and anaphylactic reactions [11,35,36]. As a consequence of these drawbacks, tremendous resources have been invested in designing next generation ESAs, which include hypoxia-inducible factor (HIF) stabilizers, hepcidin antagonists, Epo mimetics, and new iron formulations [7,37]. However, the advantages of such new approaches with regard to efficacy, safety, and cost remain unclear. Therefore improved understanding of the mechanisms underlying ACDI is critical to permit design of improved treatments.

1.3.6 Animal Models

The ability to study hematopoietic cells developing in their native milieu, and the capacity to examine the broad physiologic role of the erythropoietic iron regulatory mechanism in an animal model is ideal; however, until recently a concrete ACDI animal model has not been established. Early studies of ACDI used the sterile abscess model [38] or the *Toxoplasmosis Gondi* model [39]; however, these models only analyze an acute inflammatory response and often the anemia is transient, normalizing within 24-72 hours. In the last five years, three animal models have emerged assessing for ACDI: 1) heat- killed *Brucella abortus* (HKBA*)* treated mice, 2) CD70 transgenic (CD70TG) mice, and 3) peptidoglycan-polysaccharide (PGPS)-induced spontaneous relapsing arthritis in rats.

The HKBA mouse model was identified as a model of anemia of chronic inflammation as these mice exhibit hypoferremia with iron-limited erythropoiesis, blunted response to Epo and modest reduction in red cell survival in response to the injected *Brucella abortus*. Initially, this model showed that blocking hepcidin using either shRNA knockdown or antibody neutralization reduced anemia and potentiated responsiveness to Epo injections [17]. New studies of the HKBA model show that anemia still occurs in mice null for interleukin 6 (IL-6) and hepcidin [40], two important mediators of the inflammatory and iron sequestration response in ACDI. Furthermore, kinetic analysis of the response to *Brucella abortus* injection showed only transient changes in IL-6, hepcidin, and serum iron, with levels reverting to normal within the first 24 hours, despite the anemia lasting for up to 7 weeks [40]. Thus this model is no longer accepted as a model of ACDI, but is a valid model of anemia of acute inflammation (AI).

The CD70TG mouse model is the newest model, published in 2011. CD70TG mice overexpress TNF-superfamily member CD70 on B cells, which induces high numbers of IFNγ producing CD4 and CD8 T cells. The chronic immune activation observed in these mice disrupts hematopoiesis and induces a progressive anemia, showing significant decreases in hemoglobin (Hb) and hematocrit (Hct) at 12 weeks of age. These mice also show impairment in erythroid bone marrow output [41]. While this model is an excellent tool to fully understand the implications of a single inflammatory stimulus, IFNγ, its major weakness is that there are no observed alterations in the key ACDI contributor, hepcidin.

The rat arthritis model is the principal rodent model for ACDI due to the degree and duration of the anemia, contrasting with the mild, transient anemias in most murine models. The anemia that results from the peptidoglycan-polysaccharide (PGPS) spontaneous relasping inflammatory arthritis rat model was initially characterized by Sarot *et al*, in 1989 [42]. Six week old female Lewis rats are induced via intraperitoneal administration of PGPS fragments. These animals experience significant drop in hemoglobin at two weeks that lasts up to sixteen weeks as seen in Figure 1.8. Furthermore, the rat arthritis model had been used by Amgen for preclinical validation of Darbepoetin [43]. Characterization of this model by Weiss and colleagues revealed hematologic and ferrokinetic changes in anemic rats identical to those in human ACDI [44].

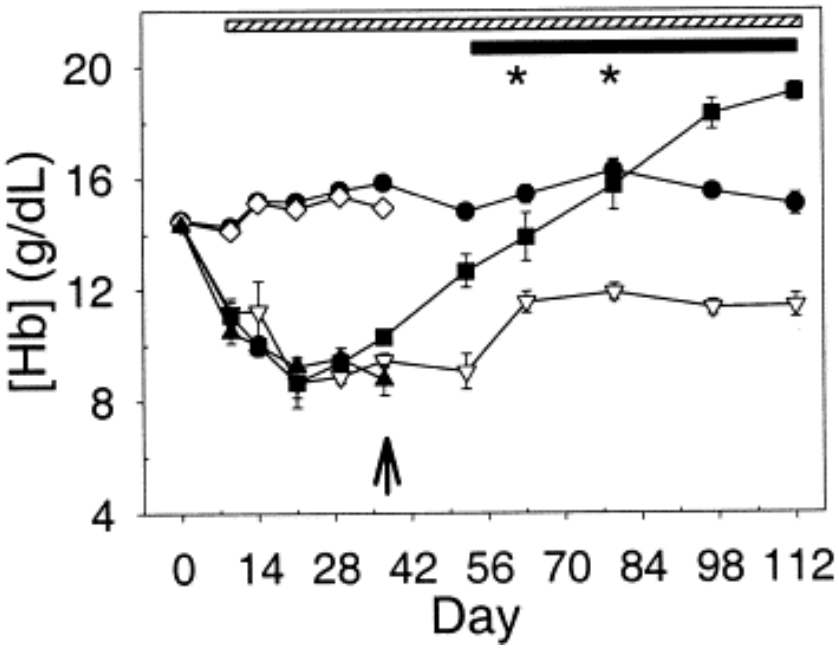

Figure 1.8 PG-APS–induced chronic ACD can be alleviated by infrequent darbepoetin alfa treatment. Diagram was retrieved from Coccia et al, *Experimental Hematology*, 2001; Volume 29 Issue 10; 1201-1209. Black circles and open diamonds: Control rats. Black triangles and open inverted triangles: Anemic rats. Black squares: darbepoetin alfa–treated rats. Arrow: Initiation of darbepoetin alfa treatment. Hatched bar: Significant anemia in the untreated rats ($p\max < 0.01$). Black bar: Significantly greater ($p\max < 0.04$) Hb levels in darbepoetin alfa–treated rats compared to anemic rats. Asterisks: Days darbepoetin alfa–treated rats achieved baseline Hb levels. Error bars are ± SEM

1.4 Thesis Objectives

The current paradigm of ACDI pathogenesis emphasizes the role of EIRR, mediated by iron sequestration increased hepcidin production [7]. In support of this paradigm, patients with ACDI consistently have diminished serum iron levels, increased stainable storage iron, and diminished stainable iron in erythroblasts [45]. The degree to which erythroid iron restriction contributes to ACDI is a question of scientific and clinical importance. Several findings argue against iron restriction as a sole causal factor. Firstly, anemias caused purely by iron deficiency manifest with red cells that are small and poorly hemoglobinized, while the red cells in ACDI typically display normal size and hemoglobin levels [46,47] Secondly, patients with ACDI have not consistently been shown to have increased serum or urinary hepcidin levels [15,48]. Thirdly, non-ACDI anemias associated with massive hepcidin overproduction due to hepatic adenomas or germline mutations in *TMPRSS6* resemble iron deficiency anemia with hypochromic microcytic red cells [49,50]. Similarly, murine models have shown differences in the anemias associated with inflammation compared to those due to transgenic hepcidin expression [51]. On the other hand, compelling evidence suggests that iron restriction plays some role in ACDI, for examples, IV iron infusion effectively ameliorates anemia in numerous patients with ACDI [7,11,13]. Furthermore, pharmacologic blockade of hepcidin induction in arthritic rats showed efficacy in reversal of anemia [52].

Thus, the two central objectives we seek to elucidate are: 1) the precise role of erythroid iron restriction response in ACDI, and 2) how to manage severe ACDI unresponsive to ESAs. In this respect, investigations using hematopoietic stem cell

culture systems and a physiologically relevant rat model of ACDI have enhanced our understanding of this common disorder.

Chapter 2:

Isocitrate ameliorates anemia by suppressing the erythroid iron restriction response

Chanté L. Richardson, Lorrie L. Delehanty, Grant C. Bullock, Claudia M. Rival, Kenneth S. Tung, Donald L. Kimpel, Sara Gardenghi, Stefano Rivella, Adam N. Goldfarb

(Modified from the version published in Journal of Clinical Investigation**)**

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ABSTRACT

Multiple soluble mediators have been implicated in erythropoietic suppression, including hepcidin [12] and various inflammatory cytokines including IFNγ and TNFα [13,53]. Their mechanism involves perturbation of iron metabolism causing erythroid iron restriction, but they also may directly inhibit erythropoiesis. The molecular basis for perturbed iron metabolism in ACDI consists of increased liver production of hepcidin, resulting in downregulation of the iron exporter ferroportin expressed on histiocytes that recycle iron from senescent red cells and on enterocytes that absorb dietary iron [14,54]. The discovery of this pathway has provided a breakthrough allowing development of new diagnostic and therapeutic approaches to ACDI [55]. In the current studies, a brief course of isocitrate treatment durably corrected anemia in the rat arthritis model of ACDI, implicating the erythroid iron restriction response, and more specifically aconitase inactivation, as essential in anemia development. Ex vivo experiments further demonstrated that the erythroid iron restriction response exerted a potent influence on the response of human erythroid precursors to certain inflammatory cytokines. Specifically, iron restriction sensitized cells to the inhibitory influence of IFNγ or TNFα, and isocitrate treatment blocked this sensitization. The signaling relationship between iron restriction and IFNγ pathways involved convergent regulation of PU.1, a myeloid transcription factor whose repression constitutes a critical commitment step in erythroid differentiation [56]. Recently, PU.1 upregulation has been identified as a critical component in a murine model of ACDI [41]. Our data show specifically that iron restriction via PKC signaling cooperated with IFNγ in upregulating PU.1 in early erythroid progenitors, an effect that was blocked by isocitrate treatment. These findings identify a pathway in which iron

restriction may contribute to ACDI through potentiating the influence of inflammatory signaling on a core component of the erythroid transcriptional program. Targeting this pathway offers new therapeutic approaches with potential advantages over current treatment regimens

RESULTS

Isocitrate treatment corrects anemia and erythropoietic defects in rodent ACDI model

Isocitrate treatment abrogates the erythroid iron restriction response in cell culture and animal models of iron deprivation [28]. Because the erythroid iron restriction response may also contribute to ACDI [53], we determined the effects of isocitrate administration in a rat arthritis model that faithfully recapitulates human ACDI [43,44]. In this model, a single injection of Streptococcal peptidoglycan-polysaccharide (PG-PS) induces chronic arthritis associated with stable normochromic, normocytic anemia presenting 2 weeks post injection [42]. The pilot trial compared 10 daily injections of trisodium isocitrate versus saline solution, beginning day 14 post PG-PS. In this trial, isocitrate treatment corrected the anemia after the initial 5 injections, and the correction was sustained for at least16 days beyond the last injection (Figure 2.1A). In a second trial, 3 injections of isocitrate sufficed for correction of anemia to the end of the study, 28 days post treatment (Figure 2.1B, Table 2.1). Associated with correction of anemia, isocitrate treatment induced a significant reticulocyte response consistent with enhancement of erythropoiesis (Figure 2.1C).

To further assess effects of isocitrate on erythropoiesis, rat marrows underwent flow cytometry 4 days post treatment as in Figures 2.1B, C. With currently available antibody reagents, normal rat erythroid progenitors can be distinguished as a discrete $CD71^{+}$ $CD11b^{-}$ marrow population, while maturing myeloid cells express bright CD11b and variable CD71, and early progenitors lack both markers (Figure 2.1D, top row). Marrows from saline-treated animals with ACDI contained decreased proportions of erythroid progenitors, increased myeloid cells, and a novel population of $CD11b^{Dim}$ $CD71^{+}$ cells (Figure 2.1D, middle row). Isocitrate treatment corrected these marrow abnormalities and restored the discrete $CD71^{+}$ $CD11b^{-}$ erythroid population seen in normal controls (Figure 2.1D, bottom row). Isocitrate significantly enhanced both percentages and absolute numbers of marrow erythroblasts in PG-PS injected animals (Figure 2.1E). An additional marrow abnormality seen in ACDI was increased erythroid cell death, which showed partial reversal by isocitrate treatment, although this effect did not attain statistical significance (Figure 2.2A). No alterations in erythroblast cell cycle distribution occurred in any of the experimental groups (Figure 2.2B). To further assess the impact of isocitrate on bone marrow erythropoiesis, we performed colony forming assays. As shown in Figure 2.2C, isocitrate treatment of PG-PS injected animals significantly enhanced both frequency and total numbers of BFU-e, as well as enhancing frequency of CFU-e**.** Enhancement of erythropoiesis promotes repression of hepcidin in the liver [57]. Consistent with a therapeutic mechanism involving enhancement of erythropoiesis, isocitrate treatment was associated with significantly decreased liver hepcidin mRNA levels (Figure 2.1F).

The results in Figure 2.1 combined with previously published in vitro data [28] suggest that isocitrate ameliorates anemia in ACDI through promoting erythropoiesis, most likely acting directly on erythroid progenitors. However, isocitrate could potentially exert indirect effects, such as induction of Epo or suppression of inflammation. Measurement of serum Epo levels in animals from Table 2.2 showed no evidence of induction by isocitrate.

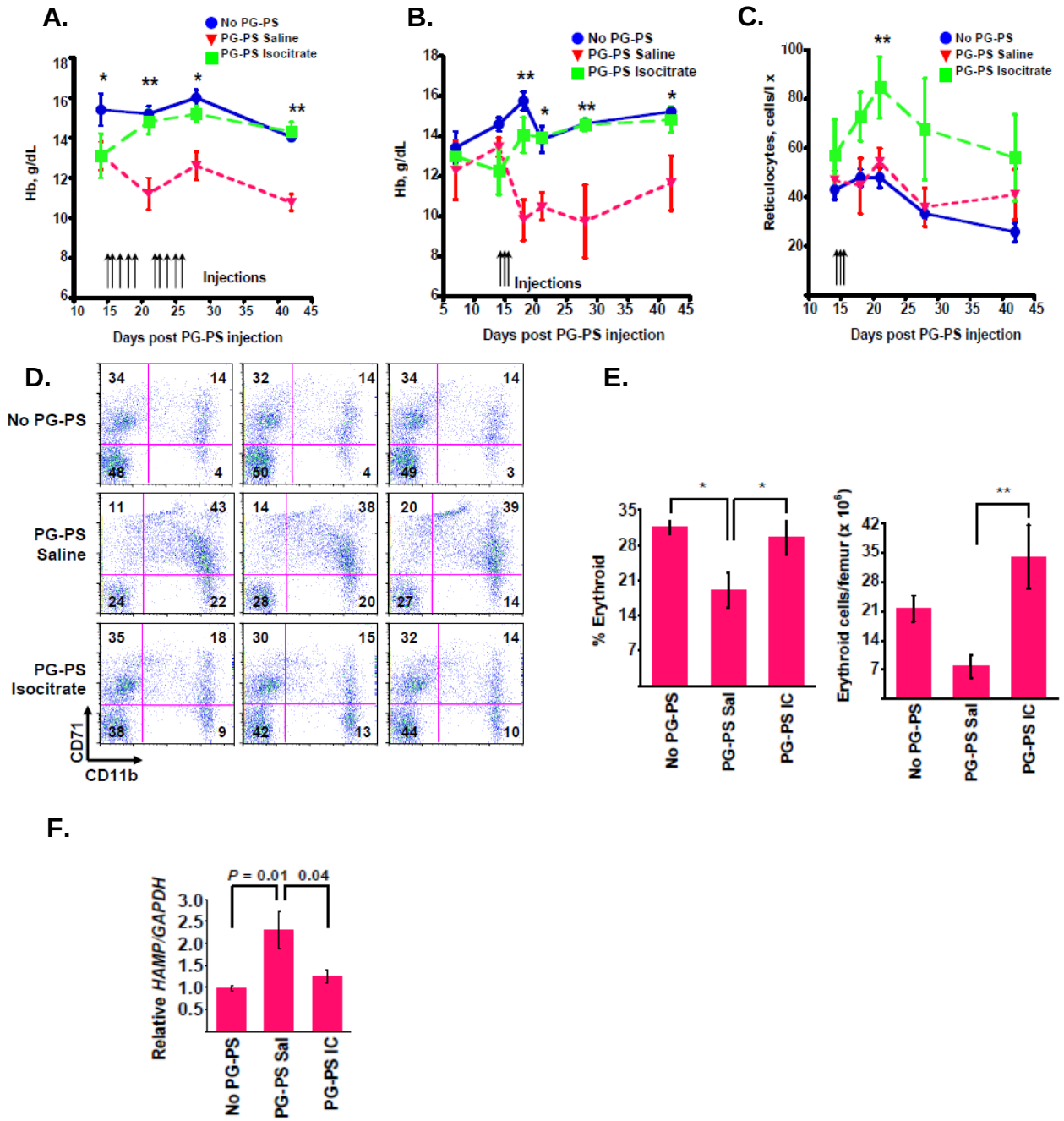


Figure 2.1 Isocitrate injections correct anemia and defective marrow erythropoiesis in a rat arthritis model of ACDI. (**A**) Normalization of peripheral blood hemoglobin levels (Hb) with 10 injections of isocitrate (IC). Arthritis was induced by injection of PG-PS on day 0, and

treatment with either IC (green) or saline (red) initiated on day 14. Non-anemic controls are shown in blue. N = 4/group for IC and 5/group for saline and non-anemic controls. (**B**) Sustained anemia correction with 3 IC injections. Experiment was conducted as in panel **A** with N = 5/group. (**C)** Peripheral blood reticulocyte counts in animals from panel **B**. (**D**) Correction of marrow erythroid defects with 3 IC injections. Animals treated as in (B) were euthanized on day 21 for marrow analysis by flow cytometry. Shown are 3 representative animals from each group. (**E**) Composite of data from panel **D**. Percentage and number of marrow erythroid cells ($CD71^+$ $CD11b^-$). N = 5/group. (**F)** Normalization of hepcidin expression with IC treatment. Animals treated as in panel **B** were euthanized on day 42 followed by qPCR analysis of liver hepcidin (*HAMP*) mRNA levels. Results are normalized to *GAPDH* and expressed relative to levels in non-inflamed controls. N = 5/group. All data are mean $\pm$ s.e.m., *$P < 0.05$, **$P < 0.01$

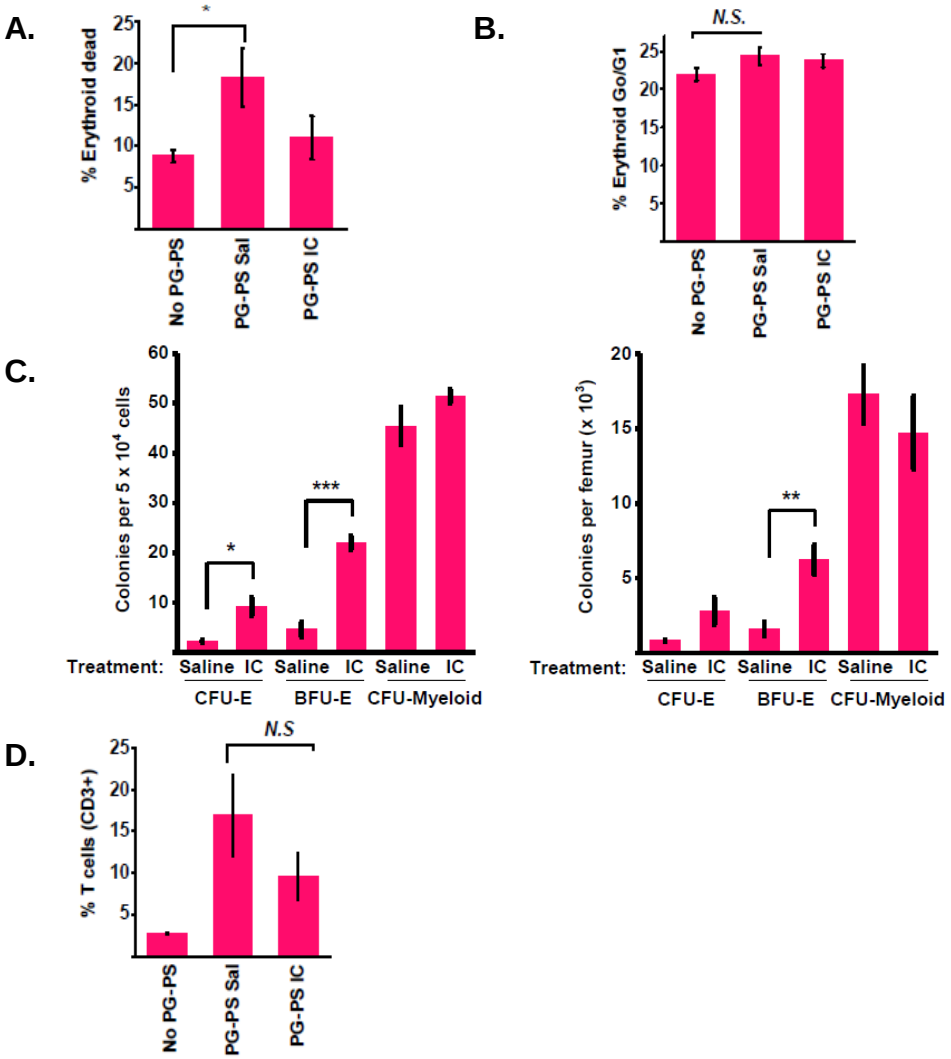


Figure 2.2 Additional studies of marrow from rats in Figure 2.1D, E. (**A**) Cell death in erythroid precursors from control and arthritic animals ± isocitrate treatment. Marrow samples (see Figure 2.1D) were costained with anti-CD71, annexin V, and 7-AAD followed by flow cytometry. Shown are percentages of $CD71^{Bright}$ SSC^{Low} cells that costain with annexin V and 7-AAD. N = 5/group. (**B**) Cell cycle profiles in erythroid precursors from control and arthritic animals ± isocitrate treatment. Marrow samples (Figure 2.1D) were stained with anti-CD71 and propidium iodide (PI) followed by flow

cytometry. Shown are percentages of $CD71^{Bright}$ SSC^{Low} cells in Go/G1-phase N = 5/group. (**C**) Isocitrate treatment of rats with ACDI enhances marrow erythroid colony forming activity. Animals treated as in Figure 2.1B were euthanized on day 21 for marrow analysis by colony forming assays. Colonies were analyzed on day 8 post seeding of 5×10^4 marrow cells in 1 mL Methocult M3434 (Stem Cell Technologies) in 35-mm plates. For each animal duplicate cultures were performed. **(D)** The animals from Figure 1D underwent assessment of marrow infiltration by T cells ($CD3^+$). For all graphs: N = 4-5/group. All data are mean $\pm$ s.e.m., $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Table 2.1: Complete blood count (CBC) parameters in ACDI rodent model

	Day 7		Day 14		Day 18			Day 35		
	Saline	PG-PS	Saline	PG-PS	Saline	PG-PS + Saline	PG-PS + IC	Saline	PG-PS + Saline	PG-PS + IC
RBC (M/µL)	8.77 ± 0.9	7.49 ± 0.1	8.03 ± 0.2	6.85* ± 0.3	7.54 ± 0.2	6.1* ± 0.2	8.11# ± 0.6	9.51 ± 1	7.51 ± 0.2	8.51# ± 0.2
HB (g/dL)	15.04 ± 0.3	13.86** ± 0.1	15.38 ± 0.2	12.68** ± 0.7	14.18 ± 0.3	10.8*** ± 0.3	14.08# ± 0.9	14.4 ± 0.3	12.04*** ± 0.2	14.18# ± 0.6
HCT (%)	54.36 ± 5.8	44.86 ± 0.8	44.92 ± 0.8	39.74* ± 2.3	46.42 ± 1	32.6*** ± 1.1	48.76 ± 3	57.06 ± 5.6	39.08* ± 0.8	48.9 ± 2.9
MCV (fL)	61.88 ± 0.3	59.85*** ± 0.4	61.3 ± 0.5	57.74** ± 1	61.6 ± 1.2	53.45*** ± 0.6	60.26### ± 0.8	60.12 ± 0.3	52.18* ± 2.2	55.23 ± 2.7
WBC (K/µL)	10.1 ± 0.9	21.53** ± 3.1	12.21 ± 0.4	28.27* ± 5.4	9.79 ± 0.6	39.14** ± 2.7	19.94 ± 7.4	9.68 ± 1.1	37.29* ± 10	14.08 ± 2.9
NEU (K/µL)	2.56 ± 0.3	14.58** ± 3.1	3.68 ± 0.3	20.72** ± 5.4	2.59 ± 0.1	29.35*** ± 1.7	11.89# ± 6.7	2.15 ± 0.4	27.15* ± 8.5	6.03 ± 2.8
MONO (K/µL)	0.63 ± 0.1	0.83 ± 0.1	0.68 ± 0.1	0.85 ± 0.2	0.46 ± 0.1	1.12* ± 0.1	0.77 ± 0.2	0.38 ± 0.1	0.99 ± 0.2	0.52 ± 0.2
PLT (K/µL)	1017 ± 387	687 ± 136	728.8 ± 13	986 ± 165	641.6 ± 44	839 ± 223	692.6 ± 96	758.2 ± 80	1256.4 ± 219	808.75 ± 136

PG-PS was injected on day 0. RBC, red blood cell count; HB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; WBC, white blood cell count; MONO, monocyte count; NEU, neutrophil count, PLTs, platelet count. N=5/group. Saline v. PG-PS,*P< 0.05, **P <0.01, ***P < 0.001; PG-PS + Saline v. PGPS + IC, #P <0.05. ##P<0.01, ###P<0.001 All data mean ± s.e.m.

Table 2.2: Serum studies in rat ACDI model

	Day 7		Day 18		
	Saline	PG-PS	Saline	PG-PS + Saline	PG-PS + IC
Serum Epo (pg/mL)	0.09 ±0	0.1 ±0	0.08 ±0	0.18 ±0	0.16 ±0
	Day 7				
	Saline	PG-PS			
Serum IFNγ (pg/mL)	0.94 ±0	2.18*** ±0			
	Day 14		Day 35		
	Saline	PG-PS	Saline	PG-PS + Saline	PG-PS + IC
Serum Fe (µg/mL)	445.9 ±81	353.4* ±40	335.7 ±34	197.24 ± 73	376.61 ±28

PG-PS was injected on day 0. N=5/group. *P< 0.05, **P <0.01, ***P < 0.001. All data are mean ± s.e.m.

Evidence that isocitrate does not act as an anti-inflammatory agent

Isocitrate potentially could promote erythropoiesis indirectly through an anti-inflammatory mechanism. However, several findings argue against this possibility. Firstly, at a time point when isocitrate significantly enhanced erythropoiesis in the rat ACDI model (Figs. 2.1C,D), it had no effects on splenic infiltration by myeloid cells (Figs. 2.3A,B.) or marrow infiltration by T cells (Fig. 2.2D.). Secondly, isocitrate injections did not affect the rapid-onset neutrophilia/anemia seen in a murine model of acute inflammation, involving intraperitoneal injection of heat-killed *Brucella abortus* [17] (Fig. 2.4D). Thirdly, isocitrate had no effect on the degree of end-organ inflammation in three different models of autoimmune disease (spontaneous autoimmune orchitis, orchitis induction by regulatory T cell depletion plus vasectomy [58], neonatal autoimmune oophoritis [59]) (Figs. 2.4A,B,C). In the rodent ACDI model, isocitrate did diminish circulating neutrophil levels (Table 2.1), but this effect is likely secondary to its enhancement of erythropoiesis. Specifically, this correction of neutrophilia may be secondary to anti-inflammatory effect of mobilizing iron [60,61], as we observe a decrease in hepcidin levels with isocitrate treatment in Fig. 2.1F, and/or due to isocitrate's potential ability to exert its effects at the level of marrow lineage reprogramming [62].

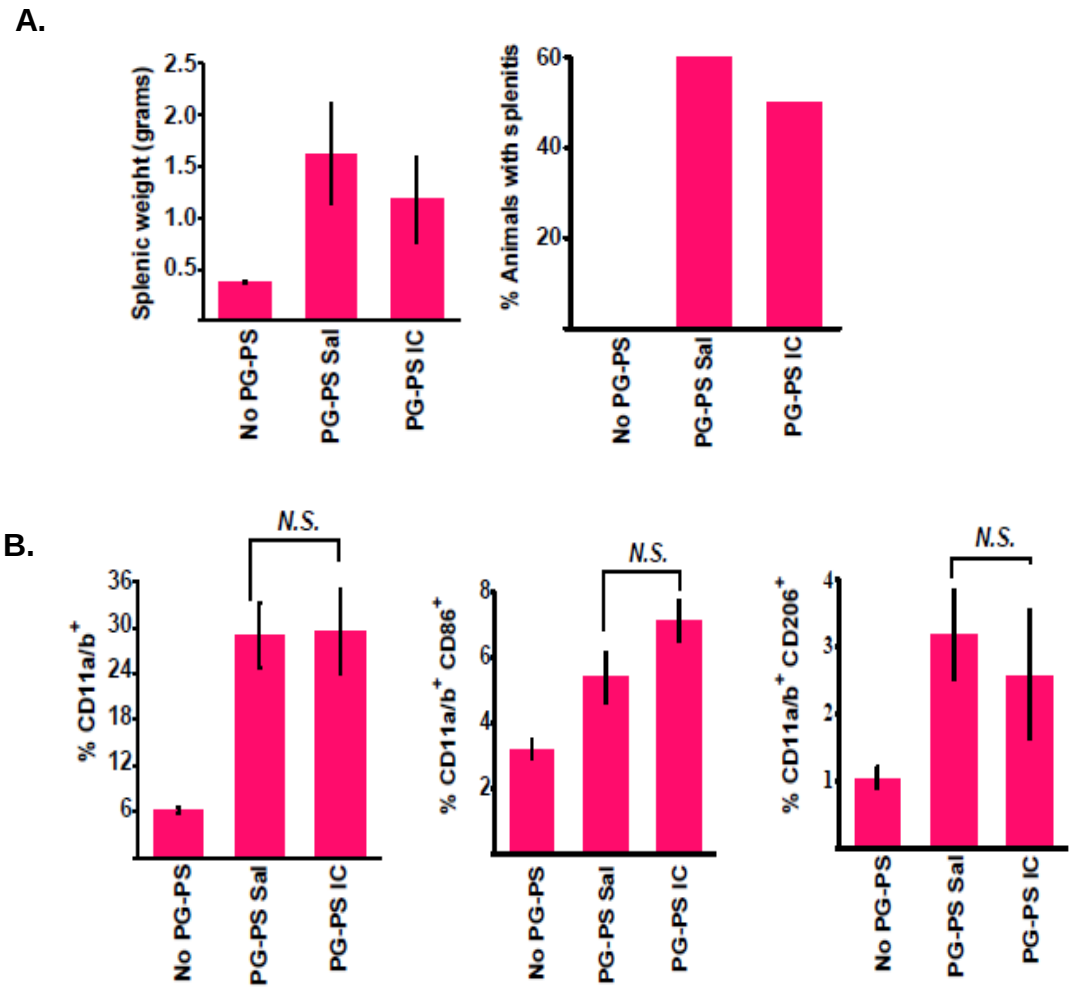


Figure 2.3 Analysis of the effects of IC treatment on splenic changes in the rat ACDI model. (**A**) The animals from Figure 2.1D underwent assessment of spleens for weight, splenitis defined by the presence of granulomatous inflammation, and extent of infiltration by myeloid cells. Granulomatous inflammation was detected by light microscopy of hematoxylin and eosin stained tissue samples. **(B)** Infiltration by total myeloid cells (CD11a/b^{+}) and by macrophage subsets M1 (CD86^{+}), and M2 (CD206^{+}) was determined by flow cytometry. For all graphs: N = 5/group. All data are mean $\pm$ s.e.m.

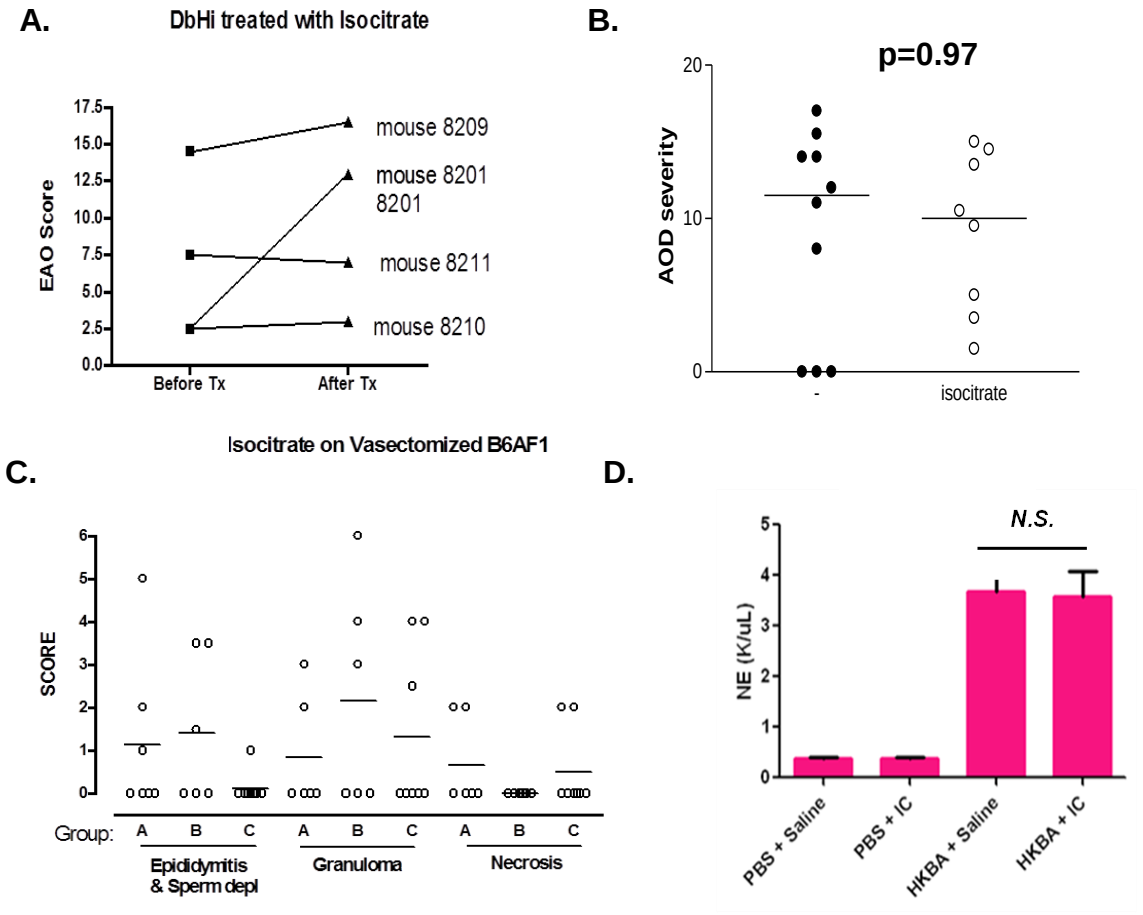


Figure 2.4 Analysis of the effects of IC treatment in multiple murine models of inflammation. (**A**) Neonatal autoimmune ovarian disease (nAOD) elicited by autoantibody injection. As described by Setiady et al [59], B6AF1 pups received injections of the anti-ZP3 antibody on post-natal days 3 and 5, followed by blinded scoring of ovarian inflammation (AOD severity) on post-natal day 14. Isocitrate treatment consisted of daily injections of 0.4 mg/animal on post-natal days 3-10. (**B**) Spontaneous autoimmune orchitis in OVA/OVA-TCR double transgenic mice (developed by Dr. Kenneth Tung, University of Virginia, Unpublished). Adult males transgenic for ovalbumin expression in male haploid germ cells and for T cell receptor specific for an ovalbumin epitope (DO11.10) underwent unilateral orchiectomy followed by a 10 day

treatment course of IC at 200 mg/kg/day followed by removal of the contralateral testis. Pre- and post-treatment specimens were blindly scored for inflammation (Experimental Autoimmune Orchitis Score) as described [58]. (**C**) Epididymitis associated with vasectomy and regulatory T cell depletion. As described by Wheeler et al [58] adult B6AF1 male mice underwent bilateral vasectomy followed by anti-CD25 mediated regulatory T cell depletion. 3 weeks post vasectomy, animals were assessed for epididymal inflammation, sperm depletion, granuloma formation, and necrosis, as described [58]. Group A received daily IC at 200mg/kg/day for 3 weeks; Group B received a similar regimen for 2 weeks; and Group C consisted of saline treated controls. (**D**) Neutrophilia caused by HKBA is unaffected by isocitrate treatment. As per Sasu et al [17] adult C57BL/6 mice received a single intraperitoneal injection of heat-killed *Brucella abortus* on day 0. Day 11-15, mice received intraperitonteal injection of 200mg/kg/day of isocitrate or equivalent volume of saline daily. For all graphs: N = 4-10/group. All data are mean $\pm$ s.e.m., $*P < 0.05$

Iron restriction sensitizes erythroid progenitors to interferon gamma

To determine whether intrinsic inflammatory signaling could cooperate with the erythroid iron restriction response in erythropoietic repression, mediators previously implicated in ACDI [13] were screened for effects on primary human progenitors in erythroid medium ± iron restriction and isocitrate. These experiments yielded three unexpected findings. Firstly, under iron replete conditions, i.e. 100% transferrin saturation (TSAT), major erythroid inhibition occurred with none of the mediators (Table 2.3). Secondly, under conditions of iron restriction (15% TSAT), IFNγ and TNFα potently inhibited erythroid development. Thirdly, isocitrate (IC) conferred resistance to IFNγ and TNFα under conditions of iron restriction (Figure 2.5A, Figure 2.6). The relevance of these findings to ACDI is suggested by prior implication of IFNγ in erythropoietic repression in human chronic kidney disease and in multiple animal models of anemia [19,41,63]. In addition, interferon signaling pathways are known to participate in erythroid inhibition by TNFα and IL-1, and in human idiopathic refractory anemia [64,65]. In the rat arthritis model of ACDI increased serum IFNγ and decreased serum iron were observed (Table 2.2).

In multiple repeat experiments**,** iron restriction reproducibly sensitized human erythroid progenitors to inhibitory effects of IFNγ on differentiation and, to a lesser extent, on proliferation. Although we did observe an inhibitory effect on viability with iron restriction alone, IFNγ did not cause any additional inhibition in viability when combined with iron restriction (Figure 2.5B). Importantly, exogenous isocitrate conferred complete IFNγ-resistance on iron deprived erythroid progenitors. These findings confirm that iron availability determines erythroid progenitor response to IFNγ and support a

direct effect of isocitrate in promoting erythropoiesis. Even transient isocitrate exposure for only the first 24 hours of culture completely rescued erythropoiesis on day 4 (Figure 2.5C), suggesting that the erythroid iron restriction response and isocitrate exert durable influences during an early developmental window.

Table 2.3: Effects of inflammatory stimuli on erythroid differentiation

Stimulus	Dose	Effects (100% TSAT)	Effects (15% TSAT)
IFNγ	1500 U/mL	NONE	*INHIBITION*
TNFα	100 ng/mL	NONE	*INHIBITION*
TRAIL	500 ng/mL	NONE	NONE
IL-1β	100 ng/mL	NONE	NONE
IL-6	100 ng/mL	NONE	NONE
IL-10	100 ng/mL	NONE	NONE
IL-15	100 ng/mL	NONE	NONE
LPS	50 µg/mL	NONE	NONE

The effects of indicated stimuli on erythroid differentiation were tested as described in **Fig. 2.5A, B**.

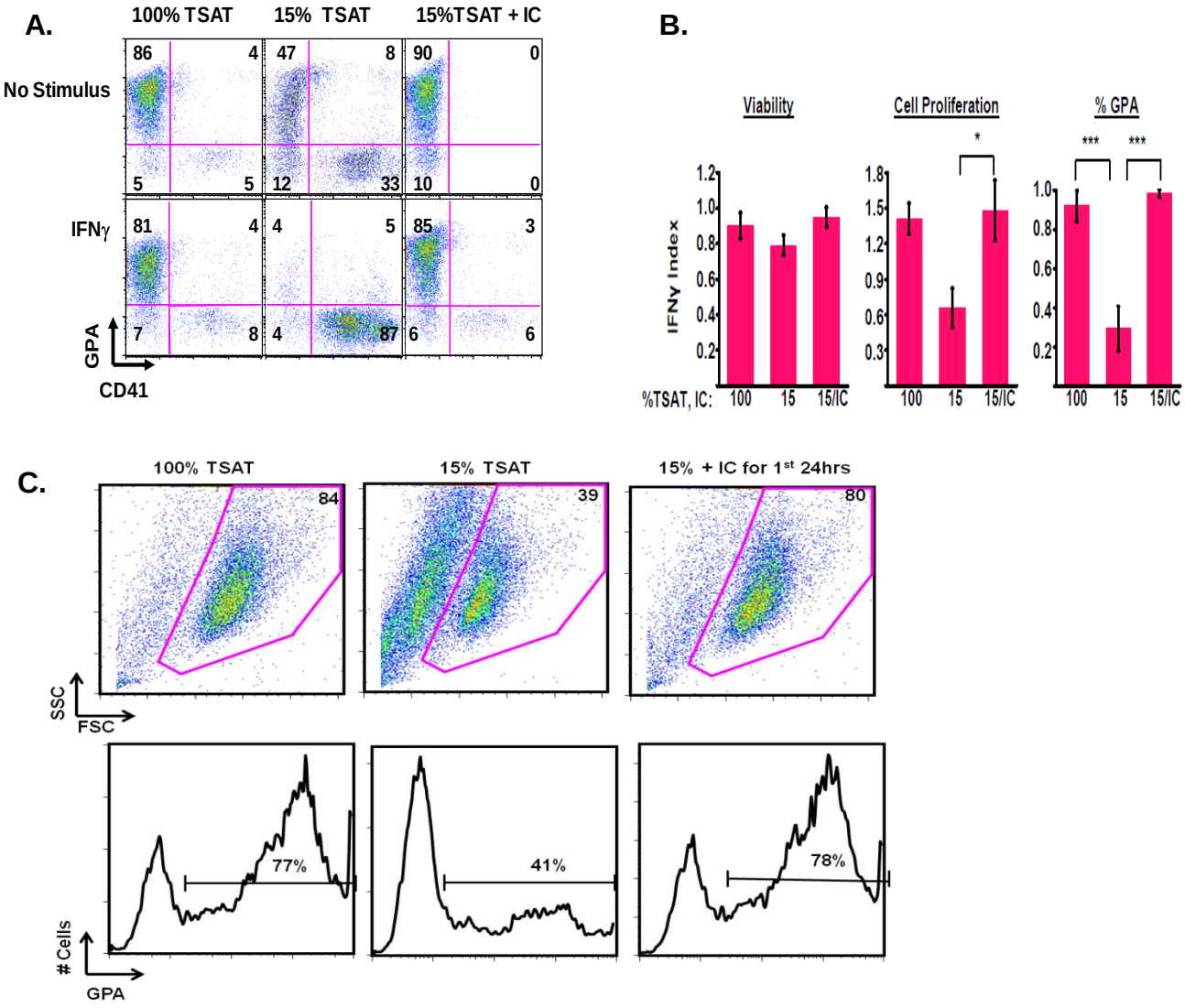


Figure 2.5 Iron restriction and isocitrate oppositely modulate the responsiveness of erythroid progenitors to the inflammatory cytokine IFNγ. Human CD34^{+} primary progenitors were cultured 5 days in erythroid medium with transferrin saturations (TSAT) of 100%, 15%, or 15% + isocitrate (IC). Where indicated, cultures also contained human IFNγ. (**A**) The cooperative inhibition of erythroid differentiation by iron restriction and IFNγ is reversed by IC treatment. Cells stained with fluorescent antibodies

to the erythroid antigen glycophorin A (GPA) and to CD41 were analyzed by flow cytometry, with percentages of positive cells indicated. (**B**) Summary graphs of 4 independent experiments conducted as in panel **A**, showing mean ± s.e.m. for IFNγ response index for viability, proliferation, and differentiation. This index consists of the ratio of values obtained in cultures with IFNγ divided by values obtained in cultures without IFNγ. Thus values >1 represent a positive effect of IFNγ, and values <1 represent an inhibitory effect. (**C**) Transient IC exposure suffices for complete rescue of differentiation. Human progenitors were cultured in erythroid medium with 15% TSAT and IFNγ. Where indicated, IC was included in the medium for the first 24 hours of culture followed by wash out and continuation in IC-free erythroid medium with 15% TSAT and IFNγ. Cells on day 5 underwent flow cytometry for GPA expression with gating on the viable fraction. N=3, All data are mean ± s.e.m., *$P < 0.05$, **P <0.01, ***$P < 0.001$.

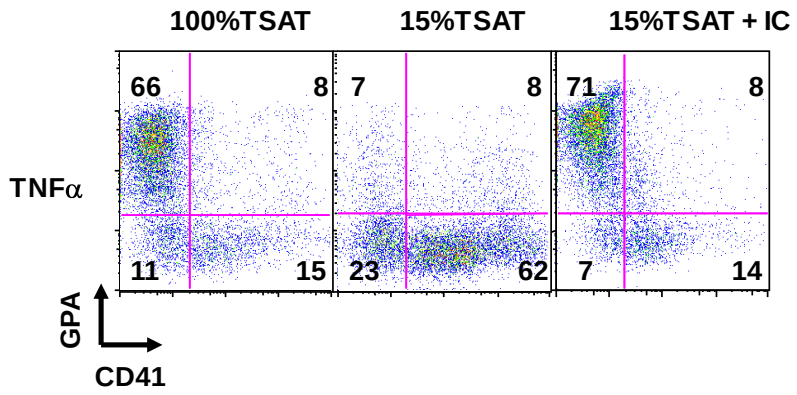
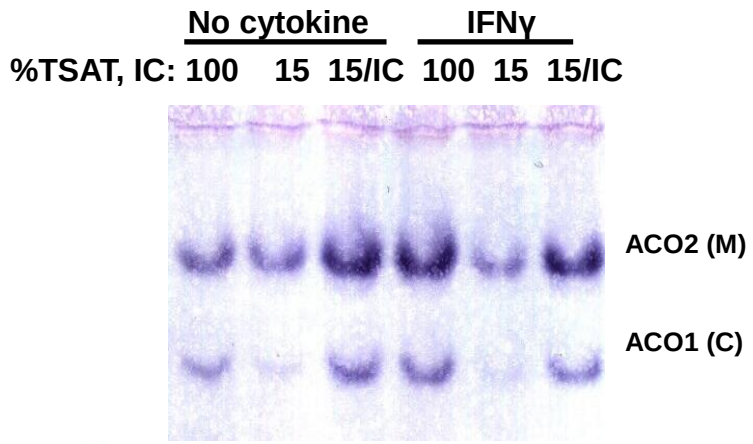


Figure 2.6 The cooperative inhibition of erythroid differentiation by iron restriction and TNFα is also reversed by isocitrate treatment This experiment was conducted exactly as in Fig. 2.3A except that TNFα rather than IFNγ was used as the inflammatory stimulus.

Distal convergence of erythroid iron restriction and interferon gamma signaling pathways

Proximal elements of the erythroid iron restriction response and IFNγ signaling pathways were analyzed to determine the basis for their cooperation in erythroid inhibition. IFNγ has been shown to promote aconitase inactivation in macrophages [66] but failed to decrease erythroid aconitase activities under various culture conditions (Figure 2.7A). This finding suggests differences between macrophage and erythroid IFN-response programs. Prior studies of the effects of iron deprivation on proximal IFNγ signaling, i.e. STAT1 activation, have shown either no influence or sensitization, depending on the cells analyzed [67-69]. In erythroid progenitors undergoing IFNγ treatment, iron restriction had no effect on STAT1 phosporylation on either tyrosine 701 or serine 727 (Figure 2.7B), the key targets of proximal kinases [70]. Furthermore, neither erythroid iron restriction nor isocitrate treatment affected IFNγ induction of STAT1, STAT2, IRF8 or IRF1 (Figure 2.7C, D, E). IFNγ activates IRF9 transcription by the gamma activated transcriptional element (GATE) pathway, an alternative STAT-independent mechanism. While IFNγ augmented IRF9 mRNA levels in erythroid cells, neither iron restriction nor isocitrate affected this induction (Figure 2.7F). In aggregate, the crosstalk between erythroid iron restriction and IFNγ signaling appears to occur distally, i.e. downstream of sensors/receptors and receptor-associated kinases.

A.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IFNγ
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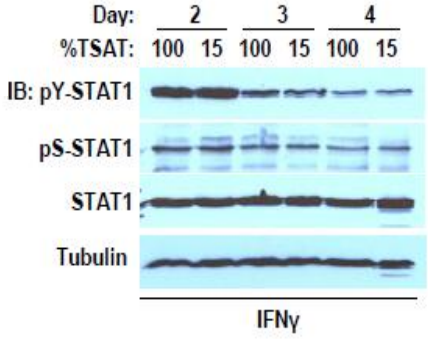
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IB: pY-STAT1
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Tubulin
IFNγ

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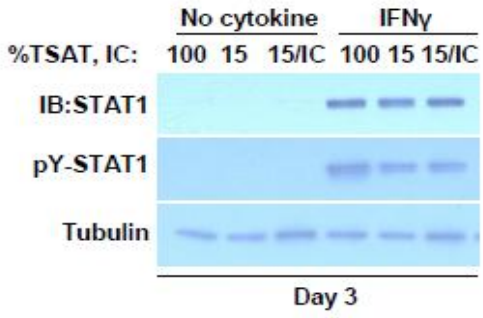
No cytokine
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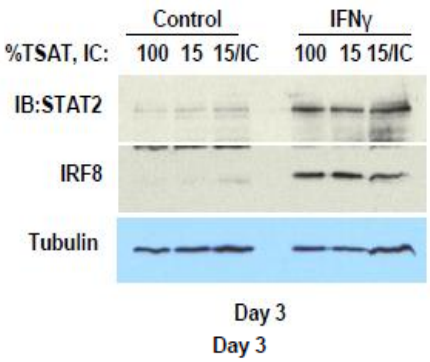
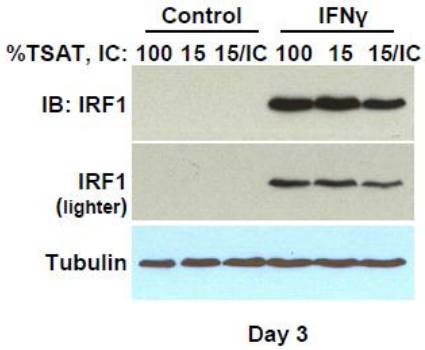
pY-STAT1
Tubulin
Day 3

D.


Control
IFNγ
%TSAT, IC: 100 15 15/IC 100 15 15/IC
IB:STAT2
IRF8
Tubulin
Day 3

Day 3

E.


Control
IFNγ
%TSAT, IC: 100 15 15/IC 100 15 15/IC
IB: IRF1
IRF1
(lighter)
Tubulin
Day 3

F.

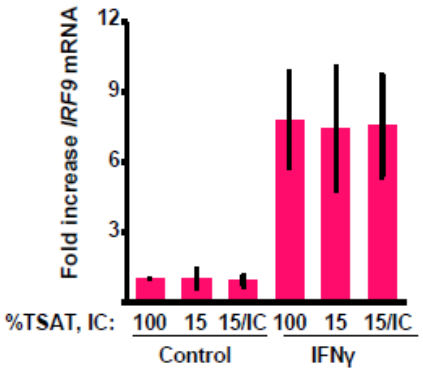

Fold increase IRF9 mRNA
12
9
6
3
%TSAT, IC: 100 15 15/IC 100 15 15/IC
Control
IFNγ

Figure 2.7 Influences of IFNγ on erythroid aconitase activity and of iron restriction and isocitrate on IFNγ-mediated signaling. (**A**) Mitochondrial (M) and cytosolic (C) aconitase activities in human progenitors subjected to IFNγ treatment ± iron restriction and isocitrate. Gel-based enzymography was performed on extracts of cells cultured 4 days in erythroid medium under the indicated conditions. (**B**) Time course analysis of the influence of iron restriction on IFNγ activation of STAT1. Whole cell lysates from human progenitors cultured in erythroid medium with IFNγ ± iron restriction underwent immunoblot (IB) analysis of STAT1 phosphorylation and expression (**C, D, E**) Influence of iron restriction and isocitrate on IFNγ signaling via JAK-STAT. Progenitors cultured 3 days in erythroid medium under the indicated conditions were analyzed as in panel B. (**F**) Influence of iron restriction and isocitrate on IFNγ signaling via the GATE pathway. Cells cultured as in **C**, **D and E** underwent qRT-PCR assessment of IRF9 mRNA levels, with normalization to *GAPDH*. Results shown as fold increase relative to levels in cells cultured without IFNγ and with 100% TSAT. All data are mean ± s.e.m., N = 3

The myeloid transcription factor PU.1 functions as a signaling node in erythropoietic regulation by iron restriction, interferon gamma, and isocitrate

Erythroid lineage commitment coincides with PU.1 downregulation, failure of which inhibits developmental progression and may ectopically activate myeloid genes [56,71]. Recently, Libregts et al. have implicated an IRF1-PU.1 signaling axis in a murine model of IFNγ-dependent anemia [41]. PU.1 levels were therefore determined in human progenitors undergoing erythroid culture as in Figure 2.7C. Under these conditions, iron restriction and isocitrate do not affect the extent of erythroid lineage commitment, reflected by CD36 upregulation and CD34 downregulation as described [72] (Figure 2.8B). Iron restriction amplified IFNγ induction of PU.1 by 2-3 fold, as well as inducing PU.1 on its own, and isocitrate abrogated PU.1 upregulation by iron restriction plus IFNγ (Figures 2.9A,B). Identical results were obtained using a starting population of purified erythroid progenitors isolated by the method of Freyssinier et al [73] (Figure 2.9C).

In situ PU.1 expression at distinct stages of human erythroid development was characterized by flow cytometry. This approach confirmed within progenitor subsets that iron restriction augmented and isocitrate blocked IFNγ induction of PU.1, with the strongest effects seen in early committed ($CD34^+$ $CD36^+$) erythroid progenitors (Figure 2.9D). In the absence of IFNγ, iron restriction also enhanced PU.1 levels preferentially within the $CD34^+$ $CD36^+$ compartment, again with complete reversal by isocitrate **(**Figure 2.8C). As an additional approach, sorted erythroid progenitors [72] underwent immunoblotting for PU.1. In early committed $CD36^+$ GPA^- cells, iron restriction promoted PU.1 upregulation in the absence and presence of IFNγ; by contrast, in the late-

stage $CD36^+$ GPA^+ population silenced PU.1 expression under all conditions (Figure 2.9E). In rat marrows harvested as in Figure 2.1D, qRT-PCR on sorted progenitors also showed evidence of increased erythroid PU.1 mRNA in ACDI, an effect consistently reversed by isocitrate treatment (3 out of 3 independent experiments) (Figure 2.8D). Isocitrate-induced downregulation of erythroid PU.1 protein expression in vivo in ACDI was demonstrated by intracellular staining of rat marrows harvested as in Figure 2.1D (Figure 2.8E).

Prior studies using a shRNA approach have demonstrated inhibitory consequences of PU.1 induction by IFNγ in human erythroid progenitors.[41] Similar experiments using lentiviral shRNA knockdowns were conducted on cells subjected to iron restriction plus IFNγ. Two hairpins causing partial PU.1 knockdown, but not a control hairpin, enhanced erythroid differentiation under these conditions (Figure 2.10A, Figure 2.11A). One factor limiting this approach, however, is the deleterious effect of prematurely downregulating PU.1 in early progenitors [56,74].

The erythroid iron restriction response exerts its effects in part through induction of PKCα/β hyperactivation, which can be blocked by isocitrate treatment (Figure 2.10B) [28]. PKC, particularly PKCα/β, in turn may regulate the expression and activity of PU.1 in early erythroid progenitors as has been shown in monocytes [75,76]. To assess PKC contribution to cooperative PU.1 induction by iron restriction plus IFNγ, cells cultured as in Figure 2.9A underwent low-dose treatment with the PKC-selective inhibitor Bisindolylmaleimide I (BIM). Notably, BIM abrogated PU.1 upregulation by iron restriction plus IFNγ (Figure 2.10C) but did not affect viability or lineage commitment within day 3 cultures (not shown). Similar results were achieved with another unrelated

PKC inhibitor Gö6976 (Figure 2.11B). These results thus support a role for PKCα/β hyperactivation by the erythroid iron restriction response [28] in cooperative induction of PU.1 by iron restriction and IFNγ.

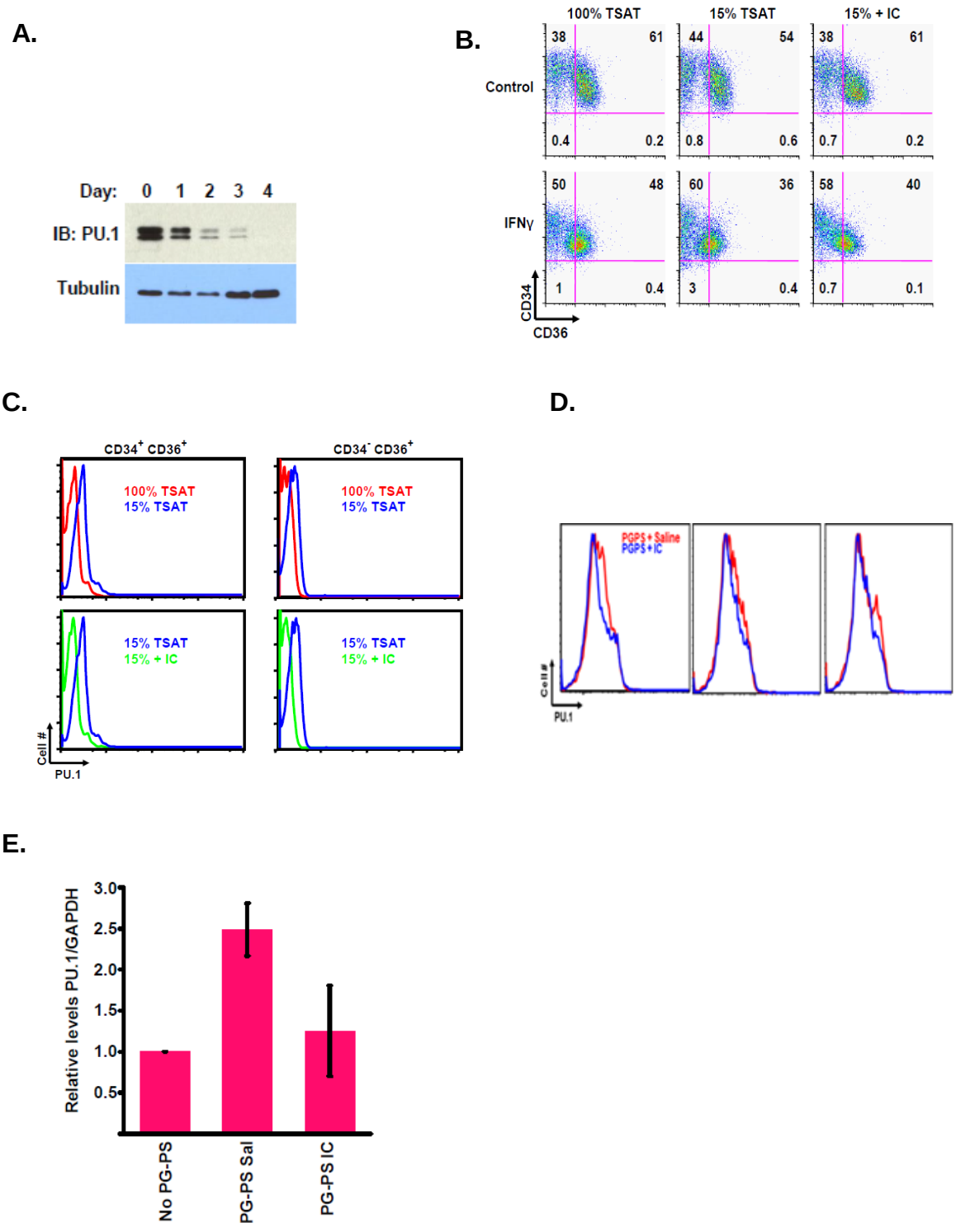


Figure 2.8 (**A**) Kinetics of PU.1 downregulation during normal erythroid differentiation. Human CD34^{+} cells were cultured in erythroid medium for the indicated days prior to

immunoblot. (**B**) No significant influence of iron restriction or isocitrate on early erythroid commitment. Human $CD34^+$ cells were cultured 3 days in erythroid medium under the indicated conditions, followed by flow cytometry. (**C**) Effects of iron restriction and isocitrate treatment on PU.1 levels in early ($CD34^+$ $CD36^+$) and later ($CD34^-$ $CD36^+$) erythroid progenitors. Human CD34+ cells cultured 3 days in erythroid medium with 100% or 15% transferrin saturation $\pm$ isocitrate treatment underwent flow cytometry for intracellular PU.1 detection. (**D**) Effects of isocitrate treatment on PU.1 levels in erythroid progenitors from rats with ACDI. Animals treated as in Figure 2.1B underwent euthanasia on day 21 followed by flow cytometry for intracellular PU.1 detection in marrow erythroid progenitors (SSC^{Low} $CD71^{High}$). Each plot shows an overlay of two different rats. **(E)** PU.1 mRNA expression in sorted erythroid progenitors from rats treated with ACDI. Rats treated as in Fig 2.1B underwent sorting of marrow erythroid precursors (CD71+ CD11b-) followed by qPCR for PU.1, with normalization to GAPDH. Shown are mean $\pm$ s.e.m for 3 independent experiments.

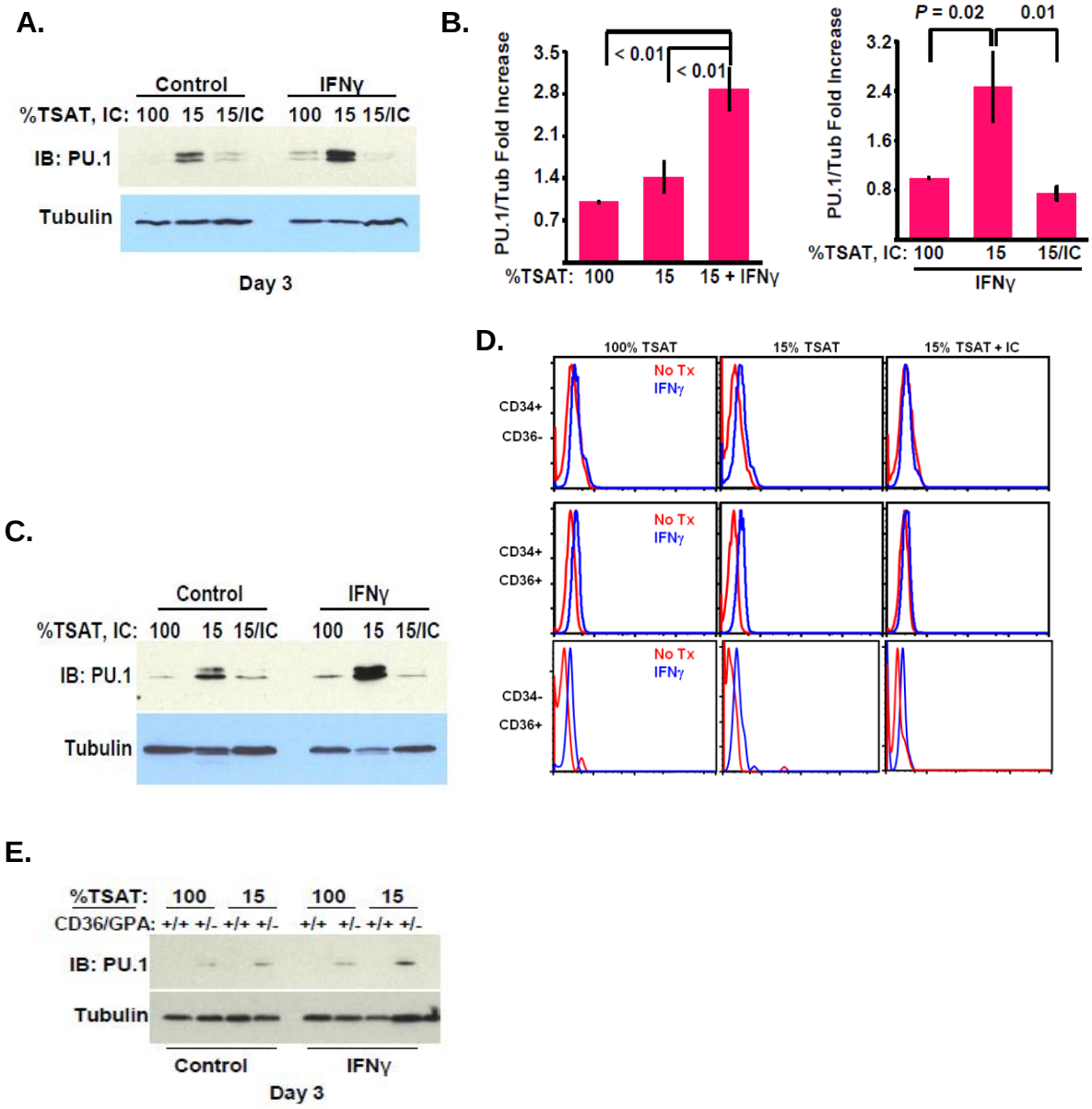


Figure 2.9 Cooperative induction of PU.1 by iron restriction and IFNγ is blocked by isocitrate. (**A**) Iron restriction and isocitrate oppositely modulate IFNγ induction of PU.1 in primary hematopoietic progenitors. Human $CD34^+$ cells cultured as indicated in erythroid medium for 3 days underwent immunoblot (IB) analysis of PU.1 expression. (**B**) Summary of 3-4 independent experiments conducted as in A. Graphs show relative PU.1 protein levels normalized to tubulin, with mean ± s.e.m. (**C**) Influences of iron

restriction, IFNγ, and isocitrate on PU.1 levels in purified erythroid progenitors. Human $CD36^+$ cells were cultured and analyzed as in A. (**D**) Influences of iron restriction, IFNγ, and isocitrate on PU.1 levels at various stages of erythroid development. Human $CD34^+$ cells cultured as in A underwent flow cytometry with intracellular staining for PU.1. (**E**) Developmental stage-dependent effects of iron restriction and IFNγ on erythroid PU.1 expression. Human $CD34^+$ cells cultured for 3 days underwent sorting for early ($CD36^+$ GPA^-) and late ($CD36^+$ GPA^+) erythroid progenitors followed by immunoblot. All data are mean $\pm$ s.e.m., N = 3

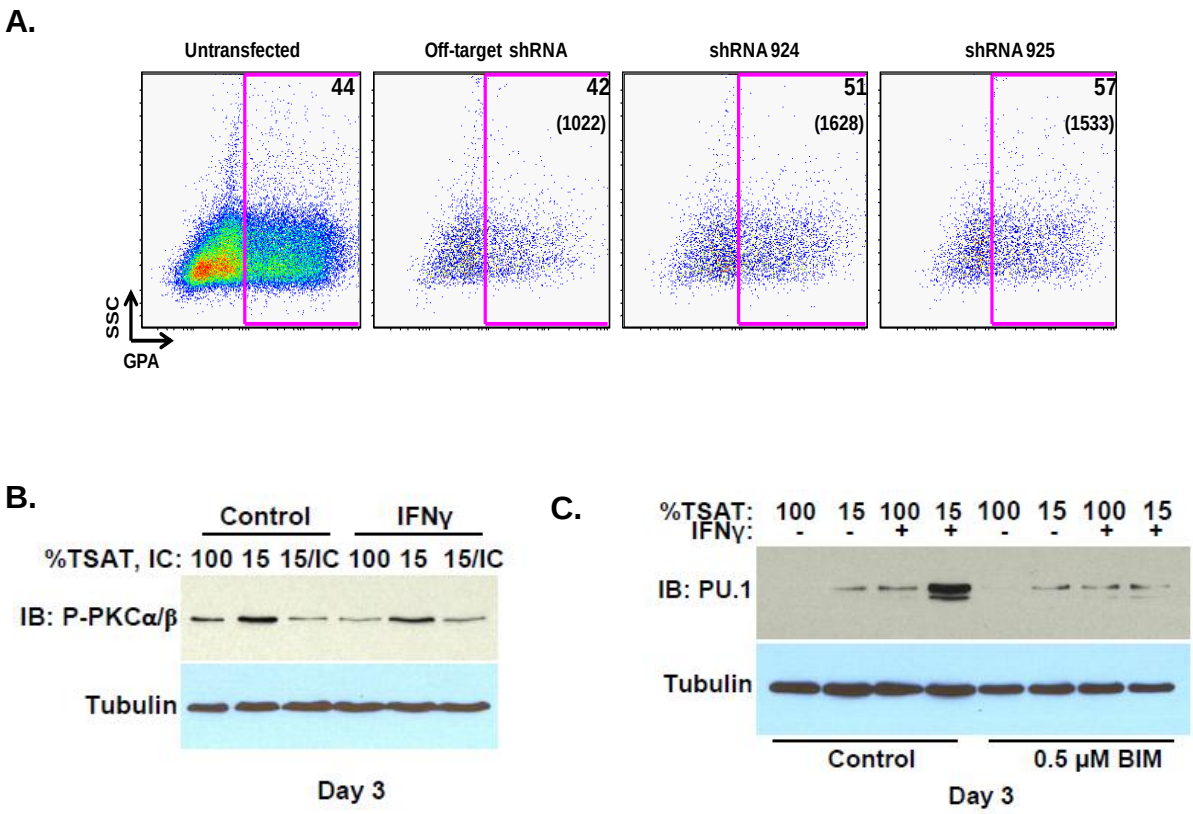


Figure 2.10 The cooperative induction of PU.1 by iron restriction and IFNγ contributes to erythroid inhibition and requires PKC signaling. (**A**) PU.1 knockdown enhances erythropoiesis in the setting of iron restriction plus IFNγ stimulation. Human $CD34^+$ cells were transduced with shRNA constructs, cultured 4 days in erythroid medium with iron restriction plus IFNγ, and analyzed by flow cytometry with gating on GFP^+ transduced cells. Relative percentage of GPA^+ cells shown in top right corner; absolute number of GPA^+ cells shown below in parentheses. Relative percentage of GFP^+ cells and absolute number of GFP^+ cells are as follows: off-target shRNA 27% (3841), shRNA #924 35% (4664), and shRNA #925 39% (3900). (**B**) Iron restriction induces PKCα/β hyperphosphorylation; isocitrate reverses this effect; and IFNγ shows no influence. Human CD34+cells were cultured as in Figure 2.5A. (**C**) PKC signaling contributes to the cooperative induction of PU.1 by iron restriction and IFNγ. Human $CD34^+$ cells

cultured as in Fig. 2.5A were treated where indicated with 0.5μM Bisindolylmaleimide I (BIM), followed by immunoblot.

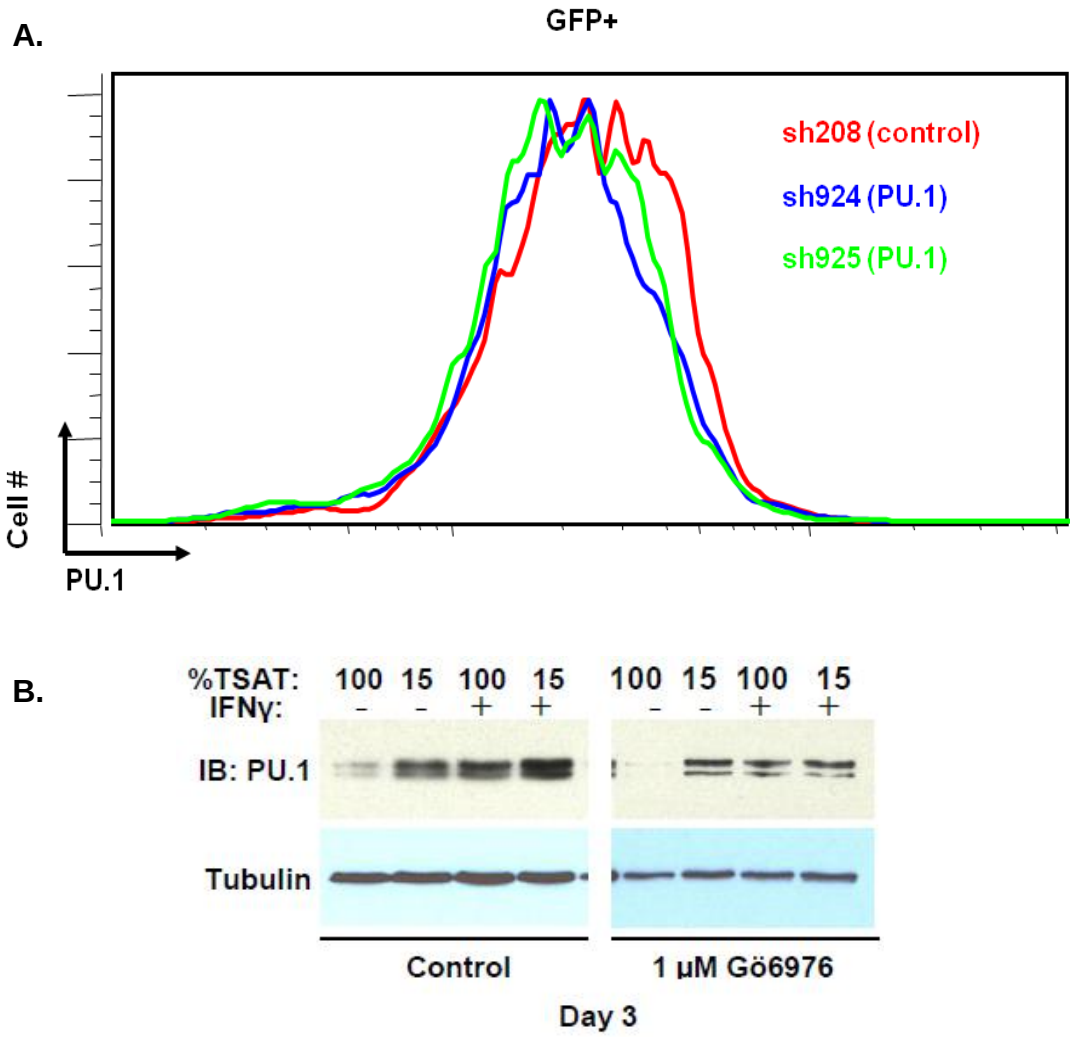


Figure 2.11 shRNA knockdown of PU.1 in human CD34+ cells and inhibition of PU.1 upregulation by PKC inhibitor. (**A**) shRNA knockdown of PU.1 in primary human progenitors subjected to iron deprivation and IFNγ treatment. Human CD34+ cells transduced with GFP-containing lentiviral shRNA constructs were cultured in erythroid medium with 15% transferrin saturation and IFNγ for 2 days, followed by intracellular staining for PU.1 and analysis by flow cytometry with gating on GFP+ cells. (**B**) Gö6976, an inhibitor of PKC that is unrelated to BIM, also inhibits PU.1 upregulation by the

combination of iron restriction and IFNγ. Human $CD34^+$ cells cultured as in Fig. 2.5A were treated where indicated with 1μM of Gö6976, followed by immunoblot.

DISCUSSION

As demonstrated in Figure 2.1, a short course of isocitrate treatment rapidly and durably corrects anemia in a rodent model previously shown to recapitulate human ACDI [44]. These results provide the first evidence that iron-regulated erythroid signaling can be therapeutically manipulated by an approach that does not involve increasing body iron stores. Such an approach offers an attractive alternative to iron provision by intravenous (IV) infusion or through hepcidin pathway antagonism. Clinical usage of IV iron has expanded due to financial and safety pressures to decrease Epo administration in patients with ACDI [77]. However, a recent study has revealed a high prevalence (84%) of hepatic iron overload in chronic kidney disease patients receiving IV iron [78]. This iron overload potentially could cause tissue damage and enhance risk of bacterial infections [77]. Hepcidin pathway antagonists have shown promise in pre-clinical models [17,79] but, their potential drawbacks conceivably also include induction of iron overload, as well as off-target effects and high cost. Isocitrate is a simple, small molecule that exerts a direct influence on early erythroid progenitors, lowering liver hepcidin expression most likely through the "erythropoietic signal"[57], and thus coupling iron uptake with erythropoietic demand.

Ex vivo analysis of erythroid inhibition by inflammatory cytokines reveals sensitization by iron restriction and desensitization by isocitrate. This novel relationship between the erythroid iron restriction response and inflammatory signaling may reconcile some of the paradoxical findings associated with human ACDI. One such finding is the normal red cell indices that occur in the majority of ACDI patients [14], despite a putative role for iron restriction as the primary cause for erythropoietic repression.

Another such finding involves the inconsistent in vitro effects of inflammatory cytokines associated with ACDI. Specifically, IFNγ and TNFα have been found to exert either negative or positive influences on erythropoiesis depending on study conditions [64,65,80]. In our culture conditions, the effects of IFNγ on erythropoiesis show a strong dependency on iron availability. Thus the effects of inflammatory cytokines on erythropoiesis most likely depend on cellular context. Our findings support a paradigm for ACDI in which sub-threshold degrees of iron restriction and inflammatory signaling, while exerting minimal effects individually, together cooperate in potent repression of erythropoiesis. According to this paradigm, interference with either the erythroid iron restriction response or inflammatory signaling may suffice to relieve this repression.

Our results identify the transcription factor PU.1 as a novel signal integration element downstream of the erythroid iron restriction response, IFNγ, and isocitrate. IFNγ recently has been shown to upregulate PU.1 by inducing the transactivator IRF1 [41] but, may also enhance PU.1 DNA binding via PKCβ-mediated signaling [75]. The erythroid iron restriction response promotes PKCα/β activation, using a pathway (Figure 1.7) that is repressed by isocitrate [28]. Once activated, PU.1 may engage in a positive autoregulatory loop designed to consolidate the myeloid transcriptional program and repress erythroid development [81]. A signaling map (Figure 2.12A) is therefore proposed in which the erythroid iron restriction response critically contributes to PU.1 activation and autoregulation via PKC activation, in a step targeted by isocitrate treatment. IFNγ signaling additionally contributes to PU.1 induction through IRF1 induction. We postulate that isocitrate inhibits PKC activation by iron restriction through binding to and stabilizing the aconitase enzymes (Figure 2.7A). The sustained therapeutic

effects of transient isocitrate treatment are most likely due to two interrelated factors: 1) the ability to target early progenitors during a key window prior to their amplification in the marrow and 2) a feed-forward mechanism in which the enhancement of erythropoiesis represses hepcidin and reverses the iron restriction.

PU.1 functions as a master regulator of myeloid development, and even transient overexpression can irreversibly alter cell fate through epigenetic reprogramming [56,82,83]. Accordingly, the erythroid repression associated with ACDI may share features with the myeloid lineage skewing identified in studies of early hematopoietic progenitors from mice with autoimmune arthritis [62]. In those studies, the marrow Kit^+ Sca^+ Lin^- (KSL) population from arthritic animals displayed upregulation of myeloid-specific transcripts combined with downregulation of erythroid genes. Our results using human erythroid cultures demonstrate PU.1 modulation by iron deprivation and isocitrate at early developmental stages ($CD34^+$ $CD36^-$ and $CD34^+$ $CD36^+$), during which progenitors may retain lineage plasticity [84]. Given the importance of graded PU.1 levels in cell fate determination [85], we propose a PU.1 threshold-based model for the mechanisms of erythroid iron restriction response and isocitrate in ACDI (Figure 2.12B). In this model, iron restriction and IFNγ separately elevate early erythroid PU.1 insufficiently to block erythropoiesis but in combination drive PU.1 above a "myeloid threshold" critical for subversion of the erythroid program. Isocitrate exposure, by retaining PU.1 below this threshold, could release early progenitors into the erythroid pathway, leading to erythropoietic repression of hepcidin and further alleviation of iron restriction. Thus, by targeting a critical early step in erythroid lineage commitment,

transient isocitrate treatment could break a vicious cycle in ACDI and elicit a durable clinical response.

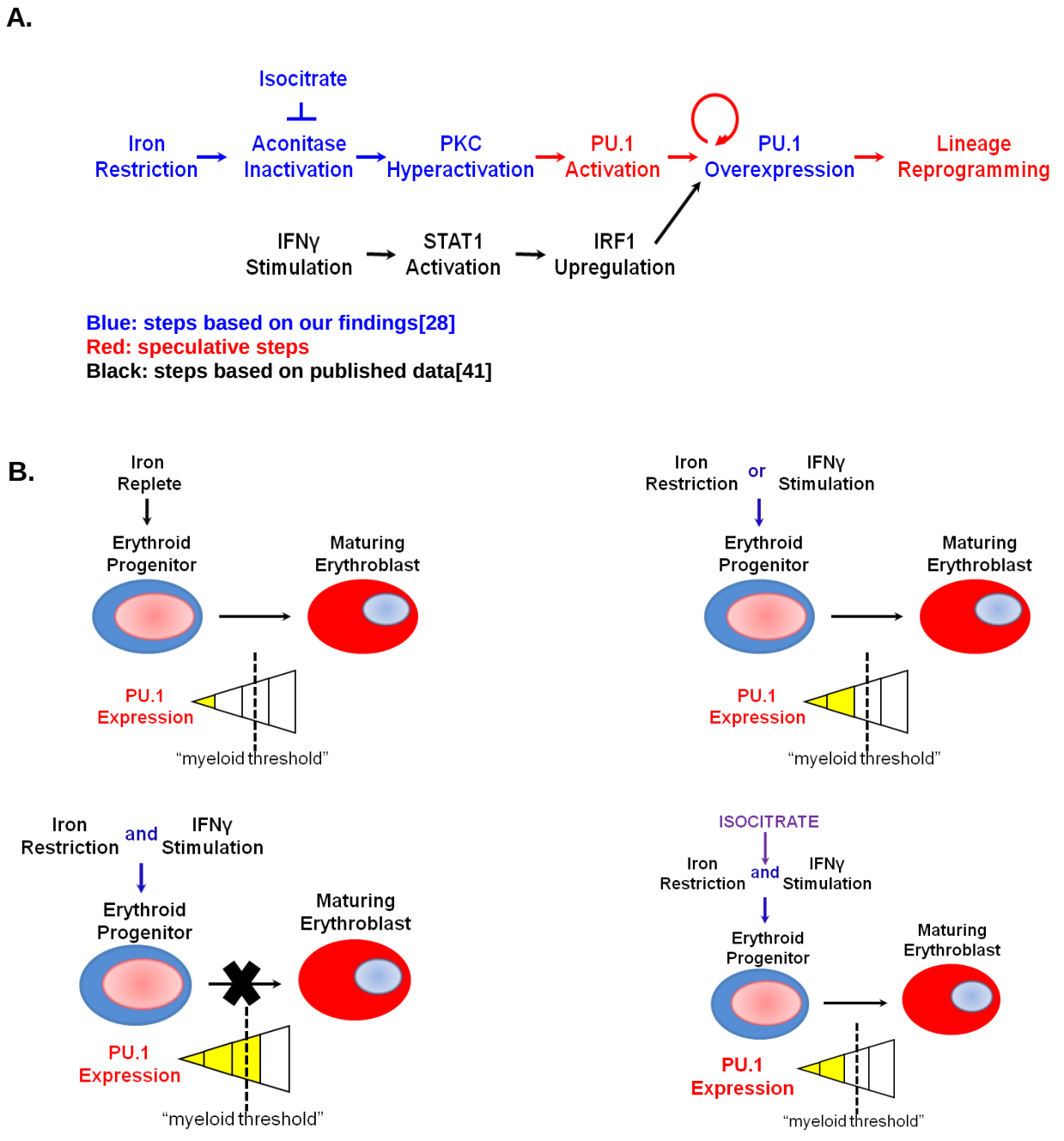


Figure 2.12 Model depicting the influences of iron restriction and isocitrate on erythropoiesis in ACDI. (**A**) Signaling diagram of the convergence of iron restriction and IFNγ on PU.1. Iron restriction is postulated to contribute through its known capacity to enhance PKCα/β activation [28], and IFNγ through a documented IRF1-PU.1

transcriptional pathway [41]. **(B)** A threshold-based mechanism, in which iron restriction and inflammatory signaling separately do not elevate PU.1 in early erythroid progenitors enough to constrain erythroid output. In combination, however, these stimuli cause PU.1 levels to exceed a critical repressive threshold and compromise erythropoietic capacity. Isocitrate restores erythropoietic capacity by retaining erythroid PU.1 levels below this critical threshold.

METHODS

Animal models

Rats were housed in a pathogen-free facility and handled in accordance with ACUC policies. 6-week-old female Lewis rats weighing 100-120 grams were purchased from Charles River Laboratories. To induce anemia of chronic disease/inflammation (ACDI), the rats received a single intraperitoneal injection of Streptococcal cell wall peptidoglycan-polysaccharide (PG-PS, Lee Laboratories/BD Biosciences) at 15 µg rhamnose/gram body weight. Blood samples were collected from tail veins into heparin coated syringes and transferred into EDTA-coated microtubes (BD Biosciences). Complete blood counts (CBC) were analyzed on the Hemavet 850FS automated analyzer (Drew Scientific). Starting 7 days post PG-PS injection, CBCs were monitored. Reticulocytes were measured by staining whole blood with thiazole orange as described [86]. Treatments, initiated on day 14 post PG-PS injection, consisted of daily intraperitoneal injections at doses of 200 mg/kg/day of trisodium isocitrate (Sigma-Aldrich) dissolved in 0.9% saline solution or equivalent volumes of 0.9% saline solution.

For serum studies, whole blood was collected into Microtainer serum separator tubes (BD Biosciences). After separation, serum was immediately stored at -80° C in single use aliquots. Serum iron analysis was performed as described [51] using the Ferene Serum Iron/UIBC kit (Thermo Scientific). Serum erythropoietin and IFNγ were measured using the Quantikine Rat EPO ELISA kit and Quantikine Rat IFNγ ELISA kit (R&D Systems).

Cell culture

Purified human $CD34^+$ progenitors derived from granulocyte-colony stimulating factor–mobilized peripheral blood cells of healthy donors were obtained as previously described [87]. These cells were grown at 37°C with 5% CO2 in serum-free medium consisting of Iscove's modified Dulbecco medium (IMDM) with β-mercaptoethanol, BIT 9500 supplement (BITS, Stem Cell Technologies), bovine serum albumin (BSA, Sigma-Aldrich), and the indicated cytokines (PeproTech). The cells initially underwent 72 hours of prestimulation with 100 ng/mL stem cell factor (SCF, PeproTech), 100 ng/mL FMS-like tyrosine kinase 3 ligand (FLT3-ligand, PeproTech), 100 ng/mL thrombopoietin (TPO, PeproTech), and 50 ng/mL interleukin-3 (IL-3, PeproTech) and were then seeded in erythroid differentiation medium, which contains recombinant human erythropoietin at 4.5U/ml (Epogen, Amgen Mfg. Ltd) with defined transferrin saturations as described [28]. Human recombinant inflammatory mediators were added to erythroid cultures as follows: IFNγ 1500U/ml, TNFα 100ng/ml, IL-1β 100ng/ml, IL-6 100ng/ml IL-10 100ng/ml, IL-15 100ng/ml, and LPS 50ug/ml (PeproTech). Purified human $CD36^+$ cells (AllCells LLC, Emeryville, CA) underwent expansion as previously described [73] for 48 hours followed by culture in erythroid medium for 3 days .

Flow cytometry

Data were collected on a FACSCalibur instrument (Becton Dickinson) and analyzed using FlowJo software Version 8.6.3 (TreeStar Inc). Fluorochrome-conjugated antibodies were purchased from BD Pharmingen, with the exceptions of PE-anti-CD71 (Dako) and Alexa Fluor 488 rabbit anti-PU.1 (Cell Signaling Technology). Bone marrow from rat

femurs was extruded into PBS supplemented with 5 mM EDTA, dissociated, and treated with hypotonic ammonium chloride to eliminate erythrocytes. Cells were then co-stained with FITC-anti-CD71 for erythroid precursors,[88] PE-anti-CD11b for myeloid cells, and APC-anti-CD3 for T cells. Erythroid precursor cell death was analyzed by co-staining marrows with FITC-anti-CD71, PE- anti-CD11b, Annexin V-phycoerythrin and 7-aminoactinomycin D (Apoptosis Detection Kit I, BD Pharmingen). For erythroid cell cycle analysis, cells were stained for CD71 and CD11b followed by ethanol fixation, RNase A treatment and propidium iodide staining. Differentiation of human erythroid progenitors was assessed by co-staining cells with fluorochrome-conjugated antibodies to CD34 (a marker of immaturity), CD36 (an early marker of erythroid lineage commitment,[72] and glycophorin A (GPA, a later marker of erythroid differentiation). Intracellular staining for erythroid PU.1 expression followed the guidelines of Koulnis et al [89]. Specifically, human progenitors stained for CD34, CD36, and GPA underwent fixation and permeabilization using the BD Cytofix/Cytoperm™ Perm/Wash kit (BD Biosciences), followed by staining in the Perm/Wash solution with Alexa Fluor 488 rabbit anti-PU.1 antibody or matched control antibody (Cell Signaling Technology). For cell sorting, human and rat precursors were isolated on a FACSVantage SE Turbo Sorter with DIVA Option (Becton Dickinson). Human cells were sorted according to CD36 and GPA expression, and rat precursors were isolated based on high levels of CD71 and low FSC/SSC [88].

Aconitase assay

Gel-based analysis of aconitase activities in progenitor extracts was performed as previously described [28,90].

Quantitative real-time PCR (qPCR)

RNA was isolated using the Qiagen RNeasy Plus Mini Kit with DNAase treatment of columns prior to RNA elution. RNA yield and quality were determined on a Thermo NanoDrop spectrophotometer. Reverse transcription was performed using the Bio-Rad iScript cDNA synthesis kit. Real time PCR was conducted using the Bio-Rad iQ SYBR Green Supermix on the Bio-Rad iCycler platform equipped with iQ real time imaging. For relative quantification of transcript levels by qPCR, we used the comparative $\Delta\Delta C_T$ formula delineated in the ABI Prism 7700 Sequence Detection System User Bulletin no. 2. All samples underwent triplicate analysis with normalization performed by subtraction of the C_T value of *GAPDH*. Human primers were: GAPDH: forward 5'-TGCCCCCATGTTTGTGATG-3', reverse 5'-TGTGGTCATGAGCCCTTCC-3'; IRF9: forward 5'-CAAGTGGAGAGTGGGCAGTT-3', reverse 5'-ATGGCATCCTCTTCCTCCTT-3'; PU.1: forward 5'-CAGCTCTACCGCCACATGGA-3', reverse 5'-TAGGAGACCTGGTGGCCAAG-3'. Rat primers were: GAPDH forward 5′-CAACTACATGGTTTACATGTTC-3′, reverse 5′-GCCAGTGGACTCCACGAC-3′; Hepcidin forward 5'-GAAGGCAAGATGGCACTAAGCA-3'; reverse 5'-TCTCGTCTGTTGCCGGAGATAG-3'; PU.1 forward 5'-

CCTTGATTGGTGGTGATGGAGAC-3', reverse 5'-CAGCTCCATGTGGCGGTAGA-3'. Primers were purchased from Integrated DNA Technologies (Coralville, IA).

Immunoblot

Whole-cell lysates underwent sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose and immunoblotting as previously described [87]. Antibodies included mouse anti-tubulin (Sigma-Aldrich); rabbit anti-STAT1, STAT2, IRF8, IRF1, PU.1 (Cell Signaling Technology); and rabbit phosphospecific antibodies to STAT1 serine 727, STAT1 tyrosine 701, and PKCα/β threonine 638/641 (Cell Signaling Technology). Densitometry data were acquired on a GS800 calibrated densitometer (Bio-Rad) and analyzed with Quantity One software (Bio-Rad).

Plasmids and Transfections

Knockdown of PU.1 expression in CD34+ cells used an off target control GIPZ shRNA construct, catalog number VGH5518-200183170 (#208) and human PU.1-targeting GIPZ shRNA constructs, catalog numbers RHS4430-100990345 (#924), and RHS4430-100990495 (#925) (Open Biosystems). Lentiviral packaging constructs pCMV-dR8.74 and pMD2.G were provided by Dr. Didier Trono (School of Life Sciences, Swiss Institute of Technology). Production of lentiviral particles by transient co-transfections of HEK293T cells was carried out using the calcium phosphate method, as previously described [91]. Spinoculation of cells, puromycin selection of transduced CD34+ cells,

and analysis of green fluorescent protein–positive (GFP^+) transduced primary progenitors were performed as previously described [91,92].

Statistical analysis

KalediaGraph software, version 4.0 (Synergy Software) was used to display the data graphically and to perform statistical analysis. Results were analyzed by unpaired two-tailed Student's t test or one-way analysis of variance (ANOVA) with Tukey post-hoc test when comparing multiple groups. P values less than or equal to 0.05 were considered significant.

Study approval

The animal experiments were approved by the University of Virginia Animal Care and Use Committee (ACUC Protocol #3545).

ACKNOWLEDGEMENTS

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and Blood Institute (K08-HL093355 to G.C.B.), the Roche Foundation for Anemia Research (A.N.G.), and the Annette Lightner Research Award (A.N.G.).

Chapter 3:
Related Data

3.1 TNFα also modulates the erythroid iron restriction response

Multiple lines of evidence implicate inflammatory cytokines as mediators of ACDI. Importantly, serum from anemic patients with chronic kidney disease, but not from normal controls, was found to inhibit autologous erythroid colony formation ex vivo. These inhibitory effects were neutralized with antibodies to IFNγ and to TNFα, suggesting a role for both cytokines in ACDI [63]. In another study, Cooper et al. showed that patterns of cytokine production by circulating T cells strongly correlated with Epo resistance, with IFNγ production by CD4 T cells being most highly predictive ($p < 0.001$) [93]. In addition, in rheumatoid arthritis patients, treatment with a neutralizing antibody to TNFα produced a mean increase of 14% in hemoglobin levels [25]. These findings suggest that both cytokines have a functional role in ACDI.

As mentioned in Chapter 2, under conditions of iron restriction (15% TSAT), TNFα potently inhibited erythroid development and isocitrate conferred resistance to TNFα (Table 2.3, Figure 2.6). Typically, iron restriction alone reduced the proportion of GPA^+ cells by 50%, whereas IFNγ or TNFα combined with iron restriction reduced the proportion of GPA^+ cells by 90%. Additionally, each cytokine had distinctive effects on morphology and differentiation. IFNγ enhanced megakaryocytic development. TNFα, on the other hand, retained cells as immature, $CD34^+$ progenitors (Figure 3.1). Strikingly, isocitrate administration not only abrogated effects due to iron restriction but also effects due to the inflammatory cytokines, leading to complete rescue of erythroid differentiation.

We previously have shown that erythroid iron restriction leads to inactivation of the aconitase enzymes, which normally convert citrate to isocitrate. Provision of the cells

with exogenous isocitrate abrogates the erythroid inhibition associated with iron deprivation. Accordingly, the participation of this pathway was assessed in the more potent erythroid inhibition paradigm associated with TNFα plus iron restriction. TNFα failed to decrease erythroid aconitase activities any further than with iron restriction alone (Figure 3.2A); a similar observation to that of IFNγ treatment plus iron restriction in Figure 2.7A. Thus, speculating that TNFα acts in a similar manner as IFNγ, we assessed TNFα's ability to modulate protein PU.1 levels. TNFα amplified PU.1 protein levels (Figure 3.2B) when combined with iron restriction. Altogether, these findings closely parallel the findings obtained with IFNγ.

Finally, in a very relevant animal model system, we recapitulated the synergistic inhibition of erythroid differentiation with iron restriction and TNFα. We induced IDA in 3 week old male C57BL6 weanlings using an Iron Deficient Diet from Harlan Teklad (Product No. TD 30396, Madison, WI), which contains 2-5 ppm iron. To examine the effects of TNFα alone two additional groups from this cohort of 7 week old C57BL6 mice were fed normal iron replete chow and treated with TNFα or saline and showed no significant differences in their RBCs. After four weeks on the iron depleted diet, the mice showed mild anemia and were treated with 1.5μg of murine recombinant TNFα. TNFα significantly decreased the RBC levels compared to levels in iron deprived mildly anemic mice treated with saline, as shown in Figure 3.3. This data shows that TNFα exacerbates the anemia of iron restriction. To fully understand TNFα's impact on the EIRR in an animal model of ACDI, repeat experiments from Chapter 2 using the PGPS rat model would need to be conducted with the experimental analysis focused on TNFα versus IFNγ.

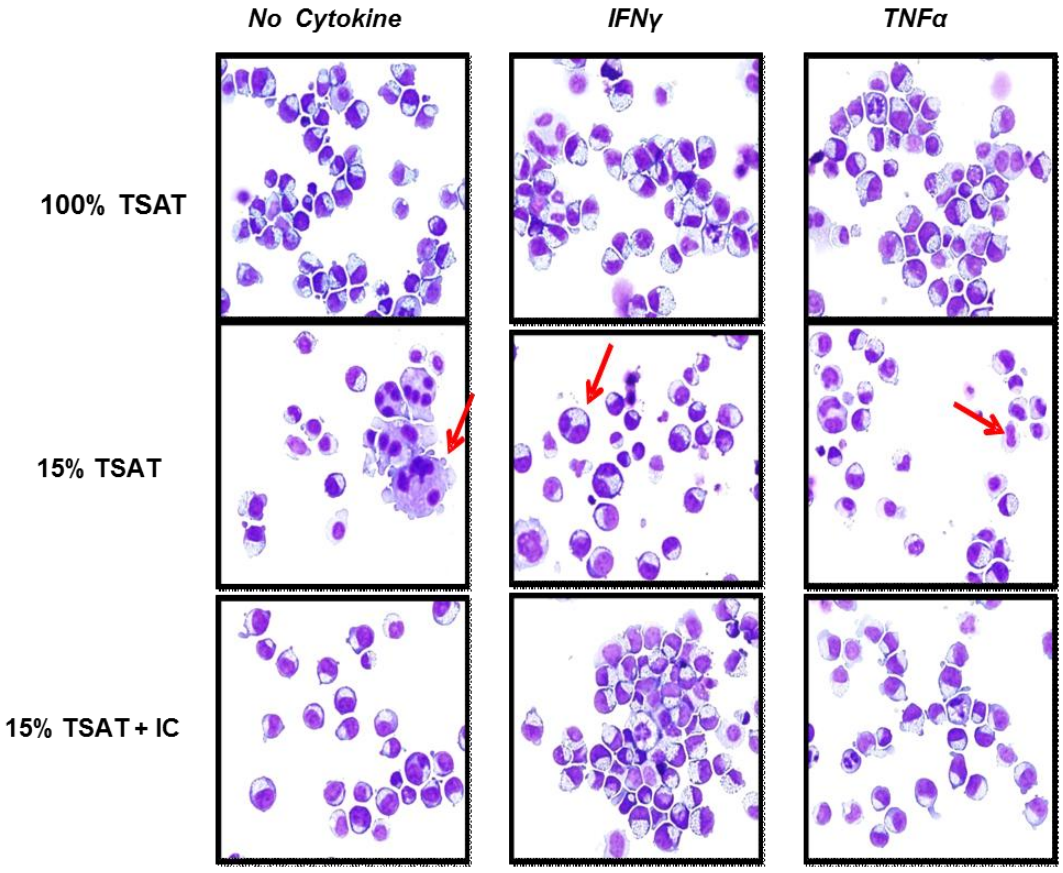


Figure 3.1 Erythroid Progenitor Morphology

Human CD34+ cells were harvested on day 5, cytospun and stained with Wright-Geimsa staining. Erythroid cells develop normally under 100% TSAT ± IFNγ or TNFα. Iron restriction alone (15% TSAT) increased generation of megakaryocytes (red arrow, 1st column). Iron restriction with IFNγ resulted in the appearance of early megarkaryotic cells (red arrow, 2nd column). Iron restriction with TNFα retained many of the cells in the immature state as well as produced abnormal cells that resemble histiocytes (red arrow, 3rd column). Isocitrate is able to prevent abnormal outgrowth of cells of other lineage and promotes normal erythroid morphology.

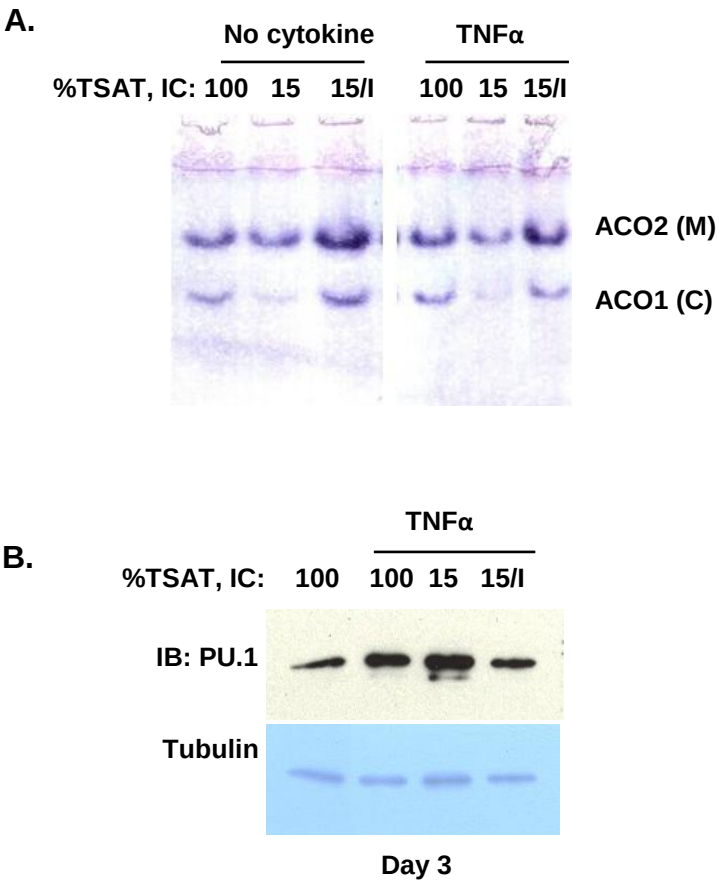


Figure 3.2 TNFα mediates signaling effects that are similar to IFNγ

A) Mitochondrial (M) and cytosolic (C) aconitase activities in human progenitors subjected to TNFα treatment ± iron restriction and isocitrate. Gel-based enzymography was performed on extracts of cells cultured 4 days in erythroid medium under the indicated conditions. **B)** Iron restriction and isocitrate oppositely modulate TNFα induction of PU.1 in primary hematopoietic progenitors. Human $CD34^+$ cells cultured as indicated in erythroid medium for 3 days underwent immunoblot (IB) analysis of PU.1 expression.

Figure 3.3 Recapitulating the interplay of TNFα and iron deprivation in murine model

Three week old male C57BL6 weanlings were placed on Iron Deficient Diet from Harlan Teklad (Product No. TD 30396, Madison, WI), After four weeks on the iron depleted diet, mice were placed on normal chow and implanted with subcutaneous mini osmotic pumps containing recombinant mouse TNFα (75µg/kg/d) or saline (0.9%) for three weeks. Data displayed is from end point retro-orbital eye blood run on the hemavet at week 7.

3.2 Alternative Molecular Pathways Mediating the Synergistic Response Between the EIRR and Inflammation

3.2.1 Alterations in Reactive Oxygen Species and Nitric Oxide

Inactivation of aconitase is the mechanistic highlight of what we understand happens under the iron restriction response. The aconitase enzymes can be inactivated in two ways: phosphorylation (ACO1, ACO2) or by reactive oxygen species (ACO2) [94]. Inflammatory responses often involve reactive oxygen species. Specifically, IFNγ induces NO and H_2O_2 production that activates IRP1 binding to the IRE-containing mRNAs. IRP1 is encoded by cytosolic aconitase, and it binds to IREs of mRNAs when it's Fe-S cluster is disrupted by iron deficiency, oxidative stress or diminished Fe-S cluster biosynthesis. Ferritin heavy chain and transferrin receptor1 are key targets of IRP1. IRP1 binds to the 5' untranslated region of ferritin heavy chain mRNA to block translation and decrease protein levels. The effect on transferrin receptor 1 is the opposite: IRP1 binds to the 3' untranslated region, stabilizing the mRNA and increasing expression. Additionally, IFNγ downregulates iron sulfur cluster scaffold protein (IscU) and cysteine desulfurase Nfs1, which are required for the initial step of the Fe-S cluster assembly for aconitase enzymes [66].

Using a flow cytometric intracellular assay in CD34+ primary cell culture system to measure both ROS and NO, we found that the levels were variable, thus leading to inconclusive results. Part of the variability may be attributed to the unsynchronized growth and stage of differentiation of the primary human hematopoietic progenitors in our cell culture model. ROS are known to play an important role in certain stages of

erythroid differentiation. ROS have been shown to activate MAPKs and HATs to induce globin expression in erythropoiesis [95].

To assess the role of ROS using a pharmacologic approach, we treated erythroid progenitors with the ROS scavengers N-acetyl cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) as described in Marinkovic et al [96]. Treatment with 100mM NAC or 500μM PDTC killed all the cultured cells under all conditions, suggesting these doses many be cytotoxic in the human primary cells. Because low amounts of ROS are involved in the differentiation of erythroid progenitors [97], we reduced the ROS scavenger dose. At reduced dose of PDTC (125μM), the viability of the cells was rescued slightly, but the PDTC effect was minimal compared to the rescue elicited by isocitrate. The NO inhibitor, L-NAME was also tested and did not show any effects with iron restriction or IFNγ. Thus inhibiting ROS or NO did not affect the erythroid response to iron restriction of IFNγ at the doses we used. These findings suggest that neither mediator plays a major role in this response.

3.2.2 Alterations in the Kinase Signaling (ERK, DAPK, JNK, & CamKII)

To address underlying mechanisms associated with erythroid cross-talk of iron and cytokine signaling, we screened pathways implicated in iron metabolism and inflammation. The four relevant pathways are: extracellular signal regulated kinases (ERK), death associated protein kinase (DAPK), c-Jun N-terminal kinase (JNK), and calmodulin-associated kinase II (CamKII).

3.2.2.1 ERK

We first conducted experiments examining the ERK pathway. To begin we assessed the upstream kinase, MEK, to see if there are changes in the protein levels in the iron restricted plus cytokine condition. Figure 3.4A shows decreased phosphorylation of MEK1/2 in iron replete conditions is activated, especially with the addition of IFN γ. In conditions of iron restriction, is not as strongly activated in comparison to iron replete conditions and IFNα and TNFγ do not modulate MEK1/2 phosphorylation any further than iron restriction alone. Total MEK1/2 protein levels were unchanged, suggesting that MEK1/2 activity is dampened in iron restricted erythroid progenitors, regardless of the cytokines. This dampening of the signal could result in block in cell proliferation. Interestingly, the immunoblot shown in Figure 3.4A demonstrated two interesting proteins at 45 and 50 kDa bands that were recognized by the monoclonal anti-phospho-MEK1/2 antibody. This same band pattern has been observed in three independent experiments. The lower band (45kDa) corresponds to phospho-MEK. The higher band (50kDa) we proposed to be either nonspecific binding of the antibody or a different isoform of MEK, possibly MEK 5. The 50kDa protein is only seen under iron restriction with or without inflammatory cytokines. A NCBI Basic Local Alignment Search Tool (BLAST) was used to identify homologous regions in MEK isoforms using the phospho-MEK1/2 antibody epitope. The search tool identified the MEK 5 isoform as having a region that was highly homologous with the MEK1/2 epitope used to create the phospho-specific antibody. MEK1/2 is a 393 amino acid protein (predicted mass of 43kDa) whereas MEK5 is 444 amino acids (predicted mass of 49kDa). Re-probing of the blots

with an anti-MEK 5 antibody was carried out but we were not able to identify the 50kDa band as MEK5.

We considered downstream targets in the signaling cascade, but analysis of ERK revealed opposing results (Figure 3.4B). We observed that phospho-MEK activity was decreased in iron restriction with or without IFNγ or TNFα in samples, whereas, phospho-activity was decreased with IFNγ plus iron restriction but increased with TNFα. Interestingly, total ERK levels also followed the same pattern as the phospho-ERK levels. However, in a later experiment (Figure 3.7A), we found phosphorylated ERK to be increased with iron restriction. This was enhanced by IFNγ treatment and reversed by isocitrate. Total levels were unchanged in this experiment regardless of treatment regimen. One possible reason for the discrepancy in the two immunoblots (Figures 3.4B, 3.7A) could be the protein loading. Protein loading in Figure 3.4B shows that tubulin levels may not be quite equal in all lanes, thus scan densitometry may be necessary to accurately assess changes. Knowing that total protein levels rarely shift in cohorts with the phosphorylated protein levels, we have biased Figure 3.7A results for IFNγ.

In view of the fact that the phosphorylation of ERK is a reflection of kinase and phosphatase activity, this result suggests that there is a defect in the upstream kinase or phosphatase. Further studies to understand the observed changes in ERK expression levels were conducted to determine whether the signal disruptions at the level of ERK were caused by a blockade or a hyperactivation in this pathway. We assessed phosphorylation of the downstream targets of ERK, such as ELK1 and p90RSK. Our anticipated results are illustrated in Table 3.1. Protein analysis of downstream targets did not confirm any of the proposed results. While we observed that ERK was

hyperactivated, phospho-ELK1 expression appeared to be upregulated with IFNγ and unchanged with TNFα under iron restriction (Figure 3.4B). Phosphorylation of p90RSK was increased with IFNγ, and unchanged by iron restriction or isocitrate. Phosphorylated p90RSK1 was undetectable with TNFα, and unchanged by iron restriction or isocitrate (Figure 3.4B); thus, these results do not permit any further conclusions to be drawn.

Because we found phosphorylated and total ERK to be hyperactivated specifically in the condition of IFNγ plus iron restriction, we used the pharmacologic MEK inhibitor, U0126, to try to correct the hyperactivation. When treating our primary CD34+ system with 10uM of U0126, we observed a marked increase in the viability of the cells, suggesting that part of the original viability defect observed with iron restriction and IFNγ is possibly due to ERK signaling. With TNFα and iron restriction, we observed that at the same dose of U0126, there was an enhancement of cell death and decreased viability, suggesting that ERK is necessary for viability and can oppose the cytotoxic effects of TNFα. Thorough analysis of the ERK pathway is necessary to make concrete conclusions and could potentially resolve published data by Talbot et al, which shows that aconitase inhibition via iron restriction impedes EPO induced ERK signaling and disrupts the ERK-aconitase interaction [27].

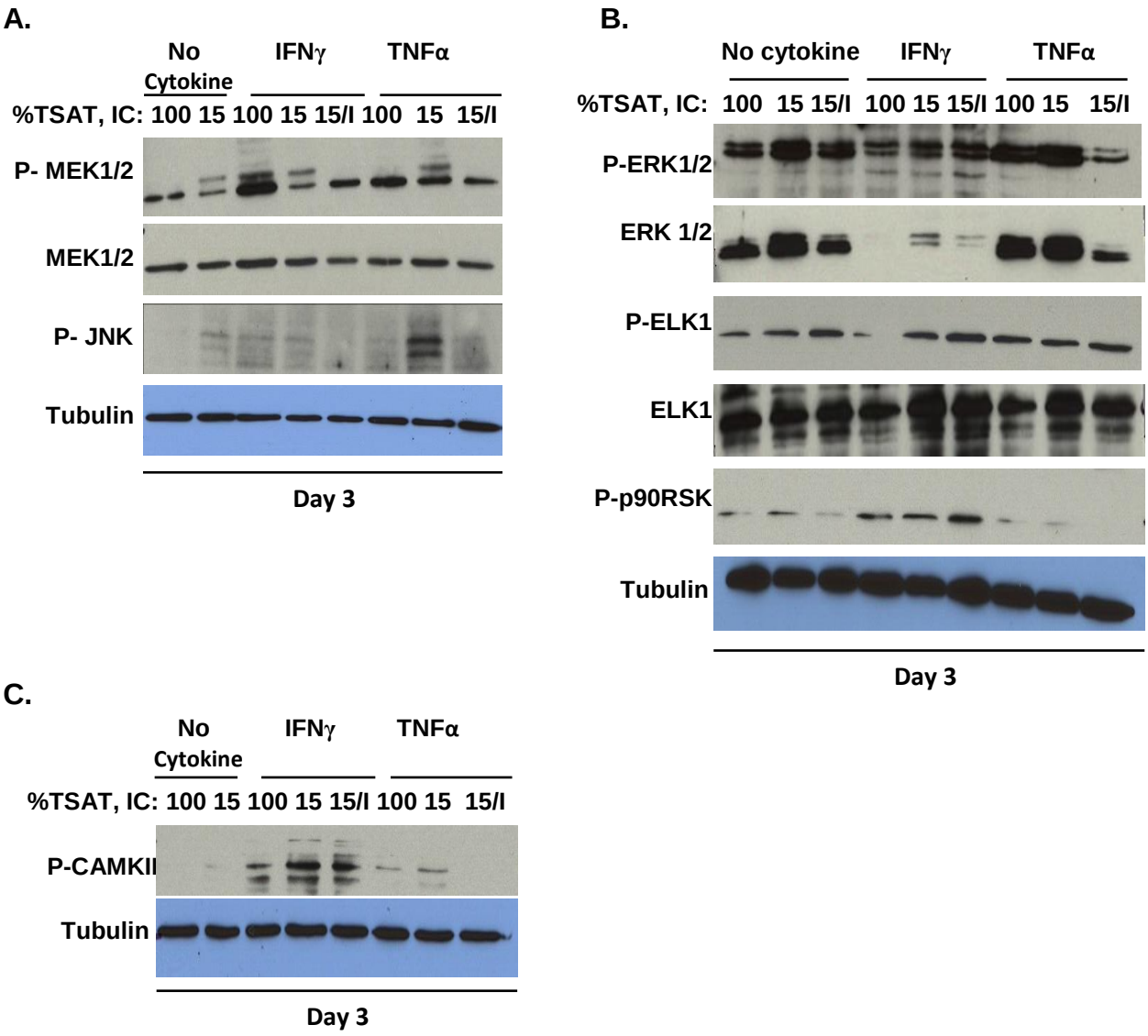


Figure 3.4 Signaling disruptions in kinase signaling implicated in iron metabolism and inflammation

Human CD34+ cells were cultured in erythroid medium + IFNγ or TNFα, + 15% transferrin saturation, and + isocitrate (I) and were harvested on day 3 for immunoblotting of whole cell lysates. **A)** Antibodies were specific for MEK1/2, phospho-MEK1/2, phospho-JNK and tubulin (Tub). **B)** Antibodies were specific for

phospho-ERK 1/2, phospho-ELK1, phosphor-p90RSK 1/2, phospho-MEK1/2, phospho-JNK , total ERK1/2, ELK1 and tubulin (Tub). **C)** Antibodies were specific for phospho-CaMKII and tubulin (Tub).

Table 3.1 Anticipated results for phosphorylation of downstream targets of ERK

Known Observation	Possible Outcomes		Meaning of Outcome
↑ p-ERK1/2	↓ p-ELK1	↓ p-p90RSK	Complete Block
↑ p-ERK1/2	↓ p-ELK1	↑ p-p90RSK	Partial Block
↑ p-ERK1/2	↑ p-ELK1	↑p-p90RSK	Hyperactive

3.2.2.2 DAPK

We conducted a literature review in attempts to find connections between kinases and the two inflammatory cytokines. We found that DAPK is regulated by IFNγ, TNFα, and surprisingly ERK. DAPK has been implicated as a stress–related kinase that is activated or inhibited by IFNγ and TNFα. Specifically, TNFα blocks apoptosis by degrading DAPK, whereas IFNγ is a positive regulator of DAPK, increasing its apoptotic and autophagic activity. Our studies show that IFNγ and TNFα might act in a similar signaling mechanism in the aconitase-PKC-pathway (Figures 2.7, 3.2), in other pathways such as DAPK these cytokines act through to different mechanism leads to opposing effects. One reason for the opposing effects is that IFNγ is much more potent than TNFα in inhibiting erythropoiesis; i.e. minute doses of IFNγ can markedly potentiate the effects of TNFα [98].

Chen, et al., in Figure 3.5 have shown the DAPK and ERK interact, in that ERK can bind to the death domain on DAPK in the cytosol, which inhibits ERK translocation to the nucleus [99]. Additionally, ERK phosphorylates DAPK in the cytosol on serine 735 which promotes apoptosis. Based on these findings, we assessed DAPK protein levels and the localization of ERK. We hypothesized that ERK activation leads to DAPK activation specifically in iron restricted conditions. Due to inconsistent results, we could not determine whether DAPK was activated or inhibited upon stimulation with iron restriction and IFNγ or TNFα. However, we did find that total ERK when under iron restricted conditions, appears to redistribute with a greater fraction going into the nuclear fraction than under iron replete conditions (Figure 3.6A). IFNγ does not appear to alter this change. ERK shifting to the nucleus may be cellular stress response to attempt to

increase cell proliferation under iron restriction. When iron restriction is combined with TNFα, this combination appeared to enhance the redistribution of ERK localization from the cytoplasmic to nuclear fraction (Figure 3.6B), indirectly suggesting that TNFα may be degrading DAPK and allowing additional ERK to shift to the nucleus.

3.2.2.3 CaMKII and JNK

IFNγ has been shown to activate CaMKII, which can act directly with STAT1 and phosphorylate STAT1 on serine 727 to achieve maximal transcriptional activation [100]. In Figure 3.4C, IFNγ did indeed activate phospho-CaMKII under all conditions; however, IFNγ plus iron restriction appeared to hyper-activate CaMKII. TNFα independently activates JNK, and caspase signaling pathways which inhibits erythroid differentiation [20]. Interestingly, JNK was found only to be activated only upon stimulation with TNFα and iron restriction (Figure 3.4C). This activation correlates with decreased cell counts in TNFα plus iron restricted cultures seen in Figure 3.4A. Again, this suggests that the two cytokines are activating different signaling cascades: TNFα and iron deprivation synergized in the activation of JNK, and IFNγ and iron restriction synergized in activating CAMKII. In both cases, isocitrate partially reversed the activation to basal levels. Altogether, the data in this chapter suggest that more research is needs to be conducted to understand the cross-talk between intracellular signaling pathways in ACDI, specifically pathways targeting aconitase and cytokine-activated kinases.

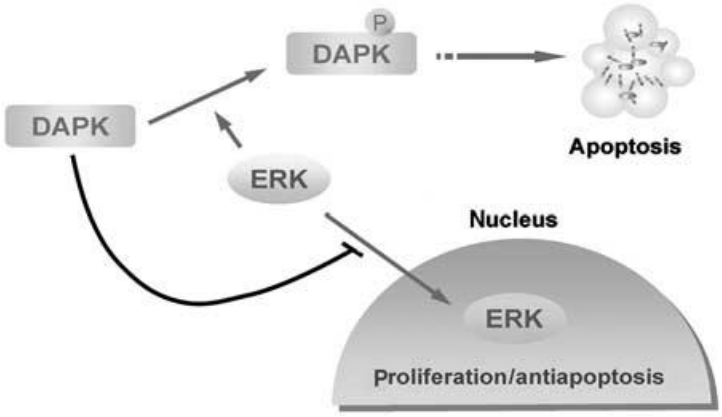


Figure 3.5 Scheme depicting the promotion of apoptosis through a feedback regulatory circuit formed by DAPK–ERK interplay

Diagram was retrieved from Chen et al, *The EMBO Journal*, 2005; Volume 24, Issue 2; 294-304

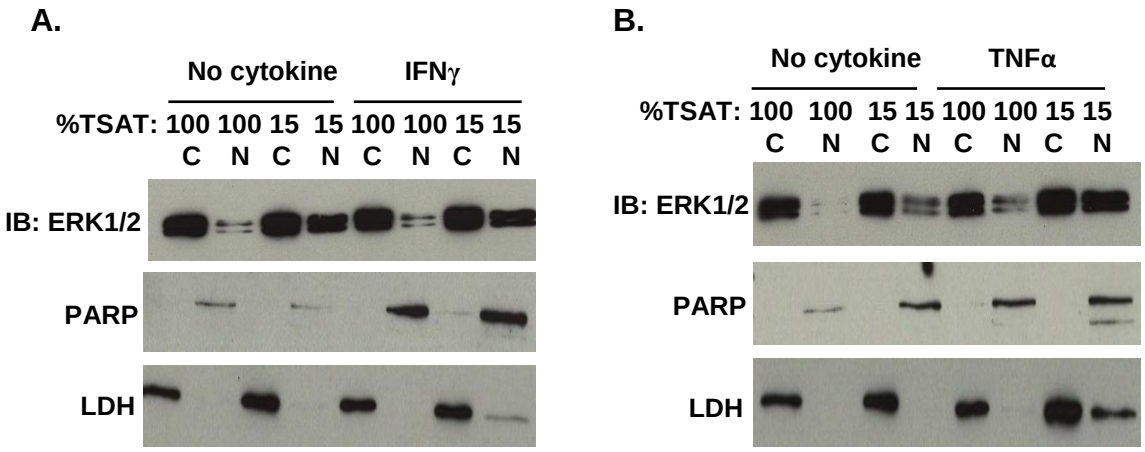


Figure 3.6 Iron restriction increases ERK nuclear localization

Human CD34+ cells were cultured in erythroid medium + IFNγ or TNFα, + 15% transferrin saturation, and + isocitrate (I), and were harvested on the indicated days for immunoblotting of whole cell lysates. Lysates were fractionated into nuclear (N) and cytoplasmic (C) fractions and run on 12% SDS page gel, followed by immunoblotting. PARP (nuclear) and LDH (cytoplasmic) were used as positive controls for localization

3.2.3 Revisiting the GATE and GAS pathways

Achieving an understanding of additional targets impacted by IFNγ in the context of iron restriction has been difficult due to the complexity of the IFN-signaling cascades. Many studies have suggested that IFNγ receptor expression may be increased as a result of iron deprivation. Regis et al reported that iron deprivation via chelating or serum deprivation upregulated IFNγ receptors surface expression, whereas the addition of iron to serum deprived T cells restored low surface expression [69]. Thus, we indirectly assessed whether iron restriction modulates IFNγ receptor surface expression. IFNγ is known to act through specific JAK/STAT molecules in different cell types. IFNγ signals directly through STAT1 in erythroid cells. In our experiments, neither iron restriction nor isocitrate had any effects on IFNγ induction of STAT1 expression and phosphorylation (Figure 2.7B, C). This would suggest that the receptors under high iron and iron restriction are able to respond to the cytokine equally well. This experiment however, does not directly prove that iron restriction does not alter the IFNγ receptor surface expression, but that signaling in this pathway is not affected.

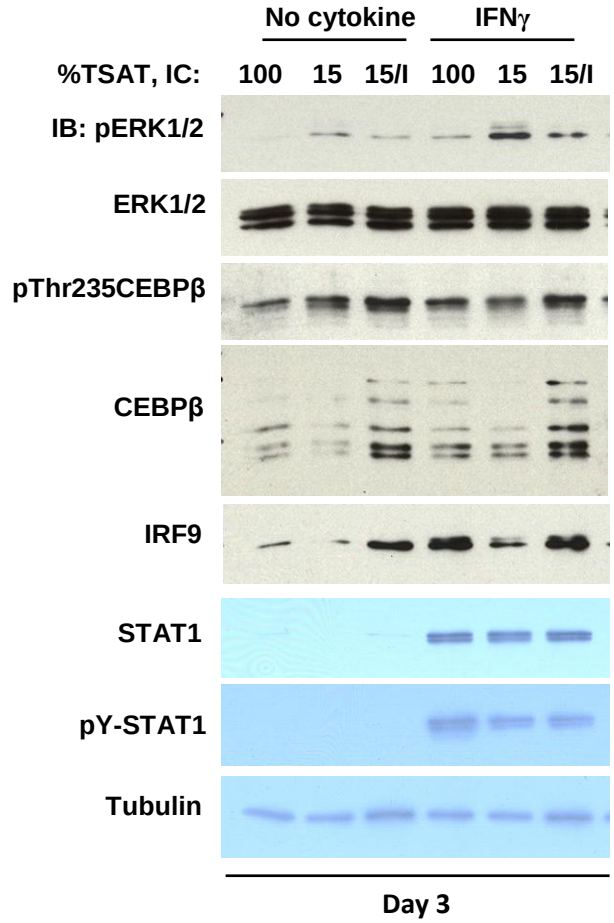
Based on our results that IFNγ signaling is still intact at the level of the JAK/STATs (Figure 2.7), we relied on the literature to point to additional IFN mediated signaling pathways. Previous work showed that IFNγ activates two defined genetic programs based on the type of cis-regulatory sequences associated with the target genes. GAS elements recruit homodimers of pY701-STAT1, while ISRE elements recruit a trimeric complex known as ISGF-3, composed of IRF9 complexed with pY-STAT1: pY-STAT2 heterodimers [70]. The ISGF3 complex is a part of the IFNγ signaling pathway that is independent of a JAK-STAT, and that transactivates a group of genes containing

GATE (g-interferon-activated transcriptional element) cis-regulatory elements, e.g. *IRF9* and *DAPK* [101,102]. In this pathway, the transcription factor C/EBPβ undergoes upregulation and phosphorylation in an ERK-dependent manner, followed by its recruitment to GATE sites [101,102]. Once recruited to these sites, C/EBPβ exerts an activating influence on the majority of target genes; however, a subset of the target genes will undergo repression [101,103]. In some instances, ISGF-3 may repress inflammatory target genes that lack ISRE elements [104,105].

Protein analysis of ISGF3 complex component IRF9 indicated it was decreased under IFNγ plus iron restriction conditions. The results in Figure 3.7A show that iron restriction causes defects in IFNγ activation of the GATE pathway, beginning on day 3 with evidence of defective IRF9 upregulation at the protein level (data not shown), but not at the mRNA level as shown in Figure 2.7F. Defects emerge in diminished C/EBPβ phosphorylation as well as expression of C/EBPβ (Figure 3.7A) at day 3 and become more pronounced by day 4 (data not shown). These signaling abnormalities were all completely corrected by isocitrate. Thus, we hypothesized that iron restriction could reconfigure the pattern of IFNγ signaling in erythroid progenitors, permitting JAK-STAT (GAS pathway) activation but blocking the GATE pathway (Figure 3.7B). The consequences of such a shift on erythropoiesis are unclear. However, Hofer et al. using a murine transgenic model of glial IFNα expression have shown strongly deleterious effects of blocking ISGF-3 activation [106]. Specifically, glial expression of IFNα in *IRF9-/-* mice, but not in wild type controls, caused rapidly fatal CNS inflammation and necrosis. In addition, IFNα treatment of purified glial cells from IRF9-/- animals elicited an altered pattern of gene regulation that resembled an IFNγ-type inflammatory response,

including aberrant activation of the GAS target gene *IRF8* [106]. Shown in Figure 2.7D, E**,** we analyzed the GAS targets, *IRF8* and *IRF1*. We found there were no changes in protein level with iron restriction.

A.


No cytokine
IFNγ
%TSAT, IC:
100 15 15/I 100 15 15/I
IB: pERK1/2
ERK1/2
pThr235CEBPβ
CEBPβ
IRF9
STAT1
pY-STAT1
Tubulin
Day 3

B.

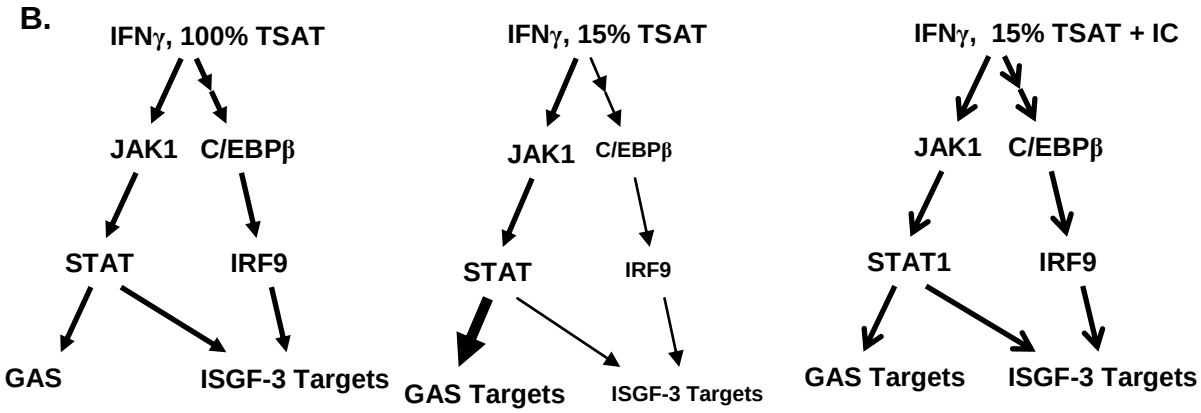

IFNγ, 100% TSAT
JAK1
C/EBPβ
STAT
IRF9
GAS
ISGF-3 Targets
IFNγ, 15% TSAT
JAK1
C/EBPβ
STAT
IRF9
GAS Targets
ISGF-3 Targets
IFNγ, 15% TSAT + IC
JAK1
C/EBPβ
STAT1
IRF9
GAS Targets
ISGF-3 Targets

Fig. 3.7 Iron restriction selectively blocks IFNγ activation of the GATE pathway. **A)** Human CD34+ cells were cultured in erythroid medium + IFN, + 15% transferrin saturation, and + isocitrate (I) were harvested on the indicated days for immunoblotting of whole cell lysates. Antibodies were directed to C/EBPβ phosphothreonine 235, total C/EBPβ, DAPK, IRF9, total STAT1 (S1), STAT1 phosphotyrosine 701 (pY-S1), and tubulin (Tub). **B)** Model for reconfigured IFNγ signaling associated with erythroid iron restriction. In iron replete conditions (100% TSAT), IFNγ is predicted to regulate both GAS and ISGF-3 targets in a balanced manner. With iron restriction (15% TSAT), IFNγ signaling is predicted to be skewed toward GAS targets. Isocitrate can restore balance through activation of C/EBPβ pathway.

Methods

Cell Culture

Purified human CD34+ progenitors derived from granulocyte-colony stimulating factor mobilized peripheral blood cells of normal donors (Hematopoietic Cell Processing Core of the Fred Hutchinson Cancer Research Center) are grown at 37ºC with 5% CO2 in serum free medium consisting of Iscove's modified Dulbecco's medium (IMDM) with β-mercaptoethanol and lot tested bovine serum albumin. To establish cultures with defined transferrin saturations, the medium is supplemented with defined ratios of the insulin/transferrin/selenium (ITS) A and B preparations from Stem Cell Technologies (Vancouver, Canada). After thawing, the CD34+ progenitors first undergo ~72 hours pre-stimulation in IMDM with β-mercaptoethanol, BIT 9500 supplement (Stem Cell Technologies) and cytokines consisting of 100 ng/ml SCF, 100 ng/ml FLT3-L, 100 ng/ml TPO, and 50 ng/ml IL-3. After prestimulation, the cells are washed and resuspended in unilineage differentiation medium. Erythroid medium contains 4.5 U/ml EPO and 25 ng/ml SCF. The indicated cytokines, human recombinant IFNγ (1500U/ml) and TNFα (100ng/ml) (Peprotech, Rocky Hill, N.J.) are added to erythroid medium. Sodium isocitrate is purchased from Sigma and used at 20mM concentration. Cell numbers and viabilities are determined manually using a hemocytometer and trypan blue staining.

Experimental Conditions:

-100% Fe (normal) ± cytokines (human IFNγ or TNFα)

-15% Fe (Iron restriction) ± cytokines (human IFNγ or TNFα)

-15% Fe (Iron restriction) ± cytokines (human IFNγ or TNFα) + 20 mM Isocitrate

Typically, cells are analyzed after 3 days in these experimental conditions. This time period is sufficient for the cells to just begin displaying phenotypic abnormalities but not sufficient for the onset of cell death, which can complicate interpretation.

Mice

Diet-induced iron deficiency anemia was initiated in 3 week old male C57BL6 weanlings (Jackson Labs) using an Iron Deficient Diet from Harlan Teklad (Product No. TD 30396, Madison, WI), which contains 2-5 ppm iron. Mice were placed on either normal chow or iron deficient chow for 4 weeks to allow anemia to develop. After being on the diet for six weeks, Alzet mini-osmotic pumps were implanted subcutaneously on the back of the mice containing either normal saline or murine recombinant TNFα at 1.5ug. Pumps were in place for 7days. CBC values were acquired from retro-orbital bleeds at the end of treatment using the Hemavet 850 FS automated CBC analyzer (Drew Scientific, Waterbury, CT).

Cytospin Preparation

For determining cell morphology, 100µL of cell suspension containing 10^5 sorted cells was used prepare cytospin preparations on coated slides, using the Thermo Scientific Shandon 4 Cytospin. The slides were stained with May-Grunwald (Sigma MG500) solution for 5 min, rinsed in 40mM Tris buffer (pH 7.2) for 90 sec, and subsequently stained with Giemsa solution (Sigma GS500).

Flow cytometry

Data were collected on a FACSCalibur instrument (Becton Dickinson) and analyzed using FlowJo software Version 8.6.3 (TreeStar Inc). Differentiation of human erythroid progenitors was assessed by co-staining cells with fluorochrome-conjugated antibodies to CD34 (a marker of immaturity), and glycophorin A (GPA, a later marker of erythroid differentiation).

Aconitase assay

Gel-based analysis of aconitase activities in progenitor extracts was performed as previously described. [28,90]

Immunoblot

Whole-cell lysates underwent sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose and immunoblotting as previously described [87]. Antibodies included mouse anti-tubulin (Sigma-Aldrich); mouse anti-IRF9 (Abcam Inc) rabbit anti-STAT1, IRF1, PU.1, ERK, C/EBPβ, ELK1, p90RSK, DAPK, CaMKII, JNK (Cell Signaling Technology); and rabbit phosphospecific antibodies to MEK1/2 (Epitomics), STAT1 serine 727, STAT1 tyrosine 701, and C/EBPβ threonine 235 (Cell Signaling Technology).

Nuclear and Cytoplasmic Fractions

Cells were harvested and the protocol from the NE-PER Nuclear and cytoplasmic Extraction Kit was followed (ThermoScientific). Cellular fractions then underwent

sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose and immunoblotting as previously described [87].

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Chapter 4:
General Discussion

ACDI afflicts millions of people and billions of dollars are spent trying to treat this anemia. ESAs, the most common therapeutic, has limitations and with continual use patient become resistant to their affects. This study had two main goals: to elucidate the precise role of erythroid iron restriction response in ACDI and to understand how to manage severe ACDI unresponsive to ESAs. We used primary human HPCs and PGPS rat model to evaluate the role of the erythroid iron restriction response in ACDI and to develop an alternative therapeutic approach to managing ACDI.

4.1 Mechanistic Insights

We showed that the compound isocitrate stimulates erythropoiesis, i.e. red cell production, under conditions of ACDI. Our data confirms that reversing the erythroid iron restriction response via isocitrate treatment corrects the elevated production of hepcidin and iron retention in macrophages, and reprograms responses of erythroid progenitors to inflammatory cytokines. In further understanding the mechanisms that underlie ACDI we have revealed that the inflammatory response produced inflammatory cytokines (IFNγ, TNFα) and synergizes with the erythroid iron restriction response to impair erythropoiesis (Figures 2.5B, 2.6). The addition of isocitrate completely abrogates this response. We show that isocitrate injection completely and durably reversed anemia in a clinically relevant rodent model of ACDI, the rat chronic arthritis model. These findings suggest that the erythroid iron restriction response plays an essential role in the pathogenesis of ACDI and that targeting this response affords a novel therapeutic opportunity.

We examined the in vitro response of primary human HPCs to inflammatory cytokines under iron replete and iron restricted conditions. IFNγ, a principle cytokine in ACDI, had no inhibitory effects on erythropoiesis in iron replete cultures but markedly inhibited erythropoiesis in iron restricted cultures, with the latter effect reversible by isocitrate. Analysis of signaling revealed no influence of iron restriction on IFNγ induction of STAT1 phosphorylation or upregulation. In analyzing how iron restriction and isocitrate affect IFNγ-mediated signaling, PU.1 was identified as a target uniquely co-regulated by iron restriction and inflammatory cytokines. Normally downregulated early in erythroid development, PU.1 is a master regulatory transcription factor whose levels dictate myeloid versus erythroid cell fate in hematopoietic progenitors as shown in Figure 4.1. Importantly, the synergistic induction of PU.1 by iron restriction and IFNγ was dependent on PKC activity and is blocked by isocitrate. Erythroid iron restriction causes the inactivation of aconitase enzymes via destabilization of the iron sulfur clusters and an associated hyperactivation of PKCα/β [28]. Isocitrate treatment blocks both aconitase inactivation and PKCα/β hyperactivation, but requires active erythropoietin signaling to exert these effects [64]. Thus, we suggest a completely new paradigm for the cellular effects of iron restriction, involving lineage reprogramming via an aconitase-PKC-PU.1 signaling pathway.

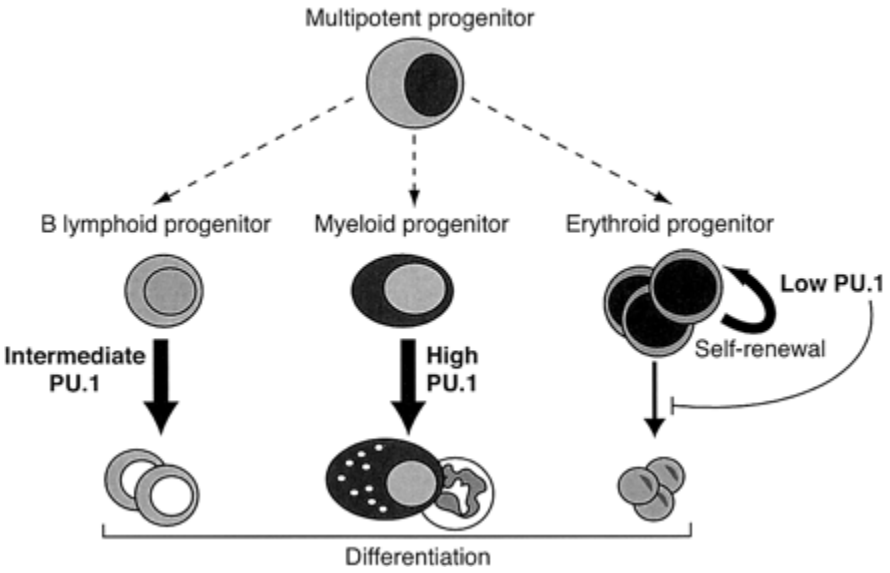

Figure 4.1 Revised model for PU.1 function in hematopoiesis

Diagram retrieved from Back et al, Blood, 2004; Volume 103, Issue 10; 3615-3623

4.2 Clinical Perspectives

There are multiple connections that link inflammatory syndromes/diseases, iron metabolism and impaired erythropoiesis. One example is rheumatoid arthritis, which causes ACDI, characterized by hepcidin-mediated iron retention in macrophages [12]. Importantly, a reciprocal relationship also exists in which erythropoiesis and iron influence arthritis activity. In particular, treatment of RA patients with recombinant human Epo significantly diminishes joint inflammation concomitant with improvement in anemia [107]. By contrast, infusion of intravenous iron causes flaring of RA disease activity [108,109]. The disease-perpetuating activity of iron was further demonstrated in an animal model of RA, in which daily administration of an iron chelating agent markedly diminished arthritis severity [61].

Our studies have established that isocitrate treatment stably and durably reverses the impaired erythropoiesis in vitro and in vivo, alleviating the anemia in ACDI. As an additional, unexpected finding, isocitrate diminished the incidence of neutrophilia associated with the arthritis in this model. Relevant to this finding, the isocitrate-treated mice showed significantly less systemic inflammation, decreased liver hepcidin expression, and diminished iron deposition in splenic macrophages. We found no evidence for direct immunosuppression by isocitrate in three different murine models of autoimmune/inflammatory disease. This indirectly answers the question of: *Is isocitrate correcting the inflammation and thus the red cells indices improve, or do increase red cell indices reduce inflammation?* In these three murine models there was no anemia, but inflammation was present, and isocitrate did not moderate inflammatory response. Thus, we believe isocitrate acts directly on the erythroid iron restriction response exerting its

effects on the marrow to stimulate erythropoiesis, leading in turn to diminished hepcidin expression followed by iron mobilization out of splenic macrophages.

Potential mechanisms for isocitrate's action could include direct redistribution of iron as shown in Figure 4.2., i.e. a "sideraphore" effect, or an indirect consequence of erythroid stimulation. Iron is mobilized out of these macrophages and is able to be used in the formation of red cells. Based on recent findings of Sindrilaru et al. that iron overloading polarizes macrophages toward an M1 proinflammatory phenotype [60], isocitrate stimulation of erythropoiesis is proposed to break a vicious cycle of codependency between chronic inflammation and anemia as depicted in Figure 4.3. As mentioned in Chapter 2, we tested the hypotheses that alterations in iron metabolism in chronic arthritis lead to accumulation of iron laden M1 type macrophages that may perpetuate inflammation and that isocitrate may prevent M1 polarization. Immunostaining studies using the M1 marker, $CD86^{+}$, in the PGPS rat model, led to observations where there was a marked increase in M1 macrophages when treated with PGPS; however, isocitrate was not able to significantly reverse the number of M1 macrophages (Figure 2.3B). Thus, additional studies are necessary to further explore the isocitrate mechanism of action. This will be critical in developing isocitrate as a novel treatment for human RA and will also elucidate a mechanism of autoimmune disease self-perpetuation.

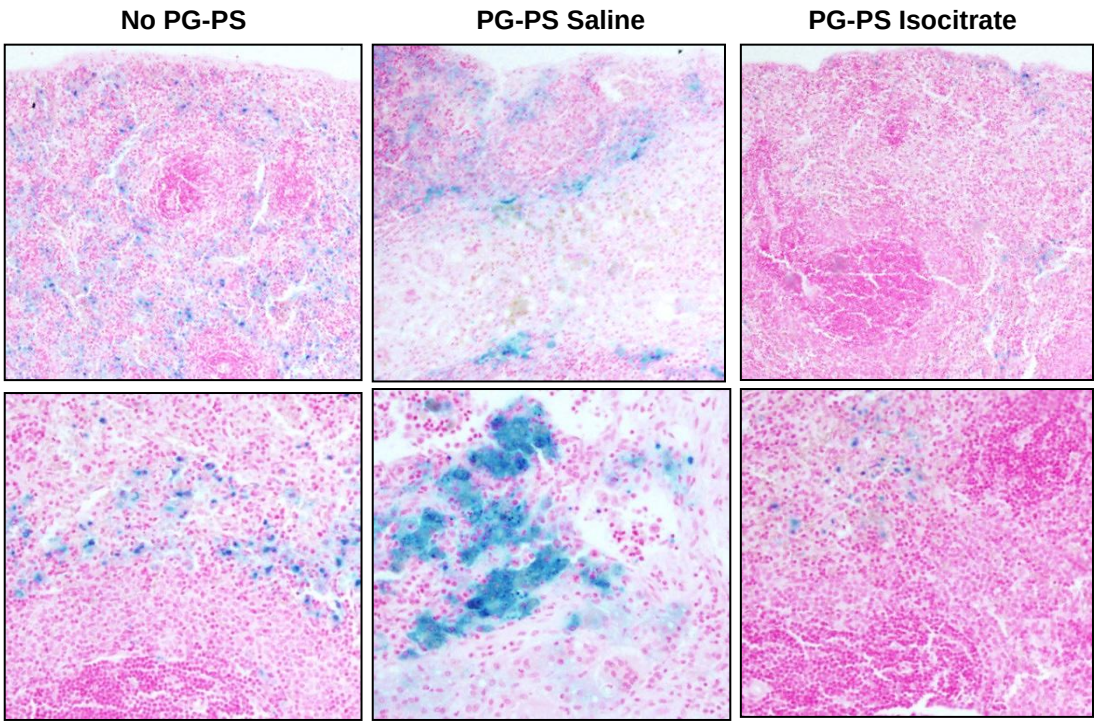

Figure 4.2 Iron Staining of Spleens

Prussian Blue Iron staining of spleens from rats harvested at 42 days. Top row is 4X objective; bottom is 10X objective.

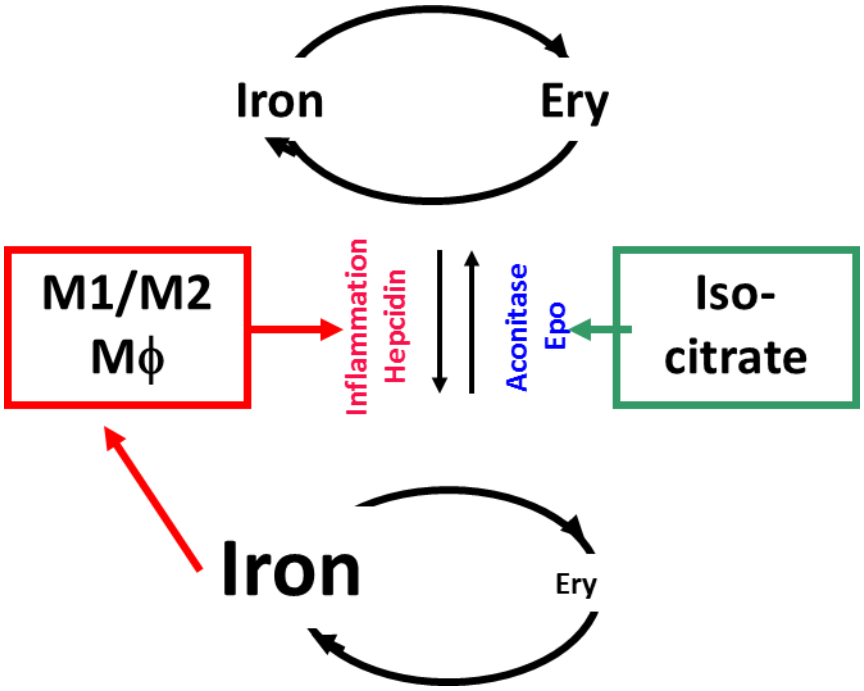

Figure 4.3: Model of codependency between chronic inflammation and anemia.

Chapter 5:
Future Directions

5.1 PU.1 Modulation by Iron Restriction

5.1.1 At what stage in erythroid development does PU.1 get induced in vivo?

PU.1 is a hematopoietic transcription factor that is required for the development of myeloid and B cells. PU.1 is also expressed in erythroid progenitors, where it blocks erythroid differentiation by binding to and inhibiting the main erythroid promoting factor, GATA-1. Our results with cultured human hematopoietic primary cells show that iron restriction potently cooperated with IFNγ signaling in the induction of PU.1 expression in an erythroid stage specific manner (Figures 2.9D,E). This cooperation was blocked by exogenous isocitrate and appears to depend on PKC signaling (Figures 2.10B,C, 2.11B). Understanding the effects of isocitrate on this relationship as well as the precise determination of the developmental stages during which PU.1 undergoes induction needs to be examined.

Future experiments should exploit existing murine anemia model systems, i.e. anemia of acute inflammation-HBKA and anemia of chronic inflammation-CD70TG strain, to characterize in vivo relationships between iron restriction, inflammation and erythroid PU.1 expression. The HBKA mice develop a transient anemia with changes in IL-6, hepcidin, and serum iron, whereas the CD70TG mice develop a chronic anemia completely dependent on IFNγ stimulus. These murine models are preferred over the PGPS rat model because they provide extremely powerful tool for determination of the developmental stages during which PU.1 undergoes induction and there are wide-range of cell surface markers available for lineage tracing.

To track the modulation of PU.1 expression during erythroid development, we will use mice with a GFP reporter controlled by the PU.1 upstream regulatory element

(URE) and promoter (available from Jackson Labs as B6;FVB-Tg(Sfpi1,-EGFP)7Dgt/J). This reporter accurately recapitulates the patterns of endogenous PU.1 expression in spleen and marrow, including low-level expression in early erythroid progenitors [110]. After development of anemia, animals we be treated with isocitrate or saline for five days as previously described. Following these five day treatments, the marrows will be harvested for flow cytometric assessment of GFP expression in erythroid precursors at various stages of development: In addition, GFP+ erythroid progenitors will undergo assessment for viability and cell cycle status.

To analyze erythroid development, marrow cells will be counted and undergo staining for CD71, Ter119, and CD44, as well as Annexin V staining for apoptotic cells. Erythroid cells will then be staged using method described in Chen et al [111]. We will then determine the extent of GFP reporter expression and Annexin V staining within each developmental stage. Absolute numbers and percentages of GFP+ cells in each erythroid stage will be compared among the experimental groups. To determine whether erythroid induction of PU.1 affects cell cycle arrest, marrow cells will undergo costaining with anti-Ter119 and DRAQ5. The distribution of cells in Go/G1, S, and G2M will be compared in GFP^{Bright}, GFP^{Dim}, and $GFP^{Negative}$ populations.

5.1.2 Does PU.1 induction change the fate of the erythroid progenitors?

Cell fate mapping studies will permit us to track in vivo fates of erythroid progenitors that have undergone PU.1 induction by the combination of iron restriction and inflammation. PU.1 is a potent master regulator with the capacity, even when transiently expressed, to reprogram the fate of hematopoietic progenitors including erythroid cells

[82,83,112,113]. Our studies will determine whether iron restriction and inflammation causes erythroid progenitors to undergo differentiation blockade, myeloid transdifferentiation, or simply cell death.

For these studies we will use erythroid Cre-mediated reporter activation to permanently mark cells that have entered the erythroid lineage. Such an approach will permit us to track erythroid progenitors that subsequently downregulate erythroid surface markers due to PU.1-induced lineage switch, as has been described in vitro [112]. For these studies we will cross EpoR-Cre mice [111] with the red fluorescent reporter strain Rosa26-floxed stop-tomato. The resultant strain will be used in the three anemia models described above. Control animals and anemic animals with and without isocitrate treatment will undergo comparison of Rosa26-Tomato in cells. We anticipate that anemic animals will show enhanced expression of the erythroid Rosa26-Tomato reporter in myeloid cells.

To verify hypothesis the marrows will be harvested and stained for erythroid panel: antibodies to CD71, Ter119, and CD44, and myeloid panel: antibodies to Gr1, CD115, CD11b, and F4/80. Gr1 vs. CD115 plots will be used distinguish neutrophil and monocytic lineages, while CD11b vs. F4/80 plots will separate macrophages from other myeloid cells. Yamada et al. previously showed PU.1-mediated induction of CD11b and F4/80 on erythroid cells to correlate with macrophage differentiation [112]. Based on this data we predict that the CD11b+ F4/80+ fraction will be particularly enriched for Tomato expression. We further predict, that isocitrate treatment will eliminate this aberrant expression pattern.

5.2 PKC's actions on the EIRR

5.2.1 Does PKC mediate PU.1 induction during iron restriction?

Our lab and others have demonstrated that mitochondrial aconitase undergoes dynamically regulated interaction with signaling factors, including ERK and PKCβ2 [27,114]. Furthermore, shRNA knockdown of mitochondrial aconitase impairs primary erythroid differentiation [28], while knockout of cytosolic aconitase has no effect on steady state or stress erythropoiesis in mice [27]. Previously, we developed a model in which mitochondrial aconitase functions to integrate information on iron availability, metabolic status, and erythropoietin signaling **(**Figure 1.7). In this model we postulate that the iron sulfur clusters in aconitase are destabilized. This destabilization induces a repressive signalosome that may act through PKC hyperactivation. Isocitrate may act to prevent assembly of the repressive signalosome through the stabilization of aconitase. We have since expanded this model (Figure 2.12A) to assign mitochondrial aconitase a central role in controlling PKC signaling, which in turn regulates PU.1 levels and ultimate cell fate. Despite its critical role in erythroid regulation, mitochondrial aconitase remains poorly characterized with regard to the molecular mechanisms by which it regulates cell signaling.

Future experiments will determine whether PKC mediates PU.1 induction during iron restriction, as suggested by our findings in huHPC cultures (Figures 2.10B,C, 2.11B). Several studies have demonstrated PU.1 regulation by PKC signaling, with one study documenting direct phosphorylation by PKCβ [75,76,115]. Furthermore, PKC signaling has been shown to alter hematopoietic cell fate in a manner similar to PU.1 induction [83,85,116]. Thus, we will directly test the model in Figure 2.12A by

determining in vivo contributions of PKC to PU.1 induction by iron restriction. PKCα and β have been targeted for study because: a) they are the isoforms that undergo hyperactivation that is induced by iron restriction and reversed by isocitrate [28]; b) PKCβ has been shown to undergo recruitment to mitochondrial aconitase [114]; c) PKCα drives macrophage differentiation in a manner similar to PU.1 [85,117]; and d) PKCβ has previously been implicated in direct regulation of PU.1 [75].

To determine the contributions of PKCα and β to PU.1 upregulation due to in vivo iron restriction, we will use PKCα and β knockout mice. Specifically, individual as well as double knockout strains will be crossed with the PU.1-EGFP reporter strain. Animals will be subjected to dietary iron deficiency or HKBA-induced acute inflammation as described above. To study chronic inflammation, we will cross PKCα knockout and PKCβ knockout mouse with the CD70TG mouse. The effects of PKC knockout will be analyzed on peripheral blood CBC, marrow erythroid development, and on PU.1-EGFP reporter expression in erythroid progenitors. We will compare the patterns of expression of the PU.1-GFP reporter in erythroid cells in various stages of development in wild type, single PKC knockout (α-/- or β-/-), and double PKC knockout (α-/- and β-/-) strains. Animals will be analyzed under resting conditions and in response to the anemic challenges + isocitrate. We will compare CBC values and overall marrow erythroid development among the various to determine whether PKC knockouts protect against anemic challenges.

Based on prior implication of PKCβ in the regulation of PU.1 and in the interaction with mitochondrial aconitase [75,114], we predict that PKCβ knockout mice will show in their response to the anemic challenges: diminished erythroid induction of

PU.1, diminished impairment of erythropoiesis, and diminished responsiveness to isocitrate treatment.

5.3 Isocitrate

5.3.1 Development

Isocitrate, a natural compound with high levels in blackberries [118], has been an alternative medicine remedy for anemia as well as inflammatory diseases such as IBS, for years. Our studies have concluded that isocitrate corrects ACDI. While our studies have been preliminary on the scale of therapeutic development, we have enough evidence to justify pharmacokinetics, metabolism and toxicology studies in the animal models. We would like to analyze different routes of administration such as oral gavage to test whether are as effective as intraperitoneal injections. In a pilot study, giving isocitrate orally resulted in a correction of anemia; however, these effects were very transient, lasting only 5-7 days post treatment. The transient correction in anemia is most likely due to different rates and efficacy of absorption between oral and intraperitoneal administration. In this study, we conducted all the treatments at the same injection dose of 200mg/kg/day for five days. In the future, we would like to get an accurate dose response curve by scaling down the current dose.

Additionally, we would like to assess the ability of isocitrate to correct anemia in other models of anemia: HBKA, CD70TG mice models, and subchronic IL-6-induced anemia model in cynomolgus monkeys [119]. Interestingly, when we treated the acute HBKA model, the results of isocitrate anemia correction were inconclusive. However, we postulate that we may need to add recombinant Epo to the isocitrate treatment regimen in

these animals to bolster the response, as we know erythroid cells need Epo to carry out erythropoiesis. In the PGPS model were observe no significant changes in serum Epo levels when comparing saline controls, PGPS treated, and PGPS plus isocitrate treated animals (Table 2.2); however, HBKA mice show changes in Epo levels in comparison to controls.

5.3.2 Mechanism

Understanding the mechanism in which isocitrate is exerting its effects is critical to its targets as well as development as a novel therapeutic. We believe isocitrate exerts its effects through the stimulation of erythropoiesis, which in turn mobilizes iron through repression of hepcidin. To test this hypothesis, first we would inject intravenously radioactive-labeled iron Fe^{59} [120,121] in combination with isocitrate and assess the location the iron in vivo; whether it gets incorporated in new red cells or is retained in the splenic macrophages. To determine whether hepcidin downregulation is required for isocitrate amelioration of inflammation, animals will receive IL-6 injections, as described [17], to enforce hepcidin expression. Lastly, to determine whether enforced suppression of erythropoiesis prevents isocitrate from ameliorating inflammation and iron deposition, we would selectively suppress marrow erythropoiesis while allowing maintenance of inflammation using thiamphenicol gavage, at 125mg/kg/day in rats. Thiamphenicol is a chloramphenicol derivative that causes an aplastic anemia. It has been shown to lower hemoglobin, reticulocyte count and thrombocyte count [122]. As described by Turton et al., this dose in rats causes a 20-fold repression of reticulocyte counts while minimally affecting neutrophil and lymphocyte counts [45]. Endpoints to be analyzed include: arthritis incidence and severity scores, complete blood counts, Fe staining of bone

marrow and spleen, macrophage subtypes/iron in spleen and joints, iron and liver hepcidin mRNA levels.

Chapter 6:
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