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Research Article

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# Isocitrate ameliorates anemia by suppressing the erythroid iron restriction response

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**The unique sensitivity of early red cell progenitors to iron deprivation, known as the erythroid iron restriction response, serves as a basis for human anemias globally. This response impairs erythropoietin-driven erythropoiesis and underlies erythropoietic repression in iron deficiency anemia. Mechanistically, the erythroid iron restriction response results from inactivation of aconitase enzymes and can be suppressed by providing the aconitase product isocitrate. Recent studies have implicated the erythroid iron restriction response in anemia of chronic disease and inflammation (ACDI), offering new therapeutic avenues for a major clinical problem; however, inflammatory signals may also directly repress erythropoiesis in ACDI. Here, we show that suppression of the erythroid iron restriction response by isocitrate administration corrected anemia and erythropoietic defects in rats with ACDI. In vitro studies demonstrated that erythroid repression by inflammatory signaling is potently modulated by the erythroid iron restriction response in a kinase-dependent pathway involving induction of the erythroid-inhibitory transcription factor PU.1. These results reveal the integration of iron and inflammatory inputs in a therapeutically tractable erythropoietic regulatory circuit.**

## Introduction

Anemias of chronic disease and inflammation (ACDI) occur frequently in patients with malignancy, autoimmunity, and kidney disease (1). Development of anemia in these patients often compromises lifestyle and may increase risk for mortality (2–4). A major factor contributing to the anemia consists of lineage-specific suppression of bone marrow erythropoiesis (5). Multiple soluble mediators have been implicated in erythropoietic suppression, including hepcidin (6) and various inflammatory cytokines, including IFN- $\gamma$  and TNF- $\alpha$  (5, 7). 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 (8, 9). The discovery of this pathway has provided a breakthrough, allowing development of new diagnostic and therapeutic approaches to ACDI (10).

The erythroid iron restriction response 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 erythropoietin signaling by transferrin receptors and by aconitase enzymes, which require iron to convert citrate to isocitrate (IC) (11–13). 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 (12, 13). Under conditions of iron deprivation, aconitase inactivation promotes PKC (PKC $\alpha/\beta$ ) hyperactivation, which in turn contributes to impaired viability and differentiation (12). Provision of exogenous IC in either cell culture or

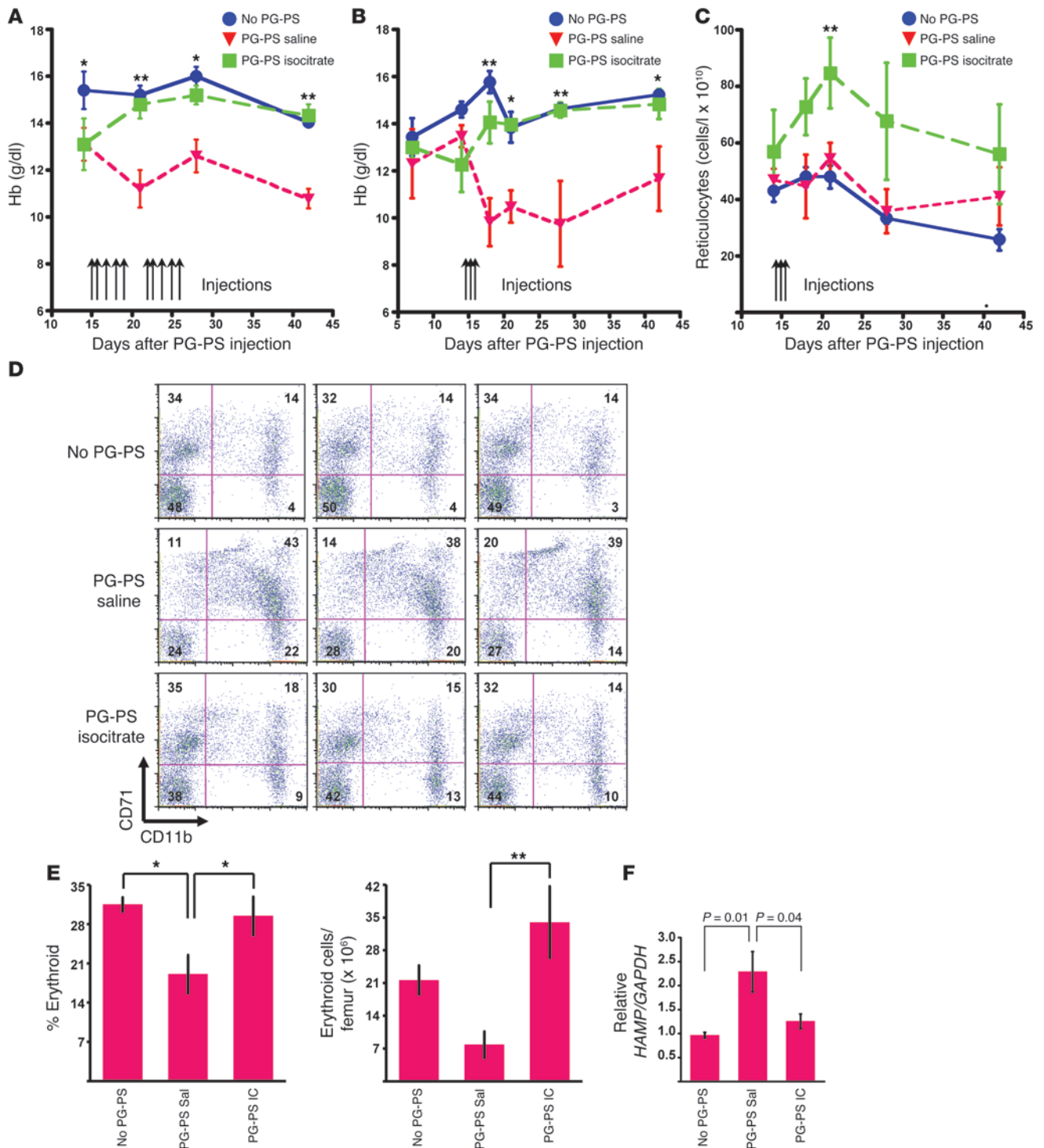
murine models of iron deficiency abrogates the erythropoietic block characteristic of the erythroid iron restriction response and prevents PKC $\alpha/\beta$  hyperactivation (12).

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. First, 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 hemoglobinization (8). Second, patients with ACDI have not consistently demonstrated increased serum or urinary hepcidin levels (14). Third, anemias caused directly by hepcidin overproduction due to hepatic adenomas or germline mutations in Tmprss6 resemble iron deficiency anemia with hypochromic microcytic red cells (15, 16). Similarly, murine models have shown differences in the anemias associated with inflammation versus those due to transgenic hepcidin expression (17). On the other hand, compelling evidence indicates that iron restriction plays some role in ACDI. Thus, intravenous iron infusion effectively ameliorates anemia in numerous patients with ACDI (1, 18, 19). Furthermore, pharmacologic blockade of hepcidin induction in arthritic rats showed efficacy in reversal of anemia (20).

In the current studies, a brief course of IC 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 IFN- $\gamma$  or TNF- $\alpha$ , and IC treatment blocked this sensitization. The signaling relationship between iron restriction and IFN- $\gamma$  pathways involved convergent regulation of PU.1, a myeloid transcription factor whose repression constitutes a critical commitment step in erythroid differentiation (21). Recently,

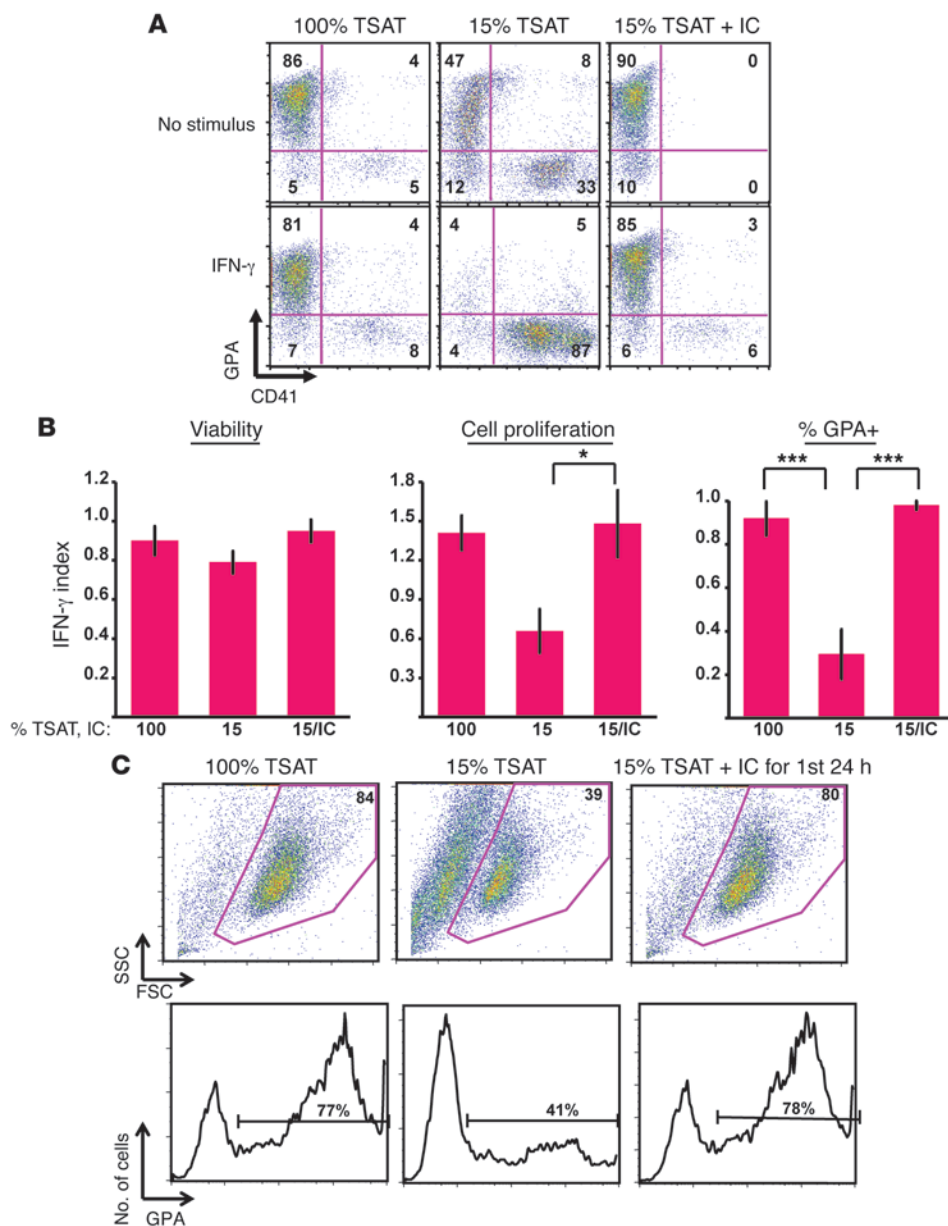
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**Figure 1**

IC 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 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. Nonanemic controls are shown in blue.  $n = 4/\text{group}$  for IC and  $5/\text{group}$  for saline and nonanemic controls. (B) Sustained anemia correction with 3 IC injections. Experiment was conducted as in A with  $n = 5/\text{group}$ . (C) Peripheral blood reticulocyte counts in animals from 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 D. Percentage and number of marrow erythroid cells (CD71<sup>+</sup>CD11b<sup>-</sup>).  $n = 5/\text{group}$ . (F) Normalization of hepcidin expression with IC treatment. Animals treated as in 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 noninflamed controls.  $n = 5/\text{group}$ . All data are mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ .



**Figure 2** Iron restriction and IC oppositely modulate the responsiveness of erythroid progenitors to the inflammatory cytokine IFN- $\gamma$ . Human CD34<sup>+</sup> primary progenitors were cultured 5 days in erythroid medium with TSATs of 100%, 15%, or 15% + IC. Where indicated, cultures also contained human IFN- $\gamma$ . **(A)** The cooperative inhibition of erythroid differentiation by iron restriction and IFN- $\gamma$  is reversed by IC treatment. Cells stained with fluorescent antibodies to the erythroid antigen 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 **A**, showing mean  $\pm$  SEM for IFN- $\gamma$  response index for viability, proliferation, and differentiation. This index consists of the ratio of values obtained in cultures with IFN- $\gamma$  divided by values obtained in cultures without IFN- $\gamma$ . Thus, values greater than 1 represent a positive effect of IFN- $\gamma$ , and values less than 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 IFN- $\gamma$ . 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 IFN- $\gamma$ . Cells on day 5 underwent flow cytometry for GPA expression with gating on the viable fraction.  $n = 3$ . All data are mean  $\pm$  SEM. \* $P < 0.05$ ; \*\*\* $P < 0.001$ .

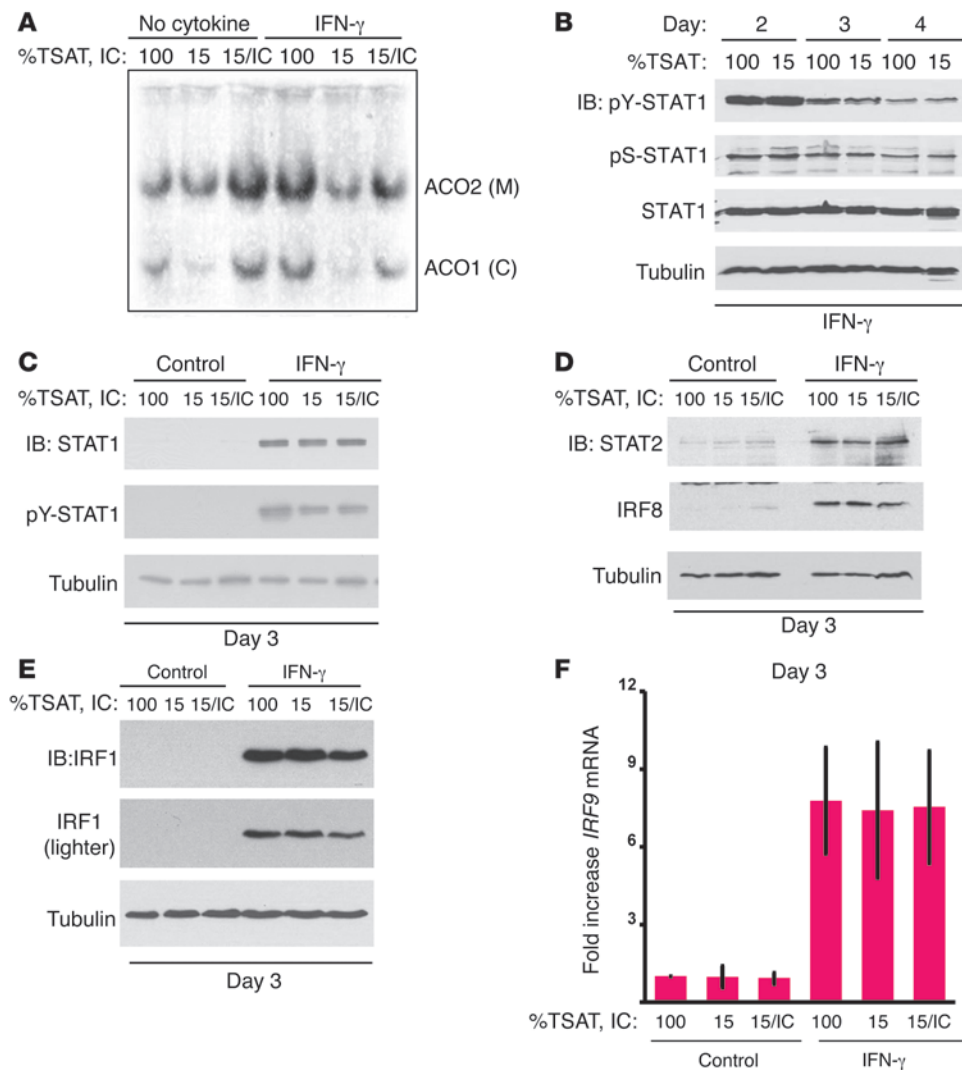
PU.1 upregulation has been identified as a critical component in a murine model of ACIDI (22). Our data show specifically that iron restriction via PKC signaling cooperated with IFN- $\gamma$  in upregulating PU.1 in early erythroid progenitors, an effect that was blocked by IC treatment. These findings identify a pathway in which iron restriction may contribute to ACIDI 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**

*IC treatment corrects anemia and erythropoietic defects in rodent ACIDI model.* IC treatment abrogates the erythroid iron restriction response in cell culture and animal models of iron deprivation (12). Because the erythroid iron restriction response may also con-

tribute to ACIDI (7), we determined the effects of IC administration in a rat arthritis model that faithfully recapitulates human ACIDI (23, 24). In this model, a single injection of streptococcal peptidoglycan-polysaccharide (PG-PS) induces chronic arthritis associated with stable normochromic, normocytic anemia persisting 2 weeks after injection (25). The pilot trial compared 10 daily injections of trisodium IC versus saline solution, beginning day 14 after PG-PS. In this trial, IC treatment corrected the anemia after the initial 5 injections, and the correction was sustained for at least 16 days beyond the last injection (Figure 1A). In a second trial, 3 injections of IC sufficed for correction of anemia to the end of the study, 28 days after treatment (Figure 1B and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI68487DS1). Associated with correction of anemia, IC treatment induced a significant reticulocyte response consistent with enhancement of erythropoiesis (Figure 1C).





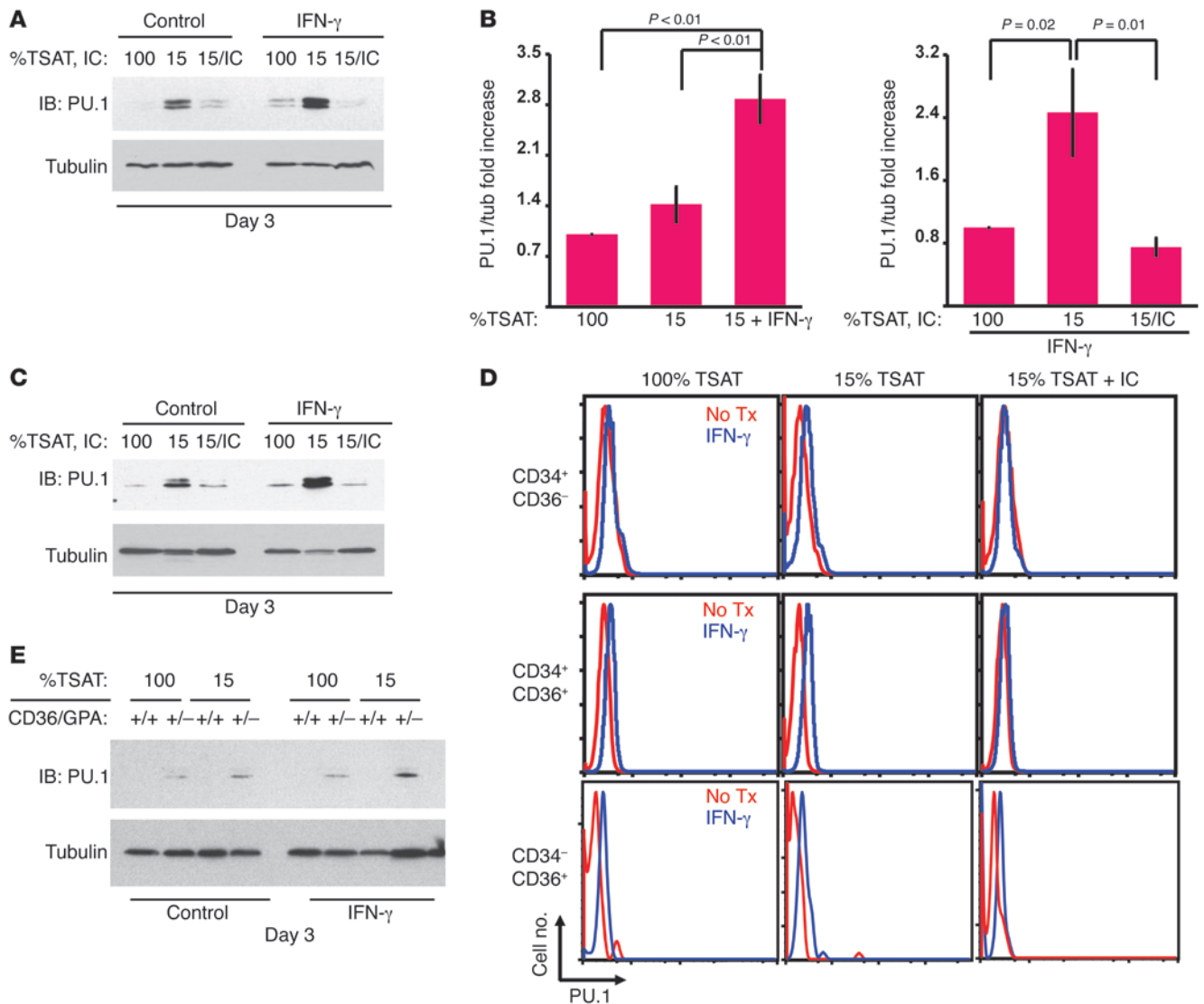
**Figure 3** Influences of IFN- $\gamma$  on erythroid aconitase activity and of iron restriction and IC on IFN- $\gamma$ -mediated signaling. (A) Mitochondrial (M) and cytosolic (C) aconitase activities in human progenitors subjected to IFN- $\gamma$  treatment  $\pm$  iron restriction and IC. Gel-based 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 IFN- $\gamma$  activation of STAT1. Whole-cell lysates from human progenitors cultured in erythroid medium with IFN- $\gamma$   $\pm$  iron restriction underwent immunoblot (IB) analysis of STAT1 phosphorylation and expression (C–E) Influences of iron restriction and IC on IFN- $\gamma$  signaling via JAK-STAT. Progenitors cultured 3 days in erythroid medium under the indicated conditions were analyzed as in B. (F) Influences of iron restriction and IC on IFN- $\gamma$  signaling via the GATE pathway. Cells cultured as in C–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 IFN- $\gamma$  and with 100% TSAT. All data are mean  $\pm$  SEM.  $n = 3$ .

To further assess effects of IC on erythropoiesis, rat marrows underwent flow cytometry 4 days after treatment as represented in Figure 1, B and C. With currently available antibody reagents, normal rat erythroid progenitors can be distinguished as a discrete CD71<sup>+</sup>CD11b<sup>-</sup> marrow population, while maturing myeloid cells express bright CD11b and variable CD71, and early progenitors lack both markers (Figure 1D). Marrows from saline-treated animals with ACDI contained decreased proportions of erythroid progenitors, increased myeloid cells, and a novel population of CD11b<sup>Dim</sup>CD71<sup>+</sup> cells (Figure 1D). IC treatment corrected these marrow abnormalities and restored the discrete CD71<sup>+</sup>CD11b<sup>-</sup> erythroid population seen in normal controls (Figure 1D). IC significantly enhanced both percentages and absolute numbers of marrow erythroblasts in PG-PS-injected animals (Figure 1E). An additional marrow abnormality seen in ACDI was increased erythroid cell death, which showed partial reversal by IC treatment, although this effect did not attain statistical significance (Supplemental Figure 1A). No alterations in erythroblast cell-cycle distribution occurred in any of the experimental groups (Supplemental Figure 1B). To further assess the impact of IC on bone marrow erythropoiesis, we performed colony forming assays. As shown in Supplemental Figure 1C, IC treatment of PG-PS-injected animals

significantly enhanced both frequency and total numbers of burst-forming unit-erythroid (BFU-e) as well as enhancing frequency of CFU-erythroid (CFU-e). Enhancement of erythropoiesis promotes repression of hepcidin in the liver (26). Consistent with a therapeutic mechanism involving enhancement of erythropoiesis, IC treatment was associated with significantly decreased liver hepcidin mRNA levels (Figure 1F).

The results in Figure 1, combined with previously published in vitro data (12), suggest that IC ameliorates anemia in ACDI through promoting erythropoiesis, most likely acting directly on erythroid progenitors. However, IC could potentially exert indirect effects, such as induction of EPO or suppression of inflammation. Measurement of serum EPO levels in animals from Supplemental Table 2 showed no evidence of induction by IC. In addition, a variety of experimental approaches revealed no direct impact of IC on acute or chronic inflammation (see Supplemental Results and associated Supplemental Figure 1D, Supplemental Figure 2, A and B, and Supplemental Figure 3, A–D).

*Iron restriction sensitizes erythroid progenitors to IFN- $\gamma$ .* To determine whether intrinsic inflammatory signaling could cooperate with the erythroid iron restriction response in erythropoietic repression, mediators previously implicated in ACDI (5) were screened for effects on



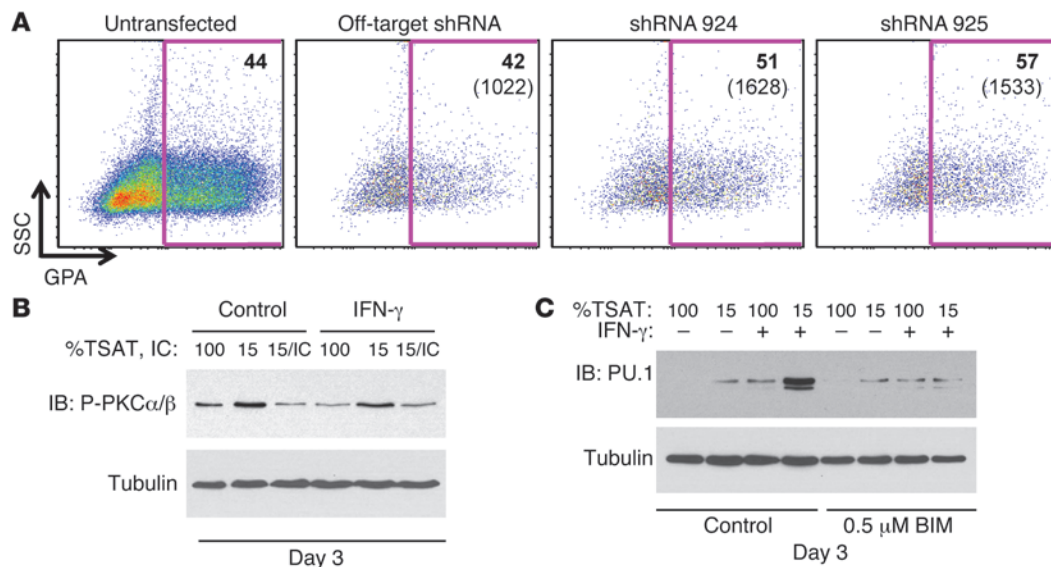
**Figure 4**

Cooperative induction of PU.1 by iron restriction and IFN- $\gamma$  is blocked by IC. (A) Iron restriction and IC oppositely modulate IFN- $\gamma$  induction of PU.1 in primary hematopoietic progenitors. Human CD34<sup>+</sup> cells cultured as indicated in erythroid medium for 3 days underwent immunoblot 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  $\pm$  SEM. (C) Influences of iron restriction, IFN- $\gamma$ , and IC on PU.1 levels in purified erythroid progenitors. Human CD36<sup>+</sup> cells were cultured and analyzed as in A. (D) Influences of iron restriction, IFN- $\gamma$ , and IC on PU.1 levels at various stages of erythroid development. Human CD34<sup>+</sup> cells cultured as in A underwent flow cytometry with intracellular staining for PU.1. (E) Developmental stage-dependent effects of iron restriction and IFN- $\gamma$  on erythroid PU.1 expression. Human CD34<sup>+</sup> cells cultured for 3 days underwent sorting for early (CD36<sup>+</sup>GPA<sup>-</sup>) and late (CD36<sup>+</sup>GPA<sup>+</sup>) erythroid progenitors followed by immunoblot. All data are mean  $\pm$  SEM. *n* = 3.

primary human progenitors in erythroid medium  $\pm$  iron restriction and IC. These experiments yielded 3 unexpected findings. First, under iron replete conditions, i.e., 100% transferrin saturation (TSAT), major erythroid inhibition occurred with none of the mediators (Supplemental Table 3). Second, under conditions of iron restriction (15% TSAT), IFN- $\gamma$  and TNF- $\alpha$  potently inhibited erythroid development. Third, IC conferred resistance to IFN- $\gamma$  and TNF- $\alpha$  under conditions of iron restriction (Figure 2A and Supplemental Figure 4). The relevance of these findings to ACIDI is suggested by prior implication of IFN- $\gamma$  in erythropoietic repression in human chronic kidney disease and in multiple animal models of anemia (22, 27, 28). In addition,

IFN- $\gamma$  signaling pathways are known to participate in erythroid inhibition by TNF- $\alpha$  and IL-1 and in human idiopathic refractory anemia (29, 30). In the rat arthritis model of ACIDI, increased serum IFN- $\gamma$  and decreased serum iron were observed (Supplemental Table 2).

In multiple repeat experiments, iron restriction reproducibly sensitized human erythroid progenitors to inhibitory effects of IFN- $\gamma$  on differentiation and, to a lesser extent, on proliferation. Although we did observe an inhibitory effect on viability with iron restriction alone, IFN- $\gamma$  did not cause any additional inhibition in viability when combined with iron restriction (Figure 2B). Importantly, exogenous IC conferred complete IFN- $\gamma$  resistance on iron-



**Figure 5**

The cooperative induction of PU.1 by iron restriction and IFN- $\gamma$  contributes to erythroid inhibition and requires PKC signaling. **(A)** PU.1 knockdown enhances erythropoiesis in the setting of iron restriction plus IFN- $\gamma$  stimulation. Human CD34<sup>+</sup> cells were transduced with shRNA constructs, cultured 4 days in erythroid medium with iron restriction plus IFN- $\gamma$ , and analyzed by flow cytometry with gating on GFP<sup>+</sup> transduced cells. Relative percentage of GPA<sup>+</sup> cells shown in top right corner; absolute number of GPA<sup>+</sup> cells shown below in parentheses. Relative percentage of GFP<sup>+</sup> cells and absolute number of GFP<sup>+</sup> cells are as follows: off-target shRNA 27%, 3841; shRNA #924 35%, 4664; shRNA #925 39%, 3900. **(B)** Iron restriction induces PKC $\alpha/\beta$  hyperphosphorylation, IC reverses this effect, and IFN- $\gamma$  shows no influence. Human CD34<sup>+</sup> cells were cultured as in Figure 4A. **(C)** PKC signaling contributes to the cooperative induction of PU.1 by iron restriction and IFN- $\gamma$ . Human CD34<sup>+</sup> cells cultured as in Figure 4A were treated where indicated with 0.5  $\mu$ M BIM, followed by immunoblot.

deprived erythroid progenitors. These findings confirm that iron availability determines erythroid progenitor response to IFN- $\gamma$  and support a direct effect of IC in promoting erythropoiesis. Even transient IC exposure for only the first 24 hours of culture completely rescued erythropoiesis on day 4 (Figure 2C), suggesting that the erythroid iron restriction response and IC exert durable influences during an early developmental window.

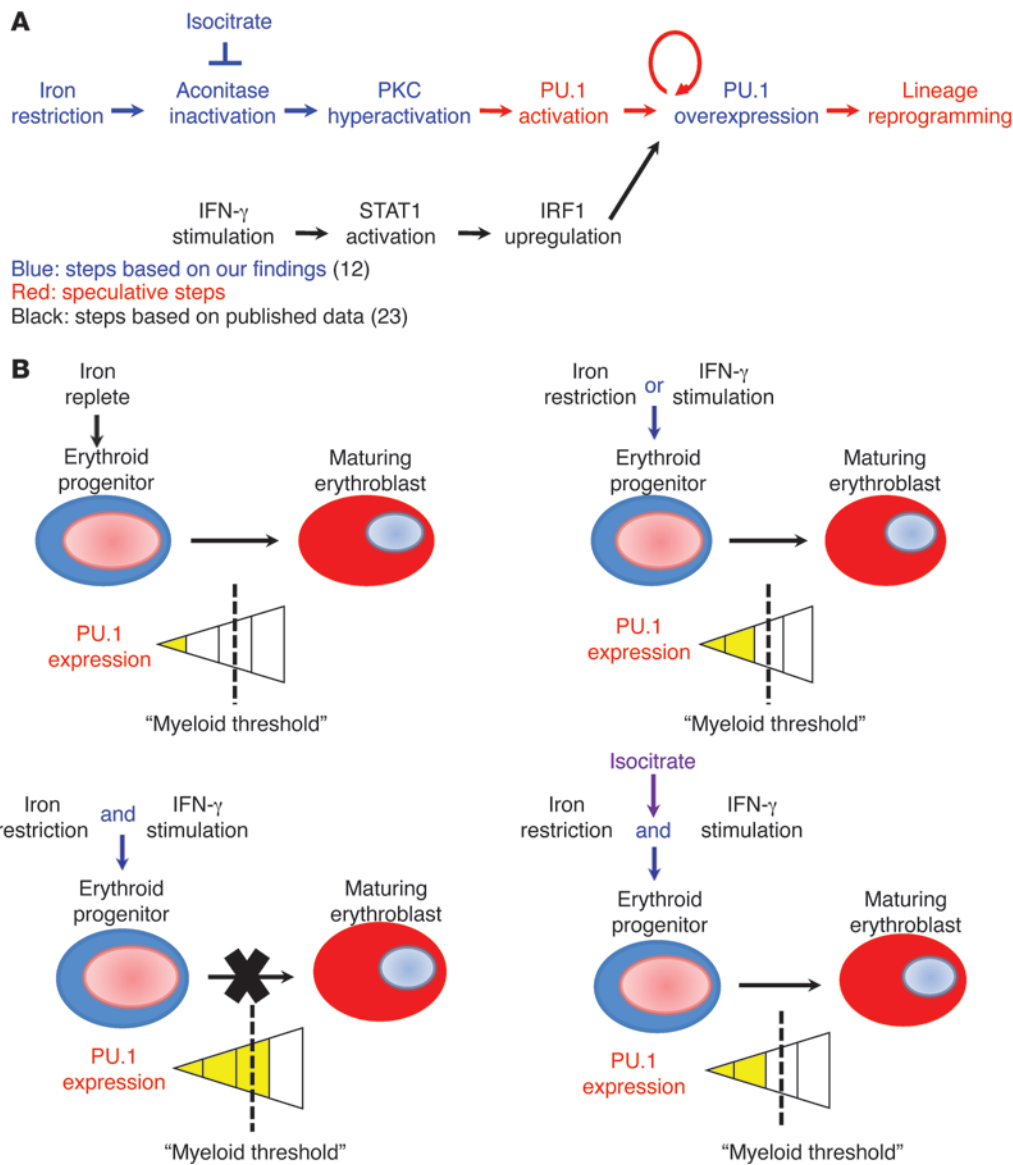
*Distal convergence of erythroid iron restriction and IFN- $\gamma$ -signaling pathways.* Proximal elements of the erythroid iron restriction response and IFN- $\gamma$ -signaling pathways were analyzed to determine the basis for their cooperation in erythroid inhibition. IFN- $\gamma$  has been shown to promote aconitase inactivation in macrophages (31), but failed to decrease erythroid aconitase activities under various culture conditions (Figure 3A). This finding suggests differences between macrophage and erythroid IFN response programs. Prior studies of the effects of iron deprivation on proximal IFN- $\gamma$  signaling, i.e., STAT1 activation, have shown either no influence or sensitization, depending on the cells analyzed (32–34). In erythroid progenitors undergoing IFN- $\gamma$  treatment, iron restriction had no effect on STAT1 phosphorylation on either tyrosine 701 or serine 727 (Figure 3B), the key targets of proximal kinases (35). Furthermore, neither erythroid iron restriction nor IC treatment affected IFN- $\gamma$  induction of STAT1, STAT2, IRF8, or IRF1 (Figure 3, C–E). IFN- $\gamma$  activates IRF9 transcription by the gamma-activated transcriptional element (GATE) pathway, an alternative STAT-independent mechanism. While IFN- $\gamma$  augmented IRF9 mRNA levels in erythroid cells, neither iron restriction nor IC affected this induction (Figure 3F). In aggregate, the crosstalk between erythroid

iron restriction and IFN- $\gamma$  signaling appears to occur distally, i.e., downstream of sensors/receptors and receptor-associated kinases.

*The myeloid transcription factor PU.1 functions as a signaling node in erythropoietic regulation by iron restriction, IFN- $\gamma$ , and IC.* Erythroid lineage commitment coincides with PU.1 downregulation, failure of which inhibits developmental progression and may ectopically activate myeloid genes (21, 36). Recently, Libregts et al. have implicated an IRF1-PU.1 signaling axis in a murine model of IFN- $\gamma$ -dependent anemia (22). PU.1 levels were therefore determined in human progenitors undergoing erythroid culture as in Figure 3C. Under these conditions, iron restriction and IC do not affect the extent of erythroid

lineage commitment, reflected by CD36 upregulation and CD34 downregulation as described (ref. 37 and Supplemental Figure 5B). Iron restriction amplified IFN- $\gamma$  induction of PU.1 by 2- to 3-fold, as well as inducing PU.1 on its own, and IC abrogated PU.1 upregulation by iron restriction plus IFN- $\gamma$  (Figure 4, A and B). Identical results were obtained using a starting population of purified erythroid progenitors isolated by the method of Freyssinier et al. (ref. 38 and Figure 4C).

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 IC blocked IFN- $\gamma$  induction of PU.1, with the strongest effects seen in early committed (CD34<sup>+</sup>CD36<sup>+</sup>) erythroid progenitors (Figure 4D). In the absence of IFN- $\gamma$ , iron restriction also enhanced PU.1 levels preferentially within the CD34<sup>+</sup>CD36<sup>+</sup> compartment, again with complete reversal by IC (Supplemental Figure 5C). As an additional approach, sorted erythroid progenitors (37) underwent immunoblotting for PU.1. In early committed CD36<sup>+</sup> GPA<sup>+</sup> cells, iron restriction promoted PU.1 upregulation in the absence and presence of IFN- $\gamma$ ; in contrast, the late-stage CD36<sup>+</sup>GPA<sup>+</sup> population silenced PU.1 expression under all conditions (Figure 4E). In rat marrows harvested as in Figure 1D, quantitative RT-PCR (qRT-PCR) on sorted progenitors also showed evidence of increased erythroid PU.1 mRNA in ACDI, an effect consistently reversed by IC treatment (3 out of 3 independent experiments) (Supplemental Figure 5E). IC-induced downregulation of erythroid PU.1 protein expression in vivo in ACDI was demonstrated by intracellular staining of rat marrows harvested as in Figure 1D (Supplemental Figure 5D).



**Figure 6**  
 Model depicting the influences of iron restriction and IC on erythropoiesis in ACDCI. (A) Signaling diagram of the convergence of iron restriction and IFN-γ on PU.1. Iron restriction is postulated to contribute through its known capacity to enhance PKCα/β activation (12), and IFN-γ through a documented IRF1/PU.1 transcriptional pathway (22). (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. IC restores erythropoietic capacity by retaining erythroid PU.1 levels below this critical threshold.

Prior studies using an shRNA approach have demonstrated inhibitory consequences of PU.1 induction by IFN-γ in human erythroid progenitors (22). Similar experiments using lentiviral shRNA knockdowns were conducted on cells subjected to iron restriction plus IFN-γ. Two hairpins causing partial *PU.1* knockdown, but not a control hairpin, enhanced erythroid differentiation under these conditions (Figure 5A and Supplemental Figure 6A). One factor limiting this approach, however, consists of the deleterious effects of prematurely downregulating *PU.1* in early progenitors (21, 39).

The erythroid iron restriction response exerts its effects in part through induction of PKCα/β hyperactivation, which can be blocked by IC treatment (Figure 5B and ref. 12). 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 (40, 41). To assess PKC contribution to cooperative PU.1 induction by iron restriction plus IFN-γ, cells cultured as in Figure 4A underwent low-dose treatment with the PKC-selective inhibitor bisindolylmaleimide I (BIM). Notably, BIM abrogated PU.1

upregulation by iron restriction plus IFN-γ (Figure 5C), but did not affect viability or lineage commitment within day-3 cultures (not shown). Similar results were achieved with another unrelated PKC inhibitor, Gö6976 (Supplemental Figure 6B). These results thus support a role for PKCα/β hyperactivation by the erythroid iron restriction response (12) in cooperative induction of PU.1 by iron restriction and IFN-γ.

**Discussion**

As demonstrated in Figure 1, a short course of IC treatment rapidly and durably corrects anemia in a rodent model previously shown to recapitulate human ACDCI (24). These results provide what we believe is 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 i.v. infusion or through hepcidin pathway antagonism. Clinical usage of i.v. iron has expanded due to financial and safety pressures to lower





EPO administration in patients with ACDI (42). However, a recent study has revealed a high prevalence (84%) of hepatic iron overload in chronic kidney disease patients receiving i.v. iron (43). This iron overload potentially could cause tissue damage and enhance risk of bacterial infections (42). Hecpudin pathway antagonists have shown promise in preclinical models (44, 45), but their potential drawbacks conceivably also include induction of iron overload as well as off-target effects and high cost. IC consists of a simple, small molecule that exerts a direct influence on early erythroid progenitors, lowering liver hepcidin expression most likely through the “erythropoietic signal” (26), 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 IC. This 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 (8) 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, IFN- $\gamma$  and TNF- $\alpha$  have been found to exert either negative or positive influences on erythropoiesis, depending on study conditions (29, 30, 46). In our culture conditions, the effects of IFN- $\gamma$  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 signal integration element downstream of the erythroid iron restriction response, IFN- $\gamma$ , and IC. IFN- $\gamma$  recently has been shown to upregulate *PU.1* by inducing the transactivator IRF1 (22), but may also enhance PU.1 DNA binding via PKC $\beta$ -mediated signaling (40). The erythroid iron restriction response promotes PKC $\alpha/\beta$  activation, using a pathway that is repressed by IC (12). Once activated, PU.1 may engage in a positive autoregulatory loop designed to consolidate the myeloid transcriptional program and repress erythroid development (47). A signaling map (Figure 6A) 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 IC treatment. IFN- $\gamma$  signaling additionally contributes to PU.1 induction through IRF1 induction. We postulate that IC inhibits PKC activation by iron restriction through binding to and stabilizing the aconitase enzymes (Figure 3A). The sustained therapeutic effects of transient IC treatment are most likely due to 2 interrelated factors: (a) the ability to target early progenitors during a key window prior to their amplification in the marrow and (b) 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 (21, 48, 49). Accordingly, the erythroid repression associated with ACDI may share features with the myeloid lineage skewing identified in studies of early hema-

topoietic progenitors from mice with autoimmune arthritis (50). In those studies, the marrow Kit<sup>+</sup>Sca<sup>+</sup>Lin<sup>-</sup> (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 IC at early developmental stages (CD34<sup>+</sup>CD36<sup>-</sup> and CD34<sup>+</sup>CD36<sup>+</sup>), during which progenitors may retain lineage plasticity (51). Given the importance of graded PU.1 levels in cell-fate determination (52), we propose a PU.1 threshold-based model for the mechanisms of erythroid iron restriction response and IC in ACDI (Figure 6B). In this model, iron restriction and IFN- $\gamma$  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. IC 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 IC treatment could break a vicious cycle in ACDI and elicit a durable clinical response.

## Methods

**Animal models.** Rats were housed in a pathogen-free facility and handled in accordance with Animal Care and Use Committee (ACUC) policies. Six-week-old female Lewis rats weighing 100–120 grams were purchased from Charles River Laboratories. To induce ACDI, the rats received a single i.p. injection of PG-PS (Lee Laboratories/BD Biosciences) at 15  $\mu$ g rhamnose/g 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 after PG-PS injection, CBCs were monitored. Reticulocytes were measured by staining whole blood with thiazole orange as described (53). Treatments, initiated on day 14 after PG-PS injection, consisted of daily i.p. injections at doses of 200 mg/kg/d of trisodium IC (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  $-80^{\circ}\text{C}$  in single-use aliquots. Serum iron analysis was performed as described (17) using the Ferene Serum Iron/UIBC kit (Thermo Scientific). Serum erythropoietin and IFN- $\gamma$  were measured using the Quantikine Rat EPO ELISA kit and Quantikine Rat IFN- $\gamma$  ELISA kit (R&D Systems).

**Cell culture.** Purified human CD34<sup>+</sup> progenitors derived from granulocyte CSF-mobilized peripheral blood cells of healthy donors were obtained as previously described (54). These cells were grown at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  in serum-free medium consisting of Iscove's modified Dulbecco's medium (IMDM) with  $\beta$ -mercaptoethanol, BIT 9500 supplement (BITS) (Stem Cell Technologies), BSA (Sigma-Aldrich), and the indicated cytokines (PeproTech). The cells initially underwent 72 hours of prestimulation with 100 ng/ml SCF (PeproTech), 100 ng/ml FMS-like tyrosine kinase 3 ligand (FLT3 ligand) (PeproTech), 100 ng/ml thrombopoietin (TPO) (PeproTech), and 50 ng/ml IL-3 (PeproTech) and were then seeded in erythroid differentiation medium, which contains recombinant human erythropoietin at 4.5 U/ml (Epogen; Amgen Mfg. Ltd) with defined TSATs as described (12). Human recombinant inflammatory mediators were added to erythroid cultures as follows: 1500 U/ml IFN- $\gamma$ , 100 ng/ml TNF- $\alpha$ , 100 ng/ml IL-1 $\beta$ , 100 ng/ml IL-6, 100 ng/ml IL-10, 100 ng/ml IL-15, and 50  $\mu$ g/ml LPS (PeproTech). Purified human CD36<sup>+</sup> cells (AllCells LLC) underwent expansion as previously described (38) for 48 hours followed by culture in erythroid medium for 3 days.



**Flow cytometry.** Data were collected on a FACSCalibur instrument (BD) and analyzed using FlowJo software version 8.6.3 (TreeStar Inc). Fluorochrome-conjugated antibodies were purchased from BD Biosciences – 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 costained with FITC-anti-CD71 for erythroid precursors (55), PE-anti-CD11b for myeloid cells, and APC-anti-CD3 for T cells. Erythroid precursor cell death was analyzed by costaining marrows with FITC-anti-CD71, PE-anti-CD11b, annexin V-phycoerythrin, and 7-aminoactinomycin D (Apoptosis Detection Kit I; BD Biosciences – 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 costaining cells with fluorochrome-conjugated antibodies to CD34 (a marker of immaturity), CD36 (an early marker of erythroid lineage commitment) (37), and glycophorin A (GPA) (a later marker of erythroid differentiation). Intracellular staining for erythroid PU.1 expression followed the guidelines of Koulis et al. (56). Specifically, human progenitors stained for CD34, CD36, and GPA underwent fixation and permeabilization using the BD Cytotfix/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 FACS Vantage SE Turbo Sorter with DIVA Option (BD). 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 (55).

**Aconitase assay.** Gel-based analysis of aconitase activities in progenitor extracts was performed as previously described. (12, 57)

**Quantitative real-time PCR.** RNA was isolated using the QIAGEN RNeasy Plus Mini Kit with DNase 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 quantitative real-time PCR (qPCR), we used the comparative  $\Delta\Delta C_t$  formula delineated in the ABI Prism 7700 Sequence Detection System user bulletin no. 2 (58). All samples underwent triplicate analysis with normalization performed by subtraction of the  $C_t$  value of *GAPDH*. Human primers were as follows: *GAPDH*: forward, 5'-TGCCCCCATGTTTGTGATG-3', reverse, 5'-TGTGGTCATGAGCCCTTCC-3'; *IRF9*: forward, 5'-CAAGTGGAGAGTGGGCAGTT-3', reverse, 5'-ATGGCATCCTCTCTCCTT-3'; and *PU.1*: forward, 5'-CAGCTCTACCGCCACATGGA-3', reverse, 5'-TAGGAGACCTGGTGCCAAAG-3'. Rat primers were as follows: *GAPDH*: forward, 5'-CAACTACATGGTTTACATGTT-3', reverse, 5'-GCCAGTGGACTCCACGAC-3'; *hepcidin*: forward, 5'-GAAGGCAAGATGGCACTAAGCA-3'; reverse, 5'-TCTGTCTGTTGCCGAGATAG-3'; and *PU.1*: forward, 5'-CCTTGATTGTGGTGATGGAGAC-3', reverse, 5'-CAGCTCCATGTGGCGGTAGA-3'. Primers were purchased from Integrated DNA Technologies.

**Immunoblot.** Whole-cell lysates underwent SDS-PAGE followed by transfer to nitrocellulose and immunoblotting as previously described (54). Antibodies included mouse anti-tubulin (Sigma-Aldrich); rabbit anti-STAT1, anti-STAT2, anti-IRF8, anti-IRF1, anti-PU.1 (Cell Signaling Technology); and rabbit phosphospecific antibodies to STAT1 serine 727, STAT1 tyrosine 701, and PKC $\alpha/\beta$  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<sup>+</sup> cells used an off-target control GIPZ shRNA construct (catalog nos. VGH5518-200183170; #208) and human PU.1-targeting GIPZ shRNA constructs (catalog nos. RHS4430-100990345 #924 and RHS4430-100990495 #925) (Open Biosystems). Lentiviral packaging constructs pCMV-dR8.74 and pMD2.G were provided by Didier Trono (École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland). Production of lentiviral particles by transient cotransfections of HEK293T cells was carried out using the calcium phosphate method, as previously described (59). Spinoculation of cells, puromycin selection of transduced CD34<sup>+</sup> cells, and analysis of GFP<sup>+</sup> transduced primary progenitors were performed as previously described (59, 60).

**Statistics.** KalediaGraph software, version 4.0 (Synergy Software) was used to display the data graphically and to perform statistical analysis. Results were analyzed by unpaired 2-tailed Student's *t* test or 1-way ANOVA with Tukey's 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 ACUC (protocol #3545).

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