SUPPLEMENTARY INFORMATION

Novel therapeutic approach targeting anemia of chronic disease and inflammation

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COMPETING FINANCIAL INTERESTS

The authors have declared that no conflict of interest exists.

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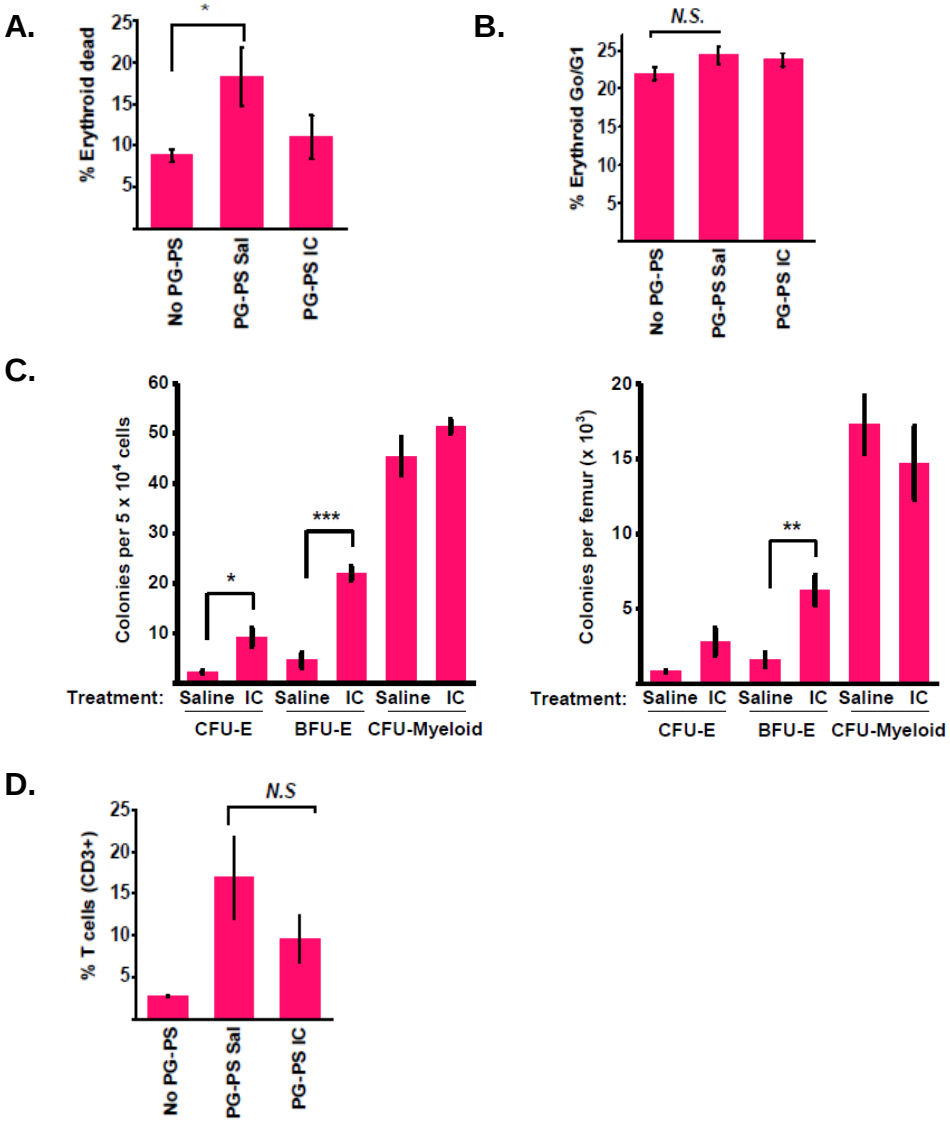
SUPPLEMENTAL RESULTS

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. 1C, D**), it had no effects on splenic infiltration by myeloid cells (**Supplemental Figs. 2A, B.**) or marrow infiltration by T cells (**Supplemental Fig. 1D.**). 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*(1) (**Supplemental Fig. 3D**). 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(2), neonatal autoimmune oophoritis(3)) (**Supplemental Figs. 3A,B,C**). In the rodent ACDI model, isocitrate did diminish circulating neutrophilia (**Supplemental Table 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,(4, 5) as we observe a decrease in hepcidin levels with isocitrate treatment in **Fig. 1F**, and/or due to isocitrate's potential ability to exert its effects at the level of marrow lineage reprogramming.(6)

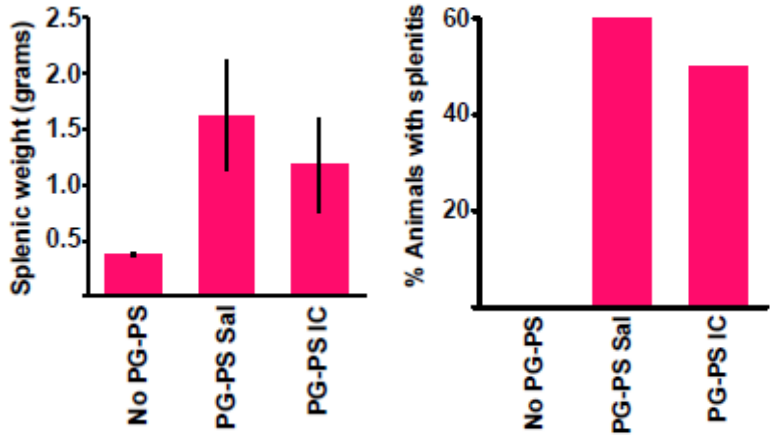
Supplemental Figure 1



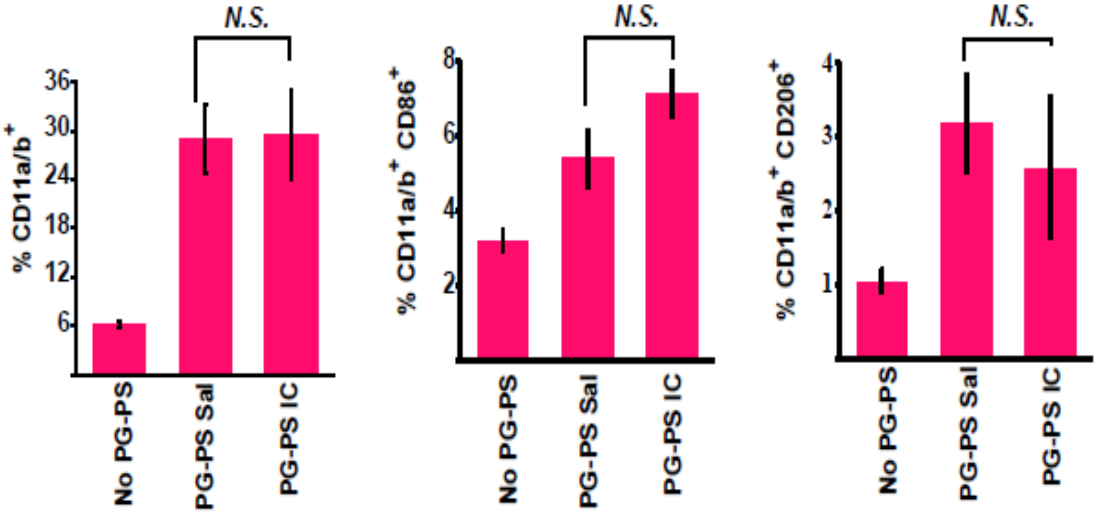
Supplemental Figure 1 Additional studies of marrow from rats in Figure 1D, E. (**A**) Cell death in erythroid precursors from control and arthritic animals ± isocitrate treatment. Marrow samples (see **Figure 1D**) were costained with anti-CD71, annexin V, and 7-AAD followed by flow cytometry. Shown 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 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 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 ± s.e.m., **P*< 0.05, ***P* <0.01,****P* < 0.001.

Supplemental Figure 2

A.

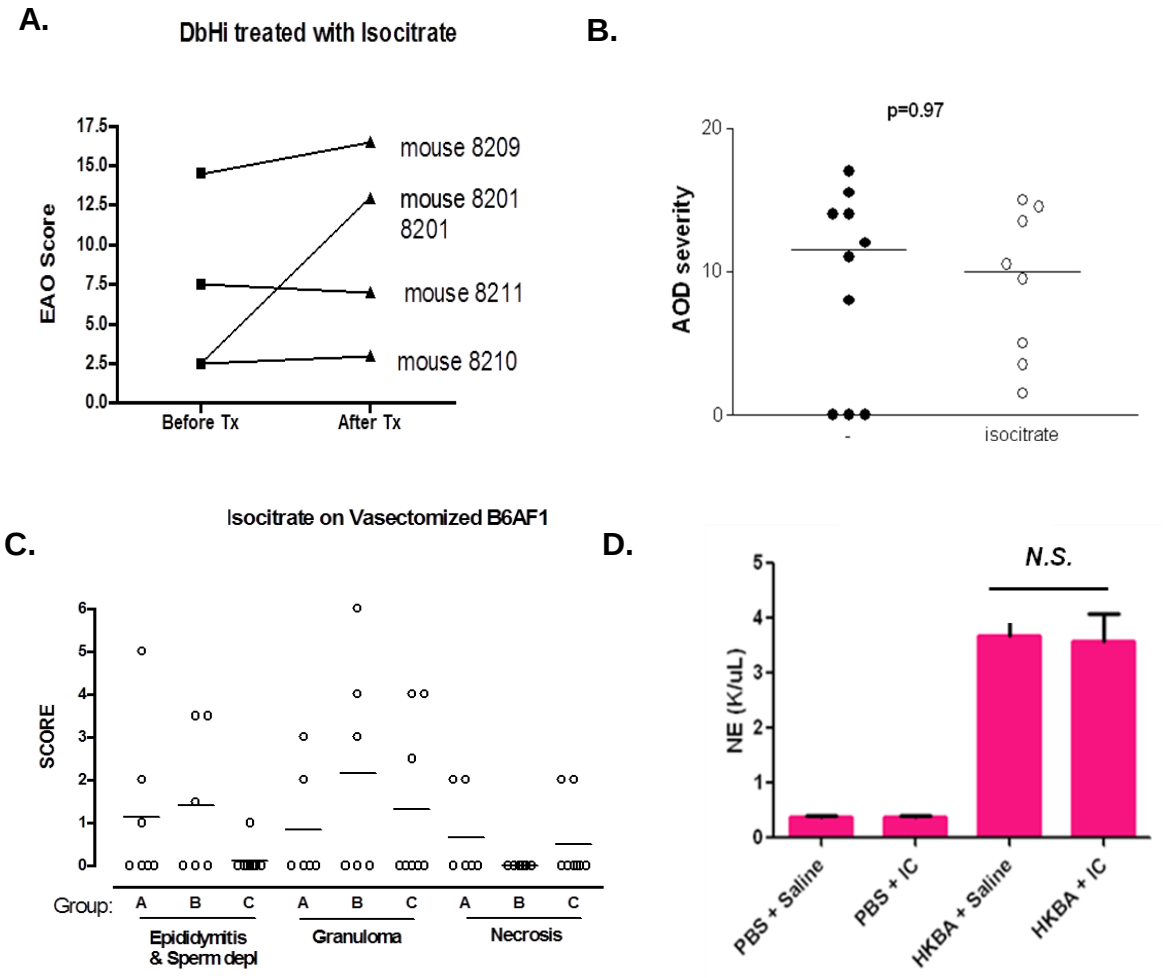


B.



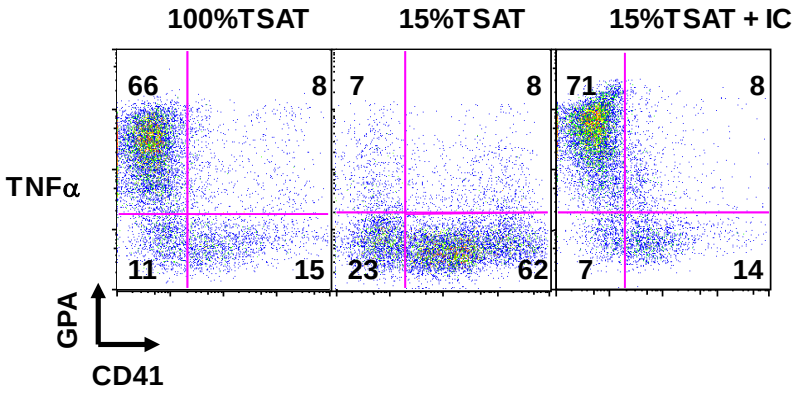
Supplemental Figure 2 Analysis of the effects of IC treatment on splenic changes in the rat ACDI model. (**A**) The animals from Figure 1**D** underwent assessment of spleens for weight, splenitis defined by the presence of granulomatous inflammation, and extent of infiltration by myeloid cells. Granulomatous 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.

Supplemental Figure 3



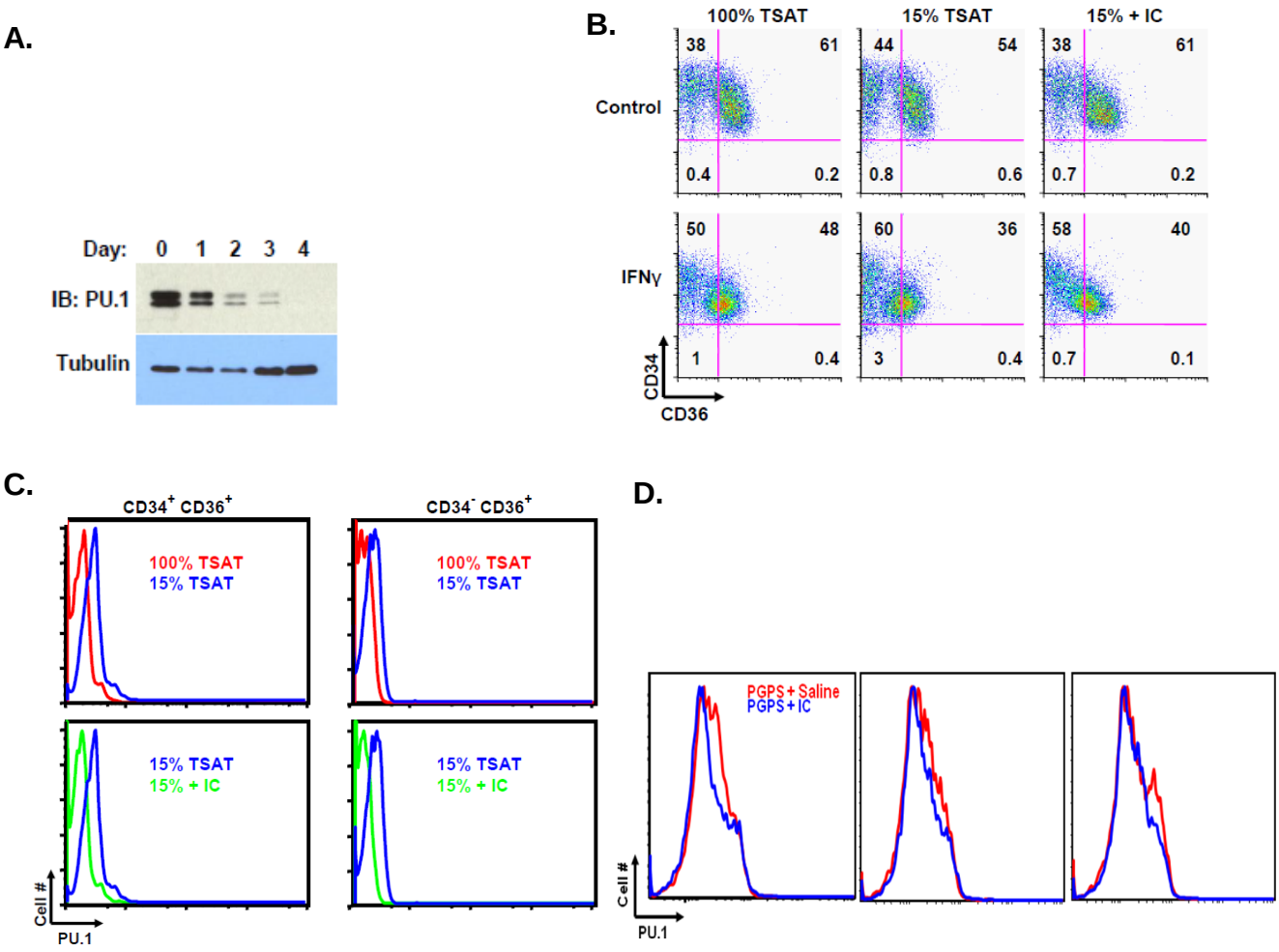
Supplemental Figure 3 Analysis of the effects of IC treatment in multiple murine models of inflammation. (**A**) Neonatal autoimmune ovarian disease (nAOD) elicited by autoantibody injection. As described by Setiady et al.(3) B6AF1 pups received injections of the anti-ZP3 antibody on post-natal days 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.(2) (**C**) Epididymitis associated with vasectomy and regulatory T cell depletion. As described by Wheeler et al.(2) 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.(2) 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**) Rapid induction of acute inflammation and neutrophilia. As per Sasu et al.(1) 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

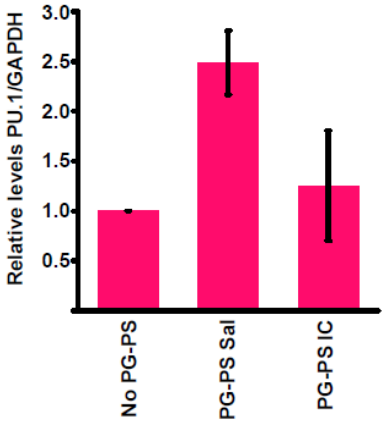
Supplemental Figure 4



Supplemental Figure 4 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. 2A** except that TNFα rather than IFNγ was used as the inflammatory stimulus.

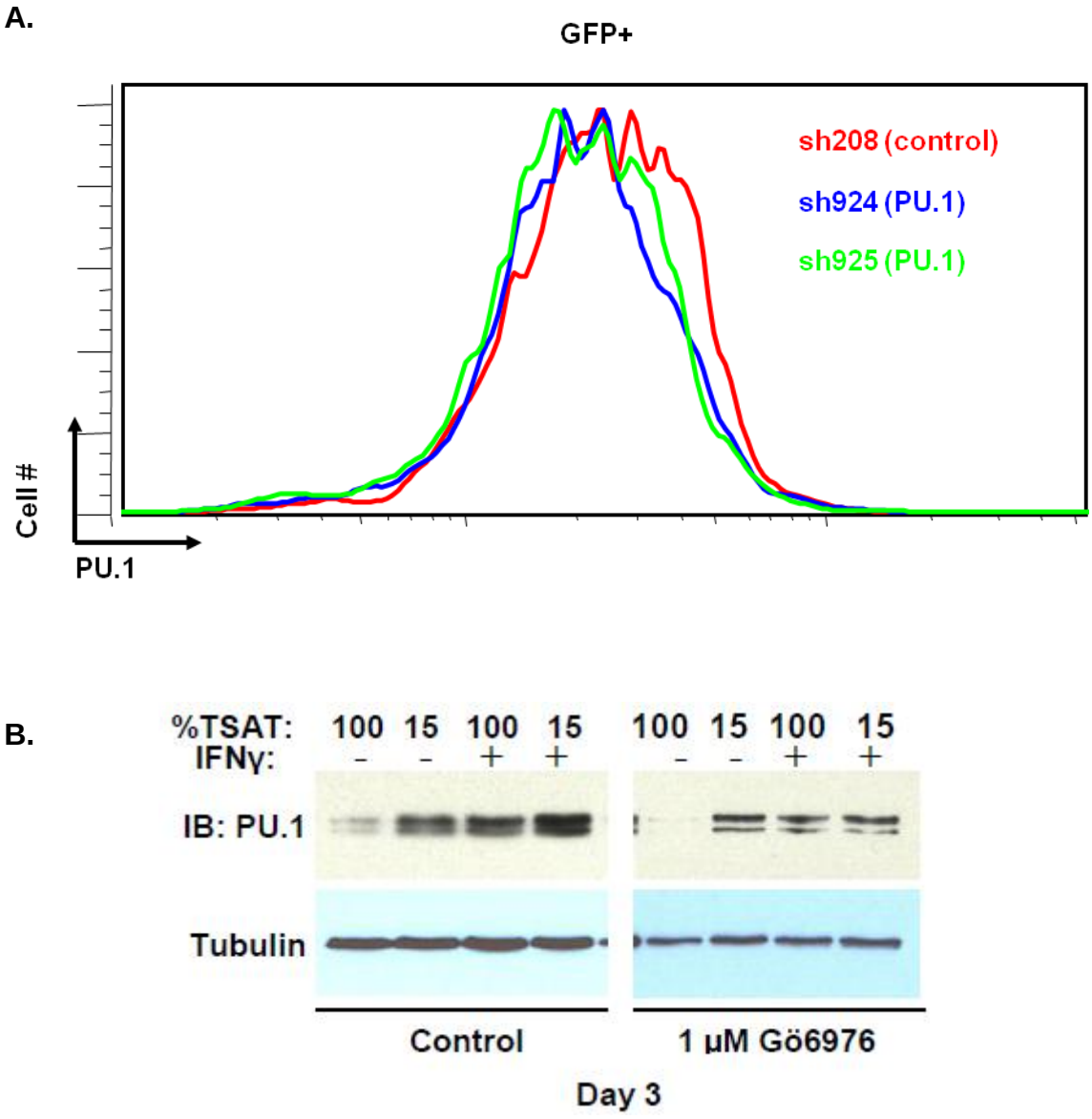
Supplemental Figure 5





Supplemental Figure 5 (**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 ± isocitrate treatment underwent flow cytometry for intracellular PU.1 detection as in **Fig. 4D**. (**D**) Effects of isocitrate treatment on PU.1 levels in erythroid progenitors from rats with ACDI. Animals treated as in **Figure 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 erythoid progenitors from rats treated with ACDI. Rats treated as in Fig 1D underwent sorting of marrow erythroid precursors (CD71+ CD11b-) followed by qPCR for PU.1, with normalization to GAPDH. Shown are mean ± s.e.m for 3 independent experiments.

Supplemental Figure 6



Supplemental Figure 6 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. 4A** were treated where indicated with 1μM of Gö6976, followed by immunoblot.

Supplemental Table 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.

Supplemental Table 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.

Supplemental Table 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 A, B**.

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