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Chronic IFN-γ production in mice induces anemia by reducing erythrocyte lifespan and inhibiting erythropoiesis through an IRF-1/PU.1-axis

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Abstract
Anemia of chronic disease (ACD) is a complication accompanying many inflammatory diseases. The pro-inflammatory cytokine IFN-γ has been implicated in this form of anemia, but the underlying mechanism remains unclear. Here we describe a novel mouse model for ACD, in which enhanced CD27-mediated costimulation strongly increases the formation of IFN-γ-producing effector T cells, leading to a progressive anemia. We demonstrate that the anemia in these mice is fully dependent on IFN-γ and that this cytokine reduces both the lifespan and the formation of red blood cells. Molecular analysis revealed that IFN-γ induces expression of the transcription factors IRF-1 and PU.1 in both murine and human erythroid precursors. We found that upon IFN-γ-stimulation, IRF-1 binds to the promoter of PU.1 and induces PU.1 expression, leading to inhibition of erythropoiesis. Notably, downregulation of either IRF-1 or PU.1 expression is sufficient to overcome IFN-γ-induced inhibition of erythropoiesis. These findings reveal a molecular mechanism by which chronic exposure to IFN-γ induces anemia.
**Introduction**

The maintenance of the number of circulating red blood cells (RBCs) is based on a tight balance between the production of new RBCs by erythroid progenitors and the removal of effete RBCs by cells of the hemophagocytic system. Several processes can negatively affect this erythroid homeostasis and subsequently lead to the development of anemia. Iron deficiency is the primary cause of anemia in the human population, but patients suffering from diseases involving chronic immune activation, such as persistent infections, cancer, and autoimmune diseases, are also commonly found to be anemic. This condition is termed Anemia of Chronic Disease (ACD) or Anemia of Inflammation and it is the second most prevalent form of anemia. Although the mechanisms involved in the development of ACD are still a matter of debate, pro-inflammatory cytokines such as IL-1, IL-6, TNFa and IFN-γ are thought to be important players in its development. Particularly IFN-γ has been extensively studied in this respect and it has been shown to have a direct suppressive effect on the formation of erythroid colonies in vitro. Exposure to IFN-γ in vitro contributes to early erythroblast death due to the induction of pro-apoptotic molecules, such as TRAIL, TWEAK and CD95(L). Whether this mechanism is also the cause of anemia following chronic IFN-γ exposure in vivo is not yet clear. Another possibility is that IFN-γ negatively affects the lifespan of RBCs due to its ability to activate macrophages and thereby the hemophagocytic system that removes RBCs from the circulation. Moreover, IFN-γ can also induce iron retention in macrophages, which negatively affects iron homeostasis and thereby the erythroid balance.

We have investigated the consequences of enhanced IFN-γ production on erythroid homeostasis in vivo using a mouse model for sterile chronic immune activation. In this model, we overexpressed the TNF-superfamily member CD70 on B cells, which induces high numbers of IFN-γ-producing effector CD4 and CD8 T cells due to enhanced costimulation through the receptor CD27 on T cells. Consequently, these CD70-transgenic (CD70TG) mice display improved T cell immunity and efficiently counter a challenge with influenza virus or tumor cells. However, the increased pool of effector T cells also seriously disrupts the hematopoietic system at different levels, as CD70TG mice gradually lose their B cells and eosinophilic granulocytes due to chronic exposure to IFN-γ, lose their NK cells, and eventually even exhaust their naive T cell pool. Here we describe that CD70TG mice also become anemic, which is fully dependent on the production of IFN-γ. We used this model to examine the cellular and molecular mechanism by which IFN-γ induces anemia.

We found that chronic production of IFN-γ in vivo profoundly shortens the lifespan of mature RBCs and also negatively affects the differentiation capacity of early erythroid progenitors in BM. Molecular analysis of the latter revealed that IFN-γ induces the expression of IRF-1 in erythroid precursor cells, which upregulates the transcription factor PU.1 and thereby inhibits erythroid differentiation. These findings demonstrate the profound impact of this cytokine on the erythroid balance in vivo and reveal the molecular mechanism by which IFN-γ inhibits erythropoiesis.
Materials and Methods

Mice
For experiments WT, CD70TG\textsuperscript{10}, IFN-γ\textsuperscript{−/−} and CD70TG\textsuperscript{+}IFN-γ\textsuperscript{−/−} mice were used. Mice were bred and maintained on a C57BL/6 background in the animal facilities of the Academic Medical Center (University of Amsterdam, Amsterdam, The Netherlands) in specific pathogen-free conditions. Mice were given standard chow and acidified drinking water ad libitum. All animal experiments were approved by the Experimental Animal Committee of the Academic Medical Center in Amsterdam, The Netherlands, according to institutional and national guidelines.

Peripheral Blood Analysis
Full blood cell analysis, measuring RBC counts, Hgb content, hematocrit, RDW, MCH, MCHC and MCV, was performed on heparinized blood with an automated hemocytometer (Vet ABC Counter, SCIL, Viernheim, Germany or Coulter Ac•T Diff2, Beckman Coulter, Woerden, The Netherlands).

Flow Cytometry and Cell Sorting
Single cell suspensions from spleen were obtained by mincing the organ through 40 μm cell strainers. To obtain splenic macrophages, spleens were first digested with Liberase / DNAse (Roche) for 30 minutes at 37°C. Single cell suspensions from BM were obtained by crushing femurs and tibiae and subsequently filtering the suspension through a 40 mm cell strainer. Where possible, cells were stained in the presence of anti-CD16/CD32 block (2.4G2; kind gift from Louis Boon, Bioceros, Utrecht, The Netherlands). Monoclonal antibodies used (all from eBioscience, unless stated otherwise) were: c-Kit-APC (2B8); CD71-PE (R17217), Ter119-Pe-Cy5.5 (Ly-76), F4/80-FITC/APC (BM8), CD11b-APC (M1/70), PU.1-Alexa\textsuperscript{488} (9G7, Cell Signaling) and MHCII-biotin\textsuperscript{10}. For identification of Common Myeloid Progenitors (CMPs) and Megakaryocyte-Erythroid Precursors (MEPs) by flow cytometry, cells were incubated with a lineage cocktail of biotin-conjugated antibodies directed against CD4 (GK1.5), CD8α (53-6.7), B220 (RA3-6B2), CD11b (M1/70), Gr1 (RB6-8C5) and Ter119 (Ly-76). After washing, cells were incubated with streptavidin-PE, CD34-FITC (RAM34), CD127-PerCP-Cy5.5 (A7R34), CD16/32-PE-Cy7 (93) and c-Kit-APC (2B8). For sorting, BM cells were first enriched with anti-CD117 microbeads (Miltenyi Biotec), stained with the antibodies described above, and CMPs and MEPs were subsequently sorted on a FACSaria (BD). Due to the upregulation of Sca-1 by IFN-γ on all BM cells in CD70TG mice\textsuperscript{15}, this marker was not included in the analysis or sorting strategy for CMPs and MEPs, which did not compromise our findings (Fig. S1a). For FACS analysis of reticulocytes, 3-5 ml of heparinized blood was washed in FACS buffer and surface stained for CD71. Cells were washed and resuspended in FACS buffer, after which Thiazole Orange (Sigma-Aldrich) was added to a final concentration of 1 ng/ml and immediately analyzed. Data acquisition was done with a FACSCalibur (BD) or FACSCanto II (BD) and data was analyzed using FlowJo software (Tree Star, Inc.).

EPO-Elisa
Serum EPO levels of mice were quantified by using the Quantikine Mouse/Rat EPO Immunoassay (R&D Systems) as described by the manufacturer.
Biotinylating of RBCs

In vivo biotinyllation was achieved by i.v. injection of 100 μl of 30 mg/ml sulfo-N-hydroxysuccinimide-long chain-biotin (sulfo-NHS-LC-biotin, Pierce). At regular time intervals, a few microliters of blood were isolated via vena saphena puncture, stained with PE-conjugated-streptavidin and erythroid specific antibodies and analyzed using FACS. For adoptive transfer of biotin-labelled-RBCs, blood was drawn from WT donor mice, washed with PBS-G (PBS supplemented with 0.1% glucose) and subsequently incubated with 0.1 mg/ml sulfo-NHS-LC-biotin in PBS-G for 15 minutes at RT. After washing, 200 μl of biotin-labelled RBCs was injected intravenously RBC turnover was analyzed as described above.

Erythrophagocytosis Assay

WT whole blood was washed with PBS, resuspended to a concentration of 2 x 10^8 cells/ml and subsequently labelled with a final concentration of 25 μM CFSE (Invitrogen), according to the manufacturer's protocol. Single cell suspensions from Liberase-digested spleens were cultured for 2 hours in 12-well plates in phenol-red free DMEM (Lonza)/10% FCS for 2 hours to allow macrophage adherence. Unbound cells were removed and CFSE-labelled RBCs were added in a concentration of 2 x 10^7 RBCs/ml and co-cultured for 2 hours. Cells were subsequently washed, non-phagocytosed RBCs were lysed with ammonium chloride and the macrophages were isolated for FACS analysis.

Semi Solid Colony Assays

For burst forming unit-erythroid (BFU-e) and colony forming unit-erythroid (CFU-e) assays, 2 x 10^5 nucleated total BM cells were plated in methylcellulose medium (MethoCult M3234, StemCell Technologies). Medium was supplemented with 4 U/ml human recombinant EPO (Janssen-Cilag), 100 ng/ml murine recombinant SCF, 20 μg/ml human holo-transferrin (SCIPAC, T101-5), 2 x 10^-4 M hemin (Sigma-Aldrich, H9039) and 1% penicillin/streptomycin/L-glutamine solution (Invitrogen). CFU-e colonies were counted on day 3, BFU-e colonies on day 8. When appropriate, mouse recombinant IFN-γ (PeproTech) was added. FACS-sorted BM CMPs and MEPs were cultured in complete methylcellulose medium (Methocult M3434, Stemcell Technologies) at 250 cells per 35 mm culture dish and colony formation was analyzed on day 8.

Microarray

CD71+ cells were MACS-enriched by labelling with biotin-conjugated CD71 antibodies (eBioscience) and streptavidin-conjugated microbeads (Miltenyi Biotech). Total RNA was extracted using Trizol (Invitrogen). Initial RNA yield and subsequent quality of the labelled fragmented cRNA was determined using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). 100-300ng of total RNA was hybridized to Mouse Gene 1.0 ST Array GeneChips, according to the manufacturer’s protocols (Affymetrix). Single Array Expression Analysis was performed using Genespring GX Software (Agilent Technologies). This platform generates a list of differentially expressed genes after filtering absent, marginal or AFFY control probe sets and applying a log2 transformation. A 1.5-fold change threshold and test statistic of p<0.05 were used as cut-off. These data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE22656. Further analysis was performed using Ingenuity Pathways Analysis (Ingenuity Systems).
Potential PU.1 and Gata1 target genes classification was obtained by comparing our list of IFN-γ-dependent differentially expressed genes with unpublished (LG), published PU.1 (GDS9011)16 and Gata1 ChIP-seq data (ERA000161)17 using Ingenuity Pathways Analysis.

**Quantitative PCR**
Total RNA was isolated using Trizol (Invitrogen) and reverse transcribed to cDNA using random hexamers and Superscript II reverse transcriptase (Roche). Quantitative PCR was performed using the StepOnePlus RT-PCR system (Applied Biosystems) using Express SYBR GreenER (Invitrogen). Primer sequences are available on request.

**Western Blot**
Total protein extracts were made according to standard methods. Proteins were separated on a 12.5% SDS-PAGE-gels and subsequently transferred onto polyvinylidene difluoride membranes (Sigma Aldrich) by electroblotting. Membranes were blocked with PBS containing 1% BSA or 3% milk and 0.25% Tween. Blots were incubated overnight at 4°C with primary antibodies, thoroughly washed with PBS containing 0.25% Tween, incubated with appropriate horseradish-peroxidase-conjugated secondary antibodies and developed in enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). Antibodies used were: PU.1 (T21, sc-352), Gata1 (N6, 265 and H200, sc-13053), IRF-1 (H8, sc-74530), GAPDH (MAB374 Chemicon Millipore), Beta-Actin (ab-6276) and Nucleophosmin (FC8229, ab-10530).

**Cell Culture of Human Erythroid Progenitors and shRNA-mediated Knockdown**
In accordance with institutional guidelines provided by the Erasmus MC Medical Ethical Committee (METC), human erythroid progenitor cells were cultured as described18, in the presence of recombinant human EPO (1 unit/ml, kind gift of Ortho-Biotech, Tilburg, The Netherlands), recombinant human SCF (50 ng/ml, kind gift of Amgen, Breda, The Netherlands) and dexamethasone (5 x 10^{-7} M; Sigma). Lentivirus was produced by transient transfection of 293T cells 19. Supernatant was harvested over three consecutive days after transfection, kept at 4°C and pooled. Pooled supernatant was filtered and concentrated by centrifugation at 20 krpm for 2h at 4°C. Human erythroid progenitor cells were transduced in 6-well plates at 2-3 x 10^6 cells per well and sufficient amounts of virus to transduce ~80% of the cells. When appropriate, puromycin (1 µg/ml final concentration) was added after 1 day, and selection was performed overnight.

For knockdown experiments, clones from The RNAi Consortium (TRC 15; Sigma) were used. The non-target SHC002 vector was used as a control. (SHC002: 5’-CAACAAGATGAAGAGCACAAA-3’). We tested 5 shRNA clones directed against PU.1 and IRF-1 mRNA, and we selected the ones with stronger downregulation for further experiments: PU.1 (TRCN0000020538: 5’-GAAGAAGCTCACCTACCAGTT-3’), and IRF-1 (TRCN0000014671: 5’-AGATGCTAAGAGCAAGGCAA-3’).

At day 1-3 after selection cells were counted and plated in triplicate in methylcellulose medium (MethoCult H4434, StemCell Technologies). We used 2 x 10^5 cells for CFU-e counts and 2 x 10^6 cells for BFU-e counts. Medium was supplemented with 1% penicillin/streptomycin solution (Gibco BRL) and when appropriate with 200 ng/ml of human recombinant IFN-γ (PeproTech). CFU-e colonies were counted on day 8 and BFU-e colonies on day 16.
**Chromatin Immunoprecipitation**

Human erythroid progenitor cells were cultured and grown in sufficient numbers and pulsed or mock pulsed overnight with 200ng/ml human recombinant IFN-γ. Cells were collected and Chromatin Immunoprecipitation (ChIP) was performed as described with IRF-1 (H8, sc-74530) and CD71 antibody (347510, BD Biosciences, San Jose, CA, USA) as a negative control. qPCR was performed on the input and immunoprecipitated samples using primers for the IRF-1 binding site at the TAP121 and PU.1 promoter. The relative fold enrichment was calculated as RFE = \(2^{\Delta\Delta C_T}\) (ChIP sample – CT input sample).

Primers used are:

- **TAP1 promoter**, amplicon size 94 bp.
  
  5’-GGCGAGAAGCTCAGCATT-3 and 5’-TAGTCTGGGCAGGCCACTTT-3’

- **PU.1 promoter**, amplicon size 121 bp.
  
  5’-CTGGTCTGAAGTGCCTTTCTTTG-3 and 5’-AAGAAGGAGTTGAGGAGCCAC-3’

**Statistical Analysis**

Results are expressed as mean ± SD. Statistical analysis between groups was performed with Graphpad Prism 5, using either a paired or non-paired 2-tailed Student’s t-test when comparing two groups or a one-way or two-way ANOVA test with Bonferroni correction when comparing more than two groups. Area under the curve analysis was performed using SPSS 15.0.1 software (SPSS Inc.). P-values <0.05 were considered to be statistically significant.

**Results**

**CD70TG mice develop an IFN-γ-dependent anemia**

As CD70-overexpression induces the formation of high numbers of IFN-γ-producing effector T cells, we used this model of chronic immune activation to examine the impact of IFN-γ on RBC homeostasis in vivo. Although CD70TG mice have normal RBC numbers, hemoglobin levels and hematocrit at four weeks of age, they develop severe anemia over time, as is evident from the significant decrease of these parameters in twelve week old mice (Fig. 1a). Cytometric analysis indicated that CD70TG mice develop a normochromic (normal mean corpuscular hemoglobin concentration) and normocytic (normal mean corpuscular volume) anemia (Table I). Importantly, this anemia is fully dependent on IFN-γ, as it does not occur in IFN-γ-deficient CD70TG (CD70TG*IFN-γ-/-) mice. Mean corpuscular hemoglobin (MCH) is slightly increased in CD70TG mice, but not in CD70TG*IFN-γ-/- mice (Table I), which correlates with an IFN-γ-dependent increase in reticulocytes in peripheral blood (Fig. 1b-c).

As mice typically respond to anemia and hypoxic stress by inducing extramedullary stress-erythropoiesis in the spleen, we examined the spleens of CD70TG mice in more detail. CD70TG mice develop a severe IFN-γ-dependent splenomegaly (Fig. 1d-e). This increase in spleen size can not be attributed to a leukocyte expansion, as CD70TG mice have reduced splenocyte numbers due to the progressive depletion of B cells. Therefore, we examined the splenic erythroid compartment, based on the differential expression of TER119 and CD71. We found that CD70TG mice have a dramatic increase of all erythroblast subsets, whereas CD70TG*IFN-γ-/- mice do not (Fig. 1f-g). Finally, CD70TG mice also displayed an
Figure 1. CD70TG mice develop IFN-γ-dependent anemia.
Analysis of WT, CD70TG, IFN-γ-/- and CD70TG*IFN-γ-/- mice. (a) Hemocytometric analysis at 4 and 12 weeks of age: RBC, red blood cell counts; Hgb, hemoglobin; Hct, hematocrit; Mean ± SD is shown in bar graphs for 3-5 mice per group. * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a two-way ANOVA with Bonferroni correction. (b, c) Representative dot plots and bar graph depicting the percentage of blood reticulocytes, i.e. CD71 + and Thiazole Orange (TO) positive cells. (d) Picture of spleens and (e) bar graph depicting spleen weight. (f) Representative dot plots of the splenic erythroid compartment. Numbers I, II, III and IV refer to the respective erythroblast subsets defined by Socolovsky et al.22. (g) Absolute numbers of indicated erythroblast subsets per spleen. Mean ± SD is shown in bar graphs for three mice per group; results are representative from three independently performed experiments. * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a one-way ANOVA. (h) Plasma EPO levels. Mean ± SD is shown in bar graphs for 5-8 mice per group; * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a one-way ANOVA with Bonferroni correction. For (b-h) mice were used in the age range of 10-16 weeks old.
IFN-γ induces anemia by reducing erythrocyte lifespan and inhibiting erythropoiesis. IFN-γ-dependent increase in plasma EPO levels (Fig. 1h), consistent with the notion that these mice have hypoxia. These data demonstrate that CD70TG mice develop an IFN-γ-dependent anemia and concomitant stress erythropoiesis in the spleen. We used this model to investigate the mechanism by which IFN-γ contributes to ACD.

**IFN-γ enhances RBC turnover and increases macrophage erythrophagocytosis**

As normocytic anemia in combination with reticulocytosis is indicative of accelerated RBC destruction, we examined whether IFN-γ affected the lifespan of RBCs in CD70TG mice. Therefore, mice were injected intravenously with biotin, which stably labels all RBCs in the circulation and allowed us to determine the turnover of RBCs (Fig. 2a). Whereas WT mice lost 50% of their biotinylated RBCs in ~23 days, CD70TG mice reached this point already after 12 days (Fig. 2b). This increased loss of biotinylated RBCs was fully IFN-γ-dependent, as RBC removal was normal or even slightly delayed in CD70TG*IFN-γ-/- mice (Fig. 2b and Fig. S2a).

Since IFN-γ is a potent activator of macrophages and the hemophagocytic system, we determined whether IFN-γ accelerated RBC turnover in CD70TG mice in an extrinsic manner. Therefore, we transferred ex vivo-biotinylated WT RBCs to the four different groups of mice and followed the fate of these cells over time. This approach revealed that WT RBCs were also removed more rapidly from the circulation of CD70TG mice, which was not seen in CD70TG*IFN-γ-/- mice (Fig. 2c and Fig. S2b). This increase in hemophagocytic capacity correlated well with the finding that splenic red pulp macrophages (F4/80hi, CD11blo) were activated in CD70TG mice, but not in CD70TG*IFN-γ-/- mice, based on the increased expression of MHCII (Fig. 2d). To establish the erythrophagocytic capacity of these cells, splenic red pulp macrophages were isolated, cocultured for 2 hours with CFSE-labeled WT RBCs, followed by lysis of the remaining non-phagocytosed RBCs. These experiments indicate that, compared to those from WT mice, red pulp macrophages from CD70TG mice phagocytose more erythrocytes, while red pulp macrophages from CD70TG*IFN-γ-/- mice phagocytose fewer (Fig. 2e). This correlates well with the degree of RBC turnover observed in vivo (Fig. 2b). Taken together, these data indicate that IFN-γ produced during chronic inflammation activates the splenic hemophagocytic compartment, thereby enhancing the turnover of RBCs.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>CD70TG</th>
<th>IFN-γ-/-</th>
<th>CD70TG*IFN-γ-/-</th>
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<tr>
<td><strong>RBC (x 10¹²/L)</strong></td>
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<td>6.58 (1.08) *</td>
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<td><strong>Hgb (mmol/L)</strong></td>
<td>9.30 (0.75)</td>
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<td>8.56 (0.59)</td>
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<td><strong>Hct (L/L)</strong></td>
<td>0.49 (0.04)</td>
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<td><strong>RDW (%)</strong></td>
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<td>18.04 (1.69)</td>
<td>19.31 (2.24)</td>
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<td><strong>MCH (fmol)</strong></td>
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<td><strong>MCHC (mmol/L)</strong></td>
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<td>20.26 (1.63)</td>
<td>19.30 (1.09)</td>
<td>19.85 (1.60)</td>
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Table I. IFN-γ-dependent normochromic and normocytic anemia in CD70TG mice.

Hemocytometric analysis of 10-16 week old WT, CD70TG, IFN-γ-/- and CD70TG*IFN-γ-/- mice. RBC, red blood cell counts; Hgb, hemoglobin; Hct, hematocrit; RDW, red cell distribution width; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration. Values indicate the average (SD) from 10-18 mice. * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a one-way ANOVA with Bonferroni correction.
IFN-γ inhibits erythroid output from BM

As IFN-γ can also negatively regulate erythropoiesis of BM progenitor cells\(^6\), we analyzed the erythroid compartment in BM from CD70TG mice and found significantly fewer erythroblasts (Ery II, III and IV) than in WT BM (Fig. 3a). For the early subsets this was to a certain extent also seen in CD70TG*IFN-γ\(^{-/-}\) mice, but the final stage of orthochromatophilic erythroblasts (Ery IV) was not affected in these mice (Fig. 3a). This correlated well with the observations that BM of CD70TG mice was paler compared to WT, IFN-γ\(^{-/-}\) and CD70TG*IFN-γ\(^{-/-}\) mice (data not shown), which is also a clear sign for a reduction in hemoglobinized cells in CD70TG mice. Furthermore, we found a relative accumulation of pro-erythroblasts in CD70TG mice (Fig. 3b), which, together with the decrease in absolute number of mature erythroid cells (Fig. 3a), indicates that erythropoiesis in CD70TG BM is hampered. We found no evidence for increased apoptosis of erythroid precursor populations in CD70TG mice, based on Annexin-V stainings of bone marrow (Fig. S3a). Yet, reduced erythropoiesis was confirmed when the erythroid forming capacity of the BM was functionally tested using colony-forming assays, as the number of BFU-e was strongly reduced in CD70TG BM compared to WT BM. This reduction was dependent on IFN-γ (Fig. 3c). Formation of more mature CFU-e was not affected, but the negative impact of IFN-γ on erythroid formation was evident from the fact that both IFN-γ\(^{-/-}\) and CD70TG*IFN-γ\(^{-/-}\) mice had more CFU-es than WT and CD70TG mice (Fig. 3d). Moreover, purified CMPs from CD70TG mice formed fewer erythroid colonies compared to WT mice, but more myeloid colonies (Fig. 3e), indicating a decreased commitment of hematopoietic progenitors to the erythroid lineage. Subsequently, we examined BM of CD70TG mice for the presence of IFN-γ-producing T cells, which revealed that CD70TG mice have 7-fold more IFN-γ-producing CD4\(^+\) T cells and 4-fold more IFN-γ-producing CD8\(^+\) T cells than WT mice (Fig. 3f-g). Serum IFN-γ levels were undetectable (data not shown) and strongly suggesting that a local increase in IFN-γ-production is responsible for the decreased BFU-e capacity in CD70TG mice. Moreover, we demonstrate that IFN-γ is sufficient to inhibit the outgrowth of BFU-e from WT BM (Fig. 3h), whereas CFU-e are less sensitive to this cytokine (Fig. 3i), which is consistent with previous findings\(^5\). In conclusion, these data imply that prolonged exposure to high IFN-γ levels in vivo causes ACD, not only by an increased turnover of RBCs, but also by a reduction in the erythroid forming capacity of hematopoietic progenitor cells in BM.

IFN-γ induces expression of PU.1

To investigate the molecular mechanism by which IFN-γ affects erythropoiesis, we performed microarray analysis on CD71\(^+\) erythroblasts from BM of WT, CD70TG, IFN-γ\(^{-/-}\) and CD70TG*IFN-γ\(^{-/-}\) mice. Unsupervised cluster analysis showed that erythroblasts from CD70TG mice clustered separately from the other mice, independently of their ability to produce IFN-γ (Fig. 4a). However, we also found a set of 191 transcripts that was differentially expressed in CD70TG mice compared to CD70TG*IFN-γ\(^{-/-}\) as well as WT and IFN-γ\(^{-/-}\) mice, and we decided to further focus on these genes, as they were directly or indirectly regulated by IFN-γ. Comparison with other datasets\(^16\) revealed that 128 out of these 191 genes (67%) are potential targets of the transcription factors PU.1 or GATA-1 (Fig. 4b and Fig. S4), which are the key regulators of myeloid and erythroid differentiation, respectively. During normal erythroid differentiation, PU.1 is downregulated, while GATA-1 upregulation mediates differentiation of erythroid precursors\(^25\). Expression of these factors has to be tightly controlled during hematopoiesis, as PU.1 and GATA-1 physically interact and thereby...
IFN-γ induces anemia by reducing erythrocyte lifespan and inhibiting erythropoiesis.

**Figure 2.** Constitutive IFN-γ exposure in vivo enhances RBC turn-over and increases macrophage erythrophagocytosis. (a) Flow cytometric analysis of in vivo biotinylated peripheral blood using fluorescently labelled streptavidin and Ter119. Representative dot plots of day 17 after transfer are depicted. (b) Turnover of in vivo biotinylated RBCs from WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice as measured by flow cytometry. (c) Turnover of adoptively transferred ex vivo biotinylated WT RBCs in WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice as measured by flow cytometry. (d) MHCII surface expression of splenic red pulp macrophages (F4/80+ CD11blow) expressed as geometric MFI. (e) Uptake of CFSE-labelled erythrocytes by splenic red pulp macrophages (F4/80+CD11b+) expressed as the ratio of CFSE geometric MFI compared to background. Data in (e) is obtained from two independent experiments. Mean ± SD is depicted for 4 mice (b, c), 3 mice (d) or duplicate analysis (e) per group. * indicates a significant difference (p<0.05) between CD70TG or CD70TG*IFN-γ−/− mice and all other groups, using a one-way ANOVA with Bonferroni correction.
Figure 3. IFN-γ production in CD70TG mice inhibits erythroid BM output.

(a) Absolute numbers of various erythroblast subsets or (b) relative contribution of pro-erythroblasts (percentage Ery I (Ter119<sup>med</sup>CD71<sup>high</sup>) from all erythroid (Ter119<sup>med/high</sup> cells)) in BM (isolated from two femurs and two tibiae) of WT, CD70TG, IFN-γ<sup>-/-</sup> and CD70TG*IFN-γ<sup>-/-</sup> mice. (c, d) BFU-e and CFU-e numbers of unfractionated BM from WT, CD70TG, IFN-γ<sup>-/-</sup> and CD70TG*IFN-γ<sup>-/-</sup> mice. (e) Colony assay of FACS-sorted CMPs from WT and CD70TG mice. (f, g) Absolute numbers of IFN-γ-producing CD4 and CD8 T cells in BM of WT and CD70TG mice, measured after PMA/ionomycin stimulation. (h, i) Effect of IFN-γ on the in vitro BFU-e and CFU-e colony forming capacity of BM cells from WT mice. Mean ± SD is depicted for 3 mice per group (a-d, f-g) or triplicate analysis (e, h-i); results are representative from three (a-d) or two (e-i) independently performed experiments. *p<0.01 (a) or p<0.05 (b-h) using a one-way ANOVA with Bonferroni correction (a-d), or a non-paired Student’s t-test (e-i).
IFN-γ induces anemia by reducing erythrocyte lifespan and inhibiting erythropoiesis

block each others function^{26-28}. Protein expression analysis of these transcription factors on Western blot revealed that PU.1 was highly upregulated in CD71+ cells of CD70TG mice, which was fully dependent on IFN-γ, as it can be seen in IFN-γ−/− samples where PU.1 levels are even lower than in WT, whereas expression of GATA-1 was not altered between the different groups of mice (Fig. 4c). Flow cytometric analysis of earlier progenitors revealed that PU.1 was also upregulated in megakaryocyte/erythrocyte lineage-restricted progenitors (MEPs) of CD70TG, but not of CD70TG*IFN-γ−/− mice (Fig. 4d). Finally, we found that IFN-γ treatment of purified CD71+ cells (Fig. 4e) or MEPs (Fig. 4f) was sufficient to upregulate PU.1 expression, whereas it did not affect GATA-1 (Fig. 4f). Since PU.1 is a known inhibitor of erythroid differentiation^{27-29}, these data strongly suggest that IFN-γ blocks erythropoiesis through the induction of PU.1.

IFN-γ upregulates PU.1 expression through induction of IRF-1

Next, we set out to determine the molecular mechanism by which IFN-γ-signaling induces the expression of PU.1. The microarray analysis revealed that IFN-γ also induced expression of the transcription factor IRF-1 (interferon regulatory factor 1) and its downstream target genes (Table II and Fig. S4). We validated by quantitative PCR that IRF-1 was upregulated in CD71+ BM cells of CD70TG mice in an IFN-γ-dependent manner (Fig. 5a). Moreover, incubation with IFN-γ was sufficient to induce IRF-1 in CD71+ cells and MEPs (Fig. 5b). Examination of the PU.1 promoter revealed a potential IRF-1/2 binding site ~15kb upstream of the transcription start site, which was highly conserved among mammals (Fig. 5c). To determine whether IRF-1 protein indeed interacted with this domain, we performed chromatin immune-precipitation (ChIP) experiments, using expanded primary human erythroid progenitor cells; these cells can be expanded to sufficient numbers required for these experiments^{18} and they enabled us to validate the effect of IFN-γ also on human erythroid precursors. We found that IFN-γ inhibited the outgrowth of BFU-e and CFU-e of human erythroid progenitor cells in vitro (Fig. 5d), which corroborates comparable experiments with progenitor cells from human BM^{3;4;6}. Moreover, IFN-γ induced the expression of both IRF-1 and PU.1 also in these primary human cells (Fig. S5a). Subsequent ChIP analysis revealed that IFN-γ treatment led to increased IRF-1 binding to the highly conserved IRF-1/2 motifs in the PU.1 locus (Fig. 5e). IFN-γ treatment also increased binding of IRF-1 to an IRF-1 binding site in the Tap1 promoter^{21}, which served as a positive control. No significant enrichment was observed when a negative control antibody (anti-CD71) was used for the immunoprecipitation.

Finally, to demonstrate that IRF-1 and PU.1 were responsible for the IFN-γ-induced inhibition of erythroid colony formation, we transduced human erythroid progenitor cells with lentiviruses expressing a shRNA to either IRF-1 or PU.1, stimulated these cells with IFN-γ and tested their ability to form erythroid colonies. These constructs strongly inhibited the upregulation of their cognate target mRNAs upon IFN-γ stimulation (Fig. S4b). In addition, inhibition of IRF-1 expression efficiently prevented the IFN-γ-mediated upregulation of PU.1 and vice versa (Fig. S5b), indicating that IRF-1 and PU.1 reciprocally enhance each others expression. Importantly, subsequent colony assays revealed that inhibition of either IRF-1 or PU.1 expression was sufficient to overcome the IFN-γ-induced reduction in CFU-e and BFU-e numbers (Fig. 5f). These data demonstrate that IFN-γ blocks erythroid differentiation by inducing the expression of PU.1 in an IRF-1 dependent manner.
Figure 4. IFN-γ induces expression of PU.1 in erythroblasts.
(a) Microarray heat map of unsupervised cluster analysis for genes expressed in CD71+ erythroblasts from BM of WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice. Red; high expression, green; low expression. Three mice were analysed per group. (b) Venn-diagram displaying potential PU.1 and GATA-1 targets amongst the IFN-γ-regulated genes in CD70TG mice. (c) Western blot analysis of PU.1 and GATA-1 expression on purified CD71+ BM cells from 2 mice per experimental group. (d) Flow cytometric analysis of PU.1 expression in MEPs of WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice. Horizontal line represents the value of the isotype control. Mean ± SD is depicted for three mice per group; results are representative from two independently performed experiments, * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a one-way ANOVA with Bonferroni correction. (e) QPCR analysis of PU.1 mRNA on WT CD71+ erythroblasts cultured overnight with or without IFN-γ. (f) QPCR analysis of PU.1 and GATA-1 mRNA on WT MEPs cultured overnight with or without IFN-γ. (e, f) Data is presented as the fold induction of expression compared to the medium control for seven (e), nine or six (f) mice per group, pooled from at least two independently performed experiments; * p<0.01 (e) or p<0.05 using a paired Student’s t-test.
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Discussion

Next to its well-established pro-inflammatory and anti-microbial role, IFN-γ can also influence the hematopoietic process in the BM, as it affects HSC quiescence and inhibits the formation of B cells and eosinophilic granulocytes. IFN-γ also has a strong inhibitory effect on erythroid colony formation in vitro and it is therefore assumed that IFN-γ plays a major role in the development of ACD in patients suffering from cancer or chronic inflammatory conditions. However, the impact of chronic IFN-γ production on erythroid homeostasis in vivo as well as the molecular mechanism by which IFN-γ inhibits erythroid differentiation has been poorly investigated. This is due to the fact that most methods used to induce chronic inflammation not only elicit the production of IFN-γ, but a whole plethora of pro-inflammatory cytokines, which can also act synergistically with IFN-γ on erythroid differentiation, as has been shown for TNFa and type I IFNs. Of interest in this respect is the recent finding that the anemia accompanying the acute phase of Toxoplasma gondii infection is dependent on the production of IL-15, which is regulated by IFN-γ. Yet, the underlying molecular mechanism linking IFN-γ to anemia in either this model or in ACD remains unclear. Using a sterile form of chronic inflammation elicited by enhanced and sustained T cell activation, we describe here that the ensuing increase in IFN-γ production induces anemia due to the concomitant inhibition of BM erythropoiesis and the enhanced turn-over of circulating RBCs. Although T cells in CD70TG mice do not have increased production of IL-2, TNFa or type I IFNs (data not shown), we cannot exclude that other pro-inflammatory mediators are induced in CD70TG mice that contribute to the severity of the anemia. Importantly, we could not detect any significant changes in the serum levels of IL-10, IL-1a, IL-6 nor TNFa in these mice (data not shown). Yet, the phenotype of CD70TG*IFN-γ-/- mice demonstrates that even if the (local) production of such factors would be increased, they are either not sufficient to induce anemia and/or stress erythropoiesis or they are induced by IFN-γ itself.

Table II. Relative expression of IFN-γ-inducible genes in erythroblasts of CD70TG mice and their relationship with IRF-1.

Selection of IRF-1 related genes (based on the microarray data described in Figure 4 and Figure S3) that were significantly upregulated in CD70TG mice compared to WT as well as CD70TG*IFN-γ-/- mice. These IFN-γ inducible genes were categorized as being either IRF-1 dependent (top half) or independent (bottom half), based on indicated literature references (right column).

<table>
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<th>Relationship with IRF-1:</th>
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Selection of IRF-1 related genes (based on the microarray data described in Figure 4 and Figure S3) that were significantly upregulated in CD70TG mice compared to WT as well as CD70TG*IFN-γ-/- mice. These IFN-γ inducible genes were categorized as being either IRF-1 dependent (top half) or independent (bottom half), based on indicated literature references (right column).
Figure 5. IFN-γ upregulates PU.1 expression in erythroblast through IRF-1.
(a) QPCR analysis of the expression of IRF-1 mRNA in CD71+ erythroblasts from WT, CD70TG, IFN-γ-/- and CD70TG*IFN-γ-/- mice. Mean ± SD is depicted for 3 mice per group, * indicates a significant difference (p<0.05) between CD70TG mice and all other groups (using a one-way ANOVA with Bonferroni correction). (b) QPCR analysis of IRF-1 mRNA in CD71+ erythroblasts or MEPs from WT mice cultured overnight with or without IFN-γ. Data is presented as the fold induction of expression compared to the medium control for seven (CD71+) or six (MEPs) mice per group, pooled from two independently performed experiments; * p<0.001 (e) or p<0.05 using a paired Student’s t-test. (c) Sequence analysis of the promoter region of the human PU.1 (SPI1) gene, displaying a conserved putative IRF-1/2 binding site ~15kb upstream of the transcription start site (based on the UCSC Genome Browser). (d) Effect of IFN-γ on the in vitro BFU-e and CFU-e colony forming potential of human erythroid precursor cells cultured overnight with or without IFN-γ. Mean ± SD from triplicate analysis; results are representative from two independently performed experiments, *p<0.05 (non-paired Student’s t-test). (e) Chromatin immunoprecipitation analysis of the binding of IRF-1 to the putative IRF-1/2 binding site in the PU.1 promoter of human erythroid precursor cells cultured overnight with or without IFN-γ. RFE, relative fold enrichment. Mean ± SD from 3 independent experiments, *p<0.05 (non-paired Student’s t-test). (f) Effect of IFN-γ on the BFU-e and CFU-e potential of human erythroid precursor cells transduced with shRNA directed against IRF-1 or PU.1. Mean ± SD from 3 independent experiments, *p<0.05 (one-way ANOVA with Bonferroni correction).
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Although we demonstrated that IFN-γ negatively affects both the formation and the lifespan of RBCs, it has been described that IFN-γ induced by in vivo administration of CpG-oligodeoxyribonucleotides does mediate erythroid suppression, but not reduction of RBC survival\(^3\). However, this study addressed the role of IFN-γ in acute inflammatory responses, while we have used a valid model for chronic inflammation. We hypothesized that a chronic response could result in macrophage activation and thereby affect RBC uptake. Concordantly, we found an IFN-γ-dependent increase of RBC uptake by activated splenic macrophages in vitro (Fig. 2d-e), as well as increased iron-storage in the spleens of CD70TG mice (data not shown). The latter observation suggests potential defects in iron release, which also impedes the course of erythropoiesis\(^3\). However, we did not find altered expression of the iron-regulating hormone hepcidin\(^3\) in the liver of CD70TG mice (Fig. S2a-b), indicating that hepcidin is not causally involved in the anemic phenotype of these mice.

Next to the clear impact on RBC lifespan, we also found that IFN-γ impaired BM erythropoiesis, as was evident from strongly reduced BFU-e numbers (Fig. 3c) and fewer mature erythroblasts (Fig. 3a) in CD70TG mice, but not CD70TG*IFN-γ-/- mice. It could though well be that the observed decrease is an underestimation and that this defect was already partly compensated in vivo by the increased EPO levels in these mice, since EPO is sufficient to increase erythropoiesis not only in the spleen, but also in BM\(^3\). Regarding the spleen, CD70TG mice suffered from splenomegaly due to the induction of stress erythropoiesis, which is a physiological response to anemia in rodents. Yet, the fact that CD70TG mice still become progressively anemic (Fig. 1a) at least demonstrates that this form of “emergency” erythropoiesis is not sufficient to prevent anemia in these mice. It could well be that stress erythropoiesis is negatively affected by the combination of increased local production of IFN-γ and enhanced RBC destruction in the spleen. This issue is currently under investigation.

At a molecular level, we demonstrate that IFN-γ inhibits erythropoiesis by activation of an IRF-1-PU.1 axis. Since type I IFNs can also induce expression of IRF-1 and efficiently suppress erythroid colony formation in vitro\(^3\), we expect that chronic production of IFNa or IFNb can elicit a comparable degree of inhibition of BM erythropoiesis. This has been indirectly achieved in mice by knocking out IRF2, a suppressor of type I IFN signaling\(^8\). These mice become anemic, which is rescued in the absence of IFNAR1, a member of the type I IFN receptor complex. However, type I IFNs are less potent activators of macrophages, which suggests that chronic production of type I IFNs has a less significant impact on RBC turnover and does not induce anemia as efficiently as IFN-γ. In steady state conditions, EPO levels in IRF2-/- mice are not increased to the same extent as in our mouse model of sterile chronic inflammation.

PU.1 is a well known transcriptional regulator of myelopoiesis\(^2\). In the erythroid lineage, PU.1 activity is repressed by GATA-1, which is essential for normal erythropoiesis\(^2\). The interplay of PU.1 and GATA-1 is relevant for lineage commitment and high expression of PU.1 is known to represses GATA-1 activity and thereby erythroid differentiation\(^2\). We identified a highly conserved IRF-1 binding site in the distal promoter of the PU.1 gene locus and showed that IFN-γ induces PU.1 expression via IRF-1. IFN-γ can induce apoptosis of erythroid progenitors through TRAIL, TWEAK and CD95\(^4\) and indeed we found that TRAIL (TNFSF10), a target of PU.1, is upregulated by IFN-γ in CD71+ cells of CD70TG mice (Fig. S4). We have found that IRF-1 and PU.1 are interdependent, since shRNA-mediated downregulation of each factor resulted in downregulation of the expression levels of the other. These findings are
supported by the fact that IRF-1-/- mice have defective myelopoiesis, which is partly caused by downregulation of PU.138, and that infection of neutrophil precursors with Anaplasma phagocytophilum results in downregulation of both IRF-1 and PU.139. A cooperative function between IRF-1 and PU.1 has also been reported during ATRA-mediated granulopoiesis40 and in IFN-γ-stimulated myeloid cells41. Furthermore, IRF-1 is naturally downregulated towards the final stages of erythroid maturation42, which overall sustains the notion that suppression of the IRF-1-PU.1 axis is a prerequisite for normal erythropoiesis. Our findings provide the molecular mechanism by which IFN-γ affects erythropoiesis, which implicates transcriptional regulation of hematopoietic-lineage differentiation in response to inflammation.

Regarding the physiological rationale behind the impact of IFN-γ on erythropoiesis, we postulate that during immune activation, IFN-γ temporarily shifts the balance of hematopoietic differentiation towards myeloid cells in order to combat an infection. Activated T cells might well play an important role in this process, as these cells can migrate to the BM and modulate hematopoiesis10,12,43. A temporary increase of myelopoiesis at the cost of erythropoiesis will not be detrimental to the host and will benefit the ongoing immune response, as the lifespan of RBCs is 10-50 fold longer than that of neutrophils or monocytes44,45. However, during chronic immune activation, the prolongation of such a shift can lead to anemia, as observed in CD70TG mice, which also have increased monocyte formation12. Our findings not only emphasize the importance of IFN-γ as a potent regulator of hematopoiesis, but also reveal the potential molecular mechanism of inflammation-induced anemia that occurs frequently in patients suffering from chronic inflammatory diseases, such as HIV-infection and rheumatoid arthritis. Interestingly, BFU-e colony formation is strongly reduced in BM of anemic patients suffering from chronic idiopathic neutropenia, which is significantly increased when the cells are cultured with IFN-γ-neutralizing antibodies46. It will be important to determine the contribution of this cytokine to the development of anemia in several chronic inflammatory diseases and whether neutralization of IFN-γ could be an effective treatment for different forms of ACD.

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We thank Ernie de Boer (EMC) for sharing Gata1 ChIP-seq data, Berend Hooibrink (AMC) for cell sorting and the staff of the animal facility of the AMC for excellent animal care. We appreciate the help from Dr Reuben Tooze (Leeds) in discovering the IRF-1/2 binding site in the PU.1 promoter and we thank Prof Dr Rene van Lier, and Dr Marieke von Lindern, Dr Esther Nolte-‘t Hoen and Dr Reuben Tooze for critical reading of the manuscript. This work was funded by a VENI (LG; 863.09.012) and VIDI grant (MAN; 917.76.310) from The Netherlands Organization of Scientific Research, an EMBO Fellowship (PP, ASTF 15-2010) and a project grant from the Landsteiner Foundation for Bloodtransfusion Research (MAN; 0607).
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Reference List


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**Supplementary Figures**

**Supplementary Figure 1. Altered sorting strategy of precursor cells due to Sca-1 upregulation.**

(a) In comparison to the traditional sorting strategy, in this study Sca-1 could not be used in the sorting strategy of CMPs and MEPs due to Sca-1 upregulation in CD70TG mice. Sca-1 was therefore excluded from the sorting strategy. (b) To look at functional capacity, WT BM CMPs or MEPs were FACS-sorted using the traditional method and the method used in this study and cultured in M3434 medium (Stemcell Inc) at 250 cells per 35 mm culture dish. After 8 days of culture, colony forming units (CFU) were scored according to the type of cells present. Mean ± SD from triplicate analysis is depicted.
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Supplementary Figure 2. IFN-γ enhances RBC turn-over.
(a) Determination of the area under the curve of the turnover of biotinylated RBCs of WT, CD70TG, IFN-γ⁻/⁻ and CD70TG⁺ IFN-γ⁺/⁻ mice as displayed in Figure 2b. (b) Determination of the area under the curve of the turnover of adoptively transferred biotinylated WT RBCs in WT, CD70TG, IFN-γ⁻/⁻ and CD70TG⁺ IFN-γ⁺/⁻ mice as displayed in Figure 2c. Mean ± SD is depicted, * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a one-way ANOVA with Bonferroni correction.

Supplementary Figure 3. Annexin-staining on erytroid precursor cells in the bone marrow reveals no direct link between IFN-γ and apoptosis.
(a-b) FACS-analysis for Annexin V-positivity was performed on total bone marrow samples of WT, CD70TG, IFN-γ⁻/⁻ and CD70TG⁺ IFN-γ⁺/⁻ mice directly ex vivo. Mean percentage ± SD is depicted for 2 mice per group in experiment 1 and 3 mice per group in experiment 2. * indicates a significant difference (p<0.05) using a one-way ANOVA with Bonferroni correction.
**Supplementary Figure 4. Microarray analysis: IFNγ dependent genes in CD71+ erythroblasts isolated from CD70TG mice.**

Grey colour tab, potential PU.1 target genes. Cream colour tab, potential GATA-1 target genes. White, neither PU.1 nor GATA-1 target genes.

The shape of each symbol represents the function of the encoded protein, whereas the color indicates whether a gene is upregulated (red) or downregulated (green) by IFN-γ.
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Supplementary Figure 5. Transcription factor expression and suppression in human erythroid precursor cells.
(a) Western blot analysis of PU.1, GATA-1 and IRF-1 expression on human erythroid precursor cells cultured overnight with or without IFN-γ. Actin and nucleophosmin (NPM) are used as loading controls. (b) QPCR analysis of the expression of PU.1 and IRF-1 mRNA of human erythroid precursor cells transduced with either a short hairpin against PU.1 or IRF-1 and cultured overnight with or without IFN-γ. Data is presented as mean of the fold enrichment IFN-γ/untreated ± SD.

Supplementary Figure 6. Unaltered hepatic hepcidin expression.
Q-PCR analysis of the expression of hepcidin mRNA levels in liver cells of WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice. Mean ± SD is depicted for 3 mice per group.