STAT6-mediated suppression of erythropoiesis in an experimental model of malarial anemia

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The online version of this article contains a supplementary appendix.

ABSTRACT

Background

The contribution of pro-inflammatory cytokines to the pathogenesis of malarial anemia has been studied extensively but the roles of Th2 cytokines remain unknown. Here, we investigated the role of signal transducer and activator of transcription (STAT)6-mediated responses in erythropoietic suppression during acute malaria infection in mice.

Design and Methods

Naïve and/or erythropoietin-treated wild-type and STAT6$^{-/-}$ mice were infected with *Plasmodium chabaudi* AS (*P. chabaudi*), and the effects on parasitemia, hematologic parameters, erythropoietin receptor, TER119, and CD71 expression, *in vitro* erythropoietin-stimulated proliferation of splenic erythroid precursors, and serum cytokine levels were analyzed. To explore the role of interleukin-4 in STAT6-dependent erythropoietic suppression, mice were treated *in vivo* with a monoclonal antibody to interleukin-4 and the effects on parasitemia, hematologic parameters, and cytokine levels were analyzed.

Results

Infected STAT6$^{-/-}$ mice developed enhanced reticulocytosis compared to wild-type mice despite higher parasitemia and a similar course of anemia. Enhanced reticulocytosis in infected STAT6$^{-/-}$ mice was associated with an increased frequency of late-stage erythroblasts, fewer leukocytes expressing CD71, and increased erythropoietin-stimulated proliferation of splenocytes compared to infected wild-type mice. Interleukin-4-depleted wild-type mice had increased levels of parasitemia and a course of reticulocytosis similar to responses observed in infected STAT6$^{-/-}$ mice. Determination of serum cytokine levels in STAT6$^{-/-}$ and wild-type mice depleted of interleukin-4 by treatment with mAb revealed significantly lower levels of interferon-γ compared to control wild-type mice during infection.

Conclusions

Together, these findings provide evidence for a STAT6-dependent mechanism in mediating erythropoietic suppression during acute blood-stage malaria and indicate a role for interleukin-4 and possibly interferon-γ in STAT6-induced erythropoietic suppression.

Key words: malaria, anemia, erythropoiesis, STAT6, interleukin-4, interferon-γ.

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Introduction

Severe malarial anemia is one of the most prevalent life-threatening complications of *Plasmodium falciparum* infection, particularly in young children and pregnant women in sub-Saharan Africa.[1-4] In addition to destruction of infected and uninfected red blood cells, insufficient erythropoiesis, due to either ineffective erythropoiesis and/or dyserythropoiesis, plays a key role in malarial anemia.[5,6] Deficient erythropoietin (EPO) production does not appear to provide an explanation for malarial anemia since most studies indicate that EPO production is adequately increased in response to the level of anemia.[6,7] Rather, sub-optimal responses of erythroid progenitor cells to EPO appear to underlie suppressed erythropoiesis in individuals with severe anemia.[6] Suppressed erythropoiesis and dyserythropoiesis have been observed in the presence of both host-derived factors such as cytokines and the parasite-derived factor hemozoin, indicating that insufficient erythropoiesis during malaria is multifactorial, but the mechanism remains unknown.[8,9]

Although findings in mouse malaria models have provided new insight into the mechanism involved in severe malarial anemia, the broad clinical spectrum of *P. falciparum* infection in humans underlies the difficulty in directly transferring results from mice to humans. Various models of *Plasmodium* infection in mice share important similarities with the clinical variability in humans and are useful to investigate a broad range of questions concerning the pathogenesis of malarial anemia.[9] *P. chabaudi* AS (*P. chabaudi*), used in the present study, causes infections in mice with blood parasitemias greater than 20% and acute anemia analogous to similar manifestations in some individuals infected with *P. falciparum*. Evans and colleagues recently developed a model in *P. berghei* ANKA-infected, semi-immune mice to study chronic malarial anemia that frequently occurs in association with low parasitemias during *P. falciparum* infections.[10]

In vitro studies in mouse models of malaria demonstrate that an as yet unidentified soluble inhibitory factor released from cultured bone marrow and spleen cells from infected mice suppresses EPO-induced proliferation of erythroid progenitor cells.[11-13] Interferon (IFN)-γ and tumor necrosis factor (TNF)-α, produced during the acute phase of malaria infection, have been considered as candidates for this suppressive factor because of their ability to inhibit the growth of erythroid colony-forming cells.[14-16] Other pro-inflammatory cytokines such as interleukin (IL)-12 and macrophage migration inhibitory factor have also been implicated in the pathogenesis of malarial anemia.[17] However, there is no conclusive evidence supporting a role for these cytokines in suppressed erythropoiesis during malaria infection.

While the contribution of pro-inflammatory cytokines to the pathogenesis of malarial anemia has been studied extensively, the roles of Th2 cytokines remain unknown. The Th2 cytokines, IL-4 and IL-13, have many similar functional properties and share a common receptor subunit, IL-4 receptor α, which activates signaling through signal transducer and activator of transcription (STAT)6.[18] A role for Th2 cytokine-mediated responses in the homeostasis of hematopoietic progenitor cells was suggested by the findings that STAT6$^{-/-}$ mice have increased numbers of myeloid progenitors in bone marrow and spleen and increased cell cycling.[19] Furthermore, IL-4 and IL-13 contribute to the diversion of iron traffic by increasing iron uptake and storage in activated macrophages, hence suppressing the development of hemoglobin-producing erythroblasts.[20]

Here, we investigated the role of STAT6-mediated responses in the development of malarial anemia by comparing erythropoietic responses in wild-type (WT) C57BL/6 (B6) and STAT6$^{-/-}$ mice infected with *P. chabaudi*. Our findings establish a critical role for STAT6-dependent signaling in suppressed erythropoiesis during acute blood-stage malaria infection and indicate a role for IL-4 and possibly IFN-γ in STAT6-induced erythropoietin suppression.

Design and Methods

Mice

Breeding pairs of STAT6$^{-/-}$ mice (generously provided by Dr. Derek McKay, McMaster University, Hamilton, ON, Canada), generated as described previously,[21] were established in the animal facility of the Research Institute of the McGill University Health Centre. Mice were on the B6 background and were age-matched with WT B6 mice purchased from Charles River Laboratories (St. Constant, QC, Canada). Female mice, 8-12 weeks old, were used in all experiments, which were performed in accordance with the guidelines of the Canadian Council for Animal Care.

Parasite, experimental infection and hematologic analyses

P. chabaudi was maintained as described previously.[22] Mice were infected intraperitoneally with 10^6 parasitized red blood cells. At the days indicated, 5 μL of tail vein blood were collected for parasitological and hematologic analyses. Total numbers of red blood cells were determined using a hemocytometer. Parasitemia and reticulocytosis were determined on blood smears stained with Diff-Quik® II Solution (Dade Behring, Dudingen, Switzerland) by counting, respectively, the percentages of parasitized red blood cells and reticulocytes per 400 cells.

In vivo *antibody treatment*

WT mice were treated intraperitoneally with anti-IL-4 (11B.11; IgG1; 5 mg; NCI Biological Resources Branch, Frederick, MD, USA) monoclonal antibody 1 day before infection and weekly thereafter. Control mice received no treatment or an equal concentration of isotype control antibody.

In vivo *erythropoietin treatment*

As described previously,[23] mice with parasitemias of 5-8% (typically on day 5 post-infection; p.i.) were treated intravenously with 10 U recombinant murine

EPO (Roche, Laval, QC, Canada) in 0.2 mL phosphate-buffered saline supplemented with 0.1% bovine serum albumin for 3 days (days 5, 6 and 7 p.i.). Naïve mice were treated with an equal dose of EPO for 3 days as control. Since the responses of naïve mice treated with 0.1% bovine serum albumin in phosphate-buffered saline were indistinguishable from those of untreated mice, only data from naïve mice are presented.

Flow cytometry

Mice were sacrificed 1 day after the completion of EPO treatment and spleens were harvested aseptically. Single cell suspensions of splenocytes were prepared and red blood cells lysed with NH_4Cl. The viability of splenocytes was assessed with trypan blue and was always greater than 90%. Splenocytes were adjusted to a concentration of 1×10^6/mL and were FcR-blocked with anti-CD16/CD32 monoclonal antibody (2.4G2; BD Biosciences, Mississauga, ON, Canada) prior to staining for flow cytometry. Early erythroid progenitors were identified by staining with rabbit anti-human EPO receptor (EPOR) antibody (H-194; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody (Sigma-Aldrich, Oakville, ON, USA).[24] Proerythroblasts, basophilic erythroblasts, polychromatic and orthochromatic erythroblasts were identified by differential staining with phycoerythrin-conjugated anti-TER119 (BD Biosciences) and fluorescein isothiocyanate-conjugated anti-CD71 (BD Biosciences) monoclonal antibodies.[25] Cells were also stained with combinations of fluorescein isothiocyanate-conjugated anti-CD71 monoclonal antibody and phycoerythrin-conjugated monoclonal antibodies (eBioscience, San Diego, CA, USA) against CD3 (145-2C11), CD45R/B220 (RA3-6B2), CD11c (HL3), CD11b (M1/70), and Gr-1 (RB6-8C5). Cells were acquired using a FACSCalibur (BD Biosciences) and gated on live cells based on forward and side scatter; data were analyzed using CellQuest Pro software (BD Biosciences). The frequencies of cells expressing TER119 and CD71 are presented as percentages of total nucleated splenocytes.

Erythropoietin proliferation assay

Spleen cells were re-suspended to 4×10^6 cells/mL in Iscove's modified Dulbecco's medium (Gibco-Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA) and 0.12% gentamicin (Sabrex, Montreal, QC, Canada). Aliquots of 100 μL were seeded into 96-well microtiter plates in the presence of EPO (0 to 1 U/mL) and incubated for 24 hours at 37°C. During the last 2 hours of incubation, 1 μCi ^{3}H-thymidine (specific activity 53.0 Ci/mmol [1.98 TBq/mmol]; Amersham Biosciences, Baie d'Urfe, QC, Canada) was added to each well. ^{3}H-thymidine incorporation was determined by scintillation counting.

Cytokine quantification

Serum IFN-γ, TNF-α, IL-12p70, IL-12p40, IL-4, and IL-10 levels were quantitated by enzyme-linked immunosorbent assay (ELISA) using paired capture and detection antibodies (BD Biosciences) as previously described.[26] IL-13 levels were determined using a DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analyses

Data are presented as means ± SEM. The statistical significance of differences between groups was analyzed by a two-tailed, unpaired Student's *t* test and multiple comparisons were analyzed by ANOVA. Statistical significance was defined as $p<0.05$.

Results

STAT6$^{-/-}$ mice display enhanced reticulocytosis compared to wild-type controls during malaria

Alleviation of malarial anemia in *P. chabaudi*-infected B6 mice is delayed by suppressed reticulocytosis, with maximal suppression around the peak parasitemia occurring between days 7-9 p.i. with 10^6 parasitized red blood cells.[6] To examine the relationship between Th2 cytokines and sub-optimal reticulocytosis during blood-stage malaria, parasitemia and hematologic parameters were monitored in *P. chabaudi*-infected WT and STAT6$^{-/-}$ mice. Infected WT mice developed moderate, acute parasitemia levels and controlled and eliminated the parasite by about 4 weeks p.i. due to the generation of protective type 1 innate and adaptive immune responses (Figure 1A).[22,27] Anemia developed rapidly in WT mice beginning on day 6 and was alleviated only following reticulocytosis beginning on day 11 p.i. (Figure 1B, C). WT mice suffered a recrudescent parasitemia of 12% on day 18 p.i. and experienced another drop in red blood cells followed by a second wave of reticulocytosis during the third week of infection.

Similarily to in WT mice, parasitemia became apparent in infected STAT6$^{-/-}$ on day 4 (Figure 1A). Parasite replication accelerated rapidly, reaching a peak on day 8 p.i.; parasitemia was significantly higher in STAT6$^{-/-}$ mice than in WT mice on days 8-10 p.i. Despite this difference, the severity of anemia was similar in STAT6$^{-/-}$ and WT mice (Figure 1B). Following a sharp drop in red blood cells, reticulocytosis occurred promptly and to a higher level in infected STAT6$^{-/-}$ mice such that the onset of reticulocytosis was earlier (day 7 versus day 11 p.i.) and the magnitude was higher on days 7-12 p.i. in STAT6$^{-/-}$ compared to WT mice (Figure 1B, C). Furthermore, reticulocytosis peaked in STAT6$^{-/-}$ mice on day 11 p.i. while the response peaked in WT mice on day 13 p.i. STAT6$^{-/-}$ mice also suffered recrudescent parasitemia but recrudescence occurred 2 days earlier on day 16 and was significantly higher than that observed in WT mice. Recrudescence was followed by another drop in red blood cells and a subsequent secondary wave of reticulocytosis in both strains; at this time, the number of red blood cells was significantly higher in infected WT than STAT6$^{-/-}$ mice.

To further explore the role of STAT6 in suppressed reticulocytosis during malaria, we compared hematologic parameters in naïve and infected WT and STAT6$^{-/-}$ mice in response to exogenous EPO treatment. The

numbers of red blood cells and percentages of reticulocytes were indistinguishable in naïve WT and STAT6$^{-/-}$ mice, and reticulocytosis occurred to a similar extent following EPO treatment in naïve mice of both strains (Table 1). On day 8 p.i., EPO treatment induced significantly lower reticulocytosis in *P. chabaudi*-infected WT mice than in EPO-treated naïve WT mice. Consistent with the data reported in Figure 1, EPO-treated infected STAT6$^{-/-}$ mice displayed significantly higher parasitemia despite similar levels of anemia, and reticulocytosis was higher compared to that in similarly treated infected WT mice. During recrudescent parasitemia, reticulocytosis was not suppressed following EPO treatment of infected WT mice (*data not shown*). These observations suggest that erythroid precursors in infected STAT6$^{-/-}$ mice are more responsive to endogenous as well as exogenous EPO than are the precursors of infected WT mice. Collectively, these findings, which are consistent with our previous observations of suppressed reticulocytosis during acute blood-stage *P. chabaudi* in susceptible A/J mice,[23] suggest a role for STAT6 signaling in suppressed erythropoiesis during malaria infection.

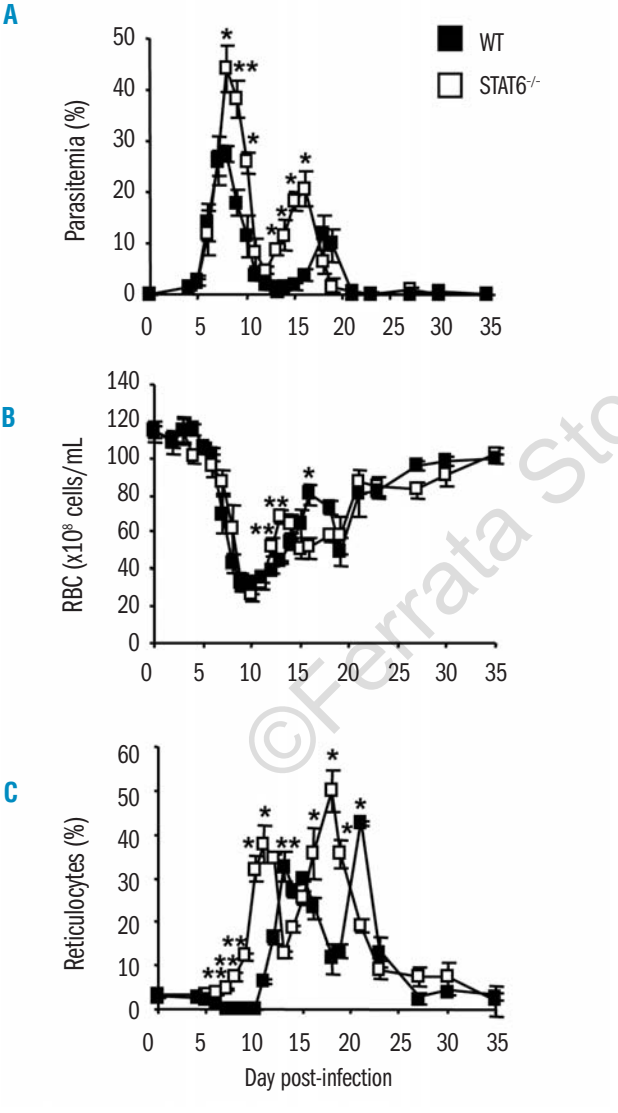

Figure 1. Course of *P. chabaudi* infection and hematologic parameters in WT and STAT6$^{-/-}$ mice. Mice were infected intraperitoneally with 10^6 parasitized red blood cells and the courses of parasitemia (A), progression of anemia (B), and reticulocytosis (C) were followed. Data are means ± SEM for four mice per group. *$p<0.05$; **$p<0.01$, WT *vs.* STAT6$^{-/-}$ mice. Similar results were obtained in three independent experiments.

Deficiency in STAT6 is associated with increased erythropoiesis during malaria

A sub-optimal erythropoietic response, evident as a deficiency in late-stage erythroid progenitors in response to exogenous EPO, contributes to suppressed erythropoiesis during *P. chabaudi* infection.[23] To investigate the role of STAT6 signaling in erythropoietic development during acute malaria infection when reticulocytosis is suppressed, the numbers of splenocytes expressing early (EPOR) and late-stage (TER119) erythroid markers were compared in naïve and *P. chabaudi*-infected WT and STAT6$^{-/-}$ mice on day 8 p.i. The generation and differentiation of erythroid progenitors were examined in mice of the two genotypes, either in naïve mice during homeostasis or in infected mice in response to endogenous EPO produced during malaria. In addition, separate groups of naïve and infected WT and STAT6$^{-/-}$ mice were treated with exogenous EPO. Consistent with our earlier findings,[23] *P. chabaudi* infection, with or without EPO treatment, resulted in significantly higher numbers of EPOR$^+$ cells in both WT and STAT6$^{-/-}$ mice compared to in their respective uninfected controls (Figure 2A, B). Importantly, there were no significant differences in EPOR$^+$ cell numbers between infected WT and STAT6$^{-/-}$ mice, with or without EPO treatment.

We also examined expression of the erythroid lineage-specific marker TER119, expressed by erythroblasts from the stages of proerythroblast to mature erythrocyte,[28] in the spleens of WT and STAT6$^{-/-}$ mice. As shown in Figure 2C, naïve WT and STAT6$^{-/-}$ mice without EPO treatment had similar low numbers of TER119$^+$ cells. Compared to their respective naïve controls, infected STAT6$^{-/-}$ mice displayed a greater than 20-fold increase in TER119$^+$ erythroblasts while WT mice had a 6-fold increase. Notably, infected STAT6$^{-/-}$ mice had significantly higher numbers of TER119$^+$ cells compared to infected WT mice. After EPO treatment, naïve mice of both strains had comparable increases in

Table 1. Hematologic parameters of naïve and *P. chabaudi*-infected wild-type and STAT6$^{-/-}$ mice[a,b].

	Parasitemia (%)	RBC (x10^8 cells)	Reticulocytes (%)
WT	ND	111.2±5.6	2.9±0.2
WT + EPO	ND	116.8±6.5	9.3±1.3[1]
WT Day 8 + EPO	41.75±3.3	52.4±10.8	2.1±0.7[2]
STAT6$^{-/-}$	ND	110.6±7.2	3.3±0.6
STAT6$^{-/-}$ + EPO	ND	115.3±1.54	8.1±0.8[1]
STAT6$^{-/-}$ Day 8 + EPO	55.8±2.0*	53.8±2.9	9.9±0.4[3]

[a]*Naïve mice or mice infected intraperitoneally with 10^6 parasitized red blood cells on day 0 were either untreated or treated with 10 U EPO on three consecutive days corresponding to days 5, 6 and 7 p.i. Hematologic parameters were determined in naïve and infected mice on day 8 p.i. (one day after completion of EPO treatment).* [b]*Data are presented as means ± SEM for four mice per group.* [1]*$p<0.01$, naïve WT vs. EPO-treated WT mice or naïve STAT6$^{-/-}$ vs. EPO-treated STAT6$^{-/-}$ mice.* [2]*$p<0.01$, EPO-treated naïve WT vs. EPO-treated infected WT mice.* [3]*$p<0.01$, EPO-treated infected WT vs. EPO-treated infected STAT6$^{-/-}$ mice. Similar results were obtained in three independent experiments.*

TER119+ cells (Figure 2D). *P. chabaudi* infection in WT mice resulted in a greater than 30% decrease in TER119+ cells in response to exogenous EPO. In contrast, EPO-treated infected STAT6$^{-/-}$ mice had similar numbers of TER119+ erythroblasts as EPO-treated naïve STAT6$^{-/-}$ mice but a significantly higher number of TER119+ cells compared to EPO-treated infected WT mice. The significant differences in splenic TER119+ erythroblasts between *P. chabaudi*-infected WT and STAT6$^{-/-}$mice, with and without EPO treatment, were not due to differences in splenomegaly, as reflected by indistinguishable weights and cellularities in the spleens of the two strains (*Online Supplementary Table S1*). These data indicate that during *P. chabaudi* infection, early erythroid progenitors in both WT and STAT6$^{-/-}$ mice responded equally to stimulation with endogenous or exogenous EPO and differentiated into EPOR+ cells. However, only infected STAT6$^{-/-}$ and not infected WT mice, with or without EPO treatment, had appropriately increased differentiation of EPOR+ cells into TER119+ erythroblasts, events which resulted in enhanced reticulocytosis and prompt alleviation of malarial anemia.

Erythropoetin-stimulated proliferation is suppressed in infected wild-type but not STAT6$^{-/-}$ mice

Our previous studies in susceptible A/J mice demonstrated that impaired proliferation of early EPO-responsive erythroid progenitors in the spleen contributes to the sub-optimal increase in TER119+ erythroblasts during *P. chabaudi* infection.[23] Splenic EPOR+ cells from infected WT mice also exhibited little proliferation to EPO stimulation *in vitro* while splenic erythroid progenitors from STAT6$^{-/-}$ mice displayed significantly increased EPO-stimulated proliferation compared to their respective naïve controls (Figure 2E,F). Furthermore, splenic EPO-responsive cells from EPO-treated infected WT mice exhibited significantly lower EPO-stimulated proliferation *in vitro* compared to that of EPO-treated naïve WT mice. In contrast, EPOR+ cells from EPO-treated infected STAT6$^{-/-}$ mice exhibited high proliferation in response to EPO stimulation *in vitro* similar to the responses of cells from EPO-treated naïve STAT6$^{-/-}$ mice over a wide range of doses (31.25-250 mUnits EPO/mL; Figure 2F). Although STAT6$^{-/-}$ and WT mice had comparable increases in splenic EPOR+ cells during malaria infection, EPO-responsive cells from STAT6$^{-/-}$ displayed higher EPO-stimulated proliferation than WT cells ($p<0.05$). This finding suggests that the robust response of EPOR+ cells from infected STAT6$^{-/-}$ mice to EPO may contribute to the prompt, efficient reticulocytosis during *P. chabaudi* infection.

FACS analysis of TER119+CD71+ and TER119-CD71+ splenocytes in P. chabaudi*-infected wild-type and STAT6$^{-/-}$ mice*

Transferrin receptor (CD71), critical for iron uptake

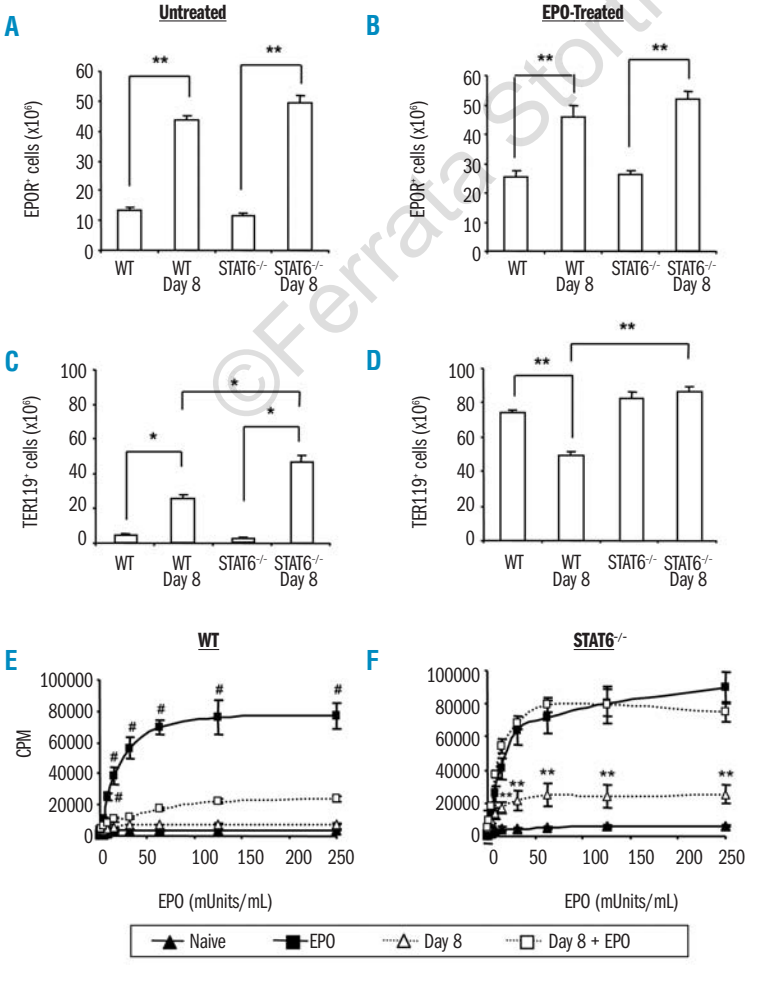

Figure 2. Flow cytometric analyses of splenic erythroid progenitors in naïve or *P. chabaudi*-infected WT and STAT6$^{-/-}$ mice. Naïve mice and mice infected intraperitoneally with 10^6 parasitized red blood cells on day 0 were either untreated or treated with 10 U EPO on three consecutive days corresponding to days 5, 6 and 7 p.i. Spleen cells were harvested from naïve and infected mice on day 8 p.i. (one day after completion of EPO treatment) and stained with rabbit anti-human EPOR antibody followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody or phycoerythrin-conjugated anti-TER119 monoclonal antibody and analyzed by FACS. Numbers of EPOR+ erythroid progenitors per spleen in (A) untreated and (B) EPO-treated mice. Numbers of TER119+ erythroblasts per spleen in (C) untreated and (D) EPO-treated mice. *In vitro* EPO-stimulated proliferation of unfractionated total splenocytes in (E) WT and (F) STAT6$^{-/-}$ mice. Data in A-D are presented as means ± SEM for four mice per group; *$p<0.05$; **$p<0.01$, naïve WT *vs.* infected WT mice, naïve STAT6$^{-/-}$ *vs.* infected STAT6$^{-/-}$ mice, or infected WT *vs.* infected STAT6$^{-/-}$ mice. Data in E and F are presented as means ± SEM for three mice per group; *$p<0.05$; **$p<0.01$, naïve *vs.* infected mice. #$p<0.01$, EPO-treated *vs.* EPO-treated infected mice. Similar results were obtained in three independent experiments.

and hemoglobin synthesis in erythroid cells, is expressed not only by erythroblasts, that is TER119$^+$ cells, but also by other rapidly dividing non-erythroid cells.[29] Similar increases in total numbers of CD71$^+$ splenocytes were apparent during infection in both WT and STAT6-deficient mice compared to their respective naïve control groups (Figure 3A). The numbers of CD71$^+$ splenocytes were also similarly increased in EPO-treated WT and STAT6$^{-/-}$ mice, regardless of infection with *P. chabaudi* (Figure 3B). Further analysis revealed that the frequency of TER119$^-$ cells expressing CD71 was higher in infected WT than STAT6$^{-/-}$ mice (p<0.05) (*Online Supplementary Figure S1*) although the numbers of splenic leukocyte subpopulations were similar in uninfected and infected WT and STAT6$^{-/-}$ mice (*Online Supplementary Figure S2*). These findings suggest that a deficiency in STAT6 signaling during malaria infection may have limited diversion of iron stores from hemoglobin-producing TER119$^+$ erythroid progenitors to rapidly expanding non-erythroid TER119$^-$ cells, thereby favoring the differentiation of erythroid progenitors.

To investigate this possibility further, we analyzed the co-expression of TER119 and CD71 on splenocytes from naïve and infected WT and STAT6$^{-/-}$ mice, with and without EPO treatment. The level of co-expression of TER119 and CD71 on early and late-stage erythroblasts defines the specific stage of their differentiation.[25] Infected WT mice, with or without EPO treatment, had 3-4 fold fewer late-stage basophilic [Region II (TER119^{high}CD71^{high})] as well as polychromatic and orthochromatic erythroblasts [Region III (TER119^{high}CD71$^{low\text{-}intermediate}$)] compared with EPO-treated naïve WT mice (Figure 3D, F *vs.* E). Infected STAT6$^{-/-}$ mice, with or without EPO treatment, also had lower frequencies of late-stage erythroblasts compared to EPO-treated naïve STAT6$^{-/-}$ mice (Figure 3H, J *vs.* I). However, infected STAT6$^{-/-}$ mice, with or without EPO treatment, had approximately twice the frequency of late-stage erythroblasts in Regions II and III as similarly treated infected WT mice (Figure 3H, J *vs.* D, F). This finding indicates that the decrease in late-stage erythroblasts in infected STAT6$^{-/-}$ mice was not as severe as that apparent in infected WT mice. Consistent with the finding that WT mice had higher numbers of non-erythroid splenocytes expressing CD71 during infection, infected WT mice, with or without EPO treatment (Figure 3D, F), had 40% or higher frequencies of TER119$^-$CD71$^{low\text{-}intermediate}$ cells (Region IV) compared to the respective group of infected STAT6$^{-/-}$ mice (Figure 3H, J). Together, these results indicate that the frequency of terminally differentiating erythroblasts was lower in WT compared to STAT6-deficient mice during *P. chabaudi* infection.

In vivo *cytokine levels in infected STAT6$^{-/-}$ mice*

To determine whether differences in the cytokine profiles of *P. chabaudi*-infected STAT6$^{-/-}$ and WT mice explained the disparity in their erythropoietic responses, cytokine levels in sera collected at peak parasitemia were analyzed. Serum IL-12p40, TNF-α, IL-10, and IL-4 levels were indistinguishable in WT and STAT6$^{-/-}$ mice (Table 2) while IL-12p70 and IL-13 were undetectable (*data not shown*). STAT6$^{-/-}$ mice had dramatically lower

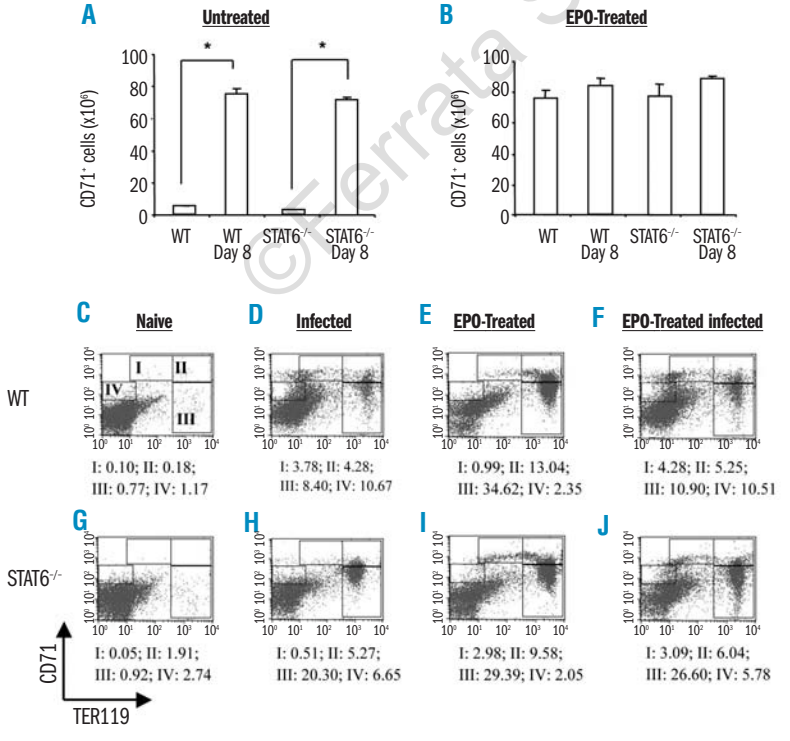

Figure 3. FACS analysis of CD71 expression and terminal differentiation of erythroblasts in the spleens of WT and STAT6$^{-/-}$ mice during *P. chabaudi* infection. Naïve mice and mice infected intraperitoneally with 10^6 parasitized red blood cells on day 0 were either untreated or treated with 10 U EPO on three consecutive days corresponding to days 5, 6 and 7 p.i. Spleen cells were harvested from naïve and infected mice on day 8 p.i. (one day after completion of EPO treatment) and red blood cells were lysed as described in the Design and Methods. Splenocytes were stained with fluorescein isothiocyanate-conjugated anti-CD71 monoclonal antibody. Numbers of CD71$^+$ cells per spleen in (A) untreated and (B) EPO-treated mice. Splenic erythroblasts at different maturation stages were identified by staining cells with phycoerythrin-conjugated anti-TER119 and fluorescein isothiocyanate-conjugated anti-CD71 monoclonal antibodies in (C, D, G, H) untreated and (E, F, I, J) EPO-treated mice. TER119^{intermediate}CD7^{high} (Region I) defines proerythroblasts; TER119^{high}CD71^{high} (Region II) defines basophilic erythroblasts; TER119^{high}CD71$^{low\text{-}intermediate}$ (Region III) defines polychromatic and orthochromatic erythroblasts; and TER119$^-$CD71$^{low\text{-}intermediate}$ (Region IV) defines the non-erythroid population expressing CD71. Values in each region are presented below and represent the frequencies of cells expressing TER119 and/or CD71 per total spleen cells. Data in A and B are presented as means ± SEM for four mice per group; *, p<0.01, naïve WT *vs.* infected WT mice or naïve STAT6$^{-/-}$ *vs.* infected STAT6$^{-/-}$ mice. Similar results were obtained in three independent experiments. Data in C-J are representative of one experiment. The same trend was observed in three separate experiments.

levels of IFN-γ in sera than WT mice during infection ($p<0.01$). Given that IFN-γ suppresses erythropoiesis,[15,30,31] this observation suggests a possible role for IFN-γ in STAT6-induced erythropoietic suppression.

Neutralization of interleukin-4 in infected wild-type mice

To determine the mechanism underlying STAT6-dependent erythropoietic suppression during malaria, the role of cytokines that activate STAT6, that is, IL-4 and IL-13, in suppressed reticulocytosis during *P. chabaudi* infection was examined. Since IL-4, but not IL-13, was increased after infection in both WT and STAT6$^{-/-}$ mice, we focused on the former mediator by treating WT mice with neutralizing anti-IL-4 monoclonal antibody or isotype control antibody and following the course of parasitemia, development of anemia, and reticulocytosis. The responses of IL-4-depleted WT (Figure 4) and STAT6$^{-/-}$ (Figure 1) mice during *P. chabaudi* infection were remarkably similar. Neutralization of IL-4 in infected WT mice resulted in a higher peak parasitemia on day 7 p.i. compared to that in mice treated with an isotype control (Figure 4A). IL-4-depleted mice experienced a marked recrudescent parasitemia of 25% on day 14 p.i. while isotype control mice experienced a small recrudescent parasitemia of 6% on day 13 p.i. The progression and severity of anemia were similar in infected anti-IL-4 monoclonal antibody-treated and iso-

Table 2. Serum cytokine levels at peak parasitemia following *P. chabaudi* infection in WT and STAT6$^{-/-}$ mice[a,b].

	Levels of serum cytokines (pg/mL)				
	IL-12p40	IFN-γ	TNF-α	IL-10	IL-4
WT	1068.5±29.7	ND	ND	ND	4.7±3.1
WT Day 8	1933.5±167.6	207.6±36.4	222.4±69.2	3062.5±647.8	24.1±2.2
STAT6$^{-/-}$	1563.5±36.5	ND	ND	ND	13.3±3.8
STAT6$^{-/-}$ Day 8	2417.3±232.8	2.9±2.6*	193.8±39.3	2457.5±602.8	28.8±0.6

[a]*Mice were infected intraperitoneally with 10^6 parasitized red blood cells on day 0. Serum levels of IL-12p70, IL-12p40, IFN-γ, TNF-α, IL-10, IL-13, and IL-4 were measured by ELISA at peak parasitemia on day 8 p.i. IL-12p70 and IL-13 were not detectable.* [b]*Data are presented as means ± SEM for three to six mice per group; *p<0.01, WT day 8 vs. STAT6$^{-/-}$ day 8 mice. Similar results were obtained in two independent experiments. ND = not detectable.*

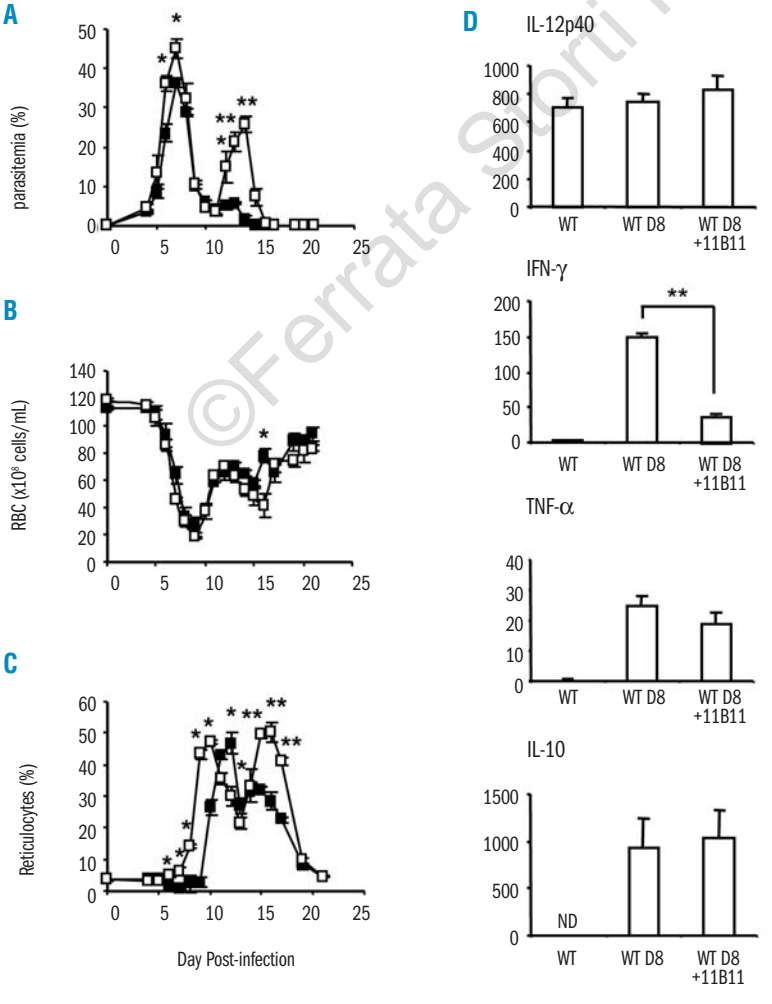

Figure 4. Hematologic parameters and serum cytokine levels following depletion of IL-4 in *P. chabaudi*-infected WT mice. Mice were infected intraperitoneally with 10^6 parasitized red blood cells on day 0. Neutralizing anti-IL-4 monoclonal antibody or isotype control antibody was administered weekly beginning 1 day before infection. (A) Course of parasitemia. (B) Progression of anemia. (C) Course of reticulocytosis. (D) Serum levels of IL-12p40, IFN-γ, TNF-α, and IL-10 were determined by ELISA at peak parasitemia on day 8 p.i., and are presented as pg/mL. Data are means ± SEM for four mice per group. *$p<0.05$; **$p<0.01$, mice treated with isotype control antibody *vs.* mice treated with anti-IL-4 monoclonal antibody-treated mice. Similar results were obtained in two independent experiments.

type control mice, except on day 16 p.i. when isotype control mice displayed a significantly higher number of red blood cells than anti-IL-4 monoclonal antibody-treated mice (Figure 4B). Moreover, IL-4-depleted WT mice experienced an earlier reticulocytosis that peaked on day 10 compared to day 12 in isotype control mice (Figure 4C). A secondary peak of reticulocytosis occurred on days 15 and 16 p.i. in isotype control and anti-IL-4 monoclonal antibody-treated mice, respectively. As in infected STAT6$^{-/-}$ mice, neutralization of IL-4 in infected WT mice resulted in dramatically lower serum IFN-γ levels compared to those in isotype control WT mice (Figure 4D). IL-12p40, TNF-α, and IL-10 levels were unchanged in infected WT mice after IL-4 neutralization while IL-12p70 and IL-13 were undetectable (*data not shown*). Together, these findings provide compelling evidence supporting a role for STAT6-dependent responses involving IL-4 and possibly IFN-γ in erythropoietic suppression during malaria infection.

Discussion

Here, we provide novel evidence for the importance of STAT6 signaling in anemia during *P. chabaudi* AS infection in mice. This model is useful for studying malarial anemia associated with high parasitemia because it encompasses many features similar to anemia during acute *P. falciparum* infection, including destruction of both infected and uninfected red blood cells and erythropoietic suppression.[9] In the absence of STAT6, *P. chabaudi*-infected mice displayed an earlier and higher reticulocytosis despite experiencing a higher peak of parasitemia and a similar level of anemia compared to infected WT mice. Moreover, reticulocytosis was significantly enhanced in infected STAT6$^{-/-}$ compared to WT mice following treatment with exogenous EPO despite higher peak parasitemia and anemia similar to that of EPO-treated infected WT mice. Together, these findings suggest that erythropoietic suppression was less severe in infected STAT6$^{-/-}$ than in WT mice.

Studies on the mechanisms underlying erythropoietic suppression have focused primarily on Th1 cytokines. Excessive production of pro-inflammatory cytokines inhibits erythropoiesis, suppresses EPO production, and triggers the diversion of iron stores away from hemoglobin-producing cells.[32] In contrast, little is known about the roles of the Th2 cytokines, IL-4 and IL-13, in regulating erythropoiesis. Repeated IL-13 injections induce anemia and extramedullary hematopoiesis in mice.[33] IL-4 synergizes with a number of cytokines to stimulate colony-formation by hematopoietic progenitor cells and induces more primitive, multipotential blast-cell colonies.[34,35] Like Th1 cytokines, IL-4 and IL-13 participate in the induction of hyperferremia during chronic inflammation by increasing CD71-mediated iron uptake and storage in activated macrophages.[20] Although these findings suggest that STAT6 signaling plays a role in hematopoietic progenitor cell homeostasis, its precise role in regulating erythropoiesis, especially during malaria infection, has hitherto been unexplored.

A recent study by Broxmeyer *et al.* demonstrated that STAT6 deficiency regulates myeloid progenitor cell homeostasis via secretion of the Th1-induced growth factor oncostatin M, an effect replicated in IL-13$^{-/-}$ but not in IL-4$^{-/-}$ mice.[19] Although STAT6$^{-/-}$ mice were observed to have increased numbers of granulocyte-macrophage (CFU-GM), erythroid (BFU-E), and multipotential (CFU-GEMM) progenitor cells, the frequencies of mature myeloid cells, including macrophages and granulocytes, as well as of lymphocytes, red blood cells and platelets, were found to be similar in STAT6$^{-/-}$ and WT mice.[19,36] Likewise, we observed that naïve WT and STAT6$^{-/-}$ mice had similar numbers of red blood cells and low percentages of reticulocytes in peripheral blood and similar numbers of EPOR^{+} and TER119^{+} cells in spleen. We also observed that naïve WT and STAT6$^{-/-}$ mice responded similarly to treatment with exogenous EPO, with comparable reticulocytosis as well as comparable increases in the numbers of splenic EPOR^{+} cells and TER119^{+} erythroblasts. In addition, splenocyte proliferation in response to EPO stimulation *in vitro* was comparable in EPO-treated naïve WT and STAT6$^{-/-}$ mice.

Based on our previous findings that reticulocytosis is suppressed during acute *P. chabaudi* infection around the time of peak parasitemia,[37] we investigated the role of STAT6 in erythropoietic suppression during acute infection. Since the spleen is the major site of extramedullary erythropoiesis in malaria-infected mice,[22,23] the frequency and numbers of early and late-stage erythroid progenitor cells were analyzed in this tissue in WT and STAT6$^{-/-}$ mice on day 8 p.i. Enhanced reticulocytosis in STAT6$^{-/-}$ mice during *P. chabaudi* infection was associated with marked increases in EPO-stimulated maturation and proliferation of splenic erythroid progenitor cells and maintenance of iron uptake within the erythroid compartment. Moreover, the differences between WT and STAT6$^{-/-}$ mice were apparent during infection when untreated as well as EPO-treated mice were compared. The effect of STAT6 deficiency was confined to the splenic erythroid compartment since leukocyte subpopulations were all similarly increased in WT and STAT6$^{-/-}$ mice in response to *P. chabaudi* infection.

CD71 is highly expressed on hemoglobin-producing cells, mainly proerythroblasts and basophilic erythroblasts, placental tissue, and other rapidly dividing cells.[29] *P. chabaudi* infection in susceptible A/J mice induces increased CD71 expression on splenocytes; however, CD71 expression is diverted from hemoglobin-producing cells to non-erythroid cells.[23] Our present findings demonstrated that CD71 expression increased similarly in the spleens of untreated and EPO-treated STAT6$^{-/-}$ and WT mice after malaria infection. Consistent with our previous findings in EPO-treated A/J mice during *P. chabaudi* infection, infected WT but not STAT6$^{-/-}$ mice displayed a severe deficiency in late-stage polychromatic and orthochromatic erythroblasts together with increased CD71 expression on splenic leukocytes in response to exogenous EPO. These data indicate that a deficiency in STAT6 limited diversion of CD71 from erythroid to non-erythroid cells, hence maintaining iron uptake in the erythroid compartment. The marked increase in CD71 expression on rapidly dividing

TER119$^-$ cells, including B cells, T cells, macrophages/monocytes, granulocytes, and dendritic cells, in infected WT compared to STAT6$^{-/-}$ mice, may explain reduced EPO-stimulated proliferation *in vitro* by splenic EPO-responsive cells from infected WT mice. Together, these data suggest that in the absence of STAT6 signaling, CD71 expression is conserved on erythroid cells, resulting in sufficient iron uptake in EPO-responsive proliferating cells and hemoglobin-producing cells and adequate production of terminally differentiating erythroblasts.

One potential explanation for the differences in erythropoietic responses in STAT6$^{-/-}$ and WT mice may be distinct cytokine profiles elicited in response to *P. chabaudi* infection. Although STAT6 is critical for signaling by IL-4 and IL-13,[38,39] its role in regulating type 1 cytokine production is unclear.[40,41] Previous findings indicate that CD4$^+$ T cells from STAT6$^{-/-}$ mice display an increased capacity to develop into IFN-γ-producing Th1 cells following infection with various pathogens.[42,43] In contrast, we observed that STAT6$^{-/-}$ mice produced less IFN-γ *in vivo* during malaria infection compared to infected WT mice. This finding was supported by the observation that STAT6$^{-/-}$ mice developed a higher peak parasitemia and is consistent with the protective effect of IFN-γ during acute blood-stage *P. chabaudi* infection.[26]

Evidence indicates that IL-4, but not IL-13, acts via a STAT6-dependent mechanism to induce IFN-γ production by natural killer cells and natural killer T cells;[44] IL-4-induced IFN-γ production has been demonstrated in mice infected with *Candida albicans* and *Leishmania major*.[45,46] As observed in STAT6$^{-/-}$ mice, IL-4 neutralization in *P. chabaudi*-infected WT mice resulted in a similar course of anemia despite a significantly higher peak parasitemia compared to that in isotype control WT mice. Moreover, infected IL-4-depleted mice, like STAT6$^{-/-}$ mice, developed an earlier reticulocyte response in association with low serum levels of IFN-γ. These findings suggest that IL-4 priming may be important to induce a protective Th1 response to control acute malaria infection as well as to regulate erythropoiesis. Langhorne and colleagues observed that *P. chabaudi*-infected IL-4$^{-/-}$ mice control and clear primary infection although IFN-γ mRNA levels in the spleen are lower during the first week of infection in IL-4$^{-/-}$ compared to WT mice.[47] The difference in IFN-γ mRNA levels between infected WT and IL-4$^{-/-}$ mice was found to be even more dramatic in CD4$^+$ T cells enriched from the spleen. Although IFN-γ has been demonstrated to suppress erythropoiesis *in vitro* and *in vivo* in animal models as well as in humans,[30,31,48,49] its role in suppressing erythropoiesis during malaria infection has been difficult to demonstrate due to the critical role of this cytokine in innate and adaptive immune responses.[27] Our data suggest a possible role for IFN-γ in STAT6-induced erythropoietic suppression during *P. chabaudi* infection and are consistent with recent data in IFN-γ$^{-/-}$ mice infected with *P. yoelii*.[50] It is of interest to note that although susceptible A/J mice exhibit low IFN-γ levels in response to *P. chabaudi* infection compared to resistant B6 mice, A/J mice display suppressed erythropoiesis and have high mortality, suggesting that erythropoietic suppression is not dependent on IFN-γ alone.[51] Whether the early reticulocyte response in STAT6$^{-/-}$ mice is dependent solely on low IFN-γ levels or on a combination of low IFN-γ levels and the absence of IL-4-dependent STAT6 signaling remains to be investigated.

In conclusion, our findings provide novel evidence for a STAT6-dependent mechanism involving IL-4 and possibly IFN-γ in mediating erythropoietic suppression during blood-stage malaria. Novel insights into the complex role of the host immune system in regulating erythropoiesis may contribute to the understanding of erythropoietic suppression during malarial anemia.

Authorship and Disclosures

NT designed and performed the research, analyzed the data and drafted the manuscript. MMS provided scientific expertise in designing experiments and edited the manuscript. MT maintained the parasite and breeding of knock-out mice and performed flow cytometric analyses and assisted in the preparation of the manuscript.

The authors reported no potential conflicts of interest.

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