Original Article
Cytokine dysregulation associated with malarial anemia in *Plasmodium yoelii* infected mice

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Abstract: The mechanisms of malaria anemia remain incompletely understood although much effort has been put on studies in both human and murine systems. Hematopoiesis is regulated by the proliferation, differentiation and maturation of erythropoietic progenitor cells into erythrocytes and is tightly controlled by a complex communication network of cytokines as signal mediators. The present study used the murine *P. yoelii* 17XNL malaria model to investigate the profile of cytokines and leukocytes throughout the entire infection. Moreover, malaria induced anemia was studied in comparison with anemia induced by hemorrhage and hemolysis. During *the P.* yoelii infection, the levels of erythropoietic-related cytokines, such as G-CSF, GMCSF, IL-7, and IL-17, were pronouncedly reduced, while those of regulatory cytokines, such as IL-10 and TNF-α, were constantly increased. This cytokine profile corresponded well with the cellular composition during the infection, such as drastically decreased levels of CD4+ and CD8+ T cells. The profiles of erythropoiesis or hematopoiesis related cytokines during malarial anemia showed striking differences from those during anemia induced by hemorrhage or hemolysis. This study demonstrates that a markedly dysregulated cytokine network occurred in this murine malaria model, which may open a new window of insight into the mechanisms of malaria related anemia.

Keywords: Malaria, *Plasmodium yoelii*, anemia, hematopoiesis, IL-7, IL-17, Epo, cytokine

Introduction

Malaria is a global disease, in which malarial anemia and cerebral malaria are two major causes of death. Malarial anemia is a multifactorial disease, involving both increased destruction of circulating red cells and an inadequate erythropoietic response to compensate for the increased red-cell destruction. Multiple mechanisms appear to be responsible for this increased rate of red-cell loss from the circulation, including sequestration of infected red cells in the microvasculature [1-3] and phagocytosis of both parasitized and non-parasitized red cells [4, 5].

In *P. falciparum* malaria, an inadequate reticulocyte response indicating a transient suppression of erythropoiesis was reported [6, 7], and

malaria infection was shown to affect the proliferation of bone marrow primitive progenitor cells, or committed precursor cells [8].

Hematopoietic stem cells, that differentiate into mature lineage-restricted blood cells, including erythrocytes and leukocytes, are under the influence of a network of cytokines. Erythropoiesis is a branch of hematopoiesis that is regulated by erythropoietic-related factors, such as erythropoietin [Epo], granulocyte colony-stimulating factor [G-CSF], and granulocyte-macrophage colony-stimulating factor [GMCSF]. Erythropoietin, a hormone produced by the kidney, plays an essential role in stimulating erythrocyte production. High levels of Epo or an Epo-like substance in malaria anemic sera have been reported in children with *P. falciparum* malaria [9, 10], while others have

reported that Epo production is impaired in mice infected with *P. berghei* [11] or in humans infected with *P. falciparum* [12-14].

T cells, in particular CD4+ cells, play a key role in the protective immune responses against the blood stages of malaria, orchestrating cellular and humoral immune mechanisms by cytokines [15, 16]. The balance between cytokines produced by T-helper [Th]1 and Th2 types of cells during different phases of the blood stage infection may determine the disease outcome [17]. Erythropoiesis is closely connected with the immune system, the T-cell produced cytokine IL-17, together with IL-7, influencing the proliferation of erythroid precursor cells [18, 19]. Malaria infections have profound effects on T cells, such as intensive T-cell loss due to apoptosis [20]. Also, T cells trigger the complicated sequences of events involved in humoral immunity by activating B cells to produce high levels of antimalarial antibodies [21, 22]. As the activation of T cells is heavily dependent on the innate immune response, such as antigen-presenting cells, analyses of the relationship between T cells, monocytes, and their cytokine products during the entire malaria infection would give a new insight into the pathogenic events occurring in malaria.

In order to get further insight into the mechanisms behind the development of malarial anemia, this study aimed at investigating the cytokine profile in mice during anemia induced by a *P. yoelii* malaria infection in comparison with anemia induced by hemorrhage or hemolysis.

Material and methods

Experimental animals and parasites

Age and sex-matched C57BL mice were used for all the experiments. In total, more than 400 C57BL mice were used, starting each time point in the experiments with 5 mice for the loss during the experiments. The parasites used were of the non-lethal *P. yoelii* 17XNL strain. For infection of mice, 1×10^6 *P. yoelii* infected red blood cells derived from a source mouse were injected intraperitoneally into the mice. Parasitemia was determined by fluorescence microscopy with acridine orange [23]. All animal work was performed according to the New York Blood Center IACUC approved protocol.

Induction of anemia by hemolysis or hemorrhage

Hemolytic anemia was induced by phenylhydrazine hydrochloride injection. A saline dosage of 1 mg per 25 g of body weight was used [24] (Sigma, Rosebrough, NJ) for every second day for a total of four times. Hemorrhage anemia was induced by tail bleeding, 0.3 ml of blood being drawn every second day for a total of four times [25]. Hematological parameters were detected by the Advia 120 Hematology System (Bayer, Tarrytown, NY) [26].
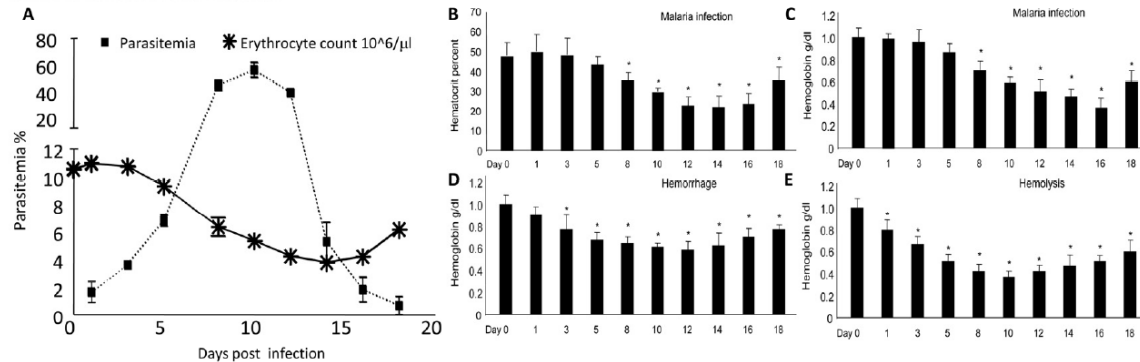
Preparation of mouse plasma and cells

Intracardiac blood was collected from each group of 5 mice at specific times at days 0, 1, 3, 5, 8, 10, 12, 14, 16, and 18. Ten μl of blood were diluted into a microcentrifuge tube with 90 μl of red-cell lysis buffer (BD Biosciences, New York City, NY) for the total leukocyte counting. Twenty-five μl of blood were diluted with EDTA solution to generate hematological parameters as detected by the Advia 120 Hematology System. Hundred μl of blood were taken for analysis of the cellular distribution by flow cytometry. Plasma was centrifuged at 800xg for 10 min, and each sample was individually stored at -80°C until cytokine analyses. Mouse spleen single-cell suspensions were prepared after digestion with Liberase Cl solution (Roche, Nutley, NJ) for 30 min, and then passage through a 100 nm cell strainer.

Measurement of plasma cytokine levels

Cytokines were measured at specific times by using Beadlyte mouse multicytokine detection system (Upstate Biotechnology, Lake Placid, NY and LINCO Research Inc, Saint Charles, MO), following the manufacturer's instructions. Briefly, 25 μl of mouse plasma were incubated with anti-multi-cytokine beads in individual wells of a 96-well plate. Twenty five μl of biotinylated anti-multi-cytokine/chemokine were added into each well, incubated for 1 h at room temperature, followed by washings (3X) with PBS. Streptavidin-phycoerythrin (PE) conjugated antibodies were added, and samples were incubated for 1 h at room temperature. The samples were then washed three times. The plates were read on a Luminex100™ instrument. The concentration of mean fluorescence intensity (MFI) of cytokine/chemokine was

Figure 1. Kinetics of parasitemia, anemia, and anemia induced by hemolysis or hemorrhage. A. Malaria parasitemia in peripheral blood (broken line) and red cell count (solid line) during the course of *P. yoelii* infection. Parasitemia was detected in three to five individual mice per time point. B. Kinetics of hematocrit and (C) kinetics of hemoglobin during malaria infection. D. Kinetics of hemoglobin induced by hemolysis and (E) kinetics of hemoglobin hemorrhage. Symbols represent the mean ± SD of 3 to 5 mice. The experiment was repeated twice with similar results.

determined from a standard curve generated with defined cytokine/chemokine reference samples provided by the companies mentioned above. Mouse plasma erythropoietin was detected by ELISA (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Flow cytometry analysis

Cells prepared as described above were incubated with anti-FcR antibodies (Santa Cruz Biotech, CA) for 20 min at 4°C to block non-specific binding of fluorochrome-labeled antibodies, and then stained with antibodies to the mouse glycophorin A associated protein Ter 119 (Invitrogen Camarillo, CA), CD45 (BD Biosciences clone: 30-F11), CD4, CD8, NK1.1, MAC1, and GR1 (R&D Systems) . The antibodies were diluted in FACS buffer and incubated with cells in each tube on ice for one hour. Thirty thousand cells were collected with a FACSCalibur instrument and data analysis was done with CellQuest program (both from Becton Dickinson). The Ter 119 antibody was used for erythroid cells to eliminate red cells in the analysis (**Figure 2C**). The antibody to CD45, a pan marker for all leukocytes, was used to enclose the leukocytes for analysis (**Figure 2C**).

Determination of total leukocyte numbers

Ten μl of mouse blood, collected as described above, were diluted into 90 μl of FACS red cell lysis buffer for 1 h, and the total cell number was counted with a hematocytometer chamber. The cell number was calculated with the exclusion of non-lysed reticulocytes, and infected reticulocytes were detected by fluorescence microscopy [23].

Statistical analysis

Data are presented in bar graphs as mean ± SD. Values were compared using Student "t" test and considered significant with "p" values of ≤ 0.05.
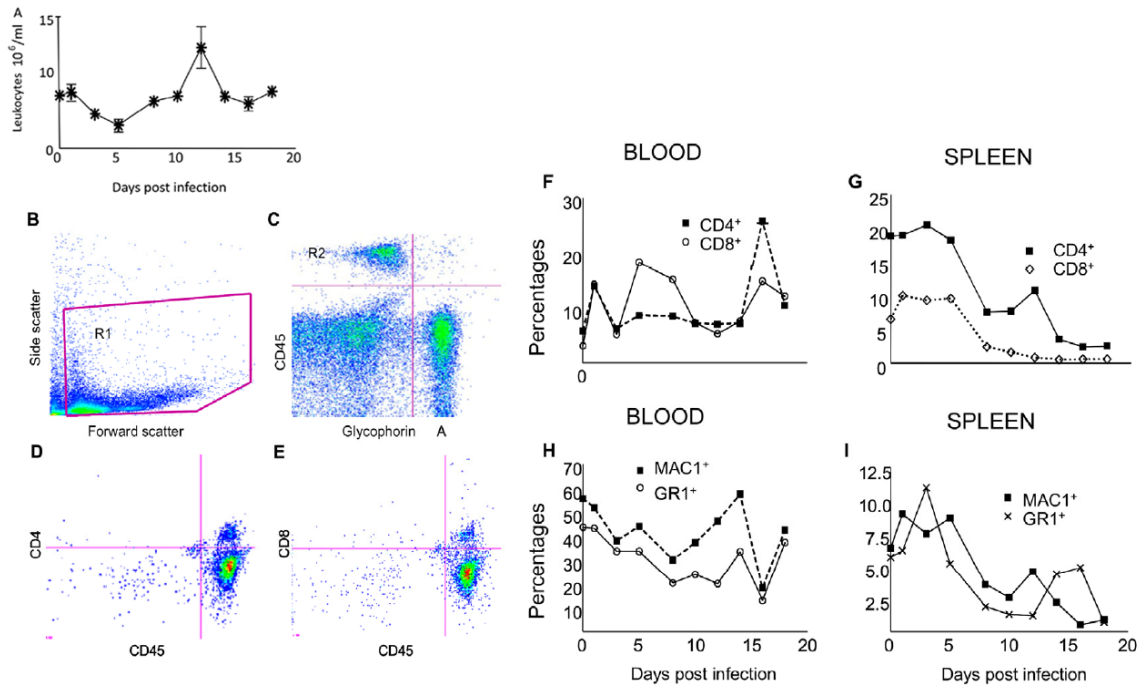
Results

Mouse malaria infection and anemia

Blood-stage *P. yoelii* 17XNL parasites induced malaria infection in C57BL mice, with the parasitemia peaking on day 10 at about 60% and then dramatically decreasing to less than 5% by day 15 (**Figure 1**). No apparent second peak of parasitemia was observed. The mice developed anemia and exhibited concurrent inactivity during days 8 to 16 (**Figure 1**). The erythrocyte counts dropped from the normal value of $10 \times 10^6/mm^3$ to $6.5 \times 10^6/mm^3$ at day 8, and reached nadir at day 14, returning to about $6.5 \times 10^6/mm^3$ at day 18 (**Figure 1**). Both the hematocrit and hemoglobin levels corresponded to the erythrocyte counts (**Figure 1**)*. The degree of anemia and the levels of parasitemia showed an inverse relationship.

Anemia induced by hemolysis or hemorrhage was also observed, and presented similarly to malaria-induced anemia by decreased hemoglobin levels. Phenylhydrazine hydrochloride induced anemia was associated with typical

Figure 2. Kinetics of the cellular compositions during malaria infection. A. Example of flow cytometry analysis and gating strategy for identification and quantification of blood CD4+, CD8+, Mac1 and GR1 cell subsets. The live cell population (R1) in whole blood is identified by a combination of forward and side scatter properties (B), followed by exclusion gating of glycophorin A+ cells in lower right quadrant (C). The cells in R2 were analyzed for sub-sets of CD4+ (D), CD8+ (E). Kinetics of sub-sets of CD4+, CD8+, Mac1, and Gr1+ in peripheral blood (F and G) and in spleen (H and I), respectively. Symbols represent the mean ± SD of 3 to 5 mice.

symptoms related to anemia, including tiredness and hematuria [27]. The hemoglobin levels decreased gradually after each injection of phenylhydrazine hydrochloride (**Figure 1**) and it reached its lowest level at day 10, compared to the level of about 30% at the beginning of the study. Similarly, hemorrhage induced anemia resulted in a reduction of the hemoglobin level to about 60% of the initial level of 11 g/dl at the start of the study (**Figure 1**).

Cellular composition in spleen and blood during P. yoelii infection

In order to investigate how different leukocyte populations were affected by the parasite infection in the *P. yoelii* malaria model, hemocytometer and flow cytometry analyses of peripheral and spleen leukocytes were performed. The total number of peripheral leukocytes increased slightly at day 1 of the infection, and then gradually decreased into the nadir at day 5 at about 2.9×10^6/ml, whereafter the leukocyte numbers increased to reach a maximum of 13 x

10^6/ml at day 12, just after the peak of parasitemia, and then they decreased to reach normal levels by day 15 (**Figure 2A**).

The proportion of CD4+ and CD8+ T cells among the peripheral CD45+ cells in peripheral blood was similar and increased slightly directly after the start of the infection, but went down to the initial levels at day 3 (**Figure 2F**). The proportion of the CD4+ T cells was steady until day 15, while there was a transient increase in the proportion of CD8+ T cells. The proportions of both CD4+ and CD8+ T cells among the CD45+ cells were increased to the levels above the initial levels directly after the peak of the parasitemia (**Figure 2F**). The ratio between CD4+ and CD8+ T-cells in the blood changed from 1:2 at the beginning of the infection to 1:1 at later time during points (**Figure 2F**). The proportion of CD4+ T cells in the spleen was higher than that of the CD8+ T cells, 18% and 6% respectively, but after day 5 both cell types decreased drastically to reach a level of about 1% after the clearance of the parasitemia (**Figure 2G**).

With regard to the innate immune cells, the proportions of Gr-1$^+$ cells (granulocytes) and Mac-1$^+$ cells (monocytes/macrophages) in the CD45$^+$ cell population were gradually reduced after the initiation of the *P. yoelii* infection and reached the nadir at day 8 post-infection in peripheral blood (**Figure 2H**), and at day 10-12 in the spleen (**Figure 2I**). On days 10 to 15 the proportion of both cell types in peripheral blood increased gradually, an increase that was less pronounced for Gr-1$^+$ cells (**Figure 2H**).

Cytokine levels during a P. yoelii infection

In order to explore whether the profiles of CD4$^+$ and CD8$^+$ T cells, monocytes/macrophages, and granulocytes during a *P. yoelii* infection also are reflected in the cytokine production, plasma levels of multiple cytokines were determined during the full course of the infection. Cytokines included in the study were such that play roles in Th1 type of responses, including IL-2, IL-12p70, IFN-γ (**Figure 3A-C**) and in Th2 type of responses, including IL-5, IL-6, IL-13 (**Figure 3D-F**). Further cytokines included were such related to antigen-presenting cells, including MCP-1, MIP-1a, IL-7, IL-1b, IL-1a, and IL-15 (**Figure 3G-L**), as well as cytokines related to regulatory immune responses, including IL-4, IL-10, IP-10 (CXCL10), and TNF-α (**Figure 3M-P**). Most of the T-cell-related cytokines, as well as the cytokines produced by antigen-presenting cells, showed a short peak of increased levels in the beginning of the infection and then a gradual decrease down to or below basal levels (**Figure 3A-C, 3E, 3G-L**). Also cytokines related to regulatory immunity (IL-4, IL-10, IP-10, and TNF-α) showed an increase in levels shortly after the initiation of the infection, and remained above the basal levels through the entire infection, although IL-4 appeared in two peaks (**Figure 3M-P**). In addition, for some cytokines, the second peak occurred more or less in association with peak parasitemia or after the parasites were cleared (**Figure 3K, 3M, 3O**).

EPO and hematopoiesis-related cytokines in malarial anemia in comparison with hemolysis or hematopoiesis induced anemia

In order to investigate whether the anemia induced by a *P. yoelii* infection may be due to altered levels of cytokines related to erythropoiesis or hematopoiesis, the levels of Epo, IL-17, IL-7, G-CSF and GMCSF during an entire infection were determined. The infection resulted in increased levels of Epo already at day 1, and then they gradually increased to reach the highest levels during the peak of parasitemia at days 10 to 12, with a 45-fold increase as compared with pre-infection level (**Figure 4A**). These high levels of Epo were largely sustained up to day 18, the last time point tested.

The *P. yoelii* infection caused a decrease in the levels of G-CSF at the onset of the infection which remained low throughout the entire course of the infection (**Figure 4B**). The levels of GMCSF, IL-7 and IL-17 exhibited similar patterns to those seen with the majority of cytokines, showing a slight increase in the very beginning of the infection, and then a subsequent decline to levels below the baseline (**Figure 4C-E**). This decline for IL-17 was particularly marked, decreasing more or less gradually throughout the infection up to day 18 (**Figure 4E**).
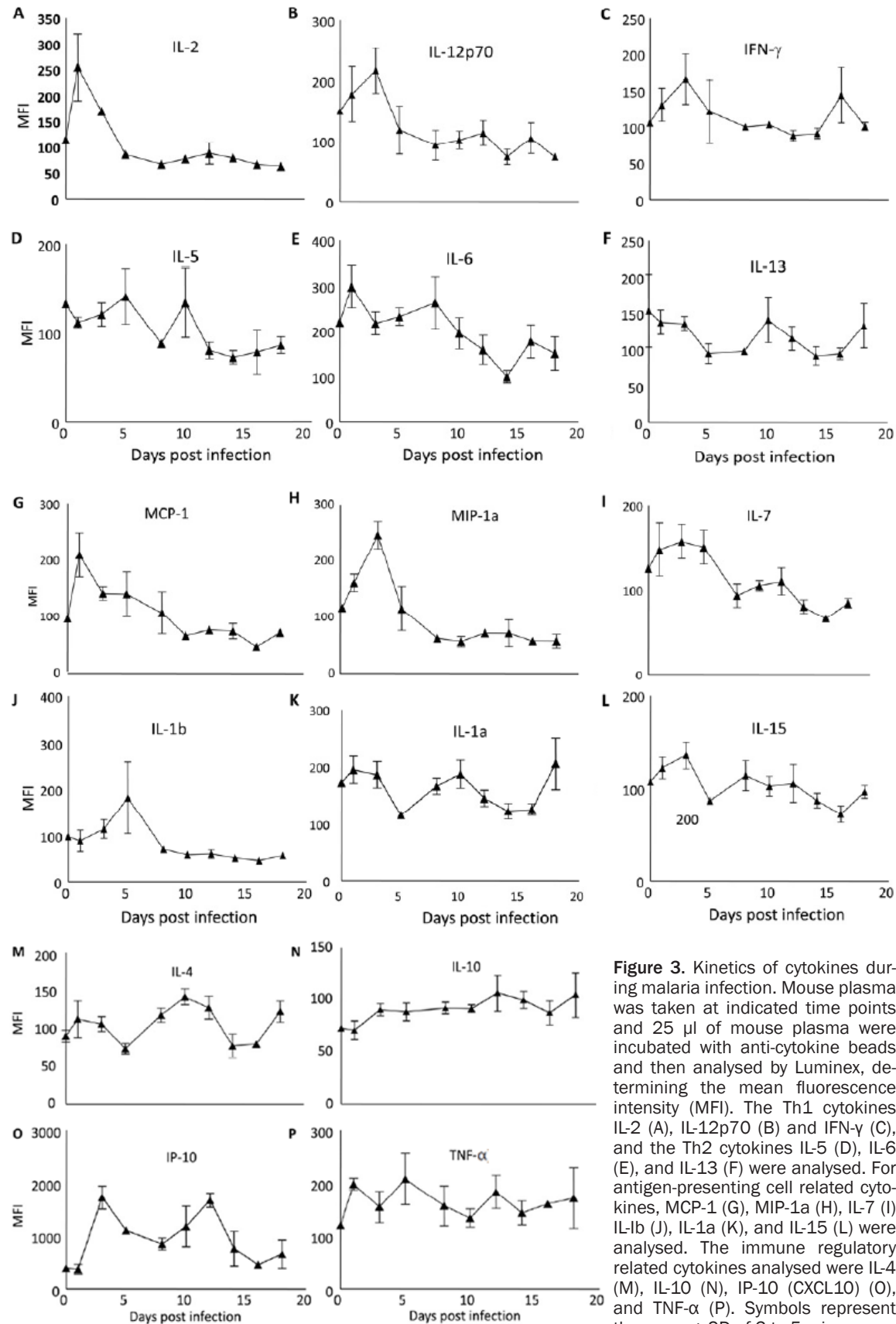
On the basis of the results above, a comparison was made between malarial anemia and anemia induced by either hemolysis or hemorrhage. The levels of selected cytokines were determined in day 10 plasma from the anemic mice, a time point when all three groups exhibited low hemoglobin levels (**Figure 1**. No differences were noted in the Epo response between the three types of anemia (**Figure 4F**). However, the levels of IL-7 and IL-17, cytokines that exert their greatest impact on hematopoiesis and erythropoiesis, were decreased only in malaria anemia when compared with hemolytic anemia or hemorrhage anemia (**Figure 4G, 4H**). In contrast, while IL-10 and TNF-α were strongly induced during malarial anemia, no such induction was seen in anemia caused by hemolysis or hemorrhage (**Figure 4I, 4J**).

Discussion

The present study confirms and extends findings in previous reports on the profound impact of malaria infections on the immune system, both regarding the cellular composition and the cytokine network [3, 7, 21, 31]. Our study provides new information about the potential underlying mechanisms associated with the destructive effects of malaria-induced anemia. A clear reciprocal relationship between parasitemia and erythrocyte count was seen during

Figure 3. Kinetics of cytokines during malaria infection. Mouse plasma was taken at indicated time points and 25 µl of mouse plasma were incubated with anti-cytokine beads and then analysed by Luminex, determining the mean fluorescence intensity (MFI). The Th1 cytokines IL-2 (A), IL-12p70 (B) and IFN-γ (C), and the Th2 cytokines IL-5 (D), IL-6 (E), and IL-13 (F) were analysed. For antigen-presenting cell related cytokines, MCP-1 (G), MIP-1a (H), IL-7 (I) IL-Ib (J), IL-1a (K), and IL-15 (L) were analysed. The immune regulatory related cytokines analysed were IL-4 (M), IL-10 (N), IP-10 (CXCL10) (O), and TNF-α (P). Symbols represent the mean ± SD of 3 to 5 mice.

the *P. yoelii* infection, resulting in malarial anemia as indicated by decreased erythrocyte counts, hematocrit, and hemoglobin during the later phase of the infection.

The cause of malarial anemia is multifactorial, including both different mechanisms of erythrocyte destruction as well as impaired regeneration of erythrocytes [1-3]. The anemia associated with an infection by a non-lethal strain *P. yoelii* is to a major part due to the massive destruction of erythrocytes, as a high parasitemia is reached during the relatively short duration of the infection. In contrast, a recent study with semi-immune *P. berghei* infected mice, showed that the severity of anemia was independent of the parasitemia, but was linked to the duration of patent infection, which arose from an accelerated turnover of uninfected erythrocytes [28]. However, the impact of the *P. yoelii* infection on the leukocyte and cytokine composition in the mice indicates that hematopoiesis and erythropoiesis may be profoundly affected.

The total peripheral leukocyte count decreased during the first phase of the *P. yoelii* infection, indicating a negative impact on the anti-malarial immune responses, which is in agreement with observations in human malaria infections [29, 30]. The decrease of the Gr-1$^+$ and Mac-1$^+$ cells in the peripheral CD45$^+$ cell population appear to have a major impact on the kinetics profile of the total leukocyte population, indicating that mainly innate immune responses were affected by this malaria infection. In contrast, the proportions of the CD4$^+$ and CD8$^+$ T cells among the peripheral CD45$^+$ cell population were variable, but consistently above the initial basal levels of these cells. However, in the spleen, the proportions of CD4$^+$ and CD8$^+$ T cells, as well as the Gr-1$^+$ and Mac-1$^+$ cells, drastically decreased during the *P. yoelii* infection. Mouse spleen T cells have been shown undergo extensive apoptosis during both *P. chabaudi* and *P. yoelii* infections, thus contributing to the loss of T cells [20, 31].

The anemia associated with the *P. yoelii* infection did not involve Epo, as the Epo levels increased and remained high during the infection. This has also been observed by studies in children with *P. falciparum* malaria [9, 10]. However, others have reported on impaired Epo
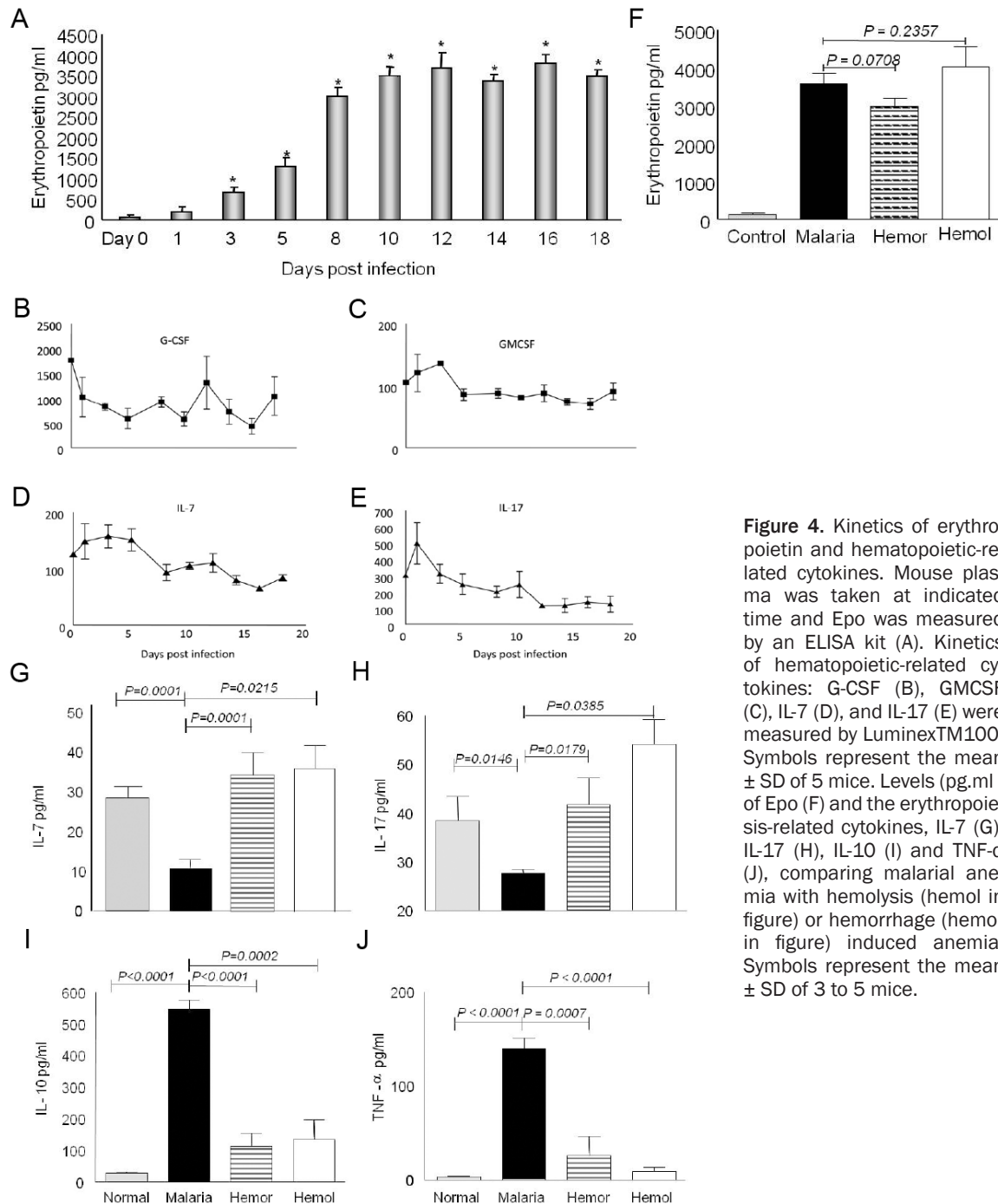
production in mice infected with *P. berghei* [29] or in humans infected with *P. falciparum* [12-14]. The levels of erythropoietic-related cytokines, such as G-CSF, GMCSF, IL-7, and IL-17, were pronouncedly reduced during the *P. yoelii* infection, while those of regulatory cytokines, such as IL-10 and TNF-α, were constantly increased. This indicates a dysregulation of the cytokine network in the infected mice, which may contribute to the dyserythropoiesis due to the imbalanced stimulatory cell signaling observed during malaria infections [32, 33].

However, *P. yoelii* parasites may also affect the erythropoiesis directly, due to parasite derived soluble proteins, which bind to mouse bone marrow nucleated cells (unpublished data). As yet undefined soluble factors derived from murine malaria parasites have been demonstrated to inhibit the function of dendritic cells [34] and erythropoiesis [35]. Furthermore, the malaria parasite *P. vivax* has been shown to suppress erythroid development *in vitro*, indicating the potential of this parasite to cause severe anemia [36].

In contrast to cytokines that play important roles in immune cell activation, cytokines that play regulatory roles in the immune system were significantly increased during the *P. yoelii* infection. Thus, the elevated regulatory cytokines, including IL-2, IL-4, IL-10, and TNF-γ might be due to the abundance of soluble parasite derived exoantigens, which may induce cross-priming, resulting in the activation CD8$^+$ T cells. This may be responsible for the change in the ratio of CD4$^+$ to CD8$^+$ T-cells from 2:1 at the beginning of the infection to 1:1 at later time points of the infection.

Malarial anemia, showed striking differences as compared with hemolysis-induced and hemorrhage-induced anemia. The levels of IL-7 and IL-17, which play important roles in erythroid precursor proliferation [37, 38], were significantly increased in hemolysis and hemorrhage-induced anemia, which was in sharp contrast to what was found among mice with malaria-induced anemia. This strongly suggests that inhibition of erythropoiesis had taken place during malaria infection. Moreover, the levels of IL-10 and TNF-α, two erythroid progenitor cell growth inhibitors, were significantly elevated throughout the course of the P. yoelii infection, while they l remained low in the other two types
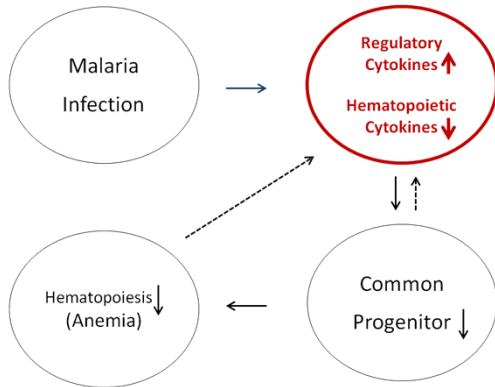
Figure 4. Kinetics of erythropoietin and hematopoietic-related cytokines. Mouse plasma was taken at indicated time and Epo was measured by an ELISA kit (A). Kinetics of hematopoietic-related cytokines: G-CSF (B), GMCSF (C), IL-7 (D), and IL-17 (E) were measured by LuminexTM100. Symbols represent the mean ± SD of 5 mice. Levels (pg.ml) of Epo (F) and the erythropoiesis-related cytokines, IL-7 (G), IL-17 (H), IL-10 (I) and TNF-α (J), comparing malarial anemia with hemolysis (hemol in figure) or hemorrhage (hemor in figure) induced anemia. Symbols represent the mean ± SD of 3 to 5 mice.

of anemia. This suggests that the imbalanced cytokine network induced by the parasites [33], had an impact on erythropoiesis. Higher levels of IL-10 are found in P. falciparum infected children [10, 39]. The data could give an explanation for the impaired erythroid progenitor cellular proliferation in malaria infected mice [40] and humans [9]. Also, our results are consistent with microarray data available on the repression of erythropoiesis [41]. Taken together, these findings are consistent with the notion that cytokines play a direct role in preventing or

Figure 5. A proposed developmental scheme of malaria infection induced anemia. Malaria infection induces increased levels of regulatory cytokines and the decreased levels of hematopoietic cytokines. The altered cytokine levels affect common progenitor cell proliferation, resulting in dyserythropoiesis (anemia). Solid lines indicate direct effects and broken lines show reduced positive effects on target cells.

slowing erythroid cell development in malaria anemia, but not in hemolysis or hematopoiesis induced anemia.

In conclusion, our experimental data suggest that the *P. yoelii* infection in mice has a profound impact on the immune system, both regarding the cellular composition and the cytokine balance. The dysregulated cytokine network, in particular in the context of hematopoiesis-related cytokines, appears to play an important role in malarial anemia induced by *P. yoelii* (**Figure 5**). Studies in progress indicate that the binding of malarial exoantigens to nucleated cells might be partly responsible for the decreases in the leukocyte population during the *P. yoelii* infection, possibly by affecting leukocytes receiving stimulatory ligand(s) and thus affecting downstream cell signaling. Further investigations in this context at molecular level are required better understand how malaria infection affects the regulated cytokine network that disconcerts the erythropoiesis and immune responses.

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