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Cell-Rather Than Antibody-Mediated Immunity Leads to the Development of Profound Thrombocytopenia during Experimental Plasmodium berghei Malaria

Irene Gramaglia, Herman Sahlin, John P. Nolan, John A. Frangos, Marcos Intaglietta, and Henri C. van der Heyde

Experimental malarial thrombocytopenia can reach life-threatening levels and is believed to be due to Abs targeting platelets for destruction by the reticuloendothelial system. However, we report that Abs account for at most 15% of platelet destruction as Plasmodium berghei-infected B cell-deficient mice exhibited profound thrombocytopenia (83%) as did C57BL/6 controls (98%). Further, no significant increase in Abs bound to intact platelets was observed during infection. P. berghei infection can enhance the activity of anti-platelet Abs as indicated by a significantly (p < 0.005) increased thrombocytopenia on day 4 of infection in mice that were administered a low dose anti-CD41 mAb compared with rat IgG1-injected controls. RAG1−/− and CD4− plus CD8− deficient mice were markedly protected from thrombocytopenia (p < 0.005) and malarial pathogenesis. CD8− or TCRγδ deficient mice were not protected from thrombocytopenia and CD4-deficient mice were modestly protected. RAG1−/− mice exhibited significantly (p < 0.05) lower levels of plasma TNF, IFN-γ, and IL-12 during infection. IFNγ−/− and IL-12−/− mice exhibited increased survival but similar thrombocytopenia to C57BL/6 controls. Collectively, these data indicate that thrombocytopenia is necessary but not sufficient for malarial pathogenesis and Abs are not the major contributors to malarial thrombocytopenia. Rather, we propose that both CD4+ and CD8+ T cell populations play key roles in malarial thrombocytopenia; a complex bidirectional interaction between cell-mediated immunity and platelets exists during experimental severe malaria that regulates both responses. The Journal of Immunology, 2005, 175: 7699–7707.
important in the pathogenesis of experimental severe malaria (ESM)\(^3\) (21–23).

To assess the contributions of Ab vs T cell-dependent Ab-independent mechanisms to malarial thrombocytopenia, we determined whether 1) malarial thrombocytopenia occurs in the absence of B cells and Ab, 2) anti-platelet Abs are elicited by \(P.\) \textit{berghei} infection, 3) experimental malaria exacerbates anti-CD41 mAb-mediated thrombocytopenia, and 4) T cells and selected proinflammatory cytokines are required for malarial thrombocytopenia.

Materials and Methods

\textbf{Infection of mice}

\textit{Plasmodium berghei} ANKA strain was stored as a frozen stabulate. B cell-deficient (\(\mu\)-MT\(^{–/–}\)), RAG1\(^{–/–}\), CD8\(^{–/–}\), IFN\(^y\)^{–/–} (GKO), IL-12p40\(^{–/–}\), BALB/c, and C57BL/6 mice (24–27) were obtained at 4–5 wk of age from The Jackson Laboratory or bred at La Jolla Bioengineering Institute (La Jolla, CA) from animals obtained from The Jackson Laboratory. All knockout animals were on the C57BL/6 background. Animals were housed in microisolator cages and provided food and water ad libitum. Experimental mice were injected i.p. with \(1 \times 10^9\) \(P.\) \textit{berghei} parasitized erythrocytes when they were between 6 and 10 wk of age. The number of infected erythrocytes among 200 and 1000 erythrocytes in Giemsa-stained thin blood films determined the \(P.\) \textit{berghei} parasitemia. Mice were scored for clinical signs of ESM on day 6, 8, 10, and 12 based on their gripping and righting ability and overall appearance (e.g., breathing and ruffled fur) on a scale of 0 to 5, with 5 reflecting no sign of ESM and a score of 0 given to mice with no gripping or righting ability. The righting and gripping scores were summed. To kill parasites in the blood, quinine (120 mg/kg) was injected i.p. daily into each mouse. The Institutional Animal Use Committee of La Jolla Bioengineering Institute approved all animal procedures.

\textbf{Injection with anti-platelet Abs}

Anti-CD41 (aIIb or glycoprotein IIb) mAb (clone MwReg30) was purchased from BD Biosciences. Anti-CD41/CD61 mAb (clone 1B5), provided by Dr. B. Coller (Rockefeller University, New York, NY), is an F(ab\(^{-}\))\(_2\) Ab that binds to platelet CD41/CD61 but does not deplete cells purchased from BD Biosciences. For verification of \(y\) T cell depletion, aliquots of splenocytes were fluoresce labeled with anti-CD3 (2C11; biotin-streptavidin-allophycocyanin), anti-CD4 (RM4; FITC), and anti-CD8 (Ly2; PE-Cy5) mAb; all Abs were purchased from BD Biosciences. For verification of \(y\) T cell depletion, aliquots of splenocytes were fluoresce labeled with anti-CD3 (2C11; biotin-streptavidin-allophycocyanin), anti-TCR\(^{\beta}\) (H57; PE-Cy5), and anti-TCR\(^{y}\) (GL3; FITC).

Cells (splenocytes and platelets) were immediately analyzed on a FACS-Calibur (BD Biosciences) and the CellQuest program was used for data acquisition. At least, 5000 cells with size (forward light scatter (FSC)) and granularity (side light scatter (SSC)) of platelets were acquired for subsequent analysis with the Attractors program. For analysis of FSC and SSC of platelets from mice, at least 10,000 CD41\(^{+}\) cells were collected for analysis. At least, 10,000 splenocytes were analyzed for the enumeration of T cell subsets. Spherotech beads, which are easily distinguished from cells based on their unique FSC, SSC, and fluorescence in all three channels, were used to calculate the number of cells per microliter using the formula: [(No. of positive events)/(No. of beads collected)]\(\times\) (sample volume in microliters).

\textbf{Surface detection of anti-platelet Ab}

One microliter of blood was obtained from the tail vein and added to 49 \(\mu\)l of citrate buffer. The samples were washed twice with 2 ml of Tyrode\’s buffer to remove any circulating Ig that might interfere with the anti-mouse Ig-Alexa Fluor 568 binding to Ab on the surface of the platelets. The washed samples were then incubated with anti-CD41-FITC and anti-mouse Ig-Alexa Fluor 568 for 30 min at 4°C. The cells were resuspended in 1 ml of Tyrode’s buffer and immediately analyzed on the FACS-Calibur. CD41\(^{+}\) events (20,000) were collected at all time points for analysis.

\textbf{In vitro platelet activation}

Plasma (20 \(\mu\)l) from selected source mice were added to 1 \(\mu\)l of PRP from an uninfected C57BL/6 mouse. After a 30 min incubation at room temperature, the number of platelets was assessed in each replicate by flow cytometry as described and compared with the number of platelets in 1 \(\mu\)l of PRP with uninfected plasma added. The reduction in platelet count provides an assessment of the activation potential of the selected plasma.

\textbf{Plasma cytokine levels}

Three replicates of 25 \(\mu\)l of plasma were analyzed in a modified ELISA using beads (BD Biosciences) for the levels of selected cytokine proteins. The levels of cytokine proteins in plasma were compared with the values of 2-fold serial dilutions of standards starting at 5000 pg/ml to obtain a plasma cytokine concentration. The lower limit of detection for this assay is 20 pg/ml. The cytokines analyzed in this multiplex assay were IFN-\(\gamma\), TNF-\(\alpha\), IL-6, IL-10, and IL-12p70.

\textbf{Statistical analysis}

ANOVA with the Statview program (SAS Institute) was performed to statistically compare all measurements with a p value cutoff of 0.05. Survival between different groups was compared with nonparametric log-rank test. The mean and SD of the parasitemia, cytokine levels, and number of platelets per microliter of blood are reported.

\textbf{Results}

C57BL/6 mice infected with \(P.\) \textit{berghei} exhibit marked weight loss, rapid breathing, neurological impairment (such as minimal righting and gripping reflex), obtundation, and then death on about day 6 of infection. BALB/c and IFN-\(\gamma\)\(^{–/–}\) mice on a C57BL/6 background are resistant to the development of ESM with few mice exhibiting the clinical symptoms of ESM or becoming moribund before day 12 of infection (31, 32). Resistant mice surviving beyond day 12 develop hyperparasitemia and succumb secondarily to anemia 21 days or more after initiation of the infection.

\(^3\) Abbreviations used in this paper: ESM, experimental severe malaria; PPP, platelet poor plasma; PRP, platelet rich plasma; FSC, forward light scatter; SCC, side light scatter.
Thrombocytopenia during P. berghei malaria occurs in the absence of B cells and Ab

To determine whether polyclonal autoimmune Abs produced during malaria are required for the development of thrombocytopenia during P. berghei infection, groups of five B cell-deficient mice and C57BL/6 controls were injected with 1 × 10⁶ parasitized erythrocytes, and the number of platelets per microliter of blood was assessed by flow cytometry during the course of the infection. The parasitemia in the two groups of animals was similar (Fig. 1A), indicating that differences in parasite replication did not affect the levels of thrombocytopenia. There was no difference in survival between the two groups of mice with all mice becoming moribund on day 7 of P. berghei infection (Fig. 1B). The number of platelets per microliter of blood declined markedly (6-fold; \( p = 0.0002 \)) during the course of P. berghei malaria in B cell-deficient mice declining from 870,974 ± 234,517 platelets/µl on day 0 to 150,108 ± 106,821 platelets/µl on day 6 of infection (Fig. 1C). The number of platelets per microliter of blood declined markedly in C57BL/6 control mice during the course of P. berghei malaria from 1,086,126 ± 384,928 platelets per microliter on day 0 to 19,118 ± 3722 platelets/µl on day 6 of infection (Fig. 1C). The extent of thrombocytopenia on day 6 of P. berghei infection was greater in C57BL/6 mice compared with B cell-deficient mice (\( p = 0.045 \)). Indeed, depletion by experimental malaria of circulating platelets was 98% in C57BL/6 mice and 83% in B cell-deficient mice. This experiment was repeated with similar results: 94% depletion of platelets in C57BL/6 controls and 89% in the B cell-deficient mice infected with P. berghei. Thus, the contribution of anti-platelet Ab to ESM thrombocytopenia is between 5 and 15%.

To determine whether there were differences in platelet size in mice with Ab compared with intact controls, we assessed the change in the FSC and SSC of at least 10,000 CD41⁺ cells (platelets) in the five B cell-deficient mice and C57BL/6 controls. Flow cytometric analysis of CD41⁺ cells with a selected FSC and SSC (i.e., platelets) in mice infected with P. berghei showed a significant (\( p < 0.05 \)) increase in the average platelet FSC (crude measure of size) and SSC (crude measure of granularity) in infected B cell-deficient mice (FSC, 25.5 ± 2.8; SSC, 100.8 ± 10.2) compared with uninfected B cell-deficient controls (FSC, 11.7 ± 0.6; SSC, 67.0 ± 2.0). The increase in platelet size was similar to that observed in C57BL/6 mice after infection (FSC, 30.5 ± 1.5; SSC, 111.5 ± 6.6) vs (FSC, 12.8 ± 0.7; SSC, 68 ± 17) in uninfected C57BL/6 mice. In vitro platelet activation assessed by flow cytometry after stimulation with 20 nM thrombin showed a marked decline in platelet number and the formation of platelets with larger FSC than platelets from unstimulated controls. The increase in the physical properties of the platelets during ESM (i.e., FSC and SSC) likely represents platelet activation; platelets from B cell-deficient mice and C57BL/6 controls were activated to a similar extent.

Because platelet particles are proposed to play a major role in malarial pathogenesis (33), we determined whether Ab increases the number of circulating particles during the course of P. berghei malaria. Platelet particles exhibit lower FSC than platelets from uninfected mice while still retaining platelet-specific proteins, such as CD41 (αIIb). The decline in the number of platelet particles detected in B cell-deficient mice (\( n = 5 \)) and C57BL/6 (\( n = 5 \)) controls paralleled each other during the course of P. berghei malaria (Fig. 1D), but there was a modestly greater number of particles in B cell-deficient mice compared with C57BL/6 controls on day 6 of infection. This finding indicates that anti-platelet Ab is not required for platelet particle formation.

Anti-platelet Ab is detected on the surface of platelet particles but not platelets during the course of P. berghei malaria

To assess whether Ig elicited by infection with P. berghei binds to platelets thereby targeting them for destruction, we fluorescence labeled CD41⁺ cells/particles (events) with anti-mouse Ig Ab in C57BL/6 mice (\( n = 5 \)) during the course of P. berghei infection and in uninfected mice (\( n = 5 \)). On day 3 of P. berghei infection, the percentage of CD41⁺ events labeled with anti-Ig Ab was significantly increased (\( p = 0.02 \)) in infected mice when compared with uninfected mice measured at the same time (Fig. 2A). Although the percentage of CD41⁺ events that are surface Ig⁺ increases from 0.2 ± 0.1% on day 0 to 20.9 ± 6.5% on day 5 of infection (Fig. 2A), the absolute number of CD41⁺ events that were labeled with anti-Ig was not significantly different (\( p > 0.5 \)) from controls at any time point due to the thrombocytopenia observed in infected mice (Fig. 2B). Subdivision of the CD41 events into cells and particles based on their FSC indicated that the CD41⁺, Ig⁺ cells (Fig. 2, D and F) exhibited lower FSC than intact platelets from uninfected mice and hence were particles (Fig. 2, C and E). These platelet particles labeled with anti-platelet Ab did not exhibit any significant increase in FSC during the course of infection, whereas CD41⁺, Ig⁺ platelets became significantly (\( p < 0.001 \)) larger. Few if any particles were detected with lower levels of CD41 on their surface or with lower SSC than conventional platelets. This experiment was repeated with similar results. These findings indicate that anti-platelet Abs are present on the surface of platelet particles but not intact platelets.

Administration of low doses of anti-platelet mAb during P. berghei malaria exacerbates thrombocytopenia

Musaji et al. (34) report that viral infection results in increased thrombocytopenia and increased clearance of platelets through phagocytosis by IFN-γ-activated macrophages if low levels (3 µg) of anti-platelet mAbs are injected upon infection. Injection of this low amount of Ab alone had no effect on platelet numbers in uninfected controls. To assess whether anti-platelet Abs possibly

FIGURE 1. B cell-deficient mice exhibit marked thrombocytopenia following P. berghei infection. The average P. berghei parasitemia ± SD (A) and cumulative survival (B) in groups of five B cell-deficient mice (■) and C57BL/6 controls (○) injected i.p. on day 0 with 1 × 10⁶ parasitized erythrocytes. The number of platelets (CD41⁺ with selected FSC and SSC) (C) and platelet microparticles (CD41⁺ with lower FSC and SSC) (D) in the two groups of mice were determined during the course of P. berghei infection by flow cytometry.
elicted by a previous viral infection exacerbate malarial thrombocytopenia potentially contributing to ESM pathogenesis, we injected a low dose of anti-CD41 mAb (3 μg) i.p. on day 1 of infection and assessed thrombocytopenia during the course of malaria. We selected depleting anti-CD41 mAb (MwReg30) because the CD41/CD61 (αIIb/β3) heterodimer is expressed on the surface of platelets and is often a target for autoantibodies. Upon platelet activation, CD41/CD61 assumes an active conformation, then binds a variety of molecules, including fibrinogen, and is important in clot formation.

Mice injected on day 1 with the anti-CD41 mAb capable of depleting platelets exhibited a significant (p = 0.01) decrease in platelet numbers on day 4 of infection compared with mice injected with a rat IgG1 isotype control. The dose of anti-CD41 mAb was not sufficient to initially deplete platelets because platelet numbers per microliter of blood on day 1 pre-Ig and 2 h post-Ig injection were not significantly different (Fig. 3A). Injection of higher doses of MwReg30 (100 μg) rapidly depletes platelets within 30 min after i.p. injection (35).

Anti-CD41/CD61 mAb clone 1B5 is a F(ab′)2 that binds to the surface of platelets but does not result in their depletion (28). Unlike MwReg30, this nondepleting anti-CD41/CD61 (1B5) mAb did not elicit increased thrombocytopenia compared with the isotype control (Fig. 3B). The experiment with depleting anti-CD41 mAb (MwReg30) was repeated twice with similar results; the 1B5 experiment was repeated once with similar results. Collectively, these results indicate that ESM will accelerate thrombocytopenia if anti-platelet Abs are present through an Fc receptor-dependent mechanism.

Plasma from infected mice activates platelets independent of Ab

Because thrombocytopenia is likely to be mediated through a systemic factor rather than cell-cell contact and to rule out anti-platelet Ab as that factor, we assessed whether plasma from infected B cell-deficient mice and C57BL/6 controls activates platelets in vitro. PPP (20 μl) from either infected or uninfected mice (n = 5) were incubated with PRP from a healthy mouse. We observed a significant increase in the mean FSC (from 9.4 ± 0.4 to 12 ± 2; p < 0.001) and SSC (from 59 ± 3 to 65 ± 3; p = 0.001) of platelets incubated with infected plasma suggesting that a factor produced during infection with P. berghei could mediate platelet activation. A comparable increase in platelet size was observed when platelets were incubated with plasma from B cell-deficient mice (FSC, 12 ± 2; SSC, 71 ± 4; p < 0.001). Further, plasma from P. berghei-infected C57BL/6 mice on day 5 of infection induced a 20% decrease in platelet numbers following incubation with platelets from a healthy mouse compared with plasma from infected with platelets from an F(ab′)2 of 1B5 clone (●) of anti-CD41/CD61 mAb.
uninfected mice. This experiment was repeated with similar results; however, the magnitude of platelet depletion by the infected plasma varied from experiment to experiment. This result indicates that platelet activation by factors in the plasma of mice with ESM does not require anti-platelet Abs.

**Thrombocytopenia reverses after quinine treatment to kill the parasite**

To determine whether thrombocytopenia reverses after killing the parasite, we injected i.p. quinine (120 mg/kg) daily into groups of uninfected mice (n = 5), or infected mice (n = 9) beginning on day 5 of ESM. A group of infected mice (n = 7) was injected i.p. with saline vehicle. The parasitemia on day 5 of ESM was similar in both groups of mice before the initiation of quinine therapy (Fig. 4A). On day 7 onward, the parasitemia was lower in quinine-treated group compared with controls injected with saline (Fig. 4A). On day 5 of ESM (before the initiation of treatment), both groups of mice exhibited similar degrees of profound thrombocytopenia (Fig. 4B). The thrombocytopenia remained profound in the saline-injected group and the animals succumbed on day 6 (n = 6) and day 7 (n = 1) of ESM. On day 6 of ESM, the levels of thrombocytopenia were similar in quinine-treated mice and saline-treated controls, but reversed rapidly on day 7 only in the quinine-treated animals and approached uninfected levels by day 8. Only one of the quinine-treated animals succumbed on day 6 of ESM. The number of platelets in blood of uninfected animals treated with quinine remained constant, indicating that quinine at this dose did not have direct effects on thrombocytopenia. Combination therapy of quinine plus trimethoprim sulfamethoxazole to increase survival compared with C57BL/6 controls with five of five RAG1−/− mice surviving beyond day 12 of *P. berghei* infection (Fig. 5B). RAG1−/− mice exhibited modest thrombocytopenia; the number of platelets per microliter of blood declined 2-fold (p = 0.03) from 704,179 ± 271,383 on day 0 to 368,041 ± 83,342 on day 6 of infection (Fig. 5C). The numbers of platelets per microliter of blood were significantly (p < 0.0001) greater in RAG1−/− mice on day 6 of *P. berghei* malaria than in C57BL/6 controls (20,189 ± 19,075). Thus, depletion by experimental malaria of circulating platelets was 90.3 ± 10.7% in C57BL/6 mice but only 43.7 ± 24.1% in T cell- and B cell-deficient mice.

The number of platelet particles per microliter of blood declined significantly during the course of *P. berghei* malaria in both RAG1−/− mice and C57BL/6 controls (Fig. 5D). Flow cytometric analysis of CD41+ cells revealed that platelets from RAG1−/− mice exhibited significantly lower FSC (26 ± 3) and SSC (107 ± 6) on day 6 of *P. berghei* infection compared with infected RAG1−/− mice exhibited larger FSC and SSC than platelets from uninfected controls (FSC, 15 ± 0.3; SSC, 83 ± 4), suggesting they were partially activated. These experiments were repeated with similar results.

To define which T cell subset or subsets are required for protection against thrombocytopenia, we assessed the level of thrombocytopenia during the course of ESM in mice lacking selected T cell subsets. Mice treated with anti-CD4 mAb were depleted because few CD3+CD4+ (<0.1%) cells were detected on day 8 of *P. berghei* infection. The average *P. berghei* parasitemia ± SD (A) and cumulative survival (B) in groups (n = 5) of RAG1-deficient mice (■) and C57BL/6 controls (●) injected i.p. on day 0 with 1 × 10^6 parasitized erythrocytes. The number of platelets (CD41+ with selected FSC and SSC) (C) and platelet microparticles (CD41+ with lower FSC and SSC) (D) in the two groups of mice were determined during the course of *P. berghei* infection by flow cytometry.

**FIGURE 4.** *P. berghei*-infected mice treated on day 5 of ESM exhibit recovery of platelet numbers. The average *P. berghei* parasitemia ± SD (A) and platelet numbers ± SD (B) in groups of C57BL/6 mice injected i.p. with 120 mg/kg quinine (●; n = 9) or with saline vehicle (○; n = 7). A third group was uninfected but injected with same dose of quinine and at the same times as the infected animals (▲; n = 5). Time points of injection of quinine dose (↓) are shown.

**FIGURE 5.** RAG1-deficient mice exhibit marked protection from thrombocytopenia following *P. berghei* infection. The average *P. berghei* parasitemia ± SD (A) and cumulative survival (B) in groups (n = 5) of RAG1-deficient mice (■) and C57BL/6 controls (●) injected i.p. on day 0 with 1 × 10^6 parasitized erythrocytes. The number of platelets (CD41+ with selected FSC and SSC) (C) and platelet microparticles (CD41+ with lower FSC and SSC) (D) in the two groups of mice were determined during the course of *P. berghei* infection by flow cytometry.
berghei infection. An anti-CD4 mAb (Rm4.4) recognizing a distinct epitope from the deleting Ab (GK1.5) was used to ensure we assessed cell depletion rather than Ab masking. As expected, CD8\(^{-/ -}\) mice exhibited few if any (<0.1%) CD3\(^+\) CD8\(^-\) cells detectable by flow cytometry. Few (<0.1%) if any CD3\(^+\) TCR\(\gamma\delta\) T cells were detected by flow cytometry in anti-TCR\(\gamma\delta\) mAb-injected mice; in addition, few (<0.1%) CD3\(^+\) TCR\(\alpha\beta\) cells were detected, indicating that depletion of \(\gamma\delta\) T cell population had occurred rather than Ab masking of TCR\(\gamma\delta\). Depletion of \(\gamma\delta\) T cells (data not shown) and CD8\(^-\) T cells did not alter the course of thrombocytopenia during ESM (Table I). Anti-CD4 mAb treatment provided some protection from thrombocytopenia particularly on day 4 of ESM, but the combination of CD4\(^+\) and CD8\(^-\) T cells provided the greatest (\(p = 0.0002\)) level of protection. Collectively, these findings indicate that cell mediated immunity comprising both CD4\(^+\) and CD8\(^-\) T cells function in eliciting malarial thrombocytopenia.

Proinflammatory cytokines elicited by P. berghei infection are markedly altered in RAG1\(^{-/ -}\) mice compared with C57BL/6 controls

To determine whether proinflammatory cytokines in the plasma of infected RAG1\(^{-/ -}\) mice were reduced compared with C57BL/6 controls, we assessed the levels of selected cytokines on day 6 of \(P.\) berghei infection in these two groups (\(n = 5\)) of mice. IFN-\(\gamma\), TNF, and IL-10 were assessed because IFN-\(\gamma\)- or TNF-R2-deficient mice and IL-10-treated mice are protected from the development of ESM (32, 36, 37). Proinflammatory or type 1 cytokines elicit ESM, whereas type 2 cytokines have no effect or are protective (32, 38). Infected RAG1-deficient mice exhibited significantly reduced levels of proinflammatory IFN-\(\gamma\), TNF, and IL-12 compared with C57BL/6 controls, but the levels of IL-6 and anti-inflammatory IL-10 were not statistically different (Table II). Both RAG1\(^{-/ -}\) and C57BL/6 control mice exhibited similar parasitemia on days 4 and 6 of infection, indicating that differences in parasite replication did not account for the results. The RAG1\(^{-/ -}\) mice exhibited few clinical signs of severe malaria (10 of 10 \(\pm\) 0), whereas the C57BL/6 mice were clearly ill (4.6 of 10 \(\pm\) 0.5). This experiment was repeated with similar results. If thrombocytopenia was necessary and sufficient to mediate the genesis of ESM, then the findings that the levels of proinflammatory cytokines IFN-\(\gamma\), TNF, and IL-12 were significantly lower in RAG1\(^{-/ -}\) mice and that mice lacking IFN-\(\gamma\) and TNF-R2 were protected from ESM suggest that these proinflammatory cytokines may be required to elicit thrombocytopenia during ESM.

Thrombocytopenia during \(P.\) berghei malaria is not significantly ameliorated in the absence of proinflammatory cytokines IFN-\(\gamma\) and IL-12

Because BALB/c and IFN\(\gamma^{-/ -}\) mice are ESM-resistant strains of mice (31, 32) and RAG1\(^{-/ -}\) exhibited decreased levels of IFN-\(\gamma\), we assessed whether the level of thrombocytopenia was similar in these two resistant strains of mice compared with susceptible C57BL/6 controls. Groups of BALB/c and C57BL/6 mice (\(n = 4\)) were infected i.p. with \(1 \times 10^6\) erythrocytes parasitized with \(P.\) berghei and the number of platelets per microliter of blood was assessed during the course of the infection. The parasitemiae were similar in ESM-resistant BALB/c and IFN\(\gamma^{-/ -}\) mice and ESM-susceptible C57BL/6 controls on days 4 and 6 of infection (Table III), indicating that differences in parasite replication did not affect the thrombocytopenia results. However, the number of platelets per microliter of blood on day 6 of infection was significantly greater in BALB/c mice (157,360 \(\pm\) 18,042) when compared with C57BL/6 controls (32,600 \(\pm\) 8074; \(p = 0.006\)). As we reported previously (32), IFN\(\gamma^{-/ -}\) mice on a C57BL/6 background were significantly (\(p = 0.005\)) protected from ESM. The platelet numbers on day 4 of infection were significantly (\(p = 0.002\)) lower in ESM-susceptible C57BL/6 mice than in ESM-resistant IFN\(\gamma^{-/ -}\), but the numbers on day 6 of infection were similar (\(p = 0.6\)) in both groups of mice. The experiments with ESM-resistant BALB/c and IFN\(\gamma^{-/ -}\) mice were repeated with similar results.

Because the RAG1-deficient mice exhibited lower levels of IL-12, and IL-12 is a proinflammatory cytokine that is often required to elicit production of IFN-\(\gamma\), it is possible that the absence of this cytokine has a greater effect than the absence of IFN-\(\gamma\) alone. To determine whether 1) IL-12-deficient mice are protected from \(P.\) berghei infection or 2) IL-12 is critical for development of malarial thrombocytopenia, we infected groups (\(n = 5\)) of IL-12\(^{-/ -}\) mice and ESM-susceptible C57BL/6 controls with \(P.\) berghei. IL-12\(^{-/ -}\) mice were significantly protected from ESM (\(p = 0.003\)) when compared with C57BL/6 controls with one in five IL-12\(^{-/ -}\) mice surviving beyond day 12 of infection and no C57BL/6 mice (\(n = 5\)) surviving. The parasitemia in both groups of mice was similar on days 4 and 6 of infection, indicating that protection cannot be attributed to differences in parasite replication. Despite the increased survival of the IL-12\(^{-/ -}\) mice, the extent of thrombocytopenia on days 4 and 6 of infection was similar in both groups of mice (Table III). The experiment assessing survival and thrombocytopenia in IL-12\(^{-/ -}\) mice was repeated and in this experiment we observed a modest and similar level of thrombocytopenia in both IL-12\(^{-/ -}\) mice and C57BL/6 controls on day 4 of ESM and profound thrombocytopenia in both groups of mice on day 6. The survival experiment alone was repeated two more times with similar results. Collectively, these findings indicate that 1) the proinflammatory cytokines IL-12 and IFN-\(\gamma\) contribute to ESM pathogenesis but are not required for the development of malarial thrombocytopenia and 2) thrombocytopenia alone is not sufficient to mediate disease.

Discussion

Assessment of thrombocytopenia in ESM-susceptible C57BL/6 mice infected with \(P.\) berghei clearly indicates that there is a

Table I. Parasitemia and percentage decrease in platelet counts\(^a\)

<table>
<thead>
<tr>
<th>Group</th>
<th>% Parasitemia</th>
<th>Platelet No. per Microliter of Blood</th>
<th>% Thrombocytopenia</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Day 4 Day 6</td>
<td>Day 0</td>
<td>Day 4 Day 6</td>
</tr>
<tr>
<td>Anti-CD4 mAb</td>
<td>8.7 (\pm) 2.1</td>
<td>20.3 (\pm) 6.2</td>
<td>1,830,341 (\pm) 334,083</td>
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<tr>
<td>CD8(^{-/ -})</td>
<td>11.2 (\pm) 2.2</td>
<td>21.6 (\pm) 2.8</td>
<td>1,673,040 (\pm) 395,512</td>
</tr>
<tr>
<td>Anti-CD4→CD8(^{-/ -})</td>
<td>6.9 (\pm) 1.7</td>
<td>17.6 (\pm) 2.7</td>
<td>1,574,813 (\pm) 321,182</td>
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<tr>
<td>C57BL/6</td>
<td>7.9 (\pm) 3.8</td>
<td>17.0 (\pm) 5.3</td>
<td>1,400,040 (\pm) 207,774</td>
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</tbody>
</table>

\(^a\) Parasitemia and percentage decrease in platelet counts on days 4 and 6 of \(P.\) berghei infection in groups (\(n = 5\)) of mice lacking selected T cell subsets (column 1) and C57BL/6 controls. Values are mean \(\pm\) SD.

\(^b\) Platelet depletion is significantly less (\(p < 0.005\)) than C57BL/6 mice.

\(^c\) A value of \(p = 0.053\) for anti-CD4 mAb-injected groups vs C57BL/6 controls on day 6 of ESM.
marked removal of platelets from the blood during ESM and that the thrombocytopenia reaches life-threatening levels, with blood platelet numbers declining between 100- and 1000-fold. Thrombocytopenia of this magnitude results in impaired clot formation and hence a predisposition to hemorrhage. Indeed, mice with ESM exhibit significantly (p < 0.05) greater clotting times (data not shown) and hemorrhaging into vital organs, such as the brain and lung. The mechanisms mediating thrombocytopenia during ESM are poorly defined but may be due to 1) decreased production, 2) increased sequestration, or 3) increased destruction of platelets. There is no apparent defect in platelet production accounting for P. berghei malarial thrombocytopenia because the number of megakaryocytes in bone marrow actually increases at the same time as consumption of radiolabeled platelets is markedly increased (16).

Increased platelet sequestration is a potential mechanism for the removal of platelets from the blood during ESM because firm adhesion of platelets to the microvasculature via ICAM-1 and P-selectin has been reported (23, 39, 40). However, it is unlikely that platelet adhesion to an inflamed microvasculature is the major contributor to malarial thrombocytopenia because 1) the level of thrombocytopenia is not altered in infected P-selectin-deficient mice that lack platelet adhesion (23, 41, 42) and 2) platelets adhere to the microvasculature primarily on day 6 of infection whereas marked thrombocytopenia is already observed on day 4 or day 5 of infection. In addition, thrombocytopenia is not ameliorated in IFN-γ−/− mice, and increases in ICAM-1 expression during ESM requires IFN-γ (43). The increase in platelet number after initiation of drug therapy on day 5 of ESM is consistent with sequestered platelets being released from the endothelium. However, the increase in number may also reflect the abatement of platelet destruction and increased platelet production levels elicited by ESM (16). Thus, destruction of platelets appears to be the primary mechanism mediating thrombocytopenia. Platelet destruction during ESM could occur via anti-platelet Abs and phagocytosis by macrophages, apoptosis, and platelet Ab independent phagocytosis in spleen.

As proposed for viral infections, and immune thrombocytopenia purpura (2, 14, 34), the mechanism for platelet depletion during malaria is believed to be the production of anti-platelet Abs that bind to platelets and then target them for destruction by activated macrophages (16). However, our results indicate that thrombocytopenia in B cell-deficient mice infected with P. berghei is marked; the difference in thrombocytopenia between B cell-deficient mice and B cell-intact controls on day 6 of P. berghei infection was 98 vs 83%, respectively, suggesting that Ab-mediated platelet depletion accounts for at the most 5–15% of the malarial thrombocytopenia.

Platelets are reportedly coated with Ab during malaria and that the number of platelets with platelet-bound Ab correlated inversely with the platelet count (16). In contrast, our flow cytometry results indicate that there is no significant increase in the number of Ab-coated CD41+ events during the course of P. berghei malaria. Further, these CD41+ events represent particles rather than intact platelets as they exhibit smaller FSC (size) than platelets. Few CD41+ events are detected with SSC much lower than platelets as observed by Piguet et al. (44–46); this may be due to the processing of platelets to PRP by these researchers, whereas we analyze blood directly.

Our in vitro results with PPP from infected mice indicates that this PPP activates platelets from uninfected mice to increase their FSC and SSC; the number of platelets declines (~20%) when PPP from infected mice is added compared with PPP from uninfected mice. There was no difference in the platelet response to PPP from B cell-deficient mice and infected C57BL/6 controls, indicating that Ab is not responsible for this in vitro activation of platelets. We are assuming that activation is a step in the process leading to platelet destruction. The marked thrombocytopenia in B cell-deficient mice infected with P. berghei, the lack of detection of Ab bound to platelets during ESM, and the similar effects of PPP from B cell-deficient mice and from C57BL/6 controls on in vitro platelet activation collectively indicate that anti-platelet Ab plays a minimal role in malarial thrombocytopenia. Although the magnitude of thrombocytopenia is markedly different, these results agree with those of Musaji et al. (34), who observe that acute viral thrombocytopenia occurs in the absence of anti-platelet Ab.

Chronic viral or other infections can ultimately elicit anti-platelet Abs whose platelet deactivating activity can then be enhanced by T cell-independent, macrophage-dependent phagocytosis resulting from subsequent viral infection; repeated viral infections has therefore been proposed to mediate childhood immune thrombocytopenia purpura (34). Similarly, we observe that P. berghei infection will exacerbate the effects of anti-CD41 mAb Abs if present. The increased platelet depletion by anti-CD41 mAb during ESM is mediated by the Fc receptor because an F(ab′)2 of an anti-CD41 mAb does not elicit increased thrombocytopenia. Experimental malaria is a much stronger stimulus for thrombocytopenia than viral infection because ESM depletes virtually all platelets (98%) from the circulation within 6 days of infection, whereas lactate dehydrogenase-elevating virus and mouse hepatitis virus result in ~50% loss of platelets (34). By day 6 of ESM, there is no difference between thrombocytopenia in mice treated with depleting anti-platelet clone or with rat IgG, nor did the mice die more rapidly.

One slight internal discrepancy with our data might be: why is malarial thrombocytopenia in B cell-deficient mice ameliorated compared with C57BL/6 controls when no anti-platelet Ab is detected on platelets circulating during P. berghei malaria? One possible explanation is that the cell-mediated immune response during malaria is altered in the absence of B cells (47, 48). Indeed, the marked amelioration of thrombocytopenia in RAG1−/− mice indicates that T cell-mediated immune responses are important in malarial thrombocytopenia. The fact that RAG1−/− mice lack both T and B cells and are resistant to the development of ESM thrombocytopenia suggests that both cell types may collaborate in mediating the destruction of platelets.

There are several possible mechanisms whereby the cell-mediated immune response might be responsible for the destruction of malarial thrombocytopenia.

### Table II. Levels of selected cytokines in plasma

<table>
<thead>
<tr>
<th>Group</th>
<th>IFN-γ</th>
<th>TNF</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>66 ± 42</td>
<td>63 ± 35</td>
<td>16 ± 7</td>
<td>79 ± 86</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>RAG1−/−</td>
<td>0 ± 0</td>
<td>18 ± 3</td>
<td>10 ± 3</td>
<td>0 ± 0</td>
<td>13 ± 8</td>
</tr>
</tbody>
</table>

*Levels of selected cytokines in the plasma of groups (n = 5) of RAG1-deficient mice and C57BL/6 controls on day 6 of P. berghei infection. Average value of cytokine (pg/ml) ± SD is shown. Statistical significance from comparison of infected RAG1−/− mice and C57BL/6 controls was: IFN-γ: p = 0.007; TNF: p = 0.02; IL-6: p = 0.2; IL-10: p = 0.06; IL-12: p = 0.05. In uninfected mice, these cytokines are not detected.
platelets during *P. berghei* malaria. The first is that factors produced through the cell-mediated immune response induce platelets to undergo apoptosis and form platelet microparticles, as proposed by Piguet et al. (46). They proposed that signaling through CD40L on activated platelets initiates the caspase cascade, resulting in apoptosis of the platelet and the formation of platelet microparticles (46). Indeed, this group reports that caspases are activated in platelets during ESM, and inhibition of the caspases protects against microparticle formation (46, 49). Signaling by TNF does not initiate apoptosis during *P. berghei* infection because malarial thrombocytopenia and formation of microparticles is similar in TNFR1- and TNFR2-deficient mice (45, 50).

In contrast to the Piguet and colleagues (46, 49) results, we detect no increases in the number of particles in RAG1−/− mice by flow cytometry in whole blood during the course of *P. berghei* malaria. Protection from thrombocytopenia in CD40−/− and CD40L−/− (CD154) mice (44) is mediated by similar mechanism to RAG1-deficient mice, namely, the absence of a cell-mediated immune response rather than the induction of apoptosis in platelets. Our depletion studies indicate that removal of CD8+ T cells or γδ T cells alone provides minimal protection against thrombocytopenia, and depletion of CD4+ T cells provides some protection (~15%). CD4+ plus CD8+ T cell subset deficiency does markedly protect against thrombocytopenia (~30%). It is well recognized that the immune response compensates for the lack of a T cell subset during malaria (48); thus, in the single T cell subset depletion studies, CD8+ T cells may compensate for the lack of CD4+ T cells and vice versa.

We have reported that depletion of platelets early during *P. berghei* infection leads to an altered immune response with significantly decreased levels of proinflammatory cytokines, such as IFN-γ and IL-2, as well as increased levels of IL-10, indicating a role for platelets in the regulation of pathogenic cytokines and cell-mediated immune responses (35). The current results with RAG1−/− mice exhibiting ameliorated malarial thrombocytopenia indicate that the interaction is bidirectional between platelets and the cell-mediated immune response. If thrombocytopenia is sufficient to mediate the genesis of ESM, the decreased levels of proinflammatory TNF, IFN-γ, and IL-12 in the RAG1−/− mice together with the protection against ESM in TNFR2−/−, IFNγ−/−, and IL-12−/− mice would suggest that IL-12 and IFN-γ might be important cytokines in the development of malarial thrombocytopenia. Because mice protected from the development of ESM (IL-12−/− and IFNγ−/− mice) still exhibit profound thrombocytopenia on day 6 of ESM when the C57BL/6 controls are moribund, these findings suggest that either thrombocytopenia is a symptom of ESM but is not essential to its pathogenesis or severe thrombocytopenia is necessary but not sufficient to mediate ESM. However, because profound thrombocytopenia occurs in all moribund mice, and moribund mice exhibit increased clotting times, vascular leak and hemorrhaging into the brain and lung, we propose that thrombocytopenia is necessary for the development of ESM. Hemorrhage often occurs due to thrombocytopenia, linking thrombocytopenia with an important mechanism of pathogenesis. In addition, platelet depletion protected mice and altered the pathogenic immune response, indicating that platelet activation and thrombocytopenia are unlikely to be merely symptoms of ESM.

In conjunction with thrombocytopenia, other factors such as disruption of the vascular wall integrity by activated immune cells and up-regulation of endothelial cell adhesion molecules by proinflammatory cytokines and chemokines may be necessary second signals to cause hemorrhage. Thus, analogous to the two-signal hypothesis for activation of T cells (51, 52), activation of two or more pathogenic mechanisms is required for lethal malaria and thrombocytopenia is likely one of the critical pathogenic mechanisms. This study indicates that the mechanism underlying malaria thrombocytopenia does not involve Abs but rather requires a cell-mediated immune response. Our results indicate that Ab-mediated mechanism of thrombocytopenia in falciparum patients suggested by the earlier studies by Kelton et al. (14) should be revisited to ensure that intact platelets and not platelet particles are indeed labeled with Ab in falciparum patients and that these Abs function to remove platelets from blood. The precise mechanism whereby cell-mediated immunity mediates thrombocytopenia is independent of IL-12 and IFN-γ. Further, these results strongly support a bidirectional link between the coagulation system and cell-mediated immunity whereby platelets alter the proinflammatory cytokine response but their activation can in turn be affected by it.

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### Disclosures

The authors have no financial conflict of interest.

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