Clinical malaria is associated with high levels of circulating cytokines (Kwiatkowski et al., 1990). Although balanced production of IFN-γ, TNF, IL-1, and IL-6 is capable of controlling parasite growth (Stevenson and Riley, 2004), their excessive production exacerbates disease severity (Grau and de Kossodo, 1994; Kwiatkowski, 1995) and contributes to the development of cerebral malaria (Grau et al., 1989; Kwiatkowski et al., 1990).

The inflammatory effector mechanisms remain ill-defined and complex. They involve innate immune responses through members of the mammalian Toll-like receptor (TLR) family that recognize parasite molecules (Coban et al., 2007), or hemozoin, which is a digestion product of hemoglobin (Coban et al., 2005). Antibody-mediated inflammatory responses may also be critical. Although the role of IgG subclasses has been characterized in some detail (Shi et al., 1996; Metzger et al., 2003), the functional implication of IgE antibodies in malaria pathogenesis needs to be clarified. Past studies have shown that patients with severe malaria show higher IgE levels against *P. falciparum* than those with uncomplicated malaria (Perlmann et al., 1994, 2000). Other studies reported a reduced risk of severe malaria (Bereczky et al., 2004) and protection from cerebral malaria of IgE antibodies (Nacher et al., 2000). IgE bind to high-affinity
IgE receptors (FceRI) on mast cells (MCs) and basophils, as well as to low-affinity IgE receptors (FceRII/CD23) on B cells, granulocytes, and many other cell types. Interaction of CD23 with IgE immune complexes activates iNOS gene transcription leading to nitric oxide production, which is thought to cause cerebral malaria (Maneerat et al., 2000). Activation of basophils (Nyakeriga et al., 2003) and MCs (Furuta et al., 2006) that express FceRI, but not CD23, may also exacerbate malaria pathogenesis through histamine secretion. High plasma and tissue histamine levels increased disease severity in humans infected with *Plasmodium falciparum* and in several animal models of *Plasmodium* infection (Srichaikul et al., 1976; Bhattacharya et al., 1988). Histidine decarboxylase KO mice exhibited a marked resistance to severe malaria induced by *Plasmodium berghei* ANKA, an effect that involved binding to H1 and H2 receptors (Beghdadi et al., 2008).

To examine the role of IgE/FceRI-mediated inflammatory processes in malaria pathogenesis, we studied mice that were genetically deficient for FceRIα (Dombrowicz et al., 1993b) and IgE (Oettgen et al., 1994) after inoculation of *PlbANKA*. The present investigations document that both mouse populations are resistant to ECM and control parasite development. Neither MCs nor basophils home to the brain, but a subset of FceRIα neutrophils that were otherwise shown to emerge in the BM of *PlbANKA*-hosting mice do home to the brain, disrupting its steady-state features.

**RESULTS**

**FceRI and IgE deficiency confers protection against ECM**

To examine whether IgE synthesis and FceRI-expressing cells are involved in ECM, we infected FceRIα-KO mice that are resistant to IgE-mediated anaphylaxis (Dombrowicz et al., 1993b) with *PlbANKA* via mosquito bites. The majority (80%) of *PlbANKA*-infected WT mice died from ECM within 7–12 d (median survival, day 8; Fig. 1 b), whereas 90% of FceRIα-KO mice survived at day 15 and did not develop ECM. Mice that resisted ECM later died of parasitemia-induced anemia without neurological signs (median survival: day 27; Fig. 1 b). Parasitemia increased over time similarly in C57BL/6 WT and FceRIα-KO mice until day 15 after infection (Fig. 1 a). After day 15, parasitemia in WT mice that had survived ECM continued to rise, whereas in FceRIα-KO mice it reached a plateau, indicating that, in contrast to WT mice, they were able to control parasite growth at later stages of infection. Quantitative RT-PCR analysis in brains of WT mice also showed an increase in FceRIα mRNA over time until day 6, when it became significant, paralleling the appearance of the first signs of ECM (Fig. 1 c).

MC numbers in peritoneum and BM showed no difference between FceRIα-KO and WT mice, ruling out any defect in MC development and function in FceRIα-KO mice (Fig. S1 a; Dombrowicz et al., 1993b). Because histamine was previously implicated in malaria pathogenesis (Beghdadi et al., 2008), we measured histamine contents in BM cells and peritoneal MCs and found no differences between the two genotypes (Fig. S1 b). Additionally, plasma histamine levels were similar in FceRIα-KO mice and WT mice (Fig. S1 c).

ECM resistance in the absence of FceRIα likely reflects a role of IgE in malaria pathogenesis, as this receptor serves as a signaling relay after ligand binding (Bryce et al., 2004) and after antigen-specific cross-linking of IgE-sensitized cells (Blank and Rivera, 2004). In concordance, IgE-KO mice were less susceptible to ECM after infection with *PlbANKA*. They survived >3 wk with 60% not developing ECM.

**Figure 1. FceRI expression and IgE are critical for the pathogenesis of ECM.** FceRIα-KO mice and C57BL/6N (WT) mice were infected with *PlbANKA* through mosquito bites (8–10 mosquitoes per mouse). (a) Parasitemia (Mann-Whitney test; *, P < 0.036; **, P < 0.008). (b) Kaplan-Meier survival plots (log-rank test, n = 52, P = 0.0004). Data are from five independent experiments. (c) C57BL/6N (WT) mice were sacrificed at indicated time points after receiving blood-stage parasites of *PlbANKA* inoculated intraperitoneally (10⁶ infected RBCs), and brain tissues were prepared for mRNA extraction. Transcription of the a chain of FceRI was evaluated by real-time RT-PCR. mRNA expression was normalized to hypoxanthine phosphoribosyltransferase expression. ***, ANOVA, P = 0.0055; followed by an posteriori Fisher’s PLSD test, P = 0.0008. Results were from two independent experiments. (d–i) EG-KO (n = 25) and IgE-KO mice (n = 39); (j) FceRIα-KO (n = 2). (d) Kaplan-Meier survival plots were recorded (log-rank test, P = 0.035). Survival data are from four independent experiments. (e) Total IgE as measured in the plasma from noninfected (d0) and *PlbANKA*-infected mice taken at indicated time points. Background value determinations of IgE were performed in sera from IgE-KO mice. * and ** indicate that differences are significant (Mann-Whitney test, n = 5–7, 0.01 < P < 0.05 and 0.001 < P < 0.01, respectively) relative to the basal level. Results are from three independent experiments. (f–i) Serum levels of IFN-γ (f) and IL-6 (h) were quantified by ELISA at day 6 after infection. Transcription of IFN-γ (g) and IL-6 (i) in the brain (n > 6/group) 6 d after infection as evaluated by real-time RT-PCR. Gene mRNA expression is normalized to the endogenous control gene GAPDH, and the relative expression levels were calculated using the uninfected animals as a calibrator. Data are presented as the means ± SD from two independent experiments. **, P < 0.02.
whereas 75% of WT controls died of ECM (Fig. 1 d). Together, these data suggest that IgE-mediated signaling via FcεRI is essential for the occurrence of ECM. Considering the role of IgE, we found that total plasma IgE levels became signifi-
cantly elevated only at day 12 after infection, as measured in surviving WT mice (Fig. 1 e).

In agreement with reports that ECM pathology in PbANKA-infected mice is associated with increased pro-inflammatory IFN-γ (Hunt and Grau, 2003) and IL-6 production (Lou et al., 1998), significantly higher levels were found in plasma and brain tissue of ECM-sensitive C57BL/6 mice than in FcεRIα-KO mice (Fig. 1, f–i). These data demonstrate that IgE and FcεRIα deficiency confers resistance to ECM and that the absence of signaling-competent receptors results in lower inflammatory cytokines in plasma and brain tissue.

Neither MCs nor basophils contribute to the development of ECM

To investigate the role of FcεRI-expressing cells, we first used MC–deficient Wth/Wth, which, like the previously used FcεRIα-KO and IgE-KO mice, are on the C57BL/6 background. MC–deficient Wth/Wth and WT mice were infected with PbANKA strain via mosquito bites. Parasitemia over time (Fig. 2 a) and survival rates (Fig. 2 b) showed no significant differences between both mouse strains excluding a role of MCs as major pathological effectors. To confirm this, FcεRIα-KO and Wth/Wth mice were reconstituted with WT MCs yielding an average of 1.2 and 1.05% FcεRIα+ MCs, respectively, in the peritoneal fluid 10 wk after reconstitution, as compared with 1.75% in WT animals (Fig. S1 d).

No effect was seen in reconstituted Wth/Wth mice and reconstituted FcεRIα–KO mice remained resistant to ECM (Fig. S1 e). These data formally rule out MCs as the cellular IgE targets in malaria pathogenesis.

Alternative FcεRI-expressing cells are basophils. To investigate their implication, mice were treated with the basophil-depleting antibody Ba103 1 d before infection. Severe depletion of DX5+/FcεRIα+ blood basophils (Fig. S2) did not improve disease susceptibility as compared with control mice and the survival rates were not significantly different compared with FcεRIα-KO or IgE-KO mice (compare Fig. 1, b and d, and Fig. 2 c). Collectively, these data suggest that FcεRIα+ cells other than MCs and basophils play a pivotal role in ECM development.

Similar infection outcomes were obtained whether mice were infected via mosquito bites or via infected RBCs (iRBCs). Henceforth, mice were infected via iRBCs.

In mice, expression of the FcεRIα is thought to be limited to MCs and basophils. However, it has been reported recently that FcεRIα and γ polypeptides are expressed in rat and mouse pinealocytes, the melatonin-secreting cell of the pineal gland (Ganguly et al., 2007). As the brain is a target tissue in ECM, we speculated that FcεRIα expressed by pinealocytes contribute to disease expression. To address this, adoptive transfer experiments were performed by injecting BM cells from FcεRIα-KO and C57BL/6 mice into irradiated FcεRIα-KO mice. Only mice that received FcεRIα+ BM cells developed ECM (Fig. 2 d), ruling out a significant contribution of pinealocytes in disease expression. Conversely, reconstitution of irradiated C57BL/6 mice with BM from syngeneic mice or from FcεRIα-KO mice showed that only the latter acquired resistance to ECM (unpublished data). Altogether, these data demonstrate that the FcεRIα-expressing cells that contribute to disease expression are of hematopoietic origin.

To exclude functional overlap between MCs and basophils, it was important to test the effect of depleting basophils in the mast cell–deficient Wsh mice. Preliminary data show that depletion of basophils in mast cell–deficient mice did not alter the phenotype of infected mice in regard to the development of ECM (unpublished data), suggesting that both cell lineages are apparently not involved in CM pathogenesis.

The reported binding of IgE to FcγRI in neutrophil activation (Hirano et al., 2007; Mancardi et al., 2008), it was necessary to preclude any possibility of this receptor to contribute to the observed effects associated with the FcεRI. To this end, C57BL/6 mice were inoculated with PbANKA-infected erythrocytes, and then treated with 200 µg of anti-FcγRI mAb (9e9 antibody) every other day from day 2 to day 6 after infection. Binding of the

**Figure 2.** Mast cells and basophils are not involved in malaria pathogenesis. Mast cell–deficient Wth/Wth and C57BL/6N (WT) mice were infected with PbANKA through mosquito bites (8–10 mosquitoes per mouse). Parasitemia (a) and Kaplan-Meier survival plots (b) were recorded (log-rank test; n = 32 of each genotype; P = 0.1). There were no significant differences in parasitemia between groups. Data are from four independent experiments. (c) C57BL/6N mice were treated with basophil-depleting BA103 antibody 1 d before PbANKA inoculation. Untreated, BA103-treated C57BL/6N, and control antibody-treated (Ab-Ctl) C57BL/6N mice were infected via mosquito bites. Kaplan-Meier survival plots were recorded (log-rank test; n = 11 of each genotype; P = 0.0179). Median mortality is 5 d for the two control groups, and 7 d for BA103-treated C57BL/6N mice. Data shown are from two independent experiments. (d) After irradiation, FcεRIα-KO mice intravenously received 5 × 106 BM cells isolated either from FcεRIα–KO mice or from WT C57BL/6N mice. The two groups of mice were infected with PbANKA with 106 PbANKA–iRBC. Kaplan-Meier survival plots were recorded (log-rank test; n = 12 of each genotype; P = 0.0026). Data shown are from two independent experiments.
antibody was assessed throughout the infection period, as shown by FACS analysis of blood leukocytes gated on CD45+ cells (preliminary results; unpublished data). Occurrences of ECM and survival data were strictly similar between mice that received the anti-FcγRIIV antibody or the isotype control antibody (preliminary results; unpublished data).

Identification of neutrophils and eosinophils as novel FcεRI-expressing cells induced during malaria disease

In search of FcεRI-expressing cells, we surmised that neutrophils and eosinophils might represent inflammatory cells, which under the particular circumstance of Plasmodium infection, could express FcεRI and cause the disease. A kinetic analysis of the frequency of 7/4+ Ly6G+ neutrophils, Siglec-F+ eosinophils, and FcεRI+ cells among blood leukocytes showed that neutrophils increased constantly over time, whereas eosinophils remained stable at around 5% (Fig. 3 a). The proportion of total FcεRI+ cells also increased steadily (Fig. 3 a). The most highly represented FcεRI+ cells among examined blood leukocytes were neutrophils reaching up to 6% (Fig. 3 b). FcεRI+ eosinophils peaked at day 4 rising from 0.2 to 4% and then decreased between d6–8 reaching 1.5% (Fig. 3 b).

In the BM, the proportion of neutrophils and eosinophils decreased from day 0 to 4, and then slightly increased until day 8 for neutrophils, while eosinophils decreased again after a short rise (Fig. 3 c). Fig. 3 d shows that naive mice expressed a small but detectable proportion of FcεRI+ neutrophils (1.8%) and eosinophils (1.9%). This proportion peaked at 8.5 and 6% for neutrophils and eosinophils, respectively, at day 4 after infection, whereas at day 6 and 8 after infection, the fraction of FcεRI+ cells declined for the two cell types. In parallel, basophils increased from 0.5% at day 0 to 2% at day 8, whereas the fraction of MCs remained unchanged.

In the brain, no cells were detected until 4 d after infection, which is when both neutrophils (4.9%) and eosinophils (2.3%) appeared, and their proportions reached 14 and 9%, respectively, at 8 d after infection (Fig. 3 e). The proportions of FcεRI+ neutrophils and eosinophils were, however, strikingly different with an accumulation of ~5% of neutrophils at 6–8 d, as compared with 1.3% of FcεRI+ eosinophils (Fig. 3 f). It must be noted that neither mast cells nor basophils were present at any given time (Fig. 3, e and f). An illustration of a FACS analysis of neutrophil populations associated with brain tissue taken at 6 d after infection and showing ECM signs is shown in Fig. 4. Double labeling of leukocyte populations with anti-7/4 and anti-Ly6G antibodies revealed a highly enriched proportion of FcεRI+ 7/4+ Ly6G+ neutrophils (55–61%) associated with brain tissue of mice showing ECM signs at 6 d after infection (Fig. 4, top).

To verify whether the increase in receptor expression occurs in the neutrophil population present in the brain, and also to determine whether this increase in expression only occurs in mouse strains susceptible to CM when infected with PbANKA, mice infected with the non–ECM-inducing PlσNK65 parasites did not show any sequestration of FcεRI+ neutrophils in their brain even at 20 d after infection (Fig. 4, bottom). It must also be emphasized that there were small amounts of leukocytes associated with the brain tissue. Indeed, the CD45+ population in the brain of PlσNK65-infected mice represents only 0.25% of total cells, whereas the same population was 20-fold higher (5%) in PbANKA-infected mice.

Collectively, these data show that a significant proportion of neutrophils and eosinophils expressed FcεRI during infection with PbANKA parasite.

Depletion of neutrophils protects mice from ECM

For depletion experiments, we used the NIPM-M14 mAb, in which staining patterns shows a complete overlap between the anti-Ly6G and the 7/4 antibodies. Administration of 200 μg of the antineutrophil mAb NIMP-R14 1 d before infection, which elicited complete depletion of neutrophils up to day 6 (unpublished data), prevented the development of neurological signs of ECM, and protected mice from death as compared with those treated with control IgG isotype (Fig. 5 a). Death occurred on average between day 20–25, without neurological signs from hyperparasitemia-induced anemia (median survival time, 21 d versus 7 d for neutrophil-depleted and control mice, respectively; n = 8; P = 0.006), with no significant difference in final parasitemia (P > 0.05; Fig. 5 b).

To examine the possible contribution of eosinophils to ECM pathogenesis, C57BL/6 mice were treated with eosinophil-depleting anti–mouse Siglec-F antibody 1 d before infection and...
at day 4 after infection. No significant differences between eosinophil-depleted and control IgG-treated mice were observed in the development of ECM (Fig. 5 c) and parasitemia (Fig. 5 d). These data demonstrate that neutrophils, but not eosinophils, play a critical role in the pathogenesis of ECM and that cell depletion does not directly affect parasitemia.

To establish if neutrophil depletion was equally effective after infection, we performed a kinetic analysis. Mice were treated before infection or at day 4, 5, or 6 after infection with anti-neutrophil-depleting antibody. Treatment at 4 d after infection was equally effective as depletion 1 d before infection (Fig. 5, a and c). Treatment on day 5 after infection was less protective, although significant (n = 12; P = 0.031; Fig. 5 f) compared with mice treated on day 6 after infection (n = 12; P = 0.074), which is when mice started to develop the first signs of ECM (Fig. 5 g). Thus, late depletion of neutrophils is no longer protective once the pathogenetic processes are established, particularly in the brain, where on day 5 these events are already in place (Beghdadi et al., 2008).

To analyze the role of FcεRI+ neutrophils in the initiation of ECM pathology, FcεRIα-KO mice were repleted with BM-derived FcεRI+ neutrophils from PbANKA-infected C57BL/6 mice 6 d after infection. FcεRIα+ cells were enriched in the low-density layers of Percoll gradient (50–45% and <45%; Fig. S3) before FACS sorting. Double-positive cells gated on 7/4 and Ly6G expression gave rise to two neutrophil populations: Ly6G+ 7/4+ FcεRI+ neutrophils (9.3%) and Ly6G+ 7/4+ FcεRIα+ neutrophils. 90.7% were obtained from PbANKA-infected mice, whereas in normal mice the Ly6G+ 7/4+ FcεRI+ neutrophil population represents only 1% (Fig. S4, b and c). Additional morphological analysis of FcεRIα+ and FcεRI+ neutrophil populations showed that they were phenotypically indistinguishable, as shown by cytospin analysis (Fig. S4, d and e). Sorted triple-positive (Ly6G+ 7/4+ FcεRI+) neutrophils or double-positive (Ly6G+ 7/4+ FcεRIα+) neutrophils were injected intravenously into recipient FcεRIα-KO mice. Before injection, these cells were analyzed for their possible contamination with other leukocytes, and as shown in Fig. S4 f, sorted cells consisted of highly pure neutrophil populations with only minor contamination from Siglec-F+ cells, CD11c+ cells, and F4/80+ cells. FcεRIα-KO mice repleted with Ly6G+ 7/4+ FcεRI+ neutrophils acquired susceptibility to ECM, as compared with those reconstituted with FcεRIα+ neutrophils (n = 15, P = 0.0087; Fig. 5 h). To analyze brain localization, naive or infected FcεRIα-KO or WT C57BL/6 mice (6 d after infection) were injected intravenously with 106 CFSE-labeled sorted neutrophils (7/4+ Ly6G+ FcεRI+) obtained from infected C57BL/6 mice. As shown in Fig. 5 i, a significant proportion of CFSE-labeled neutrophils was found to be associated to brain tissue of both infected recipient FcεRIα-KO (4.9%) and WT C57BL/6 mice (4.5%) compared with noninfected FcεRIα-KO (0.7%) and WT C57BL/6 mice (0.6%), respectively. Similar results were obtained 1 and 24 h after injection, suggesting that 7/4+ Ly6G+ FcεRI+ home rapidly and stably to the brain tissue of infected mice. These data demonstrate that the expression of FcεRI+ in neutrophils is critical for the development of ECM.

Biochemical characterization of FcεRI expressed by neutrophils

As neutrophils ordinarily do not express FcεRI, we further characterized this receptor in cells from PbANKA-infected mice. Highly homogeneous double-positive Ly6G+ 7/4+ cells obtained by FACS sorting (Fig. S4), subjected to analysis using RT-PCR, showed that neutrophils from naive mice expressed low levels of FcεRIα mRNA and expression increased in infected mice (Fig. 6 a). Flow cytometry confirmed that naive mice expressed only low levels of surface FcεRI and IgE-binding (~1%). On day 6 after infection, ~9–10% of neutrophils stained positive with anti-FcεRIα and anti-IgE, indicating that they were IgE-sensitized (Fig. 6 b). CD23,
another receptor, which may also account for IgE binding, was not expressed by neutrophils from naive or infected mice (Fig. 6 b). Confocal image analysis also confirmed that FceRIα was colocalized with the FceRIγ subunit (Fig. 6 c), whereas FceRIβ was undetectable (not depicted). Immunoblot analysis of cell lysates confirmed the presence of FceRIγ and the absence of FceRIβ, whereas FceRIβ was readily detected in RBL-2H3 and BMMC lysates (Fig. 6 d). To exclude the possibility that the lack of the FceRI β chain is caused by a proteolytic degradation, presence of the β chain was assessed at the transcriptional level. Analysis of neutrophils by real time qPCR clearly indicated that FcεRIα transcriptional level. Analysis of neutrophils by real time proteolytic degradation, presence of the possibility that the lack of the FcεRIα was readily detected in absence of FcεRIα RI, whereas FcεRIα RI was not expressed by neutrophils from naive or infected mice (Fig. 6 b). Although FcγRIV has been reported to bind IgE (Hirano et al., 2007) or both IgE and IgEβ allotypes (Mancardi et al., 2008) at high concentrations, we did not detect IgE-binding under steady-state conditions in naive mice, and IgE-binding could not be blocked with anti-FcγRIV antibody in cells from infected mice (unpublished data). We also performed coimmunoprecipitation experiments to analyze the subunit composition of FcεRI. The receptor was directly immunoprecipitated with IgE-Sepharose to avoid precipitation of murine IgG re- action of FcεRI absent. The receptor was directly immuno precipitation experiments to analyze the subunit composition of FcεRI. The receptor was directly immunoprecipitated under mild conditions (0.3% Triton X-100) revealed the specific presence of FceRIγ, whereas FceRIβ was absent. Both β and γ chains were readily coimmunoprecipitated from BMMC lysates (Fig. 6 e). The γ chain was also immunoprecipitated in the presence of anti FcγRIV blocking antibody (unpublished data) excluding that coimmunoprecipitation of the γ chain was caused by FcγRIV. Together, these data indicated that the parasite-induced neutrophil population expressed an unconventional FcεRI in the absence of FcεRI β chain. To assess the functionality of this receptor, neutrophils were passively sensitized with anti-DNP–specific IgE mAb. Stimulation of sensitized cells for 4 h with DNP-HSA elicited significant amounts of TNF and IL-6 release (Fig. 6 f).

**DISCUSSION**

In this work, we report the implication of molecular and cellular components of the IgE-mediated allergic inflammatory cascade in malaria pathogenesis. Using a murine model of ECM, we show that the inflammatory response elicited by IgE–FcεRI complexes drives development of ECM. This did not involve MCs or basophils. A major finding was that PbANKA-induced expression of FcεRI in neutrophils...
and eosinophils and that depletion of neutrophils, but not eosinophils, had a positive clinical outcome.

In humans, studies aiming at establishing the allergic nature of malaria disease report conflicting results ranging from disease-aggravating to disease-protecting roles of IgE (Perlmann et al., 1997; Bereczky et al., 2004). To clarify this issue, we used genetically deficient animals or antibody-depleted cell populations to examine crucial components of the allergic inflammatory response in malaria pathogenesis. We found that targeted disruption of IgE or the α chain of FcεRI led to resistance to the development of ECM after infection with PfANKA. These results indicate a pathological role of IgE that acts via FcεRI to promote disease development. Interestingly, FcεRI-α-KO mice showed a more marked resistant phenotype than IgE-KO mice, leaving open the possibility for additional disease-aggravating signaling events via FcεRI. This may include galectin 3, an alternative ligand for FcεRI. In agreement, deletion of the galectin-3 gene in susceptible C57BL/6 mice resulted in partial protection from ECM (Oakley et al., 2009). In both FcεRI-α-KO and IgE-KO mice, the observed clinical immunity occurred in the absence of antiparasite immunity. The association of FcεRI with the neuropathological disorders observed during ECM was further emphasized by the constant increase of the receptor α chain expression in the brain.

Figure 6. Parasite-induced neutrophils express an unconventional FcεRI.

(a) RT-PCR analysis of expression of FcεRIα mRNA in naive and 2 PbANKA-infected neutrophil preparations. RT-PCR from BMMC and PCR amplification of FcεRIα plasmid DNA were used as positive controls. Data were from two independent experiments. (b) Neutrophil population was sorted from BM cells from naive C57BL/6 mice or from mice infected 6 d earlier with 10⁶ PbANKA-iRBC. Cells were counter-stained with either anti-FcεRI, anti-IgE, anti-FcyRII, or anti-CD23 antibodies and compared was performed between neutrophils from naive and infected mice. Data were obtained from four independent experiments. (c) Percoll-enriched and 7/4-FITC-sorted sorted neutrophils (blue pseudocolor) from the BM of PbANKA-infected C57BL/6 mice were stained with anti-FcεRI (green pseudocolor) before fixation, permeabilization, and staining, with anti-FcεRI γ (red). Cells were visualized by confocal microscopy. The DIC image, single optical sections through individual cells, as well as the merge (green and red fluorescence) are presented. Bars, 5 µm. Quantitative analysis of colocalization of γ chain with FcεRI (red with green) and of FcεRI with γ chain (green with red) were, respectively, 0.48 ± 0.23 and 0.11 ± 0.10. Data were from three independent experiments. (d) Cell lysates (20 and 5 µg, respectively) from RBL-2H3, BMMC, and Ly6G-FITC-sorted neutrophils were migrated on 15% SDS-PAGE and subjected to immunoblotting with anti-β and anti-γ antibodies. (e) Lysates from indicated cells or buffer control were subjected to immunoprecipitation with BSA- or IgE-Sepharose as indicated, followed by immunoblotting with anti-β and anti-γ antibodies. The results shown are representative of three independent experiments. (f) Neutrophils, which were passively sensitized with anti-DNP IgE mAb, were stimulated with indicated concentrations of DNP-HSA, and release of TNF and IL-6 was determined by ELISA. * represents significant differences observed for TNF (Mann-Whitney test, P = 0.033) and IL-6 (Mann-Whitney test, P = 0.0117) as compared with basal levels. Data shown in d-f were from two independent experiments.
of infected mice. Conventional effector cells expressing FcεRI in mice consist of MCs and basophils. However, our data using MC-deficient mice or antibody-mediated depletion of basophils excluded MCs and basophils as relevant effector cells. To exclude functional overlap between mast cells and basophils, mast cell–deficient mice treated with the anti–basophil-depleting antibody did not result in any protection against ECM. Previously, MCs and MC-derived TNF were shown to protect against ECM (Furuta et al., 2006). This contradictory result may have been influenced by the different MC–deficient W/Wv mouse strain used, which makes the mice anemic, and thus potentially compromised in controlling a parasite infection. It was also puzzling that TNF was protective in this study, although TNF generally enhances disease severity (Grau et al., 1987).

Searching for alternative FcεRI-expressing cell types, we identified neutrophils and eosinophils, and discovered that the former had a disease enhancing. Although FcεRIα is essentially absent in neutrophils in naive mice, FcεRIα becomes induced during Plasmodium infection as demonstrated by: RT-PCR analysis, the binding of the α chain–specific MAR-1 antibody, and detection of membrane-bound IgE. Additional characterization showed that the receptor was colocalized with the FcεRIγ subunit that could be coimmunoprecipitated with IgE. In contrast, the FcεRI β chain was undetectable in neutrophils. Although FcγRIIV, which is expressed in neutrophils in both naive and infected mice, could potentially bind IgE at high concentration (Hirano et al., 2007; Mancardi et al., 2008), we did not detect significant IgE binding to this receptor. Similarly, another IgE receptor CD23, was not expressed in neutrophils, suggesting that IgE binding to FcεRI was specific. In humans, FcεRI is frequently expressed in neutrophils of atopic asthmatics as a trimeric FcεRIαγ2 receptor (Saffar et al., 2007), whereas it is absent in healthy individuals. It has therefore been postulated that FcεRI-induced inflammatory signaling and the reported antiapoptotic effect of monomeric IgE may exacerbate the pathogenetic effects of FcεRI+ neutrophils in asthma (Saffar et al., 2007). A parallel can be made in the present work as to the possible implication of FcεRI+ neutrophils in malaria pathogenesis. Indeed, cell type–specific depletion of neutrophils, but not of eosinophils was found to protect mice against ECM and mortality consistent with previous findings using the same neutrophil–depleting antibody (NIMP-R14; Senaldi et al., 1994). Furthermore, transfer of FcεRI+, but not FcεRI+, neutrophils conferred ECM susceptibility to the otherwise resistant FcεRI-α-KO mice. Likewise, irradiated C57BL/6 mice engrafted with the BM from FcεRI-α-KO mice acquired resistance to ECM, which was not the case when these mice were reconstituted with the BM from C57BL/6 mice (unpublished data). These results emphasize the critical role of FcεRI+ neutrophils in the disease expression and mortality, as the sequestration properties of this cell population in the brain tissue occurs rapidly, within an hour after cell transfer, in PhANKA-infected FcεRI-α-KO mice that are otherwise CM resistant. The fact that the FcεRI+ neutrophils did not sequester in the brain of noninfected mice suggests that the brain tissue of PbANKA-infected FcεRI-α-KO mice acquired the potential to recruit FcεRI+ neutrophils. After infection, given the absence of FcεRI in these mice, the pathogenetic effects could not be observed. A subsequent issue relates to the specific induction of FcεRI+ neutrophils by the PbANKA strain, as this cell population was absent when the non–CM-inducing PbNK65 strain was used. Indeed, it is frequently asked what distinguishes these two parasite strains in terms of their ability to cause ECM or not. One possible explanation is provided by the present work, which establishes a close relationship between the disease-associated FcεRI+ neutrophils and CM-inducing PbANKA strain. However, it remains to be determined at the molecular level by which mechanism PbANKA induces the expression of FcεRI.

Collectively, our findings provide a new concept on inflammatory response–driven malaria pathogenesis where neutrophils play a central role in inducing inflammatory responses, reminiscent of the allergic-type reactions implicated in the development of ECM. Inflammatory cytokines IFN-γ and IL-6, which have elevated expression locally in the brain and systemically in the plasma, may represent surrogate markers for this neutrophil–mediated response. Because ECM pathology was found to be associated with increased proinflammatory IFN-γ (Hunt and Grau, 2003), an attenuated production of this cytokine in FcεRI-α-KO mice raises the possibility that FcεRI+ neutrophils that sequester in the brain of infected C57BL/6 mice may represent an additional source of IFN-γ. Functional studies of FcεRI+ neutrophils via cross-linking of bound IgE results in the production of IL-6 and TNF. In infected mice, the triggering of cytokine release may occur in different tissues, including BM, blood, and brain, giving rise to both systemic and local cytokine induction. Physiologically, several possibilities exist to explain the elevated cytokine levels, such as the possibility that cytokines are released by activated neutrophils subsequent to aggregation of specific IgE antibodies on sensitized neutrophils by parasite–derived antigens. However, this mechanism is uncertain, as we were not able to detect such specific IgE antibodies in infected mice. It is also possible that IgE present in the mouse before infection exerts a priming function when binding to newly expressed FcεRI on a subset of neutrophils, rendering them more responsive to other stimuli.

Several questions remain unresolved. In particular, as mentioned above, the Plasmodium infection–associated signals required for the induction of FcεRI expression in neutrophils are unknown. However, this property seems to be specific to the ECM–inducing PhANKA parasite, as mice infected with non–ECM–inducing PbNK65 parasite or mice infected with Escherichia coli undergoing septic peritonitis as a result of cecal ligation and puncture did not show any expression of FcεRI in their neutrophils (unpublished data). In humans, it is believed that expression of FcεRI in neutrophils is regulated by Th2 cytokines (Saffar et al., 2007) and GM-CSF. We detected IL-4 in the brain and in the spleen at 6 d after...
infection (unpublished data), but a clear relationship with the induction of IgE and FcεRI+ neutrophils during *Plasmodium* infection was not formally established. Although IgE-KO mice were clinically resistant, our attempts to detect *Plasmodium* antigen-specific IgE antibodies in the plasma of infected mice have failed. It is possible that within the relatively short period of infection (6–8 d), an effective IgE antibody response may be hardly detectable. A significant increase in total IgE antibodies became detectable in surviving mice only at day 12 after infection (Fig. 1 e). One possibility is that limited amounts of IgE antibodies are sufficient to trigger neutrophil responses. Alternatively, sensitization with parasite antigen-unrelated IgE antibodies may render FcεRI+ neutrophils more prone to fulfill their effector functions. In support of this, an earlier study of MCs highlighted the role of antigen-irrelevant IgE in positively influencing hapten-specific contact hypersensitivity (Bryce et al., 2004). Therefore, it is possible that IgE present in the mouse before infection binds to newly expressed FcεRI thereby priming these cells for enhanced effector responses.

Our biochemical analysis showed that mouse neutrophils, express FcεRIαβ trimers in the absence of the β chain (Wang et al., 1992; Maurer et al., 1996). This was surprising, as previous experiments had indicated a requirement of this subunit for cell surface expression in rodents in contrast to the human receptor (Miller et al., 1989), where expression can be observed in several β-less cell types (Bieber, 2007). However, a precedent for the expression of FcεRI in the absence of FcεRIβ in mice has previously been made in lung DCs after infection with Sendai virus (Grayson et al., 2007). It is therefore conceivable that infectious events may enable expression of a trimeric FcεRIαβ receptor by masking inherent retention signals of the α chain (Blank et al., 1991; Hartman et al., 2008). Alternatively, they may induce expression of “β-like” molecules that support receptor expression. Additional studies are necessary to clarify the various aspects leading to the activation of FcεRI+ neutrophils during malaria disease.

Collectively, our data provide evidence for the implication of an unconventional FcεRI in neutrophils as being important in the expression of severe malaria mediated by an IgE-mediated inflammatory response.

**MATERIALS AND METHODS**

**Mice.** Female C57BL/6 mice 6–8 wk old were purchased from Charles River Breeding Laboratories. FcεRIαβ-KO mice have been previously described (Dombrowicz et al., 1993a). IgE single-KO mice were derived from double IgE/CD16-KO mice by backcrosses on C57BL/6 mice (provided by J.S Verbeek, Leiden University Medical Center, Leiden, the Netherlands). These mice were originally provided by H. Oettgen (Children's Hospital, Harvard Medical School, Boston, MA) C57BL/6-KaW-sh/W-sh mice were provided by P. Besmer (Sloan-Kettering Institute, New York, NY). All mice were on the C57BL/6 background. All animal care and experimentation was conducted in accordance with the Institut Pasteur animal care and use committee guidelines.

**Parasites and infection.** For all infections, non-ECM-inducing *P. berghei* (strain NK65; PbNK65) or ECM-inducing (ANKA strain) strains expressing the GFP on CS or hsp70 promoter (Ishino et al., 2006), were used allowing the detection of sporozoites and blood stage parasites by fluorescent microscopy. Parasites were provided by Dr. T. Ishino (Department of Invertebrate Zoology, Mae University School of Medicine, Edobashi, Tsu, Japan). Infection of mice with PbANKA induces ECM, characterized by paralysis, ataxia, convulsions, and coma between 7–9 d after infection. PbNK65 induces a lethal malaria around 20–25 d after infection without neurological symptoms and was a gift from R. Menard (Institut Pasteur, Paris, France). The parasites were maintained in a cycle between C57BL/6 mice and *Anopheles stephensi* (Demeure et al., 2005). The erythrocytic phases of the parasites were maintained in liquid nitrogen as iRBC in Aedes’s solution (Sigma-Aldrich) containing 10% glycerol. The infection is induced either by exposure to 8–10 infected *An. stephensi* mosquitoes (Beghadi et al., 2008) or by intraperitoneal injection of 10⁶ iRBCs.

**Flow cytometry analysis.** Single-cell suspensions from blood or BM obtained at different time points after inoculation of C57BL/6/N mice with 10⁶ PbANKA-iRBCs were stained for FACS analysis according to standard protocols in cold PBS containing 2% FCS and 0.01% sodium azide (FACS buffer). Labeling was performed as follows: for total neutrophils (PerCP-anti-CD45+ FITC-7/4+ PE-anti-Ly6G mAbs), total eosinophils (PerCP-anti-CD45 mAb+ PE-anti-Siglec-F mAb), and total FcεRIαβ leukocytes (PerCP-anti-CD45 mAb+ PE-anti-FcεRIαβ chain mAb). To unequivocally define FcεRIαβ neutrophil subpopulation by FACS analyses, cells were labeled with PerCP-antiCD45 mAb and counter-stained with antineutrophil FITC-7/4 and PE-anti-Ly6G antibodies, and with APC-anti-FcεRIα chain mAb. Detection of basophils consisted of PerCP-anti-CD45 mAb+ APC-CD56+ PE-anti-FcεRIα chain mAb. FcεRIαβ+ eosinophils were identified using PerCP-antiCD45 mAb+ PE-anti-Siglec-F mAb+ FITC-anti-FcεRIα chain mAb, and mast cells were characterized using PerCP-antiCD45 mAb+ APC-anti-CD117+ PE-anti-FcεRIα chain mAbs. All antibodies were obtained from BD, except FITC-7/4 and anti-FcεRIα chain mAbs (clone MAR-1), which were obtained from Invitrogen and Ebioscence, respectively. The hamster anti-mouse FcγRI antibody (clone 9e9) was provided by J. Ravetch (The Rockefeller University, New York, NY). Analysis was performed using a four-color FACSCalibur flow cytometer with ProCellQuest software (BD). Data were expressed as the percentage of total CD45+ BM or blood cells, or as the percentage of total FcεRIαβ+ BM or blood cells.

**Confocal microscopy and immunoprecipitation studies.** For confocal immunofluorescence microscopy, FITC-anti-Ly6G+ neutrophils, FcεRI-expressing cells (~4 × 10⁶ cells) sorted out by FACS were incubated with anti-FcεRI antibody MAR-1 (1 h on ice), followed by incubation with Dylight 649 goat anti-Armenian hamster (BioLegend) before fixation for 15 min in PBS containing 4% paraformaldehyde followed by 2 washes with PBS. Fixed cells were permeabilized in PBS containing 0.025% saponin for 20 min at room temperature, followed by blocking in PBS containing 0.025% saponin and 10% goat serum/0.2% BSA (Invitrogen) for 30 min at room temperature. Cells were then stained with rabbit anti-FcγRI in PBS containing 0.025% saponin and 10% goat serum/0.2% BSA overnight at 4°C, followed by incubation with anti-rabbit-Alexa Fluor 568 for 60 min in PBS containing 0.01% saponin and 10% goat serum/0.2% BSA. After washing, cells were mounted in Prolong-Gold anti-fading reagent (Invitrogen) and analyzed using a confocal laser-scanning microscope (LSM 510; Carl Zeiss, Inc.). Images were taken using 63× oil immersion objective lens. Colocalization was quantified using LSM 510 image software. A total of 7 cells with a minimum of 10 stacks were quantified.

**Immunoprecipitation and immunoblotting.** Ly6G-FITC–sorted positive neutrophils enriched for FcεRI-expressing cells by Percoll gradient centrifugation were washed 2 times in PBS and resuspended at 1 × 10⁶ cells/ml in PBS. 3 million cells were used for preparation of cell lysates in 50 mM Hepes, pH 7.3, containing 0.5% Triton X-100, 150 mM NaCl, 10 mM MgCl₂, 50 mM NaF, 1 mM sodium orthovanadate, and the protease inhibitors aprotonin 1,000 U/ml (Sigma-Aldrich), pepstatin 10 µg/ml, leupeptin 20 µg/ml, and AEBSF 2 µM.
(Alexis, Inc.). For immunoprecipitation, cells were lysed on ice for 30 min in 50 mM Hepes, pH 7.3, containing 0.3% Triton X-100, 150 mM NaCl, 10 mM MgCl2, 50 mM NaF, 1 mM sodium orthovanadate, and the protease inhibitors aprotonin 1000 U/ml (Sigma-Aldrich), pepstatin 10 µg/ml, leupeptin 20 µg/ml, and AEBSF 2 µM (Alexis, Inc.). After centrifugation, proteins were resolved on SDS-PAGE and transferred onto PVDF membranes (Sigma-Aldrich). Membranes were blocked with 4% BSA for 1 h, followed by incubation with primary mouse anti-FceR1β, or rabbit anti-FceR1γ (1 h at room temperature). After several washes, blots were incubated with goat anti-mouse IgG HRP (1:10,000) or donkey anti-rabbit IgG HRP (Jackson ImmunoResearch Laboratories) for 45 min and were developed by ECL (GE Healthcare).

Real-time RT-PCR and RT-PCR of the α chain of the FceRI. Gene expression in the brains from FceRIα-KO and C57BL/6 mice at various time points after infection were analyzed by the real-time RT-PCR. RNA used for these assays was isolated by means of a two-step extraction process. First, brains were surgically removed from mice as previously described and placed immediately in RNAlater (QIAGEN) at 4°C overnight. After RNAlater mini-columns were spun at high speed (10,000 g) for 2 min at a setting of 30 cycles/s. Samples are spun at high speed (10,000 g) for 3 min to remove debris and lipids. Half of the sample was transferred to a new tube and mixed with 500 µl of TRizol reagent by vortexing. After this step, RNA extraction proceeded according to the manufacturer’s protocol. Precipitated mRNA was resuspended in 100 µl of RNase-free water. The second step of this extraction was followed by QIAGEN’s protocol for RNA clean-up, including steps for removal of protein and DNA (RNAeasy kit; QIAGEN). Samples were eluted with 50 µl of RNase-free water and quality and quantity assured by photospectroscopy. Real-time RT-PCR used primer-probe sets for the α chain of mouse FceRI and standard TaqMan protocols (Applied Biosystems).

RT-PCR to amplify FceRIα was performed according to standard procedures as previously described (Roa et al., 1997). 1 µg of mRNA from BMMC, naive, and Plasmodium-infected neutrophils were reverse transcribed using Superscript RT (Invitrogen). An aliquot of the cDNA (1 µg) was amplified using primers yielding full-length (680 bp) FceRIα (5’-ACTT-GATGAAATCGCAGAAGATCTGTA-3’ and 5’-CTGATAGCTTGGTTAGAGCTCGTTTC-3’) with an annealing temperature of 53°C and 35 cycles of amplification. PCR amplification of FceRIα cDNA (Ra et al., 1991) was used as a positive control.

Detection of IgE antibodies in sera from Plasmodium-infected mice. For parasite-specific IgE antibodies, 96-well plates were coated with PbANKA freeze-thawed crude lysate in carbonate buffer, pH 9.6, for 2 h at 37°C. After the plates were saturated with 1% BSA for 2 h at 37°C. Serial dilutions of sera were incubated at ~80°C until processing. Brains were thawed in 1 ml of TRizol and subjected to bead disruption in a polytron 3 times for 2 min at a setting of 30 cycles/s. Samples are spun at high speed (10,000 g) for 3 min to remove debris and lipids. Half of the sample was transferred to a new tube and mixed with 500 µl of TRizol reagent by vortexing. After this step, RNA extraction proceeded according to the manufacturer’s protocol. Precipitated mRNA was resuspended in 100 µl of RNase-free water. The second step of this extraction was followed by QIAGEN’s protocol for RNA clean-up, including steps for removal of protein and DNA (RNAeasy kit; QIAGEN). Samples were eluted with 50 µl of RNase-free water and quality and quantity assured by photospectroscopy. Real-time RT-PCR used primer-probe sets for the α chain of mouse FceRI and standard TaqMan protocols (Applied Biosystems).

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In vivo depletion of basophils, neutrophils, and eosinophils. Basophils represent <1% of peripheral blood leukocytes and have been neglected until recently, when they were found to fulfill critical immunological functions (Karayayana et al., 2009). These cells, which generate large quantities of T helper 2 (Th2) cytokines such as IL-4, provided new insights into their possible role in allergic disorders and immunity to pathogens. A specific antibody has recently been generated, the rat monoclonal antibody Ba103, which recognizes a mouse basophil-associated protein (Obata et al., 2007). For basophil depletion, mice were given an intravenous injection of Ba103 1 d before infection with 108 RBCs. A single intravenous administration of 30 µg Ba103 drastically reduced the basophil number in the peripheral blood, and this level remained reduced for ~10 d after the single injection of Ba103, and thereafter returned to normal. Unlike the number of basophils, no significant reduction in the number of mast cells was observed (Obata et al., 2007). To determine if eosinophils and neutrophils play any role in malaria disease severity, cell-specific depletion experiments were performed. C57BL/6 mice were injected 1 d before infection with PbANKA pRBCs, with 200 µg of a rat anti–mouse neutrophils (clone NIMP-R14; Sendt et al., 1994) provided by G. Mül (Institut Pasteur, Paris), which was shown to recognize the same neutrophil population as the anti-Ly6G and the 7/4 mAbs and the double-positive (Ly6Gγ/7/4+ FceRIγ) neutrophils (2 × 106 neutrophils/mouse) were injected intravenously in recipient FcεRi-ko KO mice. Given the short half-life of neutrophils, their injection at day 6 after infection was taken as the optimal proximal time before the manifestation of disease signs.

Neutrophil activation and cytokine release. Purified neutrophils were resuspended at 106 cells/ml in RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, and then incubated for 1 h with 1 µg/ml of anti-dinitrophenyl (DNP)-specific IgE mAb obtained from culture media of the hybridoma HB-DNP-e-26.82 (Lee et al., 1980). For stimulation, the cells were incubated with DNP-HSA at 37°C for indicated times. Supernatants were then tested for IL-6 and TNF content by ELISA (BD). Lysates were tested for phosphorylation of p38 MAPK and Akt.
University of Dundee, UK; Zimmermann et al., 2008). A second injection of anti-Siglec-F antibody was provided at day 4 after infection to maintain eosinophils at a minimal level until the expression of clinical signs. At day 6, when the first clinical signs appear, no detectable neutrophils or eosinophils were present in peripheral blood. To verify if NIMP-R14 antibody specifically recognizes neutrophil cells, BM cells obtained 6 d after infection from C57BL/6 mice were first stained with NIMP-R14 mAb followed by anti-rat IgG mAb PE/Cy7 and positive cells were sorted out. NIMP-R14 + cells were then counter-stained with anti-GR-1-PE, 7/4-FITC, MAR-1-Alexa Fluor 647, CD11c-PE, DX5-PE, CD11b-PE, and Siglec-F-PE antibodies. The pattern of stainings shows a complete overlap between the NIPPM-R14 antibody and the anti-Ly6G and 7/4 antibodies.

Statistical analysis. Significant differences in survival were evaluated by generation of Kaplan–Meier plots and log rank analysis. P < 0.05 was considered statistically significant. For other analyses, after verification using a Jarque-Bera test, our data did not follow normal distribution, and thus, when differences between groups of mice were to be compared at a given time point, the Mann-Whitney test was performed with significance set at P < 0.05.

Online supplemental material. Fig. S1 shows an assessment of mast cell development and function in FcεRIα-KO mice and mast cell reconstitution of FcεRIα-KO mice with Wdβ1 mice. Fig. S2 shows in vivo depletion of basophils. Fig. S3 shows density-based distribution of FcεRIα neutrophils. Fig. S4 shows the phenotypic characterization of FcεRIα neutrophils. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110845/DC1.

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Author contributions A. Porcherie, C. Mathieu, R. Peronet, and U. Blank performed experiments and analyzed the data. H. Kiefer-Biašizzu, and J. Caver gave technical support. G. Milon, H. Karasuyma, M. Dy, J.P. Kinet, and J. Louis developed analytical tools and gave conceptual advice. U. Blank and S. Mécheri wrote the paper. S. Mécheri conceived the study. All authors discussed the results and commented the manuscript at all stages.

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