American Journal of Pathology, Vol. 166, No. 1, January 2005

Neurobiology

ABCA1 Gene Deletion Protects against Cerebral Malaria

Potential Pathogenic Role of Microparticles in Neuropathology

Valéry Combes,* Nicolas Coltel,* Mélanie Alibert,† Miranda van Eck,‡ Cécile Raymond,† Irène Juhan-Vague,§ Georges Emile Grau,* and Giovanna Chimini†

From the Laboratory of Immunopathology, the Centre d'Immunologie de Marseille Luminy,† INSERM/Centre National de la Recherche Scientifique/Université de la Méditerranée, and the Haematology Laboratories,§ Faculty of Medicine, IFR48, Marseille, France; and the Division of Biopharmaceutics,‡ Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden University, The Netherlands*

The ATP-binding cassette transporter A1 (ABCA1) modulates the transbilayer distribution of phosphatidylserine at the outer leaflet of the plasma membrane. This external exposure of phosphatidylserine is a hallmark of microparticle production and is impaired in ABCA1$^{-/-}$ mice. In this study, we report about the complete resistance to cerebral malaria of these mice. On analysis of histological and systemic parameters we evidenced an impairment of cellular responses to *Plasmodium berghei* ANKA infection in ABCA1$^{-/-}$ mice, as shown by lower plasma tumor necrosis factor levels, a weaker up-regulation of endothelial adhesion molecules in brain microvessels, a reduced leukocyte sequestration, as well as an ablated platelet accumulation. Besides, the number and the procoagulant activity of microparticles were dramatically reduced in the plasma of ABCA1$^{-/-}$ compared to ABCA1$^{+/+}$ mice. Moreover, microparticles derived from *Plasmodium berghei* ANKA-infected ABCA1$^{+/+}$ mice induced a significant increase of tumor necrosis factor release by noninfected macrophages. In ABCA1$^{-/-}$ mice platelet and macrophage responses to vesiculation agonists were ablated and reduced, respectively. Altogether, by pointing out the ABCA1 transporter as a major element controlling cerebral malaria susceptibility, these data provide a novel insight into its pathophysiological mechanisms and are consistent with a pathogenic role of microparticles in this neurological syndrome. *(Am J Pathol 2005, 166:295–302)*

The ATP-binding cassette transporter A1 (ABCA1), controls the initial steps of reverse cholesterol transport, ie, the release of cellular phospholipids to lipid-free apolipoproteins. The targeted deletion of the ABCA1 locus in mouse is an experimental model for the human Tangier dyslipidemia, a naturally occurring loss of function in man,[1] characterized by low to absent plasma high-density lipoprotein (HDL) levels and increased deposition of cholesteryl esters in several tissues.[2] From a molecular standpoint the ABCA1-dependent control of cellular lipid effluxes is a consequence of the intrinsic flippase activity of the ABCA1 transporter that modulates the transbilayer distribution of phosphatidylserine (PS) at the plasma membrane.[3] Indeed, compared to ABCA1 wild-type mice (ABCA1 WT), red blood cells from ABCA1 knockout mice (ABCA1 KO) expose reduced amount of PS after stimulation with A23187, as assessed by prothrombinase activity and microparticle (MP) production.[1]

MPs are submicron membrane elements, mainly expressing PS at their surface, as well as surface antigens from their cell of origin.[4] Originating from physiological processes, MPs are found in the circulation of healthy subjects,[5–7] but their numbers can be increased in vari-

Supported by the French Ministry of Research (PAL+ Program, to G.E.G. and G.C.), the United Nations Development Program/World Bank/World Health Organization Special Programe for Research and Training in Tropical Diseases (Tropical Disease Research, to G.E.G. and G.C.), and the Association de Lutte Contre le Cancer (grant no. 4245 to G.C.). The Centre d'Immunologie de Marseille Luminy is supported by institutional grants from INSERM and Centre National de la Recherche Scientifique.

V.C., N.C., and M.A. contributed equally to the work; and G.E.G. and G.C. directed the work equally.

Accepted for publication September 21, 2004.

Address reprint requests to Prof. Georges E. Grau, Laboratory of Immunopathology, Faculty of Medicine, IFR48, Université de la Méditerranée, 27, Bd Jean Moulin, F-13385 Marseille Cedex 05, France. E-mail: georges.grau@medecine.univ-mrs.fr.

ous pathological conditions such as thrombotic[4,5,8] or infectious diseases.[7,9–13] These MPs derive from activated blood cells and may carry at their surface procoagulant[7,13] and proadhesive properties,[14] suggesting that they could be implicated in the pathogenesis of these diseases. Such a role has indeed been recently demonstrated in mice in which the involvement of procoagulant MPs in the development and growth of thrombi was assessed *in vivo*.[15] In addition in the experimental model of mouse malarial infection, a correlation between thrombocytopenia, increased numbers of platelet-derived MPs, and expression of cerebral complications has been reported.[16]

The syndrome of cerebral malaria (CM), the major fatal complication of plasmodium infection, invariably occurs, in susceptible mice strains, at day 7 after infection with *Plasmodium berghei* ANKA (PbA) and is unrelated to parasite counts in the blood.[17] The fatal outcome is generally attributed to the sequestration of activated blood cells (notably monocytes/macrophages, parasitized erythrocytes, and platelets) in cerebral vessels consequent to immune responses in the host.[18] However, the pathogenic mechanisms underlying the occurrence of cerebral lesions are still incompletely understood. Along this line of thought and in particular considering the suggestion of a causal relationship between MPs and the clinical severity of malaria, we set out to investigate whether a reduction of cellular ability to vesiculate, as that evidenced in the absence of the ABCA1 transporter, had any effect on the course of experimental malarial infection in mice.

In this study we provide evidence that the sole absence of ABCA1 gene converts the susceptibility of the DBA/1 strain of mice to complete resistance to CM. This is accompanied by a lower reactivity of cells within the brain vessels and a reduced cellular vesiculation, as assessed both *in vivo*, by lower levels of plasma MPs during malarial infection, and *in vitro* on restimulation of platelets and macrophages. These findings are thus consistent with a pathogenic role of MPs in the development of cerebral complications.

Materials and Methods

Mice

ABCA1 WT ($ABCA1^{+/+}$) and ABCA1 KO ($ABCA1^{-/-}$) DBA/1 mice (12 to 14 weeks old) were generated in our laboratory as previously described.[1] Apo-$AI^{-/-}$ mice ($Apoa1^{tm1Unc}$) in C57BL/6J background[19] and C57BL/6J control strain mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were maintained under pathogen-free conditions in our facilities. Mice were infected by intraperitoneal injection of 10^6 pRBC. Blood was collected from the tail for determination of parasitemia, or from retro-orbital puncture under anesthesia for study of hematological parameters. On the onset of the neurological phase, ie, days 7 to 11, after anesthesia, brains were sampled, included in Tissue-Tek (Leica Microsystèmes, Rueil-Malmaison, France), snap-frozen in liquid nitrogen, and then stored at −80°C.

Immunohistochemistry

For immunostaining, 7-μm frozen sections were incubated overnight at 4°C with primary monoclonal antibodies directed against murine ICAM-1 (3E2), VCAM-1(429), CD40 (3/23), LFA-1(M17/4) (Pharmingen, San Diego, CA), and CD41 (MWReg30)[20] after saturation with normal rabbit serum. After washing, sections were incubated for 1 hour at room temperature with biotinylated goat polyclonal antibodies anti-rat or anti-hamster IgG, followed by the addition of HRPO-avidin (anti-rat or anti-hamster ABC kits; Vector, Peterborough, England). Color reaction was obtained by the addition of diaminobenzidine substrate-chromogen (Sigma, Saint-Quentin Fallavier, France). Slides were counterstained with Mayer's hematoxylin before permanent mounting with Entellan (Merck, Brussels, Belgium). Slides were pictured at ×400 magnification using an Eclipse 800 microscope (Nikon, Champigny-sur-Marne, France) and a digital camera; vessel labeling was then analyzed by quantitative digitalized image analysis using Lucia software (Nikon). At least 10 brains were sampled per group and computerized image analysis was performed on an average of 10 microphotographs per mouse.

Tumor Necrosis Factor (TNF) Assay

On the same samples, diluted 1:2 in the recommended buffer, TNF levels were measured using the Quantikine ELISA kit (R&D Systems, Lille, France). Macrophage cell culture supernatants were diluted similarly.

HDL Analysis

Lipid analysis was performed as described.[21] Briefly, serum concentrations of cholesterol were determined using enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany). The distribution of cholesterol over the different lipoproteins in serum was analyzed by fractionation of 30 μl of serum of each mouse using a Superose 6 column (3.2 × 30 mm, Smartsystem; Pharmacia, Uppsala, Sweden). Total cholesterol content of the effluent was determined using enzymatic colorimetric assays (Roche Diagnostics), taking the efficiency of recovery from the column into account.

MP Analysis

Blood samples were collected on 200 μl of 0.129 mol/L sodium citrate. Platelet-free plasma was prepared as previously described.[5] Briefly, samples were spun down at 1500 × *g* for 15 minutes, followed by a 2-minute centrifugation at 13,000 × *g*, were then labeled with fluorescein isothiocyanate (FITC)-annexin V (Beckman Coulter Immunotech, Marseille, France) for 15 minutes at room temperature, and then diluted in 500 μl of binding

buffer. Samples were then analyzed using an Epics XL flow cytometer (Beckman Coulter, Villepinte, France).

Platelet-free plasma from either uninfected or PbA-infected ABCA1$^{-/-}$ and ABCA1$^{+/+}$ was incubated with specific monoclonal antibodies directed against endothelium ($\alpha_v\beta3$, CD51; Becton Dickinson Pharmingen), monocytes (CD14, Becton Dickinson Pharmingen), and platelets (GPIIbIIIa, CD41), followed by a FITC-F(ab)$'_2$-goat anti-rat secondary antibody. After addition of phosphate-buffered saline (PBS), samples were analyzed by flow cytometry and the percentages of each cell-derived MPs were determined.

Procoagulant Activity of MPs

Plasma from PbA-infected mice on day 8 after infection were prepared as described above (ABCA1$^{+/+}$, $n = 14$; ABCA1$^{-/-}$, $n = 10$) and centrifuged twice at 20,000 $\times$ *g* for 1 hour. After each step, MP pellet was resuspended in PBS. Pellets from centrifugation 1 and 2 were pooled. The total MP number present in each pellet was quantified by FITC-annexinV labeling. Numbers of MPs of each sample were adjusted, by dilution with Owren-Koller buffer (Diagnostica Stago, France), to the lowest number obtained. Procoagulant activity of MPs was quantified as the reduction of Howell clotting time of a normal mouse plasma pool (N plasma) using a modified method published by Combes and colleagues.[5] Briefly, 75 μl of N plasma were mixed, in a glass tube containing a magnet, with 37.5 μl of Owren-Koller buffer (control) or 37.5 μl of MP suspension (assay). Clotting reaction was initiated by the addition of 37.5 μl of 100 mmol/L $CaCl_2$. The reaction was performed in a water bath under stirring at 37°C and the time for the clot to form was recorded. Procoagulant activity was expressed as the percentage of reduction of the control clotting time: (1-ratio assay/control) $\times$ 100.

Proinflammatory Potential of MPs

MP pellet were prepared as above, resuspended in Dulbecco's modified Eagle's medium-F12, and counted by flow cytometry using FITC-annexin V labeling. Noninfected mouse peritoneal exudate cells (100,000 macrophages/well) were seeded overnight in RPMI 10%-fetal calf serum medium at 37°C. Adherent cells were washed and co-cultured in 96-well flat-bottomed culture plates with either RPMI medium alone or supplemented with lipopolysaccharide (LPS) (2 μg/ml), MPs (from infected or noninfected ABCA1$^{+/+}$ mice) in a 1:7 ratio (macrophage:MP) for 3 hours. Plates were then centrifuged at 1000 $\times$ *g* for 5 minutes and supernatants were harvested and kept frozen at −20°C until TNF assay.

Platelet Activation Assay

After blood collection from noninfected mice, platelet-rich plasma was prepared by centrifugation at 200 $\times$ *g* during 15 minutes. Platelet-rich plasma was then activated 45 minutes at 37°C with either calcium ionophore (A23187, 5 μmol/L; Sigma) or ADP (10 μmol/L, Sigma). After activation, plasma was prepared by two successive centrifugations at 1500 $\times$ *g* during 15 minutes and 13,000 $\times$ *g* during 2 minutes. Plasma was then labeled with FITC-annexin V and MPs were counted by flow cytometry.

Macrophage Activation Assay

Macrophages from noninfected mice were collected 4 days after intraperitoneal injection with 3% thioglycollate (Invitrogen, Cergy-Pontoise, France). Thioglycollate-induced macrophages were purified by peritoneal cavity washes using ice-cold Hanks' balanced salt solution (Invitrogen). Contaminating red blood cells were removed by incubation for 10 minutes at 37°C in the dark in lysis buffer (NH_4Cl, 8.26 g/L^{-1}; $NaHCO_3$, 1.00 g/L^{-1}, ethylenediaminetetraacetic acid (4 Na) 0.037 g/L^{-1}, pH 7.3). After washes, cells were allowed to adhere in 24-well flat-bottom culture plates at a density of 1×10^5 cells per well, overnight, in medium (Dulbecco's modified Eagle's medium/F12 and 10% fetal calf serum, Invitrogen). Cells were then washed and placed in serum-free medium (Dulbecco's modified Eagle's medium/F12) for 2 hours. Medium was then discarded and cells were activated either overnight with LPS (10 μg/ml^{-1}) or for 6 hours with A23187 (5 μmol/L, Sigma). Activation supernatant was then removed, centrifuged 2 minutes at 13,000 $\times$ *g* to pellet cells in suspension, and labeled with FITC-annexin V before flow cytometric analysis. All these experiments were performed three times on cells from five mice per group.

Statistical Analysis

Survival curves (Kaplan-Meier), nonparametric Mann-Whitney *U*-tests, and χ^2 tests were performed with GraphPad Prism 4.0 software and $P < 0.05$ was considered significant. Results are expressed as mean ± SEM or SD as mentioned. Linear regression was performed to compare regression lines from MP procoagulant activities.

Results

ABCA1 Gene Inactivation Fully Protects against CM

Series of ABCA1 KO (ABCA1$^{-/-}$) and ABCA1 WT (ABCA1$^{+/+}$) DBA/1 mice were infected by PbA and subsequently monitored for the development of cerebral signs. The ABCA1$^{+/+}$ mice died within the same time frame as CM susceptible mice of C57BL/6 strain, ie, during the second week of infection, referred to as the "neurological" phase (Figure 1a). As expected, ABCA1 WT mice presented evident clinical signs of neurological syndrome; these were not seen in ABCA1 KO mice, despite identical levels of parasitemia on day 8 of infection (18% and 17%, respectively).[17] In ABCA1$^{-/-}$ mice, death occurred during the third week of PbA infection in the context of severe anemia (Figure 1a). The evaluation of the cumulative incidence of the neurological syndrome

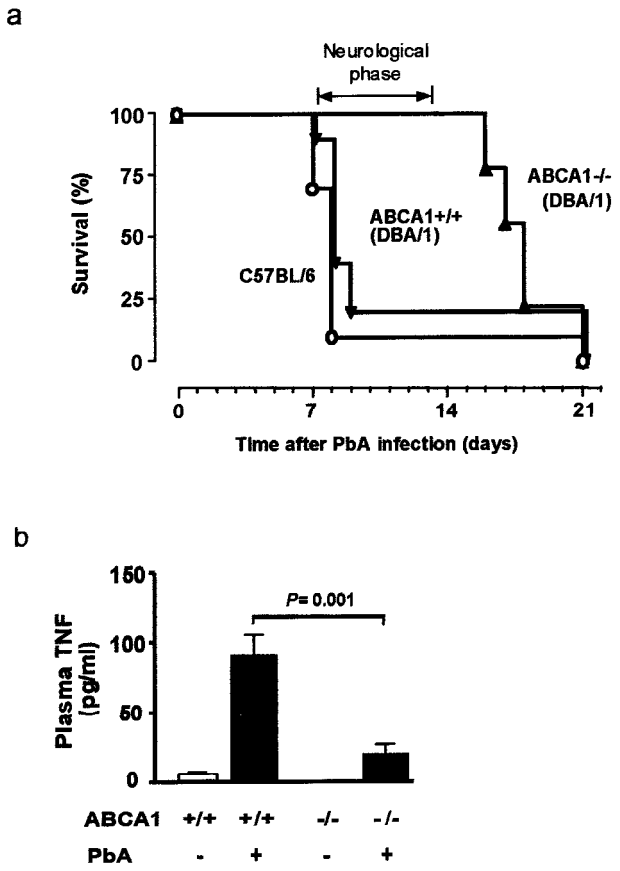

Figure 1. Susceptibility to CM: relationship to circulating MP levels and to ABCA1 gene. **a:** Survival curves show that the sole absence of the ABCA1 gene ($ABCA1^{-/-}$, $n = 27$) significantly (Kaplan-Meier, $P < 0.01$) prolonged survival because of the abolition of the cerebral pathology, responsible for deaths in the neurological phase. In contrast, the $ABCA1^{+/+}$ mice ($n = 24$), on the same DBA/1 background were as susceptible as C57BL/6 mice. **b:** At day 8 after PbA infection, plasma TNF levels were significantly lower in $ABCA1^{-/-}$ ($n = 15$) than in $ABCA1^{+/+}$ ($n = 14$) mice. Results are expressed as mean ± SEM.

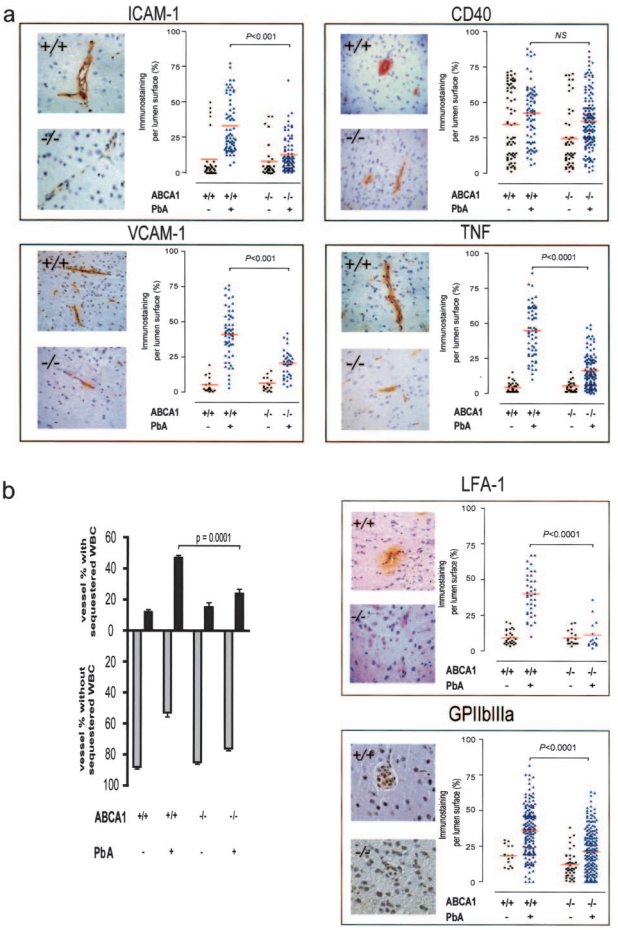

Figure 2. Phenotypic analysis of brain microvessels from $ABCA1^{+/+}$ and $ABCA1^{-/-}$ mice on infection by PbA. **a:** No up-regulation of TNF or of the endothelial adhesion molecules, ICAM-1 and VCAM-1, was detected on PbA infection in $ABCA1^{-/-}$ mice, as compared to $ABCA1^{+/+}$. In contrast, CD40 expression was comparable in $ABCA1^{-/-}$ and $ABCA1^{+/+}$ mice. **b:** Percentages of vessels (±SEM, at least 10 mice per group) with sequestered white blood cells (WBC, two or more per vessel) showed a dramatic increase on infection in $ABCA1^{+/+}$ mice, whereas this increase was not significant in $ABCA1^{-/-}$ mice. Immunohistochemistry, using standard peroxidase staining as described, and quantitative image analysis for LFA-1 and GPIIbIIIa showed a marked up-regulation of both molecules in $ABCA1^{+/+}$ mice, but not in $ABCA1^{-/-}$ mice. Original magnifications, ×400.

revealed a complete protection in $ABCA1^{-/-}$ mice, whereas $ABCA1^{+/+}$ and heterozygous $ABCA1^{+/-}$ mice showed an incidence of 88% and 75%, respectively.

Because TNF represents a key element in the development of the neurological phase, the levels of this cytokine were monitored after PbA infection. Immunoreactive plasma TNF levels were only moderately increased in PbA-infected $ABCA1^{-/-}$ mice, whereas the $ABCA1^{+/+}$ mice showed the dramatic increase known to occur in CM-S strains (0.5 ± 0.5 versus 18.3 ± 4.4, $P = 0.0002$, and 0.9 ± 0.7 versus 77.4 ± 14.7 pg/ml, $P < 0.0001$, respectively; Figure 1b). These observations suggest that contrary to their wild-type counterpart, on PbA infection, $ABCA1^{-/-}$ mice behave like other CM-R mice such as BALB/c or DBA-2.

Immunopathology of Brain Lesions of $ABCA1^{-/-}$ Mice

To better characterize the protection of $ABCA1^{-/-}$ mice, we analyzed by immunohistochemistry brain specimens sampled at day 7 after infection, to address the activation status of three cells types involved in CM pathogenesis: endothelium, leukocytes, and platelets. In infected mice endothelium, the up-regulation of ICAM-1 and VCAM-1 induced by infection was significantly lower in $ABCA1^{-/-}$ compared to $ABCA1^{+/+}$ mice ($P < 0.001$; Figure 2a, top and bottom left). The presence of intravascular TNF was also significantly reduced in $ABCA1^{-/-}$ mice ($P < 0.0001$; Figure 2a, bottom right). In contrast, the expression of CD40 (Figure 2a, top right), and of iNOS (data not shown) was comparable in $ABCA1^{+/+}$ and $ABCA1^{-/-}$ mice.

As expected from the absence of neurological signs, the incidence of perivascular hemorrhages were less abundant in $ABCA1^{-/-}$ animals (data not shown). Furthermore, there was a significantly lower percentage of vessels with sequestered cells when compared to lesions in wild-type animals (Figure 2b, top left; $P = 0.0001$). In addition, LFA-1 expression was significantly lower in mi-

Table 1. HDL Levels in ABCA1$^{-/-}$ Mice Compared to Apo-AI$^{-/-}$

Strain	Phenotype	HDL-Cholesterol levels (μg/ml)	Incidence of the syndrome
DBA/1	ABCA1$^{+/+}$	564 ± 65	90%
DBA/1	ABCA1$^{-/-}$	64 ± 1	0%
C57BL/6–129	Apo-AI$^{+/+}$	424 ± 69	85%
C57BL/6–129	Apo-AI$^{-/-}$	148 ± 33	100%

A mere modification of plasma HDL levels was not able per se to prevent CM. Apo-AI$^{-/-}$ mice showed reduced plasma HDL but were fully susceptible to CM, as indicated by the cumulative incidence (n = 7 per group).

crovessels from ABCA1$^{-/-}$ mice than in those from wild-type controls (Figure 2b, top right; $P < 0.0001$). This suggests lower numbers of sequestered leukocytes, or of platelets, because LFA-1 is also expressed on their surface.[22,23] Indeed, platelets were found in reduced numbers in the vascular lumen of infected ABCA1$^{-/-}$ mice, as quantitated by GPIIbIIIa staining (Figure 2b, bottom right; $P < 0.0001$). Overall these data indicate an impaired cellular activation in ABCA1$^{-/-}$ mice during the malarial infection.

The reduction in the sequestration of leukocytes in the brain vessels of ABCA1 KO mice on infection prompted us to investigate the role of bone marrow (BM)-derived cells into the generation of resistance in ABCA1 KO mice. We thus assessed the response to PbA infection into BM chimeric mice between the two strains. However, total body irradiation modified the survival curves in the control grafts, ie, ABCA1 WT into WT and ABCA1 KO into KO (not shown and N.H. Hunt, personal communication), thus hampering a conclusive interpretation of the results. Nonetheless, the chimeras harboring ABCA1 KO BM into ABCA1 WT recipients died between day 8 and day 22 after infection (five of seven with clinical signs of CM). Conversely, grafting BM from ABCA1 WT mice into ABCA1 KO resulted in the death of all mice (n = 7) by severe anemia without signs of CM.

Because the ABCA1 transporter plays a key role in lipid metabolism and its functional impairment results in drastically reduced plasma HDL levels, it was mandatory to investigate whether protection against CM occurred as a mere consequence of dyslipidemia. We thus studied the response to PbA infection of Apo-AI-deficient mice, which also show low levels of plasma HDL.[19] Despite similar incidence of the neurological syndrome in the wild-type corresponding strains, Apo-AI$^{-/-}$ mice developed CM similarly to these susceptible strains (Table 1), showing that the low levels of HDL cannot explain by themselves the protection of ABCA1$^{-/-}$ mice.

MP Production Is Reduced in ABCA1$^{-/-}$ Mice

Another feature of ABCA1$^{-/-}$ mice is the impaired capacity to produce erythrocytic MPs in response to agonists.[1] We first turned our attention to plasma MP levels, quantitated by flow cytometry on day 7 or 8 after infection, as a reflection of the capability of blood cells to vesiculate in response to PbA infection. We observed a

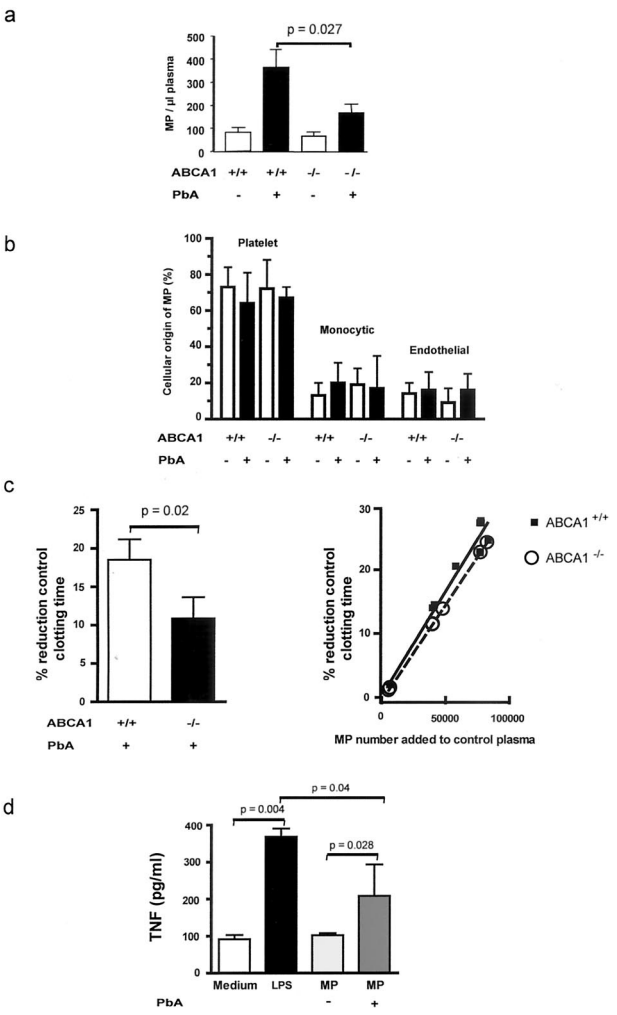

Figure 3. Parameters of the host response in ABCA1$^{+/+}$ and ABCA1$^{-/-}$ mice on PbA infection. **a:** On day 8 of PbA infection, plasma MP numbers raised significantly less in ABCA1$^{-/-}$ (n = 18) than in ABCA1$^{+/+}$ mice (n = 13). **b:** No modification was seen in cellular origins of plasma MP in ABCA1$^{+/+}$ and ABCA1$^{-/-}$ (n = 5 mice per group), before and 8 days after PbA infection. **c:** On day 8 of infection, MP isolated from plasma of ABCA1$^{+/+}$ mice (n = 14) displayed a stronger reduction of the control clotting time (**left**) than those from ABCA1$^{-/-}$ mice (n = 10). This reduction was dependent on the number of MP added but remained significantly higher for ABCA1$^{+/+}$ than for ABCA1$^{-/-}$ mice (**right**). **d:** On day 8 of infection, peritoneal macrophages were incubated with either medium (+), LPS (**black columns**, 2 μg/ml), MP isolated from plasma of ABCA1$^{+/+}$ mice (**dark gray columns**, n = 18), or from noninfected mice (**light gray columns**, n = 9) during 3 hours, and TNF released in the culture supernatant was measured by ELISA. Results are expressed as mean ± SEM.

dramatic rise in MP plasma levels in ABCA1$^{+/+}$ mice, whereas it was significantly less marked in their ABCA1$^{-/-}$ counterparts (P = 0.03 versus 0.009; Figure 3a). Using flow cytometry, we then estimated the cell origin of MPs present in the plasma. This phenotyping indicated that MPs were mainly of platelet origin, whereas monocyte and endothelial MPs accounted for less than 30% (Figure 3b). Interestingly, the cellular origin of MPs was similar in both ABCA1$^{-/-}$ mice and in ABCA1$^{+/+}$ mice. In addition, although the number of total plasma MPs was different between ABCA1$^{+/+}$ and ABCA1$^{-/-}$ mice on infection, the cell origin of these MPs was not significantly modified by this infection (Figure 3b).

MPs from ABCA1$^{-/-}$ Mice Show a Reduced Procoagulant Activity

The procoagulant activity of MPs isolated from PbA-infected mice was determined using a modified protocol of the Howell clotting time. The procoagulant activity was expressed as the capacity of a suspension of MPs to reduce the clotting time of normal plasma. MPs from PbA-infected ABCA1$^{+/+}$ mice reduced more strongly the clotting time than those from PbA-infected ABCA1$^{-/-}$ (Figure 3c, left; $P = 0.02$). Moreover when increasing numbers of MPs were added to the control plasma, we found, in both mouse strains, a linear relationship between the reduction of the clotting time and the number of MPs (Figure 3c, right). A linear regression between the two curves showed that although the slopes are not significantly different, the elevations are highly significantly different ($P = 0.0065$), indicating that the two curves are distinct and that the ABCA1$^{+/+}$ curve is above the ABCA1$^{-/-}$ one. Altogether, these data and the observed higher numbers of circulating MPs in ABCA1$^{+/+}$ mice, converge on a higher circulating MP-derived procoagulant activity in the plasma of ABCA1$^{+/+}$ mice.

MPs from PbA-Infected ABCA1$^{+/+}$ Mice Display a Higher Proinflammatory Activity than Those MPs from Noninfected Mice

When added to macrophages at a ratio of 1:7 (macrophage:MP), MPs isolated from the plasma of noninfected mice induced a significantly lower release of TNF (Figure 3d; $P = 0.028$) than MPs isolated from PbA-infected mice. As a control, macrophages were incubated with medium alone or LPS and, as expected, LPS induced a significantly higher TNF release than either medium alone or MPs from PbA-infected mice (Figure 3d; $P = 0.004$ and $P = 0.04$, respectively). These data show that MPs produced in conditions of infection display a proinflammatory potential that is not present on the MPs produced in physiological conditions.

Cellular Responses to Vesiculation Agonists

Platelets and monocyte/macrophages are two cell types known to play a crucial role in the pathogenesis of the cerebral syndrome. As we observed a reduced number of MPs in the plasma, we assessed whether these two cell types present an altered response to *in vitro* stimulation by agonists of vesiculation as it has already been shown for erythrocytes.[1] Platelets were purified from whole blood and activated with ADP and A23187. In parallel, thioglycollate-induced peritoneal macrophages from noninfected mice were restimulated *in vitro* with calcium ionophore (A23187) and LPS. Although both platelets (Figure 4a) and macrophages (Figure 4b) purified from ABCA1$^{+/+}$ mice showed the significant increase in vesiculation similar to the one known to occur in human cells,[11] in ABCA1$^{-/-}$ mice, platelets showed a complete lack of vesiculation and macrophages presented a reduced capacity to release MPs when submitted to the agonists. Moreover, stimulation-induced MP release was statistically higher in ABCA1$^{+/+}$ than in ABCA1$^{-/-}$ mice for both platelets (ADP, $P = 0.04$; A23187, $P = 0.01$) and macrophages (LPS, $P = 0.013$; A23187, $P = 0.024$). These data thus confirm and extend the previous findings of reduced membrane vesiculation from ABCA1$^{-/-}$ cells *in vitro* and evidence a similar lack of response after *in vivo* stimulation of the animals by plasmodial antigens.

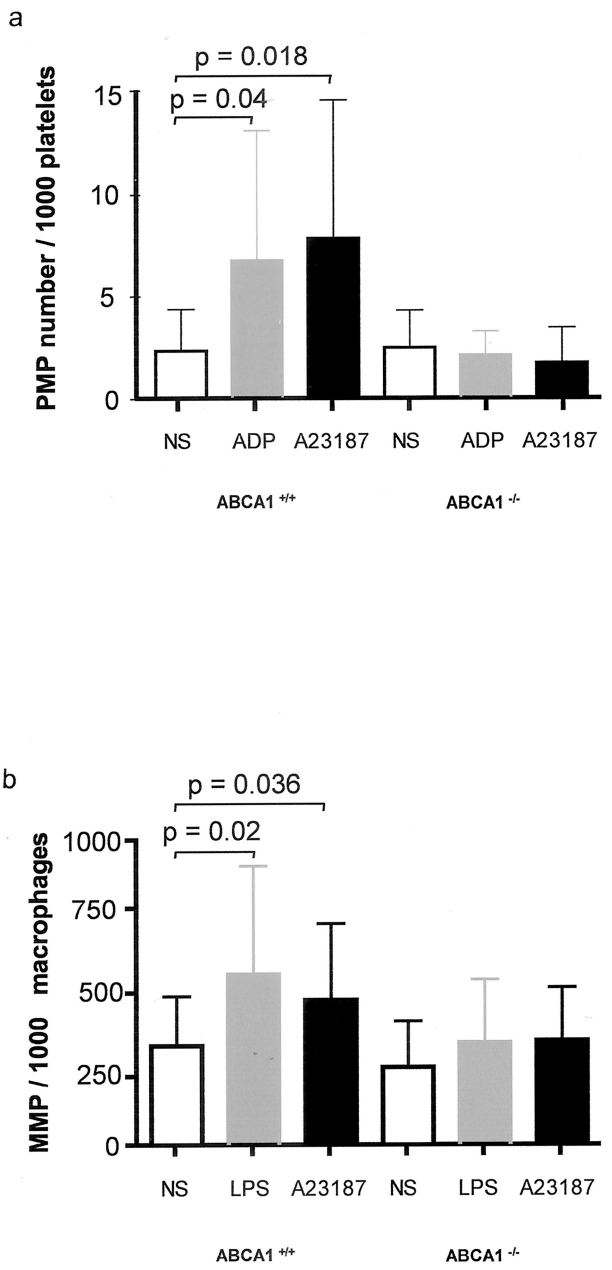

Figure 4. MP release capacity of platelets and macrophages. The release of MPs after activation with vesiculation agonists by platelets (**a**) and macrophages (**b**) was impaired in uninfected ABCA1$^{-/-}$ mice ($n = 8$) as compared to ABCA1$^{+/+}$ mice ($n = 9$). Platelets were stimulated with either ADP (**gray columns**, 10 nmol/L) or A23187 (**black columns**, 5 μmol/L) and macrophages with either LPS (**gray columns**, 10 μg/ml) or A23187 (**black columns**, 5 μmol/L). Results are expressed as mean ± SD.

Discussion

In this study we evidenced a direct relationship between the complete resistance to CM and the functional inactivation of the ABCA1 gene. We could indeed exclude any modification in susceptibility to infection by PbA consequent to the inactivation of gene, as witnessed by the similar levels of parasitemia in ABCA1$^{+/+}$ and ABCA1$^{-/-}$ animals. The ABCA1 gene is known to be a crucial determinant of reverse cholesterol transport and to greatly alter lipid metabolism *in vivo*. However, we demonstrated that low levels of plasma HDL per se are not sufficient to alter the incidence of CM. Indeed, mice with similarly reduced HDL levels, but consequent to the inactivation Apo-AI gene, another key regulator of lipid metabolism, are not protected against CM. Thus, the intrinsic activity of ABCA1 gene rather than its metabolic consequences are to be considered causal in the reversion of susceptibility of DBA1 mice to CM.

On malaria infection, in ABCA1$^{-/-}$ mice, we evidenced a lower activation status of the cells present in the brain lesion, as showed by the weaker up-regulation of endothelial adhesion molecules in brain microvessels such as ICAM-1 and VCAM-1, the reduced leukocyte sequestration, as well as the ablated platelet accumulation. The endothelial alteration, subsequent to inflammatory cytokine release, is a crucial step in the pathogenesis of CM.[18,24] The absence of elevated TNF levels in the plasma of PbA-infected ABCA1 KO mice could therefore partly explain this impaired cell reactivity. A similar lack of ICAM-1 up-regulation correlated with protection has been found in TNFR2-deficient mice[25] and, more recently, in a series of cytokine knockout (TNF, interleukin-12, or interferon-γ) and T-cell-deficient Rag1$^{-/-}$ mice, on infection by PbA.[26] Besides, ICAM-1$^{-/-}$ mice infected by PbA do not develop CM.[27] A modulation of lymphotoxin (LT) could also be envisaged since recent data have shown that LT$^{-/-}$ mice are protected against the neurological syndrome.[28] The lack of platelet accumulation in brain vessels from PbA-infected ABCA1$^{-/-}$ mice could also be explained by the fact that platelets from ABCA1$^{-/-}$ mice display an impaired platelet aggregation in whole blood on *in vitro* ADP stimulation[29] and thus present a compromised platelet function.

Because of its flippase activity, ABCA1 is one of the determinants of the lipid transbilayer remodeling, and thereby is involved in MP production. Indeed, ABCA1 deficiency led to a reduced externalization ability and consecutive membrane vesiculation of red blood cells.[1] In addition to this *in vitro* role on vesiculation, we demonstrated here that ABCA1 is necessary for an enhancement of the *in vivo* vesiculation during the course of PbA infection. This is consistent with the previous demonstration that reducing platelet-derived MP levels via caspase inhibitors lowers CM incidence.[16] Moreover, we found that platelets and macrophages of ABCA1-deficient mice are significantly less reactive in terms of MP production when restimulated *in vitro*. Indeed, the ABCA1 transporter controls the outward translocation of PS at the plasma membrane[3] and is thus likely to affect the vesiculation process. On the other hand, we provide evidence that, on infection, MPs can be envisaged as pathogenic elements in view of their procoagulant and proinflammatory properties. Indeed, we demonstrated here that, added at identical numbers, MPs derived from the plasma of PbA-infected ABCA1$^{+/+}$ mice displayed a higher procoagulant activity than those derived from the plasma of ABCA1$^{-/-}$ mice. This suggests that because the absolute number of MPs in the plasma is higher in WT than in KO mice, the global circulating procoagulant activity is therefore higher in WT. This is compatible with a higher susceptibility of these mice to develop thrombotic events within the vessels where the endothelial activation is the highest. However, ABCA1 knockout mice produce less TNF during infection (Figure 1b) and this may explain their relative resistance to CM. This finding is consistent with the importance of proinflammatory cytokines in CM.

Similarly, we demonstrated that MP isolated from PbA-infected mice display a higher proinflammatory activity than those isolated from noninfected mice. This suggests that MPs produced during malarial infection could be pathogenic also via the induction of the release of proinflammatory mediators, such as TNF, by circulating mononuclear cells. This would maintain an inflammatory atmosphere within the vessels, in a way that favors a worsening of the endothelial lesion occurring during CM. To further assess this pathogenic role of MPs, it will be worth assessing whether resistance to CM invariably accompanies the down-modulation of genes that, like ABCA1, control vesicle formation from membranes.

The finding that ABCA1 deletion confers a complete protection against CM, associated to an impaired MP production, orientates toward new therapeutic approaches to the search for candidate genes controlling vesiculation. Vesiculation also is implicated in diseases such as atherosclerosis in which one of the main goals of research is to prevent the thrombogenic effect of PS in the atherosclerotic plaque by preventing both MP release and PS exposure at the cell surfaces. Thus, lower MP levels are likely to reduce the incidence of CM and thrombotic diseases.[4,30] Although these data may appear contradictory with the known anti-atherogenic properties of the ABCA1,[31] one has to keep in mind that CM is an acute thrombotic event limited to microvessels whereas atherosclerosis is a chronic alteration of cell lipid metabolism essentially expressed in macrovessels. Taken together, our data provide new insights in CM pathogenesis and suggest that interventions on the ABCA1 gene product could be of interest, not only in severe malaria, but more widely in thrombotic disorders.

References

1. Hamon Y, Broccardo C, Chambenoit O, Luciani MF, Toti F, Chaslin S, Freyssinet JM, Devaux PF, McNeish J, Marguet D, Chimini G: ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. Nat Cell Biol 2000, 2:399–406
2. Oram JF: Tangier disease and ABCA1. Biochim Biophys Acta 2000, 1529:321–330
3. Daleke DL: Regulation of transbilayer plasma membrane phospholipid asymmetry. J Lipid Res 2003, 44:233–242
4. Freyssinet JM: Cellular microparticles: what are they bad or good for? J Thromb Haemost 2003, 1:1655–1662

5. Combes V, Simon AC, Grau GE, Arnoux D, Camoin L, Sabatier F, Mutin M, Sanmarco M, Sampol J, Dignat-George F: In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. J Clin Invest 1999, 104:93–102
6. Nieuwland R, Berckmans RJ, Rotteveel-Eijkman RC, Maquelin KN, Roozendaal KJ, Jansen PG, ten Have K, Eijsman L, Hack CE, Sturk A: Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. Circulation 1997, 96:3534–3541
7. Joop K, Berckmans RJ, Nieuwland R, Berkhout J, Romijn FP, Hack CE, Sturk A: Microparticles from patients with multiple organ dysfunction syndrome and sepsis support coagulation through multiple mechanisms. Thromb Haemost 2001, 85:810–820
8. Sabatier F, Darmon P, Hugel B, Combes V, Sanmarco M, Velut JG, Arnoux D, Charpiot P, Freyssinet JM, Oliver C, Sampol J, Dignat-George F: Type 1 and type 2 diabetic patients display different patterns of cellular microparticles. Diabetes 2002, 51:2840–2845
9. Nieuwland R, Berckmans RJ, McGregor S, Boing AN, Romijn FP, Westendorp RG, Hack CE, Sturk A: Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. Blood 2000, 95:930–935
10. Osmanovic N, Romijn FP, Joop K, Sturk A, Nieuwland R: Soluble selectins in sepsis: microparticle-associated, but only to a minor degree. Thromb Haemost 2000, 84:731–732
11. Ogura H, Kawasaki T, Tanaka H, Koh T, Tanaka R, Ozeki Y, Hosotsubo H, Kuwagata Y, Shimazu T, Sugimoto H: Activated platelets enhance microparticle formation and platelet-leukocyte interaction in severe trauma and sepsis. J Trauma 2001, 50:801–809
12. Fujimi S, Ogura H, Tanaka H, Koh T, Hosotsubo H, Nakamori Y, Kuwagata Y, Shimazu T, Sugimoto H: Activated polymorphonuclear leukocytes enhance production of leukocyte microparticles with increased adhesion molecules in patients with sepsis. J Trauma 2002, 52:443–448
13. Geisbert TW, Young HA, Jarling PB, Davis KJ, Kagan E, Hensley LE: Mechanisms underlying coagulation abnormalities in Ebola hemorrhagic fever: overexpression of tissue factor in primate monocytes/macrophages is a key event. J Infect Dis 2003, 188:1616–1629
14. Mack M, Kleinschmidt A, Bruhl H, Klier C, Nelson PJ, Cihak J, Plachy J, Stangassinger M, Erfle V, Schlondorff D: Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. Nat Med 2000, 6:769–775
15. Hrachovinova I, Cambien B, Hafezi-Moghadam A, Kappelmayer J, Camphausen RT, Widom A, Xia L, Kazazian Jr HH, Schaub RG, McEver RP, Wagner DD: Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A. Nat Med 2003, 9:1020–1025
16. Piguet PF, Kan CD, Vesin C: Thrombocytopenia in an animal model of malaria is associated with an increased caspase-mediated death of thrombocytes. Apoptosis 2002, 7:91–98
17. Lou J, Lucas R, Grau GE: Pathogenesis of cerebral malaria: recent experimental data and possible applications for humans. Clin Microbiol Rev 2001, 14:810–820
18. Hunt NH, Grau GE: Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. Trends Immunol 2003, 24:491–499
19. Williamson R, Lee D, Hagaman J, Maeda N: Marked reduction of high density lipoprotein cholesterol in mice genetically modified to lack apolipoprotein A-I. Proc Natl Acad Sci USA 1992, 89:7134–7138
20. Nieswandt B, Echtenacher B, Wachs FP, Schroder J, Gessner JE, Schmidt RE, Grau GE, Mannel DN: Acute systemic reaction and lung alterations induced by an antiplatelet integrin gpIIb/IIIa antibody in mice. Blood 1999, 94:684–693
21. Van Eck M, Herijgers N, Yates J, Pearce NJ, Hoogerbrugge PM, Groot PH, Van Berkel TJ: Bone marrow transplantation in apolipoprotein E-deficient mice. Effect of ApoE gene dosage on serum lipid concentrations, (beta)VLDL catabolism, and atherosclerosis. Arterioscler Thromb Vasc Biol 1997, 17:3117–3126
22. Piguet PF, Vesin C, Ryser JE, Senaldi G, Grau GE, Tacchini-Cottier F: An effector role for platelets in systemic and local lipopolysaccharide-induced toxicity in mice, mediated by a CD11a- and CD54-dependent interaction with endothelium. Infect Immun 1993, 61:4182–4187
23. Grau GE, Tacchini-Cottier F, Vesin C, Milon G, Lou JN, Piguet PF, Juillard P: TNF-induced microvascular pathology: active role for platelets and importance of the LFA-1/ICAM-1 interaction. Eur Cytokine Netw 1993, 4:415–419
24. Grau GE, Pointaire P, Piguet PF, Vesin C, Rosen H, Stamenkovic I, Takei F, Vassalli P: Late administration of monoclonal antibody to leukocyte function-antigen 1 abrogates incipient murine cerebral malaria. Eur J Immunol 1991, 21:2265–2267
25. Lucas R, Juillard P, Decoster E, Redard M, Burger D, Donati Y, Giroud C, Monso-Hinard C, De Kesel T, Buurman WA, Moore MW, Dayer JM, Fiers W, Bluethmann H, Grau GE: Crucial role of tumor necrosis factor (TNF) receptor 2 and membrane-bound TNF in experimental cerebral malaria. Eur J Immunol 1997, 27:1719–1725
26. Bauer PR, Van Der Heyde HC, Sun G, Specian RD, Granger DN: Regulation of endothelial cell adhesion molecule expression in an experimental model of cerebral malaria. Microcirculation 2002, 9:463–470
27. Favre N, Da Laperousaz C, Ryffel B, Weiss NA, Imhof BA, Rudin W, Lucas R, Piguet PF: Role of ICAM-1 (CD54) in the development of murine cerebral malaria. Microbes Infect 1999, 1:961–968
28. Engwerda CR, Mynott TL, Sawhney S, De Souza JB, Bickle QD, Kaye PM: Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria. J Exp Med 2002, 195:1371–1377
29. Orso E, Broccardo C, Kaminski WE, Bottcher A, Liebisch G, Drobnik W, Gotz A, Chambenoit O, Diederich W, Langmann T, Spruss T, Luciani MF, Rothe G, Lackner KJ, Chimini G, Schmitz G: Transport of lipids from Golgi to plasma membrane is defective in Tangier disease patients and Abc1-deficient mice. Nat Genet 2000, 24:192–196
30. VanWijk MJ, VanBavel E, Sturk A, Nieuwland R: Microparticles in cardiovascular diseases. Cardiovasc Res 2003, 59:277–287
31. Tall AR, Costet P, Wang N: Regulation and mechanisms of macrophage cholesterol efflux. J Clin Invest 2002, 110:899–904