Defibrotide Interferes With Several Steps of the Coagulation-Inflammation Cycle and Exhibits Therapeutic Potential to Treat Severe Malaria

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Objective—The coagulation-inflammation cycle has been implicated as a critical component in malaria pathogenesis. Defibrotide (DF), a mixture of DNA aptamers, displays anticoagulant, anti-inflammatory, and endothelial cell (EC)-protective activities and has been successfully used to treat comatose children with veno-occlusive disease. DF was investigated here as a drug to treat cerebral malaria.

Methods and Results—DF blocks tissue factor expression by ECs incubated with parasitized red blood cells and attenuates prothrombinase activity, platelet aggregation, and complement activation. In contrast, it does not affect nitric oxide bioavailability. We also demonstrated that Plasmodium falciparum glycosylphosphatidylinositol (*Pf*-GPI) induces tissue factor expression in ECs and cytokine production by dendritic cells. Notably, dendritic cells, known to modulate coagulation and inflammation systemically, were identified as a novel target for DF. Accordingly, DF inhibits Toll-like receptor ligand-dependent dendritic cells activation by a mechanism that is blocked by adenosine receptor antagonist (8-p-sulfophenyltheophylline) but not reproduced by synthetic poly-A, -C, -T, and -G. These results imply that aptameric sequences and adenosine receptor mediate dendritic cells responses to the drug. DF also prevents rosetting formation, red blood cells invasion by P. falciparum and abolishes oocysts development in Anopheles gambiae. In a murine model of cerebral malaria, DF affected parasitemia, decreased IFN-γ levels, and ameliorated clinical score (day 5) with a trend for increased survival.

Conclusion—Therapeutic use of DF in malaria is proposed. **(*Arterioscler Thromb Vasc Biol*. 2012;32:786-798.)**

Key Words: anticoagulants ■ blood coagulation ■ endothelium ■ microcirculation ■ vascular biology

Malaria caused by Plasmodium falciparum remains a deadly disease whose mechanism of pathogenesis has been intensely investigated from various angles.[1–6] Despite production of novel antimalarial agents, the mortality associated with the severe disease (eg, cerebral malaria [CM]) remains very high, even when the best intensive-care support is available.[7] Therefore, the identification of novel adjuvant therapies is needed in an attempt to improve survival. More recently, the coagulation-inflammation cycle has emerged as a critical component of malaria pathogenesis. Accordingly, tissue factor (TF) has been identified in the endothelium of children who died from CM, and parasitized red blood cells (pRBC) were shown to amplify the coagulation cascade through the support of multimolecular coagulation complex formation.[8] The combination of pathological and laboratory features found in malaria lead to the proposal of the so-called TF model (TFM) for human malaria pathogenesis.[9]

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This model takes into account compensated or decompensated disseminated intravascular coagulation as relevant compo-

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nents of the disease. This notion is particularly important because sequestration, high procoagulant tonus, and EC activation—plus increased levels of inflammatory cytokines—are also present in uncomplicated malaria. Consumption of antihemostatics,[1] decrease in nitric oxide (NO) bioavailability,[2] distinct polymorphisms,[10] parasite-derived proteins (eg, HRPII),[11] and lipids (eg, Pf-GPI),[12] and events yet to be identified, may predispose to decompensation in cerebral and placental malaria.[9,13] Notably, it has been recently reported that activation of DCs by coagulation (eg, thrombin) in the lymphatic compartment plays a major role in orchestrating systemic inflammation and decompensation in endotoxemic mice by a mechanism involving the Protease-Activated Receptors (PAR) 1 and sphingosine 1-phosphate receptor 3 (S1P3) pathways.[14] This cascade of events escalates and results in organ dysfunction, leading to increase in mortality.[15,16] It also places DCs at the interface of coagulation and inflammation and suggests that targeting DC function may be useful in an attempt to change morbidity in clinical conditions associated with dysregulated coagulation-inflammation cycle. Experimentally, this has been successfully tested by pharmacological inhibition of PAR-1, or by PAR1 knockout mice whose DCs no longer respond to thrombin.[14] DCs have also been identified as a target for activated protein C and to mediate its anti-inflammatory effects in vivo.[17]

Because of the complexity of malaria pathogenesis, several steps should be considered as potential targets for treatment. In this context, DF is a derivative extracted from mammalian organs that can be obtained from the controlled depolymerization of porcine intestinal mucosal genomic DNA. This process generates a mixture composed mostly of low molecular weight DNA that has been identified as 90% single-strand aptamers and 10% double-strand DNA with an average molecular mass of 16.5 kDa (length 9- to 80-mer, 50-mer average).[18–20] During the last few years, a number of in vivo studies in humans have shown that DF has EC protective, antiischemic, and anti-inflammatory properties without intense systemic anticoagulant effects.[18–20] The mechanism of action of DF is not yet completely clear but it is related to its polyanionic nature,[19] interaction with plasma or matrix proteins,[21] binding to adenosine receptors (ARs),[22] and protection of the vascular endothelium.[23] Accordingly, in vitro or ex vivo studies have demonstrated that DF blocks TF and plasminogen activator inhibitor-1 expression induced by lipopolysaccharide (LPS) in ECs,[24] prevents EC apoptosis caused by fludarabine,[25] promotes expression of thrombomodulin by ECs,[18–20] and attenuates leukocyte-endothelial cell interaction in the rat mesenteric vascular bed with decreased expression of P-selectin.[18–20,26] DF also prevents TF and intercellular adhesion molecule 1 expression by autologous sera from hematopoietic stem cell transplantation patients.[27] Administration of DF to healthy volunteers causes a decrease of plasminogen activator inhibitor-1[28] and an increase of TF pathway inhibitor,[23] and prostacyclin (EC markers) by a mechanism that is attenuated by the AR antagonist theophylline.[29] This is consistent with DF targeting endothelium in vivo on one hand, and ARs modulating EC function on the other.[30] In other words, DF therapeutic value is the result of a combination of effects. This has raised much interest for the use of DF in clinical conditions at high hemorrhagic risk, including severe hepatic sinusoidal obstruction syndrome, also commonly referred to as veno-occlusive disease (VOD).[31]

VOD occurs in children who have received chemotherapy in preparation for stem cell or bone marrow transplantation; it is characterized by activation of ECs of the sinusoids of the liver and procoagulant tonus.[31] While this population is typically thrombocytopenic, no major bleeding or important side effects have been reported after therapy with DF.[18–20] In fact, DF (25–40 mg/Kg/d; 200 μg/mL plasma concentration)[28] is now used for the prophylaxis or treatment of VOD with a high survival rate (35% to 45%) according to different clinical trials.[18–20] These therapeutic aspects of DF are particularly relevant in severe P. falciparum infection, because children with malaria who become comatose are also typically thrombocytopenic and often present EC activation and dysregulated coagulation.[1]

Our aim in this study has been to study the effects of DF in key events associated with malaria pathogenesis in vitro and to evaluate its therapeutic potential in vivo using a murine model of CM. Our data show that DF interrupts several steps associated with malaria infection.

Methods

Culture of P. falciparum Parasites

Mycoplasma-free parasites (3D7) were thawed and initially grown in a 5% suspension of purified human O^+ RBCs in RPMI 1640 medium supplemented with 0.5% Albumax II, 2 g/L sodium bicarbonate, 0.1 mmol/L hypoxanthine, 25 mmol/L Hepes (pH 7.4), and 10 mg/L gentamicin, at 37°C, 5% O_2, 5% CO_2, 90% N_2 as reported[8] and in the online-only Data Supplement.

Purification of P. falciparum Glycosylphosphatidylinositol

Briefly, glycolipids were extracted three times with chloroform/methanol/water (C/M/W 10:10:3, by volume). P. falciparum Glycosylphosphatidylinositol (*Pf*-GPIs) were separated from contaminants by precipitation under a stream of nitrogen and submitted to thin layer chromatography. *Pf*-GPIs metabolically labeled in a glucose-free medium (Sigma) containing 0.5 mCi [3H]glucosamine (Hartmann Analytic, Braunschweig, Germany) were used as tracers as described in the online-only Data Supplement. Absence of endotoxin was verified by *Pf*-GPI probes with the Limulus Amebocyte Lysate kit QCL-100 (Bio-Whittaker, Walkersville, MD).

Culture of Human Dermal Microvascular ECs, TF Expression, and Prothrombinase Assembly by pRBCs

Adult human pooled microvascular ECs (MVECs) were grown in the presence of EBM-2 Plus. After trypsinization, MVECs were seeded at a density of 3×10^4 cells/cm^2 (96-well format) and grown until confluence and coincubated with pRBC in the presence and absence of DF. Assembly of the extrinsic Xnase by MVECs and prothrombinase was performed as described.[8] In some experiments, EC were incubated with *Pf*-GPI, and TF expression was estimated as above.

Measurement of Thrombin Generation in Plasma, Platelet Aggregation, and Complement Assays

Thrombin formation in plasma, platelet aggregation, and complement assays were estimated as described in detail in the online-only Data Supplement.

NO Consumption Assay

A 50-mL solution of 40 μmol/L DETA NONOate in PBS, pH 7.4, was prepared in a glass vessel actively purged with helium in-line with an NO chemiluminescence analyzer. This solution produced a steady-state NO signal of ≈50 to 70 mV, which was generated by the decay of DETA-NONOate and the release of NO. When the signal became stable, 50-μL samples of standards or DF were injected into the NONOate solution with and without DF, in the presence or absence of hemoglobin (online-only Data Supplement).

Hemoglobin Spectra in the Presence of DF

Absorbance scanning of hemoglobin was performed in a Carry 100 BIO and described in detail in the online-only Data Supplement.

DCs Assays

Bone marrow-derived DCs from C57BL/6 mice were generated as described.[32,33] Culture conditions, determination of cytokines and PGE_2 production in the supernatant, and evaluation of surface markers by flow cytometry are described in detail in the online-only Data Supplement. In some assays, DCs were incubated with DF or chemically synthesized poly-A, poly-C, poly-T, poly-G (or an equimolar mixture of each; poly-A, -C, -T, and -G) followed by addition of LPS or Pam3CSK4. In other assays, DCs were incubated with *Pf*-GPI, and cytokines production and CD40 expression were estimated as described in the online-only Data Supplement.

Growth Inhibition Assays

DF, poly-CT, or poly-A were added directly to P. falciparum cultures (3D7 strain) containing trophozoite-stage parasites. After 48 hours of culture, the respective numbers of trophozoites were determined by parasite LDH. In some experiments, DF was added to ring-stage RBC and development to trophozoites was assessed using Giemsa smears as described in detail in the online-only Data Supplement.

Invasion Assay

Purified merozoites (200 μL of 2×10^7/mL) were incubated with the indicated concentration of DF in triplicate for 2 minutes at RT in a total volume of 400 μL. Prewarmed RBC (5 μL of 20% Ht) was added to the mixture and incubated at 37°C for 1 hour. The effect of DF on invasion was measured by counting the number of newly formed rings on Giemsa-stained smears, as described in detail in the online-only Data Supplement.

Erythrocyte-Binding (Rosetting) and Membrane-Feeding Assays

Erythrocyte-binding assay was carried out as described previously.[34] Feeding assay, infection, and midgut dissection are reported in the online-only Data Supplement.

Animals, Malaria Infections, and DF Treatment

P. berghei ANKA pRBCs (1×10^6) were used to infect mice ($n=10$) intraperitoneally. DF (85 μL; 7 mg) was injected IP 3 times/day, starting from day 1 or 4 postinfection. In some experiments, blood was collected at day 5 to estimate platelet number and plasma levels of interferon-γ, interleukin (IL)-10, monocyte chemoattractant protein-1, RANTES, and IL-6. Estimation of parasitemias, clinical scores, hemoglobin, weights, cutaneous temperature and survival was described in detail in the online-only Data Supplement.

Serine Protease Inhibition Assays

Enzyme proteolytic activity was performed as described in detail in the online-only Data Supplement.

Statistical Analysis

Results are expressed as means±SEM. Statistical differences among the groups were analyzed by *t* test or ANOVA using Tukey or Bonferroni as a multiple comparison posttest. Mann-Whitney–Wilcoxon was used for nonparametric distribution. Kaplan-Meyer curves were used for survival analysis. Significance was set at $P\leq0.05$ (Graph-Pad Prisma Software, La Jolla, CA).

Results

DF Interferes With Several Components of the Coagulation-Inflammation Cycle In Vitro

Incubation of pRBCs with ECs is accompanied by a series of biochemical changes including apoptosis, secretion of inflammatory cytokines, generation of microparticles, and expression of adhesion molecules and procoagulant TF.[1–6] Because DF displays EC-protective activities,[18–20] we tested its effects in TF expression by MVEC coincubated with pRBCs in vitro. Factor Xa (FXa) generation was used as a read-out of the assay. Figure 1A shows that in the absence of DF, FXa levels reached ≈35 pM,[8] while they were near basal levels with all DF concentrations tested (IC_{50} <100 μg/mL). As a control for drug DNA composition and verification of average molecular weight, DF was loaded in ethidium bromide gel and runs as <100-bp band (Figure 1A, inset).

pRBCs displays phosphatidylserine and support the prothrombinase complex in vitro at remarkably low hematocrit and parasite concentrations;[8] therefore, they may operate as activated platelets in the amplification phase of the coagulation cascade, particularly at sequestration sites where the concentration of pRBCs is very high. Figure 1B shows that incubation of pRBCs with Factor Va, FXa, prothrombin, and Ca^{2+} (prothrombinase) leads to intense thrombin generation that is linear for the first 10 minutes. In the presence of DF, prothrombinase activity is attenuated only at high concentrations of the drug (IC_{50} ≈1 mg/mL). Likewise, generation of thrombin triggered by addition of TF to plasma is also inhibited by DF (Figure 1C). This result confirms that DF displays anticoagulant activity in vitro.[18–20] Because platelets have been implicated as important players in malaria pathogenesis, through a number of mechanisms[1,35] we next tested the effects of DF in thrombin-induced human platelet aggregation. Although DF blocks thrombin-induced platelet aggregation, inhibition was attained only at high doses (IC_{50} of ≈1 mg/mL) (Figure 1D). DF (>1 mg/mL) also delays complement activation by the alternative pathway (Figure 1E), although no inhibition was observed for the classic pathway (not shown). Because enzymes contribute to the coagulation-inflammation cycle through distinct mechanisms,[15] it was of interest to study whether DF displays antiprotease activity. Figure 1F shows that DF at 100 μg/mL partially inhibits elastase and cathepsin G activities but was ineffective towards thrombin, FXa, kallikrein, chymase, trypsin, α-chymotrypsin, β-tryptase, granzyme B, and u-PA.

DF Does Not Interfere With NO Bioavailability or Hemoglobin Spectra

CM patients and mice infected with Plasmodium sp. often display high levels of free hemoglobin (hemolysis), which binds to NO, leading to impairment of NO bioavailability.[2] It has been suggested that low levels of bioactive NO contribute to disease pathogenesis by a number of mechanisms; one of them is through attenuation of inflammation.[2] Therefore, we

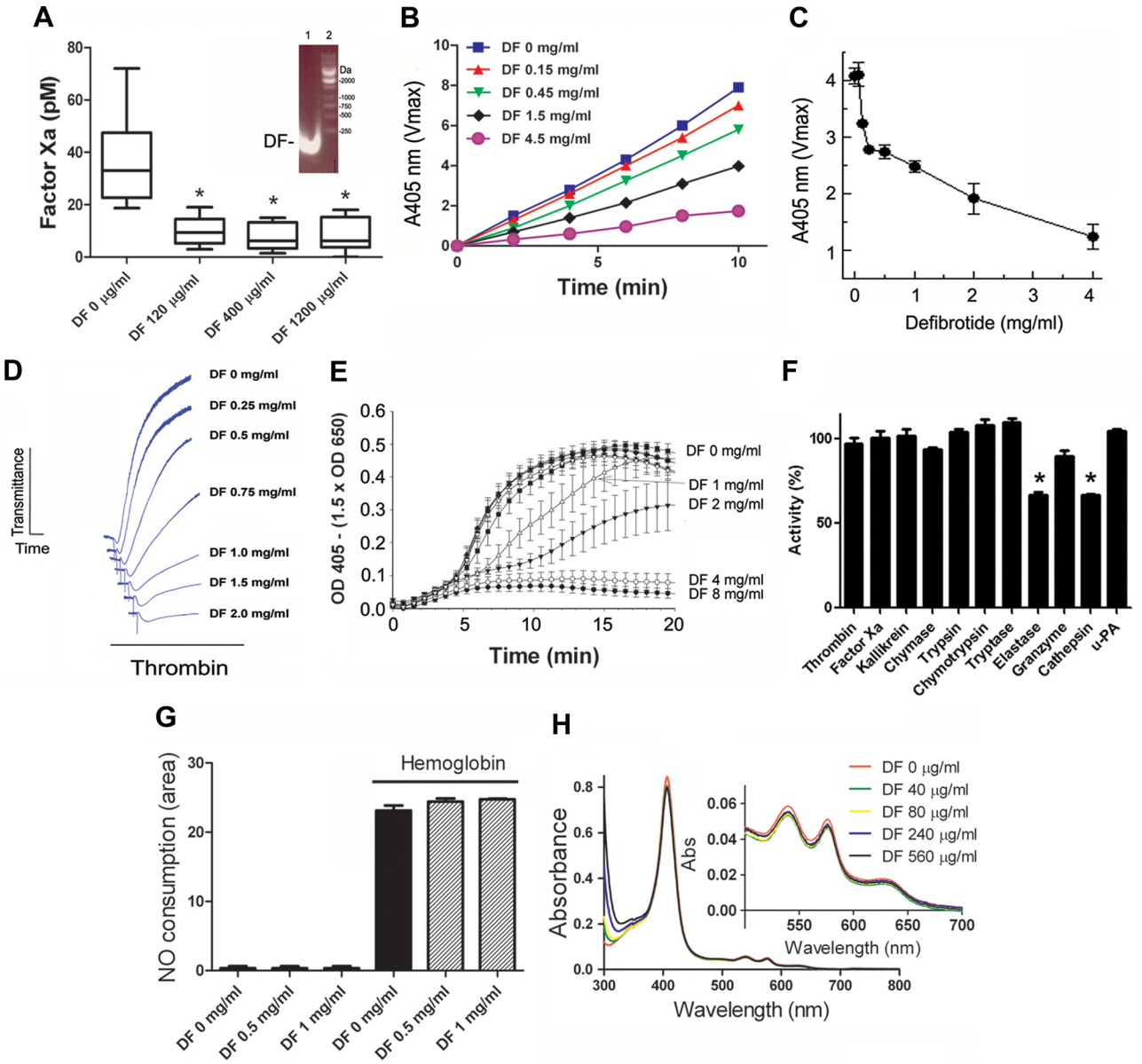

Figure 1. Defibrotide (DF) inhibits prohemostatic events associated with malaria pathogenesis but does not affect nitric oxide (NO) bioavailability or oxyhemoglobin spectra. **A**, Parasitized red blood cells (pRBC)-induced tissue factor (TF) expression in microvascular endothelial cells (MVECs). Red blood cells (RBCs) and MVEC were separately incubated for 1 hour with DF, and then coincubated for 6 hours followed by washing of the wells. A mixture of FVIIa/FX was added to the wells, followed by addition of S-2222. *$P \leq 0.05$ (ANOVA, Bonferroni posttest). **Inset**: Ln 1, DF (1.6 μg) was loaded on ethidium bromide gel and migrated <100 bp; lane 2, mol weight markers. **B**, Prothrombinase assembly. pRBC: 0.2% hematocrit at 50% parasitemia were incubated with DF for 5 minutes at 37°C in TBS-BSA-Ca^{2+} buffer; then Factor Xa (0.02 nmol/L) and Factor Va (1 nmol/L), were added to the wells; reactions were started with prothrombin (1.4 μmol/L). Thrombin formation was estimated using chromogenic substrate S-2238. **C**, Generation of thrombin in the plasma. Coagulation activation was initiated by cephalin plus kaolin, in the absence or presence of DF. Thrombin formation was estimated using chromogenic substrate S-2238. **D**, Thrombin-induced platelet aggregation. Washed human platelets were activated by thrombin (0.1 U/mL) in the presence of indicated concentrations of DF. Aggregation was determined by turbidimetry. **E**, Complement activation. DF delays alternative pathway tested by RBC lysis assay as described in Methods. DF did not affect the classical pathway (not shown). **F**, Inhibition of proteolytic activity. DF (100 μg/mL) inhibits cleavage of small fluorogenic substrates by elastase and cathepsin G. *$P \leq 0.05$ (*t* test). **G**, NO bioavailability. Samples (50 μL) of standard or DF, with or without oxy-Hb, were injected into the NONOate solution and NO estimated by chemiluminescence analyzer. **H**, Hemoglobin spectra. One milliliter of hemoglobin (2 μmol/L) in PBS was added to glass cuvettes followed by addition of DF (0–560 μg/mL) and absorbance scanning (200–800 nm). Inset details Hb spectra from 500 to 700 nm. Experiments were performed in triplicates or quadruplicates.

tested whether DF scavenges NO using a direct consumption assay. Figure 1G shows that when NO was delivered to a chamber containing DF, no change in gas concentration was observed, indicating that the drug was devoid of direct NO-scavenging properties. DF also did not affect NO consumption indirectly, when Hb was used as a scavenging molecule. Consistent with lack of DF interaction with Hb, the oxy-Hb spectrum—which is characterized by peak absorptions at 414, 540, and 578 nm—remained unchanged in the presence of DF (0–560 μg/mL) (Figure 1H).

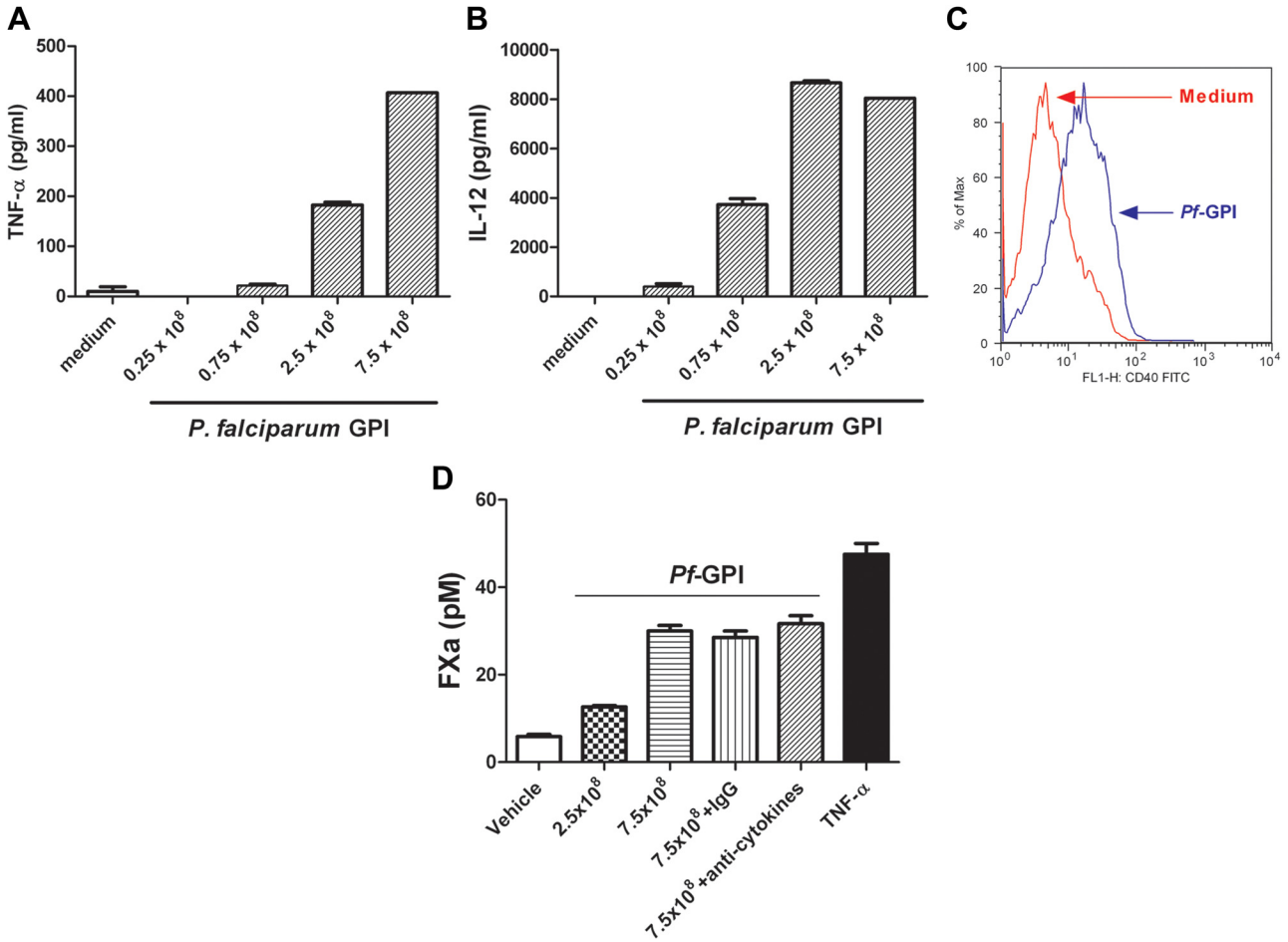

Figure 2. Purified Plasmodium falciparum glycosylphosphatidylinositol (*Pf*-GPI) modulates dendritic cell (DC) responses and induces tissue factor (TF) expression by endothelial cells (ECs). *Pf*-GPI (concentration corresponds to the number of parasites used for *Pf*-GPI extraction) was incubated with DCs overnight. Supernatants were collected, and tumor necrosis factor (TNF)-α (**A**) or interleukin (IL)-12 (**B**) production was estimated by ELISA. **C**, DCs were stimulated by *Pf*-GPI (corresponding to 7.5×10^8 parasites) and expression of CD40 estimated by flow cytometry. Results are expressed as percent of maximum expression of CD40 (left histogram, control; right histogram, *Pf*-GPI -stimulated cells). **D**, *Pf*-GPI was incubated with ECs for 6 hours and TF expression was estimated by Factor Xa (FXa) generation as reported in Figure 1A. Anti-TNFα, -IL-1β, and -IL-6 antibodies or isotype controls were used at 15 μg/mL; TNF-α (10 ng/mL) was used as control. Experiments were performed in triplicates or quadruplicates.

Pf-GPI is a Potent Inducer of DC Activation and TF Expression by ECs

DCs are critical components of the host response to infection and have recently been implicated as major orchestrators of systemic coagulation and inflammation through a PAR1-S1P3 dependent pathway.[14] Because *Pf*-GPI has been reported to modulate macrophage function through a TLR2-mediated mechanism,[12] we initially tested *Pf*-GPI as a potential modulator of DC function. Figure 2A and 2B, shows that *Pf*-GPI dose-dependently induces DC production of TNF-α and IL-12, respectively. It also promotes upregulation of costimulatory CD40 (Figure 2C). In addition, *Pf*-GPI was found to induce TF expression when added to EC in culture by a mechanism independent of cytokines such as TNF-α, IL-1, and IL-6 (Figure 2D). *Pf*-GPI-induced TF expression was comparable to TNF-α (10 ng/mL).

DF Negatively Modulates DC Function Through Aptameric Sequences and ARs

Next, we asked whether DF modulates DC response in vitro. Because purified *Pf*-GPI was available at limited amounts, we have replaced it with the TLR4 agonist LPS (ultra pure) to stimulate DC function.[32,33] Figure 3A and 3B, respectively, show that LPS-induced TNF-α and IL-12p40 production by DC is dose-dependently inhibited by incubation with DF. In addition, DF augmented LPS-induced production of anti-inflammatory IL-10 (Figure 3C) and blocked upregulation of costimulatory CD40 in response to LPS (Figure 3D) without affecting CD80 and CD86 (not shown). For cytokine production and CD40 upregulation, DF effects were attained at IC_{50} $\approx$100 μg/mL. Control experiments demonstrated that overnight incubation with DF (300 μg/mL) is not toxic to DCs based on the percentage of phosphatydilserine (PS) exposure ($<$0.03% for both saline and DF, data not shown).

It has been reported that N-ethylcarboxamide adenosine (nonselective agonist of ARs),[36] adenine nucleotides,[37] and adenosine,[33] negatively modulate DC function and promote an increase of PGE_2 production.[30,32] Consistent with adenosine-like activity, our experiments demonstrate that DF induces PGE_2 generation by DC in vitro (Figure 3E). Next, polynucleotides consisting of poly-A, poly-C, poly-G, and

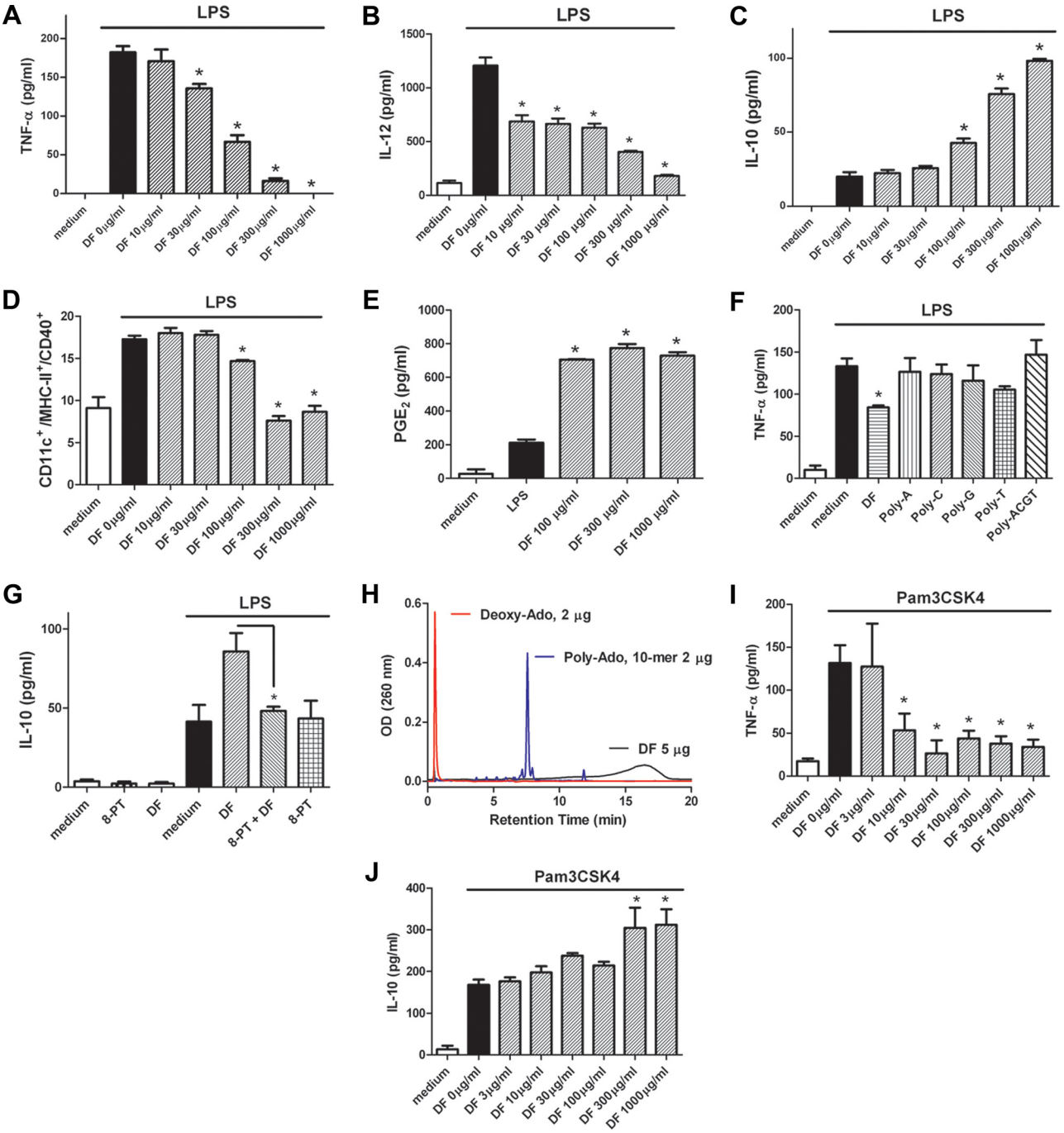

Figure 3. Defibrotide (DF) modulates dendritic cells (DC) responses. DCs were incubated overnight with DF at the indicated concentrations, followed by addition of lipopolysaccharide (LPS) (Toll-like receptor [TLR4 ligand], 50 ng/mL) for 18 hours. **A**, Then, tumor necrosis factor (TNF)-α, (**B**) interleukin (IL)-12, and (**C**) IL-10 cytokine in culture supernatants were measured by ELISA. **D**, The cells from the culture were used to evaluate the effect of DF on the expression of MHC-II and CD40 in the CD11c+DCs. **E**, DF (at the indicated concentrations) or ultrapure LPS (50 ng/mL) were incubated overnight with DCs. Supernatants were used to determine PGE_2 concentration as reported in Methods. **F**, DCs were incubated overnight with DF or with synthetic poly-A, poly-T, poly-C, poly-G, or an equimolar mixture containing poly-A, -C, -T, and -G followed by addition of LPS (50 ng/mL) for 18 hours and the supernatant used to measure TNF-α. **G**, DCs were incubated with AR nonselective antagonist 8-PT (10 μmol/L) for 1 hour before addition of DF. After overnight incubation, 8-PT (10 μmol/L) was added again to the wells, and LPS (50 ng/mL) was subsequently added for 18 hours. IL-10 production was estimated as above. All experiments were performed in triplicates or quadruplicates. **H**, DF does not contain adenosine. DF, and the standards deoxy-Ado or poly-Ado (10-mer) were loaded into DEAE column and monitored with a diode array detector as described in Methods. Ado, adenosine. For (**I**) and (**J**), DCs were incubated overnight with DF at the indicated concentrations, followed by addition of Pam3CSK4 (TLR2 ligand, 1 μg/mL) for 18 hours. Then, TNF-α (**I**) and IL-10 (**J**) in culture supernatants were measured by ELISA. *$P \leq 0.05$ (ANOVA, Bonferroni posttest).

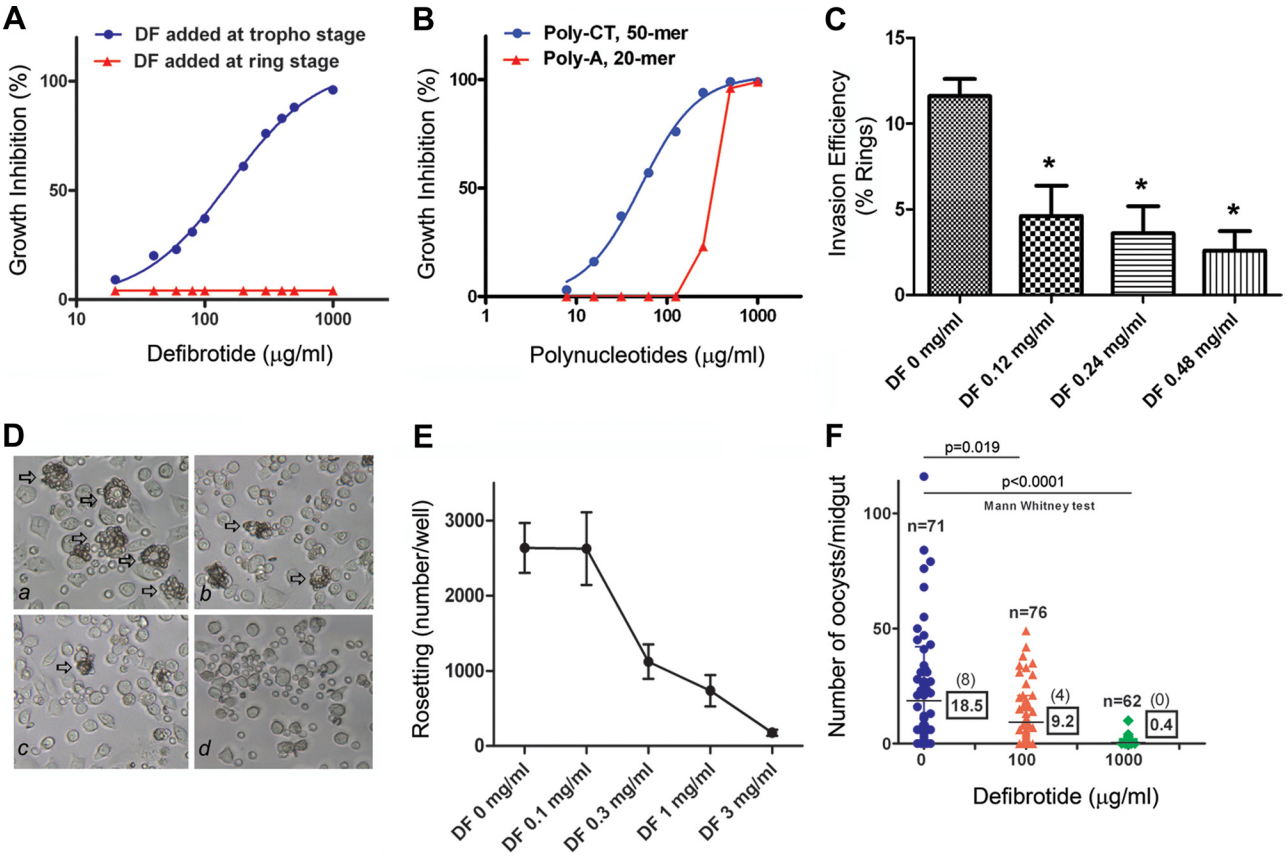

Figure 4. Defibrotide (DF) blocks parasite growth, rosetting formation, and oocyst development. **A**, DF was added at different concentrations directly to P. falciparum cultures containing trophozoite-stage parasites. After 48 hours of culture, the respective number of trophozoites present in the cultures was determined by parasite LDH (growth inhibition assay). In some experiments DF was added to ring-stage parasites and trophozoites development assessed using Giemsa smears. **B**, Poly-CT or poly-A were added to trophozoite-stage and invasion estimated as in (**A**). Poly indicates polynucleotides. **C**, DF was added to purified merozoites and invasion of RBC was estimated one hour later by Giemsa staining. *$P \leq 0.05$ (ANOVA, Bonferroni posttest). **D**, Rosetting formation. DF inhibits human erythrocyte binding assays with transfected CHO cells expressing P. falciparum EBA175 region II. Panel a, PBS; panel b, DF (0.3 mg/mL) and panel c, DF (3 mg/mL). Arrows show rosettes. Panel d shows absence of rosetting when CHO-cells were transfected with nonrosetting forming P. falciparum EMP-1 region DBL2 (negative control). **E**, Quantification of rosetting presented in (**D**). **F**, DF was added to the erythrocyte/serum mixture at designated concentrations prior to parasite addition. This infective feed was fed to Anopheles gambiae simultaneously using glass feeders, and mosquitoes were allowed to feed for 20 to 30 minutes through parafilm. Mosquitoes were maintained for 10 days at 27°C, 70% to 80% humidity, and provided with 5% glucose solution in PABA water. Midgut dissection was performed on day 10 postfeeding, with midguts examined under light microscopy for the presence of oocysts as described in Methods. The numbers in the boxes represent the average of oocysts/midgut and the number in parenthesis represents the median. n indicates the number of mosquitoes dissected per condition; n=71 (DF, 0 mg/mL); n=76 (DF, 100 μg/mL); n=62 (DF, 1 mg/mL). *$P \leq 0.05$ (Mann-Whitney-Wilcoxon test).

poly-T with similar molecular weight of DF were synthesized in an attempt to evaluate whether any aptameric sequence could mimic the effects of the drug in DCs. Figure 3F shows that although DF attenuates TNF-α production by DC stimulated with LPS, none of the polynucleotides tested separately or as an equimolar mixture of poly-A, -C, -T, and -G exhibited the same effect.

To verify whether DF acts through ARs, DCs were incubated with the 8-p-sulfophenyltheophylline (8-PT), a nonselective AR antagonist that reportedly blocks DF binding to cell membranes and also prevents inhibition by DF of K^+-induced contraction of guinea-pig smooth muscle.[22] Incubation with 8-PT was followed by addition of DF. As shown in Figure 3G, 8-PT completely inhibits DF-induced IL-10 production by DCs stimulated with LPS; this result is in line with DF behaving as an agonist of ARs.[22] In order to exclude that adenosine was present in DF, Figure 3H shows that DF loaded in a DEAE column elutes mostly around 15 to 18 minutes. In contrast, standard deoxyadenosine and synthetic poly-A (10-mer) elutes in the void and at ≈8 minutes, respectively. It is evident that adenosine does not contaminate DF.

We next examined whether DF also blocks DCs response to a pure TLR2 agonist, Pam3CSK4, which mimics closely the specificity of *Pf*-GPI.[12] Figure 3I demonstrates that DF dose-dependently attenuates TNF-α production by DCs and provokes an increase of IL-10 levels in Pam3CSK4-estimulated DCs (Figure 3J).

DF Inhibits Parasite Growth, Rosetting Formation, and Oocyst Development

DF was tested in the life cycle of P. falciparum. Figure 4A demonstrates that DF added to a parasite culture enriched with late trophozoites and schizonts prevents parasite growth in vitro with an IC_{50} ≈150 μg/mL. In contrast, no effect was

observed when DF was added to a culture containing ring stages only, suggesting that the drug inhibits RBC invasion by merozoites. A 50-mer poly-CT nucleotide also blocked parasite development with IC_{50} in the range determined for DF whereas a 20-mer poly-A is significantly less active (Figure 4B). To confirm the effects of DF in invasion, the drug was added to purified merozoites and invasion of RBC detected 1 hour later by Giemsa staining. Figure 4C demonstrates that DF blocks invasion in a dose-dependent manner with an IC_{50} ≈100 μg/mL.

Rosetting has been described in malaria and is mediated by interactions between Pf-EMP1 and other molecules of the RBC.[4] In order to gain further insight into the mechanism of blockade of RBC invasion by DF, CHO cells transfected with P. falciparum merozoite protein EBA175 (which is involved in RBC invasion) were incubated with RBC and evaluated for rosetting formation, as a surrogate pRBC-RBC interaction.[34] Figure 4D shows the pictures for rosetting in the absence of DF (panel 4Da; PBS), or in the presence of 0.3 mg/mL DF (panel 4Db), and 3 mg/mL DF (panel 4Dc). Results were expressed as the number of rosettes per well, with an IC_{50} ≈200 μg/mL (Figure 4E). A negative control using CHO cells transfected with EMP-1 (panel 4Dd), which is not involved in RBC invasion, does not show rosetting formation.

Transmission-blocking vaccines are based on the rationale that antibodies produced in the host and directed toward the vector are capable of blocking parasite invasion, once ingested by the mosquito. Figure 4F shows that DF inhibits development of oocyst formation when given to mosquitoes fed on P. falciparum (gametocytes)-infected blood with an IC_{50} ≈100 μg/mL; complete inhibition was attained at 1 mg/mL.

DF Ameliorates Clinical Score

Effects of DF were evaluated in a murine model for CM. Mice were infected IP with 10^6 pRBCs and followed for 15 days. DF was injected daily IP with 85 μL(≈ 7 mg)/mouse, 3 times/day (21 mg total), starting on day 1 or day 4 post infection. Figure 5A shows that detectable parasitemia started at day 3 and increased rapidly in the following days. DF started on day 1 slightly but significantly delayed parasitemia development, although no effects were observed when it was initiated at day 4. Control animals showed neurological symptoms of CM at day 5 or 6, and death usually occurred at day 6 or 7. Figure 5B shows that when DF treatment started at day 1, clinical scores were improved by DF at day 5; treated mice appeared healthier than saline-treated animals but in the next morning neurological symptoms were present, with an increase in the clinical score and death of some animals. At this time point, most of the mice in the saline-treated group were dead (Figure 5B). A trend in increase of survival was consistently found but did not reach significance ($P<0.05$) (Figure 5C). Survivals treated with DF from day 1 also displayed higher hemoglobin levels (Figure 5D), less weight loss (Figure 5E), and normal temperature (Figure 5F) when compared to animals given DF at day 4. Experiments performed with 170 μL (14 mg)/mouse, 3 times/day (42 mg total) yield similar results in the clinical score and no

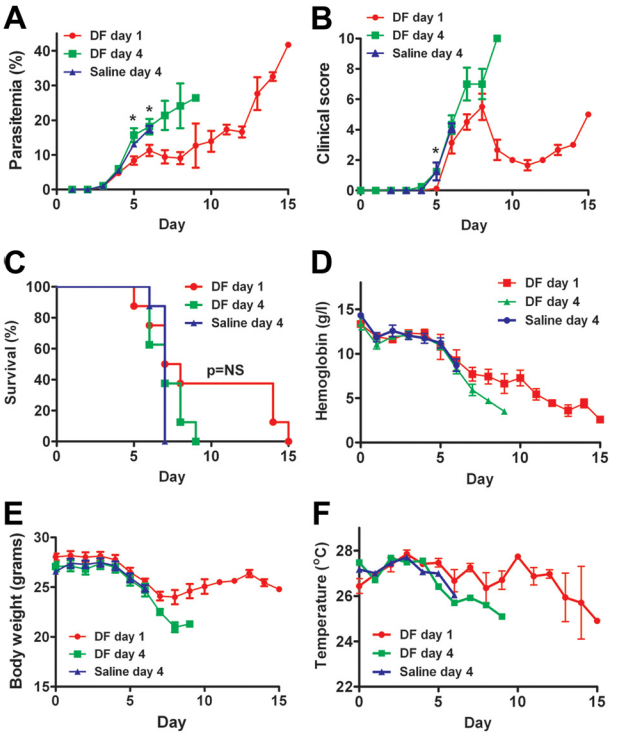

Figure 5. Defibrotide (DF) effects in a murine model of CM. P. berghei ANKA parasitized red blood cells (1×10^6) were used to infect mice (n=10) IP. DF (85 μL; 7 mg) or saline (85 μL) was injected IP, 3 times/day, starting from day 1 or 4 postinfections. **A**, Nontreated mice displayed higher parasitemias than DF-treated mice. Parasitemias were determined by Giemsa-stained smears of tail blood. **B**, Clinical scores were evaluated as described in Methods. **C**, The percent of nontreated and treated mice that survived over time is given in Kaplan–Meier curves. **D**, Hemoglobin was estimated using a Hemocue Hb 201+ Analyzer by taking ≈20 μL of blood from the tips of mice tails. **E**, The weights of mice were determined using a veterinary scale. **F**, Cutaneous temperature was estimated at the tail with an infrared thermometer according to manufacturer's instructions. For each experiment, 10 animals were used per group. *$P<0.05$ (2-way ANOVA, Bonferroni posttest, for saline vs DF day 1). NS, nonsignificant.

improvement in the survival curve was noticed (not shown; n=10/group).

We examine whether DF administration could affect the level of inflammatory cytokines and platelet number. Figure 6A shows that infection with P. falciparum is accompanied by thrombocytopenia, which was not affected by DF. An increase of IL-10, monocyte chemoattractant protein-1, RANTES, and IL-6 was observed on infection, but plasma levels were not affected by DF. On the other hand, the level of IFN-γ was significantly reduced by the drug. Figure 6B depicts the putative targets for DF.

Discussion

Our results indicate that DF has potential therapeutic value for the treatment of severe P. falciparum infection. Experiments described here demonstrate that DF blocks TF expression by MVECs coincubated with pRBCs at relatively low doses (Figure 1). These results are consistent with the endothelium-cytoprotective effect of the drug, which has been among its most known relevant therapeutic properties

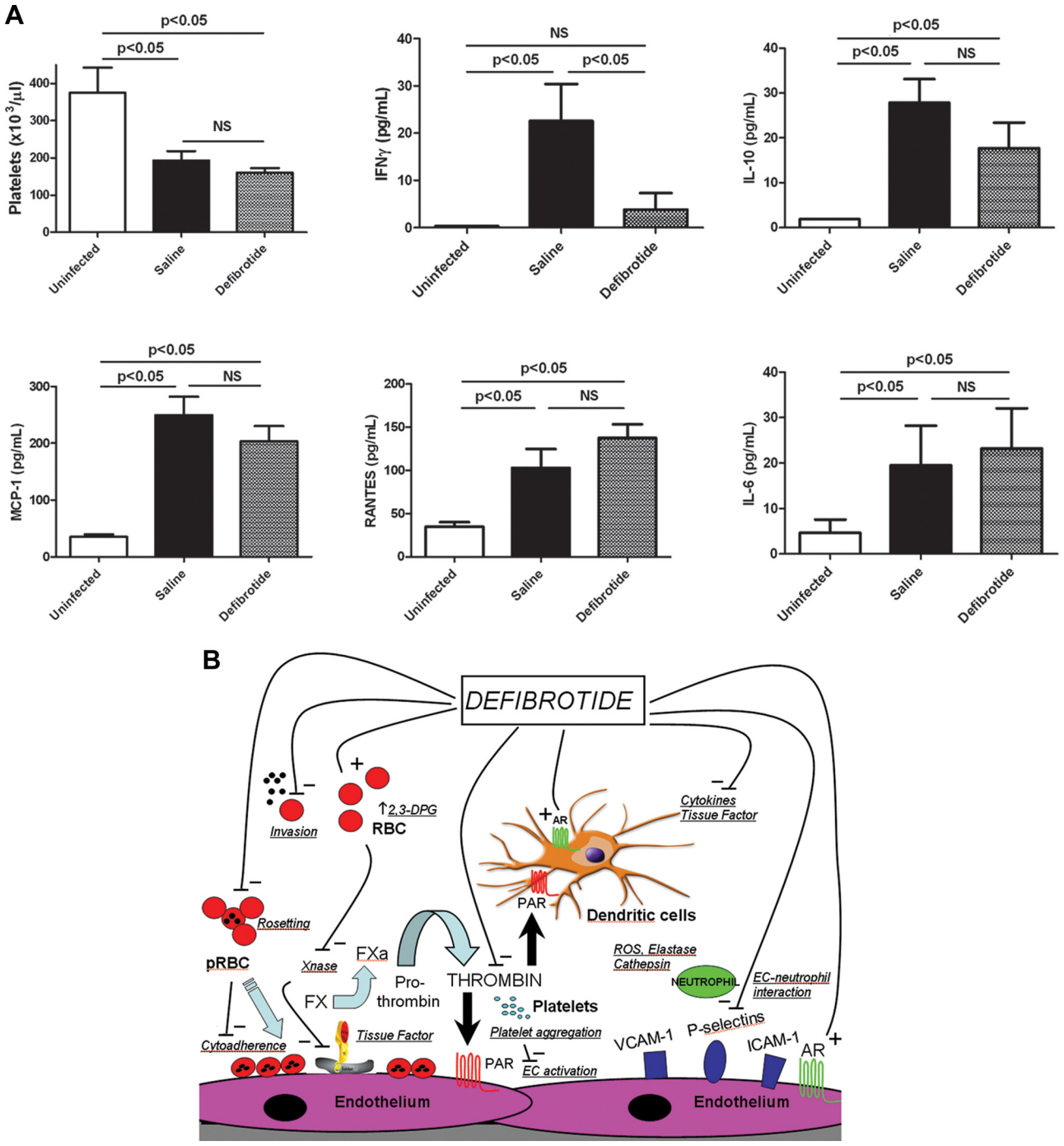

Figure 6. Defibrotide (DF) inhibits interferon (IFN)-γ production in a mice model of cerebral malaria and potentially interferes with several relevant inflammatory events associated with P. falciparum infection. **A**, P. berghei ANKA parasitized red blood cells (pRBCs) (1×10^6) were used to infect mice ($n=10$) IP. DF (85 μL; 7 mg) or saline (85 μL) was injected IP, 3 times/day, starting from day 1 postinfection. At day 5, blood was collected to estimate platelet number using a Hemavec and plasma levels of interferon (INF)-γ, interleukin (IL)-10, monocyte chemoattractant protein-1 (MCP-1), RANTES, and IL-6 as described in Methods. *$P\leq0.05$ (ANOVA, Tukey posttest). NS indicates nonsignificant. **B**, pRBC interaction with endothelium is associated with endothelial cell (EC) activation and initiation of the coagulation-inflammation cycle.[9] *Pf*-GPI,[12] DNA-hemozoin complex[41] (not shown) potentially contribute to dendritic cell (DC) activation through Toll-like receptor, whereas coagulation activation results in thrombin generation and DCs activation (in the lymphatics) and in endothelial cells (in the vascular compartment) through protease-activated receptors (PARs).[16] DF blocks rosetting, red blood cell invasion by P. falciparum, and increases 2,3-DPG.[43] Through charge effects or specific aptameric sequences DF may also interfere with enzyme activity (eg, thrombin, elastase, and cathepsin), platelet aggregation, and complement activation.[18–20] As an agonist of adenosine receptors, DF downmodulates DCs and endothelial cell function resulting in inhibition of systemic coagulation and inflammation.[16] DF also reportedly inhibits P-selectin expression by endothelium blocking neutrophil-EC interaction.[26] In addition, DF increases plasma levels of TF pathway inhibitor, PGI_2, PGE_2, and decreases plasminogen activator inhibitor-1.[18–20]

(see Introduction).[18–20] This property is particularly important because malaria is accompanied by endothelium activation, which appears to play a major role in the disease pathogenesis.[1–6] DF also exhibits anticoagulant activity (Figure 1) by a mechanism involving a specific antithrombin sequence.[21] Additionally, it has been suggested that the anticoagulant effect of DF in vivo may occur indirectly through increase of TF pathway inhibitor,[23] the physiological inhibitor of TF.[38] It is important to recognize that DF does not produce intense systemic anticoagulation; this property appears to be an advantage in the treatment of malaria and VOD patients, who are often thrombocytopenic.[1] DF is therefore distinct from heparin, a drug reportedly ineffective for the treatment of P. falciparum infection in humans[39] and with which administration is potentially accompanied by bleeding. Our results also revealed that elastase was partially blocked by DF (Figure 1). Inhibition of elastase activity in malaria[40] appears to be relevant because the enzyme degrades TF pathway inhibitor[38] and also affects thrombomodulin function, thus interfering with protein C activation.[16] Moreover, DF was found here to attenuate the activity of cathepsin G (platelet aggregation inducer) and the drug is also known to blunt superoxide generation by neutrophils.[18–20] Accordingly, inhibition of endothelial cells, platelets, and neutrophil functions, in addition to distinct enzymes involved in hemostasis, indicate that DF redundantly contributes to attenuate several aspects associated with inflammation present in P. falciparum infection.

Host response to infection is a highly complex event and represents a crosstalk between innate and adaptative immune responses where amplification of the coagulation-inflammation cycle takes place through a number of mechanisms.[15,16] Recently, DCs have been implicated as critical orchestrators of systemic inflammation, coagulation, and decompensation responses of disseminated intravascular coagulation by a mechanism involving PAR1 and S1P3.[14] Accordingly, DCs have been proposed as the primary cells at which coagulation and inflammation intersects within the lymphatic compartment.[14] Although this pathway has been characterized in endotoxemic mice, it is worth noting that P. falciparum infection shares some physiopathologic aspects with sepsis.[1,15] In fact, sequestration-related events in malaria are associated with upregulation of adhesion molecules in endothelial cell, dysregulated coagulation, and cytokine production.[1–6] In addition, P. falciparum pRBCs express *Pf*-GPI, a TLR2 agonist that—like LPS—reportedly activates macrophages, monocytes, and ECs in vitro.[12] We have expanded *Pf*-GPI properties by showing that it is a potent inducer of TF expression by ECs and that it increases TNF-α and IL-12 production and CD40 expression by DCs (Figure 2). Thus, it is conceivable that *Pf*-GPI contributes to both systemic inflammation and procoagulant tonus in P. falciparum infection. Other parasite soluble components (eg, DNA-hemozoin complex)[41] and coagulation in the lymphatics[14,16] may contribute to positively modulate DCs function in vivo.[14] In other words, it is evident that a state of high activation of DCs takes place in malaria.

To test the effects of DF in DCs, TLR2 (Pam3CSK4) and TLR4 (LPS) ligands were employed as surrogates because of the limited availability of *Pf*-GPI. Accordingly, DF attenuates LPS-induced production of TNF-α, IL-12, and CD40 expression and increases anti-inflammatory IL-10 generation by DCs. DF also inhibits a TLR2 agonist (Pam3CSK4)-induced TNF-α production by DCs (Figure 3), indicating that the drug modulates DCs response to a TLR agonist that more closely mimics *Pf*-GPI specificity. Furthermore, DF induces strong generation of PGE_2 by DCs, a prostanoid inhibitor of DC function through endoperoxide receptor and cAMP-PKA mechanisms.[32,33] These results are also consistent with agonistic activity of AR reported before for adenosine, adenine nucleotides, and AR agonist N-ethylcarboxamide adenosine.[33,36,37] These findings also help to explain why higher levels of PGE_2 are found in rodent urine or human plasma after administration of DF.[18–20] Notably, modulation of DCs occurs through ARs because it was blocked by the nonselective AR antagonist 8-PT, which prevents DF binding to cell membranes and its pharmacological activity.[22] However, the relative contribution of AR subtypes (eg, AR1, AR2A, AR2B, and AR3) mediating DF response is particularly complex[30,42] and remains to be determined. It is also conceivable that DF effects in DCs were mediated by specific sequences and not simply by charge, as it could not be reproduced by synthetic polynucleotides of the same average mol wt found in DF. Furthermore, the lack of effect of poly-A, -C, -T, and -G in DCs suggests that adenosine putatively generated from depolimerization of DNA during incubation with DCs did not take place or account for the effects of DF. These results are congruent with previous binding experiments and pharmacological assays demonstrating that DF behaves as a competitive agonist of ARs.[22]

The novel mechanism of action of DF revealed in this study thus suggests that its therapeutic use in VOD may occur through attenuation of DC function besides its well-known protective role in ECs.[18–20] Both activities are particularly relevant in P. falciparum infection which pathogenesis is better described as a disease with an important microcirculation dysfunction and immune-mediated mechanisms.[1–6] In other words, DF may protect endothelium activation in one hand, and downmodulates DC function on the other, thus contributing to diminish the procoagulant/inflammatory tonus of the disease. In regard to AR, gene knockout or pharmacological modulation of ARs protects against inflammation in numerous models of endotoxemia or vascular inflammation.[42] It is important to recognize that the relevant in vitro activities reported here for DF occurs at concentrations that are achievable in vivo. Accordingly, studies in humans treated with DF (25–40 mg/kg/d, given every 6 hours, 2-hours infusion) demonstrated constant plasma concentration of $\approx$200 μg/mL,[28] which is in the range of IC_{50} needed to inhibit ECs, DCs, rosetting, and parasite invasion (Figure 1, 3, and 4). In contrast, DF may only partially block some other activities due to high IC_{50} determined in vitro (Figure 1).

Our results also demonstrate that DF does not quench NO directly, nor changes oxyhemoglobin spectra or interferes with NO scavenging properties of hemoglobin (Figure 1). These results indicate that DF may not affect NO availability in vivo, directly or indirectly. This is a useful property, as NO bioavailability in malaria is impaired and suggested to contribute to disease pathogenesis.[2] Actually, DF reportedly

promotes ex vivo increase in nitrates in perfusates collected from the hearts of guinea pigs.[18–20] All these effects may contribute to decreased inflammatory tonus in malaria. In addition, DF increases 2,3-diphosphoglycerate in RBCs after administration to rats,[43] which is consistent with an agonist of ARs.[44] While decreasing Hb affinity for O_2 may affect sickling,[44] it may contribute to deliver oxygen in anemic patients who are often the case in P. falciparum infection.[1–6]

DF has also been tested in distinct steps of the life cycle of P. falciparum. In vitro results show that it promotes inhibition of parasite growth, RBC invasion, and rosetting formation with similar IC_{50} without affecting the development of ring stages to mature forms. Similar results were observed when a 50-mer polynucleotide composed of poly-CT was tested in the growth-inhibition assay; a 20-mer poly-A was less active. These results indicate that inhibition of invasion by DF is charge-, length-, but not sequence-specific. Conceivably, DF, other oligodeoxynucleotides[45] and glycosaminoglycans[46] block invasion or interfere with sequestration because these molecules increase the charge repulsive force between cells such as merozoites and erythrocytes, which are negatively charged. Also, oocysts did not develop in the midgut of mosquitoes who fed on infected blood supplemented with DF. It may be that some components of the sexual cycle of P. falciparum or implantation of the gametocyte across the peritrophic membrane are sensitive to charge effects or components found in DF; therefore, it appears that DF exhibits transmission blocking properties.

Consistent with the in vitro effects, results in vivo demonstrated that administration of DF started at day 1 postinfection slightly although significantly interfered with development of parasitemia in mice infected with Plasmodium berghei ANKA (Figure 5). It also ameliorates the clinical score at day 5, which was evident based on the behavior of DF- versus saline-treated animals. A trend for increased survival after IP injection of DF was also observed across experiments, without, however, reaching statistical significance. Therefore, DF appears to delay the development of the disease without being able to fully interrupt the events that lead to death in the CM model. Not surprisingly, DF effects were not evident when injections started at day 4, likely because the pathological processes leading to fatal CM were too advanced in time. Our results also demonstrated that thrombocytopenia produced by Plasmodium sp. infection was not affected by DF, as well as the plasma levels of IL-6, and RANTES; a trend for lower levels was observed for monocyte chemoattractant protein-1 and IL-10 (Figure 6). Notably, IFN-γ levels were significantly reduced after administration of the drug. This suggests that DCs—known to modulates T-cell responses and IFN-γ levels[47]—might be negatively modulated by DF in vivo (Figure 6). Although anticoagulant, endothelium protective, DCs inhibitory, and invasion-blocking properties may account for the better clinical score observed after administration of DF, the relative contribution of each activity remains to be determined. Also, it is possible that the short half-life of DF due to plasma exonucleases may have interfered with DF bioavailability and efficacy.[18–20]

At present, it is prudent to say that results with DF in an experimental model—whether positive or negative—should be contextualized before discarding or advocating it as a drug to treat severe malaria in humans. In fact, the relevance of murine model for CM has been intensely debated.[48] More importantly, large clinical experience with DF in several clinical trials for treatment of VOD shows that comatose pediatric patients, who are also common in severe malaria, benefited from the therapy with the drug without complications.[18–20] In addition, DF is stable at room temperature and active by oral, subcutaneous, or intravenous routes and is inexpensive when compared with activated protein C or other drugs that negatively motivate the coagulation-inflammation cycle.[16] In this context, treatment of P. falciparum-infected patients with phosphodiesterase inhibitor pentoxifylline—a drug whose mechanism is in contrast to AR agonist DF—has been evaluated before. The study was terminated earlier than planned because it failed to improve clinical score and it also worsened some laboratory parameters associated with inflammation such as macrophage activation.[49] Polymorphism of AR2A has also been suggested to be associated with severity in malaria.[50] These results highlight how modulation of ARs by DF in different cell types may affect inflammatory tonus in the disease, and also emphasize therapeutic applications for aptamers. Although the combined actions of DF may, in theory, redundantly interrupt the coagulation-inflammation cycle, it remains to be proven whether P. falciparum-infected patients treated with antimalarials will respond favorably to adjuvant administration of the drug.

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Disclosures

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