

σ^E responses) that a total of 50 proteins act upstream of or in the same pathway as these three stress responses: more than half of the network and most of the 77 newly discovered proteins. Stress responses constitute the largest central nodes of the network (Fig. 3A).

DSB-dependent stress-induced mutagenesis produces more than half of spontaneous base substitution and frameshift mutations in starving *E. coli* (2) and is thus important to evolution. It promotes genetic diversity specifically when cells are maladapted to their environment, that is, when they are stressed. Similar mutagenesis pathways promote antibiotic-induced resistance to ciprofloxacin (antibiotic) in *E. coli* (6) and bile-induced bile resistance of pathogenic *Salmonella* (22, 23). Mutagenic DSB repair occurs in yeast (24) and is implicated in mutation hotspots in *E. coli* (25) and human cancers (26, 27), although whether it is related to stress responses in yeast and human, such as hypoxia-induced SIM (5), is unknown. The identification of stress response regulators as central network hubs suggests to us that these may provide promising candidates as targets for new drugs to inhibit mutagenesis that allows pathogens to adapt in response to antibiotics and the immune system, both stressors. Inhibiting evolution could allow conventional antibiotics and cancer chemotherapies to work without inducing resistance and, perhaps, allow the immune system to overtake pathogens.

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Acknowledgments: We thank R. Nichols and C. A. Gross for earlier bioinformatic analysis and discussion; S. Gottesman, C. Herman, J. D. Wang, and J. Weissman for discussions; J. Casadesús, S. Gottesman, C. Herman, G. Ira, X. Pan, E. Rogers, T. Silhavy, and J. D. Wang for comments on the manuscript; and D. Satory for help with strains. Bioinformatics supported by NSF 0905536, 1062455, and NIH GM79656 and GM66099 grants (O.L., A.M.L.). R.B.N. was supported by NIH Director's Pioneer Award DP1-CA174424 (S.M.R.). Supported by NIH grants R01-GM64022 (P.J.H.), R01-GM53158 (S.M.R.) and fellowships F32-GM095267 (R.L.F.) and F32-GM19909 (M.-J.L.). The genes of mutagenic repair of DNA breaks are accessible at <http://wiki.porteco.org/tools/simnetwork>.

Supplementary Materials

www.sciencemag.org/cgi/content/full/338/6112/1344/DC1
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28 June 2012; accepted 30 October 2012
10.1126/science.1226683

Platelet Factor 4 and Duffy Antigen Required for Platelet Killing of *Plasmodium falciparum*

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Platelets restrict the growth of intraerythrocytic malaria parasites by binding to parasitized cells and killing the parasite within. Here, we show that the platelet molecule platelet factor 4 (PF4 or CXCL4) and the erythrocyte Duffy-antigen receptor (Fy) are necessary for platelet-mediated killing of *Plasmodium falciparum* parasites. PF4 is released by platelets on contact with parasitized red cells, and the protein directly kills intraerythrocytic parasites. This function for PF4 is critically dependent on Fy, which binds PF4. Genetic disruption of Fy expression inhibits binding of PF4 to parasitized cells and concomitantly prevents parasite killing by both human platelets and recombinant human PF4. The protective function afforded by platelets during a malarial infection may therefore be compromised in Duffy-negative individuals, who do not express Fy.

Platelets protect the host against death by malarial parasites. A normally resistant mouse, deprived of platelets, will die from a murine malarial infection as a result of high

circulating levels of viable parasites (1, 2). Platelet binding to the infected red cell is associated with the death of the intraerythrocytic malarial parasite (1, 3), and killing of parasites appears to be species independent. Platelets from mice or humans can kill intraerythrocytic *Plasmodium chabaudi* or *P. falciparum*, respectively (1, 3). Both human and mouse malarial infections are also accompanied by thrombocytopenia (1, 4, 5), which correlates with increased parasite density and more severe disease (6–8). Platelets bind

both infected and noninfected red cells during a malarial infection (9, 10), but a higher fraction of infected red cells bind platelets (1). However, the molecular mediators involved in the parasite-killing phenomenon remain unknown. Evolutionary selection for the Duffy-antigen (Fy) negative allele in Africa is believed to provide protection against *P. vivax* infection (11). We report here that Fy binds the platelet effector molecule PF4 and is necessary for platelet-mediated killing of *P. falciparum*.

Platelet activation is accompanied by the release of molecules from intracellular α granules. PF4 is a CXC-type chemokine and an abundant constituent of α granules (12, 13). We found, in agreement with others (14), that *P. falciparum*-infected red blood cells (iRBCs) stimulate the release of PF4 from purified human platelets (fig. S1). We conducted indirect immunofluorescent assays (IFA) to detect PF4 and observed a striking immunolocalization of PF4 on iRBCs incubated with platelets. The antigen was present on the iRBC surface and intracellularly (Fig. 1A and fig. S2A). Notably, PF4 was not detected in uninfected red cells (whether bound or not bound to platelets) or in parasitized cells cultured in the absence of platelets. The frequency of PF4-stained iRBCs depended on the duration of platelet incubation and/or the parasite stage. After addition of platelets to cultures of synchronized immature ring stage parasites (8 to 16 hours after invasion), approximately 20% of iRBCs contained

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PF4 after 9 hours. After 22 hours, the proportion of PF4-stained cells had increased and reached a plateau of 70 to 75% (Fig. 1B). The majority of parasites were mature trophozoites by this time. Platelet-expressed CD36 mediates the binding of platelets to *P. falciparum*-infected red cells (15) and is required for the iRBC-stimulated release of PF4 from platelets (14). We examined the role of CD36 in the laboratory-adapted reference strain of *P. falciparum* (3D7) and two recent clinical isolates (PF2006 and X1E) that naturally bound to CD36 (fig. S3). In our experiments, use of a soluble CD36 protein in platelet-treated *P. falciparum* cultures reduced the frequency of platelet-iRBC binding by more than a factor of two in all the strains and concomitantly prevented the platelets from inhibiting parasite growth (figs. S3 and S4), indicating that platelet binding to iRBCs (via CD36) is necessary for platelet-mediated killing of *P. falciparum*. Interestingly, the CD36 treatment also reduced the frequency of PF4-stained iRBCs by almost 50% (Fig. 1B and fig. S3). This difference can be explained almost entirely by an equivalent reduction in platelet-iRBC binding, indicating that localization of platelet-derived PF4 on iRBCs involves contact between platelets and parasitized cells. There are some PF4-positive cells that have no bound platelets; we assume these have become disassociated. A terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay was used to identify dead intraerythrocytic parasites. Consistent with previous studies (1), we observed more dead intraerythrocytic parasites after platelet treatment (Fig. 1D). After colabeling with TUNEL and antibodies to PF4, we observed platelet-bound iRBCs that were positively stained for PF4 and TUNEL (Fig. 1C and fig. S2). After 22 hours of platelet treatment, over 90% of iRBCs that contained dead, TUNEL-labeled parasites also contained PF4 (Fig. 1D). Together these data suggest that contact between platelets and parasitized red cells results in the localized release of PF4 from platelets and that this release is associated with parasite killing.

In addition to its role as a classical chemokine, PF4 also possesses antimicrobial activity and can directly kill a number of pathogenic bacteria and fungi at submicromolar concentrations (16, 17), thus prompting the hypothesis that PF4 itself is the lethal platelet effector molecule. The *P. falciparum* killing activity of whole purified platelets was partially blocked by antibodies to PF4. Addition of equivalent numbers of lysed platelets to cultured parasites resulted in a similar killing effect, and this was completely blocked by the inclusion of antibodies to PF4 (fig. S5), indicating that the lethal effect of platelets is entirely due to PF4. We further characterized this function for PF4 by using a commercially produced recombinant human PF4 (rhPF4) protein in *P. falciparum* parasite growth assays. The protein inhibited parasite growth in a specific and dose-dependent manner, with an estimated median inhibitory concentration (IC₅₀) of 0.5 μM and IC₁₀₀

of 5 μM (Fig. 2A). This matched closely with the levels of PF4 present in cultures treated with lysed platelets (0.71 ± 0.22 μM), and similar potency was observed against drug-resistant falciparum strains K1 and W2-mef. We next investigated the intraerythrocytic developmental stage of the parasite most sensitive to PF4. In one approach, rhPF4 was added to parasites synchronized at the immature ring stage or mature trophozoite stage for 4 hours, washed out, and then the effect on growth was determined. Treatment of the immature parasites for 4 hours had a negligible impact on parasite growth, whereas the same treatment of mature parasites inhibited growth as effectively as continuous rhPF4 treatment (Fig. 2B and fig. S6). In a second approach, rhPF4 was added to immature ring-stage parasites and rates of parasite death were measured using the TUNEL assay. The rhPF4 treatment resulted in a modest but significant increase in dead parasites after 6 hours (where immature rings were still the predominant form), whereas 22- and 28-hour treatments resulted in rates of death 3 to 4 times as high as control cultures; pigmented trophozoite-stage parasites predominated at these time points (Fig. 2C). We then examined the ability of PF4 to directly interact with *P. falciparum*-infected

erythrocytes. Using antibodies to PF4 and flow cytometry, we detected a population of PF4-stained iRBCs after rhPF4 treatment. The number of PF4-positive iRBCs was 3 to 4 times as great as uninfected cells. These cells were most numerous in cultures where pigmented trophozoite and schizont forms of the parasite predominated (fig. S8, A and B). Taken together, these data strongly suggest that PF4 is a *P. falciparum* killing agent. PF4 preferentially kills later stages of parasite development, coinciding with binding of the protein to the infected cell; however, the exact mechanism by which PF4 exerts its toxic effect remains to be determined.

The Duffy-antigen receptor for chemokines (Fy/DARC) is expressed on erythrocytes and has several chemokine ligands, including PF4 (18, 19). We therefore tested the hypothesis that the killing effect of PF4 may be exerted via interaction with the Fy molecule. First, addition of ligand-blocking antibodies to Fy (20) reduced the ability of both platelets and rhPF4 to kill 3D7 parasites (Fig. 3A). The PF2006 and X1E clinical isolates were also protected from platelet killing by the Fy6 antibody (fig. S3D). Second, inclusion of equimolar concentrations of chemokines with greater affinity for Fy than PF4 [GROα/CXCL1 and

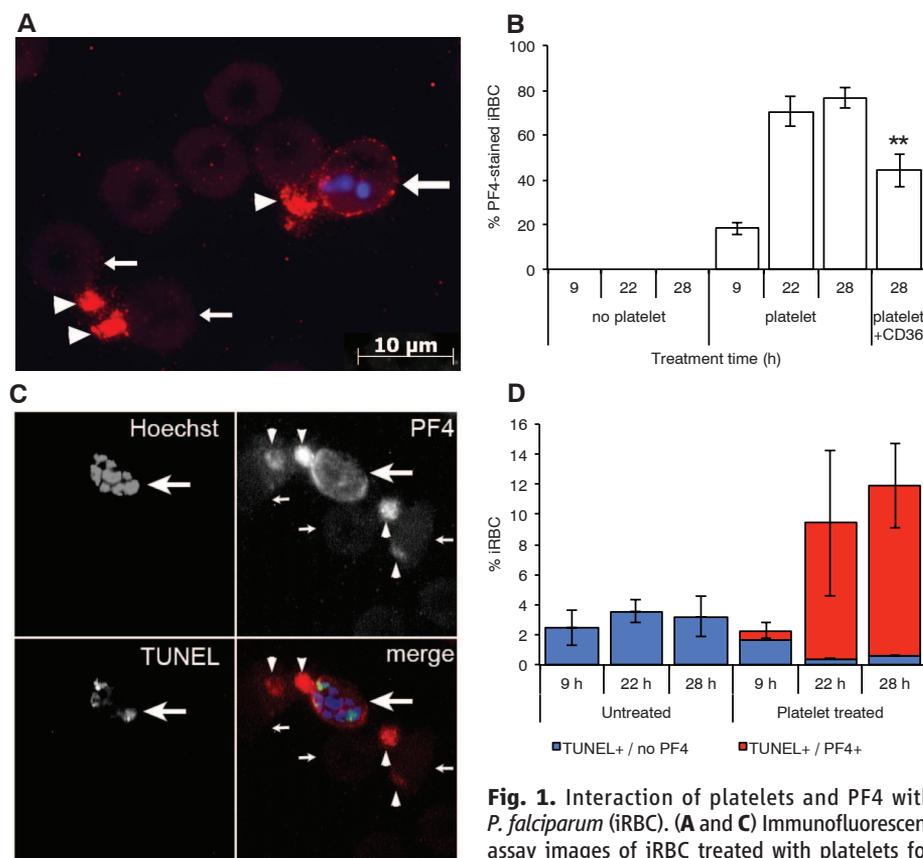


Fig. 1. Interaction of platelets and PF4 with *P. falciparum* (iRBC). (A and C) Immunofluorescent assay images of iRBC treated with platelets for 24 hours. Cells were stained with Hoechst 5769121 (blue), antibody to PF4 (red) and labeled using the TUNEL assay [green; (C) only]. Arrowheads indicate platelets, small arrows indicate uninfected RBC, and large arrows indicate platelet-bound and anti-PF4 stained [and, in (C), TUNEL-labeled] iRBC. (B) Percentage of anti-PF4-stained iRBC, and (D) TUNEL and anti-PF4 colabeled iRBC, after platelet treatment. Error bars represent mean proportions (±SEM) determined in three independent experiments. **P = 0.005 compared with 28-hour platelet treatment.

RANTES/CCL5 (16, 17)] blocked the killing activity of rhPF4, whereas a chemokine with little affinity for Fy [SDF1/CXCL12 (21)] had no effect (Fig. 3B). None of these chemokines affected the intraerythrocytic growth of parasites in control experiments (fig. S7). In a third set of experiments, we used red cells genetically deficient in Fy expression. A mutation in the GATA motif of the Fy promoter prevents erythrocyte expression of Fy, is common in African populations (22), and protects against *P. vivax* infection (11). Strikingly, neither platelets nor rhPF4 was able to prevent the growth of *P. falciparum* parasites or kill these parasites when they were propagated

in Fy-deficient cells (Fig. 3, A and C). The Fy deficiency did not affect parasite growth per se (fig. S7), and the lack of platelet-mediated killing was not associated with any change in the ability of platelets to bind Fy-deficient iRBCs (fig. S4B). However, after platelet treatment of either Fy-deficient cells infected with 3D7 or Pf2006-infected cells treated with antibody to Fy6, we observed an almost 50% reduction in the proportion of PF4-bound iRBCs (Fig. 4A and fig. S4E). In addition, treatment of Fy-deficient 3D7-infected cells with rhPF4 resulted in reduced levels of PF4 binding and fewer PF4-stained cells (fig. S8, C to E). Together, these data suggest that Fy is critical

in the pathway mediating the platelet killing and functions as a receptor for PF4 on iRBC. In agreement with previous studies (23, 24), we observed, using IFA, that Fy is localized within the *P. falciparum* parasite (fig. S9), suggesting a mechanism whereby the intraerythrocytic parasite may be directly exposed to PF4. IFA costaining for Fy and PF4 revealed colocalization of the molecules within *P. falciparum*-infected cells that were treated with rhPF4 (Fig. 4B). To confirm these observations, parasites from rhPF4-treated cultures were purified using saponin and immunoblotted for PF4. PF4 was detected in the purified parasite extracts. Interestingly, the levels of PF4 observed

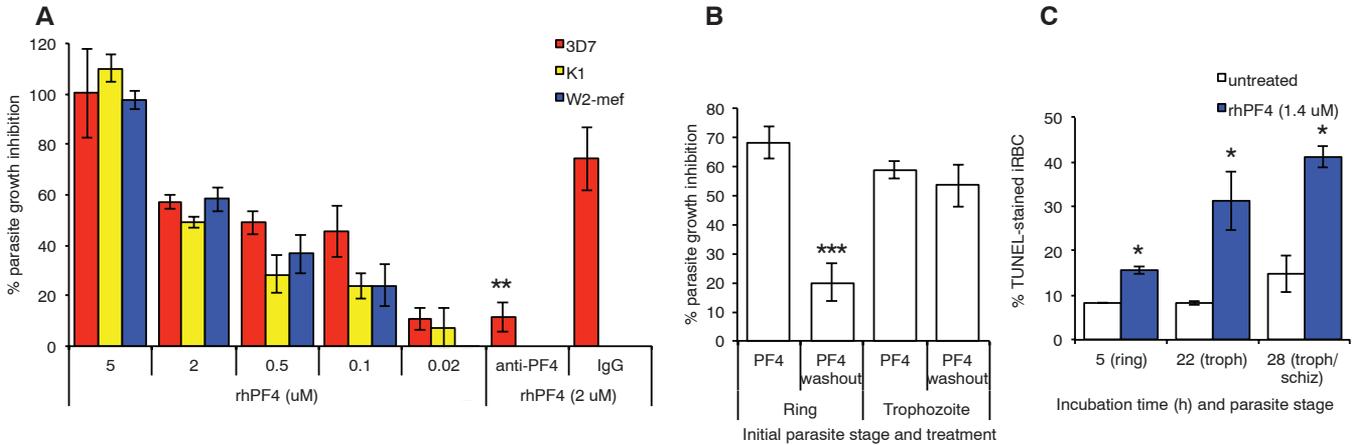


Fig. 2. The *P. falciparum* killing function of PF4. (A) Growth inhibition of *P. falciparum* by rhPF4. Data represent the mean of at least two independent assays, each performed in duplicate (\pm SEM) for three parasite strains. $**P < 0.01$ compared with nonspecific rabbit immunoglobulin G (IgG) control antibody. (B) Growth inhibition of *P. falciparum* 3D7 by rhPF4 after treatment for 4 hours and washout, or continuous treatment. Synchronized ring or trophozoite-stage parasites were used. Error bars represent the mean of at least two independent

assays, each performed in triplicate (\pm SEM). $***P < 0.001$ compared with continuous treatment of ring-stage parasites. (C) Percentage of TUNEL-labeled *P. falciparum* 3D7 iRBC after treatment with rhPF4. The predominant developmental parasite stages at each time point were either immature rings (ring), pigmented trophozoites (troph) or schizonts (schiz). Bars represent mean proportions (\pm SEM) determined in two independent experiments. $*P < 0.05$ compared with untreated control cultures at each respective time point.

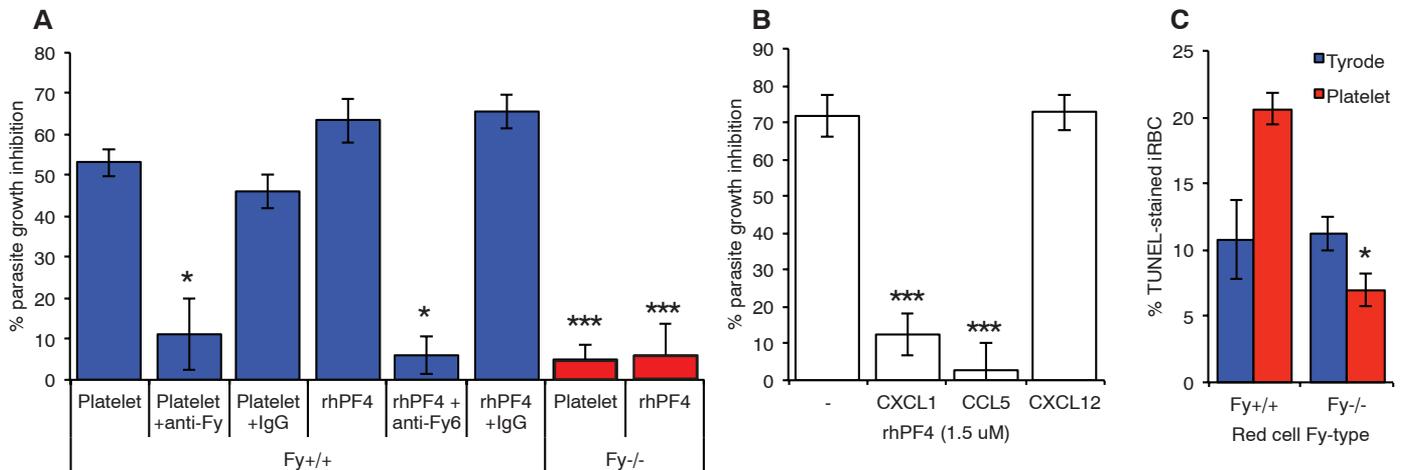


Fig. 3. Requirement of Fy for platelet- and PF4-mediated parasite killing. (A) Growth inhibition of *P. falciparum* 3D7 by platelets or rhPF4 and the effect of antibodies to Fy (anti-Fy and anti-Fy6) and Fy-deficient erythrocytes ($Fy^{-/-}$). Error bars represent the mean (\pm SEM) of at least three independent experiments, each performed in duplicate. The blood from seven different $Fy^{-/-}$ donors was tested. $*P < 0.05$ compared with isotype control IgG antibody; $***P < 0.001$ compared with respectively treated parasites grown in $Fy^{+/+}$ cells. (B) Effect of chemokine Fy ligands on *P. falciparum* growth inhibition

by rhPF4. Data represent the mean (\pm SEM) of at least two independent experiments, each performed in duplicate. $***P < 0.001$ compared with no chemokine. (C) Percentage of TUNEL-labeled *P. falciparum* 3D7 parasites grown in Fy-sufficient ($Fy^{+/+}$) or Fy-deficient ($Fy^{-/-}$) red cells after platelet treatment for 24 hours. Error bars represent mean proportions (\pm SEM) determined in three independent experiments. The blood from three different Fy-negative donors was tested. $*P = 0.02$ compared with platelet-treated $Fy^{+/+}$ cells.

in parasites grown in Fy-deficient cells were significantly reduced by a factor of almost three (Fig. 4C and D). These data suggest that infected cells internalize PF4 and that the Fy molecule largely mediates the phenomenon.

Our data show that the intraerythrocytic killing of falciparum parasites by platelets requires platelet-iRBC contact, release of PF4, and binding of PF4 to the Fy receptor. Although platelets release PF4 into the medium of cultured *P. falciparum*, the levels measured in our experiments were insufficient to kill the parasites. Instead, our data suggest that after binding to iRBCs, platelets release PF4, resulting in locally high concentrations. The released PF4 then binds to the surface of the infected cell. PF4 binding is greatest in red cells with mature parasites, and these parasites are more likely to be TUNEL positive, i.e., dead. Not all PF4-bound cells were colabeled by TUNEL, suggesting that there may be a delay between the binding of PF4, the death of parasites, and the subsequent fragmentation. Others have reported that the bactericidal activity of PF4 and related platelet antimicrobial proteins is exerted via disruption of cell metabolism and membrane potential, although the exact mechanisms remain unclear (25, 26). A similar cytotoxic or cytostatic mechanism may also operate on the metabolically active maturing parasite. Interestingly, depletion of PF4 rescues otherwise susceptible mice in the

P. berghei-induced experimental cerebral malaria model (ECM) (14). However, ECM is mediated through an unrelated pathology and is not associated with parasite death. Erythrocyte Fy is essential for the killing of *P. falciparum* by platelets and PF4. Our data show that Fy binds PF4. There is more binding in Fy-positive than Fy-negative cells. There is also more PF4 present internally in Fy-positive erythrocytes compared with Fy-deficient cells. We therefore deduce that PF4 binding to Fy mediates both the increased concentration of PF4 on the surface of the cell and the observed intraerythrocytic accumulation of the protein through transport from the surface of the cell, although we have yet to demonstrate this formally. A genetic lack of Fy renders an infected cell impervious to killing by both PF4 and platelets and is associated with less surface binding and internalized PF4. In normally resistant mice, platelet deficiency leads to a more severe outcome (1, 2), but Fy deficiency has no effect (27), possibly indicating a different effector pathway in the mouse.

The vast majority of individuals living in Western and Central equatorial Africa are Duffy-antigen negative (22). An obvious implication of our findings is the potential lack of platelet-mediated protection against malaria in these individuals and the possibility that this has an impact on disease severity and outcome. Although the available epidemiological and clinical data are

insufficient to directly compare disease severity and outcome in humans with different Duffy alleles (28–30), *P. falciparum* infections are most common in equatorial Africa and result in the highest global rates of death (31, 32).

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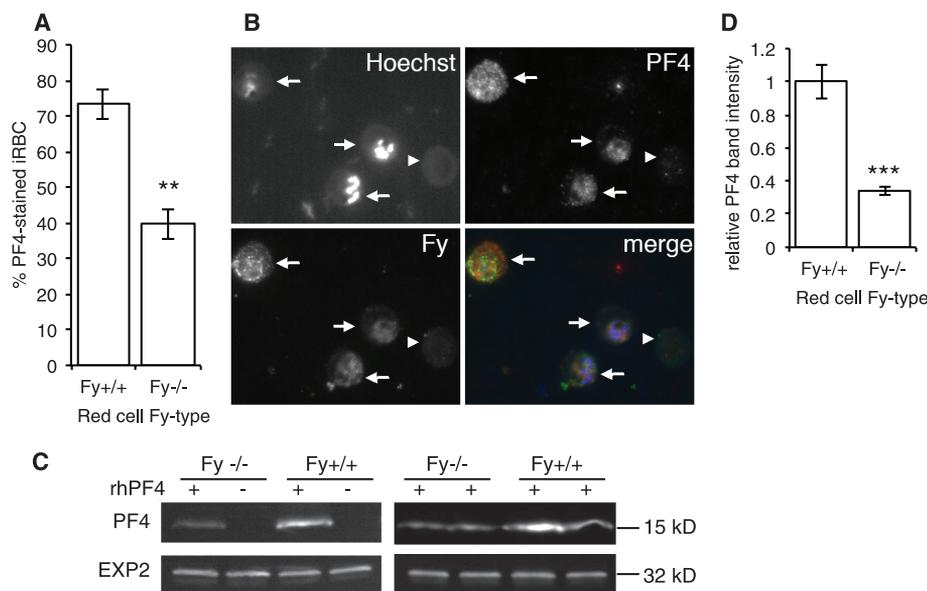


Fig. 4. Requirement of Duffy antigen for PF4 binding and internalization by iRBC. **(A)** Percentage of anti-PF4 stained *P. falciparum* 3D7 iRBC grown in Fy-expressing (Fy^{+/+}) or Fy-deficient (Fy^{-/-}) cells after treatment with human platelets for 24 hours. Error bars represent mean proportions (±SEM) determined in three independent experiments. The blood from three different Fy-negative donors was tested. ****P** = 0.002 compared with Fy^{+/+} cells. **(B)** Immunofluorescent assay images of PF4-treated iRBC stained with Hoechst (blue), antibody to PF4 (red), and antibody to Fy (green). rhPF4 was added to synchronized ring-stage parasites for 24 hours. Arrowheads indicate uninfected cells, and arrows indicate iRBC (PF4 and Fy costained). **(C)** Immunoblot analysis of PF4 in rhPF4-treated *P. falciparum* 3D7 parasites. Immunoblots (using antibodies to PF4 or EXP2) of saponin-purified trophozoites, treated before purification with or without rhPF4 (0.5 μM) for 2 hours. Parasites were grown in Fy-deficient (Fy^{-/-}) or sufficient (Fy^{+/+}) red cells. **(D)** Quantification of the PF4 immunoblot analysis. Error bars represent the mean pixel intensity (±SEM) of the PF4 immunoreactive bands relative to EXP2 [shown in (C)]. *****P** < 0.001 compared with Fy^{+/+} cells.

Acknowledgments: We thank the Australian Red Cross Blood Services in Hobart and Brisbane for providing red blood cells and antibodies, the platelet donors, and C. Flowers and R. McWhirter for phlebotomy. We also acknowledge funding support from the National Health and Medical Research Council of Australia (grants 605524 and 490037), the Australian Cancer Research Foundation, the National Collaborative Research Infrastructure Strategy of Australia, The Bill and Melinda Gates Foundation Grand Challenges Explorations, The Howard Hughes Medical Institute, Australian Research Council (grant DP120100061 and Future Fellowship to J.G.B.), and the Victorian State Government Operational Infrastructure Support Scheme. Ethics approval for the platelet donations was received from the Human Research Ethics Committee (Tasmania) Network (H0009004). Data are deposited in the Dryad Repository: <http://dx.doi.org/10.5061/dryad.8sb5h>

Supplementary Materials
www.sciencemag.org/cgi/content/full/338/6112/1348/DC1
 Materials and Methods
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 References (33–36)
 15 August 2012; accepted 25 October 2012
 10.1126/science.1228892

Platelet Factor 4 and Duffy Antigen Required for Platelet Killing of *Plasmodium falciparum*

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Science **338** (6112), 1348-1351.
DOI: 10.1126/science.1228892

Platelets Poison Parasites

Activated platelets bound to malaria parasite-infected red blood cells were once thought to contribute to pathogenesis, but recently the platelets have been found to have a protective effect. **McMorran et al.** (p. 1348; see the Perspective by Engwerda and Good) extended this discovery to show that platelet activation releases intracellular granules containing a chemokine, PF4, which is internalized by *Plasmodium falciparum*-infected red cells. Subsequently, mature parasites within the cells die. The Duffy blood-group factor on red blood cells is known to act as a nonspecific receptor for chemokines, such as PF4, as well as a receptor for cell invasion by other species of malaria parasite. When the Duffy antigen was blocked by antibody treatment, platelets and PF4 were less able to kill the *P. falciparum* parasites within.

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