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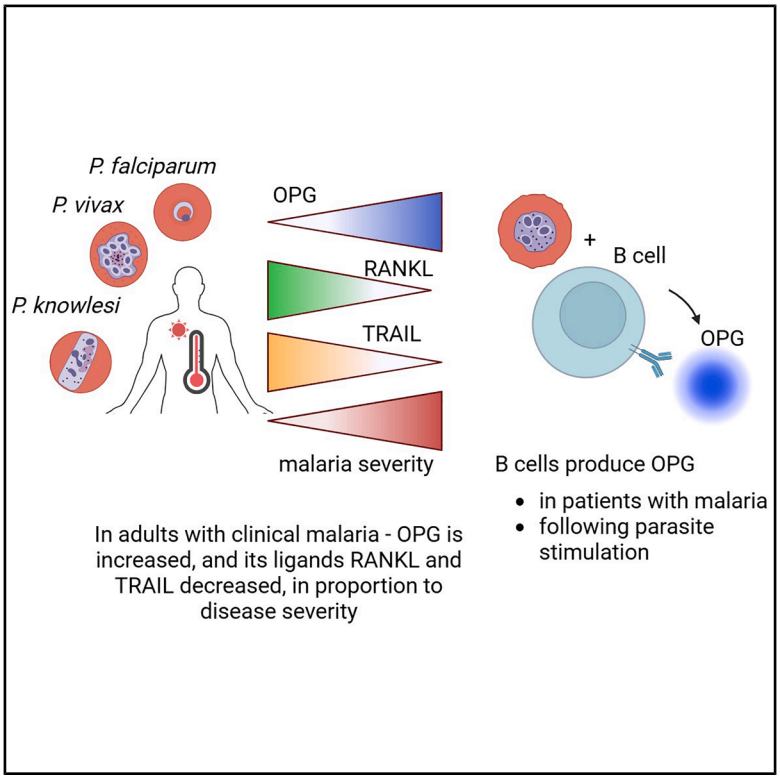
Graphical abstract



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In brief

Disease; Molecular biology; Immune response

Highlights

- In adults with malaria, OPG is increased and its ligands RANKL and TRAIL are decreased
- Increased OPG, and decreased RANKL and TRAIL, are associated with disease severity
- The malaria parasite *P. falciparum* stimulates B cells to produce OPG *in vitro*
- B cell production of OPG is increased *ex vivo* in patients with malaria

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Osteoprotegerin and its ligands RANKL and TRAIL in falciparum, vivax, and knowlesi malaria

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SUMMARY

Osteoprotegerin (OPG), a soluble decoy receptor for receptor activator of NF-ĸB ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL), is increasingly recognized as a marker of poor prognosis in cardiovascular disease. Here, we demonstrate that in adults with falciparum and vivax malaria, OPG is increased, and its ligands TRAIL and RANKL decreased, in association with validated markers of disease severity. Using longitudinal samples from malaria volunteer infection studies, we demonstrate that TRAIL is unexpectedly increased in early infection, suggesting binding of OPG to RANKL prior to TRAIL. Finally, in addition to its known vascular origin, we show that *P. falciparum* stimulates B cells to produce OPG *in vitro*, and that B cell OPG production is increased *ex vivo* in patients with falciparum, vivax, and knowlesi malaria. Our findings provide evidence of the importance of the OPG/RANKL/TRAIL pathway in pathogenesis of acute systemic inflammatory and microvascular diseases, with implications for adjunctive therapies.

INTRODUCTION

Osteoprotegerin (OPG) is a naturally circulating soluble decoy receptor for receptor activator of NF-ĸB ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL). OPG was initially identified as a key modulator of bone metabolism, produced by osteoblasts and regulating bone remodeling through its inhibition of RANKL-mediated osteoclastogenesis.[1,2] OPG, however, is also produced *in vitro* by vascular smooth muscle cells,[3] endothelial cells,[4] and immune cells including B lymphocytes and dendritic cells,[5] and its role in the cardiovascular and immune systems is increasingly recognized.[6] OPG is now well documented to be a marker of poor prognosis in cardiovascular disease, and has been associated with all-cause mortality in a number of studies.[7,8] The pathogenic effects of OPG within the cardiovascular system are incompletely understood, but are thought to relate in part to endothelial activation and dysfunction, mediated through upregulation of endothelial cell adhesion molecules, increased production of reactive oxygen species, apoptosis of human endothelial progenitor cells, and inhibition of endothelial nitric oxide (NO) synthase.[7]

Many of the pathogenic processes mediated by OPG are also key features of severe malaria.[9] Indeed, we have recently demonstrated that OPG is increased in adults with malaria from the zoonotic parasite *Plasmodium knowlesi*, and is associated with adverse effects such as microvascular dysfunction and acute kidney injury.[10] However, OPG has not been evaluated in adults with clinical malaria from *P. falciparum* and *P. vivax*, the parasite species accounting for almost 100% of malaria deaths worldwide.[11] Furthermore, the role of the OPG ligands RANKL and TRAIL have neither been evaluated in malaria, nor the cellular sources of OPG.

Here, we demonstrate that in adults with falciparum and vivax malaria, OPG is increased, and its ligands TRAIL and RANKL decreased, in association with validated markers of disease severity. Further, in addition to its known vascular origin, we demonstrate that OPG is also produced from B cells stimulated by *P. falciparum* parasites *in vitro*, and *ex vivo* from B cells from patients with *P. falciparum*, *P. vivax*, and *P. knowlesi* malaria. Our findings provide evidence of the importance of the OPG/RANKL/TRAIL pathway in pathogenesis of acute systemic inflammatory and microvascular diseases, with implications for adjunctive therapies.

Table 1. Baseline characteristics of Malaysian malaria patients and controls

	Controls (*n* = 50)	*P. falciparum* (*n* = 169)	*P. vivax* (*n* = 62)	*P. knowlesi* (*n* = 201)
Age, years (median, range)	43 (14–69)	27 (13–78)	27 (13–79)	45 (13–94)
Male sex, n (%)	34 (68)	124 (73)	48 (77)	157 (78)
Ethnicity, n (%)				
Dusun	17 (34)	84 (50)	27 (44)	61 (30)
Kadazan	7 (14)	23 (14)	4 (6)	23 (11)
Rungus	14 (28)	4 (2)	1 (2)	41 (20)
Bajau	4 (8)	19 (11)	10 (16)	23 (11)
Other	8 (16)	39 (23)	20 (32)	53 (26)
Fever duration, days		5 (3–7)	5 (3–7)	5 (3–7)
Parasite count, parasites/μL		10,500 (3425–33,571)	4735 (2848–10,336)	5550 (1596–31,862)
Severe malaria, n (%)		19 (11%)	9 (15%)	48 (24%)
Creatinine, μmol/L		87 (73–103)	87 (69–101)	100 (81–122)
Lactate, mmol/L		1.24 (0.91–1.66); *N* = 144	1.24 (0.83–1.55); *N* = 56	1.2 (0.98–1.56); *N* = 181
Angiopoietin-2, pg/mL	1182 (875–1597)	3700 (2228–5840)	5007 (3580–6863)	5083 (3288–8023)
VWF, pg/mL	1156 (843–1634); *N* = 30	5470 (3906–7424); *N* = 54	4303 (3108–5561); *N* = 38	5171 (4251–6256); *N* = 85
P-selectin, pg/mL	40 (31–52)	46 (35–69)	47 (34–62)	33 (26–42); *N* = 199
ICAM-1, pg/mL	149 (123–167)	550 (395–705)	507 (416–686)	482 (379–646)
E-selectin, pg/mL	19 (13–25)	59 (42–86)	69 (43–97)	53 (40–69)
OPG, pg/mL	986 (625–1463)	2344 (1310–4666)	2578 (1301–5642); *N* = 61	2475 (1727–4214); *N* = 199
RANKL[a], pmol/L	0.225 (0.141–0.335); *N* = 20	0.031 (0.031–0.083); *N* = 43	0.031 (0.031–0.031); *N* = 25	0.031 (0.031–0.031); *N* = 28
TRAIL, pg/mL	74 (66–99); *N* = 20	58 (45–84)	58 (45–72); *N* = 61	50 (36–63); *N* = 197

Table includes all patients who had plasma OPG and/or TRAIL measured. Numbers are median (inter-quartile range) unless otherwise stated. HRP2: histidine rich protein 2; VWF: von Willibrand factor; ICAM-1: intracerebral adhesion molecule 1; OPG: osteoprotegerin; RANKL: receptor activator of NF-kB ligand; TRAIL: tumor necrosis factor apoptosis inducing ligand.

[a]Patients with a RANKL concentration below the limit of detection were assigned a value half that of the limit of detection, which was 0.0625 pg/mL. RANKL was below the limit of detection in 27 (63%) patients with *P. falciparum*, 21 (84%) patients with *P. vivax* and 22 (79%) patients with *P. knowlesi*.

RESULTS

OPG is increased in Malaysian patients with falciparum and vivax malaria

OPG was evaluated in 429 Malaysian patients hospitalized with malaria and 50 healthy controls enrolled at the same study site. Patients with malaria included 169 with *P. falciparum* (150 non-severe, 19 severe), 61 with *P. vivax* (53 non-severe, 8 severe), and 199 with *P. knowlesi* (153 non-severe, 46 severe). Clinical and pathophysiological data from this cohort of patients have been previously reported.[12–14] Baseline demographic and clinical characteristics are shown in Table 1. Overall, 329 (76%) of the patients with malaria were male, and the median age was 34 years (range 13–94 years).

OPG was increased in patients with falciparum and vivax malaria, compared to controls ($p < 0.0001$ for both comparisons; Table 1 and Figure 1). OPG was also higher in patients with severe compared to non-severe disease, although in vivax malaria this difference was not statistically significant ($p = 0.052$; Figure 1). There was no significant difference in the concentration of plasma OPG between species. There was also no significant difference in concentration of OPG between males and females, in either falciparum or vivax malaria.

In patients with falciparum malaria, OPG was correlated with parasitemia and age (Table 2). In falciparum malaria OPG also correlated with other markers of disease severity including creatinine, platelet nadir, IL-6 and the endothelial activation markers angiopoietin-2 and E-selectin, with the correlations with creatinine, platelet nadir, IL-6, and E-selectin remaining significant after controlling for parasitemia. In patients with vivax malaria, OPG was correlated with parasitemia, IL-6, angiopoietin-2, and E-selectin, with the correlations with IL-6 and E-selectin remaining significant after controlling for parasitemia (Table 2). There was no correlation between OPG and endothelial function, in either falciparum or vivax malaria.

RANKL and TRAIL are reduced in Malaysian patients with falciparum, vivax, and knowlesi malaria

Plasma concentrations of the OPG ligand TRAIL were measured in 169 patients with falciparum malaria (150 non-severe, 19

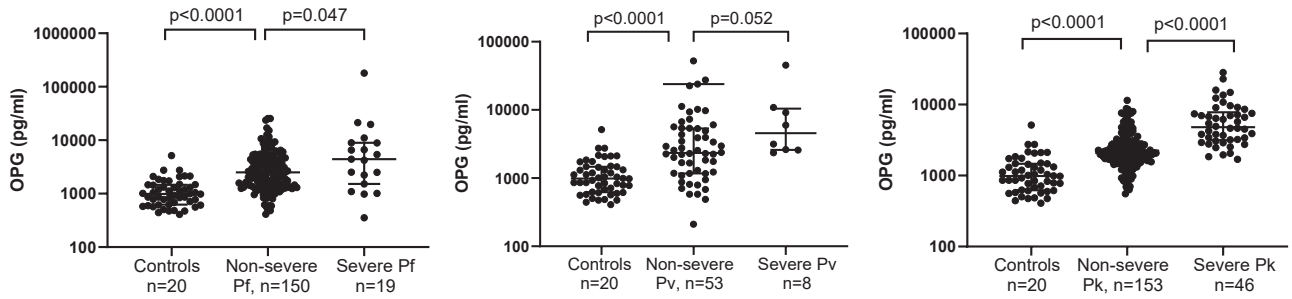


Figure 1. Plasma concentrations of osteoprotegerin (OPG) in Malaysian patients with *P. falciparum*, *P. vivax*, and *P. knowlesi* malaria

Pf = *P. falciparum*; Pv = *P. vivax*; Pk = *P. knowlesi*. Errors bars represent median and inter-quartile range. Data for patients with knowlesi malaria have been previously reported,[10] and are included here for comparison with falciparum and vivax malaria.

severe), 61 patients with vivax malaria (52 non-severe, 9 severe), and 197 patients with knowlesi malaria (149 non-severe, 48 severe). RANKL was measured in a subset of patients including 43 with *P. falciparum* (24 non-severe, 19 severe), 25 with *P. vivax* (19 non-severe, 6 severe), and 28 with *P. knowlesi* (15 non-severe, 13 severe). Consistent with the binding of OPG to its ligands, plasma concentrations of RANKL and TRAIL were both reduced in patients with malaria compared to controls (Table 1 and Figure 2). RANKL was suppressed to below the level of detection in 70/96 (73%) of all malaria patients, including 27 (63%) of those with *P. falciparum*, 21 (84%) of those with *P. vivax*, and 22 (79%) of those with *P. knowlesi*, compared to only 1 of 20 (5%) controls ($p < 0.0001$ for all comparisons between each species and controls). RANKL was undetectable in all patients with severe knowlesi and vivax malaria, although was detectable (at mostly low levels) in 8/19 (42%) of patients with severe falciparum malaria. Similar to RANKL, TRAIL was also reduced in patients with falciparum, vivax, and knowlesi malaria compared to controls ($p = 0.008$, $p = 0.0004$, and $p < 0.0001$, respectively). In patients with vivax and knowlesi malaria, TRAIL was lower in those with severe compared to non-severe disease ($p = 0.0009$ and $p < 0.0001$, respectively); however, this difference was not observed in patients with falciparum malaria. There were no differences in concentrations of RANKL or TRAIL between males and females, in any species.

In all species, TRAIL was inversely correlated with parasitemia and with age, although in *P. falciparum* and *P. knowlesi* the correlation with age did not remain significant after controlling for parasitemia (Table 3). As expected (due to the binding of TRAIL to OPG), in all species TRAIL was inversely correlated with OPG; in *P. vivax* and *P. knowlesi* this remained significant after controlling for parasitemia (Table 3). In all species TRAIL was also inversely correlated with angiopoietin-2, remaining significant after controlling for parasitemia in *P. falciparum* and *P. knowlesi*. In *P. knowlesi* TRAIL was inversely correlated with creatinine, and positively correlated with hemoglobin and platelet nadir, with these correlations remaining significant after controlling for parasitemia. In patients

Table 2. Correlations with OPG in patients with falciparum and vivax malaria

	P. falciparum ($n = 169$)				*P. vivax* ($n = 61$)			
	Univariate analysis		Controlling for parasitemia		Univariate analysis		Controlling for parasitemia	
	Correlation coefficient	*p* value	Correlation coefficient	*p* value	Correlation coefficient	*p* value	Correlation coefficient	*p* value
Parasite count	0.29	**<0.0001**			0.47	**0.0002**		
Age	0.24	**0.002**	0.20	0.008	0.05	0.67		
Creatinine	0.24	**0.002**	0.18	**0.003**	0.05	0.68		
Lactate	0.18	0.031	0.25	**0.006**	0.30	0.026	0.28	0.038
IL-6	0.47	**<0.0001**	0.39	**<0.0001**	0.61	**<0.0001**	0.47	**0.0002**
Angiopoietin-2	0.22	**0.004**	0.17	0.022	0.40	**0.001**	0.24	0.07
ICAM-1	0.17	0.023	0.23	0.014	0.19	0.15		
E-selectin	0.41	**<0.0001**	0.37	**<0.0001**	0.39	**0.002**	0.38	**0.003**
Hb nadir	0.12	0.11			−0.01	0.91		
Plt nadir	−0.32	**<0.0001**	−0.29	**0.0001**	−0.20	0.12		
Endothelial function[a]	0.01	0.96			−0.01	0.95		

[a]Measured by peripheral arterial tonometry in 88 patients with *P. falciparum* and 38 patients with *P. vivax*. ICAM-1: intracerebral adhesion molecule 1; Hb: hemoglobin; Plt: platelet. Partial correlations on log-transformed variables, controlling for parasitemia, were conducted for all variables with *p* value < 0.05 on univariate analysis. *p* values in bold are considered statistically significant ($p < 0.0045$) after using the conservative Bonferroni correction to account for multiple correlations (11 variables).

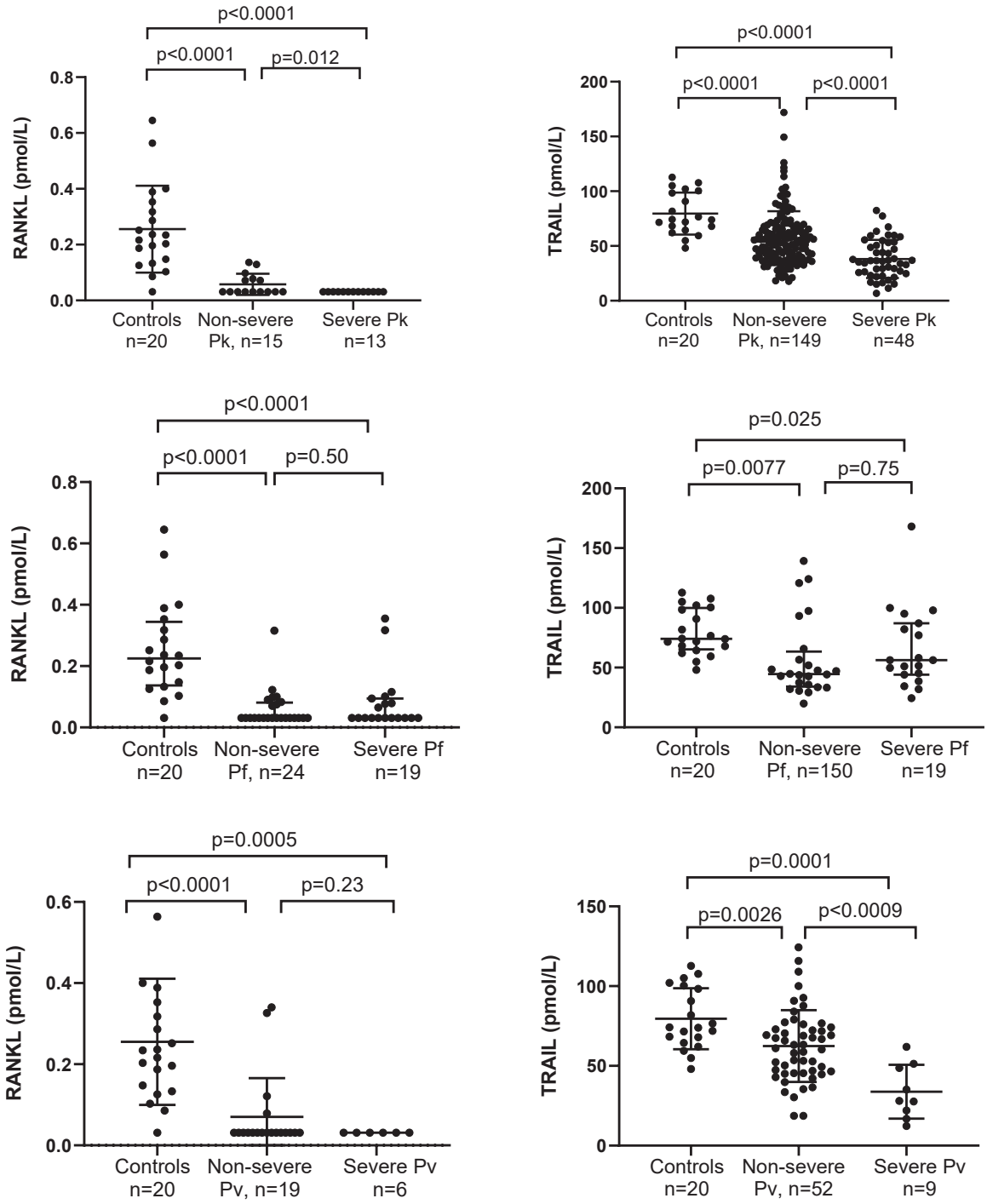


Figure 2. Plasma concentrations of RANKL and TRAIL in Malaysian patients with *P. falciparum*, *P. vivax*, and *P. knowlesi* malaria
Errors bars represent median and inter-quartile range.

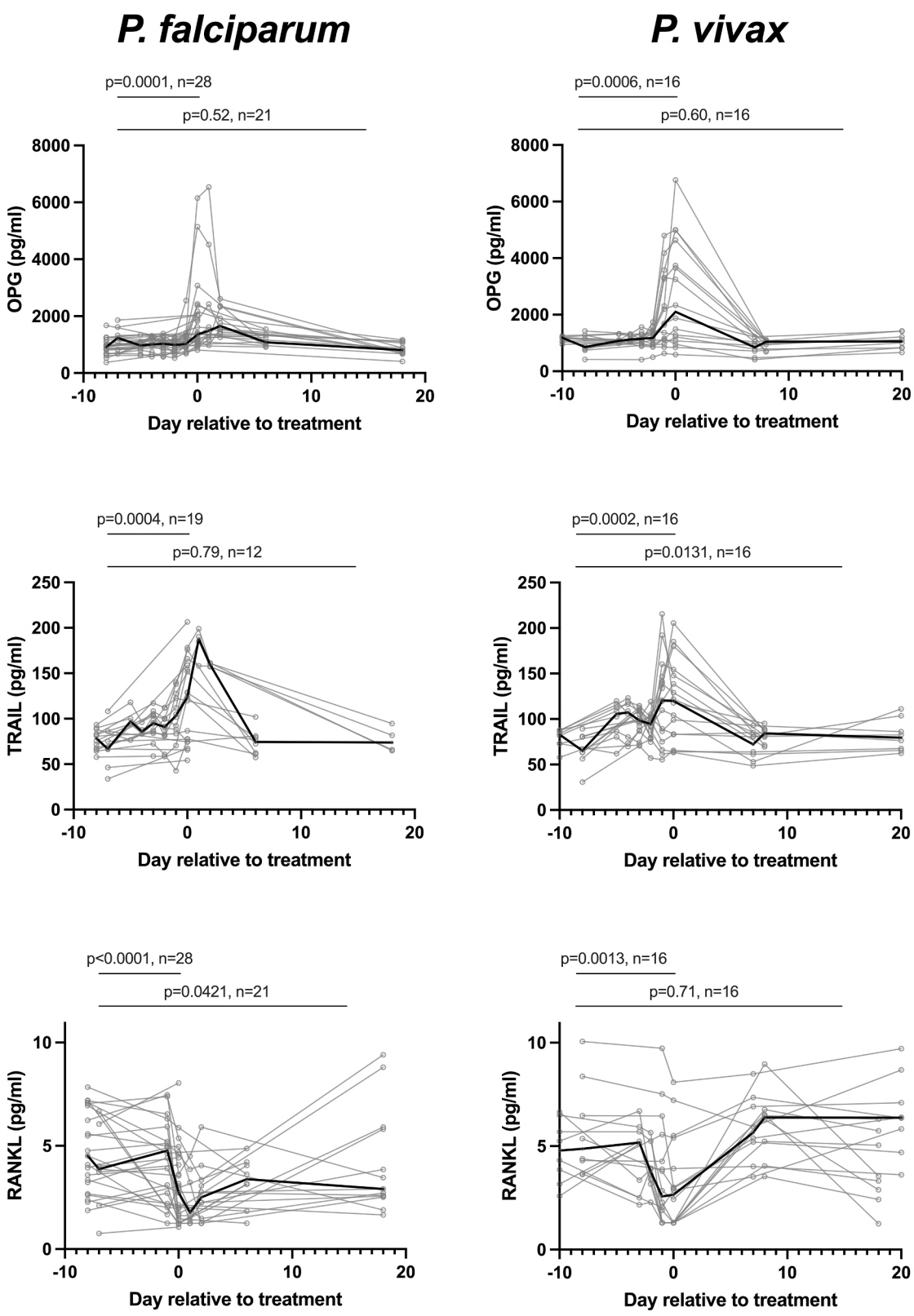
with *P. knowlesi*, in a backward stepwise linear regression model including age, parasitemia, and OPG, TRAIL was inversely proportional to risk of having acute kidney injury on admission (defined by KDIGO criteria; Table S1). However, this did not remain significant if angiopoietin-2, another known risk factor for AKI in knowlesi malaria,[10] was included in the model.

Previous studies have demonstrated that the OPG/TRAIL ratio may have greater prognostic value than either biomarker alone,[7] and we therefore also evaluated correlates of this ratio. In

Table 3. Correlations with TRAIL in patients with falciparum, vivax and knowlesi malaria

	P. falciparum (*n* = 169)				*P. vivax* (*n* = 61)				*P. knowlesi* (*n* = 197)			
	Univariate analysis		Controlling for parasitemia		Univariate analysis		Controlling for parasitemia		Univariate analysis		Controlling for parasitemia	
	Correlation coefficient	*p* value	Correlation coefficient	*p* value	Correlation coefficient	*p* value	Correlation coefficient	*p* value	Correlation coefficient	*p* value	Correlation coefficient	*p* value
Parasite count	−0.28	**0.0002**			−0.40	**0.001**			−0.35	**<0.0001**		
Age	−0.18	0.018	−0.15	0.060	−0.45	**0.0002**	−0.49	0.0001	−0.23	**0.001**	−0.14	0.053
Creatinine	−0.10	0.19			−0.30	0.019			−0.40	**<0.0001**	−0.31	**<0.0001**
Lactate	−0.17	0.045	−0.17	0.060	−0.03	0.80			−0.16	0.038	−0.06	0.33
IL-6	−0.22	**0.004**	−0.11	0.15	−0.22	0.093	−0.05	0.81	−0.35	**<0.0001**	−0.12	0.06
Ang-2	−0.39	**<0.0001**	−0.27	**0.0002**	−0.41	**0.0009**	−0.28	0.018	−0.49	**<0.0001**	−0.36	**<0.0001**
OPG	−0.26	**0.0006**	−0.15	0.024	−0.49	**0.0001**	−0.36	0.017	−0.34	**<0.0001**	−0.20	**0.001**
ICAM-1	−0.14	0.068			0.08	0.53			−0.13	0.070		
E-selectin	−0.17	0.026	−0.13	0.11	−0.09	0.49			−0.07	0.34		
Hb nadir	−0.06	0.42			0.03	0.83			0.30	**<0.0001**	0.20	**0.002**
Plt nadir	0.227	**0.003**	−0.17	0.028	0.19	0.13			0.32	**<0.0001**	0.21	**0.001**
Endothelial function[a]	0.01	0.94			0.14	0.41			0.26	0.010	0.06	0.14

[a]Measured by peripheral arterial tonometry in 88 patients with *P. falciparum*, 38 with *P. vivax,* and 96 with *P. knowlesi*. IL-6: interleukin 6; Ang-2: angiopoietin-2; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor kappa B lamda; ICAM-1: intracerebral adhesion molecule 1; Hb: hemoglobin; Plt: platelet. For *P. falciparum* and *P. knowlesi*, the correlations with Ang-2 remained significant after controlling for OPG ($p = 0.0001$, and $p < 0.0001$, respectively). Partial correlations on log-transformed variables, controlling for parasitemia, were conducted for all variables with p value < 0.05 on univariate analysis. p values in bold are considered statistically significant ($p < 0.0042$) after using the conservative Bonferroni correction to account for multiple correlations (12 variables).



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general, associations with other biomarkers of severity were indeed stronger with the OPG/TRAIL ratio than with either TRAIL or OPG alone (Table S2). In each malaria species, the OPG/TRAIL ratio was associated with IL-6, angiopoietin-2, and E-selectin, after controlling for parasitemia. In falciparum and knowlesi malaria, the OPG/TRAIL ratio was also correlated with lactate, ICAM-1 and inversely with platelet nadir. In knowlesi malaria, the OPG/TRAIL ratio was inversely correlated with endothelial function.

Clinical correlates of RANKL were not evaluated due to the low proportion of patients with detectable RANKL. However, given previous data from murine studies demonstrating increased RANKL expression following recovery from acute malaria (resulting in increased osteoclastogenesis and bone resorption),[15] we measured RANKL on samples taken on day 28 follow-up in 15 patients with *P. falciparum*, 15 with *P. vivax*, and 17 with *P. knowlesi* malaria. In all species, RANKL concentrations at day 28 were increased above enrollment levels ($p < 0.001$ for each species), but were not elevated above the levels observed in healthy controls (Figure S1).

RANKL is reduced, while TRAIL increased, in volunteers infected with *P. falciparum* and *P. vivax*

Characteristics of participants enrolled in malaria volunteer infection studies (VIS), and details of these studies, are described in Table S3. Data were included from 28 participants enrolled in *P. falciparum* VIS, and 16 participants enrolled in *P. vivax* VIS.

We previously reported that concentrations of OPG were increased early in volunteers experimentally infected with *P. falciparum* and *P. vivax*, consistent with the increase observed in Malaysian patients with clinical malaria.[16] Also consistent with data from the Malaysian patients with malaria, RANKL was reduced in volunteers infected with *P. falciparum* and *P. vivax*, being suppressed to below the level of detection in 7/28 (25%) participants infected with *P. falciparum*, and 7/16 (44%) participants infected with *P. vivax* (Figure 3). In both the *P. falciparum* and the *P. vivax* groups, RANKL was lower at the time of antimalarial treatment compared to baseline ($p < 0.0001$ and $p = 0.0013$, respectively). By the end of the studies, RANKL had returned to baseline levels in the *P. vivax* group, although remained slightly lower than baseline levels in the *P. falciparum* group ($p = 0.042$). On the day of treatment, RANKL was inversely correlated with parasitemia in participants inoculated with *P. vivax*, although this did not remain significant after controlling for OPG (Table S4). There was no correlation between RANKL and parasitemia in participants inoculated with *P. falciparum*. As expected, on the day of treatment RANKL was strongly inversely correlated with OPG in both the *P. falciparum* ($r = -0.66$, $p = 0.0002$) and *P. vivax* ($r = -0.92$, $p = <0.0001$) groups, remaining significant after controlling for parasitemia.

In contrast to Malaysian patients with clinical malaria, plasma concentrations of TRAIL were unexpectedly increased in volunteers infected with *P. falciparum* and *P. vivax*, with concentrations peaking on the day of treatment (*P. falciparum:* $p = 0.0004$, *P. vivax:* $p = 0.0002$) before falling to near baseline levels following treatment (Figure 3). On the day of treatment TRAIL was positively correlated with parasitemia in participants inoculated with *P. vivax*, but not *P. falciparum* (Table S5).

B cell production of OPG is increased following stimulation by parasitized red blood cells

Given the marked elevation in OPG observed in experimental and clinical malaria, and the correlations with disease severity in the latter, we sought to determine if *P. falciparum* parasites could stimulate B cells to produce OPG. Peripheral blood mononuclear cells (PBMCs) isolated from buffy coats from healthy donors ($n = 10$) from Australian Red Cross LifeBlood were stimulated with *P. falciparum* parasitized red blood cells (pRBCs) or uninfected RBCs (uRBCs). Expression of OPG from B cells was increased in donors after stimulation with pRBCs, compared to uRBCs ($p = 0.0039$, Figures 4A and 4B). Negligible OPG was detected from other cell lineages, including NK cells and monocytes (Figure S2). To identify the B cell subsets producing OPG in response to parasite stimulation, B cells were characterized as naive (IgD+) or non-naïve (IgD−) B cells (Figure 4C). Following stimulation with pRBCs, the large majority of OPG was produced by naive (IgD+) B cells (Figure 4D).

B cell production of OPG is increased during clinical malaria

To evaluate whether B cell expression of OPG is increased in clinical malaria, we measured *ex vivo* OPG production from B cells from Malaysian patients with malaria (6 with *P. falciparum*, 8 with *P. vivax*, and 7 with *P. knowlesi*) on enrollment and at day 28, and from 5 healthy controls recruited from the same study site. Age and sex were similar between groups (Table S6). Compared to controls, *ex vivo* OPG B cell expression was higher in patients with falciparum, vivax, and knowlesi malaria, although numbers were small and the latter did not reach statistical significance ($p = 0.017$, $p = 0.045$ and $p = 0.073$, respectively; Figures 5A, 5B, and S3A). There was limited OPG detected from other cell lineages (Figure S3B). B cell expression of OPG had decreased by day 28 in patients with falciparum, vivax and knowlesi malaria ($p = 0.062$, $p = 0.078$, and $p = 0.016$, respectively; Figure 5B). In contrast to parasite-stimulated PBMCs from blood donors, in patients with malaria OPG from B cells was detected in both IgD+ and IgD− subsets, with no difference in subset distribution between species (Figures 5C and 5D). Within IgD− cells, the majority of OPG B cells were CD27 positive/CD21 negative memory B cells.

DISCUSSION

OPG is increasingly recognized as a marker of disease severity in a wide range of conditions. We previously reported that OPG

Figure 3. Longitudinal concentrations of OPG, RANKL and TRAIL in participants inoculated with *P. falciparum* (left column) and *P. vivax* (right column)

Black line represents median. Gray lines represent individual participants. Comparisons by Wilcoxon matched pair signed rank test for baseline (day of controlled malaria infection) and day of antimalarial treatment, and baseline and end of study measurements. Data for OPG has already been reported,[16] and is included here for context.

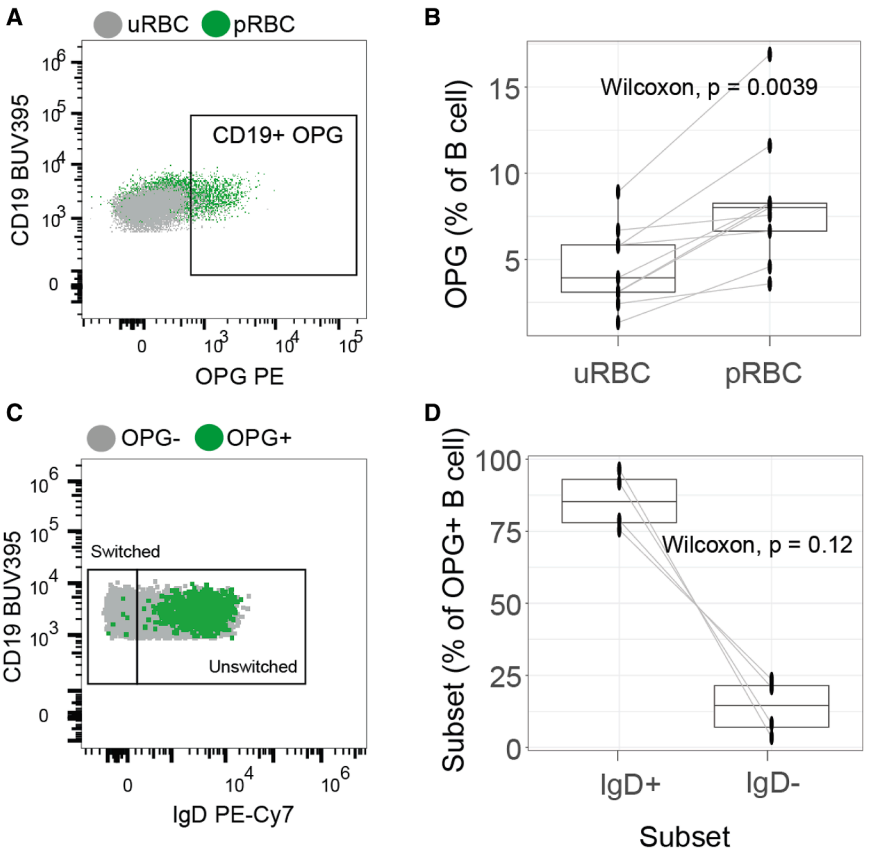

Figure 4. Malaria parasites can induce OPG expression in B cells

(A) PBMCs from buffy coats from Australian donors (n = 10) were stimulated with uninfected RBCs (uRBCs) or *P, falciparum*-infected RBCs (pRBCs) and OPG production from B cells assessed by flow cytometry. Representative gating figure of OPG from B cells following pRBC or uRBC stimulation.

(B) OPG B cell expression from malaria-naive donors, after stimulation with uRBCs or pRBCs. The p value was calculated using Wilcoxon signed-rank test. The boxplots represent the median and inter-quartile range.

(C) Analysis of B cell subsets that produce OPG. Representative gating figure of IgD expression from OPG positive cells B cells.

(D) IgD+ and IgD− B cell subsets as a proportion of all OPG+ B cells following parasite stimulation of PBMCs from malaria-naïve donors (n = 4). The p value was calculated using Wilcoxon signed-rank test. The boxplots represent the median and inter-quartile range.

See also Figure S3.

was increased in adults with knowlesi malaria, and associated with markers of disease severity.[10] In this study we extend these reports and demonstrate that OPG is also increased, and associated with disease severity, in Malaysian adults with falciparum and vivax malaria. We also demonstrate that the OPG ligands RANKL and TRAIL are reduced in adults with falciparum, vivax and knowlesi malaria, with this reduction in TRAIL being associated with markers of disease severity. Consistent with these findings in clinical malaria, RANKL was also reduced in volunteers enrolled in *P. falciparum* and *P. vivax* VIS, although TRAIL was unexpectedly increased, suggesting that binding of OPG to RANKL precedes binding to TRAIL. Finally, we show that *P. falciparum* pRBCs stimulate B cell production of OPG *in vitro*, and that B cell production of OPG is increased *ex vivo* in patients with falciparum, vivax, and knowlesi malaria.

Our finding that OPG is elevated in adults with falciparum malaria is consistent with a report of elevated OPG in African children with cerebral malaria.[17] In the current study, in adults with falciparum malaria OPG was associated with markers of inflammation and disease severity including IL-6, platelet nadir, creatinine, and the endothelial activation markers angiopoietin-2 and E-selectin. These findings are consistent with the production of OPG from endothelial Weibel-Palade bodies in response to inflammatory cytokines,[4] and the role of OPG in inhibiting endothelial NO, exacerbating endothelial activation and upregulating adhesion molecules.[7,18,19] These findings suggest that OPG may play a key role in mediating pathogenesis of falciparum malaria, particularly given the independent role of endothelial activation in predicting severe disease and death in falciparum malaria.[20]

OPG was also associated with markers of disease severity in patients with vivax malaria, including IL-6. This association between OPG and IL-6 in both falciparum and vivax malaria is consistent with murine data demonstrating that LPS-induced production of inflammatory cytokines was reduced in mice lacking OPG.[21] Pathogenic mechanisms in *P. vivax* are less well understood than in *P. falciparum* malaria[22]; however, the inflammatory response induced by *P. vivax* parasites has been shown to be more prominent than in falciparum malaria,[23] and our data suggest that OPG may contribute to this inflammatory response.

To our knowledge this is the first study that has evaluated concentrations of the OPG ligands RANKL and TRAIL in patients with malaria from any species. Consistent with the binding of these ligands to OPG, concentrations of RANKL and TRAIL were both reduced in patients with clinical malaria. This was particularly apparent for RANKL, which was suppressed to below the level of detection in the majority of patients with malaria, even in those with non-severe disease. This suppression of RANKL would be expected to lead to reduced RANKL/RANK signaling, thereby increasing downstream production of proinflammatory cytokines.[21]

As with RANKL, concentrations of TRAIL were also reduced in patients with falciparum, vivax, and knowlesi malaria, and in vivax and knowlesi malaria, were lower in patients with severe compared to non-severe disease. In all species, concentrations of TRAIL were inversely proportional to parasitemia, and with the endothelial activation marker angiopoietin-2. In knowlesi malaria, concentrations of TRAIL were associated with hemoglobin nadir, platelet nadir, and inversely with creatinine, suggesting that reduced levels

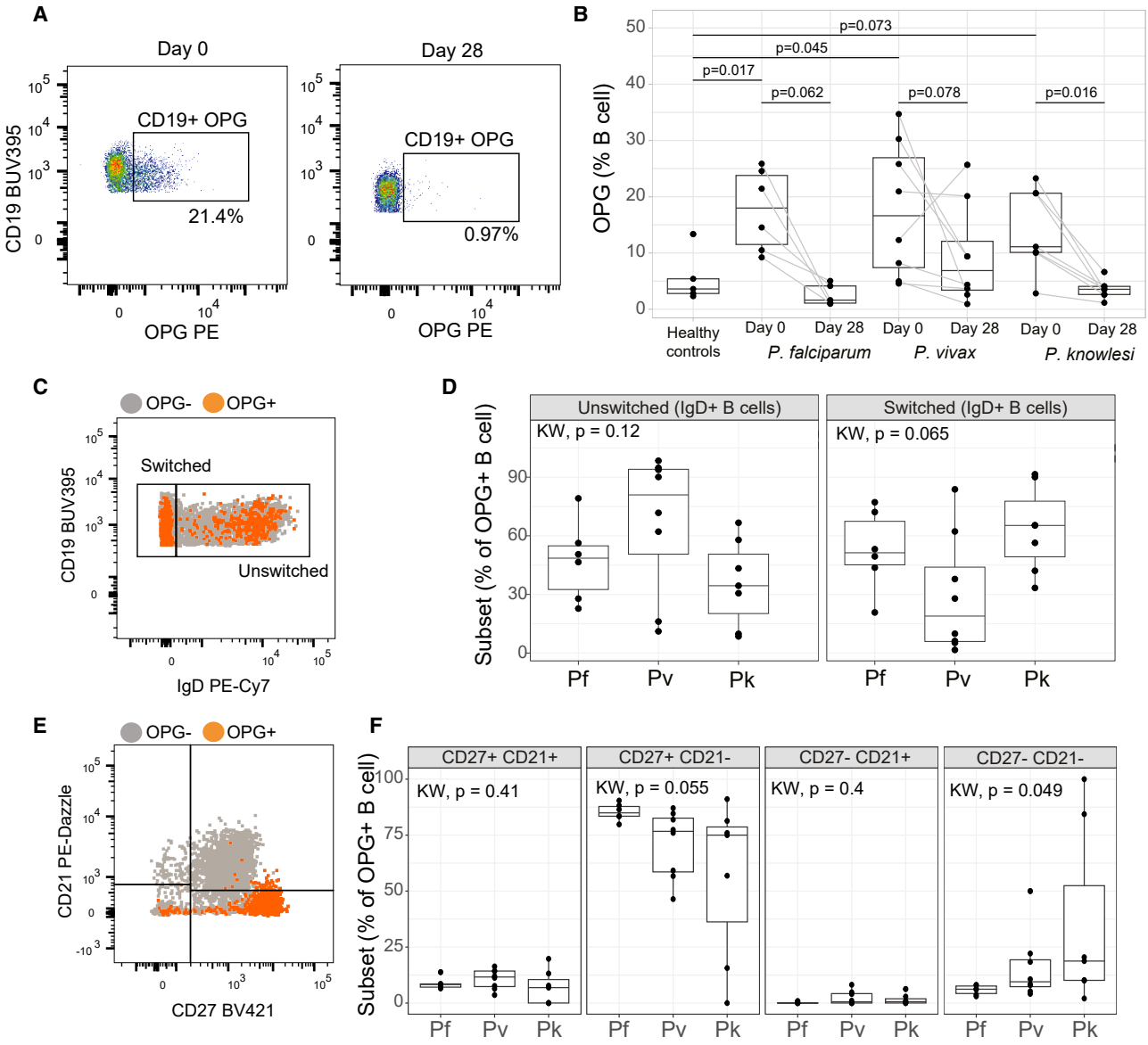

Figure 5. B cell production of OPG during clinical malaria

(A) OPG expression from B cells at enrollment (day 0) and convalescence (day 28), gating example.

(B) Proportion of B cells expressing OPG in Malaysian patients with *P. falciparum* (n = 6 day 0 and n = 5 day 28), *P. vivax* (*n* = 8) and *P. knowlesi* (*n* = 7), compared to Malaysian healthy controls (*n* = 5). *p* values between healthy controls and day 0 malaria infection were calculated using Mann Whitney. *p* values between day 0 and day 28 were calculated using Wilcox signed-rank test. The boxplots represent the median and inter-quartile range.

(C) IgD expression in OPG+ B cells, gating example.

(D) IgD+ and IgD- B cell subsets as a proportion of all OPG+ B cells at enrollment in patients with malaria. *p* values were calculated using Kruskal-Wallis (KW). The boxplots represent the median and inter-quartile range.

(E) CD27 and CD21 expression in OPG+ IgD− B cells, gating example.

(F) CD27 and CD21 B cell subsets as a proportion of all IgD− OPG+ B cells at enrollment in patients with malaria. *p* values were calculated using Kruskal-Wallis (KW). The boxplots represent the median and inter-quartile range.

See also Figure S3.

of TRAIL may contribute to disease pathogenesis. Low levels of TRAIL have also been associated with poor outcomes in cardiovascular disease.[7] While the mechanisms of the potential protective effects of TRAIL are uncertain, TRAIL has been shown to increase endothelial NO production, and to inhibit angiotensin II-induced production of reactive oxygen species, possibly leading to stabilization of activated endothelium.[24–26] Consistent with this protective effect, we found an association between the OPG/TRAIL ratio and endothelial function in patients with knowlesi malaria, remaining significant after controlling for parasitemia.

We also evaluated concentrations of RANKL and TRAIL in participants enrolled in *P. vivax* and *P. falciparum* malaria VIS. Consistent with the suppression of RANKL in patients with clinical malaria, RANKL was also decreased on the day of treatment in these participants. However, in contrast to the decreased concentrations of TRAIL observed in patients with clinical malaria, TRAIL was unexpectedly increased in participants inoculated with the *P. falciparum* and *P. vivax*. This finding suggests that TRAIL increases early in infection, but declines as disease progresses, likely due to binding of TRAIL to increasing concentrations of OPG. The fact that suppression of RANKL precedes reductions in TRAIL is consistent with the greater binding affinity of OPG to RANKL,[27] with OPG possibly only binding to TRAIL later in the disease course once free RANKL is no longer available.

Given the increase in OPG in clinical malaria and the likely role that this biomarker plays in a number of pathophysiological pathways, we sought to determine the role of immune cells in producing OPG in malaria. B cells are well-recognized as a source of OPG[5]; indeed, in a mouse study, Li et al. demonstrated that B cells accounted for >60% of all bone marrow production of OPG, and that B cell deficient mice had reduced levels of OPG.[28] In the current study, we found that *P. falciparum*-infected RBCs stimulated B cells to produce OPG *ex vivo*. Further, B cell expression of OPG was increased in patients with falciparum, vivax, and knowlesi malaria, compared to healthy controls. While OPG producing B cells *in vitro* were largely naive, during clinical infection both naive and memory B cell subsets produced OPG, consistent with activation of the adaptive immune response. Our findings suggest that B cell production of OPG may be contributing at least in part to the substantial elevation in plasma OPG in patients with malaria. Significant parasite induction of B cell OPG production is also supported by the recent recognition of the very large reservoir of replicating *P. vivax* and *P. falciparum* parasites in the B cell rich spleen.[29,30]

B cell production of OPG in malaria is also consistent with a growing body of evidence suggesting a link between OPG and immunity, with OPG shown to regulate B cell maturation and development of efficient antibody responses through CD40L signaling.[5] In a murine study, OPG deficient mice accumulated immature B cells in their spleens, and generated impaired antibody responses to a T cell dependent antigen challenge.[5] Another study demonstrated that treatment of mice with OPG increased humoral immune responses to a pneumococcal vaccine.[31] It is therefore possible that in malaria, despite possible adverse pathogenic consequences of elevated OPG, B cell production of OPG may play a role in development of antimalarial immunity. Further studies evaluating associations between OPG and antimalarial immune responses are warranted.

In summary, we found that plasma OPG was increased in adults with falciparum and vivax malaria, and associated with markers of disease severity in both species. The OPG ligands RANKL and TRAIL were both suppressed in clinical falciparum, vivax, and knowlesi malaria, with reductions in TRAIL also associated with markers of severity, suggesting that suppression of RANKL and TRAIL may contribute to OPG-mediated pathogenesis in each *Plasmodium* species. In participants enrolled in malaria VIS, RANKL was also suppressed while TRAIL was unexpectedly increased, suggesting that OPG binds to RANKL early in disease and later to TRAIL as disease progresses. Finally, we demonstrate B cell production of OPG in clinical malaria, highlighting a potential role of OPG in the host immune response to malaria. Further studies evaluating the role of OPG in antimalarial immunity, and B cell production of OPG in other conditions, are warranted.

Limitations of the study

Our study had limitations. First, the strength of many of the reported correlations with OPG and TRAIL was moderate, and it is possible that other confounding factors contributed to these associations. Second, for the experiments evaluating B cell production of OPG, sample sizes were small, limiting our ability to conduct statistical comparisons between groups. Third, although our findings suggest a role of the RANKL/OPG/TRAIL pathway in pathogenesis of malaria, we did not evaluate the mechanisms by which this may occur. Finally, while we hypothesize that B cell production of OPG may be contributing to the elevated concentrations of plasma OPG in malaria, we did not evaluate other sources of OPG.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed and will be fulfilled by the lead contact, Bridget E Barber (bridget.barber@qimrb.edu.au).

Materials availability

The study did not generate new reagents.

Data and code availability

- All data can be obtained from the lead contact, on reasonable request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

B.E.B., N.M.A., and M.J.B. conceived and designed the study. B.E.B., M.J.G., and T.W. conducted the clinical studies in Malaysia. J.W., B.E.B., and J.S.M., conducted the malaria volunteer infection studies. K.P. conducted the ELISAs. A.S.N., D.A., J.L., and F.A. conducted the B cell assays. A.S.N., J.W., J.L., M.J.B., and B.E.B. analyzed the data. A.S.N., J.W., J.L., M.J.B., and B.E.B. wrote the paper, with input from all other authors. All authors approved the final draft of the manuscript. The order of co-first authorship was determined by contribution toward generating and analyzing primary data.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Human CD19-BUV395	BD Biosciences (USA)	Clone SJ25C1;RRID: AB_2738274
Anti-Human CD3-FITC	Biolegend (USA)	Clone HIT3a;RRID: AB_314041
Anti-Human CD21- PE/Dazzle™ 594	Biolegend (USA)	Clone Bu32;RRID: AB_2750242
Anti-Human CD27-BV421	BD Biosciences (USA)	Clone M-T271;RRID: AB_11153497
Anti-Human IgD-PE-Cy7	BD Biosciences (USA)	Clone IA6-2;RRID: AB_10642457
Anti-Human CD14-BV650	BD Biosciences (USA)	Clone M5E2;RRID: AB_2744286
Anti-Human CD56-BV510	Biolegend (USA)	Clone HCD56;RRID: AB_2561385
Biological samples		
Plasma and PBMCs from Malaysian patients with malaria, and from healthy controls	Patients with malaria admitted at Queen Elizabeth Hospital, Sabah, Malaysia. Controls were relatives or friends visiting patients with malaria.	N/A
Plasma from participants enrolled in malaria volunteer infection studies	Patients enrolled in malaria volunteer infection studies at QIMR Berghofer, Brisbane, Australia	N/A
PBMCs isolated from buffy coats obtained from healthy blood donors	Australian Red Cross Lifeblood	N/A
Critical commercial assays		
Human Osteoprotegerin/TNFRSF11B DuoSet ELISA	R&D Systems	DY805
RANKL ELISA kit	Biomedica Diagnostics	BI-20462
TRAIL ELISA kit	R&D Systems	DTRL00
Ang 2 Quantikine ELISA kits	R&D Systems	DANG20
P-selectin Quantikine ELISA kits	R&D Systems	BBE6
ICAM-1 Quantikine ELISA kits	R&D Systems	DCD540
E-selectin Quantikine ELISA kits	R&D Systems	DSLE00
IMUBIND® vWF ELISA	Biomedica Diagnostics	828
Experimental models: Organisms/strains		
P. falciparum: strain 3D7	QIMR Berghofer	N/A
Software and algorithms		
FlowJo software version 10	Treestar, San Carlos CA	version 10
Stata software, version 15	College Station, TX: StataCorp LLC	version 15
GraphPad Prism, version 8.4.3	Boston, Massachusetts USA	version 8.4.3

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Malaysian study site, patients and procedures

Patients were enrolled as part of an observational study of all malaria patients admitted to Queen Elizabeth Hospital, an adult tertiary-referral hospital in Sabah, Malaysia,[13] For the current study, male and female patients enrolled between September 2010 and December 2012 were included if they had PCR-confirmed *P. falciparum, P. vivax* or *P. knowlesi* monoinfection, were non-pregnant, ≥12 years old, had no major comorbidities or concurrent illness and were within 18 h of commencing antimalarial treatment. Patient demographics are provided in Table 1. Parasite counts were determined by microscopy, and parasite species identified by PCR. Venous blood was collected in lithium heparin and citrate tubes for pathophysiological biomarkers on enrollment and at day 28. PBMCs were isolated and fractioned by Ficoll® Paque Plus (Cytiva) density-gradient centrifugation and frozen at −70 °C in 10% dimethyl sulfoxide (DMSO) with 90% fetal bovine serum (FBS). Endothelial function was measured non-invasively on enrollment

using peripheral arterial tonometry (EndoPAT), giving a reactive hyperaemia peripheral arterial tonometry (RHPAT) index.[9] Severe malaria was defined according to modified WHO criteria,[32] as previously described.[13] Healthy controls were visitors or relatives of malaria patients, with no history of fever in the past 48 h and with blood film negative for malaria parasites.

Malaria volunteer infection studies

Malaria volunteer infection studies (VIS) were conducted in Brisbane, Australia, as previously described.[16] In brief, healthy male and female malaria-naïve volunteers were inoculated with ~2800 viable *P. falciparum* 3D7-infected RBCs, or ~1,800 *P. vivax*-infected RBCs. Participant demographics are provided in Table S3. Peripheral blood parasitemia was measured by qPCR daily from day 4 until administration of antimalarial drugs, which occurred on day 7, 8 or 10, depending on the study, when parasitemia for most participants had exceeded 5,000 parasites/mL. Venous blood was collected in lithium heparin and citrate tubes for pathophysiological biomarkers prior to inoculation and prior to treatment in all studies, and at other timepoints in some of the studies (Table S1). Participants with *P. falciparum* and with samples available were enrolled in studies NCT02783820,[33] NCT02389348,[34] NCT02281344,[35] and NCT02573857,[36] Participants with *P. vivax* were enrolled in studies NCT02573857[37] and ACTRN12616000174482.[38]

Study approval

All studies were conducted in accordance with the Declaration of Helsinki and the International Committee of Harmonisation of Good Clinical Practice guidelines. All participants provided informed written consent. For the use of samples from the malaria volunteer infection studies and for the isolation of PBMCs from buffy coats obtained from Australian Red Cross Lifeblood, ethics approval was obtained from the Human Research Ethics Committee of the QIMR Berghofer Medical Research Institute (P1479 and P3444 respectively). For the clinical studies in Malaysia, ethics approval was obtained from the Malaysian Ministry of Health (NMRR10_754_6684) and the Menzies School of Health Research, Darwin, Australia (HREC 2010_1431). Informed written consent was provided by all participating adults and parents/guardians of those under 18 years of age.

METHOD DETAILS

ELISAs

For the Malaysian studies and for the malaria VIS, venous blood collected in lithium heparin and citrate tubes was centrifuged (including a second high-spin speed for the citrate tube) within 30 min of collection and plasma stored at −70 °C. Plasma concentrations of OPG were measured using a duoset ELISA from R&D Systems. Free soluble RANKL and TRAIL were measured on lithium heparin plasma by ELISA (Biomedica Diagnostics). Angiopoietin-2, P-selectin, and adhesion molecules intercellular adhesion molecule (ICAM)-1 and E-selectin were measured on lithium heparin plasma using quantikine ELISA kits from R&D Systems. Plasma von Willibrand factor (vWF) was measured on the citrated platelet-free plasma by ELISA (Biomedica Diagnostics). For logistical reasons, not all of the laboratory assays were performed on all patients or participants.

B cell stimulation, and *ex vivo* OPG production

To assess if parasite stimulation could directly induce B cell OPG production, PBMCs were isolated from buffy coats obtained from healthy blood donors (n = 10) from Australian Red Cross Lifeblood. PBMCs were isolated and fractioned by Ficoll® Paque Plus (Cytiva) and frozen in 10% DMSO with 90% FBS. PBMCs were thawed in 10% FBS/RPMI, and 1 million PBMCs stimulated with 3 million parasitized red blood cells (pRBCs) or unparasitised RBCs (uRBCs) for 3 days. Total B cells were characterised as $CD19^+$ and B cell subsets identified by IgD, CD21 and CD27. In brief, 1 million PBMCs were surface stained with NIR Live/Dead (Thermofisher), CD19-BUV395 (SJ25C1, 1/50), CD3-FITC (HIT3a, 1/25), CD21-PE-Dazzle (Bu32, 1/50), CD27-BV421 (M-T271, 1/50), IgD-PE-Cy7 (IA6-2, 1/50), CD14-BV650 (M5E2, 1/50) and CD56-BV510 (HCD56, 1/25) for 30 min at room temperature followed by 2% FBS/PBS wash. Cells were fixed and permeabilized using 1X Perm/Wash™ (BD Biosciences, USA) and incubated with anti-human polyclonal OPG-biotin (1/15 Leinco technologies, USA) at 30 min at room temperature, followed by the addition of Streptavidin-PE (0.125 mg per million cells) to quantify intracellular OPG. All cells were acquired on a Fortessa V flow cytometer (BD Immunocytometry Systems, San Jose, CA) and the data was analyzed using FlowJo software version 10 (Treestar, San Carlos CA). Surface antibodies were sourced from BD Biosciences (USA) and Biolegend (USA).

To assess if B cell OPG production was increased in clinical malaria, PBMCs from Malaysian patients with malaria and from healthy controls from the same study site were thawed in RPMI containing 10% FBS and rested overnight at 37 °C, 5% CO_2. Cells were then analyzed by flow cytometry as for parasite stimulated cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using Stata software (version 15) and GraphPad Prism 8.4.3. For continuous variables, comparisons between groups were analyzed using student's T-test or Mann-Whitney tests, depending on distribution. For comparisons made between three groups, the Kruskall-Wallis test was used. Categorical variables were compared using the χ^2 test. Associations between continuous variables were assessed using Spearman's or Pearson's correlation coefficients, depending on distribution. Partial correlations were used to adjust for parasitaemia, with non-normally distributed variables log-transformed to normality.

The conservative Bonferroni correction was used to indicate statistical significance, after accounting for multiple correlations. A backwards stepwise regression model was used to evaluate the association between TRAIL and acute kidney injury (defined by KDIGO criteria), with variables log transformed and removed if $p > 0.05$. Wilcoxon signed-rank test was used to compare baseline and follow-up measurements. Measurements below the limit of detection were assigned a value of half the lower limit of detection.

No formal sample size calculations were conducted for these exploratory analyses. For the study conducted in Malaysia, sample size was determined primarily by the number of patients hospitalised with malaria at the study site during the study period. For patients with falciparum and knowlesi malaria, the number of patients enrolled and with samples available would be expected to be sufficient to detect associations between variables with Spearman's correlation coefficients of 0.3 with a 95% CI width of 0.3; for the smaller number of patients with vivax malaria during this time period, the sample size would be expected to be sufficient to detect correlation coefficients of 0.3 with a 95% CI width of 0.5.[39] For the malaria volunteer infection studies, the sample size was determined by the number of participants enrolled in these studies who had samples available for exploratory analyses. These numbers were small; thus, the focus of this analysis was on the longitudinal trends in RANKL and OPG. For correlation analyses, the sample size would be expected to be sufficient to detect correlations of 0.7 (95% CI width 0.5) for *P. falciparum*, and 0.8 (96% CI width 0.5) for *P. vivax*. Results should be considered exploratory.

ADDITIONAL RESOURCES

This study utilised samples collected from participants enrolled in clinical trials. These included *P. falciparum* (trial registration numbers NCT02783820,[33] NCT02389348,[34] NCT02281344,[35] and NCT02573857,[36]) and *P. vivax* (trial registration numbers NCT02573857[37] and ACTRN12616000174482[38]) malaria volunteer infection studies.