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Plasmodium falciparum Malaria Carriage Associates with Reduced γδ T-Cell and NK Cell Responses to Infected Red Blood Cells *In Vitro*

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Abstract

Background: Innate immune cells including γδ T-cells and NK cells are directly activated by *Plasmodium falciparum* parasites and contribute to the control of parasitaemia. The aim of this study was to determine whether parasite carriage affects innate immune cell responses *in vitro* to *P. falciparum* infected red blood cells (PfRBC).

Methods: Peripheral blood mononuclear cells were collected from 61 Malian children aged 5 to 15 years at the start of the transmission season. Parasite carriage at the start of the transmission season was assessed by PCR and microscopy for Malian children. Peripheral blood mononuclear cells were stimulated with PfRBC to assess cytokine production and degranulation of innate lymphocytes (γδ T-cells, CD3+CD56+ cells and NK cells) by flow cytometry.

Results: Granzyme B production in response to PfRBC was observed by all three innate cell subsets in Malian children, as were IFNγ production by γδ T-cells and NK cells and γδ T-cell degranulation. Children with ongoing *P. falciparum* infection showed significantly reduced PfRBC-specific IFNγ production by γδ T-cells and NK cells and degranulation by γδ T-cells as compared with those with undetectable parasitaemia by PCR and microscopy. Reduced IFNγ responses by NK cells were already observed for children with submicroscopic parasitaemia as compared to those with negative PCR. Children with high parasite densities showed a significant reduction in degranulating γδ T-cells relative to their low parasite density counterparts.

Conclusion: *P. falciparum*-specific responses by γδ T-cells and NK cells were negatively impacted by ongoing *P. falciparum* infection.

Keywords: Malaria, *Plasmodium falciparum*, degranulation, Granzyme B, IFNγ, γδ T-cells, NK cells.

Introduction

Malaria caused by the protozoan parasite *Plasmodium falciparum* remains a major public health concern in sub-Saharan Africa, causing unacceptably high morbidity and mortality in children aged below 5 years and pregnant women [1]. Naturally acquired immunity to malaria develops with age after repeated exposure to infective mosquito bites and protects against clinical symptoms instead of inducing sterile protection from infection [2-6]. Protection from clinical symptoms requires both control of parasitaemia and a well-balanced immune response to avoid intense inflammation. The cellular mechanisms underlying acquired immunity from symptomatic disease are

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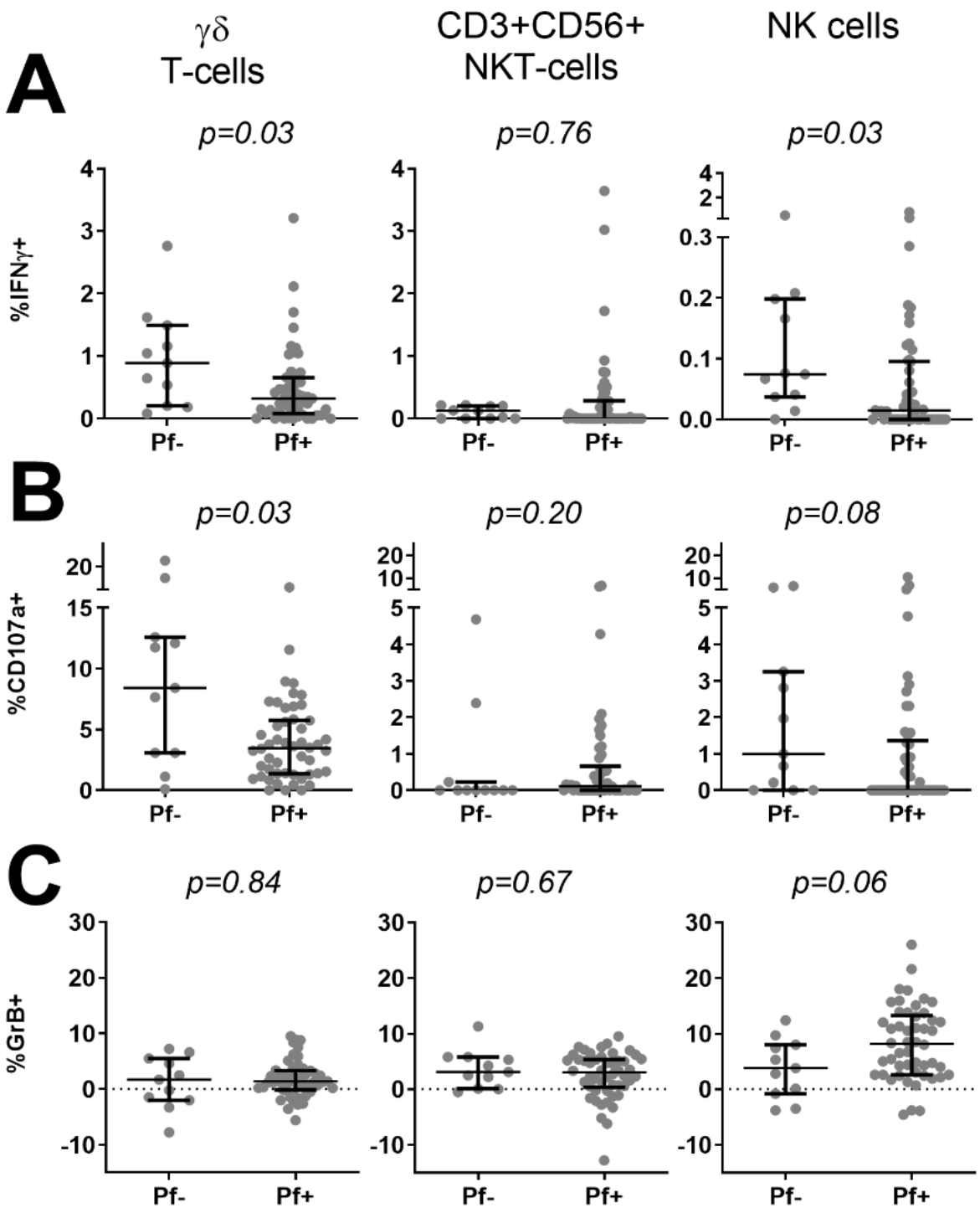

Figure 2: Comparison of PfRBC-specific innate responses based on parasite prevalence at time of blood collection. PBMC from all 61 Malian children were stimulated with either PfRBC or uRBC for 24h. (A) IFNγ production, (B) degranulation assessed by CD107a expression and (C) Granzyme B content were assessed by flow cytometry. Parasite specific responses were calculated by subtraction of uRBC background responses. Data is presented for each individual donor (grey dots) and as median with IQR (black error bars) for n=11 children who were qPCR and thick-smear negative (Pf-) and n=50 children who were qPCR positive (Pf+) at the time of blood collection for immunological analysis. Groups were compared by Mann-Whitney U test.

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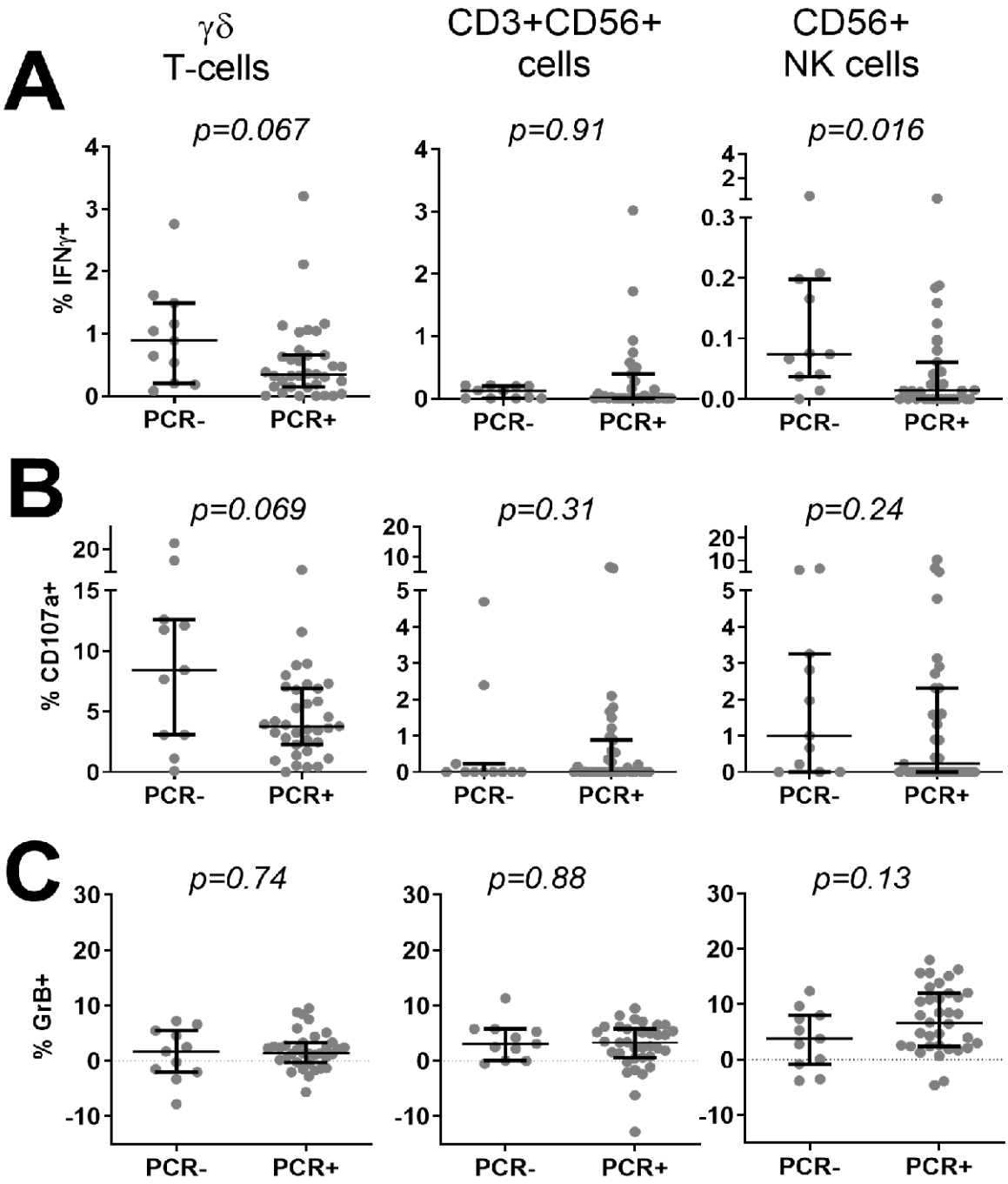

Figure 3: Comparison of PfRBC-specific innate responses based on submicroscopic parasite prevalence at time of blood collection. PBMC from 46 thick smear negative Malian children were stimulated with either PfRBC or uRBC for 24h. (A) IFNγ production, (B) degranulation assessed by CD107a expression and (C) Granzyme B content were assessed by flow cytometry. Parasite specific responses were calculated by subtraction of uRBC background responses. Data is presented for each individual donor (grey dots) and as median with IQR (black error bars) for n=11 children who were qPCR negative (PCR-) and n=35 children who were qPCR positive (PCR+) at the time of blood collection for immunological analysis. Groups were compared by Mann-Whitney U test.

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Innate and adaptive responses to PfRBC do not correlate with incidence of clinical disease during follow-up

Finally, we assessed whether we could find any evidence to support prior findings by others that the level of cellular responses at the start of the transmission season associated with incidence of subsequent clinical disease. Children who developed asymptomatic or symptomatic infection during follow-up did not differ markedly in demographic or parasitological parameters at time of blood collection, except for a trend for higher parasite load in thick smear positive children amongst those developing symptomatic disease during follow-up compared to those that did not become symptomatic (*p=0.066*, **Table 1**). There was, however, no significant difference at the start of the transmission season between these two groups based on PfRBC-specific degranulation, IFNγ and Granzyme B production by any lymphocyte subset (**Table S1**).

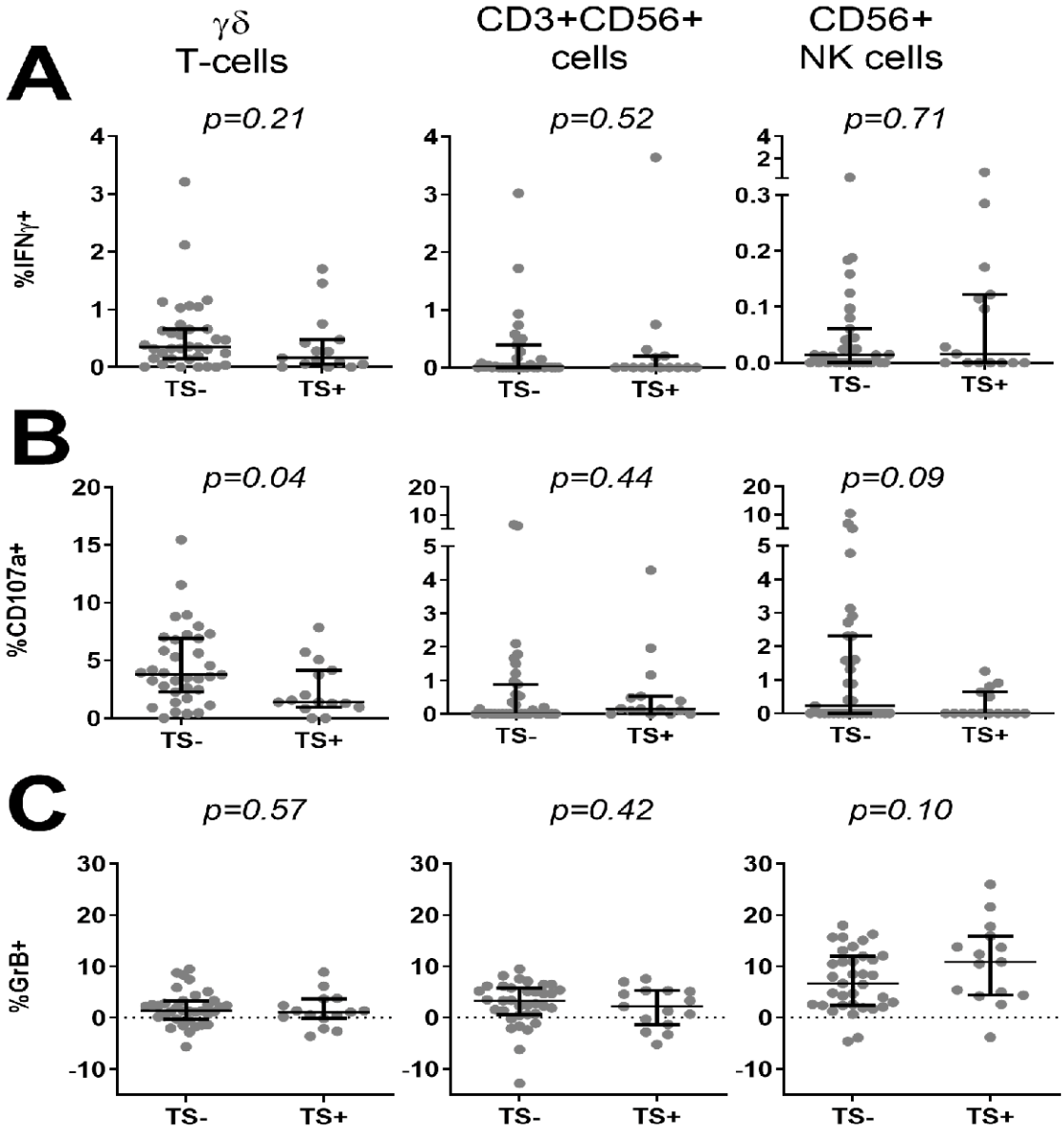

Figure 4: Comparison of PfRBC-specific innate responses based on parasite density at time of blood collection. PBMC from 50 PCR+ Malian children were stimulated with either PfRBC or uRBC for 24h. (A) IFNγ production, (B) de-granulation assessed by CD107a expression and (C) Granzyme B content were assessed by flow cytometry. Parasite specific responses were calculated by subtraction of uRBC background responses. Data is presented for each individual donor (grey dots) and as median with IQR (black error bars) for n=35 children who were thick smear negative (TS-) and n=15 children who were thick smear positive (TS+) at the time of blood collection for immunological analysis. Groups were compared by Mann-Whitney U test.

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Discussion

In this study, we assessed the impact of ongoing parasitaemia on innate immune cell responses to PfRBC. Ongoing parasitaemia in Malian children associated with reduced IFNγ and degranulation responses to PfRBC by γδ T-cells and reduced NK cell IFNγ responses. In a previous study, Tanzanian adults also showed much weaker IFNγ responses to PfRBC in a side-by-side comparison with Dutch adults both prior to and after a controlled human malaria infection [60]. This indicates that rather than age, prior repeated exposure to *P. falciparum* might be a driving factor for these impaired innate responses. Impaired pro-inflammatory cytokine production by innate cells during infection may be beneficial to the host by limiting the inflammatory response and thus promoting disease tolerance [61]. Indeed, lower levels of IFNγ and TNFα co-production by Vδ2 γδ T-cells are associated with a decreased likelihood to remain asymptomatic during an episode of *P. falciparum* infections in the following year in a Ugandan child cohort [32, 58]. In contrast, in the present study cytokine production or degranulation responses by γδ T-cells or other innate lymphocytes do not differ between children developing clinical symptoms or not during a malaria episode during follow-up. One contributing factor may be the definition of clinical immunity, which is more stringent in our study, as children were only considered asymptomatic if they did not experience any clinical episode during follow-up (as opposed to at least one asymptomatic episode in a previous study [58]). Another contributing factor may be the lower malaria incidence and hence prior malaria exposure in the area of the current study compared to the Ugandan child cohort [32, 58].

A small cohort study has previously reported that γδ T-cell cytokine production and proliferation are reduced during acute *P. falciparum* infection [62]. The four travelers examined in this study, however, had microscopically detectable parasites. Our study suggests that already submicroscopic parasitaemia can have a significant effect on innate lymphocyte function, namely γδ T-cell and NK cell IFNγ production and γδ T-cell degranulation. This finding contrasts with the findings in a Ugandan childhood cohort, where no differences between uninfected children and those with submicroscopic parasitaemia were found [32]. This is particularly relevant since a sizable part of the population carries parasites at submicroscopic levels, even in low transmission areas [63-69]. In our cohort, the proportion of children carrying submicroscopic parasites levels at the start of the transmission season was greater than 80%. Our findings on γδ T-cells are in line with previous studies reporting impaired degranulation and pro-inflammatory cytokine production in response to PfRBC in Ugandan children, which increased with cumulative episodes of malaria [32, 35, 58]. While evidence for impaired γδ T-cell directly to PfRBCs upon repeated parasite exposure is thus accumulating, the underlying mechanism remains unclear. Next to up-regulation of inhibitory receptors such as Tim-3 [32], another possibility is activation of the PPARα pathway, which has been linked to γδ T-cell desensitization upon repeated purified phosphoantigen exposure in macaques [70, 71].

As for NK cells, little is known thus far about any changes to their ability to directly respond to PfRBC following repeated exposure or ongoing infectious with *P. falciparum* except that they are functionally impaired in severe compared to uncomplicated malaria [59]. Even malaria-naïve individuals already show a great variability in their NK cell response to PfRBC, which could be linked to differences in receptors relevant for recognition of or interaction with PfRBC. Donors with NK cells capable of reducing PfRBC growth *in vitro* have been shown to express higher levels of the RNA sensor MDA5 [46]. The kinetics of NK activation (based on CD69 expression) have further been tentatively linked to NK cell expression of the activating receptor NKp30, with higher baseline expression correlating with NK activation at lower parasitaemia levels following controlled human malaria infection [72]. Additionally, NKp30 was upregulated at peak parasitaemia after controlled human malaria infection compared to baseline [72]. Finally, NK cells can be inhibited by PfRBC through interaction of P. falciparum RIFIN proteins with the inhibitory receptor LILRB1 on NK cells [73-75]. It remains to be investigated, however, whether expression of MDA5, NKp30 or LILRB1 is modulated by repeated parasite exposure and could be linked to the impaired response to PfRBC as reported herein. While reduced pro-inflammatory responses of innate lymphocytes to the malaria parasite might be beneficial in promoting disease tolerance [61], impaired cytotoxic effector function of innate immune cells may also negatively impact on disease control, since cellular cytotoxicity by both NK cells and γδ T-cells do contribute to control parasite growth [33, 37, 38, 47, 48]. In the current study, we specifically focus on direct responses of innate lymphocytes to PfRBC, using isolated PBMC and non-immune human serum, while antibody-dependent responses including antibody-dependent cytotoxicity were not investigated. As for γδ T-cells, expression of CD16 increases with repeated malaria exposure [32, 35, 58]. Notably, CD16 expression is associated with poor responsiveness of γδ T-cells to phosphoantigens [76] and instead mediates antibody-dependent cytotoxicity [50, 76]. Indeed, γδ T-cells have been shown to mediate cytotoxicity against PfRBC opsonized by hyperimmune IgG [77]. Therefore, γδ T-cells cytotoxicity may be retained in malaria exposed individuals but shifted from responses directly induced by PfRBC-inherent factors such as phosphoantigens to antibody-dependent mechanisms. Similarly, while NK cells show a trend for reduced

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degranulation activity induced by direct PfRBC recognition in our study, evidence is accumulating that their ability to mediate antibody-dependent cytotoxicity remains unaffected or is even enhanced [78]. This was shown previously for NK cells that up-regulated PD-1 expression upon exposure to PfRBC in vitro and selectively lost only their ability to kill MHC class I negative target cells, but not antibody-opsonized targets [59]. Notably, the ability of these PD-1 expressing NK cells to become activated by or kill PfRBC was not evaluated. Moreover, a subset of adaptive CD56 negative NK cells with potent antibody-dependent cytotoxic capacity have been shown to expand with repeated malaria exposure and associated with protection from clinical malaria [79, 80]. These specific NK cells have only recently been reported and were not subject of the current study. Unlike for γδ T-cells and NK cells, IFNγ and degranulation responses of CD3+CD56+ cells in Malian children were not affected by infection or parasite density status of Malian children. CD3+CD56+ cells are often referred to as NKT-like cells; however, recent studies have shown that this CD3+CD56+ T-cell population consists of multiple different subpopulations with varying polarization and cytotoxic potential [81]. CD1d restricted NKT cells expressing an invariant TCR are actually rarer and only a small subset of CD3+CD56+ cells [82, 83]. In as how far function specifically of CD1d restricted NKT cells is affected by parasite carriage has not been addressed in this present study. Finally, impaired innate responses may also inhibit induction of protective adaptive memory responses since specifically γδ T-cells are known for a variety of immune effector functions beyond cytokine production or target cell killing. These activities include the promotion of adaptive immune responses by antigen-presentation to CD4+ and CD8+ T-cells [84], which may also play a role in Plasmodium infection [85, 86]. Future studies are therefore needed to elucidate the influence of past or acute infections on a larger spectrum of γδ T-cell functions both in the presence and absence of parasite-specific antibodies, and the potential consequences for adaptive immunity.

Conclusion

In conclusion, ongoing *P. falciparum*-infection in Malian children impaired γδ T-cells and NK cell IFNγ production and γδ T-cells degranulation in direct response to PfRBC. For NK cells, these effects were already observed at submicroscopic parasite densities.

Abbreviations

IFN: Interferon

NK: Natural killer cell

PBMC: Peripheral blood mononuclear cell

Pf: *Plasmodium falciparum*

PfRBC: *Plasmodium falciparum* infected red blood cell

uRBC: uninfected red blood cell

Declarations

Consent for publication: Not applicable.

Availability of data and materials: The datasets generated and analyzed during the current study are available from the corresponding authors on reasonable request.

Competing Interests: The authors declare they have no competing interests.

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Authors' Contributions: MD, CA and NO conducted the experiments; MD, BK, CA and AS planned the experiments and analyzed the data; MD, BK, OD, and RS designed and supervised the field study; MD, BK, CA, KN, YK, SS, BO and SA performed the field studies and collected samples and clinical data; MD, BK, OD, RS, and AS interpreted the data and wrote the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY FILES

Table S1: Relationship between PfRBC re-stimulated responses and clinical disease during follow-up

Cellular responses[a]		CD4+	CD8+	γδT	CD3+CD56+	CD56+ NK
IFNγ[d]	Sympt[b]	0.008 (0.0-0.02)	0.004 (0.0-0.015)	0.39 (0.09-0.96)	0.0007 (0.0-0.15)	0.024 (0.0-0.09)
	Asympt[c]	0.007 (0.0-0.017)	0.008 (0.0-0.022)	0.31 (0.15-0.72)	0.017 (0.0-0.39)	0.029 (0.0-0.18)
		p= 0.74	*p= 0.33*	*p= 0.91*	*p= 0.43*	*p= 0.47*
CD107a[d]	Sympt	0.052 (0.0-0.14)	0.05 (0.0-0.23)	3.77 (1.42-7.28)	0.02 (0.0-0.54)	0.23 (0.0-1.59)
	Asympt	0.012 (0.0-0.07)	0.015 (0.0-0.16)	3.33 (1.37-6.50)	0.05 (0.0-0.81)	0.11 (0.0-2.11)
		p= 0.35	*p= 0.44*	*p= 0.70*	*p= 0.80*	*p= 0.71*
GrzB[d]	Sympt	0.12 (-0.024-0.19)	1.2 (0.30-1.95)	2.06 (-0.22-4.05)	4.7 (0.35-6.2)	8.5 (2.9-12.8)
	Asympt	0.04 (-0.015-0.15)	0.9 (0.09 -2.85)	1.2 (-1.38-3.35)	2.2 (0.13-4.9)	4.9 (2.6-10.8)
		p= 0.13	*p= 0.87*	*p= 0.49*	*p= 0.095*	*P= 0.30*

a. In 61 Malian children early in the transmission season (July 2012)
b. n=33 children becoming symptomatic after exposure confirmed by thick smear or PCR
c. n=28 children remaining asymptomatic despite exposure confirmed by thick smear or PCR
d. PfRBC-specific responses (shown as median percentage responding cells with IQR) were calculated by individual subtraction of uRBC background responses and analyzed by Mann-Whitney U test

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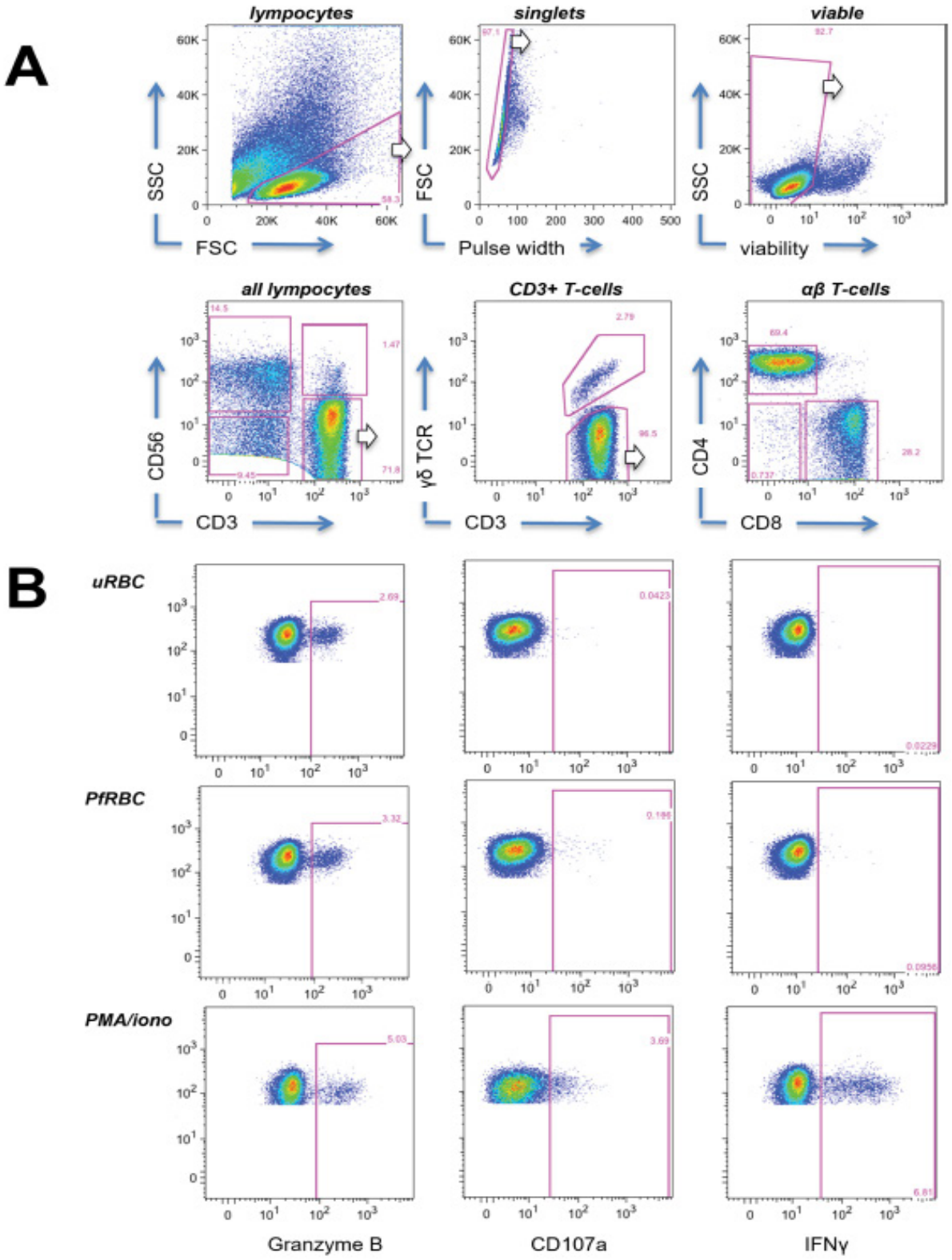

Figure S1: Flow cytometry gating strategy. (A) PBMCs were sequentially gated to remove debris, doublets and dead cells. Viable lymphocytes were then distinguished into CD3-CD56+ NK cells, CD3+CD56+ cells and CD3+CD56- T-cells. CD3+CD56- T-cells were further subdivided based on presence or absence of Pan-γδTCR expression. Non-γδ T-cells were divided into CD4+ and CD8+ T-cells. (B) Representative plots are shown for cytokine production (Granzyme B, IFNγ) and degranulation (CD107a) by total CD3+ T-cells during 24h stimulation with uRBC or PfRBC, or 4h stimulation with PMA/ionomycin.

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