EMBO reports

# Neutrophils impose strong immune pressure against PfEMP1 variants implicated in cerebral malaria

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## Review Timeline:

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|---|---|
| Transfer from Review Commons: | 16th Jul 21 |
| Editorial Decision: | 22nd Jul 21 |
| Revision Received: | 10th Feb 22 |
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*Editor: Achim Breiling*


## Transaction Report: This manuscript was transferred to EMBO Reports following peer review at Review Commons.

Dear Prof. Dzikowski,

Thank you for transferring your manuscript from Review Commons to EMBO reports. I now went through your manuscript, the referee reports (attached again below), and your revision plan. All three referees acknowledge the potential interest of the findings. Nevertheless, they have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn, which I see you are willing and able to address during a major revision of the manuscript. It would be very important, though, to address major point of referee #2 experimentally and provide data showing the interaction/binding taking place in vivo, even if this means a couple of more months of experimentation.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an

appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

# Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
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10) Please order the manuscript sections (using the indicated names) like this:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements (including funding information) - Author contributions - Conflict of interest statement - References - Figure legends - Expanded View Figure legends.

11) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right or left corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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Referee #1:

Zelter et al investigate the ability of neutrophils to recognize and kill Plasmodium falciparum infected RBCs. They show that neutrophils can detect certain Plasmodium variant surface antigens and subsequently kill infected red cells. This has important implications for both immunity to Plasmodium, as well as pathogenesis - the authors show that neutrophils can shape the Plasmodium antigenic repertoire. Importantly all work is done with human primary neutrophils and with P. falciparum, both relevant and challenging models.
The experiments are generally performed robustly and the key conclusions are convincing, except in the following instances:

Fig 1: In bacterial killing assays, it is standard practice to include a phagocytosis inhibitor (eg cytochalasin) to distinguish between binding and intracellular killing. This control should be included.

Figure 1: it would be interesting to determine what is being phagocytosed? Free merozoites, trophozoites or schizonts? This could be easily determined from blood smears

Figure 2F: methodology here is not well explained. Why are neutrophils not labeled with sybr green? It is also not clear how to interpret it. Please provide better explanation or move to supplement

2H: have the authors tested the effect of catalase on growth of parasites (in absence of neutrophils)?

Figure 3
Line 377: "implying that neutrophil interaction with iRBCs is largely PfEMP1-mediated:
PfEMP KO parasites led to approx. 32% reduction in interaction (Fig 3A), so 'largely' should be changed to 'partially'

Fig 4G; the appropriate control here is a protein other than sICAM, rather than no protein

**Minor Comments**

**Discussion:**

There is accumulating evidence that neutrophil activation contributes to severe disease. Could activation of neutrophils by ICAM binding PfEMP1 variants drive inflammation in malaria? Is PFD1235w/ PF3D7_0425800 linked to severe disease?

Abstract: 'PfEMP1s' is not grammatically correct

Line 428: "expressed primarily by endothelial cells in brain vasculature " Both ICAM and EPCR are expressed throughout various vascular beds.

Significance:

This is neglected area of research in a disease that exerts a tremendous health burden. Zelter at provide an important advance in our mechanist understanding of how neutrophils and parasites interact. This is therefore relevant to the malaria community and will help formulate future research directions.

It may also be of interest to neutrophil researchers as it expands the role of neutrophils to parasitic infections and elucidates a role for ICAM on neutrophils, which was previously underappreciated.

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Referee #2:

The present study investigated the ability of neutrophils to inhibit in vitro culture of Plasmodium falciparum.
They show that the neutrophils engage with the surface of the erythrocyte associated with expression of PfEMP1s implicated in cerebral malaria. The study provide data for interactions between neutrophils and infected RBCs and that this influenced but not solely determined by the PfEMP1. The link to cerebral malaria comes from the ICAM1 binding phenotype of the PfEMP1 expressed and ICAM1 expression on the neutrophil, but appears not substantiated by other data.

**Major**

This is a demonstration of neutrophil action in vitro in a static assay. I think there is a mission link to the in vivo conditions and whether the killing could take place there. The arteries are very different from this static environment. Did the authors look into adhesion (inhibition may be difficult) of neutrophils to iRBC/PfEMP1 under comparable to blood flow sheer stress conditions?

Also I do not recall neutrophil adhesion in connection with parasite cytoadhesion in cerebral malaria in any of the post mortem studies done, is this because no one looked for this? Any data pointing towards the interaction/binding taking place in vivo would be very welcomed to this paper.
The authors mention two modes of immune evasion by the parasite. Which mode of immune evasion would it be to express a PfEMP1 that flags killing by neutrophils?

**Minor comments**

- Did the authors investigate the effect of bloodtype? Ie. growing the parasites on the autologous red bloods to the neutrophils? so that there is no interference.
- It would be obvious to investigate a CSA adhering VAR2CSA expressing parasite especially as neutrophils accumulate in the intervillous space in the placenta during pregnancy associated malaria.
- any clue as to what the neutrophil binds in PfEMP1 KO or trypsinated iRBC?
- I find the title misleading as it may be interpreted as evolutionary selection which is not investigated.

Significance:

The data to my knowledge are novel, clearly presented and trustworthy. I find the data compelling and it is logical that neutrophils could play a role in malaria.

This should be general interest to malaria researchers and immunologists.

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Referee #3:

This manuscript provides evidence that neutrophils recognise and kill P. falciparum infected erythrocytes (at least in part) via pfemp1-ICAM interactions and provide evidence for a contribution of neutrophil ROS to the killing mechanism. The work is presented to a high standard and the conclusions, for the most part, are supported by the data. This work makes an important contribution to our understanding of the role of neutrophils in the clearance of P. falciparum.

The following pints should be addressed:

1. Neutrophil fate in the co-culture system: what is the impact of antibody dependent and independent phagocytosis of iRBC on the viability of neutrophils on this system? Is there a more rapid attrition of GFP+ neutrophils? Are apoptotic pathways induced after iRBC phagocytosis?
2. In figure 2. Do other, ROS independent mechanisms of parasite killing operate after day 1?
3. In figure 4, the authors demonstrate that D. iRBC uptake is reduced from around 24% to 24% and E. killing from 70% to 50% in the presence of anti-ICAM antibodies. Soluble ICAM-Fc reduces phagocytosis by around 20% and killing by 60-70%. EPCR seems to play little role in the system. Why is this effect only partial and what other receptors - ligand interactions are potentially mediating these processes. A separate point: does antibody mediated opsonisation overcome the impact of blockade ICAM-pfemp-1 interactions?
4. Graphs are presented as summary data. it would be useful to see the individual level variation in responses of distinct primary neytrophil donors. In this context some discussion of neutrophil functioal cappacity in the context of varying differentiation phenotype of circulating neutrophils would be beneficial.
5. There are some minor errors of syntax which should be checked throughout.

Significance:

This work is important as it increasses our understanding of the interractions required between malaria infected erythrocytes and neutrophils which facilitate paraite killing.

The manuscript is certainly of interest to a malaria / immunology research audience and has potential impacts for our understanding of differentce in innate parasite control in different arasite strains and between individual human hosts

My relevant expertise is in the study of innate immunity to malaria infection.

Dear editor,

We have revised our manuscript entitled "Neutrophils impose strong immune pressure against PfEMP1 variants implicated in cerebral malaria" according to the reviewers' comments. Please find below a summary of the changes. On behalf of all authors I would like to thank the reviewers for their thorough reading of the manuscript. Their careful criticism and constructive suggestions contributed greatly to the improvement of our paper.

The revised manuscript includes additional experiments as requested by the reviewers. These experiments emphasize the specificity of the interaction of PfEMP1 with ICAM-1 expressed on neutrophils' surface, confirm the ability of neutrophils to interact and phagocytose blood stage parasites and provides additional insight into the mechanisms of neutrophil's cytotoxicity.

We have revised figure 2 & 4 and added significant amount of supplementary data in addition to textual changes. We are happy that all reviewers found the paper interesting and important and hope that these revisions address their concerns.

Please find below a point by point answers to the comments made by the reviewers.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Zelter et al investigate the ability of neutrophils to recognize and kill Plasmodium falciparum infected RBCs. They show that neutrophils can detect certain Plasmodium variant surface antigens and subsequently kill infected red cells. This has important implications for both immunity to Plasmodium, as well as pathogenesis - the authors show that neutrophils can shape the Plasmodium antigenic repertoire. Importantly all work is done with human primary neutrophils and with P. falciparum, both relevant and challenging models.
The experiments are generally performed robustly and the key conclusions are convincing, except in the following instances:

Fig 1: In bacterial killing assays, it is standard practice to include a phagocytosis inhibitor (eg cytochalasin) to distinguish between binding and intracellular killing. This control should be included.

We thank the reviewer for the kind words and very constructive suggestions. As suggested by the reviewer we performed a neutrophil mediated killing assay in the presence or absence of cytochalasin. Specifically, we isolated neutrophils from 3 independent healthy donors and cultured them with iRBC. We show (see below) that cytochalasin dramatically and significantly reduces neutrophil cytotoxicity in 3 independent biological repeats. This new data was added to the revised version of Figure 2.

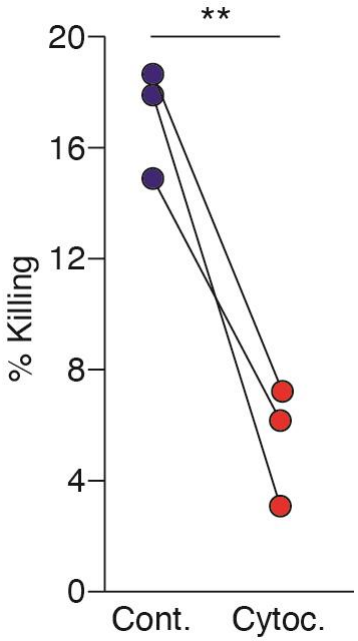


Figure 1: it would be interesting to determine what is being phagocytosed? Free merozoites, trophozoites or schizonts? This could be easily determined from blood smears

The questions raised by the reviewer are indeed interesting and deserve attention. Early reports from the 1980's and 1990's provided indications that neutrophils are able to phagocytose all blood stages: merozoites, early ring stages, trophozoites, schizonts and even gametocytes (see for example Kumaratilake et al. *Infect. Immun.* 1990 & 1992, Kumaratilake et al, *J. Immun.* 1991 and more). In our experiments we focused primarily on the neutrophil's interaction with late stage intraerythrocytic parasites that already modified the RBC by placing parasite antigens (PfEMP1) on the erythrocyte surface. To this end we synchronized our parasite cultures and isolated late stage containing iRBC prior to the addition of neutrophils. In our data, phagocytosis of schizont can be seen in figure 1A (upper panel) and phagocytosis of trophozoite can be seen in figure 1A (lower panel). Blood smears in figure 1B corroborate the observation that late stages (arrows) are phagocytosed by neutrophils. To clarify this point, we have elaborated on this in the revised manuscript.

Figure 2F: methodology here is not well explained. Why are neutrophils not labeled with sybr

green? It is also not clear how to interpret it. Please provide better explanation or move to supplement

We thank the reviewer for pointing out the mistake in the legend for figure 2F. The text mistakenly states “day 4 late stages” instead of “day 4 early stages”. This mistake was corrected in the revised version of figure 2. In this experiment neutrophils were stained with an anti-CD11b antibody and excluded from the analysis by gating on the SYBR green positive CD11b negative population – representing iRBCs. Next, we focused on the signal intensity of the iRBCs which correlates with the developmental stage – since schizonts contains larger amounts of DNA they have a significantly stronger signal and can be easily distinguished from early ring stages which have lower SYBR green signal. While the control group completed a life cycle and returned to early ring stages by day 4 (low signal population), in the neutrophil group there were parasites that did not progress beyond the schizont stage (high signal population) suggesting that neutrophils interfere with the ability of late stage parasite to successfully complete their cell cycle. We have amended the text, and incorporated this into the text and figure legends of the revised version of the manuscript.

2H: have the authors tested the effect of catalase on growth of parasites (in absence of neutrophils)?

Yes. In figure 2H we show parasite growth (as % parasitemia) cultured alone (Control, blue bars) or in the presence of neutrophils (Neut., red bars). This was done either in the presence (on the right) or absence (on the left) of catalase. This data shows that catalase in the absence of neutrophils has no significant effect on parasite growth since both blue bars have similar values.

Figure 3
Line 377: "implying that neutrophil interaction with iRBCs is largely PfEMP1-mediated: PfEMP KO parasites led to approx. 32% reduction in interaction (Fig 3A), so 'largely' should be changed to 'partially'

We have revised the text according to the suggestion made by the reviewer.

Fig 4G; the appropriate control here is a protein other than sICAM, rather than no protein

To accommodate this concern, we repeated the experiment described in figure 4G using other soluble unrelated protein (sRAGE) generated in our lab (Sionov et al., Oncoimmunology 2019). The results of this experiment are now included in the revised Figure 4G.

**Minor Comments**

**Discussion:**

There is accumulating evidence that neutrophil activation contributes to severe disease. Could activation of neutrophils by ICAM binding PfEMP1 variants drive inflammation in malaria? Is PFD1235w/ PF3D7_0425800 linked to severe disease?

There are several publications linking PFD1235w to severe malaria and in particular to cerebral malaria. The latest publication (Adams et al. *J Exp Med* 2021) shows how iRBC expressing this specific PfEMP1 binds to brain endothelial cells and enhance the expression of ICAM-1 on the surface of the brain cells. In addition to this, that study shows how PFD1235w expressing iRBC induce cell swelling and disrupts the blood brain barrier and associated with cerebral malaria. In addition, activated neutrophils were associated with cerebral malaria in children and were linked to inflammation that was suggested to contribute to the vasculopathy of cerebral malaria (Feintuch et al., mBio 2016). We have revised discussion of the manuscript to emphasize this evidence.

Abstract: 'PfEMP1s' is not grammatically correct

We thank the reviewer for spotting this mistake and have corrected this.

Line 428: "expressed primarily by endothelial cells in brain vasculature " Both ICAM and EPCR are expressed throughout various vascular beds.

ICAM-1 and EPCR are indeed expressed throughout. During inflammation ICAM-1 become upregulated and PFD1235w expressing iRBC´s are capable of inducing such an upregulation, and thus, increase cytoadherance in brain vasculature. To clarify this, we have amended the text of the revised manuscript.

Reviewer #1 (Significance (Required)):

This is neglected area of research in a disease that exerts a tremendous health burden. Zelter at provide an important advance in our mechanist understanding of how neutrophils and parasites interact. This is therefore relevant to the malaria community and will help formulate future research directions.

It may also be of interest to neutrophil researchers as it expands the role of neutrophils to parasitic infections and elucidates a role for ICAM on neutrophils, which was previously underappreciated.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

The present study investigated the ability of neutrophils to inhibit in vitro culture of Plasmodium falciparum.
They show that the neutrophils engage with the surface of the erythrocyte associated with expression of PfEMP1s implicated in cerebral malaria. The study provide data for interactions between neutrophils and infected RBCs and that this influenced but not solely determined by the PfEMP1. The link to cerebral malaria comes from the ICAM1 binding phenotype of the PfEMP1 expressed and ICAM1 expression on the neutrophil, but appears not substantiated by other data.

Here we report that neutrophils have higher efficiency of recognizing and eliminating red blood cells infected with parasite which express a particular subset of ICAM-1-binding PfEMP1. The link between these PfEMP1 subtypes and cerebral malaria has been substantiated by a number of publications. The most recent one from Adams et al. (*J Exp Med*, 2021), provided cellular insight as to how cyto-adherance of iRBC by this PfEMP1 subtype enhance ICAM-1 expression on brain endothelial cells, induce cell swelling and disrupts the Blood Brain Barrier in cerebral malaria. To accommodate for the reviewers comment we have elaborated on this in the revised manuscript.

**Major**

This is a demonstration of neutrophil action in vitro in a static assay. I think there is a mission link to the in vivo conditions and whether the killing could take place there. The arteries are very different from this static environment. Did the authors look into adhesion (inhibition may be difficult) of neutrophils to iRBC/PfEMP1 under comparable to blood flow sheer stress conditions?
Also I do not recall neutrophil adhesion in connection with parasite cytoadhesion in cerebral malaria in any of the post mortem studies done, is this because no one looked for this? Any data pointing towards the interaction/binding taking place in vivo would be very welcomed to this paper.

This comment by the reviewer highlights the limitations of *ex vivo* experimental designs where the *in vivo* conditions cannot be perfectly recapitulated. This is relevant in this context as there is no mouse model for PfEMP1 cytoadhesion as Plasmodium species that infect mice do not have *var* genes that encode for PfEMP1. These limitations also include the ability to perfectly mimic such interactions in the circulation even when performing our experiments under constant agitation as we did (and not under static conditions). While sheer stress is indeed a major factor in large blood vessels, the pressure in microvasculature, where iRBC cytoadhere, is significantly lower. Moreover, considering the parasite's sequestration in small blood vessels, causing local

inflammation, we cannot exclude the possibility that a significant proportion of the interactions between neutrophils and parasites *in vivo* occurs in near-static conditions.

We therefore agree with the reviewer that evidence for neutrophil adhesion in connection with parasite cytoadhesion in cerebral malaria would be of value as a link to the clinical situation. In this regard, Feintuch et al, (mBio, 2016), which found strong neutrophils activation in the circulation of children with severe cerebral malaria, reported that interactions of neutrophils and iRBC in brain microvasculature was rarely seen in post mortem autopsies of these kids. A more recent report by Knackstedt et. al (*Science immunology*, 2019) shows colocalization of neutrophil extracellular traps (NETs) and iRBC in the capillaries of cerebral malaria patients. These reported links and the possibility that neutrophils play a protective role in the circulation before cyto-adherence in the brain is discussed in the revised version of our manuscript.

The authors mention two modes of immune evasion by the parasite. Which mode of immune evasion would it be to express a PfEMP1 that flags killing by neutrophils?

The reviewer raises an interesting philosophical question touching upon one of the most basic questions in host-parasite co-evolution which is a continuous arm race. The case of PfEMP1 is a fantastic example, on the one hand – PfEMP1 serves to mediate iRBC cytoadhesion and sequestration leading to avoidance of splenic elimination. On the other hand – PfEMP1 is the main parasitic antigen presented to the immune system. To overcome this potential double ended sword the parasite evolved to undergo antigenic switches in expression of different PfEMP1 variants, thus creating a tremendous extent of antigenic variation. While the evolutionary advantage of sequestration in specific tissues is yet to be elucidated, our data indicates that neutrophils may serve as the first line of immune defense against a parasite subpopulation that is associated with cerebral malaria.

**Minor comments**

- Did the authors investigate the effect of bloodtype? Ie. growing the parasites on the autologous red bloods to the neutrophils? so that there is no interference.

Yes. As pointed out by the reviewer, we also suspected that blood type may have an influence on neutrophil/iRBC interaction. We therefore performed initial neutrophil mediated killing of iRBC in autologous and allogeneic blood type and found no significant differences related to blood type. This point has been emphasized more clearly in the text.

- It would be obvious to investigate a CSA adhering VAR2CSA expressing parasite especially

as neutrophils accumulate in the intervillous space in the placenta during pregnancy associated malaria.

We completely agree. The interaction between neutrophils and VAR2CSA, the PfEMP1 variant that leads to iRBC sequestration in the placenta which is the main cause for pregnancy associated malaria is interesting and important avenue for investigation. However, the role played by VAR2CSA in regulating neutrophil/iRBC interaction is a separate project currently at its initial steps, and we believe is beyond the scope of the current manuscript.

- any clue as to what the neutrophil binds in PfEMP1 KO or trypsinated iRBC?

Our data indicates that while trypsinization limits neutrophil/iRBC interaction using wild type parasites it has no significant effect on PfEMP1 KO parasites. This suggests that elements that mediate PfEMP1 independent neutrophil/iRBC interactions are either trypsin insensitive surface proteins or are non-protein moieties such as sugars. Alternatively, parasite-induced structural deformability of the iRBC may play a role in this interaction. This point is discussed in the revised manuscript.

- I find the title misleading as it may be interpreted as evolutionary selection which is not investigated.

To avoid confusion, the title was changed to: “Neutrophils impose strong immune selection against PfEMP1 variants implicated in cerebral malaria”.

Reviewer #2 (Significance (Required)):

The data to my knowledge are novel, clearly presented and trustworthy. I find the data compelling and it is logical that neutrophils could play a role in malaria.

This should be general interest to malaria researchers and immunologists.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

This manuscript provides evidence that neutrophils recognise and kill P. falciparum infected erythrocytes (at least in part) via pfemp1-ICAM interactions and provide evidence for a contribution of neutrophil ROS to the killing mechanism. The work is presented to a high standard and the conclusions, for the most part, are supported by the data. This work makes an important contribution to our understanding of the role of neutrophils in the clearance of P. falciparum.

The following pints should be addressed:

1. Neutrophil fate in the co-culture system: what is the impact of antibody dependent and independent phagocytosis of iRBC on the viability of neutrophils on this system? Is there a more rapid attrition of GFP+ neutrophils? Are apoptotic pathways induced after iRBC phagocytosis?

The reviewer raises a valid question regarding the effect of iRBC phagocytosis on neutrophil viability. To answer this question, we performed an experiment where neutrophils were incubated with GFP+ iRBC. Following this incubation, we stained the neutrophils with Annexin V/PI and analyzed by FACS. Our new data show that most (~80%) of neutrophils remain fully viable regardless of whether they have or haven't phagocytosed iRBC. Importantly, iRBC phagocytosis did not enhance either apoptosis (Annexin) or necrosis (PI) of neutrophils. This new data is now presented as supplementary figure S1.

2. In figure 2. Do other, ROS independent mechanisms of parasite killing operate after day 1?

Neutrophils may kill parasites using various mechanisms. We distinguish between ROS-dependent and ROS-independent cytotoxic mechanisms by adding catalase to the co-culture. In the original manuscript we present data from day 1 showing that catalase efficiently blocks neutrophil killing. Similar data was obtained from day 3 suggesting that this mechanism is valid also beyond day 1. The data from day 3 was added as supplementary Figure S2.

3. In figure 4, the authors demonstrate that D. iRBC uptake is reduced from around 24% to 24% and E. killing from 70% to 50% in the presence of anti-ICAM antibodies. Soluble ICAM-Fc reduces phagocytosis by around 20% and killing by 60-70%. EPCR seems to play little role in the system. Why is this effect only partial and what other receptors - ligand interactions are potentially mediating these processes.

The reviewer is correct in pointing out that the effect of modifying ICAM-1 expression on neutrophils surface presented in figure 4D is partial. This observation could be explained by several possible reasons:

1. The ICAM-1 knockdown system using shRNA is not complete.
2. Neutrophils might also recognize iRBCs via other receptors/mechanisms as evident by the trypsinization experiments in figure 3B.

The possibility of additional recognition mechanisms that mediate PfEMP1 independent neutrophil/iRBC interactions such as trypsin insensitive surface proteins or non-protein moieties such as sugars is addressed in the revised manuscript (see also comment to reviewer #2).

A separate point: does antibody mediated opsonisation overcome the impact of blockade ICAM-pfemp-1 interactions?

To accommodate this comment, we performed additional experiments testing neutrophil cytotoxicity against iRBC incubated with anti ICAM1 antibody or an unrelated antibody (CD11b), in the presence or absence of opsonizing AB+ sera. We found that opsonisation had no significant effect the ability of anti-ICAM1 antibodies to block ICAM1-PfEMP1 interactions. This data is now included in supplementary figure S3.

4. Graphs are presented as summary data. it would be useful to see the individual level variation in responses of distinct primary neytrophil donors. In this context some discussion of neutrophil functioal cappacity in the context of varying differentiation phenotype of circulating neutrophils would be beneficial.

We thank the reviewer for this comment. We have previously shown the presence of different neutrophil subpopulations (Sagiv et al., Cell Rep 2015), in our current study, we focused on the function of normal density neutrophils (NDN) that we obtained from healthy donors. This neutrophil subpopulation represents malaria-naïve non-inflammatory neutrophils, providing us with the opportunity to study the initial interaction between neutrophils and iRBCs. This point is highlighted in the revised discussion. In addition, we have collected a substantial amount of data from different donors (marked example in figure 2I), showing no significant differences between donors. This point is also emphasized in the revised version.

5. There are some minor errors of syntax which should be checked throughout.

We apologize for these errors. We read through the text thoroughly in attempt to correct these errors.

Reviewer #3 (Significance (Required)):

This work is important as it increasses our understanding of the interractions required between malaria infected erythrocytes and neutrophils which facilitate paraite killing.

The manuscript is certainly of interest to a malaria / immunology research audience and has potential impacts for our understanding of differentce in innate parasite control in different arasite strains and between individual human hosts

My relevant expertise is in the study of innate immunity to malaria infection

Dear Prof. Dzikowski,

Thank you for the submission of your revised manuscript to our editorial offices. I have received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now fully support publication of your work in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised version of the manuscript:

- Please reduce the number of keywords on the title page to five.

- Please remove the significance statement from the manuscript.

- The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. Thus, please combine the three Supplementary Figures into one Figure and name this Figure EV1. Please upload this figure as separate, individual file upon re-submission, change the callouts in the manuscript and add a figure legend in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Like this, no Appendix file is needed.

- Please call out figures and panels sequentially (or change the order of the panels). Presently, Fig. 1D is called out after 2A. Please check.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (for main and the EV figure), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and add a statement declaring your competing interests. Please name that section 'Disclosure and Competing Interests Statement' and add it after the acknowledgements section.

- Please add a 'Data availability section' (DAS) to the manuscript, also if no large datasets have been submitted to a public database. Please state there 'No large primary datasets have been generated and deposited'. Further, please indicate this clearly in the author checklist (field F18).

- Please add a paragraph defining the contributions of each author to the manuscript main text.

- Please order the manuscript sections like this (using these names):
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Author contributions - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file.

- Please add scale bars of similar style and thickness to the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- Please format the references according to our journal style (we need et al. for publications with more than 10 authors). See also:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes and queries we ask you to include in your final manuscript text. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short (one sentence each) bullet points highlighting the key findings of your study.

- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

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Referee #1:

The authors have addressed my comments

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Referee #2:

I have re-appraised this manuscript following suggested changes and comments for Review Commons. The authors have addressed my previous comments in detail, including providing additional experimental data to address these (and those of other reviewers). I am satisfied that the manuscript is now suitable for publication in EMBO reports.

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Referee #3:

The authors have adequately addressed my concerns and comments in the response and revision of the paper.

_____

The authors have addressed all minor editorial requests.

Prof. Ron Dzikowski
Hebrew University - Hadassah Medical School
Microbiology & Molecular Genetics, IMRIC
The Kuvin Center for study of Infectious and Tropical Diseases
Ein Kerem
Jerusalem 91120
Israel

Dear Prof. Dzikowski,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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| Journal Submitted to: EMBO Reports |
| Manuscript Number: EMBOR-2021-53641V2 |

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
**Please note that a copy of this checklist will be published alongside your article.**

**Abridged guidelines for figures**
**1. Data**
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

**2. Captions**
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

**Please complete ALL of the questions below.**
**Select "Not Applicable" only when the requested information is not relevant for your study.**

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| Newly Created Materials | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
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| Antibodies | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| For **antibodies** provide the following information:<br>- Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number<br>- Non-commercial: RRID or citation | Yes | Materials and Methods |

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| **Cell lines:** Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID. | Yes | Materials and Methods |
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| Include a statement about **sample size** estimate even if no statistical methods were used. | Yes | Figure legends |
| Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described? | Not Applicable | |
| Include a statement about **blinding** even if no blinding was done. | Not Applicable | |
| Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established?<br><br>If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification. | Not Applicable | |
| For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared? | Yes | Materials and Methods |

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| Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | Yes | Materials and Methods |
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