nature portfolio

Peer Review File

A broadly cross-reactive i-body to AMA1 potently inhibits blood and liver stages of Plasmodium parasites

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The manuscript titled "A broadly cross-reactive i-body to AMA1 potently inhibits blood and liver stages of Plasmodium parasites" aims to identify a single-domain antibody targeting a conserved epitope in the Apical membrane antigen 1 (AMA1) and prevent its binding to a loop in rhoptry neck protein 2 (RON2L). The authors used a phage display approach to identify a humanized single-domain antibodies, conducting detailed structural and functional studies to characterize and assess their neutralizing potential.

The study demonstrates that the single-domain antibody WD34 recognizes a conformational epitope within the conserved RON2L binding site in AMA1, which partially overlaps with the epitopes of previously characterized shark single-domain antibodies (VNAR) 14I-1, 14I1-M15, and murine antibody 1F9, respectively. Interestingly, WD34 neutralizes blood-stage parasites from various P. falciparum (Pf) strains and Pf3D7 transgenic with P. vivax AMA1 and P. knowlesi in a growth inhibition assay, indicating that WD34 epitope residues are conserved among Plasmodium species. Additionally, WD34 inhibits hepatocyte invasion by P. falciparum sporozoites.

The results presented herein are well-supported by the data, and the findings are thoroughly discussed. The Methods section sufficiently describes the experiments, ensuring reproducibility of the results.

Overall, the manuscript is well-written, and the work presented herein is a valuable resource for researchers involved in therapeutic antibodies development and malaria vaccine research.

Major Comments

Several sensograms in Figure 3c appears incorrect, and the X (Response, RU) and Y (Time, Sec.) axes are not visible in the review file. Please include the correct sensograms. I strongly suggest including sensograms for WD34 and WD33 binding to all AMA1 alleles mentioned in the Table (under Figure 3c), either in the main figure or as a supplementary figure. This addition will demonstrate the overall quality of SPR data. Additionally, incorporating the Table into Figure 3 seems unnecessary since the KD values are already present in Supplementary Table 1.

For the WD34 epitope on PfAMA1 and PvAMA1 structures, please incorporate electron density maps (2Fo-Fc map contoured at 1.0 σ level).

To illustrate the widely conserved nature of the WD34 epitope and support its cross-neutralization potential, please include a multiple sequence alignment (MSA, displaying mostly WD34 epitope residues) of AMA1 alleles from P. falciparum (covering various strains), P. vivax (with diverse strains), and other apicomplexan parasites. Highlight the WD34 epitope residues in this MSA. Although a similar analysis exists in Supplementary Figure 13, including the MSA into the main figure will help readers in quickly appreciating the conserved nature of the WD34 epitope.

In the "Structure determination of the AMA1-WD34 complexes" subsection of the methods, cite relevant references for Phaser and Coot:
Phaser: https://doi.org/10.1107/S0907444905001617, https://doi.org/10.1107/S0021889807021206, https://doi.org/10.1107/S0907444909052925
Coot: https://doi.org/10.1107/S0907444910007493

In supplementary information, Table 1, include units for ka, kd, KD, and Rmax. For association rate constant (ka) and dissociation rate constant (kd), use lowercase "k".

Minor comments

In the Figure 3d legend, please correct as follows (remove "of" before "inhibition"):
"Dose-dependent inhibition..."

In the "WD34 inhibits erythrocyte invasion by merozoites of multiple Plasmodium species" subsection of the results, please correct it to "in vitro" (line 2).

In the "Structure determination of the AMA1-WD34 complexes" subsection of the methods, specify the pH for "50 mM Tris" (first paragraph, line 2).

In the "Invasion inhibition assays for transgenic parasites" subsection of the methods, italicize "P. falciparum" and "P. vivax" (second paragraph, lines 3 and 4).

In the supplementary information, correct the Figure 2 legend as follows:
"Phage enrichment ELISA against multiple AMA1 isoforms of P. falciparum using pooled phage from W2mef-based biopanning."

Reviewer #2:
Remarks to the Author:
In the manuscript Angage et al, describe a novel iBody that inhibits merozoite and sporozoite invasion of both different Plasmodium falciparum strains and as well as Plasmodium species. This is to my knowledge the first time, such a pan-reactive invasion blocking antibody has been described, and therefore obviously interesting. The work is well described, easy to follow and experiments seems to be well conducted.

I have though some minor comments and corrections:
Since it is observed that two of your AMA1 specific antibodies could compete with the binding of WD34, there could be a risk that naturally acquired antibodies could interfere with the inhibitory effect of WD34. So, it would be very relevant to include a GIA assay where WD34 is combined with naturally acquired antibodies, to see if the WD34 iBody would increase the inhibitory capacity of the immune sera or there will be a competition, so that the immune sera reduce the effect of the WD34 ibody. This would be relevant both for future vaccine design based on the structures presented here as well as the potential for use as a therapeutic in endemic areas.
In figure 5e the effect of WD34 on hepatocyte invasion is shown, and it is concluded that WD34 significantly reduces the invasion. The only marked statistical difference is between NF54 (no antibody) and NF54+WD54. But all the values with the control antibody seems to be lower than without any antibodies as well. So, it would be good to also show the P-values when comparing the WD34 and the 21H5 mAb. Is there still a significant difference there?

Minor corrections:
On page 11, it says …..Assay with parasite cultures in vivo…..I guess it should be changed into in vitro.

Reviewer #3:
Remarks to the Author:
This manuscript by Angage et al describes the application of phage display to identify a broadly cross-reactive, engineered human Ig-like domain (WD-34) protein that binds P. falciparum AMA1. Strong data is provided showing potent inhibition of genetically distinct strains through its interaction with highly conserved amino acids in key loops (Ia, Ib, Ic and Ie) surrounding the hydrophobic pocket. They elegantly demonstrate that WD-34 inhibits merozoite and sporozoite invasion of RBCs and

hepatocytes respectively, by blocking AMA1-RON2 interaction. Furthermore, this is the first demonstration of an inhibitor that binds to AMA1 from other Plasmodium spp. (P. vivax, P.knowlesi and P.chabaudi) and block parasite invasion (P.knowlesi, PfPvAMA1).
Some concerns to be addressed by the authors,
1. The approach used here to identify WD34 is interesting. But WD34 is not a "antimalarial antibody" nor a "single-domain antibody" and the random peptides are not "CDRs". This gives a wrong connotation by invoking an antibody and distracts from the elegant approach.
2. Previously, the authors identified a 20 amino acid peptide (R1) using a random phage display library. Nevertheless, the approach used here identified a pan-AMA1 reactive molecule containing two random peptide inserts, it is still a phage display screen to identify peptide-based inhibitors.
3. Nice structural data is provided to explain the lower KD and the corresponding weaker inhibition of PvAMA1 compared to PfAMA1 expressing parasites. It is interesting to see that WD34 bound to the P.chabaudi AMA1 with KD similar to PfAMA1 (Fig. 3C). How does the interacting residues in PfAMA1 compare with PcAMA1?
4. The methods section under "Expression and purification of i-bodies" describes the purification of Fc region tagged i-bodies but not sure which experiments used these proteins.
5. Peptide inhibitors of AMA1 such as R1 and even RON2 peptide have shown to be potent inhibitors. Particularly, RON2 peptide that bind to all PfAMA1 at affinities similar to or better than WD-34 have been previously demonstrated. A key advancement at this point would be to demonstrate in vivo activity of such an approach. Since WD-34 binds strongly to PcAMA1 and it appears the authors have made WD-34 Fc fusion proteins, it is important to show if it can protect in this easily accessible P.chabaudi-mouse model. Otherwise it may be that this is only another peptide, albeit, a pan-AMA1 binding peptide.
6. Could the authors provide additional assessment of WD-34 such as thermal stability, protease protection etc, if such a molecule is proposed to be used as an antimalarial.
7. Will the insertion of two random peptides into a human protein lead to immunogenicity against the neural cell adhesion molecule? The authors could test this my injecting mice and measuring for anti-drug antibodies.
8. The colors of the amino acids color in Fig 1e is hard to see. It would also be helpful to number the starting and ending amino acids to follow the references to specific amino acids in the text. The labeling of "CDR1" and CDR3" is confusing since this is not an antibody
9. Fig 2b- The resolution of the IFA data is not good. Even at this low resolution of miscroscopy, the signal for 5G8(AMA1) antibody and the WD34,WD33 staining do not overlap within the parasite.
10. The western blots in Fig2A showing only two bands corresponding to the AMA1 full length and processed forms is string. But a IP/mass spec would be needed to know that these peptides are binding to only AMA1 in the parasite.
11. It is interesting that WD-33 does not compete for RON2 binding but still appears to inhibit multiple Pf strains. Where does it bind on AMA1? This would be important for understanding other binding sites for RON2 or even other potential parasite/rbc receptor.

Reviewer's comment	Response
Reviewer #1	
1. Several sensograms in Figure 3c appears incorrect, and the X (Response, RU) and Y (Time, Sec.) axes are not visible in the review file. Please include the correct sensograms. I strongly suggest including sensograms for WD34 and WD33 binding to all AMA1 alleles mentioned in the Table (under Figure 3c), either in the main figure or as a supplementary figure. This addition will demonstrate the overall quality of SPR data. Additionally, incorporating the Table into Figure 3 seems unnecessary since the KD values are already present in Supplementary Table 1.	Thank you for the review and feedback. All 24 sensorgrams were added to the separate supplementary file (SPR extended file) with better visibility. We have retained the table with K_D values in Figure 3 for ease of understanding for the readers.
2. For the WD34 epitope on PfAMA1 and PvAMA1 structures, please incorporate electron density maps (2Fo-Fc map contoured at 1.0 σ level).	Electron density maps were incorporated into the supplementary figures.
3. To illustrate the widely conserved nature of the WD34 epitope and support its cross-neutralization potential, please include a multiple sequence alignment (MSA, displaying mostly WD34 epitope residues) of AMA1 alleles from P. falciparum (covering various strains), P. vivax (with diverse strains), and other apicomplexan parasites. Highlight the WD34 epitope residues in this MSA. Although a similar analysis exists in Supplementary Figure 13, including the MSA into the main figure will help readers in quickly appreciating the conserved nature of the WD34 epitope.	The percentage conservation of the main WD34-interacting residues in AMA1 are plotted separately for *P. falciparum* isolates, *P. vivax* isolates, and *Plasmodium* isolates (Fig. 6e). The separation of these residues along the AMA1 polypeptide chain makes it difficult to present these adequately in the long MSA, however in Fig. 6e, we have highlighted the critical conserved residues. .

4. In the "Structure determination of the AMA1-WD34 complexes" subsection of the methods, cite relevant references for Phaser and Coot: Phaser: https://doi.org/10.1107/S0907444905001617, https://doi.org/10.1107/S0021889807021206, https://doi.org/10.1107/S0907444909052925 Coot: https://doi.org/10.1107/S0907444910007493	The reference was corrected in the revised manuscript.
5. In supplementary information, Table 1, include units for ka, kd, KD, and Rmax. For association rate constant (ka) and dissociation rate constant (kd), use lowercase "k".	Corrections were added in the revised supplementary information (Extended SPR data file-Table 1)
Minor comments	
1. In the Figure 3d legend, please correct as follows (remove "of" before "inhibition"): "Dose-dependent inhibition..."	Thank you for all the minor comments. We corrected this in the revised manuscript.
2. In the "WD34 inhibits erythrocyte invasion by merozoites of multiple Plasmodium species" subsection of the results, please correct it to "in vitro" (line 2)	We corrected this in the revised manuscript.
3. In the "Structure determination of the AMA1-WD34 complexes" subsection of the methods, specify the pH for "50 mM Tris" (first paragraph, line 2)	We corrected this in the revised manuscript.
4. In the "Invasion inhibition assays for transgenic parasites" subsection of the methods, italicize "P. falciparum" and "P. vivax" (second paragraph, lines 3 and 4).	We corrected this in the revised manuscript.
5. In the supplementary information, correct the Figure 2 legend as follows:	We corrected this in the revised manuscript.

"Phage enrichment ELISA against multiple AMA1 isoforms of P. falciparum using pooled phage from W2mef-based biopanning."	
Reviewer #2	
Minor comments only	
1. Since it is observed that two of your AMA1-specific antibodies could compete with the binding of WD34, there could be a risk that naturally acquired antibodies could interfere with the inhibitory effect of WD34. So, it would be very relevant to include a GIA assay where WD34 is combined with naturally acquired antibodies, to see if the WD34 i-body would increase the inhibitory capacity of the immune sera or there will be a competition, so that the immune sera reduce the effect of the WD34 ibody. This would be relevant both for future vaccine design based on the structures presented here as well as the potential for use as a therapeutic in endemic areas.	Thank you for the valuable feedback. This comment was discussed with the Editor. While we agree this is an important question, we would not be able to address this adequately within the timeframe for resubmission. Obtaining the appropriate ethics approval and access to blood from malaria-infected individuals would not be possible in the timeframe for resubmission. As mentioned, WD34 competes somewhat with mAb 1F9, even though the footprints on AMA1 are different. The blocking is presumably by steric hindrance. Moreover, the lack of reports of naturally occurring antibodies with cross-species reactivity suggests that if these antibodies are induced by malaria infection, they are a very small fraction of the total antibody response. We also note that in the clinical trials ofAMA1 vaccines broadly cross-reacting antibodies to AMA1 were not induced. We aim to collaborate with James Beeson's group to try and answer this question in the near future.
2. In figure 5e the effect of WD34 on hepatocyte invasion is shown, and it is concluded that WD34 significantly reduces the invasion. The only marked statistical difference is between NF54 (no antibody) and NF54+WD54. But all the values with the control antibody seems to be lower than without any antibodies as well. So, it would be good to also show the P-values when comparing the WD34 and the 21H5 mAb. Is there still a significant difference there?	We thank the reviewer for suggesting a more detailed statistical analysis of these results, which we have now carried out. We have now revised Figure 5 by including the background control (no sporozoites, No SPZ) and performing a 2-way ANOVA to enable multiple comparisons. The ANOVA showed that while NF54 invasion occurs (compare NF54 to No SPZ; P=0.0004), the addition of control 21H5 antibody had no significant inhibitory effect on invasion (NF54 vs NF54+21H5; P≥0.4006. However, the addition of 3 µM WD34 had an inhibitory effect (NF54 vs NF54+WD34; P=0.0178) (see also No SPZ vs NF54+WD43 3 uM; P=0.5831). We have now added these statistical comparisons to the figure. We also added a more detailed description into the main text: "Hepatocyte invasion by NF54 was moderately inhibited by WD34 and in a dose-dependent manner (mean difference 62.9%; p = 0.0178) but NF54 was not significantly inhibited by the control i-body (mean difference 35.1%; $p \geq 0.4006$) (Fig. 5e).".
3. On page 11, it says …..Assay with parasite cultures in vivo…..I guess it should be changed into in vitro.	We corrected this in the revised manuscript.

Reviewer #3	
1. The approach used here to identify WD34 is interesting. But WD34 is not a "antimalarial antibody" nor a "single-domain antibody" and the random peptides are not "CDRs". This gives a wrong connotation by invoking an antibody and distracts from the elegant approach.	We agree that WD34 is not a classical antibody but is a single-domain antibody-like molecule. It is analogous to the variable new antigen receptor found in sharks. It also consists of a single immunoglobulin domain where we have engineered random sequences analogous to the CDRs in the shark variable new antigen receptor[1]. We have published several reports previously describing the i-body and have referred to the small regions of variability as CDRs[2-4]. We are keen to retain this nomenclature so as to remain consistent with past literature. However, to address this point, we have clarified this based on the reviewers' comments in the introduction, where we have clearly defined the nature of the i-body as a single-domain antibody-like molecule.
2. Previously, the authors identified a 20 amino acid peptide (R1) using a random phage display library. Nevertheless, the approach used here identified a pan-AMA1 reactive molecule containing two random peptide inserts, it is still a phage display screen to identify peptide-based inhibitors.	Thank you for raising an interesting point, but as mentioned above, the i-body is analogous to previously reported single domains such as nanobodies and shark new antigen receptors. Although there are two random peptide inserts, they are held in a defined conformation due to the immunoglobulin scaffold. This is a crucial difference between a peptide and the peptide sequences of CDRs.
3. Nice structural data is provided to explain the lower KD and the corresponding weaker inhibition of PvAMA1 compared to PfAMA1-expressing parasites. It is interesting to see that WD34 bound to the P.chabaudi AMA1 with KD similar to PfAMA1 (Fig. 3C). How does the interacting residues in PfAMA1 compare with PcAMA1?	This has been addressed in the discussion, and the MSA figure has been added to the supplementary data Fig. 12d.
4. The methods section under "Expression and purification of i-bodies" describes the purification of Fc region tagged i-bodies but not sure which experiments used these proteins.	Thank you for the comment. We used Fc-tagged WD33 and WD34 to conduct Competition ELISA with mAbs 4G2 and 1F9. The purpose of the Fc conjugation was to use it as a tag for the identification of i-bodies.
5. Peptide inhibitors of AMA1 such as R1 and even RON2 peptide have shown to be potent inhibitors.	We thank the reviewer for raising this point and agree that a *P. chabaudi in vivo* model would be supportive. Currently, we do not have access to that model but included in the

Particularly, RON2 peptide that bind to all PfAMA1 at affinities similar to or better than WD-34 have been previously demonstrated. A key advancement at this point would be to demonstrate in vivo activity of such an approach. Since WD-34 binds strongly to PcAMA1 and it appears the authors have made WD-34 Fc fusion proteins, it is important to show if it can protect in this easily accessible P.chabaudi-mouse model. Otherwise it may be that this is only another peptide, albeit, a pan-AMA1 binding peptide.	revised manuscript are the results of a Proof-of-Concept study using mice infected with asexual blood-stages of *P. berghei*. This experiment shows a moderate but statistically significant inhibitory effect of WD34 in the Peter's 4-day suppressive test in n=4 WD34-treated mice compared to the parasitemia in n=3 untreated control mice (p = 0.0009). However, this suppressive effect was short, as the parasitemia was similar in all mice following cessation of i-body injections (days 1-4 only). This representative PoC experiment is provided in Figure 6 and represents a single experiment however the effect was consistent in the cohort of n=3 or n=4 mice per group and time limitations applied. We confirmed that WD34 recognized *P. berghei* AMA1 using immunoblots of uninfected and *P. berghei*-infected erythrocytes WD34 recognized a single protein in infected but not uninfected erythrocytes which had the expected mobility on SDS-PAGE for AMA1 of a rodent parasite. Importantly, reducing agents impaired recognition of the *P. berghei* protein by WD34, consistent with this i-body recognizing a disulphide bond-dependent conformational epitope as is expected of WD34 binding to AMA1.
6. Could the authors provide additional assessment of WD-34 such as thermal stability, protease protection etc, if such a molecule is proposed to be used as an antimalarial.	Yes. Results of thermal stability, serum stability and activity assays were added into supplementary Fig. 13, and results were stated in the results section.
7. The colors of the amino acids color in Fig 1e is hard to see. It would also be helpful to number the starting and ending amino acids to follow the references to specific amino acids in the text. The labeling of "CDR1" and CDR3" is confusing since this is not an antibody.	Figure 1e was corrected with a larger font and background colour.
8. Fig 2b- The resolution of the IFA data is not good. Even at this low resolution of miscroscopy, the signal for 5G8(AMA1) antibody and the WD34, WD33 staining do not overlap within the parasite.	The mAb 5G8 sees a linear epitope in the AMA1 pro-domain, whereas WD33 and WD34 see conformational epitopes in Domain I and Domain II. Therefore, it is expected that 5G8 and WD33 or WD34 signals may not overlap completely as 5G8 could only recognise unprocessed AMA1, whereas both WD33 and WD34 could recognise both versions of AMA1. Further, as 5G8 mAb recognises a linear epitope, it is possible that

	5G8 mAb can recognise unprocessed and unfolded AMA1 in parasites that WD33 and WD34 may not recognise.
9. The western blots in Fig2A showing only two bands corresponding to the AMA1 full length and processed forms is string. But a IP/mass spec would be needed to know that these peptides are binding to only AMA1 in the parasite.	While the blots are consistent with previously described antibodies, we have carried out the IP/ mass spec experiment as suggested. IP/MS clearly demonstrated that AMA1 was the main protein pulled down in all three replicates. In addition, AMA1 was not pulled down by a control i-body. These results are described in the revised manuscript's supplementary figures.
10. It is interesting that WD-33 does not compete for RON2 binding but still appears to inhibit multiple Pf strains. Where does it bind on AMA1? This would be important for understanding other binding sites for RON2 or even other potential parasite/rbc receptor.	Attempts to crystalise the AMA1-WD33 co-complex haven't been successful. Competition ELISA showed that WD33 binding was inhibited only by 4G2 mAb, suggesting that the WD33 footprint may lie in Domain II. These data were added to Extended Data Fig. 5 and mentioned in the results section.

1. Streltsov, V.A., Varghese, J.N., Carmichael, J.A., Irving, R.A., Hudson, P.J., and Nuttall, S.D. (2004). Structural evidence for evolution of shark Ig new antigen receptor variable domain antibodies from a cell-surface receptor. Proceedings of the National Academy of Sciences *101*, 12444-12449. doi:10.1073/pnas.0403509101.
2. Griffiths, K., Dolezal, O., Cao, B., Nilsson, S.K., See, H.B., Pfleger, K.D.G., Roche, M., Gorry, P.R., Pow, A., Viduka, K., et al. (2016). i-bodies, Human Single Domain Antibodies That Antagonize Chemokine Receptor CXCR4*. Journal of Biological Chemistry *291*, 12641-12657. https://doi.org/10.1074/jbc.M116.721050.
3. Griffiths, K., Binder, U., McDowell, W., Tommasi, R., Frigerio, M., Darby, W.G., Hosking, C.G., Renaud, L., Machacek, M., Lloyd, P., et al. (2019). Half-life extension and non-human primate pharmacokinetic safety studies of i-body AD-114 targeting human CXCR4. mAbs *11*, 1331-1340. 10.1080/19420862.2019.1626652.
4. Cao, Q., Huang, C., Yi, H., Gill, A.J., Chou, A., Foley, M., Hosking, C.G., Lim, K.K., Triffon, C.F., Shi, Y., et al. (2022). A single-domain i-body, AD-114, attenuates renal fibrosis through blockade of CXCR4. JCI Insight *7*. 10.1172/jci.insight.143018.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Although the authors have incorporated the suggested changes, please make sure to address the following minor corrections:

In Figures 7b, c, and d, although it is clear that residues in red represent residues involved in polar interactions and residues in blue represent residues involved in Van der Waals interactions, this is not indicated in the legends for Figures 7b, c, and d. Please indicate what the red and blue residues represent in the legend for Figure 7.

Please indicate map contoured and sigma level in extended data Figure 6 legend.

In the supplementary information, Table 1 (Extended SPR Data), for the dissociation rate constant (kd), use a lowercase "k".

Reviewer #2:
Remarks to the Author:
I think the authors have responded well to the comments raised in my initial review of the manuscript and revised accordingly. Therefore I have no further comments.

Reviewer #3:
Remarks to the Author:
The authors have been responsive and sufficiently addressed the reviewers' concerns. The manuscript is acceptable for publication.

Reviewer's comment	Response
Reviewer #1	
1. Although the authors have incorporated the suggested changes, please make sure to address the following minor corrections: In Figures 7b, c, and d, although it is clear that residues in red represent residues involved in polar interactions and residues in blue represent residues involved in Van der Waals interactions, this is not indicated in the legends for Figures 7b, c, and d. Please indicate what the red and blue residues represent in the legend for Figure 7.	Thank you for the comments. We have corrected the manuscript according to your comment.
2. Please indicate map contoured and sigma level in extended data Figure 6 legend.	Thank you. We corrected the manuscript.
Reviewer #2	
1. I think the authors have responded well to the comments raised in my initial review of the manuscript and revised accordingly. Therefore I have no further comments.	Thank you.
Reviewer #3	
1. The authors have been responsive and sufficiently addressed the reviewers' concerns. The manuscript is acceptable for publication.	Thank you.