A novel immunotherapeutic strategy to interfere with *Plasmodium falciparum* pathogenicity and transmission

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INTRODUCTION

The constant emergence of *Plasmodium falciparum (Pf)* resistance to antimalarial drugs threatens global malaria control efforts and underscores the urgent need for novel therapeutic strategies. Our group has developed an immunotherapeutic strategy based on bi-modular fusion proteins (BMFPs) that redirect a pre-existing Epstein-Barr virus (EBV) antibody response toward specific target cells[1]. Here, we adapt this technology to develop second-line treatments to reduce malaria pathogenicity and prevent the transmission of drug-resistant parasites. This will be achieved by targeting *P. falciparum* proteins expressed on the surface of infected erythrocytes (*Pf*CBP1/GEXP10 and *Pf*CBP2/ GEXP07) on trophozoites and schizonts (asexual stages) or on early gametocytes (sexual stages), as well as proteins displayed on the surface of gametes (*Pf*s230). BMFPs consist of a Nanobody (Nb) -based binding domain that specifically recognizes these parasite antigens, fused to the EBV antigen P18F3, enabling the recruitment of circulating endogenous anti-P18F3 IgG in EBV+ individuals (**Fig. 1**) to trigger diverse immune effector mechanisms in the human body (**Fig. 2**) or act as neutralizing agents in the *Anopheles* mosquito to interfere with transmission (**Fig. 3**).

- Nanobodies against *Pf*CBP1, *Pf*CBP2 were selected from an immune library **(Fig. 2.1) (Fig. 2.3)**.
- The sequences of Nanobodies targeting *Pf*s230 were retrieved from Dietrich *et al.*[2] **(Fig. 3.1)**.
- Nanobody candidates were fused to P18F3 to generate BMFPs **(Fig. 3.2)**.
- BMFPs were subjected to biochemical characterization to assess their binding affinity to their respective targets **(Fig. 2.5) (Fig. 3.3)**.
- Transgenic *P. berghei* (*Pb*) parasite lines (two expressing *Pf*CBP1 or *Pf*CBP2, and another in which the *Pb*s230-D1 domain was substituted with *Pf*s230-D1) were created to evaluate the efficacy of the BMFP candidates *in vivo* **(Fig. 2.6) (Fig. 3.4)**.

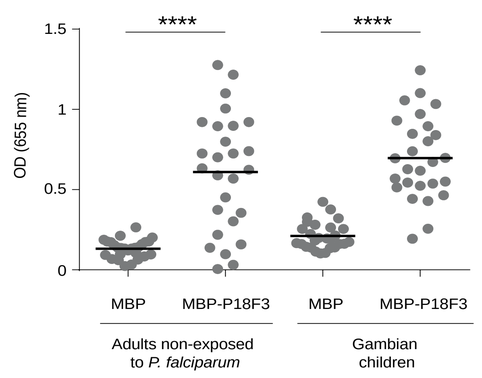
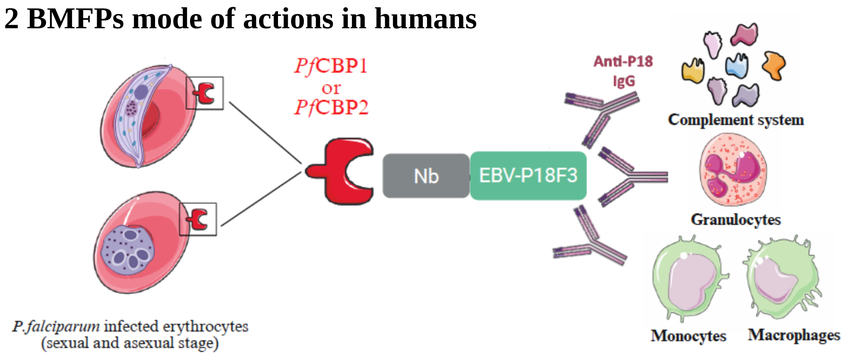


Fig. 1 Children (Mean age 9.15) living in malaria-endemic areas exhibit high levels of circulating anti-EBV-P18F3 IgG.

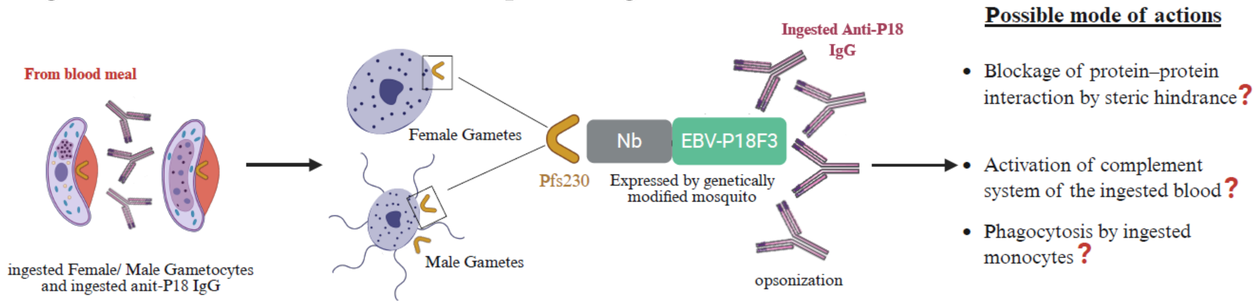
Pathogenicity / Transmission: *Pf*CBPs

Fig. 2 BMFPs mode of actions in humans



Transmission : *Pf*s230

Fig. 3 BMFPs mode of actions in mosquito midgut



Generation of an immune Nanobody library targeting *Pf*CBPs

Fig. 2.1 Overview of Nanobody generation

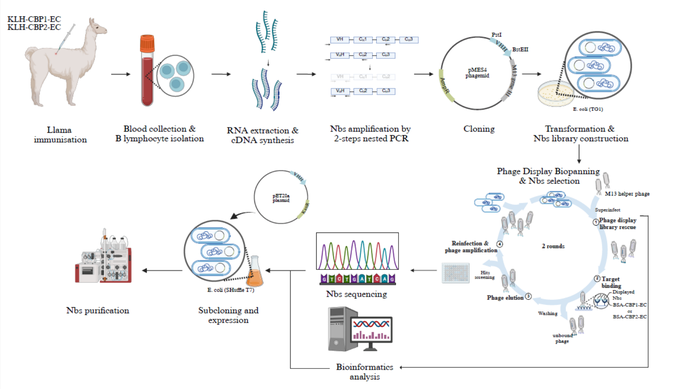


Fig. 2.2 IgG recognition following llama immunization

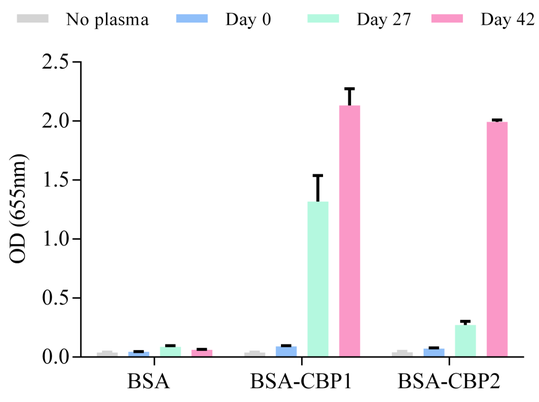
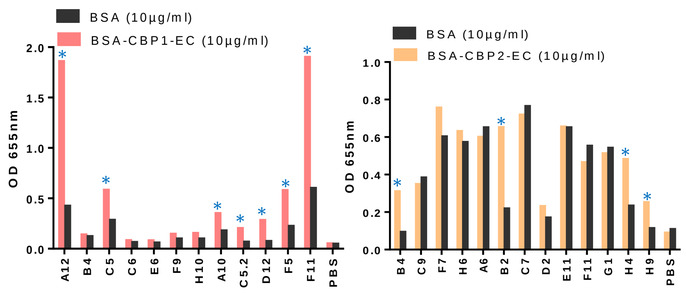
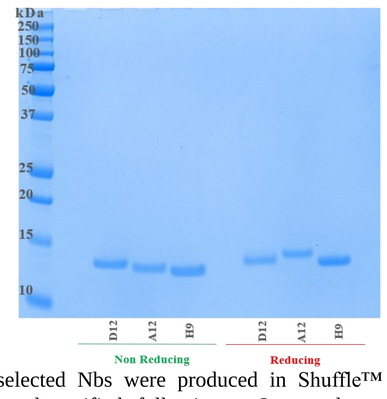


Fig. 2.3 Selection of binders



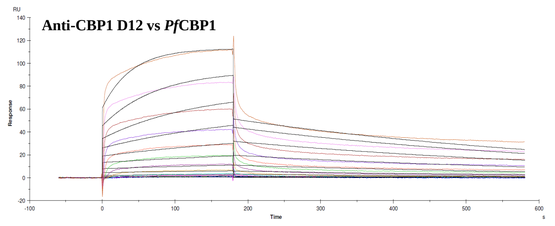
- * Nanobodies with absorbance at least twice that of the signal obtained on BSA were considered positive for binding. Anti-*Pf*CBP1, A12 and F11 shared identical sequences, as did F5 and A10. All anti-*Pf*CBP2 (B4, B2, H4, H9) were sequence-identical.

Fig. 2.4 Production of selected Anti-*Pf*CBPs



- Three selected Nbs were produced in Shuffle™ bacterial system and purified following a 2-step chromatography process. Anti-*Pf*CBP1 Nbs include D12 and A12, while H9 is specific to *Pf*CBP2.

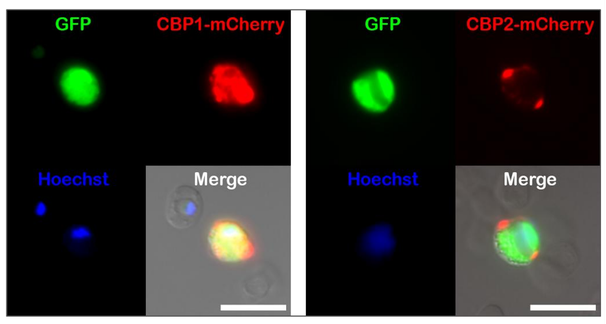
Fig. 2.5 SPR analysis of Anti-*Pf*CBPs binding to *Pf*CBPs



Targeting antigen	Nb	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (M)
*Pf*CBP1	Anti-CBP1 D12	1.167×10^4	0.001833	157.1×10^{-9}
	Anti-CBP1 A12	1.175×10^5	0.001795	10.26×10^{-9}
	Anti-CBP2 H9	1.251×10^2	0.004075	$32{,}580 \times 10^{-9}$
*Pf*CBP2	Anti-CBP1 D12	8.749×10^4	0.01279	146.2×10^{-9}
	Anti-CBP1 A12	6.236×10^3	0.002375	380.9×10^{-9}
	Anti-CBP2 H9	1.933×10^7	0.001767	0.09143×10^{-9}

- K_D values were calculated using a 1:1 kinetic model and are summarized in the table.

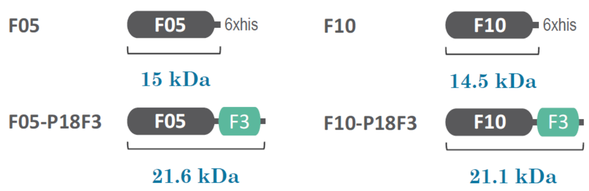
Fig. 2.6 Establishing *in vivo* murine model to evaluate Anti-*Pf*CBPs BMFPs efficacy



- Fluorescence microscopy of mouse erythrocytes infected with genetically modified *Pb*GFP parasites (green) expressing mCherry-tagged *Pf*CBP1 or *Pf*CBP2 (red). Nuclei were stained with Hoechst (blue).

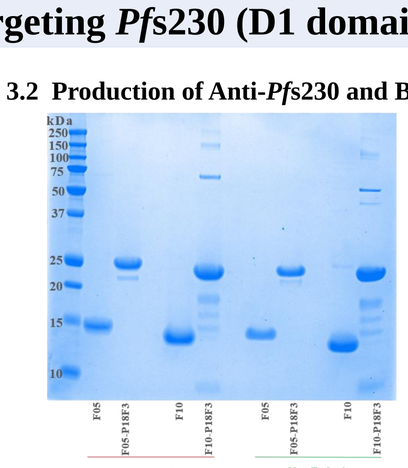
Production of Nanobody-based BMFP targeting *Pf*s230 (D1 domain)

Fig. 3.1 Anti-*Pf*s230 and BMFPs constructs



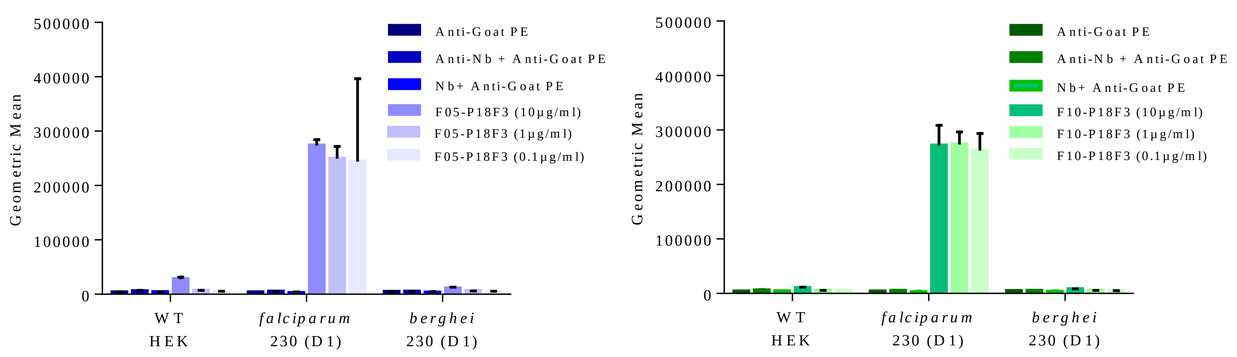
- F05 and F10 Nbs amino acid sequences were taken from Dietrich *et al.*[2]

Fig. 3.2 Production of Anti-*Pf*s230 and BMFPs



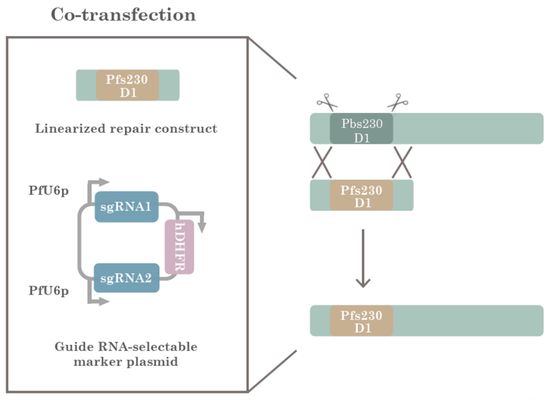
- F05 and F10 Nbs were produced in Shuffle™ bacterial system, either in their native form or fused with P18F3, then purified following a 2-step chromatography process.

Fig. 3.3 Anti-*Pf*s230 Nanobodies don't cross-react with *Plasmodium berghei* 230 (D1 domain)

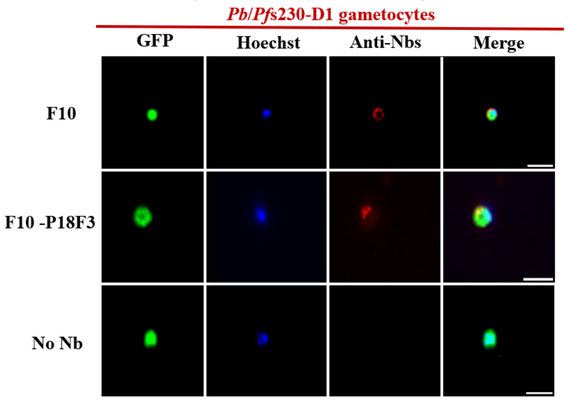


- *P. falciparum 230* D1 domain and *P. berghei* 230 D1 domain were expressed on the surface of HEK cells for 48 hours following transfection. The recognition of F05 BMFP and F10 BMFP to *Pf*s230 D1 and *Pb*s230 D1 was then assessed using flow cytometry.

Fig. 3.4 Establishing *in vivo* murine model to evaluate Anti-*Pf*s230 BMFPs efficacy



- Generation of genetically modified in which *P. berghei* 230 D1 domain was substituted by *Pf*s230 D1 using CRISPR–Cas9 technology.



- Immunofluorescence assay (IFA) shows specific binding of F10 Nb and F10-P18F3 (red) to *Pf*s230-D1 expressed on modified *P. berghei* gametocytes (green). Nuclei were stained with Hoechst (blue).

CONCLUSION & PERSPECTIVES

- Our work highlights the feasibility of engineering Nanobody-based bi-modular fusion proteins (BMFPs) as a novel immunotherapeutic strategy against malaria. By redirecting naturally acquired EBV immunity toward key parasite surface antigens, this approach offers a promising avenue for both the clearance of infected erythrocytes and the disruption of parasite transmission.
- For the clearance strategy, we successfully identified and produced BMFPs targeting *Pf*CBP1 and *Pf*CBP2. Genetically modified *P. berghei* lines expressing these antigens are now available to assess the *in vivo* efficacy of BMFPs in reducing parasite burden.
- For the transmission-blocking strategy, we generated BMFPs against *Pf*s230 and established *Pf*s230-expressing *P. berghei* transgenic parasites to evaluate whether BMFPs can interfere with parasite development in the mosquito and block transmission.
- This dual approach positions BMFPs as a promising immunotherapeutic platform with the potential to mitigate disease severity and limit the spread of drug-resistant *Plasmodium* strains.

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