bioRxiv preprint doi: https://doi.org/10.1101/147769; this version posted June 8, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Engagement rules that underpin DBL-DARC interactions for ingress of *Plasmodium knowlesi* and*Plasmodium vivax* into human erythrocytes

Manickam Yogavel, Abhishek Jamwal,

Swati Gupta and Amit Sharma[1]

Molecular Medicine Group

International Center for Genetic Engineering and Biotechnology,

New Delhi, India

Correspondence to:

Amit Sharma

Email: amitpsharma68@gmail.com

Abstract

Plasmodium parasites are unicellular organisms that cause malaria and ensuing morbidity in afflicted regions of the world. Although the most dreadful of human malaria parasites is *P. falciparum*, its variant *P. vivax* is common in India, South East Asia and Latin America. In addition, a fifth human parasite called *P. knowlesi* is able to cause infections in humans in some regions. *P. vivax* has a wide distribution with ~40% of world's population at risk of infection and 70-130 million annual cases. The molecular mechanisms by which *P. knowlesi* and *P. vivax* invade human red blood cells have long been studied. Malaria parasite erythrocytic stages comprise of repeated propagation of parasites via cyclical invasion of host RBCs using dedicated receptor-ligand interactions. A family of erythrocyte-binding proteins (EBPs) that include *P. knowlesi* and *P. vivax* Duffy-binding proteins (PvDBP and PkDBP respectively) attach to duffy antigen (DARC) on human erythrocytes for invasion via their duffy binding-like domains (DBLs). Here, we provide a comprehensive overview that presents new insights on the atomic resolution interactions that underpin the binding of Duffy antigen on human red blood cells with *P. knowlesi* and *P. vivax* DBL domains. Using extensive structural and biochemical data from the past decade, we provide a novel, testable and overarching model that fully rationalizes even contradictory pieces of evidence that have so far existed in the literature. We resolve the conundrum of how parasite-encoded DBL domains recognize human DARC and its two sulfated tyrosine residues. We provide evidence of two distinct DARC binding sites on *P. knowlesi* and *P. vivax* DBLs that together likely engage the extracellular domain of DARC. These analyses are important for both malaria vaccine and inhibitor development efforts that are targeted at abrogating DARC-DBL interactions as an avenue to prevent invasion of malaria parasites into human red blood cells.

Duffy antigen receptor for chemokines (DARC) exploited of ingress of malaria parasites

The duffy blood group antigen for chemokine (DARC) is a seven transmembrane protein present on surface of erythrocytes and endothelial cells (Fig 1) [1]. It is a promiscuous cytokine/chemokine receptor involved in pro-inflammatory processes of the immune system, where it acts as a scavenger, reducing excess amounts of toxic chemokines produced in some pathological conditions [2]. DARC is also used as an entry vehicle by the malaria parasites *P. knowelsi* and *P. vivax* (*Pk/Pv*) (Fig. 1) [3-5]. The DARC regions spanning its soluble domain from 1-60 contain two key tyrosyl residues at positions 30 and 41, where post-translational modification in the form of sulfation occurs [6]. Of tyrosines 30 and 41, it is critically the sulfation of the latter which seems essential for high affinity binding to *Pk/Pv* proteins [6]. The *Pk/Pv* erythrocyte binding proteins (PvEBPs) present on merozoite surface are responsible for binding to the DARC receptor on reticulocytes, and then mediating an irreversible junction formation that is vital for the parasite invasion process (Fig. 1) [7-9]. EBPs generally contain one or two extracellular cysteine-rich domains (region II), a second extracellular cysteine-rich domain (region VI), type I trans-membrane domain and a short cytoplasmic domain [10-12]. The key molecular players – EBPs - encoded by *Pk/Pv* for erythrocyte colonization contain Duffy-binding-like (DBL) domains that specifically recognize DARC via intermolecular interactions [11-13]. *Pk/Pv* DBLs are organized into 3 subdomains, and are typified (mostly) by twelve-cysteine residues that are disulfide linked [11, 13-16]. The importance of DBL-DARC pairing is underscored by human genetic data where DARC negative individuals tend to be protected from *P. vivax* infection [17-21]. Contrary to the established DBL–DARC invasion pathway, there is evidence for DARC-independent invasion pathway in case of *P. vivax* infections, calling for new

caution in assessing the utility of DARC recognizing Pv DBL as a *P. vivax* vaccine candidate [22, 23].

Binding sites on Pk/Pv DBLs for DARC sulfated tyrosines based on analysis of crystal structures

Pk/Pv DBL subdomains contain majority of the conserved cysteine residues that are (mostly) linked into disulfides and likely contribute to the structural integrity of DBLs [11,12,16]. In addition, conservation of hydrophobic residues within DBLs allows for a parasite-specific evolutionary motif that is both constant (in structural terms) and variable (in sequence). Metaphorically, hence, DBLs are built using same principles as antibody structures, where the overall 3D core structures remain the same but sequence variation in exposed residues and loop regions allows for surface diversity that can thus engage with plethora of biomolecular receptors[11,16].

Crystal structure of PkDBL had suggested a region on its subdomain 2 that could accommodate the DARC's sulfated tyrosine 41 [11], here referred to as Site 1 (Fig 2a). The PkDBL subdomain 2 presents a remarkably surface exposed region of highly conserved residues that arrange into distinct regions lying adjacent to each other – positively charged residues (Lys 96, Lys 100 and Arg 103 and Lys 133) and non-polars (Tyr 94, Leu 168 and Ile 175) [11]. These two dual character surfaces on Pk and Pv DBLs were proposed to bind sulfated tyrosyl 41 based on structural considerations emanating from collation of mutagenesis data from two distinct groups at the time [15, 24]. Further, the proposed residues on Pk and Pv DBLs remain fully conserved in these DARC binding DBLs, hence lending support to the proposal of their essential role in

binding to DARC's sulfated tyrosine 41 [11,15,16]. These data, coupled with the observation that sulfation of DARC's Tyr41 significantly enhanced binding to Pk/Pv DBLs led to a model of DARC's docking onto Pk/Pv DBLs via the identified site on PkDBL (now labeled as Site 1, Fig 2a-c).

The crystal structure of Pv DBL later identified another site on DARC-binding DBL (Fig 2b, d), hereafter labeled Site 2, where based on the binding of crystallization liquor phosphate/selenate (ostensibly mimicking the sulfate of DARC's tyr 30/41), it was proposed as the key site for DARC recognition [25]. The DARC-DBL engagement was further proposed to lead to dimerization of PkDBL [25]. Indeed, the existence of this putative sulphotyrosine binding site (Site 2) was proposed based on the evidence for the bound phosphate/selenate groups at the (proposed) dimer interface of PvDBP [25]. In a latter study of PvDBL-DARC peptide complex, DARC resides were indeed found in proximity to Site 2 (Fig 2b, d) but of the DARC region that contains Tyr30 (unsulfated) and not Tyr41 [26]. Hence, the conundrum of where the binding site for the DARC peptide that contains the key Tyr41 resides on Pk/Pv DBLs had remained unsolved. It is further noteworthy that although it is feasible that the DBL engagement with DARC *in vivo* drives oligomerization of the DBLs (and speculatively of the Pk/Pv EBPs), both biochemical evidence from our laboratory and PISA calculations do not support dimerization of Pk/PvDBLs regardless of the presence of either sulfates, phosphates or selenates [11, 25, 26]. Indeed, the proposed trimeric/tetrameric oligomer states for PvDBL-DARC based on crystal structure of DARC peptide with PvDBL are also unsupported by PISA [27], a gold-standard software in macromolecular crystallography that takes into account protomer packing, stability and monomer buried surface area values into consideration for assessing oligomeric states [25,26].

A new overarching model for coupling of Pk/Pv DBLs with DARC via its sulfated tyrosines

Driven by the observation of bound phosphates/selenates in Pv DBL, we investigated the Pk DBL crystal structure and observed a bound sulfate at Site 1 (Fig 2b, c) which overlaps with earlier our proposed model [11], where the highly conserved positively charged residues were earlier mapped [11], and which were identified in two different mutagnesis screens [11, 15, 23]. The presence of bound sulfate at Site 1 in Pk DBL is a striking observation and it immediately opens the possibility of reinterpreting the engagement rules for DARC recognition by Pk/Pv DBLs. Hence, based on (a) Pk DBL-sulfate complex (Site 1, Fig. 2b, c, e), (b) PvDBL structure with bound phosphate (in Site 2, Fig. 2b, d), (c) PvDBL structure with bound DARC and tyr 30 (Site 2, Fig. 2b, d) and available mutagenesis data on DARC/DBL interactions, we propose a simple, novel, testable and fully rationalized resolution to the conundrum of DARC-DBL coupling (Fig. 2e, f). We envisage that DARC peptide 1-60 may dock on to Pk/Pv DBLs via its sulphated tyro 41 on Site 1 (Fig. 2b, e, f) and on to Site 2 via its tyr 30 (Fig 2b, e, f). Based on structural, geometrical and molecular size considerations, it is both reasonable and feasible that the DARC peptide stretches from Site 1 to Site 2 on Pk/Pv DBLs and hooks with both via its sulfated tyr41 and tyr 30 (Fig 2f.) Indeed, the side chains in Pk/Pv DBLs that constitute Site 1 are identical (4/4) between Pk and Pv DBLs, and mostly (2/3) conserved for Site 2 (Fig 2). Invariance in Site 1 residues from Pk and Pv DBL - Lys96, Lys100, Arg103 and Lys 177 (Pk DBL numbering)[11], that directly contact the bound sulphate moiety (Fig 2e), and their implication via binding data based on earlier mutagenesis experiments provide very strong support for the primacy of Site 1 in DARC-DBL binding.

Our presented model resolves the puzzle of how parasite-encoded DBL domains recognize human DARC and its two sulfated tyrosine residues. Our analysis provides new evidence for two distinct DARC binding sites on *P. knowlesi* and *P. vivax* DBLs that together likely engage the extracellular domain of DARC via its tyrosine 30 and sulfated tyrosine 41. Our analyses shall be important for both malaria vaccine and inhibitor development efforts that are targeted at abrogating DARC-DBL interactions as an avenue to prevent invasion of malaria parasites into human red blood cells.

Conclusions

Structural investigations of receptor-ligand interactions that allow ingress of malaria parasites into human erythrocytes have yielded substantial information on the residue-level engagements that drive invasion of *P. knowlesi* and *P. vivax*. Over the past decade, it has become clear that the intricacies of DARC-DBL are yet to be fully revealed, despite substantial advances. We consolidated structural and biochemical data on Pk/Pv DBL interactions with DARC via its extracellular peptide spanning residues 1-60. We specifically focused on rationalizing the potentially disparate sets of binding sites for tyrosines 30 and 41 of DARC on Pk/PvDBLs. We have resolved the possible muddle in the literature on mechanics of how parasite-encoded DBL domains recognize human DARC and its two sulfated tyrosine residues. Our analysis collates structural data emanating from PK/PV DBL Sites 1 and 2, from bound sulfate in Site 1 and from bound DARC peptide in Site 2 to provide an architectural framework that suggests twin binding for DARC's tyrosines on Pk/Pv DBLs. These analyses will allow a yet deeper dissection of

DARC-DBL interactions that are vital for invasion of many strains of *P. vivax* malaria parasites into human red blood cells.

Key learning points:

- *P. knowlesi* and *P. vivax* cause malaria in humans and these parasites exploit their encoded DBL domains to recognize and bind human DARC to invade red blood cells.
- In most human infections of *P. knowlesi* and *P. vivax*, parasite ingress may be prevented if the DBL engagement with DARC can be intercepted.
- Post-translational modification of human DARC at tyrosines 30 and 41 occurs, and of these it the DARC's sulfated Tyr 41 that confers strength to binding between DARC and *Pv/Pk* DBLs.
- A resolution to the existing conundrum of how both DARC tyrosines 30 and 41 hook with DBLs is presented here. We show that Pk/Pv DBLs possess dual binding sites where Site 1 and Site 2 link with DARC's tyrosines 41 and 30.
- Our new model rationalizes available structural and biochemical data on the intricacies of DBL-DARC interactions, and presents a novel, testable and overarching framework for understanding DBL-DARC engagement.

Key papers in the field

1. Hans D, Pattnaik P, Bhattacharyya A, et al. Mapping binding residues in the Plasmodium vivax domain that binds Duffy antigen during red cell invasion. *Mol Microbiol*. 2005;55(5):1423-1434. doi:10.1111/j.1365-2958.2005.04484.x.

2. Singh SK, Hora R, Belrhali H, Chitnis CE, Sharma A. Structural basis for Duffy recognition by the malaria parasite Duffy-binding-like domain. *Nature*. 2006;439(7077):741-744. doi:10.1038/nature04443.

3. Chitnis CE, Sharma A. Targeting the Plasmodium vivax Duffy-binding protein. *Trends Parasitol*. 2008;24(1):29-34. doi:10.1016/j.pt.2007.10.004.

4. Batchelor JD, Zahm JA, Tolia NH. Dimerization of Plasmodium vivax DBP is induced upon receptor binding and drives recognition of DARC. *Nat Struct Mol Biol*. 2011;18(8):908-914. doi:10.1038/nsmb.2088.

5. Batchelor JD, Malpede BM, Omattage NS, DeKoster GT, Henzler-Wildman KA, Tolia NH. Red Blood Cell Invasion by Plasmodium vivax: Structural Basis for DBP Engagement of DARC. *PLoS Pathog*. 2014;10(1). doi:10.1371/journal.ppat.1003869.

6. Ntumngia FB, Thomson-Luque R, Pires C V., Adams JH. The role of the human duffy antigen receptor for chemokines in malaria susceptibility: Current opinions and future treatment prospects. J Receptor Ligand Channel Res. 2016;9: 1–10. doi:10.2147/JRLCR.S99725

References.

1. de Brevern AG, Wong H, Tournamille C, Colin Y, Le Van Kim C, et al. (2005) A structural model of a seven-transmembrane helix receptor: the Duffy antigen/receptor for chemokine (DARC). Biochim Biophys Acta 1724: 288-306.

2. Rot A, von Andrian UH (2004) Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. Annu Rev Immunol 22: 891-928.

3. Miller LH, Mason SJ, Dvorak JA, McGinniss MH, Rothman IK (1975) Erythrocyte receptors for (Plasmodium knowlesi) malaria: Duffy blood group determinants. Science 189: 561-563.

4. Miller LH, Mason SJ, Clyde DF, McGinniss MH (1976) The resistance factor to Plasmodium vivax in blacks. The Duffy-blood-group genotype, FyFy. N Engl J Med 295: 302-304.

5. Chitnis CE, Sharma A (2008) Targeting the Plasmodium vivax Duffy-binding protein. Trends Parasitol 24: 29-34.

6. Choe H, Moore MJ, Owens CM, Wright PL, Vasilieva N, et al. (2005) Sulphated tyrosines mediate association of chemokines and Plasmodium vivax Duffy binding protein with the Duffy antigen/receptor for chemokines (DARC). Mol Microbiol 55: 1413-1422.

7. Cowman AF, Crabb BS (2006) Invasion of red blood cells by malaria parasites. Cell 124: 755-766.

8. Adams JH, Hudson DE, Torii M, Ward GE, Wellems TE, et al. (1990) The Duffy receptor family of Plasmodium knowlesi is located within the micronemes of invasive malaria merozoites. Cell 63: 141-153.

9. Adams JH, Sim BK, Dolan SA, Fang X, Kaslow DC, et al. (1992) A family of erythrocyte binding proteins of malaria parasites. Proc Natl Acad Sci U S A 89: 7085-7089.

10. Adams JH, Blair PL, Kaneko O, Peterson DS (2001) An expanding ebl family of Plasmodium falciparum. Trends Parasitol 17: 297-299.

11. Singh SK, Hora R, Belrhali H, Chitnis CE, Sharma A (2006) Structural basis for Duffy recognition by the malaria parasite Duffy-binding-like domain. Nature 439: 741-744.

12. Singh SK, Singh AP, Pandey S, Yazdani SS, Chitnis CE, et al. (2003) Definition of structural elements in Plasmodium vivax and P. knowlesi Duffy-binding domains necessary for erythrocyte invasion. Biochem J 374: 193-198.

13. Xainli J, Adams JH, King CL (2000) The erythrocyte binding motif of plasmodium vivax duffy binding protein is highly polymorphic and functionally conserved in isolates from Papua New Guinea. Mol Biochem Parasitol 111: 253-260.

14. VanBuskirk KM, Sevova E, Adams JH (2004) Conserved residues in the Plasmodium vivax Duffy-binding protein ligand domain are critical for erythrocyte receptor recognition. Proc Natl Acad Sci U S A 101: 15754-15759.

15. Hans D, Pattnaik P, Bhattacharyya A, Shakri AR, Yazdani SS, et al. (2005) Mapping binding residues in the Plasmodium vivax domain that binds Duffy antigen during red cell invasion. Mol Microbiol 55: 1423-1434.

16. Gill J, Chitnis CE, Sharma A (2009) Structural insights into chondroitin sulphate A binding Duffy-binding-like domains from Plasmodium falciparum: implications for intervention strategies against placental malaria. Malar J 8: 67.

17. Menard D, Barnadas C, Bouchier C, Henry-Halldin C, Gray LR, et al. (2010) Plasmodium vivax clinical malaria is commonly observed in Duffy-negative Malagasy people. Proc Natl Acad Sci U S A 107: 5967-5971.

18. Ryan JR, Stoute JA, Amon J, Dunton RF, Mtalib R, et al. (2006) Evidence for transmission of Plasmodium vivax among a duffy antigen negative population in Western Kenya. Am J Trop Med Hyg 75: 575-581.

19. King CL, Adams JH, Xianli J, Grimberg BT, McHenry AM, et al. (2011) Fy(a)/Fy(b) antigen polymorphism in human erythrocyte Duffy antigen affects susceptibility to Plasmodium vivax malaria. Proc Natl Acad Sci U S A 108: 20113-20118.

20. Cavasini CE, Mattos LC, Couto AA, Bonini-Domingos CR, Valencia SH, et al. (2007) Plasmodium vivax infection among Duffy antigen-negative individuals from the Brazilian Amazon region: an exception? Trans R Soc Trop Med Hyg 101: 1042-1044.

21. Wurtz N, Mint Lekweiry K, Bogreau H, Pradines B, Rogier C, et al. (2011) Vivax malaria in Mauritania includes infection of a Duffy-negative individual. Malar J 10: 336.

22. Ntumngia FB, Thomson-Luque R, Torres Lde M, Gunalan K, Carvalho LH, et al. (2016) A Novel Erythrocyte Binding Protein of Plasmodium vivax Suggests an Alternate Invasion Pathway into Duffy-Positive Reticulocytes. MBio 7.

23. Ntumngia FB, Thomson-Luque R, Pires C V., Adams JH. The role of the human duffy antigen receptor for chemokines in malaria susceptibility: Current opinions and future treatment prospects. J Receptor Ligand Channel Res. 2016;9: 1–10. doi:10.2147/JRLCR.S99725

24. VanBuskirk KM, Sevova E, Adams JH (2004) Conserved residues in the Plasmodium vivax Duffy-binding protein ligand domain are critical for erythrocyte receptor recognition. Proc Natl Acad Sci U S A 101: 15754-15759.

25. Batchelor JD, Zahm JA, Tolia NH (2011) Dimerization of Plasmodium vivax DBP is induced upon receptor binding and drives recognition of DARC. Nat Struct Mol Biol 18: 908-914.

26. Paing MM, Tolia NH (2014) Multimeric assembly of host-pathogen adhesion complexes involved in apicomplexan invasion. PLoS Pathog 10: e1004120.

27. Krissinel E, Henrick K (2007) Inference of macromolecular assemblies from crystalline state. J Mol Biol 372: 774-797.

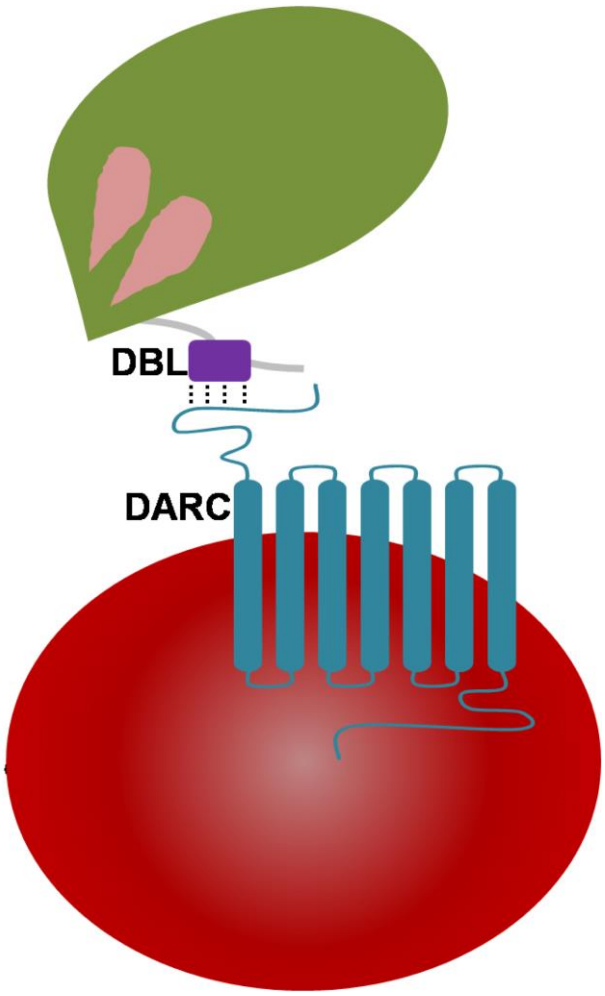


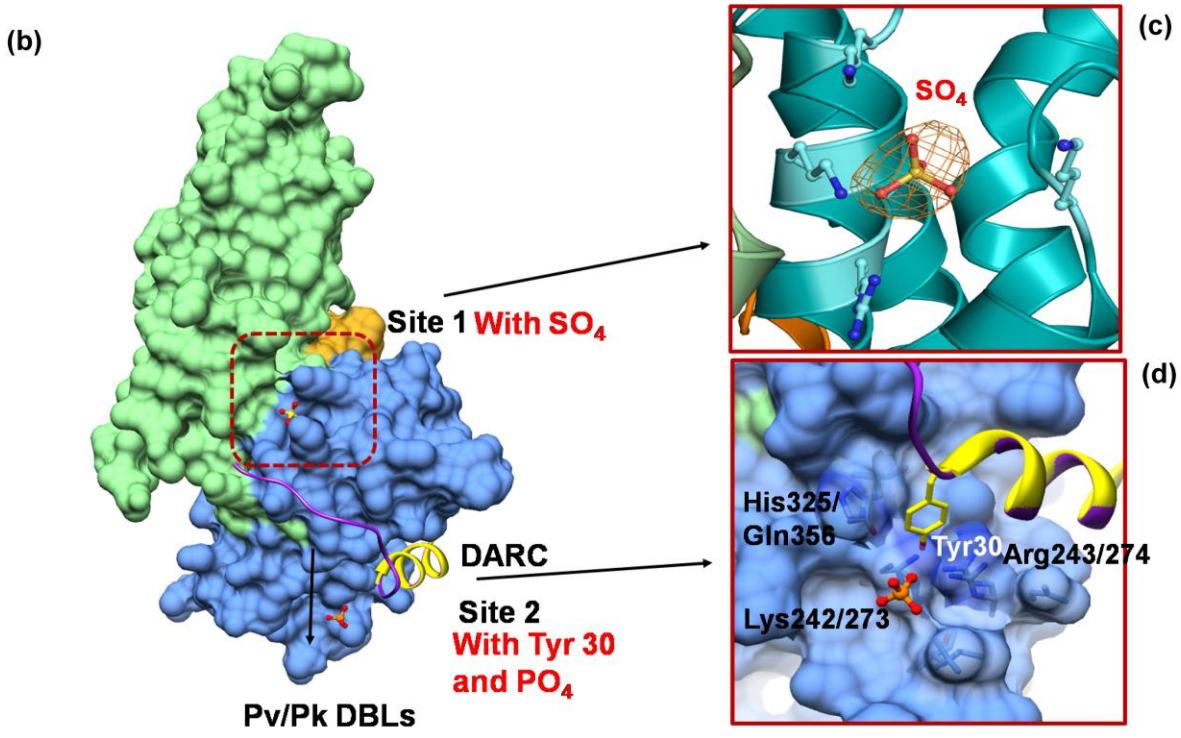
Figure 1:

The interaction of *P. knowlesi* and *P. vivax* DBL (Duffy-binding-like) domains with 7 transmembrane chemokine receptor on human Erythrocytes DARC (Duffy antigen receptor for chemokines). The DBLs are part of large parasite-encoded membrane proteins called EBPs (erythrocyte binding proteins) which contain conserved DBLs that engage with host cellular receptors. The DARC extracellular domain of residues 1-60 contains the key tyrosyl residues are positions of 30 and 41, as discussed in the text.

(a) MGNCLHRAELSPSTENSSQLDFEDVWNSSYGVNDSFPDGDYGANLEAAAPCHSCNLLDDS

30 41

DARC 1-60

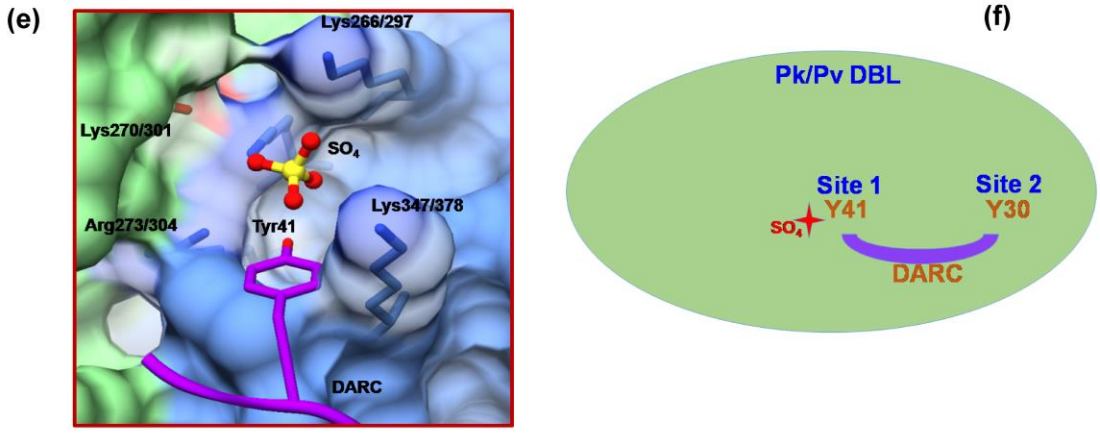


MGNCLHRAELSPSTENSSQLDFEDVWNSSYGVNDSFPDGDYGANLEAAAPCHSCNLLDDS

30 41

DARC 1-60

Site 2 Site 1



DBL binding to sulfated Y41 of DARC via Site 1

Figure 2

a) Sequence of human DARC with tyrosyl residues 30 and 41 shows in red as these are important for both chemokine DBL domain binding. The first Tyr30 is important for chemokine binding while Y41 residue sulfation is critical for strong binding of DARC to Pk/Pv DBL. **b)** DBL sub-domains 1 (orange), 2 (green) and 3 (blue) are shown in molecular surface while the N-terminal DARC peptide is shown as ribbon (yellow for residues 19-30 and purple for residues 31-43). The bound sulphate in PkDBL, phosphate in PvDBL and DARC peptide in PvDBL are shown. Site 1 was identified based on extensive mutagenesis data while site 2 was proposed based on phosphate binding and on complex with Tyr30. The yellow region of DARC is from crystal structure of PvDBL-DARC complex while the purple DARC represents modeling such that sulphated-Tyr41 is proximal to the sulfphate observed in PkDBL structure. The DARC peptide will easily reach from Tyr30 to Tyr41 as it spans DBL sites 2 and 1 respectively. **c)** The 2Fo-Fc electron density contoured at 1.5 σ level for bound SO4 in PkDBL (PDB id 5X6N). **d)** Bound PO4 and Tyr30-DARC peptide in PvDBL (PDB id 3RRC) respectively. **e)** Close view of site 1 with sulphate interacting residues which are identical between Pk/Pv DBLs. The PDB code for Pk DBL used for this analysis is 5X6N. Note that the corresponding residue numbers are Pk DBL: Lys96, Lys100, Arg103 and Lys 177. The DARC peptide in purple is modelled and shows that tyrosyl 41 that can be accommadated in ideal proximity to the sulphate observed, suggesting that sulfation of Y41 in DARC allows its engagement with the highly conserved Site 1 in DBLs from Pk/Pv. **f)** Schematic model of the overall binding interactions that likely underpin molecular surfaces of DARC and Pk/Pv DBLs. The DARC peptide engages at two sites with DARC residues Y30 and Y41, which are spaced ideally for docking on to the surface of Pk/Pv DBLs. Star, represents sulphated tyrosine.