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Immune protection-inducing protein structures (IMPIPS) against malaria: the weapons needed for beating Odysseus☆

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ABSTRACT

The review covers the functional and structural approach followed by our group for more than 34 years in the search for a methodology that allows the rational design of chemically synthesised vaccines. An analogy between Odysseus, the cunning hero of the epic poem Odyssey by Homer, and the elusive *Plasmodium* parasite has been made, to review our research group's main considerations when developing a rational methodology for designing second generation, modified peptide-based, minimal subunit, multi-antigen, multi-stage, chemically synthesised vaccines against *Plasmodium falciparum* malaria.

1. Introduction

Homer's Odyssey [1] relates the story of Odysseus, the King of Ithaca, who was the cleverest, most daring and intelligent Greek warrior who participated in the Trojan War. Known as the King of stratagems and trickery, he convinced Achilles, the King of the Myrmidons (from the Greek ants, so named for their armour and organisation in battle) to participate in the War, he designed and created the Trojan horse to open up the defences of this impenetrable city, he made the Cyclops, Polyphemus, drunk and tied his colleagues to the belly of a ram (camouflaging them) to enable them to pass as such to escape the giant's physical search after having blinded the giant's single eye, and he led Polyphemus to the cliff where the Cyclops threw himself over [1].

Microbes, particularly malarial parasites, like Odysseus, use analogous tricks and artifices to survive and escape immune pressure, such as genetic variability, blocking, inhibition, suppression, camouflage (fooling Polyphemus) [2,3], immune system dendritic cell apoptosis [4,5] (Polyphemus' suicide), gene duplications, reshuffling, translocations (Trojan horse) [6–8], interference with cell differentiation [2,9,10] and many more. However, **immunological silence** may be the subtlest of such mechanisms [11,12].

We have found that key peptides, as illustrated by *Plasmodium* protein's conserved high affinity binding peptides (cHABPs) which affix themselves to red blood cells (RBC) and hepatocytes to enable host cell invasion and infection [13–15], are immunologically silent and invisible to the immune system (blinding Polyphemus); this will be analysed in greater detail further on.

Only in-depth knowledge of these cHABPs at atomic level, aimed at modifying them (to render them immunogenic and protectogenic) will provide the weapons able to overcome such evasion mechanisms and thus allow a protection-inducing immune response to be mounted [12].

2. The malaria parasite, the Odysseus of the microbes

Human malaria, mainly that caused by the *Plasmodium falciparum* parasite, is a transmittable disease affecting around 200 million people per year and killing about 650,000 of them annually, especially children less than 5 years old living in sub-Saharan Africa [16].

The parasite's complex life-cycle, elegantly studied in depth by many groups [7,8,17–19], involves several developmental stages during which it changes its morphology and molecular expression. Inoculated by a female *Anopheles* mosquito bite, larva-like structures called sporozoites (Spz) (Fig. 1A) use gliding motility [17] (Fig. 1B) to traverse vascular endothelial, Kupffer and dendritic cells, halt in hepatocytes having high sulphate concentration in heparan sulphate proteoglycans (HSPG) [17] (Fig. 1C), and proliferate and differentiate inside infected hepatocytes. This period of susceptibility to attack by the immune system prior to

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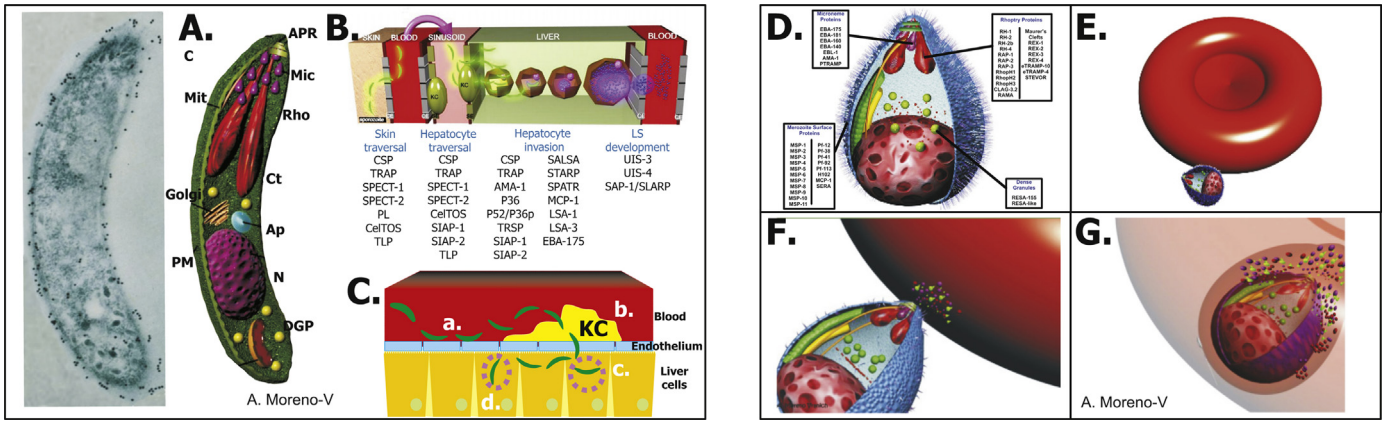

Fig. 1. (A) *Plasmodium falciparum* sporozoite structural features. Immune electron microscopy of a Spz displaying reactivity with anti-CSP antibodies (black dots on the membrane) (left) and schematic view of the Spz showing its invasion machinery and essential organelles (right). PM; plasma membrane, DGP, dense granules; Mic, micronemes; Rho, rhoptry; Ct, cytostome; APR, apical pole ring; N, nucleus; Mit, mitochondria and Ap, apicoplast. Adapted from [92,13]. (B) The sporozoite's journey inside the mammalian host (adapted from [93]). Sporozoites are injected into the skin during an infected *Anopheles* mosquito's blood meal where they make use of their gliding motility to subsequently enter the bloodstream through a capillary endothelial cell. Spz then along the fenestrated endothelia, cross the sinusoidal cell barrier by traversing a resident Kupffer cell (KC), or another hepatocyte before invading the final resident hepatocyte (seen in detail in C) [94,95]. Adapted from [93]. Massive replication and differentiation leads to erythrocyte-infectious Mrz formation; these are released into the bloodstream through merosomes [96], thereby entering the *P. falciparum* intraerythrocyte phase. The Figure also indicates some of the main parasite proteins involved in each process. (D) *P. falciparum* merozoite structural features. Schematic view of the merozoite showing its essential organelles and the proteins localised in them and involved in Mrz invasion of RBC [56]. Mrz invasion of RBCs (adapted from [15]). After an initial contact (E), the Mrz rolls over the RBC and reorientates itself toward its apical pole, releases micronemal proteins onto the RBC surface, starts to penetrate it, establishes a tight junction between the Mrz and RBC membrane and releases rhoptry proteins (F), some of which are involved in parasitophorous vacuole (PV) formation (G) [15,19,97]. Asexual replication begins after Mrz complete their invagination into the PV. Adapted from [18].

sequestration within hepatocytes, lasts from a few minutes to 1 h. Due to the small number of Spz inoculated during a mosquito bite (100–1000 per infectious bite) and this window of susceptibility, this is the most attractive stage for immune-assisted attack and therefore a preferred target for vaccine development.

Fig. 2A gives a schematic representation of the most relevant Spz proteins involved in invasion of hepatic cells. The circumsporozoite protein 1 (CSP-1), thrombospondin-related associated protein (TRAP), sporozoite threonine and asparagine rich protein (STARP), sporozoite proteins essential for cell traversal 1 and 2 (SPECT-1 and 2), cell traversal protein for ookinetes and sporozoites (CelTOS), sporozoite protein with an altered thrombospondin repeat (SPATR), multi-domain sporozoite surface protein MB-2, merozoite capping protein 1 (MCP-1), sporozoite-induced associated proteins 1 and 2 (SIAP 1 and 2) and thrombospondin-related sporozoite protein (TRSP) have been the most studied of the around 30 molecules described to date as being specific for these functions, as reviewed in [13,14].

During a single week Spz reproduce 30,000 times within hepatic cells where they are inaccessible to the immune system (camouflaging), producing molecules specific for this stage, such as liver stage antigen 1–3 (LSA 1–3), and sporozoite-associated liver stage antigen (SALSA) [13] (Fig. 2A) and changing their morphology into a piriform structure (Fig. 1D) called a merozoite (Mrz). The Mrz expresses different molecules enabling it to roll over the red blood cell (RBC) surface (Fig. 1E), reorientates towards the apical end to form a tight junction complex and releases microneme proteins (Fig. 1F) for invasion of the RBC in a receptor-ligand type interaction, a process taking about 180 s.

Each Mrz can invade a RBC to initiate its reproductive cycle; this lasts 48–72 h, produces around 30 new Mrz and causes RBC death [4,5]. So far, only an innate and/or acquired immune response or antimalarial drug treatment can stop this process. The release of detritus produced during intracellular growth (Fig. 1G), the destruction of RBC, vascular occlusion and interaction with cytokines produces symptoms and signs of the disease (fever, nausea, headache, shivering, anaemia, thrombocytopenia) and may lead to severe malaria and death [20] which, as mentioned beforehand, claims ~650,000 individuals, mainly children aged <5 years old, each year.

The molecules involved in this stage which have been most studied to date (their schematic representation is depicted in Fig. 2B) include merozoite surface proteins 1–11 (MSP-1 to -11), erythrocyte binding antigens having different molecular weight (EBA 175, 140, 181), erythrocyte binding ligand (EBL), apical merozoite antigen 1 (AMA-1), reticulocyte binding homologue proteins 1–5 (Rh 1–5), membrane bound proteins Pf 12, 38, 42, 92, 113, serine repeat antigen 5 (SERA 5), ring erythrocyte surface antigen (RESA), histidine-rich proteins I and II (HRP I and II), proteins encoded by the cytoadherence-linked asexual genes 3.2 and 9 (CLAG 3.2 and 9) and rhoptry proteins 1–5 (Rhop 1–5) [15].

When Spz and Mrz are free and have not yet invaded their respective target cells (camouflaging themselves inside the host cells, like Odysseus' colleagues in Polyphemus' cave), the plasmodia are susceptible in these few moments to attack by the immune system from the outside. The search for vaccines (weapons) targeting proteins from such stages has thus become a priority. Some examples are given in [21–26]. However, the microscopic Odysseus has many strategies in store for escaping immune pressure [2,12,27].

3. Smokescreens and genetic variability

Many soluble proteins released into the bloodstream have no recognised function regarding invasion (glutamate-rich protein (GLURP), HRP-II, etc) [28,29], whilst others, on being processed, have highly antigenic and/or immunogenic repeat amino acid sequences (tandem repeats) but no protectogenic activity since the antibodies induced by them do not impede invasion and thus infection. Many of them are thus considered **decoys or smokescreens** [30,31], this being one the main mechanisms used by the parasite for evasion. Tremendous genetic variability in unstable genetic areas (those susceptible to mutation) which enable a protein's essential structural and antigenic characteristics to change dramatically and thereby evade previously induced immune responses,

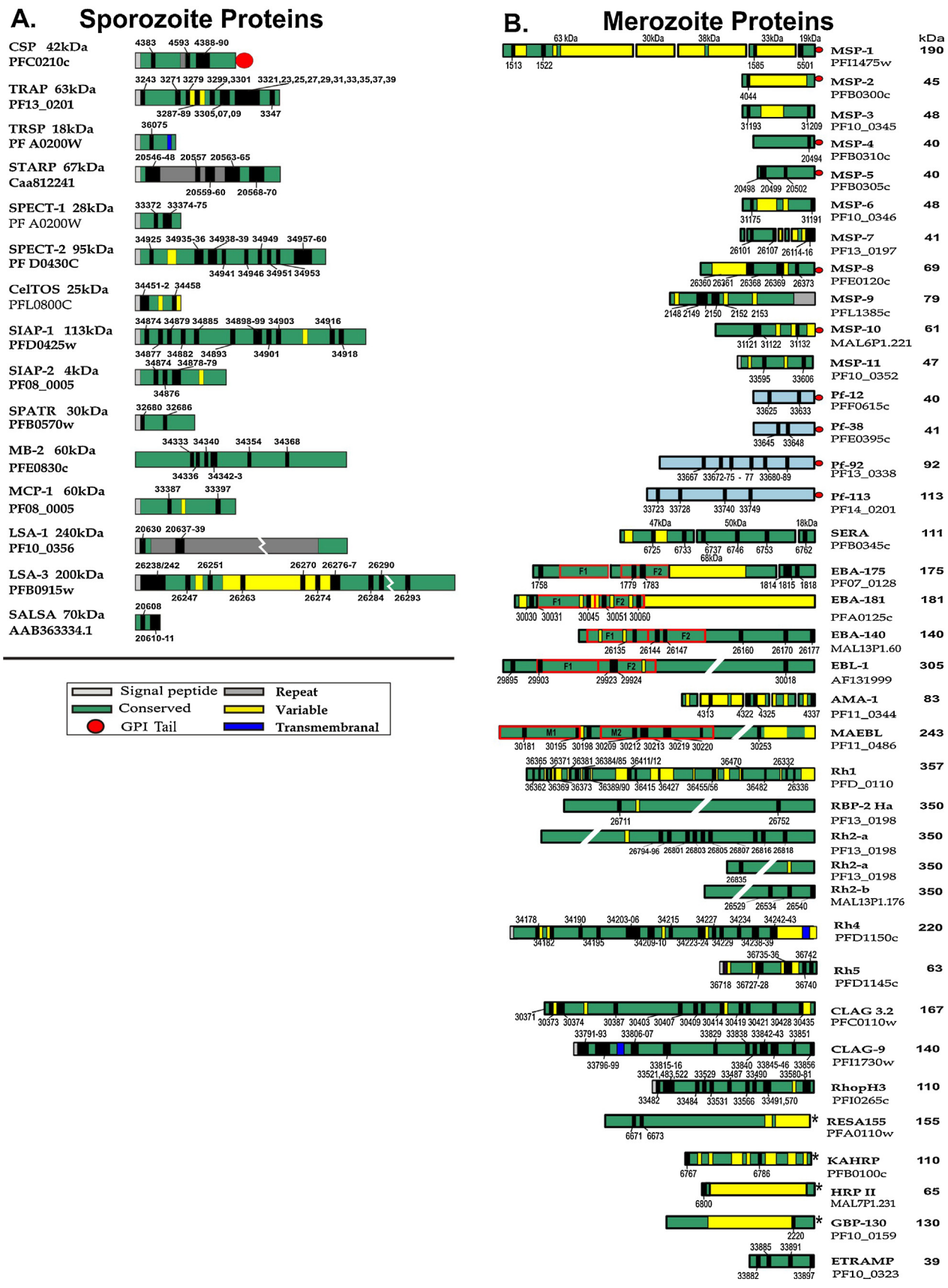

Fig. 2. *P. falciparum* Spz and Mrz proteins involved in hepatocyte and RBC invasion. (A) Schematic representation of 15 *Pf* Spz invasion-associated and cell traversal-related proteins. Adapted from [13,14]. (B) Schematic representation of 35 *Pf* Mrz proteins involved in RBC invasion (due to its length, Rh2a is displayed three times). Location of HABPs (black) regarding binding to RBCs and HepG2 cells; molecular mass and sequence accession codes are also indicated. Adapted from [12–15,98]. The native amino acid sequences for cHABPs are shown in Table 1. For A and B, the bar length corresponds to approximate molecular weights. The colour code is described in the box. GPI: glycosylphosphatidylinositol anchor.

is another mechanism frequently used by the parasite to escape immune pressure [32–36]. Based on the number of point mutations expressed, it has been calculated that *P. falciparum* has thousands of genetic variants, thereby making it very difficult to control using biologically derived vaccine strategies, including whole genetically attenuated parasites, recombinant proteins, DNA fragments, and vector-based vaccines [24,26]. Moreover, our Lilliputian Odysseus engages in yet another subtle strategy, as described below.

3.1. cHABPs are immunologically silent or invisible

Our group (HC, MAP and MEP) has developed and used a highly specific, sensitive and robust methodology throughout the last 25 years [37–39], making use of 15–20 amino acid-long, chemically synthesised, non-overlapping peptides covering the whole protein, to identify high activity binding peptides or HABPs from the most relevant functional proteins from *Plasmodium falciparum* parasite (Fig. 2 and Table 1) involved in invasion to host cells, reviewed in [13–15,38,40]. These HABPs engage in receptor–ligand interactions, as demonstrated by highly specific and strong binding capacity to host cells, invasion inhibition, susceptibility to enzyme treatment, and crosslinking with pertinent host cell membrane receptors. Many HABPs are derived from conserved protein regions, and are named conserved high activity binding peptides or cHABPs as they have no mutations or changes in the amino acid sequences in a large panel of molecules from strains from different parts of the world, as reported in the PlasmoDB database [25]. These cHABPs perform biological functions involved in *P. falciparum* invasion and infection of host cells (Fig. 2), and therefore would be ideal vaccine candidates. However, they have the property of being immunologically silent (i.e. they do not induce any type of immune response) as illustrated in Fig. 3 [41], and as will be analysed atomically and immunogenetically later on.

This **immunological silence** is different from prior work by Eli Sercarz's group on "cryptic epitopes" (from the old Greek κρυπτικός ≡ hidden; in science, dark, enigmatic or camouflaged) performed long ago, defined as conserved amino acid sequences derived from any molecule involved or not in invasion or infection of host cells (like **h**en **e**gg **l**ysozyme (HEL)), that are hidden inside, camouflaged or covered by other protein structures or even amino acids that cannot be seen by experimental models or human immune system with certain genetic background, suggesting "holes" in the immune system [42–44].

This **immunological silence** was discovered by us 25 years ago when using ELISA for analysing the reactivity of chemically synthesised 20 mer, non-overlapping, sequential peptides derived from a large panel of proteins with sera taken from individuals who had recovered from several episodes of malaria (1–3 times) caused by *P. falciparum* together with hyper-immune sera from *Aotus* infected several times (from 1 to 5) with the highly virulent *Aotus*-adapted *P. falciparum* FVO strain (making them resistant to intravenous infection with more than 500 million infected erythrocytes (iE) for this strain). It was found that all of them had developed antibodies against distinct molecules and their fragments, mainly the variable ones, but never against cHABPs (Fig. 3A, coloured in green in the MSP-2 molecule as one example, unpublished results).

This suggested that cHABPs were immunologically silent or invisible to the immune system and that protective immunity was strain-specific, since further challenge of these resistant monkeys with a different *P. falciparum* strain (FMG) induced the disease in these monkeys, even with a small dose (<10,000 iE), despite the very high antibody titres against other peptides from the same or different proteins but NOT against cHABPs from any of the analysed molecules. The seminal results by other groups from field-studies demonstrating that the immune response against malaria was strain-specific [45–50] (Fig. 3A) were thus corroborated by a different approach.

The immunological invisibility or silence (tricking Polyphemus) of cHABPs presented the problem of how to induce a strain-transcending universal immune response which would include all parasite strains.

3.2. mHABPs as breakers of immunological silence

Based on the altered peptide ligand (APL) concept postulated by Paul Allen (1989) stating that replacing some residues modified their immunological performance (increasing, reducing or even suppressing it) [51,52], cHABPs' critical residues in binding to RBC (those whose binding capacity became reduced by more than 50% when modified by glycine analogue replacement scanning methods (Fig. 5) [15,39]), our group dedicated its efforts towards identifying the universal rules for replacing cHABPs' critical binding residues to bypass the immunological silence and induce a heightened protective immune response against malaria in *Aotus* monkeys.

All amino acid physicochemical parameters were analysed (size, volume, charge, residue location in the peptide, etc) and around 40,000 (15–20 mer long) peptide analogues were synthesised, and a subset of around 4000 were tested in *Aotus* monkeys, thereby determining which amino acid should replace another for inducing **immunogenicity**, i.e. maximizing antibody responses by ELISA against native and/or modified peptides (not shown here), by immunofluorescence antibody test (IFA) against the whole parasite or by Western blot (WB) against merozoite lysate or recombinant fragments from the corresponding proteins, but more importantly, for inducing **protectivity**, i.e., inducing a protective immune response against experimental challenge with an intravenous inoculation of 100,000 freshly infected erythrocytes from the *Aotus*-adapted *P. falciparum* FVO strain [53].

This extensive and thorough chemical, biological and immunological analysis showed that the key substitutions were to replace amino acids by others having opposite polarity, such as W↔Y, F↔R, I or L↔H, M↔K, V↔Q or E, P↔D, C↔T, A↔S, which allowed preservation of critical binding residue volume, mass and surface [11,12] (Table 1). This unique, striking and straightforward strategy broke immunological silence and led to obtaining modified HABPs (mHABPs) which were highly immunogenic, as assessed by high antibody titres against the original parasite proteins by IFA, or when expressed as recombinants, or when present in Mrz lysates (as determined by WB). These mHABPs induced full protective immunity (the absolute absence of the parasite in the blood) in *Aotus* (Table 1) [53–57], and were defined as **i**mmune **p**rotection-**i**nducing **p**rotein **s**tructures (IMPIPS) [19]. Whether or not a mHABP was also an IMPIPS depended upon the stereo-chemical, and topological characteristics induced by these modifications in the peptide: some mHABPs were not immunogenic [12], others induced short-lived antibody responses but were not protective [32], others induced long-lasting antibody responses but were also not protective [33], whilst some were IMPIPS, with these various characteristics depending upon the 3D structural conformation of the peptide, to be analysed in detail further on. Therefore all IMPIPS are mHABPs but NOT all mHABPs are IMPIPS.

4. Immunogenetic analysis

The protective effect of humoral immunity against clinical malaria was described more than 50 years ago [58], with a highlight being the genetic control of such responses by major histocompatibility complex (MHC) molecules expressed by antigen-presenting cells, particularly class II molecules (MHC II, or HLA-DRβ in humans).

Table 1
Sequences for cHABPs and their immunogenic protection-inducing analogues (mHABPs) from the most relevant *P. falciparum* Mrz (left) and Spz(right) proteins involved in host cell invasion and endothelial cell adhesion (Fig.2). Critical binding residues in cHABPs and those that were changed in protective mHABPs are highlighted in bold. II and III: Antibody titres (in parenthesis) assessed by IFA 20 days after the second and third immunisations, respectively. Those monkeys displaying these antibody titres were the same ones showing strong reactivity by ELISA (not shown) and WB with schizont protein lysate or recombinant fragments containing the amino acid sequences from where the mHABPs were derived. They are the same fully protected monkeys in experimental challenge with the *Aotus*-adapted virulent *P. falciparum* FVO strain. For both tables, the prefix represents the amount of monkeys which developed these antibody titres. The big black dots indicate those native or modified HABPs for which their 3D structure was assessed by ^{1}H NMR. Only the most representative immunogenic protection-inducing mHABPs from each family are shown. For Table regarding Mrz proteins (adapted from [12]), Prot: fully protected monkeys. DR: HLA-DR β1* allele with which each HABP was found to have high binding capacity.

	PEPTIDE	SEQUENCE	II	III	Prot	DR	3D
MSP-1	1513	GYSLFQKEKMVLNEGTSGTAG	0	0	0/5	8	●
	9882	---------ET----------	0	1(160)	1/5	ND	●
	13946	---------MKT----------	2(320)	2(320)	2/12	?	●
	1522	QIPYNLKIRANELDVLKKLV	0	0	0/5	3,11	●
	9782	----------GG--GG-----	0	2(2560)	1/3	11	●
	9548	----------GG----------	0	1(1280)	1/5	11	
	1585	EVLYLKPLAGVYRSLKKQLE	0	0	0/5	?	●
	13450	-----LD----------	2(2560)	1(2560)	2/4	3	●
	10014	----HV----------	2(640)	1(640)	2/4	11	●
	11860	----HM--G----A-----	1(1280)	1(2560)	1/7	11	●
	22770	-HL--G----A-----	2(1280)	ND	2/9	ND	
	5501	MLNISQHQCVKKQCPQNS	0	0	0/5	7	
	12926	-------S----AD---	0/5	1(640)	1/5	ND	
	17944	-------T-MMMT----	0	1(160)	1/7	7	
	24148	-----ML-T-MMMT--K	1(640)	ND	1/8	7	●
MSP-2	4044	KNESKYSNTFINNAYNMSIR	0	0	0/5	?	●
	10008	----------EV----------	2(5120)	1(5120)	1/3	4	●
	13464	-IM--WG---EV----------	0	1(320)	1/6	ND	
	15502	-IM--WA---NI------N-	1(160)	1(320)	1/5	ND	
	15504	-I---WA---NI------N-	2(320)	2(320)	1/3	ND	
	22774	I---WA---EV-----VN-	4(320)	1(2560)	1/10	?	●
	24112	---------NI-----V--RSM	2(5120)	ND	2/15	4	●
	24180	--M-M---A-DI-----AN-R--	1(2560)	ND	1/9	4	●
ABRA	2150	KMNMLKENVDYIQKNQNLFK	0	0	0/5	?	
	24922	-----HL---PW-MNK------	1(320)	ND	2/7	?	
AMA-1	4313	DAEVAGTQYRLPSGKCPVFG	0	0	0/5	?	●
	10022	----------FH----S-----	0	1(5120)	1/5	7	●
	22780	----------FH----V-----	2(2560)	ND	2/10	7	●
	22782	----------WFV----V-----	1(2560)	ND	1/10	7	●
	4325	MIKSAFLPTGAFKADRYKSH	0	0	0/5	3,11	●
	13486	---AS-D-------SP------	3(2560)	2(1280)	2/5	ND	●
	15514	----A--H-------S--W----	1(160)	1(160)	1/4	7	●
	15516	----A--H-------MS--W----	1(1280)	1(160)	1/4	ND	●
	20034	----A----------M----------	2(320)	1(160)	2/8	4,7	●
	4337	WGEEKRASHTTPVLMEKPYY	0	0	0/5	?	●
	14044	YS-M----L------K------	1(160)	1(320)	1/5	7	●
	22822	HTTYS-M----L------K-----	1(1280)	ND	1/8	?	
	24926	---YSMM----L------K-----	1(320)	ND	1/7	ND	●
EBA-175	1758	KSYGTPDNIDKNMSLIHKHN	0	0	0	?	●
	13790	MA--SD--D---K--D-----	1(320)	1(320)	2/4	4	●
	24150	W---SVD---PM----------	1(1280)	ND	2/7	4	●
	1779	NIDRIYDKNLLMIKEHILAI	0	0	0 /5	11	
	9794	-GG----------G----------	0	1(320)	1/3	ND	
	22812	-N------M-H-----M------	1(2560)	ND	1/9	11	●
	23386	-NP----M-H-----M------	5(640)	ND	1/7	11	
	1783	HRNKKNDKLYRDEWWKVIKK	0	0	0/5	3, 11	●
	9928	----------Y--N----------	1(2560)	3(2560)	3/11	3	●
	9930	----------T----------	0	2(2560)	1/5	ND	
	22814	------M-Y--T---DVW	2(5120)	ND	2/10	3	●
	1815	YTNQNINISQERDLQKHGFH	0	0	0/5	?	●
	24292	L--------D--FN-M------	2(320)	ND	2/7	3	●
	1818	NNNFNNIPSRYNLYDKKLDL	0	0	0/5	?	●
	24166	----------M-P-DD	1(320)	2(640)	2/5	1,11	●
RESA-155	6671	MTDVNRYRYSNNYEAIPHIS	0	0	0/5	4,7	CD
	9948	----------EQ----------	0	1(1280)	1/4	4	●
	13492	----I----------SD---	2(5120)	0	1/6	4	●
	22720	----------V----SN--K-	0	1(5120)	1/10	4	●
HRP I	6786	KSKKHKDHDGEKKKSKKHKD	0	0	0/5	?	●
	12934	-----MI---------I--L---	0	1(160)	1/4	3	
	24224	-----M-L---MMMA-------	3(640)	ND	1/8	?	●
HRP II	6800	NNSAFNNNLCSKNAKGLNLN	0	0	0/5	?	●
	10004	--------S----M--I----	0	2(2560)	1/4	ND	
	24228	---DD--TAM--M--I--KR	1(320)	ND	1/8	7	
	24230	------DD--TAA--M--I---	1(320)	ND	2/7	7	●
SERA	6725	LKETNNAISFESNSGSLEKK	0	0	0/5	3	
	23422	----------M--A----------	1(640)	ND	1/8	11	●
	6737	YDNILVKMFKTNENNDKSELI	0	0	0/5	3	●
	22834	----H-----VI----------	1(2560)	ND	2/9	11	●
	22796	----------VI----------	1(5120)	ND	2/9	11	
	13844	-----H-----VIM---------	1(320)	ND	1/6	11	
	24096	-----H-----VI----------	1(5120)	ND	2/8	ND	
	6746	DQGNCDTSWIFASKYHLETI	0	0	0/5	3, 11	CD
	23230	----SI----R-----L-----	1(320)	0	1/9	3	●
	24214	----SI----R-A-F------	1(320)	ND	1/6	3	
	24216	----TI---NR-A-F------	5(320)	ND	2/9	11	●
	6754	KKVQNLCGDDTADHAVNIVG	0	0	0	ND	●
	23426	-------T------L-T-----	0	3(320)	1/9	4	●
	6762	NEVSERVHVYHILKHIKDGK	0	0	0/5	?	●
	22462	--T--F----------------	0	ND	1/9	ND	
	24210	-M----------M-------	1(160)	ND	2/8	ND	●
	24310	-M----------N-M-------	2(160)	ND	2/8	3	●
PfEMP-1	6505	ESAKHMFDRIGKDVYDKVKE	0	0	0	?	●
	12722	-----K----------M----	2(320)	2(320)	3/12	?	●
	23410	----K--F---I---M---R	4(1280)	ND	1/8	?	
	6583	KEWGEQFCIERLRYEQNIREA	0	0	0		●
	6584	CKRKCEKYKKYISEKKQEW	0	0	0		●
	6622	KKWWDMNKYHIWESMI	0	0	0		●

	Peptide	Sequence	II	III	DR	3D
CSP	4383	NSRSLGENDDGNNDENEKLR	0	0		●
	24258	KKISFSLGEKVIGNN	1 (640)	1 (640)	3, 7	●
	25608	----------	1 (640)	1 (640)	4	●
	4388	GNGQGHNMPNDPNRNVDENA	0	0		●
	32958a	-----L--N----F------	1 (320)	1 (1280)	1	●
	32958b	-----L--N----F------	1 (640)	1 (1280)	1	●
	4389	HNMPNDPNRNVDENANANSA	0	0		●
	13516	-------IFMS---------VKN	3 (320)	3 (640)	4	●
	13528	-------IFMS------GM--VKN	3 (640)	1 (640)	4	●
TRAP	3243	YLVNGRDVQNNIVDE	0	0		CD
	24236	DLFH-----TM-I----	1 (300)	1 (320)		nd
	24312	DLFH-----TM------	3 (320)	2 (320)	3	CD
	3277/3279	GQGINVAFNRYLVGCHPSDGKCNLY	0	0		●
	24238	-----FH--T--AP------	2 (1280)	2 (1280)	1, 4	●
	3285/3287	MKAVCVEVDKTASCGVWDEWSPYSV	0	0	3	CD
	24240	T---EM---T----NM------	1 (300)	1 (320)	3	nd
	25626	T---EM---TA--NE------	2 (1280)	2 (1280)		nd
	3289	SPCSVTCGKGTRSRK	0	0		●
	24246	--T---V---AF-F-RK	1 (1280)	1 (1280)	3	●
	3287/3289	TASCGVWDEWSPCSVTCGKGTRS	0	0		nd
	24242	-------V-TAV-M----RK	2 (1280)	2 (1280)		●
	3329	DNNGNRHVPNSEDREY	0	0		nd
	24476	SQ-V--P------A-P--T	1 (320)	1 (320)		nd
	3331/3333	SEDRETRPHGRNNENRSYNRKHNNT	0	0		nd
	10114	----------FV---	1 (320)	1 (320)		nd
	3347	YAGEPAPFDEPLGEE	0	0		●
	24254	GAATP-S---S----V-----	2 (1280)	3 (1280)	4	●
STARP	20546	VIKHNRFLSEYQSNFLGGGY	0	0	3	●
	24972	----M--HVD--AI------	nd	3 (1280)	3	●
	24320	----M--HAD--AP------	1 (320)	1 (320)	3	●
	20570	KSMINAYLDKLDLETVRKIH	0	0	3	●
	24322	-------HP----M------	1 (320)	1 (320)	1, 3	●
SIAP-1	34893	KVQQGLSYLLRRKNGTKHPVY	0	0		
	38162	-T--H--H---------V-----	1 (80)	1 (160)		
	34899	YVLNSKLLNSRSFDKFKWIQ	0	0		
	38166	----------H----------	1 (80)	1 (160)		
SIAP-2	36879	LLLYSTNSEDNLDISFGELQ	0	0		
	38156	--H----I-QP----------	1 (80)	1 (160)		
SPECT-2	34938	YTKSLSAEAKVSGSYWGIAS	0	0		
	38890	SD---A-A----K----------	1 (320)	1 (640)		
	34949	KLTPISDSFDSDDLKESYDK	0	0		
	38128	----------T---------	1 (320)	1 (320)		
	38888	----N---------KM-----	1 (320)	1 (320)		
CelTOS	34451	NVLCFRGNNGHNSSSSLYNG	0	0		
	38138	--HT---D-V-------	1 (80)	1 (80)		*
	34458	IWNYNSPDVSESEESLSDDF	0	0		
	38140	------D----------	1 (80)	1 (80)		
MB2	34312	CSRNKLNFHNIQTDNTIYKP	0	0		
	28972	T----------	1 (160)	1 (320)		

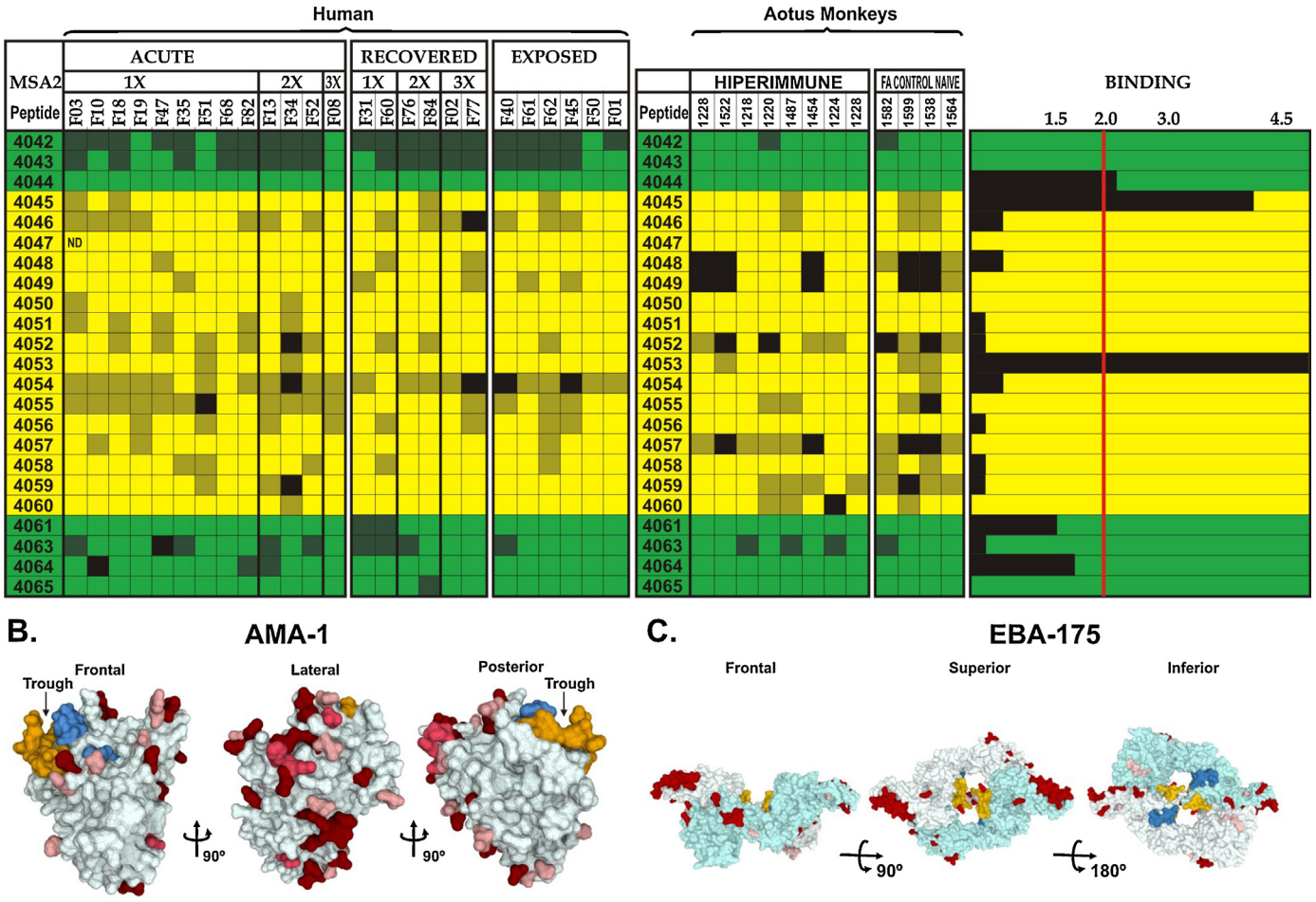

Fig. 3. (A) MSP-2 peptides antigenicity. Example of reactivity of human and *Aotus* monkeys' hyperimmune sera against PfMSP-2 peptides (left). On the right, the MSP-2 peptides binding profile to RBCs is shown. Humans were recovered from several episodes of malaria (1–3 times) caused by *P. falciparum* and *Aotus* were infected several times (from 1 to 3) with the virulent *Aotus*-adapted *P. falciparum* FVO strain. The sera (analysed by ELISA with many Spz and Mrz proteins, 20 mer long, sequential, non-overlapping peptides) recognised variable sequences (yellow), but no cHABPs (green), which allowed us to recognise the immunological silence of cHABPs. (B and C) Immune evasion mechanisms. Space filling 3D structure of AMA-1 (PDB: 1Z40) and EBA-175 (PDB: 1ZRL). Protein location of cHABPs 4313 (yellow) and 4325 (dark blue) in AMA-1 and 1783 (yellow) and 1779 (dark blue) as well as polymorphic residues localisation (in red, the darkest the most variable ones), showing their localisation in regions opposite or very distant from cHABP location [12,35,99].

Studies by one of our group (MEP) at Rockefeller University in human immunogenetics (almost 40 years ago) led to establishing an association between rheumatoid arthritis, systemic lupus erythematosus [59] and infectious diseases like chronic Lyme arthritis [60] and rheumatic fever [61] with HLA-DR complex molecules. A search for a similar association with the first chemically synthesised malaria vaccine (*SPf66*) [62,63] led to identifying a relationship between HLA-DRβ*04 genetic trait and the absence of antibody production or protection against malaria, as well as the predominance of TCRVβ10, 11 or 3 expression in these individuals [64,65].

The only methodology available at the time for determining cHABPs' experimental binding capacity to purified HLA-DRβ1* molecules was adopted, working with a limited number of these molecules (HLA-DRβ1* 0101, 0301, 0401, 0701 and 1101) which were representative of the main haplotypes (set of alleles) HLA-DR1, DR52, DR53 [66]. Most cHABPs did not bind to these molecules or did so promiscuously, binding to several of them, whilst many mHABPs adopted specific allele binding capacity (more than 50% displacement of control peptide), clearly suggesting that the modifications so made had induced structural changes regarding the specificity of binding to these molecules, making them highly specific for and having great affinity for a determined HLA-DRβ. This partly explained why these mHABPs' specific allele characteristics and their immunogenic and protection-inducing ability were revealed in just some of the immunised monkeys [11–13,53–57,67,68].

5. The *Aotus* monkey as the ideal model for anti-malarial vaccine development

This small New World primate, weighing around 800 g, is indigenous to South-America; it was discovered in Colombia by Humboldt in 1812 and identified by Carl Johnson (Rockefeller Foundation) in 1966 as a species which is highly susceptible to developing human malaria [69]. Since these monkeys can be easily infected with small doses of any human malaria infected RBCs and alternatively their infected blood can transmit the same malaria to human volunteers, one of the main Robert Koch's principles is fulfilled. It thus became the appropriate experimental model for developing drugs and vaccines against this disease [70]. Being endemic in Colombia, our Institute took this monkey as the ideal model for developing an anti-malarial vaccine and an experimental station was created in January 1980 in the Amazon Jungle, in Leticia, Amazonas, Colombia, where they have a population density of 25.9–45.0 monkeys per km^2, therefore being classified as a non-endangered species. Colombia's official environmental authority

(CorpoAmazonia) requires that monkeys received in its presence be tattooed and classified under supervision by biologist and veterinarian officials. Once experiments have been completed (immunisation and challenge), the monkeys are kept in quarantine under the oversight of an expert primatologist and then returned malaria-free to their native habitat in excellent physical condition, also under official supervision.

The seminal results following the development of *SPf66* (the first chemically synthesised vaccine, tested in large human groups in different parts of the world) [71–74] led to molecular biology (DNA sequencing) being used for characterising the genes from more than 40 of this monkey's main immune system molecules in our institute from 1990s onwards, a task undertaken by Dr. Alberto Moreno, Dr. Carlos Suarez and Dr. Manuel Alfonso Patarroyo's team, originally with the cooperation of Professor Gerd Pluschke and Dr. Claudia Daubenberger. More than 98% identity has since been found with human orthologues in some *Aotus* proteins, such as the cytokines, and other cluster of cell differentiation (CD) interacting molecules. Likewise, 84–96.5% similarity with its human counterpart has been found in other molecules having high genetic variability, such as MHCI II and α, β, γ and σ T-cell receptors [75–78], similar to the variability found amongst different human ethnic groups, supporting the relevance of using this monkey in developing vaccines for human use.

6. If you don't understand the function, analyse the structure and vice versa

Since 1996, we have identified ~300 cHABP and mHABP 3D structures, using Kurt Wütrich's 1984 methodology for determining the three dimensional (3D) structure of peptides and proteins by ^{1}H NMR [79]. These findings, combined with functional, biological and immunological studies of these molecules, have formed our approach to rational vaccine design [11,12,56]. Accordingly, "If you **don't** understand the biological and/or immunological function, analyse the structure and vice versa" has become a "workhorse" phrase in our institute. Our studies have confirmed what has been observed by circular dichroism (CD), where most cHABPs show α-helical or β-sheet, turns or random structures, whilst most mHABPs displaying IMPIPS activity have a particular polyproline type II left-handed-like ($PPII_L$) structure, a molecular conformation described in 1993 by Adzhubei and Sternberg [80]. This $PPII_L$ helix has been characterised by having an extended left-handed structure, having 3.1 Å per residue, 3 residues (2.5–4.0) 9.12 Å per pitch, the absence of intra-chain H-bonds, side-chains located perpendicularly to the peptide backbone and $-90 \pm 15^\circ$ and $+135 \pm 15^\circ$ ϕ and ψ angle rotation, respectively. A short while afterwards, Jardetzky (1996) found that this structure ($PPII_L$) had the same characteristics as the enterotoxin B peptide which fitted into the HLA-DRβ1* 0101 peptide binding region (PBR) groove [81] and, a few years later, other groups confirmed their findings [81,82]. This provided the impetus for us to begin our own ^{1}H NMR 3D studies of cHABPs and mHABPs in 1996.

Based on atomic knowledge of 3D structure (by X-ray crystallography) of HLA-DR molecules (elegantly described by structural immunologists) [81,83] and of our own peptides (determined by ^{1}H NMR), a striking finding was made: cHABP amino acids DID NOT FIT into the HLA-DRβ1* PBR groove. This was due to cHABPs' short, compact, rigid, right-handed, α-helical structure (since left-handed structures are energetically unfavourable) having 1.5 Å per residue, inter-atom H-bonds, $\phi = -60^\circ$, -90°, $\psi = -40^\circ$, -60° angle limits and 3.6 amino acids per pitch (in essence ~12 Å distance between the 9 residues the PBR can accommodate). Some other cHABPs displayed very long extended structure in the β-sheets or showed a folded β-turn structure (having 2 residues per turn and H-bonds between i + 1 and i + 2) or displayed totally unordered (random) structures, too long or not structured enough to fit into the HLA-DRβ1* PBR. Meanwhile, immune protection inducing mHABPs displayed $PPII_L$ conformations involving an appropriate 26.5 ± 3.5 Å distance (~3$PPII_L$ turns, since each turn had a 9.1 Å distance) [84] so that the most distant residues perfectly fitted in HLA-DRβ1* PBR position 1 (position is shown by p from now on) and p9, anchoring mHABPs to these class II molecules and proper N and O backbone atom orientation, as discussed later [55,56,85,86]. This data clearly showed that these mHABPs, displaying such stereochemical characteristics, could become IMPIPS since they fitted perfectly well into HLA-DRβ1* PBR to properly activate immune protective activity.

7. Charge and volume of the amino acids fitting into HLA-DRβ1 pockets

As well as the aforementioned appropriate distance for an HLA-DRβ1*-binding molecule (i.e. a mHABP), the immune response is characterised by the specificity of the stereo-electron characteristics determining such interaction. A very brief description, with pertinent examples, follows. The amino acids forming Pocket 1 of the HLA-DRβ1* PBR (**Fα24**, **Fα32**, Iα31, **Wα43**, Iβ69, Vβ85, Gβ6V, **Fβ89** and Vβ91) (Fig. 4A1 and C1), due to their hydrophobic character and to aromatic residues' resonant π bonds (in bold) making this pocket deep and hydrophobic (Fig. 4A2), cause Pocket 1 (specific pockets are referred to by P from now onwards) to display a preference for aromatic residues such as Trp, Phe and Tyr, establishing strong, resonant π–π bond interactions [83,87] and allowing a peptide to fit correctly. This pocket displays the dimorphic variant Gβ86V; taking into account that V (140.0 Å^3) is bulkier than G (60.0 Å^3), this physicochemical volumetric difference sterically impedes the fit of large aromatic amino acids like Trp and Tyr, but due to its apolarity receives large apolar residues, such as Leu, Ile, Met and Val. This variant is present in all HLA-DR* allele lineages (Fig. 4C1).

Regarding P4, where most allelic variants are present, the genetic polymorphism is most prominent in the β-chain (since the α-chain is invariable). Tremendous genetic polymorphism in the amino acid charge and volume is found, mainly in β9, β11, β13, β26, β28, β30, β70, β71, β73 and β74 (Fig. 4A1 and C2) [87–89]. Such combinations of different amino acid changes in these positions generate a huge immunological repertoire to accept the different microbial and self-epitopes. Thus, as an example, in individuals having the HLA-DRβ1* 0301 genetic trait involving amino acids Kβ71 and Rβ74 (voluminous, positively charged residues), such variants determine that this pocket be small and positively charged, only accepting small, oppositely charged (negative) amino acids, such as Asp; by the same token, individuals expressing HLA-DRβ1* 1501 (DR2) involving small apolar residues Aβ71 and Aβ74, have a spacious apolar P4, thereby allowing large apolar amino acids (Phe, Leu and Ile) to fit (Fig. 4A1 and C2) [87,88]. There are many more examples for each one of the HLA-DRβ1* allele lineages.

Similarly, regarding P6 for HLA-DRβ1* 0301 individuals, the small size of Sβ11, Sβ13 and Eβ9 forming it and the negative charge of amino acids αE11 and αD66 and the H-bond network established with Nα62 and Nα69 create a highly polar, spacious, negatively charged P6 where complementary large, positively charged amino acids such as Lys, Arg and/or His fit perfectly (Fig. 4A1 and C3) [87].

By contrast, in P6 and as a consequence of apolar amino acids Vβ11, Fβ13 and Wβ9 being voluminous in HLA-DRβ1* 0101 individuals, an extremely small and apolar niche is created in this pocket where only small, apolar amino acids such as Gly, Ala, Pro or Ser fit (characteristic for this allele) (Fig. 4B and C3).

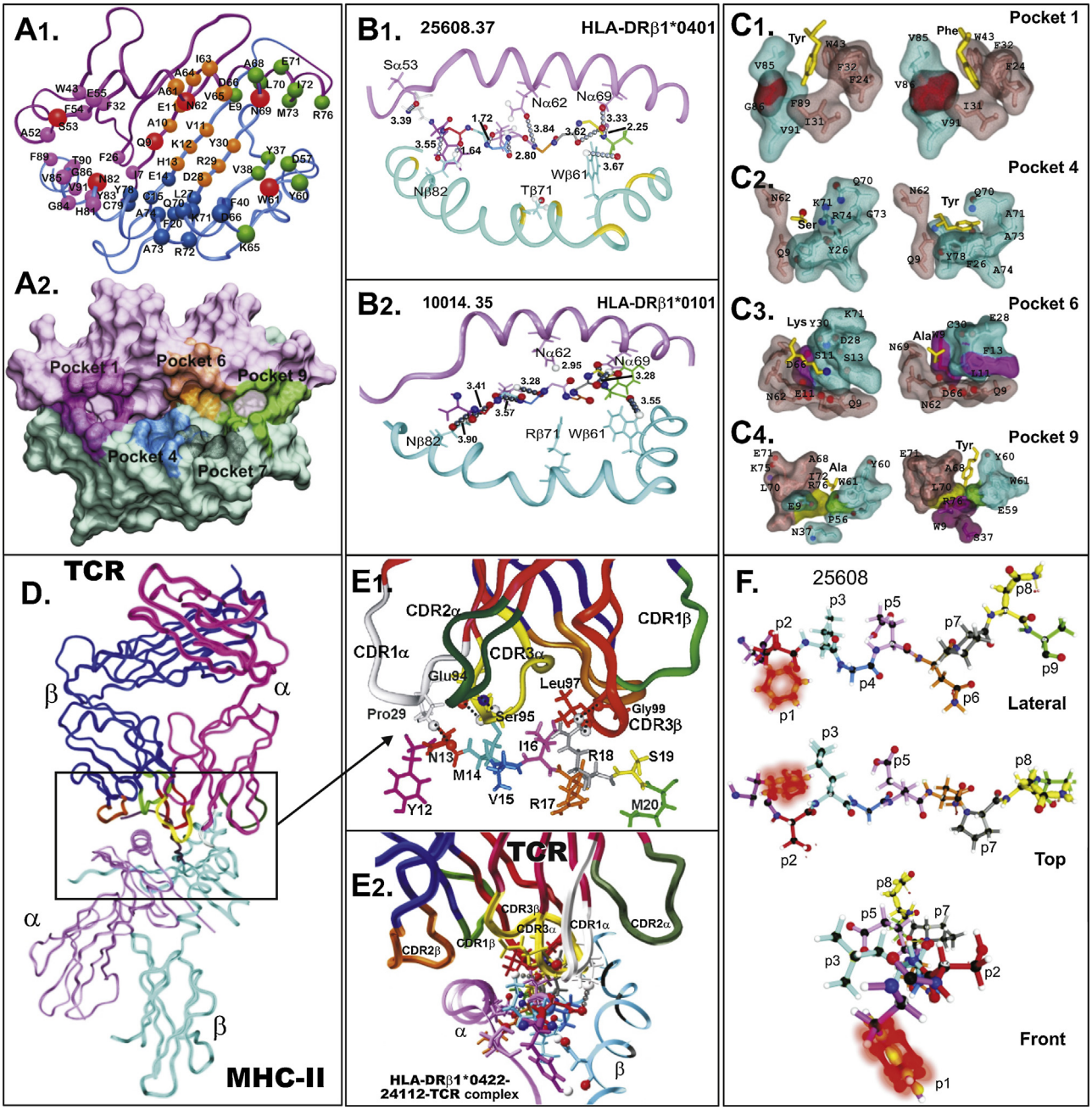

Fig. 4. Structural features of MHC-II, TCR and MHC-II/peptide/TCR interaction. (A1) Ribbon representation of the HLA-DRB1*0401 (PDB accession number 2SEB) molecule showing the α-chain in pink and the β-chain in light blue [89]. Residues forming Pocket 1 are shown in fuchsia, Pocket 4 in blue, Pocket 6 in light brown and Pocket 9 in green. Red balls show residues establishing H-bonds with peptide backbone atoms [89]. (A2) The space filling figure shows the depth of Pockets 1 (fuchsia) and 9 (green), surface localisation of Pockets 4 (blue) and 6 (light brown) on the HLA-DRβ1*0401 PBR platform [89]. (B1) Superimposition of mHABP 25608.37 (CSP HABP 4383) backbone structure (determined by ^{1}H NMR [85]) on HLA-DRβ1*0401 determined by X-ray crystallography. (B2) Superimposition of mHABP 10014.35 (MSP-1 1585 HABP) backbone structure (determined by ^{1}H NMR) on HLA-DRβ1*0101 [86]. For B1 and B2, inter-atom distances (expressed in Angstroms (Å)) between peptide backbone and HLA-DR side-chain atoms are shown as small silver balls. (C1–C4) Surface representation of HLA-DRβ1*03 (PDB accession number 1A6A) Pockets 1, 4, 6 and 9 (left) and HLA-DRβ1*15 (PDB accession number 1BX2) (right) [87,88,100]. Regarding each Pocket, it can be seen that PBR sequence variation dramatically affects its volume and therefore the size and polarity of any residue which might fit into it. The Gβ86V dimorphic difference present in all allele families is shown in C1 in red. Differences between human and *Aotus* are shown in C2–C4 in fuchsia, their peripheral location regarding peptide-binding residues led us to suggest that such substitutions do not affect peptide binding. (D) MHCII–24112 (MSP2 4044 cHABP)–TCR trimolecular complex formation associated with sterilising immunity. Docking and energy minimisation studies for this mHABP with a previously reported HLA-DRβ1*0401–HA1.7 peptide–TCR complex structure [101,102]. The HLA-DRβ1*04 and TCR Vβ CDR3 amino acid sequence variations identified in protected *Aotus* monkeys were used to determine spontaneous H-bond formation, van der Waals (vdW) interactions (small black dots), residue orientation and intermolecular distance differences between interacting atoms. (E1) H-bonds formed by mHABP24112- TCR interaction. (E2) Side view of the HLA-DRβ1*0422–24112–TCR trimolecular complex [102]. (F) Orientation and steric-electron effects for mHABP 25608.37 residues. For Phe1 hybrid sigma (σ) orbitals (yellow), π and p orbitals perpendicular to them (red, blurred balloons) are shown. Ser2 (red), Leu3 (blue), Glu5 (rose), Pro7 (grey) and Asp8 (yellow) are pointing upwards towards the TCR [90,103]. The bottom front view clearly shows these residues' orientations: p2 (Ser) towards the right-hand side, p3 (Leu) towards the left, p5 (Glu) perpendicular to the peptide backbone, p7 (Pro) towards the right and p8 (Asn) towards the left. All these stereochemical dispositions determined by their X_1 and X_2 angles' gauche^{+} orientation to allow perfect TCR diagonal orientation, were assessed experimentally by ^{1}H NMR for each one of the cHABPs and mHABPs (~300).

By the same token, the genetic dimorphism in P9 involving a salt bridge being formed between Rα76 and Dβ57 determines this pocket's size and limits; consequently, P9 in individuals having genetic variation β57D$^+$ accepts small amino acids whose polarity depends on the presence of other polar amino acids, such as Eβ9, Kβ9 or Wβ9 (Fig. 4A1 and C4), thereby receiving charged amino acids, such as Lys, Arg or His for Eβ9, or Gly, Ser or Asp for Kβ9, whilst the apolarity and large size of Wβ9 determine that the volume of an apolar amino acid fitting into P9 be small [87,88], and so on, successively for each and every HLA-DRβ1* allele variant. All these genetically determined physicochemical characteristics in P1, 4, 6 and 9 and their combinations are some of the key factors for the exquisite and huge specificity of the immune system recognition, extremely relevant for vaccine development.

8. Anchoring mHABPs to the HLA-DRβ* PBR

The distance established between the farthest atoms in P1–9 (26.5 ± 3.5 Å), together with the volume and electron charge of the residues fitting into P1, 4, 6 and 9, determine the precise location of mHABPs in the PBR, thereby enabling their stable anchoring. Such binding is mediated by H-bonds established between O and

Table 2
Conformer dihedral angle measurements for some mHABPs as determined by 1H NMR. The HLA-DRβ molecule for each mHABP is indicated (top). PPIIL, αL and β-turn regions are highlighted in grey, green and yellow, respectively. Deep purple shows the X1 and X2 rotation determining the gauche$^+$ orientation of these rotamers [85].

25608.37 HLADR *0401/0422

AA	-	~1	~2	~3	~4
K	111,6	-171,5	177,0	67,7	66,8
N	-82,5	120,0	- 74,9	-98,4	
S	-78,9	120,7	-173,8		
F	-77,7	129,0	-174,5	-95,4	
S	-82,1	114,9	-174,7		
L	-80,6	122,5	-69,9	172,8	
G	-91,8	85,1			
E	-77,9	125,5	-58,4	-63,1	-87,7
N	-78,4	128,1	-174,2	-95,6	
P	-46,5	149,4	-7,4	-21,1	
N	-61,6	119,8	80,0	-97,3	
A	61,0	44,9			
N	-81,0	131,6	-174,6	-96,1	
P	-59,3	126,5	12,7	-31,0	

32958.2 HLADR *0401/0422

AA	-	~1	~2	~3	~4
G	97,8				
N	-157,8	81,1	-172,6	-95,2	
G	-85,3	-71,6			
Q	-141,4	78,9	-70,1	-178,2	-82,6
G	-142,3	-79,3			
L	65,4	22,9	-69,0	150,1	
N	-149,1	57,7	-77,7	80,9	
M	-75,8	-3,0	-75,1	-166,5	-69,4
N	-157,0	79,1	-172,0	-93,8	
N	-79,4	133,0	-171,9	-92,0	
P	-56,0	127,9	8,9	-28,0	
P	-55,5	122,4	8,1	-27,6	
N	-158,5	79,7	-171,6	-94,2	
F	-137,1	42,1	-156,6	-105,4	
N	-166,3	83,1	-148,4	96,6	
V	-66,3	-32,1	104,1		
D	-97,5	35,2	-160,4	73,1	
E	-75,4	-3,3	66,0	-179,5	96,8
N	-159,0	81,0	-162,0	100,3	
A	-80,8	120,1			

13790.46 HLADR *0401

AA	-	~1	~2	~3	~4
M	-93,6	123,0	-70,5	-71,5	-179,2
A	-157,5	116,6			
Y	-144,8	-67,8	28,5	-90,4	
G	-69,0	-86,9			
S	82,4	135,8	-43,2		
D	-85,5	64,6	66,0	93,0	
D	-84,0	111,6	-62,5	-78,0	
N	-100,9	112,9	-173,0	1,7	
D	-101,3	97,7	-174,3	-91,5	
D	-92,8	119,2	-171,9	81,8	
K	-84,4	-10,1	85,2	-54,9	-74,1 -69,7
N	-73,3	31,8	-155,5	3,5	
K	-76,2	-69,7	-169,5	-173,1	180,0 -179,9
S	-87,7	42,9	-62,5		
L	-125,4	-55,3	-91,3	-160,9	
D	-78,9	34,2	-64,3	-33,0	
H	-141,8	159,7	-85,1	-113,9	
K	70,4	105,0	-50,2	-65,9	89,3 176,9
H	-93,7	76,7	-159,4	-73,2	
N	-127,1	-60,3	61,5	-64,9	

24112.39 HLADR *0403/0401

AA	-	~1	~2	~3	~4
S	-162,2	139,5	-171,8		
K	-128,9	30,2	72,2	-77,8	-171,9 -179,0
Y	-146,3	-65,3	-173,0	88,6	
S	39,3	65,2	64,2		
N	44,0	53,2	61,8	-95,9	
T	-61,7	-55,6	-161,4		
F	35,7	67,9	-65,8	-75,8	
N	-65,2	-83,9	-66,1	-13,2	
I	-82,4	-75,2	38,4	147,5	
N	-124,1	-62,2	-69,1	-73,8	
A	63,3	32,8			
Y	-66,2	-17,5	-170,0	-102,8	
N	-59,4	-42,2	-83,7	5,5	
M	55,7	160,6	-57,1	-175,6	-165,2
V	-93,8	116,3	-73,1		
I	-80,4	-36,4	68,1	-82,5	
R	-83,5	123,9	-166,3	178,5	-67,9 -87,4
R	-74,6	113,9	-62,3	-178,8	-73,2 106,3
S	-86,1	121,3	66,5		
M	-107,1	121,7	-85,2	-175,7	179,3

23426.35 HLADR *0403

AA	-	~1	~2	~3	~4
K	112,3	-68,4	-178,6	180,0	180,0
K	-98,8	37,5	-69,0	-164,9	-178,3 -179,7
V	-84,1	5,4	107,8		
Q	-93,0	37,5	-159,6	-176,9	82,5
N	69,3	75,4	-66,6	-92,6	
L	-117,7	55,6	-78,8	-138,0	
T	-96,7	28,4	-169,0		
G	-178,9	-75,0			
D	62,4	69,6	-172,7	-98,4	
D	63,2	66,3	-176,0	-102,0	
T	-148,7	69,3	-49,6		
A	-163,3	73,8			
D	-108,5	27,0	71,6	158,9	
L	57,0	63,1	-64,4	-87,7	
A	60,8	68,3			
T	55,9	56,9	64,8		
N	68,5	66,0	-156,5	-70,3	
I	44,6	91,2	-62,1	171,7	
V	-67,5	138,3	-126,2		
G	-179,3	-99,3			

10014.35 HLADR *0101

AA	-	~1	~2	~3	~4
E	-146,8	60,0	62,4	-175,2	93,4
V	107,1	91,3	-36,0		
L	-132,9	-74,4	64,2	-67,3	
Y	-66,3	-30,3	63,3	-87,5	
H	-149,7	88,6	-173,6	-109,1	
V	-105,1	102,5	-174,3		
P	-88,7	89,6	22,7	-11,4	
L	-108,6	16,8	65,4	121,5	
A	73,1	-0,7			
G	50,8	48,6			
V	65,1	31,2	89,9		
Y	36,5	34,2	85,6	-78,2	
R	-170,2	-37,1	-159,0	70,7	179,4 177,6
S	-64,6	-57,7	-139,6		
L	-78,0	-22,6	-69,6	170,0	
K	-70,7	-37,3	-61,0	-174,4	-175,3 -71,5
K	-68,3	-50,8	-174,4	68,8	179,6 -179,4
Q	-71,0	-74,1	-171,1	-71,6	-91,6
L	-73,1	71,3	-71,2	165,9	
E	-96,6	-72,0	-162,2	-174,9	-92,4

10022.43 HLADR 5*0102/0101

AA	-	~1	~2	~3	~4
D	-152,0	84,2	-162,0	-78,2	
A	-63,3	-52,1			
E	-40,0	-79,1	177,6	59,5	-95,5
V	76,5	-49,9	-63,0		
A	-64,7	163,1			
G	-177,4	70,8			
T	-83,5	-56,5	58,3		
Q	58,3	83,1	-174,0	56,4	79,2
Y	53,2	74,6	-162,7	81,2	
F	-140,2	131,1	-112,2	-89,3	
H	-129,1	108,3	-170,8	40,1	
P	-62,4	95,5	-24,7	33,3	
S	-90,8	115,6	-173,0		
G	-178,9	-96,4			
K	-83,7	118,0	61,5	-177,9	-178,4 -70,6
S	-137,2	99,0	-69,8		
P	-62,7	127,0	-24,8	34,8	
V	-92,4	84,1	-166,5		
F	-148,1	-78,5	-174,8	78,8	
G	170,3	101,2			

24320.18 HLADR 3*0101/0301

AA	-	~1	~2	~3	~4
V	118,0	-57,9			
I	-85,5	-79,9	-54,0	169,3	
K	61,6	41,1	-87,1	-162,1	-172,5 -179,3
H	68,2	48,9	-82,3	-58,8	
M	57,8	43,2	-76,6	-158,3	-177,4
R	62,9	29,5	-63,9	102,1	-179,5 -88,6
F	78,6	45,3	-167,2	63,7	
H	67,8	28,2	-113,0	-67,7	
A	85,5	65,4			
D	51,6	39,5	-164,9	-102,9	
Y	73,6	79,7	-166,6	67,0	
Q	-109,8	34,5	-150,9	-172,5	71,8
A	-77,6	-62,7			
P	-72,7	-12,1	19,3	-33,8	
F	-93,4	133,2	-169,6	91,1	
L	-87,8	103,7	-70,4	-178,7	
G	-92,9	100,4			
G	-179,7	-97,6			
G	-179,1	-96,2			
Y	-80,8	120,0	-67,6	-80,9	

10008.23 HLADR *12

AA	-	~1	~2	~3	~4
K	-141,0	108,9	-173,7	175,8	70,3 177,5
N	-150,7	96,2	-170,0	88,0	
E	-94,4	90,4	57,4	-179,0	-92,6
S	-103,9	81,1	59,5		
K	-109,3	107,2	-68,5	-64,7	-178,3 71,0
Y	-142,7	-58,8	-160,2	-102,2	
S	53,0	51,5	-173,7		
N	50,1	27,9	-162,6	17,8	
T	80,6	-37,0	-12,4		
F	49,9	-67,4	66,2	-86,0	
E	-115,9	86,9	-175,4	-178,9	94,5
V	-95,0	74,0	-169,3		
N	-125,4	78,8	-172,1	71,7	
A	-146,1	-62,2			
Y	-53,0	-50,6	-169,2	-98,5	
N	-67,8	-28,3	-165,9	17,8	
M	-77,9	159,9	-60,2	-77,5	106,7
S	-114,3	-76,5	-63,5		
I	-86,7	145,5	48,8	63,7	
R	-158,9	-58,0	60,1	-175,4	-70,1 172,2

24292.12 HLADR *1401

AA	-	~1	~2	~3	~4
L	-75,6	-48,5	-71,6	-168,9	
T	-80,9	-62,6	-54,0		
N	-77,4	123,2	-163,5	98,3	
Q	54,3	84,9	165,7	61,0	102,3
N	-123,7	-36,4	-170,3	-102,7	
I	-64,2	-55,7	-48,5	-62,4	
N	-62,7	-46,0	-165,7	-95,3	
I	-63,0	-33,1	55,6	82,2	
D	-67,9	-41,5	-170,7	85,3	
Q	-59,3	-46,7	-58,9	-36,2	95,0
E	-61,0	-46,7	-66,7	173,5	-91,4
F	-78,1	-75,1	-172,4	80,0	
N	-76,9	127,1	-171,4	-91,4	
L	-127,0	88,4	-162,6	-64,1	
M	-135,9	86,3	-69,7	177,5	-178,6
K	51,0	97,7	72,1	-176,4	-75,6 79,2
H	-78,2	-51,0	-71,8	-5,9	
G	91,2	96,2			
F	-75,0	126,3	-166,4	83,6	
H	-80,8	115,6	-161,2	29,6	

24246.41 HLADR *0101/0301

AA	-	~1	~2	~3	~4
S	92,5	48,9			
P	-72,3	-24,8	-10,5	23,7	
T	-93,3	14,3	-52,5		
S	53,1	96,3	-130.3		
V	-56,1	-173,8	-59,5		
T	-115,8	3,1	178,3		
V	37,0	33,8	-56,0		
G	55,6	22,5			
K	-62,1	-175,3	61,7	-155,2	-140,1 -178,2
G	62,6	-87,0			
A	54,4	44,8			
F	52,4	54,0	-116,7	93,2	
S	-150,6	-50,1	78,8		
F	-69,4	67,6	-130,5	-77,4	
K	-180,0	-25,2	52,7	139,8	-97,3 -177,5
A	-51,6	177,0	89,9	-57,4	-145,3 -102,6
E	-171,8		56,9	159,3	-39,4

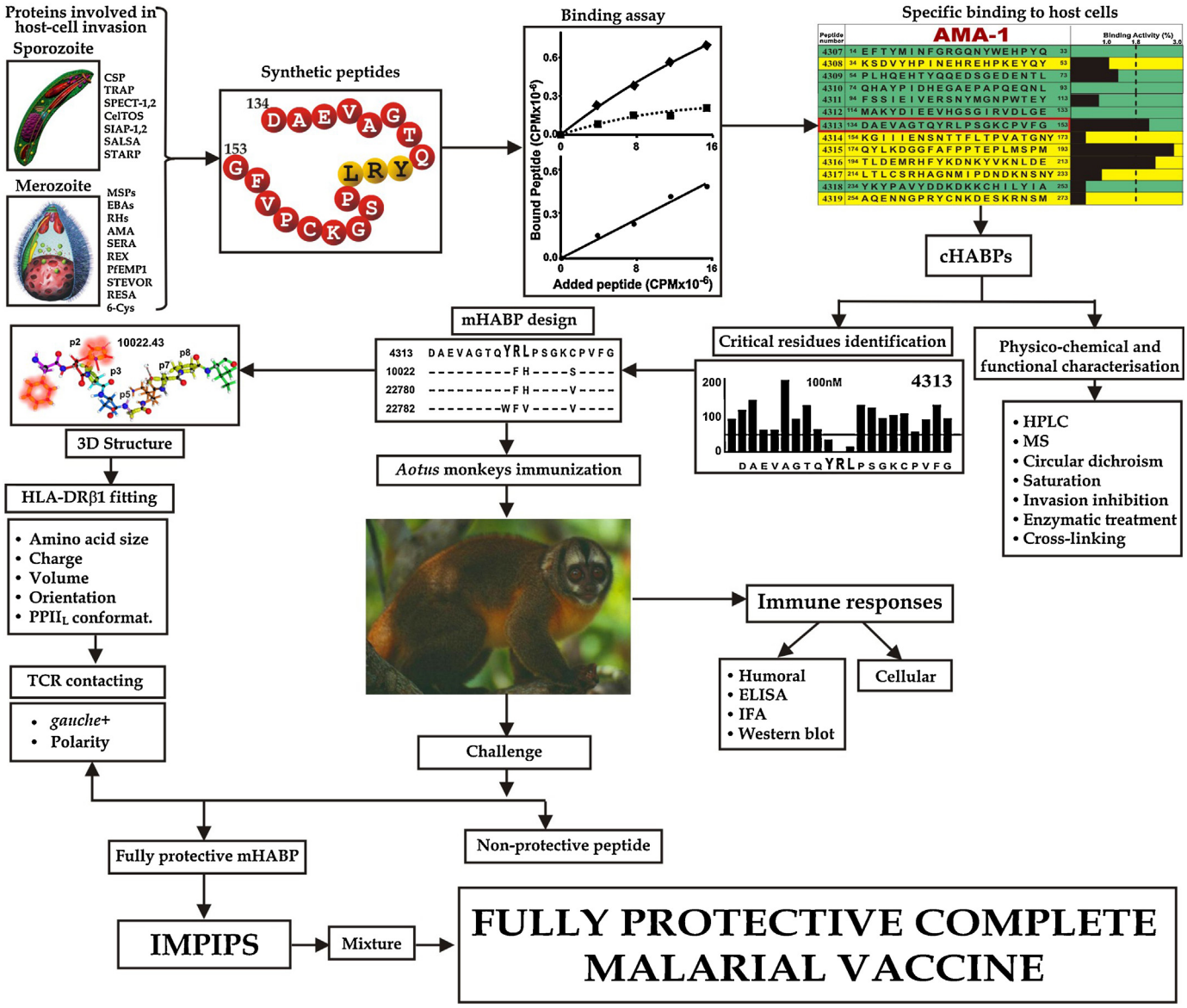

Fig. 5. Flow-chart for developing a fully protective complete malarial vaccine. The elegant work by many other groups has identified the Spz and Mrz derived proteins relevant in *P. falciparum* invasion and infection to host cells. Based on this information chemically synthesised peptides follow the path shown in this chart to obtain a fully protective complete minimal subunit based multistage multi-epitope chemically synthesised malarial vaccine.

N free electron pairs from mHABP backbone atoms with oppositely charged free electrons located in HLA-DRβ1 amino acid side-chain atoms (Qα9, Nα62, Nα69 and Nβ82). This forms 9–11 atom ring structures having double H-bond and single H-bond (Sα53, Kβ71, Wβ6) binding, establishing 9–13 H-bonds strongly anchoring mHABPs to the floor of the PBR thereby stabilising its presence for ~120 h, enabling presentation to the T-cell receptor (TCR). This stable and long-lasting HLA-DRβ1*-mHABP complex is a key factor in antigen presentation to the TCR and the induction of an appropriate immune response [12,56,83,85,86,90,91].

9. Side-chain orientation for making contact with the TCR

After binding and stabilising HLA-DRβ or MHC II molecules in the PBR, an appropriate orientation must be ensured for the side-chains of solvent-exposed or upwardly orientated amino acids to make contact with the TCR (Fig. 4D and E). After many very frustrating monkey trials, when these individually highly immunogenic and protection inducing mHABPs were mixed yielding completely negative results, we found that the rotamer orientation of TCR-contacting residues was the critical factor for generating protective, long-lasting immunity in response to a multi-epitope vaccine [12,57,91]. This meant that the rotamer orientation and charge of side chains from TCR contacting residues had to be taken into account, such that a residue located in p2 must be polar and orientated towards the right, a residue located in p3 apolar towards the left, a residue located in p5 polar and perpendicular to the axis of the mHABP backbone, a residue located in p7 apolar and towards the left and, for p8, towards the right. This is also associated with p2, p3, p5, p7 and p8 side-chain X_1, X_2 and X_3 angle orientation, specific rotamer dispositions being adopted where X_1 angles in p3 and p7 must adopt a gauche$^+$ position, the same as most X_2 angles in p5, for inducing sterilising protective immunity [90] (Fig. 4F and Table 2A). This enabled the mixing of several IMPIPS, a critical step in the pursuit of a highly protective subunit mixture malaria vaccine.

The foregoing analysis shows that successful subunit vaccine design, particularly regarding malaria caused by *P. falciparum*, relies

upon adherence to strict stereo-electron and physical-chemical rules for peptide configuration that determine biological function, immunogenetic specificities and immunological outcome (Fig. 5). We believe that this is a fundamental discovery, and that it will allow the circumvention of one of the most devious adaptations of the malaria parasite for evading host immunity. Our microscopic Odysseus likely still has many as-yet-unknown stratagems but, hopefully, in a not too distant future, we can understand them all and defeat him.

10. Conclusions and perspective:

A large body of immunological, functional and structural evidence has demonstrated that conserved high activity binding peptides, or cHABPs, which are directly involved in biological functions mediating invasion or infection of host cells, are immunologically silent structures (Aza J. and Patarroyo ME., manuscript in preparation). On the other hand, variable amino acid sequences in the same molecules are highly immunogenic, but NOT protection-inducing, partly due to their tremendous genetic variability and the strain specificity of the immune response they elicit, and partly due to their stereochemical and topological location in such invasion-relevant molecules. These highly polymorphic regions are structurally far from the cHABPs, as originally suggested by Sing et al. [41] (Fig. 3A and C gives examples) and recently demonstrated by us in several malarial proteins for which the 3D structure has been determined. To render cHABPs into highly immunogenic and/or protection inducing immunogens (IMPIPS), with a goal to achieve strain-transcending immunity, specific modification of certain residues need to be made; if done properly, this allows them to fit perfectly into the HLA-DRβ1*-mHABP-TCR complex.

We have described the structural, topological and stereo-electronic rules or principles that enable the transformation of host cell invasion functionally relevant cHABPs into potent IMPIPS. These include specifications for residue charge and volume (analysed above), a 26.5 ± 3.5 Å distance between the farthest IMPIPS residues fitting into HLA-DRβ1* molecule PBR P1–P9, and specific peptide bond ϕ and ψ angle plane rotations to form or contain $PPII_L$ structures to ensure appropriate peptide backbone and O and N atom orientation. These rules allow for appropriate anchoring to HLA-DRβ1*molecules. There must also be specific topological orientation of residues pointing away from the PBR, such as p2 towards the right-hand side, p3 perpendicular or toward the left, p5 perpendicular or lightly tilted towards the left, p7 above but not clearly defined and p8 towards the left-hand side. Furthermore, IMPIPS demand a specific electron landscape, involving non-bonding electron pairs with p orbitals in p2, p5 and p8, while p3 and p7 are dominated by residues having σ orbitals. This enables strong, specific interaction for ~120 h with the TCR to form an appropriate HLA-DRβ1*-IMPIPS-TCR complex. If these principles are not adhered to, inappropriate amino acid orientation will lead to suppression, blocking or impeding the appropriate fit of the mHABP, resulting in an unstable complex and therefore a muted non-protective immune response.

These rules or principles provide a rational methodology (Fig. 5) to design highly immunogenic, fully protective, multi-epitope, multistage, minimal subunit-based, chemically synthesised peptides for malaria vaccines, here named IMPIPS. Our findings give strong support to our **functional–structural approach**, and open the gate for developing chemically synthesised vaccines in other areas, with malaria representing just one of many infectious agents where these principles could be applied.

The appropriate mixture of IMPIPS therefore should circumvent the interference, blocking or suppression barriers erected by the malaria parasite. We propose that if an IMPIPS mixture covered the full repertoire of HLA-DRβ1* genetic variants, it could achieve the long sought-after dream of a fully protective malaria vaccine.

11. Colophon

The Odyssey narrates Odysseus' adventures up to the point of his return to Ithaca, but Homer does not tell us what happened afterwards; one could hope that, like the tale of such an astute and cunning Greek warrior, the history of malaria becomes a story of the past when the rules and principles outlined above for controlling it have been adopted.

Conflict of interest statement

The authors have declared that no conflicts of interest exist.

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