Vaccine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Novel adenovirus encoded virus-like particles displaying the placental malaria associated VAR2CSA antigen

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ARTICLE INFO

Article history:
Received 13 September 2016
Received in revised form 4 January 2017
Accepted 9 January 2017
Available online xxxx

Keywords:
Placental malaria
Adenoviral vectors
Prime-boost regimens
Virus-like particles
VAR2CSA

ABSTRACT

The malaria parasite *Plasmodium falciparum* presents antigens on the infected erythrocyte surface that bind human receptors expressed on the vascular endothelium. The VAR2CSA mediated binding to a distinct chondroitin sulphate A (CSA) is a crucial step in the pathophysiology of placental malaria and the CSA binding region of VAR2CSA has been identified as a promising vaccine target against placental malaria.

Here we designed adenovirus encoded virus-like particles (VLP) by co-encoding Simian Immunodeficiency Virus (SIV) *gag* and VAR2CSA. The VAR2CSA antigen was fused to the transmembrane (TM) and cytoplasmic tail (CT) domains of either the envelope protein of mouse mammary tumour virus (MMTV) or the hemagglutinin (HA) of influenza A. For a non-VLP incorporation control, a third design was made where VAR2CSA was expressed without TM-CT domains.

In the primary immunogenicity study in Balb/c mice, VAR2CSA fused to HA TM-CT was significantly superior in inducing ID1-ID2a specific antibodies after the first immunization. A sequential study was performed to include a comparison to the soluble VAR2CSA protein vaccine, which has entered a phase I clinical trial (NCT02647489). The results revealed the induction of higher antibody responses and increased inhibition of parasite binding to CSA using either VAR2CSA HA TM-CT or VAR2CSA MMTV TM-CT as priming vaccines for protein double-boost immunizations, compared to protein prime-double boost regimen. Analysis of pooled serum samples on peptide arrays revealed a unique targeting of several epitopes in mice that had been primed with VAR2CSA HA TM-CT. Consequently, modification of VLP anchors is an important point of optimization in virus-encoded retroviral VLP-based vaccines, and adenovirus VLPs boosted by recombinant proteins offer hope of increasing the levels of protective VAR2CSA specific antibodies.

1. Introduction

Malaria is a widely spread infectious disease and Europe is the only WHO region in the world where indigenous malaria has been eradicated [1]. The estimated number of malaria infections amounted to 214 million in 2015, of which 438,000 infections had a deadly outcome [2]. The two most vulnerable groups are women and children, who suffer especially from malaria caused mortality. Placental malaria (PM), is a particular type of malaria often occurring in primigravidae women and is primarily caused by infection with the malaria parasite *Plasmodium falciparum* (*P. falciparum*) [3]. *P. falciparum* infection in pregnant women does not only put these women at higher risk of maternal mortality, but it also greatly increases the risk of infant and newborn mortality and low birth weight [4,5]. Encouragingly, it has been observed that women develop antibodies during their first pregnancy that interfere with parasite sequestration, leaving them less prone to PM during subsequent pregnancies [6]. This suggests that a vaccine that would induce antibodies with similar functions could protect pregnant women from PM.

During its life cycle in the human host, *P. falciparum* can partly avoid being discovered and targeted by the immune system by residing inside erythrocytes. Here, the parasites express the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) proteins encoded by 60 different *var* genes, leading to the

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http://dx.doi.org/10.1016/j.vaccine.2017.01.016
0264-410X/

sequestration of the infected erythrocytes (IE) in the vasculature [7]. To evade immune recognition by antibodies, the *var* genes have evolved a very high sequence variation [8]. In PM, the *P. falciparum* parasite expresses primarily one PfEMP1 protein, VAR2CSA, to sequester the IE to the placenta where it binds to chondroitin sulfate A (CSA) [9]. The recent identification of the minimal binding region of VAR2CSA to CSA, the DBL2X domain with the flanking regions ID1 and ID2a, was a finding that encouraged the pursuit of developing a vaccine against PM [10,11].

A vaccine to protect women against malaria during pregnancy would likely be administered before the women got pregnant, hence it is particular important that such a vaccine induces high titered and long lasting responses. Ideally the vaccine would be given together with the paediatric immunization program or any future Human Papilloma Virus (HPV) mass immunization programs.

Both protein and DNA vaccines have been generated to investigate the immunogenicity of VAR2CSA domains and the functionality of the induced antibodies. Protein immunizations based on several domains have shown high functionality in inhibiting the binding of homologous IE to CSA [10,12,13]. Furthermore, cross-reactive antibodies have been induced using DNA immunizations encoding the N-terminal sequence up to the DBL2X domain [14] and protein immunization with the combined ID1-DBL2X-ID2a domains [15]. Recently, novel vaccine designs were successfully attempted using pre-assembled virus-like particles (VLPs) and subsequent coupling of the antigens by monovalent streptavidin or isopeptide bonding proteins [16–18]. Analysis of homologous parasite inhibition in serum from VLP immunized animals revealed highly functional antibodies induced by the VLP based immunogen without any adjuvant. Using VLPs as a means to optimise immune responses is not a novel concept. VLPs have been used to vaccinate against VLP forming pathogens themselves such as the licensed HPV [19] and Hepatitis B Virus [20] vaccines. In addition, the property of some pathogens to naturally form VLPs has also made them useful as carriers of other antigens of interest [21,22]. The morphology and size of VLPs make them highly immunogenic as the innate immune system is optimized to capture and display virus particles. Unfortunately, such VLPs have shown to be difficult to produce, and enveloped viral particles typically loose potency and stability in the cold chain [23]. Additionally, recombinant systems for large scale productions face difficulties in yield, formation of naturally appearing particles, and contaminants [24]. Such problems are an important drawback of the enveloped VLPs that are otherwise easily genetically pseudotyped with heterologous proteins. These difficulties can in principle be entirely circumvented by encoding enveloped based VLPs within a viral vector.

Here, we evaluated the humoral responses induced by adenovirus encoded VLPs using genetic components based on Simian Immunodeficiency Virus (SIV) *gag* [25] linked to the ID1-DBL2X-ID2a domains (ID1-ID2a) of VAR2CSA. In two studies we compared the antibody responses induced by ID1-ID2a fused to two different membrane anchors, the transmembrane and cytoplasmic tail (TM-CT) of either the envelope protein of mouse mammary tumour virus (MMTV) or hemagglutinin (HA) of influenza A virus [26], or unfused and thus secreted (S). While adenovirus encoding ID1-ID2a fused to either HA TM-CT or MMTV TM-CT showed superiority as priming vaccines in a ID1-ID2a protein double-boost regimen, evidenced by both increased antibody responses and functional antibody induction, the protein double-boost regimen revealed that ID1-ID2a HA TM-CT priming induced broader antibody responses as shown in peptide arrays. These results show that VLP incorporation of a PM associated antigen can have an important influence on the priming of humoral immune responses.

2. Materials and methods

2.1. Ethical statements

Female Balb/c mice at the age of 6–8 weeks were purchased from Taconic M&B (Ry, Denmark), and housed at the Panum Institute, Copenhagen, Denmark. All experiments were initiated after allowing the mice to acclimatize for 1 week. Experiments were approved by the national animal experiments inspectorate (Dyreforsøgstilsynet) and performed according to national guidelines.

Anonymous donors had provided their consent to the use of their donated blood for culturing of parasites.

2.2. Design and production of adenoviral vaccines

Replication-deficient E1 deleted human adenovirus type 5 (huAd5) [27] vectors were used in these studies. Formation of retroviral VLPs requires the Gag protein, which independently can form VLPs, and a membrane anchor for the antigen that is to be sorted into the secretory pathway. To deliver these components in a single expression cassette we engineered the adenoviral vectors to express SIVmac239 Gag and the ID1-ID2a domains of VAR2CSA (from the FCR3 isolate of *P. falciparum* (GenBank: GU249598)) (defined in [15]), linked to Gag via a Glycine/Serine/Glycine (GSG) linker followed by a self-cleaving porcine teschovirus-1 2A peptide (P2A) [28]. The ID1-ID2a domains were N-terminally linked to the gaussia luciferase signal peptide [29]. Three different vectors were designed: where (1) the C-terminal of ID1-ID2a was fused to the transmembrane (TM) and cytoplasmic tail (CT) regions of either the envelope protein of mouse mammary tumour virus (MMTV), (2) the C-terminal of ID1-ID2a was fused to the TM-CT of the hemagglutinin (HA) protein of influenza A virus, (3) where no fusion was performed at the C-terminal. HEK293 cells expressing the tetracycline operator (T-REx™-293 cell line) (Thermo Fischer) [30] were used for vaccine production.

The huAd5 vectors were produced by homologous recombination in BJ5183 cells [31]. The inserted antigens were flanked by the human CMV promoter (huCMV) and a simian virus 40 (SV40) polyadenylation signal. The vectors encoded tetracycline operator (TetO) [30] sequences downstream of the huCMV. Once the adenoviral genomes had been amplified, the adenoviral particles were purified using a caesium chloride gradient as described elsewhere [32].

2.3. Protein production

The boundaries of domains and sub-domains of VAR2CSA were previously defined by performing both structural and DNA sequence analysis followed by screening of truncated recombinant proteins [8,33]. The generation of recombinant Baculovirus encoding the VAR2CSA fragments, the expression of recombinant proteins from Baculovirus infected High-Five insect cells, and purification of recombinant proteins, has been described elsewhere [16].

2.4. Immunizations

In the first study mice were immunized intramuscularly (i.m.) (5 mice per group) with 1×10^{10} virus particles (VP) in a total volume of 50 µl PBS of either vaccine (day 0). At week 17, mice were boosted with the same vaccine as they had been primed with. Serum samples were harvested at week 4, 7, 11, 16, 21 and 25.

In the second study, groups of 10 mice were primed with either vaccine using 6×10^9 VP in a total volume of 50 µl PBS (day 0). At week 8, groups of 5 mice were either boosted with the same

Please cite this article in press as: Andersson A-MC et al. Novel adenovirus encoded virus-like particles displaying the placental malaria associated VAR2CSA antigen. Vaccine (2017), http://dx.doi.org/10.1016/j.vaccine.2017.01.016