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Evaluation of a novel multi-immunogen vaccine strategy for targeting 4E10/10E8 neutralizing epitopes on HIV-1 gp41 membrane proximal external region

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ABSTRACT

The membrane proximal external region (MPER) of HIV-1 gp41 is targeted by broadly neutralizing antibodies (bnAbs) 4E10 and 10E8. In this proof-of-concept study, we evaluated a novel multi-immunogen vaccine strategy referred to as Incremental, Phased Antigenic Stimulation for Rapid Antibody Maturation (IPAS-RAM) to induce 4E10/10E8-like bnAbs. Rabbits were immunized sequentially, but in a phased manner, with three immunogens that are progressively more native (gp41-28×3, gp41-54CT, and rVV-gp160$_{DH12}$). Although nAbs were not induced, epitope-mapping analyses indicated that IPAS-RAM vaccination was better able to target antibodies towards the 4E10/10E8 epitopes than homologous prime-boost immunization using gp41-28×3 alone. MPER-specific rabbit monoclonal antibodies were generated, including 9F6. Although it lacked neutralizing activity, the target epitope profile of 9F6 closely resembled those of 4E10 and 10E8 (^{671}NWFDITNWLWYIK^{683}). B-cell repertoire analyses suggested the importance of co-immunizations for maturation of 9F6, which warrants further evaluation of our IPAS-RAM vaccine strategy using an improved priming immunogen.

1. Introduction

To date, dozens of human monoclonal antibodies (mAbs) have been isolated from virus-infected patients that can neutralize a large number of HIV-1 variants from multiple clades (Huang et al., 2014, 2012; Pejchal et al., 2011; Scheid et al., 2011; Walker et al., 2011, 2009; Wu et al., 2010; Zwick et al., 2001). These broadly neutralizing antibodies (bnAbs) target a few select conserved sites of vulnerability on viral envelope glycoproteins gp120 and gp41 (for reviews, see Georgiev et al. (2013), Haynes et al. (2014), Kwong et al. (2013), Mascola and Haynes (2013), Mascola and Montefiori (2010), McCoy and Weiss (2013) and van Gils and Sanders (2013)). One of these targets is the membrane proximal external region (MPER), a highly conserved domain of ~22 amino acid residues situated at the C-terminal end of the gp41 ectodomain. The MPER is thought to play a critical role during the fusion between viral and cellular membranes (Muñoz-Barroso et al., 1999; Salzwedel et al., 1999). It is targeted by bnAbs 2F5, Z13e1, 4E10 and 10E8 (Huang et al., 2012; Purtscher et al., 1994; Stiegler et al., 2001; Zwick et al., 2001). 4E10 and 10E8 are particularly notable as they have been shown to neutralize ~98% of the HIV-1 isolates tested (Huang et al., 2012). 4E10 and 10E8 epitopes lie within the C-terminal 13 residues of the MPER (^{671}NWFDITNWLWYIK^{683}) and their crystal structures have been determined (Cardoso et al., 2007; Huang et al., 2012).

Despite having short, linear, simple alpha-helical epitopes, efforts to develop a vaccine that can induce 4E10/10E8-like bnAbs have been unsuccessful (see Banerjee et al. (2016) and Habte et al. (2015) and references therein). Since all of the immunogens evaluated could bind 4E10/10E8, the failure to induce similar bnAbs is not because antigens could not assume the correct epitope structures. Rather, it is likely due to the difficulty in inducing high levels of MPER-directed antibodies that can bind the neutralizing epitopes in the context of a whole trimeric gp120/gp41 complex. Unfortunately, this problem cannot be remedied simply by using a trimeric envelope complex because the MPER is immunorecessive compared to other epitopes that elicit type-specific or non-neutralizing antibodies. An additional challenge in

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inducing MPER neutralizing antibodies is that the structure of the gp41 subunit is highly dynamic as it undergoes significant structural changes to mediate fusion between viral and cellular membranes (Melikyan, 2008). The conformation, orientation and accessibility of the MPER neutralizing epitopes likely vary significantly at different stages of the fusion process, about which little is known at the present time. Further, the orientation of the MPER relative to the membrane surface or to the rest of the native gp120/gp41 trimeric complex is unknown. These factors make it difficult to design small, sub-domain immunogens.

Taken together, the major challenge in developing an MPER-based vaccine is designing immunogens and/or developing vaccine strategies that both force the immune system to focus antibody responses towards the MPER and also guide antibody evolution so that mature antibodies bind neutralizing epitopes on trimeric envelope spikes on the virus particles. Considering that antibody maturation will have to occur during a relatively short timeframe, we postulated that inducing bnAbs against the MPER would be impossible to accomplish with a single immunogen using typical vaccine approaches. To address this problem, we devised a novel vaccine strategy, referred to as Incremental, Phased Antigenic Stimulation for Rapid Antibody Maturation, or IPAS-RAM. The basic concept is to prime the immune system using a small MPER-derived peptide to stimulate a broad spectrum of antibodies against the MPER, then selectively amplify those that bind the native structure by boosting with progressively more native immunogens. Although a number of studies recently reported sequential immunization with different immunogens, they used only a single immunogen during each immunization (Briney et al., 2016; Escolano et al., 2016; Haynes et al., 2012; Sok et al., 2016; Tian et al., 2016). What makes our IPAS-RAM strategy unique is that the immune system is exposed to different, but related, immunogens simultaneously in a phased manner, such that B cells stimulated by a smaller immunogen can concurrently engage common epitopes on a larger, more native immunogen. By repeating this process using incrementally more native antigens, we hypothesized that the immune system can better target MPER neutralizing epitopes and that antibodies could undergo the maturation process more efficiently.

In this proof-of-concept study, we evaluated the IPAS-RAM vaccine strategy using three immunogens in rabbits: An MPER-based polypeptide, a membrane-bound gp41 mini-protein, and a full-length gp160. We hypothesized that a peptide-based priming antigen would be highly effective in eliciting antibodies against the MPER, and that boosting with progressively larger and more “native” antigens would enable select antibodies to mature into bnAbs capable of binding gp41 as it appears in the native trimer. Although we did not succeed in eliciting neutralizing antibodies, results of our study demonstrate proof-of-principle for the IPAS-RAM vaccine strategy and identify ways to improve it.

2. Results

2.1. Immunogens

To focus antibody responses towards the MPER, we generated an immunogen designated gp41-28×3, which consists of three tandem copies of the C-terminal 28 a.a. of gp41 ectodomain (Fig. 1A). The immunogen was produced initially as a fusion protein (HR1-HR2-28×3) by adjoining 28×3 to the HR1-HR2 regions of gp41, which forms a six-helix bundle (6HB) (Shi et al., 2010). This was done because we had observed that HR1-HR2 6HB allows high level protein expression in *E. coli* (Fig. 1B; unpublished data). The HR1-HR2 portion was subsequently removed by thrombin digestion (Fig. 1C). Three 28-mer peptides were linked together to increase its immunogenicity without requiring conjugation to a heterologous carrier protein. All of the bnAbs tested (2F5, Z13e1, 4E10 and 10E8) bound gp41-28×3 (Fig. 1D). However, 10E8 binding was about ~50-fold weaker than 2F5 and ~10-fold weaker than Z13e1 and 4E10, suggesting that the conformation of the 10E8 epitope may not be optimal.

The conformation, orientation, and/or accessibility of the neutralizing epitopes on the MPER are likely affected by the membrane surface as well as other proximal gp41 domains (Irimia et al., 2016; Montero et al., 2012; Rujas et al., 2015). To present the MPER in a more native-like setting, we generated a second immunogen designated gp41-54CT. It comprises the C-terminal 54 a.a. of the gp41 ectodomain that includes the HR2 domain and the MPER, along with the transmembrane domain and the cytoplasmic tail (CT). We hypothesized that this immunogen would selectively amplify a subset of antibodies induced by gp41-28×3 that could bind the MPER in the context of the membrane surface and HR2. HEK-293T cells transfected with the plasmid encoding gp41-54CT could be detected using 2F5, Z13e1 and 4E10 by flow cytometry analyses (Fig. 1E), indicating cell surface expression of the protein and correct conformation of neutralizing epitopes. Both gp41-54CT and gp41-28×3 are based on M group consensus sequence (Gao et al., 2005). For the final boost, we generated a recombinant vaccinia virus expressing the full-length gp160 of the HIV-1_{DH12} strain (rVV-$gp160_{DH12}$). A schematic diagram showing the relative sizes of the three immunogens are illustrated in Fig. 1F.

2.2. Immunization and evaluation of antibody responses

To evaluate IPAS-RAM vaccine strategy, rabbits (R1, R2 and R3) were first immunized with gp41-28×3 only (Fig. 2A). Four weeks later, a combination of gp41-28×3 and gp41-54CT was administered, with the latter delivered by DNA electroporation. Instead of immunizing with just gp41-54CT, gp41-28×3 was also included because we postulated that immunizing with both immunogens would preferentially stimulate antibody responses towards epitopes present on both immunogens (*i.e.* the C-terminal 28 a.a.). Similarly, on week 11, a combination of gp41-54CT and rVV-$gp160_{DH12}$ was administered. After the first immunization, the antibody titers reached $>10^3$ and the titers increased by about 100 fold after the second immunization (Fig. S1A). However, because significant increases in antibody responses were not observed after the third immunization, a fourth immunization was given on week 29 using gp41-28×3 and rVV-$gp160_{DH12}$. The long resting period was given so that antiviral immune responses against the vaccinia virus vector induced after the third immunization would subside. Antibody responses increased only slightly after the fourth immunization.

To identify immunogenic linear epitopes, ELISAs were conducted with overlapping 10-mer peptide sets biotinylated either at the N- or C-terminal ends as we previously reported (Banerjee et al., 2016; Habte et al., 2015). After the first immunization, very little response was detected (Fig. 2B). However, after the second immunization, strong antibody responses were detected against peptides in the cluster II immunodominant region just upstream of the 2F5 epitope (peptides 653 and 656; also peptide 650 for rabbit R2). After the third immunization with gp41-54CT and rVV-$gp160_{DH12}$, the cluster II region still remained immunodominant. However, antibody responses appeared against other linear epitopes. R2 showed good binding to peptide ^{662}ALDKWASLWN^{671} containing the 2F5 epitope. This rabbit also showed low level reactivity against other C-terminal peptides (665, 668, 671 and 674). Antiserum from R3 bound to peptides ^{668}SLWNWFDITN^{677} and ^{671}NWFDITNWLW^{680} that contain parts of the 4E10 and 10E8 epitopes. R1 and R2 also recognized additional peptides within the HR2 domain (peptides 629 and 638). Except for peptides 653 and 656, antibody responses against peptides exhibited animal-to-animal variation. The fourth immunization with gp41-28×3 and rVV-$gp160_{DH12}$ further enhanced anti-MPER antibodies in R2 (peptides 668 and 671) and R3 (peptides 671 and 674). Interestingly, antibody responses against many of the upstream peptides (629, 638, 650, 653 and 656) diminished significantly for R2.

To examine whether antibodies induced by the IPAS-RAM vaccine strategy are different from those induced by a typical homologous prime-