Vaccine Scaffolds

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An Epitope-Specific Respiratory Syncytial Virus Vaccine Based on an Antibody Scaffold

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Abstract: *Respiratory syncytial virus (RSV) is a leading cause of lower respiratory tract infections in children. We have generated an epitope-specific RSV vaccine by grafting a neutralizing epitope (F-epitope) in its native conformation into an immunoglobulin scaffold. The resulting antibody fusion exhibited strong binding affinity to Motavizumab, an RSV neutralizing antibody, and effectively induced potent neutralizing antibodies in mice. This work illustrates the potential of the immunoglobulin molecule as a scaffold to present conformationally constrained B-cell epitopes.*

Respiratory syncytial virus (RSV) is a negative-sense, single-stranded RNA virus that belongs to the *Paramyxoviridae* family. RSV is the cause of more than 30 million lower respiratory tract infections annually and more than 60 thousand deaths worldwide.[1] It is also the major cause of hospitalization in children under 5 years of age. Young children at risk are currently treated with multiple injections of the antibody Palivizumab, a neutralizing antibody directed against an epitope of the RSV F protein. A vaccine-based approach would be far more effective in reducing RSV infections, especially in developing countries where treatment with anti-RSV antibodies is difficult. However, in early clinical trials, a formalin-inactivated whole virus vaccine caused enhanced disease severity upon natural RSV infection. DNA-based, live-attenuated or whole-inactivated virus-based, and protein subunit vaccines have been explored during the past 50 years without success,[2] partially because of the poor induction of neutralizing antibodies.[3] Immunization with linear peptides corresponding to the neutralizing epitope failed to elicit neutralizing antibodies in a mouse study, which underlines the critical role of the native conformation of the epitope.[4] Recently it was shown that protective antibodies can be elicited with an epitope-focused vaccination strategy, wherein the active structural epitope was grafted onto a computationally designed protein scaffold which stabilized its wild-type helix-turn-helix conformation.[5] This work highlights the importance of presenting epitopes in their native conformation in a vaccine candidate. Moreover, it encourages the development of additional protein scaffolds to present foreign epitopes to the immune system in defined conformations.

We previously identified a family of natural bovine antibodies (Figure 1 A) with an ultralong heavy chain complementary determining region 3 (CDR3) consisting of an extended β-sheet structure terminated in a disulfide-bonded "knob" domain.[6] On the basis of this structure, we have designed both β-sheet and helical CDR architectures that present fused growth factors, cytokines, and bioactive peptides in their native conformations. The resulting antibody fusion proteins retain their biological activity but have increased stability and serum half-lives.[7] Here, we demonstrate that a similar approach can be used to present an immunogenic RSV epitope in its active helical conformation. The resulting fusion protein elicits protective neutralizing antibodies in mice.

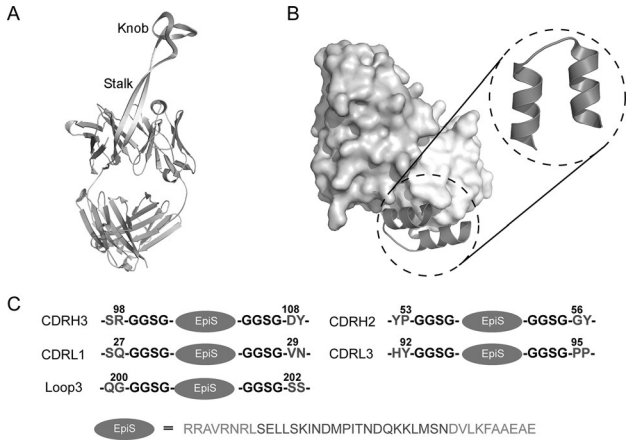

Figure 1. Vaccine design. A) Crystal structure of bovine antibody BLV1H12 (PDB code 4K3D) shows an ultralong CDR3 with a disulfide cross-linked knob domain on top of a solvent-exposed β-strand stalk. B) Crystal structure of Mota in complex with its peptide epitope (dark, enlarged) from RSV F protein (PDB code 3IXT). C) Map of the key elements of EpiS and its fusion to Herceptin. Numbers indicate the fusion sites.

Of the eleven proteins encoded in the RSV genome,[8] antibodies directed against the fusion (F) and attachment (G) glycoproteins confer neutralization and protection to RSV in animal models.[9] Because the RSV F glycoprotein is conserved among RSV A and B strains, antibodies targeting the F

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protein have the potential to provide cross protection.[10] The crystal structure of the neutralizing antibody Motavizumab (Mota, a more potent second-generation Palivizumab) complexed to the F protein reveals a 22-amino acid epitope in a helix-turn-helix conformation (Figure 1B).[11] A stable helix-turn-helix mimic of the epitope (SELLSKINDMPITNDQKKLMSN) has been shown to elicit neutralizing antibodies against RSV virus in rhesus macaques.[5] Similarly, we reasoned that one can graft this F-epitope into an appropriately designed CDR of an antibody and maintain its native conformation. The resulting antibody-epitope fusion is expected to have a long circulating half-life, present antigen in a bivalent fashion, and minimize induction of off-target antibodies.

To test whether one can substitute the Mota-binding F-epitope into an antibody CDR and retain its active conformation, we fused this peptide to various CDRs of Herceptin, an anti-Her2 antibody with low immunogenicity used clinically for the treatment of breast cancer.[12] Previously we have fused various conformationally constrained peptides, such as CXCR4 receptor antagonist peptide, glucagon-like peptide receptor agonist peptide, and protease inhibitor peptides to various CDRs of Herceptin in their biologically active conformations.[7a,13] The engineered F-epitope (EpiS) consisted of the 22-amino acid Mota-binding peptide core, flanked by short helices consisting of 8 N-terminal and 10 C-terminal residues to further stabilize the fused helical peptide (Figure 1C). To identify the best site for presenting the epitope, we screened different CDR and constant loops in the antibody scaffold. The EpiS epitope was inserted between R98 and D108 of CDRH3 (hH3-EpiS), P53 and G56 of CDRH2 (hH2-EpiS), Q27 and V29 of CDRL1 (hL1-EpiS), Y92 and P95 of CDRL3 (hL3-EpiS), and between G200 and S202 of light chain constant loop3 (hLoop3-EpiS) (Figure 1C). EpiS is predicted to have T-cell epitopes using the IEDB analysis resource consensus tool (Supporting Information, Figure S1), therefore, we reasoned the antibody EpiS fusion is likely to elicit a T-cell-dependent antibody response. All fusion proteins were transiently expressed in FreeStyle 293 cells as secreted proteins and purified by protein G chromatography. hH3-EpiS, hL3-EpiS, and hLoop3-EpiS were produced in good yields of 8.4 $mg\,L^{-1}$, 6.7 $mg\,L^{-1}$, and 8.5 $mg\,L^{-1}$, respectively; These purified antibody fusions were characterized by SDS-PAGE and MS (Supporting Information, Figure S2).

To confirm that the epitope retained its correct conformation when grafted into the antibody scaffold, we determined the binding affinities of all purified fusion proteins to Mota. A qualitative enzyme-linked immunosorbent assay (ELISA) was performed and the results revealed that all fusion proteins bound tightly with Mota while Herceptin itself showed no binding (Figure 2A). The apparent binding constant of hH3-EpiS with Mota was determined by a quantitative ELISA to be 154 ± 18 pM (Figure 2B), which is consistent with the binding affinity of F-epitope to Mota (reported values of 30 pM to 800 pM).[5] A K_d value of 240 pM obtained from biolayer interferometry (BLI) further validated the high affinity of hH3-EpiS to Mota (Supporting Information, Figure S3).

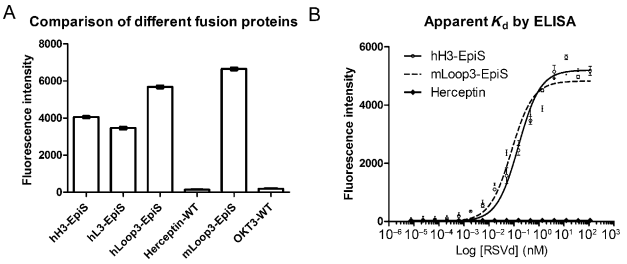

Figure 2. Binding affinities of the antibody-epitope fusion proteins with Mota. A) ELISA analysis for hH3-EpiS, hL3-EpiS, hLoop3-EpiS, and mLoop3-EpiS fusion proteins binding with Mota. B) ELISA determination of the apparent K_d for hH3-EpiS and mLoop3-EpiS with Mota, wild-type Herceptin as a control. A plate coated with Mota Fab at 1 μg per well was blocked with 2% BSA in PBS and incubated with (A) different fusion proteins, or wild-type Herceptin and OKT3 as controls, at 0.5 $\mu g\,mL^{-1}$ or (B) increasing concentration of hH3-EpiS, mLoop3-EpiS, or Herceptin, followed by the addition of HRP-conjugated anti-human IgG (Fc-specific) or anti-mouse IgG (Fc-specific). After incubation and wash, 100 μL QuantaBlu fluorogenic ELISA substrate was added to each well and the signals were monitored using a plate reader (EX 325 nm/EM 420 nm). Assays were performed in triplicate and error bars represent the standard deviation.

Next, we examined this immunoglobulin-based vaccine in a mouse model by monitoring antibody titers in response to immunization with the immunoglobulin-epitope fusion. Because human antibodies are immunogenic in mice,[14] a mouse immunoglobulin scaffold was used to present the F protein epitope. OKT3 is a fully mouse antibody with good stability, which makes it a suitable surrogate for immunization studies in mice. hLoop3-EpiS was translated into OKT3 based on sequence alignment by inserting the EpiS epitope between T199 and T201 in Loop3 to yield mLoop3-EpiS. The engineered antibody was transiently expressed in FreeStyle 293 cells with a yield of 3.0 $mg\,L^{-1}$. The purified antibody fusion was characterized by SDS-PAGE and MS (Supporting Information, Figure S2). The apparent binding constant of mLoop3-EpiS with Mota was assessed by a quantitative ELISA to be 80 ± 13 pM (Figure 2B), which was further validated by BLI ($k_{on} = 1.3 \times 10^5\,M^{-1}s^{-1}$, k_{off} is below the detection limit; Supporting Information, Figure S3).

Two groups of BALB/c mice ($n = 10$) were immunized three times with either 10 μg mLoop3-EpiS or 10 μg of wild-type OKT3 as a negative control on a biweekly schedule in weeks 0, 2, and 4. Serum samples from weeks 3, 5 and 7 were collected, and their antibody titers against the mLoop3-EpiS, RSV F protein and OKT3 were measured by ELISA at 1:100 dilution. Antibodies against the fusion protein mLoop3-EpiS were observed from week 3 onward and continuously increased in weeks 5 and 7, while there was no detectable antibody response towards the OKT3 control up to week 7 (Figure 3A; Supporting Information, Figure S4). The antibodies elicited by mLoop3-EpiS cross-react with RSV F glycoprotein by ELISA (Figure 3A). Finally, we demonstrated the neutralizing activity of our vaccine in an RSV virus protection assay. A widely used RSV neutralization assay was adopted to determine whether antibodies generated by this epitope-focused fusion protein can neutralize the active virus.[15] RSV (10 μL at 10^6 $cfu\,mL^{-1}$) pretreated with 10 μL

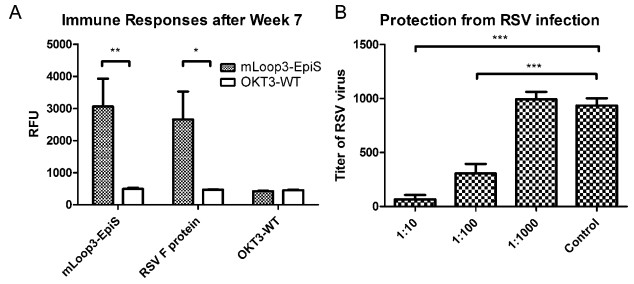

Figure 3. A) Mouse vaccination study. Immune responses 7 weeks after the first immunization (3 weeks after the third immunization) with mLoop3-EpiS showed significant differences in titers against the fusion protein mLoop3-EpiS and the RSV F protein, but no difference for OKT3-WT. A plate coated with Mota Fab at 1 μg per well was blocked with 2% BSA in PBS and incubated with 1:100 diluted serum samples at room temperature for 1 hour, followed by the addition of HRP-conjugated anti-mouse IgG (Fc-specific). After incubation and wash, 100 μL QuantaBlu fluorogenic ELISA substrate was added to each well and the signals were monitored using a plate reader (EX 325 nm/EM 420 nm). X-axis represents the coating agents. $*P<0.05$, $**P<0.01$ vs. control group; $n=10$ indicates the number of mice per group. B) Serum from mice immunized with mLoop3-EpiS significantly block the viral infection of Hep-2 cells. Serum dilutions (1:10, 1:100, 1:1000) were mixed 1:1 with RSV virus and the mixture was inoculated on Hep-2 cell monolayer. After a 4 hour incubation, the inoculum was removed and 3 mL overlay of DMEM/F12 with 0.3% ICN immunodiffusion grade agarose was added. The plates were fixed after 7 days incubation and the plaques were counted after visualizing by the addition of 0.05% neutral red. $***P<0.001$ vs. control group; $n=10$ indicates the number of mice per group.

of serum dilutions (1:10, 1:100, 1:1000) from the vaccinated or control groups were allowed to infect mono-layer Hep-2 cells for four hours before the medium was removed and the cells were overlaid with DMEM/F12 in 0.3% agarose. After 6 days, the monolayers were fixed and stained. Plaques were counted and the neutralizing rates were calculated accordingly. Significant differences were observed in RSV infectivity with 1:10 and 1:100 serum dilutions of the vaccinated versus control group. (Figure 3B).[16] This result demonstrates that the antibodies raised to the antibody-epitope fusion protein bind the intact viruses and neutralize their infectivity in vitro. The presence of neutralizing antibodies is a key step in evaluation of a vaccine candidate and has been proven to be crucial for the protection of the respiratory tract against RSV infection.[17] The titers of neutralizing antibodies present in the vaccinated mice strongly suggest that the antibody-epitope vaccine will protect in animal models of RSV infection. To this end, we will next investigate the magnitude and durability of these responses as well as the in vivo protection in non-human primates.

In conclusion, we have demonstrated that the RSV F-epitope peptide can be fused to the immunoglobulin domain which supports its native-like helix-turn-helix conformation. The fusion protein is capable of eliciting a strong antibody response with cross-reactivity towards the wild-type RSV F antigen. Moreover, serum from immunized mice contain RSV neutralizing antibodies which block RSV infection in vitro. This work suggests that the immunoglobulin scaffold can serve as a universal and non-immunogenic scaffold for the development of next generation epitope-focused vaccines, including fusion of two or more epitopes.

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