Potent single-domain antibodies that arrest respiratory syncytial virus fusion protein in its prefusion state

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Human respiratory syncytial virus (RSV) is the main cause of lower respiratory tract infections in young children. The RSV fusion protein (F) is highly conserved and is the only viral membrane protein that is essential for infection. The prefusion conformation of RSV F is considered the most relevant target for antiviral strategies because it is the fusion-competent form of the protein and the primary target of neutralizing activity present in human serum. Here, we describe two llama-derived single-domain antibodies (VHHs) that have potent RSV-neutralizing activity and bind selectively to prefusion RSV F with picomolar affinity. Crystal structures of these VHHs in complex with prefusion F show that they recognize a conserved cavity formed by two F protomers. In addition, the VHHs prevent RSV replication and lung infiltration of inflammatory monocytes and T cells in RSV-challenged mice. These prefusion F-specific VHHs represent promising antiviral agents against RSV.

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Human respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infections in children under the age of five throughout the world. It is estimated that RSV infects about 33.8 million children in this age group annually, of which more than 3 million require hospitalization due to severe bronchiolitis or pneumonia. Reinfections occur regularly throughout life because natural infection offers only limited immunity. RSV is also recognized as a major pathogen for the elderly, with a disease burden similar to that of seasonal influenza. Thus, there is an urgent need for therapeutics that can reduce disease caused by RSV.

Despite its medical importance and decades of intense research, there is still no licensed RSV vaccine nor an effective antiviral. The humanized monoclonal antibody (mAb) palivizumab (Synagis) reduces hospitalizations when administered prophylactically, but its high cost and limited efficacy restrict its use to high-risk infants. Palivizumab neutralizes RSV by binding to the fusion (F) protein and preventing fusion of the viral membrane with the host-cell membrane. RSV F is a class I fusion protein that is expressed as an inactive precursor, F0, which is cleaved at two sites by a furin-like protease, leading to the formation of the disulfide-linked F2 (N-terminal) and F1 (C-terminal) subunits, which associate and trimerize to form the mature prefusion F protein. Upon triggering, prefusion F partially refolds and inserts its hydrophobic fusion peptide into the membrane of the target cell. Fusion of the viral and host-cell membranes is facilitated by further refolding of the F protein into the stable postfusion conformation. Small molecules that bind to RSV F and prevent its structural remodeling, or F-specific antibodies that interfere with membrane fusion, can block RSV infection. Such compounds are being clinically developed.

Palivizumab binds to antigenic site II on RSV F, which is one of two well-characterized antigenic sites that are present on both the pre- and postfusion conformations. However, intensive screening for human mAbs that potently neutralize RSV has resulted in the isolation of prefusion F-specific antibodies with more robust neutralizing activity than palivizumab. Recently, RSV F was successfully stabilized in its prefusion conformation through the introduction of an intraprotomeric disulfide bond, cavity-filling mutations and a trimerization motif. This reagent, called DS-Cav1, has been instrumental in revealing that the vast majority of RSV-neutralizing immunoglobulins in human sera selectively bind to F in its prefusion conformation.

In addition to conventional antibodies, heavy-chain-only antibodies also exist in nature, for example, in both camels and sharks. The isolated antigen-recognition domains of these unusual antibodies are known as single-domain antibodies (VHHs). VHHs are very well suited for the development of therapeutics because of their small size, ease of production and physical stability that allows alternative routes of administration such as pulmonary delivery by nebulization. A number of clinical trials are already ongoing with recombinant VHHs for the treatment of rheumatoid arthritis, cancer and infectious diseases.

ALX-0171 is an RSV-neutralizing VHH that binds to an epitope on RSV F that is similar to that of palivizumab. In a phase I/IIa trial, hospitalized RSV-infected children were treated daily for three consecutive days with ALX-0171 delivered by an inhalation device. The treatment was safe and did not lead to any treatment-related serious adverse events. Interestingly, the study also revealed a trend towards a therapeutic effect, based on reduced viral loads in nasal swabs and clinical symptoms. In contrast, a similar trial with motavizumab—an affinity matured version of palivizumab—did not alter viral replication or improve clinical symptoms when administered after infection. This different outcome might be explained by the direct delivery of ALX-0171 to the lungs whereas only about 0.2% of systemically administered antibody ends up in the lung lumen.

We hypothesized that a prefusion-specific VHH would have a much stronger antiviral effect than a conformation-independent VHH like ALX-0171. Here, we present the isolation and characterization of two llama-derived VHHs that potently neutralize RSV A and B subtypes and selectively bind prefusion F with picomolar affinity. Structural studies reveal that these two VHHs bind to a conserved quaternary epitope composed of two F protomers. Furthermore, prophylactic treatment of mice with these VHHs potently controlled RSV infection and associated inflammation.

Results
Isolation of VHHs with exceptional RSV-neutralizing activity. To obtain VHHs that selectively bind to the prefusion conformation of RSV F, a llama was immunized with recombinant F protein that was stabilized in the prefusion conformation, that is, DS-Cav1. Plasma prepared after six weekly immunizations with prefusion F protein had potent RSV subtype A and B neutralizing activity (Supplementary Fig. 1). Peripheral blood mononuclear cells were isolated from the prefusion F-immunized llama and used to generate a VHH phage-display library. One round of panning on immobilized prefusion F protein resulted in a 140-fold enrichment in candidate prefusion F-binding phages. In a first approach to select RSV-neutralizing VHHs, we randomly selected 90 clones from this enriched population and tested binding to RSV F protein by ELISA. Thirty-seven clones expressed VHH that bound to either pre-or postfusion F protein (FAFP); twenty-eight of these had a unique sequence and were subsequently expressed in Pichia pastoris. RSV A2-neutralizing activity was present in the crude growth medium of P. pastoris clones 1, 4, 8, 13 and 44, which were retained for further analysis (Supplementary Fig. 2a).

In an alternative approach, the VHH cDNA inserts obtained after one round of panning on prefusion F were cloned as a pool into the P. pastoris expression vector and the resulting library was used to transform P. pastoris. The crude culture growth medium of individual P. pastoris transformants was then tested for RSV-neutralizing activity. One candidate, named F-VHH-L66, displayed clear RSV A2-neutralizing activity (Supplementary Fig. 2b). We purified the VHHs from the culture supernatant of the selected P. pastoris transformants for further analysis. All six purified VHHs (F-VHH-1, -4, -8, -13, -44 and -L66) neutralized RSV A and RSV B subtypes in vitro, with F-VHH-4 and F-VHH-L66 exhibiting the most potent neutralization, with an IC50 value below 0.1 nM for both RSV strains (Fig. 1a,b). These two VHHs were therefore selected for further characterization. Sequence analysis of F-VHH-4 and -L66 revealed an identical complementarity-determining region (CDR) 1, a similar CDR2, but a very different CDR3 (Fig. 1c and Supplementary Fig. 3). F-VHH-4 and -L66 both have a cysteine residue at position 100c in the CDR3 and at position 50 in the CDR2. A disulfide bond between the CDR3 and a residue at position 33 or 50 is a hallmark of VHHs and helps to stabilize the extended CDR3 loop conformation.

To assess the antiviral potency of the VHHs in more detail, RSV-neutralization assays were performed in which F-VHH-4 and -L66 were compared with several F-specific mAbs (palivizumab, motavizumab, D25 and AM22) and ALX-0171. Remarkably, despite their monovalent character, F-VHH-4 and -L66 neutralized RSV A and B with picomolar IC50 values, which are similar to or lower than those for the bivalent prefusion...
F-specific mAbs D25 and AM22 (Fig. 1d,e). The VHHs were also tested in a neutralization assay against a set of clinical RSV isolates and a set of molecular clones built on the RSV Line 19 backbone, but with F and G derived from primary clinical isolates. These strains were as susceptible as the lab strains to neutralization by the VHHs (Supplementary Table 1 and Supplementary Fig. 4).

In HEp-2 cells the F protein is involved in both viral attachment and membrane fusion. We therefore investigated if the VHHs could prevent infection after attachment of viral particles to the cells. Similar to palivizumab and D25, F-VHH-4 and F-VHH-L66 prevented infection when added after virion attachment (Fig. 1f). However, mixing of RSV with the neutralizing VHHs before infection did not hinder viral attachment to HEp-2 target cells, indicating that F-VHH-4 and -L66 inhibit RSV in vitro by blocking membrane fusion post-attachment (Supplementary Fig. 5).

**Figure 1 | F-VHHs with potent RSV-neutralizing activity.** RSV-neutralizing activity by F-VHHs. (a) RSV A2 (50 pfu per well) or (b) RSV B49 (40 pfu per well) was preincubated with different concentrations of VHH before infection of Vero cells. Three days later, the viral plaques were stained with polyclonal anti-RSV serum. (c) Amino acid sequences of F-VHH-4 and F-VHH-L66 with Kabat numbering. The complementarity-determining regions (CDRs) are boxed. Cysteines in CDR2 and CDR3 are in bold. (d) RSV A2 (30 pfu per well) plaque-reduction activity of F-VHH-4 and F-VHH-L66 compared with mAbs. The Ctrl-VHH is specific for an irrelevant target. (e) IC_{50} values of F-VHHS and mAbs against RSV A2 and RSV B49 as determined by plaque-reduction assay. Mean values ± s.d. from four (VHHs), three (D25) or two (palivizumab, motavizumab and AM22) repeat experiments are depicted for RSV A. Mean values ± s.d. from two experiments are depicted for RSV B. ND: Not determined. (f) Dilution series of VHHs and mAbs were added to HEp-2 cells that had been preincubated with RSV A2 (25 pfu per well) at 4°C for 2 h, allowing viral attachment. After 2 h at 37°C, the VHHs or antibodies were washed away and infection was allowed during two days at 37°C after which the plaques were stained. Antibody 65 is a mouse IgG2a monoclonal antibody that is specific for an irrelevant antigen.
VHHs bind a unique prefusion F-specific epitope. To investigate whether the neutralizing VHHs specifically recognize the prefusion conformation of RSV F, the binding kinetics to recombinant pre- and postfusion F proteins were measured by surface plasmon resonance (SPR). The VHHs bound tightly to prefusion-stabilized F, whereas no binding to postfusion F could be detected (Fig. 2a and Supplementary Fig. 6). F-VHH-4 bound to immobilized prefusion RSV F with an equilibrium dissociation constant (K_D) lower than 18 pM. The off-rate was extremely slow and outside the range measurable by SPR, therefore, the upper limit of the K_D was estimated using the measured association rate constant (5.7 \times 10^5 M^{-1} s^{-1}) and the lower limit of detection for the dissociation rate constant (1 \times 10^{-5} s^{-1}). F-VHH-L66 also bound to prefusion F with sub-nanomolar affinity. However, compared with F-VHH-4, the F-VHH-L66 association rate constant was lower (3.3 \times 10^5 M^{-1} s^{-1}) and the dissociation rate constant was higher (5 \times 10^{-5} s^{-1}), resulting in a K_D of 154 pM. Binding of the VHHs to cells transfected with a codon-optimized F-expression plasmid and to RSV-infected cells was also observed (Supplementary Figs 7 and 8), demonstrating that the VHHs recognize full-length F in the context of biological membranes.

To gain more insight into the epitopes that are targeted by F-VHH-4 and -L66, we performed cross-competition binding experiments using biolayer interferometry and a set of mAbs with known epitopes. This analysis revealed that the two VHHs competed with each other for binding to immobilized prefusion F and thus likely bound to overlapping epitopes (Fig. 2b). Binding by D25, which recognizes site Ø on prefusion F, was not hindered by F-VHH-4 or -L66, which suggests that neither VHH bound to this site. In contrast, palivizumab,
AM14, MPE8 and 101F interfered with binding of the two VHHs to prefusion F and vice versa, suggesting that the VHH epitope is located in the vicinity of the binding sites of these four antibodies, approximately midway between the apex of the trimer and the viral membrane.

To further narrow down the binding site of the VHHs, we selected for viruses resistant to F-VHH-4 and -L66. Two rounds of selection in the presence of F-VHH-L66 and four rounds of selection in the presence of F-VHH-4 led to the isolation of a single type of escape variant that had a threonine to asparagine substitution at position 50 in the F2 subunit. F-VHH-L66 failed to neutralize this mutant virus, whereas this virus was still susceptible to inhibition by F-VHH-4, although this required 300-fold more F-VHH-4 compared with the parental virus (Fig. 3). Thr50 resides at the floor of a cavity in prefusion F that is surrounded by the epitopes of palivizumab, AM14 and 101F, suggesting that both VHHs bind to this cavity.

F-VHHs bind a cavity formed by two F protomers. To precisely define the epitopes recognized by F-VHH-4 and -L66, their crystal structures in complex with prefusion F were determined to 3.3 and 3.8 Å resolution, respectively (Table 1). In addition, the crystal structure of unbound F-VHH-4 was determined to 1.9 Å resolution (Table 1 and Supplementary Fig. 9). The complex structures revealed that despite substantial differences in CDR3 sequence, F-VHH-4 and -L66 bind in a nearly identical manner to a cavity formed by the boundary of two F protomers (Fig. 4a). This cavity is bordered by antigenic site II of one protomer and antigenic site IV of the neighbouring protomer, and the location of this epitope between the binding sites of palivizumab, 101F and AM14 explains the ability of the VHHs to compete with each of these antibodies (Fig. 2b and Supplementary Fig. 10). This epitope has also been mapped by mutagenesis as the likely epitope for MPE8, which cross-neutralizes RSV, human metapneumovirus (hMPV) and pneumonia virus of mice (PVM). The VHHs, however, while being broadly neutralizing for RSV, did not neutralize hMPV (Supplementary Table 2).

Table 1 | Crystallographic data collection and refinement statistics.

|                      | F-VHH-4 | F-VHH-4–prefusion F | F-VHH-L66–prefusion F |
|----------------------|---------|---------------------|-----------------------|
| PDB ID               | 5TP3    | 5TOJ                | 5TOK                  |
| Data collection      |         |                     |                       |
| Space group          | P212121 | P3221               | P3121                 |
| Cell dimensions      | 47.7, 47.8, 149.8 | 173.2, 173.2, 153.3 | 138.9, 139.9, 221.9   |
| Resolution (Å)       | 34.5–1.9 | 38.0–3.3             | 50.4–3.8              |
| Rmerge               | (1.92–1.87) | (3.43–3.30)         | (4.06–3.80)           |
| Rmerge/Rfree (%)     | 0.118 (1.125) | 0.188 (1.081)       | 0.332 (1.246)         |
| I/σ                   | 11.1 (2.1)   | 9.2 (2.1)           | 5.2 (1.5)             |
| CC1/2                | 0.995 (0.465) | 0.995 (0.545)       | 0.975 (0.549)         |
| Completeness (%)     | 100 (100) | 99.9 (100)          | 99.9 (100)            |
| Redundancy (%)       | 6.6 (5.6)  | 7.4 (7.5)           | 5.3 (5.5)             |
| Refinement           |         |                     |                       |
| Resolution (Å)       | 34.5–1.9 | 38.0–3.3             | 50.4–3.8              |
| Rmerge/Rfree (%)     | (1.94–1.87) | (3.38–3.30)         | (3.95–3.80)           |
| Unique reflections   | 28,958 (2,824) | 40,277 (2,821)     | 24,981 (2,726)        |
| No. atoms            | 17.0/20.8 | 18.4/23.4           | 24.8/28.6             |
| Protein              | 1,910    | 14,082              | 13,952                |
| Glycan (NAG)         | 0        | 42                  | 42                    |
| Ion (PO4)            | 0        | 0                   | 5                     |
| Water                | 143      | 0                   | 0                     |
| B-factors            |         |                     |                       |
| Protein              | 25.7     | 94.5                | 124.0                 |
| Glycan (NAG)         | —        | 97.3                | 117.9                 |
| Ion (PO4)            | —        | —                   | 149.0                 |
| Water                | 29.4     | —                   | —                     |
| R.m.s. deviations    |         |                     |                       |
| Bond lengths (Å)     | 0.007    | 0.005               | 0.007                 |
| Bond angles (°)      | 0.81     | 1.20                | 1.13                  |
| Ramachandran         |         |                     |                       |
| Favoured (%)         | 97.2     | 96.5                | 96.6                  |
| Allowed (%)          | 2.9      | 3.3                 | 3.0                   |
| Outliers (%)         | 0        | 0.2                 | 0.4                   |

Values in parentheses are for the highest-resolution shell.
1,000-fold weaker than the affinity measured for trimeric prefusion F. No binding of F-VHH-L66 to monomeric prefusion F was detected, even at the highest concentration tested (500 nM) (Supplementary Fig. 11). Both VHHs were also able to bind and trap wild-type F protein ectodomain in the trimeric prefusion conformation (Supplementary Fig. 12). Therefore, both VHHs have a high selectivity for the trimeric state of prefusion F.

The CDR3s of F-VHH-4 and -L66 are submerged in the interprotomeric cavity and therefore mediate the majority of the interactions with RSV F (Fig. 5 and Supplementary Table 3). The main chain of both F-VHH-4 and -L66 forms a hydrogen bond with Thr50 of F2, the residue mutated in the escape viruses isolated for both VHHs. A number of hydrogen bonds are also formed between the CDR3 and the antiparallel β-strands that neighbour antigenic site IV. The CDR3 of each VHH is disulfide-linked to CDR2, which hydrogen bonds with the α7 helix of antigenic site II (Fig. 5).

Upon membrane fusion, the domains of each RSV F protomer contacted by theFigure 4 | F-VHHS bind an epitope formed by two protomers of prefusion F. (a) Superposition of the crystal structures of three F-VHH-4 s (blue) and three F-VHH-L66s (orange) bound to prefusion RSV F, viewed from the side (left) and the top (right). (b) Close-up of the VHH-binding cavity, viewed from the side (left) and from the bottom (right). The VHH epitope is outlined with a dotted black line in the side view. The CDR2s and CDR3s of both VHHs are shown as tubes.

Figure 5 | F-VHHS make direct contacts with residues on two neighbouring protomers of prefusion F. Close-up of the CDR3 and CDR2 from (a) F-VHH-4 and (b) F-VHH-L66 bound to prefusion F. CDR2 and CDR3 are shown as tubes with the N- and C-termini labelled. Side-chains and main-chain involved in hydrogen bonding are shown as sticks, with hydrogen bonds depicted as black dotted lines. Residues of the F protein are labelled.
VHHs undergo a rigid-body movement that causes their relative positions to change substantially between the prefusion and postfusion states. Superposition of the postfusion F structure onto the VHH-bound prefusion structures reveals a significant clash between the loop connecting α6 and α7 of postfusion F and the CDR3 of either VHH (Supplementary Fig. 13). Therefore, our structural data suggest that F-VHH-4 and -L66 neutralize RSV and block fusion by preventing the movement of domains required for conversion of prefusion RSV F to the postfusion state. In addition, both VHHs bury approximately 100 Å² on the β4 strand, which dramatically rearranges to become a portion of the α5 helix of postfusion F. Although the buried surface area in this region is relatively small, these contacts may also contribute to the prefusion specificity of the VHHs by preventing the refolding of β4 during the transition from prefusion to postfusion F.

F-VHHs reduce replication of RSV in vivo. In a final set of experiments, we evaluated the protective potential of F-VHH-4 and -L66 in vivo. Both F-VHHs strongly reduced virus replication when administered intranasally to BALB/c mice at a dose of 30 μg (1.5 mg kg⁻¹) before challenge with RSV (Supplementary Fig. 14). We selected F-VHH-4 for subsequent animal experiments because it has a slightly higher neutralizing activity than F-VHH-L66. Intranasal administration of 10 μg (0.5 mg kg⁻¹) F-VHH-4 or palivizumab to BALB/c mice 4 h before challenge with 1 × 10⁶ PFU of RSV A2 resulted in undetectable levels of virus in the lungs and the bronchoalveolar lavage on day five after challenge (Fig. 6a and Supplementary Fig. 15). This apparent lack of virus in the lungs of F-VHH-4- or palivizumab-treated mice could be due to an in vitro effect of the remaining VHH or antibody in the lung homogenates. Therefore, viral RNA was quantified by an RSV-specific RT-qPCR. The lungs of mice treated with F-VHH-4 were negative or borderline positive for RSV RNA, whereas the relative amount of RSV RNA indicated at least a 170-fold higher viral load in samples from palivizumab-treated mice compared with Ctrl-VHH 4 h before infection with 1 × 10⁶ RSV A2 or PBS (mock). Five days after infection, the pulmonary RSV load was determined by (a) plaque assay (dashed line represents the detection limit) or (b) by quantification of the amount of lung viral RNA using RT-qPCR. Horizontal lines indicate medians. *P < 0.05, **P < 0.01 (Mann–Whitney U-test).

Discussion

Our aim was to obtain molecules with very high RSV-neutralizing activity that could be developed as a therapeutic for the treatment of RSV infections. We describe a new class of VHVs that bind specifically to the prefusion conformation of the RSV F protein. Such VHVs were obtained by the immunization of a llama with a soluble prefusion-stabilized F protein combined with an RSV-neutralization-based screening strategy. The resulting VHVs display broad RSV-neutralizing activity, bind specifically and with high affinity to the prefusion conformation of F and inhibit RSV replication in vivo. The potent RSV-neutralizing activity of the F-VHVs correlates with their ultra-high affinity for the prefusion F protein. By tightly binding to an epitope that is only present on the trimeric prefusion conformation, the VHVs presumably interfere with the transition of the F protein from the prefusion to the postfusion state, thereby preventing fusion with the host cell.

F-VHH-4 and F-VHH-L66 have only 5 out of 15 amino acids in common in their CDR3. Yet, F-VHH-4 and -L66 bind closely overlapping epitopes in a nearly identical way. On the nucleotide level, the VHVs are 94% identical, indicating that they probably emerged from the same recombined germline sequences. If so, it is remarkable that two very different paths of affinity maturation converge to highly similar modes of binding. Crystallization studies showed that the CDR3 of both VHVs bind into a cavity formed by two protomers. This illustrates the tendency of VHVs to preferably bind clefts or cavities, whereas conventional antibodies in general bind either flat surfaces or extruding epitopes²⁵. The epitope of F-VHH-4 and F-VHH-L66 overlaps with the epitope mapped by mutagenesis for the broadly neutralizing antibody MPE8, which has neutralizing activity against human and bovine...
RSV, hMPV and PVM. Nevertheless, the VHVs do not neutralize hMPV. RSV and hMPV F proteins have only 33% overall amino acid sequence identity, yet a substantial area of the surmounted MPE8 epitope is conserved between the two viruses. Although the VHVs bind to a similar region, they make crucial contacts with a number of residues that are not conserved between RSV F and hMPV F, explaining the lack of hMPV neutralization by the F-VHVs. Thus, in this case there appears to be a trade-off between the potency of the VHVs and the breadth of MPE8.

We addressed the in vitro escape potential of RSV in the presence of the VHVs. F-VHH-4 and F-VHH-L66 escape viruses had a single mutation resulting in a substitution of Thr50, which is located at the bottom of the cavity bound by the VHVs. Although resistant to F-VHH-L66, this virus could still be neutralized by F-VHH-4, albeit with 300-fold lower efficiency. The presence of three consecutive serine residues in the CDR3 of F-VHH-4 could lead to more flexibility of this loop compared with that of F-VHH-L66 and allow F-VHH-4 to bind to an altered conformation of prefusion F. Although this possibility of selecting for an escape mutant could raise concerns for the clinical use of these VHVs, the escape virus was severely attenuated as it was impossible to grow the virus to substantial amounts to make high titre virus stocks.

Other VHVs against RSV F have been described recently Nb017, for example, is a VH developed by Ablynx that binds to antigenic site II of RSV F19. F-VHH-4 outperforms Nb017 by 15,000- and 180,000-fold on RSV A and RSV B neutralization respectively, reflecting the higher potency of prefusion-specific antibodies compared with antibodies that bind both conformations of F. To increase its neutralizing activity, Nb017 was re-formatted into a trivalent VH named ALX-0171. However, monovalent F-VHH-4 and -L66 already display higher RSV A- and B-neutralizing activity than that reported for ALX-0171. In addition, the small size of monovalent VHVs may reduce their potential immunogenicity and facilitate penetration of the mucus layer that lines the respiratory epithelium to reach RSV-infected cells. It is also straightforward to link a monovalent VH to other carriers, such as a VH that binds to another epitope in RSV or to a protein with an effector function of interest.

A phase I/IIa clinical trial with inhaled ALX-0171 in hospitalized RSV-infected children indicated that the drug was safe. The trial also indicated that treatment with this trivalent pre- and postfusion F-reactive VH could reduce viral replication and symptoms in the infected children. By selectively targeting prefusion F, for example with the VHVs described here, the antiviral impact in patients may be improved, which could result in a more robust clinical benefit in a treatment setting of RSV-infected patients. As the vast majority of RSV-infected infants are hospitalized at or after the peak of viral replication and disease, early RSV detection followed by rapid antiviral treatment remains key to reduce symptoms and prevent hospitalization of patients. Nebulization of F-VHH-4 as soon as possible after RSV confirmation offers the additional advantage that the antiviral is delivered directly to the site of infection.

Developing new antibody-based drugs for respiratory infections in a VHH format has a number of advantages. Production and purification are inexpensive, straightforward and can be performed in organisms that are generally regarded as safe and without the use of animal-derived products. In addition, due to their small size, high stability and solubility, VHVs can be easily administered via inhalation, directly to the site of viral infection. The in vitro and in vivo results reported here support further testing of F-VHH-4 and F-VHH-L66 as novel therapeutics against RSV.
contains a Zeocine resistance marker for selection in bacterial as well as in yeast cells. The vectors were linearized by Pmel before transformation of P. pastoris strains GS115 using the condensed transformation protocol described by Lin-Cereghino et al.37

Purification of VHHs produced by Pichia pastoris. Expression of VHH by transformed P. pastoris clones was first analysed in 2 ml cultures. On day one, individual transformants were used to inoculate 2 ml of YPNG medium (2% pepton, 1% Bacto yeast extract, 1.34% YNB, 0.1 M potassium phosphate pH 6, 0.00004% biotin, 1% glycerol) with 100 µg ml⁻¹ Zeocin (Life Technologies) and incubated while shaking at 28 °C for 24 h. Next, cells were pelleted and the YPNG medium was replaced by YPNNM medium (2% pepton, 1% Bacto yeast extract, 1.34% YNB, 0.1 M potassium phosphate pH 6, 0.00004% biotin, 1% methanol) to induce VHH expression. Cultures were incubated at 28 °C while shaking for 48 h. Fifty microliters of 50% methanol was added to the cultures at 16, 24 and 40 h. After 48 h, the yeast cells were pelleted and the supernatant was retained to assess the presence of VHH. To select VHHs with RSV-neutralizing activity, serial dilutions of the crude YPNNM supernatant from individual P. pastoris transformants were tested in a plaque-reduction assay.

P. pastoris transformants that yielded high levels of VHH in the medium and with high RSV-neutralizing activity were selected for scaling up using 100 or 300 ml P. pastoris cultures. Growth and methanol induction conditions and harvesting of medium were similar as mentioned above for the 2 ml cultures. The cleared medium was subjected to ammonium sulfate precipitation (80% saturation) for 4 h at 4 °C. The insoluble fraction was pelleted by centrifugation at 20,000g and solubilized in 10 ml HiTrap binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) before purification on a 1 ml HiTrap HP column (GE Healthcare). Relevant fractions containing the VHH were pooled, and concentrated with a Vivaspin column (5 kDa cutoff, GE Healthcare) and then subjected to size-exclusion chromatography (Superdex 75). Fractions containing the VHH were again pooled and concentrated and the concentration was determined. The purified VHHs were aliquoted and stored at −80 °C until further use.

Cells and viruses. HEp-2 cells (ATCC, CCL-23), Vero cells (ATCC, CCL-81), HEK-293 T cells (a gift from Dr M. Hall, University of Birmingham, Birmingham, UK) and A549 cells (ATCC, CCL-185) were grown in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM l-glutamine, non-essential amino acids (Invitrogen, Carlsbad, CA, USA) and 1 mM sodium pyruvate at 37 °C in the presence of 5% carbon dioxide.

Fluorescence plate reader neutralization assay. Fluorescence plate reader neutralization assay was performed as previously described15. A total of 2.4 × 10³ HEP-2 cells per well in 30 µl culture medium were fed 384-well black optical bottom plate (Nunc 384-well plates, Thermo Scientific). Serum samples or antibodies (1 mg ml⁻¹) were diluted four-fold for 12 dilutions starting from 1:10, equal volume of recombinant mKate-RSV (subtype A and subtype B) was added and mixed, incubated at 37 °C for 1 h, then 50 µl mixture of sample and virus was added to cells and incubated at 37 °C for 24–22 h. After incubation, assay plate was measured for fluorescence intensity in microplate reader at Ex 588 nm and Em 635 nm (SpectraMax Paradigm, molecular devices). For the 96-well plate format, 5 × 10^4 cells per well in 100 µl of culture medium were seeded in a 96-well plate. 100 µl mixtures of sample, virus and 1:100 diluted serum were added to the wells. Fluorescence plate reader neutralization assay was performed as the reduction of fluorescence of samples comparing to virus control. Data was analysed and EC₅₀ was calculated by curve fitting with GraphPad Prism software (GraphPad Software).

Attachment assay. RSV A2 (1 x 10⁸ PFU) was incubated with 1 µM VHH/antibody or 2.5 µM dextran sulfate (MP Biomedicals) for 30 min at room temperature. The mixtures were added to chilled HEp-2 cells and incubated for 2 h at 4 °C. The cells were washed five times with cold PBS with 0.5% BSA (PBS-BSA). Half of the cells were fixed with 2% PFA, stained with goat anti-RSV polyclonal serum (AB1128, Chemicon International) and subsequently with AlexaFluor 488 labelled donkey anti-goat antibody (Invitrogen, 1/600 dilution). After washing, the cells were analysed using a FACSCalibur flow cytometer. The other half of the cells was incubated at 37 °C in DMEM with 2% FCS. After 48 h, the cells were collected, fixed and analysed as described above.

VHH binding to cells expressing F. HEK293T cells were transfected with pCAGGS-F, which encodes a codon-optimized RSV F cDNA, with the FuGene HD transfection reagent (Promega) according to the manufacturer’s protocol. To trace transfected cells, transfusions were performed in the presence of pEGFP-NLS. Control transfusions were performed with only pEGFP-NLS. Forty-two hours after transfection the cells were detached, washed and blocked. Subsequently, the cells were incubated with a 1/10 dilution series of VHH or mAb in PBS-BSA. One hour later the cells were washed and stained with mouse anti-Histidine Tag antibody (MCA1396, Abd Serotec) followed by anti-mouse IgG Alexa 633 (Invitrogen) (for the VHH samples) or anti-human IgG Alexa 633 (Invitrogen) (for the antibody samples). The stained cells were analysed using a FACSCalibur flow cytometer. All procedures were performed on ice or at 4 °C.

Binding to infected cells was assessed in a similar manner. Vero cells were inoculated with RSV A2 at a MOI of 0.1 and after 48 h the cells were collected and fixed with 2% PFA. The cells were stained with 1 µg ml⁻¹ of VHH or antibody. The rest of the staining procedure was performed as described above.

Immunostaining of infected cells. A549 cells, grown on glass plates for confocal microscopy or 12-well imaging dishes (ibidi) for TIRF-imaging, were mock-infected or infected with RSV A2 (MOI 1) for 24 h and fixed with 2% PFA. The cells were blocked and stained with 1 µg ml⁻¹ of F-VHH-4, F-VHH-L6 or Ctrl-VHH in PBS-BSA. One hour later the cells were washed and fixed again with 2% PFA and stained with polyclonal goat anti-RSV serum (or polyclonal rabbit anti-G serum (Sino Biological Inc. 11070-V08H) for the TIRF images) and mouse anti-Histidine Tag antibody. VHH binding was detected with anti-mouse IgG Alexa 488 and anti-RSV serum with anti-goat Alexa 633 (or anti-rabbit Alexa 568 for the TIRF images). After washing the samples were mounted and confocal images were recorded with a SPS Leica confocal microscope. TIRF images were recorded with a Zeiss TIRF Observer. The recorded images were processed with Velocity software (Perkin Elmer).

Cross-competition analysis with biolayer interferometry. Antibody cross-competition was performed as described previously11 with some modifications. Briefly, DS-Cav1 protein (10 µg ml⁻¹) was immobilized in a random orientation onto streptavidin-coated biosensors (ForteBio) pre-activated with N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) through amine coupling reaction in acetate buffer (pH 5). The reaction was quenched by 1 M ethanolamine (pH 8) and DS-Cav1-immobilized biosensors were then coupled reaction in acetate buffer (pH 5). The reaction was quenched by 1 M ethanolamine (pH 8) and DS-Cav1-immobilized biosensors were then coupled reaction in acetate buffer (pH 5). The reaction was quenched by 1 M ethanolamine (pH 8) and DS-Cav1-immobilized biosensors were then coupled reaction in acetate buffer (pH 5). The reaction was quenched by 1 M ethanolamine (pH 8) and DS-Cav1-immobilized biosensors were then coupled reaction in acetate buffer (pH 5). The reaction was quenched by 1 M ethanolamine (pH 8) and DS-Cav1-immobilized biosensors were then coupled reaction in acetate buffer (pH 5). The reaction was quenched by 1 M ethanolamine (pH 8) and DS-Cav1-immobilized biosensors were then coupled reaction in acetate buffer (pH 5). The reaction was quenched by 1 M ethanolamine (pH 8) and DS-Cav1-immobilized biosensors were then coupled reaction in acetate buffer (pH 5). The reaction was quenched by 1 M ethanolamine (pH 8) and DS-Cav1-immobilized biosensors were then.
F-VHH escape mutant viruses. A dilution series of RSV A Long was used to infect Vero cells in the presence of a dose corresponding to 20, 80 or 320 times the IC50 of F-VHH-4 or F-VHH-L66. After 6 days of infection, the proportion of the cells infected with the highest dilution of RSV for which virus could be detected (observed by plaque assay), was used to infect a subsequent series of Vero cells in the presence of VHH or palivizumab. After two and four passages in presence of F-VHH-L66 and F-VHH-4, respectively, single RSV isolates were obtained by serial dilution which were amplified on Vero cells. Total RNA was isolated from the resulting virus stocks, cDNA was prepared and the F gene was amplified and sequenced with following FW and RV primers (5′ATCAAGGCTTAAACATGGGATGGAATTCCTA5′, 5′AACCCTGCGATTGTTACTAATATTGCAATAT5′). A plaque-reduction assay with these virus isolates was performed.

Surface plasmon resonance. Purified DS-Cav1 with a C-terminal Strep-tag II and 6X HisTag was captured on an NTA sensor chip to ~530 response units (RU) each cycle using a Biacore X100 (GE Healthcare). The NTA sensor chip was regenerated between cycles using 0.25 M EDTA followed by 0.5 mM NaCl. A buffer-only sample was injected over the DS-Cav1 and reference flow cells, followed by F-VHH-4 or F-VHH-L66 2-fold serially diluted from 5 nM to 39.1 pM in HBS-P+, with a duplication of the 1.25 nM concentration. The data were double-reference subtracted and fit to a 1:1 binding model using Scrubber.

Protein production for crystallization. Freestyle 293-F cells (R79007, Invitrogen) were transfected with plasmid encoding stabilized prefusion RSV F (DS-Cav1)13. Proteins were expressed in the presence of kinfumine (5 μM) and purified from cell supernatants using Strept-Tactin resin (IBA). Tags were removed by thrombin digestion and glycans were removed by digestion with Endo H (10% w/v) for 2 h at room temperature. Purified DS-Cav1 was combined with a 1.5-fold and 2-fold molar excess of Pichia-expressed F-VHH-4 and F-VHH-L66, respectively, and separated from excess F-VHH using a Superose 6 column (GE Healthcare Biosciences). For optimization trials of the bound VHHs, vector encoding F-VHH-4 or -L66 with a C-terminal 3C cleavage site, Twin-Strep-tag and 8X HisTag were transfected into FreeStyle 293-F cells. Proteins were purified from cell supernatants using Strept-Tactin resin before tag removal by HRV 3C digestion. VHHs were separated from cleaved tags using a Superdex 75 column.

Crystalization data collection. F-VHH-4 crystals were produced by sitting-drop vapour diffusion by mixing 1 μl of F-VHH-4 (4 μg/ml) with 1 μl of reservoir solution containing 3% (v/v) PEG 8000, 2.2 M ammonium sulphate, 0.2 M sodium chloride and 0.1 M HEPES pH 7.5. Crystals were soaked in reservoir solution lacking 0.2 M sodium chloride and supplemented with 25% 2R,3R-butanediol and frozen in liquid nitrogen. Diffraction data were collected to 1.87 Å at SBC beamline 19-ID (Advanced Photon Source, Argonne National Laboratory).

Crystals of F-VHH-4 in complex with DS-Cav1 were produced by hanging-drop vapour diffusion by mixing 2 μl of the complex (2.45 mg/ml) with 1 μl of reservoir solution containing 0.2 M ammonium citrate pH 4.5, 14.75% (v/v) PEG 3350 and 8.85% (v/v) isopropanol. Crystals were soaked in reservoir solution supplemented with 30% (v/v) ethylene glycol and frozen in liquid nitrogen. Diffraction data were collected to 3.2 Å at GM/CA beamline 3-1D (APS, Argonne National Laboratory).

Crystals of F-VHH-66 in complex with DS-Cav1 were produced by free-interface diffusion by mixing 2 μl of the complex (4.79 mg/ml) with 2 μl of 0.05 M sodium citrate, 0.1 M KCl and 20% (w/v) PEG 4000 in a CryoOptimization Chip (Microlytic). Crystals were soaked in crystallization solution supplemented with 30% (v/v) ethylene glycol and frozen in liquid nitrogen. Diffraction data were collected to 3.8 Å at SBC beamline 19-ID.

Structure determination. All data were indexed and integrated in iMOSFLM43 and scaled and merged using AIMLESS44. F-VHH-4 crystals formed in space group P212121 and scaled and merged using AIMLESS. F-VHH-4 and -L66 crystals formed in space group P212121 and scaled and merged using AIMLESS. F-VHH-4 and -L66 crystals were built manually in Coot and refined with PHENIX using NCS torsion restraints and reference-model restraints to an Rmerge/Rfree of 18.4/23.4% and 24.8/28.6%, respectively. Complete data collection and refinement statistics are presented in Table 1 and stereo views of a portion of the electron density for each structure are shown in Supplementary Fig. 18.

HMPV microneutralization test. Predetermined amounts of GFP-expressing hMPV recombinant viruses (NL/1/00 A1 sublineage or NL/1/99 B1 sublineage, a kind gift of Bernadette van den Hoogen and Ron Fouchier, Rotterdam, the Netherlands) or GFP-pRSV (A2 strain, a kind gift of Mark Peeples, Columbus, Ohio, USA) were mixed with serial dilutions of VHH or monoclonal antibodies before being added to cultures of Vero-118 cells. Forty-eight hours later, the medium was removed, PBS was added and the amount of GFP per well was measured with a Tecan microplate reader M200. Fluorescence values were represented as per cent of a virus control without antibody and IC50 values were calculated from the plotted curve.

Mice. Specific pathogen-free BALB/c mice were obtained from Charles River (Charles River Wiga, Sulzfeld, Germany). The animals were housed in a temperature-controlled environment with 12 h light/dark cycles; food and water were provided ad libitum. The animal facility operates under the Flemish Government License Number LA1400536. All experiments were done under conditions specified by law and authorized by the Institutional Ethical Committee on Experimental Animals (Ethical application EC2015-019).

Administration of VHHs and RSV A2 infection in mice. Mice, seven weeks old, were randomly distributed in experimental groups of six animals. They were slightly anesthetized by isoflurane before intranasal administration of VHHs, palivizumab or RSV challenge virus. Viruses were administered intranasally in a total volume of 5 μl PBS. Each group received 10 μg of F-VHH-4, 10 μg of Ctrl-VHH or 10 μg of palivizumab 4 h before infection with 104 PFU of RSV A2. The mock-infected group received 10 μg of Ctrl-VHH that is specific for a Junin virus protein.

Determination of lung viral titres by plaque assay. Five days after challenge, mice were euthanized, lungs were removed and homogenized by vigorous shaking. RSV A Long was used to determine the lung RSV load by qRT-PCR. To determine the lungs RSV load by qRT-PCR, total RNA from the cleared lung homogenates was prepared using the high-throughput RNA Tissue Kit (Roche). cDNA was synthesised using random hexamer primers and the High Pure RNA Tissue Kit (Roche) according to the manufacturer’s instructions. Next, cDNA was prepared by the use of random hexamer primers and the Transcriptor First Strand cDNA Synthesis Kit (Roche). The relative levels of genomic RSV M cDNA were determined by qRT-PCR using primers specific for the RSV A2 M gene (5′TCACCGAGCTCCACATAC5′ and 5′GCAAGTTCTTTC3′) and 5′TCATCGTCTTTTTC3′ in a CrystalOptimization Chip (Microlytic). The cDNA was then amplified and sequenced with following FW and RV primers (5′ACCCATGGGATGGAATTCCTA5′, 5′AACCCTGCGATTGTTACTAATATTGCAATAT5′). A plaque-reduction assay with these virus isolates was performed.

Analysis of pulmonary cell infiltration. The immune cell composition of bronchoalveolar lavages (BAL) was determined by analysing surface expression of MHC-II, CD3e, Siglec-F, CD4, CD8, CD11b and CD11c. Briefly, high-affinity Fc receptors (FcRs) were blocked by incubation with purified affinity Fc receptors (Fc Block, BD Pharmingen, 553142) for 30 min at 4°C. Subsequently, the cells were stained with MHC-II-εFluor450 (eBioscience, 48-5321-82, 1/500), CD3e-AlexaFluor488 (BD Pharmingen, 557666, 1/300), Siglec-F-PE (BD Pharmingen, 562068, 1/200), CD4-PerCP-Cy5.5 (BD Pharmingen, 555026, 1/300), CD8-PE-Cy7 (eBioscience, 25-0081-81, 1/300), CD11b-APC-Cy7 (BD Pharmingen, 557657, 1/500) and CD11c-APC (BD Pharmingen, 550261, 1/200) for 1 h at 4°C. After staining, samples were measured on an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA), and analysed using FlowJo X (TreeStar) software.

Testing lower doses of F-VHH-4 in mice. BALB/c mice, 7-weeks-old, were slightly anesthetized by isoflurane before intranasal administration of VHH or RSV challenge virus. VHH and RSV virus were formulated in PBS and administered in a total volume of 50 μl, which was distributed equally over the two nostrils. Each group of six mice received either 1, 3 or 10 μg of F-VHH-4 or 10 μg of Ctrl-VHH 4 h before infection with 106 PFU of RSV A2. The mock-infected group received 10 μg of Ctrl-VHH.

HMPV microneutralization test. Predetermined amounts of GFP-expressing hMPV recombinant viruses (NL/1/00 A1 sublineage or NL/1/99 B1 sublineage, a kind gift of Bernadette van den Hoogen and Ron Fouchier, Rotterdam, the Netherlands) or GFP-pRSV (A2 strain, a kind gift of Mark Peeples, Columbus, Ohio, USA) were mixed with serial dilutions of VHH or monoclonal antibodies before being added to cultures of Vero-118 cells. Forty-eight hours later, the medium was removed, PBS was added and the amount of GFP per well was measured with a Tecan microplate reader M200. Fluorescence values were represented as per cent of a virus control without antibody and IC50 values were calculated from the plotted curve.
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**Author contributions**
I.R., J.S.M., B.S.G., B.S. and X.S. planned the study. I.R., M.S.A.G., S.C.K., K.S., D.W., M.K., M.C., V.M. and J.S. performed the research. I.R., M.S.A.G., B.S., J.S.M. and X.S. wrote the manuscript with contributions from J.A.M. and B.S.G. All authors reviewed the manuscript before submission.

**Additional information**
**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** X.S., B.S., I.R., J.S.M., M.S.A.G. and B.S.G. are named as inventors on a patent pending entitled: ‘Single-domain antibody against RSV F protein’, US 62/181,522. The remaining authors declare no competing financial interests.

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Corrigendum: Potent single-domain antibodies that arrest respiratory syncytial virus fusion protein in its prefusion state

Iebe Rossey, Morgan S.A. Gilman, Stephanie C. Kabeche, Koen Sedeyn, Daniel Wrapp, Masaru Kanekiyo, Man Chen, Vicente Mas, Jan Spitaels, José A. Melero, Barney S. Graham, Bert Schepens, Jason S. McLellan & Xavier Saelens

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In this Article, the clinical isolate MAD/GM3_10/14 of respiratory syncytial virus (RSV) is incorrectly described as an RSV A isolate. MAD/GM3_10/14 has been identified as an RSV B isolate on the basis of its fusion protein sequence. The fusion protein sequences of the clinical isolates used in this study have been deposited in GenBank under accession codes MF361899 (MAD/GM2_2/12), MF361900 (MAD/GM2_12/12), MF361901 (MAD/GM2_13/12), MF361902 (MAD/GM2_14/12), MF361903 (MAD/GM3_10/14), MF361904 (MON/9/92), and MF361905 (MAD/GM3_7/13).