Nanobodies® Specific for Respiratory Syncytial Virus Fusion Protein Protect Against Infection by Inhibition of Fusion

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(see the editorial commentary by Graham et al, on pages xxx-xxx)

Despite the medical importance of respiratory syncytial virus (RSV) infections, there is no vaccine or therapeutic agent available. Prophylactic administration of palivizumab, a humanized monoclonal RSV fusion (F) protein–specific antibody, can protect high-risk children. Previously, we have demonstrated that RSV can be neutralized by picomolar concentrations of a camelid immunoglobulin single-variable domain that binds the RSV protein F (F-VHHb nanobodies). Here, we investigated the mechanism by which these nanobodies neutralize RSV and tested their antiviral activity in vivo. We demonstrate that bivalent RSV F–specific nanobodies neutralize RSV infection by inhibiting fusion without affecting viral attachment. The ability of RSV F–specific nanobodies to protect against RSV infection was investigated in vivo. Intranasal administration of bivalent RSV F–specific nanobodies protected BALB/c mice from RSV infection, and associated pulmonary inflammation. Moreover, therapeutic treatment with these nanobodies after RSV infection could reduce viral replication and reduced pulmonary inflammation. Thus, nanobodies are promising therapeutic molecules for treatment of RSV.

Infection by respiratory syncytial virus (RSV) is the leading cause of acute lower respiratory tract disease in children worldwide. RSV infects approximately 90% of all children at least once before the age of 2 [1]. As RSV infections themselves do not evoke long-living immune protection, they recur throughout life, causing also considerable morbidity and mortality in the elderly [2]. It has been estimated that RSV infects about 64 million people annually and results in 160 000 deaths. Although most RSV infections cause only moderate symptoms, about 0.5% of children with a primary RSV infection require hospitalization because of bronchiolitis or pneumonia. RSV infections in early life are associated with long-term respiratory distress [3].

Despite the major importance of RSV infections, no vaccine or effective antiviral therapy is available. However, a particular prophylactic treatment can reduce RSV-associated hospitalization of high-risk infants by 55% [4]. This prophylactic therapy is based on monthly intramuscular administration of large amounts (15 mg/kg) of a humanized monoclonal antibody, palivizumab (Synagis), which is directed against an epitope in the antigenic region II of the RSV fusion protein. Because this treatment is very expensive, it cannot be used generally [5].

Camelids and sharks express not only conventional antibodies but also functional antibodies composed only of heavy chains (HcAbs) [6]. In these antibodies, antigen binding is confined to 1 single-variable domain (VHH). Recombinant VHH molecules, called nanobodies, can

bind to epitopes with high affinity and specificity. Because of their small size and extended CDR3 loops, nanobodies can bind to epitopes that are normally inaccessible for conventional antibodies or their fragments. Nanobodies are attractive tools for therapy and research because they can be produced by microorganisms, they are easy to format, and they remain stable even in stringent conditions [7]. Previously, we described a panel of nanobodies that can bind RSV F protein with high affinity and at subnanomolar concentrations inhibit RSV replication in vitro [8]. Here, we investigated the molecular mechanism by which the most potent of these nanobodies inhibits RSV replication and tested its ability to suppress RSV replication in mice.

MATERIALS AND METHODS

Nanobodies, Cells, and Virus

RSV F protein (F-VHHm and F-VHHb)–specific and H5N1-HA (H5-VHHb)–specific nanobodies used in this study have been described previously [8, 9]. Hep-2, Vero, and A549 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, and 2 mM L-glutamine at 37°C in the presence of 5% carbon dioxide. RSV A2 was propagated on Hep-2 cells and quantified on Vero cells by plaque-reduction assay using goat anti-RSV serum (AB1128, Chemicon International).

Plaque Reduction Assay

Nanobodies or antibodies were diluted in serum-free medium and incubated with RSV for 30 minutes at 37°C, and used to infect Vero cells. After 3 hours, the cells were washed 3 times with growth medium containing 2% FCS and incubated for 72 hours in growth medium containing 2% FCS and 0.6% avicel RC-851 (FMC Biopolymers). We tested viral infection by immunostaining of the viral plaques with goat anti-RSV serum. Alternatively, to assess inhibition of virus–cell fusion, we added the nanobodies or antibodies to cells that had been preincubated with RSV at 4°C for 2 hours.

Attachment Assay

RSV (5×10^6 plaque-forming units [PFU]; multiplicity of infection [MOI] = 500) was incubated with nanobodies, palivizumab, or dextran sulfate for 30 minutes at room temperature and then chilled for 15 minutes on ice. The mixtures were added to chilled Vero cells and incubated for 2 hours at 4°C. After washing 5 times with cold phosphate-buffered saline (PBS), the cells were fixed with 4% paraformaldehyde. The binding of RSV virus to the cell surface was tested by immunostaining with goat anti-RSV serum and the nuclear stain 4',6-diamidino-2-phenylindole (DAPI).

In Vivo Prophylaxis and Therapeutics

Female BALB/c mice at the age of 7–8 weeks and housed in a specific pathogen–free (SPF) facility were treated with cyclophosphamide (CP) by subcutaneous injections: 3 mg/kg

9 days before death, and 2 mg/kg 6, 4, and 2 days before death. At the indicated times, mice were slightly anesthetized by isoflurane, and 50 μL of PBS, nanobodies, antibodies, or RSV A2 was administered intranasally. We killed the mice 5 days postchallenge; we removed the lungs and homogenized them with a Heidolph RZR 2020 homogenizer in 1.0 or 1.5 mL PBS. We cleared lung homogenates by centrifugation ($1000 \times g$) for 15 minutes at 4°C and used to titrate the virus by plaque-reduction assay. We set lung homogenates in which no virus was detected as the detection limit of the assay.

Immunostaining

We mock-infected or infected A549 cells with 0.5 MOI of RSV A2 in serum-free medium. After 4 hours, we washed away free virus and incubated the cells in growth medium containing 1% FCS. After 16 hours, the cells were washed, fixed with 2% paraformaldehyde, and blocked in PBS containing 1% BSA (PBS/BSA) for 1 hour. Then, we added RSV F nanobodies to a final concentration of 5 μg/mL and continued incubation for 1 hour. We washed cells and added goat anti-RSV serum. We detected nanobodies with polyclonal rabbit anti-nanobody antiserum followed by anti–rabbit immunoglobulin G (IgG) antibodies labeled with Alexa 488. Goat anti-RSV antibodies were revealed with anti-goat IgG antibodies labeled with Alexa 568. Confocal images of the stained cells were recorded with a Zeiss confocal microscope.

Analysis of Pulmonary Cell Infiltration

For this analysis, we used normal immune-competent BALB/c mice. Bronchoalveolar lavage (BAL) immune cell composition was determined on a LSR-II flow cytometer (BD Biosciences) by analyzing cellular autofluorescence and surface expression of CD3ε, CD4, CD8a, CD11b, CCR3, MHC-II, and CD11c, similar to the protocol described in Bogaert et al [10]. All antibodies were from Pharmingen (BD Biosciences) except for CCR3 (R&D Systems).

Statistical Analysis

We used GraphPad Prism 4 for statistical analyses. We used the Mann–Whitney U test to evaluate differences between 2 groups.

RESULTS

Nanobodies Specific for RSV F Protein Do Not Inhibit RSV Attachment to Cells

F-VHHb (RSV-D3(15GSS)2 in [8]) is a bivalent nanobody composed of 2 identical nanobodies fused by a 15 amino acid–long glycine–serine (GS) linker. We previously demonstrated that this nanobody, which binds to immunogenic region II of the F protein, is a potent inhibitor of RSV replication in vitro [8]. Here, we studied the mechanism by which this RSV-F–specific nanobody neutralizes RSV infection. To test whether F-VHHb nanobodies can neutralize RSV by blocking viral entry,

we performed a plaque-reduction assay in which the inoculums, containing the virus premixed with PBS, F-VHHb, palivizumab, or a negative control antibody H5-VHHb, were washed away 3 hours after infection. Figure 1A shows that F-VHHb and palivizumab readily inhibited viral entry with a half maximal inhibitory concentration (IC_{50}) of, respectively, 0.056 ± 0.012 nM ($\sim 1.7 \pm 0.36$ ng/mL) and 1.03 ± 0.75 nM ($\sim 154.5 \pm 112.5$ ng/mL).

In vitro, RSV entry is the result of viral attachment and subsequent fusion of the viral membrane with the plasma membrane of the cell. Viral attachment can be prevented by antibodies that either coat or cross-link virions, or specifically block the interaction between a viral membrane protein and its receptor on the cell [11]. Although the RSV G protein has been shown to be involved in viral attachment, the RSV F protein can also mediate attachment independently of the G protein [12–14].
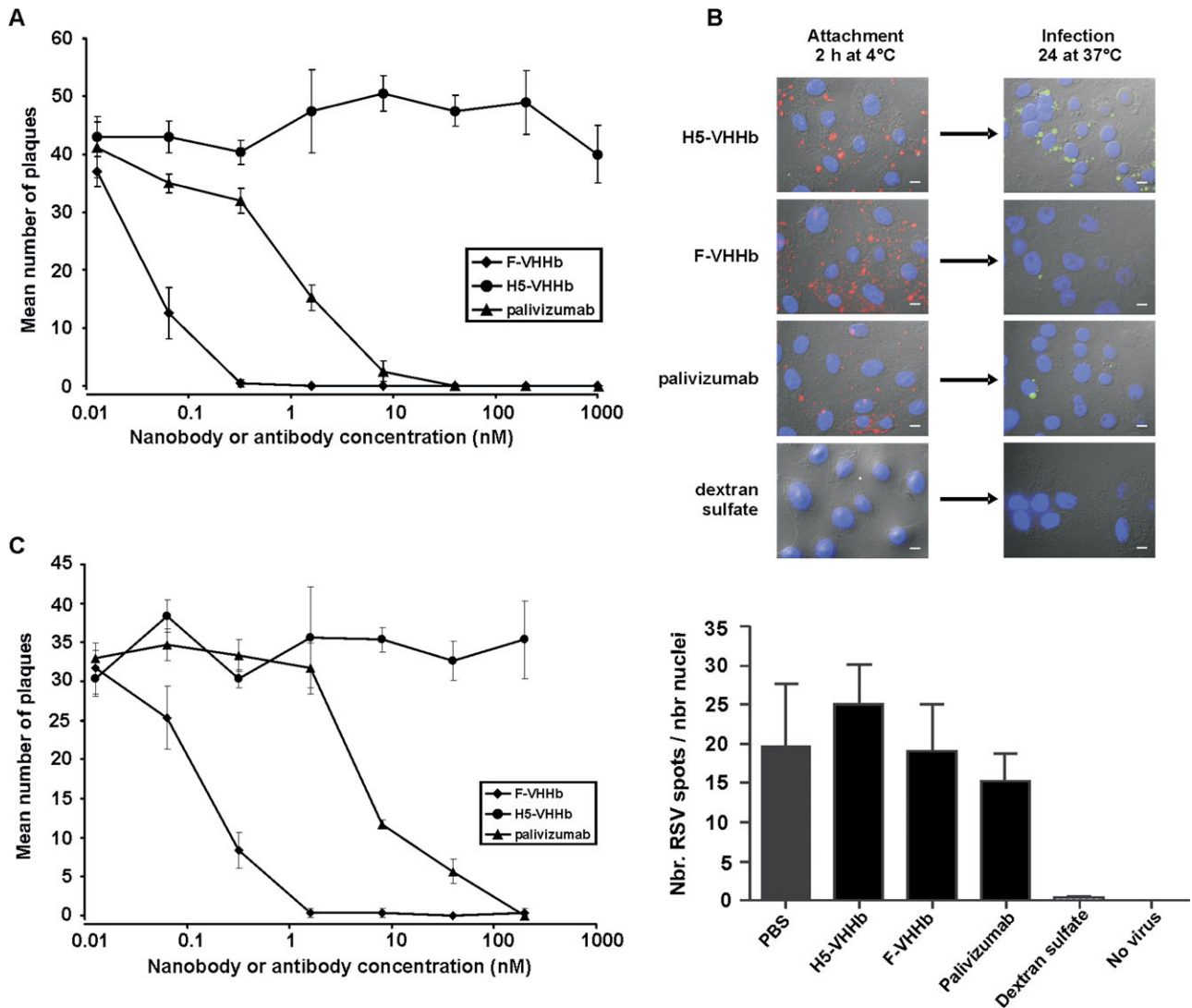

Figure 1. Respiratory syncytial virus (RSV) F–specific nanobodies inhibit RSV replication by blocking viral fusion but not viral attachment. A, An RSV plaque-reduction assay was performed in which RSV inoculum virus was preincubated with the indicated nanobodies or palivizumab before infection. B, RSV virions were allowed to attach to Vero cells at 4°C in the presence of nanobodies, palivizumab, or dextran sulfate, as indicated. Inocula were washed away 2 hours after infection and cells were fixed and immunostained with an RSV-specific polyclonal serum (red, left panels). In a parallel setup, infection was continued for 22 hours at 37°C and was followed by immunostaining with anti-nucleoprotein (green, right panels). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue), scale bar = 10 μm. For quantification of cell-bound RSV virions, 3 images were taken for each condition and analyzed by Volocity software (Perkin Elmer). For each image, the ratio between the number of RSV-positive spots as detected by the use of goat anti-RSV serum and the number of nuclei was calculated. The bars represent the average calculated ratios ± the standard deviation of 3 images. C, A plaque-reduction assay in which the nanobodies or palivizumab was added to Vero cells that had been preincubated with RSV at 4°C for 2 hours to allow viral attachment. The inocula were subsequently washed away and the cells were treated with the indicated concentrations of nanobodies or palivizumab for 30 minutes at 4°C and an extra 2 hours at 37°C. Subsequently, infection was allowed to proceed for 3 days.

To test whether F-VHHb nanobodies could inhibit RSV infection by preventing RSV attachment to the target cells, an attachment assay was performed. RSV was incubated with a high concentration of nanobodies or palivizumab. As positive control, we used dextran sulfate (30 μg/mL), a polyanion that prevents RSV attachment [15]. After incubation, the mixtures were added to the cells and incubated for 2 hours at 4°C, and then binding was assessed by immunostaining. In parallel, after incubation at 4°C, the same setup was incubated further for 22 hours at 37°C to allow infection. Figure 1B shows that although F-VHHb nanobodies and palivizumab blocked RSV infection, they did not impair RSV attachment to target cells. These data indicate that F-VHHb nanobodies neutralize RSV infection after virus attachment.

RSV F Protein Specific Nanobodies Block Viral Fusion

Following viral attachment, the RSV F protein mediates membrane fusion [16]. To investigate whether nanobodies neutralize RSV by blocking viral fusion, we tested a dilution series of F-VHHb nanobodies in a fusion-inhibition assay. We incubated chilled Vero cells with RSV A2 at 4°C to allow viral attachment to the cells without membrane fusion. We added different F-VHHb, H5-VHHb nanobodies, or palivizumab to the cells 2 hours later. After incubation for another 30 minutes at 4°C and 2 hours at 37°C, we washed the cells and incubated them at 37°C for 3 days to allow infection and plaque formation. Addition of F-VHHb nanobodies or palivizumab after viral attachment resulted in inhibition of viral infection: IC_{50} of 0.146 nM and 5.76 nM, respectively (Figure 1C). Because blocking RSV infection by nanobodies after viral attachment was almost as efficient as before attachment, we conclude that F-VHHb nanobodies prevent RSV entry mainly by blocking viral fusion.

RSV F–Specific Nanobodies Inhibit Syncytium Formation

In addition to fusion of viral membranes with the plasma membrane of target cells, the RSV F protein can also induce the formation of multinucleated syncytia by fusion of the membrane of an infected cell with that of a neighboring cell. To determine whether binding of F-VHHb nanobodies to F protein expressed at the surface of infected cells (Supplementary Figure 1A) can prevent cell–cell fusion, syncytium formation assays were performed. At 8 hours after infection, we incubated cells with different amounts of nanobodies or antibodies for 2 days to allow syncytium formation and replication. The number of syncytia and the number of nuclei per syncytium were determined after immunostaining with anti-RSV serum, a membrane stain, and DAPI. Addition of F-VHHb nanobodies or palivizumab after infection efficiently reduced the number of syncytia and their size (Figure 2). Remarkably, F-VHHb nanobodies (IC_{50}, 0.063 nM ± 0.026 nM) and palivizumab (IC_{50}, 5.57 nM ± 0.245 nM) inhibited syncytium formation as efficiently as in the RSV neutralization assay.

Intranasal Administration of Nanobodies Specific for RSV F Protein Protects Against RSV Infection In Vivo

We next tested whether RSV F–specific nanobodies can protect against RSV infection *in vivo*. We used a BALB/c mouse RSV infection model. To enhance RSV replication, we treated mice with the immunosuppressive drug cyclophosphamide (CP) [17, 18]. We infected CP-treated mice with RSV 4 hours after intranasal administration of 100 μg (5 mg/kg) F-VHHb, H5-VHHb, palivizumab, or PBS. We killed the mice 5 days later and determined RSV replication levels in the lungs. In contrast to mice treated with PBS or H5-VHHb nanobody, mice treated with 100 μg of F-VHHb or palivizumab showed no detectable RSV in lung homogenates (Figure 3A). Similarly, we detected no RSV 3 days after infection in the lungs of mice treated with F-VHHb or palivizumab (Supplementary Figure 1B). Lung samples prepared 3 days after intranasal nanobody administration still contained nanobodies that can neutralize RSV in vitro (Supplementay Figure 1C) and hence could interfere with the RSV plaque-reduction assay used to determine the RSV pulmonary titer. We did not observe this for mice treated with palivizumab. However, we confirmed protection against RSV infection 3 days after challenge by RSV-specific quantitative real-time polymerase chain reaction (qPCR; Supplementary Figure 1B).

To compare the efficacy of intranasally administered monovalent and bivalent nanobodies, we administered to CP-treated mice different amounts of F-VHHm or F-VHHb nanobodies intranasally 4 hours before RSV challenge. Previously, we have shown that the monovalent counterpart of the bivalent F-VHHb (F-VHHm) nanobody is about 1/4000 as effective in blocking RSV infection in vitro [8]. Figure 3B shows that in the lungs of mice treated with 60 μg (3 mg/kg) or 12 μg (0.6 mg/kg) F-VHHb, residual RSV level was below detection level or at least one-hundredth the level found in mice that had received a similar molar amount of F-VHHm. We also observed a significant but more moderate reduction in viral titer in mice that were treated with 2.4 μg (0.12 mg/kg) or 0.48 μg (0.024 mg/kg) F-VHHb. In addition, prophylactic nanobody treatment prevented the accumulation of viral RNA (Supplementary Figure 1D).

Protection Against RSV Infection by Intranasal Administration of Nanobodies Is Long Lasting

Next, we examined the duration of protection by a single prophylactic dose of nanobodies. We infected CP-treated mice with RSV 24, 48, or 72 hours after intranasal administration of a single dose of 60 μg (3 mg/kg) of F-VHHb. Intranasal administration of RSV F–specific nanobodies up to 48 hours before RSV challenge strongly reduced viral replication in the lungs. More specifically, no replicating virus was detected in the lungs of 5 of 5 and 4 of 6 mice that had been treated with nanobodies 24 or 48 hours before infection, respectively (Figure 4). Administration of RSV F–specific nanobodies 72 hours before RSV challenge significantly reduced viral replication but did not
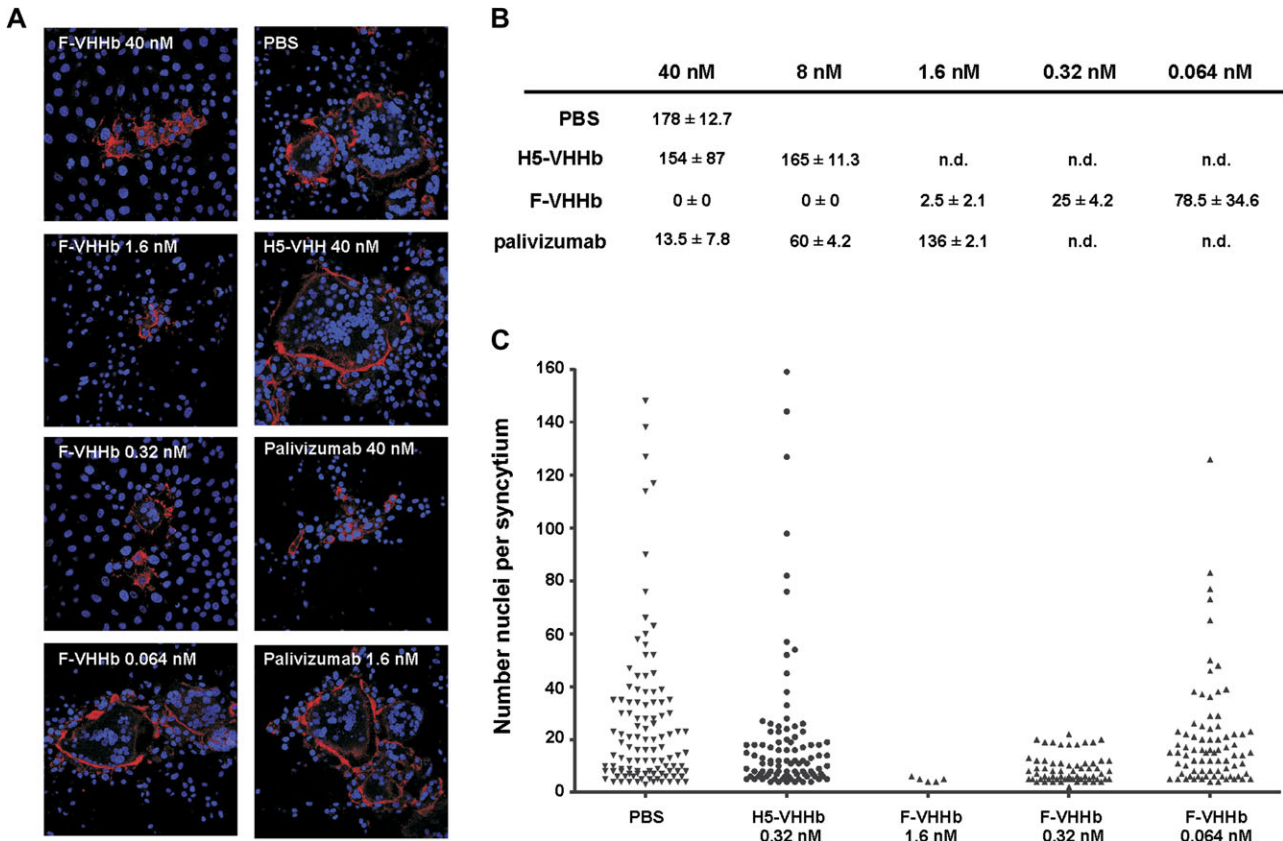
Figure 2. Respiratory syncytial virus (RSV) fusion protein–specific nanobodies inhibit syncytium formation and growth. *A*, Different amounts of nanobodies or palivizumab were added to Vero cells that had been infected with RSV for 8 hours. After an additional 48 hours, the cells were fixed and stained with anti-RSV serum (red) and 4',6-diamidino-2-phenylindole (DAPI; blue) to visualize syncytia. *B*, Quantification of the number of syncytia per well; and *C*, the number of nuclei per syncytium.

The table in panel B:

	40 nM	8 nM	1.6 nM	0.32 nM	0.064 nM
PBS	178 ± 12.7				
H5-VHHb	154 ± 87	165 ± 11.3	n.d.	n.d.	n.d.
F-VHHb	0 ± 0	0 ± 0	2.5 ± 2.1	25 ± 4.2	78.5 ± 34.6
palivizumab	13.5 ± 7.8	60 ± 4.2	136 ± 2.1	n.d.	n.d.

prevent it. Whereas treatment at 24 and 48 hours prior to infection also extensively reduced the amount of RSV RNA in the lungs, the reduction of RSV RNA levels was smaller and not significant when the F-VHHb nanobodies were administered 72 hours prior to infection (Supplementary Figure 1E). These data indicate that pulmonary delivery of nanobodies can protect against RSV infection for at least 48 hours.

Therapeutic Efficacy of RSV F–Specific Nanobodies

RSV-specific therapeutics that are effective after the onset of RSV infection are urgently needed. Therefore, we also investigated whether RSV F–specific nanobodies can hamper RSV replication in mice when administered after infection. We administered bivalent RSV F–specific nanobodies (60 μg) intranasally to CP-treated BALB/c mice 4, 24, or 48 hours after RSV infection. We killed the mice 5 days later to determine the pulmonary viral titer. Figure 5 shows that we detected a virus level of one-hundredth the original level in the lungs of mice that were treated with RSV F–specific nanobodies either 4 or 24 hours after infection. Mice treated with RSV F–specific nanobodies 48 hours after RSV challenge displayed even lower levels of RSV (Supplementary Figure 1F). However, the

reduction in RSV lung titer in these mice might in part be explained by the presence of sufficient residual nanobodies in the lung homogenates to neutralize RSV infection in the plaque-reduction assay used to determine the pulmonary viral titer (Supplementary Figure 1F).

RSV-Neutralizing Nanobodies Can Reduce Pulmonary Inflammation and Morbidity

To test if prophylactic or therapeutic treatment with RSV F–specific nanobodies can affect RSV-induced pulmonary inflammation and morbidity, BALB/c mice (not treated with cyclophosphamide) were infected with 1.10^7 PFU RSV either 5 hours after or 24 hours before intranasal administration of nanobodies (20 μg). Figure 6A illustrates that prophylactic and therapeutic treatment respectively, could prevent and reduce RSV induced body weight loss. In addition prophylactic treatment of mice with RSV neutralizing nanobodies could prevent the infiltration of T cells and neutrophils in the lungs of RSV infected mice (Figure 6B, C). Cellular infiltration was partially reduced by RSV–neutralizing nanobodies administered 24 hours after infection. Although prophylactic treatment almost completely blocked pulmonary RSV replication,
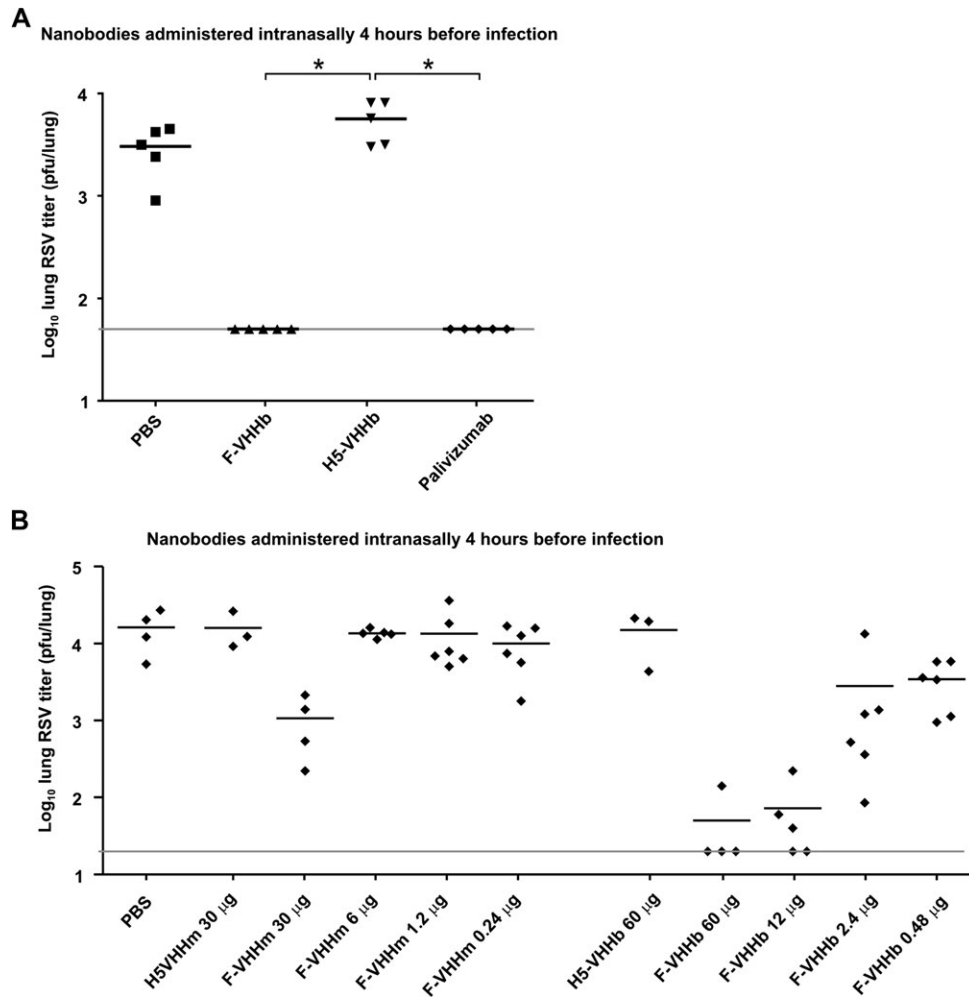
Figure 3. Prophylactic intranasal administration of respiratory syncytial virus (RSV) fusion protein–specific nanobodies protects mice against RSV infection. *A*, Phosphate-buffered saline or 100 µg of F-VHHb nanobody, H5-VHHb nanobody, or palivizumab was administered intranasally to cyclophosphamide-treated BALB/c mice 4 hours before RSV infection. We killed the mice 5 days after RSV challenge determine the pulmonary RSV titer by plaque-reduction assay (detection limit = 50 plaque-forming units [PFU]). *P < .01. *B*, The indicated amounts of bivalent (F-VHHb) or monovalent (F-VHHm) nanobodies were administered 4 hours before RSV infection. We killed the mice 5 days after RSV challenge to determine the pulmonary RSV titer by plaque-reduction assay (detection limit = 20 PFU). P < .01 for F-VHHm (30 µg) vs H5-VHHm (30 µg) and for F-VHHb (60 µg), F-VHHb (12 µg), F-VHHb (2.4 µg), and F-VHHb (0.48 µg) vs H5-VHHb (60 µg).

in this experiment therapeutic administration of nanobodies only slightly reduced the levels of infectious RSV in the BAL fluid (Figure 6D).

DISCUSSION

We investigated how RSV F–specific VHHb nanobodies that bind to the antigenic site II of the RSV fusion protein neutralize RSV infection. Although the RSV F protein can also mediate RSV attachment [12–14] our data indicate that RSV F–specific VHHb nanobodies do not interfere with viral attachment but neutralize RSV by blocking membrane fusion. First, high concentrations of RSV F–specific VHHb nanobodies do not hamper the binding of virions to cells. Second, RSV F–specific VHHb nanobodies block viral infection when administered after viral attachment as

efficiently as when applied before infection. Third, RSV F–specific VHHb nanobodies can prevent the fusion of infected cells with neighboring noninfected cells with an efficiency (IC$_{50}$ ≈ 65 pM) that is comparable to viral neutralization. Viral neutralization by antibodies that block viral fusion has also been reported for other class I fusion proteins, such as those of Influenza viruses and HIV [19, 20]. Moreover, palivizumab, motavizumab, and mAb 47, which, like RSV F–specific VHHb nanobodies, target the antigenic region II of the RSV F protein, have recently been reported to neutralize RSV by blocking viral fusion [21, 22].

The RSV F protein mediates the fusion of the viral lipid membrane with the lipid membrane of the target cell by undergoing a series of conformational changes from the metastable prefusion form to the stable post fusion form [23]. To which of these RSV F conformations do nanobodies bind? The RSV
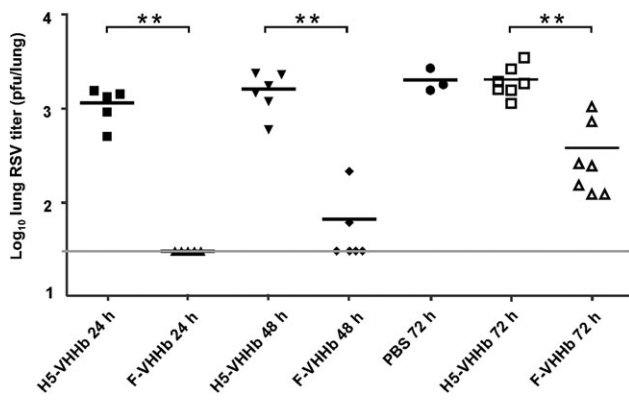
Figure 4. Nanobodies administered intranasally at indicated time points before infection. Suppression of respiratory syncytial virus (RSV) replication by prophylactic intranasal administration of nanobodies is long lasting. Phosphate-buffered saline (PBS) or 60 μg of F-VHHb or H5-VHHb was administered intranasally to cyclophosphamide-treated mice at the indicated time points before RSV infection. 5 days after RSV challenge, the mice were killed to determine the pulmonary RSV titer by plaque-reduction assay (detection limit = 30 PFU). **$P < .005$.

F–specific nanobodies are derived from lamas immunized with recombinant F proteins lacking the transmembrane region, F_{TM-}. Preparations of F_{TM-} resemble paramyxovirus postfusion F trimers [24]. Two recent reports demonstrated that the motavizumab epitope is maintained in the postfusion form of the RSV F protein [25, 26]. This explains why immunization with recombinant F protein in postfusion conformation can elicit nanobodies that could recognize the F protein in its prefusion or intermediate form. It is possible that our nanobodies bind to the F postfusion form, but also that binding to the F prefusion form or intermediates between the 2 states inhibits membrane fusion.
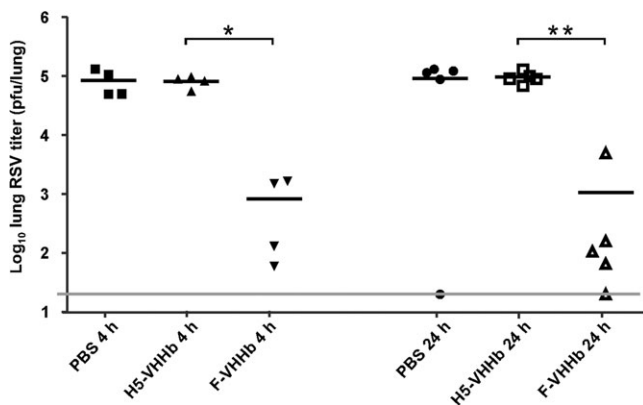

Figure 5. Nanobodies administered intranasally at indicated time points after infection. Therapeutic efficacy of intranasally administered respiratory syncytial virus (RSV) fusion protein–specific nanobodies against RSV infections. Phosphate-buffered saline (PBS) or 60 μg of F-VHHb or H5-VHHb nanobody was administered intranasally to cyclophosphamide-treated mice at the indicated time points after RSV infection. 5 days after RSV challenge, the mice were killed to determine the pulmonary RSV titer by plaque-reduction assay (detection limit = 20 PFU). *$P < .05$ and **$P < .005$.

Based on the reported structure of the prefusion form of the PIV5 F protein trimer, which likely resembles that of the RSV F protein, it is unlikely that Fab fragments or antibodies can bind to the antigenic region II without extensive clashes and thus considerable flexibility of the F trimer [27]. In this way, binding of Fab fragments or antibodies might block fusion merely by inducing and freezing conformational changes in the F protein trimer. Nanobodies might block fusion in the same way. However, because nanobodies are small ($\approx$15 kDa), their binding to antigenic region II might involve lesser clashes with the F trimer. If nanobodies could bind to the F trimer without inducing conformational changes, they might stabilize the F prefusion form and thereby prevent the conformational changes required for fusion. Alternatively, bound nanobodies might shield or interact with the fusion peptide and thereby prevent its insertion into the cell membrane.

RSV F–specific VHHb nanobodies are consistently more efficient in inhibiting viral and cell–cell fusion than palivizumab antibodies. Several factors might contribute to the high activity of these nanobodies. The antigenic region II within the RSV F trimer is likely not directly accessible for binding antibodies or Fab fragments [27]. Nanobodies are much smaller than antibodies and Fab fragments, they have an extended CDR3 loop, and they mediate antigen binding by 3 CDR loops instead of 6. So the antigenic region II within the RSV F trimer might be more accessible for nanobodies than for Fab fragments or antibodies. In addition, as the antigenic region II is likely located at the side of the F trimer head, on the dense surface of virions this site might be more accessible for small nanobodies [24]. Moreover, the 15 amino acid–long GS linker is likely more flexible than the hinge of an antibody. This might allow bivalent F-VHHb nanobodies to bind 2 F trimers more readily than conventional antibodies do. This is in agreement with the observation that bivalent F-VHHb nanobodies can neutralize RSV in vitro up to about 4000 times more efficiently than their monovalent counterparts, whereas palivizumab IgG is only about 180-fold more efficient than palivizumab Fab fragments [8, 28].

We examined whether RSV F–specific VHHb nanobodies can protect mice against RSV infection. As RSV replication is restricted to the epithelial cells of the respiratory tract, nanobodies were delivered intranasally. We demonstrate that intranasal administration of 0.6 mg/kg of RSV F–specific bivalent nanobodies strongly reduced RSV replication in mice, and this reduction was more moderate with monovalent RSV F–specific nanobodies. These data demonstrate that also *in vivo* bivalent RSV F–specific nanobodies are significantly more efficient inhibitors of RSV replication than their monovalent counterparts.

An effective and practical prophylactic intervention should preferentially have a long-lasting effect after a single administration. In our model, bivalent RSV F–specific nanobodies readily reduced RSV replication even when administered up to 48 hours before challenge, and RSV pulmonary titer was reduced five-fold
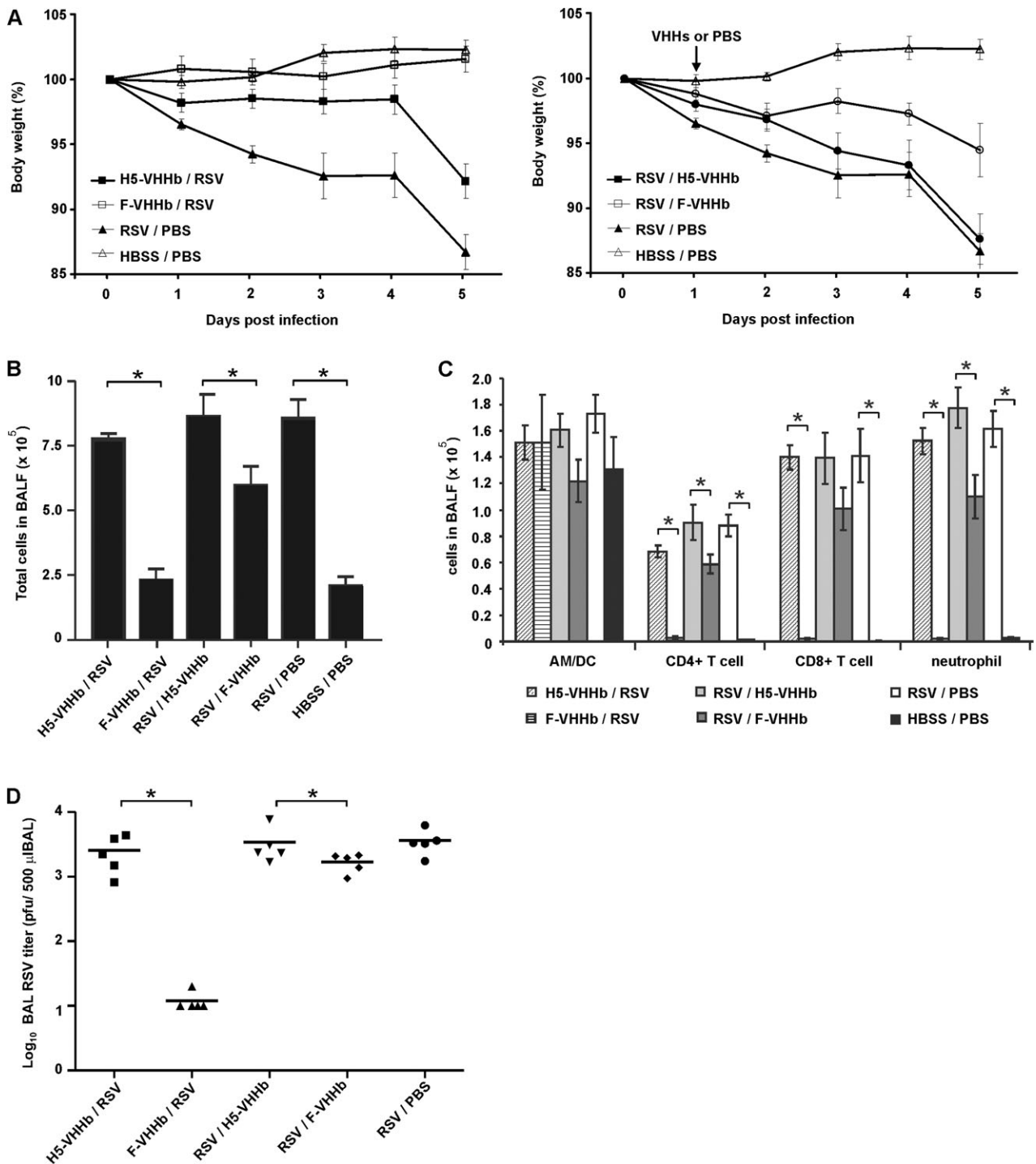
Figure 6. Respiratory syncytial virus (RSV)–neutralizing F-VHH nanobodies can prevent or reduce RSV-induced pulmonary inflammation and morbidity. Groups of 5 mice were treated intranasally with 20 μg F-VHHb or H5-VHHb nanobody either 5 hours before (F-VHHb/RSV and H5-VHH/RSV) or 24 hours after RSV infection (RSV/H5-VHHb and RSV/F-VHHb). RSV-infected mice that were therapeutically treated with phosphate-buffered saline (PBS) (RSV/PBS) and mock-infected mice (intranasal administration of 50 mL Hanks Balanced Salt Solution [HBSS]: HBSS/PBS) were used as additional controls. *A,* The relative body weight was monitored daily and presented as percentage of the initial body weight. The left panel presents the relative body weight ± standard error of the mean (SEM; n = 5) of the mice treated with nanobodies before infection and of mice treated with PBS 24 hours after infection (RSV/PBS) or of mock-infected mice (HBSS/PBS). At day 5 the body weight of F-VHHb treated mice is significantly different from the body weight of mice treated with RSV neutralizing F-VHH nanobodies: $P < .005$ (Mann–Whitney U test). The right panel presents the relative body weight ± SEM (n = 5) of the mice treated with nanobodies after infection (F-VHHb/RSV and H5-VHH/RSV), and again mice treated with PBS 24 hours after infection (HBSS/PBS) or mock-infected mice (HBSS/PBS). At days 3, 4, and 5, the body weight of F-VHHb–treated mice is significantly different from the relative

in mice treated up to 72 hours before infection (Figure 4). This agrees with our other observation that, 72 hours after intranasal administration, titers of residual nanobodies in lung homogenates are sufficient to neutralize virus in vitro. In addition, we also demonstrate that intranasal administration of nanobodies specific for RSV F 1 or 2 days after experimental RSV infection strongly reduced the pulmonary RSV titer (Figure 5). Finally we demonstrated that prophylactic and therapeutic nanobody treatment respectively, can prevent and reduce RSV induced pulmonary inflammation and morbidity in mice (Figure 6).

Several nanobodies have entered clinical trials. An RSV-neutralizing nanobody, delivered via the lungs, is expected to enter phase I clinical trial during 2011 (www.ablynx.com). A potential concern for using nanobodies is of course immunogenicity because of the llama origin. However, to reduce the risk of immunogenicity, nanobodies can be humanized. This is a straightforward procedure because nanobodies already display relatively high sequence homology to human heavy chain variable domains, typically between 80% and 90% when comparing the framework regions. However, as is the case for that of conventional antibodies, immunogenicity of nanobodies must be assessed case by case.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Potential conflicts of interest. W. V. and P. V. are employees of Ablynx NV, which has provided the recombinant VHH antibody fragments. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Figure 6 continued. body weight of mice treated with RSV-neutralizing F-VHH nanobodies: $P < .05$ (Mann–Whitney U test). *B*, 5 days after RSV infection, 6 mL bronchoalveolar lavage (BAL) fluid was prepared from each mouse. The total number of cells present in the BAL fluid was determined by manual counting. Bars present the average number of cells $\pm$ SEM (n = 5). *C*, The presence of neutrophils, CD4+ and CD8 + T cells, the count of alveolar macrophages plus dendritic cells in the BAL fluid was determined by flow cytometry: *$P < .05$ (Mann–Whitney U test). *D*, The levels of infectious RSV in the first 500 μL BAL fluid recovered from the mice was determined by plaque-reduction assay (detection limit is 10 plaque-forming units/500 mL BAL fluid; Mann–Whitney U test). *$P < .05$.

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