Protection and mechanism of action of a novel human respiratory syncytial virus vaccine candidate based on the extracellular domain of small hydrophobic protein

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Abstract

Infections with human respiratory syncytial virus (HRSV) occur globally in all age groups and can have devastating consequences in young infants. We demonstrate that a vaccine based on the extracellular domain (SHe) of the small hydrophobic (SH) protein of HRSV, reduced viral replication in challenged laboratory mice and in cotton rats. We show that this suppression of viral replication can be transferred by serum and depends on a functional IgG receptor compartment with a major contribution of FcγRI and FcγRIII. Using a conditional cell depletion method, we provide evidence that alveolar macrophages are involved in the protection by SHe-specific antibodies. HRSV-infected cells abundantly express SH on the cell surface and are likely the prime target of the humoral immune response elicited by SHe-based vaccination. Finally, natural infection of humans and experimental infection of mice or cotton rats does not induce a strong immune response against HRSV SHe. Using SHe as a vaccine antigen induces immune protection against HRSV by a mechanism that differs from the natural immune response and from other HRSV vaccination strategies explored to date. Hence, HRSV vaccine candidates that aim at inducing protective neutralizing antibodies or T-cell responses could be complemented with a SHe-based antigen to further improve immune protection.

Keywords alveolar macrophages; Fcγ receptor; human respiratory syncytial virus; small hydrophobic protein; vaccine

Subject Categories Immunology; Microbiology; Virology & Host Pathogen Interaction

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Introduction

Human respiratory syncytial virus (HRSV) is the most important viral cause of acute lower respiratory tract infections (ALRI) in infants worldwide (Liu et al, 2012). About 66,000–199,000 children die every year due to complications caused by HRSV infection (Nair et al, 2010). By the age of 2 years, most children have been infected at least once by HRSV (Glezen et al, 1986; Hall et al, 1991). Although in most children HRSV replication remains restricted to the upper respiratory tract, infection regularly spreads to the lower respiratory tract, causing bronchiolitis. This inflammation of the bronchioles is thought to result from massive HRSV infection of the bronchial and alveolar epithelial cells, resulting in sloughing of these cells and formation of clumps that occlude the small airways of the developing infant lungs (DeVincenzo et al, 2005; Welliver et al, 2008). In high-risk infants, severe HRSV bronchiolitis can be prevented by prophylactic treatment with palivizumab, a HRSV-neutralizing monoclonal antibody (IMpact-RSV Study Group, 1998). Palivizumab, or its affinity-matured variant motavizumab, has however no therapeutic benefit (Ramilo et al, 2014). A Cochrane study concluded that therapeutic treatment with aerosolized ribavirin, a guanosine analogue with antiviral activity against both RNA and DNA viruses, might reduce mortality and days of hospitalization in infants with severe HRSV infection (Ventre & Randolph, 2007). Due to its potential teratogenicity, ribavirin is not generally used to treat HRSV-associated illness. As there is no effective antiviral or anti-inflammatory therapy for HRSV, treatment in hospitals is mainly supportive and includes fluid and oxygen supply, and mechanical ventilation. Besides causing acute bronchiolitis, severe HRSV infections in infants can evoke recurrent wheezing at a later age and correlate with a predisposition to allergic asthma (Sigurs et al, 2000; Andreakos, 2012; Blanken et al, 2013).
The global burden caused by HRSV infections extends beyond very young children. A study performed by Nair et al (2013) estimated that annually, HRSV causes about 33.8 million cases of ALRI and 3.3 million cases of severe ALRI requiring hospitalization in children younger than 5 years. In industrialized countries, deaths due to HRSV ALRI are rare (0.7% of all severe ALRI) and occur almost exclusively in children younger than 1 year. However, in developing countries, fatal HRSV infections are more frequent (2.1% of all severe HRSV cases) and remain frequent at later ages (Nair et al, 2013). Furthermore, HRSV is increasingly being recognized as a major pathogen in elderly and immunocompromised adults, and even in previously healthy adults (Hall et al, 2001; Falsey et al, 2005; Luchsinger et al, 2012).

Despite the medical importance of HRSV and decades of intensive research, there is at present no licensed vaccine for this virus. A major obstacle and puzzle facing the development of a vaccine with long-lasting protection is the apparent inability of natural HRSV infections to elicit protective immunity. This is illustrated by the recurrence of HRSV infections in all age groups and the high rate of HRSV infections in infants with maternally derived HRSV-neutralizing antibodies (Henderson et al, 1979; Hall et al, 2001; Collins & Graham, 2008). Even healthy individuals with high levels of neutralizing serum antibodies can be reinfected, even with the same HRSV strain within, 2–6 months (Hall et al, 1991).

Vaccines used to prevent or treat infectious diseases aim at mimicking at least part of the host immune response that accompanies recovery from natural infection. In many cases, this implies the induction of neutralizing antibodies directed against major surface proteins of the pathogen. Likewise, most HRSV vaccines being developed aim at inducing HRSV-neutralizing antibodies directed against either the HRSV attachment protein (G) or the fusion protein (F) (Graham, 2011). This strategy has not yet produced an effective HRSV vaccine, but is reasonably still further explored.

We have explored an alternative, unconventional vaccination strategy to control HRSV infection. Next to the F and G proteins, HRSV also expresses a third membrane protein, the small hydrophobic (SH) protein (Olmsted & Collins, 1989; Collins et al, 1990). Although the exact function of SH remains poorly understood, it folds into pentameric cation-selective ion channels that can activate the NLRP3 inflammasome (Carter et al, 2010; Gan et al, 2012; Triantafillou et al, 2013). The importance of these functions remains unknown as recombinant HRSV that lacks SH expression is not attenuated in vitro and only slightly attenuated in mice and non-human primates (Bukreyev et al, 1997; Whitehead et al, 1999). Due to its small size and low abundance on HRSV virions, SH is poorly immunogenic upon natural infections (Connors et al, 1991; Akerlind-Stopner et al, 1993). We generated immunogens that allowed induction of IgG antibodies directed against the SH ectodomain (SHe) in laboratory mice and cotton rats. These SHe-specific antibodies lacked virus-neutralizing activity, readily bound to the surface of HRSV-infected cells and reduced HRSV replication in these two animal models. Based on selective immune cell depletion studies and passive transfer experiments, we also identified the mechanism of action of this novel HRSV vaccination approach.

**Results**

**SHe-specific immunity reduces HRSV replication**

Experimental or natural infection with HRSV leads to a poor humoral immune response toward the SH ectodomain (Akerlind-Stopner et al, 1993; Connors et al, 1991 and see below). Therefore, we first improved the immunogenicity of a peptide corresponding to SHe by linking it chemically to keyhole limpet hemocyanin (KLH). The SHe-KLH conjugate was used to immunize BALB/c mice in combination with incomplete Freund’s adjuvant (IFA). Using a SHe peptide-based ELISA, we found that immunization with SHe-KLH induced SHe-specific serum IgG1 and IgG2a antibodies and that the levels of IgG antibodies were increased by booster immunizations (Fig 1A–C). In contrast, little or no SHe-specific IgG antibodies were detected in mice infected with live HRSV (Fig 1C; Supplementary Fig S2C).

We then investigated whether SHe-specific serum antibodies from immunized mice could neutralize HRSV infections in an in vitro plaque reduction assay. In contrast to sera derived from HRSV A2 infected mice, high-titer SHe immune serum failed to neutralize this virus in vitro (Fig 1D). To test whether SHe-based immunization could counteract HRSV infections, mice were challenged with 1 × 10⁶ plaque-forming units (PFU) of HRSV A2. Compared to control vaccinated animals, all SHe-KLH-immunized mice displayed significantly lower pulmonary HRSV titers at 5 days post-infection (Fig 1E). Furthermore, following challenge, SHe-KLH-immunized mice had a slightly higher body weight compared to both control groups (Fig 1F). SHe-specific antibodies were also induced by SHe peptides conjugated to virus-like particles derived from Hepatitis B core (HBC) protein and by SHe linked genetically to recombinant tetrameric and pentameric scaffold proteins, although these responses were less robust than those induced by SHe-KLH (data not shown).

To investigate whether the reduction of HRSV replication in SHe-KLH-vaccinated mice is short living or long living, BALB/c mice were vaccinated with KLH or SHe-KLH in combination with either IFA or Sigma Adjuvant System (SAS). As a negative control, mice were mock-vaccinated with PBS without adjuvant. Immunizations with IFA were performed three times, whereas immunizations with SAS were performed twice. Figure 2A and B show that mice immunized with SHe-KLH with either adjuvant had high levels of SHe-specific serum IgG1 and moderate levels of serum IgG2a at 3 weeks before viral challenge. Six weeks after the last immunization with IFA and 8 weeks after the last immunization with SAS adjuvant, the mice were challenged with 1 × 10⁶ PFU of HRSV A2. At six days post-challenge, all mice that were vaccinated with SHe-KLH had significantly lower lung HRSV titers as compared to KLH- or PBS-vaccinated mice (Fig 2C). Up till 6 days post-infection, no significant differences in body weight were observed, although there was a trend toward somewhat higher relative body weight for SHe-KLH-immunized mice as compared to KLH-immunized mice (Fig 2D). In a separate experiment, HRSV challenge was postponed to eleven weeks after the last immunization with KLH or SHe-KLH in combination with IFA. Supplementary Fig S1 shows that at eleven weeks after the last immunization, all mice had high SHe-specific IgG serum titers that were slightly lower than serum titers at 4 weeks after the last immunization. Supplementary Fig S1C illustrates that also when challenge with 1 × 10⁶ PFU, HRSV A2 is performed...
Figure 1. Immunization with SHe conjugate vaccine reduces HRSV replication in BALB/c mice.

SHe-KLH immunization induces SHe-specific serum IgG antibodies. BALB/c mice were immunized 3 times intraperitoneally with 20 µg KLH or SHe-KLH combined with incomplete Freund’s adjuvant or with PBS. Serum was collected one day before the first immunization and 20 days after each immunization.

A Endpoint IgG titers of pooled sera from each group (n = 6, except PBS group: n = 3) tested in a SHe peptide ELISA.
B Individual SHe-specific serum IgG titers obtained 20 days after the second boost immunization. Horizontal bars represent the median.
C SHe-specific serum IgG, IgG1, and IgG2 endpoint titers in pooled sera after the second boost immunization.
D SHe-KLH immune serum does not neutralize HRSV in vitro. An HRSV A2 in vitro neutralization assay using the indicated dilutions of pooled sera, obtained after the second boost vaccination of BALB/c mice immunized as in (A), was performed. RSV: serum from BALB/c mice that were previously infected with HRSV A2. The amount of viral antigen was quantified by ELISA using goat anti-HRSV immune serum. The graph shows the O.D. for each sample.
E Vaccination with SHe-KLH reduces HRSV A2 replication in the lungs of challenged BALB/c mice. The graph shows the number of PFU per lung for each mouse, sampled 5 days after challenge with 10^6 PFU of HRSV A2. Horizontal bars depict the median value (Dunn’s multiple comparisons test).
F Challenged SHe-KLH-immunized BALB/c mice do not display weight loss. The graphs are representative for two independent experiments, and horizontal bars depict the mean value.

11 weeks after the last immunization, SHe-KLH-vaccinated mice had significantly lower lung HRSV titers as compared to KLH-vaccinated mice. Together, these data indicate that the protection afforded by SHe-based vaccination is relatively long living.

The amino acid sequence of SHe is highly conserved among the group A HRSV but differs substantially from SHe in group B HRSV where it is also sequence conserved (Supplementary Figs S10 and S11) (Collins et al., 1990). Consequently, it is likely that vaccination against HRSV B subgroup viruses would require a subgroup-B-matched SHe (SHe-B). Immunization of mice with an SHe-B peptide conjugated to HBC-VLP-induced SHe-B-specific serum IgG that reacted poorly with SHe of subgroup A HRSV (Supplementary Fig S2A–C). These sera however readily bound to the surface of cells infected with HRSV B (Supplementary Fig S2D). To test whether SHe-B immunization affects HRSV B replication in mice, we investigated whether replicating virus could be isolated from the lungs of BALB/c mice infected with a laboratory strain of HRSV B (HRSV B1) or with HRSV B clinical isolates (JX576729, JX576730, JX576731 in Supplementary Fig S11) (Tan et al., 2013a,b). Despite several attempts, we could not isolate replicating virus from the lungs of mice that had been infected with any of these HRSV B strains. However, infection of BALB/c mice with clinical HRSV B isolates caused significant body weight loss and pulmonary infiltration of leukocytes (data not shown). Therefore, as a read out for protection against HRSV B, we monitored body weight loss and cellular infiltration of the lungs of mice infected with clinical HRSV B isolate JX576731. BALB/c mice were vaccinated three times with HBC-SHeB, with unconjugated HBC, both in combination with IFA, or were mock vaccinated with PBS (Supplementary Fig S2). Due to the high degree of conservation of the F protein between HRSV A and HRSV B subgroup viruses, HRSV A infections induce HRSV B neutralizing antibodies (White et al., 2005). Therefore, prior infection with HRSV A2 was included as a positive control. Three weeks after the last immunization, the mice were challenged with 1.5 x 10^6 PFU of HRSV B. Seven days after infection, there was a trend toward lower body weight loss, less pulmonary CD8+ T-cell infiltration, and lower levels of HRSV B-specific RNA in the lungs of HBC-SHeB-vaccinated mice as compared to HBC-vaccinated control mice (Supplementary Fig S2E–G). Prior infection with HRSV A strongly reduced the levels of HRSV B-specific RNA in the lungs but failed to prevent body weight loss and pulmonary cell infiltration. These data suggest that SHe-B based vaccination might also hamper HRSV B replication in vivo. To test the potential cross-reactivity between SHe of HRSV A and HRSV B viruses, KLH, SHe-KLH, and HBC-SHeB immune sera were tested in a SHeA and SHeB peptide ELISA. Supplementary Fig S2I–J show that SHeA peptide is bound by SHeA-KLH immune serum and not by HBC-SHeB immune serum. Accordingly, SHeB peptide can only be bound by HBC-SHeB.
immune serum and not by SheA-KLH immune serum. Hence to cover both HRSV A and HRSV B viruses, a She-based vaccine should contain both the SheA and SheB antigen.

She-based vaccination reduces HRSV replication in cotton rats

Cotton rats are more permissive for HRSV infection than laboratory mice (Byrd & Prince, 1997). Therefore, we also evaluated She-based immune protection in this animal model. Cotton rats (six animals per group) were immunized three times intraperitoneally with KLH or She-KLH in combination with Sigma Adjuvant System (KLH SAS and She-KLH SAS). A third immunization, the latter two groups received PBS without adjuvant. As negative control, one group of six mice was vaccinated with PBS without adjuvant (PBS). All immunizations were performed every 2 weeks. Serum was collected 20 days after each immunization. Six weeks after the last immunization with incomplete Freund’s adjuvant and 8 weeks after the last immunization with Sigma Adjuvant System, the mice were challenged with $1 \times 10^6$ PFU HRSV A2. Six days after challenge, the lungs were collected to determine the pulmonary HRSV titer by plaque assay.

A She-KLH immunization induces She-specific serum IgG1 antibodies. The graph shows the She-specific IgG1 serum endpoint titers of each mouse at 3 weeks before viral challenge as determined by She peptide ELISA. Horizontal bars indicate the mean IgG1 titers.

B She-KLH immunization induces She-specific serum IgG2a antibodies. The graph shows the She-specific IgG2a serum endpoint titers of each mouse at 3 weeks before viral challenge as determined by She peptide ELISA. Horizontal bars indicate the mean IgG2a titers.

C Vaccination with She-KLH reduces HRSV A2 replication in the lungs of challenged BALB/c mice. The graph shows the number of PFU per lung for each mouse, sampled 6 days after challenge with $1 \times 10^6$ PFU of HRSV A2. Horizontal bars represent the median (one-way ANOVA Dunn’s multiple comparisons test).

D Vaccination with She-KLH is not associated with enhanced body weight loss upon HRSV infection. The graph shows the relative body weight at day 6 post-infection calculated as the ratio between body weight at day 6 and body weight at day 0. Horizontal bars represent the median.

Figure 2. The reduction of HRSV in She-KLH-immunized mice is not short living.

Two groups of 8 BALB/c mice were immunized three times intraperitoneally with KLH or She-KLH combined with incomplete Freund’s adjuvant (KLH IFA and She-KLH IFA). In parallel, two groups of 7 mice were immunized two times intraperitoneally with KLH or She-KLH in combination with Sigma Adjuvant System (KLH SAS and She-KLH SAS). As a negative control, one group of 8 mice was vaccinated with PBS without adjuvant (PBS). All immunizations were performed every 2 weeks. Serum was collected 20 days after each immunization. Six weeks after the last immunization with incomplete Freund’s adjuvant and 8 weeks after the last immunization with Sigma Adjuvant System, the mice were challenged with $1 \times 10^6$ PFU HRSV A2. Six days after challenge, the lungs were collected to determine the pulmonary HRSV titer by plaque assay.

A She-KLH immunization induces She-specific serum IgG1 antibodies. The graph shows the She-specific IgG1 serum endpoint titers of each mouse at 3 weeks before viral challenge as determined by She peptide ELISA. Horizontal bars indicate the mean IgG1 titers.

B She-KLH immunization induces She-specific serum IgG2a antibodies. The graph shows the She-specific IgG2a serum endpoint titers of each mouse at 3 weeks before viral challenge as determined by She peptide ELISA. Horizontal bars indicate the mean IgG2a titers.

C Vaccination with She-KLH reduces HRSV A2 replication in the lungs of challenged BALB/c mice. The graph shows the number of PFU per lung for each mouse, sampled 6 days after challenge with $1 \times 10^6$ PFU of HRSV A2. Horizontal bars represent the median (one-way ANOVA Dunn’s multiple comparisons test).

D Vaccination with She-KLH is not associated with enhanced body weight loss upon HRSV infection. The graph shows the relative body weight at day 6 post-infection calculated as the ratio between body weight at day 6 and body weight at day 0. Horizontal bars represent the median.

Because our She-based vaccine approach does not mimic natural immunity and does not induce an in vitro neutralizing response, we wanted to ascertain that this approach was safe. As a positive control for exacerbation of disease following HRSV A infection, one group of cotton rats was immunized with alum-adjuvanted formalin-inactivated HRSV A Bernett (GA1 genotype). In agreement with our findings in laboratory mice, She-KLH elicited high levels of She-specific serum IgG antibodies in cotton rats. Also in cotton rats, HRSV A infection did not elicit detectable She-specific serum IgG, nor could She-specific IgG be detected in sera from cotton rats that had been immunized with FI-HRSV (Fig 3A and B). HRSV infection or immunization with FI-HRSV plus alum-induced protective levels of HRSV A neutralizing serum antibodies (Fig 3C). In contrast, HRSV A neutralizing activity was undetectable in sera from cotton rats immunized with She-KLH (Fig 3C). Despite the absence of HRSV A neutralizing antibodies, challenged She-immunized cotton rats displayed a tenfold lower pulmonary HRSV A titer as compared to KLH-immunized cotton rats, a difference that was statistically
Figure 3. SHe-KLH immunization reduces pulmonary HRSV replication in cotton rats.

Cotton rats (n = 6 per group) were immunized three times intraperitoneally with 20 μg of KLH or SHe-KLH combined with incomplete Freund’s adjuvant or with PBS. Additional groups of animals were infected once intranasally with live HRSV or immunized once intramuscularly with formalin-inactivated HRSV (FI-HRSV) at the same time of priming of the other groups. Serum was collected one day before the first immunization and 20 days after each immunization (boost one and two).

A Endpoint titer of SHe-specific IgG in pooled sera after each immunization.
B Individual SHe-specific serum IgG endpoint titers, sampled 20 days after the second boost. Horizontal bars represent the mean.
C SHe-KLH immune cotton rat serum does not neutralize HRSV in vitro. The graph shows the HRSV-neutralizing titer in serum isolated 20 days after boost immunization of each cotton rat. Note: One animal that was vaccinated with FI-RSV died before HRSV challenge. Horizontal bars represent the mean.
D Vaccination with SHe-KLH reduces HRSV replication in the lungs of challenged cotton rats. The graph shows the number of PFU/g of lung tissue for each animal, sampled 5 days post-infection with 2.25 × 10² PFU of HRSV Tracy. Horizontal bars represent the mean (unpaired 2-sided Mann–Whitney U-test). Note: One animal in the KLH and one animal in the PBS-vaccinated group died after HRSV challenge.
E HRSV titers in the upper respiratory tract of challenged cotton rats. The graph shows the number of PFU per nasal wash for each animal, sampled 5 days post-infection with 2.25 × 10² PFU of HRSV Tracy. Horizontal bars represent the mean (unpaired 2-sided Mann–Whitney U-test).
F Alveolitis area score for the lungs of each animal (one-way ANOVA Dunn’s multiple comparisons test).
G Alveolitis severity score for the lungs of each animal (one-way ANOVA Dunn’s multiple comparisons test).
H Representative Micrographs (20×) of H&E-stained lung sections. Scale bars: 100 μm.
significant (Fig 3D). SHe-KLH-immunized cotton rats also displayed a trend toward reduced HRSV A titers in the nasal tract compared to KLH control animals (Fig 3E). Importantly, and in contrast to challenged FI-HRSV immunized cotton rats, HRSV A challenge of SHe-KLH-immunized animals was not associated with alveolitis or pulmonary infiltration of eosinophils upon HRSV A infection, indicating that vaccination with SHe-KLH in combination with IFA does not exacerbate pulmonary pathology upon infection in cotton rats (Fig 3F–H; Supplementary Fig S3A–J).

Adoptive transfer of SHe-specific immune serum reduces HRSV replication and associated morbidity in mice

Because SHe-based immunization is associated with reduced HRSV replication upon challenge of mice and cotton rats in the absence of demonstrable neutralizing antibodies, we first investigated whether SHe-specific antibodies contributed to protection. We administered serum from mice immunized with PBS, KLH, or SHe-KLH intranasally into naïve mice 1 day before and 1 day after HRSV A2 challenge with 5 × 10^5 PFU of HRSV A2. Five days after infection, the pulmonary HRSV titer was determined. Figure 4A shows that lung virus titers were significantly lower in mice that had been treated with SHe-KLH immune serum compared to mice that had received PBS or KLH immune serum. To investigate the dose effect of passive immunization with SHe immune serum, different amounts ranging from 0 to 50 μL of SHe-KLH serum were administered intranasally to mice 1 day before and 1 day after HRSV challenge. All administrated sera were adjusted to a final volume of 50 μL using KLH immune serum. Whereas a dose of 5 μL SHe immune serum could reduce the lung HRSV titer by threefold, 25 and 50 μL SHe immune serum reduced the lung HRSV titer by 11 and 17-fold, respectively, on day 5 after infection (Supplementary Fig S4A and B). None of the mice displayed body weight loss during the experiment (Supplementary Fig S4C and D). To test whether next to intranasal administration also parenteral passive immunization with SHe immune serum could reduce HRSV replication, KLH or SHe-KLH immune serum or HRSV convalescent serum was injected intraperitoneally 16 h before HRSV challenge. Supplementary Fig S4C and D illustrates that also parenteral administration of SHe-KLH immune serum can hamper HRSV replication although to a lesser extent than HRSV convalescent serum.

Although HRSV replicates poorly in mice, challenge of BALB/c mice with a high viral dose is often associated with considerable body weight loss (Graham, 2011). To test whether SHe-specific antibodies can reduce this type of HRSV-induced morbidity, mice were treated with SHe-KLH or control KLH immune serum or with convalescent serum from mice that had been challenged with HRSV. Mice that were not treated and not infected (NI) were used as negative control for HRSV-associated body weight loss. Next, we challenged the animals with a dose of 10^7 PFU of HRSV A2 and monitored body weight. From day five after infection onwards, all challenged mice started to lose weight (Fig 4B). However, from day 6 after challenge, mice that had received SHe or HRSV immune serum started to recover, whereas mice that had been treated with KLH immune serum continued to lose weight and recovered significantly more slowly (Fig 4B). We conclude that SHe-KLH immune serum reduces HRSV replication and associated body weight loss in mice.

SHe-antibody-based immune protection depends on Fcγ receptors

SHe-specific antibodies failed to neutralize HRSV in vitro, yet SHe-specific immune serum reduced HRSV replication in vivo, indicating that an alternative mechanism of protection was operating in vivo. Antibodies that are directed against virus surface antigens can cooperate with leukocytes to kill or remove virus-infected cells by mechanisms that require Fc receptors on the surface of different types of leukocytes, including NK cells, macrophages, dendritic cells, neutrophils, and eosinophils (Bruhns, 2012; Jiang et al., 2011). To test whether Fcγ receptors are involved in the control of HRSV
replication mediated by SHe immune serum, we performed adoptive serum transfer experiments in BALB/c mice with a targeted disruption of the activating receptors FcγR I and FcγR III (Fig 5A) (Hazenbos et al., 1996; Meyer et al., 1998; Nimmerjahn & Ravetch, 2006). HRSV challenge of wild-type and (FcγR I, FcγR III)−/− BALB/c mice that had received KLH control immune serum had comparable levels of HRSV in their lungs, indicating that these two mouse strains are equally susceptible to HRSV (Fig 5B) (Bukreyev et al., 2008). As expected, SHe immune serum significantly reduced HRSV titers in the lungs of wild-type BALB/c mice. In contrast, adoptively transferred SHe immune serum failed to reduce pulmonary HRSV titers in (FcγR I, FcγR III)−/− mice. As Fcγ receptors are involved in the pharmacokinetics of IgG, we investigated whether the failure of SHe immune serum to reduce HRSV replication in (FcγR I, FcγR III)−/− mice might be due to lower levels of SHe-specific IgG antibodies in the lungs (Wang et al., 2008). SHe peptide ELISA of lung homogenates revealed that the levels of SHe-specific IgG were comparable in wild-type and (FcγR I, FcγR III)−/− mice (Fig 5C). We conclude that SH-specific IgG require FcγRI and or FcγRIII to reduce pulmonary HRSV replication in mouse.

SHe-antibody-based immune protection depends on alveolar macrophages

There are three activating Fcγ receptors in mouse: FcγR I, FcγR III, and FcγR IV (Nimmerjahn & Ravetch, 2006). These as well as the inhibitory receptor FcγR II are differentially expressed on myeloid and lymphoid cells (Bruhns, 2012). NK cells, for example, can kill infected cells through FcγR III-mediated antibody-dependent cell-mediated cytotoxicity. As NK cells infiltrate the lungs early during HRSV infection (Hussell & Openshaw, 1998; Moore et al., 2011). Prior intranasal administration of clodronate-loaded liposomes reduced the number of alveolar macrophages by approximately 70% on the day of infection (Fig 6B). As expected, in HRSV-challenged mice that were treated with PBS, intranasal administration of SHe immune serum significantly reduced the

**Figure 5. Reduction of HRSV replication by SHe immune serum depends on Fcγ receptors I and/or III.**

A Schematic overview of the protocol used to investigate the role of Fcγ receptors in SHe-antibody-mediated reduction of HRSV replication. One day before and one day after HRSV A2 infection, wild-type (WT, 6 mice per group) or Fcγ receptor I and III double knockout (FcγR II−/−, 5 mice per group) BALB/c mice were treated intranasally with 35 μl of PBS (PBS), KLH (KLH) or SHe-KLH immune serum (SHe-KLH). Five days after challenge with 5 × 10^5 PFU of HRSV A2, the lungs were collected and HRSV A2 titers were determined by plaque assay.

B Transfer of SHe-KLH immune serum reduces HRSV replication in wild-type, but not in FcγR III−/− mice. The graph shows the number of PFU per lung of each mouse, and horizontal bars represent the mean (one-way ANOVA Dunn’s multiple comparisons test).

C SHe-specific IgG levels in lung homogenates of wild-type and FcγR III−/− mice are comparable. The graph shows the SHe-specific endpoint IgG titer in lung homogenates prepared 5 days after infection of each mouse treated with SHe-KLH immune serum, with horizontal bars representing the mean. The graphs are representative for two independent experiments.
pulmonary HRSV titer as compared to KLH immune serum and was associated with a slightly higher body weight after challenge (Fig 6C and D). In mice that had received control KLH immune serum, depletion of monocytes by clodronate-loaded liposomes did not significantly affect the HRSV pulmonary titer (Fig 6C). In contrast, treatment with clodronate-loaded liposomes partially but significantly restored HRSV replication in the lungs of mice that had been treated with SHe immune serum (Fig 6C). Importantly, pulmonary SHe-specific IgG antibodies were not lower but rather higher in the mice with depleted alveolar macrophages (Fig 6E). This is in line with the role of macrophages in the clearance of IgG (Ordas et al., 2012; Wang et al., 2008). To investigate the contribution of alveolar macrophages at different time points during HRSV infection, the previous experiment was repeated but instead of at 5 days post-infection, the lungs were collected at 4 and 6 days post-infection. As expected in mice that were not treated with clodronate-loaded liposomes, intranasal administration of SHe-KLH immune serum significantly reduced the lung HRSV titer at 4 days post-infection by

Figure 6. Suppression of HRSV replication by SHe immune serum depends on alveolar macrophages.

A Schematic overview of the protocol used to investigate the role of alveolar macrophages in SHe-antibody-mediated protection against HRSV. Three days before infection, anesthetized BALB/c mice were treated intranasally with PBS (PBS) or clodronate-loaded liposomes (Cl. Lip.). One day before and one day after HRSV A2 infection, the mice received KLH (KLH) or SHe-KLH immune serum (SHe-KLH) via the intranasal route. On day 5 after challenge with $5 \times 10^5$ PFU of HRSV A2, mice were sacrificed and the pulmonary viral load was determined by plaque assay.

B Treatment with clodronate-loaded liposomes selectively reduces the number of alveolar macrophages in the lungs. Mice were treated with PBS or clodronate-loaded liposomes 3 days before BAL fluid collection. Two days later, these mice were additionally treated with PBS or KLH immune serum. The number and type of cells in the BAL fluid was determined by flow cytometry. The graph represents the number of CD8+ T cells (CD8+), CD4+ T cells (CD4+), neutrophils (neut.), eosinophils (eos.), resident alveolar macrophages (rAM), infiltrating monocytes (Mon.), and dendritic cells (DC) in BAL fluid. PBS/PBS: passive transfer of PBS and treatment with PBS; PBS/Cl. lip.: passive transfer of PBS and treatment with clodronate-loaded liposomes; serum/PBS: passive transfer of KLH immune serum and treatment with PBS; Serum/Cl. lip.: passive transfer of KLH immune serum and treatment with clodronate-loaded liposomes.

C Treatment of mice with clodronate-loaded liposomes impairs SHe-immune serum-mediated suppression of HRSV replication. The graph shows the number of PFU per lung of each mouse on day 5 after challenge. Horizontal bars represent the means (one-way ANOVA Dunn’s multiple comparisons test). The graph is representative for two independent experiments.

D Depletion of alveolar macrophages does not affect body weight upon HRSV infection. The graph shows the relative body weight of each animal on day 5 after infection and horizontal bars depict the mean.

E Depletion of alveolar macrophages does not decrease the amount of SHe-specific IgG in the lung. The graph shows the SHe-specific IgG endpoint titers in cleared lung homogenates prepared on day 5 after challenge with HRSV A2, as determined by a SHe peptide ELISA. Horizontal bars represent the mean.

F, G BALB/c mice ($n = 16$ per group) were immunized three times with KLH or SHe-KLH in combination with incomplete Freund’s adjuvant. Immunizations were performed intraperitoneally with 3-week interval. Nineteen days after the last immunization, half of the mice were treated with, respectively, PBS (KLH PBS and SHe-KLH PBS) or clodronate-loaded liposomes (KLH Cl. Lip. and SHe-KLH Cl. Lip.). Three days later, all mice were challenged with $1 \times 10^6$ PFU RSV A2. Five days after challenge, the lungs were collected for HRSV titration. (F) SHe-specific IgG endpoint titers of sera collected 2 weeks after the last immunization, as tested by SHe peptide ELISA. Horizontal bars represent the mean. (C) The reduction of HRSV replication in SHe-KLH-vaccinated mice depends on alveolar macrophages. The graph shows the number of PFU per lung for each mouse. Horizontal bars represent the means (one-way ANOVA Tukey’s multiple comparisons test).
approximately tenfold (Supplementary Fig S7A). In contrast, in mice in which the resident alveolar macrophages were depleted by clodronate-loaded liposomes, there was only a twofold, statistically not significant, reduction in lung HRSV titer in mice that were treated with SHe-KLH immune serum as compared to mice that were treated with KLH immune serum (Supplementary Fig S7A). At 6 days after infection, the reduction (approximately 110-fold) of HRSV replication in the lungs of SHe-KLH immune serum treated mice was more pronounced than at 4 days post-infection (Supplementary Fig S7C). In mice in which the resident alveolar macrophages were depleted by clodronate-loaded liposomes, treatment with SHe-KLH immune serum still reduced the lung viral titer by approximately 15-fold. Supplementary Fig S7B and D illustrate that the reduced effect of SHe immune serum in mice with depleted alveolar macrophages is not due to lower SHe-specific IgG titers in the lungs of these mice. These data indicate that at later time points during HRSV infections, other, possibly infiltrating, leukocytes aid at reducing HRSV replication in the lungs.

Because the intranasal route of serum administration might affect the mechanism by which SHe-specific antibodies hamper HRSV replication in the lungs, the role of alveolar macrophages was also investigated when SHe immune serum was administered parenterally. Supplementary Fig S8A and B illustrate that depletion of alveolar macrophages abrogated the reduction of HRSV replication in mice that were treated with SHe immune serum by intraperitoneal injection. To further investigate the role of alveolar macrophages, we tested whether depletion of alveolar macrophages also impairs protection afforded by active SHe vaccination. Mice were vaccinated with KLH or SHe-KLH in combination with IFA. Nineteen days after protection afforded by active SHe vaccination. Mice were vaccinated tested whether depletion of alveolar macrophages also impairs the mechanism by which SHe-specific antibodies hamper HRSV replication during HRSV infections, other, possibly infiltrating, leukocytes aid at reducing HRSV replication in the lungs.

Because the F protein is readily accessible at the surface of infected HRSV A2-infected A549 cells was compared to bind- ing to HRSV A2 virions attached to the surface of A549 cells at 4°C. Because the F protein is readily accessible at the surface of infected cells and virions, a HRSV F-specific IgG monoclonal antibody was used as positive control (Magro et al., 2010). Polyclonal anti-HRSV goat immune serum was used to identify the HRSV-infected cells and HRSV virions. Polyclonal anti-HRSV serum recognized the surface of the infected cells, including the long filaments and especially the sprouts of these filaments (Fig 7A and B). Reactivity of the F protein-specific monoclonal antibody was largely confined to the cell surface and filament tips. Since these F-rich tips are of similar size and have an F protein staining pattern that is similar to HRSV virions that are attached to the target cells (Fig 7C), it is likely that these sprouts correspond to budding virions. Similar to the F-specific monoclonal antibody and polyclonal anti-HRSV immune serum, SHe immune serum readily recognized the surface of HRSV-infected cells. However, in contrast to the F-specific monoclonal antibody, SHe immune serum barely bound to the filament tips (Fig 7A and D). In a second approach, performed in parallel, we studied the binding of SHe-specific antibodies to HRSV virions that were attached to but not yet fused with A549 cells (Schepens et al., 2011). HRSV virions were recognized by both polyclonal anti-HRSV serum and the F protein-specific monoclonal antibody (Fig 7C and D). More specifically, all virions that were stained by the polyclonal HRSV serum were also recognized by the monoclonal anti-F protein antibody. In contrast, SHe immune serum largely failed to bind to HRSV virions (Fig 7A, C and D). Taken together, these results suggest that SHe immune serum reduces HRSV replication in vivo by a mechanism that targets HRSV-infected cells rather than virions.

**Human sera lack high levels of SHe-specific antibodies**

Many of the current HRSV vaccine approaches aim at inducing HRSV-neutralizing antibodies directed against the F or G protein. However, most adults already have high levels of HRSV-neutralizing serum antibodies. To investigate the levels of SHe-specific IgG in sera of adults, we used SHe peptide ELISA to examine a panel of reference sera with varying levels of HRSV-neutralizing antibodies, as well as purified human serum IgG (Yang et al., 2007). In contrast to serum from SHe-KLH-vaccinated mice, sera from HRSV-infected or KLH-vaccinated mice and all human sera lacked detectable SHe-specific IgG antibodies (Fig 8A and C). Still, all tested human sera and sera from HRSV-infected or SHe-KLH-immunized mice readily recognized HRSV proteins in lysates of HRSV-infected cells (Fig 8B and D). These data suggest that HRSV infections in humans do not induce long-living high levels of SHe-specific IgG.

**Discussion**

Although the identification of HRSV as an important cause of bronchiolitis in infants was made more than 55 years ago, there is still no licensed vaccine for this important respiratory pathogen (Collins & Graham, 2008). Most attempts to develop such a vaccine aim at inducing neutralizing antibodies directed against the major surface proteins, F and G (Graham, 2011). Inducing neutralizing serum antibodies is a reasonable approach because different reports have illustrated that high levels of such antibodies correlate with reduced disease (Piedra et al., 2003; Luchsinger et al., 2012; Glezen et al., 1986). Moreover, prophylactic passive immunization of high-risk infants with the humanized monoclonal antibody palivizumab,
which is F specific and virus neutralizing, reduces the risk of HRSV-associated hospitalization by approximately 50% (IMPact-RSV Study Group, 1998). However, natural HRSV infection does not induce long-lasting immunity. Unlike human influenza, this lack of protection against reinfection occurs without significant antigenic drift (Collins & Graham, 2008). Apart from a recently described neutralizing epitope that is unique to the prefusion conformation of F, the fusion protein of HRSV is highly conserved (McLellan et al., 2013). Healthy adults with high levels of neutralizing antibodies in circulation can be experimentally reinfected with HRSV (Hall et al., 1991). In addition, some studies even reported a lack of demonstrable correlation between high levels of serum neutralizing antibodies and protection against severe disease caused by HRSV (Friedewald et al., 1968; Mills et al., 1971; Falsey & Walsh, 1992; Brandenburg et al., 1997; Falsey et al., 1999; Wright et al., 2002). Taken together, it remains to be determined whether vaccination strategies that mimic the host immune response following natural infection with HRSV could provide significant protection.

We developed an alternative vaccination strategy that is not based on natural correlates of protection. In contrast to the major
surface proteins, F and G, the SH protein does not elicit high levels of antibodies or contribute to protection upon infection (Connors et al., 1991; Akerlind-Stopner et al., 1993). SH is a pentameric membrane protein that is not absolutely required for in vitro virus growth but presumably modulates the host innate immune response and it is essential for in vivo viral fitness (Bukreyev et al., 1997; Gan et al., 2012; Triantafilou et al., 2013).

The inability of HRSV infections to induce high levels of SH-specific antibodies can be explained by the close proximity of this 24 amino acid long ectodomain to the cell and viral membrane and because of its limited incorporation into budding virions (Rixon et al., 2004). We confirmed the findings of Rixon et al., which were obtained by immune electron microscopy, by immunostaining HRSV virions attached to the surface of target cells.

In agreement with the weak immunogenicity of the SH ectodomain upon HRSV infection and the weak or absent binding of SH IgC to HRSV virions, SH immune sera failed to neutralize HRSV in vitro. Passive immunization of mice with heat-inactivated SH immune serum revealed that SH-specific antibodies were associated with reduced pulmonary HRSV replication, suggesting that these antibodies are prime mediators of immune protection by this novel SH-based HRSV vaccination approach. Next to SH-specific antibodies, SH-specific T cells might also be involved in SH-vaccination-mediated reduction of viral replication. In mouse, a CD4+ T-cell epitope within SH, and in humans, a CD8+ T cells epitope within the SH protein have been reported (Nicholas et al., 1989; Cherrie et al., 1992). However, we could not detect SH-specific T cells in the spleens of HRSV-infected mice by ELISPOT or by intracellular cytokine staining, whereas F-specific CD8+ T cells were readily detected in the spleens of these HRSV-infected mice (unpublished data). Moreover, depletion of CD8 cells upon HRSV challenge did not abolish the reduction of HRSV in SH vaccinated mice (Supplementary Fig S9).

Apart from direct neutralization of viral particles, antibodies can also combat viral infections by opsonizing viral particles, marking them for uptake by phagocytes or complement-dependent lysis, or by initiating the elimination of infected cells through antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), or complement-dependent cytotoxicity (CDC) (Hogarth & Pietersz, 2012). ADCC and ADCP depend on activating Fc receptors FcγRI, FcγRII, or FcγRIII in mouse and on FcγRI, FcγRIIA, FcγRIIC, and FcγRIIIB in human (Nimmerjahn & Ravetch, 2006; Bruhns, 2012). These receptors are expressed alone or combined with other activating and/or silencing Fcγ receptors.

Figure 8. Human sera with high HRSV-neutralizing activity lack high levels of SH-specific IgG.

A–D SH peptide ELISA of human (A) and mouse (C) sera. Human reference sera to HRSV were obtained from the NIH Biodefense and Emerging Infections Research Resources Repository (NIAID, NIH). Antiserum NR-4020: pooled human reference serum; NR-4021: pooled human sera with high HRSV neutralization titer; NR-4022: pooled human sera with intermediate HRSV neutralization titer; NR-4023: pooled human sera with low HRSV neutralization titer; and NR-21973: human reference Ig to HRSV (1% in PBS). The mouse sera represent pooled pre-immune sera (pre.), pooled immune sera from mice immunized three times intraperitoneally with SHe-KLH or KLH in combination with incomplete Freund’s adjuvant, and pooled sera of mice infected with HRSV A2 (HRSV). HRSV-specific ELISA of human (B) and mouse (D) sera. The ELISA plates in (B) and (D) were coated with the supernatant of HRSV A2 infected cells and tested with the sera used in (A) and (C).
In infants, HRSV pathology is characterized by airway occlusion caused by sloughed HRSV-infected cells (Welliver et al., 2008). This feature of HRSV pathology in infants can be mimicked in HRSV-infected mice in which alveolar macrophages were depleted by clodronate liposomes (Reed et al., 2008; Welliver et al., 2008). This indicates that macrophages might help to avert airway occlusion by clearing HRSV-infected cells that are sloughed off as well as apoptotic leukocytes. Hence, it was suggested that vaccination strategies that stimulate local macrophage function might be particularly effective in infants (Reed et al., 2008).

For a vaccine or antibody treatment based on a small antigen, it is crucial that the antigen is highly conserved in time and among strains. Recently, a small number of studies described SH coding sequences or complete HRSV genome sequences of clinical isolates. These studies included isolates collected between 1998 and 2011 in Europe, Brazil, and the USA and belonging to different HRSV A genotypes (GA1, GA2, GA4, GA5, and GA7) (Kumaria et al., 2011; Rebuffo-Scheer et al., 2011; Lima et al., 2012; Tan et al., 2013a,b). To evaluate the conservation of the ectodomain of HRSV A subgroup SH, we aligned the SH sequences from these studies (Supplemental Fig S10 and S11). Of the 135 retrieved sequences, 5 isolates (3.7%) displayed one amino acid substitution compared to the consensus sequence. None of the sequences displayed more than one amino acid substitution. No deletions or insertions were observed. In addition, SHs of the HRSV A laboratory strains RSV A2 (M74568) and RSV long (AY911262), respectively, isolated in 1961 in Australia and in 1956 in the USA, and the RSV-2 (NC001803) line-19 (FJ614813) and HRSV A Tracy used in our cotton rat model, are identical to the consensus amino acid sequence. Importantly, the sequence of SHs from group A HRSV and group B HRSV differs substantially (Chen et al., 2000). Therefore, a SH comprising HRSV vaccine candidate should contain both the SHeA and the SHeB antigen.

(FCγRIIb in mouse and FCγRIIB in human) on the surface of innate immune effector cells such as monocytes, macrophages, NKs, DCs, neutrophils, eosinophils, and mast cells (Bruhns, 2012). Using (FCγRI, FCγRIII)−/− BALB/c mice, we demonstrated that the reduction of pulmonary HRSV replication mediated by SHe immune serum strongly depends on FCγRI and/or FCγRIII. In addition, conditional depletion of resident alveolar macrophages (rAM) significantly but not completely abrogated the effect of SHe immune serum on HRSV pulmonary titers. The residual antiviral effect of SHe immune serum could be explained by the observation that rAM depletion was not complete (Fig 6B). Alternatively, this may indicate that other FCγ R expressing cells such as infiltrating monocytes, NK cells, and neutrophils could also be involved, especially at later time points during infection (Supplementary Fig S7).

Therefore, we propose a mechanism in which rAM cooperate with SHe-specific IgG to target infected cells rather than free virions. rAM might also indirectly contribute to SHe immune serum-mediated inhibition of viral replication. Recently, it has become clear that the crosstalk between macrophages and NK cells is important for the host response to pathogens and tumors (Nedvetzki et al., 2007; Lapaque et al., 2009; Klezovich-Benard et al., 2012). Depletion of rAM by clodronate liposomes was shown to impair early NK infiltration and activation in the lungs of HRSV-infected mice (Pribul et al., 2008). In this way, macrophages might also promote SHe-specific, antibody-dependent, NK-mediated ADCC of HRSV-infected cells. However, our observation that depletion of NK cells does not impact the activity of SHe immune serum argues against a pivotal role for NK cells.

It is clear that in both experimental models, mice and cotton rats, the impact of SHe protein vaccination on HRSV replication (6 to 70-fold reduction) does not seem to match up to the sterilizing immunity that some of the F protein-based vaccine candidates elicit, including the FI-HRSV vaccine used here in cotton rats. However, one has to take into account that both BALB/c mice and cotton rats are only semi-permissive for HRSV infection, and so high-titer inocula delivered directly to the lower respiratory tract are required for productive infection in these animals. In humans, HRSV infection starts in the upper respiratory tract and viral spread to the lower respiratory tract probably involves multiple rounds of infections. In such a situation, when the number of infected cells increases gradually over time, antibodies that reduce HRSV replication by eliminating HRSV-infected cells could have a greater impact. SHe-specific antibodies were not detected in human reference sera with HRSV-neutralizing activity. Whereas F protein-based vaccination strategies aim at increasing the level of HRSV-neutralizing antibodies, which are already significantly present in sera from adults, a SHe-based vaccination strategy would induce antibodies against an additional immune-protective antigen. Taken into account that SHe-based vaccination does not provide sterile immunity, clinical implementation of an HRSV vaccine based solely on SHe antigen is unlikely. Adding SHs antigen to vaccine candidates that are based on HRSV F and G, in order to induce neutralizing antibodies, or to vaccines that aim at inducing protective T cells, would be an interesting approach to increase immune protection.

There is increasing evidence that non-neutralizing antibodies that can mediate ADCC contribute significantly to protection against HIV infections. The recent RV144 phase III trial for the first time resulted in some level of protection (31% compared to placebo) (Rerks-Ngarm et al., 2009). Surprisingly, this protection did not correlate with the induction of neutralizing antibodies but with high levels of non-neutralizing antibodies capable of mediating ADCC (Rerks-Ngarm et al., 2009; Bonsignori et al., 2012; Haynes et al., 2012; Liao et al., 2013). Likewise, not the presence of neutralizing antibodies but the presence of high levels of antibodies capable of ADCC in breast milk correlates with protection against vertical transmission of HIV via breastfeeding (Mabuka et al., 2012). Moreover, the protective activity of a broadly neutralizing HIV antibody in macaques relies largely on its interaction with Fe receptors (Hessell et al., 2007). In addition, ADCC exerted by cross-reactive HA-specific antibodies is associated with cross-protective immunity against the 2009 pandemic H1N1 in macaque monkeys that had been previously infected with seasonal H1N1 (Jegaskanda et al., 2013). Moreover, in animal models, protection against influenza infections by antibodies directed against the conserved ectodomain of the influenza M2 protein strongly depends on FCγ receptors on macrophages (El Bakkouri et al., 2011). Next to the non-neutralizing M2e antibodies, also the recently discovered human ‘broadly neutralizing’ antibodies that bind to the conserved stalk of influenza HA require on FCγ receptors for their protective activity in vivo (Corti & Lanzavecchia, 2013; Dilillo et al., 2014). These examples indicate that induction of antibodies able to eliminate infected cells by FC receptor-expressing host cells could be an effective strategy for development of vaccines against intracellular pathogens.

TheAuthors
In conclusion, SHe-based immunity is not based on natural immunity and entails an ADCC-type or ADCP-type of protective mechanism and therefore can be considered complementary to F-, G- and N-based subunit immunization strategies that are being explored (Graham, 2011; Remot et al, 2012). Additional preclinical development of this SHe-based vaccine might benefit from additional evaluation in an animal model that is more permissive for HRV or demonstration of its efficacy in natural hosts for HRV-related paramyxoviruses that also encode SH. In addition, we propose that a viable path for further clinical evaluation of SHe would be to include this antigen in other HRV vaccine candidates that aim at inducing neutralizing antibodies and protective T-cell responses.

Materials and Methods

Ethics statement

All mouse experiments described in this study were conducted according to the national (Belgian Law 14/08/1986 and 22/12/2003, Belgian Royal Decree 06/04/2010) and European (EU Directives 2010/63/EU, 86/609/EEG) animal regulations. All experiments on mice were animal protocols approved by the ethics committee of Ghent University ( Permit number LA1400091, approval ID 2007/027, 2010/025, and 2013/025). All efforts were made to avoid or ameliorate suffering of animals. The cotton rat experiment was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental protocols were approved by the BCM Investigational Animal Care and Use Committee.

Cells and viruses

Hep-2 cells (ATCC, CCL-23), Vero cells (ATCC, CCL-81), and A549 cells ATCC, CCL-185) were grown at 37°C in the presence of 5% CO2 in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin, 1% streptomycin, 2 mM L-glutamine, non-essential amino acids (Invitrogen, Carlsbad, California), and 1 mM sodium pyruvate. HRV A2 (VR-1540) and HRV B1 (VR-1580) were obtained from ATCC (ATCC, Rockville). The clinical HRV B isolates were obtained from Gasthuisberg University Medical Hospital of Leuven, Belgium, and described in Tan et al. (2013a,b) (JX576729, JX576730, and JX576731, see Supplementary Fig S8). All HRVs were propagated on sub-confluent Hep-2 cells infected with 0.1–0.01 MOI. Three days after infection, the culture medium was collected and cleared by centrifugation (1,000 × g) for 15 min. The supernatant was adjusted to 10% PEG and incubated at 4°C with agitation for 4 h. Subsequently, HRV was isolated by centrifugation (4,000 × g) for 30 min at 4°C. The HRV pellet was resuspended in HBSS containing 20% sucrose, aliquoted, and stored at –80°C. The infectious units in the HRV stocks were quantified in duplicate or triplicate on Vero cells by plaque assay.

Mice

Specific pathogen-free female BALB/c mice at the age of 7–8 weeks were purchased from Charles River, and (FcγRI, FcγRIII)−/− mice (BALB/c genetic background) were originally obtained from Dr. Verbeek and bred in the specific pathogen-free (SPF) animal facility of DMBR (Hazenbos et al, 1996). All mice were genotyped by a PCR protocol using genomic DNA. The mice were housed in a SPF temperature-controlled environment with 12-h light/dark cycles and given water and food ad libitum. Mice were used at 9 weeks of age after adaptation in the animal room for 1 week.

Production of SHe–KLH antigen

For chemical conjugation of SH to keyhole limpet hemocyanin (KLH), we used a SHe peptide variant (CGGGS-NKLSEYNVFNKT-FELPRARVNT) in which the cysteine at position 4 in the natural SH amino acid sequence was substituted with a serine to prevent conjugation at this position. An N-terminal CGGGS linker was added as a flexible spacer separating SHe from the carrier. KLH-peptide conjugation and isolation was performed using the Imject Maleimide-Activated mcKLH Kit (Thermo Fisher Scientific, Rockford, USA) according to the manufacturer’s instructions.

Production of HBC-SHeB antigen

A peptide corresponding to the SH ectodomain of HRV B with an N-terminal CGGGS linker and cysteine at position 10 replaced by a serine (CGGGS-NKLSEHKTFSNKTLEQQMYYQINT) was chemically conjugated to Hepatitis B core protein virus-like particles (HBc-SHeB) by using the heterobifunctional crosslinker Sulfo-MBS according to the manufacturer’s instructions (Pierce, Rockford, USA). The production and purification of recombinant HBc particles in which a single lysine was inserted in the immune dominant loop between amino acids 76 and 77 to allow chemical conjugation is described in De Filette et al (2005).

Mice immunization and infection

At the age of 9 weeks, the mice were immunized intraperitoneally with 20 μg antigen dissolved in PBS to a volume of 100 μl PBS and mixed with 100 μl incomplete Freund’s adjuvant or the Sigma Adjuvant System (Sigma-Aldrich, St. Louis, USA). Immunizations were performed three times at 3-week intervals, unless indicated otherwise. Two weeks after each immunization, blood samples were collected from the lateral tail vein. Blood was left to clot at 37°C for 30 min, and serum was collected by taking the supernatant from two consecutive centrifugations. The serum titer of SHe-specific IgG, IgG1, and IgG2a were determined by peptide ELISA. Three weeks after the final immunization, the mice were sedated with isoflurane and challenged intranasally with 1 × 106 PFU of HRV or a different dose as specified. Five days after infection, the mice were killed by cervical dislocation. The lungs were collected and homogenized with a Heidolph RZR 2020 homogenizer in 1.0 ml HBSS containing 20% sucrose. The lung homogenates were cleared by centrifugation (1,000 × g) for 15 min at 4°C and used to determine the viral titer by plaque assay on Vero cells.

Immunization and infection of cotton rats

This experiment was performed at Baylor College by Dr. PA. Piedra and Dr. B. Gilbert. Cotton rats (Sigmoden hispidus) of both
genders were bred and housed in the Baylor College of Medicine (Houston, TX, USA) vivarium in cages covered with barrier filters and given food and water ad libitum. Cotton rats were immunized intraperitoneally with PBS or with 100 μg KLH or SHE-KLH dissolved in 100 μl PBS and mixed with 100 μl incomplete Freund’s adjuvant. Immunizations were performed three times at 3-week intervals. At the time of the first immunization, one group of cotton rats were infected intranasally with 2.04 × 10⁵ PFU of HRSV Tracy (Hep-2 grown) under light sedation with isoflurane, and one group were immunized in the left tibialis anterior with formalin-inactivated HRSV-Bernett grown in Vero cells (Piedra et al., 1989). Blood was collected 3 weeks after each immunization. Twenty-four days after the last immunization, all cotton rats were challenged with 2.25 × 10⁵ PFU of HRSV Tracy (100 μl) under light sedation with isoflurane. Five days after challenge, the cotton rats were euthanized with CO₂. The left lung was tied off and used for histopathology. The remaining right lobes from the lung were removed, rinsed in sterile water to remove external blood contamination, and weighed. The right lobes were transversely lavaged using 3 ml of Iscove’s medium with 15% glycerol mixed with 2% FBS-MEM (1:1, v:v) injected at multiple sites to totally inflate the lobes. Lavage fluid was recovered by gently flattening the inflated lobes and stored on ice until titered. For nasal washes of the upper respiratory tract, the jaws were disarticulated. The head was then removed, and 1 ml of Iscove’s media with 15% glycerol mixed with 2% FBS-MEM (1:1, v:v) was pushed through each nare (total of 2 ml). The effluent was collected from the posterior opening of the pallet and stored on ice until titered. The virus titer in the lung lavage fluids and nasal washes was determined by plaque assay using Hep-2 cells. Six days after infection with duplicate dilution series of the prepared lung lavage fluids and nasal washes, the viral plaques were stained with 0.1% crystal violet in 10% formalin solution and counted. For histopathology, the left lung was perfused with 10% neutral buffered formalin (for 2 h). Tissues were stored in 10% neutral buffered formalin and sent to the Center for Comparative Medicine, BCM, where the pathologist who assigned the histopathological scores was blinded to the group assignments.

Adoptive serum transfer experiments

SHe-KLH, KLH, and PBS immune sera were obtained from mice that had been immunized 3 times with 20 μg SHe-KLH or KLH in combination with incomplete Freund’s adjuvant (Merck Milipore, Billerica, USA), with PBS or from immunologically naive mice that had been infected with 1 × 10⁶ PFU of HRSV to obtain serum with virus-neutralizing activity. Three weeks after the third immunization, blood was obtained from the orbital sinus of mice anesthetized with an intraperitoneal injection of avertin (2.5% in PBS). Blood was left to clot at 37°C for 30 min, and serum was obtained by taking the supernatant from two consecutive centrifugations. The serum was heat-inactivated by incubation at 56°C for 30 min. In passive immunization experiments, 35 μl of heat-inactivated serum was administered intranasally under isoflurane sedation 24 h before and 24 h after HRSV challenge. Five days after challenge with 10⁶ PFU HRSV, the mice were sacrificed and their lungs were excised to determine viral load.

ELISA

ELISA was used to determine antibody titers in sera from individual mice, or pooled sera. To determine SHe-specific IgG in mouse, cotton rat, and human sera, microtiter plates (type II F96 Maxisorp, Nunc) were coated with 0.2 μg SHe peptide (NKLCYEVNFHKNT-FELPRARVNT) in 100 μl of 50 mM sodium bicarbonate buffer, pH 9.7, and incubated overnight at 37°C. A peptide corresponding to the influenza M2 ectodomain (SLITEVETPIRNEGCRCONDSSD) was used as negative control. Alternatively, microtiter plates were coated at 4°C with supernatant of cultured Vero cells that had been infected with HRSV A2. After washing, the plates were blocked for 1 h with 200 μl of 1% BSA in PBS. After 1-h incubation, the plates were washed again. Unless specified otherwise, a series of threefold dilutions of different mouse, cotton rat, or human serum samples, starting with a 1/100 dilution, were loaded on the coated plates. The following human serum samples were obtained from the NIH Biodefense and Emerging Infections Research Resources Respiratory: NR-40210, human reference antisera to respiratory syncytial virus; NR-4021, human antisera to respiratory syncytial virus, high control; NR-4022, human antisera to respiratory syncytial virus, medium control; NR-4023, human antisera to respiratory syncytial virus, low control; and NR-21973, human reference immune globulin to respiratory syncytial virus (Yang et al., 2007). The bound antibodies were detected with individual or a mixture of peroxidase-labeled antibodies directed against mouse isotypes IgG1, IgG2a, IgG2b, or IgG3 (individually diluted 1/6,000) (Southern Biotechnology Associates, Inc., Birmingham, AL, USA), or against human IgG1, IgG2, IgG3, and IgG4 (individually diluted 1/2,000) (Southern Biotechnology Associates, Inc.), or against cotton rat IgG (diluted 1/1,000) (Immunochemistry Consultants Laboratory Inc., Portland, USA) in PBS + 1% BSA + 0.05% Tween 20. After washing, the microtiter plates were incubated for 5 min with TMB substrate (tetramethylbenzidine, Sigma-Aldrich). The reaction was stopped by adding an equal volume 1 M H₂SO₄, and absorbance at 450 nm was measured. Endpoint titers were defined as the highest dilution producing an O.D. value twice that of background (pre-immune serum).

Plaque assay

Monolayers of Vero cells were infected with 50 μl of serial threefold dilutions of the lung homogenates in a 96-well plate in serum-free OptiMEM medium (Invitrogen) supplemented with penicillin and streptomycin. After 3 h, the medium was removed and the cells were washed twice with PBS. After adding 150 μl of growth medium containing 2% FCS and 0.6% avicel RC-851 (FMC Biopolymers), the cells were incubated for 4–5 days at 37°C. After infection, the cells were washed twice with PBS and subsequently fixed in 2% paraformaldehyde. After overnight fixation at 4°C, the paraformaldehyde solution was removed and the cells were washed twice with PBS. Subsequently, the cells were permeabilized with PBS containing 0.2% Triton X-100 for 5 min and blocked with PBS containing 1% BSA. The viral plaques were stained with a polyclonal goat anti-HRSV serum (AB1128, Chemicon International) (1/4,000). After washing three times with 1% BSA in PBS, the cells were incubated with HRP-conjugated anti-goat IgG antibodies (SC2020, Santa Cruz) for 30 min. Non-binding
antibodies were removed by washing four times with PBS containing 1% BSA and 0.01% Triton X-100 and once with PBS. Finally, the plaques were visualized by using TrueBlue peroxidase substrate (KPL, Gaithersburg). The plaques of different dilutions were counted, and for each dilution, the number of PFU per lung (1 ml of lung homogenate) was calculated as the number of plaques present in the dilution × the factor of dilution × 20 (20 = 1,000 µl total supernatant volume/50 µl of supernatant used to infect the first well of the dilution series). The number of PFU/lung was then calculated as the average number of PFU/lung calculated for the different dilutions. As each supernatant of the homogenized lungs was tested in duplicate, the final number of PFU/lung was calculated as the average of these duplicates.

In vitro neutralization using mouse sera

Virus neutralization activity of mouse sera was tested by plaque reduction assay. Mouse sera (not heat inactivated) were diluted in serum-free medium and incubated with approximately 50 PFU HRNV for 30 min at 37°C in a final volume of 50 µl. These samples were then used to infect a confluent cell layer of Vero cells grown in a 96-well plate. After 3 h incubation at 37°C, 150 µl of growth medium containing 2% FCS and 0.6% avicel RC-851 (FMC Biopolymers) was added. Three days after infection, plaque formation was detected by immunostaining with goat anti-HRSV serum and HRP-coupled anti-goat IgG. The stained plaques were visualized by using TrueBlue peroxidase substrate (KPL, Gaithersburg, USA) and counted visually. Alternatively, viral antigen was detected by adding 100 µl TMB substrate. After incubation for a few min, 50 µl of colorized TMB substrate of each sample was mixed with an equal volume of 1 M H2SO4 in a 96-well plate and absorbance was measured at 450 nm.

In vitro neutralization of cotton rat sera

Tests for serum neutralizing antibodies to HRNV Tracy were performed with HEp-2 cells grown in 96-well microtiter plates. Serial twofold dilutions in duplicates starting at 3 log2 were performed to determine the neutralizing antibody (Ab) titer for each sample. The neutralizing antibody titer was defined as the serum dilution resulting in >50% reduction in viral cytopathic effect (CPE). CPE is determined visually after the wells are fixed with 10% formalin and stained with crystal violet. Neutralizing antibody titers are categorical log numbers and not continuous values. The lowest detectable N titers is 2.5 log2. Samples with non-detectable neutralizing antibody titers were assigned a value of 2 log2.

QPCR of HRNV B RNA in BALF of infected mice

The relative levels of HRNV B N cDNA were determined by qRT–PCR using primers specific for the HRNV B N RNA (RSVB-N-Fw: 5'-GGCTCCAGAATATAGCCATGATTC-3' and RSVB-N-Rev 5'-TGTTATTACAAAGACGTATTACACAT-3') and an HRNV B N-specific FAM-conjugated nucleotide probe (5'-TATCATCCACAGCTG-3'). The relative RNA level calculated as 1/2^N (N is the number of PCR cycles) with the calculated value for the mock-infected mice was set as 1.

Detection of FcγRI on the surface of resident alveolar macrophages

BALF of 4 wild-type mice and 3 FcγRI−/− mice were prepared and, respectively, pooled. After fixation with 1% PFA for 10 min at 4°C, the BALF cells were washed and blocked with PBS containing 1% BSA and 1 µg/ml anti-Cd16/Cd32 Fc-block for 1 h. Subsequently, the cells were stained with PE/Texas Red-conjugated anti-Cd11c in combination with either Alexa647-conjugated anti-FcγRI antibody or a APC-conjugated mouse IgG1k isotype control antibody. For unstained and single stain controls, a mixtures of wild-type and FcγRI−/− BALF cells were used. Resident alveolar macrophages were detected as highly autofluorescent (FITC channel), Cd11c-positive single cells. The samples were measured on a LSRII flow cytometer and analyzed with Flowjo.

Depletion of alveolar macrophages and flowcytometric analysis of BALF cells

Liposomes containing dichloromethylene dipiphosphonate (clodronate) or PBS were prepared as described previously (Van Rooijen & Sanders, 1994; Pribul et al., 2008). Fifty microliters of PBS or clodronate-loaded liposomes were administered intranasally to isoflurane sedated BALB/c mice 3 days before HRNV challenge. Depletion of alveolar macrophages (AM) was ascertained by determining the cell content of bronchoalveolar lavage (BAL) isolated 72 h after liposome administration under anesthesia with an intraperitoneal injection of avertin (2.5% in PBS). A 23-gauge cannula was inserted into the trachea, and cells were collected by washing the airway lumen twice with 0.5 ml PBS containing 0.05 mM EDTA. Total numbers of BAL cells were counted by using a Bürker chamber (Marienfeld, Lauda-Königshofen, Germany). Trypan Blue was added to exclude dead cells. BAL immune cell composition was determined on an LSR-II flow cytometer (BD Biosciences) by analyzing cellular autofluorescence and surface expression of CD3ε, CD4, CD8α, CD11b, CCR3, MHC-II, and CD11c, similar to the protocol described in Schepens et al. (2011). All antibodies were purchased from Pharmingen (BD Biosciences) except for CCR3 (R&D Systems). For challenge experiments, clodronate liposomes were administered 3 days before HRNV challenge. One day before and 1 day after HRNV challenge, 35 µl of heat-inactivated SHE-KLH or KLH mouse immune serum was administered intranasally to mice sedated with isoflurane.

Immunostaining HRNV-infected cells and cells to which HRNV is attached

On day 1, 7 × 104 A549 cells were seeded on glass cover slips in two different 24-well plates. On day 2, the cells of 1 plate were infected with 1 MOI of HRNV A2. On day 3, the cells of the second plate were washed once with cold PBS and then incubated with cold OptiMEM medium containing either 5 MOI HRNV A2 or no virus for 2 h on ice. Subsequently, all cells were washed twice with medium containing 10% FCS and twice with cold PBS. Next, the cells were fixed with 2% paraformaldehyde in PBS for 30 min on ice. After fixation, the cells were washed twice with PBS and blocked with 1% BSA in PBS. The cells were then stained with goat anti-HRSV serum (diluted 1/2,000 in 1% BSA in PBS) (AB1128, Millipore, Massachusetts, USA) in combination with either an HRNV F-specific mouse monoclonal
Problem

Human respiratory syncytial virus (HRSV) is the major respiratory virus in young infants. In adults and the elderly, HRSV closely follows influenza virus as a cause of severe lower respiratory tract disease. Despite decades of research, there is no licensed HRSV vaccine available. Conventional HRSV vaccine candidates are mainly based on the induction of neutralizing antibodies directed against the two major HRSV surface proteins F and G. However, although high levels of HRSV-neutralizing antibodies correlate with reduced incidence of HRSV-associated hospitalization, humans with high levels of such antibodies can still be infected. Moreover, boosting pre-existing HRSV-neutralizing antibodies in humans by vaccination appears to be difficult. Therefore, we have explored an unconventional approach that does not rely on the induction of virus-neutralizing antibodies or mimics immune responses following natural infection, to develop an HRSV vaccination strategy.

Results

We used the extracellular domain (SHe) of the small hydrophobic domain of HRSV as a vaccine antigen. This domain is displayed on the surface of infected cells and to a lesser extent on the surface of virions. We found that antibodies directed against this domain could be generated by a vaccine comprising this domain. Immunization of mice or cotton rats with this novel vaccine antigen resulted in significantly reduced virus replication upon HRSV infection. We also propose a likely mechanism of action of SHe-specific immunity. Using cellular depletion experiments and mice with targeted disruption of FcγRI and FcγRIII, we show that antibodies directed against SHe reduce HRSV replication via IgG binding receptors and alveolar macrophages. Finally, pre-existing humoral immunity against SHe is very low in the human population, suggesting that HRSV has evolved in the absence of SHe-based immunity.

Impact

Our work proposes a novel HRSV vaccination approach that targets an HRSV antigen that is naturally not readily recognized by the host adaptive immune system. This vaccine protects against HRSV by a mechanism that is different from that of all other HRSV vaccine approaches that have been explored to date. Hence, this new HRSV vaccination approach might complement vaccination strategies based on the induction of neutralizing antibodies.

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Author contributions

Conceived and designed the experiments: BS, BG, PP, and XS. Performed the experiments: BS, KS, SDB, MS, KR, LH, and BG. Contributed reagents/materials/analysis tools: NVR. Analyzed the data: BS, MS, KR, LH, MVR, BG, PP, and XS. Wrote the manuscript: BS, WF, and XS.

Conflict of interest

VIB and UGhent hold patent rights on SHe-based vaccines and treatment options for RSV: patent application WO2012/065997 (24.05.2012) with Bert Schepens, Walter Fiers, and Xavier Saelens as inventors.

Supplementary information for this article is available online: http://embomolmed.embopress.org

The paper explained

antibody (diluted 1/2,000 in 1% BSA in PBS) (MAB858-1, Millipore, Massachusetts, USA) or serum (precleared overnight with detached A549 cells and diluted 1/500 in 1% BSA) of KLH or SHe-KLH-immunized mice. After washing three times with 1% BSA in PBS, the bound goat and mouse IgG antibodies were detected with, respectively, Alexa594-conjugated donkey anti-goat IgG and Alexa488-conjugated donkey anti-mouse IgG antibodies (Invitrogen Molecular Probes, Paisley, UK). The nucleus was stained with DAPI nuclear stain. Images were recorded with a Leica TCS SP5 confocal microscope. The infection and attachment samples were recorded with the same settings. Images were processed with Volocity software (Perkin Elmer, Massachusetts, USA). For the quantitative analysis of antibody binding to HRSV virions attached to cells, seven images were used for the samples stained with SHe-KLH serum and the F-specific monoclonal antibody. One image was used for the control sample stained with KLH serum.

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