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Antibody Response to the Central Unglycosylated Region of the Respiratory Syncytial Virus Attachment Protein in Mice

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Abstract

We examined the humoral immune response to the unglycosylated central region of the respiratory syncytial virus (RSV) attachment (G) protein in mice following intranasal challenge at day 0 (primary) and day 21 (secondary) with subtype A (A2 strain) or B (B1 strain) RSV preparations. Our serological screening reagents included bacterially derived glutathione S-transferase (GST) fusion proteins, each bearing a portion of the RSV G central core (CC; residues 151–190), proximal central core (PCC; residues 151–172), and the distal central core (DCC; residues 173–190) and purified RSV G proteins from subtype A and B viruses. Convalescent sera collected on day 21 following primary RSV infection bore robust IgG response primarily against the homosubtypic RSV G DCC with relatively modest antigen affinity/avidity as demonstrated by brief incubation with 6M urea. In contrast, sera collected on day 42 following secondary homosubtypic RSV infection bore IgG titers of higher magnitudes and antigen affinity/avidity against the homosubtypic RSV G CC, PCC, and/or the DCC regions and full-length RSV G protein but not against the heterosubtypic RSV G protein or recombinant CC subdomains. In contrast, heterosubtypic secondary RSV infection elicits a broad array of IgG responses with titers of varying magnitudes to homo- and heterosubtypic RSV G CC regions as well as to purified F, Ga, and Gb proteins with the notable exception of minimal response to the RSV G DCC domain associated with the secondary RSV challenge. Our results have implications for RSV G-based serological assays as well as prophylactic immunotherapy and RSV vaccine development.

Keywords

respiratory syncytial virus; attachment protein; humoral immune response; viral subtype

1. Introduction

RSV is an important cause of lower respiratory tract infections in the pediatric and elderly adult populations.[1] Based on antigenic/genetic differences, RSV strains are categorized as subtype A or B.[2] The major targets of host adaptive immune response following RSV infection are the RSV F (fusion) and G (attachment) proteins.[3] While the neutralizing RSV

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F epitopes are conserved among circulating strains, i.e. subtype-independent, many RSV G epitopes recognized by monoclonal antibodies (MAbs) and RSV convalescent sera are subtype-specific.[4–9]

Previous studies of pediatric RSV convalescent sera suggest that the unglycosylated central core (CC; residues 151–190) of the RSV G protein bears subtype-specific determinants of anti-G humoral immune response.[6–8,10–12] We have further partitioned the RSV G CC region into the proximal conserved core (PCC; residues 151–172) and distal conserved core (DCC; residues 173–190 bearing structural homology to the fractalkine CX3C motif; Fig. 1A).[13,14] While screening RSV convalescent sera from elderly adults, we noted that the PCC-specific IgG responses were subtype-independent while DCC-specific responses were subtype-specific.[13,14]

In the mouse model of RSV infection, many key aspects of RSV G-specific antibody responses, including viral subtype-dependent and –independent nature of anti-CC domain antibodies and their antigen affinity/avidity profiles, remain incompletely understood. We now describe our efforts to characterize anti-RSV G IgG response in mice following primary and secondary RSV challenge.

2. Materials and Methods

Mammalian cells and RSV strains

HEp-2 and Vero cells and RSV A2 and B1 strains were obtained from American Type Culture Collection. Upon receipt, the viruses were propagated in Vero cells (unless otherwise specified) for 4 passages prior to mouse challenges. Cells were maintained in Modified Eagle's Media + 10% fetal calf serum + L-glutamine + penicillin-streptomycin (Invitrogen).[15,16] Clarified extracts of infected cells were titered for RSV and stored at –80°C until use within 2 months.[17,18]

DNA constructions

Plasmids encoding GST–RSV A2 or B1 CC, PCC, and DCC have been described.[13] For this study, we re-engineered plasmids encoding GST-G A2 and B1 PCC and CC so that all fusion proteins in this study now bear the residues ...GSGSG... as the linker between the GST and RSV G moieties. The linker modifications did not affect their serum reactogenicities as compare to those of previously utilized proteins with ...GSG... linkers (data not shown).

Bacterial manipulations and protein purification

GST-G proteins were purified as described.[13] RSV Fa and Ga proteins (subscript = viral subtype) were fractionated from RSV A2-infected HEp-2 cell lysates as described except that RSV Fa was purified using anti-RSV F MAb affinity chromatography while RSV Ga fractions were treated with anti-RSV F MAb-Sepharose and purified using lectin-Sepharose (GE Healthcare).[19,20] RSV B1-derived Gb protein was obtained from Edward Walsh, MD (University of Rochester).

Mouse experiments

Female 6 week-old BALB/c mice (Jackson Laboratories) were fed and housed in a pathogen-free environment within the University of Rochester School of Medicine and Dentistry Vivarium and in accordance with Institutional Animal Care and Use Committee requirements.

For determination of RSV A2 and B1 titers in lung homogenates, mice were anesthetized with isofluorane (Butler) and intranasally challenged with 50 μ l of RSV preparations containing $\sim 1 \times 10^5$ plaque forming units (pfu) of A2 or B1 strain. On day 4 post-challenge, each animal was sacrificed and lung homogenates were plated onto HEp-2 cells for plaque immunostaining assay with anti-RSV F antibodies.[17,21] Bronchoalveolar lavages (BAL) with phosphate buffered saline (PBS) were performed on day 6 post-RSV challenge and the harvested cells were counted, cytopun, and stained using Hema 3 (Fisher).[22] For each sample, differential counts were obtained by counting 200 leukocytes at 500 $\times$ magnification.

For serological screening, mice were challenged with RSV as above on day 0 and were terminally bled at day 21 (primary infection) or bled via the submandibular route two days prior to re-challenge (i.e. day 19) at day 21 and terminally bled at day 42 (secondary infection) (Fig. 1B). For mice subjected to secondary infections, pooled day 19 sera yielded similar ELISA results to day 21 terminal bleeds of mice used for primary infections (data not shown).

Enzyme-linked immunosorbent assays (ELISAs)

Mouse sera were screened against each GST-G derivative using 100 ng/well (for GST derivatives) or ~ 50 ng/well (for RSV glycoproteins) and PBS/0.05% Tween-20 for washes. [23] Each serum was diluted to 1:25–1:50 (Figure Legends) and serially diluted two-fold. The antigen-IgG complexes were visualized and quantitated as described. End point serum titers for each serum dilution series were calculated at an absorbance of 0.3 (i.e. three-fold higher than background levels) and expressed as reciprocal $\log_2$ dilutions. For serum dilutions with maximum absorbance values of < 0.3 , the reciprocal $\log_2$ titer of the highest serum dilution (e.g. 1:50 = titer of 5.64) was imputed. For antibody affinity/avidity measurements, pooled sera from each RSV-infected mouse group were tested in triplicate in ELISAs with a 5-minute incubation at ambient temperature with either PBS or 6M ultrapure urea (Fisher)/PBS between the primary and secondary antibody incubations.[24]

Graphical/statistical analyses

Data were analyzed using Prism 5.0 (Graphpad) with means compared using Wilcoxon rank-sum tests.

3. Results

3.1 Viral replication and pulmonary immune cell migration following RSV challenge

To characterize our RSV mouse model, we challenged mice with RSV A2 or B1 strain and determined RSV lung homogenate titers and the BAL cell number/types on day 4 and 6 post-challenge, respectively. RSV A2 and B1 titers (mean $\pm$ SEM) were 4.18 ± 3.35 and $3.28 \pm 2.71 \log_{10}$ pfu/g lung tissue (Fig. 2A), which are consistent with previous observations that RSV subtype A strains replicate to ~ 10 fold higher lung homogenate titers than do subtype B strains in mice.[22,25] However, the total number and differential cell count in BALs were similar between the two experimental groups (Fig. 2B/C), suggesting that the levels of RSV B1 replication is sufficient to elicit pulmonary immune cell migration comparable to that induced by higher lung titers of RSV A2.

3.2 Antibody responses following primary infection

We then characterized the IgG response against the RSV G CC region and purified F and G proteins at day 21 following primary RSV A2 or B1 infection in mice (Fig. 3). Among individual sera from RSV A2-challenged mice ($n = 10$), we observed robust responses (8.25 ± 0.07 mean $\pm$ SEM reciprocal $\log_2$ titers) against the homosubtypic A2 DCC domain and to a lesser degree, against the A2 CC and PCC domains (6.38 ± 0.08 and 6.26 ± 0.17 ,

respectively; Fig. 3A). There was a statistically significant ($p < 0.05$) difference between the mean A2 DCC titer and those against the A2 PCC and the A2 CC domains, but not between the A2 PCC and CC-specific titers. In contrast, we noted minimal reactivity against all three heterosubtypic RSV B1-derived GST-G derivatives. While we observed robust RSV Fa-specific titers (8.68 ± 0.22), there were minimal responses against the RSV Ga and Gb proteins following primary RSV A2 infection. Immunoblots using pooled convalescent sera also failed to detect purified Ga under denaturing and reducing/non-reducing conditions (data not shown). The magnitudes and trends of serum reactivity against purified F and G proteins as well as homo/heterosubtypic CC, DCC, and PCC-specific responses did not appreciably differ between sera from mice infected with HEP2 or Vero-derived RSV A2, thus implying the absence of host cell dependence on humoral immune responses against our screening reagents (data not shown).[26,27]

We then performed antigen affinity/avidity assays in which antigen:antibody complexes were subjected to a brief incubation with PBS alone or PBS + 6M urea prior to detection of remaining complexes (Fig. 3B).[24,28,29] Using pooled A2 convalescent sera, we noted detectable titers against each of the A2-derived central unglycosylated region (A2 DCC: 7.78 ± 0.03 ; A2 PCC: 6.00 ± 0.03 ; and A2 CC: 6.54 ± 0.14) as well as to B1 CC (5.65 ± 0.01) that withstood PBS incubation but were substantially reduced (to 5.26 ± 0.30 , or ~ 2.5 fold decline in reciprocal $\log_2$ titers for A2 DCC) or eliminated to undetectable levels (for A2 PCC, A2 CC, and B1 CC) upon 6M urea incubation. We also noted similar changes in the Fa-specific titers between PBS and urea incubations (8.50 ± 0.15 vs. 5.81 ± 0.16 , respectively). Thus, the RSV G central unglycosylated region-specific IgG responses following primary RSV A2 infection is of relatively low affinity that can be disrupted by brief 6M urea incubation.

We also screened individual convalescent sera from RSV B1 infected mice ($n = 8$; Fig. 3C) and observed strongly homosubtypic responses against the B1 DCC domain (7.14 ± 0.30) and to a lesser extent against the B1 CC and PCC domains (6.97 ± 0.27 and 5.82 ± 0.11 , respectively). In general, titers against the RSV B1-derived CC subdomains, particularly against the B1 PCC, were lower than the corresponding homosubtypic response vs. A2 derived CC subdomains and correlate with lower lung homogenate B1 vs. A2 titers (Fig. 2A). The difference in the mean titers for B1 DCC and PCC were statistically significant ($p < 0.05$) but not for B1 DCC vs. B1 CC and B1 PCC vs. B1 CC. There were minimal responses vs. RSV A2-derived CC residues. We observed robust titers against purified RSV Fa protein (8.81 ± 0.38) but not against either of the purified Ga or Gb proteins. Antigen avidity/affinity testing also revealed similar patterns as those in A2 convalescent mouse sera (Fig. 3D). We conclude that primary RSV infection in mice elicits primarily homosubtypic IgG response to the RSV G unglycosylated region.

3.3. Antibody responses following secondary infection

We then analyzed the RSV G CC-specific responses in secondary RSV infections. We intranasally challenged mice with RSV A2 or B1 and then three weeks later rechallenged with either homosubtypic or heterosubtypic RSV preparations and characterized day 42 sera (Fig. 1B).

When we screened homosubtypic convalescent sera from mice ($n = 9$) infected with RSV A2 at d0 and d21 (Fig. 4A), we noted several differences as compared to our results with sera after primary A2 infection. First, the magnitude of IgG response following secondary A2 infection was generally higher than those seen after the primary infection. The homosubtype-specific responses were highest against A2 DCC (12.22 ± 0.34) followed by A2 CC and PCC (10.63 ± 0.47 and 7.89 ± 0.71 , respectively). For these three RSV A2-derived domains, each of the two-way paired comparisons of mean titers was statistically

significant ($p < 0.05$). Second, the B1-specific response remained much lower than those vs. homosubtypic A2-derived residues. Third, we observed higher titers against purified RSV glycoproteins, including against RSV Fa protein (12.05 ± 0.31), RSV Ga protein (9.16 ± 0.21), and to a lesser extent, against the Gb protein (6.00 ± 0.18).

Secondary RSV A2 infection also led to higher antigen-specific titers following urea incubation as compared to those following primary RSV A2 infection. For A2-derived residues, the urea treatment led to the following mean $\pm$ SEM titer changes: A2 DCC: $12.99 \pm 0.19 \rightarrow 11.08 \pm 0.24$; A2 CC: $11.41 \pm 0.24 \rightarrow 9.37 \pm 0.06$; and A2 PCC: $8.75 \pm 0.14 \rightarrow 8.07 \pm 0.18$. Similar titer changes of 1.5–1.75 reciprocal $\log_2$ titer were noted for B1 CC as well as for Fa and Ga. The low level reactivity vs. Gb was rendered below the limits of detection with urea.

Our ELISA screening of mice ($n = 9$) infected twice with RSV B1 revealed reactivity vs. homosubtypic B1 CC (9.41 ± 0.23) and B1 DCC (8.41 ± 0.61) domains and to a lesser extent, against the B1 PCC domain (6.25 ± 0.30) (Fig. 4C). The difference between the B1 PCC-specific titer and that for B1 DCC and B1 CC were statistically significant ($p < 0.05$) but not between B1 CC and DCC-specific titers. The responses against heterosubtypic A2-derived residues were minimal. The reactivity against the purified RSV Fa protein was again robust (13.20 ± 0.47) and the mean RSV Gb-specific titer (6.73 ± 0.31) was higher than that for Ga (5.72 ± 0.24) in a statistically significant manner ($p < 0.05$). In antigen affinity/avidity assays, we observed similar homosubtypic-specific trends as in the A2-A2 convalescent sera (Fig. 4D).

We extended our analysis to heterosubtypic secondary infections in which mice ($n = 6$ for both A2-B1 and B1-A2 groups) were challenged with RSV A2 on day 0 and challenged with RSV B1 on day 21 and vice versa (Fig. 2B). Among individual day 42 sera, we noticed a greater range of reactivity to our screening reagents as compared to those from mice subjected to homosubtypic secondary infections (Fig. 5A and 5C). We did note the following trends: 1) the degree of reactivity vs. the DCC domain appears to be closely related to the subtype of the initial strain of RSV challenge, i.e. A2-B1 infected mice demonstrated anti-A2 DCC reactivity but minimal response to the RSV B1 DCC residues, and vice versa; 2) except for this DCC-specific observation, the reactivity of A2-B1 infected mouse sera demonstrated higher titers against A2 and B1-derived RSV G unglycosylated subdomains than those from B1-A2 infected mice; and 3) there were detectable reactivity vs. purified RSV Fa, Ga, and Gb proteins although the mean titers for Ga were higher than those of Gb with and without the presence of urea incubation in pooled sera (Fig. 5B and 5D).

4. Discussion

We systematically screened mouse convalescent sera for IgG response to the central unglycosylated region of the RSV G protein following primary and secondary RSV infections. We utilized structurally partitioned CC subdomains from homo- and heterosubtypic RSV G proteins and measured antigen affinity/avidity. We note similarities and differences between our results and previous serological screening of human and rodent sera.

Primary RSV infection in mice elicited robust, homosubtypic IgG response against the DCC. Our findings are consistent with pediatric convalescent sera screening in which the RSV G core peptide (residues 158–189) has been shown to be more sensitive for serological detection than purified RSV G; we surmise that this may be primarily due to the DCC-specific IgG responses.[11] Another pediatric retrospective study utilized peptides bearing subtype-specific RSV G cysteine “noose” (residues 164–189) to screen RSV convalescent

sera, but reported that only 11 out of 26 (42%) and 16/26 (62%) of paired sera from RSV-infected infants elicited 4-fold titer increase against the Ga and Gb cysteine noose, respectively.[30] This observation suggests that the magnitude and/or the frequency of DCC/cysteine “noose” specific response among infants may be different than those of mice following primary RSV infection.

Previous human or mouse serum screening studies have not characterized the antigen affinity/avidity of RSV G-specific IgG response. Following primary RSV infection, we consistently noted that all IgG responses, including those against DCC, were decreased by > 4-fold (i.e. > 2 reciprocal log₂ titers) with urea washes. Thus, the initial exposure (via primary RSV infection) to the RSV G unglycosylated region elicits antigen-antibody interactions of relatively low affinity/avidity.

In mice, primary RSV infection elicited minimal IgG response against full-length RSV G regardless of the subtype of the G protein or RSV strain, or cells used to generate the RSV strain (data not shown). These results are consistent with previous studies in which IgG titers against purified RSV G and transfected cell extracts bearing full-length RSV G were reported as < 1:40 and 1:10–1:100, respectively, in RSV A2 convalescent mouse sera.[31,32] In contrast, primary RSV infection in cotton rats elicited titers of 1:1000–1:4000 against homosubtypic RSV G and ~1:128 for heterosubtypic RSV G protein.[33] Similarly, the anti-RSV G titers in RSV convalescent sera are typically ~6.5–7 reciprocal log₂ titers and also demonstrate a strongly homosubtype-specific IgG response.[34,35] The differences between mouse and cotton rat/human sera reactogenicity vs. G may be multifactorial in nature, including epitope inaccessibility and/or the lower levels of RSV replication in mice – particularly that of subtype B strain, and the correspondingly lower titers of homosubtypic PCC response than subtype A strain – than in humans and cotton rats.[25,36] The weak serological recognition of full-length Ga or Gb in mice may correlate poorly with a neutralizing or fusion inhibiting response *in vivo*.

In contrast to the murine IgG responses elicited by primary RSV infection, those elicited by secondary RSV infections are more complex. Homosubtypic secondary RSV infection in mice increased the magnitude and affinity/avidity of homosubtypic humoral immune response to the RSV G CC, PCC, and/or the DCC regions and purified G protein but not against the heterosubtypic RSV G protein or recombinant CC subdomains. In contrast, heterosubtypic secondary RSV infection elicited a broad array of IgG responses with titers of varying magnitudes to homo- and heterosubtypic RSV G CC regions as well as to purified F, Ga, and Gb proteins except to the RSV G DCC domain associated with the secondary RSV challenge. Thus, among mice infected twice with RSV and in a heterosubtypic manner, the DCC-specific IgG response correlates with the subtype of the RSV strain used in primary infection.

These findings are notable given that secondary RSV infections in cotton rats (i.e. RSV challenges at days 0 and 21) and in mice cause abortive viral replication with no detection of RSV titers in lung homogenates.[17,37] Our results imply that such limited viral replication following secondary RSV challenge may be sufficient to alter the IgG profile against our panel of screening reagents in mice. Furthermore, the initial RSV exposure appears to “imprint” or restrict the subsequent heterosubtypic anti-DCC response.

What is the target of homosubtypic RSV G DCC-specific immune response following primary RSV infection? We speculate that it is secreted G (Gs), the predominant RSV G species that is released from RSV infected cells cultured *ex vivo* and primes CTL response and serves as “antigen decoy” *in vivo*. [38–41] The monomeric Gs structure likely exposes epitopes within the CC region to the host immune system, and the DCC structure may be

more surface-exposed and immunogenic than the PCC with its predicted hydrophobic residues.[42] The DCC-specific antibodies may then counter the effects of Gs and block endotoxin-mediated cytokine production and leukocyte migration due to the CXCR3 motif within the DCC region.[43–45] The therapeutic effects of anti-RSV G MAb 131-2G that blocks G protein interaction with CX3CR1 validates the targeting of RSV G DCC region as a potential target for prophylactic therapy and vaccine development.[21,46] Our data also suggest that the anti-DCC antibodies in response to primary RSV infection possess relatively low antigen affinity/avidity and do not efficiently recognize purified G protein, perhaps due to steric hindrance of DCC regions within the homotetrameric RSV G protein. [47]

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References

1. Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, et al. The burden of respiratory syncytial virus infection in young children. *N Engl J Med*. 2009; 360(6):588–98. [PubMed: 19196675]
2. Melero JA, Garcia-Barreno B, Martinez I, Pringle CR, Cane PA. Antigenic structure, evolution and immunobiology of human respiratory syncytial virus attachment (G) protein. *J Gen Virol*. 1997; 78(Pt 10):2411–8. [PubMed: 9349459]
3. Collins PL, Graham BS. Viral and host factors in human respiratory syncytial virus pathogenesis. *J Virol*. 2008; 82(5):2040–55. [PubMed: 17928346]
4. Walsh EE, Brandriss MW, Schlesinger JJ. Immunological differences between the envelope glycoproteins of two strains of human respiratory syncytial virus. *J Gen Virol*. 1987; 68(Pt 8):2169–76. [PubMed: 3302101]
5. Walsh EE, Hall CB, Schlesinger JJ, Brandriss MW, Hildreth S, Paradiso P. Comparison of antigenic sites of subtype-specific respiratory syncytial virus attachment proteins. *J Gen Virol*. 1989; 70(Pt 11):2953–61. [PubMed: 2479715]
6. Cane PA, Thomas HM, Simpson AF, Evans JE, Hart CA, Pringle CR. Analysis of the human serological immune response to a variable region of the attachment (G) protein of respiratory syncytial virus during primary infection. *J Med Virol*. 1996; 48(3):253–61. [PubMed: 8801286]
7. Cane PA. Analysis of linear epitopes recognised by the primary human antibody response to a variable region of the attachment (G) protein of respiratory syncytial virus. *J Med Virol*. 1997; 51(4):297–304. [PubMed: 9093944]
8. Palomo C, Cane PA, Melero JA. Evaluation of the antibody specificities of human convalescent-phase sera against the attachment (G) protein of human respiratory syncytial virus: influence of strain variation and carbohydrate side chains. *J Med Virol*. 2000; 60(4):468–74. [PubMed: 10686032]
9. Lopez JA, Bustos R, Orvell C, Berois M, Arbiza J, Garcia-Barreno B, et al. Antigenic structure of human respiratory syncytial virus fusion glycoprotein. *J Virol*. 1998; 72(8):6922–8. [PubMed: 9658147]
10. Langedijk JP, Middel WG, Schaaper WM, Melen RH, Kramps JA, Brandenburg AH, et al. Type-specific serologic diagnosis of respiratory syncytial virus infection, based on a synthetic peptide of the attachment protein G. *J Immunol Methods*. 1996; 193(2):157–66. [PubMed: 8699029]
11. Langedijk JP, Brandenburg AH, Middel WG, Osterhaus A, Melen RH, van Oirschot JT. A subtype-specific peptide-based enzyme immunoassay for detection of antibodies to the G protein of human respiratory syncytial virus is more sensitive than routine serological tests. *J Clin Microbiol*. 1997; 35(7):1656–60. [PubMed: 9196168]

12. Jones LP, Zheng HQ, Karron RA, Peret TC, Tsou C, Anderson LJ. Multiplex assay for detection of strain-specific antibodies against the two variable regions of the G protein of respiratory syncytial virus. *Clin Diagn Lab Immunol.* 2002; 9(3):633–8. [PubMed: 11986272]
13. Murata Y, Lightfoote PM, Bear JN, Falsey AR, Walsh EE. Humoral response to the central unglycosylated region of the respiratory syncytial virus attachment protein. *Vaccine.* 2010; 28(38): 6242–6. [PubMed: 20655403]
14. Murata Y, Lightfoote PM, Falsey AR, Walsh EE. Identification of and human serum reactivity to neutralizing epitopes within the central unglycosylated region of the respiratory syncytial virus attachment protein. *Clin Vaccine Immunol.* 2010; 17(4):695–7. [PubMed: 20164253]
15. Walsh EE, Brandriss MW, Schlesinger JJ. Purification and characterization of the respiratory syncytial virus fusion protein. *J Gen Virol.* 1985; 66(Pt 3):409–15. [PubMed: 3838336]
16. Walsh EE, Hall CB, Briselli M, Brandriss MW, Schlesinger JJ. Immunization with glycoprotein subunits of respiratory syncytial virus to protect cotton rats against viral infection. *J Infect Dis.* 1987; 155(6):1198–204. [PubMed: 3553346]
17. Boukhvalova MS, Yin KC, Prince GA, Blanco JC. Methods for monitoring dynamics of pulmonary RSV replication by viral culture and by real-time reverse transcription-PCR in vivo: Detection of abortive viral replication. *Curr Protoc Cell Biol.* 2010; Chapter 26(Unit26.6)
18. Mok H, Lee S, Utley TJ, Shepherd BE, Polosukhin VV, Collier ML, et al. Venezuelan equine encephalitis virus replicon particles encoding respiratory syncytial virus surface glycoproteins induce protective mucosal responses in mice and cotton rats. *J Virol.* 2007; 81(24):13710–22. [PubMed: 17928349]
19. Roder C, Krusat T, Reimers K, Werchau H. Purification of respiratory syncytial virus F and G proteins. *J Chromatogr B Biomed Sci Appl.* 2000; 737(1–2):97–106. [PubMed: 10681046]
20. Walsh EE, Hruska J. Monoclonal antibodies to respiratory syncytial virus proteins: identification of the fusion protein. *J Virol.* 1983; 47(1):171–7. [PubMed: 6345804]
21. Zhang W, Choi Y, Haynes LM, Harcourt JL, Anderson LJ, Jones LP, et al. Vaccination to induce antibodies blocking the CX3C-CX3CR1 interaction of respiratory syncytial virus G protein reduces pulmonary inflammation and virus replication in mice. *J Virol.* 2010; 84(2):1148–57. [PubMed: 19864390]
22. Tripp RA, Moore D, Jones L, Sullender W, Winter J, Anderson LJ. Respiratory syncytial virus G and/or SH protein alters Th1 cytokines, natural killer cells, and neutrophils responding to pulmonary infection in BALB/c mice. *J Virol.* 1999; 73(9):7099–107. [PubMed: 10438795]
23. Murata Y, Lightfoote PM, Rose RC, Walsh EE. Antigenic presentation of heterologous epitopes engineered into the outer surface-exposed helix 4 loop region of human papillomavirus L1 capsomeres. *Virol J.* 2009; 6:81. [PubMed: 19538743]
24. Polack FP, Hoffman SJ, Crujeiras G, Griffin DE. A role for nonprotective complement-fixing antibodies with low avidity for measles virus in atypical measles. *Nat Med.* 2003; 9(9):1209–13. [PubMed: 12925847]
25. Nguyen TN, Power UF, Robert A, Haeuw JF, Helffer K, Perez A, et al. The respiratory syncytial virus g protein conserved domain induces a persistent and protective antibody response in rodents. *PLoS One.* 2012; 7(3):e34331. [PubMed: 22479601]
26. Kwilas S, Liesman RM, Zhang L, Walsh E, Pickles RJ, Peeples ME. Respiratory syncytial virus grown in Vero cells contains a truncated attachment protein that alters its infectivity and dependence on glycosaminoglycans. *J Virol.* 2009; 83(20):10710–8. [PubMed: 19656891]
27. Garcia-Beato R, Martinez I, Franci C, Real FX, Garcia-Barreno B, Melero JA. Host cell effect upon glycosylation and antigenicity of human respiratory syncytial virus G glycoprotein. *Virology.* 1996; 221(2):301–9. [PubMed: 8661440]
28. Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. *Nat Med.* 2009; 15(1):34–41. [PubMed: 19079256]
29. Feng J, Gulati U, Zhang X, Keitel WA, Thompson DM, James JA, et al. Antibody quantity versus quality after influenza vaccination. *Vaccine.* 2009; 27(45):6358–62. [PubMed: 19840673]

30. Shinoff JJ, O'Brien KL, Thumar B, Shaw JB, Reid R, Hua W, et al. Young infants can develop protective levels of neutralizing antibody after infection with respiratory syncytial virus. *J Infect Dis.* 2008; 198(7):1007–15. [PubMed: 18702606]
31. Routledge EG, Willcocks MM, Samson AC, Morgan L, Scott R, Anderson JJ, et al. The purification of four respiratory syncytial virus proteins and their evaluation as protective agents against experimental infection in BALB/c mice. *J Gen Virol.* 1988; 69(Pt 2):293–303. [PubMed: 3339327]
32. Murawski MR, McGinnes LW, Finberg RW, Kurt-Jones EA, Massare MJ, Smith G, et al. Newcastle disease virus-like particles containing respiratory syncytial virus G protein induced protection in BALB/c mice, with no evidence of immunopathology. *J Virol.* 2010; 84(2):1110–23. [PubMed: 19889768]
33. Murphy BR, Sotnikov A, Paradiso PR, Hildreth SW, Jenson AB, Baggs RB, et al. Immunization of cotton rats with the fusion (F) and large (G) glycoproteins of respiratory syncytial virus (RSV) protects against RSV challenge without potentiating RSV disease. *Vaccine.* 1989; 7(6):533–40. [PubMed: 2692334]
34. Muelenaer PM, Henderson FW, Hemming VG, Walsh EE, Anderson LJ, Prince GA, et al. Group-specific serum antibody responses in children with primary and recurrent respiratory syncytial virus infections. *J Infect Dis.* 1991; 164(1):15–21. [PubMed: 2056202]
35. Hendry RM, Burns JC, Walsh EE, Graham BS, Wright PF, Hemming VG, et al. Strain-specific serum antibody responses in infants undergoing primary infection with respiratory syncytial virus. *J Infect Dis.* 1988; 157(4):640–7. [PubMed: 3346563]
36. Blanco JCG, Richardson JY, Darnell MER, Rowzee A, Pletneva L, Porter DD, et al. Cytokine and chemokine gene expression after primary and secondary respiratory syncytial virus infection in cotton rats. *J Infect Dis.* 2002; 185:1780–5. [PubMed: 12085325]
37. Schuurhof A, Bont L, Pennings JL, Hodemaekers HM, Wester PW, Buisman A, et al. Gene expression differences in lungs of mice during secondary immune responses to respiratory syncytial virus infection. *J Virol.* 2010; 84(18):9584–94. [PubMed: 20592085]
38. Johnson TR, Johnson JE, Roberts SR, Wertz GW, Parker RA, Graham BS. Priming with secreted glycoprotein G of respiratory syncytial virus (RSV) augments interleukin-5 production and tissue eosinophilia after RSV challenge. *J Virol.* 1998; 72(4):2871–80. [PubMed: 9525607]
39. Johnson TR, Graham BS. Secreted respiratory syncytial virus G glycoprotein induces interleukin-5 (IL-5), IL-13, and eosinophilia by an IL-4-independent mechanism. *J Virol.* 1999; 73(10):8485–95. [PubMed: 10482601]
40. Bukreyev A, Yang L, Fricke J, Cheng L, Ward JM, Murphy BR, et al. The secreted form of respiratory syncytial virus G glycoprotein helps the virus evade antibody-mediated restriction of replication by acting as an antigen decoy and through effects on Fc receptor-bearing leukocytes. *J Virol.* 2008; 82(24):12191–204. [PubMed: 18842713]
41. Hendricks DA, McIntosh K, Patterson JL. Further characterization of the soluble form of the G glycoprotein of respiratory syncytial virus. *J Virol.* 1988; 62(7):2228–33. [PubMed: 3373568]
42. Gorman JJ, McKimm-Breschkin JL, Norton RS, Barnham KJ. Antiviral activity and structural characteristics of the nonglycosylated central subdomain of human respiratory syncytial virus attachment (G) glycoprotein. *J Biol Chem.* 2001; 276(42):38988–94. [PubMed: 11487583]
43. Haynes LM, Jones LP, Barskey A, Anderson LJ, Tripp RA. Enhanced disease and pulmonary eosinophilia associated with formalin-inactivated respiratory syncytial virus vaccination are linked to G glycoprotein CX3C-CX3CR1 interaction and expression of substance P. *J Virol.* 2003; 77(18):9831–44. [PubMed: 12941892]
44. Harcourt J, Alvarez R, Jones LP, Henderson C, Anderson LJ, Tripp RA. Respiratory syncytial virus G protein and G protein CX3C motif adversely affect CX3CR1+ T cell responses. *J Immunol.* 2006; 176(3):1600–8. [PubMed: 16424189]
45. Oshansky CM, Zhang W, Moore E, Tripp RA. The host response and molecular pathogenesis associated with respiratory syncytial virus infection. *Future Microbiol.* 2009; 4(3):279–97. [PubMed: 19327115]
46. Radu GU, Caidi H, Miao C, Tripp RA, Anderson LJ, Haynes LM. Prophylactic treatment with a G glycoprotein monoclonal antibody reduces pulmonary inflammation in respiratory syncytial virus

- (RSV)-challenged naive and formalin-inactivated RSV-immunized BALB/c mice. *J Virol.* 2010; 84(18):9632–6. [PubMed: 20592094]
47. Escribano-Romero E, Rawling J, Garcia-Barreno B, Melero JA. The soluble form of human respiratory syncytial virus attachment protein differs from the membrane-bound form in its oligomeric state but is still capable of binding to cell surface proteoglycans. *J Virol.* 2004; 78(7): 3524–32. [PubMed: 15016875]

Highlights

- We measured RSV G central core-specific IgG in RSV convalescent mouse sera.
- Primary RSV infection elicits virus subtype-specific IgG vs. aa 173–190.
- Such subtype specificity remains unaffected by subtype of secondary infection.
- Homosubtypic secondary RSV infection elicits homosubtypic IgG vs. aa 151–190.
- RSV G-specific IgG antigen avidity/affinity increases after secondary infection.

Fig. 1. Serological screening reagents and animal immunization schemes used in the study. (A) Shown are the residues comprising the central unglycosylated region of the RSV A2 or B1 G protein. The invariant residues 164–176 that are conserved among clinical isolates are boxed and the cysteines are shown in bold. Residues comprising the conserved core (CC), proximal conserved core (PCC), and the distal conserved core (DCC) domains are underlined; for each domain, the relevant residue positions are numbered at the respective line margins. (B) Schematic diagram of animal immunization scheme for this study. For primary RSV infection, mice were intranasally challenged with either RSV A2 or B1 strain (see text for details) on day 0 (d0) and terminally bled on d21. For secondary RSV infections, mice were challenged with either RSV A2 or B1 strain on d0 and on d21 (either homosubtypically, i.e. A2/A2 or B1/B1, or heterosubtypically, i.e. A2/B1 or B1/A2) and terminally bled on d42.

Fig. 2.

Viral replication and pulmonary immune cell migration following RSV A2 or B1 primary infection in mice. (A) RSV lung titers following intranasal challenge. Mice ($n=5$ /group) were intranasally challenged with $1E5$ pfu of RSV A2 or B1 and four days later sacrificed for lung homogenate preparation. The resulting RSV plaques on Hep2 cell monolayers were detected using anti-RSV F MAbs and counted. Individual RSV titer points are plotted and bars indicate mean $\pm$ SEM (A2: 4.18 ± 3.35 ; and B1: 3.28 ± 2.71) $\log_{10}$ pfu/g lung tissue and plotted on a semi-log scale. The difference between the mean RSV A2 titer vs. RSV B1 titer was statistically significant ($p < 0.05$). (B) and (C) Analysis of BAL cells following RSV challenge. Mice ($n=5$ /group and distinct from those used in Panel (A)) were challenged with RSV A2 or B1 as above and on day 6 post-challenge, sacrificed and subjected to BAL with PBS. The total number of cells in each BAL sample was counted and plotted as mean $\pm$ SEM (Panel B); values were $197,000 \pm 18,765$ and $197,000 \pm 15,379$ for RSV A2 or B1 infected mice, respectively. The BAL cells were then stained and counted (Materials and Methods), and expressed as a percentage of total cells counted (200 cells/sample). The results from each of the two groups of mice were pooled and the mean $\pm$ SEM were plotted for the three major cell types. None of the BAL samples bore $> 1\%$ of cells that were categorized as eosinophils or basophils, and thus were not plotted or analyzed further. All comparisons of variables in (B) or (C) for A2 vs. B1 virus were deemed not to be statistically significant ($P > 0.05$).

Fig. 3.

Serum reactivity against GST-RSV G proteins and purified RSV F and G glycoproteins following primary RSV infection. RSV A2 strain or B1 strain were prepared from infected Vero cells (see text for details) and used to intranasally challenge mice ($n = 10$ for A2 strain; $n = 8$ for B1) on d0. On d21 following challenge, serum was collected from each mouse and tested individually (panels A and C) against GST alone, various GST-RSV G preparations, or purified RSV Fa, Ga, or Gb proteins in ELISAs followed by end point titers (expressed as reciprocal log₂ titers). Pooled sera from each group of mice were used to measure antigen-specific titers in triplicate with PBS (shaded bars) or 6M urea (open bars) incubation in between the primary and secondary antibody steps (panels B and D). Results were expressed as mean $\pm$ SEM (represented by bar above each column) reciprocal log₂ titers. Note that given the minimal reactivity of individual sera against purified Ga and Gb proteins, d21 pooled sera were not tested against Ga/Gb in the antigen affinity/avidity assays. Note also that for the experiments shown in Panels B and D, the initial starting serum dilution was 1:25 = 4.64 reciprocal log₂ titer. For assays summarized in Panels A and C, and elsewhere in this report, the starting serum dilution was 1:50.

Fig. 4.

Serum reactivity against GST-RSV G proteins and purified RSV F and G glycoproteins following homosubtypic secondary RSV infection. RSV A2 strain or B1 strain were prepared from infected Vero cells (see text for details) and used to intranasally challenge mice ($n = 9$ for both groups) on d0 and d21. On d42 following the initial RSV challenge, serum was collected from each mouse and tested individually (panels A and C) against GST alone, various GST-RSV G preparations, or purified RSV Fa, Ga, or Gb proteins in ELISAs followed by end point titers (expressed as reciprocal log₂ titers). Pooled sera from each group of mice were used to measure antigen-specific titers in triplicate with PBS (shaded bars) or 6M urea (open bars) incubation in between the primary and secondary antibody steps (panels B and D). Results were expressed as mean $\pm$ SEM (represented by bar above each column) reciprocal log₂ titers. For all experiments shown, the initial starting serum dilution was 1:50.

Fig. 5.

Serum reactivity against GST-RSV G proteins and purified RSV F and G glycoproteins following heterosubtypic secondary RSV infection. RSV A2 strain or B1 strain were prepared from infected Vero cells (see text for details) and used to intranasally challenge mice ($n = 6$ for both groups) on d0 and then challenged with the heterosubtypic virus (i.e. A2 $\rightarrow$ B1, and B1 $\rightarrow$ A2) on d21. On d42 following the initial RSV challenge, serum was collected from each mouse and tested individually (panels A and C) against GST alone, various GST-RSV G preparations, or purified RSV Fa, Ga, or Gb proteins in ELISAs followed by end point titers (expressed as reciprocal log₂ titers). Pooled sera from each group of mice were used to measure antigen-specific titers in triplicate with PBS (shaded bars) or 6M urea (open bars) incubation in between the primary and secondary antibody steps (panels B and D). Results were expressed as mean $\pm$ SEM (represented by bar above each column) reciprocal log₂ titers. For all experiments shown, the initial starting serum dilution was 1:50.