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Rhinovirus infections and immunisation induce cross-serotype reactive antibodies to VP1

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ABSTRACT

Rhinoviruses (RVs) are ubiquitous human respiratory viruses, the major cause of common colds, acute exacerbations of asthma and other respiratory diseases. The development of antibodies to RV following primary infection is poorly understood and there is currently no RV vaccine available. We therefore used mouse models of intranasal RV infection and immunisation to determine the induction, magnitude and specificity of antibody responses. Strong cross-serotype RV-specific IgG responses in serum and bronchoalveolar lavage were induced towards the RV capsid protein VP1. IgA responses were weaker, requiring two infections to generate detectable RV-specific binding. Similarly two or more RV infections were necessary to induce neutralising antibodies. Immunisation strategies boosted homotypic as well as inducing cross-serotype neutralising IgG responses. We conclude that VP1 based antigens combined with adjuvants may permit successful antibody-mediated vaccine design and development.

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1. Introduction

RVs are ubiquitous human pathogens of the respiratory tract. They are the major cause of common colds and acute exacerbations of airway diseases such as asthma and chronic obstructive pulmonary disease (COPD), are responsible for approximately 50% of virus-induced respiratory infections and half to two-thirds of virus-induced asthma exacerbations (Greenberg, 2007; Johnston et al., 1995). RV belongs to the single-stranded positive sense RNA virus *Picornaviridae* family. The RNA genome is surrounded by an icosahedral protein capsid comprising 60 copies of 4 structural proteins, the surface exposed VP1, VP2, VP3 and the buried VP4 (Rossmann et al., 1985). One hundred distinct serotypes of RV numbered 1–100 have been well characterised (Rhinoviruses: a numbering system, 1967; Conant and Hamparian, 1968a,b) and classified based on antibody neutralisation properties with guinea

pig antisera (Schieble et al., 1974). RV can be further grouped based upon receptor usage with approximately 90% of serotyped strains (major group) using ICAM-1 as the receptor to enter host epithelial cells (Greve et al., 1989) whereas the minor group is known to exploit members of the low-density lipoprotein (LDL) receptor family (Hofer et al., 1994).

In humans, protection against re-infection with RV is associated with high levels of serotype-specific neutralising antibody both in serum and respiratory secretions (Barclay et al., 1989). Vaccination with RV preparations also induces neutralising antibodies in humans with IgA being the dominant antibody found in nasal secretions whereas in serum IgG dominates (Knopf et al., 1970; Perkins et al., 1969). In addition, high levels of RV-binding serum IgA correlates well with total serum neutralising antibody levels, the lack of development of a cold, and reduced viral shedding following experimental RV infection (Barclay and Al-Nakib, 1987; Barclay et al., 1988). The relative contributions of the various human IgG and IgA subclasses to RV neutralisation has not been investigated extensively although RV-binding IgG1, IgG4, and IgA1 levels rise in response to RV infection and neutralising titres are correlated with both IgG1 and IgA1 levels (Carey et al., 1992). In most cases, neutralising antibodies generated to RV following immunisation or infection are highly serotype-specific (Conant and Hamparian, 1968a,b), nevertheless in some cases, antibodies persisting from

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previous encounters with other RV serotypes have been demonstrated to cross-react with closely related RV serotypes and rarely to result in cross-neutralisation (Fox, 1976). Thus, there are limited data from humans supporting the development of vaccine strategies that induce cross-serotype neutralising antibodies against RV. However, the lack of a practical mouse model of RV infection has been a major barrier for vaccine development and the investigation of the fundamentals of RV antibody generation.

Complicating the study of antibody generation following RV infection is the fact that humans experience frequent RV infections throughout life (Turner, 1997) and therefore develop RV-specific antibodies from an early age. Recently a mouse model of RV infection was developed (Bartlett et al., 2008). Intranasal infection of minor (BALB/c) and major group (transgenic BALB/c expressing mouse-human ICAM-1 chimaera) RV induced airway inflammatory responses and viral replication was demonstrated in airway epithelium (Bartlett et al., 2008). The major advantage of using a mouse model of RV infection for our studies is, unlike humans, mice are immunologically naive to RV and the development of the humoral immune responses can be directly studied without interference from cross reactive antibodies. We have now used this model to characterise the induction of humoral immune responses generated after single and multiple i.n. RV infections, studies that are difficult to perform in humans. We determined that antibody responses were predominantly IgG, were directed against the RV capsid protein VP1 and multiple RV infections were required to generate neutralising antibodies. We also report that these responses can be augmented by mucosal CpG, as well as by systemic immunisation with inactivated virus and adjuvant. The latter approach boosted serotype-specific as well cross-serotype neutralising antibody. Our findings demonstrate the utility of the mouse model for investigating anti-RV humoral immunity and for determining the effect of vaccine strategies on the response to RV infection. VP1 based antigens combined with adjuvants have promise as candidate vaccines to boost serotype-specific as well as cross-serotype neutralising antibodies.

2. Materials and methods

2.1. Rhinovirus preparations

Serotypes RV1B, RV29 and RV16 were produced by culture in HeLa cells as described previously (Bartlett et al., 2008). To investigate virus-specificity of antibody binding, control HeLa lysate was prepared in an identical fashion from uninfected cells. RV preparations were inactivated by exposing them to UV light (1,200 mJ/cm² for 30 min) as previously described (Bartlett et al., 2008). Recombinant VP1 from RV14 and RV89 was expressed and purified as described (Edlmayr et al., 2009, 2011).

2.2. Animals and experimental protocols

All animal work was in accordance with Project Licence PPL70/6387, and performed according to regulations outlined by the Home Office, UK, in agreement with the Animals (Scientific Procedures) Act 1986. Female 6–8 week old BALB/c mice were purchased from Harlan and housed under appropriate SPF conditions. RV infections were performed as described previously (Bartlett et al., 2008). Briefly, purified RV (1×10^6 TCID₅₀) in a 50 μ L volume of PBS was introduced i.n. to groups of 4–6 isoflurane anaesthetised mice.

2.2.1. Primary infection

Mice were infected i.n. with RV and sacrificed at time points up to 18 days later.

2.2.2. Secondary infection

Mice were infected i.n. with RV, left to resolve the infection for 30 days then re-infected i.n. with homologous or heterologous RV serotypes and sacrificed at time points up to 16 days later. In some experiments, mice were also administered 20 μ g CpG oligonucleotides (ODN 1826, Invivogen, Nottingham UK) i.n. 2 days before and at the same time as the first RV infection, left for 30 days and re-infected i.n. with RV with analyses performed 7 days later.

2.2.3. Multiple infections

Mice were i.n. infected with RV1B on 5 separate occasions at 14–20 day intervals and sacrificed for endpoint analyses 4 days after the final infection.

2.2.4. Hyper-immunisation

Mice were immunised subcutaneously (s.c.) with 0.2 mL of a solution containing equal volumes of Freund's complete adjuvant (FCA, Sigma Aldrich UK) and RV1B (2×10^6 TCID₅₀). Mice were then further immunised in a similar fashion using Freund's incomplete adjuvant (IFA, Sigma Aldrich UK) and RV1B 4 further times at monthly intervals. Control animals received adjuvant immunisations alone. Mice were infected i.n. with RV1B 1 month after the final immunisation and sacrificed 4 days later.

2.3. Elisa

Plates (96-well, Nunc MaxiSorp) were coated overnight at 4 °C with the purified RV preparations used for i.n. infections and HeLa cell lysates diluted to a protein concentration of 5 μ g/mL in PBS or with purified recombinant VP1 proteins diluted to 1 μ g/mL in PBS. Plates were blocked for 2 h at room temperature by adding PBS containing 0.05% Tween20 and 5% milk powder (PBST-milk) before the addition of diluted serum or BAL samples and incubation overnight at 4 °C. Plates were washed with PBST and bound immunoglobulins were detected using biotinylated rat anti-mouse IgG1, IgG2a, IgG2b or IgA (BD Biosciences UK, Oxford UK) diluted 1:1000 before the addition of streptavidin-peroxidase (Invitrogen, Paisley UK). Plates were developed using tetramethyl benzidine (TMB) solution (Invitrogen, Paisley UK) as substrate and the optical density read using a spectrophotometer at 450 nm with 570 nm reference.

2.4. Immunoblotting

RV protein preparations were separated by reducing SDS-PAGE, transferred to nitrocellulose membranes, blocked with PBST-milk for 1 h at room temperature before diluted mouse or guinea pig serum (V-113-501-558, purchased from ATCC) was added and incubated overnight with shaking at 4 °C. Bound serum IgG was analysed using anti-mouse IgG or anti-guinea pig IgG coupled to peroxidase (Sigma Aldrich UK). ECL advance (Amersham Biosciences UK) was added to washed membranes which were then exposed to X-ray film and developed.

2.5. In vitro RV neutralisation assay

Mouse serum was serially diluted in a 96-well tissue culture plate in DMEM containing 4%FCS then 100 TCID₅₀ of RV1B or RV16 was added before incubation with shaking at room temperature for 1 h. 2×10^4 HeLa cells were added to each well and the plates incubated at 37 °C for 3 days before the cytopathic effect (CPE) was inspected microscopically. CPE was measured by staining with crystal violet as described (Edlmayr et al., 2011). Guinea pig antiserum specific for RV1B (V-113-501-558) or RV16 (V-105-501-558) were purchased from ATCC and served as a positive control for virus neutralisation.

2.6. IgG depletion from antiserum

Mouse serum was diluted in PBS and allowed to mix with protein G Sepharose overnight at 4 °C. After centrifugation to remove the Sepharose beads and bound IgG, the unbound fraction containing IgA was retained and used in ELISA experiments. Depletion of IgG from serum was confirmed by showing loss of binding to RV by ELISA.

2.7. Flow cytometry

Leukocytes from BAL and lungs were prepared as described previously (Bartlett et al., 2008). Approximately 5×10^5 cells were suspended in FACS buffer (PBS containing 1% bovine serum albumin and 0.01% sodium azide), Fc receptors blocked with rat anti-mouse CD16/CD32 (BD Biosciences UK, Oxford UK) for 20 min on ice then stained with Pacific Blue-conjugated rat anti-mouse CD3 (anti-CD3-PB) and fluorescein isothiocyanate-conjugated rat anti-mouse CD19 (anti-CD19-FITC) for 30 min on ice. The cells were then washed several times before flow cytometric analysis.

2.8. B cell ELISpot

Lung and mediastinal lymph node (MLN) leukocytes were prepared as described previously (Bartlett et al., 2008). Detection of total and RV-specific IgG and IgA production by B cells was performed by ELISpot assay using kits purchased from Mabtech AB (Nacka Strand, Sweden) and following manufacturer instructions.

3. Results

3.1. Intranasal infection of mice with RV rapidly induces cross serotype reactive IgG responses

We investigated the IgG responses generated to RV by BALB/c mice following primary RV infection and re-challenge with homologous (RV1B) or heterologous (RV29) RV serotypes. Mice infected with RV1B or RV29 made RV-specific serum IgG2a and IgG2b that was detectable by ELISA after 8–16 days (upper panels of Fig. 1A–D). RV-specificity was confirmed by showing that both IgG2a (Fig. 1A and C) and IgG2b (Fig. 1B and D) responses in wells coated with RV preparations were significantly greater than responses observed in wells coated with HeLa cell lysates. The use of HeLa lysate as an antigen was important given that RV was grown in these cells and contained HeLa- and serum-derived proteins against which antibodies may be generated. We also observed RV-specific IgG that were serotype cross reactive since both serum IgG2a and IgG2b were generated that bound RV1B after a single RV29 infection (Fig. 1A and B). Following a single RV1B infection only cross reactive serum IgG2b that recognised RV29 was detected (Fig. 1C and D). These data reflect the high degree of capsid protein homology (77% identity within VP1-VP2-VP3) that exists between RV1B and RV29 which are both type A RV's. Re-challenge of mice by infection with RV1B or RV29 one month after the initial RV1B infection generated increased RV-specific serum IgG responses that was detectable as early as 1 day after the second infection and reached maximal levels after 4–8 days (lower panels of Fig. 1A–D).

3.2. RV capsid protein VP1 is a major antibody target

To characterise the IgG binding to RV proteins by serum from RV1B infected mice we performed western blot analyses. Proteins within HeLa lysate, RV1B, RV29 and RV16 preparations were separated by electrophoresis and probed with serum from RV1B in-

fecting mice or commercially available guinea pig antisera to RV1B. Detection of the bound mouse IgG revealed a virus-specific band of approximately 39 kDa common to RV1B, RV29 and RV16 (Fig. 2A left panel). The 39 kDa band was also observed in each of the three RV preparations when probed with a commercially available guinea pig antisera raised against RV1B (Fig. 2A right panel). It is likely that the 39 kDa band common to each RV preparation is VP1 since it has been shown previously to migrate at this molecular weight (Skern et al., 1987). To confirm the presence of antibodies against VP1 in sera from mice infected twice with RV1B we performed ELISA using purified recombinant VP1 from RV serotypes RV14 and RV89 (Edlmayr et al., 2011) as the antigen since recombinant VP1 from RV1B was unavailable. We observed that mouse IgG2a from RV1B infected animals bound to the more similar VP1 from RV89 (66% identity between RV1B and RV89 VP1) but not to VP1 from RV14 (43% identity between RV1B and RV14 VP1) (Fig. 2B upper panels). IgG1 responses were not significant (Fig. 2B lower panels). These data support our notion that antisera from RV infected mice primarily binds to the capsid protein VP1.

3.3. Multiple RV infections induce IgA responses

Two RV1B infections were required to generate significant IgA binding to RV1B as detected by ELISA (Fig. 3A). We observed that both bronchoalveolar lavage (BAL) and serum contained detectable RV1B-specific IgA 4–8 days after the second RV1B infection whereas a single RV1B infection did not generate significant IgA response to RV1B (Fig. 3A upper panel). RV1B serotype-specific binding was detected in BAL by day 4 post second infection whereas cross reactive BAL IgA responses to RV29 were lower with significant binding not observed until 8 days after two RV1B infections (Fig. 3A lower panel). For IgG isotype antibody responses no RV-specific IgG was found in BAL after a single RV infection but significant binding of IgG2a and IgG1 to RV1B was detected 8–16 days after two infections (Fig. 3B). These data imply that mice infected with RV generate weak mucosal antibody responses or simply they may reflect the fact that lower concentrations of IgA than IgG are found in these samples. To determine if the difficulty in detecting IgA was due to larger amounts of RV-specific IgG blocking access of IgA to antigenic sites we treated serum from RV1B-infected mice with protein G to remove IgG. This reduced the levels of IgG2a that bound RV and permitted the easier detection of RV-specific IgA in serum samples from mice infected multiple times with RV1B (Fig. 3C). These data are consistent with the notion that mice infected with RV generate both systemic and mucosal RV-specific antibody responses and that the higher IgG levels can mask detection of IgA levels.

3.4. Lung and mediastinal lymph node B lymphocytes produce RV-specific IgG

To investigate the source of RV-specific antibodies we analysed B lymphocytes and their production of RV-specific antibodies following a single RV infection. We first determined by flow cytometric analyses that CD3⁺CD19⁺ B lymphocytes were found normally in the lungs and that RV infection induced their appearance in BAL after 4–7 days (SFig. 1A and B). Approximately 8.8% of BAL lymphocytes were B cells and their numbers were 100-fold lower than found in lungs (SFig. 1A and 1B). By ELISpot, we were unable to detect RV-specific IgG or IgA production by BAL cells, reflecting the low frequency of B cells found in this anatomical location. However, similar analyses of unfractionated lung (Fig. 4A) and mediastinal lymph node (Fig. 4B) leukocytes showed that RV-specific IgG was produced in a time-dependent manner. Thus, RV-specific IgG production by B cells was maximal 10–18 days after a single

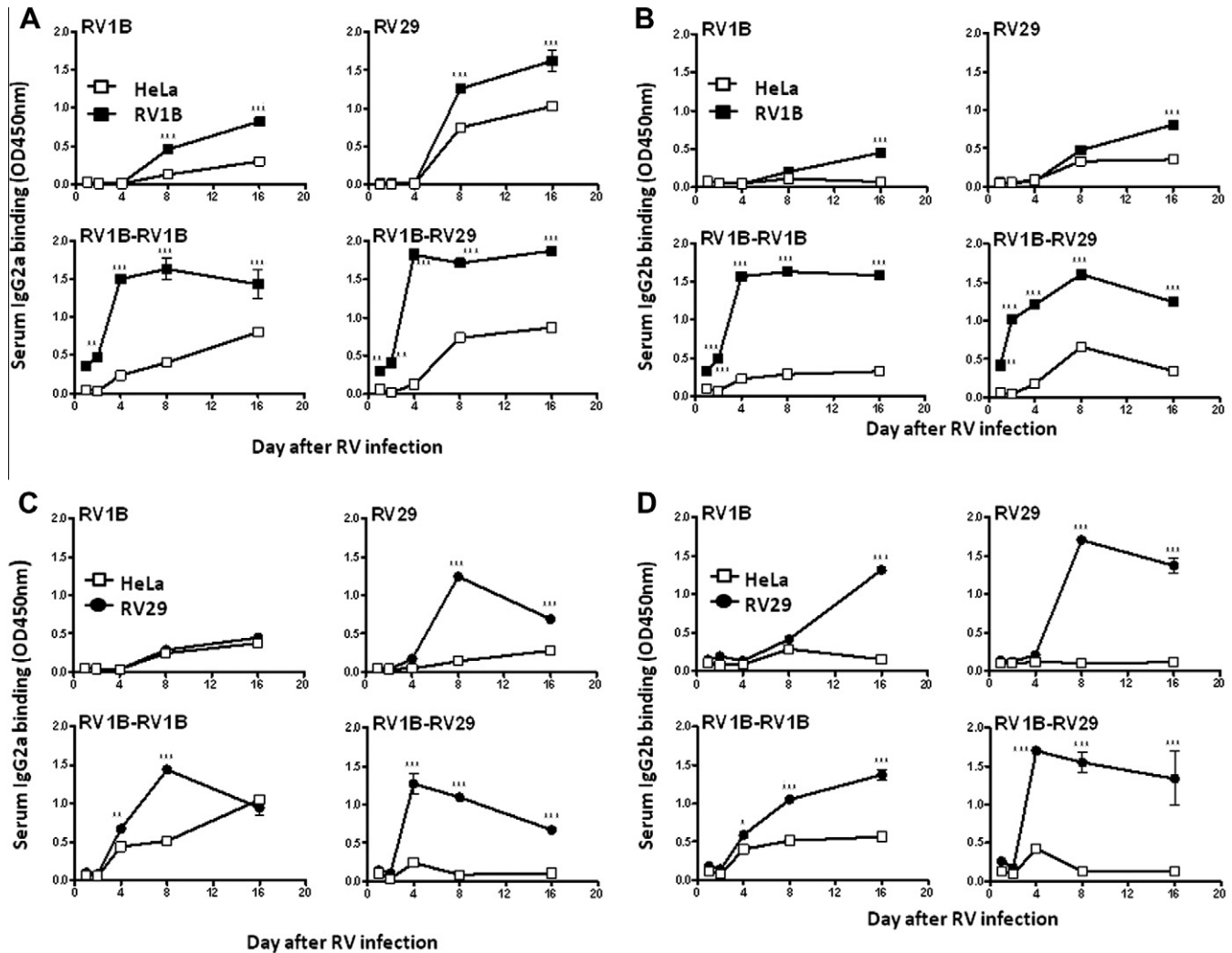


Fig. 1. Primary RV infections and homologous or heterologous secondary RV infections induce RV-specific serum IgG2a and IgG2b. BALB/c mice were infected i.n. with RV1B or mock infected i.n. with PBS, left for 30 days then infected i.n. with RV1B or RV29, sacrificed at the indicated time points and serum obtained. Diluted mouse antiserum (1:50) was added to ELISA plates coated with HeLa cell lysate (open symbols), purified RV1B (closed squares; A and B), or purified RV29 (closed circles; C and D) and binding of IgG2a (A and C) and IgG2b (B and D) was assessed. Data are presented as the mean \pm SEM ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for HeLa vs. RV).

RV1B infection in both lung and mediastinal lymph node, with a greater frequency of production by cells from the lymph nodes. RV-specific IgA was similarly produced albeit at much lower levels than IgG (Fig. 4A and B), reflecting the masking effect of higher IgG levels or the modest IgA responses observed in serum and BAL.

3.5. Mucosal CpG administration enhances IgG2a and RV-neutralising antibody responses in response to RV infection

To promote neutralising antibody generation, we also treated the mice infected with RV with Th1-promoting CpG oligonucleotides (Fig. 5A). We determined the level of RV1B-specific serum IgG by ELISA following two RV1B i.n. infections 30 days apart, the first infection directly following CpG or mock i.n. treatment. Antisera from mice that were treated with CpG and infected with RV1B displayed significantly increased IgG2a responses but unaltered IgG1 responses to RV1B (Fig. 5B). These data are consistent with the known ability of CpG to promote Th1 immunity (Krieg, 2002) and suggest that memory Th1 immunity to RV1B was encouraged by mucosal CpG administration at the time of first encounter with RV1B. To determine if the increased Th1-associated IgG2a antibody responses promoted by CpG resulted in production of neutralising

antibodies, we performed *in vitro* RV neutralisation tests. Antisera from RV-infected and CpG-treated animals contained antibodies that neutralised RV1B infectivity whereas antisera from mice infected twice with RV1B in the absence of CpG failed to neutralise (Fig. 5C). The neutralising property of the CpG-adjuvanted response was serotype specific as neutralisation of RV16 was not observed (Fig. 5C).

3.6. Immunisation with RV in Freund's adjuvant generates cross-serotype neutralising IgG

Having determined that mucosal CpG administration with live RV1B infection promoted serotype-specific neutralising antibody responses, we investigated whether systemic immunisations might induce cross-serotype neutralising responses (Fig. 6A). Following subcutaneous immunisation with inactivated RV1B in Freund's adjuvant followed by a single intranasal infection with live RV1B we observed high-titre RV-specific serum IgG2a and IgG1 (Fig. 6B). RV-specific serum IgA was not detected following this immunisation protocol. Similarly to i.n. RV1B infections, immunisation with RV1B in adjuvant generated strong serum IgG2a responses to recombinant RV89 VP1 but not to RV14 VP1 (Fig. 6C).

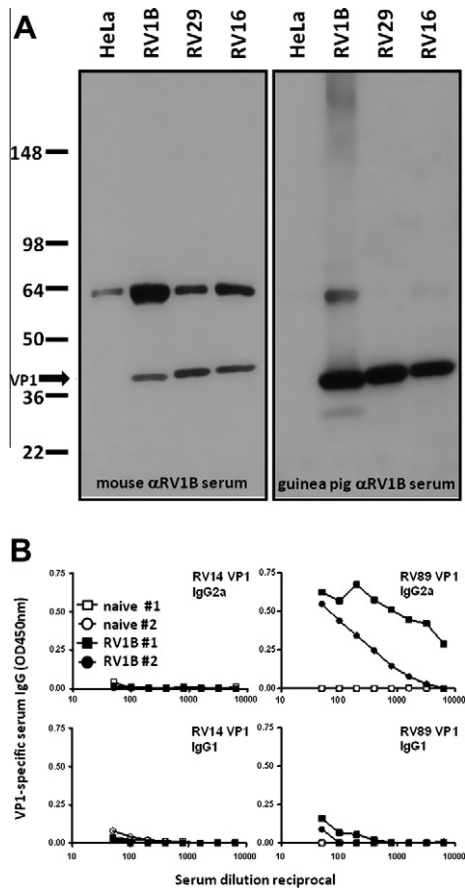


Fig. 2. Antisera from RV infected mice binds to the RV capsid protein VP1. (A) Western blotting was performed to analyse IgG binding to HeLa lysate, RV1B, RV16, and RV29 preparations using diluted antisera (1:500) from mice infected twice with RV1B or diluted guinea pig antisera (1:1000) against RV1B. RV-specific band suspected to be VP1 due to predicted molecular weight of 39 kDa is denoted by arrow. (B) Antisera from naive mice (open symbols) or mice infected with RV1B (closed symbols) were analysed by ELISA for IgG2a and IgG1 binding to purified recombinant RV14 or RV89 VP1. Data are presented as serial dilutions of individual antisera ($n = 2$, naive and RV1B infected).

Interestingly, this immunisation strategy differed from RV infections in that the production of IgG1 that bound strongly to RV89 VP1 was induced (Fig. 6C). Antisera from immunised mice was also analysed for binding to RV preparations by Western blot and was shown to react similarly to sera from RV infected mice in that mouse IgG bound to a RV-specific 39 kDa protein common to RV1B, RV29 and RV16 preparations (arrow) consistent with VP1 (Fig. 6D). UV treatment of the RV preparations promoted loss of antigenic epitopes by protein cross-linking/aggregation and did not allow recognition by the antisera. We also determined the ability of these sera to neutralise RV *in vitro*. The antisera neutralised RV1B at an endpoint dilution of 1:640 and also neutralised RV16 at an endpoint dilution of 1:40 whereas sera from mice administered Freund's adjuvant alone contained no neutralising activity (Fig. 6E). RV1B challenge of immunised mice increased the numbers of CD3⁺CD19⁺ B lymphocytes found in BAL, when compared to challenge of mice receiving adjuvant alone, without altering the numbers of these cells found in lungs (SFig. 1C).

4. Discussion

Antibody responses to RV infection are not completely understood and are difficult to study in adults and children due to the fact that humans are infected within months after birth and then

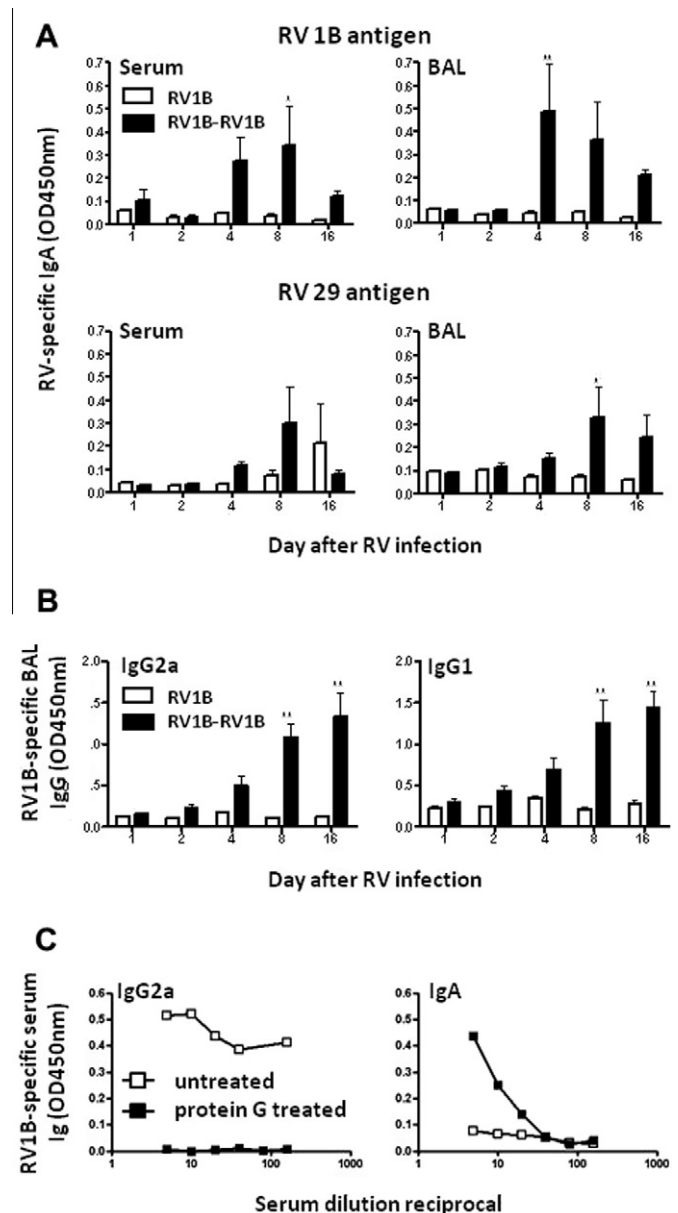


Fig. 3. Induction of RV-specific IgA and mucosal antibody responses requires two RV infections. BALB/c mice were infected with RV1B or mock infected with PBS, left for 30 days then re-infected with RV1B, sacrificed at the indicated time points and serum and BAL obtained. (A) Diluted serum (1:50) or BAL (1:4) from single (white bars) or double (black bars) RV1B infected mice was added to ELISA plates coated with purified RV1B (A, upper panel) or purified RV29 (A, lower panel) and assessed for IgA binding. (B) Diluted BAL (1:4) from single infected (white bars) or double RV1B infected (black bars) mice was also assessed for IgG2a and IgG1 binding to RV1B. Data are presented as the mean \pm SEM ($^*p < 0.05$, $^{**}p < 0.01$, for single RV1B infection vs. double RV1B infections). (C) Serum from BALB/c mice infected five times with RV1B was pooled and allowed to bind with protein G Sepharose to deplete IgG. The unbound fraction (closed squares) was compared with untreated serum (open squares) for levels of IgG2a and IgA binding to RV1B.

continuously throughout life (Turner, 1997). As a result all humans possess high levels of RV-specific antibodies that complicate studies into RV antibody-mediated immunity and vaccine development. To avoid these problems, in this study we have characterised the generation of antibody responses to RV following infection and immunisation of mice. To our knowledge this is the first study to examine antibody responses to RV *in vivo* in an immunologically naive host. Our results showed that significant cross serotype IgG responses in serum and BAL are generated in

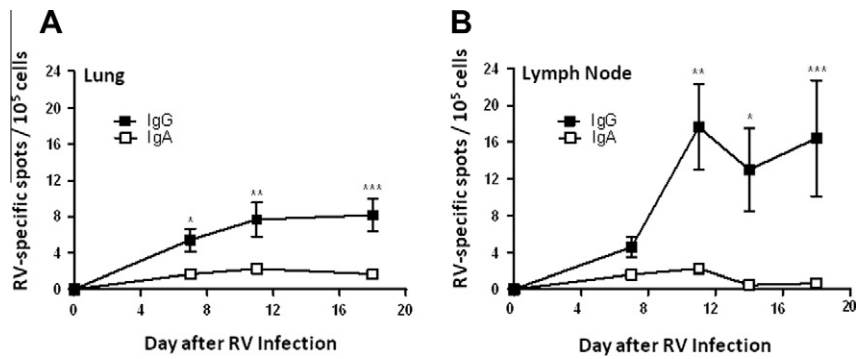


Fig. 4. The frequency of RV-specific IgG producing B cells in lungs and lymph nodes are increased after RV infection. The frequency of RV1B-specific IgG and IgA producing B lymphocytes in preparations of lung cells (A) and mediastinal lymph node cells (B) was determined by ELISpot assay at the indicated times following single i.n. RV1B infection and in naive mice (Day 0). Data are presented as the mean \pm SEM (* p < 0.05, ** p < 0.01, *** p < 0.001, for IgG or IgA vs. time 0).

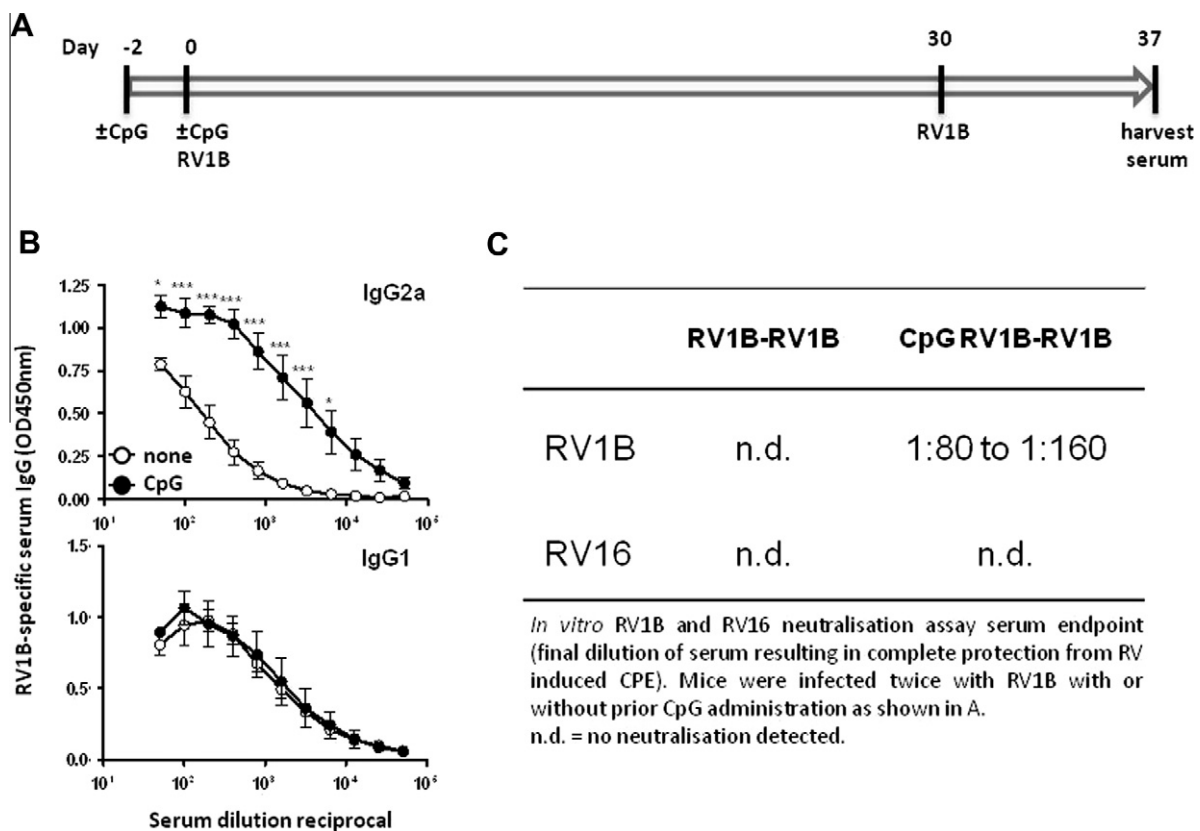


Fig. 5. Mucosal CpG administration augments RV-specific IgG2a responses. (A) BALB/c mice were administered i.n. with CpG 2 days prior to and on the day of RV1B i.n. infection, left for 30 days then re-infected with RV1B and sacrificed 7 days later to obtain serum. (B) RV1B-specific IgG2a and IgG1 were determined by ELISA using antisera from RV infected mice with (closed circles) and without (open circles) prior CpG treatment. Data are presented as the mean \pm SEM of pooled antiserum from four animals (* p < 0.05, *** p < 0.001, for RV1B infections vs. CpG/ RV1B infections). (C) Anti-RV sera generated in mice infected twice with RV1B with prior CpG treatment (CpG RV1B-RV1B) or not (RV1B-RV1B) were titrated for *in vitro* neutralisation of HeLa cell infection by RV1B or RV16. Cultures were inspected microscopically and scored for the endpoint dilution of sera that displayed complete protection from cytopathic effect (CPE).

mice following i.n. RV infection although the production of robust IgA responses and protective neutralising antibodies required repeat or multiple infections. The dominant antibody target for cross-serotype recognition appeared to be the RV capsid protein VP1. The mouse model also allows for investigations of specific antibody responses following adjuvant administration, immunisation and RV challenge demonstrating its utility for the analyses and development of RV vaccine approaches.

RV infections are common throughout life in humans making RV one of the most successful human viral pathogens. It has been estimated that children can get up to 12 RV infections per year and

adults on average 2–5 infections (Turner, 1997). RVs exist as more than 100 serotypes and serological findings demonstrate that protective antibodies generated following infection with one serotype neutralise that serotype but provide little to no cross-protection to other serotypes (Gwaltney, 1985). Because many serotypes of RV are capable of circulating in the population simultaneously and protective neutralising antibody is highly serotype specific, humans are frequently infected by RV serotypes even though they may possess antibodies that are cross-serotype binding. Similarly, as we have shown here with immunologically naive mice, single RV infections generated cross-serotype binding but non-neutralis-

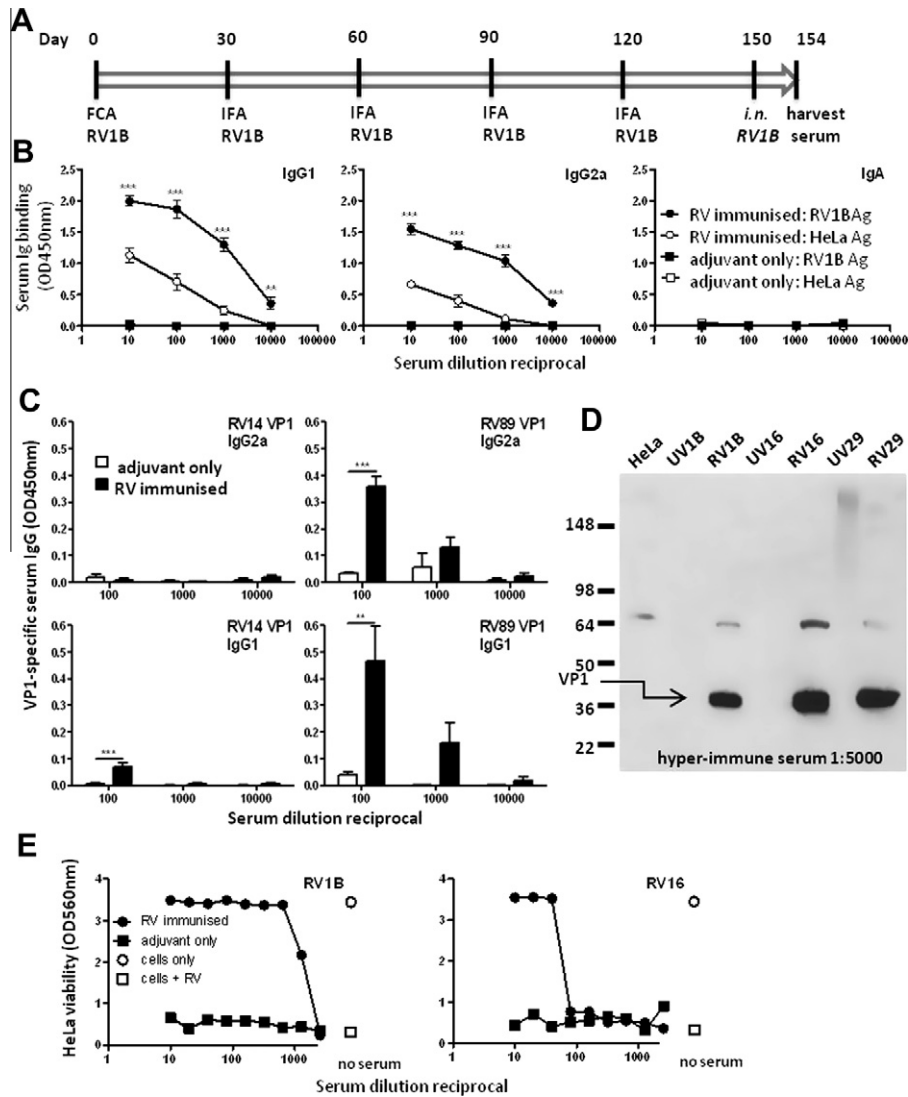


Fig. 6. Systemic immunisation of mice with RV generates cross-serotype neutralising serum IgG. (A) Antisera were generated by hyper-immunisation of mice with adjuvant-immersed RV1B preparations or adjuvant alone before i.n. infection with RV1B. (B) Sera from adjuvant-treated (square symbols) and RV1B hyper-immunised BALB/c mice (circular symbols) were analysed for IgG2a, IgG1 and IgA binding to HeLa lysate (open symbols) and purified RV1B (closed symbols) as antigens (Ag) by ELISA. Data are presented as the mean \pm SEM (** $p < 0.01$, *** $p < 0.001$, for hyper-immune sera binding RV1B vs. HeLa lysate). (C) IgG binding to recombinant VP1 was performed by titration of serum and analysis by ELISA. Serum IgG2a (upper graphs) and IgG1 (lower graphs) from adjuvant-treated (open bars) and hyper-immunised mice (closed bars) were assessed for binding to recombinant VP1 of RV14 (left) and RV89 (right). (D) Western blotting was performed to analyse IgG binding to HeLa lysate, RV1B, RV16, RV29, and UV-inactivated RV preparations (UV1B, UV16, UV29) using diluted hyper-immune antisera (1:5000). (E) *In vitro* neutralisation of RV1B and RV16 was performed by serial titration of pooled adjuvant-treated (filled squares) and hyper-immune (filled circles) BALB/c mouse antisera. Culture wells were inspected microscopically for cytopathic effect which was quantitated by crystal violet staining and comparisons were made to wells containing HeLa cells alone (open circles) and HeLa cells infected with RV (open squares). Data are presented as the mean \pm SEM (** $p < 0.01$, *** $p < 0.001$, for hyper-immune serum vs. adjuvant only).

ing antibodies. To generate higher avidity IgG binding, IgA and neutralising antibody responses, two RV infections, Th1-promoting adjuvants or immunisation with RV preparations in adjuvant were required. This is in contrast to humans where a single infection is often sufficient to generate systemic and mucosal antibody responses capable of serotype-specific neutralisation (Douglas et al., 1967), however since humans are frequently infected with RV, it is likely that pre-existing pools of cross-serotype CD4 helper T cells generated to these prior RV infections may help prime the response to infection with a new RV serotype. This priming effect is presumably also influenced by age, with young children and infants having been exposed to significantly fewer RV infections and also being susceptible to more frequent RV infections (Taylor-Robinson et al., 1963).

In humans, nasal antibodies of the IgA class have been shown to be critical for protection against RV infection (Barclay and Al-Nakib, 1987; Barclay et al., 1988) and patients with hypogamma-

globulinemia develop persistent RV infections despite adequate intravenous immunoglobulin (IVIg) replacement therapy further highlighting the importance of secretory IgA (Kainulainen et al., 2010). RV-specific secretory IgA is rarely present in the absence of serum IgA and rarely absent when serum titres are high, therefore serum IgA should be a useful marker for the development of mucosal humoral immunity to RV. We identified both mucosal and systemic IgA responses to RV in mice but these required more than one RV infection. These IgA responses were lower than the IgG responses found in both serum and lung washes and are consistent with our detection of a reduced frequency of B cells producing RV-specific IgA compared to IgG in mediastinal lymph nodes and lungs. Systemic immunisation of mice with RV in Freund's adjuvant did not promote IgA responses despite generating high-titre neutralising IgG responses, suggesting that the route of RV administration and virus replication might be critical for optimal IgA responses. However, Barnett et al. have previously demonstrated

that IgA-producing hybridomas specific to RV are generated after immunisation of mice with adjuvanted RV peptides (Barnett et al., 1995, 1993). Nevertheless, a stronger IgG response in mice may still be biologically significant for RV infection since it is known that secretory IgA protects the upper respiratory tract from influenza but IgG is more important for protection of the lungs (Renegar et al., 2004).

Our results indicate that the RV capsid protein VP1 is a major target of the antibody response in infected mice. We did not detect IgG to VP2 or VP3 despite the fact that we used infectious RV preparations made from HeLa cell cultures in our assays and preparations made in this way have been shown to contain VP1, VP2 and VP3 (Johnston et al., 1998). For our studies, recombinant VP2 and VP3 were not available and the western blot analyses relied on denatured and reduced viral antigen which may have lost potential conformational epitopes within VP2 and VP3. Most antibodies generated to RV infection in humans are directed against epitopes found on surface-exposed structures of VP1, VP2 and VP3 (Carey et al., 1992). Although there is evidence that the N-terminus of VP4 may be transiently exposed at the capsid surface in a process known as capsid breathing (Lewis et al., 1998) and antibodies to this region of VP4 are cross-serotype protective (Katpally et al., 2009), naturally occurring human antibodies to VP4 have not been described. A recent study by Niespodziana et al. revealed that children with RV-induced respiratory symptoms primarily generate IgG1 and IgA to N-terminal regions of VP1 and that responses to VP2, VP3 and VP4 were significantly lower (Niespodziana et al., 2011). Interestingly the major epitope of these VP1-specific antibodies is located inside the viral capsid and as such is a non-neutralising epitope, suggesting that misdirection of antibody responses in addition to strain variability can explain the escape of RV to protective immunity (Niespodziana et al., 2011). In the current study we have shown by western blotting of denatured RV preparations and by immunoassay of recombinant purified VP1 that mice infected with RV generate IgG that binds to VP1 of multiple RV serotypes. However this binding was sufficient for neutralisation of just the homologous RV serotype suggesting the importance of fine specificity for VP1. Furthermore, the IgG subclass IgG2a was found to be the dominant antibody class interacting with recombinant VP1 even though we demonstrated both IgG2a and IgG1 responses to intact viral preparations. IgG2a has been reported to be commonly produced by mice in response to viral infections (Coutelier et al., 1987) and may be a more effective neutralising antibody due to its ability to fix complement (Neuberger and Rajewsky, 1981). Our data is highly suggestive of IgG2a targeting a linear cross reactive epitope of VP1 following RV infection of mice. Since VP1 is the dominant and most external of the RV capsid proteins and contains receptor binding sites for both the major and minor group RV (Vlasak et al., 2003), it is therefore not surprising that mice infected with RV predominantly generate antibodies to this region of the capsid. VP1 is a useful antibody target as a recent study by Edlmayr et al., (Edlmayr et al., 2011) has revealed that immunisation of rabbits with recombinant VP1 generates antisera with broad cross-serotype neutralising properties. Significantly, there is a high degree of amino acid identity between VP1 within both type A and B RV serotypes and VP1 also contains several conserved domains, the drug binding pocket for pleconaril and two of the four neutralising immunogenic sites targeted by antibodies (Laine et al., 2006; Ledford et al., 2004; Rossmann et al., 1985; Sherry et al., 1986).

It is important to understand the development of antibody responses to RV as young children are obvious vaccine candidates for prevention of early recurrent RV infections which have been reported to be associated with increased risk of asthma development (Jackson et al., 2008) and RV-induced exacerbations of asthma (Murray et al., 2006). In this regard, the study of antibody

responses in mice infected or immunised with RV add to the current limited knowledge regarding binding and neutralising antibody responses to RV in humans. We have established the utility of the mouse model by demonstrating the induction of cross-serotype RV-specific antibodies following infection and the use of two different adjuvant approaches to promote neutralising responses. This is the first *in vivo* study of the priming of antibody responses to RV infection and taken together, these results and our model system have important implications for RV vaccine design. Our studies suggest that RV vaccine approaches need to target the lung using defined highly conserved RV epitopes to generate cross-serotype local mucosal IgA responses which may be more suitable for protection than the systemic IgG responses often favoured. Further studies, such as different strains of mice (e.g. C57Bl/6), the use of mice transgenic for specific antibody genes (e.g. human Ig V regions) or with disrupted IgG genes, other adjuvants, and the transfer of immune serum to assess protection and subsequent analyses in our model system will increase the understanding of cross-protective antibody responses to RV and lead to the generation of a vaccine for this important human respiratory viral pathogen.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.antiviral.2012.06.006>.

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