



Epitope mapping of antibodies induced with a conserved rhinovirus protein generating protective anti-rhinovirus immunity



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ABSTRACT

Human rhinovirus (RV) infections are the principle cause of common colds and precipitate asthma and chronic obstructive pulmonary disease (COPD) exacerbations. Currently there is no vaccine for RV which is largely due to the existence of ~160 serotypes/strains. We demonstrated previously that immunising mice with highly conserved VP4 and VP2 regions of the RV polyprotein (RV-A16 VP0) generated cross-reactive immunity to RV *in vivo*. The current study investigated and mapped the epitopes of RV-A16 VP0 that are targets for antibodies in serum samples from VP0 immunisation and RV challenge studies in mice. Recombinant capsid proteins, peptide pools and individual peptides spanning the immunogen sequence (RV-A16 VP0) were assessed for IgG binding sites to identify epitopes. We found that peptide pools covering the C-terminus of VP4, the N-terminus of VP2 and the neutralising NIm-II site within VP2 were bound by serum IgG from immunised mice. The NIm-II site peptide pool blocked IgG binding to the immunogen RV-A16 VP0 and individual peptides within the pool binding IgG were further mapped. Thus, we have identified immunodominant epitopes of RV vaccine candidate RV-A16 VP0, noting that strong IgG binding antibodies were observed that target a key neutralising epitope that is highly variable amongst RV serotypes.

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1. Introduction

Human rhinovirus (RV) infections cause a self-limiting illness of the upper respiratory tract called the common cold, however in people with chronic airway disease (asthma and COPD), these viral infections involve the lower respiratory tract and cause serious exacerbations that can be life threatening [1]. There are very limited options for treatment of RV infections and fifty years of research has failed to produce a reliable and specific antiviral [2]. Likewise, only slight progress has been made in harnessing the adaptive immune system to produce an effective vaccine. The difficulty has been attributed to the large number of serologically

distinct virus types which necessitate finding new broadly cross-reactive immunogens [3].

Improving understanding of the correlates of protection from infection and resultant disease is key to developing a RV vaccine. Antibody (Ab, Abs) responses have been well studied, with neutralising epitopes having been defined and the ability of neutralising Ab to protect from disease having been established [4–6]. Similarly, Abs are well established as a key correlate of protection for influenza virus [7]. RV-specific Abs are directed against epitopes found on the surface exposed areas of capsid proteins VP1, VP2 and VP3 [8], 60 copies of each being arranged structurally into an icosahedron, with VP4 buried beneath the surface [9]. Abs are critical for protection against re-infection with the same serotype since defence is associated with high levels of neutralising antibody both in serum and respiratory secretions [4] but in general, neutralising Abs to RV are serotype-specific [10,11]. Interestingly, Abs persisting from prior encounters with other RV serotypes may cross-react with closely related RV serotypes and sometimes

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can cross-neutralise [12]. Structural studies of neutralising Abs bound to RV have found that effective neutralising activity depends on Ab binding that; (1) blocks virus interaction with the major group ICAM-1 entry receptor [13]; (2) stabilises the virus capsid preventing uncoating and release of virus nucleic acid [14,15]; (3) aggregates virions [16]; or (4) induces virus uncoating extracellularly [17]. Studies of major group RV-B14 and minor group RV-A02 identified two neutralising immunogenic sites (NIm-Ia and NIm-Ib) on VP1 and two further neutralising sites, NIm-II on VP2 and NIm-III on VP3 [6,8,18]. The majority of the NIm sites are discontinuous epitopes forming protrusions from the capsid surface and all are highly variable regions of the VP sequences amongst the RV serotypes. This variability explains the high level of serotype specificity and poor cross protective abilities of neutralising Abs. Furthermore, analogous NIm's have not been functionally described on other RV serotypes. Another feature reflecting their wide diversity is that distinct RVs use different entry receptors during infection of cells. Thus, intercellular adhesion molecule 1 (ICAM-1) is used by all RV type B and most type A, low density lipoprotein receptor (LDLR) by 12 type A, and cadherin-related family member 3 (CDHR3) is used by RV type C [19]. The understanding of RV-specific T cell immunity is much more limited than what is known for Abs. Studies in humans have identified predominantly CD4+ Th1-cytokine-secreting T cells that are capable of cross-reactivity to diverse RV serotypes [20,21] and more recent studies have defined potential cross-reactive RV-induced memory T cell epitopes within RV structural proteins [22,23]. Supporting these observations are our prior studies in mice immunised with highly conserved VP4 and VP2 regions of the RV polyprotein (RV-A16 VPO) that also showed the generation of cross-reactive T cell immunity [24]. Nevertheless the role of T cells in protective immunity to RV remains poorly understood.

A successful RV vaccine must induce immunological memory from cross-serotype reactive B cells secreting Abs and/or T cells. Human clinical trials in the 1960s and 1970s investigated the use of a single inactivated serotype [25–29], expanded to 10 serotypes [30] but neither approach generated significant cross-protective responses. With the advent of small animal immunisation and challenge models, suitable vaccine candidates can now be evaluated thoroughly before translation to humans. Our studies in mice [24,31] and those of others in cotton rats [32,33] have demonstrated that different vaccination strategies can induce some cross-protective immunity *in vivo*. The multivalent inactivated approach has also been re-evaluated in mice and rhesus macaques, where it was shown that neutralising Ab responses could be generated to 25 and 50 serotypes respectively [34]. However, these neutralising Ab responses were restricted to only the serotypes included in the vaccine formulation and no challenge studies were performed to define the protective capabilities *in vivo*.

The current study was undertaken to address our understanding of RV-specific neutralising Ab during immunisation and RV challenge and the role played by capsid binding sites. Therefore, we measured Ab responses in antisera to overlapping peptides representing the structural proteins within the RV-A16 polyprotein to define immunodominant epitopes. We characterised *in vitro* the antisera obtained from a previous study that had demonstrated cross-serotype binding properties and serotype-specific neutralisation [24]. In this prior study we showed that RV-specific IgG and IgA were found in serum and bronchoalveolar lavage fluid (BALF) respectively obtained from mice immunised with RV-A16 VPO and that the strongest binding was observed with immunised and RV challenged animals. The serum IgG was also shown to bind to VPO of distinct serotypes RV-A29, RV-A16 and RV-A01 by western blot and *in vitro* was capable of neutralising the serotype of RV used for challenge *in vivo* [24]. Now we have gone further, mapping and comparing the binding patterns of Abs in antisera induced by

VPO-immunisation and RV challenge. IgG was found to target minimal regions of the immunogen sequence with the immunodominant region structurally mapped to an area coinciding with the NIm-II analogous neutralising site of VP2.

2. Methods

2.1. Mouse immunisation and challenge studies

Four *in vivo* immunisation and challenge experiments were performed using C57BL/6 6–8 week old mice as described previously [24]. Mice were divided into 3 treatment groups with n = 4 per group: (**A-RV**) immunised with adjuvant alone (incomplete Freund's adjuvant and CpG (IFA/CpG; obtained from Sigma-Aldrich and Invivogen respectively)) and challenged with RV-A01 or RV-A29; (**I-P**) immunised with RV-A16 VPO in IFA/CpG and challenged with phosphate buffered saline (PBS); (**I-RV**) immunised with RV-A16 VPO in IFA/CpG and challenged with RV-A01 or RV-A29 [24]. Two studies each involved RV-A01 challenge (**RV-A01 #1 & RV-A01 #2**) or RV-A29 challenge (**RV-A29 #1 & RV-A29 #2**). All animal studies were conducted according to UK home office legislation (Animals (Scientific Procedures) Act 1986), project licence number PPL 70/7234. Serum was obtained as described previously [24]. Mice were immunised twice over three weeks subcutaneously (SC) and challenged IN four weeks after the second SC immunisation with RV-A01 or RV-A29. Fourteen days after challenge, serum was obtained to determine Ab levels, specificity and neutralising capabilities.

2.2. RV-A16 structural protein peptide pools

Overlapping 15mer peptides (overlapping by 11 amino acids), covering the full RV-A16 capsid sequences were generated based on published sequences (NCBI accession Q82122) and obtained from Mimotopes Pty Ltd. Peptides were suspended in DMSO and combined into pools of 10 peptides for initial Ab mapping experiments. Eight peptide pools span the VPO region of RV-A16, 6 peptide pools span the VP3 region and 7 peptide pools cover VP1. Thus, peptide pool 1 covers the VP4 sequence, peptide pool 2 overlaps the C-terminus of VP4 and the N-terminus of VP2 and peptide pools 3 through 8 cover the remainder of VP2 with peptide pool 9 also covering part of the VP2 C-terminus. Peptide pools 9 to 15 cover the VP3 region. Part of peptide pool 15 and peptide pools 16 through 21 cover VP1. Peptide pools sequences and a representation of the regions covered by each peptide pool in the RV-A16 capsid sequences are shown in [Supplementary Figs. 1 and 2](#).

2.3. ELISA

ELISA was performed as described previously [24,31] with modifications. For indirect ELISA, plates were coated with purified RV capsid proteins (RV-A89 VP1, VP2, VP3, VP4 [35]; RV-A16 VPO) at 1 µg/ml, peptide pools at 4 µg/ml, or individual peptides (pool 6) at 1 µg/ml diluted in PBS. Mouse sera were diluted 1:100 in PBS containing 0.05% tween 20 (Sigma-Aldrich) and 5% Marvel skimmed milk powder (Tesco) (PBST-milk). For competition ELISA, plates were coated with recombinant VPO at 0.2 µg/ml in PBS (equivalent to 10 ng per well). Mouse sera were diluted 1:25000 in PBST-milk and mixed with peptide pools or VPO diluted to 0.1 mg/ml, incubated for 2 h at 37 °C before addition to blocked and coated plates (equivalent to 5 µg per well). This corresponds in each well of the competition assays to a 500-fold mass excess of soluble peptide pools or VPO to the available plate-bound VPO. All assays were developed with peroxidase-labelled goat anti-mouse IgG or IgA (Southern Biotechnology Associates) followed

by 3,3',5,5'-Tetramethylbenzidine (Invitrogen) substrate before optical density was read at 450 nm.

2.4. RV capsid proteins structure images

Protein data bank submission 1AYM was used as the source of coordinates for the RV-A16 capsid proteins. Images were constructed using UCSF Chimera molecular graphics program [36] and Pymol molecular graphics program (PyMOL: The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

3. Results

3.1. VP0-binding IgG also binds VP4 and VP2 from a distinct RV serotype

To determine the specificity and cross-serotype nature of the antibodies induced by immunisation and challenge with greater detail, we performed ELISA experiments using the recombinant capsid proteins VP1, VP2, VP3 and VP4 of RV-A89 [35] and the immunising recombinant protein VP0 of RV-A16 as antigens. Fig. 1 shows that sera from four independent *in vivo* experiments of immunised alone (I-P) and immunised and challenged mice (I-RV) contained IgG that bound strongly and consistently to RV-A16 VP0 (inset) and to both VP2 and VP4 of RV-A89, whereas mock-immunised but challenged mice (A-RV) had no significant IgG that bound to these proteins. Binding to VP1 and VP3 of RV-A89 was not significant and very weak across all groups except that *in vivo* experiment RV-A01 #1 showed detectable binding to VP1 that was not statistically significant. These results, as expected, confirm that the sera from VP0 immunised mice contains

IgG that binds to the components of the immunogen, VP4 and VP2, in a cross-serotype manner. No binding of serum IgA to these proteins by ELISA was detected (data not shown) similarly to previous results [24].

3.2. IgG binds to peptides covering the VP4 C-terminus, VP2 N-terminus and the VP2 N1m-II site

We designed overlapping peptide pools that span the 4 capsid proteins of RV-A16. The sequences of peptides within each pool and their location within each capsid protein are shown in Supplementary Fig. 1. Twenty one peptide pools were generated that span VP4, VP2, VP3 and VP1 (Supplementary Fig. 2) with each pool containing 10 peptides of 15 amino acid length that have an 11 amino acid overlap with the next peptide. Thus, pools 1 and 2 covered VP4 and the beginning of VP2, pools 3–9 covered the remainder of VP2 and beginning of VP3, pools 10–15 covered VP3 and the beginning of VP1, and pools 16–21 covered the remainder of VP1 (Supplementary Figs. 1 and 2). To determine IgG binding to smaller regions of the RV capsid proteins, peptide pools 1–21 were used as antigens in ELISA experiments. We found that specific IgG binding was restricted to peptide pools that covered the VP0 region (pools 1–8, VP2 and VP4) and was only observed in VP0-immunised mice (I-P and I-RV) with no detectable binding found in sera from unimmunised but RV challenged mice (A-RV) (Fig. 2A). Consistent with prior results obtained with recombinant RV capsid proteins, serum IgG was unable to bind peptide pools 9–21 which span the VP3 and VP1 capsid proteins. Closer inspection of the peptide pools revealed that sera from immunised mice contained IgG that bound peptide pools 2, 3 and 6 consistently with peptide pool 8 showing detectable binding in one instance only (Fig. 2B). Thus, sera from

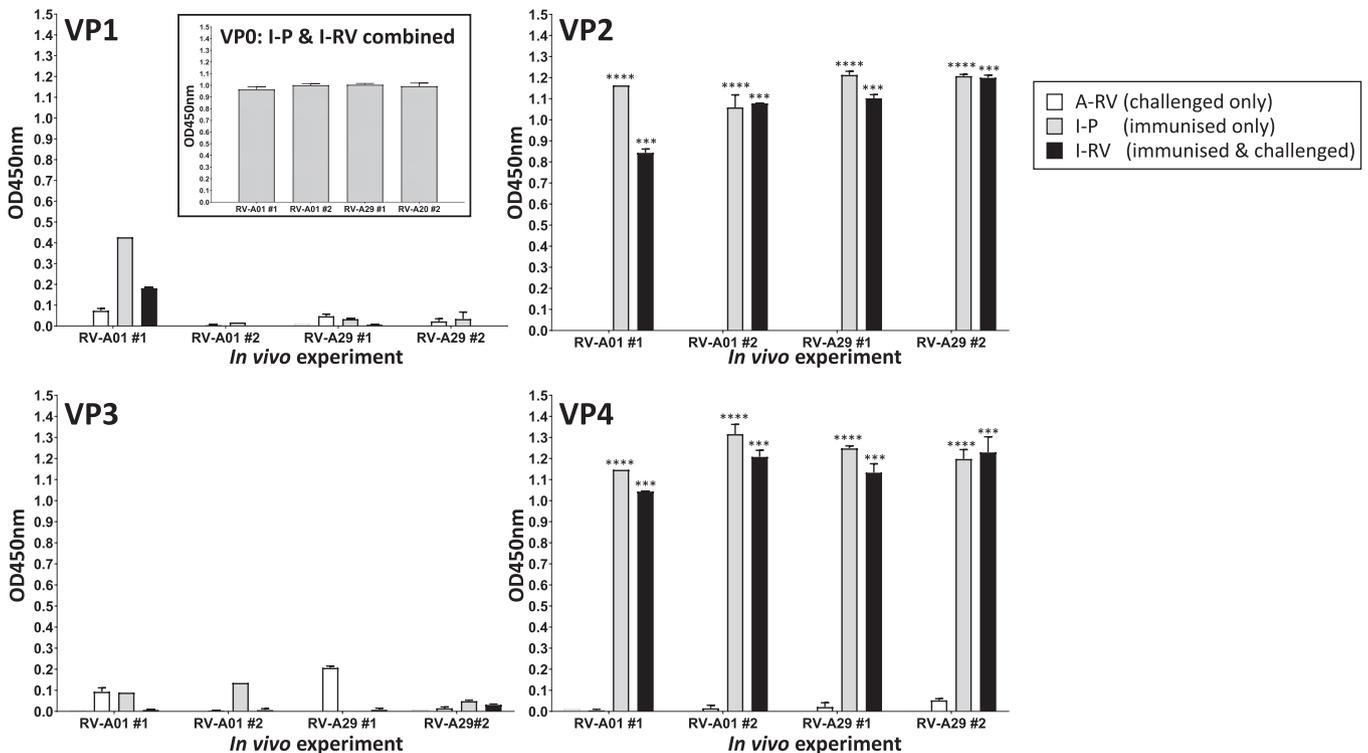


Fig. 1. Serum IgG binds to recombinant RV capsid proteins by ELISA. The RV recombinant proteins VP0 from RV-A16 and VP1, VP2, VP3 and VP4 from RV-A89 were coated onto ELISA plates and serum from 4 independent experiments allowed to bind. Detecting antibody was against mouse IgG. For VP1, VP2, VP3 and VP4 open bars represent A-RV, shaded bars represent I-P, and black bars represent I-RV. For VP0 (inset graph) shaded bars represent I-P and I-RV combined sera binding. Data is expressed as the mean \pm SEM of 4 individual sera within each group and were compared by one way ANOVA with each bar compared to the negative control. **** $p < 0.0001$, *** $p < 0.005$, no asterisk is not significantly different and $p > 0.05$.

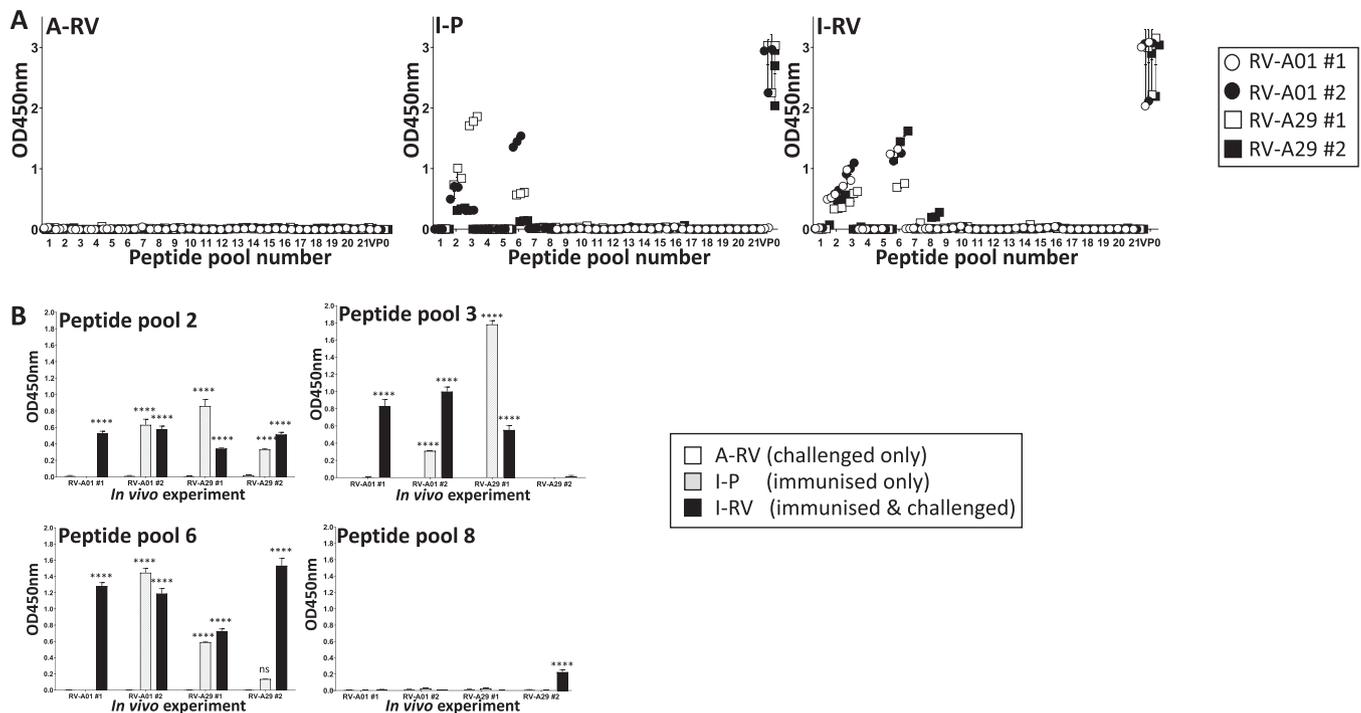


Fig. 2. Serum IgG binds to specific peptide pools that cover VP0. (A) Groups of overlapping peptides covering the RV16 capsid proteins and recombinant VP0 were coated onto ELISA plates and serum from 4 independent experiments (open circle, *in vivo* experiment RV-A01 #1; closed circle, *in vivo* experiment RV-A01 #2; open square, *in vivo* experiment RV-A29 #1; closed square, *in vivo* experiment RV-A29 #2) allowed to bind. Detecting antibody was against mouse IgG. Data are expressed as 4 individual serum samples within each group with the mean \pm SEM indicated. (B) Data from peptide pools 2, 3, 6 and 8 are shown as bar graphs for the 4 independent immunisation experiments with open bars representing A-RV, shaded bars representing I-P and black bars representing I-RV. Data is presented as the mean \pm SEM of 4 individual sera within each group and were compared to each other by two way ANOVA. **** $p < 0.0001$, ns = not significant $p > 0.05$.

each of the four independent immunisation experiments contained IgG that could bind to RV-A16 peptides within pools 2, 3 and 6 which cover the VP4 C-terminus, VP2 N-terminus and the VP2 N1m-II site respectively. Binding was variable, as expected for polyclonal responses to short linear peptide pools, but statistically significant in each case. Furthermore, there was no consistent differences when comparing immunised and mock challenged mice (I-P) with immunised and challenged mice (I-RV), suggesting that live virus challenge did not appreciably alter the spectrum of IgG that VP0 immunisation alone induced. As before with the recombinant capsid proteins, no detectable binding of serum IgG was found to these peptide pools (data not shown).

3.3. The N1m-II site and proximal regions of VP2 are immunodominant

To confirm the IgG binding to peptides of VP0 and to determine the immunodominant sites of VP0 targeted by IgG we performed inhibition experiments using the peptide pools to interfere with IgG binding to immobilised VP0. We used a 500-fold mass excess of the soluble peptide pool compared to plate-bound VP0. IgG binding to plate-bound VP0 was consistently inhibited by peptide pool 6 in each experiment and antisera tested (Fig. 3A). Thus, significant inhibition of IgG binding to VP0 was observed with excess peptide pool 6, by approximately 45% in each experiment using sera from both I-P and I-RV mice suggesting that a large proportion of IgG in these sera bound epitopes within this region of VP2. As expected, the peptide pools that did not display IgG binding in Fig. 2A (pools 1, 4, 5, 7 and 8) had no inhibitory effect on the binding of serum IgG to VP0. Interestingly, the peptide pools 2 and 3 that were shown in Fig. 2 to bind IgG within the sera, did not significantly interfere with IgG binding to VP0 in most experiments. However, weak but statistically significant inhibition was seen with peptide pool 2 in I-P mice from *in vivo* experiment RV-A01

#2 and with peptide pool 3 in I-RV mice from *in vivo* experiment RV-A29 #1. In all cases, as an internal positive control to determine specificity, excess soluble VP0 was able to inhibit IgG binding to plate-bound VP0 by greater than 95% (Fig. 3A). Doubling the peptide pool concentration to 1000-fold mass excess did not improve inhibition of IgG binding to VP0 above 45% for pool 6 and also did not generate further significant inhibition with pools 2 and 3 (data not shown).

We next determined whether combinations of the peptide pools allowed improved inhibition of serum IgG binding to VP0. The combination of peptide pools 2 & 6 was consistently able to inhibit up to 80% of IgG binding to VP0 whereas other combinations (pools 3 & 6; pools 8 & 6) were generally weaker inhibitors and in one case was unable to significantly block IgG binding (Fig. 3B). Conversely, the combination of peptide pools 2 & 3 did not significantly inhibit IgG binding, except in one experiment. Collectively, these data indicate that much of the serum IgG generated by VP0 immunisation targets the region of VP0 covered by peptide pool 6 (VP2 N1m-II site) with a lesser contribution of IgG that recognises the VP4 C-terminus and VP2 N-terminus covered by peptide pool 2.

The data obtained with sera binding to the peptide pools and their use to block IgG binding to VP0 are summarised in Table 1 and help establish that the region of VP0 covered by peptide pool 6 was immunodominant, inducing the strongest IgG binding. This peptide pool spanned regions of VP2 that have previously been shown to be a neutralising epitope of RV-B14 [6]. Thus, IgG binding to individual peptides from this pool was determined next to assist defining the fine specificity of binding. Fig. 4 displays the profile of IgG binding to the individual 10 peptides contained within pool 6. Binding to peptides (51, 59 and 60) at the extremities of the region was either very weak or undetectable in all cases whereas significant binding to the 7 core peptides (52, 53, 54, 55, 56, 57, and 58)

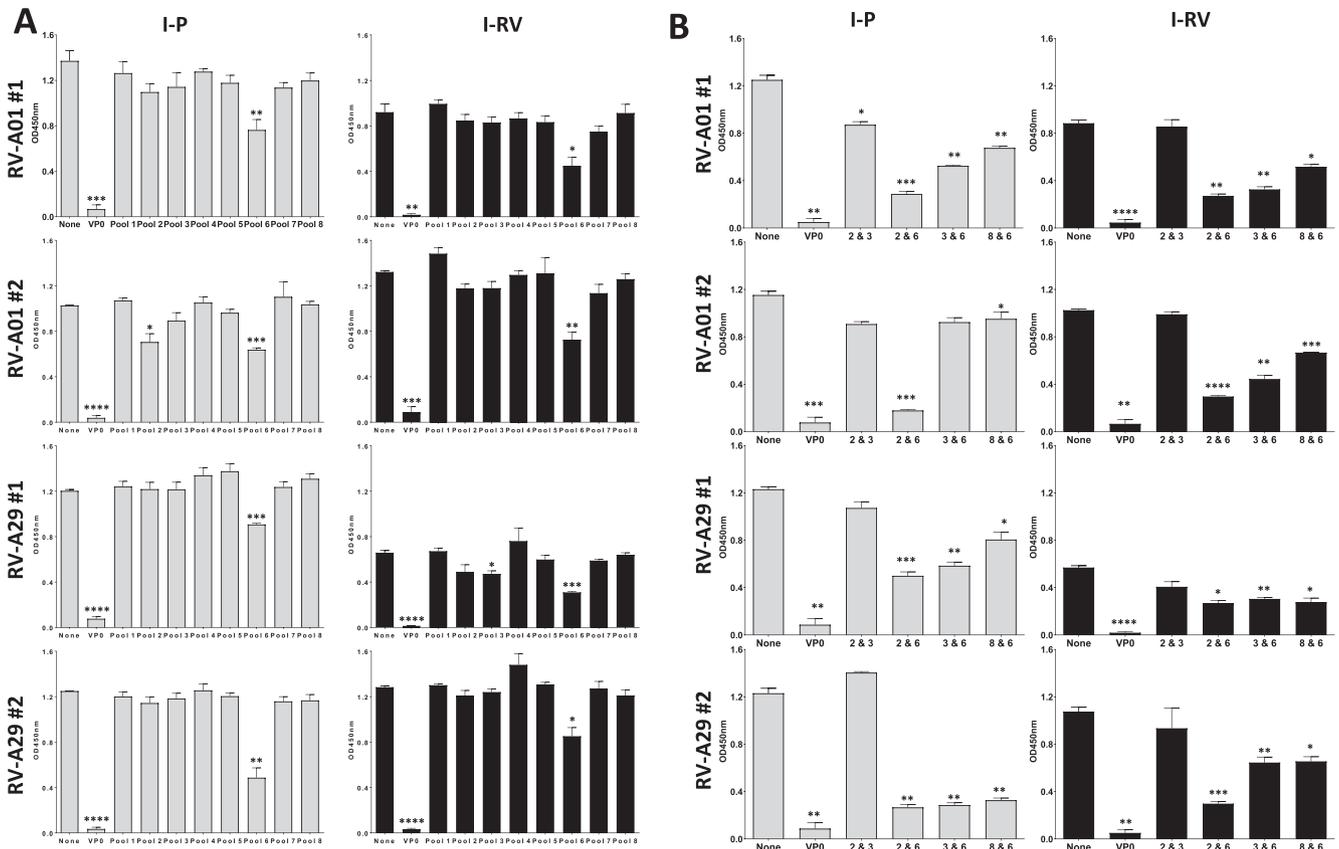


Fig. 3. Specific peptide pools of VP0 inhibit serum IgG binding to VP0 by ELISA. (A) ELISA plates were coated with recombinant VP0 and sera from experiments allowed to bind in the presence of 500-fold excess of peptide pools, recombinant VP0 or absence of peptides (None). Detecting antibody was against mouse IgG. Data are expressed as the mean \pm SEM of 4 individual sera that are grouped by immunisation experiment and whether I-P or I-RV. Each data bar were compared to None by two way ANOVA. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, no asterisk is not significant $p > 0.05$. (B) Similarly to (A) peptide pools in the indicated combinations, VP0 or no peptides (None) were added to sera samples and allowed to bind to coated VP0. Data are grouped by experiment and condition (I-P and I-RV) and are expressed as the mean \pm SEM of 4 individual sera. Each data bar were compared to None by two way ANOVA. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, no asterisk is not significant $p > 0.05$.

was observed with most of the sera obtained from the 4 independent *in vivo* experiments. Peptide 52 (KGNVNAGYKYT) was consistently bound the strongest by IgG in all cases and the remaining 6

peptides of this core recognition region were also bound well however there were notable exceptions with sera obtained from certain experiments. For example, sera I-RV from *in vivo* experiments RV-A29 #1 & RV-A29 #2 did not bind peptides 55 and 56 well, and antisera I-P from experiments 1 and 4 displayed weaker binding to numerous core peptides. No discernible differences in binding between sera I-P and I-RV that could be attributed to the RV challenge were noted. A summary of these data, indicating the regions of VP2 covered by the various individual peptides are shown in Table 2, where it is shown that IgG binding tends to favour peptides containing the conserved amino acid sequences that span the predicted NIm-II epitope regions. These regions are shown in Supplementary Fig. 3 where alignments of the RV type A homologous VP2 sequences surrounding the NIm-II site are highlighted.

Table 1
summary of peptide pool IgG mapping experiments.

Peptide pool	Location in VP0 sequence	Binds IgG ^a	Inhibits IgG binding to VP0 ^b
1	VP4 N-terminus 1–51 (1–51 of VP0)	No	No
2	VP4 C-terminus 52–68 VP2 N-terminus 1–23 (41–91 of VP0)	Yes	Minimal ^c
3	VP2 N-terminus 13–63 (81–131 of VP0)	Yes	Minimal ^c
4	VP2 53–103 (121–171 of VP0)	No	No
5	VP2 93–143 start of NIm-II (161–211 of VP0)	No	No
6	VP2 133–183 NIm-II site (201–251 of VP0)	Yes	Yes
7	VP2 173–223 (241–291 of VP0)	No	No
8	VP2 C-terminus 213–262 (281–331 of VP0)	No ^d	No

^a Binding to peptide pools by ELISA shown in Fig. 2.

^b Peptide pools inhibition of IgG binding to VP0 by ELISA shown in Fig. 3.

^c In one experiment shown in Fig. 3 the peptide pool weakly inhibited, however in combination with peptide pool 6 was more effective.

^d In one experiment significant binding was observed as shown in Fig. 2.

3.4. Imaging of the IgG-binding immunodominant region in the VP2 structure

We used molecular imaging of the solved structure of RV-A16 [37] to identify the precise location on the capsid and VP2 of the core peptides within pool 6 targeted by serum IgG. Protein data bank submission 1AYM was used as the source of coordinates for the RV-A16 capsid proteins for the generation of structural images. An image of the VP1-VP2-VP3 protomer and its location within the RV capsid structure is shown in Fig. 5A and 5B. To determine the location of the immunodominant region we highlighted the

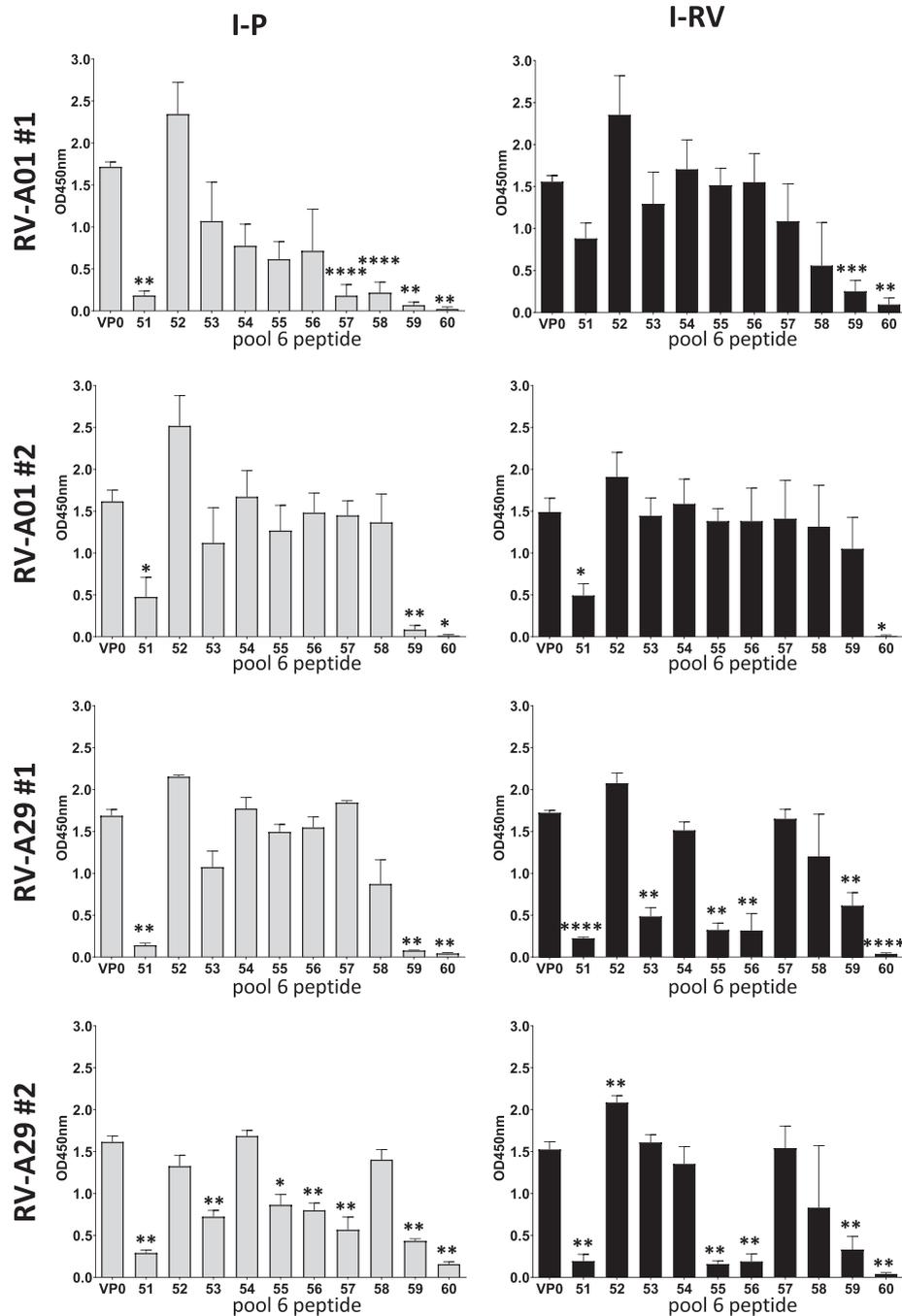


Fig. 4. Fine specificity of NIm-II peptides bound by serum IgG. Individual peptides from peptide pool 6 were coated onto ELISA plates and IgG from sera allowed to bind. Detecting antibody was against mouse IgG. Data are the average of two assays and are expressed as the mean \pm SEM of 4 individual sera grouped from 4 independent immunisation experiments and whether I-P or I-RV. Data with significantly different binding compared to VP0 were determined by two way ANOVA. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, no asterisk is not significant $p > 0.05$.

regions of VP2 covered by peptides within pool 6 (VP2 133–183). Fig. 5C shows that this region covers a relatively large proportion of the VP2 surface bordering VP3 and VP1, consisting of core regions with defined secondary structure and surface-exposed polypeptide loops with less secondary structural order. Not surprisingly the NIm-II site is also found within the pool 6 footprint and forms the surface-exposed protrusions (Fig. 5C and D). The NIm-II region is highly diverse amongst the type A RVs which can explain serotype specificity of neutralising Ab's binding to this region, however there are also highly conserved regions flanking the core NIm-II site (SFig 3).

4. Discussion

This study has investigated details of the Ab response generated to the RV vaccine candidate VP0 in mouse models. This experimental vaccine, previously shown to generate cross-serotype reactive cell-mediated immunity and protective humoral responses [24], is in pre-clinical development. This study set out to define better the cross-serotype and neutralising Ab responses by determining the immunodominant site(s) of the RV capsid targeted. We determined that serum IgG responses are specific for fragments of VP4 and VP2 with the dominant site surrounding the VP2 NIm-II neu-

Table 2
Summary of peptide pool 6 IgG mapping data.

Peptide #	Sequence	IgG binding ^a	Location in RV-A16 VP0 ^b
51	ATVNKGNVNAVGYKYT	Weak	VP2 133–147 Part 1 of the N1m-II epitope
52	KGNVNAVGYKYTHPGE	Strong	VP2 137–151 Conserved region between the two N1m-II sequences
53	NAGYKYTHPGEAGRE	Medium	VP2 141–155 Conserved region between the two N1m-II sequences
54	KYTHPGEAGREVTGQ	Medium	VP2 145–159 Conserved region between the two N1m-II sequences and part 2 of the N1m-II epitope
55	PGEAGREVTGQVENE	Medium ^c	VP2 149–163 Conserved region between the two N1m-II sequences and part 2 of the N1m-II epitope
56	GREVTGQVENEKQPS	Medium ^c	VP2 153–167 Part 2 of N1m-II epitope
57	GTQVENEKQPSDDNW	Medium	VP2 157–171 Part 2 of N1m-II epitope and conserved region after N1m-II epitope
58	ENEKQPSDDNWLNF	Medium	VP2 161–175 Conserved region after the N1m-II epitope
59	QPSDDNWLNFDTLL	Weak	VP2 165–179 Conserved region after the N1m-II epitope
60	DNWLNFDTLLGNLL	None	VP2 169–183 Conserved region after the N1m-II epitope

^a Binding to peptide pools by ELISA shown in Fig. 4.

^b Peptides spanning the VP2 amino acid sequence shown and the N1m-II site first identified in RV B14.

^c Not generalizable as some antisera did not bind.

tralisng site. Binding to the immunodominant site could not be linked to virus neutralisation, therefore monoclonal Ab production will be required to allow linkage between immunogen binding site, neutralisation mechanism and cross serotype recognition ability.

Human infection with RV generates neutralising Abs to RV but these are serotype specific [4]. Studies in animals experimentally infected have produced similar results [31]. Immunisation of humans with inactivated RV [25,27,28] likewise induces serotype specific neutralising Abs, whereas animal immunisations have been shown to generate some limited cross-serotype neutralising abilities [31,38,39]. The critical correlate of protection of a successful RV vaccine would be the ability of Abs to cross-serotype neutralise but this is notoriously difficult due to the large number of immunologically distinct RV serotypes [3]. RV vaccine formulations containing 10 inactivated serotypes fail to generate cross-protective Abs when administered to humans [30] and the thought of packaging a larger number of serotypes into a single vaccine formulation has been dismissed due to the presence of upwards of 160 RV strains [40]. However, recent attempts have renewed hope by demonstrating that a 50-valent inactivated RV vaccine administered to rhesus macaques can induce broadly reactive neutralising Abs, but only to those 50 serotypes contained within the vaccine formulation [34].

Using a very different immunisation approach by formulating a single recombinant RV polypeptide predicted to induce cross-serotype immunity, we have established that VP0 immunisation with subsequent RV infection significantly induces neutralising Abs compared to RV infection alone, but again Ab induced is serotype specific [24]. Thus, one of the initial aims of this study was to better define the Abs resulting in RV neutralisation that are generated following RV challenge subsequent to VP0 immunisation. IgG

binding experiments did not readily identify unique Ab specificities that could be linked to neutralisation, which may reflect the fact that linear peptides and bacterially produced recombinant polypeptides that are unlikely to recreate the exact 3D structure of the viral capsid were used as antigens and inhibitors in the study. In addition, *in vivo* experiments often show significant variation between animals and the use of sera containing Abs of numerous specificities also makes these analyses more difficult. Variable responses between experiments are therefore somewhat expected. Thus, in conjunction with the use of non-native antigens for *in vitro* analyses, it is possible that an important and potentially neutralising proportion of the IgG in the sera that favours discontinuous or native epitopes may not be detected by these experiments. Nevertheless, our results have narrowed the most immunogenic region of the immunogen to a stretch of less than 50 amino acids that can potentially elicit the neutralising Abs and possibly the cross-serotype reactions. Importantly, this immunodominant region was identified as the major IgG target by both direct binding experiments and inhibition experiments. Thus, in all cases the sera generated from immunisation favoured this smaller, surface-exposed region that contains an established neutralising epitope and highly conserved sequences found in distinct RV serotypes. Other regions identified within VP0 that were bound by IgG include the VP4 C-terminus and the VP2 N-terminus – regions that are very highly conserved among RV serotypes. Binding to these regions can also help explain cross-serotype recognition by the antisera but Abs binding these regions have not been demonstrated to neutralise RV. Furthermore, inhibition experiments indicate that these sites are less immunodominant than the N1m-II region. The fact that our polypeptide immunogen VP0 is bacterially-produced and is therefore unlikely to fold in the native configuration found in RV-A16 can explain why immunisation of mice with the VP0 immunogen alone did not induce neutralising Abs. The generation of neutralisation ability requires exposure to live RV which argues for a requirement of conformational or discontinuous epitopes to induce the correct Abs. In addition, these epitopes may not have been mimicked by the peptides used in the current study since we observed no quantifiable differences in the Ab binding spectrum when comparing IgG from immunised mice to that of immunised and RV challenged mice. Further suggesting that such neutralising Abs are very rare and are therefore a minor fraction of the IgG found in these sera, making their identification in these experiments complicated.

Previous studies identified a peptide mimotope (VKAETRLNPDLPQTEC) unique to and covering the second part of the N1m-II site of RV-A02 that when used for immunisation induces Abs that bind to VP2 and neutralises RV-A02 *in vitro* [41]. This argues that in some cases short peptides do fold in configurations that prime for Abs that recognise neutralising epitopes. In fact, the N1m-II site is the only known neutralisation site of RV where Abs have been shown to react with a peptide mimotope. The monoclonal Ab 8F5 [42] binds this region and its uncomplexed 3D structure has been solved [43] and also in complex with the N1m-II peptide [15]. The neutralisation mechanism of 8F5 is to stabilise the RV capsid structure and prevent uncoating and release of viral RNA. Although this peptide sequence is unique to RV-A02, an analogous region is found within the immunodominant VP2 domain identified here for RV-A16, suggesting that the Abs we describe here binding this region will neutralise RV with a similar mechanism. The concept of peptide immunisation conferring protective immunity to RV was also confirmed by immunisations with short conserved peptides of VP1 (VVQAMYVPPGAPNPKEC) and VP3 (KLILAYTPPGARGPQDC) of RV-B14 [44], sequences that form the RV capsid canyon structure and are critical for interaction with the host cell receptor intercellular adhesion molecule 1 (ICAM-1) [9]. Immunisation of rabbits with similar peptides induced cross-

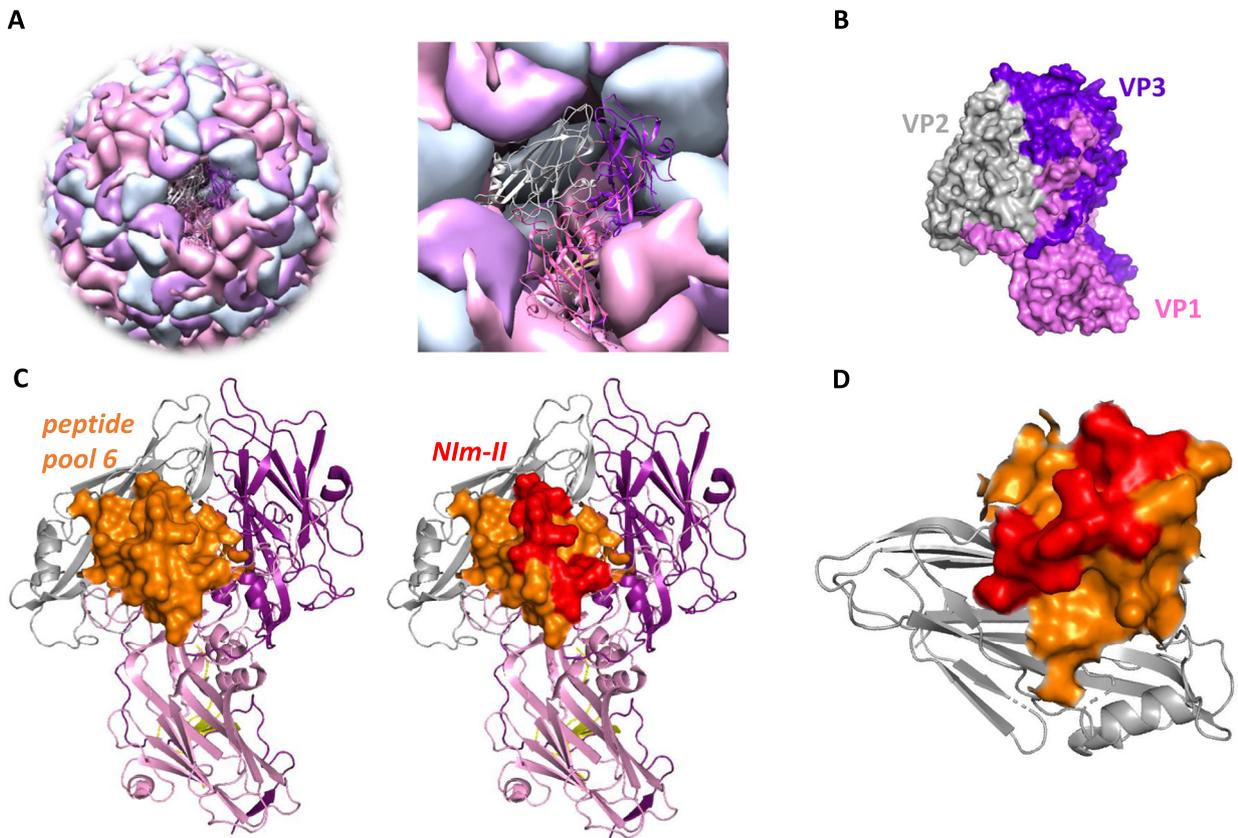


Fig. 5. Colocalization of the immunodominant region and NIm-II site within VP2 structure of the RV A16 capsid. Images were created using the PDB entry 1AYM of the RV A16 capsid structure solved by X-ray diffraction [37] using UCSF Chimera molecular graphics program (A) or Pymol molecular graphics program (B, C, D). (A) The entire virus structure as molecular surface image, with ribbons view of the repeating unit and a close up view of the interaction. VP1 is shown in pink, VP2 is shown in grey, VP3 is shown as purple and VP4 is in cream and located within the core of the virus. (B) Molecular surface for interaction of VP1, VP2 and VP3. Colour scheme as for A. (C) Superimposed on VP2 ribbon diagram the molecular surface shown in orange is the peptide pool 6 – residues 133–183. VP1 is shown as pink, VP2 is shown as grey, VP3 is shown as purple and VP4 is shown in yellow. The second image is similar with NIm-II residues (134–138 and 155–162) shown in red. (D) The image shows just the VP2 ribbon diagram in grey oriented to display molecular surface and the topography of the protrusion covered by peptide pool 6 shown in orange and NIm-II in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

serotype binding and neutralising Abs without the requirement for live virus challenge [44]. Taken together these results highlight that careful immunogen selection can result in functional and broadly reactive Abs to RVs. It is interesting to note that in our experiments, immunisation with a much larger immunogen still generates IgG's that target a smaller and highly serotype-specific NIm-II region but that these Abs are also cross-serotype reactive, binding to polypeptides from at least four distinct type A RV serotypes (RV-A16, RV-A01, RV-A29, [24] and RV-A89). We have not expanded our investigations to explore the molecular nature of the cross-serotype reactive IgG experimentally but bioinformatic analyses of the equivalent region of numerous type A RV VP2 sequences has revealed significant areas of amino acid identity flanking the highly variable NIm-II minimal epitope. Indeed, our RV-A16 molecular imaging studies of VP2 show that these conserved sequences form a semi-exposed IgG-accessible basal surface from which the NIm-II epitope protrudes and help explain the IgG cross-serotype binding but serotype specificity of neutralisation.

Although Abs that bind to NIm-II are thought to neutralise RV by stabilising the capsid structure and preventing uncoating, there may be additional mechanisms that depend on the Ab spectrum generated by infection or immunisation and the RV serotype specificity. It is possible that NIm-II binding Abs are carried into cells by RV and activate neutralisation through engagement of TRIM21 [45], an Fc receptor found in the cytoplasm of cells. In fact, Ab

opsonised RV-A02 was shown recently to be neutralised by a TRIM21 dependent mechanism in HeLa cells [46]. The binding site of Abs that neutralise RV by this novel mechanism have yet to be explored but the Abs themselves require a slow dissociation rate for their antiviral activity [47]. We propose that the NIm-II binding Abs described here that are induced by VP0 immunisation and RV challenge may neutralise RV by a similar mechanism. Our data therefore support future studies involved in generating targeted neutralising monoclonal antibodies through rational immunogen design to uncover novel neutralising mechanisms and the molecular basis of cross-serotype recognition.

Declaration of interests

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and performed experiments. CMN created structural images. KN and RV provided reagents. JSN, GRM, SLJ and RV analysed data and wrote the paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2019.04.018>.

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