

1 **Introduction**

2 Human rhinovirus (RV) infections cause a self-limiting illness of the upper respiratory tract
3 called the common cold, however in people with chronic airway disease (asthma and COPD),
4 these viral infections involve the lower respiratory tract and cause serious exacerbations that
5 can be life threatening [1]. There are very limited options for treatment of RV infections and
6 fifty years of research has failed to produce a reliable and specific antiviral [2]. Likewise, only
7 slight progress has been made in harnessing the adaptive immune system to produce an
8 effective vaccine. The difficulty has been attributed to the large number of serologically distinct
9 virus types which necessitate finding new broadly cross-reactive immunogens [3].

10 Improving understanding of the correlates of protection from infection and resultant disease is
11 key to developing a RV vaccine. Antibody (Ab, Abs) responses have been well studied, with
12 neutralising epitopes having been defined and the ability of neutralising Ab to protect from
13 disease having been established [4-6]. Similarly, Abs are well established as a key correlate of
14 protection for influenza virus [7]. RV-specific Abs are directed against epitopes found on the
15 surface exposed areas of capsid proteins VP1, VP2 and VP3 [8], 60 copies of each being
16 arranged structurally into an icosahedron, with VP4 buried beneath the surface [9]. Abs are
17 critical for protection against re-infection with the same serotype since defence is associated
18 with high levels of neutralising antibody both in serum and respiratory secretions [4] but in
19 general, neutralising Abs to RV are serotype-specific [10, 11]. Interestingly, Abs persisting
20 from prior encounters with other RV serotypes may cross-react with closely related RV
21 serotypes and sometimes can cross-neutralise [12]. Structural studies of neutralising Abs bound
22 to RV have found that effective neutralising activity depends on Ab binding that; 1) blocks
23 virus interaction with the major group ICAM-1 entry receptor [13]; 2) stabilises the virus capsid
24 preventing uncoating and release of virus nucleic acid [14, 15]; 3) aggregates virions [16]; and
25 4) induces virus uncoating extracellularly [17]. Studies of major group RV-B14 and minor
26 group RV-A02 identified two neutralising immunogenic sites (NIm-Ia and NIm-Ib) on VP1
27 and two further neutralising sites, NIm-II on VP2 and NIm-III on VP3 [6, 8, 18]. The majority
28 of the NIm sites are discontinuous epitopes forming protrusions from the capsid surface and
29 all are highly variable regions of the VP sequences amongst the RV serotypes. This variability
30 explains the high level of serotype specificity and poor cross protective abilities of neutralising
31 Abs. Furthermore, analogous NIm's have not been functionally described on other RV
32 serotypes. Another feature reflecting their wide diversity is that distinct RVs use different entry
33 receptors during infection of cells. Thus, intercellular adhesion molecule 1 (ICAM-1) is used

34 by all RV type B and most type A, low density lipoprotein receptor (LDLR) by 12 type A, and
35 cadherin-related family member 3 (CDHR3) is used by RV type C [19]. On the other hand,
36 understanding of RV-specific T cell immunity is much more limited. Studies in humans have
37 identified predominantly CD4⁺ Th1-cytokine-secreting T cells that are capable of cross-
38 reactivity to diverse RV serotypes [20, 21] and more recent studies have defined potential
39 cross-reactive RV-induced memory T cell epitopes within RV structural proteins [22, 23].
40 Supporting these observations are our prior studies in mice immunised with highly conserved
41 VP4 and VP2 regions of the RV polyprotein (RV-A16 VP0) that also showed the generation
42 of cross-reactive T cell immunity [24]. Nevertheless the role of T cells in protective immunity
43 to RV remains poorly understood.

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

56 The current study was undertaken to address our understanding of RV-specific neutralising Ab
57 during immunisation and RV challenge and the role played by capsid binding sites. Therefore,
58 we measured Ab responses in antisera to overlapping peptides representing the structural
59 proteins within the RV-A16 polyprotein to define immunodominant epitopes. We characterised
60 *in vitro* the antisera obtained from a previous study that had demonstrated cross-serotype
61 binding properties and serotype-specific neutralisation [24]. In this prior study we showed that
62 RV-specific IgG and IgA were found in serum and bronchoalveolar lavage fluid (BALF)
63 respectively obtained from mice immunised with RV-A16 VP0 and that the strongest binding
64 was observed with immunised and RV challenged animals. The serum IgG was also shown to
65 bind to VP0 of distinct serotypes RV-A29, RV-A16 and RV-A01 by western blot and *in vitro*
66 was capable of neutralising the serotype of RV used for challenge *in vivo* [24]. Now we have

67 gone further, mapping and comparing the binding patterns of Abs in antisera induced by VP0-
68 immunisation and RV challenge. IgG was found to target minimal regions of the immunogen
69 sequence with the immunodominant region structurally mapped to an area coinciding with the
70 NIm-II analogous neutralising site of VP2.

71

72 **Methods**

73 *Mouse Immunisation and Challenge Studies*

74 Four *in vivo* immunisation and challenge experiments were performed using C57BL/6 6-8
75 week old mice as described previously [24]. Mice were divided into 3 treatment groups with
76 n=4 per group: (**A-RV**) immunised with adjuvant alone (incomplete Freund's adjuvant and
77 CpG (IFA/CpG; obtained from Sigma-Aldrich and Invivogen respectively)) and challenged
78 with RV-A01 or RV-A29; (**I-P**) immunised with RV-A16 VP0 in IFA/CpG and challenged
79 with phosphate buffered saline (PBS); (**I-RV**) immunised with RV-A16 VP0 in IFA/CpG and
80 challenged with RV-A01 or RV-A29 [24]. Two studies each involved RV-A01 challenge (**RV-**
81 **A01 #1 & RV-A01 #2**) or RV-A29 challenge (**RV-A29 #1 & RV-A29 #2**). All animal studies
82 were conducted according to UK home office legislation (Animals (Scientific Procedures) Act
83 1986), project licence number PPL 70/7234. Serum was obtained as described previously [24].
84 Mice were immunised twice over three weeks subcutaneously (SC) and challenged IN four
85 weeks after the second SC immunisation with RV-A01 or RV-A29. Fourteen days after
86 challenge, serum was obtained to determine Ab levels, specificity and neutralising capabilities.

87

88 *RV-A16 Structural Protein Peptide Pools*

89 Overlapping 15mer peptides (overlapping by 11 amino acids), covering the full RV-A16 capsid
90 sequences were generated based on published sequences (NCBI accession Q82122) and
91 obtained from Mimotopes Pty Ltd. Peptides were suspended in DMSO and combined into
92 pools of 10 peptides for initial Ab mapping experiments. Eight peptide pools span the VP0
93 region of RV-A16, 6 peptide pools span the VP3 region and 7 peptide pools cover VP1. Thus,
94 peptide pool 1 covers the VP4 sequence, peptide pool 2 overlaps the C-terminus of VP4 and
95 the N-terminus of VP2 and peptide pools 3 through 8 cover the remainder of VP2 with peptide
96 pool 9 also covering part of the VP2 C-terminus. Peptide pools 9 to 15 cover the VP3 region.

97 Part of peptide pool 15 and peptide pools 16 through 21 cover VP1. Peptide pools sequences
98 and a representation of the regions covered by each peptide pool in the RV-A16 capsid
99 sequences are shown in Supplementary Figures 1 and 2.

100

101 *ELISA*

102 ELISA was performed as described previously [24, 31] with modifications. For indirect
103 ELISA, plates were coated with purified RV capsid proteins (RV-A89 VP1, VP2, VP3, VP4
104 [35]; RV-A16 VP0) at 1µg/ml, peptide pools at 4µg/ml, or individual peptides (pool 6) at
105 1µg/ml diluted in PBS. Mouse sera were diluted 1:100 in PBS containing 0.05% tween 20
106 (Sigma-Aldrich) and 5% Marvel skimmed milk powder (Tesco) (PBST-milk). For competition
107 ELISA, plates were coated with recombinant VP0 at 0.2µg/ml in PBS (equivalent to 10ng per
108 well). Mouse sera were diluted 1:25000 in PBST-milk and mixed with peptide pools or VP0
109 diluted to 0.1mg/ml, incubated for 2hr at 37 °C before addition to blocked and coated plates
110 (equivalent to 5µg per well). This corresponds in each well of the competition assays to a 500-
111 fold mass excess of soluble peptide pools or VP0 to the available plate-bound VP0. All assays
112 were developed with peroxidase-labelled goat anti-mouse IgG or IgA (Southern Biotechnology
113 Associates) followed by 3,3',5,5'-Tetramethylbenzidine (Invitrogen) substrate before optical
114 density was read at 450nm.

115

116 *RV Capsid Proteins Structure Images*

117 Protein data bank submission 1AYM was used as the source of coordinates for the RV-A16
118 capsid proteins. Images were constructed using UCSF Chimera molecular graphics program
119 [36] and Pymol molecular graphics program (PyMOL: The PyMOL Molecular Graphics
120 System, Version 1.8 Schrödinger, LLC).

121

122

123

124

125 **Results**

126 *VP0-binding IgG also binds VP4 and VP2 from a distinct RV serotype*

127 To determine the specificity and cross-serotype nature of the antibodies induced by
128 immunisation and challenge with greater detail, we performed ELISA experiments using the
129 recombinant capsid proteins VP1, VP2, VP3 and VP4 of RV-A89 [35] and the immunising
130 recombinant protein VP0 of RV-A16 as antigens. Figure 1 shows that sera from four
131 independent *in vivo* experiments of immunised alone (I-P) and immunised and challenged mice
132 (I-RV) contained IgG that bound strongly and consistently to RV-A16 VP0 (inset) and to both
133 VP2 and VP4 of RV-A89, whereas mock-immunised but challenged mice (A-RV) had no
134 significant IgG that bound to these proteins. Binding to VP1 and VP3 of RV-A89 was not
135 significant and very weak across all groups except that *in vivo* experiment RV-A01 #1 showed
136 detectable binding to VP1 that was not statistically significant. These results, as expected,
137 confirm that the sera from VP0 immunised mice contains IgG that binds to the components of
138 the immunogen, VP4 and VP2, in a cross-serotype manner. No binding of serum IgA to these
139 proteins by ELISA was detected (data not shown) similarly to previous results [24].

140 *IgG binds to peptides covering the VP4 C-terminus, VP2 N-terminus and the VP2 NIm-II site*

141 We designed overlapping peptide pools that span the 4 capsid proteins of RV-A16. The
142 sequences of peptides within each pool and their location within each capsid protein are shown
143 in Supplementary Figure 1. Twenty one peptide pools were generated that span VP4, VP2, VP3
144 and VP1 (Supplementary Figure 2) with each pool containing 10 peptides of 15 amino acid
145 length that have an 11 amino acid overlap with the next peptide. Thus, pools 1 and 2 covered
146 VP4 and the beginning of VP2, pools 3-9 covered the remainder of VP2 and beginning of VP3,
147 pools 10-15 covered VP3 and the beginning of VP1, and pools 16-21 covered the remainder of
148 VP1 (Supplementary Figures 1 and 2). To determine IgG binding to smaller regions of the RV
149 capsid proteins, peptide pools 1-21 were used as antigens in ELISA experiments. We found
150 that specific IgG binding was restricted to peptide pools that covered the VP0 region (pools 1-
151 8, VP2 and VP4) and was only observed in VP0-immunised mice (I-P and I-RV) with no
152 detectable binding found in sera from unimmunised but RV challenged mice (A-RV) (Figure
153 2A). Consistent with prior results obtained with recombinant RV capsid proteins, serum IgG
154 was unable to bind peptide pools 9-21 which span the VP3 and VP1 capsid proteins. Closer
155 inspection of the peptide pools revealed that sera from immunised mice contained IgG that
156 bound peptide pools 2, 3 and 6 consistently with peptide pool 8 showing detectable binding in

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

166 *The NIm-II site and proximal regions of VP2 are immunodominant*

167 To confirm the IgG binding to peptides of VP0 and to determine the immunodominant sites of
168 VP0 targeted by IgG we performed inhibition experiments using the peptide pools to interfere
169 with IgG binding to immobilised VP0. We used a 500-fold mass excess of the soluble peptide
170 pool compared to plate-bound VP0. IgG binding to plate-bound VP0 was consistently inhibited
171 by peptide pool 6 in each experiment and antisera tested (Figure 3A). Thus, significant
172 inhibition of IgG binding to VP0 was observed with excess peptide pool 6, by approximately
173 45% in each experiment using sera from both I-P and I-RV mice suggesting that a large
174 proportion of IgG in these sera bound epitopes within this region of VP2. As expected, the
175 peptide pools that did not display IgG binding in Figure 2A (pools 1, 4, 5, 7 and 8) had no
176 inhibitory effect on the binding of serum IgG to VP0. Interestingly, the peptide pools 2 and 3
177 that were shown in Figure 2 to bind IgG within the sera, did not significantly interfere with IgG
178 binding to VP0 in most experiments. However, weak but statistically significant inhibition was
179 seen with peptide pool 2 in I-P mice from *in vivo* experiment RV-A01 #2 and with peptide pool
180 3 in I-RV mice from *in vivo* experiment RV-A29 #1. In all cases, as an internal positive control
181 to determine specificity, excess soluble VP0 was able to inhibit IgG binding to plate-bound
182 VP0 by greater than 95% (Figure 3A). Doubling the peptide pool concentration to 1000-fold
183 mass excess did not improve inhibition of IgG binding to VP0 above 45% for pool 6 and also
184 did not generate further significant inhibition with pools 2 and 3 (data not shown).

185 We next determined whether combinations of the peptide pools allowed improved inhibition
186 of serum IgG binding to VP0. The combination of peptide pools 2 & 6 was consistently able
187 to inhibit up to 80% of IgG binding to VP0 whereas other combinations (pools 3 & 6; pools 8
188 & 6) were generally weaker inhibitors and in one case was unable to significantly block IgG

189 binding (Figure 3B). Conversely, the combination of peptide pools 2 & 3 did not significantly
190 inhibit IgG binding, except in one experiment. Collectively, these data indicate that much of
191 the serum IgG generated by VP0 immunisation targets the region of VP0 covered by peptide
192 pool 6 (VP2 NIm-II site) with a lesser contribution of IgG that recognises the VP4 C-terminus
193 and VP2 N-terminus covered by peptide pool 2.

194 The data obtained with sera binding to the peptide pools and their use to block IgG binding to
195 VP0 are summarised in Table 1 and help establish that the region of VP0 covered by peptide
196 pool 6 was immunodominant, inducing the strongest IgG binding. This peptide pool spanned
197 regions of VP2 that have previously been shown to be a neutralising epitope of RV-B14 [6].
198 Thus, IgG binding to individual peptides from this pool was determined next to assist defining
199 the fine specificity of binding. Figure 4 displays the profile of IgG binding to the individual 10
200 peptides contained within pool 6. Binding to peptides (51, 59 and 60) at the extremities of the
201 region was either very weak or undetectable in all cases whereas significant binding to the 7
202 core peptides (52, 53, 54, 55, 56, 57, and 58) was observed with most of the sera obtained from
203 the 4 independent *in vivo* experiments. Peptide 52 (KGNVNAGYKYT) was consistently bound
204 the strongest by IgG in all cases and the remaining 6 peptides of this core recognition region
205 were also bound well however there were notable exceptions with sera obtained from certain
206 experiments. For example, sera I-RV from *in vivo* experiments RV-A29 #1 & RV-A29 #2 did
207 not bind peptides 55 and 56 well, and antisera I-P from experiments 1 and 4 displayed weaker
208 binding to numerous core peptides. No discernible differences in binding between sera I-P and
209 I-RV that could be attributed to the RV challenge were noted. A summary of these data,
210 indicating the regions of VP2 covered by the various individual peptides are shown in Table 2,
211 where it is shown that IgG binding tends to favour peptides containing the conserved amino
212 acid sequences that span the predicted NIm-II epitope regions. These regions are shown in
213 Supplementary Figure 3 where alignments of the RV type A homologous VP2 sequences
214 surrounding the NIm-II site are highlighted.

215 *Imaging of the IgG-binding immunodominant region in the VP2 structure*

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

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

230

231 **Discussion**

232 This study has investigated details of the Ab response generated to the RV vaccine candidate
233 VP0 in mouse models. This experimental vaccine, previously shown to generate cross-serotype
234 reactive cell-mediated immunity and protective humoral responses [24], is in pre-clinical
235 development. This study set out to define better the cross-serotype and neutralising Ab
236 responses by determining the immunodominant site(s) of the RV capsid targeted. We
237 determined that serum IgG responses are specific for fragments of VP4 and VP2 with the
238 dominant site surrounding the VP2 NIm-II neutralising site. Binding to the immunodominant
239 site could not be linked to virus neutralisation, therefore monoclonal Ab production will be
240 required to allow linkage between immunogen binding site, neutralisation mechanism and
241 cross serotype recognition ability.

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

253 vaccine administered to rhesus macaques can induce broadly reactive neutralising Abs, but
254 only to those 50 serotypes contained within the vaccine formulation [34].

255 Using a very different immunisation approach by formulating a single recombinant RV
256 polypeptide predicted to induce cross-serotype immunity, we have established that VP0
257 immunisation with subsequent RV infection significantly induces neutralising Abs compared
258 to RV infection alone, but again Ab induced is serotype specific [24]. Thus, one of the initial
259 aims of this study was to better define the Abs resulting in RV neutralisation that are generated
260 following RV challenge subsequent to VP0 immunisation. IgG binding experiments did not
261 readily identify unique Ab specificities that could be linked to neutralisation, which may reflect
262 the fact that linear peptides and bacterially produced recombinant polypeptides that are unlikely
263 to recreate the exact 3D structure of the viral capsid were used as antigens and inhibitors in the
264 study. In addition, *in vivo* experiments often show significant variation between animals and
265 the use of sera containing Abs of numerous specificities also makes these analyses more
266 difficult. Variable responses between experiments are therefore somewhat expected. Thus, in
267 conjunction with the use of non-native antigens for *in vitro* analyses, it is possible that an
268 important and potentially neutralising proportion of the IgG in the sera that favours
269 discontinuous or native epitopes may not be detected by these experiments. Nevertheless, our
270 results have narrowed the most immunogenic region of the immunogen to a stretch of less than
271 50 amino acids that can potentially elicit the neutralising Abs and possibly the cross-serotype
272 reactions. Importantly, this immunodominant region was identified as the major IgG target by
273 both direct binding experiments and inhibition experiments. Thus, in all cases the sera
274 generated from immunisation favoured this smaller, surface-exposed region that contains an
275 established neutralising epitope and highly conserved sequences found in distinct RV
276 serotypes. Other regions identified within VP0 that were bound by IgG include the VP4 C-
277 terminus and the VP2 N-terminus - regions that are very highly conserved among RV
278 serotypes. Binding to these regions can also help explain cross-serotype recognition by the
279 antisera but Abs binding these regions have not been demonstrated to neutralise RV.
280 Furthermore, inhibition experiments indicate that these sites are less immunodominant than the
281 NIm-II region. The fact that our polypeptide immunogen VP0 is bacterially-produced and is
282 therefore unlikely to fold in the native configuration found in RV-A16 can explain why
283 immunisation of mice with the VP0 immunogen alone did not induce neutralising Abs. Since
284 the generation of neutralisation ability requires exposure to live RV, which argues for a
285 requirement of conformational or discontinuous epitopes to induce the correct Abs. In addition,

286 these epitopes may not have been mimicked by the peptides used in the current study since we
287 observed no quantifiable differences in the Ab binding spectrum when comparing IgG from
288 immunised mice to that of immunised and RV challenged mice. Further suggesting that such
289 neutralising Abs are very rare and are therefore a minor fraction of the IgG found in these sera,
290 making their identification in these experiments complicated.

291 Previous studies identified a peptide mimotope (VKAETRLNPDLQPTEC) unique to and
292 covering the second part of the NIm-II site of RV-A02 that when used for immunisation
293 induces Abs that bind to VP2 and neutralises RV-A02 *in vitro* [41]. This argues that in some
294 cases short peptides do fold in configurations that prime for Abs that recognise neutralising
295 epitopes. In fact, the NIm-II site is the only known neutralisation site of RV where Abs have
296 been shown to react with a peptide mimotope. The monoclonal Ab 8F5 [42] binds this region
297 and its uncomplexed 3D structure has been solved [43] and also in complex with the NIm-II
298 peptide [15]. The neutralisation mechanism of 8F5 is to stabilise the RV capsid structure and
299 prevent uncoating and release of viral RNA. Although this peptide sequence is unique to RV-
300 A02, an analogous region is found within the immunodominant VP2 domain identified here
301 for RV-A16, suggesting that the Abs we describe here binding this region will neutralise RV
302 with a similar mechanism. The concept of peptide immunisation conferring protective
303 immunity to RV was also confirmed by immunisations with short conserved peptides of VP1
304 (VVQAMYVPPGAPNPKEC) and VP3 (KLILAYTPPGARGPQDC) of RV-B14 [44],
305 sequences that form the RV capsid canyon structure and are critical for interaction with the
306 host cell receptor intercellular adhesion molecule 1 (ICAM-1) [9]. Immunisation of rabbits
307 with similar peptides induced cross-serotype binding and neutralising Abs without the
308 requirement for live virus challenge [44]. Taken together these results highlight that careful
309 immunogen selection can result in functional and broadly reactive Abs to RVs. It is interesting
310 to note that in our experiments, immunisation with a much larger immunogen still generates
311 IgG's that target a smaller and highly serotype-specific NIm-II region but that these Abs are
312 also cross-serotype reactive, binding to polypeptides from at least four distinct type A RV
313 serotypes (RV-A16, RV-A01, RV-A29, [24] and RV-A89). We have not expanded our
314 investigations to explore the molecular nature of the cross-serotype reactive IgG
315 experimentally but bioinformatic analyses of the equivalent region of numerous type A RV
316 VP2 sequences has revealed significant areas of amino acid identity flanking the highly
317 variable NIm-II minimal epitope. Indeed, our RV-A16 molecular imaging studies of VP2 show
318 that these conserved sequences form a semi-exposed IgG-accessible basal surface from which

319 the NIm-II epitope protrudes and help explain the IgG cross-serotype binding but serotype
320 specificity of neutralisation.

321 Although Abs that bind to NIm-II are thought to neutralise RV by stabilising the capsid
322 structure and preventing uncoating, there may be additional mechanisms that depend on the Ab
323 spectrum generated by infection or immunisation and the RV serotype specificity. It is possible
324 that NIm-II binding Abs are carried into cells by RV and activate neutralisation through
325 engagement of TRIM21 [45], an Fc receptor found in the cytoplasm of cells. In fact, Ab
326 opsonised RV-A02 was shown recently to be neutralised by a TRIM21 dependent mechanism
327 in HeLa cells [46]. The binding site of Abs that neutralise RV by this novel mechanism have
328 yet to be explored but the Abs themselves require a slow dissociation rate for their antiviral
329 activity [47]. We propose that the NIm-II binding Abs described here that are induced by VP0
330 immunisation and RV challenge may neutralise RV by a similar mechanism. Our data therefore
331 support future studies involved in generating targeted neutralising monoclonal antibodies
332 through rational immunogen design to uncover novel neutralising mechanisms and the
333 molecular basis of cross-serotype recognition.

334

335 **Declaration of interests**

336 All authors approve this submission. The authors acknowledge this work was supported in part
337 by grants from Sanofi Pasteur (to SLJ), the European Union (to SLJ and RV), grant P29398 of
338 the Austrian Science Fund (to KN) and Dunhill Medical Trust serendipity award (to GRM).
339 The funders had no input into study design. Authors NG, SLJ and GRM are co-inventors of a
340 US patent regarding the rhinovirus vaccine. Authors RV and KN are co-inventors of a patent
341 application regarding rhinovirus diagnostics by antibodies. JSN, NG and GRM designed and
342 performed experiments. CMN created structural images. KN and RV provided reagents. JSN,
343 GRM, SLJ and RV analysed data and wrote the paper.

344

345 **Figure Legends**

346 *Figure 1: Serum IgG binds to recombinant RV capsid proteins by ELISA.* The RV
347 recombinant proteins VP0 from RV-A16 and VP1, VP2, VP3 and VP4 from RV-A89 were
348 coated onto ELISA plates and serum from 4 independent experiments allowed to bind.

349 Detecting antibody was against mouse IgG. For VP1, VP2, VP3 and VP4 open bars represent
350 A-RV, shaded bars represent I-PBS, and black bars represent I-RV. For VP0 (inset graph)
351 shaded bars represent I-PBS and I-RV combined sera binding. Data is expressed as the mean
352 +/- SEM of 4 individual sera within each group and were compared by one way ANOVA with
353 each bar compared to the negative control. **** p < 0.0001, *** p < 0.005, no asterisk is not
354 significantly different and p > 0.05.

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

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

378 **Figure 4: Fine specificity of Nim-II peptides bound by serum IgG.** Individual peptides from
379 peptide pool 6 were coated onto ELISA plates and IgG from sera allowed to bind. Detecting
380 antibody was against mouse IgG. Data are the average of two assays and are expressed as the

381 mean +/- SEM of 4 individual sera grouped from 4 independent immunisation experiments and
382 whether I-P or I-RV. Data with significantly different binding compared to VP0 were
383 determined by two way ANOVA. **** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05, no
384 asterisk is not significant p > 0.05.

385 **Figure 5: Colocalization of the immunodominant region and NIm-II site within VP2**
386 **structure of the RV A16 capsid.** Images were created using the PDB entry 1AYM of the RV
387 A16 capsid structure solved by X-ray diffraction [37] using UCSF Chimera molecular graphics
388 program (A) or Pymol molecular graphics program (B, C, D). (A) The entire virus structure
389 as molecular surface image, with ribbons view of the repeating unit and a close up view of the
390 interaction. VP1 is shown in pink, VP2 is shown in grey, VP3 is shown as purple and VP4 is
391 in cream and located within the core of the virus. (B) Molecular surface for interaction of VP1,
392 VP2 and VP3. Colour scheme as for A. (C) Superimposed on VP2 ribbon diagram the
393 molecular surface shown in orange is the peptide pool 6 – residues 133-183. VP1 is shown as
394 pink, VP2 is shown as grey, VP3 is shown as purple and VP4 is shown on yellow. The second
395 image is similar with NIm-II residues (134-138 and 155-162) shown in red. (D) The image
396 shows just the VP2 ribbon diagram in grey oriented to display molecular surface and the
397 topography of the protrusion covered by peptide pool 6 shown in orange and NIm-II in red.

398

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