

A systematic review and meta-analysis of animal studies investigating the relationship between serum antibody, T lymphocytes and respiratory syncytial virus (RSV) disease

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Abstract

Background: Respiratory syncytial virus (RSV) infections occur in human populations around the globe, causing disease of variable severity, disproportionately affecting infants and older adults (>65 years). Immune responses can be protective but also contribute to disease. Experimental studies in animals enable detailed investigation of immune responses, provide insights into clinical questions and accelerate the development of passive and active vaccination. We aimed to review the role of antibody and T cell responses in relation to RSV disease severity in animals.

Method: Systematic review and meta-analysis of animal studies examining the association between T-cell responses/phenotype or Ab titres and severity of RSV disease. PubMed, Zoological Record and Embase databases were screened from January 1980 to May 2018 to identify animal studies of RSV infection which assessed serum Ab titre or T lymphocytes with disease severity as an outcome. Sixty-three studies were included in the final review.

Results: RSV-specific antibody appears to protect from disease in mice, but such an effect was less evident in bovine RSV. Strong T-cell, Th1, Th2, Th17, CD4/8 responses, and weak Treg responses accompany severe disease in mice.

Conclusion: Murine studies suggest that measures of T lymphocyte activity (particularly CD4 and CD8 T-cells) may be predictive biomarkers of severity. Further enquiry is merited to validate these results and assess relevance as biomarkers for human disease.

51 **Introduction**

52 Nearly all children are infected with RSV by the age of two years[1], resulting in measurable immune
53 responses. Despite this, reinfections occur throughout life, demonstrating incompletely protective
54 immunity [2]. RSV disease in infants usually causes a mild upper respiratory tract infection (URTI),
55 but cases of more severe lower respiratory tract infections (LRTI) also occur. In a recent study
56 hospitalisation due to RSV LRTI occurred in 35.6 out of every 1000 children in the industrialised
57 world[3]. Severe RSV infection occurs in only a small percentage of those that are infected; while some
58 risk factors have been identified (e.g. prematurity[4]), there is not yet a comprehensive understanding
59 of the host factors that influence which children will develop more severe disease. Biomarkers that can
60 aid in prediction of children likely to develop severe RSV disease are therefore desirable to more
61 directly target treatment and resources to the children at greatest risk of severe infection.

62
63 RSV disease has been extensively studied in animal models, which recapitulate many (but not all)
64 aspects of human infection. The inflammatory cell infiltration of the lung in mice can resemble that
65 seen in infantile bronchiolitis [5], and the cotton rat is well established as semi-permissive for infection
66 with human RSV strains [6]. Calves infected with bovine RSV (bRSV) recapitulate some aspects of
67 human disease but also have distinctive features (such as acute emphysema) but there are logistical
68 limitations of working with large animal species. Other animals that have been used to study RSV
69 include lambs[7][8] and chimpanzees[9], but non-human primates have major cost and ethical
70 limitations.

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72
73 This study focussed on surveying the effects of serum antibody (Ab), T lymphocyte frequency and
74 phenotype on the immune response to RSV and on the identification of putative biomarkers of
75 protection and disease. Human infant studies demonstrate that neutralising Ab titre correlates inversely
76 with susceptibility to reinfection[10] and there is an inverse correlation between maternally-derived Ab
77 titre (MDA) and RSV severity [11][12][13][14][15][16]. In addition, monoclonal Ab prophylaxis can
78 prevent RSV disease in the lower respiratory tract [17]. However, low levels of neutralising Abs

79 correlate with enhanced disease severity after RSV infection[15][18][14]. Isotypes of serum Ab and
80 both self-derived and maternally-derived serum Ab titres are reviewed separately.

81
82 This study also investigates the pathological and protective functions of T lymphocytes in RSV
83 infection [19]. Children with a defective T-cell response have higher viral loads, delayed viral clearance
84 and prolonged disease[20][21]. However, T-cell reconstitution in immunodeficient patients can
85 enhance disease[22]. Measures of T lymphocyte frequency or phenotype in the periphery may not
86 predict disease severity because the relevant cells are concentrated in the site of disease.

87
88 This study aims to systematically review the animal model literature on the roles of T lymphocytes and
89 RSV-specific antibody in protection and pathogenesis of RSV disease. We assess the potential value of
90 these readouts in animal studies as predictive or concurrent markers of RSV disease severity in humans.

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Materials and methods

Selection criteria: We included studies that either investigated Ab titres (any isotype) or T lymphocyte activity or numbers as a variable. *Participants:* any animal model of any age (human, *in vitro* or epidemiological studies were excluded); *interventions:* human or bovine RSV infection; *comparisons:* within (versus controls) and between studies; *outcomes:* disease severity measures (any quantitative severity scoring); *studies:* controlled trials, cohort studies and case-control studies.

Search strategy: A search was conducted on 28th May 2018 using Embase, Pubmed and Zoological Record databases from January 1980 to May 2018. 1980 was used as a cut-off the relative youth of animal research in RSV at the time (bovine RSV had only been discovered in 1970) means few studies exist in mice or in cattle during this period. The search terms were 'RSV' or 'Respiratory Syncytial Virus' using filters: 'Journals only', 'Animal experiments' and 'English language'. The inclusion criteria were applied via title and abstract screening, followed by full-text screening. All screening was conducted by two independent researchers, and contradictions resolved by a third independent researcher. Full texts of identified articles were gathered.

Variable measures: T lymphocyte measures: cell number, proportion, activity and molecular markers, were measured from samples of bronchoalveolar lavage fluid (BALF), lung tissue and serum. Data from the following cell types were analysed separately: CD4 (Th1, Th2, Th17), Treg, CD8, T-cells (both CD4 and CD8) and lymphocytes (T and B cells, and natural killer cells). Antibody measure was isotype titre via optical density measurements from serum samples.

Severity Measures: *Primary measures* were weight loss, airway function, lung histopathology and illness severity scores, and were assumed to be proportional to disease severity. Weight loss is a common, reproducible and objective measure of disease severity in animal models[23]. Airway function measures reflect the reduced lung function of infants with severe RSV infection. Lung resistance (RL) gives consistent results for airway function in both mild and severe inflammation models[24], and lung dynamic compliance (Cdyn) is a reflection of peripheral lung function[25]. Some studies dichotomised their severity measure as high or low severity, using a cut-off of '2 or more symptoms present' for bovine respiratory disease (BRD), which is severe, or 'fewer than 2 symptoms' for 'non-BRD', which is mild or sub-clinical.

121 Data analysis: Ab titres, severity scores and study meta-information were extracted where available. The
122 studies were divided and analysed in subgroups based on animal type, Ab isotype or T-cell
123 measurement. Forest plots were created using either standardised mean difference (for continuous
124 outcome measures of severity) or odds ratio (for dichotomous outcome measures of severity) to analyse
125 outcomes for high and low Ab groups and high or low cell count groups. Where studies reported
126 continuous outcomes (temperature, weight loss or symptom scores), forest plots were drawn using
127 standardised mean difference (SMD) of severity measures to make different outcomes comparable.
128 Where studies reported a dichotomous outcome measure (i.e. high or low disease severity), effect sizes
129 were calculated as odds ratios and these values are shown in a separate forest plot. Bias was assessed
130 for each study and bias assessment methods are described in supplemental results. A predictive
131 biomarker was defined as a biological variable that was measured at least 24h hours prior to the
132 measurement of disease severity. A concurrent biomarker was defined as a biological variable that was
133 measured at the same time as the measurement of disease severity.

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135
136 Scatter graphs were also plotted for the analysis of T lymphocytes as many lymphocyte and severity
137 measures were common across studies. Spearman's rank correlation coefficient (r_s) was calculated to
138 indicate the association between each cell and severity measure. Cell number and activity data were
139 taken at least one day earlier than severity measures to make the plots predictive. For meta-analysis
140 purposes predictive and concurrent biomarkers were considered separately and placed in the relevant
141 category based on time of variable measurement relative to measurement of disease severity (as a
142 variable). If the variable was measured before the time of disease severity measurement it was
143 considered predictive, if it was measured at the same time it was considered concurrent.

144 **Results**

145 The literature search yielded 5023 studies, which were screened and assessed for eligibility by two
146 reviewers. Twenty-six (Supp figure 1A, Supp figure 3) studies met the eligibility criteria regarding Ab
147 titres and 37 (supp figure 1B) for T lymphocyte frequency or activity.

148

1. Effect of T lymphocytes on severity

The number of studies reporting positive and negative associations of lymphocytes with RSV disease severity were assessed (table 1). Four studies considered CD4⁺ T cells (and not any specific subtype) and all of them demonstrated increased CD4⁺ T cell abundance to be associated with more severe disease. Twenty-one studies contained some measurement of CD8⁺ T cells and their association with more severe RSV disease, of which 17 showed a positive association, two showed a negative association, and two showed an inconclusive association. Thirteen studies measured total T lymphocytes association with RSV disease, of which nine showed a positive association, two showed a negative association, and two showed an inconclusive association.

Th1 responses are characterised by expression of IFN γ and TNF α . IFN γ ⁺ and/or TNF α ⁺ CD4 cells were measured in six studies, five of which exhibited positive correlation with viral replication, weight loss[26][27][28], AHR and lung pathology[29] across a range of interventions. However, one study found that the reduced IFN γ +CD4⁺ cell numbers also corresponded to an increase in weight loss[30].

Th2 markers include IL-4 and IL-13. All 5 studies that measured Th2 markers found a positive correlation with disease severity. Increased IL4+CD4 cells corresponded to greater weight loss[30], worse lung function, and greater inflammation[31], eosinophilia and mucus secretion[32]. CD4-specific deletion of IL-4Ra attenuated these pathologies, suggesting Th2 cells have a role in disease[32]. In addition, increased IL-13⁺ CD4 and CD8 levels in BALF corresponded to greater lung resistance abolished by anti-IL13 Ab[33].

Increased Th17 cells (defined by ROR γ T expression or IL-17+CD4⁺ numbers) were associated with more severe lung pathology and increased weight loss in all studies[29][34][35].

Some studies utilised time points prior to the onset of RSV disease, so analysis for these studies were considered separately to understand which elements of the immune system existing before infection have an influence on disease severity upon infection. A time point before the onset of disease or

176 measurement of symptoms is required to assess earlier states that may lead to more severe disease later
177 in disease. These biomarkers identified from data from earlier time points can be considered
178 “predictive” of likelihood of developing severe disease upon subsequent infection. For predictive
179 biomarkers, scatter plots showed there were significant correlations between CD4 and CD8 lung cell
180 number and weight loss (Fig. 2E and 2F), with r_s values of -0.83 and -0.68, respectively (Spearman's
181 $\rho p < 0.05$).

182
183 In order to robustly assess the effects of lymphocyte types on disease severity, standardised mean
184 difference was calculated for studies using these cell types. Forest plots of standardised mean difference
185 illustrated statistically significant increases in disease severity with elevated CD4 T cell (Fig. 1A), CD8
186 (Fig. 1B) and both CD4 and CD 8 T-cell (Fig. 1C) numbers, and significant decreases in disease severity
187 with increased Treg numbers (Fig. 1D). Plots of mean difference (Supp. Fig. 4, 5) gave the percentage
188 change in weight loss as 6.32 (95% CI: 3.27, 9.38) for CD4, 7.90 (95% CI: 5.49, 10.32) for CD8, 8.73
189 (95% CI: -0.65, 18.10) for T-cells and -5.99 (95% CI: -8.69, -3.29) for Treg cells when cell numbers
190 were high. Given mean weight loss was $\approx 10\%$ and maximal weight loss was 23.3%, high or low cell
191 numbers could therefore have a major impact on disease severity.

192
193 With respect to correlations between measured cell abundances in mice and disease severity metrics,
194 there was no statistically significant correlation between CD4 populations in the lung and weight loss
195 (Fig. 2A) (Spearman's $\rho = -.168$, $P = 0.32$), but there was a statistically significant correlation with
196 CD4+ proportion in lungs and weight loss (Fig. 2B) (Spearman's $\rho = 0.94$, $p < 0.05$), as determined
197 by sensitivity analysis where neonatal data were excluded because these mice exhibited weight gain
198 (342% and 502%) rather than weight loss (75-105%) (combined $r_s = -0.17$). Moreover, the statistically
199 significant r_s value of -0.31 for CD8 number in lungs versus weight loss (Fig. 2C) (Spearman's $\rho p <$
200 0.05, 2-tailed) supports that of the predictive plot. Finally, lymphocyte number was found to positively
201 correlate with Cdyn (Fig. 2D).

202

203 2. Effect of virus-specific antibody on severity

204

205 Calf studies

206 Most preclinical studies on RSV Ab titres (17 of 26, 65%) (Supp.Table 1) were conducted in bovine
207 RSV (bRSV) infected calves, while 9 (35%) (Table 2) were conducted in mice infected with hRSV.
208 Plots of mean difference were generated to demonstrate the effects of antibody subtypes on disease
209 severity. Meta-analyses of calf studies (Figure 3) did not demonstrate any significant association
210 between Ab titre measured at the time of severity measurements and disease severity over the course of
211 disease, with the exception of maternally derived antibodies, which was associated with decreased
212 disease severity.

213

214 Mouse Studies

215 A significant association with severity was observed for 'total RSV-specific antibody' (all serum
216 isotypes) demonstrating high Ab titres protect from severe symptoms ($P < 0.005$; figure 4). No significant
217 association with severity was observed for 'RSV-specific IgG titre' or 'IgE titre' (total and RSV-specific
218 IgE). For the purpose of the Ab titre analyses no distinction was made between "predictive" and
219 "correlative" as relatively few studies with Ab as an outcome measurement had depletion of cells or
220 measurements taken prior to infection, so all studies were considered together for a correlative analysis.

221

222 Discussion

223 Based on published data, markers of CD4 lymphocytic responses (especially, markers of highly active
224 or Th2-skewed lymphocytic response and a relative lack of immunoregulatory Treg responses) are
225 associated with severe disease in RSV-infected sensitized mice. With respect to CD8+ lymphocytes, it
226 seems that CD8+ T cells may enhance pathogenic effects early during infection, while being assisting
227 viral clearance in the later stages. RSV neutralising antibody is a potential biomarker or protection
228 against severe disease, especially in the lower respiratory tract. However, evidence from bovine studies
229 is less clear, only maternally derived antibody showing predictive effects in our metanalysis.

230
231 There is clear evidence that strong inflammatory responses in the airway are associated with more
232 severe disease in mice and that lymphocytes are central to the pathogenesis of lung disease. Higher
233 lymphocyte numbers are significantly correlated with lower lung dynamic compliance (Cdyn), which
234 is associated with increased airway occlusion. In humans and in bovines this can be attributed to
235 increased populations of apoptotic or necrotic cells in the airway during a heightened immune response,
236 however, this has not yet been demonstrated in mice[36]. It has been shown that pathogenesis in
237 neonatal mice can be reduced by knockout of IL-4 α , and that expression of IL-4 α in response to RSV
238 infection contributes towards a further Th2 bias[37] and increased disease severity.

239
240 Increased activation and numbers of CD8 T cells in the lungs of RSV infected animals is associated
241 with more severe disease, but also with viral clearance. Moreover, a statistically significant positive
242 correlation between lung CD8 number and weight loss supports the further investigation of low CD8 T
243 cell numbers as a potential biomarker to predict severe RSV disease. Based on this evidence, CD8 T
244 cells have a role in enhanced disease severity in acute disease but also in eventual viral clearance.

245
246 The proportions of lymphocyte subtypes in the mouse model are among the most promising biomarker
247 candidates for disease severity. Increased Th1, Th2 and Th17 responses may all be associated with
248 greater disease severity. Th2-skewed responses in general were more severe than Th1-skewed

249 responses, but this may need additional study. Th1 responses having a stronger capacity to control and
250 limit infection than other T lymphocyte subtypes and accordingly have a lower correlation with disease
251 severity than other T lymphocyte subtypes that are not as capable of controlling or limiting viral
252 infection. Treg numbers were generally associated with accelerated recovery and reduced disease
253 severity in mice. Regulatory T cells inhibit the development of other T cell subtypes that are associated
254 with more severe disease in mice.

255
256 It has been observed in humans that frequency of activated Tregs in the blood is reduced in RSV
257 infection[38]. Dampened Treg responses correlate with increased Th2 responses[39], which are
258 associated with more severe disease in humans[40]. Decreased Treg numbers may result in more severe
259 infection primarily due to a decreased capacity to control inflammation in infection.

260
261 The most prominently identified association between antibodies and disease was between total RSV-
262 specific Ab titre and more severe RSV infection in mice. However, Abs are not only protective against
263 reinfection but are also involved in preventing severe disease through reducing viral replication prior to
264 disease onset and cellular immune responses[41]. Indeed, some associations between Ab titres and
265 severe disease were observed,[42] but age, particularly, was a confounder in many studies. Neutralising
266 antibodies do not appear to be an appropriate biomarker for disease severity in the bovine model.

267
268 The collective bovine data presented here shows little or no association between RSV specific Ig and
269 disease severity. However, the studies that revealed a positive correlation between neutralising antibody
270 and severe disease had notable confounders, such as age or allergic sensitisation, making them less
271 reliable to assess biomarkers for disease severity[42][43][44]. Conversely to the ambiguous role of RSV
272 neutralising antibody, maternally derived antibodies in the bovine model demonstrate a consistently
273 protective role and exist primarily in the group at the highest risk of disease.

274
275 From our analysis, the mouse offers a better model for understanding the role of antibodies in human
276 disease severity than the bovine model, since consistently protective antibody response observed in this

277 study more closely resembles that in humans[11][12][13][14][15][16][41] than the inconsistent bovine
278 neutralising antibody response.

279

280 There were some limitations to this study. It is conceivable that some associations investigated in this
281 review were masked by poor methodology or reporting in some studies. Mice are only semi permissive
282 to hRSV replication, and do not naturally experience severe respiratory disease on hRSV exposure.
283 bRSV, however, can result in fatal respiratory disease in calves and thus potentially offers a
284 representation of the pathology closer to that of humans. While mice do not appear to suffer from the
285 same negative effects of lymphopenia that humans do in the context of hRSV infection, there is some
286 shared association between more severe disease and heightened activity, particularly where there is a
287 Th2 skew in the CD4 cells. It is likely that mice do not suffer as highly in the context of lymphopenia
288 when compared to humans due to their lack of competence as a host for hRSV.

289

290 Conclusions drawn from meta-analyses in these studies may potentially have issues in translation to
291 humans or more modern methods of measuring cell and cytokine populations due to the heterogeneity
292 of many of the methods used in generating these data. This may result in a limited power to draw
293 conclusions from these data in particular where there are low numbers of studies, and a random effects
294 estimate cannot be robustly generated for meta-analyses. Additionally, many of the studies used in this
295 analysis treated mice with immunogenic compounds or cytokines such as to induce or dampen immune
296 responses in specific ways. Due to the heterogeneity of the compounds used across these studies there
297 may be confounding effects due to effects of these molecules that have been attributed to the effect of
298 RSV infection. Additionally, many human studies have shown the importance of specific lymphocyte
299 subtypes in RSV disease[45][46][38][47][48][49], that the design of the present study was not capable
300 of fully elucidating. Follow up work with a greater focus on lymphocyte subtypes would help in greater
301 elucidating the role of these cell types in model organisms.

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304 Conclusions

305 We present a systematic review and meta-analysis of the relationship between serum Ab titre or T
306 lymphocytes and disease severity in animal studies of RSV infection. Bovine models of RSV infection
307 do not show a clear association between serum Ab titres and ensuing severity of RSV disease. Studies
308 in mice suggest that low Ab concentrations are predictive of greater disease severity, but these studies
309 may be confounded and show risk of bias. The most promising biomarkers of disease severity in mice
310 are measures of lymphocyte number and differentiation, especially if measured directly at the site of
311 infection. These findings suggest that T lymphocyte numbers, activation and differentiation are possible
312 biomarkers that might be explored also in human studies. Due to differences in both pathogenesis of
313 disease, and measurements of disease between these models and humans, applicability, and relevance
314 of these biomarkers in a clinical setting is difficult to predict and requires further targeted investigations,
315 especially those focused on mucosal responses. The animal data can suggest hypotheses to be tested in
316 clinical studies, but for some weaker biomarkers more evidence from animal model studies is required.
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325 Figures and tables
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	Severity	Murine models	Citations	Bovine models	Citations
Lymphocyte responses	↑	CD4 ⁺ [4/4] CD8 ⁺ [17/21] Total T cells [9/13] Th17[3/3] γδ T-cells[2/2] Th1[5/6] Th2[5/5]	[50][51][52][26][27][31] [53][54][29][34][35][30] [32][33] [28][55][56][57] [58][59][60][61] [62]		
	No effect	Tregs [1/8] Total T cells[2/13] CD8 ⁺ [2/21]	[63][54][64][27][26]		
	↓	Tregs [7/8] Total T cells [2/13] CD8 ⁺ [2/21]	[50][51][26][27][30] [65][66][67][68]		
Antibody responses	↑	IgG [1/2] IgE [2/3]	[69][70][71]	RSV specific Ig [5/8] IgE [2/3] IgA [1/1] IgG [1/1] Maternally derived antibody [3/3]	[42][72][44] [73] [74][75][76][77] [78][79][80][81]
	No effect				
	↓	RSV specific Ig [3/3] IgG[1/2] IgE [1/3]	[65][82][52][83][84]	IgM [1/1] RSV specific Ig [3/8] IgE [1/3]	[77][85][86][72] [43]

327 Table 1- Summary of reported associations with disease severity in papers included in the systematic review.
328 Each reviewed cell type or antibody isotype is listed as contributing positively to severity in the discussed
329 animal model, with number of studies that support that relationship out of total studies with information on that
330 cell type or antibody isotype. Where different subtypes of CD4 T cells were considered independently they were
331 not counted towards the total number of CD4 studies, similarly different isotypes of RSV specific antibodies are
332 considered separately from studies that look at general specific antibodies, references to the studies data were
333 acquired from are also listed
334
335
336 Figure 1 - Predictive forest plots presenting random-effects meta-analysis with standard mean difference of
337 disease severity measures between high cell and low cell number groups in mice, in cases where data on abundance
338 of cell types is known prior to infection, such as in knockout mouse models generated with Review Manager 5.3

software. The 4 separate plots are for CD4 cells (A, $p=0.001$, $I^2=58\%$), CD8 cells (B, $p<0.00001$, $I^2=73\%$), T cells (C, $p=0.0007$, $I^2=0\%$) and Treg cells (D, $p=0.0009$, $I^2=57\%$). For panels B and D, * refers to studies using illness score as their severity measure, as opposed to weight loss in unindicated studies, and ** refers to a second experiment by Loebbermann et al, 2012[27], which uses IL-2 complex stimulation to alter cell numbers and disease severity, rather than cell deletion.

Figure 2 - Scatter graphs showing the relationship between various T lymphocytes (x-axis) and disease severity measures (y-axis, commonly weight loss but also Cdyn). Rho value was calculated for each plot. Increasing weight loss, with a smaller % of initial weight, indicates more severe disease, as does a smaller Cdyn. Plots A-D use all study groups where cell measurements are taken at the 4-8 days post infection, whilst E and F involve studies where lymphocyte measurements were taken prior to infection. Plot D refers to 12.5-15mg/ml of acetylcholine or methacholine used to challenge mice in whole body plethysmography, to calculate lung dynamic compliance (Cdyn). All studies measuring weight loss or Cdyn as an outcome measure studied mice between the age of 6-10 weeks of age. Each data point indicates one study group. Multiple groups are included from each study, points are coloured according to study.

Figure 3 - Forest plots of disease severity outcome data from calf studies that used; A: a continuous outcome measure of disease severity, such as a multifactorial scoring system, or a measure with some other non-binary outcome such as temperature; B: a dichotomous outcome measure of disease severity, where cattle were defined as severe or mild based on the development of any observable form of respiratory disease

Figure 4 - Forest plot of disease severity outcome versus neutralising antibody levels data from mouse studies that used a continuous severity outcome such as a multifactorial scoring system, or a measure with some other non-binary outcome such as temperature.

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388 **Conflicts of interest**

389 SBD acts on behalf of St George's, University of London as an Investigator on studies sponsored
390 and/or funded by vaccine manufacturers including Janssen and Medimmune. He receives no personal
391 financial benefit for this work. AJP is Chair of UK Dept. Health and Social Care's (DHSC) Joint
392 Committee on Vaccination & Immunisation (JCVI) & the European Medicines Agency (EMA)
393 scientific advisory group on vaccines and is a member of the WHO's SAGE. PJMO is the president of

394 the International RSV Society and has been a member of scientific advisory panels for Janssen (J&J),
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691 **Supplementary figures and tables**

692

Study	Variable	Primary outcome (continuous / dichotomic data)	Association (P<0.05)	Study limitations	Number of animals (cattle)	Day of Antibody measurement	Age of cattle
Gaeta 2018[1]	Specific Ig Titre	BRD	No	Dichotomous outcome	115	Time of recruitment	1-12 months
Antonis 2010[2]	Specific Ig Titre	Symptom score	Yes	Confounding (high/low Ab groups were different ages)	10	Days 0, 3,6,9, 13	0-6wks
Kalina 2006[3]	Specific Ig Titre	Symptom score	Yes	Confounding (coinfection with Alternaria)	14	Days 0 - 120	8-10wks
Bingham 2000[4]	Specific Ig Titre	BRD	Yes	Dichotomous outcome	254	Days 0, 28 post infection	N/A
	Specific Ig Titre	Weight gain	No				
Thomas 1998	Specific Ig titre	Pneumonic consolidation	Yes		21	Day 5 post infection	1-2wks
Elvander 1998[5]	Specific Ig Titre	Rectal temp	No	Confounding (coinfection with Bovine viral diarrhoea virus (BVDV))	12	Days 0 – 11 post infection	14-17wks
Gershwin 1994[6]	Specific Ig Titre	Symptom score	No	Confounding (coinfection with Micropolyspora faeni)	NA	NA	NA
Allen 1992[7]	Specific Ig Titre	BRD	No	Dichotomous outcome	136	Day 28 post infection	6-8wks
Blodorn 2015[8]	MDA titre	Symptom scores	No		6	Day 7 post infection	< 1 month

Uttenthal 2000 [9]	MDA titre	Rectal temp	No		5	Days 0 – 30 post infection	3- 12wks
Belknap 1991 [10]	MDA titre	Respiratory Rate Rectal Temp	Yes	Temperature data were not shown	17	Days 0 – 12 post infection	0-2wks
Orro 2011 [11]	Specific IgG ₁ Titre	Symptom score	No		10	Days 0 – 42	9-32 wks
Stewart 1990 [12]	Specific IgM Titre	Symptom score	No		15	Day 14 post infection	7-8wk
Kalina 2004 [13]	Specific IgE Titre	Symptom score	Yes	Correlation testing used ranked data	35	Days 7 - 52	6-8wks
Gershwin 1990 [14]	Specific IgE Titre	Symptom score	Yes		NA	NA	NA
Stewart 1989a [15]	Specific IgE Titre	Symptom score	No	Poor reporting of data	15	Day 14	N/A
Stewart 1989 [16]	Specific IgE Titre	Symptom score	Yes		6	Days 0 -14	NA

Supp Table 1 - A summary of calf studies, with details of observed correlations and study limitations. BRD denotes 'bovine respiratory disease', defined as a symptom score of 2 or more, and is the more severe outcome. MDA denotes maternally derived antibodies, Days indicates number of days post (or prior) to infection that outcome variable measurements were taken. Outcome measurements include both measurements of antibody and cell abundances as well as measurements of disease severity.

Study	Variable	Outcome	Association? (P<0.05)	Limitation	Number of mice	Days of outcome measurement	Age of mice
Chang 2004[17]	Specific Ig Titre	Weight loss Symptom scores	Yes	Confounding (IL-2 overexpression in one group)	32	Days 0, 2, 8, 45	6wks
Graham 1991[18]	Specific Ig titre	Weight loss	Yes	Confounding (B cells and therefore all antibody depleted)	44	Days 0, 4,5,7,8	4-8wks
Graham 1991a[19]	Specific Ig titre	Weight loss	No	Poor data reporting	24	Days 7, 10, 28, 42	6-8wks
Graham 1988[20]	Specific Ig titre	Weight loss	No		15	Days 1 - 11	4-32wks
Shao 2015[21]	Specific IgG titre	Lung pathology	Yes	Confounding (Anti-CD25 antibody given to one group)	10	Day 0, 4, 8, 28	6-8wks
Harker 2010[22]	Specific IgG titre	Weight loss	Yes	Confounding (IL- 18 overexpression in one group)	15	0, 2,4,6,8,12	7-8wks
Dakhama 2009[23]	Specific IgE titre	Airway resistance	Yes	Confounding (IL- 4/IL-13 knockout)	62	Day -1, 0, 2,3,6	8-10wks
Dakhama 2004[24]	Specific IgE titre	Airway resistance	Yes	Confounding (anti- IgE antibody given to one group)	114	Days 7, 14, 21, 28	8-10wks
Barends 2002[25]	Total IgE titre	Weight loss	No	Confounding (ovalbumin allergy)	24	Day 0, 4, 6	7-9wks
Matsuse 2000[26]	Total IgE titre	Lung pathology	Yes Yes	Confounding (co- infection with	24	Day 4,7,8,10,32,91	6-8wks

		Airway resistance		Dermatophagoides farinae (Df))			
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Supp Table 2 - A summary of calf studies, with details of observed correlations and study limitations. Days indicates number of days post (or prior) to infection that outcome variable measurements were taken. Outcome measurements include both measurements of antibody and cell abundances as well as measurements of disease severity.

Supplementary Figure 1 - Literature Search Flowchart, based on PRISMA methods for conducting systematic reviews for A: systematic literature search for contribution of lymphocyte and cellular responses and their relevance to animal disease severity and B: systematic literature search for contribution of immunoglobulin responses and their influence on animal disease severity

Study	Animal	Sex	Age	No. animals per group	Infection protocol and RSV strain	Main intervention/ observation	Sample	Control	Outcomes reported
Alwan 1992[27]	BM	F	3-4M	3-8	Primary, A2	T: CD4, CD8, TC	N	T: irradiated cells	WEIGHT LOSS, H, C, VL
Bukreyev 2001[28]	BM	F	11Wks	6	Primary, rRSV/mGM-CSF	N: CD4	L	rRSV, mock-infected	VL, C
Culley 2002[29]	BM	U	1D-12Wks	U	Recurrent, A2	P: CD4, CD8	L	UV-inactivated RSV	WEIGHT LOSS, VL, C, H
Dakhama 2005[30]	BM	U	0-3Wks	4-8	Recurrent, A2	N: lymphocytes	B	UV-inactivated RSV	RL, H, C, VL
Durant 2013[31]	CM	U	6-10Wks	11-13	Primary, A2	D: Treg. N: CD4, CD8	M	Uninfected	WEIGHT LOSS, H, C, VL
Fulton 2010[32]	BM	U	6-8Wks	4	Primary, A2	D: Tregs. N+P: Treg, CD8	M	Non-depleted	VL, IS, H, WEIGHT LOSS, Penh
Graham 1991[19]	BM	F	8-10Wks	24	Recurrent, A2	Lymphocyte aggregates	N	None	WEIGHT LOSS, VL, H
Harker 2007[33]	BM	F	4-8Wks	≥4	Recurrent, rRSV/IFN γ , rRSV/IL-2	P: lymphocytes. N: CD4, CD8	M	WT A2 RSV	VL, WEIGHT LOSS, H, C
Jiang 2012[34]	BM	F	8Wks	U	Primary, A2	N: lymphocytes	B	OVA/PBS only	RL, Cdyn, C, H
Jiang 2009[35]	BM	F	6-10Wks	10	Primary, A2	D: CD8. N: lymphocytes. P: TC, CD4, CD8	B	Control Ab, OVA/PBS only	RL, H, C
Krishnamoorthy 2012[36]	BM	B	3-8Wks	4-5	Recurrent, A2	N: lymphocytes, Treg. T: Treg	B	UV-inactivated RSV, uninfected	RL, C, H

Lee 2010[37]	BM	F	8Wks	5	Primary, A2	D: Treg. P: Treg. N: lymphocytes, CD4, CD8	M	PBS, control Ab	WEIGHT LOSS, VL, H, C
Loebbermann 2012[38]	BM	U	6-10Wks	4-5	Primary, A2	D: Treg. IL-2cx stimulation. N: CD4, CD8	B	Uninfected. Non-depleted	WEIGHT LOSS, H, C, VL
Munoz 1991[39]	CM	M	U	13	Primary, A2	T: CD8	N	No transfer	VL
Nagata 2015[40]	CM	F	6-10Wks	4	Primary, A	D: Treg (via SMYD3 KO). M: Th17	N	Non-depleted	C, H
Ostler 2003[41]	BM	U	6-12Wks	3-6	Primary, A2	N: CD8	B	UV-inactivated RSV	WEIGHT LOSS, C, VL
Peebles 1999[42]	BM	F	8Wks	6-10	Primary RSV+OVA, A2	N: lymphocytes	B	OVA only. RSV only	RL, H
Rutigliano 2004[43]	BM	F	8-10Wks	30	Primary, A2	D+N: CD8	L	Control Ab	WEIGHT LOSS, IS, VL, C, H
Schwarz 1999[44]	BM	F	8-12Wks	12	Primary, A	D: CD8	N	Mock-infected, no depletion	Penh, H, C
Schwarz 1999[45]	BM	F	8-12Wks	12	Primary, A	T: CD4, CD8, TC. D: CD4, CD8	N	Mock-infected, no transfer/ depletion	Penh, H, C
Shao 2015[21]	BM	U	6-8Wks	5	Primary, B1	D: Treg. P: Treg, CD8	L	Control Ab	H
Sun 2011[46]	BM	U	9-12Wks	5-10	Primary, A2	IL-10 KO, anti-IL-10R. N: CD8. P: CD4, CD8	L	WT animal, control Ab	WEIGHT LOSS, C, Cdyn, RL, VL, H
Tregoning 2010[47]	BM	F	6-10Wks	≥3	Primary, A2	Anti-CCL3 Ab and CCL3 KO. N: CD4, CD8	L	Control Ab. WT mouse	WEIGHT LOSS, VL, H, C

Tregoning 2008[48]	BM	F	4D-6Wks	≥4	Recurrent, A2	D/N/P: CD4, CD8.	L	Mock-infected	WEIGHT LOSS, C, Penh, H
Wang 2014[49]	BM	F	4Wks	20-40	Primary, A2	N: lymphocyte. P: Th17, Tregs	B	Mock-infected. OVA only	WEIGHT LOSS, VL, RL, Cdyn, H
Wang 2014[50]	BM	F	4Wks	20	Primary OVA/RSV, A2	N: lymphocytes. M: Th1, Th2, Th17	B	OVA only. RSV only	VL, RL, Cdyn, H, C,
Weiss 2011[51]	BM	F	6-8Wks	8	Primary, A2	IL-10 KO, anti-IL-10R. N: CD4, CD8	M	Control Ab. WT mouse	C, WEIGHT LOSS, VL, H, Penh
Yao 2015[52]	BM	U	8-10Wks	≥4	Primary, A2	Anti-PD-L1 Ab. N: CD4, CD8	L	PBS only, control Ab	WEIGHT LOSS, VL, C
You 2013[53]	BM	F	6-8Wks	5	Recurrent, A2	IL4Ra deletion on TCs. N: lymphocytes, CD4	B	WT animal. Mock infection	RL, C, H
Zeng 2014[54]	BM	F	8Wks	5	Primary, A2	T: γδ TC	L	PBS only. OVA only	C
Zhang 2013[55]	BM	F	6-10Wks	5	Primary, A2	T: γδ TC. N: lymphocytes, γδ TCs	M	Mock-infected, OVA	Penh, H, C
Zhang 2002[56]	BM	F	2-22M	10-12	Primary, A2	P: CD4, CD8	M	Uninfected	VL
Zhou 2008[57]	BM	F	8-12Wks	8	Primary, A2	D: nude mouse. N: lymphocytes	B	Mock-infected. WT mouse	VL, H, C

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749 Supplementary table 3 – Table of all studies used in analysis of relationship of lymphocytes with disease
750 severity in this review. Abbreviations are as follows: ANIMAL - BM (BALB/c mouse), CM (C57BL/6 mouse;
751 SEX - F (female animal), U (unknown sex), M (male), B (both male and female animals); AGE - W (weeks), M
752 (months), D (days), U (unknown age); INFECTION AND RSV STRAIN – primary (single infection), recurrent
753 (multiple infections), A/A2/ B1 (refers to the strain of virus), rRSV (recombinant RSV); MAIN
754 INTERVENTION/ OBSERVATION – N (number of cells), P (proportion of cells), D (depletion of cells), T
755 (transfer of cells), KO (knockout), TC (both CD4 and CD8 cells); SAMPLE – N (no sample), L (lung sample),
756 B (bronchoalveolar fluid sample), M (both lung and bronchoalveolar fluid samples); CONTROLS – WT (wild-
757 type), OVA (ovalbumin, used to immunologically challenge animals), PBS (phosphate-buffered saline control

758 medium); OUTCOMES REPORTED – WL (weight loss), VL (viral load), H (lung histopathology), C (cytokine
759 responses), RL (lung resistance), Cdyn (lung dynamic compliance), Penh (enhanced pause)

Supplemental methods

Search strategy: A search was conducted on 28th May 2018 using Embase, Pubmed and Zoological Record databases from January 1980 to May 2018. The search terms were ‘RSV’ or ‘Respiratory Syncytial Virus’ using filters: ‘Journals only’, ‘Animal experiments’ and ‘English language’. The inclusion criteria were applied via title and abstract screening, followed by full-text screening. Full texts of identified articles were gathered.

Selection criteria: *interventions:* human or bovine RSV infection; *comparisons:* within (versus controls) and between studies; *outcomes:* disease severity measures (any quantitative severity scoring); *studies:* controlled trials, cohort studies and case-control studies.

Data extraction: A data extraction form was created to ensure consistently extracted data from articles, including authors, year, title, objective, population, design (groups), intervention, results, interpretation, and quality metrics.

Meta-analysis: Severity outcome effect sizes were gathered from each study with sufficient data. For studies concerning Ab titre, animals in each study were grouped by Ab titre. For studies considering T lymphocytes, meta-analysis for total T-cells, CD4, CD8 and Treg cells was undertaken separately to determine the effects of high or low cell numbers on disease severity. When results were portrayed graphically, data were extracted using GetData Graph Digitiser. Forest plots were created using Cochrane’s ‘Review Manager’ Software (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). The tabled observations are independent and are self-reported correlations from the authors of the studies.

Data analysis: Where multiple independent groups from single studies were available results from both groups were presented on forest plots. Where studies reported a dichotomous outcome measure (i.e. high or low disease severity), effect sizes were calculated as odds ratios and these values are shown in a separate forest plot, as the axis uses a different scale to the effect sizes of continuous outcome.

Assessment of risk bias – Each individual study was assessed for quality of evidence using the Cochrane Library’s GRADE (Grading of Recommendations, Assessment, Development and Evaluations) risk of bias scoring system[58][59]. The assessed categories were: appropriateness of control group, presence of confounding variables, appropriateness of variable/outcome measures, and duration of follow-up.

Each study was given a score of low, moderate or high risk for each category and a stacked column graph was generated to depict the risk of bias across all studies. Scores were also considered in data analysis and interpretation where studies disagreed, since the results of lower quality studies merited less weight.

Supplementary results

Calf Studies

Fourteen of the 17 calf studies measured infant Ab and the remaining 3 examined the effect of maternal Ab (MDA) on disease severity, using colostrum feeding or deprivation. Seven studies observed a significant correlation between a given Ab titre and a severity measure. We examined each isotype independently: whereas 'bRSV-specific antibody titre' (all isotypes) was infrequently associated with severity measures (2 of 7, 29%), 'bRSV-specific IgE titre' was associated with disease severity in three out of four studies with the fourth study suffering from poor data reporting. MDA titre was associated with severity in only one of three studies, but all studies comprised only small numbers of animals. There were few studies that analysed RSV specific antibody isotypes (with the exception of IgE) in isolation (Supp table 1), and these were not found to be associated with severity by any studies (Table 1).

Mean difference analyses calves

In the studies with a continuous severity outcome, no overall association can be drawn with Ab titre, nor did any isotype variable indicate a substantial association with severity. Combining data from studies that shared outcome measures (e.g. weight loss) was not possible due to incomparable measures of Ab titre. Although most studies included a small number of animals (averaging 5 animals per group), the three experiments with dichotomous outcome measures (i.e. high or low severity) had higher sample size (>100). Those outcomes (Figure 1) showed no overall significant effect of a different Ab titre on the incidence of BRD (a severity score of 2+).

Three articles reported titres of maternally-derived Ab (MDA) in young calves in relation to RSV infection severity. These studies were conducted in colostrum-fed and colostrum-deprived calves to simulate high and low MDA titres in calves. Overall MDA did not significantly affect severity of disease in these studies (Figure 3). However, 2 out of 3 studies analysing MDA reported an association between more severe disease and lower antibody titres (table 1), with the third study showing a high variance in measured disease severities, and no statistical significance.

Summary of characteristics of studies

A variety of cell types have been studied, including total lymphocytes (n=13 articles), as well as subsets of lymphocytes, such as CD4 (n=18) and CD8 (n=22) T cells and Tregs (n=8). In these studies, many different immunological variables were measured, including cell number and/or proportion (n=30), adoptive transfer of RSV infected lymphocytes to uninfected mice (n=7), immunodeficiencies (n=10), activity of lymphocytes in response to infection (n=9) and various molecular markers (n=19). Samples were from BALF (n=12), lung tissue (n=11), both (n=6) or serum (n=1) and were collected at variable time-points post-infection. A diversity of severity measures was used in these studies, including weight loss (n=16), lung resistance (RL) (n=11), lung dynamic compliance (Cdyn) (n=4) and a composite severity score (n=2). Various model organisms were studied, including mice (n=33), guinea-pigs (n=2) and rats (n=2), with ages ranging from 6 to 10 weeks old. For our analysis, studies were included that focused on both primary and recurrent infection.

Cell number and proportion studies: 5 of 7 studies investigating T lymphocyte numbers indicated a positive correlation with severity (Table 1). Of these, 4 showed positive correlation with the duration of airway hyperreactivity (AHR) across mouse and rat models and in both primary and recurrent infection[60][42][61][30]. One article demonstrated an association between T lymphocyte numbers, illness severity and viral load[48]. Two of 7 studies of T lymphocyte numbers reported a negative correlation with RSV titre, illness severity[19], and weight loss[41](Table 1). In 6 of 7 studies that investigated CD8 cell number a positive correlation was found between CD8 cell number and various measures of disease severity, included RL, Cdyn[34], weight loss[29][33] (Table 1), airway restriction, pulmonary inflammation, and renal injury[62]. These studies included animals with both primary and secondary infection and various interventions, including recombinant RSV (rRSV)/ IFN γ [33] and anti-TNF-treatment[47]. The results of one study conflicted with the results of the other studies that used CD8 T cells as a variable, reporting that reduced RSV-specific CD8 cell numbers corresponded with lower weight loss, yet greater viral load[47]. All four studies of Treg proportions found a negative correlation with disease severity, despite no significant difference in RSV titre[38][51][40][49](Table 1).

Cell transfer studies: Studies considered for this category involved transfer of lymphocytes into mice that were infected with RSV. CD8 T cell transfer in 2 of 3 studies was associated with increased disease severity, as measured by weight loss[27] and AHR[45]; the third CD8 T cell transfer study, however, found increased lung viral clearance[39]. Both CD4 T cell transfer studies found greater weight loss[27], eosinophilia and AHR[45], and more severe immunopathology than CD8 T Cell transfer. One of the two studies of Tregs found increased cell numbers suppressed airway inflammation and accelerated recovery (based on weight loss)[38]. However, another study showed mixed results, as Treg transfer from uninfected tolerised mice suppressed inflammation, whilst transfer from infected mice exacerbated airway inflammation[36]. This suggests normal Treg cells have a protective, suppressive activity that is dysfunctional in infected mice. $\gamma\delta$ T-cell transfer was associated with increased Th2 cytokines, airway inflammation, and allergic asthma in both studies[54][55].

Cell depletion studies: In studies considered for this category the experiments involved the depletion of the capacity of the mouse to produce immune cells, often by irradiation of lymph nodes, or using immunodeficient mouse strains without the capacity to produce certain types of cells. One study of immunodeficient nude mice with depleted lymphocytes found increased viral titres and more severe histopathology compared with wild type (WT) mice[57]. Five of six Treg depletion studies found reduced Treg numbers correlated with increased weight loss, airway restriction and inflammatory responses[31][32][38][40][37]. However, reports had mixed effects on viral clearance, with evidence for unchanged[38], delayed[32][21] and enhanced[38] clearance. In three of five studies, CD8 T cell depletion was associated with increased weight loss, AHR, illness, viral load, and eosinophilia[48][45][43]. Two of five studies had mixed results, with increased AHR, but reduced airway inflammation[35] and eosinophilia[44]. CD4 T cell depletion also reduced AHR[45] and weight loss[48], and depletion of both CD4 and CD8 cells completely abrogated weight loss[48].

Cell activity: Two studies indicated positive correlation between CD8 cell responses (e.g. proliferation, cell-mediated killing and IFN γ production) and illness, but negative correlation with viral titre[43][56]. Similarly, four of six studies measuring T-cell activation exhibited an association with illness severity, weight loss, and RL[48][46], as measured by Granzyme B, IFN γ [38] and CD11a[31] markers. However, one of those studies found a correlation with increased viral load[48], whilst another found no such

association[46]. Moreover, two studies showed no association between T-cell activation and weight loss based on T-cell CD69 expression[38][37].

Cell molecular markers: All 9 studies investigating CD8 T cells found a positive correlation between IFN γ + and/or TNF α + CD8 cells and weight loss[52], airway restriction[33][48][31][38][47][37], viral titre and illness[41][43].

Downregulated Th2/Th17 (GATA3, STAT6, ROR γ T) and upregulated Th1 (T-bet) responses were associated with decreased AHR, lung inflammation and RSV replication, although this was in cell culture, not *in vivo*[50].

Supplementary discussion

It is conceivable that some associations investigated in this review were masked by poor methodology or reporting in some studies. Mice are only semi permissive to hRSV replication, and do not naturally experience severe respiratory disease on hRSV exposure. bRSV, however, can result in fatal respiratory disease in calves and thus potentially offers a representation of the pathology closer to that of humans. Another complicating factor is the time at which biomarkers were assessed in animal models in comparison to humans. For example, many of the studies of Ab in infants measured Ab soon after birth to predict RSV disease over the next few months. Ab was often tested in animal models just prior to challenge with RSV (usually no more than 24 hours prior to challenge). These differences in timing of measurement of biomarkers may significantly influence the findings from each study.

Half (9/17) of the studies analysing the role of Abs in disease severity in cattle reported a positive correlation of Abs with disease severity, while the other half support a protective role. In the mouse model, however, the evidence strongly supports neutralising Ab as a potential correlative biomarker. Most (14 of 17, 82%) calf studies had sufficient data on relevant outcomes to perform meta-analysis, of

which 11 of 14 (79%) used continuous measures of severity (Figure 1) and the other three used dichotomous measures (Figure 1).

Interestingly, the results of this study show that the different isotypes of Igs are poor biomarkers of disease severity on their own in both cattle and in mice, due to inconsistent relationships with disease severity. This is unexpected in particular for IgE, which has frequently been associated with airway hypersensitivity and Th2 responses in RSV infection[63][23]. As IgE is more often implicated in airway hypersensitivity, it is possible that the metrics of disease severity used in these studies do not capture the associated longer-term allergic symptoms. Similarly, it is not unexpected that evidence for IgM or IgA as a biomarker in either model would be conflicting or insubstantial. These isotypes have relatively poorly characterised roles in RSV disease, although they are consistently detectable after primary RSV infection in humans[64].

Measures of disease severity used in animal models are not necessarily reflective of the human experience of disease, and as such biomarkers for disease in these models do not necessarily reflect disease in other species with differing manifestations of disease. The most commonly used elements of severity scores in animal models were weight loss and histopathological features of the respiratory tract, which do not have commonly used counterpart disease severity readouts in humans. Aspects of the pathogenesis of disease also differ between models, as despite being one of the primary cell types driving severe RSV disease in humans, neutrophils may have a more protective role in mice[65]. Nevertheless, these models remain important for the improvement of our understanding of RSV disease as they allow studies to be undertaken that are unethical or impossible in humans.

Many mouse experiments used genetically altered mice infected with human RSV to assess various cell functions. Due to the regulatory role of certain cells (in particular CD4 lymphocytes) in regulating other aspects of the immune response (for example antibody development) it may be questionable to attribute the effects of these interventions to these cells alone. While the importance of these cells as regulators of RSV disease based on the evidence shown here cannot be denied, the fact that they have

regulatory roles in other processes creates difficulties in understanding which conditions created by changes to these relatively central regulators are in fact responsible for changes in disease severity.

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