Early-Life Commensal Lung Bacteria Modulate Immune Responses and Susceptibility to RSV Infection in Neonatal Mice

Quentin Marquant[§1,2,3], Claire Chottin[§1], Cécile Ferret[1,3], Eloi Poucet[1], Vinciane Saint-Criq[4], Daphné Laubreton[1], Carole Drajac[1], Edwige Bouguyon[1], Aude Remot[5], Samuel Constant[6], Song Huang[6], Edouard Sage[1,2,3], Isabelle Schwartz-Cornil[1,3], Muriel Thomas[4], Sabine Riffault[1,3], and Delphyne Descamps[¶1,3]

[§] Quentin Marquant and Claire Chottin should be considered joint first authors.
[¶]Corresponding author

[1]Université Paris-Saclay, INRAE, UVSQ, VIM, 78350, Jouy-en-Josas, France
[2]Hôpital Foch, Suresnes, France
[3]ERL U1369, INSERM, V2I, 78350, Jouy-en-Josas, France
[4]Université Paris-Saclay, INRAE, Micalis, 78350, Jouy-en-Josas, France
[5]Université de Tours, INRAE, ISP, 37380, Nouzilly, France
[6]Epithelix, 18, Chemin des Aulx, CH-1228 Plan-les-Ouates, Geneva, Switzerland

Correspondence to: Delphyne Descamps, Tel: (33) 01 34 65 26 10, Email: delphyne.descamps@inrae.fr. Virologie et Immunologie Moléculaires, INRAE, UVSQ, INSERM Domaine de Vilvert, 78350 Jouy-en-Josas, France.

Abstract

The lung mucosa at birth and shortly after is particularly vulnerable to respiratory syncytial virus (RSV) infection. Respiratory disease severity is strongly influenced by the age at first exposure, which may also affect the trajectory of airway function. Early-life represents a critical window for lung development and initial microbiota colonization, both of which shape mucosal immune responses to RSV. The impact of the initial establishment of the lung microbiota by its primo-colonizing strains on susceptibility to RSV infection in neonatal mice remains poorly described. In the present study, we showed that early-life primo-colonizing bacterial strains in the mouse lung differentially induce innate immune responses and influence RSV susceptibility in *ex vivo* models using lung explants and alveolar macrophages (AMs). We identified a specific bacterial strain (strain 17) whose prior exposure enhances type I interferon (IFN-I) responses in AMs upon RSV infection and reduces viral replication both *ex vivo* and *in vivo* in lung tissues. Intranasal administration of this strain during early life prevented the development of immunopathological responses upon RSV reinfection in adult mice. Finally, using a translational human airway epithelium model, we demonstrated that pre-exposure to strain 17 restricts RSV spread without cytotoxicity, likely *via* enhanced β-defensin 2 production. These findings highlight the potential of early-life microbiota modulation as a promising intervention for preventing RSV disease and its long-term respiratory consequences.

Keywords

Respiratory Syncytial Virus (RSV), Early-life, Lung microbiota, Primo-colonizing bacteria, Mucosal immunity, Immune imprinting**,** Interferon, Alveolar macrophages, lung explants

49 # 1 Introduction

50 The respiratory syncytial virus (RSV), known as the primary cause of bronchiolitis in infants, is a
51 major contributor to lower respiratory tract infections (LRTIs), and remains the leading cause of child
52 mortality worldwide [1]. Moreover, severe RSV infection in early-life has been associated with long-
53 term respiratory problems, such as repeated LRTI, wheezing, asthma, and reduced lung function,
54 which can continue into adulthood in the form of chronic respiratory disease [2]. New passive
55 immunisation strategies, such as maternal vaccination or administration of monoclonal antibodies,
56 have been approved to reduce the burden of acute RSV infection in high-risk infants. However, some
57 complications have been reported, including an increase in preterm births [3]. Direct vaccination in
58 children remains a challenge, particularly after the FDA recently suspended all RSV vaccine trials in
59 infants following reports of increased cases of severe illness in one trial [4]. The mechanisms
60 underlying pulmonary hypersensitivity to RSV in early-life and its long-term health effects remain to
61 be elucidated in order to develop effective prophylactic strategies. An increasing number of studies
62 on RSV-infected neonatal mice (less than 7 days old at initial infection) have validated this model as
63 relevant for understanding RSV infection in human infants [5]. It has been shown that the age at first
64 infection plays a key role in shaping subsequent immune responses, notably by inducing epigenetic
65 modifications [6]. This may increase the severity of later RSV infections [7], potentially by driving a
66 type 2-biased immunity [8], which promotes allergic responses later in life [6]. Moreover, the early-
67 life in mice is characterized by an immediate permissiveness to RSV replication, which gradually
68 decreases with age as the ability to mount type I interferon (IFN-I) responses improves [9]. IFN-I are
69 rarely detected in nasal washes from RSV-infected children [10]. However, IFN-α and IFN-β control
70 viral pathogenesis by inducing interferon-stimulated genes that provide an early antiviral defense and
71 shape inflammatory and adaptive immune responses [11]. IFN-I responses appear to play a key role
72 in the outcome of RSV infection [11], as treatments that enhance IFN-I levels in infected neonatal
73 mice have been shown to reduce viral replication and long-term RSV-induced lung effects [12, 13].
74 Thus, modulation of pulmonary innate immune defenses during the perinatal period can affect
75 susceptibility to RSV infection, and determine long-term respiratory outcomes.

76 Early-life is indeed a crucial period for lung mucosa development, during which
77 environmental factors influence the maturation of three interconnected components, namely, the
78 airway epithelium, immune cells, and the resident microbiota. When the tuning between these three
79 components is inadequate, it can ultimately affect susceptibility to infections or predispose to chronic
80 respiratory diseases [14]. There is increasing evidence that postnatal lung mucosa is biased toward a
81 type 2 immune response, as indicated by the spontaneous accumulation of type 2 innate lymphoid

82 cells (ILC2), eosinophils, and basophils, along with the production of cytokines such as IL-33 and
83 IL-13 [15], which are typically involved in anti-inflammatory responses and tissue repair. Such a
84 tissue environment is prone to the early polarization of tissue-resident alveolar macrophages (AMs)
85 toward a tolerogenic, anti-inflammatory M2 phenotype [16]. Moreover, the lung microbiota
86 establishes rapidly after birth and it shaped by factors linked to the host and the environment [17-19].
87 A recent prospective pediatric cohort study showed that between birth and 3 months of age, rapid
88 changes in the airway bacterial microbiota are accompanied by a major shift in the JAK/STAT and
89 interferon immune pathways, revealing a link between bacterial communities and early-life
90 respiratory immunity [20]. In addition, the lung microbiota contributes to immune tolerance in early-
91 life according to an asthma model [17] and attenuates innate immune reactivity to PAMPs [21].

92 The incidence of respiratory infections and the occurrence of dysbiosis in the lung microbiota
93 are closely related [22]. The severity of RSV infection, [23, 24] and the persistence of symptoms in
94 children may be influenced by the bacterial ecosystem [25]. Conversely, the presence of *Lactobacillus*
95 in the nasopharynx is associated with a reduced risk of childhood wheezing illnesses at 2 years of age
96 [26]. In mice, viable commensal bacteria are detected in the lungs as early as 3 days of age, with their
97 number and diversity increasing over time [17] and reaching a plateau by weaning at around 21 days
98 of age [19]. A collection of primo-colonizing bacteria was established *via* their isolation from lung
99 homogenates of neonatal mice using aerobic or anaerobic culture conditions [19]. Intranasal
100 administration of certain strains of this collection during early life influenced the outcome of an
101 allergic asthma model [19], while instillation of other strains in adult mice attenuated pulmonary
102 inflammation following *Mycobacterium tuberculosis* infection [27]. These findings suggest that lung
103 primo-colonizing commensal bacteria can modulate pulmonary immunity and influence
104 susceptibility to respiratory diseases. However, the mechanisms by which early bacterial colonization
105 of the lungs modulates immune responses and determines both immediate and long-term respiratory
106 outcomes, including during early-life RSV infection, remain largely unexplored [28]. In the present
107 study, we demonstrated that early-life commensal lung bacteria modulate immune responses and RSV
108 susceptibility in neonatal mice. We established a proof of concept demonstrating that it is possible to
109 select a single strain from a panel of lung primo-colonizing commensal bacteria based on its
110 immunomodulatory and antiviral properties on the neonatal lungs. One bacterial strain in particular,
111 hereafter referred to as strain 17, promoted IFN-I antiviral pathways and reduced RSV replication
112 both *ex vivo* and *in vivo* in lung tissues. Finally, its intranasal administration to neonatal mice
113 prevented early-life immunopathological priming, which became apparent after a second exposure to
114 RSV in adulthood.

115 # 2 Materials and methods

116 ## 2.1 Bacterial strains' identification and growth

117 Bacterial strains (Supplemental Table 1) previously isolated from 3-day-old mouse lungs were stored
118 at −80°C [19]. Strains were revived in 10 mL yeast-heminBHI or MRS medium and subcultured (100
119 µL/10 mL). Growth was monitored hourly for 8h by spectrophotometry to determine exponential and
120 stationary phases. Bacterial counts were obtained using a BD Accuri™ C6 flow cytometer, and strain
121 identity was confirmed by 16S PCR (J4/J7 primers) and MALDI-TOF mass spectrometry (CIRM,
122 INRAE, Nouzilly, France).

123 ## 2.2 Virus

124 Human RSV strain A2 and recombinant RSV-Long expressing mCherry (RSV-mCherry) or
125 luciferase (RSV-Luc) were produced, titrated on HEp-2 cells, and stored at -80°C [29].

126 ## 2.3 *Ex vivo* lung explants, stimulation and infection

127 Pregnant BALB/cJrj mice were purchased from Janvier (Le Genest, France). Lungs from 6-day-old
128 neonatal mice were filled *via* tracheotomy with RPMI containing 1.5% low-melting agarose (37°C).
129 After solidification, lungs were placed in a Krumdieck tissue slicer (MD6000) chamber containing
130 PBS with 1× antibiotic-antimycotic and cut into 150 µm slices to obtain precision-cut lung slices
131 (PCLS) [19, 21]. PCLS were stimulated for 16h at 37 °C, 5% CO_2 with strains (50 CFU/well in RPMI
132 with 5% FBS and L-glutamine). Inoculations were verified by CFU counts on agar plates. Three hours
133 later, PCLS were infected with RSV-Luc (7.19×10^4 PFU/well) or Mock control. Supernatants were
134 collected. PCLS were lysated in lysis buffer (5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, 1%
135 Triton X100, pH 7,4) or Passive Lysis Buffer (30 mM Tris, 10 mM $MgCl_2$, 1% Triton 100X, 2%
136 Glycerol) using a Precellys 24-bead grinder homogenizer (Bertin Technologies) for 2 × 15s at 6,000
137 x g. Protein levels were quantified with DC™ or RC-DC™ Protein Assays (Bio-Rad).

138 ## 2.4 Experiments with differentiated human primary bronchial epithelial cells

139 MucilAir® from healthy donors were purchased from Epithelix. Cells (≈5 x 10^5 cells/transwell) were
140 stimulated with bacterium 17 (10^3 CFU/transwell) in 20 µL in apical side. After 3h, cells were
141 infected with RSV-mCherry (MOI = 0.1) or Mock control during 3h. The inoculum was removed and
142 medium replaced by strain 17 containing fresh medium. RSV-mCherry replication was visualized
143 using the ZOE Fluorescent Cell Imager (Bio-Rad) and quantified with a fluorescence plate reader
144 (Tecan Infinite 200 Pro). Supernatants and cell lysates were then collected.

145 ## 2.5 *Ex vivo* AM culture and infection

146 Bronchoalveolar lavages (BALs) were performed on 6-days-old mice to isolate AM, as previously
147 described [9, 30, 31]. Cells (1 x 10^5) were plated, 24h after seeding, incubated with bacterial strains

148 (50 CFU/well) for 3h before exposure to RSV-Long (MOI = 5) or a mock control. Supernatants and
149 cells were collected 24h post-infection.

150 **2.6 Cell cytotoxicity**

151 Cytotoxicity was measured by lactate deshydrogenase (LDH) activity, using the Non-Radioactive
152 Cytotoxicity Assay kit (Promega).

153 **2.7 Cytokine, IFN, and RSV-specific Ab assays**

154 Cytokine production was measured using the 15-plex or IL-6/TNF-α/IFN-α/IFN-β custom 4-Plex
155 mouse ProcartaPlex™ immunoassay (ThermoFisher Scientific). Human IL-6, TNF-α or IP-10 were
156 measured by Luminex and human β-defensin-2 by ELISA (NB-50-1634-1000, NeoBiotech). Data
157 were read on MagPix instrument (Luminex), and analyzed using Bio-Plex Manager software (Bio-
158 Rad). HEK-Blue™ IFN-λ (InvivoGen) cells were used for the detection of bioactive human IFN-λ.
159 Individual mouse sera were assayed for specific Ab (IgG1, IgG2a) against RSV lysates by ELISA
160 [13].

161 **2.8 *In vivo* bacterial strains inoculation and RSV infection**

162 Neonate mice (age 3 and 5 days) received 10 µL of strain 17 intranasally (i.n.) route. At 6 days old,
163 mice were infected with RSV-Luc (7.187 x 10^6 PFU/mL, 10 µL, i.n) or a Mock control. In other
164 experiments, neonate mice were first infected by RSV-A2 (2.3 × 10^6 PFU/mL, 10 µL, i.n.), and were
165 kept until 8 weeks of age when they were all infected with RSV-A2 (100 µL, i.n.) [13]. BAL and lung
166 tissues were collected.

167 **2.9 Bioluminescence measurements**

168 Photon emission was measured in lysates of the nasal cavity or lung tissues and PCLS using the IVIS
169 system (Xenogen Biosciences) and Living Image software (version 4.0, Caliper Life Sciences),
170 following a previously described method [9], and was normalized to protein content.

171 **2.10 Cell infiltration**

172 Cells were collected from BALs and centrifugated at 400 x g for 10min at 4°C. May-Grunwald-
173 Giemsa coloration was performed to assess the differential cell counts using light microscopy. Two
174 hundred cells were counted to determine relative and absolute cell numbers.

175 **2.11 RT-qPCR**

176 Total RNA was extracted from lung lysates using a NucleoSpin® RNA midi column (Macherey
177 Nagel) and reverse transcribed using random primers and SuperScript II (Invitrogen), according to
178 the manufacturer's instructions. The primers (Sigma–Aldrich) used are listed (Supp. Table 2). RT-
179 qPCR was performed in duplicate for each gene using the CFX Connect™ Real-Time PCR Detection
180 System (Bio-rad) and SYBRGreen PCR Master Mix (Eurogenetec). Data were analyzed using CFX

181 Manager™ Software (Bio-rad) to determine the quantification cycle (Cq) values. Results were
182 determined using the formula $2^{-\Delta Cq}$, with $\Delta CQ = Cq_{gene} - Cq_{(mean\ (bActine, Gapdh, Hprt))}$.

183 **2.12 Data and statistical analysis**

184 Data are expressed as mean ± SEM. Statistical significance was set at $*P < 0.05$, $**P < 0.01$, $***P <$
185 0.001, and $****P < 0.0001$ using GraphPad Prism 8 (GraphPad Software Inc.). Data normality was
186 assessed using the D'Agostino–Pearson and Shapiro–Wilk tests. Depending on the distribution,
187 differences between groups were analyzed using a parametric *t*-test or a nonparametric Mann–
188 Whitney test (two groups), or by one-way ANOVA/Kruskal–Wallis or two-way ANOVA with
189 Tukey's multiple comparisons test.
190

191 # 3 Results

192 ## 3.1 Primo-colonizing bacteria exert distinct immunostimulatory effects on neonatal lung
193 mucosa *ex vivo* model.

194 The goal was to test the concept that a commensal strain can promote a protective, antiviral type 1
195 immunity in the lungs during early-life. To evaluate the immunostimulatory effects of primo-
196 colonizing bacterial strains (Supplemental Table 1) on the neonatal lung, we established a PCLS-
197 based screening assay using lung tissue from 6-day-old mice, an *ex vivo* model that recapitulates
198 pulmonary cytokine production [32]. PCLS were exposed to each strain at MOI=50 (Figure 1A).
199 Among 20 strains tested, two (strains 1 and 11) caused significant cytotoxicity after 16h and were
200 excluded, while most showed cytotoxicity levels comparable to the control medium (mean = 21.3%
201 ± 1.1%), indicating no harmful effects (Supplemental Figure S1A). Cytokines were quantified using
202 a 15-plex assay and grouped by functional category (pro-inflammatory, type 1 immunity, type 2
203 immunity, Th17/Th22/Th9-responses, and lymphocyte proliferation, Supplemental Figure S1B-F).
204 Both the nature and magnitude of cytokine production by PCLS varied across bacterial strains. Strains
205 2, 19 and 16 induced high levels of pro-inflammatory cytokines such as TNF-α and IL-6, together
206 with robust a type 2 immune response, including IL-10 (Supplemental Figures S1B and S1D). In
207 contrast, most other strains induced low cytokine production. A bacterial strain, the strain 17, stood
208 out from the others. Strain 17, identified as *Escherichia coli* by 16S sequencing and mass
209 spectrometry (Supplemental Table 1), also inducing type 2 cytokines (Supplemental Figure S1D) but
210 was unique in triggering a strong type 1 profile, characterized by a high production of IFN-γ and IL-
211 12p70 (Figure 1B), and increased IL-22 and IL-9 secretion (Supplemental Figure S1E). To highlight
212 strain-specific immune signatures, cytokine levels were expressed as fold-change relative to control
213 PCLS and visualized as heat map (Figure 1C), with hierarchical clustering revealing groups of strains
214 with similar immunostimulatory patterns. Overall, primo-colonizing lung bacteria displayed distinct
215 immunostimulatory capacities in PCLS from neonatal mice, with strain 17 standing out for its
216 strongly type 1-skewed immune profile and lack of cytotoxicity.

217 ## 3.2 Strain 17 exerts *ex vivo* antiviral activity against RSV by limiting viral replication and
218 enhancing the innate immune response.

219 We next focused on the early events following RSV infection to explore how strain 17 affects early
220 innate antiviral response. To assess whether strain 17 influences early-life RSV susceptibility, we
221 performed co-exposure experiments using PCLS from 6-day-old mice. PCLS were first exposed to
222 strain 17 (50 CFU/well as previously used [19]) to elicit its immunostimulatory effects, and then
223 infected with luciferase-expressing RSV (RSV-Luc) or mock-treated for 16h to evaluate viral

224 replication (Figure 2A). Strains 16, 24, and 25, displaying distinct cytokine profiles (see Supplemental
225 Figure S1), were included as comparators. Cytotoxicity induced by these strains, RSV-Luc, or their
226 combination did not exceed 21% and was comparable to that of the control medium. Thus, co-
227 exposure did not affect PCLS viability and supporting the safe use of these bacteria during RSV
228 infection (Figure 2B). Viral replication was quantified in PCLS lysates using a luciferase activity
229 assay (Figure 2C). PCLS exposed to strain 25 showed a slight increase in luciferase activity compared
230 to the control condition (not statistically different), suggesting a potential enhancement of RSV
231 replication associated with the presence of strain 25. Pre-exposure to strains 16 or 24 resulted in only
232 a slight reduction in RSV replication (non stastistically significant). In contrast, exposure to strain 17
233 resulted in a strong significant reduction in luciferase activity compared to both control and PCLS
234 exposed to strain 16 or 24 conditions. Altogether, these results suggest that pre-exposure to lung
235 primo-colonizing commensal bacteria can modulate RSV replication in PCLS from 6-day-old mice,
236 with strain 17 emerging as the most effective strain in reducing RSV replication.

237 We also investigated the effect of strain 17 on AMs, the main pulmonary resident immune
238 cells, involved in IFN-I production during RSV infection [33]. AMs isolated from BAL of 6-day-old
239 mice were first exposed to strain 17 (50 CFU/well as previously used [19]) or control medium,
240 followed by exposure to RSV (Figure 3A). After 16h of incubation, exposure of AMs to strain 17
241 alone induce a slight but unsignificant production of IL-6, TNF-α, and IFN-I in the supernatants
242 compared to basal control conditions (no strain, no RSV, Figures 3B-C). When AMs exposed to strain
243 17 were subsequently infected with RSV, they produced markedly more inflammatory (IL-6 and
244 TNF-α, Figure 3B) and antiviral (IFN-α and IFN-β, Figure 3C) cytokines than RSV-infected AMs
245 without bacterial pre-exposure. Notably, RSV-infected AMs produced 3-fold higher levels of IFN-I
246 when exposed to strain 17. Thus, neonatal AMs exposure to strain 17 improved their ability to mount
247 an innate antiviral immune response.

248 **3.3 Pulmonary exposure to strain 17 decreases the susceptibility of neonatal mice to RSV**
249 **infection.**

250 Collectively, these *in vitro* results identify strain 17 as non cytotoxic strain capable of enhancing IFN-
251 I production and limiting RSV replication. We therefore evaluated whether pulmonary exposure to
252 strain 17 confers protection against RSV infection in neonatal mice. To assess the *in vivo* relevance
253 of its effects, we first investigated whether intranasal (i.n.) administration of this commensal strain
254 (10^6 CFU; dose and method previously shown to deliver sufficient bacteria to the lungs [19]) was
255 non-pathogenic in neonatal mice. No mortality was observed in neonatal mice following
256 administration of strain 17 (data not shown). A modest increase in neutrophil counts was detected in
257 BALs, although total cell numbers remained unchanged (Supplemental Figure S2). Next, we

258 performed a dose-response study to determine the minimal amount of strain 17 required to achieve
259 its *in vivo* immunomodulatory effect. Neonatal mice received three different amounts of strain 17 or
260 PBS diluent intranasally at 3- and 5-days-old to modulate the neonatal lung mucosa before infection
261 with RSV-Luc at day 6 (Figure 4A). Mice were euthanized 1 or 2 d.p.i. to assess viral replication in
262 the upper and lower respiratory tracts, as well as the associated immune response in lung tissues. As
263 previously shown [9, 13], i.n. RSV-Luc infection of neonatal mice led to rapid viral replication in the
264 nasal cavity, reaching $1.42 \times 10^5 \pm 0.38 \times 10^5$ and $0.89 \times 10^5 \pm 0.28 \times 10^5$ photons/mg at 1 and 2
265 d.p.i., respectively (Figure 4B). In PBS-treated neonates, lung replication was detectable at 1 d.p.i.
266 ($1.02 \times 10^4 \pm 0.38 \times 10^4$ photons/mg) and increased by 2 d.p.i. ($2.09 \times 10^4 \pm 0.32 \times 10^4$ photons/mg),
267 confirmed by RSV *N gene* expression (Figures 4C-D). Strain 17 significantly reduced nasal viral
268 replication at 1 d.p.i. at all doses (3-4-fold, Figure 4B), whereas only the 10^6 CFU dose significantly
269 lowered lung replication ($0.098 \times 10^4 \pm 0.047 \times 10^4$ photons/mg, Figure 4C). The 10^5 CFU dose
270 showed delayed lung protection at 2 d.p.i., while the 10^3 CFU dose had no effect, indicating that upper
271 airway protection requires lower doses, whereas lung protection needs higher or delayed dosing
272 (Figures 4B-D). RSV infection in neonatal mouse lungs induced low *IFN-I* gene expression at 1 d.p.i.
273 (Figure 5A), and led to a slight, non-significant, increase in BAL cellularity between 1 and 2 d.p.i.
274 (Figure 5B), primarily due to neutrophil recruitment (Figure 5D), as previously shown [9, 12, 13]. In
275 neonates treated with high doses of strain 17, *IFN-I gene* expressions, and notably IFN-α, were
276 increased at 1 d.p.i. (Figure 5A), while total BAL cells (Figure 5B) and macrophages (Figure 5C)
277 remained unchanged. In contrast, neutrophil recruitment was significantly enhanced from 1 to 2 d.p.i.,
278 with an ~8-fold increase at 2 d.p.i. in RSV-infected mice pre-treated with the highest doses of strain
279 17 (Figure 5D). Overall, these results demonstrate that neonatal exposure to strain 17 enhances early
280 innate immune responses, leading to reduced RSV replication, dose-dependent protection of the lungs
281 and a strong protective effect in the nasal cavity.

282 **3.4 Strain 17 treatment of neonates attenuates the long-term pulmonary consequences of early-**
283 **life RSV infection upon rechallenge in adulthood.**

284 *In vitro*, bacterial strain 17 demonstrated a pronounced enhancement of type 1 immune responses.
285 Thus, we employed a previously described model of adult RSV re-exposure to investigate the ability
286 of strain 17 to promote protective immunity and counteract the immunopathological lung priming
287 induced by early-life RSV infection [8]. Three-day-old mice were treated intranasally with strain 17
288 or PBS vehicle at days 3, 5, 8, and 10 days of age. At 6-days-old, mice were infected intranasally
289 with RSV, followed by a second exposure in adulthood (Figure 6A). Weight change was monitored
290 (Figure 6B), and antibody responses (Figures 6C-D) and cell infiltration in BALs were assessed
291 (Supplemental Figures S3B-E). As expected, mice initially infected as pups and re-exposed to RSV

292 in adulthood (PBS/RSV/RSV group) lost weight for over the 14 days post-infection (d.p.i), whereas
293 mice treated with strain 17 and infected with RSV as neonates (strain 17/RSV/RSV group) lost little
294 weight and regained their initial weight by 8 d.p.i., consistently showing a better although not
295 stastically significant, maintenance of body weight compared with controls (Figure 6B). We next
296 investigated whether neonatal strain administration would impact the anti-RSV IgG1 response
297 indicative of type 2 immunity and expected for adults upon RSV reexposure [8]. As expected, the re-
298 exposure to RSV in adulhood induced a significant production of anti-RSV-IgG1 at 14 d.p.i. in the
299 PBS/RSV/RSV group (Figure 6C). In contrast, strain 17-treated neonates showed an early induction
300 of anti-RSV-IgG2a antibody production as soon as 7 d.p.i. (Figure 6D) and no significant increase in
301 IgG1 at 14 d.p.i. (Figure 6C). Finally, we monitored cellular inflammation. At 7 d.p.i., total BAL cell
302 numbers were significantly higher in mice first infected as neonates and re-exposed as adults
303 (PBS/RSV/RSV, $2.51 \times 10^5 \pm 0.38 \times 10^5$ cells) than in mice infected only as adults (PBS/Mock/RSV,
304 $1.26 \times 10^5 \pm 0.33 \times 10^5$ cells ; Supplemental Figure S3B, $p < 0.05$). BAL cell counts in strain
305 17/RSV/RSV-treated mice did not differ significantly from the PBS/RSV/RSV group (Supplemental
306 Figure S3B). Macrophage and neutrophil numbers were similar across groups at 7 and 9 d.p.i.
307 (Supplemental Figures S3C–D), while the overall leukocyte increase was mainly due to lymphocyte
308 recruitment ($0.98 \times 10^5 \pm 0.19 \times 10^5$ cells in PBS/RSV/RSV *vs.* $0.81 \times 10^5 \pm 0.24 \times 10^5$ cells in strain
309 17/RSV/RSV; Supplemental Figure S3E), which persisted until 9 d.p.i. despite declining over time.
310 Significant eosinophil recruitment was observed in the BAL of PBS/RSV/RSV mice at 7 d.p.i. (0.08
311 $\times 10^5 \pm 0.012 \times 10^5$ cells) and persisted at 9 d.p.i. ($0.065 \times 10^5 \pm 0.019 \times 10^5$ cells), but was absent in
312 PBS/Mock/RSV mice, reflecting type 2 immune priming from early-life RSV infection (Figures 6E–
313 F). Notably, eosinophil infiltration was nearly abolished in mice treated with strain 17 prior to the
314 first RSV infection ($0.006 \times 10^5 \pm 0.004 \times 10^5$ cells at 7 d.p.i.). Accordingly, the strain 17/RSV/RSV
315 group had 9.6- and 3.4-fold fewer eosinophils in the BAL compared to PBS/RSV/RSV animals at 7
316 and 9 d.p.i., respectively. Neonatal treatment with strain 17 did not affect overall leukocyte infiltration
317 upon adult RSV re-exposure but prevented immunopathologic eosinophilia. Altogether, these data
318 indicate that the early-life presence of strain 17 during the first RSV infection of mice promoted a
319 shift in the RSV-specific antibody toward a type 1 profile (higher IgG2a, lower IgG1), associated
320 with minimal eosinophil recruitment and improved disease outcomes (trend to maintain their weight).

321 **3.5 Strain 17 limits RSV spread in a translational human airway model.**

322 Finally, to validate the beneficial anti-RSV effect of strain 17 in a translational model, human primary
323 airway epithelial cells cultured at the air-liquid interface were pre-exposed to strain 17 and
324 subsequently infected with mCherry-expressing RSV to visualize cellular RSV replication (Figure
325 7A). Without bacterial pre-exposure, RSV replication was detectable at 48h post-infection, as shown

326 by mCherry fluorescence (Figure 7B). In contrast, strain 17 exposure markedly reduced mCherry
327 activity compared to the control (Figures 7B-C). No cytotoxicity was detected (Figure 7D), and
328 neither RSV exposure nor treatment with strain 17 induced the production of cytokines (IL-6, TNF-
329 α or IP-10, Supplemental Figure S4) in these experimental conditions. Type III interferons (IFN-λ)
330 are produced by airway epithelial cells infected with influenza virus earlier than IFN-I, helping to
331 restrict viral spread without provoking inflammation [34]. As expected, RSV infection induced IFN-
332 λ production in epithelial cells (Figure 7E). Strain 17 alone did not induce IFN-λ and did not enhance
333 the IFN-λ response to RSV infection; if anything, it tended to reduce secretion. Overall, the effect of
334 strain 17 is therefore unlikely to be explained by an increase in IFN-λ. We then measured another
335 antiviral factor, the antimicrobial peptide human β-defensin 2, in epithelial cells (Figure 7F). This
336 cationic molecule, secreted by lung epithelial cells, blocks RSV entry by destabilizing its envelope
337 [35]. Strain 17 strongly induced β-defensin 2 (2040 ± 571 pg/mL *vs* 65 ± 19 pg/mL in controls),
338 whereas RSV alone triggered low levels (120 ± 32 pg/mL). Thus, pre-exposure to strain 17 markedly
339 reduced RSV propagation in a human airway epithelium model without cytotoxicity, likely *via*
340 enhanced β-defensin 2 secretion.
341

342 **4 Discussion**

343 The lung microbiota and its influence on RSV infection severity have recently been identified as
344 critical research areas in the fight against bronchiolitis in young children [23, 24] [21]. While
345 modulation of the lung microbiota or metabolites appears to be a promising strategy for strengthening
346 immune defenses in adults [36, 37], the impact during the critical period of lung mucosal development
347 and susceptibility to RSV infection is yet unknown [28]. In this study, we demonstrated that the
348 immune responses of neonatal mice to RSV infection, as well as their long-term consequences, could
349 be favorably modulated by lung primo-colonizing bacterial strains given shortly after birth. In
350 particular, using complementary cellular and *in vivo* approaches, we identified a bacterial strain 17
351 (*E. coli*), that promoted protective type 1 immune responses and limited RSV replication in both
352 mouse neonatal models and human epithelial cells.

353 We employed a PCLS-based screening platform from neonatal mice to assess cytokine responses
354 to a collection of primo-colonizing bacterial strains isolated from mouse neonatal lungs. This strain
355 collection encompasses major bacterial families, ranging from *Staphylococcus* to *Lactobacillus*.
356 While most *Lactobacillus* strains exhibited low immunostimulatory activity on neonatal PCLS, the
357 majority of *Staphylococcus* strains induced a pro-inflammatory cytokine profile. In contrast, strain
358 17, identified as *Escherichia coli*, uniquely induced a strong polarized type-1 cytokine response,
359 particularly IFN-γ and IL-12p70, without causing cytotoxicity, suggesting its potential to prime
360 antiviral immunity in the neonatal lung. However, strain 17 also induced the production of IL-9, a
361 cytokine linked to asthma and detected in airway aspirates from patients with severe bronchiolitis
362 [38]. Its role in early-life pulmonary mucosa and RSV pathology remains poorly understood [39].
363 Interestingly, strain 17 also induced the secretion of IL-22, which is known to promote epithelial
364 barrier regeneration [40], and has a beneficial inhibitory effect on RSV infection has been
365 demonstrated in a neonatal mouse model [41]. Although cytokine accumulation during 24-hour
366 PCLS-based screening prevents the evaluation of secretion kinetics or autocrine effects, this approach
367 nevertheless reveals the broad potential of early-life lung primo-colonizing bacteria to induce immune
368 stimulation and polarization. Strain 17 differed markedly from the other lung primo-colonizing strains
369 tested due to its unique ability to limit RSV replication in both PCLS and a human airway epithelium
370 model, underscoring its translational relevance. It is interesting to note that strains taken individually
371 may display disease-specific immunobeneficial effects. Indeed, strain 24, previously shown to be
372 protective in a juvenile asthma model [19], had neither a protective nor a detrimental effect on RSV
373 replication in this study. These findings underscore the diverse immunomodulatory potential of
374 bacteria within the pulmonary microbiota. Functionally, the antiviral activity of strain 17 in human
375 epithelial cells appeared to be independent of IFN-λ and was associated with increased production of

376 β-defensin 2, a known host defense peptide capable of destabilizing the viral envelope, thereby
377 indirectly interfering with RSV entry and/or propagation [35]. Our study also demonstrated that strain
378 17 can prime anti-RSV immunity in neonatal AMs by enhancing their production of IFN-I. Thus,
379 strain 17 confers innate protective immunity to the lung mucosa in early-life.

380 Our *in vivo* studies confirmed the ability of strain 17 to reduce RSV replication in the upper and
381 lower respiratory tracts of neonatal mice. The degree of protection was dose-dependent and associated
382 with an induction of IFN-I expression, as well as increased neutrophil recruitment. The role of the
383 neutrophils recruited in strain 17-treated mice, with or without RSV infection, remains unclear,
384 particularly regarding their protective *versus* pathological effects in neonates [42]. Nevertheless,
385 early-life exposure to strain 17 mitigated long-term immunopathology upon re-exposure to RSV in
386 adulthood. Mice treated with strain 17 exhibited a relatively stable body weight curve with no major
387 alterations following viral reinfection, a shift toward type 1 immunity (increased IgG2a, decreased
388 IgG1), and reduced eosinophilic infiltration. These data indicate that early-life administration of strain
389 17 induces protective immunomodulation in the lung mucosa, mitigating the long-term consequences
390 of type 2 immune imprinting established by neonatal RSV infection. Although colonization was not
391 assessed, our study highlights the importance of dosing relative to the targeted respiratory region. For
392 instance, the instillation of 10^6 CFU into the lungs of a neonatal mouse represents a substantial
393 bacterial load compared to the naturally sparse neonatal lung microbiota [19]. Higher doses were
394 required to confer early protection in the lungs, whereas lower doses sufficed for the upper airways.
395 This likely reflects the larger tissue surface of the lungs that must be exposed to the bacteria, rather
396 than differences in microbiota density, which is already low in the pulmonary mucosa [43]. However,
397 before proposing this bacterial strain as a therapeutic candidate for early immunomodulation to
398 prevent acute and chronic RSV-related diseases, it is essential to elucidate the molecular pathways
399 underlying its immunostimulatory effects in both epithelial and immune cells. Future studies should
400 investigate whether the beneficial effects of strain 17 require live microorganisms, which colonize
401 tissues and are likely more effective than inert components to produce metabolites. Testing
402 conditioned supernatants could then clarify the contribution of bacterial metabolic activity, such as
403 acetate production *in vitro* (data not shown), a metabolite known to enhance antiviral responses in
404 AMs during RSV infection [44, 45]. Indeed, in an adult context, airway administration of
405 *Lactobacillus* or *Corynebacterium* strains [46, 47], or a standardized bacterial lysate derived from
406 human respiratory strains [48] is associated with improved resistance of mice to RSV challenge.
407 Morever, higher acetate levels boosts the IFN-β response of AMs and epithelial ISG expression,
408 reducing viral load and pulmonary inflammation in RSV-infected adult mice [44, 45]. It has been
409 shown that *E. coli* probiotic strains, can have both deleterious and beneficial effects on digestive

health [49]. So, before exploiting a such promising strain as a probiotic to enhance respiratory defenses, a global safety assessement is required.

5 Conclusion

Overall, this study reveals that the immune responses of neonatal mice to RSV infection, as well as their long-term consequences, can be favorably modulated by early-life lung-colonizing bacterial strains. These results reinforce the notion that the neonatal lung microbiota plays a key role in shaping pulmonary immune responses, and that its disruption, for example by antibiotics, can have profound and lasting effects on host immunity and respiratory vulnerability [50]. A recent study in pediatric RSV cohorts indicates that persistent respiratory symptoms during the convalescence phase are associated with disruptions to the respiratory microbiota [25]. It would be interesting to explore further the potential benefits of primo-colonizing strains during recovery.

Author Contibutions

Q.M. and C.C.: Methodology, Validation, Investigation, Data curation, Visualization, Writing – review & editing. C.F.: Validation, Data curation, Resources. E.P. and E.B.: Validation, Data curation. V.S-C. and D.L.: Methodology, Writing – review & editing. C.D.: Methodology, Investigation. A.R.: Methology, Conceptualization. S.C., S.H. and E.S.: Resources. I.S-C., M.T. and S.R.: Resources, Conceptualization, Writing – review & editing. D.D.: Conceptualization, Methodology, Investigation, Validation, Formal analysis, , Writing – Original Draft, Writing – review & editing, Supervision, Visualization, Project administration, Funding acquisition.

Acknowledgements

We greatly acknowledge M. Prost and A. Mialet for their assistances with experiments. We are grateful to M-A. Rameix-Welti (INSERM, U1173, UVSQ) and J-F Eléouët (INRAE, UVSQ, VIM) for providing recombinant RSV-Cherry. We are grateful to the IERP unit, INRAE (Infectiology of fishes and rodent facility, doi: 10.15454/1.5572427140471238E12). We thank the Emerg'in platform for access to IVIS-Spectrum that was financed by the Region Ile De France (DIM One Health). We thank E Helloin and C Darrigo (CIRM, INRAE, Nouzilly) for bacterial strains identification by mass spectrometry.

Fundings

C. Drajac. and Q. Marquant were the recipient of a Ph.D. and post-doctoral fellowships of Région Ile-de-France (DIM-Malinf or DIM-OneHealth, respectively). C. Chottin was the recipient of a Ph.D. fellowship from the French research ministry. D. Laubreton and experiments were supported by a grant of the French national research agency "Agence Nationale de la Recherche" (ANR-13-BSV3-0016 and ANR-24-NEOMIS). This project was supported by the Fondation Air Liquide, Vaincre la Mucoviscidose and by Association Grégory Lemarchal.

Ethics Statement

All experiments were approved by the ethics committee COMETHEA (Ethical Committee for Animal Experimentation, authorization number: APAFIS#14654-2018032116445169).

Conflict of Interest statement

The authors declare no competing financial interests. The authors declare that all the other data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon request.

Data Availability Statement

Data are available from the corresponding author upon reasonable request.

456 **References.**

457 1. Liu, L., et al., *Global, regional, and national causes of under-5 mortality in 2000-15: an*
458 *updated systematic analysis with implications for the Sustainable Development Goals.* Lancet,
459 2016. **388**(10063): p. 3027–3035.
460 2. Zar, H.J., et al., *Early-life respiratory syncytial virus disease and long-term respiratory*
461 *health.* Lancet Respir Med, 2024. **12**(10): p. 810–821.
462 3. Dieussaert, I., et al., *RSV Prefusion F Protein-Based Maternal Vaccine - Preterm Birth and*
463 *Other Outcomes.* N Engl J Med, 2024. **390**(11): p. 1009–1021.
464 4. Mahase, E., *FDA pauses all infant RSV vaccine trials after rise in severe illnesses.* BMJ,
465 2024. **387**: p. q2852.
466 5. Cormier, S.A., D. You, and S. Honnegowda, *The use of a neonatal mouse model to study*
467 *respiratory syncytial virus infections.* Expert Rev Anti Infect Ther, 2010. **8**(12): p. 1371–80.
468 6. Morris, S.B., et al., *Long-term alterations in lung epithelial cells after EL-RSV infection*
469 *exacerbate allergic responses through IL-1beta-induced pathways.* Mucosal Immunol, 2024. **17**(5):
470 p. 1072–1088.
471 7. Malinczak, C.A., et al., *Early-life pulmonary viral infection leads to long-term functional and*
472 *lower airway structural changes in the lungs.* Am J Physiol Lung Cell Mol Physiol, 2024. **326**(3): p.
473 L280–L291.
474 8. Culley, F.J., J. Pollott, and P.J. Openshaw, *Age at first viral infection determines the pattern*
475 *of T cell-mediated disease during reinfection in adulthood.* J Exp Med, 2002. **196**(10): p. 1381–6.
476 9. Laubreton, D., et al., *Regulatory B Lymphocytes Colonize the Respiratory Tract of Neonatal*
477 *Mice and Modulate Immune Responses of Alveolar Macrophages to RSV Infection in IL-10-*
478 *dependant Manner* Viruses, 2020. **12**(822).
479 10. Hall, C.B., et al., *Interferon production in children with respiratory syncytial, influenza, and*
480 *parainfluenza virus infections.* J Pediatr, 1978. **93**(1): p. 28–32.
481 11. Hijano, D.R., et al., *Role of Type I Interferon (IFN) in the Respiratory Syncytial Virus (RSV)*
482 *Immune Response and Disease Severity.* Front Immunol, 2019. **10**: p. 566.
483 12. Cormier, S.A., et al., *Limited type I interferons and plasmacytoid dendritic cells during*
484 *neonatal respiratory syncytial virus infection permit immunopathogenesis upon reinfection.* J Virol,
485 2014. **88**(16): p. 9350–60.
486 13. Remot, A., et al., *Flt3 ligand improves the innate response to respiratory syncytial virus and*
487 *limits lung disease upon RSV reexposure in neonate mice.* Eur J Immunol, 2016. **46**(4): p. 874–84.
488 14. Torow, N., et al., *Neonatal mucosal immunology.* Mucosal Immunol, 2017. **10**(1): p. 5–17.
489 15. de Kleer, I.M., et al., *Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2*
490 *Immunity in the Developing Lung.* Immunity, 2016. **45**(6): p. 1285–1298.
491 16. Saluzzo, S., et al., *First-Breath-Induced Type 2 Pathways Shape the Lung Immune*
492 *Environment.* Cell Rep, 2017. **18**(8): p. 1893–1905.
493 17. Gollwitzer, E.S., et al., *Lung microbiota promotes tolerance to allergens in neonates via PD-*
494 *L1.* Nat Med, 2014. **20**(6): p. 642–7.
495 18. Pattaroni, C., B.J. Marsland, and N.L. Harris, *Early-Life Host-Microbial Interactions and*
496 *Asthma Development: A Lifelong Impact?* Immunol Rev, 2025. **330**(1): p. e70019.
497 19. Remot, A., et al., *Bacteria isolated from lung modulate asthma susceptibility in mice.* ISME
498 J, 2017. **11**(5): p. 1061–1074.
499 20. Pattaroni, C., et al., *Bacterial communities co-develop with respiratory immunity early in life,*
500 *linking dysbiosis to systemic monocyte signature and wheezing.* Sci Adv, 2025. **11**(42): p.
501 eadw1410.
502 21. Marquant, Q., et al., *The microbiota plays a critical role in the reactivity of lung immune*
503 *components to innate ligands.* FASEB J, 2021. **35**(4): p. e21348.
504 22. de Steenhuijsen Piters, W.A.A., et al., *Early-life viral infections are associated with*
505 *disadvantageous immune and microbiota profiles and recurrent respiratory infections.* Nat
506 Microbiol, 2022. **7**(2): p. 224–237.
507 23. Schippa, S., et al., *Nasal Microbiota in RSV Bronchiolitis.* Microorganisms, 2020. **8**(5).
508 24. Yagi, K., et al., *Early-Life Lung and Gut Microbiota Development and Respiratory Syncytial*
509 *Virus Infection.* Front Immunol, 2022. **13**: p. 877771.

510 25. Kristensen, M., et al., *The respiratory microbiome is linked to the severity of RSV infections*
511 *and the persistence of symptoms in children.* Cell Rep Med, 2024. **5**(12): p. 101836.
512 26. Rosas-Salazar, C., et al., *Nasopharyngeal Lactobacillus is associated with a reduced risk of*
513 *childhood wheezing illnesses following acute respiratory syncytial virus infection in infancy.* J
514 Allergy Clin Immunol, 2018. **142**(5): p. 1447–1456 e9.
515 27. Bernard-Raichon, L., et al., *A Pulmonary Lactobacillus murinus Strain Induces Th17 and*
516 *RORgammat(+) Regulatory T Cells and Reduces Lung Inflammation in Tuberculosis.* J Immunol,
517 2021. **207**(7): p. 1857–1870.
518 28. de Steenhuijsen Piters, W.A.A., J. Binkowska, and D. Bogaert, *Early Life Microbiota and*
519 *Respiratory Tract Infections.* Cell Host Microbe, 2020. **28**(2): p. 223–232.
520 29. Rameix-Welti, M.A., et al., *Visualizing the replication of respiratory syncytial virus in cells*
521 *and in living mice.* Nat Commun, 2014. **5**: p. 5104.
522 30. Descamps, D., et al., *Toll-like receptor 5 (TLR5), IL-1beta secretion, and asparagine*
523 *endopeptidase are critical factors for alveolar macrophage phagocytosis and bacterial killing.* Proc
524 Natl Acad Sci U S A, 2012. **109**(5): p. 1619–24.
525 31. Drajac, C., et al., *Control of IFN-I responses by the aminopeptidase IRAP in neonatal*
526 *C57BL/6 alveolar macrophages during RSV infection.* Mucosal Immunol, 2021. **14**(4): p. 949–962.
527 32. Liu, G., et al., *Use of precision cut lung slices as a translational model for the study of lung*
528 *biology.* Respir Res, 2019. **20**(1): p. 162.
529 33. Makris, S., et al., *Alveolar Macrophages Can Control Respiratory Syncytial Virus Infection*
530 *in the Absence of Type I Interferons.* J Innate Immun, 2016. **8**(5): p. 452–63.
531 34. Galani, I.E., et al., *Interferon-lambda Mediates Non-redundant Front-Line Antiviral*
532 *Protection against Influenza Virus Infection without Compromising Host Fitness.* Immunity, 2017.
533 **46**(5): p. 875–890 e6.
534 35. Kota, S., et al., *Role of human beta-defensin-2 during tumor necrosis factor-alpha/NF-*
535 *kappaB-mediated innate antiviral response against human respiratory syncytial virus.* J Biol Chem,
536 2008. **283**(33): p. 22417–29.
537 36. Drajac, C., et al., *Pulmonary Susceptibility of Neonates to Respiratory Syncytial Virus*
538 *Infection: A Problem of Innate Immunity?* J Immunol Res, 2017. **2017**: p. 8734504.
539 37. Fonseca, W., N.W. Lukacs, and C. Ptaschinski, *Factors Affecting the Immunity to*
540 *Respiratory Syncytial Virus: From Epigenetics to Microbiome.* Front Immunol, 2018. **9**: p. 226.
541 38. McNamara, P.S., et al., *Interleukin 9 production in the lungs of infants with severe*
542 *respiratory syncytial virus bronchiolitis.* Lancet, 2004. **363**(9414): p. 1031–7.
543 39. Dodd, J.S., et al., *IL-9 regulates pathology during primary and memory responses to*
544 *respiratory syncytial virus infection.* J Immunol, 2009. **183**(11): p. 7006–13.
545 40. Dean, L.S., et al., *I don't know about you, but I'm feeling IL-22.* Cytokine Growth Factor
546 Rev, 2024. **80**: p. 1–11.
547 41. Das, S., et al., *Interleukin-22 Inhibits Respiratory Syncytial Virus Production by Blocking*
548 *Virus-Mediated Subversion of Cellular Autophagy.* iScience, 2020. **23**(7): p. 101256.
549 42. Tahamtan, A., et al., *Neutrophils in respiratory syncytial virus infection: From harmful*
550 *effects to therapeutic opportunities.* Br J Pharmacol, 2021. **178**(3): p. 515–530.
551 43. Ozcam, M. and S.V. Lynch, *The gut-airway microbiome axis in health and respiratory*
552 *diseases.* Nat Rev Microbiol, 2024. **22**(8): p. 492–506.
553 44. Antunes, K.H., et al., *Microbiota-derived acetate protects against respiratory syncytial virus*
554 *infection through a GPR43-type 1 interferon response.* Nat Commun, 2019. **10**(1): p. 3273.
555 45. Ji, J.J., et al., *Probiotics protect against RSV infection by modulating the microbiota-*
556 *alveolar-macrophage axis.* Acta Pharmacol Sin, 2021. **42**(10): p. 1630–1641.
557 46. Villena, J., et al., *Orally administered Lactobacillus rhamnosus modulates the respiratory*
558 *immune response triggered by the viral pathogen-associated molecular pattern poly(I:C).* BMC
559 Immunol, 2012. **13**: p. 53.
560 47. Kanmani, P., et al., *Respiratory Commensal Bacteria Corynebacterium*
561 *pseudodiphtheriticum Improves Resistance of Infant Mice to Respiratory Syncytial Virus and*
562 *Streptococcus pneumoniae Superinfection.* Front Microbiol, 2017. **8**: p. 1613.
563 48. Antunes, K.H., et al., *Airway Administration of Bacterial Lysate OM-85 Protects Mice*
564 *Against Respiratory Syncytial Virus Infection.* Front Immunol, 2022. **13**: p. 867022.

565 49. Nougayrede, J.P., et al., *A Toxic Friend: Genotoxic and Mutagenic Activity of the Probiotic*
566 *Strain Escherichia coli Nissle 1917.* mSphere, 2021. **6**(4): p. e0062421.
567 50. Perdijk, O., et al., *Antibiotic-driven dysbiosis in early life disrupts indole-3-propionic acid*
568 *production and exacerbates allergic airway inflammation in adulthood.* Immunity, 2024. **57**(8): p.
569 1939–1954 e7.

570

571

572 **Figure legends**

573 **Figure. 1:** Cytokine production by precision-cut lung slices (PCLS) from neonatal mice varies in
574 response to exposure to lung primo-colonizing bacteria. **A**. Experimental design. The lungs from 6-
575 day-old mice were isolated and cut with a Krumdieck tissue slicer to obtain PCLS. They were next
576 exposed to bacteria (50 CFU/well) or cultured in medium control. Supernatants were collected 16h
577 after stimulation with bacterial strains and PCLS were lysed. **B**. Secretion of cytokines associated
578 with type 1 immune response (IFN-β, IL-12p70). Results are expressed as mean ± SEM and represent
579 a pool of five independent experiments (n = 2-5 biological replicate samples/condition). $*P < 0.05$,
580 $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$ between the control medium and the strain-exposed
581 condition (ANOVA, Kruskal-Wallis test followed by Dunn's post-hoc test). **C**. The heat map
582 illustrates the fold-change (FC) of cytokine production induced after bacterial exposure. For each
583 strain and cytokine secretion, the FC were calculated using the following formula: FC = (average of
584 the stimulated conditions)/(average of the control conditions). FC were entered into Morpheus online
585 software (Broad Institute) to obtain the heatmaps.
586

587 **Figure. 2:** RSV replication in neonatal PCLS is differentially affected by primo-colonizing
588 commensal bacteria. **A**. Experimental design. The lungs from 6-day-old mice were isolated and cut
589 with a Krumdieck tissue slicer to obtain PCLS. They were next exposed to strains 16, 17, 24 or 25
590 (50 CFU/well) or cultured in control medium. PCLS were infected with RSV expressing luciferase
591 (RSV-Luc, 7.187 x 10^4 PFU/well) or mock control (-) 3h after bacterial stimulation. Supernatants
592 were collected 16h after bacterial stimulation and PCLS were lysed. **B**. Cytotoxicity observed in
593 PCLS was measured by LDH activity assay. Results are expressed as the mean ± SEM and represent
594 a pool of four independent experiments (n = 5-18 per group in simplicate). **C**. RSV-Luc replication
595 was assessed by measuring luciferase activity in lysates of PCLS exposed or unexposed to strains.
596 Results are expressed as mean ± SEM and represent a pool of four independent experiments (n = 6-
597 21 per group in duplicate). $*P < 0.05$ and $****P < 0.0001$ between the control medium or the strain-
598 exposed condition (ANOVA, Kruskal-Wallis test followed by Dunn's post-hoc test).
599

600 **Figure. 3:** Cytokines and type I interferon (IFN-I) production by RSV-infected alveolar macrophages
601 (AMs) from neonatal mice is enhanced in response to exposure to strain 17. **A**. Experimental design.
602 AMs isolated from BAL of neonatal mice were exposed to strain 17 (50 CFU/well) or cultured in
603 medium control. AMs were infected with rRSV-long (MOI = 5) 3h after bacterial stimulation.
604 Supernatants were collected 16h after bacterial stimulation and AMs were lysed. The production of
605 pro-inflammatory cytokines (IL-6 and TNF-α, **B**) and IFN-I (IFN-α and IFN-β, **C**) was measured in

606 supernatants using multiplex immunoassay. Data are representative of two independent experiments.
607 Means ± SEM are represented. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ (2way
608 ANOVA with Tukey's multiple comparisons test).
609
610 **Figure. 4:** Early-life airway administration of strain 17 restricts RSV replication in neonatal mice. **A**.
611 Experimental design. Neonatal mice were treated with different quantities of strain 17 (10^3, 10^5 or
612 10^6 CFU) or PBS vehicle at 3 and 5-days old (i.n., 10 µL). At 6-days old, mice were infected with
613 RSV-Luc (7.2 x 10^4 PFU, 10 µL, i.n) and were euthanized at 1 d.p.i or 2 d.p.i. to assess viral
614 replication by luciferase activity assay in the lysats of nasal cavities (**B**) and lungs (**C**). **D**. The viral
615 replication was evaluated by quantification of the RSV *N gene* expression. The level of expression
616 was calculated by the formula $2^{-\Delta Cq}$ with $\Delta CQ = Cq_{N\ gene}-Cq_{reference\ genes}$. Results are expressed as mean
617 ± SEM. Data are representative of two independent experiment (n = 5-8 per group). *$P < 0.05$, **P
618 < 0.01, ***$P < 0.001$, Mann-Whitney test.
619
620 **Figure. 5:** Early-life airway administration of strain 17 enhances neonatal pulmonary immunity
621 against RSV. Neonatal mice were treated with different quantities of strain 17 (10^3, 10^5 or 10^6 CFU)
622 or PBS vehicle at 3 and 5-days old (i.n., 10 µL). At 6 days old, mice were infected with RSV-Luc
623 (7.2 x 10^4 PFU, 10 µL, i.n) and were euthanized at 1 or 2 d.p.i. **A**. *IFN-I gene* expression was
624 measured by qRT-PCR in lung lysates at 1 d.p.i.. Gene expression levels were calculated using the 2^{-}
625 $^{\Delta Cq}$ method, with Cq values normalized to housekeeping genes. **B**. Cell counts were performed on
626 BAL samples at 1 or 2 d.p.i.. Cell infiltration in BALs was assessed by May-Grünwald-Giemsa
627 staining to identify and differentiate macrophages (**C**) and polynuclear neutrophils (**D**) at 1 or 2 d.p.i..
628 Results are expressed as mean ± SEM. Data are representative of two independent experiments (n =
629 5-8 per group). *$P < 0.05$, **$P < 0.01$, Mann-Whitney test.
630
631 **Figure. 6 :** Early-life airway administration of strain 17 attenuates the long-term pulmonary
632 consequences of neonatal RSV infection during reinfection in adulthood. **A**. Experimental design.
633 Three-day-old mice were treated intranasally with bacterium 17 (10^6 CFU, i.n., 10 µL) or PBS vehicle
634 at days 3, 5, 8, and 10 days of age. At 6 days old, mice were infected intranasally with RSV-A2 (2.3
635 × 10^4 PFU in 10 µL), followed by a second infection with RSV-A2 (2.3 × 10^5 PFU in 100 µL, i.n.) at
636 8 weeks of age. Mice were euthanized at 7, 9 or 14 days post-reinfection (d.p.i.) to assess cellular
637 infiltration in the BALs and blood was collected. **B**. Weight evolution after the second infection.
638 Results are expressed as mean ± SEM and presented as a percentage of the starting weight (n = 4 per
639 group). **C-D**. Anti-RSV antibody assays. Serum was used to quantify anti-RSV IgG1 (**C**) or IgG2a

640 (**D**) antibodies by ELISA. Results are expressed as the mean ± SEM (n = 4-5 per group). *$P < 0.05$,
641 **$P < 0.01$, Mann-Whitney test. **E-F**. The infiltration of eosinophils in BALs was assessed by May-
642 Grünwald-Giemsa staining at 7 or 9 d.p.i.. Results are expressed as mean ± SEM. Data are
643 representative of two independent experiments (n = 5-9 per group). *$P < 0.05$, **$P < 0.01$, Mann-
644 Whitney test.
645
646 **Figure. 7:** RSV replication in human primary airway epithelial cells is reduced by the pre-exposure
647 to strain 17. **A**. Experimental design. Human primary bronchial epithelial cells cultured at the air
648 liquid interface (MucilAir™) were first exposed to strain 17 (10^3 CFU/well) for 3h, followed by RSV
649 expressing mCherry (RSV-mCherry, MOI = 0.1) infection or an incubation with mock for a further
650 3h. Supernatants and cell lysates were collected 48h after incubation. RSV replication was evaluated
651 by detecting mCherry using microscopy (**B**, three images representative of six inserts) or a
652 fluorescence plate reader (**C**). Results are expressed as the mean fluorescence intensity (MFI)
653 observed in lysats of RSV-mCherry-infected cells, after subtraction of the background MFI from
654 mock-infected controls (± SEM), and represent a pool of two independent experiments (n = 3 inserts
655 per group per experiment, **$P < 0.01$, Mann-Whitney test). **D**. Cytotoxicity observed in MucilAir™
656 was measured by LDH activity assay. Results are expressed as mean ± SEM. **E**. Bioactive IFN-λ was
657 evaluated by measuring the response of HEK-Blue™ IFN-λ cells to the supernatants. SEAP activity
658 was assessed using QUANTI-Blue™ Solution. The optical density (OD) at 630 nm is shown as mean
659 ± SEM. **F**. β-defensin 2 secretion in supernatants was evaluated by ELISA. **E-F**. Data represent a
660 pool of two independent experiments (n = 9 inserts per group, ***$P < 0.001$, ****$P < 0.0001$, 2way
661 ANOVA with Tukey's multiple comparisons test).
662

Early-Life Commensal Lung Bacteria Modulate Immune Responses and Susceptibility ito RSV Infection in Neonatal Mice

Figures

Figure 1

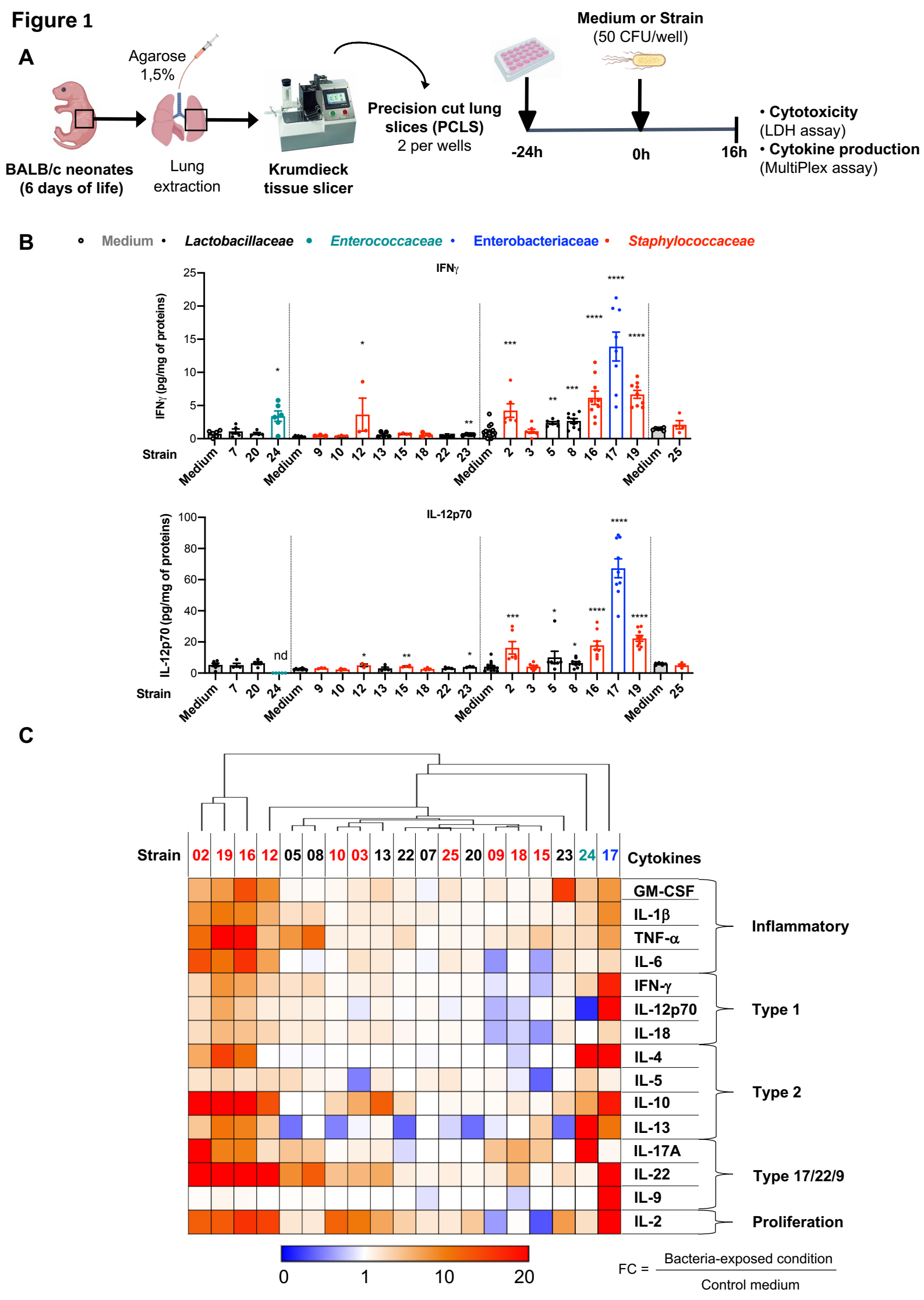

A
Agarose 1,5%
BALB/c neonates (6 days of life)
Lung extraction
Krumdieck tissue slicer
Precision cut lung slices (PCLS)
2 per wells
Medium or Strain
(50 CFU/well)
-24h
0h
16h
• Cytotoxicity
(LDH assay)
• Cytokine production
(MultiPlex assay)
B
Medium
Lactobacillaceae
Enterococcaceae
Enterobacteriaceae
Staphylococcaceae
IFNγ
IFNγ (pg/mg of proteins)
IL-12p70
IL-12p70 (pg/mg of proteins)
nd
Strain
Medium 7 20 24 Medium 9 10 12 13 15 18 22 23 Medium 2 3 5 8 16 17 19 Medium 25
C
Strain
02 19 16 12 05 08 10 03 13 22 07 25 20 09 18 15 23 24 17
Cytokines
GM-CSF
IL-1β
TNF-α
IL-6
IFN-γ
IL-12p70
IL-18
IL-4
IL-5
IL-10
IL-13
IL-17A
IL-22
IL-9
IL-2
Inflammatory
Type 1
Type 2
Type 17/22/9
Proliferation
0 1 10 20
FC = Bacteria-exposed condition / Control medium

Figure 2

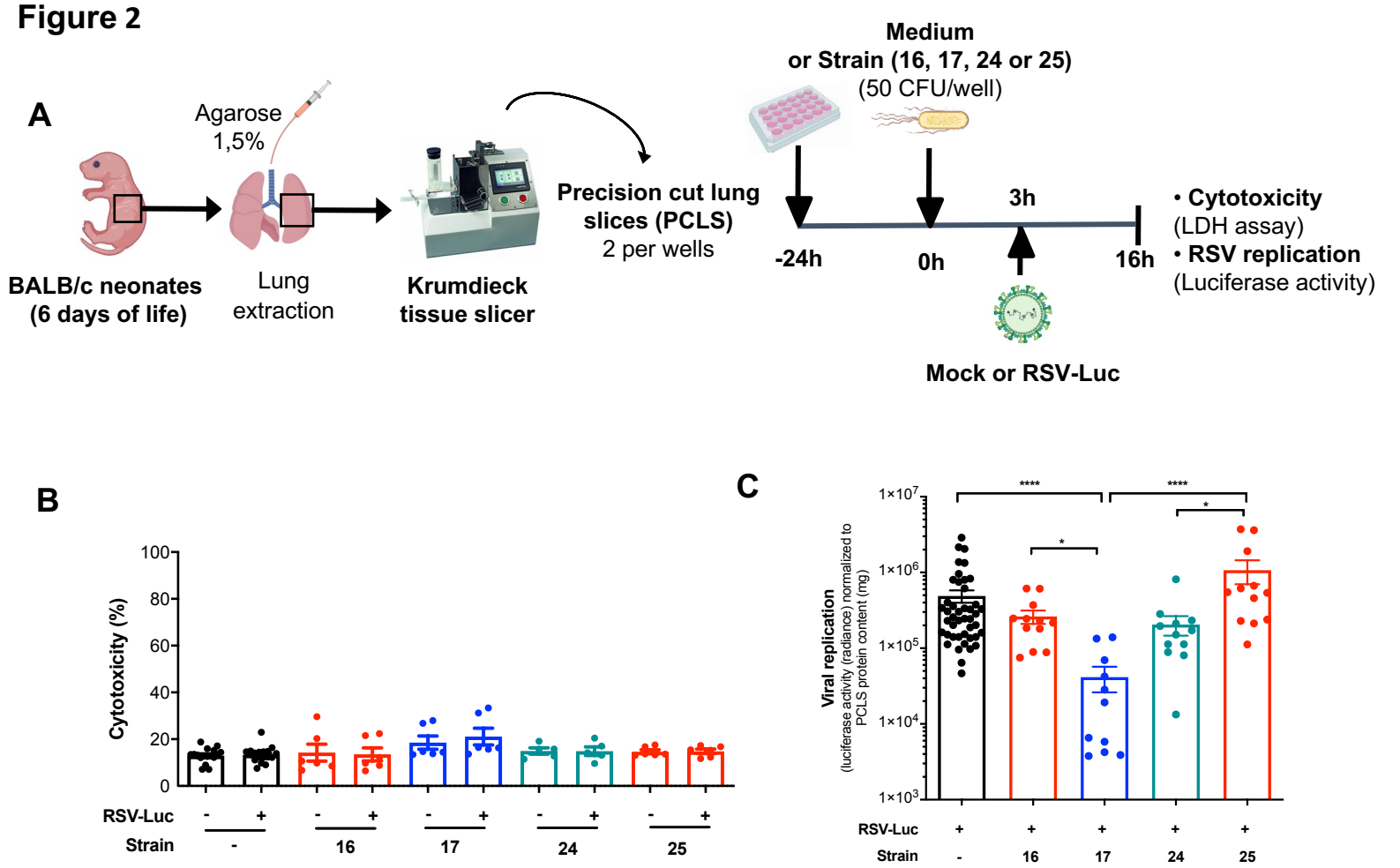

A
Agarose
1,5%
BALB/c neonates
(6 days of life)
Lung
extraction
Krumdieck
tissue slicer
Precision cut lung
slices (PCLS)
2 per wells
Medium
or Strain (16, 17, 24 or 25)
(50 CFU/well)
-24h
0h
3h
16h
Mock or RSV-Luc
• Cytotoxicity
(LDH assay)
• RSV replication
(Luciferase activity)
B
Cytotoxicity (%)
100
80
60
40
20
0
RSV-Luc
Strain
-
16
17
24
25
C
Viral replication
(luciferase activity (radiance) normalized to
PCLS protein content (mg)
1×10^7
1×10^6
1×10^5
1×10^4
1×10^3
****
*
RSV-Luc
Strain

Figure 3

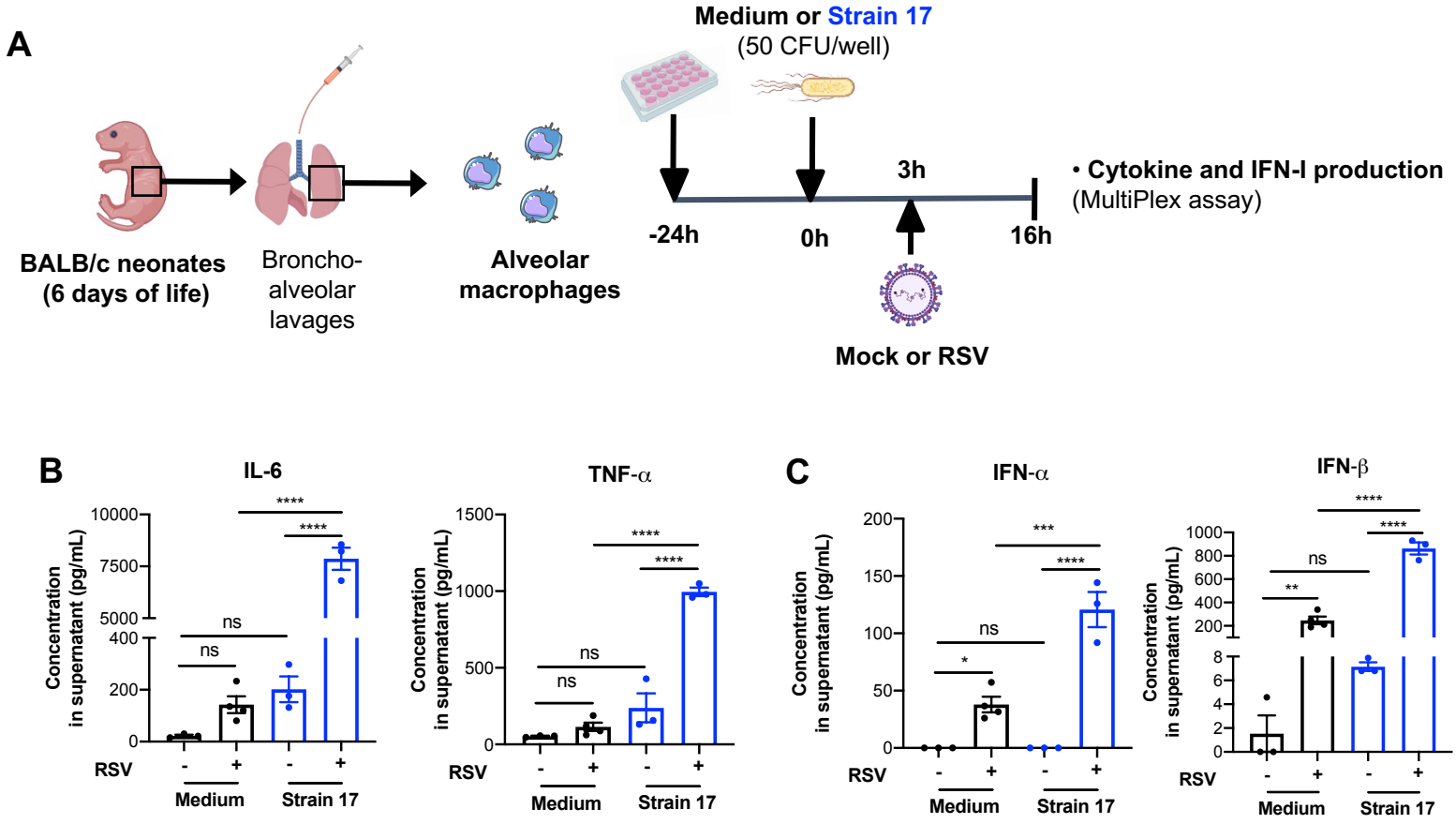

A
BALB/c neonates
(6 days of life)
Broncho-
alveolar
lavages
Alveolar
macrophages
Medium or Strain 17
(50 CFU/well)
-24h
0h
3h
16h
Mock or RSV
• Cytokine and IFN-I production
(MultiPlex assay)
B
IL-6
TNF-α
C
IFN-α
IFN-β
Concentration
in supernatant (pg/mL)
RSV
Medium
Strain 17
ns
****
***
*
**

Figure 4

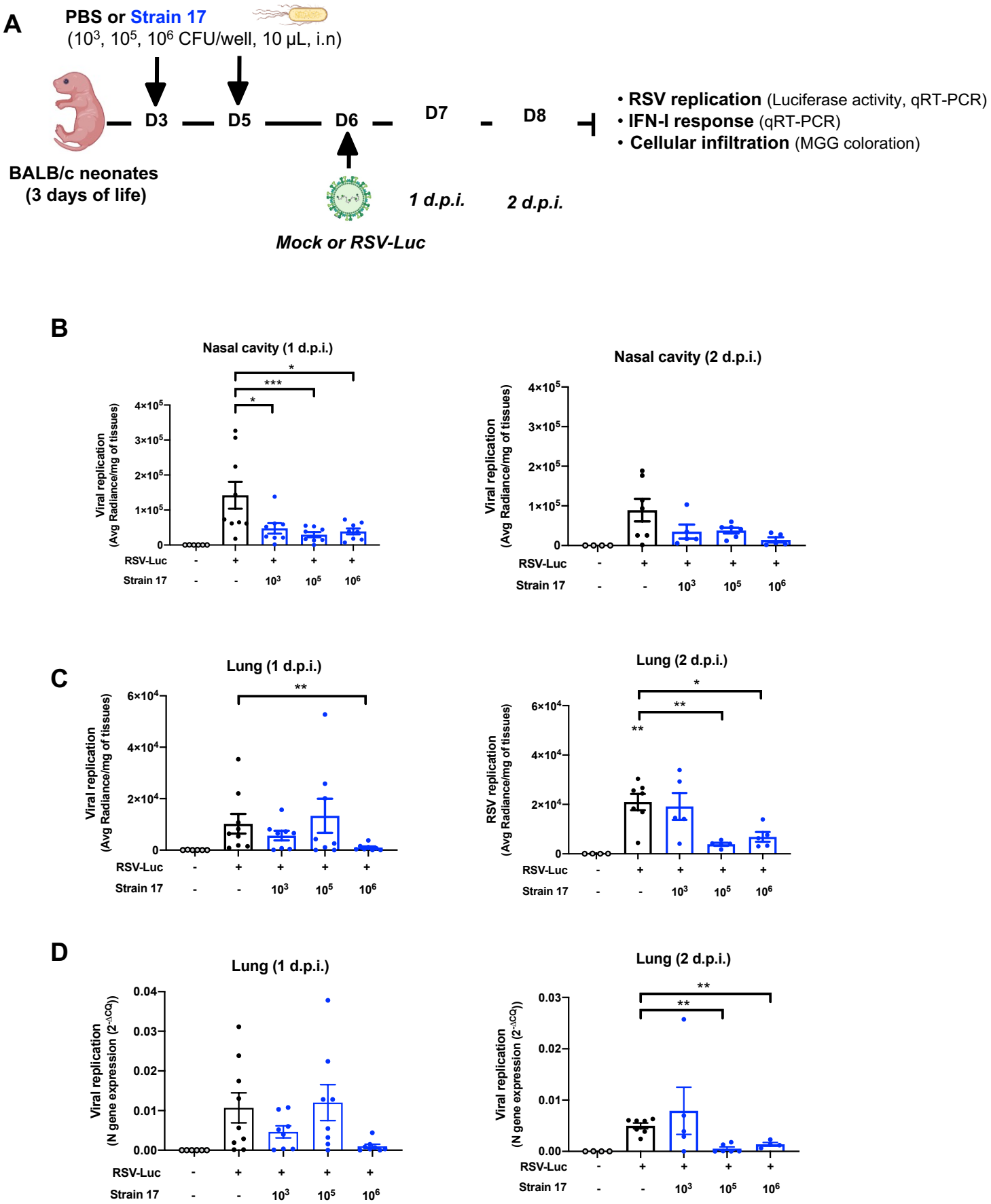

A
PBS or Strain 17
(10³, 10⁵, 10⁶ CFU/well, 10 µL, i.n)
D3
D5
D6
D7
D8
BALB/c neonates
(3 days of life)
1 d.p.i.
2 d.p.i.
Mock or RSV-Luc
• RSV replication (Luciferase activity, qRT-PCR)
• IFN-I response (qRT-PCR)
• Cellular infiltration (MGG coloration)
B
Nasal cavity (1 d.p.i.)
Nasal cavity (2 d.p.i.)
Viral replication
(Avg Radiance/mg of tissues)
RSV-Luc
Strain 17
C
Lung (1 d.p.i.)
Lung (2 d.p.i.)
RSV replication
(Avg Radiance/mg of tissues)
D
Viral replication
(N gene expression (2^-ΔCQ))

Figure 5

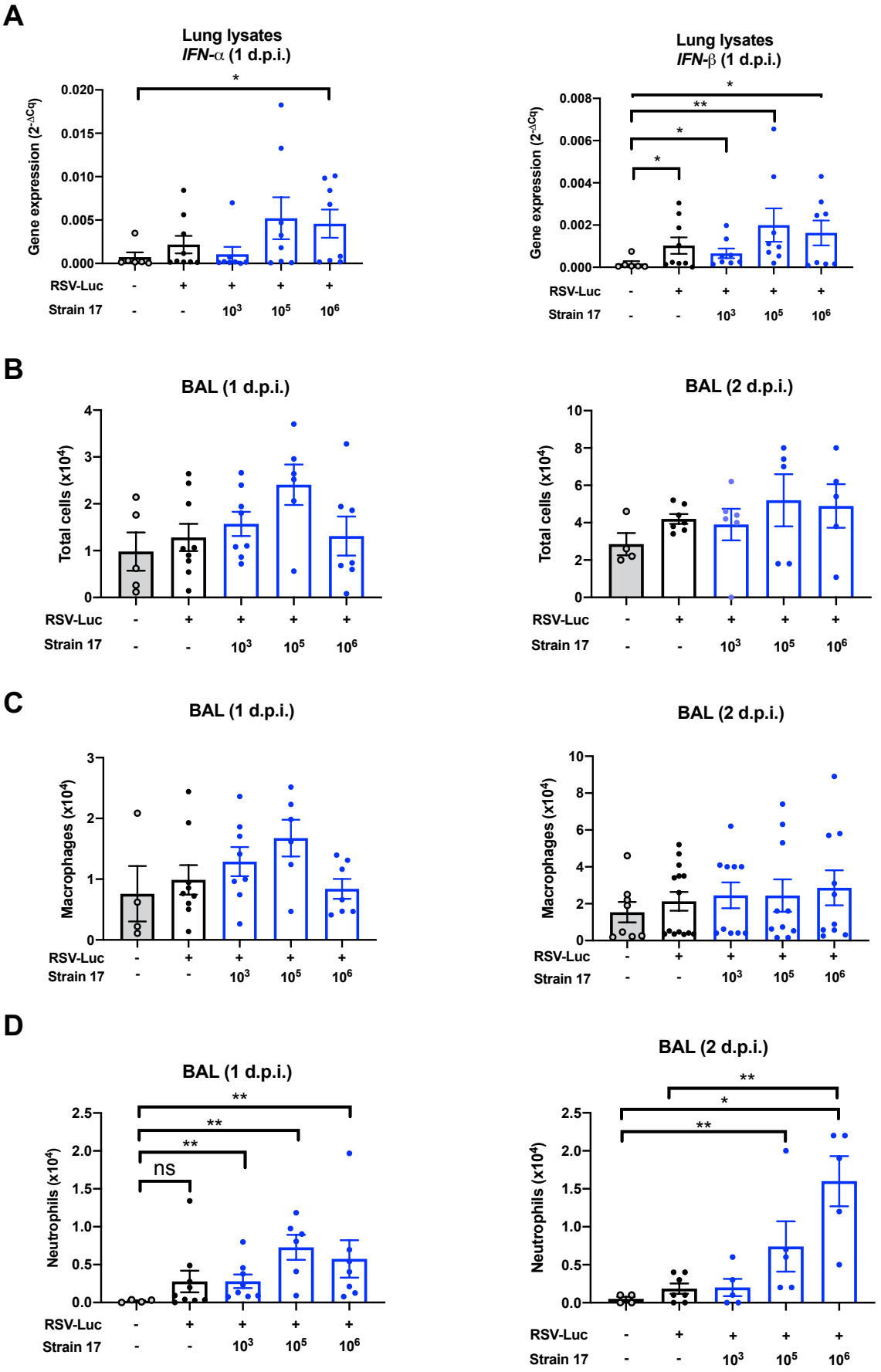

A
Lung lysates
IFN-α (1 d.p.i.)
Gene expression (2^-ΔCq)
0.020
0.015
0.010
0.005
0.000
*
RSV-Luc - + + + +
Strain 17 - - 10^3 10^5 10^6
Lung lysates
IFN-β (1 d.p.i.)
Gene expression (2^-ΔCq)
0.008
0.006
0.004
0.002
0.000
*
**
*
*
RSV-Luc - + + + +
Strain 17 - - 10^3 10^5 10^6
B
BAL (1 d.p.i.)
Total cells (x10^4)
4
3
2
1
0
RSV-Luc - + + + +
Strain 17 - - 10^3 10^5 10^6
BAL (2 d.p.i.)
Total cells (x10^4)
10
8
6
4
2
0
RSV-Luc - + + + +
Strain 17 - - 10^3 10^5 10^6
C
BAL (1 d.p.i.)
Macrophages (x10^4)
3
2
1
0
RSV-Luc - + + + +
Strain 17 - - 10^3 10^5 10^6
BAL (2 d.p.i.)
Macrophages (x10^4)
10
8
6
4
2
0
RSV-Luc - + + + +
Strain 17 - - 10^3 10^5 10^6
D
BAL (1 d.p.i.)
Neutrophils (x10^4)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
ns
RSV-Luc - + + + +
Strain 17 - - 10^3 10^5 10^6
BAL (2 d.p.i.)
Neutrophils (x10^4)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
**
RSV-Luc - + + + +
Strain 17 - - 10^3 10^5 10^6

Figure 6

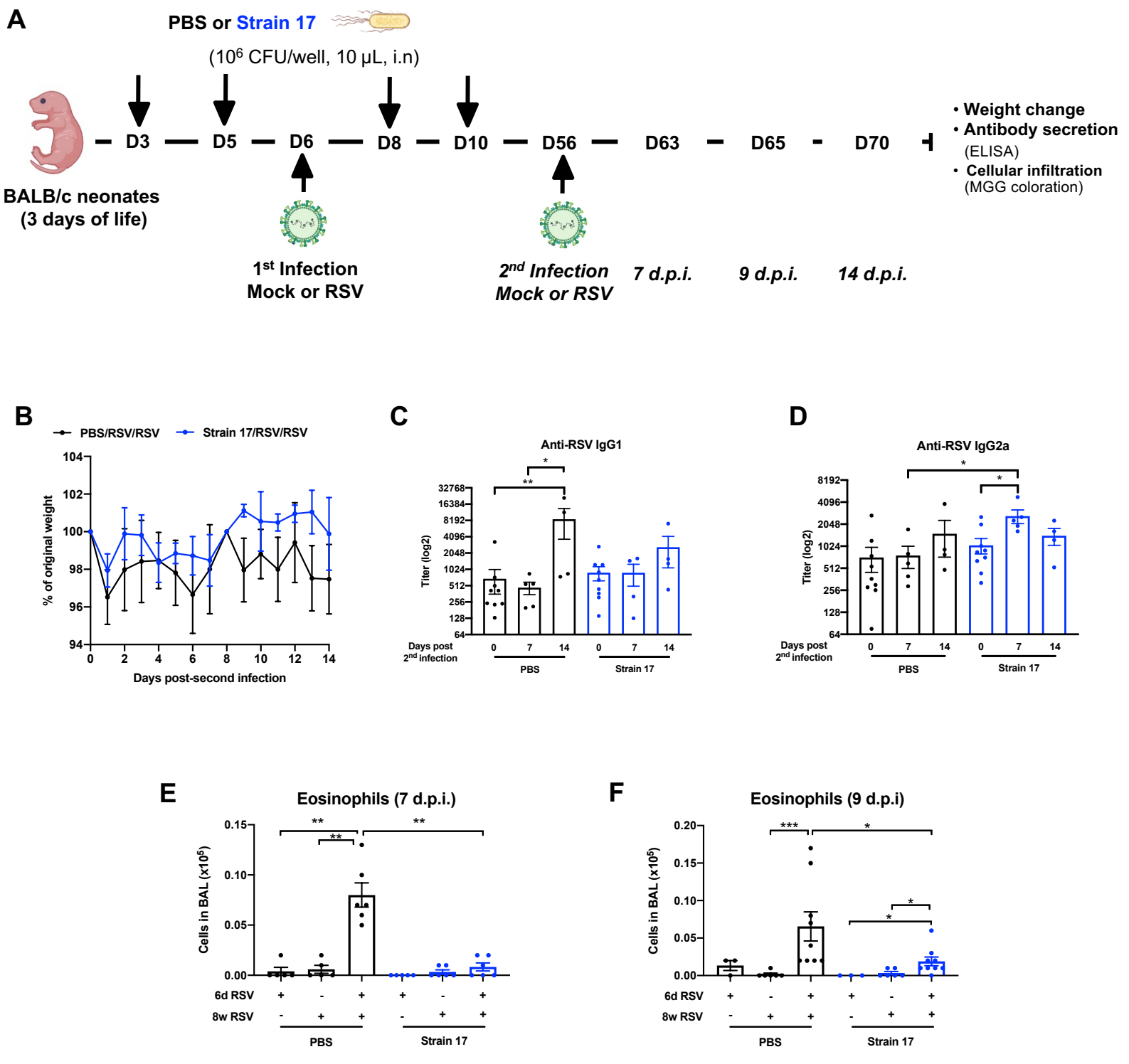
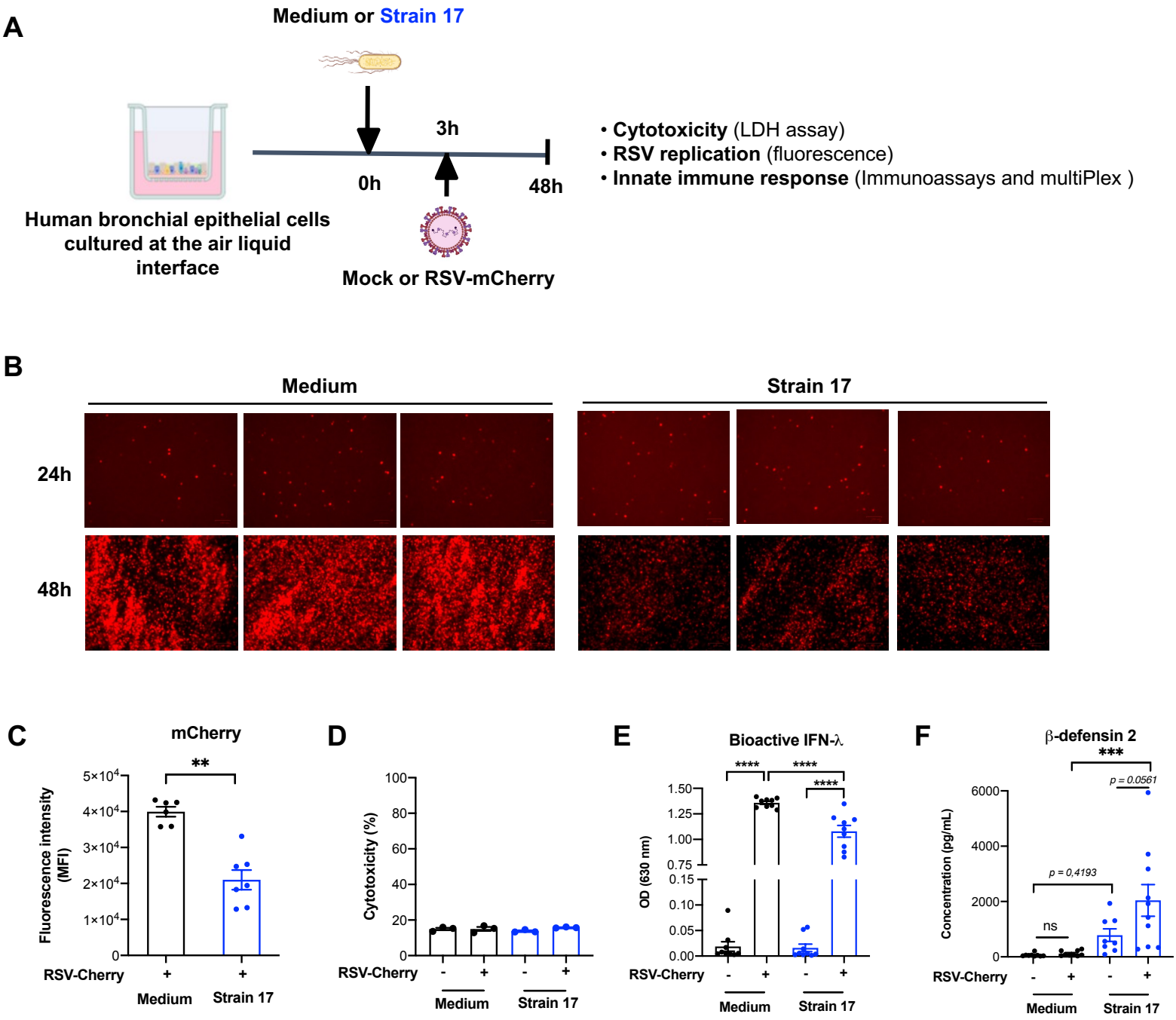

A
PBS or Strain 17
(10^6 CFU/well, 10 µL, i.n)
BALB/c neonates
(3 days of life)
D3
D5
D6
D8
D10
D56
D63
D65
D70
• Weight change
• Antibody secretion (ELISA)
• Cellular infiltration (MGG coloration)
1st Infection
Mock or RSV
2nd Infection
Mock or RSV
7 d.p.i.
9 d.p.i.
14 d.p.i.
B
PBS/RSV/RSV
Strain 17/RSV/RSV
% of original weight
Days post-second infection
C
Anti-RSV IgG1
Titer (log2)
Days post 2nd infection
PBS
Strain 17
D
Anti-RSV IgG2a
Titer (log2)
Days post 2nd infection
PBS
Strain 17
E
Eosinophils (7 d.p.i.)
Cells in BAL (x10^5)
6d RSV
8w RSV
PBS
Strain 17
F
Eosinophils (9 d.p.i)
Cells in BAL (x10^5)
6d RSV
8w RSV
PBS
Strain 17

Figure 7


A
Medium or Strain 17
3h
0h
48h
Human bronchial epithelial cells cultured at the air liquid interface
Mock or RSV-mCherry
• Cytotoxicity (LDH assay)
• RSV replication (fluorescence)
• Innate immune response (Immunoassays and multiPlex)
B
Medium
Strain 17
24h
48h
C
mCherry
Fluorescence intensity (MFI)
5×10⁴
4×10⁴
3×10⁴
2×10⁴
1×10⁴
0
**
RSV-Cherry
+
+
Medium
Strain 17
D
Cytotoxicity (%)
100
80
60
40
20
0
RSV-Cherry
-
+
-
+
Medium
Strain 17
E
Bioactive IFN-λ
OD (630 nm)
1.50
1.25
1.00
0.75
0.15
0.10
0.05
0.00
****
****
****
RSV-Cherry
-
+
-
+
Medium
Strain 17
F
β-defensin 2
Concentration (pg/mL)
6000
4000
2000
0
***
p = 0.0561
p = 0,4193
ns
RSV-Cherry
-
+
-
+
Medium
Strain 17