

1 **Magnitude and kinetics of T cell and antibody responses during**
2 **H1N1pdm09 infection in outbred and inbred Babraham pigs**
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27 **Abstract**

28 We have used the pig, a large natural host animal for influenza with many physiological
29 similarities to humans, to characterize $\alpha\beta$, $\gamma\delta$ T cell and antibody (Ab) immune responses to
30 the 2009 pandemic H1N1 virus infection. We evaluated the kinetic of virus infection and
31 associated response in inbred Babraham pigs with identical MHC (Swine Leucocyte Antigen)
32 and compared them to commercial outbred animals. High level of nasal virus shedding
33 continued up to day 4-5 post infection followed by a steep decline and clearance of virus by
34 day 9. Adaptive T cell and Ab responses were detectable from day 5-6 post infection reaching
35 a peak at 9-14 days. $\gamma\delta$ cells produced cytokines *ex vivo* at day 2 post infection, while virus
36 specific IFN γ producing $\gamma\delta$ T cells were detected from day 7 post infection. Analysis of NP
37 tetramer specific and virus specific CD8 and CD4 T cells in blood, lung, lung draining lymph
38 nodes and broncho-alveolar lavage (BAL) showed clear differences in cytokine production
39 between these tissues. BAL contained the most highly activated CD8, CD4 and $\gamma\delta$ cells
40 producing large amounts of cytokines, which likely contribute to elimination of virus. The weak
41 response in blood did not reflect the powerful local lung immune responses. The immune
42 response in the Babraham pig following H1N1pdm09 influenza infection was comparable to
43 that of outbred animals. The ability to utilize these two swine models together will provide
44 unparalleled power to analyse immune responses to influenza.

45 **Introduction**

46 Influenza viruses are a global health threat to humans and pigs, causing considerable
47 morbidity and mortality. Frequent zoonotic crossover between pigs and humans contributes
48 to the evolution of influenza viruses and can be a source for novel pandemic strains (Nelson
49 and Vincent, 2015; Kaplan et al., 2020; Sun et al., 2020). The emergence of the 2009
50 pandemic H1N1 (H1N1pdm09) virus, which is now globally endemic in both pigs and humans,
51 illustrates the importance of pigs in new outbreaks in humans (Smith et al., 2009). Influenza A
52 virus (IAV) infection in pigs causes significant economic loss due to reduced weight gain,
53 suboptimal reproductive performance and secondary infections. Immunization with inactivated
54 influenza virus is currently the most effective way of inducing strain-specific neutralizing
55 antibodies, directed against the surface glycoprotein haemagglutinin (HA). Because of the
56 constant evolution of the virus, broadly cross-protective vaccines would be highly desirable
57 and central to the control of influenza in both pigs and humans.

58 Animal models are essential to develop better vaccines and control strategies and to
59 provide insight into human disease. Most models have limitations in recapitulating the full
60 range of disease observed in humans. Mice, guinea pigs and non-human primates are not
61 generally susceptible to natural routes of influenza infection and may require adapted strains,
62 physiologic stressors and/or unnatural inoculation procedures (Bouvier and Lowen, 2010;
63 Margine and Krammer, 2014; Hemmink et al., 2018; Mifsud et al., 2018). In contrast, pigs are
64 an important, natural, large animal host for IAV and are infected by the same subtypes of
65 H1N1 and H3N2 viruses as humans (Watson et al., 2015; Lewis et al., 2016). Pigs have a
66 longer life span, are genetically, immunologically, physiologically and anatomically more like
67 humans than small laboratory animals and have a comparable distribution of sialic acid
68 receptors in the respiratory tract (Janke, 2014; Rajao and Vincent, 2015). Pigs exhibit similar
69 clinical manifestations and pathogenesis when infected with IAV making them an excellent
70 model to study immunity to influenza. Furthermore, we have defined the dynamics of
71 H1N1pdm09 influenza virus transmission in pigs and demonstrated the utility of the pig model
72 to test therapeutic antibody delivery platforms and vaccines (Canini et al., 2020; McNee et al.,
73 2020).

74 Several inbred miniature pig breeds have been developed, including NIH and Yucatan,
75 with defined swine leukocyte antigens (SLA type, the swine major histocompatibility complex)
76 (Sachs et al., 1976; Choi et al., 2016). However, the inbred Babraham is the only example of
77 a full-size inbred strain of pig, closely related to commercial breeds, making them an
78 appropriate model to study diseases important to commercial pig production (Signer et al.,
79 1999; Schwartz et al., 2018). The sharing of IAV strains between pigs and humans makes it
80 an obvious species in which to study immunity to influenza and to test vaccines or therapeutic

81 strategies prior to human clinical trials. In addition we have developed a toolset to study
82 immune responses in Babraham, including adoptive cell transfer and peptide SLA tetramers
83 allowing us to study the fine specificity of immune responses (Binns et al., 1981; Tungatt et
84 al., 2018).

85 Despite extensive knowledge of the role of T cells in protection against IAV in mice
86 and humans, few studies in pigs have evaluated this in depth. The duration and magnitude of
87 T cell and humoral responses has been assessed after swine H1N1, H1N2 and H3N2
88 challenges in pigs (Heinen et al., 2000; Larsen et al., 2000; Khatri et al., 2010; Talker et al.,
89 2015; Talker et al., 2016). The frequency and activation status of leucocytes in local and
90 systemic tissues was also determined after H1N1pdm09 infection (Schwaiger et al., 2019).
91 However no detailed analysis of T cell immune responses in broncho-alveolar lavage (BAL)
92 have been performed, a location which we have shown to contain tissue resident memory
93 cells that are essential for heterosubtypic protection (Holzer et al., 2018a). Neither has there
94 been a detailed analysis of T cell and antibody (Ab) immune responses to H1N1pdm09,
95 although this continues to cross the species barrier from humans to pigs. H1N1pdm09
96 circulating in swine herds maintains antigenic similarity to human seasonal strains, providing
97 a unique opportunity to use a virus affecting both humans and swine to examine immune
98 responses induced by infection.

99 Here we characterized $\alpha\beta$, $\gamma\delta$ T cell and Ab immune responses to H1N1pdm09 in local
100 lung and systemic tissues in Babraham pigs and compared them to commercial outbred
101 animals. These two pig models together will allow fine grain dissection of immune responses
102 to IAV in a species which is a natural host for the virus and similar in many respects to humans.
103

104 **Materials and methods**

105 **Animals and influenza H1N1pdm09 challenge.** The animal experiments were approved by
106 the ethical review processes at the Pirbright Institute and Bristol and conducted according to
107 the UK Government Animal (Scientific Procedures) Act 1986 under project licence
108 P47CE0FF2. Both Institutes conform to the ARRIVE guidelines.

109 Thirty two outbred old Landrace x Hampshire cross (from a commercial high health
110 status herd) and 56 inbred Babraham pigs (bred at Animal Plant Health Agency, APHA
111 Weybridge, UK) were screened for absence of influenza A infection by matrix gene real time
112 RT-PCR and for antibody-free status by HAI using four swine influenza virus antigens -
113 H1N1pdm09, H1N2, H3N2 and avian-like H1N1. The average age of the outbred pigs 7 days
114 before the challenge was 8.7 weeks and of the Babraham 8.3 weeks. Pigs were challenged
115 intra-nasally with 1×10^7 PFU of MDCK grown swine A(H1N1)pdm09 isolate,
116 A/swine/England/1353/2009, derived from the 2009 pandemic virus, swine clade 1A.3.

117 (H1N1pdm09) in a total of 4 ml (2 ml per nostril) using a mucosal atomisation device MAD300
118 (MAD, Wolfe-Tory Medical). Two experiments with outbred (OB) pigs (referred to as OB1 and
119 OB2) and two with inbred Babraham (BM) pigs (referred to as BM1 and BM2) were performed
120 (**Fig. 1A**). In each experiment one pig was culled on days 1 to 7, 9, 11 and 13 post infection
121 and a *post-mortem* examination performed with collection of tissue samples. Uninfected
122 controls were sampled: two on the day prior to infection and two at day 8 post infection. Two
123 naïve pigs (referred to as in-contact) were co-housed with the directly challenged pigs in
124 experiments OB1, OB2, BM1, BM2 and culled at days 11 and 13 post infection together with
125 the last two directly challenged pigs. A fifth experiment was performed with Babraham pigs
126 (experiment BM3) in which 3 were culled on days 6, 7, 13, 14, 20 and 21 post infection (**Fig.**
127 **1A**). In the BM3 experiment 6 control animals were included, 3 of which were culled one day
128 before and 3 on the day of infection.

129

130 **Tissue sample and processing** Two nasal swabs (one per nostril) were taken from all
131 surviving pigs following infection with H1N1pdm09 (**Fig. 1**) on days 1 to 7, 9, 11 and 13 in
132 OB1, OB2, BM1 and BM2, and on days 1 to 9 in BM3. Animals were humanely euthanized at
133 the indicated times with an overdose of pentobarbital sodium anaesthetic. Peripheral blood
134 (PBMC), tracheobronchial lymph nodes (TBLN), lung, bronchial alveolar lavage (BAL) were
135 processed as previously described (Morgan et al., 2016b; Holzer et al., 2018b). The tissue
136 homogenate was washed, red blood cells lysed and cell suspension passed through 100µM
137 cell strainer twice. Cells were cryopreserved in FBS containing 10% DMSO.

138

139 **Plaque assays.** Virus titer in nasal swabs was determined by plaque assay on MDCK cells
140 (Central Service Unit, The Pirbright Institute, UK). Samples were 10-fold serially diluted in
141 Dulbecco's Modified Eagle's Medium (DMEM) and 200µl overlayed on confluent MDCK cells
142 in 12 well tissue culture plates. After 1 hour, the plates were washed and overlayed with 2ml
143 of culture medium containing 0.66 % Agar. Plates were incubated at 37°C for 48 to 72 hours
144 and plaques visualized using 0.1% crystal violet. plaques were counted at the appropriate
145 dilution and expressed as plaque forming units (PFU) per ml of nasal swab.

146

147 **IFN γ ELISpot assay.** Frequencies of IFN γ spot forming cells (SFC) were determined using
148 cryopreserved cells a previously described (Morgan et al., 2016a; Holzer et al., 2018a). Cells
149 were stimulated with live MDCK-grown H1N1pdm09 (MOI 1), medium control, or 4 µg/ml Con
150 A (Sigma-Aldrich). Results were expressed as number of IFN γ producing cells per 10⁶ cells
151 after subtraction of the average number of spots in medium control wells.

152

153 **Flow cytometry.** Cryopreserved single cell suspensions from blood, TBLN, BAL and lung
154 were thawed, rested for 1-2 hours and aliquoted into 96 well plates at 1×10^6 cells/ well. Cells
155 were stimulated with live MDCK-grown H1N1pdm09 (MOI 1) or medium control and incubated
156 at 37°C for 18 hours. Golgi plug (BD Biosciences) was added for the last 4 hours of stimulation.
157 PMA Ionomycin (Biolegend) was added to appropriate control wells as a positive control at
158 the same time as the Golgi plug. Following incubation cells were washed at 1000 x g for 5
159 minutes and re-suspended followed by addition of primary antibodies, Near-Infrared Fixable
160 LIVE/DEAD stain (Invitrogen) and secondary antibodies (**Table 3**). Cells were fixed and
161 permeabilised with BD Fix and perm buffer (BD Biosciences) as per the manufacturer's
162 instructions prior to the addition of internal cytokine antibodies. Cells were washed and re-
163 suspended in PBS prior to analysis using a MACSquant analyser10 (Miltenyi).

164 The NP₂₉₀₋₂₉₈ SLA tetramer binding was performed as previously described (Tungatt et
165 al., 2018). Briefly, biotinylated NP peptide loaded SLA monomers, were freshly assembled
166 into tetramer with streptavidin BV421 (Biolegend, UK) and diluted with PBS to a final
167 concentration of 0.1 µg/µl. Two million mononuclear cells were incubated with protease kinase
168 inhibitor (Dasatinib, Axon Medchem) in PBS for 30 minutes at 37°C and 0.3 µg of tetramer
169 was added to the cells on ice for another 30 minutes. Surface staining with optimal antibody
170 concentrations in FACS buffer (PBS supplemented with 2% FCS and 0.05% sodium azide)
171 was performed on ice for 20 minutes (**Table 1**). Samples were washed twice with FACS buffer
172 and fixed in 1% paraformaldehyde before analysis on MACSquant analyser10 (Miltenyi). All
173 flow cytometry data was analysed by Boolean gating using FlowJo v10.6 (TreeStar, US).

174

175 **Serological assays.** ELISA was performed using live H1N1pdm09 virus or recombinant
176 haemagglutinin from H1N1pdm09 (pH1) containing a C-terminal thrombin cleavage site, a
177 trimerization sequence, a hexahistidine tag and a BirA recognition sequence as previously
178 described (Huang et al., 2015). Cut-off values determined as average naïve values plus three-
179 fold standard deviation at optimal starting dilution. Starting dilutions were 1:20, 1:2 and 1:4 for
180 serum, BAL and nasal swab respectively. Hemagglutination inhibition (HAI) Ab titers were
181 determined using 0.5% chicken red blood cells and H1N1pdm09 at a concentration of 4 HA
182 units/ml. Microneutralization (MN) was performed using standard procedures as described
183 previously (Powell et al., 2012; McNee et al., 2020).

184 The porcine sera were also tested for binding to MDCK-SIAT1 cells stably expressing
185 pH1 from H1N1pdm09 (A/England/195/2009), H1 from A/Puerto Rico/8/1934 (PR8, H1N1)
186 and H5 HA (A/Vietnam/1203/2004). Confluent cell monolayers in 96-well microtiter plates
187 were washed with PBS and 50 µl of the serum dilution was added for 1 h at room temperature.
188 The plates were washed three times with PBS and 100 µl of horseradish peroxidase (HRP)-

189 conjugated goat anti-pig Fc fragment secondary antibody (Bethyl Laboratories, diluted in PBS,
190 0.1% BSA) was added for 1 h at room temperature. The plates were washed three times with
191 PBS and developed with 100 μ l/well TMB high sensitivity substrate solution (Biolegend). After
192 5 to 10 min the reaction was stopped with 100 μ l 1M sulfuric acid and the plates were read at
193 450 and 570 nm with the Cytation3 Imaging Reader (Biotek). The cut off value was defined as
194 the average of all blank wells plus three times the standard deviation of the blank wells.

195

196 **Enzyme-linked lectin Assay (ELLA).** Neuraminidase inhibiting Ab titres were determined in
197 serum and BAL fluid using an Enzyme-linked lectin assay (ELLA). Ninety six-well microtiter
198 plates (Maxi Sorp, Nunc, Sigma-Aldrich, UK) were coated with 50 μ l/well of 25 μ g/ml fetuin
199 and incubated at 4°C overnight. Heat inactivated sera samples were serially diluted in a
200 separate 96-well plate. An equal volume of (H7(Net219) N1(Eng195) S-FLU (H7N1 S-FLU)
201 (kindly provided by Professor Alain Townsend, University of Oxford) was added to each well
202 and incubated at room temperature on a rocking platform for 20 minutes. The H7N1 S-FLU
203 was titered beforehand in the absence of serum to determine optimal concentration for the
204 assay. Fetuin plates were washed with PBS four times before 100 μ l/well of the serum/virus
205 mix was transferred and incubated overnight at 37°C. The serum/virus mix was removed, and
206 the plate washed four times with PBS before adding 50 μ l/well of Peanut Agglutinin-HRP at 1
207 μ g/ml and incubating for 90 minutes at room temperature on a rocking platform. Plates were
208 washed and 50 μ l/well of TMB High Sensitivity substrate solution (BioLegend, UK) was added.
209 Plates were developed for 6 minutes, the reaction stopped with 50 μ l of 1M H₂SO₄ and the
210 plates were read at 450 and 630 nm using a Biotek Elx808 reader. Samples were measured
211 as end titre representing the highest dilution with signal greater than cut-off. The cut off value
212 was defined as the average of all blank wells plus three times the standard deviation of the
213 blank wells.

214

215 **B cell ELISpot.** B cell ELISpots were performed for the detection and enumeration of
216 antibody-secreting cells in single cell suspensions prepared from different tissues and
217 peripheral blood. ELISpot plates (Multi Screen-HA, Millipore, UK) were coated with 100 μ l per
218 well of appropriate antigen or antibody diluted in carbonate/bicarbonate buffer for 2h at 37°C.
219 To detect HA-specific spot-forming cells, plates were coated with 2.5 μ g per well of
220 recombinant pH A from H1N1pdm09 (A/England/195/2009) and for the enumeration of total
221 IgG-secreting cells with 1 μ g per well of anti-porcine IgG (mAb, MT421, Mabtech AB, Sweden)
222 or with culture medium supplemented with 10% FBS (media background control). The coated
223 plates were washed with PBS and blocked with 200 μ l/well 4% milk (Marvel) in PBS. Frozen
224 cell suspensions from different tissues were filtered through sterile 70 μ M cell strainers, plated
225 at different cell densities in culture medium (RPMI, 10% FBS, HEPES, Sodium pyruvate,

226 Glutamax and Penicillin/Streptomycin) on the ELISPOT plates and incubated for a minimum
227 of 18 h at 37°C in a 5% CO₂ incubator. After incubation the cell suspension was removed, the
228 plates washed once with ice-cold sterile H₂O and thereafter with PBS/0.05 % Tween 20, before
229 incubation with 100 µl per well of 0.5 µg/ml biotinylated anti porcine IgG (mAb, MT424,
230 Mabtech AB, Sweden) diluted in PBS/0.5 % FBS for two hours at room temperature. Plates
231 were washed with PBS/0.05% Tween 20 and incubated with streptavidin – alkaline
232 phosphatase conjugate (Strep-ALP, Mabtech AB, Sweden). After a final wash, the plates were
233 incubated with AP Conjugate Substrate (Bio-Rad, UK) for a maximum of 30 min. The reaction
234 was stopped by rinsing the plates in tap water and dried before spots were counted.

235

236 **Statistical analysis.** All statistical analysis was performed using Prism 8.1.2. The kinetics of
237 viral shedding were analysed using a linear mixed model. The model included viral titre (log₁₀
238 PFU/ml) as the response variable, day post infection (as a categorical variable) and pig type
239 (OB or BM) and an interaction between them as fixed effects and pig ID nested in experiment
240 as random effects. The model was implemented using the lme4 package (Bates et al., 2015)
241 in R (version 3.6.1) (<https://www.R-project.org/>)

242 ELISpot data were analysed using a linear model. The model included log₁₀ SFC/10⁶
243 cells+1 as the response variable and day post infection (as a categorical variable), source
244 (BAL, lung, PBMC, TBLN) and pig type (OB or BM) and two- and three-way interactions
245 between them as fixed effects. Model simplification proceeded by stepwise deletion of non-
246 significant (P>0.05) terms as judged by *F*-tests. The model was implemented in R (version
247 3.6.1).

248 Because of possible non-normality and non-constant variance the percentage of
249 different T cells (NP₂₉₀₋₂₉₈ CD8, IFN γ CD8 β , IL-2 CD8 β , TNF CD8 β , IFN γ CD4, IL-2 CD4, TNF
250 CD4, IFN γ CD2 $\gamma\delta$ ex vivo, TNF CD2 $\gamma\delta$ ex vivo, IFN γ /TNF CD2 $\gamma\delta$ ex vivo, IFN γ CD2 $\gamma\delta$, TNF
251 CD2 $\gamma\delta$, IL-17A CD2 $\gamma\delta$) from each source (BAL, lung, PBMC, TBLN) and pig type (OB and
252 BM) at each day post infection were analysed using Kruskal-Wallis tests. If significant
253 (P<0.05), pairwise Mann-Whitney-Wilcoxon tests were used to compare groups. These
254 analyses were implemented in R (version 3.6.1). A similar approach was used to compare the
255 percentage of different T cells in all sources from inoculated and uninfected control pigs at
256 each time point and in BAL from in-contact and experimentally-inoculated pigs (in this case
257 observations from 6-11 dpi were combined).

258 The dynamics of antibody responses were analysed by fitting logistic growth curves to
259 the data, $y=\kappa/(1+\exp(-\beta(t-\delta)))$, where y is the log₁₀ antibody titre, t is days post infection, κ is
260 the upper asymptote, β is the rate of increase and δ is the time of maximum increase. The

261 parameters (i.e. κ , β and δ) were allowed to vary between BM and OB pigs. Model fitting using
262 the nlme package (<https://CRAN.R-project.org/package=nlme>) in R (version 3.6.1).

263

264 **Results**

265 **Experimental design, virus shedding and lymphocyte dynamics during H1N1pdm09**
266 **infection.** Five experiments were performed to characterise local and systemic immune
267 responses. In the first four experiments ten pigs were infected intranasally with H1N1pdm09
268 virus and monitored for clinical signs. One infected pig was culled on each of days 1 to 7, 9,
269 11 and 13 post infection. A full post-mortem examination was performed and BAL, lung, TBLN
270 and PBMC samples collected. Four uninfected controls were sampled in parallel, two on the
271 day prior to infection and two at day 8 post infection. Two experiments with outbred (OB) pigs
272 (referred to as experiments OB1 and OB2) and two with inbred Babraham (BM) pigs (referred
273 to as BM1 and BM2) were performed (**Fig. 1A**). In addition, 2 naïve pigs (referred to as in-
274 contact pigs) were co-housed with the directly challenged pigs in experiments OB1, OB2,
275 BM1, BM2 and culled at days 11 and 13 post contact. A fifth experiment was carried out with
276 18 BM (experiment BM3) in which 3 pigs were culled on each of days 6, 7, 13, 14, 20 and 21
277 post infection (**Fig. 1A**). In the BM3 experiment six uninfected controls were sampled, 3 one
278 day before and 3 on the day of infection.

279 Viral load was determined in daily nasal swabs taken from both the directly challenged
280 and in-contact pigs (**Fig. 1B**). In directly challenged pigs, peak virus load was reached 1 to 3
281 days post infection (DPI), declined sharply after 4 DPI and was not detectable after 7 DPI. No
282 differences in virus shedding between OB and BM were detected ($p=0.65$). Although the onset
283 of viral shedding was delayed, most in-contact pigs showed similar kinetics to directly
284 challenged ones, indicating that the natural contact infection is very similar to intra-nasal
285 challenge with mucosal atomization device (MAD).

286 We determined the proportion of CD8 β , CD4 and $\gamma\delta$ cells over the time course in BAL,
287 lung, TBLN and PBMC (**Fig. 1C, Suppl Fig1**). BM animals had a significantly lower proportion
288 of CD8 β T cells than OB, apparent in all tissues in naïve unexposed animals (6.6% in BM vs
289 24.2% in OB in BAL, 4.2% vs 16.2% in lung, 3.2% vs 9.4% in PBMC and 7.6% vs 11.5% in
290 TBLN) (**Suppl Fig. 1A**). BM animals also showed a significantly higher proportion of $\gamma\delta$ T cells
291 in BAL, lung and PBMC (**Suppl Figure 1A**). No significant differences in CD4 T cells were
292 detected between OB and BM. The proportion of CD4, CD8 and $\gamma\delta$ cells did not change
293 significantly over the time course of H1N1pdm09 infection, although an increase in the
294 proportion of CD8 β in the BAL for the BM animals was observed, as previously reported
295 (Khatri et al., 2010).

296 Overall the kinetic of virus infection and shedding were similar between BM and OB,
297 although there were differences in the proportions of proportions of CD8 and $\gamma\delta$ cells.

298

299 **T cell responses during H1N1pdm09 infection in pigs.** As T cells are crucial for control of
300 virus replication, we examined in detail the CD8 and CD4 responses during H1N1pdm09
301 infection (McMichael et al., 1983; Sridhar et al., 2013; Hayward et al., 2015; Holzer et al.,
302 2019). First, we enumerated IFN γ secreting cell by ELISpot following re-stimulation with
303 H1N1pdm09 (**Figs. 2A and B**). IFN γ spot forming cells (SFC) were detectable from 6 DPI and
304 maintained in all tissues until 21 DPI. During the early stage of infection the strongest
305 responses were in the TBLN (mean 474 SFC/10⁶ cells at 7 DPI), whereas from 14 to 21 DPI
306 the highest number of IFN γ secreting cells was detected in the lung, with SFC continuing to
307 expand in this tissue (mean 368 SFC/10⁶ cells at 14 DPI and 972/10⁶ cells SFC at 21 DPI).
308 The response in the BAL was lower than lung (p=0.04), due to the low proportion of T cells
309 present in the BAL (**Fig. 1C**). The IFN γ ELISpot response in the PBMC was low with a peak
310 of 296 SFC/10⁶ cells at 13 DPI. No differences in responses between the same tissues in OB
311 and BM were detected (p>0.11).

312 To further dissect the T cell response, we enumerated antigen specific cytotoxic
313 CD8 β T cells against the nuclear protein (NP) using peptide NP₂₉₀₋₂₉₈ (DFEREGYSL) tetramer,
314 which we have previously shown to be dominant in BM animals infected with H1N1pdm09
315 (Tungatt et al., 2018). Tetramer responses were measured in experiments BM1, BM2 and
316 BM3 (**Suppl Fig 1B, Figs. 2C and D**). NP₂₉₀₋₂₉₈ responses were detected in BAL and lung at
317 6 DPI, reaching a peak at 9 DPI and still present at 20 - 21 DPI. In TBLN one animal responded
318 at 5 DPI, but the peak was at 9 - 11DPI and still present at 21 DPI. The responses in PBMC
319 were low (0.2% at 6 DPI) and there were no detectable responses at 20-21 DPI (**Table 1**).
320

321 **Cytokine production by CD4 and CD8 T cells.** We analysed production of IFN γ , TNF and
322 IL-2 by CD8 β and CD4 cells by intracellular staining (ICS) (**Suppl Fig. 2A**). The kinetics of the
323 CD8 cytotoxic T cell response was similar when analysed by ICS, ELISpot and tetramer
324 binding. There was a minimal response up to 5 - 6 DPI, followed by a marked increase in
325 cytokine-producing T cells particularly in the BAL (peak of 7.9% IFN γ and 7.6% TNF at 9 DPI)
326 and lung (peak of 1.3% IFN γ and 0.6% TNF at 9 DPI). CD8 T-cells produced minimal IL-2 in
327 all tissues except for BAL, where 0.7% to 1.3% positive cells were detected between 7-13 DPI.
328 PBMC had much lower proportion of cytokine producing CD8 cells with maximum 0.3% IFN γ
329 and 0.2% TNF production in PBMC at 9 DPI. The high cytokine responses were maintained
330 in BAL and lung until 21 DPI, with lower responses in the TBLN and none in PBMC (**Fig. 3**).

331 We next determined the quality of cytokine responses of CD8 β cells. The CD8 β T-cell
332 cytokine response was dominated by IFN γ single producing cells with some IFN γ /TNF double
333 producing cells also present in all tissues (**Fig. 3A**). However, the highest proportion of double
334 IFN γ /TNF producing cells was present in the BAL (**Table 1**). A triple secreting IFN γ / IL-2/TNF
335 population was detected only in the BAL and these cells produced greater levels of IFN γ per
336 cell as measured by MFI (**Suppl Fig. 2B**). The individual cytokine profiles of the BM and OB
337 were similar during the time course of H1N1pdm09 infection and shown in **Suppl Fig. 3**. We
338 also analysed the responses in the in-contact animals from experiments OB1, OB2, BM1 and
339 BM2. These animals had the same profiles of cytokine production in BAL (**Suppl Fig. 4**) and
340 in the other tissues (data not shown) as directly challenged animals (**Table 2**).

341 The CD4 response was lower than the CD8 and developed earlier at 4 -5 DPI in some
342 animals (**Fig. 4**). It was greatest in the BAL and peaked at 9 DPI similarly to CD8 (1.6% IFN γ
343 and 2.1% TNF) and almost disappeared by 21 DPI. The CD4 response was lower in TBLN,
344 lung and PBMC (**Table 1**). CD4 cytokine secretion differed between tissues. Single cytokine-
345 secreting IFN γ and TNF CD4 cells were dominant in the lung and TBLN respectively, while in
346 the BAL and PBMC both single IFN γ , single TNF and double IFN γ /TNF were present. The
347 individual cytokine profiles of the BM and OB animals were comparable (**Suppl Fig. 3**). The
348 in-contacts also showed a similar pattern of cytokine production except for TNF (**Suppl Fig.**
349 **4, Table 2**).

350 These results demonstrate that there is a strong antigen specific CD8 response in the
351 local lung tissues and in particularly in the BAL. Cytokine production by CD8 was dominated
352 by IFN γ and TNF, but the BAL also had a significant population of IL-2-producing cells and
353 more double- and triple-producing cells, compared to TBLN, lung and PBMC. The CD4 T-cell
354 response was also greatest in the BAL, although much lower and declining more rapidly than
355 the CD8 response. The cytokine responses were similar between the in-contact and directly
356 infected animals, indicating the similarities between experimental intra-nasal challenge and
357 natural infection. No differences in magnitude, kinetic and quality of cytokine responses were
358 observed between the OB and BM animals.

359
360 **$\gamma\delta$ T cell responses during H1N1pdm09 infection in pigs.** The importance of $\gamma\delta$ T cells in
361 control of influenza infection has been demonstrated in mice and humans (Carding et al.,
362 1990; Li et al., 2013a; Xue et al., 2017; Palomino-Segura et al., 2020). In pigs $\gamma\delta$ T cells are a
363 prominent population in blood and secondary lymphoid organs and can produce IFN γ , TNF
364 and IL-17A following polyclonal stimulation (Takamatsu et al., 2006; Gerner et al., 2009;
365 Sedlak et al., 2014). Porcine $\gamma\delta$ T cells have been divided into different subsets based on the
366 expression of CD2 and CD8 α (Stepanova and Sinkora, 2013).

367 We measured IFN γ , TNF and IL-17A production in CD2 $^+$ $\gamma\delta$ cells immediately *ex vivo*
368 and following H1N1pdm09 re-stimulation. The cytokine production by CD2 $^-$ $\gamma\delta$ T cells was very
369 low (data not shown) and thus we focused on CD2 $^+$ $\gamma\delta$ T cells. *Ex vivo*, BAL CD2 $^+$ $\gamma\delta$ cells,
370 without H1N1pdm09 stimulation, secreted IFN γ and TNF early post infection with the highest
371 frequency of 0.6% IFN γ and 1.6% TNF at 3 DPI. The cells co-produced IFN γ /TNF at low levels
372 (**Fig. 5**). A minimal amount of IL-17A was detected in BAL and no IFN γ , TNF or IL-17 in the
373 other tissues *ex vivo* (data not shown).

374 We also measured cytokine production after H1N1pdm09 stimulation *in vitro* and the
375 highest proportion of IFN γ producing cells was detected in BAL and lung at 7 DPI and
376 maintained until 13 DPI (0.4% at 11 DPI for BAL) while in lung the highest frequency was 0.4%
377 at 7 DPI (**Fig. 6**). TNF showed similar pattern in BAL: increased at 9 DPI reaching a peak at
378 11 DPI with a mean of 0.7%. At later stages of infection, the frequency of cytokine producing
379 cells were much lower. The majority of the H1N1pdm09 stimulated cells were IFN γ /TNF co-
380 producing (**Suppl Fig. 5**). The responses in the contacts (0.5% for IFN γ and more than 1% for
381 TNF) were similar to those in the directly challenged animals following H1N1pdm09 stimulation
382 (**Table 2**). The proportion of IL-17A secreting CD2 $^+$ cells was much lower compared to IFN γ
383 and TNF with the greatest response in the BAL at 11 DPI (0.2%).

384 Overall these data demonstrate that $\gamma\delta$ cells produce cytokines *ex vivo* early post
385 infection, but that H1N1pdm09 *in vitro* stimulation increases cytokine production in CD2 $^+$ $\gamma\delta$ T
386 cells from 7 to 13 DPI. No difference between OB and BM pigs were detected of response of
387 *ex vivo* or stimulated $\gamma\delta$ cells.

388

389 **Antibody and B cell responses during H1N1pdm09 infection in pigs.** The antibody
390 response after H1N1pdm09 infection was determined in serum, BAL and nasal swabs. Virus
391 specific IgG and IgA were measured by end point titer ELISA against H1N1pdm09 virus or
392 recombinant HA from H1N1pdm09/A/England/195/2009 (pH1) (**Figs. 7A and B**). Serum IgG
393 against H1N1pdm09 virus was detectable at 5-6 DPI, reached its peak at 14 DPI (1:13,650)
394 and was maintained until 21 DPI (1:8,530). IgA titers were lower compared to IgG. In contrast
395 in BAL, IgG and IgA against H1N1pdm09 were present at the same levels. BAL IgG reached
396 a peak of 1:2,370 at 13 DPI which was maintained up to 21 DPI. IgG and IgA were also
397 measured in nasal swabs from experiment BM3 up to 9 DPI. Responses were detected at 6
398 DPI reaching a peak of 1:48 and 1:28 respectively by 9 DPI. We measured the ELISA
399 response to pH1, which had a similar kinetic as the response to H1N1pdm09 virus but with
400 approximately a log lower titer (**Fig. 7B**). No significant differences in the upper asymptote,
401 rate of increase in titre or time of maximum increase were detected for IgG or IgA between

402 OB and BM, except for serum IgA H1N1 ELISA (upper asymptote OB 1:2,700 > BM 1:2,100,
403 $p=0.05$).

404 To assess the breadth and cross-reactivity of the Ab, we tested the binding of sera
405 from 21 DPI to MDCK cells expressing pH1, H5 (from A/Vietnam/1203/2004) and HA from
406 PR8 in which, unlike in ELISA, the natural conformation of HA is maintained. There was strong
407 binding to the MDCK expressing pH1 and weaker binding to H5 and HA from PR8 suggesting
408 that H1N1pdm09 induces cross reactive responses to other group 1 H1 and H5 viruses (**Fig.**
409 **7C**).

410 The function of antibodies in serum and BAL was tested by microneutralization (MN)
411 assessing inhibition of virus entry, inhibition of hemagglutination (HAI) and inhibition of
412 neuraminidase activity by enzyme-linked lectin assay (ELLA) (**Fig. 8A**). MN was first detected
413 in serum at 5 or 6 DPI mirroring Ab production in the tissues, increasing to 1:140 at 11 DPI at
414 and 1:480 at 21 DPI. HAI and ELLA followed a similar pattern reaching 1:746 HAI or 1:160
415 ELLA at 21 DPI. BAL showed much lower MN, HAI and ELLA responses compared to serum.
416 MN and ELLA titres in BAL peaked at 13 DPI and were maintained until 20 DPI. HAI reached
417 a peak at 11 DPI and was undetectable at DPI 21. No significant differences in MN, HAI and
418 ELLA in the upper asymptote, rate of increase in titre or time of maximum increase between
419 OB and BM animals were detected

420 To determine the major sites of Ab production following H1N1pdm09 infection BAL,
421 lung, TBLN, spleen and PBMC from experiment BM3 were tested for total IgG and HA-specific
422 Ab secreting cells (ASC) (**Fig. 8B**). IgG producing cells were detected in all tissues with a
423 trend for increasing numbers over time up to 21 DPI. TBLN showed the highest frequency of
424 HA specific ASC reaching 200 ASC/ 10^6 cells at 20/21 DPI. Lung demonstrated a similar pattern
425 but with 18 ASC/ 10^6 cells at 20/21 DPI.

426 In summary a strong Ab response was detected in serum, which was dominated by
427 IgG, while in BAL the ELISA titers of IgG and IgA were comparable. Antibodies cross reacted
428 with HA from H1 and H5 viruses. Microneutralization, HAI and ELLA titers were much higher
429 in serum than BAL. HA specific ASC were detected in TBLN and lung. No differences were
430 observed in the Ab responses between OB and BM animals.

431

432 **Discussion**

433 In this study we investigated the kinetic and magnitude of T cell and Ab responses in
434 respiratory tissues and blood in outbred Landrace x Hampshire cross and inbred Babraham
435 pigs following H1N1pdm09 infection. The relationship between these parameters and the virus
436 load is illustrated in **Fig. 9**. After experimental infection with H1N1pdm09 virus shedding
437 plateaued between 1 and 4 - 5 DPI, followed by a steep decline so that by 9 DPI no virus could

438 be detected in any animal. An *ex vivo* $\gamma\delta$ cell IFN γ and TNF response was apparent from 2
439 DPI, although this declined by 7 DPI. In contrast, virus specific IFN γ producing $\gamma\delta$ cells were
440 detected at 7 DPI and maintained to 13 DPI. Significant virus specific CD4 and CD8 T cell
441 response were present at 6 DPI. Similarly, virus-specific IgG and IgA were detected in serum
442 and BAL at 5 - 6 DPI by which time the viral load had declined by 2-3 logs. By the time of the
443 peak of the T cell and Ab responses (9-14 DPI), no virus was detectable. These kinetics
444 suggest that innate mechanisms, including perhaps early $\gamma\delta$ cell cytokine secretion, contain
445 viral replication at a plateau level in the first 4-5 days post infection, while adaptive T and Ab
446 responses contribute to the complete clearance of virus after 5 DPI in primary infection and
447 prevent future infections by a more rapid secondary immune response.

448 Similar kinetics of adaptive T cell responses have been reported in mice, with antigen
449 specific cells detected as early 4-5 days post infection, increasing in number between 5 -12
450 DPI in lung tissues (Lawrence et al., 2005; Miao et al., 2010). Experiments in mice have shown
451 that depletion of B or CD8 T cells results in delayed clearance of IAV (Eichelberger et al.,
452 1991; Bender et al., 1992; Sarawar et al., 1994; Graham and Braciale, 1997). CD4 T cells also
453 contribute to control of influenza infection, although depletion of this cell subset alone only
454 slightly delayed viral clearance (Topham et al., 1996; Román et al., 2002; Jolley-Gibbs et al.,
455 2005). The strong CD8 and Ab responses detected in the present study suggests that these
456 cell types are also important for viral clearance in pigs. This could be confirmed by depletions
457 studies or cell transfer in inbred Babraham pigs.

458 Few studies have analysed in depth the conventional T cell response in pigs. The most
459 comprehensive study showed a low frequency of virus specific IFN γ producing CD4 and CD8
460 in the lung as early as 4 DPI after H1N2 intratracheal challenge, reaching a peak at 9 DPI,
461 with the highest response in lung compared to TBLN or PBMC (Talker et al., 2016). Here, for
462 the first time, we have analysed the cytokine responses in BAL as well as lung interstitial
463 tissues, which showed a similar kinetic. However, the response in the BAL was much stronger
464 in terms of frequency of cytokine producing T cells. The BAL T cells produced multiple
465 cytokines and more per cell, indicating that they may be most efficient in clearing the virus.
466 Cytokine production differed between CD8 and CD4 cells and between BAL, lung and TBLN,
467 perhaps reflecting the extensive tissue compartmentalization in the respiratory tract and
468 differential localization of CD4 and CD8 T cells (Topham and Reilly, 2018). Whether
469 specialized CD4 and CD8 cells are compartmentalized due to the migration of different
470 subsets to specific sites, as has been proposed in mice, or because tissue environments alter
471 cytokine production remains to be established (Strutt et al., 2013).

472 An important difference between the present study and Talker *et al* is that they used
473 the more pathogenic swine H1N2 virus, which was delivered in a large volume and high dose

474 (15 ml of 10^7 TCID₅₀/ml) intratracheally (Talker et al., 2016). This might explain the stronger
475 and more prolonged lung, TBLN and PBMC responses they observed. The pigs in the present
476 study were infected intranasally with a MAD and the response here was similar to the in-
477 contact animals, suggesting that this method is more similar to natural infection. Furthermore
478 our scintigraphy study also indicates that this method of challenge targets both the upper and
479 lower respiratory tract (Martini, 2020).

480 We detected a 27 times lower proportion of CD8 antigen-specific cells in the blood
481 compared to BAL. Similarly, antigen-specific CD8 cells responses were much higher in the
482 BAL of patients with H1N1pdm09 compared to blood (Zhao et al., 2012). This indicates that
483 sampling blood is not reflective of the true response in the lung and local tissues, which has
484 implications for the design and analysis of clinical trials for T-cell targeted vaccines. In contrast,
485 CD4 responses were more similar in magnitude in blood and BAL, although less long lived
486 than CD8.

487 The contributions of $\gamma\delta$ cells to lung homeostasis and influenza immunity remain
488 incompletely explored. In pigs, $\gamma\delta$ cells comprise up to 50% of lymphocytes in the blood
489 (particularly in young animals) in contrast to humans where they usually represent 1-5% of
490 lymphocytes (Roden et al., 2008; Schwaiger et al., 2019). $\gamma\delta$ T cell have previously been
491 reported to increase late after IAV infection in mice, although an early increase in $\gamma\delta$ cells in
492 mice and pigs has also been reported (Carding et al., 1990; Khatri et al., 2010; Palomino-
493 Segura et al., 2020). Human $\gamma\delta$ cells can expand in a TCR-independent manner in response
494 to IAV, and the human V γ 9V δ 2 T cell subset kills IAV-infected A549 airway cells (Li et al.,
495 2013b). Although we did not observe a significant increase in $\gamma\delta$ cells after H1N1pdm09
496 infection, we showed that $\gamma\delta$ cells produce IFN γ and TNF as early as day 2 post infection ex
497 vivo, in agreement with studies in mice (Xue et al., 2017). $\gamma\delta$ cells are a major source for IL-
498 17 production, which has been shown to play a role in IAV infection, but we detected only low
499 levels of IL-17A after H1N1pdm09 stimulation of BAL cells (Crowe et al., 2009; Li et al., 2012;
500 Palomino-Segura et al., 2020). Surprisingly, we demonstrated that *in vitro* stimulation with
501 H1N1pdm09 induces IFN γ and TNF production in CD2 $^+$ $\gamma\delta$ T cells from 7 to 13 DPI. This is
502 reminiscent of an adaptive T cell response. Recombinant hemagglutinin from H5N1 has been
503 previously demonstrated to activate human PBMC $\gamma\delta$ T cells *in vitro* and this was not mediated
504 by TCR or pattern recognition receptors (Lu et al., 2013). Further studies will elucidate the
505 mechanisms of cytokine induction and whether it is TCR dependent.

506 H1N1pdm09 infection was characterised by high IgG and IgA titers in serum and BAL,
507 and a detectable antibody titer in nasal swabs. The IgG titer was higher than IgA in serum,
508 while similar levels of IgA and IgG were detected in BAL and nasal swabs, suggesting local
509 production of this isotype or more efficient translocation. Neutralization, HAI and

510 neuraminidase inhibition titers peaked at 11 - 21 DPI. Our findings are in agreement with
511 previous studies showing that in experimentally H1N1 infected pigs HA-specific antibodies
512 peaked at 2-3 weeks (Larsen et al., 2000). Similarly we detected HA-specific antibody-
513 secreting cells in the local TBLN and lung tissues, but not PBMC (Larsen et al., 2000).
514 However, it might be that antibody-secreting cells are largely lost in these liquid nitrogen frozen
515 and thawed samples.

516 Despite centuries of agricultural selective breeding, the pig has maintained a
517 significant level of SLA genetic diversity, with 227 class I and 211 class II alleles identified for
518 *Sus scrofa* in the Immuno-Polymorphism Database (IPD) MHC database to date, making
519 analysis of the fine specificity of immune responses extremely difficult (Maccari et al., 2017).
520 The inbred Babraham line of pigs, on the other hand, is SLA homozygous for class I SLA-
521 1*14:02; SLA-2*11:04 and SLA-3*04:03 and class II DRB1-*05:01, DQA-*01:03 and DQB1-
522 *08:01 (Schwartz et al., 2018). This homozygosity enabled the use of peptide-SLA tetramers
523 to the dominant NP antigen to track the CD8 response in tissues in this study (Tungatt et al.,
524 2018). The $\alpha\beta$, $\gamma\delta$ T-cell and Ab responses in the OB and BM animals were comparable,
525 although there was lower proportion of CD8 cells and higher proportion of $\gamma\delta$ cells in the BM
526 pigs. This may be due to a genetic difference, although it may also be a result of different
527 housing conditions, since the Babraham pigs are maintained under specific pathogen free
528 conditions, whereas the outbred pigs were obtained from a commercial breeder.

529 Our detailed analysis of immune responses in pigs showed that the viral load is
530 contained in the period before the adaptive response is detectable, indicating the importance
531 of innate immune mechanisms in influenza infection. As in other species however it appears
532 that the adaptive response is essential for elimination of virus. BAL contains the most highly
533 activated CD8, CD4 and $\gamma\delta$ cells producing large amounts of cytokines, which likely contribute
534 to clearance of virus. We further show clear differences between the function of CD4, CD8
535 and $\gamma\delta$ T cells between the lung, BAL and TBLN, while the blood is a poor representation of
536 the local immune response. The immune response in the Babraham pig following H1N1pdm09
537 influenza infection was comparable to that of outbred animals. The availability of fine grain
538 immunologic tools in Babraham pigs will allow the unraveling of immune mechanisms and
539 confirm and extend findings in outbred populations.

540

541 **Funding:** This work was funded by the UKRI Biotechnology and Biological Sciences Research
542 Council (BBSRC) grants: sLoLa BB/L001330/1, BBS/E/I/00007031, BBS/E/I/00007038 and
543 BBS/E/I/00007039.

544

545 **Acknowledgements.** We are grateful to animal staff at Langford School of Veterinary for
546 providing excellent animal care. We are grateful to Alain Townsend for providing recombinant
547 HAs and H7N1 virus. We thank APHA for providing the swine A/Sw/Eng/1353/09 influenza
548 virus strain (DEFRA surveillance programme SW3401).

549

550 **Authors contribution.** BC, MB, ET conceived, designed and coordinated the study. BC,
551 MB, ET, ME, AM, EP, EV, BP, VM OF, RH, AT, RB, SM designed and performed experiments,
552 processed samples and analyzed the data. SG performed statistical analysis. AF and AS
553 generated SLA tetramers. ET, ME, EV, AM wrote and revised the manuscript and figures. All
554 authors reviewed the manuscript.

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748

749 **Table 1.** Comparison of different populations of T cells from Babraham pigs infected with
 750 H1N1pdm09 virus.

% T cells binding to tetramer or /cytokine producing		Days Post Infection (DPI)		
		6 DPI	7 DPI	13 DPI
CD8β	NP ₂₉₀₋₂₉₈	no significant (P>0.05) differences*	BAL†>PBMC (P=0.01) lung>PBMC (P=0.04) lung>TBLN (P=0.04)	BAL>lung (P=0.04) BAL>TBLN (P=0.01) BAL>PMBC (P=0.01) lung>PMBC (P=0.01) TBLN>PMBC (P=0.01)
CD8β	IFN γ	BAL>PBMC (P=0.01) BAL>TBLN (P=0.01) lung>PBMC (P=0.01) lung>TBLN (P=0.03)	BAL>PBMC (P=0.01) BAL>TBLN (P=0.01) lung>PBMC (P=0.01) lung>TBLN (P=0.03)	BAL>PBMC (P=0.01) BAL>TBLN (P=0.01) lung>PBMC (P=0.01) lung>TBLN (P=0.03)
	IL-2	no significant (P>0.05) differences	BAL>lung (P=0.06) BAL>PBMC (P=0.02) BAL>BM TBLN (P=0.02)	BAL>lung (P=0.01) BAL>PBMC (P=0.01) BAL>TBLN (P=0.01)
	TNF	BAL>lung (P=0.01) BAL>PBMC (P=0.01) BAL>TBLN (P=0.01) lung>PBMC (P=0.02)	BAL>lung (P=0.02) BAL>PBMC (P=0.01) BAL>TBLN (P=0.01) lung>PBMC (P=0.02)	BAL>lung (P=0.01) BAL>PBMC (P=0.01) BAL>TBLN (P=0.01) lung>PBMC (P=0.02) lung>TBLN (P=0.06)
CD4	IFN γ	no significant (P>0.05) differences	lung>TBLN (P=0.02) lung>PBMC (P=0.01)	BAL>PBMC (P=0.06) lung>PBMC (P=0.06)
	IL-2	BAL>PBMC (P=0.01) lung>PBMC (P=0.03) TBLN>PBMC (P=0.01)	BAL>PBMC (P=0.01) lung>PBMC (P=0.01) TBLN>PBMC (P=0.01)	BAL>PBMC (P=0.01) lung>PBMC (P=0.01) TBLN>PBMC (P=0.01)
	TNF	no significant (P>0.05) differences	no significant (P>0.05) differences	BAL>PBMC (P=0.06) TBLN>lung (P=0.06) TBLN>PBMC (P=0.02)

751 * P-values based on pairwise Mann-Whitney-Wilcoxon tests following a significant (P<0.05) Kruskal-
 752 Wallis test

753 † BAL: broncho-alveolar lavage; PBMC: peripheral blood mononuclear cell; TBLN: tracheobronchial
 754 lymph node

755

756

757 **Table 2.** Comparison of T cells in broncho-alveolar lavage from experimentally-inoculated (I)
758 and in-contact (C) Babraham (BM) and outbred (OB) pigs infected with H1N1pdm09 swine
759 influenza virus.

T cells	% cells producing		
	IFN γ	TNF	IL-2 or IL-17a*
CD8 β	no significant (P>0.05) differences†	no significant (P>0.05) differences†	no significant (P>0.05) differences†
CD4	no significant (P>0.05) differences†	OB, C>BM, C (P=0.03) OB, C>BM, I (P=0.02)	no significant (P>0.05) differences†
$\gamma\delta$ ex vivo	no significant (P>0.05) differences†	no significant (P>0.05) differences†	not tested
$\gamma\delta$ H1N1pdm09 stimulated	no significant (P>0.05) differences†	no significant (P>0.05) differences†	no significant (P>0.05) differences†

760 * IL-2 for CD8 β and CD4 T cells; IL-17a for $\gamma\delta$ T cells

761 † P-values based on pairwise Mann-Whitney-Wilcoxon tests following a significant (P<0.05)
762 Kruskal-Wallis test

763

764 **Table 3.** Antibodies used

Antigen	Clone	Isotype	Fluorochrome	Source of primary Ab	Details of secondary Ab
Staining for conventional T cells					
CD4	74-12-4	IgG2b	PerCP-Cy5.5	BD Biosciences	
CD8b	PPT23	IgG1	FITC	Bio-Rad Laboratories	
TNF	MAb11	IgG1	BV421	BioLegend	
IFN γ	P2G10	IgG1	APC	BD Biosciences	
IL-2	A150D 3F1 2H2	IgG2a	PE-Cy7	ThermoFisher	rat-anti-mouse, IgG2a, BioLegend
Staining for $\gamma\delta$ T cells					
TCR $\gamma\delta$	PGBL22A	IgG1	PE-Cy7	Cambridge bioscience	rat-anti-mouse, IgG1, BioLegend
CD8 α	76-2-11	IgG2a	FITC	BD Biosciences	
CD2	MSA4	IgG2a	PerCP-Cy5.5	Cambridge bioscience	rat-anti-mouse, IgG2a, BioLegend
TNF	MAb11	IgG1	BV421	BioLegend	
IFN γ	P2G10	IgG1	APC	BD Biosciences	
IL-17A	SCPL1362	IgG1	PE	BD Biosciences	

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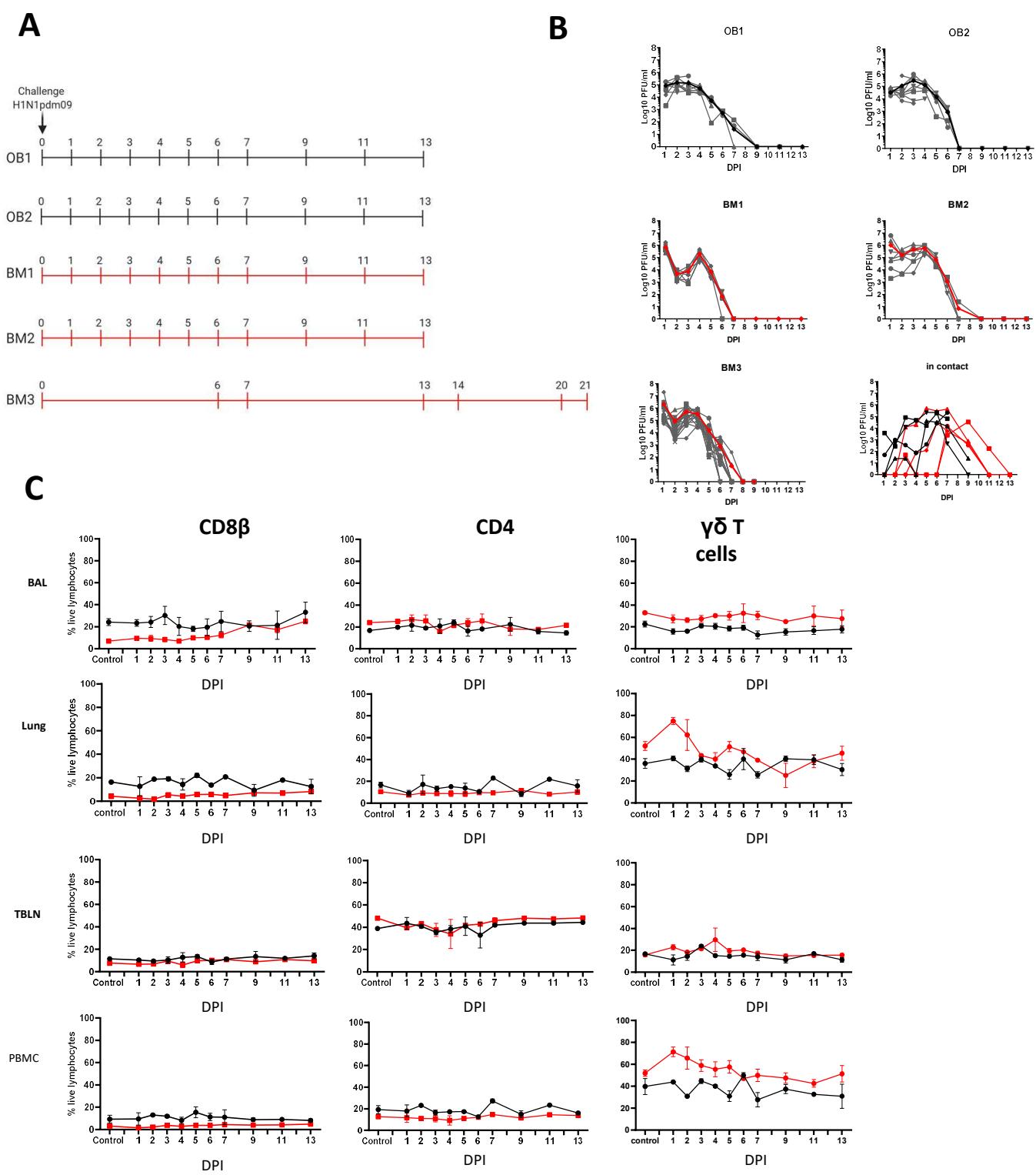


Figure 1. Experimental design, viral load and cell subset dynamics following H1N1 pmd09 infection. (A) Pigs were infected with H1N1pdm09 and culled on the days indicated. Two experiments with outbred (OB1 and OB2, black line) and two with inbred Babraham pigs (BM1 and BM2, red line) were performed. Two in-contact animals were included in each experiment one culled at day 11 and one at day 13 post infection. An extended time course of 21 days was performed with 18 inbred Babraham pigs (BM3, red line) with animals culled on the indicated days. (B) Virus load was determined by plaque assay of daily nasal swabs at the indicated time points. The thick line indicate the mean. (C) Proportions of CD4, CD8 and $\gamma\delta$ cells were determined by flow cytometry at the indicated time points.

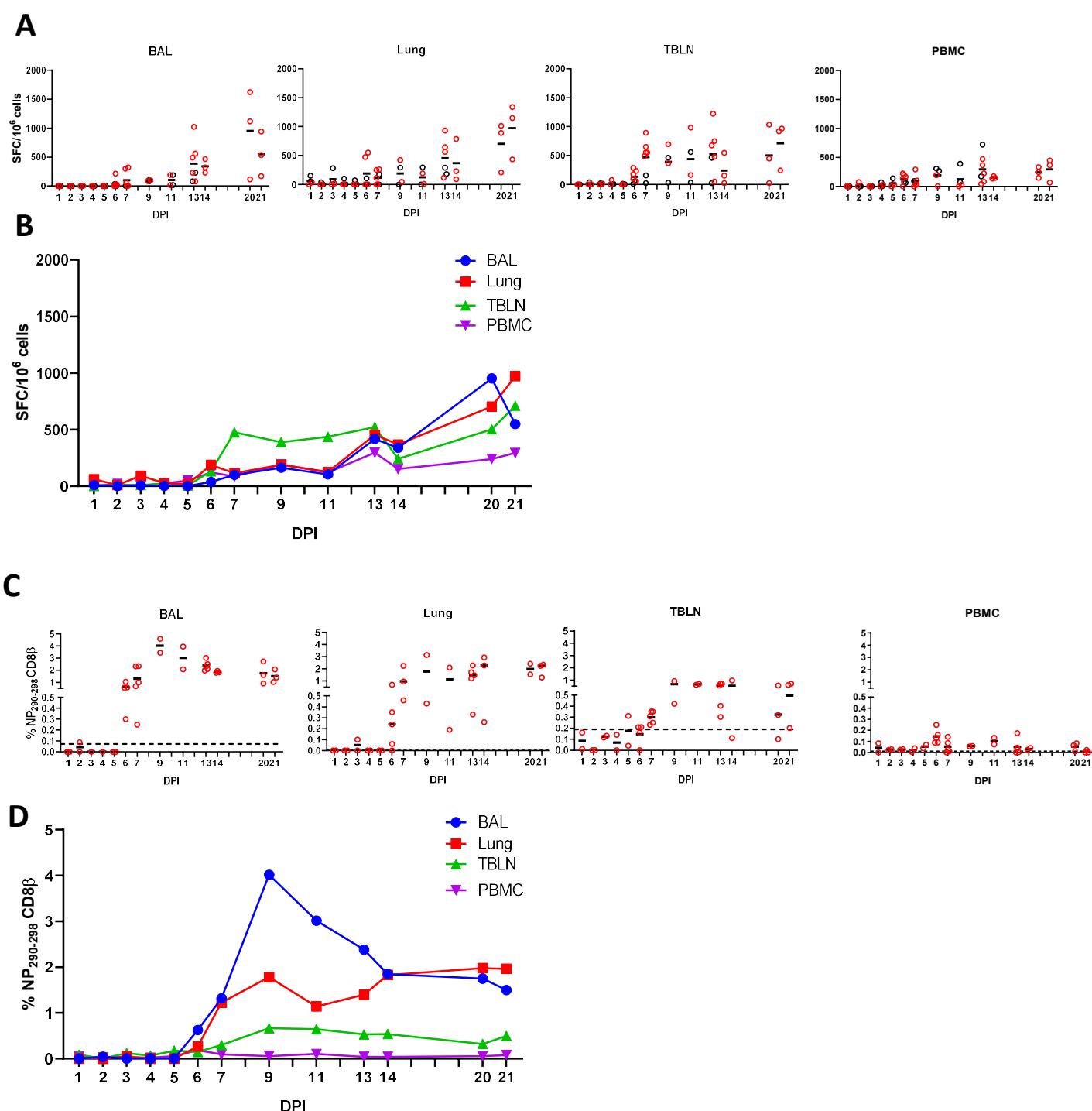


Figure 2. IFN γ ELISpot and tetramer responses. (A,B) IFN γ secreting spot forming cells (SFC) in BAL, lung, TBLN and PBMC in outbred (black circles) and inbred (red circles) pigs were enumerated after stimulation with H1N1pdm09 or medium control. (B) The mean percentages for each population are shown. DPI 1 to 7, 9, 11 and 13 each show results from 2 outbred and 2 inbred pigs. DPI 6, 7, 13, 14, 20 and 21 also include results from 3 additional inbred pigs. (C, D) Proportions of NP₂₉₀₋₂₉₈ CD8 T cells in tissues. Background staining with SLA matched tetramers containing irrelevant peptide has been subtracted. Dotted lines indicate proportions of tetramer positive cells in uninfected animals. Data from 2 outbred and 2 inbred pigs is shown for days 1 to 5 and 9 to 11. DPI 6, 7, 13, 14, 20 and 21 show data from 3 additional inbred pigs. Data in C and D is from 2 pigs (DPI 1-5, 9, 11), 3 pigs (DPI 14, 20, 21) or 5 pigs (DPI 6, 7 and 13).

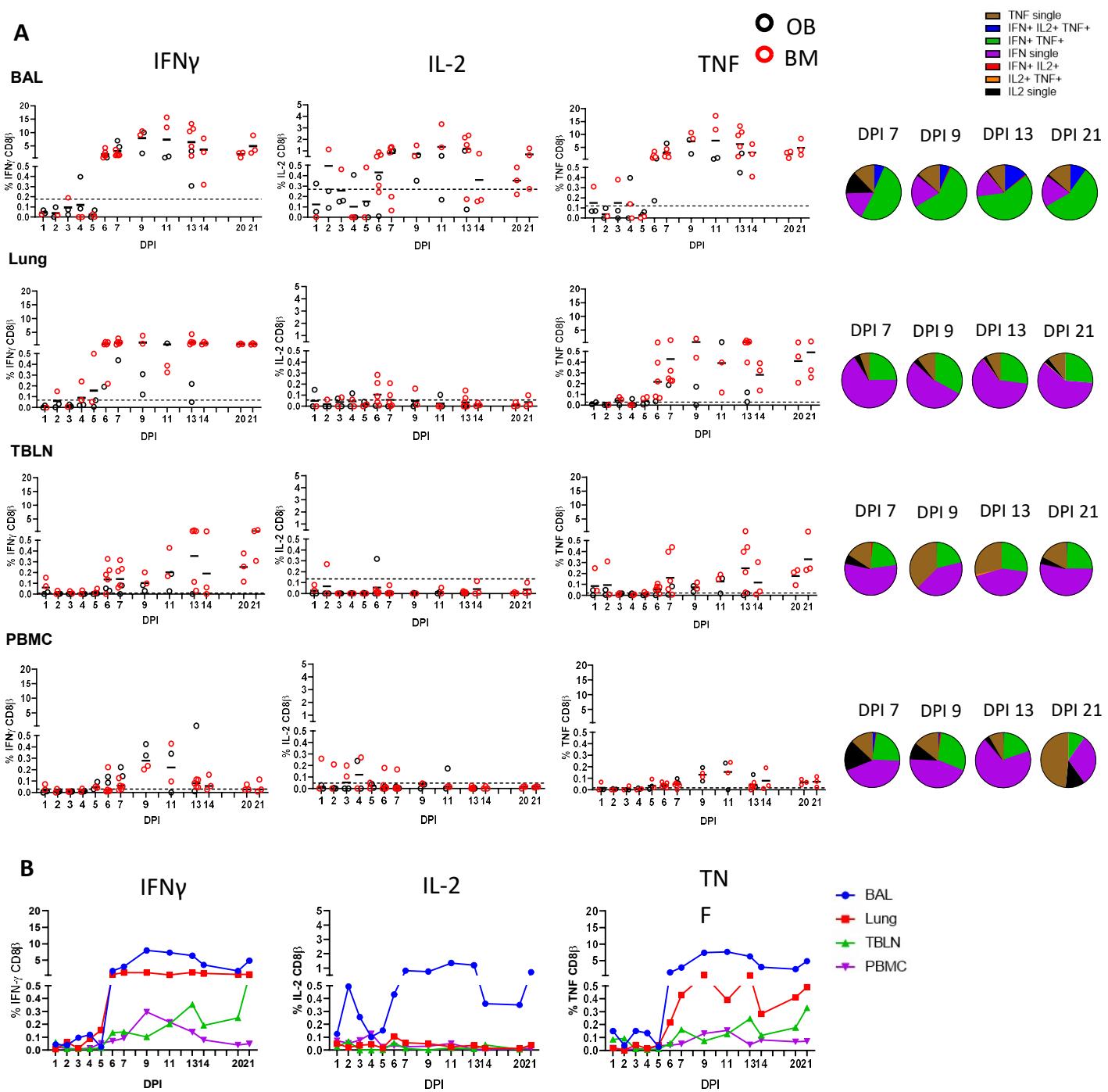
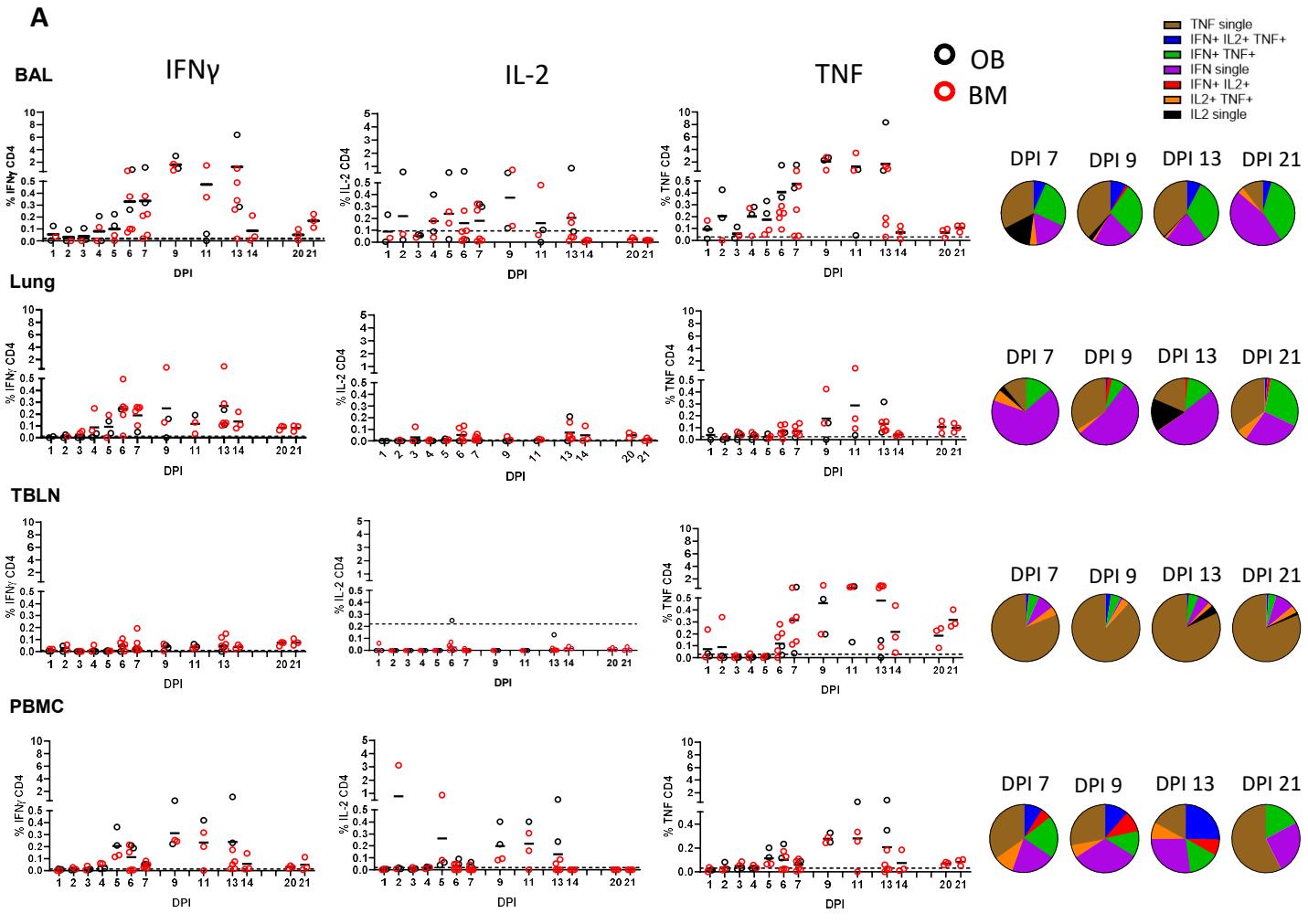


Figure 3. CD8 β T cell cytokine responses. (A) Cytokine response of CD8 β T cells in outbred (black circles) and inbred (red circles) pigs at each time point following influenza infection. BAL, lung, TBLN or PBMC cells were stimulated with H1N1pdm09 and cytokine secretion measured using intracytoplasmic staining. The mean of the 22 uninfected control animals is represented by a dotted line. DPI 1 to 7, 9, 11 and 13 each show results from 2 outbred and 2 inbred pigs. DPI 6, 7, 13, 14, 20 and 21 also include results from 3 additional inbred pigs. Pie charts show the proportion of single, double and triple cytokine secreting CD8 T cells for IFN γ , TNF and IL-2 at 7, 9, 13 and 21 DPI. (B) The mean percentages for IFN γ , TNF and IL-2 in each tissue for both OB and BM together are shown over the time course.

A



B

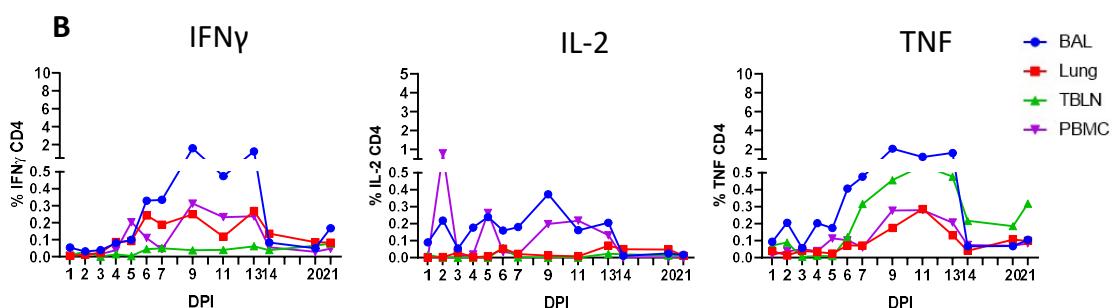


Figure 4. CD4 T cell cytokine responses. (A) Cytokine response of CD4 T cells in outbred (black circles) and inbred (red circles) pigs at each time point following influenza infection. BAL, lung, TBLN or PBMC cells were stimulated with H1N1pdm09 and cytokine secretion measured using intra-cytoplasmic staining. The mean of the 22 uninfected control animals is represented by a dotted line.

DPI 1 to 7, 9, 11 and 13 each show results from 2 outbred and 2 inbred pigs. DPI 6, 7, 13, 14, 20 and 21 also include results from 3 additional inbred pigs. Pie charts show the proportion of single, double and triple cytokine secreting CD8 T cells for IFNγ, TNF and IL-2 at 7, 9, 13 and 21 DPI. **(B)** The mean percentages for IFNγ, TNF and IL-2 in each tissue for OB and BM together are shown over the time course.

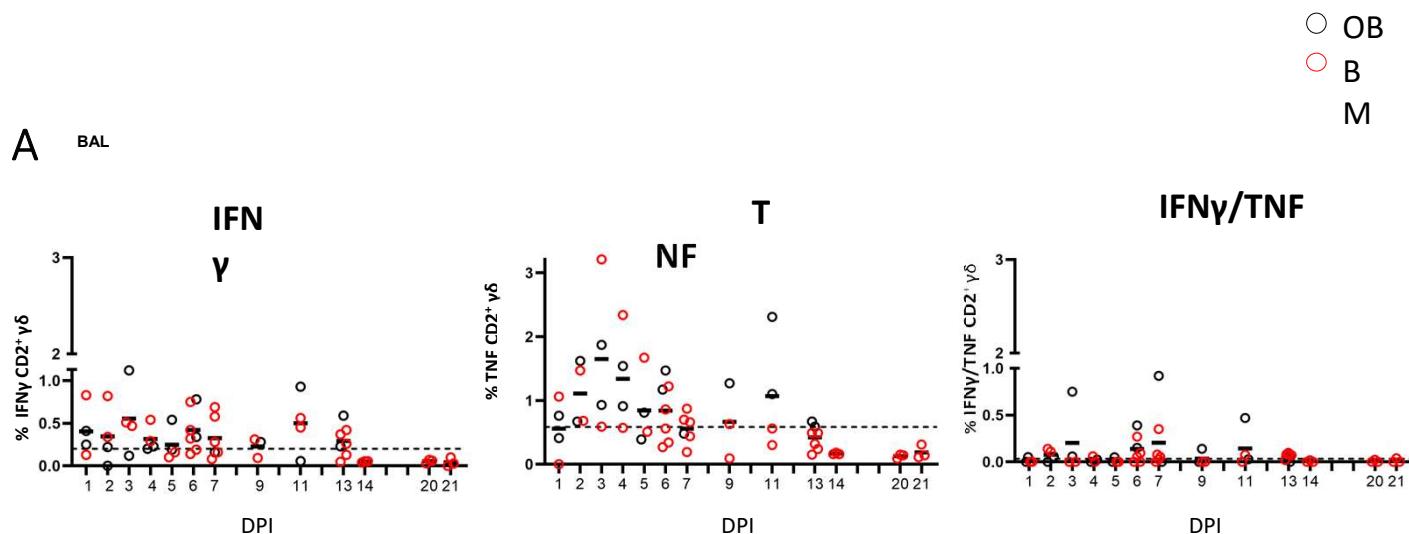


Figure 5. $\gamma\delta$ T cell *ex vivo* responses in BAL. Cytokine response of $\gamma\delta$ T cells in outbred (black circles) and inbred (red circles) pigs at each time point following H1N1pdm09 infection. IFN γ and TNF production in BAL cells *ex vivo* without stimulation was measured using intracellular staining. The right hand panel shows the IFN γ /TNF double producing cells. The mean of the 22 uninfected control animals is represented by a dotted line. DPI 1 to 7, 9, 11 and 13 each show results from 2 outbred and 2 inbred pigs. DPI 6, 7, 13, 14, 20 and 21 also include results from 3 additional inbred pigs.

A

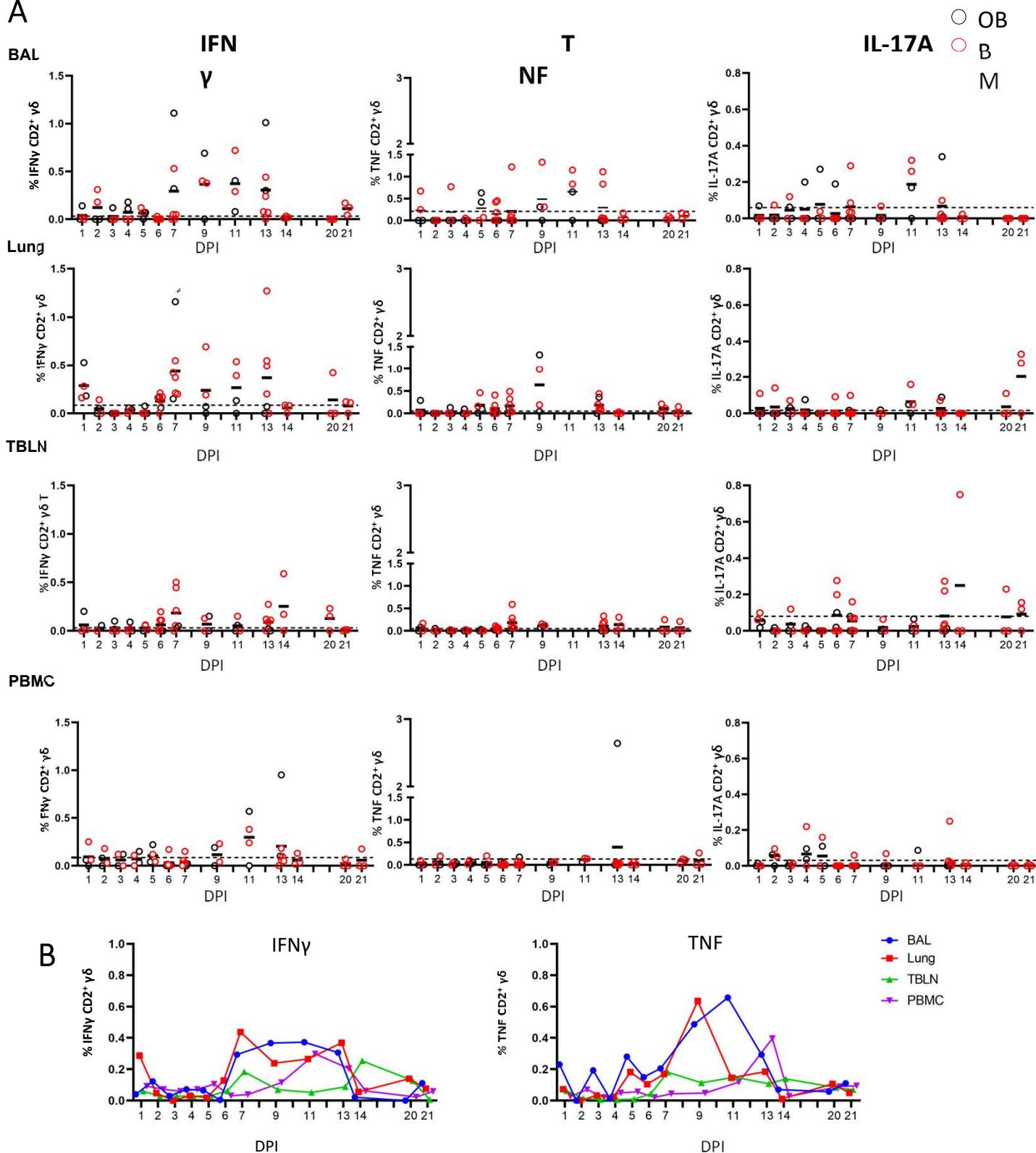


Figure 6. $\gamma\delta$ T cell responses after H1N1pdm09 stimulation. (A) Frequencies of IFN γ , TNF and IL-17A producing CD2 $^{+}$ $\gamma\delta$ T cells in outbred (black circles) and inbred (red circles) pigs following influenza infection. BAL, lung, TBLN and PBMC were stimulated with H1N1pdm09 and cytokine secretion measured using intra-cytoplasmic staining. The mean of the 22 uninfected control animals is represented by a dotted line. DPI 1 to 7, 9, 11 and 13 each show results from 2 outbred and 2 inbred pigs. DPI 6, 7, 13, 14, 20 and 21 also include results from 3 additional inbred pigs. **(B)** Mean percentages for IFN γ and TNF in each tissue are shown over the time course.

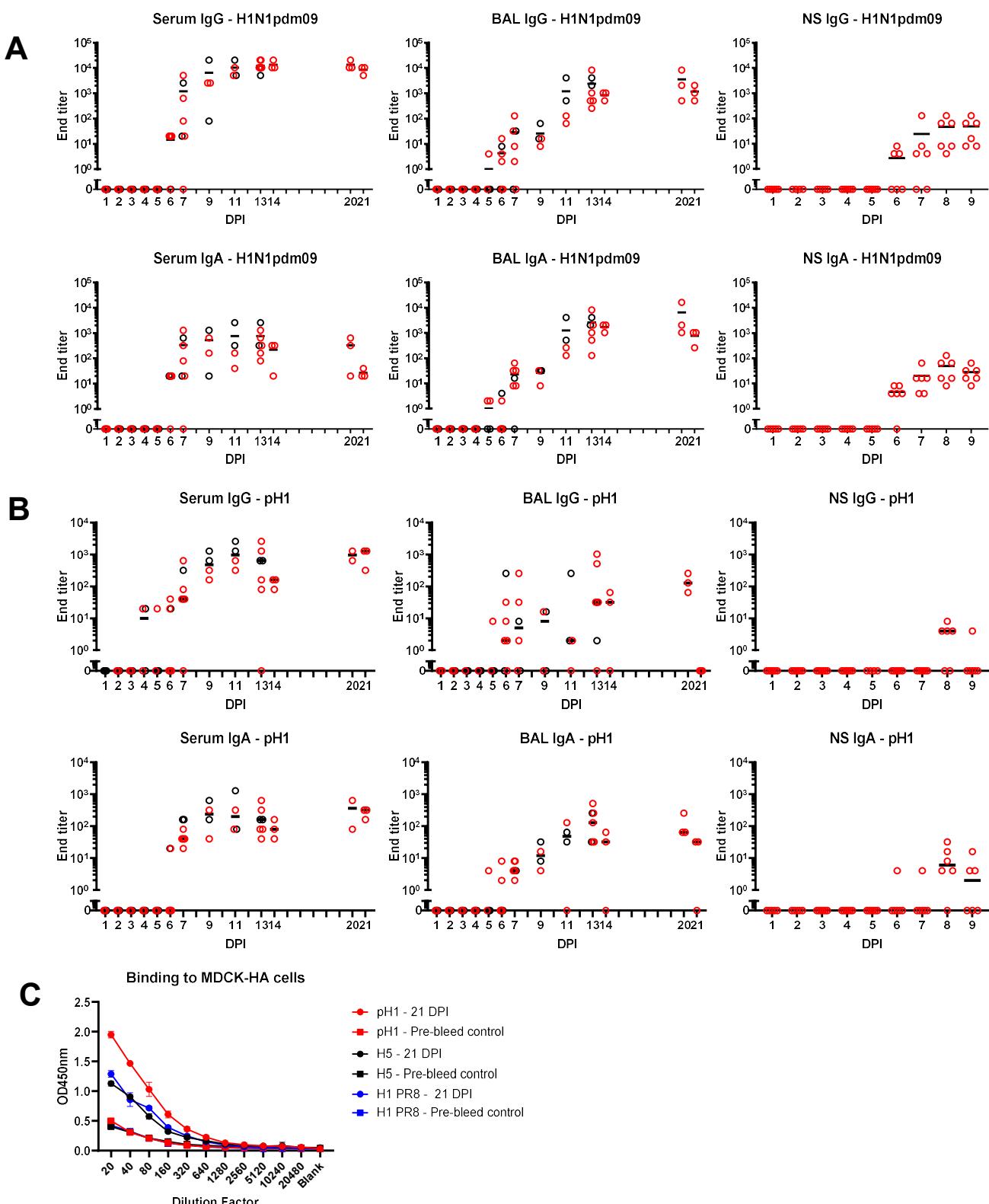
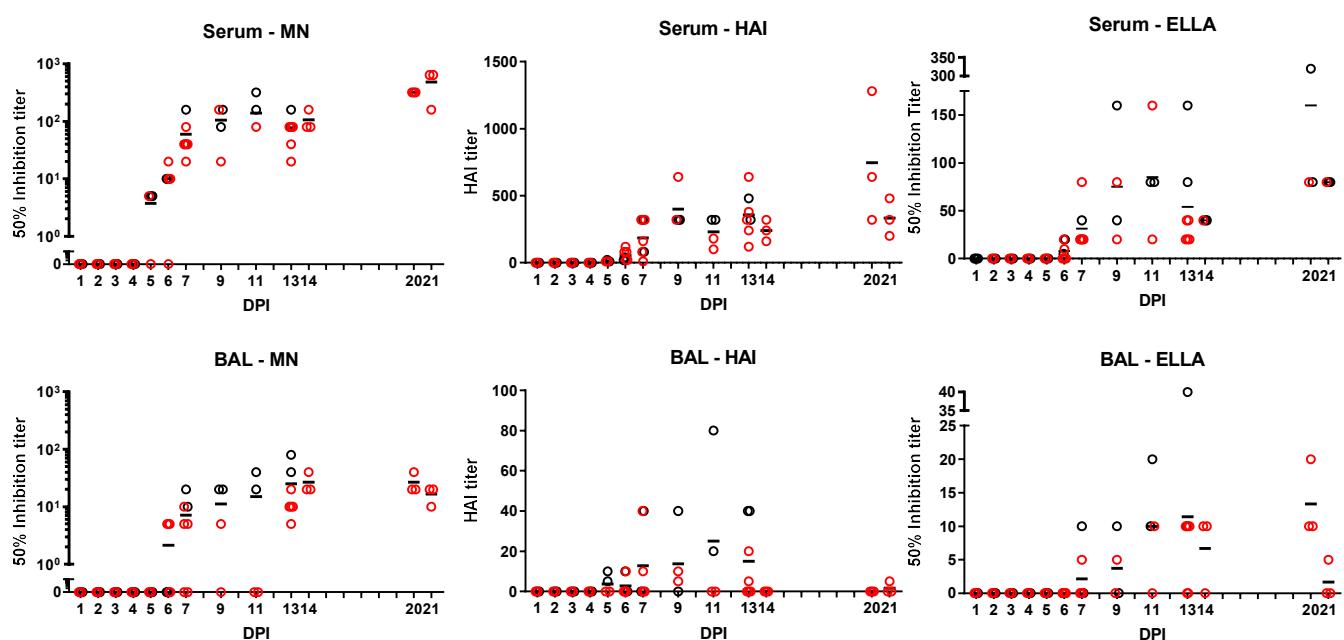


Figure 7. Ab ELISA responses and binding to MDCK-HA expressing cells. Influenza H1N1pdm09 virus specific IgA and IgG (A) and haemagglutinin (pHA) specific (B) responses in serum, BAL and nasal swabs (NS) were determined by ELISA and shown as black (for OB) and red (for BM) circles. (C) Binding of serum at 21 DPI to MDCK-pH1, MDCK-H1 PR8 and MDCK-H5 expressing cells.

A



B

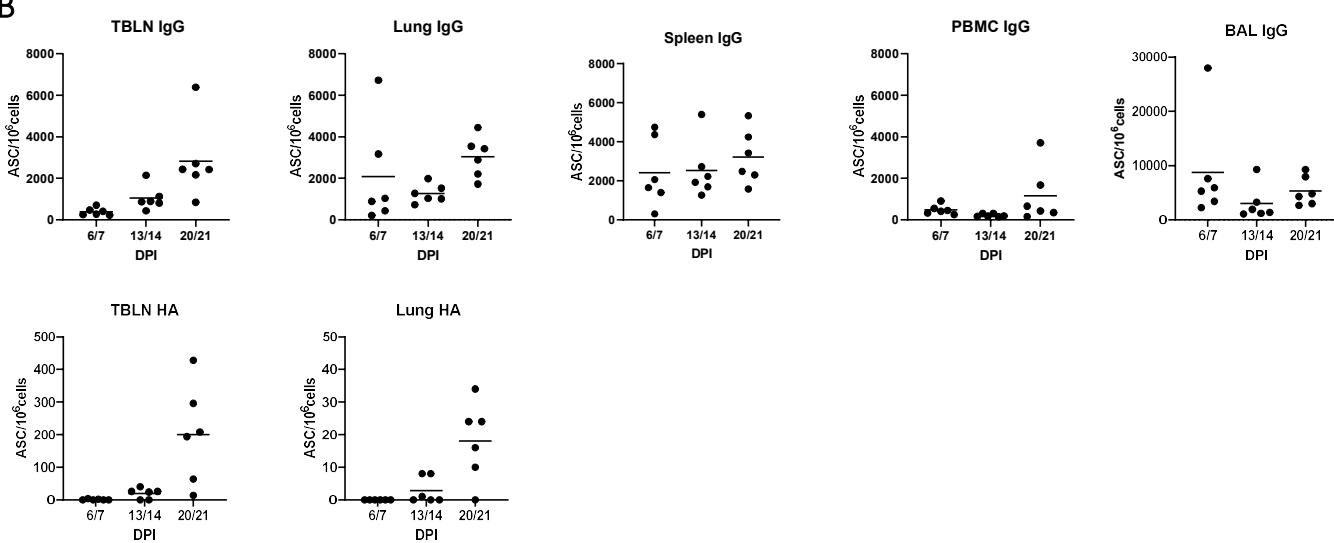


Figure 8. Ab function and antibody secreting cells in tissues. (A) The mean neutralisation (MN), hemagglutination inhibition (HAI) and ELLA titers in serum and BAL over time are shown as red (BM) and black (OB) circles. (B) IgG and pHA specific spot forming cells (SFC) were enumerated in blood of animals from experiment BM3 at the indicated time points in TBLN, lung, spleen, BAL and PBMC.

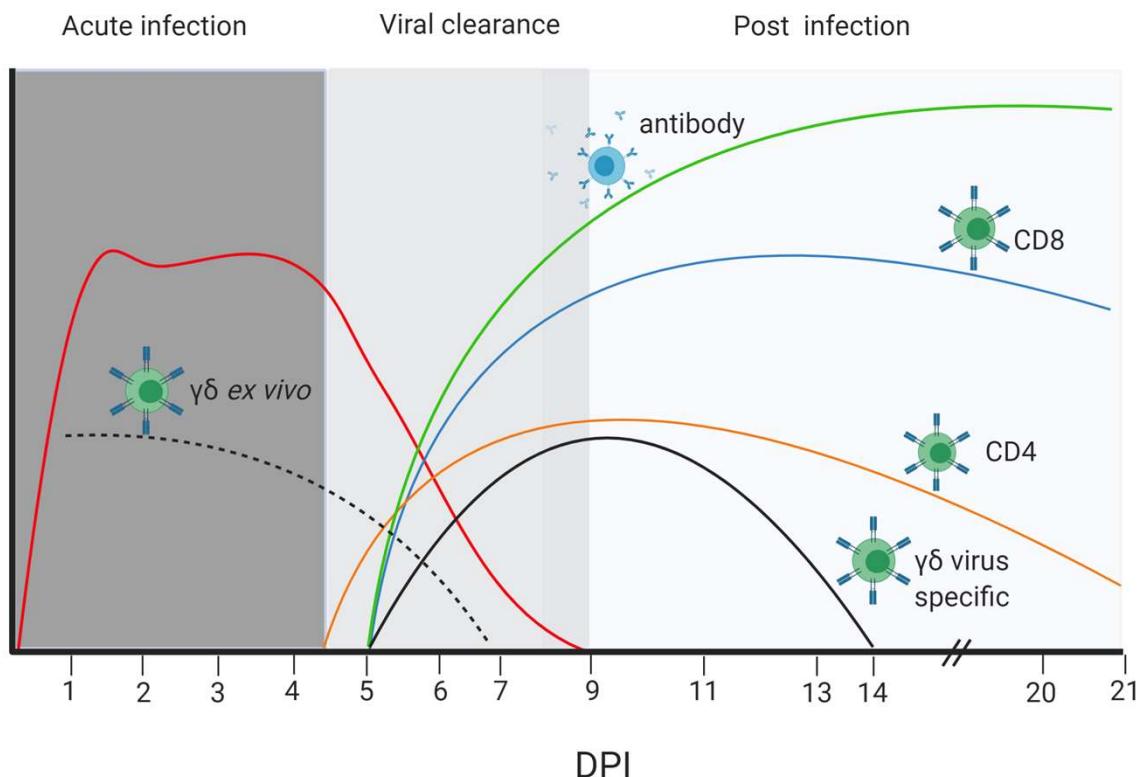
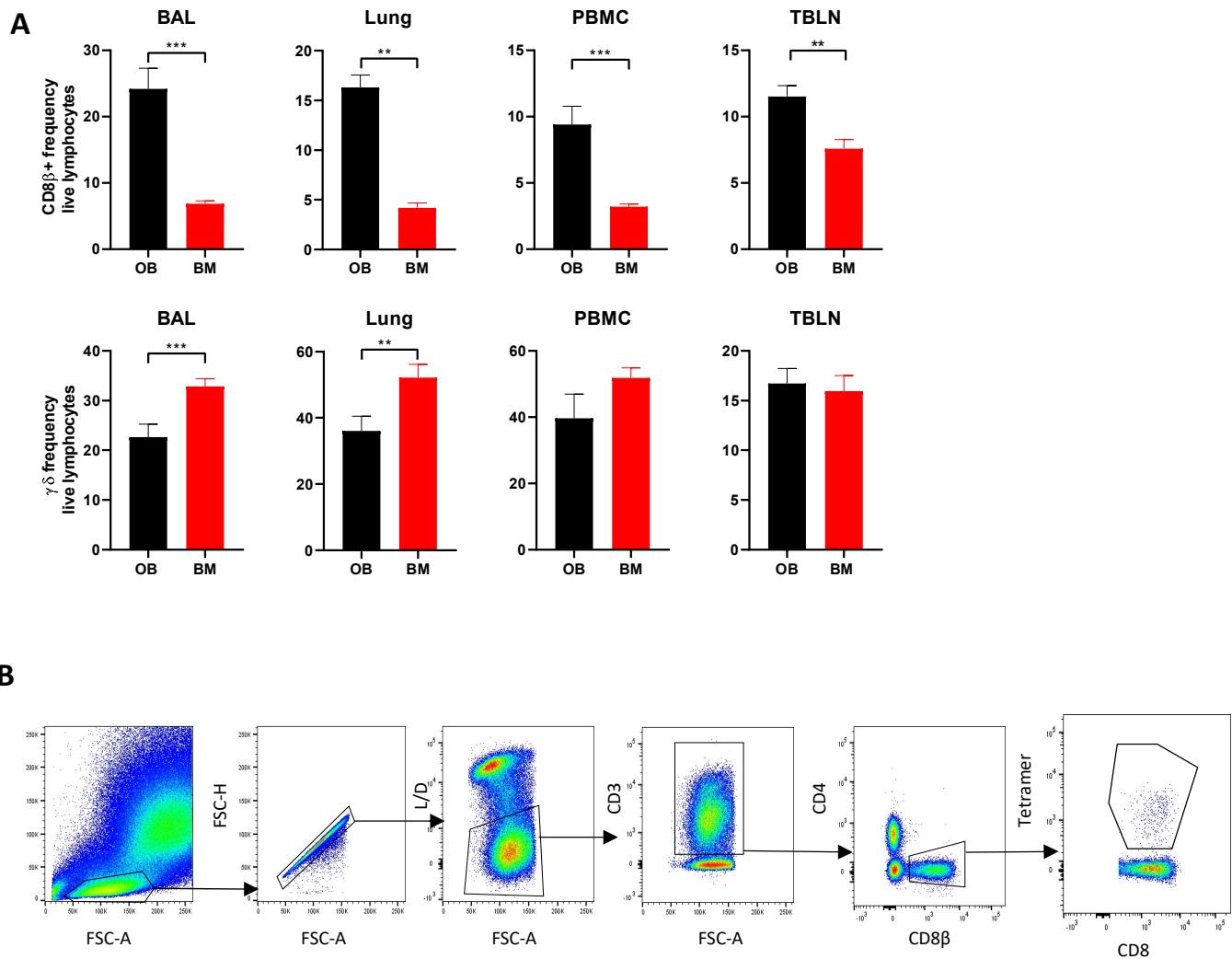


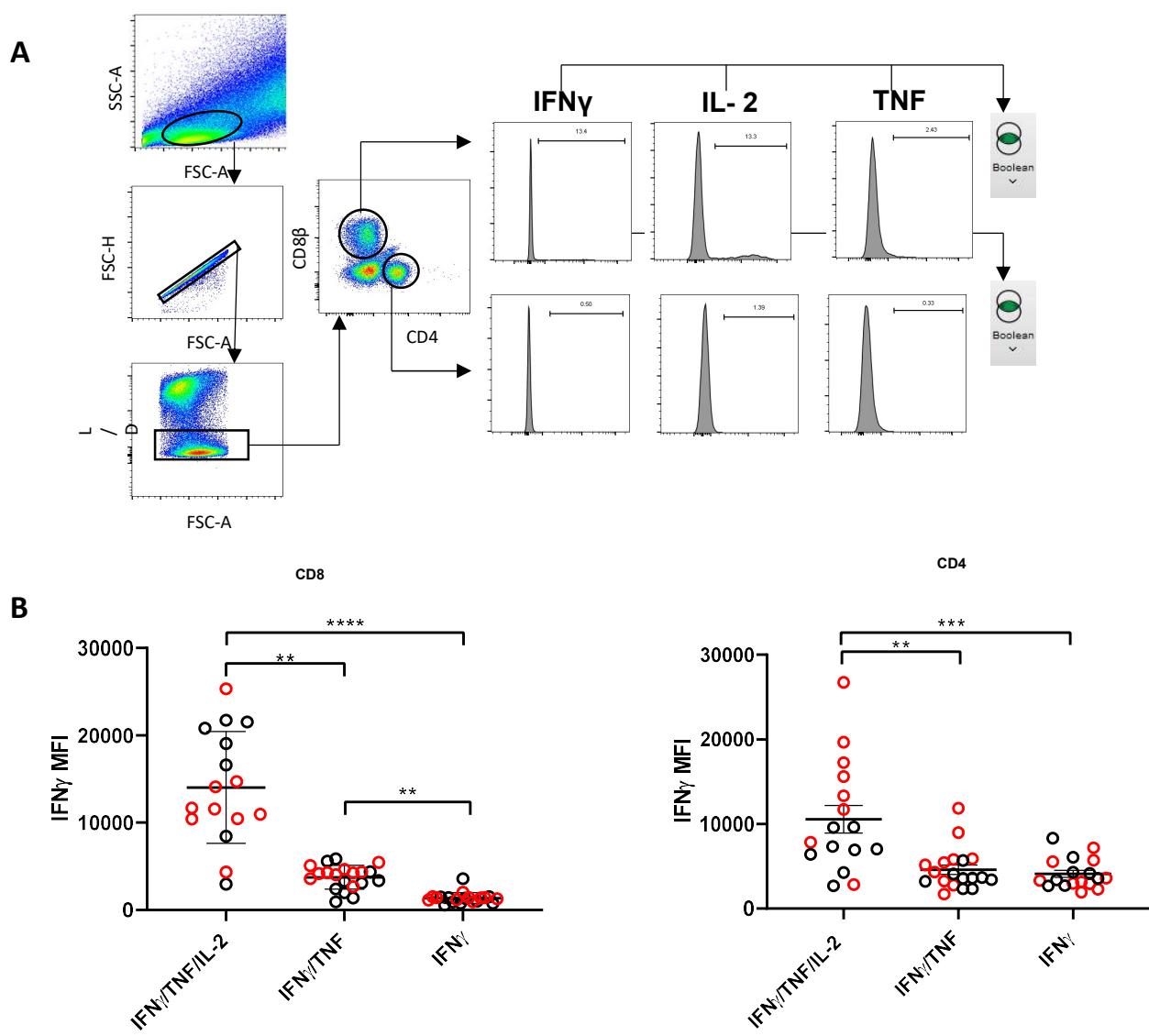
Figure 9. Dynamics of viral load, T cell and Ab responses. Stylised presentation of kinetics and magnitude of viral load and immune responses. The different colour lines represent the antibody and cellular responses as indicated, and the red line the virus load. The dotted black line represents ex vivo cytokine production by $\gamma\delta$ cells, while the solid black line is the cytokine production by $\gamma\delta$ cells re-stimulated with H1N1pdm09 virus *in vitro*. The figure was created with [BioRender.com](https://biorender.com).

Supplementary Figure 1



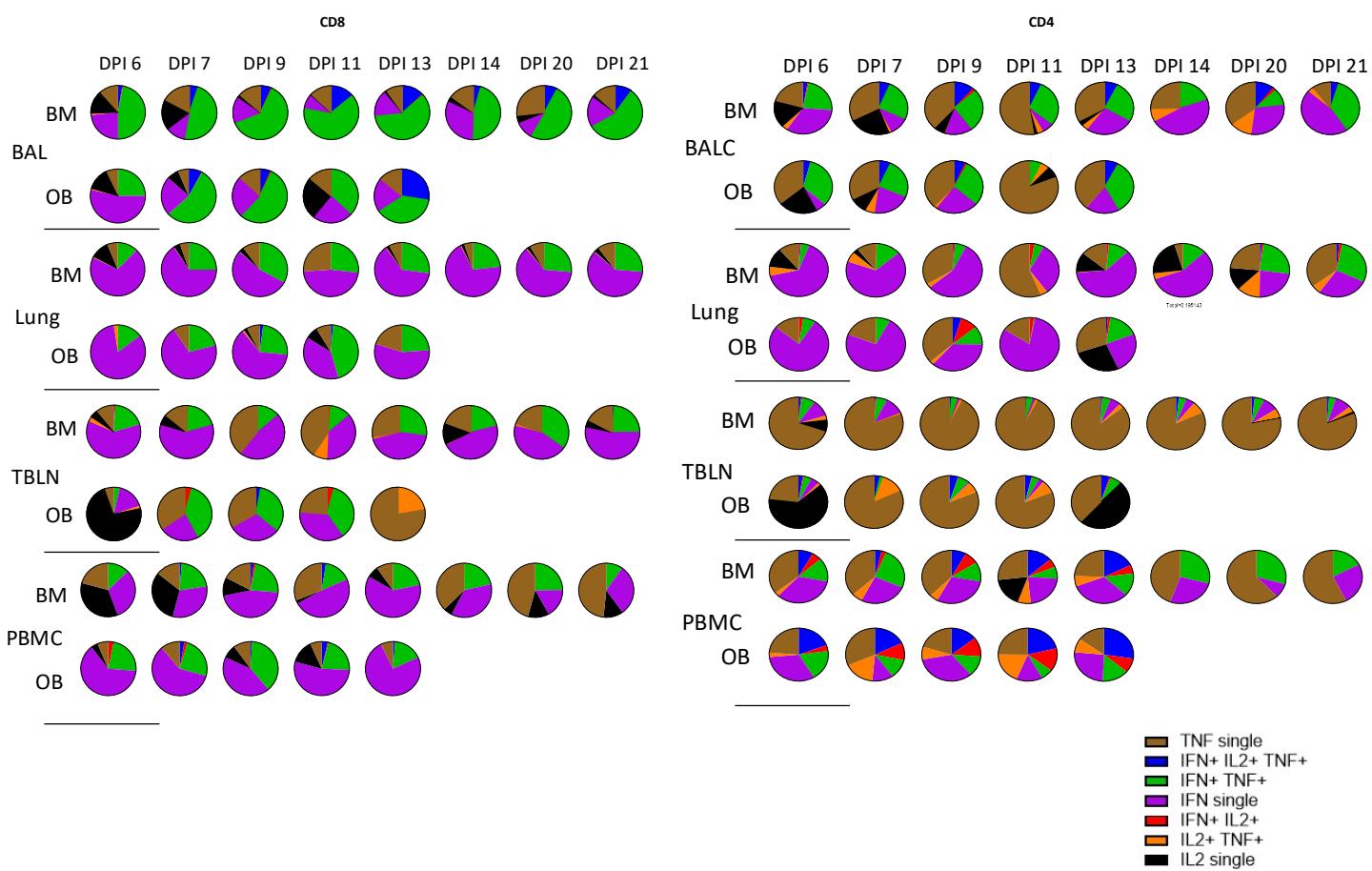
Supplementary Figure 1. Proportion of CD8 cells in OB and BM and gating strategy for tetramer identification. **(A)** Comparison of proportions of CD8 β and $\gamma\delta$ cells in control uninfected outbred (experiments OB1 and OB2) and inbred Babraham (experiments BM1 and BM2) pigs. Asterisks denote * $p \leq 0.05$, ** ≤ 0.005 , *** ≤ 0.0001 . **(B)** Gating strategy for identification of NP₂₉₀₋₂₉₈ tetramer T cells. BAL, lung, TBLN and PBMC were stained with the relevant antibodies. Lymphocytes were gated by light scatter, followed by exclusion of doublets and dead cells. CD3+, CD4- CD8 β + T cells were gates and the Tetramer+ population enumerated as a percentage of CD8 β + T cells.

Supplementary Figure 2

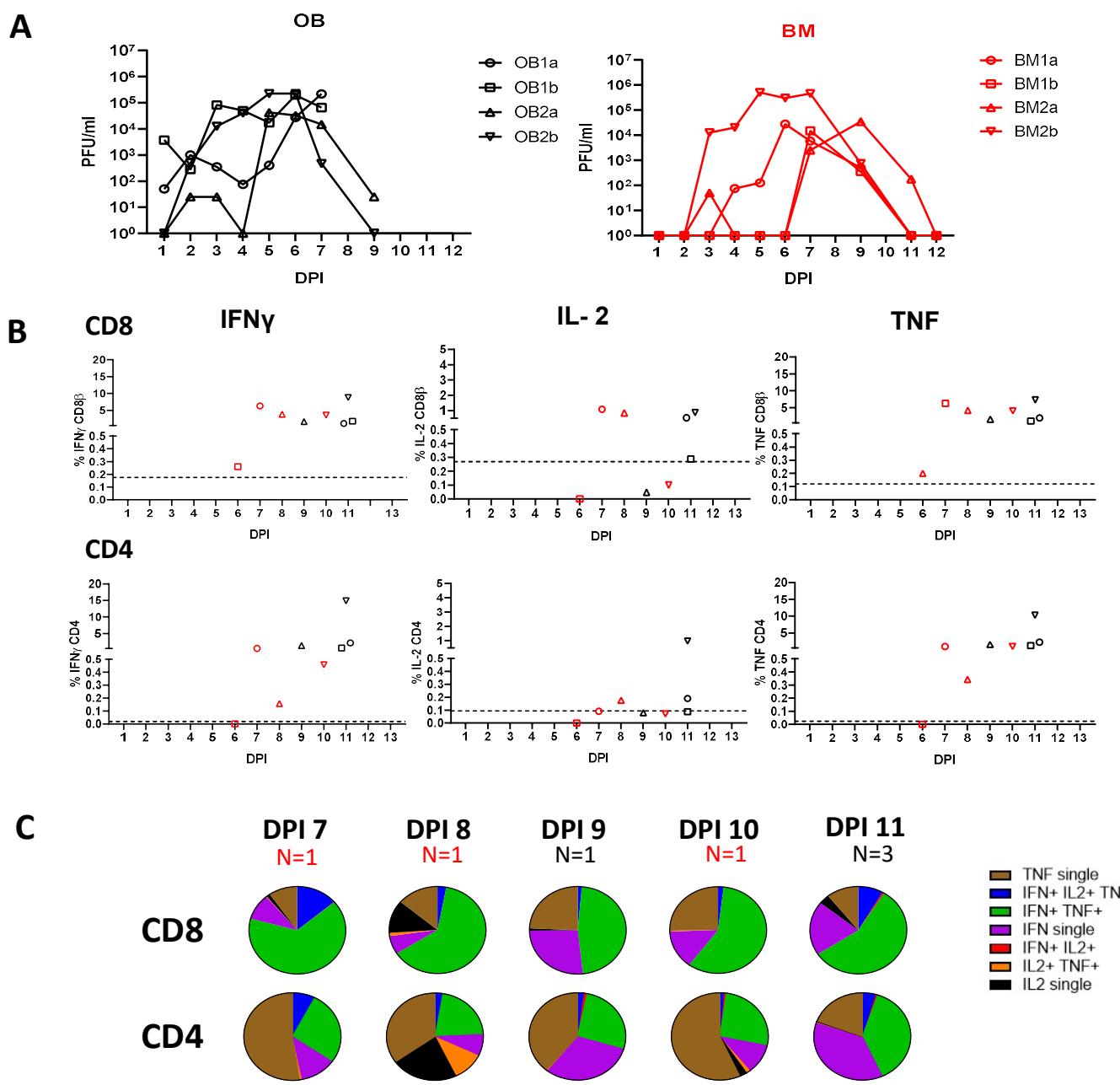


Supplementary Figure 2: Gating strategy and cytokine production by CD8 and CD4 cells. (A) Gating strategy for identification of CD8 β and CD4 cytokine producing cells. BAL, lung, TBLN and PBMC were stimulated with H1N1pdm09, followed by intracytoplasmic staining. Lymphocytes were gated by light scatter and further sub-gated for exclusion of dead cells with a live/ dead discrimination dye. CD8 β or CD4 cells were gated and expression of IFN γ , TNF or IL-2 was determined by histogram. Boolean gating of all cytokine positive cells was performed and cytokine responses determined by summing the total cytokine production in single, double and triple producing cells. (B) MFI of IFN γ secretion was determined in CD8 β and CD4 cells identified as IFN γ single, IFN γ /TNF double or IFN γ /TNF/IL-2 triple secreting T cells. IFN γ MFI is plotted for BM (red circles) and OB (black circles) animal. Asterisk indicates * $p\leq 0.05$, ** $p\leq 0.01$ and *** $p\leq 0.001$.

Supplementary Figure 3

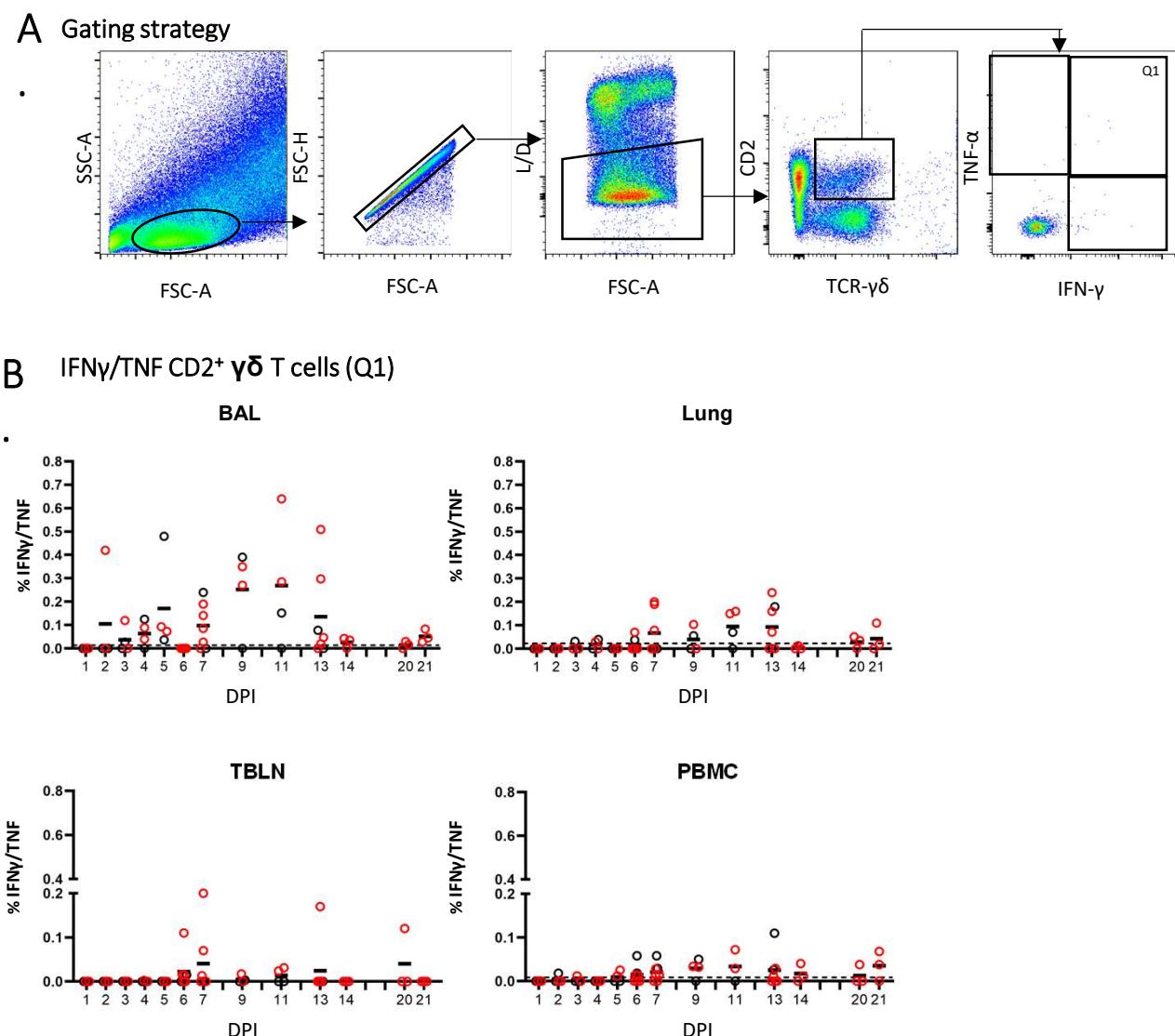


Supplementary Figure 3. Cytokine production by CD8 β and CD4 cells. Boolean gating of cytokine producing CD8 and CD4 T cells in tissues. Pie charts depict the proportion of single, double and triple IFN γ , IL-2 and TNF cytokine producing cells as a proportion of the total cytokine production in BAL, lung, TBLN and PBMC. Cytokine response for 6, 7, 9, 13 and 21 DPI for both OB and BM are shown. Medium control was subtracted from each stimulated population.



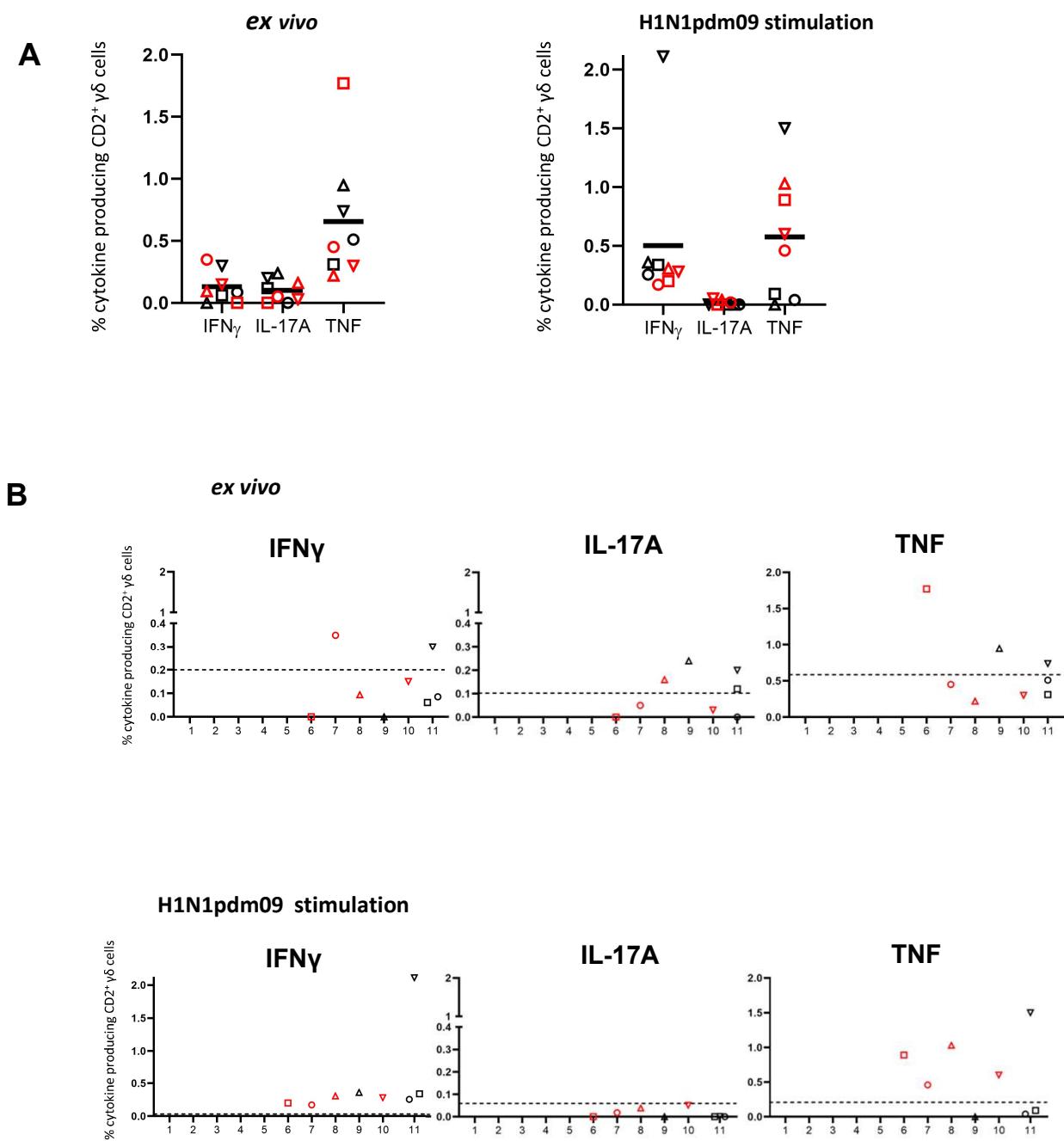
Supplementary Figure 4: CD8 β and CD4 cytokine response in BAL of in-contact animals. **(A)** Virus load in in-contact animals was determined by plaque assay of daily nasal swabs at the indicated time points for outbred (black) and inbred Babraham (red) pigs. **(B)** BAL CD4 and CD8 cytokine responses were determined by intracytoplasmic staining and shown as the days post infection counting from the first day the animal shed virus. **(C)** Pie charts show the proportion of single, double and triple cytokine secreting CD8 T cells for IFN γ , TNF and IL-2.

Supplementary Figure 5



Supplementary Figure 5. Double cytokine producing $\gamma\delta$ cells after H1N1pdm09 stimulation. (A) Lymphocytes were gated by light scatter properties and further sub-gated for exclusion of doublets and dead cells with a live/ dead discrimination dye. The CD2 $^+$ $\gamma\delta$ T cells were analyzed for co-production of IFN γ /TNF (Q1). (B) Frequency of IFN γ /TNF co-producing CD2 $^+$ $\gamma\delta$ T cells in outbred (black circles) and inbred (red circles) pigs following influenza infection. BAL, lung, TBLN and PBMC cells were stimulated overnight with H1N1pdm09 followed by intracellular cytokine staining. DPI 1 to 7, 9, 11 and 13 each show results from 2 outbred and 2 inbred pigs. DPI 6, 7, 13, 14, 20 and 21 also include results from 3 additional inbred pigs. The mean of the 22 uninfected control animals is represented by a dotted line.

Supplementary Figure 6



Supplementary Figure 4: BAL cytokine response in $\gamma\delta$ cells of in-contact animals. (A) Proportions of IFN γ , TNF and IL-17 cytokine producing cells in all in-contacts and (B) at each time point after infection for the individual in-contact animals shown as the days post infection counting from the first day the animal shed virus.