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► To cite this version:

Damien Garrido, Andreas Alber, Emmanuel Kut, Nathalie Katy Chanteloup, Adrien Lion, et al.. The role of type I interferons (IFNs) in the regulation of chicken macrophage inflammatory response to bacterial challenge. *Developmental and Comparative Immunology*, 2018, 86, pp.156-170. 10.1016/j.dci.2018.04.025 . hal-02627348

HAL Id: hal-02627348

<https://hal.inrae.fr/hal-02627348v1>

Submitted on 23 Feb 2024

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Citation for published version:

Garrido, D, Alber, A, Kut, E, Chanteloup, NK, Lion, A, Trotureau, A, Dupont, J, Tedin, K, Kaspers, B, Vervelde, L, Trapp, S, Schouler, C & Guabiraba, R 2018, 'The role of type I interferons (IFNs) in the regulation of chicken macrophage inflammatory response to bacterial challenge', *Developmental and Comparative Immunology*, vol. 86, pp. 156-170. <https://doi.org/10.1016/j.dci.2018.04.025>

Digital Object Identifier (DOI):

[10.1016/j.dci.2018.04.025](https://doi.org/10.1016/j.dci.2018.04.025)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Developmental and Comparative Immunology

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Accepted Manuscript

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PII: S0145-305X(18)30101-0

DOI: [10.1016/j.dci.2018.04.025](https://doi.org/10.1016/j.dci.2018.04.025)

Reference: DCI 3161

To appear in: *Developmental and Comparative Immunology*

Received Date: 6 March 2018

Revised Date: 29 April 2018

Accepted Date: 29 April 2018

Please cite this article as: Garrido, D., Alber, A., Kut, E., Chanteloup, N.K., Lion, A., Trotereau, Angé., Dupont, Joë., Tedin, K., Kaspers, B., Vervelde, L., Trapp, S., Schouler, C., Guabiraba, R., The role of type I interferons (IFNs) in the regulation of chicken macrophage inflammatory response to bacterial challenge, *Developmental and Comparative Immunology* (2018), doi: 10.1016/j.dci.2018.04.025.

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1 **The role of type I interferons (IFNs) in the regulation of chicken**
2 **macrophage inflammatory response to bacterial challenge**

3

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21

22 **Abstract**

23 Mammalian type I interferons (IFN α/β) are known to modulate inflammatory processes in
24 addition to their antiviral properties. Indeed, virus-induced type I interferons regulate the
25 mammalian phagocyte immune response to bacteria during superinfections. However, it
26 remains unresolved whether type I IFNs similarly impact the chicken macrophage immune
27 response. We first evidenced that IFN α and IFN β act differently in terms of gene expression
28 stimulation and activation of intracellular signaling pathways in chicken macrophages. Next,
29 we showed that priming of chicken macrophages with IFN α increased bacteria uptake,
30 boosted bacterial-induced ROS/NO production and led to an increased transcriptional
31 expression or production of *NOS2/NO*, *IL1B/IL-1 β* , and notably *IFNB/IFN β* . Neutralization
32 of IFN β during bacterial challenge limited IFN α -induced augmentation of the pro-
33 inflammatory response. In conclusion, we demonstrated that type I IFNs differently regulate
34 chicken macrophage functions and drive a pro-inflammatory response to bacterial challenge.
35 These findings shed light on the diverse functions of type I IFNs in chicken macrophages.

36

37 **Keywords:** Avian pathogenic *E. coli*; chicken; inflammation; interferon stimulated genes;
38 macrophages; type I interferons

39 1. Introduction

40 Interferons (IFNs) are key cytokines within the innate immune response. They were first
41 discovered in 1957 due to their capacity to inhibit influenza virus replication in embryonated
42 chicken eggs (Isaacs and Lindenmann, 1957). IFNs are divided into three subgroups: type I,
43 II, and III IFNs. Type I IFNs, including various subtypes of IFN α , IFN β , and some “minor”
44 IFNs (i.e. IFN δ , IFN ϵ , IFN κ , IFN τ , and IFN ω), are produced during viral and bacterial
45 infections (Bogdan et al., 2004; de Weerd and Nguyen, 2012; Ivashkiv and Donlin, 2014). In
46 mammals, most cell types are able to produce IFN β , including non-immune cells, while IFN α
47 is mainly produced by hematopoietic cells, especially plasmacytoid dendritic cells (Ivashkiv
48 and Donlin, 2014). IFN α and IFN β bind to the same receptor, Interferon-alpha Receptor
49 (IFNAR), which is composed of two subunits (IFNAR1 and IFNAR2) and expressed in the
50 majority of tissues (de Weerd and Nguyen, 2012).

51
52 Binding of type I IFNs to IFNAR entails the rapid activation of different signalling pathways
53 for the regulation of Interferon-Stimulated Genes (ISGs) (Hervas-Stubbs et al., 2011), many
54 of which play a critical role in the limitation of viral replication (Schneider et al., 2014). In
55 addition, type I IFNs have been shown to enhance antigen-presentation, regulate
56 inflammasome activation and upregulate pro-inflammatory cytokines production in
57 mammalian species (Hervas-Stubbs et al., 2011; Malireddi and Kanneganti, 2013; Simmons
58 et al., 2012). In humans, dysregulated type I IFNs responses were shown to be associated to
59 immune disorders such as chronic infection, autoimmune and inflammatory diseases
60 (Ivashkiv and Donlin, 2014; Trinchieri, 2010). Therefore, a tight regulation is required to
61 shape the outcome of type I IFN responses in order to achieve the balance between IFN-
62 mediated protective immunity and exacerbated IFN signalling (Trinchieri, 2010).

63

64 Virus-induced type I IFNs have been associated to impaired host immune responses such as
65 decreased bactericidal functions of phagocytic cells (Shepardson et al., 2016), granulocyte
66 apoptosis (Merches et al., 2015; Navarini et al., 2006), over-activation of the Nod1/Nod2
67 pathway (Kim et al., 2011), decreased chemokine secretion (Nakamura et al., 2011;
68 Shahangian et al., 2009), and attenuation of antimicrobial peptides expression (Lee et al.,
69 2015). Consequently, certain cell populations such as macrophages may become affected by
70 an enriched type I IFNs environment that is typical for viral infections (Shepardson et al.,
71 2016). These cellular and molecular events are at the origin of the well-established principle
72 that primary viral infections may predispose the host to bacterial superinfections (McCullers,
73 2014; Metzger and Sun, 2013). This observation is not restricted to humans and experimental
74 mammalian models, since poultry species, including galliform birds, are often impacted by
75 viral/bacterial co-infections (Ariaans et al., 2008; Gross, 1990; Kodihalli et al., 1994; Matthijs
76 et al., 2009; Nakamura et al., 1994). However, cell populations and cytokines involved in the
77 pathogenesis of co-infections affecting poultry are still poorly characterized.

78
79 In galliform birds, viral infections such as those caused by low pathogenic avian influenza
80 virus (LPAIV) may lead to a type I IFN response (Adams et al., 2009; Cornelissen et al.,
81 2012). However, it remains unclear whether this response contributes to predisposing animals
82 to bacterial superinfection through a dysregulated macrophage function. Nevertheless, it is
83 well established that macrophages play a key role during LPAIV infection and that they are
84 efficiently responding to type I IFNs (Kodihalli et al., 1994; Qu et al., 2013). In turkeys, it has
85 been demonstrated that LPAIV infection compromises pulmonary macrophages function,
86 which would likely predispose birds to secondary bacterial infections (Kodihalli et al., 1994).
87 These studies underscore the relevance of macrophages and their crucial role in the early
88 phases of infection for the priming of an efficient antiviral host response to limit viral

89 dissemination (Abdul-Cader et al., 2017; Duan et al., 2017; Fujisawa et al., 1987). In addition,
90 macrophages appear to have an important role in the control of avian colibacillosis, which is
91 caused by avian pathogenic *E. coli* (APEC) strains (Guabiraba and Schouler, 2015; Mellata et
92 al., 2003). Colibacillosis is the most relevant opportunistic bacterial infection of poultry. Its
93 severity is frequently correlated to a primary viral infection, notably in regard to its
94 pulmonary manifestation (Ariaans et al., 2008; Matthijs et al., 2009; Mosleh et al., 2017;
95 Nakamura et al., 1994; Nolan et al., 2008; Umar et al., 2018; Umar et al., 2017).

96

97 In the present study, we provided first insights on how chicken macrophages respond to type I
98 IFNs. In addition, we have set up an *in vitro* model based on IFN α priming of chicken
99 macrophages followed by stimulation with *E. coli* lipopolysaccharide (LPS) or infection with
100 avian pathogenic *E. coli* (APEC) to assess the impact of type I IFNs on the macrophage
101 response to bacterial challenge. Our data provide new information on the cellular and
102 molecular determinants of chicken macrophage functions in an inflammatory milieu likely to
103 be encountered during viral/bacterial co-infection in poultry.

104

105 **2. Materials and methods**

106 **2.1. Reagents**

107 Lipopolysaccharide (LPS, from *E. coli* O55:B5) was purchased from Sigma-Aldrich, UK.
108 Chicken IFN α and IFN β were produced in *E. coli* and purified as previously described
109 (Schultz et al., 1995a; Schultz et al., 1995b). Purified rabbit anti-chicken IFN β antiserum was
110 obtained as previously described (Schwarz et al., 2004). Recombinant chicken type I IFNs
111 and IFN β antiserum were tested negative for endotoxin contamination using HEK-Blue™
112 TLR4 cells designed for studying the stimulation of TLR4 by monitoring the activation of

113 NF- κ B and AP-1 (InvivoGen, USA). SB-203580 (p38 MAP Kinase inhibitor) and
114 Wortmannin (PI3-kinase/Akt inhibitor) were purchased from Tocris Bioscience, UK. BAY11-
115 7082 (I κ B- α inhibitor) and BX795 (TBK1/IKK ϵ inhibitor) were purchased from InvivoGen,
116 USA. During the experiments, LPS, IFN α , IFN β and IFN β -antiserum were diluted in RPMI
117 1640 medium (Gibco, UK). Inhibitors were diluted in dimethyl sulfoxide (DMSO, Sigma-
118 Aldrich, UK) and RPMI 1640 medium. Final concentration of DMSO in cell culture wells
119 never exceeded 0.1%.

120 **2.2. Cell culture**

121 HD11, an avian myelocytomatosis virus (MC29)-transformed chicken macrophage-like cell
122 line (Beug et al., 1979), was cultured in RPMI 1640 medium (Gibco, UK), supplemented with
123 10% heat-inactivated fetal calf serum (FCS, Gibco, UK), 25 mM HEPES, 2 mM L-glutamine,
124 100 U/ml penicillin and 100 μ g/ml streptomycin (GE Healthcare, USA). HD11 cells were
125 routinely grown in 75-cm² flasks (Corning, USA) at 41°C and 5% CO₂.

126 An HD11-NF κ B luciferase reporter cell line was constructed by infection of cells with
127 replication-incompetent, lentivirus-based pseudoviral particles harboring a vector containing a
128 basal promoter element (TATA box) and tandem repeats of an NF κ B consensus sequence
129 fused to a luciferase reporter gene (Cignal Lenti Reporters, SABiosciences, Frederick,
130 Maryland, USA). Cell lines expressing the reporter fusion were selected under puromycin
131 selection according to the manufacturer's instructions, and individual clones purified by
132 limited dilution. Clones were subsequently screened for NF κ B activation in response to LPS
133 and those showing high induction ratios with a low signal/noise ratio were retained. Cells
134 were routinely cultured in DMEM F-12 (1:1) medium (Gibco, UK), supplemented with 10%
135 heat-inactivated FCS, 15 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml

136 streptomycin and 5 µg/ml puromycin (Sigma-Aldrich, UK), and incubated as described
137 above.

138 The CEC32-Mx-Luc and the CEC32-NFκB-Luc reporter cell lines are quail fibroblast cell
139 lines carrying the luciferase gene under the control of chicken Mx promoter (Schwarz et al.,
140 2004) or carrying an NFκB-regulated luciferase reporter gene (Gyorfy et al., 2003),
141 respectively. CEC32-Mx-Luc and the CEC32-NFκB-Luc were kindly provided by Prof. Peter
142 Stäheli (University of Freiburg, Germany). CEC32 luciferase reporter cells were cultivated in
143 DMEM GlutaMAX™-I supplemented with 8% heat-inactivated FCS, 2% heat-inactivated
144 chicken serum (Gibco, UK), 4.5 g/l D-glucose, 100 U/ml penicillin, 100 µg/ml streptomycin
145 and 50 µg/ml geneticin (G418) (Gibco, UK) and grown in 25-cm² flasks (Corning, USA) at
146 41°C and 5% CO₂.

147 Chicken bone marrow derived macrophages (chBMDM) were generated from bone marrow
148 cells using recombinant chicken colony-stimulating factor 1 (CSF-1) (Garceau et al., 2010)
149 produced in COS-7 cells (fibroblast-like cell line derived from monkey kidney tissue, ATCC,
150 USA) transfected with a pTarget vector (Promega, UK) expressing chicken CSF-1 (kindly
151 provided by Prof. Pete Kaiser, The Roslin Institute, UK). Briefly, femurs and tibias of 4
152 week-old White Leghorn B13/B13 histocompatible chickens were removed, both ends of the
153 bones were cut and the bone marrow was flushed with RPMI 1640 supplemented with 100
154 U/ml penicillin and 100 µg/ml streptomycin. Cells were washed and re-suspended in RPMI
155 1640 medium then loaded onto an equal volume of Histopaque-1077 (Sigma-Aldrich, UK)
156 and centrifuged at 400 g for 20 min. Cells at the interface were collected and washed twice in
157 RPMI 1640 medium. Purified cells were seeded at 1x10⁶ cells/ml in sterile 60 mm
158 bacteriological petri dishes in RPMI 1640 medium supplemented with 10% heat-inactivated
159 FCS, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and
160 COS-7 supernatant containing chicken CSF-1 at 41°C and 5% CO₂. Half of the medium was

161 replaced with fresh medium containing chicken CSF-1 at day 3. At day 6 adherent cells were
162 harvested and washed in phosphate buffered saline (PBS, Gibco, UK) supplemented with 2
163 mM Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, UK) and re-suspended in RPMI
164 1640 supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 2 mM L-glutamine, 100
165 U/ml penicillin and 100 µg/ml streptomycin.

166 Chicken lung macrophages were obtained from transgenic birds expressing the fluorescent
167 mApple reporter under control of promoter and enhancer elements of the chicken CSF1R
168 locus (Balic et al., 2014) as described previously (Jansen et al. 2010). Briefly, lungs from 4
169 week-old MacRed chickens were removed, cut into pieces, and incubated with a DNase
170 I/collagenase A mix (1 mg/ml and 3 mg/ml, respectively; Sigma-Aldrich, UK) diluted in
171 supplement free RPMI 1640 medium for 30 min at 41°C and 5% CO₂. The digested tissue
172 suspension was filtered through a 70 µm strainer, washed with PBS, and leukocytes were
173 purified using a density gradient as described above. Cells at the interface were collected and
174 washed twice in PBS. Cells were cultured at 1.5x10⁶ cells/ml in 6-well plates in a final
175 volume of 5 ml with RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2
176 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 200 ng/ml of CSF-1 at
177 41°C and 5% CO₂ for 24h. The next day, culture medium containing non-adhered cells was
178 removed and replaced with fresh complete RPMI 1640 medium without CSF-1 for the
179 experimental treatment. The number of adherent macrophages was evaluated in parallel by
180 flow cytometry and was approximately 5x10⁵ cells per well.

181 **2.3. *In vitro* stimulation protocol**

182 HD11 and chBMDM cells were seeded in 12-well plates at 5x10⁵ cells/well and 7.5x10⁵
183 cells/well, respectively, and incubated at 41°C and 5% CO₂ overnight prior to stimulation.
184 Chicken lung macrophages at 5x10⁵ cells/well were obtained as described above and not re-

185 seeded. Next, the cells were pretreated for 16h with chicken recombinant IFN α (50 ng/ml)
186 unless otherwise indicated. Cells were gently washed with PBS prior to stimulation with IFN α
187 (50 ng/ml), LPS (10 ng/ml), purified IFN β -antiserum (17.5 μ g/ml) or IFN β (50 ng/ml) at
188 different treatment combinations and at different time-points. In all experiments, mock
189 controls were treated with medium or medium with 0.1% DMSO (when pharmacological
190 inhibitors were used). Supernatants were harvested and stored at -20°C after stimulation for
191 further analysis. HD11 cells and chBMDM were washed in PBS and lysed with RNA lysis
192 buffer (Macherey-Nagel, Germany) containing 2-mercaptoethanol (Merck Millipore,
193 Germany), snap frozen in liquid nitrogen and stocked at -80°C until RNA extraction. Chicken
194 lung macrophages were washed in PBS and lysed with RNA lysis buffer (Qiagen, Germany)
195 containing 2-mercaptoethanol. For protein dosage and western blot analysis, cells were
196 washed in PBS followed by cell lysis using Laemmli Sample Buffer (BioRad, USA)
197 containing a proteases inhibitors cocktail (Santa Cruz Biotechnology, USA) and 2.5% 2-
198 mercaptoethanol, and stocked at -20°C.

199 **2.4. Experimental design for *in vitro* infection**

200 HD11 were seeded in 12-well plates at 5×10^5 cells/well and chicken lung macrophages were
201 used at a final number of 5×10^5 cells/well in 6-well plates (in which they were obtained) in
202 complete RPMI 1640 medium, and incubated at 41°C under 5% CO $_2$ overnight. The APEC
203 strains used for infections were BEN2908 (O2:K1:H5), a nalidixic acid-resistant derivative of
204 strain MT78 which was isolated from the trachea of a chicken with respiratory infection (Dho
205 and Lafont, 1982) or BEN2908 harboring pFPV25.1 (a plasmid expressing GFP) (Valdivia
206 and Falkow, 1996). Bacteria were diluted at the appropriate concentration in supplement-free
207 RPMI 1640 medium, and cells were infected at a multiplicity of infection (MOI) of 10
208 followed by incubation at 41°C under 5% CO $_2$. The mock control group received supplement-
209 free RPMI 1640 medium without bacteria. After 1h (adhesion period), one group of APEC-

210 infected HD11 cells were washed with PBS then lysed with PBS containing 0.1% Triton X-
211 100 (Sigma-Aldrich, UK). Bacteria in the cell lysates were plated onto LB agar plates to
212 evaluate the number of adherent bacteria (colony-forming units). For the other groups, HD11
213 or chicken lung macrophages were gently washed with PBS and remaining extracellular
214 bacteria were killed by incubating cells with FCS-free medium containing gentamicin (100
215 $\mu\text{g/ml}$) for 1h 30 min. Cells were then lysed with 1X PBS containing 0.1% Triton X-100 or
216 harvested in FACS buffer (PBS supplemented with 2% heat-inactivated FCS and 2 mM
217 EDTA) for further flow cytometry analysis. Remaining intracellular viable bacteria were
218 plated onto LB agar to determine the number of intracellular bacteria. For gene expression
219 analysis, an additional group of HD11 cells were infected as described above, and incubated
220 with medium containing gentamicin (10 $\mu\text{g/ml}$) for 6h. Cells were washed with PBS then
221 lysed with RNA lysis buffer (Macherey-Nagel, Germany).

222 **2.5. Flow cytometry analysis**

223 Cell viability following different stimuli was assessed using the chicken Annexin V
224 Fluorescein kit (Kingfisher Biotech, USA) and the fluorescent DNA intercalator 7-
225 aminoactinomycin D (7-AAD, BD Biosciences, USA) as markers of apoptosis and necrosis,
226 respectively. HD11 cells were seeded at 2×10^5 cells/well in 24-well plates and pretreated with
227 IFN α (50 ng/ml) (unless otherwise indicated). Following stimulation with IFN α (50 ng/ml) or
228 LPS (10 ng/ml) for 6h, supernatants were discarded and the cells were harvested and washed
229 in PBS. Cells were stained according to the manufacturer's protocol and the viability was
230 analyzed by flow cytometry (BD FACS Calibur). Data were expressed as the percentage of
231 cells undergoing late apoptosis/necrosis (Annexin V⁺ 7-AAD⁺) over total acquired events
232 (50,000 cells).

233 Expression of co-stimulation markers on the chicken macrophage HD11 cell line was also
234 assessed. HD11 cells were treated as described above and harvested in FACS buffer (PBS
235 supplemented with 2% heat-inactivated FCS and 2 mM EDTA). Cells were washed and
236 incubated for 45 min at 4°C with a mouse anti-chicken CD40 antibody (BioRad, USA) at a
237 1:200 dilution or with the respective isotype control (BioRad) at a 1:200 dilution. Cells were
238 again washed and incubated for 45 min with a rat anti-mouse IgG-specific secondary antibody
239 coupled to fluorescein isothiocyanate (FITC, Thermo Fisher Scientific, USA). In a separate
240 staining protocol, cells were also stained with a mouse anti-chicken MHC class II FITC
241 (BioRad) or with the respective isotype control (BioRad) at a 1:200 dilution for 45 min. HD11
242 cells were washed and re-suspended in FACS buffer prior to analysis. Data were expressed as
243 Mean Fluorescence Intensity (MFI) from FITC⁺ cell populations over total acquired events
244 (50,000 cells).

245 Bacterial fluorescence was assessed by flow cytometry. Briefly, chicken lung macrophages
246 (5×10^5 cells/well in 6-well plates) were pretreated with the different stimuli and infected as
247 described above. Lung macrophages were detached from the plate using TrypLE Express
248 (Invitrogen, USA) for 15 min, washed, and harvested in FACS buffer prior to analysis (BD
249 LSRFortessa™). SYTOX Blue Dead Cell Stain (Invitrogen, USA) was added to discriminate
250 live and dead cells. Data were expressed as Mean Fluorescence Intensity (MFI) from GFP⁺
251 cell populations over total acquired events (50,000 live cells).

252 **2.6. Gene expression analysis**

253 Total RNA from HD11 cells and chBMDM was extracted from frozen cell lysates using the
254 NucleoSpin[®] RNA kit (Macherey-Nagel, Germany) according to the manufacturer's
255 instructions, while total RNA from lung macrophages was extracted using the RNeasy[®] Mini-
256 Kit (Qiagen, Germany). Both protocols contained a DNase treatment step. RNA quality and

257 concentration were determined by NanoDrop spectrophotometric measurement (Thermo
258 Scientific, USA). Total RNA (up to 1 µg per reaction) was reverse transcribed using iScript
259 Reverse Transcription Supermix for RT-qPCR (Bio-Rad, USA). Quantitative Real-time PCR
260 (qRT-PCR) was performed on a CFX96 machine (Bio-Rad, USA). The reaction mixture was
261 composed of cDNA, iQ SYBR Green Supermix (Bio-Rad, USA), primer pairs (Eurogentec,
262 Belgium) and nuclease-free water (Sigma-Aldrich, UK) in a total volume of 10 µl. qRT-PCR
263 data were analyzed using the CFX Manager software 3.1 (Bio-Rad, USA). Gene expression
264 for each target gene was normalized to gene expression levels of chicken hypoxanthine-
265 guanine phosphoribosyltransferase (HPRT), β-2-microglobulin (β2M) and/or glyceraldehyde-
266 3-phosphate dehydrogenase (GAPDH). A list of primer pairs utilized in the present study is
267 given in **Table 1**. Relative normalized expression was calculated using the $2^{-\Delta\Delta C_t}$ method
268 and data are represented as fold increase as compared to control (or mock) groups. Baseline
269 cycle threshold (Ct) values for the target genes in HD11 cells, chBMDM and lung
270 macrophages are shown in **Supplementary Table 1**.

271 **2.7. NO and ROS production**

272 Nitrite (NaNO₂) concentration, as an index of nitric oxide (NO) production, was determined
273 by spectrophotometry in cell culture supernatants using a standard Griess assay according to
274 the manufacturer's instructions (Promega, UK). The absorbance was read at 550 nm in a
275 Multiskan Ascent plate reader (Thermo Fisher Scientific, USA). The nitrite concentration was
276 calculated using a sodium nitrite standard curve.

277 ROS (Reactive Oxygen Species) production was evaluated by flow cytometry using the
278 CellROX[®] Green Reagent kit (Invitrogen, USA) according to the manufacturer's instructions.
279 Briefly, HD11 cells were seeded at 2×10^5 cells/well in 24-well plates and pretreated with
280 IFNα (50 ng/ml) for 16h (unless otherwise indicated). Next, cells were gently washed with

281 PBS then incubated with fresh medium containing LPS (10 ng/ml) or IFN α (50 ng/ml) for 6h
282 before the addition of CellROX[®] Green Reagent for 30 min. Cells were washed, harvested in
283 FACS buffer and ROS production was determined by flow cytometry analysis (BD FACS
284 Calibur). Data were expressed as Mean Fluorescence Intensity (MFI) from green fluorescent⁺
285 cell population over total acquired events (50,000 cells).

286 **2.8. Western blot**

287 Total protein was quantified using a Quick Start[™] Bradford Protein Assay (Bio-Rad, USA).
288 15 μ L of protein-containing lysates were separated on a 12% polyacrylamide gel in Tris-
289 Glycin-SDS buffer (25 mM, 200 mM and 0.5% respectively) and transferred to Porablot[®]
290 nitrocellulose membranes (0.45 μ m) (Macherey-Nagel, Germany) using a Mini Trans-Blot[®]
291 cell (Bio-Rad) in 1X CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) buffer. Following
292 overnight immersion at 4°C in a blocking solution (3% non-fat milk powder in buffer
293 containing 10 mM Tris, 150 mM NaCl and 0.1% Tween 20), the membranes were washed
294 and incubated for 1h at RT with a mouse anti-GAPDH antibody (MAB374, Millipore, USA)
295 at a 1:500 dilution (3% milk powder in Tris-NaCl-Tween buffer), a rabbit anti-p38 antibody
296 (#9212, Cell Signaling, USA) at a 1:1000 dilution, a rabbit anti-phospho-p38 antibody
297 (#9211, Cell Signaling) at a 1:1000 dilution, a mouse anti-STAT1 antibody (1/Stat1, BD
298 Bioscience, USA) at a 1:1000 dilution, a rabbit anti-phospho-STAT1 antibody (15H13L67,
299 Life Technologies, USA) at a 1:1000 dilution, a rabbit anti-Akt (pan) antibody (#4691, Cell
300 Signaling) at a 1:1000 dilution, a rabbit anti-phospho-Akt1/2/3 antibody (sc-7985-R, Santa
301 Cruz Biotechnology) at a 1:1000 dilution, a mouse anti-phospho-I κ B α (Ser32/36) antibody
302 (#9246, Cell Signaling) at a 1:1000 dilution, a rabbit anti-p44/42 MAPK (Erk1/2) antibody
303 (#4695, Cell Signaling) at a 1:1000 dilution or a rabbit anti-phospho-p44/42 MAPK (Erk1/2)
304 antibody (#4377, Cell Signaling) at a 1:1000 dilution. Membranes were washed three times
305 and incubated for 1h at RT with a mouse or rabbit IgG-specific secondary antibody coupled to

306 horseradish peroxidase (HRP, Sigma) at a dilution of 1:10000 (3% milk powder in Tris-NaCl-
307 Tween buffer). After washing, the membranes were overlaid with the WesternBright® ECL
308 peroxidase substrate (Advansta) and chemiluminescence was visualized using a Fusion-FX
309 imaging platform (Vilber Lourmat, France).

310 **2.9. Luciferase reporter assays**

311 Type I IFN and IL1 β production in supernatants of stimulated chicken HD11 cells or
312 chBMDM was measured using luciferase-based Mx- or NF κ B-reporter bioassays,
313 respectively (Gyorfy et al., 2003; Schwarz et al., 2004). Briefly, CEC32-Mx or CEC32-NF κ B
314 cells were seeded at 2.5×10^5 cells/well in 24-well plates and incubated at 41°C under 5% CO₂
315 overnight. The next day, cells were incubated for 6h with the diluted supernatants (1/10 of
316 total volume) from stimulated HD11 or chBMDM cell cultures. Medium was removed and
317 cells were washed twice with PBS. Cells were lysed using the Cell Culture Lysis Reagent
318 (Promega, USA), according to the manufacturer's instructions, and luciferase activity was
319 measured using the Luciferase assay reagent (Promega, USA) and a GloMax-Multi Detection
320 System (Promega, USA). Data were expressed as IFN β or IL1 β activity (fold increase as
321 compared to control group).

322 For NF κ B activity measurement in HD11 cells, HD11-NF κ B reporter cells were seeded at
323 2.5×10^5 cells/well in 24-well dishes and incubated at 41°C under 5% CO₂ overnight. The next
324 day, HD11-NF κ B cells were incubated for 6h with the indicated stimuli and inhibitors at the
325 appropriate concentration. Next, medium was removed and cells were treated as described for
326 CEC32-Mx or CEC32-NF κ B cells. Data are expressed as NF κ B activity (fold increase
327 relative to the control or mock group).

328 **2.10. Phagocytosis and endocytosis assay**

329 Phagocytosis or endocytosis (fluid-phase pinocytosis and receptor-mediated endocytosis)
330 capacity of HD11 cells were evaluated by flow cytometry using pHrodo™ Green *E. coli*
331 BioParticles® Conjugate or pHrodo™ Green Dextran (Invitrogen, USA), respectively.
332 Briefly, HD11 cells were seeded in 48-well plates at 2×10^5 cells/well and incubated at 41°C
333 under 5% CO₂ overnight. Then, cells were pretreated with IFN α (50 ng/ml) for 16h (unless
334 otherwise indicated). The next day, cells were gently washed with PBS then incubated with
335 fresh incomplete RPMI 1640 containing pHrodo™ Green Dextran at 50 μ g/ml or with
336 incomplete RPMI 1640 containing unopsonized pHrodo™ Green *E. coli* BioParticles®
337 Conjugate at 333 μ g/ml for 30 min and 1h, respectively. Cells were washed then harvested
338 with pre-warmed FACS buffer and subsequently analyzed by flow cytometry (BD FACS
339 Calibur). Data were expressed as the percentage of pHrodo green⁺ cell populations over total
340 acquired events (50,000 cells).

341 **2.11. Statistical analysis**

342 Comparisons between two groups were performed using a two-tailed unpaired Student's t test.
343 Multiple groups were compared using a one-way ANOVA analysis followed by a Tukey
344 multiple comparison post-hoc test. Values for all measurements are expressed as mean \pm
345 SEM. $P < 0.05$ was considered statistically significant. Data are representative of at least two
346 independent experiments unless otherwise indicated. Statistical analysis was performed using
347 the GraphPad Prism 6.0 software (GraphPad Software, USA).

348

349 **3. Results**

350 **3.1. IFN α and IFN β elicit different intracellular responses in chicken**
351 **primary macrophages**

352 To gain first insights into the functional roles played by type I IFNs in chicken primary
353 macrophages, we compared the expression profiles of various ISGs, IFN-regulatory
354 transcription factors and the pro-inflammatory responses elicited by IFN α and IFN β . At 2h
355 (data not shown) and 6h following type I IFNs stimulation, chBMDM showed increased
356 expression of the ISGs 2'-5' oligoadenylate synthetase (*OAS1*), myxovirus resistance protein
357 (*MX1*), double-stranded RNA-activated protein kinase (*EIF2AK2*) and signal transducer and
358 activator of transcription 1 (*STAT1*) as compared to the mock control group (**Figure 1A**).
359 Among the ISGs and associated transcription factors, only the expression of interferon
360 regulatory factor 7 (*IRF7*) proved to be more elevated after IFN β stimulation at the tested
361 time-points, while all other ISGs were more efficiently induced by IFN α . Yet, pro-
362 inflammatory genes such as *IL1B*, *IFNB*, *IL6* and *IL8L2* were markedly up-regulated upon
363 IFN β treatment as compared to cells treated with IFN α (**Figure 1B**). Our data therefore
364 demonstrated that, at least at early time-points, both type I IFNs play a role in triggering ISGs
365 expression, but IFN β has a more pronounced effect on pro-inflammatory cytokine gene
366 expression in chBMDM.

367 In mammals, STAT1, mitogen-activated protein kinases (MAPKs) p38 and p44/42 (Erk1/2),
368 PI3K/Akt, and NF κ B pathways have been shown to be involved in type I IFN signalling
369 (Hervas-Stubbs et al., 2011). We therefore performed western blot analyses to investigate
370 whether some of these signalling pathways were triggered by type I IFNs in chBMDM.
371 STAT1 expression was strongly induced from 6h to 24h upon exposure to IFN α as compared
372 to the mock control group (**Figure 2A**). These results confirmed that the upregulation of
373 STAT1 protein is correlated to the gene expression data (**Figure 1A**). Furthermore, IFN α
374 stimulation markedly induced early phosphorylation and activation of STAT1 (pSTAT1),
375 which lasted for up to 24h, as compared to the mock control group. In contrast to the situation

376 in cells stimulated with IFN α , STAT1 and pSTAT1 were only slightly induced by IFN β , both
377 displaying a modest, yet sustained induction at 24h post-stimulation (**Figure 2A**).

378 We also found that p38 MAPK, p44/42 MAPK and Akt were constitutively expressed in
379 chBMDM (**Figure 2A**). The p38 MAPK phosphorylated form (pp38) was expressed from 30
380 min to 6h following stimulation with IFN α or IFN β . Only low levels of pp38 were detected
381 24h after stimulation with both type I IFNs, suggesting that the p38 MAPK pathway is
382 activated only at early time-points following stimulation. In contrast to the rapid and clear
383 expression pattern observed for pp38, pp44/42 expression was weakly induced from 2h to 24h
384 following stimulation with IFN α or IFN β . This might suggest a minor (or indirect) role of
385 these cytokines in the activation of this pathway in chBMDM. In regard to the PI3K/Akt
386 pathway, pAkt expression was weakly induced by IFN α . However, stimulation with IFN β
387 markedly upregulated pAkt expression as early as 1h, with a quick decrease to mock control
388 group levels after 2h. Therefore, the PI3K/Akt pathway is likely to be better activated by
389 IFN β as compared to IFN α in chBMDM. Finally, we investigated the role of type I IFNs in
390 the expression of proteins involved in the activation of NF κ B. Activation occurs via
391 phosphorylation of I κ B α at Ser32 and Ser36 followed by proteasome-mediated degradation
392 that results in the release and nuclear translocation of active NF κ B (Hayden and Ghosh,
393 2008). Although we could not detect the expression of I κ B α in chBMDM using the present
394 western blot protocol and commercially available antibodies (data not shown), we
395 demonstrated that IFN β , but not IFN α , induced a marked expression of pI κ B α (Ser32/36) at
396 6h post-stimulation (**Figure 2A**).

397 To better characterize the potential activation NF κ B by type I IFNs, we used an HD11-NF κ B
398 luciferase reporter cell line. Cells were stimulated for 6h with IFN α or IFN β , and LPS
399 treatment was included as a positive control. As expected, and consistent with its incapacity to
400 directly induce a pro-inflammatory profile, the NF κ B pathway was not triggered by IFN α . In

401 contrast, it was strongly activated by IFN β as compared to the mock control group (**Figure**
402 **2B**), which is in line with its ability to induce phosphorylation of I κ B α at 6h (**Figure 2A**). In
403 addition, we observed that pharmacological inhibition of the intracellular signalling pathways
404 p38 MAPK, PI3K/Akt and TBK1/IKK ϵ partially reduced the activation of NF κ B induced by
405 IFN β (**Figure 2B**). The 7-AAD staining protocol revealed that none of the inhibitors were
406 found to be cytotoxic (necrotic cell death) at the concentrations used (data not shown). These
407 data suggest that the signalling pathways studied are likely to be involved in the activation of
408 NF κ B following stimulation with IFN β .

409 Taken together, our results revealed that IFN α and IFN β play different roles in the induction
410 of chicken macrophage intracellular signalling pathways upstream of the transcriptional
411 regulation of ISGs or pro-inflammatory genes.

412

413 **3.2. IFN α elicits similar response patterns in a chicken macrophage cell line** 414 **and in lung macrophages**

415 IFN α is the best studied type I IFN in birds (Giotis et al., 2016; Goossens et al., 2013; Roll et
416 al., 2017; Santhakumar et al., 2017). We therefore complemented our findings by assessing
417 the impact of IFN α in the well-established chicken macrophage cell line HD11 (Beug et al.,
418 1979). Incubation of HD11 cells with IFN α for 6h led to a marked increase in the expression
419 of the ISGs *OAS1*, *MX1* and *EIF2AK2* as compared to the mock control group (**Figure 3A**).
420 The interferon-regulated transcription factors *IRF7* and *STAT1* likewise showed a significant
421 increase in their transcriptional expression (3 and 7 fold, respectively) (**Figure 3A**). IFN α
422 stimulation did not alter *IL1B*, *NOS2*, *IFNA* and *IFNB* gene expression and nitric oxide (NO)
423 production in HD11 supernatants (**Figure 3B**). Furthermore, we confirmed by flow cytometry

424 analysis using Annexin V and 7-AAD staining that IFN α was not cytotoxic (late
425 apoptosis/necrosis) to HD11 cells after 6h or 16h of stimulation (**Figure 3C**).

426 To better improve our knowledge on the responses elicited by IFN α in chicken macrophages,
427 gene expression data for HD11 cells were compared to those obtained from chicken lung
428 macrophages at a matching time-point (6h). Except for *OAS1*, ISGs expression in lung
429 macrophages was significantly higher (307%, 154%, 300% and 100% for *EIF2AK2*, *MX1*,
430 *IRF7* and *STAT1*, respectively) than those found in HD11 cells (**Figure 3D**). Interestingly, the
431 baseline Ct values for the aforementioned ISGs (except for *OAS1*) are very similar between
432 HD11 cells and lung macrophages (**Supplementary Table 1**). Similarly to HD11 cells, no
433 increase in pro-inflammatory gene expression was observed (data not shown).

434 Consequently, the HD11 cell line was used in most experiments of the present study, because
435 of its easy accessibility, handling, and maintenance. Nevertheless, the most relevant findings
436 were further confirmed using chicken primary macrophages.

437

438 **3.3. IFN α priming potentiates the pro-inflammatory response to *E. coli* LPS** 439 **in chicken macrophages**

440 As demonstrated in chicken lung macrophages and in a cell line, IFN α strongly induced ISGs
441 but a negligible pro-inflammatory gene expression profile. Indeed, IFN α has been used in
442 priming strategies to assess the impact of type I IFNs in cellular responses to cytokines,
443 pathogen-associated molecular patterns (PAMPs) or pathogens in chickens and mammalian
444 species (Doughty et al., 2001; Jiang et al., 2011; Pei et al., 2001; Sharif et al., 2004). We
445 therefore asked whether a type I IFN enriched environment, likely to be encountered during
446 viral infections, could modulate the chicken macrophage inflammatory response to *E. coli*
447 LPS. We stimulated HD11 cells by incubating them with IFN α for 16h (priming time).

448 Shorter and longer exposition times were tested and showed to be less effective in inducing a
449 non-cytotoxic priming activity in this macrophage cell line (data not shown). HD11 cells were
450 subsequently stimulated for 6h with *E. coli* LPS (**Figure 4A**), a time point where gene
451 expression or NO production were peaking or produced consistently reproducible data.

452 We observed that IFN α priming followed by LPS stimulation (IFN α prm + LPS) markedly
453 upregulated *IL1B*, *NOS2* and *IFNB* gene expression, concomitant with NO and ROS
454 production, as compared to the group treated with LPS alone (**Figure 4B and 4C**). The same
455 stimulating effect was observed for the expression of the genes of interest at 2h (data not
456 shown). As expected, IFN α priming (IFN α prm) alone and IFN α stimulation (6h) had no
457 effect on pro-inflammatory gene expression and were ineffective in promoting ROS or NO
458 production (**Figure 4B and 4C**). We next used a bioassay to verify whether the *IFNB*
459 upregulation was associated to IFN β production by chicken macrophages. Under all treatment
460 conditions tested, *IFNA* gene expression was assed using different primer pairs (data not
461 shown) and was found to be never induced in HD11 cells, suggesting that the type I IFN
462 bioactivity determined in the bioassay would largely rest on IFN β production. Corroborating
463 the gene expression data, we observed that more IFN β was produced following LPS
464 stimulation when HD11 cells were previously primed with IFN α (**Figure 5A**). Although IFN α
465 priming potentiated the pro-inflammatory response to LPS, no additional cytotoxic effect was
466 observed when both molecules were added to the cells as compared to the group receiving
467 LPS alone (**Figure 5B**).

468 In addition, we evaluated the impact of IFN α and/or LPS on the expression of co-stimulation
469 markers by HD11 cells. Only LPS was able to upregulate CD40 expression, whereas IFN α
470 priming had no potentiating effect (**Figure 5C**). However, MHC II expression was increased
471 by IFN α priming (16h), but not by IFN α stimulation (6h). The addition of LPS did not

472 increase MHC II expression in priming or mock conditions at the same time-points (**Figure**
473 **5C**).

474 We confirmed these results by using chBMDM and applying the same experimental approach.
475 *IL1B*, *NOS2*, *IFNB* expression, and NO, IFN β and IL-1 β production (as assessed using the
476 CEC32-NF κ B reporter cell line), were all upregulated when chBMDM were primed with
477 IFN α for 16h and then challenged with LPS for 2h (data not shown) or 6h (**Supplementary**
478 **Figure 1A and 1B**). Interestingly, we observed that IFN α stimulation induced *IL1B* and
479 *NOS2* expression concomitant with NO production, which rapidly decreased to control group
480 levels. Notably, neither gene expression nor NO production was observed in the IFN α primed
481 group (16h), therefore excluding any potential cumulative effects on the priming approach.
482 Strikingly, the bioassay revealed that IFN β was also produced in the IFN α primed group.
483 However, HD11 cells were washed with PBS after the priming to avoid any contamination of
484 IFN α in the culture supernatant, therefore suggesting a sustained secretion of IFN β by these
485 cells (**Supplementary Figure 1B**).

486 In conclusion, IFN α priming favours the development of an increased pro-inflammatory
487 response to *E. coli* LPS in chicken macrophages without entailing cytotoxic effects.
488 Moreover, IFN α increases MHC II expression on the HD11 cell line, suggesting an increased
489 antigen presentation potential.

490

491 **3.4. IFN β mediates the increased pro-inflammatory response to *E. coli* LPS** 492 **in chicken macrophages following IFN α priming**

493 We observed that IFN β production induced by LPS was strongly enhanced by IFN α priming
494 in chicken macrophages. Previous studies have demonstrated that IFN β is involved in
495 different pro-inflammatory processes in mammals and chickens (de Weerd and Nguyen,

496 2012; Hervas-Stubbs et al., 2011; Santhakumar et al., 2017). We therefore speculated that
497 IFN β could be involved in the onset of a pro-inflammatory state induced by IFN α priming. To
498 test this, HD11 cells were treated as described earlier and a rabbit anti-chicken IFN β -
499 antiserum was added along with LPS in order to neutralize IFN β released into the medium. As
500 shown in **Figure 6A**, in the group primed with IFN α and subsequently challenged with LPS
501 for 2h (data not shown) and 6h, the addition of IFN β -antiserum (right bars) virtually
502 abrogated the potentiation effects of the priming protocol on *IL1B*, *NOS2* and *IFNB*
503 expression, and on NO and IFN β production. Importantly, IFN β -antiserum did not affect the
504 pro-inflammatory response induced by LPS alone (**Figure 6A**), and IFN β -antiserum was not
505 cytotoxic for chicken macrophages (**Figure 6B**).

506 The same experiments were also performed using chBMDM, in which IFN β -antiserum
507 addition similarly abrogated the potentiating effect of IFN α priming on the LPS-induced pro-
508 inflammatory response (**Supplementary Figure 2**). To corroborate our findings, we also
509 incubated chBMDM with LPS and IFN β . We observed that, when added together, LPS and
510 IFN β mimicked the potentiating pro-inflammatory effect induced by IFN α priming (**Figure**
511 **6C**).

512 Our data thus suggest that IFN β is a key mediator of the increased pro-inflammatory response
513 to LPS observed in chicken macrophages previously primed with IFN α .

514

515 **3.5. IFN α priming potentiates chicken macrophage pro-inflammatory**
516 **responses to APEC infection paralleled by an increased phagocytosis**
517 **capacity**

518 Since IFN α priming potentiates the chicken macrophage pro-inflammatory response to *E. coli*
519 LPS, we next explored whether IFN α priming modulates the macrophage response to avian
520 pathogenic *E. coli* (APEC) infection. HD11 cells were primed with IFN α for 16h then
521 infected with the highly adhesive/invasive APEC strain BEN2908 at an MOI of 10 for 6h.
522 Cells primed with IFN α and infected with the APEC strain (IFN α prm + APEC) showed an
523 up-regulation of *IFNB* expression, concomitant with an increased NO and IFN β production,
524 when compared to the non-primed BEN2908-infected group (APEC) (**Figure 7A**). *IL1B* was
525 also up-regulated in the primed group, but no statistical difference was seen compared to the
526 non-primed APEC group (**Figure 7A**).

527 In addition, we counted intracellular bacteria at 1h and 2h 30 min post infection in order to
528 assess the number of adhered and intracellular bacteria, respectively. Bacterial adhesion was
529 not affected by IFN α priming (**Figure 7B**) whereas the number of viable intracellular bacteria
530 was significantly increased when macrophages were primed with IFN α (**Figure 7C**).

531 We next asked whether the increased intracellular bacterial load could be mediated by an
532 IFN α -dependent enhancement of the endocytic or phagocytic capacity of HD11 cells.
533 Subsequent to several pilot experiments to identify the best time-points and reagents
534 concentrations to be used in these experiments (data not shown), endocytosis (fluid-phase
535 pinocytosis and receptor-mediated endocytosis) and phagocytosis were evaluated by flow
536 cytometry using fluorescent particles. We found that IFN α priming did not impact
537 endocytosis (**Figure 7D**) but it was able to significantly enhance the phagocytosis capacity of
538 chicken macrophages (20% increase) (**Figure 7E**).

539 The increase in intracellular bacterial uptake observed in HD11 cells following IFN α priming
540 was confirmed in experiments with chicken lung macrophages using a BEN2908 strain
541 expressing the GFP (**Figure 7F**). In addition, intracellular bacterial fluorescence was assessed

542 by flow cytometry and found to be higher in lung macrophages primed with IFN α (**Figure**
543 **7G**).

544 Altogether, these results evidenced that IFN α priming potentiates the pro-inflammatory
545 response to APEC infection in chicken macrophages paralleled by an increased phagocytic
546 activity, without detectable bactericidal consequences.

547

548 **4. Discussion**

549 Macrophage functions such as pathogen recognition, phagocytosis and cytokine expression
550 have been shown to be greatly impacted by type I IFNs in mammalian systems (Lee et al.,
551 2015; Nakamura et al., 2011; Shahangian et al., 2009; Shepardson et al., 2016). In chickens,
552 although extensive work have been done to understand type I IFN biology and their inhibitory
553 effects on virus replication (Giotis et al., 2016; Jiang et al., 2011; Mo et al., 2001; Pei et al.,
554 2001; Roll et al., 2017), many aspects of the type I IFN response remain unexplored, notably
555 in regard to macrophages and their inflammatory response. Our data revealed that for chicken
556 primary macrophages IFN α was a more potent inducer of ISGs expression (*OAS*, *MX1*, *PKR*
557 and *STAT1*) when compared to IFN β at 6h post stimulation. In contrast, at the same time point
558 post stimulation, IFN β proofed to be a better inducer of pro-inflammatory cytokine gene
559 expression (*IL1B*, *IFNB*, *IL6* and *IL8L2*). A previous study using the DF-1 chicken fibroblast
560 cell line showed that IFN α stimulation entails a strong antiviral profile, mainly through a
561 marked upregulation of ISGs associated to robust antiviral activity (Qu et al., 2013). In
562 contrast, IFN β appears to rather drive an immune modulatory response. Our data obtained
563 with chicken macrophages are in good agreement with this observation. Several hypothesis
564 have been made to explain these differential effects, including putative different affinities of
565 type I IFNs to the subunits of their cognate receptor (IFNAR1 and IFNAR2) (Santhakumar et

566 al., 2017). However, type I IFN signalling and the resulting gene expression patterns are
567 likely to be different between chicken macrophages and fibroblasts, due to the different
568 biological functions of these cells.

569 The distinct activity profiles observed for type I IFNs in the present study might be explained
570 by differences in the activation of intracellular signalling pathways. In mammals, STAT1 is
571 an important mediator of the JAK/STAT pathway in type I IFNs signalling, leading to the
572 transcription of ISGs (Hervas-Stubbs et al., 2011). In chicken macrophages, STAT1
573 phosphorylation was only weakly induced by IFN β , but remained on a steady level
574 throughout the stimulation period. In contrast, IFN α rapidly induced STAT1 phosphorylation.
575 We speculate that the differences in ISGs expression induced by the two type I IFNs is
576 directly linked to differential STAT1 pathway activation. Conversely, the p38 MAPK
577 pathway exhibited the same activation kinetics in response to both IFN α and IFN β . The role
578 of this signalling pathway for the induction of ISGs and other genes downstream of the type I
579 IFN receptor IFNAR has been demonstrated in mice (Li et al., 2004), but remains unclear in
580 birds.

581 Although 6h and 16h stimulation with IFN α alone was not found to strongly induce
582 transcriptional expression of *IL1B*, *NOS2*, and *IFNB* in HD11 cells and primary macrophages,
583 it potentiated the pro-inflammatory response to *E. coli* LPS, APEC LPS (data not shown) and
584 APEC infection. We hypothesize that IFN α may prime or modify intracellular events in the
585 macrophages, such as increasing the expression, production and/or activation of so far
586 unrecognized transcription factors, thereby promoting the IFN β over-production observed
587 after challenge with bacterial molecular patterns. In mammals, type I IFNs act through
588 JAK/STAT, CRB, PI3K/Akt, NF κ B and MAPK signalling pathways (Hervas-Stubbs et al.,
589 2011), which are still largely unexplored in chickens due to the lack of species-specific
590 pharmacological inhibitors and antibodies. One study has previously demonstrated that

591 chicken IFN α and IFN β promoters share binding regions for transcription factors of the IRF
592 family, and that the IFN β promoter has an additional NF κ B binding site (Sick et al., 1998).
593 We demonstrated here that only *IFNB* gene expression was induced after bacterial challenge,
594 in agreement with previously published data (Barjesteh et al., 2014). IFN β is known to induce
595 NF κ B activation (Hervas-Stubbs et al., 2011). We therefore assume that the NF κ B pathway
596 may be involved in IFN β production induced by LPS and in the inflammatory responses
597 elicited by IFN β . In fact, when we compared the effects of IFN α and IFN β using the HD11-
598 NF κ B reporter cell line and by assessing the phosphorylation of I κ B α , we observed that IFN α
599 did not activate this pathway, contrary to what was observed for IFN β . On the other hand, in
600 chicken primary macrophages, IFN α stimulation led to increased *IL1B* and *NOS2* gene
601 expression, all of which have been shown to be induced by the NF κ B pathway upon LPS
602 stimulation in mammals and chickens (Aktan, 2004; Contassot et al., 2012; He and Kogut,
603 2003; Weining et al., 1998). This suggests that the regulation of the NF κ B pathway in an
604 immortalized cell line and in primary macrophages seems to be different.

605 NF κ B activation by IFN β in chicken macrophages is partially dependent on the p38 MAPK,
606 PI3K/Akt and/or TBK1/IKK ϵ signalling pathways. To our knowledge, this is the first time
607 that such a mechanism of action has been described for a type I IFN in chickens. Since
608 pharmacological inhibition of these pathways did not completely abrogate NF κ B activation in
609 the HD11 cell line, other pathways involved in this activation remain to be identified. In
610 addition, IFN β induced the expression of *IRF7* and could therefore potentially activate this
611 transcription factor. Since both NF κ B and IRF7 may bind to specific binding motifs present in
612 the IFN β promoter (Sick et al., 1998), we assume that IFN β might be able to enhance its own
613 expression through an amplification loop involving the aforementioned signalling pathways.
614 Bacterial LPS activates Toll-like receptor 4 (TLR4) and triggers intracellular signalling
615 pathways leading to NF κ B activation via Myd88-dependent or the Myd88-independent

616 TRIF/TRAM-dependent pathways in mammals (Takeda and Akira, 2007). It is well
617 established in mammals that the TRIF/TRAM-dependent pathway also activates IRF3, which
618 results in IFN β induction (Kawai and Akira, 2010). Previous studies have demonstrated that
619 LPS-induced IFN β is crucial for LPS-dependent NO production (Vadiveloo et al., 2000), pro-
620 inflammatory cytokine and chemokine expression (Thomas et al., 2006), and LPS-derived
621 ISGs expression (Sheikh et al., 2014) in murine macrophages. In chickens, little is known
622 regarding the contribution of IFN β to LPS-induced inflammatory responses. Moreover, an
623 orthologue of the *Ticam2* gene (encoding TRAM) appears to be missing from the chicken
624 genome, suggesting that TLR4 signalling through TRIF/TRAM might not be functional or
625 ineffective in LPS-stimulated chicken cells (Keestra and van Putten, 2008). Nevertheless, our
626 findings demonstrated that *E. coli* LPS or APEC are able to induce IFN β production in
627 chicken macrophages, corroborating previous data obtained with the MQ-NCSU chicken
628 macrophage cell line (Barjesteh et al., 2014).

629 Although all the molecular events involved in IFN α -induced macrophage reprogramming
630 could not be identified, we demonstrated that the IFN α -activated STAT1 and p38 MAPK
631 signalling pathways are likely to play a role in rendering macrophages more prone to mount
632 an inflammatory response (via IFN β production) after bacterial challenge. Based on our data,
633 we assume that the cross-talk between signalling pathways downstream of TLR4 (e.g. NF κ B,
634 p38 MAPK and IRF7) and IFNAR leads to the potentiated production of IFN β , which in turn
635 activates NF κ B via p38 MAPK, PI3K/Akt, TBK1/IKK ϵ , and eventually other unidentified
636 pathways, ultimately resulting in the potentiated production of pro-inflammatory mediators.

637 Previous studies demonstrated that IFN α stimulation increases mouse peritoneal macrophage
638 phagocytosis and bacterial adhesion through potential modifications of membrane surface
639 receptors such as Fc or C3b receptors (Rollag et al., 1984). In the present study, we observed
640 that bacterial adhesion remained unchanged upon IFN α stimulation. Therefore, we surmise

641 that specific and non-specific bacterial receptors (e.g. scavenger receptors) may not account
642 for the enhancement in macrophage phagocytosis. In addition, Fc receptors do not appear to
643 be involved in this phenomenon since neither bacteria nor bio-particles were opsonized. We
644 thus assume that IFN α stimulation is able to induce unrecognized metabolic modifications
645 within chicken macrophages that led to an increased phagocytic activity. This mechanism is
646 likely to be IFN β -independent since IFN β -antiserum treatment did not reduce phagocytic
647 activity of IFN α -primed macrophages (data not shown).

648 A recent study demonstrated that a preceding LPAIV H9N2 infection increased innate
649 immunity-related gene expression in response to LPS challenge in the HD11 cell line (Qi et
650 al., 2016). Here we showed that a type I IFN enriched environment, likely to be present in
651 mucosal surfaces (respiratory and intestinal tract) infected with LPAIV, was sufficient to
652 reproduce this phenomenon in chicken macrophages. However, in the LPAIV infection-LPS
653 challenge system, *IFNA* and *TLR4* expression was found to be upregulated, a phenomenon
654 that was not observed in the present work, suggesting that these responses could be type I
655 IFN-independent. It would be challenging to test whether this pro-inflammatory response
656 might also occur *in vivo* in an LPAIV-APEC superinfection model. Yet, previous studies
657 indicated that LPAIV infection may pave the way for clinical colibacillosis in poultry (Bano
658 et al., 2003; Mosleh et al., 2017; Umar et al., 2018). Even though type I IFNs are produced
659 during viral infections, including low pathogenic avian influenza, the pathogenesis of
660 viral/bacterial co-infections may be very different depending on the viral pathogen or strain.
661 For example, during infectious bronchitis virus (IBV) infection, the type I IFN response
662 remained unchanged between co-infected and *E. coli* infected groups, suggesting that a type I
663 IFN response was not involved in the exacerbation of colibacillosis (Ariaans et al., 2008).

664 Altogether, our data provide the first evidence for the role of type I IFNs in modifying
665 chicken macrophage homeostasis, which may translate into a prominent pro-inflammatory

666 phenotype mediated by IFN β when these cells encounter bacteria. Our findings point to an
667 eminent role of this mechanism in the pathogenesis of viral/bacterial co-infections in the
668 chicken that warrants further investigation by an in-depth analysis of the innate immune
669 response in experimentally (co-)infected animals.

670

671 **Acknowledgments**

672 We thank the animal experimental unit PFIE (Plateforme d'Infectiologie Expérimentale,
673 Centre INRA Val de Loire, Nouzilly, France) and The National Avian Research Facility,
674 Roslin Institute, UK, for providing the animals used for the isolation of primary macrophages.
675 This study received financial support from the European program ERA-NET ANIHW
676 (Animal Health and Welfare) through funding of the MICHIC project (Understanding
677 mucosal immunology and co-infections in the chicken to drive vaccine strategies) and from
678 The Roslin Institute Strategic Grant funding from the BBSRC (BB/P013740/1). DG was
679 supported by a PhD fellowship from the Région Centre Val de Loire, France.

680

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876

877 **Figure Legends**

878 **Figure 1. IFN α and IFN β elicit different responses in chicken primary macrophages.**

879 chBMDM were stimulated with IFN α (50 ng/ml) or IFN β (50 ng/ml) for 6h before qRT-PCR

880 analysis. (A) OAS/OAS1, Mx/MX1, PKR/EIF2AK2, STAT1/STAT1 and IRF7/IRF7 gene

881 expression. (B) IL-1 β /IL1B, IFN β /IFNB, IL-6/IL6, CXCLi2/IL8L2 gene expression. qRT-

882 PCR data are expressed as relative normalized expression (as compared to mock control

883 group). Values are \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ or **** $P < 0.0001$ when
884 compared to mock control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ or #### $P < 0.0001$
885 when compared to IFN α group. Data are representative of two independent experiments
886 performed in triplicates.

887

888 **Figure 2. IFN α and IFN β differently modulate chicken primary macrophages signalling**
889 **pathways.** (A) chBMDM were stimulated with IFN α (50 ng/ml) or IFN β (50 ng/ml) for 30
890 min, 1h, 2h, 6h or 24h, then western blot analysis was performed on cell lysates.
891 Representative immunoblotting revelations of STAT1, pSTAT1, p38, ppP38, p44/42,
892 pp44/42, Akt, pAkt and pI κ B α are shown. Protein molecular weight is indicated with black
893 arrows. GAPDH was used as loading control. (B) HD11-NF κ B reporter cells were incubated
894 with IFN α (50 ng/ml), LPS (10 ng/ml) or IFN β (50 ng/ml) alone or in combination with
895 pharmacological inhibitors (SB-203580 at 10 μ M, Wortmannin at 2 μ M, BX795 at 2 μ M and
896 BAY11-7082 at 10 μ M) for 6h then luciferase activity was measured. Data are expressed as
897 fold increase in NF κ B activity, as compared to control groups, and values are \pm SEM. ** $P <$
898 0.01, *** $P < 0.001$ or **** $P < 0.0001$ when compared to mock control group. # $P < 0.05$, ## P
899 < 0.01 , ### $P < 0.001$ or #### $P < 0.0001$ when compared to the IFN α group (left graph) or the
900 IFN β group (right graph). Data are representative of two (A) or three (B) independent
901 experiments performed in triplicates.

902

903 **Figure 3. IFN α elicits similar response patterns in a chicken macrophage cell line and in**
904 **lung macrophages.** HD11 cells (A, B, and C) or macrophages from chicken lungs (D) were
905 stimulated with IFN α (50 ng/ml) for 6h before qRT-PCR analysis, nitric oxide (NO) dosage
906 or flow cytometry analysis for cell viability. (A) PKR/*EIF2AK2*, Mx/*MX1*, OAS/*OAS1*,

907 IRF7/*IRF7* and STAT1/*STAT1* gene expression, (B) IL-1 β /*IL1B*, IFN α /*IFNA*, IFN β /*IFNB*,
908 iNOS/*NOS2* gene expression (left Y axis) and NO production (right Y axis). (C)
909 Representative dot plot of HD11 double positive population for Annexin V and 7AAD,
910 canonical markers of cell death, and histogram showing the percentage of double positive
911 populations as compared to mock control group. (D) PKR/*EIF2AK2*, Mx/*MX1*, OAS/*OAS1*,
912 IRF7/*IRF7* and STAT1/*STAT1* gene expression. Values are \pm SEM. qRT-PCR data are
913 expressed as relative normalized expression (as compared to mock control group). *P < 0.05,
914 **P < 0.01, ***P < 0.001 or ****P < 0.0001 when compared to mock control group. Data are
915 representative of two (C and D) or three (A and B) independent experiments performed in
916 duplicates (A, B and C) or triplicates (D).

917

918 **Figure 4. IFN α priming potentiates the pro-inflammatory response to *E. coli* LPS in**
919 **chicken macrophages.** (A) Experimental protocol: HD11 cells were primed with IFN α (50
920 ng/ml) or mock treatments for 16h. The medium was removed and cells were washed before
921 receiving LPS (10 ng/ml), IFN α (50 ng/ml) or mock stimulation for 6h. (B) IL-1 β /*IL1B*,
922 iNOS/*NOS2*, IFN α /*IFNA*, IFN β /*IFNB* gene expression and NO production. qRT-PCR data are
923 expressed as relative normalized expression (as compared to mock control group). (C)
924 Reactive Oxygen Species (ROS) production measured by flow cytometry using a fluorescent
925 probe. Data are expressed as Mean Fluorescent Intensity (MFI). All values are \pm SEM. ***P
926 < 0.001 or ****P < 0.0001 when compared to mock control group. ###P < 0.001 or #####P <
927 0.0001 when compared to LPS group. Data are representative of four (B) or two (C)
928 independent experiments performed in triplicates.

929

930 **Figure 5. IFN α priming boosts MHC class II expression and IFN β production in**
931 **response to *E. coli* LPS stimulation without exerting any measurable cytotoxic effect on**
932 **chicken macrophages. (A)** IFN β production was quantified in HD11 supernatants through a
933 bioassay using a CEC32-Mx luciferase reporter cell-line. Data are expressed as fold increase
934 in IFN β activity as compared to control groups. **(B)** Cell viability was assessed by flow
935 cytometry using 7-AAD. Data are expressed as the percentage of dead cells (7AAD positive).
936 **(C)** CD40 or MHC class II expression was analysed by flow cytometry. Data are expressed as
937 Mean Fluorescence Intensity (MFI) from FITC⁺ cells. All values are \pm SEM. *P < 0.05, **P <
938 0.01, ***P < 0.001 or ****P < 0.0001 when compared to mock control group. #####P <
939 0.0001 when compared to LPS group. Data are representative of two independent experiments
940 performed in triplicates.

941

942 **Figure 6. IFN β mediates the increased pro-inflammatory response to *E. coli* LPS in**
943 **chicken macrophages following IFN α priming.** HD11 cells **(A and B)** or chBMDM **(C)**
944 were primed with IFN α (50 ng/ml) or mock treatments for 16h, then stimulated for 6h with
945 LPS (10 ng/ml) and/or IFN β -antiserum (17.5 μ g/ml), and/or IFN β (50 ng/ml) or IFN α (50
946 ng/ml). IL-1 β /*IL1B*, IFN β /*IFNB*, iNOS/*NOS2* gene expression and NO production in HD11
947 **(A)** and in chBMDM **(C)**. qRT-PCR data are expressed as relative normalized expression (as
948 compared to mock control group). **(B)** Cell viability was assessed by flow cytometry using 7-
949 AAD. Data are expressed as the percentage of dead cells (7AAD positive). Values are \pm SEM.
950 *P < 0.05, **P < 0.01 or ****P < 0.0001 when compared to mock control group. #P < 0.05 or
951 #####P < 0.0001 when compared to LPS group. Data are representative of two **(B and C)** or
952 three **(A)** independent experiments performed in duplicates.

953

954 **Figure 7. IFN α priming potentiates the chicken macrophage pro-inflammatory response**
955 **to APEC infection paralleled by an increased phagocytosis capacity.** HD11 cells (**A, B, C,**
956 **D and E**) or chicken lung macrophages (**F and G**) were primed with IFN α (50 ng/ml) or mock
957 treatments for 16h, then cells were infected with 10 MOI of APEC BEN2908 or BEN2908
958 GFP respectively, for 1h (adhesion assessment). Next, cells were treated with gentamycin to
959 kill extracellular bacteria for 1h 30 min (intracellular bacterial load assessment) and incubated
960 for up to 6h (for transcriptomic analysis). (**A**) IL-1 β /*IL1B* and IFN β /*IFNB* gene expression, as
961 assessed by qRT-PCR, and NO and IFN β production as assessed through nitrite dosage and
962 CEC32-Mx bioassay, respectively. The number of adhered bacteria (**B**) and intracellular
963 bacteria (**C and F**) was evaluated through colony-forming units (CFU) counts. Endocytosis
964 (**D**) and phagocytosis (**E**) capacities were evaluated by flow cytometry. Cells were pre-treated
965 with IFN α (50 ng/ml) or mock treatments for 16h, and incubated for 30 min with pHrodo[™]
966 Green dextran (endocytosis) or for 1h with pHrodo[™] Green *E. coli* BioParticles[®] Conjugate
967 (phagocytosis) at 50 μ g/ml and 333 μ g/ml, respectively. Data are expressed as the percentage
968 of pHrodo Green⁺ cells. (**G**) The fluorescence from intracellular GFP-expressing bacteria was
969 evaluated by flow cytometry and is expressed as Mean Fluorescence Intensity (MFI) from
970 GFP⁺ cells. Values are \pm SEM. *P < 0.05, **P < 0.01, ***P < 0.001 or ****P < 0.0001 when
971 compared to mock control group. #####P < 0.0001 when compared to APEC group. Data are
972 representative of two (F and G) or three (A, B, C, D and E) independent experiments
973 performed in triplicates.

974

975

Target genes	Forward primers 5'-3'	Reverse primers 5'-3'
<i>B2M</i> (β 2M)	CGTCCTCAACTGCTTCGCG	TTCGTGCTCCACCTTGC
<i>HPRT</i> (HPRT)	TGGTGGGGATGACCTCTCAA	GGCCGATATCCCACACTTCG
<i>GAPDH</i> (GAPDH)	GTCCTCTCTGGCAAAGTCCAAG	CCACAACATACTCAGCACCTGC
<i>EIF2AK2</i> (PKR)	GGGACATGATTGAGCCAAAGCAAGA	GAGCGTGGGGGTCTCCGGTA
<i>MX1</i> (Mx)	ACGTCCCAGACCTGACACTA	TTTAGTGAGGACCCCAAGCG
<i>OAS1</i> (OAS)	CTTCGGAGTCAGCATCACCA	TCCTGAATCACCTGCCCCAG
<i>IRF7</i> (IRF7)	TGCCTCAGGCGTCCCAATG	TGTGTGCCACAGGGTTGGC

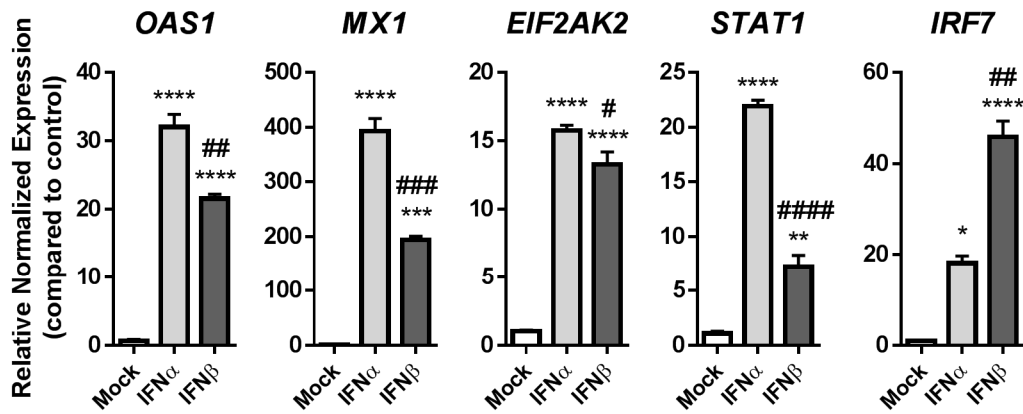
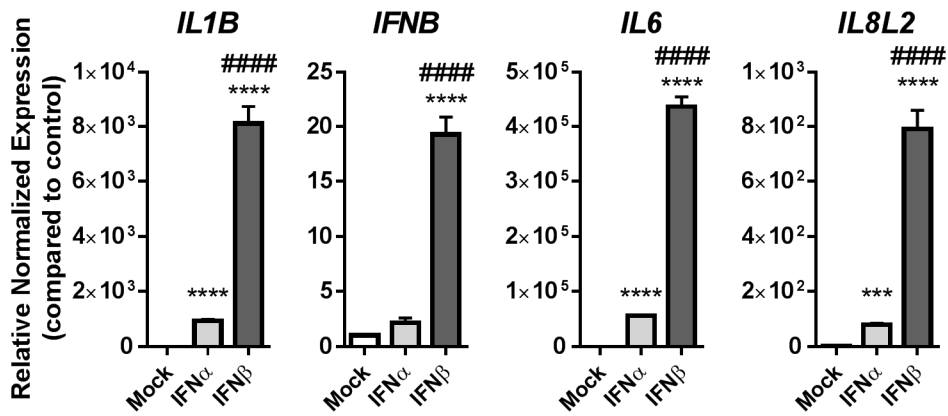
976

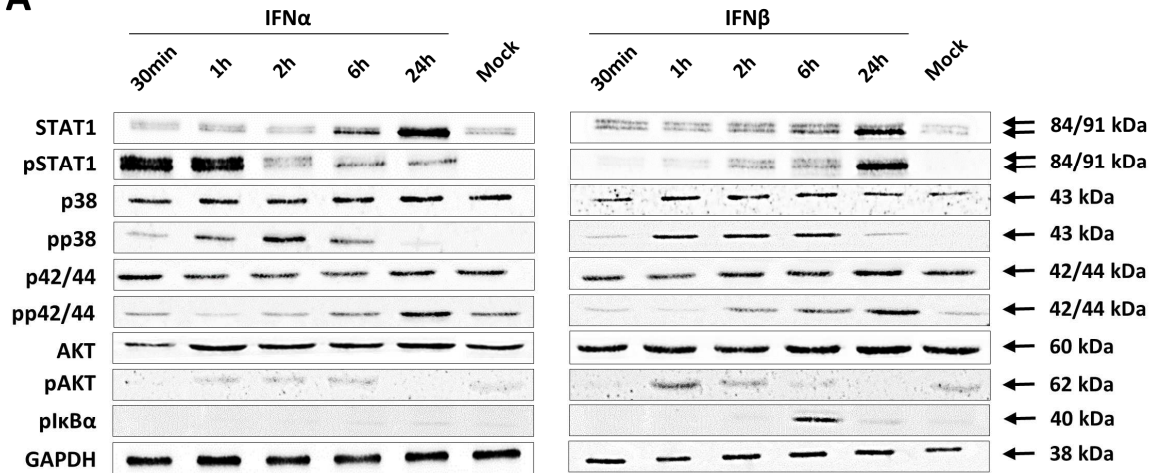
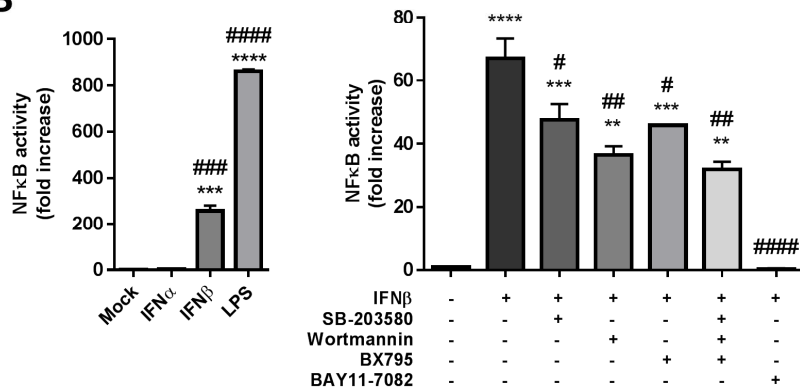
<i>STAT1</i> (STAT1)	AAGCAAACGTAATCTTCAGGATAAC	TTTCTCTCCTCTTTCAGACAGTTG
<i>IL1B</i> (IL1 β)	AGGCTCAACATTGCGCTGTA	CTTGTAGCCCTTGATGCCCA
<i>IFNA</i> (IFN α)	CAACGACACCATCCTGGACA	GGGCTGCTGAGGATTTTGAA
<i>IFNB</i> (IFN β)	TCCTGCAACCATCTTCGTCA	CACGTCTTGTTGTGGGCAAG
<i>NOS2</i> (iNOS)	CCACCAGGAGATGTTGAACTATGTC	CCAGATGTGTGTTTTCCATGCA
<i>IL6</i> (IL6)	GCTTCGACGAGGAGAAATGC	GCCAGGTGCTTTGTGCTGTA
<i>IL8L2</i> (CXCLi2)	CTGCGGTGCCAGTGCATTAG	AGCACACCTCTCTTCCATCC

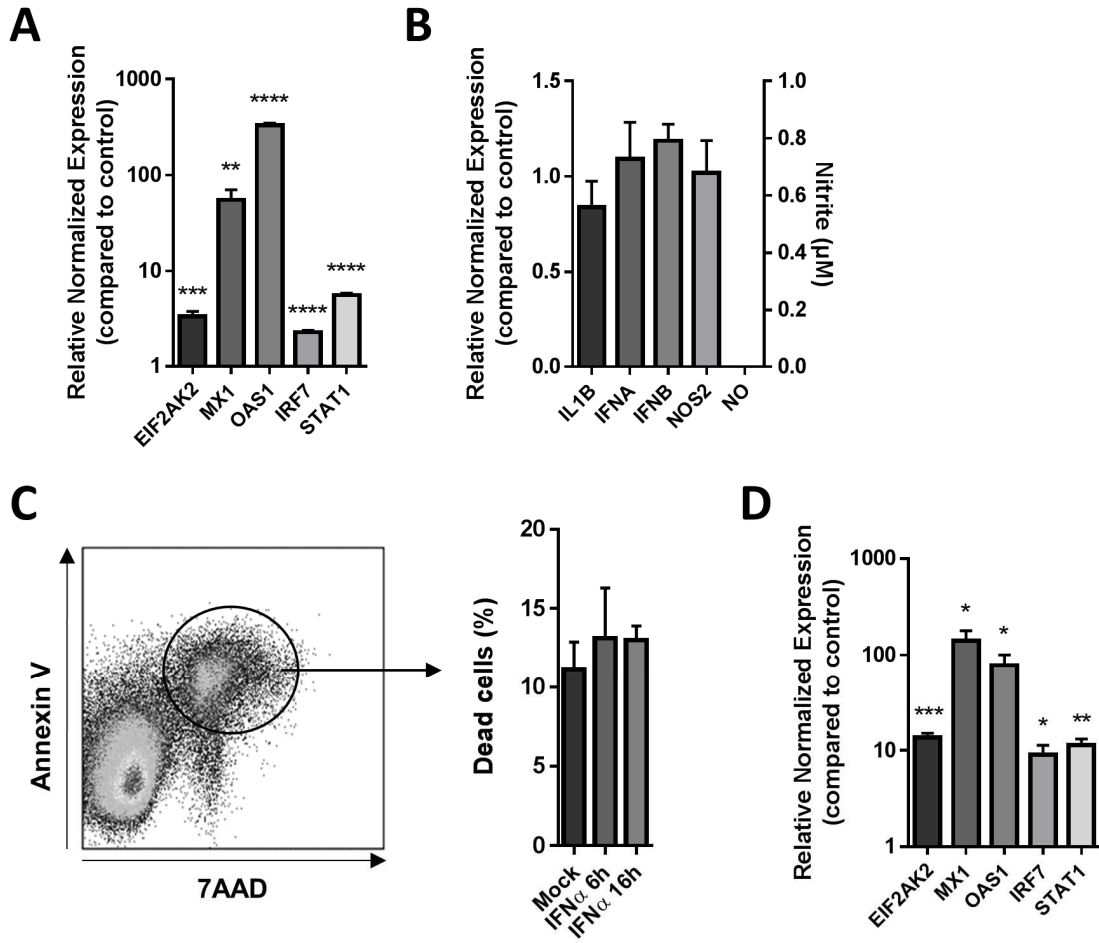
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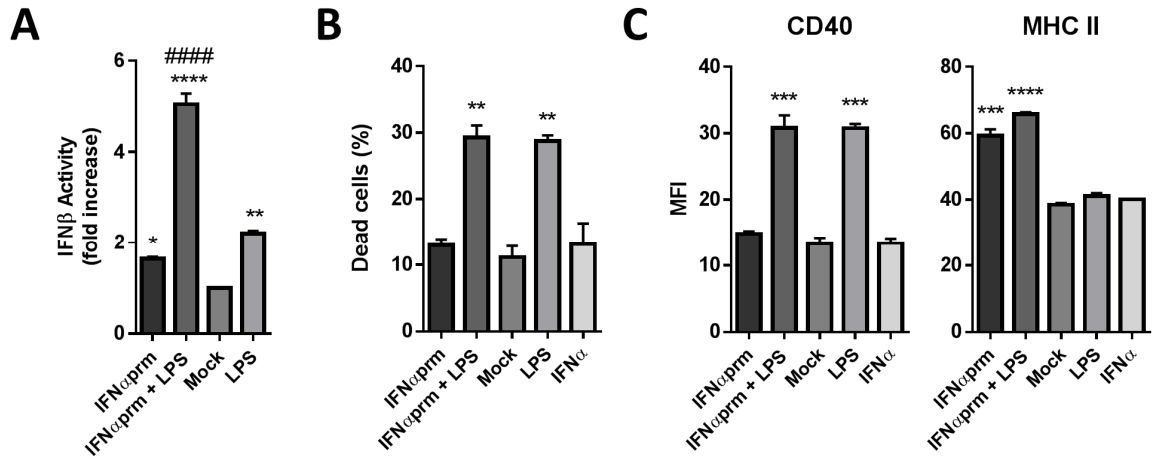
978 **Table 1.** Primer pairs used in the present study for qRT-PCR analysis

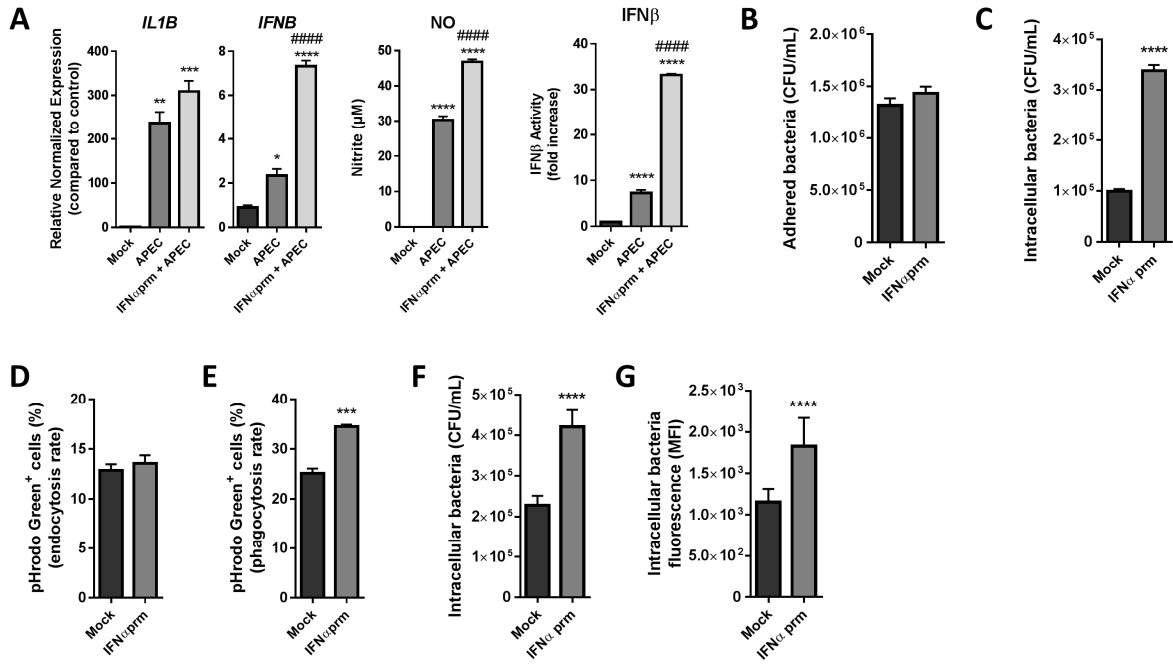
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A**B**

A**B**







Highlights:

- Type I IFNs differently regulate intracellular events in chicken macrophages
- IFN α priming boosts the macrophage inflammatory response to bacterial challenge
- This boost in the inflammatory response is mediated by IFN β
- Bacterial uptake is increased if chicken macrophages are primed with IFN α