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Susceptibility of bone marrow derived macrophages to influenza virus infection is dependent on macrophage phenotype

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Abstract:	The role of the macrophage in influenza virus infection is complex. Macrophages are critical for resolution of influenza virus infections but implicated in morbidity and mortality in severe infections. They can be infected with influenza virus and consequently macrophage infection is likely to have an impact on the host immune response. Macrophages display a range of functional phenotypes from the prototypical pro-inflammatory classically activated cell to alternatively activated anti-inflammatory macrophages involved in immune regulation and wound healing. We were interested in how macrophages of different phenotype respond to influenza virus infection and therefore have studied the infection of bone marrow derived macrophages (BMDMs) of classical and alternative phenotype in vitro. Our results show that alternatively activated macrophages are more readily infected and killed by the virus than classically activated. Classically activated BMDMs express the proinflammatory markers inducible nitric oxide synthase (iNOS) and TNFα and TNFα expression was further up-regulated following infection. Alternatively activated macrophages express Arginase-1 and CD206, however, following infection, expression of these markers is down regulated while expression of iNOS and TNFα is upregulated. Thus, infection can override the anti-inflammatory state of alternatively activated macrophages. Importantly, however, this results in lower levels of pro-inflammatory markers than those produced by classically activated cells. Our results show that macrophage phenotype affects the inflammatory macrophage response following infection and indicate that modulating the macrophage phenotype may provide a route to develop novel strategies to prevent and treat influenza virus infection.

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1

2

3 # Susceptibility of bone marrow derived macrophages to influenza
4 # virus infection is dependent on macrophage phenotype

5

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22 **Summary**

23 The role of the macrophage in influenza virus infection is complex. Macrophages are critical
24 for resolution of influenza virus infections but implicated in morbidity and mortality in severe
25 infections. They can be infected with influenza virus and consequently macrophage infection
26 is likely to have an impact on the host immune response. Macrophages display a range of
27 functional phenotypes from the prototypical pro-inflammatory classically activated cell to
28 alternatively activated anti-inflammatory macrophages involved in immune regulation and
29 wound healing. We were interested in how macrophages of different phenotype respond to
30 influenza virus infection and therefore have studied the infection of bone marrow derived
31 macrophages (BMDMs) of classical and alternative phenotype *in vitro*. Our results show that
32 alternatively activated macrophages are more readily infected and killed by the virus than
33 classically activated. Classically activated BMDMs express the proinflammatory markers
34 inducible nitric oxide synthase (iNOS) and TNFα and TNFα expression was further up-
35 regulated following infection. Alternatively activated macrophages express Arginase-1 and
36 CD206, however, following infection, expression of these markers is down regulated while
37 expression of iNOS and TNFα is upregulated. Thus, infection can override the anti-
38 inflammatory state of alternatively activated macrophages. Importantly, however, this results
39 in lower levels of pro-inflammatory markers than those produced by classically activated cells.
40 Our results show that macrophage phenotype affects the inflammatory macrophage response
41 following infection and indicate that modulating the macrophage phenotype may provide a
42 route to develop novel strategies to prevent and treat influenza virus infection.

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45 **Introduction**

46 Influenza A viruses impose a considerable burden on human health. Seasonal influenza virus
47 infections range from mild to life-threatening with the outcome dependent on both strain of
48 virus and host response. They are associated with significant morbidity and mortality,
49 particularly in the elderly and the very young. The emergence of highly pathogenic avian
50 strains that can infect humans, albeit with a limited ability to spread human to human, presents
51 an additional threat (Webster & Govorkova, 2014). A key feature of these strains is an
52 overreactive immune response that fails to control the infection, resulting in excessive
53 production of cytokines and chemokines, influx of immune cells and severe immunopathology
54 which contributes to high mortality (Cheung *et al.*, 2002; Kobasa *et al.*, 2007; Korteweg & Gu,
55 2008).

56

57 Influenza virus primarily infects epithelial cells in the respiratory track but also has a well-
58 recognized ability to infect macrophages (Rodgers & Mims, 1982; Tumpey *et al.*, 2005).
59 Macrophages play a central role in initiating and controlling the immune response to infections
60 and infection of these innate immune cells is highly likely to have consequences for the
61 outcome of infection. It is clear, however, that the ability to infect and replicate in macrophages
62 is dependent on both virus strain and origin of the macrophage and that the results of infection
63 are highly variable (Nicol & Dutia, 2014; Short *et al.*, 2012). Infection of monocyte derived
64 macrophages *in vitro* with highly pathogenic viruses leads to production of higher levels of
65 inflammatory cytokines and chemokines than are produced in response to infection with low
66 pathogenicity seasonal viruses (Cheung *et al.*, 2002; Zhou *et al.*, 2006) suggesting that
67 infection of macrophages may be, at least in part, responsible for the increased pathogenicity
68 of these viruses. *In vivo* studies, however, have shown that a virus strain that readily infects
69 macrophages *in vitro* is less pathogenic in mice than a related virus that fails to infect
70 macrophages (Tate *et al.*, 2010). In this instance, depletion of macrophages increases the
71 virulence suggesting that macrophage infection can attenuate pathogenesis.

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72 Macrophages are not, however, a single homogeneous population. They are highly
73 pleiomorphic cells with a range of phenotypes and functions (Gordon & Taylor, 2005). At the
74 extremes of the spectrum of macrophage phenotypes are the "classically activated" or M1
75 macrophage, generally considered to be pro-inflammatory, producing TNFα, IL-6, IL-1β and
76 inducible nitric oxide synthase (iNOS), while the "alternatively activated" or M2 macrophage
77 has up-regulated expression of the macrophage mannose receptor CD206 and MHC class II,
78 produces high levels of Arginase-1 (Arg-1) and endocytic function and is considered to be
79 associated with wound healing and repair. IFNγ and TNFα drive classical macrophage
80 activation while alternative macrophage activation is driven by the Th2 cytokines IL-4 and IL-
81 13. Between these two extremes lies a range of subtly different phenotypes which orchestrate
82 and regulate the immune response (Gordon, 2003).
83
84 We hypothesized that susceptibility and subsequent response of macrophages to influenza
85 virus infection may depend on their phenotype, and that it would be possible to alter the extent
86 of infection and thus influenza-associated pathology by manipulating macrophage phenotype.
87 Here we show that alternatively activated BMDMs are more susceptible to infection with the
88 A/WSN/33 strain of influenza virus than classically activated BMDMs and are more readily
89 killed by infection. Infection of alternatively activated BMDMs overrides their anti-inflammatory
90 state, inducing a pro-inflammatory macrophage phenotype. However, infection of alternatively
91 activated BMDMs results in production of lower levels of pro-inflammatory markers including
92 iNOS and TNFα than infection of classically activated BMDMs. Overall, our study supports
93 the hypothesis that alternatively activated macrophages have a protective role in highly
94 pathogenic virus infection.
95
96

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97 **Results**

98 **129Sv/Ev bone marrow derived macrophages can be infected with influenza virus strain**
99 **A/WSN/33**

100 Previous studies have reported that influenza viruses can infect macrophages with varying
101 efficiency in a virus strain dependent manner (Reading *et al.*, 2000; Rodgers & Mims, 1981;
102 Tate *et al.*, 2010). We wished to investigate the ability of A/WSN/33 to infect macrophages
103 from mice on the 129Sv/Ev background, therefore we derived macrophages from femurs of 6-
104 8 week old female 129Sv/Ev mice by culture for 7 days in medium containing M-CSF. FACS
105 analysis showed that >95% of the cells expressed the macrophage markers CD11b and F4/80
106 (data not shown) and therefore were of macrophage phenotype (Misharin *et al.*, 2013). The
107 bone marrow derived macrophages (BMDMs) were infected with varying multiplicities of
108 infection (MOI) using viral titres determined on MDCK cells, incubated for various lengths of
109 time and stained for viral antigen using a polyclonal antibody directed against purified H1N1
110 virus. No antigen positive cells were detected at 1h post-infection (Fig1b) but positive cells
111 were detected from 6h post-infection (fig 1c,d) indicating that viral protein synthesis was
112 required for antibody staining. An MOI of 10 resulted in infection of around 60% of cells but
113 increasing the amount of input virus did not increase the percentage of cells infected further.
114 Poisson distribution predicts that, if BMDMs were as infected with the same efficiency as
115 MDCKs, an MOI of 5 should result in infection rate of > 99% of cells. Thus, although BMDM
116 can be infected with A/WSN/33, they are less readily infected than MDCK cells.
117

118 **Effect of macrophage phenotype on infection**

119 In order to produce polarized macrophage populations, we treated macrophages derived from
120 wild type 129Sv/Ev mice and mice on the same genetic background lacking the IFNγ receptor
121 (IFNγR$^{-/-}$) with IFNγ or IL-4 for 16 hours and measured levels of iNOS and Arg-1 by qRT-PCR
122 and biochemical assay. Figure 2 shows that treatment of both 129Sv/Ev and IFNγR$^{-/-}$ BMDMs
123 with IL-4 (Fig 2a,b) for 16 hours led to induction of Arg-1 mRNA and arginase activity, indicating

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124 that the macrophages had differentiated to an alternatively activated –like phenotype.
125 Treatment of 129Sv/Ev BMDMs with IFNγ resulted in production of iNOS mRNA and iNOS
126 activity (Fig 2c,d) confirming that these BMDMs had differentiated to a classical phenotype.
127 IFNγR$^{-/-}$ BMDM cannot respond to IFNγ and therefore were not able to produce a classical
128 macrophage response, ie they did not upregulate iNOS upon IFNγ treatment (Fig 2c,d). We
129 next infected the polarized macrophages with A/WSN/33 and stained with antibody to H1N1
130 virus (Fig 3). Treatment of 129Sv/Ev and IFNγR$^{-/-}$ BMDMs with IFNγ or IL-4 and infection with
131 virus did not affect the cell density (Fig 3a-c, f-h). However, it was clear from 6hr post-infection
132 that IL-4 treated macrophages were more readily infected than IFNγ treated macrophages (Fig
133 3d & i). Similar results were observed at 48h post-infection (Fig 3e & j) and intervening time
134 points. Thus, alternatively activated macrophages from both strains of mice are more readily
135 infected than classically activated.
136
137 Consistent with the higher levels of viral antigen present in alternatively activated
138 macrophages, qRT-PCR showed significantly higher amounts of M1 mRNA synthesized in
139 these cells indicating higher levels of viral infection ($p<0.001$) (Fig 4).
140
141 **Survival of influenza virus infected macrophages**
142 The survival of influenza virus infected BMDMs was assessed by use of the CellTiter-Blue
143 viability assay. Figures 5 a & b show that by 20h post-infection, infection of alternatively
144 activated macrophages resulted in a significantly lower rate of viability than seen in infected
145 classically activated macrophages. Interestingly, 129Sv/Ev IFNγ treated macrophages show a
146 trend towards higher viability than untreated BMDMs from the same mice (Fig 5a). This
147 difference is not apparent for the IFNγR$^{-/-}$ BMDMS which cannot respond to IFNγ suggesting
148 that classical activation confers protection against virus induced cell death. These data
149 suggest that IL-4 activation renders BMDM more permissive for A/WSN/33 and that once
150 infected, these cells are more readily killed by the virus than classically activated cells. In order

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151 to determine whether higher levels of productive virus infection occurred in alternatively
152 activated macrophages, we measured the infectious virus present in the cell supernatants (Fig
153 5c). At 48h post-infection, the amount of virus in supernatants was higher than at 1h post-
154 infection indicating that there was a low level replication in the BMDMs. However, the amount
155 of virus recovered was less than the amount of input virus and we found no evidence that
156 alternative activation resulted in production of higher levels of infectious virus by the BMDMs.
157
158 **Cytokine response to influenza virus infection**
159 In order to assess the effect of influenza virus infection on macrophage phenotype, we
160 analysed the expression of phenotypic markers in infected/polarized BMDMs. iNOS, TNFα
161 and IL-12p40 were chosen as markers of classically activated macrophages. iNOS is
162 associated with inflammatory macrophage response and the secreted cytokines TNFα and IL-
163 12p40 contribute to macrophage driven inflammatory response. In addition to Arg-1, which is
164 highly expressed in alternatively activated macrophages (Fig 2), we measured expression of
165 the mannose receptor, CD206 which is upregulated on alternatively activated macrophages
166 (Gordon, 2003). BMDMs were treated with IFNγ or IL-4 and infected with A/WSN/33 at an MOI
167 of 10. Cells were harvested at 48 hours post-infection and the expression of markers was
168 measured by qRT-PCR. Treatment of 129Sv/Ev macrophages with IFNγ upregulated iNOS
169 by $>10^4$ fold and no further upregulation was induced by infection with virus (Fig 6a). Similar
170 results were found for IL-12p40 (data not shown). In contrast, TNFα expression in these
171 macrophages, although elevated by IFNγ alone, was significantly higher in cells which had
172 been infected with virus ($p<0.05$, Fig 6e). Thus virus infection can drive expression of this pro-
173 inflammatory cytokine. Arg-1 and CD206 expression in the IFNγ treated 129Sv/Ev
174 macrophages was low and virus infection did not alter this (Fig 6c,g). As expected, IFNγ
175 treatment of macrophages grown from IFNγ$R^{-/-}$ mice did not result in significant changes in
176 expression of the classical markers iNOS and TNFα (Fig 6a,e). Infection of IFNγ$R^{-/-}$
177 macrophages with influenza virus upregulated iNOS expression (Fig 6a) indicating the virus

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178 alone could switch on expression of this inflammatory marker. TNFα expression was clearly
179 completely dependent on the IFNγ activity as there was no change in expression following
180 virus infection of the IFNγR$^{-/-}$ macrophages (Fig 6e). Similarly, there was no significant change
181 in IL-12p40 expression (data not shown). Thus, infection of classically activated macrophages
182 leads to increased expression of TNFα enhancing the pro-inflammatory state of these
183 macrophages. In the absence of IFNγ responsiveness infection led to enhanced expression
184 of iNOS but did not induce synthesis of secreted pro-inflammatory cytokines (TNFα and IL-12)
185 indicating that these macrophages did not enter a pro-inflammatory state.
186
187 Alternative activation of BMDMs from both wild type 129Sv/Ev and IFNγR$^{-/-}$ mice resulted in
188 upregulation of Arg-1 and CD206 compared to untreated BMDMs (Fig 6d,h). Classical markers
189 were low or undetectable in these cells (Fig 6b,f). However, infection of alternatively activated
190 129Sv/Ev BMDMs induced expression of iNOS and TNFα indicating that virus infection could
191 override the alternative, anti-inflammatory state of these macrophages, produce a pro-
192 inflammatory state within the cells (iNOS) and led to secretion of pro-inflammatory cytokines
193 (Fig 6b,f). Similarly, iNOS was significantly upregulated following infection of alternatively
194 activated IFNγR$^{-/-}$ BMDMs (Fig 6b; $p<0.001$) but levels were lower than those produced in
195 alternatively activated 129Sv/Ev BMDMs. Again, expression of TNFα was completely
196 dependent on responsiveness to IFNγ (Fig 6f). Infection of alternatively activated wild type
197 129Sv/Ev and IFNγR$^{-/-}$ BMDMs led to decreased expression of Arg-1 and CD206 (Fig 6 d,h).
198 Thus, infection of alternatively activated macrophages led to down regulation of alternative
199 markers and induction of the pro-inflammatory mediators iNOS and TNFα. However, the levels
200 of these cytokines produced by alternatively activated macrophages are less than those
201 produced by classically activated macrophages. Whilst infection can clearly override the anti-
202 inflammatory state, infection of alternatively activated macrophages leads to lower levels of
203 pro-inflammatory cytokine production than those observed following infection of classically
204 activated macrophages.

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205 **Discussion**

206 Our results show that alternatively activated macrophages are more susceptible to infection
207 with A/WSN/33 than classically activated cells. A higher percentage of cells express viral
208 antigens and higher levels of M1 mRNA are produced in alternatively activated cells than in
209 classically activated cells. Similarly, Hoeve *et al* (Hoeve *et al.*, 2012) showed that following
210 infection with the H3N2 virus Udorn, a significantly higher number of human monocyte derived
211 macrophages with anti-inflammatory characteristics contained viral antigen than those with
212 proinflammatory phenotype. The effect of IL-4 on uptake of antigens by macrophages is
213 dependent on both antigen and pathway. Treatment of macrophages with IL-4 leads to
214 increased uptake of soluble antigen as well as increased mannose receptor dependent uptake
215 of antigen (Montaner *et al.*, 1999; Raveh *et al.*, 1998). However, alternative activation of
216 macrophages with IL-4 has been shown to impair phagocytosis of bacteria and microbial
217 particles (Varin *et al.*, 2010). The higher levels of M1 mRNA in IL-4 treated cells argue that
218 the presence of virus antigen in cells is not simply due to increased phagocytosis of viral
219 antigens but rather is due to increased infection of these macrophages. Influenza viruses
220 usually enter cells by endocytosis following initial binding of the HA to sialic acids on the cell
221 surface (Matlin *et al.*, 1981; Skehel & Wiley, 2000). However, there is evidence that influenza
222 A virus can use other cell surface molecules including the macrophage mannose receptor
223 CD206, macrophage galectin type lectins DC-SGN and L-SIGN to bind to and enter
224 macrophages (Londrigan *et al.*; Reading *et al.*, 2000; Upham *et al.*, 2010). Alternatively
225 activated macrophages express a different range of cell surface proteins to those found on
226 classically activated macrophages. For example, CD206, is more highly expressed on the
227 surface of alternatively activated macrophages than on classically activated and is indeed
228 considered a marker for alternative activation (Gordon, 2003; Stein *et al.*, 1992). At this point,
229 further work is required to understand the mechanisms by which alternatively activated
230 macrophages are more readily infected but this may have important implications for influenza
231 virus pathogenesis.

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232 Interestingly, alternatively activated macrophages are more readily killed by infection with
233 A/WSN/33 than classically activated macrophages. Influenza virus infection leads to cell death
234 hence it is likely that the difference reflects the level of infection. qRT-PCR data show that at
235 48h post-infection there is up to 100 fold more M1 mRNA in alternatively activated cultures
236 than in classically activated. This, together with the fact that a higher percentage of
237 alternatively activated macrophages is infected is consistent with the higher level of cell death
238 found in alternatively activated cultures. It is notable, however, that the apparently more
239 permissive state of alternatively activated macrophages did not lead to production of higher
240 levels of infectious virus than are found in classically activated cells. Although there is
241 evidence for productive influenza virus infections in human macrophages (Hoeve *et al*., 2012;
242 Perrone *et al*., 2008; van Riel *et al*., 2011; Yu *et al*., 2011), a number of publications have
243 reported that influenza virus infection is abortive in murine macrophages (Rodgers & Mims,
244 1981; Tate *et al*., 2011; Tate *et al*., 2010). Our data show that whilst there is some replication
245 of A/WSN/33 in murine BMDMs the ability to produce infectious virus is not related to the
246 activation state of the macrophage. The ease with which alternatively activated macrophages
247 become infected has important implications, i.e. manipulation of phenotype *in vivo* may allow
248 macrophages to act as a sink for virus.

249

250 Infection of both classically and alternatively activated 129Sv/Ev macrophages resulted in up-
251 regulation of TNFα, a cytokine associated with severe influenza virus infections *in vivo*.
252 Macrophages can produce IFNγ (Gessani & Belardelli, 1998; Schroder *et al*., 2004) and
253 therefore it is likely that autocrine production of this cytokine contributes to the ability of
254 alternatively activated macrophages to override the IL-4 response and produce an
255 inflammatory response. Infection also resulted in upregulation of iNOS and IL12p40 in
256 alternatively activated 129Sv/Ev macrophages. Type I interferons and IL-1β can induce
257 synthesis of iNOS (Gao *et al*., 1998; Geller *et al*., 1995) and it is likely these cytokines together
258 with TNFα and IFNγ are involved in induction of iNOS and IL-12p40 following virus infection

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259 (Drapier *et al.*, 1988; Farrell & Blake, 1996; Ma *et al.*, 1996). The upregulation of
260 proinflammatory markers together with the down regulation of Arg-1 and CD206 expression
261 demonstrates that infection results in a switch in cell phenotype. However, although both
262 classes of macrophage produced a pro-inflammatory response following virus infection,
263 alternatively activated macrophages produced lower levels of proinflammatory markers than
264 classically activated macrophages. Thus, the data support the hypothesis that manipulation
265 of the macrophage phenotype could have an impact on influenza virus pathogenesis.

266

267 IFNγ$R^{-/-}$ macrophages were significantly compromised in pro-inflammatory responses and
268 TNFα production was severely limited. Influenza virus infection did induce iNOS and IL-12p40
269 in these macrophages, most likely due to the action of type I interferons and IL-1β, but levels
270 were 1000 fold less than in wild type classically activated infected macrophages. Non-
271 activated IFNγ$R^{-/-}$ BMDMs have significantly higher expression of CD206 than 129Sv/Ev
272 BMDMs. Given that CD206 has been shown to act as a receptor for influenza virus, it is
273 interesting to speculate that this may play a role in the increased susceptibility to infection
274 found in these macrophages. Overall, these data provide evidence IFNγ responsiveness is
275 critical to the macrophage response to influenza virus infection and highlight the role of IFNγ
276 as a critical cytokine in pathogenesis of influenza virus infections.

277

278 We have chosen to investigate the infection of BMDM and have successfully demonstrated
279 that alternatively activated macrophages and those which cannot effectively mount a classical
280 response (IFNγ$R^{-/-}$) are more susceptible to influenza virus infection. Whilst these studies were
281 carried out with BMDMs rather than alveolar macrophages, they provide important clues for
282 understanding the function of macrophages in control of influenza virus infections. Recently it
283 has been shown that alternatively activated alveolar macrophages can protect against lethal
284 challenge in a mouse model (Wang *et al.*, 2013). That study did not address macrophage

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285 infection but our data would suggest that infection of alternatively activated macrophages per

286 se is likely to play a role in this protective effect.

287

288 Macrophages clearly play a critical role in influenza virus infection. Depletion of macrophages

289 in animal models leads to exacerbation of infection indicating their importance in an effective

290 host response to infection (Tate *et al.*, 2011; Tumpey *et al.*, 2005). Similarly, transfer of

291 macrophages accounts for the protective effect of prior infection with a herpesvirus (Saito *et*

292 *al.*, 2013). However, they are major producers of inflammatory cytokines and hence have been

293 implicated in the development of the severe pathology associated with fatal infections (Cheung

294 *et al.*, 2002; Perrone *et al.*, 2008). Our study shows that macrophages of different phenotypes

295 respond very differently to influenza virus infection. Given the diversity and plasticity of

296 macrophages, understanding the interaction between macrophage phenotype and virus

297 infection is likely to be fundamental to the development of novel strategies to prevent and treat

298 severe influenza virus infections.

299

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300 **Methods**

301 **Cell culture**

302 L929 murine fibroblasts were grown in tissue culture flasks (Nunc) in Rosewell Park Memorial
303 Institute Medium (RPMI) supplemented with 10% foetal calf serum, 100units/ml penicillin,
304 100μg/ml streptomycin and 2mM L-glutamine (Invitrogen). Supernatant from confluent
305 cultures was pooled, clarified by centrifugation at 8000g and stored in aliquots at -20ºC as a
306 source of M-CSF for bone marrow derived macrophages (BMDM).

307

308 Madin Darby canine kidney cells (MDCKs) were grown in Dulbecco's Modified Eagles Medium
309 (DMEM, Invitrogen) supplemented as for RPMI.

310

311 **Virus growth and assay**

312 A/WSN/33 was propagated on MDCKs at a multiplicity of infection (MOI) of 0.001 for 48 hours
313 before harvest of supernatant containing the virus. Supernatant was clarified by centrifugation
314 at 3000g and stored in aliquots at -80°C. Titre was determined by plaque assay as previously
315 described (Nicol *et al.*, 2012).

316

317 **Macrophage isolation, activation and infection**

318 129Sv/Ev and IFNγR$^{-/-}$ mice on the 129Sv/Ev background (Huang *et al.*, 1993) were purchased
319 from B & K Universal and bred in-house. Femurs from 6-8 week old female mice were
320 removed, cleaned in alcohol and the bone marrow flushed out with supplemented RPMI, using
321 a 25G needle and syringe. Bone marrow cells were plated onto 100mm bacteriological dishes
322 (Sterilin) in 50% supplemented RPMI: 50% L929 fibroblast conditioned media (complete
323 macrophage media). Cultures were initially set up by seeding cells from one femur in each
324 plate. Bone marrow derived macrophages were passaged on day four by the following
325 method: medium was removed and retained, the adherent cells were incubated with
326 Dulbecco's Phosphate Buffered Saline (D-PBS; Life Technologies) for 5 minutes then
327 detached from the plastic by washing vigorously with D-PBS using an 18 gauge needle and

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328 syringe and recovered by centrifugation at 8000g. Cells from each plate were then reseeded
329 into two new plates in complete macrophage medium supplemented with 5ml original medium.
330 On day 7, BMDM were harvested, counted and seeded into 100mm dishes, 96 well plates or
331 onto 8 well glass chamber slides (BD Falcon) at $1x10^5$ cells/ml. BMDM were activated with
332 either 1ng/ml IFNγ or 4ng/ml IL-4 (Peprotech) in complete macrophage media for 16 hours
333 prior to infection with A/WSN/33. Activating media was removed from activated BMDM and
334 kept at 37°C while infection was carried out. BMDM were infected at an MOI of 10 by
335 incubation with virus diluted in serum free DMEM for 1 hour. The inoculum was then removed
336 and activating media replaced onto the cells to allow continued exposure to cytokine. After 48
337 hours, medium was removed for assay of iNOS and Arg-1 the cells were washed by incubation
338 in Ca2+ Mg+ free PBS (Invitrogen) for 5 minutes, flushed from the dishes as described above,
339 counted and pelleted by centrifugation at 1500g. Cell pellets were stored at -80°C.
340
341 **Cell viability assays**
342 Activated and non-activated BMDMs in 96 well plates were infected at an MOI of 10 and, at
343 appropriate time points, cell viability was measured using the CellTiter-Blue cell Viability Assay
344 (Promega). Samples were assayed in triplicate.
345
346 **Immunostaining**
347 Chamber slides were washed with PBS and fixed for 30mins with 4% (w/v) paraformaldehyde
348 (PFA). After fixation, slides were washed with PBS and either stained immediately or stored
349 at 4°C until required. Before staining, stored slides were washed with PBS and blocked for
350 30mins at room temperature with CAS block (Invitrogen). After extensive washing, slides were
351 probed with 1 in 500 dilution of polyclonal goat anti–influenza A H1N1 strain USSR antibody
352 (AbD Serotec) in CAS block. After 30 minutes incubation at room temperature, slides were
353 washed with PBS and bound antibody was detected by incubation for 30 minutes with rabbit
354 anti-goat/sheep alexafluor-488 conjugated secondary antibody, diluted 1 in 1000 in CAS block

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355 (Invitrogen). Unbound conjugate was removed by washing with PBS and the slides were
356 counterstained with DAPI and mounted in Prolong Gold (Life Techologies) mounting medium.
357

358 **iNOS assays**

359 Active inducible nitric oxide synthase (iNOS) was determined by the Greiss reagent bioassay,
360 which results in production of nitrite and a colour change from colourless to pink in the
361 presence of enzyme. 100µL Greiss reagent, 5.8% (v/v) H3PO4, 1% (w/v) sulphanilamide,
362 0.1% (w/v) N-(1-Naphthyl) ethylenediamine dihydrochloride was added to 100µL BMDM
363 supernatant or 100µL sodium nitrite standard (Sigma) and absorbance read at 540nm.
364

365 **Arginase-1 bioassay**

366 Bioactive Arginase-1 was measured by conversion of L-Arginine to urea as follows. 1x105
367 BMDM were plated onto 96 well flat bottomed plates (Nunc), washed with PBS and lysed with
368 0.1% Triton-X (Sigma). The lysate was then removed to sterile 1.5ml tubes. After addition of
369 100µL 25mM Tris-HCl and 20µL 10mM MnCl2, tubes were incubated at 56°C for 10 minutes.
370 100µL of each sample was transferred to fresh tubes and incubated with 100µL 0.5M L-
371 Arginine for 2 hours. During this time a standard dilution series of urea was made. Following
372 the incubation step, 800µL 10% (v/v) sulphuric/30% (v/v) phosphoric acid solution was added
373 along with 40µL isonitropropiophenone, mixed by vortexing and incubated at 95°C for 30
374 minutes. Once cooled, samples and standards were placed in a 96 well plate and absorbance
375 read at 540nm.
376

377 **Quantitative RT- PCR**

378 RNA was extracted from frozen BMDM using an RNeasy minikit and Qiashredders (Qiagen),
379 as per manufacturer's guidelines. Genomic DNA was removed by treatment with DNA-free
380 (Ambion) according to the manufacturer's instructions. 1-2µg RNA was reverse transcribed to
381 cDNA with Superscript III (Invitrogen). cDNA was routinely diluted 1 in 20 for quantitative
382 reverse transcriptase PCR (qRT-PCR) analysis. Primers were designed as follows for each

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383 gene of interest, along with reference genes succinate dehydrogenase A (SDHA) and calnexin
384 (CNX). SDHA and CNX were chosen from a panel of 12 housekeeping genes (Quantace)
385 which were tested to determine the genes with the most stable expression in BMDMs. Optimal
386 amplification conditions were determined for each gene of interest to ensure >95% efficiency
387 of single products. qRT-PCR was carried out using a Rotorgene 3000 cycler (Qiagen).
388 Primers sets are as follows: calnexin F ttagttgaccagtctgttg, R cctttcatcccaatcttcag; succinate
389 dehydrogenase A F gctcctactgatgaaacctg, R aactcaatcccttacagcaa; iNOS F
390 tgctactgagacagggaag, R gacagtctccattcccaa; TNFα F caccaccatcaaggactcaa, R
391 gacagaggcaacctgaccac; IL-12p40 F ggaagcacggcagcagaata, R ttgagggagaagtaggaatgg; M1
392 F ctctctatcgtcccgtcagg, R gagcgtgaacac aaatccta.
393 Each gene of interest was normalized to the reference genes using Genex software (MultiD)
394 and relative expression of infected to mock controls was calculated.
395
396 **Statistical methods**
397 Statistical analysis of the differences in the various parameters of interest (the percentage of
398 macrophages infected, normalised copy number, percentage survival and gene expression)
399 following classical and alternative activation of macrophages in both 129Sv/Ev and IFNγR$^{-/-}$
400 mice was performed using standard linear-mixed effect models (Pinheiro & Bates 2009),
401 with individual mouse entered as the random effect to take account of the repeated measures
402 taken from them. All statistical analyses were carried out on log10 transformed data to
403 normalise the residuals and were performed in R (v 3.1.1 © 2014 The R Foundation for
404 Statistical Computing), using the package '*nlme*' (v 3.1-117). Statistical significance was
405 graded as * P<0.05, ** P<0.005, *** P<0.001.
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415

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416 **Figure legends**

417 **Figure 1. Influenza virus infection of 129Sv/Ev BMDMs**. BMDM cultured from femurs of
418 female 129Sv/Ev mice were mock-infected or infected with A/WSN/33 and stained with
419 antiserum to viral antigens (a) mock-infected cells; (b) 1h post-infection, MOI 10; (c) 6h post-
420 infection, MOI 10; (d) 48h post-infection, MOI 10.

421 **Figure 2. Treatment of 129Sv/Ev bone marrow derived macrophages with IFNγ or IL-4**
422 **leads to polarization of the macrophages to classical or alternative phenotypes.** BMDMs
423 derived from 129Sv/Ev and IFNγ$R^{-/-}$ mice were treated for 16h with 1ng/ml IFNγ or 4ng/ml IL-
424 4. Expression of Arg-1 (a) or iNOS (c) mRNA was measured by qRT-PCR or by biochemical
425 assay for Arg-1 activity (b) or iNOS activity (d).

426 **Figure 3. Alternatively activated macrophages are more readily infected with A/WSN/33**
427 **than classically activated macrophages.** BMDMs derived from 129Sv/Ev (a-e) and IFNγ R^{-}
428 $^{/-}$ (f-j) mice were cultured in medium containing M-CSF alone (a,f) or treated with IFNγ (b,g) or
429 IL-4 (c,h) for 16h and infected with 10pfu/cell A/WSN/33 followed by staining with antiserum to
430 virus antigens. The percentage of cells positive for antigen was quantitated at 6h (d,i) and 48h
431 (e,j) * $p<0.05$ *** $p<0.001$.

432 **Figure 4. Alternatively activated BMDMs produce more M1 mRNA than classically**
433 **activated macrophages.** BMDMs were activated with IFNγ (classically activated) or IL-4
434 (alternatively active) and infected with 10pfu/cell A/WSN/33. 48h post-infection, M1 mRNA
435 levels were measured by qRT-PCR. *** indicates $p<0.001$.

436 **Figure 5. Alternatively activated BMDMs are more susceptible to cell death following**
437 **infection with A/WSN/33 but do not support higher levels of virus replication.** BMDMs
438 from 129Sv/Ev and IFNγ$R^{-/-}$ mice were untreated or activated with IFNγ or IL-4 and infected
439 with 10pfu/cell. Cell viability was measured by the CellTiter-Blue assay at various times after
440 infection. (a) 129Sv/Ev BMDMs; (b) IFNγ$R^{-/-}$ BMDMs, *** $p<0.001$. Virus titres in cell

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441 supernatants at 1h and 48h post-infection were measured by titration on MDCK cells (c).
442 Results are representative of 3 independent experiments.

443 **Figure 6. Expression of pro- and anti-inflammatory markers in classically and**
444 **alternatively activated macrophages infected with influenza virus A/WSN/33.** BMDMs
445 were treated with 1ng/ml IFNγ or 4ng/ml IL-4 for 16hours and then infected with 10pfu/cell
446 A/WSN/33. Cells were harvested at 48 hours post-infection and expression of cellular
447 markers was monitored by qRT-PCR. Results are representative of 3 independent
448 experiments. (a,b) INOS; (c,d) Arg-1; (e,f) TNFα; (g,h) CD206. * $p<0.05$; ** $p<0.005$,
449 *** $p<0.001$.

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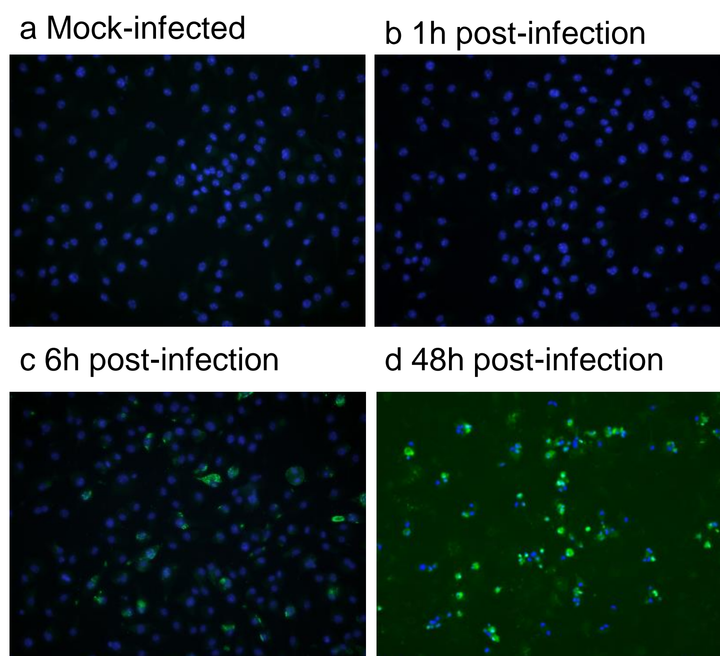

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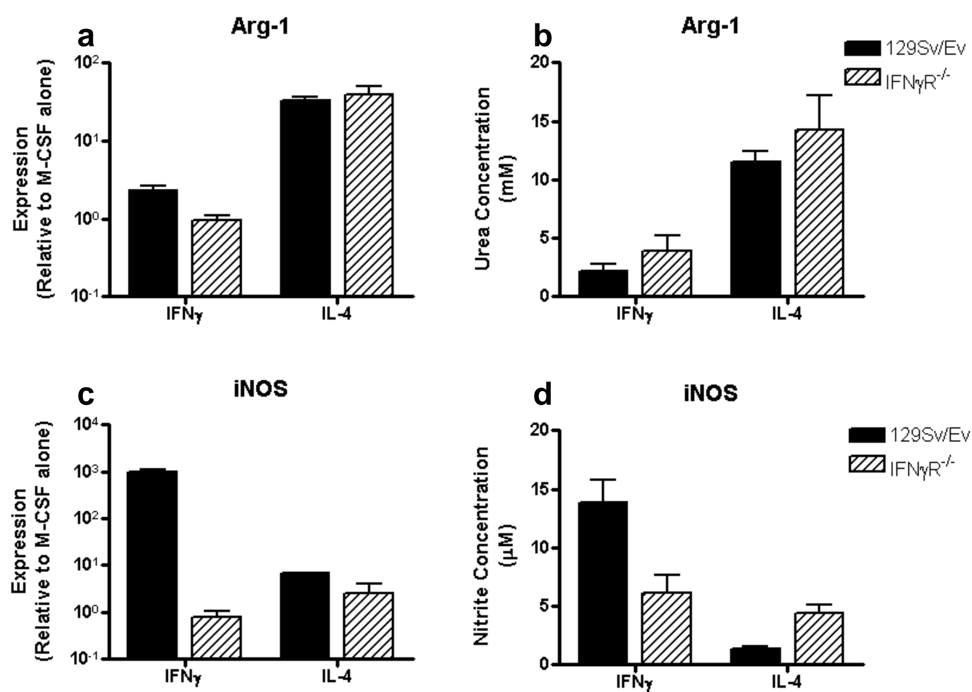

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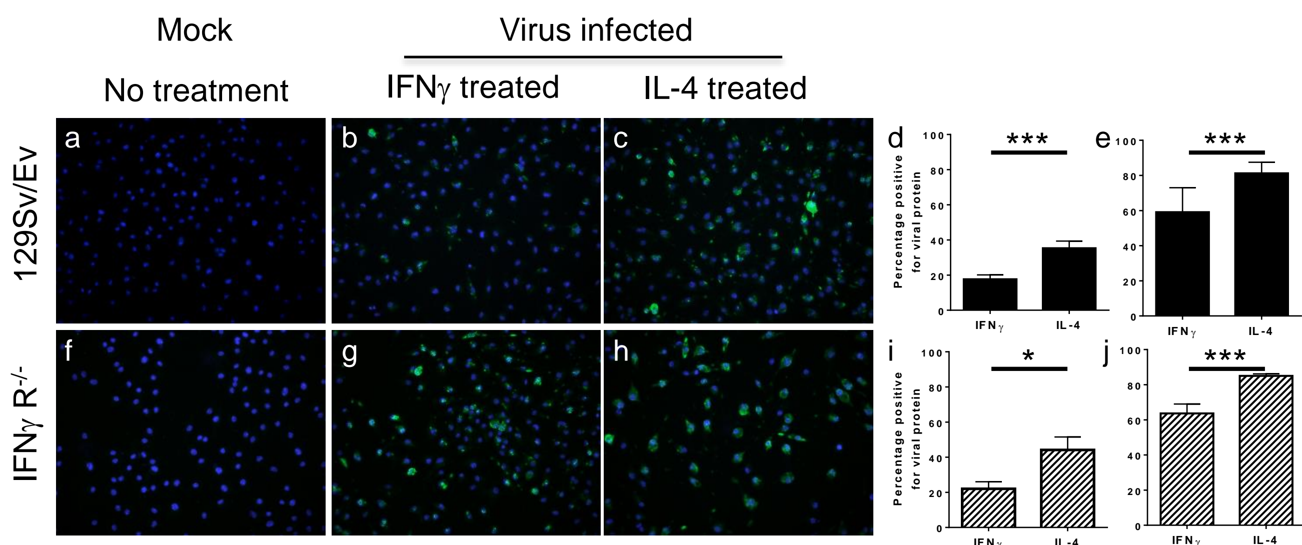

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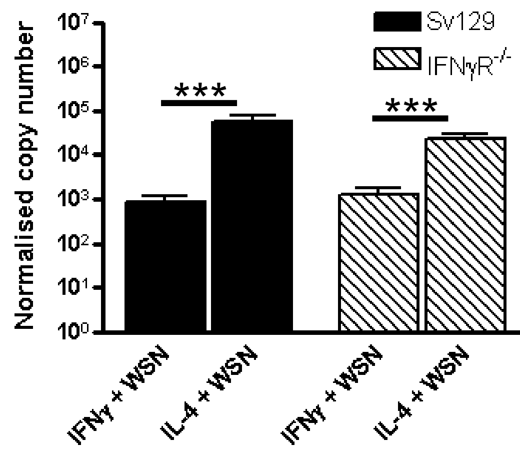

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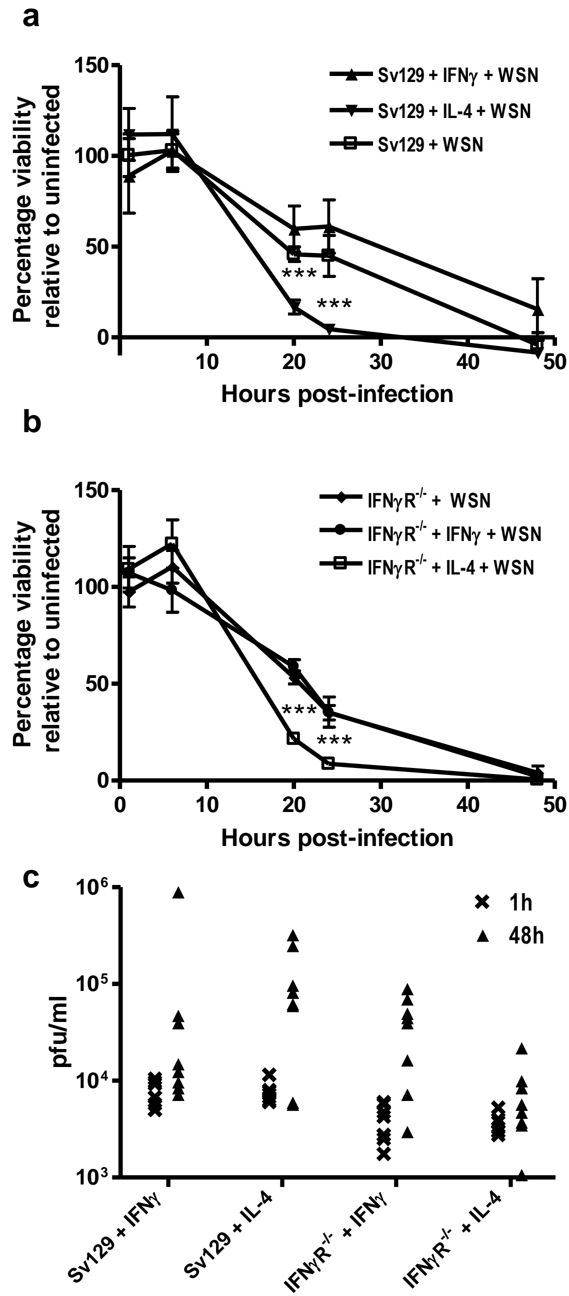

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Figure 6