Functionally distinct neutrophils sequentially promote Th1-type responses upon antiviral antibody therapy

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SUMMARY

Antiviral monoclonal antibodies (mAbs) can generate protective immunity through immune complexes (IC)-FcγRs interactions. We have shown the essential role of neutrophils in mAb-induced immunity of retrovirus-infected mice. Using this model, here we addressed how viral infection, with or without mAb therapy, affects neutrophils' functional activation. We found that neutrophils activated by viral ICs secreted high levels of chemokines able to recruit monocytes and neutrophils themselves. Moreover, inflammatory cytokines potentiated chemokines and cytokines release by IC-activated cells and induced FcγRs upregulation. Similarly, infection and mAb-treatment upregulated FcγRs expression on neutrophils and enhanced their cytokines and chemokines secretion. FcγRs upregulation allowed to identify *in vivo* two splenic neutrophils subpopulations with distinct phenotypic and functional properties that differentially and sequentially collaborate with inflammatory monocytes to induce Th1-type responses in mAb-treated mice. Our work provides novel findings on the heterogeneity and the immunomodulatory role of neutrophils in the enhancement of immune responses upon antiviral mAb therapy.

INTRODUCTION

The development of powerful antiviral monoclonal antibodies (mAbs) has provided new therapeutic opportunities to treat severe viral infections (1–4). Fc-dependent mechanisms are crucial for efficient antiviral activity of neutralizing mAbs through the engagement of IgG receptors (FcγRs) expressed on immune cells. These Fc-FcγR interactions lead to the elimination of viral particles and virus-infected cells through phagocytic and cytotoxic mechanisms (i.e. antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cell-mediated cytotoxicity (ADCC),…) (5,6). Moreover, studies in different animal models of viral infection, including ours, have provided evidence that mAbs can also enhance antiviral immune responses (so called "vaccinal effects") in a Fc-dependent manner (7). These vaccinal effects have been recently reported in HIV-infected patients treated with broadly neutralizing mAb (bnAbs) (8–10) although the mechanisms involved have not been identified thus far. The elucidation of the molecular and cellular mechanisms driving Fc-dependent, mAb-mediated immunomodulation is therefore an important issue that will be key to achieving protective immunity against severe viral infections by mAbs.

While several Fc-mediated effector functions (i.e. ADCC, ADCP, ….) have been shown to be required for antibody-mediated antiviral protection (11–16) whether and how FcγR engagement by antiviral mAbs affects the immunomodulatory properties of different FcγR-expressing cells (i.e. cytokines/chemokines secretion, activation markers expression, …) has been little studied. In addition, the specific contribution of different FcγRs-expressing cells in the induction of vaccinal effects by mAbs still remains ill-understood. These issues are all the more important to address given that both virus-driven inflammation and Fc-mediated clearance of opsonized virus/infected cells by immune effector cells generate multiple danger signals able to further modulate the host immune response. However, multiple restrictions (i.e. technical and ethical issues, costs, …) largely limit those studies in humans and non-human primates (NHP). As an alternative, *in vivo* studies in immunocompetent mice infected with the Murine Leukemia Virus FrCasE allowed the identification of several immunological mechanisms that drive protective immunity upon mAb therapy (7,17). We have previously showed that treatment of FrCasE-infected mice with the neutralizing mAb 667 (recognizing the retroviral envelope glycoprotein, expressed in virions and infected cells) elicits protective adaptive antiviral immunity through the engagement of FcγRs. Notably, mAbs form immune complexes (ICs) with viral determinants that enhance antiviral T-cell responses through FcγR-mediated binding to dendritic cells (DCs) (18–21). We have recently shown a key immunomodulatory role of neutrophils in the induction of protective humoral responses *via* the acquisition of B-cell helper functions upon engagement of FcγRs by the therapeutic mAb (22). Thus, the immunomodulatory actions of mAbs rely on their Fc fragment binding to multiple FcγR-expressing cells such as DCs and neutrophils. While the role of IC-activated DCs in the enhancement of antiviral immune responses has been addressed in several studies

101 (17,23,24) the role of IC-activated neutrophils has mostly been overlooked. Evidence shows that
102 neutrophils, in addition to being key effector cells to fight against invading pathogens, are also endowed
103 with immunomodulatory properties through the secretion of a plethora of chemokines and cytokines
104 (25–27). Yet, the functional activation of neutrophils by viral ICs and the resulting effect on their
105 immunomodulatory properties have poorly been studied in the context of antiviral mAbs therapies.
106 Furthermore, recent studies have highlighted a vast heterogeneity of neutrophils phenotypes and
107 functions in both homeostatic and pathological conditions (28–32). As these phenotypic and functional
108 variants of neutrophils depend on their context-dependent stimulation (inflammatory environment,
109 timing, disease progression, …), it is important to dissect how the viral infection and mAb therapy shape
110 the phenotype and functional properties of neutrophils.
111
112 Here, we used the FrCasE retroviral model to address how viral infection, with or without mAb therapy,
113 affects the phenotypical and functional activation of neutrophils. Neutrophils activated by viral
114 determinants secreted high levels of monocytes- and neutrophils-recruiting chemokines. We have shown
115 that the inflammatory environment resulting from the ongoing infection and mAb-treatment modulates
116 the functional properties of neutrophils and monocytes. We have also identified two neutrophils
117 subpopulations with distinct functional activation states that sequentially participate in the induction of
118 Th1-type responses in mAb-treated mice in collaboration with inflammatory monocytes. Our work
119 provides hitherto unreported findings on both the heterogeneity and immunomodulatory properties of
120 neutrophils in a context of antiviral mAb-therapy. These findings might help to improve mAb-based
121 antiviral therapies by tailoring therapeutic interventions aiming at harnessing the immunomodulatory
122 properties of neutrophils.
123
124
125

126 **RESULTS**
127
128 *Neutrophils activated by viral determinants, free or in the form of ICs, secrete high levels of chemokines*
129 *able to recruit monocytes and neutrophils*. We have previously shown a key role of neutrophils in the
130 induction of long-term protective antiviral immunity upon mAb therapy of infected mice (22). To better
131 characterize the phenotypical and functional activation of neutrophils by viral determinants, free or in
132 the form of ICs, we isolated bone marrow (BM) neutrophils from naive mice and stimulated them for
133 24h *in vitro* with free FrCasE virions or opsonized with the 667 mAb (ICs) (Figure 1A). Free 667 mAb
134 was used as control. Both, FrCasE virions and ICs induced a strong activation of neutrophils as shown
135 by a higher expression of the CD11b molecule as well as an increased frequency of $CD11b^{hi}$ $CD62L^{lo}$
136 neutrophils (Figure 1B). However, IC-mediated phenotypic activation was significantly higher.
137
138 We next assessed the functional activation of virus- and IC-stimulated neutrophils by measuring their
139 chemokines and cytokines secretion capacity. Both stimuli poorly induced the secretion of the 12
140 cytokines analyzed, with no significant differences between unstimulated neutrophils *versus* virus or
141 IC-stimulated neutrophils (Supplemental Figure 1A). This contrasted with the high secretion of IL-6
142 and TNFα pro-inflammatory cytokines observed upon lipopolysaccharide (LPS) neutrophils stimulation
143 despite similar CD11b upregulation induced by LPS and viral determinants (Supplemental Figures 1B-
144 1C). These data show that bacterial-related pathogen-associated molecular patterns (PAMPs) induce a
145 functional activation of neutrophils different from that of viral stimuli. In contrast to cytokine secretion,
146 both virus and ICs lead to high secretion levels of several chemokines such as CCL2, CXCL1, CXCL5
147 and to a lesser extent CCL3 and CCL4 (Figure 1C). In addition, LPS-stimulated neutrophils only
148 secreted a low amount of CCL3 and CCL4 chemokines, with no secretion of CCL2, CXCL1, CXCL5
149 chemokines thus providing further evidence of a pathogen-dependent secretion profile of neutrophils
150 (Supplemental Figure 1D). Interestingly, the chemokines which were more strongly secreted by
151 neutrophils upon viral stimuli (but not by LPS) have been shown to be involved in the recruitment of
152 monocytes (CCL2) (33–36) and neutrophils themselves (CXCL1, CXCL5) (37–40).
153
154 *Inflammatory monocytes activated by viral determinants, free or in the form of ICs, secrete high levels*
155 *of chemokines able to recruit neutrophils and monocytes*. Taking into account the high amounts of the
156 monocytes-recruiting CCL2 chemokine secreted by viral determinants-activated neutrophils, we next
157 assessed the phenotypical and functional activation of monocytes by these stimuli. $Ly6C^{hi}$ monocytes
158 were isolated from naive mice BM and stimulated for 24h with virus or ICs (Figure 2A). Phenotypical
159 activation was evaluated by measuring the CD86 co-stimulatory molecule expression on the cell surface.
160 Free virions and ICs significantly increased CD86 expression but no significant differences were
161 observed between both stimuli (Figure 2B).
162

163 Virus- and ICs induced a weak secretion of most of the 12 cytokines analyzed, except for IL6 and TNFα.
164 However, no significant differences between unstimulated monocytes *versus* virus- or IC-stimulated
165 monocytes were detected (Supplemental Figure 2A). This contrasted with high level secretion of IL-6,
166 TNFα and IFNγ observed upon LPS stimulation (Supplemental Figures 2B-2D). The quantification of
167 the chemokine release by activated monocytes showed some interesting similarities with neutrophils.
168 Following virus or ICs stimulation, monocytes produced CXCL5, CXCL1 and CCL2, with no
169 significant differences between virus *versus* ICs (Figure 2C). As compared to neutrophils, higher
170 amounts of the neutrophil-recruiting chemokine CXCL1 were detected as well as lower amounts of
171 CXCL5 and CCL2. Interestingly, LPS stimulation induced a wider and different panel of chemokine
172 release (Supplemental Figure 2D), notably with the secretion of high amounts of CCL3, CCL4, CCL5,
173 and to a lesser extent CXCL1 and CXCL10. As observed in neutrophils, these data show that viral
174 stimuli induce a functional activation of monocytes different from that of bacterial-related PAMPs.
175
176 *Inflammatory conditions potentiate the activation of neutrophils and monocytes by viral ICs*. As the
177 inflammatory microenvironment resulting from the viral infection and mAb therapy might affect the
178 antiviral immune response, we next assessed the phenotypic and functional activation of neutrophils and
179 monocytes by viruses and ICs in the presence of proinflammatory/immunomodulatory cytokines, such
180 as TNFα, IFN-I and IFNγ.
181
182 As depicted by an increased expression of CD11b, TNFα and IFNγ significantly enhanced the
183 phenotypic activation of neutrophils by ICs (but not by free virus) (Figure 3A). Priming conditions also
184 modulated the secretion profile of virus- and IC-activated cells in a cytokine-specific manner. TNFα
185 enhanced the secretion of CXCL1 and TNFα itself by IC-activated neutrophils but not by virus-activated
186 neutrophils (Figure 3B, Supplemental figure 3A). IFN-I and IFNγ (Figure 3B, Supplemental figure 3A)
187 significantly potentiated CCL4 secretion by IC-activated neutrophils. Both types of IFN induced the
188 production of CXCL10, consistent with the IFN-dependent induction of this chemokine. However,
189 CXCL10 secretion was significantly increased in neutrophils activated by virus or ICs upon IFN
190 priming. IFNγ also significantly enhanced CCL5 and CXCL1 secretion by IC-stimulated neutrophils. In
191 contrast, TNFα-, IFN-I- or IFNγ priming did not modulate the secretion of CCL2, CXCL5 and CCL3
192 by neither virus- nor IC-activated neutrophils (not shown).
193
194 As for monocytes, IFN-I and IFNγ significantly potentiated the phenotypic activation of IC-stimulated
195 monocytes (as depicted by an increased expression of CD86 molecule) (Figure 3C) but not that of virus-
196 activated cells. TNFα, IFN-I or IFNγ significantly potentiated the secretion of CCL2 and CXCL1
197 (Figures 3D and Supplemental figures 3B) by IC-activated monocytes. Similar to neutrophils, IFNγ and
198 IFN-I enhanced the secretion of CXCL10 by IC-stimulated monocytes and IFN-I significantly increased

199 the production of TNFα. Priming conditions also modulated the cytokine/chemokine secretion profile
200 of virus-activated monocytes, but in a more restricted way than in IC-activated cells. Thus, CXCL1
201 secretion was enhanced by TNFα and IFNγ and CCL2 by IFN-I. Both types of IFN also increased the
202 release of CXCL10 by virus-activated monocytes (Figure 3D). Similar secretion levels of CXCL5 virus-
203 and IC-activated monocytes were observed in the presence or in the absence of TNFα-, IFN-I- and
204 IFNγ- priming (not shown).
205
206 Altogether, these data highlight that inflammatory conditions strongly potentiate the functional
207 activation of IC-stimulated neutrophils and monocytes, although with different immune outcomes
208 depending on the priming condition and the responding cell type (Figure 3). Our data describe a distinct
209 enhancement of neutrophils and monocytes secretion profile by TNFα-, IFN-I- and IFNγ- priming
210 showing thus a cytokine-specific effect on the functional activation of these IC-stimulated cells. Such
211 cytokine/chemokine secretion enhancement is also cell type specific (i.e. increased secretion of CCL4,
212 CCL5, CXCL1 and TNFα by IC-activated neutrophils and CCL2, CXCL1 and TNFα by IC-activated
213 monocytes). In contrast, inflammatory conditions hardly modify the secretion profile of free virus-
214 activated cells, except for the secretion of the CXCL10 IFN-inducible chemokine.
215
216 *Inflammatory conditions upregulate the expression of FcγRIV on in vitro activated neutrophils and*
217 *monocytes*. We next assessed whether the activation of neutrophils and monocytes by virus and IC
218 affected their FcγRs expression, both in the absence and in the presence of inflammatory cytokines.
219 Indeed, FcγRs expression might differ between steady-state *versus* inflammatory/pathological
220 conditions (41), however little is known about modulation of FcγRs expression in the context of viral
221 infections and mAb-therapy. Neither virus nor IC upregulated the expression of FcγRIV on neutrophils
222 and monocytes (Figure 4A-4D). On the contrary, IFNγ and IFN-I priming (but not TNFα) upregulated
223 the expression of FcγRIV on neutrophils (IFNγ) (Figure 4B) and monocytes (IFNγ and IFN-I) (Figure
224 4D). Both IFNs also enhanced FcγRI expression on monocytes whereas a decreased FcγRII-III
225 expression was observed following IC stimulation and cytokine-priming (Supplemental Figure 4). No
226 FcγRI expression was detected on neutrophils neither upon viral determinants stimulation nor cytokine
227 priming.
228
229 *Viral infection and mAb therapy upregulate FcγRIV expression on two neutrophils subpopulations and*
230 *on inflammatory monocytes*. We next assessed *in vivo* whether the inflammatory environment resulting
231 from FrCasE viral infection and 667 mAb therapy modulated the expression of FcγRIV. The modulation
232 of this FcγR is all the more relevant to be studied in this experimental model as (i) it is a high affinity
233 receptor for IgG2a, (which is the isotype of the 667 mAb) and (ii) it is strongly expressed in neutrophils,

234 the latter having a key immunomodulatory role in mAb-mediated protection of retrovirus-infected mice
235 (22).
236
237 To this end, mice were infected and treated, or not, with the therapeutic mAb (infected/treated and
238 infected/non-treated, respectively). Then, the expression of FcγRIV on neutrophils and inflammatory
239 monocytes from the spleen (one of the main sites of viral replication) was evaluated at different time
240 points post-infection (p.i.). Age-matched naive mice were used as controls. The cell populations of
241 interest were defined by flow cytometry (Figure 5A) based on the expression of CD11b, Ly6G and Ly6C
242 to gate neutrophils ($CD11b^{+}$, $Ly6G^{hi}$) and inflammatory monocytes ($CD11b^{+}Ly6G^{-}Ly6C^{hi}$).
243 Interestingly, we identified another population of neutrophils displaying a lower expression of the cell
244 surface marker Ly6G ($CD11b^{+}Ly6G^{int}$) (Figure 5A). No expression of the eosinophils cell surface
245 marker Siglec F was detected in this population (not shown). The frequency of both populations of
246 neutrophils $CD11b^{+}Ly6G^{hi}$ and $CD11b^{+}Ly6G^{int}$ was significantly increased in the spleen of
247 infected/non-treated mice at 8 and 14 days p.i. This increased neutrophil frequency was associated with
248 a higher percentage of spleen infected cells as compared to infected/treated mice (22). On the contrary,
249 the frequency of inflammatory monocytes ($CD11b^{+}Ly6G^{-}Ly6C^{hi}$) remained unchanged in infected mice
250 at day 8 p.i. but was significantly increased at day 14 p.i. (Figure 5B), suggesting an earlier recruitment
251 of neutrophil than inflammatory monocytes upon infection.
252
253 In steady-state conditions, FcγRIV was highly expressed on $CD11b^{+}Ly6G^{hi}$ and $CD11b^{+}Ly6G^{int}$
254 neutrophils. Lower FcγRIV expression was detected in inflammatory monocytes at the different time
255 points assessed (Figure 5C). Notably, upon viral infection and mAb-treatment, FcγRIV expression was
256 upregulated in the three myeloid cells populations although, to a different magnitude depending on the
257 cell type, time p.i. and mAb-treatment (Figure 5C). At day 8 p.i. FcγRIV expression was significantly
258 upregulated on neutrophils and monocytes in infected/treated and infected/non-treated mice, the latter
259 showing a stronger FcγRIV upregulation. In contrast, at day 14 p.i. infected/treated mice showed a
260 stronger upregulation of FcγRIV on the three myeloid cells populations as compared to infected/non-
261 treated and control mice. $CD11b^{+}Ly6G^{int}$ neutrophils displayed the strongest expression of FcγRIV as
262 well as the highest magnitude of FcγRIV upregulation after infection and mAb-treatment. No detectable
263 FcγRI expression was observed at day 8 p.i. in any of the three cell types assessed. However, at day 14
264 p.i. infected/treated mice showed a significant upregulation of this inducible FcγR receptor in
265 $CD11b^{+}Ly6G^{int}$ neutrophils and monocytes (Supplemental Figure 5).
266
267 These results highlight that viral infection and mAb therapy results in an evolving inflammatory
268 environment that dramatically modulates the expression of FcγRs in neutrophils and monocytes, leading

269 to a stronger upregulation of FcγRIV at 8 days p.i. in infected/non treated mice and at 14 days p.i. in
270 infected/treated mice.
271
272 *High* Fc*γRIV-expressing CD11b*$^{+}$*Ly6G*^{int} *neutrophils subpopulation displays phenotypic properties*
273 *different from those of CD11b*$^{+}$*Ly6G*^{hi} *neutrophils*. High level expression and strong upregulation of
274 FcγRIV on CD11b^{+}Ly6G^{int} (Figure 5C and 5D) suggested different and specific properties of this
275 neutrophil subpopulation. We thus characterized it further. We observed neutrophils Ly6G^{int} to be
276 smaller than neutrophils Ly6G^{hi} and displaying a lower granulosity (Figure 5D). To determine the
277 specificities of this population, we studied the expression pattern of neutrophils and monocytes cell-
278 surface markers in these cells at steady-state conditions (Figure 5E). Similar to CD11b^{+}Ly6G^{hi}
279 neutrophils, CD11b^{+}Ly6G^{int} cells expressed low levels of the monocyte cell surface markers Ly6C and
280 CCR2 (Figure 5E). They also expressed the CXCR4 chemokine receptor although at intermediate levels
281 as compared to CD11b^{+}Ly6G^{hi} neutrophils and inflammatory monocytes. However, they weakly
282 express the chemokine receptor CXCR2 which is highly expressed on CD11b^{+}Ly6G^{hi} neutrophils.
283
284 These results revealed that Ly6G^{int} cells display a neutrophil-like phenotype but with specific
285 characteristics as compared to classical Ly6G^{hi} neutrophils, notably (i) smaller size and granulosity, (ii)
286 higher viral-induced FcγRIV expression, and (iii) a pattern expression of cell surface markers
287 characteristic of neutrophils (Ly6C^{lo}, CCR2^{-}, CXCR4^{int}) except for the lack of expression of the CXCR2
288 receptor. This suggests that CD11b^{+}Ly6G^{int} cells are a phenotypic variant of neutrophils. However,
289 whether CD11b^{+}Ly6G^{int} cells represent a *bona fide* novel neutrophils subset or neutrophils displaying
290 a different activation/maturation state could not be discriminated by these phenotypic properties as this
291 will require integrative high-dimensional analyses including not only functional and phenotypic
292 characterization but also deep genomic profiling of the neutrophil subpopulations identified (29).
293
294 *FcγRIV upregulation in infected/treated mice is associated with enhanced expression of MHC-II and*
295 *co-stimulatory molecules on neutrophils and monocytes*. We next addressed *in vivo* the functional
296 activation of splenic neutrophils and inflammatory monocytes in infected mice with or without
297 immunotherapy at days 8 and 14 p.i. by monitoring cell surface activation markers. We were able to
298 show that the activation state of the cells evolved over time. At day 8 p.i., (Figure 6A) CD11b was
299 upregulated on neutrophils and monocytes upon viral infection either in the absence or in the presence
300 of immunotherapy. However, infected/treated mice showed a significantly higher CD86 upregulation in
301 CD11b^{+}Ly6G^{hi} and CD11b^{+}Ly6G^{int} neutrophils than infected/non-treated mice. MHC-II molecule was
302 also upregulated on CD11b^{+}Ly6G^{int} neutrophils and monocytes upon viral infection. At day 14 p.i.
303 (Figure 6B), CD11b^{+}Ly6G^{hi} splenic neutrophils were similarly activated in infected/treated- and

304 infected/non-treated mice, as deduced from an increased expression of CD11b and CD86. However,
305 CD86 expression on CD11b^{+}Ly6G^{int} neutrophils was significantly increased in infected/treated mice,
306 suggesting a mAb-mediated upregulation of these costimulatory molecules on this neutrophil
307 subpopulation. Likewise, inflammatory monocytes from infected/treated mice also showed a
308 significantly increased expression of CD86 co-stimulatory molecules as compared to infected/non-
309 treated mice. Notably, MHC-II expression was significantly increased in infected/treated mice in both
310 neutrophil populations as well as in monocytes as compared to infected/non-treated mice.
311
312 Consistent with a FcγRIV upregulation in neutrophils and monocytes in infected/treated mice (Figure
313 5C), these data demonstrate a mAb-mediated upregulation of MHC-II and costimulatory molecules in
314 CD11b^{+}Ly6G^{int} neutrophils and monocytes as well as a significant upregulation of MHC-II in
315 CD11b^{+}Ly6G^{hi} neutrophils at 14 days p.i. (Figure 6B).
316
317 *CD11b^{+}Ly6G^{hi}, CD11b^{+}Ly6G^{int} neutrophils and inflammatory monocytes are differentially and*
318 *sequentially activated upon antiviral mAb treatment*. To further characterize the functional activation
319 state of the two neutrophils populations (CD11b^{+}Ly6G^{hi} and CD11b^{+}Ly6G^{int}) and inflammatory
320 monocytes upon viral infection and mAb-therapy, we next addressed the cytokine and chemokine
321 secretion profile of splenic neutrophils and monocytes sorted from infected mice treated, or not, with
322 the 667 mAb, at days 8 and 14 p.i. Cells sorted from age-matched naive mice were used as controls. We
323 found that the secretion profile evolved over time and was cell type- and stimulus specific, with globally
324 enhanced cytokine/chemokines secretion in cells isolated from infected/treated mice (Figure 7A and
325 7B), in particular at day 14 p.i.
326
327 At day 8 p.i., the CD11b^{+}Ly6G^{hi} neutrophils subpopulation from both infected/non-treated and
328 infected/treated mice showed a broad chemokine and cytokine secretion profile as deduced from an
329 increased secretion of most of the 13 chemokines and 12 cytokines assessed, although at weak levels
330 (Figure 7B and 7C). CD11b^{+}Ly6G^{int} neutrophils showed enhanced expression of several chemokines
331 mostly in cells isolated from infected/non-treated mice notably with a strong enhanced secretion of
332 CCL3, CCL5 and CXCL5. In addition, CD11b^{+}Ly6G^{int} neutrophils also showed a slight secretion of
333 multiples cytokines in infected/treated mice, but to a lesser extent than CD11b^{+}Ly6G^{hi} neutrophils
334 (lower fold-increase) (Figure 7B and 7C). In contrast, infection and mAb-treatment hardly affected the
335 chemokine/cytokine secretion of Ly6C^{hi} monocytes. They only showed a weak increase in CXCL1
336 secretion in infected/treated mice as well as a slight increase in cytokines secretion (both in terms of
337 diversity and fold increase) mainly in infected/non-treated mice (Figure 7B).
338

339 Interestingly, at day 14 p.i., the functional activation of the three different cell types assessed completely
340 differed from that observed at day 8 p.i. (Figure 7B and 7C). We found a more restricted but stronger
341 induction of chemokines/cytokines secretion, mostly in cells isolated from infected/treated mice. In
342 contrast to the broad cytokines and chemokines secretion profile observed at day 8 p.i., $CD11b^{+}Ly6G^{hi}$
343 neutrophils only secreted CCL4 and CCL5 chemokines and showed no cytokine secretion at day 14 p.i.
344 In addition, $CD11b^{+}Ly6G^{int}$ neutrophils showed a strong enhanced expression of 6 chemokines (CCL3,
345 CCL4, CCL5, CCL22, CXCL9 and CXCL10) and 3 Th1-polarizing cytokines (IL-6, TNFα, IFNγ) in
346 infected/treated mice. $Ly6C^{hi}$ monocytes showed a proinflammatory chemokine/cytokine secretion
347 profile similar to $CD11b^{+}Ly6G^{int}$ neutrophils, except for the secretion of CXCL9, that was specific to
348 $CD11b^{+}Ly6G^{int}$ neutrophils and CXCL1 that was only secreted by monocytes.
349
350 In summary, FrCasE infection and 667 mAb-treatment induce the secretion of multiple chemokines and
351 cytokines by neutrophils and monocytes. Interestingly, the secretion profile evolves over time and
352 differs among the three different cell types assessed, providing further evidence of a different functional
353 activation state of the two neutrophils subpopulations ($Ly6G^{hi}$ and $Ly6G^{int}$). In addition, these data show
354 an enhanced secretion of Th1-polarizing cytokines and chemokines by cells isolated from
355 infected/treated mice at 14 p.i, showing a role for the therapeutic mAb in the functional activation of
356 these FcγRIV highly-expressing cells.
357

358 **DISCUSSION**
359

360 We have previously shown that neutrophils have a key immunomodulatory role in the induction of
361 protective immunity by antiviral mAbs through the acquisition of B-cell helper functions (22). Here we
362 provide a new insight into both the heterogeneity and immunomodulatory role of neutrophils in a context
363 of antiviral mAb-therapy. Our work shows that two functionally distinct neutrophil subpopulations can
364 secrete multiple cytokines and chemokines able to recruit and activate multiple cells of the innate and
365 the adaptive immune system. The cytokine/chemokine secretion profile: differs between both neutrophil
366 subpopulations, is stimulus-dependent, evolves over time upon viral infection and mAb-treatment and
367 suggests a potential cooperation with inflammatory monocytes in the induction of Th1 immune
368 responses upon mAb-treatment of infected mice.
369

370 Our data show that neutrophils cytokine/chemokine secretion profile differs between viral *versus*
371 bacterial stimulus. This is important to highlight as most of the studies investigating the
372 immunomodulatory role of neutrophils have been conducted in bacterial infection models. Thus, *in vitro*
373 stimulation of neutrophils by viral determinants led to a poor production of cytokines but to a wide and
374 strong release of chemokine, with notably high secretion levels of the monocytes-and neutrophils-
375 recruiting chemokines (CCL2, CXCL1, CXCL5) that was not observed upon LPS stimulation. On the
376 contrary, LPS-stimulated neutrophils produced high amounts of proinflammatory cytokines but a
377 narrower and weaker chemokine release. As for neutrophils, stimulation of monocytes by viral
378 determinants (but not LPS) led to the secretion of high amounts of the neutrophils-recruiting chemokine
379 CXCL1 and to a lesser extent the monocytes-recruiting chemokine CCL2. These results suggest a self-
380 sustaining mechanism of neutrophils and monocytes recruitment upon viral infection, and raise the
381 hypothesis of an early cooperation between neutrophils and monocytes in the modulation/induction of
382 the antiviral immune response. This secretion profile of neutrophils induced by viral determinants is in
383 agreement with an increased CCL2 release observed upon *in vitro* HIV stimulation of neutrophils (42).
384 Increased levels of CCL2 and CXCL1 have also been reported in neutrophil- and monocytes-infiltrated
385 tissues in different viral infections, (43–46). However, chemokine increase was mostly assessed in total
386 tissue extracts but not directly in neutrophils or monocytes isolated cells, which prevented the
387 identification of the cell origin of chemokines. It is worth noting that a very early but transient expression
388 has also been reported (i.e. 24-48h p.i.) of CCL2 and CXCL1 upon infection by RSV, CMV and
389 influenza virus (43,44,47). Such early and transient expression would be in agreement with the very low
390 secretion of these chemokines detected at day 8 p.i. in infected mice treated, or not, with the therapeutic
391 mAb.
392

393 Our data also highlight the effect of inflammatory conditions on the modulation of the phenotypic and
394 functional activity of neutrophils and monocytes. Thus, *in vitro* priming with

395 inflammatory/immunomodulatory cytokines clearly potentiated the release of several chemokines and
396 cytokines by IC-activated neutrophils and monocytes (Figure 3) while having a less pronounced or no
397 effect on virus-activated cells. This suggests that cytokine-priming associated to FcγR-triggering leads
398 to enhanced immune responses. Although not formally shown, these results support a role for the
399 inflammatory environment in the enhancement of chemokine/cytokine secretion by neutrophils and
400 monocytes observed in infected-treated mice. Furthermore, our *in vitro* observations also allowed us to
401 dissect the specific effect of IFN-γ, IFN-I and TNF-α on the enhancement of the functional properties
402 of IC-activated neutrophils and monocytes. Notably, IFN-γ and IFN-I priming (but not TNF-α), led to
403 the upregulation of FcγRs in neutrophils and/or monocytes, in agreement with previous reports in other
404 experimental settings (48–51). Consistent with these results, our *in vivo* work shows that the
405 inflammatory environment resulting from ongoing infection and mAb-treatment also modulates FcγRs
406 expression on neutrophils and monocytes (in particular FcγRIV). Thus, FcγRIV expression is more
407 strongly enhanced in infected/non-treated mice at day 8 p.i. and in infected/treated mice at day 14 p.i.,
408 suggesting a different and evolving inflammatory environment in both groups of mice. These results are
409 in agreement with previous work showing FcγRs modulation by the inflammatory conditions resulting
410 from bacterial and IC-mediated autoimmune pathologies (41,52); and provide new evidence on the
411 regulation of FcγRs expression in the specific inflammatory context of antiviral mAb-based
412 immunotherapies, not hitherto reported. Notably, our work shows that the magnitude of FcγRIV
413 upregulation upon viral infection and mAb-treatment is different in neutrophils and monocytes. This
414 differential FcγRIV expression together with a different expression level of the Ly6G neutrophil cell
415 surface marker ($Ly6G^{hi}$ and $Ly6G^{int}$) contributed to the identification and characterization of two
416 neutrophils subpopulation displaying distinct functional properties as deduced from their different (i)
417 phenotype (size, granulosity, cell surface molecules) (Figures 5 and 6), (ii) magnitude of expression and
418 upregulation of FcγRIV, costimulatory molecules and MHC-II (Figures 5 and 6) and (iii)
419 cytokine/chemokine secretion profile (Figure 7) upon viral infection and mAb-treatment. Recent studies
420 have shown neutrophils heterogeneity in healthy tissues as well as in different pathological conditions
421 (29,32) through the identification of different neutrophils subpopulations. Such neutrophil heterogeneity
422 is context-dependent as it is influenced by the nature and magnitude of the stimuli. In the particular
423 context of infectious diseases, neutrophils heterogeneity is still poorly characterized and it has mostly
424 been assessed in bacterial infections (53,54). Similar to our observations Deniset and collaborators (53)
425 reported two distinct splenic neutrophils subpopulations displaying different Ly6G expression levels
426 ($Ly6G^{hi}$ and $Ly6G^{int}$) in *Streptococcus pneumoniae* infected mice. This study evidenced some
427 phenotypic differences (cell surface markers of maturation, nuclear morphology, ….) between both
428 neutrophil subpopulations but their functional activation was not assessed. Importantly, we describe
429 here two phenotypic and functional variants of neutrophils displaying distinct immunomodulatory
430 properties that might be key and complementary for inducing antiviral protective immunity. Our work

431 provides thus novel findings on the heterogeneity and function of neutrophils upon viral infection and
432 mAb-treatment.
433
434 Our work provides new mechanistic insights into the hitherto underestimated role of neutrophils as key
435 cells in the modulation of adaptive antiviral immunity upon mAb treatment. Interestingly, at steady-
436 state conditions, neutrophils express high levels of FcγRIV. Its expression is higher than that observed
437 in inflammatory monocytes. Thus, it is tempting to speculate that upon viral infection and mAb-
438 treatment, the early neutrophils recruitment, together with their high expression of FcγRIV, result in
439 potent Fc-triggering by ICs in these cells. This might be key to initiate the modulation of immune
440 responses through the production of multiple cytokines and chemokines able to recruit and activate
441 multiple innate immune cells such as monocytes, NK cells, DC and neutrophils themselves (26). This
442 suggest a potential role for neutrophils as early drivers of the induction of vaccinal effects by mAbs.
443 Supporting this hypothesis, at day 8 p.i. both neutrophils populations showed a higher and a wider
444 induction of chemokines and cytokines release than monocytes. Subsequently, the inflammatory
445 environment resulting from viral infection and mAb-mediated antiviral effects induces the upregulation
446 of FcγRs in the different innate immune cells that might further increase Fc-triggering by ICs leading to
447 improved immune responses, both in terms of magnitude and quality. In agreement with this, the FcγRIV
448 upregulation in neutrophils and monocytes in infected/treated mice observed at 14 days p.i. is associated
449 with (i) higher expression of costimulatory molecules and MHC-II and (ii) higher secretion of cytokines
450 and chemokines. In keeping with this, enhanced secretion of Th1-polarizing cytokines (IL-6, TNFα,
451 IFNγ) (55–59) and chemokines (CXCL9 and CXCL10) (60–63) by $CD11b^{+}Ly6G^{int}$ neutrophils and
452 monocytes from infected/treated mice at 14 p.i, argues in favor of a mAb-mediated Th1 polarization of
453 the antiviral immune response by these cells. Consistent with such Th1 polarization, we previously
454 reported strong CD8 T-cells responses at day 14 p.i. in infected/treated mice (19,22,64) as well as long-
455 lasting protective humoral immune responses predominantly of the IgG2a isotype (19,22,65). Finally,
456 it is also worth mentioning the upregulation of MHC-II molecules observed in $CD11b^{+}Ly6G^{hi}$ and
457 $CD11b^{+}Ly6G^{int}$ neutrophils sorted from infected/treated mice. This observation broadens the
458 immunomodulatory role of neutrophils upon mAb-treatment as it suggests that neutrophils might
459 acquire antigen-presenting cell features and induce antigen-specific T-cells responses (66–68). In
460 agreement with this, it has been shown that upon *ex vivo* stimulation with IgG immune complex (IC) or
461 viral cognate antigens, neutrophils upregulated expression of MHCII and costimulatory molecules and
462 increased T cell activation.
463
464 Our work show that mAb-mediated activation of neutrophils and monocytes might be essential to induce
465 strong Th1-polarized early immune responses that might be key to induce protective immunity by mAb.
466 In addition, the distinct secretion profile of $Ly6G^{hi}$ and $Ly6G^{int}$ neutrophils and $Ly6C^{hi}$ monocytes, in

467 terms of type, amount and kinetics of chemokines/cytokines secretion, shows a differentially and
468 sequentially activation of theses FcγRIV-expressing cells upon antiviral mAb treatment and suggests
469 their key and complementary action in the induction of protective immune responses by mAb. Thus, the
470 identification of neutrophils as key players in the induction of protective immunity by antiviral mAbs
471 and their potential collaboration with inflammatory monocytes might have therapeutic implications.
472 These findings might help to improve mAb-based antiviral therapies by tailoring therapeutic
473 interventions aiming at harnessing the immunomodulatory properties of these cells. To this end,
474 different approaches could be envisaged: (i) the use of appropriate immunostimulatory host-directed
475 therapies and (ii) the design of antiviral mAbs engineered to enhance their affinity for FcγRs expressed
476 on human neutrophils and monocytes, such as FcγRIIa and FcγRIIIa (12,69,70) .Thus, in addition to
477 allow superior antibody-mediated phagocytosis, these Fc-engineered mAbs could also modulate
478 cytokine/chemokine production to ultimately lead to more effective adaptive immune responses.

479

480 **METHODS**
481

482 ***Mice:*** Inbred 129/Sv/Ev mice (H-2D^b haplotype) were used and maintained under conventional,
483 pathogen-free facilities at the Institut de Génétique Moléculaire de Montpellier (RAM-ZEFI). They have
484 been used without distinction as to sex and at different ages according to experiments.
485

486 ***Viral stocks***: FrCasE (71) viral stocks were produced and stored as previously described in (65).
487

488 ***Viral infection and immunotherapy:*** Eight-day-old 129/Sv/Ev mice were infected by intraperitoneal
489 (i.p.) administration with 50 µl of a viral suspension containing 50,000 focus-forming units (FFU) and
490 treated, or not, with 30 µg 667 mAb targeting gp70 protein of viral envelope, (72), 1-hour p.i. and on
491 days 2 and 5 p.i. by i.p. administration. Mice were euthanized and spleens collected at days 8 and 14 p.i.
492

493 ***Phenotypical and functional activation of FcγRIV-expressing cell from spleen ex vivo:***
494 Single-cell suspensions of splenocytes were obtained from naive, infected/non-treated and
495 infected/treated mice at 8 and 14 days p.i. and immunotherapy. Spleen cell suspensions were obtained
496 by mechanical dissociation in PBS, then filtered in 0,70 μm strainer. 20 % of each spleen was used for
497 immunophenotyping by FACS, 80% left was dedicated to main FcγRIV-expressing cell sorting. Red
498 blood cells were lysed (ACK, Lonza) and an enrichment with biotinylated anti-B220 (BD Biosciences),
499 anti-CD3 (BD Biosciences), following of anti-biotin Ab magnet-bead coupled (Miltenyi) and magnetic
500 LS-columns (Miltenyi) was performed to remove spleen lymphocytes and increase the sorting efficacy.
501 Cells were stained with specific marker of populations of interest (Ly6G-BD BioSciences, Ly6C-
502 BioLegend, CD11b-BD BioSciences) and neutrophils (Ly6G^{hi} and Ly6G^{int}) and inflammatory
503 monocytes (Ly6C^{hi}) were sorted (>97-98% pure) using the BD Biosciences FACSAria device. Sorted
504 cells were cultured in 96-well plates at a density of 4x10^6 cells/ml (1x10^6 cells/well) in 10% FBS-
505 containing RPMI medium for 24h, and cell-free supernatants were collected and stored at -20°C, to
506 allow cytokines and chemokines protein release quantification.

507 ***Flow cytometry:*** Organs of interest were collected to realize immunophenotyping of immune cells.
508 Spleen cell suspensions were obtained by mechanical dissociation in PBS. BM cell suspensions were
509 obtained by dissection and PBS-2%-FBS flushing of tibias and femurs. Cells were stained at 4°C using
510 fluorochrome-conjugated antibodies against CCR2 (SA203G11, BioLegend), CD11b (M1/70,
511 eBioscience), CD11c (HL3, BD Biosciences), CD16/32 (93, BD Biosciences), CD45.2 (104, BD
512 Biosciences), CD62L (MEL-14, BD Biosciences), CD64 (x54-5/7.1, BD Biosciences), CD86 (GL1, BD
513 Biosciences), CXCR2 (SA044G4, BioLegend), CXCR4 (2B11, Life Technologies), Ly6G (1A8, BD
514 Biosciences), Ly6C (AL-21, BioLegend) and Siglec F (E50-2440, BD Pharmingen). FcγRIV expression

515 was determined using 9E9 antibody (kindly provided by Dr.Pierre Bruhns, Institut Pasteur), produced
516 by BioXcell, and then labeled with Alexa Fluor 647. FrCasE-infected cells were assayed using an anti-
517 Gag mAb (H34) (73) kindly provided by Pr. Ulf Dittmer, University of Duisburg-Essen labeled with
518 Alexa Fluor 647 or incubated with an anti-IgG2b labeled PE. Forward scatter area and forward scatter
519 time-of-flight, as well as side scatter, were used to remove doublets from flow cytometry analyses. Cells
520 were analyzed on FACS LSR Fortessa (BD Bioscience), and the data were analyzed using the FlowJo
521 software.

522 ***Neutrophils and monocytes isolated from BM used in in vitro experiments:*** Neutrophils and
523 monocytes were purified from 8 to 11-week-old naive mice BM. After dissection of lower limbs, BM
524 cell suspensions were collected by PBS-2% FBS EDTA (2 mM) flushing (25G needle) of tibias and
525 femurs. BM cell suspensions were filtered with a 0,40 μm nylon strainer. Two magnetic-based cell
526 sorting (MACS) isolation kits were used to purified either neutrophils (Miltenyi Biotec) and monocytes,
527 (Miltenyi Biotec), both with high purity (>97-98%), determined by FACS (LSR Fortessa, BD
528 Bioscience). Cells were placed in culture in U bottom 96-well plates at a concentration of 1 million/ml
529 in 10% FBS-containing RPMI medium.
530
531 ***Stimulation of neutrophils and monocytes in vitro***: Purified neutrophils and monocytes were
532 seeded at 150 000 cells/ well in 150 μl of RPMI, then incubated for 24h with LPS (1μg/ml, Sigma), or
533 FrCasE virus (MOI 5: 5 viral particles /cell), or ICs (virus FrCasE and 1μg 667 mAb), or 1μg 667 mAb
534 alone. In parallel, the same experiments were performed adding inflammatory cytokines, TNFα 100
535 UI/ml (Peprotech), IFN γ 100 UI/ml (eBioScience), IFNα11 a 1000 UI/ml, produced and generously
536 provided by Dr. Gilles Uzé (DIMP, CNRS). After 24h of stimulation, supernatants were collected and
537 stored at -20°C to quantify chemokines and cytokines protein release secretion (describe below).
538 Phenotypical activation of both neutrophils and monocytes was measured using surface markers by flow
539 cytometry.
540
541 ***Chemokines and cytokines protein release quantification:*** Soluble chemokines and cytokines
542 secretion were quantified from cell-free collected supernatants of *in vitro* cultured neutrophils and
543 monocytes and of sorted splenic neutrophils Ly6G^{hi} and Ly6G^{int}, and inflammatory monocytes (of
544 naive, infected/non-treated and infected/treated mice 8 and 14 days p.i. and immunotherapy), using
545 bead-based immunoassays (LegendPLex, BioLegend) and analyzed on the BD Bioscience-LSR
546 Fortessa device. The protein release quantification was established by the appropriate software
547 (LEGENDplex^{TM} data analysis).
548

549 ***Statistics:*** Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). Data
550 were expressed as mean ± SEM, and statistical significance was established using a parametric 1-way
551 ANOVA test with Bonferroni's multiple comparisons post-tests or non-parametric Kruskal-Wallis test
552 with Dunn's multiple comparisons post-test for multiple comparisons or paired Student's *t* tests when
553 two groups were compared. *P* values lower than 0.05 were considered as statistically significant.

554 ***Study approval***. All experimental procedures were performed in accordance with the French national
555 animal care guidelines (CEEA-LR-12146 approval, Ethics committee of the Languedoc-Roussillon
556 Region, Montpellier).

557

558

AUTHOR CONTRIBUTIONS

Jennifer Lambour (JL), Mar Naranjo-Gomez (MN-G) and Mireia Pelegrin (MP) defined the research program. JL, MN-G, and Myriam Boyer-Clavel (MB-C) performed the experiments. JL, MN-G and MP carried out the data analyses. JL and MP wrote the manuscript. Grants to MP funded the study.

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CONFLICT OF INTEREST DISCLOSURES

The authors declare no competing financial interests.

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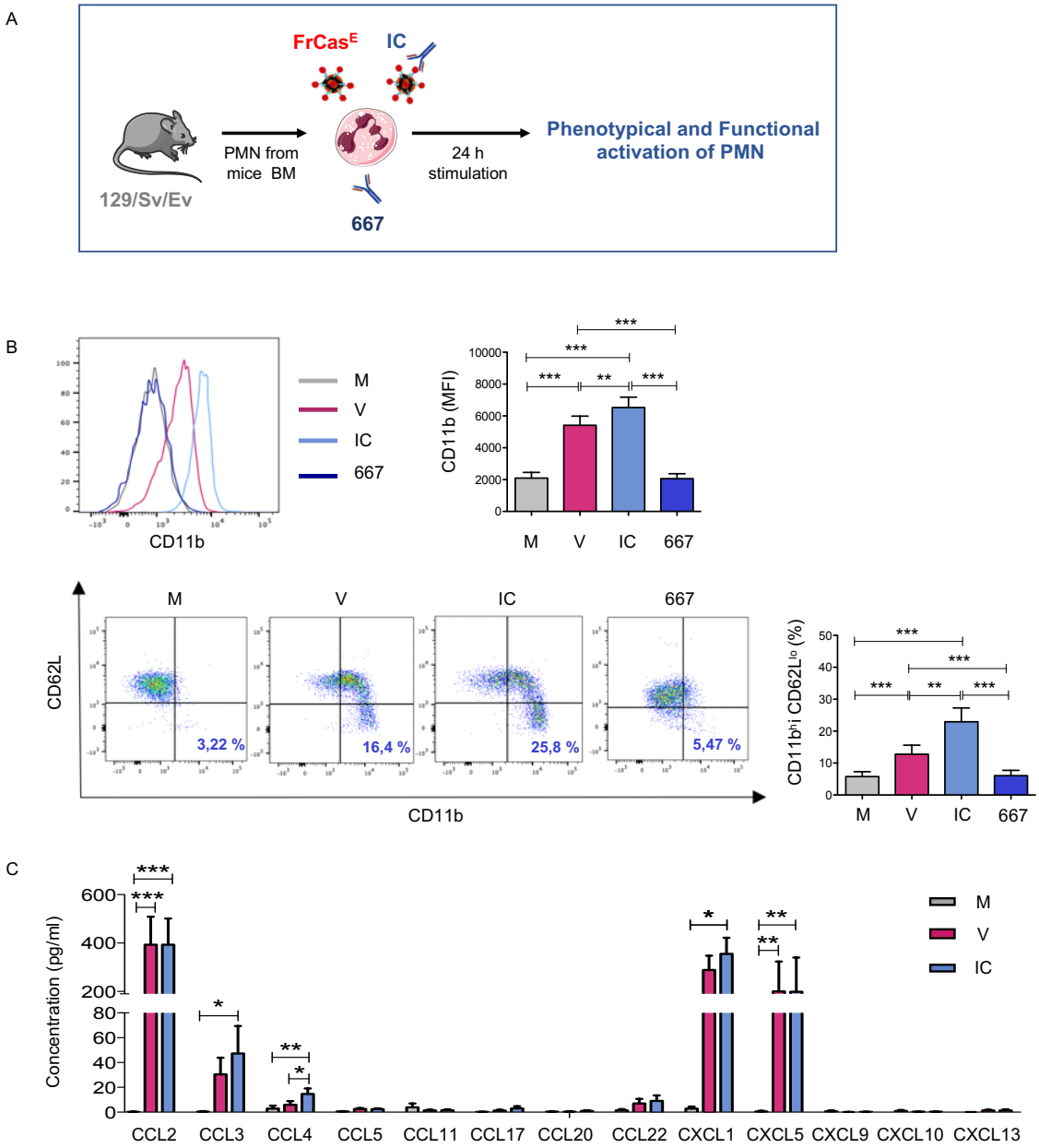


Figure 1: **Phenotypic and functional activation of neutrophils stimulated with FrCasE virions and ICs. A.** *Experimental scheme*. **B**. *Phenotypic activation of neutrophils stimulated by FrCasE virions or viral ICs made with the 667 mAb*. V, free virions; IC, viral ICs; M, culture medium. Free 667 mAb was used as control. Activation was assessed by monitoring CD11b expression and frequency of CD11b^{hi} CD62L^{lo} neutrophils. The data represent 12 independent experiments. **C**. *Functional activation of neutrophils stimulated by FrCasE virions (V) or viral ICs made with the 667 mAb (IC)*. Chemokines release was assessed in supernatants of neutrophils isolated from BM of naive mice (>97-98% purity) and stimulated for 24 h by FrCasE virions (red) or viral ICs (blue) or left unstimulated (grey). The data represent 5 independent experiments. Data are expressed as means +/- SEM. Statistical significance was established using a parametric 1-way ANOVA test with Bonferroni's multiple comparisons post-tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

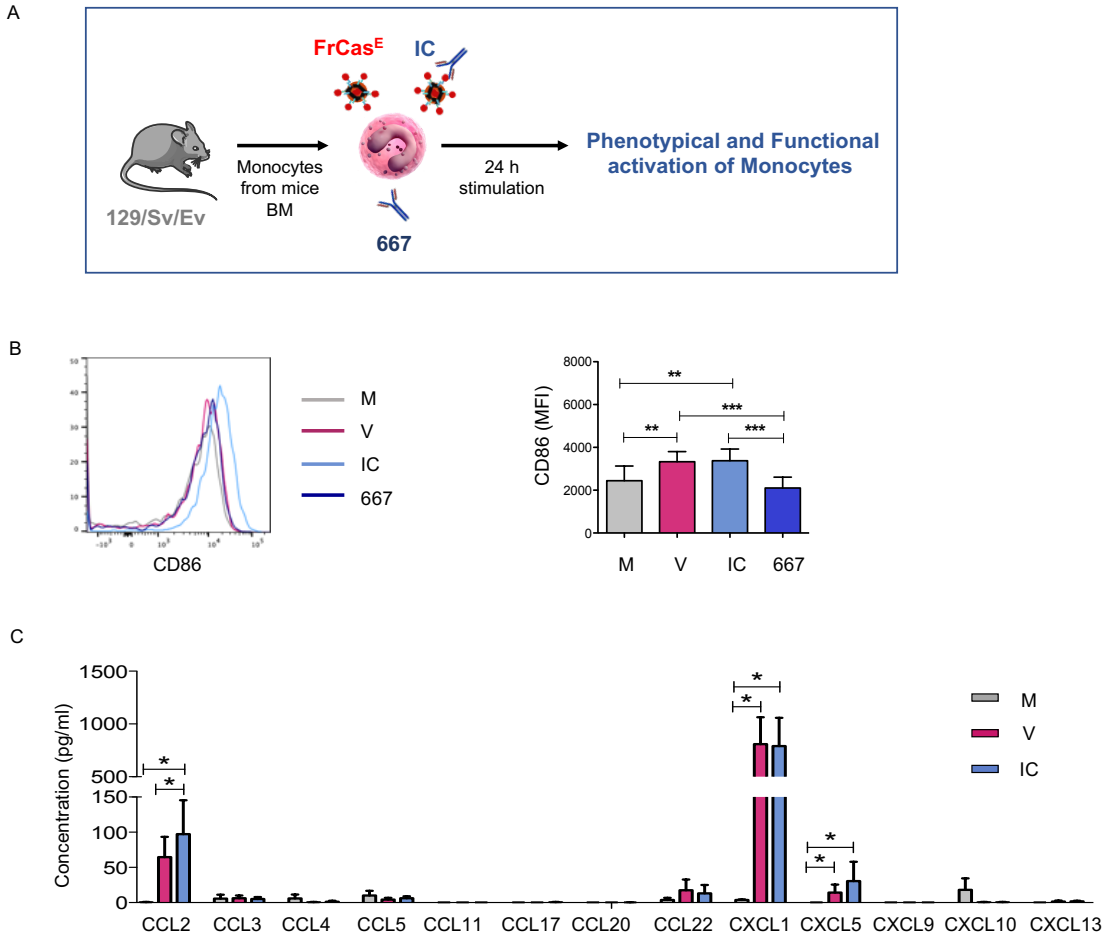


Figure 2: **Phenotypic and functional activation of monocytes stimulated with FrCasE virions and ICs**. **A.** *Experimental scheme*. **B**. *Phenotypic activation of monocytes stimulated by FrCasE virions or viral ICs made with the 667 mAb*. Free 667 mAb was used as control. V, free virions; IC, viral ICs; M, culture medium. Activation was assessed by monitoring CD86 expression. The data represent 7 independent experiments **C**. *Functional activation of monocytes stimulated by FrCasE virions (V) or viral ICs made with the 667 mAb (IC)*. Chemokines release was assessed in supernatants of monocytes isolated from BM of naive mice (>97-98% purity) and stimulated for 24 h by FrCasE virions (red) or viral ICs (blue) or left unstimulated (grey). The data represent 5 independent experiments. Data are expressed as means +/- SEM. Statistical significance was established using a parametric 1-way ANOVA test with Bonferroni's multiple comparisons post-tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

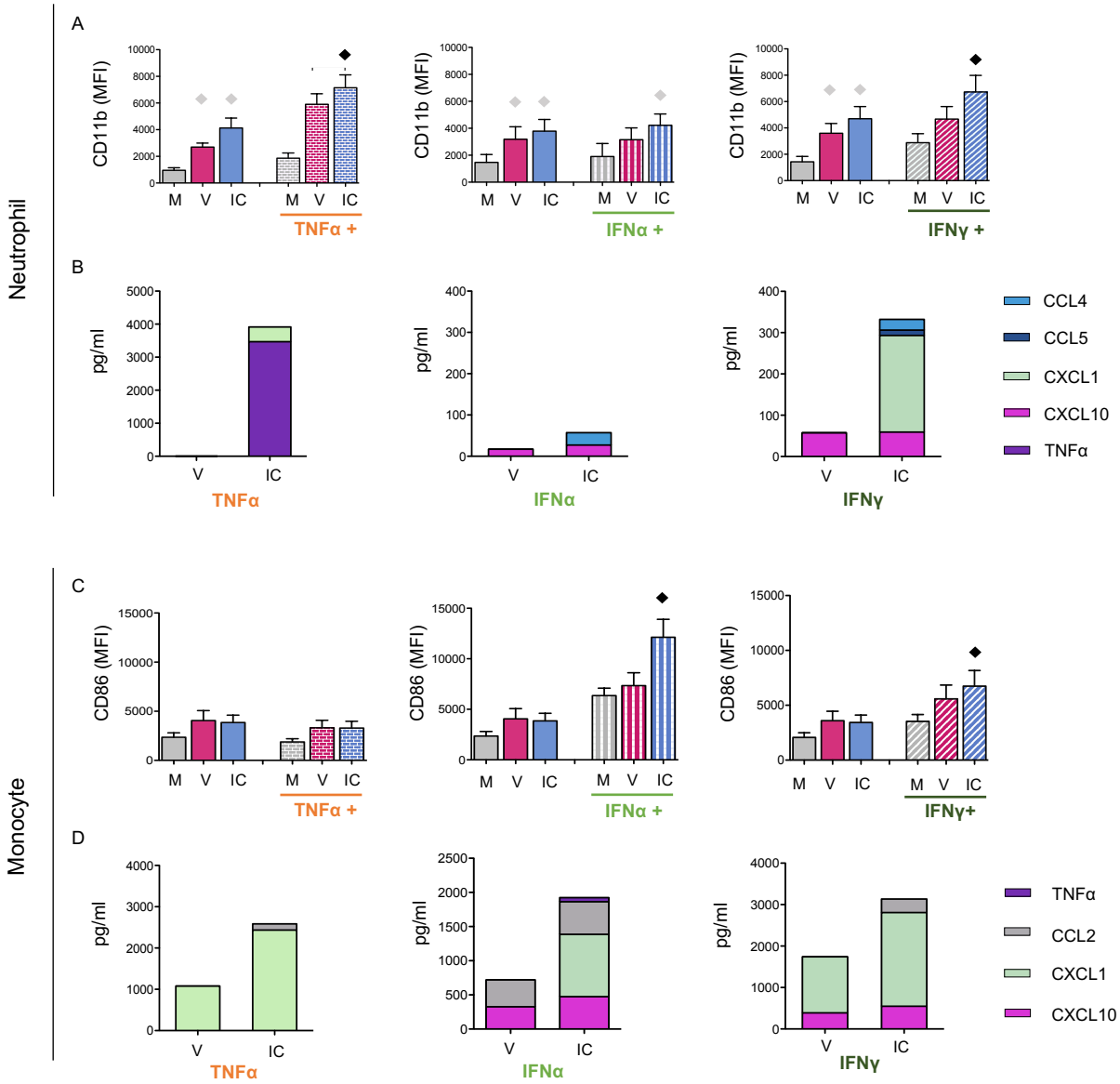


Figure 3: **Cytokine priming potentialize the functional activation of neutrophils and monocytes by ICs.** BM-derived neutrophils and monocytes were isolated from naive mice and activated as in **Figures 1 and 2** in the presence, or in the absence, of *TNFα, IFN-I or IFNγ*. V, free virions; IC, viral ICs; M, culture medium. **(A, C).** *Phenotypic activation of activated neutrophils (**A**) and monocytes (**C**) by TNFα, IFN-I or IFNγ.* Activation was assessed by monitoring CD11b (**A**) and CD86 expression **(C)**. *Modulation of the functional activation of neutrophils and monocytes by TNFα, IFN-I or IFNγ.* **(B, D).** Chemokines and cytokines release were assessed in supernatants of activated neutrophils and monocytes. Histograms depict the increase in cytokine/chemokine release upon priming of virus- and IC-activated neutrophils by *TNFα, IFN-I or IFNγ* (calculated as the difference in the amount of chemokine/cytokine secretion by V- or IC-stimulated neutrophils in the absence or in the presence of priming; **Supplemental figure 3**). Only those chemokines/cytokines significantly enhanced by cytokine priming are depicted. The data represent 6 independent experiments for **A**, 4 independent experiments for **B** and 3 independent experiments for **C** and **D**. Data are expressed as means +/- SEM. Diamonds indicate significant differences to all the other stimulation conditions (black diamond) or to corresponding medium without virus or IC stimuli (grey diamond) as determined by Kruskal-Wallis test with Dunn's multiple comparisons post-tests ($p < 0.05$).

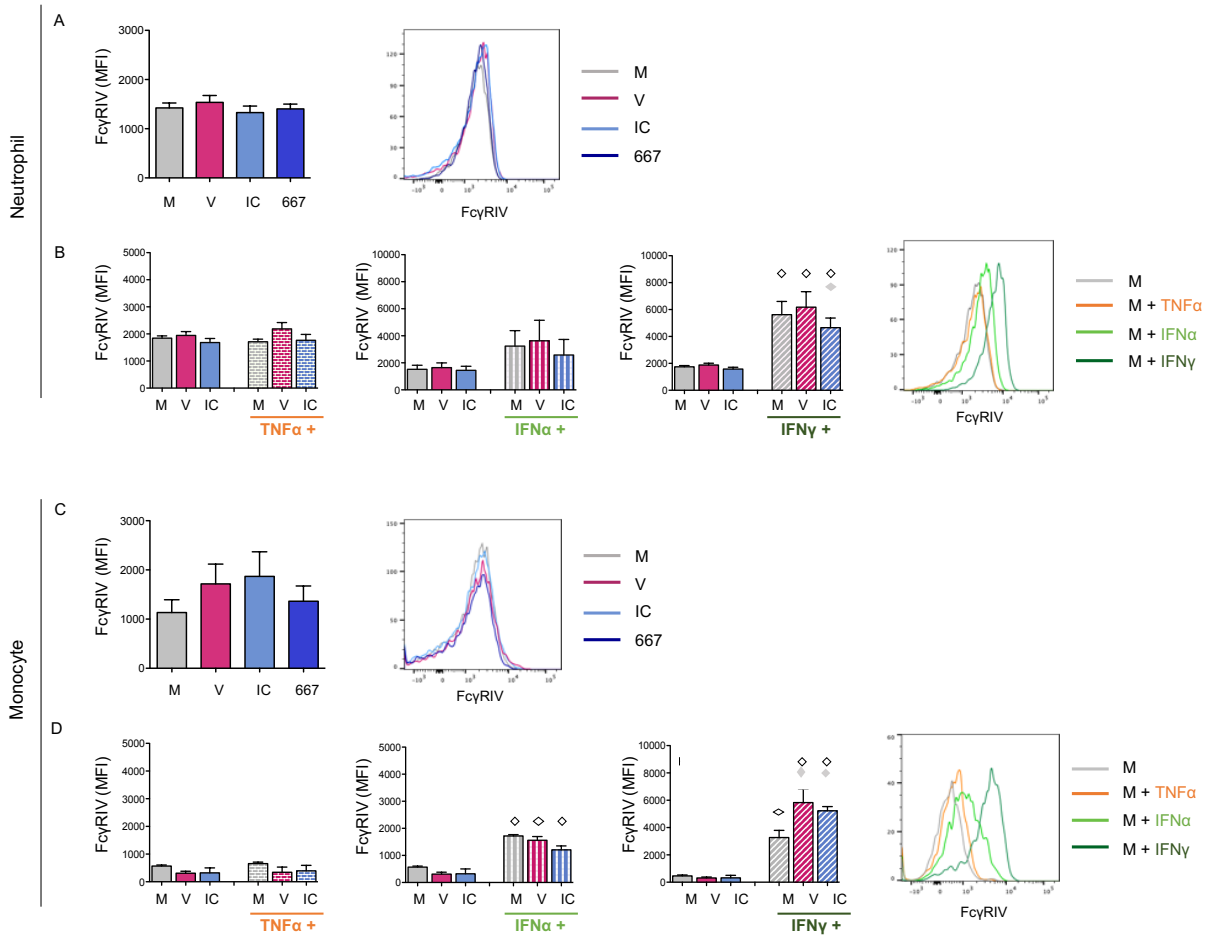
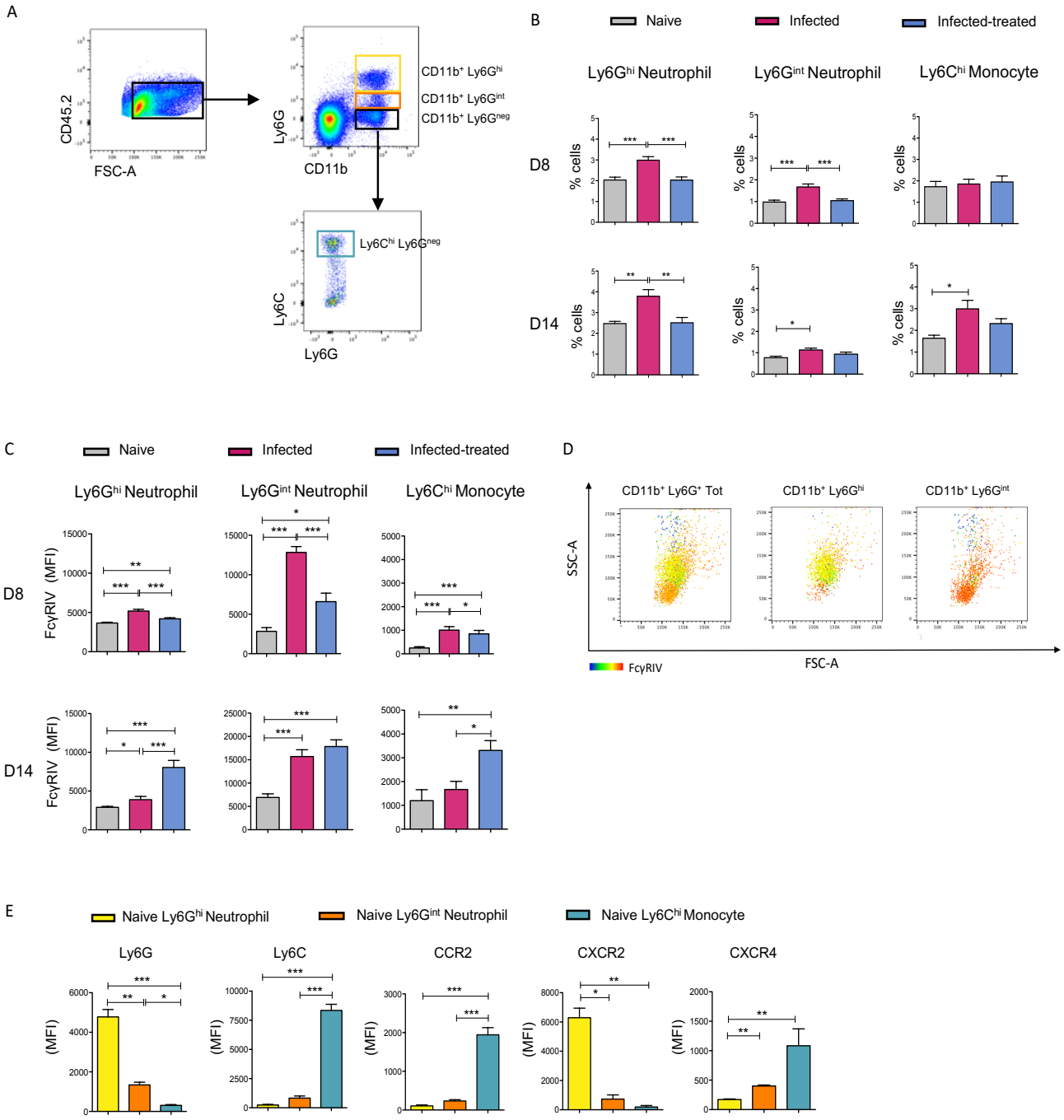


Figure 4: **FcγRIV is upregulated by IFN priming on both neutrophils and monocytes cell surface.** Neutrophils and monocytes were isolated from naive mice and stimulated as in **Figure 3**. FcγRIV expression was evaluated on neutrophils. V, free virions; IC, viral ICs; M, culture medium. **(A, B)** and monocytes **(C, D)** in absence (**A, C**) or in the presence of TNFα, IFN-I or IFNγ (**B, D**). The data represent 5 independent experiments for neutrophils (**A, B**) and 3 independent experiments for monocytes (**C, D**). Data are expressed as means +/- SEM. Diamonds indicate significant differences to the corresponding stimuli in the absence of cytokine priming (open diamond) or to corresponding medium without virus or IC stimuli (grey diamond) as determined by Kruskal-Wallis test with Dunn's multiple comparisons post-tests ($p < 0.05$).



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942 ***Figure 5:*** **FcγRIV is upregulated *in vivo* on two different splenic neutrophils subpopulations and on**
943 **inflammatory monocytes.** Splenocytes from naive (grey), infected/non-treated (I/NT; red) and infected/treated
944 (I/T;blue) mice were analyzed on days 8 (D8) and 14 (D14) p.i. for (i) neutrophils and monocytes recruitment and
945 (ii) for FcγRIV expression. **(A)** Gating strategy used to define neutrophils and monocytes populations. **(B)**
946 Frequencies of *CD11b*$^{+}$*Ly6G*^{hi} and *CD11b*$^{+}$*Ly6G*^{int} neutrophils subpopulations as well as *Ly6C*^{hi} monocytes in the
947 CD45.2^{+} leukocytic population. The data represent 5 independent experiments at D8 p.i. and 6 independent
948 experiments at D14 p.i with at least 6-9 mice per group (I/NT and I/T) and 3-5 mice per group (naive mice). **(C-**
949 **D)** FcγRIV expression on *CD11b*$^{+}$*Ly6G*^{hi} and *CD11b*$^{+}$*Ly6G*^{int} neutrophils subpopulations and *Ly6C*^{hi} monocytes.
950 The data represent 5 independent experiments at D8 p.i. and 6 independent experiments at D14 p.i with at least 6-
951 9 mice per group (I/NT and I/T) and 3-5 mice per group (naive mice). **(E)** Cell surface markers expression of
952 *CD11b*$^{+}$*Ly6G*^{hi} and *CD11b*$^{+}$*Ly6G*^{int} neutrophils and *Ly6C*^{hi} monocytes isolated from 15-days old naive mice, with
953 3-5 mice per group. Data are expressed as means +/- SEM. Statistical significance was established using a
954 parametric 1-way ANOVA test with Bonferroni's multiple comparisons post-tests (*$p < 0.05$; **$p < 0.01$; ***$p <$
955 0.001).

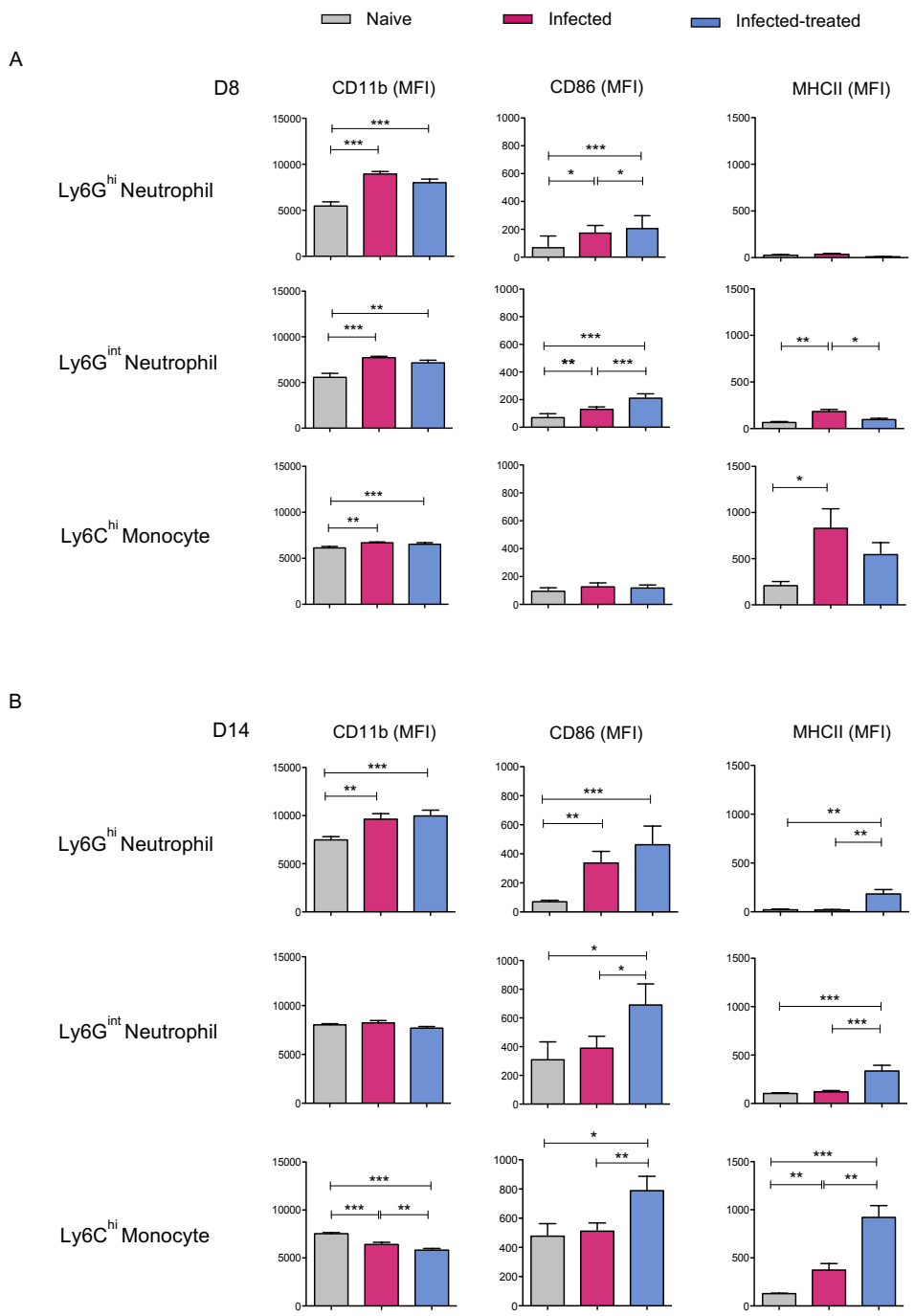

Figure 6: **Cell surface activation markers expression on two different splenic neutrophils subpopulations and on inflammatory monocytes.** Splenocytes from naive (grey), infected/non-treated (I/NT; red) and infected/treated (I/T;blue) mice were analyzed on days 8 (**A**) and 14 p.i. (**B**) for neutrophils and monocytes expression of CD11b, CD86 and MHC-II. Data represent 5 independent experiments at D8 p.i. and 6 independent experiments at D14 p.i with at least 6-9 mice per group (I/NT and I/T) and 3-5 mice per group (naive mice). Data are expressed as means +/- SEM. Statistical significance was established using a parametric 1-way ANOVA test with Bonferroni's multiple comparisons post-tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

A
D8
D14
Chemokines (pg/ml)
Cytokines (pg/ml)
1500
1000
500
0
I IT
Ly6Ghi
Ly6Gint
Ly6Chi
B
Chemokines
Cytokines
CCL2
CCL3
CCL4
CCL5
CCL11
CCL17
CCL20
CCL22
CXCL1
CXCL5
CXCL9
CXCL10
CXC13
IL1α
IL1β
IL6
IL10
IL12p70
IL17A
IL23
IL27
IFNβ
IFNγ
TNFα
GM-CSF
Naive
0 1 10 50 100 >500
C
Chemokines
Cytokines
CCL2 CCL3 CCL4 CCL5 CCL11 CCL17 CCL20 CCL22 CXCL1 CXCL5 CXCL9 CXCL10 CXCL13
IL1α IL1β IL6 IL10 IL12p70 IL17A IL23 IL27 IFNβ IFNγ TNFα GM-CSF

1000
1001 ***Figure 7:*** **Cytokine and chemokine secretion profile of two different splenic neutrophils subpopulations and**
1002 **on inflammatory monocytes.** Splenic neutrophils subpopulations and inflammatory monocytes from naive,
1003 infected/non-treated (I) and infected/treated mice (IT) were isolated at days 8 p.i. and 14 p.i. for assaying their
1004 chemokine and cytokine secretion profile in supernatants of sorted cells cultured at a density of $4x10^6$ cells/ml
1005 ($1x10^6$ cells/well) for 24 h. Data represent 3 independent experiments on D8 p.i. and 4 independent experiments
1006 on D14 p.i. with at least 6-8 mice per group. (**A**) Total quantity of chemokines and cytokines secreted by
1007 neutrophils subpopulations and inflammatory monocytes from infected/non-treated and infected/treated mice. (**B**)
1008 Chemokine and cytokine secretion of neutrophils subpopulations and inflammatory monocytes isolated from
1009 naive, infected/non-treated (I) and infected/treated mice (IT). The color code shows fold-increase compared to
1010 secretion detected in cells isolated from naive mice (the raw chemokine and cytokine release values are given in
1011 brackets, expressed in pg/ml). (**C**) Chemokine and cytokine secretion of neutrophils subpopulations and
1012 inflammatory monocytes isolated from infected/non-treated (I) and infected/treated mice (IT) expressed as
1013 percentage of chemokine and cytokine showing a fold-increase ≥1.4 as compared to cells isolated from naive mice.