Differential and sequential immunomodulatory role of neutrophils and Ly6C^{hi} inflammatory monocytes during antiviral antibody therapy

Authors:

Jennifer Lambour[1], Mar Naranjo-Gomez[1,2], Myriam Boyer-Clavel[3] and Mireia Pelegrin[1,2] *

Address:

[1] IGMM, Univ Montpellier, CNRS, Montpellier, France

[2] Current address: IRMB, Univ Montpellier, INSERM, CNRS, Montpellier, France

[3] Montpellier Ressources Imagerie, Biocampus, Univ Montpellier, CNRS, Montpellier, France

***Corresponding author and Lead contact:**

Mireia Pelegrin

Institute of Molecular Genetics of Montpellier

1919, route de Mende

34293 Montpellier Cedex 5

France

mireia.pelegrin@inserm.fr

Keywords : antiviral immune responses, monoclonal antibodies, immunotherapy, vaccinal effects, immune complexes, neutrophils, monocytes, FcγR.

Abstract

Antiviral monoclonal antibodies (mAbs) can generate protective immunity through Fc-FcγRs interactions. Using a mouse model of retroviral infection, we previously showed a crucial role for immune complexes (ICs) in the enhancement of T-cell responses through FcγR-mediated activation of dendritic cells (DCs). However, IC-FcγR interactions involve different cells of the immune system other than DCs such as neutrophils and monocytes. These two myeloid cell-types are innate effector cells rapidly recruited to sites of infection. In addition to being key cells to fight against invading pathogens, they are also endowed with immunomodulatory properties. While the role of DCs in enhancing antiviral immune responses upon mAb treatment has been addressed in several studies, the role of neutrophils and monocytes has been much less studied. Here we addressed how mAb therapy affects the functional activation of neutrophils and inflammatory monocytes in retrovirus-infected mice. We found that both cell-types activated *in vitro* 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 FcγRIV upregulation. Similarly, infection and mAb-treatment upregulated FcγRIV expression on neutrophils and inflammatory monocytes and enhanced their cytokines and chemokines secretion. Notably, upon antibody therapy neutrophils and inflammatory monocytes displayed distinct functional activation states and sequentially modulated the antiviral immune response through the secretion of Th1-type polarizing cytokines and chemokines. Our work provides novel findings on the immunomodulatory role of neutrophils and monocytes in the enhancement of immune responses upon antiviral mAb therapy.

56 **Introduction**

57 The development of powerful antiviral monoclonal antibodies (mAbs) has provided new
58 therapeutic opportunities to treat severe viral infections [1,2]. Fc-dependent mechanisms are
59 crucial for efficient antiviral activity of neutralizing mAbs through the engagement of IgG
60 receptors (FcγRs) expressed on immune cells. These Fc-FcγR interactions lead to the
61 elimination of viral particles and virus-infected cells through phagocytic and cytotoxic
62 mechanisms (i.e. antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cell-
63 mediated cytotoxicity (ADCC),…) [3]. Moreover, studies in different animal models of viral
64 infection, including ours, have provided evidence that mAbs can also enhance antiviral immune
65 responses (so called "vaccinal effects") in a Fc-dependent manner [4]. These vaccinal effects have
66 been recently reported in HIV-infected patients treated with broadly neutralizing mAb (bnAbs)
67 [5–7] although the mechanisms involved have not been identified thus far. The elucidation of the
68 molecular and cellular mechanisms driving Fc-dependent, mAb-mediated immunomodulation
69 is therefore an important issue that will be key to achieving protective immunity against severe
70 viral infections by mAbs.

71

72 While several Fc-mediated effector functions (i.e. ADCC, ADCP, ….) have been shown to be
73 required for antibody-mediated antiviral protection, [8–11] whether and how FcγR engagement by
74 antiviral mAbs affects the immunomodulatory properties of different FcγR-expressing cells
75 (i.e. cytokines/chemokines secretion, activation markers expression, …) has been little studied.
76 In addition, the specific contribution of different FcγRs-expressing cells in the induction of
77 vaccinal effects by mAbs still remains ill-understood. These issues are important to be
78 addressed due to their potential clinical implications. However, multiple restrictions (i.e.
79 technical and ethical issues, costs, …) largely limit those studies in humans and non-human
80 primates (NHP). As an alternative, *in vivo* studies in immunocompetent mice infected with the

81 Murine Leukemia Virus FrCasE allowed the identification of several immunological
82 mechanisms that drive protective immunity upon mAb therapy [4,12]. We have previously shown
83 that treatment of FrCasE-infected mice with the neutralizing mAb 667 (recognizing the
84 retroviral envelope glycoprotein, expressed on virions and infected cells) elicits protective
85 adaptive antiviral immunity through the engagement of FcγRs [13,14]. Notably, mAbs form
86 immune complexes (ICs) with viral determinants that enhance antiviral T-cell responses
87 through FcγR-mediated binding to dendritic cells (DCs) [13,15–17]. However, IC-FcγR interactions
88 may involve different cells of the immune system other than DCs (i.e. neutrophils, monocytes,
89 ….). We have recently shown a key immunomodulatory role of neutrophils in the induction of
90 protective humoral responses *via* the acquisition of B-cell helper functions (i.e. B-cell activating
91 factor secretion) upon engagement of FcγRs by the therapeutic mAb [18]. While the role of IC-
92 activated DCs in the enhancement of antiviral immune responses has been addressed in several
93 studies [12,19,20] the role of IC-activated neutrophils has mostly been overlooked. Neutrophils are
94 innate effector cells rapidly recruited to sites of infection. Evidence shows that, in addition to
95 being key effector cells to fight against invading pathogens, neutrophils are also endowed with
96 immunomodulatory properties through the secretion of a plethora of chemokines and cytokines
97 [21–23]. Yet, the functional activation of neutrophils by viral ICs and the resulting effect on their
98 immunomodulatory properties have poorly been studied in the context of antiviral mAbs
99 therapies. Similar to neutrophils, inflammatory Ly6C^{hi} monocytes are also rapidly recruited to
100 sites of infection and are key players to fight against viral infections [24]. Recently, it has been
101 reported that the therapeutic effect of antiviral mAb requires the engagement of FcγR on
102 monocytes [10]. This depends on Fc-FcγR interactions that are crucial to control viral spread
103 through the enhancement of ADCP. However, the potential contribution of monocytes to the
104 induction of vaccinal effects by antiviral mAb has not been reported. As both neutrophils and
105 inflammatory monocytes display multiple immunomodulatory functions and can mediate

106 protective immunity, immunopathology or immunosuppression in a context dependent manner,
107 it is important to dissect how antiviral mAb therapy shapes the phenotype and functional
108 properties of these FcγR-expressing cells as this might have important therapeutic implications.
109
110 Here, we used the FrCasE retroviral model to address how viral infection, with or without mAb
111 therapy, affects the phenotypical and functional activation of neutrophils and inflammatory
112 monocytes. Both, neutrophils and monocytes activated *in vitro* by viral determinants secreted
113 high levels of monocyte- and neutrophil-recruiting chemokines, suggesting a self-sustaining
114 mechanism of neutrophils and monocytes recruitment upon viral infection. *In vivo*, we have
115 shown that viral infection and mAb-treatment shape the immunomodulatory properties of
116 neutrophils and inflammatory monocytes. Our data show that the functional activation of both
117 cell types differs in terms of cytokine and chemokine secretion, evolves overtime and is
118 different in the presence or the absence of mAb-treatment. Importantly, antibody therapy leads
119 to increased secretion of proinflammatory cytokines and chemokines that are potent inducers
120 of Th1-biased immune responses. Our work provides hitherto unreported findings on the
121 immunomodulatory properties of neutrophils and inflammatory monocytes in a context of
122 antiviral mAb-therapy. These findings might help to improve mAb-based antiviral therapies by
123 tailoring therapeutic interventions aiming at harnessing the immunomodulatory properties of
124 these cells.
125

126 **Materials and methods**

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

131

132 ***Viral stocks***: FrCasE [25] viral stocks were produced and stored as previously described in [26].

133

134 ***Viral infection and immunotherapy:*** Eight-day-old 129/Sv/Ev mice were infected by
135 intraperitoneal (i.p.) administration with 50 µl of a viral suspension containing 50,000 focus-
136 forming units (FFU) and treated, or not, with 30 µg 667 mAb targeting gp70 protein of viral
137 envelope, [27], 1-hour p.i. and on days 2 and 5 p.i. by i.p. administration. Mice were euthanized
138 and spleens collected at days 8 and 14 p.i.

139

140 ***Phenotypical and functional activation of FcγRIV-expressing cell from spleen ex vivo:***
141 Single-cell suspensions of splenocytes were obtained from naive, infected/non-treated and
142 infected/treated mice at 8 and 14 days p.i. Spleen cell suspensions were obtained by mechanical
143 dissociation in PBS, then filtered in 0,70 µm strainer. 20 % of each spleen was used for
144 immunophenotyping by FACS, 80% left was dedicated to neutrophils and monocytes sorting.
145 Red blood cells were lysed (ACK, Lonza) and an enrichment with biotinylated anti-B220 (BD
146 Biosciences), anti-CD3 (BD Biosciences), following of anti-biotin Ab magnet-bead coupled
147 (Miltenyi) and magnetic LS-columns (Miltenyi) was performed to remove spleen lymphocytes
148 and increase the sorting efficacy. Cells were stained with specific marker of populations of
149 interest (Ly6G-BD BioSciences, Ly6C-BioLegend, CD11b-BD BioSciences) and neutrophils
150 (Ly6G^{hi}) and inflammatory monocytes (Ly6C^{hi}) were sorted (>97-98% pure) using the BD

151 Biosciences FACSAria device. Sorted cells were cultured in 96-well plates at a density of $4x10^6$
152 cells/ml ($1x10^6$ cells/well) in 10% FBS-containing RPMI medium for 24h, and cell-free
153 supernatants were collected and stored at -20°C, to allow cytokines and chemokines protein
154 release quantification.

155 ***Flow cytometry:*** Organs of interest were collected to realize immunophenotyping of immune
156 cells. Spleen cell suspensions were obtained by mechanical dissociation in PBS. BM cell
157 suspensions were obtained by dissection and PBS-2%-FBS flushing of tibias and femurs. Cells
158 were stained at 4°C using fluorochrome-conjugated antibodies against, CD11b (M1/70,
159 eBioscience), CD45.2 (104, BD Biosciences), CD62L (MEL-14, BD Biosciences), CD86
160 (GL1, BD Biosciences), Ly6G (1A8, BD Biosciences) and Ly6C (AL-21, BioLegend). FcγRIV
161 expression was determined using 9E9 antibody (kindly provided by Dr. Pierre Bruhns, Institut
162 Pasteur), produced by BioXcell, and then labeled with Alexa Fluor 647. Forward scatter area
163 and forward scatter time-of-flight, as well as side scatter, were used to remove doublets from
164 flow cytometry analyses. Cells were analyzed on FACS LSR Fortessa (BD Bioscience), and
165 the data were analyzed using the FlowJo software.

166 ***Neutrophils and monocytes isolated from BM used in in vitro experiments****:* Neutrophils and
167 monocytes were purified from 8 to 11-week-old naive mice BM. After dissection of lower
168 limbs, BM cell suspensions were collected by PBS-2% FBS EDTA (2 mM) flushing (25G
169 needle) of tibias and femurs. BM cell suspensions were filtered with a 0,40 µm nylon strainer.
170 Two magnetic-based cell sorting (MACS) isolation kits were used to purified either neutrophils
171 (Miltenyi Biotec) and monocytes, (Miltenyi Biotec) by negative selection, both with high purity
172 (>97-98%), determined by FACS (LSR Fortessa, BD Bioscience). Cells were placed in culture
173 in U bottom 96-well plates at a concentration of 1 million/ml in 10% FBS-containing RPMI
174 medium.

175

176 ***Stimulation of neutrophils and monocytes in vitro***: Purified neutrophils and monocytes were
177 seeded at 150 000 cells/ well in 150 µl of RPMI, then incubated for 24h with LPS (1µg/ml,
178 Sigma), or FrCasE virus (MOI 5: 5 viral particles /cell), or ICs (virus FrCasE and 1µg 667
179 mAb), or 1µg 667 mAb alone. The MOI and 667 mAb concentration used to form ICs were
180 previously identified by dose-response experiments involving different MOI and different 667
181 mAb quantities. In parallel, the same experiments were performed adding inflammatory
182 cytokines, TNFα 100 UI/ml (Peprotech), IFN γ 100 UI/ml (eBioScience), IFNα11 a 1000
183 UI/ml, produced and generously provided by Dr. Gilles Uzé (DIMP, CNRS). After 24h of
184 stimulation, supernatants were collected and stored at -20°C to quantify chemokines and
185 cytokines protein release secretion. The viability of neutrophils and monocytes 24h post-
186 stimulation was high (> 85%). No significant differences in cell viability were observed among
187 the different stimulation conditions. Phenotypic activation of both neutrophils and monocytes
188 was measured using surface markers by flow cytometry.

189

190 ***Chemokines and cytokines protein release quantification:*** Soluble chemokines and cytokines
191 secretion were quantified from cell-free collected supernatants of *in vitro* cultured neutrophils
192 and monocytes and of sorted splenic neutrophils and inflammatory monocytes (of naive,
193 infected/non-treated and infected/treated mice 8 and 14 days p.i. and immunotherapy), using
194 bead-based immunoassays (LegendPLex, BioLegend) and analyzed on the BD Bioscience-LSR
195 Fortessa device. The protein release quantification was established by the appropriate software
196 (LEGENDplex^{TM} data analysis).

197

198 ***Statistics:*** Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software).
199 Data were expressed as mean ± SEM, and statistical significance was established using a

200 parametric 1-way ANOVA test with Bonferroni's multiple comparisons post-tests or non-
201 parametric Kruskal-Wallis test with Dunn's multiple comparisons post-test for multiple
202 comparisons or paired Student's *t* tests when two groups were compared. *P* values lower than
203 0.05 were considered as statistically significant.

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

207

208 **Results**

209 ***Neutrophils activated by viral determinants, free or in the form of ICs, secrete high levels of***
210 ***chemokines able to recruit monocytes and neutrophils***. We have previously shown a key role
211 of neutrophils in the induction of long-term protective antiviral immunity upon mAb therapy
212 of infected mice [18]. To better characterize the phenotypical and functional activation of
213 neutrophils by viral determinants, free or in the form of ICs, we isolated bone marrow (BM)
214 neutrophils from naive mice and stimulated them for 24h *in vitro* with free FrCasE virions or
215 opsonized with the 667 mAb (ICs) (Figure 1A). Free 667 mAb was used as control. Both,
216 FrCasE virions and ICs induced a strong activation of neutrophils as shown by a higher
217 expression of the CD11b molecule as well as an increased frequency of $CD11b^{hi}$ $CD62L^{lo}$
218 neutrophils (Figure 1B). However, IC-mediated phenotypic activation was significantly higher.
219

220 We next assessed the functional activation of virus- and IC-stimulated neutrophils by measuring
221 their capacity to secrete chemokines and cytokines. Both stimuli led to high secretion levels of
222 several chemokines such as CCL2, CXCL1, CXCL5 and to a lesser extent CCL3 and CCL4
223 (Figure 1C), the secretion of the latter chemokine being significantly enhanced in IC-stimulated
224 neutrophils. However, both the virus and ICs poorly induced the secretion of the 12 cytokines
225 analyzed (Supplemental Figure 1A). This contrasted with the high secretion of IL-6 and TNFα
226 pro-inflammatory cytokines observed upon lipopolysaccharide (LPS) stimulation despite
227 similar CD11b upregulation induced by LPS and viral determinants (Supplemental Figures 1B-
228 1C). In addition, LPS-stimulated neutrophils only secreted a low amount of CCL3 and CCL4
229 chemokines, with no secretion of CCL2, CXCL1, CXCL5 chemokines (Supplemental Figure
230 1D). These data show that bacterial-related pathogen-associated molecular patterns (PAMPs)
231 induce a functional activation of neutrophils different from that of viral stimuli. Interestingly,
232 the chemokines which were more strongly secreted by neutrophils upon viral stimuli (but not

233 by LPS) have been shown to be involved in the recruitment of neutrophils themselves (CXCL1,
234 CXCL5) and inflammatory monocytes (CCL2).
235
236 ***Inflammatory monocytes activated by viral determinants, free or in the form of ICs, secrete***
237 ***high levels of chemokines able to recruit neutrophils and monocytes***. We next assessed the
238 activation of Ly6C^{hi} monocytes isolated from naive mice BM and stimulated for 24h with virus
239 or ICs (Figure 2A). Both stimuli significantly activated monocytes (as depicted by an increased
240 CD86 expression) (Figure 2B) and induced the secretion of CXCL5, CXCL1 and CCL2
241 chemokines, the secretion of the latter chemokine being significantly enhanced in IC-stimulated
242 monocytes (Figure 2C). As compared to neutrophils, higher amounts of the neutrophil-
243 recruiting chemokine CXCL1 were detected as well as lower amounts of CXCL5 and CCL2.
244 Virus- and ICs induced a weak secretion of most of the 12 cytokines analyzed (Supplemental
245 Figure 2A). This contrasted with high level secretion of IL-6, TNFα and IFNγ observed upon
246 LPS stimulation (Supplemental Figure 2C). LPS stimulation also induced a wider and different
247 panel of chemokine release (Supplemental Figure 2D), notably with the secretion of high
248 amounts of CCL3, CCL4, CCL5, and to a lesser extent CXCL1 and CXCL10. As observed in
249 neutrophils, these data show that viral stimuli induce a functional activation of monocytes
250 different from that of bacterial-related PAMPs.
251
252 ***Inflammatory conditions potentiate the activation of neutrophils and monocytes by viral ICs***.
253 As the inflammatory microenvironment resulting from the viral infection and mAb therapy
254 might affect the antiviral immune response, we next assessed the phenotypic and functional
255 activation of neutrophils and monocytes by viruses and ICs under an inflammatory environment
256 (i.e. in the presence of proinflammatory/immunomodulatory cytokines, such as TNFα, IFN-I
257 and IFNγ).

258

259 TNFα and IFNγ significantly enhanced the phenotypic activation of neutrophils by ICs (but not
260 by free virus) (Figure 3A). We also showed that TNFα, IFNγ and IFN-I enhanced the secretion
261 of different cytokines and chemokines by IC-stimulated neutrophils (Figures 3B-3E). Notably,
262 we observed an enhanced secretion of TNFα (by TNFα itself), CXCL1 (by TNFα and IFNγ),
263 CCL4 and CXCL10 (by IFNγ and IFN-I) and CCL5 (by IFNγ). In contrast, inflammatory
264 conditions hardly modified the secretion profile of free virus-activated neutrophils as only the
265 secretion of the CXCL10 was induced in virus stimulated cells, consistent with the IFN-
266 dependent induction of this chemokine (Figure 3E).

267

268 With regard to monocytes, IFN-I and IFNγ significantly potentiated the phenotypic activation
269 of IC-stimulated monocytes (as depicted by an increased expression of the CD86 molecule)
270 (Figure 4A) but not that of virus-activated cells. Similar to neutrophils, TNFα, IFNγ and IFN-
271 I also enhanced the secretion of different cytokines/chemokines by IC-stimulated monocytes,
272 leading to increased secretion of CCL2 and CXCL1 (enhanced by the 3 cytokines), TNFα
273 (enhanced by IFN-I) and CXCL10 (enhanced by both types of IFN) (Figure 4B-4E). TNFα,
274 IFNγ and IFN-I stimulation also modulated the cytokine/chemokine secretion profile of virus-
275 activated monocytes (i.e. CCL2, CXCL1), although to a lesser extent than IC-activated cells
276 (Figure 4E).

277

278 Altogether, these data highlight that inflammatory conditions enhance the phenotypic and
279 functional activation of IC-stimulated neutrophils and monocytes. The cytokine/chemokine
280 secretion enhancement observed in IC-activated cells differs depending on the stimulating
281 cytokine and the responding cell type (i.e. increased secretion of TNFα, CCL4, CCL5 and
282 CXCL1 by IC-activated neutrophils and TNFα, CCL2 and CXCL1 by IC-activated

283 monocytes). It is worth to note that inflammatory conditions mainly enhanced the secretion
284 profile of IC-activated cells but not that of virus-activated cells (notably in neutrophils).
285
286 ***Inflammatory conditions upregulate the expression of FcγRIV on in vitro activated***
287 ***neutrophils and monocytes.*** FcγRs expression might differ between steady-state *versus*
288 inflammatory/pathological conditions [28], however little is known about modulation of FcγRs
289 expression in the context of viral infections and mAb-therapy. We thus assessed whether the
290 activation of neutrophils and monocytes by virus and ICs affected the expression of the
291 activating receptor FcγRIV, both in the absence and in the presence of inflammatory cytokines.
292 The modulation of this FcγR is all the more relevant to be studied in this experimental model
293 as (i) it is a high affinity receptor for IgG2a, (which is the isotype of the 667 mAb) and (ii) it is
294 highly expressed on neutrophils, the latter having a key immunomodulatory role in mAb-
295 mediated protection of retrovirus-infected mice [18]. We found that IFNγ and IFN-I stimulation
296 (but not TNF-α) led to the upregulation of FcγRIV expression on neutrophils (IFNγ) (Figure
297 5A) and monocytes (IFNγ and IFN-I) (Figure 5B). In contrast, in the absence of inflammatory
298 cytokines, neither the virus nor the ICs significantly modulated the expression FcγRIV on
299 neutrophils (Figure 5A) and monocytes (Figure 5B). However, IFNγ stimulation enhanced the
300 upregulation of FcγRIV expression in virus- and IC-stimulated monocytes. These results show
301 the specific effect of the different inflammatory cytokines (TNF-α, IFN-I, IFN-γ) on the
302 modulation of FcγRIV expression.
303
304 ***Viral infection and mAb therapy upregulate FcγRIV expression on neutrophils and on***
305 ***inflammatory monocytes***. We next assessed *in vivo* whether the inflammatory environment
306 resulting from FrCasE viral infection and 667 mAb therapy modulated the expression of

307 FcγRIV on neutrophils and inflammatory monocytes. To this end, mice were infected and
308 treated, or not, with the therapeutic mAb (infected/treated and infected/non-treated,
309 respectively). Then, the expression of FcγRIV on neutrophils and inflammatory monocytes
310 from the spleen (one of the main sites of viral replication) was evaluated at different time points
311 post-infection (p.i.): at day 8 p.i. (i.e. corresponding to the peak of viremia) and day 14 (i.e.
312 corresponding to the peak of primary cytotoxic T-cell responses) [13]. Age-matched naive mice
313 were used as controls. The cell populations of interest were defined by flow cytometry (Figure
314 6A) based on the expression of CD11b, Ly6G and Ly6C to gate neutrophils ($CD11b^{+}$, $Ly6G^{hi}$)
315 and inflammatory monocytes ($CD11b^{+}Ly6G^{-}Ly6C^{hi}$).
316
317 In steady-state conditions, FcγRIV was highly expressed on splenic neutrophils. Lower FcγRIV
318 expression was detected in $Ly6C^{hi}$ monocytes at the different time points assessed (Figure 6B).
319 At day 8 p.i. FcγRIV expression was significantly upregulated on neutrophils and monocytes
320 in infected/non-treated mice. In contrast, at day 14 p.i. infected/treated mice showed a stronger
321 upregulation of FcγRIV on both cell-types as compared to infected/non-treated and control
322 mice.
323
324 These results suggest a distinct inflammatory environment in infected/non-treated mice *versus*
325 infected/treated mice that evolves over time and differentially modulates the expression of the
326 activating FcγRIV in neutrophils and monocytes, leading to a stronger upregulation at 8 days
327 p.i. in infected/non- treated mice and at 14 days p.i. in infected/treated mice.
328
329 ***FcγRIV upregulation in infected/treated mice is associated with enhanced expression of***
330 ***MHC-II and co-stimulatory molecules on neutrophils and monocytes.*** We next addressed the
331 activation of splenic neutrophils and inflammatory monocytes in infected mice with or without

332 immunotherapy at days 8 and 14 p.i. by monitoring cell surface activation markers. Similar to
333 FcγRIV expression, we showed that the activation state of the cells evolved over time. At day
334 8 p.i. (Figure 6C), CD11b was upregulated on neutrophils and inflammatory monocytes upon
335 viral infection either in the absence or in the presence of immunotherapy. However,
336 infected/treated mice showed a significantly higher CD86 upregulation in neutrophils than
337 infected/non-treated mice. At day 14 p.i. (Figure 6C), CD11b and CD86 expression was
338 increased in splenic neutrophils isolated from both infected/treated- and infected/non-treated
339 mice, with no significant differences between these two groups of mice. In contrast, MHC-II
340 upregulation was only induced in infected/treated mice, suggesting a mAb-mediated
341 upregulation of this molecule. Similarly, we observed a significantly higher upregulation of
342 CD86 co-stimulatory molecule and MHC-II in inflammatory monocytes from infected/treated
343 mice as compared to infected/non-treated mice.
344
345 These data show that mAb treatment leads to the upregulation of MHC-II in neutrophils and
346 monocytes at 14 days p.i. as well as the upregulation of the CD86 costimulatory molecule in
347 monocytes (Figure 6C). This is associated with the FcγRIV upregulation observed in
348 neutrophils and Ly6C^{hi} monocytes from infected/treated mice at this time point (Figure 6B).
349
350 ***Neutrophils and inflammatory monocytes are differentially and sequentially activated upon***
351 ***antiviral mAb treatment.*** To further characterize the activation state of neutrophils and Ly6C^{hi}
352 monocytes upon viral infection and mAb-therapy, we addressed the cytokine and chemokine
353 secretion profile of splenic neutrophils and monocytes sorted from infected mice treated, or not,
354 with the 667 mAb, at days 8 and 14 p.i. Cells sorted from age-matched naive mice were used
355 as controls. We found that the secretion profile of neutrophils and monocytes was distinct but
356 it was globally enhanced in both cell-types in infected/treated mice as compared to

357 infected/non-treated mice (Figure 7A and 7B), in particular at day 8 p.i. in neutrophils and at
358 14 p.i. in monocytes.
359
360 At day 8 p.i., neutrophils from both infected/non-treated and infected/treated mice showed a
361 broad chemokine and cytokine secretion profile as deduced from an increased secretion of most
362 of the 13 chemokines and 12 cytokines assessed, although at weak levels (Figure 7B and 7C).
363 Notably, neutrophils from infected/treated mice showed a higher increase in IFN-I (IFNβ)
364 secretion (8,3-fold increase) than neutrophils from infected/non-treated mice (4,8-fold
365 increase). In contrast, infection and mAb-treatment hardly affected the chemokine/cytokine
366 secretion of Ly6C^{hi} monocytes as they only showed a weak increase (1.4-fold increase) in
367 CXCL1 secretion in infected/treated mice as well as a slight increase in cytokines secretion
368 (both in terms of diversity and fold increase) mainly in infected/non-treated mice (Figure 7B).
369
370 Interestingly, at day 14 p.i., the functional activation of both cell-types completely differed from
371 that observed at day 8 p.i. (Figure 7B and 7C). We found a more restricted but stronger
372 induction of chemokines/cytokines secretion, mostly in cells isolated from infected/treated
373 mice. In contrast to the broad cytokines and chemokines secretion profile observed at day 8 p.i.,
374 neutrophils only secreted CCL4 and CCL5 chemokines and showed no cytokine secretion at
375 day 14 p.i. It is worth noting that CCL4 secretion was strongly enhanced in infected/treated
376 mice consistent with the enhanced secretion of this chemokine observed upon neutrophils IC-
377 stimulation *in vitro*. In addition, Ly6C^{hi} monocytes showed a higher secretion of 6 chemokines
378 (CCL3, CCL4, CCL5, CCL22, CXCL1 and CXCL10) and 3 Th1-polarizing cytokines (IL-6,
379 TNFα, IFNγ) in infected/treated mice as compared to infected/non-treated mice, with notably
380 strong induction of IL-6 and IFNγ.
381

382 In summary, FrCasE infection and 667 mAb-treatment induce the secretion of multiple
383 chemokines and cytokines by neutrophils and monocytes. The secretion profile evolves over
384 time in both cell-types and it is mostly increased upon antibody therapy, with notably an
385 enhanced secretion of Th1-polarizing cytokines and chemokines by Ly6C^{hi} monocytes isolated
386 from infected/treated mice at 14 p.i, Overall, these data suggest a role for the therapeutic mAb
387 in the functional activation of these FcγRIV-expressing cells.
388

389 **Discussion**

390 We have previously shown that neutrophils have a key immunomodulatory role in the induction
391 of protective immunity by antiviral mAbs through the acquisition of B-cell helper functions [18].
392 Here we provide a new insight into the immunomodulatory role of neutrophils in a context of
393 antiviral mAb-therapy. Our work shows that antibody therapy shapes neutrophils properties,
394 notably it leads to the upregulation of MHC-II expression and the enhanced secretion of
395 multiple cytokines and chemokines. Our work also show that mAb-treatment strongly enhances
396 the functional activation of inflammatory monocytes leading to the secretion of Th1-type
397 polarizing cytokines and chemokines. These enhanced immunomodulatory functions of
398 neutrophils and monocytes observed in infected/treated mice are associated with the
399 upregulation of FcγRIV upon mAb treatment.

400

401 Our data show that neutrophils' cytokine/chemokine secretion profile differs between viral
402 *versus* bacterial stimulus. This is important to highlight as most of the studies investigating the
403 immunomodulatory role of neutrophils have been conducted in bacterial infection models.
404 Thus, *in vitro* stimulation of neutrophils by viral determinants led to a poor production of
405 cytokines but to a wide and strong release of chemokine, with notably high secretion levels of
406 the monocytes-and neutrophils-recruiting chemokines (CCL2, CXCL1, CXCL5) that was not
407 observed upon LPS stimulation. On the contrary, LPS-stimulated neutrophils produced high
408 amounts of proinflammatory cytokines but a narrower and weaker chemokine release. As for
409 neutrophils, viral determinants also lead to different functional activation of monocytes than
410 LPS. Thus, stimulation of monocytes by viral determinants (but not LPS) led to the secretion
411 of high amounts of the neutrophils-recruiting chemokine CXCL1 and to a lesser extent the
412 monocytes-recruiting chemokine CCL2. These results suggest a self-sustaining mechanism of
413 neutrophils and monocytes recruitment upon viral infection, and raise the hypothesis of an early

414 cooperation between neutrophils and monocytes in the induction of the antiviral immune
415 response. This secretion profile of neutrophils induced by viral determinants is in agreement
416 with an increased CCL2 release observed upon *in vitro* HIV stimulation of neutrophils [29].
417 Increased levels of CCL2 and CXCL1 have also been reported in neutrophil- and monocytes-
418 infiltrated tissues in different viral infections [30–33]. However, chemokine increase was mostly
419 assessed in total tissue extracts but not directly in neutrophils or monocytes isolated cells, which
420 prevented the identification of the cell origin of chemokines. It is worth noting that a very early
421 (i.e. 24-48h p.i.) but transient expression of CCL2 and CXCL1 has also been reported upon
422 infection by RSV, CMV and influenza virus [30,31,34]. Such early but transient expression would
423 be in agreement with the very low secretion of these chemokines detected at day 8 p.i. in
424 infected mice treated, or not, with the therapeutic mAb.
425
426 Our data also highlight the effect of inflammatory conditions on the modulation of the
427 functional activity of neutrophils and monocytes. Thus, the *in vitro* stimulation with
428 inflammatory/immunomodulatory cytokines potentiated the release of several chemokines and
429 cytokines by IC-activated neutrophils and monocytes (Figure 3) (i.e. CCL4, CCL5, CXCL1,
430 TNFα) while having a less pronounced or no effect on virus-activated cells. This is consistent
431 with the upregulation of FcγRIV also observed in cytokine-stimulated neutrophils and
432 monocytes. Although not formally shown, these results support a role for the inflammatory
433 environment and IC-stimulation in the enhancement of chemokine/cytokine secretion by
434 neutrophils and monocytes observed in infected-treated mice. Our *in vitro* observations also
435 allowed us to dissect the specific effect of IFNγ, IFN-I and TNFα on the enhancement of the
436 functional properties of IC-activated neutrophils and monocytes (both in terms of secretion
437 profile and FcγRIV modulation). Notably, IFNγ and IFN-I priming (but not TNF-α), led to the
438 upregulation of FcγRIV in neutrophils and/or monocytes, in agreement with previous reports

439 in other experimental settings [35–37]. This suggests a role for both types of IFN in the upregulation
440 of FcγRIV on neutrophils and monocytes observed *in vivo* upon infection and mAb-treatment.
441 However, FcγRIV expression was more strongly enhanced in infected/non-treated mice at day
442 8 p.i. and in infected/treated mice at day 14 p.i. suggesting a different and evolving
443 inflammatory environment in both groups of mice. Similarly, the neutrophils and monocytes
444 secretion profile was also distinct at days 8 p.i. and 14 p.i., The different activation state of
445 these cells observed between infected/treated versus infected/non-treated mice (notably at day
446 14 p.i.) suggests that the control of viral propagation by the therapeutic mAb dramatically
447 changes the inflammatory environment and the subsequent immune outcome. Thus, in addition
448 to blunt viral propagation, Fc-mediated clearance of opsonized virus/infected cells by immune
449 effector cells (i.e. ADCC, CDC, ADCP, …) might generate danger signals able to induce
450 inflammation. This, together with FcγR-triggering might lead to enhanced activation of FcγR-
451 expressing cells. Our *in vivo* results showing FcγRIV upregulation on neutrophils and
452 monocytes are in agreement with the FcγRs modulation induced by the inflammatory conditions
453 resulting from bacterial and IC-mediated autoimmune pathologies [28,38]; and provide new
454 evidence on the regulation of FcγRs expression in the specific inflammatory context of antiviral
455 mAb-based immunotherapies, not reported thus far.
456
457 Our work provides new mechanistic insights into the hitherto underestimated role of neutrophils
458 as key cells in the modulation of adaptive antiviral immunity upon mAb treatment.
459 Interestingly, at steady-state conditions, neutrophils express high levels of FcγRIV. Its
460 expression is higher than that observed in inflammatory monocytes. Thus, it is tempting to
461 speculate that upon viral infection and mAb-treatment, the early recruitment of neutrophils,
462 together with their high expression of FcγRIV, result in potent Fc-triggering by ICs in these
463 cells. This might be key to initiate the modulation of immune responses through the production

464 of multiple cytokines and chemokines able to recruit and activate multiple innate immune cells
465 such as monocytes, NK cells, DCs and neutrophils themselves [22]. This suggests a potential role
466 for neutrophils as early drivers of the induction of vaccinal effects by mAbs. Supporting this
467 hypothesis, at day 8 p.i. neutrophils showed a higher and a wider induction of chemokines and
468 cytokines release than monocytes, while monocytes secreted strong quantities of Th-1
469 polarizing cytokines and chemokines at day 14 p.i. Consistent with this, a differential and
470 sequential functional activation of myeloid cells has been recently reported by Zhang and
471 collaborators in a model of influenza infection [39]. By using single-cell RNA sequencing, they
472 showed two waves of inflammation, with neutrophils being the major contributor to the first
473 wave while macrophages generated a second wave of proinflammatory factors. However, the
474 contribution of the different myeloid cells to the inflammation process was only assessed at the
475 transcription level.
476
477 Our work suggests that the upregulation of FcγRIV on neutrophils and inflammatory monocytes
478 induced by the viral infection and mAb-treatment might increase Fc-triggering by ICs leading
479 to improved immune responses, both in terms of magnitude and quality. In agreement with this,
480 the FcγRIV upregulation on neutrophils and monocytes in infected/treated mice observed at 14
481 days p.i. is associated with (i) higher expression of CD86 costimulatory molecule and MHC-II
482 and (ii) higher secretion of cytokines and chemokines, which might enhance antiviral immune
483 responses. The high secretion of Th1-polarizing cytokines (IL-6, TNFα, IFNγ) [40–44] and
484 chemokines (CXCL10) [45–48] by inflammatory monocytes from infected/treated mice at day 14
485 p.i, argues in favor of a mAb-mediated Th1 polarization of the antiviral immune response by
486 these cells. According to this, we previously reported that 667-mAb treatment of FrCasE
487 infected mice leads to a Th1-biaised immune response as observed by the development of
488 strong CD8 T-cells responses at day 14 p.i. [13,18,49] as well as long-lasting protective humoral

489 immune responses predominantly of the IgG2a isotype [13,18,26]. This Th1-type immune response
490 observed in infected/treated mice contrasts to the non-protective immunosuppressive responses
491 observed in infected/non-treated mice [50]. Our results are also consistent work of Fox and
492 collaborators showing that the therapeutic activity of mAbs against chikungunya virus (CHIK-
493 V) requires Fc-FcγR interaction on monocytes [10]. In agreement with our data, mAb-treatment
494 led to higher levels of proinflammatory chemokines and cytokines in ankles of CHIK-V
495 infected mice. However, chemokines and cytokines were assessed in total ankle samples
496 containing multiples myeloid cell-types (including neutrophils and monocytes) but not in
497 isolated myeloid cells. Thus, neither the effect of mAb-treatment on the secretion profile of
498 monocytes nor the cell origin of chemokines and cytokines were addressed. Finally, it is also
499 worth mentioning the upregulation of MHC-II molecules observed in neutrophils sorted from
500 infected/treated mice. This observation broadens the immunomodulatory role of neutrophils
501 upon mAb-treatment as it suggests that neutrophils might acquire antigen-presenting cell
502 features and induce antigen-specific T-cells responses. In agreement with this, it has been
503 shown that upon *ex vivo* stimulation with IgG immune complex (IC) or viral cognate antigens,
504 neutrophils upregulated expression of MHCII and costimulatory molecules and increased T cell
505 activation [51–53].
506
507 Our work shows a distinct secretion profile of neutrophils and Ly6C^{hi} monocytes, in terms of
508 type, amount and kinetics of chemokines/cytokines secretion. This highlights a differentially
509 and sequentially activation of these FcγRIV-expressing cells upon antiviral mAb treatment and
510 suggests their key and complementary action in the induction of protective immune responses
511 by mAb. This is important to keep into consideration as thus far, mostly DC have been
512 considered as key cells involved in the induction of vaccinal effects by mAbs [13,16,17]. Thus, the
513 identification of neutrophils and inflammatory monocytes as players in the induction of

514 protective immunity by mAbs might have therapeutic implications. These findings might help
515 to improve mAb-based antiviral therapies by tailoring therapeutic interventions aiming at
516 harnessing the immunomodulatory properties of these cells. To this end, different approaches
517 could be envisaged: (i) the use of cell-type specific, appropriate immunostimulatory host-
518 directed therapies and (ii) the design of antiviral mAbs engineered to enhance their affinity for
519 FcγRs expressed on human neutrophils and monocytes, such as FcγRIIa and FcγRIIIa [17,54–56] .
520 Thus, in addition to allow superior antibody-mediated phagocytosis, these Fc-engineered mAbs
521 could also modulate cytokine/chemokine production to ultimately lead to more effective
522 adaptive immune responses.

523

524 **Author contributions**

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

529

530 **Acknowledgments**

531 This work was supported by grants from the ANRS (France REcherche Nord&sud Sida-hiv
532 Hépatites; ECTZ46143; ECTZ47079), the Ligue Régionale Contre le Cancer (157935,
533 R19063FF- RAB19013FFA), Sidaction (BI25-1-02278, A014-2-AEQ-08-01) and the
534 Fondation pour la Recherche Médicale (SPF20120523949). M. Naranjo-Gomez, J. Lambour,
535 and M. Pelegrin are members of the "MabImprove Labex", a public grant overseen by the
536 French National Research Agency (ANR) as part of the "Investments for the future" program
537 (reference: ANR-10-LABX -53-01) that also supported this work. We thank the imaging
538 facility MRI, which is part of the UMS BioCampus Montpellier and a member of the national
539 infrastructure France-BioImaging, supported by the French National Research Agency (ANR-
540 10-INBS-04, "Investments for the future"). We are grateful to the animal facility of the Institut
541 de Génétique Moléculaire de Montpellier (RAM-ZEFI) which is part of the "Réseau des
542 Animaleries Montpelliéraines" RAMIBiSA Facility for animal experiments. We are grateful to
543 S. Gailhac from MRI for support in cytometry experiments, to Thierry Gostan (SERENAD
544 Complex Biological Data Analysis Service) for support in statistical analyses, to Helen Phillips
545 Bevis for English editing services and to Drs. Valerie Dardalhon, Gilles Uzé and Pascale Plence
546 for critical reading of the manuscript.

547

548

549 **Conflict of interest disclosures**

550 The authors declare no competing financial interests.

551

552 **References**

553 1. Dibo M, Battocchio EC, dos Santos Souza LM, et al. Antibody Therapy for the Control of
554 Viral Diseases: An Update. *CPB*. 2019;20(13):1108-1121.
555
556 2. Salazar G, Zhang N, Fu T-M, An Z. Antibody therapies for the prevention and treatment of
557 viral infections. *NPJ Vaccines*. 2017;2:19.
558
559 3. Lu LL, Suscovich TJ, Fortune SM, Alter G. Beyond binding: antibody effector functions in
560 infectious diseases. *Nat Rev Immunol*. 2018;18(1):46-61.
561
562 4. Pelegrin M, Naranjo-Gomez M, Piechaczyk M. Antiviral Monoclonal Antibodies: Can They
563 Be More Than Simple Neutralizing Agents? *Trends Microbiol*. 2015;23(10):653-665.
564
565 5. Naranjo-Gomez M, Pelegrin M. Vaccinal effect of HIV-1 antibody therapy: *Current Opinion*
566 *in HIV and AIDS*. 2019;14(4):325-333.
567
568 6. Niessl J, Baxter AE, Mendoza P, et al. Combination anti-HIV-1 antibody therapy is
569 associated with increased virus-specific T cell immunity. *Nat Med*. 2020;26(2):222-227.
570
571 7. Schoofs T, Klein F, Braunschweig M, et al. HIV-1 therapy with monoclonal antibody
572 3BNC117 elicits host immune responses against HIV-1. *Science*. 2016;352(6288):997-1001.
573
574 8. Bournazos S, Klein F, Pietzsch J, Seaman MS, Nussenzweig MC, Ravetch JV. Broadly
575 Neutralizing Anti-HIV-1 Antibodies Require Fc Effector Functions for In Vivo Activity. *Cell*.
576 2014;158(6):1243-1253.

577

578 9. Earnest JT, Basore K, Roy V, et al. Neutralizing antibodies against Mayaro virus require Fc
579 effector functions for protective activity. *Journal of Experimental Medicine*.
580 2019;216(10):2282-2301.

581

582 10. Fox JM, Roy V, Gunn BM, et al. Optimal therapeutic activity of monoclonal antibodies
583 against chikungunya virus requires Fc-FcγR interaction on monocytes. *Sci Immunol*.
584 2019;4(32):eaav5062.

585

586 11. Gunn BM, Yu W-H, Karim MM, et al. A Role for Fc Function in Therapeutic Monoclonal
587 Antibody-Mediated Protection against Ebola Virus. *Cell Host & Microbe*. 2018;24(2):221-
588 233.e5.

589

590 12. Lambour J, Naranjo-Gomez M, Piechaczyk M, Pelegrin M. Converting monoclonal
591 antibody-based immunotherapies from passive to active: bringing immune complexes into play.
592 *Emerg Microbes Infect*. 2016;5(8):e92.

593

594 13. Michaud H-A, Gomard T, Gros L, et al. A crucial role for infected-cell/antibody immune
595 complexes in the enhancement of endogenous antiviral immunity by short passive
596 immunotherapy. *PLoS Pathog*. 2010;6(6):e1000948.

597

598 14. Nasser R, Pelegrin M, Michaud H-A, Plays M, Piechaczyk M, Gros L. Long-lasting
599 protective antiviral immunity induced by passive immunotherapies requires both neutralizing
600 and effector functions of the administered monoclonal antibody. *J Virol*. 2010;84(19):10169-
601 10181.

602

603 15. Celis E, Chang TW. HBsAg-serum protein complexes stimulate immune T lymphocytes
604 more efficiently than do pure HBsAg. *Hepatology*. 1984;4(6):1116-1123.

605

606 16. Yamamoto T, Iwamoto N, Yamamoto H, et al. Polyfunctional CD4+ T-cell induction in
607 neutralizing antibody-triggered control of simian immunodeficiency virus infection. *J Virol*.
608 2009;83(11):5514-5524.

609

610 17. Bournazos S, Corti D, Virgin HW, Ravetch JV. Fc-optimized antibodies elicit CD8
611 immunity to viral respiratory infection. *Nature*. Published online October 8, 2020.

612

613 18. Naranjo-Gomez M, Lambour J, Piechaczyk M, Pelegrin M. Neutrophils are essential for
614 induction of vaccine-like effects by antiviral monoclonal antibody immunotherapies. *JCI*
615 *Insight*. 2018;3(9).

616

617 19. Wang X-Y, Wang B, Wen Y-M. From therapeutic antibodies to immune complex vaccines.
618 *npj Vaccines*. 2019;4(1):2.

619

620 20. Wen Y-M, Mu L, Shi Y. Immunoregulatory functions of immune complexes in vaccine and
621 therapy. *EMBO Mol Med*. 2016;8(10):1120-1133.

622

623 21. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and
624 regulation of innate and adaptive immunity. *Nat Rev Immunol*. 2011;11(8):519-531.

625

626 22. Tecchio C, Cassatella MA. Neutrophil-derived chemokines on the road to immunity. *Semin*

627 *Immunol*. 2016;28(2):119-128.

628

629 23. Tamassia N, Bianchetto-Aguilera F, Arruda-Silva F, et al. Cytokine production by human
630 neutrophils: revisiting the "dark side of the moon." *Eur J Clin Invest*. Published online May 17,
631 2018:e12952.

632

633 24. Stegelmeier AA, van Vloten JP, Mould RC, et al. Myeloid Cells during Viral Infections and
634 Inflammation. *Viruses*. 2019;11(2):168.

635

636 25. Portis JL, Czub S, Garon CF, McAtee FJ. Neurodegenerative disease induced by the wild
637 mouse ecotropic retrovirus is markedly accelerated by long terminal repeat and gag-pol
638 sequences from nondefective Friend murine leukemia virus. *J Virol*. 1990;64(4):1648-1656.

639

640 26. Gros L, Dreja H, Fiser AL, Plays M, Pelegrin M, Piechaczyk M. Induction of long-term
641 protective antiviral endogenous immune response by short neutralizing monoclonal antibody
642 treatment. *J Virol*. 2005;79(10):6272-6280.

643

644 27. McAtee FJ, Portis JL. Monoclonal antibodies specific for wild mouse neurotropic
645 retrovirus: detection of comparable levels of virus replication in mouse strains susceptible and
646 resistant to paralytic disease. *J Virol*. 1985;56(3):1018-1022.

647

648 28. Wang Y, Jönsson F. Expression, Role, and Regulation of Neutrophil Fcγ Receptors. *Front*
649 *Immunol*. 2019;10:1958. doi:10.3389/fimmu.2019.01958

650

651 29. Yoshida T, Kobayashi M, Li X-D, Pollard RB, Suzuki F. Inhibitory effect of glycyrrhizin

652 on the neutrophil-dependent increase of R5 HIV replication in cultures of macrophages.
653 *Immunol Cell Biol*. 2009;87(7):554-558.
654
655 30. Hokeness KL, Kuziel WA, Biron CA, Salazar-Mather TP. Monocyte Chemoattractant
656 Protein-1 and CCR2 Interactions Are Required for IFN- / -Induced Inflammatory Responses
657 and Antiviral Defense in Liver. *The Journal of Immunology*. 2005;174(3):1549-1556.
658
659 31. Miller AL, Bowlin TL, Lukacs NW. Respiratory Syncytial Virus–Induced Chemokine
660 Production: Linking Viral Replication to Chemokine Production In Vitro and In Vivo. *J*
661 *INFECT DIS*. 2004;189(8):1419-1430.
662
663 32. Rubio N, Sanz-Rodriguez F. Induction of the CXCL1 (KC) chemokine in mouse astrocytes
664 by infection with the murine encephalomyelitis virus of Theiler. *Virology*. 2007;358(1):98-108.
665
666 33. Seo S-U, Kwon H-J, Ko H-J, et al. Type I interferon signaling regulates Ly6C(hi)
667 monocytes and neutrophils during acute viral pneumonia in mice. *PLoS Pathog*.
668 2011;7(2):e1001304.
669
670 34. Wareing MD, Lyon AB, Lu B, Gerard C, Sarawar SR. Chemokine expression during the
671 development and resolution of a pulmonary leukocyte response to influenza A virus infection
672 in mice. *Journal of Leukocyte Biology*. 2004;76(4):886-895.
673
674 35. Dahal LN, Dou L, Hussain K, et al. STING Activation Reverses Lymphoma-Mediated
675 Resistance to Antibody Immunotherapy. *Cancer Res*. 2017;77(13):3619-3631.
676

677 36. Lehmann B, Biburger M, Brückner C, et al. Tumor location determines tissue-specific
678 recruitment of tumor-associated macrophages and antibody-dependent immunotherapy
679 response. *Sci Immunol*. 2017;2(7):eaah6413.
680
681 37. Pricop L, Redecha P, Teillaud JL, et al. Differential modulation of stimulatory and
682 inhibitory Fc gamma receptors on human monocytes by Th1 and Th2 cytokines. *J Immunol*.
683 2001;166(1):531-537.
684
685 38. Bruhns P. Properties of mouse and human IgG receptors and their contribution to disease
686 models. *Blood*. 2012;119(24):5640-5649.
687
688 39. Zhang J, Liu J, Yuan Y, et al. Two waves of pro-inflammatory factors are released during
689 the influenza A virus (IAV)-driven pulmonary immunopathogenesis. Sant AJ, ed. *PLoS*
690 *Pathog*. 2020;16(2):e1008334.
691
692 40. Czarniecki CW. The role of tumor necrosis factor in viral disease. *Antiviral Research*.
693 1993;22(4):223-258.
694
695 41. Hildenbrand B, Lorenzen D, Sauer B, et al. IFN-y enhances T(H)1 polarisation of
696 monocyte-derived dendritic cells matured with clinical-grade cytokines using serum-free
697 conditions. *Anticancer Res*. 2008;28(3A):1467-1476.
698
699 42. Jin P, Zhao Y, Liu H, et al. Interferon-γ and Tumor Necrosis Factor-α Polarize Bone Marrow
700 Stromal Cells Uniformly to a Th1 Phenotype. *Sci Rep*. 2016;6(1):26345.
701

702 43. Uciechowski P, Dempke WCM. Interleukin-6: A Masterplayer in the Cytokine Network.
703 *Oncology*. 2020;98(3):131-137.
704
705 44. Wang JP, Kurt-Jones EA, Shin OS, Manchak MD, Levin MJ, Finberg RW. Varicella-Zoster
706 Virus Activates Inflammatory Cytokines in Human Monocytes and Macrophages via Toll-Like
707 Receptor 2. *Journal of Virology*. 2005;79(20):12658-12666.
708
709 45. Gaylo-Moynihan A, Prizant H, Popović M, et al. Programming of Distinct Chemokine-
710 Dependent and -Independent Search Strategies for Th1 and Th2 Cells Optimizes Function at
711 Inflamed Sites. *Immunity*. 2019;51(2):298-309.e6.
712
713 46. Kelly-Scumpia KM, Scumpia PO, Delano MJ, et al. Type I interferon signaling in
714 hematopoietic cells is required for survival in mouse polymicrobial sepsis by regulating
715 CXCL10. *The Journal of Experimental Medicine*. 2010;207(2):319-326.
716
717 47. Tannenbaum CS, Tubbs R, Armstrong D, Finke JH, Bukowski RM, Hamilton TA. The
718 CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse
719 RENCA tumor. *J Immunol*. 1998;161(2):927-932.
720
721 48. Tokunaga R, Zhang W, Naseem M, et al. CXCL9, CXCL10, CXCL11/CXCR3 axis for
722 immune activation – A target for novel cancer therapy. *Cancer Treatment Reviews*. 2018;63:40-
723 47.
724
725 49. Gros L, Pelegrin M, Michaud H-A, et al. Endogenous cytotoxic T-cell response contributes
726 to the long-term antiretroviral protection induced by a short period of antibody-based

727 immunotherapy of neonatally infected mice. *J Virol*. 2008;82(3):1339-1349.

728

729 50. Nasser R, Pelegrin M, Plays M, Gros L, Piechaczyk M. Control of regulatory T cells is
730 necessary for vaccine-like effects of antiviral immunotherapy by monoclonal antibodies. *Blood*.
731 2013;121(7):1102-1111.

732

733 51. Lok LSC, Dennison TW, Mahbubani KM, Saeb-Parsy K, Chilvers ER, Clatworthy MR.
734 Phenotypically distinct neutrophils patrol uninfected human and mouse lymph nodes. *Proc Natl*
735 *Acad Sci USA*. 2019;116(38):19083-19089.

736

737 52. Meinderts SM, Baker G, van Wijk S, et al. Neutrophils acquire antigen-presenting cell
738 features after phagocytosis of IgG-opsonized erythrocytes. *Blood Advances*. 2019;3(11):1761-
739 1773.

740

741 53. Vono M, Lin A, Norrby-Teglund A, Koup RA, Liang F, Loré K. Neutrophils acquire the
742 capacity for antigen presentation to memory CD4+ T cells in vitro and ex vivo. *Blood*.
743 2017;129(14):1991-2001.

744

745 54. Bournazos S, DiLillo DJ, Goff AJ, Glass PJ, Ravetch JV. Differential requirements for
746 FcγR engagement by protective antibodies against Ebola virus. *Proc Natl Acad Sci USA*.
747 2019;116(40):20054-20062.

748

749 55. Chen TF, Sazinsky SL, Houde D, et al. Engineering Aglycosylated IgG Variants with Wild-
750 Type or Improved Binding Affinity to Human Fc Gamma RIIA and Fc Gamma RIIIAs. *Journal*
751 *of Molecular Biology*. 2017;429(16):2528-2541.

752

753 56. DiLillo DJ, Ravetch JV. Differential Fc-Receptor Engagement Drives an Anti-tumor

754 Vaccinal Effect. *Cell*. 2015;161(5):1035-1045.

755

756

757 **FIGURE LEGENDS**

758 ***Figure 1:*** **Phenotypic and functional activation of neutrophils stimulated with FrCasE**
759 **virions and ICs. A.** *Experimental scheme*. **B**. *Phenotypic activation of neutrophils stimulated*
760 *by FrCasE virions or viral ICs made with the 667 mAb.* V, free virions; IC, viral ICs; M, culture
761 medium. Free 667 mAb was used as control. Activation was assessed by monitoring CD11b
762 expression and frequency of $CD11b^{hi}$ $CD62L^{lo}$ neutrophils. The data represent 12 independent
763 experiments. **C**. *Functional activation of neutrophils stimulated by FrCasE virions (V) or viral*
764 *ICs made with the 667 mAb (IC).* Chemokines release was assessed in supernatants of
765 neutrophils isolated from BM of naive mice (>97-98% purity) and stimulated for 24h by FrCasE
766 virions (red) or viral ICs (blue) or left unstimulated (grey). The data represent 5 independent
767 experiments. Data are expressed as means +/- SEM. Statistical significance was established
768 using a parametric 1-way ANOVA test with Bonferroni's multiple comparisons post-tests (*p
769 < 0.05; **p < 0.01; ***p < 0.001).

770

771 ***Figure 2:*** **Phenotypic and functional activation of monocytes stimulated with FrCasE**
772 **virions and ICs**. **A.** *Experimental scheme*. **B**. *Phenotypic activation of monocytes stimulated*
773 *by FrCasE virions or viral ICs made with the 667 mAb.* Free 667 mAb was used as control. V,
774 free virions; IC, viral ICs; M, culture medium. Activation was assessed by monitoring CD86
775 expression. The data represent 7 independent experiments **C**. *Functional activation of*
776 *monocytes stimulated by FrCasE virions (V) or viral ICs made with the 667 mAb (IC).*
777 Chemokines release was assessed in supernatants of monocytes isolated from BM of naive mice
778 (>97-98% purity) and stimulated for 24h by FrCasE virions (red) or viral ICs (blue) or left
779 unstimulated (grey). The data represent 5 independent experiments. Data are expressed as
780 means +/- SEM. Statistical significance was established using a parametric 1-way ANOVA test
781 with Bonferroni's multiple comparisons post-tests (*p < 0.05; **p < 0.01; ***p < 0.001).

782

783 ***Figure 3: C*ytokine stimulation potentialize the functional activation of neutrophils by ICs.**
784 BM-derived neutrophils were isolated from naive mice and activated as in **Figure 1** in the
785 presence, or in the absence, of *TNFα, IFN-I or IFNγ.* V, free virions; IC, viral ICs; M, culture
786 medium. **(A).** *Phenotypic activation of activated neutrophils by TNFα, IFN-I or IFNγ.*
787 Activation was assessed by monitoring CD11b (**A**). *Modulation of the functional activation of*
788 *neutrophils by TNF*α (**B**), IFN-I (**C**) or IFN*γ* **(D)**. Chemokines and cytokines release were
789 assessed in supernatants of activated neutrophils (**B-D**). (**E**) Histograms depict the increase in
790 cytokine/chemokine release upon stimulation of virus- and IC-activated neutrophils by *TNFα,*
791 *IFN-I or IFNγ* (calculated as the difference in the amount of chemokine/cytokine secretion by
792 V- or IC-stimulated neutrophils in the presence or in the absence of cytokine stimulation). Only
793 those chemokines/cytokines significantly enhanced by *TNF*α (**B**), IFN-I (**C**) or IFN*γ* **(D)**
794 stimulation are depicted. The data represent 6 independent experiments for **A**, 4 independent
795 experiments for **B-E**. Data are expressed as means +/- SEM. Diamonds indicate significant
796 differences to all the other stimulation conditions (black diamond), or to the corresponding
797 stimuli in the absence of cytokine stimulation (open diamond), to corresponding medium
798 without virus or IC stimuli (grey diamond) as determined by Kruskal-Wallis test with Dunn's
799 multiple comparisons post-tests ($p < 0.05$).

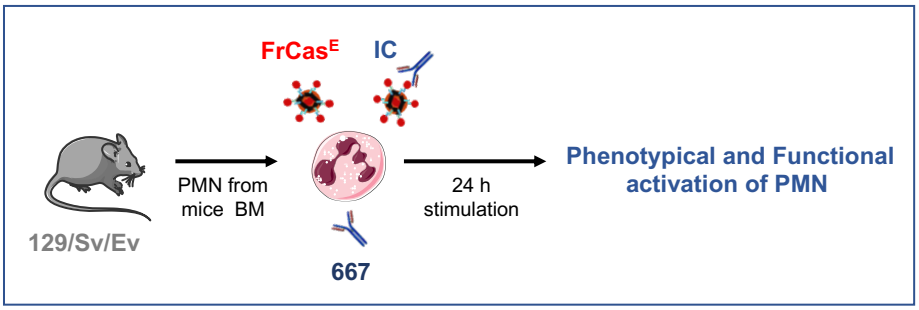
800

801 ***Figure 4: C*ytokine stimulation potentialize the functional activation of monocytes by ICs.**
802 BM-derived monocytes were isolated from naive mice and activated as in **Figure 2** in the
803 presence, or in the absence, of *TNFα, IFN-I or IFNγ.* V, free virions; IC, viral ICs; M, culture
804 medium. **(A).** *Phenotypic activation of activated monocytes (A) by TNFα, IFN-I or IFNγ.*
805 Activation was assessed by monitoring CD86 expression **(A)**. *Modulation of the functional*
806 *activation of monocytes by TNF*α (**B**), IFN-I (**C**) or IFN*γ* **(D).** Chemokines and cytokines

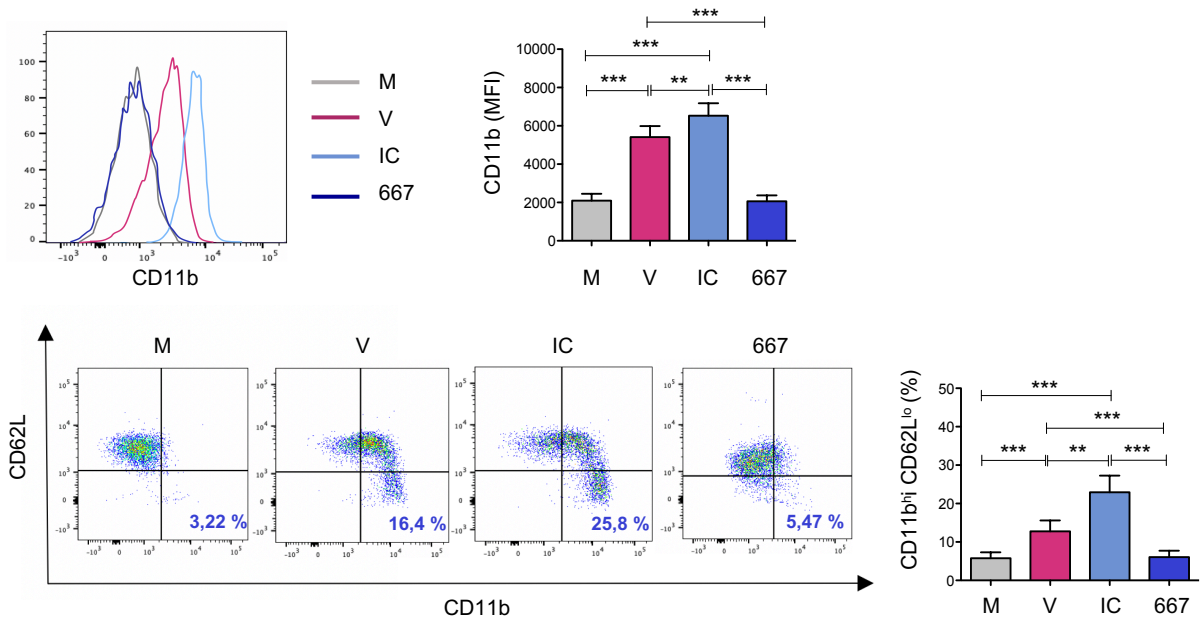
807 release were assessed in supernatants of activated neutrophils (**B-D**). (**E**) Histograms depict the
808 increase in cytokine/chemokine release upon stimulation of virus- and IC-activated monocytes
809 by *TNFα, IFN-I or IFNγ* (calculated as the difference in the amount of chemokine/cytokine
810 secretion by V- or IC-stimulated monocytes in the presence or in the absence of cytokine
811 stimulation). Only those chemokines/cytokines significantly enhanced by *TNFα* (**B**), IFN-I (**C**)
812 or IFN*γ* **(D)** stimulation are depicted. The data represent 3 independent experiments for **A** and
813 3 independent experiments for **B-E**. Data are expressed as means +/- SEM. Diamonds indicate
814 significant differences to all the other stimulation conditions (black diamond), or to the
815 corresponding stimuli in the absence of cytokine stimulation (open diamond), or to
816 corresponding medium without virus or IC stimuli (grey diamond) as determined by Kruskal-
817 Wallis test with Dunn's multiple comparisons post-tests ($p < 0.05$).
818
819 ***Figure 5:*** **FcγRIV is upregulated by IFN stimulation on both neutrophils and monocytes**
820 **cell surface.** Neutrophils and monocytes were isolated from naive mice and stimulated as in
821 **Figures 3 and 4**. FcγRIV expression was evaluated on neutrophils **(A)** and monocytes **(B)**. V,
822 free virions; IC, viral ICs; M, culture medium. The data represent 6 independent experiments
823 for neutrophils (**A**) and 3 independent experiments for monocytes (**B**). Data are expressed as
824 means +/- SEM. Diamonds indicate significant differences to the corresponding stimuli in the
825 absence of cytokine stimulation (open diamond) or to corresponding medium without virus or
826 IC stimuli (grey diamond) as determined by Kruskal-Wallis test with Dunn's multiple
827 comparisons post-tests ($p < 0.05$).
828
829 ***Figure 6:*** **FcγRIV is upregulated *in vivo* on splenic neutrophils and on inflammatory**
830 **monocytes.** Splenocytes from naive (grey), infected/non-treated (I/NT; red) and
831 infected/treated (I/T; blue) mice were analyzed on days 8 (D8) and 14 (D14) p.i. for FcγRIV

832 expression. **(A)** Gating strategy used to define neutrophil and monocyte populations. **(B)**
833 FcγRIV expression on *CD11b*$^{+}$*Ly6G*^{hi} neutrophils and *Ly6C*^{hi} monocytes. **(C)** Cell surface
834 markers expression (CD11b, CD86, MHCII) on *CD11b*$^{+}$*Ly6G*^{hi} neutrophils and *CD11b*$^{+}$*Ly6C*^{hi}
835 monocytes surface. The data represent 5 independent experiments at D8 p.i. and 6 independent
836 experiments at D14 p.i with at least 6-8 mice per group (I/NT and I/T) and 3-5 mice per group
837 (naive mice). Data are expressed as means +/- SEM. Statistical significance was established
838 using a parametric 1-way ANOVA test with Bonferroni's multiple comparisons post-tests (*p
839 < 0.05; **p < 0.01; ***p < 0.001).
840
841 ***Figure 7:*** **Cytokine and chemokine secretion profile of splenic neutrophils and**
842 **inflammatory monocytes.** Splenic neutrophils and inflammatory monocytes from naive,
843 infected/non-treated (I) and infected/treated mice (IT) were isolated at days 8 p.i. (D8) and 14
844 p.i. (D14) for assaying their chemokine and cytokine secretion profile in supernatants of sorted
845 cells cultured at a density of $4x10^6$ cells/ml ($1x10^6$ cells/well) for 24 h. Data represent 3
846 independent experiments on D8 p.i. and 4 independent experiments on D14 p.i. with at least 6-
847 8 mice per group. (**A**) Total quantity of chemokines and cytokines secreted by neutrophils and
848 inflammatory monocytes from infected/non-treated (I) and infected/treated mice (I/T). (**B**)
849 Chemokine and cytokine secretion of neutrophils and inflammatory monocytes isolated from
850 naive, infected/non-treated (I) and infected/treated mice (IT). The color code shows fold-
851 increase compared to secretion detected in cells isolated from naive mice (the raw chemokine
852 and cytokine release values are given in brackets, expressed in pg/ml). (**C**) Chemokine and
853 cytokine secretion of neutrophils and inflammatory monocytes isolated from infected/non-
854 treated (I) and infected/treated mice (IT) expressed as percentage of chemokine and cytokine
855 showing a fold-increase ≥1.4 as compared to cells isolated from naive mice.
856

A


FrCas^E
IC
129/Sv/Ev
PMN from mice BM
24 h stimulation
667
Phenotypical and Functional activation of PMN

B


M
V
IC
667
CD11b
CD11b (MFI)
CD62L
3,22 %
16,4 %
25,8 %
5,47 %
CD11b^{hi} CD62L^{lo} (%)

C

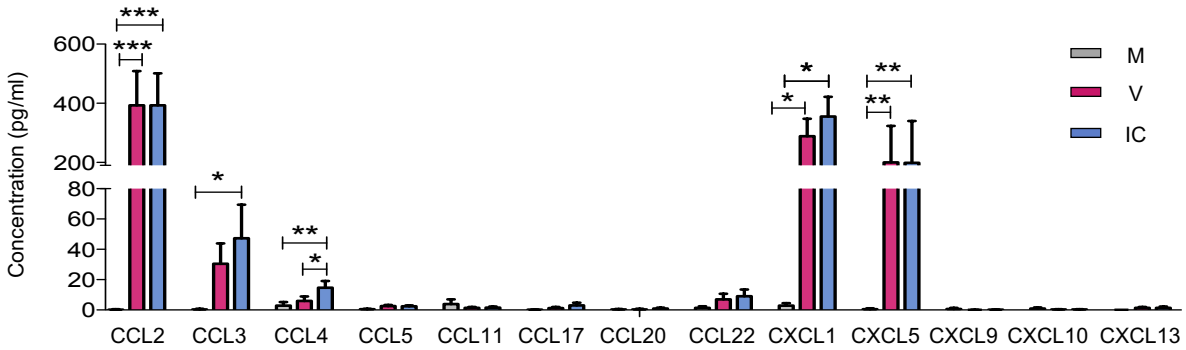
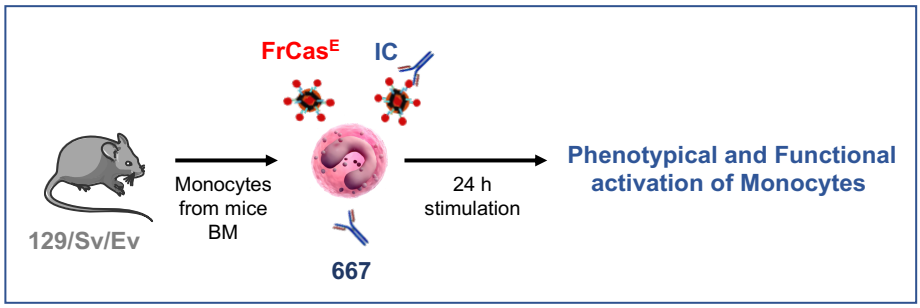
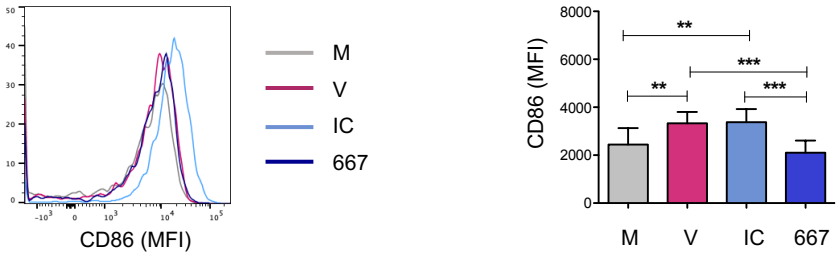

Concentration (pg/ml)
M
V
IC
CCL2
CCL3
CCL4
CCL5
CCL11
CCL17
CCL20
CCL22
CXCL1
CXCL5
CXCL9
CXCL10
CXCL13

Figure 1

A


FrCas^E
IC
129/Sv/Ev
Monocytes from mice BM
667
24 h stimulation
Phenotypical and Functional activation of Monocytes

B


CD86 (MFI)
M
V
IC
667
CD86 (MFI)
8000
6000
4000
2000
0
**
***
**
***
M
V
IC
667

C

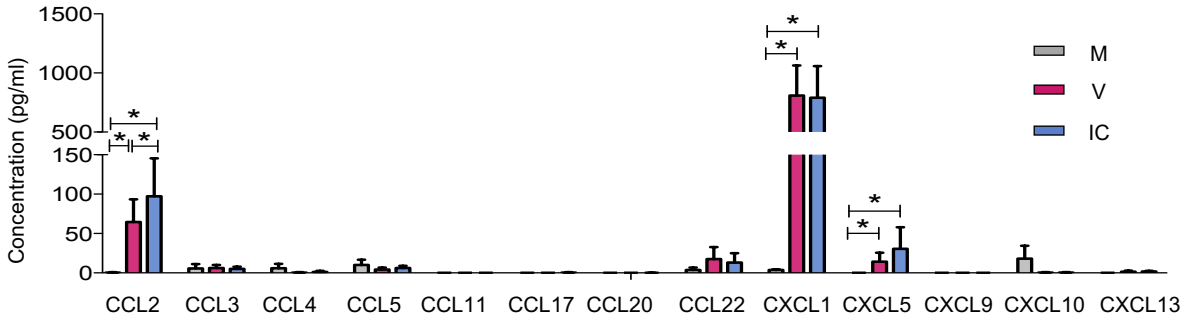

Concentration (pg/ml)
1500
1000
500
150
100
50
0
M
V
IC
CCL2
CCL3
CCL4
CCL5
CCL11
CCL17
CCL20
CCL22
CXCL1
CXCL5
CXCL9
CXCL10
CXCL13

Figure 2

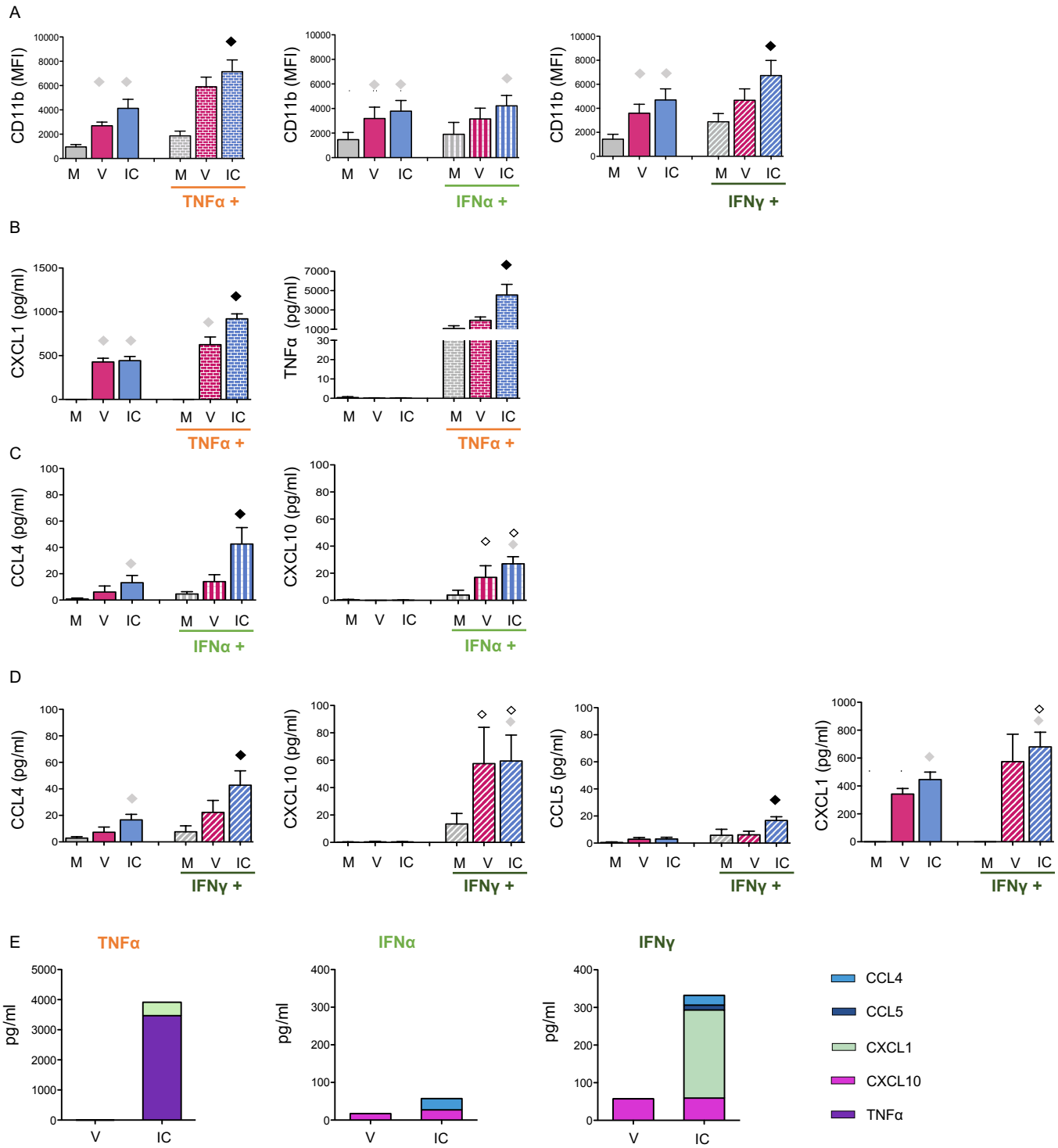

A
CD11b (MFI)
M V IC M V IC
TNFα +
IFNα +
IFNγ +
B
CXCL1 (pg/ml)
TNFα (pg/ml)
C
CCL4 (pg/ml)
CXCL10 (pg/ml)
D
CCL5 (pg/ml)
CXCL1 (pg/ml)
E
TNFα
IFNα
IFNγ
pg/ml
V IC
CCL4
CCL5
CXCL1
CXCL10
TNFα

Figure 3

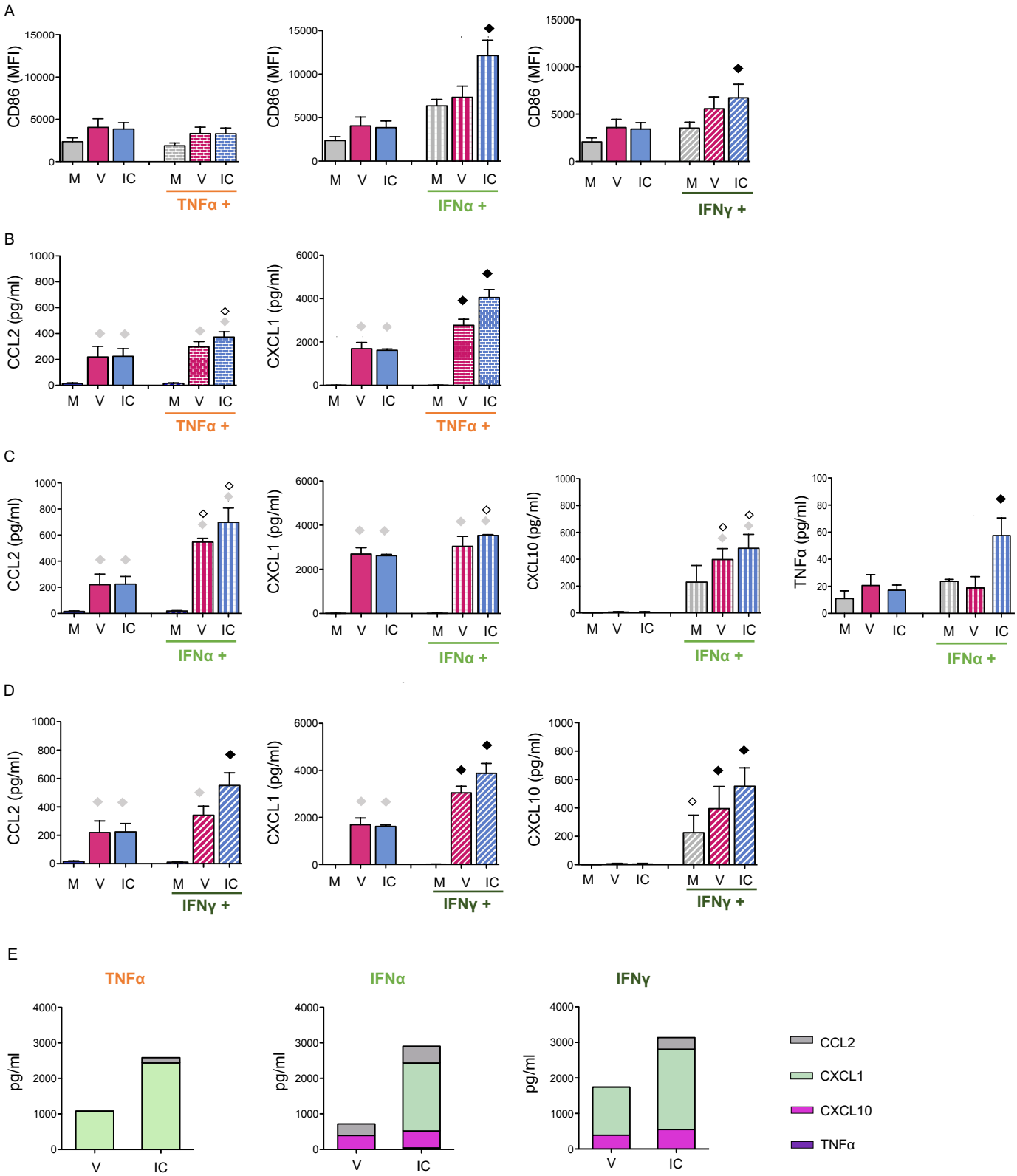
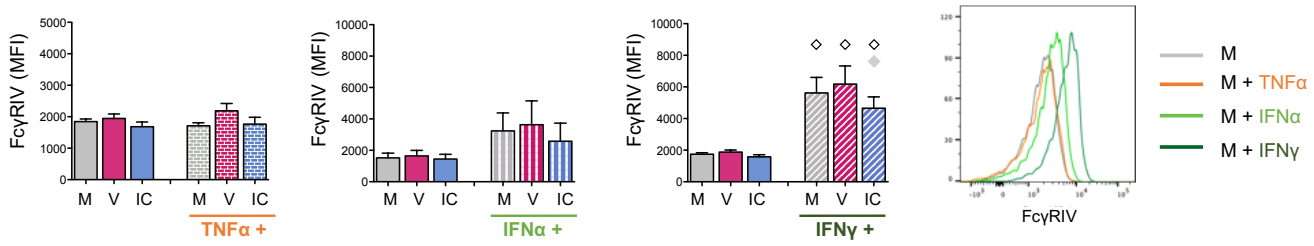

A
CD86 (MFI)
15000
10000
5000
0
M V IC M V IC
TNFα +
IFNα +
IFNγ +
B
CCL2 (pg/ml)
1000
800
600
400
200
CXCL1 (pg/ml)
6000
4000
2000
C
CXCL10 (pg/ml)
TNFα (pg/ml)
100
80
60
40
20
D
E
TNFα
IFNα
IFNγ
pg/ml
V IC
CCL2
CXCL1
CXCL10
TNFα

Figure 4

A


FcγRIV (MFI)
M V IC M V IC
TNFα +
IFNα +
IFNγ +
FcγRIV
M
M + TNFα
M + IFNα
M + IFNγ

B

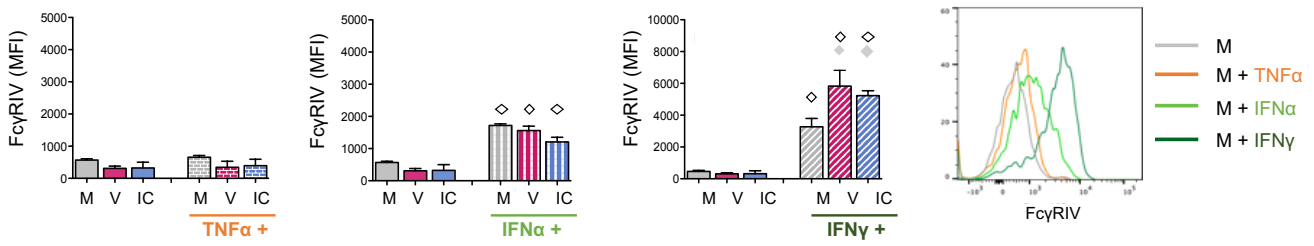

FcγRIV (MFI)
M V IC M V IC
TNFα +
IFNα +
IFNγ +
FcγRIV
M
M + TNFα
M + IFNα
M + IFNγ

Figure 5

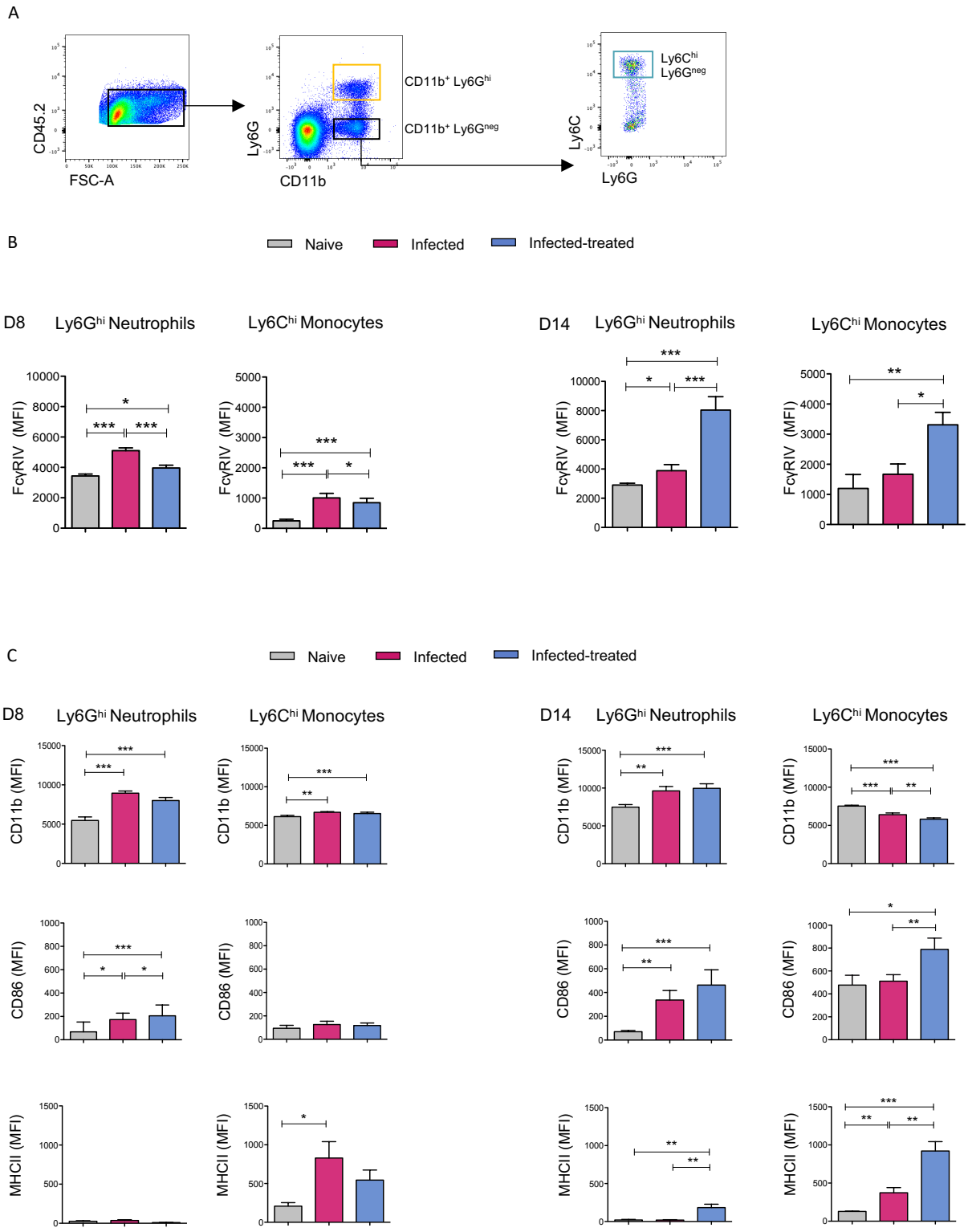
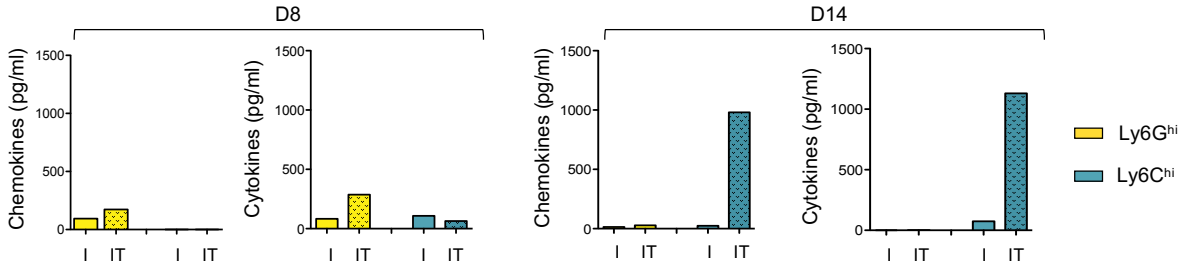

A
CD45.2
FSC-A
Ly6G
CD11b
CD11b+ Ly6Ghi
CD11b+ Ly6Gneg
Ly6C
Ly6G
Ly6Chi
Ly6Gneg
B
Naive
Infected
Infected-treated
D8
Ly6Ghi Neutrophils
Ly6Chi Monocytes
D14
Ly6Ghi Neutrophils
Ly6Chi Monocytes
FcγRIV (MFI)
C
CD11b (MFI)
CD86 (MFI)
MHCII (MFI)

Figure 6

A



B

D8	Ly6G^hi Naive	Ly6G^hi I	Ly6G^hi IT	Ly6C^hi Naive	Ly6C^hi I	Ly6C^hi IT
Chemokines						
CCL2	1 (7)	1,4	1,4	1 (17)	0,8	0,8
CCL3				1 (21)	0,7	1,1
CCL4	1 (18)	2,1	2,7	1 (272)	0,3	0,4
CCL5	1 (8,4)	1,9	1,9	1(375)	0,4	0,3
CCL11	1(6,4)	2	1,7	1(18)	0,8	0,7
CCL17	1 (10)	2	2,2	1 (45)	0,7	0,7
CCL20	1 (4,5)	2,4	1,7	1 (12)	0,8	0,7
CCL22	1 (7,3)	1,7	1,8	1 (19)	0,9	0,9
CXCL1	1 (7,3)	0,8	3,2	1 (24)	0,4	1,4
CXCL5	1 (29)	1,3	2,7	1 (202)	0,5	0,8
CXCL9	1 (6,3)	1,5	1,4	1 (16)	0,7	0,6
CXCL10				1 (151)	0,9	1,1
CXC13	1 (6,1)	1,2	1,4	1(16,7)	0,6	0,6
Cytokines						
IL1α				1 (4,1)	2,3	0,9
IL1β	1 (8,7)	2,2	2,7	1 (9,4)	2,2	1,6
IL6	1 (8,7)	1,2	2,2	1 (66)	0,5	0,7
IL10	1 (1,2)	15	12,7	1 (16)	0,9	0,4
IL12p70	1 (3,3)	2,7	2,7	1 (11)	1,4	0,8
IL17A	1 (6,1)	1,9	2,7	1 (13)	1,6	0,8
IL23	1 (3,2)	2,4	2,8	1 (9,6)	1	1
IL27	1 (13)	2,2	3,1	1 (11)	3,7	1,5
IFNβ	1 (3,4)	4,8	8,3	1 (73)	0,7	1,1
IFNγ	1 (8)	1,2	0,8	1 (6,1)	2,1	1,3
TNFα	1 (6)	2,3	3,4	1(611)	0,5	0,2
GM-CSF	1 (7,2)	1,9	2,4	1 (16)	1,2	0,6

D14	Ly6G^hi Naive	Ly6G^hi I	Ly6G^hi IT	Ly6C^hi Naive	Ly6C^hi I	Ly6C^hi IT
Chemokines						
CCL2						
CCL3				1 (3,7)	0,4	1,9
CCL4	1 (1)	7,1	26,6	1 (10)	1,4	2,8
CCL5	1 (2,5)	1,1	1,4	1(100)	1,1	8,5
CCL11						
CCL17						
CCL20						
CCL22				1 (4,8)	1,8	15,9
CXCL1				1 (2,5)	0,9	8,5
CXCL5						
CXCL9						
CXCL10				1 (8,2)	2,1	13,4
CXC13						
Cytokines						
IL1α						
IL1β						
IL6				1 (1,9)	33,4	296,4
IL10						
IL12p70						
IL17A						
IL23						
IL27						
IFNβ						
IFNγ				1 (0,8)	1,6	616
TNFα				1 (42)	1,1	2,9
GM-CSF						

0 1 10 50 100 >500

C

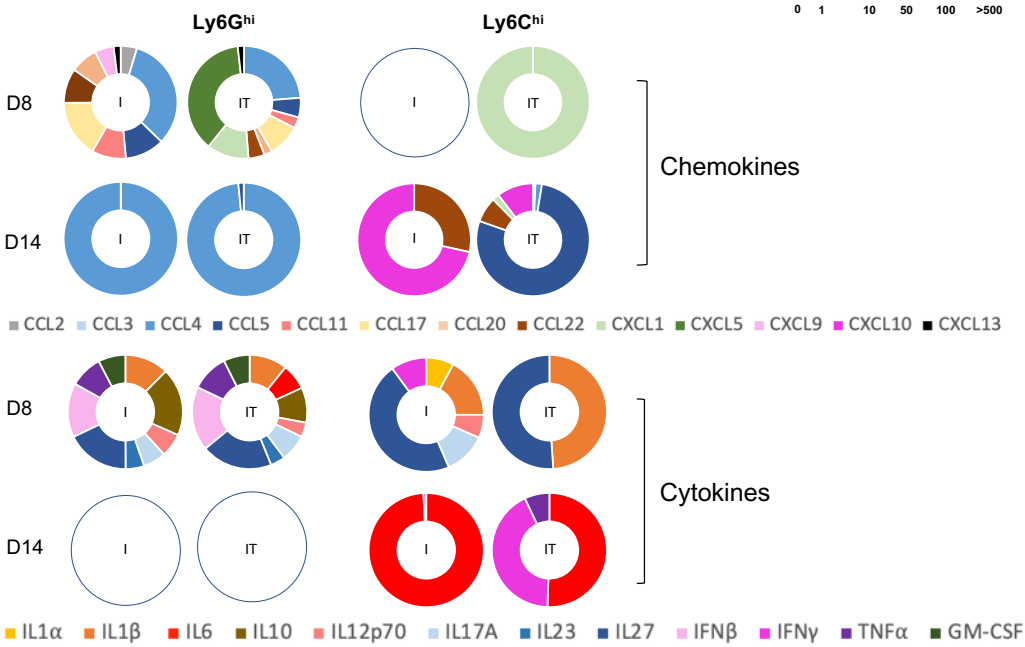


Figure 7