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1 **Neutrophils are essential for induction of vaccine-like effects by antiviral monoclonal**
2 **antibody immunotherapies**

3

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25 **KEY WORDS**

26 Antiviral immunity, antiviral monoclonal antibodies, immunotherapy, B-cell helper neutrophils,
27 vaccine-like effects.

28

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30

31 **ABSTRACT**

32 Using a mouse retroviral model, we have shown that monoclonal antibody (mAb)-based
33 immunotherapy can induce life-long endogenous protective immunity (vaccine-like effects). This
34 observation has potentially important consequences for treating life-threatening human viral
35 infections. Here, we investigated the role of neutrophils in this effect. Neutrophils are innate immunity
36 effector cells with well-established microbe-killing activities that are rapidly mobilized upon infection.
37 They are also emerging as orchestrators of innate and adaptive immunities. However, their
38 immunomodulatory activity during antiviral mAb immunotherapies has never been studied. Our data
39 reveal that neutrophils have an essential role in immunotherapy-induced immune protection of
40 infected mice. Unexpectedly, neutrophils have a limited effect in controlling viral propagation upon
41 passive immunotherapy administration, which is mostly mediated by natural killer cells (NKs).
42 Instead, neutrophils operate as essential inducers of a potent host humoral antiviral response. Thus,
43 neutrophils play an unexpected key role in protective immunity induction by antiviral mAbs. Our
44 work opens new approaches to improve antiviral immunotherapies as they suggest that preserving
45 neutrophil functions and counts might be required for achieving mAb-induced protective immunity.

46

47

48 INTRODUCTION

49 Neutralizing monoclonal antibodies (mAbs) are now considered as a potential therapeutic approach for
50 the prevention and treatment of chronic and acute viral infections, including newly emerging viral
51 infections (1). In recent years, there has been a dramatic increase in the development of new mAbs
52 with improved neutralizing activity (1–14). Notably, in addition to anti-respiratory syncytial virus
53 (RSV) mAb used to treat infant respiratory disease, several mAbs directed against human
54 cytomegalovirus, human immunodeficiency- (HIV), influenza-, Ebola- and rabies virus are currently
55 engaged in promising clinical trials (1, 3, 9, 14). While the direct effects of antiviral mAbs on viral
56 propagation have been studied extensively, little attention has been paid to their potential
57 immunomodulatory effects until recently (10, 15, 16).

58 Using a mouse model of persistent retroviral infection, the erythroleukemia induced by the Murine
59 Leukemia Virus FrCasE, we have shown that treatment of infected mice with a highly neutralizing
60 mAb can induce life-long protective immunity (vaccine-like effects) (see 10). This observation may
61 have major therapeutic implications for humans. Indeed, increased antiviral immune responses after
62 mAb treatments were recently observed in preclinical models of HIV, RSV and henipavirus infections
63 (see 10). Moreover, passive immunotherapy with broadly neutralizing mAbs stimulated antiviral
64 antibody responses in HIV-1-infected patients (14). Similarly, vaccine-like effects have also been
65 documented in preclinical models of cancer immunotherapies (17–19), as well as in clinical trials
66 using anti-CD20, anti-EGFR or anti-HER2 mAbs (20–23). Yet, neither the mechanisms at play nor the
67 possible long-term protective consequences have been addressed in such clinical trials.

68 In humans, technical-, ethical- and cost limitations strongly limit investigations. Instead,
69 immunocompetent animals offer the possibility to identify the cellular and molecular actors of
70 antiviral mAb-induced vaccine-like effects. The FrCasE-induced erythroleukemia is a model system
71 permitting extensive analysis of the endogenous immune response after passive mAb-based
72 immunotherapy under conditions of both chronic infection and pathological development. We found
73 that a 5-day treatment of FrCasE-infected mice with the neutralizing mAb 667 (an IgG2a recognizing
74 the retroviral envelope glycoprotein; Env) shortly after infection provides long-lasting (> 1 year)

75 protective antiviral immunity of the Th1 type with enhanced IgG2a humoral- and cytotoxic T cell
76 (CTL) responses and reduced regulatory T-cell activity (24–29). The immunomodulatory action of
77 mAb 667 depends on its ability to interact with receptors for IgGs (Fc γ R). Notably, immune
78 complexes (ICs) formed between the administered mAb and viral determinants enhance antiviral CTL
79 responses through Fc γ R-mediated binding to dendritic cells (DCs) (27).

80 Importantly, IC-Fc γ R interactions concern several Fc γ R-bearing innate effector cells other than DCs,
81 including neutrophils that are rapidly recruited at sites of infections. While they have long been
82 viewed as simple and direct scavengers of extracellular pathogens, there is recent evidence that
83 neutrophils can be key cells in the orchestration of innate and adaptive immunity via the interaction
84 with different myeloid and lymphoid cells and the modulation of their functions (30, 31). However,
85 their role in antiviral immunity is still largely unknown. Indeed, most investigations have studied
86 neutrophil functions in viral pathogenesis or in the control of viral propagation through their effector
87 functions, i.e. phagocytosis, reactive oxygen species production, neutrophil extracellular traps
88 formation, etc. (32, 33). In contrast, the immunomodulatory properties of neutrophils have been poorly
89 studied in viral infections and not addressed in the context of antiviral immunotherapies.

90 Here, we used the FrCasE model to address the general question of neutrophils participation in the
91 induction of protective immunity by antiviral mAbs. We found that they are essential for the
92 protection of infected, 667-treated mice. Unexpectedly, and unlike NKs, neutrophils had a limited
93 effect on viral propagation control. Instead, their protective effect relied on their capacity to induce
94 potent humoral responses due to the acquisition of B-cell-helper activity upon mAb treatment. Our
95 findings should facilitate improved design of mAb-based antiviral therapies as they suggest that
96 preserving neutrophil functions and counts might be required for achieving optimal protection by
97 mAb.

98

99 **RESULTS**

100 *Neutrophils are necessary for protection of infected mice by antiviral mAb without affecting viral*
101 *propagation.* We first addressed neutrophil mobilization in FrCasE-infected mice with, or without, 667
102 mAb treatment (infected/treated vs infected/non-treated). Passive immunotherapy was administered on
103 the same day after establishment of viral infection (27) (Figure 1A, upper part) and at days 2 and 5 p.i.
104 Age-matched naive mice were used as controls. Neutrophil recruitment was assessed in the spleen, one
105 of the most viremic organs, at day 8 p.i, when viral replication is maximal in infected/non-treated mice
106 (27). Neutrophil abundance in infected/treated mice was comparable to that observed in naive mice,
107 whereas it was significantly higher in infected/non-treated animals (Figure 1B). Interestingly, this
108 increased neutrophil frequency was associated with a higher percentage of spleen infected cells
109 (Figure 1C), as assessed by flow cytometry using the H34 antibody (recognizing a Gag protein epitope
110 expressed on the surface of FrCasE-infected cells) (34–36).

111 Next, we assessed the role of neutrophils in the control of viral propagation, as well as in the
112 protection against leukemia in infected mice, with or without 667 mAb treatment. To this end,
113 neutrophils were depleted by administering a mAb (1A8) directed to their specific Ly6G cell surface
114 marker (37, 38) or an isotype control mAb (2A3). Depletion started 1 day before infection (Figure 1A,
115 lower part), was efficient and specific (Supplemental Figure 1), and was maintained for 21 days, i.e.
116 the time necessary to eliminate the therapeutic 667 mAb (27). Neutrophil elimination accelerated
117 disease development in infected/non-treated mice and drastically reduced protection provided by 667
118 to infected mice (Figure 1D). We next assessed viral propagation in the different groups of mice. In
119 infected/non-treated animals, neutrophil depletion (Figure 2A) was associated with a significant
120 increase in the percentage of infected spleen cells at days 8 (Figure 1E) and 14 p.i. (Supplemental
121 Figure 2A) as well as with a higher viremia (Supplemental Figure 2B). In contrast, in infected/treated
122 mice, viral propagation was not significantly affected at days 8- (Figure 1E) and 14- p.i. (Figure 2B
123 and Supplemental Figure 2) upon neutrophil depletion and remained lower than in infected/non-treated
124 mice. This suggested that viral control by 667 mAb involved other innate immunity effector cells.

125 Thus, neutrophils exert different antiviral effects on FrCasE-infected mice depending on
126 immunotherapy. In animals undergoing simple infection, neutrophils participate in the control of viral
127 propagation. Instead, in infected/treated mice, they are crucial during the immunotherapy period for
128 generation of long-term protection against leukemia, despite their limited effect on viral propagation.

129

130 *NKs control viral propagation in infected/treated mice.* As NKs can exert antibody-dependent cellular
131 cytotoxicity (ADCC) activity against infected cells exposing determinants such as the retroviral Env
132 protein (27, 39, 40), we asked whether NKs were involved in the control of viral propagation in
133 infected/treated mice. To this end, NK cells were depleted using an anti-asialo-GM1 antibody (41–44)
134 (Figure 2A). Contrasting to neutrophil depletion, the absence of NKs in infected/treated mice led to a
135 significantly increased viral propagation at day 14 p.i. (Figure 2B). Next, we compared 667-mediated
136 ADCC activity of NKs and neutrophils against infected cells using an *in vivo* antibody-mediated
137 killing assay relying on the administration of 667-opsonized FrCasE-infected splenocytes in naive
138 mice (27) (Figure 2C). Depletion of NKs, but not neutrophils, led to reduced mAb-mediated infected
139 cell lysis (Figure 2D). Finally, as NKs are crucial for viral propagation control during the
140 immunotherapy period, we assessed their role in the protection against leukemia in infected/treated
141 mice. Importantly, NKs depletion drastically reduced survival of immunotreated mice (Figure 2E),
142 indicating that antibody-mediated control of viral propagation by NKs is necessary for long-term
143 antiviral protection.

144 Thus, NKs are crucial for protection of infected/treated mice via efficient control of viral propagation
145 by 667-mediated ADCC.

146

147 *Neutrophils differentially alter innate lymphoid cells biology in infected/treated- and infected/non-*
148 *treated mice.* Innate lymphoid cells (ILC) are a heterogeneous population of immune cells that
149 includes NK cells and ILC1, ILC2 and ILC3. As NK biology can be affected by neutrophils (45), we
150 first assessed the effect of neutrophil depletion on NKs. To this end, we measured the frequency of
151 CD3⁻NKp46⁺ cells in the spleen of mice from the different groups. CD3⁻NKp46⁺ population mostly

152 identifies NKs but it may also include ILC1 and a subpopulation of ILC3 cells. NK cells can be
153 distinguished from such ILCs using the cell surface marker CD49b (46). Notably, over 95 % of CD3⁻
154 NKp46⁺ cells were CD49b⁺ (Supplemental Figure 3) indicating that the vast majority of CD3⁻NKp46⁺
155 cells in the spleen displayed a NK phenotype. As shown in Figure 3A, CD3⁻NKp46⁺ cells recruitment
156 in spleens of both infected/non-treated and infected/treated mice at day 14 p.i. was similar and stronger
157 than in naive mice. We also observed that neutrophils were involved in CD3⁻NKp46⁺ cells
158 mobilization in infected/non-treated mice but neither in infected/treated- nor in naive mice (Figure
159 3A). We next assessed the frequency of splenic ILCs, other than NKs, by quantifying CD117 and
160 CD127 expression in the NKp46⁺ spleen cell population lacking the common lymphoid and myeloid
161 lineage (Lin)-associated markers (Lin⁻) (47). The recruitment of those ILCs in spleens of both
162 infected/non-treated and infected/treated mice at day 14 p.i. was not significantly different from that
163 observed in naive mice (Figure 3B). In addition, neutrophil depletion did not significantly alter the
164 frequency of such ILCs (Figure 3B) in any group of mice.

165 These results show an enhanced recruitment of CD3⁻NKp46⁺ cells, which are mostly NK cells, in both
166 infected/treated and infected/non-treated mice but these effects are neutrophil-dependent only in the
167 latter. They also show that neutrophil depletion does not affect the frequency of splenic Lin⁻
168 CD117⁺CD127⁺ ILCs, in agreement with the lack of effect of anti-Ly6G-mediated-neutrophil
169 depletion on splenic ILCs reported in other experimental settings (47).

170 To better characterize the effect of neutrophils on splenic CD3⁻NKp46⁺ cells, we assessed their
171 maturation by monitoring CD11b and CD27 expression at day 14 p.i. These markers identify different
172 stages of NK maturation in mice (45). Relative to naive mice, both infected/non-treated and
173 infected/treated mice showed similar higher frequencies of CD11b⁺ CD3⁻NKp46⁺ cells (including fully
174 mature CD11b⁺CD27⁻ and semi-mature CD11b⁺CD27⁺ cells) (Figure 3C) at the expense of immature
175 ones (CD11b⁻) (not shown). Moreover, depletion of neutrophils entailed a strong reduction of the
176 frequency of CD11b⁺ CD3⁻NKp46⁺ cells only in infected/non-treated mice. Further characterization of
177 CD3⁻NKp46⁺ cells indicated higher neutrophil-dependent IFN- γ production in infected/treated mice
178 but low and similar IFN- γ levels in naive and infected/non-treated animals, whether the latter were

179 neutrophil proficient or deficient (Figure 3D). Finally, we assessed whether neutrophils affected the
180 ADCC activity of NKs in infected/treated mice. Notably, ablation of neutrophils did not alter 667-
181 mediated ADCC activity of NK cells (Figure 3E), consistent with the lack of effect on viral
182 propagation (Figure 1E and 2B).

183 Thus, CD3⁻NKp46⁺ maturation is similarly enhanced in both infected/treated and infected/non-treated
184 mice but these effects are neutrophil-dependent only in the latter. Nevertheless, neutrophils might
185 participate in functional activation of CD3⁻NKp46⁺ cells in infected/treated animals, as our results
186 show that neutrophils modulate their IFN- γ secretion capacity in infected/treated mice.

187

188 *Neutrophils are crucial for inducing the humoral, but not the CD8⁺ T-cell, antiviral response in*
189 *immunotherapy-treated mice.* Since neutrophils exert no control of viral propagation during the
190 immunotherapy period (Figure 1E and 2B) but are necessary for long-term protection of mice (Figure
191 1D), we asked whether they could be key for the induction of vaccine-like effects.

192 We first addressed cellular adaptive immunity by assaying the primary virus-specific CD8⁺ T-cell
193 response in infected/non-treated and infected/treated mice at its peak (i.e. 14 days p.i.) (27) with or
194 without neutrophils depletion. Consistent with our previous work, infected/treated mice showed an
195 increased virus-specific CD8⁺ T-cell response relative to infected/non-treated mice. In neither case the
196 frequency of virus-specific CD8⁺ T cells was altered by neutrophil depletion (Figure 4A). Similarly,
197 neither case showed any difference in the frequency of CD8⁺ T cells expressing IFN- γ (Figure 4B).
198 These data ruled out a major role for neutrophils in the 667 mAb-induced anti-viral CD8⁺ T-cell
199 response.

200 Next, we addressed humoral immunity in infected/non-treated and infected/treated mice, depleted or
201 not in neutrophils. To achieve this, anti-FrCasE serum immunoglobulins (Ig) from mice of the
202 different groups were assayed by ELISA. Virus-specific IgM titers were assayed at 14 days p.i. (i.e. at
203 the peak of the IgM response upon FrCasE infection and 667 treatment, Supplemental Figure 4) and
204 were not significantly different between infected/non-treated and infected/treated mice (Figure 5A).

205 Interestingly, neutrophils depletion did not alter virus-specific IgM titers in either infected/non-treated
206 or infected/treated mice (Figure 5A). In agreement with published studies in other experimental
207 settings, the absence of neutrophils did not affect the levels of IgM (48). On the contrary, and
208 consistent with our previous work (27), infected/treated mice displayed a much higher level of anti-
209 FrCasE IgGs than infected/non-treated animals (Figure 5B), with a peak (700 $\mu\text{g/ml}$) by day 68 post-
210 infection and a still high level (100 $\mu\text{g/ml}$) at the end of the experiment. Interestingly, in the absence of
211 neutrophils, serum concentration of antiviral IgGs dramatically decreased in infected/treated mice
212 (Figure 5B and Supplemental Figure 5). In contrast, neutrophils showed no role in the regulation of the
213 poor non-protective antibody response elicited in infected/non-treated mice (Figure 5B). Importantly,
214 high anti-FrCasE IgG seric levels correlated with longer survival times, supporting a role for the high
215 humoral antiviral response in protection against disease (Figure 5C). To further characterize the long-
216 term virus-specific humoral response, we tested whether infected/treated mice, depleted or not in
217 neutrophils, could respond to a virus challenge performed 3 months after the first infection (i.e. a time
218 point at which the primary humoral response has strongly declined). Infected/treated mice, depleted or
219 not in neutrophils, were inoculated with FrCasE and serum samples were collected 1 week later to
220 assay the generation of endogenous anti-FrCasE antibodies. In the presence of neutrophils, 3 out of 5
221 infected/treated mice showed an increase in the virus-specific humoral response (Figure 5 D) while, in
222 the absence of neutrophils, none of the infected/treated mice responded to the viral challenge.
223 Altogether, these results show that neutrophil depletion at early time points after infection and
224 treatment affects humoral immunity with an effect on both primary and memory virus-specific B-cell
225 responses.

226 As marginal zone (MZ) B cells are known to contribute to the mounting of antibody responses (49, 50)
227 and their function might be modulated by neutrophils (51), we next addressed the frequencies of
228 splenic MZ (CD21^{high}IgM^{high} CD19⁺ cells)- and follicular (FO) (CD23⁺IgM^{low} CD19⁺ cells) B
229 cells. MZ B-cells frequency was enhanced in a neutrophil-dependent manner in infected/treated mice
230 but unchanged in infected/non-treated animals relative to naive mice (Figure 6A). In contrast, the
231 frequency of FO B cells was significantly enhanced in infected/treated mice relative to naive mice,

232 with however no role for neutrophils (Figure 6B). Interestingly, consistent with a role of MZ B cells in
233 the generation of plasma cells (49), the higher frequency of splenic MZ B cells at day 14 p.i. in
234 infected/treated mice was associated with a higher frequency of CD138⁺ plasma cells in bone-marrow
235 (BM), as compared to infected/non-treated animals. This effect was neutrophil-dependent (Figure 6C).
236 Finally, histological analyses of spleens of infected mice at 14 days p.i. revealed larger germinal
237 centers (GC, defined by staining of MZ CD169⁺ macrophages and B220⁺ cells) in infected/treated
238 versus infected/non-treated mice (Figure 6D). In agreement with a role of neutrophils in the
239 enhancement of the humoral response, neutrophil depletion in infected/treated mice led to smaller GCs
240 (Figure 6D).

241 Thus, neutrophils are essential for the vaccine-like effects induced by the 667 immunotherapy through
242 the stimulation of the humoral, but not the CD8⁺ T-cell, antiviral response. This effect is associated
243 with a neutrophil-dependent increase in both splenic MZ B cells and BM plasma cells.

244

245 *Neutrophils are differently activated in infected/-treated- and -non-treated mice.* Finally, we addressed
246 the functional activation of splenic neutrophils in infected mice with or without 667 immunotherapy
247 at day 8 p.i. by monitoring cell surface activation markers and quantifying various cytokine mRNA
248 and protein levels. In infected/treated- and infected/non-treated mice, splenic neutrophils were
249 similarly activated, as deduced from CD62L shedding and increased expression of CD11b (Figure
250 7A). However, neutrophils from infected/treated mice showed a significantly stronger expression of
251 two cytokines involved in B-cell activation, B cell-activating factor (BAFF) and lymphotoxin α
252 (LT α), compared to infected/non-treated animals (Figure 7B), suggesting a role for the therapeutic
253 mAb in the functional activation of neutrophils. Splenic neutrophils sorted from infected/treated mice
254 also showed a trend for higher secretion capacity of BAFF and LT α , as compared to infected/non-
255 treated mice neutrophils, even though such a trend did not reach statistical significance. To further
256 assess whether mAb-triggering (through Fc γ R cross-linking) could enhance BAFF and LT α release by
257 neutrophils, we isolated BM neutrophils from naive mice and stimulated them for 24 h in plates coated
258 with the 667 mAb. Experiments were conducted in the presence or the absence of the pro-

259 inflammatory cytokine IFN- γ to assess the extent to which inflammatory conditions could synergize
260 with 667 mAb-triggering. Interestingly, in the presence of IFN- γ , 667 mAb triggering led to a
261 significantly increased release of BAFF and LT α by neutrophils (Figure 7C).

262 In summary, FrCasE infection activates neutrophils independently of immunotherapy. However, the
263 activation state significantly differs between the two groups of animals, notably with a significantly
264 stronger expression of LT α and BAFF in immunotherapy-treated mice. In addition, inflammatory
265 conditions synergize with mAb-mediated activation of neutrophils leading to an enhanced release of
266 BAFF and LT α .

267

268 **DISCUSSION**

269
270 We have previously shown that antiviral mAb-based therapies can induce life-long protective
271 immunity. This finding potentially has important therapeutic implications, as evidence suggests that it
272 may also apply to diverse severe human viral diseases (10, 14). A paramount task is now to identify
273 the mechanisms at the origin of the mAb-induced vaccine-like effects and exploit them for more
274 efficient mAb-based treatment of patients.

275 Here, we report that neutrophils are essential during the immunotherapy period for long-term survival
276 of FrCasE-infected mice, not because they control viral propagation but because they are crucial for
277 inducing a protective humoral response without an effect on the CD8⁺ T-cell response. In contrast, we
278 show that, upon 667 mAb treatment, NKs are crucial for the elimination of infected cells by 667-
279 mediated ADCC activity and are necessary for long-term survival of infected/treated mice. Thus, our
280 work indicates that both innate effector cells have distinct but complementary roles in the protection of
281 infected mice by mAb; NKs have an early and predominant role in the control of viral spread while
282 neutrophils are essential for the emergence of a potent host antiviral humoral response. Our findings
283 contrast with the current view of neutrophils usually considered as simple frontline agents against
284 invading pathogens and highlight the hitherto unreported role of neutrophils as key cells in the
285 modulation of adaptive antiviral immunity upon mAb treatment.

286 Our data indicate that survival of infected/treated mice depends on an efficient anti-FrCasE humoral
287 response. Infected/treated mice show neutrophil-dependent (i) increased frequency of MZ B cells, (ii)
288 enhanced formation of GCs, (iii) increased plasma cell generation and (iv) enhanced production of
289 antiviral IgGs. Interestingly, the absence of neutrophils during the immunotherapy period in infected
290 mice also leads to impaired development of secondary humoral responses upon viral challenge.
291 Overall our data suggest that neutrophils are essential for antiviral protection due to their B-cell-helper
292 activity. Similarly, such a helper function has already been documented under homeostatic conditions
293 (51), in autoimmunity disease-prone mice (52), during emergency granulopoiesis (53) and in bacterial
294 infection (54). Importantly, our findings show that the acquisition of B-cell helper functions in

295 infected mice is dependent on immunotherapy, as no modulation of the anti-FrCasE humoral response
296 was detected in infected/non-treated mice upon neutrophil depletion.

297 Though not excluding the role of other factors, our study suggests potential roles for BAFF and LT α
298 in this B-cell helper function. As concerns BAFF, it is interesting to note that its secretion by splenic
299 neutrophils can contribute to the activation of splenic MZ B cells and the acceleration of plasma cell
300 generation (51, 55). Moreover, BAFF administration to mice increases both the frequency of MZ B
301 cells and antibody production (56, 57) and constitutes a signal for both MZ B cell survival and
302 differentiation into plasmablasts (47, 58–61) . Together with the fact that MZ B cells can favor the
303 generation of plasma cells upon microbial infection (49), it is reasonable to speculate that BAFF
304 induction in neutrophils of infected/treated mice may also favor the MZ B cell response and the
305 subsequent generation of plasma cells. Interestingly, in agreement with our in vitro results showing
306 enhanced BAFF release by neutrophils upon combination of IFN- γ stimulation and Fc γ R cross-link
307 (Figure 7C), different pro-inflammatory stimuli, including ICs, have been shown to act as
308 secretagogues and to synergize with IFN- γ to enhance BAFF secretion by human neutrophils (62).
309 Moreover, neutrophil depletion in autoimmunity-prone mice led to a reduction of auto-antibodies titers
310 that correlated with decreased serum levels of IFN- γ and BAFF (52). Thus, it can reasonably be
311 hypothesized that inflammatory conditions synergize with ICs formed after 667 mAb-treatment of
312 FrCasE-infected mice to enhance BAFF release by neutrophils.

313 Although not formally shown, our data suggest that LT α release by neutrophils might play a role in
314 the enhancement of the antiviral humoral response in infected/treated mice. LT α is involved in the
315 formation of secondary lymphoid organs, it is expressed by lymphocytes and mediates a large variety
316 of inflammatory and antiviral responses. It has also been reported to play a role in the development of
317 GC formation and to be required for IgG responses (63–65). Here, we show that this cytokine is
318 expressed and released by neutrophils upon mAb treatment of infected mice. Interestingly, although
319 LT α is not known to be expressed by neutrophils, it has been proposed that non-lymphocytic murine
320 splenic cells are able to produce it (66). We also report that Fc γ R cross-linking by immobilized 667

321 mAb synergizes with IFN- γ to enhance LT α release by neutrophils. This suggests that, similarly to
322 BAFF, inflammatory conditions and mAb-triggering might lead to increased LT α secretion by
323 neutrophils. Further studies will be required to address whether BAFF and LT α combine their actions
324 to stimulate the antiviral humoral response upon mAb immunotherapy. Finally, as IFN- γ potentiates
325 both BAFF and LT α release, it will also be important to assess whether and how neutrophil-dependent
326 IFN- γ production by NKs in infected/treated mice contributes to the stimulation of antibody responses,
327 as this cytokine affects class-switching and long-term maintenance of neutralizing antibody titers in
328 retrovirally-infected mice (34) and humoral autoimmunity in humans (67).

329 Identifying neutrophils as key players in the induction of protective immunity by antiviral mAbs has
330 important therapeutic implications for several reasons. First, if combination therapies are considered, it
331 will be of utmost importance that the agent(s) co-administered with the passive immunotherapy do not
332 alter neutrophil functions and counts to avoid inhibiting efficient antiviral humoral immune responses.
333 Second, in pathological situations leading to neutropenia and/or impaired neutrophils functions, such
334 as certain viral infections (68, 69) and/or drug-induced neutropenia (70), it will be essential to restore
335 them. This could, for example, be achieved through administration of granulocyte colony-stimulating
336 factor (G-CSF) a cytokine already used in the clinic to treat neutropenic patients. Interestingly, this
337 cytokine might also be used in combination with treatments aiming at enhancing the generation of
338 functional neutrophils (70). Furthermore, beyond stimulating neutrophil activation and/or
339 mobilization, G-CSF enhances neutrophil BAFF-secretion capacity and thereby their ability to
340 stimulate B cells (71). Similarly, reduction of viral load by antiretroviral therapies also permits partial
341 restoration of the impaired functions of neutrophils observed in HIV-infected patients (68).
342 Interestingly, neutrophils have been shown to mediate immunosuppression via the PD-L1/PD-1
343 pathway in HIV-infected patients (72). Antiviral passive immunotherapies might therefore benefit
344 from combination with the administration of mAbs targeting this immune checkpoint. Finally,
345 alternative approaches, such as engineering the Fc fragment of antiviral mAbs, merit consideration.
346 Increasing their affinity for the Fc γ Rs expressed by neutrophils might, at first, allow superior antibody-
347 mediated phagocytosis, as recently reported in the case the Fc-modified VR01 anti-HIV mAb (73).

348 This could then alter cell signaling and cytokine/chemokines production to ultimately lead to more
349 effective adaptive immune responses. Thus, stimulating neutrophil activity, restoring their impaired
350 functions and/or counteracting their immunosuppressive actions should improve the vaccine-like
351 effects of antiviral mAb-based immunotherapies. This might also apply to cancer treatment as
352 enhancement of antitumoral immune responses has also been observed in mAb-based anticancer
353 immunotherapies. This is all the more important to take into consideration as neutrophils have been
354 shown to play a role in the therapeutic activity of anticancer mAb (74–78).

355

356 **METHODS**

357 *Ethical statement.* Mice were bred and maintained under conventional, pathogen-free facilities at the
358 Institut de Génétique Moléculaire de Montpellier. All experimental procedures were performed in
359 accordance with the French national animal care guidelines (CEEA-LR-12146 approval).

360
361 *Viral stocks.* FrCasE viral stocks were produced, assayed and stored as described previously (27).

362
363 *Viral infection, immunotherapy and mice follow-up.* Eight day-old 129/Sv/Ev mice were infected
364 intraperitoneally (i.p.) with 50 µl of a virus suspension containing 50,000 ffu (focus-forming units)
365 and treated, or not, with 30 µg of 667 mAb 1 hour post-infection (p.i.) and on days 2 and 5 p.i. by i.p.
366 administration. Mice were examined at regular intervals for clinical signs of erythroleukemia
367 (reduction of hematocrits). They were euthanized when their hematocrits reached 35% (experimental
368 endpoint).

369
370 *Flow cytometry.* Spleen single-cell suspensions were obtained by mechanical dissociation of the
371 organs in PBS. Bone marrow (BM) cell suspensions were obtained by dissection and PBS-flushing of
372 tibias and femurs. Cells were stained at 4°C using fluorochrome-conjugated antibodies to: CD3e (145-
373 2C11), CD4 (RM4-5), CD8 (Ly2, 53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD21/35
374 (eBio8d9), CD23 (B3B4), CD27 (LG3A10), CD45.2 (104), CD45R/B220 (RA3-6B2), CD49b (DX5),
375 CD62L (Ly22, MEL-14), CD117 (2B8), CD127 (SB/199), CD138 (281-2), F4/80 (BM8), Gr1 (RB6-
376 8C5), IFN-γ (XMG1.2), IgM (eB121-15F9), Ly6G (1A8), lineage (Ter119; Gr1; CD45R/B220;
377 CD11c; F4/80; CD3e), NKp46 (29A1.4) (BD Bioscience, eBioscience or BioLegend). FrCasE-
378 infected cells were assayed using an anti-Gag mAb (H34) (35) labelled with Alexa Fluor 647. Forward
379 scatter area and forward scatter time-of-flight, as well as side scatter, were used to remove doublets
380 from flow cytometry analyses. Cells were analyzed on FACSCanto II flow cytometer (BD Bioscience)
381 and the data were analyzed using the FlowJo software (Tree Star).

382

383 *ELISA of anti-FrCasE antibodies.* Plasma anti-FrCasE immunoglobulins were assayed by ELISA as
384 already described (26, 27). Peroxidase-conjugated anti-mouse IgG or IgM rabbit antisera (Serotec)
385 were used as secondary antibodies.

386 *Virus challenge experiments.* Infected/treated mice, depleted or not in neutrophils, were injected i.v.
387 with 300 μ l of a 5×10^4 FFU/ml FrCasE suspension mixed to 2×10^6 FrCasE-infected splenocytes 3
388 months after the first infection. Blood samples were collected 1 week post-challenge to assay
389 endogenous anti-FrCasE IgG concentrations by ELISA.

390 *In vivo depletion of Ly6G⁺ and NK cells.* Neutrophils were depleted by administering a rat anti-Ly6G
391 antibody (1A8; BioXcell), (37, 38) injected i.p. at different time-points (150 μ g/injection) or isotype
392 control rat IgG (2A3; BioXcell). Neutrophils depletion was monitored by flow cytometry analysis of
393 Gr1^{high} and CD11b⁺ cells. NKs were depleted using the anti-asialo GM1 antibody (Wako Pure
394 Chemical Industries, Ltd), injected i.p. at different time-points (50 μ l/injection). This antibody has
395 been used to study the in vivo functions of NKs in mouse strains lacking the NK1.1 allotype, which is
396 a feature of 129 Sv/Ev mice (42).

397
398 *In vivo cytotoxic activity.* Experiments were conducted as described in (27, 79). Briefly, red blood cell-
399 free splenocytes were recovered from 10 day-old FrCasE-infected-, or non-infected, pups. Splenocytes
400 from non-infected mice were labelled with the vital dye carboxy-fluorescein succinimidyl ester
401 (CFSE; Molecular Probes) at a concentration of 0.5 μ M (CFSE^{low} cells). Splenocytes from infected
402 mice were labelled with 5 μ M CFSE (CFSE^{high} cells) and pre-incubated, or not, with the 667 mAb (the
403 absence of 667 allows to quantify spontaneous cell death). Both cell populations were mixed at a 1:1
404 ratio before retroorbital administration to recipient mice. Cytotoxic activity against infected splenocytes
405 was calculated from the ratio of CFSE^{low}/CFSE^{high} cells in spleen assayed by flow cytometry 5 hours
406 later. To assess the contribution of NKs and neutrophils to antibody-mediated cytotoxicity, 50 μ l of the
407 anti-asialo GM1 or 200 μ g of the anti-Ly6G 1A8 mAb were administered 1 day prior to the assay.

408

409 *Flow cytometry assay of CD8⁺ T cells specific for FrCasE-infected cells.* Splenocytes were labelled
410 with both an APC-conjugated anti-CD8⁺ T cell antibody and a PE-conjugated MHC class I H-2D^b
411 tetramer (Beckman Coulter) displaying the immunodominant Friend virus GagL epitope (27) (80) (D^b-
412 GagL tetramers) as previously described (27).

413
414 *Assay of IFN- γ production.* 10⁶ splenocytes were incubated at 37°C for 5 hours in 12-well plates in
415 500 μ l of RPMI culture medium containing phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin
416 (500 ng/ml) in the presence of brefeldin A (10 μ g/ml; Sigma-Aldrich). IFN- γ production was flow
417 cytometry-assayed using the intracellular Cytofix/Cytoperm Fixation/Permeabilization staining kit
418 (Becton Dickinson).

419
420 *Histological analyses.* Spleens from infected/non-treated- and infected/treated mice (depleted or not in
421 neutrophils) were recovered at day 14 p.i. and prepared as previously described (81). Briefly, spleens
422 were initially fixed at 4°C overnight with a PLP (Paraformaldehyde-lysine-periodate) solution and
423 then embedded in 4% low temperature-gelling agarose (type VII-1; Sigma-Aldrich) prepared in PBS
424 (Phosphate Buffered Saline). 300 μ m slices were cut with a vibratome (VT 1000S; Leica) in a bath of
425 ice-cold PBS. Sections of tissues were submerged in PBS and transferred to 0.4 μ m organotypic
426 culture inserts (Millicell; Millipore) for staining with an anti-B220 (RA3-6B2, Becton Dickinson) and
427 an anti-CD169 antibody (MOMA-1, Biorad) at 37°C for 20 minutes. The images were captured using
428 a Leica SP8-UV confocal scanning microscope.

429
430 *RT-qPCR quantification of gene expression.* Single-cell suspensions of splenocytes were prepared
431 from naive, infected/non-treated and infected/treated mice 8 days p.i. and immunotherapy. Neutrophils
432 (CD11b⁺Ly6G^{high} expression) were sorted (>98% pure) using a BD Biosciences FACSARIA device.
433 RNA was extracted from 1-2 x 10⁶ sorted neutrophils using the RNeasy micro kit (Qiagen). RNA
434 quality and integrity were verified using the Agilent 2100 bioanalyzer. cDNAs were synthesized using
435 the RT2 First Strand Kit (Qiagen). All quantitative PCRs were performed following protocols
436 optimized for the RT2 quantitative Profiler PCR array using SYBR Green mix (Qiagen) and

437 LightCycler 480 II machine (Roche). All data were normalized to β -actin. Results were expressed as
438 fold increases with respect to naive cells using the $\Delta\Delta$ Ct method.

439

440 *BAFF and LT α protein release quantification.* Soluble BAFF and LT α from cell-free supernatants of
441 cultured neutrophils were assayed using BAFF (R&D Systems) and LT α ELISA (NeoBiotech),
442 respectively. Supernatants were collected from sorted splenic neutrophils (from naive, infected/non-
443 treated and infected/treated mice at 8 days p.i. and immunotherapy) cultured in 96-well plates at a
444 density of 2×10^5 cells/well for 24 h. Alternatively, neutrophils were isolated from naive mice BM
445 using a magnetic-based cell-sorting (MACS) neutrophil isolation kit (>95% purity; Miltenyi Biotec)
446 and cultured for 24 h in 667mAb-coated 24-well plates at a density of 2×10^6 cells in 500 μ l of
447 medium, in the presence or in the absence of IFN- γ (100 μ g/mL). 667 mAb-non coated plates were
448 used as controls. G-CSF (R&D Systems) was added at a concentration of 10 ng/ml to neutrophil
449 cultures to maintain cell viability

450

451 *Statistical analyses.* Statistical analyses were performed using GraphPad Prism 5 (GraphPad
452 Software). Data were expressed as means \pm SEM and statistical significance was established using a
453 parametric one-way ANOVA test with a Bonferroni correction for multiple comparisons or unpaired
454 Student's t tests when two groups were compared. p values lower than 0.05 were considered as
455 statistically significant.

456

457

458 **AUTHOR CONTRIBUTIONS**

459 Mireia Pelegrin (MPe), MN-G and Marc Piechaczyk (MPi) defined the research program. MN-G, JL
460 and MPe performed the experiments and carried out the data analyses with a contribution by MPi.
461 MPe, MN-G and MPi wrote the manuscript. Grants to MPe and MPi funded the study.

462

463

464

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481

482 **CONFLICT OF INTEREST DISCLOSURES**

483 The authors declare no competing financial interests.

484

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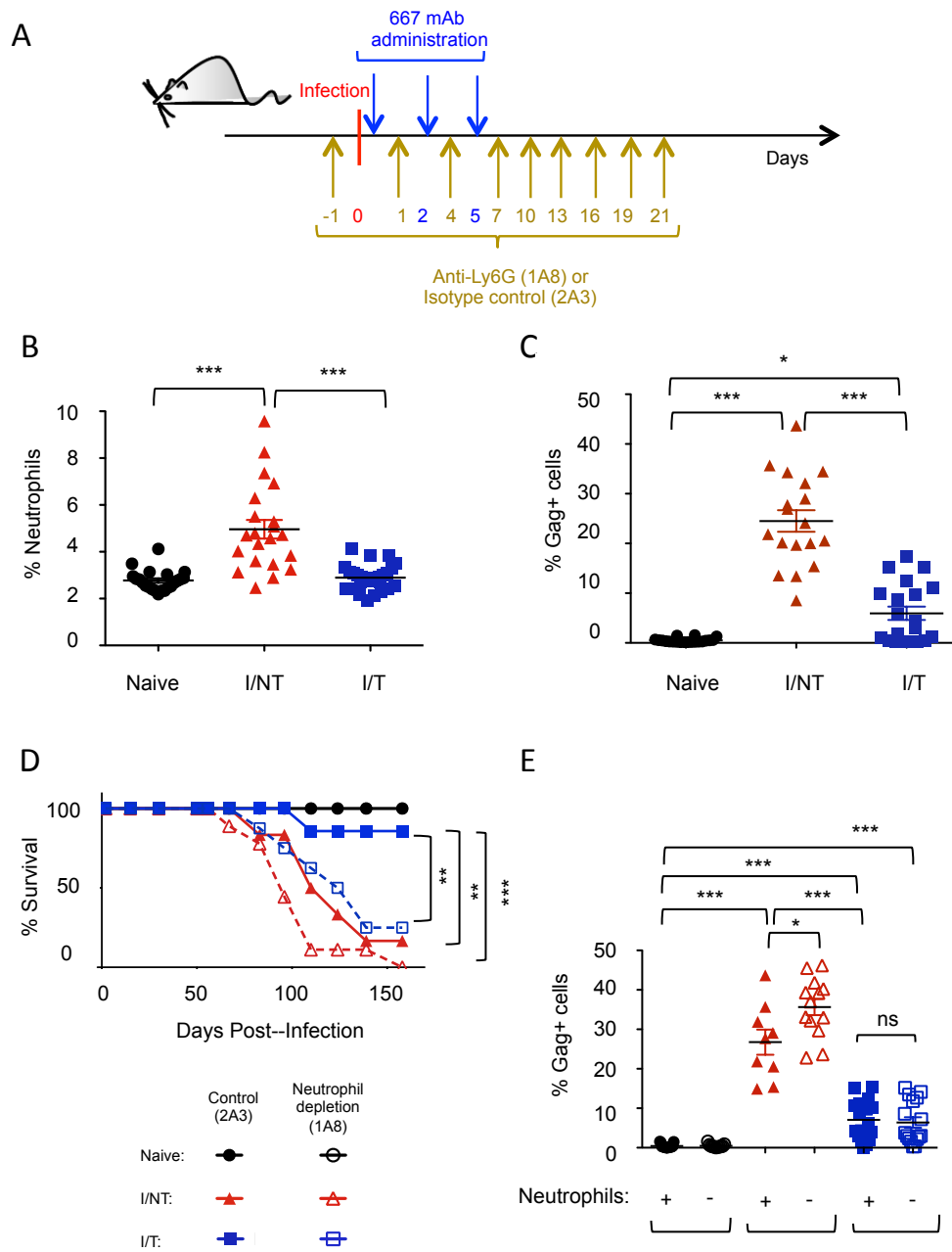
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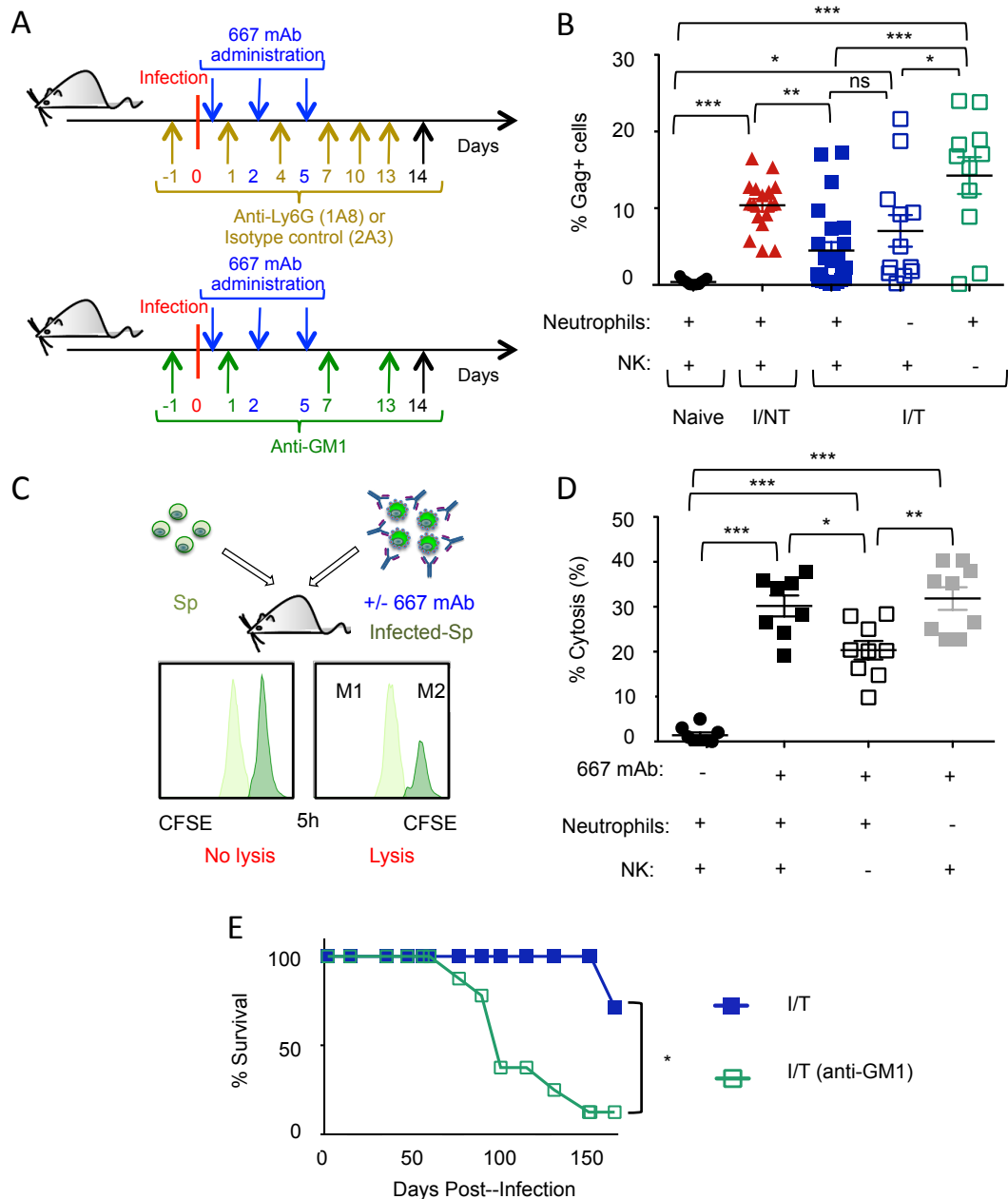
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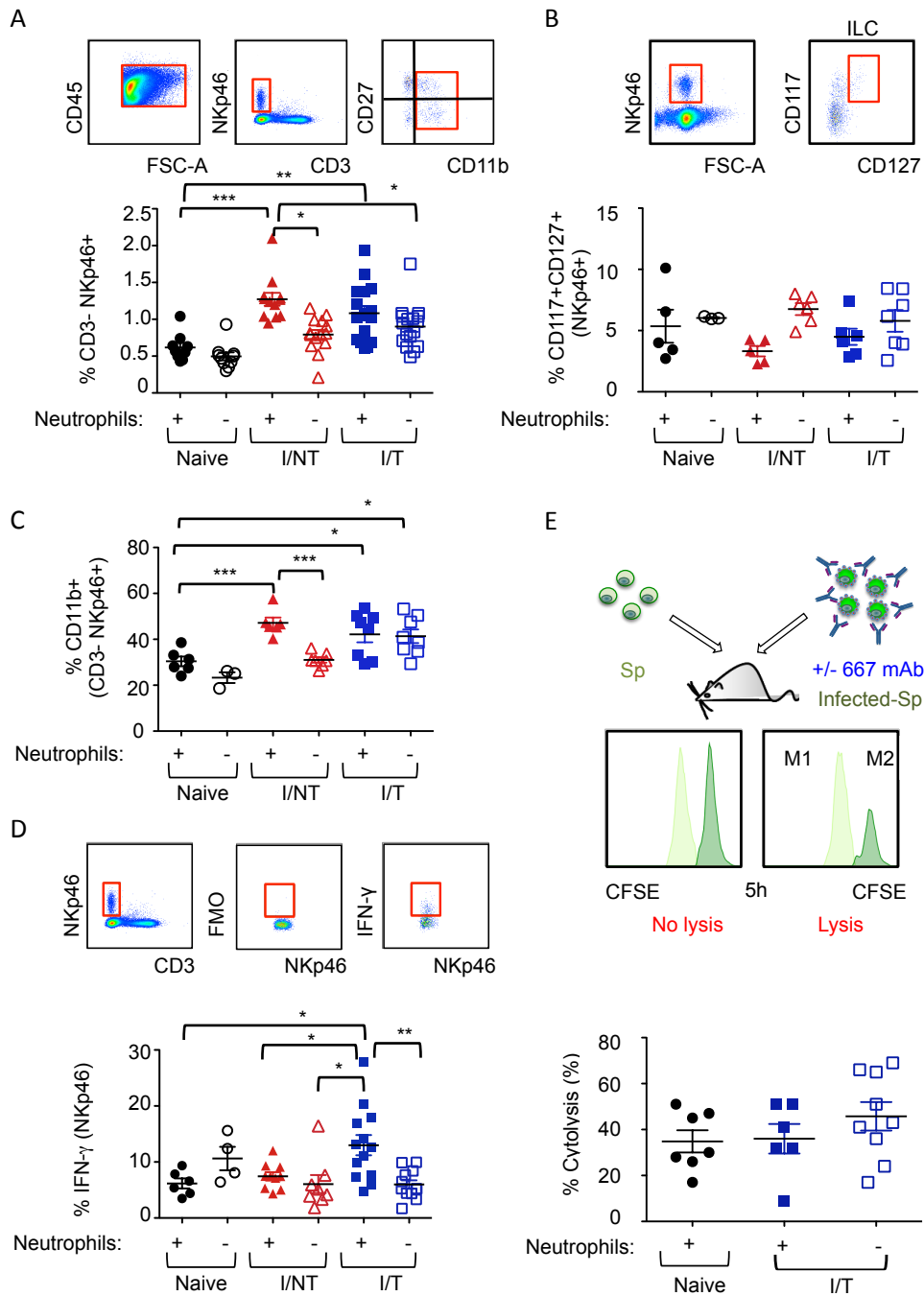
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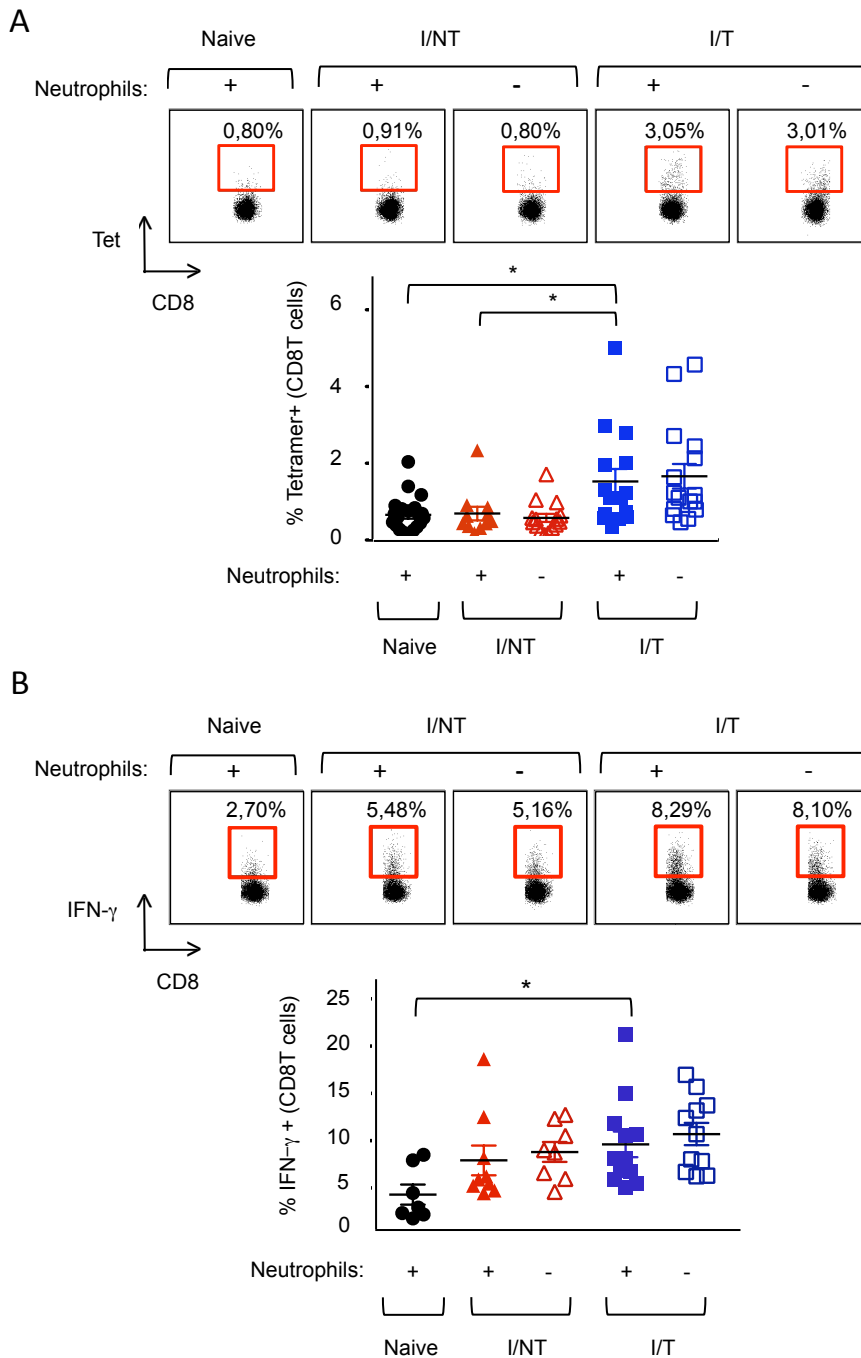
672 **Figure 1. Antiviral effects of neutrophils.** (A) *Experimental scheme.* Upper part: 8 day-old pups were infected
 673 and treated with the 667 mAb as indicated. Lower part: mice were treated as indicated with the anti-Ly6G 1A8
 674 mAb or the isotype control 2A3 mAb in neutrophil depletion experiments. (B-C) *Neutrophil recruitment and*
 675 *infected cells rate in spleen.* Splenocytes from naive, infected/non-treated (I/NT) and infected/treated (I/T) mice
 676 were analyzed by flow cytometry on day 8 p.i. for (i) neutrophil recruitment (% of Ly6G⁺ cells) and (ii) for
 677 retroviral positivity of splenocytes (% of Gag⁺ cells) gated in the CD45.2⁺ population. The data presented
 678 correspond to 5 independent experiments with at least 15 mice per group. (D) *Mouse survival.* Naive, I/NT and
 679 I/T mice were treated with either the anti-neutrophil- (1A8) or the control (2A3) mAb as indicated in (A) and
 680 followed up for leukemic death. The data represent 2 independent experiments with 6-9 mice per group. (E)
 681 *Infected cells rate upon neutrophil depletion.* Neutrophils of naive, I/NT and I/T mice were depleted, or not,
 682 as indicated in (A) and infected splenocytes were assayed as in (C) on day 8 p.i. The data represent 4 independent
 683 experiments with 9-15 mice per group. Data are expressed as means +/- SEM. Statistical significance was
 684 established using a parametric one-way ANOVA test with a Bonferroni correction. (*p < 0.05; **p <
 685 0.01; ***p < 0.001).
 686



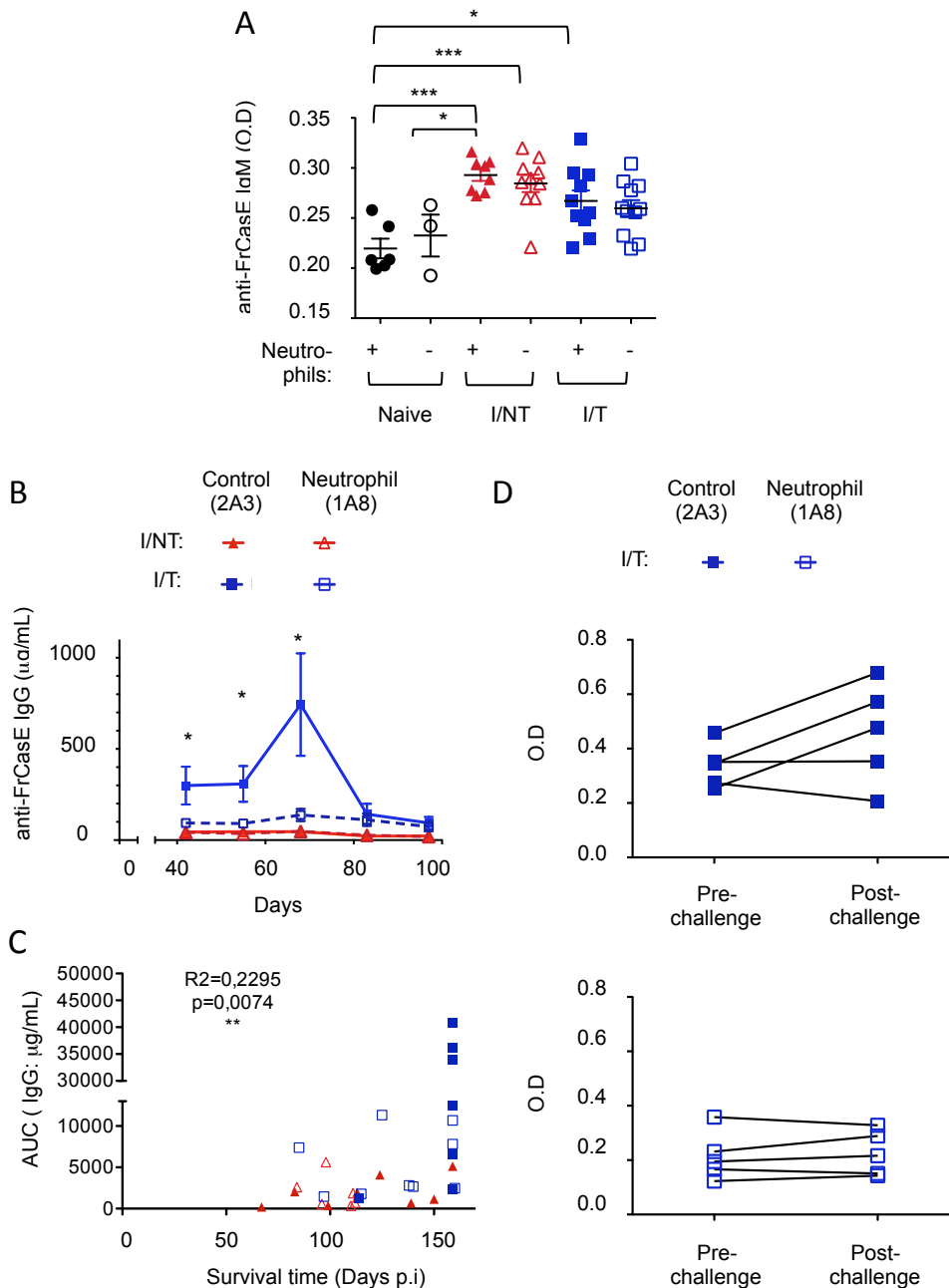
687 **Figure 2. Antibody-mediated control of viral propagation by NKs.** (A) *Experimental scheme.* Mice were
688 infected and mAb-treated as in Fig 1A. Upper part: infected/treated mice were treated as indicated with the anti-
689 Ly6G 1A8 mAb or the isotype control 2A3 mAb to deplete neutrophils. Lower part: infected/treated mice were
690 treated as indicated with the anti-asialo-GM1 antibody to deplete NKs. (B) *Effect of neutrophils or NKs*
691 *depletion in viral spread in infected/treated mice.* Percentage of infected cells at day 14 p.i in the spleen of
692 naive-, I/NT- and I/T mice, depleted or not in neutrophils or NKs assessed as in Fig 1C. The data represent 3
693 independent experiments with at least 8 mice per group. Data are expressed as means \pm SEM. (* $p < 0.05$, ** p
694 < 0.01 , *** $p < 0.001$). (C-D) *In vivo* cytolysis activity of 667 in naive mice after depletion of neutrophils or NKs.
695 Splenocytes from non-infected mice (Sp) were labelled using $0.5 \mu\text{M}$ of the vital dye CFSE (CFSE^{low} cells; M1)
696 and mixed at a 1:1 ratio with splenocytes from infected mice (Infected-Sp) labelled using $5 \mu\text{M}$ CFSE (CFSE^{high}
697 cells; M2) and pre-incubated, or not, with the 667 mAb. Mixed cell populations were administered to naive mice
698 1 day after depletion of either neutrophils or NKs with the 1A8 mAb or the anti-asialo-GM1 antibody,
699 respectively. Cytolysis was quantified 5 hours later as described in Methods section. The data are presented as
700 mean \pm SEM of 2 independent experiments with at least 8 mice per group. Statistical significance was
701 established using a parametric one-way ANOVA test with a Bonferroni correction (panels B and D).
702 (E) *Effect of NKs depletion in the survival of infected/treated mice.* I/T, NKs-depleted or not as indicated in (A)
703 were followed up for leukemic death. The data represent 2 independent experiments with 7 mice per group.
704 Statistical significance was established using an unpaired Student's test.



705 **Figure 3. Effects of neutrophil depletion on innate lymphoid cells recruitment and biology.** (A-D)
 706 Neutrophils of naive, I/NT and I/T mice were depleted, or not, as indicated in Figure 1A and ILC in the spleen
 707 were assayed 14 days p.i. by flow cytometry. (A) Frequency of CD3⁻NKp46⁺ cells in the CD45.2⁺ leukocytic
 708 population. (B) Frequency of CD117⁺/CD127⁺ cells in the Lin⁻NKp46⁺ population. (C) Maturation (CD11b⁺
 709 cells) and (D) expression of IFN- γ in the CD3⁻NKp46⁺ population. (E) In vivo cytotoxicity activity of 667 in
 710 infected/treated mice after depletion of neutrophils. The 1A8, or the 2A3 isotype control mAb, were
 711 administered to I/T mice and 667 ADCC activity was quantified at 30 days p.i. as in Fig 2C-2D. The data
 712 represent at least 2 independent experiments. Data are expressed as means \pm SEM. Statistical
 713 significance was established using a parametric one-way ANOVA test with a Bonferroni correction
 714 (* p < 0.05; ** p < 0.01; *** p < 0.001).



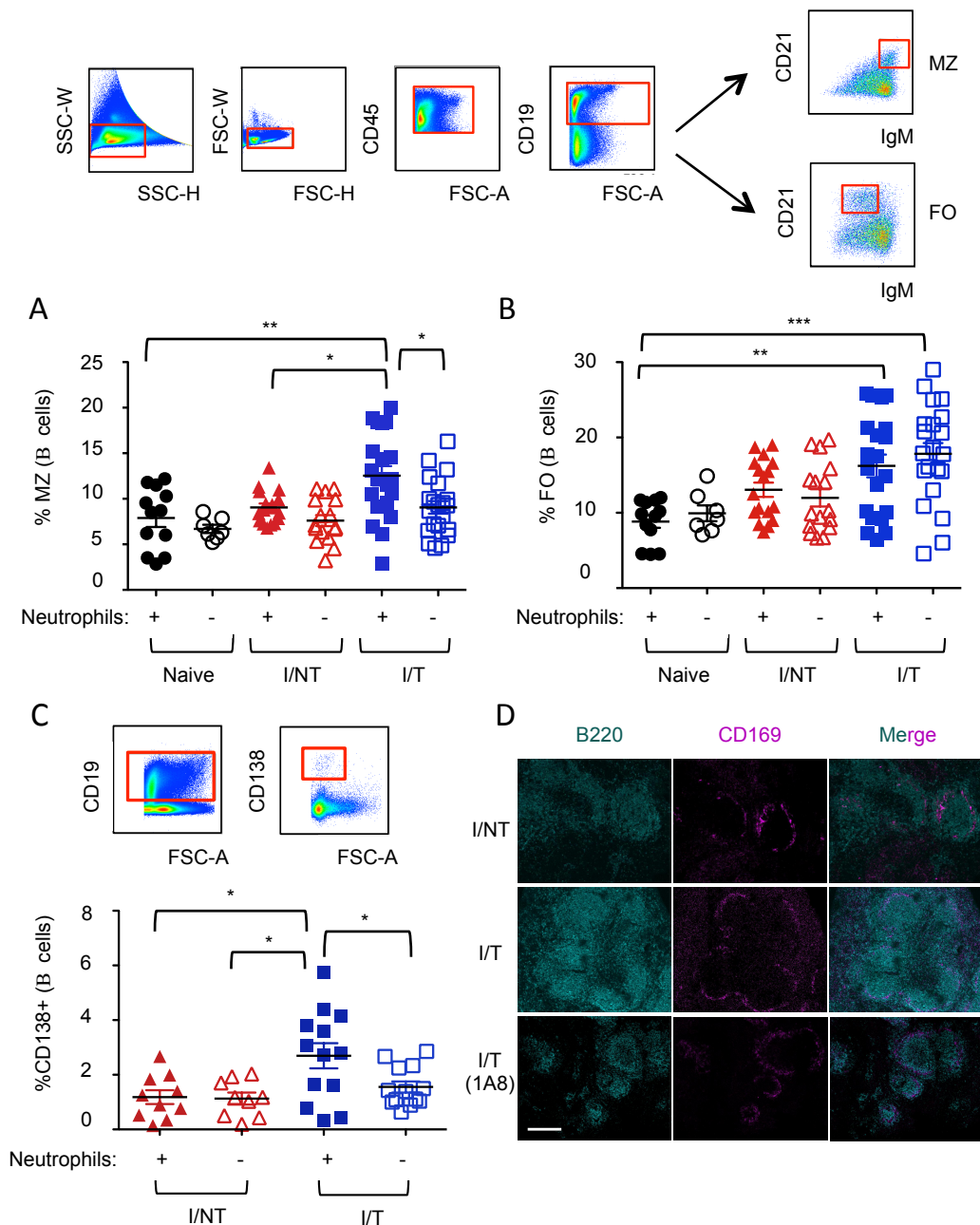
715 **Figure 4. Assay of FrCasE-specific CD8⁺ T cells in the presence and absence of neutrophils.** Neutrophils of
 716 naive, I/NT and I/T mice were depleted, or not, as indicated in (A). *Frequency of FrCasE-specific CD8⁺ T cells.*
 717 Spleen cells were isolated at day 14 p.i. and the frequency of virus-specific CD8⁺ T cells in the total CD8⁺ T-
 718 cells population was assayed by flow cytometry using the H2D^b-GagL MHC tetramer. The data represent 4
 719 independent experiments with at least 11 mice per group. (B) *Expression of IFN- γ by CD8⁺ T cells.* Splenic
 720 CD8⁺ T cells were flow cytometry-analyzed for the expression of IFN- γ . The data presented represent 3
 721 independent experiments with at least 7 mice per group. Data are expressed as means \pm SEM. Statistical
 722 significance was established using a parametric one-way ANOVA test with a Bonferroni correction
 723 (* p < 0,05).
 724



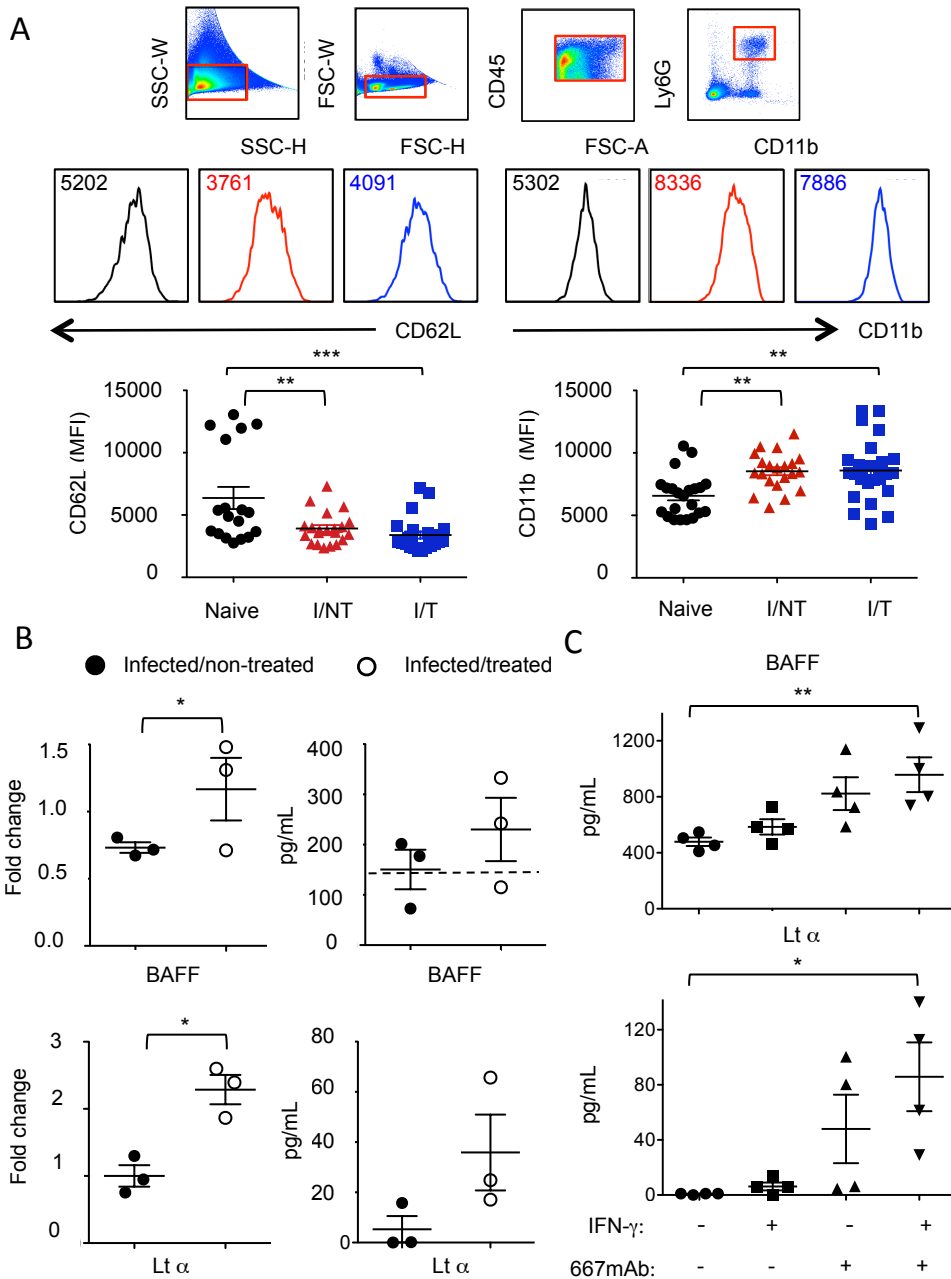
725 **Figure 5. Enhancement of the humoral antiviral response by neutrophils.** Neutrophils of naive, I/NT and I/T
726 mice were depleted, or not, as indicated in Figure 1A. (A, B) *Serum concentration of FrCasE-specific Igs.* (A)
727 Seric FrCasE-specific IgM levels were assayed by ELISA at 14 days p.i. The data represent 2 independent
728 experiments with 8-11 mice per group (for I/NT and I/T mice) and 3 to 6 mice per group (for naive mice). (B)
729 Seric FrCasE-specific IgG concentration was assayed by ELISA at the indicated times. The data represent 2
730 independent experiments with 7-9 mice per group. Data are expressed as means +/- SEM. Statistical
731 significance was established using a parametric one-way ANOVA test with a Bonferroni correction
732 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (C) *Correlation between serum anti-FrCasE IgG levels (evaluated as the*
733 *area under the curve; AUC) and survival times, analyzed using the Pearson correlation test.* AUC was evaluated
734 until the last time point at which all mice were still alive (day 68 p.i). All infected/non-treated- (n = 8),
735 infected/treated- (n = 9) mice, depleted or not in neutrophils (n = 9 and n = 7, respectively), showed in Fig 1D
736 were evaluated for such a correlation. (D) *FrCasE-specific secondary humoral response.* Seric FrCasE-specific
737 IgG levels in I/T mice (depleted or not in neutrophils) were assayed by ELISA before and 1 week after a viral
738 challenge performed at day 93 p.i. The data represent 2 independent experiments with 5 mice per group.
739 Statistical significance was established using a paired Student's t test.

740

741



742 **Figure 6. Effects of neutrophil depletion on B-cell responses.** (A-D) Neutrophils of naive, I/NT and I/T mice
 743 were depleted, or not, as indicated in Figure 1A. (A-B) Frequency of MZ and follicular (FO) B cells. Spleen
 744 cells were isolated at day 14 p.i. and flow cytometry-analyzed for the frequency of MZ (CD21^{high}IgM^{high}) (A)
 745 and FO (CD23⁺IgM^{low}) (B) CD19⁺ B cells. (C) Frequency of plasma cells. BM cells were isolated at day 14 p.i.
 746 and flow cytometry-analyzed for the frequency of CD138⁺ (CD19⁺) B cells. The data represent 5 independent
 747 experiments with 7-12 mice per group for naive mice and 17-21 per group for I/NT and I/T mice. Data are
 748 expressed as means +/- SEM. Statistical significance was established using a parametric one-way
 749 ANOVA test with a Bonferroni correction. (*p < 0.05; **p < 0.01; ***p < 0.001). (D) Histological analyses
 750 of spleen sections. Immunolabelling of B cells (B220⁺) and macrophages of the MZ (CD169⁺) was performed in
 751 sections from spleens of infected/non-treated and infected/treated mice (depleted or not in neutrophils)
 752 recovered at 14 days p.i. to visualize germinal centers. The images are representative of four separate mice for each
 753 experimental condition. Scale bar 200 μm.



754 **Figure 7. Activation of splenic and BM-isolated neutrophils.** (A) Expression of CD11b and CD62L. Spleen
 755 cells from naive, I/NT and I/T mice were isolated at day 8 p.i. and flow cytometry-analyzed for assaying cell
 756 surface expression of CD11b and CD62L. The data represent 5 independent experiments with at least 18 mice per
 757 group. Data are expressed as means \pm SEM. (B) Expression and protein release of BAFF and LT α by
 758 neutrophils. Neutrophils from naive, I/NT and I/T mice were sorted from the spleen at day 8 p.i. and assessed for
 759 cytokine expression or protein release. Cytokine expression (left) was assessed by RT-qPCR normalized to β -actin.
 760 The data show fold changes in cytokine expression by neutrophils from I/NT and I/T mice as compared to naive
 761 mice and are representative of 3 independent experiments with 8-10 mice per group. Protein release (right) was
 762 assessed by ELISA in supernatants of sorted neutrophils cultured at a density of 10^5 cells/well for 24 h. The data
 763 show BAFF and LT α release by neutrophils from I/NT and I/T and are representative of 3 independent
 764 experiments with 8-10 mice per group. The dashed line represents the level of BAFF released by neutrophils
 765 sorted from naive mice. No LT α release was detected from neutrophils sorted from naive mice. (C) BAFF and
 766 LT α release by BM-isolated neutrophils. BAFF and LT α release was assessed by ELISA in supernatants of
 767 neutrophils isolated from BM of naive mice (>95% purity) and cultured for 24 h in 667-mAb coated 24-well plates
 768 at a density of 2×10^6 cells in 500 μ l of medium. Experiments were done in the presence and in the absence of the
 769 pro-inflammatory cytokine IFN- γ (100 μ g/ml). 667 mAb non-coated plates were used as control. The data
 770 represent 4 independent experiments. Data are expressed as means \pm SEM. Statistical significance was
 771 established using a parametric one-way ANOVA test with a Bonferroni correction (panels A and C) or a
 772 paired Student's t test (panel B). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).