

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

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25 **KEY WORDS**

26 Antiviral immunity, antiviral monoclonal antibodies, immunotherapy, B-cell helper neutrophils,

- 27 vaccine-like effects.
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- 29
- 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

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.

In humans, technical-, ethical- and cost limitations strongly limit investigations. Instead, immunocompetent animals offer the possibility to identify the cellular and molecular actors of antiviral mAb-induced vaccine-like effects. The FrCasE-induced erythroleukemia is a model system permitting extensive analysis of the endogenous immune response after passive mAb-based immunotherapy under conditions of both chronic infection and pathological development. We found that a 5-day treatment of FrCasE-infected mice with the neutralizing mAb 667 (an IgG2a recognizing the retroviral envelope glycoprotein; Env) shortly after infection provides long-lasting (> 1 year) protective antiviral immunity of the Th1 type with enhanced IgG2a humoral- and cytotoxic T cell (CTL) responses and reduced regulatory T-cell activity (24–29). The immunomodulatory action of mAb 667 depends on its ability to interact with receptors for IgGs (Fc γ R). Notably, immune complexes (ICs) formed between the administered mAb and viral determinants enhance antiviral CTL responses through Fc γ R-mediated binding to dendritic cells (DCs) (27).

80 Importantly, IC-FcyR interactions concern several FcyR-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.

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.

Thus, neutrophils exert different antiviral effects on FrCasE-infected mice depending on immunotherapy. In animals undergoing simple infection, neutrophils participate in the control of viral propagation. Instead, in infected/treated mice, they are crucial during the immunotherapy period for 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 propagation145 by 667-mediated ADCC.

146

Neutrophils differentially alter innate lymphoid cells biology in infected/treated- and infected/nontreated mice. Innate lymphoid cells (ILC) are a heterogeneous population of immune cells that includes NK cells and ILC1, ILC2 and ILC3. As NK biology can be affected by neutrophils (45), we first assessed the effect of neutrophil depletion on NKs. To this end, we measured the frequency of 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.

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

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

Next, we addressed humoral immunity in infected/non-treated and infected/treated mice, depleted or not in neutrophils. To achieve this, anti-FrCasE serum immunoglobulins (Ig) from mice of the different groups were assayed by ELISA. Virus-specific IgM titers were assayed at 14 days p.i. (i.e. at the peak of the IgM response upon FrCasE infection and 667 treatment, Supplemental Figure 4) and 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 μ g/ml) by day 68 post-210 infection and a still high level (100 µg/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.

As marginal zone (MZ) B cells are known to contribute to the mounting of antibody responses (49, 50) and their function might be modulated by neutrophils (51), we next addressed the frequencies of splenic MZ (CD21highIgMhigh CD19+ cells)- and follicular (FO) (CD23+IgMlow CD19+ cells) B cells. MZ B-cells frequency was enhanced in a neutrophil-dependent manner in infected/treated mice but unchanged in infected/non-treated animals relative to naive mice (Figure 6A). In contrast, the 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).

Thus, neutrophils are essential for the vaccine-like effects induced by the 667 immunotherapy through the stimulation of the humoral, but not the CD8⁺ T-cell, antiviral response. This effect is associated 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 a 252 (LTa), 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 LTa, 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 pro259 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 .

268 269

B **DISCUSSION**

We have previously shown that antiviral mAb-based therapies can induce life-long protective immunity. This finding potentially has important therapeutic implications, as evidence suggests that it may also apply to diverse severe human viral diseases (10, 14). A paramount task is now to identify the mechanisms at the origin of the mAb-induced vaccine-like effects and exploit them for more 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 infected mice is dependent on immunotherapy, as no modulation of the anti-FrCasE humoral responsewas 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 LTa 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-y stimulation and FcyR 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 $LT\alpha$ release by neutrophils might play a role in 314 the enhancement of the antiviral humoral response in infected/treated mice. $LT\alpha$ 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 LTa 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 FcyR 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 LTa 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-y potentiates 325 both BAFF and LTa release, it will also be important to assess whether and how neutrophil-dependent 326 IFN-y 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 FcyRs 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).

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

356 **METHODS**

- *Ethical statement*. Mice were bred and maintained under conventional, pathogen-free facilities at the
 Institut de Génétique Moléculaire de Montpellier. All experimental procedures were performed in
 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).

ELISA of anti-FrCasE antibodies. Plasma anti-FrCasE immunoglobulins were assayed by ELISA as
already described (26, 27). Peroxidase-conjugated anti-mouse IgG or IgM rabbit antisera (Serotec)
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 x 10⁴ FFU/ml FrCasE suspension mixed to 2 x 10⁶ 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 cytolysis 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 µM (CFSE^{low} cells). Splenocytes from infected mice were labelled with 5 µM CFSE (CFSE^{high} cells) and pre-incubated, or not, with the 667 mAb (the 402 403 absence of 667 allows to quantify spontaneous cell death). Both cell populations were mixed at a 1:1 404 ratio before retrorbital administration to recipient mice. Cytolysis activity against infected splenocytes was calculated from the ratio of CFSE^{low}/CFSE^{high} cells in spleen assayed by flow cytometry 5 hours 405 406 later. To assess the contribution of NKs and neutrophils to antibody-mediated cytolysis, 50 µl of the 407 anti-asialo GM1 or 200 µg of the anti-Ly6G 1A8 mAb were administered 1 day prior to the assay.

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

413

414 *Assay of IFN-\gamma production.* 10⁶ splenocytes were incubated at 37°C for 5 hours in 12-well plates in 415 500 µl 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 µg/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 µm 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 µm 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 LTa 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 density of $2x10^5$ cells/well for 24 h. Alternatively, neutrophils were isolated from naive mice BM 444 445 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 µl of medium, in the presence or in the absence of IFN- γ (100 µg/mL). 667 mAb-non coated plates were 447 448 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 +/- 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

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

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481

482 CONFLICT OF INTEREST DISCLOSURES

483 The authors declare no competing financial interests.

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671 FIGURES



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, as 682 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 < 0.05; 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 +/- 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 $0.5 \,\mu$ 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 µM 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 cytolysis 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 +/- 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 +/- 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 independent experiments with 7-9 mice per group. Data are expressed as means +/- SEM. Statistical 730 731 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.



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) recovered 752 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 +/- 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 10⁵ cells/well for 24 h. The data 763 show BAFF and LTa 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 $LT\alpha$ 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 µl 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 +/- 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).