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

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KEY WORDS
Antiviral immunity, antiviral monoclonal antibodies, immunotherapy, B-cell helper neutrophils, vaccine-like effects.
ABSTRACT

Using a mouse retroviral model, we have shown that monoclonal antibody (mAb)-based immunotherapy can induce life-long endogenous protective immunity (vaccine-like effects). This observation has potentially important consequences for treating life-threatening human viral infections. Here, we investigated the role of neutrophils in this effect. Neutrophils are innate immunity effector cells with well-established microbe-killing activities that are rapidly mobilized upon infection. They are also emerging as orchestrators of innate and adaptive immunities. However, their immunomodulatory activity during antiviral mAb immunotherapies has never been studied. Our data reveal that neutrophils have an essential role in immunotherapy-induced immune protection of infected mice. Unexpectedly, neutrophils have a limited effect in controlling viral propagation upon passive immunotherapy administration, which is mostly mediated by natural killer cells (NKs). Instead, neutrophils operate as essential inducers of a potent host humoral antiviral response. Thus, neutrophils play an unexpected key role in protective immunity induction by antiviral mAbs. Our work opens new approaches to improve antiviral immunotherapies as they suggest that preserving neutrophil functions and counts might be required for achieving mAb-induced protective immunity.
Neutralizing monoclonal antibodies (mAbs) are now considered as a potential therapeutic approach for the prevention and treatment of chronic and acute viral infections, including newly emerging viral infections (1). In recent years, there has been a dramatic increase in the development of new mAbs with improved neutralizing activity (1–14). Notably, in addition to anti-respiratory syncytial virus (RSV) mAb used to treat infant respiratory disease, several mAbs directed against human cytomegalovirus, human immunodeficiency- (HIV), influenza-, Ebola- and rabies virus are currently engaged in promising clinical trials (1, 3, 9, 14). While the direct effects of antiviral mAbs on viral propagation have been studied extensively, little attention has been paid to their potential immunomodulatory effects until recently (10, 15, 16).

Using a mouse model of persistent retroviral infection, the erythroleukemia induced by the Murine Leukemia Virus FrCasE, we have shown that treatment of infected mice with a highly neutralizing mAb can induce life-long protective immunity (vaccine-like effects) (see 10). This observation may have major therapeutic implications for humans. Indeed, increased antiviral immune responses after mAb treatments were recently observed in preclinical models of HIV, RSV and henipavirus infections (see 10). Moreover, passive immunotherapy with broadly neutralizing mAbs stimulated antiviral antibody responses in HIV-1-infected patients (14). Similarly, vaccine-like effects have also been documented in preclinical models of cancer immunotherapies (17–19), as well as in clinical trials using anti-CD20, anti-EGFR or anti-HER2 mAbs (20–23). Yet, neither the mechanisms at play nor the 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 (FcγR). Notably, immune
complexes (ICs) formed between the administered mAb and viral determinants enhance antiviral CTL
responses through FcγR-mediated binding to dendritic cells (DCs) (27).

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

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

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

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

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

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

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

To better characterize the effect of neutrophils on splenic CD3’NKp46+ cells, we assessed their
maturation by monitoring CD11b and CD27 expression at day 14 p.i. These markers identify different
stages of NK maturation in mice (45). Relative to naive mice, both infected/non-treated and
infected/treated mice showed similar higher frequencies of CD11b+ CD3’NKp46+ cells (including fully
mature CD11b’CD27- and semi-mature CD11b’CD27- cells) (Figure 3C) at the expense of immature
ones (CD11b-) (not shown). Moreover, depletion of neutrophils entailed a strong reduction of the
frequency of CD11b+ CD3’NKp46+ cells only in infected/non-treated mice. Further characterization of
CD3’NKp46+ cells indicated higher neutrophil-dependent IFN-γ production in infected/treated mice
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 667-mediated ADCC activity of NK cells (Figure 3E), consistent with the lack of effect on viral propagation (Figure 1E and 2B).

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

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

We first addressed cellular adaptive immunity by assaying the primary virus-specific CD8 T-cell response in infected/non-treated and infected/treated mice at its peak (i.e. 14 days p.i.) (27) with or without neutrophils depletion. Consistent with our previous work, infected/treated mice showed an increased virus-specific CD8 T-cell response relative to infected/non-treated mice. In neither case the frequency of virus-specific CD8 T cells was altered by neutrophil depletion (Figure 4A). Similarly, neither case showed any difference in the frequency of CD8 T cells expressing IFN-γ (Figure 4B). These data ruled out a major role for neutrophils in the 667 mAb-induced anti-viral CD8 T-cell 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).
Interestingly, neutrophils depletion did not alter virus-specific IgM titers in either infected/non-treated or infected/treated mice (Figure 5A). In agreement with published studies in other experimental settings, the absence of neutrophils did not affect the levels of IgM (48). On the contrary, and consistent with our previous work (27), infected/treated mice displayed a much higher level of anti-FrCasE IgGs than infected/non-treated animals (Figure 5B), with a peak (700 µg/ml) by day 68 post-infection and a still high level (100 µg/ml) at the end of the experiment. Interestingly, in the absence of neutrophils, serum concentration of antiviral IgGs dramatically decreased in infected/treated mice (Figure 5B and Supplemental Figure 5). In contrast, neutrophils showed no role in the regulation of the poor non-protective antibody response elicited in infected/non-treated mice (Figure 5B). Importantly, high anti-FrCasE IgG seric levels correlated with longer survival times, supporting a role for the high humoral antiviral response in protection against disease (Figure 5C). To further characterize the long-term virus-specific humoral response, we tested whether infected/treated mice, depleted or not in neutrophils, could respond to a virus challenge performed 3 months after the first infection (i.e. a time point at which the primary humoral response has strongly declined). Infected/treated mice, depleted or not in neutrophils, were inoculated with FrCasE and serum samples were collected 1 week later to assay the generation of endogenous anti-FrCasE antibodies. In the presence of neutrophils, 3 out of 5 infected/treated mice showed an increase in the virus-specific humoral response (Figure 5 D) while, in the absence of neutrophils, none of the infected/treated mice responded to the viral challenge. Altogether, these results show that neutrophil depletion at early time points after infection and treatment affects humoral immunity with an effect on both primary and memory virus-specific B-cell 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,
with however no role for neutrophils (Figure 6B). Interestingly, consistent with a role of MZ B cells in the generation of plasma cells (49), the higher frequency of splenic MZ B cells at day 14 p.i. in infected/treated mice was associated with a higher frequency of CD138⁺ plasma cells in bone-marrow (BM), as compared to infected/non-treated animals. This effect was neutrophil-dependent (Figure 6C). Finally, histological analyses of spleens of infected mice at 14 days p.i. revealed larger germinal centers (GC, defined by staining of MZ CD169⁺ macrophages and B220⁺ cells) in infected/treated versus infected/non-treated mice (Figure 6D). In agreement with a role of neutrophils in the enhancement of the humoral response, neutrophil depletion in infected/treated mice led to smaller GCs (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.

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

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

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

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

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

Although not formally shown, our data suggest that LTα release by neutrophils might play a role in the enhancement of the antiviral humoral response in infected/treated mice. LTα is involved in the formation of secondary lymphoid organs, it is expressed by lymphocytes and mediates a large variety of inflammatory and antiviral responses. It has also been reported to play a role in the development of GC formation and to be required for IgG responses (63–65). Here, we show that this cytokine is expressed and released by neutrophils upon mAb treatment of infected mice. Interestingly, although LTα is not known to be expressed by neutrophils, it has been proposed that non-lymphocytic murine splenic cells are able to produce it (66). We also report that FcγR cross-linking by immobilized 667...
mAb synergizes with IFN-γ to enhance LTα release by neutrophils. This suggests that, similarly to BAFF, inflammatory conditions and mAb-triggering might lead to increased LTα secretion by neutrophils. Further studies will be required to address whether BAFF and LTα combine their actions to stimulate the antiviral humoral response upon mAb immunotherapy. Finally, as IFN-γ potentiates both BAFF and LTα release, it will also be important to assess whether and how neutrophil-dependent IFN-γ production by NKs in infected/treated mice contributes to the stimulation of antibody responses, as this cytokine affects class-switching and long-term maintenance of neutralizing antibody titers in retrovirally-infected mice (34) and humoral autoimmunity in humans (67).

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

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

Viral infection, immunotherapy and mice follow-up. Eight day-old 129/Sv/Ev mice were infected intraperitoneally (i.p.) with 50 µl of a virus suspension containing 50,000 ffu (focus-forming units) 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. administration. Mice were examined at regular intervals for clinical signs of erythroleukemia (reduction of hematocrits). They were euthanized when their hematocrits reached 35% (experimental endpoint).

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

**Virus challenge experiments.** Infected/treated mice, depleted or not in neutrophils, were injected i.v. with 300 µl of a 5 x 10⁴ FFU/ml FrCasE suspension mixed to 2 x 10⁶ FrCasE-infected splenocytes 3 months after the first infection. Blood samples were collected 1 week post-challenge to assay endogenous anti-FrCasE IgG concentrations by ELISA.

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

**In vivo cytolysis activity.** Experiments were conducted as described in (27, 79). Briefly, red blood cell-free splenocytes were recovered from 10 day-old FrCasE-infected-, or non-infected, pups. Splenocytes from non-infected mice were labelled with the vital dye carboxy-fluorescein succinimidyl ester (CFSE; Molecular Probes) at a concentration of 0.5 µM (CFSE<sub>low</sub> cells). Splenocytes from infected mice were labelled with 5 µM CFSE (CFSE<sub>high</sub> cells) and pre-incubated, or not, with the 667 mAb (the absence of 667 allows to quantify spontaneous cell death). Both cell populations were mixed at a 1:1 ratio before retrorbital administration to recipient mice. Cytolysis activity against infected splenocytes was calculated from the ratio of CFSE<sub>low</sub>/CFSE<sub>high</sub> cells in spleen assayed by flow cytometry 5 hours later. To assess the contribution of NKs and neutrophils to antibody-mediated cytolysis, 50 µl of the 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-2Dᵇ tetramer (Beckman Coulter) displaying the immunodominant Friend virus GagL epitope (27) (80) (Dᵇ-GagL tetramers) as previously described (27).

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

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

RT-qPCR quantification of gene expression. Single-cell suspensions of splenocytes were prepared from naive, infected/non-treated and infected/treated mice 8 days p.i. and immunotherapy. Neutrophils (CD11b⁺Ly6G_high expression) were sorted (>98% pure) using a BD Biosciences FACSaria device. RNA was extracted from 1-2 x 10⁶ sorted neutrophils using the RNeasy micro kit (Qiagen). RNA quality and integrity were verified using the Agilent 2100 bioanalyzer. cDNAs were synthesized using the RT2 First Strand Kit (Qiagen). All quantitative PCRs were performed following protocols optimized for the RT2 quantitative Profiler PCR array using SYBR Green mix (Qiagen) and
LightCycler 480 II machine (Roche). All data were normalized to β-actin. Results were expressed as fold increases with respect to naive cells using the ΔΔCt method.

BAFF and LTα protein release quantification. Soluble BAFF and LTα from cell-free supernatants of cultured neutrophils were assayed using BAFF (R&D Systems) and LTα ELISA (NeoBiotech), respectively. Supernatants were collected from sorted splenic neutrophils (from naive, infected/non-treated and infected/treated mice at 8 days p.i. and immunotherapy) cultured in 96-well plates at a density of 2×10^5 cells/well for 24 h. Alternatively, neutrophils were isolated from naive mice BM using a magnetic-based cell-sorting (MACS) neutrophil isolation kit (>95% purity; Miltenyi Biotec) 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 used as controls. G-CSF (R&D Systems) was added at a concentration of 10 ng/ml to neutrophil cultures to maintain cell viability.

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

AUTHOR CONTRIBUTIONS

Mireia Pelegrin (MPe), MN-G and Marc Piechaczyk (MPi) defined the research program. MN-G, JL and MPi 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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Figure 1. Anti-viral effects of neutrophils. (A) Experimental scheme. Upper part: 8 day-old pups were infected and treated with the 667 mAb as indicated. Lower part: mice were treated as indicated with the anti-Ly6G 1A8 mAb or the isotype control 2A3 mAb in neutrophil depletion experiments. (B-C) Neutrophil recruitment and infected cells rate in spleen. Splenocytes from naive, infected/non-treated (I/NT) and infected/treated (I/T) mice were analyzed by flow cytometry on day 8 p.i. for (i) neutrophil recruitment (% of Ly6G+ cells) and (ii) for retroviral positivity of splenocytes (% of Gag+ cells) gated in the CD45.2+ population. The data presented correspond to 5 independent experiments with at least 15 mice per group. (D) Mouse survival. Naive, I/NT and I/T mice were treated with either the anti-neutrophil- (1A8) or the control (2A3) mAb as indicated in (A) and followed up for leukemic death. The data represent 2 independent experiments with 6-9 mice per group. (E) Infected cells rate upon neutrophil depletion. Neutrophils of naive, I/NT and I/T mice were depleted, or not, as indicated in (A) and infected splenocytes were assayed as in (C) on day 8 p.i. The data represent 4 independent experiments with 9-15 mice per group. Data are expressed as means +/- SEM. Statistical significance was established using a parametric one-way ANOVA test with a Bonferroni correction. (*p < 0.05; **p < 0.01; ***p < 0.001).
Statistical significance was established using a parametric one-way ANOVA test with a Bonferroni correction (panels B and D).
Figure 3. Effects of neutrophil depletion on innate lymphoid cells recruitment and biology. (A-D)

Neutrophils of naive, I/NT and I/T mice were depleted, or not, as indicated in Figure 1A and ILC in the spleen were assayed 14 days p.i. by flow cytometry. (A) Frequency of CD3\(^\ast\)NKp46\(^+\) cells in the CD45.2\(^+\) leukocytic population. (B) Frequency of CD117\(^+\)/CD127\(^+\) cells in the LinNKp46\(^+\) population. (C) Maturation (CD11b\(^+\) cells) and (D) expression of IFN-\(\gamma\) in the CD3NKp46\(^+\) population. (E) In vivo cytolysis activity of 667 in infected/treated mice after depletion of neutrophils. The 1A8, or the 2A3 isotype control mAb, were administered to I/T mice and 667 ADCC activity was quantified at 30 days p.i. as in Fig 2C-2D. The data represent at least 2 independent experiments. Data are expressed as means +/- SEM. Statistical significance was established using a parametric one-way ANOVA test with a Bonferroni correction (*p < 0.05; **p < 0.01; ***p < 0.001).
Figure 4. Assay of FrCasE-specific CD8⁺ T cells in the presence and absence of neutrophils. Neutrophils of naive, I/NT and I/T mice were depleted, or not, as indicated in (A). Frequency of FrCasE-specific CD8⁺ T cells. Spleen cells were isolated at day 14 p.i. and the frequency of virus-specific CD8⁺ T cells in the total CD8⁺ T-cells population was assayed by flow cytometry using the H2Dᵇ-GagL MHC tetramer. The data represent 4 independent experiments with at least 11 mice per group. (B) Expression of IFN-γ by CD8⁺ T cells. Splenic CD8⁺ T cells were flow cytometry-analyzed for the expression of IFN-γ. The data presented represent 3 independent experiments with at least 7 mice per group. Data are expressed as means +/- SEM. Statistical significance was established using a parametric one-way ANOVA test with a Bonferroni correction (*p<0.05).
Figure 5. Enhancement of the humoral antiviral response by neutrophils. Neutrophils of naive, I/NT and I/T mice were depleted, or not, as indicated in Figure 1A. (A, B) Serum concentration of FrCasE-specific IgGs. (A) Serum FrCasE-specific IgM levels were assayed by ELISA at 14 days p.i. The data represent 2 independent 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) Serum 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 significance was established using a parametric one-way ANOVA test with a Bonferroni correction (*p < 0.05; **p < 0.01; ***p < 0.001). (C) Correlation between serum anti-FrCasE IgG levels (evaluated as the area under the curve; AUC) and survival times, analyzed using the Pearson correlation test. AUC was evaluated until the last time point at which all mice were still alive (day 68 p.i.). All infected/non-treated- (n = 8), infected/treated- (n = 9) mice, depleted or not in neutrophils (n = 9 and n = 7, respectively), showed in Fig 1D were evaluated for such a correlation. (D) FrCasE-specific secondary humoral response. Serum FrCasE-specific IgG levels in I/T mice (depleted or not in neutrophils) were assayed by ELISA before and 1 week after a viral challenge performed at day 93 p.i. The data represent 2 independent experiments with 5 mice per group. Statistical significance was established using a paired Student’s t test.
Figure 6. Effects of neutrophil depletion on B-cell responses. (A-D) Neutrophils of naive, I/NT and I/T mice were depleted, or not, as indicated in Figure 1A. (A-B) Frequency of MZ and follicular (FO) B cells. Spleen cells were isolated at day 14 p.i. and flow cytometry-analyzed for the frequency of MZ (CD21^high^IgM^low^) (A) and FO (CD23^IgM^low^) (B) CD19^+^ B cells. (C) Frequency of plasma cells. BM cells were isolated at day 14 p.i. and flow cytometry-analyzed for the frequency of CD138^+^CD19^+^ B cells. The data represent 5 independent experiments with 7-12 mice per group for naive mice and 17-21 per group for I/NT and I/T mice. Data are expressed as means +/- SEM. Statistical significance was established using a parametric one-way ANOVA test with a Bonferroni correction. (*p < 0.05; **p < 0.01; ***p < 0.001). (D) Histological analyses of spleen sections. Immunolabelling of B cells (B220^+^) and macrophages of the MZ (CD169^+^) was performed in sections from spleens of infected/non-treated and infected/treated mice (depleted or not in neutrophils) recovered at 14 days p.i. to visualize germinal centers. The images are representative of four separate mice for each experimental condition. Scale bar 200 µm.
Figure 7. Activation of splenic and BM-isolated neutrophils. (A) Expression of CD11b and CD62L. Spleen cells from naive, I/NT and I/T mice were isolated at day 8 p.i. and flow cytometry-analyzed for assaying cell surface expression of CD11b and CD62L. The data represent 5 independent experiments with at least 18 mice per group. Data are expressed as means +/- SEM. (B) Expression and protein release of BAFF and LTα by neutrophils. Neutrophils from naive, I/NT and I/T mice were sorted from the spleen at day 8 p.i. and assessed for cytokine expression or protein release. Cytokine expression (left) was assessed by RT-qPCR normalized to β-actin. The data show fold changes in cytokine expression by neutrophils from I/NT and I/T mice as compared to naive mice and are representative of 3 independent experiments with 8-10 mice per group. Protein release (right) was assessed by ELISA in supernatants of sorted neutrophils cultured at a density of 10^6 cells/well for 24 h. The data show BAFF and LTα release by neutrophils from I/NT and I/T and are representative of 3 independent experiments with 8-10 mice per group. The dashed line represents the level of BAFF released by neutrophils sorted from naive mice. No LTα release was detected from neutrophils sorted from naive mice. (C) BAFF and LTα release by BM-isolated neutrophils. BAFF and LTα release was assessed by ELISA in supernatants of neutrophils isolated from BM of naive mice (>95% purity) and cultured for 24 h in 667-mAb coated 24-well plates at a density of 2 x 10^6 cells in 500 µl of medium. Experiments were done in the presence and in the absence of the pro-inflammatory cytokine IFN-γ (100 µg/ml). 667 mAb non-coated plates were used as control. The data represent 4 independent experiments. Data are expressed as means +/- SEM. Statistical significance was established using a parametric one-way ANOVA test with a Bonferroni correction (panels A and C) or a paired Student’s t test (panel B). (*p < 0.05; **p < 0.01; ***p < 0.001).