Different immunomodulatory role of Ly6G\textsuperscript{hi} and Ly6G\textsuperscript{int} neutrophils during antiviral antibody therapy

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SUMMARY

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

The development of powerful antiviral monoclonal antibodies (mAbs) has provided new therapeutic opportunities to treat severe viral infections (Dibo et al., 2019; Hooft van Huijsduijnen et al., 2020; Salazar et al., 2017; Wang and Zhang, 2020). Fc-dependent mechanisms are crucial for efficient antiviral activity of neutralizing mAbs through the engagement of IgG receptors (FcγRs) expressed on immune cells. These Fc-FcγR interactions lead to the elimination of viral particles and virus-infected cells through phagocytic and cytotoxic mechanisms (i.e. antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cell-mediated cytotoxicity (ADCC),…) (Bournazos and Ravetch, 2017a; Lu et al., 2018). Moreover, studies in different animal models of viral infection, including ours, have provided evidence that mAbs can also enhance antiviral immune responses (so called “vaccinal effects”) in a Fc-dependent manner (Pelegrin et al., 2015). These vaccinal effects have been recently reported in HIV-infected patients treated with broadly neutralizing mAb (bnAbs) (Naranjo-Gomez and Pelegrin, 2019; Niessl et al., 2020; Schoofs et al., 2016) although the mechanisms involved have not been identified thus far. The elucidation of the molecular and cellular mechanisms driving Fc-dependent, mAb-mediated immunomodulation is therefore an important issue that will be key to achieving protective immunity against severe viral infections by mAbs.

While several Fc-mediated effector functions (i.e. ADCC, ADCP, …) have been shown to be required for antibody-mediated antiviral protection (Bournazos et al., 2014, 2019; Earnest et al., 2019; Fox et al., 2019; Gunn et al., 2018; Liu et al., 2017) whether and how FcγR engagement by antiviral mAbs affects the immunomodulatory properties of different FcγR-expressing cells (i.e. cytokines/chemokines secretion, activation markers expression, …) has been little studied. In addition, the specific contribution of different FcγRs-expressing cells in the induction of vaccinal effects by mAbs still remains ill-understood. These issues are all the more important to address given that both virus-driven inflammation and Fc-mediated clearance of opsonized virus/infected cells by immune effector cells generate multiple danger signals able to further modulate the host immune response. However, multiple restrictions (i.e. technical and ethical issues, costs, …) largely limit those studies in humans and non-human primates (NHP). As an alternative, in vivo studies in immunocompetent mice infected with the Murine Leukemia Virus FrCasE allowed the identification of several immunological mechanisms that drive protective immunity upon mAb therapy (Lambour et al., 2016; Pelegrin et al., 2015). We have previously showed that treatment of FrCasE-infected mice with the neutralizing mAb 667 (recognizing the retroviral envelope glycoprotein, expressed in virions and infected cells) elicits protective adaptive antiviral immunity through the engagement of FcγRs. Notably, mAbs form immune complexes (ICs) with viral determinants that enhance antiviral T-cell responses through FcγR-mediated binding to dendritic cells (DCs) (Celis and Chang, 1984; Michaud et al., 2010; Nasser et al., 2010; Yamamoto et al., 2009). We have recently shown a key
immunomodulatory role of neutrophils in the induction of protective humoral responses via the acquisition of B-cell helper functions upon engagement of FcγRs by the therapeutic mAb (Naranjo-Gomez et al., 2018). Thus, the immunomodulatory actions of mAbs rely on their Fc fragment binding to multiple FcγR-expressing cells such as DCs and neutrophils. While the role of IC-activated DCs in the enhancement of antiviral immune responses has been addressed in several studies (Lambour et al., 2016; Wang et al., 2019; Wen et al., 2016) the role of IC-activated neutrophils has mostly been overlooked. Evidence shows that neutrophils, in addition to being key effector cells to fight against invading pathogens, are also endowed with immunomodulatory properties through the secretion of a plethora of chemokines and cytokines (Mantovani et al., 2011; Tamassia et al., 2018; Tecchio and Cassatella, 2016). Yet, the functional activation of neutrophils by viral ICs and the resulting effect on their immunomodulatory properties have poorly been studied in the context of antiviral mAbs therapies. Furthermore, recent studies have highlighted a vast heterogeneity of neutrophils phenotypes and functions in both homeostatic and pathological conditions (Deniset and Kubes, 2018; Ng et al., 2019; Scapini et al., 2016; Silvestre-Roig et al., 2016, 2019). As these phenotypic and functional variants of neutrophils depend on their context-dependent stimulation (inflammatory environment, timing, disease progression, …), it is important to dissect how the viral infection and mAb therapy shape the phenotype and functional properties of neutrophils.

Here, we used the FrCasE retroviral model to address how viral infection, with or without mAb therapy, affects the phenotypical and functional activation of neutrophils. Neutrophils activated by viral determinants secreted high levels of monocytes- and neutrophils-recruiting chemokines. We have shown that the inflammatory environment resulting from the ongoing infection and mAb-treatment modulates the functional properties of neutrophils and monocytes. We have also identified two neutrophils subpopulations with distinct functional activation states that differentially participate in the induction of Th1-type responses in mAb-treated mice in collaboration with inflammatory monocytes. Our work provides hitherto unreported findings on both the heterogeneity and immunomodulatory properties of neutrophils in a context of antiviral mAb-therapy. These findings might help to improve mAb-based antiviral therapies by tailoring therapeutic interventions aiming at harnessing the immunomodulatory properties of neutrophils.
RESULTS

Neutrophils activated by viral determinants, free or in the form of ICs, secrete high levels of chemokines able to recruit monocytes and neutrophils. We have previously shown a key role of neutrophils in the induction of long-term protective antiviral immunity upon mAb therapy of infected mice (Naranjo-Gomez et al., 2018). To better characterize the phenotypical and functional activation of neutrophils by viral determinants, free or in the form of ICs, we isolated bone marrow (BM) neutrophils from naive mice and stimulated them for 24h in vitro with free FrCasE virions or opsonized with the 667 mAb (ICs) (Figure 1A). Free 667 mAb was used as control. Both, FrCasE virions and ICs induced a strong activation of neutrophils as shown by a higher expression of the CD11b molecule as well as an increased frequency of CD11bhi CD62Llo neutrophils (Figure 1B). However, IC-mediated phenotypic activation was significantly higher.

We next assessed the functional activation of virus- and IC-stimulated neutrophils by measuring their chemokines and cytokines secretion capacity. Both stimuli poorly induced the secretion of the 12 cytokines analyzed, with no significant differences between unstimulated neutrophils versus virus or IC-stimulated neutrophils (Supplemental Figure 1A). This contrasted with the high secretion of IL-6 and TNFα pro-inflammatory cytokines observed upon lypopolisaccharide (LPS) neutrophils stimulation despite similar CD11b upregulation induced by LPS and viral determinants (Supplemental Figures 1B-1C). These data show that bacterial-related pathogen-associated molecular patterns (PAMPs) induce a functional activation of neutrophils different from that of viral stimuli. In contrast to cytokine secretion, both virus and ICs lead to high secretion levels of several chemokines such as CCL2, CXCL1, CXCL5 and to a lesser extent CCL3 and CCL4 (Figure 1C). In addition, LPS-stimulated neutrophils only secreted a low amount of CCL3 and CCL4 chemokines, with no secretion of CCL2, CXCL1, CXCL5 chemokines thus providing further evidence of a pathogen-dependent secretion profile of neutrophils (Supplemental Figure 1D). Interestingly, the chemokines which were more strongly secreted by neutrophils upon viral stimuli (but not by LPS) have been shown to be involved in the recruitment of monocytes (CCL2) (Fan et al., 2018; Kim et al., 2011; Leonard and Yoshimura, 1990; Robben et al., 2005) and neutrophils themselves (CXCL1, CXCL5) (Haider et al., 2019; Pruenster et al., 2009; Ritzman et al., 2010; Rovai et al., 1998).

Inflammatory monocytes activated by viral determinants, free or in the form of ICs, secrete high levels of chemokines able to recruit neutrophils and monocytes. Taking into account the high amounts of the monocytes-recruiting CCL2 chemokine secreted by viral determinants-activated neutrophils, we next assessed the phenotypical and functional activation of monocytes by these stimuli. Ly6Chi monocytes were isolated from naive mice BM and stimulated for 24h with virus or ICs (Figure 2A). Phenotypical activation was evaluated by measuring the CD86 co-stimulatory molecule expression on the cell
surface. Free virions and ICs significantly increased CD86 expression but no significant differences were observed between both stimuli (Figure 2B).

Virus- and ICs induced a weak secretion of most of the 12 cytokines analyzed, except for IL6 and TNFα. However, no significant differences between unstimulated monocytes versus virus- or IC-stimulated monocytes were detected (Supplemental Figure 2A). This contrasted with high level secretion of IL-6, TNFα and IFNγ observed upon LPS stimulation (Supplemental Figures 2B-2D). The quantification of the chemokine release by activated monocytes showed some interesting similarities with neutrophils. Following virus or ICs stimulation, monocytes produced CXCL5, CXCL1 and CCL2, with no significant differences between virus versus ICs (Figure 2C). As compared to neutrophils, higher amounts of the neutrophil-recruiting chemokine CXCL1 were detected as well as lower amounts of CXCL5 and CCL2. Interestingly, LPS stimulation induced a wider and different panel of chemokine release (Supplemental Figure 2D), notably with the secretion of high amounts of CCL3, CCL4, CCL5, and to a lesser extent CXCL1 and CXCL10. As observed in neutrophils, these data show that viral stimuli induce a functional activation of monocytes different from that of bacterial-related PAMPs.

Inflammatory conditions potentiate the activation of neutrophils and monocytes by viral ICs. As the inflammatory microenvironment resulting from the viral infection and mAb therapy might affect the antiviral immune response, we next assessed the phenotypic and functional activation of neutrophils and monocytes by viruses and ICs in the presence of proinflammatory/immunomodulatory cytokines, such as TNFα, IFN-I and IFNγ.

As depicted by an increased expression of CD11b, TNFα and IFNγ significantly enhanced the phenotypic activation of neutrophils by ICs (but not by free virus) (Figure 3A). Priming conditions also modulated the secretion profile of virus- and IC-activated cells in a cytokine-specific manner. TNFα enhanced the secretion of CXCL1 and TNFα itself by IC-activated neutrophils but not by virus-activated neutrophils (Figure 3B, Supplemental figure 3A). IFN-I and IFNγ (Figure 3B, Supplemental figure 3A) significantly potentiated CCL4 secretion by IC-activated neutrophils. Both types of IFN induced the production of CXCL10, consistent with the IFN-dependent induction of this chemokine. However, CXCL10 secretion was significantly increased in neutrophils activated by virus or ICs upon IFN priming. IFNγ also significantly enhanced CCL5 and CXCL1 secretion by IC-stimulated neutrophils. In contrast, TNFα-, IFN-I- or IFNγ priming did not modulate the secretion of CCL2, CXCL5 and CCL3 by neither virus- nor IC-activated neutrophils (not shown).
As for monocytes, IFN-I and IFNγ significantly potentiated the phenotypic activation of IC-stimulated monocytes (as depicted by an increased expression of CD86 molecule) (Figure 3C) but not that of virus-activated cells. TNFα, IFN-I or IFNγ significantly potentiated the secretion of CCL2 and CXCL1 (Figures 3D and Supplemental figures 3B) by IC-activated monocytes. Similar to neutrophils, IFNγ and IFN-I enhanced the secretion of CXCL10 by IC-stimulated monocytes and IFN-I significantly increased the production of TNFα. Priming conditions also modulated the cytokine/chemokine secretion profile of virus-activated monocytes, but in a more restricted way than in IC-activated cells. Thus, CXCL1 secretion was enhanced by TNFα and IFNγ and CCL2 by IFN-I. Both types of IFN also increased the release of CXCL10 by virus-activated monocytes (Figure 3D). Similar secretion levels of CXCL5 virus- and IC-activated monocytes were observed in the presence or in the absence of TNFα-, IFN-I- and IFNγ- priming (not shown).

Altogether, these data highlight that inflammatory conditions strongly potentiate the functional activation of IC-stimulated neutrophils and monocytes, although with different immune outcomes depending on the priming condition and the responding cell type (Figure 3). Our data describe a distinct enhancement of neutrophils and monocytes secretion profile by TNFα-, IFN-I- and IFNγ-priming showing thus a cytokine-specific effect on the functional activation of these IC-stimulated cells. Such cytokine/chemokine secretion enhancement is also cell type specific (i.e. increased secretion of CCL4, CCL5, CXCL1 and TNFα by IC-activated neutrophils and CCL2, CXCL1 and TNFα by IC-activated monocytes). In contrast, inflammatory conditions hardly modify the secretion profile of free virus-activated cells, except for the secretion of the CXCL10 IFN-inducible chemokine.

Inflammatory conditions upregulate the expression of FcγRIV on in vitro activated neutrophils and monocytes. We next assessed whether the activation of neutrophils and monocytes by virus and IC affected their FcγRs expression, both in the absence and in the presence of inflammatory cytokines. Indeed, FcγRs expression might differ between steady-state versus inflammatory/pathological conditions (Wang and Jönsson, 2019), however little is known about modulation of FcγRs expression in the context of viral infections and mAb-therapy. Neither virus nor IC upregulated the expression of FcγRIV on neutrophils and monocytes (Figure 4A-4D). On the contrary, IFNγ and IFN-I priming (but not TNFα) upregulated the expression of FcγRI on neutrophils and monocytes (IFNγ and IFN-I) (Figure 4D). Both IFNs also enhanced FcγRII expression on monocytes whereas a decreased FcγRII-III expression was observed following IC stimulation and cytokine-priming (Supplemental Figure 4). No FcγRI expression was detected on neutrophils neither upon viral determinants stimulation nor cytokine priming.
Viral infection and mAb therapy upregulate FcγRIV expression on two neutrophils subpopulations and on inflammatory monocytes. We next assessed in vivo whether the inflammatory environment resulting from FrCasE viral infection and 667 mAb therapy modulated the expression of FcγRIV. The modulation of this FcγR is all the more relevant to be studied in this experimental model as (i) it is a high affinity receptor for IgG2a, (which is the isotype of the 667 mAb) and (ii) it is strongly expressed in neutrophils, the latter having a key immunomodulatory role in mAb-mediated protection of retrovirus-infected mice (Naranjo-Gomez et al., 2018).

To this end, mice were infected and treated, or not, with the therapeutic mAb (infected/treated and infected/non-treated, respectively). Then, the expression of FcγRIV on neutrophils and inflammatory monocytes from the spleen (one of the main sites of viral replication) was evaluated at different time points post-infection (p.i.). Age-matched naive mice were used as controls. The cell populations of interest were defined by flow cytometry (Figure 5A) based on the expression of CD11b, Ly6G and Ly6C to gate neutrophils (CD11b+, Ly6Ghi) and inflammatory monocytes (CD11b+Ly6G-Ly6C+). Interestingly, we identified another population of neutrophils displaying a lower expression of the cell surface marker Ly6G (CD11b+Ly6Gint) (Figure 5A). No expression of the eosinophils cell surface marker Siglec F was detected in this population (not shown). The frequency of both populations of neutrophils CD11b+Ly6Ghi and CD11b+Ly6Gint was significantly increased in the spleen of infected/non-treated mice at 8 and 14 days p.i. This increased neutrophil frequency was associated with a higher percentage of spleen infected cells as compared to infected/treated mice (Naranjo-Gomez et al., 2018). On the contrary, the frequency of inflammatory monocytes (CD11b+Ly6G+Ly6C+) remained unchanged in infected mice at day 8 p.i. but was significantly increased at day 14 p.i. (Figure 5B), suggesting an earlier recruitment of neutrophil than inflammatory monocytes upon infection.

In steady-state conditions, FcγRIV was highly expressed on CD11b+Ly6Ghi and CD11b+Ly6Gint neutrophils. Lower FcγRIV expression was detected in inflammatory monocytes at the different time points assessed (Figure 5C). Notably, upon viral infection and mAb-treatment, FcγRIV expression was upregulated in the three myeloid cells populations although, to a different magnitude depending on the cell type, time p.i. and mAb-treatment (Figure 5C). At day 8 p.i. FcγRIV expression was significantly upregulated on neutrophils and monocytes in infected/treated and infected/non-treated mice, the latter showing a stronger FcγRIV upregulation. In contrast, at day 14 p.i. infected/treated mice showed a stronger upregulation of FcγRIV on the three myeloid cells populations as compared to infected/non-treated and control mice. CD11b+Ly6Gint neutrophils displayed the strongest expression of FcγRIV as well as the highest magnitude of FcγRIV upregulation after infection and mAb-treatment. No detectable FcγRI expression was observed at day 8 p.i. in any of the three cell types assessed.
However, at day 14 p.i. infected/treated mice showed a significant upregulation of this inducible FcγR in CD11b+Ly6Gint neutrophils and monocytes (Supplemental Figure 5).

These results highlight that viral infection and mAb therapy results in an evolving inflammatory environment that dramatically modulates the expression of FcγRs in neutrophils and monocytes, leading to a stronger upregulation of FcγRIV at 8 days p.i. in infected/non treated mice and at 14 days p.i. in infected/treated mice.

High FcγRIV-expressing CD11b+Ly6Gint neutrophils subpopulation displays phenotypic properties different from those of CD11b+Ly6Ghi neutrophils. High level expression and strong upregulation of FcγRIV on CD11b+Ly6Gint (Figure 5C and 5D) suggested different and specific properties of this neutrophil subpopulation. We thus characterized it further. We observed neutrophils Ly6Gint to be smaller than neutrophils Ly6Ghi and displaying a lower granulosity (Figure 5D). To determine the specificities of this population, we studied the expression pattern of neutrophils and monocytes cell-surface markers in these cells at steady-state conditions (Figure 5E). Similar to CD11b+Ly6Ghi neutrophils, CD11b+Ly6Gint cells expressed low levels of the monocyte cell surface markers Ly6C and CCR2 (Figure 5E). They also expressed the CXCR4 chemokine receptor although at intermediate levels as compared to CD11b+Ly6Ghi neutrophils and inflammatory monocytes. However, they weakly express the chemokine receptor CXCR2 which is highly expressed on CD11b+Ly6Ghi neutrophils.

These results revealed that Ly6Gint cells display a neutrophil-like phenotype but with specific characteristics as compared to classical Ly6Ghi neutrophils, notably (i) smaller size and granulosity, (ii) higher viral-induced FcγRIV expression, and (iii) a pattern expression of cell surface markers characteristic of neutrophils (Ly6Clo, CCR2, CXCR4int) except for the lack of expression of the CXCR2 receptor. This suggests that CD11b+Ly6Gint cells are a phenotypic variant of neutrophils. However, whether CD11b+Ly6Gint cells represent a bona fide novel neutrophils subset or neutrophils displaying a different activation/maturation state could not be discriminated by these phenotypic properties as this will require integrative high-dimensional analyses including not only functional and phenotypic characterization but also deep genomic profiling of the neutrophil subpopulations identified (Ng et al., 2019).

FcγRIV upregulation in infected/treated mice is associated with enhanced expression of MHC-II and co-stimulatory molecules on neutrophils and monocytes. We next addressed in vivo the functional activation of splenic neutrophils and inflammatory monocytes in infected mice with or without immunotherapy at days 8 and 14 p.i. by monitoring cell surface activation markers. We were able to
show that the activation state of the cells evolved over time. At day 8 p.i., (Figure 6A) CD11b was upregulated on neutrophils and monocytes upon viral infection either in the absence or in the presence of immunotherapy. However, infected/treated mice showed a significantly higher CD86 upregulation in CD11b^+Ly6G^hi and CD11b^+Ly6G^int neutrophils than infected/non-treated mice. MHC-II molecule was also upregulated on CD11b^+Ly6G^int neutrophils and monocytes upon viral infection. At day 14 p.i. (Figure 6B), CD11b^+Ly6G^hi splenic neutrophils were similarly activated in infected/treated- and infected/non-treated mice, as deduced from an increased expression of CD11b and CD86. However, CD86 expression on CD11b^+Ly6G^int neutrophils was significantly increased in infected/treated mice, suggesting a mAb-mediated upregulation of these costimulatory molecules on this neutrophil subpopulation. Likewise, inflammatory monocytes from infected/treated mice also showed a significantly increased expression of CD86 co-stimulatory molecules as compared to infected/non-treated mice. Notably, MHC-II expression was significantly increased in infected/treated mice in both neutrophil populations as well as in monocytes as compared to infected/non-treated mice.

Consistent with a FcγRIV upregulation in neutrophils and monocytes in infected/treated mice (Figure 5C), these data demonstrate a mAb-mediated upregulation of MHC-II and costimulatory molecules in CD11b^+Ly6G^hi neutrophils and monocytes as well as a significant upregulation of MHC-II in CD11b^+Ly6G^int neutrophils at 14 days p.i. (Figure 6B).

*CD11b^+Ly6G^hi, CD11b^+Ly6G^int neutrophils and inflammatory monocytes are differentially and sequentially activated upon antiviral mAb treatment.* To further characterize the functional activation state of the two neutrophils populations (CD11b^+Ly6G^hi and CD11b^+Ly6G^int) and inflammatory monocytes upon viral infection and mAb-therapy, we next addressed the cytokine and chemokine secretion profile of splenic neutrophils and monocytes sorted from infected mice treated, or not, with the 667 mAb, at days 8 and 14 p.i. Cells sorted from age-matched naive mice were used as controls. We found that the secretion profile evolved over time and was cell type- and stimulus specific, with globally enhanced cytokine/chemokines secretion in cells isolated from infected/treated mice (Figure 7A and 7B), in particular at day 14 p.i.

At day 8 p.i., the CD11b^+Ly6G^hi neutrophils subpopulation from both infected/non-treated and infected/treated mice showed a broad chemokine and cytokine secretion profile as deduced from an increased secretion of most of the 13 chemokines and 12 cytokines assessed, although at weak levels (Figure 7B and 7C). CD11b^+Ly6G^int neutrophils showed enhanced expression of several chemokines mostly in cells isolated from infected/non-treated mice notably with a strong enhanced secretion of CCL3, CCL5 and CXCL5. In addition, CD11b^+Ly6G^int neutrophils also showed a slight secretion of multiples cytokines in infected/treated mice, but to a lesser extent than CD11b^+Ly6G^hi neutrophils.
(lower fold-increase) (Figure 7B and 7C). In contrast, infection and mAb-treatment hardly affected the chemokine/cytokine secretion of Ly6C<sup>hi</sup> monocytes. They only showed a weak increase in CXCL1 secretion in infected/treated mice as well as a slight increase in cytokines secretion (both in terms of diversity and fold increase) mainly in infected/non-treated mice (Figure 7B).

Interestingly, at day 14 p.i., the functional activation of the three different cell types assessed completely differed from that observed at day 8 p.i. (Figure 7B and 7C). We found a more restricted but stronger induction of chemokines/cytokines secretion, mostly in cells isolated from infected/treated mice. In contrast to the broad cytokines and chemokines secretion profile observed at day 8 p.i., CD11b<sup>+</sup>Ly6G<sup>hi</sup> neutrophils only secreted CCL4 and CCL5 chemokines and showed no cytokine secretion at day 14 p.i. In addition, CD11b<sup>+</sup>Ly6G<sup>int</sup> neutrophils showed a strong enhanced expression of 6 chemokines (CCL3, CCL4, CCL5, CCL22, CXCL9 and CXCL10) and 3 Th1-polarizing cytokines (IL-6, TNFα, IFNγ) in infected/treated mice. Ly6C<sup>hi</sup> monocytes showed a proinflammatory chemokine/cytokine secretion profile similar to CD11b<sup>+</sup>Ly6G<sup>int</sup> neutrophils, except for the secretion of CXCL9, that was specific to CD11b<sup>+</sup>Ly6G<sup>int</sup> neutrophils and CXCL1 that was only secreted by monocytes.

In summary, FrCasE infection and 667 mAb-treatment induce the secretion of multiple chemokines and cytokines by neutrophils and monocytes. Interestingly, the secretion profile evolves over time and differs among the three different cell types assessed, providing further evidence of a different functional activation state of the two neutrophils subpopulations (Ly6G<sup>hi</sup> and Ly6G<sup>int</sup>). In addition, these data show an enhanced secretion of Th1-polarizing cytokines and chemokines by cells isolated from infected/treated mice at 14 p.i., showing a role for the therapeutic mAb in the functional activation of these FcγRIV highly-expressing cells.
DISCUSSION

We have previously shown that neutrophils have a key immunomodulatory role in the induction of protective immunity by antiviral mAbs through the acquisition of B-cell helper functions (Naranjo-Gomez et al., 2018). Here we provide a new insight into both the heterogeneity and immunomodulatory role of neutrophils in a context of antiviral mAb-therapy. Our work shows that two functionally distinct neutrophil subpopulations can secrete multiple cytokines and chemokines able to recruit and activate multiple cells of the innate and the adaptive immune system. The cytokine/chemokine secretion profile: differs between both neutrophil subpopulations, is stimulus-dependent, evolves over time upon viral infection and mAb-treatment and suggests a potential cooperation with inflammatory monocytes in the induction of Th1 immune responses upon mAb-treatment of infected mice.

Our data show that neutrophils cytokine/chemokine secretion profile differs between viral versus bacterial stimulus. This is important to highlight as most of the studies investigating the immunomodulatory role of neutrophils have been conducted in bacterial infection models. Thus, in vitro stimulation of neutrophils by viral determinants led to a poor production of cytokines but to a wide and strong release of chemokine, with notably high secretion levels of the monocytes-and neutrophils-recruiting chemokines (CCL2, CXCL1, CXCL5) that was not observed upon LPS stimulation. On the contrary, LPS-stimulated neutrophils produced high amounts of proinflammatory cytokines but a narrower and weaker chemokine release. As for neutrophils, stimulation of monocytes by viral determinants (but not LPS) led to the secretion of high amounts of the neutrophils-recruiting chemokine CXCL1 and to a lesser extent the monocytes-recruiting chemokine CCL2. These results suggest a self-sustaining mechanism of neutrophils and monocytes recruitment upon viral infection, and raise the hypothesis of an early cooperation between neutrophils and monocytes in the modulation/induction of the antiviral immune response. This secretion profile of neutrophils induced by viral determinants is in agreement with an increased CCL2 release observed upon in vitro HIV stimulation of neutrophils (Yoshida et al., 2009). Increased levels of CCL2 and CXCL1 have also been reported in neutrophil- and monocytes-infiltrated tissues in different viral infections, (Hokeness et al., 2005; Miller et al., 2004; Rubio and Sanz-Rodriguez, 2007; Seo et al., 2011). However, chemokine increase was mostly assessed in total tissue extracts but not directly in neutrophils or monocytes isolated cells, which prevented the identification of the cell origin of chemokines. It is worth noting that a very early but transient expression has also been reported (i.e. 24-48h p.i.) of CCL2 and CXCL1 upon infection by RSV, CMV and influenza virus (Hokeness et al., 2005; Miller et al., 2004; Wareing et al., 2004). Such early and transient expression would be in agreement with the very low secretion of these chemokines detected at day 8 p.i. in infected mice treated, or not, with the therapeutic mAb.
Our data also highlight the effect of inflammatory conditions on the modulation of the phenotypic and functional activity of neutrophils and monocytes. Thus, in vitro priming with inflammatory/immunomodulatory cytokines clearly potentiated the release of several chemokines and cytokines by IC-activated neutrophils and monocytes (Figure 3) while having a less pronounced or no effect on virus-activated cells. This suggests that cytokine-priming associated to FcγR-triggering leads to enhanced immune responses. Although not formally shown, these results support a role for the inflammatory environment in the enhancement of chemokine/cytokine secretion by neutrophils and monocytes observed in infected-treated mice. Furthermore, our in vitro observations also allowed us to dissect the specific effect of IFN-γ, IFN-I and TNF-α on the enhancement of the functional properties of IC-activated neutrophils and monocytes. Notably, IFN-γ and IFN-I priming (but not TNF-α), led to the upregulation of FcγRs in neutrophils and/or monocytes, in agreement with previous reports in other experimental settings (Dahal et al., 2017; Lehmann et al., 2017; Morquin et al., 2017; Pricop et al., 2001). Consistent with these results, our in vivo work shows that the inflammatory environment resulting from ongoing infection and mAb-treatment also modulates FcγRs expression on neutrophils and monocytes (in particular Fcγ(RIV)). Thus, FcγRIV expression is more strongly enhanced in infected/non-treated mice at day 8 p.i. and in infected/treated mice at day 14 p.i., suggesting a different and evolving inflammatory environment in both groups of mice. These results are in agreement with previous work showing FcγRs modulation by the inflammatory conditions resulting from bacterial and IC-mediated autoimmune pathologies (Bruhns, 2012; Wang and Jönsson, 2019); and provide new evidence on the regulation of FcγRs expression in the specific inflammatory context of antiviral mAb-based immunotherapies, not hitherto reported. Notably, our work shows that the magnitude of FcγRIV upregulation upon viral infection and mAb-treatment is different in neutrophils and monocytes. This differential FcγRIV expression together with a different expression level of the Ly6G neutrophil cell surface marker (Ly6Ghi and Ly6Gint) contributed to the identification and characterization of two neutrophils subpopulation displaying distinct functional properties as deduced from their different (i) phenotype (size, granulosity, cell surface molecules) (Figures 5 and 6), (ii) magnitude of expression and upregulation of FcγRIV, costimulatory molecules and MHC-II (Figures 5 and 6) and (iii) cytokine/chemokine secretion profile (Figure 7) upon viral infection and mAb-treatment. Recent studies have shown neutrophils heterogeneity in healthy tissues as well as in different pathological conditions (Ng et al., 2019; Silvestre-Roig et al., 2019) through the identification of different neutrophils subpopulations. Such neutrophil heterogeneity is context-dependent as it is influenced by the nature and magnitude of the stimuli. In the particular context of infectious diseases, neutrophils heterogeneity is still poorly characterized and it has mostly been assessed in bacterial infections (Deniset et al., 2017; Tsuda et al., 2004). Similar to our observations Deniset and collaborators (Deniset et al., 2017) reported two distinct splenic neutrophils subpopulations displaying different
Ly6G expression levels (Ly6G\textsuperscript{hi} and Ly6G\textsuperscript{int}) in \textit{Streptococcus pneumoniae} infected mice. This study evidenced some phenotypic differences (cell surface markers of maturation, nuclear morphology, ...) between both neutrophil subpopulations but their functional activation was not assessed. Importantly, we describe here two phenotypic and functional variants of neutrophils displaying distinct immunomodulatory properties that might be key and complementary for inducing antiviral protective immunity. Our work provides thus novel findings on the heterogeneity and function of neutrophils upon viral infection and mAb-treatment.

Our work provides new mechanistic insights into the hitherto underestimated role of neutrophils as key cells in the modulation of adaptive antiviral immunity upon mAb treatment. Interestingly, at steady-state conditions, neutrophils express high levels of Fc\textgamma{RIV}. Its expression is higher than that observed in inflammatory monocytes. Thus, it is tempting to speculate that upon viral infection and mAb-treatment, the early neutrophils recruitment, together with their high expression of Fc\textgamma{RIV}, result in potent Fc-triggering by ICs in these cells. This might be key to initiate the modulation of immune responses through the production of multiple cytokines and chemokines able to recruit and activate multiple innate immune cells such as monocytes, NK cells, DC and neutrophils themselves (Tecchio and Cassatella, 2016). This suggest a potential role for neutrophils as early drivers of the induction of vaccinal effects by mAbs. Supporting this hypothesis, at day 8 p.i. both neutrophils populations showed a higher and a wider induction of chemokines and cytokines release than monocytes. Subsequently, the inflammatory environment resulting from viral infection and mAb-mediated antiviral effects induces the upregulation of Fc\textgamma{Rs} in the different innate immune cells that might further increase Fc-triggering by ICs leading to improved immune responses, both in terms of magnitude and quality. In agreement with this, the Fc\textgamma{RIV} upregulation in neutrophils and monocytes in infected/treated mice observed at 14 days p.i. is associated with (i) higher expression of costimulatory molecules and MHC-II and (ii) higher secretion of cytokines and chemokines. In keeping with this, enhanced secretion of Th1-polarizing cytokines (IL-6, TNF\textalpha{}, IFN\gamma{}) (Czarniecki, 1993; Hildenbrand et al., 2008; Jin et al., 2016; Uciechowski and Dempke, 2020; Wang et al., 2005) and chemokines (CXCL9 and CXCL10) (Gaylo-Moynihan et al., 2019; Kelly-Scumpia et al., 2010; Tannenbaum et al., 1998; Tokunaga et al., 2018) by CD11b\textsuperscript{+}Ly6G\textsuperscript{int} neutrophils and monocytes from infected/treated mice at 14 p.i., argues in favor of a mAb-mediated Th1 polarization of the antiviral immune response by these cells. Consistent with such Th1 polarization, we previously reported strong CD8 T-cells responses at day 14 p.i. in infected/treated mice (Gros et al., 2008; Michaud et al., 2010) as well as long-lasting protective humoral immune responses predominantly of the IgG2a isotype (Gros et al., 2005; Michaud et al., 2010; Naranjo-Gomez et al., 2018). Finally, it is also worth mentioning the upregulation of MHC-II molecules observed in CD11b\textsuperscript{+}Ly6G\textsuperscript{hi} and CD11b\textsuperscript{+}Ly6G\textsuperscript{int} neutrophils sorted from infected/treated mice. This observation broadens the immunomodulatory role...
of neutrophils upon mAb-treatment as it suggests that neutrophils might acquire antigen-presenting cell features and induce antigen-specific T-cells responses (Lok et al., 2019; Meinderts et al., 2019; Vono et al., 2017). In agreement with this, it has been shown that upon ex vivo stimulation with IgG immune complex (IC) or viral cognate antigens, neutrophils upregulated expression of MHCII and costimulatory molecules and increased T cell activation.

Our work show that mAb-mediated activation of neutrophils and monocytes might be essential to induce strong Th1-polarized early immune responses that might be key to induce protective immunity by mAb. In addition, the distinct secretion profile of Ly6Ghi and Ly6Gint neutrophils and Ly6Chi monocytes, in terms of type, amount and kinetics of chemokines/cytokines secretion, shows a differentially and sequentially activation of these FcγRIV-expressing cells upon antiviral mAb treatment and suggests their key and complementary action in the induction of protective immune responses by mAb. Thus, the identification of neutrophils as key players in the induction of protective immunity by antiviral mAbs and their potential collaboration with inflammatory monocytes might have therapeutic implications. These findings might help to improve mAb-based antiviral therapies by tailoring therapeutic interventions aiming at harnessing the immunomodulatory properties of these cells. To this end, different approaches could be envisaged: (i) the use of appropriate immunostimulatory host-directed therapies and (ii) the design of antiviral mAbs engineered to enhance their affinity for FcγRs expressed on human neutrophils and monocytes, such as FcγRIIa and FcγRIIIa (Bournazos et al., 2019; Chen et al., 2017; DiLillo and Ravetch, 2015). Thus, in addition to allow superior antibody-mediated phagocytosis, these Fc-engineered mAbs could also modulate cytokine/chemokine production to ultimately lead to more effective adaptive immune responses.
METHODS

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

Viral stocks: FrCas<sup>E</sup> (Portis et al., 1990) viral stocks were produced and stored as previously described in (Gros et al., 2005).

Viral infection and immunotherapy: Eight-day-old 129/Sv/Ev mice were infected by intraperitoneal (i.p.) administration with 50 μl of a viral suspension containing 50,000 focus-forming units (FFU) and treated, or not, with 30 μg 667 mAb targeting gp70 protein of viral envelope, (McAtee and Portis, 1985), 1-hour p.i. and on days 2 and 5 p.i. by i.p. administration. Mice were euthanized and spleens collected at days 8 and 14 p.i.

Phenotypical and functional activation of FcγRIV-expressing cell from spleen ex vivo:
Single-cell suspensions of splenocytes were obtained from naive, infected/non-treated and infected/treated mice at 8 and 14 days p.i. and immunotherapy. Spleen cell suspensions were obtained by mechanical dissociation in PBS, then filtered in 0.70 μm strainer. 20 % of each spleen was used for immunophenotyping by FACS, 80% left was dedicated to main FcγRIV-expressing cell sorting. Red blood cells were lysed (ACK, Lonza) and an enrichment with biotinylated anti-B220 (BD Biosciences), anti-CD3 (BD Biosciences), following of anti-biotin Ab magnet-bead coupled (Miltenyi) and magnetic LS-columns (Miltenyi) was performed to remove spleen lymphocytes and increase the sorting efficacy. Cells were stained with specific marker of populations of interest (Ly6G- BD Biosciences), anti-CD3 (BD Biosciences), anti-CD11b (BD Biosciences) and neutrophils (Ly6G<sup>hi</sup> and L6G<sup>int</sup>) and inflammatory monocytes (Ly6C<sup>hi</sup>) were sorted (>97-98% pure) using the BD Biosciences FACS Aria device. Sorted cells were cultured in 96-well plates at a density of 4x10<sup>6</sup> cells/ml (1x10<sup>6</sup> cells/well) in 10% FBS-containing RPMI medium for 24h, and cell-free supernatants were collected and stored at -20°C, to allow cytokines and chemokines protein release quantification.

Flow cytometry: Organs of interest were collected to realize immunophenotyping of immune cells. Spleen cell suspensions were obtained by mechanical dissociation in PBS. BM cell suspensions were obtained by dissection and PBS-2%-FBS flushing of tibias and femurs. Cells were stained at 4°C using fluorochrome-conjugated antibodies against CCR2 (SA203G11, BioLegend), CD11b (M1/70, eBioscience), CD11c (HL3, BD Biosciences), CD16/32 (93, BD Biosciences), CD45.2 (104, BD Biosciences), CD62L (MEL-14, BD Biosciences), CD64 (x54-5/7.1, BD Biosciences), CD86 (GL1, BD Biosciences), CXCR2 (SA044G4, BioLegend), CXCR4 (2B11, Life Technologies), Ly6G (1A8, which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission. bioRxiv preprint doi: https://doi.org/10.1101/2020.04.22.055533; this version posted April 24, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.
BD Biosciences), Ly6C (AL-21, BioLegend) and Siglec F (E50-2440, BD Pharmingen). FcγRIV expression was determined using 9E9 antibody (kindly provided by Dr. Pierre Bruhns, Institut Pasteur), produced by BioXcell, and then labeled with Alexa Fluor 647. FrCasE-infected cells were assayed using an anti-Gag mAb (H34) (Chesebro et al., 1981; kindly provided by Pr. Ulf Dittmer, University of Duisburg-Essen) labeled with Alexa Fluor 647 or incubated with an anti-IgG2b labeled PE. 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 FACS LSR Fortessa (BD Bioscience), and the data were analyzed using the FlowJo software.

**Neutrophils and monocytes isolated from BM used in in vitro experiments**: Neutrophils and monocytes were purified from 8 to 11-week-old naive mice BM. After dissection of lower limbs, BM cell suspensions were collected by PBS-2% FBS EDTA (2 mM) flushing (25G needle) of tibias and femurs. BM cell suspensions were filtered with a 0.40 µm nylon strainer. Two magnetic-based cell sorting (MACS) isolation kits were used to purified either neutrophils (Miltenyi Biotec) and monocytes, (Miltenyi Biotec), both with high purity (>97-98%), determined by FACS (LSR Fortessa, BD Bioscience). Cells were placed in culture in U bottom 96-well plates at a concentration of 1 million/ml in 10% FBS-containing RPMI medium.

**Stimulation of neutrophils and monocytes in vitro**: Purified neutrophils and monocytes were seeded at 150 000 cells/well in 150 µl of RPMI, then incubated for 24h with LPS (1µg/ml, Sigma), or FrCasE virus (MOI 5: 5 viral particles /cell), or ICs (virus FrCasE and 1µg 667 mAb), or 1µg 667 mAb alone. In parallel, the same experiments were performed adding inflammatory cytokines, TNFα 100 UI/ml (Peprotech), IFN γ 100 UI/ml (eBioScience), IFNα11 a 1000 UI/ml, produced and generously provided by Dr. Gilles Uzé (DIMP, CNRS). After 24h of stimulation, supernatants were collected and stored at -20ºC to quantify chemokines and cytokines protein release secretion (describe below). Phenotypical activation of both neutrophils and monocytes was measured using surface markers by flow cytometry.

**Chemokines and cytokines protein release quantification**: Soluble chemokines and cytokines secretion were quantified from cell-free collected supernatants of in vitro cultured neutrophils and monocytes and of sorted splenic neutrophils Ly6Ghi and Ly6Gint, and inflammatory monocytes (of naive, infected/non-treated and infected/treated mice 8 and 14 days p.i. and immunotherapy), using bead-based immunoassays (LegendPLex, BioLegend) and analyzed on the BD Bioscience-LSR Fortessa device. The protein release quantification was established by the appropriate software (LEGENDplex™ data analysis).
**Statistics:** Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). Data were expressed as mean ± SEM, and statistical significance was established using a parametric 1-way ANOVA test or non-parametric Kruskal-Wallis test for multiple comparisons or paired Student’s t tests when two groups were compared. P values lower than 0.05 were considered as statistically significant.

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

**AUTHOR CONTRIBUTIONS**

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

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**CONFLICT OF INTEREST DISCLOSURES**

The authors declare no competing financial interests.
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FIGURE LEGENDS

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

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

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

Figure 4: FcγRIV is upregulated by IFN priming on both neutrophils and monocytes cell surface. Neutrophils and monocytes were isolated from naive mice and stimulated as in Figure 3. FcγRIV expression was evaluated on neutrophils (A, B) and monocytes (C, D) in absence (A, C) or in the presence of TNFα, IFN-1 or IFNγ (B, D). The data represent 5 independent experiments for neutrophils (A, B) and 3 independent experiments for monocytes (C, D). Data are expressed as means +/- SEM. Diamonds indicate significant differences to the corresponding stimuli in the absence of cytokine priming (open diamond) or to corresponding medium without virus or IC stimuli (grey diamond) as determined by Kruskal-Wallis tests (p < 0.05).

Figure 5: FcγRIV is upregulated in vivo on two different splenic neutrophils subpopulations and on inflammatory monocytes. Splenocytes from naive (grey), infected/non-treated (I/NT; red) and infected/treated (I/T; blue) mice were analyzed on days 8 (D8) and 14 (D14) p.i. for (i) neutrophils and monocytes recruitment and (ii) for FcγRIV expression. (A) Gating strategy used to define neutrophils and monocytes populations. (B) Frequencies of CD11b^hiLy6G^hi and CD11b^hiLy6G^int neutrophils subpopulations as well as Ly6C^hi monocytes in the CD45.2^+ leukocytic population. The data represent 5 independent experiments at D8 p.i. and 6 independent experiments at D14 p.i with at least 6-9 mice per group (I/NT and I/T) and 3-5 mice per group (naive mice). (C-D) FcγRIV expression on CD11b^hiLy6G^hi and CD11b^hiLy6G^int neutrophils subpopulations and Ly6C^hi monocytes. The data represent 5 independent experiments at D8 p.i. and 6 independent experiments at D14 p.i with at least 6-9 mice per group (I/NT and I/T) and 3-5 mice per group (naive mice). (E) Cell surface markers expression of CD11b^hiLy6G^hi and CD11b^hiLy6G^int neutrophils and Ly6C^hi monocytes isolated from 15-days old naive mice, with 3-5 mice per group. Data are expressed as means +/- SEM. Statistical significance was established using a parametric 1-way ANOVA test (*p < 0.05; **p < 0.01; ***p < 0.001).

Figure 6: Cell surface activation markers expression on two different splenic neutrophils subpopulations and on inflammatory monocytes. Splenocytes from naive (grey), infected/non-treated (I/NT; red) and infected/treated (I/T; blue) mice were analyzed on days 8 (A) and 14 p.i. (B) for neutrophils and monocytes expression of CD11b, CD86 and MHC-II. Data represent 5 independent experiments at D8 p.i. and 6 independent experiments at D14 p.i with at least 6-9 mice per group (I/NT and I/T) and 3-5 mice per group (naive mice). Data are expressed as means +/- SEM. Statistical
significance was established using a parametric 1-way ANOVA test (*p < 0.05; **p < 0.01; ***p < 0.001).

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

**SUPPLEMENTAL FIGURE LEGENDS**

**Supplemental Figure 1.** Functional activation of neutrophils stimulated with viral determinants or LPS. BM-derived neutrophils were isolated from naive mice and stimulated for 24h in vitro with viral determinants (virus or ICs) or LPS (1µg/ml). A. Cytokine secretion profile of neutrophils stimulated by virus (red) or viral ICs (blue) or unstimulated (grey). Cytokine release was assessed in supernatants of neutrophils isolated from BM of naive mice (>97-98% purity). Data represent 5 independent experiments and are expressed as means +/- SEM. Statistical significance was established using a parametric 1-way ANOVA test (*p < 0.05; **p < 0.01; ***p < 0.001). B-D. Functional activation of neutrophils stimulated by LPS. Activation was assessed by monitoring CD11b expression and frequency of CD11b^hi CD62L^- neutrophils (B) as well as the cytokines (C) and chemokines (D) released in supernatants of neutrophils stimulated for 24 h by LPS (green) or left unstimulated (grey). Data represent 12 independent experiments and are expressed as means +/- SEM. Statistical significance was established using a paired Student’s t test (*p < 0.05; **p < 0.01; ***p < 0.001).

**Supplemental Figure 2:** Functional activation of monocytes stimulated with viral determinants or LPS. BM-derived monocytes were isolated from naive mice and stimulated for 24h in vitro with viral determinants (virus or IC) or LPS (1µg/ml). A. Cytokine secretion profile of monocytes...
stimulated by virus (red) or viral ICs (blue) or unstimulated (grey). Cytokine release was assessed in supernatants of monocytes isolated from BM of naive mice (>97-98% purity) and stimulated for 24 h. The data represent 6 independent experiments and are expressed as means +/- SEM. Statistical significance was established using a parametric 1-way ANOVA test (*p < 0.05; **p < 0.01; ***p < 0.001).

**B-D. Functional activation of monocytes stimulated by LPS.** Activation was assessed by monitoring the CD86 expression level (B) as well as the cytokines (C) and chemokines (D) release monitored in supernatants of monocytes stimulated for 24 h by LPS (green) or left unstimulated (grey). Data represent 5 independent experiments and are expressed as means +/- SEM. Statistical significance was established using a paired Student’s t test (*p < 0.05; **p < 0.01; ***p < 0.001).

**Supplemental Figure 3.** Cytokine priming enhance chemokine/cytokine release by IC-activated neutrophils and monocytes. BM-derived neutrophils and monocytes were isolated from naive mice and activated as in **Figures 1 and 2** in the presence, or in the absence, of TNFα, IFN-I or IFNγ. Chemokines and cytokines release were assessed in supernatants of activated neutrophils (A) and monocytes (B). Only those chemokines/cytokines significantly enhanced by cytokine priming are depicted. The data represent 4 independent experiments for A and 3 independent experiments for B. Diamonds indicate significant differences to all the other stimulation conditions (black diamond), or to corresponding medium without virus or ICs stimuli (grey diamond), or to the corresponding stimuli in the absence of cytokine priming (open diamond), as determined by Kruskal Wallis test (p < 0.05).

**Supplemental Figure 4: FcγRI and FcγRII-III in vitro expression on neutrophils and monocytes stimulated with viral determinants, in the presence and the absence of TNFα, IFN-I or IFNγ.** FcγRII-III and FcγRI expression was monitored on neutrophils (A) and monocytes (B). No FcγRI expression was detected on neutrophils. The data represent 6 independent experiments for A et 4 independent experiments for B. Data are expressed as means +/- SEM. Diamonds indicate significant differences from the corresponding stimuli in the absence of cytokine priming (open diamond), or from all the other stimulation conditions (black diamond) or from corresponding medium without virus or ICs stimuli (grey diamond) as determined by Kruskal Wallis test (p < 0.05).

**Supplemental Figure 5: FcγRI and FcγRII-III in vivo expression on two different splenic neutrophils subpopulations and on inflammatory monocytes.** Splenocytes from naïve (grey, infected/non-treated I/NT;red) and infected/treated (I/T; blue) mice were analyzed on days 8 (A) and 14 p.i. (B) for FcγRII-III and FcγRI expression on CD11b<sup>+</sup>Ly6G<sup>+</sup> and CD11b<sup>+</sup>Ly6G<sup>-</sup> neutrophils subpopulations and Ly6C<sup>hi</sup> monocytes. Data represent 5 independent experiments at D8 p.i. and 6 independent experiments at D14 p.i with at least 6-9 mice per group (I/NT and I/T) and 3-5 mice per
group (naive mice). Data are expressed as means +/- SEM. Statistical significance was established using a parametric 1-way ANOVA test (*p < 0.05; **p < 0.01; ***p < 0.001).
129/Sv/Ev PMN from mice BM 24 h stimulation 24 h stimulation Phenotypical and Functional activation of PMN

CD11b (MFI)

FrCas\textsuperscript{E} IC

CD11b

CD62L

M V IC 667

CD11b (MFI)

M V IC 667

CD62L

M V IC 667

M V IC 667

Concentration (pg/ml)

CCL2 CCL3 CCL4 CCL5 CCL11 CCL17 CCL20 CCL22 CXCL1 CXCL5 CXCL9 CXCL10 CXCL13

Figure 1
Figure 2

A

129/Sv/Ev
Monocytes from mice
BM
FrCasE
IC
24 h stimulation
Phenotypical and Functional activation of Monocytes

B

CD86 (MFI)

M
V
IC
667

C

Concentration (pg/ml)

CCL2 CCL3 CCL4 CCL5 CCL11 CCL20 CCL22 CXCL1 CXCL5 CXCL9 CXCL10 CXCL13

M V IC
Figure 3

**Neutrophil**

- **A**
  - CD11b (MFI) for TNFα +, IFNα +, and IFNγ +

- **B**
  - pg/ml for TNFα, IFNα, and IFNγ

**Monocyte**

- **C**
  - CD86 (MFI) for TNFα +, IFNα +, and IFNγ +

- **D**
  - pg/ml for TNFα, IFNα, and IFNγ

Legends:
- CCL4
- CCL5
- CXCL1
- CXCL10
- TNFα
- IFNα
- IFNγ

Note: This figure shows the cytokine expression and cell surface marker levels in neutrophils and monocytes treated with various cytokines.
**Figure 4**

A graph showing the expression of FcγRIV (MFI) on neutrophils in response to different stimuli.

- **A**: Graph showing FcγRIV expression on neutrophils treated with TNFα, IFNα, and IFNγ.
- **B**: Graph showing FcγRIV expression on neutrophils treated with NS and various cytokines.
- **C**: Graph showing FcγRIV expression on monocytes treated with TNFα, IFNα, and IFNγ.
- **D**: Graph showing FcγRIV expression on monocytes treated with NS and various cytokines.

The graphs are labeled with specific treatments and show FcγRIV expression levels as a function of M, V, IC, and 667.
Figure 6

A

D8

CD11b (MFI)

**

Ly6G<sup>hi</sup> Neutrophil

Ly6G<sup>int</sup> Neutrophil

Ly6C<sup>hi</sup> Monocyte

CD86 (MFI)

***

MHCII (MFI)

***

B

D14

CD11b (MFI)

**

Ly6G<sup>hi</sup> Neutrophil

Ly6G<sup>int</sup> Neutrophil

Ly6C<sup>hi</sup> Monocyte

CD86 (MFI)

*

MHCII (MFI)

***

*
Figure 7