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Limitations of neutrophil depletion by anti-Ly6G antibodies in two heterogenic immunological models

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ABSTRACT

Neutrophil-depleting antibodies, such as anti-GR1 (RB6-8C5) and anti-Ly6G (1A8), are commonly used to study the *in vivo* function of neutrophils in murine disease models. Anti-Ly6G antibodies became the standard, because in contrast to anti-GR1, these do not bind Ly6C. The efficiency of the depletion needs to be carefully analysed as flow cytometry plots may be misinterpreted. For example, the staining intensity of GR1 on neutrophils ($CD11b^+$ $GR1^{hi}$) drops upon anti-Ly6G administration. We show that this drop is due to competition between anti-GR1 and anti-Ly6G antibodies. Neutrophil depletion with anti-Ly6G in naive mice was organ- and strain-specific. Furthermore, an incomplete anti-Ly6G-dependent neutrophil depletion was obtained in two immune-mediated mouse models, *i.e.* in malaria-infected C57BL/6 mice and in complete Freund's adjuvant (CFA)-challenged BALB/c mice. BrdU-incorporation studies show a slight increase in proliferating bone marrow neutrophils upon depletion in naive mice. Strikingly, depletion with anti-Ly6G in CFA-challenged BALB/c mice resulted in a significant increase in proliferating splenic neutrophils, causing a fast rebound of new immature neutrophils. In conclusion, our results emphasize the importance of careful panel design, gating strategies and duration of neutrophil depletion and highlight the context-dependent Ly6G depletion efficiency. It furthermore underlines the need for new tools to understand the *in vivo* role of neutrophils in immunological models.

1. Introduction

Neutrophils are the most abundant white blood cells in the circulation. These short-lived cells have the capacity to infiltrate into sites of inflammation and are rapidly replenished in adult bone marrow pools by myelopoiesis [1]. In mice, neutrophils are defined as $CD11b^+$ $Ly6G^+$ $Ly6C^{int}$ cells and have a variety of functions [2–4]. Many disease models are characterised by an expansion of neutrophil numbers in the circulation and/or in different organs, where they are considered as important drivers of immune responses and critical players in the resolution process. Nevertheless, their exact role is often poorly understood. One way to study the *in vivo* function of neutrophils is by using neutrophil-depleting antibodies, which can be administered at different time points during the disease process. Two different antibodies are commonly used for this approach in mice: anti-Ly6G (clone 1A8) and anti-GR1 (clone RB6-8C5). Ly6G expression is restricted to neutrophils and is involved in recruitment and transmigration. Ly6C, another member of the Ly6 family, is expressed on neutrophils and other leukocyte populations, and plays a role in the adhesion to the endothelium [4,5]. The anti-GR1 antibodies bind both to Ly6G and

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CFA, complete Freund's adjuvants; i.p., intraperitoneally; MA-ARDS, malaria-associated acute respiratory distress syndrome; *Pb*NK65, *Plasmodium berghei* NK65; p.i., post infection; RBCs, red blood cell; s.c., subcutaneously; SPF, specific pathogen-free

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Ly6C and may thereby also deplete subpopulations of dendritic cells, monocytes, macrophages and lymphocytes [5,6]. Since the anti-Ly6G antibodies are more specific for neutrophils compared to the anti-GR1 antibodies, they became the standard approach for neutrophil depletion experiments.

In tumour biology, different concerns have been raised in using neutrophil-depleting antibodies. In an editorial by Ma and Greten, the authors stressed the importance of properly checking the depletion efficacy [7]. These authors underlined the fact that neutrophil depletion efficacy may be misinterpreted by the method, timing and location of neutrophil depletion analysis. However, despite the release of this editorial, Ly6G-depleting antibodies are still widely used, which may be related to the lack of appropiate alternatives. Here, we describe some experimental pitfalls of Ly6G-depleting antibodies in two immunological models to warn for misinterpretations.

The first model is a mouse model to study malaria-associated acute respiratory distress syndrome (MA-ARDS). Thereby, C57BL/6 mice are infected with the *Plasmodium berghei* NK65-Edinburgh line (*Pb*NK65) [8], resulting in the development of experimental MA-ARDS at day 9 post infection (p.i.). The pathology in these mice resembles the histopathological findings in MA-ARDS patients [9]. MA-ARDS pathology in both patients and mice is characterised by severe interstitial and alveolar edema and a vast infiltration of mononuclear leukocytes and neutrophils [10]. In this mouse model, $CD8^+$ T cells were shown to be essential for the pathogenesis [11], but the role of neutrophils remains unclear. In another model for malaria-associated acute lung injury (*i.e.* the infection of DBA/2 mice with *P. berghei* ANKA), neutrophils were suggested to play an essential role in the pathogenesis based on depletion experiments with the less specific anti-GR1 antibody [12].

The second model is based on the injection of complete Freund's adjuvants (CFA) in BALB/c mice [13]. CFA consists of an oil emulsion containing heat-killed mycobacteria [14]. It is the most commonly used immunoadjuvant in animal models for the production of (auto)antibodies and for the induction of experimental autoimmune diseases [14,15]. A single subcutaneous (s.c.) injection of CFA is sufficient to induce a local and systemic inflammation in the mice. The mycobacterial component of CFA elicits myelopoiesis in both bone marrow and in spleen (designated as extramedullary myelopoiesis) and is associated with neutrophilia [14,15]. This model is used to study activated hematopoiesis or myelopoiesis [13].

In this research letter, we show incomplete neutrophil depletion in two heterogenic immunological models. We also report organ-specific and genetic background-related differences in the efficacy of neutrophil depletion with anti-Ly6G, and demonstrate the importance of adequately verifying the neutrophil depletion efficacy.

2. Materials and methods

2.1. Mice and cell isolation

Seven to eight weeks old C57BL/6 mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and housed in a conventional or specific pathogen-free (SPF) facility. Six to eight weeks old SPF BALB/c mice were bred in the animal house of the Rega Institute for Medical Research, KU Leuven. All mice were housed in individually ventilated cages. All experiments were performed at the KU Leuven according to the regulations of the European Union (directive 2010/63/EU) and the Belgian Royal Decree of 29 May 2013, and were approved by the Animal Ethics Committee of the KU Leuven (License LA1210186, Belgium). Murine blood samples were obtained by cardiac punction in heparinized (LEO, Pharma, Lier, Belgium) syringes followed by transcardial perfusion. Ciculating leukocytes were obtained from blood after lysis of red blood cells (RBC) with 0.83% ammoniumchloride (NH_4Cl) in 10 mM Tris (pH 7.2). Bone marrow was extracted from the tibia of one of the hind legs. Single cell leukocytes of the lungs were obtained using the gentle MACS dissociator (Miltenyi Biotec, Leiden, The Netherlands) followed by a collagenase D (2 mg/ml, Roche, Mannheim, Germany) and DNase (40 U/ml, Roche) digest for 30 min at 37 °C in HEPES buffer. Next, pulmonary leukocytes were purified using density gradient centrifugation with 40% and 72% Percoll solutions (GE healthcare, Upsala, Sweden). Spleens were passed through a cell strainer (70 µm) and RBCs were lysed with 0.83% NH_4Cl. Cell numbers were manually counted with a Bürker chamber upon staining with trypan-blue (VWR, Heverlee, Belgium) to exclude dead cells.

2.2. Antibody competition test

To test competition between anti-Ly6G and anti-GR1, splenocytes from C57BL/6 mice were incubated with or without non-labeled anti-Ly6G (200 µg/ml, corresponding to depletion concentration, clone 1A8, Bio X cell, Huisen, The Netherlands) prior to staining with fluorescently labeled anti-GR1 (clone RB6-8C5, Biolegend, London, UK). In parallel, competition between fluorescently labeled anti-Ly6C (2.4 µg/ml, concentration as used for Ly6C staining, clone AL-21, BD Biosciences, Erembodegem, Belgium) and anti-GR1 was tested in the same manner.

2.3. Ly6G depletion in naive mice

Naive C57BL/6 and BALB/c mice were intraperitoneally (i.p.) injected with 200 or 500 µg of anti-Ly6G (clone 1A8, Bio X cell) or with IgG2a isotype control (anti-β-Gal, clone GL117). Mice were euthanised two days later.

2.4. Ly6G depletion in the PbNK65 infection-model

Mice were infected with *Pb*NK65 by i.p. injection of 10^4 infected RBCs as described previously [16]. Infected C57BL/6 mice received *ad libitum* high-energy food (Ssniff Spezialdiäte GMBH, Soest, Germany) and water, which was supplemented with 0.422 mg/ml 4-amino-benzoic acid sodium (PABA) (Sigma-Aldrich, Bornem, Belgium) for optimal parasite growth. On day 9 p.i., mice were euthanised. Age- and sex-matched non-infected littermates were included as controls. Mice were i.p. treated with 200 µg or 500 µg of anti-Ly6G (clone 1A8, Bio X cell) or with an IgG2a isotype control (anti-β-Gal, clone GL117) on days -1, 3 and 7 after infection.

2.5. Ly6G depletion in the CFA-based immunisation model

BALB/c mice were s.c. injected with CFA (Difco, Franklin Lakes, NJ, USA) together with heat-killed mycobacteria (1.5 mg/ml) as previously described [13]. Mice were euthanised and analysed on day 21 post CFA-challenge. Age- and sex-matched non-injected littermates were included as controls. Mice were treated i.p. with 250 µg or 600 µg of anti-Ly6G (clone 1A8, Bio X cell) or with an IgG2a isotype control (anti-β-Gal, clone GL117) on day 14 and 17 post CFA-challenge (unless specified otherwise).

2.6. Flow cytometry

Cells were incubated with FcR-block (Miltenyi Biotec) and stained extracellularly in Brilliant stain buffer (BD Biosciences). The following mouse antibodies were used in this study: anti-CD45 (clone 30-F11, Biolegend), anti-Ly6C (clone AL-21, BD Biosciences), anti-CD11b (clone M1/70, eBioscience, San Diego, CA, USA), anti-GR1 (clone RB6-8C5, Biolegend), anti-CD3 (clone 17A2, Biolegend), anti-CD19 (clone 6D5, Biolegend), anti-NK1.1 (clone PK136, Biolegend) and anti-CXCR2 (clone SA045E1, Biolegend). Forward and side scatter analysis was used to limit debris and doublets. All leukocyte populations were gated on $CD45^+$ live cells. Cells positive for lineage-specific markers were excluded (with CD3, CD19 and NK1.1 as exclusion markers). Neutrophils were gated as specified. Cells were run on a LSRFortessa X20 (BD Biosciences) equipped with the DIVA software and analysed with

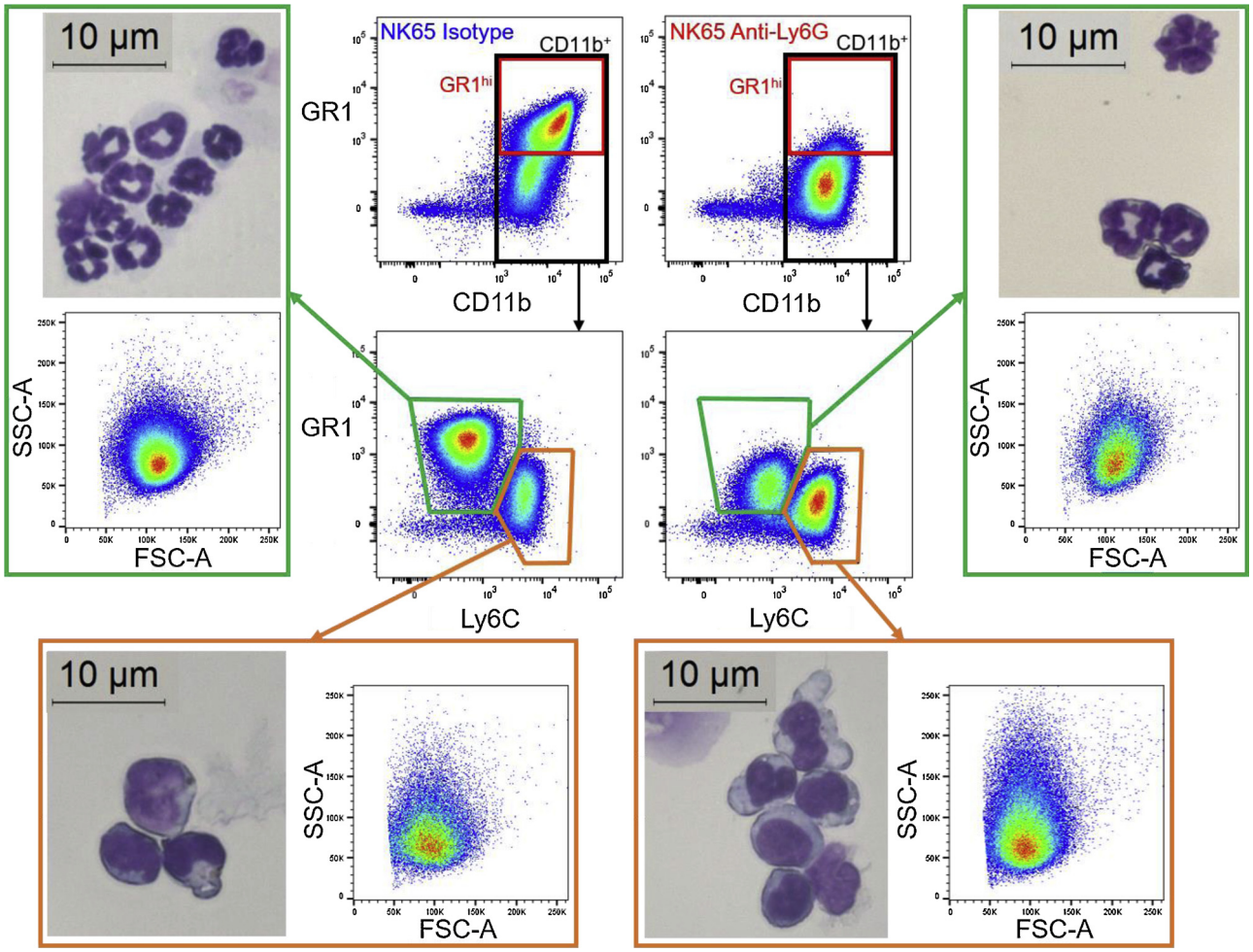

Fig. 1. Gating strategy used to evaluate the efficiency of neutrophil depletion.
Representative flow cytometry plots show the gating strategy of neutrophils from pulmonary leukocytes of *Pb*NK65-infected C57BL/6 mice that were either i.p. injected on day -1, 3 and 7 p.i. with 500 µg isotype control (IgG2a) or 500 µg anti-Ly6G (1A8). First, live $CD45^+$ $CD11b^+$ cells were gated (black gate). Neutrophils were gated as $GR1^{int/hi}$ $Ly6C^{int}$ (green gate). Monocytes were gated as $Ly6C^{hi}$ (orange gate). Both were gated after exclusion of $CD3^+$, $CD19^+$ or $NK1.1^+$ cells. Red gate represents live $CD45^+$ $CD11b^+$ $GR1^{hi}$ cells. Gated $GR1^{int/hi}$ $Ly6C^{int}$ and $Ly6C^{hi}$ cells were sorted and representative microscopic pictures and FSC/SSC plots were shown. The graphs show data from one representative sample out of 2 independent experiments.

FlowJo software (LLC, V10, Ashland, OR, USA).

2.7. Fluorescence-activated cell sorting of gated neutrophils and inflammatory monocytes

*Pb*NK65-infected C57BL/6 mice were i.p. injected with 500 µg anti-Ly6G (clone 1A8) or isotype control (anti-β-Gal, clone GL117) on days -1, 3 and 7 after infection. On day 9 p.i., mice were sacrificed and lung cells were isolated. The pulmonary leukocytes were stained as described above with anti-CD45 (clone 30-F11, Biolegend), anti-Ly6C (clone HK1.4, eBioscience), anti-GR1 (clone RB6-8C5, eBioscience), anti-CD3 (clone 17A2, Biolegend), anti-CD19 (clobe 6D5, Biolegend) and anti-NK1.1 (clone PK136, eBioscience). Neutrophils and inflammatory monocytes were sorted on a BD Melody (BD Biosciences) equipped with the BD FACS Chorus software 1.0 according to the gating as shown in Fig. 1. Cytospins were prepared from the sorted cells using the Shandon cytospin 2 (Thermo Fischer Scientific, Aalst, Belgium). After the slides were air-dried, they were stained with Hemacolor (Merck, Darmstadt, Germany). Pictures were made under a 40x magnification using a Leica DM2000 LED microscope (Leica microsystems, Diegem, Belgium).

2.8. BrdU incorporation assay

One day before analysis, mice received a single i.p. injection of 1.5 mg 5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich). BrdU positive cells were visualised with the BrdU flow kit from BD as described by the manufacturer's protocol. In brief, cells were extracellularly stained, fixed and permeabilized. To detect incorporated BrdU, cells were treated with DNase (300 µg/ml) and stained with a PE-labeled anti-BrdU antibody (BD Biosciences).

2.9. Statistical analysis

GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. The non-parametric Kruskal-Wallis test followed by Dunn's nonparametric comparison was used to calculate significance levels for the differences between groups. Significance levels were indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Non-siginificant differences were not indicated.

3. Results

3.1. Optimizing a gating strategy to evaluate the efficiency of neutrophil depletion after injection with anti-Ly6G (clone 1A8)

In this study, flow cytometry was used to determine the efficiency of depletion in naive mice and two murine immunological models. For instance, in the lungs of *Pb*NK65-infected C57BL/6 mice, anti-Ly6G depletion resulted in the apparent disappearance of neutrophils, when defined as $CD11b^+$ $GR1^{hi}$ double positive (Fig. 1, upper dot plots, red gate). However, GR1/Ly6C plots of the $CD11b^+$ subset clearly showed that neutrophils ($GR1^{int/hi}$ $Ly6C^{int}$) were not fully depleted but that instead, the GR1 intensity decreased (Fig. 1, lower dot plots, green gate). Therefore, determination of depletion efficiency in GR1/CD11b plots results in wrong conclusions, since the neutrophil population is masked by the $Ly6C^{hi}$ inflammatory monocytes ($CD11b^+$ $Ly6C^{hi}$) upon anti-Ly6G depletion. The drop in GR1 intensity was independent of the dosage used (200 µg or 500 µg, data not shown) and is most likely explained by competition between staining anti-GR1 and depleting anti-Ly6G antibodies. In an *in vitro* competition experiment, anti-Ly6G at 200 µg/ml, which approximates the concentration that might be reached in an *in vivo* depletion experiment, clearly competed for the binding of the detection anti-GR1 antibody (Suppl. Fig. 1). Furthermore, a parallel competition experiment showed that the anti-Ly6C antibody, at 2.4 µg/ml used for detection of Ly6C in our gating strategy, does not compete with the detection anti-GR1 antibody. Therefore, to enumerate the neutrophils in a correct way, these were gated as $GR1^{int/hi}$ $Ly6C^{int}$ $CD11b^+$ cells. Microscopy analysis of sorted $GR1^{int/hi}$ $Ly6C^{int}$ $CD11b^+$ and $Ly6C^{hi}$ $CD11b^+$ cells, confirmed that these cells are, respectively, neutrophils and inflammatory monocytes (Fig. 1). In contrast to other studies, forward scatter (FSC) and side scatter (SSC) could not be used upon *Pb*NK65 infection to assess the depletion efficacy, as the inflammatory monocytes and neutrophils overlap in the FSC/SSC plot (Fig. 1) [17].

3.2. Depletion efficacy in naive BALB/c and C57BL/6 mice

In naive C57BL/6 and BALB/c mice, the efficacy of neutrophil depletion after anti-Ly6G injection was evaluated in different organs, including lungs, spleen, blood and bone marrow. Depletion with 200 µg of anti-Ly6G resulted in a reduction of pulmonary neutrophils of ± 43% in C57BL/6 mice and ± 96% in BALB/c mice (Fig. 2A). Comparable results were found in the spleen: a reduction of ± 67% and ± 96% in C57BL/6 and BALB/c mice, respectively, upon depletion with 200 µg of anti-Ly6G (Fig. 2B). Depletion of neutrophils with 200 µg anti-Ly6G was efficient in blood, with a reduction of ± 87% and ± 95% in C57BL/6 and BALB/c mice, respectively, after depletion with 200 µg anti-Ly6G (Fig. 2C). Similar depletion efficacies were found after injection of 500 µg anti-Ly6G in all three organs (Fig. 2A–C). The decrease in neutrophils after depletion with 200 µg or 500 µg anti-Ly6G was significant in all three organs in both C57BL/6 and BALB/c mice, with only a trend in lungs ($p = 0.0540$) and blood ($p = 0.0669$) after depletion with 500 µg anti-Ly6G in C57BL/6 mice. In contrast, neutrophil depletion in the bone marrow was incomplete, since the number of neutrophils was at best decreased with ± 33% in C57BL/6 mice and ± 66% in BALB/c mice (Fig. 2D). Interestingly, in spleen, blood and lungs, the depletion appeared to be less efficient for $CXCR2^-$ neutrophils (Suppl. Fig. 2). In all four organs, we observed a higher GR1 expression on neutrophils of BALB/c mice compared to C57BL/6 mice (Fig. 2E–H), despite a similar Ly6C expression (data not shown). This suggests that the lower percentual depletion efficiency in C57BL/6 mice *versus* BALB/c mice might be related to a lower Ly6G expression on the neutrophils or due to lower number of neutrophils in the different organs of naive C57BL/6 mice.

3.3. Incomplete depletion of neutrophils in both PbNK65-infected C57BL/6 mice and CFA-challenged BALB/c mice

Using the above described gating procedure, we quantified the absolute number of neutrophils in the target organs of two immunological models and calculated the neutrophil depletion efficiency of the anti-Ly6G antibody. Infection of C57BL/6 mice with *Pb*NK65 resulted in an increased number of neutrophils in the lungs (Fig. 3A). Challenging BALB/c mice with CFA caused increased neutrophil numbers in the spleen, a consequence of myelopoiesis as described before (Fig. 3B) [14,15]. Depletion of neutrophils was incomplete and independent of anti-Ly6G dosage. Upon administration of 200 or 500 µg anti-Ly6G on days -1, 3 and 7 p.i. in *Pb*NK65-infected C57BL/6 mice, the number of pulmonary neutrophils was only reduced by ± 48% or ± 59%, respectively (Fig. 3A). Also, in CFA-challenged BALB/c mice, depletion was incomplete. When 250 or 600 µg of anti-Ly6G was administrated on day 14 and 17 post CFA-challenge, a splenic neutrophil reduction of less than ± 65% was seen at day 21 (Fig. 3B). In contrast, when neutrophils would have been gated as $CD11b^+$ $GR1^{hi}$ (Fig. 1, red gating), more than 90% depletion would have been apparently observed in lungs of *Pb*NK65-infected C57BL/6 mice and in the spleen of CFA-challenged BALB/c mice, demonstrating the risk of wrong interpretations.

3.4. Depletion of neutrophils is associated with increased proliferation

To understand the importance of a proper timing of the administration of depleting antibodies, we performed a BrdU incorporation test in naive mice. 24 h after BrdU injection, approximately 10% of the bone marrow neutrophils ($GR1^{int/hi}$ $Ly6C^{int}$ $CD11b^+$) incorporated BrdU in isotype control-treated mice (Fig. 4A). Upon single-dose neutrophil depletion 24 h before BrdU injection, bone marrow neutrophils showed a significantly increased BrdU incorporation in both C57BL/6 and BALB/c mice (non-significant trend in case of 500 µg anti-Ly6G in C57BL/6, $p = 0.0709$) (Fig. 4A). In the spleen of naive C57BL/6 and BALB/c mice, no significant differences were found upon depletion with anti-Ly6G (Fig. 4B). However, CFA-challenged BALB/c mice that received 600 µg anti-Ly6G on days 14 and 17 post CFA-challenge showed a significant increase in the percentage of proliferating splenic neutrophils (Fig. 4C). This increased proliferation seen in the spleen, may explain the limited and temporary depletion capacity of the antibody in this model. When mice were treated with 250 µg of anti-Ly6G antibodies twice a week, starting immediately after CFA administration (on day -1, 3, 6, 10, 14, 17 and 20), the number of splenic neutrophils increased profoundly on day 21 post CFA-injection (Suppl. Fig. 3A). These neutrophils had an immature phenotype which is characterised by a banded nuclear morphology, suggesting a rapid infiltration of new neutrophils (Suppl. Fig. 3B and C) [18].

4. Discussion

Neutrophil-depleting antibodies are frequently used *in vivo* to assess the role of neutrophils in disease models. Often, the neutrophil depletion efficacy is inadequately verified, for example by only determining the depletion in the circulation [6]. Moreover, a common mistake is the use of anti-Ly6G (clone 1A8) for both detection and depletion of the neutrophils [6,19]. One can better opt for a different antibody, for example anti-GR1 (clone RB6-8C5), for the detection of neutrophils after depletion with anti-Ly6G. The use of a different antibody clone is also a good solution. However, we demonstrated that competition exists between the anti-Ly6G and anti-GR1 antibodies, which resulted in a lower GR1 staining and an apparent disappearance of the $GR1^{hi}$ population. Such staining procedure may lead to the misinterpretation that depletion was successful, while neutrophils were still present but masked by other $GR1^{int/lo}$ populations, *e.g.* the inflammatory $Ly6C^{hi}$ monocytes. These results emphasize the importance of careful panel design and gating strategies during neutrophil depletion studies. Using

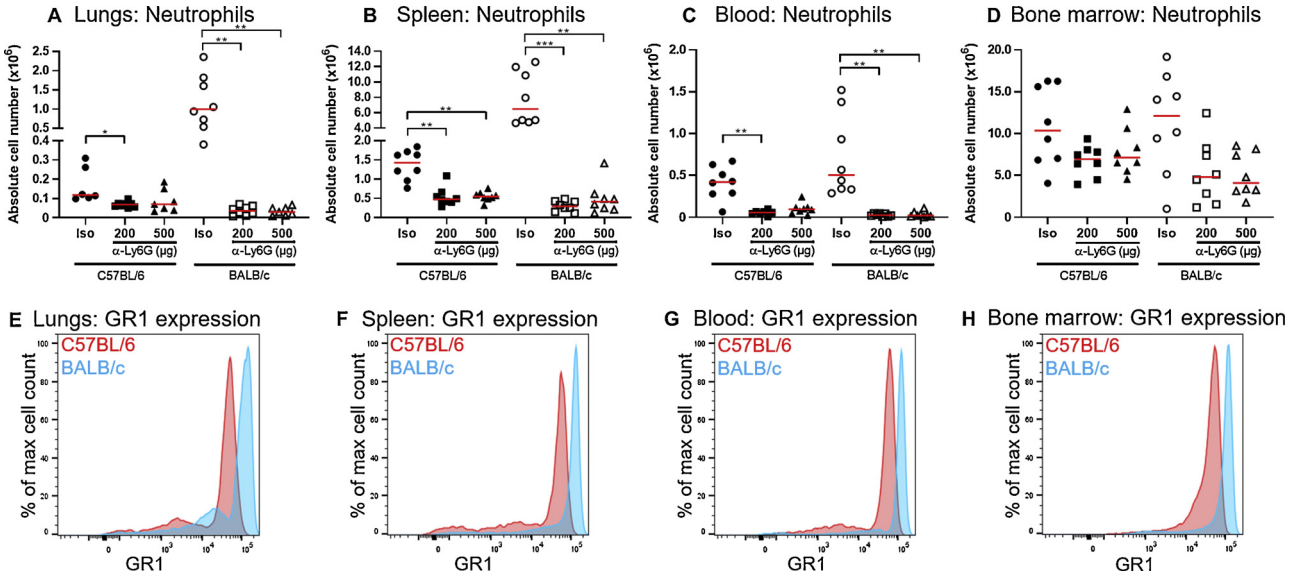

Fig. 2. Efficiency of neutrophil depletion with anti-Ly6G in naive C57BL/6 and BALB/c mice.
C57BL/6 and BALB/c mice were injected i.p. with 200 or 500 μg of anti-Ly6G (1A8) or isotype control (IgG2a). Two days after administration, lung, spleen, blood and bone marrow leukocytes were collected and analysed with flow cytometry. A–D) Absolute cell number of neutrophils (GR1$^{int/hi}$ Ly6C^{int} CD11b^{+}) present in lungs (A), spleen (B), blood (C) and bone marrow (D) (n = 8). Results pooled from 2 independent experiments. E–H) Representative plots of the intensity of GR1 expression of CD11b^{+} ly6C$^{int/-}$ cells in lungs (E), spleen (F), blood (G) and bone marrow (H) of isotype injected C57BL/6 (red) and BALB/c mice (blue). The graphs show data from one representative experiment out of 2 independent experiments.

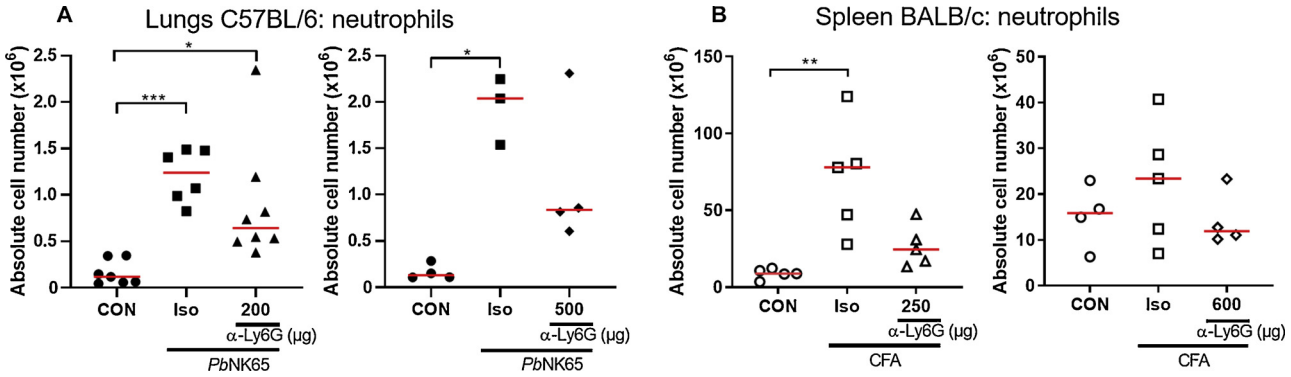

Fig. 3. Incomplete depletion of neutrophils with anti-Ly6G in *Pb*NK65-infected C57BL/6 mice and CFA-challenged BALB/c mice.
A) *Pb*NK65-infected C57BL/6 mice were injected i.p. on day -1, 3 and 7 p.i. with 200 or 500 μg of anti-Ly6G (1A8) or with the corresponding amount of isotype control (IgG2a). On day 9 p.i., mice suffered from MA-ARDS and were sacrificed. Absolute numbers of pulmonary neutrophils were determined (n = 3–7). B) CFA-challenged BALB/c mice were injected i.p. with 250 or 600 μg of anti-Ly6G (1A8) or with isotype control (IgG2a) on day 14 and 17 post challenge. On day 21, mice were sacrificed and the number of neutrophils in the spleen were determined (n = 4–5). A–B) Graphs represent the absolute number of neutrophils after gating as described in Fig. 1. Panel A left graph, pooled data from 2 independent experiments; panel A right graph and panel B, graphs show data from one representative experiment out of 2 independent experiments.

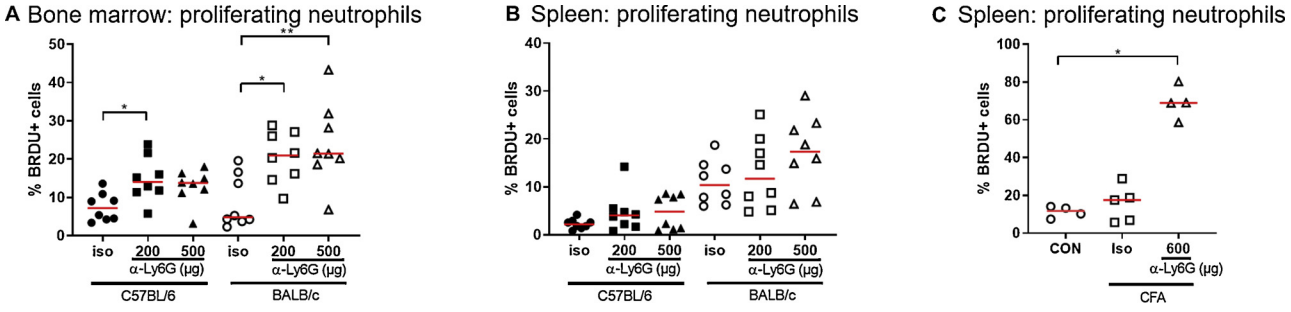

Fig. 4. Proliferation of neutrophils upon depletion with anti-Ly6G.
A–B) Percentage of BrdU^{+} bone marrow (A) and splenic (B) neutrophils (GR1$^{int/hi}$ Ly6C^{int} CD11b^{+}) in naive C57BL/6 and BALB/c mice two days after depletion with 200 or 500 μg of anti-Ly6G (1A8) or upon injection with isotype control (IgG2a). BrdU was injected 24 h prior to dissection (*i.e.* one day after depletion) (n = 8). Results pooled from 2 independent experiments. C) Percentage of BrdU^{+} splenic neutrophils (GR1$^{int/hi}$ Ly6C^{int} CD11b^{+}) in CFA-challenged BALB/c mice that were depleted with 600 μg of anti-Ly6G (1A8) or injected with isotype control (IgG2a) on day 14 and 17 and dissected on day 21. BrdU was injected 24 h prior to dissection (day 20) (n = 4–5). Data from one representative experiment out of 2 independent experiments.

naive mice, we demonstrated that an efficient depletion (> 80%) of neutrophils was obtained in the blood, lungs and spleen but not in the bone marrow of BALB/c mice. In contrast, efficient depletion of neutrophils was only reached in the blood of naive C57BL/6 mice and not in the organs, such as lungs, spleen and bone marrow. These findings stress the importance of confirming the depletion efficacy in the organ of interest, rather than only in the blood. Interestingly, the depletion appeared less efficient for the $CXCR2^-$ neutrophils, which may have either an immature or aged character, in blood, spleen and lungs. CXCR2 is upregulated during maturation and regulates neutrophil egress from the bone marrow [20]. Aged neutrophils decrease their CXCR2 expression, facilitating them to reverse-migrate back to the bone marrow, where they can be eliminated by the macrophages [21].

Interestingly, increased numbers of neutrophils and higher GR1-staining were found in BALB/c compared to C57BL/6 mice. This suggested that the higher percentual depletion efficiency seen in BALB/c mice might be related to a higher Ly6G expression or elevated neutrophil numbers before depletion. However, other factors might also play a role, such as potential differences in complement activation or phagocytosis, as it was shown that Ly6G depletion depends on the presence of macrophages [22]. In our study, we were unable to obtain efficient depletion of neutrophils in both *Pb*NK65-infected C57BL/6 mice and CFA-challenged BALB/c mice, and showed that this limited depletion was dosage independent. In normal situations, the turnover of neutrophils is tightly regulated [1,23]. Depletion of neutrophils causes a G-CSF-dependent positive feedback loop that triggers the expansion of new neutrophils in the bone marrow at the expense of thrombopoietic and red cell precursors [24]. The BrdU incorporation studies indeed document that, upon depletion, an increased proliferation of neutrophils occured in the bone marrow of naive mice, two days after anti-Ly6G depletion. In the CFA-induced inflammation model, the duration of the depletion is an important limiting factor. In this model, long persistent depletion, twice a week starting one day prior to CFA challenge, resulted in a profound increased number of immature neutrophils in the spleen, associated with an increased number of $BrdU^+$ splenic neutrophils. The increased number of proliferating splenic neutrophils was also found in the CFA-challenged BALB/c mice that were depleted with 600 µg of anti-Ly6G on day 14 and 17, and dissected on day 21. This nicely illustrated the temporary depletion capacity of the antibodies in this CFA-based model.

In conclusion, neutrophil depletion is organ-, strain- and timing-specific and should thus be analysed carefully. This study describes the incomplete depletion in two immunological models and underlines the need for new tools to understand the role of neutrophils *in vivo*.

Author contributions

EP, BM, LV, TM, TP and HP performed the experiments. EP and BM analysed the data. PM, GO and PVDS conceived the study. LB provided the IgG2a isotype control antibodies. EP, BM, PM and PVDS wrote the first drafts of the manuscript. All authors critically read and edited the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.06.006.

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