Title: Protocols for circulating neutrophil depletion in neonatal C57Bl/6 mice

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ABSTRACT:

Background: Murine neonatal neutrophil depletion strategies have problems with achieving deep circulating neutrophil clearance and accurate residual neutrophil fraction detection. Boivin et al. demonstrated an isotype switch method of achieving profound neutrophil clearance using a combination of anti-Ly6G and anti-rat κ Ig light chain antibodies in adult C57Bl/6 mice, proven by extra- and intracellular Ly6G detection by flow cytometry. We adapted this technique to neonatal mice testing four neutrophil depletion strategies.

Methods: Four protocols were tested: P3 Ly6G and P1-3 Ly6G (anti-Ly6G on postnatal days (P) 3 and 1-3 respectively), and P3 Dual and P1-3 Dual (anti-Ly6G and anti-rat κ Ig light chain on P3 and P1-3 respectively). Intracellular and extracellular Ly6G presence was detected using flow cytometry. Isotype control antibodies were used as controls.

Results: P1-3 Dual achieved significantly better neutrophil depletion than the P1-3 Ly6G or P3 Ly6G protocols (97% vs 74% and 97% vs 50%, respectively). The P3 Dual protocol alone was enough to achieve significantly better neutrophil clearance (93%) than any of the Ly6G alone protocols. The Ly6G alone protocols led to near-total elimination of extracellular Ly6G, but there was a significant presence of intracellular Ly6G in the CD45+ cell population, which evaded detection by extracellular Ly6G antibody-based detection methods.

Discussion: Anti-Ly6G antibody alone is ineffective in producing effective circulating neutrophil clearance in neonatal mice. Dual antibody-based neutrophil depletion strategies achieve>90% clearance in P4 mice. A single day of dual antibody treatment achieves 93% neutrophil depletion in neonatal mice and is an alternative to daily injections.

Keywords: Ly6G, isotype switch, neonatal neutrophils

Summary Sentence: Dual antibody-based neutrophil depletion effectively induces circulating neutrophil clearance in neonatal mice.

INTRODUCTION:

Neonates, especially those born preterm, have a significantly higher risk of mortality and morbidity from sepsis than any other age group [1, 2]. This has been attributed to an early exit from a sterile intra-uterine environment to a microbe-laden external environment, a naïve adaptive immune system, and a developmentally immature innate immune system [3]. Neutrophils form the largest fraction of the innate immune system, and neonatal neutrophils are structurally and functionally different from adult neutrophils [4]. Elucidating neutrophil-dependent mechanisms contributing to increased sepsis-related mortality in neonates is critical in developing novel therapeutic strategies. Depletion of neutrophils is often used in murine models of sepsis to characterize the role and contribution of the circulating neutrophil compartment. Neutrophil depletion strategies include pharmacologic agents such as cyclophosphamide and vinblastine, as well as antineutrophil antibodies [5, 6]. Pharmacologic neutrophil depletion has fallen out of favor due to effects on other cell lines and other cellular processes such as microtubule assembly [7].

Murine models that use antibody-mediated neutrophil depletion use either anti-Ly6G or anti-Gr1 antibodies [6]. Anti-Gr1, RB6-8C5 clone is a rat IgG2b, which leads to neutrophil depletion by complement-mediated cell cytotoxicity, in addition to the classical pathway, and is thus faster and more pronounced [8]. However, the anti-Gr1 antibody also recognizes Ly6C receptors, expressed by monocytes, macrophages, and a subpopulation of CD8+ T cells [6, 9]. This leads to the non-specific depletion of several different cell lines [10]. Anti-Ly6G antibody is a rat IgG2a isotype and binds specifically to the Ly6G receptors on neutrophils and leads to neutrophil depletion by Fc-opsonization and macrophage phagocytosis, which is a slower method than the complement-mediated cytotoxicity seen in anti-Gr1 depletion [8].

Boivin et al. have developed a new model for sustained and more durable depletion of neutrophils in adult mice where they injected anti-Ly6G daily and anti-rat IgG2a antibody every other day [8]. The authors mention that this “Combo” treatment creates an isotype switch in

which the anti-Ly6G depleting antibody (rat IgG2a isotype) is bound by the anti-rat IgG2a (mouse IgG2a isotype). Mouse IgG2a is orthologous to rat IgG2b, thus creating an isotype switch and leading to faster and more profound neutropenia by complement-mediated processes. This group circumvented the issue of antigen masking during the detection of the residual circulating neutrophils to measure the efficacy of neutrophil depletion. Although extracellular Ly6G staining would detect circulating neutrophils using flow cytometry under standard conditions, those circulating neutrophils to which the anti-Ly6G depleting antibodies are bound are not detected by this technique due to antigen masking. This hurdle was overcome using a combination of extracellular Ly6G staining, intracellular Ly6G staining, and extracellular rat IgG2a staining [8]. Using this method, rat IgG2a staining can detect those residual circulating neutrophils still bound to the anti-Ly6G depleting antibody and evade detection by fluorophore-conjugated anti-Ly6G antibodies.

Neonatal neutrophil depletion studies commonly use anti-Ly6G depleting antibodies to study the role of the neutrophil [11-13]. These studies generally report >90% neutrophil depletion using fluorophore-conjugated extracellular Ly6G detection on flow cytometry, which does not detect the circulating neutrophils bound to the depleting antibody. Data on the accurate degree of neutrophil depletion using extra- and intracellular staining methods are absent. In addition, no studies have replicated the isotype switch in neonatal mice to demonstrate whether this leads to more profound neutrophil depletion or whether an isotype switch model is unnecessary in neonatal mice. Here, we describe a modified Boivin neutrophil depletion strategy for postnatal day 4 (P4) neonatal mice. Further, we tested the combined staining method for evidence of neutrophil depletion in the conventional anti-Ly6G depletion and the Boivin “Combo” depletion strategy, adapted for neonatal mouse pups.

MATERIALS AND METHODS:

Animals: All animal experiments were conducted following the approved protocols of the Case Western Reserve University (CWRU) Institutional Animal Care and Use Committee. Mice colonies were housed in a pathogen-free animal facility. C57Bl/6 mice procured from The Jackson Laboratory (Bar Harbor, Maine, USA) were used for all experiments. Neonatal mice were anesthetized using isoflurane following CWRU Animal Resource Center protocols for peripheral blood sampling from the external jugular vein. Pups were euthanized after extraction of blood using cervical dislocation as per CWRU and NIH guidelines for rodents < 10 days of age.

In vivo antibodies: The anti-Ly6G antibody (clone 1A8, #BP0075-1), the anti-rat kappa immunoglobulin light chain antibody (clone MAR18.5, #BE0122), and the corresponding isotype controls were procured from BioXCell (Lebanon, NH, USA). Anti-Ly6G was used at a dose of 100ug/gm, and anti-rat k IgG light chain antibody was used at a dose of 50ug/gm. Intraperitoneal injections were administered to mice using 31-gauge syringes.

Neutrophil depletion protocols (Fig. 1A):

One-day anti-Ly6G (P3 Ly6G) – Anti-Ly6G antibody injection on P3, followed by peripheral blood (PB) sampling on P4 after 24 hours (h).

Three-day anti-Ly6G (P1-3 Ly6G): Daily anti-Ly6G antibody injections were administered from P1 to P3, followed by peripheral blood (PB) sampling on P4 24 h after the last injection.

One-day dual-antibody treatment (P3 Dual) – Anti-Ly6G and anti-rat kappa immunoglobulin light chain antibody on P3, followed by PB sampling after 24 h.

Three-day dual-antibody treatment (P1-3 Dual) – Daily anti-Ly6G and anti-rat kappa immunoglobulin light chain antibody injections (separated by four h) from P1 – P3, followed by PB sampling on P4, 24 hrs after last injection.

Anti-Ly6G and anti-rat k IgG light chain antibody injections were spaced out by a 4 – 6 h interval.

Flow cytometry – Peripheral whole blood at P4 was used for all experiments. RBC lysis was performed using hypotonic 0.2% saline for 30 seconds, reconstituted to normal saline by adding an equal volume of 1.8% saline, centrifuged, and resuspended in flow sorting buffer (PBS + 1% fetal bovine serum + 2mM EDTA). For extracellular staining, cells were incubated with antibodies - CD45-APC (Biolegend), Ly6G-FITC (eBioscience), anti-rat IgG2a-PE (Biolegend) at a concentration of 1:1000. For intracellular Ly6G staining, cells were fixed and permeabilized (Biolegend FluoroFix buffer, Biolegend Perm/Wash buffer), incubated with anti-Ly6G-PE antibody at 1:1000 for 30 minutes, followed by two washes. All flow cytometry experiments were conducted using BD LSR Fortessa cytometer at the CWRU Cytometry Core. Flow cytometry results were analyzed, and representative images were built using FlowJo (BD Biosciences, Oregon, USA).

Statistical analysis: GraphPad Prism was used for all statistical analyses, with significance considered when $p < 0.05$.

RESULTS:

Administering Anti-Ly6G antibody alone does not induce profound neutrophil depletion in P4 pups: We found no difference between previously reported circulating neutrophil counts in neonatal C57Bl/6 mice [14] and the circulating fraction of CD45+ cells in P4 PB, which were positive for either intracellular or extracellular Ly6G or both after isotype control injections in our studies (Fig. 1B (i) and Table 1). Both P3 Ly6G and P1-3 Ly6G protocols effectively removed any extracellular presence of Ly6G on circulating CD45+ cells, to 1.3% and 0.3%, respectively, from a baseline level of 19.6% (Fig. 1B). Using only extracellular Ly6G-based detection techniques to detect residual neutrophils would thus show >98% neutrophil depletion using either protocol. However, using intracellular Ly6G staining, we demonstrate that the P3 Ly6G

protocol increased antigen masking by increasing the fraction of cells with only internal Ly6G expression and no detectable extracellular Ly6G expression from 2.1% to 9.67% ($p = 0.002$) (Fig. 1 B and C). P1-3 Ly6G leads to fewer neutrophils with only intracellular Ly6G expression (5.08%), but it still did not achieve >98% neutropenia based on intracellular + extracellular Ly6G detection (Fig. 1 B and C). In our studies, both P3 Ly6G and P1-3 Ly6G protocols achieved ~50% and ~74% neutrophil depletion, contrary to what has been reported previously using only extracellular Ly6G detection. We use fluorophore-conjugated anti-rat IgG2a antibodies to detect the circulating neutrophils to which anti-Ly6G antibodies were already bound, and this ranged between 10-12% of all CD45+ cells in both P3 Ly6G and P1-P3 Ly6G protocols, despite both protocols showing <2% extracellular Ly6G+ cells, further proving that the depleting antibody itself impairs detection of the Ly6G receptor, similar to what Boivin et al. report.

<u>P1-3 Dual induces more significant neutrophil depletion than P1-3 Ly6G:</u> Adapting the established “Combo” depletion strategy for neonatal mice, we show that dual antibody treatment with anti-mouse-Ly6G followed by anti-rat IgG2a leads to more profound neutrophil depletion ($p<0.0001$) (Fig 1B and Table 1). Unlike Boivin et al.’s methodology of every other day anti-rat IgG2a antibody, we injected the mouse pups with both antibodies daily, separated by 4-6h. Our primary rationale was that, unlike adult experiments where cell depletion can be carried out for days to weeks before endpoint experiments, we only had three days to produce neutrophil depletion in neonatal pups and wanted to maximize the effect of the depleting antibodies. P1-3 Dual produced ~97% neutrophil depletion in P4 mouse pups based on intracellular and extracellular Ly6G detection methods (Table 1). The fraction of CD45+ cells expressing only intracellular Ly6G, typically evading detection, was 0.4% (Fig. 1B (v) and Fig. 1C).

<u>P3 Dual is a better alternative than P1-3 Ly6G and P3 Ly6G:</u> Administering two i.p. injections daily to neonatal mice can be challenging. We modified our approach to only P3 injections 24 hours before PB sampling. The P3 Dual protocol induced more profound neutrophil depletion

than the P1-3 Ly6G protocol (~93% vs. ~74%, p=0.0074), the more commonly reported protocol for neonatal neutrophil depletion. This significant reduction in circulating neutrophil count was primarily attributable to a decrease in the neutrophils with intracellular Ly6G expression, similar to the results in the P1-3 Dual protocol (Fig 1B (iv) and Fig. 1C). P3 Dual is more effective than P1-3 Ly6G, as expected, in depleting cells expressing Ly6G intra- or extracellularly or both (Table 1).

DISCUSSION:

Adapting an existing adult murine protocol described by Boivin et al. for neonatal studies adds significant knowledge to neonatal neutrophil biology. Neutrophil depletion models are fraught with the problems of accurate and specific neutrophil population estimation after antibody treatment and the development of antibodies against the depleting antibody. The latter is not a problem with neonatal experiments, as mice take ~seven days to develop anti-rat antibodies [8]. The production of the anti-Ly6G 1A8 clone antibody has improved the specificity of neutrophil depletion experiments, and most current studies use this antibody instead of the anti-Gr1 antibody, which also recognizes Ly6C expressed by other myeloid cells. Neonatal studies employing neutrophil depletion techniques use daily anti-Ly6G injections, and most of these studies have shown near-total circulating neutrophil depletion. These studies identify the residual neutrophil population by extracellular Ly6G staining, which runs the risk of incorrect estimation of neutrophils as they cannot detect the antigen-masked population. In our study, using the reported technique of dual Ly6G detection (intra- + extracellular), we show that the previously published strategy of using daily anti-Ly6G antibody injections to induce neutrophil depletion in P4 neonatal mice leads to only ~75% neutrophil depletion, instead of the reported >90% depletion [11-13]. Although anti-Ly6G antibodies can effectively remove extracellular Ly6G+ cells from circulation, neutrophils can internalize the Ly6G receptor as an adaptive mechanism against depleting antibodies, thus exhibiting decreased surface expression of the

Ly6G receptor, preventing effective depletion by anti-Ly6G antibody treatment alone [15]. The increased time in depletion by only anti-Ly6G-based protocols may allow for this internalization to occur and lead to efflux of neutrophil precursors from the bone marrow, which might not have robust Ly6G expression extracellularly [16].

Boivin et al. have demonstrated that by creating an isotype switch from rat IgG2a (anti-Ly6G, 1A8) to mouse IgG2a (anti-rat IgG2a), which is orthologous to rat IgG2b, faster and durable neutrophil depletion can be achieved [8, 17]. We show that a single dose of dual antibody treatment on P3 leads to more pronounced neutrophil depletion than three days of anti-Ly6G antibody treatment. This is particularly useful as injecting P1 mouse pups can be technically challenging and avoids daily injections in neonatal mice. The novelty of our technique lies in adapting this approach with modifications in the dosing schedule specifically for neonatal mice, which demonstrates highly effective neutrophil clearance compared to prior protocols.

The two major limitations of this protocol are outlined below. First, our study is restricted to neonatal mice. We chose P4 specifically as many neonatal studies on regulating the immune response are conducted at this age [11, 12]. Hence, we wanted to verify that adequate and complete neutrophil depletion can be achieved at this age. We urge investigators to test similar antibody combination strategies for experiments planned at other neonatal ages.

Further, we did not test for the bone marrow or tissue neutrophil fraction, as this was not the primary focus of the study. The circulating neutrophil fraction is the primary response to bacterial antigens and is the fraction mobilized first in response to any antigenic stimuli in peripheral tissues. Our protocols did not precisely measure the other myeloid cell types. It has been well established that anti-Ly6G-1A8 clone antibody does not affect other myeloid cell lines and is specific for mature neutrophil populations. Our experiments were also carried out in C57Bl/6 mice, which do not express the gene for mouse IgG2a and hence cannot produce IgG2a subclass antibodies [18]. We encourage investigators using BALB/c and Swiss Webster

mice, which produce IgG2a antibodies, to explore this protocol and verify the degree of neutrophil clearance before conducting experiments.

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Authors' contributions: DM conceptualized experimental techniques, performed experiments, and wrote and reviewed the manuscript. SS contributed to all experimental methods, flow cytometry interpretation, statistical analyses, and manuscript editing and reviewing. AT and YL contributed to experimental design and figure creation and reviewed and edited the manuscript. LN provided conceptual expertise and funding and reviewed and edited the manuscript.

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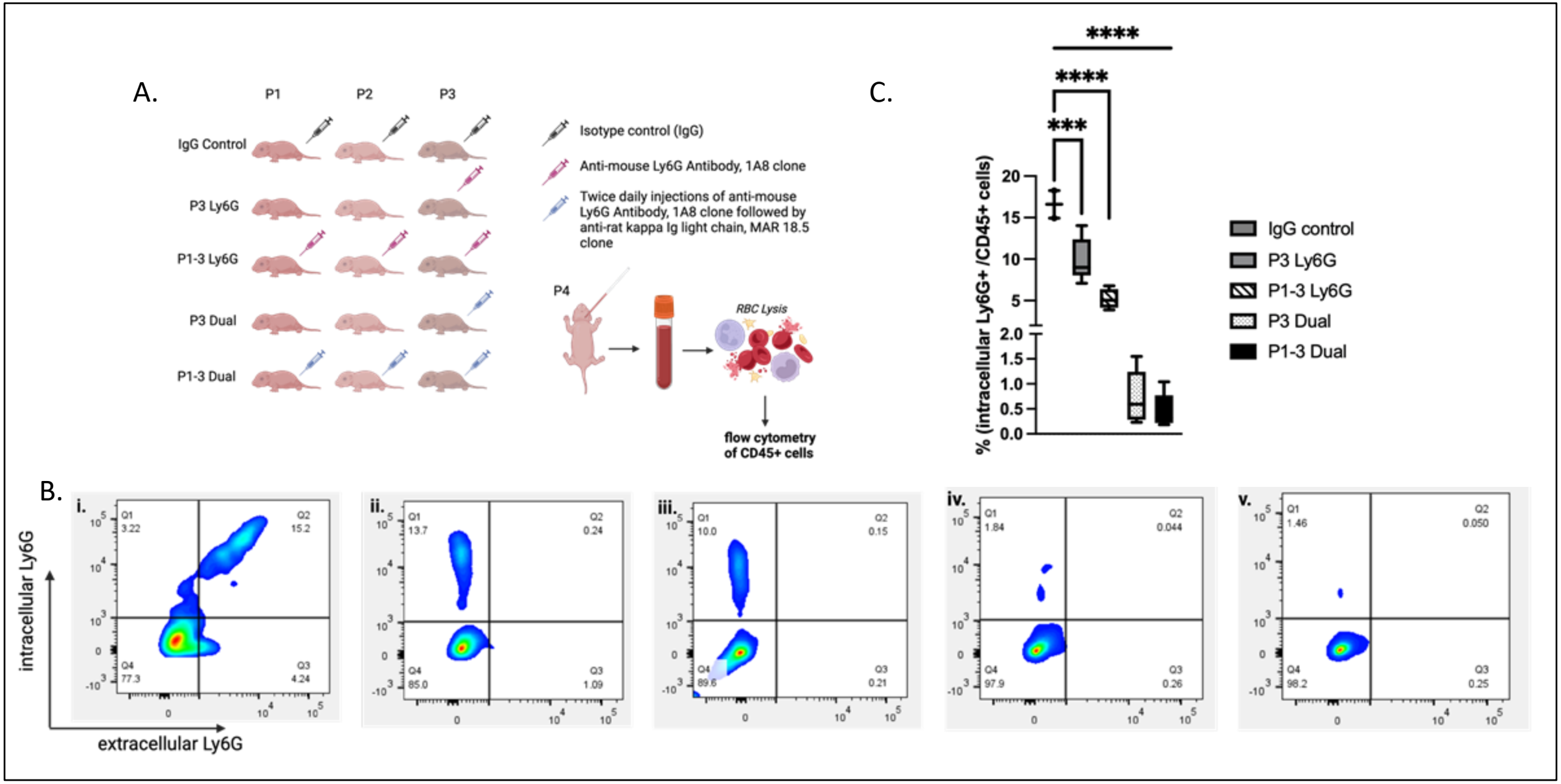
	Only intracellular Ly6G expression (% of CD45+ cells)	Only extracellular Ly6G expression (% of CD45+ cells)	Both intra- and extracellular Ly6G expression (% of CD45+ cells)	Total neutrophil count (% of CD45+ cells)	Neutrophil depletion percentage based on isotype control arm
Isotype control (n=2)	2.10 ± 0.64	3.40 ± 0.60	14.50 ± 1.70	20.00 ± 2.93	0%
P3 Ly6G (n=5)	9.67 ± 2.62	0.36 ± 0.48	0.17 ± 0.25	10.32 ± 3.02	50%
P1-3 Ly6G (n=4)	5.08 ± 1.09	0.09 ± 0.06	0.11 ± 0.13	5.28 ± 1.15	73%
P3 Dual (n=6)	0.50 ± 0.46	0.75 ± 0.49	0.23 ± 0.27	1.49 ± 0.63	93%
P1-3 Dual (n=5)	0.42 ± 0.35	0.173 ± 0.09	0.04 ± 0.03	0.63 ± 0.30	97%

Table 1: Peripheral blood circulating cells expressed as a percentage of live CD45+ cells positive for either intracellular or extracellular Ly6G expression and those cells that express Ly6G both intra- and extracellularly. The rightmost column gives the neutrophil counts for each protocol based on whether the cell expressed Ly6G (intra- or extracellular).

Figure Legends:

Figure 1. (A) P3 protocols included intraperitoneal injections administered on postnatal day three only, and P1-3 protocols included intraperitoneal injections administered daily on postnatal days 1-3. All protocols were followed by peripheral blood sampling on P4 24 hours after the last injection. Red blood cell lysis was performed using hypotonic saline, reconstituted to normal saline, and cells were resuspended in flow cytometry buffer. Extracellular staining was performed by APC-CD45, FITC-Ly6G (1:1000), cells were fixed and permeabilized, and intracellular staining was performed by PE-Ly6G (1:1000). (B) Flow cytometry was performed on BD LSF Fortessa cytometer for all protocols (i) IgG control (n=2), (ii) P3 Ly6G (n=5), (iii) P1-3 Ly6G (n=4), (iv) P3 Dual (n=6), and (v) P1-3 Dual (n=5). We included quarters 1 – 3 for a total neutrophil count (CD45+ cells staining either intracellularly or extracellularly for Ly6G). As compared to the mean neutrophil count in isotype control injected mice, P3 Ly6G mice exhibited ~50% reduction, P1-3 Ly6G mice showed 74% reduction, P3 Dual mice showed 93% reduction, and P1-3 Dual mice exhibited 97% reduction in the circulating neutrophil counts. (C) Percentage of intracellular Ly6G positive cells out of all CD45+ cells. As compared to those in isotype control injected mice (16.6 ± 2.34), this percentage decreased only by 6.63% in P3 Ly6G

injected mice and by 11.41% in P1-P3 Ly6G injected mice ($p<0.0001$). However, this reduction was far more effective in both protocols involving anti-rat kappa Ig light chain antibodies. The percentage of CD45+ cells positive for intracellular Ly6G reduced by 15.86% in P3 Dual mice ($p<0.0001$) and by 16.14% in P1-3 Dual mice ($p<0.0001$), thus showing the effectiveness of the dual antibody treatment protocols in preventing antigen masking and Ly6G receptor internalization.


A.
P1
P2
P3
IgG Control
P3 Ly6G
P1-3 Ly6G
P3 Dual
P1-3 Dual
Isotype control (IgG)
Anti-mouse Ly6G Antibody, 1A8 clone
Twice daily injections of anti-mouse Ly6G Antibody, 1A8 clone followed by anti-rat kappa Ig light chain, MAR 18.5 clone
P4
RBC Lysis
flow cytometry of CD45+ cells
C.
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****
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% (intracellular Ly6G+ /CD45+ cells)
IgG control
P3 Ly6G
P1-3 Ly6G
P3 Dual
P1-3 Dual
B.
intracellular Ly6G
extracellular Ly6G
i.
Q1 3.22
Q2 15.2
Q4 77.3
Q3 4.24
ii.
Q1 13.7
Q2 0.24
Q4 85.0
Q3 1.09
iii.
Q1 10.0
Q2 0.15
Q4 89.6
Q3 0.21
iv.
Q1 1.84
Q2 0.044
Q4 97.9
Q3 0.26
v.
Q1 1.46
Q2 0.050
Q4 98.2
Q3 0.25