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Julien Stackowicz, Caitlin Gillis, Ophélie Godon, Bruno Iannascoli, Eva Conde, Edouard Leveque, William Worrall, Stephen Galli, Pierre Bruhns, Laurent Lionel Reber, et al.

► To cite this version:

Julien Stackowicz, Caitlin Gillis, Ophélie Godon, Bruno Iannascoli, Eva Conde, et al.. Conditional neutrophil depletion challenges their contribution to mouse models of anaphylaxis. *Allergy*, In press, 10.1111/all.15738 . pasteur-04143597v1

HAL Id: pasteur-04143597

<https://pasteur.hal.science/pasteur-04143597v1>

Submitted on 27 Jun 2023 (v1), last revised 28 Jun 2023 (v2)

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1 **Conditional neutrophil depletion challenges their contribution to mouse**
2 **models of anaphylaxis**

3

4 Julien Stackowicz^{1,2}, Caitlin M. Gillis^{1,3}, Ophélie Godon¹, Bruno Iannascoli¹, Eva Conde^{1,2},
5 Edouard Leveque⁴, William P. M. Worrall⁴, Stephen J. Galli^{5,\$}, Pierre Bruhns^{1,\$}, Laurent L.
6 Reber^{1,4,\$}, and Friederike Jönsson^{1,6,\$}

7

8 ¹Institut Pasteur, Université de Paris Cité, Unit of Antibodies in Therapy and Pathology,
9 INSERM UMR1222, F-75015 Paris.

10 ²Sorbonne Université, College Doctoral, Paris, France.

11 ³now at: Centre for Inflammation, Centenary Institute and University of Technology Sydney,
12 Faculty of Science, Sydney, Australia

13 ⁴Toulouse Institute for Infectious and Inflammatory Diseases (Infinity), INSERM UMR1291,
14 CNRS UMR5051, University Toulouse III, F-31024 Toulouse.

15 ⁵Departments of Pathology and of Microbiology and Immunology, Sean N. Parker Center for
16 Allergy and Asthma Research, Stanford University School of Medicine, Stanford, CA, USA.

17 ⁶CNRS, F-75015 Paris.

18 ^{\$}co-senior authorship

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20 **Corresponding authors:**

21

22 Friederike Jönsson PhD, Unit of Antibodies in Therapy and Pathology, Institut Pasteur, 25 rue
23 du Docteur Roux, 75015 Paris, France. E-mail: joensson@pasteur.fr

24

25 Laurent L. Reber PhD, Toulouse Institute for Infectious and Inflammatory Diseases
26 (INFINITY), CHU Purpan – BP 3028, 31024 Toulouse CEDEX 3, France. E-mail:

27 laurent.reber@inserm.fr

28 To the Editor,

29

30 Anaphylaxis is an acute and potentially lethal systemic allergic reaction. In humans, it is
31 largely accepted that anaphylaxis relies predominately on IgE antibodies¹. However, IgG
32 might also contribute to anaphylaxis induced by infused drugs¹. Several mouse models have
33 been developed to identify key effector cells and mediators of anaphylaxis. Consequently, two
34 main pathways have been identified in mice: a “classical” pathway consisting of IgE, FcεRI,
35 histamine and mast cells, and an “alternative” pathway involving IgG, FcγRIII, platelet-
36 activating factor (PAF) and - depending on the anaphylaxis model studied - macrophages,
37 basophils and/or neutrophils¹⁻³.

38

39 We and others have reported that neutrophils are potentially important drivers of IgG
40 anaphylaxis in mice, based on experiments using neutrophil-depleting mAbs^{1,4}. ~~However, the~~
41 ~~use of such mAbs has caveats, considering potential off target effects.~~ In contrast, Strait et al.³
42 showed that injection of anti-Gr-1 mAb 2 days before antigen challenge failed to suppress
43 IgG-mediated anaphylaxis, indicating that the effect of these mAbs likely depends on the
44 dose, timing of injection and specific model used. We recently described an inducible,
45 antibody-independent, neutrophil depletion mouse model (*PMN^{DTR}* mice), relying on the
46 selective expression of the diphtheria toxin (DT) receptor on neutrophils⁵. Injection of DT in
47 *PMN^{DTR}* mice leads to a marked depletion of blood, spleen and bone marrow neutrophils (**Fig.**
48 **S1A-D**)⁵. We therefore used *PMN^{DTR}* mice to re-evaluate the contribution of neutrophils to
49 anaphylaxis models using *PMN^{DTR}* mice.

50

51 To elicit active systemic anaphylaxis (ASA), mice were immunized with BSA emulsified in
52 Freund’s adjuvant, and challenged i.v. with BSA (**Fig. 1A**), a model that mostly relies on

53 IgG⁴. As expected, pre-treatment of mice with anti-Ly6G or anti-Gr-1 neutrophil-depleting
54 mAbs markedly reduced hypothermia and mortality in this model (**Fig. 1B-C**). Strikingly,
55 however, DT-treated neutrophil-deficient *PMN^{DTR}* mice exhibited similar anaphylaxis
56 severity to neutrophil-sufficient controls (**Fig. 1D-E**). We obtained similar results in models
57 of IgG2a- or IgG2b-induced passive systemic anaphylaxis (PSA) (**Fig. 1F-J**).

58
59 Interestingly, while performing the initial treatment to deplete neutrophils, we observed that
60 injection of either anti-Ly6G or anti-Gr-1 mAbs induced a transient hypothermia in WT mice,
61 whereas injection of DT in *PMN^{DTR}* mice did not induce any apparent side effects (**Fig. 2A-**
62 **B**). We therefore questioned whether engagement of FcγRs and/or complement by the
63 depleting mAbs might induce an initial anaphylactic-like event that could desensitize mice to
64 subsequent IgG-mediated anaphylaxis. To test this, we administered anti-Ly6G mAb to
65 FcγR^{Null} mice that do not express FcγRs, and to FcγR^{Null}/C1q^{KO} mice that lack both FcγRs and
66 complement component C1q. As expected, neutrophil depletion by mAbs was impaired (**Fig.**
67 **S1E-G**), which suggests that this depletion occurs at least partly through antibody-dependent
68 cellular cytotoxicity or phagocytosis mediated by FcγRs, and potentially via complement-
69 dependent cytotoxicity. Moreover, transient hypothermia after mAb injection was abolished
70 in FcγR and complement deficient animals (**Fig. 2C**).

71
72 PAF is a key driver of anaphylaxis^{1,2}. We therefore assessed the role of PAF in anti-Ly6G
73 mAb-induced hypothermia using PAF receptor-deficient mice (PtafR^{KO}) which are resistant
74 to PAF-induced shock (**Fig. S2A**). Neutrophil depletion efficacy was unaltered in PtafR^{KO}
75 mice (**Fig. S2B-D**), however these mice exhibited reduced hypothermia upon injection of
76 anti-Ly6G mAbs (**Fig. 2D**). In agreement with these results, pre-treatment of mice with a PAF
77 receptor antagonist (ABT-491) also largely prevented hypothermia upon injection of anti-

78 Ly6G mAbs (**Fig. 2E-F**), without affecting neutrophil depletion efficacy (**Fig. S2G-I**). ABT-
79 491 has a short half-life *in vivo* (**Fig. S2E-F**). We could then demonstrate that when the PAF-
80 induced hypothermia after anti-Ly6G mAb injection is blocked, then the protective effect of
81 this mAb on IgG anaphylaxis is reduced (**Fig 2G**).

82

83 Our data demonstrate that IgG-anaphylaxis can still occur in neutropenic mice and that
84 neutrophil-targeting mAbs likely protect from anaphylaxis through their capacity to
85 desensitize effector cells by engaging FcγRs and inducing the release of PAF, rather than via
86 neutrophil depletion *per se*. Neutrophils are therefore dispensable for IgG-anaphylaxis in
87 mice. They may nevertheless be activated during IgG-anaphylaxis, since their FcγRs are
88 engaged^{6,7}, as in other IgG-mediated inflammatory processes.

89

90 In addition, we used *PMN^{DTR}* mice to confirm that neutrophils do play a pivotal role in two
91 other models of IgG-mediated inflammation: immune complex-induced airway inflammation
92 (**Fig. S3A-E**) and autoantibody-induced arthritis (**Fig. S3F-H**). This extends the validation of
93 *PMN^{DTR}* mice as a meaningful model in neutrophil research.

94

95 This study highlights that that the use of depleting mAbs *in vivo*, and especially in antibody-
96 driven reactions such as anaphylaxis, should be carefully evaluated to avoid overinterpretation
97 of the actual contribution of neutrophils to disease models. Not only should we consider the
98 potential off-target effects of mAbs, but also the ramifications of on-target depletion
99 strategies which engage Fc receptors and consequently affect other immune activation
100 pathways.

101 Funding source

102 This work was supported by a Jeunes Chercheuses/Jeunes Chercheurs grant from the Agence
103 National de la Recherche (ANR-16-CE15-0012, to FJ). The Phenomin project receives funds
104 from the ANR under the framework “Investissements d’Avenir” labelled ANR-10-INBS-07
105 PHENOMIN. PB acknowledges support from the European Research Council (ERC)–
106 Seventh Framework Program (ERC-2013-CoG 616050), Institut Pasteur and INSERM
107 (Institut National de la Santé et de la Recherche Médicale), SJG from NIH grant R01
108 AI165373, CMG was supported by a stipend from the Pasteur - Paris University (PPU)
109 International PhD program and by the Institut Carnot Pasteur Maladies Infectieuses, and LLR
110 from an ATIP-Avenir grant from the INSERM and the French Medical Foundation (Equipe
111 FRM 2021).

112

113 Authorship Contributions

114 JS together with CMG, OG, FJ, EL, WW and EC performed all experiments, BI prepared
115 essential reagents, JS, CMG, PB, LR and FJ analyzed and discussed results, and SJG, PB, LR
116 and FJ supervised the research. JS, CMG, SJG, PB, LR, and FJ prepared the figures and wrote
117 the manuscript. All authors were given the opportunity to read and make amendments to the
118 manuscript.

119

120 Conflicts of interest

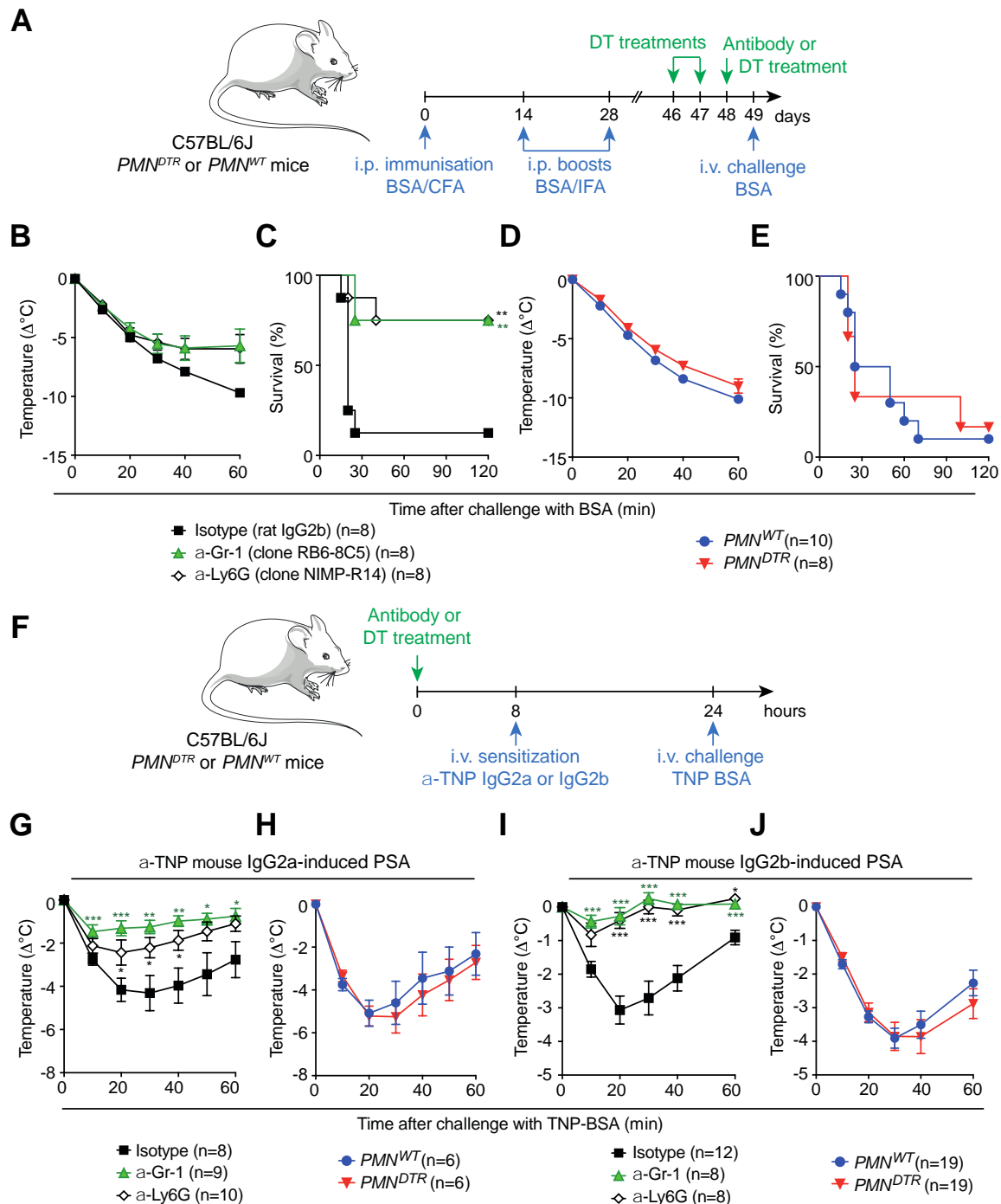
121 PB is a paid consultant to Regeneron Pharmaceuticals. The rest of the authors declare that
122 they have no relevant conflicts of interest.

123 Acknowledgements

124 We are grateful to Lynn E. MacDonald and Andrew L Murphy, working at Regeneron
125 Pharmaceuticals, for providing VG1505 (Fc γ RI^{only}) and VG598 (C1q^{KO}) mouse strains.

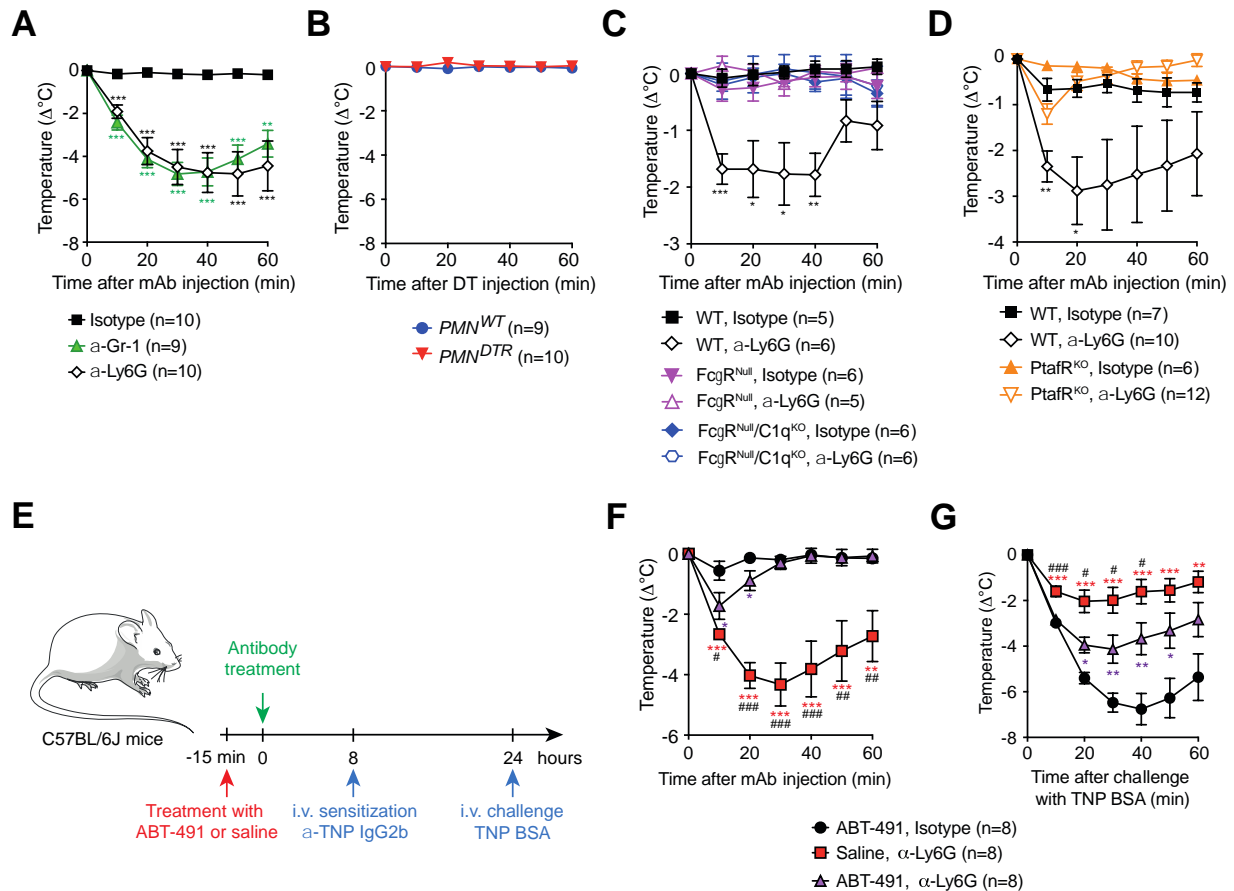
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145

146 **Figure 1. Neutrophil depletion using antibodies, but not the PMN^{DTR} model, protects**
 147 **mice from active or passive anaphylaxis. (A-E)** Active systemic anaphylaxis (ASA) in WT
 148 mice pre-treated with neutrophil-depleting α -Gr-1 or α -Ly6G mAbs (or an isotype control),
 149 and in PMN^{DTR} and PMN^{WT} mice pre-treated with DT. (A) Protocol outline. (B, D) Changes
 150 in body temperature ($\Delta^{\circ}\text{C}$) and (C, E) survival after challenge with BSA. (F) IgG2a- or
 151 IgG2b-mediated passive systemic anaphylaxis (PSA) protocol outline. (G-J) Changes in body
 152 temperature ($\Delta^{\circ}\text{C}$) after challenge with TNP-BSA. Data are pooled from at least two

153 independent experiments, means \pm s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, using Mann-
 154 Whitney test (two-tailed) or Mantel-Cox log-rank test.



155

156 **Figure 2. α -Ly6G mAbs induce $Fc\gamma R$ - and PAF-dependent hypothermia which protects**
 157 **mice from subsequent IgG anaphylaxis. (A-B)** Changes in body temperature ($\Delta^{\circ}\text{C}$) after
 158 injection of (A) neutrophil-depleting α -Ly6G or α -Gr-1 mAbs, or a rat IgG2b isotype control
 159 (Isotype) in C57BL/6J mice, or (B) DT into PMN^{WT} or PMN^{DTR} mice. (C-D) Changes in body
 160 temperature following injection of α -Ly6G mAbs or a rat IgG2b isotype control (Isotype) in
 161 (C) WT, $Fc\gamma R^{Null}$ and $Fc\gamma R^{Null}/C1q^{KO}$, or (D) controls vs. $PtafR^{KO}$ mice. (E-G) Mice were
 162 pre-treated with ABT-491 or saline 15 minutes prior to injection of α -Ly6G mAbs or isotype
 163 control. Mice were then sensitized with α -TNP IgG2a and challenged 16 hours later with
 164 TNP-BSA (E). Changes in body temperature were monitored after injection of α -Ly6G mAbs
 165 (F) and after challenge with TNP-BSA (G). Data are pooled from at least two different
 166 experiments, means \pm s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ vs. respective isotype
 167 control group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.005$ between saline, α -Ly6G and ABT-491, α -
 168 Ly6G groups (in F and G) using Mann-Whitney test (two-tailed).