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1 **Preventive nasal administration of flagellin restores antimicrobial effect of gentamicin and**
2 **protects against a multidrug resistant strain of *Pseudomonas aeruginosa***

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12 Running Title: Treatment with flagellin has anti-pseudomonal effect

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16
17 *# In this work, A.R. Maia and A. Cezard are co-first authors and M. Si-Tahar and V. Hervé are
18 co-senior authors. The order of authors was established according to their involvement in the
19 assembly and refinement of the manuscript.

20

21 **Abstract**

22 The increasing prevalence of multidrug-resistant *Pseudomonas aeruginosa* (PA) is a significant
23 concern for chronic respiratory disease exacerbations. Host-directed drugs such as flagellin, an agonist
24 of Toll-like receptor 5 (TLR5), have emerged as a promising solution. In this study, we evaluated the
25 prophylactic intranasal administration of flagellin against a multidrug-resistant strain of PA (PA^{MDR}) in
26 mice and assessed the possible synergy with the antibiotic gentamicin (GNT). The results indicated that
27 flagellin treatment before infection decreased bacterial load in the lungs, likely due to an increase in
28 neutrophil recruitment, and reduced signs of inflammation, including proinflammatory cytokines. The
29 combination of flagellin and GNT showed a synergistic effect, decreasing even more the bacterial load
30 and increasing mice survival rates, in comparison with mice pre-treated only with flagellin. These
31 findings suggest that preventive nasal administration of flagellin could restore the effect of GNT against
32 MDR strains of PA, paving the way for the use of flagellin in vulnerable patients with chronic
33 respiratory diseases.

34 **Introduction**

35 *Pseudomonas aeruginosa* (PA) is one of the leading causes of hospital-acquired infections
36 globally and the most common pathogen associated with respiratory chronic illnesses, such as cystic
37 fibrosis (CF) (1–3). Overuse of antimicrobial agents during the past decades has resulted in the emergence
38 of PA resistant strains, particularly to β -lactams, carbapenems, and aminoglycosides antibiotics (4).
39 Multidrug-resistant (MDR) strains of PA are even harder to eradicate and cause higher mortality rates (5,
40 6). As a consequence, the World Health Organization (WHO) has included MDR strains of PA in a list
41 of pathogens requiring urgent attention for the development of new therapeutic strategies (7).

42 Approaches that can enhance and rescue antibiotic efficacy are of significant interest and are
43 increasingly being studied (8). In this context, host-directed molecules in combination with antibiotics
44 are a promising strategy for preventing infections and restoring antibiotic effectiveness. Flagellin, a
45 structural component of the flagellum of Gram-negative bacteria, has emerged as a promising host-
46 directed immunomodulatory drug (9). In particular, the recombinant form of flagellin (flagellin $\Delta_{174-400}$),
47 which lacks its antigenic part, has been described for its immuno-stimulatory capacity and antimicrobial
48 properties against intestinal infections caused by *Enterococcus faecium*, *Clostridium difficile*, and
49 *Escherichia coli* (10–12), and against respiratory infections caused by *Streptococcus pneumoniae* (13).

50 In the airways, the mechanism of action of flagellin $\Delta_{174-400}$ (hereafter only designated as flagellin)
51 is well-described. Flagellin binds to Toll-like receptor 5 (TLR5), a pattern recognition receptor (PRR)
52 present in epithelial cells (ECs), alveolar macrophages (AM), conventional dendritic cells (cDCs), and
53 lymphocytes (14). This interaction strongly activates the transcription factor nuclear factor-kappa B
54 (NF κ B), Mitogen-activated protein kinases (MAPK), and interferon (IFN)-regulatory factor (IRF)
55 pathways, resulting in the transcription of genes encoding several immune mediators (15). This includes
56 chemokines such as CXCL1, CXCL2, CXCL5, CCL2 and CCL20, cytokines such as Interleukin-6 (IL-
57 6), Granulocyte-macrophage colony-stimulating factor (GM-CSF), and Interleukin-1 beta (IL-1 β) as well
58 as mucins and several antimicrobial peptides such as β -defensins (16–18).

59 It has been demonstrated that flagellin triggers a short-lived response (peaking 2 hours after
60 administration and decaying 2-3 hours after the peak) due to strong feedback regulatory mechanisms
61 (López-Gálvez *et al.*, 2021). This controlled response is crucial because it allows the inflammation to
62 resolve towards homeostasis. Interestingly, the same study also shows that a second stimuli with flagellin
63 (either with soluble protein or PA infection) leads to a less intense proinflammatory state, suggesting that
64 a single prophylactic flagellin instillation can reduce the inflammatory response to PA (20).

65 Previous studies have also shown that nasal administration of flagellin has a synergistic effect
66 with the antibiotic amoxicillin in controlling antibiotic-resistant pneumococcal infections (13). Other data
67 have suggested that prophylactic nasal administration of flagellin can decrease the bacterial load of pigs
68 infected with the *P. aeruginosa* (PAK) strain (20). However, flagellin has never been tested in
69 combination with an antibiotic to fight PA and was never evaluated against a MDR strain of PA.

70 Here, using an *in vivo* mouse model, we show that while prophylactic flagellin alone attenuates
71 infection caused by a MDR strain of PA (PA^{MDR}), its combination with the antibiotic gentamicin (GNT)
72 leads to a strong decrease in bacterial load in the lung and a significant reduction in cell infiltration and
73 inflammatory cytokines. Moreover, mice receiving flagellin in combination with GNT showed a 100%
74 survival rate, a result that GNT alone was unable to provide against PA^{MDR}.

75

76 **Materials and Methods**

77 **1.1 Microbial strain and culture conditions**

78 *Pseudomonas aeruginosa* multidrug-resistant strain was isolated from a clinical patient and
79 kindly provided by Dr. Katy Jeannot (CHRU Besançon, France). This strain is multidrug resistant
80 (denominated in this study as PA^{MDR}) including a resistance to gentamicin (MIC > 4 µg/mL), and
81 its characterization is in Supplementary Table 1 (done by microbiology service of CHRU
82 Bretonneau (Tours, France)) and Supplementary Figure 1. *Pseudomonas aeruginosa* PAO1 strain
83 was kindly provided by Dr. Sophie Bleves (CNRS & Aix-Marseille Université, Laboratoire
84 d'Ingénierie des Systèmes Macromoléculaires (UMR7255), Institut de Microbiologie de la
85 Méditerranée, Marseille, France)

86 **1.2 Flagellin**

87 The custom-designed flagellin used in the present study, i.e., FLAMOD (recombinant
88 flagellin FliC Δ 174-400 as described in Nempont *et al.*, 2008 and harboring one extra amino acid
89 at the N terminus) derives from *Salmonella enterica* serovar Typhimurium FliC. It was produced
90 in inclusion bodies in *Escherichia coli* by the Vaccine Development department at Statens Serum
91 Institut, Denmark. FLAMOD (in this work only denominated flagellin) was purified by filtration
92 and chromatography and resuspended in NaPi buffer (10 mM phosphate, 145 mM NaCl,
93 polysorbate 80 0.02 % (w/v) pH 6.5). The immunostimulatory activity was validated using the
94 HEK-DualTM hTLR5 cell assay (Invivogen). The low endotoxin content in the protein preparation
95 was assessed with a *Limulus* assay (Pyrochrome, kinetic LAL assay from Associates of Cape Cod
96 Inc.).

97 **1.3 Susceptibility testing**

98 Broth microdilution method was used for the determination of minimal inhibitory (MIC) of
99 gentamicin against PA^{MDR}. Minimal inhibitory concentrations were determined in accordance with
100 the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Briefly, PA strain was
101 cultured on Tryptic Soy Agar (TSA) plates overnight. Three isolated colonies were suspended in 3
102 mL of LB medium and grown overnight at 37°C, under agitation (200 rpm). Then several dilutions
103 of this fresh suspension were prepared and incubated at 37°C for 4 hours, under agitation (200
104 rpm). The suspension with OD₆₀₀ between 0.3 and 0.6, representing the exponential phase of
105 growth, was centrifuged 10 minutes at 3000 g. Bacteria were suspended in MH medium to obtain
106 approximately 2x10⁵ CFU/mL (CFU for colony forming unit). The inoculum size was verified by
107 plating 5-fold dilutions on TSA plates and incubating overnight at 37°C for CFU counts. Hundred
108 microliters/well of the bacterial suspension were inoculated into 96-well microtiter plate and 100
109 µL/well of MH (control) or gentamicin were added in duplicate for each condition. The microtiter
110 plate was incubated in a plate reader (TECAN Infinite 200, Lyon, France) for 24 h at 37°C. The
111 absorbance at OD₆₀₀ was read at 30-min intervals.

112 **1.4 Animal care and handling**

113 C57Bl/6 female mice were purchased from the Centre d'Elevage R. Janvier (Le Genest Saint-
114 Isle, France) and were used at about 8 weeks of age. All procedures were in accordance with the
115 European animal welfare regulations. All mice were housed under specific-pathogen-free
116 conditions at the PST "Animaleries" animal facility (Université de Tours, France) and had access
117 to food and water *ad libitum*. All experiments complied with the French government's ethical and
118 animal experiment regulations (APAFIS#201604071220401.V2-4885 and 2016111512369894 V3
119 - 7590).

120 Mice were treated with 10 µg of recombinant flagellin (FLAMOD) 24h before infection with
121 5x10⁵ or 8x10⁵ CFU/mouse of PA^{MDR} by the nasal route (Figure 1A). One-hour post-infection, mice
122 were treated by intraperitoneal injection (i.p.) with 15 mg/kg of gentamicin purchased from Sigma
123 Aldrich (Sant-Quentin Fallavier, France). The choice of gentamicin dose was based on previous
124 experiments *in vivo* showing that a single dose of 15 mg/kg i.p. was the minimum able to decrease
125 PA^{MDR} bacterial load (in mice infected with 10⁵ CFU/mouse). Sixteen hours after challenge with
126 PA^{MDR}, mice were sacrificed and airways were washed four times with 0.5 mL of Phosphate
127 Buffered Saline (PBS) for bronchioalveolar lavage (BAL) collection. After centrifugation at 400g
128 for 5 min, BAL fluids were stored at -80°C for subsequent measurement of inflammatory mediators
129 and pellets were recovered in PBS 2% Fetal Bovine Serum (FBS). The pellet was resuspended and
130 filtered, and erythrocytes were discarded using a red blood cell lysis buffer. Leukocytes were
131 counted and analyzed by flow cytometry. After BAL recovery, lungs were perfused with 10 mL
132 PBS injected into the heart. The lungs were photographed for inflammation assessment based on
133 lung morphology and color. The right lungs were homogenized with 1 mL of PBS using the Gentle
134 MACSTM Octo Dissociator (Miltenyi Biotech) and 100 µL of lung suspension was plated for
135 bacterial count.

136 **1.5 Analysis of synergy between flagellin and gentamicin**

137 The effect of treatments (standalone Flagellin, standalone GNT or combination
138 Flagellin+GNT) against PA^{MDR} was quantified as the percentage of bacterial growth (%_{growth}),
139 corresponding to the ratio of the mean bacterial load in the lungs of infected and treated mice to
140 that of infected and untreated mice (control). For example, the effect of treatment A was calculated
141 as follows: %growth_[A] = (mean CFU_[A]/mean CFU_[control]) × 100. The predicted additive effect (or
142 predicted %_{growth}) of a combination treatment was calculated as described previously (22). Briefly,
143 the predicted %_{growth} of a treatment combining compounds A and B is the product of the
144 experimentally defined %_{growth} values for each standalone treatment (predicted %_{growth}[A+B]
145 = %_{growth}[A] × %_{growth}[B]). If the experimental %_{growth} for the combination treatment is lower
146 or higher than the predicted %_{growth}, then the two drugs are synergistic or antagonistic,
147 respectively. When the experimental and predicted %_{growth} values are identical, the two drugs'
148 effects are additive.

149 **1.6 Flow cytometry analysis**

150 Bronchioalveolar lavage from all mice was dispensed into round bottomed 96-well plates
151 and were centrifuged at 500 g at 4°C for 5 min. Samples were further stained using specific
152 antibodies and appropriate isotype controls. To identify total leukocytes, neutrophils, total dendritic
153 cells and alveolar macrophages, cells were stained with a lineage cocktail containing anti-CD45
154 (APCVio770 - clone 30-f11, BD cat.no. 45-04551-82), anti-CD11b (PE - clone M1/70, BD cat.no.
155 553311), anti-CD11c (PEVio770 - (clone N418, Bio cat.no. 117318), anti-Ly6G (APC - clone 1A8,
156 BD cat.no. 5605599), anti- SiglecF (VioBlue - clone E50-2440, BD cat.no. 565934), anti-MHCII
157 (PerCP - clone AF6-120.1, BD cat.no. 46-5320-82), and LIVE/DEAD™ Fixable Aqua Dead Cell
158 Stain Kit (Thermo Fisher Scientific, cat.no. L34966). Flow cytometry data were acquired on a
159 MACSQuant® Analyzer and analyses were performed using the VenturiOne software according
160 to the gating strategy described in Supplementary Figure 2.

161 **1.7 ELISA**

162 BAL were centrifuged 5 min at 500 g and supernatants were stored at -80°C. DuoSet ELISA
163 (Mouse IL-6, IL-1β, TNF-α and GM-CSF) were performed according to the manufacturer's (R&D
164 Systems) instructions.

165 **1.8 Statistical analysis**

166 Sample size estimation was based on previous animal studies conducted by our group using
167 *P. aeruginosa*-infection mice model. There was no randomization or blinding procedure. Statistical
168 analyses were performed using GraphPad Prism. Data are reported as mean ± SEM. Statistical
169 values, including the number of replicates and the statistical test used, can be found in the figure
170 legends. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.

171 **Results**

172 **The combination of preventive flagellin and the antibiotic gentamicin displays synergistic**
173 **therapeutic activity against a MDR strain of *P. aeruginosa***

174 To evaluate the impact of preventive intranasal flagellin in combination with the antibiotic
175 gentamicin against PA^{MDR} we compared mice that received both prophylactic flagellin (24 hours
176 before infection) and GNT 1-hour post-infection (“Flagellin+GNT group”) with groups of mice
177 that received either prophylactic flagellin alone (“Flagellin group”), or mice only treated with GNT
178 (“GNT group”), or infected non-treated mice (“Untreated group”). We also considered animals that
179 were neither treated nor infected as healthy (“Healthy group”) (see Figure 1A). We assessed
180 bacterial load and inflammatory status in the BAL fluids of these mice 16 hours post-infection (i.e.
181 40 hours after flagellin administration).

182 As expected, infected untreated mice presented a high bacterial load in the lungs 16 hours
183 after PA^{MDR} challenge (4.5×10^6 CFU \pm 1.7×10^6) (see Figure 1B). Although GNT treatment was not
184 able to significantly decrease the bacterial load of infected mice, prophylactic administration of
185 flagellin significantly reduced PA^{MDR} CFU ($p < 0.05$). Interestingly, when mice received the
186 combination of flagellin and GNT, they had much lower bacterial CFU (a 400-fold reduction
187 compared to infected non-treated mice; $p < 0.001$). This result suggests a significant therapeutic
188 advantage for the combination treatment compared to flagellin or GNT treatments alone.

189 When two drugs are administered in combination, their effect may have an additive mode of
190 action, or they may work in synergy to improve the host's condition. Hence, the interaction between
191 two drugs is classified as synergistic when the total effect of the two drugs combined is greater
192 than that predicted by their individual potencies. Using the equation described in the materials and
193 methods, the expected percentage of bacterial growth when combining flagellin and GNT should
194 be 9.4% (55% growth for GNT x 17% growth for flagellin = 9.4%). However, Flagellin+GNT
195 combination therapy led to only 0.2% of PA^{MDR} growth compared to the control (infected untreated
196 mice). Since the experimental percentage of growth is lower than the theoretical predicted value,
197 there is a synergistic effect between flagellin and GNT in controlling *P. aeruginosa* growth.

198
199 **Preventive intranasal administration of flagellin decreases proinflammatory cytokines and**
200 **cell infiltration but increases neutrophils and dendritic cells in the BAL of PA^{MDR}-infected**
201 **mice**

202 Using the same experimental design as described above, we examined the inflammatory
203 status of mice. We analyzed the macroscopic aspect of their lungs (Figure 2A), immune cell
204 populations recruitment (Figure 2B), and the secretion of proinflammatory cytokines in BAL
205 (Figure 2C), at 16 hours post-infection, for all experimental groups.

206 Figure 2A shows the difference between representative lungs of a mouse from each group.
207 As expected, untreated mice infected with PA^{MDR} showed a higher extent of gross pathology and

208 hemorrhage. Interestingly, mice only treated with GNT experienced similar macroscopic lung
209 pathology than did the untreated mice. While animals pre-treated with flagellin before infection
210 exhibited a clear reduction in hemorrhage, the reduction was even more reduced in animals that
211 received the biotherapy of flagellin and GNT.

212 The total number of cells and leukocytes (CD45+) in the BAL (Figure 2B.1 and 2B.2,
213 respectively) was analyzed by flow cytometry using the gating strategy described in Supplementary
214 Figure 2. We observed that animals treated with flagellin, with or without GNT, presented
215 significantly fewer cells in the BAL at 16 hours post-infection, compared to infected non-treated
216 or those only treated with GNT ($p < 0.05$ for group Flagellin vs. group Untreated and $p < 0.001$ for
217 group Flagellin+GNT vs. group GNT). Interestingly, among the cells present in the BAL,
218 significantly more were CD45+ immune cells for the animals that received preventive flagellin
219 ($p < 0.01$ for group Flagellin vs. group Untreated and $p < 0.001$ for group Flagellin+GNT vs. group
220 GNT).

221 According to the study of Fougeron *et al.* (2015), the number of neutrophils and total DCs in
222 the lungs of mice remain elevated for approximately 72 hours after being challenged nasally with
223 flagellin. In this context, we investigated both immune cell populations (neutrophils identified as
224 CD45⁺CD11b⁺Ly6G⁺ and total DCs as CD45⁺CD11c⁺SiglecF⁺MHCII⁺) in the BAL (Figure 2B.3
225 and 2B.4, respectively). As expected, *P. aeruginosa* infection induced neutrophil recruitment to
226 the airways. The mucosal delivery of flagellin (in combination or not with GNT) induced an even
227 higher increase in the number of neutrophils ($p < 0.01$ for group Flagellin vs. group Untreated and
228 $p < 0.001$ for group Flagellin+GNT vs. group GNT). Flagellin instillation also caused an increase in
229 total DCs ($p < 0.01$ for group Flagellin vs. group Untreated and $p < 0.001$ for group Flagellin+GNT
230 vs. group GNT). Regarding AM (identified as CD45⁺CD11c⁺SiglecF⁺; Figure 2B.5), infection with
231 PA^{MDR} decreased the number of this cell population in BAL (a 5-fold reduction compared to the
232 non-infected group) and treatment with the antibiotic GNT was not enough to revert such drop.
233 Pre-treatment with flagellin (in combination or not with GNT) preserved the level of AM ($p < 0.01$
234 for group Flagellin vs. group Untreated and $p < 0.001$ for group Flagellin+GNT vs. group GNT).

235 Sixteen hours after challenge with PA^{MDR}, infected non-treated animals presented high levels
236 of IL-6, Tumor Necrosis Factor alpha (TNF- α), GM-CSF and IL-1 β 16h post-infection (Figure
237 2C). Whereas GNT-treated mice also presented high proinflammatory cytokines, mice that
238 received flagellin (in combination or not with GNT) showed a significant reduction for all tested
239 cytokines ($p < 0.01$ or $p < 0.001$). Thus, flagellin pretreatment reduced the expression of
240 proinflammatory cytokines triggered by PA^{MDR} infection. The combination of flagellin and GNT
241 fully restores the level of pro-inflammatory mediators released from the lungs of PA^{MDR}-infected
242 mice to a homeostatic basal level.

243 **Combination of prophylactic flagellin and therapeutic gentamicin protects mice against a** 244 **lethal dose of a MDR strain of *P. aeruginosa***

245 We evaluated the effectiveness of the Flagellin+GNT combination treatment in protecting
246 mice against a lethal PA^{MDR} infection. To this end, we monitored mice survival over 60 hours post

247 bacterial infection (Figure 3A). As anticipated for a MDR strain of PA, most infected non-treated
248 mice succumbed to the infection within 24 hours (Figure 3B). Similarly, the mice only treated with
249 GNT showed similar susceptibility to bacterial infection. However, prophylactic flagellin treatment
250 alone was sufficient to significantly protect the mice against the PA infection (80% survival for
251 group “Flagellin” vs. 20% survival for group “GNT”, $p < 0.001$). Moreover, the combination of
252 prophylactic flagellin with therapeutic GNT resulted in full protection compared to non-treated
253 animals (Figure 3B).

254

255 **Discussion**

256 In the present study we used an *in vivo* mouse model to investigate the effect of prophylactic
257 nasal administration of flagellin in combination, or not, with the antibiotic GNT against a MDR
258 strain of *P. aeruginosa* (PA^{MDR}). We demonstrated that (i) a single prophylactic respiratory
259 administration of flagellin was sufficient to decrease bacterial load in the lungs and, when
260 combined with GNT 1h post-infection, this reduction was even more accentuated, which suggests
261 a synergistic effect between the two treatments; (ii) pre-treatment with flagellin, in combination or
262 not with GNT, reduced inflammation but increased the number of neutrophils, total DCs and
263 maintained AM levels in the BAL, 16h post-infection.; and (iii) combination of prophylactic
264 flagellin and GNT 1h post-infection led to 100% survival, contrary to the control groups.

265 Several studies have shown that nasal administration of flagellin can reduce bacterial load
266 in an animal infection model (11, 17, 24, 25). For example, the study from Muñoz N., *et al.*
267 demonstrated that respiratory treatment with flagellin leads to *S. pneumoniae* clearance in the lungs
268 and promotes survival of infected mice. They attributed such results to increased neutrophil
269 infiltration into the airways as a result of strong chemokine secretion by flagellin-stimulated ECs.
270 Our findings are consistent with such studies. We observed that animals pre-treated with flagellin
271 24 hours before infection, receiving or not GNT 1-hour after challenge, presented significantly less
272 PA^{MDR} bacterial load and higher neutrophil counts in the BAL, compared with infected non-treated
273 mice or only treated with GNT. Neutrophils play a critical role in combating infections through
274 their antimicrobial functions. Particularly in the context of *P. aeruginosa*, it has been demonstrated
275 that early neutrophil influx to the respiratory airways is essential for the clearance of the pathogen
276 (12, 26, 27). Accordingly, we hypothesize that, the rise in neutrophil counts in flagellin-treated
277 mice could play a role in the decrease of *P. aeruginosa* CFU in those same mice. The secretion of
278 antimicrobial compounds by ECs (Kinnebrew *et al.*, 2010 and Yu *et al.*, 2010) may also contribute
279 to the reduction of PA^{MDR}. To fully comprehend the effect of antimicrobial effectors in our model,
280 additional experiments should be carried out.

281 While standalone flagellin was effective in clearing PA^{MDR}, our study showed that the
282 combination of prophylactic flagellin and therapeutic GNT confers an additional advantage. Mice
283 treated with this combination showed an even greater reduction in lung bacterial load compared to
284 those treated with flagellin alone, suggesting a synergy between the antibiotic and flagellin. Since

285 we did not find any significant difference in number of immune cell populations, particularly
286 neutrophils, between mice treated with flagellin alone and those treated with Flagellin+GNT, the
287 observed additional advantage in the combination group may be attributed to the direct antibiotic's
288 impact, despite GNT's limited efficacy against PA^{MDR} when used alone. We hypothesize that the
289 prophylactic nasal administration of flagellin may have contributed not only to the recruitment of
290 phagocytes to the site of infection but also to the secretion of multiple antimicrobial peptides, which
291 in turn lead to the elimination of, at least, some of the bacteria present in the respiratory tract. When
292 GNT was administered, 1h after infection, less viable bacteria in the flagellin-regulated lung
293 mucosa enabled the antibiotic to efficiently clear most of the remaining bacteria.

294 Thus, our work is reminiscent of previous studies showing that a host-directed
295 immunomodulator can contribute to the clearance of a pathogen, facilitating the action of an
296 antibiotic (13, 28–31). Secoeudesma sesquiterpenes lactone A (SESLA) is an anti-inflammatory
297 molecule proved to modulate NFκB and PI3K/Akt signaling pathways (31). When in simultaneous
298 combination with the antibiotic meropenem, it promotes bacterial clearance and survival in mice
299 infected with carbapenem-resistant *Klebsiella pneumoniae*. Recently, a similar strategy was found
300 to be efficacious in the context of intestinal infections with *Salmonella* Typhimurium (28). Indeed,
301 the combination of systemic administration of ciprofloxacin and a TLR4 or TLR9 agonist was able
302 to further reduce the number of antibiotic-tolerant *Salmonella* in the gut and in the associated
303 draining lymph nodes, compared with standalone treatments (28). Another study also showed that
304 the simultaneous combination of flagellin and the antibiotic amoxicillin, administered 12 hours
305 post-infection, can provide additional protection by effectively eliminating a resistant strain of *S.*
306 *pneumoniae* (13).

307 A host-directed immunomodulator, when paired with an antibiotic, should also promote a
308 balanced inflammatory response and confer long-lasting immunity (32, 33). In our study,
309 respiratory administration with flagellin 24-hours before challenge, either alone or in combination
310 with GNT, effectively reduced excessive local inflammation caused by PA^{MDR} infection. Mice
311 receiving the combination of flagellin and GNT exhibited even lower tissue hemorrhage, a decrease
312 in total cell infiltration in the BAL, and a return of proinflammatory cytokine levels to steady state
313 by 16 hours post-infection. These findings are in alignment with a recent investigation conducted
314 by López-Gálvez R. *et al* (2021). Using airway epithelial cells and a pig model, their research
315 demonstrates that flagellin serves as an immunostimulant molecule capable of eliciting a transient
316 proinflammatory reaction. This proinflammatory stimulus plays a pivotal role initiating an efficient
317 host immune response towards a forthcoming pathogen. However, because flagellin's
318 proinflammatory stimulus is short-lived and the molecule itself is also rapidly degraded within the
319 airway mucosa, a few hours after its administration there is no longer inflammation driven by
320 flagellin. They also show that, particularly in the context of *P. aeruginosa* infection, a first
321 prophylactic stimulus with flagellin is enough to attenuate *P. aeruginosa*-triggered inflammatory
322 response. Although the detailed mechanism is still unclear, López-Gálvez R. *et al* (2021) suggest
323 that epigenetic changes or trained immunity caused by flagellin (also present in *P. aeruginosa*
324 flagellum) may also contribute to restrain inflammation upon *P. aeruginosa* challenge (34, 35). For
325 example, the research of Bigot and colleagues demonstrated that bronchial epithelial cells pre-

326 exposed with flagellin modify their inflammatory response to a second stimulus due to epigenetic
327 regulation (35). The observed decreased inflammatory response could also be related to TLR5
328 signalling desensitization due to a decrease in receptor availability or tachyphylaxis, as observed
329 when repeated doses of TLR ligands are administered to mice (36). In addition, it has been shown
330 that flagellin treatment increased concentration of TNFAIP3. This key negative regulator of the
331 innate immune response, can decrease the signalling of both TLR4 and TLR5 pathways, thereby
332 modifying the ability of airway epithelial cells to respond to a second stimulation by *P. aeruginosa*
333 (37, 38).

334 Our findings also demonstrate that preventive administration of flagellin can enhance the
335 preservation of AM, which were diminished following *P. aeruginosa* infection. Such apparent
336 reduction has also been previously reported in the context of Influenza and SARS-Cov-2 infection
337 (39, 40). While we have yet to fully elucidate the precise mechanisms underlying this decrease in
338 our PA^{MDR} infection model, our data strongly suggests that nasal flagellin administration
339 effectively mitigates AM reduction. The advantages of preserving AM in the context of respiratory
340 infections are vast (41). These cells are the first to encounter pathogens in the alveoli. After an
341 antigen exposure, AM become activated and not only are able to internalize and kill pathogens but
342 also produce several proinflammatory cytokines and chemokines that recruit neutrophils to the site
343 of infection, helping with clearance. Thus, flagellin-dependent AM preservation may contribute to
344 the reduction of *P. aeruginosa* infections.

345 It has been shown that total DCs, which include monocyte derived-DCs and conventional
346 DCs are increased in the airway mucosa after stimulus with flagellin. Although we did not
347 discriminate DC subtypes in the current study, we also observed an increase in these immune cell
348 population in animals that were nasally challenged with flagellin, 24h before infection, (receiving
349 or not GNT 1h post-infection). These results may indicate possible long-lasting immunity driven
350 by prophylactic nasal flagellin against a MDR strain of *P. aeruginosa*, although further studies
351 would have to be conducted to confirm such hypothesis.

352 Finally, the beneficial synergy between flagellin and gentamicin was evidenced in a
353 survival study. The combination between flagellin and GNT protected all infected mice, which was
354 not observed in any other group. The observed survival outcome may be attributed to the
355 synergistic effects of flagellin and the restoration of GNT effectiveness, involving several
356 mechanisms: (i) flagellin enhances the influx of neutrophils into the lungs, (ii) these cells aid in
357 bacterial clearance, (iii) the reduced bacterial burden increases the antibiotic-to-bacteria ratio,
358 thereby restoring the efficacy of GNT, and (iv) flagellin's prophylactic effect mitigates excessive
359 inflammation in the lungs, ultimately contributing to improved host survival. Such results highlight
360 the advantageous synergy between the two molecules and underline the importance of investigating
361 such combination further. Moreover, our work extends the current knowledge about the beneficial
362 effect of flagellin as host-directed immunomodulator to MDR strains of *P. aeruginosa*, which
363 present a significant challenge for patients with chronic respiratory conditions and limited
364 treatment options. Although more research is required to confirm the feasibility of prophylactic
365 flagellin administration in patients, this study provides a basis for a novel dual therapy against *P.*
366 *aeruginosa* in the context of increasing antimicrobial resistance.

367

368 **Author Contributions**

369 A.R.M., A.C., J.-C.S., V.H. and M.S.-T. designed the study. A.R.M., A.C., D.F., V.V. and B.B.
370 performed the experiments. A.R.M., A.C., V.H. and M.S.-T. wrote the manuscript. All authors
371 analysed the data, contributed to the article and approved the submitted version.

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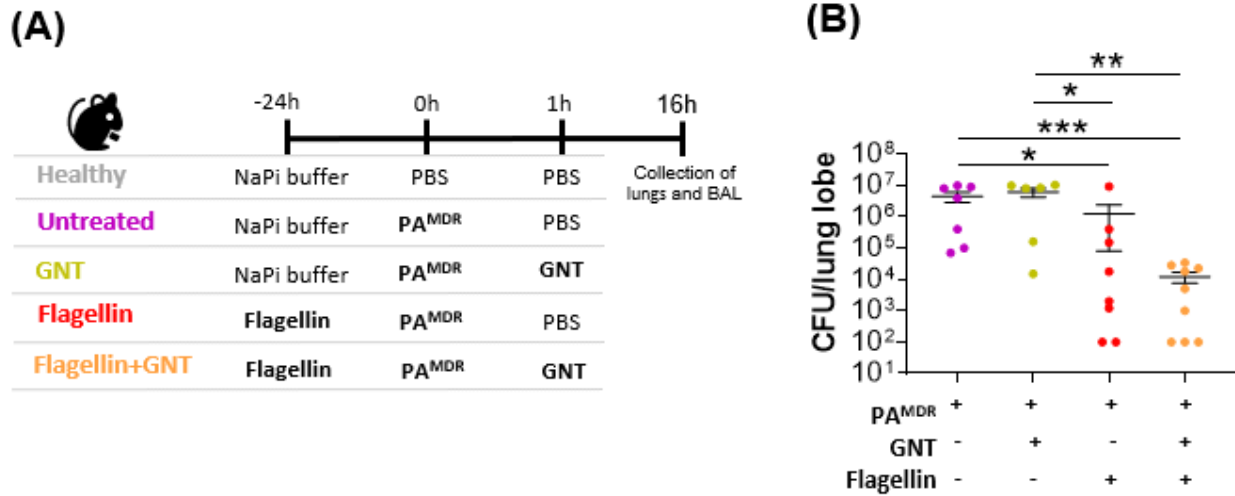
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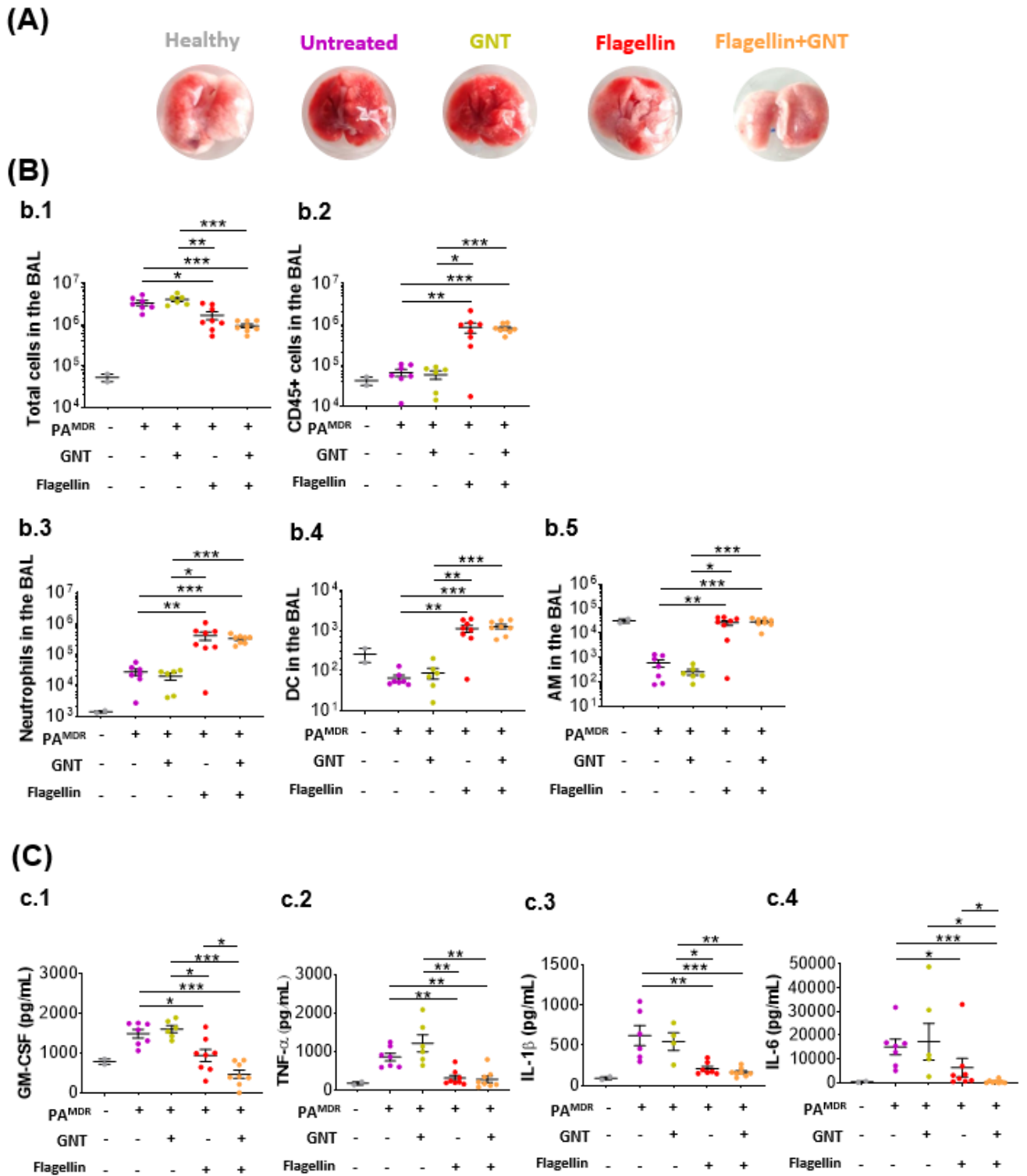
593 **FIGURE 1.**



594 **FIGURE 1. Timeline for treatment and infection of mice and bacterial load 16h post-infection.**

595 (A) Twenty-four hours before infection mice in group “Untreated” (n=7) and “GNT” (n=6) received NaPi
 596 buffer and mice in groups “Flagellin” (n=8) and “Flagellin+GNT” (n=9) received FliC (10µg), resuspended
 597 in NaPi buffer, by the nasal route. At T0 all animals (with the exception of controls in “Healthy” group)
 598 were intranasally infected with 5x10⁵ CFU of a MDR strain of *P. aeruginosa*. One hour after infection,
 599 mice in group GNT and Flagellin+GNT received GNT 15mg/Kg by intraperitoneal injection. Healthy mice
 600 were used as control (group “Healthy”). All mice were sacrificed 16h p.i. (B) Colony forming units
 601 (CFU)/lung lobe were counted in all infected mice. Each symbol represents an individual animal in 2
 602 independent experiments. Differences between groups were analysed with Mann-Whitney test. Data are
 603 reported as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

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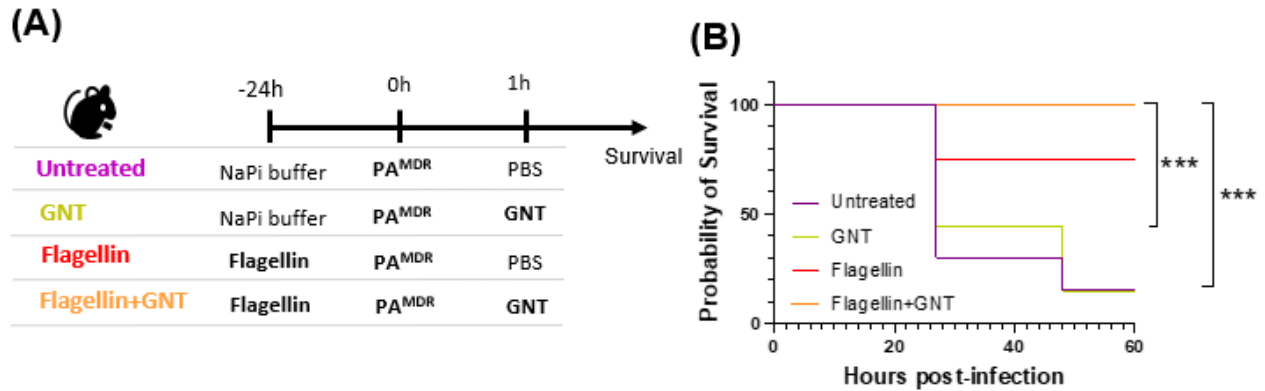
FIGURE 2.

612 **Figure 2. Macroscopic aspect of lungs, immune cell populations recruitment and the secretion of proinflammatory cytokines in BAL.**

Twenty-four hours before infection with 5×10^5 CFU of a MDR *P. aeruginosa* strain, mice were treated or not with flagellin (10 μ g) intranasally. Then, 1-hour post infection, mice were treated intraperitoneally or not with Gentamicin (GNT, at 15mg/Kg body weight). All mice were sacrificed 16h p.i.. Lungs were perfused for visual assessment of inflammation (**A**) and BAL fluids were collected to determine the number of immune and inflammatory cells by flow cytometry (**B**) and the levels of GM-CSF, TNF- α , IL-1 β and IL-6 (**C**). All data are represented as the mean \pm SEM and are cumulative of 2 independent experiments. The number of mice per group is indicated by symbols (grey (n=2), purple (n=7), green (n=6), red (n=8) and orange (n=8)). Statistical analysis was performed using the Mann-Whitney test. *p < 0.05, **p < 0.01, ***p < 0.001, .****p < 0.0001

613 **FIGURE 3.**

614



615 **Figure 3. Timeline for treatment and infection of mice and survival study.**

616 (A) Twenty-four hours before infection, C57Bl/6 mice received flagellin (10µg) (red (n=5) and orange (n=7)
 617 groups) resuspended in NaPi buffer, or only NaPi buffer (purple (n=7) and green (n=7) groups) by the nasal
 618 route. At T0 all animals were intranasally infected with 8x10⁵ CFU of a MDR strain of *P. aeruginosa*. One
 619 hour after infection mice received GNT 15mg/kg by intraperitoneal injection (green and orange groups) or
 620 PBS (purple and red groups). (B) Animal survival were monitored daily. All data are represented are
 621 cumulative of 2 independent experiments. Statistical analysis was performed using the Log-rank (Mantel-
 622 Cox) test. *p < 0.05, **p < 0.01, ***p < 0.001, .****p < 0.0001

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