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

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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***

3  
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11  
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<sup>MDR</sup>) 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  $\beta$ -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 $\kappa$ 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 $\beta$ ) as well  
58 as mucins and several antimicrobial peptides such as  $\beta$ -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<sup>MDR</sup>), 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<sup>MDR</sup>.

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<sup>MDR</sup>) 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 $\Delta$ 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-Dual<sup>TM</sup> 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<sup>MDR</sup>. 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 OD<sub>600</sub> 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 2x10<sup>5</sup> 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 OD<sub>600</sub> 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 5x10<sup>5</sup> or 8x10<sup>5</sup> CFU/mouse of PA<sup>MDR</sup> 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<sup>MDR</sup> bacterial load (in mice infected with 10<sup>5</sup> CFU/mouse). Sixteen hours after challenge with  
126 PA<sup>MDR</sup>, 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<sup>MDR</sup> was quantified as the percentage of bacterial growth (%<sub>growth</sub>),  
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<sub>[A]</sub> = (mean CFU<sub>[A]</sub>/mean CFU<sub>[control]</sub>) × 100. The predicted additive effect (or  
142 predicted %<sub>growth</sub>) of a combination treatment was calculated as described previously (22). Briefly,  
143 the predicted %<sub>growth</sub> of a treatment combining compounds A and B is the product of the  
144 experimentally defined %<sub>growth</sub> values for each standalone treatment (predicted %<sub>growth</sub>[A+B]  
145 = %<sub>growth</sub>[A] × %<sub>growth</sub>[B]). If the experimental %<sub>growth</sub> for the combination treatment is lower  
146 or higher than the predicted %<sub>growth</sub>, then the two drugs are synergistic or antagonistic,  
147 respectively. When the experimental and predicted %<sub>growth</sub> 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<sup>MDR</sup> 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<sup>MDR</sup> challenge ( $4.5 \times 10^6$  CFU  $\pm$   $1.7 \times 10^6$ ) (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<sup>MDR</sup> 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<sup>MDR</sup> 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<sup>MDR</sup>-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<sup>MDR</sup> 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<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup> and total DCs as CD45<sup>+</sup>CD11c<sup>+</sup>SiglecF<sup>+</sup>MHCII<sup>+</sup>) 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<sup>+</sup>CD11c<sup>+</sup>SiglecF<sup>+</sup>; Figure 2B.5), infection with  
231 PA<sup>MDR</sup> 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<sup>MDR</sup>, infected non-treated animals presented high levels  
236 of IL-6, Tumor Necrosis Factor alpha (TNF- $\alpha$ ), GM-CSF and IL-1 $\beta$  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<sup>MDR</sup> infection. The combination of flagellin and GNT  
241 fully restores the level of pro-inflammatory mediators released from the lungs of PA<sup>MDR</sup>-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<sup>MDR</sup> 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<sup>MDR</sup>). 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<sup>MDR</sup> 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<sup>MDR</sup>. 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<sup>MDR</sup>, 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<sup>MDR</sup> 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<sup>MDR</sup> 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<sup>MDR</sup> 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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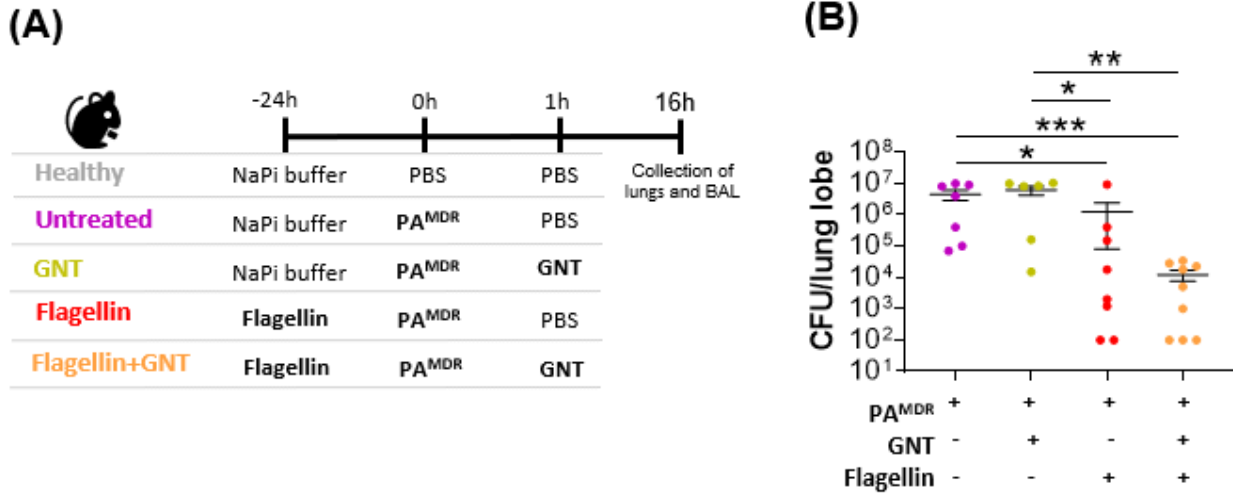
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593 **FIGURE 1.**

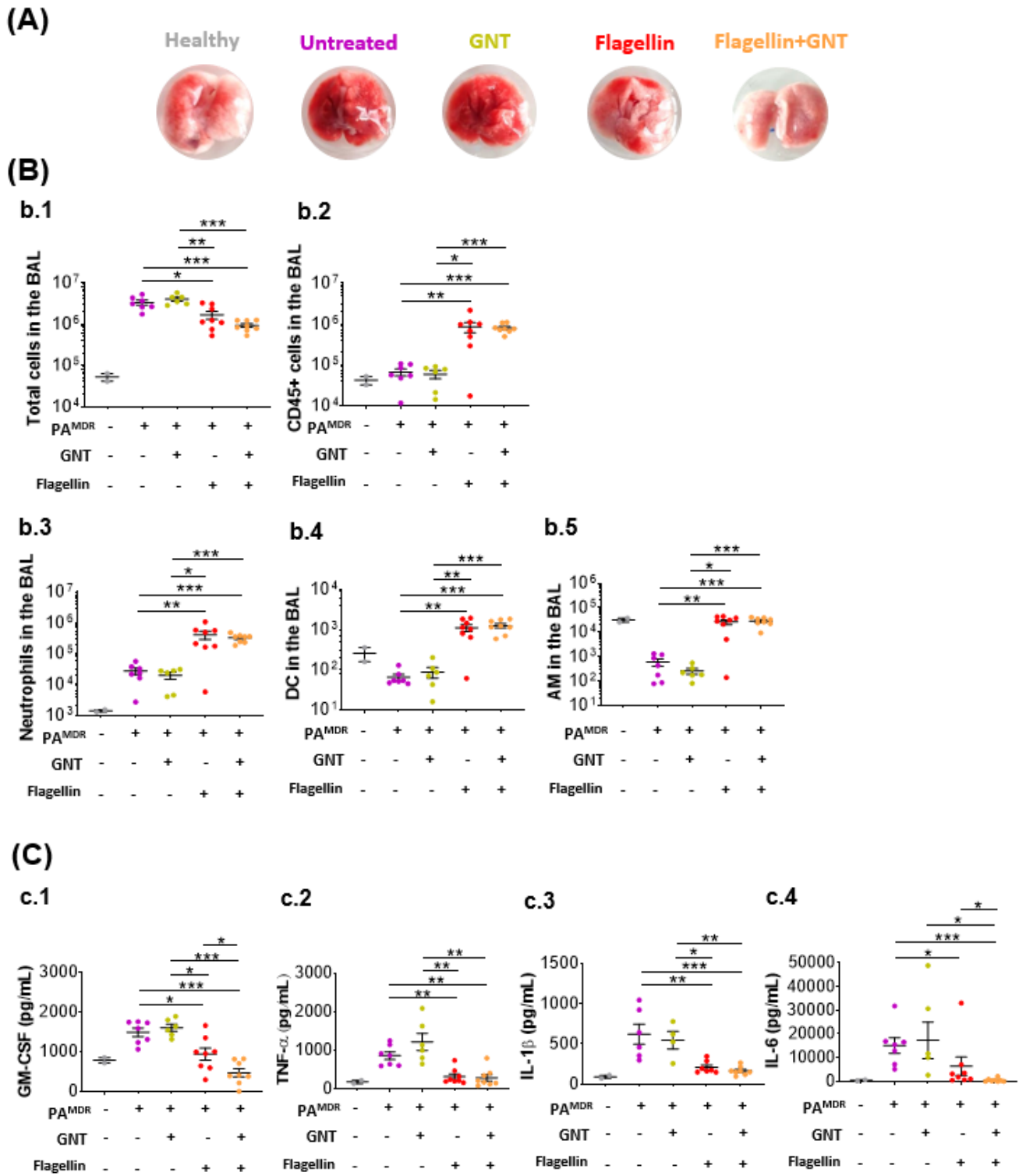


594 **FIGURE 1. Timeline for treatment and infection of mice and bacterial load 16h post-infection.**

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 5x10<sup>5</sup> 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.**



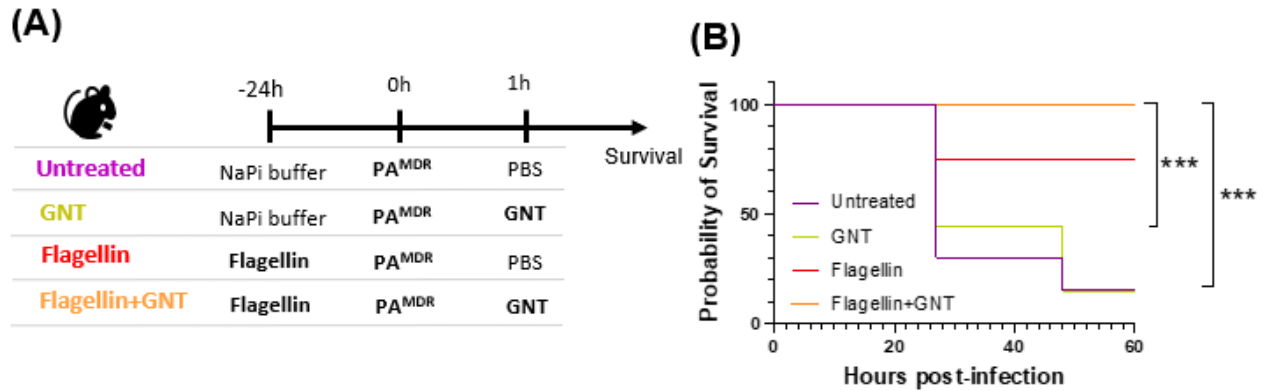


612 **Figure 2. Macroscopic aspect of lungs, immune cell populations recruitment and the secretion of proinflammatory cytokines in BAL.**

Twenty-four hours before infection with  $5 \times 10^5$  CFU of a MDR *P. aeruginosa* strain, mice were treated or not with flagellin (10 $\mu$ 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- $\alpha$ , IL-1 $\beta$  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.**

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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 8x10<sup>5</sup> 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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