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Bacteriophages immunomodulate the response of monocytes:

Short running title: Immunogenicity of bacteriophages

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ABSTRACT

Bacteriophages are present in fluids from cirrhosis patients. However, their effect on the immune response is unknown. In this work, we explore the role of phages in the phenotype, function and cytokine production of monocytes. We stimulated healthy monocytes with five different butanol-purified phage suspensions infective for Gram-negative and Gram-positive bacteria. We studied the expression of the monocyte markers involved in lipopolysaccharide recognition (LPS; CD14), antigen presentation (HLA-DR) and co-stimulation (CD86), and the concentration of induced cytokines (TNF-α, IFN-α and IL-10) by phages. To confirm the direct role of phages without the interference of contaminating soluble LPS in phage suspensions, polymyxin B was added to the cell cultures. Phagocytosis experiments were assessed by flow cytometry using labelled phage suspensions. We observed that butanol-purified phages reduced the surface levels of CD14 and CD86 in monocytes and increased the secreted levels of TNF-α and IL-10 compared with the control sample containing only butanol buffer. All phage suspensions showed downregulation of HLA-DR expression but only *Staphylococcus aureus* phage contaminated with *Escherichia coli* reached statistical significance. The addition of polymyxin B did not restore the monocytic response induced by phages, suggesting that the effect was not caused by the presence of LPS. Monocytes were able to phagocyte phages in a dose- and time-dependent manner. To conclude, the phagocytosis of butanol-purified phages altered the phenotype and cytokine production of monocytes suggesting they become tolerogenic.

Keywords: Bacteriophages; Immunity; Cirrhosis

49 **IMPACT STATEMENT**

50 It is known that patients with liver cirrhosis have phages in their ascitic fluids. Our work demonstrates

51 that phages induce an immune response in healthy monocytes, in terms of phenotype, production of

52 soluble factors and phagocytosis function. Furthermore, our work validates the usefulness of

53 polymyxin B and 1-butanol protocol as easy strategies to discard the interference of bacterial products

54 in the phage suspensions.

55

56 **INTRODUCTION**

57 Bacteriophages, also called phages, are viruses present in 45% of the ascitic fluid (AF) samples from

58 patients with spontaneous bacterial peritonitis (SBP).[1, 2] SBP, which is the most severe complication

59 in patients with liver cirrhosis, is generated by the translocation of bacteria from the gut to the

60 peritoneal cavity caused by an increased intestinal permeability.[3] The diagnosis of SBP is based on a

61 positive bacteriological culture result and a count of polymorphonuclear cells above 250 cells/mm^3 in

62 the AF. However, bacteria are not detected in a half of the patients with >250 cells/mm^3 in AF.

63 Interestingly, these patients have a similar severity and prognosis than those patients with positive

64 bacteriological result, [4] suggesting that not only the presence of bacteria in AF induces an immune

65 response.

66 Bacterial cells are infected **by specific phages through lysogenic cycles and then, bacteria are**

67 **lysed through lytic cycles.**[5] Although phages do not infect eukaryotic cells, immune cells can be

68 stimulated directly by phages or indirectly by the massive pathogen-associated molecular patterns

69 (PAMPs), released after the lysis of phage-induced or phage-infected bacteria.[6] Phagocytes

70 (monocytes and neutrophils) are the main cell populations found in AF from patients with cirrhosis.

71 We have previously reported that ascitic monocytes from SBP expressed low levels of antigen

72 presentation (HLA-DR) and activation (CD86) markers. Furthermore, they produced high levels of

73 TNF-α and IL-10 and they have a reduced ability to phagocytose *Escherichia coli*.[7] Surprisingly,

74 ascitic monocytes from SBP patients with either positive or negative bacteriological culture show a

75 similar immune response.[7]

76 In the present study, we hypothesized that the immune response displayed by ascitic monocytes in

77 cirrhosis is not only due to the presence of bacteria but also to the presence of phages in the AF. To

78 confirm our hypothesis, first, healthy monocytes were stimulated with butanol-purified phage

79 suspensions and we studied the markers related with LPS receptor (CD14), antigen presentation

80 (HLA-DR) and co-stimulation (CD86), and the soluble factors (TNF-α, IFN-α and IL-10) observed in

81 the ascitic monocytes. Second, it was analyzed whether the immune response observed was phage-

82 host specific. Third, the interference of soluble LPS in the phage suspensions was discarded through

83 two strategies: by the addition of polymyxin B and using phage suspensions infecting Gram-positive

84 ***Staphylococcus aureus***, which do not contain LPS in their bacterial cell wall. Finally, the

85 phagocytosis was studied by flow cytometry using labelled phages in dose- and time-dependent

86 assays.

87

88 **MATERIALS AND METHODS**

89 **Bacteriophages and bacterial strains**

90 Five different phage suspensions were studied. They included three virulent phages infecting *E. coli*
91 WG5 (ATCC 700078) (phages SOM1, SOM3 and SOM4)[8] and one phage infecting *S. aureus* strain
92 RN4220 (phage ϕ11)[9] (Table 1). The fifth phage suspension contained the same *S. aureus* phage but
93 propagated in a *S. aureus* RN4220 culture contaminated with a sonicated culture of *E. coli* strain
94 WG5 (prepared as described in the following section). This phage suspension was included to confirm
95 that the butanol protocol removes the contaminating soluble lipopolysaccharide (LPS) from *E. coli*.
96 This is in contrast with the non-contaminated ϕ11 phage suspension where LPS could not be present
97 as *S. aureus* lacks LPS in its cell wall.

98 **Butanol-purification of phage suspensions**

99 Phage preparations were purified following the butanol protocol described by Szermer-Olearnik B et
100 al.[10] For it, bacteria culture in Luria broth was carried at 37°C for 8–16 hours, until the optical density
101 (OD, 600 nm) reached 0.3, which corresponded to about 10^8 bacterial cells/ml.[8] At this point the
102 culture was infected with phage in a proportion of 0.1 PFU/bacterial cells, and incubated at 37°C for 8
103 h. Crude bacterial lysates (5 ml–20 l) were filtered through 0.22 μm low protein binding
104 polyethersulfone (PES) membranes (Millex-GP, Merck). 1-butanol was added (about 40% v/v) to the
105 bacterial lysate and shaken for 1–3 hours at room temperature. Then, the two-phase mixture was
106 cooled to 4°C for 1–3 hours and separated by centrifugation at 4000 ×g, 10 min. The collected
107 aqueous phases were dialyzed in a buffer containing NaCl 0.15M and concentrated with Amicon
108 Ultra-15 Centrifugal Filters 50K (Millipore) to a final volume of 5 mL in NaCl 0.15M. A buffer
109 control using the same bacterial cultures in the absence of phages was processed in parallel and
110 included in the analysis. In parallel, one phage ϕ11 suspension was contaminated with *E. coli* to

111 include LPS in the suspensions and monitor its removal by the butanol protocol. An overnight culture

112 of *E. coli* strain WG5 containing 10^9 bacterial cells/ml was sonicated for 30 sec and placed on dry ice

113 for 30 sec in four consecutive steps to disrupt the cells. One ml of this culture was added to 200 ml

114 culture of *S. aureus* RN4220 containing phage ϕ11 culture in a proportion of 0.1 plaque-forming unit

115 (PFU)/bacterial cells, and incubated at 37°C for 8 h. Purification of phages was performed after

116 incubation as described above. After purification and concentration of the phage suspensions, phage

117 titer was determined using the double layer agar technique.[11]

118 **SYBR-Gold staining of phage suspensions**

119 The phage SOM1 and SOM3 suspensions were stained with SYBR-Gold (Molecular probes,

120 Thermofisher) as previously reported.[12] Briefly, 20 µl of SYBR-Gold 100X was added per ml of

121 phage suspension (109 PFU/ml). Suspensions were gently mixed and incubated 1h in the dark.

122 Suspensions were washed four times with NaCl 0.15M using Amicon Ultra-15 Centrifugal Filters

123 50K (Millipore) to remove the excess of SYBR-Gold and the suspension was obtained in a final

124 volume of 1 ml using NaCl 0.15M. After purification and concentration of the phage suspensions, the

125 total number of labelled phages was counted by flow cytometry and adjusted to ca 10^7 phage

126 particles/ml using buffer NaCl 0.15M.

127 A buffer control using culture media in the absence of phages was processed in parallel for SYBR

128 Gold staining and purification and included in the analysis.

129 **PBMCs isolation and stimulation with phage suspensions**

130 PBMCs were isolated from 10 ml of peripheral blood of healthy donors using a Lymphoprep gradient

131 (AXIS-SHIELD, PoCAs, Oslo, Norway). The total number of cells was counted by flow cytometry

132 and adjusted to $2x10^5$ monocytes/ml with RPMI medium supplemented with 25mM HEPES buffer

133 (hereafter referred as RPMI-HEPES medium; Sigma-Aldrich, St.Louis, MO). Subsequently, 100.000

134 PBMCs were stimulated with 1/100 of phage suspensions diluted in RPMI-HEPES medium in a total

135 volume of 200 µl in 96-well culture plates. As a negative control, PBMCs were also stimulated with

136 1/100 of the butanol buffer. Furthermore, to discard the possibility that soluble LPS molecules

137 remained in the purifications of phage infecting ***E. coli***, PBMCs were pre-cultured with 10 µg/ml of

138 polymyxin B (Sigma-Aldrich) for 30 minutes at 37ºC before the stimulation with phage suspensions.

139 Then, PBMCs were incubated for 24 hours at 37ºC. After incubation, cells were harvested from wells,

140 stained for 15 minutes at room temperature and darkness with anti-CD14 PECy7 (BioLegend), anti-

141 CD86 PE (BioLegend) and anti-HLA-DR APC (Immunotools), and washed with Phosphate Buffered

142 Saline (PBS 1X) before the acquisition by flow cytometry (MACSQuant Analyzer; Miltenyi,

143 Germany).

144 **Soluble factors measured in supernatants of phage stimulated PBMCs**

145 Supernatants of PBMCs stimulated with phage suspensions for 24 hours were collected and the levels

146 of TNF-α, IFN-α (BD Biosciences, San Diego, CA) and IL-10 (ImmunoTools, Friesoythe, Germany)

147 were measured by ELISA. The limit of detection was 30 pg/ml for TNF-α, 7 pg/ml for IFN-α and 9.4

148 pg/ml for IL-10. Supernatants were diluted 1/2 for TNF-α, 1/20 for IFN-α, and 1/10 for IL-10.

149 **Phagocytosis assay with labelled phage**

150 Phage suspensions containing 10^7 PFU/ml labelled with SYBR-Gold as described above were used

151 for the phagocytosis experiments. Two ml of whole blood were lysed using red blood cell lysis

152 (BioLegend, San Diego, CA). Phagocytes (monocytes and neutrophils) were counted by flow

153 cytometry and adjusted to 200.000 cells with RPMI-HEPES medium. Phagocytes were incubated in

154 96-well culture plates with 1/5, 1/10 and 1/20 labelled phage SOM1 and SOM3 for periods of 20, 40

155 and 120 minutes at 37ºC and at 4ºC to inhibit the phagocytosis process. After the incubation, cells

156 were harvested from wells, stained for 15 minutes at room temperature and darkness with anti-CD14-

157 PECy7 (BioLegend) and washed with PBS 1X before the acquisition by flow cytometry. Phagocytic

158 monocytes were defined as CD14 positive, low granularity and SYBR-Gold positive.

159 **Statistical analysis**

160 Experimental groups were compared by t-test for paired data with the respective negative controls.

161 Correlations were analysed by Spearman test. Significance was established at $p < 0.05$. Values were

162 expressed as mean ± SD.

163

164 **RESULTS**

165 **Effect of phages on the expression of monocyte markers**

166 It was first confirmed that either the butanol buffer used for the phage purification or the phage

167 suspensions did not affect to the viability, size, and granularity of CD14+ cells (monocytes) (**Figure**

168 **S1**). Upon stimulation, we found that all phage suspensions significantly reduced the percentage and

169 the expression of CD14 and CD86 compared with monocytes stimulated with buffer in the absence of

170 phages. **Only ϕ11 phage contaminated with *E. coli* significantly down-regulated HLA-DR**

171 **expression compared with buffer in absence of phages** (**Figure 1**).

172 To validate that the observed changes in monocytes were not due to contaminating LPS from Gram-

173 negative bacteria in the phage suspensions, we performed the monocyte stimulation in the presence of

174 polymyxin B (polyB). PolyB neutralizes the effect of LPS but at higher concentrations can also down-

175 regulate CD14 [13]. Therefore, we first tested the concentration of polyB to counteract the effect of LPS

176 without down-regulating CD14. At 10 µg/ml, polyB maintained the expression of CD14 and reverted

177 the downregulation of HLA-DR and CD86 produced by LPS (**Figure S2**). However, polyB did not

178 revert the reduction of CD14, HLA-DR and CD86 expression on monocytes after phage stimulus,

179 confirming that the specificity of immune response in monocytes was induced by the phages.

180 Furthermore, we did not observe significant differences between the *S. aureus* ϕ11 phage suspension

181 and *S. aureus* ϕ11 phage contaminated with *E. coli* (**Figure 1**). This observation validates that butanol

182 protocol removed efficiently soluble LPS from the phage suspensions, either those propagated in *E.*

183 *coli* or those in *S. aureus* that were contaminated with *E. coli*. Only the phage suspension SOM4

184 cultured in presence of polyB increased the levels of CD14 in monocytes compared with the culture

185 without polyB, but without reaching de CD14 levels observed under buffer conditions. However, the

186 effect of polyB in this phage suspension was not observed neither in HLA-DR nor in CD86 levels,

187 suggesting that only a minimal source of residual LPS could exist in this phage suspension.

188 **Effect of phages on the production of soluble factors by PBMCs**

189 The concentration of IFN-α, TNF-α and IL-10 produced by PBMCs stimulated with the five phage

190 suspensions was measured in the supernatants. IFN-α levels were undetectable in all the conditions in

191 PBMCs cultured neither with buffer alone nor upon stimulation with phage suspensions (**Figure 2A**).

192 All phage suspensions increased the TNF-α and IL-10 production by PBMCs compared with buffer

193 alone (**Figure 2B-C**). We have also demonstrated using butanol-purified phages that the presence of

194 polyB during the stimulation with phage suspensions did not revert the TNF-α and IL-10 levels.

195 Taking together the results about phenotype and soluble factors induced by phages, a negative

196 correlation between the expression of CD86 on monocytes stimulated with phage suspensions and the

197 TNF-α levels in their supernatants was observed (rho=-0.66, p=0.007) (**Figure S3**).

198 **Ability of monocytes to phagocytose phages**

199 The phage-monocyte interaction using labelled phages infecting Gram-negative (SOM4) and Gram-

200 positive (ϕ11) bacteria was assessed by flow cytometry. We observed an increased phagocytosis at the

201 highest phage concentration used (1/5) compared with the lowest phage concentration (1/20) and at 40

202 minutes of incubation (**Figure 3A**). Since phagocytosis and unspecific binding of phages to the cell

203 surface cannot be distinguished by flow cytometry, we repeated the experiment at 4ºC, a temperature

204 at which phagocytosis activity does not take place, to confirm that monocytes efficiently phagocyte

205 phages in an early and dose-dependent process (**Figure 3B**).

206

207 **DISCUSSION**

208 In the present study, by using butanol-purified phages, we have demonstrated that phages are able to

209 induce in monocytes a tolerant immune response after being phagocytosed. This response is similar to

210 that observed in our previous paper about ascitic monocytes from cirrhotic patients. It is mainly

211 characterized by a reduction in the expression of CD14 and CD86 and an increase in the soluble TNF-

212 α and IL-10 levels. Furthermore, we have validated the butanol purification as a useful protocol to be

213 used in cell stimulation assays without any interference of free endotoxin in phage suspensions.

214 The reduction in CD14 and CD86 observed on monocytes stimulated by phages suggest a strategy of

215 phages to avoid being removed by the immune system through turning cells into tolerogenic state.

216 HLA-DR expression was also down-regulated by ϕ11 phage contaminated with *E. coli*. During an
217 infection, it is likely that the tolerogenic monocytes induced by phages favors the infection
218 progression. Our previous results in ascitic monocytes from infected SBP patients showed a similarly
219 reduced expression of CD14, HLA-DR and CD86 [7] than the response observed in healthy monocytes
220 stimulated by phages. Particularly, low HLA-DR levels in the ascitic monocytes from SBP with the
221 negative bacteriological result were associated with a high bacterial DNA burden.[14] However, studies
222 with isolated phages from infected fluids are needed to better understand their role during the
223 infection. It is also well known that soluble LPS from Gram-negative bacteria is a potent inductor of
224 the immune response. Our results demonstrate that polyB did not revert the changes induced by phage
225 suspensions in monocytes. Therefore, our work highlight the use of polyB as an easy strategy to
226 validate that the changes observed in immune cells after phage stimulations are not affected by
227 contaminating soluble LPS in phage suspensions.

228 We did not observe significant differences in the immune response induced in monocytes by the
229 different phages infecting the different bacterial hosts. Van Belleghem JD *et al.* have also observed
230 comparable induced immune responses by Gram-negative and Gram-positive phages.[15] One
231 possibility is that the changes in CD14, HLA-DR and CD86 expression are induced by phage proteins
232 common in all these phages. In any case, differences attributable to remaining **LPS** fragments from
233 the host bacteria in the phage suspensions can be ruled out.

234 In our assays, we were not able to detect IFN-α levels. This fact could be surprising since monocytes
235 and dendritic cells are able to produce type I IFN during viral infections. However, it could be

236 explained by the fact that IFN-α needs shorter stimulation times to be detected.[16] We found that phage
237 suspensions increased the TNF-α and IL-10 levels. IL-10 results are in line with the findings in
238 cirrhosis, since SBP patients had also an increased ascitic IL-10 levels compared with patients without
239 ascitic infection. However, patients with SBP did not show any difference in TNF-α levels compared
240 with patients without ascitic infection.[7] This finding can be explained by the elevated production of
241 inflammatory mediators that patients with cirrhosis display regardless of any ascitic infection.[17]
242 According to our results, both pro- and anti-inflammatory gene expression profiles of PBMCs
243 stimulated with CsCl-purified phages are reported,[15] supporting the results about the immunogenicity
244 of phages. Taking together the phenotypic changes and the soluble factors induced, we found a
245 negative correlation between CD86 and TNF-α levels. This finding is consistent with the
246 downregulation of CD86 on monocytes by TNF-α described in patients infected by human
247 immunodeficiency virus (HIV). This phenotypic change induces an altered production of IL-2 and,
248 consequently, results in a deficient proliferative response of lymphocytes.[18] It is likely that phages in
249 SBP induce soluble factors that contribute to the tolerant state of ascitic monocytes and, consequently,
250 favor the infection.

251 We have validated a flow cytometry assay to study the phage-monocyte interaction through
252 phagocytosis assays with labelled phage suspensions. We have observed the phagocytosis of phages
253 by monocytes at short times. According to other reports using microscopic analysis, phagocytosis of
254 phages is an early process since, at longer times of incubation, the intracellular destruction of phages
255 begins.[19, 20] In line with these results, we did not detect phagocytosis of phages at longer times of
256 incubation (120 minutes). In cirrhosis, we have previously shown that ascitic monocytes from patients

with SBP had impaired phagocytosis of *E. coli.*[7] It is possible that the tolerant state induced by phages also contributes to the impaired phagocytosis of monocytes against bacterial infection. The outcome for the phages is to reduce their own elimination by monocytes while promoting the survival of their bacterial host. This hypothesis was demonstrated by Sweere JM *et al.* when observed that the presence of phages reduced the phagocytosis of bacteria.[21]

Finally, our work suggests that phages infecting Gram-negative and Gram-positive bacteria turn healthy monocytes into a tolerogenic state. We can speculate that in certain infection-associated pathologies, such as cirrhosis, ~~that~~ not only bacteria but also phages ~~in AF~~, could induce an immune response in monocytes to avoid the clearance of the infection. However, further studies using isolated phages from infected fluids are needed to support our hypothesis.

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282
283 **FIGURE LEGENDS**
284 **Figure 1. Changes in monocyte markers expression induced by butanol-purified Gram-negative**
285 **and Gram-positive phages. (A)** Percentage of $CD14^{+}$, **(B)** $CD14^{+}HLA\text{-}DR^{+}$ and **(C)** $CD14^{+}CD86^{+}$
286 cells from PBMCs stimulated with 1/100 butanol buffer and 1/100 butanol-purified phages without
287 adding polymixin B (polyB; solid bar) or after adding polyB (stripe pattern). **(D)** Representative flow
288 cytometry image of $CD14^{+}CD86^{+}$ cells from PBMCs stimulated with 1/100 butanol buffer or 1/100
289 SOM1 phage with or without polyB. *<0.05; **<0.01; ***<0.001; *Buffer vs Phage; $^{\#}$Buffer with polyB
290 vs Phage with polyB; $^{\$}$Phage vs Phage with polyB.
291 **Figure 2. Inflammation induced by butanol-purified Gram-negative and Gram-positive phages**.
292 **(A)** IFN-α **(B)** TNF-α and **(C)** IL-10 levels measured by ELISA assays in the supernatants of PBMCs
293 stimulated with 1/100 butanol-purified phages without (solid bar) or after adding polyB (stripe
294 pattern). *<0.05; **<0.01; ***<0.001; *Buffer vs Phage; $^{\#}$Buffer with polyB vs Phage with polyB;
295 $^{\$}$Phage vs Phage with polyB.
296 **Figure 3. Phagocytosis of gram-negative and gram-positive phages by monocytes. (A)** Kinetic
297 assay of phagocytosis: Percentage of phagocytic monocytes cultured with 1/5, 1/10 and 1/20 of
298 SYBR-Gold -labeled Gram-negative (SOM4) and Gram-positive (ϕ11) phage for 20 and 40 minutes

299 at 37ºC. Phagocytosis was determined using flow cytometry and was expressed as the percentage of

300 phagocytic monocytes stained with anti-CD14. *<0.05, Phagocytosis 1/5 vs 1/10 or 1/20. **(B)**

301 Temperature assay to determine the non-specific phagocytosis of phages: monocytes were cultured

302 with 1/5, 1/10 and 1/20 of SYBR-Gold -labeled gram-negative (SOM4) and gram-positive (ϕ11)

303 phage for 40 minutes at 4ºC to inhibit the mechanism of phagocytosis. *<0.05, **<0.01, Phagocytosis

304 at 37ºC vs phagocytosis at 4ºC.

305

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364 **Table 1. Characteristics of the different phages used in the cell stimulations.**

Bacteriophage	Phage family	Tail type	Source	Bacterial host strain	Titer after purification (PFU/ml)	Reference
SOM1	*Siphoviridae*	Curly	Sewage	*E. coli* WG5 ATCC 700078	8.0×10^7	(8) Muniesa et al., 2003
SOM3	*Myoviridae*	Contractile	Sewage	*E. coli* WG5 ATCC 700078	7.0×10^8	(8) Muniesa et al., 2003
SOM4	*Siphoviridae*	Flexible	Sewage	*E. coli* WG5 ATCC 700078	4.2×10^8	(8) Muniesa et al., 2003
ϕ11	*Siphoviridae*	Flexible	*S. aureus* RN451	*S. aureus* RN4220	3.2×10^8	(22) Ubeda et al., 2005

365 [1] PFU, Plaque-forming unit

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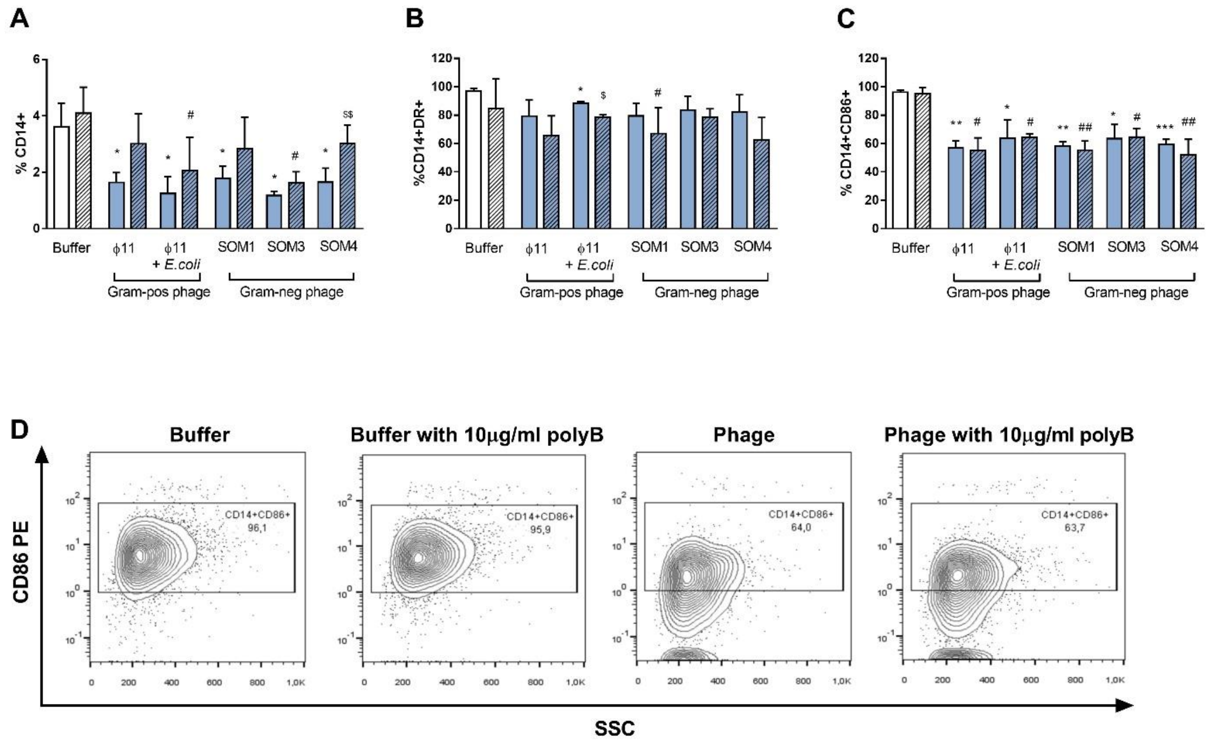
376 **FIGURES**



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378 **Figure 1**

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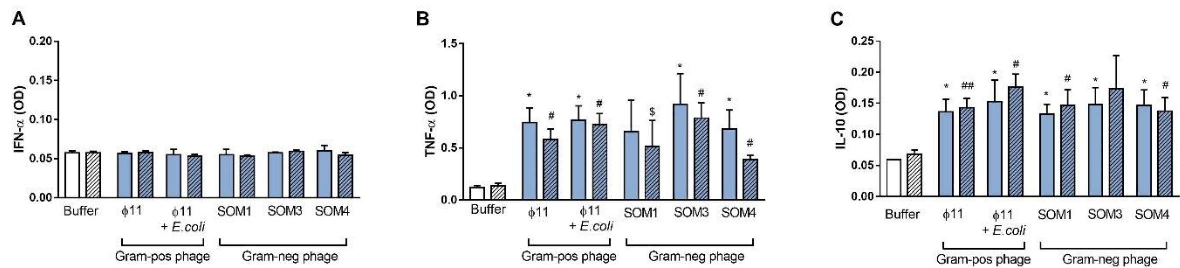

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382 **Figure 2.**

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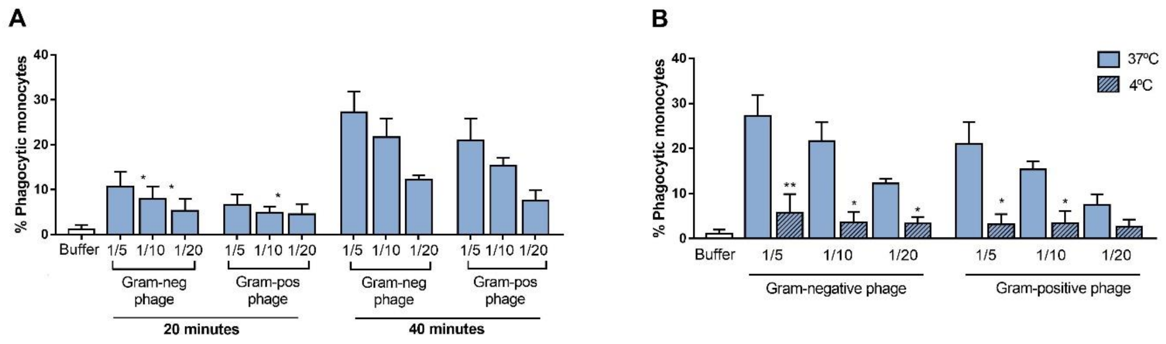

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387 **Figure 3.**

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