Bacteriophages immunomodulate the response of monocytes

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Abstract: (1) Background: Bacteriophages are present in the ascitic fluid of patients with cirrhosis. We reported that ascitic monocytes from these patients have characteristic phenotypes, functions, and cytokine responses. Here, we explore whether phages induce the immune response observed in ascitic monocytes. **(2) Methods**: We stimulated healthy monocytes with five different butanol-purified phage suspensions infective for gram-negative and gram-positive bacteria. We studied the expression of markers involved in the lipopolysaccharides recognition (LPS; CD14), antigen presentation (HLA-DR) and co-stimulation (CD86), and the cytokines induced in presence of phages (TNF-α, IFN-α and IL-10). Polymyxin B was added to the cell cultures to discard the interference of soluble LPS in phage suspensions. Phagocytosis using labelled phages was assessed by flow cytometry. **(3) Results**: Upon stimulation, 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 containing only butanol buffer. Polymyxin B did not restore the monocytic response induced by phages. Monocytes were able to phagocyte phages in a dose- and time-dependent manner. **(4) Conclusions**: Butanol-purified phages are phagocytosed by monocytes and induce a phenotype and cytokine production similar to the response observed in ascitic monocytes from patients with cirrhosis.

Keywords: Bacteriophages; Immunity; Cirrhosis

1. Introduction

Bacteriophages, also called phages, are viruses present in 45% of the ascitic fluid (AF) samples from patients with spontaneous bacterial peritonitis (SBP) [1,2]. SBP, which is the most severe complication in patients with liver cirrhosis, is generated by the translocation of bacteria from the gut to the peritoneal cavity caused by an increased intestinal permeability [3]. The diagnosis of SBP is based on a positive bacteriological culture result and a count of polymorphonuclear cells above 250 cells/mm^3 in the AF. However, bacteria are not detected in a half of the patients with >250 cells/mm^3 in AF. Interestingly, these patients have a similar severity and prognosis than those patients with positive bacteriological result [4], suggesting that not only the presence of bacteria in AF induces an immune response.

Bacterial cells are infected and lysed by specific phages through lysogenic and lytic cycles [5]. Although phages do not infect eukaryotic cells, immune cells can be stimulated directly by phages or indirectly by the massive pathogen-associated molecular patterns (PAMPs), released after the lysis

47 of phage-induced or phage-infected bacteria [6]. Phagocytes (monocytes and neutrophils) are the
48 main cell populations found in AF from patients with cirrhosis. We have previously reported that
49 ascitic monocytes from SBP expressed low levels of antigen presentation (HLA-DR) and activation
50 (CD86) markers. Furthermore, they produced high levels of TNF-α and IL-10 and they have a reduced
51 ability to phagocytose *Escherichia coli* [7]. Surprisingly, ascitic monocytes from SBP patients with
52 either positive or negative bacteriological culture show a similar immune response [7].
53 In the present study, we hypothesized that the immune response displayed by ascitic monocytes
54 in cirrhosis is not only due to the presence of bacteria but also to the presence of phages in the AF. To
55 confirm our hypothesis, first, healthy monocytes were stimulated with butanol-purified phage
56 suspensions and we studied the markers related with LPS receptor (CD14), antigen presentation
57 (HLA-DR) and co-stimulation (CD86), and the soluble factors (TNF-α, IFN-α and IL-10) observed in
58 the ascitic monocytes. Second, it was analyzed whether the immune response observed was phage-
59 host specific. Third, the interference of soluble LPS in the phage suspensions was discarded through
60 two strategies: by the addition of polymyxin B and using phage suspensions infecting Gram-positive
61 *Staphylococcus aureus*, which do not contain LPS in their bacterial cell wall. Finally, the phagocytosis
62 was studied by flow cytometry using labelled phages in dose- and time-dependent assays.

63 **2. Materials and Methods**

64 *2.1. Bacteriophages and bacterial strains*

65 Five different phage suspensions were studied. They included three virulent phages infecting *E.*
66 *coli* WG5 (ATCC 700078) (phages SOM1, SOM3 and SOM4) [8] and one phage infecting *S. aureus*
67 strain RN4220 (phage ϕ11) [9] (**Table 1**). The fifth phage suspension contained the same *S. aureus*
68 phage but propagated in a *S. aureus* RN4220 culture contaminated with a sonicated culture of *E. coli*
69 strain WG5 (prepared as described in the following section). This phage suspension was included to
70 confirm that the butanol protocol removes the contaminating soluble lipopolysaccharide (LPS) from
71 *E. coli*. This is in contrast with the non-contaminated ϕ11 phage suspension where LPS could not be
72 present as *S. aureus* lacks LPS in its cell wall.
73

74 **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)[1]	Reference
SOM1	*Siphoviridae*	Curly	Sewage	*E. coli* WG5 ATCC 700078	8.0 x10^7	[8]
SOM3	*Myoviridae*	Contractile	Sewage	*E. coli* WG5 ATCC 700078	7.0 x10^8	[8]
SOM4	*Siphoviridae*	Flexible	Sewage	*E. coli* WG5 ATCC 700078	4.2 x10^8	[8]
ϕ11	*Siphoviridae*	Flexible	*S. aureus* RN451	*S. aureus* RN4220	3.2 x10^8	[10]

75 [1] PFU, Plaque-forming unit

76 *2.2. Butanol-purification of phage suspensions*

77 Phage preparations were purified following the butanol protocol described by Szermer-Olearnik
78 B *et al.* [11]. For it, bacteria culture in Luria broth was carried at 37°C for 8–16 hours, until the optical
79 density (OD, 600 nm) reached 0.3, which corresponded to about 10^8 bacterial cells/ml [8]. At this point
80 the culture was infected with phage in a proportion of 0.1 PFU/bacterial cells, and incubated at 37°C

for 8 h. Crude bacterial lysates (5 ml–20 l) were filtered through 0.22 μm low protein binding polyethersulfone (PES) membranes (Millex-GP, Merck). 1-butanol was added (about 40% v/v) to the bacterial lysate and shaken for 1–3 hours at room temperature. Then, the two-phase mixture was cooled to 4°C for 1–3 hours and separated by centrifugation at 4000 ×g, 10 min. The collected aqueous phases were dialyzed in a buffer containing NaCl 0.15M and concentrated with Amicon Ultra-15 Centrifugal Filters 50K (Millipore) to a final volume of 5 mL in NaCl 0.15M. A buffer control using the same bacterial cultures in the absence of phages was processed in parallel and included in the analysis.

In parallel, one phage ϕ11 suspension was contaminated with *E. coli* to include LPS in the suspensions and monitor its removal by the butanol protocol. An overnight culture of *E. coli* strain WG5 containing 10^9 bacterial cells/ml was sonicated for 30 sec and placed on dry ice for 30 sec in four consecutive steps to disrupt the cells. One ml of this culture was added to 200 ml culture of *S. aureus* RN4220 containing phage ϕ11 culture in a proportion of 0.1 plaque-forming unit (PFU)/bacterial cells, and incubated at 37°C for 8 h. Purification of phages was performed after incubation as described above.

After purification and concentration of the phage suspensions, phage titer was determined using the double layer agar technique [12].

2.3. SYBR-Gold staining of phage suspensions

The phage SOM1 and SOM3 suspensions were stained with SYBR-Gold (Molecular probes, Thermofisher) as previously reported [13]. Briefly, 20 μl of SYBR-Gold 100X was added per ml of phage suspension (10^9 PFU/ml). Suspensions were gently mixed and incubated 1h in the dark. Suspensions were washed four times with NaCl 0.15M using Amicon Ultra-15 Centrifugal Filters 50K (Millipore) to remove the excess of SYBR-Gold and the suspension was obtained in a final volume of 1 ml using NaCl 0.15M. After purification and concentration of the phage suspensions, the total number of labelled phages was counted by flow cytometry and adjusted to ca 10^7 phage particles/ml using buffer NaCl 0.15M.

A buffer control using culture media in the absence of phages was processed in parallel for SYBR Gold staining and purification and included in the analysis.

2.4. PBMCs isolation and stimulation with phage suspensions

PBMCs were isolated from 10 ml of peripheral blood of healthy donors using a Lymphoprep gradient (AXIS-SHIELD, PoCAs, Oslo, Norway). The total number of cells was counted by flow cytometry and adjusted to $2x10^5$ monocytes/ml with RPMI medium supplemented with 25mM HEPES buffer (hereafter referred as RPMI-HEPES medium; Sigma-Aldrich, St.Louis, MO). Subsequently, 100.000 PBMCs were stimulated with 1/100 of phage suspensions diluted in RPMI-HEPES medium in a total volume of 200 μl in 96-well culture plates. As a negative control, PBMCs were also stimulated with 1/100 of the butanol buffer. Furthermore, to discard the possibility that soluble LPS molecules remained in the purifications of phage infecting *E. coli*, PBMCs were pre-cultured with 10 μg/ml of polymyxin B (Sigma-Aldrich) for 30 minutes at 37ºC before the stimulation with phage suspensions. Then, PBMCs were incubated for 24 hours at 37ºC. After incubation, cells were harvested from wells, stained for 15 minutes at room temperature and darkness with anti-CD14 PECy7 (BioLegend), anti-CD86 PE (BioLegend) and anti-HLA-DR APC (Immunotools), and washed with Phosphate Buffered Saline (PBS 1X) before the acquisition by flow cytometry (MACSQuant Analyzer; Miltenyi, Germany).

2.5. Soluble factors measured in supernatants of phage stimulated PBMCs

128 Supernatants of PBMCs stimulated with phage suspensions for 24 hours were collected and the
129 levels of TNF-α, IFN-α (BD Biosciences, San Diego, CA) and IL-10 (ImmunoTools, Friesoythe,
130 Germany) were measured by ELISA. The limit of detection was 30 pg/ml for TNF-α, 7 pg/ml for IFN-
131 α and 9.4 pg/ml for IL-10. Supernatants were diluted 1/2 for TNF-α, 1/20 for IFN-α, and 1/10 for IL-
132 10.
133

134 *2.6. Phagocytosis assay with labelled phage*

135 Phage suspensions containing 10^7 PFU/ml labelled with SYBR-Gold as described above were
136 used for the phagocytosis experiments. Two ml of whole blood were lysed using red blood cell lysis
137 (BioLegend, San Diego, CA). Phagocytes (monocytes and neutrophils) were counted by flow
138 cytometry and adjusted to 200.000 cells with RPMI-HEPES medium. Phagocytes were incubated in
139 96-well culture plates with 1/5, 1/10 and 1/20 labelled phage SOM1 and SOM3 for periods of 20, 40
140 and 120 minutes at 37ºC and at 4ºC to inhibit the phagocytosis process. After the incubation, cells
141 were harvested from wells, stained for 15 minutes at room temperature and darkness with anti-CD14-
142 PECy7 (BioLegend) and washed with PBS 1X before the acquisition by flow cytometry. Phagocytic
143 monocytes were defined as CD14 positive, low granularity and SYBR-Gold positive.
144

145 *2.7. Statistical analysis*

146 Experimental groups were compared by t-test for paired data with the respective negative
147 controls. Correlations were analysed by Spearman test. Significance was established at $p < 0.05$.
148 Values were expressed as mean ± SD.

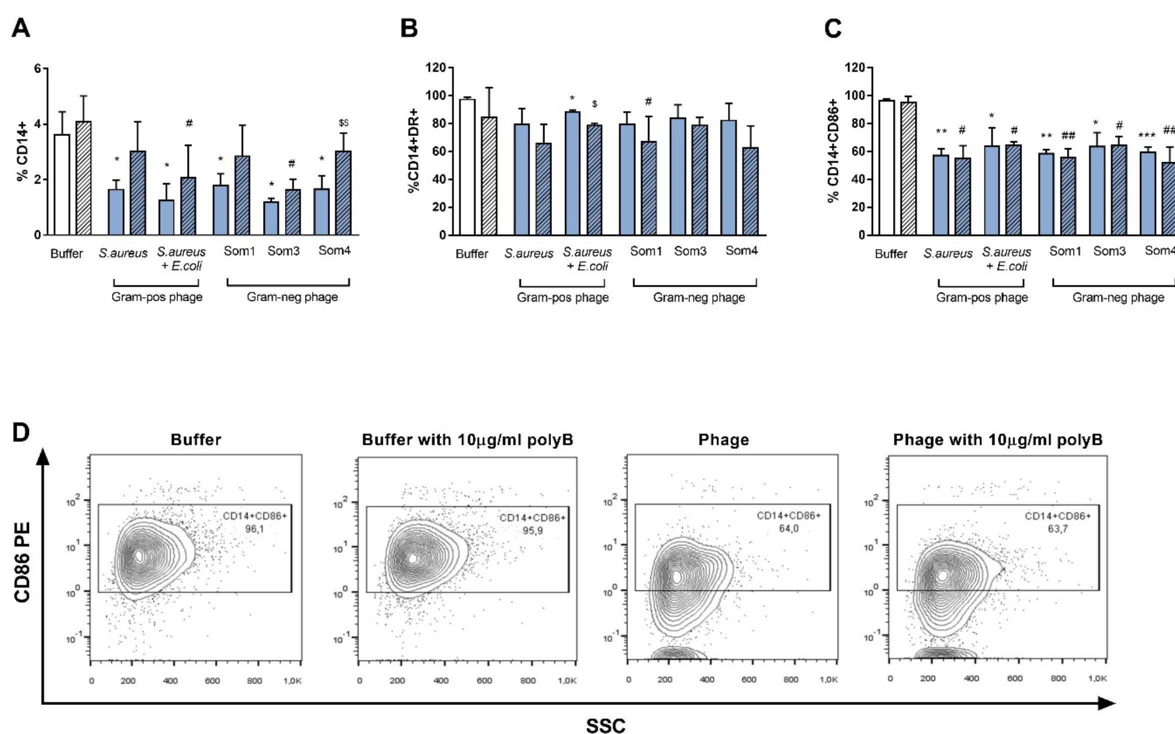
149 **3. Results**

150 *3.1. Effect of phages on the expression of monocyte markers*

151 It was first confirmed that either the butanol buffer used for the phage purification or the phage
152 suspensions did not affect to the viability, size, and granularity of CD14+ cells (monocytes) (data not
153 shown). Upon stimulation, we found that all phage suspensions significantly reduced the percentage
154 and the expression of CD14 and CD86 compared with monocytes stimulated with buffer in the
155 absence of phages. All phage suspensions also tended to down-regulate HLA-DR expression but only
156 ϕ11 phage contaminated with *E. coli* reached a statistical significance (**Figure 1**).

Figure 1. Changes in monocyte markers expression induced by butanol-purified Gram-negative and Gram-positive phages. (**A**) Percentage of CD14+, (**B**) CD14+HLA-DR+ and (**C**) CD14+CD86+ cells from PBMCs stimulated with 1/100 butanol buffer and 1/100 butanol-purified phages without adding polymyxin B (polyB; solid bar) or after adding polyB (stripe pattern). (**D**) Representative flow cytometry image of CD14+CD86+ cells from PBMCs stimulated with 1/100 butanol buffer or 1/100 SOM1 phage with or without polyB. *<0.05; **<0.01; ***<0.001; *Buffer vs Phage; #Buffer with polyB vs Phage with polyB; $Phage vs Phage with polyB

To validate that the observed changes in monocytes were not due to contaminating LPS from Gram-negative bacteria in the phage suspensions, we performed the monocyte stimulation in the presence of polymyxin B (polyB). PolyB neutralizes the effect of LPS but at higher concentrations can also down-regulate CD14 [14]. Therefore, we first tested the concentration of polyB to counteract the effect of LPS without down-regulating CD14. At 10 μg/ml, polyB maintained the expression of CD14 and reverted the downregulation of HLA-DR and CD86 produced by LPS (**Figure S1**). However, polyB did not revert the reduction of CD14, HLA-DR and CD86 expression on monocytes after phage stimulus, confirming that the specificity of immune response in monocytes was induced by the phages. Furthermore, we did not observe significant differences between the S. *aureus* ϕ11 phage suspension and *S. aureus* ϕ11 phage contaminated with *E. coli* (**Figure 1**). This observation validates that butanol protocol removed efficiently soluble LPS from the phage suspensions, either those propagated in *E. coli* or those in *S. aureus* that were contaminaded with *E. coli*. Only the phage suspension SOM4 cultured in presence of polyB increased the levels of CD14 in monocytes compared with the culture without polyB, but without reaching de CD14 levels observed under buffer conditions. However, the effect of polyB in this phage suspension was not observed neither in HLA-DR nor in CD86 levels, suggesting that only a minimal source of residual LPS could exist in this phage suspension.



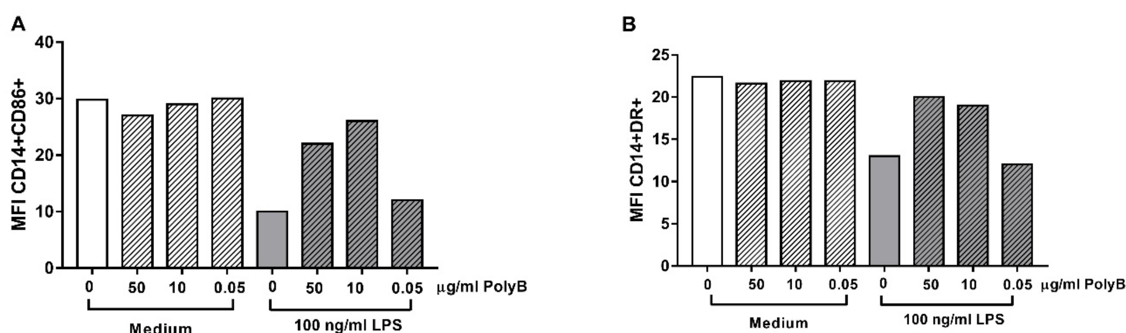


Figure S1. Confirmation of the efficiency of polymyxin B against LPS. PBMCs were cultured with RPMI-HEPES alone (white bar) or with 50, 10 and 0.05 µg/ml of polymyxin B (stripe pattern) for 30 minutes at 37ºC before the stimulation with 100 ng/ml LPS (grey bar) for 24h at 37ºC. The expression of (**A**) CD86 and (**B**) HLA-DR were analysed in CD14+ (monocytes).

3.2. Effect of phages on the production of soluble factors by PBMCs

The concentration of IFN-α, TNF-α and IL-10 produced by PBMCs stimulated with the five phage suspensions was measured in the supernatants. IFN-α levels were undetectable in all the conditions in PBMCs cultured neither with buffer alone nor upon stimulation with phage suspensions (**Figure 2A**). All phage suspensions increased the TNF-α and IL-10 production by PBMCs compared with buffer alone (**Figure 2B-C**). We have also demonstrated using butanol-purified phages that the presence of polyB during the stimulation with phage suspensions did not revert the TNF-α and IL-10 levels.

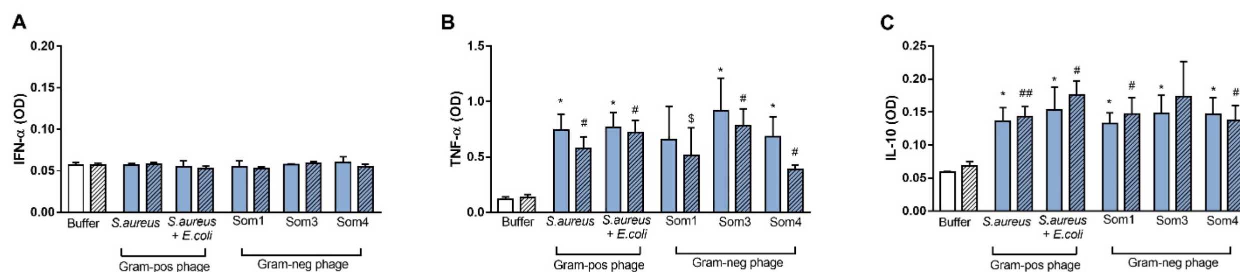


Figure 2. Inflammation induced by butanol-purified Gram-negative and Gram-positive phages. (**A**) IFN-α (**B**) TNF-α and (**C**) IL-10 levels measured by ELISA assays in the supernatants of PBMCs stimulated with 1/100 butanol-purified phages without (solid bar) or after adding polyB (stripe pattern). *<0.05; **<0.01; ***<0.001; *Buffer vs Phage; #Buffer with polyB vs Phage with polyB; $Phage vs Phage with polyB.

Taking together the results about phenotype and soluble factors induced by phages, a negative correlation between the expression of CD86 on monocytes stimulated with phage suspensions and the TNF-α levels in their supernatants was observed (rho=-0.66, p=0.007) (**Figure S2**).

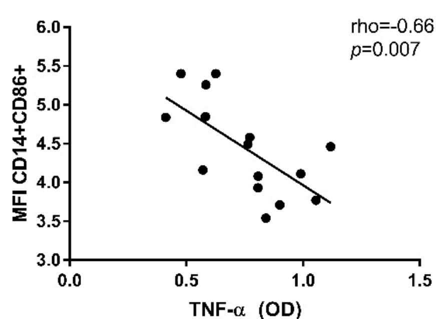


Figure S2. Relationship between the expression of CD86 in monocytes stimulated with the five phage suspensions and the levels of TNF-α produced in their supernatants.

3.3. Ability of monocytes to phagocytose phages

The phage-monocyte interaction using labelled phages infecting Gram-negative (SOM4) and Gram-positive (ϕ11) bacteria was assessed by flow cytometry. We observed an increased phagocytosis at the highest phage concentration used (1/5) compared with the lowest phage concentration (1/20) and at 40 minutes of incubation (**Figure 3A**). Since phagocytosis and unspecific binding of phages to the cell surface cannot be distinguished by flow cytometry, we repeated the experiment at 4ºC, a temperature at which phagocytosis activity does not take place, to confirm that monocytes efficiently phagocyte phages in an early and dose-dependent process. (**Figure 3B**).

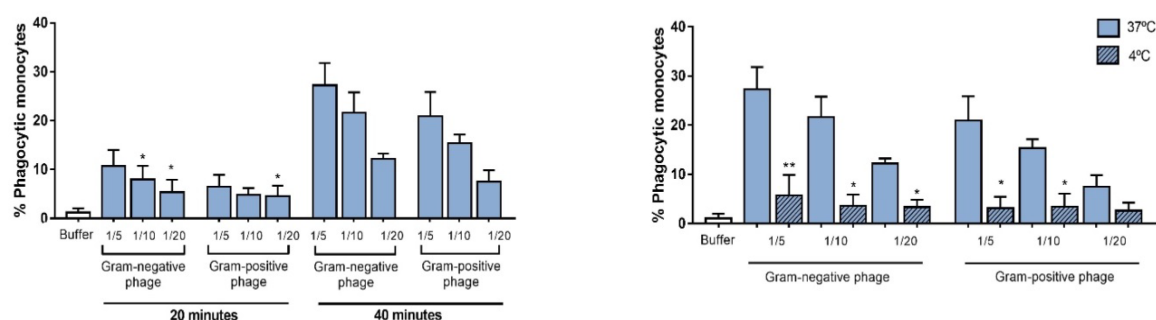


Figure 3. Phagocytosis of gram-negative and gram-positive phages by monocytes. (**A**) Kinetic assay of phagocytosis: Percentage of phagocytic monocytes cultured with 1/5, 1/10 and 1/20 of SYBR-Gold -labelled Gram-negative (SOM4) and Gram-positive (ϕ11) phage for 20 and 40 minutes at 37ºC. Phagocytosis was determined using flow cytometry and was expressed as the percentage of phagocytic monocytes stained with anti-CD14. *<0.05, Phagocytosis 1/5 vs 1/10 or 1/20. (**B**) Temperature assay to determine the non-specific phagocytosis of phages: monocytes were cultured with 1/5, 1/10 and 1/20 of SYBR-Gold -labelled gram-negative (SOM4) and gram-positive (ϕ11) phage for 40 minutes at 4ºC to inhibit the mechanism of phagocytosis. *<0.05, **<0.01, Phagocytosis at 37ºC vs phagocytosis at 4ºC.

4. Discussion

In the present study, by using butanol-purified phages, we have demonstrated that phages are able to induce in monocytes a tolerant immune response after being phagocytosed. This response is similar to that observed in our previous paper about ascitic monocytes from cirrhotic patients. It is mainly characterized by a reduction in the expression of CD14 and CD86 and an increase in the soluble TNF-α and IL-10 levels. Furthermore, we have validated the butanol purification as a useful protocol to be used in cell stimulation assays without any interference of free endotoxin in phage suspensions.

The reduction in CD14 and CD86 observed on monocytes stimulated by phages suggest a strategy of phages to avoid being removed by the immune system through turning cells into tolerogenic state. HLA-DR expression was also down-regulated by phages but only ϕ11 phage contaminated with *E. coli* reached a statistical significance. In cirrhosis, we have reported that ascitic monocytes from SBP patients had similarly reduced expression of CD14, HLA-DR and CD86 [7] than the response observed in healthy monocytes stimulated by phages. Particularly, low HLA-DR levels

in the ascitic monocytes from SBP with the negative bacteriological result were associated with a high bacterial DNA burden [15]. These data suggest that, during bacterial ascitic infection, the tolerant state of ascitic monocytes do not contribute to the removing of bacteria from the AF. It is well known that soluble LPS from Gram-negative bacteria is a potent inductor of the immune response. PolyB did not revert the changes induced by phage suspensions in monocytes. Therefore, our results highlight the use of polyB as an easy strategy to validate that the changes observed in immune cells after phage stimulations are not affected by contaminating soluble LPS in phage suspensions.

We did not observe significant differences in the immune response induced in monocytes by the different phages infecting the different bacterial hosts. Van Belleghem JD *et al.* have also observed comparable induced immune responses by Gram-negative and Gram-positive phages [16]. One possibility is that the changes in CD14, HLA-DR and CD86 expression are induced by phage proteins common in all these phages, but in any case, differences attributable to remaining fragments of the host bacteria in the phage suspensions can be ruled out.

In our assays, we did not detect IFN-α levels. This fact could be surprising since monocytes and dendritic cells are able to produce type I IFN during viral infections. However, it could be explained by the fact that IFN-α needs shorter stimulation times to be detected [17]. We found that phage suspensions increased the TNF-α and IL-10 levels. IL-10 results are in line with the findings in cirrhosis, since SBP patients had also an increased ascitic IL-10 levels compared with patients without ascitic infection. However, patients with SBP did not show any difference in TNF-α levels compared with patients without ascitic infection [7]. This finding can be explained by the elevated production of inflammatory mediators that patients with cirrhosis display regardless of any ascitic infection [18]. According to our results, both pro- and anti-inflammatory gene expression profiles of PBMCs stimulated with CsCl-purified phages are reported [16], supporting the results about the immunogenicity of phages. Taking together the phenotypic changes and the soluble factors induced, we found a negative correlation between CD86 and TNF-α levels. This finding is consistent with the downregulation of CD86 on monocytes by TNF-α described in patients infected by human immunodeficiency virus (HIV). This phenotypic change induces an altered production of IL-2 and, consequently, results in a deficient proliferative response of lymphocytes [19]. In the context of SBP, the presence of phages induced factors that will contribute to the tolerant state of ascitic monocytes, favoring the ascitic infection.

We have validated a flow cytometry assay to study the phage-monocyte interaction through phagocytosis assays with labelled phage suspensions. We have observed the phagocytosis of phages by monocytes at short times. According to other reports using microscopic analysis, phagocytosis of phages is an early process since, at longer times of incubation, the intracellular destruction of phages begins [20,21]. In line with these results, we did not detect phagocytosis of phages at longer times of 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 [22].

Finally, our work suggests that not only bacteria but also phages in AF could induce an immune response in monocytes to avoid the clearance of ascitic infection. In cirrhosis, the immunogenicity of phages could be particularly of interest in patients with SBP that have a negative bacteriological result. The immune response observed in healthy monocytes stimulated with phages infecting Gram-negative and Gram-positive bacteria in our study is comparable to the response observed in ascitic monocytes from SBP patients with the negative bacteriological result. These patients have a similar severity and prognosis than patients with the positive bacteriological result and are treated with

antibiotics despite there is no viable bacterial growth in the culture [4]. It seems plausible that the immune response observed in the ascitic monocytes from these patients could be due to the presence of phages in the AF. Therefore, it would be relevant to further study isolated phages from AF to fully understand the monocytic immune response of patients with SBP that had a negative bacteriological result.

5. Conclusions

Monocytes are able to phagocytose butanol-purified phages and, consequently, phages induce phenotypic changes and a cytokine production, similar to those observed in the ascitic SBP environment.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Confirmation of the efficiency of polymyxin B against LPS, Figure S2: Relationship between the expression of CD86 in monocytes stimulated with the five phage suspensions and the levels of TNF-α produced in their supernatants.

Author Contributions: Conceptualization, X.X. and Y.Y.; methodology, X.X.; software, X.X.; validation, X.X., Y.Y. and Z.Z.; formal analysis, X.X.; investigation, X.X.; resources, X.X.; data curation, X.X.; writing—original draft preparation, X.X.; writing—review and editing, X.X.; visualization, X.X.; supervision, X.X.; project administration, X.X.; funding acquisition, Y.Y. All authors have read and agreed to the published version of the manuscript.

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