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The effects of T4 and A5/80 phages on the expression of immunologically important genes in differentiated Caco-2 cells*

Wpływ bakteriofagów T4 i A5/80 na ekspresję ważnych immunologicznie genów w zróżnicowanych komórkach Caco-2

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Bacteriophages are an abundant component of the mucosal microbiota in humans and some animal species. Intestinal epithelial cells (IECs) are the key element responsible for the induction and regulation of immune responses in the gut mucosa. The objective of this study was to evaluate the effects of T4 and A5/80 bacteriophages on the expression of immunologically important genes in Caco-2, a model cell line for IECs.

Materials & Method: Bacteriophages were added to cultures of differentiated Caco-2 cells for 12 hours, while control cultures were treated with phosphate-buffered saline (PBS). Expression of genes in Caco-2 cells was determined using custom-made RT^2 Profiler PCR Arrays, which allow for the evaluation of gene expression with the sensitivity and specificity of real-time PCR. We evaluated the expression of 21 genes which are important for the immune functions of IECs, including *IL1B, IL6, IL7, IL10, IL15, IL18, IL25, IL33, TGFB1, TNF, CXCL8, CCL2, TSLP, FCER2, PIGR, DEFB4A, CAMP, REG3G, TNFSF13, TNFSF13B*, and *MUC2*.

Results: Both examined phages significantly influenced the expression of a number of genes compared with control cultures. In particular, T4 significantly increased the expression of the *CCL2* and *DEFB4A* genes, while A5/80 induced the expression of the *PIGR* gene.

Discussion: Together with the findings from previous studies, our results suggest that by modulating the expression of some genes, bacteriophages may affect immune responses in the gut mucosa.

Keywords: **bacteriophage, intestinal epithelial cell, Caco-2, inflammatory bowel disease, defensin, gene expression**

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INTRODUCTION

Until recently, research into the gut microbiota has focused on bacteria. However, a growing number of studies point to viruses as another important component of the microbiota in both humans and various animal species [14, 29, 32]. Most viruses found in the gut are bacteriophages (phages) – viruses that infect solely bacterial cells. Phages identified in the gut include mostly non-enveloped DNA viruses, both dsDNA *Caudovirales* and ssDNA *Microviridae*, as well as ssDNA filamentous phages from the family *Inoviridae* [29]. A substantial proportion of phages present in the gut – between 20 and 50% – are temperate [26]. Interestingly, the majority of gut phages seem to be unique to individual hosts from which they were isolated [19]. Furthermore, it was shown that healthy individuals tend to conserve the same phages over time, especially the most abundant ones [31]. However, it needs to be underscored that research into gut phages remains at a relatively early stage; for instance, it is estimated that 75% to 99% of intestinal phage genomes are not significantly similar to any known viral genome [1]. Furthermore, the hosts for most of the phages present in the gut have not been identified yet [6].

According to our hypothesis, phages present in the gut might not only affect physiological flora but also regulate the immune responses in the gut mucosa and other tissues [10]. Since then, a number of studies have shown that phages can indeed induce such effects. For instance, Yang et al. suggested that viruses including phages could modulate inflammatory reactions in the gut by inducing TLR3- and TLR7-dependent production of interferon (IFN)-β [37]. However, in another study, oral administration of a phage cocktail aggravated dextran sulfate sodium (DSS)-induced colitis in a TLR9- and IFN-γ-dependent manner; the authors of the latter study also showed the expansion of IFN-γ-producing CD4+ T cells and of CD8+ T cells in the Peyer's patches of germ-free mice orally treated with *Escherichia coli* phages [8]. Overall, experimental data about the immunomodulatory effects of gut phages are extremely scant and to some extent conflicting.

The objective of the present study was to evaluate the effects of two bacteriophages – T4 (infecting *E. coli*) and A5/80 (infecting *Staphylococcus*) on the expression of a panel of immunologically important genes in differentiated Caco-2 cells. Caco-2 is a model cell line for intestinal epithelial cells (IECs) which are the major component of the gut immune system [15, 23]. By modulating the expression of some genes in IECs, phages might affect immune responses in the gut mucosa.

MATERIALS AND METHODS

Purified phage preparations

E. coli T4 phage [38] was obtained from th American Type Culture Collection (ATCC; USA). Staphylococcal A5/80 phage [16] was obtained from the bacteriophage collection of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET PAS). Bacterial strains for phage propagation (*E. coli* B and *Staphylococcus aureus* 80 for T4 and A5/80, respectively) were obtained from the Polish Collection of Microorganisms at the HIIET PAS. Crude bacterial lysates of both phages were prepared as reported in [31]. Purified preparations of both phages were prepared as reported in detail elsewhere [2, 17]. The concentration of lipopolisaccharide (LPS) in purified phage preparations was measured using the QLC 1000 Endpoint Chromogenic LAL test kit (Lonza, Switzerland) according to the manufacturer's instructions. Phage titer in purified phage preparations was determined by the double layer method [13]. Stock preparations of both phages (10^{10} plaque forming units (PFU)/ml) were diluted with a culture medium just before the experiments.

Preparation of LPS from *E. coli* B

LPS from *E. coli* B was isolated as described elsewhere [3 and references therein]. In brief, *E. coli* B cells were grown for 24 h at 37°C in liquid LB medium, pH 7.0 in 10-liter BioFlo415 fermenter (Eppendorf, Germany). LPS was isolated by the PCP (phenol/chlorophorm/petroleum) method and purified by ultracentrifugation (105.000 x g, 4 h, 4°C). Then LPS was diluted with PBS and dispersed using a Sonopuls HD 2070 ultrasonic homogenizer connected to a UW 2070 HF-generator (both from Bandelin, Germany). LPS activity in the stock suspension measured in the LAL test was 5000 EU/ml. LPS was dispersed again just before it was diluted with a culture medium and added to the cell culture. To test the influence of LPS due to its presence in phage preparations LPS suspension was used as a control.

Cell line and cell culture

Caco-2 cell line was obtained from the American Type Culture Collection (ATCC; USA). Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM with 4500 mg/l

glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate; Merck) supplemented with 10% Foetal Bovine Serum (Thermo Fisher Scientific), 2 mM L-glutamine, 1% Non-Essential Amino Acids (Thermo Fisher Scientific) and Pen-Strep antibiotic solution (Thermo Fisher Scientific) at a final concentration of 100 U/ml penicillin G and 100 mg/ml streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. To obtain differentiated cells, cells in suspension were seeded in 24-well microplates (Nunclon Surface, Nunc) at a density of 50,000 of cells/1 ml of the culture medium supplemented as described above per well and incubated for 21 days to obtain cell monolayer with a final density of ca. 4×10^5 cells /well. The culture medium was changed every 2–4 days (a break between changes was gradually reduced during culture time). Then the cell culture medium was replaced with 900 µl/well of a fresh medium, and cells were treated (in triplicates) for 12 h with one of the following additives at a volume of 100 µl: purified T4 phage preparation, purified A5/80 phage preparation, PBS (untreated control), and *E. coli* B LPS (LPS control) at the final activity of 0.5 endotoxin units (EU)/ml which corresponded to the final endotoxin activity in cultures incubated with phages. The titers of both phages were adjusted so as to obtain the ratio of 250 phage particles per one cell. After incubation, the cell culture medium was discarded, cells were washed with PBS, and 1 ml of RNAlater Storage Solution (Sigma) was added to each well. Then the plates were kept overnight at +4°C and stored at –20°C until RNA isolation.

Measurement of gene expression

RNA was isolated from Caco-2 cells using a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity was determined with NanoDrop spectrophotometer (Thermo Fisher Scientific). Gene expression was determined with reverse-transcripted real-time PCR (RT-qPCR) using a custom-made RT^2 Profiler PCR Array Format F (Qiagen) by Roche Light Cycler 480 according to the manufacturer's protocol. This assay allows one to determine gene expression with the specificity and sensitivity of quantitative real-time PCR. The custom-made panel developed with a view to our project included the following genes: *IL1B, IL6, IL7, IL10, IL15, IL18, IL25, IL33, TGFB1, TNF, CXCL8, CCL2, TSLP, FCER2, PIGR, DEFB4A, CAMP, REG3G, TNFSF13, TNFSF13B*, and *MUC2*. The level of genomic DNA contamination, possibility of inhibition of the reverse-transcription reaction, the presence of PCR amplification inhibitors, the cycling conditions, and the relative sensitivity of the instrument were validated according to the RT^2 Profiler PCR Array manufacturer's guide.

Data analysis

The relative transcript levels of the genes being investigated were analyzed using the $\Delta\Delta C_T$ method according to the RT^2 Profiler PCR Array manufacturer's guide. The average C_T of three housekeeping genes (GAPDH, B2M and G6PD) was used for normalization. Differences in mRNA expression between T4-, A5/80- or LPS-treated cultures and the untreated control cells were presented as fold-change (f-c) values ($2^{-\Delta\Delta CT}$). The range of f-c was indicated by $2^{-(\Delta\Delta C\ T + SD)}$ and $2^{-(\Delta\Delta CT\ -\ SD)}$, where SD for $\Delta\Delta C_T$ was calculated based on standard deviations of ΔC_T values for treated and untreated cultures [11]. In order to exclude potential bias resulting from averaging data transformed through the $2\text{-}\Delta\Delta C_T$ equation, a one-way analysis of variance (ANOVA) of $\Delta\Delta C_T$ values obtained for each experiment was done using the SigmaPlot 12.3 software (Systat Software, Germany). The significance for this test was set at 5% (p-value < 0.05).

RESULTS

To evaluate the effects of T4 and A5/80 phages on the expression of immunologically-important genes in differentiated Caco-2 cells we used a custom-made RT^2 Profiler PCR Array. This assay allowed us to simultaneously measure the expression of 21 genes. Generally, RT^2 Profiler PCR Arrays evaluate the expression of the examined genes with the sensitivity and specificity of quantitative real-time PCR. Expression of genes in differentiated Caco-2 cells was determined after 12-h incubation with purified phage preparations. Compared with control cultures, both investigated preparations significantly affected the expression of a number of genes; of note, the effects of T4 and A5/80 were different from one another (Tab. 1).

The most remarkable effect was a high increase in the expression of the *DEFB4A* gene in Caco-2 cells treated with T4 phage compared with control cultures (f–c = 15.643; p <0.001). Unlike T4 phage, LPS suspension did not significantly increase the expression of this gene compared with cultures treated with PBS (f–c = 1.9; p >0.05). Thus increase in the expression of the *DEFB4A* gene induced by T4 was not mediated by residual LPS present in the phage preparation. The other examined phage – A5/80 – did not significantly affect the expression of the *DEFB4A* gene, either (Tab. 1).

T4 phage significantly increased the expression of the *CCL2* gene compared with control cultures treated with PBS (f–c = 7.365; p = 0.022). This effect was probably caused by phage particles because LPS suspension had no significant effect on the expression of *CCL2*. The effect of the A5/80 phage on the expression of this gene was also negligible (Tab. 1). Moreover, we observed more than a two-fold increase in the expression of *IL7, TNF, FCER2, PIGR, MUC2, TSLP*, and *IL25* genes in cultures to which the T4 phage was added compared with control cultures; however, none of those effects was statistically significant (Tab. 1).

We also found that A5/80 significantly induced the expression of the *PIGR* gene compared with control cultures (f–c = 2.969; p = 0.03). Unlike A5/80, neither T4 nor LPS suspension had any significant effect on the expression of this gene (Tab. 1). Other genes whose expression was increased more than twofold in cultures treated with A5/80 compared with control cultures included *IL10, CCL2, MUC2, and CAMP*. However, all those changes fell short of statistical significance (Tab. 1).

Table 1. The effects of T4 and A5/80 phages on expression of immunologically important genes in Caco-2 cells

No.	Gene	Fold–change value		
		T4 vs control	A5/80 vs control	LPS vs control
1	*IL1B*	0.805 (0.273–2.373)	0.843 (0.286–2.487)	0.947 (0.341–2.627)
2	*IL6*	0.825 (0.208–3.276)	0.692 (0.174–2.745)	0.847 (0.153–4.685)
3	*IL10*	0.579 (0.017–19.317)	**2.445** (0.073–81.486)	**3.600** (0.313–41.306)
4	*IL7*	**2.089** (0.092–47.187)	1.691 (0.074–38.173)	1.052 (0.143–7.7)
5	*IL15*	1.591 (0.629–4.025)	1.467 (0.580–3.71)	1.136 (0.477–2.699)
6	*IL18*	0.928 (0.5–1.689)	0.899 (0.493–1.636)	0.837 (0.546–.281)
7	*IL33*	1.665 (0.372–7.442)	1.480 (0.331–6.614)	0.616 (0.11–3.435)
8	*TSLP*	**3.256** (1.556 – 6.811)	1.294 (0.618–2.706)	1.131 (0.493–2.592)
9	*IL25*	**3.434** (1.489–7.916)	1.941 (0.841 – 4.473)	**2.084** (0.964 – 4.499)
10	*TGFB1*	1.182 (0.637–2.194)	1.118 (0.602–2.074)	0.963 (0.518–1.787)
11	*TNF*	**2.637** (0.728–9.553	1.314 (0.362–4.76)	1.285 (0.509–3.244)
12	*IL8*	1.446 (0.731–2.860)	1.497 (0.756–2.96)	1.107 (0.501–2.444)
13	*CCL2*	**7.365 *** (1.219–44.489)	4.255 (0.704–25.7)	1.99 (0.327–12.063)
14	*FCER2*	**3.432** (0.417–28.218)	1.315 (0.159–10.81)	1.553 (0.254–9.481)
15	*PIGR*	**2.197** (1.017–4.747)	**2.969*** (1.374–6.413)	1.573 (0.802–3.083)
16	*DEFB4A*	**15.643 *** (8.876–27.569)	1.725 (0.978–3.04)	**1.914** (1.328–2.756)
17	*CAMP*	1.226 (0.732–2.053)	**2.240** (1.337–3.751)	1.195 (0.450–3.166)
18	*REG3G*	1.746 (1.105–2.757)	1.739 (1.101–2.746)	1.362 (0.777–2.386)
19	*TNFSF13*	0.512 (0.189–1.383)	1.204 (0.446–3.251)	0.931 (0.316–2.738)
20	*TNFSF13B*	1.460 (0.626–3.408)	1.835 (0.786–4.281)	**3.034** (1.469–6.265)
21	*MUC2*	**6.129** (0.139–270.277)	**3.095** (0.070–136.472)	**6.749 *** (0.734–61.983)

Shown are fold-changes (±2SD) compared with untreated control cultures. Fold-change values suggesting gene overexpression (f–c ≥ 2.0) or expression inhibition (f–c ≤ 0.5) are marked in bold. Significance of the difference in gene expression following treatment of Caco-2 cells with T4 phage preparation, A5/80 phage preparation, or *E. coli* B LPS: * ($p < 0.05$ as determined by ANOVA).

DISCUSSION

The objective of this work was to evaluate the effects of T4 and A5/80 phages on the expression of immunologically-important genes in differentiated Caco-2 cells, a model cell line for IECs [21]. By modulating the expression of those genes, bacteriophages could affect immune responses in the gut mucosa. We selected T4 and A5/80 because both phages displayed activity in our previous study performed on A549 cell line [24].

Overall, we found that both the T4 and A5/80 phage significantly increased the expression of a number of genes. Those effects were different for T4 and A5/80, what suggests that individual phages can impact the gut cells gene expression in different ways. Our findings are in line with the results of previous research that also showed that individual phages can induce different immunomodulatory effects [9].

One of the most important effects found in our study is a significant induction of the expression of the *DEFB4A* gene by the T4 phage. This gene encodes for β-defensin-2 (BD2), a potent antimicrobial peptide produced by epithelial cells, which has a very important role in inducing innate immune responses against bacteria and other microbes [28]. Recently, it was shown that recombinant BD2 ameliorated an inflammatory reaction in three different animal models of inflammatory bowel disease (IBD) [12]. In another study, transfection of Caco-2 cells with a gene encoding for BD2 resulted in down-regulated expression of pro-inflammatory cytokines in response to infection by *Salmonella typhimurium* [7]. Thus, our findings may suggest a novel mechanism of anti-inflammatory activity of phages. Such activity might be exhibited by endogenous phages from the microbiota as well as exogenous phages administered during phage therapy. Moreover, our results may provide a starting point for further studies to evaluate potential anti-inflammatory effects of phages in animal models of IBD.

We also found that the T4 phage displayed a tendency to increase the expression of the *TNF* gene. This result deserves further research because the product of this gene – TNF-α – is a key cytokine implicated in the pathogenesis of IBD [30]. However, it needs to be stressed that while T4 increased the expression of *TNF* more than twofold compared with control cultures, this effect fell short of statistical significance.

Moreover, we found that neither of the examined phage preparation significantly induced the expression of the *IL15* gene. While the role of IL-15 in the pathogenesis of IBD has not been fully elucidated yet, most studies have shown that this cytokine can display pro-inflammatory activity in this context [34].

A number of studies published over recent years showed substantial differences in the composition of the gut virome, including bacteriophages, between patients with IBD and healthy individuals [4; 22; 36]. Some authors suggested that phages might contribute to inflammatory reactions in the gut not only by inducing dysbiosis of the microbiota, but also by exerting direct pro-inflammatory effects [22]. However, our results show that some bacterial viruses could in fact exert contrary (anti-inflammatory) effects. Further studies are necessary to elucidate the actual role of bacteriophages in the pathogenesis of IBD.

We also found that A5/80, but not T4 phage, significantly induced the expression of the *PIGR* gene. This gene encodes for the polymeric immunoglobulin receptor (pIgR), a key protein involved in the transport of dimeric immunoglobulin A and polymeric immunoglobulin M from the lamina propria across the epithelial barrier to mucosal surfaces. Secretion of polymeric Igs is one of the main mechanisms mediating antimicrobial immune responses in the gut [35]. Thus, it is possible that some phages might contribute to the elimination of pathogenic bacteria and viruses in the gut by facilitating the transport of polymeric Igs across the epithelial barrier.

An important question is what mechanism(s) underlie phage-mediated modulation of gene expression in Caco-2 cells. The results of other studies showed that some bacteriophages could penetrate the cell membranes of different cell lines (including Caco-2) as a result of transcytosis [25]. Thus phages can interact with both extra- and intracellular receptors. However, it remains to be elucidated what class of receptors mediated the effects observed in this study.

In conclusion, both examined phages significantly induced the expression of genes with potentially beneficial activities, especially *DEFB4A* and *PIGR*. These findings imply a possibility for the immunomodulatory role of phages from the gut microbiota, and may provide a starting point for novel applications of phages (phage repurposing), especially in the treatment of IBD.

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