Comparative Immunology, Microbiology and Infectious Diseases 66 (2019) 101328

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Comparative study of immune responses elicited by outer membrane vesicles of different *Pseudomonas aeruginosa* strains

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ARTICLE INFO

Keywords:
Outer membrane vesicles
Pseudomonas aeruginosa
Caco2
Toll like receptors
Cytokines
Caco2

ABSTRACT

Background: *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic mucosal human pathogen that naturally releases outer membrane vesicles (OMVs) in different environments, as do other Gram-negative bacteria. The intestinal tract infections caused by *P. aeruginosa* increase the risk of respiratory infections and mortality of other diseases related to gut infections. Therefore, in this study, we attempted to investigate toll-like receptor (TLR) signaling pathways and immune response profiles of human colon adenocarcinoma (Caco2) cell line exposed to the OMVs of three different *P. aeruginosa* strains (i.e., antibiotic-susceptible, multi-drug resistant (MDR), and standard lab ATCC 17933).

Materials and methods: Real-time quantitative reverse transcription PCR array was carried out to determine mRNA expression in 84 TLRs signaling pathway genes, and the production of specific cytokines was measured by ELISA.

Results: OMVs of different strains could induce unique changes in regulating TLRs signaling pathways, such that there were remarkable differences in pro-inflammatory effects and anti-inflammatory responses among the three strains.

Conclusion: The more complete immune responses observed through the MAMPs caused by MDR strain OMVs interactions with Caco2 lead us to the conclusion that the use of MDR or cystic fibrosis strain OMVs for better known the host immune responses seems preferable.

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a typical Gram-negative opportunistic pathogen common in water and soil [1]. This pathogen can cause multiple types of infections such as nosocomial pneumonia, hospital-acquired infections in immunocompromised individuals [2], particularly those with cystic fibrosis [1], and a variety of life-threatening infections in ventilated and burns patients and those with pulmonary diseases [3]. Moreover, the intestinal tract infections caused by *P. aeruginosa* increase the risk of respiratory infections and mortality of other diseases related to gut infections [4].

It is worth mentioning that the gut is a good repository for *P. aeruginosa* [5]. Importantly, the mortality rate of *Pseudomonas* infections is very high due to its resistance to disinfectants [6,7]. *P. aeruginosa* releases outer membrane vesicles (OMVs) as do other pathogenic and non-pathogenic Gram-negative bacteria [1], which has been identified as an important mechanism of interaction with hosts [8].

OMVs are bilayered structures ranging from 50 to 250 nm in diameter and are naturally generated from the outer membrane of Gram-negative bacteria as a response to envelope stress and diffuse into the environment both in vitro and in vivo [9,10]. OMVs contain almost all the biological constituents in a live bacterium such as peptidoglycan, phospholipid, lipopolysaccharide (LPS) [11,12], bacterial proteins, virulence factors [13,14], signaling molecules of the quorum sensing, and genetic materials [15]. Since the discovery of OMVs (more than 40 years ago), many roles have been assigned to them. Initially, they were assumed to have no role and were viewed as artifacts of bacterial growth. However, in recent studies they have been considered as the cause of infection, inflammation progression, and immune system's maturity [15–17].

In addition to the crucial roles that OMVs play in different aspects of bacterial physiology, they can remarkably modulate immune responses in their hosts through a variety of signaling events [18–20]. They are capable of fusing with host cells through lipid rafts in cytoplasmic

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https://doi.org/10.1016/j.cimid.2019.101328
Received 5 May 2019; Received in revised form 13 June 2019; Accepted 26 June 2019

membrane [21] and thus, being implicated in many host cellular functions through the delivery of their contents [22,23]. Research interest in clarifying the signaling pathways through which OMVs affect the immune system is on the rise, and the findings may help improve therapy in the future.

The OMVs generated by *P. aeruginosa* have been shown to activate IL-8 release [24] and the production of other inflammatory chemokines and cytokines from epithelial cells [25], suggesting that OMVs play a considerable role in pathogenicity and host immunity [18]. Moreover, it has been reported that *P. aeruginosa* OMVs may mount remarkable innate immune responses through toll-like receptor 4 (*TLR4*) [26] and *TLR2* [25]. Known as the most important pattern recognition receptors (PRRs), the TLRs family is expressed in a broad variety of host cells such as epithelial cells and recognize a structural diversity of microbial-associated molecular patterns (MAMPs) [27], but their regulation by OMVs from pathogenic bacteria remains unexplored and little is known about the host immune responses [26]. OMVs content is strain-dependent [15,28], and we hypothesized that OMVs have the ability to stimulate most TLRs with respect to their various MAMPs content. Thus, in the present study we aimed to compare the TLR-signaling pathways and cytokine responses activated in Caco2 epithelial cell line treated individually with the OMVs of different *P. aeruginosa* strains.

2. Materials and methods

2.1. Bacterial strains

In this experimental study, the *P. aeruginosa* standard lab strain (ATCC 17933) was obtained from Pasture Institute of Iran. Antibiotic-susceptible and MDR strains were supplied by Loghman Hospital. All strains were grown in Luria-Bertani (LB) broth medium.

2.2. OMVs isolation

OMVs produced by *P. aeruginosa* strains were purified through using a modified differential centrifugation and discontinuous protocol described by Siadat et al. Briefly, 500 ml of each *P. aeruginosa* strain was grown (at 37 °C) separately up to the early stationary phase. Purification of OMVs was carried out using Tris-HCl, EDTA and 100 g/l deoxycholate followed by their consecutive centrifugation at 20,000 *g* for 30 min. Finally, OMVs were pelleted by ultracentrifugation at 125,000 *g* for 2 h. The OMV pellet were suspended in sucrose (pH 7.4) and were kept in -20 °C before use [27].

2.3. Protein and LPS measuring of extracted OMVs

OMV protein was quantified by measuring the absorbance at 280 nm using a NanoDrop instrument. LPS content of the different OMVs samples was evaluated by a Limulus assay according to the U.S. standard (Thermo Scientific, USA).

2.4. Cell culture and OMV inoculation

Human colon adenocarcinoma (Caco-2) cell line was kindly donated by Pasture Institute of Iran. The cells grown at 37 °C in standard media of Dulbecco's Modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA.) plus 20% fetal bovine serum (FBS) (Gibco), 100 u/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). Caco2 cells were seeded (2×10^5) in 6-well plates, and the tests were administered when the cells reached 85–90% confluency. In order to investigate the immune responses activated by isolated *P. aeruginosa* different strains OMVs, Caco-2 cells were exposed with 50 µg ml^{-1} (which is the appropriate concentration required to elicit a statistically significant increase in IL-8 secretion) [29] of OMVs from each strains separately (24 h in 37 °C). The cell supernatants collected and stored at − 80 °C for the cytokine assays.

2.5. RNA isolation and cDNA synthesis

The total RNA was isolated using RNA extraction kit (Roche, Germany). cDNA Synthesis kit (Roche, Germany) and 1 µg of total RNA were utilized to synthesize cDNA according to the instructions from the manufacturing company before conducting PCR array experiments.

2.6. Real time PCR array

The transcriptomic profile expressions of TLR signaling pathway were analyzed using RT^2 PCR Array Human Toll-Like Receptor Signaling Pathway kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, the PCR array was done on the Light Cycler Real time PCR and PCR master mix for SYBR Green detection for each reaction. qRT-PCR conditions were defined as follows: precycling was held at 95 °C for 10 min, and then it was followed by 40 cycles of shuttle heating at 95 °C for 15 s and 1 min at 60 °C. All assays were done in triplicate.

2.7. Cytokine/chemokine measurements

The supernatants of Caco2 cells incubated with *P. aeruginosa* OMVs were collected after 24 h and kept at − 80 °C until used. Determination of IFN γ, IL4 and IL-10 were assessed by enzyme-linked immunosorbent assay (ELISA) kits (Mabtech, Sweden), according to the instructions. Each supernatant was assayed in duplicate.

2.8. Statistical analysis

RT2 Profiler PCR Array Data Analysis Web portal (https://www.qiagen.com/ir/resources/geneglobe/) was applied to analyze the differences in gene expression as well as Path Visio software, KEGG String, and Wiki Pathways online website (https://www.wikipathways.org/index.php/WikiPathways). The transcript levels were compared using student's t-test. GraphPad Prism 6 (GraphPad, La Jolla, CA, USA) and path Visio software were employed for statistical analyses for the cytokine assays. p values < 0.05 were considered as statistically significant.

3. Results

3.1. Protein and LPS containing of extracted OMVs

The protein concentration of the OMVs obtained from each strain was measured by Nano-drop. MDR, antibiotic-susceptible, and standard lab strains had 3.191, 2.823 and 0.731 mg/ml protein content and the LPS amounts in OMVs estimated by Limulus assay were 3, 2.9 and 2.8 EU/ml, respectively.

3.2. Gene expression

To determine the effects of *P. aeruginosa* different strains OMVs on regulation of TLR signaling pathway genes in Caco-2 cells, 84 TLR-related genes were evaluated using quantitative PCR array. The expression profiles of 30 out of the 84 genes changed in Caco-2 in response to the *P. aeruginosa* standard lab strain OMVs. Moreover, 18 genes were up-regulated, while 12 genes were down-regulated. Notably, as illustrated in Table 1, some genes were highly expressed such as *TLR9*, *TICAM2*, *IRF1*, *CXCL8, CXCL10* whose expression increased by more than 5 folds ($p < 0.05$). The expression levels of 33 genes out of the total 84 ones changed in Caco-2 exposed to antibiotic-susceptible strain OMVs, 28 genes were up-regulated, and five genes were down-regulated. As shown in Table 1, the expression levels of *TLR1*, *CSF3*, *CXCL10*, *IL8* genes increased by more than 8 folds ($p < 0.05$) in this case. The TLRs pathways expression profiles in Caco-2 indicated that 43 of the 84 genes changed in response to MDR strain OMVs; 40 genes

Table 1
Fold regulation comparison in Caco-2 cells treated with various *P. aeruginosa* strains OMVs (standard, antibiotic susceptible and MDR). Fold Regulation (comparing to control group) cut off 2 and p-Value cut off 0.05.

symbol	standard strain OMV Fold regulation	Antibiotic susceptible strain OMV Fold regulation	MDR strain OMV Fold regulation	symbol	standard strain OMV Fold regulation	Antibiotic susceptible strain OMV Fold regulation	MDR strain OMV Fold regulation
BTK	3.4058	8.8643	3.6503	MAP2K4	0	0	0
CASP8	0	0	0	MAP3K1	0	0	0
CCL2	−3.0994	−2.7359	0	MAP3K7	0	0	0
CD14	−3.0994	0	0	MAP4K4	−2.1023	0	0
CD180	0	0	17.976	MAPK8	0	0	0
CD80	0	0	11.0655	MAPK8IP3	0	0	0
CD86	0	0	10.3245	MYD88	0	0	0
CHUK	0	0	0	NFKB1	−2.4318	0	0
CLEC4E	0	0	8.988	NFKB2	3.0272	3.5016	3.0908
CSF2	0	2.7283	15.649	NFKBIA	2.247	3.7529	2.5105
CSF3	2.6537	10.9132	6.8116	NFKBIL1	0	7.1503	−2.2069
CXCL10	9.4349	8.8643	7.3005	NFRKB	0	0	0
ECSIT	0	0	0	NR2C2	0	0	2.7856
EIF2AK2	0	0	0	PELI1	−2.0028	0	0
ELK1	0	0	0	PPARA	0	0	0
FADD	0	0	−2.0449	PRKRA	0	0	0
FOS	4.4015	2.0677	7.8245	PTGS2	0	0	0
HMGB1	−2.4487	−2.3817	−2.0449	REL	0	0	0
HRAS	0	0	0	RELA	0	2.6537	0
HSPA1A	3.7529	12.536	0	RIPK2	−2.3983	0	0
HSPD1	0	0	0	SARM1	−7.423	−5.3964	0
IFNA1	0	0	11.0655	SIGIRR	0	0	0
IFNB1	0	0	6.3555	TAB1	0	0	0
IFNG	−2.2377	0	8.803	TBK1	0	0	0
IKBKB	0	0	0	TICAM1	0	2.2008	2.5633
IL10	0	0	13.9095	TICAM2	7.5058	2.3916	3.6757
IL12A	0	2.4419	4.0222	TIRAP	0	0	0
IL1A	2.4419	3.2445	2.4932	TLR1	3.4058	8.8643	11.0655
IL1B	2.6354	2.2161	4.193	TLR10	0	4.7174	13.5292
IL2	0	2.0534	6.7646	TLR2	0	0	0
IL6	0	0	5.9299	TLR3	−2.9526	0	2.3262
IL8	6.0126	20.7926	14.9078	TLR4	2.247	−2.117	3.8317
IRAK1	−2.7741	0	0	TLR5	0	2.9856	4.5884
IRAK2	2.0111	3.8852	2.2626	TLR6	2.0392	0	9.1769
IRAK4	−2.4829	0	0	TLR7	0	3.0063	21.2295
IRF1	2.0111	4.2812	3.6503	TLR8	0	0	8.0445
IRF3	0	0		TLR9	5.8078	2.9856	7.9889
JUN	10.8378	12.4494	6.3116	TNF	0	0	10.4686
LTA	0	0	2.9241	TNFRSF1A	0	0	0
LY86	0	0	12.536	TOLLIP	0	0	0
LY96	0	4.5253	15.9778	TRAF6	0	2.0111	0
MAP2K3	0	0	0	UBE2N	0	0	0

were up-regulated, and three genes were down-regulated. As shown in Table 1, the expression levels of *CSF2*, *IL8*, *IL10, LY86*, *LY96*, *TLR10*, and *TLR7* genes increased by more than 12 folds ($p < 0.05$) in caco2 treated by MDR OMVs. Also, for visualization of gene regulation in different treatments, heatmap graphs (Fig. 1) were drawn with respect to the mean log2 fold change in genes of the treated Caco-2 cell line.

3.3. ELISA results

The statistical test for the ELISA results performed with the graphpad prism 6 software. The expression of IL-4, IL-10, and IFN-γ significantly increased in Caco-2 cells treated with OMVs of MDR strain versus the control group ($P < 0.0001$). Moreover, Caco-2 cells treated with OMVs of antibiotic-susceptible (sensitive) strain increased secretion of IL-4 ($P < 0.00075$) and IFN-γ ($P < 0.0012$), while expression of IL-10 by cells treated with OMVs of antibiotic-susceptible and secretion of IL-4, IL-10, and IFN-γ by cells treated with OMVs of standard lab strain showed no significant differences with the control group (Fig. 2).

4. Discussion

The data presented by previous studies demonstrate that the OMVs generated by *P. aeruginosa* are able to induce inflammation without the need to use living bacteria [25]. They also reported that *P. aeruginosa* OMVs could elicit immune responses via activating TLRs [25,26], which in turn, trigger a distinct, strain-specific pattern of cytokines and chemokines production [24,25]. Despite the growing interest in this research topic, the characteristics of immune responses and the stimulatory role of MAMPs presented in *P. aeruginosa* OMVs remain to be fully determined. Through this pioneering study, we demonstrated that intestinal epithelial cells exposure to OMVs of different *P. aeruginosa* strains could induce strain-specific changes in the expression profiles of TLRs signaling pathway-related genes, leading to different levels of pro-inflammatory cytokines and chemokines secretions.

Ellis et al. reported that vesicle proteins stimulate IL-6 production as a response to intact *P. aeruginosa* vesicles in macrophages [24]. In the present study protein measuring demonstrated that the OMVs sample of the MDR strain had the highest protein content, and the up-regulation of *IL-6* was only detected in response to the MDR strain OMVs (Table 1) which is may be associated with the Ellis findings but needs further study.

Earlier studies have demonstrated that the MAMPs composition of OMVs enables them to induce potent innate immune responses through their recognition by host TLRs [30,31]. It has also been found that toll-like receptor 4 (*TLR4*) is the main extracellular PRR which is

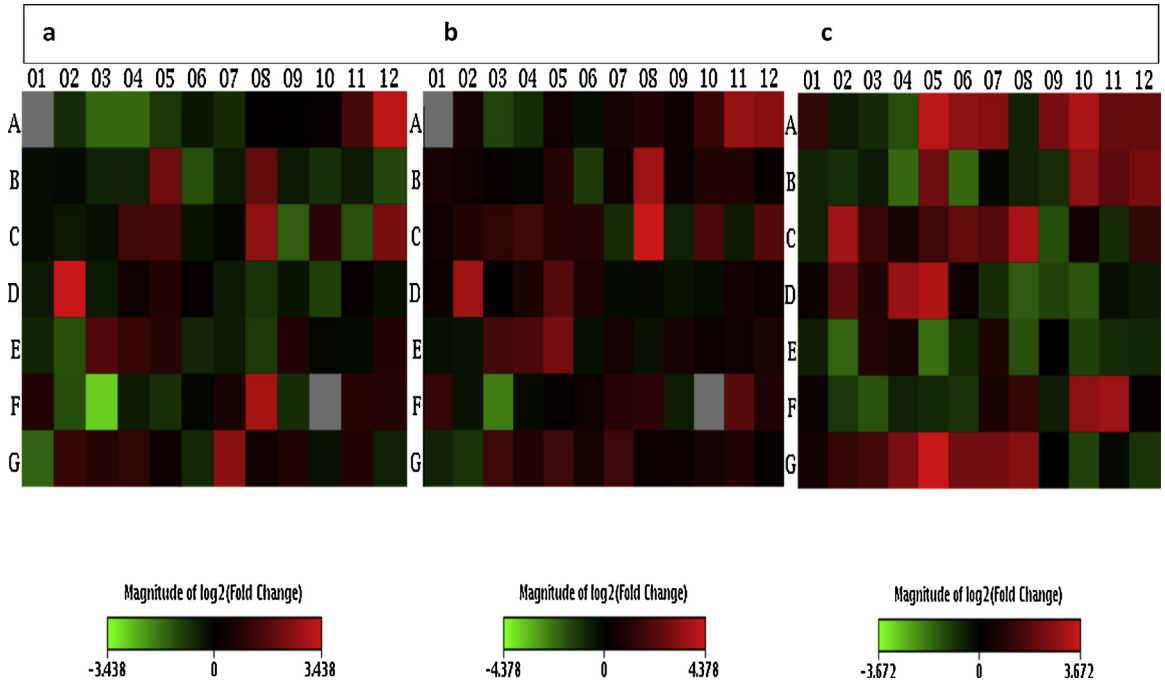

Fig. 1. Heatmap graph, the mRNA expression profile of genes involved in TLRs signaling pathway from Caco-2 cells treated with *P. aeruginosa* standard strain OMVs (a) antibiotic-susceptible OMVs (b) and MDR strain OMVs (c) of gene expression data. The minimum and maximum values of the heat map in (a) were − 3.438 and 3.438, in (b) were − 4.378 and 4.378 and in (c) were -3.672 and 3.672 respectively.

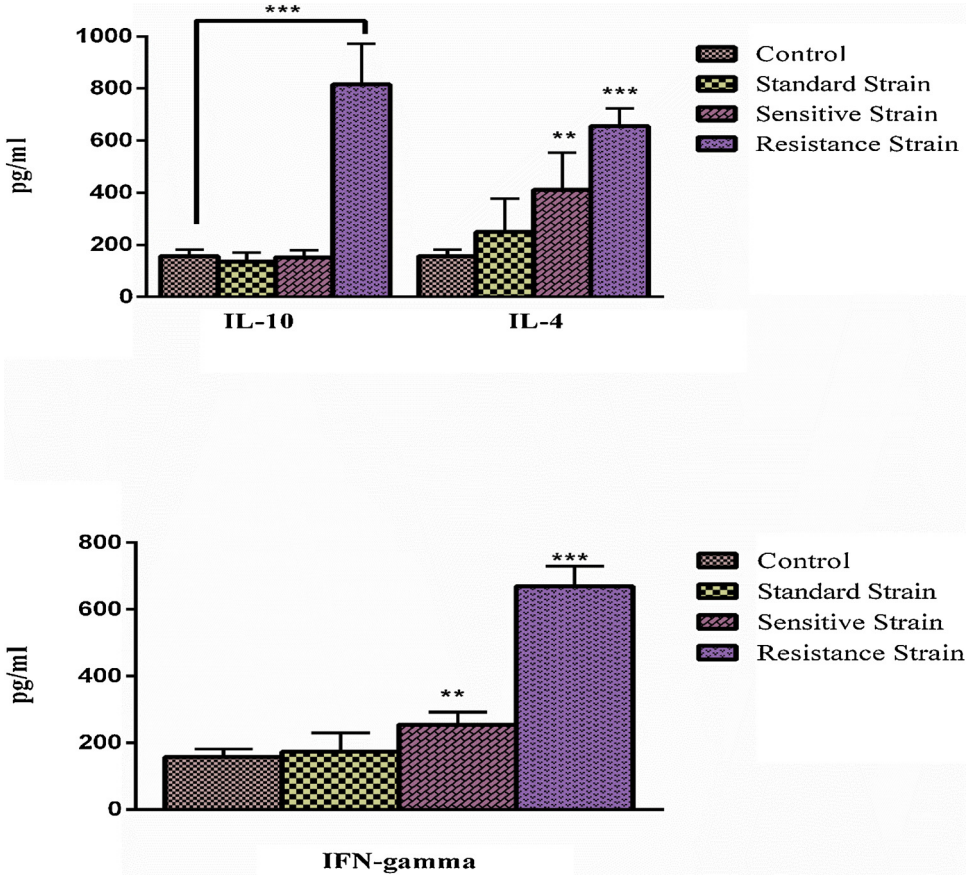

Fig. 2. Production of IFN-γ, IL-10 and IL-4 were measured by ELISA within 24 h after Caco2 OMVs exposure. (∗∗p < 0.001, and ∗∗∗p < 0.0001). The results are shown as the mean + SD of duplicate measurements.

extensively up-regulated in the interaction with LPS presented on *P. aeruginosa* OMVs in alveolar epithelial cells [25,26,32]. However, Park et al. used *TLR2* and *TLR4* knockout mice in their experiments and reported that *TLR2* and *TLR4* are partly involved in the inflammatory responses to OMVs derived from *P. aeruginosa* [25]. Our results are consistent with those of Park et al. study as our results showed *TLR2* did not undergo any changes in response to OMVs of various strains of *P. aeruginosa* and *TLR4* was slightly up-regulated in response to standard and MDR strains OMVs and was even down-regulated in response to antibiotic-susceptible strain OMVs.

Park et al. suggested that other TLRs play more significant roles in response to OMVs. In detail, *TLR1*, *TLR5-6-7-8-9*, and *TLR10* for MDR, *TLR1* and *TLR10* for antibiotic-susceptible, and *TLR9* for standard lab strains were the significantly up-regulated TLRs in Caco2 after being challenged by these OMVs compared to controls. Some other TLRs were also expressed, though at a lower level (2 folds). One plausible explanation for the extensive up-regulation of intracellular TLRs in response to MDR OMVs especially *TLR7* more than 21 folds, could be that *P. aeruginosa* OMVs can potentially fuse with the lipid rafts localized in host cells plasma membrane and deliver their content directly to the cytoplasm [33]. Furthermore, confocal microscopy and immunohistochemistry analysis illustrated that the amount of OMV molecules decreased and significant amounts of *P. aeruginosa* OMVs were visible inside the host cell 6 h after OMV introduction [25,33]. After 24 h of OMV exposure to Caco2, the total RNA was harvested, and the reduction of extracellular TLRs and the high levels of intracellular TLRs might be associated with the OMVs fusion with the host cells and also the intense induction of *TLR7* might be related with the presence of ssRNA in MDR OMVs [34].

As a ligand for unmethylated DNA, *TLR9* showed a four-fold up-regulation in response to standard and MDR strain OMVs. Electrophoresis of OMVs demonstrated the presence of DNA in our study. Due to the poor quality of the image, it is not reported in the findings. Indeed, these results are inconsistent with previous findings reporting that cytosine-guanine DNA may not induce immune responses to *P. aeruginosa* OMVs [24].

Our findings revealed that the genes related to *TLR4*, such as *LY96*, *TICAM-2,* and *TICAM-1,* in a TRIF-dependent manner were highly up-regulated in response to MDR strain OMVs, mildly in response to antibiotic-susceptible strain OMVs, and slightly in response to standard strain OMVs (Table 1), which was highly consistent with the amount of LPS measured in OMVs. In addition, sterile alpha and TIR motif containing 1 (*SARM1*), a negative regulator of MyD-independent pathway, was strongly down-regulated in response to all three types of OMVs. Considering this profile and the reduction of *IRAK-1* and *IRAK-4* genes associated with TLR4/MyD88-dependent manner, as well as the strong activation of other TLRs in this study, our results are in line with those of Zhao et al., who reported that *TLR4* with LPS ligand primarily activates the host immune system in response to *P. aeruginosa* OMVs [26], and Ramphal et al., who suggested that *Pseudomonas* LPS plays an adjuvant role but is not a key virulence factor triggering a significant pro-inflammatory response [35]. It also agrees with the results of Behrouzi et al. in that they proposed OMVs activate the *TLR4* in a MyD88-independent manner [36].

IL-8 was highly up-regulated in response to all three types of OMVs (Table 1). In this regard, Bauman and Kuehn suggested that strain-dependent differences in LPS and protein content of OMVs of different strains of *P. aeruginosa* may account for the different levels of *IL-8* secretion from lung epithelial cells [18]. Park et al. observed *CXCL1*, *CCL2*, *IL-1β*, *TNF-ά*, *IL-6*, and *IFN-γ* up-regulation in response to *P. aeruginosa* OMVs both in vivo and in vitro in a mouse model [25]. Our data demonstrated that pro-inflammatory responses induced by OMVs of *P. aeruginosa* are strain-specific [24].

The induction of almost all TLRs by the MDR strain OMVs strongly up-regulated the majority of the pro-inflammatory and antiviral cytokines in Caco2, such as *TNF-ά*, *IL-6*, *IL-2*, *IL-1β*, *IL-8*, *IL-12A*, and *IFN-γ*, while the OMVs of standard lab and antibiotic-susceptible strains induce expression of pro-inflammatory cytokines *IL-8* and *CXCL10*, as indicated in Table 1. In fact, we detected many more significant changes in the expression profiles (in about 40 genes) of TLR pathways in Caco2 cell lines after treatment with the MDR strain OMVs when compared to OMVs of standard lab and antibiotic-susceptible strains. Variations in the immune response to MDR strain OMVs compared to OMVs from the standard lab strain may be accounted for by strain-dependent differences in LPS and/or protein components [18].

The up-regulation of *CD80*, *CD86*, *IFNγ*, *IL-10*, *IL-12A*, *IL-1B*, *IL-2*, *TNF-α*, *CSFs*, and *CXCL10*, as regulatory genes of adaptive immunity, in response to MDR OMVs (Table 1) provides an advantage for the development of adaptive immune responses to protect against infections. For example, *IL-12A* is a pro-inflammatory cytokine that induces the production of *IFNs* type I, the differentiation of T-cell and associated innate resistance, and adaptive immunity [37], which was up-regulated only in response to MDR OMVs (Table 1). IL-10 is a potent suppressor of *IL-12* production [38]. IL-10, as our results confirmed, has an inhibitory effect on *TNF-ά*, *IL-1B*, and *IL-8*, and it is highly released in response to MDR OMVs in a cell line (Fig. 2). Besides the up-regulation of inflammatory cytokines such as *IFN*-γ(Fig. 2) and activation of adaptive immunity (Table 1), increasing anti-inflammatory cytokines such IL-10 and IL-4 in response to MDR OMVs (Fig. 2), can probably display the immunomodulatory role of OMVs, which is a crucial factor in striking a balance between efficient immunity against pathogens and inflammation disadvantages, of course looking at a larger cytokine panel might give a better idea whether the overall effect of MDR OMVs is pro- or anti-inflammatory.

TNF, *FADD,* and *CASP8* are effector genes contributing to apoptosis [39]. In our study, besides *TNF* up-regulation in response to the MDR OMVs, *FADD* expression was down-regulated, and no changes were observed in *CASP8* expression in response to OMVs of any strain. Therefore, according to our findings, *P. aeruginosa* OMVs infection is not considered the cause of cell death. In addition, *HMGB1*, which plays a role in autophagy regulation, was down-regulated in response to all the three OMVs; thus, autophagy pathway activation is less likely to be induced in response to OMVs of different strains of *P. aeruginosa*. This study showed that *P. aeruginosa* OMVs provoked distinct gene expression profiles of TLR-signaling pathways inducing the production of pro-inflammatory molecules. Nonetheless, additional studies are warranted to determine the exact molecular mechanisms of OMVs-TLRs interaction.

5. Conclusion

We identified that TLRs signaling pathways expression profile of intestinal epithelial cell (Caco2) changes in response to OMVs of various strains of *P. aeruginosa*, especially in the case of OMVs from the MDR strain. Pro-inflammatory cytokines such as *IL-6*, *IL-2*, *TNF-ά, IFN-γ*, and adaptive immunity regulatory genes were up-regulated through the activation of almost all TLRs by the MDR strain OMVs. Further studies of *Pseudomonas* OMV signaling immunization may be illuminate the exact molecular mechanisms of OMVs-TLRs interaction. OMVs might be used in isolation or in tandem with antibiotics due to the serious role of OMVs in stimulating the immune system [25]. However, so far few studies have investigated the immune protection of *P. aeruginosa* OMVs for vaccine development considering their potential to elicit cytokines in macrophages and epithelial cells [24]. The more complete immune responses observed through the MAMPs caused by MDR strain OMVs interactions with Caco2 lead us to the conclusion that the use of MDR or cystic fibrosis strain OMVs for this kind of research seems preferable.

Funding

This work was supported by Iran Biotech Fund [grant Number 94/

10243] and Pasteur Institute of Iran.

Authors' contributions

S.D.S., F.V.; design the experiments and data analysis. F.S.; Wrote the manuscript and carried out the experiment. T.N.; Edited the manuscript. All authors read and approved the final manuscript.

Declarations of Interest

None.

Acknowledgments

We would like to thank Dr. Ava Behrouzi for her help and other members of Mycobacteriology and Pulmonary Research Department, Pasteur Institute of Iran for their contributions in this study. All authors declare that there are no conflicting interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.101328.

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