Heliyon 9 (2023) e13846

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

The effect of different *C. difficile* MLST strains on viability and activity of macrophages

Gewa Saad [a], Maya Azrad [b], Meral Aias [a], Tamar Leshem [b], Zohar Hamo [a], Layan Abu Rahmoun [a], Avi Peretz [a,b,*]

[a] *Azrieli Faculty of Medicine, Bar Ilan University, Safed, 1311502, Israel*
[b] *Clinical Microbiology Laboratory, Tzafon Medical Center, Poriya, Tiberias 1528001, Israel*

ARTICLE INFO

Keywords:
C. difficile
MLST
Strains
Toxins levels
Macrophages activity
Cytokine levels

ABSTRACT

Objectives: Clostridioides difficile is the most common infectious agent of nosocomial diarrhea. *C. difficile* infection (CDI) pathogenesis and disease severity depend on its toxins (toxins A, B and binary) and on the host's immune response, especially the innate immune system. The current study examined the efficacy of macrophage activity, macrophages viability and cytokine secretion levelsin response to different sequence type (ST) strains of *C. difficile*.

Methods: RAW 264.7 macrophages were exposed to six different strains of *C. difficile* as well as to both toxins A and B and macrophage viability was measured. The levels of four secreted cytokines were determined by RT-PCR and ELISA. Morphological changes to the macrophages were investigated by fluorescent microscopy.

Results: Strains ST37 and ST42 affected macrophages' vitality the most. Toxins A and B led to a significant reduction in macrophages' vitality at most time points. In addition, starting at 30-min post-exposure to 5 ng/μl of both toxins led to significant differences in macrophage viability versus at lower concentrations. Furthermore, cytokine secretion levels, including IL-12, IL-6 and TNF-α, increased dramatically when macrophages were exposed to strains ST42 or ST104. Finally, gene expression surveys point to increases in IL-12 gene expression in response to both ST42 and ST104.

Conclusions: C. difficile strains with higher toxins levels induced an increased activation of the innate immune system and may activate macrophages more profoundly resulting in secretion of higher levels of pro-inflammatory cytokines. However, higher toxin levels may also damage macrophages' normal skeletal structure, reducing macrophage viability.

1. Introduction

Clostridioides difficile (*C. difficile*) is the most common agent responsible for nosocomial diarrhea, called *Clostridioides difficile infection* (CDI).

The pathogenesis of CDI and disease severity depend, among other factors, on bacteria's ability to produce toxins (toxins A and B) [1]. *C. difficile* strains may produce toxin A and toxin B, toxin A/toxin B alone or no toxin at all. Additionally, some strains can produce a third toxin, named the binary toxin, which has been associated with hyper virulent strains characterized by high rates of antibiotic

* Corresponding author. Hanna Senesh 818/2 Tiberias, Israel.
E-mail address: aperetz@tzmc.gov.il (A. Peretz).

https://doi.org/10.1016/j.heliyon.2023.e13846
Received 23 June 2022; Received in revised form 13 February 2023; Accepted 14 February 2023
Available online 17 February 2023

resistance, elevated levels of toxins A and B and increased rates of mortality [2].

C. difficile strains have been recently classified by different molecular methods, in particular multilocus sequence typing (MLST), which was developed to study the genetic relationship and the population structure of *C. difficile* isolates from various patients. MLST is performed by DNA sequence analysis of seven housekeeping genes (*aro*E, *ddl, dut*A, *tpi, rec*A, *gmk,* and *sod*A) [3,4].

Another factor affecting CDI severity and progression is the host immune response, especially the innate system, which is considered the body's second detention barrier against pathogens [5].

The immune response's intensity is correlated with macrophage-mediated phagocytosis efficacy, since phagocytosis in pluricellular organisms is a complex process that has a dual role in addition to its importance in maintaining tissue homeostasis: ingestion and destruction of pathogens and elimination of apoptotic cells [6–8].

Macrophages are the first specialized cells involved in the detection, phagocytosis, and destruction of bacteria and other harmful organisms. In addition, they can also present antigens to T cells and release molecules known as cytokines such as IL-10, IL-12, TGF-β and IL-22 which activate other cells, and enhance complement-mediated bacterial phagocytosis [6,7].

It is still unclear how some patients quickly recover from infection while others do not even develop symptoms at all. Several recent studies associated this to the regulation of diverse host physiological functions including metabolism, the nervous system, homeostasis of immunity and particularly the efficiency of innate immune system mediation [9]. Also, there are toxigenic strains of *C. difficile* that are more resistant to phagocytosis compared to non-toxigenic strains [3,8]. However, previous studies have not investigated different strains of *C. difficile* classified by MLST and compared the differences in the host macrophages response they induce, leading us to conduct the current study in which we compared the viability and activity of macrophages, and cytokine production in response to different *C. difficile* ST strains.

2. Methods

2.1. Study isolates

Thirty isolates were chosen from a bank of *C. difficile* isolates collected at the clinical microbiology laboratory of the Tzafon Medical center. These isolates were previously isolated from stool samples of patients aged ≥18 years diagnosed with CDI during hospitalization at the Tzafon Medical Center, and were enrolled in a previous study approved by the Tzafon Medical Center Helsinki Committee, POR-0085-15. Five isolates of each sequence type (ST) strain (ST2, ST4, ST13, ST37, ST42, and ST104) were included in the study. These strains are the most common strains in our medical center [10,11].

2.2. Bacterial culture

Frozen isolates were inoculated on selective CHROMagar medium ChromID™ *C. difficile* (CDIF), (BioMérieux, Durham, NC), and incubated at 37 °C, in a Bactron 300 anaerobic chamber (Sheldon Manufacturing, Cornelius, OR, USA) for 48 h. *C. difficile* colonies appear asymmetric, black in color, and with an irregular border. Final identification was performed using the Bruker Biotyper system (Bruker Daltonics, Bremen, Germany), which is based on the matrix-assisted laser desorption ionization-time of flight (MALDI TOF) technique [12].

For the experiments, thioglycolate broth (Becton Dickinson, Heidelberg, Germany) supplemented with L-cysteine 0.5 g/L was inoculated with colonies of each bacterial strain. The broths were incubated overnight at 37 °C, under anaerobic conditions, in a Bactron 300 anaerobic chamber (Sheldon Manufacturing).

2.3. Macrophages RAW 264.7 cell culture

RAW 264.7 macrophages cells (ATCC TIB-71) were cultured in Dulbecco's modified Eagle medium (DMEM; ATCC 30–2002) supplemented with 4 mM L-glutamine and 1% Pen-Strep (50 μg/ml penicillin and 50 μg/ml streptomycin). Then the medium was incubated for 24 h at 37 °C, 90% humidity and 5% CO_2. Approximately 5 × 10 [6] cells/well were seeded in 6-wells plates for gene expression experiment and 5 × 10 [4] were seeded in 96-wells plates for the other experiments.

2.4. Macrophages RAW 264.7 cells' viability test

Viability following exposure to different STs: Bacteria of each ST were grown overnight in thioglycolate broth (Becton Dickinson) supplemented with L-cysteine 0.5 g/L. Then, a sample was taken from each bacterial suspension for turbidity measurement and adjustment to 0.5 McFarland (equivalent to 1.5×10^8 CFU/mL).

50 μl of bacteria (grown overnight) of each ST at an MOI of 1:50 suspended in thioglycolate fluid was added to each well. Then, the plate was incubated at 37 °C, 90% humidity and 5% CO_2 for 5 min, 10 min, 1 h and 4 h.

Viability following exposure to toxins: 50 μl of toxin A/toxin B/both toxins A + B at different concentrations were added to each well. Then, the plate was incubated at 37 °C, 90% humidity and 5% CO_2 for 30 min, 6 h, 12 h and 24 h. In order to prepare the desirable toxins concentrations, native *C. difficile* Toxin A and B, (Abcam, Toronto, Canada) were dissolved in DDW and serial diluted in DDW to the following concentrations: 5 ng/μl, 1 ng/μl, 0.5 ng/μ and 0.05 ng/μl.

Following macrophages exposure to the different ST strains or to bacterial toxins, macrophages' viability was tested using the Cell Proliferation Kit -XTT based (Biological industries, Beit Haemek, Israel); following addition of XTT reagent solution, OD_{450} was

measured using an ELISA reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.5. Cytokines levels measurements

Macrophages were seeded in a 96 well plate were exposed to different strains for 24 h 50 μl of bacteria of each ST at an MOI of 1:50 suspended in thioglycolate fluid was added to each well. Then, the plate was incubated at 37 °C, 90% humidity and 5% CO_2 for 24 h. Following incubation, four cytokines (IL-12, TNF-α, IL-1β and IL-6) were quantified in the growth medium of RAW264.7 cells, using ELISA kits for each cytokine (Abcam, Toronto, Canada), according to the manufacturer's instructions. The plate was read at OD_{450} using a spectrophotometer ELISA reader.

2.6. Cytokine gene expression

Macrophages were seeded in a 6-well plate were exposed to different strains for 24 h 50 μl of bacteria from each ST at an MOI of 1:50 suspended in thioglycolate fluid was added to each well. Then, the plate was incubated at 37 °C, 90% humidity and 5% CO_2 for 24 h. Following incubation, the expression levels of the following cytokine genes: IL-12, TNF-α, IL-1β and IL-6 were quantified. Macrophages infected with different STs for 24 h were scraped by trypsinization and the suspension was centrifuged at 250×*g* for 5 min. RNA was extracted from the pellets using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Ottawa, Ontario, Canada), according to the manufacturer's instructions. Then, cDNA was synthesized from the purified RNA using the SuperScript™ III First-Strand Synthesis Kit (Thermo Fisher Scientific, Carlsbad, CA, USA), according to the manufacturer's instructions. Finally, the cDNA was amplified by RT-PCR. To this end, 2 μL of cDNA, 1 μL of primers for each cytokine (0.5 μl forward primer and 0.5 μl reverse primer, 10 μM each), 10 μL SYBR Green Master Mix (Thermo Fisher Scientific) and 17 μL water were added to a PCR tube loaded on a CFX96 Real-Time PCR System (Bio- Rad). PCR reaction conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. GAPDH gene was used as a reference gene. Table 1 presents the primers used in the current study. Changes in cytokines levels following exposure to the different STs were evaluated in comparison to control unexposed cells, using the delta-delta Ct method.

2.7. Macrophages' morphological changes

Macrophages (1 × 10 [4]) were seeded in a 6-well plate and incubated at 37 °C, 90% humidity and 5% CO_2 for 24 h. Then, 5 × 10 [5] bacteria (MOI 1:50) were added to each well, following which the wells were stained for actin by adding two drops of Actin Red (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated at 37 °C, 90% humidity and 5% CO_2 for 16 h in the dark [13]. Then, images were acquired with a fluorescent microscope at X100 (Axio observer, ZEISS, Germany). The excitation/emission was set to 488 nm/510 nm.

2.8. Statistical analysis

Each experiment was perfumed 5 times with 3 replicates per each isolate. Kruskal–Wallis one-way analysis of variance (ANOVA) with Dunn's post-hoc test were used to analyze differences in the effects of different *C. difficile* ST strains or toxins. The data was analyzed using GraphPad Prism version 9.3.0.463 (GraphPad company, San Diego, USA).

3. Results

3.1. The effect of different ST strains on macrophages vitality

As presented in Fig. 1A–C, there were no significant differences between the viability of macrophages exposed to *C. difficile* cells compared to control unexposed cells following a short exposure period (5 min- 1 h, Fig. 1A–C, respectively). In contrast, following a longer, 4 h exposure, a significant reduction (20% reduction, $p = 0.0320$) in macrophages' viability was seen in macrophages exposed to ST42 compared to unexposed cells (Fig. 1D). All strains led to a reduction in macrophages' viability after a 4-h exposure, however

Table 1
Primers used for cytokines genes.

Gene	Primer sequence (5'-3')	Reference
GAPDH	F: ATGGTGAAGGTCGGAGTGAACG R: TGTAGTGAAGGTCAATGAAGGGGTC	9
IL-12	F: TTAATTGAGGTCGTGGTAGAAGCTG R: GGTCTCAGTTGCAGGTTCTTGG	10
TNF-α	F: GGTGCCTATGTCTCAGCCTCTT R: GCCATAGAACTGATGAGAGGGAG	11
IL-1β	F: TGGACCTTCCAGGATGAGGACA R: GTTCATCTCGGAGCCTGTAGTG	12
IL-6	F: CCTCTGGTCTTCTGGAGTACC R: CCTCTGGTCTTCTGGAGTACC	12

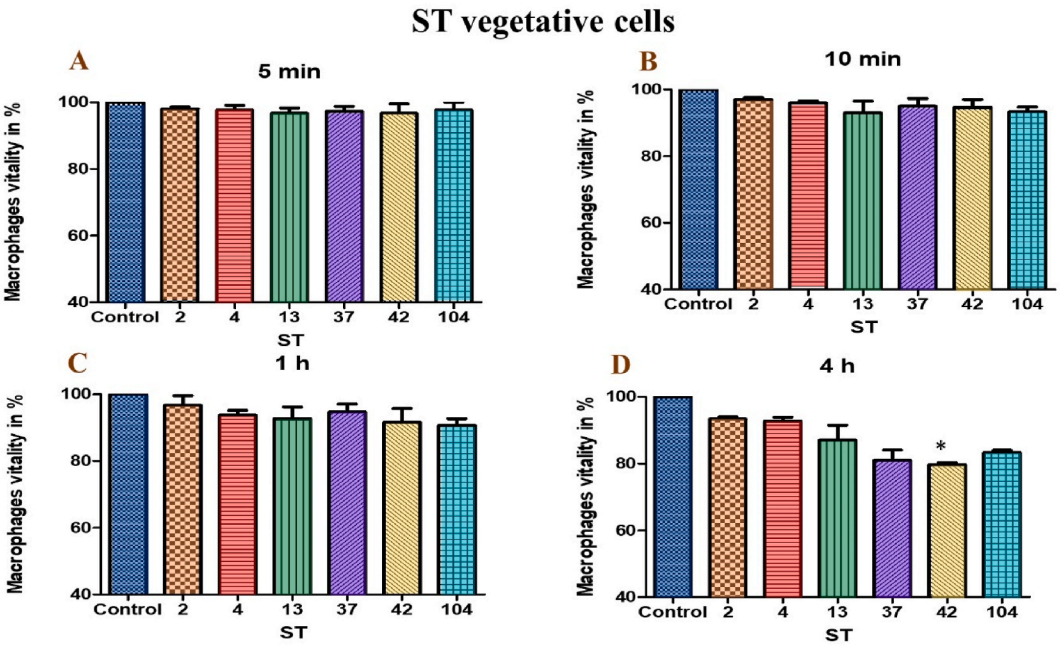

Fig. 1. Macrophages' viability was measured following exposure to different ST vegetative cells for various time periods: A. 5 min, B. 10 min, C. 1 h and D. 4 h. Cell viability is presented as mean percentage of control ± standard deviation. (n = 5). The Kruskal–Wallis test was used to analyze differences between cell viability of exposed and unexposed macrophages, (*$p < 0.05$).

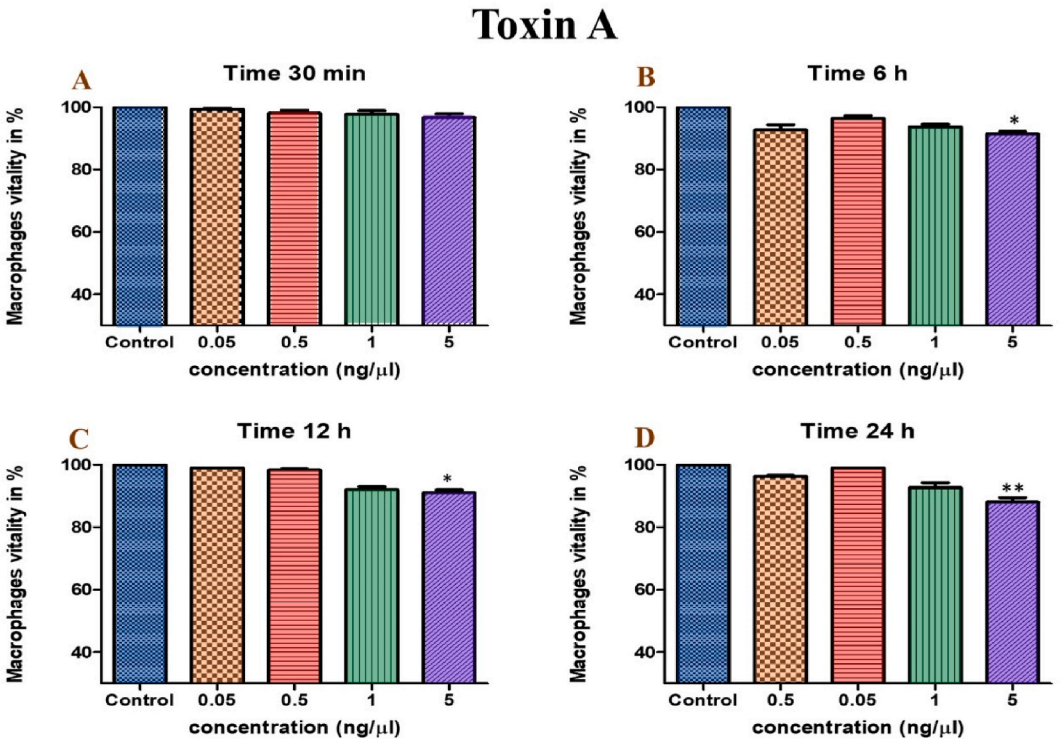

Fig. 2. Macrophages' viability was measured following exposure to different concentrations of toxin A (5 ng/µl, 1 ng/µl, 0.5 ng/µl and 0.05 ng/µl) for various time periods: A. 30 min, B. 6 h, C. 12 h and D. 24 h. Cell viability is presented as mean percentage of control ± standard deviation; (n = 5). Kruskal–Wallis was used to analyze differences between cell viability of exposed and unexposed macrophages, (*$p < 0.05$, **$p < 0.01$).

only ST42 displayed a statistically significant reduction; for example, ST2 and ST4 caused a 7% reduction, ST13 caused a 13% reduction, ST37 caused a 19% reduction and ST104 caused a 17% reduction.

3.2. Toxins effect on macrophages vitality

RAW 264.7 macrophages were exposed to four different concentrations of native toxin A (5 ng/μl, 1 ng/μl, 0.5 ng/μl and 0.05 ng/μl). Then, macrophages' viability was measured at four different time points (30 min, 6 h, 12 h and 24 h, Fig. 2A–D, respectively). As presented in Fig. 2A–D, there were no significant differences between macrophages exposed to 0.05 ng/μl and 0.5 ng/μl toxin A, compared to unexposed cells, in all exposure periods. Exposure of macrophages to 1 ng/μl toxin A induced a reduction in macrophages vitality, however the differences noted were not statistically significant. In contrast, exposure to 5 ng/μl toxin A induced a significant reduction in macrophages' vitality in most of the time points; a 9% reduction was seen after a 6 h exposure to 5 ng/μl toxin A ($p = 0.0320$) (Fig. 2B), a 10% reduction after 12 h ($p = 0.0162$) (Fig. 2C), and a 12% reduction after 24 h ($p = 0.0088$) (Fig. 2D).

Next, we performed the same assay with native toxin B. As presented in Fig. 3A–D, there were no significant changes in macrophages' viability when exposed to toxin B (at concentrations of 0.05 ng/μl −1 ng/μl), compared to unexposed cells, regardless of the period of exposure. On the contrary, exposure to 5 ng/μl toxin caused a significant decline in cells viability seen after only 6 h (Fig. 3B). It should be noted that there were reductions in viability in macrophages that were exposed to concentration 0.5 or 1 ng/μl of the toxin for 12 or 24 h (Fig. 3C and D, respectively), however these changes were non-significant.

Since most strains secrete both toxins, we exposed macrophages to combinations of native toxin A and B. No significant differences were found between macrophages exposed to toxin A and B (at concentrations of 0.05 ng/μl - 1 ng/μl) in each of the time periods, compared to unexposed cells (Fig. 4A–D). In contrast, exposure to 5 ng/μl of toxin A and B caused a significant decline in cellular viability already after 30 min.

3.3. Cytokine protein levels

We compared the levels of cytokines secreted by the macrophages following exposure to different *C. difficile* ST strains. We found that macrophages exposed to *C. difficile* strains secreted significantly higher levels of cytokines compared to unexposed macrophages (Fig. 5A–D). In particular, ST42 induced a 8.5-fold increase in IL-1β levels (7.812 pg/ml compared to 1 pg/ml in control cells, $p = 0.035$) (Fig. 5A), a 62.57-fold increase in IL-12 levels (1814.6 pg/ml compared to 29 pg/ml in control cells, $p = 0.0003$) (Fig. 5B), a 213.88-fold increase in TNF-α levels (5274.8 pg/ml compared to 24.7 pg/ml, $p = 0.0018$) (Fig. 5C), and a 39.81-fold increase in IL-6

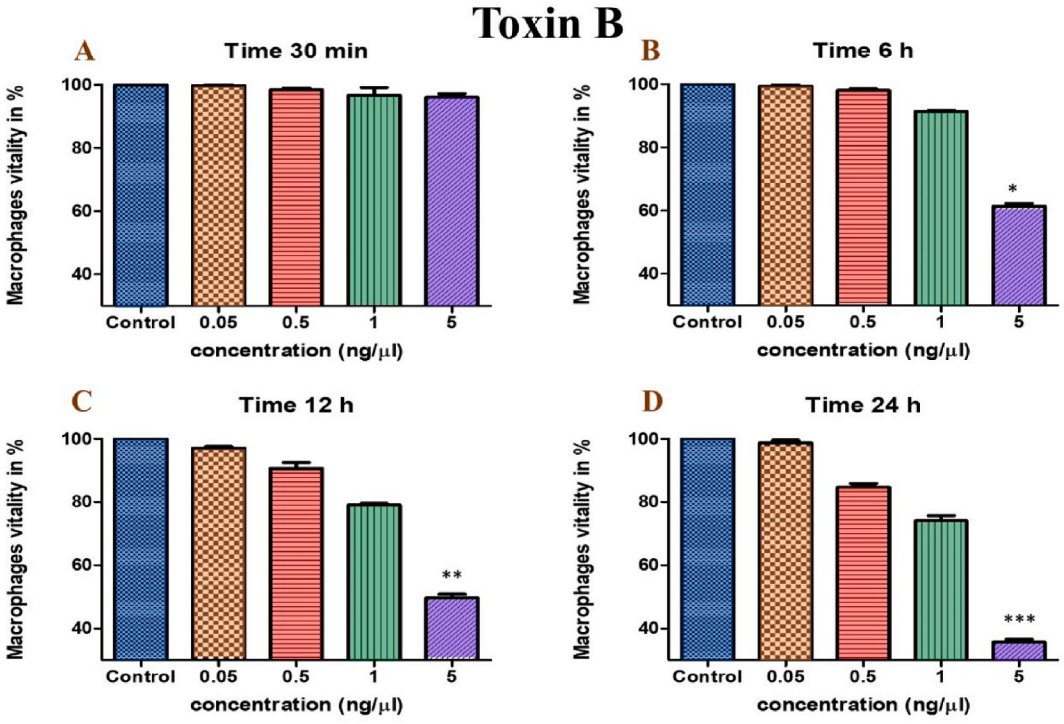

Fig. 3. Macrophages' viability was measured following exposure to different concentrations of toxin B (5 ng/μl, 1 ng/μl, 0.5 ng/μl and 0.05 ng/μl) for various time periods: A. 30 min, B. 6 h, C. 12 h and D. 24 h. Cell viability is presented as mean percentage of control ± standard deviation; (n = 5). The Kruskal–Wallis test was used to analyze differences between cell viability of exposed and unexposed macrophages, (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

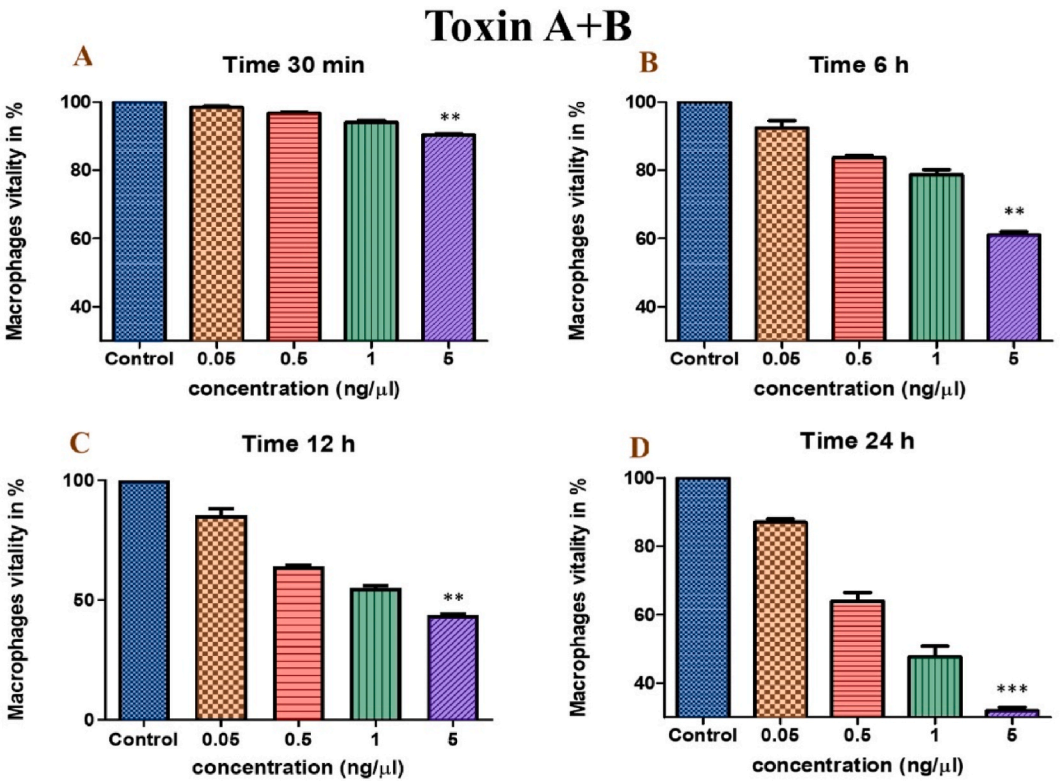

Fig. 4. Macrophages' viability was measured following exposure to different concentrations of toxin A and B (0.05–5 ng/µl) for various time periods: A. 30 min, B. 6 h, C. 12 h and D. 24 h. Cell viability is presented as mean percentage of control ± standard deviation; (n = 5). The Kruskal–Wallis test was used to analyze differences between cell viability of exposed and unexposed macrophages, (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

levels (198.2 pg/ml compared to 5 pg/ml in control cells, $p = 0.0101$) (Fig. 5D). ST104 caused a significant increase in cytokines' secretion; a 39.78-fold increase in IL-12 levels (1153.8 pg/ml compared to 29 pg/ml, $p = 0.0044$) (Fig. 5B), and a 211.93-fold increase in TNF-α levels (5226.6 pg/ml compared to 24.7 pg/ml, $p = 0.0090$) (Fig. 5C). Also, ST37 caused a 36.34-fold increase in IL-12 levels (1054 pg/ml, $p = 0.0126$) (Fig. 5B). When comparing the different STs, we found that IL-12 levels were significantly different between macrophages exposed to ST42 (1814.6 pg/ml) and ST4 (509.2 pg/ml) ($p = 0.044$) (Fig. 5B).

3.4. Cytokines gene expression

We performed a RT-PCR for numerous cytokines to monitor gene expression. Significant increases in cytokines' gene levels were measured in macrophages exposed to *C. difficile* strains, compared to unexposed cells (Fig. 6A–D); for example, ST13 caused a 16.4-fold increase in IL-1β gene levels ($p = 0.0080$) (Fig. 6A). ST42 caused a 2.9-fold increase in IL-12 gene levels ($p = 0.00305$) (Fig. 6B) and also a 4.98-fold increase in TNF-α gene levels ($p = 0.0126$) (Fig. 6C); ST2 caused a 33.2-fold increase and ST104 caused an 18.37-fold increase in IL-6 gene levels ($p = 0.0055$, $p = 0.0220$, respectively) (Fig. 6D). Mostly, no statistical differences were seen in cytokines levels expressed in macrophages exposed to different strains. However, ST13 caused a 14.5–fold increase in IL-1β gene levels compared to ST37 ($p = 0.038$) (Fig. 6A), and ST42 caused a 6.84–fold increase in IL-12 gene expression compared to ST4 ($p = 0.0071$) (Fig. 6B).

3.5. Morphological changes in macrophage cells

The last aim was to monitor the effect of ST strains on macrophages' morphology. Macrophages were exposed to the different strains for 4 h, following which the macrophages were stained in order to distinguish their cytoskeleton morphological changes. Macrophages exposed to ST13 and ST42 presented more extensions (Fig. 7C and D, respectively), compared to control (Fig. 7A) and to ST2 (Fig. 7B), indicating macrophage activation.

4. Discussion

The present study dealt with one of the most common nosocomial infection worldwide, CDI [14]. Due to the differences in disease severity among patients, it is believed that disease progress depends both on the pathogen and the patient. Therefore, it is very

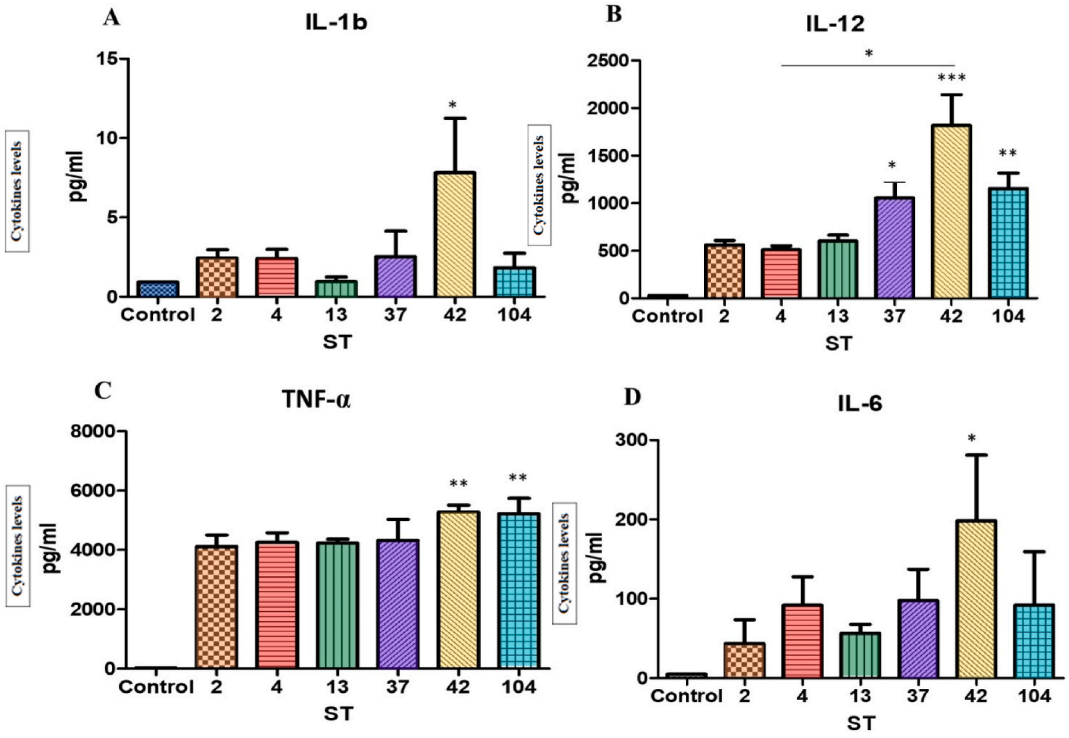

Fig. 5. The effect of *C. difficile* strains on cytokines levels was examined using an ELISA assay for the measurement of A. IL-1β, B. IL-12, C. TNF-α and D. IL-6. Cytokines levels are presented as mean levels in pg/ml ± standard deviation; (n = 5). The Kruskal–Wallis test was used to analyze differences between macrophages infected with STs and uninfected macrophages, (*p < 0.05, **p < 0.01, ***p < 0.001).

interesting to examine the immune response as it may also affect disease severity.

4.1. The effect of C. difficile STs on macrophages vitality

We found that the STs induced a reduction in cells' viability. Specifically, 20%, 19% and 17% reduction in cellular viability were seen following a 4 h exposure of the macrophages to ST42, ST37 and ST104, respectively.

These reductions in cell viability may be associated to the toxins secreted by *C. difficile*, since previous reports have showed that exposure of macrophages to *C. difficile*'s toxins A or B induced cell death [15–17]. For example, 30% cell death was induced in macrophages exposed to toxin A at a concentration of 1 μg/ml for 24 h. 20% and 60% cell death were measured in macrophages exposed to toxin B at concentrations of 0.1 and 1 μg/ml, respectively [15].

Therefore, we assume that the reduction in cell viability seen following exposure to the bacteria is a result of its exposure to the bacterial toxins. In this regard, a previous study which evaluated toxins' concentrations secreted by different STs found that ST42 and ST104 secreted the highest toxin levels; both ST strains induced the most significant cytopathic effects and reductions in cell proliferation of Vero cells tested using the XTT assay, compared to other strains. ST37 also showed a more severe impact on cellular morphology, compared to other strains [10]. ST42 (Ribotype 106- RT 106) was also previously associated with increased rates of poor clinical outcomes compared to RT 014/020 and comparable poor clinical outcomes to RT 02 which is known ashyper virulent strain [18]. An additional study reported that a representative RT 106 strain induced 100% lethality in a hamster model of acute CDI [18,19].

Another factor that may be responsible for the reduction in cell viability following exposure of macrophages to *C. difficile* strains is the surface layer proteins (SLPs) of the bacteria. It was previously shown that these SLPs are recognized by the TLR4 [20]. A previous study reported that *C. difficile* SLPs activated a clearance response in murine macrophages, including induction of pro-inflammatory cytokines production, macrophage migration, and phagocytosis [20]. Therefore, future studies should focus on the relationship between SLPs and macrophage activation.

4.2. The effect of toxins A and B on macrophages vitality

We found that high concentration (5 ng/μl) of either toxin A or B induced the most significant reduction in cell viability, compared to lower concentrations, at each time point. This result was seen even after a short 6 h' exposure to the toxins.

It is known that both toxins are cytotoxic to epithelial and immune cells [21], and that toxin B is more potent than toxin A [16]. As mentioned above, previous reports have shown that exposure of macrophages to *C. difficile*'s toxins A or B induced cell death [10,16,

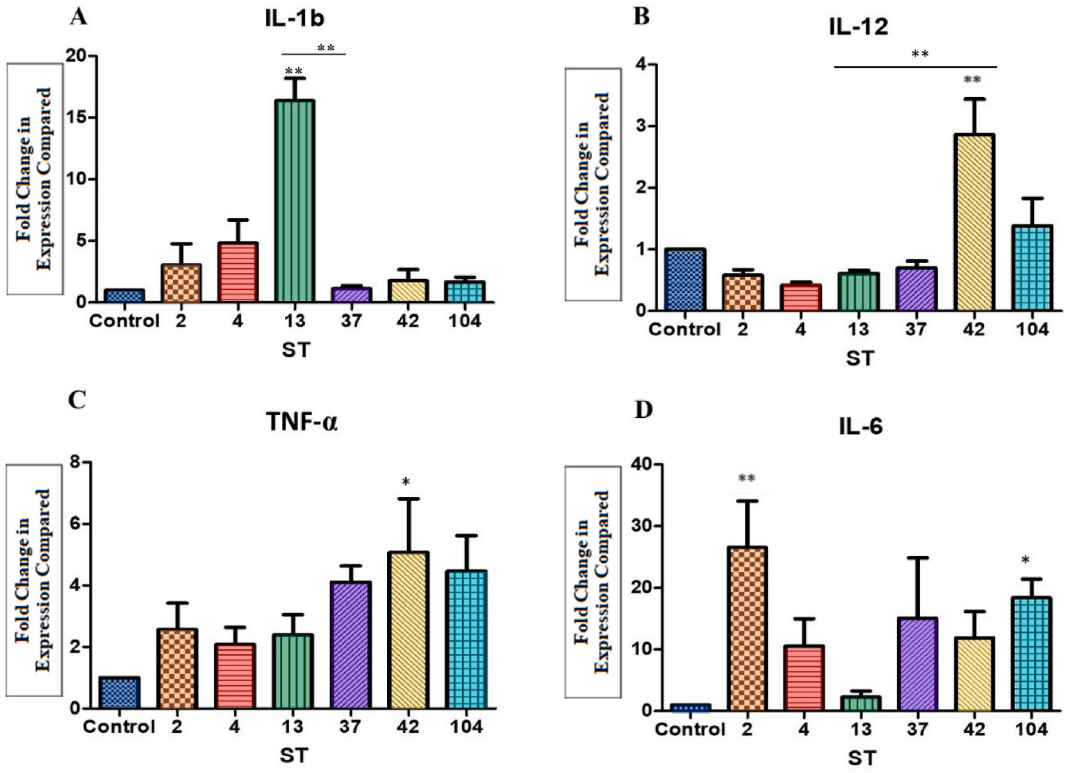

Fig. 6. The effect of *C. difficile* strains on cytokines gene expression was examined using the rt-PCR assay to determine A. IL-1β, B. IL-12, C. TNF-α and D. IL-6. Cytokines' gene expression is presented as mean change fold of control ± standard deviation; (n = 5). The Kruskal–Wallis test was used to analyze differences between macrophages infected with STs and uninfected macrophages, (*$p < 0.05$, **$p < 0.01$).

17]. For example, a study which investigated the effect of *C. difficile* toxin A on isolated colonic lamina propria cell preparations, showed that toxin A induced an early loss of macrophages in a dose- and time-dependent fashion [17]. Lactate dehydrogenase levels of THP-1 macrophages exposed to toxin A, B, or both, increased in a time- and dose-dependent manner [22]. The minimal concentration inducing cell death in THP-1 macrophages was 2.5 μg/ml of toxin A, B, or both. The discrepancy in the minimal concentration leading to cell death in the above study compared to our results is probably due to the different types of cells used in each study.

We also found that toxin B was more potent compared to toxin A. This result was also reported by other studies that indicated that toxin B has a greater effect on cellular function, compared to toxin A, as indicated by the cytopathic effect [16,21,23]. For example, one study showed that low concentrations of toxin B are sufficient to cause serious damage to the skeletal structure of YAMC cells, and to induce the mechanism that switches from apoptosis to necrosis, while higher concentrations were required for toxin A-medicated cell damage and death [23].

4.3. The effect of C. difficile ST's on macrophages cytokines levels

Previous studies that investigated the immune response to *C. difficile*, showed that cytokine production was induced following macrophages' exposure to the bacteria [24] or to bacterial proteins such as the SLP [20,25,26] the flagella, HSP42, HSP60 [25], and toxins A and B [24,25,27].

The interesting finding in the current study was that ST42 and ST104 caused the most significant increase in cytokine levels compared to control cells. These results show that immune response is strain specific, and may be related to the toxins levels secreted by each strain. As mentioned above, a previous study, which evaluated the levels of secreted toxins among 63 *C. difficile* isolates, found that ST42 and ST104 secreted the highest toxins' levels, compared to other strains [10,28]. These strains induced the most significant cytopathic effect in Vero cells [10], indicating that a strain with higher toxin levels leads to a more significant development of inflammation and secretion of pro-inflammatory cytokines [29,30]. It should be noted that ST42 and ST104 increased the secretion of IL-1β and TNF-α, both of which are pro-inflammatory cytokines, leading to the intensification of the inflammation. A previous study showed that both Il-1β and TNFα secreted from macrophages induced the chemotaxis of neutrophils to the rat peritoneal cavity [31]. This may explain the severe disease caused by ST42 and ST104. Furthermore, previous reports have shown that *C. difficile* toxins induce the production of several proinflammatory cytokines including Il-1α, Il-1β, Il-6, IL-8 and TNFα [25,27,31]. It is known that the toxins' glucosyltransferase activity mediates the TNFα-dependent induction of both local and systematic inflammatory responses [32].

Other bacterial components may also trigger cytokine production; for example, a previous study reported that cell surface-

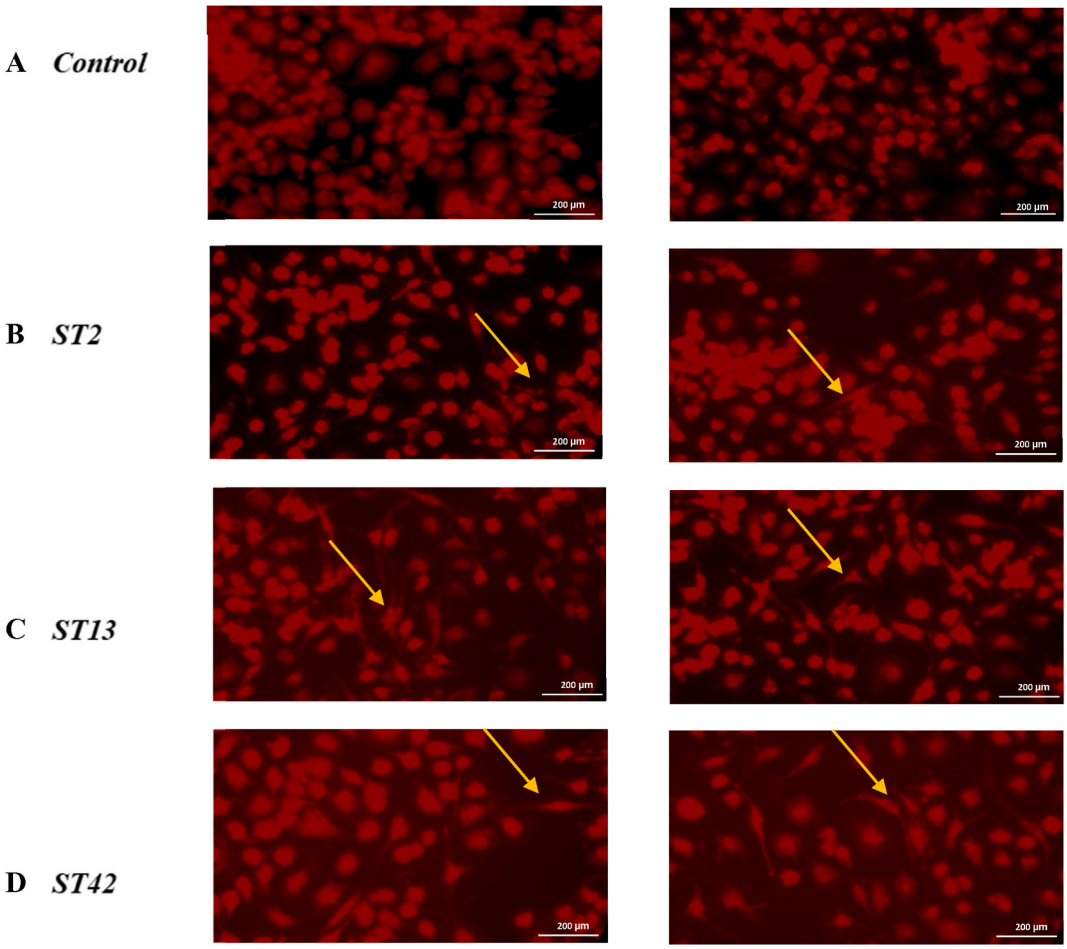

Fig. 7. The effect of *C. difficile* ST2 (B), ST13 (C) and ST42 (D) vegetative cells on morphological changes of the macrophages was determined using actin staining in fluorescent microscopy, comparing infected to uninfected cells (A). Fluorescent Microscope × 100, Scale: 50 µm.

associated proteins extracted from 5 different *C. difficile* strains induced cytokine production in THP-1 macrophage cells [25]. Exposure of THP-1 cells to these proteins, including the SLPs, the flagella, HSP42, and HSP60, increased the production of various cytokines. IL-8 was the most common induced cytokine, and its levels were similar following exposure to the different proteins. All proteins induced similar levels of IL-1β and IL-6. On the other hand, specific cytokines levels were different with regard to the protein to which the cells were exposed; for example, the SLPs triggered the most significant increase in IL-12p70, and HSP60 triggered the highest production of TNFα [25].

In contrast, a study that exposed macrophages to SLPs of different ribotypes revealed a more potent immune response to SLPs from RT 027 and RT 078, known to be hyper-virulent [20]. Additionally, it is possible that different strains differ in the presence and levels of specific components (such as flagella and SLPs) and this may explain the differences in the cytokines induced by each strain. Therefore, more studies should be conducted in order to understand whether the macrophages' immune response is strain-dependent and determine the mechanism responsible for the intensity of the response.

All the cytokines secreted by macrophages play a significant role in the immune response against *C. difficile*; IL-1β plays a role in adaptive immunity, which enhances differentiation and expansion of T cells. IL-1β also induces the differentiation of T cells into TH17 cells, which are involved in bacterial clearance. IL-12 affects macrophages to increase phagocytosis and cytokine production in order to activate T cells and NK cells. IL-6 is secreted as a response to PAMPs recognition and mainly causes fever and local inflammation. TNFα induces the production of additional pro-inflammatory cytokines leading to enhanced apoptosis during bacterial or viral infection.

Cytokine gene expression was investigated using RT-PCR assay and the results showed significant differences between *C. difficile*-exposed macrophages to control cells. Additionally, there were differences between macrophages exposed to different STs.

The expression levels of IL-12 and TNFα genes following exposure to the different STs overall correlated with protein levels patterns. Surprisingly, the expression levels of IL-1β, a pro-inflammatory cytokine with pleiotropic activities including differentiation and growth of T and B cells, were the highest following infection with ST13 rather than ST42, as we expected based on the ELISA assay. Likewise, IL-6 gene was also expressed differently compared to its protein levels. This may be related to degradation of either the RNA or the protein. Further investigation is required to understand the differences in RNA and protein levels.

4.4. The effect of C. difficile ST's on macrophages morphological changes

The first and most obvious impact on cell physiology is the loss of structural integrity, which results from the decline in F-actin in cells exposed to TcdA and TcdB secreted by the bacteria causing alterations in cell surface projections and rearranged microvilli; the distinction between the STs could be due to their toxin production levels (ST42 vs. ST2). Moreover, TcdA and TcdB-intoxicated cells demonstrated a noticeable cell retraction phenotype linked to changes in the microfilament system, marginalization of the nucleus and grave changes in the cytoskeleton. Furthermore, cytopathic effects observed are due to the presence of both toxins similarly. Mechanistically, the loss of structural integrity in toxin treated cells is expected, since Rho and Rac regulate structural processes dependent on actin polymerization and cells with considerable loss of actin cytoskeleton integrity are unlikely to carry out their roles) within the host [33].

The study has several limitations. First, we used limited number of different STs and total number of isolates. A future study should include more strains. Second, we did not demonstrate the phagocytosis process, which is the main activity of macrophages.

5. Conclusions

In conclusion, we observed that specific strains induced a more significant decrease in macrophages' cell viability and induced an increased production of cytokines. Some of these strains were previously characterized with higher toxins levels, compared to other strains, which may explain the more profound effect on the macrophages. Further investigation regarding other virulent components of the strains such as flagella and SLPs, is needed to gain further insight behind the diverse macrophages' response to the different strains of *C. difficile*.

Author contribution statement

Gewa Saad: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Maya Azrad: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Meral Aias; Zohar Hamo; Layan Abu Rahmoun: Performed the experiments.

Tamar Leshem: Performed the experiments; Analyzed and interpreted the data.

Avi Peretz: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no competing interests.

Additional information

No additional information is available for this paper.

References

[1] N.M. Khardori, Toxin B is essential for virulence of Clostridium difficile, Yearb. Med. (2009) 85–86.
[2] D.J. Reinert, T. Jank, K. Aktories, G.E. Schulz, Structural basis for the function of Clostridium difficile toxin B, J. Mol. Biol. 351 (2005) 973–981.
[3] L. Lemee, A. Dhalluin, M. Pestel-Caron, J.F. Lemeland, J.L. Pons, Multilocus sequence typing analysis of human and animal Clostridium difficile isolates of various toxigenic types, J. Clin. Microbiol. 42 (2004) 2609–2617.
[4] P. Shoaei, et al., Clostridium difficile infection in cancer patients with hospital acquired diarrhea at the teaching hospitals in Iran: multilocus sequence typing analysis (MLST) and Antimicrobial resistance pattern, Ann. Ig 31 (2019) 365–373.
[5] J. Benveniste, G. Lespinats, C. Adam, J.-C. Salomon, Immunoglobulins in intact, immunized, and contaminated axenic mice: study of serum IgA, J. Immunol. 107 (1971).
[6] J. Czepiel, et al., Clostridium difficile infection: review, Eur. J. Clin. Microbiol. Infect. Dis. (2019), https://doi.org/10.1007/s10096-019-03539-6.
[7] Buonomo, E. L. & Petri, W. A. The microbiota and immune response during Clostridium difficile infection. Anaerobe doi:10.1016/j.anaerobe.2016.05.009.
[8] D.C. Dailey, A. Kaiser, R.H. Schloemer, Factors influencing the phagocytosis of *Clostridium difficile* by human polymorphonuclear leukocytes, Infect. Immun. 55 (7) (1987) 1541–1546.
[9] E.M. Sternberg, Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens, Nat. Rev. Immunol. 6 (4) (2006) 318–328.
[10] Z. Hamo, M. Azrad, B. Fichtman, A. Peretz, The cytopathic effect of different toxin concentrations from different *Clostridioides difficile* sequence types strains in Vero cells, Front. Microbiol. 12 (2021) 1–8.
[11] L.A. Rahmoun, M. Azrad, A. Peretz, Antibiotic resistance and biofilm production capacity in *Clostridioides difficile*, Front. Cell. Infect. Microbiol. 4 (11) (2021), 683464, https://doi.org/10.3389/fcimb.2021.683464. PMID: 34422678; PMCID: PMC8371447.Layan.

[12] N. Singhal, M. Kumar, P.K. Kanaujia, J.S. Virdi, MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis, Front. Microbiol. 6 (2015) 791.
[13] S. Wang, F. Liu, Z. Zeng, H. Yang, H. Jiang, The protective effect of bafilomycin A1 against cobalt nanoparticle-induced cytotoxicity and aseptic inflammation in macrophages in vitro, Biol. Trace Elem. Res. 169 (1) (2016) 94–105.
[14] B.K. Sandhu, S.M. McBride, Clostridioides difficile, Trends Microbiol. 26 (2018) 1049–1050.
[15] A.A. Melo Filho, M.H.L.P. Souza, D.M. Lyerly, F.Q. Cunha, A.A.M. Lima, R.A. Ribeiro, Role of tumor necrosis factor and nitric oxide in the cytotoxic effects of Clostridium difficile toxin A and toxin B on macrophages, Toxicon 35 (5) (1997) 743–752.
[16] J. Ng, et al., Clostridium difficile toxin-induced inflammation and intestinal injury are mediated by the inflammasome, Gastroenterology 139 (2010) 542–552. e3.
[17] Y.R. Mahida, et al., Effect of Clostridium difficile toxin A on human colonic lamina propria cells: early loss of macrophages followed by T-cell apoptosis, Infect. Immun. 66 (1998) 5462–5469.
[18] M. Saeed, et al., Anaerobe Comparative clinical outcomes evaluation of hospitalized patients infected with Clostridioides difficile ribotype 106 vs . other toxigenic strains, Anaerobe 72 (2021), 102440.
[19] B.A.P. Roxas, et al., Phylogenomic analysis of Clostridioides difficile ribotype 106 strains reveals novel genetic islands and emergent phenotypes, Sci. Rep. (2020) 1–17, https://doi.org/10.1038/s41598-020-79123-2.
[20] L.E. Collins, et al., Surface layer proteins isolated from Clostridium difficile induce clearance responses in macrophages, Microb. Infect. 16 (2014) 391–400.
[21] K. Aktories, C. Schwan, T. Jank, Clostridium difficile toxin biology, Annu. Rev. Microbiol. 71 (2017) 281–307.
[22] K. Solomon, J. Webb, N. Ali, R.A. Robins, Y.R. Mahida, Monocytes are highly sensitive to clostridium difficile toxin A-induced apoptotic and nonapoptotic cell death, Infect. Immun. 73 (3) (2005) 1625–1634.
[23] N.M. Chumbler, M.A. Farrow, L.A. Lapierre, J.L. Franklin, D.B. Lacy, Clostridium difficile toxins TcdA and TcdB cause colonic tissue damage by distinct mechanisms, Infect. Immun. 84 (2016) 2871–2877.
[24] N.V. Jafari, et al., Clostridium difficile modulates host innate immunity via toxin-independent and dependent mechanism(s), PLoS One 8 (2013) 1–10.
[25] P. Vohra, I.R. Poxton, Induction of cytokines in a macrophage cell line by proteins of Clostridium difficile, FEMS Immunol. Med. Microbiol. 65 (2012) 96–104.
[26] M. Lynch, et al., Surface layer proteins from virulent Clostridium difficile ribotypes exhibit signatures of positive selection with consequences for innate immune response, BMC Evol. Biol. 17 (2017) 90.
[27] X. Sun, X. He, S. Tzipori, R. Gerhard, H. Feng, Essential role of the glucosyltransferase activity in Clostridium difficile toxin-induced secretion of TNF-α by macrophages, Microb. Pathog. 46 (2009) 298–305.
[28] S.A. Kuehne, et al., The Role of Toxin A and Toxin B in Clostridium difficile Infection, 2010, https://doi.org/10.1038/nature09397.
[29] Batah, J. et al. Clostridium difficile flagella induce a pro-inflammatory response in intestinal epithelium of mice in cooperation with toxins. Sci. Rep. doi: 10.1038/s41598-017-03621-z.
[30] A. Nicholas, et al., Clostridium Difficile-Derived Membrane Vesicles Induce the Expression of Pro-inflammatory Genes and Cytotoxicity in Colonic Epithelial Cells in Vitro, 2017, https://doi.org/10.1016/j.micpath.2017.03.006.
[31] R. Madan, W.A. Petri, Immune responses to Clostridium difficile infection, Trends Mol. Med. 18 (2012) 658–666.
[32] P.J. Chiu, J. Rathod, Y.P. Hong, P.J. Tsai, Y.P. Hung, W.C. Ko, I.H. Huang, Clostridioides difficile spores stimulate inflammatory cytokine responses and induce cytotoxicity in macrophages, Anaerobe 70 (2021), 102381.
[33] D. Lyras, J.R. O'Connor, P.M. Howarth, S.P. Sambol, G.P. Carter, T. Phumoonna, J.I. Rood, Toxin B is essential for virulence of Clostridium difficile, Nature 458 (7242) (2009) 1176–1179.