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Yessotoxin inhibits phagocytic activity of macrophages

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ABSTRACT

Yessotoxin (YTX) is a sulphated polyether compound produced by some species of dinoflagellate algae, that can be accumulated in bivalve mollusks and ingested by humans upon eating contaminated shellfish. Experiments in mice have demonstrated the lethal effect of YTX after intraperitoneal injection, whereas its oral administration has only limited acute toxicity, coupled with an alteration of plasma membrane protein turnover in the colon of the animals. *In vitro* studies have shown that this effect is due to the inhibition of endocytosis induced by the toxin. In this work, we investigated the effects of YTX on phagocytosis by using the J774 macrophage cell line. We found that macrophages exposed to 10 or 1 nM YTX display a reduced phagocytic activity against *Candida albicans*; moreover, phagosome maturation is also inhibited in these cells. Such results were confirmed with resident peritoneal macrophages from normal mice. The inhibition of both phagocytosis and phagosome maturation likely involves cytoskeletal alterations, since a striking rearrangement of the F-actin organization occurs in YTX-treated J774 macrophages. Surprisingly, YTX also enhances cytokine production (TNF-α, MIP-1α and MIP-2) by J774 macrophages. Overall, our results show that low doses of YTX significantly affect both effector and secretory functions of macrophages.

1. Introduction

Yessotoxin (YTX) is a sulphated polyether toxin originally isolated from the digestive gland of scallops *Patinopecten yessoensis* (Murata et al., 1987). YTX is produced by several algal species (Draisci et al., 1999; Rhodes et al., 2006; Satake et al., 1997) and it has been found to contaminate bivalve molluscs in several countries (Amzil et al., 2008; Draisci et al., 1999; Morton et al., 2007; Murata et al., 1987; Ramstad et al., 2001).

Studies carried out on animal models have shown that the toxicity of YTX differs significantly, depending on its route of administration. Specifically, YTX has been found to be lethal if injected intraperitoneally (ip) in mice at doses higher than 80 μg/kg (Aune et al., 2002, 2008; Murata et al., 1987; Ogino et al., 1997; Terao et al., 1990; Tubaro et al., 2003). However, when orally administered, YTX does not kill the animals either after a single dose, as high as 50 mg/kg (Aune et al., 2002; Munday et al., 2008; Murata et al., 1987; Ogino et al., 1997; Terao et al., 1990; Tubaro et al., 2003), or following multiple doses of up to 5 mg/kg repeated for 1–3 weeks (Callegari et al., 2006; Tubaro et al., 2004, 2008). Although it has been speculated that the low oral toxicity of YTX is due to the lack of toxin absorption by the gastro-intestinal tract of the mice, a recent study has shown that after oral administration a low, albeit measurable, absorption of YTX occurs, since the blood concentrations of toxin in these mice were in the range of 10^{-9}–10^{-8} M (Tubaro et al., 2008). Histological analysis revealed

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mainly heart and intestine alterations. In the heart of YTX-treated animals, ultrastructural changes have been detected by electron microscopy (Aune et al., 2002; Tubaro et al., 2003, 2004), while in the colon decreased levels of proteolytic fragments of the cell adhesion protein E-cadherin have been found (Callegari et al., 2006).

The mechanistic basis of alterations found in animal studies are largely undetermined, and few processes have been shown to be affected by 10^{-9}–10^{-8} M. By *in vitro* studies distinct effects have been reported, depending on the duration of toxin treatment and the cell line used in the investigation. Short term cell exposure to 10^{-9}–10^{-8} M YTX concentrations induces a limited increase in intracellular Ca^{2+} levels in primary cultures of neuronal cells (Perez-Gomez et al., 2006) and alters protein disposal in epithelial cells and fibroblasts (Pierotti et al., 2003; Ronzitti et al., 2004; Malagoli et al., 2006a; Callegari and Rossini, 2008). Prolonged cellular treatments with these low YTX concentrations for several days, in turn, causes cell death (Ogino et al., 1997; Leira et al., 2002; Malaguti et al., 2002; Malagoli et al., 2006a; Perez-Gomez et al., 2006; Suárez Korsnes et al., 2006; Dell'Ovo et al., 2008).

With regard to the alteration of protein disposal, we have shown that treatment of epithelial cells with 1 nM YTX blocks both the internalization and the complete degradation of the plasma membrane E-cadherin, via the impairment of clathrin-dependent endocytosis (Callegari and Rossini, 2008). However, since the toxin concentrations inhibiting endocytosis *in vitro* (Callegari and Rossini, 2008) were lower than those detected in the blood of orally treated mice (Tubaro et al., 2008), in this study we evaluated if YTX has an effect on the phagocytic activity of macrophages.

Endocytosis and phagocytosis represent the main processes by which the cells of innate immunity dispose off foreign materials, such as invading pathogens (Pizarro-Cerda and Cossart, 2006). Macrophages are the first line of defence in host tissues, since they engulf invading microorganisms within a membrane-bound compartment called phagosome (Kwiatkowska and Sobota, 1999; Rabinovitch, 1995). The phagocytic process requires the assembly of actin microfilaments underneath the plasma membrane, at site where the contact with pathogen occurs, followed by ingestion in an intracellular vacuole, called phagosome. The resulting phagosome undergoes a series of modification processes (like acidification) resulting in its maturation to a phagolysosome. This process allows eventually the killing of the ingested microrganism (Damiani and Colombo, 2003; Vieira et al., 2002).

In order to investigate the effect of YTX on macrophage-mediated phagocytosis, both the J774 cell line and mouse peritoneal macrophages have been employed. We found that low concentrations (10^{-9}–10^{-8} M) of YTX significantly impair their capacity to phagocytose *Candida albicans*.

2. Materials and methods

2.1. Yessotoxin

Yessotoxin (YTX) was obtained from the Institute of Environmental Science and Research Limited (Lower Hutt, New Zealand). The YTX stock solution, dissolved in absolute ethanol (EtOH) at a final concentration of 10 μM, was stored in glass vials protected from light at −20 °C. Dilutions of the YTX stock solution were carried out in absolute EtOH.

2.2. Macrophage cell line

The murine macrophage cell line J774 was maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Defined Hyclone, Logan, Utah, USA), 50 μg/ml gentamicin (Bio Whittaker, Verviers, Belgium) and 2 mM L-glutamine (EuroClone, Milan, Italy). Biweekly, the cells were detached by vigorous shaking and the day before the experiment fresh cultures were started at a cell concentration of 5×10^5/ml.

2.3. Mouse peritoneal macrophages

Resident peritoneal cells (PC) from adult 129S6 mice (Charles River, Italy) were collected after ip injection of 6–8 ml of Hank's Buffered Salt Solution (HBSS) medium without calcium and magnesium, containing gentamicin (50 μg/ml). PC were washed and resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated FBS, gentamicin (50 μg/ml) and L-glutamine (2 mM). The percentage of macrophages in the PC population was about 70, as evaluated by examination of cytospin Diff-Quik preparations. PC (2.8×10^5/well) were plated for 2 h on Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL, USA) pre-treated with poly-L-lysine (Sigma–Aldrich, Saint Louis, MO, USA), as described below. Two hours later, the non-adherent cells were removed by extensive washings and the adherent peritoneal macrophages were exposed to YTX for 12 h.

2.4. C. albicans

The *C. albicans* PCA2 strain used in this study was a poorly virulent, echinocandin-resistant mutant of the parental strain 3153A, which has lost the ability to produce hyphae (Mattia et al., 1982). Long-term storage of the yeast was done in 20% glycerol at −20 °C. On the day of the assay, cultures were transferred in Sabouraud dextrose broth (Oxoid, Hampshire, England), under shaking, for 1 h. When required, staining of *C. albicans* was performed by incubating the yeast cells with 2 μg/ml of Oregon green 488 (Og) (Molecular Probes, Eugene, OR, USA) (Levitz et al., 1999; Wozniak et al., 2006), in the dark at 37 °C for 1 h. After labelling, cells were washed twice with PBS and then resuspended in RPMI 1640 medium at the desired concentration.

2.5. YTX treatment of macrophages

J774 macrophages and mouse peritoneal macrophages (2×10^5/well) were incubated with 10, 1 or 0.1 nM YTX in 1% EtOH for 12 h at 37 °C in 5% CO_2. In order to evaluate the effects of EtOH on cells, control macrophages were incubated with 1% (v/v) EtOH-containing RPMI medium.

2.6. Phagocytosis assay

Lab-Tek II chamber slides were treated with poly-L-lysine, 10 μg per well, for 30 min. After two washes with PBS, macrophages (10^6/ml in RPMI complete medium, 100 μl per well) were plated on chamber slides and exposed for 12 h to 10, 1 or 0.1 nM YTX. At the end of the incubation, cells were infected for 0.5, 1.5 and 3 h with PCA2 yeast cells (5×10^6/ml) prelabelled with Og. Fifteen minutes before the end of each incubation time, Uvitex 2B (Polysciences, Inc., Warrington, PA, USA) was added to each sample (Chaka et al., 1995). Macrophages were then washed with PBS to remove the Candida cells that had not been internalized, and fixed with 4% formaldehyde (FA) freshly made from paraformaldehyde solution in PBS pH 7.4 (JT Baker, Deventer, Holland). As previously described (Chaka et al., 1995), the staining of yeast cell wall with Uvitex allows to discriminate between adherent or phagocytized fungi. When visualized by fluorescence microscopy, the non-internalized yeasts appeared blue, while all the yeast cells retained the Og fluorescence (independently of the yeast localization). Finally, by merging Og and Uvitex images, the extracellular yeast cells could be definitely excluded. At least 200 macrophages from each sample were examined and any cell containing one or more yeast was considered as phagocytic. The percentage of phagocytic cells was calculated according to the following formula:

$$\text{Phagocytic cells (\%)} = \frac{\text{Number of macrophages containing} \geq 1 \text{yeast cell}}{\text{Total number of macrophages inspected}} \times 100$$

2.7. Phagolysosome acidification assay

Acidification of phagosome in J774 macrophages and mouse peritoneal macrophages, treated in Lab-Tek II chamber slides as described in Section 2.6, was assessed by using the acidotropic dye LysoTracker Red DND-99 (Molecular Probes, Eugene, OR, USA) (Binker et al., 2007). Briefly, cells pre-loaded with 75 nM LysoTracker were infected with Og-labelled fungi for 0.5, 1.5 and 3 h. After counterstaining of PCA2 cells with Uvitex 2B, samples were fixed with 4% FA and immediately examined by fluorescence microscopy. A minimum of 200 fungi-containing phagosomes was scored and the percent of acid phagolysosome was determined as the ratio between the number of LysoTracker-labelled red phagosomes and the total number of yeast-containing phagosomes.

2.8. Evaluation of J774 cytoskeletal modification following YTX treatment

J774 macrophages (10^6/ml in RPMI complete medium, 100 μl per well) in Lab-Tek II chamber slides, treated as described in Section 2.6, were exposed for 12 h to 1 nM YTX at 37 °C in 5% CO_2. At the end of incubation time, cells were fixed with 4% FA, permeabilized with 0.2% Triton X-100 (10 min at 37 °C) and then labelled with the nuclear stain 4,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich, St. Louis, MO, USA) for 10 min, at room temperature, in the dark. Subsequently, macrophages were treated for 20 min at 23 °C with Oregon green 488-phalloidin (Molecular Probes, Eugene, OR, USA) and then visualized by means of epifluorescence microscopy. In parallel, the samples were assessed also for the amount of F-actin. The measurement of F-actin was carried out following the method originally described by Cunningham (1995), with minor modifications. Briefly, cells were scraped in PBS, washed twice with PBS, centrifuged for 10 min at $800 \times g$ and the cellular sediment was resuspended in 100 μl of PBS containing 0.5% of Triton X-100 and 100 nM Oregon green-phalloidin. The incubation lasted 30 min at room temperature in the dark. The cell extracts were next centrifuged for 60 min at $20{,}000 \times g$. The pellet was then resuspended in 1 ml of absolute methanol and incubated for 90 h at −20 °C in the dark. The content of Oregon green-phalloidin of each sample was then determined in a fluorimeter (Perkin–Elmer Corp, USA), using a $\lambda_{ecc} = 490$ nm and a $\lambda_{em} = 517$ nm.

2.9. Cytokine secretion

J774 cells (10^6/ml) were exposed to 10, 1 or 0.1 nM YTX for 12 h in 24 well plates. Positive and negative controls were represented by cells stimulated with LPS (1 μg/ml) and 1% EtOH-containing RPMI medium, respectively. At the end of the stimulation period, cell supernatants were collected and frozen at −80 °C until used. TNF-α, MIP1-α and MIP-2 were measured by sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's procedure (R&D Systems, MN, USA). OD values were measured by a Microplate Reader (Sunrise, Tecan, Salzburg, Austria) and results were expressed as pg/ml of cytokines. Experiments were repeated at least three times and each sample was run in triplicate.

2.10. Lipopolysaccharide (LPS) detection

The presence of endotoxin in YTX preparations was evaluated by using the gel-clot Limulus Amebocyte Lysate *in vitro* assay (LAL, PBI International, Milan, Italy) according to the manufacturer's recommendations. Briefly, standard series of the control endotoxin (CSE, from 0.250 EU to 0.03 EU per ml) and endotoxin-free water were evaluated as positive and negative control, respectively. LAL preparation was incubated for 1 h at 37 °C with 1% EtOH, 10 nM YTX in 1% EtOH or control samples. At the end of the incubation period, all tubes were evaluated for the formation of a gel-clot, indicating the presence of endotoxin. The sensibility of the test used in this study was 0.125 EU/ml.

2.11. Epifluorescence microscopy studies

Images were generated and captured with a Nikon Eclipse 90i system, equipped with Nomarski differential interference contrast (DIC) optics. The overlapping signals of Og-labelled yeasts (green fluorescence) with either the Uvitex 2B (blue fluorescence) or the LysoTracker (red fluorescence) was always interpreted as colocalization. At each time point, samples were photographed with a DS-5Mc Nikon digital camera and the resulting photographs were analyzed by using the Nikon AcU2 software program.

2.12. Statistical analysis

Statistical analysis was performed by one-way ANOVA with a Bonferroni's correction post-test. The results shown in figures and tables represent the mean ± standard deviation (SD) of 3–5 experiments performed. * $p < 0.01$ and ** $p < 0.005$.

3. Results

To investigate the effect of YTX on functional activity of J774 macrophages, we have first evaluated the ability of cells, exposed to the algal toxin, to internalize *C. albicans*. To this end, J774 macrophages were treated with different toxin concentrations and then infected with Og-prelabelled fungi. At the end of incubation time, the unphagocytosed yeast cells were counterstained with Uvitex 2B. This approach allowed us to discriminate between intracellular and extracellular Candida, and thus, to accurately quantify the phagocytic cells (Orsi et al., 2009). As depicted in Fig. 1, the incubation of J774 macrophages with either 1 or 10 nM YTX caused a significant inhibition of phagocytosis after 1.5 and 3 h of exposure to the yeast. The reduction observed was dose-dependent and it was never found with 0.1 nM YTX.

In order to investigate the effects of YTX on phagosome maturation, J774 macrophages pre-exposed to YTX, were infected with Candida in the presence of LysoTracker, a marker of phagosome acidification. Fig. 2 shows fields representative of the various experimental conditions. Both the J774 macrophages and the yeast cells were distinctly observed by DIC (A and F); the total yeast cells were detectable by Og-staining (B and G); the extracellularly localized fungi were evidenced by Uvitex 2B (C and H); the LysoTracker staining identified acidic intracellular compartments (D and I). As shown in panel D, the internalized yeasts were clearly detected in acidic compartments of control macrophages, whereas no acidotropic LysoTracker staining was evident in cells treated with YTX (I). These differences were confirmed by the merged images (E and L). A quantitative analysis of these data was then performed and the results were expressed as percentage of acidic phagolysosomes, as detailed in Section 2 As shown in Fig. 3, a dramatic inhibition of phagosome maturation was observed in macrophages pre-exposed to 10 or 1 nM YTX at all the time points tested. In contrast, no significant differences were observed in macrophages incubated with either 0.1 nM YTX or EtOH.

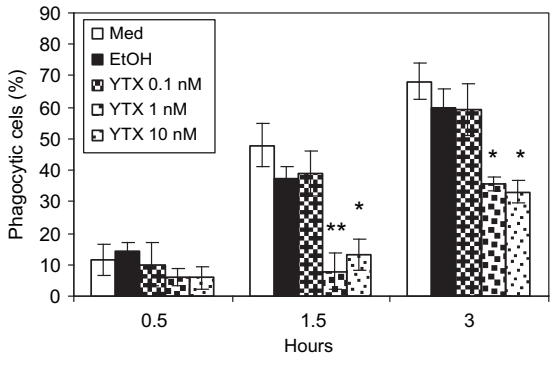

Fig. 1. Phagocytic activity of J774 macrophages incubated with *C. albicans*. J774 cells were treated with 0.1, 1 or 10 nM YTX for 12 h and then exposed to *C. albicans* for 0.5, 1.5 and 3 h [Effector to Target (E:T) ratio 1:5)]. Bars represent the SD and asterisks indicate statistically significant differences (* $p < 0.01$ ** $p < 0.005$).

J774 cells, pre-exposed to YTX and then infected with *C. albicans*, were also evaluated for their morphology. Fig. 4 shows that, unlike controls, macrophages that had been treated with either 10 nM (C) or 1 nM (D) YTX have lost their typical elongated shape, becoming rounded and smaller. Moreover, morphological alterations were also observed in yeast cells ingested by YTX-treated macrophages, as these fungi displayed many long germinal tubes (C and D), that could not be observed in fungi internalized by untreated macrophages (A and B).

Phagocytosis being a complex and dynamic process, depending on actin rearrangement, we next evaluated the effects of YTX on J774 macrophages cytoskeleton. To this end, cells exposed to the toxin were treated with the blue nuclear stain DAPI and then incubated with Og-labelled phalloidin, which specifically binds to cellular F-actin. An altered distribution pattern of F-actin microfilaments (uniformly dispersed in the cytoplasm of YTX-treated cells) was displayed by the fluorescence microscope analysis (Fig. 5A). On the contrary, structured bundles of F-actin filaments, organized in a well-defined cytoskeleton, were observed in the control macrophages (Fig. 5B). These findings prompted us to check whether YTX treatment could change the F-actin levels in J774 macrophages. However, by direct fluorimetric measurements of the F-actin content, we did not find any significant change in the levels of F-actin between the YTX and the vehicle-exposed J774 cells (data not shown). These findings indicate that YTX can significantly influence the integrity of the cytoskeleton, by altering the F-actin organization.

To further investigate the effects of the toxin on the functional activity of macrophages, we analyzed the pattern of cytokine production following YTX treatment. For this purpose, J774 macrophages were treated for 12 h with different YTX concentrations and then their supernatants were collected and tested for the levels of TNF-α, MIP-1α and MIP-2. As depicted in Table 1, the amounts of cytokines secreted by YTX-exposed J774 cells were significantly higher than those observed in untreated cells. Interestingly, in the case of MIP-2 the level of cytokine produced was even higher than that observed after LPS stimulation, used as positive control. This phenomenon

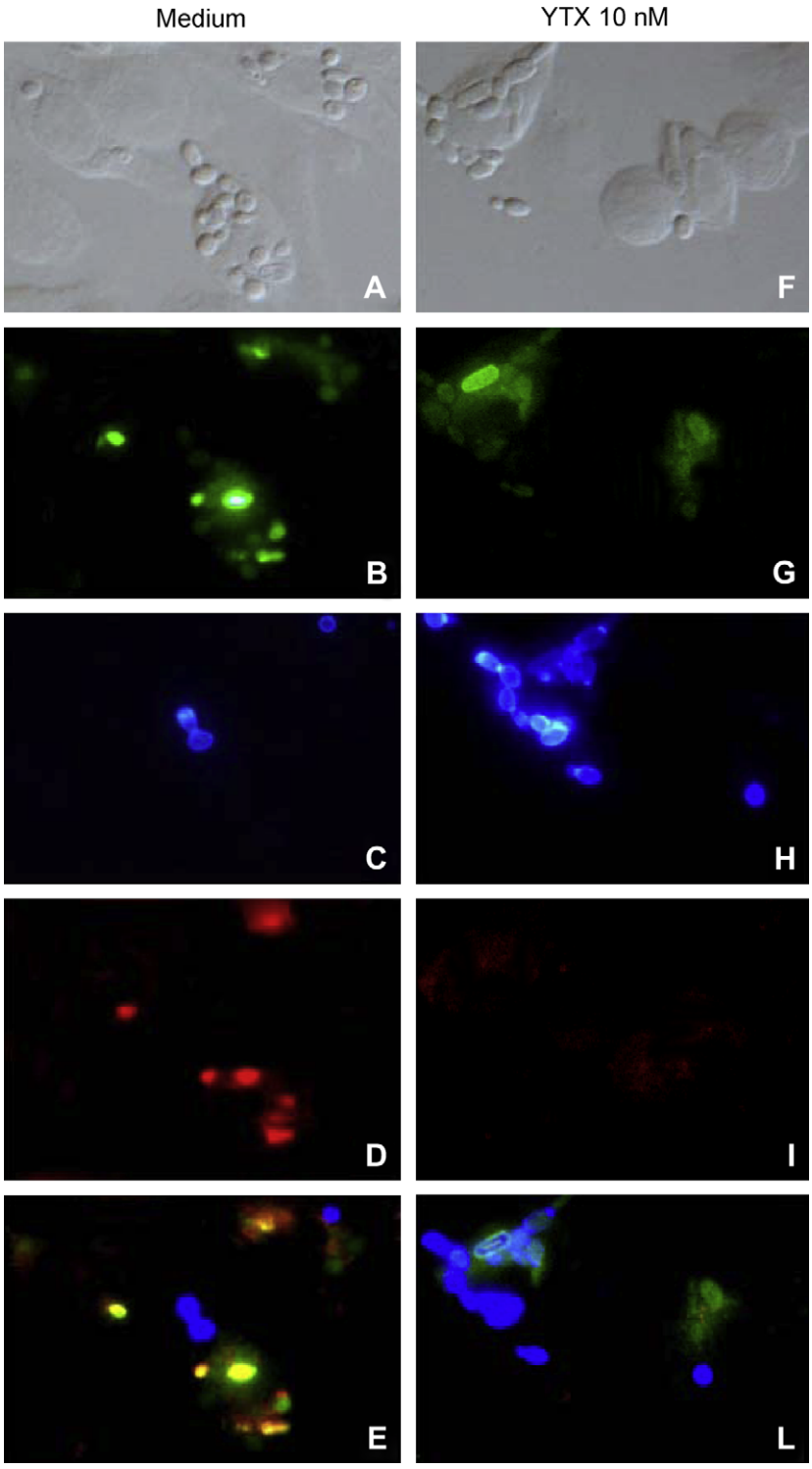

Fig. 2. Colocalization of yeast cells with the acidotropic LysoTracker dye. J774 cells, treated with 1 nM YTX for 12 h and then infected for 3 h with *C. albicans* (E:T ratio 1:5). The acidic phagolysosomes were then labelled with LysoTracker DND-99 and visualized by epifluorescence microscopy. DIC images (A and F); Oregon green-labelled *C. albicans* (B and G); Uvitex 2B-labelled *C. albicans* (C and H); red LysoTracker-labelled acidic phagolysosomes (D and I); merge images (E and L).

was dose-dependent and it became undetectable when cells had been exposed to 0.1 nM YTX. In order to exclude that the YTX effect on cytokine secretion might have been caused by the presence of contaminating LPS in our toxin preparation, both the YTX stock preparation and EtOH were tested for the presence of endotoxin. The amount of LPS

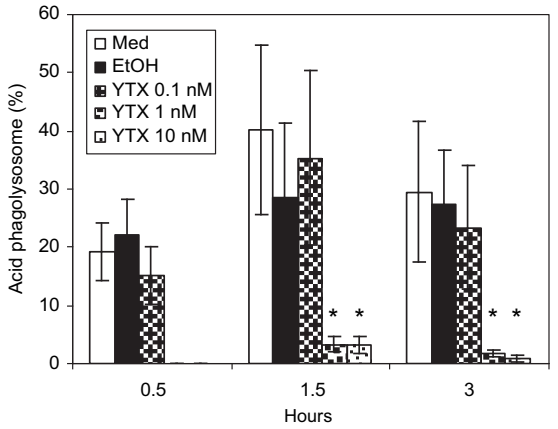

Fig. 3. Percentage of colocalization of yeast cells with acidotropic LysoTracker dye. J774 macrophages, treated with 0.1, 1 or 10 nM YTX for 12 h, were exposed to *C. albicans* for 0.5, 1.5 and 3 h (E:T ratio 1:5). Acid phagolysosomes were then labelled with LysoTracker DND-99 and visualized by epifluorescence microscopy. Bars represent the SD and asterisks indicate statistically significant differences (* $p < 0.01$).

found in these samples was negligible, being lower than 0.125 EU/ml (the sensitivity threshold of the assay), indicating that the stimulatory effect on cytokine secretion in J774 macrophages was actually due to YTX exposure.

To confirm the results obtained with the macrophages cell line J774, we next performed phagocytosis experiments by using resident macrophages from the peritoneum of naive mice. We found that the *in vitro* treatment of peritoneal macrophages with 10 and 1 nM YTX markedly impaired their ability to ingest *C. albicans*; in addition, their exposure to the algal toxin also prevented the phagosome maturation (Fig. 6). Overall, these results indicate that YTX is able to exert inhibitory effects on different phagocytic cells, suggesting that this toxin could also have an immunomodulatory effect *in vivo*.

4. Discussion

In this work we show that the *in vitro* treatment of macrophages with 10^{-9}–10^{-8} M YTX causes a significant reduction in their capacity to phagocytose *C. albicans*. The inhibition observed is dose-dependent and it becomes detectable after 1.5 h of phagocytosis. Moreover, not only the ability of macrophages to internalize yeasts is reduced, but also their phagosome maturation is prevented. The demonstration that YTX impairs phagocytosis is in accordance with the evidence that YTX inhibits the endocytosis of E-cadherin in human adenocarcinoma MCF-7 cells (Callegari and Rossini, 2008). The capacity of 1 nM YTX to inhibit endocytosis in MCF-7 cells and to alter protein degradation has been originally shown by Rossini's group (Callegari and Rossini, 2008; Pierotti et al., 2003; Ronzitti et al., 2004; Ronzitti and Rossini, 2008). In such studies, they showed that in YTX-treated cells, a fragment of the adhesion protein E-cadherin accumulates at plasma membrane level or in early endosomes, but it does not reach the lysosomes for its complete disposal. Such results and the present data with mouse macrophages allow us to conclude that YTX impairs the endocytic machinery; this, in turn, leads to a block of different, although mechanistically related, cellular processes, such as pathogen internalization and degradation of selected plasma membrane proteins. Since these evidences have been obtained with cells belonging to different histological types, we may speculate that the impairment of vesicular transport from the plasma membrane to intracellular compartments could also occur in other cells exposed to the toxin.

The alteration of protein degradation by cell treatment with YTX has been reported to occur in other systems (Malagoli et al., 2006a).

The molecular events by which YTX impairs phagocytosis and phagosome maturation in macrophages remain to be established, yet our results suggest that the alteration of the cytoskeleton may be a key event in these processes. YTX has been reported to alter the cell cytoskeleton in a cell-specific way. Disorganization of the actin cytoskeleton and decreased levels of F-actin have been detected by fluorescence microscopy in primary cultures of neuronal cells (Perez-Gomez et al., 2006), as well as in insect fat body cells and mouse 3T3 fibroblasts (Malagoli et al., 2006a). In other investigations, the fluorimetric quantification of F-actin has provided evidence that YTX reduces the levels of F-actin in MCF-7 adenocarcinoma cells (Ronzitti et al., 2007), but not in other cells, like BE(2)-M17 neuroblastoma (Leira et al., 2003), rabbit enterocytes (Ares et al., 2005) and Caco-2 cells (Ronzitti et al., 2007).

Since phagocytosis is a dynamic process, dependent on actin rearrangement, it is likely that the mechanism by which YTX impairs the phagocytic activity of macrophages involves the alteration of the cytoskeleton. By using J774 macrophages, we show that YTX affects the cytoskeleton by

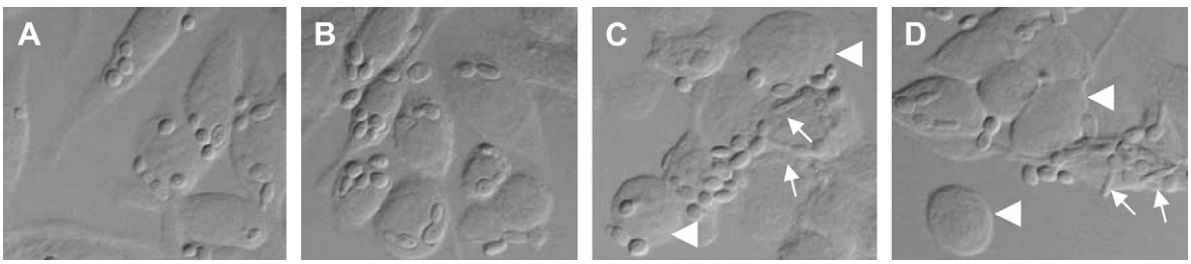

Fig. 4. Effects of YTX on the morphology of J774 macrophages and *C. albicans*. J774 cells were exposed for 12 h to YTX and then infected with the yeast. The morphological changes on both populations were analyzed by fluorescence microscopy of J774 cells exposed to: medium (A), EtOH (B), 10 nM YTX (C) and 1 nM YTX (D). Arrowheads indicate altered morphology of macrophage cells, thin arrows indicate the pseudhypha of *C. albicans*.

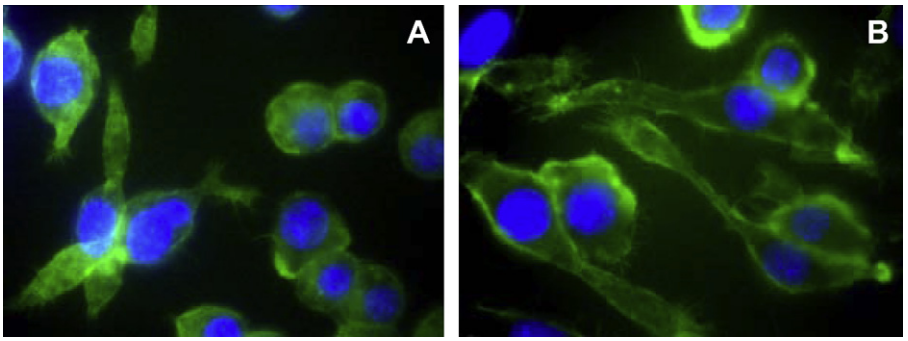

Fig. 5. Modification of F-actin organization in J774 cells exposed to YTX, as shown by nuclear DAPI labelling and counterstaining with Oregon green 488-phalloidin. J774 cells incubated with 1 nM YTX (A); J774 control cells (B).

altering the organization of F-actin, and this, in turn, leads to changes in cell shape, without a detectable reduction in its F-actin content. The molecular processes that determine the structural changes that we have detected are presently unknown, and further studies are needed to clarify them.

The action of YTX on J774 macrophages is not confined to the inhibition of their phagocytic activity. Surprisingly, the induction of inflammatory cytokines, such as TNF-α, MIP-1α and MIP-2, is also significantly enhanced in YTX-treated macrophages. It is reported that TNF-α plays a primary role in the regulation of immune response by causing apoptosis, cellular proliferation, inflammation, viral replication and tumorigenesis (Wajant et al., 2003). Since it is known that TNF-α also stimulates phagocytosis on macrophages (Wajant et al., 2003), the high levels of TNF-α secreted in our experimental model may represent an adaptive response of macrophages to the inhibitory effect of YTX.

Interestingly, in previous studies Franchini et al. (2004) reported that the ip injection of mice with YTX induces morpho-functional alterations in the thymus and the duodenum of the animals. Specifically, in the latter compartment a higher number of infiltrated blood cells between the epithelial cells of villi were observed. In addition, the pattern of cytokines produced in YTX-treated mice was also different, since a higher number of TNF-α immunoreactive cells were seen in the connective tissue, and these were granulocytes and macrophages. Further evidences indicate that human lymphocytes exposed *in vitro* to YTX produced more interleukin-2 (Alfonso et al., 2003).

Table 1
Levels of TNF-α, MIP-1α and MIP-2 produced by J774 cells treated with YTX.

Treatment	Levels of TNF-α	Levels of MIP-1α	Levels of MIP-2
None	nd	276.1 ± 12.3	nd
EtOH 1%	nd	293.0 ± 15.6	nd
LPS	480.5 ± 21.2	1029.2 ± 94.8	19.7 ± 5.6
YTX 0.1 nM	nd	459.3 ± 62.1	nd
YTX 1 nM	77.7 ± 5.3	799.2 ± 85.2	335.5 ± 56.3
YTX 10 nM	87.9 ± 8.7	803.6 ± 62.2	300.5 ± 70.5

J774 cells were stimulated with YTX or LPS for 12 h at 37 °C. At the end of the incubation time, the culture supernatants were harvested and the levels of cytokines produced were measured, as detailed in Section 2. Values were expressed as pg/ml. nd = not detectable (<7 pg/ml).

Overall, the cells of the immune system are responsive to YTX in a wide concentration range (10^{-9}–10^{-6} M), depending on the effect evaluated in different studies (De la Rosa et al., 2001; Alfonso et al., 2003; Malagoli et al., 2006a,b), suggesting the possibility that low YTX concentrations might adversely affect the host immune function.

In the last years, the toxicological relevance of YTX has been thoroughly investigated, because of the significant differences observed in the severity of symptoms and their outcome in the mouse model, following ip injection or oral administration (Aune et al., 2002, 2008; Callegari et al., 2006; Murata et al., 1987; Ogino et al., 1997; Terao et al., 1990; Tubaro et al., 2003, 2004, 2008). Although the results of these studies indicate that the oral administration of YTX could have limited toxicity on animal, based on histopathological examination of organs, evidence suggests that more subtle adverse effects might ensue in mice exposed to the toxin. According to this, several lines of evidence would call for caution, since: *i*) 10^{-9}–10^{-8} M YTX concentrations

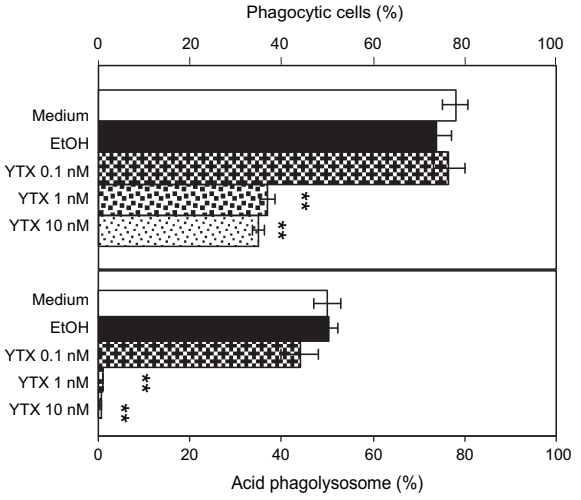

Fig. 6. Phagocytic activity and phagosome maturation in mouse peritoneal macrophages treated with YTX. Resident peritoneal macrophages from normal mice were treated *in vitro* with 0.1, 1 or 10 nM YTX and then incubated for 3 h with *C. albicans* (E:T ratio 1:5). Bars represent the SD and asterisks indicate statistically significant differences (* $p < 0.01$; ** $p < 0.005$).

have been detected in the blood of orally treated mice (Tubaro et al., 2008); *ii*) 10^{-10}–10^{-8} M concentrations have been shown to be cytotoxic in different cell lines (Leira et al., 2002; Malagoli et al., 2006a; Malaguti et al., 2002; Perez-Gomez et al., 2006; Ronzitti et al., 2004), including primary cultures of rat cardiomyocytes (Dell'Ovo et al., 2008); moreover, the same concentrations are effective in blocking endocytosis of E-cadherin (Callegari and Rossini, 2008) and in inhibiting phagocytosis (as shown in the present study); *iii*) decreased levels of degraded E-cadherin are found in mice orally administrated with 1 mg YTX/kg body weight (Callegari et al., 2006), indicating that disposal of plasma membrane proteins might be impaired in some organs under those conditions. Such evidences, as well as the present results, strongly suggest the need to carry out further investigations in order to evaluate the effects exerted by 10^{-9}–10^{-8} M YTX concentrations in animals and other experimental systems. This will allow a better understanding of the molecular and functional consequences of YTX ingestion by humans.

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Conflict of interest

There are no conflicts of interest.

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