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Inhibition of interferon-γ-induced nitric oxide production in endotoxin-activated macrophages by cytolethal distending toxin

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Introduction: Cytolethal distending toxin (CDT) is a DNA-targeting agent produced by certain pathogenic gram-negative bacteria such as the periodontopathogenic organism *Aggregatibacter actinomycetemcomitans*. CDT targets lymphocytes and other cells causing cell cycle arrest and apoptosis, impairing the host immune response and contributing to the persistence of infections caused by this microorganism. In this study we explored the effects of CDT on the innate immune response, by investigating how it affects production of nitric oxide (NO) by macrophages.
Methods: Murine peritoneal macrophages were stimulated with *Escherichia coli* sonicates and NO production was measured in the presence or not of active CDT.
Results: We observed that CDT promptly and significantly inhibited NO production by inducible nitric oxide synthase (iNOS) in a dose-dependent manner. This inhibition is directed towards interferon-γ-dependent pathways and is not mediated by either interleukin-4 or interleukin-10.
Conclusion: This mechanism may constitute an important aspect of the immunosuppression mediated by CDT and may have potential clinical implications in *A. actinomycetemcomitans* infections.

Key words: *Actinobacillus actinomycetemcomitans*; cytolethal distending toxin; macrophages; nitric acid

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The cytolethal distending toxins (CDT) are multi-component cytotoxins produced by several gram-negative pathogens associated with mucosal surfaces, including *Helicobacter hepaticus*, *Shigella dysenteriae*, *Haemophilus ducreyi*, certain *Campylobacter* species and *Escherichia coli* strains (5), and the oral organism *Aggregatibacter (Actinobacillus) actinomycetemcomitans* (5). *A. actinomycetemcomitans* is associated with localized aggressive periodontitis and with several systemic infections. Most *A. actinomycetemcomitans* isolates produce CDT although the toxicity may vary among strains (5, 13, 24).

The toxin is encoded by three different genes: *cdtA*, *cdtB*, and *cdtC* (5, 19) and the active subunit, CDTB, is reported to share structural and functional homology with mammalian DNase I. This could explain its ability to cleave the host's DNA, triggering the DNA damage response (5, 12). Therefore, one proposed model for the toxicity of CDT is by activating the ataxia telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) kinases, consequently inducing a cell cycle arrest mediated by Cdc2 and Cdc25, similar to other DNA-damaging agents (2, 9). Some studies however pointed out similarities between CDTB and phosphatases (11) and proposed a mode of action that involves dephosphorylation of negative regulators of mitosis and inactivation of

Cdc2 (5, 8). More recently, it was suggested that CDTB, like Src homology 2-containing inositol-5′-phosphatase 1 (SHIP-1), dephosphorylates phosphatidyl inositol-3,4,5-triphosphate and thereby modulates the activity of pleckstrin-homology-containing proteins such as Akt (26).

CDT targets many different cell types, including certain human periodontal ligament cells and gingival fibroblasts, resulting in a characteristic cell cycle arrest in the G2/M phase (5, 8). Lymphocytes also undergo cell cycle arrest and eventually apoptotic death as a result of CDT activity. This results in impaired host immunity (5, 26, 28), which ultimately contributes to the persistence of infection characteristic of CDT-producing bacteria (25, 31). A specific immunosuppressive effect could also be demonstrated *in vitro* by the ability of CDT to induce apoptosis in primary human peripheral blood mononuclear cells and cultured T-cell lines without affecting circulating human monocytes and polymorphonuclear cell viability (1, 5, 26, 28). In addition, CDT may be able to interfere with the proper mounting of an adaptive immune response by partially inhibiting the production of tumor necrosis factor-α, interleukin-6 (IL-6), IL-8, and IL-12 by human monocyte-derived dendritic cells (DCs) and macrophages. Persistent CDT intoxication results in progressive apoptosis of DCs and loss of their capacity to activate T cells (30). Conversely, in some instances CDT may exhibit proinflammatory properties because the toxin was reported to potentiate the release of proinflammatory cytokines by host cells and enhance RANKL (receptor activator of nuclear factor-κB ligand) expression in human gingival fibroblasts and periodontal ligament cells (1, 3, 5).

Macrophages are central to the defense against microbial infections. As such, they can directly recognize exogenous ligands expressed by microorganisms, such as lipopolysaccharide (LPS) and stimulate T cells and natural killer cells, which in turn can greatly enhance the microbicidal activity of macrophages. To better characterize the immunomodulatory role of *A. actinomycetemcomitans* CDT on the innate immune response, we investigated how it may interfere with macrophage functions; specifically nitric oxide (NO) release, one of the most relevant effector mechanisms during a bacterial infection.

Material and methods

CDT preparation

Aliquots of frozen *E. coli* DH5α cells hosting a recombinant plasmid (pcdt1) containing the three open reading frames for the fully active toxin: *cdtA*, *cdtB*, and *cdtC* (19) and a control *E. coli* p*Bluescript* were inoculated on Luria–Bertani medium containing 100 μg/ml ampicillin, and were incubated overnight at 37°C. After growth, the cells were washed twice, suspended in phosphate-buffered saline (pH 7.3) and lyzed by sonication: two 1-min strokes (Branson Sonifier 450, Branson Ultrasonics Co, Danbury, CT). The resulting lysates were centrifuged at 10,000 ***g*** for 10 min at 4°C and the supernatants were sterilized by filtration through 0.22-μm pore size filters (Millipore, Bedford, MA). Protein concentration was determined by the Lowry method (Pierce, Rockford, OH). Sonicates were then split into aliquots and kept frozen at −80°C until use.

Media and reagents

Resident peritoneal cells and L929 murine fibroblasts were maintained in RPMI-1640 culture medium supplemented with 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum. The reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Animals

C57BL/6, BALB/c, BALB/c Xid, C3H/HePas, and C3H/HeJ isogenic mice aged 8 weeks, except where noted otherwise, were used. All the mice came from our own animal facilities and this study was previously approved by the Animal Research Ethics Committee of the Institute of Biomedical Sciences, University of São Paulo.

Resident peritoneal cells

Peritoneal exudate cells (PECs) from five animals of each strain were obtained by lavage of the peritoneal cavity with 5-ml injections of phosphate-buffered saline. The cells were centrifuged, resuspended in complete medium, and adjusted to 2×10^6/ml. One-hundred microliters of the cell suspensions was plated in each well of 96-well flat-bottom plates (Corning Costar Co., Cambridge, MA) in quadruplicate. The percentages of macrophages in the mixtures were 60–80% for peritoneal cells and 90–95% for adherent peritoneal cells. To obtain adherent cells, the microplates were incubated at 37°C for 2 h, and non-adherent cells were removed by three washes with warm medium.

Treatment with bacterial sonicates

PECs (2×10^6 cells/ml) and L929 fibroblasts (2×10^5 cells/ml) were treated with different concentrations of supernatants from bacterial lysates (sonicates) from *E. coli* p*Bluescript* (control) and *E. coli* p*cdt1* (CDT-producing) strains for 15 min on ice. The cells were then washed twice with RPMI-1640 (9) and the microplates were incubated at 37°C in a humidified atmosphere under 5% CO_2 for the indicated periods of time.

Measurement of nitrite concentrations

NO production was determined by measuring nitrite concentration (Griess Reagent standard reaction). Briefly, 50 μl of supernatant from PEC cultures (treated with CDT and control sonicates) were incubated with 50 μl Griess reagent (sulfanilamide 1%, *N*-1-naphthylethylene diamine dihydrochloride 0.1%, H_3PO_4 2.5%) at room temperature for 10 min. Absorbance of the samples was determined at 540 nm, employing a 620 nm reference filter, using a microplate reader (Dynatech, Boonville, IN). Nitrite concentration was estimated from a sodium nitrite standard curve.

Cell viability

At the end of each incubation period, peritoneal cells were stained with 0.5% crystal violet solution (Sigma Chemical Co) in acetic acid (30%) for 15 min at room temperature and rinsed thoroughly with distilled water. After air-drying, crystal violet was solubilized in 200 μl ethanol for 30 min, and the optical density at 570 nm was measured using a microplate reader (Dynatech).

Cell cycle analysis

Cell cycle arrest in L929 cells treated with CDT was performed as previously described (23). Briefly, cells were plated at a density of 2×10^5 cells/ml in 24-well plates and incubated as described above. Then, the cells were harvested and incubated in the dark for 3 h at 4°C with hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate) containing 50 μg/ml propidium iodide (PI, Sigma) for cell lysis and nucleus staining. Isolated nuclei (10^4/well) were analyzed using a FacsCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Statistical analysis

The Tukey–Krammer test was used to compare NO production among the groups. Differences were considered significant at the 5% level. All experiments were performed in quadruplicate in three assays, although a single representative experiment is presented in each figure.

Results

CDT was expressed in *E. coli* by transforming the bacteria with a plasmid containing the three genes necessary to form a fully functional toxin. Partially purified lysates from sonicated bacteria were characterized and subsequently used in the experiments described below (as previously described by Mayer et al. (19). To confirm the presence and activity of the holotoxin in the *E. coli* sonicates, L929 fibroblasts were treated for different periods of time with control and CDT *E. coli* sonicates and the cells were analyzed under a light microscope for morphological changes. The peculiar effects of CDT, represented by cell distension, could be promptly observed in cells treated with CDT-containing sonicate; whereas no effect on cell morphology was observed in cells exposed to the control bacterial sonicate (Fig. 1A and B). A pronounced cell cycle arrest at the G2/M phases was also observed in CDT-treated cells (Fig. 1C).

As an initial characterization of our system, we delineated the pathways involved in the response of PECs to the control *E. coli* sonicates. PECs from C3H/HePas (wild-type; WT) mice responded in a dose–response manner to the lysates, but no NO production whatsoever was observed for cells from C3H/HeJ mice (Fig. 2A), which are genetically deficient for Toll-like receptor 4 (TLR-4) (7). These results show that the response is triggered by the recognition of LPS and perhaps other bacterial components present in the lysates. The outcome of this process is specific induction of inducible nitric oxide synthase (iNOS). PECs from iNOS knockout (KO) C57BL/6 animals or WT PECs treated with the iNOS selective inhibitor aminoguanidine (21) therefore failed to produce NO in response to the lysates (Fig. 2B). IL-12 and interferon-γ (IFN-γ) KO PECs had a diminished response compared to WT cells (Fig. 2C), indicating that the pathway for NO production is partially dependent on these cytokines, as expected for a T helper type 1 (Th1) response. Since the effects of IL-12 and IFN-γ are similar, it is likely that the sole action of IL-12 is through IFN-γ production. On the other hand, there was residual NO production in IFN-γ KO cells, revealing an iNOS-dependent but IFNγ-independent pathway that could represent an intrinsic response triggered by LPS on macrophages.

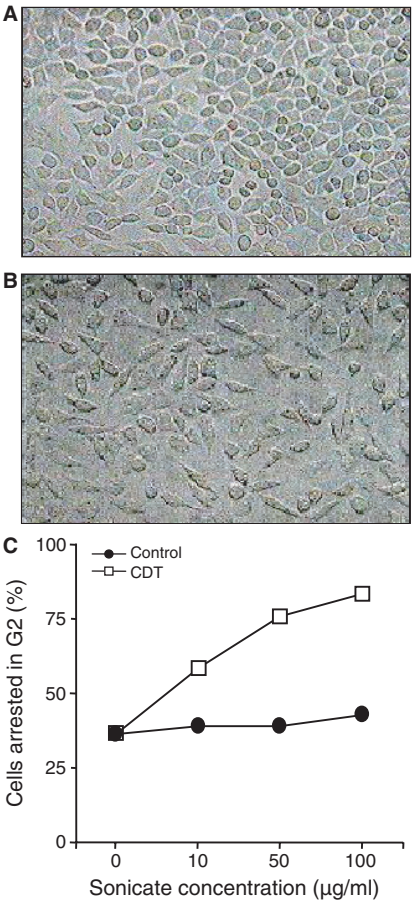

Fig. 1. Characterization of cytolethal distending toxin (CDT)-containing bacterial sonicates. The presence of active CDT was evaluated in L929 cells exposed to sonicates (20 μg/ml) prepared from recombinant *Escherichia coli* (DH5α), transformed with the pBluescript control vector (A) or from bacteria transformed with the pcdt1 plasmid (B). Cells were photographed under a light microscope after 48 h (magnification × 200). (C) L929 cells were analyzed by flow cytometry for DNA content after staining of the nuclei with propidium iodide. The percentage of cells arrested at the G2 phase after a 24-h treatment with various concentrations of sonicates is shown.

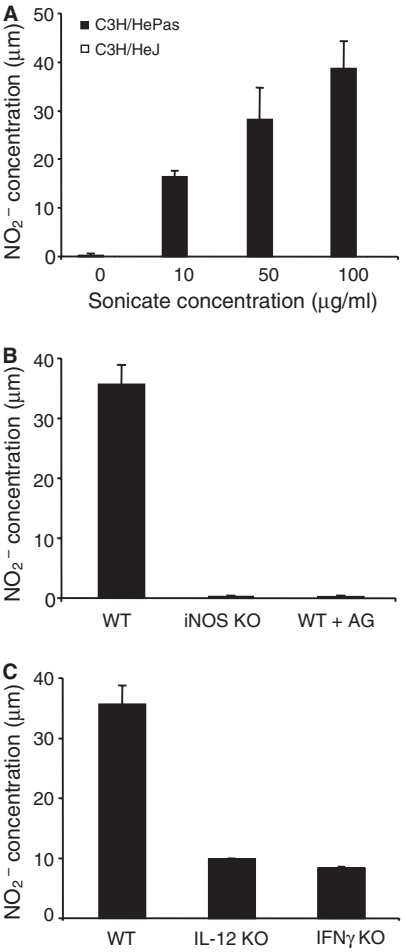

Fig. 2. Effector signals and cytokines involved in nitric oxide (NO) production by peritoneal exudate cells in response to *Escherichia coli* sonicates. Nitrite concentrations (NO_2^-) as a measurement of NO release were evaluated by the Griess reaction after 48 h. Peritoneal exudate cells (PECs) obtained from C3H/HePas and C3H/HeJ mice, which are genetically deficient in (A) Toll-like receptor 4, (B) C57BL/6 wild-type and iNOS KO, and (C) IL-12 KO and IFN-γ KO mice were treated with control or CDT-containing *E. coli* sonicates (50 μg/ml). In (B) C57BL/6 WT cells were pretreated with 10 mM aminoguanidine (AG), a selective iNOS inhibitor. Bars represent mean values ($n = 4$).

The presence of CDT in the *E. coli* sonicates resulted in a significant inhibition of NO production by the exudate cells, of about 30% (Fig. 3A and Table 1). The viability of the peritoneal cells treated with both sonicates was tested using the crystal violet assay and was similar in all experiments (data not shown); therefore it is unlikely that the results are the consequence of selective cell death in the CDT-treated samples. Adherent peritoneal cells, which are enriched for macrophages (90–95% of the total), exhibited an even more pronounced inhibition of NO production by CDT, of approximately 60% (Fig. 3B), possibly indicating a direct activity of CDT on the physiology of these cells. These effects were independent of the distending activity of CDT, because these cells did not show any morphological changes after treatment with the CDT sonicates (data not shown). All the results presented here were obtained after 48 h of

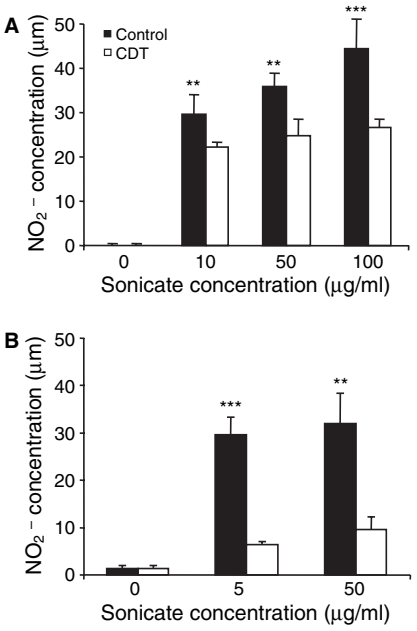

Fig. 3. Effect of cytolethal distending toxin (CDT) on nitric oxide (NO) release by peritoneal exudate cells (PECs) and murine macrophages. Peritoneal exudate cells (A) and adherent peritoneal cells (B) from C57BL/6 mice were exposed to the indicated concentrations of control or CDT sonicates. Nitrite concentrations were measured by the Griess reaction after 48 h. Bars represent mean and standard deviation ($n = 4$); ***$P < 0.001$, **$P < 0.01$.

incubation, when NO concentrations reached their peak, but the effects persisted for even longer periods of time.

To further characterize the cytokines and pathways involved in our model, we employed C57BL/6 animals knocked-out for IL-4 and IL-10, besides the IL-12 and IFN-γ already mentioned. While PECs from IL-10 KO animals were no different from WT cells regarding NO production, IL-4 KO cells presented an enhanced overall response to the sonicates (Fig. 4A and B). Although we could not detect IL-4 (or IL-10) in the supernatants of cell cultures from WT mice (data not shown), the presence of even small amounts of IL-4 produced by Th2 cells probably dampens the response to bacterial endotoxins; nevertheless the results show that the inhibitory effects of CDT are not dependent on IL-4 or IL-10 production (Fig. 4A and B and Table 1).

As previously observed, the NO production depends on IL-12 and IFN-γ. However the inhibitory effect of CDT was completely lost in IFN-γ KO cells but not in IL-12 KO cells (Fig. 4C and D), revealing that the toxin may act on IFN-γ-dependent pathways, without clear compromise of IL-12 production. Despite the fact that NO levels produced by IL-12 KO cells were much lower than by WT, we could still see inhibition promoted by CDT (Fig. 4C and Table 1).

BALB/c mice are known to have a shift towards a Th2 type of immune response, resulting in an inhibition of Th1 cells and the IFN-γ pathway, which in turn makes the animals very susceptible to infections (18). PECs from these animals showed poor production of NO and did not respond to the toxin. On the other hand, cells from BALB/Xid animals showed a degree of response similar to that of C57BL/6 and a similar modulation by CDT (Fig. 5A and Table 1). BALB/Xid mice are deficient in B1 cells (16), which are a source for IL-10 production and consequently have higher levels of IFN-γ and are more resistant to infections compared to WT BALB/c (20). These results once again show the dependency on IFN-γ in our model and modulation by Th1 cytokines.

Aging is also associated with a less efficient response to infections, possibly resulting from a defective response to IFN-γ, culminating with lower production of NO by macrophages (10). As expected, aged C57BL/6 mice showed a decline in the NO response to *E. coli* lysates and no modulation by CDT could be observed (Fig. 5B and Table 1).

Discussion

Evasion of host immunity is a key aspect in the virulence of periodontopathogenic organisms. The gram-negative bacterium *A. actinomycetemcomitans* produces at least two factors that modulate the host's immune response: a potent leukotoxin and a CDT (5, 29), both of which can be related to the chronicity of infections promoted by this organism. Part of the immunosuppressive role of CDT may be the result of its ability to induce cell cycle arrest and apoptosis in human B and T lymphocytes, thereby inhibiting cell proliferation and immunoglobulin production. Therefore, it can be suggested that the T-cell and B-cell clones that are responding to the pathogen during an infection, and consequently in active proliferation, are being targeted and specifically deleted by CDT. In this manner the pathogen may be able to restrict an adaptive immune response raised against it (5, 26).

Table 1. Cytolethal distending toxin effects on nitric oxide release by murine peritoneal exudate cells (the data are summarized for all mouse strains used in this study)

Strain	Phenotype	Nitrite concentration (μM)[1]		Inhibition (%)[2]	Main observation(s)
		Control	CDT		
C3H/HePas	WT	21.54 ± 1.05	14.18 ± 1.11	34	NO production is dependent on TLR-4 and iNOS
C3H/HeJ	TLR-4-deficient (7)	0.00 ± 0.02	0.00 ± 0.01	N/A	
C57BL/6	iNOS KO	0.16 ± 0.03	0.12 ± 0.01	N/A	
	WT	35.67 ± 3.16	24.64 ± 3.90	31	CDT inhibits macrophage NO production triggered by bacterial LPS
	IL-10 KO	28.54 ± 3.37	19.08 ± 0.54	33	No role in CDT-mediated inhibition
	IL-4 KO	49.93 ± 0.72	35.34 ± 3.22	29	IL-4 can modulate the overall responsiveness to the lysates but plays no role in CDT-mediated inhibition
	IL-12 KO	9.62 ± 0.27	4.61 ± 0.24	52	The inhibition does not result from diminished IL-12 production
	IFN-γ KO	8.07 ± 1.32	8.01 ± 0.88	<1	CDT inhibits IFN-γ-dependent NO production but not IFN-γ-independent pathways
BALB/c	WT	11.70 ± 1.24	10.15 ± 1.93	13	
BALB/Xid	B1 deficient (16)	41.37 ± 5.26	30.10 ± 0.11	27	
C57BL/6	WT[3]	1.60 ± 0.27	1.5 ± 0.46	6	

CDT, cytolethal distending toxin; IFN-γ, interferon-γ; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; KO, knock out; NO, nitric oxide; PEC, peritoneal exudate cells; TLR-4, Toll-like receptor 4; WT, wild-type.

[1]Cells were exposed to 50 μg/ml of control or CDT-containing sonicates and NO production was measured after 48 h. Values were obtained by the Griess reaction and represent mean ± SD.

[2]Values are approximate.

[3]Forty-week-old mice. All other values were obtained for 8-week-old mice.

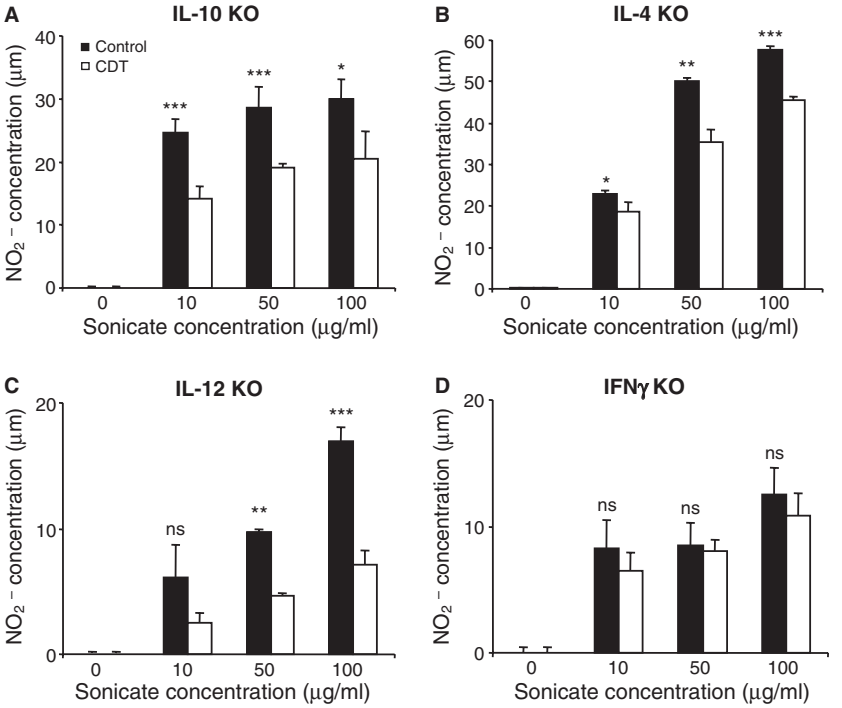

Fig. 4. Role of the main T helper type 1 and type 2 cytokines on cytolethal distending toxin (CDT)-mediated inhibition of nitric oxide (NO) release by peritoneal exudate cells (PECs). NO release was measured for PECs derived from C57BL/6 interleukin-10 knockout (IL-10 KO) (A), IL-4 KO (B), IL-12 KO (C), and IFN-γ KO (D) mice exposed to both control and CDT-containing sonicates. Nitrite concentration was measured by the Griess reaction after 48 h. Bars represent mean and standard deviation ($n = 4$); ***$P < 0.001$, *$P < 0.05$, ns: not significant.

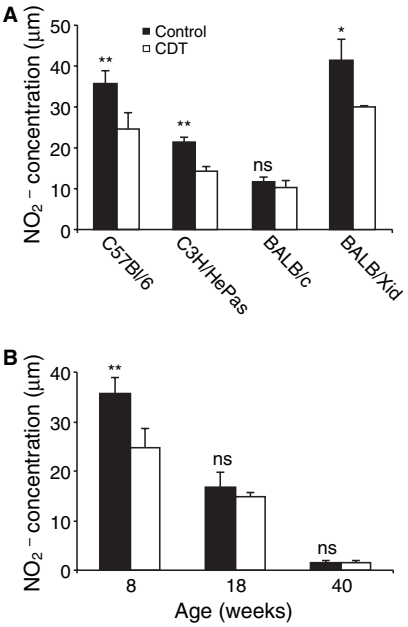

Fig. 5. Effect of cytolethal distending toxin (CDT) on interferon-γ (IFN-γ)-dependent nitric oxide (NO) production by peritoneal exudate cells (PECs). The actions of CDT were evaluated in mouse models with intact or inhibited IFN-γ pathways. NO release by PECs from 8-week-old C57BL/6, C3H/HePas, BALB/c, and BALB/Xid mice (A) or C57BL/6 mice of different ages (B) was measured as described above. Cells were exposed to 50 μg/ml of control and CDT-containing sonicates. Bars represent mean and standard deviation ($n = 4$); **$P < 0.01$, *$P < 0.05$; ns, not significant.

The first response to an infection by gram-negative bacteria comes from macrophages and other cells through their recognition by pattern-recognition receptors, such as TLR-4. As a result there is production of inflammatory cytokines and microbicidal amounts of reactive oxygen and reactive nitrogen species (ROS/RNS), a response that is further enhanced by activated Th1 cells. So far, the effects of CDT on the effector mechanisms of innate immunity are poorly understood. In an effort to better characterize the role of CDT in infections caused by *A. actinomycetemcomitans* or other CDT-producing pathogens, we focused on how CDT might modulate NO production by activated macrophages.

In this study, *E. coli* sonic extracts were used to induce NO production and secretion. This response was dependent on the recognition of bacterial LPS by TLR-4 and iNOS expression, because cells from C3H/HeJ or iNOS KO mice did not show any response to bacterial sonicates. This experimental model comprehends several aspects of a real infection by gram-negative bacteria, while allowing the evaluation of the isolated effects of CDT without the interference of other virulence factors, like leukotoxin. Using this system, we were able to demonstrate that NO production by PECs from C57BL/6, BALB/Xid, and C3H/HePas mice was promptly and significantly inhibited by the presence of CDT and that this inhibition was completely lost in IFN-γ KO animals. These results implicate CDT as a modulator of the inflammatory response by suppressing NO (produced by iNOS) in IFN-γ-dependent pathways. These observations were also supported by the data obtained using animals of a more advanced age (18 weeks). In these animals, the IFN-γ activation pathway was inhibited compared to younger mice (8 weeks old) probably because of the inability of the macrophages to activate mitogen-activated protein kinase pathways in response to receptor binding by the cytokine (10, 27). Cells from aged animals were still able to produce low amounts of NO through IFN-γ-independent pathways, but this production was not inhibited by CDT.

IL-12, a cytokine produced mainly by macrophages, is one of the most potent inducers of IFN-γ secretion by natural killer and Th1 cells. Consequently, IL-12-deficient mice have impaired NO production and increased susceptibility to infections. As expected, cells from IL-12 KO animals showed an overall reduction in NO levels produced after stimulation with control bacterial sonicate. Nevertheless, the inhibitory effects of CDT could still be observed in these cells. Although IL-12 is the main inducer of IFN-γ production in this model, a small production of NO could be detected in its absence, probably as the result of IL-18, which is also secreted during a Th1 response and synergizes with IL-12 to induce IFN-γ (14).

Given that Th2-derived cytokines can counteract the effects of IL-12 and suppress the IFN-γ/iNOS response, we then investigated whether CDT could be regulating the response through an increased production of IL-10 or IL-4, two major Th2 cytokines. Experiments performed with cells from either IL-4 or IL-10 KO mice demonstrated that CDT displayed a similar degree of suppression of NO production in the absence of these cytokines (Table 1). Overall, the data demonstrated that the effects of CDT were independent of these cytokines, despite the fact that their presence was able to modulate the general response.

As mentioned above, the primary source of IFN-γ in the PEC cultures could be natural killer cells, lymphocytes, or other cell types, which may all be present to some extent in the peritoneal exudates. Cultures of adherent peritoneal cells were employed instead of total PECs in some assays, which are greatly enriched for macrophages. No significant difference in the amount of NO produced by PECs or macrophages in response to control sonicates was detected between both cultures, implicating that the macrophages were the main, if not the sole, NO producers in this system. Strikingly, however, the degree of suppression mediated by CDT in the macrophage cultures was much more pronounced. Taken together, the results support a model in which CDT influences NO secretion and macrophage activation through inhibition of IFN-γ-dependent pathways downstream of IL-12/IL-18 production. Furthermore, they suggest that the main target of CDT could be the macrophages themselves.

Macrophages can produce IFN-γ autonomously upon stimulation with LPS, which could then act in an autocrine way to induce iNOS and NO production (15). The signaling pathway initiated by IFN-γ binding to its receptor is mediated by a number of tyrosine kinases, including Janus kinase 1/2 and phosphoinositide 3-kinase/Akt (4). Although further detailed mechanistic studies are necessary to verify and dissect a possible role of CDT in macrophages, an interesting possibility is that it might interfere with phosphorylation events at the various steps of the IFN-γ signaling pathway. A widely demonstrated effect of CDT is the inhibition of tyrosine phosphorylation (5).

In fact, some authors consider CDTB to be a phosphatase capable of inactivating the Akt pathway (11, 26) and a similar evasion mechanism is already well described for *Leishmania* species (22). This intracellular parasite makes use of the endogenous phosphatase SHIP-1 to down-regulate IFN-γ-activated kinases and to inhibit NO production to maintain an intracellular milieu suitable for its survival (22).

Targeting of endogenous pathways leading to NO production has also been described for other pathogens, such as *Candida albicans* (6) and *Cryptococcus neoformans* (17). Whether inhibition of NO production by CDT actually occurs *in vivo* remains unknown. However, the CDT-induced blockade of NO production via the IFN-γ pathway could represent an important factor in the development of periodontal diseases by *A. actinomycetemcomitans*, by facilitating the establishment and maintenance of infectious foci.

Acknowledgments

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