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Abstract

Cytolethal distending toxins (Cdt) are a family of toxins produced by several human pathogens which infect mucocutaneous tissue and induce inflammatory disease. We have previously demonstrated that the *Aggregatibacter actinomycetemcomitans* Cdt induces a pro-inflammatory response from human macrophages which involves activation of the NLRP3 inflammasome. We now demonstrate that in addition to activating caspase-1 (canonical inflammasome), Cdt treatment leads to caspase-4 activation and involvement of the noncanonical inflammasome. Cdt-treated cells exhibit pyroptosis characterised by cleavage of gasdermin-D (GSDMD), release of HMGB1 at 24 hr and LDH at 48 hr. Inhibition of either the canonical (caspase-1) or noncanonical (caspase-4) inflammasome blocks both Cdt-induced release of IL-1 β and induction of pyroptosis. Analysis of upstream events indicates that Cdt induces Syk phosphorylation (activation); furthermore, blockade of Syk expression and inhibition of pSyk activity inhibit both Cdt-induced cytokine release and pyroptosis. Finally, we demonstrate that increases in pSyk are dependent upon Cdt-induced activation of GSK3 β . These studies advance our understanding of Cdt function and provide new insight into the virulence potential of Cdt in mediating the pathogenesis of disease caused by Cdt-producing organisms such as *A. actinomycetemcomitans*. © 2020 John Wiley & Sons Ltd

Keywords

Aggregatibacter actinomycetemcomitans; inflammasome; mechanism of action; microbial–cell interaction; toxins

Disciplines

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RESEARCH ARTICLE

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Cytotoxic distending toxin-induced release of interleukin-1 β by human macrophages is dependent upon activation of glycogen synthase kinase 3 β , spleen tyrosine kinase (Syk) and the noncanonical inflammasome

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Abstract

Cytotoxic distending toxins (Cdt) are a family of toxins produced by several human pathogens which infect mucocutaneous tissue and induce inflammatory disease. We have previously demonstrated that the *Aggregatibacter actinomycetemcomitans* Cdt induces a pro-inflammatory response from human macrophages which involves activation of the NLRP3 inflammasome. We now demonstrate that in addition to activating caspase-1 (canonical inflammasome), Cdt treatment leads to caspase-4 activation and involvement of the noncanonical inflammasome. Cdt-treated cells exhibit pyroptosis characterised by cleavage of gasdermin-D (GSDMD), release of HMGB1 at 24 hr and LDH at 48 hr. Inhibition of either the canonical (caspase-1) or noncanonical (caspase-4) inflammasome blocks both Cdt-induced release of IL-1 β and induction of pyroptosis. Analysis of upstream events indicates that Cdt induces Syk phosphorylation (activation); furthermore, blockade of Syk expression and inhibition of pSyk activity inhibit both Cdt-induced cytokine release and pyroptosis. Finally, we demonstrate that increases in pSyk are dependent upon Cdt-induced activation of GSK3 β . These studies advance our understanding of Cdt function and provide new insight into the virulence potential of Cdt in mediating the pathogenesis of disease caused by Cdt-producing organisms such as *A. actinomycetemcomitans*.

KEYWORDS

Aggregatibacter actinomycetemcomitans, inflammasome, mechanism of action, microbial–cell interaction, toxins

1 | INTRODUCTION

Cytotoxic distending toxins (Cdt) represent a family of heat-labile protein cytotoxins produced by over 30 γ - and ϵ -Proteobacteria that are human and/or animal pathogens. These bacteria share the common ability to colonise mucocutaneous tissue such as oral, gastrointestinal, urinary and respiratory tracts (DiRienzo, 2014a, 2014b; Gargi,

Reno, & Blanke, 2012; Ge et al., 2005; Ge et al., 2007; Ge et al., 2017; Haghjoo & Galan, 2004; Jinadasa, Bloom, Weiss, & Duhamel, 2011; Lara-Tejero & Galan, 2001; Scuron, Boesze-Battaglia, Dlakic, & Shenker, 2016; Shenker et al., 1999; Thelestam & Frisan, 2004; Whitehouse et al., 1998; Young et al., 2004). Moreover, Cdt-producing bacteria are known to induce disease in these mucocutaneous niches that are characterised by sustained infection and inflammation.

Collectively, our studies have demonstrated that Cdt, produced by the periodontal pathogen, *Aggregatibacter actinomycetemcomitans*, is capable of impairing lymphocyte proliferation and survival while also promoting production of pro-inflammatory mediators from macrophages (Shenker et al., 1999; Shenker et al., 2001; Shenker et al., 2005; Shenker et al., 2015; Shenker, Demuth, & Zekavat, 2006; Shenker, Hoffmaster, McKay, & Demuth, 2000; Shenker, Walker, Zekavat, Dlakic, & Boesze-Battaglia, 2014). Moreover, we have shown that these toxic effects are dependent upon the active Cdt subunit, CdtB, and its ability to function as a phosphatidylinositol (PI)-3,4,5-triphosphate (PIP3) phosphatase which depletes cells of PIP3 and induces blockade of the PI-3K signalling pathway (Shenker, Boesze-Battaglia, Alexander, Dlakic, & Shenker, 2016; Shenker et al., 2007; Shenker, Walker, Zekavat, & Boesze-Battaglia, 2016b; Shenker et al., 2014).

Cdt is a heterotrimeric complex that functions as an AB₂ toxin; in addition to CdtB, the toxin consists of two additional subunits, CdtA and CdtC, that are involved in toxin-host cell interactions (reviewed in (Gargi et al., 2012; Scuron et al., 2016). These interactions are dependent upon CdtC (and CdtB) binding to cholesterol within the plasma membrane (Boesze-Battaglia et al., 2009; Boesze-Battaglia et al., 2015; Boesze-Battaglia et al., 2016; Boesze-Battaglia et al., 2017; Guerra et al., 2005; Lai et al., 2013; Zhou, Zhang, Zhao, & Jin, 2012). As noted above, internalisation of CdtB leads to PI-3K signalling blockade within 2 hr in both lymphocytes and macrophages (Shenker, Boesze-Battaglia et al., 2016; Shenker et al., 2007; Shenker et al., 2014). Toxin-treated lymphocytes undergo cell cycle arrest (G2/M) and apoptosis. In contrast, Cdt-mediated PI-3K blockade in non-proliferating cells such as macrophages does not lead to apoptosis; instead, the toxin induces a pro-inflammatory cytokine response. This response includes increased synthesis and release of TNF α and IL-6 as well as synthesis, maturation and release of IL-1 β and IL-18 (Shenker et al., 2014). Recently, we also demonstrated that Cdt-mediated maturation and release of IL-1 β and IL-18 were dependent upon activation of the NLRP3 inflammasome which in turn requires Cdt-dependent increases in extracellular ATP, activation of the P2X7 purinergic receptor and K⁺ efflux (Shenker et al., 2015).

Recent studies have demonstrated that release of mature IL-1 β is not only regulated by canonical inflammasomes which involves caspase-1 activation, but can also involve stimulation of noncanonical inflammasomes leading to activation of other caspases such as caspase-4 and caspase-5 (caspase-11 in mice; Platnich & Muruve, 2019; Vigano et al., 2015). In light of these observations, we extended our studies on the pro-inflammatory effects of Cdt on macrophages and now report that Cdt-induced IL-1 β release is also dependent upon caspase-4 activation. Furthermore, we demonstrate that Cdt-mediated activation of both the canonical and noncanonical inflammasome is regulated upstream by activation of spleen tyrosine kinase (Syk) and GSK3 β . Activation of both inflammasomes leads downstream to pyroptosis exemplified by the release of both the high mobility group box 1 protein (HMGB1) and lactic dehydrogenase (LDH) along with cleavage of gasdermin D (GSDMD).

2 | RESULTS

2.1 | Cdt activates the noncanonical inflammasome leading to pyroptosis

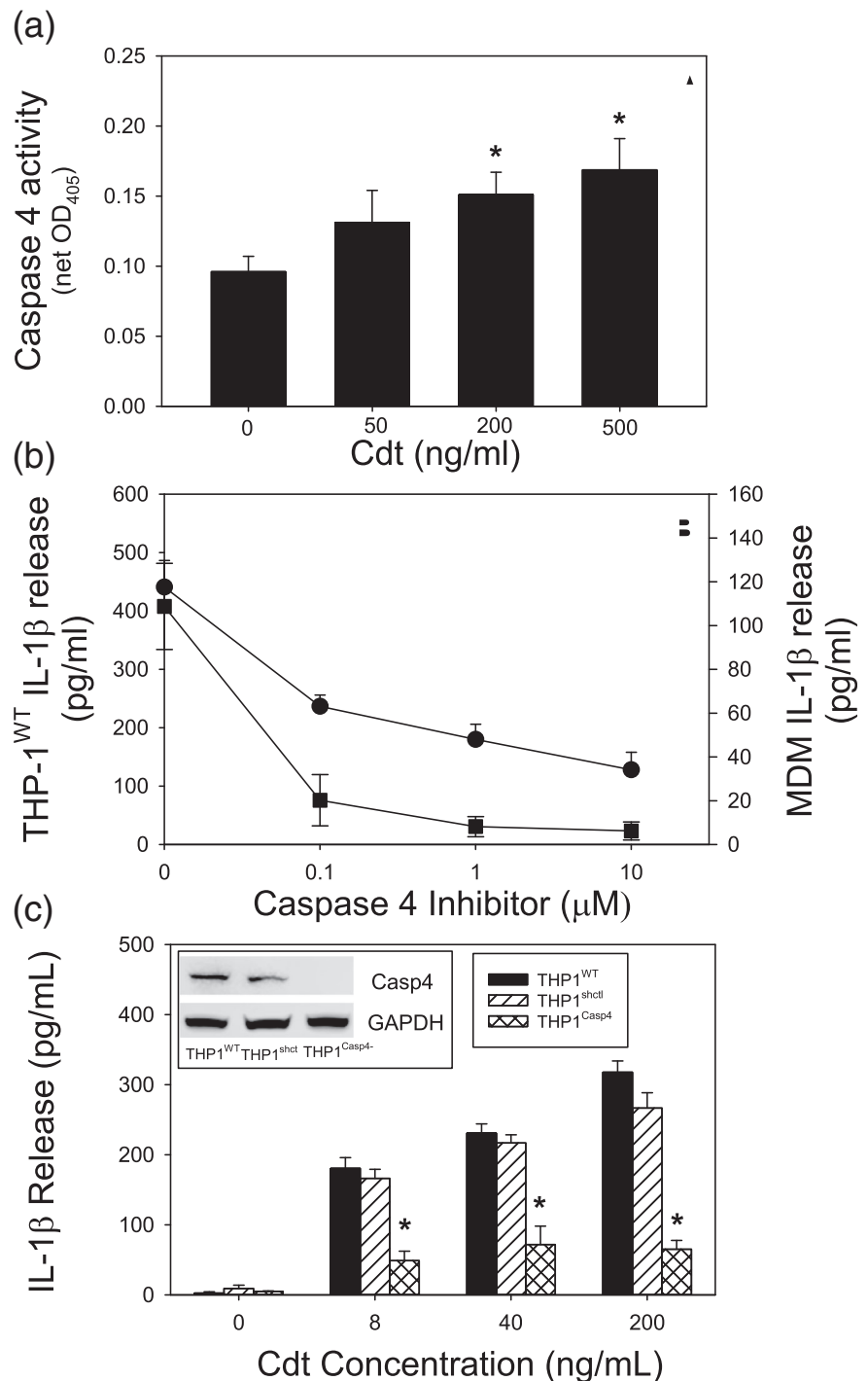
The goal of this study was to explore the contribution of noncanonical inflammasome pathways in mediating the Cdt-induced pro-inflammatory response in human macrophages derived from both THP-1 cells and monocyte-derived macrophages (MDM). In this regard, the most studied mediator of the noncanonical inflammasome is caspase-11 in murine macrophages and caspase-4 (and -5), the human analog of caspase-11. Therefore, we focused our study on caspase-4 in macrophages derived from the human leukaemia monocytic cell line (wild type), THP-1^{WT}. THP-1^{WT}-derived macrophages were treated with 0–500 ng/mL Cdt for 2 hr and the cells analysed for caspase-4 activation (Figure 1a). Caspase 4 activity was monitored by measuring the net change in OD₄₀₅ resulting from the release of the chromophore *p*-NA from LEVD-*p*NA as described in Materials and Methods. Caspase-4 activity exhibited a statistically significant dose-dependent increase over baseline levels representing increases of 44%, 67% and 89% in the presence of 50, 200 and 500 ng/mL Cdt, respectively. The magnitude of the increase in caspase-4 activity was comparable to the increment observed with LPS transfection (Figure 1s).

In order to determine if the increase in caspase-4 activity was critical to Cdt-induced release of mature IL-1 β , THP-1^{WT} macrophages were pre-treated with 0–10 μ M of the cell permeable caspase-4 inhibitor (LEVD-CHO) (Figure 1b). In the presence of 50 ng/mL Cdt, IL-1 β levels increased to 441 \pm 46 pg/mL versus 41.7 \pm 2.9 pg/mL in untreated cells. Pre-treatment of macrophages with 0.1, 1.0 and 10 μ M inhibitor reduced IL-1 β release to 54%, 41% and 29% of levels observed with Cdt alone. The requirement for caspase-4 in mediating Cdt-induced release of IL-1 β was also assessed in MDM; as shown in Figure 1b, 200 ng/mL Cdt-induced an increase in IL-1 β release to 108.8 \pm 19.7 pg/mL over baseline levels of 0.2 \pm 0.2 pg/mL. In the presence of 0.1, 1.0 and 10 μ M caspase-4 inhibitor the amount of cytokine release was significantly reduced to <20% of levels observed in cells treated with toxin alone.

To rule out possible off-target effects of the caspase-4 inhibitor and more directly implicate caspase-4 in the pro-inflammatory response to Cdt, THP-1 cells deficient in caspase-4 expression (THP-1^{Casp4⁻}) were assessed for their susceptibility to Cdt-induced release of IL-1 β . As shown in Figure 1c(inset), Western blot analysis of the protein levels of caspase-4 were not detectable in the THP-1^{Casp4⁻} cells; in contrast, caspase-4 was present in THP-1^{WT} cells. Cells were then treated with Cdt for 5 hr and the supernatants analysed for IL-1 β levels by ELISA. THP-1^{WT} macrophages exhibited a dose-dependent increase in IL-1 β release when challenged with 8, 40 and 200 ng/mL Cdt. In contrast, THP^{Casp4⁻} cells were severely impaired in their ability to produce mature IL-1 β as they released 48.9 \pm 13.5, 72.0 \pm 26.1 and 65.2 \pm 12.4 pg/mL in the presence of 8, 40 and 200 ng/mL Cdt, respectively. It should be noted that caspase-4 deficient cells retained the capacity to exhibit canonical inflammasome activation in response

FIGURE 1 Cdt-induced release of IL-1 β is dependent upon caspase-4 activation.

(A) THP-1^{WT}-derived macrophages were treated with Cdt and analysed for caspase-4 activation. Results are plotted as Cdt concentration vs caspase-4 activity (net OD₄₀₅) and are the mean \pm SEM of four experiments. (B) THP-1^{WT}- (circle) and monocyte (square)-derived macrophages were pre-treated with varying amounts of the caspase-4 inhibitor LEVD-CHO followed by the addition of 200 ng/mL Cdt. Supernatants were harvested 5 hr later and analysed for IL-1 β by ELISA. Results are plotted as mean \pm SEM pg/ml IL-1 β vs caspase-4 inhibitor concentration. Results are the mean of three experiments; note: samples receiving caspase-4 inhibitor were significantly lower at all concentrations than samples receiving Cdt alone ($p < .05$). (C) Macrophages derived from THP-1^{WT} (solid bar), THP-1^{shctl} (hatched bars) and THP-1^{Casp4⁻} (cross-hatched bars) were treated with varying concentrations of Cdt for 5 hr. Supernatants were harvested and analysed by ELISA for IL-1 β . Results are plotted as the mean \pm SEM pg/ml IL-1 β for four experiments versus Cdt concentration. Inset shows caspase-4 protein levels (Western blot) in cell extracts obtained from THP-1^{WT}, THP-1^{shctl} and THP-1^{Casp4⁻} derived macrophages. The asterisk indicates statistical significance ($p < .05$ relative to THP-1^{WT}-derived macrophages treated with the same concentration of Cdt



to Cdt as previously reported (Shenker et al., 2015); Cdt-treated THP-1^{Casp4⁻} derived macrophages demonstrated both an increase in pro-IL-1 β and active caspase-1 (p20) in response to Cdt treatment (Figure 2s).

Our observations demonstrate that Cdt-induced release of IL-1 β is dependent upon caspase-4 activation; this finding is significant as this caspase is involved in not only, the noncanonical inflammasome, but also pyroptosis, a form of inflammatory caspase-mediated lytic cell death (Martel, Lai, Ko, Young, & Ojcius, 2016; Taabazuing, Okondo, & Bachovchin, 2017; Xia, Wang, Zheng, Jiang, & Hu, 2019). In the next

series of experiments, we focused on the relationship between Cdt, caspase-4 activation, pyroptosis and ultimately the release of cytokines. First, we assessed Cdt's ability to induce the release of LDH and HMGB1. THP-1^{WT} derived macrophages were treated with Cdt for 48 hr and then cell supernatants were assessed for LDH activity. As shown in Figure 2a, THP-1^{WT} cells exhibited a 24% net increase in the release of LDH in the presence of Cdt (50 ng/mL). It should be noted that in preliminary experiments, LDH release was not observed at earlier time points. In contrast, THP-1^{Casp4⁻} cells exhibited a 7% net increase in LDH; this was statistically significantly less than the

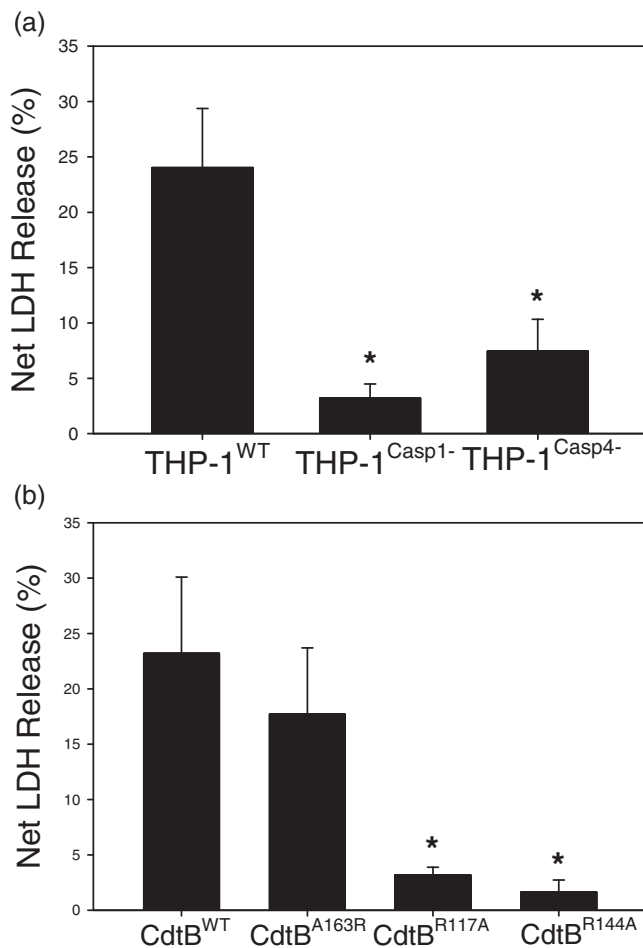


FIGURE 2 Cdt-induced LDH release from macrophages. (A) macrophages derived from THP-1^{WT}, THP-1^{Casp1-/-} and THP-1^{Casp4-/-} cells were incubated with 50 ng/mL Cdt; supernatants were harvested 48 hr later and analysed for LDH activity. Results are plotted as the net release of LDH (%) mean \pm SEM obtained from four experiments vs Cdt concentration. (B) THP-1^{WT}-derived macrophages were treated with 50 ng/mL Cdt containing CdtB^{WT}, CdtB^{A163R}, CdtB^{R117A} or CdtB^{R144A}. Supernatants were obtained at 48 hr and assayed for LDH activity. Results are plotted as in (A) and are the mean \pm SEM of four experiments. The asterisk indicates statistical significance $p < .05$ when compared to respective control (0 Cdt)

amount of LDH release observed with Cdt-treated THP-1^{WT} cells. In addition to the noncanonical inflammasome, it has been reported that pyroptosis may also be induced by the canonical inflammasome (Shi, Gao, & Shao, 2017). Therefore, LDH release was also assessed in caspase-1 deficient cells. THP-1^{Casp1-/-}-derived macrophages were treated with Cdt for 48 hr and the supernatants assessed for enzymatic activity; these cells released 3% net LDH which was also statistically significantly less than the amount observed with Cdt-treated THP-1^{WT} cells.

In another series of experiments we determined whether CdtB associated lipid phosphatase activity was required to induce LDH release from THP-1^{WT}-derived macrophages. Cells were treated with 50 ng/mL Cdt containing either CdtB^{WT} or CdtB mutant proteins that

retain (CdtB^{A163R}) or lack (CdtB^{R117A} and CdtB^{R144A}) lipid phosphatase activity; these mutants have previously been characterised and it should be noted that the latter two mutants have also been shown to be deficient in toxic activity (Shenker, Boesze-Battaglia et al., 2016). Supernatants were harvested 48 hr later and assessed for LDH activity; as shown in Figure 2b, THP-1^{WT} cells treated with Cdt containing CdtB^{WT} exhibited 23% LDH release. Cells treated with Cdt containing the CdtB^{A163R} mutant protein, which retains both phosphatase and toxic activities, exhibited 18% LDH release; this was not statistically different from the release observed with THP-1^{WT} cells treated with Cdt containing CdtB^{WT}. In contrast, cells treated with the enzymatically deficient CdtB mutants, CdtB^{R117A} or CdtB^{R144A}, exhibited low levels of LDH release which were statistically significantly lower than the amount released by cells treated with toxin containing CdtB^{WT} subunit.

In addition to LDH, supernatants were assessed for the release of HMGB1, a pro-inflammatory mediator whose release is associated with pyroptosis and often employed as a marker for the activation of this process (Bui et al., 2016; Johnson et al., 2015). As shown in Figure 3a, THP-1^{WT} cells treated with 25 ng/mL Cdt were analysed by Western blot for HMGB1. Cells exhibited a significant increase in release of HMGB1 at 24 hr relative to untreated (control) cells. Furthermore, as we observed with LDH release, the release of HMGB1 was dependent upon CdtB's ability to function as a lipid phosphatase. Cells treated with Cdt containing the active CdtB^{A163R} subunit exhibited significant HMGB1 release; these levels were comparable to the release observed with cells treated with Cdt containing CdtB^{WT}. In contrast, cells treated with Cdt containing the phosphatase deficient subunit, CdtB^{R117A}, exhibited much lower levels of HMGB1 release. It should also be noted that cells treated with 25 ng/mL Cdt containing CdtB^{WT} also exhibited a 40% reduction in total cellular HMGB1 (Figure S3).

The requirement for caspase-1 and caspase-4 activation leading to the release of HMGB1 was also assessed (Figure 3b). THP-1^{WT}-derived macrophages were treated with 50 ng/mL Cdt and the supernatants analysed 24 hr later for HMGB1. Results are plotted as a percentage of HMGB1 observed in the supernatants of Cdt-treated THP-1^{WT} cells (100%); as observed above, Cdt induced a significant increase in HMGB1 release in THP-1^{WT} cells. In contrast, both THP-1^{Casp1-/-} and THP-1^{Casp4-/-} cells exhibited low levels of HMGB1 in their supernatants: 1.6 ± 3.2 and $8.6 \pm 5.9\%$ of toxin-treated THP-1^{WT} cells, respectively; these levels were not significantly higher than that observed with the untreated control cells.

One of the key elements in caspase-1 and -4-mediated activation of pyroptosis and the resulting release of pro-inflammatory cytokines is the cleavage of GSDMD. Caspase-1 and caspase-4 cleavage removes the C-terminal fragment; this releases and activates the N-terminal fragment, GSDMD-NT. This fragment is directly responsible for pore formation in the plasma membrane which in turn enhances cytokine release and ultimately leads to cell death as indicated by LDH release (Liu et al., 2016). As shown in Figure 4a,b, THP-1^{WT}-derived macrophages treated with Cdt for 24 hr exhibited an increase in the GSDMD-NT fragment. The levels of GSDMD-NT increased in

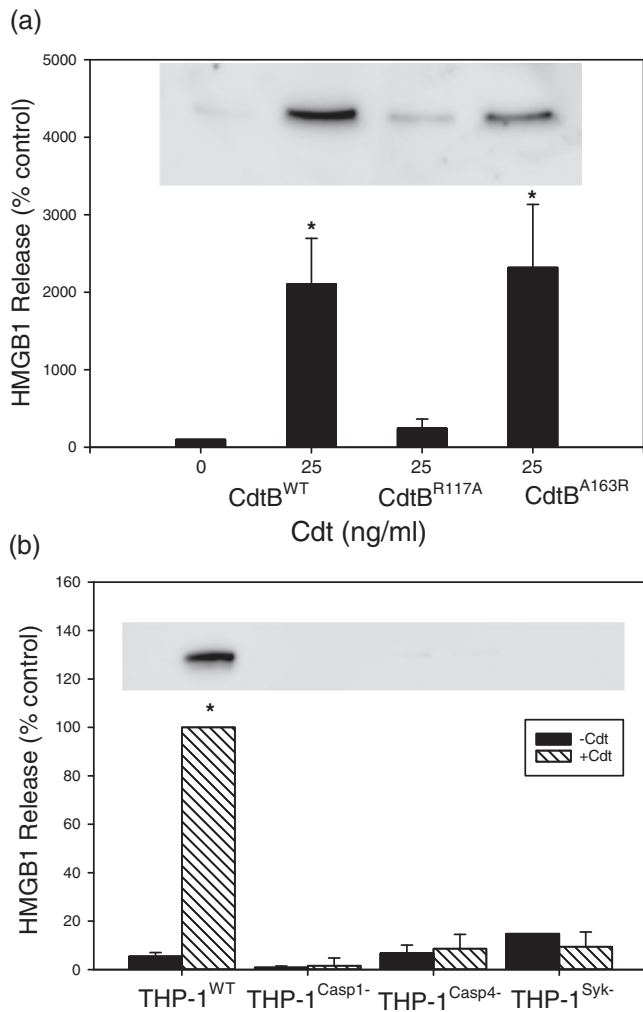


FIGURE 3 Cdt-induced HMGB1 release from macrophages. (A) THP-1^{WT}-derived macrophages were incubated with Cdt containing CdtB^{WT}, CdtB^{R117A} or CdtB^{A163R}. Following incubation for 24 hr, supernatants were harvested and analysed by Western blot for the presence of HMGB1. Results are plotted as HMGB1 levels (% of levels released by control cells; 0 Cdt) versus Cdt concentration and CdtB subunit type; results represent the mean \pm SEM of four experiments. A representative Western blot is shown for HGMB1. (B) Macrophages derived from THP-1^{WT}, THP-1^{Casp1}, THP-1^{Casp4} and THP-1^{Syk} cells incubated with medium (solid bars) or 50 ng/mL Cdt (hatched bars); 24 hr later, supernatants were harvested and analysed for the presence of HMGB1. Results are plotted as HMGB1 release (% of release observed in THP-1^{WT}-derived macrophages incubated with Cdt) vs cell type. Results are the mean \pm SEM of three experiments. The asterisk indicates statistical significance ($p < .05$) when compared to control cells incubated in medium alone (A) or to THP-1^{WT}-derived macrophages incubated with Cdt. A representative full blot is shown in Figure 5s

cells treated with Cdt to 401 (50 ng/mL Cdt), 501 (100 ng/mL Cdt) and 443 (200 ng/mL Cdt) percent over that observed with cells incubated in medium alone; these increases were statistically significant. Further evidence of GSDMD-NT involvement in Cdt-induced cytokine release was demonstrated by its pharmacologic inhibition. NSA

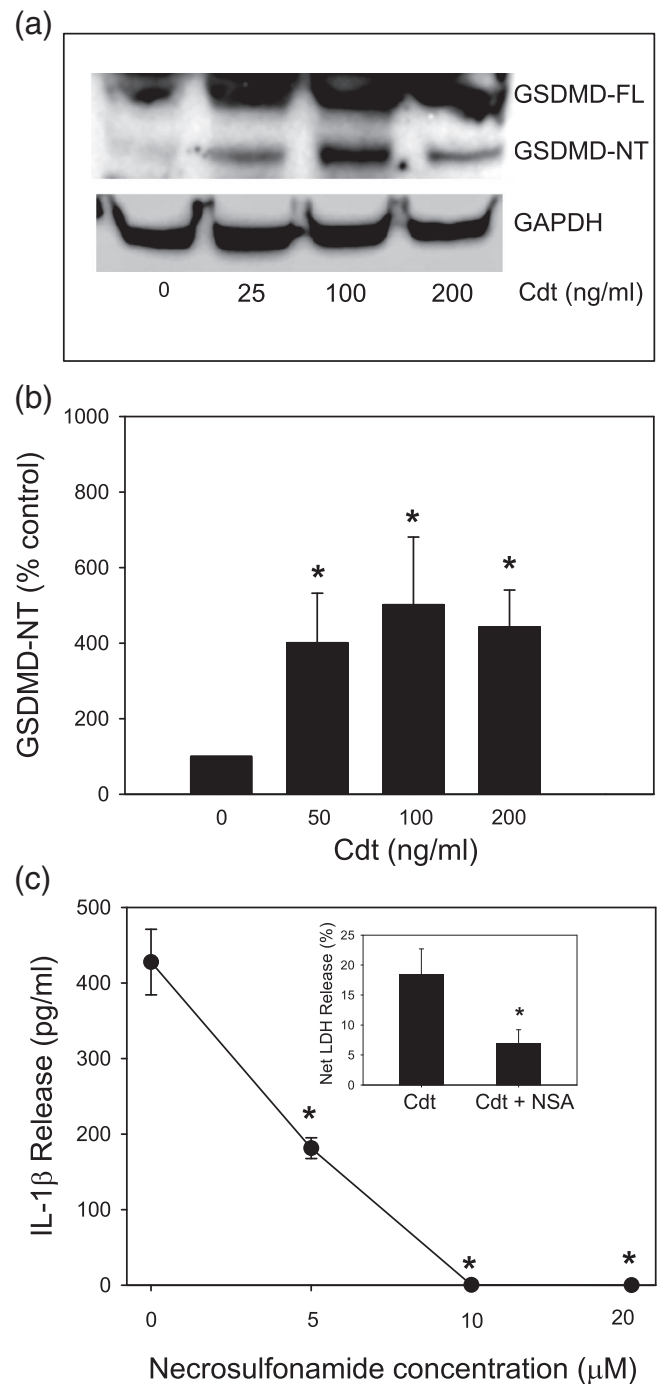


FIGURE 4 Cdt-treated macrophages exhibit GSDMD cleavage. (A) Macrophages derived from THP-1^{WT} cells were incubated with varying amounts of Cdt for 24 hr. Cell extracts were analysed by Western blot for the presence of GSDMD-NT. (A) shows a representative blot. (B) shows the results of three experiments: data are plotted as percent release (relative to control cells) mean \pm SEM. (C) THP-1^{WT}-derived macrophages were pre-incubated with varying amounts of NSA for 60 min followed by the addition of 200 ng/mL Cdt. Supernatants were analysed 5 hr later for the presence of IL-1 β by ELISA. Results are plotted as the mean \pm SEM of three experiments vs NSA concentration. The asterisk indicates statistical significance $< .05$. A representative full blot is shown in Figure 5s

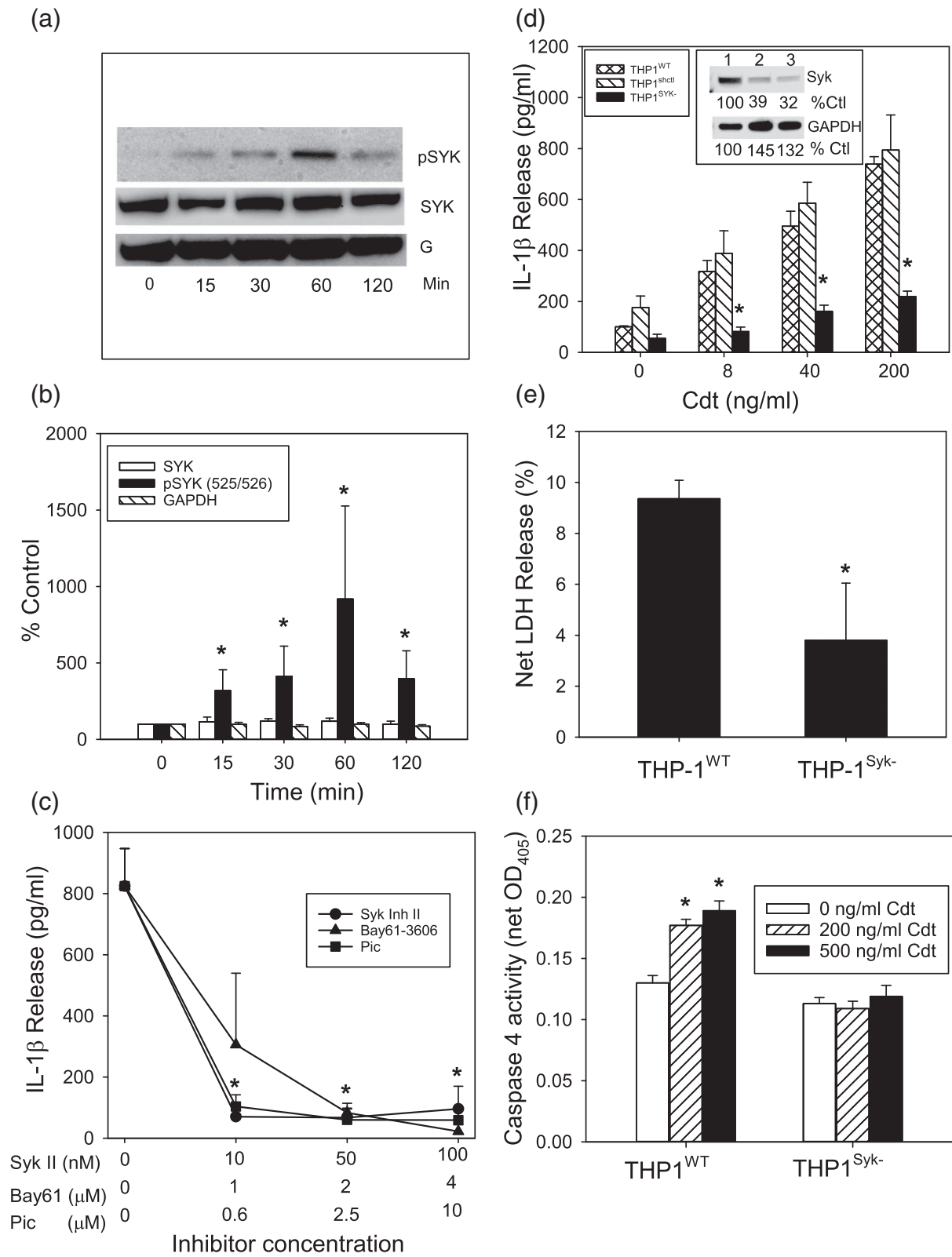


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has recently been shown to bind to GSDMD and inhibit both pore formation by GSDMD-NT and the subsequent onset of pyroptosis (Rathkey et al., 2018). In Figure 4c, we demonstrate that THP-1^{WT}-derived macrophages pre-treated with 5, 10 and 20 μ M NSA blocked Cdt-induced release of IL-1 β ; in the presence of toxin alone, cells released 427 pg/mL cytokine. In contrast, pre-treatment with NSA reduced IL-1 β release to 181, 0.2 and 0 pg/mL IL-1 β . Furthermore, pre-treatment with 10 μ M NSA reduced LDH release from 18% with Cdt alone to 6.9% (inset, Figure 4c).

Preparations of recombinant proteins expressed in *E. coli* typically contain some level of LPS contamination. While our preparations typically contain low levels of LPS, we routinely prepare samples of Cdt in which we have significantly reduced the levels of LPS (Cdt-LD). This preparation of toxin is employed in select experiments to rule out contribution of LPS contamination as a significant contributor to our experimental observations. As shown in Figure S4, we compared our standard Cdt preparation to Cdt-LD for their ability to activate caspase-4, induce the release of both HMGB1 and LDH as well as the cleavage of GSDMD. In each instance, we failed to detect a significant difference in the potency of the two Cdt preparations.

2.2 | Upstream events critical to Cdt-induced activation of noncanonical pyroptosis

In the next series of experiments we explored the role of the non-receptor protein tyrosine kinase, Syk, as an upstream mediator of Cdt-induced activation of both canonical and noncanonical inflammasomes. Cdt-treated macrophages were first assessed for changes in both total Syk and its activated form, phosphorylated Syk (pSyk; 525/526). THP-1^{WT}-derived macrophages were treated with Cdt (200 ng/mL) and analysed by Western blot over time (0–120 min); Figure 5a,b). The levels of Syk and pSyk, respectively, are presented as a percentage of the amount of each protein observed in untreated control cells. Total Syk levels remained relatively constant over the 2 hr time period. In contrast, pSyk levels exhibited a statistically

significant increase (318% of control values) at 15 min; these levels continued to rise to 412% (30 min) and 917% (60 min) above control levels. The levels remained elevated at 120 min but lower than the peak levels observed at 60 min (395%).

To determine if Syk is critical to Cdt-mediated inflammasome activation and the release of IL-1 β , we utilised both a pharmacologic and molecular approach to block its activation. First, three Syk inhibitors were employed: Syk Inhibitor II [(Syk II); 0–100 nM], Bay61-3606 [(Bay61); 0–4 μ M] and piceatanol [(pic); 0–1 μ M] (Figure 5c). THP-1^{WT}-derived macrophages were pre-incubated with each inhibitor for 1 hr followed by the addition of 200 ng/mL Cdt; after an additional 5 hr incubation, supernatants were collected and analysed for the presence of IL-1 β by ELISA. Cells treated with Cdt in the absence of inhibitor released 824 \pm 122 pg/mL IL-1 β as compared to 108 \pm 10 pg/mL for cells exposed to medium only. Pre-treatment of cells with the lowest dose of each inhibitor resulted in significant reductions in IL-1 β release: 70 \pm 19, 306 \pm 233 and 104 \pm 38 pg/mL in the presence of Syk II inhibitor (10 nM), Bay61-3606 (1 μ M) and piceatanol (0.6 μ M), respectively. IL-1 β release continued to decline in the presence of 50 and 100 nM Syk II inhibitor to 67 \pm 28 and 95 \pm 74 pg/mL. No further decreases were observed with Bay61-3606 and piceatanol.

Lentivirus particles containing Syk shRNA sequences were used to transfect THP-1 cells (THP-1^{Syk⁻}) and reduce Syk expression (Figure 5d, inset). These cells were employed to further analyse the requirement for Syk in Cdt-induced IL-1 β maturation and release. As shown in Figure 5d, treatment of THP-1^{WT} cells with increasing concentrations of Cdt (0–200 ng/mL) resulted in a dose-dependent increase in the release of IL-1 β into the medium: 100 \pm 4.3 (0 Cdt), 318 \pm 43 (8 ng/mL Cdt), 496 \pm 58 (40 ng/mL Cdt) and 738 \pm 29 pg/mL (200 ng/mL Cdt). In contrast, THP-1^{Syk⁻} cells were severely compromised in their ability to respond to Cdt as these cells released 55 (0 Cdt), 82 (8 ng/mL Cdt), 161 (40 ng/mL Cdt) and 219 pg/mL IL-1 β (200 ng/mL Cdt).

In addition to Cdt-induced release of IL-1 β in THP-1^{Syk⁻} cells, the release of both LDH and HMGB1 into cell supernatants was assessed. As shown in Figure 5e, the supernatants of THP-1^{WT} cells contained

FIGURE 5 Cdt-induced pro-inflammatory response and noncanonical inflammasome activation is dependent upon Syk. (A, B) THP-1^{WT}-derived macrophages were incubated with 200 ng/mL Cdt for 0–120 min; cell extracts were analysed for the Syk and pSyk levels by Western blot. A representative blot including GAPDH (G) is shown in (A) and pooled results from three experiments in (B); data are plotted as a percentage of untreated (time 0) levels and represent the mean \pm SEM of four experiments. The asterisk indicates statistical significance ($p < .05$) when compared to cells exposed to medium only. (C) THP-1^{WT}-derived macrophages were pre-incubated with varying amounts of one of three Syk inhibitors: Syk II (circle), Bay61 (triangles) or piceatanol (Pic) for 60 min followed by the addition of 200 ng/mL Cdt. Supernatants were harvested 5 hr later and analysed by ELISA for IL-1 β . Results are plotted as mean \pm SEM of IL-1 β (pg/ml) based on three experiments vs Syk inhibitor concentration. Note: all samples treated with inhibitor and Cdt were statistically significantly lower when compared to cells treated with Cdt alone. (D) Macrophages derived from THP-1^{WT} (cross hatched bars), THP-1^{shctrl} (hatched bars) and THP-1^{Syk⁻} (solid bars) cells were incubated with varying amounts of Cdt. Supernatants were harvested 5 hr later and analysed by ELISA for IL-1 β . Results are plotted as mean \pm SEM of IL-1 β (pg/ml) of three experiments vs Cdt concentration. The asterisk indicates statistical significance ($p < .05$) when compared to control cells (0 Cdt). (E) THP-1^{WT}- and THP-1^{Syk⁻}-derived macrophages were incubated with medium or 200 ng/mL Cdt (cross-hatched bars) for 48 hr; supernatants were analysed for LDH activity and plotted as net LDH release (%) (mean \pm SEM for four experiments). The asterisk indicates statistical significance ($p < .05$) when compared to Cdt-treated THP-1^{WT} control cells (0 Cdt). (F) THP-1^{WT}- and THP-1^{Syk⁻}-derived macrophages were incubated with medium (open bars), 200 ng/mL Cdt (hatched bars) and 500 ng/mL Cdt (solid bars) for 2 hr. Cell extracts were analysed for caspase-4 activity (net OD₄₀₅). Results are the mean \pm SEM for four experiments; * indicates statistical significance when compared to control cells (0 Cdt)

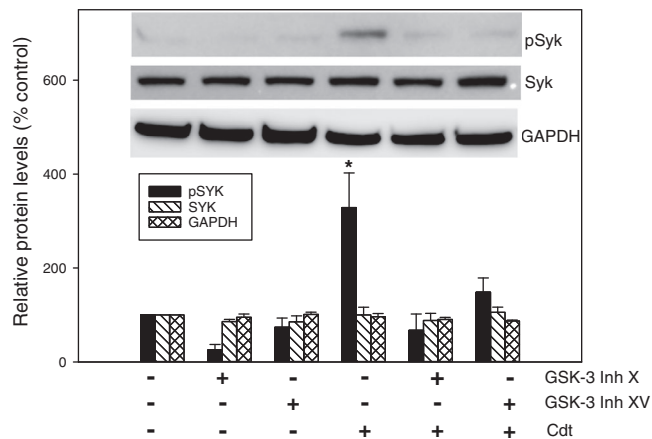


FIGURE 6 GSK3 β inhibitors block Cdt-induced Syk phosphorylation. THP-1^{WT}-derived macrophages were pre-incubated with medium, 10 μ M GSK-3 Inh X or 1.0 μ M GSK-3 Inh XV for 60. Medium or 200 ng/mL Cdt was then added to cell cultures and cells harvested 2 hr later. Cell extracts were analysed for Syk and pSyk by western blot. GAPDH was used as a loading control. Results are plotted as the relative protein levels (% control) and represent the mean \pm SEM of three experiments. A representative blot is shown in the inset. The asterisk indicates statistical significance relative to cells treated with medium

LDH (9%) in the presence of 200 ng/mL Cdt. In contrast, macrophages derived from THP-1^{Syk⁻} cells exhibited significantly less LDH (3.8%) in the cell supernatants. Similarly, THP-1^{Syk⁻}-derived macrophages failed to release significant amounts of HMGB1 (Figure 3b).

In the next series of experiments the requirement for Syk in mediating Cdt-induced caspase-4 activation was assessed. As shown in Figure 5f, THP-1^{WT}-derived macrophages cells exhibited an increase in caspase-4 activity in the presence of Cdt (net OD₄₀₅): 0.13 \pm 0.006 (0 Cdt), 0.18 \pm 0.005 (200 ng/mL Cdt) and 0.190 \pm 0.009 (500 ng/mL Cdt). In comparison, THP-1^{Syk⁻}-derived macrophages did not exhibit an increase in caspase-4 activity over levels observed with control cells.

In previous studies we established that the action of *A. actinomycetemcomitans* Cdt on lymphocytes and macrophages was dependent upon CdtB's ability to function as a PIP3 phosphatase (Shenker, Boesze-Battaglia et al., 2016; Shenker et al., 2007; Shenker et al., 2014). Depletion of PIP3 results in PI-3K signalling blockade: reduced PIP3, reduced pAkt (loss of kinase activity) and reduced pGSK3 β (activation of kinase activity). The significance of these observations was demonstrated by the ability of GSK3 β inhibitors to block Cdt toxicity including activation of the canonical NLRP3 inflammasome (Shenker, Boesze-Battaglia et al., 2016; Shenker et al., 2014). In order to build upon our previous observations and link them with our current studies, we next explored the possibility that the observed increases in Cdt-mediated Syk phosphorylation might also be linked to PI-3K signalling. Therefore, we assessed the ability of two of these inhibitors, GSK-3 inhibitor X and XV, to block Cdt-induced increases in pSyk. As shown in Figure 6, both inhibitors reduced background levels of pSyk to: 25.4% (GSK-3 X) and 73.8% (GSK-3 XV) of

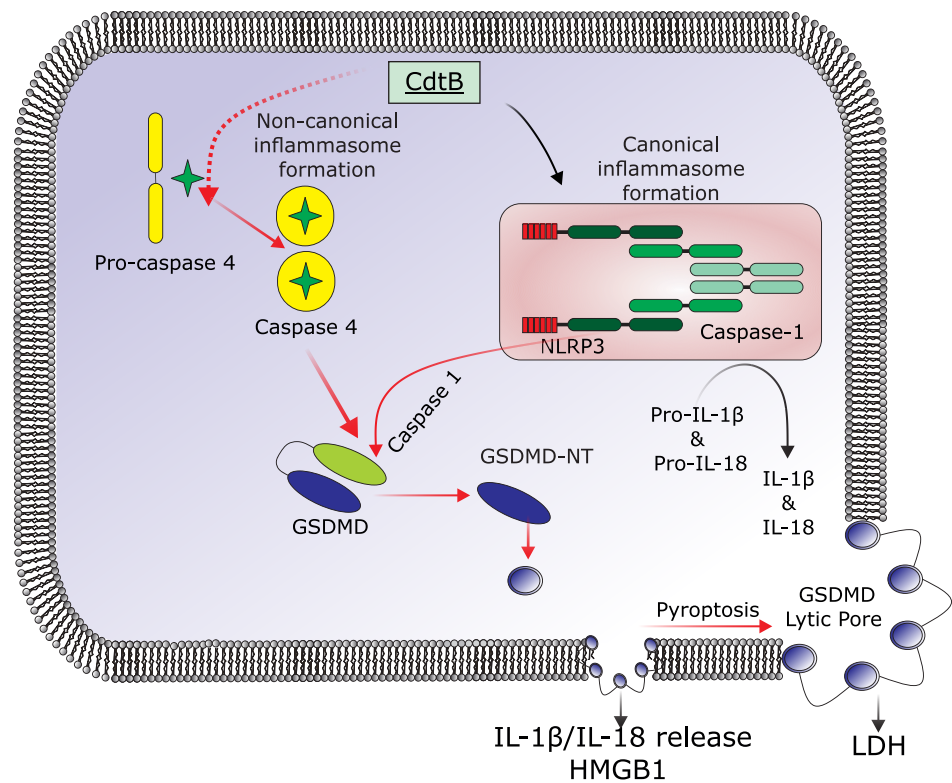
levels observed in untreated control cells. In the presence of Cdt, pSyk levels increased to 328.8% above control cell levels. The inhibitors blocked the effects of Cdt on pSyk levels as they remained below levels observed with Cdt alone. Total Syk levels were not significantly altered by either of the inhibitors.

3 | DISCUSSION

Cdt toxicity is typically associated with induction of irreversible cell cycle arrest and eventually apoptotic cell death in proliferating cells such as lymphocytes (reviewed in Gargi et al., 2013; Scuron et al., 2016). In previous studies, however, we have demonstrated that non-proliferating cells, such as human monocytes and macrophages, are resistant to *A. actinomycetemcomitans* Cdt-induced apoptosis (Shenker et al., 2014; Shenker et al., 2015). Instead, the toxin induces macrophages derived from either human monocytes or from the acute monocytic leukaemia cell line, THP-1, to synthesise and release pro-inflammatory cytokines (IL-1 β , IL-18, TNF α and IL-6). Additionally, we have shown that the Cdt-induced pro-inflammatory response involves activation of the canonical NLRP3 inflammasome (Figure 7). In those studies we employed both specific inhibitors and stable transfection (shRNA) of cell lines to demonstrate requirements for NLRP3, ASC as well as caspase-1. Furthermore, Cdt-mediated inflammasome activation was found to be dependent upon upstream danger signals including: reactive oxygen species (ROS) and Cdt-induced increases in extracellular ATP. The latter contributed to the activation of the P2X₇ purinergic receptor and K⁺ efflux. Additionally, we have demonstrated that induction of this pro-inflammatory response was dependent upon CdtB associated PIP3 phosphatase activity. Specifically, enzymatic depletion of PIP3 results in blockade of the PI-3K signalling pathway leading to both Akt inactivation and GSK3 β activation (Shenker et al., 2016a). In the current study we demonstrate that, in addition to the canonical inflammasome, the Cdt-induced pro-inflammatory response is also dependent upon activation of the noncanonical inflammasome and further that activation of both inflammasomes contribute to cytokine release which is dependent upon GSDMD cleavage which in turn leads to pyroptosis (summarised in Figure 7).

Pyroptosis was first recognised as a form of cell death that was distinct from apoptosis as it was independent of caspase-3 (Tang, Kang, Berghe, Vandenabeele, & Kroemer, 2019; Xia et al., 2019). The two forms of cell death exhibit other distinguishing characteristics. Morphologically, pyroptotic cells display loss of plasma membrane integrity which results from pore formation within the plasma membrane and ultimately leads to cell swelling and release of cytoplasmic content. Biochemically, pyroptosis is mediated by inflammatory caspases-1, -4, -5 and -11 (murine cells) (Casson et al., 2015; Solberger, Strittmatter, Kistowska, French, & Beer, 2012; Taabazuing et al., 2017; Tang et al., 2019; Vigano et al., 2015). As noted above, caspase-1 is typically activated by a multi-protein complex, the inflammasome, consisting of a NOD-like receptor (NLR), a linker protein such as ASC and pro-caspase-1 and is often referred to as the canonical inflammasome (He, Hara, & Nunez, 2016). Activation of the

FIGURE 7 Schematic showing an overview of the proposed activation of the noncanonical inflammasome and pyroptosis by Cdt. Internalisation of CdtB leads to activation of caspase-4 and caspase-1; the later involves activation of the canonical inflammasome that we have previously described in detail (Shenker et al., 2015). Both caspase-4 and caspase-1 cleave GSDMD to release the pore-forming fragment, GSDMD-NT. Initially, this leads to the formation of small pores that facilitate the release of IL-1 β , IL-18 and HMGB1. Eventually, these pores enlarge and become lytic allowing LDH to escape from the dying cells



canonical inflammasome leads to cleavage and activation of caspase-1 and, in turn, maturation of IL-1 β . Cell death mediated by this complex is referred to as canonical pyroptosis and typically occurs in monocytes, macrophages and dendritic cells (Russo, Behl, Banerjee, & Rathinam, 2018; Shi et al., 2017). In contrast, noncanonical pyroptosis is mediated by activated caspases-4, -5, -11 and occurs in a variety of cell types (Shi et al., 2017; Xia et al., 2019). Activation of this pathway was initially identified as occurring as a result of direct interaction between intracellular bacterial lipopolysaccharide (LPS) and caspase-4/5; cell surface interactions involving TLR4-LPS do not activate these caspases (Shi et al., 2014). Whereas activated caspase-1 directly cleaves pro-IL-1 β and pro-IL-18 leading to the formation of the mature cytokine, caspases -4, -5 and -11 do not promote cleavage. However, each of these caspases, along with caspase-1, cleave GSDMD releasing the active, pore forming, N-terminal fragment, GSDMD-NT (Evavold & Kagan, 2019; Feng, Fox, & Man, 2018a; Liu et al., 2016; Shi et al., 2017).

We now demonstrate that in addition to caspase-1, caspase-4 is activated in Cdt-treated macrophages and, further, the release of IL-1 β is also dependent upon caspase-4. Cells pre-treated with the caspase-4 inhibitor LEVD-CHO or deficient in caspase-4 expression were resistant to Cdt-induced cytokine release. Cdt-treated macrophages become pyroptotic within 48 hr and exhibit both GSDMD cleavage and an increase in membrane permeability (Kovacs & Miao, 2017; Sborgi et al., 2016; Shi et al., 2015). The latter was demonstrated by the release of both LDH and HMGB1 into culture supernatants. The release of HMGB1 is particularly significant as it not only represents a marker for the onset of pyroptosis but is also a potent inducer of pro-inflammatory responses (Lotze & Tracey, 2005). In

contrast, macrophages derived from either THP-1^{Casp1-} or THP-1^{Casp4-} cells did not exhibit GSDMD cleavage or increased membrane permeability as evinced by the failure to release either LDH or HMGB1. These results suggest that not only are these cells resistant to Cdt-induced pyroptosis but also that both caspase-1 and caspase-4 were required for this lytic form of cell death. These caspases are thought to act in a synergistic role in mediating GSDMD cleavage and in turn, pyroptosis. Therefore, it was surprising that depletion of either caspase-1 or caspase-4 was sufficient to block pyroptosis and IL-1 β release. These results suggest a cooperative role such that the activation of either caspase alone is insufficient to induce pyroptosis and facilitate cytokine release or alternatively, that caspase-4 is upstream of caspase-1.

GSDMDs are a family of cytosolic proteins with diverse function and expression profiles; one member of this family, GSDMD, as noted above is cleaved by caspases-1 and -4. The liberated N-terminus, GSDMD-NT, is the pore forming fragment (Evavold & Kagan, 2019; Feng, Fox, & Man, 2018a; Liu et al., 2016; Shi et al., 2017). Indeed, we demonstrate that not only do Cdt-treated macrophages contain significant increases in GSDMD-NT when exposed to Cdt, but further, that NSA, an inhibitor of GSDMD cleavage, blocks the release of both IL-1 β and LDH (Rathkey et al., 2018). Following cleavage, GSDMD-NT has been shown to bind to phosphatidylinositol (PI) phosphates on the inner leaf of plasma membranes as well as on mitochondrial, lysosomal, phagocytic and endosomal membranes (reviewed in Feng, Fox, & Man, 2018a). This interaction is critical to subsequent pore formation as it promotes oligomerization of GSDMD-NT. One of the phosphoinositides that GSDMD-NT binds to is PI-3,4-P₂; this PI-disphosphate is of particular relevance to our study as it is the

enzymatic breakdown product of CdtB-mediated PIP3 degradation (Shenker et al., 2007). Thus, it is interesting to speculate that a secondary effect of CdtB on macrophages is to not only induce PI-3K signalling blockade and activate the canonical and noncanonical inflammasomes, but to further potentiate pore formation by providing additional docking sites for the GSDMD-NT. These potential interactions are the subject of ongoing investigation.

One critical feature of pores formed by GSDMD-NT is that they are selective and further that selectivity is determined by pore size (Liu et al., 2016). Initially, the pores are small (10–15 nm inner diameter), large enough for the release of cytokines and the danger signal HMGB1. This is critical for the release of mature IL-1 β and IL18 (~4.5 nm diameter) which lack N-terminal signal sequences necessary for secretion via biosynthetic vesicle-mediated pathways. Furthermore, it has been proposed that the formation of these small GSDMD-NT-dependent pores is consistent with macrophages being in a hyperactive/pro-inflammatory state (Evavold & Kagan, 2019). Under these conditions the pores are not lytic and cells are able to survive. Our findings are consistent with this scheme as we observe the release of IL- β and HMGB1 during the first 24 hr of exposure to Cdt; we do not observe the release of LDH at this time. LDH requires larger lytic pores to escape cells as it exists as a tetramer in the cytosol. Eventually, the pores enlarge and reach a threshold that leads to the lytic phase of pyroptosis characterised by the release of LDH and other intracellular components (Evavold & Kagan, 2019; Shi et al., 2017; Taabazuing et al., 2017). Indeed, we do not observe LDH release in Cdt-treated cells until 48 post treatment with the toxin. The mechanism leading to transition to larger lytic pores is not clear, but it has been proposed that initially the number and/or size of the GSDMD-NT pores is maintained by an endosomal sorting complex required for transport (ESCRT)-III repair mechanism (Ruhl et al., 2018). It has been suggested that failure of this repair mechanism ultimately contributes to pyroptotic cell death.

Previously, we have detailed the relationship between CdtB associated lipid phosphatase activity, PI-3K signalling and Cdt toxicity and, as noted above, one of the key outcomes of this blockade is the activation of GSK3 β (i.e., reduced phosphorylation). Thus, it is consistent that the ability of Cdt to activate canonical and noncanonical inflammasomes and thereby induce pyroptosis requires that the toxin complex contain a CdtB subunit that retains PIP3 phosphatase activity (e.g., CdtB^{WT} or CdtB^{A163R}). Likewise, it was not surprising that CdtB mutants that lack lipid phosphatase and toxic activity (CdtB^{R117A} and CdtB^{R144A}) were unable to induce LDH and HMGB1 release as well as activate caspase-4. These observations point to a potential linkage between the induction of pyroptosis and CdtB-mediated blockade of the PI-3K signalling pathway. The activation of this kinase and the potential cross-talk between PI-3K signalling and inflammasome activation is further discussed below as it may also relate to Syk activation.

Syk is a nonreceptor tyrosine kinase; its activation is typically considered to be the result of interaction with immunoreceptor tyrosine-based motif (ITAM)-containing proteins. It is becoming increasingly clear that Syk is activated in macrophages following stimulation via

pathogen recognition receptors (PRRs) and further that activation of this tyrosine kinase has been shown to be critical for pro-inflammatory cytokine gene expression (reviewed in Mocsai, Ruland, & Tybulewicz, 2010). Additionally, canonical inflammasome activation has been shown to be regulated by pSyk via phosphorylation of ASC, a requirement for its oligomerization. Indeed, we demonstrate that within 15 min of exposure to Cdt, there is a significant increase in pSyk. Moreover, pre-incubation of cells with Syk inhibitors blocks Cdt-induced release of mature IL-1 β . Similarly, macrophages derived from Syk deficient cells (THP-1^{Syk⁻}) were resistant to Cdt-induced IL-1 β release. These results are consistent with the reported role of Syk in mediating activation of the NLRP3 inflammasome (Feng et al., 2018b; Fitzpatrick, 2015; Said-Sadier, Padilla, Langsley, & Ojcius, 2010). We also observed that THP-1^{Syk⁻}-derived macrophages failed to exhibit an increase in caspase-4 activity in the presence of Cdt and further that the toxin did not induce the release of either HMGB1 or LDH in these cells. Thus, in addition to canonical inflammasome activation, Syk appears to also regulate Cdt-induced activation of the noncanonical inflammasome and ultimately pyroptosis.

In order to account for CdtB-dependent Syk activation, we explored the possibility that GSK3 β might be involved. As noted earlier, we have previously demonstrated that Cdt-induced PI-3K signalling blockade in both lymphocytes and macrophages results in GSK3 β activation (Scuron et al., 2016; Shenker, Boesze-Battaglia et al., 2016; Shenker et al., 2007; Shenker et al., 2015; Shenker et al., 2014). Moreover, we have shown that GSK3 β inhibitors blocked toxin-induced IL-1 β release, clearly indicating a role for this kinase in canonical and/or noncanonical inflammasome activation. We now report that these same inhibitors block Cdt-induced Syk phosphorylation. These studies demonstrate a potential link between GSK3 β activation and/or a downstream PI-3K signalling component, Syk activation and IL-1 β release via canonical and/or noncanonical pathways.

In conclusion, we propose that pyroptosis, in general, and Cdt-induced pyroptosis, in particular, is critical to the toxins virulence potential as this form of cell death has been implicated to play a critical role in microbial infection and host defence. One key aspect of this lytic cell death pathway is that it provides a mechanism for the release of IL-1 β and IL-18, cytokines that lack signal sequences for secretion, as well as other pro-inflammatory mediators such as HMGB1 and eicosonoids (Figure 7). Collectively, these pro-inflammatory mediators potentially benefit the host by promoting microbial clearance through both bactericidal and phagocytic mechanisms. In this context, it is also important to note that in addition to creating pores in the plasma membranes of host cells, GSDMD-NT is lethal to bacteria. Pyroptosis also serves to eliminate intracellular pathogens as host cell death releases these organisms where they face elimination by both innate and acquired host defence (Xia et al., 2019). Alternatively, pyroptotic amplification of the release of inflammatory mediators contributes to development of chronic inflammation and tissue injury. It is in this context that we propose that Cdt-hijacks this critical host defence mechanism, amplifies the pro-inflammatory response and eventually contributes to the destruction of tooth supporting tissue, a clinical feature of *A. actinomycetemcomitans* infection. Moreover,

A. actinomycetemcomitans Cdt is capable of functioning as a triperditous toxin (virulence factor) by altering host defence mechanisms where it impairs acquired immunity, activates innate immunity and damages epithelial barriers (reviewed in Scuron et al., 2016). These properties are consistent with the current view of the role for *A. actinomycetemcomitans* in the pathogenesis of localised aggressive periodontitis. Rather than serve as the causative agent, it has been proposed that *A. actinomycetemcomitans* is an early coloniser that modifies local host defence; this perturbation facilitates both its own survival and in turn contributes to dysbiosis by advancing the survival of other microbes (Fine, Patil, & Velusamy, 2019). The ability to function in this manner and differentially affect a spectrum of cell types is due to two properties of this AB₂ toxin. The binding unit, CdtC, binds to cholesterol, a ubiquitous component of all cell membranes and the active subunit, CdB, is a lipid phosphatase capable of depleting cells of PIP3 resulting in blockade of the PI-3K signalling pathway, a ubiquitous signalling cascade, functional in all cells.

4 | EXPERIMENTAL PROCEDURES

4.1 | Cells and reagents

The human acute monocytic leukaemia cell line, THP-1, was obtained from ATCC; cells were maintained in RPMI 1640 containing 10% FBS, 1 mM sodium pyruvate, 20 μM 2-mercaptoethanol and 2% penicillin-streptomycin at 37°C with 5% CO₂ in a humidified incubator. THP-1 cells stably expressing shRNA against caspase-1 (THP-1^{Casp1-}) and Syk (THP-1^{Syk-}) were provided by D. M. Ojcius and previously described (Abdul-Sater, Said-Sadier, Padilla, & Ojcius, 2010; Chen et al., 2012); caspase-4 deficient cells (THP-1^{Casp4-}) were provided by P.R. Huang and similarly prepared with lentiviral particles containing the following sequence: 5'-GCAACGTATGGCAGGACAAAT-3'. The cells were prepared by transducing THP-1 cells with lentiviral particles, along with nontarget controls, as previously described (Abdul-Sater et al., 2010; Chen et al., 2012). Expression levels were recently reported to be reduced >95% for THP-1^{Casp1-}, >70% THP-1^{Syk-} and caspase-4 was not detectable by Western blot in THP-1^{Casp4-} cells with no effect in the nontarget controls (Zhu et al., 2015). THP-1 cells were differentiated into macrophages by incubating cells in the presence of 50 ng/mL PMA for 48 hr at which time the cells were washed and incubated an additional 24 hr in medium prior to use.

Human peripheral blood mononuclear cells (HPBMC) were obtained from venous blood of healthy donors as previously described (Shenker et al., 2004). Monocytes were further purified from HPBMC by positive selection using anti-CD14 mAb according to the manufacturers directions (Dynabeads® FlowComp™ Human CD14; ThermoFisher Invitrogen, Waltham, MA). Anti-CD14 mAb-bead complexes were removed from the positively selected monocytes using a release buffer (Invitrogen). Monocytes were routinely found to be >96% pure as determined by immunofluorescent staining and analysed by flow cytometry. Monocyte-derived macrophages (MDM) were generated by incubating monocytes in DMEM

containing 10% fetal bovine serum and 2% penicillin/streptomycin for 6–8 days.

THP-1 derived macrophages and MDM were incubated as described below (or in Figure legends) and treated with recombinant Cdt holotoxin containing CdtB wildtype (CdtB^{WT}) or mutant (CdtB^{A163R}, CdtB^{R117A} or CdtB^{R144A}) which were expressed and purified as described below. In some experiments cells were also treated with: necrosulfonamide (NSA), Syk inhibitor II, Bay61-3606, picceatanol, GSK inhibitor XV (MilliporeSigma, Burlington, MA) and GSK inhibitor X (Santa Cruz Biotechnology, Dallas, TX).

4.2 | Expression and purification of Cdt holotoxin

Construction and expression of the plasmid containing the *cdt* genes for the holotoxin (pUCAcdtABC^{his}) have previously been reported (Shenker et al., 2004). The plasmids were constructed so that the *cdt* genes were under control of the *lac* promoter and transformed into *E. coli* DH5α. Cultures of transformed *E. coli* were grown in 1 L LB broth and induced with 0.1 mM IPTG for 2 hr; bacterial cells were harvested, washed and resuspended in 50 mM Tris (pH 8.0). The cells were frozen overnight, thawed and sonicated. The histidine-tagged peptide holotoxin was isolated by nickel affinity chromatography as previously described (Shenker et al., 2004).

4.3 | Analysis of IL-1β release

IL-1β release was measured in THP-1 derived macrophages (2 × 10⁵ cells) or MDM incubated for 5 hr in the presence or absence of varying amounts of Cdt as previously described (Shenker et al., 2015). Briefly, culture supernatants were collected and analysed by ELISA for IL-1β (Quantikine Elisa Kit; R and D Systems, Minneapolis, MN). In each instance, the amount of cytokine present in the supernatant was determined using a standard curve.

4.4 | Western blot analysis

Replicate wells of THP-1 derived macrophages (4 × 10⁶ cells/well) were incubated as described with Cdt. The cells were washed and treated with 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate and protease and phosphatase inhibitors (ThermoFisher Pierce Protein Biology, Pittsburgh, PA); replicate wells were pooled and protein concentration determined. HMGB1 release into culture supernatants was determined following precipitation of supernatants in 20% TCA. Samples were separated on 12% SDS-PAGE and then transferred to nitrocellulose. The membrane was blocked with BLOTTO and then incubated with anti-pro-IL-1β, anti-caspase-4, anti-Syk, anti-pSyk, anti-GSDMD or anti-GAPDH antibody (Santa Cruz Biotechnology) for 18 hr at 4°C (Shenker et al., 1999). Membranes were washed, incubated with either goat anti-rabbit IgG or goat-anti-mouse IgG conjugated to

horseradish peroxidase. The Western blots were developed using chemiluminescence and analysed (Licor).

4.5 | Analysis of LDH release

THP-1-derived macrophages (5×10^4) were incubated as described in Figure Legends. The supernatants were harvested and LDH activity was detected using a commercially available kit (LDH Cytotoxicity Assay Kit; ThermoFisher Pierce Protein Biology). Extracellular LDH in the supernatant was quantified by a coupled enzymatic reaction in which LDH catalyses the conversion of lactate to pyruvate via NAD⁺ reduction to NADH. NADH is then utilised by diaphorase to reduce tetrazolium salt to red formazan which is measured at OD₄₉₀ nm. LDH release is presented as % net LDH release: $100 \times (\text{experimental OD}_{490} \text{ release} - \text{spontaneous OD}_{490} \text{ release}) / \text{maximum OD}_{490} \text{ release} - \text{spontaneous OD}_{490} \text{ release}$.

4.6 | Analysis of caspase-4 activity

THP-1-derived macrophages (4×10^6) were incubated as described in the figure legends. Caspase-4 activity was measured using a commercially available kit (Caspase-4 Colorimetric Assay Kit; BioVision, Milpitas, CA). Cells were harvested, lysed and following centrifugation the supernatants containing the cytosolic extract were assessed for caspase-4 activity. Briefly, caspase-4 activity was determined by monitoring the release of the chromophore *p*-nitranilide (pNA) from the labelled substrate, LEVD-pNA at OD₄₀₅ nm. Background OD₄₀₅ from cell lysates and buffers were subtracted from measurements obtained from both control and treated samples (net OD₄₀₅).

4.7 | Statistical analysis

Mean \pm standard error of the mean were calculated for replicate experiments. Significance was determined using a Student's *t*-test; differences between multiple treatments were compared by ANOVA paired with Tukeys HSD posttest; a *P*-value of less than .05 was considered to be statistically significant.

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CONFLICT OF INTEREST

Authors declare no potential conflict of interest.

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