Multiple Roles of CLAN (Caspase-Associated Recruitment Domain, Leucine-Rich Repeat, and NAIP CIIA HET-E, and TP1-Containing Protein) in the Mammalian Innate Immune Response

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Multiple Roles of CLAN (Caspase-Associated Recruitment Domain, Leucine-Rich Repeat, and NAIP CIIA HET-E, and TP1-Containing Protein) in the Mammalian Innate Immune Response

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NAIP CIIA HET-E and TP1 (NACHT) family proteins are involved in sensing intracellular pathogens or pathogen-derived molecules, triggering host defense responses resulting in caspase-mediated processing of proinflammatory cytokines and NF-κB activation. Caspase-associated recruitment domain, leucine-rich repeat, and NACHT-containing protein (CLAN), also known as ICE protease-activating factor, belongs to a branch of the NACHT family that contains proteins carrying caspase-associated recruitment domains (CARDs) and leucine-rich repeats (LRRs). By using gene transfer and RNA-interference approaches, we demonstrate in this study that CLAN modulates endogenous caspase-1 activation and subsequent IL-1β secretion from human macrophages after exposure to LPS, peptidoglycan, and pathogenic bacteria. CLAN was also found to mediate a direct antibacterial effect within macrophages after Salmonella infection and to sensitize host cells to Salmonella-induced cell death through a caspase-1-independent mechanism. These results indicate that CLAN contributes to several biological processes central to host defense, suggesting a prominent role for this NACHT family member in innate immunity.

involve oligomerization via their corresponding NACHT do-
main, triggered by binding of specific pathogen-derived mol-
cules to the LRRs (15, 16).

A subset of CARD-containing proteins within the greater NACHT family shares considerable amino acid sequence similarity and includes Nod1/CARD4, Nod2/CARD15, and CARD, LRR, and NACHT-containing protein (CLAN), also known as ICE pro-
tease-activating factor (17–20). Interest in this branch of the NACHT family has propagated since the discovery of a correlation between hereditary mutations in the Nod2/CARD15 gene and sus-
cceptibility to inflammatory bowel disorders such as Crohn’s dis-
ease and Blau syndrome (10, 11). Additional research has identi-
fied muramyl dipeptide, the minimal bioactive peptidoglycan motif common to all bacteria, as the ligand recognized nonredundantly by the LRRs of Nod2 (21). The inactivating truncation mutations found within the LRR domain of Nod2 have been hypothesized to contribute to defective NF-kB-initiated inflammatory responses in the gut after exposure to invasive bacteria. Although much em-
phasis has been placed on the importance of Nod2 in the innate immune response to specific bacterial cell wall components, this protein appears to play a nonessential or redundant role in host defense, as evidenced by a knockout mouse model (22, 23). How-
ever, deletion of the murine Nod2 gene did provide limited protection against LPS-induced death of mice, suggesting a contribut-
ing role in sepsis (23). Similarly, Nod1 has been shown to recog-
ize a unique muropeptide found in Gram-negative bacteria (22, 24), but a critical role in host defense has not yet been estab-
lished for this protein. It remains to be determined whether other NACHT family proteins with similar domain architectures are essential for bacterial pattern recognition and inflammatory responses within macrophages and other host defense cells.

The Nod1/Nod2-like protein CLAN (also known as ICE pro-
tease-activating factor or CARD12) was originally described as a CARD, LRR, and NACHT-containing protein capable of binding and activating procaspase-1 after overexpression in HEK293T cells (17, 25). The function of CLAN in human macrophages (where it is endogenously expressed) has not been reported previ-
ously, nor have the specific ligands responsible for its activation been identified. In this study we used gene transfer and RNA inter-
terference (RNAi) to explore the functions of CLAN in the human monocytic cell line THP-1, differentiated into macrophages by 

\[ \text{Materials and Methods} \]

\textbf{Cell lines and bacterial strains}

The THP-1 monocytic leukemia cell line was obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 sup-
plemented with 10% FCS, 1% (v/v) penicillin (100 U/ml), streptomycin (100 μg/ml), and t-glutamine. Cells were maintained at 37°C in a 5% CO₂-
95% air atmosphere and were subcultured every 6 days. Salmonella enteri-
tidis strain LK5 was a gift from Dr. S. Maloy (San Diego State Uni-
versity, San Diego, CA); Salmonella typhimurium strain SL1344, S. typhimurium SL1344 SpoB⁻ and Shigella flexneri were gifts from Dr. S. Falkow (Stanford University, Stanford, CA).

\textit{Generation of stably transfected THP-1 cell lines}

The pLXR retroviral vector (BD Pharmingen, San Diego, CA) containing a neomycin resistance cassette and a CDNA encoding full-length CLAN (with a C-terminal c-Myc tag) or empty plXRN was transduced along with a vector encoding the stomatitis virus glycoprotein (VSV-G) into the 293-GP packaging cell line using Superfect (Qiagen, Valencia, CA). At 24 h post-transfection, virus-containing supernatants were collected, passed through 0.45-μm pore size, syringe-tip filters, and applied to 5 × 10⁶ THP-1 cells in the presence of 5 μg/ml polybrene in 24-well plates. Cells were sedimented by centrifugation for 45 min at 800 × g. After three rounds of infection, cells were incubated at 32°C for 24 h and provided fresh medium containing 800 μg/ml gentamicin the following day. To ge-
nenerate CLAN-deficient monocytes, oligonucleotides encoding a short RNAi hairpin construct specific for the CLAN coding sequence were subcloned into pSUPER (gift from D. Billadeau, Mayo Clinic, Rochester, MN), an RNAi-expressing plasmid based on pSUPER, containing the RNA Pol III H1 promoter. Annealed oligonucleotides were cloned upstream of the RNA polymerase III promoter (primer 1 sequence, 5’-GATCCCCAGGACAGACATCATGCGCTTCAGAAGAGGCGATGAATGTCTGGTTTTGGAAGGA-3’; primer 2 sequence, 5’-AGCTTTTCTAAAAGGACGCAACATTCAATGGCGTTCACGGTTGGAGGCGATGAATGTCTGGTTTTGGAAGGA-3’). The cassette containing the promoter and CLAN sequence was then sub-
cloned into pLXRN, and the sequence was verified and used to create packaged virus as described above. A THP-1 cell line stably expressing a control RNAi hairpin (scrambled CLAN sequence (SCR)) was created sim-
ilarly. The levels of vector-derived CLAN mRNA expression in infected THP-1 cells were determined by RT-PCR analysis (in the linear range of amplification) using conditions described previously (17). The expression of CLAN protein in infected THP-1 cells was also confirmed by immuno-
blotting using an Ab specific for the c-Myc epitope tag, as previously described (17).

\textbf{Macrophage infections}

S. enteritidis strain LK5 and mouse-virulent S. typhimurium strain SL1344 were grown standing in high salt Luria Bertoni broth overnight until mid-
log growth phase was achieved. S. flexneri was grown overnight in tryptic-
soy broth, then subcultured 1:50 for 2 h before infection. THP-1 cells (3 × 10⁶) were differentiated for 16 h with PMA (50 ng/ml), washed, and ex-
posed to bacteria at a multiplicity of infection (MOI) of 5–50 for 1 h in antibiotic-free RPMI 1640 containing 5% FCS at 37°C. Cells were washed in HBSS, then fresh medium containing gentamicin (100 μg/ml) was added for 1 h to kill extracellular bacteria. After supernatant collection and ex-
tensive washing, cells were incubated for additional time periods in me-
dium containing 10 μg/ml gentamicin, after which they were lysed in 1% Triton X-100 to release intracellular bacteria. Serial dilutions of lysates were spread on Luria Bertoni-agar plates and incubated overnight at 37°C to assess levels of viable intracellular bacteria. Cytotoxicity was evaluated by measuring cytosolic lactate dehydrogenase (LDH) release into the su-
pernatant using a colorimetric assay (Cytotoxicity Detection Kit, Promega, Madison, WI). Secreted IL-1β levels were analyzed using an ELISA (BD Pharmingen). In some experiments, differentiated cells were preincubated for 1 h with 100 μM of the caspase-1 inhibitor z-WEHD-fmk (Alexis, San Diego, CA), 50 μM of the pan-caspase inhibitor z-VAD-fmk (Calbiochem, La Jolla, CA), or vehicle control (DMSO) before bacterial exposure.

\textbf{Determination of phagocytic index}

To determine whether differences in Salmonella uptake accounted for the effect of CLAN on intracellular bacterial levels during gentamicin protec-
tion assays, an enhanced cyan fluorescent protein (ECPF)-expressing S. typhimurium strain was generated using electroporant S. typhimurium and pECPF plasmid (Invitrogen Life Technologies, San Diego, CA). THP-
1/Neo or THP-1/CLAN cells (10⁵) were differentiated on four-well glass 

\textbf{Pathogen-associated molecular pattern (PAMP) exposure}

THP-1 cells (3 × 10⁶) were differentiated for 16 h, washed, and exposed to LPS isolated from Escherichia coli strain 055:B5 (Sigma-Aldrich, St. Louis, MO; used at 1–1000 ng/ml), PGN from Staphylococcus aureus (Fluka, Buchs, Switzerland; used at 0.5–10 μg/ml), lipoteichoic acid (LTA) from S. aureus (Fluka; used at 10 μg/ml), or unmethylated CpG DNA (6410 oligonucleotides; gift from ISIS Pharmaceuticals; used at 10 μM). In some experiments, cells were preincubated with caspase inhibitors before PAMP exposure, as described. After 6 h, supernatants were collected and analyzed for secreted LDH and IL-1β.
Results

Manipulating CLAN expression levels in THP-1 cells

To study the functions of CLAN, we generated a THP-1 cell line overexpressing CLAN and a THP-1 cell line deficient in endogenous CLAN using retroviral gene insertion of the full-length CLAN cDNA (THP-1/CLAN) or a construct encoding an RNAi hairpin specific for CLAN (THP-1/HP), respectively. As controls, two additional cell lines were created: one expressing only the neomycin resistance gene (THP-1/Neo) and one expressing a nonspecific RNAi hairpin construct corresponding to the scrambled CLAN RNAi sequence (THP-1/SCR). Expression levels of CLAN mRNA were assessed by RT-PCR using control cells (THP-1/Neo) as a reference (Fig. 1A). THP-1 cells containing the integrated CLAN expression vector had ~2-fold higher levels of CLAN mRNA than Neo control cells. Conversely, THP-1 cells containing the CLAN RNAi vector expressed ~50% lower levels of CLAN mRNA compared with THP-1/Neo control cells, whereas CLAN expression levels in THP-1/SCR were approximately equivalent to those seen in THP-1/Neo (Fig. 1A). The lack of anti-CLAN Abs precluded assessment of the effects of gene transfer and RNAi on levels of CLAN protein, but the expression of the Myc-tagged CLAN protein in retrovirus-infected THP-1 cells was verified by immunoblotting (not shown).

Endogenous CLAN mRNA levels were also analyzed in wild-type THP-1 cells after exposure to LPS, the monocyte-differentiating agents PMA and CSF-1, and proinflammatory cytokines IFN-γ and TNF-α (Fig. 1B). In comparison with untreated control cells, CLAN expression was up-regulated at 16 h after stimulation with LPS and TNF-α, suggesting a role for endogenous CLAN in the macrophage responses to these inflammatory mediators. By comparison, PMA had relatively little effect on CLAN mRNA levels in THP-1 cells despite inducing these monocytic leukemia cells to undergo differentiation from small round suspension cells to large adherent macrophages. The cytokines IFN-γ and CSF-1 also had little or no effect on CLAN expression in these experiments.

The increase in the expression of CLAN mRNA appears to represent a relatively late response to these proinflammatory factors, because no alterations in expression were observed 4 h after stimulation (data not shown).

CLAN-dependent IL-1β secretion in macrophages after PAMP exposure

To identify potential ligands for CLAN, a number of PAMPs were used to assess the inflammatory response of the genetically manipulated THP-1 cell lines. The concentrations of PAMPs used were within the range typically required for bioactivity, as previously reported (14). In comparison with the control cell line THP-1/Neo, differentiated THP-1/CLAN cells secreted significantly higher amounts of processed IL-1β after exposure to LPS derived from E. coli and to PGN isolated from S. aureus (Fig. 2A). To exclude nonspecific suppression of PAMP response caused by introduction of the hairpin vector, a control cell line expressing a nonspecific (scrambled sequence) RNAi hairpin construct (THP-1/SCR) was also used in these experiments. After exposure to LPS or PGN, IL-1β secretion was not inhibited compared with that from THP-1/Neo control cells. In fact, the levels of IL-1β secreted from this cell line in response to PAMPs were significantly higher than those from THP-1/Neo cells. Addition of exogenous IFN-β to cultures before PAMP exposure also augmented PAMP responses by THP-1 cells (data not shown). Thus, nonspecific induction of IFN production by RNAi hairpin vectors cannot account for the robust suppression of PAMP responses observed in THP-1 cells expressing the CLAN siRNA vector.

Dose-response analysis indicated that CLAN enhances IL-1β secretion after exposure to as little as 1 ng/ml LPS and 1 μg/ml PGN (Fig. 2, B and C). CLAN-overexpressing cells also secreted slightly more IL-1β after LTA exposure compared with control cells. In contrast to LPS, PGN, and LTA, another PAMP not typically associated with the induction of IL-1β secretion in human macrophages, unmethylated CpG DNA (26), did not stimulate the release of this cytokine from any of the cell lines examined.

Although the overexpression of CLAN enhanced THP-1 cell secretion of IL-1β in response to certain PAMPs, CLAN RNAi-expressing THP-1/HP cells exhibited a marked defect in IL-1β generation after exposure to LPS and PGN at all concentrations tested (Fig. 2, A–C). These findings suggest that endogenous CLAN plays a critical role in IL-1β production by THP-1 cells when confronted with bacterial cell wall components.

To explore the mechanism by which overexpression of CLAN leads to enhanced IL-1β secretion, we tested the effects of caspase inhibitory, cell-permeable peptides. In both THP-1/Neo and THP-1/CLAN cells, PAMP-induced IL-1β secretion was found to be dependent on caspase-1 activation, as shown by the ability of an irreversible inhibitor of this protease (z-WEHD-fmk) to abolish cytokine secretion (Fig. 2D). Preincubation with the pan-caspase inhibitor z-VAD-fmk also completely blocked IL-1β release after LPS and PGN exposure (data not shown). As measured by LDH release into the supernatant, no cytotoxicity was observed in treated cells during the course of these experiments (data not shown), suggesting that PAMP exposure selectively activates proinflammatory caspses, but not apoptotic caspses (reviewed in Ref. 27).

CLAN-dependent IL-1β secretion in THP-1 macrophages after bacterial infection

We next investigated whether modifying CLAN expression levels alters IL-1β secretion after bacterial infection. THP-1/Neo, THP-1/CLAN, and THP-1/HP cells were differentiated for 16 h using PMA, then infected with pathogenic Gram-negative bacteria in

FIGURE 1. Altered CLAN levels in stably transfected and stimulated THP-1 cells. A, THP-1 cells were infected with recombinant retroviruses engineered to express full-length CLAN with a C-terminal Myc tag, a RNAi hairpin construct specific for CLAN (HP), or a nonspecific SCR. Polyclonal cell populations were selected with G418, and CLAN mRNA levels were analyzed by RT-PCR using primers that detect both endogenous and retrovirus-induced CLAN mRNA. THP-1 cells stably infected with empty vector and selected in G418 (Neo) served as a control for comparison of CLAN mRNA expression. RT-PCR analysis was performed using 2 μg of RNA, oligo(dT) priming, and primers specific for either CLAN (top) or GAPDH (bottom). PCR performed without template cDNA (neg) excluded a contribution from contaminating DNA. B, Wild-type THP-1 cells were treated for 16 h with LPS (200 ng/ml), PMA (50 ng/ml), IFN-γ (1000 U/ml), TNF-α (50 ng/ml), or CSF-1 (20 ng/ml). RNA was isolated, and 2 μg was analyzed by RT-PCR, using primers specific for either CLAN (top) or GAPDH (bottom).
log-phase growth at an MOI of 5. At 1 and 9 h after bacterial addition, cell supernatants were collected, and mature IL-1β levels were analyzed using ELISA.

After infection with *S. enteritidis* (LK5 strain), macrophages overexpressing CLAN secreted significantly higher amounts of IL-1β compared with control cells (Fig. 3A). In contrast to the enhanced response of THP-1/CLAN cells, THP-1/HP macrophages expressing CLAN RNAi consistently secrcted slightly less IL-1β after *Salmonella* infection, although the results did not reach statistical significance. Similar results were obtained in experiments in which differentiated THP-1 cells were infected with *S. typhimurium* or *S. flexneri*, an invasive pathogen that (unlike *Salmonella*) does not reside in intracellular vacuoles (Fig. 3, B and C). *Salmonella*-induced IL-1β processing and secretion were rapid, occurring within 1 h of infection, most likely due to the efficient intracellular delivery of bacterial components and activation of a post-translational mechanism of cytokine regulation rather than to differences based on altered pre-IL-1β message levels. Elevated IL-1β release from THP-1/CLAN cells was observed at 9 h post-infection as well (Fig. 3, A–C).

Bacteria-induced secretion of IL-1β from THP-1/Neo cells was completely blocked by preincubating THP-1 cells with the caspase-1 inhibitor z-WEHD-fmk or the broad-spectrum caspase inhibitor z-VAD-fmk (Fig. 3D). Preincubation of THP-1/CLAN with z-WEHD-fmk reduced most, but not all, bacteria-induced IL-1β secretion, whereas z-VAD-fmk completely blocked IL-1β secretion for THP-1/CLAN in these experiments. These results suggest that after exposure to live bacteria, both caspase-dependent and -independent mechanisms of pro-IL-1β activation function in CLAN-overexpressing macrophages. Alternatively, these data may simply reflect differences in effectiveness of these pharmacological caspase inhibitors under the experimental conditions used.

**Antibacterial effects of CLAN**

To determine the effects of CLAN on intracellular bacteria levels within macrophages after infection, a gentamicin protection assay was used. THP-1 cells were differentiated for 16 h, then exposed to *Salmonella* species at an MOI of 5. After the killing of extracellular bacteria with gentamicin, cells were incubated for additional periods, then lysed to assess the number of viable intracellular bacteria capable of forming colonies when plated on Luria Bertoni/agar.

Overexpression of CLAN in THP-1 macrophages was associated with significantly reduced levels of surviving intracellular *S. enteritidis* compared with that in THP-1/Neo control cells (Fig. 4A). Similar observations were made using another strain of pathogenic bacteria, *S. typhimurium* (Fig. 4B), and a less virulent *S. enteritidis* strain (data not shown). Although THP-1/HP macrophages expressing CLAN RNAi often contained higher levels of intracellular bacteria in these experiments, the results did not reach statistical significance (not shown).

To further investigate the mechanism of the antibacterial effects of CLAN, THP-1 macrophages were pretreated with caspase inhibitors for 1 h before infection with *S. enteritidis*. Although the preadministration of z-WEHD-fmk or z-VAD-fmk lowered the overall levels of intracellular bacteria, no significant inhibition of the antibacterial effects of CLAN was observed, indicating a caspase-independent process. We also observed that the CFUs recovered from THP-1/Neo and THP-1/CLAN were approximately equivalent immediately after the infection (1 h), indicating that CLAN most likely does not significantly affect bacterial entry into...
these experiments (Figs. 4 and 5). To confirm this observation, THP-1 macrophages were grown on glass slides and infected with ECFP-expressing S. typhimurium for 1 h before extensive washing and microscopic determination of the percentage of macrophages with ingested bacteria. These studies indicated that the levels of initial phagocytosis of Salmonella by THP-1/Neo and THP-1/CLAN cells were approximately equal, thus ruling out altered bacterial uptake as a mechanism for the antibacterial phenotype of CLAN-overexpressing cells.

CLAN potentiates Salmonella-induced cell death

In pilot experiments involving Salmonella infection, we noted that THP-1/CLAN cells were rapidly killed after exposure to very high levels of bacteria (MOI \( \geq 10 \)). Cytotoxicity assays revealed that CLAN overexpression increased macrophage susceptibility to cell death induced by S. enteritidis and S. typhimurium at an MOI of 50, as determined by cytosolic LDH release into culture supernatants (Fig. 6, A and B). Infections of THP-1 cell lines with Salmonella species at an MOI of 10 produced similar results (data not shown). In contrast to live bacteria, heat-killed S. typhimurium applied at MOIs as high as 200 failed to induce any amount of cytotoxicity in THP-1/CLAN macrophages (data not shown).

Preincubation of THP-1 cell lines with z-WEHD-fmk failed to inhibit cell death after infection at a high MOI, suggesting a caspase-1-independent mechanism of cell death. In contrast, preincubation of THP-1/CLAN with the broad-spectrum caspase inhibitor z-VAD-fmk completely abolished cell death induced by S. enteritidis at a high MOI while greatly reducing cell death induced by high MOI S. typhimurium infection (Fig. 6, A and B). Despite the possibility of a caspase-1-independent cell death mechanism, an isogenic mutant of S. typhimurium lacking the caspase-1-activating SipB gene was also used and failed to effectively induce the death of any of the THP-1 cell lines tested (Fig. 6C).

Discussion

In this study we show that overexpression of CLAN in THP-1 macrophages enhances caspase-1-dependent IL-1\( \beta \) secretion induced by exposure to the bacterial cell wall components LPS and PGN, whereas ablation of CLAN expression using RNAi impairs IL-1\( \beta \) production by cells exposed to these PAMPs. Infection of CLAN-overexpressing macrophages with S. enteritidis or S. typhimurium also results in hyperssecretion of IL-1\( \beta \) compared with that by control cells, demonstrating the ability of CLAN to modulate cytokine responses to live invading bacteria. When exposed at a modest MOI, CLAN overexpression provides a bacteriocidal or bacteriostatic effect, reducing the accumulation of live bacteria in infected macrophages. In contrast, when the intracellular bacterial burden is high, CLAN promotes macrophage cell death. Taken together, these results indicate that CLAN contributes to several biological processes that are central to host defense: modulating the relative sensitivity of macrophages to LPS and PGN, and impacting host-pathogen interactions.

During revision of this manuscript, Mariathasan et al. (28) demonstrated that macrophages from CLAN-deficient mice are defective in IL-1\( \beta \) secretion after infection by S. typhimurium. Thus, our data, using human cells and RNAi techniques, corroborate these findings from mice, establishing evolutionary conservation of mechanism. However, in contrast to results obtained using murine cells, where macrophages isolated from CLAN-deficient mice were found to secrete similar levels of IL-1\( \beta \) in response to purified PAMP molecules such as LPS and PGN (28), we found that genetic manipulation of CLAN expression in human cells did alter IL-1\( \beta \) production in response to individual PAMPs. This discrepancy may be due to the interspecies variation and warrants further investigation. Nevertheless, our findings suggest that endogenous CLAN plays a critical role in IL-1\( \beta \) production by human THP-1 cells when confronted with bacterial cell wall components.
Cytotoxicity levels were assessed by LDH release (murium SipB-deficient mutant (both at an MOI of 50). The activation of caspase-1 and its subsequent processing of proinflammatory cytokines represent critical events in the inflammatory process (13, 29) and may be associated with the pathogenesis of a variety of inflammatory diseases (30). In mammals, multiple proteins that control caspase-1 activation have been identified (17, 31, 32). Among the caspase-1-activating proteins are intracellular proteins that respond to bacterial products, suggesting a role in innate immunity. CLAN, a macrophage-expressed protein with domain architecture similar to that of Nod1 and Nod2, is known to bind and activate caspase-1 in overexpression systems, but until now its functions have not been put into a physiological context.

The expression of endogenous CLAN is up-regulated in macrophages following exposure to the proinflammatory cytokine TNF-α, similar to observations made previously with regard to Nod2 expression in intestinal epithelial cells (33). Additionally, expression levels of CLAN were found to be up-regulated by the bacterial cell wall component LPS. The ability of TNF-α and LPS to increase CLAN expression indicates that it may be acutely increased in macrophages in preparation for or in response to bacterial challenge.

To explore the functions of CLAN in macrophages, we used gene transfer and RNAi-mediated gene ablation methods to examine the effects of manipulating CLAN expression in THP-1 macrophages. Our data indicate that the levels of CLAN determine the magnitude of THP-1 cell responses to several bacterial cell wall components (LPS, PGN, and LTA) with respect to release of IL-1β. The overexpression of CLAN also markedly enhanced the secretion of this cytokine from THP-1 macrophages after infection by live bacteria. Furthermore, these effects were suppressed by the caspase inhibitor z-WEHD-fmk, implying a caspase-1-dependent mechanism. Reducing CLAN expression by RNAi significantly diminished IL-1β production in response to bacterial cell wall components, but did not abolish the cellular response to live bacteria. We interpret these results to mean that either residual CLAN expression in RNAi-expressing cells was sufficient for retention of IL-1β production, or redundancy exists in the intracellular molecules that are capable of sensing the presence of bacteria and triggering caspase-1 activation and IL-1β production.

The observation that CLAN-overexpressing macrophages are hyper-responsive to LPS, PGN, and LTA suggests either that the LRR domain of CLAN recognizes structural elements common to all these molecules, or this NACHT family protein operates downstream of intracellular signaling pathways activated by all three PAMPs. Despite the large size of the NACHT protein family (with 20 members identified in the human genome) (34), to date only bacterial ligands have been identified for NACHT family members Nod1 and Nod2. The ability of CLAN to enhance IL-1β release in response to LPS, PGN, and LTA is significant in that these microbial components are believed to synergize in generating the inflammatory responses associated with septic toxic shock (1, 35). Thus, NACHT family members such as CLAN should be examined with respect to their potential to serve as targets for drug discovery, in particular, taking advantage of the putative nucleotide-binding NACHT domains, which are thought to mediate activation of these proteins via NACHT-NACHT oligomerization (15, 36, 37).

By monitoring levels of viable Salmonella in THP-1 macrophages after infection with these invasive bacteria, a role was discovered for CLAN in suppressing their intracellular growth or survival. Gentamicin protection assays using three different Salmonella species conclusively demonstrated that THP-1 cells

![Image](http://www.jimmunol.org/DownloadedFromhttp://www.jimmunol.org/)
overexpressing CLAN display enhanced antibacterial properties compared with control cells. No difference in *Salmonella* invasion was observed between THP-1/CLAN and THP-1/Neo cells. These results show that CLAN impacts processes that contribute to bacterial eradication or intracellular bacterial replication and are similar to the effects of Nod2 overexpression on *S. typhimurium* survival, as previously reported for intestinal epithelial cells (38). It remains to be determined whether various NACHT family proteins are responsible for recognizing individual pathogens and triggering subsequent antimicrobial activities or if they possess overlapping specificities. In an attempt to explain the inhibitory effects of CLAN on intracellular bacterial survival or proliferation, the levels of several endogenous antibacterial factors were analyzed in the THP-1 model. Specifically, the levels of human β-defensin-1 and -2 (natural antibiotic peptides previously shown to be regulated by LPS in macrophages) (39) were assessed in THP-1/Neo, THP-1/CLAN, and THP-1/HP cells by RT-PCR analysis. In untreated conditions and after exposure to *Salmonella* or LPS for various time periods, the expression levels of these genes were unchanged (not shown), thus excluding them as a possible explanation for the antimicrobial effects of CLAN.

Invasive pathogenic bacteria can find shelter from the host immune system by invading and replicating in macrophages, where they are protected from effectors of the humoral and innate immune defense systems. Bacteria such as *Salmonella* produce several virulence proteins associated with type III secretion systems that enable these microorganisms to subvert host antibacterial processes in the cytosol of phagocytes (40, 41). Another hypothesized benefit of residing within the macrophage is that these host cells carry organisms through the lymph and blood to other tissues, thus facilitating their in vivo dissemination (42, 43). Our observation that the overexpression of CLAN predisposes macrophages to cell death upon exposure to large bacterial loads suggests that this protein may serve to counter the “hitch-hiking” effect exploited by intracellular pathogens, similar to the hypersensitive response of plants (9). Conversely, it has been hypothesized that microbes such as *Salmonella* may trigger the apoptosis of host macrophages to induce tissue damage and facilitate pathogen spreading within the lymphatic system (44, 45). The fact that cell death, in addition to inflammation, is believed to be another dominant feature of septic shock lends further credence to the idea of targeting CLAN as part of a novel anti-inflammatory treatment (46).

The cytotoxicity experiments presented in this study demonstrate that *Salmonella*-induced cell death in CLAN-overexpressing human macrophages is rapid, with the majority of death occurring within the first hour of infection. The failure of CLAN RNAi expression in THP-1 cells to repress cell death induced by *Salmonella* raises the possibility that residual amounts of CLAN expression in siRNA-expressing cells may be sufficient to detect intracellular bacteria and induce cell death. Definitive evidence supporting a role for CLAN in inducing macrophage cell death was obtained recently by others using CLAN-deficient murine macrophages, showing failure to undergo cell death after *Salmonella* infection (28). Interestingly, the enhanced bacteria-induced cytotoxicity observed in CLAN-overexpressing THP-1 cells was refractory to suppression by the caspase-1 inhibitor, z-WEHD-fmk, implying a caspase-1-independent mechanism. Nevertheless, *Salmonella*-mediated killing of CLAN-overexpressing THP-1 macrophages is caspase-dependent, as demonstrated by its complete negation by the pan-caspase inhibitor z-VAD-fmk. In this regard, it may be relevant that CLAN has previously been reported to promote apoptosis by binding the bipartite adapter protein ASC (apoptosis-associated speck-like protein containing a CARD) and enhancing activation of procaspase-8 (47). Thus, CLAN may be capable of indirectly activating other members of the caspase family, besides caspase-1, accounting for our experimental observations. Interestingly, a role for caspase-2 in *S. typhimurium*-induced cell death has been suggested, with activation of this protease occurring in conjunction with caspases-3, -6, and -8 in murine bone marrow-derived macrophages (48). Given that exposure to high concentrations of LPS or heat-killed *Salmonella* did not induce cell death in CLAN-overexpressing macrophages, it seems that highly efficient intracellular presentation of PAMPs and/or a heat-labile factor are needed for cytotoxicity. The failure of a SipB-defective *Salmonella* strain to induce macrophage cell death may also indicate that a functional SipB-related virulence protein is required as a cofactor for *Salmonella*-induced killing of CLAN-overexpressing macrophages.

The influence of CLAN on multiple arms of the innate immune system indicates that this NACHT family protein may have diverse effects on invading pathogens at both the cellular level (through its antibacterial and host cell death-inducing effects) as well as at the level of the whole organism through proinflammatory cytokine secretion (summarized in Fig. 7). More detailed structural studies may elucidate the specific components of LPS and PGN to which CLAN-overexpressing cells respond, analogous to recent studies involving Nod1 and Nod2 in which the minimal elements of bacterial PGN were dissected (21, 24). The further characterization of genetically engineered mice lacking CLAN will help to conclusively define the role of CLAN in host defense against invading bacteria as well as in sepsis and inflammatory disease models.

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**References**


