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Cutting Edge: ASC Mediates the Induction of Multiple Cytokines by Porphyromonas gingivalis via Caspase-1-Dependent and -Independent Pathways

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Porphyromonas gingivalis (Pg) is a major etiologic agent for chronic periodontitis. Tissue destruction by Pg results partly from induction of host inflammatory responses through TLR2 signaling. This work examines the role of apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), an adaptor molecule important for TLR-mediated caspase-1 activation. Results demonstrate that ASC levels are stable upon infection of human THP1 monocytic cells with Pg but decrease after cytokine induction. Using short hairpin RNA, we demonstrate an essential role for ASC in induction of IL-1β by TLR2, 4, and 5 agonists, live Escherichia coli, and Pg. Induction of IL-6, IL-8, IL-10, and TNF also requires ASC, but this induction is not inhibited by IL-1 receptor antagonist or caspase-1 inhibitor. Similar results in U937 indicate broad applicability of these findings. Pg-infected ASC knockdown THP1 cells exhibit reduced transcript levels and NF-κB activation. These results suggest a role for ASC in cytokine induction by Pg involving both caspase-1-dependent and -independent mechanisms. The Journal of Immunology, 2006, 177: 4252–4256.

The Gram-negative anaerobe Porphyromonas gingivalis (Pg) is a major etiologic agent in periodontal disease, a chronic inflammatory disorder that leads to slow but steady destruction of the supporting structures of the teeth and ultimately to tooth loss. Pg surface structures, including LPS, induce host inflammatory responses that result in local tissue destruction (1). LPS from Pg differs from Escherichia coli LPS structurally and signals primarily through TLR2 instead of TLR4 (2, 3). However, both are strong inducers of IL-1β. Since IL-1β and other inflammatory cytokines cause periodontal tissue destruction, understanding how live Pg interacts with host cells and induces cytokine release will help clarify disease processes and identify treatment strategies.

Generation of mature IL-1β requires proteolytic processing of pro-IL-1β by the IL-1β-converting enzyme, caspase-1 (4). Caspase-1 is a component of the inflammasome, a cytosolic multiprotein complex that also contains apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC; also known as TMS1 or PYCARD) (5–7). ASC is a 21.5-kDa protein that contains a pyrin domain and caspase-recruitment domain; shRNA, short hairpin RNA.

A number of recent studies to determine the role of ASC in NF-κB-mediated induction of inflammatory cytokines have yielded conflicting results. Exogenously expressed ASC can activate, repress, or have no effect on NF-κB depending on dose and coexpression of CATERPILLER NBD-LRR proteins (9–15). Since these studies were performed using an overexpression system, the results may not reflect the normal physiological function of ASC. Studies using macrophages from ASC−/− mice suggest a role for ASC in caspase-1 activation but not in IκB degradation in response to TNF or LPS (16, 17). In contrast, studies using small interfering RNA and the human monocytic cell line THP1 indicate that ASC represses NF-κB (10). These same authors pointed out the difficulty in using this system due to low efficacy of their small interfering RNA (<50% knockdown) (11).

To address the role of human ASC in Pg response in a physiological setting, we have created stable THP1 cells expressing different short hairpin RNA (shRNA) for ASC. Studies using live Pg demonstrate that ASC is required for the induction of IL-1β, but also for the induction of other cytokines as a consequence of reduced NF-κB activation.

Materials and Methods

Cell culture

THP1 and U937 monocytic cells (American Type Culture Collection) were cultured in RPMI 1640 and 10% FCS. Where indicated, cells were treated with 50 multiplicity of infection Pg, 0.5 multiplicity of infection E. coli, 20 ng/ml rIL-1β (PeproTech), 200 ng/ml Pam3Cys-Ser-Lys (InvivoGen), 1 μg/ml Ultrapure Pg or E. coli 0111:B4 LPS, or 100 ng/ml flagellin (InvivoGen), BD ApoAlert VAD-fmk (pan-caspase inhibitor) or YYAD-cmk (caspase-1 inhibitor) (BD Clontech), DMSO (solvent control), or the IL-1 receptor antagonist Anakinra (Kineret; Amgen) were added 1 h before

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3 Abbreviations used in this paper: Pg, Porphyromonas gingivalis; ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; shRNA, short hairpin RNA.
Bacterial culture

P. g. strain AT436 was cultured anaerobically (3) and E. coli strain DH-5α aerobically until late exponential phase (OD 0.8–1.2 at 660 nm). Aliquots were frozen in medium containing 20% glycerol at −80°C.

Real-time PCR

Real-time PCR was performed as previously described (18) using the following primers, listed as (forward, reverse): ASC (AACCCCAAGCGATCGGGAAG, TTAGGGCTGGAGGAGCGAAG); IL-1β (ACATGGCGAATGAGGATGC, CCATGCGCAACAAAGCCTGA); IL-6 (TGCGTCTCCTTTGGTCCTCTCAG, GGTACATCGTGAGGAGCGAAG); IL-8 (TTCGTGTGTTGGGCGGCACTG); TNF (TCTGTTGGCTGCTGACACT, GGTACAGGCTGCTACT); and 18s (CCGGTACCAACATCGAGG, GCTGTGGCCACCGACTT). Values represent averages + SD of triplicates for RNA isolated on different days standardized to 18s RNA expression.

Western blot analyses

Immunoblots were performed using Abs APO-25SN-014-R100 (Immunodiagnostic) for ASC, sc-109 for total p65 (Santa Cruz Biotechnology), and mAb 374 (Chemicon International) for GAPDH nos. 2022, 9242, and 30315 (Cell Signaling Technology) for IL-1β, IκB, and phospho-p65 (Ser536). All results are representative of three independent experiments.

Preparation of shRNA plasmids and cell lines for ASC knockdown

Plasmids for shRNA expression were made by inserting a histone H1 promoter, shRNA, and termination sequence into a GFP-containing pHSPG retroviral shuttle vector. Detailed methods for shRNA production have been described previously (18). The shRNA target sequences are as follows: shASC#1-GCTCTTCATTGTTTCACACCA; mutshASC#1-GCTCTTCCTTCCTGA; shASC#2-GCTGGAACTTGAGTCGACCA; and shASC#2-GCTGGAACTTGAGTCGACCA. ShRNA transduction was described previously (19). IFN response was not activated by shRNA as assessed by OAS1 expression.

ELISA

Supernatants were assayed 18–24 h, following stimulation using human ELISA kits (BD Biosciences). Samples were assayed within linear range. IGF-1 was assayed using the human IGF-1 Quantikine ELISA kit (R&D Systems). All values represent averages + SD of triplicates from different days of stimulation.

EMSA and supershift analysis

EMSA was performed as described previously (20). Competition assays were done using 20X oligonucleotide corresponding to the consensus for NF-κB (19) or Oct-1 (TTCGTATGGGATGCTGACCA) (IDT). For supershift analysis, samples were subsequently incubated with preimmune sera (Vector Laboratories) or Abs specific to RelB (C-19), NF-κB p50 (N-19), c-Rel (N-460), NF-κB p52 (447), or p65 (A) (Santa Cruz Biotechnology).

Results and Discussion

ASC and cytokine expression in THP1 cells is modulated by Pg infection

RNA was isolated from THP1 cells following a time course of infection with Pg. Pg induces expression of IL-1β and IL-8 peaking at 2 h postinfection and IL-6 peaking at 6–24 h (Fig. 1A, first three panels). Expression of ASC mRNA was initially stable but dropped dramatically by 6 h postinfection, while ASC protein was easily detectable up to 18 h postinfection (Fig. 1, A, bottom panel, and B). This finding indicates that ASC expression in Pg-infected cells is sustained until cytokines are induced. Later reduction in ASC could serve as a mechanism for shutting down inflammation, thus avoiding overwhelming immune response. These observations are in contrast to increased ASC expression reported by another group using E. coli LPS as a stimulant (11). Differences could be due to the use of different infectious agents as Pg causes a chronic disorder, but also may be due to their use of TPA to differentiate the cells before stimulation. Nevertheless, the dynamic range of ASC expression is consistent with a role of ASC in the induction of inflammatory cytokines.

IL-1β induction by live Pg is reduced in ASC knockdown cells

To determine the function of human ASC, we prepared two knockdown cell lines expressing shRNA that target ASC at different sites (shASC#1 and shASC#2) and compared expression against THP1 cells and control lines expressing empty vector, shRNA against mouse plexin (shmPlex) (19), or a 5-bp mutant version of shASC#1 (mutshASC#1). shASC#1 represents a near complete knockdown of ASC expression, and shASC#2 represents >70% knockdown at both the RNA and protein level (Fig. 2, A and B).

ASC is required for the activation of IL-1β in mouse macrophages in response to TLR agonists and at least three pathogens known to signal through TLR (12, 21, 22). To determine
whether ASC is required for IL-1β induction by live Pg. Western blot analysis was performed. Pro-IL-1β was detected 4 h after Pg infection, and cleaved IL-1β was apparent by 7 h in control THP1 and mutshASC#1 cell lines (Fig. 2C, first six lanes). Levels of pro-IL-1β were reduced in shASC#1, and cleaved IL-1β was undetectable (lanes 7–9). These findings are consistent with other studies indicating that ASC can have differing effects depending on dose (11, 12).

Reduced IL-6, IL-8, IL-10, and TNF in ASC knockdown cells in response to TLR agonists and Pg
Previous studies in mice demonstrate a role for ASC in the induction of IL-1β, but not TNF-α, by Francisella tularensis, L. monocytogenes, and a panel of TLR agonists (17, 21, 22). To test if ASC is required for the induction of additional inflammatory cytokines and chemokines in human cells, supernatants from Pg-infected THP1 cells were assessed by ELISA. Our results demonstrated that in addition to IL-1β, high-level induction of IL-6, IL-8, IL-10, and TNF by Pg required ASC, while the expression of IGF-1 was ASC independent (Fig. 3A). The role for ASC in cytokine induction is broad, as we observed reduced cytokine levels in ASC shRNA cells induced by live E. coli, the synthetic TLR2 agonist Pam3Cys; purified Pg LPS, which signals through TLR2, and to a lesser extent TLR4 and TLR1 (2, 3); the TLR4 agonist E. coli LPS; and the TLR5 agonist flagellin (Fig. 3B). ASC is required at the level of transcription and over a time course of Pg induction (Fig. 3C). Moreover, similar findings with shRNA in another human monocytic cell line, U937, indicate broad applicability (Fig. 3D). ASC is thought to regulate IL-1β expression by regulating caspase-1. However, IL-6, IL-8, IL-10, and TNF are not known to be regulated by this pathway. These results suggest that ASC exhibits caspase-1-independent functions.

Reduced IL-6 and IL-8 induction in ASC knockdown cells is not explained by reduced autocrine activation by IL-1β or caspase-1 activity
IL-1β can induce cytokine expression by autocrine stimulation (23). To test the possibility that ASC-dependent cytokine production is due to autocrine stimulation by IL-1β, cells were exposed to 20 ng/ml rIL-1β, a level 10–50 times higher than secreted in Pg-infected THP1 cells. Even at this exaggerated level, IL-1β stimulated <3000 pg/ml IL-8 (Fig. 4A, first two bars). This is <1/200 the amount of IL-8 that was stimulated by Pg infection (compare Fig. 4A vs Figs. 3, A and B, and 4B). This finding suggests that levels of secondary induction of IL-8 by IL-1β in THP1 cells are not appreciable.

To examine the autocrine role of IL-1β, cells were pretreated with the IL-1 receptor antagonist Anakinra (24). As expected, Anakinra completely blocked induction of IL-8 by rIL-1β (Fig. 4C).
controls to show that ASC plays a crucial role in IL-1
THP1 and U937 cells. ELISA results are shown for IL-1β, IL-6, and IL-8 for THP1 cells following stimulation with Pg.
Pan-caspase inhibitor VAD-fmk, caspase-1 inhibitor YVAD-cmk, or equal volume of DMSO solvent was added 1 h before infection at the concentrations indicated.

4A, bars 3 and 4). Anakinra did not affect IL-8 or IL-6 levels in response to Pg (Fig. 4, B and C), indicating that the reduced induction of IL-6 and IL-8 in ASC knockdown cells cannot be explained by an IL-1β feedback mechanism.

To corroborate these findings, THP1 cells were pretreated with the caspase inhibitors VAD-fmk or YVAD-cmk, which would be expected to block IL-1β maturation (Fig. 5). The induction of IL-1β was reduced by both inhibitors, whereas DMSO had no effect (top panel). Induction of IL-6 and IL-8 by Pg was not affected by either inhibitor. Thus, effects on IL-6 and IL-8 induction are caspase-independent, and autocrine activation by IL-1β does not play a significant role in the induction of IL-6 and IL-8 by Pg.

ASC knockdown correlates with reduced NF-κB activation
NF-κB is activated in response to many infectious agents and is known to contribute to periodontitis (25). EMSA was used to determine whether Pg activates NF-κB in THP1 cells. A shifted band was observed in extracts from Pg-infected cells (Fig. 6A, lane 2). This band was supershifted by Abs to both the p50 and p65 forms of NF-κB but not an isotype control Ab (lanes 3–5). Complexes were competed by unlabeled oligonucleotides for NF-κB but not Oct-1 (lanes 6 and 7).

To compare levels of NF-κB activation in control and ASC knockdown cells, EMSA was repeated over a time course of Pg infection (Fig. 6B). NF-κB was activated in both mutshASC#1 and shASC#1 cells. However, NF-κB binding activity was reproducibly less in shASC#1 cells at all time points. Consistently, shASC cells displayed delayed IκB degradation and p65 phosphorylation in response to Pg (Fig. 6C). Reduced release of NF-κB from IκB in ASC knockdown cells could explain the lower levels of secreted cytokines in Pg-stimulated shASC THP1 and U937 cells.

In summary, we used two shRNA and multiple specificity controls to show that ASC plays a crucial role in IL-1β induction by Pg infection and a host of other microbial products. Most importantly, we find that ASC enhances induction of other cytokines via a caspase-1, IL-1β-independent pathway(s). The latter finding reveals new functions for ASC in human cells that were not revealed in previous mouse studies. Reduced cytokine expression is correlated with diminished NF-κB activity; however, other mechanisms remain to be revealed. Effects on MAPK or other signaling pathways also could potentially contribute to the reduction in cytokine levels, and these possibilities are currently under investigation.

Disclosures
The authors have no financial conflict of interest.

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