Mechanism and repertoire of ASC-mediated gene expression

Mechanism and Repertoire of Apoptosis-Associated Speck-Like Protein Containing a Caspase Recruitment Domain-Mediated Gene Expression[1]

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Abstract

Apoptosis associated speck-like protein containing a caspase recruitment domain (ASC) is an adaptor molecule that mediates inflammatory and apoptotic signals. Although the role of ASC in caspase-1-mediated IL-1β and IL-18 maturation is well known, ASC also induces NF-κB activation and cytokine gene expression in human cells. Here we investigated the molecular mechanism and repertoire of ASC-induced gene expression in human cells. We found that the specific activation of ASC induced AP-1 activity, which was required for optimal *IL8* promoter activity. ASC activation also induced STAT3- but not STAT1-, interferon-stimulated gene factor 3-, or NFAT-dependent reporter gene expression. The ASC-mediated AP-1 activation was NF-κB-independent and primarily cell-autonomous response, while the STAT3 activation required NF-κB activation and was mediated by a factor that can act in a paracrine manner. ASC-mediated AP-1 activation was inhibited by chemical or protein inhibitors for caspase-8, caspase-8-targeting small interfering RNA (siRNA), and p38- and JNK-inhibitors, but not by a caspase-1 inhibitor, caspase-9- or FADD -dominant-negative mutants, FADD or RICK-targeting siRNAs, or a MEK inhibitor, indicating that the ASC-induced AP-1 activation is mediated by caspase-8, p38, and JNK, but does not require caspase-1, caspase-9, FADD, RICK, or ERK. DNA microarray analyses identified 75 genes that were induced by ASC activation. A large proportion of them were related to transcription (23%), inflammation (21%), or cell death (16%), indicating that ASC is a potent inducer of inflammatory and cell death-related genes. This is the first report of ASC-mediated AP-1 activation and the repertoire of genes induced downstream of ASC activation.

Introduction

A caspase recruitment domain (CARD[4])-containing protein, apoptosis associated speck-like protein containing a CARD (ASC, also called TMS-1), was originally identified as a protein that forms large aggregates (called specks) in HL-60 human leukemia cells treated with chemotherapeutic agents (1), and as the product of a gene that is silenced in cancer cells by DNA methylation (2). Thus, ASC has been implicated in apoptosis and tumor suppression. ASC consists of an N-terminal pyrin domain and a C-terminal CARD. The pyrin domain and CARD belong to the death domain-fold domains, which engage in homophilic protein-protein interactions. In this context, ASC resembles FADD, an adaptor protein that recruits caspase-8 to death receptors. In fact, ASC has been found to recruit caspase-1 to several members of the nucleotide-binding domain and leucine-rich repeats (LRRs)-containing proteins (NLRs), including cryopyrin (also called NLRP3), and to induce caspase-1-mediated IL-1β maturation (3-6). Because these NLRs function as cytoplasmic sensors for invading bacteria and viruses in cells, and because macrophages from ASC-deficient mice are defective in the production of IL-1β upon microbial infection, ASC is considered to be an important molecular component of the innate immune system (7-10).

In terms of the pro-inflammatory activity of ASC, it has been demonstrated that ASC by itself or in combination with NLRs including cryopyrin and CARD12 (also called NLRC4, IPAF, CLR2.1, and CLAN) induces NF-κB activation when expressed in HEK293 cells by genetic reconstitution (3, 11, 12). Consistent with this, the knockdown of ASC expression by RNA interference in human monocytic/macrophagic cell lines results in reduced NF-κB activation as well as diminished IL-8 and TNF-α production upon *Porphyromonas gingivalis* infection (13), indicating that ASC plays an essential role in the transcriptional activation of inflammatory cytokine genes in human

macrophages, although studies involving ASC-deficient mice have indicated that ASC is not essential for the expression of cytokine genes and NF-κB activation induced by microbial infection in murine macrophages (7, 14, 15).

To investigate the molecular mechanism of ASC-mediated NF-κB activation, we previously developed a chimeric protein (C12N2) consisting of the CARD from CARD12 and the nucleotide-binding oligomerization domain (NOD) and LRRs from NOD2 (also called NLRC2), a sensor for muramyl dipeptide (MDP) (12). The C12N2 and ASC-expressing HEK293-derived cell line (MAIL8) produces IL-8 upon MDP stimulation in a manner dependent on NF-κB activation. Using this cell line, we demonstrated that caspase-8 is required for ASC-mediated NF-κB activation and IL-8 production (12).

In this study, to clarify the mechanism of ASC-mediated transcriptional regulation of cytokine genes, we explored the ASC-mediated activation of the *IL8* promoter and transcription factors. In addition, we performed microarray analyses to obtain an overview of the genes that are up- or down-regulated upon ASC activation.

Materials and Methods

Reagents

MDP was purchased from Sigma (Saint Louis, MO), recombinant TNF-α from Genzyme (Cambridge, MA). Z-VAD-fluoromethylketone (fmk), z-AAD-chloromethyl-ketone (cmk) and the MAPK inhibitor set (PD98059, SB202190, SB203580, and SB202474) from Calbiochem (La Jolla, CA), and the JNK inhibitor SP600125 from Alexis Biochemicals (San Diego, CA).

Cell lines

The KBG cell line is a HEK293-derived stable transfectant of an NF-κB-GFP reporter construct. The MAIL8 cell line is a KBG-derived stable transfectant expressing ASC and the FLAG-tagged C12N2 chimeric protein (12). In this study, we used an apoptosis resistant subline (MAIL8-102).

Plasmids

Luciferase reporter plasmids carrying the *IL8* promoter (-133-Luc) and its mutants were described previously (16, 17). Reporter plasmids carrying TPA-responsive elements (TREs) (2xTRE-Luc), acute phase response elements (APREs) (5xAPREsp-Luc), and NFAT-binding sites (pNFAT72-Luc) were kindly provided by Dr. Katsuji Yoshioka (Kanazawa University Cancer Research Institute, Kanazawa, Japan), Dr. Kouichi Nakajima (Osaka City University Medical School, Osaka, Japan), and Dr. Shoichiro Miyatake (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), respectively. Reporter plasmids carrying NF-κB-binding sites (pNF-κB-Luc) were purchased from Stratagene (La Jolla, CA), and those carrying interferon-stimulated response elements (ISREs) (pISRE-TA-Luc) and gamma-activated sequences (GAS)

(pGAS-TA-Luc) were purchased from Clontech (Palo Alto, CA). Expression plasmids for a short isoform of caspase-like apoptosis regulatory protein (CLARP-S), a constitutive active mutant of CARD12 (CARD12ΔLRR, amino acids 1-457), dominant-negative mutants of FADD (amino acids 80-208), caspase-8B (C377S), caspase-9 (C287S), and c-Jun (amino acids 123-334), and a proteasome-resistant IκBα mutant (S32A, S36A) were described previously (12, 17, 18).

Small interfering RNAs (siRNAs)

The FADD-, caspase-8-, and RICK-targeting siRNAs were described previously (17). ASC-targeting siRNA and control siRNA were purchased from Dharmacon (Chicago, IL; si-GENOM D-004378-03 and D-001210-05, respectively) or Invitrogen (Carlsbad, CA; Stealth Select RNAi HSS147064 and Stealth RNAi Negative Control Low GC Duplex, respectively). MAIL8 cells were transfected with double stranded siRNA using Lipofectamine 2000 or RNAiMAX (Invitrogen) according to the manufacturer's protocol. In some experiments, cells were transfected with reporter plasmids using linear polyethyleneimine 12 h later. Forty-eight hours after the initial transfection, the cells were used for assays unless otherwise specified.

Reporter assay

Reporter assays were performed as described previously (12). In brief, cells were transfected with one of the firefly luciferase reporter plasmids and a control plasmid (pRL-TK) constitutively expressing *Renilla* luciferase, using linear polyethyleneimine. The firefly and *Renilla* luciferase activities were measured 24 hr after the transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The fold induction of luciferase activity = experimental relative luciferase activity / relative

luciferase activity of unstimulated cells or vector control; where relative luciferase activity = firefly luciferase activity / *Renilla* luciferase activity.

Western blotting

Western blotting was carried out as described previously (17, 18).

Measurement of IL-8

The amount of IL-8 in the culture supernatant was determined using the BD OptEIA Set (PharMingen, San Diego, CA).

Measurement of caspase-8 activity

Caspase-8 activity in cell lysates was determined using caspase-8 fluorometric assay kit (MBL, Nagoya, Japan) according to the manufacture protocol.

Microarray analysis

Microarray analyses were performed according to the Agilent 60-mer Oligo Microarray Processing Protocol (Agilent Technologies, Santa Clara, CA). Total RNA was purified from MAIL8 or KBG cells cultured with or without 10 ng/ml MDP or TNF-α for 16 h. The quality of RNA samples was assessed with the RNA 6000 Nano LabChip Kit using Bioanalyzer 2100 (Agilent). Cy3 (for unstimulated samples)- and Cy5 (for stimulated samples)-labeled cRNAs were prepared using the Low RNA Input Linear Amplification Kit (Agilent). Various mixtures of Cy3- and Cy5-labeled cRNAs were purified using the RNeasy RNA purification Kit (Qiagen), and then applied onto the Human 1A (v2) 22 k Oligo Microarray (Agilent) loaded with 20,173 DNA 60-mers, including those from 18,716 human genes. Hybridized and washed arrays were scanned using a

microarray scanner G2565BA (Agilent). Microarray images were analyzed with Feature Extraction Software v7.5 (Agilent). The significance of the log ratio was assessed by computing the most conservative log ratio error and significance value (*P* value), using a standard error propagation algorithm (Agilent) and a universal error model (Rosetta Biosoftware, Seattle, WA). Single linkage hierarchical clustering with the Euclidean distance metric was performed using MultiExperiment Viewer v4.0 (19).

RT-PCR

DNA-free RNA was purified from cells using the RNeasy Mini Kit and RNase-free DNase Set (Qiagen, Hilden, Germany), and cDNA was prepared using an oligo dT primer and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The following primers were used for PCR: TNF forward, 5'-aaggacaccatgagcactga-3'; reverse, 5'-cgtttgggaaggttggatgtt-3'; IL6 forward, 5'-ctccaggagcccagctatga-3'; reverse, 5'-cccatgctacatttgccgaa-3'; IL8 forward, 5'-cagttttgccaaggagtgctaa-3'; reverse, 5'-aacttctccacaaccctctgc-3'; CXCL2 forward, 5'-aaccgcctgctgagccccat-3'; reverse, 5'-ccttcaggaacagccaccaa-3'; ACTB forward, 5'-tccctggagaagagctacga-3'; reverse, 5'-aaagccatgccaatctcatc-3'; FOS forward, 5'-caccgacctgcctgcaagat-3'; reverse, 5'-cagctcgggcagtggcactt-3'; JUNB forward, 5'-ccacctcccgtttacaccaa-3'; reverse, 5'-gccttgagcgtcttcacctt-3'; JUN forward, 5'-cgaactgcacagccagaaca-3'; reverse, 5'-tcctgggactccatgtcgat-3'; RELB forward, 5'-agctacggcgtggacaagaa-3'; reverse, 5'-atcgcggggctacgtggctt-3'; NFKBIA forward, 5'-ccgagactttcgaggaaata-3'; reverse, 5'-tcagccccttgcactcataa-3'; NFKBIZ forward, 5'-gcacaggtgaacaccacaga-3'; reverse, 5'-gagctaatacggtggagctct-3'.

Results

ASC induces AP-1 activation, leading to IL8 promoter activation.

We previously established HEK293 cell-derived stable cell line (MAIL8) that expresses ASC and C12N2, and found that MDP stimulation induces the C12N2-mediated oligomerization of ASC and ASC-mediated NF-κB activation, leading to IL-8 production in this cell line (12). In this response, ASC activation and NF-κB activation occur primarily in a same cell, so that the NF-κB activation does not depend on externalization or secretion of a certain factor that can act in a paracrine manner. In this meaning, the ASC-mediated NF-κB activation is a "cell-autonomous" response. In the current study, we first sought to clarify further the molecular mechanisms of ASC-mediated *IL8* gene expression. Although some sublines of MAIL8 are killed by apoptosis upon MDP stimulation (20), many MAIL8 sublines as well as the parental HEK293 cell line are resistant to ASC-mediated apoptosis. To avoid the influence of cell death on ASC-mediated gene expression, we used an apoptosis-resistant subline (MAIL8-102) throughout this study.

The *IL8* promoter region contains three important *cis*-acting elements for *IL8* gene transcription, namely the NF-κB-, AP-1-, and NF-IL-6-binding sites (16), and *IL8* transcription requires either the combination of the NF-κB- and NF-IL-6- or of the NF-κB- and AP-1-binding sites, depending on the type of cell or stimulation (21, 22). To determine which elements contribute to the ASC-mediated *IL8* promoter activation, we used a luciferase reporter gene construct under the control of the minimal essential promoter region of the *IL8* gene (–133 to +44 bp), which contains all three elements, and a series of constructs with a mutation in each of the elements. Consistent with our above-described previous findings, ASC activation (induced by MDP stimulation) resulted in potent transcriptional activity of the wild-type *IL8* promoter in MAIL8 cells,

and a mutation in the NF-κB-binding site diminished the promoter activity. In addition, a mutation in the AP-1- but not the NF-IL-6-binding site caused a severe reduction in the ASC-induced *IL8* promoter activation (*Fig*. 1*A*). Furthermore, a c-Jun dominant-negative mutant that inhibits AP-1 activity inhibited the ASC-induced *IL8* promoter activity, whereas the Gal4 DNA-binding domain used as a control did not significantly affect the activity (Fig. 1*B*). Consistent with these results, MDP stimulation also induced the expression of an AP-1-reporter construct carrying tandem TREs (Fig. 1*C*). MDP induced the AP-1 activation in HEK293 cells only when ASC and C12N2 were expressed simultaneously, but not when these components were expressed separately (Fig. 1*D*), indicating that C12N2 and ASC cooperatively transduce the MDP signal into AP-1 activation. Furthermore, knockdown of ASC expression using ASC-targeting siRNA abrogated MDP-induced AP-1 activation in MAIL8 cells (Fig 1*E*), confirming that the observed AP-1 activation was mediated by ASC.

MDP stimulation induced NF-κB and AP-1 activation also in an apoptosis-sensitive subline of MAIL8 cells (supplemental Fig. S1),[5] indicating that these responses are not restricted to apoptosis-resistant sublines. To exclude the possibility that ASC could mediate AP-1 activation only in the experimental system using the C12N2 chimera protein, a LRR-truncated form of CARD12 (CARD12ΔLRR) that can constitutively activate ASC was used in place of C12N2 (Fig. 1*F*). As expected, coexpression of CARD12ΔLRR and ASC in HEK293 cells induced AP-1 activation, while either one alone did not. These results further support the notion that ASC has a potential to mediate AP-1 activation.

Pretreatment with the proteasome inhibitor MG132 and expression of a proteasome-resistant mutant of IκBα (IκBα-SR) that inhibits NF-κB activation failed to inhibit the MDP-induced AP-1 activation in MAIL8 cells (Fig. 1, *G* and *H*). Conversely,

the c-Jun dominant-negative mutant failed to inhibit NF-κB activation under the same conditions (data not shown). Thus, the MDP-induced NF-κB and AP-1 activation were independent of each other.

To clarify whether MDP induces AP-1 activation in a manner that is cell-autonomous or dependent on a factor externalized on the cell surface or in the medium, MAIL8 or HEK293 cells were transfected with an AP-1 reporter plasmid, and these cell lines were then co-cultured in the presence or absence of MDP. MDP potently induced AP-1 activation in MAIL8 cells, but only weak AP-1 activation was observed in HEK293 cells under these conditions (Fig. 1*I*), indicating that MDP induced AP-1 activation largely in a cell-autonomous manner.

These data together with our previous results (12) indicate that the oligomerization of ASC induced by C12N2 upon MDP stimulation causes the cell-autonomous activation of AP-1 and NF-κB independent of each other, and AP-1 and NF-κB then synergistically induce the transcriptional activity of the *IL8* promoter.

ASC-mediated STAT3 activation depends on an externalized factor.

To investigate whether the activation of ASC induces other inflammatory transcription factors, we introduced reporter genes carrying tandem APREs (STAT3-binding sites), ISREs (ISGF3-binding sites), GAS (STAT1-binding site), or NFAT-binding sites in MAIL8 cells. MDP induced the expression of the APRE reporter gene but not of the other reporter genes (Fig. 2*A*), indicating that the activation of ASC induces STAT3 but not STAT1, ISGF3 (a complex of STAT1, STAT2, and IRF9), or NFAT activity. Unlike the AP-1 activation, the APRE activation was strongly inhibited by MG132 and IκBα-SR (Fig. 2*B* and data not shown). Furthermore, potent APRE activity was induced in HEK293 cells cocultured with MAIL8 cells upon MDP stimulation (Fig. 2*C*). These

results indicate that the ASC-mediated APRE activation was mainly mediated by an externalized factor, whose production depended on NF-κB activation.

Caspase-8 is involved in the ASC-mediated AP-1 activation.

Because caspase-8 plays an important role in ASC-mediated NF-κB activation (12), we investigated whether it is also involved in the ASC-mediated AP-1 activation. The expression of a caspase-8 dominant-negative mutant or of CLARP (also called FLIP, a natural inhibitor protein for caspase-8), but not of dominant-negative mutants of caspase-9 or FADD, inhibited the MDP-induced AP-1 activation in MAIL8 cells (Fig. 3, *A* and *B*). Consistent with this, the knockdown of caspase-8 but not of FADD expression by their corresponding siRNAs inhibited this response (Fig. 3*C*). In addition, the knockdown of RICK, which is essential for NOD2-mediated NF-κB activation (23, 24), did not affect this response. Furthermore, the pan-caspase inhibitor z-VAD-fmk and caspase-8 inhibitor z-IETD-fmk inhibited this response, while the caspase-1 inhibitor Ac-YVAD-cmk and granzyme B inhibitor z-AAD-cmk did not significantly affected (Fig. 3*D*). These results indicate that the catalytic activity of caspase-8 is required for the ASC-mediated AP-1 activation, whereas caspase-1, caspase-9, FADD, and RICK are not essential for this response.

Role of MAPKs in the ASC-mediated AP-1 activation and IL-8 production

Different MAPKs play important roles in AP-1 activation in different contexts. Therefore, we investigated which MAPKs are activated in MAIL8 cells upon MDP stimulation. Western blots using a pair of phosphorylated form-specific and pan-specific antibodies against each of the three major types of MAPKs (namely ERK1/2, JNK1/2, and p38) indicated that MDP induced phosphorylation of JNK and p38 but not of ERK

(Fig. 4*A*). To determine the contribution of these MAPKs to ASC-mediated AP-1 activation and IL-8 production, we tested the effects of MAPK- and MAPK kinase-inhibitors. Consistent with the observed phosphorylation of JNK and p38, inhibitors for these MAPKs (SB202190 and SB203580 for p38, and SP600125 for JNK) but not a control substance (SB202474) inhibited the MDP-induced AP-1 activation and IL-8 production in MAIL8 cells (Fig. 4, *B*-*E*), whereas an inhibitor for MAP/ERK kinase (PD98059) failed to inhibit these responses (Fig. 4, *C* and *E*). These results indicate that JNK and p38 are involved in the ASC-mediated AP-1 activation that is required for optimal IL-8 production. None of these inhibitors affected the MDP-induced NF-κB activation in MAIL8 cells (Fig. 4*F*), confirming that ASC-mediated NF-κB activation is independent of AP-1 activation.

Because ASC-mediated AP-1 activation was dependent on both caspase-8 and MAPKs, we investigated how these dependencies inter-related. In MAIL8 cells, pan-caspase-inhibitor z-VAD-fmk inhibited MDP-induced JNK phosphorylation (Fig. 4*G*), whereas none of MAPK inhibitors inhibited MDP-induced caspase-8 activation detected using fluorogenic substrate of caspase-8 (Fig. 4*H*). These results indicate that caspase-8 activation is upstream of MAPK activation.

Profiling of the gene expression induced by ASC activation

Finally, the profiling of ASC-induced genes by microarray analyses was performed {original data sets of microarray analyses are available at CIBEX (http://cibex.nig.ac.jp/index.jsp, accession # CBX41) upon publication}. The expression of 75 and 23 genes was found to be increased and decreased more than two fold, respectively, in MAIL8 cells but not in control KBG cells (HEK293-derived cell line stably expressing an NF-κB-GFP reporter construct) upon MDP stimulation

(supplemental Table S1). The top 30 genes that were upregulated in MDP-stimulated MAIL8 cells but not in MDP-stimulated KGB cells are listed in Table 1. On the other hand, 81 genes and 46 genes were, respectively, increased and decreased more than two fold, when the KBG cells were stimulated with TNF-α (supplemental Table S2). Cluster analysis indicated that some but not all of the genes upregulated by ASC activation were also upregulated by TNF-α stimulation (Fig. 5*A*, See also Table 1 and supplemental Table S1). The genes upregulated by both ASC activation and TNF-α stimulation included components or inhibitors of NF-κB, and inflammatory cytokines. In contrast, the genes that were specifically induced by ASC activation included components of AP-1, and stress-related genes. The gene descriptions and/or gene ontology terms provided by the manufacturer suggested that about 23%, 21%, and 16% of the 75 genes upregulated in MAIL8 after MDP stimulation were related to transcription, inflammation, and apoptosis/cell death, respectively (Fig. 5*B*). The induction of mRNAs for inflammatory cytokines (TNF, IL-6, IL-8, and CXCL2) and components or regulators of AP-1 and NF-κB (Fos, c-Jun, JunB, RelB, IκBα, and IκBζ) was confirmed by RT-PCR analyses (Fig. 5, *C* and *D*). MDP-induced expression of these mRNAs was markedly reduced when ASC expression was suppressed using ASC-targeting siRNA (Fig. 5*E*), indicating that the MDP-induced gene expression in this system is ASC-dependent.

Discussion

One of the obstacles to investigate the molecular mechanisms of the ASC-induced gene expression under physiological conditions is that there is no specific stimulus for ASC. Actually, many stimuli for ASC also activate toll-like receptors that have a potential to induce a variety of genes. In addition, although macrophages has been used to investigate physiological functions of ASC, activation of ASC induces caspase-1-mediated IL-1β production, and thus an observed ASC-mediated response may be secondary to IL-1β production in this type of cells. To overcome these problems, we previously established MAIL8 cells. Importantly, the parental HEK293 cells do not express NOD2, and do not respond to MDP. Furthermore, all the MDP-induced responses observed so far (i.e. apoptosis, NF-κB activation, and AP-1 activation) in MAIL8 cells were ASC dependent (12, 20, and Fig. 1*D*). In addition, MAIL8 cells as well as HEK293 cells express no or little toll-like receptors or caspase-1. Thus, our experimental system is more suitable for investigating the molecular mechanism of ASC-mediated responses than are more physiological experimental systems in which macrophages are stimulated with microbial components or microbes, which can activate other pattern-recognition receptors, such as toll-like receptors.

We previously demonstrated that activation of ASC induces NF-κB activation, which is essential for the MDP-induced IL-8 production in MAIL8 cells. Here, we showed for the first time that the activation of ASC also induces a cell-autonomous AP-1 activation. This response is critical for the MDP-induced IL-8 production in MAIL8 cells. Because NF-κB and AP-1 act synergistically to induce expression of a variety of genes involved in immune responses, it is likely that the function of ASC to mediate coordinated activation of these transcription factors in response to pathogen recognition by pattern recognition receptors such as CARD12 and cryopyrin play an

important role in the innate immune system. This notion is consistent with a recent report demonstrated that ASC plays an important role in NF-κB activation and cytokine production in human monocytic/macrophagic cell lines infected by *Porphyromonas gingivalis* (13). In contrast, it has been demonstrated that macrophages isolated from ASC-deficient mice produces normal levels of TNF-α and/or IL-6 upon LPS stimulation or microbial infection, suggesting that ASC is dispensable for cytokine gene expression in mouse macrophages (7, 14, 15). However, the role of ASC in cytokine production from other cell types in mice, especially those cells do not express Toll-like receptors still remains to be determined.

The activation of ASC also induced the expression of an APRE-reporter gene, indicating STAT3 activation. A cell-autonomous assay (Fig. 2*C*) revealed that the latter response is not cell-autonomous but mediated by a factor that can act in a paracrine manner. In addition, this response was suppressed by inhibiting NF-κB (Fig. 2*B* and data not shown) or AP-1 (data not shown), suggesting that the factor is produced in an NF-κB- and AP-1-dependent manner. Although IL-6 is a well known inducer for STAT3 activation, and IL-6 mRNA was actually induced upon MDP stimulation in MAIL8 cells (Table 1 and Fig. 5*C*), the MDP-induced APRE-reporter gene expression in MAIL8 cells was not inhibited by neutralization with an anti-IL-6 mAb, and recombinant IL-6 failed to induce the APRE-reporter gene expression in MAIL8 cells (data not shown). Thus, some other factor(s) must be responsible for the STAT3 activation.

Consistent with the potent activation of three major inflammation-associated transcription factors, NF-κB, AP-1, and STAT3, microarray analyses indicated that the expression of a number of genes encoding inflammatory cytokines as well as components or regulators of NF-κB and AP-1 was upregulated upon ASC activation.

These results further supported the idea that ASC plays an important role in inflammatory responses through, not only caspase-1 activation, but also the transcriptional regulation of a variety of inflammatory factors in human cells. Interestingly, a subset of ASC-induced genes, including *FOS*, *JUNB*, and *JUN*, all of which encode components of AP-1, was not significantly induced upon TNF-α stimulation in MAIL8 cells. One likely reason for TNF-α's failure to induce some ASC-inducible genes is that, unlike ASC activation, TNF-α stimulation induces only marginal AP-1 activation in HEK293-derived cell lines, including MAIL8 cells (data not shown). The microarray analyses also indicated that ASC induces a number of cell death-related genes, including TNF, NRA41 (also called NUR77, Table 1) and BBC3 (also called PUMA, Fig. 5*B*). Thus, ASC may be involved in apoptosis not only though the activation of caspase-8 but also by inducing the expression of proapoptotic genes.

Here, we found that the activation of ASC induces AP-1 in a caspase-8-dependent manner. Caspase-8 also plays important roles in ASC-mediated apoptosis and NF-κB activation (11, 12, 20). Thus, this ASC-caspase-8 axis branches in at least three directions. This signal transduction system is strikingly similar to the Fas ligand-induced one, which is mediated by the Fas-FADD-caspase-8 axis, in that the same three pathways are activated downstream of caspase-8 (17). Nonetheless, FADD is not involved in the ASC-mediated responses. Considering the structural similarity of FADD and ASC, it is likely that ASC plays an equivalent role to FADD as an adaptor protein that acts as a bridge between an upstream molecule (i.e., CARD12) and caspase-8. This notion is also supported by our previous finding that endogenous caspase-8 colocalizes with ASC upon MDP stimulation in MAIL8 cells (12). ASC activation induced a significantly higher level of AP-1 activation than did Fas ligand stimulation in MAIL8 cells (data not shown), although caspase-8 plays an essential role

in both cases. This is probably because ASC activation induced the phosphorylation of both p38 and JNK, which were involved in AP-1 activation (Fig. 5), whereas the Fas ligand-induced AP-1 activation solely depends on JNK, and not on p38 (18). Thus, ASC and FADD mediate similar signaling pathways, both using caspase-8, but they are not exactly the same. This difference between their signals may be explained by the different components of the signaling complexes recruited by ASC and FADD.

In conclusion, our results demonstrate a novel function for ASC in the transcriptional regulation of inflammatory and cell-death-related genes in human cells and thus provide new insights into the role of ASC in the innate immune system.

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Disclosures

The authors have no financial conflict of interest.

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Footnotes

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[4] Abbreviations used in this paper: CARD, caspase recruitment domain; APRE, acute phase response element; ASC, Apoptosis associated speck-like protein containing a CARD; cmk, chloromethylketone; CLARP-S, a short isoform of caspase-like apoptosis regulatory protein; FADD, Fas-associated death domain protein; fmk, fluoromethylketone; GAS, γ-activated sequences; ISRE, interferon-stimulated response element; LRRs, leucine-rich repeats; MDP, muramyl dipeptide; NLR, nucleotide-binding domain and LRRs-containing protein; NOD, nucleotide-binding oligomerization domain; siRNA, Small interfering RNA; TRE, 12-*O*-tetradecanoylphorbol-13-acetate-responsive element

[5] The online version of this article contains supplemental material.

Figure legends

FIGURE 1. ASC activates the *IL8* promoter through AP-1 activation in MAIL8 cells, and the ASC-induced AP-1 activation is an NF-κB-independent and cell-autonomous response. *A*, MAIL8 cells were transfected with a reporter plasmid carrying a luciferase gene driven by the wild-type *IL8* promoter or this promoter mutated at the NF-κB-, AP-1-, or NF-IL-6-binding site, and were stimulated with 10 ng/ml MDP or left unstimulated during the last 16 h of a 32-h culture. *B*, MAIL8 cells were co-transfected with the wild-type *IL8* promoter reporter plasmid and an expression plasmid for a dominant-negative mutant of c-Jun (cJun-DN) or the DNA-binding domain of Gal4 (Gal4-DB), and stimulated as in (*A*). *C*, MAIL8 cells were transfected with an AP-1 reporter plasmid and stimulated as in (*A*). *D*, HEK293 cells were transfected with an empty vector, or expression vectors for C12N2 and/or ASC as indicated together with an AP-1 reporter plasmid, and then stimulated as in (*A*). *E*, MAIL8 cells were sequentially transfected with siRNA (control or ASC-targeting siRNA) and an AP-1 reporter plasmid with a 12-h interval. Cells were then stimulated as in (*A*). Knockdown of ASC expression was confirmed by Western Blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as an internal control to show equal loading. *F*, HEK293 cells were transfected with an empty vector, or expression vectors for CARD12ΔLRR and/or ASC as indicated together with an AP-1 reporter plasmid. AP-1 activity was determined 24 h after the transfection. *G*, MAIL8 cells were transfected with an AP-1 reporter plasmid and stimulated as in (*A*), except that the cells were pretreated with or without 10 μM MG132 for 1 h before MDP stimulation. *H*, MAIL8 cells were co-transfected with the AP-1 reporter plasmid together with either empty vector or an expression plasmid for IκBα-SR, and stimulated as in (*A*). *I*, MAIL8 cells

and HEK293 cells were cultured separately in two wells and transfected with an empty vector or an AP-1 reporter plasmid as indicated. Twelve hours later, the cells in the two wells were mixed and stimulated with 10 ng/ml MDP or left unstimulated during the last 16 h of a further 24-h culture. *A-I*, *IL8* promoter and AP-1 activation was evaluated by a luciferase reporter assay.

FIGURE 2. ASC activates APRE-reporter gene expression by a non-cell-autonomous mechanism involving NF-κB activation. *A*, MAIL8 cells were transfected with a reporter plasmid carrying APREs, ISREs, GAS, or NFAT-binding sites, and stimulated with 10 ng/ml MDP or left unstimulated during the last 16 h of a 32-h culture. The observed weak induction of the NFAT reporter gene by MDP was not reproducible. *B*, MAIL8 cells were transfected with the APRE reporter plasmid and stimulated as in (*A*), except that the cells were pretreated with or without 10 μM MG132 for 1 h before MDP stimulation. *C*, MAIL8 cells and HEK293 cells were cultured separately in two wells and transfected with an empty vector or an APRE reporter plasmid as indicated. Twelve hours later, the cells in the two wells were mixed and stimulated with 10 ng/ml MDP or left unstimulated during the last 16 h of a further 24-h culture. *A-C*, Reporter activity was determined by luciferase assay.

FIGURE 3. Caspase-8 is essential for the ASC-induced AP-1 activation. *A*, MAIL8 cells were co-transfected with the AP-1 reporter plasmid and an expression plasmid for a dominant-negative mutant of caspase-8 or -9 (casp8-DN or casp9-DN), and stimulated with 10 ng/ml MDP or left unstimulated during the last 16 h of a 32-h culture. *B*, MAIL8 cells were co-transfected with the AP-1 reporter plasmid and an expression plasmid for CLARP-S or a dominant-negative mutant of FADD (FADD-DN), and

stimulated as in (*A*). *C*, MAIL8 cells were co-transfected with the AP-1 reporter plasmid and siRNA targeting caspase-8, FADD, or RICK (10 nM), and stimulated with 10 ng/ml MDP or left unstimulated during the last 16 h of a 48-h culture. *D*, MAIL8 cells were transfected with the AP-1 reporter plasmid and stimulated as in (*A*), except that the cells were pretreated with or without Z-VAD-fmk (pan-caspase inhibitor), Ac-YVAD-cmk (caspase-1 inhibitor), z-IETD-fmk (caspase-8 inhibitor), or Z-AAD-cmk (granzyme B inhibitor) for 1 h before MDP stimulation (20 μM). *A-D*, AP-1 activation was evaluated by a luciferase reporter assay.

FIGURE 4. JNK and p38 activities are required for the ASC-induced AP-1 activation and IL-8 production. *A*, MAIL8 cells were stimulated with 10 ng/ml MDP for the indicated periods. The whole-cell lysates were then assayed by western blotting using antibodies against phosphorylated or entire JNK1/2, p38 or ERK. Whole-cell lysates after UV treatment (500 J/m^2) were used as a positive control for phosphorylated JNK1/2 and p38. *B* and *C*, MAIL8 cells were transfected with the AP-1 reporter plasmid and pretreated with the indicated MAPK inhibitors (20 μM) for 1 h, and further stimulated with MDP (10 ng/ml) for 16 h. AP-1 activation was evaluated by a luciferase reporter assay. *D* and *E*, MAIL8 cells were pretreated with the indicated MAPK inhibitors (20 μM) for 1 h and further stimulated with MDP (10 ng/ml) for 16 h. The amount of IL-8 in the culture supernatant was determined by ELISA. *F*, MAIL8 cells were transfected with the NF-κB reporter plasmid and then treated as in (*B*). NF-κB activity was evaluated by a luciferase reporter assay. *G*, MAIL8 cells were pretreated with the indicated caspase inhibitors (20 μM) for 1 h and further stimulated with MDP (10 ng/ml) for 16 h. The whole-cell lysates were then assayed by western blotting using antibodies against phosphorylated or entire JNK1/2. *H*, MAIL8 cells were pretreated

with the indicated MAPK or caspase inhibitors (20 μM) for 1 h and further stimulated with MDP (10 ng/ml) for 16 h. Cell lysates were then analyzed for caspase-8 activity using fluorogenic caspase-8 substrate, IETD-7-amino-4-trifluoromethyl coumarin. RFU, relative fluorescent units. *B-H*, SP600125, JNK inhibitor; PD98059, MEK inhibitor; SB202190 and SB203580, p38 inhibitors; SB202474, control substance; z-VAD-fmk, pancaspase-inhibitor; z-IETD-fmk, caspase-8 inhibitor.

FIGURE 5. Profiling of the gene expression induced by ASC activation. *A*, Single linkage hierarchical clustering with the Euclidean distance metric of the microarray data shown in Table 1 and supplemental Table S1 was performed for those genes that were induced more than two-fold by MDP stimulation in MAIL8 cells but not in KBG cells, using MultiExperiment Viewer v4.0 (19). *B*, Sets of genes that are related to transcription, inflammation and/or cell death among 75 genes upregulated upon ASC-activation. *C* and *D*, The total RNA was purified from MAIL8 cells cultured with or without 10 ng/ml MDP for the indicated periods in (*C*) or 12 h in (*D*). Induction of the mRNAs for inflammatory cytokines, AP-1, and NF-κB-related genes was confirmed by RT-PCR analyses. *E*, MAIL8 cells were transfected with control and ASC-targeting siRNA twice at a 36-h interval. Cells were then stimulated with or without 10 ng/ml MDP during the last 12 h of another 36-h culture. The expression levels of indicated genes were then examined by RT-PCR. ACTB, β-actin; NFKBIA, IκBα; NFKBIZ, IκBζ.