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TNF-α induces caspase-1 activation independently of simultaneously induced NLRP3 in 3T3-L1 cells

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Abstract

The intracellular cysteine protease caspase-1 is critically involved in obesity-induced inflammation in adipose tissue. A substantial body of evidence from immune cells, such as macrophages, has shown that caspase-1 activation depends largely on a protein complex, called the NLRP3 inflammasome, which consists of the NOD-like receptor (NLR) family protein NLRP3, the adaptor protein ASC, and caspase-1 itself. However, it is not fully understood how caspase-1 activation is regulated within adipocytes upon inflammatory stimuli. In this study, we show that TNF-α-induced activation of caspase-1 is accompanied by robust induction of NLRP3 in 3T3-L1 adipocytes but that caspase-1 activation may not depend on the NLRP3 inflammasome. Treatment of 3T3-L1 cells with TNF-α induced mRNA expression and activation of caspase-1. Although the basal expression of NLRP3 and ASC was undetectable in unstimulated cells, TNF-α strongly induced NLRP3 expression but did not induce ASC expression. Interestingly, inhibitors of the ERK MAP kinase pathway strongly suppressed NLRP3 expression but did not suppress the expression and activation of caspase-1 induced by TNF-α, suggesting that NLRP3 is dispensable for TNF-α-induced caspase-1 activation. Moreover, we did not detect the basal and TNF-α-induced expression of other NLR proteins (NLRP1a, NLRP1b, and NLRC4), which do not necessarily require ASC for caspase-1 activation. These results suggest that TNF-α induces caspase-1 activation in an inflammasome-independent manner in 3T3-L1 cells and that the ERK-dependent expression of NLRP3 may play a role independently of its canonical role as a component of inflammasomes.
Introduction

Accumulating evidence has shown that low-grade but chronic inflammation in adipose tissue is strongly associated with obesity and type 2 diabetes (T2D) (Donath and Shoelson, 2011). Proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, are secreted from macrophages that are recruited into obese adipose tissue and are known to play a major role in inflammation in adipose tissue (Weisberg et al., 2003). Indeed, in mice, ablation of the protein complex NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing 3) inflammasome, which mediates IL-1β maturation and secretion, prevents obesity-induced inflammation and insulin resistance, a major risk factor for T2D (Stienstra et al., 2011; Vandanmagsar et al., 2011; Wen et al., 2011; Zhou et al., 2010).

NLRP3, a member of the NOD-like receptor (NLR) family, binds to the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD)) and pro-caspase-1, the latent form of the cysteine protease caspase-1, in response to a variety of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), composing the NLRP3 inflammasome (Guo et al., 2015). In the inflammasome, processing and activation of pro-caspase-1 are induced, followed by caspase-1-mediated cleavage of the latent form of IL-1β. The NLR proteins other than NLRP3 also form their own inflammasomes, but caspase-1 is their common component (Sollberger et al., 2014; Wen et al., 2013).

Much attention has been paid to the role of caspase-1 in adipocytes as a mediator of inflammation in adipose tissue. It has been shown that expression of caspase-1 is upregulated during adipocyte differentiation and modulates adipocyte function (Stienstra et al., 2010). Thus, to
gain a better understanding of the roles of caspase-1 in adipose tissue inflammation, it is important to distinguish the activation mechanism and roles of caspase-1 in adipocytes from those in other types of cells, such as macrophages. To this end, cultured adipocytes, such as primary adipocytes and differentiated mouse 3T3-L1 adipocytes, are important tools. It has been reported that caspase-1 induces cleavage of the NAD+-dependent deacetylase SIRT1 and thus inhibits its role as a regulatory sensor of nutrient availability in TNF-α-treated 3T3-L1 cells (Chalkiadaki and Guarente, 2012). It has also been reported that caspase-1 may induce degradation of peroxisome proliferator-activated receptor γ (PPARγ), a transcription factor critical for differentiation and function of adipocytes, in 3T3-L1 cells treated with TNF-α and cycloheximide (CHX) (He et al., 2008). Despite such evidence of active adipocyte caspase-1, it is not fully understood how caspase-1 activation upon inflammatory stimuli is regulated in adipocytes.

In this study, we found that TNF-α induced expression and activation of caspase-1 but that the simultaneously induced NLRP3 unexpectedly appeared to be dispensable for TNF-α-induced caspase-1 activation in 3T3-L1 cells. Thus, the activation mechanism of caspase-1 and the role of NLRP3, both independent of inflammasomes, may exist in TNF-α-treated 3T3-L1 cells.
Materials and Methods

Reagents

PD0325901, SP600125, ATP and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant mouse TNF-α was purchased from Peprotech, Inc. (Rocky Hill, NJ). SB203580 and BAY11-7082 were purchased from Merck Millipore (Darmstadt, Germany). PD184352 was synthesized as described previously (Tanimura et al., 2003).

Cell culture

Mouse 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 units/mL of penicillin G and 0.1 mg/mL of streptomycin (DMEM) containing 10% calf serum. Differentiation of 3T3-L1 preadipocytes into adipocytes was achieved by the following procedure: cells were cultured in the initiation medium of DMEM containing 10% fetal calf serum (FCS), 5 µg/mL insulin (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Wako Pure Chemical, Osaka, Japan), and 0.25 µM dexamethasone (Sigma-Aldrich) for 2 days. After 3 days of culture in the progression medium (DMEM containing 10% FCS and 5 µg/mL insulin), cells were cultured in the maintenance medium (DMEM containing 10% FCS), which was exchanged every other day. Cells were used for experiments at days 10-14 after the induction of differentiation. RAW264 (RIKEN BioResource Center, Tsukuba, Japan) and J774A.1 (American Type Culture Collection) macrophage-like cell lines were cultured in DMEM containing 10% FCS. Mouse peritoneal exudate cells were prepared as described previously (Schneider, 2013).
Quantitative reverse transcription (RT)- PCR

Total RNA was isolated from 3T3-L1 cells or C57BL/6J mouse spleen using the TRI-reagent (Molecular Research Center, Cincinnati, OH), and cDNA was synthesized using the PrimeScript RT reagent kit with the gDNA Eraser kit (Takara Bio, Kyoto, Japan). Quantitative RT-PCR was performed by the Takara Thermal Cycler Dice (Takara Bio) using TaqMan Gene expression assays (pro-caspase1, Mm00438023_m1; ASC, Mm00445747_g1; NLRP3, Mm00840904_m1; GAPDH, Mm99999915_g1) (Applied Biosystems, Foster City, CA) or SYBR Green-based detection assays using SYBR premix Ex Taq II (Tli RNaseH Plus) (Takara Bio). In the SYBR Green-based assays, the oligonucleotide primers for NLRP1a, NLRP1b, NLRP3, and NLRC4 were described previously (Lech et al., 2010), and the following were used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5’-TGTGTCCGTCGTGGA TCTGA-3’; reverse, 5’-TTGCTGTTGAAGTCGCAGGAG-3’. The relative expression of each mRNA was calculated and normalized to GAPDH mRNA in the same sample using the comparative CT method (Schmittgen and Livak, 2008).

Immunoblot analysis

The cells were lysed with a lysis buffer containing 25 mM Tris-HCl [pH 7.5], 25 mM NaCl, 0.5 mM EGTA, 5 µg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM Na pyrophosphate, 1 mM Na-o-vanadate, 25 mM p-NPP, 25 mM β-glycerophosphate, 0.2 mM Na molybdate, 20 nM okadaic acid, and 1% Triton X-100; this was followed by sonication for 1 min. The lysates were centrifuged for 30 min at 15,000 x g, and the resulting supernatants were fractionated by
SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes. The membranes were probed with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI). Protein bands were visualized by the enhanced chemiluminescence system and analyzed by an ImageQuant LAS4000 (GE Healthcare Bio-sciences, Piscataway, NJ). The following primary antibodies were used in this study: Caspase-1 antibody (Adipogen, San Diego, CA); β-actin antibody (Cell signaling, Danvers, MA); phospho-ERK1/2 antibody (Sigma-Aldrich) that detects the activation phosphorylation of ERK1 and ERK2, the two mammalian isoforms of ERK; and ERK1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). In some experiments, the intensity of each band was quantified using an image analysis software ImageQuant TL (GE Healthcare).

**Measurement of caspase-1 activity**

The cells were lysed with RIPA buffer containing 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. The lysates were centrifuged for 15 min at 15,000 x g. Caspase-1 activity was assayed by incubating the supernatants in a buffer containing 50 µM of the fluorometric peptide substrate Ac-YVAD-AFC (Enzo Life Sciences, Inc. Farmingdale, NY), 100 mM HEPES [pH 7.5], 10% glycerol, and 10 mM DTT for 2 hr at 37 °C. Fluorescence was measured with an excitation wavelength of 400 nm and an emission wavelength of 505 nm by a Cytation 3 cell imaging multi-mode reader (BioTek Instruments, Inc., Winooski, VT).

**Statistical analyses**

Student’s t-test (unpaired, two-tailed) was used to compare two groups of independent samples.
Results

TNF-α induces expression and activation of caspase-1 in 3T3-L1 cells

To address how caspase-1 reacts to inflammatory stimuli in adipocytes, we began by examining expression and activation of caspase-1 in 3T3-L1 cells treated with TNF-α. TNF-α-dependent mRNA expression of pro-caspase-1 was detected as early as 3 hr after stimulation with 20 ng/mL TNF-α and further increased thereafter (Fig. 1A). Consistently with this, TNF-α-dependent protein expression of pro-caspase-1 was first detected at 6 hr and almost plateaued 12 hr after stimulation (Fig. 1B). At 24 hr after stimulation, protein expression of pro-caspase-1 was induced in response to TNF-α in a dose-dependent manner at doses ranging from 5 to 20 ng/mL (Fig. 1C). At 6 hr after stimulation, caspase-1 activity, which was assayed as cleavage activity of the fluorometric peptide substrate Ac-YVAD-AFC, was higher in the lysate from cells treated with 20 ng/mL TNF-α for 6 hr than in untreated cells (Fig. 1D). These results indicate that TNF-α induces expression and activation of caspase-1 in 3T3-L1 cells.

TNF-α induces expression of NLRP3 but not ASC in 3T3-L1 cells

Because caspase-1 is activated in the NLRP3 inflammasome in various contexts, we examined mRNA expression of its components in 3T3-L1 cells. Whereas expression of NLRP3, ASC, and pro-caspase-1 was easily detected in macrophage-like J774.A.1 cells, none of them were detected in unstimulated 3T3-L1 cells (Fig. 2A). Consistently with a previous report (Pelegrin et al., 2008), another macrophage-like cell line, RAW264, lacked ASC expression, serving as a negative control. Intriguingly, TNF-α strongly induced expression of NLRP3 as early as 1 hr after stimulation, and
TNF-α-dependent induction peaked at 3 hr after stimulation (**Fig. 2B, upper graph**). On the other hand, TNF-α did not induce ASC expression throughout the 6 hr stimulation period (**Fig. 2B, lower graph**). Also at the protein level, TNF-α did not induce ASC throughout the 24 hr stimulation period (**Fig. 2C**).

**TNF-α-induced expression of NLRP3 depends on the ERK pathway**

To determine whether TNF-α induces expression of NLRP3 and pro-caspase-1 through a common mechanism in 3T3-L1 cells, we first examined the involvement of the NF-κB and MAP kinase pathways, which are known to be main pathways downstream of receptors for TNF-α (Sabio and Davis, 2014), in TNF-α-induced NLRP3 expression. Consistent with the finding that NLRP3 expression induced by LPS, a major PAMP of Gram-negative bacteria, largely depended on the NF-κB pathway in mouse macrophages (Bauernfeind et al., 2009), TNF-α-induced expression of NLRP3 was suppressed to some extent by 10 μM of the NF-κB inhibitor BAY11-7082 (**Fig. 3A**). On the other hand, TNF-α-induced expression of NLRP3 was strongly suppressed by the same dose of the MEK inhibitor PD184352, which inhibits the ERK MAP kinase pathway, but by neither SB203580 nor SP600125, inhibitors of p38 MAP kinase and c-Jun N-terminal kinase (JNK), respectively, which, together with ERK, comprise the three independent MAP kinase pathways in mammals. TNF-α-induced expression of NLRP3 was similarly suppressed by the lower dose (2 μM) of another MEK inhibitor PD0325901, which has been found to be more potent than PD184352 and indeed strongly suppressed TNF-α-induced activation, as well as basal activity, of ERK1 and ERK2 (Sebolt-Leopold and Herrera, 2004) (**Figs. 3B and 3C**). Thus, the ERK pathway may play a major role in TNF-α-induced NLRP3 expression in 3T3-L1 cells, probably in
Neither expression nor activation of caspase-1 induced by TNF-α depends on the ERK pathway.

We next examined the requirement of the NF-κB and ERK pathways for TNF-α-induced pro-caspase-1 expression. Similar to NLRP3 expression, TNF-α-induced mRNA expression of pro-caspase-1 was suppressed to some extent by BAY11-7082 (Fig. 4A), suggesting that the NF-κB pathway is commonly required for TNF-α-induced expression of pro-caspase-1 and NLRP3. However, PD0325901 exerted no inhibitory effects on TNF-α-induced pro-caspase-1 expression at both the mRNA and protein levels (Figs. 4B and 4C). PD0325901 also did not suppress TNF-α-induced caspase-1 activation (Fig 4D). These results suggest that TNF-α-induced expression and activation of caspase-1 is regulated independently of the ERK-mediated induction of NLRP3 in 3T3-L1 cells. Together with the result that ASC was not detected even in the presence of TNF-α (Fig. 2), the NLRP3 inflammasome does not appear to be involved in TNF-α-induced activation of caspase-1 in 3T3-L1 cells.

NLR proteins that can activate caspase-1 independently of ASC are not induced by TNF-α in 3T3-L1 cells

The possibility that other NLR proteins, particularly those that can activate caspase-1 independently of ASC, might induce TNF-α-induced activation of caspase-1 in 3T3-L1 cells still remained. The corresponding NLR proteins reported so far are NLRP1a, NLRP1b, and NLRC4 (Broz et al., 2010; Masters et al., 2012; Van Opdenbosch et al., 2014). We thus examined their expression at the
mRNA level in 3T3-L1 cells after we determined the experimental conditions under which their expression was detected in RNA extracted from mouse spleen (Fig. 5A). However, neither the basal nor the TNF-α-induced expression of these NLR proteins was detected in 3T3-L1 cells (Fig. 5B).

Thus, TNF-α may induce caspase-1 activation in an inflammasome-independent manner in 3T3-L1 cells.
Discussion

In this study, we found that TNF-\(\alpha\)-induced caspase-1 activation in 3T3-L1 adipocytes did not appear to depend on inflammasomes, which are generally thought to be essential for caspase-1 activation (Sollberger et al., 2014). To date, no definite mechanisms of inflammasome-independent caspase-1 activation have been proposed.

Consistently with the requirement of inflammasomes for the maturation and secretion of IL-1\(\beta\) (Guo et al., 2015), we could not detect any IL-1\(\beta\) secretion from TNF-\(\alpha\)-treated 3T3-L1 cells (data not shown), suggesting that caspase-1 induced and activated by TNF-\(\alpha\) in these cells has functions different from inducing IL-1\(\beta\) secretion. As mentioned in the Introduction, it has been shown that caspase-1 induces the cleavage of SIRT1 in the same context (TNF-\(\alpha\)-treated 3T3-L1 cells), and this cleavage of a key metabolic regulator may contribute to the regulation of adipose tissue inflammation (Chalkiadaki and Guarente, 2012). Caspase-1 has also been proposed to induce the cleavage of PPAR\(\gamma\). Nevertheless, PPAR\(\gamma\) cleavage induced by caspase-1 occurred only when 3T3-L1 cells were treated with TNF-\(\alpha\) in combination with CHX (He et al., 2008). Another group has reported that the other caspases, mainly caspase-3 and -6, are responsible for PPAR\(\gamma\) cleavage in 3T3-L1 cells treated only with TNF-\(\alpha\) (Guilherme et al., 2009). Thus, further evidence is required to clarify whether PPAR\(\gamma\) indeed is a substrate of caspase-1 in inflammatory-stimulated adipocytes.

Of note, previous reports have suggest that pro-caspase-1 binds to receptor interacting protein 2 (RIP2), a CARD-containing kinase, and promotes NF-\(\kappa\)B activation independently of the protease activity of caspase-1 (Lamkanfi et al., 2004). Interestingly, ASC has been shown to compete with RIP2 for binding to caspase-1 (Sarkar et al., 2006), suggesting that this protease activity-independent function of caspase-1 is augmented in 3T3-L1 cells that appear to lack ASC.
Thus, TNF-α-induced expression of caspase-1 may be sufficient to induce an inflammatory response in adipocytes at least to some extent.

We also found that TNF-α-induced mRNA expression of NLRP3 was detected as early as 1 hr after stimulation (Fig. 2B), suggesting that the Nlrp3 gene is an immediate early gene responsive to TNF-α in 3T3-L1 cells. This is consistent with a previous report that in 3T3-L1 cells, NLRP3 expression was induced by 24-hr treatment with TNF-α or IL-1β, whereas ASC expression did not change, even in the presence of various pro-inflammatory cytokines including TNF-α and IL-1β (Yin et al., 2014). Intriguingly, the responsiveness of the Nlrp3 gene to TNF-α was found to be largely dependent on the ERK pathway because MEK inhibitors strongly suppressed the TNF-α-induced expression of NLRP3 (Fig. 3). Mice deficient in ERK1, one of two ERK isoforms in mammals, that are challenged with a high-fat diet or are crossed with leptin-deficient (ob/ob) mice, have been shown to exhibit reduced insulin resistance compared with wild-type mice (Bost et al., 2005; Jager et al., 2011), although the molecular functions of ERK in this context are still unknown. These findings strongly suggest that the ERK pathway accelerates the inflammatory response of adipose tissue to various pro-inflammatory cytokines including TNF-α, and that ERK-dependent induction of NLRP3 in adipocytes may play some roles in this process.

Our results that NLRP3 appears to be dispensable for caspase-1 activation in TNF-α-treated 3T3-L1 cells suggest the existence of inflammasome-independent roles of NLRP3 in the adipocyte inflammatory response. It has recently been shown that regulation of chemokine-mediated functions of neutrophils, which contributes to hepatic ischemia-reperfusion injury, and promotion of TGF-β signaling in kidney epithelium are both regulated by NLRP3 independently of inflammasomes (Inoue et al., 2014; Wang et al., 2013). More recently, NLRP3 has
also been shown to function independently of inflammasomes as a transcriptional regulator of T
helper type 2 (T\(_h2\)) differentiation (Bruchard et al., 2015). Thus, caspase-1 and NLRP3 may
function independently from each other in 3T3-L1 cells stimulated with pro-inflammatory cytokines,
and therefore, the elucidation of their respective functions in adipocytes will shed new light on the
regulatory mechanism of adipose tissue inflammation.

Acknowledgments

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1 Literature Cited


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Figure legends

Figure 1. TNF-α induces expression and activation of caspase-1 in 3T3-L1 cells

(A) TNF-α-induced mRNA expression of pro-caspase-1. 3T3-L1 cells were treated with or without 20 ng/mL TNF-α for the indicated time periods. The relative expression of pro-caspase-1 was examined by quantitative RT-PCR. Data are shown as the mean ± SEM (n=3).

(B, C) TNF-α-induced protein expression of pro-caspase-1. 3T3-L1 cells were treated with or without 20 ng/mL TNF-α for the indicated time periods (B) or treated with the indicated doses of TNF-α for 24 hr (C). Cell lysates were subjected to immunoblot analysis with the indicated antibodies. The quantified relative expression levels of pro-caspase-1 are indicated between the upper and lower panels.

(D) TNF-α-induced activation of caspase-1. 3T3-L1 cells were treated with 20 ng/mL TNF-α for 6 hr. Cell lysates were used to measure the caspase-1 activity using the fluorometric peptide substrate Ac-YVAD-AFC. Data are shown as the mean ± SEM (n=3). **p < 0.01, compared with the untreated cells.

Figure 2. TNF-α induces mRNA expression of NLRP3 in 3T3-L1 cells

(A) mRNA expression of NLRP3, ASC, and pro-caspase-1 in J774.A.1, RAW264, and 3T3-L1 cells. The relative gene expression was examined by quantitative RT-PCR. Data are shown as the mean ± SEM (n=3).

(B) TNF-α-induced mRNA expression of NLRP3 (upper graph) and ASC (lower graph). 3T3-L1 cells were treated with or without 20 ng/mL TNF-α for the indicated time periods. The relative gene
expression was examined by quantitative RT-PCR. Data are shown as the mean ± SEM (n=3).

(C) Protein expression of ASC in 3T3-L1 cells. 3T3-L1 cells were treated with or without 20 ng/mL TNF-α for the indicated time periods. As a positive control, mouse peritoneal exudate cells (PEC) were pre-treated with 100 ng/mL LPS for 4 hr and then treated with 5 mM ATP for 30 min. The cell lysates were subjected to immunoblot analysis using the indicated antibodies.

Figure 3. TNF-α-induced mRNA expression of NLRP3 depends on the ERK pathway in 3T3-L1 cells

(A, B) Effects of inhibitors of the MAP kinase and NF-κB pathways on TNF-α-induced mRNA expression of NLRP3. 3T3-L1 cells were pre-treated with 10 µM each of PD184352, SB203580, SP600125, or BAY11-7082 (A) and with 2 µM PD0325901 or 10 µM PD184352 (B) for 30 min. The cells were then treated with 20 ng/mL TNF-α for 3 hr. The relative expression of NLRP3 was examined by quantitative RT-PCR. Data are shown as the mean ± SEM (n=3). **p < 0.01, compared with the cells treated with TNF-α but not with any inhibitor (the leftmost column).

(C) Effect of PD0325901 on TNF-α-induced activation of ERK1 and ERK2. The cells were pre-treated with or without 2 µM PD0325901 for 30 min and then treated with 20 ng/mL TNF-α for 6 hr, and the cell lysates were subjected to immunoblot analysis using the indicated antibodies.

Figure 4. Neither expression nor activation of caspase-1 induced by TNF-α depends on the ERK pathway

(A, B) Effects of BAY11-7082 and PD0325901 on TNF-α-induced mRNA expression of pro-caspase-1. 3T3-L1 cells were pre-treated with or without 10 µM BAY11-7082 (A) or 2 µM
PD0325901 (B) for 30 min and then treated with 20 ng/mL TNF-α for 3 hr. The relative expression of pro-caspase-1 was examined by quantitative RT-PCR. Data are shown as the mean ± SEM (n=3). *p < 0.05, n.s. = not significant.

(C) Effect of PD0325901 on TNF-α-induced protein expression of pro-caspase-1. The cells were pre-treated with or without 2 μM PD0325901 for 30 min and then treated with 20 ng/mL TNF-α for 12 hr, and the cell lysates were subjected to immunoblot analysis using the indicated antibodies.

(D) Effect of PD0325901 on TNF-α-induced activation of caspase-1. The cells were pre-treated with or without 2 μM PD0325901 for 30 min and then treated with 20 ng/mL TNF-α for 6 hr, and caspase-1 activity was measured in the cell lysates. Data are shown as the mean ± SEM (n=3). *p < 0.05, **p < 0.01.

Figure 5. NLR proteins that can activate caspase-1 independently of ASC are not induced by TNF-α in 3T3-L1 cells

(A) Validation of quantitative RT-PCR for detecting mRNA of various NLR proteins using mouse spleen RNA. Expression of the indicated NLR was examined by quantitative RT-PCR using SYBR Green-based detection assays. Data are shown as the mean ± SEM (n=3). (B) TNF-α-induced mRNA expression of various NLR proteins. 3T3-L1 cells were treated with or without 20 ng/mL TNF-α for 3 hr. The relative mRNA expression of the indicated NLR proteins was examined by quantitative RT-PCR using SYBR Green-based detection assays. Data are shown as the mean ± SEM (n=3). N.D. = not detected.
Fig. 1

A. Pro-caspase-1

Relative mRNA expression

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<td>-</td>
<td>-</td>
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B. Pro-caspase-1 and β-actin

Time (hr) 1 3 6 12 24

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C. Pro-caspase-1 and β-actin

TNF-α (ng/mL) 0 0.1 1 5 10 20 50

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D. Relative caspase-1 activity

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Fig. 2

A

NLRP3

ASC

Pro-caspase-1

Relative mRNA expression

JT74.A-1  RAW264  3T3-L1

JT74.A-1  RAW264  3T3-L1

JT74.A-1  RAW264  3T3-L1

B

NLRP3

Relative mRNA expression

TNF-α

1 hr 3 hr 6 hr

TNF-α

1 hr 3 hr 6 hr

ASC

PEC

C

TNF-α

3 hr 6 hr 24 hr

PEC

ASC

β-actin
Fig. 3

A

Relative mRNA expression

\[ \text{NLRP3} \]

(-) PD184352 SB203580 SP600125 BAY11-7082

B

Relative mRNA expression

\[ \text{NLRP3} \]

(-) PD0325901 PD184352

C

PD0325901 - + - +

TNF-\(\alpha\) - - + +

Phospho-ERK1/2

ERK1/2
**Fig. 4**

**A**

Relative mRNA expression

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**B**

Relative mRNA expression

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**C**

Pro-caspase-1

|          |          |          |          |          |
|----------|----------|----------|----------|
| PD0325901 |          |          |          |
| TNF-α    |          |          |          |

**D**

Relative caspase-1 activity

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Fig. 5

A

Relative mRNA expression

NLRP3  NLRP1a  NLRP1b  NLRC4

B

Relative mRNA expression

N.D.  N.D.  N.D.  N.D.

TNF-α

-  +  -  +  -  +  -  +