

1 **TNF- $\alpha$  induces caspase-1 activation independently of simultaneously induced NLRP3**  
2 **in 3T3-L1 cells**

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1 **Abstract**

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

20

21

## 1 **Introduction**

2 Accumulating evidence has shown that low-grade but chronic inflammation in adipose tissue is  
3 strongly associated with obesity and type 2 diabetes (T2D) (Donath and Shoelson, 2011).  
4 Proinflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$ , are  
5 secreted from macrophages that are recruited into obese adipose tissue and are known to play a  
6 major role in inflammation in adipose tissue (Weisberg et al., 2003). Indeed, in mice, ablation of the  
7 protein complex NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin  
8 domain-containing 3) inflammasome, which mediates IL-1 $\beta$  maturation and secretion, prevents  
9 obesity-induced inflammation and insulin resistance, a major risk factor for T2D (Stienstra et al.,  
10 2011; Vandanmagsar et al., 2011; Wen et al., 2011; Zhou et al., 2010).

11 NLRP3, a member of the NOD-like receptor (NLR) family, binds to the adaptor protein  
12 ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment  
13 domain (CARD)) and pro-caspase-1, the latent form of the cysteine protease caspase-1, in response  
14 to a variety of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular  
15 patterns (DAMPs), composing the NLRP3 inflammasome (Guo et al., 2015). In the inflammasome,  
16 processing and activation of pro-caspase-1 are induced, followed by caspase-1-mediated cleavage  
17 of the latent form of IL-1 $\beta$ . The NLR proteins other than NLRP3 also form their own  
18 inflammasomes, but caspase-1 is their common component (Sollberger et al., 2014; Wen et al.,  
19 2013).

20 Much attention has been paid to the role of caspase-1 in adipocytes as a mediator of  
21 inflammation in adipose tissue. It has been shown that expression of caspase-1 is upregulated  
22 during adipocyte differentiation and modulates adipocyte function (Stienstra et al., 2010). Thus, to

1 gain a better understanding of the roles of caspase-1 in adipose tissue inflammation, it is important  
2 to distinguish the activation mechanism and roles of caspase-1 in adipocytes from those in other  
3 types of cells, such as macrophages. To this end, cultured adipocytes, such as primary adipocytes  
4 and differentiated mouse 3T3-L1 adipocytes, are important tools. It has been reported that caspase-1  
5 induces cleavage of the NAD<sup>+</sup>-dependent deacetylase SIRT1 and thus inhibits its role as a  
6 regulatory sensor of nutrient availability in TNF- $\alpha$ -treated 3T3-L1 cells (Chalkiadaki and Guarente,  
7 2012). It has also been reported that caspase-1 may induce degradation of peroxisome  
8 proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), a transcription factor critical for differentiation and  
9 function of adipocytes, in 3T3-L1 cells treated with TNF- $\alpha$  and cycloheximide (CHX) (He et al.,  
10 2008). Despite such evidence of active adipocyte caspase-1, it is not fully understood how  
11 caspase-1 activation upon inflammatory stimuli is regulated in adipocytes.

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

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18

## 1 **Materials and Methods**

2

### 3 ***Reagents***

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

8

### 9 ***Cell culture***

10 Mouse 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were cultured in  
11 Dulbecco's modified Eagle's medium supplemented with 100 units/mL of penicillin G and 0.1  
12 mg/mL of streptomycin (DMEM) containing 10% calf serum. Differentiation of 3T3-L1  
13 preadipocytes into adipocytes was achieved by the following procedure: cells were cultured in the  
14 initiation medium of DMEM containing 10% fetal calf serum (FCS), 5  $\mu$ g/mL insulin  
15 (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Wako Pure Chemical, Osaka, Japan), and  
16 0.25  $\mu$ M dexamethasone (Sigma-Aldrich) for 2 days. After 3 days of culture in the progression  
17 medium (DMEM containing 10% FCS and 5  $\mu$ g/mL insulin), cells were cultured in the maintenance  
18 medium (DMEM containing 10% FCS), which was exchanged every other day. Cells were used for  
19 experiments at days 10-14 after the induction of differentiation. RAW264 (RIKEN BioResource  
20 Center, Tsukuba, Japan) and J774A.1 (American Type Culture Collection) macrophage-like cell  
21 lines were cultured in DMEM containing 10% FCS. Mouse peritoneal exudate cells were prepared  
22 as described previously (Schneider, 2013).

1

## 2 ***Quantitative reverse transcription (RT)- PCR***

3 Total RNA was isolated from 3T3-L1 cells or C57BL/6J mouse spleen using the TRI-reagent  
4 (Molecular Research Center, Cincinnati, OH), and cDNA was synthesized using the PrimeScript RT  
5 reagent kit with the gDNA Eraser kit (Takara Bio, Kyoto, Japan). Quantitative RT-PCR was  
6 performed by the Takara Thermal Cycler Dice (Takara Bio) using TaqMan Gene expression assays  
7 (pro-caspase1, Mm00438023\_m1; ASC, Mm00445747\_g1; NLRP3, Mm00840904\_m1; GAPDH,  
8 Mm99999915\_g1) (Applied Biosystems, Foster City, CA) or SYBR Green-based detection assays  
9 using SYBR premix Ex Taq II (Tli RNaseH Plus) (Takara Bio). In the SYBR Green-based assays,  
10 the oligonucleotide primers for NLRP1a, NLRP1b, NLRP3, and NLRC4 were described previously  
11 (Lech et al., 2010), and the following were used for glyceraldehyde-3-phosphate dehydrogenase  
12 (GAPDH): forward, 5'-TGTGTCCGTCGTGGATCTGA-3'; reverse,  
13 5'-TTGCTGTTGAAGTCGCAGGAG-3'. The relative expression of each mRNA was calculated  
14 and normalized to GAPDH mRNA in the same sample using the comparative CT method  
15 (Schmittgen and Livak, 2008).

16

## 17 ***Immunoblot analysis***

18 The cells were lysed with a lysis buffer containing 25 mM Tris-HCl [pH 7.5], 25 mM NaCl, 0.5  
19 mM EGTA, 5 µg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM Na pyrophosphate, 1  
20 mM Na-o-vanadate, 25 mM p-NPP, 25 mM β-glycerophosphate, 0.2 mM Na molybdate, 20 nM  
21 okadaic acid, and 1% Triton X-100; this was followed by sonication for 1 min. The lysates were  
22 centrifuged for 30 min at 15,000 x g, and the resulting supernatants were fractionated by

1 SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride  
2 membranes. The membranes were probed with primary antibodies and horseradish  
3 peroxidase-conjugated secondary antibodies (Promega, Madison, WI). Protein bands were  
4 visualized by the enhanced chemiluminescence system and analyzed by an ImageQuant LAS4000  
5 (GE Healthcare Bio-sciences, Piscataway, NJ). The following primary antibodies were used in this  
6 study: Caspase-1 antibody (Adipogen, San Diego, CA);  $\beta$ -actin antibody (Cell signaling, Danvers,  
7 MA); phospho-ERK1/2 antibody (Sigma-Aldrich) that detects the activation phosphorylation of  
8 ERK1 and ERK2, the two mammalian isoforms of ERK; and ERK1 antibody (Santa Cruz  
9 Biotechnology, Santa Cruz, CA). In some experiments, the intensity of each band was quantified  
10 using an image analysis software ImageQuant TL (GE Healthcare).

11

### 12 ***Measurement of caspase-1 activity***

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

20

### 21 ***Statistical analyses***

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

1 **Results**

2

3 **TNF- $\alpha$  induces expression and activation of caspase-1 in 3T3-L1 cells**

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

15

16 **TNF- $\alpha$  induces expression of NLRP3 but not ASC in 3T3-L1 cells**

17 Because caspase-1 is activated in the NLRP3 inflammasome in various contexts, we examined  
18 mRNA expression of its components in 3T3-L1 cells. Whereas expression of NLRP3, ASC, and  
19 pro-caspase-1 was easily detected in macrophage-like J774.A.1 cells, none of them were detected in  
20 unstimulated 3T3-L1 cells (**Fig. 2A**). Consistently with a previous report (Pelegrin et al., 2008),  
21 another macrophage-like cell line, RAW264, lacked ASC expression, serving as a negative control.  
22 Intriguingly, TNF- $\alpha$  strongly induced expression of NLRP3 as early as 1 hr after stimulation, and



1 TNF- $\alpha$ -dependent induction peaked at 3 hr after stimulation (**Fig. 2B, upper graph**). On the other  
2 hand, TNF- $\alpha$  did not induce ASC expression throughout the 6 hr stimulation period (**Fig. 2B, lower**  
3 **graph**). Also at the protein level, TNF- $\alpha$  did not induce ASC throughout the 24 hr stimulation  
4 period (**Fig. 2C**).

5

### 6 **TNF- $\alpha$ -induced expression of NLRP3 depends on the ERK pathway**

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

1 coordination with the NF- $\kappa$ B pathway.

2

3 **Neither expression nor activation of caspase-1 induced by TNF- $\alpha$  depends on the ERK**  
4 **pathway**

5 We next examined the requirement of the NF- $\kappa$ B and ERK pathways for TNF- $\alpha$ -induced  
6 pro-caspase-1 expression. Similar to NLRP3 expression, TNF- $\alpha$ -induced mRNA expression of  
7 pro-caspase-1 was suppressed to some extent by BAY11-7082 (**Fig. 4A**), suggesting that the NF- $\kappa$ B  
8 pathway is commonly required for TNF- $\alpha$ -induced expression of pro-caspase-1 and NLRP3.  
9 However, PD0325901 exerted no inhibitory effects on TNF- $\alpha$ -induced pro-caspase-1 expression at  
10 both the mRNA and protein levels (**Figs. 4B** and **4C**). PD0325901 also did not suppress  
11 TNF- $\alpha$ -induced caspase-1 activation (**Fig 4D**). These results suggest that TNF- $\alpha$ -induced expression  
12 and activation of caspase-1 is regulated independently of the ERK-mediated induction of NLRP3 in  
13 3T3-L1 cells. Together with the result that ASC was not detected even in the presence of TNF- $\alpha$   
14 (**Fig. 2**), the NLRP3 inflammasome does not appear to be involved in TNF- $\alpha$ -induced activation of  
15 caspase-1 in 3T3-L1 cells.

16

17 **NLR proteins that can activate caspase-1 independently of ASC are not induced by TNF- $\alpha$  in**  
18 **3T3-L1 cells**

19 The possibility that other NLR proteins, particularly those that can activate caspase-1 independently  
20 of ASC, might induce TNF- $\alpha$ -induced activation of caspase-1 in 3T3-L1 cells still remained. The  
21 corresponding NLR proteins reported so far are NLRP1a, NLRP1b, and NLRC4 (Broz et al., 2010;  
22 Masters et al., 2012; Van Opendenbosch et al., 2014). We thus examined their expression at the

1 mRNA level in 3T3-L1 cells after we determined the experimental conditions under which their  
2 expression was detected in RNA extracted from mouse spleen (**Fig. 5A**). However, neither the basal  
3 nor the TNF- $\alpha$ -induced expression of these NLR proteins was detected in 3T3-L1 cells (**Fig. 5B**).  
4 Thus, TNF- $\alpha$  may induce caspase-1 activation in an inflammasome-independent manner in 3T3-L1  
5 cells.

## 1 **Discussion**

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

6 Consistently with the requirement of inflammasomes for the maturation and secretion of  
7 IL-1 $\beta$  (Guo et al., 2015), we could not detect any IL-1 $\beta$  secretion from TNF- $\alpha$ -treated 3T3-L1 cells  
8 (data not shown), suggesting that caspase-1 induced and activated by TNF- $\alpha$  in these cells has  
9 functions different from inducing IL-1 $\beta$  secretion. As mentioned in the Introduction, it has been  
10 shown that caspase-1 induces the cleavage of SIRT1 in the same context (TNF- $\alpha$ -treated 3T3-L1  
11 cells), and this cleavage of a key metabolic regulator may contribute to the regulation of adipose  
12 tissue inflammation (Chalkiadaki and Guarente, 2012). Caspase-1 has also been proposed to induce  
13 the cleavage of PPAR $\gamma$ . Nevertheless, PPAR $\gamma$  cleavage induced by caspase-1 occurred only when  
14 3T3-L1 cells were treated with TNF- $\alpha$  in combination with CHX (He et al., 2008). Another group  
15 has reported that the other caspases, mainly caspase-3 and -6, are responsible for PPAR $\gamma$  cleavage in  
16 3T3-L1 cells treated only with TNF- $\alpha$  (Guilherme et al., 2009). Thus, further evidence is required to  
17 clarify whether PPAR $\gamma$  indeed is a substrate of caspase-1 in inflammatory-stimulated adipocytes.

18 Of note, previous reports have suggest that pro-caspase-1 binds to receptor interacting  
19 protein 2 (RIP2), a CARD-containing kinase, and promotes NF- $\kappa$ B activation independently of the  
20 protease activity of caspase-1 (Lamkanfi et al., 2004). Interestingly, ASC has been shown to  
21 compete with RIP2 for binding to caspase-1 (Sarkar et al., 2006), suggesting that this protease  
22 activity-independent function of caspase-1 is augmented in 3T3-L1 cells that appear to lack ASC.

1 Thus, TNF- $\alpha$ -induced expression of caspase-1 may be sufficient to induce an inflammatory  
2 response in adipocytes at least to some extent.

3 We also found that TNF- $\alpha$ -induced mRNA expression of NLRP3 was detected as early as  
4 1 hr after stimulation (**Fig. 2B**), suggesting that the *Nlrp3* gene is an immediate early gene  
5 responsive to TNF- $\alpha$  in 3T3-L1 cells. This is consistent with a previous report that in 3T3-L1 cells,  
6 NLRP3 expression was induced by 24-hr treatment with TNF- $\alpha$  or IL-1 $\beta$ , whereas ASC expression  
7 did not change, even in the presence of various pro-inflammatory cytokines including TNF- $\alpha$  and  
8 IL-1 $\beta$  (Yin et al., 2014). Intriguingly, the responsiveness of the *Nlrp3* gene to TNF- $\alpha$  was found to  
9 be largely dependent on the ERK pathway because MEK inhibitors strongly suppressed the  
10 TNF- $\alpha$ -induced expression of NLRP3 (**Fig. 3**). Mice deficient in ERK1, one of two ERK isoforms  
11 in mammals, that are challenged with a high-fat diet or are crossed with leptin-deficient (ob/ob)  
12 mice, have been shown to exhibit reduced insulin resistance compared with wild-type mice (Bost et  
13 al., 2005; Jager et al., 2011), although the molecular functions of ERK in this context are still  
14 unknown. These findings strongly suggest that the ERK pathway accelerates the inflammatory  
15 response of adipose tissue to various pro-inflammatory cytokines including TNF- $\alpha$ , and that  
16 ERK-dependent induction of NLRP3 in adipocytes may play some roles in this process.

17 Our results that NLRP3 appears to be dispensable for caspase-1 activation in  
18 TNF- $\alpha$ -treated 3T3-L1 cells suggest the existence of inflammasome-independent roles of NLRP3 in  
19 the adipocyte inflammatory response. It has recently been shown that regulation of  
20 chemokine-mediated functions of neutrophils, which contributes to hepatic ischemia-reperfusion  
21 injury, and promotion of TGF- $\beta$  signaling in kidney epithelium are both regulated by NLRP3  
22 independently of inflammasomes (Inoue et al., 2014; Wang et al., 2013). More recently, NLRP3 has

1 also been shown to function independently of inflammasomes as a transcriptional regulator of T  
2 helper type 2 (T<sub>H</sub>2) differentiation (Bruchard et al., 2015). Thus, caspase-1 and NLRP3 may  
3 function independently from each other in 3T3-L1 cells stimulated with pro-inflammatory cytokines,  
4 and therefore, the elucidation of their respective functions in adipocytes will shed new light on the  
5 regulatory mechanism of adipose tissue inflammation.

6

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10

1 **Literature Cited**

2

3 Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri  
4 T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E. 2009. Cutting edge: NF-kappaB  
5 activating pattern recognition and cytokine receptors license NLRP3 inflammasome  
6 activation by regulating NLRP3 expression. *J Immunol* 183:787-791.

7 Bost F, Aouadi M, Caron L, Even P, Belmonte N, Prot M, Dani C, Hofman P, Pages G,  
8 Pouyssegur J, Le Marchand-Brustel Y, Binetruy B. 2005. The extracellular  
9 signal-regulated kinase isoform ERK1 is specifically required for in vitro and in vivo  
10 adipogenesis. *Diabetes* 54:402-411.

11 Broz P, von Moltke J, Jones JW, Vance RE, Monack DM. 2010. Differential requirement for  
12 Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. *Cell*  
13 *Host Microbe* 8:471-483.

14 Bruchard M, Rebe C, Derangere V, Togbe D, Ryffel B, Boidot R, Humblin E, Hamman A,  
15 Chalmin F, Berger H, Chevriaux A, Limagne E, Apetoh L, Vegran F, Ghiringhelli F. 2015.  
16 The receptor NLRP3 is a transcriptional regulator of TH2 differentiation. *Nat Immunol*  
17 16:859-870.

18 Chalkiadaki A, Guarente L. 2012. High-Fat Diet Triggers Inflammation-Induced Cleavage of  
19 SIRT1 in Adipose Tissue To Promote Metabolic Dysfunction. *Cell Metab* 16:180-188.

20 Donath MY, Shoelson SE. 2011. Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol*  
21 11:98-107.

22 Guilherme A, Tesz GJ, Guntur KV, Czech MP. 2009. Tumor necrosis factor-alpha induces  
23 caspase-mediated cleavage of peroxisome proliferator-activated receptor gamma in  
24 adipocytes. *J Biol Chem* 284:17082-17091.

25 Guo H, Callaway JB, Ting JP. 2015. Inflammasomes: mechanism of action, role in disease, and  
26 therapeutics. *Nat Med* 21:677-687.

27 He F, Doucet JA, Stephens JM. 2008. Caspase-mediated degradation of PPARgamma proteins  
28 in adipocytes. *Obesity* 16:1735-1741.

29 Inoue Y, Shirasuna K, Kimura H, Usui F, Kawashima A, Karasawa T, Tago K, Dezaki K,  
30 Nishimura S, Sagara J, Noda T, Iwakura Y, Tsutsui H, Taniguchi S, Yanagisawa K, Yada  
31 T, Yasuda Y, Takahashi M. 2014. NLRP3 regulates neutrophil functions and contributes  
32 to hepatic ischemia-reperfusion injury independently of inflammasomes. *J Immunol*

1 192:4342-4351.

2 Jager J, Corcelle V, Gremeaux T, Laurent K, Waget A, Pages G, Binetruy B, Le  
3 Marchand-Brustel Y, Burcelin R, Bost F, Tanti JF. 2011. Deficiency in the extracellular  
4 signal-regulated kinase 1 (ERK1) protects leptin-deficient mice from insulin resistance  
5 without affecting obesity. *Diabetologia* 54:180-189.

6 Lamkanfi M, Kalai M, Saelens X, Declercq W, Vandenabeele P. 2004. Caspase-1 activates  
7 nuclear factor of the kappa-enhancer in B cells independently of its enzymatic activity. *J*  
8 *Biol Chem* 279:24785-24793.

9 Lech M, Avila-Ferruffino A, Skuginna V, Susanti HE, Anders HJ. 2010. Quantitative expression  
10 of RIG-like helicase, NOD-like receptor and inflammasome-related mRNAs in humans  
11 and mice. *International immunology* 22:717-728.

12 Masters SL, Gerlic M, Metcalf D, Preston S, Pellegrini M, O'Donnell JA, McArthur K, Baldwin  
13 TM, Chevrier S, Nowell CJ, Cengia LH, Henley KJ, Collinge JE, Kastner DL,  
14 Feigenbaum L, Hilton DJ, Alexander WS, Kile BT, Croker BA. 2012. NLRP1  
15 inflammasome activation induces pyroptosis of hematopoietic progenitor cells.  
16 *Immunity* 37:1009-1023.

17 Pelegrin P, Barroso-Gutierrez C, Surprenant A. 2008. P2X7 receptor differentially couples to  
18 distinct release pathways for IL-1beta in mouse macrophage. *J Immunol* 180:7147-7157.

19 Sabio G, Davis RJ. 2014. TNF and MAP kinase signalling pathways. *Seminars in immunology*  
20 26:237-245.

21 Sarkar A, Duncan M, Hart J, Hertlein E, Guttridge DC, Wewers MD. 2006. ASC Directs NF- B  
22 Activation by Regulating Receptor Interacting Protein-2 (RIP2) Caspase-1 Interactions.  
23 *J Immunol* 176:4979-4986.

24 Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative C(T) method.  
25 *Nat Protoc* 3:1101-1108.

26 Schneider M. 2013. Collecting resident or thioglycollate-elicited peritoneal macrophages.  
27 *Methods Mol Biol* 1031:37-40.

28 Sebolt-Leopold JS, Herrera R. 2004. Targeting the mitogen-activated protein kinase cascade to  
29 treat cancer. *Nat Rev Cancer* 4:937-947.

30 Sollberger G, Strittmatter GE, Garstkiewicz M, Sand J, Beer HD. 2014. Caspase-1: the  
31 inflammasome and beyond. *Innate immunity* 20:115-125.

32 Stienstra R, Joosten LA, Koenen T, van Tits B, van Diepen JA, van den Berg SA, Rensen PC,  
33 Voshol PJ, Fantuzzi G, Hijmans A, Kersten S, Muller M, van den Berg WB, van Rooijen



1 N, Wabitsch M, Kullberg BJ, van der Meer JW, Kanneganti T, Tack CJ, Netea MG. 2010.  
2 The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and  
3 insulin sensitivity. *Cell Metab* 12:593-605.

4 Stienstra R, van Diepen JA, Tack CJ, Zaki MH, van de Veerdonk FL, Perera D, Neale GA,  
5 Hooiveld GJ, Hijmans A, Vroegrijk I, van den Berg S, Romijn J, Rensen PC, Joosten LA,  
6 Netea MG, Kanneganti TD. 2011. Inflammasome is a central player in the induction of  
7 obesity and insulin resistance. *Proc Natl Acad Sci U S A* 108:15324-15329.

8 Tanimura S, Asato K, Fujishiro SH, Kohno M. 2003. Specific blockade of the ERK pathway  
9 inhibits the invasiveness of tumor cells: down-regulation of matrix  
10 metalloproteinase-3/-9/-14 and CD44. *Biochem Biophys Res Commun* 304:801-806.

11 Van Opdenbosch N, Gurung P, Vande Walle L, Fossoul A, Kanneganti TD, Lamkanfi M. 2014.  
12 Activation of the NLRP1b inflammasome independently of ASC-mediated caspase-1  
13 autoproteolysis and speck formation. *Nature communications* 5:3209.

14 Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, Ravussin E,  
15 Stephens JM, Dixit VD. 2011. The NLRP3 inflammasome instigates obesity-induced  
16 inflammation and insulin resistance. *Nat Med* 17:179-188.

17 Wang W, Wang X, Chun J, Vilaysane A, Clark S, French G, Bracey NA, Trpkov K, Bonni S, Duff  
18 HJ, Beck PL, Muruve DA. 2013. Inflammasome-independent NLRP3 augments  
19 TGF-beta signaling in kidney epithelium. *J Immunol* 190:1239-1249.

20 Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. 2003. Obesity is  
21 associated with macrophage accumulation in adipose tissue. *J Clin Invest*  
22 112:1796-1808.

23 Wen H, Gris D, Lei Y, Jha S, Zhang L, Huang MT, Brickey WJ, Ting JP. 2011. Fatty acid-induced  
24 NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nat Immunol*  
25 12:408-415.

26 Wen H, Miao EA, Ting JP. 2013. Mechanisms of NOD-like receptor-associated inflammasome  
27 activation. *Immunity* 39:432-441.

28 Yin Z, Deng T, Peterson LE, Yu R, Lin J, Hamilton DJ, Reardon PR, Sherman V, Winnier GE,  
29 Zhan M, Lyon CJ, Wong ST, Hsueh WA. 2014. Transcriptome analysis of human  
30 adipocytes implicates the NOD-like receptor pathway in obesity-induced adipose  
31 inflammation. *Mol Cell Endocrinol* 394:80-87.

32 Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. 2010. Thioredoxin-interacting protein links  
33 oxidative stress to inflammasome activation. *Nat Immunol* 11:136-140.

1 **Figure legends**

2

3 **Figure 1. TNF- $\alpha$  induces expression and activation of caspase-1 in 3T3-L1 cells**

4 (A) TNF- $\alpha$ -induced mRNA expression of pro-caspase-1. 3T3-L1 cells were treated with or without  
5 20 ng/mL TNF- $\alpha$  for the indicated time periods. The relative expression of pro-caspase-1 was  
6 examined by quantitative RT-PCR. Data are shown as the mean  $\pm$  SEM (n=3).

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

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

16

17 **Figure 2. TNF- $\alpha$  induces mRNA expression of NLRP3 in 3T3-L1 cells**

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

21 (B) TNF- $\alpha$ -induced mRNA expression of NLRP3 (upper graph) and ASC (lower graph). 3T3-L1  
22 cells were treated with or without 20 ng/mL TNF- $\alpha$  for the indicated time periods. The relative gene

1 expression was examined by quantitative RT-PCR. Data are shown as the mean  $\pm$  SEM (n=3).  
2 (C) Protein expression of ASC in 3T3-L1 cells. 3T3-L1 cells were treated with or without 20  
3 ng/mL TNF- $\alpha$  for the indicated time periods. As a positive control, mouse peritoneal exudate cells  
4 (PEC) were pre-treated with 100 ng/mL LPS for 4 hr and then treated with 5 mM ATP for 30 min.  
5 The cell lysates were subjected to immunoblot analysis using the indicated antibodies.

6

7 **Figure 3. TNF- $\alpha$ -induced mRNA expression of NLRP3 depends on the ERK pathway in**  
8 **3T3-L1 cells**

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

15 (C) Effect of PD0325901 on TNF- $\alpha$ -induced activation of ERK1 and ERK2. The cells were  
16 pre-treated with or without 2  $\mu$ M PD0325901 for 30 min and then treated with 20 ng/mL TNF- $\alpha$  for  
17 6 hr, and the cell lysates were subjected to immunoblot analysis using the indicated antibodies.

18

19 **Figure 4. Neither expression nor activation of caspase-1 induced by TNF- $\alpha$  depends on the**  
20 **ERK pathway**

21 (A, B) Effects of BAY11-7082 and PD0325901 on TNF- $\alpha$ -induced mRNA expression of  
22 pro-caspase-1. 3T3-L1 cells were pre-treated with or without 10  $\mu$ M BAY11-7082 (A) or 2  $\mu$ M

1 PD0325901 **(B)** for 30 min and then treated with 20 ng/mL TNF- $\alpha$  for 3 hr. The relative expression  
2 of pro-caspase-1 was examined by quantitative RT-PCR. Data are shown as the mean  $\pm$  SEM (n=3).  
3 \* $p$  < 0.05, n.s. = not significant.

4 **(C)** Effect of PD0325901 on TNF- $\alpha$ -induced protein expression of pro-caspase-1. The cells were  
5 pre-treated with or without 2  $\mu$ M PD0325901 for 30 min and then treated with 20 ng/mL TNF- $\alpha$  for  
6 12 hr, and the cell lysates were subjected to immunoblot analysis using the indicated antibodies.

7 **(D)** Effect of PD0325901 on TNF- $\alpha$ -induced activation of caspase-1. The cells were pre-treated  
8 with or without 2  $\mu$ M PD0325901 for 30 min and then treated with 20 ng/mL TNF- $\alpha$  for 6 hr, and  
9 caspase-1 activity was measured in the cell lysates. Data are shown as the mean  $\pm$  SEM (n=3). \* $p$  <  
10 0.05, \*\* $p$  < 0.01.

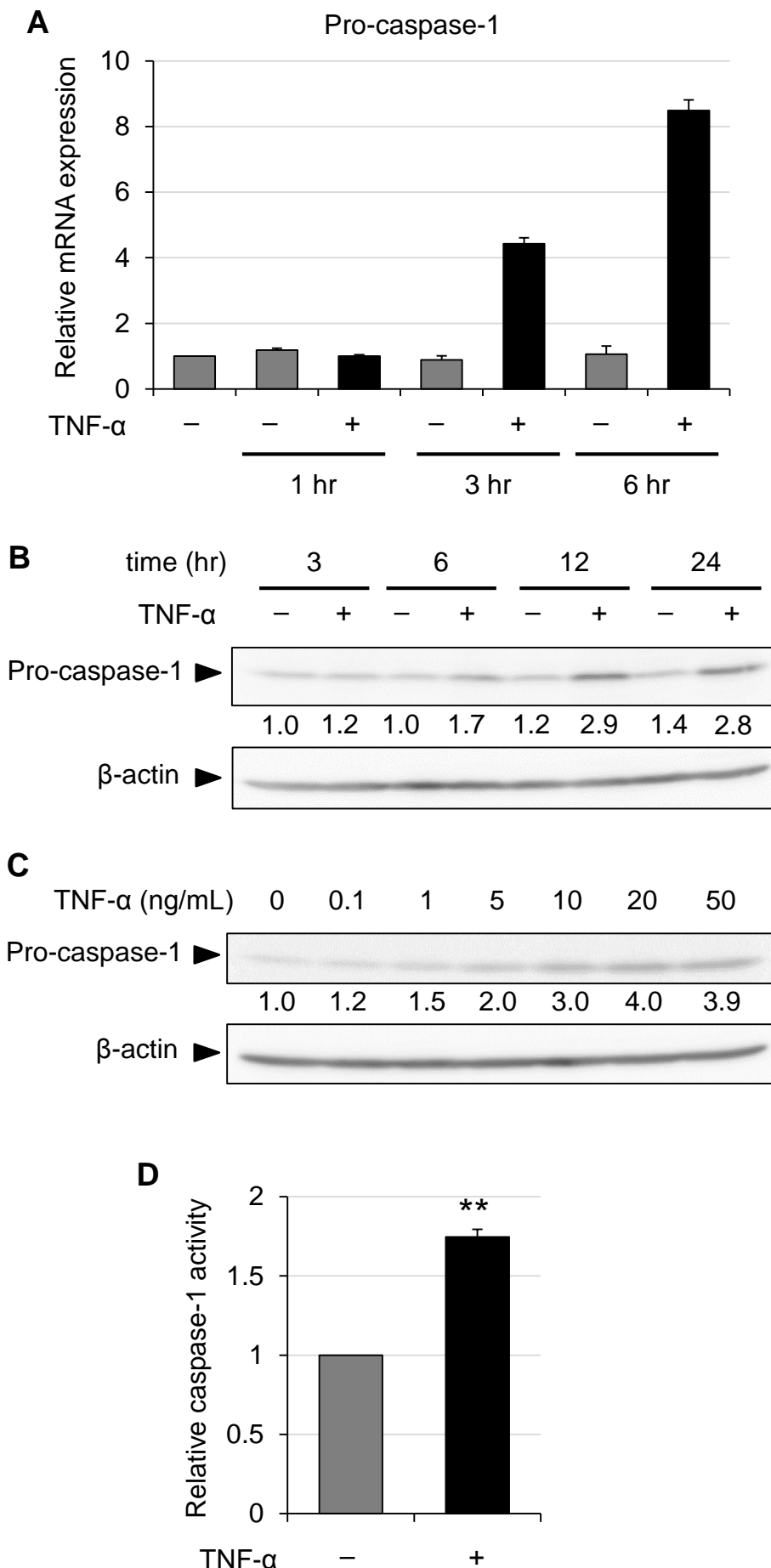
11

12 **Figure 5. NLR proteins that can activate caspase-1 independently of ASC are not induced by**  
13 **TNF- $\alpha$  in 3T3-L1 cells**

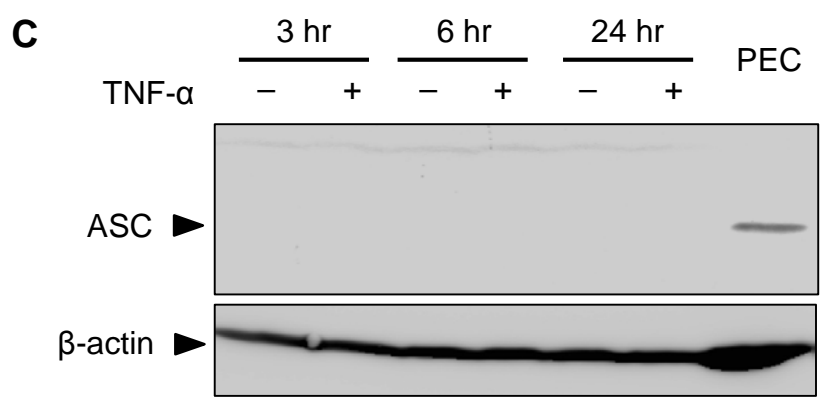
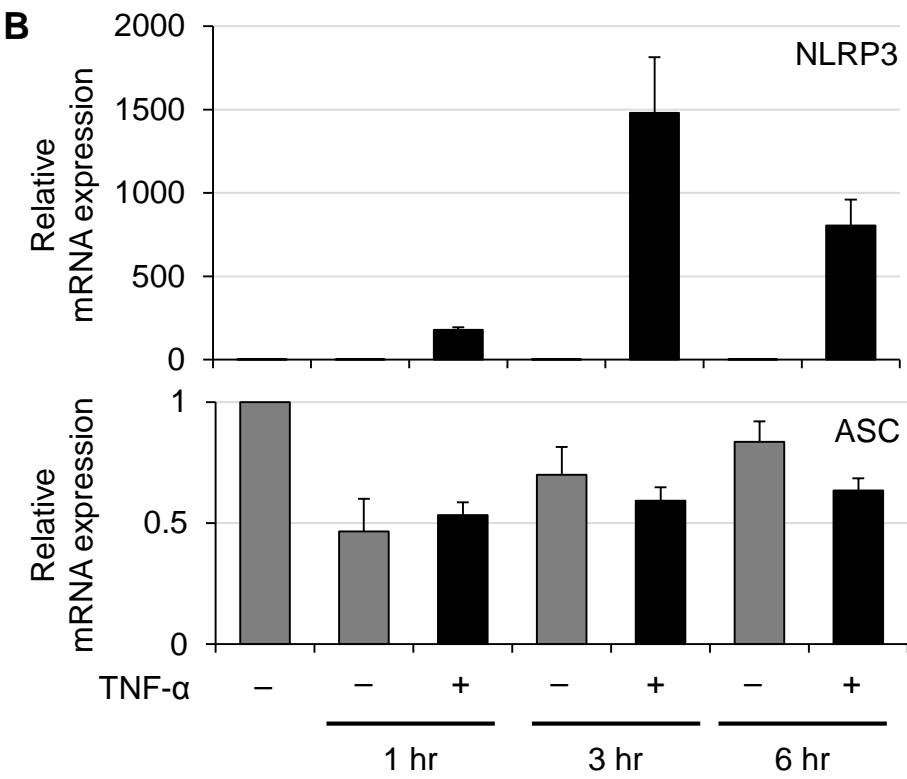
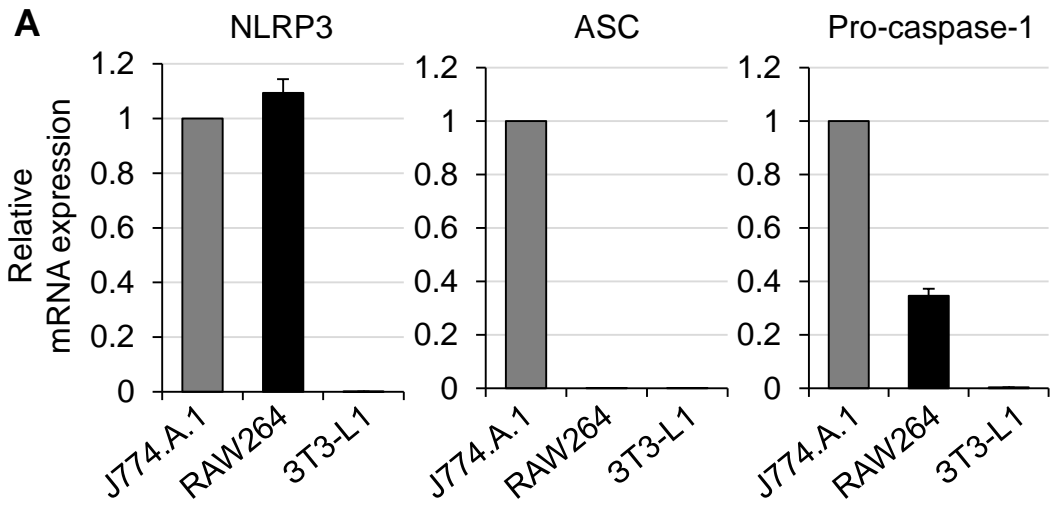
14 **(A)** Validation of quantitative RT-PCR for detecting mRNA of various NLR proteins using mouse  
15 spleen RNA. Expression of the indicated NLR was examined by quantitative RT-PCR using SYBR  
16 Green-based detection assays. Data are shown as the mean  $\pm$  SEM (n=3). **(B)** TNF- $\alpha$ -induced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

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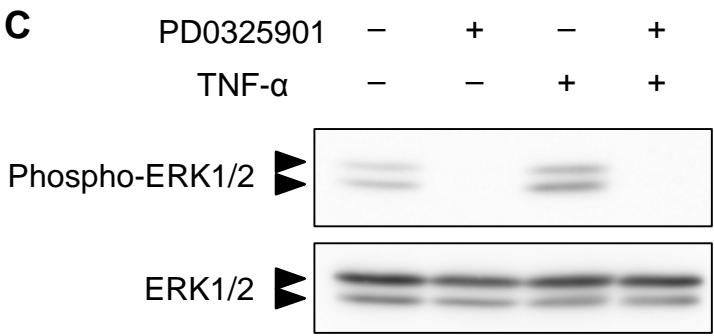
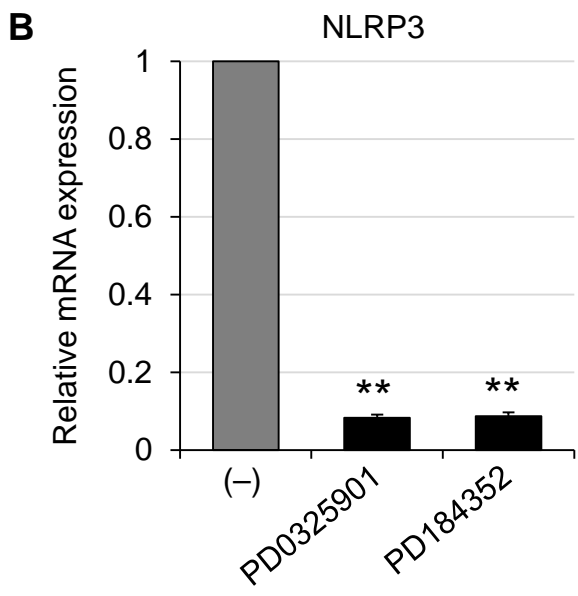
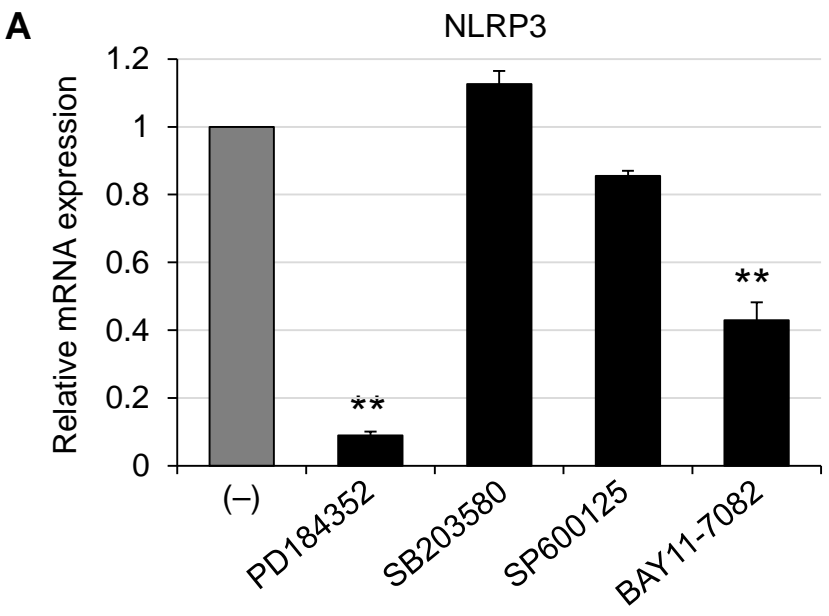
**Fig. 1**



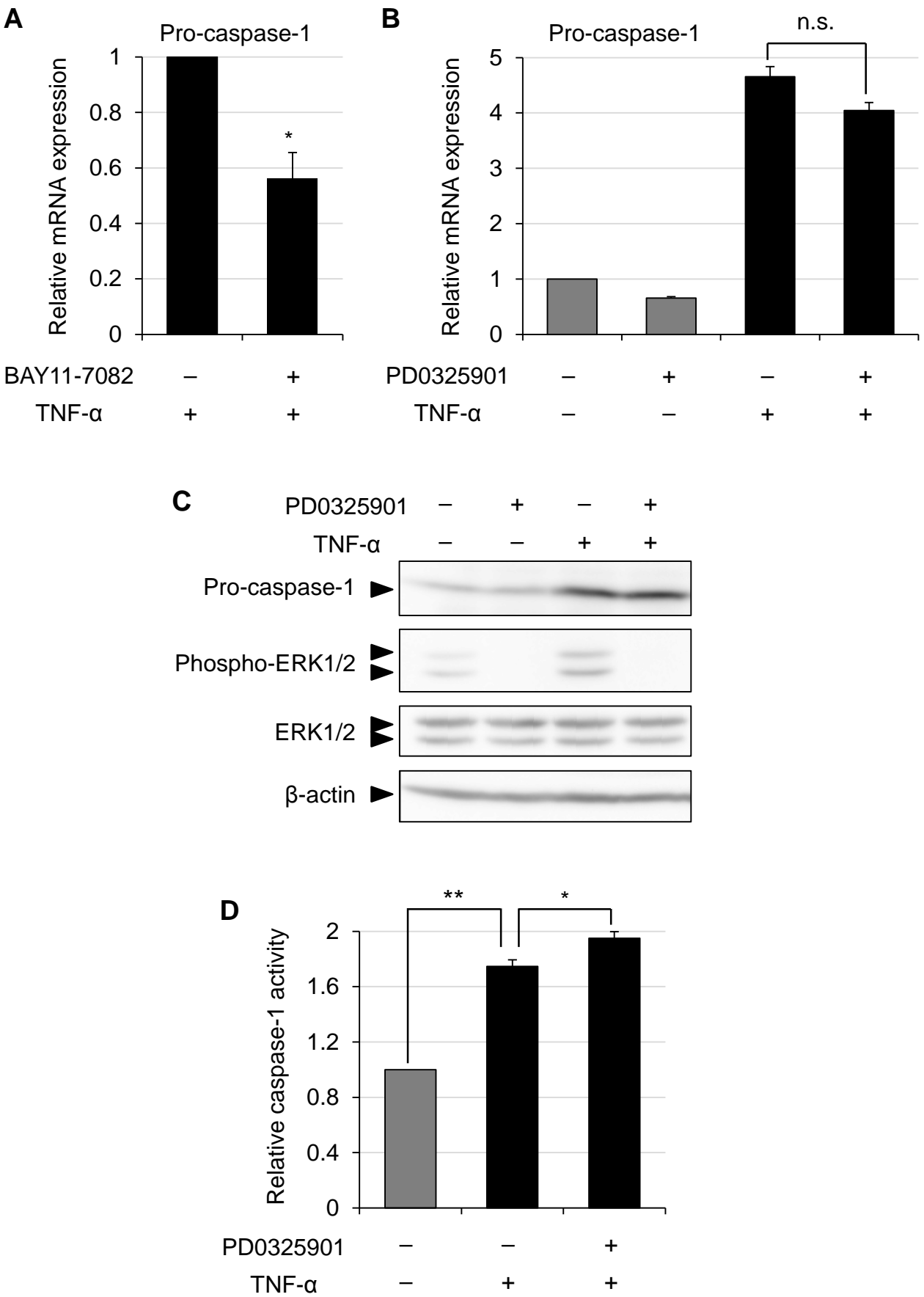
**Fig. 2**



**Fig. 3**



**Fig. 4**





**Fig. 5**

