Effects of IL-1 receptor-associated kinase (IRAK) expression on IL-1 signaling are independent of its kinase activity

J. Knop, M.U. Martin*

Institut für Molekularpharmakologie OE 5320, Medizinische Hochschule Hannover, D-30623 Hannover, Germany

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Abstract Interleukin-1 (IL-1) stimulates the association of the IL-1 receptor-associated protein kinase (IRAK) with the heterodimer of IL-1RI and IL-1RAcP via the adapter protein MyD88. In the receptor complex IRAK becomes heavily phosphorylated and concomitantly activated. Here we show that overexpression of a kinase-inactive mutant of IRAK (K239S) inhibits neither IL-1-stimulated activation of the transcription factor NF-κB, nor that of the c-Jun N-terminal kinase nor IL-2 production in murine EL-4 cells, but enhances these effects in a manner comparable to wild type IRAK. This strongly suggests that the intrinsic kinase activity is not required for downstream signaling via IRAK.

Key words: Interleukin-1 signaling;
Interleukin-1 receptor-associated protein kinase

1. Introduction

Interleukin-1 (IL-1) is an important pro-inflammatory cytokine centrally involved in the activation of the immune response [1]. IL-1 binds to two specific plasma membrane receptors which both belong to the IL-1 receptor/Toll family [2]. Active signaling complexes are formed by the association of IL-1RAcP with ligated IL-1RI [3,4]. The cytoplasmic domains of both IL-1RI and IL-1RAcP are required for IL-1 signaling to occur [5–7]. Very rapidly after IL-1 stimulation of cells, a serine/threonine-specific protein kinase activity can be coprecipitated with the IL-1RI [8–10]. This kinase was cloned and termed IL-1 receptor-associated kinase (IRAK) [11]. IRAK associates with the IL-1RI/IL-1RAcP heterodimer via the adaptor protein MyD88 involving the interaction of the 'death domains' in IRAK and MyD88, respectively [12–14]. In the active IL-1 receptor complex IRAK becomes heavily phosphorylated, strongly suggesting that IRAK auto- or crossphosphorylates and thus activates itself for downstream signaling events, namely the interaction with TRAF6 [15], leading to activation of the transcription factor NF-κB.

On the other hand, it was suggested that hyperphosphorylation of IRAK is a signal for rapid proteolytic degradation by a proteasome-dependent mechanism [16]. Thus, it is currently unclear whether the kinase activity of IRAK is indispensable for the initiation of downstream signaling, i.e. by phosphorylating subsequent target molecules, or whether the phosphorylation is only involved in regulating the stability of IRAK molecules.

In order to elucidate the requirement of IRAK's kinase activity in IL-1 signaling we employed a kinase-inactive mutant of human IRAK (K239S). Here we show that overexpression of IRAK (K239S) over a background of endogenous murine IRAK surprisingly neither negatively affects IL-1-stimulated activation of the transcription factor NF-κB, nor the activation of the c-Jun N-terminal kinase (JNK), nor the IL-1-induced IL-2 production of EL-4 cells. In contrast, IRAK (K239S) behaves like wild type IRAK with respect to the IL-1 signaling events determined, suggesting that the intrinsic kinase activity of IRAK and hyperphosphorylation of IRAK are not essential for IL-1-stimulated signaling.

2. Materials and methods

2.1. Expression vectors and transfection

Mammalian expression vectors encoding wild type human IRAK (pRK5-IRAK) and human IRAK with a defect ATP binding site (IRAK K239S) were kind gifts of Z. Cao (Tularik Inc., South San Francisco, CA, USA) and have been described [13]. The control plasmid pRK5* was generated from pRK5-IRAK by excising the insert. The NF-κB-luciferase reporter gene construct 5×NF-κB-luc was kindly provided by W. Falk (Regensburg, Germany). The plasmid pHA-JNK1 encoding N-terminal HA-tagged human JNK1 [17] was generated by the polymerase chain reaction (PCR) cloning technique.

EL-4 6.1 were maintained at 37°C, 5% CO_2 in RPMI 1640 supplemented with 5% (v/v) fetal calf serum and transiently transfected using the DEAE-dextran procedure exactly as described previously [18]. In all experiments, the amount of transfected DNA was kept constant by adding control plasmid.

2.2. Detection of IRAK protein and kinase activity

For immunoprecipitation of IRAK 1×10^7 cells were transfected with the indicated amounts of expression constructs. 24 h after transfection cells were either left untreated or stimulated with rhIL-1β (10 ng/ml, Dompé, L'Aquila, Italy) at 37°C for the times indicated. Cells were collected, washed and lysed for 30 min in a lysis buffer containing 50 mM HEPES pH 7.9, 250 mM NaCl, 20 mM glycerophosphate, 5 mM *p*-nitrophenylphosphate, 1 mM EDTA-Na_2, 1 mM Na-*ortho*-vanadate, 0.5% (v/v) Nonidet P-40, 10% (v/v) glycerol, 5 mM DTT and protease inhibitors (Complete, Roche Diagnostics, Mannheim, Germany). Nuclei and cellular debris were removed by centrifugation (13 000×*g*, 30 min, 4°C). IRAK was precipitated with 0.5 μl of a polyclonal serum (kindly provided by Z. Cao) and 25 μl of 50% (v/v) protein G-Sepharose slurry (Pharmacia, Freiburg, Germany). Immunoprecipitates were subjected to an in vitro kinase assay using myelin basic protein (MBP, Sigma, Deisenhofen, Germany) as exogenous substrate as previously described [8]. Phosphorylated proteins were separated by SDS-PAGE and visualized by phosphoimaging.

2.3. Western blotting

Immunoprecipitates were heated in SDS sample buffer, proteins separated by SDS-PAGE, and transferred to PVDF membranes. Membranes were incubated with the indicated antibodies and reactive bands were visualized with horseradish peroxidase-coupled secondary antibodies by an enhanced chemoluminescence detection system (Pierce, Rockford, IL, USA).

*Corresponding author. Fax: (49) (511) 532 4081.
E-mail: martin.michael@mh-hannover.de

Abbreviations: IL-1, interleukin-1; IRAK, IL-1 receptor-associated kinase; wt, wild type; rh, recombinant human

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2.4. Reporter gene assays

For detection of NF-κB-dependent reporter gene activity, 5×10^6 cells were cotransfected with the indicated expression constructs and 0.5 μg 5×NF-κB-luc. After 4 h, cells were either left untreated, or stimulated with 10 ng/ml rhIL-1β or 100 ng/ml rhTNFα (BASF AG, Ludwigshafen, Germany) for an additional 20 h before harvest. Luciferase activity was determined using the Luciferase Assay System (Promega, Mannheim, Germany) and normalized on the basis of protein concentration as measured according to Bradford [19] with reagents from Bio-Rad (Munich, Germany).

2.5. In vitro SAP kinase assay

As a representative of SAP kinases the activity of cotransfected c-Jun N-terminal kinase was measured. 1×10^7 cells were cotransfected with the indicated expression constructs and 0.5 μg pHA-JNK1. After 24 h, cells were either left untreated, or stimulated with 10 ng/ml rhIL1β or 0.5 M sorbitol for 15 min. Cells were lysed as described in Section 2.2 and JNK1 was precipitated with 0.5 μg of the monoclonal anti-HA antibody (12CA5, ATCC) and 25 μl of 50% (v/v) protein G-Sepharose slurry (Pharmacia, Freiburg). One half of the immunoprecipitates was subjected to an in vitro kinase assay using recombinant GST-c-Jun(1–79) (Stratagene, Heidelberg, Germany) as substrate exactly as described [4]. The second half of the immunoprecipitates was used to control JNK1 expression by immunoblotting with the antibody 12CA5.

2.6. Quantification of IL-2 production by ELISA technique

5×10^6 cells were transfected with the indicated expression constructs. After 4 h, the cells were left untreated or stimulated with 10 ng/ml rhIL-1β for an additional 20 h. Supernatants were collected and IL-2 was measured using antibody pairs for mouse IL-2 according to the manufacturer's instructions (BioSOURCE Europe, Fleurus, Belgium).

3. Results

3.1. Mutant human IRAK (K239S) is not enzymatically active

An in vitro kinase assay was performed with IRAK immunoprecipitated from transiently transfected EL-4 cells in the presence of the exogenous substrate MBP. While background phosphorylation in untreated cells was barely detectable, IL-1 stimulation of endogenous murine IRAK (which was also precipitated by the antiserum) for 10 or 60 min resulted in autophosphorylation (Fig. 1A, control, upper panel) and phosphorylation of MBP (Fig. 1A, control, lower panel). Overexpression of human IRAK (wt) resulted in a pronounced phosphorylation in the range of 80–100 kDa upon IL-1 stimulation most likely due to auto- or crossphosphorylation of the immunoprecipitated human IRAK (Fig. 1A, IRAK (wt), upper panel). Furthermore, the exogenous substrate MBP became phosphorylated (Fig. 1A, IRAK (wt), lower panel). If mutant IRAK (K239S) was overexpressed in EL-4 cells only the weak phosphorylation of endogenous murine IRAK was detectable after IL-1 stimulation and no additional phosphorylation of mutant IRAK (K239S) was observed (Fig. 1A, IRAK (K239S), upper panel).

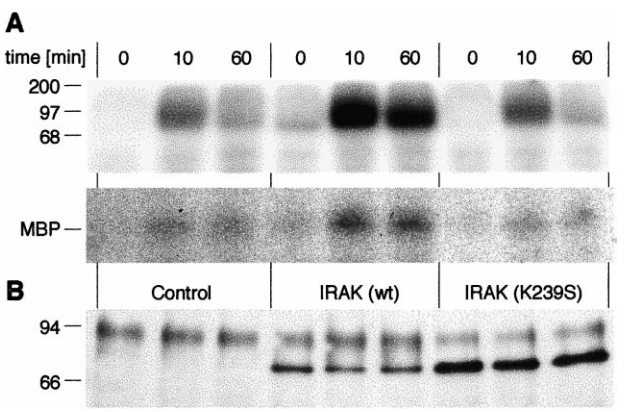

Fig. 1. Detection of IRAK activity (in vitro kinase assay, A) and IRAK protein (immunoblot, B). 1×10^7 EL-4 cells were transfected with 0.1 μg (A) or 0.5 μg (B) control plasmid or vectors encoding IRAK (wt) or IRAK (K239S), respectively. After 24 h cells were stimulated with 10 ng/ml rhIL-1β for 0, 10 or 60 min. IRAK was precipitated from cell lysates using an anti-IRAK antiserum. Immunoprecipitates were subjected to an in vitro kinase assay with MBP as exogenous substrate (A) or immunoblotted with the anti-IRAK antiserum (B).

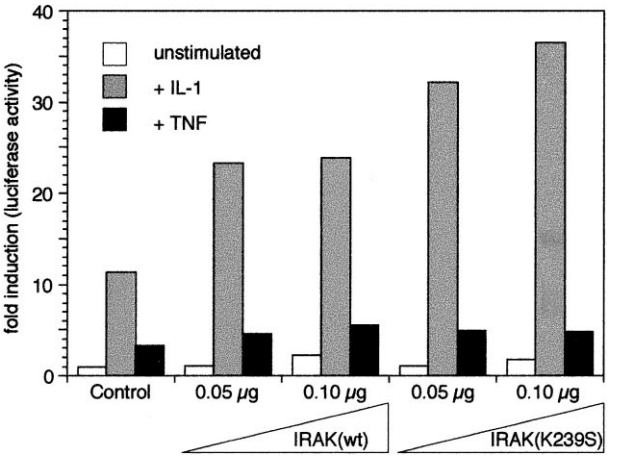

Fig. 2. Effect of IRAK (K239S) expression on IL-1-stimulated NF-κB activation. 5×10^6 EL-4 cells were transiently transfected with 0.5 μg of the reporter plasmid 5×NF-κB-luc and the indicated vector constructs. After 4 h, cells were either left untreated or were stimulated with 10 ng/ml rhIL-1β or 100 ng/ml TNF-α for 20 h. Luciferase activities were determined and normalized on the basis of protein concentration. Three independent experiments produced similar results.

Protein expression of human IRAK (wt) and IRAK (K239S) in transiently transfected cells was checked by Western blotting. While no signal was detectable in vector-only transfected cells (expression of endogenous murine IRAK in EL-4 cells is below the detection limit, Fig. 1B, control), signals were observed in EL-4 cells transfected with either human IRAK (wt) or IRAK (K239S) (Fig. 1B). Consistently, more protein of IRAK (K239S) was detected than of IRAK (wt) if the same concentration of plasmids were used for transfection.

3.2. Kinase-inactive IRAK (K239S) is not dominant negative for IL-1-stimulated NF-κB transactivating activity but can activate NF-κB in an IL-1-dependent manner

IL-1 and tumor necrosis factor (TNF) stimulated the activation of the transcription factor NF-κB in EL-4 cells as measured by a NF-κB-dependent luciferase reporter system (Fig. 2, control). Transient overexpression of human IRAK (wt) resulted in a strong increase in IL-1-stimulated reporter gene expression, whereas TNF-stimulated NF-κB activity remained unchanged (Fig. 2, IRAK (wt)). Overexpression of kinase-inactive IRAK (K239S) yielded the same result. While TNF-stimulated NF-κB activity remained unaffected, IL-1-stimulated NF κB activity by far exceeded the level achieved in mock-transfected cells.

3.3. Kinase-inactive IRAK (K239S) does not inhibit IL-1-stimulated activation of c-Jun N-terminal kinase but activates JNK in an IL-1-dependent manner

In EL-4 cells transfected with vector only IL-1 and osmotic stress (0.5 M sorbitol) stimulated the activation of JNK. Transient overexpression of IRAK (wt) resulted in an increase in IL-1-stimulated GST-c-Jun phosphorylation (Fig. 3A, upper panel, Fig. 3B, left panel). Overexpression of kinase-inactive IRAK (K239S) also led to a strong IL-1-dependent stimulation of JNK activity (Fig. 3A, lower panel, Fig. 3B, right panel). The stress-induced phosphorylation of GST-c-Jun was unaffected by overexpression of either IRAK (wt) or IRAK (K239S).

3.4. Overexpression of IRAK (wt) or kinase-inactive IRAK (K239S) leads to an increase in IL-1-dependent IL-2 synthesis and release

Unstimulated EL-4 cells do not produce IL-2. IL-1 weakly induced the synthesis and release of IL-2 (Fig. 4, control). This IL-1-dependent release of IL-2 was greatly enhanced by overexpression of IRAK (wt) (Fig. 4, IRAK (wt)), an effect previously described for permanent EL-4 clones expressing human IRAK (wt) [18]. Overexpression of IRAK (K239S) did not abolish but enhanced IL-1-stimulated IL-2 synthesis in a fashion qualitatively comparable to IRAK (wt) (Fig. 4, IRAK (K239S)).

In all parameters used as readout, IL-1-stimulated signals from EL-4 cells transiently transfected with IRAK (K239S) tended to exceed those achieved with IRAK (wt). This effect is most likely due to the higher protein levels detected for IRAK (K239S) by Western blotting.

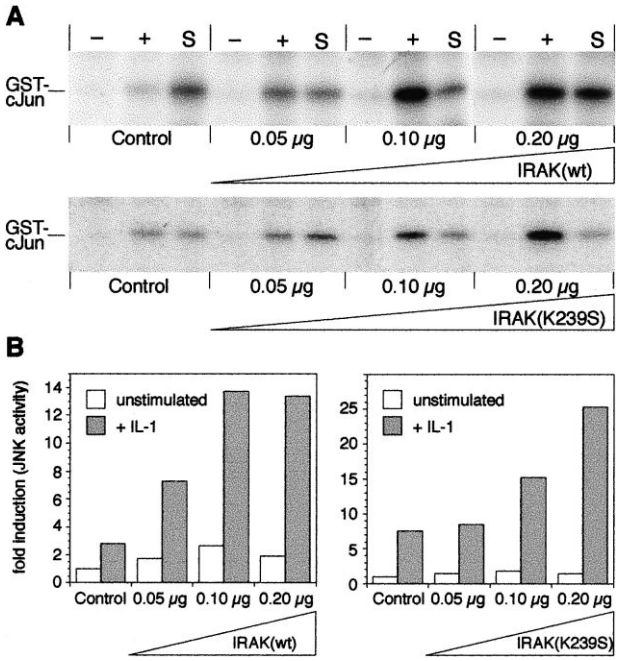

Fig. 3. Influence of IRAK (K239S) expression on IL-1-stimulated JNK activation. 5×10^6 EL-4 cells were transfected with 0.5 μg of the plasmid pHA-JNK1 and the indicated expression constructs. After 24 h, cells were either left untreated (−) or were stimulated with 10 ng/ml rhIL-1β (+) or 0.5 M sorbitol (S) for 15 min. Epitope-tagged JNK1 was precipitated from cell lysates using the anti-HA antibody 12CA5. Immunoprecipitates were subjected to an in vitro kinase assay with GST-c-Jun as exogenous substrate (A). Phosphorylation signals were quantitated densitometrically (B) and normalized on the basis of JNK1 expression as detected by Western blot analysis of the immunoprecipitates with the anti-HA antibody (data not shown). Three independent experiments produced similar results.

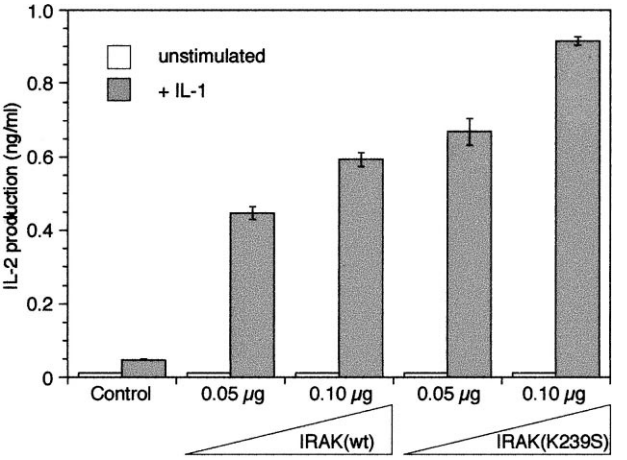

Fig. 4. For quantification of IL-1-stimulated IL-2 production, cells were transiently transfected with the indicated expression vectors for 4 h and were subsequently left untreated or were stimulated with 10 ng/ml rhIL-1β for 18 h. IL-2 released into the supernatant was measured by ELISA (IL-2 production of unstimulated cells was below the detection limit of 15.6 pg/ml). Bars express the means of quadruplicate values from one out of two experiments with similar results.

4. Discussion

IRAK is a central element in IL-1 signaling. Permanent overexpression of IRAK enhanced and prolonged IL-1-stimulated activation of NF-κB and JNK in EL-4 cells [18], while IRAK-deficient murine embryonal fibroblasts showed a drastically reduced response to IL-1 [20]. Upon IL-1 stimulation, IRAK is recruited to the IL-1 receptor complex and auto- or crossphosphorylates itself, leading to a pronounced shift in electrophoretic mobility of IRAK from 80 to 100 kDa [11]. This hyperphosphorylation precedes interaction with TRAF6, a molecule coupling IRAK to downstream effectors such as NIK [15]. By analogy to other protein kinases in cytokine signaling, it is commonly assumed that the enzymatic activity of IRAK is required for IL-1-dependent activation of itself, allowing phosphorylation of downstream components responsible for relaying the IL-1 signal to the level of transcription factors such as NF-κB and AP-1. However, no endogenous substrate for IRAK has been molecularly identified yet, which is required for IL-1 signaling. Recently, a homologue of IRAK, IRAK-2, was identified by EST database search [12], which is not active as a kinase (Muzio and Wesche, personal communication) but is able to activate NF-κB if overexpressed. In addition, a recently published abstract [21] suggests that IRAK's kinase activity may not be required for IL-1 signaling.

In order to address the question whether IRAK's enzymatic activity is required to allow downstream signaling, we expressed a mutant form of human IRAK (K293S) in murine EL-4 cells. In this mutant, the lysine residue 239 in the ATP binding site was mutated to serine. It is well known that overexpression of the N-terminal half of IRAK abrogates IL-1-stimulated NF-κB activation in human 293 [12] and mouse

EL-4 cells [18]. If kinase activity is required for IL-1 signaling, one would expect a kinase-inactive IRAK molecule to behave as dominant negative inhibitor upon overexpression.

Overexpression of human IRAK (wt) resulted in strong autophosphorylation after IL-1 treatment. This is in accordance with results obtained in EL-4 clones permanently expressing human IRAK (wt) [18], showing that intact human IRAK can be activated by the murine IL-1 receptor system and will phosphorylate itself and the exogenous substrate MBP in vitro. The mutant form of IRAK (K239S) was not capable of autophosphorylating itself nor was IRAK (K239S) protein phosphorylated by endogenous murine IRAK in this in vitro kinase assay.

Surprisingly, in none of the parameters used as readout for IL-1 signaling events, kinase-inactive IRAK (K239S) behaved as dominant negative inhibitor. In contrast, upon overexpression of IRAK (wt) as well as IRAK (K239S), we observed a strong enhancement of IL-1-stimulated NF-κB-dependent reporter gene activity. The same results were obtained for the activation of JNKs, which was clearly not reduced but enhanced by kinase-inactive IRAK (K239S). Moreover, IL-1-stimulated IL-2 production was not inhibited but clearly enhanced by overexpressing IRAK (K239S). No qualitative difference was seen between expression of IRAK (wt) or IRAK (K239S), underlining the fact that kinase-inactive IRAK (K239S) was definitely not dominant negative in this complex bioresponse of EL-4 cells.

The question arises what the role of the kinase activity of IRAK (wt) may be if it is not required for initiating downstream signaling events. In the normal situation IRAK (and presumably IRAK-2) will be recruited to the heterodimer of IL-1RI and IL-1RAcP by MyD88 upon IL-1 stimulation. One assumes that IRAK autophosphorylates itself and crossphosphorylates IRAK-2. The observation that only unphosphorylated IRAK can be coprecipitated with MyD88 [13] suggests that phosphorylation of IRAK may be the signal to leave the receptor complex, allowing interaction with TRAF6 [15]. It remains to be seen whether kinase-inactive IRAK (K239S) can interact with TRAF6 in a similar way. On the other hand, it is conceivable that unphosphorylated kinase-inactive IRAK (K239S) may activate components of the NF-κB pathway independently of TRAF6. Thus it was recently described that casein kinase II influences IL-1-mediated NF-κB activation [22] and phosphatidylinositol 3-kinase is discussed to be involved in IL-1 signaling [23,24]. In both cases, the coupling mechanisms to the activated IL-1 receptor complex and IRAK remain elusive. To this end it is noteworthy that IL-1 responses (activation of NF-κB and JNKs) were drastically reduced but not completely abolished in IRAK-deficient embryonal fibroblasts [20] suggesting that either IRAK2 or yet to be discovered members of the IRAK family can compensate for the lack of IRAK to a certain extent. Another possibility is that other pathways may lead to partial activation of NF-κB and JNKs independently of IRAK's presence.

Recently, it was shown that after association of IRAK to the activated IL-1 receptor complex hyperphosphorylated IRAK disappears not only from this complex but also from the cytosolic pool. This process was inhibited by proteasome inhibitors, suggesting that hyperphosphorylation of IRAK is the signal to remove IRAK from the receptor complex by proteolytic cleavage [16]. It is conceivable that under physiological conditions the availability of IRAK to replenish hyperphosphorylated molecules removed from the receptor complex is limited, thus regulating subsequent IL-1-stimulated signaling events. This would explain our observation that permanent overexpression of IRAK leads to prolonged kinetics of NF-κB and JNK activation [18]. If autophosphorylation is the signal for initiating the removal and proteolytic breakdown of IRAK, kinase-inactive IRAK (K239S), which is not phosphorylated upon IL-1 stimulation, should persist longer in the active signaling complex. This possibly explains the observed differences in expression of IRAK protein and the additional increase in IL-1 responses caused by IRAK (K239S) in comparison to IRAK (wt), which readily autophosphorylates itself and therefore is prone to the downregulatory pathway.

In conclusion, growing evidence points to the fact that the molecular way of action of IRAK may be different from that of other kinases involved in cytokine signaling: IRAK seems to be a highly sophisticated adaptor protein which in concert with its partner TRAF6 can activate the downstream kinase NIK in a manner currently unclear. For this function its kinase activity may not be required. In addition IRAK autophosphorylates itself at multiple residues and thus initiates its own removal from the receptor complex and its subsequent degradation by proteolytic cleavage. By this mechanism it may terminate signaling through the active IL-1 receptor complex. Thus IRAK, once activated, may be a self-limiting adaptor of IL-1 signaling possibly protecting target cells from prolonged activation. This would be a novel mechanism of autocatalyzed downregulation of the IL-1 signaling machinery.

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