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SHORT COMMUNICATION

Frontline

The protein kinase PKR is critical for LPS-induced iNOS production but dispensable for inflammasome activation in macrophages

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Inflammasomes are multi-protein platforms that drive the activation of caspase-1 leading to the processing and secretion of biologically active IL-1β and IL-18. Different inflammasomes including NOD-like receptor (NLR) family pyrin domain-containing 3 (NLRP3), NLR caspase-recruitment domain-containing 4 (NLRC4) and absent in melanoma 2 (AIM2) are activated and assembled in response to distinct microbial or endogenous stimuli. However, the mechanisms by which upstream stimuli trigger inflammasome activation remain poorly understood. Double-stranded RNA-activated protein kinase (PKR), a protein kinase activated by viral infection, has been recently shown to be required for the activation of the inflammasomes. Using macrophages from two different mouse strains deficient in PKR, we found that PKR is important for the induction of the inducible nitric oxide synthase (iNOS). However, PKR was dispensable for caspase-1 activation, processing of pro-IL-1β/IL-18 and secretion of IL-1β induced by stimuli that trigger the activation of NLRP3, NLRC4 and AIM2. These results indicate that PKR is not required for inflammasome activation in macrophages.

Keywords: Caspase-1 · IL-1β · Inflammasome · Macrophages · PKR

Introduction

PKR, known as double-stranded RNA-activated protein kinase, is activated by viral infection and plays an important role in controlling viral spreading within the host [1, 2]. PKR contains an N-terminal dsRNA binding domain and a C-terminal kinase domain [3]. After activation by binding to viral dsRNA, PKR phosphorylates the translation initiation factor EIF2A to inhibit cellular RNA translation leading to the inhibition of viral protein synthesis [1]. PKR can also modulate NF-κB signaling and cellular apoptosis [4, 5]. In addition, stimulation of TLR4 can trigger PKR-mediated apoptosis of macrophages, which allow some pathogens such as *Bacillus anthracis* to escape immune clearance [6]. PKR can also link pathogen sensing to stress responses in metabolic disease [7]. Notably, PKR has been recently implicated in the processing of caspase-1 and IL-1β secretion in response to diverse stimuli [8], suggesting that this kinase acts in a common step required for inflammasome activation.

Inflammasomes are intracellular multi-protein complexes that drive the activation of the protease caspase-1 [9, 10]. To date, four bona fide inflammasomes have been identified, NOD-like receptor (NLR) family pyrin domain-containing 1 (NLRP1), NLRP3, NLR caspase-recruitment domain-containing 4 (NLRC4) and absent in melanoma 2 (AIM2), that link specific microbial or endogenous stimuli to caspase-1 activation [9, 10]. Once activated, caspase-1 proteolytically cleaves pro-IL1β and pro-IL-18 into their biologically active cytokines [11]. Although there is clear evidence that the activation mechanism of each inflammasome is different [9–11], a recent study reported that PKR is required for the activation of NLRP3, NLRC4 and AIM2 [8]. The latter study suggested that PKR is a common regulator of the inflammasomes.

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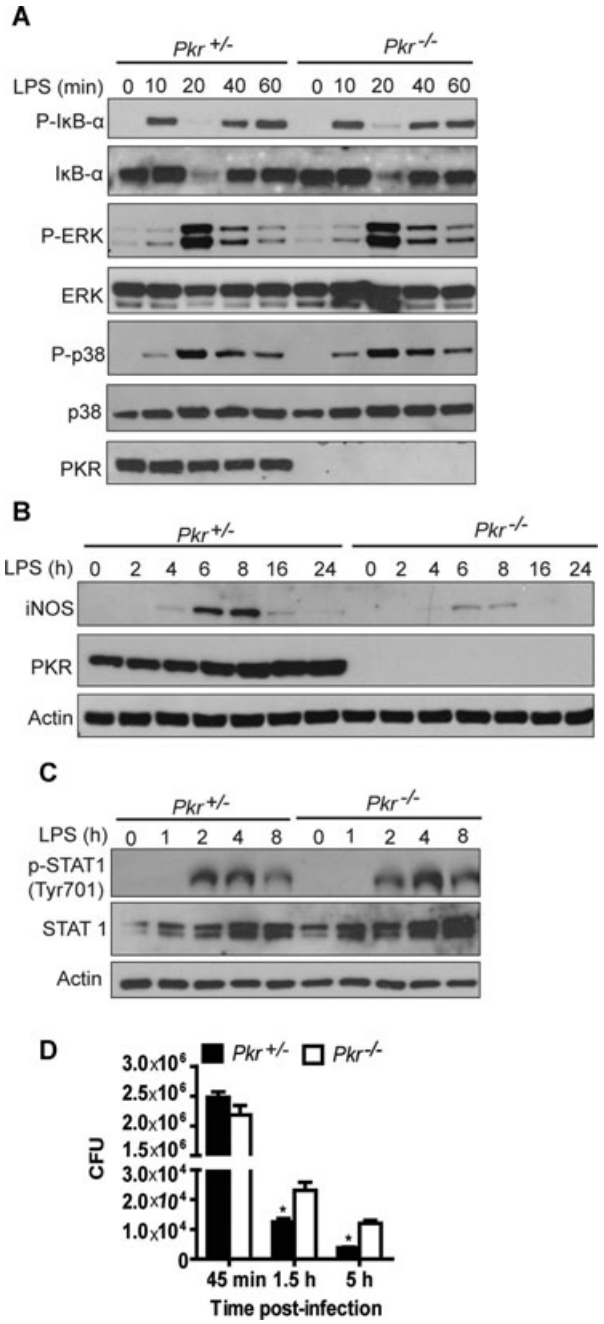

Figure 1. PKR is critical for LPS-induced iNOS production in macrophages. (A) BMDMs from heterozygous ($Pkr^{+/-}$) and homozygous ($Pkr^{-/-}$) mice were stimulated with 100 ng/mL LPS for indicated time. Cell extracts were immunoblotted with antibodies reacting with phosphorylated forms of IκBα, p38 or ERK. The immunoblots were stripped and re-probed for total IκBα, p38 or ERK. Probing for PKR protein confirmed the genotype of $Pkr^{-/-}$. (B) BMDMs were stimulated with 100 ng/mL LPS for indicated time. Cell lysates were immublotted for iNOS and PKR. β-actin was used as a loading control. (C) BMDMs were stimulated with 100 ng/mL of LPS for indicated time. Cell lysates were immunoblotted for STAT1 and p-STAT1 (Tyr701). β-actin was used as a loading control. (D) Live intracellular bacteria from BMDMs after indicated time of infection with *E. coli*. Data shown are from one experiment representative of three independent experiments. $^*p < 0.05$, Student's *t*-test.

To further understand the role of PKR in caspase-1 activation, we studied the activation of the NLRP3, NLRC4 and AIM2 in macrophages from mice deficient in PKR. In contrast to published results [8], we found that PKR is dispensable for inflammasome activation.

Results and discussion

PKR is critical for LPS-induced iNOS production in macrophages

PKR is phosphorylated in macrophages after LPS stimulation [6,12]. To determine the potential role of PKR in the TLR4 signaling pathway, we treated BM-derived macrophages (BMDMs) from $Pkr^{+/-}$ and $Pkr^{-/-}$ mice with LPS for different times, and analysed the phosphorylation status of IκBα, ERK and p38 (Fig. 1A). The phosphorylation levels of these proteins was indistinguishable in LPS-stimulated $Pkr^{+/-}$ and $Pkr^{-/-}$ macrophages, suggesting that PKR protein is not required for NF-κB, ERK and p38 activation in response to LPS. Notably, the production of iNOS, an enzyme catalysing NO which is involved in host defense against microbes [13], was markedly reduced in $Pkr^{-/-}$ macrophages when compared with that of $Pkr^{+/-}$ macrophages (Fig. 1B). Several transcription factors, including NF-κB, AP-1 and STAT1, have been shown to regulate iNOS expression [13]. LPS-induced phosphorylation of STAT1 at Tyr 701, a site essential for its activation, was not altered by PKR deficiency, indicating that it is unlikely that PKR is involved in the upstream signaling pathway of STAT1 activation (Fig. 1C). Consistent with the reduction of iNOS expression, the bacteria-killing capacity after exposure to *Escherichia coli* was reduced in $Pkr^{-/-}$ macrophages (Fig. 1D). Our results confirm and extend previous findings that PKR plays a role in LPS-induced iNOS production and bacteria-killing function of macrophages.

PKR is dispensable for inflammasome activation in macrophages

Next, we investigated the involvement of PKR in inflammasome activation. LPS-primed $Pkr^{+/-}$ and $Pkr^{-/-}$ macrophages were treated with known activators of NLRP3, NLRC4 and AIM2. In contrast to a recent report [8], the amounts of processed caspase-1 (p20 and p10), and IL-1β/IL-18 maturation in the cell supernatant in response to activators of NLRP3 including ATP, nigerin and silica particles were comparable in $Pkr^{+/-}$ and $Pkr^{-/-}$ macrophages (Fig. 2A). No role for PKR was also found in the activation of caspase-1 and pro-IL-1β/IL-18 processing after infection of macrophages with *Salmonella thyphimurium* that activates the NLRC4 inflammasome (Fig. 2B). Furthermore, caspase-1 activation and IL-1β processing induced by poly (dA:dT) that triggers AIM2 activation [14–16], was comparable in $Pkr^{+/-}$ and $Pkr^{+/-}$ macrophages (Fig. 2C). ELISA analysis showed robust IL-1β release in $Pkr^{-/-}$ macrophages after stimulation with ATP,

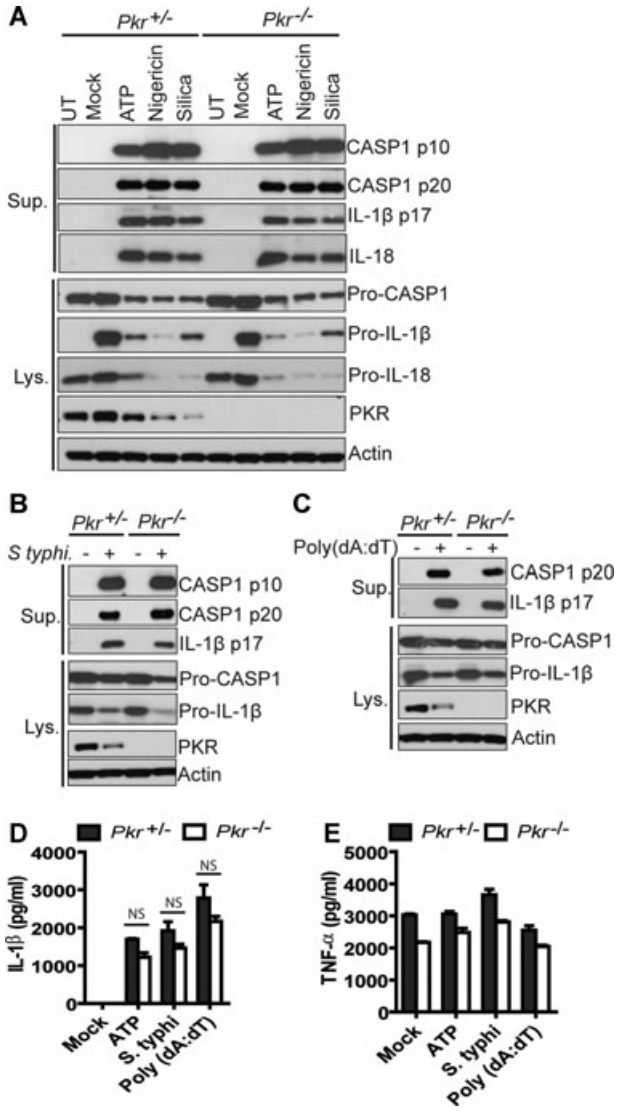

Figure 2. PKR-deficient BMDMs show normal inflammasome activation. (A) BMDMs were un-stimulated (UT), or primed with LPS (100 ng/mL) for 4 h before stimulated with NLRP3 activators, ATP (5 mM) for 30 min, Nigericin (5 μM) for 1 h, Silica (500 μg/mL) for 6 h. (B) BMDMs were primed with LPS (100 ng/mL) for 4 h, followed by infection with *Salmonella typhimurium* (*S. typhi.*, MOI = 10) for 1 h, or left uninfected. (C) LPS-primed BMDMs were stimulated with 2 μg/mL poly (dA:dT) for 6 h or left unstimulated. After stimulation or infection, supernatant (Sup.) and cell lysates (Lys.) were prepared and immunoblotted for indicated proteins. (D, E) The secretion of (D) IL-1β and (E) TNF-α from BMDMs was analysed after inflammasome activation. Data are shown as the mean ± SD of triplicate wells and all data shown are representative of three independent experiments.

Salmonella and poly (dA:dT), although the amounts were slightly reduced in $Pkr^{-/-}$ macrophages when compared with those in $Pkr^{+/-}$ macrophages (Fig. 2D). However, similar reduction was observed in TNF-α secretion (Fig. 2E), suggesting that the slight reduction in IL-1β secretion in $Pkr^{-/-}$ macrophages is not related to inflammasome activation.

Our initial studies were performed with PKR-deficient mice in which the N-terminal RNA binding domain of PKR was deleted [17]. In contrast, Lu et al. studied PKR using KO mice with deletion of the catalytic domain of PKR [18]. Although both KO mice lack expression of full-length PKR, some conflicting results have been reported for these two mouse mutant strains [19]. Therefore, we also studied inflammasome activation in macrophages from mutant mice with deletion of the catalytic domain of PKR. Analysis of macrophages from this $Pkr^{-/-}$ mouse strain also revealed comparable caspase-1 activation and pro-IL-1β/IL-18 processing in response to activators of the NLRP3 inflammasome when compared with that of WT macrophages (Fig. 3A). As expected, caspase-1 activation and pro-IL-1β/IL-18 procession were abrogated in macrophages from $Nlrp3^{-/-}$ mice (Fig. 3A). Likewise, caspase-1 activation and pro-IL-1β maturation induced by aluminum salts (Alum), another activator of NLRP3, were unimpaired in $Pkr^{-/-}$ macrophages, but abolished in $Nlrp3^{-/-}$ macrophages (Fig. 3B). 2-aminopurin (2-AP), a potent inhibitor of PKR, was reported to inhibit ATP-induced NLRP3 inflammasome activation at millimolar concentration [8]. Notably, addition of 2-AP at this high concentration inhibited ATP-induced NLRP3 inflammasome activation in both WT and PKR-deficient macrophages (Fig. 3C). This result suggests at this high concentration, 2-AP inhibits the inflammasome through off-target effects. Furthermore, caspase-1 activation in response to *Salmonella* or poly (dA:dT) were unaffected by deletion of the catalytic domain of PKR (Fig. 3D and E). Consistent with these results, IL-1β and TNF-α release induced by ATP, *Salmonella* and poly (dA:dT) were unimpaired in $Pkr^{-/-}$ macrophages (Fig. 3F and G).

Concluding remarks

Our results indicate that the protein kinase PKR plays a critical role in regulating iNOS production by macrophages after LPS challenge, which correlated with reduced intracellular killing of *E. coli*. However, we found no detectable role for PKR in the activation of the NLRP3, NLRC4 or AIM2 inflammasomes in macrophages. We do not have a clear explanation for the difference in results between our studies and those of Lu et al. [8]. It is possible that subtle variation in experimental conditions may account, at least in part, for the differences in results. In our studies, parallel experiments were performed using macrophages from mice deficient in NLRP3 and NLRC4 that showed requirement for these inflammasomes, but not PKR, for caspase-1 activation triggered by specific stimuli. In addition, our conclusions regarding PKR were obtained from experiments using two independently generated PKR-deficient mice that targeted two critical regions of PKR. Additional studies are needed to clarify the role of PKR in inflammasome activation.

Materials and methods

Mice and cells

WT and Nlrp3-deficient mice have been described previously [20]. Two different types of PKR targeted mutations have been reported

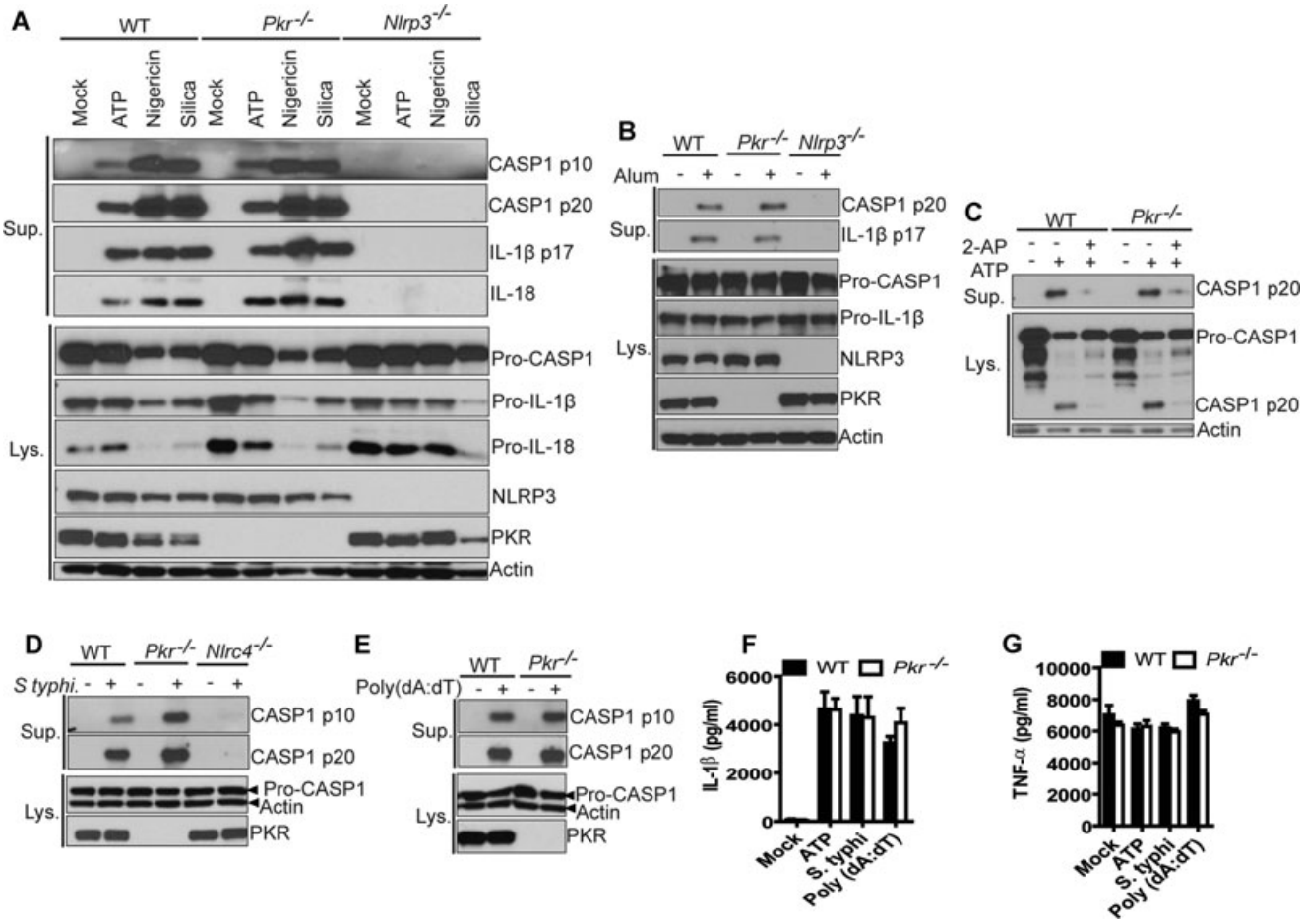

Figure 3. Inflammasome activation was analysed in BMDMs with a targeted deletion of the catalytic domain of PKR. (A, B) BMDMs from WT mice, *Pkr*$^{-/-}$ mice and *Nlrp3*$^{-/-}$ mice were primed with LPS (500 ng/mL) for 4 h before being stimulated with (A) NLRP3 activators, ATP (5 mM) for 30 min, Nigericin (5 μM) for 1 h, Silica (U.S. Silica, 500 μg/mL) or (B) Alum (500 μg/mL) for 6 h. (C) LPS-primed BMDMs from WT mice and *Pkr*$^{-/-}$ mice were pre-treated with 1 mM 2-aminopurin (2-AP) or PBS (control) for 30 min before stimulation with 5 mM ATP for 30 min. (D) LPS-primed BMDMs were infected with *Salmonella typhimurium* (*S. typhi*., MOI = 10) for 1 h, or uninfected. Caspase-1 cleavage was analysed by immunoblotting. (E) LPS-primed BMDMs were stimulated with 2 μg/mL poly (dA:dT) for 6 h or left unstimulated. After stimulation, supernatant (Sup.) and cell lysates (Lys.) were prepared and immunoblotted for indicated proteins. (F, G) The secretion of (F) IL-1β and (G) TNF-α from BMDMs was measured after inflammasome activation. Data are shown as the mean + SD of triplicate wells and all data shown are representative of three independent experiments.

in mice, targeted deletion of the PKR RNA-binding domain and targeted deletion of PKR catalytic domain [17, 18]. Leg bones of *Pkr*$^{+/-}$ and *Pkr*$^{-/-}$ mice with targeted deletion of the RNA-binding domain of PKR, which were originally generated from on a mixed 129 SvEv x C57Bl6 background and backcrossed to C57BL/6 one time [21], were a gift of Randal Kaufman (Sanford-Burham Medical Research Institute, La Jolla). Leg bones of *Pkr*$^{-/-}$ mice with targeted deletion of the catalytic domain of PKR were generated on a 129Sv background and backcrossed to BALB/c mice at least six times (a gift of Yingjie Chen, University of Minnesota, Minneapolis). BMDMs were prepared and cultured as previously described [22]. Cells were seeded overnight in 12-well plate with 1×10^6 cells per well.

Reagents

Ultrapure LPS from *E. coli* 0111:B4, Alum, 2-aminopurin (2-AP) and poly(dA:dT)/lyovec were purchased from Invivogen. ATP was purchased from Sigma. Nigericin was purchased from Calbiochem. *Salmonella enterica* serovar *typhimurium* strain SL1344 was a gift from Denise Monack (Stanford University, Stanford, CA). Antibodies for IκBα, p-IκBα, p38, p-p38, Erk, p-Erk, iNOS, STAT1 and p-STAT1 (Tyr 701) were purchased from Cell Signaling. Murine IL-1β antibody (AF-401-NA) was purchased from R&D Systems. Actin antibody was purchased from GenScript. Antibodies for PKR (sc-6282) and caspase-1 (sc-514) were purchased from Santa Cruz. Caspase-1 antibody for the cleaved p20 of caspase-1 was

generated in our laboratory. IL-18 antibody (5180R-100) was purchased from BioVision. Rabbit anti-mouse-Nlrp3 antibody was generated by immunizing rabbits with mouse Nlrp3 protein (amino acids 1–194) expressed in *E. coli* and purified by affinity chromatography using a nickel column.

Bacteria-killing assay

BMDMs were incubated with *E. coli* strain at MOI of 10 for 30 min. Extracellular bacteria were killed by treatment with gentamicin (100 μg/mL) for 15 min. At indicated time points, cells were lysed with 0.1% Trinton X-100 and serial dilutions of cell extract were spread on LB agar plates. Live intracellular bacteria were counted after overnight incubation in 37°C.

Immunoblotting

Cells were lysed in ice-cold PBS buffer containing 1% NP-40 supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany). The proteins from cell-free supernatants were precipitated by choloform/methanol method as previously described [23]. Protein samples were separated by SDS-PAGE and transferred to PVDF membranes by electroblotting (Bio-Rad) and membranes were immunoblotted with respective antibodies.

Measurements of cytokines

Mouse IL-1β and TNF-α in culture supernatants were measured by ELISA kits (R&D Systems). Assays were performed in triplicate for each independent experiment.

Statistical analysis

Student's *t*-test was used to determine statistically significant difference between two groups. A *p*-value of <0.05 was considered significant.

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Conflict of interest: Luigi Franchi is an employee of Lycera, a biotechnology company specializing in the field of inflammation.

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Abbreviations: **AIM2**: absent in melanoma 2 · **BMDM**: BM-derived macrophage · **NLRC4**: NLR caspase-recruitment domain-containing 4 · **NLRP3**: NOD-like receptor (NLR) family pyrin domain-containing 3 · **PKR**: dsRNA-activated protein kinase

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