LETTER

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Novel role of PKR in inflammasome activation and HMGB1 release

Ben Lu[1,2], Takahisa Nakamura[3], Karen Inouye[3], Jianhua Li[1], Yiting Tang[4], Peter Lundbäck[5], Sergio I. Valdes-Ferrer[1,2], Peder S. Olofsson[1], Thomas Kalb[1], Jesse Roth[1], Yongrui Zou[4], Helena Erlandsson-Harris[5], Huan Yang[1], Jenny P.-Y. Ting[7], Haichao Wang[8], Ulf Andersson[6], Daniel J. Antoine[9], Sangeeta S. Chavan[1], Gökhan S. Hotamisligil[3] & Kevin J. Tracey[1,2]

The inflammasome regulates the release of caspase activation-dependent cytokines, including interleukin (IL)-1β, IL-18 and high-mobility group box 1 (HMGB1)[1–5]. By studying HMGB1 release mechanisms, here we identify a role for double-stranded RNA-dependent protein kinase (PKR, also known as EIF2AK2) in inflammasome activation. Exposure of macrophages to inflammasome agonists induced PKR autophosphorylation. PKR inactivation by genetic deletion or pharmacological inhibition severely impaired inflammasome activation in response to double-stranded RNA, ATP, monosodium urate, adjuvant aluminium, rotenone, live *Escherichia coli*, anthrax lethal toxin, DNA transfection and *Salmonella typhimurium* infection. PKR deficiency significantly inhibited the secretion of IL-1β, IL-18 and HMGB1 in *E. coli*-induced peritonitis. PKR physically interacts with several inflammasome components, including NOD-like receptor (NLR) family pyrin domain-containing 3 (NLRP3), NLRP1, NLR family CARD domain-containing protein 4 (NLRC4), absent in melanoma 2 (AIM2), and broadly regulates inflammasome activation. PKR autophosphorylation in a cell-free system with recombinant NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC, also known as PYCARD) and pro-caspase-1 reconstitutes inflammasome activity. These results show a crucial role for PKR in inflammasome activation, and indicate that it should be possible to pharmacologically target this molecule to treat inflammation.

HMGB1, a ubiquitous nuclear and cytosolic protein, is released into the extracellular space during sterile inflammation and infection[6]. Immunocompetent cells secrete HMGB1 after stimulation with microbial products and other danger signals[4–6]. Ischaemic and injured somatic cells also release HMGB1, but in this case the release mechanisms are passive[6]. Extracellular HMGB1 signals through Toll-like receptor 4 (TLR4) receptors in macrophages to induce cytokine release[6,7] and mediate cell migration by interacting with receptor for advanced glycation end-products (RAGE)[8]. The importance of these mechanisms in disease pathogenesis has been established, because neutralizing HMGB1 attenuates disease severity in infectious and sterile injury syndromes such as arthritis, colitis, sepsis and ischaemia reperfusion[6]. The mediator role of HMGB1 at the intersection of both sterile and infectious inflammation highlights the importance of mechanisms that regulate the secretion of HMGB1 from immune cells[6].

Early observations suggested that secretion of HMGB1 from macrophages requires inflammasome and caspase activity[3–5,9,10], and is highly associated with HMGB1 translocation from the nucleus to the cytoplasm[4,6]. HMGB1 is also a universal sentinel to viral invasion, and its release can be stimulated by polyinosinic:polycytidylic acid (poly(I:C)), a double-stranded RNA (dsRNA) mimetic[11,12]. Because we were interested in studying HMGB1 release mechanisms from macrophages exposed to poly(I:C), we first considered earlier observations that PKR is phosphorylated in these conditions[13,14]. As PKR is also activated by prototypical pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) known to stimulate HMGB1 release[15,16] and activate the inflammasome, we reasoned that PKR activation might participate in inflammasome activation.

Accordingly, we measured HMGB1 release by peritoneal macrophages obtained from PKR-deficient ($Pkr^{-/-}$) mice, in which the PKR kinase domain had been functionally inactivated by genetic deletion[16]. HMGB1 secretion after poly(I:C) exposure in $Pkr^{-/-}$ macrophages was significantly lower than in wild-type ($Pkr^{+/+}$) macrophages (Fig. 1a). Pharmacological inhibition of PKR in $Pkr^{+/+}$ macrophages dose-dependently, and significantly, inhibited poly(I:C)-induced HMGB1 release and PKR phosphorylation (Fig. 1b and Supplementary Fig. 1). Other prototypical danger signals, including ATP, monosodium urate (MSU), adjuvant aluminium (ALU) and live *E. coli*, also significantly increased PKR phosphorylation and HMGB1 release (Fig. 1c–e). Addition of potassium to the extracellular space significantly inhibited ATP-induced PKR activation (Fig. 1c). Inactivation of macrophage PKR by genetic deletion (Fig. 1d, e) or pharmacological inhibition (Fig. 1f and Supplementary Fig. 2) also significantly inhibited HMGB1 release.

Pyroptosis, a form of programmed, inflammatory cell death, occurs with macrophage inflammasome activation, and we observed that deletion of PKR significantly inhibited lactate dehydrogenase (LDH) release (Fig. 1g). Analysis by tandem mass spectrometry of HMGB1 released in response to ATP, MSU or ALU indicated that HMGB1 was highly acetylated in the nuclear location sequence (Fig. 1h and Supplementary Figs 3–6). By contrast, HMGB1 released from macrophages subjected to freeze/thaw cycles was not acetylated in the nuclear location sequence (Fig. 1h). Together with evidence that inflammasome activation participates in the nuclear translocation of HMGB1 (ref. 4), these results indicated that HMGB1 hyperacetylation and release, and inflammasome activation, are regulated by PKR.

To address the role of PKR in activating the NLRP3 inflammasome, we measured caspase-1 activation and IL-1β cleavage in peritoneal macrophages from $Pkr^{+/+}$ and $Pkr^{-/-}$ mice. Caspase-1 activation and IL-1β cleavage were significantly inhibited in $Pkr^{-/-}$ macrophages stimulated by exposure to ATP, MSU and ALU (Fig. 2a). Similar results were obtained in bone marrow-derived dendritic cells (BMDCs) (Supplementary Fig. 7) and bone marrow-dervied macrophages (BMDMs) (Supplementary Fig. 8). The expression of NLRP3 and pro-IL-1β did not differ significantly in $Pkr^{-/-}$ or $Pkr^{+/+}$ macrophages (Fig. 2a and Supplementary Fig. 9), but IL-1β secretion by macrophages exposed to live *E. coli*. was significantly inhibited in

[1]Laboratory of Biomedical Science, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, New York 11030, USA. [2]The Elmezzi Graduate School of Molecular Medicine, North Shore-LIJ Health system, 350 Community Drive, Manhasset, New York 11030, USA. [3]Department of Genetics & Complex Diseases, Harvard School of Public Health, Boston, Massachusetts 02115, USA. [4]Center for Autoimmunity and Musculoskeletal Diseases, Feinstein Institute for Medical Research, Manhasset, New York, New York 11030, USA. [5]Department of Medicine, Karolinska Institute, Karolinska University Hospital, Stockholm S-17176, Sweden. [6]Women's and Children's Health, Karolinska Institute, Karolinska University Hospital, Stockholm S-17176, Sweden. [7]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA. [8]Department of Emergency Medicine, North Shore University Hospital, Manhasset, New York 11030, USA. [9]MRC Centre for Drug Safety Science, Department of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool L69 3GE, UK.

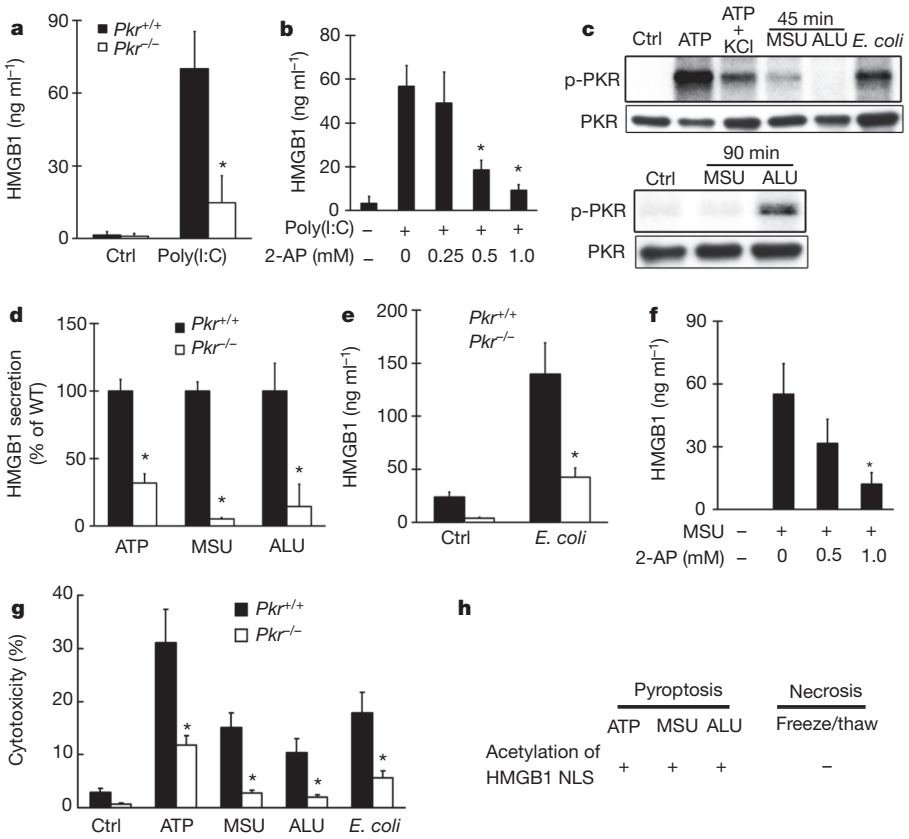

Figure 1 | Role of PKR in pyroptosis-mediated HMGB1 release. **a**, **b**, Macrophages from $Pkr^{+/+}$ or $Pkr^{-/-}$ mice (**a**) or from $Pkr^{+/+}$ mice treated with indicated doses of the PKR inhibitor 2-AP (**b**) were stimulated with poly(I:C). Ctrl, control. **c**, Lipopolysaccharide (LPS)-primed $Pkr^{+/+}$ macrophages were stimulated or treated with or without potassium-substituted medium (KCl) as indicated. Cells were lysed at indicated time points and PKR activation was monitored by autophosphorylation (denoted by p-PKR). **d**–**g**, LPS-primed $Pkr^{+/+}$ or $Pkr^{-/-}$ macrophages were stimulated or treated with 2-AP as indicated. HMGB1 levels in the supernatant were determined by western blot. WT, wild type. Cytotoxicity (**g**) was determined by LDH assay. Data are mean and s.d. of three independent experiments. $*P < 0.05$ versus wild-type-stimulated groups. **h**, Mass-spectrometric analysis of the acetylation status of nuclear location sequences (NLS) of HMGB1.

$Pkr^{-/-}$ macrophages as compared with $Pkr^{+/+}$ macrophages (Fig. 2b). Tumour necrosis factor (TNF-α) release in the $Pkr^{+/+}$ and $Pkr^{-/-}$ macrophages was comparable, indicating that the decreased release of IL-1β in the $Pkr^{-/-}$ macrophages is not attributable to a global defect in signal transduction (Supplementary Fig. 10).

Transfection of BMDCs with poly(I:C) and *E. coli* RNA significantly activated caspase-1 and stimulated IL-1β cleavage in $Pkr^{+/+}$ but not $Pkr^{-/-}$ cells (Supplementary Fig. 11). Similar observations were obtained in $Pkr^{-/-}$ and $Pkr^{+/+}$ macrophages stimulated by rotenone, which induces mitochondrial reactive oxygen species production and PKR phosphorylation (Supplementary Figs 12 and 13). Pharmacological inhibition of PKR dose-dependently suppressed MSU-induced caspase-1 activation and IL-1β cleavage. The observed half-maximum inhibitory concentrations (IC_{50} values) of 2-aminopurine (2-AP) (Fig. 2c) and $C_{13}H_8N_4OS$ (Supplementary Fig. 14) were 0.5 mM and 0.25 μM, respectively, which agree closely with their known IC_{50} against PKR. PKR inhibition significantly reduced ATP- and ALU-induced inflammasome activation in mouse macrophages (Supplementary Figs 15 and 16), and in human monocytic THP-1 cells (Supplementary Fig. 17). IL-18 release was significantly lower in $Pkr^{-/-}$ macrophages than in $Pkr^{+/+}$ macrophages stimulated with ATP, MSU or ALU, whereas TNF-α and IL-6 were not suppressed (Supplementary Fig. 18). The addition of 2-AP reduced MSU-induced IL-18 release, but not TNF-α and IL-6 (Supplementary Fig. 18). 2-AP failed to inhibit MSU-induced caspase-1 activation and IL-1β cleavage in $Pkr^{-/-}$ macrophages (Supplementary Fig. 19). Collectively, these findings establish a crucial role for PKR in activating the NLRP3 inflammasome.

$Pkr^{+/+}$ and $Pkr^{-/-}$ mice were then exposed to live *E. coli*, to activate the NLRP3 inflammasome *in vivo*[17]. Serum levels of IL-1β, IL-18 and HMGB1 were significantly reduced in $Pkr^{-/-}$ mice as compared with $Pkr^{+/+}$ mice (Fig. 2d). In agreement with previous evidence suggesting that peritoneal neutrophil infiltration requires inflammasome activation[18], we observed significantly fewer neutrophils in peritoneal lavage from $Pkr^{-/-}$ mice than in $Pkr^{+/+}$ controls (Fig. 2e). Serum IL-6, an inflammasome-independent cytokine, was comparable in both groups (Fig. 2d). Exposure of $Pkr^{-/-}$ mice to endotoxemia produced significantly lower serum IL-1β and IL-18 levels, but quantitatively similar serum levels of TNF-α and IL-6 as compared with $Pkr^{+/+}$ controls (Supplementary Fig. 20). Thus, PKR regulates inflammasome-dependent cytokine release *in vivo*.

To study the mechanisms of PKR regulation, HEK293A cells, which do not normally express the NLRP3 components, were transfected with plasmids that co-express ASC, pro-caspase-1 and NLRP3. These modified cells were then co-transfected with plasmids that express PKR, or specific short hairpin RNA (shRNA) to suppress endogenous PKR. Overexpression of PKR significantly enhanced caspase-1 activation and IL-1β cleavage, and knockdown of endogenous PKR by shRNA abolished caspase-1 cleavage in the NLRP3 inflammasome-reconstituted cells (Fig. 2f, g). IL-1β cleavage was significantly abrogated by pharmacological inhibition of PKR (Supplementary Fig. 21). PKR overexpression failed to activate caspase-1 directly in the absence of NLRP3 (Supplementary Fig. 22), and did not enhance HMGB1 release in the absence of caspase-1 (Supplementary Fig. 23). Knockdown of endogenous eIF2α, a PKR substrate[13,14], did not

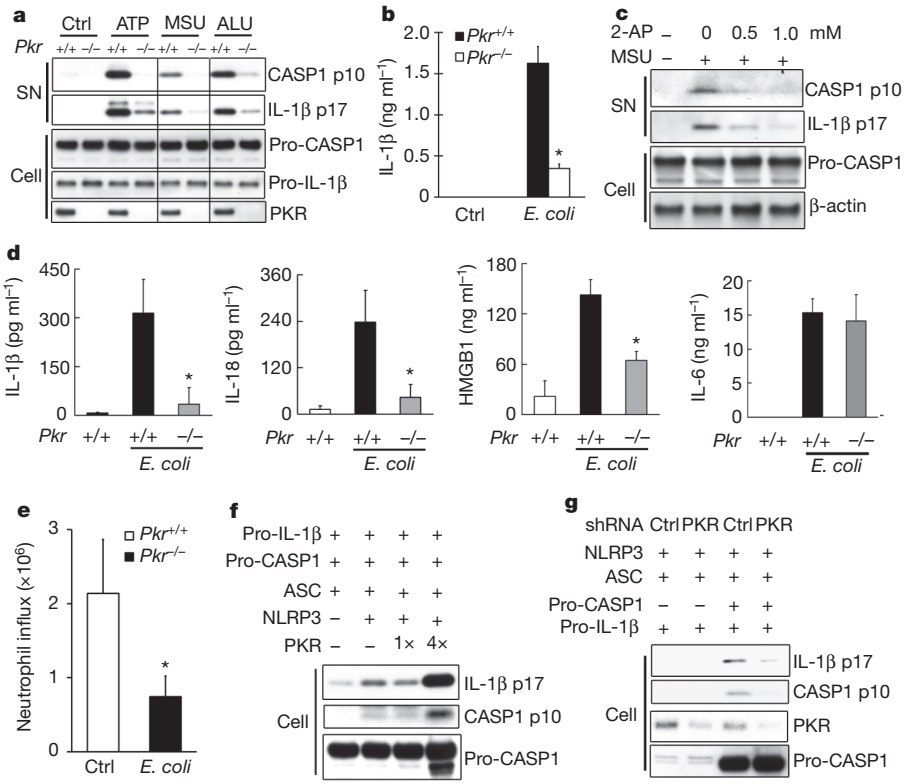

Figure 2 | PKR is important for inflammasome activation. **a**, **b**, LPS-primed $Pkr^{+/+}$ or $Pkr^{-/-}$ macrophages were stimulated as indicated. SN, supernatant. **c**, $Pkr^{+/+}$ macrophages were stimulated or treated with 2-AP as indicated. **d**, **e**, $Pkr^{+/+}$ or $Pkr^{-/-}$ mice ($n = 5$) were injected with live *E. coli*. **f**, **g**, HEK293A cells were transfected as indicated. Caspase-1 (CASP1) activation and IL-1β cleavage (as measured by the p10 and p17 subunits, respectively) were assessed by western blot. Data are representative of at least three independent experiments. Levels of IL-1β, IL-18, HMGB1 and IL-6 in the supernatant (**b**) or serum (**d**) were determined by ELISA. Peritoneal lavage fluid was collected and neutrophil content was measured by flow cytometry (**e**). Data are mean and s.d. $*P < 0.05$ versus wild-type-infected groups.

alter HMGB1 release and IL-1β cleavage in the NLRP3 inflammasome-reconstituted cells (Supplementary Fig. 24).

We therefore reasoned that PKR physically interacts with the NLRP3 inflammasome, and prepared mouse macrophage lysates for immunoprecipitation using anti-PKR antibodies. PKR pulled down NLRP3, and reciprocal immunoprecipitation of NLRP3 pulled down PKR (Supplementary Fig. 25). Similar observations were obtained in human THP-1 cells (Supplementary Fig. 26). PKR–NLRP3 complexes could not be detected in the $Pkr^{-/-}$ macrophages (Supplementary Fig. 27). Co-expression of PKR and NLRP3 in HEK293A cells, followed by immunoprecipitation, confirmed the physical interaction between PKR and NLRP3. PKR failed to associate physically with the other cytosolic receptors or inflammasome family members NOD1, NLRP12 or NLRX1 (Supplementary Fig. 28). PKR co-immunoprecipitated with truncated forms of NLRP3 containing the pyrin domain (PYD), NACHT domain and leucine-rich repeat (LRR) domain (Supplementary Fig. 29), and with the Walker A mutant form of NLRP3 (Supplementary Fig. 30), which cannot bind ATP[19]. Together with direct evidence that recombinant PKR forms a heteromeric protein complex with NLRP3 (Fig. 3a), these results indicate that PKR physically interacts with the inflammasome.

Exposure of $Pkr^{+/+}$ macrophages to ATP significantly enhanced the formation of NLRP3 and PKR complexes (Fig. 3b). Pharmacological inhibition of PKR phosphorylation by 2-AP and $C_{13}H_8N_4OS$ significantly suppressed the formation of NLRP3–PKR complexes (Fig. 3b). The addition of poly(I:C) and ATP to NLRP3 and PKR in a cell-free system significantly induced PKR phosphorylation and increased the formation of NLRP3–PKR complexes (Fig. 3a).

Next, the NLRP3 inflammasome was reconstituted in a cell-free system with recombinant NLRP3, ASC, pro-caspase-1 and PKR. Combining NLRP3 and ASC with pro-caspase-1 failed to increase caspase-1 activity (Fig. 3c). The addition of PKR and poly(I:C) or ATP significantly increased caspase-1 activity, which was inhibited by the addition of 2-AP (Fig. 3c). PKR activity is required in this cell-free system, because poly(I:C)/ATP failed to increase NLRP3 inflammasome activity in the absence of PKR. Moreover, replacing PKR with a recombinant, mutant PKR carrying the Lys296Arg mutation and thus devoid of kinase activity[13,16] failed to activate the NLRP3 inflammasome (Fig. 3c). Although PKR is an autophosphorylating kinase, it was theoretically possible that PKR might phosphorylate inflammasome components directly. Phosphorylation of NLRP3, ASC and caspase-1 could not be detected, although poly(I:C)/ATP significantly stimulated autophosphorylation of PKR (data not shown). To confirm that PKR autophosphorylation is involved in NLRP3 inflammasome activation, the PKR(K296R) mutant and NLRP3 inflammasome components were co-expressed in HEK293A cells. The PKR(K296R) mutant failed to bind NLRP3, whereas wild-type PKR did bind NLRP3 (Supplementary Fig. 31). Overexpression of the PKR(K296R) mutant failed to activate the reconstituted NLRP3 inflammasome in HEK293A cells (Supplementary Fig. 31). Together, these data indicate that PKR physically interacts with NLRP3 and is crucial for inflammasome activation.

Reasoning that PKR might associate with the inflammasome components during assembly, ATP-stimulated macrophage lysates were subjected to gel filtration chromatography. We observed that PKR and NLRP3 eluted together with ASC and caspase-1 in the high molecular mass fraction of the ATP-stimulated macrophage extract (Fig. 3d and Supplementary Fig. 32). Inhibition of PKR activation by 2-AP reduced PKR in the high molecular mass inflammasome fraction (Fig. 3d and Supplementary Fig. 33). Expression of the PKR(K296R) mutant

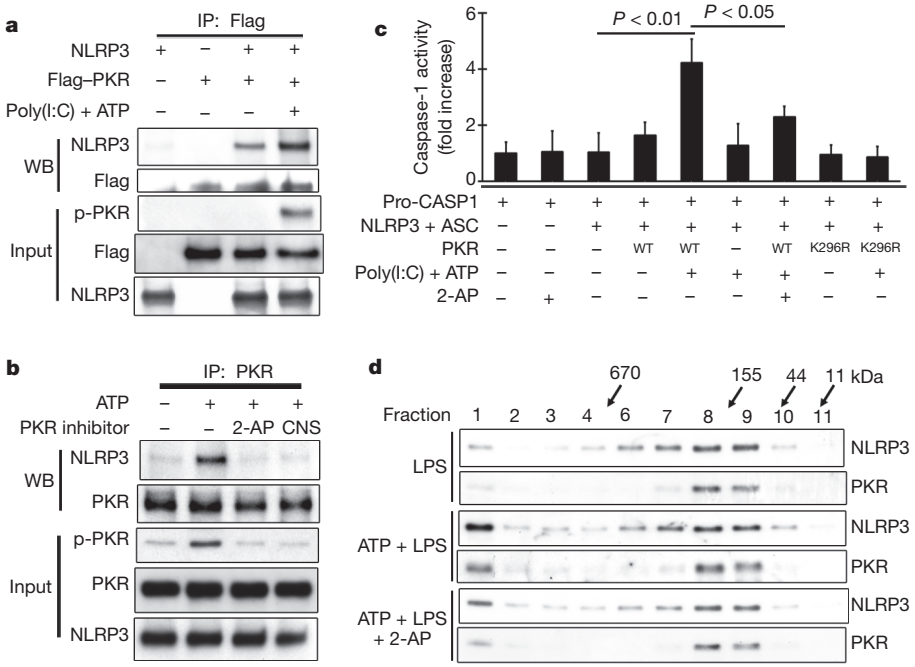

Figure 3 | PKR physically interacts with NLRP3 and facilitates inflammasome activation. **a**, **b**, Immunoprecipitation (IP) and western blot (WB) analysis of the physical interaction of PKR and NLRP3 in a cell-free system using recombinant proteins (**a**) or LPS-primed macrophages stimulated with ATP or treated with 2-AP or $C_{13}H_8N_4OS$ (CNS) as indicated (**b**). **c**, The NLRP3 inflammasome was reconstituted using recombinant proteins and ATP and poly(I:C) as indicated. Caspase-1 activity was measured by hydrolysis of the synthetic peptide WEHD-pNA. **d**, $Pkr^{+/+}$ macrophages were stimulated or treated with 2-AP as indicated. Cell lysates were subjected to gel-filtration chromatography and western blot. Results are representative of three independent experiments.

significantly reduced NLRP3 in the high molecular mass inflammasome fraction, without significantly changing total NLRP3 expression (Supplementary Fig. 34). Rendering cells deficient in PKR also significantly impaired the formation of high molecular mass protein NLRP3 inflammasome complexes, without altering total NLRP3 protein expression levels (Supplementary Fig. 35). Activation of macrophages by exposure to ATP, MSU, ALU or *E. coli* significantly shifted caspase-1 from the low molecular mass, monomeric fraction, to the high molecular mass protein complexes in $Pkr^{+/+}$ macrophages, but not in $Pkr^{-/-}$ macrophages (Supplementary Fig. 35). Thus, activated PKR is an integral component of the assembled inflammasome.

Unexpectedly, PKR also physically interacted with NLRP1, AIM2 and NLRC4 when co-expressed in HEK293A cells (Fig. 4a). To address whether PKR might also regulate NLRP1, AIM2 and NLRC4 inflammasomes, macrophages were stimulated with anthrax lethal toxin, transfected with poly(dA-dT)•poly(dA-dT) (hereafter termed poly(dA:dT)) or infected with *S. typhimurium* to activate the NLRP1, AIM2 or NLRC4 inflammasomes, respectively. This significantly enhanced PKR autophosphorylation (Supplementary Fig. 36). Inactivation of PKR by genetic deletion (Fig. 4b–d and Supplementary Figs 37 and 38) or by pharmacological inhibition (Fig. 4e and Supplementary Fig. 39) significantly inhibited caspase-1 activation, IL-1β cleavage and HMGB1 secretion. LDH release and IL-18 production were also significantly decreased in $Pkr^{-/-}$ macrophages compared with $Pkr^{+/+}$ macrophages, whereas TNF-α production was not suppressed in these conditions (Supplementary Figs 40 and 41). Overexpression of PKR significantly enhanced NLRP1, AIM2 and NLRC4 inflammasome-induced caspase-1 activation and IL-1β cleavage in HEK293A cells (Fig. 4f and Supplementary Fig. 42).

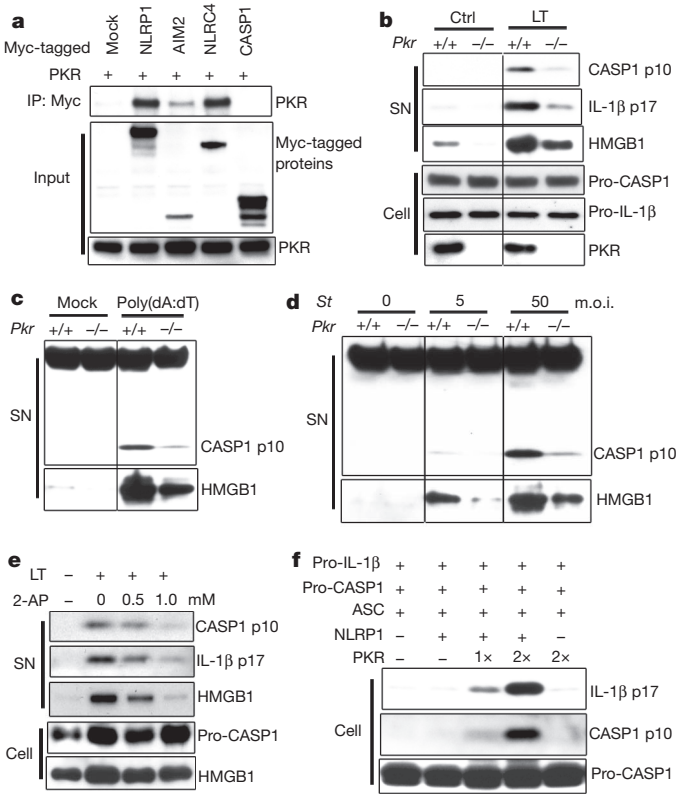

Figure 4 | PKR regulates NLRP1, AIM2 and NLRC4 inflammasome activation. **a**, HEK293A cells were transfected as indicated. The physical interaction of PKR and Myc-tagged proteins was analysed by immunoprecipitation and western blot analysis. **b**–**d**, LPS-primed (**b**) or unprimed (**c**, **d**) $Pkr^{+/+}$ or $Pkr^{-/-}$ macrophages were stimulated with anthrax lethal toxin (LT), transfected with poly(dA:dT), or infected with *S. typhimurium* (*St*). m.o.i., multiplicity of infection. **e**, $Pkr^{+/+}$ macrophages were stimulated or treated with 2-AP as indicated. **f**, HEK293A cells were transfected as indicated. Caspase-1 activation, IL-1β cleavage and HMGB1 secretion were assessed by western blot. Results are representative of at least three independent experiments.

Thus, PKR regulates the activation of the NLRP3, NLRP1, AIM2 and NLRC4 inflammasomes, but its activity does not determine which inflammasome will be activated in response to a specific stimulus.

Previous observations suggested that macrophage death by pyroptosis might initiate deleterious inflammatory responses and compromise immunity during bacterial infection[5,15], and that PKR can participate in bacteria-induced macrophage death[15]. To assess whether PKR deficiency impairs bacterial clearance, $Pkr^{+/+}$ and $Pkr^{-/-}$ mice were exposed to live *E. coli* (bearing a genetic marker) into the peritoneal cavity. Titres of the genetically labelled bacteria in the spleen and peritoneal cavity 24 h later were significantly lower in $Pkr^{-/-}$ mice than in $Pkr^{+/+}$ controls (Supplementary Fig. 43), indicating that the animals maintain the capacity to clear bacteria despite PKR deficiency.

This study establishes that PKR activity is integral to inflammasome assembly and activation. PKR physically interacts with inflammasome components, is important for caspase-1 activation, IL-1β cleavage and HMGB1 release, and mediates inflammasome activity in a cell-free system. Although originally studied as an intracellular sensor of viral dsRNA, a broader role of PKR was recently suggested as a danger-sensing molecule activated by cellular and metabolic stress[15,16,20]. Together with the established role of the inflammasome and HMGB1 in sterile and infection-associated inflammation[3,6,21–23], the identification here of PKR as a crucial regulator of inflammasome activity adds to our understanding of the mechanisms of innate immune responses to environmental, metabolic and invasive signals.

Rapidly growing evidence links the inflammasome to the pathogenesis of disease syndromes such as obesity, type 2 diabetes, atherosclerosis, gout, sepsis and colitis[4,5,10,18,24,25]. The development of therapeutics targeting the inflammasome requires knowledge of specific upstream signalling pathways that regulate inflammasome assembly and activation[25,26]. It should now be possible to design and develop therapeutics to inhibit inflammasome activity by specifically targeting PKR, without broadly impairing immunity, in the treatment of non-resolving inflammatory and meta-inflammatory syndromes.

METHODS SUMMARY

Cell stimulation. Peritoneal macrophages, BMDMs or BMDCs from $Pkr^{-/-}$ and wild-type $Pkr^{+/+}$ mice were primed with ultra-pure LPS (500 ng ml^{-1}), and then stimulated with inflammasome agonists. PKR inhibitors were added before exposure to inflammasome stimuli, where applicable. HMGB1 release, caspase-1 activation and IL-1β maturation were assessed by western blot.

Reconstituted inflammasome in HEK293A cells. Cells were transfected with plasmids expressing pro-IL-1β and inflammasome components, such as ASC, pro-caspase-1, NLRP3 and NLRP1. These modified cells were then co-transfected with plasmids that express PKR, or specific shRNA to knockdown the endogenous PKR. After 24 h, cell lysates were collected and analysed for IL-1β maturation and caspase-1 activation by western blot.

Reconstituted inflammasome in a cell-free system. Recombinant NLRP3, ASC and pro-caspase-1 were incubated with recombinant PKR and with ATP (2.5 mM) or poly(I:C) (1 ng ml^{-1}) in the PKR reaction buffer at 37 °C for 1 h. Caspase-1 activity was measured by hydrolysis of the synthetic peptide WEHD-pNA.

***E. coli*-induced peritonitis.** $Pkr^{-/-}$ and $Pkr^{+/+}$ mice were injected intraperitoneally with live *E. coli* (2×10^9 per mouse). After 6 h, blood was collected and the serum levels of IL-1β, IL-18, HMGB1 and IL-6 were measured by ELISA assay.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to K.J.T. (kjtracey@nshs.edu) or B.L. (blu@nshs.edu).

METHODS

Mice. The *Pkr*$^{-/-}$ mice and their littermate wild-type controls are originally in the mixed 129Sv/BALB/C background as described previously[16]. In *E. coli* infection and endotoxemia experiments, we used the mice that had been bred into the C57Bl/6J genetic background. Genome-wide single nucleotide polymorphism analysis confirmed that about 98% of the genetic background of these mice is of C57Bl/6J. Animal care and experimental procedures were performed with approval from the Institutional Animal Care and Use Committees of the Feinstein Institute for Medical Research and Harvard University. *Pkr*$^{-/-}$ and *Pkr*$^{+/+}$ mice are genotyped with PCR primers (*Pkr*$^{-/-}$, forward, 5′-GGAA CTTTGGAGCAATGGA-3′, reverse, 5′-TGCCAATCACAAATCTAAAAC-3′; *Pkr*$^{+/+}$, forward, 5′-TGTTCTGTGGCTATCAGGG-3′, reverse, 5′-TGACGAGT TCTTCTGAGGG-3′) yielding a 240-base-pair (bp) wild-type DNA band and a 460-bp mutant DNA band after electrophoresis in 2% agarose gel in TAE buffer.

Reagents. Ultra-pure LPS and the NLRP3 inflammasome agonists ATP, MSU and ALU were obtained from Invivogen. The anti-NLRP3 (cryo-2) antibodies were from Adipogen. The rabbit monoclonal anti-PKR (phospho T446) antibodies (E120) were from Abcam. Mouse HMGB1 monoclonal antibody IgG2b 2G7 was described previously[7,10]. *eif2a* short interfering RNA (siRNA) molecules and their negative controls were obtained from Life Technologies. Recombinant NLRP3 protein was obtained from Abnova. Recombinant Flag-tagged PKR protein was purified from *Pkr*$^{-/-}$ mouse embryonic fibroblast cells stably expressing Flag-tagged PKR. Recombinant Flag-tagged ASC and pro-caspase-1 protein were purified from HEK293A cells overexpressing Flag-tagged ASC and pro-caspase-1 (Origene). Anti-human cleaved IL-1β (D116) was from Cell Signaling. Anti-mouse IL-1β (sc-1251), anti-mouse caspase-1 (sc-514) and anti-human caspase-1 (sc-622) antibodies were from Santa Cruz.

Cell preparation and stimulation. Peritoneal macrophages were isolated and cultured as described previously[7]. Primary BMDMs and BMDCs were cultured and differentiated *in vitro* as described previously[18]. Human THP-1 cells were differentiated for 3 h with 100 nM phorbol-12-myristate-13-acetate (PMA). One million peritoneal macrophages, BMDMs, BMDCs or PMA-differentiated THP-1 cells plated in 12-well plates were primed with ultra-pure LPS (500 ng ml^{-1}) for 4 h, and then stimulated with MSU (150 μg ml^{-1}) or ALU (200 μg ml^{-1}) for 5 h, or live *E. coli* (m.o.i. = 20) for 6 h (bacteria growth is stopped by adding antibiotics into culture medium 1 h after infection), or pulsed with ATP (5 mM) for 30 min or 1 h as indicated. Ultra-pure LPS (1 ng ml^{-1})-primed mouse peritoneal macrophages were stimulated with poly(I:C) (50 μg ml^{-1}) for 16 h. For *Salmonella* infection, wild-type *S. typhimurium* was grown overnight in Luria–Bertani (LB) broth, then reinoculated at a dilution of 1:100 and grown to mid-exponential phase (3 h) to induce expression of the *Salmonella* pathogenicity island 1 type III secretion system. To minimize the involvement of NLRP3 inflammasome activation during *Salmonella* infection, unprimed *Pkr*$^{+/+}$ and *Pkr*$^{-/-}$ macrophages were infected with wild-type *S. typhimurium* (m.o.i. is from 5 to 100). The supernatant samples were collected 1 h after infection. To study AIM2 inflammasome activation, *Pkr*$^{+/+}$ and *Pkr*$^{-/-}$ macrophages were transfected with poly(dA:dT) using Lipofectamine 2000 at a concentration of 1 μg DNA plus 3.5 μl lipofectamine 2000 per ml. The supernatant samples were collected 6 h after transfection. Caspase-1 activation, HMGB1 and LDH release were analysed. To study the DNA-transfection-induced IL-1β cleavage, LPS-primed *Pkr*$^{+/+}$ and *Pkr*$^{-/-}$ macrophages were transfected with poly(dA:dT)/lyovec (Invivogen) at 5 μg ml^{-1}. There is minimum cell death with this approach. Cell lysates and precipitated supernatants were analysed by western blot analysis.

Pharmacological inhibition of PKR. The PKR inhibitor 2-AP was purchased from Sigma and dissolved in PBS:glacial acetic acid (200:1) at 60 °C for 60 min. The PKR inhibitor $C_{13}H_8N_4OS$ was purchased from Calbiochem. Ultra-pure LPS-primed peritoneal mouse macrophages, BMDMs, BMDCs or PMA-differentiated THP-1 cells were pre-treated with 2-AP (0.5 or 1.0 mM) or $C_{13}H_8N_4OS$ (0.25 or 0.5 μM) for 1–4 h before stimulating with various inflammasome activators. Higher concentrations of the above PKR inhibitors are not recommended owing to off-target effects. Cell lysates and precipitated supernatants were analysed by western blot.

ELISA. Cell culture supernatants and mouse serum samples were assayed for mouse IL-1β, IL-6, TNF-α, IL-18 (R&D Systems) and HMGB1 (IBL International) according to the manufacturer's instructions.

Transfection. One million BMDCs from *Pkr*$^{+/+}$ or *Pkr*$^{-/-}$ mice were primed with ultra-pure LPS for 2 h, and then transfected with 5 μg of poly(I:C) by lipofectamine 2000 for 6 h. In another set of experiment, ultra-pure LPS-primed BMDCs (10^6 cells) from *Pkr*$^{+/+}$ or *Pkr*$^{-/-}$ mice were transfected with 5 μg of poly(I:C) or *E. coli* RNA (Ambion) by electroporation using Nucleofector (Program V-01) and cell line Nucleofector kit V (Amaxa AG). After 3 h, cell extracts and precipitated supernatants were obtained and analysed by western blot analysis.

Reconstituted inflammasome in HEK293A cells. For the human NLRP3 inflammasome reconstitution assays, 0.2×10^6 HEK293A cells were seeded onto 24-well plates in cell culture media lacking antibiotics. After 24 h, cells were transfected with plasmids expressing pro-IL-1β and inflammasome components (Origene), such as ASC, pro-caspase-1, NLRP3 or NLRP1, using lipofectamine 2000. These modified cells were then co-transfected with plasmids that express PKR (Invivogen) or specific shRNA (Invivogen) to knockdown the endogenous PKR. The total amount of DNA was standardized to 600 ng per well using a control plasmid. Twenty-four hours later, cell lysates were collected and analysed for IL-1β maturation and caspase-1 cleavage by western blot analysis.

Immunoprecipitation. Antibodies against the PKR, NLRP3, Flag or Myc were used to precipitate proteins from cell lysis in the presence of 20 μl protein A/G beads (Santa Cruz) overnight at 4 °C. Protein complexes were washed four times with lysis buffer, and then incubated at 95 °C for 5 min and resolved by western blot analysis.

Western blot. Proteins from cell-free supernatants were extracted by methanol/chloroform precipitation as described previously[18]. Cell extracts were prepared as described previously[7]. Samples were separated by 4–20% SDS–PAGE or 4–20% native-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The relative levels of HMGB1 in the culture medium were determined by western blot analysis using recombinant HMGB1 protein to make a standard curve.

PKR autophosphorylation assay. PKR phosphorylation were assessed by PKR autophosphorylation assay as described previously[15,16].

Gel filtration chromatography. As described previously[19], fresh soluble lysates from mouse macrophages were prepared by hypotonic lysis and shearing through a 27-gauge needle. Soluble lysate (0.6 mg of total protein) was run on a Biosil 400 gel filtration column using a Bio-Rad Duoflow chromatography system in PBS with 2 mM dithiothreitol (DTT).

Reconstituted inflammasome in a cell-free system. Recombinant NLRP3, ASC and pro-caspase-1 were incubated with recombinant PKR and ATP (2.5 mM) or poly(I:C) (1 ng ml^{-1}) in PKR reaction buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 40 mM KCl and 2 mM DTT) at 37 °C for 1 h. Caspase-1 activity was measured by hydrolysis of WEHD-pNA.

***E. coli*-induced peritonitis and endotoxemia model.** *Pkr*$^{-/-}$ and *Pkr*$^{+/+}$ mice were injected intraperitoneally with live *E. coli* (2×10^9 per mouse). Six hours later, blood was collected and serum levels of IL-1β, IL-18, HMGB1 and IL-6 were measured by ELISA. Meanwhile, peritoneal cavities were washed with 10 ml of PBS. The lavage fluids were analysed for neutrophil influx by flow cytometry using the neutrophil marker GR-1. For bacterial titre experiments, *Pkr*$^{+/+}$ and *Pkr*$^{-/-}$ mice received live 2×10^9 *E. coli* into the peritoneal cavity. Bacterial titres in both the peritoneal cavity and the spleen were measured 24 h after *E. coli* challenge. To prevent other bacterial contamination during sample collection process, the *E. coli* had been transformed with an ampicillin-resistant gene. Bacterial titres were determined using ampicillin-containing LB agar plates. For endotoxemia experiments, *Pkr*$^{-/-}$ and *Pkr*$^{+/+}$ mice received 20 mg kg^{-1} of LPS and blood was collected 2.5 h later. Serum IL-1β, IL-18, TNF-α and IL-6 levels were determined by ELISA.

Analysis of HMGB1 by tamdem mass spectrometry. Detailed methods are available in Supplementary Figures and Methods.

Statistical analysis. A Student's *t*-test and one-way analysis of variance (ANOVA) were used for comparison among all different groups. $P < 0.05$ was considered statistically significant.