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The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4

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Macrophages are pivotal constituents of the innate immune system, vital for recognition and elimination of microbial pathogens[1]. Macrophages use Toll-like receptors (TLRs) to detect pathogen-associated molecular patterns—including bacterial cell wall components, such as lipopolysaccharide or lipoteichoic acid, and viral nucleic acids, such as double-stranded (ds)RNA—and in turn activate effector functions, including anti-apoptotic signalling pathways[2]. Certain pathogens, however, such as *Salmonella* spp., *Shigellae* spp. and *Yersiniae* spp., use specialized virulence factors to overcome these protective responses and induce macrophage apoptosis[3]. We found that the anthrax bacterium, *Bacillus anthracis*, selectively induces apoptosis of activated macrophages[4] through its lethal toxin, which prevents activation of the anti-apoptotic p38 mitogen-activated protein kinase[4]. We now demonstrate that macrophage apoptosis by three different bacterial pathogens depends on activation of TLR4. Dissection of anti- and pro-apoptotic signalling events triggered by TLR4 identified the dsRNA responsive protein kinase PKR as a critical mediator of pathogen-induced macrophage apoptosis. The pro-apoptotic actions of PKR are mediated both through inhibition of protein synthesis and activation of interferon response factor 3.

At least ten TLRs are known, and some of the pathogen-associated molecular patterns that cause their activation were identified[2]. Lethal toxin (LT) or p38 inhibitors induce apoptosis in macrophages incubated with either lipopolysaccharide (LPS) or lipoteichoic acid (LTA), derived from Gram-negative and Gram-positive bacteria, respectively[4]. We have now found that heat-inactivated *B. anthracis*, a Gram-positive bacterium, induces extensive macrophage apoptosis in the presence of SB202190, a p38 inhibitor, but bone-marrow-derived macrophages (BMDMs) from C3H/HeJ mice, whose TLR4 is inactive[5], are resistant to such killing (Fig. 1a). The apoptotic response to heat-killed *B. anthracis* was also seen with bacteria grown in LPS-free medium (data not shown). In wild-type BMDMs, a strong apoptotic response dependent on p38 inhibition was detected only upon treatment with the TLR4 agonist, LPS (Fig. 1b). Little or no apoptosis was seen after incubation with the TLR2 agonist synthetic bacterial lipopeptide (Pam_3CSK_4) or the TLR9 agonist immunostimulatory (CpG-containing) DNA. The TLR3 agonist, synthetic dsRNA—poly(IC)—induced a weak apoptotic response even without p38 inhibition. Transient expression of a CD4–hToll chimaera[6], in which the intracellular Toll-IL-1 receptor (TIR) domain of TLR4 was fused to the extracellular and transmembrane domains of CD4, also resulted in apoptosis after p38 inhibition (Fig. 1c). Consistent with the critical role of TLR4, BMDMs from C3H/HeJ mice, but not from the equivalent wild-type strain, C3H/HeOuJ, were resistant to apoptosis induced by LPS plus SB202190 (Fig. 1d).

TLR4 uses several adaptor proteins, including MyD88, MAL/TIRAP, TRIF and TRAM to engage downstream signalling proteins and eventually activate IκB kinase (IKK) and mitogen-activated protein kinases (MAPKs)[7,8] (Fig. 2a). Macrophages from mice lacking MyD88 (ref. 9) or TRAF6, a signalling protein that acts downstream of MyD88 and TIRAP[10], still undergo apoptosis after LPS stimulation and p38 inhibition (Fig. 2b, c). In fact, both $MyD88^{-/-}$ and $TRAF6^{-/-}$ macrophages exhibit an increased apoptotic response. NF-κB activation and IκB degradation, which depend on IKKβ[11], are reduced in both $MyD88^{-/-}$ and $TRAF6^{-/-}$ macrophages (Fig. 2d and data not shown). As NF-κB activates anti-apoptotic genes[11], these defects may explain the enhanced apoptosis of $MyD88^{-/-}$ and $TRAF6^{-/-}$ macrophages. Indeed, IKKβ-deficient macrophages generated by crossing $Ikk\beta^{F/F}$ mice[12] with mice expressing Cre recombinase from the IFN-inducible MX1 promoter[13], were defective in NF-κB activation (data not shown) and underwent apoptosis upon incubation with LPS, LTA or TNF-α, even without p38 inhibition (Fig. 2e). IKKβ-deficient macrophages were also more susceptible to poly(IC)-induced apoptosis. Deletion of IKKβ in macrophages did not affect p38 expression (Fig. 2e) or activation (data not shown).

Although TLR4 activation results in TNF-α production, the apoptosis observed in TLR4-activated and p38-inhibited macrophages was not prevented by ablating type I TNF-α receptor (unpublished results). Furthermore, unlike apoptosis induced by TNF-α, caspase-8 was not activated during LPS-induced apoptosis

(Supplementary Fig. 1a). Yet, cleavage of caspases 3, 6, 7 and 9 and cytochrome *c* release were readily observed (Supplementary Fig. 1b; data not shown), and apoptosis was inhibited by a pan-caspase inhibitor (Supplementary Fig. 1c). Hence, inhibition of p38 or IKK unleashes the ability of TLR4 to deliver a cell death signal through the mitochondrial-dependent pathway[11].

Another protein involved in TLR signalling is the dsRNA-responsive kinase PKR[14]. PKR was suggested to mediate apoptosis in fibroblasts in response to viral infection and inflammatory cytokines[15]. However, PKR also activates IKK and NF-κB[16] and thereby suppresses apoptosis. To determine the role of PKR in macrophage apoptosis, we examined its regulation and found it to be rapidly activated by either LPS or poly(IC) (Fig. 3a). Activation of PKR by either stimulus depended on TRIF (Fig. 3b). Ablation of PKR did not affect p38 or IKK activation in response to LPS (Fig. 3c) and with the exception of decreased IFNβ (Fig. 3d) or inducible NO synthase (iNOS; Fig. 3e) expression, $PKR^{-/-}$ BMDMs did not exhibit reduced induction of numerous NF-κB target genes, including those coding for anti-apoptotic proteins, such as c-IAP2, c-FLIP, A1a, A20, and Gadd45β (Fig. 3d). $PKR^{-/-}$ BMDMs also exhibited defective STAT1 phosphorylation in response to LPS (Fig. 3f), which depends on autocrine production of type I IFNs because it was not observed in macrophages deficient in type I IFN receptor (IFNRI) (Supplementary Fig. 2).

Most importantly, $PKR^{-/-}$ macrophages did not undergo apop-

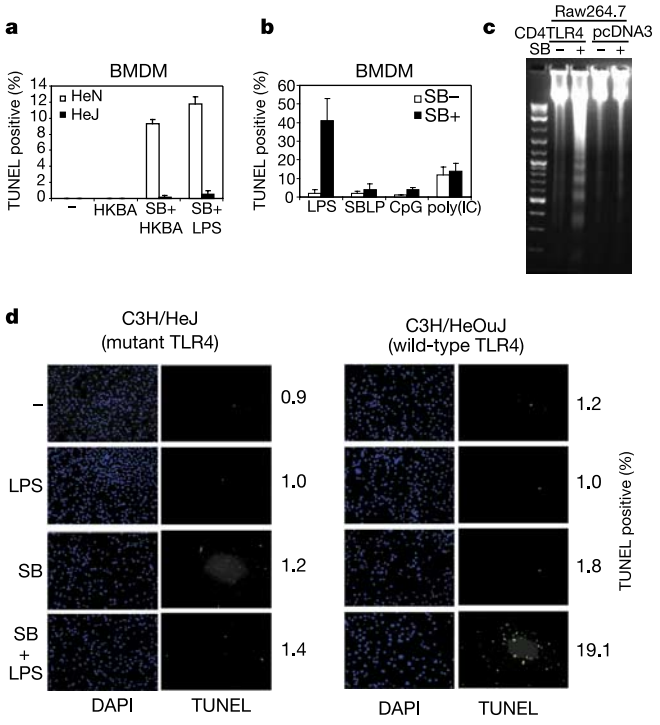

Figure 1 Heat-killed *B. anthracis* (HKBA) and LPS induce macrophage apoptosis through TLR4. **a**, HKBA induces macrophage apoptosis in a TLR4-dependent manner. BMDMs from C3H/HeN (*Tlr4* wild-type) or C3H/HeJ (*Tlr4* mutant) mice were incubated with HKBA or LPS (100 ng ml^{-1}) with or without p38 inhibitor (SB202190, 10 μM). After 18 h, apoptosis was scored by TUNEL staining. Results in this and all similar experiments were repeated several times and one representative done in triplicates is shown. Values represent averages ± s.d. **b**, TLR4 agonists induce apoptosis in the presence of a p38 inhibitor. C57BL/6 BMDMs were treated with different TLR agonists: LPS, synthetic bacterial lipopeptide (1 μg ml^{-1}), synthetic CpG-containing DNA (1 μM), or poly(IC) (10 μg ml^{-1}), with or without SB202190. **c**, TLR4 cytoplasmic domain transduces an apoptotic signal. RAW264.7 cells were transfected with a vector encoding a CD4–hTLR4 fusion protein, or an empty vector (pcDNA3). After 24 h, transfectants were incubated with or without SB202190, genomic DNA was isolated after 18 h and analysed by agarose gel electrophoresis for a nucleosomal ladder indicative of apoptosis. **d**, TLR4 is required for induction of apoptosis. C3H/HeJ or C3H/HeOuJ BMDMs were incubated with or without LPS in the presence or absence of SB202190 for 18 h and analysed by DAPI (blue) and TUNEL (green) staining. The percentage of TUNEL-positive cells is shown on the right.

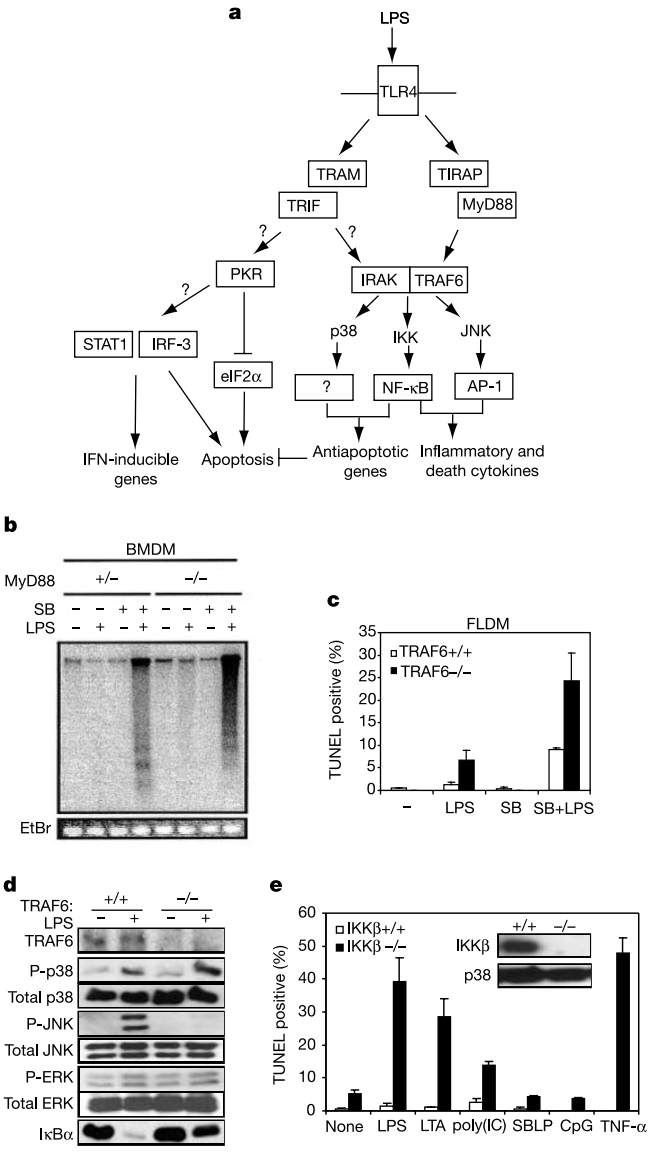

Figure 2 Role of effector molecules in TLR4-induced apoptosis. **a**, A diagram of currently known TLR4-stimulated signalling pathways in macrophages. Question marks denote yet-to-be established connections. **b**, Role of MyD88. $MyD88^{+/-}$ and $MyD88^{-/-}$ BMDMs were incubated with or without LPS in the presence or absence of SB202190. After 18 h, genomic DNA was isolated and end-labelled with [α-P^{32}]-dATP and Taq polymerase, followed by agarose gel electrophoresis and autoradiography. Gel loading was examined by EtBr staining. **c**, Role of TRAF6. Fetal-liver-derived $TRAF6^{+/+}$ and $TRAF6^{-/-}$ macrophages (FLDMs) were stimulated as above, and apoptotic cell death was quantified by TUNEL staining. **d**, Characterization of LPS signalling. $TRAF6^{+/+}$ and $TRAF6^{-/-}$ FLDMs were untreated or treated with LPS. After 20 min, cell lysates were prepared and analysed by immunoblotting with antibodies specific to TRAF6, IκBα, different MAPKs and their phosphorylated forms. **e**, Role of IKKβ. BMDMs from *Ikkβ* $^{F/F}$ ($IKK\beta^{+/+}$) or *MX1Cre-Ikkβ* $^{F/F}$ ($IKK\beta^{-/-}$) mice were untreated or treated with LPS, LTA from *B. subtilis* (10 μg ml^{-1}), poly(IC) (10 μg ml^{-1}), synthetic bacterial lipopeptide, SBLP (1 μg ml^{-1}), CpG-containing DNA (1 μM), or mouse TNF-α (10 ng ml^{-1}). After 12 h, apoptotic cell death was quantified by TUNEL staining. Inset, macrophage lysates were analysed by immunoblotting with antibodies specific to IKKβ and p38.

tosis upon incubation with LPS and either SB202190 or LT (Fig. 4a) or after prolonged incubation with LPS alone (Supplementary Fig. 3a). $PKR^{-/-}$ macrophages were, however, fully sensitive to other pro-apoptotic stimuli, such as doxorubicin or 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (Supplementary Fig. 3b). PKR activation contributes to induction of type I IFNs, such as IFNβ, which can further increase its expression[17]. Type I IFNs sensitize myeloid cells to LPS, LTA or bacterial-induced apoptosis[18]. Whereas IFNβ alone did not induce apoptosis in BMDMs, it rendered them susceptible to LPS-induced apoptosis even without SB202190 (Fig. 4b). IFNβ also potentiated the apoptosis of wild-type BMDMs exposed to LPS + SB202190, but $PKR^{-/-}$ BMDMs were resistant to these effects of IFNβ (Fig. 4b). Nevertheless, BMDMs from $IFNRI^{-/-}$ mice[19] still underwent apoptosis when incubated with LPS and SB202190 (Fig. 4c) and exhibited normal PKR activation by LPS (Supplementary Fig. 4). Thus, although PKR contributes to IFNβ production, which can potentiate macrophage apoptosis, IFNβ signalling itself is not essential for TLR4-induced PKR activation or macrophage apoptosis.

PKR is activated by poly(IC), which induces macrophage apoptosis even without p38 inhibition (Fig. 1b). Activated PKR phosphorylates eukaryotic translation initiation factor 2α (eIF2α) at serine 51 and thereby inhibits protein synthesis[20]. Although the kinase activity of PKR is not required for NF-κB activation, it is needed for induction of apoptosis (Supplementary Fig. 5). In addition, macrophages are very sensitive to protein synthesis inhibition (Supplementary Fig. 6). Hence, PKR activation may cause macrophage apoptosis by inhibiting synthesis of anti-apop-

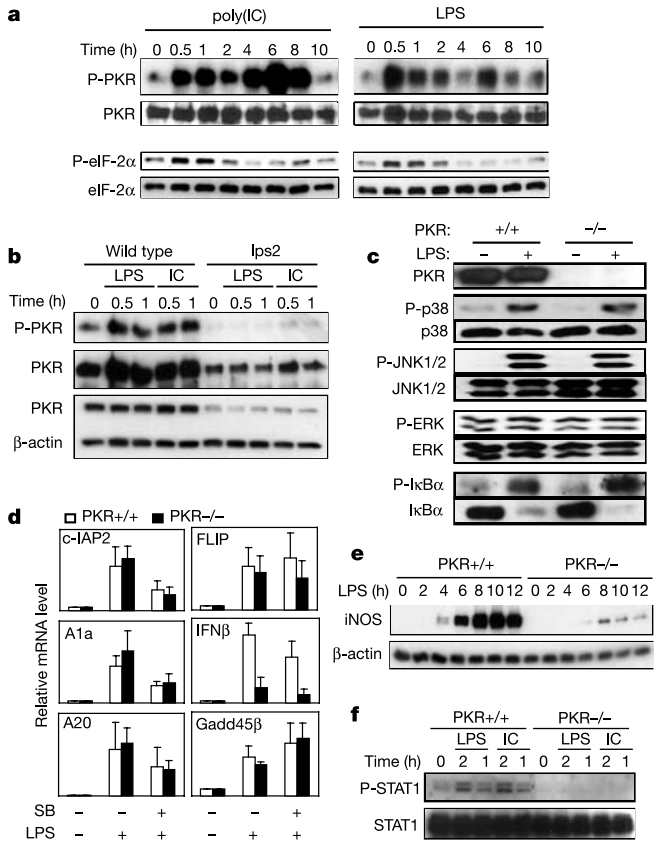

Figure 3 PKR is necessary for LPS-induced interferon signalling pathway in macrophages. **a**, Activation of PKR by LPS. BMDMs were stimulated with LPS (100 ng ml^{-1}) or poly(IC) (10 μg ml^{-1}). At the indicated time points cells were lysed and PKR activation was monitored by autophosphorylation. Gel loading was controlled by immunoblotting for PKR. The lysates were monitored for eIF-2α phosphorylation by immunoblotting with antibodies specific for phosphorylated eIF-2α (P-eIF-2α) and total eIF-2α. **b**, PKR acts downstream of TRIF. Wild-type and Ips2 (TRIF-deficient) BMDMs were stimulated with LPS or poly(IC). PKR activation was monitored by autophosphorylation. The same lysates were examined for PKR and β-actin content by immunoblotting. **c**, Normal MAPK and IKK activation in $PKR^{-/-}$ macrophages. $PKR^{+/+}$ and $PKR^{-/-}$ BMDMs were left unstimulated or stimulated with LPS. After 20 min, cell lysates were prepared and immunoblotted with antibodies to different MAPKs or IκBα and their phosphorylated forms. **d**, PKR is required for IFNβ induction but is dispensable for induction of anti-apoptotic genes. $PKR^{+/+}$ and $PKR^{-/-}$ BMDMs were incubated with or without LPS in the absence or presence of SB202190. After 4 h, total cellular RNA was isolated, and relative gene expression was determined by real-time PCR. The results are averages of three separate experiments normalized to the level of cyclophilin mRNA. **e**, **f**, PKR is required for iNOS induction (**e**) and STAT1 phosphorylation (**f**). $PKR^{+/+}$ and $PKR^{-/-}$ BMDMs were incubated with LPS or poly(IC). At the indicated time points, cell lysates were prepared and iNOS expression and STAT1 phosphorylation were examined by immunoblotting.

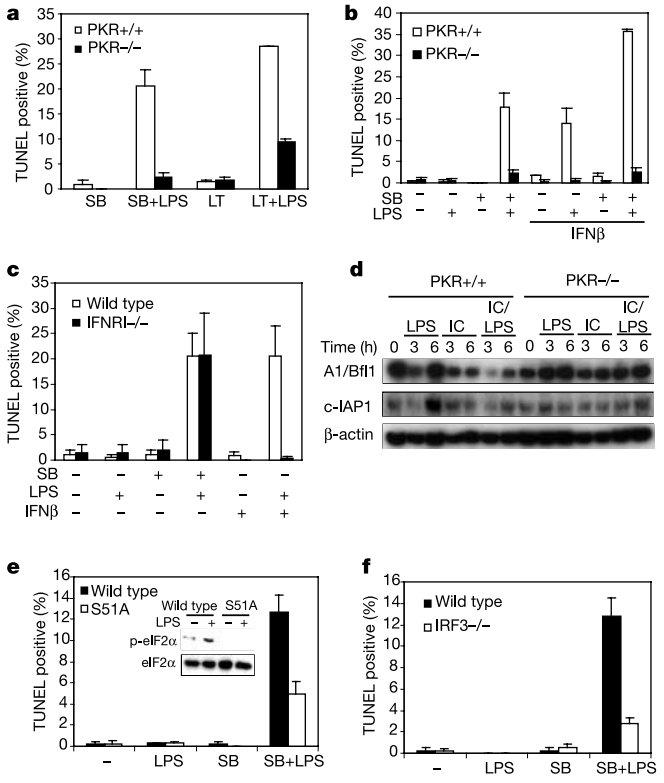

Figure 4 PKR is required for LPS-induced macrophage apoptosis. **a**, PKR-deficient BMDMs are resistant to LPS-induced apoptosis. $PKR^{+/+}$ and $PKR^{-/-}$ BMDMs were left unstimulated or stimulated with either LPS in the presence or absence of either SB202190 or LT (500 ng ml^{-1} LF and 2.5 μg ml^{-1} protective antigen, PA), and the extent of apoptosis was determined after 18 h. **b**, IFNβ sensitizes BMDMs to LPS-induced apoptosis. $PKR^{+/+}$ and $PKR^{-/-}$ BMDMs were incubated with or without LPS in the absence or presence of SB202190 or IFNβ (1,000 U ml^{-1}) for 18 h and the extent of apoptosis was determined. **c**, Type I IFN signalling is not required for LPS-induced macrophage apoptosis. BMDMs from $IFNR1^{+/+}$ or $IFNR1^{-/-}$ mice were incubated with or without LPS in the presence or absence of SB202190 and IFNβ for 18 h and the extent of apoptosis was determined. **d**, PKR activation inhibits A1/Bfl1 expression. $PKR^{+/+}$ and $PKR^{-/-}$ BMDMs were transfected with or without poly(IC) using Lipofectamine. After 6 h, LPS was added and the levels of A1/Bfl1 and cIAP-1 were examined by immunoblotting. **e**, eIF2α phosphorylation is required for induction of macrophage apoptosis. BMDMs derived from lethally irradiated mice reconstituted with fetal liver stem cells from either wild-type or *eIF2α(S51A)* mice[22] were incubated with LPS with or without SB202190 and the extent of apoptosis was analysed. The inset shows the absence of eIF2α phosphorylation in knockin macrophages. **f**, IRF3 is required for induction of macrophage apoptosis. BMDMs from wild-type or $IRF3^{-/-}$ mice were incubated with or without LPS in the presence or absence of SB202190 for 18 h and the extent of apoptosis was determined.

totic proteins. To enhance PKR activation and bypass TLR3, which may activate anti-apoptotic pathways, we introduced poly(IC) into macrophages by transfection[21]. This resulted in higher levels of PKR activity and caused a higher level of apoptosis (data not shown). Transfection of wild-type BMDMs with poly(IC) followed by LPS treatment inhibited accumulation of A1/Bfl1, an anti-apoptotic member of the Bcl2 family known to inhibit LPS-induced apoptosis of neutrophils[11], and to a lesser extent c-IAP1. The same treatment of PKR$^{-/-}$ BMDMs did not reduce the level of either protein (Fig. 4d).

To examine directly the role of eIF2α phosphorylation in macrophage apoptosis, we transplanted fetal liver haematopoietic progenitors from *eIF2α(S51A)* knockin mice, which die shortly after birth, to lethally irradiated wild-type mice, in order to obtain macrophages that express an eIF2α variant in which serine 51, the major PKR phosphorylation site, was replaced with an alanine[22]. *eIF2α(S51A)* BMDMs were considerably less sensitive than wild-type BMDMs to apoptosis caused by incubation with LPS and SB202190 (Fig. 4f). The residual apoptotic response in *eIF2α(S51A)* macrophages suggested the existence of another PKR-dependent pro-apoptotic pathway. It was proposed that interferon response factor 3 (IRF3), a transcription factor activated by dsRNA, is an important mediator of virus-induced apoptosis[23]. We found that BMDMs from IRF3$^{-/-}$ mice[24] showed increased resistance to LPS + SB202190 (Fig. 4f).

The role of TLR4 and PKR in apoptosis induced by live pathogenic bacteria was investigated using BMDMs infected with *B. anthracis*, *Yersinia* and *Salmonella*. PKR$^{-/-}$ macrophages showed markedly reduced levels of apoptosis compared with PKR$^{+/+}$ cells (Fig. 5a, b). The result with live anthrax bacilli was similar to the one obtained with LT regarding the PKR dependence of apoptosis, but did not require addition of LPS (compare Fig. 5a to Fig. 4a). Pathogens that induce macrophage apoptosis activate TLR4 through cell wall components, but apoptosis also requires a specific contribution from the bacteria. *Yersinia* spp. injects YopJ, an inhibitor of MAPK and IKK activation[25], into the host cell cytoplasm. As expected, a *yopJ* mutant of *Yersinia pseudotuberculosis* did not induce apoptosis in PKR$^{+/+}$ macrophages (data not shown).

For *Salmonella* infections, PKR-dependent macrophage apoptosis requires the SPI2 locus (data not shown), which is responsible for translocation of bacterial virulence proteins from the phagosome into the macrophage cytoplasm[26]. However, the *Salmonella* SipB protein, a caspase 1 activator[26], was dispensable for PKR-dependent apoptosis (data not shown), a finding consistent with the distinct mechanism of SipB-mediated cell death that differs from classical apoptosis[26]. Consistent with the results described above, *eIF2(S51A)* and IRF3$^{-/-}$ macrophages were also less susceptible to *Salmonella*-induced apoptosis (Fig. 5c, d). BMDMs from TLR4-deficient mice exhibited a dramatically reduced apoptotic response after infection with *B. anthracis* (Supplementary Fig. 7) and as shown in Fig. 1a, the *Tlr4* mutation in the C3H/HeJ strain prevented macrophage apoptosis by heat-killed *B. anthracis*. The apoptotic response to *Salmonella* and *Yersinia* was also considerably reduced in BMDMs from C3H/HeJ mice (Fig. 5e). Thus, the TLR4 to PKR pathway is crucial for macrophage apoptosis elicited by both Gram-positive and Gram-negative pathogens.

Microbial-induced macrophage apoptosis may represent a major mechanism allowing pathogenic bacteria to avoid detection and destruction by the innate immune system[3]. Virulence factors used by certain pathogens to dismantle host defences were identified and in some cases shown to act through inhibition of anti-apoptotic signalling pathways[4,25,26]. The results described above shed further light on this phenomenon and identify what appears to be a general mechanism used by three different bacterial pathogens, *B. anthracis*, *Yersinia* and *Salmonella*, to specifically kill activated macrophages. We find that macrophage apoptosis by either Gram-positive (*B. anthracis*) or Gram-negative (*Yersinia*, *Salmonella*) pathogens requires activation via TLR4. Curiously, however, TLR4 is not a typical death receptor with death domains that cause caspase-8 activation. In fact, TLR4 engagement results in activation of both anti-apoptotic and pro-apoptotic signalling pathways. Normally, the anti-apoptotic pathways, which depend on MyD88 and TIRAP/

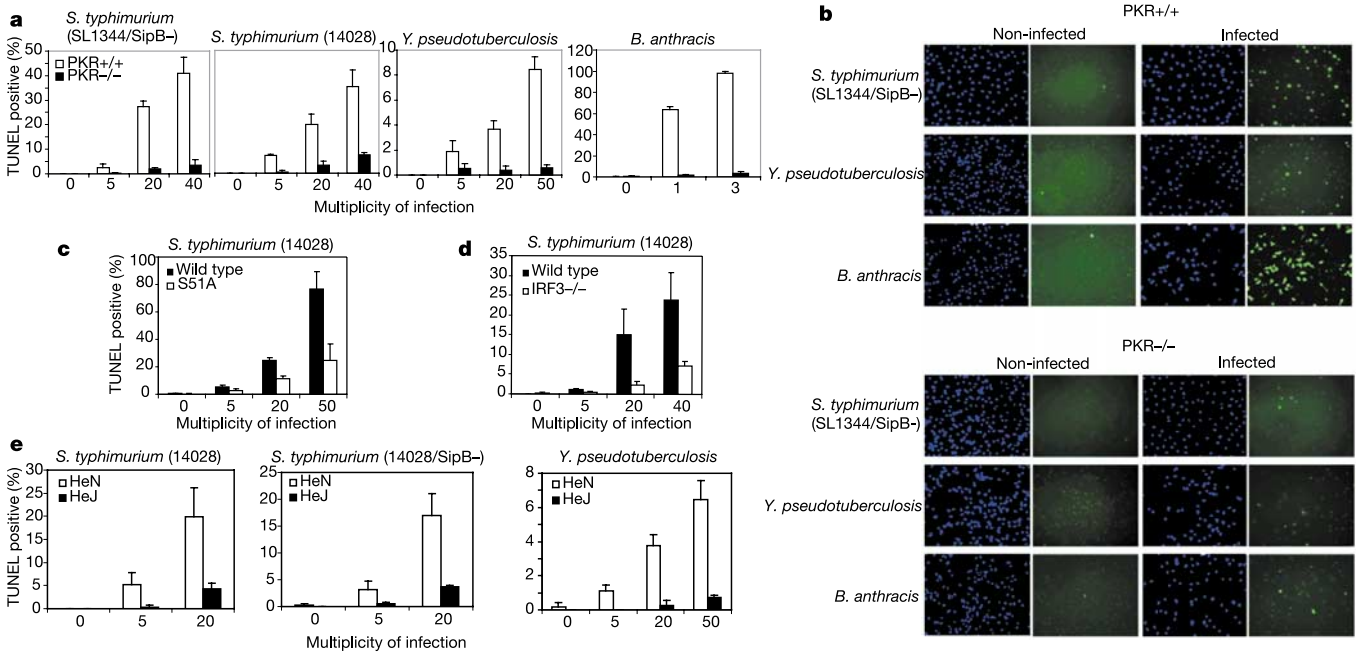

Figure 5 PKR-deficient macrophages are resistant to pathogen-induced apoptosis. **a**, PKR$^{+/+}$ and PKR$^{-/-}$ BMDMs were infected with *S. typhimurium* (SL1344/SipB^{-}), *S. typhimurium* (14028), *Y. pseudotuberculosis*, or *B. anthracis* at the indicated multiplicity of infection. Apoptotic TUNEL-positive cells were scored 18 h post-infection. **b**, Representative DAPI (blue) and TUNEL (green) staining of PKR$^{+/+}$ and PKR$^{-/-}$ BMDMs 18 h post-infection with the indicated pathogens. **c**, **d**, wild-type and *eIF2α(S51A)* (**c**), or IRF3$^{-/-}$ (**d**) BMDMs were infected with *S. typhimurium* (14028) as above and the extent of apoptosis was determined 18 h later. **e**, BMDMs from C3H/HeN (*Tlr4* wild-type) and C3H/HeJ (*Tlr4* mutant) mice were infected with the indicated pathogens and the extent of apoptosis was determined after 18 h.

MAL, dominate, and exposure of macrophages to bacterial cell wall components, such as LPS, does not result in considerable cell death. However, at least two of the pathogens we examined produce specific virulence factors that inhibit survival pathways (p38, IKK/NF-κB) and thereby tilt the balance in favour of cell killing.

We identified PKR as an essential component of the TLR4-triggered macrophage apoptosis pathway. On the basis of phenotypic and biochemical similarities in the behaviour of PKR- and TRIF- or TRAM-deficient macrophages[7,8] and the requirement of TRIF for PKR activation, we propose that TLR4 activates PKR and triggers apoptosis through these newly described adaptors. Although PKR is an important contributor to antiviral defences under certain circumstances[27], its overall contribution to host defences in the case of bacterial infections has not been explored. The results described above suggest that PKR-inhibition may strongly augment macrophage-mediated anti-bacterial responses that do not depend on production of type I IFN and induction of IFN-responsive genes such as *iNOS*. The decreased expression of iNOS, a major inducer of vasodilation upon PKR inhibition, may also be taken advantage of for prevention of septic shock. □

Methods

Mice and bone-marrow-derived macrophages

To delete IKKβ in macrophages, $Ikk\beta^{F/F}$ mice[12] were crossed with MX1-Cre mice (Jackson Laboratory). $PKR^{-/-}$ mice[28], $IFNRI^{-/-}$ (A129) and wild-type mice of the same genetic background (129/SvEv)[19] were obtained from E. Raz. C3H/HeJ, C3H/HeOuJ, C3H/HeN mice[5] and bone marrow from lps2 mice[7] were obtained from B. Beutler. $TRAF6^{+/-}$, $MyD88^{+/-}$ and $IRF3^{-/-}$ mice were received from J. Inoue, S. Akira and T. Taniguchi, respectively. C57BL/6J and C57BL/10ScCr ($TLR4^{-/-}$) mice were from the Jackson Laboratory. Unless otherwise mentioned, all knockout mice were of the C57BL/6 background, which is resistant to LT-induced necrosis. BMDMs were prepared and cultured as described[4].

Analysis of gene expression and cell signalling

Total cellular RNA was prepared using TRIzol (Invitrogen), quantified by ultraviolet absorption and analysed by real-time polymerase chain reaction (PCR)[4]. Primer sequences are available upon request. All values were normalized to the level of cyclophilin messenger RNA expression. Whole-cell extracts were prepared and PKR activity was measured by autophosphorylation[20] after immunoprecipitation with anti-PKR antibody (Santa Cruz). PKR recovery was assessed by immunoblotting. Phosphorylation of eIF2α was detected by immunoblotting with antibody against phosphorylated eIF2α (Biosource). IKK and MAPK activation were measured as described[4]. Phosphorylation of STAT1 was monitored by immunoblotting with anti-phospho-STAT1 antibody (Cell Signalling).

Bacterial strains, macrophage infections and TUNEL assay

The wild-type *Salmonella typhimurium* strains used were SL1344 and 14028. *S. typhimurium* 14028 *ssaV* and *sipB* contain mutations in genes that code for components of the SPI2 type III protein secretion system and SipB, respectively. *Y. pseudotuberculosis* strains YP126 (wild type) and YP26 ($YopJ^-$) were obtained from J. Bliska. *S. typhimurium* BMDM infection was as described[29], while *Y. pseudotuberculosis* infection was done as described[30] with slight modifications: BMDMs were infected with bacteria for 1 h, and then cultured in fresh medium containing gentamicin ($20\,\mu g\,ml^{-1}$) for another 18 h. The *B. anthracis* Sterne strain was grown overnight on BHI (brain–heart infusion) agar. A single colony was inoculated into BHI broth or RPMI medium + 10% FCS (endotoxin-free) in disposable tubes and grown with vigorous shaking to an OD_{600} of 0.4. Bacteria were washed with PBS and resuspended in PBS. To prepare heat-killed *B. anthracis*, bacterial suspensions in PBS were heated to 65 °C for 30 min. Macrophage cultures were infected as indicated and incubated for 1 h at 37 °C in 5% CO_2/95% air. Gentamicin was added to a final concentration of $20\,\mu g\,ml^{-1}$. After 20 h, the medium was removed and cells were fixed with 4% paraformaldehyde in PBS. TUNEL or DNA fragmentation assays were performed as described[4].

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