

# Involvement of xanthine oxidase and hypoxia-inducible factor 1 in Toll-like receptor 7/8-mediated activation of caspase 1 and interleukin-1 $\beta$

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**Abstract** Inflammatory reactions to ssRNA viruses are induced by the endosomal Toll-like receptors (TLRs) 7 and 8. TLR7/8-mediated inflammatory reaction results in activation of the Nalp3 inflammasome via an unknown mechanism. Here we report for the first time that TLR7/8 mediate activation of xanthine oxidase (XOD) in an HIF-1 $\alpha$ -dependent manner. XOD produces uric acid and reactive oxygen species, which could activate Nalp3 and therefore induce activation of caspase 1, known to convert inactive pro-IL-1 $\beta$  into active IL-1 $\beta$ . Specific inhibition of the XOD activity attenuates TLR7/8-mediated activation of caspase 1 and IL-1 $\beta$  release. These results were obtained using human THP-1 myeloid macrophages. The findings were verified by conducting *in vivo* experiments on mice.

**Keywords** Inflammation · Toll-like receptors · Inflammasome · HIF-1 · IL-1 $\beta$  · Caspase 1

## Introduction

ssRNA viruses [human immunodeficiency virus 1 (HIV-1), influenza, hepatitis C, many hemorrhagic fevers, etc.] cause severe illnesses, resulting in significant morbidity and mortality in both industrialised and developing countries [1]. Inflammatory reactions to ssRNA viruses are

induced by the endosomal Toll-like receptors (TLRs) 7 and 8 expressed by various cell types, including myeloid macrophages and neutrophils [2]. Ligand-induced TLR7/8-mediated inflammatory reactions result in the production of pro-inflammatory cytokines including interleukin 1 beta (IL-1 $\beta$ ), interleukin 6 (IL-6), interleukin 12 (IL-12) and tumour necrosis factor alpha (TNF- $\alpha$ ). Among these cytokines only generation of IL-1 $\beta$  is induced indirectly via activation of the multiprotein complex known as the inflammasome. Production of other cytokines is controlled directly by the transcription factors activated by TLR7/8 downstream pathways [1–3]. IL-1 $\beta$  is a highly inflammatory cytokine, which is considered as a major contributor to further development of the host immune defence [3]. IL-1 $\beta$  is activated via caspase 1-dependent conversion of the inactive pro-IL-1 $\beta$ . Caspase 1 is activated in this case by the inflammasome formed by the Nod-like receptor Nalp3. Active Nalp3 complexed to the apoptosis-associated speck-like protein (ASC) interacts with the caspase 1 forming the inflammasome, which induces the proteolytic caspase activity of the enzyme [3–5]. However, the biochemical mechanisms of the cross-talk between TLR7/8 and the Nalp3 inflammasome remain unknown. It is, however, known that Nalp3 could be activated by uric acid (UA), formed by the enzyme xanthine oxidase (XOD), which converts hypoxanthine into xanthine and further into UA [6, 7]. This enzyme is activated in response to some TLR ligands (for example, LPS—the ligand of TLR4 [8, 9]) as well as to pro-inflammatory cytokines and interferons [8]. These factors promote XOD expression. Both expression and assembly of XOD require energy [8, 9]. Energy metabolism is, however, supported during the TLR7/8-mediated inflammatory reaction by the hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ), the inducible subunit of the HIF-1 transcription complex, which promotes glycolysis and

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protects the cells against TLR-induced ATP depletion [10, 11]. The regulatory region of the XOD gene contains HIF-1 $\alpha$  cognate hypoxia-responsive elements [8]. Ligand-induced TLR7/8-mediated intracellular signalling events include activation of HIF-1 $\alpha$  in a redox-dependent manner involving the reactive oxygen species (ROS) produced by the NADPH oxidase complex [10]. UA was also reported to stimulate the activity of NADPH oxidase [12]. We therefore hypothesised that cross-regulation of XOD-dependent formation of UA and accumulation/activation of HIF-1 $\alpha$  protein could lead to the TLR7/8-mediated formation of the Nalp3 inflammasome, followed by the activation of caspase 1.

## Materials and methods

### Materials

Medium, fetal calf serum and supplements, DOTAP transfection reagent, enhanced avian HS RT-PCR kit, GenElute<sup>TM</sup> mammalian total RNA miniprep kit, and all pharmacological kinase/phosphatase inhibitors, HIF-1 $\alpha$ -specific siRNA and the uridine-rich ssRNA fragment of the HXB2 region of HIV-1 (the sequence is UUGUUAAGU-GUUCAAUUGU [13]) were purchased from Sigma (Suffolk, UK). Maxisorp<sup>TM</sup> microtitre plates were obtained from Nunc (Roskilde, Denmark). The ELISA-based assay kits for detection of human and mouse IL-1 $\beta$  as well as caspase 1 assay kit were bought from R&D Systems (Abingdon, UK). Mouse monoclonal antibody to HIF-1 $\alpha$ , mouse monoclonal antibody to TLR7, mouse monoclonal antibody to  $\beta$ -actin as well as rabbit polyclonal HRP-labelled antibody to mouse IgG and goat polyclonal HRP-labelled antibody to rabbit IgG were from Abcam (Cambridge, UK). All other chemicals were of the highest grade of purity and commercially available.

### Cell culture

THP-1 human leukemia monocytic macrophages were purchased from European Collection of Cell Cultures (Salisbury, UK). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (50 IU/ml) and streptomycin sulfate (50  $\mu$ g/ml).

### Mice

Twenty-one male wild type B6-albino inbred mice were used for the experiments. The experiments using mice were carried out at the specialised Immunology Unit of Odessa State Medical University (Odessa, Ukraine). The age of mice was 2 months, weight  $20 \pm 2$  g. All experiments

with mice were approved by the Institutional Animal Committee and handled in accordance with the Helsinki declaration. Blood serum and leukocyte fraction were isolated as described before [14].

### Transfer of ssRNA fragment and Nalp3/HIF-1 $\alpha$ siRNA to THP-1 cells

siRNAs specific to Nalp3 and HIF-1 $\alpha$  were selected as described previously and purchased from Sigma (Suffolk, UK) together with mutated forms of both siRNAs (which were used as inactive controls) [10, 11]. In all cases mutated siRNAs did not impact the investigated processes (data not shown), confirming that the effects observed were caused by knocking down specific genes when respective specific siRNAs were used. siRNAs as well as the ssRNA fragment were transfected into THP-1 cells using DOTAP transfection reagent according to the manufacturer's protocol.

### Western blot analysis

HIF-1 $\alpha$  proteins were detected by Western blot analysis [10, 15]. Briefly, 100  $\mu$ g of cell lysate protein was added to the same volume of 2 $\times$  sample buffer (125 mM Tris/HCl, 2% SDS, 10% glycerin, 1 mM DTT, 0.002% bromophenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 7.5% SDS-polyacrylamide gels and blotted to nitrocellulose membrane. Molecular weights were calibrated in proportion to the running distance of rainbow markers. Further analysis was performed as described previously [10, 15]. Actin was used as a protein loading control in all Western blot experiments. The Western blot data for HIF-1 $\alpha$  protein were analysed by densitometry (ImageJ programme was used for analysis) and normalised against respective levels of actin.

### Measurement of HIF-1 $\alpha$ and Nalp3 mRNAs by RT-PCR

Total RNA was isolated using GenElute<sup>TM</sup> mammalian total RNA miniprep kit, followed by HIF-1 $\alpha$  mRNA reverse transcriptase-polymerase chain reaction (RT-PCR) [10, 16]. Primer selection was as follows: HIF-1 $\alpha$ , 5'-CTC AAAGTCGGACAGCCTCA-3', 5'-CCCTGCAGTAGGT TTCTGCT-3'; Nalp3, 5'-CTGTGTGTGGGACTGAAG CAC-3', 5'-GCAGCCCTGCTGTTTCAGCAC-3'; actin, 5'-TGACGGGGTCAACCCACACTGTGC-CCATCTA-3', 5'-CTAGAAGCATTGCGGTTCGACGATG-GAGGG-3'. Amplification program was as follows: 95°C, 30 s; 56°C, 30 s; 72°C, 1 min; 20 cycles; 72°C, 10 min. Products were separated on 2% agarose gels and visualised with ethidium bromide.

### Measurement of caspase 1 activity

The activity of caspase 1 was assayed by the colorimetric method based on the hydrolysis of the peptide substrate. A commercially available R&D caspase 1 assay kit was used for analysis according to the manufacturer's protocol.

### Detection of the XOD activity and the UA

XOD activity was detected spectrophotometrically as described previously [17]. UA was detected by thin-layer chromatography (TLC) [18]. Blood serum UA was analysed by both TLC and the colorimetric assay [18].

### Detection of ROS generation

Generation of ROS was analysed by luminol-dependent chemiluminescence as described previously [19]. TRS were detected as indicated elsewhere [20].

### Measurement of IL-1 $\beta$ production

The levels of IL-1 $\beta$  produced by THP-1 and in blood serum of mice were analysed by ELISA, using R&D kits according to the manufacturer's protocols.

### Detection of phosphoinositide 3 kinase activity

Activity of phosphoinositide 3 (PI3) kinase was analysed using the well-described method [21, 22].

### Cell viability assay

Cell viability was analysed by the MTS test. For that purpose we used the MTS cell viability assay kit manufactured by Promega (Southampton, UK) according to the manufacturer's protocol.

### Statistical analysis

Each experiment was performed at least three times and statistical analysis was done using the two-tailed Student's *t* test. The statistical probability (*P*) was expressed as  $*P < 0.01$ . The normal distribution of data was checked.

## Results

To confirm that Nalp3 is involved in the TLR7/8-induced IL-1 $\beta$  production we exposed normal and Nalp3-knockdown (achieved by the transfection of the Nalp3-specific siRNA; successful knockdown was verified by the RT-PCR) THP-1 human myeloid macrophage cells (expressing

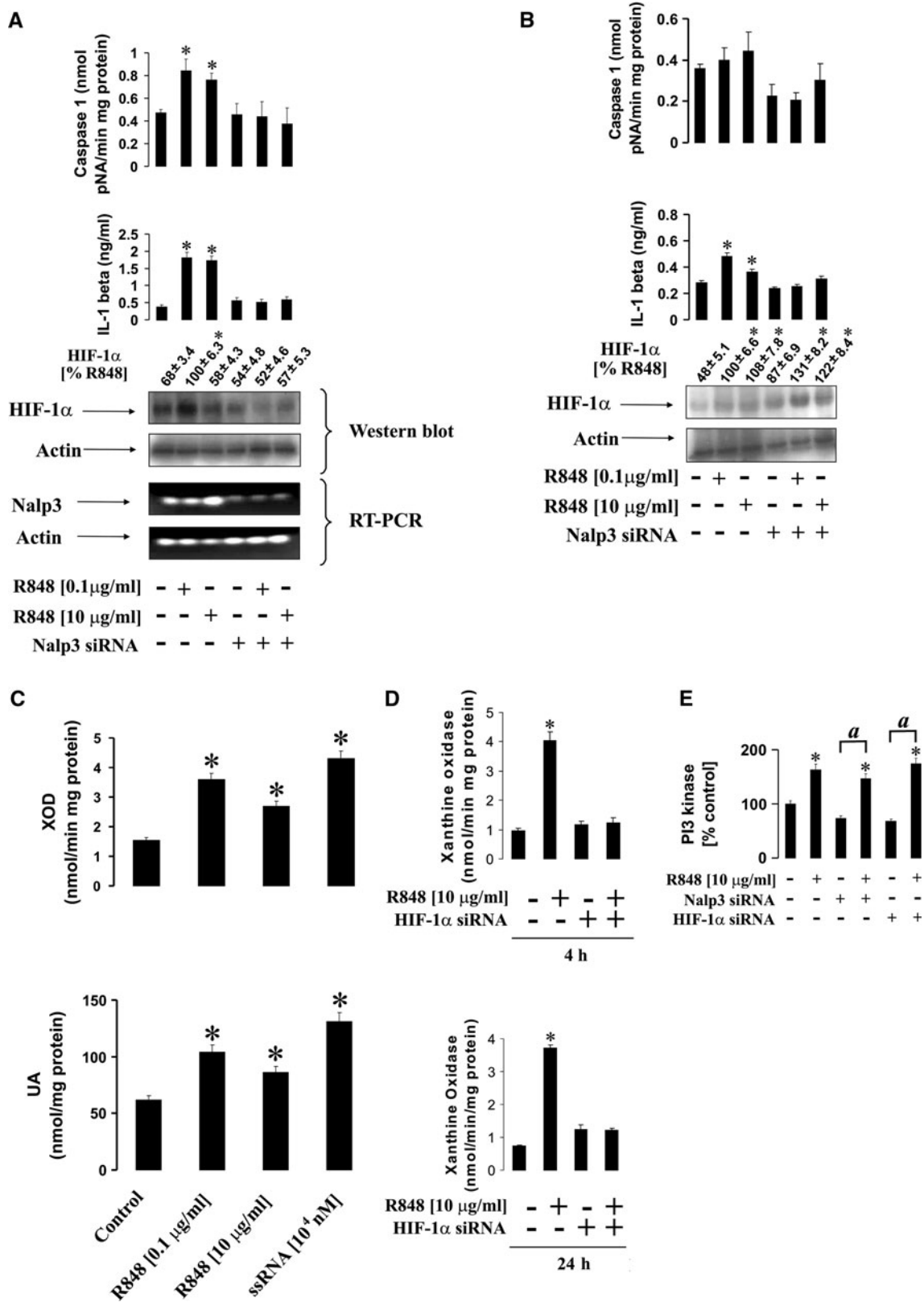
both TLR 7 and 8) to different concentrations (0.1 and 10  $\mu\text{g/ml}$ ) of the specific TLR7/8 ligand R848 [10] for 24 h. We found that exposure of the cells to the R848 led to a significant increase in the caspase 1 activity as well as the amount of IL-1 $\beta$  released. This was consistent with an increase in HIF-1 $\alpha$  accumulation. All these effects did not take place in the Nalp3 knockdown THP-1 cells (Fig. 1a). Since HIF-1 $\alpha$  protein induction is supported by IL-1 $\beta$  [23], we studied whether accumulation of HIF-1 $\alpha$  protein is attenuated at earlier stages of exposure of the cells to the R848 when the amount of IL-1 $\beta$  is much lower and therefore insufficient to significantly influence intracellular signalling events. By exposing both normal and Nalp3 knockdown THP-1 cells to 0.1 and 10  $\mu\text{g/ml}$  R848 for 4 h, we found that release of IL-1 $\beta$  was still attenuated in Nalp3 knockdown THP-1 cells, but that accumulation of HIF-1 $\alpha$  was not affected (Fig. 1b). This suggests that most likely at the later stages of the inflammatory response, IL-1 $\beta$  contributes to the accumulation of the HIF-1 $\alpha$  protein.

To find out whether TLR7/8 could mediate XOD activation we exposed THP-1 cells for 24 h to 0.1 and 10  $\mu\text{g/ml}$  R848 or 10<sup>4</sup> nM ssRNA fragment [10, 13] (see "Materials and methods" for details). We have found that XOD was significantly activated in all cases, which correlated with a significant increase in UA—the final product of purine catabolism in humans (Fig. 1c).

To study if XOD activation is dependent on HIF-1 $\alpha$  we exposed normal and HIF-1 $\alpha$  knockdown (achieved by transfection of the cells with HIF-1 $\alpha$  specific siRNA) THP-1 cells to 10  $\mu\text{g/ml}$  R848 for 4 or 24 h. To rule out the possibility of purine-dependent XOD potentiation, the highest concentration (10  $\mu\text{g/ml}$ ) of R848 (a purine-containing compound) was used. XOD activation took place for both time points in normal but not HIF-1 $\alpha$  knockdown THP-1 cells (Fig. 1d), suggesting the involvement of this protein in TLR7/8-mediated XOD activation.

Performed cell transfections did not affect the ability of the intracellular TLR7/8 to react to the ligand. The activity of PI3 kinase (whose activation by TLR7/8 does not depend on Nalp3 or HIF-1 $\alpha$ ) [24] was similarly increased in both normal and Nalp3/HIF-1 $\alpha$  knockdown cells in response to R848 stimulation (Fig. 1e). In addition, according to the results of the MTS test, the viability of the cells was not significantly affected by the transfections performed (data not shown).

To investigate whether XOD is involved in the activation of caspase 1, we pre-treated THP-1 cells for 60 min with 250  $\mu\text{g/ml}$  allopurinol (XOD specific inhibitor) and exposed them to 10  $\mu\text{g/ml}$  R848 or 10<sup>4</sup> nM ssRNA for 24 h. In both cases the ligand-induced activation of the caspase 1 and IL-1 $\beta$  release were attenuated. ROS production and HIF-1 $\alpha$  accumulation were affected as well (Fig. 2a, b). However, similar treatments performed for 4 h



led to attenuation of IL-1 $\beta$  production, but did not lead to a decrease in ROS generation and accumulation of HIF-1 $\alpha$  (Fig. 2c).

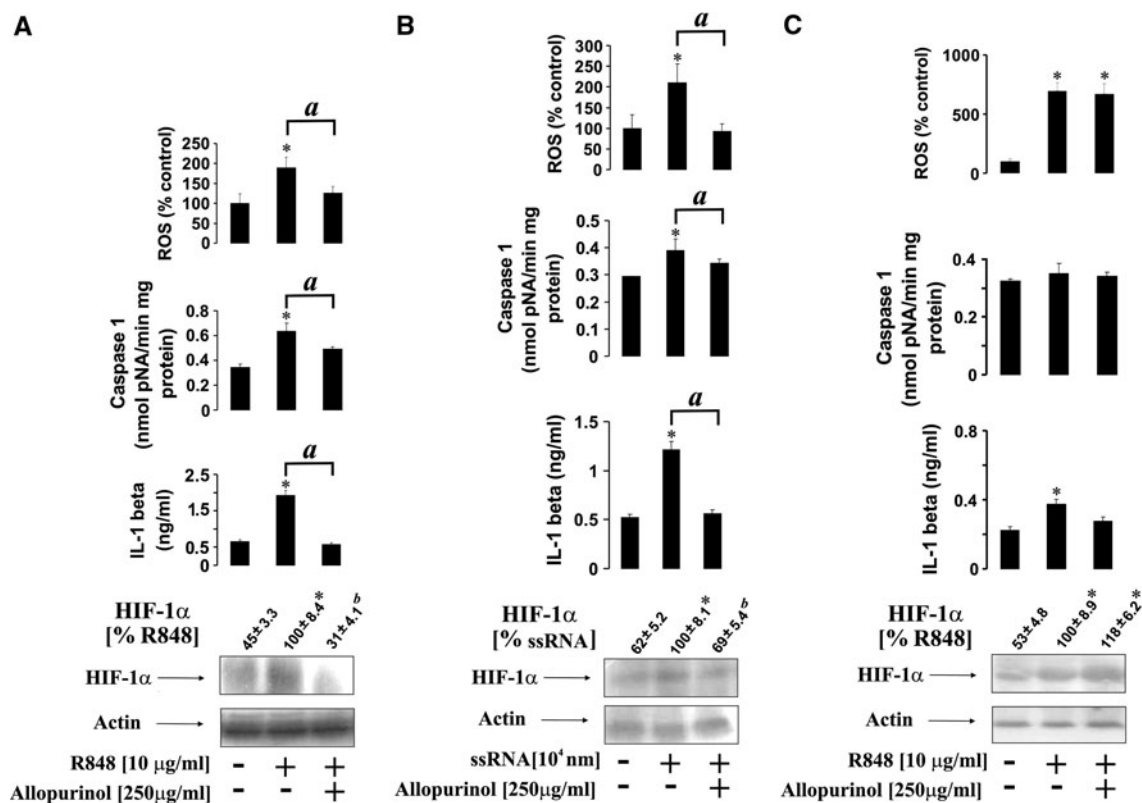
To confirm our findings utilising *in vivo* studies we used wild type inbred mice ( $n = 7$  per group). The control group received 0.9% NaCl solution via intra-peritoneal injections.

◀ **Fig. 1a–e** The involvement of Nalp3 and HIF-1 $\alpha$ -dependent XOD induction in R848-induced TLR7/8-mediated activation of caspase 1, IL-1 $\beta$  release in THP-1 cells. Normal and Nalp3 knockdown THP-1 cells were exposed to different concentrations of the R848 for 24 h (a) and 4 h (b). The accumulation of HIF-1 $\alpha$  protein was analysed by Western blot. Equal loading of the protein was controlled by the actin staining. Caspase 1 activity was analysed by the colorimetric assay. Release of IL-1 $\beta$  was measured with ELISA. Where applicable, the Nalp3 mRNA levels were determined by RT-PCR. The level of actin mRNA was used as a loading control. c Cells were exposed to different concentrations of R848 and to 10<sup>4</sup> nM ssRNA fragment for 24 h. XOD activity and intracellular UA were then detected. d Normal and HIF-1 $\alpha$  knockdown THP-1 cells were exposed to 10  $\mu$ g/ml R848 for 4 and 24 h. XOD activity was then analysed. e Normal, HIF-1 $\alpha$  knockdown and Nalp3 knockdown THP-1 cells were exposed to 10  $\mu$ g/ml R848 for 24 h. PI3K activity was then analysed. Digital data are mean values  $\pm$  SD of at least four individual experiments. \**P* < 0.01 versus control; *a* represents significant differences between two indicated values (*P* < 0.01). All Western blot and RT-PCR data are from one experiment representative of three that gave similar results

A second group received 75  $\mu$ g/kg R848 (in 0.9% NaCl) via intra-peritoneal injections. The third group received a combination of 75  $\mu$ g/kg R848 and 500  $\mu$ mol/kg allopurinol

(in 0.9% NaCl) [3, 6, 7, 10, 18]. Single injections were used to investigate the immediate rather than the chronic inflammatory reaction. Blood was collected 4 h after injection (to investigate immediate non-chronic inflammatory reaction), and the serum as well as the leukocyte fraction was isolated and subjected to further analysis. We found that in the leukocytes of mice that received R848 the accumulation of HIF-1 $\alpha$  was significantly higher compared to the control. The level of the leukocyte TLR7 was not affected. This correlated with an increase in the intracellular caspase 1 and XOD activities as well as in thiobarbiturate-reactive species (TBRS) used to characterise the level of the intracellular oxidative burst. In the blood serum, the levels of UA and IL-1 $\beta$  were significantly increased. All the effects discussed did not take place in the cells/serum obtained from animals injected with the combination of the R848 and allopurinol (Fig. 3a).

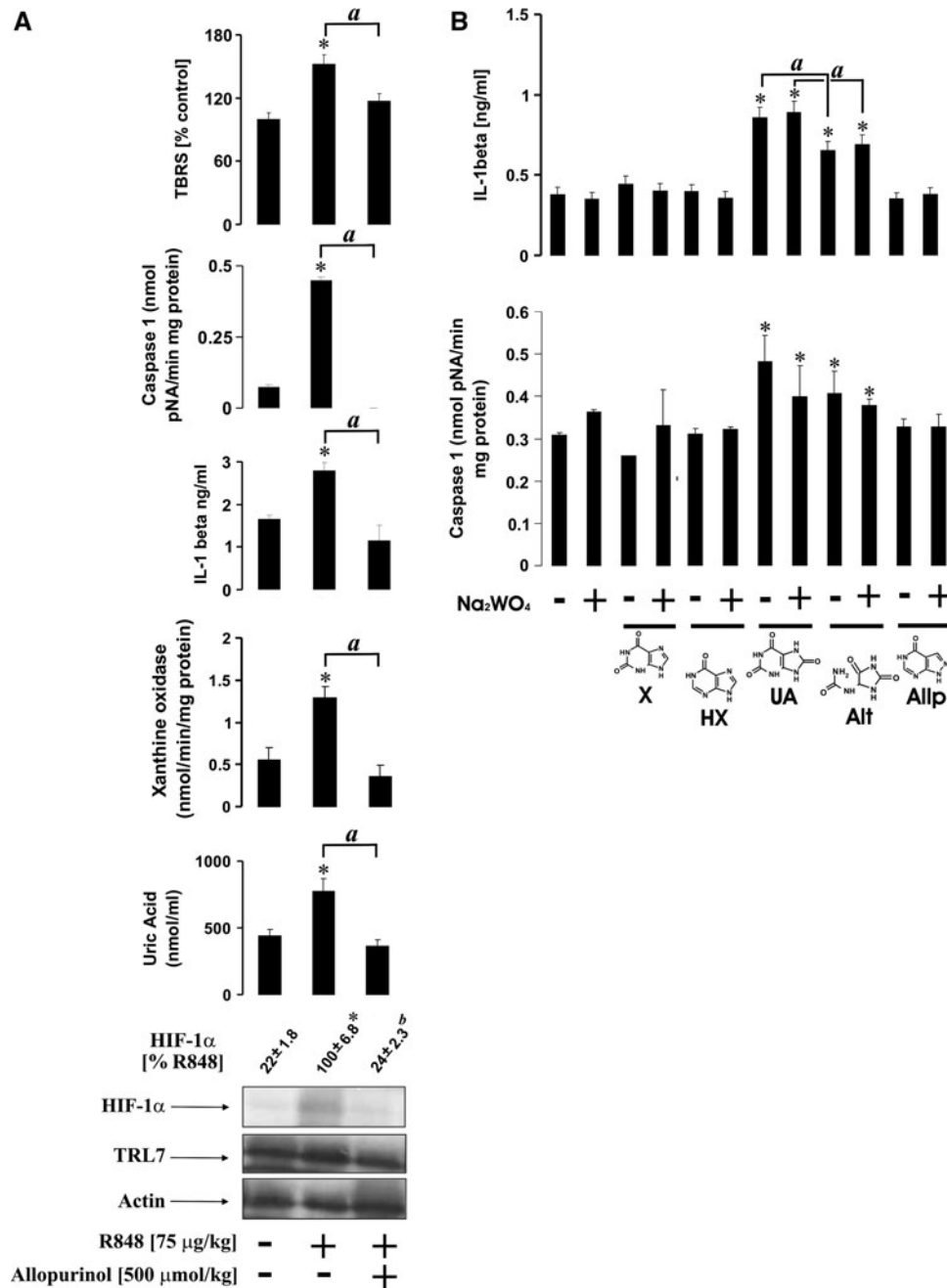
To investigate which purines could directly influence the activity of caspase 1 and production of IL-1 $\beta$ , we exposed normal THP-1 cells and those pre-treated for 1 h with 100  $\mu$ g/ml sodium tungstate (tungsten replaces



**Fig. 2a–c** The involvement of XOD in TLR7/8 ligand-induced activation of caspase 1 and IL-1 $\beta$  release in THP-1 cells. THP-1 cells were exposed to 10  $\mu$ g/ml R848 (a) or 10<sup>4</sup> nM ssRNA fragment (b) for 24 h with or without 1 h pre-treatment with 250  $\mu$ g/ml allopurinol. Additional cells were exposed to 10  $\mu$ g/ml R848 for 4 h with or without 1 h pre-treatment with 250  $\mu$ g/ml allopurinol (c). The accumulation of HIF-1 $\alpha$  protein was analysed by Western blot. Equal loading of the protein was controlled by the actin staining. Caspase 1

activity was analysed by the colorimetric assay. Release of IL-1 $\beta$  was measured with ELISA. ROS production was detected by the luminometric assay. Digital data are mean values  $\pm$  SD of at least four individual experiments. \**P* < 0.01 versus control; *a* represents significant differences between two indicated values (*P* < 0.01); *b* indicates *P* < 0.01 versus R848-treated (or ssRNA-treated) cells. All Western blot data are from one experiment representative of three that gave similar results





**Fig. 3a, b** The involvement of XOD in the R848-induced activation of caspase 1 and release of IL-1 $\beta$  in vivo. **a** Wild type male inbred mice ( $n = 7$  per group) were used in these experiments. Control group received 0.9% NaCl solution via intra-peritoneal injections. A second group received 75  $\mu$ g/kg R848 (in 0.9% NaCl) via intra-peritoneal injections. The third group received a combination of 75  $\mu$ g/kg R848 and 500  $\mu$ mol/kg allopurinol (in 0.9% NaCl). Single injections were used to investigate the immediate rather than the chronic inflammatory reaction. Blood was collected 4 h after injection, and serum as well as leukocyte fraction was isolated and subjected to further analysis. The accumulation of HIF-1 $\alpha$  protein and TLR7 was detected by Western blot. Equal loading of the protein was controlled by actin staining. Caspase 1 activity was analysed by the colorimetric assay. Serum IL-1 $\beta$  was measured with ELISA.

Intracellular XOD activity and the serum UA were also detected as outlined in “Materials and methods”. The intracellular TBRS were detected by using colorimetric assay. **b** To investigate which purines could directly influence caspase 1/IL-1 $\beta$  activation normal THP-1 cells and those pre-treated for 1 h with 100  $\mu$ g/ml sodium tungstate were exposed to 250  $\mu$ g/ml of xanthine (X), hypoxanthine (HX), uric acid (UA), allantoin (Alt) and allopurinol (Allp) for 24 h. The activity of caspase 1 and the IL-1 $\beta$  release were then analysed. Digital data are mean values  $\pm$  SD of at least seven individual experiments. \* $P < 0.01$  versus control; *a* represents significant differences between two indicated values ( $P < 0.01$ ); *b* indicates  $P < 0.01$  versus R848-injected mice. All Western blot data are from one experiment representative of seven that gave similar results

molybdenum in the active site of XOD leading to the attenuation of its catalytic activity [25]) to different purines—xanthine, hypoxanthine, UA, allantoin and allopurinol for 24 h. In all cases the concentration of the purine corresponded to 250  $\mu\text{g/ml}$ . We found that independently of the presence of tungstate, uric acid and allantoin led to an increase in both caspase 1 activity and IL-1 $\beta$  release. Other ligands (also independently of the presence of tungstate) did not impact caspase 1 and the level of IL-1 $\beta$  released (Fig. 3b).

## Discussion

Our results demonstrate the involvement of Nalp3 in the activation of caspase 1 and release of IL-1 $\beta$  induced in a TLR7/8-dependent manner. TLR7/8-mediated HIF-1 $\alpha$  accumulation was clearly independent of Nalp3 at the earlier stages (4 h). However, at later stages (24 h), Nalp3 knockdown THP-1 macrophages displayed reduced accumulation of HIF-1 $\alpha$  protein, which most likely means involvement of the released IL-1 $\beta$  in accumulation/activation of this protein.

Similarly to the TLR4-mediated signalling events [8], the TLR7/8-induced response led to an increase in XOD activity. This increase was dependent on HIF-1 $\alpha$ , since knocking this protein down via siRNA led to attenuation of the XOD activity. As the regulatory region of the XOD gene contains hypoxia-responsive elements, and as its expression also depends on the AP-1 (accumulation of this factor is an energy consuming signalling process and therefore requires HIF-1 $\alpha$  [8, 15, 26]), it is likely that attenuation of HIF-1 $\alpha$  expression would negatively influence TLR7/8-induced XOD activity.

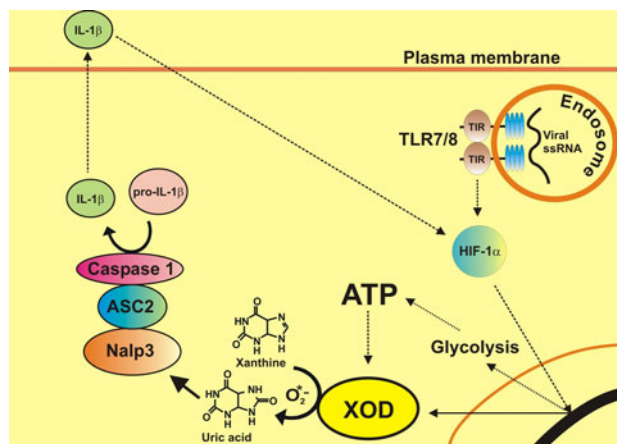
The specific XOD inhibitor allopurinol attenuated both R848 and ssRNA-induced activation of caspase 1 as well as the release of IL-1 $\beta$  in human THP-1 macrophages. In this case allopurinol is the best inhibitor, as the well-known XOD inhibitor sodium tungstate contains heavy metal (tungsten), which could impact endosomes and down-regulate the function of TLR7/8. This allopurinol-induced decrease in caspase 1 and IL-1 $\beta$  correlated with attenuation of the ROS production and HIF-1 $\alpha$  accumulation. UA, produced by XOD, positively influences and is therefore quite important for activation of the NADPH oxidase complex [12]. On the other hand, XOD is able to produce superoxide and hydrogen peroxide itself (although in the inflammatory cells XOD cannot be considered as a major contributor to the intracellular ROS generation) [8, 9]. However, in this particular case, IL-1 $\beta$  most likely has an impact on ROS production, since ROS were not attenuated when exposed for 4 h. In addition, from our previous work we know that a redox-dependent

mechanism underlies TLR7/8-mediated HIF-1 $\alpha$  accumulation [10].

Similar effects were observed *in vivo*, where allopurinol was found to attenuate R848-induced activation of caspase 1, release of IL-1 $\beta$  and accumulation of HIF-1 $\alpha$  in the leukocytes of wild-type mice. This correlated with a decrease in the intracellular XOD/serum UA as well as in the intracellular oxidative burst (concluded based on the significant decrease in the intracellular TRS).

In the case of the mouse organism, one could also expect allantoin (the product of UA degradation catalysed by urate oxidase [8]) to display an effect similar to that observed for UA. Exposure of the normal and tungstate pre-treated THP-1 cells to xanthine, hypoxanthine, UA, allantoin and allopurinol demonstrated that, independently of the XOD activity, UA and allantoin could induce activation of caspase 1 and release of IL-1 $\beta$ . However, the impact of UA was stronger compared to that observed in the cells exposed to allantoin. These data suggest that only UA and allantoin (the compounds which depend on XOD for physiological generation) [8, 9, 17], but not other natural (hypoxanthine and xanthine) and synthetic (allopurinol) purine catabolites act as activators of caspase 1/IL-1 $\beta$ .

Our results therefore suggest that the cross-regulation of XOD and the HIF-1 $\alpha$  protein form the “bridge” between TLR7/8 downstream signalling pathways and formation of the Nalp3 inflammasome, which is crucial for activation/release of the highly inflammatory IL-1 $\beta$ . This concept is summarised in the scheme presented in Fig. 4.



**Fig. 4** Scheme summarising the concept of cross-regulation between HIF-1 $\alpha$  protein and XOD during ligand-induced TLR7/8-mediated IL-1 $\beta$  activation/release. Ligand-induced TLR7/8-mediated signalling leads to the accumulation/activation of HIF-1 $\alpha$  protein. HIF-1 $\alpha$  up-regulates glycolysis and thus supports ATP generation. On the other hand, HIF-1 $\alpha$  up-regulates expression of XOD (which also depends on the intracellular ATP level). Poorly soluble UA and possibly ROS generated by XOD might lead to activation of Nalp3 that forms the inflammasome, recruiting ASC protein and activating caspase 1. This converts pro-IL-1 $\beta$  into the active IL-1 $\beta$  which has been reported [23] to trigger HIF-1 $\alpha$  accumulation

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