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Extracellular Acidosis Is a Novel Danger Signal Alerting Innate Immunity via the NLRP3 Inflammasome[S]

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Kristiina Rajamäki[‡1], Tommy Nordström[§], Katariina Nurmi[‡], Karl E. O. Åkerman[§], Petri T. Kovanen[‡], Katariina Öörni[‡], and Kari K. Eklund[‡]

From the [‡]Wihuri Research Institute, Biomedicum Helsinki, Haartmaninkatu 8, FI-00290 Helsinki, Finland and the [§]Institute of Biomedicine/Physiology, University of Helsinki, Biomedicum Helsinki, P.O. Box 63, FI-00014 Helsinki, Finland

Background: Local acidosis has been demonstrated in ischemic tissues and at inflammatory sites.
Results: Acidic extracellular pH triggers NLRP3 inflammasome activation and interleukin-1β secretion in human macrophages.
Conclusion: Acidic pH represents a novel danger signal alerting the innate immunity.
Significance: Local acidosis may promote inflammation at ischemic and inflammatory sites.

Local extracellular acidification has been demonstrated at sites of ischemia and inflammation. IL-1β is one of the key pro-inflammatory cytokines, and thus, its synthesis and secretion are tightly regulated. The NLRP3 (nucleotide-binding domain leucine-rich repeat containing family, pyrin domain containing 3) inflammasome complex, assembled in response to microbial components or endogenous danger signals, triggers caspase-1-mediated maturation and secretion of IL-1β. In this study, we explored whether acidic environment is sensed by immune cells as an inflammasome-activating danger signal.

Human macrophages were exposed to custom cell culture media at pH 7.5–6.0. Acidic medium triggered pH-dependent secretion of IL-1β and activation of caspase-1 via a mechanism involving potassium efflux from the cells. Acidic extracellular pH caused rapid intracellular acidification, and the IL-1β-inducing effect of acidic medium could be mimicked by acidifying the cytosol with bafilomycin A1, a proton pump inhibitor. Knocking down the mRNA expression of NLRP3 receptor abolished IL-1β secretion at acidic pH. Remarkably, alkaline extracellular pH strongly inhibited the IL-1β response to several known NLRP3 activators, demonstrating bipartite regulatory potential of pH on the activity of this inflammasome. The data suggest that acidic environment represents a novel endogenous danger signal alerting the innate immunity. Low pH may thus contribute to inflammation in acidosis-associated pathologies such as atherosclerosis and post-ischemic inflammatory responses.

Stringent control of both extra- and intracellular pH is essential for maintaining cellular biochemical reactions and tissue homeostasis. Despite the efficient pH control mechanisms evolved in human body, local and even systemic pH fluctuations occur frequently under pathological conditions. For example, ischemia during myocardial infarction and stroke causes a rapid drop in pH to approximately pH 6.5–6.0 in the affected tissue (1–4), and the ischemic period is promptly followed by a potentially detrimental inflammatory response (5, 6). Local acidification may develop also during acute and chronic inflammation. During inflammation, the drop in pH is a result of infiltration and activation of inflammatory cells in the tissue, which leads to increased energy and oxygen demand, accelerated glucose consumption via glycolysis and thus increased lactic acid secretion (7–11). Examples of local acidosis in chronic inflammation include atherosclerotic lesions (12, 13), airways of patients suffering from chronic inflammatory obstructive lung disease (14), and the joint fluids in patients with gouty or rheumatoid arthritis (15–17). In freshly removed human carotid atherosclerotic plaques, marked pH heterogeneity was evident, and pH values down to 6.8 were recorded (12). In acute exacerbations of asthma, the exhaled breath condensate had an average pH of 5.2 compared with pH 7.7 in healthy subjects, and anti-inflammatory corticosteroid therapy normalized the airway pH (18). Synovial fluid pH in rheumatic or gouty joints may reach values of 6.8–7.1, whereas the pH of normal synovial fluid ranges from 7.4 to 7.8 (15–17, 19). Importantly, in rheumatoid arthritis patients, the low synovial pH correlates with high disease activity and radiological joint destruction (17, 20).

Macrophages are integral cells of the innate immune system, and they play a critical role in the pathophysiology of several chronic inflammatory diseases as well as in post-ischemic inflammation (5, 6, 21–23). Activated macrophages secrete a myriad of proinflammatory cytokines and chemokines, among which IL-1β is one of the most potent in promoting inflammation and disease progression. The maturation and secretion of IL-1β, as well as that of IL-18, is controlled by the inflammasome pathway. Inflammasomes are large intracellular protein complexes that recruit and activate the caspase-1 protease, which in turn cleaves the proforms of IL-1β and IL-18 into their biologically active, secreted forms (24, 25). Inflammasome assembly is initiated by activation and self-oligomerization of certain NLR (nucleotide-binding domain leucine-rich repeat containing) or PYHIN (pyrin and HIN200 domain containing) family pattern recognition receptors in the cytoplasm (25). The most studied inflammasome-forming receptor,

[S] This article contains supplemental Table S1 and Figs. S1–S6.

[1] To whom correspondence should be addressed: Wihuri Research Institute, Biomedicum Helsinki, Haartmaninkatu 8, FI-00290 Helsinki, Finland. Tel.: 358-9-681-4128; Fax: 358-9-637476; E-mail: kristiina.rajamaki@helsinki.fi.

NLRP3,[2] is activated not only by microbial components but also by endogenous danger signals such as urate crystals formed in gouty arthritis (26) and cholesterol crystals formed in atherosclerosis (27, 28). Activation of NLRP3 is dependent on intracellular stress signals caused by the activators, but the mechanism remains poorly defined. Thus, lysosomal membrane destabilization, increased intracellular reactive oxygen species (ROS) formation, and potassium efflux from the cells may alone or in combination mediate NLRP3 activation (26). In addition to potassium efflux, also other changes in intracellular ion homeostasis may be involved in NLRP3 activation, as implied by ion substitution experiments of the extracellular medium (29, 30).

We hypothesized that acidic environment, created by actively metabolizing immune cells or hypoxia, may represent an endogenous danger signal for macrophages and trigger inflammasome activation via alterations of the intracellular milieu. To test this, custom-manufactured cell culture media covering the pH range 7.5–6.0 were designed with a single buffer system. Here, we show that extracellular acidosis triggers pH-dependent secretion of mature IL-1β from cultured human macrophages via a mechanism involving cellular potassium depletion and inflammasome activation.

EXPERIMENTAL PROCEDURES

Reagents—UltraPure LPS was purchased from InvivoGen. Caspase-1 inhibitor z-YVAD-fmk was from Santa Cruz Biotechnology. Cathepsin B inhibitor CA-074-Me was from Calbiochem. *N*-Acetyl-L-cysteine (NAC), tetraethylammonium chloride, 4-aminopyridine, oxidized ATP, KN-62, bafilomycin A1, and 5-(*N*-ethyl-*N*-isopropyl)amiloride were purchased from Sigma. Recombinant human serum amyloid A (1α isotype) was from Peprotech. Cholesterol crystals were prepared as described (27), except that only sterile containers and filters were used to obtain endotoxin-free crystals without autoclaving. Monosodium urate crystals were prepared as described (31). Endotoxin levels in the cholesterol and urate crystal preparations were below detection limit (<0.03 endotoxin units/ml) in Pyrogent Gel Clot LAL Assay (Lonza).

Cell Culture—Buffy coats were obtained from healthy human donors (Red Cross Finland Blood Service, Helsinki, Finland), and monocytes were isolated and differentiated into macrophages in Macrophage Serum-Free Medium (Invitrogen) as described (32), except that macrophage colony-stimulating factor (50 ng/ml; Nordic Biosite) was used instead of granulocyte macrophage colony-stimulating factor. Each experiment with primary human macrophages was performed using macrophages derived from a single donor. The human monocytic leukemia cell line THP-1 was obtained from the American Type Culture Collection (catalog no. TIB-202) and maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 25 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin (reagents by Lonza). To induce monocyte-to-macrophage differentiation, THP-1 cells were cultured for 72 h in culture medium supplemented with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma).

Preparation of the Acidic and Alkaline Media—To study the effects of acidic pH, we used custom RPMI 1640 media at pH 7.5, 7.0, 6.5, and 6.0 (Athena Enzyme Systems) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The RPMI formulation was modified by lowering the $NaHCO_3$ concentration from 24 to 5 mM, and by adding 20 mM PIPES buffer. PIPES at 20–40 mM is well tolerated in cell culture by several mammalian cell lines (33) and displays negligible metal ion binding and surfactant activity (34). Cell viability and morphology of differentiated primary and THP-1 macrophages were not altered in custom RPMI, pH 7.5, compared with normal RPMI (supplemental Fig. S1). To study the effects of alkaline pH, a custom bicarbonate-free DMEM (HyClone Laboratories) was buffered to pH of 7.5, 8.0, or 8.5 with 40 mM Tricine and supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Tricine is a buffer well tolerated in cell culture at 20–40 mM by several mammalian cell lines (33). The experiments involving alkaline pH were conducted at +37 °C in a non-CO_2 incubator to avoid rapid equilibration of alkaline pH toward neutral in 5% CO_2 environment.

Treatment of Cells with LPS and Inhibitors—Primary human macrophages were incubated with 1 μg/ml LPS for 4 h (medium, pH 7.5) to induce pro-IL-1β expression. Subsequently, the cells were washed in PBS, followed by exposure to the inflammasome-activating stimuli.

Stock solutions of NAC, 4-aminopyridine, and tetraethylammonium chloride were neutralized before use to ensure that they do not change the pH of the culture media during the experiments. Z-YVAD-fmk and CA-074-Me were present both in a 1-h preincubation at pH 7.5 and during the exposure to acidic pH, whereas NAC was present only during a 30-min preincubation at pH 7.5. These inhibitors exert their effects intracellularly, and the preincubation at pH 7.5 ensures efficient loading of the inhibitors into cells before introduction of acidic pH. KCl, CsCl, $BaCl_2$, tetraethylammonium chloride, 4-aminopyridine, bafilomycin A1, and 5-(*N*-ethyl-*N*-isopropyl-)amiloride were added simultaneously with the pH stimulus. Of these, the simple salts are highly water-soluble and not affected by the moderately acidic pH environment used in the experiments. 4-Aminopyridine (35), bafilomycin A1 (36), and 5-(*N*-ethyl-*N*-isopropyl)amiloride (37) have been shown to efficiently block their respective targets also under extracellular acidosis. Oxidized ATP was introduced as a 2-h preincubation at pH 7.5, which ensures covalent binding of the inhibitor to $P2X_7$ receptor (38). KN-62 retains effective inhibitor function also under extracellular acidosis (39).

ELISA Assays—IL-1β, IL-1α, IL-18, and TNF-α were analyzed in neutralized culture medium supernatants by using commercial sandwich ELISA assays (IL-18 assay by Medical & Biological Laboratories; others by R&D Systems) according to the manufacturer's instructions.

Western Blotting—Medium supernatants from THP-1 macrophages were neutralized and subsequently concentrated at +4 °C using Vivaspin 6 centrifugal concentrators with a

[2] The abbreviations used are: NLRP3, nucleotide-binding domain leucine-rich repeat containing family pyrin domain containing 3; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; TLR, Toll-like receptor; NAC, *N*-acetyl cysteine; CHC, cholesterol crystals; SAA, serum amyloid A; MSU, monosodium urate crystals; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethylester.

5-kDa cut-off (Sartorius). Cells were lysed with ice cold radioimmune precipitation assay buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS) supplemented with 1× protease inhibitor mixture EDTA-free (Roche Applied Science). Medium samples and cell lysates were run in SDS-PAGE and transferred onto Hybond-C Extra nitrocellulose membrane (Amersham Biosciences). Antibodies raised against human IL-1β and caspase-1 were from Santa Cruz Biotechnology (sc-7884 and sc-515) and the goat-anti-rabbit IgG (H+L) HRP conjugate was purchased from Zymed Laboratories Inc..

Quantitative Real-time RT-PCR—The protocol used in quantitative real-time RT-PCR has been described in our previous work and was followed here without alterations (27). The gene-specific primers and probes are listed in supplemental Table S1.

siRNA Transfections—The siRNA transfection protocol has been described in our previous work and was followed here without alteration (27). After transfection, the cells were washed with PBS and incubated for 6 h in RPMI, pH 7.5 or pH 6.5. Finally, the media were collected for cytokine analysis, and RNA was isolated from the cells for quantitative real-time RT-PCR.

Measurement of Intracellular pH—THP-1 macrophages were differentiated on 25-mm round coverslips and intracellular pH was measured by using the cell-permeable probe 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethylester (BCECF-AM; Molecular Probes) and the InCytIm2 fluorescence imaging system (Intracellular Imaging, Inc.) as described previously (40), except that the dye loading and the pH recordings here were performed in RPMI media. To compensate for the weak intrinsic fluorescence present in the medium (at 440 nm), a correction factor was determined using the free acid form of BCECF. Fifty cells per coverslip were manually selected for each analysis.

Thin Layer Chromatography—Analysis of intracellular cholesteryl ester content in macrophages was performed by thin layer chromatography as described (27).

Statistics—Statistical analysis was performed using the PASW Statistics software (version 20.0, SPSS, Inc.) and the non-parametric Mann-Whitney's *U* test. Data are presented as means ± S.E. Statistical significance was set to $p < 0.05$.

RESULTS

Acidic pH Triggers IL-1β Secretion and Caspase-1 Activation—Incubation of LPS-primed primary human macrophages, LPS-primed mouse bone marrow-derived macrophages, and PMA-differentiated human THP-1 macrophages in low pH custom media induced a pH-dependent secretion of the inflammasome-controlled cytokines IL-1β (Fig. 1, *A* and *B*) (supplemental Fig. S2*A*) and IL-18 (Fig. 1*C*). Secretion of IL-1β was below the detection limit in nonprimed human and mouse primary macrophages at acidic pH. LPS priming is required in these cells for induction of pro-IL-1β expression via Toll-like receptor (TLR)4 and nuclear factor-κB, whereas in THP-1 cells, the PMA treatment induces stable expression of pro-IL-1β, and priming with LPS is thus not required (24, 41). Caspase-1 blots from concentrated culture medium supernatants revealed the presence of the 10-kDa active subunit at pH 6.5 and pH 6.0,

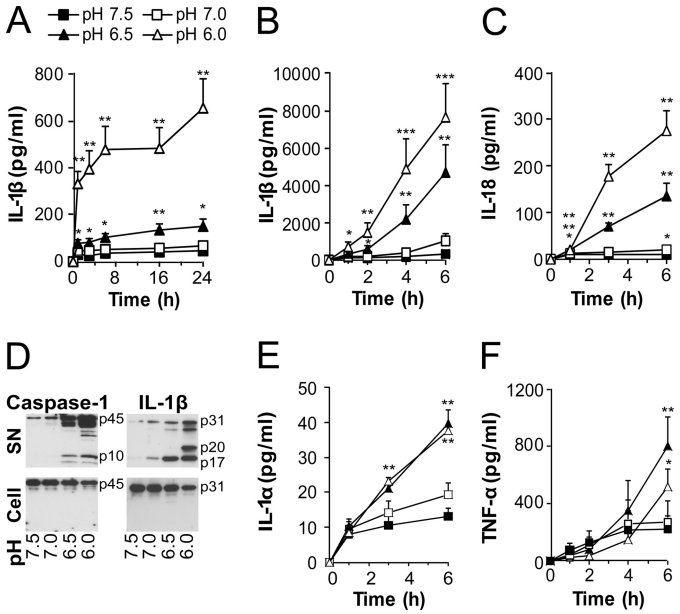

FIGURE 1. **Acidic pH triggers the secretion of proinflammatory cytokines in LPS-primed primary human macrophages (*A*) and human THP-1 macrophages (*B–F*).** *A–C*, *E*, and *F*, cytokine secretion into culture medium was analyzed by ELISA, and the data are means ± S.E. from 6 (*A*), 5 (*B*), or 3 (*C*, *E*, and *F*) experiments, performed in duplicate in *B*, *C*, *E*, and *F*. *D*, Western blots after a 6-h incubation in custom RPMI representative of three experiments. Procaspase-1 (p45), the active p10 subunit of caspase-1, pro-IL-1β (p31), mature p17 IL-1β, and a p20 fragment of IL-1β are indicated. *SN*, culture medium supernatant. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, compared with control at pH 7.5.

directly demonstrating acidic pH-induced caspase-1 activation (Fig. 1*D*, *left panel*). Moreover, Western blotting confirmed secretion of the 17-kDa mature IL-1β at pH 7.0–6.0, but not at pH 7.5 (Fig. 1*D*, *right panel*). Interestingly, also a 20-kDa IL-1β fragment was secreted at pH 6.0. A partially active IL-1β fragment of ~20 kDa also has been described previously in macrophages (42–44).

In addition to IL-1β and IL-18, secretion of two inflammasome-independent cytokines, IL-1α and TNF-α, was studied (Fig. 1, *E* and *F*). A statistically significant increase in IL-1α secretion was observed at acidic pH, although the amount of cytokine secreted in 6 h was marginal (Fig. 1*E*). Low pH induced TNF-α secretion in THP-1 macrophages (Fig. 1*F*) but not in nonprimed primary macrophages (data not shown).

Inflammasome activation is often associated with pyroptosis, a caspase-1-dependent proinflammatory form of cell death that causes the release of cytoplasmic contents to extracellular space (45). Acidic pH also caused cell death, as shown by time course of lactate dehydrogenase release from macrophages (supplemental Fig. S2*B*). However, measurement of IL-1β release from the same culture medium supernatants demonstrated that at each acidic pH value, IL-1β secretion was triggered at an earlier time point than the lactate dehydrogenase release (supplemental Fig. S2*C*), and thus, the secretion of IL-1β to culture medium was not due to cell death.

Messenger RNA Expression of IL-1 Family and Non-IL-1 Family Proinflammatory Cytokines Is Induced by Acidic pH—The effect of acidic pH on proinflammatory cytokine mRNA expression was analyzed in THP-1 macrophages. As also reported previously (41), pro-IL-1β mRNA expression was

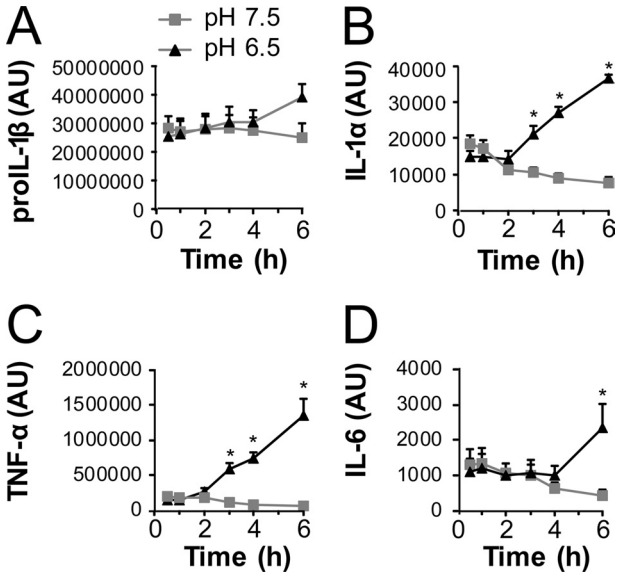

FIGURE 2. **Acidic pH induces the mRNA expression of proinflammatory cytokines in THP-1 macrophages.** *A–D*, the results from quantitative real-time RT-PCR are expressed as the means ± S.E. of relative expression (in arbitrary units, *AU*) from four experiments. *, $p < 0.05$, compared with control at pH 7.5.

strongly induced in PMA-differentiated THP-1 macrophages (Fig. 2*A*). Exposure of the cells to acidic environment did not significantly change the pro-IL-1β expression levels. Although IL-1α protein was only marginally secreted, IL-1α mRNA was induced 6-fold after 6 h at pH 6.5 compared with pH 7.5 (Fig. 2*B*). TNF-α mRNA was strongly induced at pH 6.5 after 3–6 h of incubation (Fig. 2*C*). The expression of IL-6 remained constant until the 4-h time point but was induced by 6-fold after 6 h incubation at pH 6.5 when compared with pH 7.5 (Fig. 2*D*).

Acidic pH-induced IL-1β Secretion Is Caspase-1-dependent and Proceeds via a Mechanism Involving Potassium Efflux—Next, we began to explore the mechanisms of acidic pH -induced IL-1β secretion. The caspase-1 inhibitor z-YVAD-fmk significantly inhibited IL-1β secretion at pH 6.5 both in THP-1 macrophages and in primary human macrophages (Fig. 3, *A* and *E*). Intracellular ROS formation, cathepsin B leakage from lysosomes, and potassium efflux from cells have all been implicated as events upstream of inflammasome activation (26). The general ROS inhibitor NAC did not inhibit low pH -induced IL-1β secretion (Fig. 3*B*). Inhibition of cathepsin B activity by CA-074-Me caused a partial inhibition of IL-1β secretion at pH 6.5 (Fig. 3*C*). In contrast, inhibition of potassium efflux from macrophages, achieved by balancing extracellular K^+ concentration with intracellular, strongly inhibited the acidic pH-induced IL-1β secretion both in THP-1 macrophages and in primary human macrophages (Fig. 3, *D* and *F*). This effect was not caused by increased osmolarity, as addition of 260 mM sorbitol had no inhibitory effect. Furthermore, non-selective potassium channel blockage by tetraethylammonium, Cs^+, and Ba^{2+} significantly inhibited the IL-1β response to acidic pH (Fig. 3*G*).

As demonstrated by Western blots, the caspase-1 inhibitor z-YVAD-fmk markedly inhibited secretion of the 17 kDa IL-1β at pH 6.5 and 6.0, but not that of the 20-kDa IL-1β, which was secreted only at pH 6.0 (Fig. 3*H*). This is in agreement with previous results showing that the generation of 20-kDa IL-1β in macrophages is caspase-1-independent (43). Inhibition of potassium efflux completely abolished the secretion of both 17-kDa and 20-kDa IL-1β at acidic pH, implicating potassium efflux as a major switch in activation of IL-1β-targeting proteases (Fig. 3*H*). In contrast to the ELISA results, cathepsin B inhibitor had no apparent effect on the low pH-induced 17-kDa or 20-kDa IL-1β bands (Fig. 3*H*). In addition, no differences were observed in lysosomal acridine orange staining pattern of macrophages incubated at pH 7.5 or pH 6.5, indicating that lysosomes retained their acidity, membrane integrity, and morphology despite the low pH (supplemental Fig. S3*A*). Secretion of active cathepsin B was, however, increased by 4-fold at acidic pH (supplemental Fig. S3*B*).

The caspase-1 blots from acidic medium samples revealed a ~7-kDa caspase-1 fragment co-migrating with the active 10-kDa subunit (Fig. 3*I*). The 7-kDa band was enhanced by z-YVAD-fmk, whereas the 10-kDa band was diminished by the inhibitor. This suggests that the inhibitory mode of action of z-YVAD-fmk is to induce cleavage of the 10-kDa caspase-1. In support of this idea, studies of purified recombinant caspase-1 have identified a functional autocleavage site in the 10-kDa subunit (46). We further tested the effect of 130 mM KCl on caspase-1 activation and observed complete disappearance of the 10-kDa active subunit, whereas CA-074-Me caused only a minute decrease in active caspase-1 (Fig. 3*I*).

NLRP3 Receptor Is Required for the Acidic pH-induced Inflammasome Activation—Next, we sought to identify the specific inflammasome-forming receptor activated by acidic environment. The NLRP3 receptor is activated by several endogenous danger signals (26), which made this receptor the strongest candidate. As shown in Fig. 4, mRNA encoding the NLRP3 receptor but not mRNA for NLRP1 was significantly expressed in THP-1 macrophages, and NLRP3 mRNA was further induced in acidic environment (Fig. 4, *A* and *B*). We thus silenced the expression of NLRP3 receptor by transfecting siRNA into the macrophages. Transfection efficiency, determined using a fluorescently labeled negative control siRNA, was on average 71%. NLRP3 siRNA reduced the expression of NLRP3 receptor by ~50% when compared with mock or to negative control siRNA transfections (Fig. 4*C*). Moreover, the NLRP3 siRNA abolished IL-1β secretion at pH 6.5, whereas TNF-α secretion was not significantly reduced (Fig. 4, *D* and *E*). These data indicate that the NLRP3 inflammasome, formed by the NLRP3 receptor and the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase-recruitment domain), is the low pH-responsive inflammasome in human macrophages.

Notably, the delayed cell death response to acidic environment (supplemental Fig. S2, *B* and *C*) could have caused ATP leakage from damaged/dying cells and secondary NLRP3 inflammasome activation via ligation of the $P2X_7$ receptor by ATP. To test this possibility, we employed a general P2X receptor antagonist, oxidized ATP, and a specific $P2X_7$ receptor antagonist, KN-62. Despite the relatively high concentrations used (38, 47), oxidized ATP or KN-62 had no effect on acidic pH -induced IL-1β secretion (supplemental Fig. S4, *A* and *B*), a finding in accord with previously demonstrated strong inhibition of $P2X_7$ receptor activity at acidic pH (48).

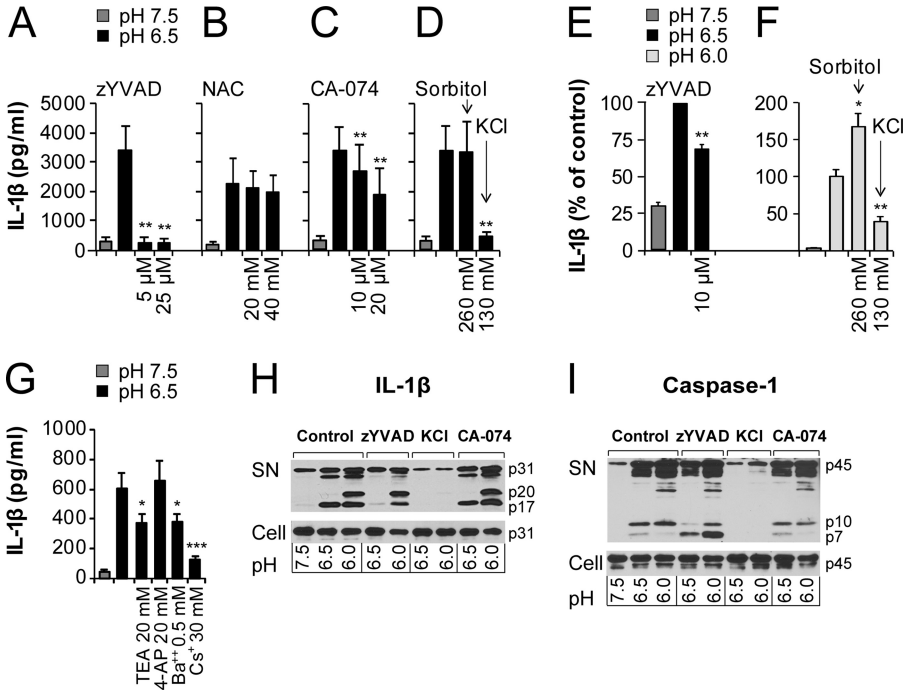

FIGURE 3. **The mechanism of low pH -induced IL-1β secretion in THP-1 macrophages (*A–D* and *G–I*) and LPS-primed primary human macrophages (*E* and *F*).** Duration of the pH stimulus was 1 h (*F*), 3 h (*G*), 4 h (*A–D*), or 6 h (*E*, *H*, and *I*). In the primary macrophages, the control values of IL-1β secretion at acidic pH were 60 ± 8 pg/ml (*E*) and 239 ± 29 pg/ml (*F*). *A–G*, the data are means ± S.E. from three to seven experiments, performed in duplicate in *B*, *F*, and *G*. The Western blots of IL-1β (*H*) and caspase-1 (*I*) are representative of four experiments. Pro-IL-1β (p31), mature p17 IL-1β, a p20 fragment of IL-1β, procaspase-1 (p45), and the active p10 subunit and a p7 fragment of caspase-1 are indicated. *SN*, culture medium supernatant; *TEA*, tetraethylammonium chloride; *4-AP*, 4-aminopyridine. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, compared with untreated control at pH 6.5/6.0. See "Experimental Procedures" for details on the use of inhibitors.

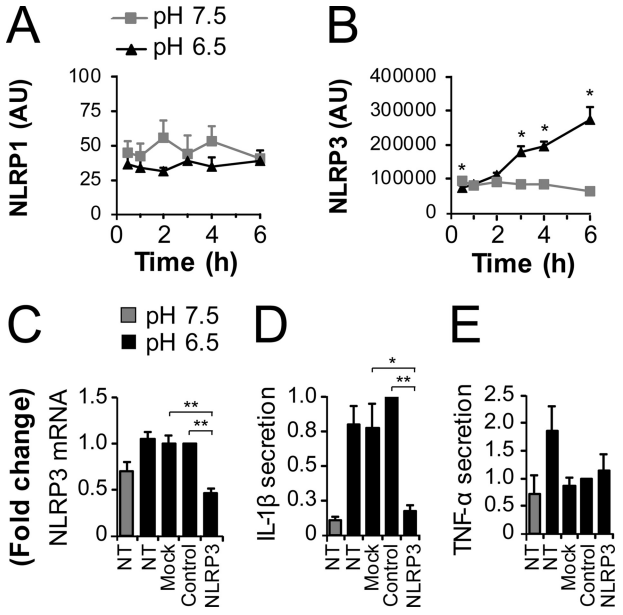

FIGURE 4. **Acidic pH activates the NLRP3 inflammasome.** The relative mRNA expressions of NLRP1 (*A*) and NLRP3 (*B*) receptors are means ± S.E. from four experiments in THP-1 macrophages. In THP-1 macrophages transfected with NLRP3 siRNA, the mRNA expression data (*C*) and cytokine secretion data (*D* and *E*) are expressed as fold changes compared with the negative control siRNA transfection. The data are means ± S.E. from five to six experiments (*C*) or from four to five experiments performed in duplicate (*D* and *E*). *AU*, arbitrary units; *NT*, nontransfected; *Mock*, transfection reagent without siRNA; *Control*, negative control siRNA; *NLRP3*, NLRP3 siRNA. *, $p < 0.05$ and **, $p < 0.01$.

Intracellular Acidification Is Essential for Inflammasome Activation Triggered by Acidic Extracellular pH—The major regulators of intracellular pH in macrophages are the plasmalemmal V-type H^+ ATPases and Na^+/H^+ exchangers, which extrude metabolic H^+ by using energy from ATP and sodium ion gradient, respectively (49). Inhibition of V-type H^+ ATPases in primary human macrophages and THP-1 macrophages by bafilomycin A1 enhanced IL-1β secretion at pH 6.5 and induced IL-1β secretion even at physiological pH after a prolonged 16-h exposure (Fig. 5, *A* and *B*). Of note, bafilomycin A1 targets both plasmalemmal and lysosomal V-type H^+ ATPases, but the plasmalemmal form is more sensitive in macrophages (50). Very similar results were obtained by using the Na^+/H^+ exchanger inhibitor 5-(*N*-ethyl-*N*-isopropyl-)amiloride (supplemental Fig. S5*A*). As the inhibition of these proton extrusion mechanisms lowers intracellular pH (pH_i), whereas extracellular pH (pH_e) remains unaltered (51, 52), the data imply that a drop in pH_i is essential for inflammasome activation caused by acidic environment.

To measure pH_i in live THP-1 macrophages, the cell-permeable fluorescent pH indicator BCECF-AM was employed (53). At pH_e 7.5, the base-line pH_i of THP-1 macrophages was ~7.2 (Fig. 5*C*). Addition of 130 mM KCl at pH_e 7.5 caused a slight increase in pH_i, but this change was not significant at the end point of the recording. In agreement with this observation, hyperosmolarity has been shown to induce intracellular alkalinization in certain cell types (54). Lowering of pH_e from 7.5 to 6.5 caused a drop in pH_i to 6.85 ± 0.03 ($p < 0.01$; Fig. 5*C*). In the presence of 130 mM KCl at pH_e 6.5, the drop in pH_i was diminished with pH_i of 6.96 ± 0.01 measured at the end point of the recording ($p < 0.01$ compared with pH_e 6.5 without KCl). However, the effect of potassium at pH_e 6.5 was only transient, as

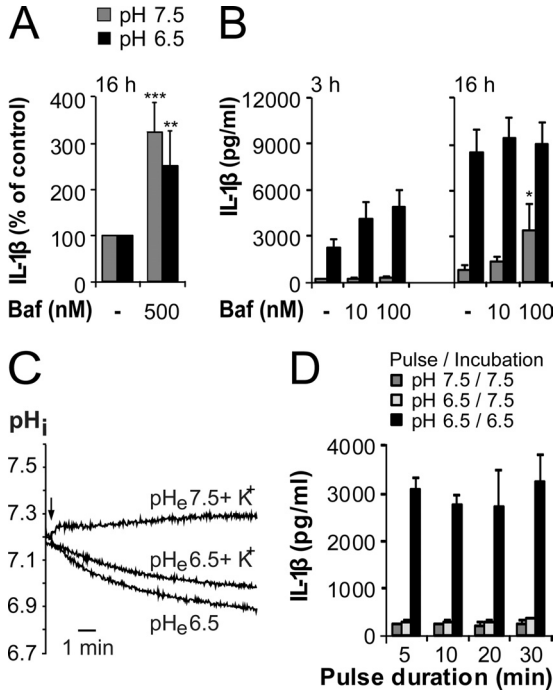

FIGURE 5. **Intracellular acidification is essential for inflammasome activation caused by low pH environment.** Bafilomycin A1 (*Baf*) induces IL-1β secretion in primary human macrophages (*A*) and THP-1 macrophages (*B*). In primary macrophages, the control values of IL-1β secretion were 27 $\pm$ 8 pg/ml and 66 $\pm$ 20 pg/ml at pH 7.5 and 6.5, respectively. The data are means $\pm$ S.E. from six to nine (*A*) and five (*B*) experiments. *C*, initial pH_i in THP-1 macrophages was recorded at pH_e 7.5, followed by perfusion of the indicated test medium (*arrow*). The data are means from three experiments (50 cells/experiment). *D*, THP-1 macrophages were pulsed in a medium of pH 7.5 or 6.5, washed, and subsequently incubated for 4 h at indicated pH for measurement of cytokine secretion. The data are means $\pm$ S.E. from four experiments performed in duplicate. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, compared with untreated control.

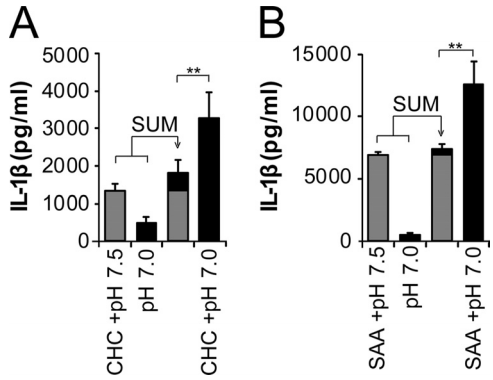

FIGURE 6. **Mildly acidic pH synergizes with other NLRP3 inflammasome activators.** THP-1 macrophages were incubated for 4 h at pH 7.5 or pH 7.0 in the absence or presence of 0.5 mg/ml CHC (*A*) or 0.5 μg/ml SAA (*B*). The background secretion of IL-1β at pH 7.5 has been subtracted from all values. The data are means $\pm$ S.E. from five experiments performed in duplicate. *SUM*, activator at pH 7.5 + release at pH 7.0 without activator. **, $p < 0.01$.

after a 3-h incubation, the pH_i of macrophages was 6.9 both in the absence and presence of 130 mM KCl (supplemental Fig. S5*B*).

To study the reversibility of inflammasome activation by acidic pH, we exploited the information given by the pH_i measurements. THP-1 macrophages were pulsed for 5–30 min at pH_e 6.5 to induce a transient drop in pH_i, and subsequently transferred to pH_e 7.5 for 4 h to measure IL-1β secretion. The short pulsing at acidic pH was not sufficient to induce sustained IL-1β secretion, implying that the effect of acidic pH on inflammasome assembly was fully reversible (Fig. 5*D*).

Mild Acidification Strongly Enhances Inflammasome Activation by Other NLRP3 Activators—To study the potential synergy between low pH and other inflammasome activators, two different types of NLRP3 activators were employed: 1) cholesterol crystals (CHC) that trigger phagocytosis-dependent inflammasome activation, and 2) serum amyloid A (SAA), which induces cell surface receptor-dependent inflammasome activation (27, 28, 55). IL-1β secretion initiated by these activators was measured both at pH 7.5 and at pH 7.0 representing mild acidification. To identify whether the effect of mild acidification with other inflammasome activators was additive or synergistic, the difference between measured (activator at pH 7.0) and summed (activator at pH 7.5 + release at pH 7.0 without activator) IL-1β response was determined. The spontaneous background IL-1β secretion at pH 7.5 was subtracted from all values. If the measured and the summed effects are equal, the effects are additive, whereas if the measured effect is significantly higher than the summed, synergy between low pH and other NLRP3 activators does exist.

The results showed that the measured IL-1β response to CHC at pH 7.0 was 1.8-fold compared with the summed one, indicating marked synergy between these two inflammasome activators (Fig. 6*A*). Very similar results were obtained using SAA, with 1.7-fold measured response compared with the summed one (Fig. 6*B*).

Alkaline pH Inhibits Inflammasome Activation Induced by NLRP3 Activators—The potential effect of alkaline pH on IL-1β response was next studied in the absence and presence of different NLRP3 activators. Alkaline pH 8.0–8.5 did not trigger IL-1β secretion but was instead found to be a potent inhibitor of NLRP3 inflammasome activation. Thus, IL-1β response to monosodium urate crystals (MSU) was dramatically reduced at alkaline pH both in primary human macrophages and in THP-1 macrophages (Fig. 7, *A* and *B*). Similar results were obtained using SAA as the stimulus (Fig. 7*C*). Measurement of lactate dehydrogenase release showed that alkaline pH did not cause cell death; rather, it effectively decreased cell death induced by MSU and SAA (supplemental Fig. S6*A*).

The conformation of SAA and/or its receptors involved in triggering inflammasome activation (TLR2, TLR4, and $P2X_7$ (55)) could be sensitive to modifications by alkaline pH, causing inhibition of receptor binding and/or activation. However, the robust TLR-dependent TNF-α response to SAA observed at pH 7.5 was not inhibited by alkaline pH (Fig. 7*D*), indicating that SAA is fully capable of receptor interactions and that TLR conformation and signaling are unaffected by alkaline pH. Furthermore, others have previously shown that $P2X_7$ receptor activity is maximal at pH 8.0–8.5 (48). In addition, incubation of macrophages with SAA did not acidify pH_i (supplemental Fig. S6*B*), indicating a more indirect effect by alkaline pH. Regarding the crystalline inflammasome activators, inhibition of phagocytosis by alkaline pH remained a possibility. Cholesterol crystals induce intracellular cholesteryl ester accumulation in macrophages, which is effectively blocked by inhibition of their

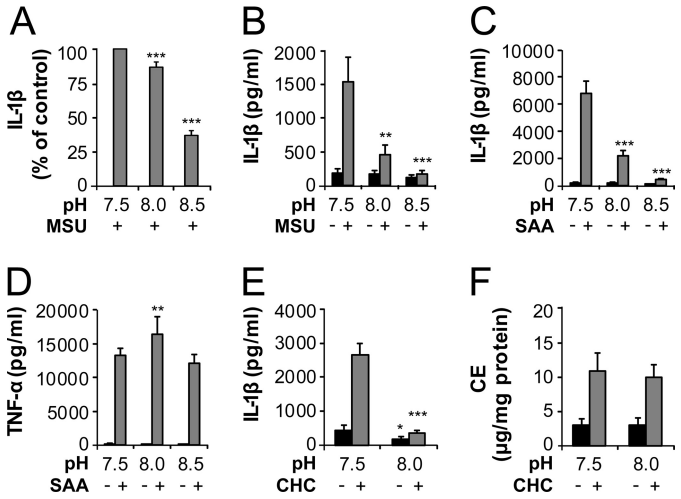

FIGURE 7. **Alkaline pH strongly inhibits NLRP3 inflammasome activation in LPS-primed primary human macrophages (*A*) and in THP-1 macrophages (*B–F*).** Duration of the pH stimulus was 10 h (*A*), 4 h (*B–D*), or 16 h (*E* and *F*), in the absence or presence of 0.25 mg/ml MSU, 2 μg/ml SAA, or 1 mg/ml CHC. In the primary macrophages, alkaline pH alone did not induce IL-1β secretion, and MSU-induced IL-1β secretion at pH 7.5 was 381 $\pm$ 61 pg/ml. The data are means $\pm$ S.E. from eight experiments (*A*) or from five experiments performed in duplicate (*B–F*). *CE*, cholesteryl ester. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, compared with control at pH 7.5.

phagocytosis by cytochalasin D (27). We thus utilized cholesteryl ester accumulation as a marker for CHC phagocytosis. Although the CHC-induced IL-1β secretion was completely blocked at pH 8.0, there was no significant difference in CHC-induced cholesteryl ester accumulation at pH 7.5 and 8.0 (Fig. 7, *E* and *F*). Finally, we analyzed the expression of pro-IL-1β and NLRP3 inflammasome components, NLRP3 and ASC, after a 0.5–6-h incubation at pH 7.5 or 8.0 and found no significant differences in their mRNA levels (supplemental Fig. S6*C*).

DISCUSSION

In this study, we show that low pH, which frequently occurs at sites of inflammation and ischemia, represents a novel danger signal alerting the innate immunity. Human macrophages respond to acidification of their environment by activating the NLRP3 inflammasome, which leads into activation of caspase-1 and consequent maturation and secretion of the potent proinflammatory cytokines IL-1β and IL-18. Thus, low pH may significantly contribute to the vicious cycle of inflammation associated with chronic inflammatory diseases such as rheumatoid arthritis and atherosclerosis. Furthermore, ischemia-induced acidification may alert resident macrophages and thus contribute to the post-ischemic inflammatory responses.

The NLRP3 inflammasome, sensitive also to other endogenous activators (26), was identified as the inflammasome responsive to acidic environment. Silencing of NLRP3 receptor expression by siRNA abolished low pH -induced IL-1β but not TNF-α secretion. Moreover, low pH enhanced the expression of NLRP3 receptor, a step that has been previously shown to be a prerequisite for NLRP3 inflammasome activation (56). Several stress signals have been proposed to act upstream NLRP3 activation (26). Of these, potassium efflux was strongly involved in low pH-induced NLRP3 activation. High extracellular K^+ concentrations and non-selective blocking of K^+ channels by tetraethylammonium, Cs^+, and Ba^{2+} (but not by 4-aminopyridine) markedly inhibited inflammasome activation by acidic pH. In agreement with our data, THP-1 macrophages after PMA differentiation express potassium channels sensitive to tetraethylammonium, Cs^+, and Ba^{2+}, but 4-aminopyridine-sensitive potassium currents are found only in undifferentiated THP-1 cells (57, 58). Increased ROS formation does not seem to participate in low pH-induced NLRP3 activation, as the general ROS inhibitor NAC, an efficient blocker of MSU and asbestos-induced NLRP3 activation (59), did not affect IL-1β secretion at acidic pH. In fact, intracellular acidification may even inhibit ROS production in macrophages (49, 60).

Acidic extracellular environment may trigger cellular signaling events either via the cell surface or via changes in intracellular milieu. Regarding the cell surface, acid-sensing ion channels activated directly by protons are intriguing candidates as mediators of acidosis-triggered cellular responses (61). However, acid-sensing ion channels are expressed mainly in neurons, and the single available study exploring their expression in macrophages (mouse peritoneal) found no expression at the protein level (62). Importantly, we could show that acidic environment causes rapid and persistent cytosolic acidification and that intracellular acidification via inhibition of V-type H^+ ATPases or Na^+/H^+ exchangers is sufficient to trigger IL-1β secretion even at an extracellular pH of 7.5. These data suggest that intracellular acidification is an essential signal in inflammasome activation triggered by low pH environment. In support of this idea, a considerable drop in intracellular pH has been shown to occur during early apoptosis and to enhance the activation of apoptotic caspases by the apoptosome complex (63, 64). Both structural and functional parallels exist between the apoptosome and the inflammasomes (26), implying that intracellular pH may regulate the assembly of several types of caspase-activating platforms.

IL-1β secretion occurs via an unconventional route, which has been proposed to involve exocytosis of endolysosome-related vesicles (65). Interestingly, intracellular acidification in macrophages has been shown to induce the movement of endolysosomal vesicles from perinuclear region to cell periphery, whereas alkalinization enhances their perinuclear localization (66). Thus, it is conceivable that the induction and inhibition of inflammasome activation by acidic and alkaline pH, respectively, is directly coupled with the movements of vesicular IL-1β secretion machinery. We did not find any evidence of lysosomal membrane destabilization at acidic pH. This is not surprising because lysosomes are highly acidic organelles and thus resistant to low pH by nature. Therefore, lysosomal damage and cathepsin B leakage to cytoplasm, suggested to mediate NLRP3 activation by silica and alum crystals (67), seems unlikely to occur in response to extracellular acidosis. Nevertheless, low pH-induced IL-1β secretion was partially dependent on cathepsin B activity, and low pH also enhanced the secretion of active cathepsin B. Taken together, these data imply that lysosomal cathepsin B may have a role in the IL-1β secretion machinery downstream inflammasome activation instead of participation in upstream events.

Even a mild extracellular acidification from pH 7.5 to pH 7.0 caused a nearly 2-fold enhancement of the IL-1β secretion

induced by SAA and CHC. This marked synergistic effect of mild acidification with other NLRP3 activators may have a major impact on inflammatory reactions *in vivo*, as acidic environments do co-exist with other inflammasome activators, *e.g.* in atherosclerosis (CHC), rheumatoid arthritis (SAA), and gout (MSU) (21–23). Importantly, we found that alkaline pH strongly inhibits the IL-1β response to both crystalline (MSU, CHC) and receptor-dependent (SAA) NLRP3 activators. The effect of extracellular alkalinization was independent of pro-IL-1β and NLRP3 inflammasome expression, and both crystal phagocytosis and receptor interactions of SAA were unaffected by alkaline pH. These data indicate that the inhibitory effect exerted by alkaline pH on NLRP3 inflammasome is mediated via an intracellular mechanism directly affecting inflammasome assembly or the events upstream.

IL-1β is a key proinflammatory cytokine with potent local (*e.g.* induction of adhesion molecules, chemokines, IL-6, and inducible nitric oxide synthase) and systemic (fever, vasodilatation) effects (68). The importance of IL-1 signaling in chronic inflammatory diseases is best exemplified by good clinical responses to IL-1 blocking therapies reported in autoinflammatory syndromes (69), rheumatoid arthritis (70), and gout (23). IL-1 blocking therapies are being evaluated also in treatment and secondary prevention of myocardial infarction and stroke, both consequences of advanced atherosclerosis (71, 72). In addition to IL-1β and IL-18, low pH induced also the expression and/or secretion of several inflammasome-independent proinflammatory cytokines (IL-1α, TNF-α, IL-6) and synergized with other NLRP3 inflammasome activators, suggesting a robust proinflammatory potential for local acidosis. Acidification at least down to pH 6.8 in chronic inflammation and down to pH 6.0 in acute ischemia has been demonstrated (1–4, 12, 15–17), suggesting significant pathophysiological relevance for the current findings.

In conclusion, we have shown here that acidic environment alerts the innate immunity by activating the NLRP3 inflammasome in human macrophages. Alkalinization of macrophage environment strongly inhibits NLRP3 inflammasome activation induced by structurally distinct activators, demonstrating the bipartite regulatory potential of extracellular pH on the inflammasome. Thus, local extracellular acidosis in the body seems to represent a novel endogenous danger signal and a powerful regulator of immune functions. Acidosis associated with inflammation could also represent an amenable target for development of novel anti-inflammatory therapies aiming at restoration of normal tissue pH.

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