# Glyburide inhibits the Cryopyrin/Nalp3 inflammasome

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Inflammasomes activate caspase-1 for processing and secretion of the cytokines interleukin-1β (IL-1β) and IL-18. Cryopyrin/NALP3/NLRP3 is an essential component of inflammasomes triggered by microbial ligands, danger-associated molecular patterns (DAMPs), and crystals. Inappropriate Cryopyrin activity has been incriminated in the pathogenesis of gouty arthritis, Alzheimer's, and silicosis. Therefore, inhibitors of the Nalp3 inflammasome offer considerable therapeutic promise. In this study, we show that the type 2 diabetes drug glyburide prevented activation of the Cryopyrin inflammasome. Glyburide's cyclohexylurea group, which binds to adenosine triphosphatase (ATP)–sensitive K+ (K$_{ATP}$) channels for insulin secretion, is dispensable for inflammasome inhibition. Macrophages lacking K$_{ATP}$ subunits or ATP-binding cassette transporters also activate the Cryopyrin inflammasome normally. Glyburide analogues inhibit ATP- but not hypothermia-induced IL-1β secretion from human monocytes expressing familial cold-associated autoinflammatory syndrome–associated Cryopyrin mutations, thus suggesting that inhibition occurs upstream of Cryopyrin. Concurrent with the role of Cryopyrin in endotoxemia, glyburide significantly delays lipopolysaccharide-induced lethality in mice. Therefore, glyburide is the first identified compound to prevent Cryopyrin activation and microbial ligand-, DAMP-, and crystal-induced IL-1β secretion.

## Introduction

Glyburide is the most widely used sulfonylurea drug for the treatment of type 2 diabetes in the United States (Riddle, 2003). The drug works by inhibiting ATP-sensitive K+ (K$_{ATP}$) channels in pancreatic β cells (Ashcroft, 2005). K$_{ATP}$ channels are octameric complexes of four Kir6.x (Kir6.1 or Kir6.2) and four sulfonylurea receptor (SUR; SUR1 or SUR2) subunits (Clement et al., 1997). The SUR subunits belong to the ATP-binding cassette (ABC) transporter family (Aguilar-Bryan et al., 1995) and function as a regulatory subunit, endowing the Kir6.x channel with sensitivity to inhibition by sulfonylureas such as glyburide and glipizide (Ashcroft, 2005). In addition to K$_{ATP}$ channels, the ABC transporter ABCA1 was proposed as a putative glyburide

target (Hamon et al., 1997). Glyburide's pharmacological properties are summarized in Fig. S1 A.

The cystein protease caspase-1 mediates the proteolytic maturation of the cytokines interleukin-1β (IL-1β) and IL-18 after its recruitment in protein complexes termed inflammasomes (Lamkanfi and Dixit, 2009). Cryopyrin/NALP3/NLRP3 is an essential component of inflammasomes triggered by pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs), and crystalline substances (Kanneganti et al., 2006, 2007; Mariathasan et al., 2006; Sutterwala et al., 2006; Lamkanfi and Dixit, 2009). Inappropriate Cryopyrin activity has been incriminated in the pathogenesis of several diseases, including gouty arthritis, Alzheimer's, and silicosis (Martinon et al., 2006; Cassel et al., 2008; Dostert et al., 2008; Halle et al.,

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Abbreviations used in this paper: ABC, ATP-binding cassette; BMDM, bone marrow–derived macrophage; DAMP, danger-associated molecular pattern; FCAS, familial cold-associated autoinflammatory syndrome; IL, interleukin; K$_{ATP}$, ATP-sensitive K+; LPS, lipopolysaccharide; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; SUR, sulfonylurea receptor.
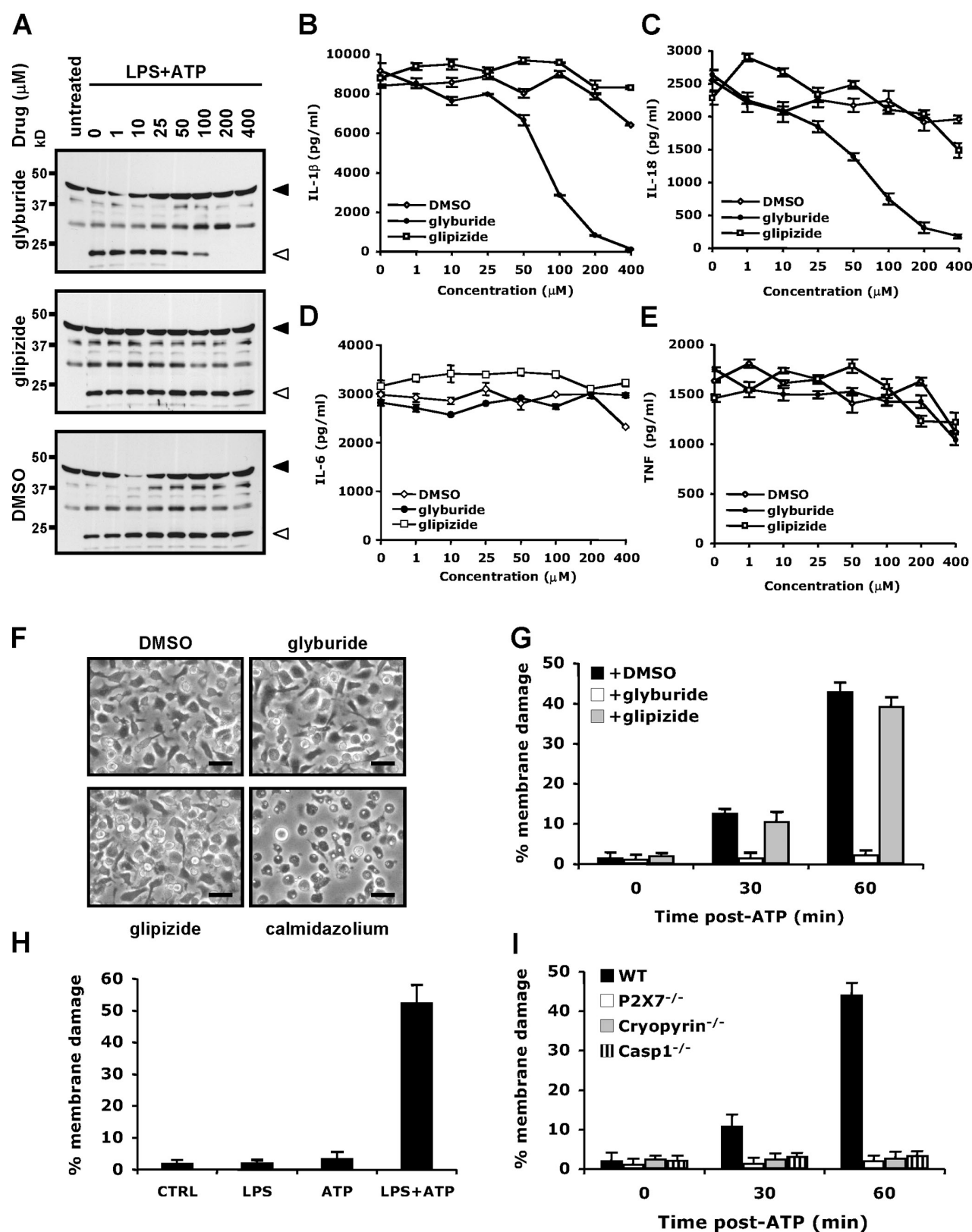
Figure 1. **Glyburide inhibits LPS+ATP-induced caspase-1 activation, secretion of IL-1β and IL-18, and macrophage cell death.** (A–E) LPS-primed BMDMs were treated with glyburide, glipizide, or DMSO for 15 min before 5 mM ATP was added for 30 min. Cell extracts were immunoblotted for caspase-1 (A), and culture supernatants were analyzed for secreted IL-1β (B), IL-18 (C), IL-6 (D), and TNF (E). Black arrowheads indicate procaspase-1, and white arrowheads mark the p20 subunit. (F) BMDMs were incubated with 200 µM glyburide, 200 µM glipizide, 200 µM DMSO, or 50 µM calmidazolium for 2 h before brightfield photographs were taken. (G) LPS-primed BMDMs were treated with 200 µM glyburide, glipizide, or DMSO for 15 min followed by 5 mM ATP for the indicated durations. Membrane damage was measured using Live/Dead assay. Bars, 20 µm. (H) BMDMs were left untreated (CTRL), stimulated with 10 µg/ml LPS for 3 h, treated with 5 mM ATP for 1 h, or treated with LPS and ATP. Membrane damage was measured using Live/Dead assay.

2008; Hornung et al., 2008), so inhibitors of the Cryopyrin inflammasome offer considerable therapeutic promise.

In this study, we show that glyburide prevented activation of the Cryopyrin inflammasome by a variety of stimuli. Concurrent with the role of Cryopyrin in endotoxemia, glyburide delayed lipopolysaccharide (LPS)-induced lethality in mice. Therefore, glyburide is the first compound identified to act upstream of Cryopyrin to prevent PAMP-, DAMP-, and crystal-induced IL-1β secretion.

# Results and discussion

### Glyburide inhibits LPS+ATP-induced caspase-1 activation, IL-1β secretion, and macrophage death

Glyburide prevents LPS+ATP-induced secretion of IL-1β from human and murine macrophages (Hamon et al., 1997; Laliberte et al., 1999; Perregaux et al., 2001) and from murine Schwann cells (Marty et al., 2005). To determine whether caspase-1 activation is impaired by glyburide, LPS-primed bone marrow–derived macrophages (BMDMs) were incubated with glyburide for 15 min before ATP was added for another 30 min. In contrast to the related sulfonylurea glipizide, glyburide inhibited caspase-1 processing in a dose-dependent fashion (Fig. 1 A), and this prevented secretion of the caspase-1–dependent cytokines IL-1β (Fig. 1 B) and IL-18 (Fig. 1 C). Secretion of IL-6 (Fig. 1 D) and TNF (Fig. 1 E) was not impaired by glyburide, ruling out a general defect in macrophage responsiveness. Inhibition was evident up to 3 h post-ATP (Fig. S1 B), indicating that glyburide did not merely delay caspase-1 activation.

Significantly, BMDMs cultured for 3 h in glyburide, glipizide, or DMSO looked morphologically normal (Fig. 1 F) and displayed no significant membrane damage (Fig. S1 C). As a positive control, macrophage death was induced with the calmodulin inhibitor calmidazolium (Fig. 1 F and Fig. S1 C). Inhibition of caspase-1 activation by glyburide was reversible because caspase-1 was activated if glyburide was removed from the culture medium before ATP addition (Fig. S1 D). Glyburide also blocked the rapid, caspase-1–dependent cell death that occurs when BMDMs are treated with LPS and ATP (Fig. 1, G–I). As expected, glipizide and DMSO did not prevent this death. Of note, neither LPS nor ATP alone affected BMDM viability (Fig. 1 H). Like caspase-1$^{-/-}$ BMDMs, cells lacking the P2X$_7$ receptor or Cryopyrin were not killed by LPS+ATP (Fig. 1 I). These results demonstrate that the Cryopyrin inflammasome is essential for LPS+ATP-induced macrophage death and that glyburide inhibits LPS+ATP-induced caspase-1 activation, IL-1β secretion, and macrophage death.

### Glyburide's sulfonyl and benzamido groups are required for optimal inhibition of the Cryopyrin inflammasome

Glyburide inhibits K$_{ATP}$ channels on pancreatic β cells. Its cyclohexylurea group is necessary for high affinity binding to SUR1 (Meyer et al., 1999), which is the SUR subunit of these K$_{ATP}$

channels. Interestingly, the cyclohexylurea group (Fig. 2 A, compound G1) was dispensable for inhibition of LPS+ATP-induced caspase-1 activation (Fig. 2 B) and IL-1β secretion (Fig. 2 C). As expected, glyburide and compound G1 did not affect IL-6 secretion (Fig. 2 D). Glipizide also contains a cyclohexylurea group (Fig. 2 A) and inhibits SUR1-containing K$_{ATP}$ channels, but it failed to inhibit caspase-1 activation (Fig. 2 B). These data suggest that inhibition of the Cryopyrin inflammasome is independent of SUR1-containing K$_{ATP}$ channels.

Additional structure activity experiments demonstrated that both the benzamido and sulfonyl groups of glyburide (Fig. 2 A, compounds G2–G4) were required for optimal inhibition. Analogue G2 comprising only the benzamido group (Fig. 2 A, compound G2) inhibited LPS+ATP-induced caspase-1 activation (Fig. 2 E) and IL-1β secretion (Fig. 2 F), albeit less effectively than compound G1. Thus, the benzamido group contributes but is not sufficient for optimal inhibition. Indeed, a G1 analogue lacking the benzamido group (Fig. 2 A, compound G3) did not affect caspase-1 activation and IL-1β secretion (Fig. 2, E and F). In addition to the benzamido group, the sulfonyl moiety is required for optimal inhibition, as deletion of the sulfonyl group from compound G1 (Fig. 2 A, compound G4) was less effective at inhibiting caspase-1 activation and IL-1β secretion (Fig. 2, E and F). Like glyburide, compounds G1–G4 permitted normal IL-6 secretion (Fig. 2 G), demonstrating the specificity of these results.

### Glyburide inhibits the Cryopyrin inflammasome downstream of the P2X$_7$ receptor

Like ATP, the cation ionophore nigericin engages the Cryopyrin inflammasome (Mariathasan et al., 2006), but nigericin signals independently of the P2X$_7$ receptor (Fig. S1, E–G; Solle et al., 2001). Glyburide potently blocked LPS+nigericin-induced caspase-1 activation (Fig. 3 A). As expected, glipizide and DMSO vehicle did not prevent LPS+nigericin-induced caspase-1 activation. Notably, substituting lipid A, lipoteichoic acid, peptidoglycan, or Pam3-CSK4 for LPS did not prevent glyburide from inhibiting caspase-1 activation (Fig. 3 B).

In agreement with previous studies (Cassel et al., 2008; Dostert et al., 2008; Hornung et al., 2008), silica and the lysosomotrophic molecule H-LL-OMe also promoted Cryopyrin-dependent caspase-1 activation and IL-1β secretion in LPS-primed BMDMs (Fig. S2, A and B). Caspase-1 activation and IL-1β secretion in response to these stimuli were independent of the P2X$_7$ receptor (Fig. S2, C and D) but required TLR4 signaling (Fig. S2 E). Glyburide effectively blocked caspase-1 activation and IL-1β secretion by these stimuli too (Fig. 3, C and D). These data suggest that glyburide targets a signaling component downstream of the P2X$_7$ receptor. We first considered the hemichannel pannexin-1, which was proposed to signal both LPS+ATP- and LPS+nigericin-induced caspase-1 activation (Pelegrin and

(I) LPS-primed BMDMs from wild-type (WT), P2X$_7$$^{-/-}$, Cryopyrin$^{-/-}$, and caspase-1$^{-/-}$ mice were treated with 5 mM ATP for the indicated durations. Membrane damage was measured with Live/Dead assay. Cytokine and cell death data represent the mean ± SD of triplicate samples from a single experiment, and all results are representative of at least three independent experiments.
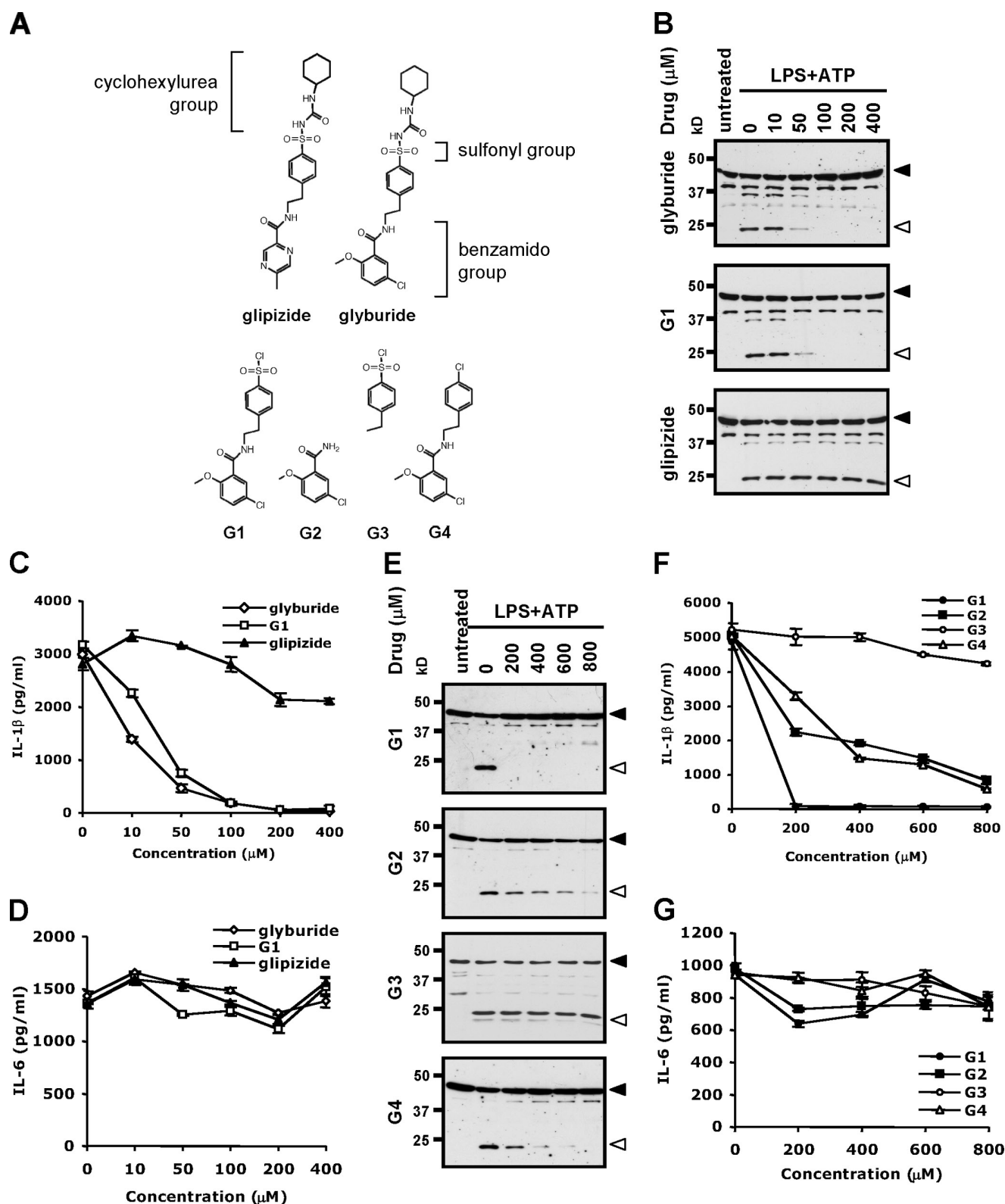
Figure 2. **Glyburide's cyclohexylurea moiety is dispensable for inflammasome inhibition.** (A) Chemical structure of glyburide, glipizide, and glyburide-derived analogues G1–G4. (B–G) LPS-primed BMDMs were treated with glyburide, glipizide, or analogues G1–G4 for 15 min before 5 mM ATP was added for 30 min. Cell extracts were immunoblotted for caspase-1 (B and E), and culture supernatants were analyzed for secreted IL-1β (C and F) and IL-6 (D and G). Black arrowheads indicate procaspase-1, and white arrowheads mark the p20 subunit. Cytokine data represent the mean ± SD of triplicate samples from a single experiment, and all results are representative of at least three independent experiments.
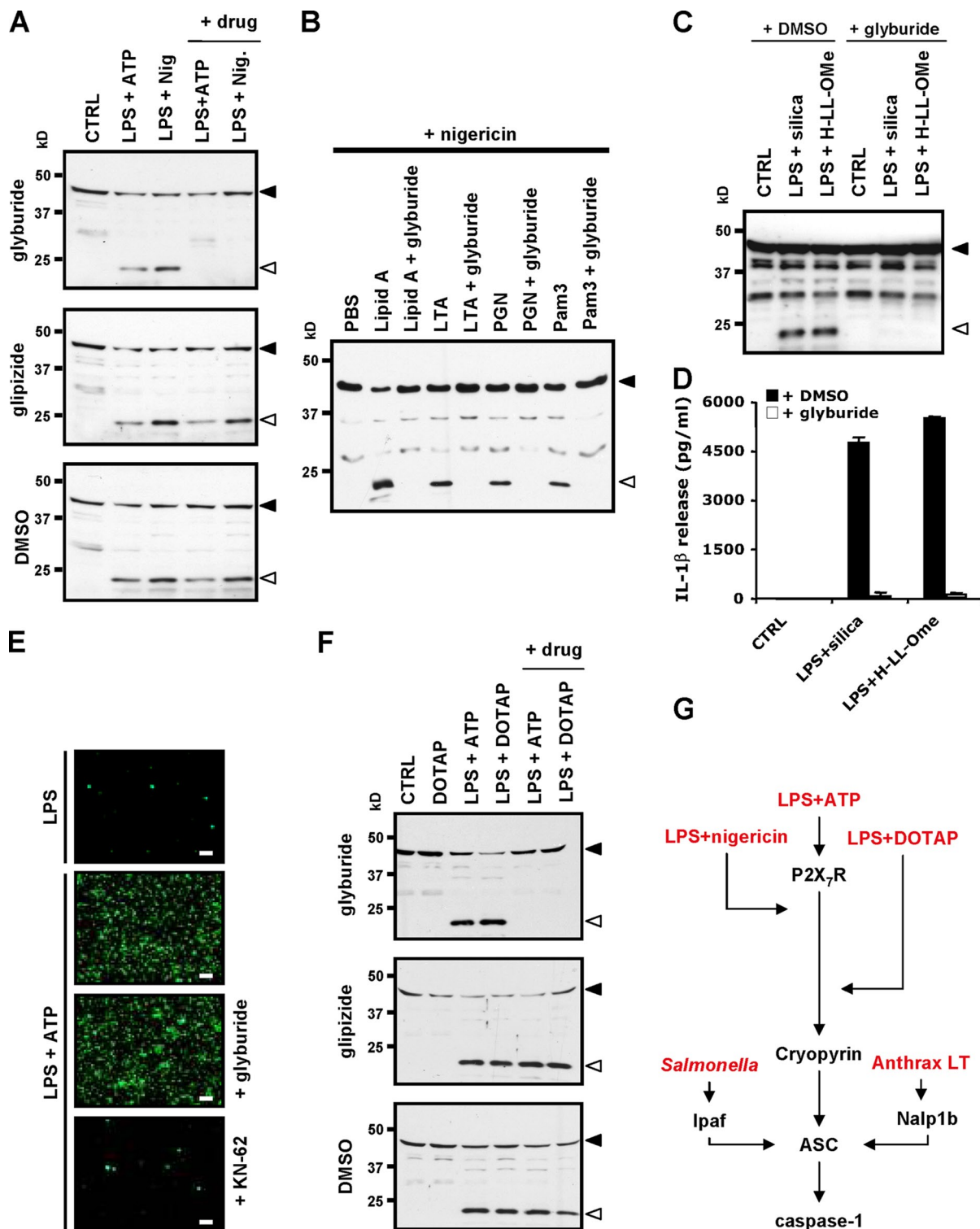
Figure 3. **Glyburide inhibits PAMP-, DAMP-, and crystal-induced activation of the Cryopyrin inflammasome.** (A) LPS-primed BMDMs were incubated with 200 µM glyburide, glipizide, or DMSO for 15 min before 20 µM nigericin was added for 30 min. Cell extracts were immunoblotted for caspase-1. (B) BMDMs were stimulated with the indicated PAMPs for 3 h, incubated with 200 µM glyburide for 15 min, and stimulated with 20 µM nigericin for 30 min. Cell extracts were immunoblotted for caspase-1. (C and D) LPS-primed BMDMs were incubated with 200 µM glyburide or DMSO for 15 min before 500 µg/ml silica or1 mM of the lysosomotrophic peptide H-LL-OMe was added for 3 h. Cell extracts were immunoblotted for caspase-1 (C), and culture supernatants were analyzed for secreted IL-1β (D). (E) LPS-primed BMDMs were left untreated or incubated with 200 µM glyburide or 10 µM KN-62 for 15 min. 2 µM YoPro-1 was subsequently added, and its uptake was visualized before and after a 5-min ATP pulse. Bars, 50 µm. (F) BMDMs were left untreated (CTRL), transfected with DOTAP alone for 4 h, stimulated with 10 µg/ml LPS for 3 h and subsequently with 5 mM ATP (LPS+ATP), or transfected with DOTAP and 30 µg/ml LPS for 4 h (DOTAP+LPS) in the presence or absence of 200 µM glyburide, glipizide, or DMSO. Cell extracts were immunoblotted for caspase-1. (G) Overview and mapping of inflammasome-activating stimuli and upstream signaling pathways. Black arrowheads indicate procaspase-1, and white arrowheads mark the p20 subunit. Cytokine data represent the mean ± SD of triplicate samples from a single experiment, and all results are representative of at least three independent experiments.
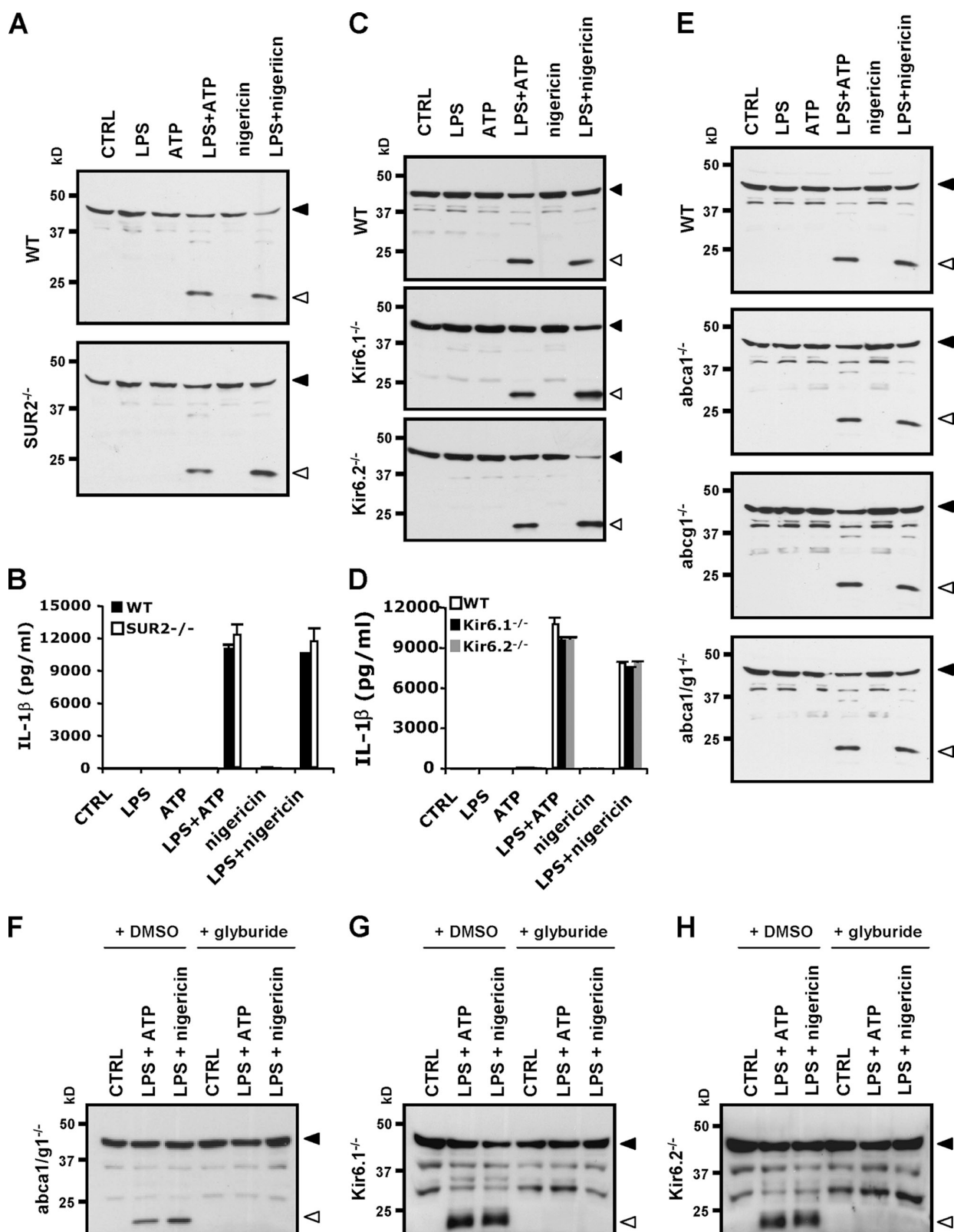
Figure 4. **$K_{ATP}$ channels and ABC transporters are dispensable for activation of the Cryopyrin inflammasome.** (A–E) BMDMs from wild-type (WT) and SUR2$^{-/-}$ (A and B), Kir6.1$^{-/-}$ and Kir6.2$^{-/-}$ (C and D), or abca1$^{-/-}$, abcg1$^{-/-}$, and abca1$^{-/-}$/abcg1$^{-/-}$ (abca1/g1$^{-/-}$) mice (E) were left untreated (CTRL), stimulated with 10 µg/ml LPS for 3 h, treated with 5 mM ATP or 20 µM nigericin for 30 min, or stimulated with LPS and treated with ATP (LPS+ATP) or nigericin (LPS+nigericin). Cell extracts were immunoblotted for caspase-1 (A, C, and E), and culture supernatants were analyzed for secreted IL-1β (B and D). (F–H), LPS-primed BMDMs from abca1$^{-/-}$/abcg1$^{-/-}$ (F), Kir6.1$^{-/-}$ (G), and Kir6.2$^{-/-}$ (H) mice were treated with 200 µM glyburide or DMSO for 15 min before 5 mM ATP or 20 µM nigericin was added for an additional 30 min. Cell extracts were immunoblotted for caspase-1. Black arrowheads indicate procaspase-1, and white arrowheads mark the p20 subunit. Cytokine data represent the mean ± SD of triplicate samples from a single experiment, and all results are representative of three independent experiments.

Surprenant, 2006, 2007; Kanneganti et al., 2007). Pannexin-1 forms a nonselective pore for molecules <1 kD, such as the fluorescent dye YoPro-1, within seconds to minutes of P2X$_7$ receptor stimulation (Pelegrin and Surprenant, 2006, 2007; Locovei et al., 2007). Unlike the P2X$_7$ receptor inhibitor KN-62, glyburide did not prevent ATP-induced YoPro-1 uptake into LPS-primed macrophages (Fig. 3 E), suggesting that pannexin-1 is not inhibited by glyburide. Concurrently, glyburide inhibited caspase-1 activation by LPS+DOTAP (Fig. 3 F), a stimulus that engages the Cryopyrin inflammasome independently of the P2X$_7$ receptor and pannexin-1 (Kanneganti et al., 2007). Collectively, these results indicate that glyburide inhibits engagement of the Cryopyrin inflammasome by diverse stimuli and that inhibition occurs downstream of the P2X$_7$ receptor (Fig. 3 G).

### K$_{ATP}$ channels and ABC transporters are dispensable for activation of the Cryopyrin inflammasome

The structure–activity relationship experiments (Fig. 2) suggest that glyburide inhibits the Cryopyrin inflammasome independently of SUR1-containing K$_{ATP}$ channels. Certain tissues such as heart and muscle express SUR2 instead of SUR1 K$_{ATP}$ channels. However, SUR2$^{-/-}$ BMDMs activated caspase-1 and secreted normal amounts of IL-1β in response to LPS+ATP and LPS+nigericin (Fig. 4, A and B). In addition, BMDMs from Kir6.1$^{-/-}$ and Kir6.2$^{-/-}$ mice also demonstrated normal LPS+ATP- and LPS+nigericin-induced caspase-1 activation (Fig. 4 C) and IL-1β secretion (Fig. 4 D). These results show that K$_{ATP}$ channels are not required for activation of the Cryopyrin inflammasome.

Previous studies also suggested that glyburide and the ABC transporter inhibitor DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium) target ABCA1 to inhibit LPS+ATP-induced IL-1β secretion (Hamon et al., 1997; Marty et al., 2005). We confirmed that DIDS inhibited LPS+ATP-induced IL-1β secretion in a dose-dependent manner (Fig. S3 A). However, DIDS also inhibited P2X$_7$ receptor-mediated currents (Ma et al., 2009) and caspase-1 activation (Fig. S3 B), suggesting that the drug prevents IL-1β secretion by directly antagonizing the P2X$_7$ receptor rather than ABCA1. Concurrently, BMDMs from mice lacking ABCA1 and/or ABCG1 activated caspase-1 normally in response to LPS+ATP and LPS+nigericin (Fig. 4 E). Caspase-1 activation also was normal in macrophages lacking the glyburide receptor cystic fibrosis transmembrane conductance regulator (unpublished data).

To complement these experiments, we analyzed whether glyburide prevented caspase-1 activation in macrophages lacking ABC transporters or K$_{ATP}$ channels. As in wild-type macrophages (Fig. 1 A), glyburide abolished LPS+ATP- and LPS+nigericin-induced caspase-1 activation in macrophages lacking ABCA1 and ABCG1 (Fig. 4 F) or the K$_{ATP}$ channel subunits Kir6.1 (Fig. 4 G) and Kir6.2 (Fig. 4 H). In summary, these experiments indicate that known targets of glyburide are dispensable for activation of the Cryopyrin inflammasome.

### Inflammasome inhibition occurs upstream of Cryopyrin

Ipaf rather than Cryopyrin is critical for inflammasome assembly and caspase-1 activation by *Salmonella typhimurium* infection

(Mariathasan et al., 2004). Interestingly, caspase-1 activation in *S. typhimurium*–infected BMDMs was not affected by glyburide (Fig. 5 A). Furthermore, anthrax lethal toxin, which engages the NALP1b inflammasome (Boyden and Dietrich, 2006), also activated caspase-1 normally in the presence of glyburide (Fig. 5 B). Concurrently, glyburide did not affect caspase-1 activity in vitro (Fig. 5 C). These data suggest that glyburide works upstream of caspase-1 and ASC, the shared adaptor in the Cryopyrin, Ipaf, and NALP1b inflammasomes (Fig. 3 G).

We next studied the affect of glyburide and compound G1 on the ATPase activity of Cryopyrin, which is required for caspase-1 activation and IL-1β secretion (Duncan et al., 2007). Neither glyburide nor compound G1 reduced the ATPase activity of recombinant Cryopyrin (Fig. 5, D and E; and Fig. S3, C and D), placing glyburide action upstream of Cryopyrin. We sought confirmatory evidence using human peripheral blood mononuclear cells (PBMCs) from individuals afflicted with familial cold-associated autoinflammatory syndrome (FCAS), a disorder resulting from a temperature-sensitive (L353P) mutation within Cryopyrin (Hoffman et al., 2001). Similar to LPS+ATP at 37°C, cold-induced conformational changes in FCAS monocytes are presumed to assemble the Cryopyrin inflammasome at the permissive temperature of 32°C. It is worth noting that FCAS monocytes secreted a small amount of IL-1β at 37°C in response to either ATP or LPS alone (Fig. S3 E), suggesting that FCAS monocytes are hyperresponsive. Compound G1 inhibited LPS+ATP-induced IL-1β secretion at 37 and 32°C (Fig. 5 F and not depicted) but did not prevent cold-induced IL-1β secretion from FCAS PBMCs at 32°C (Fig. 5 G). Therefore, compound G1 and glyburide likely act upstream of Cryopyrin in inhibiting caspase-1 activation. We also assessed the affect of glyburide on LPS-induced lethality in mice based on the observation that Cryopyrin-deficient mice are resistant to LPS-induced lethality (Mariathasan et al., 2006; Sutterwala et al., 2006). Although 90% of vehicle-treated mice died within 20 h after LPS dosage, all mice in the glyburide-treated group were alive at this time point (Fig. 5 H). Mortality in the glyburide-treated arm was significantly delayed (P < 0.0001), but in line with glyburide's relatively short half-life in vivo (∼1 h; unpublished data), the mice eventually succumbed to endotoxic shock.

Collectively, these results demonstrate that glyburide acts upstream of Cryopyrin and downstream of the P2X$_7$ receptor to block Cryopyrin-dependent inflammasome activation by PAMPs, DAMPs, and crystalline substances. These results may have significant therapeutic ramifications for the treatment of gouty arthritis, silicosis, and Alzheimer's, in which excessive Cryopyrin-dependent IL-1β production was proposed as a major cause of pathology (Cassel et al., 2008; Dostert et al., 2008; Halle et al., 2008; Hornung et al., 2008).

## Materials and methods

### Mice and macrophages
*Cryopyrin*$^{-/-}$, *caspase-1*$^{-/-}$, *Abca1*$^{-/-}$, *Abcg1*$^{-/-}$, *Abca1*$^{-/-}$*Abcg1*$^{-/-}$, *Kir6.1*$^{-/-}$, *Kir6.2*$^{-/-}$, and *SUR2*$^{-/-}$ mice have been described previously (Miki et al., 1998, 2002; Schott et al., 2004; Mariathasan et al., 2006; Stoller et al., 2007; Yvan-Charvet et al., 2008). *P2X$_7$*$^{-/-}$ mice were obtained from Lexicon Pharmaceuticals. Mice were housed in a pathogen-free facility. All experiments were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and
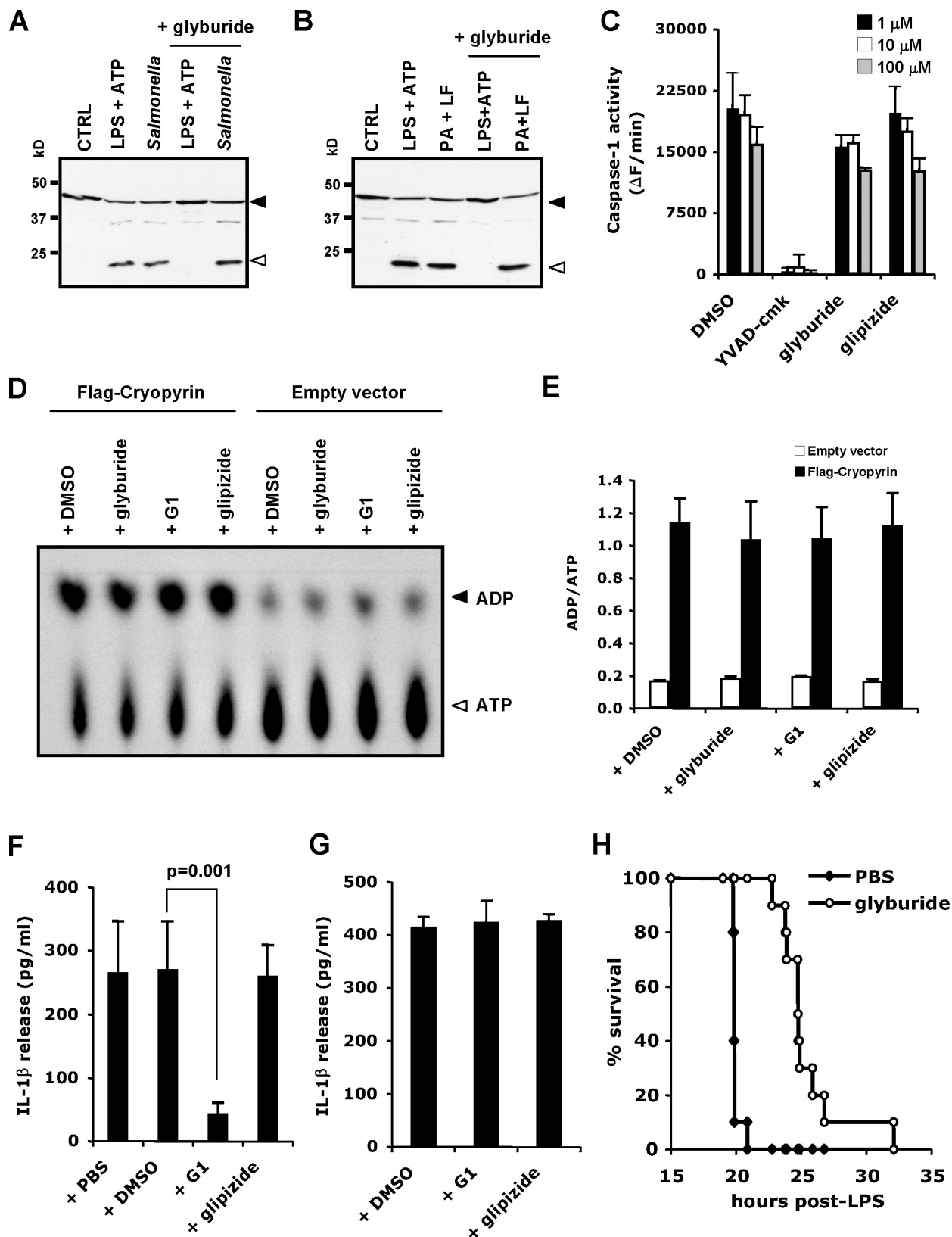
Figure 5.  **Glyburide inhibits the inflammasome upstream of Cryopyrin.** (A and B) BMDMs from C57BL/6 (A) or BALB/c mice (B) were stimulated with 10 µg/ml LPS for 3 h and treated with 5 mM ATP for 30 min (LPS+ATP), infected with *S. typhimurium* for 4 h, or treated with 10 µg/ml anthrax lethal toxin (PA+LF) for 4 h in the presence or absence of 200 µM glyburide. Cell extracts were immunoblotted for caspase-1. Black arrowheads indicate procaspase-1, and white arrowheads mark the p20 subunit. (C) In vitro enzymatic activity of 1 IU mouse caspase-1 incubated with glyburide, glipizide, or the caspase-1 inhibitor Ac-YVAD-cmk. Data represent the mean ± SD of triplicate samples from one out of three independent experiments. (D and E) ATPase activity of purified recombinant Cryopyrin and control eluates in the presence of 100 µM glyburide, compound G1, or glipizide (D). (E) ATPase activity quantified by

were approved by the Institutional Animal Care and Use Committee at Genentech. BMDMs were prepared as described previously (Lamkanfi et al., 2007). In brief, BMDMs were isolated from femurs of 6–12-wk-old mice and were cultured in Iscove's modified Dulbecco's medium containing 10% heat-inactivated FBS, 20% L cell–conditioned medium, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% $CO_2$. After 5–7 d of incubation, cells were collected and plated in 6- or 24-well plates in Iscove's modified Dulbecco's medium containing 10% heat-inactivated FBS and 100 mg/ml thymidine and antibiotics. Macrophages were cultured for an additional 24 h before use.

### LPS-induced endotoxemia
8-wk-old male C57BL/6 mice (Charles River) were maintained in micro-isolator cages and received food and water ad libitum according to the American Association of Laboratory Animal Care guidelines. Mice were randomly divided into two groups ($n$ = 10/group). The mice were injected intraperitoneally bid daily with 500 mg/kg glyburide or the formulation vehicle (DMSO/10% HP-b-CD [2.5:97.5]) in 360 µl PBS. 4 h after the first dosing, endotoxic shock was induced by intraperitoneal injection of 18 mg/kg LPS (*Escherichia coli* O111:B4; Sigma-Aldrich). Mice were monitored hourly for survival up to 36 h. Differences in group survival were analyzed with the Kaplan–Meier test. $P < 0.05$ was considered statistically significant.

### Patients
The University of California San Diego Human Research Protection Program Committee approved this study, and informed consent was obtained from all subjects. Three related patients with FCAS (L353P mutation) and five normal controls were included. The FCAS patients had a classical clinical presentation, met diagnostic criteria, and were being treated with Rilonacept, an IL-1β inhibitor approved by the Food and Drug Administration, but held their dose for >2 wk before the experiment.

### Preparation of human adherent monocytes
Venous blood was drawn into EDTA/Vacutainer tubes (BD) early in the morning when FCAS patients are generally least symptomatic. PBMC fractions were collected and resuspended in serum-free media and transferred to 24-well plates at a concentration of 1 × 10⁶/ml. Cells were incubated for 4 h at 37°C, and nonadherent cells were removed leaving adherent monocytes as previously described (Rosengren et al., 2007).

### Bacteria, ligands, and inhibitors
*S. enterica* serovar *typhimurium* strain SL1344 was provided by D. Monack (Stanford University, Palo Alto, CA). Single colonies were inoculated into 3 ml of brain-heart infusion medium and grown overnight at 30°C with shaking. Anthrax lethal factor and protective antigen (List Biological Laboratories) were used at 10 µg/ml. Ultrapure LPS, lipid A, lipoteichoic acid, peptidoglycan, and Pam3-CSK4 (InvivoGen) were used at 10 µg/ml. ATP (Roche) was used at 5 mM, and nigericin (Sigma-Aldrich) was used at a final concentration of 20 µM. Glyburide, glipizide, and calmidazolium were obtained from Sigma-Aldrich and used at the indicated concentrations. Compounds G1–G4 were purchased from Ryan Scientific. DOTAP was obtained from Roche and used according to the manufacturer's instructions. Silicia (Min-U-Sil 5) was provided by US Silica, and H-LL-OMe was purchased from Chem-Impex International. Infection and stimulation of BMDMs were performed as described in Figs. 1–5.

### Microscopy
Bright-field and fluorescence microscopy were performed in culture media at room temperature under a long-distance Plan Neofluar 20×/0.4 Ph2 Korr objective (Carl Zeiss, Inc.) on a microscope (Axiovert 200M; Carl Zeiss, Inc.) equipped with a charge-coupled device digital camera (Axiocam; Carl Zeiss, Inc.) and AxioVision software (Rel.4.6; Carl Zeiss, Inc.). For dye uptake assays, 2 µM YoPro-1 (Invitrogen) was present 10 min before macrophages were stimulated with ATP for 5 min. Fluorescence signals in digital black and white fluorographs were artificially colored green with

Photoshop (Adobe). Macrophage membrane damage was measured with a Live/Dead assay (Invitrogen).

### Cytokines, antibodies, and Western blotting
Human and mouse cytokines in culture supernatants were measured by enzyme-linked immunoabsorbent assay (R&D Systems) and Luminex assay. Data were analyzed with Student's *t* test, and $P < 0.05$ was considered statistically significant. The caspase-1 antibody used for Western blotting was raised against recombinant mouse caspase-1 and was used as described previously (Lamkanfi et al., 2007). In brief, immunoblots were incubated overnight with the caspase-1 antibody (1:1,000), and a goat anti–rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) was used to detect proteins by enhanced chemiluminescence (Thermo Fisher Scientific).

### In vitro caspase-1 activity assay
In vitro caspase-1 activity was determined by incubating 1 IU recombinant mouse caspase-1 (BioVision) with 50 mM fluorogenic caspase-1 substrate peptide Ac-WEHD-amc in 200 ml CFS buffer (10 mM Hepes, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM $KH_2PO_4$, 0.5 mM EGTA, 2 mM $MgCl_2$, 0.5 mM sodium pyruvate, 0.5 mM L-glutamine, and 10 mM DTT). The release of fluorescent 7-amino-4-methylcoumarin in the presence of the indicated concentrations of glyburide, glipizide, or the caspase-1 inhibitor Ac-YVAD-cmk was measured for 30 min at 1-min intervals by fluorometry (excitation at 360 nm and emission at 480 nm) on a Cytofluor (PerSeptive Biosystems), and the maximal rate of increase in fluorescence was calculated ($\Delta F$/min).

### ATPase assay
ATP hydrolysis was measured by visualizing the conversion of $\alpha$-[$^{32}$P]ATP to $\alpha$-[$^{32}$P]ADP using TLC. A total of 5 µl purified Cryopyrin-Flag was incubated with 0.1 µM $\alpha$-[$^{32}$P]ATP (3,000 Ci/mmol; PerkinElmer) in a total volume of 20 µl reaction buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, and EDTA-free Complete protease inhibitor cocktail tablets) containing 100 µM glyburide, compound G1, glipizide, or vehicle control (DMSO) for 2 h. The reaction was quenched by adding an equal volume of TLC development solvent (1 M formic acid and 0.5 M LiCl). A total of 2 µl reaction mixture was spotted on a polyethylene-imine cellulose TLC plate and developed with 1 M formic acid and 0.5 M LiCl in a TLC chamber. The TLC plate was exposed to x-ray film or used for quantification with a phosphoimager (Typhoon Trio; GE Healthcare).

### Online supplemental material
Fig. S1 shows that LPS+ATP-induced caspase-1 activation and macrophage death are glyburide sensitive and, unlike LPS+nigericin, require the P2X₇ receptor. Fig. S2 shows that LPS+H-LL-OMe– and LPS+silica-induced caspase-1 activation and IL-1β secretion require Cryopyrin and TLR4 but not the P2X₇ receptor. Fig. S3 shows inhibition of caspase-1 activation by DIDS, immunopurification of recombinant Cryopyrin-Flag, and IL-1β secretion from FCAS monocytes. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200903124/DC1.

phosphoimaging. Data represent the mean ± SD of triplicate samples from one out of three independent experiments. (F) Adherent monocytes from FCAS patients ($n$ = 3) were stimulated with 100 ng/ml LPS (4 h) and treated with 5 mM ATP (30 min) in the presence of vehicle controls or 100 µM compound G1 or glipizide before IL-1β levels in culture supernatants were determined. Data represent the mean ± SD of triplicate samples. (G) Adherent monocytes from FCAS patients ($n$ = 3) were cultured at 32°C for 12 h in the presence of vehicle controls or 100 µM compound G1 or glipizide. IL-1β levels were determined in culture supernatants. Data represent the mean ± SD of triplicate samples. (H) Protection against LPS-induced lethality in C57BL/6 mice ($n$ = 10) injected intraperitoneally bid daily with 500 mg/kg glyburide or formulation vehicle. Results are representative of two independent experiments.

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