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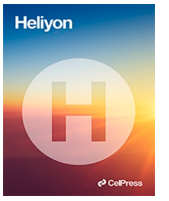

Research article

Differential effects of acute and chronic hydrocortisone treatment on pyroptosis

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ABSTRACT

Pyroptosis is a programmed and inflammation-inducing cell death that occurs predominantly in macrophages. It is characterized by the inflammasome-mediated activation of caspase-1, leading to cell lysis. During pyroptosis, pro-inflammatory mediators such as IL-1β are released extracellularly to further recruit and activate other immune cells. Thus, pyroptosis plays a crucial role in the prevention of the spread of pathogens. The clinically applied synthetic glucocorticoid, hydrocortisone (HC), has strong immunoregulatory properties. It may act as an immunosuppressive agent by negatively regulating pro-inflammatory gene transcription but has also shown immune-sensitizing properties. The conditions that determine the immunosuppressive or immune-sensitizing actions of HC during an infection are not fully clear. We hypothesized that the outcome may differ depending on the onset and duration of its administration. Therefore, we investigated the impact of acute (treatment upon infection) and chronic (24 h pre-treatment before infection) HC treatment on pyroptosis induction and execution in THP-1 macrophage-like cells. The focus was on pyroptosis-associated signaling pathways, inflammasome assembly and activation, IL-1β, and cell death. Physiological HC concentration and HC deprivation were used as controls. Compared to the physiological concentration, cells displayed augmented inflammasome activation and IL-1β release following acute HC treatment. Conversely, the whole pyroptosis machinery was suppressed by chronic HC administration.

These *in vitro* investigations demonstrate pro-inflammatory actions of acute HC exposure and the immunosuppressive effects of chronic treatment. These differential effects on pyroptosis emphasize the importance of individualized HC medication in patients upon infection, and suggest the inclusion of IL-1β as a marker for current immune capacities.

1. Introduction

Pyroptosis, a form of programmed inflammation-inducing cell death, plays a crucial role in preventing the spread of pathogens [1]. It was originally described as an inflammasome-mediated, caspase-1-dependent cell death that occurs in cells after *Salmonella typhimurium* infection or after treatment with *Bacillus anthracis* toxins [1,2]. Ultimately, pyroptosis involves pore formation on the cell membrane, leading to cell lysis and the release of pyrogenic cytokines such as IL-1β and IL-18, and *damage-associated molecular patterns*

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such as ATP, DNA, and HMGB1 [3] with further immune cell recruitment and activation [4]. This leads to direct elimination of pathogens [5] and rapid initiation of adaptive immunity [3]. While it is predominantly observed in macrophages to sense pathogens or to control pathogens from spreading [6,7], other immune cell types and epithelial cells are also capable of pyroptosis [8,9].

Pyroptosis can be induced by lipopolysaccharide (LPS) and ATP *in vitro*, followed by a multi-step process [10,11]. The first step involves a priming signal, when the binding of LPS to Toll-like receptors (TLRs), particularly TLR2 and TLR4 [12,13], induces downstream activation of the NF-κB signaling pathway, leading to translocation into the nucleus and transcription of pro-proteins such as pro-IL-1β, pro-IL-18, pro-caspase-1, and pro-gasdermin D, as well as inflammasome components such as NLRP3 and ASC [12,14]. The second step involves inflammasome activation through P2X7 receptor ligation with ATP, leading to ion channel opening and potassium efflux, which trigger the assembly and activation of the NLRP3 inflammasome [15]. The activated inflammasome then cleaves pro-form proteins into their mature forms (IL-1β, IL-18, gasdermin D) [16,17]. In the third step, pyroptosis is executed by gasdermin D oligomerization and translocation into the plasma membrane, leading to pore formation on the cell membrane and the consequent release of IL-1β and IL-18 into the extracellular space. Concurrent water influx results in cell swelling and cell membrane rupture [18].

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that is mainly released by macrophages during inflammation. Acting as an endogenous pyrogen, IL-1β induces fever, activates leukocytes, promotes migration, and triggers the release of other cytokines [19]. Pyroptosis and the associated release of IL-1β are essential for combating pathogenic infections and limiting their spread; however, they can also lead to severe acute and chronic inflammation. Thus, IL-1β plays a significant role in sepsis, inflammatory diseases, and autoimmune conditions such as rheumatoid arthritis and Type I diabetes mellitus [19].

Hydrocortisone (HC), a synthetic preparation of stress-induced adrenal glucocorticoid (GC) cortisol, is widely used in therapies to reduce inflammation in diseases such as asthma, rheumatoid arthritis, and systemic inflammatory response syndrome (SIRS) [20–24]. It exerts its anti-inflammatory activity by binding to GC receptors on immune cells with subsequent negative transcriptional regulation of proinflammatory genes encoding IL-1β, IL-6, TNF, ECAM-1, and ICAM-1 [25,26]. Additionally, GCs have been shown to inhibit NF-κB [27,28], p38-MAPK [27,29], and other signaling pathways associated with pro-inflammatory immune responses to suppress leukocyte chemotaxis, migration, differentiation, and activation [30,31].

However, in addition to their known anti-inflammatory effects, GCs also exert pro-inflammatory or immune-sensitizing properties. For instance, treatment of LPS/IFN-γ-activated macrophages with low-dose GC induces the expression of pro-inflammatory cytokines like IL-1β, IL-6, IL-12, CXCL1, CXCL10, and TNF [32]. In a transcriptional expression study in peripheral blood mononuclear cells (PBMCs), low-dose GCs promoted the expression of TLR2, TLR4, NLRP3, and the purine receptor PY2R2, which in turn activated downstream pro-inflammatory cytokines such as IL-1β, IL-6, and TNF [25,33–35]. The precise mechanism underlying the (up- or down-) regulation of pro-inflammatory cytokines remains unclear.

Although HC is widely used as a therapeutic agent against inflammatory diseases, its effects are yet to be distinguished between acute use for inflammation caused by infection and chronically high HC concentrations, such as in highly stressed individuals or patients under continuous immunosuppressive treatment.

In this study, we investigated whether acute HC administration or chronically high HC doses exert different effects on pyroptosis. Using an *in vitro* approach, we examined the association between the different steps of pyroptosis induction and execution in THP-1 macrophage-like cells. These findings may add to our understanding of the specific effects of HC on macrophages during inflammation.

2. Results

2.1. Pyroptosis-mediated cell lysis is alleviated by chronically high hydrocortisone exposure while acute concentration increase has no effect

To investigate the impact of HC exposure on pyroptosis-mediated cell lysis, cell culture supernatants were measured for lactate

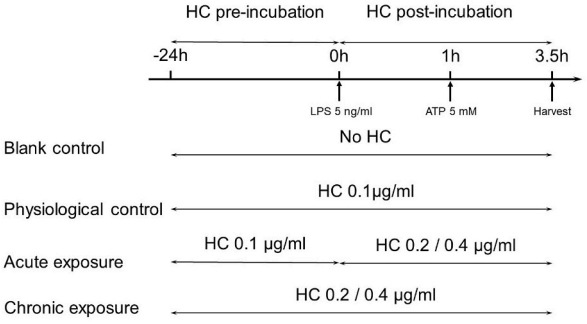

Fig. 1. Treatment scheme of hydrocortisone (HC) and LPS/ATP-induced pyroptosis. THP-1 macrophage-like cells were continuously incubated with 0.1 µg/ml HC to mimic physiological levels or HC was increased in the acute approach to 0.2 or 0.4 µg/ml HC upon pyroptosis induction. For chronically increased HC exposure, cells were continuously incubated with 0.2 or 0.4 µg/ml HC. No HC was used as blank control. Pre-incubation time with HC was 24 h. Pyroptosis was induced by 1 h LPS (5 ng/ml) priming followed by an additional incubation with ATP (5 mM) for further 2.5 h.

dehydrogenase (LDH) release and cells were analyzed for propidium iodide (PI) intercalation. Both assays provided insights into the cell membrane integrity.

In comparison to the blank control lacking HC, the physiological control supplemented with 0.1 μg/ml HC showed decreased LDH release after LPS/ATP treatment and a reduced proportion of PI-stained cells, indicating that physiological HC concentrations already exert protective effects against cell death. Notably, both LDH release and PI incorporation remained unaffected by acutely increased HC concentrations (Fig. 2 A & B - Acute exposure), compared to the physiological control. However, under chronic exposure conditions, high HC concentrations resulted in a further decrease in LDH release and proportion of PI-positive cells (Fig. 2 A & B - Chronic exposure). This indicates that high HC concentrations protect cells from lysis when applied before pyroptosis induction. Acute treatment with high HC doses has no protective effect when pyroptosis was already initiated.

2.2. *Pyroptosis-mediated release of IL-1β is promoted under acute hydrocortisone exposure, while chronic administration suppresses IL-1β release*

We determined IL-1β mRNA levels by RT-qPCR to assess the impact of HC in different doses and exposure patterns on its expression. Intracellular pro-IL-1β and cleaved IL-1β levels were quantified by western blotting, and mature IL-1β release was measured in cell culture supernatants using ELISA.

IL-1β mRNA levels were reduced in comparison to the blank control; however, this was not statistically significant. This decrease was independent of the different HC concentrations and treatment sequences (Fig. 3 A), indicating that HC had no immediate genomic effect on IL-1β production within the studied timeframe.

Cleavage of IL-1β, as detected by western blotting, revealed a strongly decreased ratio of cleaved and pro-IL-1β under acute HC exposure, demonstrating low intracellular cleaved IL-1β levels compared to the pro-form (Fig. 3 B). When pyroptosis was induced under chronic HC exposure, the cleaved/pro-IL-1β ratio also decreased in a dose-dependent manner, but to a lesser extent than under acute HC treatment (Fig. 3 B). Compared to the blank control, the physiological control exhibited a significant reduction in this ratio, suggesting that chronic exposure to this low HC dose suppresses IL-1β maturation.

The release of mature IL-1β into the extracellular space, as determined by ELISA, increased in a dose-dependent manner in the acute HC approach, reaching nearly blank control values at 0.4 μg/ml (Fig. 3C). Conversely, chronic HC exposure upon pyroptosis induction resulted in a decrease in the IL-1β concentration (Fig. 3C - Chronic exposure), indicating suppression of IL-1β release due to the anti-inflammatory effects of HC on macrophages. This effect was also observed in the physiological control group compared to the blank control.

2.3. *Acute exposure of hydrocortisone does not affect pyroptosis protein expression while chronic exposure induces down-regulation*

Inflammasomes serve as the central components that activate pyroptotic cell death. In *in vitro* induced pyroptosis by LPS and ATP, the proteins NLRP3, ASC, and caspase-1 assemble to the NLRP3 inflammasome, which cleaves –among others-the proteins IL-1β and gasdermin D. Western blot analysis of NLRP3, gasdermin D (gasdermin-D-NT when cleaved) and activated caspase-1 revealed that the expression of these three proteins remained unaffected compared to the physiological control when HC was administered acutely, (Fig. 4A–D). However, following chronic HC exposure, the expression of these three proteins was dose-dependently decreased (Fig. 4A–D).

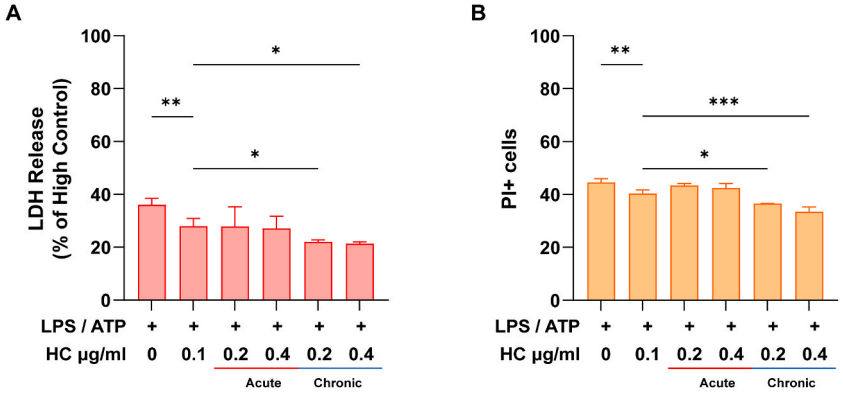

Fig. 2. Pyroptosis-mediated cell lysis under different HC treatment scenarios of THP-1 macrophage-like cells. Blank control with no HC; physiological control with continuous HC concentration of 0.1 μg/ml; acute exposure with HC increase from 0.1 μg/ml to 0.2 or 0.4 μg/ml upon pyroptosis induction; chronic exposure with continuous HC concentrations of 0.2 or 0.4 μg/ml (A) LDH release proportion in relation to the high control (whole cell lysate). (B) Percentage of PI-positive cells within all measured cells. N = 5, *P < 0.05, **P < 0.01, ***P < 0.005.

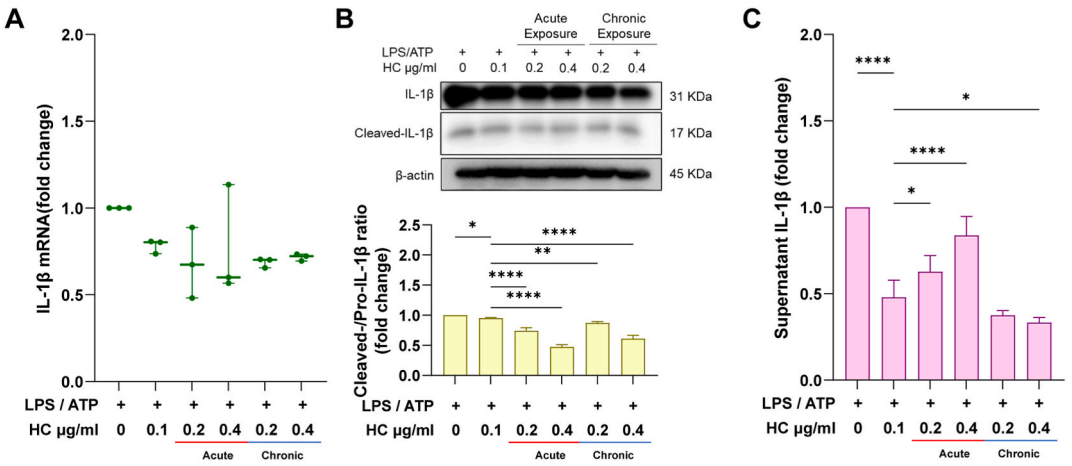

Fig. 3. Impact of different HC treatment scenarios on pyroptosis-mediated IL-1β production and release. Blank control with no HC; physiological control with continuous HC concentration of 0.1 μg/ml; acute exposure with HC increase from 0.1 μg/ml to 0.2 or 0.4 μg/ml upon pyroptosis induction; chronic exposure with continuous HC concentrations of 0.2 or 0.4 μg/ml (A) IL-1β mRNA fold change to blank control, (B) Representative western blot of cleaved and mature IL-1β and semiquantitative analysis, fold change to blank control, (C) Levels of released IL-1β in the cell culture medium, fold change increase to blank control. N = 3, *P < 0.05, **P < 0.01, ****P < 0.001.

2.4. Acute exposure to hydrocortisone promotes pyroptosis while chronic hydrocortisone alleviates pyroptosis

Upon NLRP3 inflammasome assembly, caspase-1 is cleaved and activated, and its presence in cell culture supernatants serves as an evidence for caspase-1 activation. Consistent with the extracellular IL-1β levels, caspase-1 concentration decreased in a dose-dependent manner following chronic HC exposure (Fig. 4 E). Furthermore, the physiological control also exhibited lower caspase-1 concentrations compared to the blank control. The inhibitory effect of the physiological HC concentrations was reversed by acute HC exposure (Fig. 4 E).

To detect pyroptosis-related protein expression and to determine the quantity of pyroptotic cells, flow cytometry was used to measure caspase-1 and PI double-positive cells, which are indicative of pyroptotic cells. Approximately 30 % of blank control cells underwent pyroptosis after LPS/ATP treatment, which displayed a significant reduction under physiological concentrations. Under acutely high HC concentrations, the values were comparable to those of the blank control and higher than in the physiological control (Fig. 4 F). Conversely, under escalating chronic HC exposures, the percentages of caspase-1/PI double-positive cells were significantly reduced compared to those in the blank and physiological controls (Fig. 4 F). As a result, applying doses of high HC at the onset of pyroptosis negates the protective effect of physiological (0.1 μg/ml) HC on this kind of cell death in macrophages, whereas it is further reduced by chronically elevated HC concentrations.

2.5. Acute exposure of hydrocortisone does not impact NLRP3 inflammasome assembly while chronic exposure inhibited NLRP3 inflammasome formation

To evaluate the impact of different HC treatment regimens on NLRP3 inflammasome assembly in more detail, we conducted co-immunoprecipitation (Co-IP) experiments of NLRP3 with ASC. ASC precipitation was significantly lower in the physiological control group than in the blank control group. When pyroptosis was induced under acute HC exposure, the ASC signal remained unchanged and was comparable to that of the physiological control (Fig. 5 A - IP lane 2, 3, 4, and B). Fewer ASC was co-precipitated with NLRP3 upon chronic HC exposure (Fig. 5 A - IP lane 2, 5, 6, and B). Immunocytochemistry provided similar results, demonstrating that under acute HC exposure, the extent of inflammasome formation did not change when compared with the physiological control (Fig. 5C - ②③④). In contrast, chronically high HC exposure significantly reduced the inflammasome formation (Fig. 5C - ②⑤⑥).

2.6. Acute exposure to hydrocortisone did not affect NF-κB and p38-MAPK signaling while chronic exposure reduced their activation

The NF-κB and p38-MAPK signaling pathways play critical roles in inflammation by inducing the expression of pro-inflammatory cytokines, such as IL-1β [36,37]. Here, we investigated the effects of both acute and chronic HC exposure on the activation of these signaling pathways in pyroptosis by assessing the phosphorylation state of the NF-κB subunit p65 and p38 by western blot analysis.

Acute treatment with HC didn't affect phosphorylation of p65 or p38 significantly. However, phosphorylation levels were dose-dependently suppressed by chronic HC exposure, which indicates diminished activation of the NF-κB and p38-MAPK signaling pathways (Fig. 6 A - C Chronic exposure). Additionally, we analyzed the nuclear translocation of NF-κB p65 phosphorylation using immunocytochemical staining. By confocal microscopy we could show, that acutely administered HC had no effect on the nuclear translocation of the NF-κB p65 subunit compared with the controls (Suppl. Fig. 1), which corresponds to the unaffected

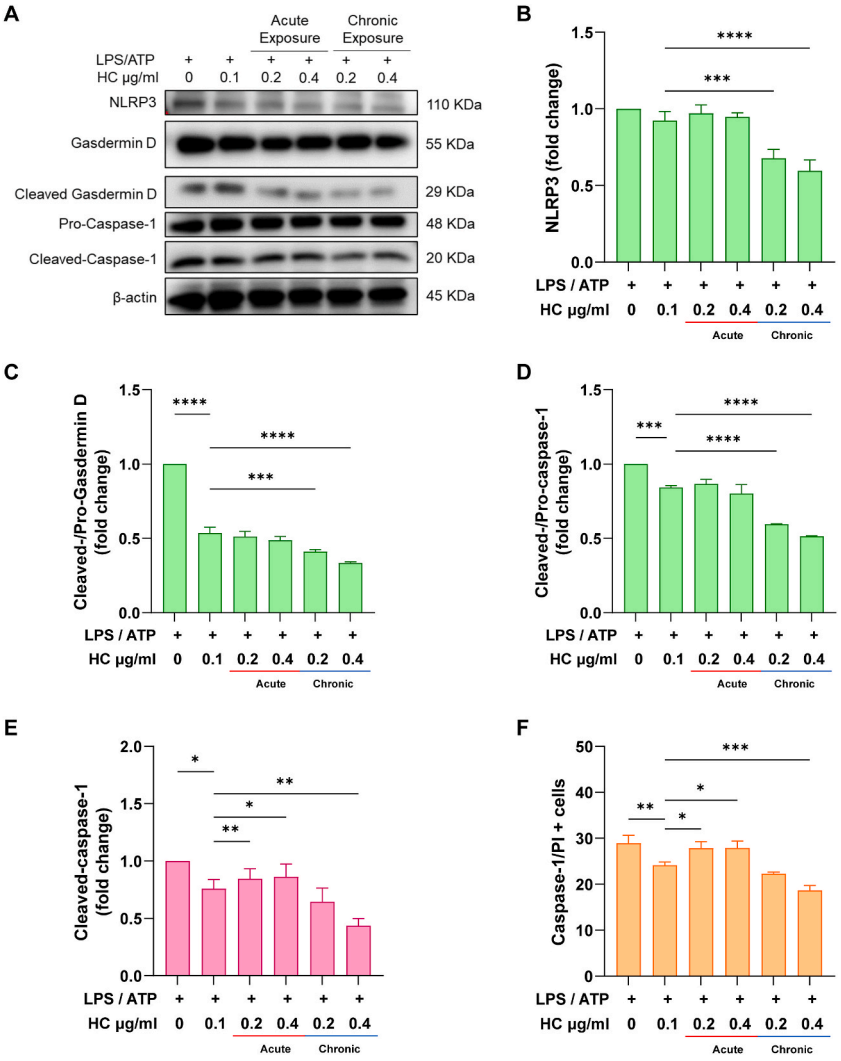

Fig. 4. Expression of pyroptosis-related proteins and extent of pyroptosis under different HC treatment scenarios. Blank control with no HC; physiological control with continuous HC concentration of 0.1 µg/ml; acute exposure with HC increase from 0.1 µg/ml to 0.2 or 0.4 µg/ml upon pyroptosis induction; chronic exposure with continuous HC concentrations of 0.2 or 0.4 µg/ml. (A) Representative western blot and semi-quantitative analysis of (B) NLRP3, (C) cleaved/pro-caspase-1 ratio, (D) cleaved/pro-gasdermin D ratio; fold change compared to blank control, (E) Caspase-1 levels in cell culture supernatants, fold change to blank control, (F) Percentage of caspase-1/PI double-positive cells among all measured cells. N = 3, *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001.

phosphorylation of p65 in western blot. However, chronic HC exposure resulted in reduced p65 translocation, which was again dependent of the applied dose (Suppl. Fig. 1). The results regarding these two signaling pathways indicate that exposure to HC in high doses lowers the activation of NF-κB signaling, but only under chronic and not under acute conditions.

To explore other underlying mechanisms beyond the NF-κB and p38-MAPK signaling pathways, we used phosphokinase protein arrays to detect proteins involved in various biological functions (Suppl. Fig. 2). In these analyses, p53 phosphorylation at Ser46 was augmented in the acute approach and reduced in chronic HC (Fig. 6 D), which resembled the phosphorylation pattern of NF-κB p65 and p38.

3. Discussion

In this *in vitro* study, we aimed to investigate the effect of the physiological glucocorticoid (GC) hydrocortisone (HC) on the induction and execution of pyroptosis by focusing on distinct acute and chronic stress exposure scenarios in THP-1 macrophage-like cells. For this, pyroptosis was induced in these cells with additional treatment regimens of HC, which should mirror either physiological cortisol levels (0.1 µg/ml), conditions of an acute moderate (0.2 µg/ml) or severe (0.4 µg/ml) HC increase as it is the case for medication after infection [38], chronically elevated HC levels as for stressed or immunosuppressed patients [38] or with no HC at all

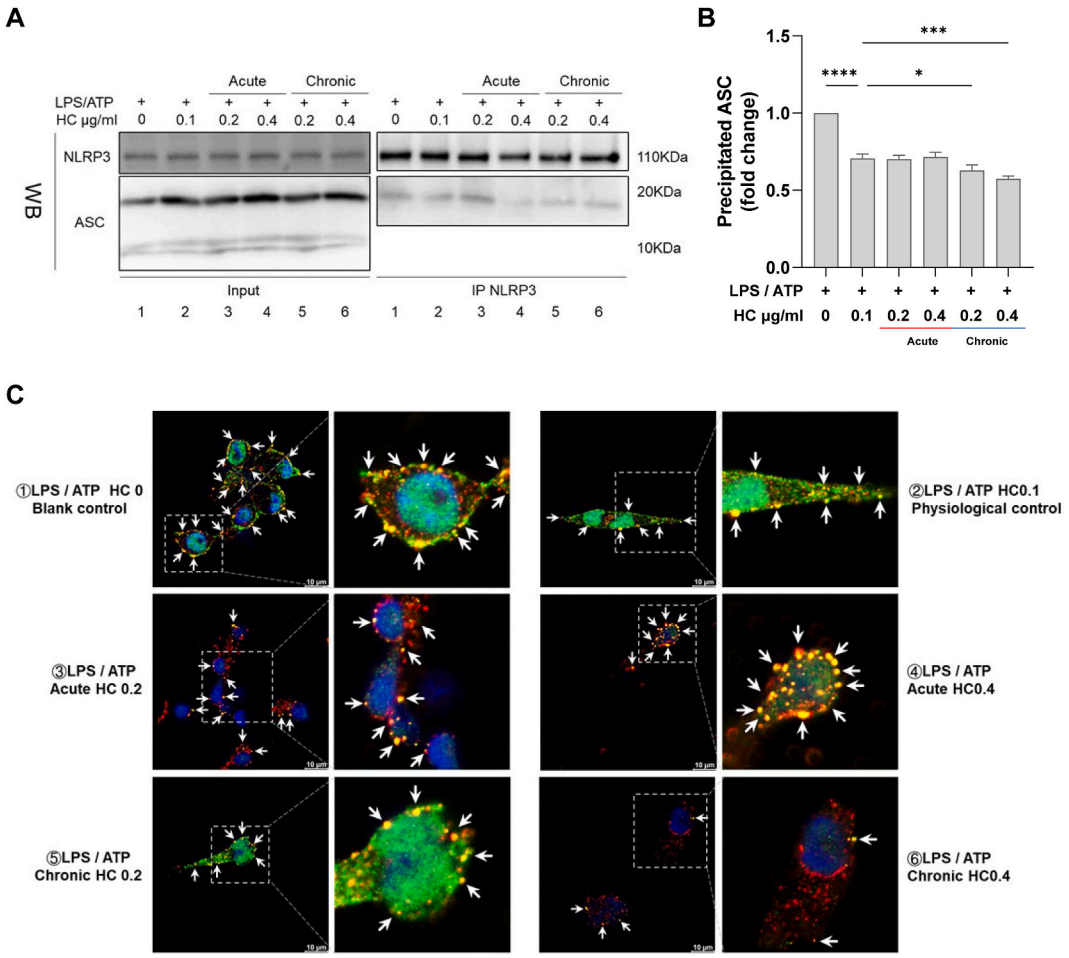

Fig. 5. Inflammasome formation under different HC treatment scenarios. Blank control with no HC; physiological control with continuous HC concentration of 0.1 μg/ml; acute exposure with HC increase from 0.1 μg/ml to 0.2 or 0.4 μg/ml upon pyroptosis induction; chronic exposure with continuous HC concentrations of 0.2 or 0.4 μg/ml. (A) Representative co-immunoprecipitation blot and (B) semiquantitative analysis of precipitated ASC by NLRP3 expressed as fold change to blank control. (C) Representative immunocytochemical stainings of NLRP3 (red) and ASC (green), arrows indicate co-localization of NLRP3 and ASC, which are regarded as inflammasome (yellow). N = 3, *P < 0.05, ***P < 0.005, ****P < 0.001, scale bar 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

like in Addison's disease [39].

The latter revealed that, in contrast to physiological HC concentrations where protein expression, inflammasome assembly, and release of IL-1β were remarkably reduced, an entire lack of HC availability results in only weak or low regulation of pyroptosis. Consequently, patients with adrenal gland insufficiency might be at a higher risk for inappropriately high cytokine secretion, such as IL-1β, upon bacterial infection.

From the perspective of designing cell biological experiments, the addition of HC to an experimental setup has been shown to have the potential to strongly influence the outcome. In particular, when investigating immune system-related questions, the effects of physiological HC concentrations should not be neglected. In an allergy model, HC favors Th2 responses and inhibits Th1 responses when treatment occurs during dendritic cell-induced T cell activation, leading to pro-allergic responses [40]. Another study demonstrated that supplementing the culture medium of THP-1 macrophage-like cells with HC resulted in the induction of CD163, promoting an M2-like phenotype, regardless of LPS stimulation [41]. Conversely, in the absence of cortisol, polarization towards an M1-like macrophage phenotype occurs [41]. In the present study, we have shown that under physiological HC concentrations, the extent of pyroptosis was much weaker than under maximally artificial conditions lacking HC. Thus, the immune response of THP-1 macrophage-like cells under HC-deficient conditions may be overestimated or inaccurate. Altogether, these few examples clarify that ignoring the effects of HC in cell culture models could lead to incomparable results between different studies, especially in *in vitro* and subsequent *in vivo* investigations.

An acute increase of HC from physiological (0.1 μg/ml) to moderate (0.2 μg/ml) or high (0.4 μg/ml) concentrations resulted in an increased maturation and release of IL-1β and caspase-1 into the extracellular space, which is a common indicator for pyroptosis [42,43]. However, in comparison to the physiological control, NF-κB signaling, inflammasome assembly, and cell death levels were not

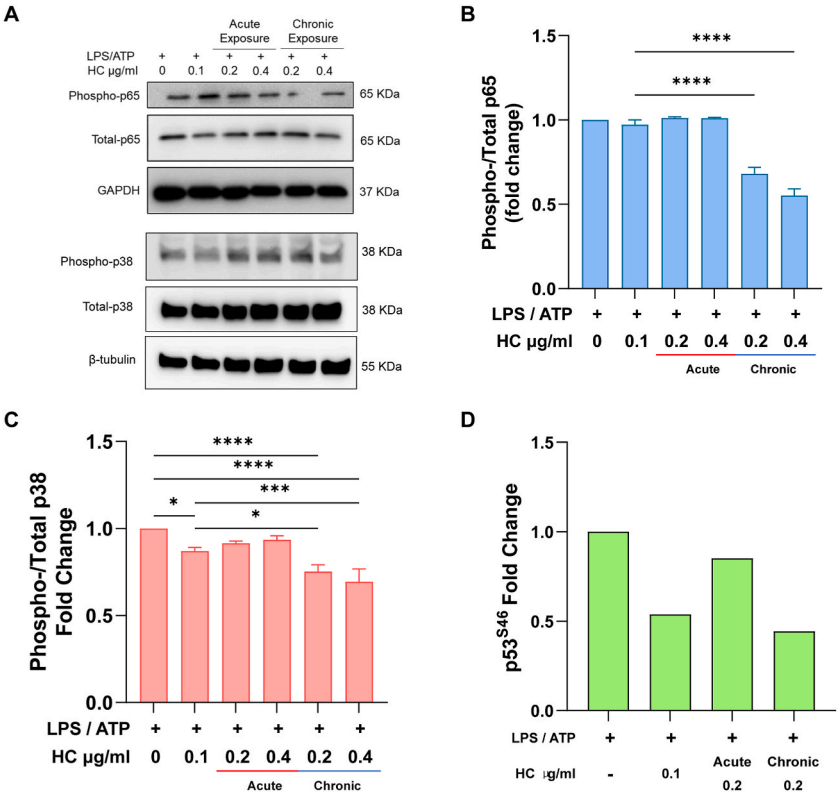

Fig. 6. Pyroptosis-associated cell stress signaling under different HC treatment scenarios. Blank control with no HC; physiological control with continuous HC concentration of 0.1 µg/ml; acute exposure with HC increase from 0.1 µg/ml to 0.2 or 0.4 µg/ml upon pyroptosis induction; chronic exposure with continuous HC concentrations of 0.2 or 0.4 µg/ml. (A) Representative western blot of (phosphorylated) p65 and p38 and semi-quantitative analysis of (B) phospho/pan-p65 ratio and (C) phospho/pan-p38 ratio; fold change compared to blank control. (D) $p53^{S46}$ phosphorylation derived from phospho-kinase array. Western blot was performed in N = 3, *P < 0.05, ***P < 0.005, ****P < 0.001. Phospho-kinase array was performed in N = 2.

increased, suggesting a hyperactive state of some of these cells, which could have been induced by DAMPs that were secreted from other dying cells around [42,44] or by the sudden increase of HC itself [45]. Hyperactive macrophages are capable of releasing IL-1β through gasdermin D pores without dying, which corresponds to the results observed in the present study.

The HC-mediated dose-dependent increase of IL-1β in the extracellular space might also be explained by the dual immune-modulating effects of HC. It was reported that HC has both anti- and pro-inflammatory attributes [46,47], which have been shown to be associated with stress mode (acute or chronic) [46]. Thus, acute stress, which is associated with suddenly increasing cortisol levels [48], can induce proinflammatory cytokines and signaling pathways [46]. The mechanisms underlying these inflammation-enhancing effects are yet to be fully elucidated. However, sociological experiments revealed doubling of IL-1β levels in blood immediately after the stressful situation has begun [49]. Similarly, investigations of blood parameters in boxers after boxing matches demonstrated a more than doubling of cortisol and IL-1β levels compared to pre-match [50]. At the molecular level, glucocorticoid receptors, which are activated in response to acute stress, can potentially induce the NF-κB, p38-MAPK, or STAT signaling pathways, which in turn results in TLR and IL-1β upregulation [35,51]. In the present study, an increase in activation of these signaling pathways was also observed, which further supports the role of GCs in mediating enhanced inflammatory responses and pathogen clearance [25].

When HC was administered chronically to the cell culture medium 24 h before LPS/ATP treatment, hereby mimicking stressful conditions or HC therapy prior to infection, pyroptosis induction and execution were effectively and dose-dependently inhibited in THP-1 macrophage-like cells. As a result, extracellular IL-1β levels and cell death proportions were remarkably reduced, which we expect to negatively influence subsequent immune cell recruitment and activation, as well as pathogen clearance processes [3–5]. This also brings IL-1β into the focus. It is unique among secreted proteins as it lacks a signal peptide that guides secretion via the endoplasmic reticulum/Golgi complex pathway. Instead, IL-1β is mainly secreted via a mechanism involving its cleavage, maturation, and release via a pore formed on the cell membrane [52]. IL-1β is cleaved by the NLRP3 inflammasome-mediated activation of caspase-1 [53,54]. Chronic exposure to THP-1 macrophage-like cells resulted in a dose-dependent reduction of NLRP3 production and inflammasome assembly, which subsequently diminished activation of caspase-1, cleavage of gasdermin D, release of IL-1β, and death of the affected cell. Notably, IL-1β mRNA levels were not changed by HC, suggesting that inhibitory effects downstream of NF-κB signaling might be involved in this cascade, such as the inhibition of NLRP3 [55–57] or the reduction of caspase-1 activation, as

observed for other GCs [58].

We have shown that chronic exposure to HC leads to a dose-dependent suppression of the phosphorylation state of p38 and p65, and consequently its translocation into the nucleus. Conversely, within an acutely high HC increase, p38 and NF-κB p65 subunit phosphorylation were, in comparison to the physiological control, tendentially increased. Interestingly, the activation of p53 followed a comparable regulation pattern. The tumor suppressor p53 is strongly involved in DNA repair and apoptosis [59]. In macrophages, p53 has also been shown to regulate inflammasome formation and induce an inflammatory response [60].

Taken together, these observations further support the occurrence of pro-inflammatory actions following acute HC exposure. Thus, our results suggest a complex interplay between HC exposure and the signaling pathways involved in pro-inflammatory responses. The differential effects of acute and chronic HC exposure on p38 and NF-κB p65 subunit phosphorylation, subsequent pyroptosis induction, execution, and IL-1β release highlight the importance of considering the duration and dosage of HC administration in patients. In this context, IL-1β may be considered as a valuable marker for individual HC medication upon bacterial infection. In highly stressed or immunosuppressed patients, where IL-1β levels are expected to be low, additional treatment with HC might further impede the patients' endogenous defense. In an acute scenario, where IL-1β levels are already increased owing to inflammasome activation by the pathogen and DAMPs from surrounding cells, treatment with HC could further elevate IL-1β levels, thereby inducing excessive and potentially harmful inflammation.

In conclusion, this investigation unravels the role of HC's dual impact on pyroptosis, demonstrating its role as both an enhancer and a suppressor of inflammation. These insights not only expand our fundamental understanding of immune modulation but also hold immense promise for future therapeutic interventions in infection.

4. Methods

4.1. Cell culture and THP-1 cell differentiation

The human monocyte-like cell line THP-1 (TIB-202, ATCC, Manassas, VA, USA) was cultured in RPMI-1640 medium (21875-034, Thermo Fisher, Waltham, MA, USA) supplemented with 10 % (v/v) Fetal Bovine Serum (P30-3306, Pan Biotech, Aidenbach, Germany), Penicillin (100 U/ml) and Streptomycin (100 μg/ml) (P06-07050, Pan Biotech, Aidenbach, Germany). Differentiation of THP-1 cells into macrophage-like cells was achieved by 50 ng/ml phorbal 12-myristate 13-acetate (PMA, P1585, Sigma-Aldrich, Steinheim, Germany) for 48 h in complete cell culture medium.

4.2. Hydrocortisone treatment and pyroptosis induction

After differentiation, adherent THP-1 macrophage-like cells were washed twice with PBS and pre-incubated with hydrocortisone (HC) (Pfizer, New York, NY, USA) for 24 h. Concentrations were set to 0.1 μg/ml, which resembles physiological HC concentrations [38]. To mimic moderate or high HC concentrations, such as in stressed patients or under immunosuppression, concentrations were set to 0.2 μg/ml or 0.4 μg/ml [38]. On the following day, HC treatment was continued either as an acute increase in concentration (from 0.1 μg/ml to 0.2 or 0.4 μg/ml) or in continuously increased concentrations (chronic; 0.2 or 0.4 μg/ml). No or low (0.1 μg/ml) HC served as blank or physiological controls, respectively (Fig. 1). In addition, 5 ng/ml Lipopolysaccharide (LPS) (L2654, Sigma-Aldrich, Steinheim, Germany) was added to cell culture RPMI-1640 medium (without phenolred) (11835-063, Thermo Fisher, Waltham, MA, USA) to prime the cells for pyroptosis induction. After 1 h LPS priming 5 mM ATP (A6419, Sigma-Aldrich, Steinheim, Germany) was added to the cells for further 2.5 h to induce pyroptosis.

4.3. Lactate dehydrogenase (LDH) assay and ELISA

After treatment, cell culture supernatants were collected and centrifuged at 300×*g* for 5 min. Cleared supernatants were transferred to a 96-well plate for CytoTox 96® Non-Radioactive Cytotoxicity Assay (G1780, Promega, Madison, WI, USA) to determine lactate dehydrogenase (LDH) release according to manufacturer's protocol. Concentrations of IL-1β and caspase-1 in supernatants were measured using IL-1 beta Human ELISA Kit (KAC1211, Thermo Fisher, Waltham, MA, USA) or Human Caspase-1 ELISA Kit (EH70RB, Thermo Fisher, Waltham, MA, USA) according to the manufacturer's protocols. Measurements of optical density were performed on a microplate reader (Molecular Devices, San Jose, CA, USA) at 450 nm (ELISAs) or 492 nm (LDH assay).

4.4. Western blot analysis, co-immunoprecipitation and protein array

After stimulation, cells were washed twice with cold PBS, and directly lysed with Cell Lysis Buffer (9803, Cell Signaling Technology, Essex County, MA, USA) on ice. Protein concentration was determined by BCA assay (23325, Thermo Fisher, Waltham, MA, USA). For western blot, 20 μg of protein lysates were boiled at 95 °C for 5 min in Laemmli buffer. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blots were incubated overnight at 4 °C with antibodies against gasdermin D (96458), cleaved-caspase-1 (4199), caspase-1 (3866), cleaved-IL-1β (83186), IL-1β (12703), NLRP3 (15101), NF-κB p65 (8242), phosphorylated-NF-κB $p65^{S562}$ (3033), p38 (9212), phosphorylated-$p38^{T180/Y182}$ (9211); for loading control antibodies against GAPDH (5174), β-actin (4967) and β-tubulin (2146) were used. HRP-anti-rabbit (7074) was used as secondary antibody. Incubation was performed for 1 h at room temperature. All antibodies were obtained from Cell Signaling Technology (Essex County, MA, USA). Membranes were developed with ECL detection reagent (RPN2232, GE Healthcare, Chicago, IL, USA) and visualized under a ChemiDoc Imaging System

(Biorad, Hercules, CA, USA).

For Co-immunoprecipitation, mouse-anti-NLRP3 antibodies (AG-20B-0014, AdipoGen, San Diego, CA, USA) were added to 100 μg protein lysate and incubated overnight at 4 °C, followed by 3 h co-incubation with Protein-A agarose beads (9863, Cell Signaling Technology, Essex County, MA, USA). Beads were washed five times with cell lysis buffer and boiled for 5 min at 95 °C with Laemmli buffer. Samples for Co-immunoprecipitation were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were then incubated overnight at 4 °C with anti-ASC- (13833) and anti-NLRP3 (15101) antibodies (both from Cell Signaling Technology, Essex County, MA, USA), followed by HRP-anti-rabbit secondary antibody incubation. Blots were developed with ECL detection reagent and visualized under ChemiDoc Imaging System.

For protein array, 100 μg of protein lysate was incubated on each membrane of the Proteome Profiler Human Phospho-Kinase Array Kit (ARY003C, R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Protein array membranes were then developed with ECL detection reagent and visualized under ChemiDoc Imaging System.

Semiquantitative analyses were done by ImageJ (NIH, Bethesda, MD, USA). For protein arrays, heatmap was generated with pheatmap package under R software (4.2.2). Full blots and protein array can be found in Supplementary Files.

4.5. Flow-cytometry for pyroptosis detection

Treated cells were harvested by accutase detachment. For pyrotosis detection, FAM-FLCA Pyroptosis Detection Kit, containing caspase-1 detection reagents and propidium iodide (PI) (SKU97, Immunochemistry, Davis, CA, USA), was used according to the manufacturers protocol and measured by flow cytometry recording 10,000 events (FASCan Flow-cytometer, Beckton Dickson, Franklin Lakes, NJ, USA). PI-positive cells were regarded as dead cells and FAM/PI double-positive cells were regarded as pyroptotic.

4.6. Immunostaining and fluorescence microscopy

Cells were grown on cover slips and treated as indicated in Fig. 1. Afterwards, cells were fixed with 2 % paraformaldehyde and permeabilized with 0.1 % Trion X-100. Samples were stained for 1 h at room temperature with rabbit-anti-NF-κB p65 (8242, Cell Signaling Technology, Essex County, MA, USA) for p65 translocation or rabbit-anti-ASC (13833 Cell Signaling Technology, Essex County, MA, USA) and mouse-anti-NLRP3 (AG-20B-0014, AdipoGen, San Diego, CA, USA) for inflammasome visualization. Afterwards, samples were incubated with anti-rabbit-FITC- (F2765) or anti-mouse-AlexaFluor546- (A11030) conjugated secondary antibodies (both from Thermo Fisher, Waltham, MA, USA) for 1 h at room temperature. Nuclei were stained with 0.2 mg/ml DAPI (D9542, Sigma-Aldrich, Steinheim, Germany). Coverslips were mounted on slides with Vectashield (H-1000, Vector Laboratories, Burlingame, CA, USA). Fluorescence images were acquired with a Leica TCS SP5 II laser confocal scanning microscope system (Leica, Wetzlar, Germany).

4.7. Quantitative reverse transcriptase PCR for IL-1β

RNA was isolated with the Nucleospin RNA kit (740955, MACHEREY-NAGEL, Düren, Germany) according to manufacturer's protocol, and reversely transcribed to cDNA using Maxima™ H Minus cDNA SynthesisMaster Mix (M1661, Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions. Real-Time PCR was done with PowerUP SYBR® Green PCR Master Mix (A25741, Thermo Fisher, Waltham, MA, USA), amplification temperature program was 95 °C for 15 s, followed by 62 °C (IL-1β) or 64 °C (18S rRNA) annealing and extension for 1 min for 40 cycles. Data was analyzed with ΔΔCT (cycle threshold) method, and normalized to the endogenous reference gene 18S rRNA. Primer sequences for IL-1β were: Forward 5′-TTACAGTGGCAATGAGGATGAC-3′, Reverse 5′-GTCGGAGATTCGTAGCTGGAT-3'; For 18S rRNA: Forward 5′-GGACAGGATTGACAGATTGATAG-3′, Reverse 5′-CTCGTTCGTTATCGGAATTAAC-3'.

4.8. Statistical analysis

All data were presented as mean ± standard deviation (SD) and analyzed by one-way ANOVA with Tukey's multiple comparison post hoc test unless indicated otherwise using Graphpad Prism 9.0 software (GraphPad, La Jolla, CA, USA). Differences with $P < 0.05$ were considered statistically significant. Every experiment was performed three times or as indicated.

Data availability statement

Supplementary information accompanies this manuscript and is attached as single files. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Bing Han: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alexander Choukér:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Dominique Moser:** Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Bing Han reports financial support was provided by China Scholarship Council. Alexander Chouker reports financial support was provided by Federal Ministry for Economic Affairs and Climate Action. Dominique Moser reports financial support was provided by Federal Ministry for Economic Affairs and Climate Action. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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