Anti-inflammatory Effects of Novel P2X4 Receptor Antagonists, NC-2600 and NP-1815-PX, in a Murine Model of Colitis

Vanessa D’Antongiovanni¹, Carolina Pellegrini¹, Laura Benvenuti¹, Matteo Fornai¹,6,7, Clelia Di Salvo¹, Gianfranco Natale², Larisa Ryskalin², Lorenzo Bolognani², Elena Lucarini³, Lorenzo Di Cesare Mannelli³, Carla Ghelardini³, Zoltan H. Nemeth⁴,5, György Haskó⁵ and Luca Antonioli¹

Received 23 December 2021; accepted 14 March 2022

Abstract—The pharmacological blockade of P2X4 receptors has shown potential benefits in the management of several immune/inflammatory diseases. However, data regarding the involvement of P2X4 receptors in the pathophysiology and mechanisms of action in intestinal inflammation are not well defined. We aimed to evaluate the anti-inflammatory effects of two novel and selective P2X4 receptor antagonists, NC-2600 and NP-1815-PX, and characterize the molecular mechanisms of their action in a murine model of 2,4-dinitrobenzene sulfonic acid (DNBS)-induced colitis. The two drugs and dexamethasone (DEX) were administered orally for 6 days, immediately after the manifestation of DNBS. The body weight decrease, resulting from colitis, was attenuated by NC-2600 and NP-1815-PX, but not DEX. However, all three drugs attenuated the increase in spleen weight and ameliorated macroscopic and microscopic colonic tissue damage. Furthermore, all three compounds decreased tissue IL-1β levels and caspase-1 expression and activity. Colonic tissue increase of tumor necrosis factor was downregulated by DEX, while both NC-2600 and NP-1815-PX were ineffective. The reduction of occludin associated with colitis was ameliorated by NC-2600 and NP-1815-PX, but not DEX. In THP-1 cells, lipopolysaccharide and ATP upregulated IL-1β release and NLRP3, caspase-1, caspase-5, and caspase-8 activity, but not of caspase-4. These changes...
INTRODUCTION

Inflammatory bowel diseases (IBDs), comprising ulcerative colitis (UC), and Crohn’s disease (CD) are chronic intestinal inflammatory disorders, characterized by massive inflammation of various parts of the digestive tract [1]. In UC, the inflammatory process is limited to the intestinal mucosa and submucosa in a continuous pattern, while in CD, the tissue inflammation is generally transmural and often discontinuous [1]. Clinically, UC and CD share similar symptoms, including diarrhea, weight loss, rectal bleeding, and abdominal pain, which may be debilitating and lead to life-threatening complications [1].

It is widely recognized that in genetically susceptible hosts, pathologic immune responses with influenced interactions with luminal microbes, compromise the intestinal epithelial barrier, triggering the development of chronic intestinal inflammation [2].

With the ever increasing incidence of IBD, a large body of evidence has highlighted the often insufficient efficacy and/or safety of traditional drugs (including corticosteroids, immunosuppressants, and salicylates [2–4]. Likewise, more recent biotechnological drugs including anti-TNF alpha and other biologic treatments can prove ineffective or over the years of administration often loose effectiveness in many IBD patients [2–4]. It has become clear that the currently available treatment options for IBD are far from being truly satisfactory, spurring the interest of several pharmaceutical companies to find novel molecular targets for clinical treatments with even better benefit/risk profiles [5, 6].

A number of evidences revealed the critical involvement of extracellular adenosine triphosphate (ATP) in modulating immune responses in several inflammatory disorders, including IBDs [7–9]. It has been widely demonstrated that during intestinal inflammation, ATP is released massively from immune and parenchymal cells, acting as a “danger” and a “find-me” signal aimed to fine-tune phagocyte activation and migration via autocrine or paracrine pathways [10]. Over the years, several purinergic receptors have been identified as crucial players in orchestrating the pro-inflammatory activity of ATP [11].

Recently, increasing attention has been focused on the P2X4 receptor, a trimeric ATP-gated cation channel, that is widely expressed in central and peripheral neurons, microglia, immune cells, epithelial cells, and even in glandular tissues [11]. This purinergic receptor, once activated by ATP in nanomolar concentrations, elicits a pro-inflammatory response with the release of pro-inflammatory cytokines, such as IL-1β and IL-18, with consequent exacerbation of the inflammatory response [11, 12].

Several evidences show the involvement of P2X4 receptors in many disorders characterized by immune dysfunctions, including post-ischemic inflammation, rheumatoid arthritis, asthma, neurodegenerative, and cardiovascular diseases as well as metabolic syndrome [13, 14]. At present, the involvement of P2X4 receptors in the pathophysiological mechanisms of intestinal inflammation is not well characterized. However, the pivotal role of this receptor subtype in driving IL-1β and IL-18 release, and other pro-inflammatory cytokines in gut tissue of IBD patients [15, 16], leads us to hypothesize a significant role of P2X4 in the pathophysiology of IBD.

Therefore, the present study was designed to investigate the role of P2X4 receptors in a murine model of 2,4-dinitrobenzene sulfonic acid (DNBS)-induced colitis, and to evaluate the potentially beneficial therapeutic effect of two novel P2X4 receptor antagonists, NC-2600 and NP-1815-PX.

MATERIALS AND METHODS

Experiments on Animals

Animals

Albino male Sprague–Dawley rats, 200–225 g in body weight, were employed throughout the study. The
animals were fed with standard laboratory chow and tap water ad libitum and were given at least 1 week to acclimatize after their delivery to the animal facility. They have been housed, three in a cage, in temperature-controlled rooms at 22–24 °C and 50–60% humidity with a 12-h light cycle. Animal care and handling were in accordance with the provisions of the European Community Council Directive 210/63/UE, recognized and adopted by the Italian Government. The experiments have been approved by the Ethical Committee for Animal Experimentation of the University of Pisa and by the Italian Ministry of Health (authorization n 674/2016-PR).

Induction of Colitis and Drug Treatments

Rats were weighed and randomly distributed into separate experimental groups of six rats in each. Colitis was induced in accordance with the method described previously by Pellegrini et al., in 2018 [17]. Briefly, during a short anesthesia with isoflurane (Abbott Labs, Pomezia, Italy), 15 mg of 2,4-dinitrobenzenesulfonic acid (DNBS) in 0.25 ml of 50% ethanol has been administered intrarectally via a polyethylene PE-60 catheter inserted 8 cm proximal to the anus. Control rats received 0.25 ml of 50% ethanol. Following the DNBS administration, rats developed full-scale colonic inflammation that became evident 6 days later.

Immediately after the DNBS treatment, test drugs (NC-2600 3, 10, 30 mg/kg; NP-1815-PX 3, 10, 30 mg/kg, and Dexamethasone (DEX) 1 mg/kg) were orally administered to the animals for 6 days. DNBS-untreated animals (control group) and DNBS-treated rats (DNBS group) received only methocel (drug vehicle). When these treatments were initiated, at the same time, we started the daily monitoring of daily weight of the rats.

The doses of NC-2600 (3, 10, 30 mg/kg) and NP-1815-PX (3, 10, 30 mg/kg) were selected based on our preliminary experiments designed to measure the effects of increasing doses of these compounds on food intake, body weight, spleen weight, and macroscopic score in the model of NCBS-induced colitis. Effective dosing of NC-2600 (10 mg/kg) and NP-1815-PX (10 mg/kg) was then chosen to further study the effects of these compounds on inflammasome pathway and tight junction expression. The dose of DEX was selected according to a previous study using a rat model of colitis [18]. Macroscopic and histological scores were assessed on the entire length of colon, while biochemical assays were performed on colonic segments collected from inflamed regions adjacent and distal to grossly necrotic tissue areas.

Assessment of Colitis

Colonic tissue samples were graded for macroscopic damage, as reported previously [17]. The criteria for macroscopic grading of colonic damage were as follows: (1) consistency of colonic fecal material (0, formed; 1, loose; 2, liquid stools); (2) presence of abdominal adhesions between colonic tissue and other organs (0, none; 1, minor; 2, major adhesions); and (3) presence of mucosal ulceration (0, none; 1, hyperemia; 2, ulceration without hyperemia; 3, ulceration with inflammation at one side; 4, ≥2 sites of ulceration and inflammation; 5, major sites of damage; and 6, major sites of damage extending > 2 cm). The score was then increased by one unit for each millimeter of colonic wall thickness. All parameters of macroscopic damage were recorded and scored for each rat by two observers blinded to the treatment. At the time of experiment, the weight of each spleen was also measured.

Microscopic Tissue Injury Scores

Microscopic tissue injury and inflammation were assessed by light microscopy on hematoxylin/eosin-stained histological sections obtained from whole gut specimens. In detail, upon removal, colonic tissue samples were cut longitudinally, rinsed with cold PBS, and immediately placed in 10% formalin solution for 24 h. Afterward, colon samples were gradually dehydrated with ethanol, embedded into paraffin blocks, and sliced longitudinally into three consecutive serial 7–8 μm sections. Each slice was placed on a glass slide and routinely processed for staining with hematoxylin and eosin. The slides were cut at two different points of the block, starting from the upper towards lower levels. The histological criteria included mucosal architecture loss (0–3), cellular infiltration (0–3), muscle layer thickening (0–3), crypt abscess (0, absent; 1, present), and goblet cell depletion (0, absent; 1, present). All parameters of histological damages were recorded and scored for each rat by two observers blinded to the treatment.

Cytokine Assays

Tumor necrosis factor (TNF) and interleukin (IL)-1β were measured with enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Camarillo, CA), as described previously [19, 20]. For this purpose, colonic tissue samples (40 mg), stored previously at −80 °C, were weighed, thawed, and homogenized in 0.4 ml of PBS, pH 7.2 and centrifuged at 13,400 g for 20 min. Aliquots (100 μL) of the supernatants were then used for assay.
The concentrations of TNF and IL-1β were expressed as picograms per milligram of tissue protein.

**Protein Extraction and Western Blot Analysis**

The colon was collected from rats and flushed of fecal content with ice-cold phosphate-buffered saline (PBS), as described previously [21, 22]. Tissues were minced and homogenized using a Potter–Elvehjem Grinder homogenizer on ice in 20% (w/v) TNE lysis buffer (50 mM Tris–HCl pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 1% NP-40, 1% SDS, 0.1% DOC) with various protease and phosphatase inhibitors. Samples were then sonicated and boiled for 5 min at 95 °C. Protein concentrations were measured with the Bradford assay. Total lysates were run on 4–20% Criterion™ TGX™ Precast Midi Protein Gels (Bio-Rad, Hercules, CA, USA) and then transferred to PVDF membranes (Trans-Blot Turbo™ PVDF Transfer packs, Biorad). Then, membranes were blocked with 3% bovine serum albumin (BSA) diluted in Tris-buffered saline (TBS, 20 mM Tris–HCl, pH 7.5, 150 mM NaCl) with 0.1% Tween 20. Primary antibodies against caspase-1 (ab1872, Abcam) and occludin (ab167161, Abcam) were used. Secondary antibodies were obtained from Abcam (anti-mouse ab97040 and anti-rabbit ab6721). Protein bands were detected with ECL reagents (Clarity Western ECL Blotting Substrate, Biorad). Densitometry was performed by the IBright Analysis software.

**Caspase-1 Activity Assay**

Caspase-1 activity in colonic tissues was measured by using the colorimetric protease assay kit (BioVision Research Products, Mountain View, CA), following the protocol provided by the manufacturer. For this purpose, colonic tissue samples (40 mg), stored previously at −80 °C, were weighed, thawed, and homogenized in 0.4 ml of PBS, pH 7.2, and centrifuged at 13,400 g for 20 min. Aliquots (100 μl) of these supernatants were then used for the assay.

**Experiments on Cell Cultures**

**Cell Culture**

Human monocytic THP-1 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 100 unit/ml penicillin–streptomycin at 37 °C in a humidified atmosphere of 5% CO₂.

**Cell Treatments**

THP-1 cells were seeded at density of 3 × 10⁵ cells well in 24-well plates and treated with phorbol 12-myristate 13-acetate (PMA, 0.5 μM). After 3 h, the medium was removed, fresh media was added, and cells were incubated overnight (37 °C, 5% CO₂).

In the first series of experiments, lipopolysaccharide (LPS)-primed (1 µg/mL, 3 h) cells were treated for 1 h with vehicle (0.03% dimethyl sulfoxide, DMSO) before the addition of ATP (5 mM, 1 h).

In the second series of experiments, LPS-primed (1 µg/ml, 3 h) cells were treated for 1 h with NC-2600 (0.03–0.3–3 μM, 1 h) before the addition of ATP (5 mM, 1 h).

In the third series of experiments, LPS-primed (1 µg/ml, 3 h) cells were treated for 1 h with NP-1815-PX (0.1–1–10 μM, 1 h) before the addition of ATP (5 mM, 1 h). Controls were run in parallel.

A schematic representation of all treatments is shown Fig. 1.

After LPS stimulation, THP-1 cells were lysed, and the medium were collected and centrifuged for 5 min at 800 rpm to obtain cell-free supernatants. Cell supernatants were used to evaluate IL-1β release, while cells were lysed for analysis of inflammasome subunits and caspase-1/-4/-5 and -8 expression.

**Western Blot**

Cells were lysed as previously described [23, 24]. Proteins were quantified with the Bradford assay. Proteins were separated onto a pre-cast 4–20% polyacrylamide gel (Mini-PROTEAN® TGX gel, Biorad) and transferred to PVDF membranes (Trans-Blot® TurboTM PVDF Transfer packs, Biorad). Membranes were blocked with 3% BSA diluted in Tris-buffered saline (TBS, 20 mM Tris–HCl, pH 7.5, 150 mM NaCl) with 0.1% Tween 20. Primary antibodies against β-actin (ab8227, Abcam), nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain containing protein 3 (NLRP3) (ab214185, Abcam), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (D2W8U, Cell Signaling), caspase-1 (ab1872, Abcam), caspase-4 (#4450, Cell Signaling), caspase-5 (#46680, Cell Signaling), and caspase-8 (ab227430, Abcam) were used. Secondary antibodies were obtained from Abcam (anti-mouse ab97040 and anti-rabbit ab6721). Protein bands were detected with ECL reagents (Clarity Western
Stimulation protocol

LPS

3 hours

Vehicle or NC-2600 or NP-1815-PX

1 hour

ATP

1 hour

Fig. 1 Schematic representation of the design of experiments on human monocytic THP-1 cells. THP-1 cells were treated for 3 h with lipopolysaccharide (LPS, 1 µg/mL). Then, cells were incubated for 1 h with vehicle (0.03% dimethyl sulfoxide, DMSO), NC-2600 (0.03–0.3–3 µM) or NP-1815-PX (0.1–1–10 µM) before the addition of ATP (5 mM, 1 h). At the end of experimental protocol, cells were lysed for analysis of inflammasome subunits and caspase-1/-4/-5 and -8 expression and the culture media were collected for analysis of IL-1β release.

ECL Blotting Substrate, Biorad). Densitometry was performed by the IBright Analysis software.

Assessment of IL-1β Release in THP-1 Cells

The release of IL-1β was measured by ELISA kit (R&D system), as previously described [25]. After cell stimulation, the medium was collected and centrifuged for 5 min at 800 rpm to obtain cell-free supernatants. Aliquots of 100 µL were used for the test. IL-1β concentration was expressed as percentage versus THP-1 control cells (Ctrl).

Drugs and Reagents

ATP, DEX, DMSO, DNBS, FBS, LPS, PBS, and PMA were purchased from Sigma Aldrich (St. Louis, MO, USA). The synthesis of NC-2600 and NP-1815-PX were performed by Nippon Chemiphar Co., Ltd. For cell culture experiments, we dissolved the compound NC-2600 in sterile DMSO and subsequently diluted it in culture medium. For the experiments on animals, solid dispersion form of NC-2600 was used, as NC-2600 is hardly dissolved.

Statistical Analysis

Data are presented as mean ± SEM and analyzed by the GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical significances were determined by one-way ANOVA followed by Tukey’s post hoc test. A P value < 0.05 was considered statistically significant.
A

Body weight change (%)

Control  
DNBS  
+ NC-2600 3 mg/Kg  
+ NC-2600 10 mg/Kg  
+ NC-2600 30 mg/Kg  
+ NP-1815-PX 3 mg/Kg  
+ NP-1815-PX 10 mg/Kg  
+ NP-1815-PX 30 mg/Kg  
+ Dex 1 mg/Kg

B

Spleen weight change (%)

NC-2600  
NP-1815-PX

C

Macroscopic tissue injury score

NC-2600  
NP-1815-PX

*  

RETRACTED ARTICLE
RESULTS

Body Weight and Spleen Weight

Six days after DNBS administration, rats displayed a significant decrease in their body weight in comparison with control animals ($-36.67 \pm 4.41\%$ and $+26.62 \pm 3.22\%$, respectively) (Fig. 2A). The body weight loss was significantly attenuated in the rats treated with NC-2600 (3, 10, 30 mg/kg) or NP-1815-PX (3, 10, 30 mg/kg), but not in animals with DEX treatment (1 mg/kg) (Fig. 2A).

The induction of colitis with DNBS resulted in a significant increase of spleen weight ($+44.33\%$ on average) (Fig. 2B), that was counteracted by all tested drugs, such as NC-2600 (3, 10, 30 mg/kg), NP-1815-PX (3, 10, 30 mg/kg), and DEX (1 mg/kg) (Fig. 2B).

Furthermore, the administration of the above compounds did not elicit significant effects in DNBS-vehicle treated animals (Control group; data not shown).

Macroscopic Damage

The administration of DNBS was associated with colonic thickening and ulcerations, with marked areas of transmural inflammation. Adhesions were often present, and the bowel was occasionally dilated, with a macroscopic tissue injury grade of 7.83 $\pm$ 0.61 (Fig. 2C). In this setting, the macroscopic colonic injury was reduced significantly by treatment with the indicated concentrations of NC-2600 (3, 10, 30 mg/kg), NP-1815-PX (3, 10, 30 mg/kg), and DEX (Fig. 2C).

Microscopic Damage

Histological examination of colonic specimens from DNBS-treated rats showed that inflammation was evident and associated with large areas of mucosal necrosis, where the glandular architecture of tissue was completely destroyed, and only amorphous material could be observed. Submucosa was thickened due to the presence of oedema and marked infiltration with inflammatory cells associated with vasodilation. These processes occasionally involved the muscular layer which appeared to be thickened as well. The mucosa and submucosa surrounding the necrotic area exhibited inflammation marked with cellular infiltration, compared with tissues obtained from control animals (Fig. 3). Colonic sections from rats with colitis displayed a severe tissue damage 6 days after DNBS treatment (Fig. 3). NC-2600, but not NP-1815-PX, dose-dependently reduced the histological damage in colonic tissues obtained from inflamed rats (Fig. 3). DEX (1 mg/kg) determines a significant improvement in macroscopic scores (Fig. 3).

TNF and IL-1β Levels in Colonic Tissues

Rats with colitis displayed a significant increase in colonic TNF levels (12.12 $\pm$ 1.78 pg/mg tissue), in comparison with control animals (5.39 $\pm$ 0.60 pg/mg tissue) (Fig. 4A). Administration of NC-2600 or NP-1815-PX did not affect TNF concentration in colonic tissues from DNBS-treated rats (Fig. 4A). By contrast, DEX significantly counteracted the increase in colonic TNF levels associated with experimental colitis (Fig. 4A).

DNBS-induced colitis was associated with a significant increase in tissue IL-1β levels (32.73 $\pm$ 1.88 pg/mg tissue), in comparison with control rats (9.53 $\pm$ 5.43 pg/mg tissue) (Fig. 4B). This response was significantly downregulated by NC-2600 or DEX (Fig. 4B). By contrast, treatment with NP-1815-PX counteracted, although not significantly, the increment of IL-1β levels (Fig. 4B).

Pro-Caspase-1 and Cleaved Caspase-1 in Colonic Tissues

Expression of pro-caspase-1 in colonic tissues did not differ among the experimental groups (Fig. 5A). In parallel, colonic tissues obtained from rats with colitis showed a significant increase in cleaved caspase-1 expression (p20, an auto-processed fragment of caspase-1) when compared with control animals (Fig. 5B). Treatment with NC-2600 (10 mg/kg) or NP-1815-PX (10 mg/kg) did not exert significant effects on cleaved caspase-1 (Fig. 5B). By contrast, DEX significantly counteracted the increase in cleaved caspase-1 expression associated with experimental colitis (Fig. 5B).

Caspase-1 Activity in Colonic Tissues

Rats with DNBS-induced colitis displayed a significant increase in caspase-1 activity, in comparison...
with control animals (Fig. 5D). Treatment with NC-2600 (10 mg/kg), NP-1815-PX (10 mg/kg), or DEX (1 mg/kg) significantly countered the increment of colonic caspase-1 activity associated with DNBS administration (Fig. 5D).

**Occludin Expression in Colonic Tissues**

Western blot analysis showed that the expression of occludin (integral plasma-membrane protein located at the tight junctions) was significantly reduced in colonic mucosa from DNBS-treated rats in comparison with control animals (Fig. 5E). Rats with colitis administered with NC-2600 (10 mg/kg) or NP-1815-PX (10 mg/kg) displayed a significant increase in occludin expression, whereas those subjected to DEX administration did not experience this phenomenon (Fig. 5E).

---

**In Vitro Assays on NLRP3 Inflammasome: Canonical and Non-canonical Pathways**

A set of in vitro experiments were performed to characterize the molecular mechanisms underlying the anti-inflammatory effects of P2X4 receptor antagonists, NC-2600 and NP-1815-PX. In particular, we focused our attention on the effects of P2X4 antagonists on the expression of inflammasome components, including NLRP3, ASC, caspase-1, caspase-4, caspase-5, and caspase-8, as well as IL-1β release in THP-1, a human monocytic cell line.

**Expression of NLRP3 and ASC**

LPS-primed THP-1 cells incubated with ATP were characterized by a significant increase of expression of
Fig. 4  TNF (A) and IL-1β levels (B) in colonic tissue from control rats and animals treated with DNBS alone or in combination with NC-2600 (3, 10, 30 mg/kg), NP-1815-PX (3, 10, 30 mg/kg), and DEX (1 mg/kg). Each column represents the mean ± SEM (n=6). One-way ANOVA followed by Tukey post hoc test results: *p<0.05, significant difference vs. the control group; a p<0.05, significant difference vs. the DNBS group. Abbreviations: DEX, Dexamethasone; DNBS, 2,4-dinitrobenzene sulfonic acid; IL-1β, interleukin 1β; TNF, tumor necrosis factor.
RETRACTED ARTICLE
NLRP3 (114 KDa) whereas ASC (25 KDa) was not affected (Fig. 6A, B). Treatment with NC-2600 or NP-1815-PX counteracted the increase in the expression of both NLRP3 and ASC in a dose-dependent fashion (Fig. 6A, B).

Expression of Pro-Caspase-1 and Cleaved Caspase-1 (Canonical NLRP3 Pathway)

NC-2600 and NP-1815-PX did not affect the expression of pro-caspase-1 (45 KDa) in LPS-primed THP-1 cells incubated with ATP (Fig. 6C, E). By contrast, both P2X4 antagonists significantly counteracted the increase in the expression of caspase-1 in LPS-primed THP-1 cells incubated with ATP (Fig. 6D, E).

Expression of Caspase-4, Caspase-5, and Caspase-8 (Non-canonical NLRP3 Pathway)

LPS-primed THP-1 cells incubated with ATP showed a significant increase of caspase-5 and caspase-8 expression, whereas no significant variations were observed for caspase-4, as compared with control cells (Ctrl) (Fig. 7A-C). Treatment with NC-2600 and NP-1815-PX significantly counteracted the increase in the expression of caspase-5 and caspase-8 (Fig. 7A-C).

IL-1β Processing and Release

Incubation of LPS-primed THP-1 cells with ATP significantly increased the release of IL-1β, as compared with control cells (Ctrl) (Fig. 7D). Such an increase was dose-dependently reduced by treatment with NC-2600 (0.03–3 µM) (Fig. 7D). Of note, despite NP-1815-PX (0.1 and 1 µM) did not alter IL-1β processing and release, incubation with 10 µM NP-1815-PX reduced significantly the IL-1β production induced by the co-treatment with LPS and ATP (Fig. 7D).

DISCUSSION

For several years, pharmacological management of IBD was limited to aminosalicylates, thiopurines, and steroids, which, despite providing symptomatic relief, affected scarcely the disease course [2]. Subsequently, advances in understanding the molecular mechanisms underlying the pathogenesis of IBD allowed us to identify novel therapies based on biological entities. These therapies aimed at curbing the action of pro-inflammatory cytokines pivotal in the onset and progression of IBDs [3]. Even though biological drugs undoubtedly represent an evolution in the therapeutic approach to IBD, these are often characterized by a high degree of therapeutic failure and in many cases by heavy adverse effects [3]. For these reasons, the identification of new therapeutic targets with immunomodulating/anti-inflammatory properties represents a field of extreme interest for the scientific community.

In this regard, increasing attention has been paid to the design and synthesis of novel pharmacological entities acting on the purinergic system as viable way to regulate the development and course of several immunemediated inflammatory diseases (IMIDs) [10]. In particular, the active role of P2X4 receptor subtype has been well defined in driving the immunostimulating effects of extracellular ATP, eliciting pro-inflammatory responses in both myeloid and lymphoid cells [13]. Therefore, this receptor subtype represents an ideal target for pharmacological management several immune/inflammatory diseases [10, 11, 13, 14].

The aims of our study were as follows: (a) to evaluate the putative anti-inflammatory effects of NC-2600 and NP-1815-PX, two novel and selective P2X4 receptor antagonists, and (b) to characterize the molecular mechanisms underlying their immunomodulatory effect in a murine model of DNBS-induced colitis. DEX was employed as a comparative agent with known anti-inflammatory activity to evaluate the relative anti-inflammatory effects of both P2X4 ligands.

The DNBS-induced colitis closely mimics the pathology of Crohn’s disease as it is characterized by body weight loss, diarrhea, colon ulceration and bleeding, the depletion of goblet cells, and the formation of granulomas within
A  B  C  D  E

\[ \text{Ctrl} \]

\[ \text{LPS plus ATP} \]

\[ + \text{NC-2600 0.03 \mu M} \]

\[ + \text{NC-2600 0.3 \mu M} \]

\[ + \text{NC-2600 3 \mu M} \]

\[ + \text{NP-1815-PX 0.1 \mu M} \]

\[ + \text{NP-1815-PX 1 \mu M} \]

\[ + \text{NP-1815-PX 10 \mu M} \]

\[ \text{Actin (42 KDa)} \]

\[ \text{NLRP3 (114 KDa)} \]

\[ \text{Pro-caspase-1 (37 KDa)} \]

\[ \text{Cleaved caspase-1 (20-22 KDa)} \]

\[ \text{ASC (25 KDa)} \]

**RETRACTED ARTICLE**
the gut wall [26, 27]. This represents a predictive model to assess the efficacy of novel anti-inflammatory agents. In this regard, the suitability of this model was corroborated by the efficacy of DEX to ameliorate the macroscopic and histological features of colitis as well as to reduce tissue TNF and IL-1β levels, in line with previous reports showing similar beneficial effects of this glucocorticoid drugs [18, 28].

In the first set of experiments, we observed that the pharmacological blockade of P2X4 receptor via NC-2600 and NP-1815-PX elicited a significant improvement in several inflammatory parameters, including body weight, spleen weight, and macroscopic and microscopic tissue injury score, as well as down-regulated IL-1β levels in DNBS-rats, thus providing evidence that the pharmacological modulation of this receptor is a viable option to manage enteric inflammatory processes. This is in line with a number of recent studies describing the critical role of P2X4 receptor in protecting against infections, inflammation, and organ injury [13]. Indeed, this receptor has been shown to mediate a variety of pathophysiological processes, characterized by an excessive immune cell activity such as autoimmunity and neuroinflammatory diseases [13]. In particular, P2X4, widely expressed on cells of both innate and adaptive immune cells, is emerging as a pivotal pathway in mediating the immunostimulating/pro-inflammatory effect of ATP [13], thus representing an attractive therapeutic target in multiple diseases, especially in chronic inflammation and neuropathic pain [29–31].

Interestingly, in our experimental model, both P2X4 antagonists failed to reduce tissue TNF levels. By contrast, NC-2600 and NP-1815-PX were able to counteract the increase of IL-1β concentration in colonic tissues from rats with DNBS-induced colitis. IL-1β is a potent inflammatory cytokine centrally involved in the development of colitis and T(H)17-associated immune responses in the gut [32]. On the same line, patients with Crohn’s disease showed a positive correlation between the severity of mucosal inflammation and the levels of IL-1β, thus corroborating a relevant role for this cytokine in the pathophysiology of IBDs [33].

Despite the mechanism of IL-1β release has proven to be complex, a set of molecular and biochemical investigations allowed to demonstrate that the bioactive form of this cytokine is produced by the inflammasome NLRP3, a caspase-1 activating molecular platform, in response to selected danger-associated molecular patterns and pathogen-associated molecular patterns [34]. In particular, the NLRP3 inflammasome complex, including NLRP3, ASC, and pro-caspase-1, trigger the processing and release of IL-1β and IL-18, acts as a key player both in shaping the central and/or peripheral immune/inflammatory responses in several inflammatory diseases, including IBDs [35]. Such pathway is currently designated as “canonical NLRP3 inflammasome activation.” In addition, a “non-canonical NLRP3 inflammasome activation,” which depends on caspase-11 in mice (caspase-4 and caspase-5 in human) and caspase-8, has been reported to play a pivotal role in the pathophysiological events underlying bowel inflammation [36].

Based on these findings, we designed several experiments to evaluate the putative effect of NC-2600 and NP-1815-PX in counteracting inflammasome NLRP3 activation, especially focusing on the effect of tested drugs on the expression and activity of caspase-1, the canonical activation pathway of NLRP3 complex. Our findings showed that treatment with NC-2600 and NP-1815-PX, despite not reducing the caspase-1 expression, was able to decrease its activity in colonic tissues from rats with colitis. This evidenced the anti-inflammatory activity of this novel P2X4 antagonists by inhibiting the canonical caspase-1-dependent NLRP3 inflammasome signaling pathway. This is in line with previous studies, performed in murine models of diabetic nephropathy, bladder inflammation, and acute kidney injury, showing that the pharmacological blockade of P2X4 receptors blunted the IL-1β release through the inhibition of NLRP3 inflammasome pathway [31, 37, 38].

In order to corroborate the results obtained from in vivo experiments, we designed a set of in vitro experiments to evaluate the effect of the P2X4 antagonists on modulating the canonical and non-canonical pathways involved in NLRP3 activation. Therefore, we performed experiments in LPS-primed and PMA-differentiated THP-1 cells treated with ATP, an established model to
investigate the functions of purinergic receptors in monocyte/macrophage cells [39–41]. THP-1 cells were incubated with LPS, a well-recognized activator of the first step of NLRP3 signaling as well as a hallmark of altered intestinal permeability, a condition typically observed in the murine model of colitis and IBD patients [42, 43]. We observed that co-treatment THP-1 cell line with LPS and ATP determined an increase in the expression of inflammasome components, including NLRP3 and caspase-1, followed by marked IL-1β release. Under this condition, the incubation with NC-2600 and NP-1815-PX counteracted the reduction of tight junction components, including zonula occludens (ZO)-1 and claudin-2, suggesting the pharmacological blockade of P2X4 receptors/NLRP3 interplay represent the logical continuation of this research topic. IL-1β, beyond being involved in the initiation and amplification of inflammatory responses, plays a critical role in the apoptosis of epithelial cells causing tissue damage and barrier dysfunction. This, in turn, leads to increased intestinal permeability typically associated with IBDs [51]. Consistently with this evidence, our experimental model showed a decreased expression levels of colonic tight junctions. Interestingly, treatment with NC-2600 and NP-1815-PX counteracted the reduction of tight junction expression associated with experimental colitis, suggesting that the pharmacological blockade of P2X4 receptors can exert beneficial effect in the maintenance of intestinal epithelial barrier integrity. Despite the fact that no data are available about the molecular mechanisms underlying the beneficial effects of this novel P2X4 antagonists in the maintenance of intestinal epithelial barrier, it is conceivable that these likely depend, at least in part, on the action of these drugs on the NLRP3/caspase-1/IL-1β axis.

In conclusion, the present study expands current knowledge about the beneficial effects of the P2X4 receptor blockade in modulating immune/inflammatory responses. Our results demonstrated here for the first time that the direct and selective inhibition of P2X4 receptors represent a viable approach for the management of bowel inflammation via the inhibition of the canonical and non-canonical NLRP3 inflammasome signaling pathways (Fig. 8). According to the present results, the blockade of P2X4 receptors allows an effective control of experimental intestinal inflammation, and novel and selective P2X4 antagonists can provide a basis for development of anti-inflammatory drugs suitable for treatment of IBD.
Intestinal inflammation

Bacterial translocation

NC-2600
NP-1815-PX

P2X4 receptor

Canonical NLRP3 activation

Non-canonical NLRP3 activation

Caspase-1 active

Caspase-4/5 active

Caspase-8 active

IL-1β

Pro-IL-1β

Pro-IL-1β

Pro-IL-1β

Pro-IL-1β

Fig. 8 Diagram showing the molecular mechanisms through which NC-2600 and NP-1815-PX inhibit NLRP3 inflammasome signaling and counteract intestinal inflammation. In the chronic phase of inflammation, canonical, and non-canonical pathways of NLRP3 inflammasome are associated with an increase in IL-1β release that are harmful to the host. In this setting, NC-2600 and NP-1815-PX are able to counteract the increase of IL-1β release through the inhibition of canonical (NLRP3/Caspase-1/IL-1β) and non-canonical (NLRP3/Caspase-4–5-8/IL-1β) pathways of NLRP3 inflammasome. Abbreviations: IL-1β, interleukin-1β; NLRP3, nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain containing protein 3; pro-IL-1β, pro-interleukin-1β.
ACKNOWLEDGEMENTS

We respectfully remember and thank our mentor, Prof. Corrado Blandizzi for being a scientific role model and guide for all the authors. We dedicate this manuscript to his memory.

AUTHOR CONTRIBUTION

VD, CP, and LA write original draft preparation; VD, CP, LB, CDS, and GS performed the research; LB, MF, CDS, and GS collected and analyzed the data. VD, CP, MF, and ZHN interpreted the data; GH, CB, and LA coauthored the writing of the manuscript and edited the manuscript.

All authors read and approved the final manuscript.

FUNDING

Open access funding provided by Università di Pisa within the CRUI-CARE Agreement. This work was performed with the financial support granted by Nippon Chemiphar Co. LTD.

DATA AVAILABILITY

All data generated or analysed during the current study are included in this article.

DECLARATIONS

Ethics Approval and Consent to Participate The experiments have been approved by the Ethical Committee for Animal Experimentation of the University of Pisa and by the Italian Ministry of Health (authorization n 674/2016-PR).

Consent for Publication All the authors have read the manuscript and agreed to submit the paper to the journal.

Competing Interests The authors declare no competing interests.

Author’s Information The use of P2X4 antagonists as medication for preventing or treating irritable bowel syndrome or inflammatory bowel diseases has been patented (PCT/IB2020/053313).

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to gain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

REFERENCES

1. Sairenji, Tomoko, Kimberly L Collins, and David V Evans. 2017. An update on inflammatory bowel disease. Primary Care 44. United States: 675–692. https://doi.org/10.1016/j.pop.2017.07.010.
2. Kim, Duk Hwan, and Jee Hee Choe. 2017. Pathogenesis of inflammatory bowel disease: recent advances in biologic therapies. Immuno- phosphorylation 17: 25-30. https://doi.org/10.4110/in.2017.17.1.25.
3. Antoniolì, Luca, Mauro Fornai, Barbara Romano, Carolina Pellegrini, and Corrado Blandizzi. 2020. Editorial: BMD management—novel targets and therapeutic perspectives. Frontiers in Pharmacology. Frontiers Media S.A. https://doi.org/10.3389/phar.2020.00448.
4. Tran, Vivy, Berkeley N. Limketkai, and Jenny S. S auk. 2019. IBD in the elderly: Management challenges and therapeutic considerations. Current Gastroenterology Reports: Springer. https://doi.org/10.1007/s11894-019-0720-7.
5. Katz, Seymour. 2007. “Mind the gap”: an unmet need for new therapy in IBD. Journal of clinical gastroenterology 41. United States: 799–809. https://doi.org/10.1097/MCG.0b013e318033d71d.
6. Ben, Dal, Luca Antonioli Diego, Catia Lambertucci, Andrea Spinaci, Matteo Fornai, Vanessa D'Antongiovanni, Carolina Pellegrini, Corrado Blandizzi, and Rosaria Volpini. 2020. Approaches for designing and discovering purinergic drugs for gastrointestinal diseases. Expert opinion on drug discovery: Taylor and Francis Ltd. https://doi.org/10.1080/17460441.2020.1743673.
7. Kurashima, Yosuke, Hiroshi Kiyono, and Jun Kunisawa. 2015. Physio-physiological role of extracellular purinergic mediators in the control of intestinal inflammation. Mediators of inflammation 2015: 427125. https://doi.org/10.1155/2015/427125.
8. Vuerich, Marta, Samiran Mukherjee, Simon C. Robson, and Maria Serena Longhi. 2020. Control of gut inflammation by modulation of purinergic signaling. Frontiers in Immunology 11. https://doi.org/10.3389/fimmu.2020.01882.
9. Longhi, Maria Serena, Alan Moss, Zhenghui Gorden Jiang, and Simon C. Robson. 2017. Purinergic signaling during intestinal inflammation. Journal of molecular medicine (Berlin, Germany) 95. J Mol Med (Berl): 915–925. https://doi.org/10.1007/S00109-017-1545-1.
10. Antoniolì, Luca, Corrado Blandizzi, Pal Pacher, and György Haskó. 2019. The purinergic system as a pharmacological target for the treatment of immune-mediated inflammatory diseases. Pharmacological Reviews 71. American Society for Pharmacology and Experimental Therapy: 345–382. https://doi.org/10.1124/pr.117.114478.
11. SurváI, Iaunus, Pierre Boudinot, Jean Kanellopoulos, and Sirje Rüütel. Boudinot. 2017. P2X4: A fast and sensitive purinergic receptor. Bio- medical journal 40: 245–256. https://doi.org/10.1016/bj.2017.06.010.
12. Sakaki, Hayato, Takuya Fujiwaki, Mitsutoshi Tsukimoto, Ayumi Kawano, Hitoshi Harada, and Shuji Kojima. 2013. P2X4 receptor regulates P2X7 receptor-dependent IL-1β and IL-18 release in mouse bone marrow-derived dendritic cells. Biochemical and
biophysical research communications 432. United States: 406–411. https://doi.org/10.1016/j.bbrc.2013.01.135.
13. Antonioli, Luca, Corrado Blandizzi, Matteo Fornai, H. Pål Pacher, Thomas Lee, and György. Haskó. 2019. P2X4 receptors, immunity, and sepsis. Current opinion in pharmacology 47: 65–74. https://doi.org/10.1016/j.coph.2019.02.011.
14. Kawano, Ayumi, Mitsutoshi Tsukimoto, Daisuke Mori, Taisei Noguchi, Hiroshi Harada, Takato Takenouchi, Hiroshi Kitani, and Shuji Kojima. 2012. Regulation of P2X7-dependent inflammatory functions by P2X4 receptor in mouse macrophages. Biochemical and biophysical research communications 420. United States: 102–107. https://doi.org/10.1016/j.bbr.2012.02.122.
15. Sivakumar, P. V., G. M. Westrich, S. Kanaly, K. Garka, T. L. Born, J. M. J. Derry, and J. L. Viney. 2002. Interleukin 18 is a primary mediator of the inflammation associated with dextran sulphate sodium induced colitis: blocking interleukin 18 attenuates intestinal damage. Gut 50. Gut: 812–820. https://doi.org/10.1136/gut.50.6.812.
16. Mao, Liming, Atsushi Kitani, Warren Strober, and Ivan J. Fuss. 2018. The role of NLRP3 and IL-1β in the pathogenesis of inflammatory bowel disease. Frontiers in Immunology. Frontiers Media S.A. https://doi.org/10.3389/fimmu.2018.02566.
17. Pellegrini, Carolina, Matteo Fornai, Rocchina Colucci, Laura Benvenuti, Vanessa D’Antongiovanni, Gianfranco Natale, Federica Fulceri, et al. 2018. A comparative study on the efficacy of NLRP3 inflammasome signaling inhibitors in a pre-clinical model of bowel inflammation. Frontiers in pharmacology 9: 1405. https://doi.org/10.3389/fphar.2018.01405.
18. Nakase, Hiroshi, Kazuichi Okazaki, Yashuhiko Tabata, Naotaka Seo, Masaya Ohana, Kazushige Uchida, Toshiki Nishi et al. 2001. Oral drug delivery system targeting immune-regulating cells ameliorates mucosal injury in trinitrobenzenesulfonic acid–induced colitis. Journal of Pharmacology and Experimental Therapeutics 297. J Pharmacol Exp Ther. 1122–1131.
19. Pellegrini, Carolina, Simona Daniele, Luca Antonioli, Laura Benvenuti, Vanessa D’Antongiovanni, Rebecca Pizzuti, Deborah Pietrobono, et al. 2020. Prodomal intestinal events in Crohn’s disease (Ad): colonic dysmotility and inflammation are associated with enteric ad-related protein deposition. International Journal of Molecular Sciences 21. MDPI AG. https://doi.org/10.3390/ijms21103523.
20. Antonioli, Luca, Eifion Robinson, Vijay Chudasama, Stephen Cadwallader, Luca Fornai, Caroline Pellegrini, et al. 2020. Colonic mucosal dysmotility associated with high-fat diet-induced obesity model of colitis. Frontiers in Pharmacological Sciences 96. J Pharmacol Sci: 307–313. https://doi.org/10.1254/jfps.2017.00048.
21. Jurga, Agnieszka M., Anna Piotrowska, Wioletta Makuch, Barbara Przewlocka, and Joanna Mika. 2017. Blockade of P2X4 receptors inhibits neuropathic pain-related behavior by preventing MMP-9 activation and, consequently, pronociceptive interleukin release in a rat model. Frontiers in Pharmacology 8. Frontiers Research Foundation. https://doi.org/10.3389/fphar.2017.00048.
22. Csóka, Balázs, Zoltán H. Németh, Ildikó Szabó, Daryl L. Davies, Zoltán V. Varga, János Pálóczi, Simonetta Falzoni, et al. 2018. Macrophase P2X4 receptors augment bacterial killing and protect against sepsis. JCI insight 3. NLM (Medline). https://doi.org/10.1172/jci.insight.99431.
23. Han, Sang Jun, Marriana Lovasz, Mihwa Kim, Vivette D’Agati, György, Haskó, H. Thomas, and Lee. 2020. P2X4 receptor exacerbates ischemic AKI and induces renal proximal tubular NLRP3 inflammasome signaling. FASEB Journal : Official publication of the Federation of American Societies for Experimental Biology 34: 5465–5482. https://doi.org/10.1096/fj.201903287R.
24. Coccia, Margherita, Oliver J. Harrison, Chris Schiering, Mark J. Asquith, Burkhard Becher, Fiona Powrie, and Kevin J. Maloy. 2012. IL-1β mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4+ Th17 cells. Journal of Experimental Medicine 209. J Exp Med: 1595–1609. https://doi.org/10.1084/jem.20111453.
25. McIntee, Craig P., Conor M. Finlay, and Ed C. Lavelle. 2019. Divergent roles for the IL-1β family in gastrointestinal homeostasis and inflammation. Frontiers in Immunology. Frontiers Media S.A. https://doi.org/10.3389/fimmu.2019.01266.
26. Pellegrini, Carolina, Matteo Fornai, Luca Antonioli, Corrado Blandizzi, and Vincenzo Calderone. 2019. Phytochemicals as novel therapeutic strategies for nlrp3 inflammasome-related neurological, metabolic, and cognitive disorders. Frontiers in Pharmacology 10. Frontiers Research Foundation. https://doi.org/10.3389/fphar.2019.00121.
27. Zhang, Xuewen, Ilaria Piano, Andrea Messina, Vanessa D’Antongiovanni, Fabiana Crò, Giovanni Provenzano, Yuri Bozzi, Claudia Gargini. 2019. A nutraceutical strategy to slowing down the progression of bone disease (Ad): colonic dysmotility and inflammation are associated with enteric ad-related protein deposition. International Journal of Molecular Sciences 21. MDPI AG. https://doi.org/10.3390/ijms21103523.
28. Antonioli, Luca, Eifion Robinson, Vijay Chudasama, Stephen Cadwallader, Luca Fornai, Caroline Pellegrini, et al. 2020. Colonic mucosal dysmotility associated with high-fat diet-induced obesity model of colitis. Frontiers in Pharmacological Sciences 96. J Pharmacol Sci: 307–313. https://doi.org/10.1254/jfps.2017.00048.
29. Jurga, Agnieszka M., Anna Piotrowska, Wioletta Makuch, Barbara Przewlocka, and Joanna Mika. 2017. Blockade of P2X4 receptors inhibits neuropathic pain-related behavior by preventing MMP-9 activation and, consequently, pronociceptive interleukin release in a rat model. Frontiers in Pharmacology 8. Frontiers Research Foundation. https://doi.org/10.3389/fphar.2017.00048.
30. Csóka, Balázs, Zoltán H. Németh, Ildikó Szabó, Daryl L. Davies, Zoltán V. Varga, János Pálóczi, Simonetta Falzoni, et al. 2018. Macrophase P2X4 receptors augment bacterial killing and protect against sepsis. JCI insight 3. NLM (Medline). https://doi.org/10.1172/jci.insight.99431.
31. Han, Sang Jun, Marriana Lovasz, Mihwa Kim, Vivette D’Agati, György, Haskó, H. Thomas, and Lee. 2020. P2X4 receptor exacerbates ischemic AKI and induces renal proximal tubular NLRP3 inflammasome signaling. FASEB Journal : Official publication of the Federation of American Societies for Experimental Biology 34: 5465–5482. https://doi.org/10.1096/fj.201903287R.
32. Coccia, Margherita, Oliver J. Harrison, Chris Schiering, Mark J. Asquith, Burkhard Becher, Fiona Powrie, and Kevin J. Maloy. 2012. IL-1β mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4+ Th17 cells. Journal of Experimental Medicine 209. J Exp Med: 1595–1609. https://doi.org/10.1084/jem.20111453.
33. McIntee, Craig P., Conor M. Finlay, and Ed C. Lavelle. 2019. Divergent roles for the IL-1β family in gastrointestinal homeostasis and inflammation. Frontiers in Immunology. Frontiers Media S.A. https://doi.org/10.3389/fimmu.2019.01266.
34. Lopez-Castejon, Gloria, and David Brough. 2011. Understanding the mechanism of IL-1β secretion. Cytokine and Growth Factor Reviews. Cytokine Growth Factor Rev. https://doi.org/10.1016/j.cytogfr.2011.01.001.
35. Pellegrini, Carolina, Matteo Fornai, Luca Antonioli, Corrado Blandizzi, and Vincenzo Calderone. 2019. Phytochemicals as novel therapeutic strategies for nlrp3 inflammasome-related neurological, metabolic, and cognitive disorders. Frontiers in Pharmacology 10. Frontiers Research Foundation. https://doi.org/10.3389/fphar.2019.00121.
inflammatory diseases. *International Journal of Molecular Sciences.* MDPI AG. https://doi.org/10.3390/IJMS20122876.
36. Pellegrini, Carolina, Luca Antonioli, Gloria Lopez-Castejon, Corrado Blandizzi, and Matteo Fornai. 2017. Canonical and non-canonical activation of NLRP3 inflammasome at the crossroad between immune tolerance and intestinal inflammation. *Frontiers in Immunology.* Frontiers Media S.A. https://doi.org/10.3389/fimmu.2017.00036.
37. Chen, Kehong, Jianguo Zhang, Weiwei Zhang, Jinhua Zhang, Junrong Yang, Kai-long Li, and Yani He. 2013. ATP-P2X4 signaling mediates NLRP3 inflammasome activation: a novel pathway of diabetic nephropathy. *The international journal of biochemistry & cell biology* 45. Netherlands: 932–943. https://doi.org/10.1016/j.biocel.2013.02.009.
38. Dunton, Cody L., J. Todd Purves, M. Francis Jr., Huixia Jin Hughes, and Jiro Nagatomi. 2018. Elevated hydrostatic pressure stimulates ATP release which mediates activation of the NLRP3 inflammasome via P2X(4) in rat urothelial cells. *International urology and nephrology* 50: 1607–1617. https://doi.org/10.1111/1532-187X.13563.
39. Tu, Yun Ming, Cheng Xin Gong, Lu Ding, Xing Zi Liu, Tao Li, Fang Fang Hu, Shuo Wang, Chao Peng Xiong, Shang Dong Liang, and Ronald Sluyter. 2020. P2Y2 and P2X4 receptors mediate Ca2+ influx in model human monocytes and macrophages. *International Journal of Molecular Sciences* 21. MDPI AG. https://doi.org/10.3390/IJMS21022876.
40. Layhadi, Janice A., and Samuel J. Fouquain. 2017. P2X4 receptor-dependent Ca2+ influx in macrophages and macrophage-stimulated ATP release which mediates activation of the NLRP3 inflammasome in 18. *Molecular Journal of Cell Research.* MDPI AG. https://doi.org/10.3390/MJCR18112261.
41. Sophocleous, Reece Andrew, Nicole Ashley, Emma, Léanne Oei, and Ronald Slyter. 2020. P2X4 receptors mediate Ca2+ mobilization in DH82 canine macrophage cells. *International Journal of Molecular Sciences* 21. MDPI AG. 1–22. https://doi.org/10.3390/IJMS21125784.
42. Hung, Yung Li, Shu Chen, Wang, Krasniko, and Shuhua Wang. 2016. Bacterial activators LPS induces inflammatory response and inhibits the activation of NLPR3 inflammasome in macrophages. *Phytomedicine* 59. Elsevier GmbH. https://doi.org/10.1016/j.phymed.2016.12.008.
43. D’Antongiovanni, Vanessa, Carolina Pellegrini, Matteo Fornai, Roscina Cozzi, Corrado Blandizzi, Luca Antonioli, and Nunzia Fornaciari. 2020. Intestinal epithelial barrier and neuromuscular component in health and disease. *World Journal of Gastroenterology.* Baishideng Publishing Group Co., Limited. https://doi.org/10.3748/wjg.v26.i14.1564.
44. de Rivero Vaccari, Juan Pablo, Dominic Bastien, Geoffrey Yurcisin, Isabelle Pineau, W. Dalton Dietrich, Yves De Koninck, Robert W. Keane, and Steve Lacroix. 2012. P2X4 receptors influence inflammasome activation after spinal cord injury. *Journal of Neuroscience* 32. J Neurosci: 3058–3066. https://doi.org/10.1523/JNEUROSCI.4930-11.2012.
45. Kayagaki, Nobuhiko, Sören Warnmark, Pujang Lamkanfi, Lieselotte Vande Walle, Salina Jamshidi, Jennifer Dower, Kim Newton, et al. 2011. Non-canonical inflammasome activation targets caspase-11. *Nature* 479. Nature: 117–121. https://doi.org/10.1038/nature10558.
46. Knodler, Leigh A., Stephanie M. Crowley, Ho Pan Sham, Hyungjin Yang, Monica Wrande, Chi Ma, Robert K. Ernst, Olivia Steele-Mortimer, Robert C. Bell, and Bruce A. Vallance. 2014. Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens. *Cell Host and Microbe* 16. Cell Host Microbe: 249–256. https://doi.org/10.1016/j.chom.2014.07.002.
47. Chiang, Prajwal, and Thirumala Devi Kanneganti. 2015. Novel role for caspase-8 in IL-1β and inflammasome regulation. *American Journal of Pathology.* Elsevier Inc. https://doi.org/10.1016/j.ajpath.2014.08.025.
48. Antonopoulos, Christina, Hana M. Russo, Caroline El Sanadi, Bradley N. Martin, Xiaoxia Li, William J. Kaiser, Edward S. Mocarski, and George R. Dubay. 2015. Caspase-8 as an effector and regulator of NLRP3 inflammasome signaling. *Journal of Biological Chemistry* 290. American Society for Biochemistry and Molecular Biology Inc.: 20167–20184. https://doi.org/10.1074/jbc.M115.652321.
49. Huang, Tsung Teng, Kowit Yu Chong, David M. Ojcius, Yi Hui Wu, Yun Fei Ko, Cheng Ye Wu, Ian Martel, Chi Chia Lin, Hsin Chih Lai, and John D. Young. 2013. Hirsutella sinensis mycelium suppresses interleukin-1β and interleukin-18 secretion by inhibiting both canonical and non-canonical inflammasomes. *Scientific Reports* 3. Sci Rep. https://doi.org/10.1038/srep01374.
50. Viganó, Elena, Catherine Emma Diamond, Roberto Spreafo, Akhila Balachander, Radoslaw M. Sobota, and Alessandra Mortellaro. 2015. Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes. *Nature Communications* 6. Nature Publishing Group. https://doi.org/10.1038/ncomms9761.
51. Al-Sadi, Rana M., and Thomas Y. Ma. 2007. IL-1β Causes an increase in intestinal epithelial tight junction permeability. *The Journal of Immunology* 178. The American Association of Immunologists: 4641–4649. https://doi.org/10.4049/jimmunol.178.7.4641.

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.