Discovery and characterization of small molecule inhibitors of NLRP3 and NLRC4 inflammasomes

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Table S1. Primers for quantitative PCR analysis.

Gene	Forward sequence (5´→3´)	Reverse sequence (5´→3´)
HPRT	CCCCAAAATGGTTAAGGTTGC	AACAAAGTCTGGCCTGTATCC
NLRP3	GCCATCATCAGCTCCTGTGT	ACTGGCTGACTGAACGACTG
Caspase-1	ACTGCTATGGACAAGGCACG	GCAAGACGTGTACGAGTGGT
IL-1β	CTCCATGAGCTTTGTACAAGG	TGCTGATGTACCAGTTGGGG
TNF-α	ATGAGCACAGAAAGCATGA	AGTAGACAGAAGAGCGTGGT
NLRC4	CAGGTCACAGAAGAAGACCTGA	ACTTCCCTTTGCCAGACTCG
IL-6	GAAAAGAGTTGTGCAATGGC	TATGGTACTCCAGAAGACCA

Table S2. IL-1β percentage, referred to controls average, released during the first screening by human macrophages in presence of 100 μM of the hits from the virtual screening. NLRP3 activation was induced through treatment with 400 ng/ml during 3 hours, followed by addition of 4.5 mM ATP for 45 minutes. All the experiments were conducted by triplicate.

	%IL-1β release (relative to control)			
	<25	**25-50**	**50-75**	**75-100**
Compound Number	C75, C97	C29, C33, C50, C53, C87, C94, C95	C9, C21, C25, C54, C56, C61, C62, C73, C78, C84	C1-C8, C10-C20, C22-C24, C26, C27, C28, C30, C31, C32, C34-C49, C51, C52, C55, C57-C60, C62-C72, C74, C76, C77, C79-C83, C85, C86 C88-C93, C96, C98

Table S3. Percentage of inhibition reached by the positive compounds in the primary i*n-vitro* screening. THP1 human macrophages were pretreated with 100 μM of the hits from the virtual screening prior to addition of 400 ng/ml LPS (3 hours) and 4.5 mM ATP (45 min).

Compound number	**% IL-1β (relative to control)**	**Standard Deviation**	**Statistical significance**[a]
C9	71.5	1.9	*
C21	66.2	33.3	*
C25	56.4	13.0	*
C29	42.3	2.5	***
C33	45.3	5.1	**
C50	49.3	12.8	*
C53	49.5	3.5	*
C54	69.5	17.2	*
C56	51.1	7.0	***
C61	57.2	6.4	***
C62	74.1	9.2	*
C73	40.3	20.4	*
C75	20.9	9.3	**
C78	62.4	5.8	*
C84	57.0	6.8	*
C87	38.1	7.9	***
C94	50.1	4.2	*
C95	47.3	7.7	*
C97	21.3	9.9	***

[a] n=3, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

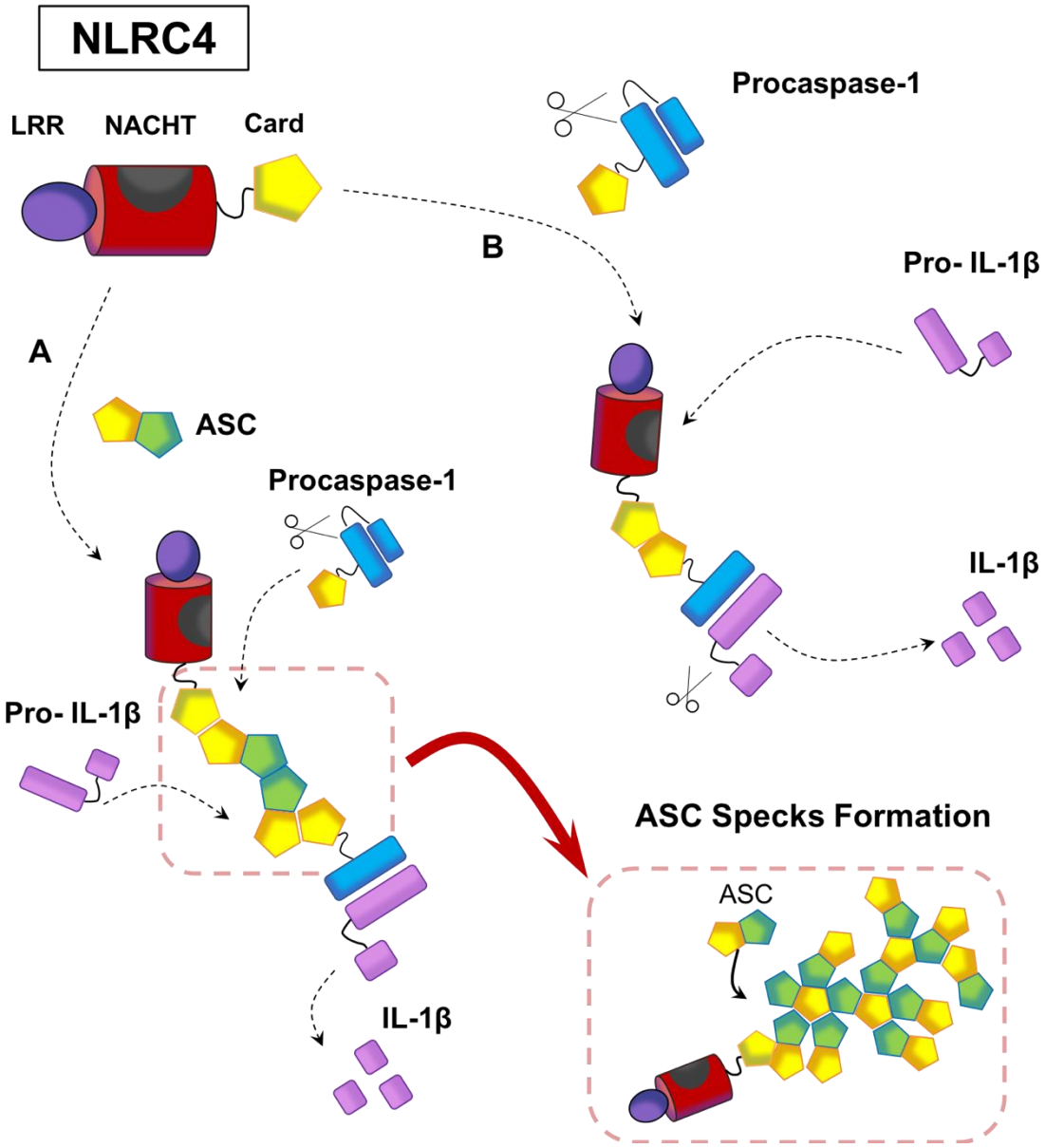

Figure S1. Activation of the NLRC4 inflammasome. The NLRC4 inflammasome is organized in three different domains; a leucine rich repeat domain (LRR), a nucleotide binding and oligomerization domain, and a card domain. Following activation, through its card domain, NLRC4 can directly interact with procaspase-1 (B), or can recruit the scaffolding protein ASC. ASC recruits procaspase-1, which induce its autoproteolytic activation and the subsequent binding and cleavage of Il-1β. Additionally, ASC homo-oligomerizes forming large specks, which propagates inflammation and present prionoid properties.

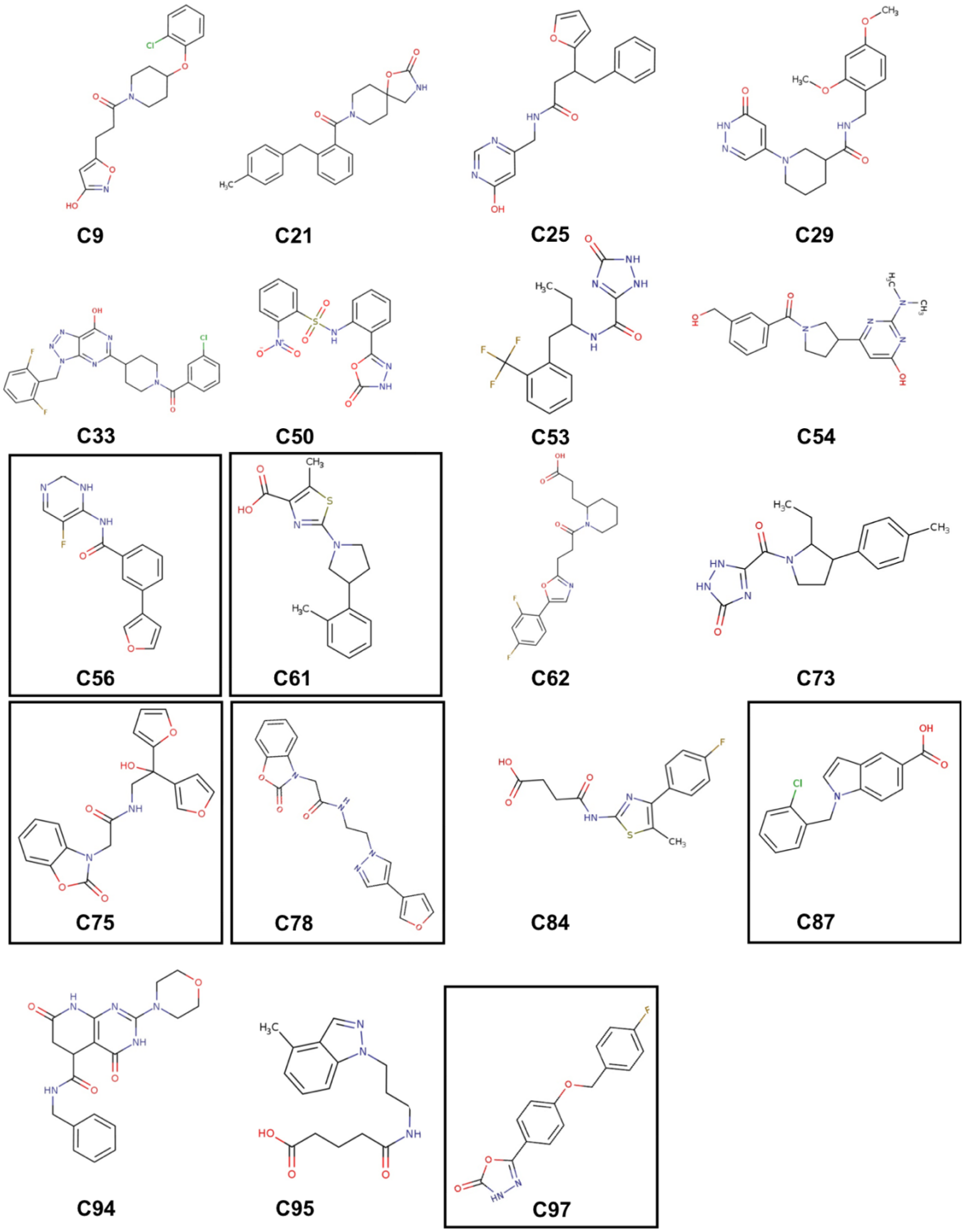

Figure S2. Chemical structure of the positive compounds from the primary screening.

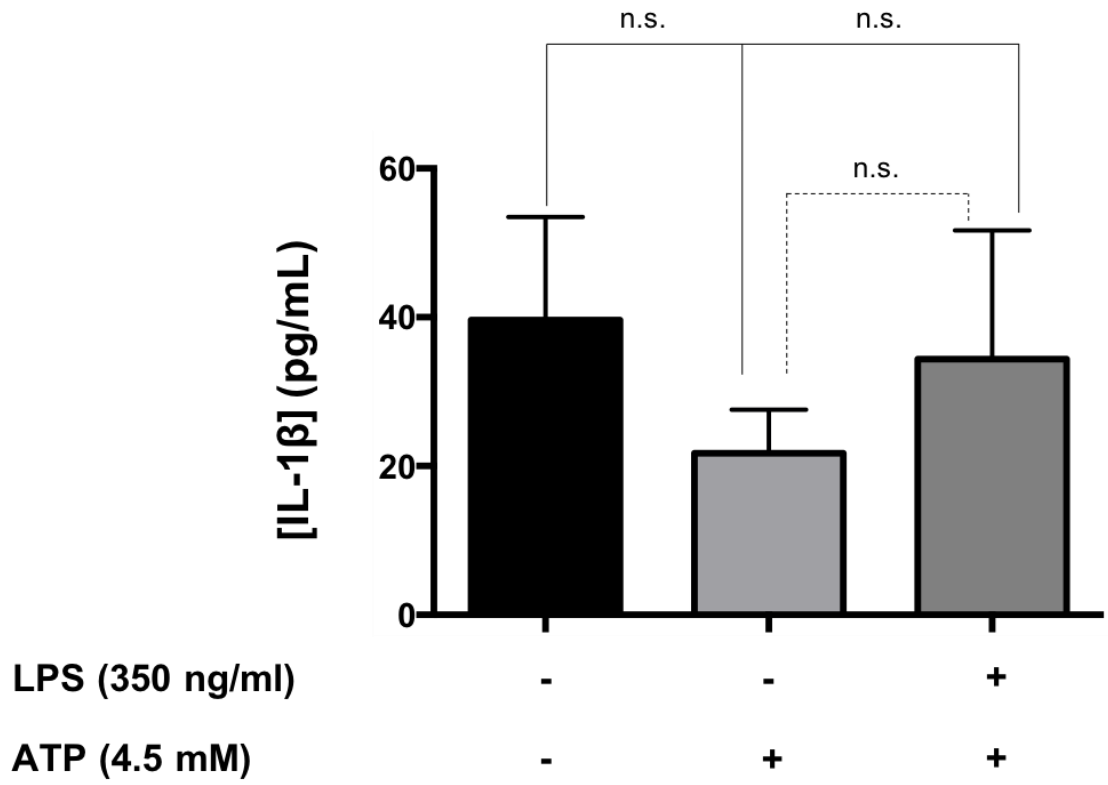

Figure S3. Treatment with LPS and ATP specifically activates NLRP3. Microglia cultures from NLRP3 KO mice was primed with 350 ng/ml LPS for 3 hours and subsequently activated with 4.5 mM ATP for 45 minutes. Data sets are compared with control samples (untreated cells).

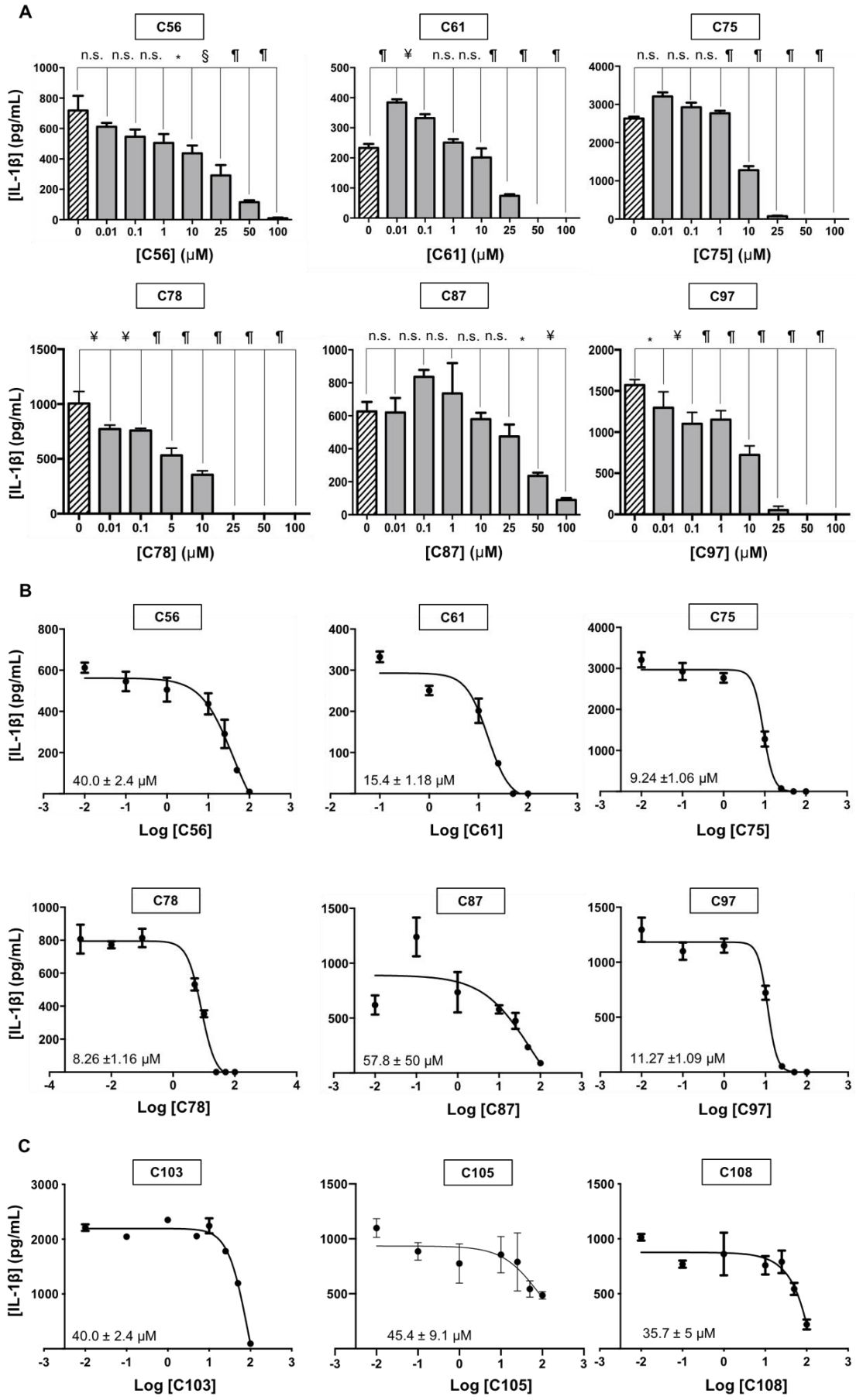
A
C56
C61
C75
C78
C87
C97
[IL-1β] (pg/mL)
[C56] (μM)
[C61] (μM)
[C75] (μM)
[C78] (μM)
[C87] (μM)
[C97] (μM)
B
Log [C56]
Log [C61]
Log [C75]
Log [C78]
Log [C87]
Log [C97]
40.0 ± 2.4 μM
15.4 ± 1.18 μM
9.24 ±1.06 μM
8.26 ±1.16 μM
57.8 ± 50 μM
11.27 ±1.09 μM
C
C103
C105
C108
Log [C103]
Log [C105]
Log [C108]
40.0 ± 2.4 μM
45.4 ± 9.1 μM
35.7 ± 5 μM

Figure S4. Dependency of the inhibitory effect with the concentration of the six compounds selected from the secondary *in-vitro* screening, as well as for the benzoxazolone acetamidyl derived compounds. Experiments were carried out in triplicate, and each sample was run in duplicate. Data sets are compared with controls (microglia cultures pretreated with the same DMSO %, and activated with LPS+ATP). Significance levels are indicated as * $p \leq 0.05$, ¥ $p \leq 0.01$, § $p \leq 0.001$, ¶ $p \leq 0.0001$.

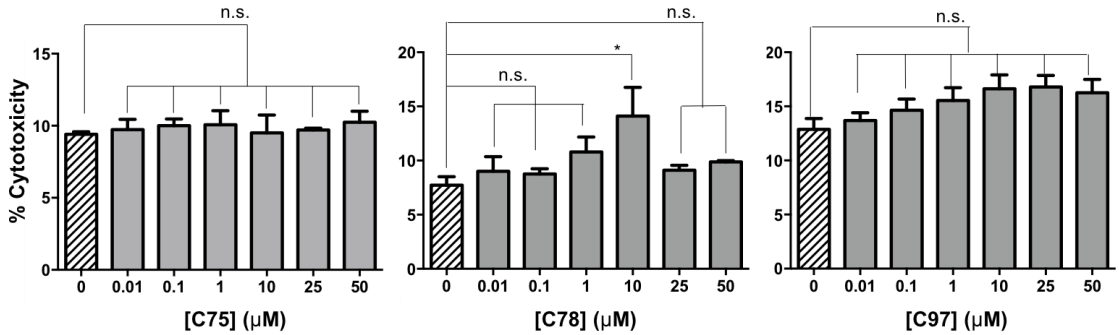

Fig S5. Cytotoxicity exerted by different concentrations of C75, C78 and C97. C78 presents a statistically significant increase in the cytotoxicity at 10 μM when compared with vehicle-treated cells ($p \leq 0.05$). However, since at higher concentrations, C78 is not toxic, and due to the significant standard deviation between replicates for this concentration, we consider this single point as an artifact.

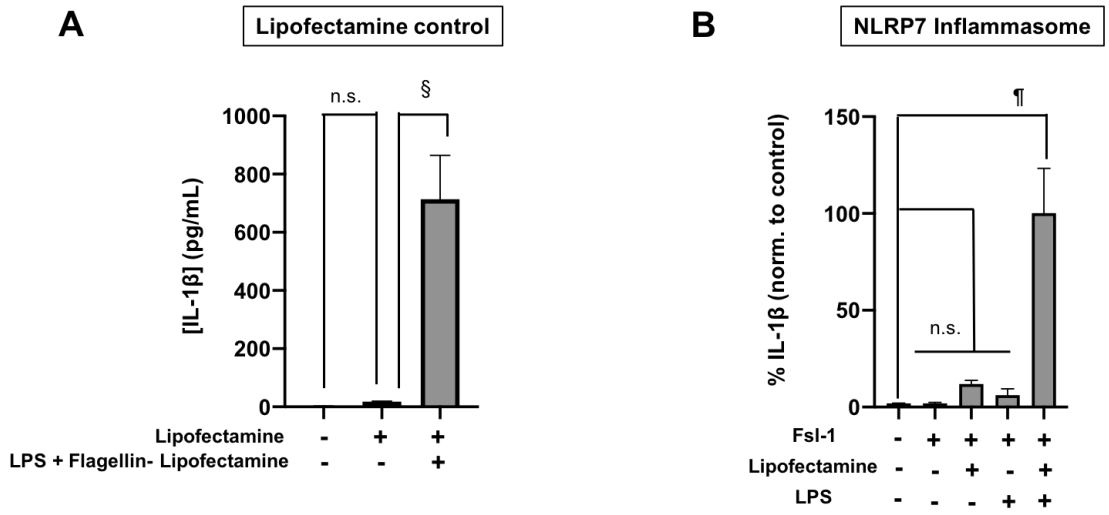

Figure S6. A) Effect of lipofectamine on microglia cultures. Primary microglia were treated with the same lipofectamine amount that used for flagellin transfection. Lipofectamine does not modify the IL-1β released to the extracellular medium. B) Conditions assayed for NLRP7 activation. Microglia cultures were kept untreated, as control, treated with 400 ng/ml fsl-1 for 7 hours; fsl-1 400 ng/ml transfected with lipofectamine, 150 ng/ml LPS for 1 hour prior to treatment with 400 ng/ml fsl-1, or 150 ng/ml LPS for 1 hour prior to 400 ng/ml transfection with lipofectamine. Experiments were carried out in biological triplicates, and samples were run in duplicate. Data sets are compared with control. Significance levels are indicated as * $p \leq 0.05$, ¥ $p \leq 0.01$, § $p \leq 0.001$, ¶ $p \leq 0.0001$.

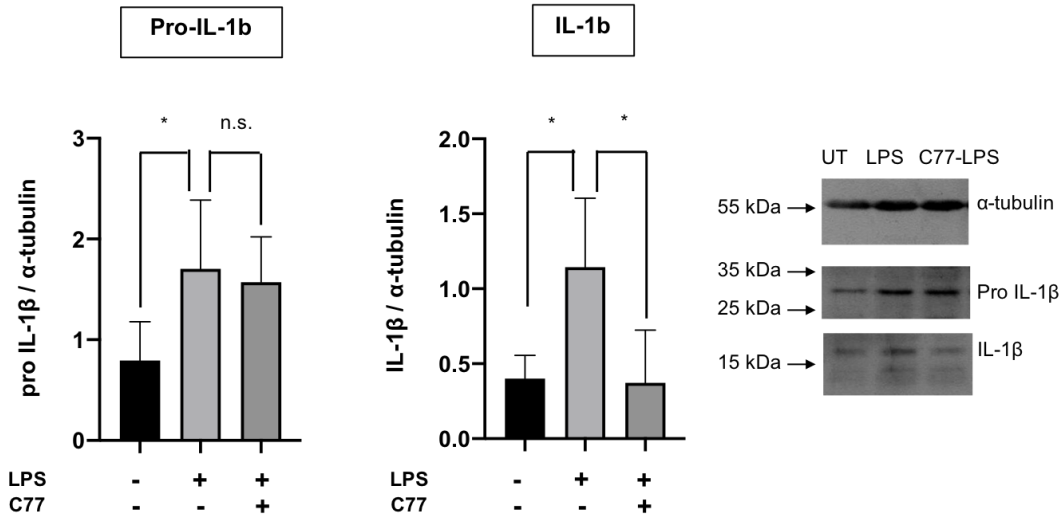

Figure S7. Western-blot quantification of pro IL-1β and IL-1β in the FC of mice treated with DMSO/PBS as vehicle, DMSO/LPS (15 mg/kg), or C77 (50 mg/kg)/LPS 15 mg/kg. n=4 animals. Significance levels are indicated as * p≤0.05, ¥ p≤0.01, § p≤0.001, ¶ p≤0.0001.

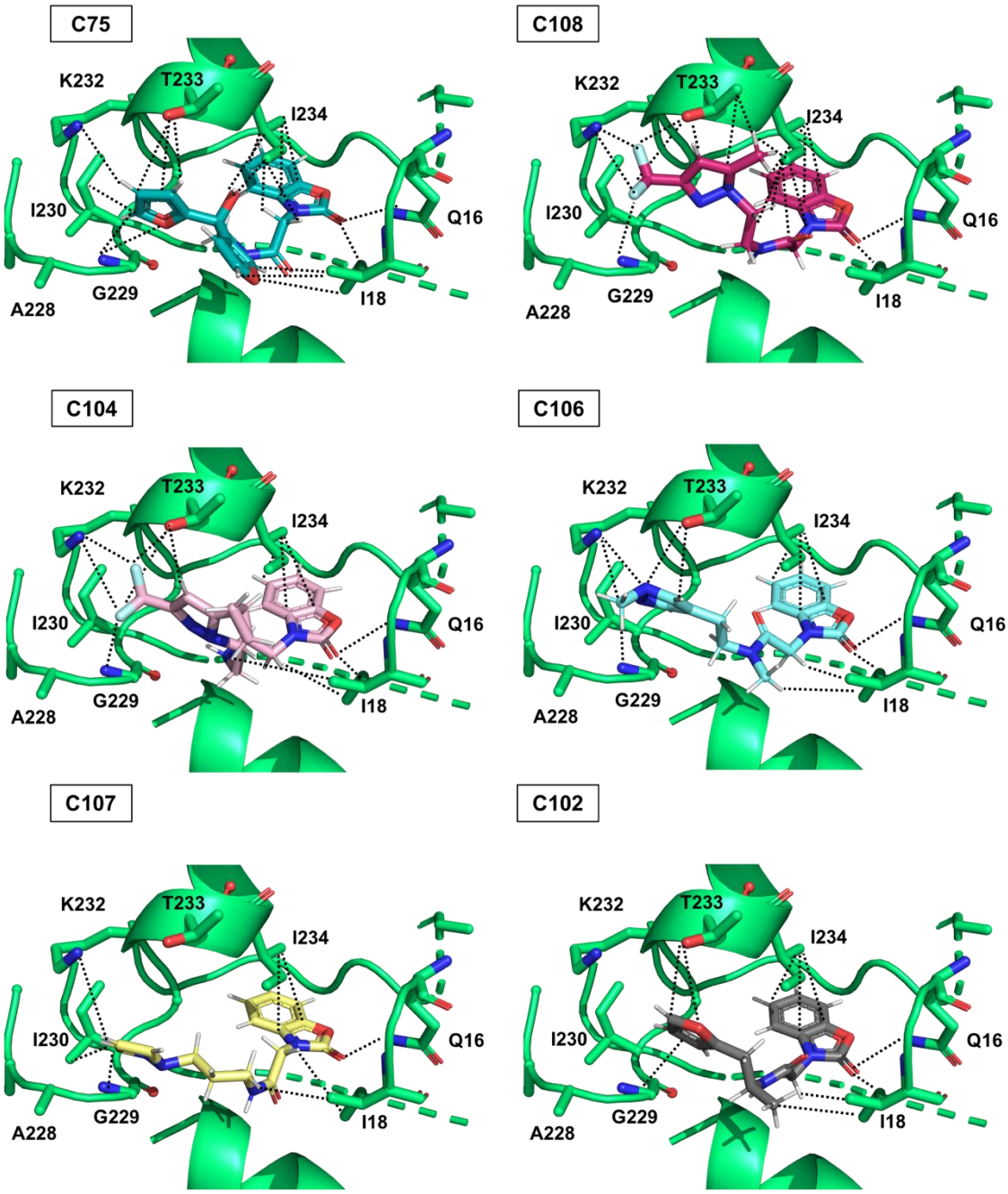

Fig S6. Structural models for the interaction of the analogs within the ATP binding pocket. The compounds are ordered according to their in-vitro ability to inhibit NLRP3-mediated IL-1β secretion, starting from C75. Thus, C75>C108> C104>C106>C107~C102. The models were obtained through computational docking, (http://www.swissdock.ch/) and represented using pymol (https://pymol.org/2). Residues stabilizing the inhibitors are shown in sticks and colored in CPK code. Interactions between side chains of these residues and the compounds are displayed in dotted lines.

Supplementary methods

Calculation of IC50 values

The IC50 value for an inhibitory process is defined as the concentration of an inhibitor required to half-reduced the measured parameter. In this manuscript, it refers to the concentration of a specific compound to reduce by the 50% the IL-1β concentration in cell supernatant (for inflammasome inhibition), or the ATP transformed into ADP (in the case of the ATPase activity). IC50 values were calculated through non-lineal regression of the values obtained in dose-response curves to a "log [Cx] vs response" model implemented in GraphPad 8, according to Eq. S1.

Eq. S1 Y=Bottom + (Top-Bottom)/(1+10^(X-LogIC50))

Where Y represents the measured magnitude, Top is the highest value for the measured magnitude, Bottom is the lowest value for the measured magnitude.