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Tyrosine phosphatase inhibition induces an ASC-dependent pyroptosis

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ABSTRACT

Pyroptosis is a type of cell death in which danger associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) induce mononuclear phagocytes to activate caspase-1 and release mature IL-1β. Because the tyrosine kinase inhibitor AG126 can prevent DAMP/PAMP induced activation of caspase-1, we hypothesized that tipping the tyrosine kinase/phosphatase balance toward phosphorylation would promote caspase-1 activation and cell death. THP-1 derived macrophages were therefore treated with the potent specific tyrosine phosphatase inhibitor, sodium orthovanadate (OVN) and analyzed for caspase-1 activation and cell death. OVN induced generalized increase in phosphorylated proteins, IL-1β release and cell death in a time and dose dependent pattern. This OVN induced pyroptosis correlated with speck formations that contained the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). Culturing the cells in the presence of extracellular K^+ (known to inhibit ATP dependent pyroptosis), a caspase inhibitor (ZVAD) or down regulating the expression of ASC with stable expression of siASC prevented the OVN induced pyroptosis. These data demonstrate that pyroptotic death is linked to tyrosine phosphatase activity providing novel targets for future pharmacologic interventions.

1. Introduction

Pyroptosis is a novel type of inflammatory programmed cell death that is mediated by the inflammatory enzyme, caspase-1 [1]. This type of cell death was initially described with *Salmonella typhimurium* infected macrophages [2,3]. Pyroptosis is distinguished from apoptosis and necrosis by its dependency on caspase-1 resulting in a cell death pattern that is a blend of the apoptosis and necrosis [4]. Triggers of pyroptosis in macrophages fall into two categories: pathogen associated molecular patterns (PAMPs), such as viral pathogens and bacterial toxins, and endogenous danger associated molecular patterns (DAMPs), such as monosodium urate and silica [5].

The key hallmark of pyroptosis is the formation of a supramolecular complex of proteins, termed the pyroptosome, which assembles in the cytoplasm immediately preceding the death of the cell [6,7]. This protein aggregate contains the adapter, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1 [8–10]. The formation of this ASC speck is also associated with the death of the host cell and the release of IL-1β and active caspase-1 [4,8].

Caspase-1 is responsible for cleaving the proinflammatory cytokines proIL-1β and proIL-18 into their active mature forms, which are then released into the extracellular environment [11,12]. Caspase-1 is synthesized in its zymogen form and its activation requires the formation of a cytoplasmic multi-protein complex called the inflammasome [5,13,14]. The inflammasome is composed of several proteins, including caspase-1, the adaptor protein ASC, and an intracellular sensor of PAMP/DAMPs. Inflammasomes are named based on the nature of their sensor. Currently, NOD-Like Receptors (NLRP1, NLRP3, NLRC4, NLRC5) [14–16], pyrin [10,17–19], absent-in-melanoma 2 (AIM2) [20–22], and the retinoic acid-inducible gene 1 (RIG-I) [23] have been identified as intracellular sensors that aggregate to form inflammasomes and activate caspase-1.

The aim of this study was to determine whether tyrosine phosphorylation plays a role in pyroptosis. Tyrosine phosphorylation plays a critical role in a large number of fundamental physiological functions [24,25]. Tyrosine phosphorylation of certain proteins is controlled by a delicate balance between two opposing types of enzymes: protein tyrosine kinase (PTKs) and protein tyrosine phosphatase (PTPs) [26]. We previously found that the tyrosine kinase inhibitor, AG126, blocked caspase-1 activation and IL-1β processing and release [27]. Towards our goal to investigate the role of tyrosine

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phosphorylation in inflammasome functioning and pyroptosis, we used a PTP inhibitor, sodium orthovanadate (OVN). OVN maintains proteins in their tyrosine-phosphorylated state. We found that OVN induces pyroptotic cell death of THP-1 derived macrophages and the associated release of the mature form of IL-1β. Thus, inhibition of tyrosine phosphatases activates caspase-1 and ASC-dependent cell death along with IL-1β processing and release.

2. Materials and methods

2.1. Construction of THP-1 cells stably expressing siASC

THP-1 cells were purchased from ATCC (lot 385653). THP-1 derivatives, stably over expressing inflammasome protein ASC (YFP-ASC) were generated with lentivirus constructs, as we described earlier [28]. To generate cells with stably knocked down ASC, we used pGreenPuro (SBI, System Biosciences) and pLenti/V5 (Invitrogen Life Technologies) vectors. siASC sequence (5′-GGCCTGCACTTTATAGACC-3′), scrambled siASC control (5′-TTCCTTACTACACCCTTGG-3′) and control (siEGFP) siRNA (5′-AAGCTGACCCTGAAGTTCA-3′) were synthesized as pGreenPuro-compatible (GATCC-sense-CTTCCTGTCAGA-antisense-TTTTTG and AATTCAAAAA-sense-TCTGACAGGAAG-antisense-G) and pLenti-compatible (CTAGCCC-sense-TTCAAGAGA-antisense-TTTTTGGAAA and CGTTTCCAAAAA-sense-TCTCTTGAA-antisense-GGG) oligonucleotides (Integrated DNA Technologies, Inc.). Corresponding oligonucleotide pair was annealed and ligated into pGreenPuro or pLenti vectors, using *BamHI*/*EcoRI* or *NheI*/*ClaI* restriction sites, respectively. Resulting plasmids were verified by sequencing and used in transfection of packaging cells HEK293FT to generate lentivirus, as we described earlier [10,19]. Co-expression of EGFP or RFP proteins in siRNA constructs were used to sort cells by flow cytometry (FACSAria, Beckton Dickinson). After two rounds of sorting we obtained a nearly homogenous cell line stably knocking down ASC. Levels of RNA and protein expression were reduced by 90%, as was verified by qPCR and immunoblot.

2.2. Cell stimulations and reagents

THP-1 derived macrophages (TDM) were produced as described [19]. Briefly, THP-1 cells were treated with 200 or 500 nM of PMA (Sigma) for 3 h, washed three times and plated in 12 wells plate for 3 days for differentiation. THP-1 and TDM were cultured in RPMI 1640 (MediaTech, Inc) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals) and 1% penicillin-streptomycin (Invitrogen Life Technologies). All cells were regularly checked for the absence of Mycoplasma contamination [29]. Cells were treated with the protein tyrosine phosphatase inhibitor, orthovanadate (Sigma–Aldrich), and stimulated with LPS (from *Escherichia coli* strain 0111:B4; Alexis Biochemicals, San Diego). Cell culture medium was used for detection of LDH and mature IL-1β released while cells pellet was lysed and analyzed for proteins by immunoblots.

2.3. Preparation of cell lysate, immunoblots and ELISA

Cells were lysed in RIPA buffer (50 mM Tris–HCl (pH7.5), 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% NP-40 and 0.25% Na-deoxycholate) supplemented with complete protease inhibitor cocktail (Sigma), 1 mM PMSF and 100 μM N-(methoxysuccinyl)-Ala-Ala-Pro-Val chloromethylketone - CMK). The protein concentrations were determined using Bio-Rad Dc protein Lowry assay (Bio-Rad). After SDS–PAGE gel separation, samples were transferred to a nitrocellulose membrane, probed with the antibody of interest and developed by ECL (Amersham Biosciences). Rabbit polyclonal antibodies against IL-1β were developed in our laboratory, as described [30]. Anti-phosphotyrosine antibodies (P-Tyr-100 and P-Tyr-102) were purchased from Cell Signaling. Released IL-1β was quantified using ELISA from R&D Systems, according to the manufacturer's protocol. In addition, IL-1β in the cell culture medium was detected by immunoblot of cell culture medium with our anti-IL-1β antibody.

2.4. Cell death detection by quantification of lactate dehydrogenase (LDH) release in cell culture medium

LDH release into cell culture medium was used as an indicator of cell death using NAD^+ reduction assay (Roche Applied Science). Cells were plated in 12-well plate at the density 1×10^6/ml and stimulated with OVN, K^+ and LPS for various time points. Cell culture medium was collected, clarified by centrifugation, and used for LDH assay. Total LDH content in cells (positive control) was measured in cells lysed with Triton X-100 (1% final concentration). Cell culture medium alone was used as a blank and OD values were subtracted from readings of samples and positive control. LDH concentration in the medium was detected at wavelength 490 nm. Cell death was calculated by the formula: [cytotoxicity (%) = (sample/positive control) × 100], as described earlier [31].

2.5. Pyroptosome counting by microscopy

TDM stably expressing YFP-ASC were differentiated in 24-well plate at density 5×10^5/ml and stimulated with OVN, K^+ and LPS overnight (15–16 h). Next morning live cell images were acquired with Olympus IX50 inverted microscope at 10 and 20× magnification, and ASC specks were counted in three random areas in each well.

2.6. Statistical analysis

All experiments were performed a minimum of three independent times and expressed as mean values ± SE. Comparison of groups for statistical difference were done using Student's *t* test or ANOVA with post hoc measures where indicated. p value $\leqslant 0.05$ was considered significant.

3. Results

3.1. OVN induces processing and release of IL-1β in a time and dose dependent manner

The detailed events that result in activation of the caspase-1 inflammasome remain incompletely understood. Because we and others had previously shown that a tyrosine kinase inhibitor can suppress the ability of ATP to induce monocyte IL-1β processing and release [27,32,33], we elected to characterize the general role that tyrosine phosphatases might play in this process. Toward this goal, THP-1 derived macrophages (TDM) were stimulated with OVN and supernatants analyzed for processed IL-1β. As shown in Fig. 1A, OVN in the absence of additional stimuli can induce a robust, dose dependent processing and release of mature IL-1β.

The time course of the phosphatase inhibition effect was also dependent upon OVN dose. Fig. 1B shows the time course for 100 μM OVN. Low dose OVN (50 μM) required 9 h to see a significant IL-1β response whereas 200 μM OVN induced release as early as 4 h after stimulation (data not shown).

3.2. IL-1β release associated with LDH release

Pyroptosis is a form of cell death that depends upon caspase-1 activation in the context of the release of IL-1β [34]. We therefore

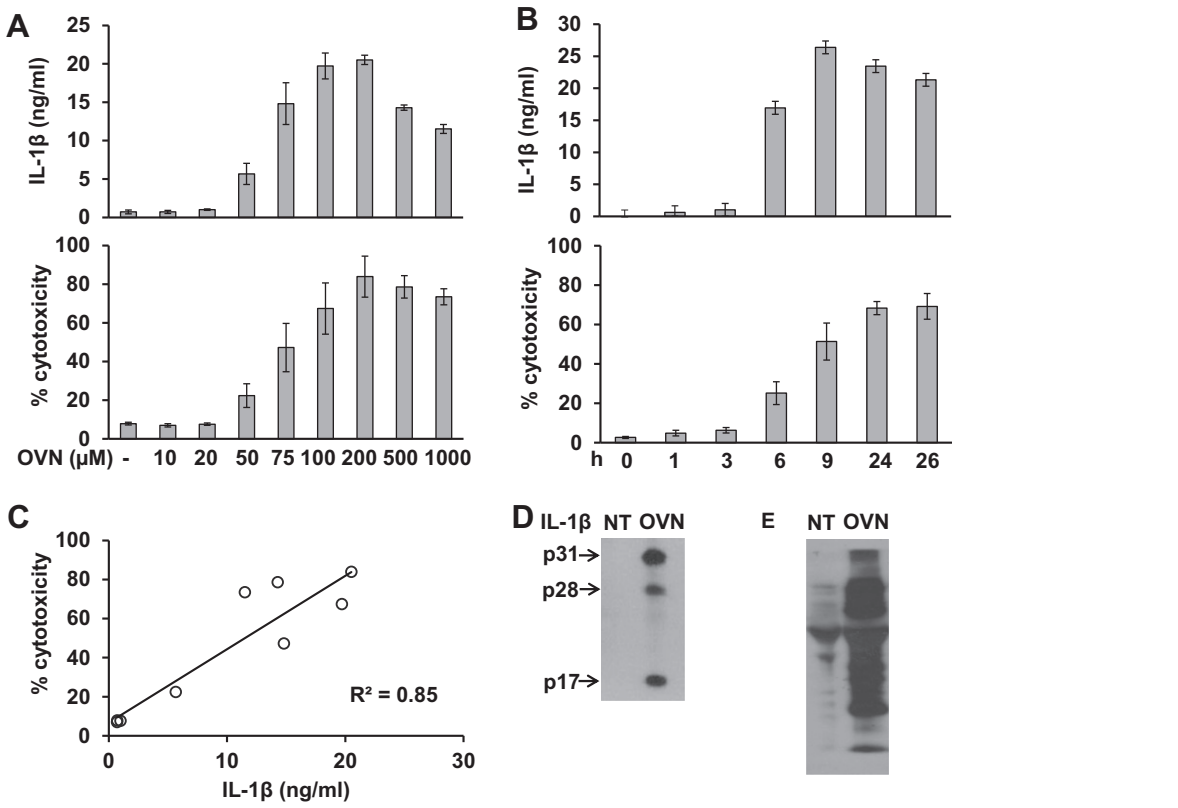

Fig. 1. Phosphatase inhibitor orthovanadate induces inflammasome activation and pyroptosis in a dose and time dependent manner. THP-1 derived macrophages (10^6/well), were treated with different concentrations of OVN (10–1000 μM) overnight and analyzed for IL-1β release and percentage of cell death (A) and the correlation between the two (C). Time course for L-1β release and cell death by THP-1 derived macrophages (10^6/well) treated with 100 μM OVN for different time points (1–26 h) (B). IL-1β Immunoblot of cell culture media to determine the forms of IL-1β released from OVN stimulated cells (D). Immunoblot of cell extracts from these cells with P-TYR-102 antibody shows the effect of OVN treatment on the total pool of tyrosine phosphorylated proteins (E).

compared the release of lactate dehydrogenase (LDH) with the release of IL-1β in response to OVN. Consistent with the induction of pyroptosis, the processing and release of mature IL-1β was highly associated with the release of LDH (Fig. 1C; $r^2 = 0.85$).

To confirm that the OVN did induce caspase-1 activation we analyzed TDM supernatants for IL-1β. Fig. 1D shows the cleavage of proIL-1β into two known products, 28 and 17 kDa IL-1β. Finally, to confirm an OVN effect on tyrosine phosphorylation, OVN treated TDMs were compared to untreated cells for the generation of phosphoproteins as detected by a phosphotyrosine specific antibody (Fig. 1E).

3.3. ASC specks induced by OVN

The strong correlation between IL-1β processing and release and LDH release suggested the process of pyroptosis, i.e. cell death associated with caspase-1 activation. ASC speck formation is a characteristic hallmark of pyroptosis [6,8,35,36]. To analyze the role of OVN in the induction of the ASC speck we utilized TDMs that stably expressed yellow-fluorescent protein ASC (YFP-ASC) and exposed them to overnight incubation with LPS or OVN to observe for the generation of ASC specks. As shown, OVN effectively induced the generation of ASC specks (Fig. 2A and B).

3.4. OVN enhances lipopolysaccharide effect

TDMs stimulated with LPS induce processing and release of IL-1β and OVN alone induces even more. However, when LPS was combined with OVN, the pyroptosome formation and release of IL-1β were further augmented (Fig. 2); indicating that phosphatases are likely involved in the regulation of the LPS induced activation events.

3.5. Extracellular K^+, siASC and caspase inhibition suppress OVN induced pyroptosis

That this process was dependent upon inflammasome activation was further confirmed by pre-incubating the TDMs with K^+, which is known to inhibit activation of the NLRP3 inflammasome [37]. Extracellular K^+ (100 mM) significantly suppressed the release of processed IL-1β, speck formation and the release of LDH consistent with the hypothesis that OVN is having a direct effect on the regulation of caspase-1 activity (Fig. 3A–C) [38,39].

Since most, if not all, inflammasome structures depend on the adaptor ASC, we tested the ability of stable siASC knockdown in THP-1 cells to suppress the ability of OVN to induce cell death. As shown in Fig. 4A siASC expressing cells effectively suppressed ASC protein expression. Furthermore, as expected for a cell death dependent upon inflammasome function, ASC knockdown prevented the OVN induced cell death and concomitant IL-1β (Fig. 4B and C).

Finally, to verify the role of caspases in OVN induction of inflammasome activation and pyroptosis, TDM were stimulated either with OVN or LPS + ATP (a classic inducer of inflammasome activation) in the presence and absence of pan-caspase inhibitor ZVAD-cmk. As shown in Table 1, the caspase inhibitor completely abolishes LPS + ATP and OVN induction of cell death and IL-1β processing and release, further confirming that tyrosine phosphorylation plays important role in inflammasome activation and pyroptosis.

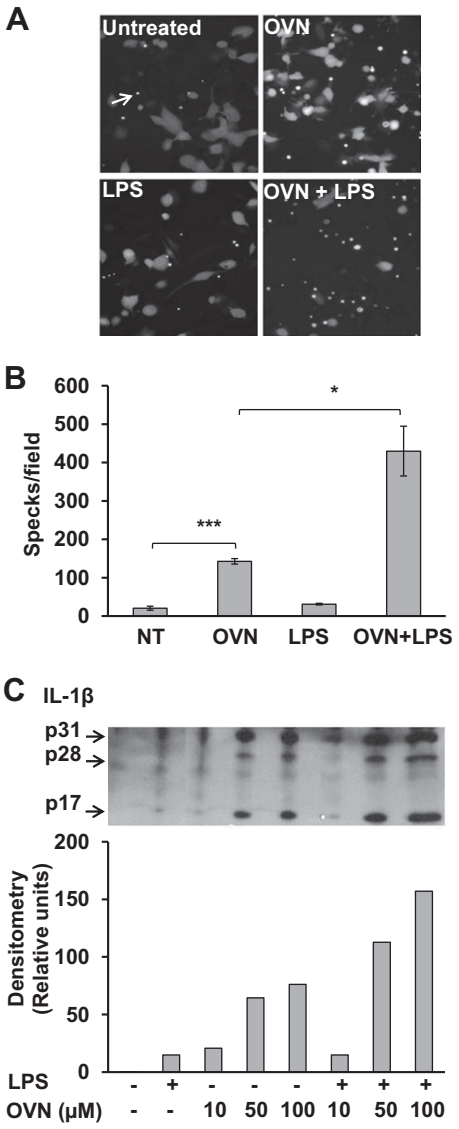

Fig. 2. OVN and LPS lead to synergism between pyroptosome formation and IL-1β release. THP-1 derived macrophages stably over expressing YFP-ASC were treated either with OVN (100 μM) and LPS (1 μg/ml) alone or OVN together with LPS overnight and then YFP-ASC specks (pointed by white arrow) were pictured and counted per field ($n = 6$ independent experiments) (A and B). Release of IL-1β correlates with speck formation as shown by an immunoblot of cell culture medium (C) and relative densitometry of mature IL-1β bands (D). $^{*}p < 0.05$, $^{***}p < 0.001$.

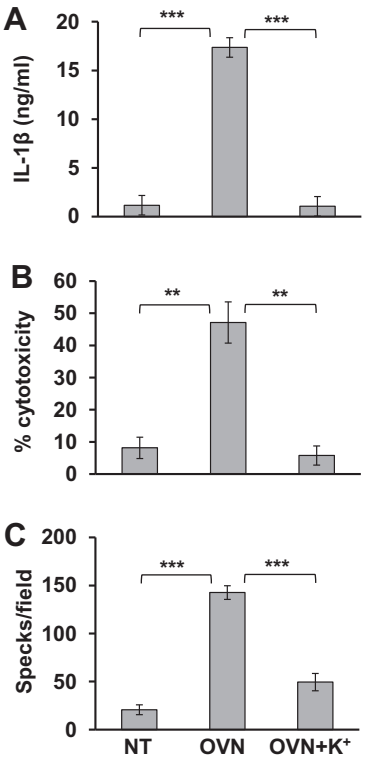

Fig. 3. Potassium inhibits inflammasome activity induced by OVN. TDM were pretreated with 100 mM potassium for 30 min followed by OVN (100 μM) overnight and analyzed for IL-1β release, cell death and ASC speck formation, respectively (A–C), $n = 3$ independent experiments for (A) and (B) and $n = 6$ independent experiments for (C). $^{**}p < 0.01$, $^{***}p < 0.001$.

4. Discussion

Sodium orthovanadate (OVN) is a phosphate analogue that consists of a core vanadium atom in place of phosphorous, and has been shown to specifically inhibit PTP [40]. As shown OVN causes unrestrained tyrosine phosphorylation, which correlates with significantly enhanced secretion of mature IL-1β from the cell, an increase in the number of "ASC specks" and the death of the monocyte. OVN induced cell death occurs by a specific pathway since inhibition of inflammasome formation, down regulation of ASC or inhibiting caspase-1 is not cytotoxic to the cell, as demonstrated by extracellular K^+ treatment, siASC knockdown and ZVAD-cmk inhibition, respectively.

How caspase-1 induces a coordinated form of cell death is unclear. Similar to apoptosis, pyroptosis induces DNA fragmentation; however the resulting DNA is diffuse and not condensed. Contrary to apoptosis and similar to necrosis, pyroptosis results in membrane rupture and release of cytosolic contents which is specifically due to activated caspase-1 [34]. This unique form of cell death is preceded by the aggregation of inflammasome proteins into an "ASC speck" and the release of mature IL-1β. The mechanisms that trigger inflammasome formation and activation of caspase-1 to process proIL-1β into its mature form are also poorly understood.

What is evident is that this inflammatory event is triggered in response to a PAMP/DAMP and this activation is tightly regulated at a minimum of two fundamental levels. Both, the enzyme and its cytokines, are synthesized in their pro-forms and require formation of a complex of proteins to scaffold the caspase-1 enzyme which will subsequently cleave and activate the effecter cytokines. We hypothesized that this regulation extends further upstream and involves the phosphorylation of proteins involved in a signaling cascade which enhances inflammasome formation. In agreement with this concept, we have previously shown that the tyrosine kinase inhibitor, AG126, decreases caspase-1 activation and IL-1β release response to LPS and ATP [27].

In addition to inhibiting inflammasome function by inhibiting tyrosine kinases, we show here that inflammasome function is enhanced and pyroptosis is induced by inhibiting tyrosine phosphatases. The balance between kinase and phosphatase activity is tightly regulated to maintain homeostatic function within cells. However, many questions remain about how tyrosine phosphorylation events trigger inflammasome activity and pyroptosis. Are the inflammasome proteins directly phosphorylated or do they lie downstream of a signaling pathway that is triggered in response to a PAMP/DAMP?

There have been prior linkages that support a role for phosphatases in inflammasome activity, For example, Alnemri et al. showed in their work on pyroptosis that the intracellular receptor pyrin

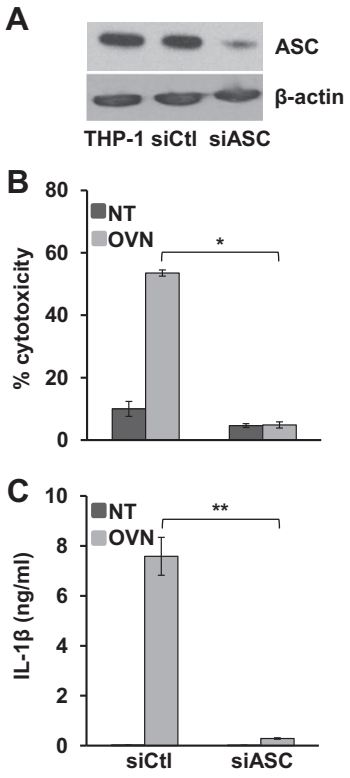

Fig. 4. Knocking down ASC inhibits inflammasome activation and pyroptosis induced by OVN. Effect of stable expression of siASC on THP-1 ASC protein expression as compared to untreated cells and cells expressing siControl. Shown is immunoblot for ASC (top) and actin control (bottom) (A). Effect of stable siASC expression on THP-1 cell death as measured by LDH release (B) and inflammasome activation as measured by mature IL-1β release (C) $n = 3$ independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 1
Caspase inhibition eliminates increased IL-1β release and TDM pyroptosis induced by OVN.[a]

Stimulus	IL-1β (ng/ml)	% cytotoxicity
Untreated TDM	1.41 ± 0.23	7.71 ± 0.06
OVN	17.7 ± 1.01*	53.2 ± 6.93*
OVN + ZVAD-cmk	0.96 ± 0.11**	6.45 ± 0.57**
LPS + ATP	4.67 ± 0.99	13.6 ± 2.71
LPS + ATP + ZVAD-cmk	0.61 ± 0.13***	6.99 ± 1.83***

[a] Data represent the mean ± SE of 3 independent experiments. TDM treated with OVN or LPS + ATP were compared to untreated cells and effect of pan caspase inhibitor ZVAD was compared to corresponding treatment groups. Asterisks indicate $p < 0.05$ using ANOVA with Tukey's post hoc comparisons for * (OVN vs. untreated); ** (OVN vs. OVN + ZVAD); *** (LPS + ATP vs. LPS + ATP + ZVAD).

assembles into an ASC speck through its binding to the proline serine threonine phosphatase-interacting protein 1 (PSTPIP1). This event triggers IL-1β release and pyroptosis [8]. This link between pyrin and PTSTPIP1 connected two inherited auto-inflammatory disease, familial Mediterranean fever (FMF) and pyogenic arthritis, pyoderma gangrenosum (PAPA syndrome), which have mutations in the respective proteins and are responsive to IL-1β antagonist treatments.

Of relevance to our findings here, PSTPIP1 interacts with PEST-type protein tyrosine phosphatase (PTP-PEST). Shoham et al. over expressed c-Abl kinase which increased PSTPIP1 phosphorylation, as well as its association with pyrin, ultimately resulting in enhanced IL-1β secretion [41]. The association of kinases and phosphatases with the pyrin inflammasome and ASC pyroptosome appears to be a critical regulatory mechanism.

PSTPIP1 is involved in cytoskeletal organization, and ASC has been shown to co-localize with the actin cytoskeleton throughout the cytoplasm [42,43]. Upon the induction of pyroptosis, ASC aggregates into one location forming an ASC speck, followed by lysis of the cell. We have shown that the formation of an ASC speck uses a phosphorylation pathway. The identity of the proteins involved in regulating phosphorylation is critical to our understanding of how PAMP/DAMPs induce inflammasome activation and pyroptosome formation.

PAMP/DAMPs trigger the formation of the inflammasome through intracellular receptors. It is interesting to note that in our study, OVN alone triggered pyroptosis in the absence of any PAMP/DAMP. When combined with the classic inflammasome primer and TLR4 agonist, LPS, OVN enhanced the secretion of mature IL-1β and induction of pyroptosis. This finding implicates an indirect connection between classical inflammasome ligands and the inflammasome, through a phosphorylated intermediate. Alternatively, the over activation of kinases may result in the formation of an endogenous DAMP which is detected by inflammasome receptors. A screen of specific PTK and PTP inhibitors is needed to determine which pathways are connected to this caspase-1/IL-1β axis. Lastly, it is intriguing to hypothesize that the generation of reactive oxygen species in response to DAMP/PAMPs may directly activate the inflammasome by inhibiting tyrosine phosphatases.

Thus, the identification of the specific upstream kinase(s) that regulate the caspase-1/IL-1β axis is a target for future studies that should generate novel therapeutic opportunities to treat a broad range of inflammatory disorders, particularly the IL-1β-associated auto-inflammatory diseases.

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