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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