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Research paper

A bioluminescent caspase-1 activity assay rapidly monitors inflammasome activation in cells☆

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ABSTRACT

Inflammasomes are protein complexes induced by diverse inflammatory stimuli that activate caspase-1, resulting in the processing and release of cytokines, IL-1β and IL-18, and pyroptosis, an immunogenic form of cell death. To provide a homogeneous method for detecting caspase-1 activity, we developed a bioluminescent, plate-based assay that combines a substrate, Z-WEHD-aminoluciferin, with a thermostable luciferase in an optimized lytic reagent added directly to cultured cells. Assay specificity for caspase-1 is conferred by inclusion of a proteasome inhibitor in the lytic reagent and by use of a caspase-1 inhibitor to confirm activity. This approach enables a specific and rapid determination of caspase-1 activation. Caspase-1 activity is stable in the reagent thereby providing assay convenience and flexibility. Using this assay system, caspase-1 activation has been determined in THP-1 cells following treatment with α-hemolysin, LPS, nigericin, gramicidin, MSU, R848, Pam3CSK4, and flagellin. Caspase-1 activation has also been demonstrated in treated J774A.1 mouse macrophages, bone marrow-derived macrophages (BMDMs) from mice, as well as in human primary monocytes. Caspase-1 activity was not detected in treated BMDMs derived from Casp1$^{-/-}$ mice, further confirming the specificity of the assay. Caspase-1 activity can be measured directly in cultured cells using the lytic reagent, or caspase-1 activity released into medium can be monitored by assay of transferred supernatant. The caspase-1 assay can be multiplexed with other assays to monitor additional parameters from the same cells, such as IL-1β release or cell death. The caspase-1 assay in combination with a sensitive real-time monitor of cell death allows one to accurately establish pyroptosis. This assay system provides a rapid, convenient, and flexible method to specifically and quantitatively monitor caspase-1 activation in cells in a plate-based format. This will allow a more efficient and effective assessment of inflammasome activation as well as enable high-throughput screening for inflammasome modulators.

1. Introduction

Our innate immune system rapidly responds to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) with inflammation. Inflammasomes are protein complexes induced by these diverse PAMPs and DAMPs that recruit and activate caspase-1. Inflammasomes are initiated by the Nod-like receptor (NLR) or PYHIN family of proteins and recruit the adaptor protein, ASC, and caspase-1 resulting in oligomerization and auto-activation of caspase-1 (Lamkanfi and Dixit, 2014). Inflammasome formation and caspase-1 activation have two important consequences: processing and release of the cytokines IL-1β and IL-18, and pyroptosis, an inflammatory caspase-dependent form of programmed cell death (Fink and Cookson, 2005). Recent studies are beginning to delineate the details of inflammasome activation, including non-canonical inflammasome pathways (Kayagaki et al., 2011, 2013; Hagar et al., 2013) and

Abbreviations: IL-1β, interleukin 1-beta; IL-18, interleukin-18; LPS, lipopolysaccharide; MSU, monosodium urate crystals; Pam3CSK4, synthetic triacylated lipopeptide; BMDM, bone marrow-derived macrophage; PBMC, peripheral blood mononuclear cell; DAMP, damage-associated molecular pattern; PAMP, pathogen-associated molecular pattern; NLRP, nod-like receptor protein; ASC, Apoptosis-associated Speck-like protein containing CARD; TLR, Toll-like receptor; XIAP, X-linked inhibitor of apoptosis protein; PMA, phorbol 12-myristate 13-acetate; P2X7R, P2X purinoceptor 7; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer.

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'alternative canonical' pathways (Gaidt et al., 2016). However, in all pathways, caspase-1 becomes activated (Man and Kanneganti, 2016), thus caspase-1 remains the essential mediator of inflammasome function and its activity is a direct marker of inflammasome activation.

Caspase-1 activity is typically monitored by western blot analysis of cleaved caspase-1 or by ELISA for released caspase-1. Western blots of processed IL-1β and IL-18 or ELISA of released cytokines are frequently used as downstream indicators for inflammasome activation and presumed caspase-1 activation. However, western blots and ELISAs do not directly monitor active caspase-1 and autoproteolysis of caspase-1 is not necessary for activity (Broz et al., 2010). Furthermore, assessing inflammasome activation indirectly with an IL-1β ELISA can sometimes be problematic due to assay cross-reactivity with pro-IL-1β. Pro-IL-1β can be released from cells when there is cytotoxicity (Lopez-Castejon and Brough, 2011; van de Veerdonk et al., 2011). Another method to monitor active caspases, FLICA (Fluorescently Labeled Inhibitors of CAspase), requires a wash step, an inherent problem for capturing active caspase-1 since it is typically released from cells following inflammasome activation. Genetic constructs used as biosensors for caspase-1 activity have also been described, but require being transfected/transduced into cells and may impact the endogenous pathway (Bartok et al., 2013; Liu et al., 2014).

We wanted to develop a simple and rapid method to directly monitor active caspase-1 in cells. A bioluminescent, coupled-enzyme assay utilizing a *Z*-WEHD-aminoluciferin substrate for caspase-1 was tested and shown to be capable of measuring caspase-1 activity in a variety of inflammasome activation models. Total caspase-1 activity can be monitored by adding the lytic reagent directly to the cell culture well. Alternatively, caspase-1 activity released into the medium can be measured independently from intracellular caspase-1 activity by transferring the culture supernatant to a new well. Despite some cross-reactivity between caspases and tetrapeptide substrates (McStay et al., 2008), we show that by including the inhibitor, Ac-YVAD-CHO, at a caspase-1 specific concentration, we can distinguish caspase-1 activity from the activity of other caspases, and pyroptosis from apoptosis.

The role of caspase-1 in processing of IL-1β and IL-18 is well defined (Thornberry et al., 1992; Martinon et al., 2002), but how the cytokines are released is not completely understood (Zhang et al., 2015). In some cases, evidence suggests that pyroptosis is the mechanism for IL-1β and IL-18 release (Liu et al., 2014), but there is also evidence indicating that IL-1β and IL-18 are processed and released in the absence of pyroptosis (Pelegrin et al., 2008; Gaidt et al., 2016), particularly in neutrophils (Chen et al., 2014). There is also evidence that indicates that pyroptosis is an effective defense mechanism against certain intracellular pathogens even in the absence of IL-1β and IL-18 release (Miao et al., 2010; Jorgensen and Miao, 2015). How caspase-1 and other inflammatory caspases regulate pyroptosis is just beginning to be understood (Shi et al., 2015; Kayagaki et al., 2015; Russo et al., 2016). Having a cell-based caspase-1 activity assay that can be conveniently multiplexed with assays for cell death and cytokine release is important for gaining a better understanding of inflammasome engagement and its various outcomes.

Some inflammasomes are activated by NLR recognition of specific triggers, e.g. NLRP1 assembles in response to anthrax toxin and NLRC4 responds to intracellular flagellin (Levinsohn et al. 2012; Franchi et al., 2006; Miao et al., 2006; Zhao et al., 2011). However, a mechanism for NLRP3 inflammasome activation has been more difficult to define due to the diversity among its agonists, which include ATP, nigericin, pore-forming toxins, uric acid crystals, and alum salts as well as LPS, peptidoglycans, and viral and bacterial RNA and DNA (Franchi et al., 2012; Vanaja et al., 2015; Elliott and Sutterwala, 2015). A two-signal mechanism for NLRP3 activation has been proposed whereby signal 1 is required to upregulate expression of NLRP3 and proIL-1β (Bauernfeind et al., 2009). Then signal 2 can induce NLRP3 inflammasome activation leading to caspase-1 activation, maturation of proIL-1β to IL-1β, and release extracellularly. However, a non-transcriptional process has also been proposed to be the basis of signal 1 (Juliana et al., 2012).

While there is wide consensus that signal 1 is provided by PAMPs recognized by toll-like receptors (TLR), the nature of signal 2 remains a subject of debate. Considerable evidence supports K^+ efflux as a common signal 2 that activates NLRP3 (Pétrilli et al., 2007; Muñoz-Planillo et al., 2013), but many other mechanisms have been proposed (Elliott and Sutterwala, 2015). It has also been suggested that signal 2 is simply a cytotoxic signal, thus facilitating IL-1β release (Cullen et al., 2015). Numerous studies have also indicated that two signals are not always required for NLRP3 inflammasome activation, depending on the cell type and inducer (Netea et al., 2009; Vigano et al., 2015; Gaidt et al., 2016). By assessing caspase-1 activity directly, we confirm that mouse J774A.1 macrophages adhere to the two-signal hypothesis, but human THP-1 monocytes do not. In THP-1 cells, caspase-1 can be activated with either signal 1 or signal 2 only, leading to pyroptosis. We combine a time course study of caspase-1 activation with a sensitive real-time assay for cell death to define the kinetics of caspase-1 activation and pyroptosis in cell populations. We further show caspase-1 activity, cell death, and IL-1β release assessed from the same well of cultured cells. In addition, we use cell-permeable inhibitors to investigate the role of caspase-1 in driving pyroptosis in nigericin-treated THP-1 cells (signal 2 only model), demonstrating that caspase-1 inhibition significantly delays cell death but does not completely prevent it. This supports the idea that caspase-1 drives a unique and very rapid form of cell death, but much like apoptotic caspases, compensatory cell death mechanisms emerge in its absence (Galluzzi et al., 2015).

2. Materials and methods

2.1. Synthesis of luminescent substrates

The *Z*-WEHD-aminoluciferin and Z-YVAD-aminoluciferin were synthesized using Fmoc chemistry as previously described (O'Brien et al., 2005). The resulting *Z*-WEHD-aminoluciferin or Z-YVAD-aminoluciferin were purified by preparative reverse-phase high-performance liquid chromatography (HPLC) to >95% purity.

2.2. Reagents

All recombinant caspases, the fluorogenic caspase-1 substrates, Ac-YVAD-AMC and Ac-WEHD-AMC, and the inhibitors, Ac-YVAD-CHO, Ac-VEID-CHO, and *Z*-YVAD-FMK were obtained from ENZO Life Sciences (Farmingdale, NY). Ac-YVAD-CHO from MP Biomedicals (Solon, OH) and Z-YVAD-FMK from BioVision (Milpitas, CA) were also used. Inflammasome inducers used were the potassium ionophore, nigericin (EMD Millipore, Danvers, MA), the pore-forming toxin, α-hemolysin (from *S. aureus*, Sigma, St. Louis, MO), the TLR agonists, LPS (TLR4) and Flagellin (TLR5), (Ultrapure grade, InvivoGen, San Diego, CA), Resiquimod (R848) (TLR7/8) and Pam3CSK4 (TLR2) (BioVision). Monosodium urate crystals (MSU) (US Biological, Salem, MA and InvivoGen), known to cause lysosomal rupture and inflammasome activation, were also used. The proteasome inhibitor, MG-132 (ENZO Life Sciences and EMD Millipore), was used at a final concentration of 60 μM unless noted otherwise. Additional inhibitors used were Ac-YVAD-CMK and CA-074ME (Sigma), VX-765 (SelleckChem, Houston, TX), and XIAP (SignalChem, Richmond, BC, Canada). Staurosporine (Sigma), Paclitaxel and Puromycin (EMD Millipore) were used to induce apoptosis. Ionomycin (Sigma) was used to induce necrosis.

2.3. Caspase-1 activity assay

Z-WEHD-aminoluciferin caspase-1 substrate is combined with components necessary for the luciferase reaction and lyophilized. This lyophilized mixture is reconstituted with a lytic buffer for the caspase-1 assay (Caspase-Glo 1 Inflammasome Assay, Promega, Madison, WI; O'Brien et al., 2005). MG-132 is added to the reconstituted substrate at 2× for a final concentration of 60 μM in the assay, except when testing

recombinant caspases. The resultant 2× reagent is combined 1:1 with the test sample. For data shown in Figs. 4–9, a second 2× reagent was made with the addition of 2 μM Ac-YVAD-CHO. The concentration of Z-WEHD-aminoluciferin is 40 μM in the reagent and 20 μM in the final assay. Z-YVAD-aminoluciferin and Z-WEHD-aminoluciferin were initially tested with the same components but without lyophilizing the mixture. All luminescence was recorded on a GloMax® Multi+ Detection System unless otherwise noted.

2.4. Cell culture

THP-1 and J774A.1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). THP-1 cells were maintained as suspension in RPMI-1640 containing HEPES, high glucose, sodium pyruvate, and L-glutamine (Gibco, Grand Island, NY) and 10% heat-inactivated fetal bovine serum (FBS-HI, Gibco) at 37 °C and 5% CO_2. Where noted, THP-1 cells were differentiated with phorbol 12-myristate 13-acetate (PMA, Sigma) at 20 nM for 2–3 days. J774A.1 cells were maintained as adherent cultures in Dulbecco's medium (DMEM, Gibco) containing 10% FBS-HI. Both THP-1 and J774A.1 cells were added to clear-bottom, white 96-well plates at 50,000 cells/well in 100 μl for all assays unless otherwise noted.

To obtain human primary cells, mobilized human peripheral blood mononuclear cells (PBMCs) were isolated from leukapheresed blood and $CD14^+$ monocytes were purified via negative selection. Monocytes were cryopreserved until ready for use. Frozen CD14+ monocytes were thawed, washed, and cultured at 37 °C for 1–2 h before counting and plating for treatment in 96-well plates in RPMI medium containing 10% FBS-HI, 1% L-glutamine, 1% NEAA, 1% Pen/Strep and 20 mM HEPES Buffer.

To obtain mouse primary cells, the femurs of 6–12 week old mice were flushed and their bone marrows cultured for 5–7 days in Iscove's modified Dulbecco's medium containing 10% FBS-HI, 30% L cell–conditioned medium, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO_2. After differentiation, bone marrow-derived macrophages (BMDMs) were harvested, plated in 96 well plates (1×10^5 cells per well) and used within the next day. BMDMs were primed for 4.5 h with 500 ng/ml LPS before stimulation with inflammasome inducers.

2.5. Mice

Nlrp3$^{-/-}$ mice and *Casp-1*$^{-/-}$ mice in C57BL/6 background have been described (Kanneganti et al., 2006; Kuida et al., 1995). NLRP3 (R258W) mice were originally provided by Warren Strober (NIH). C57BL/6 mice were originally purchased from Jackson Laboratories and maintained in the University of Michigan Animal Facility. All animal use was approved by the University of Michigan Committee on Use and Care of Animals.

2.6. Caspase-1 western blot

The caspase-1 Ab was generated for the laboratory of Gabriel Nunez by Lampire Biological Labs (Ottsville, PA). Western blots were prepared as previously described (Muñoz-Planillo et al., 2013). Total cell lysates were prepared by adding 5× Laemmli buffer to the culture medium.

2.7. Cell death assay

Cell death was monitored with the impermeable DNA dye, CellTox® Green (Promega) as per the manufacturer's instructions. All fluorescence was recorded on a GloMax® Multi + Detection System with Ex: 490 nm Em: 510–570 nm.

2.8. IL-1β measurement

After some cell treatments, half of the culture medium was removed and frozen overnight for IL-1β analysis using the human IL-1β Quantikine ELISA (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions.

3. Results

3.1. Single-step assay detects caspase-1 activity in THP-1 cells

Two bioluminescent substrates for caspase-1 were made and evaluated. Z-WEHD-aminoluciferin incorporated the optimal caspase-1 recognition tetrapeptide and Z-YVAD-aminoluciferin incorporated a known caspase-1 recognition sequence similar to the endogenous IL-1β cleavage site (Thornberry et al., 1992, 1997). Both substrates were tested with recombinant caspase-1 in a coupled-enzyme system using a stabilized luciferase. An equilibrium is reached between caspase cleavage of the substrate and luciferase utilization of the released aminoluciferin substrate, resulting in a stable luminescent signal proportional to the caspase activity (Fig. 1). The luminogenic substrates gave linear responses with a titration of caspase-1 and had significantly improved sensitivity compared to the fluorogenic substrates, Ac-WEHD-AMC and Ac-YVAD-AMC (Fig. 2). The results also indicated Z-WEHD-aminoluciferin was a more sensitive substrate for caspase-1 than Z-YVAD-aminoluciferin (Fig. 2).

We next tested Z-WEHD-aminoluciferin against two known inflammasome activators, α-hemolysin and nigericin in human monocyte THP-1 cells (Craven et al., 2009; Hentze et al., 2003). Initial results indicated non-specific processing of the substrate whether or not cells were treated with an inflammasome inducer, with less of the non-specific activity in cells treated with the cytotoxic agent. Previous work had indicated that proteasome activity can readily process similar tetrapeptide-conjugated aminoluciferin substrates (unpublished), so Z-WEHD-aminoluciferin was re-evaluated with and without the proteasome inhibitor, MG-132. In the coupled-enzyme format, the proteasome inhibitor is added with the lytic assay reagent rather than to the cells during the experiment, minimizing the possibility of artifacts. In the presence of MG-132, non-specific signal had become negligible within approximately 60 min when proteasome inhibition was complete. Subsequently, the significant difference in assay signal between either α-hemolysin or nigericin-treated cells and vehicle controls, corresponding to inflammasome activation, was stable for several hours in the lytic reagent (Fig. 3A, B). In contrast, Z-YVAD-aminoluciferin

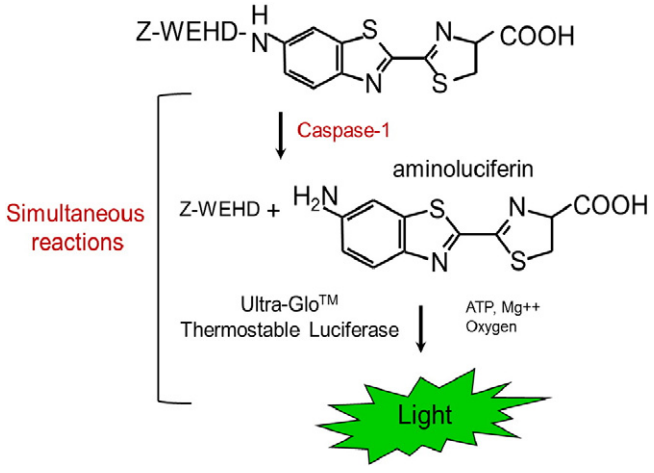

Fig. 1. Coupled-enzyme system for caspase-1 assay. Following caspase cleavage of Z-WEHD-aminoluciferin, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and light production. In the assay, the two reactions occur simultaneously resulting in a stable signal.

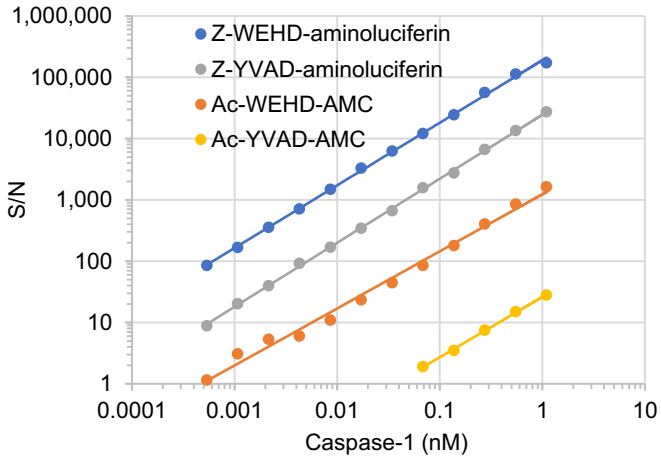

Fig. 2. A comparison between luminogenic and fluorogenic caspase-1 substrates. Recombinant caspase-1 was serially diluted in HEPES buffer (10 mM, pH 7.2 + 0.1% Prionex® +10 mM DTT) to the indicated concentrations in white 96-well plates. For luminescent assays, *Z*-WEHD-aminoluciferin or Z-YVAD-aminoluciferin was added to the luciferase-containing reagent at a concentration of 40 μM. The substrate reagents were added at a 1:1 ratio (v/v) to the titrated caspase-1. For fluorescent assays, the substrates Ac-WEHD-AMC and Ac-YVAD-AMC were diluted to 40 μM in HEPES buffer (100 mM, pH 7.2 + 0.1% Prionex® +10 mM DTT+ 0.2% CHAPS) and added at a 1:1 ratio (v/v) to the caspase-1. Readings were taken over 1 h and 5 h for luminescence and fluorescence, respectively. Luminescence results shown were recorded after 15 min and fluorescence results shown were recorded at 2.5 h, which yielded the highest signal to noise ratio. (S:N ratio = Signal – background/standard deviation of background).

used under identical conditions did not have sufficient sensitivity to detect caspase-1 activity induced by α-hemolysin (Fig. S1). The apparent Km of *Z*-WEHD-aminoluciferin for recombinant caspase-1 is consistent with that observed with nigericin-treated THP-1 cells (Fig. 3C, D), providing further evidence that the assay is monitoring caspase-1 activity in this cell-based model for inflammasome activation.

3.2. Caspase-1 inhibitor in reagent confirms detection of caspase-1 activity

Tetrapeptide caspase substrates generally have considerable cross-reactivity across the family of caspases (McStay et al., 2008). Although *Z*-WEHD-aminoluciferin appeared to be detecting caspase-1 in these well-established models of NLRP3 inflammasome activation, the highly selective caspase-1 inhibitor, Ac-YVAD-CHO, was used to confirm this observation (Garcia-Calvo et al., 1998; Pereira and Song, 2008). Following α-hemolysin treatment of THP-1 cells, Ac-YVAD-CHO was titrated in the lytic assay reagent to determine its inhibition efficiency in the cell culture reagent milieu (Fig. 4A). A majority of the activity induced by α-hemolysin was inhibited at all Ac-YVAD-CHO concentrations, but 1 μM was required to inhibit 99% of the activity within 60 min of reagent addition. A time course of luminescence indicated that inhibition was complete 60 min after reagent addition (Fig. 4B). To verify that 1 μM Ac-YVAD-CHO could inhibit caspase-1 activity present in a wide range of cell densities, THP-1 cells were titrated from 80,000 cells/well to 625 cells/well and treated with α-hemolysin or a vehicle control. The α-hemolysin-induced signal was effectively inhibited by 1 μM Ac-YVAD-CHO in the final assay at all cell concentrations, with the signal at 80,000 cells/well inhibited >95% within 60 min (Fig. 4C). This cell concentration titration also demonstrated assay linearity and a good dynamic range for detection of caspase-1 activity in cells. The effectiveness of this assay system to specifically detect caspase-1 activity was further tested in PMA-differentiated THP-1 cells treated with a panel of known inflammasome inducers. This evaluation, performed in 384-well microplate format, included the assessment of caspase-1 activity released into culture medium, previously reported to occur alongside release of IL-1β and IL-18 (Laliberte et al., 1999; Sarkar et al., 2009, Gaidt et al., 2016).

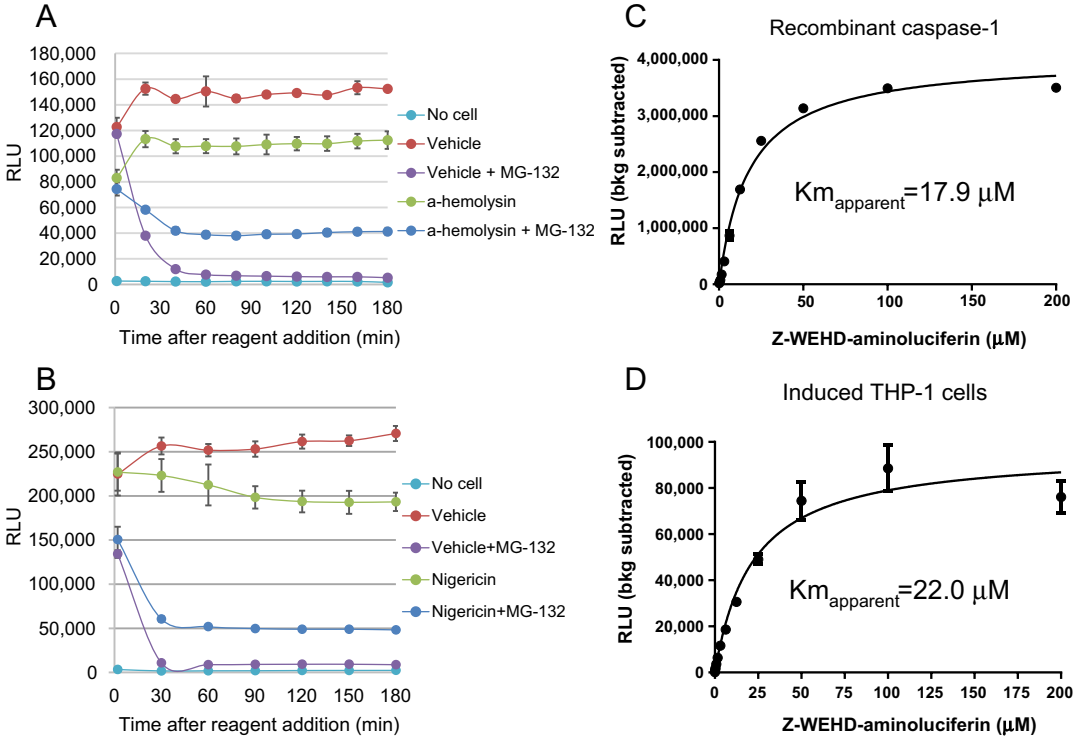

Fig. 3. MG-132 proteasome inhibitor is required with Z-WEHD-aminoluciferin in reagent to detect inflammasome specific signal. THP-1 cells were added to clear-bottom, white 96-well plates at 50,000 cells/well in 100 μl. Cells were treated with A) α-hemolysin (2 μg/ml) for 2.5 h or B) nigericin (20 μM) for 2 h and the Z-WEHD-aminoluciferin-containing caspase-1 assay reagent with or without MG-132 (60 μM) was added at a 1:1 volume ratio. Signal remains high for both treated and untreated cells in the absence of MG-132. Treated cells have a lower signal than untreated cells due to cell death and lower proteasome activity. In the presence of MG-132, the signal decreases rapidly removing proteasome activity. The signal stabilizes at 1 h and is significantly higher in the inflammasome induced cells. C) A titration of *Z*-WEHD-aminoluciferin in reagent was tested with recombinant caspase-1 (50 pM) and D) THP-1 cells (50,000 cells/well) treated with nigericin (20 μM) or vehicle. The apparent Kms are similar.

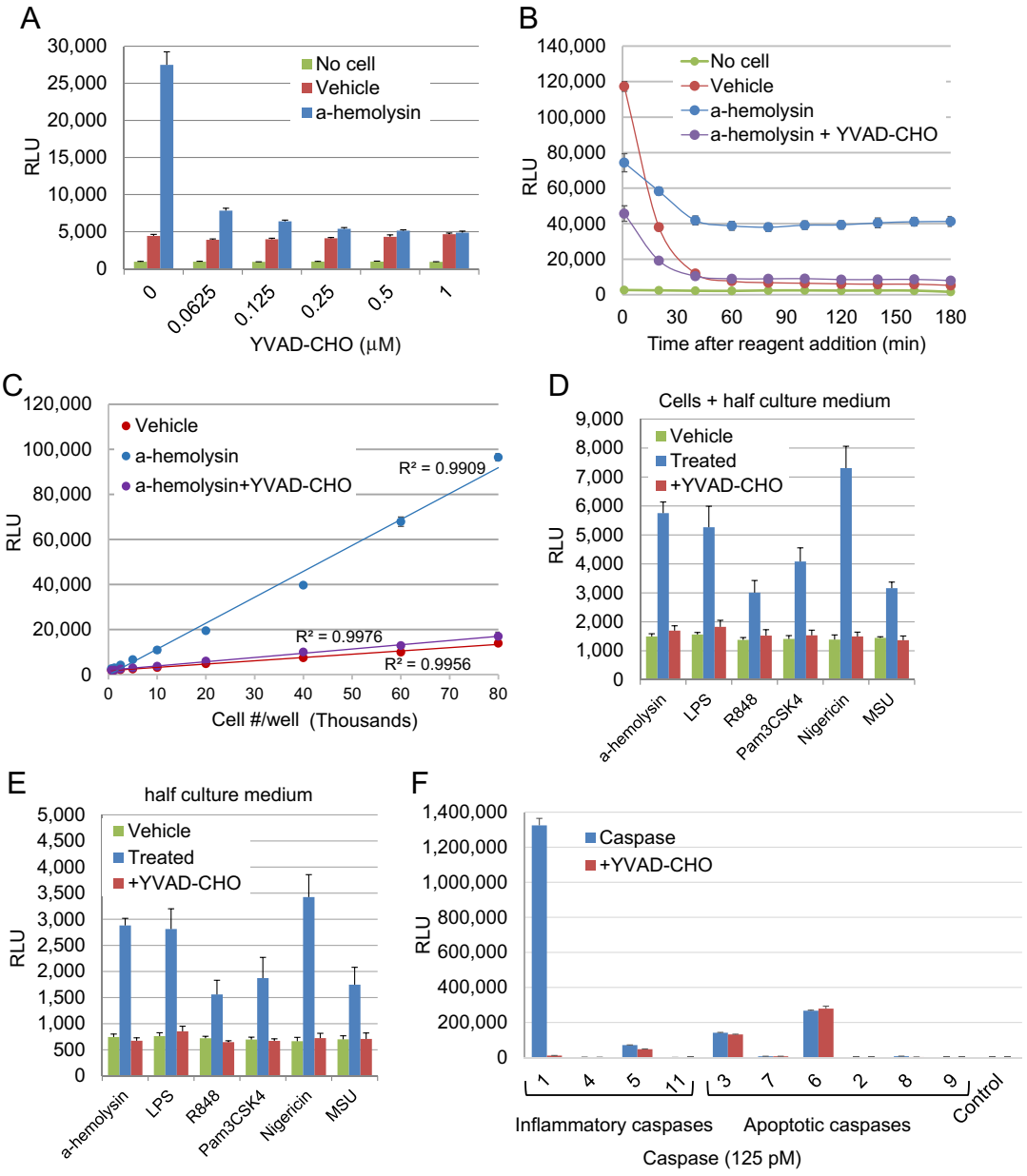

Fig. 4. The Ac-YVAD-CHO inhibitor in the Z-WEHD-aminoluciferin reagent confirms caspase-1 activity. A) THP-1 cells were treated with α-hemolysin (1 μg/ml, 3 h) or vehicle and caspase-1 assay reagent was added (1:1 volume ratio) with a titration of Ac-YVAD-CHO. Luminescence was recorded after 60 min. B) THP-1 cells were treated with α-hemolysin (2 μg/ml, 2.5 h). After treatment and caspase-1 reagent addition, luminescence was recorded every 20 min for 3 h. **C)** THP-1 cells were titrated, added into 96-well plates in 100 μl, and treated with α-hemolysin (2 μg/ml, 2 h). After treatment, caspase-1 reagent with/without Ac-YVAD-CHO at 1 μM final was added and luminescence recorded after 60 min. D) THP-1 cells were added to white 384-well plates at 12,500 cells/well in 25 μl, differentiated with PMA (20 nM for 3 days) and treated with various inflammasome inducers. After treatment, half of the medium (12.5 μl) was transferred and caspase-1 reagent was added to the cells with half of the medium or E) to the transferred culture medium (1:1 volume ratio) and luminescence was recorded at 60 min. F) Ten recombinant caspases were diluted in HEPES buffer (10 mM, pH 7.2; 10 mM DTT, 0.1% Prionex®) and added to 96-well plates containing either Ac-YVAD-CHO inhibitor in buffer or buffer only. Caspases and inhibitor were incubated for 10 min before adding caspase-1 reagent at a 1:1 volume ratio. All caspases were at a final concentration of 125 pM and the Ac-YVAD-CHO was at a final concentration of 1 μM. Luminescence results shown were recorded at 20 min. Caspase-11 was the only mouse caspase, all others were human. Caspase-8 is also a non-canonical inflammation caspase.

Caspase-1 activity was detected in the culture medium after treatment with all inflammasome inducers (Fig. 4D, E). In all cases, Ac-YVAD-CHO completely inhibited the inflammasome-induced signal.

Assay specificity was analyzed further by testing recombinant caspases at equimolar concentrations with the *Z*-WEHD-aminoluciferin substrate at its apparent Km concentration for caspase-1 (Fig. 4F). Recombinant caspases were pre-incubated with and without 1 μM Ac-YVAD-CHO before the assay. Among all caspases tested, caspases 3, 5 and 6 were the only ones that cleaved *Z*-WEHD-aminoluciferin to any significant degree, but this activity was not significantly inhibited by Ac-YVAD-CHO (Fig. 4F). Even though WEHD is the optimal tetrapeptide substrate for all of the inflammatory caspases (Thornberry et al., 1997), caspases-4 and 11 did not cleave the *Z*-WEHD-aminoluciferin substrate to any significant degree and caspase-5 cleaved the substrate only weakly compared to caspase-1 (Fig. 4F). The catalytic efficiencies (kcat/Km) of caspases 4 and 5 are at least 20-fold less than caspase-1 despite similar substrate specificities (Garcia-Calvo et al., 1999). Under some conditions, the catalytic efficiencies of caspases 4 and 5 are 100-fold lower than caspase-1 (Garcia-Calvo et al., 1998, 1999). The lack of cleavage of the *Z*-WEHD-aminoluciferin substrate by caspase-11 is consistent with caspase-11 being the mouse homolog of human caspases-4/5 (Shi et al., 2015). The Ki of Ac-YVAD-CHO for caspase-1 is at least

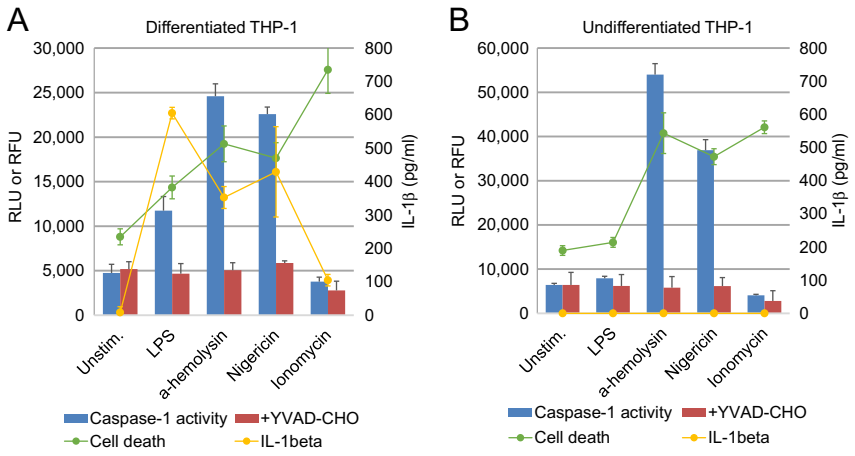

Fig. 5. *Multiplexing for caspase-1 activity, pyroptosis, and IL-1β release from THP-1 cells.* A) THP-1 cells were differentiated with PMA (20 nM) for 2 days. After differentiating, the medium was replaced and CellTox Green® diluted in medium was added. B) At this time, undifferentiated THP-1 cells were added to a second 96-well plate with CellTox Green added. Cells in both plates were treated with LPS (100 ng/ml, 3.25 h), α-hemolysin (1 μg/ml, 2.75 h), nigericin (20 μM, 2.25 h), ionomycin (200 μM, 1.25 h) or vehicle. Cell death was monitored during treatments by recording fluorescence. Just before adding caspase-1 assay reagent, half of the culture medium was removed from wells in both plates and transferred to another plate and frozen overnight at −20 °C for IL-1β ELISA the next day. Immediately following the culture medium transfer, the caspase-1 reagent was added to the remaining cells + half of the culture medium in both plates. The frozen culture medium was thawed the next day and tested for released IL-1β by ELISA.

200-fold times lower than the Ki for any other caspase (Garcia-Calvo et al., 1998). Consistent with the Ki of Ac-YVAD-CHO for different caspases, this inhibitor at 1 μM completely blocked caspase-1 activity (99%), but did not inhibit caspases 3, 5, or 6 significantly (Fig. 4F). A log scale graph of all the caspases ± Ac-YVAD-CHO with readings over time shows in detail the relative efficiencies of *Z*-WEHD-aminoluciferin processing (Fig. S3). Altogether, these results indicate that the Z-WEHD-aminoluciferin substrate in combination with the inhibitor Ac-YVAD-CHO is an excellent system to assay caspase-1 activity.

3.3. Caspase-1 activity in relation to IL-1β release and pyroptosis

To further confirm the biological significance of caspase-1 activity detected with this assay, we next assessed caspase-1 activity, IL-1β release, and cell death concurrently in both PMA-differentiated and undifferentiated THP-1 cells, since caspase-1 activity was previously detected in both models (Fig. 4A, D). Cells were treated with the known inflammasome inducers, LPS, α-hemolysin, or nigericin. Ionomycin, a calcium ionophore known to induce necrosis, served as a positive control for cell death. During the treatments, cell death was monitored with the cell-impermeant DNA dye, CellTox™ Green. After cell treatments, half of the culture medium was removed for determination of IL-1β release. The cells and remaining culture medium were tested for caspase-1 activity. In differentiated THP-1 cells, all of the treatments induced cell death, but only LPS, α-hemolysin, and nigericin induced caspase-1 activity indicative of pyroptosis (Fig. 5A). Ionomycin induced significant cell death without any caspase-1 activation, as expected for a known inducer of necrosis (Fig. S6). Substantial IL-1β levels were detected in the culture medium of differentiated THP-1 cells wherever caspase-1 activation was observed (Fig. 5A). In contrast, in undifferentiated THP-1 cells, no IL-1β was detected with any of the treatments, even though α-hemolysin and nigericin induced caspase-1 activity as well as pyroptotic cell death (Fig. 5B). PMA differentiation significantly upregulates pro-IL-1β in THP-1 cells (Bürckstümmer et al., 2009), explaining the lack of IL-1β released from undifferentiated THP-1 cells. The IL-1β detected in the culture medium of differentiated cells treated with LPS, α-hemolysin, and nigericin is consistent with caspase-1 activation causing processing of pro-IL-1β and release of IL-1β. The higher ELISA signal with LPS stimulation, despite lower caspase-1 activity relative to α-hemolysin and nigericin, may be due to additional upregulation of pro-IL-1β with LPS (Bauernfeind et al., 2009). However, the relatively low but detectable ELISA signal for IL-1β in the culture

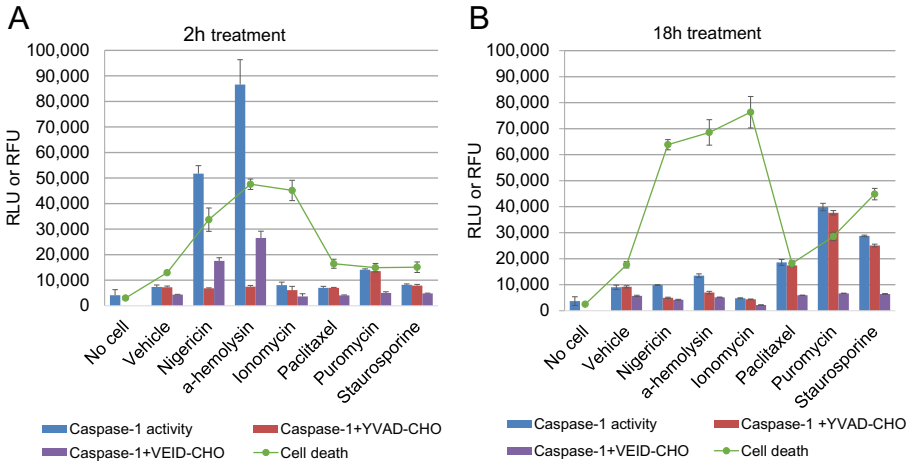

Fig. 6. Caspase-1 activity is detected in pyroptotic cells but not apoptotic cells. THP-1 cells were added to 96-well plates and CellTox Green was added. Cells were treated with either an inflammasome inducing agent, nigericin (20 μM) or α-hemolysin (2 μg/ml) or an apoptosis inducer, paclitaxel (1 μg/ml), puromycin (20 μg/ml), or staurosporine (1 μM). Ionomycin (100 μM) was used to induce necrosis. Vehicle controls for all inducers were included and averaged. Just before adding caspase-1 reagent, cell death was monitored by recording fluorescence. A) Caspase-1 reagent with or without inhibitors (1 μM) was added after 2 h or B) after 18 h. Results show luminescence recorded 90 min after reagent addition.

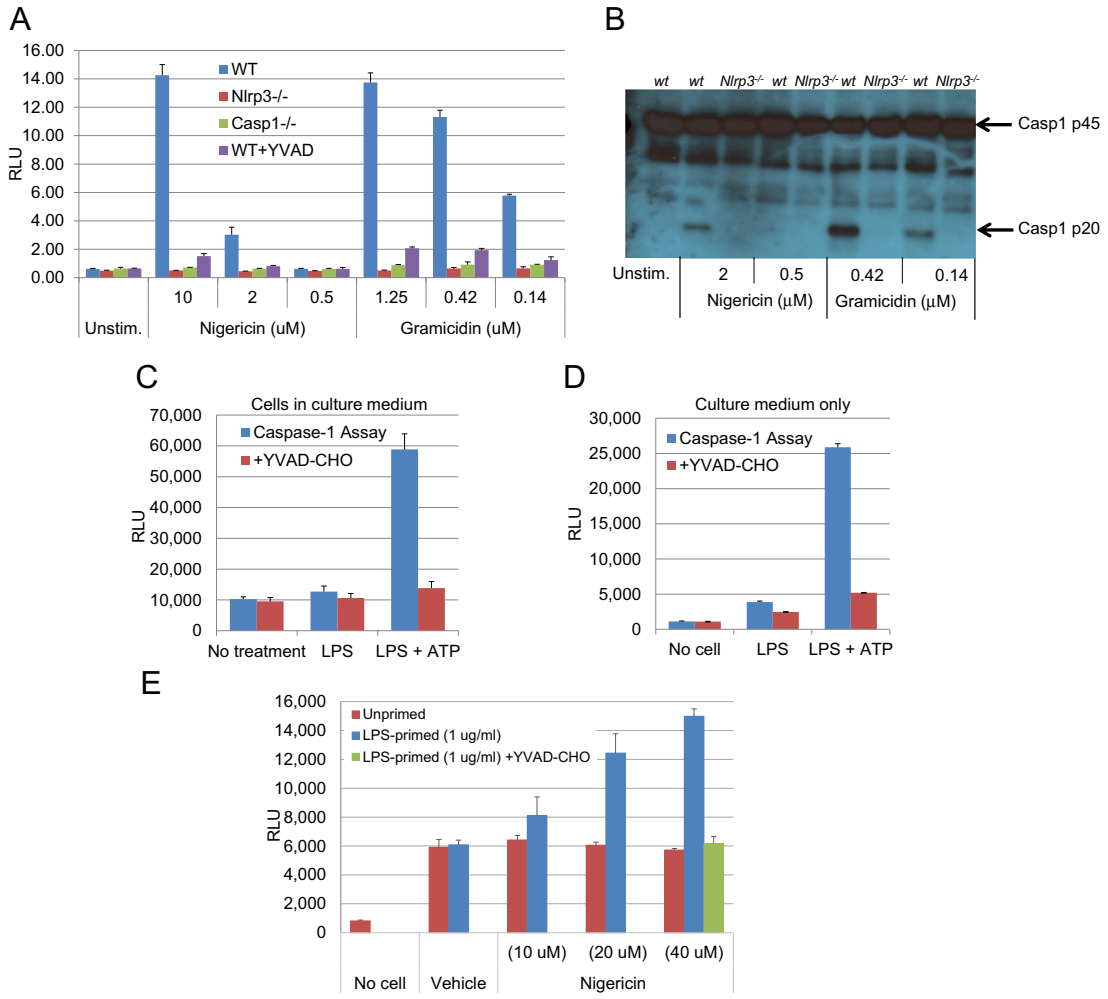

Fig. 7. Caspase-1 detected in mouse and human primary cells and a mouse cell line. A) Bone marrow-derived macrophages from wild type mice, caspase-1$^{-/-}$ and Nlrp3$^{-/-}$ mice, were added to plates at 1×10^5 cells/well in 100 μl, primed with LPS (500 ng/ml for 4.5 h), then stimulated for 30 min with nigericin or gramicidin. Caspase-1 reagent was added and luminescence read at 60 min on a Molecular Devices luminometer, showing caspase-1 activity in wild type mice only. B) Parallel wells with the same treated BMDMs were used for western blots. 25 μl of 5× Laemmli buffer with protease inhibitors was added to cells treated with the lower two doses of inducers. Lysates were frozen at −20 °C until use. 20 μl of lysate were added per lane for immunoblotting. An anti-caspase-1 Ab was used to detect the unprocessed (p45) and processed (p20) forms of caspase-1. p20 caspase-1 was detected in induced WT cell lysates, but not in Nlrp3$^{-/-}$ cell lysates. C) PBMCs (CD14+ monocytes) were added to 96-well plates at 50,000 cells/well, primed with 1 μg/ml LPS for 4 h 10 min and then pulsed with 3 mM ATP for 50 min. Caspase-1 reagent was added and luminescence recorded at 60 min. D) PBMCs were primed with 100 ng/ml LPS for 3.25 h and pulsed with 2.5 mM ATP for 45 min. Culture medium was transferred, caspase-1 reagent was added, and luminescence was recorded at 30 min. E) J774A.1 cells were added to 96-well plates at 50,000 cells/well, primed with LPS for 3 h or left unprimed, and treated with nigericin at different concentrations or vehicle. Luminescence was recorded at 90 min.

medium of ionomycin-treated cells in the absence of caspase-1 activation is consistent with cross-reactivity of assay antibodies for pro-IL-1β released due to necrotic cell death.

3.4. Assay distinguishes caspase-1 from other caspase activity and pyroptosis from apoptosis

The assay detected caspase-1 activity in cells stimulated with inflammasome inducers, confirmed by activity inhibition with the caspase-1 inhibitor, Ac-YVAD-CHO. However, since experiments with a panel of recombinant caspases indicated that *Z*-WEHD-aminoluciferin might also detect the activity of some apoptotic caspases (Fig. 4F), we performed the assay in THP-1 cells treated with the apoptosis inducers paclitaxel, puromycin and staurosporine. The apoptosis inducers were compared to the inflammasome inducers, nigericin and α-hemolysin, as well as to the necrosis inducer ionomycin. Both caspase activity and cell death were monitored at an early and a late time point for all treatments (2 h and 18 h, Fig. 6A and B). As expected, treatment with inflammasome inducers led to caspase-1 activation within 2 h and this activity was completely inhibited by addition of Ac-YVAD-CHO to the assay reagent (Fig. 6A). At this same time point, there was significant cell death in the nigericin, α-hemolysin, and ionomycin-treated cells. In contrast, the cells treated with apoptosis inducers showed little or no caspase activity and no cell death at 2 h. By 18 h, all of the apoptosis inducers produced activity detected with *Z*-WEHD-aminoluciferin, but this activity was not inhibited by Ac-YVAD-CHO, demonstrating that the apoptosis inducers do not activate caspase-1 (Fig. 6B). In contrast, this apoptosis-related activity was blocked by addition to the reagent of another caspase inhibitor, Ac-VEID-CHO, thereby confirming that *Z*-WEHD-aminoluciferin was detecting apoptotic caspases (Fig. 6B). VEID-CHO inhibits several caspases, including all of the apoptotic caspases and caspase-1 (Pereira and Song, 2008), but it is very potent against caspase-6 with a Ki of 7 nM (Heise et al., 2012). Cells treated with inflammasome inducers and ionomycin for 18 h were virtually all dead, giving a strong CellTox™ Green signal, and the caspase-1 activity induced by inflammasome agonists was not detectable anymore (Fig. 6B). There was significant apoptotic cell death following 18 h treatment with puromycin and staurosporine, although paclitaxel-treated cells had not yet undergone secondary necrosis. These results are consistent with results from the in vitro caspase panel (Fig. 4F) and demonstrate the Z-WEHD-aminoluciferin assay can detect caspase activity in apoptotic cells. However, caspase-1

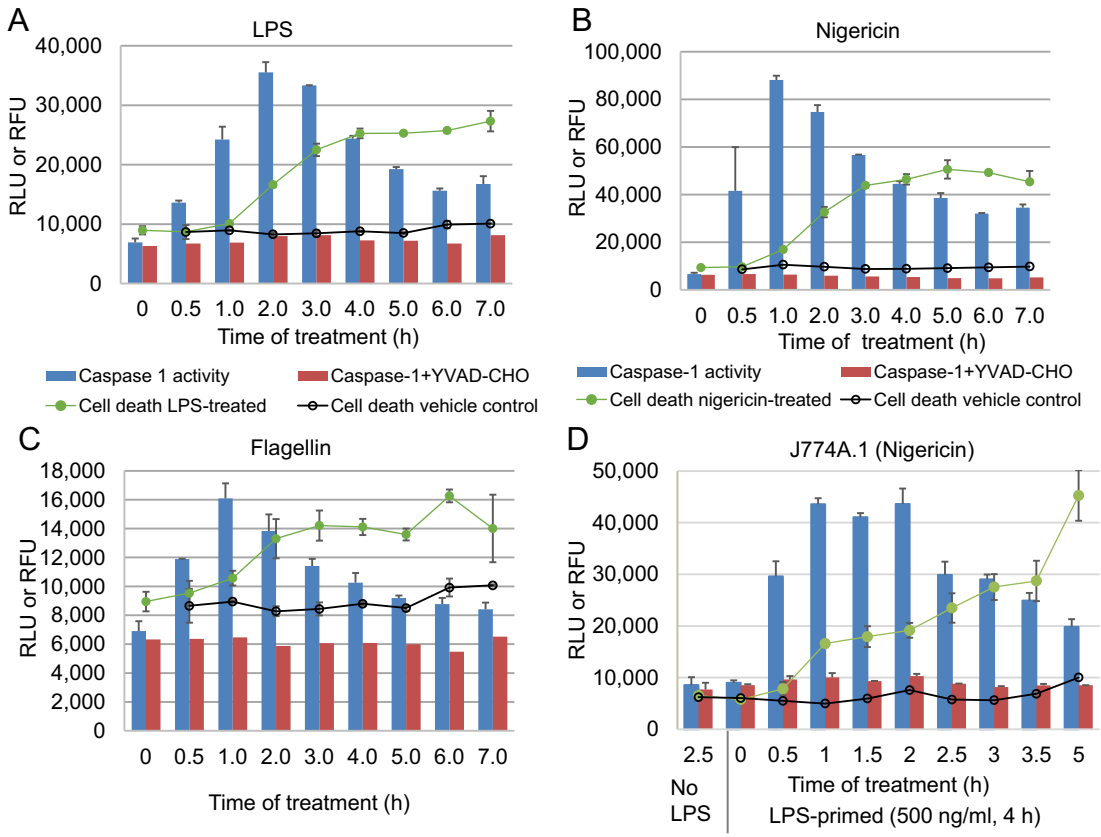

Fig. 8. Time course of caspase-1 activity and cell death. THP-1 cells were differentiated with PMA (20 nM) for 2 days. After differentiating, half of the medium was removed and CellTox Green was added and cells were treated with A) LPS (1 μg/ml), B) nigericin (20 μM), and C) flagellin (1 μg/ml) for various times. D) J774A.1 cells were added to 96-well plates at 50,000 cells/well, primed with LPS or left unprimed and treated with nigericin (20 μM) for various times. Fluorescence was recorded just before adding caspase-1 reagent.

activity induced by inflammasome activators is easily distinguished from apoptotic caspase activity by adding Ac-YVAD-CHO to the assay due to the differential sensitivity of caspases to this inhibitor (Fig. 6).

To further assess which particular caspases were responsible for the *Z*-WEHD-aminoluciferin assay signal generated by apoptotic cells, we tested additional apoptosis inducers and employed XIAP, an endogenous inhibitor specific for caspases 3, 7, and 9 (Fig. S4). Altogether, both cell-free and cell-based experiments indicate that the apoptotic signal appears to be due to a combination of the apoptotic caspases, 3 and 6, but this apoptotic signal is distinguished from caspase-1 activity since it is not inhibited by Ac-YVAD-CHO (Fig. S4).

3.5. Caspase-1 activity is detected in multiple cell types and is abolished in BMDMs from NLRP3 or caspase-1 knockout mice

In order to confirm our assay's ability to detect caspase-1 activity in a variety of experimental systems, murine primary bone marrow-derived macrophages (BMDMs), human primary peripheral blood monocytes (PBMCs), and J774A.1 mouse macrophages were evaluated. To further assess the specificity of the assay, experiments were done using murine BMDMs derived from *wildtype* (*wt*), *Nlrp3* $^{-/-}$ and *caspase-1* $^{-/-}$ mice. Nigericin and gramicidin were used to induce the inflammasome in LPS-primed BMDMs from *wt* and knockout mice. Strong signal, inhibited by Ac-YVAD-CHO, was detected in *wt* BMDMs, but no such caspase-1 signal was detected in either of the knockout BMDM cells (Fig. 7A). A caspase-1 western blot of lysates from *wt* and *Nlrp3* $^{-/-}$ BMDMs revealed the presence of processed caspase-1 only in *wt* BMDMs and only under treatment conditions exhibiting caspase-1 activity in the bioluminescent assay (Fig. 7B). The correspondence between the western blot results and the activity assay data, as well as the absence of activity in BMDMs derived from *Nlrp3* $^{-/-}$ and *caspase-1* $^{-/-}$ mice, further confirmed the plate-based assay is effectively monitoring caspase-1 activity.

Significant caspase-1 activity was also detected in human PBMCs primed with LPS and subsequently stimulated with ATP (Fig. 7C) (Callaway et al., 2015). This included caspase-1 activity released into the culture medium (Fig. 7D). This activity was completely inhibited by Ac-YVAD-CHO in the reagent (Fig. 7C, D). J774A.1 mouse macrophages, with and without LPS-priming, were tested with different doses of nigericin. Only LPS-primed J774A.1 cells showed caspase-1 activity with nigericin treatment, and this activity was abolished by adding Ac-YVAD-CHO into the assay reagent (Fig. 7E). The LPS-priming requirement for caspase-1 activation in J774A.1 macrophages stimulated with nigericin (Fig. 7E), contrasts with nigericin-stimulated caspase-1 activation in unprimed, undifferentiated THP-1 monocytes (Fig. 5B). These results show that the assay sensitivity and specificity enables caspase-1 detection in multiple cell types.

3.6. Kinetics of caspase-1 activity and pyroptosis

Recently, a genetic FRET-based sensor was used to monitor caspase-1 activity at the single cell level in peritoneal macrophages (Liu et al., 2014). This study revealed considerable variability among cells in the timing of caspase-1 activation after inflammasome induction. To assess the kinetics of inflammasome activation at the population level, we used our plate-based assay to monitor caspase-1 activity over time in both THP-1 and J774A.1 cells. PMA-differentiated THP-1 cells were stimulated with the inflammasome activators LPS, nigericin, and flagellin (Fig. 8A, B, C). J774A.1 cells were primed with LPS and subsequently stimulated with nigericin (Fig. 8D). Caspase-1 activity in THP-1 cells increased rapidly, peaking at 1 h for nigericin and flagellin stimulated cells, and 2 h for LPS stimulated cells. The activity declined steadily

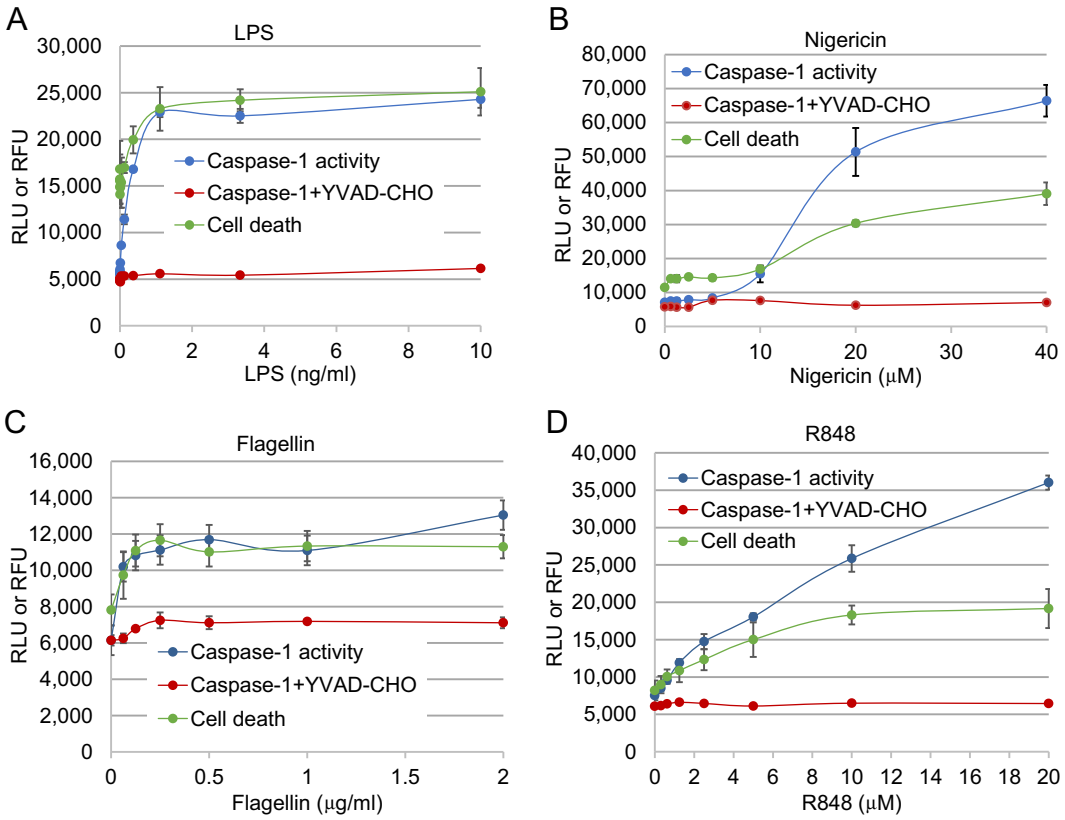

Fig. 9. Caspase-1 activity and cell death induced with dose responses of inflammasome activators. THP-1 cells were differentiated with PMA (20 nM) for 2–3 days. After differentiating, half of the medium was removed and CellTox Green was added and cells were treated with titrations of A) LPS for 3.25 h, B) nigericin for 2 h, C) flagellin for 4 h, and D) R848 for 3.2 h. Fluorescence was recorded just before adding caspase-1 reagent. Luminescence was recorded between 60 and 90 min.

after peak activation and by 7 h had decreased by at least 60% for all three treatments. A similar time course of activity was observed in LPS-primed J774A.1 cells stimulated with nigericin. Caspase-1 activity peaked at 2 h and by 5 h post-treatment, the caspase-1 activity had decreased significantly (Fig. 8D). Inflammasome activation was detected within 30 min in these populations of induced cells and after maximal caspase-1 activity in 1–2 h, caspase-1 activity steadily declined in these cell populations.

Liu et al. (2014) demonstrated a precise coincidence between caspase-1 activity and cell death at the single-cell level in peritoneal macrophages. Multiplexing assays for caspase-1 activity and cell death demonstrated that pyroptosis was detected within 30–60 min of caspase-1 activation in populations of THP-1 and J774A.1 cells (Fig. 8). Furthermore, dose responses of several inflammasome inducers revealed a close correlation between caspase-1 activity and cell death induced by each agent (Fig. 9A–D). The tight temporal correlation between caspase-1 activity and the initiation of cell death as well as the correspondence between the level of caspase-1 activity and the level of cell death provoked by each agent is consistent with previous evidence that caspase-1 drives pyroptosis (Liu et al., 2014; Schmid-Burgk et al., 2015). The transient nature of the caspase-1 activity may be a result of cell death and degradation.

3.7. Effect of caspase inhibition on IL-1β release and pyroptosis

Previous reports have indicated conflicting results on the requirement for caspase-1 in pyroptosis of nigericin-treated THP-1 cells (Hentze et al., 2003; Schmid-Burgk et al., 2015; Cullen et al., 2015). To assess the role of caspase-1 in pyroptosis and IL-1β release, we used *Z*-VAD-FMK, a pan caspase inhibitor, and Z-YVAD-FMK, a more selective caspase-1 inhibitor, to pre-treat both differentiated and undifferentiated THP-1 cells before inflammasome induction with LPS, α-hemolysin and nigericin (Fig. 10). Ionomycin was included as a cell death control. Pre-treatment with *Z*-VAD-FMK completely inhibited IL-1β release and significantly blocked cell death in inflammasome-activated differentiated cells, whereas it had no effect on ionomycin-treated cells (Fig. 10C, E). Pre-treatment with *Z*-VAD-FMK also inhibited the cell death in undifferentiated cells induced by α-hemolysin and nigericin, but not ionomycin-induced cell death (Fig. 10D). Surprisingly, Z-YVAD-FMK (20 μM) had minimal impact on cell death and IL-1β release (Fig. 10C, D, E). Z-YVAD-FMK was functional since upon cell lysis, the caspase-1 activity was completely inhibited (Fig. 10A, B). Z-YVAD-FMK may not completely block intracellular caspase-1 activity under our experimental conditions due to limited cell permeability, thus allowing both IL-1β release and pyroptosis. Alternatively, in the absence of caspase-1, other caspases may compensate to cause cell death and IL-1β release, or caspase-1 may not be absolutely required for pyroptosis in inflammasome induced THP-1 cells.

To investigate further the role of caspase-1 in pyroptosis, we studied the kinetics of this process using four inhibitors and the simple IL-1β-independent model of undifferentiated, nigericin-treated THP-1 cells. Cells were pre-treated with different doses of either *Z*-VAD-FMK or Z-YVAD-FMK and then treated with nigericin or vehicle and monitored for cell death up to 6 h (Fig. 11A). Between 1 and 6 h of nigericin treatment, cell death steadily increased. At 1–2 h, when caspase-1 activity peaks in these cells, 20 μM Z-VAD-FMK completely inhibited cell death, but 10 μM Z-VAD-FMK only partially inhibited cell death. Neither dose of Z-VAD-FMK completely inhibited cell death at later time points. As in the previous experiment, Z-YVAD-FMK at 20 μM was not effective at inhibiting cell death, although at 40 μM, Z-YVAD-FMK partially inhibited cell death (Fig. 11A). We next tested a potent and selective caspase-1 inhibitor, VX-765, (Stack et al., 2005; Wannamaker et al., 2007) and a cathepsin B inhibitor, CA-074ME, (Hentze et al., 2003) at different doses and monitored nigericin-induced pyroptosis up to

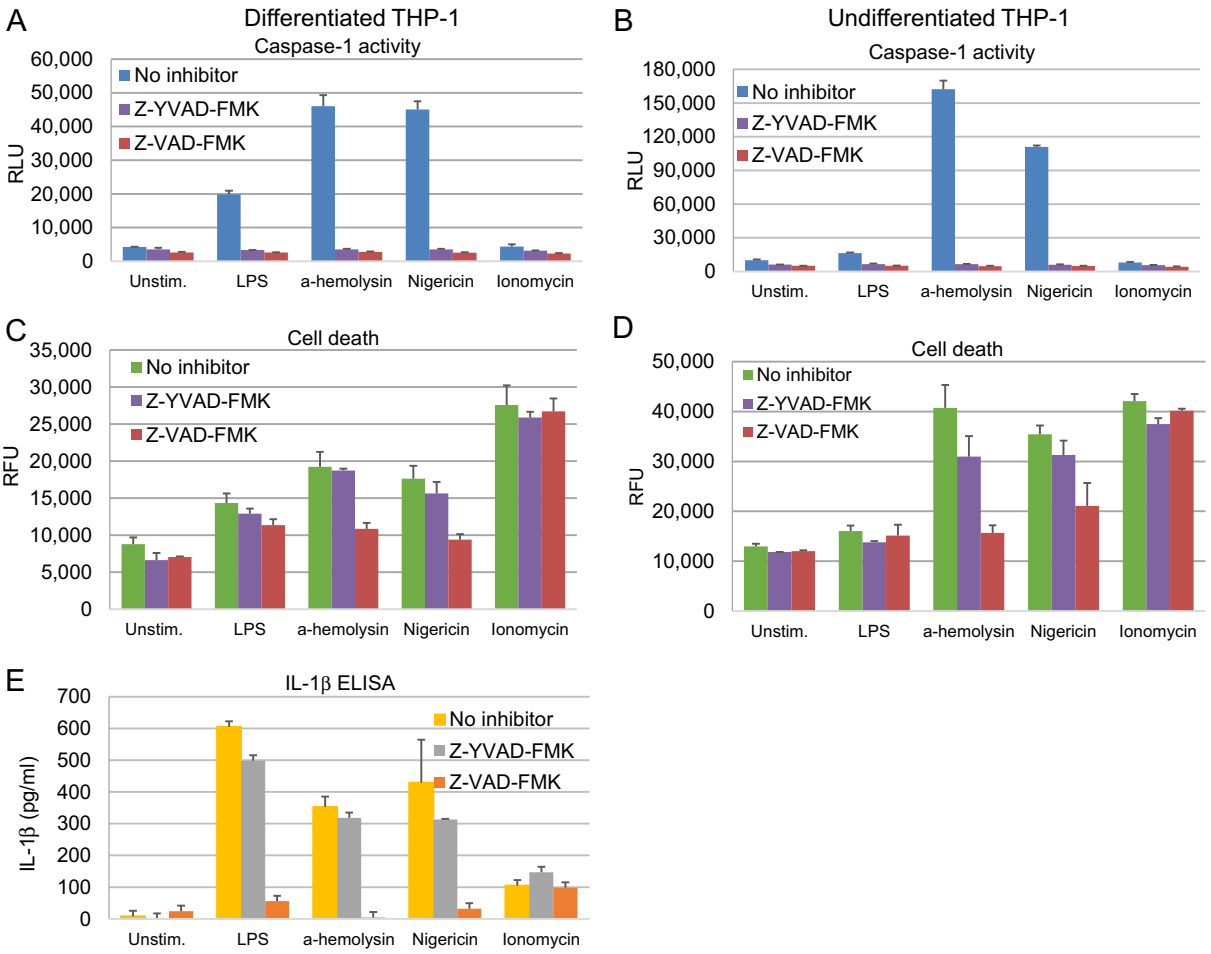

Fig. 10. Effect of cell-permeable caspase inhibitors on IL-1β release and pyroptosis. THP-1 cells were differentiated with PMA (20 nM) for 2 days. After differentiating, the medium was replaced and CellTox® Green plus Z-VAD-FMK (20 μM), Z-YVAD-FMK (20 μM) or vehicle was added. At this time, undifferentiated THP-1 cells were added to a second 96-well plate at 50,000 cells/well in 100 μl with CellTox® Green plus Z-VAD-FMK (20 μM), Z-YVAD-FMK (20 μM) or vehicle added. Cells were pre-incubated with inhibitors for 1 h before cells in both plates were treated with LPS (100 ng/ml, 3.25 h), α-hemolysin (1 μg/ml, 2.75 h), nigericin (20 μM, 2.25 h), ionomycin (200 μM, 1.25 h) or vehicle. Cell death was monitored during treatments by recording fluorescence. Just before adding caspase-1 assay reagent, half of the culture medium was removed from some wells in both plates and transferred to another plate and frozen overnight at -20 °C for IL-1β ELISA the next day. Caspase-1 reagent was added to the cells that did not have half of the culture medium removed for IL-1β ELISA. The frozen culture medium was thawed the next day and tested for released IL-1β by ELISA. A) Caspase-1 activity in differentiated cells or B) undifferentiated cells. C) Cell death as monitored with CellTox® Green just before adding caspase-1 reagent in differentiated cells and D) undifferentiated cells. E) Released IL-1β measured with ELISA.

21 h. We verified caspase-1 activation at 2 h (not shown). VX-765 inhibited cell death up to 9 h after nigericin treatment at 40 and 20 μM (Fig. 11B). Likewise, CA-074ME partially inhibited cell death, but at 9 h there was some cell death occurring at both inhibitor concentrations (Fig. 11C). Neither inhibitor at any of the tested doses completely prevented cell death at later time points (Fig. 11B, C).

4. Discussion

Tools for monitoring inflammasome activation have relied primarily on antibody-based methods such as western blots for processed caspase-1 or IL-1β and ELISA for released caspase-1 or IL-1β. Both methods are low throughput, require multiple steps, and do not directly monitor active caspase-1. Herein, we have described a plate-based caspase-1 activity assay that provides a one-step, high throughput method to directly monitor inflammasome activity using a bioluminescent substrate in a coupled-enzyme system. The sensitivity of the assay enabled measuring caspase-1 activity directly in cultured cells or released into the medium of treated cells.

Assay specificity is critical since caspases are well known to exhibit significant cross-reactivity against tetrapeptide substrates (McStay et al., 2008). In addition, the caspase-like catalytic sites of the proteasome can cleave peptides after an aspartate residue. Specificity in our caspase-1 assay was achieved by means of adding a proteasome inhibitor and a specific caspase-1 inhibitor to the reagent. The inclusion of a proteasome inhibitor in the assay reagent was necessary to block proteasome processing of the substrate. Verifying that the signal is due to caspase-1 activity and not another caspase required inclusion of the caspase-1 selective inhibitor, Ac-YVAD-CHO, in the reagent for testing in parallel wells. These two inhibitors were added with the lytic reagent after cell treatments were completed so that they had no impact on the cells during the experiment. It is important that the verification of caspase-1 activity is done at the reagent stage rather than by adding inhibitor directly to cells prior to treatment because inhibiting one caspase can lead to compensatory activity with other caspases, thereby confounding interpretations. Additionally, concentrations of cell-permeable caspase inhibitors typically used to block a particular caspase activity are high enough that specificity becomes questionable. Using the Ac-YVAD-CHO inhibitor at 1 μM in the lytic reagent only targeted caspase-1 and did not alter the cellular response. Caspase-1 activity was verified by comparing the luminescence with and without Ac-YVAD-CHO, which confirmed that the luminescence generated was indeed a result of caspase-1 activity due to inflammasome activation.

The assay was quantitative and linear as shown by titrations of both recombinant caspase-1 (Fig. 2) and treated cell numbers (Fig. 4). The quantitative robustness was also reflected by well-defined dose

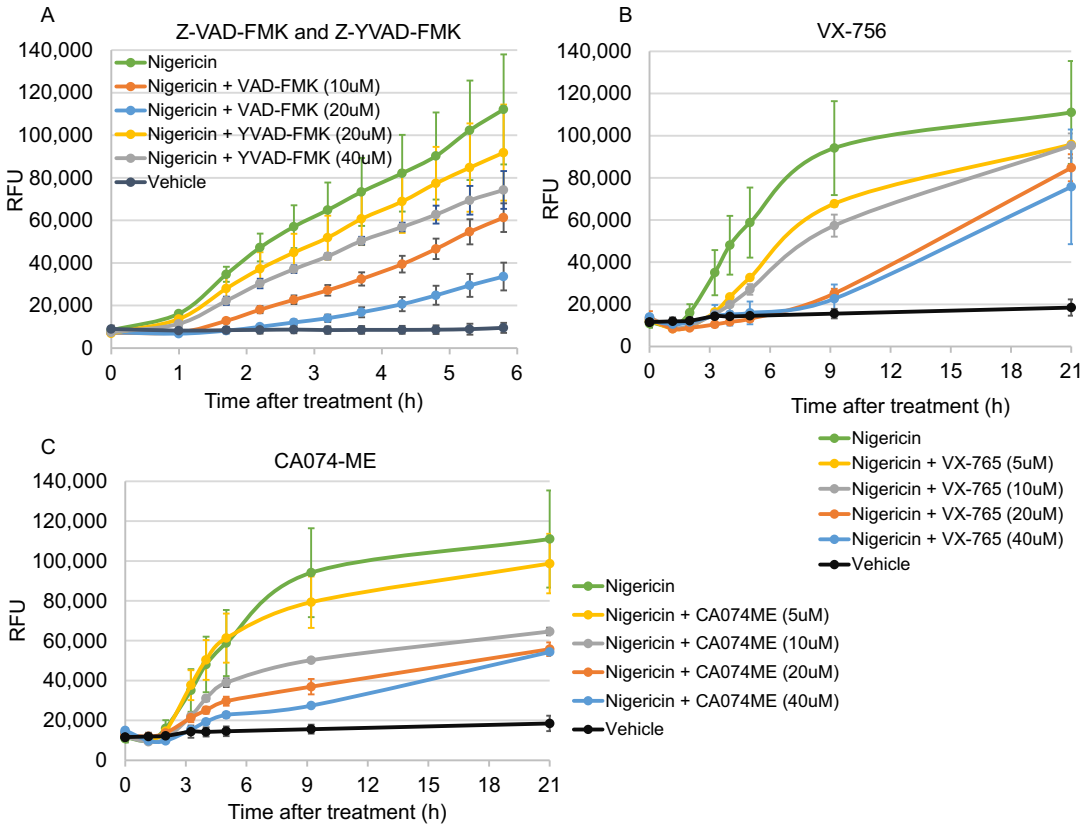

Fig. 11. Caspase-1 inhibition and cathepsin B inhibition significantly delay cell death in nigericin-treated THP-1 cells. A) THP-1 cells were added to 96-well plates with CellTox Green and either Z-VAD-FMK (10 or 20 μM) or Z-YVAD-FMK (20 or 40 μM) or vehicle. Cells were incubated with the inhibitors for 1 h before adding nigericin (20 μM) or vehicle. Fluorescence was monitored over time. B) THP-1 cells were plated and combined with CellTox Green and either VX-765 (caspase-1 inhibitor) or C) CA-074ME (cathepsin B inhibitor) at various concentrations. Cells were incubated with the inhibitors or vehicle controls for 1 h before adding nigericin (20 μM) or vehicle.

responses of inflammasome inducers (Fig. 9). The assay also enabled a precise time course determination of caspase-1 activation. As the first line of defense against pathogens, the innate immune system needs to respond rapidly. Results here demonstrated that within 30 min of stimulation with several inflammasome inducers, activated caspase-1 could be detected (Fig. 8). Human THP-1 monocytes and mouse J774A.1 macrophages both responded to inflammasome induction with a similarly rapid response. Importantly, once the inflammasome was induced, the caspase-1 activity was fairly transient. Whether the loss of caspase-1 activity results from cell death and general degradation or from an inherent enzyme inactivation mechanism is not known. Contradictory results of rapid inactivation of caspase-1 and stable activity of released caspase-1 have both been reported. A half-life of 9 min for caspase-1 activity was demonstrated in a cell-free system (Walsh et al., 2011), while stability >12 h for released caspase-1 was reported using very high concentrations of Z-YVAD-CMK (50 μM) to demonstrate specificity (Shamaa et al. 2015). However, with our sensitive, specific assay we show the time course of caspase-1 activation and inactivation in mouse and human cell culture models of inflammasome induction with specificity confirmed at all time points (Fig. 8). The half-life of caspase-1 activity in cell culture was not in minutes, but was approximately 3 h. This reflects the half-life in a population of cells, and does not give a precise half-life for caspase-1 activity from individual cells. However, it provides general information on the transience of active caspase-1. Regardless of the mechanism, once the innate immune system has responded to a danger signal, it may be advantageous to inactivate the inflammasome pathway fairly quickly to protect the host from excessive damage.

The bioluminescent assay was readily multiplexed with a sensitive fluorescent assay for cell death to monitor pyroptosis in real time, revealing a close correspondence between caspase-1 activation and cell death. The time courses of inflammasome activation revealed that cell death followed caspase-1 activation within 30–60 min in both THP-1 and J774A.1 cells (Fig. 8). In addition to a close temporal correlation between caspase-1 activation and subsequent cell death, we also found a correspondence between the level of caspase-1 activity and the amount of cell death for different inducers (Fig. 9, S6). Understanding the relationship between inflammasome activation and pyroptosis is important for several reasons. Pyroptosis is the presumed mechanism for IL-1β/IL-18 release in some inflammasome activation paradigms. These cytokines do not have classical secretory signals and their release is not well understood. In macrophages, pyroptosis appears to be the mechanism for IL-1β release in an all-or-none fashion (Liu et al., 2014). However, in neutrophils, inflammasome activation and IL-1β release have been reported in the absence of pyroptosis (Chen et al., 2014). Likewise, an alternative NLRP3 inflammasome pathway recently has been described in primary PBMCs induced by LPS only (signal 1 only) that triggers IL-1β release, but not pyroptosis (Gaidt et al., 2016). Macrophage pyroptosis, independent of IL-1β or IL-18, has also been shown to serve an important role in bacterial clearance by allowing exposure of released bacteria to phagocytic neutrophils (Miao et al., 2010, 2011).

Pyroptosis is defined as an inflammatory caspase-dependent cell death, and we show a close correspondence between caspase-1 activity and cell death. Nevertheless, the mechanism remained a mystery until recently, since no endogenous substrates of inflammatory caspases with direct involvement in pyroptosis had been identified. However, the recent identification of Gasdermin D as a substrate of inflammatory caspases required for pyroptosis (Baker et al., 2015; Kayagaki et al., 2015; Rühl and Broz, 2015; Schmid-Burgk et al., 2015; Shi et al., 2015), and the subsequent findings that the cleaved N-terminal fragment of Gasdermin D forms pores in the membrane (Liu et al., 2016; Sborgi et al., 2016; Ding et al., 2016) has greatly advanced our

understanding of inflammasome regulation of pyroptosis. Interestingly, the role of gasdermin D in pyroptosis in canonical caspase-1 inflammasome signaling is less conclusive. Pyroptosis was delayed but not prevented in Gsdmd$^{-/-}$ BMDMs treated with canonical inflammasome inducers (Kayagaki et al., 2015). We also found that blocking caspase-1 with cell-permeable inhibitors delayed cell death but did not completely prevent it in nigericin-treated THP-1 cells (Fig. 11). A recent report demonstrated blocked IL-1β release and blocked cell death in caspase-1 deficient THP-1 cells treated with nigericin, implicating caspase-1 with a required regulatory role in pyroptosis (Schmid-Burgk et al., 2015). In contrast, Cullen et al. (2015) suggested that nigericin-induced cell death (as well as other signal 2-only paradigms) in unprimed, undifferentiated THP-1 cells was inflammasome-independent and thus necrosis, but this is inconsistent with our results demonstrating clear caspase-1 activation in this same model, as well as inconsistent with Schmid-Burgk et al. (2015). Our results are also consistent with an earlier study showing a delay of pyroptosis in undifferentiated, nigericin-treated THP-1 cells pre-treated with *Z*-VAD-FMK or the cathepsin B inhibitor, CA-074ME (Hentze et al., 2003). Cathepsin B is thought to function upstream of NLRP3 activation in some inflammasome models (Guo et al., 2015). Although the earlier Hentze study used high doses of Z-VAD-FMK and CA-074ME, we obtained a similar delay in cell death using lower doses of CA-074ME as well as low doses of VX-765, a selective caspase-1 inhibitor (Stack et al., 2005; Wannamaker et al., 2007). In the case of apoptosis, it is becoming widely understood that in mammalian systems, inhibiting activation of executioner caspases that regulate programmed cell death, does not result in cytoprotection but only alters the kinetics of cell death (Galluzzi et al., 2015). Likewise for pyroptosis, inhibition of caspase-1 may alter the timing and biochemical manifestations of cell death, but not completely prevent it.

The direct caspase-1 assay enabled multiplexing for cytokine release, as well as pyroptosis. Monitoring all three parameters—caspase-1 activation, pyroptosis, and cytokine release –simultaneously from the same cell culture wells, provided a convenient method for investigating the requirements for inflammasome activation and its outcomes. A two-step mechanism for NLRP3 inflammasome activation and IL-1β maturation in macrophages is generally accepted (Netea et al., 2009; Franchi et al., 2012; Lamkanfi and Dixit, 2014; Guo et al., 2015; Elliott and Sutterwala, 2015). However, the two-step mechanism is not always a requirement for NLRP3 inflammasome activation, as has been most clearly shown for LPS stimulation of IL-1β release in human monocytes (Kahlenberg and Dubyak, 2004; Netea et al., 2009; Vigano et al., 2015; Gimenes et al., 2015; Gaidt et al., 2016). Our caspase-1 activity assay verified this dichotomy of one and two-signal mechanisms of NLRP3 inflammasome activation. Mouse J774A.1 macrophages required two signals for inflammasome activation (Figs. 7, 8), whereas THP-1 cells did not. Caspase-1 was activated in PMA-differentiated THP-1 cells using signal 1-only paradigms, including stimulation with the TLR agonists, LPS, R848, Pam3CSK4, and flagellin (Figs. 4, 8). While PMA-differentiation of THP-1 cells is generally thought to make them more macrophage-like (Daigneault et al., 2010), our results indicate they behave more like human primary monocytes regarding direct inflammasome activation with TLR agonists. Similarly, direct activation of the NLRP3 inflammasome with TLR stimulation alone was shown for mouse dendritic cells (He et al., 2013). Gaidt et al. (2016) describe a TRIF-RIPK1-FADD-Caspase-8 dependent mechanism in primary human monocytes that links LPS stimulation via TLR4 to NLRP3 activation and IL-1β release. Interestingly, this "alternative" one-signal NLRP3 inflammasome pathway does not result in pyroptosis, whereas in our PMA-differentiated THP-1 model, LPS alone triggered pyroptosis. How PMA-differentiation enables a direct link between TLR stimulation and inflammasome/caspase-1 activation is not clear, but upregulation of NLRP3 and proIL-1β are likely important. Endogenous release of ATP, acting through P2X7 receptors, in combination with "constitutive" caspase-1 activity has been proposed as the mechanism for IL-1β processing and release in LPS-stimulated human monocytes (Netea et al., 2009). However, this does not appear to be the case in mouse dendritic cells (He et al., 2013), nor THP-1 monocytes (Grahames et al., 1999). We did not detect constitutive caspase-1 activity nor did a P2X7R antagonist have any effect on LPS-induced caspase-1 activation in THP-1 cells (Fig. S5). Caspase-1 was also activated with signal 2-only paradigms, demonstrated for nigericin and α-hemolysin stimulation of undifferentiated, unprimed THP-1 cells. Both of these stimuli would result in potassium efflux, a proposed common trigger of the NLRP3 inflammasome (Muñoz-Planillo et al., 2013). Even in the absence of NLRP3 upregulation, K^+ efflux alone can activate caspase-1 in THP-1 cells (Fernandes-Alnemri et al., 2007). We show this is a specific K^+ efflux response and not a non-specific response to membrane disruption since the calcium ionophore, ionomycin, did not trigger caspase-1 activation (Fig. 5).

There are many remaining questions regarding the regulation of inflammasome activation in different cell types and the physiological outcomes of activation. A homogeneous, specific, caspase-1 activity assay provides an important tool to address these questions. Monitoring the most proximal marker of inflammasome activation, i.e., caspase-1 activation, and being able to conveniently combine this activity measurement with assessment of cell death and cytokine release addresses substantial technical hurdles that have hampered these types of studies. Furthermore, having a high throughput assay for monitoring modulators of inflammasome activation adds to the screening toolbox.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2017.03.004.

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