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Caspase-1 activity is required to bypass macrophage apoptosis upon *Salmonella* infection

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Here we report AWP28, an activity-based probe that can be used to biochemically monitor caspase-1 activation in response to proinflammatory stimuli. Using AWP28, we show that apoptosis is triggered upon *Salmonella enterica* var. Typhimurium infection in primary mouse bone marrow macrophages lacking caspase-1. Furthermore, we report that upon *Salmonella* infection, inflammasome-mediated caspase-1 activity is required to bypass apoptosis in favor of proinflammatory pyroptotic cell death.

The cysteine protease caspase-1 initiates a proinflammatory cell death program termed pyroptosis in response to a variety of insults, including metabolic stress and bacterial infection[1–3]. Pyroptosis is characterized by rapid loss of membrane integrity, resulting in cell lysis and release of proinflammatory components. Unlike apoptosis, pyroptosis is not immunologically silent and is initiated by formation of an intracellular multiprotein complex called the inflammasome[1,3–5]. Inflammasomes contain cytosolic pattern recognition receptors, caspase-1 and, often, the adaptor protein ASC[5]. These receptors oligomerize in response to pathogen- or danger-associated molecular patterns, triggering autoproteolytic activation of the procaspase-1 zymogen into its active, tetrameric form.

Because caspases are activated after translation, activity-based probes are powerful tools for studying their regulation and function[6]. To biochemically characterize inflammasome-mediated caspase-1 activation, we synthesized the fluorescently labeled probe AWP28 (**Supplementary Methods**), which contains an acyloxymethyl ketone electrophile linked to an optimized scaffold for caspase-1 recognition[7] (**Fig. 1a**). When intact primary bone marrow macrophages (BMMs) from mice were infected with the Gram-negative intracellular bacterial pathogen *S.* Typhimurium AWP28 primarily labeled multiple forms of caspase-1, as confirmed by immunoprecipitation (**Fig. 1b**,**c**). Labeling included the p45 and p35 proforms[8], revealing that higher-molecular-weight forms of caspase-1 are active in response to *S.* Typhimurium infection before being processed to the final catalytic p20 subunit in the p20/p10 heterotetramer. Notably, AWP28 labeled the p35 form of caspase-1 to a greater extent than the p20 and p22 forms, despite reports that these smaller forms are up to 130-fold more active[8,9]. Remarkably, AWP28 showed markedly reduced nonspecific background labeling compared to the commercially available caspase-1 probe FLICA, which contains the highly reactive fluoromethyl ketone electrophile[10,11] (**Supplementary Results**, **Supplementary Fig. 1**). AWP28 also labeled a small amount of active executioner caspase-7 in response to *S.* Typhimurium infection, consistent with its reported activation during pyroptosis[12] (**Fig. 1c**).

Because AWP28 was capable of labeling caspase-7, we wanted to determine whether it could be used to visualize caspase activation upon initiation of classical apoptotic pathways. In BMMs undergoing staurosporine-induced intrinsic apoptosis, AWP28 labeled the executioner caspases 3 and 7, as confirmed by immunoprecipitation (**Fig. 1d**,**e**). Characterization by *in vitro* inhibition kinetics showed that AWP28 is over ten-fold more potent against caspase-1 than against other tested caspases (**Supplementary Table 1**), yet it can be used to simultaneously visualize activation of caspase-1 and downstream executioner caspases.

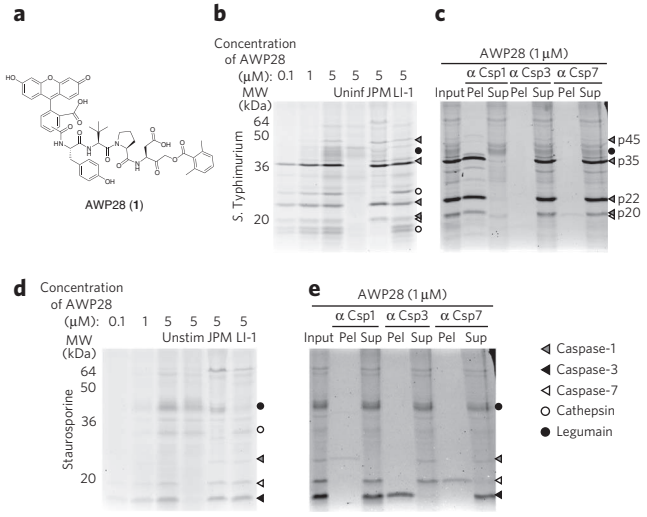

Figure 1 | AWP28 is an optimized caspase activity-based probe. (**a**) Structure of AWP28 (**1**). (**b**) AWP28 labeling of intact primary mouse BMMs infected with *S.* Typhimurium (10:1) for 1 h. Uninf, uninfected; MW, molecular weight; JPM, cathepsin inhibitor to identify off-target cathepsin labeling; LI-1, legumain inhibitor to identify off-target legumain labeling. (**c**) Labeled protein identification in *S.* Typhimurium–infected samples by immunoprecipitation. The identities of the labeled proteins as determined by immunoprecipitation are indicated by symbols defined in the key. Pel, pellet; Sup, supernatant. (**d**) AWP28 labeling of intact primary mouse BMMs stimulated with 500 nM staurosporine for 4 h. Cells were labeled with the indicated probe concentration for the final hour before harvest. Labeled proteins that are competed away upon pretreatment with 50 μM JPM-OEt (JPM) or 10 μM LI-1 reveal off-target lysosomal cysteine proteases. Unstim, unstimulated. (**e**) Labeled protein identification in staurosporine-stimulated samples by immunoprecipitation. The identities of the labeled proteins as determined by immunoprecipitation are indicated by symbols defined in the key at lower right.

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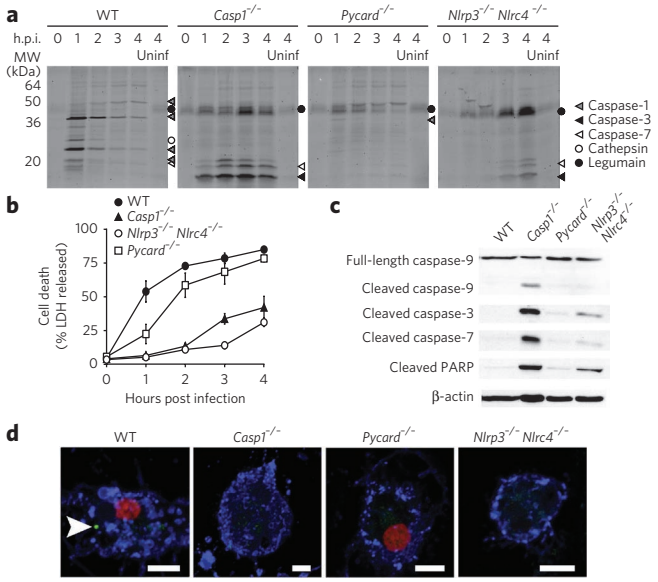

Figure 2 | *S.* Typhimurium infection triggers apoptosis in the absence of inflammasome-mediated caspase-1 activation. (**a**) BMMs missing different inflammasome components were infected with *S.* Typhimurium (10:1) and labeled with 1 μM AWP28. The identities of the various labeled proteins are indicated with symbols defined in the key. MW, molecular weight; h.p.i., hours post infection; uninf, uninfected. (**b**) Cell death was measured by release of lactate dehydrogenase. Data represent the mean of three samples ± s.d. (**c**) BMMs were infected with *S.* Typhimurium (10:1). Whole-cell lysates were blotted for caspase-9, cleaved caspases 3 and 7, and cleaved PARP. β-actin serves as a loading control. Full blots are shown in **Supplementary Figure 8**. (**d**) BMMs were infected with *S.* Typhimurium (10:1). Cells were labeled with 1 μM AWP28 (green) and stained with annexin V (blue) and propidium iodide (red). Uninfected and staurosporine controls are shown in **Supplementary Figure 9**. Inflammasome focus is indicated with white arrowhead. WT, wild type. Scale bars, 5 μm.

AWP28 can also be used in microscopy to show colocalization of active caspase-1 with the adaptor protein ASC, which oligomerizes into large macromolecular inflammasome foci that serve as an activating platform for procaspase-1 (ref. 13) (**Supplementary Fig. 2**). We found that the fraction of AWP28-labeled ASC foci saturated at concentrations as low as 100 nM, whereas FLICA produced much higher levels of background and failed to completely label all ASC foci even at the highest concentration tested (**Supplementary Fig. 2b**). Notably, despite this level of potency, AWP28 does not inhibit downstream physiological effects of infection such as cell death or interleukin-1β processing and release (**Supplementary Fig. 3**).

We next used AWP28 to dissect the kinetics of caspase-1 activation upon *S.* Typhimurium infection in macrophages lacking different components of the inflammasome complex. Active forms of caspase-1 could be detected by AWP28 for several hours following infection of wild-type BMMs (**Fig. 2a**). This activity was lost over time because the active protease was secreted and the cells died via pyroptosis[14]. To our surprise, when the probe was used to visualize caspase activation in *Casp1*$^{-/-}$ BMMs, robust activation of the executioner caspases 3 and 7 was observed (**Fig. 2a** and **Supplementary Fig. 4**). We also found that activation of the executioner caspases was delayed in infected BMMs lacking the receptors for *S.* Typhimurium, NLRP3 and NLRC4 (ref. 13), compared to activation in *Casp1*$^{-/-}$ cells. In contrast, when ASC-deficient BMMs were infected with *S.* Typhimurium, none of the caspases were robustly activated (**Fig. 2a**). These cells undergo pyroptosis similarly to wild-type BMMs but do not produce detectable amounts of cleaved caspase-1 (ref. 15). Activity of the p45 procaspase-1 has been shown to be essential for this cell death; however, this activity seems to be below the detection limit of AWP28. *S.* Typhimurium infected wild-type BMMs, and *Pycard*$^{-/-}$ BMMs (lacking ASC) showed rapid release of lactate dehydrogenase (LDH), which is a hallmark of pyroptosis[1–3] (**Fig. 2b**). In contrast, infected cells lacking caspase-1 or the inflammasome receptors released LDH at a much slower rate, consistent with a primarily apoptotic cell death. Together, these results suggest that caspases 3 and 7 are activated in response to *S.* Typhimurium infection in the absence of inflammasome-mediated caspase-1 activation.

We wanted to better characterize the delayed cell death observed in both the *Casp1*$^{-/-}$ and *Nlrp3*$^{-/-}$*Nlrc4*$^{-/-}$ genotypes. Activation of executioner caspases 3 and 7 in *S.* Typhimurium–infected macrophages correlated with cleavage of initiator caspase-9 as well as with cleavage of the executioner caspase substrate poly(ADP-ribose) polymerase (PARP; **Fig. 2c**). These cells also retained membrane integrity 2 h after infection, as shown by their lack of propidium iodide staining, but they were stained by annexin V, indicating a loss of membrane asymmetry characteristic of early apoptosis (**Fig. 2d**). In contrast, both infected wild-type and *Pycard*$^{-/-}$ BMMs were propidium iodide and annexin V positive owing to membrane damage associated with pyroptosis[2]. Infected wild-type cells also formed AWP28-labeled inflammasome foci (**Fig. 2d**). We did not detect cleaved caspases 3, 7 or 9 or PARP in infected wild-type BMMs undergoing pyroptosis (**Fig. 2c**). Taken together, these results suggest that BMMs infected with *Salmonella* undergo apoptosis in the absence of inflammasome-mediated caspase-1 activation. This phenomenon was also observed upon infection with the Gram-negative coccobacillus *Francisella novicida*, consistent with recently published work[16] (**Supplementary Fig. 5**).

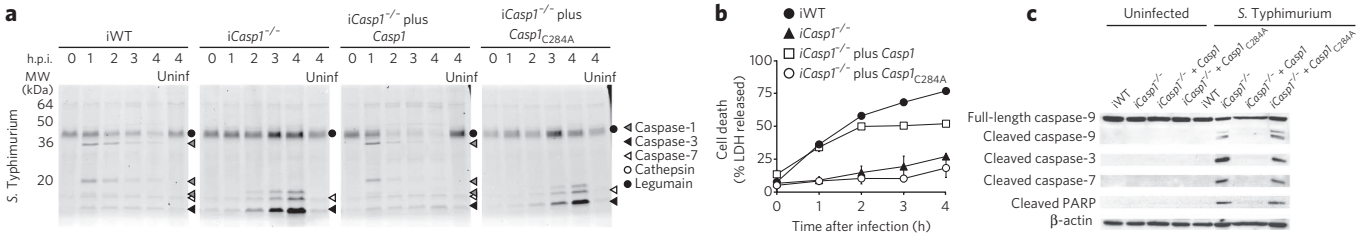

Figure 3 | Caspase-1 activity is required to bypass apoptosis upon infection. Immortalized BMMs (indicated by 'i') stably transfected with wild-type or catalytically dead (C284A) caspase-1 were infected with *S.* Typhimurium (10:1). (**a**) Time course of caspase activation. BMMs were labeled with 1 μM AWP28. The identities of the various labeled proteins are indicated with symbols defined in the key. h.p.i., hours post infection; uninf, uninfected; WT, wild type; MW, molecular weight. (**b**) Time course of *S.* Typhimurium–induced cell death as measured by LDH release. (**c**) Characterization of cell death by blotting whole-cell lysates for caspase-9, cleaved caspases 3 and 7, and cleaved PARP. β-actin serves as a loading control. Full blots are shown in **Supplementary Figure 10**.

We examined mitochondrial cytochrome *c* release as a marker of apoptosis upon bacterial infection. Cytochrome *c* was detected in the cytosol of *Casp1*$^{-/-}$ BMMs and in a delayed manner in *Nlrp3*$^{-/-}$*Nlrc4*$^{-/-}$ cells infected with *S.* Typhimurium (**Supplementary Fig. 6**). Unexpectedly, cytochrome *c* was also rapidly released in the wild-type and *Pycard*$^{-/-}$ macrophages, which undergo pyroptosis instead of apoptosis; however, these pyroptotic cells were not able to effectively induce caspase-9 activation (**Fig. 2c** and **Supplementary Fig. 5**). Because active caspases are known to induce cytochrome *c* release[17], it is possible that cytochrome *c* release during pyroptosis happens at a late stage of death and is not capable of inducing apoptosome formation.

Because caspase-1 acts in a complex with other proteins, we wanted to determine whether the presence of the protein alone was sufficient to block the apoptotic death observed in the caspase-1–deficient cells. Therefore, we infected immortalized *Casp1*$^{-/-}$ BMMs stably expressing active or catalytically dead (C284A) caspase-1 (ref. 15) with *S.* Typhimurium (**Fig. 3a**). We observed faint labeling of active caspases 3 and 7 by AWP28 in uninfected wild-type and *Casp1*$^{-/-}$ control cells; however, no background cytochrome *c* release was observed in these cells (**Supplementary Fig. 7**). *Casp1*$^{-/-}$ cells expressing wild-type caspase-1 showed caspase-1 labeling by AWP28 upon infection and underwent pyroptosis with kinetics similar to that of immortalized wild-type BMMs (**Fig. 3a**,**b**). However, expression of the catalytically dead mutant caspase-1 failed to block the robust activation of the apoptotic executioner caspases 3 and 7 seen in control *Casp1*$^{-/-}$ cells (**Fig. 3a**). Immortalized *Casp1*$^{-/-}$ BMMs expressing catalytically dead caspase-1 also retained markers of apoptosis, including caspase-9 and PARP cleavage (**Fig. 3c**). Together, these results indicate that caspase-1 proteolytic activity is required to bypass apoptosis in response to *Salmonella* infection. *Casp1*$^{-/-}$ BMMs also lack caspase-11 (ref. 18); however, our ability to restore pyroptosis by expressing only caspase-1 confirms that the interplay between apoptosis and pyroptosis is solely dependent on caspase-1 activity.

In conclusion, we used AWP28 to show that intracellular bacterial pathogens trigger apoptosis in the absence of caspase-1 activation. Pyroptosis provides an effective mechanism for the clearance of intracellular bacteria[19], explaining why many intracellular pathogens have evolved strategies to prevent inflammasome-mediated caspase-1 activation[20,21]. It would, therefore, be beneficial for the host to evolve a backup cell death pathway to destroy the replicative niche for intracellular pathogens capable of inhibiting pyroptosis. The bacterial pathogen-associated molecular patterns that transduce the proapoptotic signal are unknown. However, we show that apoptosis triggered by *S.* Typhimurium infection is delayed in the absence of the inflammasome receptors NLRP3 and NLRC4, suggesting possible overlap between proapoptotic and propyroptotic signals (**Fig. 2** and **Supplementary Fig. 6**). In some instances, proinflammatory stimuli have been reported to trigger apoptosis in the absence of caspase-1 (refs. 16,22,23). It is now clear from our results that caspase-1 activity is required to suppress apoptosis upon *Salmonella* infection. It has recently been shown that, during embryogenesis and T-cell proliferation, active proapoptotic caspase-8 blocks necroptosis by processing the deubiquitinase CYLD to generate a survival signal[24,25], which is consistent with our findings of cross-regulation of death pathways by caspase-1 activity. It is not yet clear whether caspase-1 directly cleaves a substrate that prevents the activation of the apoptotic cascade. The identification of specific points of feedback between proinflammatory and immunologically silent cell death programs may enable innate immune responses to be therapeutically modulated in pathologies such as autoinflammatory diseases and cancer.

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Author contributions

A.W.P., P.B., A.S., D.M.M. and M.B. designed the experiments. A.W.P. and P.B. performed the experiments. A.W.P., P.B., A.S., D.M.M. and M.B. analyzed the data. A.W.P. and M.B. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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