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Technical note

Applying caspase-1 inhibitors for inflammasome assays in human whole blood

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ABSTRACT

Caspase-1 processes pro-IL-1β and pro-IL-18 into bioactive forms. Caspase-1 activity is regulated by a multiprotein complex known as an inflammasome. Multiple danger and damage associated signals drive inflammasome formation. Currently, evaluation of inflammasome activity is of particular interest as its role in chronic and acute inflammatory pathologies becomes evident. Specific inhibitors are therefore required to evaluate the contributions of the inflammasome and IL-1β to these disease processes. While several inhibitors are available for caspase-1 blocking experiments, in this study we show effects of two commonly used caspase inhibitors: z-VAD-fmk and ac-YVAD-cmk on secretion of pro-inflammatory cytokines: IL-1β, TNFα, IL-8 and IL-6 in whole blood stimulated with LPS. We demonstrate ac-YVAD-cmk is a specific caspase-1 inhibitor resulting in pronounced decreases in IL-1β and less suppression of TNFα, IL-6 and IL-8, while pan-caspase inhibitor, z-VAD-fmk, only weakly suppressed Il-1β while acting strongly on the other three cytokines. Furthermore, we demonstrated that simultaneous treatment of whole blood cultures with inhibitor and LPS fails to attenuate the IL-1β response. In contrast, pretreatment with inhibitors prior to LPS stimulation is required to achieve marked decreases in IL-1β production. Thereby also demonstrating IL-1β release by cells in whole blood culture stimulated with LPS is a rapid response. Thus studying inflammasome/caspase-1/IL-1β axis requires appropriate selection and application of inhibitors.

1. Introduction

Caspases (cysteinyl aspartate proteases) represent the class of intracellular proteases evolved in multicellular organisms. They are essential mediators of cell death and inflammation (Siegel, 2006). Pro-apoptotic and pro-inflammatory caspases can be distinguished based on their substrate specificity and involvement in diverse signaling cascades. For instance, caspases-3, -6 and -7 play roles as effectors of apoptosis (Slee et al., 2001). Conversely, caspases-1, -4, -5, -11 and -12 regulate the proteolytic processing of the inflammatory cytokines precursors to their mature forms (Siegel, 2006).

Caspase-1, specifically is an important member of multiple adaptor complexes known as inflammasomes, which are assembled in response to microbial and/or damage-associated signals (Osuka et al., 2012). Activation of caspase-1 leads to proteolytic processing of immature pro-IL-1β and pro-IL-18 to their bioactive forms. Furthermore, activation of caspase-1 stimulates pyroptosis, a form of cell death characterized by rapid pore formation in the plasma membrane followed by osmotic cell lysis (Satoh et al., 2013).

Caspase-1 inhibitors are commonly used to investigate inflammasome activity (Halle et al., 2008; Dostert et al., 2009; Netea et al., 2009; Osuka et al., 2012). Often utilized, z-VAD-fmk, is a cell-permeable, irreversible pan-caspase inhibitor. Importantly, z-VAD-fmk also has affinity to caspases-3, -7 and -8, thereby stabilizing Beclin1 and promoting autophagy (Zhu et al., 2010). Also of use is ac-YVAD-cmk, a specific irreversible inhibitor of caspase-1, however it also exerts some activity

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against caspase-4 and caspase-5 (Santa Cruz Biotechnology I., 2013).

In order to demonstrate inflammasome/caspase-1 involvement in disease processes, caspase-1 inhibitors are utilized to block pre-IL-1β processing, however additional targets for these inhibitors are not always considered. In a simple model of whole blood culture stimulated with LPS, we demonstrate profound effects of z-VAD-fmk on other cytokines not processed by the inflammasome (IL-6, TNFα and IL-8), whereas ac-YVAD-cmk preferentially blocked IL-1β secretion. Therefore we strongly urge investigators to utilize the most specific caspase inhibitors available when trying to block caspase-1 mediated post-translational processing of IL-1 family of pro-cytokines.

2. Materials and methods

Briefly, we prepared whole blood cultures by diluting sodium heparinized blood obtained from healthy volunteers with RPMI media (1:1) and incubated in the absence or presence of 2 ng/ml lipopolysaccharide (LPS from *Escherichia coli* O111:B4) at 37 °C, 5% CO_2 for only 3 h (in order to limit the contribution of *de novo* transcription of cytokines in this analysis). Culture supernatant was collected and assayed for IL-1β, TNFα, IL-6 and IL-8 by MSD (Mesco Scale Discovery) or ELISAs (R&D systems) as indicated. To evaluate post-translation processing of IL-1β by caspase-1, inhibitors z-VAD-fmk (Invivogen) or ac-YVAD-cmk (Sigma-Aldrich) were applied at a final concentration of 100 μM/per 1 ml of whole blood, prior to dilution.

3. Results and discussion

In whole blood, primary monocytes respond to LPS stimulation directly driving IL-1β secretion (Netea et al., 2009). In our culture model, LPS induces significant secretion of IL-1β in 3 h compared to unstimulated control cultures (Fig. 1), although peak levels typically occur approximately 6 h post stimulation (data not shown). Caspase inhibitors are reportedly effective in concentrations of 10–100 μM in different cell culture conditions. Initial evaluation of the dose required for z-VAD-fmk inhibition of IL-1β release from LPS stimulated whole blood cultures yielded little to no inhibition of IL-1β responses (Fig. 1, top panel, and additional data not shown). Thus further evaluation continued with the highest reported concentration of inhibitors (100 μM). While simultaneous treatment of the cultures with caspase inhibitor, z-VAD-fmk (100 μM) and LPS (2 ng/ml) did not significantly inhibit IL-1β release, one hour pre-treatment of whole blood with z-VAD-fmk (100 μM) prior to LPS stimulation resulted in attenuation of IL-1β release (Fig. 1, bottom panel).

Although z-VAD-fmk pre-treatment reduced IL-1β secretion to some extent after LPS stimulation in several but not all subjects, we also compared this response with a more specific caspase-1 inhibitor, ac-YVAD-cmk (Fig. 2). In this comparison, sodium heparinized blood samples from additional healthy volunteers were pre-treated for 1 with caspase inhibitors as indicated prior to LPS stimulation. In addition to IL-1β measures, other pro-inflammatory cytokines not post-translationally regulated by inflammasome/caspase-1 activity (IL-6, IL-8, and TNFα) were also assessed to determine off-target effects. These results are summarized in Table 1. Ac-YVAD-cmk strongly inhibited IL-1β production (on average 92% ± 2%), consistently

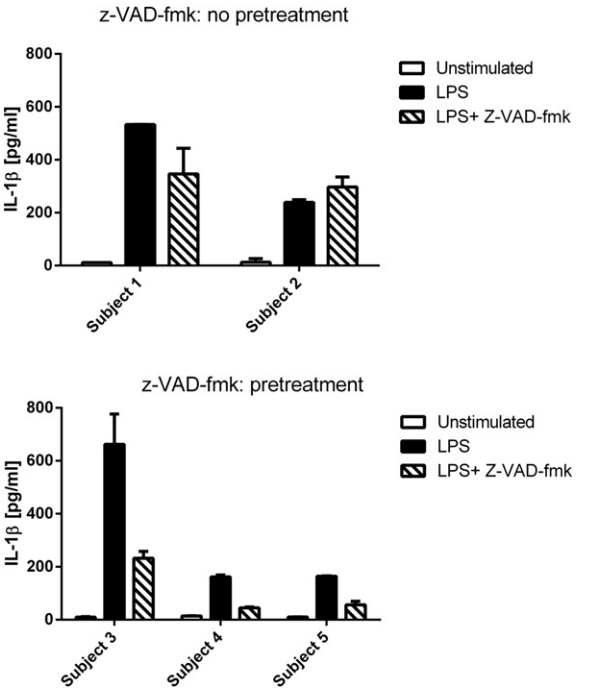

Fig. 1. IL-1β response following simultaneous treatment with caspase inhibitor, z-VAD-fmk and LPS stimulation *versus* pre-treatment with the inhibitor and subsequent stimulation with LPS. (Upper part): Whole blood, donated by two healthy volunteers was simultaneously treated with LPS and z-vad-fmk and incubated for 3 h. (Lower part): whole blood was pre-treated for 1 h with z-VAD-fmk and then stimulated with LPS and incubated for 3 h. IL-1β was measured by R&D Quantiglo ELISA.

in all 6 subjects. Importantly, the inhibitory effect of ac-YVAD-cmk on the other cytokines measured was markedly less (on average 28% ± 9%, 31% ± 15% and 45% ± 10%, for TNFα, IL-6 and IL-8, respectively). In contrast, z-VAD-fmk inhibited IL-1β release only by 21% (SD 35%, Table 1). Whereas the release of TNFα, IL-6 and IL-8 was strongly and consistently inhibited by z-VAD-fmk pre-treatment (on average 71% ± 9%, 96% ± 3% and 81% ± 10%, respectively) (Table 1, Fig. 2).

This data clearly demonstrates different effects on inflammatory cytokine secretion with caspase inhibitors of differing specificity. For investigations of inflammasome/caspase-1 mediated production of IL-1β, ac-YVAD-cmk is preferred by its strong inhibition and specificity.

The effects on TNFα, IL-8 and IL-6 may be due to indirect roles of inflammatory caspases in influencing the secretion of other proinflammatory cytokines. It is also possible that the dramatic reduction in IL-1β seen with ac-YVAD-cmk inhibition, contributes indirectly to reduce TNFα, IL-6 and IL-8 secretion due to the lack of IL-1β signaling, even during the short duration of these experiments. Interestingly it has been demonstrated *in vitro* that NF-κB can also serve as a target for caspase-1 (Lamkanfi et al., 2004), which could also lead to the reductions in cytokine secretion as observed in these experiments. Notably the requirement for specific caspase inhibitor pre-treatment, demonstrates caspase-1 activation and thereby assembly of the inflammasome, is a rapid effect of LPS-mediated priming in primary human leukocytes.