Dengue virus-infected human monocytes trigger late activation of caspase-1, which mediates pro-inflammatory IL-1β secretion and pyroptosis

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Dengue virus (DENV) infection affects millions of people annually and has the potential to cause fatal haemorrhagic fever and shock. Although the underlying pathogenesis of severe dengue illness is still unclear, current evidence suggests that severe disease progression has an immunological basis. In this study, we investigated the role of caspase-1 during host–pathogen interactions within DENV-infected human monocytes. Using DENV-infected primary monocytes, we examined caspase-1 at various levels of gene expression and probed for potential immune consequences mediated by caspase-1 such as secretion of pro-inflammatory IL-1β and pyroptotic cell death. We report that DENV-infected monocytes upregulated functional caspase-1 mRNA and pro-caspase-1 activation as a late response to infection. In addition, we found that caspase-1 is responsible for IL-1β secretion and pyroptosis of DENV-infected monocytes. Together, our results show that late caspase-1 activation within DENV-infected monocytes can contribute to pro-inflammatory outcomes that might play a role in dengue immunopathogenesis.

Dengue is an arthropod-borne viral disease that affects approximately 50–100 million people annually (Leong et al., 2007). Dengue virus (DENV), the pathogen responsible for this disease, is an enveloped, positive-sense, ssRNA virus with four known serotypes (DENV1–DENV4). After transmission of DENV to a human host, the infected individual first appears asymptomatic but subsequently develops an acute febrile phase of dengue fever (DF). Although DF is a mild and self-limiting disease, this condition may worsen in a minority of patients, resulting in the more severe and life threatening dengue haemorrhagic fever (DHF)/dengue shock syndrome (DSS).

To date, the pathogenesis of DHF/DSS is not well understood and the exact determinants that trigger severe illness are still unclear. No single mechanism has succeeded in fully describing the underlying cause of DHF/DSS. However, current available evidence suggests a strong immunological basis behind the manifestation of severe dengue disease (Pang et al., 2007; Ong & Chu, 2011).

In line with the immunological basis of DHF/DSS, the innate immune gene caspase-1 was found to be upregulated in DENV-infected cells in our pilot quantitative reverse transcriptase-PCR (qRT-PCR) screening arrays. Caspase-1 is a cytokine maturation caspase known to proteolytically activate the pro-forms of inflammatory cytokines IL-1β, IL-18 and IL-33. In addition, caspase-1 was recently found to mediate lipid biogenesis and a novel form of programmed cell death known as pyroptosis (Brennan & Cookson, 2000; Hilbi et al., 1998; Gurcel et al., 2006).

The role of caspase-1 during DENV infection was virtually unknown until a recent study by Wu et al. (2013), who found elevation of the activated caspase-1 p20 subunit released by granulocyte–macrophage colony-stimulating factor-stimulated macrophages after DENV infection. Further investigation by this group discovered the critical role of CLEC5A during inflammasome assembly, which is necessary for caspase-1 activation within DENV-infected macrophages (Wu et al., 2013). Given that a cytokine storm response is often associated with the development of DHF/DSS, their results suggested that DENV-infected macrophages could potentially contribute to an exaggerated cytokine response through unregulated caspase-1 activation.

Primary monocytes of the mononuclear phagocyte lineage are regarded as a good in vitro cell-culture model for the immunopathogenesis of dengue because: (i) human monocytes have been recognized as the primary targets and disseminators of DENV in vivo (Jessie et al., 2004; Kou et al., 2008; Alhoot et al., 2011); and (ii) monocytes are circulating immune cells known to mediate chronic inflammation through the secretion of pro-inflammatory cytokines such as IL-1β (Agarwal et al., 1995; Tilton et al., 2006). In this study, we investigated whether caspase-1 activation could be triggered within DENV-infected monocytes prior to their differentiation into macrophages.
Using DENV-infected primary monocytes, we examined caspase-1 at various levels of gene expression and probed for potential downstream immune consequences mediated by caspase-1 such as IL-1β secretion and pyroptosis.

In our initial experiments, we sought to confirm the in vitro permissivity of primary human monocytes to DENV-2 infection. Using whole blood from healthy blood donors (NUS Institutional Review Board ethic approval: 10-072E), primary monocytes of 94.2 ± 2.2% purity were isolated from purified PBMCs using a Monocyte Isolation kit II, human (Miltenyi Biotec). Freshly isolated monocytes were then either mock-infected with L-15 medium or inoculated with DENV-2 at an m.o.i. of 10, after which the infectious viral titre was ascertained by plaque assay at 24 h intervals. As expected for mock-infected controls, no infectious viral particles could be detected (data not shown). In contrast, infectious DENV-2 particles could be detected from human monocytes by 24 h post-infection (p.i.) and the viral titre increased steadily, peaking on day 3 at 4.26 log10 p.f.u. ml⁻¹ (Fig. 1a). Taken together, our results are consistent with previous studies and indicated that primary human monocytes are indeed susceptible to productive DENV-2 infection in vitro (King et al., 2002; Chao et al., 2008).

To decipher the role of caspase-1 during host–pathogen interactions, we first performed qRT-PCR to analyse the endogenous levels of functional caspase-1 mRNA splice variants within DENV-infected monocytes. Total RNA was extracted at 24 h intervals from primary monocytes that were either mock-infected or inoculated with DENV at an m.o.i. of 10. qRT-PCR was then carried out using primers for caspase-1 (5’-CAGCCCTTGTTGGTGTG-3’ and 5’-AAAATCCTTCTCTATGTTGGGCTTTC-3’) and actin (5’-AGGCGGCTACAGCCTCA-3’ and 5’-GGCGACGTAGCACTACGTCTT-3’). We observed that functional caspase-1 mRNA splice variants increased generally over time, albeit with no statistically significant increase during the first 3 days p.i. (Fig. 1b). However, a notable fold change of 2.15 was achieved by day 4, indicating late upregulation of functional caspase-1 transcripts p.i. (Fig. 1b).

Using Western blotting, we next examined caspase-1 expression and proteolytic activation at the protein level over 4 days. Four parallel experiments were carried out, each involving a 2 h treatment period with: (i) L-15 medium (control); (ii) DENV at an m.o.i. of 10; (iii) UV-inactivated DENV; or (iv) 100 ng lipopolysaccharide (LPS) ml⁻¹ (positive control). Time point zero was taken to

![Fig. 1. (a) Replication kinetics of DENV-infected monocytes (n=3) (b) qRT-PCR analysis of functional caspase-1 mRNA splice variants. Statistical analyses were carried out using a one-tailed Student’s t-test (n=3); *P<0.05. (c–f) Representative Western blot analysis of human monocytes. Three independent experiments were carried out each time with a different PBMC donor.](image-url)
begin at the end of the 2 h treatment. The primary antibodies used were anti-caspase-1 (Santa Cruz), anti-caspase-1 p10 (Santa Cruz) and anti-pan-actin (Millipore). The results from our mock-infected controls were consistent with previous studies, showing that caspase-1 was expressed constitutively in unchallenged human monocytes across all five time points and mainly accumulated as a pro-enzyme (Fig. 1c) (Schumann et al., 1998; Lin et al., 2000). When inoculated with DENV, pro-caspase-1 levels remained relatively constant for 48 h but dropped on days 3 and 4 (Fig. 1d). This was correlated with an increase in activated caspase-1 p10 subunits, as evident by a distinctly intense band on day 4 p.i. (Fig. 1d). The comparatively low level of activated caspase-1 p10 observed on the first 3 days p.i. suggested that the induction of caspase-1 activity occurred mainly as a late response in DENV-infected monocytes.

Interestingly, results from both qRT-PCR and Western blot experiments demonstrated an upregulation of caspase-1 at the transcriptional and post-translational levels during late DENV infection. Together, both pieces of evidence support the notion that caspase-1 may have a potential role in the advanced stages of DENV infection. Whether the delayed upregulation of caspase-1 gene expression is a host- or virus-induced event is currently unknown. As caspase-1 is an innate immune gene, it is likely that the upregulation of caspase-1 is part of a host-induced immune response.

Possible explanations for the initial delay in caspase-1 upregulation were obtained when we exposed human monocytes to UV-inactivated DENV. Surprisingly, we found that virtually all pro-caspase-1 was cleaved into its activated p10 form after just 2 h treatment with UV-inactivated DENV (Fig. 1e). These results indicated that UV-inactivated DENV is capable of triggering caspase-1 activation without involving an initial delay; in addition, the fact that UV-inactivated DENV could provoke pro-caspase-1 activation implies that activation of the pro-enzyme is independent of DENV replication. LPS treatment is included as positive control for this part of the study (Fig. 1f).

Interestingly, DENV antigens were also present in the DENV-infected set-up. However, pro-caspase-1 activation predominately occurred after a significant delay of several days. This suggests that ‘live’ DENV may be suppressing initial pro-caspase-1 activation in the host cell. As caspase-1 has a role in regulating innate immune defences, suppressing pro-caspase-1 activation could be a strategy to evade host immune defences while the pathogen establishes a productive infection.

Activation of pro-caspase-1 after DENV infection prompted us to investigate the possible immune consequences mediated by it. As caspase-1 is known to cleave pro-IL-1β into its biologically active form, we examined the secretion of this pro-inflammatory cytokine using ELISA (BD Bioscience). Four parallel experiments were carried out with the same treatment groups used in our earlier Western blot experiments, and the supernatants were analysed every 24 h to track the release of IL-1β. Our data showed that both DENV infection at an m.o.i. of 10 and exposure to UV-inactivated DENV were capable of triggering IL-1β secretion, mirroring the trend observed for the LPS positive control (Fig. 2a). The sharp rise in IL-1β by 24 h p.i. was surprising given that our Western blot data showed a predominant late activation of caspase-1 after DENV infection. However, our ELISA results seem consistent with previous studies reporting that IL-1 secretion could be detected from monocytes as early as 4 h after DENV infection (Chang & Shaio, 1994).

The issue of which protease is responsible for processing IL-1β for secretion has become further complicated with the discovery that caspase-1-independent pathways exist. Neutrophil elastase, chymase and several matrix metalloproteases have been shown to have the capacity to catalyse IL-1β maturation and release (Schönbeck et al., 1998; Guma et al., 2009). It is thus possible that any one or more of these enzymes could be responsible for the rise in IL-1β secretion following DENV infection. Whether caspase-1 is a key player mediating the initial rise in IL-1β comes into question.

In order to decipher whether caspase-1 contributes to IL-1β secretion following DENV infection, we blocked caspase-1 activity using the irreversible caspase-1-specific inhibitor acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-cmk; Bachem). An alamarBlue Cell Viability Assay (Invitrogen) was first performed to determine the cytotoxicity of the inhibitor and to optimize a working range of inhibitor concentration (Fig. 2b). When caspase-1 activity was inhibited following DENV infection at an m.o.i. of 10, we found that IL-1β secretion decreased significantly in a dose-dependent manner (Fig. 2c). This result indicated that caspase-1 was mediating IL-1β secretion from DENV-infected human monocytes but not entirely, as IL-1β could be detected even when 100 µM inhibitor was applied.

Interestingly, we noted that both DENV and UV-inactivated DENV were capable of triggering similar levels of IL-1β secretion by 24 h post-treatment (Fig. 2a). This result suggested that the induction of IL-1β secretion may be triggered through binding of DENV onto cell-surface receptors such as CLEC5A, in a signalling pathway independent of DENV replication (Wu et al., 2013). In view of the low infectability of primary monocytes to DENV, we hypothesize that such a ‘bystander’ effect on uninfected monocytes could account for the occurrence of caspase-1 activation despite the absence of active DENV infection.

In addition to mediating cytokine maturation, caspase-1 has been reported to promote cell survival in response to bacterial pore-forming toxins such as aerolysin. In a study by Gurcel et al. (2006), cells exposed to aerolysin were found to activate caspase-1, which subsequently went on to mediate lipid biogenesis via the processing of sterol regulatory element-binding protein. The induction of lipid biogenesis was necessary to restore plasma membrane integrity, which enhanced cell survival and bacterial toxin resistance. Intrigued by the possibility that DENV might exploit this function of caspase-1 to enhance viral assembly...
and release, we blocked caspase-1 activity following DENV infection and indeed observed a drop in DENV replication on the day of peak viral titre (Fig. 2d). Although these decreases were modest at best (half log), our results nevertheless suggest that caspase-1 may have a minor role in promoting virus replication.

Pyroptosis is a pro-inflammatory mode of programmed cell death that has until now mostly been documented during infection of macrophages. Induction of pyroptosis has two important immunological functions: first, eradication of the intracellular pathogen replication niche, and secondly, mediating inflammation through the release of intracellular contents. To date, the only established biochemical hallmark of pyroptosis is caspase-1 dependency (Fink & Cookson, 2005). Morphologically, pyroptotic cell death is known to exhibit features such as plasma membrane pore formation, cell lysis and DNA fragmentation (Bergsbaken et al., 2009).

To investigate the occurrence of pyroptosis in DENV-infected human monocytes, we incorporated caspase-1 inhibitor treatment into sub-G1 cell-cycle analysis. This technique allowed us to specifically detect the presence of DENV-induced pyroptotic cell death via two hallmarks of pyroptosis namely, caspase-1 dependency and DNA fragmentation. From our flow cytometry results, we found that inhibition of caspase-1 activity significantly reduced DENV-induced cell death on days 4 and 5 p.i. by 14.06 and 11.23%, respectively (Fig. 3a). As pyroptosis is the only mode of cell death known to be dependent on caspase-1 and also to mediate genomic fragmentation, this result is, to our knowledge, the first evidence that DENV infection can induce pyroptosis of human monocytes.

To further our biochemical evidence for pyroptosis, transmission electron microscopy imaging was used to detail the morphological characteristics of DENV-induced cell death. A similar ultrastructural study was reported almost a decade ago by Mosquera et al. (2005) who examined DENV-infected monocytes over a period of 6 h p.i. However, as caspase-1-dependent cell death seems to occur late after DENV infection, we extended our investigation over 4 days. Data from the earlier study was consistent with the established notion that DENV infection
induced apoptotic cell death (Mosquera et al., 2005). When we performed the experiment by infecting primary monocytes at an m.o.i. of 10, apoptosis could indeed be detected as early as 2 h after infection with DENV (Fig. 3b–e). Many classical morphological hallmarks of apoptosis such as cell shrinkage, plasma membrane blebbing, apoptotic body formation and nuclear fragmentation were observed (Fig. 3c). Together, these results are in concordance with the earlier report and also add to the body of data supporting DENV-induced apoptosis.

Interestingly, by 24 h p.i. we found that some monocytes were undergoing cellular lysis, a feature that is not associated with apoptosis. Upon closer inspection, there seemed to be an ordered series of events that culminate in the rupturing of DENV-infected monocytes. Beginning as an intact cell, monocytes that were destined to undergo lysis were found to have a highly condensed nucleus with many intracellular vesicles (Fig. 3f). Fusion of these vesicles made the cell extremely membranous (Fig. 3g). Few organelles were left by the time the unstable cell ruptured and spilled its intracellular contents into the extracellular milieu (Fig. 3h).

Although cellular lysis is typically associated with passive necrosis, membrane rupture is also a hallmark of pyroptosis. Furthermore, the orderly sequence of events suggested an active mode of programmed cell death instead of passive cell death. Despite the resemblance to pyroptosis, morphological data alone is insufficient to implicate with certainty the occurrence of pyroptotic cell death. Hence our present ultrastructural data indicate the presence of an alternative mode of cell death that shares similarities with pyroptosis.

In this study, we found that DENV-infected monocytes upregulated functional caspase-1 mRNA splice variants and pro-caspase-1 activation as a late response to infection. In addition, we showed that caspase-1 activation within DENV-infected monocytes was responsible for IL-1β secretion and the induction of pyroptotic cell death. Together, our results show that caspase-1 activation within DENV-infected monocytes can lead to pro-inflammatory
outcomes that, if unregulated, can potentially lead to the development of severe dengue disease.

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References


