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Caspases are key regulators of inflammatory and innate immune responses mediated by TLR3 *in vivo*

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ABSTRACT

Understanding the key regulators which impact the innate immune response during initial phases of tissue injury, can advance the use of therapeutic approaches which aim at attenuating inflammation and organ damage. Recognition of microbial components by TLRs, initiates the transcription of innate immune signal pathways, that induce the expression of key inflammatory mediators: cytokines, chemokines and adhesion molecules. Beside regulating apoptotic cell death, recent studies have revealed distinct roles for caspases in the optimal production of inflammatory cytokines and host defense against injurious infections. Whether caspases can play an immune regulatory role *in vivo* has not been sufficiently investigated. This study aims to explore whether the pan caspase inhibitor z-VAD-fmk can control inflammation and cytokine production subsequent to challenging the innate immunity of the exocrine secretory tissues *in vivo*. Submandibular glands (SMGs) of the C57BL/6 mice were challenged with the TLR3 stimulant: polyinosinic-polycytidylic acid (poly (I:C)). Results obtained from the current study provide evidence that caspases can control immune responses downstream of TLR3 ligation. The present work proposes a novel mechanism that can prevent overactivation of the innate immunity, which typically leads to fatal immune disorders.

1. Introduction

Apoptosis has been considered as one of the main factors that may be related to loss of SG secretory function (Hayashi, 2011). In fact, death of acinar and ductal cells is considered to be a major mechanism leading to salivary gland dysfunction in patients with Sjögren's syndrome (Horai et al., 2016) and following irradiation of salivary glands (Acauan et al., 2015). While apoptosis was classically described as an immunologically silent mode of cell death, several studies have shown that cells and tissues stimulated to undergo apoptosis produce moderate amounts of chemoattractant proteins and cytokines (Cullen et al., 2013), suggesting that apoptosis and inflammation are inseparably linked. Moreover, recent studies indicate that caspases, the initiators of the apoptotic cell death pathway, are situated at the nexus of vital networks that balance inflammation, antiviral immunity and cell death (Chen et al., 2017).

Caspases are a family of proteases that have been subdivided functionally into those involved in either apoptosis (caspases-2, −3, −6, −7, −8, −9 and −10 in mammals) or inflammation (caspases-1, −4, −5 and −12 in humans and caspases-1, −11 and −12 in mice) (Cohen, 1997). While the apoptotic caspases function in the initiation and execution of programmed cell death, inflammatory caspases mediate innate immune responses by cleaving proinflammatory cytokine precursors (for example, pro-IL-1β and pro-IL-18) to initiate inflammation (Man and Kanneganti, 2016).

Emerging studies have shown that caspase inhibition prevents inflammation via preventing T cell activation, Th2 cytokine production and inflammatory cell infiltration (Iwata et al., 2003). More recent studies showed that pre-treatment with the cell permeable pan-caspase inhibitor z-Val-Ala-Asp (Ome) fluoro-methyl-ketone (z-VAD-fmk) (which irreversibly binds to the catalytic site of caspase proteases (Gregoli and Bondurant, 1999)), significantly inhibited the activation of myeloperoxidase (MPO), TNF-α, IL-1β and decreased lung injury in a rat model of severe acute pancreatitis (Liu et al., 2016). The role played by all mammalian caspases in the control of inflammatory and immune reactions has been comprehensively reviewed (Galluzzi et al., 2016).

Toll-like receptors (TLRs) are transmembrane pattern recognition receptors, which play key roles in sensing microbial pathogen associated molecular patterns (PAMPs) and mounting an immune response. Indeed, TLRs induce the expression of inflammatory pro-survival factors and cytokines (Janeway and Medzhitov, 2002), that recruit polymorphonuclear leukocytes (PMNs) to the infection site (Kumar et al., 2006). TLR3 has been identified as a key sensor for viral dsRNA or its synthetic double stranded (ds)RNA analogue poly (I:C) (Blasius and

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Beutler, 2010). In turn, poly (I:C) can elicit apoptosis directly in a TLR3 dependent manner (Salaun et al., 2006). Despite the recent studies and reviews that propose caspases as the central regulators of apoptosis, inflammation and key effectors of immune responses against microbial infections (Chen et al., 2017; Man and Kanneganti, 2016; McIntire et al., 2009; Philip et al., 2016; Uchiyama and Tsutsui, 2015; Sagulenko et al., 2016), it is unclear if caspases play an immune regulatory role during acute injury phases *in vivo*. In the current study, we stimulated the TLR3-mediated innate immune response of the C57BL/6 SMGs via the synthetic dsRNA poly (I:C). The induction of a caspase-dependent apoptotic signal in the glandular cells was substantiated as early as nine hours post poly (I:C) intraductal infusion. Moreover, pre-treatment of the mice with z-VAD-fmk, blocked the activated caspases and verified their participation in the TLR3-mediated innate immune signalling and cytokine production.

Our results provide, for the first time, evidence that caspases are central in regulating inflammatory cell infiltration and activation of innate immune responses *in vivo* following TLR3 stimulation.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice weighing 18-21 g (Harlan Labs Ltd., Loughborough, UK) and aged 10–12 weeks were housed in a temperature-controlled environment under a 12 h light–dark cycle, with free access to food and water. All procedures were approved by the local ethics committee and performed under general anaesthesia under a Home Office license.

2.2. Poly (I:C) injury model

The C57BL/6 mouse SMGs were cannulated as previously performed in rats (Correia et al., 2010) and mice (Bombardieri et al., 2012). Initially, a glass cannula (Supelco, 25715, PA- USA) was stretched over a flame and fitted into a polyethylene tube with 0.28 mm inner diameter. Polyinosinic–polycytidylic acid sodium salt (P1530-25MG, Sigma-Aldrich) was then diluted in 0.9% saline solution to a final concentration of 4 mg/ml. Towards consistent, visualised and flawless SMG injection, poly (I:C) was pre-mixed with Trypan blue (T8154-100ML-Sigma- Aldrich) prior to injection. Eighty micrograms of poly (I:C) in 20 µls were loaded into a 0.3 ml syringe (6134900, VWR International). For recovery experiments, mice were anaesthetised intraperitoneally (i.p) with 0.1 ml of combined 5 mg Ketamine/1 mg Xylazine. Under a stereomicroscope, the glass cannula was inserted into Wharton's duct and poly (I:C) was injected slowly and constantly into the left SMG (Fig. 1A). The same volume of the vehicle (0.9% saline and Trypan blue) was delivered to the right SMG as a contralateral negative control.

2.3. Assessment of submandibular secretory function

Animals were anaesthetized with 150 µl of Pentobarbital Sodium (Euthatal, Merial) 1 mg/ml (i.p.), followed by endotracheal intubation. Each submandibular duct was exposed by dissection from the ventral surface through the mylohyoid muscle. Individual submandibular ducts were cut and saliva was collected using polyethylene tubes connected to insulin syringes (Fig. 1B). Saliva collection proceeded for 5 min following onset of secretion after stimulation with pilocarpine (0.5 mg/kg i.p.). Following collection into pre-weighed Eppendorf tubes, the tubes were re-weighed, the volume of saliva was calculated as 1 mg = 1 µl saliva and results were expressed as µL saliva/min.

2.4. z-VAD-fmk and apoptosis inhibition model

Cell-permeable z-VAD-fmk (BD Pharmingen™, 550377) was dissolved in DMSO, aliquoted and stored at −80 °C, and then diluted as needed in PBS for experiments. Mice were pre-treated intraperitoneally with z-VAD-fmk (10 mg/kg), 30 min prior to intraductal injection of poly (I:C) (Equils et al., 2009). In the current study, this group will be referred to as z-VAD-fmk + PIC. The contralateral submandibular gland was injected with the vehicle (0.9% saline and Trypan blue). Tissues were excised and saliva was collected 9 h following poly (I:C) intraductal injection.

2.5. TLR3/dsRNA complex inhibitor model

The TLR3/dsRNA complex inhibitor ((*R*)-2-(3-Chloro-6-fluorobenzo [b] thiophene-2 −carboxamido)-3-phenylpropanoic acid, Calbiochem Merck Millipore, 614310) was used to competitively disrupt poly (I:C) binding to TLR3 (Cheng et al., 2011). The inhibitor was dissolved in DMSO and diluted in PBS. 1 mg/mouse of the inhibitor was intraperitoneally injected into mice (Takemura et al., 2014). At the same time, 4 µg/µl poly (I:C) was combined to 50 ng TLR3/dsRNA and 20 µl of this solution was loaded in a 0.3 ml syringe. Mice were anaesthetised as previously mentioned and poly (I:C)-TLR3/dsRNA inhibitor were injected slowly and consistently into the SMG. In the current study, this group will be referred to as TLR3-I + PIC. The contralateral SMG was injected with the vehicle (0.9% saline, Trypan blue and 50 ng TLR3/dsRNA complex inhibitor). Tissues were excised and saliva was collected 9 h following poly (I:C) intraductal injection.

2.6. Histopathologic examination

Harvested SMGs were fixed in 10% neutral buffer formalin, processed and embedded in paraffin for long term storage. Poly-(I:C) induced histomorphometric changes and immune cell infiltration were examined using conventional H&E stain and contrasted versus the saline injected control glands.

2.7. Immunohistochemical analysis

Three µm tissue sections were deparaffinized, rehydrated, and unmasked in a single step using Trilogy™ (Cell Marque, Rocklin, CA, 920P-06). To block endogenous peroxidase activity and non-specific background staining sections were incubated in 3% hydrogen peroxide solution for 20–30 min. To block all epitopes on the tissue samples and prevent nonspecific antibody binding, sections were incubated with 1% BSA in 1X TBS, pH7.6 for 5 min. Primary antibody (Table 1) was applied at the appropriate working dilution overnight at 4 °C followed by secondary antibody (Table 1) which was incubated for 60 mins at room temperature. Colour was developed for 5 mins in DAB solution (Pierce™ 34002) and slides were counterstained in Mayer haematoxylin and DPX-mounted for light microscopy.

2.8. Western blotting

Tissues stored in RNAlater® were retrieved, homogenized in cell lysis buffer (AA-LYS-10 ml- RayBiotech, Inc., Norcross, GA) plus protease inhibitor cocktail (1:10 dilution, Calbiochem, UK) using a FastPrep™ tissue homogenizer (MP Biomedicals Santa Ana, CA). Protein concentration was measured using the Qubit® protein assay kit (Q33211, Invitrogen™, UK) and Qubit® 3.0 Fluorometer (Q33216, Invitrogen™, UK) and a total of 15–20 µg/lane of the different lysates were separated by SDS-PAGE on a 4–12% Novex polyacrylamide gel (Invitrogen, UK). Electro-transfer of proteins was done for 1 h to 0.2 µm pore–size nitrocellulose membrane (1620112, Bio-Rad, UK) according to standard protocol (Invitrogen, UK, Paisley), followed by membrane blocking with 5% bovine serum albumin. Membranes were incubated at 4 °C overnight with the appropriate antibody (Table 2) in blocking buffer then washed and incubated with the HRP conjugated anti-rabbit secondary antibody in blocking buffer at room temperature for 1 h. For