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The major membrane nuclease MnuA degrades neutrophil extracellular traps induced by *Mycoplasma bovis*

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ABSTRACT

Mycoplasma bovis has been increasingly recognised worldwide as an economically important pathogen of cattle, causing a range of diseases, including pneumonia, mastitis, polyarthritis and otitis media. It is believed that *M. bovis* utilises a range of cell surface proteins, including nucleases, to evade the host immune response and survive. However, despite the importance of neutrophils in controlling pathogenic bacteria, the interaction between these cells and *M. bovis* is not well-characterised. In addition to phagocytosis, neutrophils combat pathogens through the release of neutrophil extracellular traps (NETs), which are composed of their nuclear and granular components, including DNA. Here we investigated the effect of the major membrane nuclease MnuA of *M. bovis,* which *in vitro* is responsible for the majority of the nuclease activity of *M. bovis,* on NET formation. We quantified NET formation by bovine neutrophils 4 h after stimulation with wild-type *M. bovis*, an *mnuA* mutant and a *mnuA*-pIRR45 complemented *mnuA* mutant. NETs were detected following stimulation of neutrophils with the *mnuA* mutant but not after exposure to either the wild-type or the *mnuA*-pIRR45 complemented mutant, and NETs were degraded in the presence of even low concentrations of wild type *M. bovis*. Surprisingly, there was no increase in levels of intracellular reactive oxygen species (ROS) production in neutrophils stimulated with *M. bovis,* even though these neutrophils produced NETs. These results clearly demonstrate that *M. bovis* can induce NET formation in bovine neutrophils, but that the major membrane nuclease MnuA is able to rapidly degrade NETs, and thus is likely to play a significant role in virulence. In addition, *M. bovis* appears to induce NETs even though ROS production seems to be suppressed.

1. Introduction

Since its isolation in 1961 (Hale, 1962), *Mycoplasma bovis* has been recognized as an important and emerging pathogen of cattle worldwide (Adamu et al., 2013; Nicholas, 2011). It is often implicated in cases of respiratory disease, mastitis, conjunctivitis, arthritis and otitis media in cattle (Maunsell et al., 2011). Infections caused by *M. bovis* cause substantial economic losses in intensive cattle production systems as a consequence of poor growth, morbidity, deaths and costs associated with treatment and control (Rosales et al., 2015). However, there is limited information available about the mechanisms used by *M. bovis* to establish infection, persist and disseminate within the host. The variable surface proteins in *M. bovis* contribute significantly to immune evasion (Behrens et al., 1994; LeGrand et al., 1996) and membrane proteins have been reported to be involved in the modulation of the host's immune system (van der Merwe et al., 2010), either through the suppression of the immune response by inducing expression of IL-10, an anti-inflammatory cytokine, and/or through the down regulation of the pro-inflammatory cytokines INF-γ and TNF-α (Mulongo et al., 2014). However, despite the importance of neutrophils in innate immunity to bacterial pathogens, little is known about the interactions between *M. bovis* and neutrophils.

In addition to phagocytosis and degranulation, neutrophils can also deploy the antimicrobial mechanism known as neutrophil extracellular traps (NETs). These large, extracellular, web-like structures are released from neutrophils and contain decondensed chromatin and neutrophil granule proteins from azurophilic, specific and tertiary granules (Brinkmann et al., 2004). As DNA is a major component of NETs, nucleases can influence their stability and can enhance their clearance. Several studies have demonstrated these effects, and the consequent

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contribution of these enzymes to the pathogenicity of the organisms that produce them. The expression of a potent DNase, Sda1, by Group A *Streptococcus* species allows these organisms to effectively resist the effect of NETs and contributes to their virulence (Buchanan et al., 2006). Nuclease expression also contributes to the capacity of *Staphylococcus aureus* to escape NETs (Berends et al., 2010), and a cell wall anchored nuclease of *Streptococcus sanguinis* digests NET DNA and renders this pathogen more resistant to NET mediated killing (Morita et al., 2014).

Recently, several studies have investigated interactions between mycoplasmas and neutrophils. *M. bovis* has been shown to enhance neutrophil apoptosis, induce production of the pro-inflammatory cytokines, IL-12 and TNF-α, inhibit nitric oxide production and promote elastase release (Jimbo et al., 2017). *Mycoplasma agalactiae* has been shown to induce NET formation in the mammary glands of naturally infected sheep (Cacciotto et al., 2016). In *Mycoplasma pneumoniae* Mpn491, a secreted extracellular nuclease, has been shown to be responsible for NET degradation and escape from NET mediated killing (Yamamoto et al., 2017). Recombinant nuclease derived from the *M. bovis* Hubei strain could degrade NETs produced by neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA), but the ability of *M. bovis* itself to induce or clear NETs was not assessed (Zhang et al., 2016), while Gondaira et al (2017) found that stimulation of neutrophils with wild type *M. bovis* did not result in observable NET production, and suggested that this may be due to nuclease activity, but did not investigate this further.

As nucleases are thought to be important contributors to virulence in mycoplasma species (Somarajan et al., 2010), and DNA is a major component of NETs (Brinkmann et al., 2004), we hypothesised that the major membrane nuclease MnuA was likely to play a key role in the stability of NET formation in response to *M. bovis*. To test this, we characterised the interactions between neutrophils and wild type *M. bovis* and both a *M. bovis mnuA* mutant and an *mnuA* complemented mutant strain, as well as a second mutant lacking a second (putative) nuclease (the Δ0310 mutant).

2. Methods

2.1. Bacterial strains and culture conditions

M. bovis PG45 (ATCC 25523) and derivative strains were routinely cultured aerobically at 37 °C in modified Frey's broth (21g PPLO, 37 ml yeast extract, 100 ml inactivated swine serum, 4 ml 1.6% phenol red solution, 300 mg penicillin G, 859 ml distilled water, pH adjusted to 7.8), with 50 μg gentamicin/ml and 5 μg tetracycline/ml included when required to maintain selection pressure for the *Tn4001* transposon and the *oriC* plasmid, respectively. Viable counts of mycoplasma wild type and mutant strains were performed based on colour changing units (CCU) as described previously (Masukagami et al., 2017). In previous studies by our laboratory (Sharma et al., 2014), Tn4001 based transposon constructs were used to create a *M. bovis* PG45 insertional mutant library. Mutants MBOVPG45_0215 (ΔmnuA) and MBOVPG45_0310 (Δ0310) were shown by direct genome sequencing to have insertions in lipoprotein genes predicted to encode nucleases. To confirm that *mnuA* encoded the nuclease activity that had been lost by MBOVPG45_0215, a complete *mnuA* gene with its upstream sequence was introduced into the MBOVPG45_0215 mutant of *M. bovis* on the *oriC* plasmid pIRR45 (Sharma et al., 2015). The nuclease activities of the mutant and *mnuA* complemented strains were demonstrated in this previous study. Immediately prior to use in neutrophil stimulation experiments, mycoplasma cells were harvested by centrifugation (11,000*g*, 4 °C, 20 min) and resuspended in RPMI medium (ThermoFisher Scientific) for use in the assays.

2.2. Neutrophil isolation

Neutrophils were isolated using a previously described method, with slight modifications (Aulik et al., 2010). Healthy cows housed at the University of Melbourne Veterinary Clinical Centre, Werribee, were used in all experiments, and all procedures used were approved by the Faculty of Veterinary and Agricultural Sciences Animal Ethics Committee (University of Melbourne Animal Ethics Approval Number 1714126.1). Blood was collected by jugular venipuncture into EDTA tubes (Vacutainer, Becton Dickinson, Sydney, Australia), transferred to 50 ml tubes (BD Bioscience) and centrifuged (830*g*, 30 min, room temperature) to separate plasma and cellular fractions. The plasma and buffy coat were removed and the red blood cells (RBC) resuspended in 5 volumes of RBC lysis buffer (0.16 M NH_4Cl, 0.17 M Tris-HCl pH 7.2) and centrifuged immediately upon observation of colour change (400 x *g*, 4 °C, 5 min). The neutrophil pellet was washed twice with phosphate buffered saline (PBS) containing 1% v/v foetal bovine serum (FBS) and 0.105 mM EDTA and recovered by centrifugation at 400*g* at 4 °C for 5 min). The remaining cells were then resuspended in RPMI containing 0.5% or 2% v/v FBS, depending on the assay to be performed. Viability was assessed by dye exclusion using 0.1% w/v Trypan blue (Sigma-Aldrich), and only cell preparations with a viability of ≥ 99% were used in experiments. Finally, the number of viable neutrophils was adjusted to 10^5–10^6 cells/ml (depending on the assay) in RPMI supplemented with FBS and the cell suspension used immediately.

2.3. Measurement of nuclease activity of M. bovis and mutant derivatives

To assess the nuclease activity of *M. bovis* and mutant derivatives the zymogram technique was used, as described previously (Minion et al., 1993; Sharma et al., 2015). Ten millilitre cultures of wild-type *M. bovis,* the Δ0215 and Δ0310 mutants and the *mnuA*-pIRR45 complemented strain were grown to late logarithmic phase and the bacteria harvested by centrifugation (11,000*g*, 20 min, 4 °C). The cell pellets were washed twice with PBS (11,000*g*, 20 min, 4 °C), resuspended in equal volumes of Laemmli's sample buffer (2% w/v sodium dodecyl sulphate (SDS), 10% v/v glycerol, 0.625 M Tris-HCl pH 6.8, 715 mM β-mercaptoethanol, 0.04% w/v bromophenol blue), boiled for 5 min and the proteins separated by SDS-polyacrylamide gel electrophoresis (4 h at 12 mA at 4 °C) in a gel containing 12% w/v acrylamide and 10 μg/ml sheared salmon sperm DNA (Sigma). After electrophoresis, gels were washed and incubated overnight in incubation buffer (0.04 M Tris, pH 7.5, 1% w/v skim milk powder, 0.04% v/v β-mercaptoethanol) at room temperature to allow the proteins to renature. The gels were then incubated in incubation buffer supplemented with 2 mM $CaCl_2$ and 2 mM $MgCl_2$ for 7 h at 37 °C. The gels were stained with ethidium bromide (0.5 mg/ml) and the DNA visualised using a UV transilluminator. The nuclease activity in the gels appears as zones of clearance, as a result of hydrolysis of the salmon sperm DNA in the gel.

2.4. Induction of neutrophil extracellular traps (NETs)

Neutrophil extracellular trap assays were performed in 24-well tissue culture plates (Fisher Scientific) containing poly-L-lysine coated sterile 13 mm round glass coverslips (Menzel-Glaser). Neutrophils (2×10^5 in 500 μl RPMI medium) were seeded onto the coverslips and incubated for 30 min at 37 °C in a humidified atmosphere of 5% v/v CO_2 in air to facilitate attachment to coverslips. *M. bovis* wild-type, mutant or complemented strains were then added at varying multiplicities of infection (MOI of 1, 10, 50, or 100) and incubated with the neutrophils for up to 4 h. Wells with neutrophils incubated with 100 nm phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) were used as positive controls, and neutrophils with medium only were used as negative controls. Following incubation, coverslips were fixed in 4% w/v paraformaldehyde (PFA), washed twice with PBS, stained with 300 nm 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and mounted on