Veterinary Immunology and Immunopathology 164 (2015) 1–9

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Blocking the mitogen activated protein kinase-p38 pathway is associated with increase expression of nitric oxide synthase and higher production of nitric oxide by bovine macrophages infected with *Mycobacterium avium* subsp *paratuberculosis*

Cleverson D. Souza *

From the Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA 99163, USA

ARTICLE INFO

Article history:
Received 14 August 2014
Received in revised form
19 November 2014
Accepted 26 January 2015

Keywords:
Paratuberculosis
Bovine macrophages
Nitric oxide
iNOS
Mycobacterium

ABSTRACT

This study evaluated the role of the mitogen-activated protein kinase (MAPK)-p38 pathway in the nitric oxide synthase (iNOS) expression and nitric oxide (NO) production by bovine monocyte-derived macrophages ingesting *Mycobacterium avium* subsp. *paratuberculosis* (MAP) organisms *in vitro*. Bovine monocyte-derived macrophages were incubated with MAP organisms with or without a specific inhibitor of the MAPKp38 pathway and activation of the MAPKp38, interleukin - (IL) IL-10, IL-12, iNOS mRNA expression and NO production were evaluated. Incubation of macrophages with MAP organisms activates the MAPKp38 pathway at early time points post infection. Chemically inhibition of MAPKp38 before incubation of bovine macrophages with MAP resulted in increased expression of IL-12 mRNA at 2, 6 and 24 h, decreased expression of IL-10 mRNA at 2, 6 and 24 h and increased expression of iNOS mRNA at 2 and 6 h. Nitric oxide was evaluated to indirectly determine the effects of MAPKp38 pathway on the anti-microbial activity of bovine macrophages. Incubation of bovine macrophages with MAP resulted in modest increased production of NO at 4 and 6 h post infection. Pretreatment of bovine macrophages with the MAPKp38 inhibitor SB203580 before addition of MAP organisms resulted in increased production of NO at 2, 4, 6 and 24 h post infection. This study expanded our knowledge of the importance of the MAPKp38 pathway in limiting an appropriate macrophage response to MAP and suggested how activation of MAPKp38 pathway may be a target of this organism to disrupt earlier antimicrobial mechanisms of macrophages. These findings raises the interesting possibility that the cellular manipulation of MAPKp38 may be useful in designing novel vaccines against MAP.

1. Introduction

Paratuberculosis is a chronic progressive granulomatous disease of the small intestine of ruminants that is caused by *Mycobacterium avium* subsp *paratuberculosis* (MAP) (Manning, 2001; Olsen et al., 2002). The prevalence in U.S dairy herds is very high and efforts to control this disease have been hindered by the lack of understanding of the immune response to MAP and also the lack of a vaccine that induces sterile immunity (Bannantine et al., 2014; Settles et al., 2014). The major source of infection is ingestion of feces excreted by infected animals (Olsen et al.,

* Tel.: +1 509 335 0726.
E-mail addresses: souza@vetmed.wsu.edu, deso0017@gmail.com

http://dx.doi.org/10.1016/j.vetimm.2015.01.007
0165-2427/

2002). Thereafter, MAP is endocytosed in enterocytes and Peyer's patches of the ileum with subsequent infection of local macrophages and dendritic cells (Ponnusamy et al., 2013). Both, naturally occurring and experimental lesions develop during the latent stage of disease and are characterized by aggregates of epitheloid macrophages and giant cells (Huda and Jensen, 2003; Veazey et al., 1995). Few mature lymphocytes are associated with the lesions and tubercle formation and cell necrosis, characteristic of other mycobacterial infections, are less clearly demonstrated histologically (Huda and Jensen, 2003; Olsen et al., 2002). Clinical disease, characterized by emaciation and diarrhea, develops in a subset of chronically infected animals years after infection (Manning, 2001; Olsen et al., 2002).

It is believed that disease progression is associated with a breakdown in protective immunity although the triggering event remains unknown (Manning, 2001; Olsen et al., 2002; Stabel, 1998; Weiss et al., 2002, 2005a). Current research suggests that immune response against MAP is regulated by a network of cytokines and chemokines secreted by antigen presenting cells and different lineages of lymphocytes (Allen et al., 2012; Park et al., 2011; Thakur et al., 2013). These networks are targeted by mycobacteria to prevent immune elimination in part through differential receptor usage and the modulation of specific cell signaling pathways (Souza et al., 2006a, 2008; Srinivasan et al., 2014; Xu et al., 2014). The mechanisms involved in the failure of macrophages to contain MAP infection are only partially understood but studies suggest that; (1) modulation of cytokine secretion and cell signaling pathways, (2) interference with phagosomes acidification and apoptosis, (3) altered antigen presentation and (4) production of reactive oxygen species plays important roles (Weiss and Souza, 2008; Xu et al., 2014). Nitric oxide (NO) is a highly reactive signaling molecule and an important inflammatory mediator, which acts as a cytotoxic agent in addition to modulate immune responses and inflammation through multiple immune networks (Korhonen et al., 2005). Large amounts of NO are produced by inducible nitric oxide synthase (iNOS) in response to proinflammatory cytokines and bacterial products (Jagannath et al., 1998; Kapoor et al., 2010; Korhonen et al., 2005; Li and Poulos, 2005). Nitric oxide has been implicated as having a role in mycobacterial infections by supporting killing mechanisms within macrophages and co-regulating the Th1/Th2 balance (Beisiegel et al., 2009; Bose et al., 1999; Dumarey et al., 1994). *M. avium* subsp *paratuberculosis* has been shown to induce NO production by MAP *in vitro* and *in vivo* (Khalifeh et al., 2009; Weiss et al., 2005a). An *in vivo* study showed that sub-clinically infected animals up-regulates NO production and that progression to clinical disease was associated with up-regulation of IL-10 and TGF-beta expression and decreased production of NO by macrophages (Khalifeh et al., 2009). The mechanism leading to low production of NO in clinically infected animals was not determined but a linkage with the concurrent high expression of anti-inflammatory mediators as possible cause was discussed (Khalifeh et al., 2009).

Previous studies suggested that a limited number of cell signaling pathways appear to be involved with the ability of MAP to circumvent the microbicidal activity of macrophages (Souza et al., 2006a,b, 2007a,b; Weiss et al., 2008). One emerging pathway implicated in MAP pathogenesis is the Mitogen Activated Protein Kinase pathway (MAPK). These kinases belong to a family of highly conserved serine/threonine proteins that are activated by upstream kinases through a Thr-XXX-Try phosphorylation motif (Keshet and Seger, 2010). Three major and well characterized MAPKs have been identified including p42 and p44 extracellular signal-regulating kinases 1 and 2 (MAPK-ERK), MAPKp38, and p46 and p54 c-Jun-NH2-terminal kinases (MAPK-JNK). The MAPKs acts primarily by activating a variety of transcription factors including ATF2, Elk-1, c-Jun, and nFkB (Cuenda and Rousseau, 2007; Keshet and Seger, 2010; Roskoski, 2012; Symons et al., 2006). Several studies have addressed the role MAPK signaling in macrophages during mycobacterial infection (Schorey and Cooper, 2003; Weiss and Souza, 2008). In one study, using human macrophages, both MAPKp38 and MAPK-ERK1/2 were essential for *Mycobacterium tuberculosis* (TB)-induced TNF-α production, whereas, only MAPKp38 was necessary for IL-10 production (Song et al., 2003). Studies of the role of MAPK in bovine macrophages have shown that blocking MAPKp38 kinase activity results in a marked decrease in IL-10 production and a significant increase in IL-12 expression after infection of macrophages with MAP organisms (Souza et al., 2006a). Inhibition of MAPKp38 activity was associated with enhanced antimicrobial capacity of bovine macrophages demonstrated by increased acidification of phagosomes and a greater ability of macrophages to kill intracellular bacteria (Souza et al., 2006a).

The objective of this study was to evaluate the activation of the MAPKp38 pathway at earlier time points after MAP infection of bovine macrophages *in vitro* and to investigate the role of MAPKp38 in MAP-induced macrophages NO production and iNOS expression.

2. Methods

2.1. Ethics statement

All work involving animals was conducted in accordance with the recommendations in the institutional guidelines and approved animal care and use committee (IACUC) protocols at Washington State University. All other experiments were carried out in accordance with the Washington State Universities' Institutional Biosafety Committee (IBC) approved protocol number 1190.

2.2. Bovine monocyte isolation and in vitro macrophage generation

Blood samples used for isolation of monocytes were collected from five healthy adult Holstein dairy cows that tested negative for paratuberculosis as determined by culture of fecal samples and polymerase chain reaction (PCR) assay of blood and fecal samples. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Percoll (Sigma-Aldrich, USA) density gradient. Briefly, blood was layered onto 50 mL conical tubes containing Histopaque 1077 (Sigma-Aldrich, USA), and following density gradient centrifugation (500 × *g* for 20 min) at room temperature, PBMC were collected. The