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Research paper

Mycobacterium avium subspecies *paratuberculosis* suppresses expression of IL-12p40 and iNOS genes induced by signalling through CD40 in bovine monocyte-derived macrophages

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a facultative intracellular organism that resides in host macrophages. MAP causes a fatal wasting syndrome in ruminants, typified by granulomatous enteritis in the small intestine. MAP has also been suspected as a causative or exacerbating factor in some cases of human Crohn's disease. In MAP infections, a cytotoxic and proinflammatory Th1-like response is essential to control disease. While such a response may initially develop, this typically gives way to a Th2-like response later in infection. Interaction between CD40 receptors on macrophages and CD154 (CD40L) on activated T cells is crucial for maintaining a Th1 response and activation of macrophages. In this report, we investigated the hypothesis that CD40 signalling is impaired in MAP-infected macrophages. Uninfected bovine monocyte-derived macrophages (MDM) responded to CD40L by up-regulating expression of genes encoding IL-6, TNFα, IL-8, iNOS, IL-10, and IL-12p40. In contrast, MDM cells infected with MAP failed to up-regulate expression of iNOS and IL-12p40 genes in response to CD40L. CD40L stimulation caused a transient activation of the mitogen-activated protein kinase (MAPK) family member extracellular signal-regulated kinases (ERK) 1/2, stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) and p38 in MDM cells. In uninfected cells, inhibition of MAPK revealed that CD40L-mediated increase in IL-6 gene expression was dependent on activation of ERK1/2, while increases in IL-12p40, iNOS, and IL-10 gene expression were dependent on activation of p38. Because early activation of p38 was unimpaired in MAP-infected macrophages, we propose that MAP interferes with gene expression of iNOS and IL-12p40 genes downstream of p38.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease, a chronic, debilitating enteritis in ruminants. Although disease associated with MAP is largely restricted to ruminants, this organism has been implicated as a casual or exacerbating agent in human Crohn's disease (Chamberlin et al., 2001; Nasser et al., 2002), and can infect monogastric species (Beard et al., 2001; Chiodini and Buergelt, 1993).

Johne's disease ranks as one of the most costly infectious diseases of dairy cattle (Sweeney, 1996). In

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Abbreviations: CD40L, CD40 ligand; ERK, extracellular signal-regulated kinase; iNOS, inducible nitric oxide synthase; MAP, *Mycobacterium avium* subspecies *paratuberculosis*; MAPK, mitogen-activated protein kinases; MDM, monocyte-derived macrophages; MEK, mitogen-activated protein kinase kinase; MS, *Mycobacterium smegmatis*; NO, nitric oxide; SAPK/JNK, stress-activated protein kinase/Jun N-terminal kinase.

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cattle, infection with MAP leads to localized inflammation and formation of granulomatous lesion within the gut (Harris and Barletta, 2001), and, ultimately, after a 2–5 year subclinical phase, to the animals death. Once MAP is taken up, mainly *via* fecal-oral transfer, it is suspected that it crosses the epithelial lining of the gut through membranous epithelial cells, and is subsequently phagocytosed by subepithelial macrophages and dendritic cells (Clarke, 1997; Stabel, 2000). Once inside the naïve host macrophage, normal processing of the phagocytosed organisms through the endocytic pathway appears to be arrested. MAP-containing phagosomes fail to acquire significant amounts of late endosomal markers, such as Rab7, fail to be acidified, and fail to fuse with lysosomes, thus facilitating survival and proliferation of the pathogen inside macrophages (Hostetter et al., 2003; Kuehnel et al., 2001). Because MAP is largely restricted to growth within macrophages, a proinflammatory and cytotoxic Th1 response, characterized by proinflammatory cytokines such as IL-12 and IFNγ, and production of nitric oxide (NO), is essential to control the infection. Early after exposure to MAP, an appropriate Th1 response develops (Coussens, 2001). However, MAP is able to evade this early Th1 response through mechanisms that are not yet understood. Indeed, during the subclinical phase of MAP infection, the proinflammatory Th1-like response is lost and a Th2-like response, characterized by production of antibodies, predominates (Coussens, 2001; Stabel, 2000).

Interestingly**,** T cells derived from CD40 ligand (CD40L)-deficient mice are impaired in their ability to induce effector functions of macrophages, such as secretion of IL-12, and production of NO (Stout and Suttles, 1996). Consequently, these mice are highly susceptible to intracellular pathogens such as mycobacteria that would otherwise have been cleared by an appropriate Th1 response induced by T cell-macrophage interaction (Soong et al., 1996), demonstrating a critical role of CD40 signalling to control these infections. CD40L (CD154) is transiently expressed on activated T cells (Castle et al., 1993). Its ligation to the cell surface receptor CD40 on monocytes and macrophages induces intracellular signalling events including activation of mitogen-activated protein kinases (MAPK) (van Kooten and Banchereau, 2000), and subsequent expression of Th1 response genes (Kiener et al., 1995; Tian et al., 1995; van Kooten and Banchereau, 1996).

Since the development of a Th1 immune response is critical in clearance of infection with intracellular pathogens, but MAP is able to survive and to persist even under an early Th1 immune response in infected animals, we hypothesized that bovine monocyte-derived macrophages (MDM) infected with MAP would show impaired CD40-mediated gene expression of macrophage effector molecules, due to a breakdown in normal macrophage-T cell interaction. Our novel results demonstrate that MAP has a profound effect on CD40L-mediated expression of IL-12p40 and iNOS encoding genes, whereas CD40L-mediated expression of genes, such as IL-8 is not altered by MAP infection. Apparently, MAP infection of MDM cells *in vitro* causes a block in the ability of infected cells to respond normally to T cell-mediated activation. This effect may be related to persistence and virulence of MAP in bovine cells. Importantly, interference with CD40L-mediated increases in gene expression of IL-12p40 and iNOS genes does not appear to be due to prevention of early activation of MAPK, suggesting the block is downstream of these kinases.

2. Material and methods

2.1. Bacterial cultures

M. avium subspecies *paratuberculosis* (MAP) strain K10 was kindly provided by Dr. Srinand Sreevatsan (University of Minnesota, St. Paul, MN). *Mycobacterium smegmatis* (MS) strain mc^2155 was obtained from the American Type Culture Collection (#700084™; ATCC, Manassas, VA). Both strains were grown at 37 °C in Middlebrook 7H9 media (Difco Laboratories, Detroit, MI) with 10% BBL™ Middlebrook OADC enrichment (BD Biosciences, Sparks, MD), and, for MAP only, 2 mg/ml Mycobactin J (Allied Monitor, Lexana, KS). MAP cultures were grown for 12–16 weeks, and MS cultures were grown for 3 days. MAP and MS were serially diluted and counted on a bacterial hemocytometer.

Before infection of macrophage cells, bacterial suspensions were vigorously vortexed for 5 min to disperse clumps. Bacteria were diluted in PBS, and bacterial viability was evaluated by fluorescence microscopy using the Bac*Light*™ RedoxSensor™ Green Vitality Kit (Invitrogen Life Technologies, Carlsbad, CA) as recommended by the manufacturer.

2.2. Experimental animals

Healthy donor Holstein cattle ranging in age from 12 to 48 months were used as a source of cells in this study. The immune status of donor cattle with regard to infection with MAP had been monitored by serum ELISA, periodic IFN-γ testing (Bovigam; Biocor Animal Health, Omaha, NE) and fecal culture testing (Michigan State University, Diagnostic Center for Animal and Population Health, East Lansing, MI) before initiation of experiments. All animal handling procedures were in accordance with guidelines of the Michigan State University Committee on Animal Use and Care.

2.3. Preparation of bovine monocyte-derived macrophages

Isolation of primary bovine monocyte-derived macrophages (MDM) and functional characterization of these cells has been recently described (Chiang et al., 2007). Isolated mononuclear cells were plated at 1.5×10^7 cells in 6-well culture dishes for RNA extraction, or 5×10^7 cells in 100 mm culture dishes for whole cell lysate extraction, respectively. After 2 h of incubation in RPMI 1640 tissue culture medium (GIBCO®, Invitrogen Life Technologies) supplemented with 10% FBS (GIBCO®) at 39 °C and 5% CO_2, nonadherent cells were removed by washing three times with warm (39 °C) PBS. Adherent monocyte cells were allowed to differentiate in culture for 7 days in RPMI medium supplemented with 10% FBS at 39 °C and 5% CO_2. Following differentiation, MDM were checked for morphology under a light microscope. The final yield of MDM was approximately 1×10^6 cells per well or 5×10^6 cells