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Mycobacterium avium subspecies induce differential expression of pro-inflammatory mediators in a murine macrophage model: Evidence for enhanced pathogenicity of *Mycobacterium avium* subspecies *paratuberculosis*

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Abstract

Mycobacterium avium subspecies (ssp.) *paratuberculosis* (MAP) is the etiological agent of paratuberculosis, a chronic, non-treatable granulomatous enteritis of ruminants. MAP is the only mycobacterium affecting the intestinal tract, which is of interest since it is presently the most favoured pathogen linked to Crohn's disease (CD) in humans due to its frequent detection in CD tissues. MAP is genetically closely related to other *M. avium* ssp. such as *M. avium* ssp. *avium* (MAA) and *M. avium* ssp. *hominissuis* (MAH) which can cause mycobacteriosis in animals and immunocompromised humans.

We have recently shown that murine macrophage cell lines represent suitable systems to analyse *M. avium* ssp. patho-mechanisms and could show that MAP, but not MAA, specifically inhibited the antigen-specific stimulatory capacity for $CD4^+$ T-cells. In the present study, we compared gene expression profiles of murine RAW264.7 macrophages in response to infections with MAP or MAA using murine high-density oligonucleotide Affymetrix microarrays. A comparison of MAP and MAA infection revealed 17 differentially expressed genes. They were expressed at a much lower level in MAP-infected macrophages than in MAA-infected macrophages. Among these were the genes for IL-1β, IL-1α, CXCL2, PTGS2 (COX2), lipocalin (LCN2) and TNF, which are important pro-inflammatory factors. The microarray data were confirmed for selected genes by quantitative real-time reverse transcription PCR and, by protein array analyses and ELISA. Similar to MAA, infection with MAH also showed robust induction of IL-1β, CXCL2, COX2, LCN2 and TNF. Taken together, our results from *M. avium* ssp.-infected

Abbreviations: CC chemokines, cysteine cysteine chemokines; CCL2, CC chemokine ligand 2; CXC chemokines, cysteine × cysteine chemokines; CXCL2, CXC chemokine ligand 2 (also designated as MIP-2-alpha); G-CSF, granulocyte colony stimulating factor; IL, interleukin; IL-1RA, interleukin 1 receptor antagonist; LCN2, lipocalin 2; MIP2, macrophage inflammatory protein 2; PGE2, prostaglandin-endoperoxide 2; PTGS2, prostaglandin-endoperoxide synthase 2 (also designated as COX2, cyclooxygense 2); SBP, selenium binding protein; RPS9, ribosomal protein S9; TNF, tumor necrosis factor alpha/lymphotoxin.

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murine macrophages provide evidence that MAP in contrast to MAA and MAH specifically suppresses the pro-inflammatory defence mechanisms of infected macrophages.

Keywords: cDNA-microarray; Cytokine/chemokine expression; Pathogenic mycobacteria; Quantitative real-time reverse transcription PCR; RAW264.7

Introduction

Paratuberculosis (Johne's disease) is a chronic granulomatous enteritis in ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). MAP is therefore the only mycobacterial species responsible for an intestinal disease. In cattle, MAP is transmitted primarily via the faecal-oral route to neonatal calves. During the subsequent preclinical phase (2–5 years) bacteria persist and multiply in subepithelial macrophages causing a chronic transmural inflammatory reaction (Clarke, 1997; Harris and Barletta, 2001). Intermediate faecal shedding of MAP into the environment has also been observed during this preclinical phase when the animals do not show any overt disease.

The clinical stage of paratuberculosis is characterised by symptoms such as persistent diarrhoea, weight loss and progressive emaciation. During this period, MAP in faeces can reach numbers of $10^8\,g^{-1}$ and more (Cocito et al., 1994). In addition, excretion of MAP into milk has been reported (Streeter et al., 1995; Sweeney et al., 1992). Outside the host, MAP can survive for months or even years in soil and water, although they appear not to be able to multiply in the environment.

MAP has long been suggested to be associated with Crohn's disease (CD) in human (Greenstein, 2003). CD is a chronic inflammatory bowel disease with an unknown etiology. The incidence in the human population is increasing in Western Europe and North America (Loftus et al., 1998, 2002). In 1913, Dalziel reported for the first time a clinical and pathological resemblance between paratuberculosis in cattle and CD (Dalziel, 1913). Interestingly, recent studies revealed that a high percentage of CD patients were infected with MAP (Feller et al., 2007; Grant, 2005; Greenstein, 2003). In most cases, MAP was detected in intestinal tissues samples. Nevertheless, there is no direct experimental evidence for MAP as the primary etiological agent for CD thus far.

MAP is closely related to other *M. avium* subspecies such as *M. avium* subspecies *avium* (MAA) and *M. avium* subspecies *hominissuis* (MAH). This is based on an identical 16S rRNA sequence and on 97% homology of their genomes (Bannantine et al., 2004; Mijs et al., 2002; Thorel et al., 1990). The genome of MAP is 4.83 Mbp in size. Thus, it is approx. 0.6 Mbp smaller than the genome of MAH (5.48 Mbp). However, MAP-specific genes do exist (Li et al., 2005; Stratmann et al., 2004; Strommenger et al., 2001). Some of which have been associated with MAP iron metabolism (Stratmann et al., 2004).

In contrast to MAP, MAA serotypes 1–3 are responsible for avian tuberculosis. However, they can also lead to chronic mycobacteriosis in other animals such as cattle (de Lisle et al., 1998; Dvorska et al., 2004) and, rarely, in humans (Thegerstrom et al., 2005). MAH strains are found mainly in the environment, particularly in soil and water. They could be isolated from tuberculoid lesions in cattle (Dvorska et al., 2004) and are the most frequent cause of systemic mycobacteriosis in immunocompromised humans (Appelberg, 2006).

Similar to MAP, MAA and MAH use the intestine as preferred port of entry. After breaching the mucosal barrier, subepithelial macrophages are the major target cells, in which they are able to persist and multiply (Momotani et al., 1988; Sangari et al., 2001).

Murine infection models have been used in numerous studies to investigate interactions between bacterial pathogens and the host immune system. For instance, mice or murine cells can be used to distinguish MAA strains of different virulence (Pedrosa et al., 1994; Sarmento and Appelberg, 1995). Using a macrophage cell line, we could show that MAP and MAA are able to survive in such cells by inhibiting the maturation process of the phagosome (Kuehnel et al., 2001). In addition, infection with MAP, in contrast to MAA, inhibited the antigen-specific stimulatory capacity of murine macrophages for an antigen-specific $CD4^+$ T-cell line (Zur Lage et al., 2003).

MAP preferably colonizes the distal ileum of ruminants and humans which represents the most affected site in both paratuberculosis and CD (Greenstein, 2003). Other *M. avium* subspecies do not exhibit this property. Based on our previous work, we hypothesised that in contrast to MAA, MAP escapes the recognition of the host immune system after infection of intestinal macrophages. This would allow local persistence and multiplication in gut associated tissue and might be the reason for the enhanced pathogenicity of MAP.

In the present study, we used a murine macrophage cell line model to compare gene expression of macrophages in response to MAP and a MAA serotype 2 strain at 24 h post-infection. Murine high-density oligonucleotide micro-expression arrays were employed to this end and the results were compared with those obtained by quantitative real-time reverse transcription