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Effects of the non-commensal *Methylococcus capsulatus* Bath on mammalian immune cells

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ABSTRACT

Dietary inclusions of a bacterial meal consisting mainly of the non-commensal, methanotrophic bacteria *Methylococcus capsulatus* Bath have been shown to ameliorate symptoms of intestinal inflammation in different animal models. In order to investigate the molecular mechanisms causing these effects, we have studied the influence of this strain on different immune cells central for the regulation of inflammatory responses. Effects were compared to those induced by the closely related strain *M. capsulatus* Texas and the well-described probiotic strain *Escherichia coli* Nissle 1917.

M. capsulatus Bath induced macrophage polarization toward a pro-inflammatory phenotype, but not to the extent observed after exposure to *E. coli* Nissle 1917. Likewise, dose-dependent abilities to activate NF-κB transcription in U937 cells were observed, with *E. coli* Nissle 1917 being most potent. High levels of CD141 on human primary monocyte-derived dendritic cells (moDCs) were only detected after exposure to *E. coli* Nissle 1917, which collectively indicate a superior capacity to induce Th1 cell responses for this strain. On the other hand, the *M. capsulatus* strains were more potent in increasing the expression of the maturation markers CD80, CD83 and CD86 than *E. coli* Nissle 1917. *M. capsulatus* Bath induced the highest levels of IL-6, IL-10 and IL-12 secretion from dendritic cells, suggesting that this strain generally the post potent inducer of cytokine secretion.

These results show that *M. capsulatus* Bath exhibit immunogenic properties in mammalian *in vitro* systems which diverge from that of *E. coli* Nissle 1917. This may provide clues to how *M. capsulatus* Bath influence the adaptive immune system *in vivo*. However, further *in vivo* experiments are required for a complete understanding of how this strain ameliorates intestinal inflammation in animal models.

1. Introduction

Precise regulation of the intestinal barrier function is important for the maintenance of mucosal homeostasis and prevents the onset of uncontrolled inflammation (Pastorelli et al., 2013). A fine-tuned cross-talk between intestinal epithelial cells (IECs), immune cells and the bacterial community enables discrimination between pathogenic and commensal bacteria in the gut lumen (Artis, 2008). Such discrimination plays a cardinal role in influencing the function of innate mononuclear cells and lymphocytes and the subsequent onset of an appropriate inflammatory response. Alterations in this inter-cell communication may disrupt intestinal homeostasis and provoke inflammation and injury leading to intestinal disorders such as ulcerative colitis (UC) and Crohn's disease (CD), collectively known as inflammatory bowel diseases (IBDs) (Dignass et al., 2004; Clayburgh et al., 2004).

A bacterial meal called BioProtein (BP®), assumed to be a suitable protein source in fodder for the fish farming industry, has been reported to prevent soybean meal (SBM)-induced enteritis in Atlantic salmon in a dose dependent manner (Romarheim et al.,

Abbreviations: BP, BioProtein; DC, dendritic cells; DSS, dextran sulfate sodium; IECs, intestinal epithelial cells; IBD, inflammatory bowel diseases; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; moDCs, monocyte-derived DCs; MNC, mononuclear cells; PRR, pattern recognition receptors; TLR, toll-like receptor.

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2011, 2013). Similar observations have been reported in mammals where intake of BP attenuated symptoms of dextran sulfate sodium (DSS)-induced UC in mice (Kleiveland et al., 2012a). Prophylactic treatment with BP reduced colitis-induced parameters such as reduced body weight, shortening of the colon and epithelial damage. Increased IEC-proliferation and enhanced mucin 2 gene transcription were also reported, suggesting that BP affects the mechanisms implicated in the maintenance of the intestinal barrier function.

The main constituent of BP (88%) is the methanotrophic bacteria *Methylococcus capsulatus* Bath (Ward et al., 2004). Supplementary experiments have confirmed that the BP-induced effects observed in DSS-treated mice were due to *M. capsulatus* Bath and not any of the minor constituents in the preparation (Kleiveland et al., 2012a). This proposes *M. capsulatus* Bath as an interesting candidate for studying the molecular mechanisms involved in the development and treatment of IBD.

Methanotrophs are Gram-negative bacteria that grow aerobically using methane as the sole carbon and energy source (Hakemian and Rosenzweig, 2007). The biochemical components and pathways enabling *M. capsulatus* Bath to oxidize methane have been widely studied (Hakemian and Rosenzweig, 2007; Lieberman and Rosenzweig, 2005; Myronova et al., 2006; Khmelenina et al., 2011), and the concomitant ability to assimilate carbon into useful biomass at the formaldehyde level has given this strain further attention.

M. capsulatus Bath is not, to the best of our knowledge, a part of the mammalian microbiota. However, a human- or animal origin is commonly considered as a requirement for microorganisms to confer health benefits on mammalian hosts (Dunne et al., 2001). Non-commensals have not been subject to selection pressure in the mammalian gut during evolution and may retain unknown, and possibly beneficial, immunomodulatory properties. It is therefore of particular interest to understand the mechanisms by which *M. capsulatus* Bath ameliorate symptoms of intestinal inflammation in mammals. By studying the consequences of bacteria–host immune cell interactions we may not only increase our understanding of how a non-commensal can affect mammalian immunology, but also elucidate the molecular mechanisms involved in epithelial regulation.

Dendritic cells (DCs) are professional antigen-presenting cells central to the regulation of both innate and adaptive immune responses at mucosal surfaces (Coombes and Powrie, 2008). Immature monocyte-derived DCs (moDCs) reside in peripheral tissues, such as the gut mucosa, and continuously sample the intestinal lumen and the microenvironment *via* pattern recognition receptors (PRRs), such as the Toll-like receptors (Rakoff-Nahoum et al., 2004). Activation of PRRs induces moDC maturation, and, importantly, modulates moDC maturation and differentiation in such a way that both tolerance toward commensals and generation of protective immune responses against pathogens are enabled (Coombes and Powrie, 2008). Thus, bacterial effects on immature moDCs are essential to understand the outcome of bacteria–host cell interactions in the intestinal mucosa (Christensen et al., 2002; Zoumpopoulou et al., 2009; Hart et al., 2004).

Likewise, macrophages play a pivotal role in mucosal immune responses (Mowat and Bain, 2011; Hume, 2008), and may differentiate into pro- or anti-inflammatory phenotypes after antigen stimulation, indicated by an increase in inducible nitric oxide synthase- (iNOS) or arginase-1 gene expression, respectively (Lawrence and Natoli, 2011; Benoit et al., 2008).

We have studied the consequences of exposing immature moDCs to *M. capsulatus* Bath in regards to the expression of co-stimulatory and maturation markers, and the subsequent secretion of cytokines. These outcomes, known to affect the adaptive immune system such as T-cell development and polarization, were compared with those induced by other Gram-negative strains. Similarly, polarization of macrophages upon bacterial exposure was studied by quantifying iNOS and arginase-1 gene expression. Bacterial effects on NF-κB transcription and moDC morphology are also described herein which increase the understanding how a non-commensal bacteria is able to affect the immune system in a mammalian host.

2. Materials and methods

2.1. Cells and culture conditions

M. capsulatus Bath (NCIMB 11132, Aberdeen, UK) and *M. capsulatus* Texas (NCIMB 11851, Aberdeen, UK) were grown in batches of 5–20 ml nitrate minimal salt solution at 45 °C, with shaking (200 rpm), in an atmosphere containing methane, CO_2 and air (19:1:80 v/v), as previously described (Whittenbury et al., 1970). *Escherichia coli* Nissle 1917 (Villena et al., 2012) (Mutaflor, DSM 6601, serotype O6:K5:H1) was kindly provided by Ardeypharm GmbH, Herdecke, Germany and grown in Luria-Bertani Broth (Oxoid, UK) at 37 °C without shaking. UV-inactivation of *E. coli* Nissle 1917 was performed for 60 min prior to experiments.

Human blood mononuclear cells (MNC) were isolated from buffy coats of healthy volunteers obtained from Ostfold Hospital Trust, Fredrikstad, Norway, in accordance to institutional ethical guidelines. Both primary MNCs, the human monocytic leukemia cell line THP-1 (ATCC TIB-202, Rockville, USA) and the murine macrophage cell line RAW 267.4 (ATTC TIB-71, Rockville, USA) were maintained in RPMI 1640 medium with L-glutamine further supplemented with 100 μM non-essential amino acids, 1 mM sodium pyruvate (all from PAA Laboratories, Austria), 10% heat-inactivated fetal calf serum (Gibco Life Technologies, UK) and 24 μg/ml gentamicin (Lonza, Walkersville, ML). Tissue culture maintenance and experiments were carried out at 37 °C in a 5% CO_2 humidified air atmosphere.

The human myeloblastic cell line U937, stably transfected with a construct containing the luciferase gene regulated by a promoter containing NF-κB binding sites, was a kind gift from Rune Blomhoff, Faculty of Medicine, University of Oslo, Norway (Austenaa et al., 2009). Stably transfected U937 cells were cultured in supplemented RPMI 1640 medium.

2.2. Scanning electron microscopy (SEM)

Samples were gently washed with phosphate buffered saline (PBS) (PAA Laboratories, Austria) and fixed using 5% glutaraldehyde in 0.1 M sodium cacodylate and 0.1 M sucrose (pH 7.4) at room temperature for 45 min. The fixative was then replaced with 0.1 M sodium cacodylate and 0.1 M sucrose (pH 7.4) for 30 min in room temperature. Samples were then washed, dehydrated in graded ethanol series and dried using a critical-point dryer (CDP 030, BAL-TEC GmBH, Germany). Dry samples were mounted on aluminum stubs using double-faced carbon tape (Agar Scientific, UK), and coated with approximately 500 Å platinum using a sputter coater (Polaron SC7640, Quorum Technologies, UK). Microscopic analyses were performed using an EVO-50 Zeiss microscope (Carl Zeiss AG, Germany).

2.3. Transmission electron microscopy (TEM)

Samples were embedded in 0, 5% low-melting agarose to produce an agarose-plug. The plug was washed twice in distilled water prior to fixation in 2.5% glutaraldehyde/4% paraformaldehyde at room temperature for 45 min. Samples were washed twice in PBS and twice in 0.1 M saline cacodylate buffer and treated