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Differential macrophage function in Brown Swiss and Holstein Friesian cattle

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ABSTRACT

There is strong evidence that high yielding dairy cows are extremely susceptible to infectious diseases, and that this has severe economic consequences for the dairy industry and welfare implications. Here we present preliminary functional evidence showing that the innate immune response differs between cow breeds. The ability of macrophages (MØ) to kill pathogens depends in part on oxygen-dependent and independent mechanisms. The oxygen-dependent mechanisms rely on the generation of reactive oxygen and nitrogen species (ROS/RNS, respectively). ROS production has been shown to activate the inflammasome complex in MØ leading to increased production of the pro-inflammatory cytokine Interleukin-1β (IL-1β). Conversely RNS inhibits inflammasome mediated IL-1β activation, indicating a division between inflammasome activation and RNS production. In the present study MØ from Brown Swiss (BS) cattle produce significantly more RNS and less IL-1β when compared to cells from Holstein Friesian (HF) cattle in response to bacterial or fungal stimuli. Furthermore, BS MØ killed ingested *Salmonella typhimurium* more efficiently, supporting anecdotal evidence of increased disease resistance of the breed. Inhibition of autophagy by 3-methyladenine (3-MA) stimulated IL-1β secretion in cells from both breeds, but was more pronounced in HF MØ. Blocking RNS production by L-arginase completely abolished RNS production but increased IL-1β secretion in BS MØ. Collectively these preliminary data suggest that the dichotomy of inflammasome activation and RNS production exists in cattle and differs between these two breeds. As pattern recognition receptors and signaling pathways are involved in the assessed functional differences presented herein, our data potentially aid the identification of *in vitro* predictors of appropriate innate immune response. Finally, these predictors may assist in the discovery of candidate genes conferring increased disease resistance for future use in combination with known production traits.

1. Introduction

Macrophages (MØ) are the mature form of circulating monocytes (Mo) derived from a common myeloid progenitor. Inflammatory cytokines trigger adherence and rolling of circulating Mo along the endothelium of adjacent blood vessels before extravasation, maturation to MØ and migration to sites of inflammation, where they have both pro-inflammatory and inflammation resolution activity (Aguilar-Ruiz et al., 2011; Cros et al., 2010; Hussen et al., 2013; Shi and Pamer, 2011; Wong et al., 2011, 2012). In humans Mo can be typified into three subsets based on the expression of CD14 and CD16: CD14++CD16−, CD14++CD16+ and CD14+CD16++. These are referred to as classical (cM), intermediate (intM) and non-classical (ncM), respectively (Ziegler-Heitbrock et al., 2010). It has been indicated that the three subsets have different roles (Grage-Griebenow et al., 2001) and there may be a developmental pathway from cM through intM to ncM (Wong et al., 2011; Zawada et al., 2011). Exposure to the cytokine Macrophage-Colony Stimulating Factor (M-CSF) may trigger this development (Korkosz et al., 2012).

In cattle, approximately 89% of monocytes are cM (Hussen et al., 2013). These express greater phagocytic activity than intM or ncM, whilst also producing anti-inflammatory cytokines such as IL-10 (Grage-Griebenow et al., 2001; Ziegler-Heitbrock, 2007; Ziegler-Heitbrock et al., 2010) and being involved in repair of damage tissue (Wong et al., 2011, 2012). Both subsets of CD16+ Mo

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increase with infection and disease, extravasating more efficiently and producing cytokines including TNFα and IL-1β, thus being described as inflammatory Mo (Aguilar-Ruiz et al., 2011; Ancuta et al., 2003; Belge et al., 2002; Grage-Griebenow et al., 2001; Tacke and Randolph, 2006; Thieblemont et al., 1995; Wong et al., 2012; Zawada et al., 2011; Ziegler-Heitbrock et al., 2010), although some findings indicate anti-inflammatory actions for ncM (Cros et al., 2010; Hussen et al., 2013). A range of pro-inflammatory actions are exhibited by murine and human intM, from inflammatory cytokine production to antigen presentation, requiring prior phagocytosis (Aguilar-Ruiz et al., 2011; Belge et al., 2002; Shi and Pamer, 2011; Thomas and Lipsky, 1994; Ziegler-Heitbrock, 2007).

Correlating with their role in antigen presentation, human intM display the highest MHC II expression levels of the three monocyte subsets (Wong et al., 2011), indicating a strong involvement in antigen presentation. In addition to the production of cytokines and their function as antigen presenting cells, MØ constantly sample the local environment and have antigen presenting capacity (Chesnut and Grey, 1985; Gordon, 1998; Gordon and Taylor, 2005).

Responses to pathogens by MØ are mediated by pattern recognition receptors (PRR) such as Toll-like Receptors (TLR) which recognise and interact with pathogen associated molecular patterns (PAMP) (Akira et al., 2001; Taylor and Gordon, 2003). PAMPs include, but are not restricted to pathogen surface structures, genetic material and secreted or released products (Taylor and Gordon, 2003). MØ are characteristically bactericidal in nature and utilise several approaches to mediate bacterial killing but mainly employing two mechanisms which are closely interlinked: production of reactive oxygen species and reactive nitrogen species (ROS and RNS, respectively). Their release results in cell- and DNA damage and thus shows antimicrobial effects (Bogdan, 2001; Forman and Torres, 2001; Nathan, 1992, 1987). Interaction of a PAMP with the corresponding PRR stimulates a variety of pathways, resulting in the activation with the subsequent cleavage of pro-IL-1β into its active, secreted form by caspase-1 (Lamkanfi et al., 2007), assembly of the NADPH oxidase complex, electron transfer from NADPH to oxygen and a series of enzymatic reactions to produce ROS (Bokoch and Knaus, 2003; Lambeth, 2004; Quinn and Gauss, 2004). Additionally, PAMP association with PRR results in transcription of inducible Nitric Oxide Synthase (iNOS) in MØ which interacts with NADPH and ROS intermediates to create RNS in bovine and murine MØ (Jungi et al., 1996a; Nathan, 1992; Green et al., 1990).

However, there are some infectious diseases where – due to the anatomical barrier present – the anti-bacterial activity of MØ is not sufficient to eliminate the pathogen. One such organ is the bovine udder, where mechanical, chemical and pathological irritation can lead to an increased somatic cell count (SCC) (Schukken et al., 2003). However, it has to be emphasised that somatic cells in the milk consist of a pleiotropha of cells, including MØ, neutrophils, lymphocytes and epithelial cells (Boulanger et al., 2001). In humans, the majority of cells in milk have been identified as unique breast milk MØ (Yagi et al., 2010). These have been shown to be more metabolically active compared with those in peripheral blood (Johnson et al., 1980). However, the killing mechanism was shown to involve the oxidation of glucose via the hexose-monophosphate (HMP) shunt, showing that cytotoxic effects are using the same mechanism as blood derived cells by the production of hydrogen peroxide and superoxide (Johnson et al., 1980). Indeed, bovine mammary MØ challenged with phagocytic stimuli produced ROS similarly to those produced by other MØ (Harmon and Adams, 1987). Thus milk-derived MØ are capable of ROS/RNS production, has been described in milk-derived MØ (Denis et al., 2006), and seems to be an approach of the innate immune cells to combat invading bacteria in the udder.

However, despite the fact that 1/4 of cows are suffering from mastitis at any given time, not all breeds show the same mastitis incidence rate (Begley et al., 2009). Indeed, cattle breed susceptibility or resistance to infection has long been studied to improve breeding strategies (Warner et al., 1987), and differences in the genetic resistance to infection have been identified for *Mycobacterium tuberculosis* (O'Reilly and Daborn, 1995; Vordermeier et al., 2012) and *Brucellosis* (Paixao et al., 2006).

However, it has to be emphasised that research results, especially analysis of immune responses, may not translate between breeds of the same species. Indeed, observations by farmers and veterinarians have noted that Brown Swiss (BS) cattle tend to have a lower somatic cell counts (SCC) than Holstein-Friesian (HF) cattle, and are thought to be less susceptible to mastitis. This anecdotal evidence has been reinforced by studies suggesting differences in SCC could be the result of improved microbial killing mechanisms in the innate cells of BS cattle (Busato et al., 2000; Kizilkaya, 2009; Rupp and Boichard, 2003). Work by Norimatsu et al. (2004), showing low RNS production from *Salmonella typhimurium* stimulated HF MØ compared to data published by Werling et al. (2004) demonstrating high RNS production from BS MØ with the same bacterium highlights that difference in disease resistance may be conferred by the micro-bicidal capabilities of diverse breeds. Furthermore, work by Jann et al. (2008, 2009) suggests that polymorphisms in TLR genes may be involved in disease resistance or susceptibility traits in domestic animals (Jann et al., 2009, 2008). Despite estimations in heritability of production traits varying (Rupp and Boichard, 2003), Lund et al. (1994) have shown the estimated heritability of SCC to be moderately high (Lund et al., 1994). This suggests the possibility of integrating low SCC into breeding strategies, particularly if lower SCCs in milk could be linked to an increased MØ killing ability.

2. Materials and methods

2.1. Sample collection

Blood for PBMC isolation and subsequent MØ generation was collected by puncture of the jugular vein from clinically healthy HF and BS cows housed at either the RVC Boltons Park Farm (Hertfordshire, UK) or at Cancourt Farm (Wiltshire, UK). Animals used were age- and lactation-matched (2nd or 3rd lactation, respectively), unless otherwise stated. All procedures were carried under Home Office Project licence which was approved by the College's Ethics and Welfare Committee. For biological assays, blood was drawn into sterile glass vacuum bottles containing 10% Acid Citrate Dextrose (ACD) as anticoagulant. For whole blood flow cytometric analysis, blood was collected from three cows of each breed into 10 ml EDTA Vacutainers (Beckton Dickinson, Oxford, UK) by puncture of the jugular vein. Whole milk was collected into sterile glass bottles from individual cows housed at either farm. All assays were performed in at least four breed matched pairs apart from bacterial killing assays, blood MØ NO induction and preliminary IL-1β stimulations, milk derived responses which were performed in 3, 2 and 1 breed matched pairs, respectively.

2.2. Isolation peripheral blood mononuclear cells and generation of macrophages

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood as previously described (Jungi et al., 1996b). Briefly, blood was centrifuged at $700 \times g$ for 20 min before buffy coats were removed and washed in citrate buffer. RBC lysis was performed using ammonium-chloride lysis buffer. Resultant cells were suspended in RPMI media (Life Technologies, UK) before being underlayed with Histopaque ($d = 1.083\ g\ l^{-1}$, Sigma–Aldrich, UK) to isolate PBMCs by density centrifugation. PBMC were washed in PBS and counted by Trypan blue exclusion then incubated in Teflon bags