Veterinary Immunology and Immunopathology 140 (2011) 130–139

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Research paper

Transcriptional profiling of immune genes in bovine monocyte-derived macrophages exposed to bacterial antigens

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ARTICLE INFO

Article history:
Received 9 September 2010
Received in revised form
30 November 2010
Accepted 3 December 2010

Keywords:
Cattle
Macrophage
Monocyte
Toll-like receptor
Real time quantitative reverse transcription PCR
Purified protein derivative
Lipopolysaccharide

ABSTRACT

The involvement of Toll-like receptors (TLRs) and other immune signalling genes during challenge of bovine macrophages with bacterial products derived from disease-causing bacteria in cattle was investigated. An *in vitro* cell culture model of bovine monocyte derived macrophages (MDM) was established and these cells were exposed to purified protein derivative (PPD-b) derived from *Mycobacterium bovis* and to lipopolysachharide (LPS) derived from *Escherichia coli*. Following 24 h incubation, total RNA was extracted and expression of immune related genes was determined by real time quantitative reverse transcription PCR (qRT-PCR). Expression of a selection of genes spanning the TLR-2 and TLR-4 pathways, from the initial activation of the receptors to the production of pro-inflammatory cytokines and chemokines was determined. Results from repeat experiments using MDM from seven different age-matched dairy cattle showed that PPD-b treatment caused significant up-regulation of the *TLR2* and *TLR4* genes and the expression profile of TLR adaptor molecules suggested that this signalling is MYD88-dependent. Conversely, LPS caused significant up-regulation of *TLR4* via a MYD88-independent signalling pathway. Significant up-regulation of genes involved with NF-κB signalling was also detected in PPD-b- and LPS-treated samples accompanied by the expression of pro-inflammatory cytokine (*TNF*, *IL1B*, *IL6*) and chemokine genes (*IL8*, *CCL5*, *CCL3*). Overall, LPS challenge resulted in a more marked up-regulation of immune-related genes. Furthermore, the magnitude fold-change difference in gene expression suggests, at least in part, that bovine macrophages produce IFN-γ as a result of LPS challenge.

1. Introduction

Macrophages play a crucial role during the innate immune response against pathogens. These cells are equipped with different immune receptors, including the evolutionarily conserved Toll-like receptors (TLRs) that recognise foreign non-self material and trigger an inflammatory response against invading pathogens (Pluddemann et al., 2006). Vertebrate immune cells possess a range of TLR receptors, with each receptor recognising specific microbial components that are collectively known as pathogen-associated molecular patterns (PAMPs). Lipopolysaccharide (LPS), bacterial DNA, peptidoglycan, flagellar components, and bacterial lipoproteins

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doi:10.1016/j.vetimm.2010.12.002

are examples of bacterial PAMPs shared among different pathogens and are ligands for specific TLR molecules. For example, LPS, a prototypical component of Gram-negative bacteria, is the ligand for TLR-4, bacterial peptidoglycan and lipoproteins are ligands for TLR-2 and bacterial flagellin is recognised by TLR-5 (Bryant et al., 2009; Hayashi et al., 2001; Zahringer et al., 2008).

Following TLR activation by microbial components, intracellular signalling through specific pathways leads to the transcriptional up-regulation of a number of genes including cytokines, chemokines and adhesion molecules that are involved in generating an environment where infection can be contained and subsequently cleared (Carmody and Chen, 2007; Gerold et al., 2007; Kawai and Akira, 2005; Lu et al., 2008).

The bovine genome sequence has recently been completed and annotated DNA sequence information is available for genes encoding TLRs 1–10 (Elsik et al., 2009). An improved understanding of the expression and function of bovine TLR genes is important as they have an essential role in the recognition of pathogenic bacteria such as *Mycobacterium bovis* (the causative agent of bovine tuberculosis) and *Escherichia coli* [one of the causative agents of mastitis] (De Schepper et al., 2008; Werling and Coffey, 2007; Werling et al., 2006).

In the present study, we have used an *in vitro* system of cultured bovine monocyte-derived macrophages (MDM) to investigate the expression of: (1) specific TLR genes (*TLR2* and *TLR4*) together with the *TLR1* and *TLR6* genes, the products of which interact with the TLR-2 protein (Wetzler, 2003); (2) genes encoding TLR adaptor proteins (*MYD88*, *TICAM2* and *TICAM1*) responsible for the initiation of downstream signalling pathways; (3) downstream genes involved with NF-κB intra-cellular signalling (*IKBKB*, *IRAK1*, *NFKB1*, *TOLLIP* and *TRAF6*); and (4) downstream inflammatory and chemokine genes (*CCL3*, *CCL5*, *IFNG*, *IL1B*, *IL6*, *IL8*, *IL10*, *IL12B*, *IL18*, *NOS2* and *TNF*), in response to two types of bacterial antigens derived from *M. bovis* and *E. coli*. Bovine MDM were challenged with *M. bovis* purified protein derivative (PPD-b) and with *E. coli* LPS. PPD-b is widely used as a diagnostic reagent in the single intradermal cervical comparative tuberculin test (SICCT) for bovine tuberculosis (Monaghan et al., 1994).

Real time quantitative reverse transcription PCR (qRT-PCR) was used to estimate the expression of a range of genes within specific TLR signalling pathways spanning the initial activation of receptors to the production of pro-inflammatory cytokine and chemokine molecules at 24 h post-challenge. This approach is particularly pertinent to the bovine model because there are limited conventional immunological reagents available for cattle in comparison to humans and rodents (Entrican et al., 2009).

2. Materials and methods

2.1. Animals

Throughout this study, seven age-matched (3-year old) Holstein-Friesian cows were used. All animals were maintained under uniform housing conditions and nutritional regimens at the UCD Lyons Research Farm (Newcastle, County Kildare, Ireland). These animals were selected from an experimental herd without a recent history of tuberculosis and were SICTT and IFN-γ test negative. These cattle also underwent comprehensive testing for the following infections as part of regular experimental herd surveillance: brucellosis (*Brucella abortus*), Johne's disease (*M. avium* subsp. *paratuberculosis*), infectious bovine rhinotracheitis (*Bovine herpesvirus 1*), salmonella (*Salmonella typhimurium*) and bovine viral diarrhoea (*Pestivirus*)—all animals tested negative for these infections. All animal procedures detailed in this study were carried out according to the provisions of the Cruelty to Animals Act (Department of Health and Children licence no. B100/3939) and ethics approval for the study was obtained from the UCD Animal Ethics Committee.

2.2. Monocyte extraction and culture of bovine MDM

For monocyte isolation, 200 ml of blood was collected in acid citrate dextrose buffer (Sigma–Aldrich Ireland Ltd., Dublin, Ireland) in sterile bottles. Blood was layered onto Accuspin™ tubes containing Histopaque® 1077 (Sigma–Aldrich Ireland Ltd., Dublin, Ireland) and following density gradient centrifugation, peripheral blood mononuclear cells (PBMC) were collected. Contaminating red blood cells (RBC) were removed by a lysis step (RBC lysis buffer contained 10 mM $KHCO_3$, 150 mM NH_4Cl, 0.1 mM EDTA pH 8.0). PBMC were washed twice by resuspending in phosphate-buffered saline (PBS) followed by centrifugation at 1600 rpm. Following the two wash steps PBMC were resuspended in 3.6 ml FACS buffer [PBS containing 1% bovine serum albumin (BSA)].

Monocytes were isolated using the MACS® protocol and human anti-CD14 antibody MACS® microbeads (Miltenyi Biotec Ltd., Surrey, UK)—this antibody has been shown to cross-react with bovine monocytes (Jacobsen et al., 1993). For this, all 3.6 ml resuspended PBMC were mixed with 0.4 ml magnetically charged anti-CD14-labelled MACS® microbeads and then incubated at room temperature for 10 min. The PBMC-anti-CD14 mixture was then washed twice in FACS buffer as described above and then loaded on a MS column (Miltenyi Biotec Ltd., Surrey, UK) pre-washed with 3 ml FACS buffer. $CD14^+$ monocytes were positively selected from the PBMC population following application of a magnetic field to the PBMC-anti-CD14 mixture by placing the MS column on a MACS® separator (Miltenyi Biotec Ltd., Surrey, UK).

The identity and purity of $CD14^+$ monocytes was confirmed by flow cytometry using an anti-CD14 FITC labelled antibody. The purity of $CD14^+$ cells was estimated at $\geq$99%. Purified monocytes were seeded at 1×10^6/ml in 24-well tissue culture plates in RPMI 1640 medium (Invitrogen Ltd., Dublin, Ireland) containing 15% heat inactivated foetal calf serum (FCS; Invitrogen Ltd., Dublin, Ireland), 1% non-essential amino acids (NEAA, Sigma–Aldrich Ireland Ltd., Dublin, Ireland), gentamicin (5 μg/ml; Sigma–Aldrich Ireland Ltd., Dublin, Ireland) and incubated at 37 °C, 5% CO_2. Following 24 h incubation (day 1) media was changed to remove any non-adhered cells and on day 3, media was replaced with antibiotic-free culture media. To ensure that the same number of MDM were subjected to different