Molecular Immunology 47 (2009) 365–372

Contents lists available at ScienceDirect

Molecular Immunology

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

Transcriptional analysis of Toll-like receptors expression in M cells

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ARTICLE INFO

Article history:
Received 7 July 2009
Accepted 3 September 2009
Available online 24 September 2009

Keywords:
Peyer's patches
FAE
M cells
TLR
UEA-1
TLR9

ABSTRACT

M cells are located in the follicle associated epithelium (FAE) of Peyer's patches (PPs) in the small intestine, where they mediate the uptake and transcytosis of luminal antigens to the underlying lymphoid tissue. Toll-like receptors (TLRs) have emerged as key mediators in the innate immune response by recognising pathogen associated molecular patterns (PAMPs) expressed by microorganisms. TLRs have previously been shown to be differentially expressed in the gastrointestinal tract. In this study PP were harvested from BALB/c mice. *Ulex europaeus agglutinin* 1 (UAE-1) positive M cells were isolated from FAE and the expression of TLR1–9 transcripts in M cells, FAE and villus epithelium (VE) was compared by quantitative real-time PCR. Transcripts for TLR1, TLR2 and TLR4 were found to be expressed at a high level in M cells in comparison to VE, with no transcripts being detected in the FAE. TLR3 and TLR6 were not found to be expressed in M cells or in the FAE. TLR5 and TLR7 were found to be expressed at a higher level in FAE compared to M cells. TLR9, which recognises unmethylated CpG DNA of bacteria and viruses and TLR8, which recognises ssRNA, were found to be preferentially expressed in M cells compared to FAE and VE.

1. Introduction

The epithelium overlying the gastrointestinal tract (GIT) is continuously exposed to the external environment thus, being exposed to a limitless range of antigens. These antigens are eliminated by the non-specific and specific defence mechanisms mediated by the gut-associated lymphoid tissue. Aggregates of lymphoid follicles known as Peyer's patches (PPs) are found in the small intestine. The epithelium overlaying these lymphoid follicles, follicle associated epithelium (FAE), contains specialised antigen sampling M cells which mediate the transcytosis of antigens from the lumen to the underlying lymphoid tissue, leading to an induction of the adaptive immune response. Antigen sampling by M cells is proposed to be the first step in the development of protective mucosal and systemic immune response (Neutra et al., 1996). The mechanisms of M cells transcytosis of microorganisms is as yet largely undefined. M cells are presumed to express receptors on their apical membrane that microorganisms adhere to. The transcytotic activity of M cells is thus being exploited in the development of oral mucosal vaccines (Brayden et al., 2005). The lack of a universal M cell marker or a human M cell marker has impeded M cells characterisation and targeting.

M cells have been shown to constitute 10% of the FAE in mice (Bye et al., 1984) and can be distinguished from the surrounding enterocytes in the FAE by several characteristics. M cells show decreased expression of apically expressed digestive enzymes such as, alkaline phosphatase and sucrose isomaltase (Brown et al., 1990; El Bahi et al., 2002; Smith, 1985). M cells can be morphologically distinguished from the surrounding FAE enterocytes, as apically they lack an organised brush border and glycocalyx (Frey et al., 1996; Owen and Jones, 1974). The absence of an extensive glycocalyx allows greater access to the apical membrane of M cells for, luminal particulate matter, bacteria and viruses. The basolateral membrane is deeply invaginated to form an intraepithelial pocket which contains B lymphocytes, $CD4^+$ T lymphocytes, macrophages and dendritic cells (Farstad et al., 1994; Johansson and Kelsall, 2005; Owen and Jones, 1974). This structural characteristic decreases the distance that transcytotic vesicles have to travel from the apical to the basolateral surface of the M cell. Adherence to M cells has been shown to greatly enhance the transcytosis of macromolecules into the PP (Neutra et al., 2001). It has been shown that transcytosis affected by M cells is mediated by the binding of specific ligands by pathogens such as Yersinia, Salmonella and poliovirus. Transcytosis of *Yersinia enterocolytica* by M cells is mediated by invasion-β1 intergrin interactions (Grassl et al., 2003). A family of innate immune receptors, Toll-like receptors (TLRs) have recently been implicated in the transcytosis of pathogenic microorganism by M cells (Tyrer et al., 2006). The enhanced transcytosis of microspheres in the presence of peptidoglycan has been shown to be mediated by TLR2 in PP M cells (Chabot et al., 2006).

Pattern recognition receptors (PRRs) play a central role in the innate immune response by recognising conserved PAMP in microorganisms. The TLR family is one of the most studied PRR's. They are type 1 transmembrane proteins with a divergent

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doi:10.1016/j.molimm.2009.09.007

Table 1
Oligonucleotide primers used in quantitative PCR for analysis of gene expression.

Gene	Forward primer	Reverse primer	Source
TLR1	gttggtgaagaactcaggcg	tcagcttggacaatgagagg	Matsushima et al. (2004)
TLR2	tgctttcctgctggagattt	tgtaacgcaacagcttcagg	This study
TLR3	tggattcttctggtgtcttcc	agttcttcacttcgaaacgc	Matsushima et al. (2004)
TLR4	tcacctctgccttcactac	caaagatacaccaacggctc	Hollestelle et al. (2004)
TLR5	gccacatcatttccactcct	acagccgaagttccaagaga	Olson and Miller (2004)
TLR6	atatctgagcttcggatgcc	atggagaacggtggtattgg	Matsushima et al. (2004)
TLR7	ccaccagacctcttgattcc	ccagatggttcagcctacg	This study
TLR8	gaagcatttcgagcatctcc	gaagacgatttcgccaagag	Olson and Miller (2004)
TLR9	caacctcagccacaacattc	cacacttcacaccattagcc	This study
MRP	tcttgtgctgctgtgcctagtgg	ggggtttggccattaaaagt	This study
GP2	gtttgcagcctctctggac	ctccaggatgttcccacagt	This study
Clusterin	gagcttcatgacccccacta	aatcaacgtctgggaactgg	This study
CCL20	cgactgttgcctctcgtaca	aggaggttcacagccctttt	This study
GAPDH	accacagtccatgccatcac	tccaccaccctgtggctgta	This study

extracellular domain containing leucine-rich repeats responsible for binding various PAMP and an intracellular Toll/interleukin 1 domain responsible for initialising signalling (Medzhitov et al., 1997). Stimulation of a TLR by its PAMP leads to activation of a signalling cascade through recruitment of adaptor molecules (O'Neill and Bowie, 2007). TLR's have been shown to be expressed on cells of the innate immune system such as dendritic cells, macrophages and antigen-presenting cells, and have been shown to be involved in phagocytosis and in the development of a pro-inflammatory immune response (Blander and Medzhitov, 2004). To date 12 TLRs have been identified in mice and 10 TLRs in man, with TLR1–9 being the most studied (Beutler, 2005). The various TLRs recognise different PAMP such as, TLR2 which recognises peptidoglycan of Gram positive bacteria (Takeuchi et al., 1999). TLR4 and TLR5 recognise lipopolysaccharide (LPS) of Gram negative bacteria and flagellin, respectively (Hayashi et al., 2001; Poltorak et al., 1998). TLR3 and TLR7–9 detect nucleic acid derived from viruses and bacteria during infection (Alexopoulou et al., 2001; Heil et al., 2004; Hemmi et al., 2000). TLR3 and TLR7–9 have been shown to be expressed intracellularly in the endosomal–lysosomal compartment (Ahmad-Nejad et al., 2002; Nishiya et al., 2005).

Recent studies have investigated the expression of a select number TLR receptors in the FAE and M cells. TLR2 and TLR9 were found to be expressed in FAE in pigs with both TLR9 and TLR2 being preferentially expressed on M cells (Shimosato et al., 2005; Tohno et al., 2005). TLR4 has been shown to be preferentially expressed in M cells *in vitro* and *in vivo* (Tyrer et al., 2006). Murine FAE has been shown to express TLR2, TLR5 and TLR9 (Chabot et al., 2006). To date the expression of TLR transcripts in isolated M cells has not been described. In this study we isolated murine M cells from surrounding FAE using UEA-1 which binds specifically to murine M cells, allowing M cells to be differentiated from the FAE (Clark et al., 1995). The expression of TLR1–9 in UEA-1$^+$ M cells, FAE and villus epithelium (VE) was compared using quantitative real-time PCR.

2. Materials and methods

2.1. Animals and Peyer's patch isolation

Female BALB/c mice aged between 12 and 20 weeks were housed in the Biological Services Unit, University College Cork. Mice were housed under standard pathogen free conditions with food and water *ad libitum*, and were sacrificed by cervical dislocation. Sections of the small intestine containing PP were removed and stored in phosphate buffer saline (PBS) (4 °C) (Gibco BRL). The PP was excised from the surrounding VE using a sterile scalpel under Leica Zoom 2000 dissecting microscope. The isolated PP were placed in an enzymatic digest, containing 250 U/ml hyaluronidase (Sigma) and 1 U/ml dispase I (Roche), at 37 °C for 30 min under agitation (100 rpm). The resultant supernatant was centrifuged for 10 min at 180 × *g*. The cells were resuspended in 0.02% ethylenediaminetetraacetic acid (EDTA) solution and incubated for 5 min at room temperature with gentle agitation (100 rpm). The cells were passed through a 30 μm filter (Miltenyi Biotec). The single cell suspension was enumerated using an improved Neubauer haemocytometer and cell viability determined using 0.4% trypan blue (Gibco BRL).

2.2. Isolation of Ulex europaeus agglutinin 1 positive M cells, FAE and VE

The single cell suspension was centrifuged at 180 × *g* for 10 min. The cell pellet was resuspended in 200 μl sample buffer (PBS supplemented with 5% bovine serum albumin (BSA) and 2 mM EDTA) kept at 4 °C and incubated with 20 μg/ml fluorescein isothiocyanate (FITC) conjugated UEA-1 (Vector Laboratories) at room temperature for 20 min. Cells were washed with 4 °C sample buffer by centrifugation 3 times at 180 × *g* for 10 min. The cells were resuspended in 90 μl of sample buffer and incubated with 10 μl anti-FITC-microbeads at 4 °C for 20 min. The cell suspension was separated using MACS MS+ (Miltenyi Biotec) in the presence of a MiniMACS high energy magnet (Miltenyi Biotec) as per manufacturer's instructions. Briefly cells were resuspended in 500 μl degassed sample buffer. The labelled cell suspension was applied to the column and the negative fraction (non-FITC-UEA-1) was allowed to flow through the column. The column was flushed through 5 times with the degassed sample buffer. The negative fraction (FAE) was collected. The MACS MS+ column was removed from the magnet and the positive fraction was eluted from the column with degassed sample buffer. The positive and negative fractions were enumerated and cell viability determined using 0.4% trypan blue. Samples were analysed using a Beckman Coulter Epics Elite cell sorter equipped with an argon laser (488 nm) to measure fluorescence and light scatters of the fractioned cells, FITC fluorescence was detected through a 525 nm filter.

VE was isolated using a modified version of the method previously described by Iimura et al. (2000). Briefly, the small intestine was dissected longitudinally and washed 3 times with PBS. Fragments were washed with PBS by centrifugation at 200 × *g* for 10 min. The intestine fragments were incubated with 1.5 U/ml dispase I (Roche) and 0.02% EDTA at 37 °C for 30 min with agitation (100 rpm). Samples were allowed to stand thus, allowing the larger fragment to fall to the bottom and the sheets of epithelium to be separated.

2.3. RNA isolation, reverse transcription and PCR

mRNA was isolated from the three isolated cell types using Dynabeads® mRNA DIRECT™ Microkit from Dynal as per manu-