Veterinary Immunology and Immunopathology 153 (2013) 134–139

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

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

Short communication

Expression of TLR4 in swine as assessed by a newly developed monoclonal antibody

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ARTICLE INFO

Article history:
Received 11 October 2012
Received in revised form
13 December 2012
Accepted 28 December 2012

Keywords:
Swine
TLR4
Monocytes
Monoclonal antibody

ABSTRACT

Toll-like receptors (TLRs) constitute an ancient family of pattern recognition receptors for conserved microbial structures that allow rapid detection of invading pathogens, triggering immune responses. TLR4 binds lipopolysaccharides (LPS) being involved in the recognition of Gram-negative bacteria. Herein we describe the generation and characterisation of a monoclonal antibody, named 3H3, against porcine TLR4. Its specificity was confirmed by reactivity with TLR4 expressing CHO cell transfectants. On peripheral blood leukocytes TLR4 was preferentially expressed on myelomonocytic cells, with monocytes expressing higher levels than granulocytes. Staining of lung tissue sections showed that TLR4 is also expressed on epithelial cells lining the bronchial tract, a distribution consistent with a surveillance function of bacterial invasion.

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1. Introduction

Pattern recognition receptors (PRRs) bind conserved microbial structures, leading to the activation of different leucocyte subsets and the development of inflammatory and immune responses. Among the various PRRs so far known, Toll-like receptors (TLRs) represent one of the groups best characterised. They are type I transmembrane proteins, with an extracellular domain containing multiple leucine-rich repeats (LRRs), with a horseshoe-like shape, and a cytoplasmic domain homologous to that of the interleukin 1 (IL-1) receptor, the TIR domain, that engages two main signalling pathways via the TIR-containing adaptors MyD88 (common to all TLRs except TLR3) and TRIF (only used by TLR3 and TLR4) leading to the secretion of type I IFNs, pro-inflammatory cytokines and chemokines, and increased expression of co-stimulatory molecules (Kumar et al., 2009; Takeuchi and Akira, 2009).

TLR4 is the key receptor for the LPS component of Gram-negative bacteria. Mice deficient in TLR4 are LPS hyporesponsive (Takeuchi et al., 1999). It also recognises structures from mycobacteria, fungi, parasites and viruses, as well as endogenous molecules such as heat-shock proteins, fibrinogen, fibronectin or β-defensin (Kumar et al., 2009).

Detection of LPS requires other molecules in addition to TLR4. LPS binds to LPS-binding protein (LBP), present in the serum, and this LPS–LBP complex is subsequently recognised by CD14, which transfer it to a receptor complex formed by TLR4 and MD2, which undergoes oligomerisation and activates intracellular signalling pathways (Miyake, 2004).

Previous studies on cell expression of TLR4 in swine have been done with polyclonal antibodies (Moue et al., 2008; Murakami et al., 2011; Wassef et al., 2004). Here, we describe the generation of a monoclonal antibody (mAb) to

Abbreviations: APC, allophycocyanin; DC, dendritic cell; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; LBP, LPS-binding protein; LPS, lipopolysaccharide; PE, phycoerythrin; PerCP, peridinin–chlorophyll–protein complex; PRR, pattern recognition receptor; TLR, Toll-like receptor.

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http://dx.doi.org/10.1016/j.vetimm.2012.12.020

porcine TLR4, which detects TLR4 expression on immune cell subsets as well as on porcine TLR4 transfected CHO cells.

2. Materials and methods

2.1. Tissues and cells

Tissue samples for immunohistochemical analyses were collected from healthy, conventionally reared, 3-month-old Large-White pigs that had been euthanised with azaperone and ketamine. Samples were cut in pieces of about 0.5 cm^3, embedded in Tissue-Tek OCT compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, NL), snap frozen in isopentane/liquid nitrogen and stored at −80 °C until used.

Blood samples were obtained from 6- to 12-month-old outbred Large-White pigs. Peripheral blood mononuclear cells (PBMC) were isolated on Percoll discontinuous gradients after blood sedimentation through dextran, as previously described (Gonzalez et al., 1990). Granulocytes were recovered from the lower Percoll phase after lysis of erythrocytes by hypotonic treatment. Alveolar macrophages were collected by bronchoalveolar lavage as described (Carrascosa et al., 1982). Dendritic cells (DC) were derived from monocytes, which were magnetically isolated using anti-CD172a mAb BA1C11 and the VarioMACS cell sorting technique (Miltenyi Biotec, Bergisch-Gladbach, Germany), by culturing them in the presence of rpGM-CSF and rpIL-4 (BioSource, Camarillo, CA, USA), as described elsewhere (Chamorro et al., 2004). On day 5, rpTNF-α (BioSource) was added to induce their maturation. Cells were resuspended in RPMI 1640 medium (Bio-Whittaker, Verviers, Belgium), containing 10% foetal calf serum (FCS, Bio-Whittaker), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and 50 μg/mL gentamicin (Lonza, Walkersville, MD, USA).

CHO and mouse embryo NIH/3T3 cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Bio-Whittaker) supplemented with 50 μg/mL gentamicin, 2 mM L-glutamine and 10% FCS.

The reported experiments have been executed in full compliance with guidelines by the ethical committee of the institute.

2.2. Expression of TLR4-GFP construct and analysis by flow cytometry

NIH/3T3 or CHO cells were transiently transfected with plasmid pTLR4-GFP, encoding the porcine TLR4 tagged with the green fluorescent protein (GFP) on the carboxy-terminal end, by using the LipofectAMINE PLUS reagent (Invitrogen, San Diego, CA, USA), as previously described (Alvarez et al., 2006). Cells were harvested at 24 h post-transfection and analysed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Non-transfected cells were used as negative control.

2.3. Monoclonal antibody production

To raise monoclonal antibodies (mAb) against porcine TLR4 10-week-old BALB/c mice were i.v. immunised with pTLR4-GFP-transfected NIH/3T3 mouse cells (2×10^6 cells/0.1 mL of sterile PBS), and boosted 15 and 30 days later with the same amount of cells. Serum from immunised mice was collected 7–10 days after each boost, and the presence of specific Abs was tested in flow cytometry using pTLR4-GFP-transfected CHO cells. Cells transfected with a plasmid encoding the full-length pTLR2-GFP fusion protein (Alvarez et al., 2008) or non-transfected cells were used as negative control. Selected mice were boosted i.v. with 2×10^6 cells in 0.1 mL sterile PBS four days before fusion of spleen lymphocytes with the SP2/0 murine plasmacytoma, using polyethylene glycol 4000 (Merck, West Point, PA, USA) according to established protocols (Alvarez et al., 2008).

Class and subclass of mAbs were determined by ELISA with a mouse monoclonal antibody isotyping test kit from BD Biosciences (Erembodegem, Belgium). MAb 3H3 is isotype IgM,κ.

MAbs to porcine CD3 (BB23-8E, IgG2b), CD8α (76-2-11, IgG2a) and CD172a (74-22-15a, IgG2b) were kindly provided by M. Pescovitz (Indiana University, Indianapolis, IN, USA) and J. Lunney (USDA, Beltsville, MD, USA). MAbs to CD14 (My4, IgG2b), CD16 (G7, IgG1) and CD21 (B-Ly4; IgG1) were purchased from Beckman Coulter GmbH (Krefeld, Germany), AbD-Serotec (Oxford, UK), and BD Biosciences, respectively. MAbs to porcine CD163 (2A10/11, IgG1) and CD172a (BA1C11, IgG1) were developed and produced in our laboratory.

For multi-colour immunofluorescence assays, anti-CD163 mAb 2A10/11 was purified by affinity chromatography on Protein G-Sepharose CL-4B (GE Healthcare, Uppsala, Sweden), and labelled with biotin (Molecular Probes, Eugene, OR, USA), following the manufacturer's protocol.

2.4. Flow cytometry

For single-colour staining, cells ($2–5 \times 10^5$ $well^{-1}$) were incubated with 50 μL hybridoma supernatant for 30 min at 4 °C. After two washes in PBS containing 0.1% bovine serum albumin (BSA) and 0.01% sodium azide (FACS buffer), cells were incubated with FITC or phycoerythrin (PE)-conjugated rabbit F(ab′)2 anti-mouse Ig (Dako, Glostrup, Denmark) or APC-labelled rat anti-mouse IgM mAb (eBioscience, San Diego, CA, USA). Then, they were washed and fixed in 0.1% formaldehyde prior to analysis on a FACSCalibur flow cytometer (Becton Dickinson).

For two-colour staining, cells ($2–5 \times 10^5$ $well^{-1}$) were incubated with 50 μL of unlabelled mAb (hybridoma supernatant) for 30 min at 4 °C. After washing, cells were incubated with FITC-conjugated goat anti-mouse IgM and the appropriate PE-conjugated goat anti-mouse IgG subclass (IgG1 and IgG2a, Southern Biotech, Birmingham, AL, USA; IgG2b, Invitrogen, Carlsbad, CA, USA). Subsequently, cells were washed in FACS buffer and fixed in 0.1% formaldehyde prior to analysis in the cytometer.

For three-colour staining, cells were incubated with 3H3 and 74-22-15a (anti-CD172a) (hybridoma supernatants) and biotin-labelled anti-CD163 mAb 2A10/11 for 30 min at 4 °C. After washing, cells were incubated for 30 min at 4 °C with FITC-conjugated goat anti-mouse IgM, APC-conjugated goat anti-mouse IgG2b (Southern Biotech) and

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