Veterinary Immunology and Immunopathology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Effects of a pathogenic ETEC strain and a probiotic *Enterococcus faecium* strain on the inflammasome response in porcine dendritic cells

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ARTICLE INFO

Keywords:
Dendritic cell
Enterococcus faecium
Escherichia coli
Inflammasomes
Pig
Interleukin-1β

ABSTRACT

Dendritic cells (DC) are crucial for maintaining intestinal homeostasis and generating proper immune responses to bacteria occurring in the gut. Microbial stimuli can be recognized by intracellular receptors called inflammasomes, e.g., nucleotide oligomerization domain (NOD)-like receptor protein 3 (NLRP3). The aim of the present study was to unravel the inflammasome response of porcine monocyte-derived DC (MoDC). We investigated the capacity of probiotic *Enterococcus faecium* NCIMB 10415 (*E. faecium*) and enterotoxigenic *Escherichia coli* (ETEC) to elicit inflammasome activation. Since inflammasome activation normally requires a two-step process, MoDC were initially incubated with lipopolysaccharide (LPS) in order to prime cells. Primed and unprimed cells were then stimulated with the aforementioned bacterial strains. We also assessed whether preincubation with the probiotic prior to ETEC infection modified the immune response *via* the inflammasome pathway.

Phenotypical analysis by flow cytometry showed that monocytes and MoDC expressed the surface markers CD14, CD16, and CD1 continuously, whereas swine leucocyte antigen (SLA) II was upregulated during differentiation.

Following LPS priming, NLRP3, interleukin (IL)-1β and IL-18 mRNA expression, and IL-1β protein release increased. In unprimed cells, ETEC upregulated the expression of inflammasome components at later time points than in LPS-primed MoDC. Preincubation with the probiotic did not influence NLRP3 inflammasome activation in comparison with cells infected with ETEC alone.

We conclude that ETEC, but not *E. faecium*, was able to stimulate inflammasome components in porcine MoDC. The present experimental conditions revealed no NLRP3 inflammasome-dependent protective effects of *E. faecium* during a pathogenic ETEC challenge.

1. Introduction

In the intestine, DC sense and sample antigens of pathogenic and commensal microbiota and help maintaining the balance between immunogenic or tolerogenic immune responses (Johansson and Kelsall, 2005; Niess and Reinecker, 2005; Mann et al., 2013). To sense microorganisms, DC utilize innate pattern recognition receptors. In contrast to extracellular Toll-like receptors, NOD-like receptors (NLR) represent a subset of pattern recognition receptors that is located in the cytoplasm (Takeuchi and Akira, 2010). NLR recognize microbial structures like

Abbreviations: APC, allophycocyanin; ASC, apoptosis-associated speck-like protein; ATP, adenosine triphosphate; BMDC, bone-marrow-derived dendritic cells; BSA, bovine serum albumin; CFU, colony-forming units; DC, dendritic cells; *E. coli*, *Escherichia coli*; EDTA, ethylenediamine tetra-acetic acid; *E. faecalis*, *Enterococcus faecalis*; *E. faecium*, *Enterococcus faecium*; EHEC, enterohemorrhagic *Escherichia coli*; ELISA, enzyme-linked immunosorbent assay; ETEC, enterotoxigenic *Escherichia coli*; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FMO, fluorescence-minus-one; FSC, forward scatter; GM-CSF, granulocyte-macrophage colony-stimulating factor; IEC, intestinal epithelial cells; IgG, immunglobulin G; IL, interleukin; LB, Luria-Bertani; LPS, lipopolysaccharide; *L. rhamnosus*, *Lactobacillus rhamnosus*; MHC, major histocompatibility complex; MoDC, monocyte-derived dendritic cells; NLR, NOD-like receptors; NLRC4, NOD-like receptor CARD domain-containing protein 4; NLRP3, NOD-like receptor protein 3; NLRP6, NOD-like receptor protein 6; NOD, nucleotide oligomerization domain; OD, optical density; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PE, phycoerythrin; PI, propidium iodide; rp, recombinant porcine; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of the means; SLA, swine leucocyte antigen; SSC, side scatter; TBP, TATA-binding protein

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https://doi.org/10.1016/j.vetimm.2018.08.004
Received 18 December 2017; Received in revised form 18 July 2018; Accepted 12 August 2018

pathogen-associated molecular patterns and danger-associated molecular patterns (Schroder and Tschopp, 2010). Some NLR form multiprotein complexes termed inflammasomes upon activation, which finally results in an inflammatory immune response. Increasing evidence suggests that inflammasomes are directly involved in the pathogenesis of several inflammatory and metabolic diseases (De Nardo and Latz, 2011; Haneklaus and O'Neill, 2015; Opipari and Franchi, 2015).

The best characterized inflammasome in humans and mice is the NLRP3 inflammasome (Lissner and Siegmund, 2011; Rathinam et al., 2012; Shao et al., 2015). It consists of three elements: the NLRP3 protein, the adaptor protein ASC (apoptosis-associated speck-like protein), and pro-caspase-1 (Lissner and Siegmund, 2011). In most cell types, the NLRP3 inflammasome is activated by a two-step process consisting of a first priming step and a second step resulting in the formation of a functional multiprotein complex (Sutterwala et al., 2014). During priming, the transcription of inactive pro-forms of the proinflammatory cytokines IL-1β and IL-18 (Lissner and Siegmund, 2011) and of NLRP3 itself (Bauernfeind et al., 2009) is triggered. The second signal, which can be displayed by, for example, adenosine triphosphate (ATP) or pore-forming bacterial toxins (Bauernfeind et al., 2009), leads to the activation of caspase-1, which cleaves precursors of IL-1β and IL-18 into their mature forms (Franchi et al., 2012). Apart from endogenous and exogenous non-microbial signals, NLRP3 can be stimulated by bacteria and viruses (Bauernfeind et al., 2011a).

A detailed understanding of inflammasome activation attributable to bacterial infection is still limited (Franchi et al., 2009) and while inflammasomes are best studied in humans and mice, inflammasome research in human-relevant large animal models, such as the pig, has just begun (Tohno et al., 2011; Kim et al., 2014).

In pigs, NLRP3 has been detected at the mRNA level in gut-associated lymphoid tissues (Tohno et al., 2011). Well-known NLRP3 stimulants, such as ATP and nigericin, have been successfully used in porcine macrophages to induce the activation of the NLRP3 inflammasome (Kim et al., 2014). Other than these data, however, almost no information exists about the activation of porcine NLRP3 or regarding other inflammasomes in pigs.

Evidence has been obtained illustrating that probiotic bacteria are able to induce inflammasome activation. Probiotic *Lactobacillus* strains have been shown to induce NLRP3 expression in porcine gut-associated lymphoid tissues (Tohno et al., 2011). Wu et al. (2015) have found that *Lactobacillus rhamnosus* (*L. rhamnosus*) GR-1 is capable of modulating *Escherichia coli* (*E. coli*)-induced inflammation in bovine mammary epithelial cells by reducing NLRP3 activation. Since *E. faecium* has previously been shown to influence immunological functions and to reduce the incidence of *E. coli*-induced diarrhea (Taras et al., 2006), we hypothesized that the NLRP3 inflammasome might contribute to the probiotic effects of *E. faecium*. The purpose of the present study therefore was to examine different components of the inflammasome in porcine DC and to assess inflammasome expression under the influence of probiotic *versus* pathogenic bacteria. We have analyzed the capacity of a probiotic *E. faecium* strain and an ETEC strain associated with post-weaning diarrhea to elicit inflammasome activation in porcine MoDC.

2. Material and methods

2.1. Blood samples and generation of monocyte-derived dendritic cells

Blood samples were taken from healthy, conventionally reared Danbred × Pietrain pigs (10 to 12 weeks of age) kept at the Institute of Animal Nutrition (Freie Universität Berlin, Germany) and collected in blood tubes (S-Monovette®, SARSTEDT, Nürnbrecht, Germany) containing ethylenediamine tetra-acetic acid (EDTA) as an anticoagulant. All procedures were conducted in accordance with the guidelines for animal welfare and were approved by the ethics committee for animal welfare "Landesamt für Gesundheit und Soziales" (LaGeSo Berlin, no. T 0264/15).

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque™ PLUS (1.077 *g*/L, GE Healthcare, Uppsala, Sweden) density gradient centrifugation (400 × *g* for 30 min at room temperature) and washed three times in phosphate-buffered saline (PBS, Biochrom, Berlin, Germany) supplemented with 2 mM EDTA (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). $CD14^+$ monocytes were positively selected by magnetic activated cell sorting by using CD14 MicroBeads and LS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were resuspended in RPMI-1640 (Biochrom) supplemented with 10% fetal calf serum (FCS, Biochrom), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich Chemie GmbH). Cells were seeded at a density of 8×10^5 cells/mL and 1 mL per well into 24-well cell culture plates (TPP, Faustlab, Schaffhausen, Germany). In order to induce DC differentiation, 20 ng/mL recombinant porcine (rp) granulocyte-macrophage colony-stimulating factor (GM-CSF) and 50 ng/mL rp IL-4 (both from R&D Systems, Minneapolis, MN, United States) were added to the cells, which were grown at 37 °C in a humidified atmosphere of 5% CO_2 for 6 days. On day 3 of culture, another 1 mL fresh differentiation medium was added. After 6 days, adherent cells were used as immature MoDC. Differentiation of DC was monitored by microscopic examination of cell morphology using phase contrast microscopy (Leica DMI 6000 series, Leica Microsystems, Heidelberg, Germany) and phenotypical characterization by flow cytometry (described in the following section).

2.2. Flow cytometry

For the phenotyping of monocytes and MoDC, monoclonal antibodies against the following cell surface markers were used: anti-pig CD1 (clone 76-7-4, isotype Immunglobulin G [IgG]2ακ, biotinylated, SouthernBiotech, Cambridge, United Kingdom), anti-human CD14 (clone REA599, isotype recombinant human IgG1, phycoerythrin [PE]-conjugated, Miltenyi Biotec), anti-pig CD16 (clone G7, isotype IgG1, fluorescein isothiocyanate [FITC]-conjugated, Bio-Rad Laboratories GmbH, Munich, Germany), and anti-pig SLA II (clone K274.3G8, isotype IgG1, unconjugated, major histocompatibility complex [MHC] II, Bio-Rad Laboratories GmbH). Streptavidin (allophycocyanin [APC]-conjugated, eBioscience, San Diego, CA) and anti-mouse IgG1 (clone X-56, APC Vio770-conjugated, Miltenyi Biotec) served as second step reagents for anti-pig CD1 and anti-pig SLA II, respectively. Propidium iodide (PI, Miltenyi Biotec) was used as a viability dye at a final concentration of 1 µg/mL. Single-color-stained cells and unstained cells were used to correct for fluorescence spill-over. Fluorescence-minus-one (FMO) control samples were used as gating controls. In addition, control samples were stained with isotype controls or only relevant second step reagents, where applicable. Cells were incubated with the appropriate antibodies for 10 or 30 min at 4 °C in the dark according to the manufacturer's instructions. After being washed with cold PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin (BSA, GIBCO, Invitrogen, Auckland, New Zealand), cells were centrifuged and resuspended in the aforementioned buffer solution. Cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences, Heidelberg, Germany). Data were analyzed by using FlowJo (Tree Star, San Carlos, CA; version 10.2) software.

2.3. Bacterial strains

The probiotic strain *E. faecium* (Cylactin®, DSM, Kaiseraugst, Switzerland) or enterotoxigenic *E. coli* IMT4818 (isolated from a two-week-old piglet with enteritis, O149:K91:K88 [F4]) were cultivated as described in Klingspor et al. (2015). Briefly, *E. faecium* was grown in brain-heart infusion broth (OXOID GmbH, Wesel, Germany) and ETEC in Luria-Bertani (LB) medium containing 10 *g*/L tryptone, 5 *g*/L yeast extract, and 10 *g*/L NaCl, at pH 7.0 (Roth, Karlsruhe, Germany). Bacteria were cultivated until mid-log phase, centrifuged, and washed twice in cold PBS. Bacterial cells were resuspended in RPMI-1640 at a