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Toll-like receptor 2 suppresses Toll-like receptor 9 responses in Peyer's patch dendritic cells

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ABSTRACT

In the intestine, immune responses to commensal microbes should be regulated precisely. This regulation is achieved partly by dendritic cells (DCs), which recognize microbes through Toll-like receptors (TLRs). Although TLR responses have been intensely studied, cross-talk between individual TLRs remains unclear. The present study shows that TLR2 suppressed TLR9-induced *Il12b* gene expression and subsequent interleukin (IL)-12 and IL-23 production in DCs from Peyer's patch, a lymphoid tissue in the small intestine. The DCs expressed *Il12b* gene and produced IL-12 and IL-23 in response to TLR9 stimulation, and these responses were suppressed when the DCs were stimulated simultaneously with TLR2. The suppression was also observed in the non-intestinal DCs, such as spleen DCs and bone marrow-derived DCs. Peyer's patch DCs expressed *Il12b* gene also in response to TLR7 or CD40 stimulation, but these responses were not suppressed by simultaneous TLR2 stimulation. In addition, TLR9-induced *Tnf* and *Il6* gene expression was not suppressed by TLR2. Furthermore, the supernatant of TLR2-stimulated DCs could not suppress TLR9-induced *Il12b* gene expression. These results suggest that TLR2 suppress TLR9-induced responses selectively, and this suppression is not mediated by secretory factors. The suppressive TLR cross-talk might play a certain role in preventing excess inflammatory responses to commensal microbes in the intestine and may have implications for the therapeutic strategies for intestinal inflammatory diseases, autoimmune diseases and cancer.

Introduction

The intestine harbors a great number of commensal bacteria besides orally invading pathogens. The commensal bacteria interact profoundly with the host immune system and are related to the health and disease of the host. Whereas the intestinal immune system requires the commensals for normal development and function, excessive immune responses to the commensals could induce inflammatory diseases (Kamada et al., 2013). Hence, the immune responses to the bacteria should be regulated precisely to prevent destructive inflammation while eliminate the pathogens to protect the host from infectious diseases.

Peyer's patches (PPs), second lymphoid organs in the small intestine, are known as the sites for uptake of luminal antigens and subsequent induction of immune responses (Schulz and Pabst, 2013). PPs are covered by specialized epithelial cells called follicle-associated epithelium (Neutra et al., 2001). These cells have several characteristics, compared with other intestinal epithelial cells, such as thinner mucosa and lower poly immunoglobulin (Ig) receptors, which means lower ability to secrete IgA antibodies into the lumen. These characteristics make PPs easily accessible to the luminal bacteria. Hence, the immune responses in PPs may need further precise regulation than in other sites in the intestine.

Dendritic cells (DCs) play a pivotal role in detecting antigens and regulating the immune responses. It has been reported that PP DCs extend their dendrites to the lumen and capture antigens (Lelouard et al., 2012). Another study indicated that PP DCs retain live commensal bacteria and migrate to mesenteric lymph node (MLN) to induce IgA response (Macpherson and Uhr, 2004). These suggest that PP DCs are in close contact with the intestinal microbes and may regulate the immune responses to them.

DCs detect microbial stimuli *via* various pattern recognition receptors such as Toll-like receptors (TLRs). TLRs function in the

Abbreviations: BM DC, bone marrow-derived dendritic cell; CpG, non-methylated CpG oligo DNA; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GM-CSF, granulocyte–macrophage colony stimulating factor; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MLN, mesenteric lymph node; PGN, peptidoglycan; PP, Peyer's patch; P3C, Pam_3CSK_4; qPCR, quantitative polymerase chain reaction; SPL, spleen; Th, T helper type; TLR, Toll-like receptor; WT, wild type.

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form of homo- or hetero-dimer. Distinct dimers recognize distinct molecular patterns. For instance, TLR2 recognizes lipoproteins on bacterial cell walls whereas TLR9 recognizes DNA, especially, which contain non-methylated CpG motifs. Generally, one bacterium has several TLR-stimulating molecules and therefore is recognized by DCs probably *via* several TLRs (Trinchieri and Sher, 2007). Hence, the responses to multiple simultaneous TLR stimulation should be unveiled to understand the interaction between the host immune system and bacteria. There are some studies which have reported that stimulating a certain combination of TLRs results in synergistic crosstalk (Hirata et al., 2008; Krummen et al., 2010; Mäkelä et al., 2009; Mitchell et al., 2010; Napolitani et al., 2005; Ouyang et al., 2007), and suppressive crosstalk (Liu et al., 2012; Simmons et al., 2010). However, crosstalk between TLRs remains much to be elucidated.

Upon TLR stimulation, DCs secrete various cytokines to regulate immune responses. Among those, interleukin (IL)-12 and IL-23 are known as proinflammatory cytokines (Vignali and Kuchroo, 2012). These two cytokines are associated to T helper type 1 (Th1) and Th17 response, respectively, and contribute to eliminating pathogens, while they have also been reported to mediate intestinal inflammation (Davidson et al., 1998; Hue et al., 2006; Neurath et al., 1995; Yen et al., 2006). In the intestine, IL-12 and IL-23 are produced mainly by DCs (Becker et al., 2003; Kinnebrew et al., 2012). Collectively, DCs need to be regulated precisely to produce these cytokines properly in the intestine.

Both of IL-12 and IL-23 are classified into IL-12 family cytokines, which consist of α- and β-chain subunits (Goriely et al., 2008; Vignali and Kuchroo, 2012). IL-12 and IL-23 are composed of a common β-chain, IL-12/23p40, and a distinct α-chain, IL-12p35 and IL-23p19, respectively. These IL-12/23p40, IL-12p35 and IL-23p19 subunits are encoded by distinct genes, *Il12b*, *Il12a* and *Il23a*, respectively. These genes are under distinct transcriptional control and, therefore, exhibit specific expression patterns in response to distinct stimuli (Goriely et al., 2008).

The present study investigated the regulation of IL-12 and IL-23 in PP DCs upon multiple simultaneous TLR stimulation. First, we compared the subunit gene expression in PP DCs stimulated with individual TLR ligands and selected those which induced strong responses. Subsequently, the selected ligands were used simultaneously to stimulate DCs, and this revealed suppressive crosstalk between TLR2 and TLR9. Finally, we attempted to unveil the mechanism underlying the suppression.

Materials and methods

Animals

BALB/c mice were purchased from CLEA Japan. *Tlr2*$^{-/-}$ and *Tlr9*$^{-/-}$ mice with BALB/c backgrounds were obtained from Oriental Bioservice and the wild type (WT) control mice were purchased from Charles River Japan. All mice were housed under specific pathogen-free environment and were sacrificed for use in experiments when 7–12 weeks old. All experiments were performed in accordance with guidelines for animal use and care of the University of Tokyo and were approved by the Animal Use Committee of the Faculty of Agriculture at The University of Tokyo.

Media

RPMI1640 (Nissui Pharmaceutical) containing 100 U/mL Penicillin G potassium (Meiji Seika Pharma), 100 μg/mL Streptomycin sulfate (Meiji Seika Pharma), 50 μM 2-mercaptoethanol (Tokyo Chemical Industry), 0.03% L-glutamine (Wako Pure Chemical Industries) and 0.2% sodium hydrogen carbonate (Wako Pure Chemical Industries) was prepared with or without heat-inactivated fetal calf serum (FCS) in 10% or 20% concentration. These media are described below as RPMI, 10% FCS-RPMI and 20% FCS-RPMI.

Reagents

Pam_3CSK_4 (P3C), peptidoglycan (PGN) from *Staphylococcus aureus*, purified lipoteichoic acid (LTA) from *S. aureus*, ultrapure lipopolysaccharide (LPS) from *Escherichia coli* 0111: B4 strain and R848 were purchased from Invivogen. Non-methylated CpG oligo DNA (CpG; 5′-ggG GGA CGA TCG TCg ggg gg-3′, the bases described in small letters have a phosphorothioate backbone) was synthesized with high performance liquid chromatography-purification grade by Hokkaido System Science. Purified anti-mouse CD40 antibody (1C10), purified anti-mouse IL-10 antibody (JES5-16E3) and purified anti-mouse CD210 (IL-10 receptor) antibody (1B1.3a) were purchased from BioLegend.

Peyer's patch and spleen dendritic cell preparation

PPs, excised from the small intestine, and spleens (SPLs), cut into small pieces, were disrupted by collagenase (Wako Pure Chemical Industries) in 10% FCS-RPMI with 10 ng/mL DNase I (Roche). The cell suspension was filtered with a 70 μm nylon mesh and was washed with RPMI to obtain whole cells. From the whole cells, $CD11c^+$ cells were purified with MACS separation (Miltenyi Biotec) according to the manufacturer's instructions. To achieve high purification, the cells were purified with MACS columns twice. The purified $CD11c^+$ cells were washed twice with RPMI and then were used as DCs in subsequent experiments.

Bone marrow-derived dendritic cell preparation

Femur and tibia were removed from mice and were cut off at both ends. Subsequently, bone marrow was forced out into 10% FCS-RPMI and the suspension was filtered with a 70 μm nylon mesh. The obtained cells were suspended in aqueous solution of 10 mM Tris–HCl and 150 mM NH_4Cl and put for 2 min at room temperature to deplete red blood cells. The cells were washed twice with RPMI, and bone marrow cells were obtained. To generate bone marrow-derived DCs (BM DCs), the bone marrow cells were cultured in 10% FCS-RPMI containing 10 ng/mL mouse granulocyte–macrophage colony stimulating factor (GM-CSF; Miltenyi Biotec) and 10 ng/mL recombinant murine IL-4 (PeproTech) in a humidified 5% CO_2 atmosphere at 37 °C. The medium was replenished at day 3, 6 and 8 and the cells were collected at day 10 according to the protocol reported by Lutz et al. (1999). The generated BM DCs were washed twice with RPMI and were used in subsequent experiments.

TLR stimulation

For TLR stimulation, DCs were incubated in 20% FCS-RPMI with reagents for indicated time in a humidified 5% CO_2 atmosphere at 37 °C. After the incubation, the cells or the supernatants were collected for quantitative polymerase chain reaction (qPCR) or enzyme-linked immunosorbent assay (ELISA).

qPCR

Total RNA was extracted with RNeasy mini kit (QIAGEN). cDNA was synthesized with SuperScript VILO MasterMix (Life Technologies) or SuperScript II Reverse Transcriptase (Life Technologies) with Oligo $(dT)_{12-18}$ Primer (Life Technologies). Subsequently, real time PCR was performed for measuring relative gene expression with QuantiTect SYBR Green PCR kit (QIAGEN) and LightCycler (Roche). The relative gene expression was calculated assuming that targeted cDNA was doubled at one cycle. Results were normalized