Cholera toxin disrupts oral tolerance via NF-κB-mediated downregulation of indoleamine 2,3-dioxygenase expression

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Cholera toxin (CT) is an ADP-ribosylating bacterial exotoxin that has been used as an adjuvant in animal studies of oral immunization. The mechanisms of mucosal immunogenicity and adjuvanticity of CT remain to be established. In this study, we investigated the role of indoleamine 2,3-dioxygenase (IDO), which participates in the induction of immune tolerance, in CT-mediated breakdown of oral tolerance. When IDO-deficient (IDO−/−) mice and their littermates were given oral ovalbumin, significant changes in antibody responses, footpad swelling and CD4+ T cell proliferation were not observed in IDO−/− mice. Feeding of CT decreased IDO expression in mesenteric lymph nodes (MLN) and Peyer's patch (PP). CT-induced downregulation of IDO expression was reversed by inhibitors of nuclear factor-kappa B (NF-κB), pyrrolidine dithiocarbamate and p50 small interfering RNA. IDO expression was downregulated by the NF-κB inducers lipopolysaccharide and tumor necrosis factor-α. CT dampened IDO activity and mRNA expression in dendritic cells from MLN and PP. These data indicate that CT disrupts oral tolerance by activating NF-κB, which in turn downregulates IDO expression. This study betters the understanding of the molecular mechanism underlying CT-mediated abrogation of oral tolerance.

Key Words: Cholera toxin, Oral tolerance, Indoleamine 2,3-dioxygenase, Mesenteric lymph node, Peyer's patch, NF-κB

INTRODUCTION

Oral tolerance is classically defined as the specific suppression of cellular and/or humoral immune response to dietary antigen and commensal enteric bacteria or substances administered orally (Howard and Wiener, 2000). Oral tolerance has been a major obstacle to the development of efficient oral vaccines. Many studies have sought to develop mucosal vaccine adjuvants. The most commonly used adjuvants in animal studies of oral immunization are ADP-ribosylating bacterial exotoxins, such as cholera toxin (CT) and Escherichia coli heat labile toxin (Williams et al., 1999). Oral administration of CT induces antigen-specific immunity that protects against subsequent intestinal challenge (Elson and Ealding, 1984; Lycke and Holmgren, 1986; Hornqvist et al., 1991; Anjuère et al., 2004). However, the mechanisms through which CT promotes immune responses remain controversial and unclear.

The nuclear factor-kappa B (NF-κB) family of transcription factors exists as homodimers or heterodimers of five distinct proteins (p50/NF-κB1, p52/NF-κB2, p65/RelA, RelB, and cRel) (Ghosh et al., 1998). NF-κB can be activated via two distinct signal transduction pathways. The canonical (also
known as the classical) NF-κB pathway requires activation of the IKK complex, consisting of the catalytic subunits IKKα and IKKβ, and the regulatory subunit NEMO/IKKχ, and controls NF-κB activation in response to proinflammatory stimuli such as that of lipopolysaccharide, TNF, or CD40L (Li et al., 1999; Sentfleben et al., 2001b). Activation of this pathway results predominantly in the activation, nuclear translocation, and DNA binding of the classical NF-κB dimer p50-RelA. In contrast, the non-canonical pathway (also known as the alternative pathway) is strictly dependent on IKKα homodimers and requires neither IKKβ nor NEMO/IKKχ (Sentfleben et al., 2001a). Activating IKKα results in the release and nuclear translocation of mainly p52-RelB dimers. This pathway can be triggered by activating members of the TNF-receptor superfamily such as the lymphoxygen receptor, B-cell activating factor belonging to the TNF family receptor, and CD40 (which also induces canonical NF-κB signaling) (Hayden and Ghosh., 2004). Regarding the action mechanism of CT, we have previously reported that CT induces canonical activation of nuclear factor-kappa B (NF-κB) in intestinal lymphoid cells, which plays a key role in mucosal immunogenicity and adjuvanticity (Kim et al., 2013). Indoleamine 2,3-dioxygenase (IDO) is the first and rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway. It causes depletion of tryptophan, which can cause halt the growth of microbes as well as T cells (Munn and Mellor, 2016).

IDO is expressed in various tissues, including the lung, small and large intestine, colon, spleen, kidney, stomach and brain. It has broad substrate specificity for various indoleamines including l-Trp, p5-OH-Kyn, l-5OH-Kyn, tryptamine and serotonin (Hayaishi 1996; Yamamoto and Hayaishi 1967; Shimizu et al., 1978). It is induced by pathological conditions, including virus infections and endotoxic shock (Jung et al., 2009), which may reflect interferon induction. Tryptophan starvation by IDO inhibits T-cell activation and induces generation of interleukin-10-producing T regulatory (Treg) cells (Munn and Mellor, 2016). Tryptophan catabolites, such as kynurenine derivatives and O2 free radicals, regulate T-cell proliferation and survival, as they induce T cell apoptosis and exert cytotoxic actions on T cells (Babcock and Carlin, 2000; Fallarino et al., 2006; Mellor et al., 2002; Frumento et al., 2002). As a result, IDO can play a role in the induction of immune tolerance during infection, pregnancy, transplantation, autoimmunity and neoplasia including hematologic malignancies (Uyttenhove et al., 2003; Curti et al., 2007; Mellor and Uyttenhove, 2004). However, the role of IDO in CT-induced oral tolerance has not been defined.

In this study, we investigated the relationship between CT-induced oral tolerance and IDO. We found that CT disrupts oral tolerance by activating NF-κB, which, in turn downregulates IDO expression.

MATERIALS AND METHODS

Animals

Specific, pathogen-free, female C57BL/6 mice were purchased from Damul Science (Daejeon, Republic of Korea). IDO-knockout (IDO−/−) mice in the C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All mice were used at 6 to 8 weeks of age and were housed in clean, pathogen-free rooms in an environment with controlled temperature (26°C), humidity (55%) and an alternating 12 hr light/dark cycle. All experiments were conducted in accordance with the guidelines of the Chonnam National University Institutional Animal Care and Use Committee.

Reagents

Albumin from chicken egg white (ovalbumin, OVA; grade V with low endotoxin content), CT (composed of the A and B subunits) from Vibrio cholerae, pyrrolidine dithiocarbamate (PDTC) and N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). InjectAlum was purchased from Thermo Scientific (Waltham, MA, USA). Primary antibodies (Abs) against p65 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and secondary Abs (goat-anti-rabbit IgG and goat-anti-rat IgG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies (Abs) against p65 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and secondary Abs (goat-anti-rabbit IgG and goat-anti-rat IgG) were purchased from Santa Cruz Biotechnology Biotechnology (Dallas, TX, USA). Anti-CD3 and anti-CD28 Abs were purchased from eBioscience (San Diego, CA, USA). Phycoerythrin (PE) hamster anti-mouse CD11c, fluorescein isothiocyanate (FITC) rat anti-mouse CD4, PE rat anti-mouse CD25 and PE rat anti-mouse Foxp3 were pur-
chased from BD Bioscience (San Jose, CA, USA).

**Induction of oral tolerance**

Twenty milligrams OVA dissolved in 200 μl of Dulbecco's phosphate buffered saline (DPBS) was intragastrically (i.g.) administered using an oral Zonde needle (Dae Jong Instrument Industry Co., Seoul, Korea) for 3 consecutive days. To induce the occurrence of oral tolerance, mice were intraperitoneally (i.p.) immunized on days 9 and 16 with 100 μg OVA and 4 mg Al(OH)₃ (OVA-Alum; Thermo Fisher Scientific.) in PBS and were later assessed for OVA-specific immunity.

**Measurement of OVA-specific serum Ab levels**

To determine antibody responses, mice were sacrificed and blood was centrifuged for 10 min at 7,500 rpm. Serum was collected from these samples and stored frozen at -70°C until assayed. Serum levels of OVA-specific IgG were measured by an enzyme-linked immunosorbent assay as described previously (Kim et al., 2013).

**Measurement of delayed-type hypersensitivity (DTH) response**

Seven days after last immunization, mice were challenged subcutaneously (s.c.) with 10 μg of heat-aggregated OVA into the left footpads and with PBS into the right footpads. The thickness of the injected paws was measured by using a micrometer (Mitutoyo Corporation, Kawasaki, Japan) 24 hr after the injections and was recorded as the difference between the left and right footpads.

**Preparation of mesenteric lymph node (MLN) and Peyer's patch (PP) cells**

Visible PPs were carefully excised from the wall of the small intestine. MLNs and PPs were placed in an ice-cold complete RPMI 1640 (GIBCO Invitrogen, Carlsbad, CA, USA) and slit using a surgical blade. The single cell suspension was passed through a 70 μm nylon mesh (cell strainer; BD Bioscience, San Jose, CA, USA) to remove cell debris. After red-blood cells lysis, MLN and PP-cells were washed twice with complete RPMI 1640 medium. Cell viability was assessed by the trypan blue exclusion assay (Freshney, 1987).

**Purification of CD4⁺ T cells**

Single-cell suspensions were prepared from MLNs and PPs of experiment mice. CD4⁺ T cells were purified by negative selection using MagCellect Mouse CD4⁺ T cell isolation kit (R&D system, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

**Cell proliferation assay**

Purified CD4⁺ T cells from pooled PPs and MLNs were incubated with 5 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA, SE; CFSE, Molecular Probes, Eugene, OR, USA) for 8 min in 5% heat-inactivated FBS (GIBCO Invitrogen, Carlsbad, CA, USA) containing PBS at room temperature (RT). Cell proliferation assay was performed as described previously (Kim et al., 2013).

**Western blot analysis**

Mice were sacrificed by cervical dislocation and the PPs and MLNs were collected, frozen immediately in liquid nitrogen and stored at -75°C until analysis. PP and MLNs specimens were homogenized in PhosphoSafe Extraction Reagent (Novagen Merck, Darmstadt, Germany) with phenylmethylsulfonyl fluoride protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Western blot analysis was performed as described previously (Kim et al., 2012).

**Real-time reverse transcription polymerase chain reaction (PCR)**

Total RNA was isolated from MLNs and PPs using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using 1 μl of total RNA in 10 μl of reaction mixture (Promega, Madison, WI, USA) containing oligo (dT) and avian myeloblastosis virus reverse transcriptase. Real-time PCR was performed on the Roter-Gene 3000 System (Corbett Research, Morklake, Australia) using the SYBR Green PCR Master Mix Reagent Kit (Qiagen, Valencia, CA, USA). The primers were: mouse IDO; 5'-CGG ACT GAG ACT GCA CAG GTT AC-3' and 5'-ACA CAT ACG CCA TGG TGA TGT AC-3', β-actin 5'-GAT CTG GCA
CCA CAC CTT CT-3’ and 5’- GGG GTG TTG AAG GTC TCA AA-3’ All quantities were expressed as the fold-change relative to the expression of β-actin.

Immunohistochemistry

Mice were i.g. intubated with OVA or OVA plus CT for 3 consecutive days. After 24 hr, PPs and MLNs were excised and fixed in 10% (w/v) neutral buffered formalin (Sigma-aldrich, St. Louis, MO, USA) for 24 hr at RT. Formalin-fixed tissues were processed into paraffin and cut into 4-μm sections on plain slides. After deparaffinization, they were subjected to a microwave antigen retrieval procedure in 0.01 M sodium citrate buffer for 10 min. They were then incubated in methanol containing 0.3% hydrogen peroxide, at RT for 20 min to block the endogenous peroxidase. The samples were blocked by CAS-Block™ (Invitrogen Life Technologies, Carlsbad, CA) for 30 min at RT and incubated overnight at 4℃ with IDO Abs (diluted in blocking solution) at a 1:200 dilution. After washing, the samples were incubated with biotin-labeled goat anti-rabbit IgG (Biosource, Camarillo, CA, USA) for 1 hr at RT and finally with streptavidin-horseradish peroxidase (Jackson Immuno Research, West Grove, PA, USA) for 30 min at RT. Samples were stained with 3,3’-diaminobenzidene (Zymed, South San Francisco, CA, USA), counter-stained with hematoxylin for 1 min and mounted with GVA mount (Invitrogen, Carlsbad, CA, USA).

In vivo small interfering RNA (siRNA) interference

In vivo delivery of siRNA was performed using in vivo-jet polyethylene imine (PEI) (Polyplus-transfection; Qbiogene, Carlsbad, CA, USA), according to the instructions of manufacturer. In brief, p50 siRNA or control siRNA and PEI dissolved in 5% glucose were mixed in a volume of 400 μl for i.p. injection at RT for 20 min and the mixture was administered 24 hr before OVA oral intubation. The mixture containing control siRNA and PEI dissolved in 5% glucose without siRNA were used as controls. To confirm that the p50 siRNA used really block the synthesis of its target, Western blot analysis was performed. p50 siRNA consisting of four double-stranded siRNAs were commercially synthesized by Genolution (Seoul, South Korea). The pool of siRNAs contained the p50-specific sequences: 5’-CAA GCA GGA AGA UGU AGU AUU-3’, 5’-CCA CUG CUA UCU CUG AAC AUU-3’, 5’-GUC ACU CGA UUU CAU UCA AUU-3’, 5’-UUU UAC ACG CCU CUG UCA UUC GUG CUU-3’. The negative control sequence was 5’-CCU ACG CCA CCA AUU UCG U-3’.

Purification of dendritic cells (DC) from MLNs and PPs

DCs from MLNs and PPs of experiment mice were purified by negative selection using Dynabeads Mouse DC Enrichment kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Measurement of IDO enzymatic activity

IDO activity was quantified by measuring the production of L-kynurenine in the culture supernatant spectrophotometrically. In brief, freshly isolated DCs from MNL and PP were washed, resuspended in complete RPMI 1640 media containing 500 μM Trp (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37℃ for 4 hr to permit IDO to convert Trp to N-formylkynurenine. The supernatants were harvested and assayed for kynurenine. The reaction was stopped by adding 50 μl of 30% tricholoroacetic acid to 100 μl of culture supernatant and the mixture was subsequently incubated at 50℃ for 30 min to hydrolyze N-formylkynurenine produced by IDO to kynurenine. The sample was centrifuged at 12,000 rpm for 10 min. One hundred microliters of supernatant were added to an equal volume of Ehrlich's reagent (0.4% p-dimethylaminobenzaldehyde in glacial acetic acid; Sigma-Aldrich, St. Louis, MO, USA) in a 96-well microtiter plate and incubated at RT for 10 min. The optical density (OD) was measured at 450 nm. A standard curve of defined Purified kynurenine concentration (0–500 μM; Sigma-Aldrich, St. Louis, MO, USA) permitted analysis of unknowns.

Statistical analysis

Data are represented as the mean ± S.E. Statistical significance was determined by one-way ANOVA test (StatView; Abacus Concepts Inc., Berkeley, CA, USA). All experiments were conducted at least twice. Reproducible results were obtained and representative data are, therefore, shown in
RESULTS

Oral tolerance does not occur in IDO⁻/⁻ mice

To establish the relationship between oral tolerance and IDO, IDO⁻/⁻ mice and their littermates were given oral PBS or OVA, and were subsequently i.p. immunized with OVA twice at 7-day intervals. Oral tolerance again took place in OVA-fed normal mice; serum IgG responses (Fig. 1A), footpad swelling (Fig. 1B) and CD4⁺ T cell proliferation (Fig. 1C) were lower compared with those of PBS-fed mice. In contrast, significant changes in Ab responses, footpad swelling and CD4⁺ T cell proliferation were not observed in IDO⁻/⁻ mice compared to OVA-fed normal mice. These results demonstrated that IDO is required for the induction of oral tolerance.

CT downregulates IDO expression in intestinal lymphoid cells

We investigated how CT regulates IDO expression. First, MLNs and PPs from mice fed OVA with or without CT were analyzed for the IDO expression at the protein level (Fig. 2A). IDO expression was higher in MLNs and PPs from OVA-fed mice than in OVA plus CT-fed mice. These data were confirmed by measuring the mRNA level of IDO in MLNs and PPs obtained from mice fed OVA with or without CT. The IDO transcripts were upregulated markedly in OVA-fed mice compared with OVA plus CT-fed mice (Fig. 2B). Next, the expression of IDO in situ at the MLNs and PPs after oral application of OVA with or without CT was determined. MLNs and PPs sections were stained with IDO-specific Ab. IDO expression was decreased in mice fed with CT compared with mice fed without CT (Fig. 2C).

NF-κB inhibitors reverse CT-mediated downregulation of IDO expression

The transcription factor NF-κB modulates multiple physiological and pathological processes ranging from innate and adaptive immune response, inflammation, organogenesis, tumorigenesis, synaptic plasticity and memory (Lenardo and Baltimore, 1989; Courtois and Gilmore, 2006). This transcription factor is ubiquitously expressed and its nuclear-cytoplasmic localization is intricately controlled. It regulates a large number of genes that control diverse cellular responses (Yanagita et al., 1999; Sen and Baltimore, 1986). The NF-κB family is composed of five members in mammals:
RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100). We previously reported that NF-κB is involved in the CT breakdown of oral tolerance (Kim et al., 2013). Presently, we examined the role of NF-κB on CT-mediated downregulation of IDO expression in MLNs and PPs. Mice were treated with NF-κB inhibitors before OVA feeding with or without CT intubation. CT-induced downregulation of IDO expression was reversed in a dose-dependent manner by the NF-κB inhibitor, PDTC (Fig. 3A). Additionally, p50 siRNA, but not control siRNA, abrogated the CT-mediated downregulation of IDO expression (Fig. 3B). These data suggest that NF-κB activity is associated with downregulated IDO expression and that CT downregulates IDO expression by activating NF-κB. Therefore, to delineate the relationship between NF-κB and IDO, we examined the effect of NF-κB activation on IDO expression. The NF-κB inducer, LPS, increased p65 phosphorylation and reduced IDO expression. Both were reversed by NF-κB inhibitors NAC and PDTC (Fig. 3C). Another NF-κB inducer, TNF-α, displayed very similar findings compared to LPS (Fig. 3D). The data indicate that NF-κB activity is associated with downregulates IDO expression.

CT decreases IDO activity and mRNA expression in DC of MLNs and PPs

DCs are key inducers of immune tolerance. One mechanism by which DCs regulate T cells is through the expression of IDO, which plays a key role in maintaining immune tolerance (Belz et al., 2002; Munn et al., 2004). We isolated DCs from MLN and PP, and examined how CT regulates IDO activity and mRNA expression in these cells. The culture supernatant from MLN and PP DCs of OVA-fed mice contained higher level of kynurenine than those from MLN and PP DCs of OVA with CT-fed mice (Fig. 4A). Likewise, mRNA expression of IDO was significantly reduced in DCs of OVA plus CT-fed mice (Fig. 4B). The data suggest that CT dampened IDO activity and mRNA expression in DC from MLN and PP.

DISCUSSION

The present study focused on the effects of IDO on oral tolerance induction by oral feeding Ag, and the relationship between CT-mediated abrogation of oral tolerance and IDO expression. CT-mediated abrogation of oral tolerance was associated with downregulation of IDO expression. Oral tolerance achieved by OVA immunization did not occur in IDO−/− mice, but did occur in their littermates (Fig. 1). IDO expression was evident in MLNs and PPs from OVA-fed mice, but not in OVA plus CT-fed mice (Fig. 2). IDO, an
enzyme that degrades the essential amino acid tryptophan, can be synthesized by many cell types (Grohmann et al., 2002; Munn and Mellor, 2007). Accumulating evidence indicates that IDO plays an important role in maternal tolerance to the allogeneic fetus (Mellor et al., 2001), self-tolerance in non-obese diabetic mice (Alexander et al., 2002) and in certain pathological conditions, including inflammatory bowel disease (Gurtner et al., 2003). Additionally, IDO has been implicated in mucosal tolerance (van der Marel et al., 2007). Consistent with these observations, our data suggest the involvement of IDO in OVA-induced tolerance and that CT disrupts the tolerance by IDO downregulation.

Regarding the mechanism of CT-mediated downregulation of IDO, pretreatment of NF-κB inhibitors abrogated CT-induced downregulation of IDO expression. We confirmed that the NF-κB inducers, LPS and TNF-α, downregulated IDO expression (Fig. 3). This suggests that NF-κB activity is required for downregulation of IDO. We have previously reported that CT is capable of inducing NF-κB activation, which is crucial for CT-mediated abrogation of oral tolerance (Kim et al., 2013). Therefore, the fact that NF-κB inhibitors abrogated CT-induced downregulation of IDO expression...
strengthens the notion that NF-κB is crucial in CT abrogation of oral tolerance. There are conflicting reports in the literature with regard to the role of NF-κB in IDO expression (Yang et al., 2014; Manches et al., 2012; Volpi et al., 2012), with canonical (Yang et al., 2014) and noncanonical NF-κB family members are involved in IDO expression. Further studies are required to address how NF-κB activity precisely affects IDO expression.

IDO expression in DCs is considered crucial in the suppression of T cell proliferation and survival (Fallarino et al., 2003b). Furthermore, regulatory antigen presenting cells may form a bridge between Treg cells and effecter T cells, and this has been proposed as a mechanism contributing to the phenomenon of linked suppression and dominant tolerance (Munn et al., 2004). Experimental evidence indicates that murine Treg cells can induce the expression of IDO (Fallarino et al., 2003a) and IDO regulates the differentiation of a subset of Foxp3+ Treg cells in vitro (Mellor et al., 2001). However, the regulatory DC subset that induces tolerance in an IDO-dependent manner that remains unclear. In this study, CT dampened IDO activity and mRNA expression in DCs from MLN and PP. This suggests that CT negatively regulates Treg generation by downregulating IDO activity and expression. This may be one of the mechanisms underlying CT-mediated breakdown of oral tolerance.

Collectively, these results suggest that CT induces the breakdown of oral tolerance and downregulation of IDO expression via the activation of canonical NF-κB. The present findings will lay the groundwork for further studies that will elucidate the precise role of IDO in oral tolerance, and will lead to a better understanding of the molecular mechanism underlying CT-mediated abrogation of oral tolerance.

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CONFLICT OF INTEREST
No conflicts of interest, financial or otherwise, are declared by the authors.

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