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Smad3 Deficiency in Mast Cells Provides Efficient Host Protection against Acute Septic Peritonitis¹

Yutaka Kanamaru,* Koji Sumiyoshi,* Hiroko Ushio,* Hideoki Ogawa,* Ko Okumura,**† and Atsuhito Nakao²*‡

Mast cells play an important role in innate immunity as well as in allergic reaction. However, regulatory mechanisms underlying mast cell-mediated innate immune responses remain largely unknown. Here we determined whether Smad3, a major signal transducer of TGF-β, regulates innate immune response by mast cells against Gram-negative bacteria. Bone marrow-derived mast cells (BMMC) obtained from Smad3 null mutant mice showed augmented capacity to produce proinflammatory cytokines upon stimulation with a Gram-negative bacteria-associated product, LPS. In acute septic peritonitis model induced by cecal ligation and puncture, mast cell-deficient W/W<sup>+</sup> mice reconstituted with Smad3 null BMMC had significantly higher survival rate than W/W<sup>+</sup> mice reconstituted with wild-type BMMC, which was associated with higher production of proinflammatory cytokines in the peritoneal cavity. These in vitro and in vivo results suggest that Smad3 in mast cells functions as inhibitory for mast cell-mediated innate immune response against Gram-negative bacteria. Suppression of Smad3 expression in mast cells may thus have therapeutic potential for Gram-negative bacterial infection such as acute septic peritonitis by augmenting innate immune responses of mast cells. *The Journal of Immunology, 2005, 174: 4193–4197.

Materials and Methods

Mice

The generation of Smad<sup>ΔNterEx8</sup> null mice by homologous recombination was described previously (9) and kindly provided by Dr. C. Deng (National Cancer Institute, Bethesda, MD). In this line of mice, exon 8 of the Smad3 gene is deleted. The deletion removes the L3 loop, which is necessary for interaction with the TGF-β receptors, and the COOH-terminal serine-valine-serine (SSVS) consensus phosphorylation site. Smad3 null mice were backcrossed for six generations to C57BL/6 mice. Mouse heterozygous for the targeted disruption were intercrossed to produce homozygous offspring and kept under specific pathogen-free conditions in the animal facility of Juntendo University. WBB6F1-W/W<sup>+</sup> mice were purchased from Japan SLC. All animal experiments were performed according to the approved manual of the Institutional Review Board of Juntendo University.

Generation of bone marrow-derived mast cells (BMMC)<sup>3</sup>

BMMC were generated from the femoral bone marrow cells of mice and maintained in the presence of 10% pokeweed mitogen-stimulated spleen-conditioned medium as a source of mast cell growth factors as previously described (4, 15). After 4 wk of culture, >99% of the cells were identifiable as mast cells as determined by toluidine blue staining and FACs analysis of cell surface expression of c-kIt and FcεRI. May-Giemsa and Alcian safranin stainings of BMMC were performed as previously described (4).

Abbreviations used in this paper: BMMC, bone marrow-derived mast cell; CLP, cecal ligation and puncture; IRAK, IL-1R-associated kinase.

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Measurement of cytokine concentrations

BMMC (1 × 10⁶ cells/ml) in complete culture medium were stimulated with the indicated concentration of LPS (Sigma-Aldrich). Cells were incubated at 37°C for 6 h for TNF-α and IL-6. Our previous and preliminary experiments showed that these time points were optimal for the production of each cytokine from mast cells (4). The level of each cytokine in supernatant was measured by ELISA kit (Genzyme Techen). In some experiments, neutralizing Ab against TGF-β1 (100 µg/ml) (R&D Systems) or isotype control Ab was added to the BMMC culture 1 h before LPS stimulation and cytokine concentrations were measured.

Reconstitution of W/W⁰ mice with BMMC

Mast cell deficiency of W/W⁰ mice in the peritoneal cavity was selectively reconstituted 4 wk after starting the culture by the injection of 2 × 10⁶ BMMC from Smad3 null mutant or respective wild-type mice, into the peritoneal cavity as previously described (4, 5). The mice were used for experiments 5 wk after injection of BMMC. Reconstitution of mast cells was confirmed by toluidine blue or Alcian blue safranin staining of the cytopsin preparation of peritoneal cells. Functional relevance of reconstituted mast cells was confirmed by the β-hexosaminidase release assay using purified peritoneal mast cells 5 wk after reconstitution through the stimulation by FceRI cross-linking or calcium ionophore.

Cecal ligation and puncture (CLP)

Cecal ligation and puncture (CLP) was performed as previously described (4, 5, 16) with some modification. Briefly, mice were anesthetized with sodium pentobarbital (Abbott Laboratories) in 200 µl of sterile PBS. A 1-cm midline incision on the anterior abdominal wall was made. The cecum was exposed and filled with feces by squeezing stool back from the ascending colon. The cecum was 50% ligated below the ileocecal valve and then punctured twice with a 21-gauge needle followed by gentle squeezing of the cecum. Mice were observed for mortality at least five times daily over a period of 10 days. Before CLP was performed, the mice were coded so that the CLP was done without notifying an individual group.

Estimation of cytokine concentrations in peritoneal exudates

Peritoneal exudates were collected from CLP-induced mice at the indicated time points, and the levels of cytokines in peritoneal fluids were determined by ELISA kits according to the manufacturer’s instruction (Genzyme Techen). Cytospin preparations were made from the exudates of each mouse, and differential cell counts of infiltrating leukocytes were done by counting 500 leukocytes under oil immersion fields after staining with Diff-Quik (International Reagents).

RT-PCR

Total RNA was extracted from BMMC by using the Isogen solution (Nippon Gene) as recommended by the manufacturer’s instructions. cDNA was synthesized from 2 µg of total RNA using the first strand cDNA synthesis kit (Ready To Go; Amersham Biosciences). PCR amplification was performed as previously described (4, 17). The PCR products were separated by 2.0% agarose gel electrophoresis and stained with 0.5 µg/ml ethidium bromide.

Immunoblotting

Cells (1 × 10⁶) were suspended in RIPA buffer (20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10% glycerol, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS) supplemented with 0.1 mM PMSF, 100 µM Na₃VO₄, and 20 mM β-glycerophosphate. Extracts were cleared by centrifugation. Whole cell extracts (20 µg of protein) were separated on 9–15% SDS-polyacrylamide gels and transferred onto Immobilon-P membrane (Millipore). Detection of specific protein was performed by using Ab specific for TLR4 (M-16; Santa Cruz Biotecnology) and β-actin (Santa Cruz Biotecnology). Immunoreactive bands were detected by ECL (ECL-plus; Amersham Biosciences) using HRP-linked anti-goat IgG (Amersham Biosciences).

Data analysis

Statistical analysis was performed using an unpaired Student’s t test. Statistical analysis of survival data in the CLP experiment was performed using the log rank test.

Results

Smad3 deficiency enhances LPS-induced proinflammatory cytokine production by mast cells in vitro

To determine whether Smad3 regulated innate immune response by mast cells against infectious organisms, we first examined the effect of Smad3 deficiency on mast cell response to a major Gram-negative bacteria-associated pathogen, LPS. We have previously shown that LPS induces proinflammatory cytokine production by BMMC via TLR4 (4, 5). BMMC derived from Smad3 null mice showed similar staining pattern to those from wild-type mice by Alcian blue safranin, suggesting that BMMC derived from Smad3 null mice were at a similar developmental stage as those from wild-type mice (Fig. 1). Then, BMMC derived from Smad3 null mice or wild-type mice were stimulated with LPS, and cytokine concentrations in the culture supernatants were measured. As shown in Fig. 2, LPS-induced IL-6 and TNF-α production were enhanced in BMMC derived from Smad3 null mice in a dose-dependent manner when compared with BMMC derived from wild-type mice. These results indicated that Smad3 deficiency enhanced LPS-induced proinflammatory cytokine production by mast cells.

W/W⁰ mice reconstituted with BMMC from Smad3 null mice show increased protection against acute septic peritonitis induced by CLP

To clarify the role of Smad3 in mast cell-mediated innate immunity against Gram-negative bacterial infection in vivo, the peritoneal cavity of mast cell-null W/W⁰ mice were reconstituted with BMMC from wild-type mice (Smad3+/⁺) or Smad3 null mutant mice (Smad3⁻⁻) and subjected to cecal ligation and puncture (CLP), a model for acute septic peritonitis, as previously described by us and others (4, 5, 16). It is well accepted that mast cells and mast cell-derived proinflammatory cytokines are essential for protection against acute septic peritonitis induced by CLP (16). As shown in Fig. 3, 95% of mast cell-deficient W/W⁰ mice died...
within 5 days after CLP, and 50% of W/Wv mice reconstituted with wild-type BMMC (W/Wv/Smad3−/−) survived 5 days after CLP. Importantly, 5 days after CLP, W/Wv mice reconstituted with Smad3 null BMMC (W/Wv/Smad3−/−) showed a significantly higher survival rate than W/Wv/Smad3−/− mice (p < 0.05). Thus, Smad3 deficiency in mast cells provided efficient host protection from early enterobacterial infection.

Increased proinflammatory cytokine production in the peritoneal cavity of W/Wv/Smad3−/− mice after CLP

To investigate mechanisms underlying the increased survival rate in W/Wv/Smad3−/− mice against acute septic peritonitis induced by CLP, we examined the number of leukocytes and the level of proinflammatory cytokines in peritoneal exudates. As shown in Fig. 4, the number of neutrophils in peritoneal exudates was significantly higher in W/Wv/Smad3−/− mice than in W/Wv/Smad3−/+ mice. In addition, the levels of TNF-α and IL-6 in peritoneal cavities of W/Wv/Smad3−/− mice 8 h after CLP were significantly higher than those of W/Wv/Smad3−/+ mice (Fig. 4). Thus, the increased survival rate in W/Wv/Smad3−/− mice against acute septic peritonitis induced by CLP was associated with increased number of neutrophils and increased level of proinflammatory cytokines in peritoneal exudates.

Neutralizing TGF-β activity enhances LPS-induced cytokine production from BMMC

Lindstedt et al. (18) recently reported that TGF-β1 was stored as latent form in secretory granules of mast cells, that was rapidly released and converted into active form by co-released chymase upon mast cell activation. It is therefore possible that LPS induced endogenous TGF-β1, which could then suppress mast cell responses to LPS in an autocrine manner. To investigate this possibility, we examined the effect of neutralizing Ab against TGF-β1 on LPS-induced TNF-α and IL-6 production by BMMC. BMMC derived from wild-type mice showed increased production of TNF-α and IL-6 upon LPS stimulation in the presence of anti-TGF-β1 Ab when compared with those in the presence of control Ab (Fig. 5). The results indicated that endogenously produced...
FIGURE 5. Neutralizing TGF-β1 activity enhances LPS-induced cytokine production from wild-type BMMC. BMMC obtained from wild-type mice were stimulated with indicated concentration of LPS in the presence of 100 μg/ml neutralizing Ab against TGF-β1 (△) or isotype control Ab (□), and the concentrations of TNF-α and IL-6 in the supernatant were determined by ELISA. The data presented are representative of three independent experiments. Values represent the mean ± SD (n = 4). *, p < 0.05 compared with corresponding control.

FIGURE 6. Expression of TLR4, IRAK, and IRAK-M mRNAs and TLR4 protein in BMMC obtained from Smad3 null mice. A, RNA was extracted from Smad3 null BMMC (KO) or wild-type BMMC (WT), and RT-PCR analysis was then performed with specific primers for mouse TLR4, IRAK, IRAK-M, TGF-β type I receptor (TβRII), TGF-β type II receptor (TβRII), Smad2, or GAPDH. B, Cell lysates of Smad3 null BMMC (KO) or wild-type BMMC (WT) were immunoblotted with anti-TLR4 Ab (top panel) or anti-β-actin Ab (bottom panel). The data presented are representative of three independent experiments.

TGF-β1 upon LPS stimulation functioned as inhibitory for TNF-α and IL-6 release.

Expression of TLR-associated signaling molecules in Smad3 null BMMC

LPS signals via TLR4, followed by recruitment of cytoplasmic adaptor molecule MyD88, then IL-1R-associated kinase (IRAK), and finally induces activation of NF-κB and MAPK (19). IRAK-M, a member of IRAK family, is a negative regulator of TLR signaling (20). We therefore asked whether expression of these molecules were altered or not in Smad3 null BMMC. As shown in Fig. 6A, RT-PCR analysis showed that there was no significant difference in TLR4, IRAK, and IRAK-M expression between wild-type BMMC and Smad3 null BMMC. Expressions of TGF-β type I and type II receptors, and Smad2 in Smad3 null BMMC were also not significantly different from wild-type BMMC (Fig. 6A). We also found that TLR4 protein expression was comparable between wild-type BMMC and Smad3 null BMMC (Fig. 6B).

Discussion

In this study, we demonstrated that mast cell-deficient W/Wv mice reconstituted with Smad3 null BMMC enhanced protection against acute septic peritonitis induced by CLP (Fig. 3), which was associated with increased number of leukocytes and enhanced cytokine production in the peritoneal exudates (Fig. 4). In addition, Smad3 deficiency enhanced LPS-induced proinflammatory cytokine production by BMMC in vitro (Fig. 2). Echtenacher et al. (16) reported that mast cell-deficient W/Wv mice showed an increased mortality in a model of CLP-induced acute septic peritonitis when compared with wild-type mice. They also showed that anti-TNF-α Ab injected after CLP suppressed the protection against CLP-induced acute septic peritonitis by mast cells (16). Collectively, the current results suggest that Smad3 null BMMC conferred efficient protection against CLP-induced acute septic peritonitis, at least in part, through increased production of proinflammatory cytokines including TNF-α.

Recently, McCartney-Francis et al. (21) demonstrated that TGF-β signaling pathway antagonized LPS/TLR4 signaling. They showed that TGF-β or Smad3 null mutant mice died by intraperitoneal injection of non-lethal dose of LPS to wild-type mice associated with increased proinflammatory cytokines and NO production (21). The current results were consistent with their findings in that Smad3 null BMMC showed hyperresponsiveness to LPS stimulation. Mast cells distributed widely through the body may be a key player responsible for hyperresponsiveness to LPS observed in Smad3 null mice.

How Smad3 deficiency enhances LPS-induced proinflammatory cytokine production by BMMC remains uncertain. The experiment using neutralizing Ab against TGF-β1 (Fig. 5) suggested that endogenous TGF-β1 functioned as inhibitory in LPS-induced TNF-α and IL-6 production from BMMC. Recently, Sly et al. (22) showed that LPS induced TGF-β1 production by BMMC, which in turn up-regulated SHIP expression leading to LPS tolerance. Taken together, we speculate that Smad3 null BMMC might escape from the negative feedback regulation by endogenous TGF-β1 produced upon LPS stimulation. It remains to be determined whether Smad3 is involved in TGF-β-induced SHIP expression in BMMC.

Smad pathway can interfere with NF-κB signaling pathway in certain cell types (23, 24). Thus, although Smad3 deficiency did not affect TLR4 expression in BMMC (Fig. 6), it is also possible that Smad3 deficiency up-regulates LPS/TLR4/NF-κB signaling...
pathway in BMMC by unknown mechanisms, resulting in increased proinflammatory cytokine production.

The inflammatory cytokines produced as a result of LPS/TLR4 signaling, when released in excess, induce endotoxin shock, which is associated with high mortality rate (19). Therefore, our approach that acquires LPS hyperresponsiveness selectively in mast cells by targeting Smad3 may have some advantage in that it may reduce the risk of endotoxin shock by reducing the cellular source of inflammatory cytokines and selectively augmenting local inflammatory responses.

In summary, we demonstrated that Smad3 null mast cells showed enhanced production of proinflammatory cytokines upon LPS stimulation and conferred efficient protection against acute peritonitis induced by cecal ligation and puncture in mice.

The data suggest that Smad3 in mast cells negatively regulates innate immune responses by mast cells against Gram-negative bacteria. Modulation of Smad3 expression in mast cells may thus become a novel therapeutic target by regulating mast cell responses against Gram-negative bacterial infections.

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Disclosures
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