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TLR2/MyD88-Dependent and -Independent Activation of Mast Cell IgE Responses by the Skin Commensal Yeast Malassezia sympodialis

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Atopic eczema (AE) is a chronic inflammatory skin disease. Approximately 50% of adult AE patients have allergen-specific IgE reactivity to the skin commensal yeast Malassezia spp. Due to the ruptured skin barrier in AE, it is likely that Malassezia can come into contact with mast cells, which are known to be involved in AE. We therefore hypothesized that Malassezia spp. can activate mast cells. Bone marrow-derived mast cells (BMMCs) were generated from wild type, TLR2, TLR4, and MyD88 gene-deleted mice and cocultured with Malassezia sympodialis extract. We recorded that M. sympodialis induced release of cysteinyl leukotrienes in a dose-dependent manner in nonsensitized and IgE-anti-trinitrophenyl-sensitized BMMCs, respectively, with three times higher levels in the latter type of cells. IgE-sensitized BMMCs also responded by degranulation as assessed by release of β-hexosaminidase, increased MCP-1 production through a MyD88-independent pathway, and activated phosphorylation of the MAPK ERK1/2. Furthermore, M. sympodialis enhanced the degranulation of IgE receptor cross-linked wild-type BMMCs and altered the IL-6 release dose-dependently. This degranulation was independent of TLR2, TLR4, and MyD88, whereas the IL-6 production was dependent on the TLR2/MyD88 pathway and MAPK signaling. In conclusion, M. sympodialis extract can activate nonsensitized and IgE-sensitized mast cells to release inflammatory mediators, to enhance the IgE-mediated degranulation of mast cells, and to modulate MAPK activation and by signaling through the TLR2/MyD88 pathway to modify the IL-6 production of IgE receptor cross-linked mast cells. Collectively, these findings indicate that M. sympodialis can activate mast cells and might thus exacerbate the inflammatory response in AE.
in mast cell activation. Our aims were therefore to investigate: 1) whether *Malassezia* can activate mast cells and 2) if so, to define which receptor(s) and signaling pathway(s) through which the activation occurs. We determined that *M. sympodialis* can indeed activate mast cells, enhance the mast cell IgE response, and modulate MAPK activation and by signaling through the TLR2/MyD88 pathway after IL-6 production in a dose-dependent manner.

### Materials and Methods

#### Mast cell cultures

Bone marrow-derived mast cells (BMMCs) were obtained by culturing mouse bone marrow cells from wild-type (Wt), TLR2−/−, TLR4−/−, and MyD88−/− mice with the C57BL/6 genetic background (from the Karolinska Institutet mouse breeding facility, Department of Microbiology, Tumor and Cell Biology, Stockholm, Sweden), originally provided by Prof. Shizuo Akira (Osaka University, Osaka, Japan). The cells were cultured in Falcon culture flasks (BD Biosciences) at a concentration of 0.5 × 10⁶ cells/ml in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS (Invitrogen), 10% IL-3-conditioned medium (produced by X63/0 myeloma cells transfected with a mouse IL-3 expression construct) (18), 100 μg/ml penicillin/streptomycin (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 25 mM HEPES (Sigma-Aldrich), 4 mM L-glutamine (Sigma-Aldrich), 0.1 mM nonessential amino acids (Sigma-Aldrich), and 0.01–100 μg/ml of LPS from *Escherichia coli* (in air) with the culture medium being changed weekly. Mast cell development was evaluated by toluidine blue staining (19) and the purity of mast cells used in the experiments exceeded 98%. Cell surface FcεRI expression was confirmed by flow cytometry analysis (FACScalibur flow cytometer; BD Biosciences) using a hamster anti-mouse FcεRI-FITC Ab (BD Biosciences).

#### M. sympodialis extract

*M. sympodialis* extract was prepared from strain 42132 (American Type Culture Collection) according to a previously described protocol (20), with the modification of culture plates and incubation temperature. In brief, *M. sympodialis* yeast cells were harvested after 4 days of culture at 32°C on Dixon agar plates (21), freeze-dried, taken up in PBS, and sonicated. After overnight incubation at 4°C, centrifugation, and sterile filtration, the preparation of the culture was determined by flow cytometry (FACScalibur flow cytometer; BD Biosciences) using a hamster anti-mouse FcεRI-FITC Ab (BD Biosciences).

#### Mast cell activation

BMMCs were sensitized in a humidified incubator overnight at 37°C using a monoclonal mouse anti-trinitrophenyl (TNP) IgE Ab (IgE1-b4; American Biosciences) for 1 h at room temperature. The cells were washed twice in PBS and seeded into 48-well plates (BD Biosciences) at a final concentration of 1 × 10⁶ cells/ml in RPMI 1640 medium supplemented as described above. The nonsensitized cells were cultured in medium alone or subjected to treatment with different stimuli: 0.5 μM of the calcium ionophore calcimycin A23187 (Sigma-Aldrich), 1 μg/ml LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich), 1 μg/ml zymosan from *Saccharomyces cerevisiae* (Sigma-Aldrich), or 0.01–100 μg/ml *M. sympodialis* extract. The IgE-sensitized cells were cultured with 0.01–100 μg/ml *M. sympodialis* extract either alone or in combination, for cross-linking, with 100 ng/ml TNP-BSA (coupling ratio 9; Biosearch Technologies). After overnight incubation at 37°C in a humidified incubator overnight at 37°C using a monoclonal mouse anti-trinitrophenyl (TNP) IgE Ab (IgE1-b4; American Biosciences) for 1 h at room temperature. The cells were washed twice in PBS and seeded into 48-well plates (BD Biosciences) at a final concentration of 1 × 10⁶ cells/ml in RPMI 1640 medium supplemented as described above. The nonsensitized cells were cultured in medium alone or subjected to treatment with different stimuli: 0.5 μM of the calcium ionophore calcimycin A23187 (Sigma-Aldrich), 1 μg/ml LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich), 1 μg/ml zymosan from *Saccharomyces cerevisiae* (Sigma-Aldrich), or 0.01–100 μg/ml *M. sympodialis* extract. The IgE-sensitized cells were cultured with 0.01–100 μg/ml *M. sympodialis* extract either alone or in combination, for cross-linking, with 100 ng/ml TNP-BSA (coupling ratio 9; Biosearch Technologies). All cells were incubated at 37°C in a humidified incubator. Culture supernatants were harvested after 0.5 and 24 h after stimulation, respectively, and stored at −20°C until analysis. The expression of FcεRI after 24 h of incubation with or without 0.01–100 μg/ml *M. sympodialis* extract was determined by flow cytometry analysis as described above.

#### N-acetyl-β-D-hexosaminidase release assay

To detect the granular enzyme β-hexosaminidase release into the supernatant, cell activation was performed for 0.5 h in RPMI 1640 medium (Sigma-Aldrich) supplemented with 100 μg/ml penicillin/streptomycin (Sigma-Aldrich), 4 mM L-glutamine (Sigma-Aldrich), 50 μM 2-MAE, and 0.5% BSA (Sigma-Aldrich) at 37°C in a humidified incubator. An enzymatic colorimetric assay was used to analyze the amount of β-hexosaminidase as previously described (22). Briefly, 60 μl of supernatant was added in duplicates into a 96-well plate (BD Biosciences) and mixed with an equal volume of substrate solution (7.5 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma-Aldrich) dissolved in 80 mM citric acid, pH 4.5) and incubated with gentle agitation (200 rpm) for 2 h at 37°C. As a standard, the corresponding amount of lysed mast cells was used and medium alone served as a negative control. The incubation was stopped by the addition of 120 μl of glycine (0.2 M, pH 10.7) to each well, and the absorbances were measured at 405 nm and 490 nm using a Multiskan RC reader (Labsystems). Results are expressed as the percentage of total N-acetyl-β-D-hexosaminidase content mean ± SEM.

#### Cysteinyl leukotriene enzyme immunoassay

An enzyme immunoassay (Amersham Biosciences) with a sensitivity of 10 pg/ml was used to determine the release of cysteinyl leukotrienes into the supernatants after 0.5 h of culture. All assays were set up in duplicates and the results are presented as mean ± SEM.

#### Cytokine and chemokine ELISAs

Supernatants harvested after 24 h of culture were analyzed using ELISA for the production of IL-6 (CytoSet kit; BioSource International) and MCP-1 (Duoset; R&D Systems), respectively, according to the manufacturers‘ instructions. Recombinant mouse IL-6 (BioSource International) and recombinant mouse MCP-1 (R&D Systems) were used as standards. The detection level of the IL-6 assay was 3 pg/ml and that of MCP-1 was 9 pg/ml. All assays were set up in duplicate wells and the results are presented as mean ± SEM.

#### Western blotting

To analyze MAPK activation by *M. sympodialis*, IgE-sensitized Wt BMMCs were cultured with medium alone or 0.01–100 μg/ml *M. sympodialis* extract either alone or in combination with 100 ng/ml TNF-BSA. Cells were harvested at 10 min after stimulation and lysed in SDS sample buffer (125 mM Tris-HCl, pH 6.8), 4% w/v SDS, 20% glycerol, 0.02% w/v bromophenol blue, and 50 mM DTT, added just before use) before sonication on ice. Western blotting was performed using NuPAGE Bis-Tris Western gels (Invitrogen). Following electrophoresis, proteins were electroblotted onto nitrocellulose membranes (Hybond ECL; Amersham Biosciences). Membranes were then blocked for 1 h in TBS containing 5% w/v nonfat dry milk and 0.1% Tween 20. Membranes were subsequently incubated overnight at 4°C with the primary Ab, either a rabbit anti-ERK1/2 Ab as a loading control (Santa Cruz Biotechnology) or a rabbit anti-phosphorylated-ERK1/2 Ab (Cell Signaling Technology), washed, and subsequently incubated with a HRP-conjugated secondary goat-anti-rabbit Ab (Cell Signaling Technology) for 1 h at room temperature. The proteins were visualized using an ECL System LumiGLO and exposed to Hybond ECL film (Amersham Biosciences).

#### Statistical analysis

The Wilcoxon-matched pairs test was performed using the Statistica 7.1 software package (StatSoft Scandinavia). Values of *p* < 0.05 were considered to be statistically significant.

#### Results

*M. sympodialis* induces release of cysteinyl leukotrienes, but not degranulation, IL-6, or MCP-1 release from nonsensitized mast cells

*M. sympodialis* extract contains a variety of proteins, a number of which have been identified as IgE-binding allergens (7, 20, 23). The extract was first tested for its ability to induce degranulation of Wt BMMCs as assessed by release of β-hexosaminidase. Wt BMMCs were treated with increasing concentrations of *M. sympodialis* extract (0.01–100 μg/ml) for 0.5 h. As depicted in Fig. 1A, we could not observe any degranulation of Wt BMMCs following addition of either *M. sympodialis* extract, LPS, or zymosan, respectively. The cells responded as expected to the positive control A23187 (Fig. 1A).

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The *M. sympodialis* extract was next tested for its ability to induce release of cysteinyl leukotrienes but not degranulation, IL-6, or MCP-1 production from mast cells. Wt BMMCs were incubated with increasing concentrations of *M. sympodialis* extract, 1 μg/ml LPS, 1 μg/ml zymosan, medium alone (−) as a negative control, or 0.5 μM A23187 as a positive control, respectively. The concentration of β-hexosaminidase and cysteinyl leukotrienes in the supernatants after 0.5 h of incubation was determined using a β-hexosaminidase assay or an enzyme immunoassay, respectively (A and B). Release of IL-6 (C) and MCP-1 (D) after a 24 h incubation was measured by ELISA. Values are expressed as mean ± SEM from three independently conducted experiments performed in duplicate.

**FIGURE 1.** *M. sympodialis* extract induces release of cysteinyl leukotrienes but not degranulation, IL-6, or MCP-1 production from mast cells. Wt BMMCs were incubated with increasing concentrations of *M. sympodialis* extract, 1 μg/ml LPS, 1 μg/ml zymosan, medium alone (−) as a negative control, or 0.5 μM A23187 as a positive control, respectively. The concentration of β-hexosaminidase and cysteinyl leukotrienes in the supernatants after 0.5 h of incubation was determined using a β-hexosaminidase assay or an enzyme immunoassay, respectively (A and B). Release of IL-6 (C) and MCP-1 (D) after a 24 h incubation was measured by ELISA. Values are expressed as mean ± SEM from three independently conducted experiments performed in duplicate.
expected LPS-induced IL-6 production and the positive response to A23187 (Fig. 1, C and D).

**Mast cells sensitized with IgE are activated by *M. sympodialis* extract through a MyD88-independent pathway**

We next investigated the effect of *M. sympodialis* on IgE-sensitized mast cells, since mast cells in the skin of AE patients express IgE on their surface (26). BMMCs from Wt mice were sensitized with IgE-anti-TNP and thereafter treated with 0.01–100 μg/ml *M. sympodialis* extract. The highest concentration of *M. sympodialis* extract induced a significant mast cell degranulation (Fig. 2A). Moreover, *M. sympodialis* extract induced within 0.5 h release of cysteinyl leukotrienes from IgE-sensitized Wt BMMCs in a dose-dependent manner (Fig. 2B). Notably, the amount of cysteinyl leukotrienes released from IgE-sensitized mast cells stimulated with *M. sympodialis* extract was approximately three times higher than the levels released from nonsensitized mast cells following stimulation with *M. sympodialis* extract (Figs. 1B and 2B). We also measured the effect of *M. sympodialis* on the secretion of IL-6 and MCP-1. No significant increase of IL-6 secretion was detected following addition of the *M. sympodialis* extract (Fig. 2C). In contrast, similar to the degranulation response, we could measure a significant release of MCP-1 upon addition of the highest concentration of *M. sympodialis* extract (100 μg/ml; Fig. 2D). Chemokine production in mast cells is mediated through MAPK signaling (27). We therefore investigated whether *M. sympodialis* could cause activation of MAPK in IgE-sensitized mast cells. We determined that IgE-sensitized Wt BMMCs treated for 10 min with the highest concentration of *M. sympodialis* extract activated phosphorylation of the MAPK ERK1/2 (Fig. 2E), indicating that *M. sympodialis* can activate MAPK signaling in IgE-sensitized mast cells.

Because BMMCs express several TLRs (16), we explored whether *M. sympodialis* could interact through a TLR-dependent pathway that mediates degranulation and MCP-1 release. BMMCs from MyD88<sup>−/−</sup> mice were generated, since MyD88 is a protein involved in the signaling pathway of most TLRs (28). We recorded that IgE-sensitized MyD88<sup>−/−</sup> mast cells degranulated, released MCP-1, and did not produce IL-6 (Fig. 3, A–C) in a similar fashion to Wt BMMCs (Fig. 2, A, C, and D), indicating a MyD88-independent activation in *M. sympodialis*-exposed, IgE-sensitized mast cells.

**M. sympodialis enhances the IgE receptor cross-linked degranulation of mast cells independently of TLR2, TLR4, and MyD88**

A recent study reported that *E. coli* can interfere with mast cell responses and can negatively affect IgE-mediated activation (29). We therefore studied the effect of *M. sympodialis* extract on the sensitized Wt BMMCs were incubated with increasing amounts of *M. sympodialis* extract, medium alone (IgE) as a negative control, or TNP-BSA (IgE/TNP) as a positive control, respectively. Degranulation and cysteinyl leukotriene release were measured in the culture supernatants after 0.5 h incubation using a β-hexosaminidase assay or an enzyme immunoassay, respectively (A and B). IL-6 (C) and MCP-1 (D) release were measured in the supernatants 24 h after stimulation. Values are expressed as mean ± SEM from five independently conducted experiments performed in duplicate. *, p < 0.05 compared with IgE-sensitized mast cells (IgE). The level of phosphorylation of ERK1/2 (E) was analyzed in Wt BMMCs 10 min after stimulation by Western blotting (see Materials and Methods). Comparable results were obtained in two separate experiments.
FcεRI expression of Wt BMMCs. We noted similar FcεRI expression after 24 h of incubation with or without *M. sympodialis* extract (data not included). We further analyzed whether *M. sympodialis* would affect mast cell degranulation induced by aggregation of IgE receptors. IgE-anti-TNP-sensitized Wt BMMCs were therefore activated by addition of TNP-BSA along with increasing amounts of *M. sympodialis* extract, medium alone (IgE) as a negative control, or TNP-BSA (IgE/TNP) as a positive control, respectively. Degranulation was measured in the culture supernatants after a 0.5 h incubation using a β-hexosaminidase assay (A). IL-6 (B) and MCP-1 (C) release were measured in the supernatants 24 h after stimulation by ELISA. Values are expressed as mean ± SEM from five (A and B) to four (C) independently conducted experiments performed in duplicate. *, p < 0.05 compared with IgE-sensitized mast cells.

**FIGURE 4.** *M. sympodialis* extract enhances IgE-mediated degranulation of IgE receptor cross-linked BMMCs. IgE-anti-TNP-sensitized Wt (A), TLR4−/− (B), TLR2−/− (C), and MyD88−/− (D) BMMCs were incubated with TNP-BSA along with increasing amounts of *M. sympodialis* extract. Medium alone (IgE) served as a negative control and TNP-BSA (IgE/TNP) as a positive control. Degranulation of BMMCs was measured 0.5 h after stimulation using a β-hexosaminidase assay (A–D). Values are expressed as mean ± SEM of eight (A) to five (B–D) independently conducted experiments performed in duplicate. *, p < 0.05 compared with IgE/TNP-activated mast cells.
M. sympodialis enhancement of IL-6 secretion in IgE receptor cross-linked mast cells is dependent on the TLR2/MyD88 pathway

To address the question whether M. sympodialis could also influence the cytokine release of IgE receptor cross-linked mast cells, we investigated the effect of M. sympodialis extract on the secretion of the proinflammatory cytokine IL-6. Wt BMCCs pretreated with IgE-anti-TNP were coactivated with TNP-BSA and increasing amounts of M. sympodialis extract for 24 h, and the release of IL-6 was assessed by ELISA. M. sympodialis extract modified the production of IL-6 by IgE- and Ag-activated mast cells in a dose-dependent manner, whereby addition of low concentrations of M. sympodialis extract led to a significant increase in the IL-6 production and high concentrations led to a significant decrease (Fig. 5A).

Since cytokine production in mast cells has been shown to require MAPK signaling (17), we next studied whether M. sympodialis extract influenced activation of MAPK in IgE receptor cross-linked Wt BMCCs cultured with or without M. sympodialis extract. We determined that higher concentrations of extract inhibited phosphorylation of the MAPK ERK1/2 10 min after stimulation (Fig. 5B), which could reflect the observed inhibitory effect of high doses of M. sympodialis extract on IL-6 release from IgE receptor cross-linked mast cells (Fig. 5A).

A synergistic activation through FcεRI and either of TLR2 or TLR4 has been reported to facilitate IL-6 production in BMCCs (17), and we thus proceeded to investigate how M. sympodialis extract influenced IgE receptor cross-linked BMCCs from TLR2−/−, TLR4−/−, and MyD88−/− mice, respectively. Similarly to Wt BMCCs (Fig. 5A), the IL-6 release from TLR4−/− BMCCs was influenced by the addition of M. sympodialis extract, excluding LPS contamination of the extract (Fig. 5C). In contrast, M. sympodialis extract exerted no significant effect on IL-6 production in IgE receptor cross-linked BMCCs derived from TLR2−/− or MyD88−/− mice, respectively (Fig. 5, D and E), indicating a dependence on signaling through the TLR2/MyD88 pathway and a possible synergistic effect between TLR2 and FcεRI coactivation.

Discussion

We herein demonstrate that extract from the skin commensal yeast M. sympodialis activates nonsensitized, IgE-sensitized, and IgE receptor cross-linked mast cells. When culturing nonsensitized Wt BMCCs with M. sympodialis extract alone, the cells released cysteinyl leukotrienes, but we could not observe any activation in terms of degranulation, chemokine release, or cytokine release (Fig. 6A). We also determined that IgE-sensitized mast cells degranulate, release cysteinyl leukotrienes, and produce the chemokine MCP-1 upon addition of M. sympodialis extract (Fig. 6, B and C). Furthermore, our results indicate that M. sympodialis is able to activate IgE-sensitized mast cells through the MAPK pathway and

FIGURE 5. M. sympodialis modifies the IL-6 production of IgE receptor cross-linked BMCCs via the TLR2/MyD88 pathway and inhibits MAPK activation. IgE-anti-TNP-sensitized Wt BMCCs (A) were incubated with TNP-BSA along with increasing amounts of M. sympodialis extract. Medium alone (IgE) and TNP-BSA (IgE/TNP) served as negative and positive controls, respectively, and the release of IL-6 was measured after 24 h in the culture supernatants. Values are expressed as mean ± SEM of eight independently conducted experiments performed in duplicate. *, p < 0.05 compared with IgE/TNP-BSA (IgE/TNP)-activated mast cells. The level of phosphorylation of ERK1/2 (B) was analyzed by Western blotting (see Materials and Methods) in Wt BMCCs cocultured for 10 min as in A. Comparable results were obtained in two separate experiments. BMCCs obtained from TLR4−/− (C), TLR2−/− (D), and MyD88−/− (E) mice were cocultured with M. sympodialis extract as Wt BMCCs described above and the release of IL-6 was measured 24 h after stimulation. Values are expressed as mean ± SEM of five (C–E) independently conducted experiments performed in duplicate. *, p < 0.05 compared with IgE/TNP-BSA (IgE/TNP)-activated mast cells.
by signaling through the TLR2/MyD88 pathway can modify the IL-6 production of IgE receptor cross-linked mast cells.

The ability of *M. sympodialis* to induce an increase in β-hexosaminidase release could play a role in AE, since it has been reported that β-hexosaminidase is released by mast cells in combination with the serine protease tryptase (30). Tryptase can induce itching through the PAR-2 receptor, which has been shown to be involved during AE itch (31). An increase in MCP-1 release from IgE-sensitized mast cells could exacerbate inflammation in AE by recruiting monocytes (25) to the site of *M. sympodialis* invasion. The levels of cysteinyl leukotrienes released after coculture with *M. sympodialis* extract were increased in IgE-sensitized mast cells compared with in nonsensitized mast cells, indicating that the IgE sensitization increases the mast cells’ susceptibility to release cysteinyl leukotrienes in response to *M. sympodialis* activation. These findings concord with a study by Genovese et al. (32) which demonstrates that the interaction between IgE on mast cells and bacterial Ags results in an enhanced release of cysteinyl leukotrienes. Interestingly, enhanced releasability of cysteinyl leukotrienes upon activation has previously been reported in leukocytes from patients with AE compared with healthy controls (33). The observed Ag-independent activation of the mast cells indicates that some component of the *M. sympodialis* extract acts on IgE-anti-TNP-sensitized mast cells through what might be described as an “IgE-superantigen-like” effect. This is supported by the fact that mast cells can be activated independently of pathogen-specific Abs through the action of pathogen-derived Ig-binding proteins (16, 32). Several bacterial proteins have also been demonstrated to bind to different domains of FcεRI-bound IgE and thereby to act as IgE superantigens (34), two examples being *Staphylococcus aureus* protein A and *Peptostreptococcus magnus* protein L (34).

When the *M. sympodialis* extract was added to IgE receptor cross-linked mast cells, we observed that it increased the degranulation of Wt, TLR2−/−, TLR4−/−, and MyD88−/− mast cells in a dose-dependent manner. *M. sympodialis* thus acted as an amplifier of the IgE-activated mast cells by increasing the degranulation response through a TLR2−/−, TLR4−/−, and MyD88−/−-independent mechanism. To further elucidate the mechanism involved in the amplified degranulation of IgE receptor cross-linked BMMCs by *M. sympodialis*, we investigated the involvement of the fungal recognition receptor dectin-1. Olynych et al. (35) have previously demonstrated that the fungal product zymosan can induce dectin-1-dependent release of cysteinyl leukotrienes from mast cells. When IgE receptor cross-linked dectin-1−/− BMMCs (provided by Prof. Gordon D. Brown, University of Cape Town, Cape Town, South Africa) were cultured with increasing amounts of *M. sympodialis* extract, the enhanced degranulation was independent of dectin-1, as was the enhanced cysteinyl leukotriene production caused by *M. sympodialis* in nonsensitized and IgE-sensitized mast cells (data not included). Another explanation for the amplified degranulation of IgE receptor cross-linked BMMCs by the extract could be up-regulation of FcεRI expression since an earlier study by Kulka et al. (29) reported that *E. coli* can regulate FcεRI expression in mast cells. However, we observed no change in FcεRI expression following stimulation with *M. sympodialis* for 24 h, and therefore the increased degranulation observed in our study could not be due to enhanced FcεRI expression.

We also demonstrated that *M. sympodialis* extract affects IgE receptor cross-linked BMMCs to alter their IL-6 production in a dose-dependent manner by signaling through the TLR2/MyD88 pathway (Fig. 6, D–F), implying that cytokine release could be affected by a synergistic coactivation of the high-affinity IgE receptor and TLR2. A synergistic coactivation of TLR2 and FcεRI was previously reported by Qiao et al. (17) with TLR2 ligands substantially enhancing Ag-induced production of cytokines from

![Figure 6](http://www.jimmunol.org/DownloadedFrom/2013/04/23/17)

**FIGURE 6.** A schematic model for activation of mast cells by *M. sympodialis* extract. Nonsensitized mast cells release cysteinyl leukotrienes following activation with *M. sympodialis* extract (A). IgE-sensitized mast cells release cysteinyl leukotrienes, degranulate, and produce MCP-1 but not IL-6 upon addition of *M. sympodialis* extract, as indicated in bold letters (B and C). *M. sympodialis* extract enhances the degranulation of IgE receptor cross-linked mast cells by signaling through TLR2 and modifies their IL-6 production, as indicated in bold letters (D–F).
mast cells. Furthermore, the higher concentrations of M. sympodialis extract caused inhibition of phosphorylated-ERK1/2 in IgE receptor cross-linked mast cells, indicating that the modulation of the IL-6 release might be mediated through the ERK1/2 pathway. Our findings that M. sympodialis can activate mast cells via the TLR2/MyD88 pathway corroborate with the work of Baroni et al. (36) who showed that human keratinocytes increase their gene expression of TLR2 and MyD88 following activation with Malassezia furfur. In another study, suppressed degranulation was determined upon combined stimulation of BMMCs with increasing concentrations of the TLR2 ligand Pam3CSK4 and IgE receptor cross-linking (37). The mechanism underlying the suppression in that study was the electrostatic binding of DNP by Pam3CSK4, thereby reducing the amount of available Ag for IgE receptor cross-linking. Our findings demonstrate an inhibitory effect of M. sympodialis on IL-6 release from IgE receptor cross-linked BMMCs and this effect was fully abolished in TLR2- and MyD88-deficient BMMCs, thereby excluding a suppressive effect of the extract on TNP-BSA as Fehrenbach et al. (37) previously observed with Pam3CSK4 and DNP.

The observed difference in influence of M. sympodialis on mast cell IL-6 release and degranulation might be explained by considering what distinguishes the degranulation pathway from the pathway resulting in cytokine production in mast cells. An increase in cytosolic calcium levels is an essential signal for degranulation (17), whereas the generation of cytokines follows the activation of the MAPK pathway in murine mast cells (38). TLR ligands do not seem to influence the calcium levels or to cause degranulation (17), but they have been shown to stimulate the phosphorylation of MAPK (17). Our findings concur with this, since 1–100 μg/ml M. sympodialis extract could inhibit the phosphorylation of ERK1/2 in IgE receptor cross-linked BMMCs (Fig. 5B), which follows the observed TLR2/MyD88-dependent inhibition of IL-6 caused by M. sympodialis extract in IgE receptor cross-linked BMMCs.

The possible involvement of dectin-1 in the mechanisms of TLR2 in the response to M. sympodialis has to be considered. According to the proposed scheme of Medzhitov and Janeway, TLR2 and dectin-1 might cooperate in regulating the release of proinflammatory cytokines and chemokines, which would be relevant to the exacerbation of allergic inflammation. In in vitro assays, M. sympodialis (2, 14) and other members of the Malassezia genus (47) have been shown to stimulate mast cells in a TLR2-dependent manner. TLR2/MyD88-dependent cytokine release from IgE receptor cross-linked BMMCs was not affected by the addition of the dextrinase inhibitor 1-deoxylactosamine, which indicates that TLR2 engagement was sufficient to trigger cytokine release even in the absence of dextrin. The role of dextrin-1 in the response to M. sympodialis therefore remains to be fully elucidated.

In conclusion, our study demonstrates that extracts of M. sympodialis inhibit the IL-6 release from IgE receptor cross-linked BMMCs and this effect was fully abolished in TLR2- and MyD88-deficient BMMCs, thereby excluding a suppressive effect of the extract on TNP-BSA as Fehrenbach et al. (37) previously observed with Pam3CSK4 and DNP.

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Disclosures

The authors have no financial conflict of interest.

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