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TLR3-, TLR7-, and TLR9-Mediated Production of Proinflammatory Cytokines and Chemokines from Murine Connective Tissue Type Skin-Derived Mast Cells but Not from Bone Marrow-Derived Mast Cells

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Recent studies have revealed that murine bone marrow-derived cultured mast cells (BMMC), which are phenotypically immature mast cells, express functional TLR2 and TLR4 that recognize distinct pathogen-associated molecules. However, it remains relatively uncertain whether mast cells express other TLR. We recently established a method to obtain large numbers of murine fetal skin-derived cultured mast cells (FSMC); these cells exhibit important features of connective tissue type mast cells. Working with FSMC and BMMC, the TLR mRNA expression profiles were compared between both cell types. Although TLR2 and TLR4 mRNA were detected in both cells at comparable levels, TLR3, TLR7, and TLR9 mRNA were expressed by FSMC at higher levels than by BMMC, suggesting distinct TLR expression profiles among different mast cell populations. With respect to their functional aspects, FSMC, but not BMMC, dose dependently produced proinflammatory cytokines (TNF-α and IL-6) and chemokines (RANTES, MIP-1α, and MIP-2) in response to poly(I:C), R-848, and CpG oligodeoxynucleotide, which are TLR3, TLR7, and TLR9 activators, respectively. Interestingly, these TLR activators failed to induce degranulation and IL-13 production by both mast cells, although peptidoglycan and LPS (TLR2 and TLR4 activators, respectively) induced IL-13 production by both cells. Mast cells, thus, may have potential to recruit other immune cells to the infected sites by responding to various bacterial and viral components through TLR signaling pathways, presumably being involved in initiating innate immunity and subsequently linking innate and acquired immune responses. The Journal of Immunology, 2004, 173: 531–541.
and TLR9 recognize viral dsRNA (19), LPS (20), and unmethylated consensus DNA sequences, including the CpG motif in bacteria and viruses (21), respectively. Likewise, using TLR7-knockout mice, TLR7 has been recently demonstrated to recognize synthetic imidazoquinoline compounds, which are low m.w. immune response modifiers that have the potentials to serve as therapeutic agents against a variety of virus and parasites and as adjuvants in immunotherapies (22, 23). More recently, virus-derived single-stranded GU-rich RNA was identified as a natural ligand for murine TLR7 and human TLR8 (24, 25).

Recent studies revealed distinct TLR expression profiles among different types of cells, including dendritic cells (DC), macrophages, and lymphocytes (26, 27). The distinct immunological outcomes in acquired immunity have been well documented in response to different pathogens. For example, some viruses (e.g., Influenza virus) and bacteria (e.g., Mycobacterium tuberculosis) induce the differentiation of CD4+ T cells to Th1 cells, whereas some parasites (e.g., Schistosoma mansoni and Acanthocheilonema viteae) induce Th2 differentiation (28–30). However, the decision-making mechanisms that determine the type of immune response against a given pathogen are poorly understood. With this regard, a growing body of evidence indicates that these distinct outcomes evoked by different pathogens are thought to depend in part on the distinct TLR expression profiles among immune cells (31–36). Different subsets of DC and their maturation status have been reported to show distinct TLR expression profiles (37, 38). Recent studies have revealed that distinct TLR ligands instruct DC to induce Th1 or Th2 responses (33, 34, 39). For example, E. coli-derived LPS, flagellin, and CpG oligodeoxynucleotides (ODN), which trigger TLR4, TLR5, and TLR9, respectively, in- struct DC to stimulate Th1 responses through IL-12p70 (33, 34, 39, 40), whereas a Th2 activator, Pam3Cys, induces Th2 responses (33, 34, 39, 40). With respect to TLR expression profiles of mast cells, it has been demonstrated that TLR2 and TLR4 are expressed by murine BMMC and human cord blood-derived cultures of connective tissue type mast cells as shown by staining relatively large numbers of fetal skin-derived cultured mast cells (37, 38). However, the precise mechanisms of how TLR expression by peritoneal mast cells, crude peritoneal cells were obtained by peritoneal lavage with 10 ml of cold PBS plus 10% FBS from C57BL/6 mice.

RT-PCR

Recent studies revealed distinct TLR expression profiles among different types of cells, including dendritic cells (DC), macrophages, and lymphocytes (26, 27). The distinct immunological outcomes in acquired immunity have been well documented in response to different pathogens. For example, some viruses (e.g., Influenza virus) and bacteria (e.g., Mycobacterium tuberculosis) induce the differentiation of CD4+ T cells to Th1 cells, whereas some parasites (e.g., Schistosoma mansoni and Acanthocheilonema viteae) induce Th2 differentiation (28–30). However, the decision-making mechanisms that determine the type of immune response against a given pathogen are poorly understood. With this regard, a growing body of evidence indicates that these distinct outcomes evoked by different pathogens are thought to depend in part on the distinct TLR expression profiles among immune cells (31–36). Different subsets of DC and their maturation status have been reported to show distinct TLR expression profiles (37, 38). Recent studies have revealed that distinct TLR ligands instruct DC to induce Th1 or Th2 responses (33, 34, 39). For example, E. coli-derived LPS, flagellin, and CpG oligodeoxynucleotides (ODN), which trigger TLR4, TLR5, and TLR9, respectively, in- struct DC to stimulate Th1 responses through IL-12p70 (33, 34, 39, 40), whereas a Th2 activator, Pam3Cys, induces Th2 responses (33, 34, 39, 40). With respect to TLR expression profiles of mast cells, it has been demonstrated that TLR2 and TLR4 are expressed by murine BMMC and human cord blood-derived cultured mast cells (41–45). However, the detailed TLR expression profiles of mast cells remain relatively unclear. Particularly, the profiles of connective tissue type mast cells (CTMC) are totally unknown.

We have recently established a culture method that generates relatively large numbers of fetal skin-derived cultured mast cells (FSMC) at high purity (46). FSMC retain many characteristic features of connective tissue type mast cells as shown by staining patterns with Alcian blue, Safranin red, and berberine sulfate; higher histamine content in their granules than BMMC; and their reactivity to substance P and compound 48/80 (46). In this study, we examined and compared TLR expression profiles of FSMC and BMMC and determined functional responsiveness of these cells to each TLR activator. Our data indicated that there are distinct TLR expression profiles and functional responsiveness to the TLR activators between these two mast cell types.

Materials and Methods

Preparations of mast cells

All of the experimental procedures using mice were approved by the Institutional Review Board in Yamanashi University. FSMC were prepared from day 16 fetal skin of C57BL/6 mice (SLC Japan, Hamamatsu, Japan), as described previously (46). Briefly, single cell suspensions were prepared from excised trunk skin specimens by limited trypsinization with 0.25% Ca2+/Mg2+-free Hank’s balanced salt solution (HBSS) containing Ca2+ (Invitrogen, Carlsbad, CA) for 20 min at 37°C. The crude cells (5 × 107 cells/ml) were cultured in the presence of murine rIL-3 (10 ng/ml) and recombinant stem cell factor (10 ng/ml) (PeproTech, Boston, MA). RPMI 1640 (Invitrogen) supplemented with heat-inactivated 10% FCS (Sigma-Aldrich, St. Louis, MO), 2 mM l-glutamine, 10 mM nonessential amino acids, 1× penicillin/streptomycin, 10 mM sodium pyruvate, 25 mM HEPES, and 50 μM 2-ME served as the growth medium (complete RPMI 1640). Nonadherent cells and loosely adherent cells were collected 14 days after this initial culture, and enriched for mast cells by density gradient centrifugation in 1.0519 g/ml Percoll (Amersham Life Science). The resulting mast cell preparations containing >95% CD117+ cells were used as FSMC without further purification. These cells stained red with Alcian blue and Safranin red, indicating the predominance of heparin, which is characteristic of CTMC (data not shown). BMMC were prepared from bone marrow cell suspensions from C57BL/6 mice, as described previously (46). Briefly, crude bone marrow cells (4 × 107 cells/ml) were cultured in complete RPMI 1640 in the presence of murine rIL-3 (10 ng/ml) and recombinant stem cell factor (10 ng/ml). Nonadherent cells were recovered twice per week, and further expanded in fresh medium for 4 wk. The resulting cell preparations, containing >95% CD45+ CD117+ cells, were used as BMMC without further purification. In contrast to FSMC, these mast cells stained blue with Alcian blue and Safranin red, indicating the predominance of chondroitin. These cells stained red after this initial culture, and enriched for mast cells by density gradient centrifugation. These cells were then expanded in fresh medium for 6 wk. This culture method, BMMC without further purification. In contrast to FSMC, these mast cells stained blue with Alcian blue and Safranin red, indicating the predominance of chondroitin. These cells stained red after this initial culture, and enriched for mast cells by density gradient centrifugation. These cells were then expanded in fresh medium for 6 wk.
FIGURE 1. TLR mRNA expression profiles of FSMC and BMMC. Total RNA isolated from FSMC and BMMC was analyzed by RT-PCR for TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, and TLR9 mRNA expression. Equality of the reverse-transcription reaction of isolated RNA between samples was confirmed by amplification of β-actin. PCR products were visualized by staining with ethidium bromide. Data are representative of three independent experiments.

FIGURE 2. Relative comparison of TLR gene expression between FSMC and BMMC. mRNA purified from FSMC and BMMC was subjected to quantitative real-time RT-PCR using SYBR Green I for the indicated TLR. The relative gene expression levels were determined in triplicate by differences of Ct numbers, as described in Materials and Methods. Data are representative of three independent experiments.

anti-CD49b mAb (BD Pharmingen). After washing cells twice with staining buffer, the cells were fixed for 1 h with 2% paraformaldehyde in PBS and subsequently permeabilized with 0.3% saponin in PBS. For TLR4 staining, the cells were then stained with FITC-conjugated TLR4 mAb (MBL, Nagoya, Japan) or control mAb. For TLR9 staining, the cells were incubated with biotinylated anti-TLR9 mAb (Hycult Biotechnology, Uden, the Netherlands) or control mAb. For TLR9 staining, the cells were first incubated for 18 h with 0.3 μg/ml mouse IgE anti-DNP mAb (clone SPE7) (Sigma-Aldrich) depleted of dimeric or other complex forms of IgE by centrifugation at 20,000 × g for 45 min. These cells were washed three times, and then stimulated with the indicated concentrations of DNP-human serum albumin (DNP-HSA) (Sigma-Aldrich) for an additional 24 h. To study the requirement of endosomal acidification in TLR signaling, the cells were pretreated for 2 h with 100 nM bafilomycin A1 (Sigma-Aldrich), which is a specific inhibitor of the V-type ATPase responsible for the acidification of endosomes and lysosomes (48, 49), before stimulating the cells with TLR activators. The culture supernatants were collected after centrifugation, and stored at −80°C for cytokine and chemokine measurements. The concentrations of cytokines (TNF-α, IL-6, and IL-13) and chemokines (MIP-1α, MIP-2, and RANTES) in the culture supernatants were measured using Quantikine ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

Degranulation assay

Mast cells were plated at 2 × 10⁶ cells/100 μl for FSMC or 1 × 10⁶ cells/100 μl for BMMC in Tyrode’s buffer (Sigma-Aldrich) in triplicate into 96-well U-bottom plates and allowed to equilibrate for 10 min at 37°C before the addition of CpG ODN, non-CpG ODN, poly(I:C), R-848, LPS, or ionomycin at graded concentrations, or the addition of 100 μg/ml PGN, zymosan (the yeast cell wall component), Pam3Cys (synthetic lipopeptide), or lipoteic acid (LTA). Zymosan, Pam3Cys, and LTA were purchased from InvivoGen. Thirty minutes after stimulating the cells with these reagents, the plate was spun at 290 × g for 5 min at 4°C. To determine the degranulation of FSMC and BMMC by FcεRI aggregation, these cells were then incubated with mouse IgE anti-DNP mAb (0.3 μg/ml) for 18 h. The cells were washed three times with Tyrode’s buffer, and then stimulated with the indicated concentrations of DNP-HSA for 30 min. β-hexosaminidase activity of the culture supernatants was determined, as described previously (46). Briefly, aliquots (50 μl) of the supernatants were transferred in new plates together with 100 μl of 2.5 mM p-nitrophenyl-N-acetyl β-D glucosaminide (Sigma-Aldrich) solubilized in 0.04 M citrate buffer adjusted with disodium phosphate to pH 4.5. After incubation for 90 min at 37°C, the reactions were terminated by the addition of 50 μl of 0.4 M glycine, adjusted with sodium hydroxide to pH 10.7. The colored products were measured using an ELISA plate reader (Molecular Devices, Sunnyvale, CA).
CA) at 405 nm using a reference filter of 570 nm. The percentage of /H9252-hexosaminidase release was determined as: (the ratio of activities in the supernatant of tested cells and in the frozen-thawed lysate) /H11003 100.

Results
Comparison of TLR mRNA expression profiles between FSMC and BMMC
As described in the introduction, murine BMMC have been reported to express several different TLR mRNA, including TLR2 and TLR4 (41, 42). We first compared TLR mRNA expression profiles between FSMC and BMMC using RT-PCR. FSMC strongly expressed TLR1, TLR3, and TLR7 mRNA, and modestly expressed TLR9 mRNA, whereas BMMC reproducibly showed only marginal expression of these TLR mRNA. In contrast, both FSMC and BMMC expressed TLR2, TLR4, and TLR6 at comparable levels. TLR5 signals were undetectable in either FSMC or BMMC. These results indicated that FSMC exhibited distinct TLR mRNA expression profiles compared with BMMC (Fig. 1).

Relative comparison of TLR gene expression between FSMC and BMMC
We then compared the relative gene expression levels of each TLR between FSMC and BMMC using real-time PCR. As shown in Fig. 2, FSMC expressed higher levels (>10 times) of TLR3 (10.2-fold), TLR7 (183-fold), and TLR9 (60-fold) mRNA than BMMC, corresponding to the results observed by conventional RT-PCR (Fig. 1). FSMC also expressed modestly higher levels of TLR2 (4.7-fold) and TLR4 (4.5-fold), and lower levels of TLR6 (0.4-fold) compared with BMMC. These results quantitatively supported our visual estimation of the expression levels observed in Fig. 1.

Flow cytometry analyses of TLR4 and TLR9 expression by mast cells
Currently, it is still technically challenging to detect TLR at the protein level among relatively rare cell populations in a given tissue because of the lack of appropriate Abs against TLR. Among the currently available Abs against TLR, TLR4 and TLR9 have

![FIGURE 3. TLR4 and TLR9 expression by PMC. A, Crude peritoneal cells were stained with allophycocyanin-conjugated anti-CD117 mAb and PE-conjugated anti-CD49b mAb. B, Crude peritoneal cells were first stained with allophycocyanin-conjugated anti-CD117. After fixation and permeabilization, the cells were stained with anti-TLR4 mAb or anti-TLR9 mAb. CD117<sup>-</sup>-gated cells in crude peritoneal cells were analyzed for TLR expression (filled histograms). Background staining profiles with an isotype-matched control IgG are shown with open histograms. FSMC and BMMC were similarly stained with these mAbs, as described above.](http://www.jimmunol.org/)

![FIGURE 4. Proinflammatory cytokine and chemokine production by mast cells in response to a TLR4 activator, LPS. FSMC and BMMC were stimulated for 24 h with LPS at the indicated concentrations. Culture supernatants were tested for the indicated cytokines (A) and chemokines (B) by ELISA. Data are representative of two independent experiments, showing the means ± SD from triplicate cultures.](http://www.jimmunol.org/)
been successfully detected by FACS analyses using the respective Ab (50, 51). Because TLR expression by freshly isolated mast cells or tissue-resident mast cells has not been reported, we sought to examine TLR4 and TLR9 expression by PMC (considered as CTMC) in crude peritoneal cells as well as by FSMC and BMMC. As shown in Fig. 3A, 3–4% of crude peritoneal cells expressed both CD117 and CD49b, and these double-positive cells were considered as PMC, as previously reported by us and others (46, 52). As shown in Fig. 3f, PMC and FSMC expressed both TLR4 and TLR9, whereas BMMC expressed TLR4, but only marginally, if any, TLR9. Obviously, we need to examine TLR9 expression by the other CTMC to know whether TLR9 is preferentially expressed in CTMC.

**Proinflammatory cytokine and chemokine production by FSMC and BMMC in response to a TLR4 activator**

Murine BMMC have been previously reported to produce proinflammatory cytokines (i.e., IL-6 and TNF-α) in response to LPS (42). Because TLR4 expression by FSMC was comparable to that of BMMC (Figs. 1–3), we examined whether LPS (a TLR4 ligand) would induce the secretion of proinflammatory cytokines and chemokines from FSMC as well as from BMMC. BMMC produced TNF-α and IL-6 in response to LPS in a dose-dependent manner, corresponding to a previous report (42) (Fig. 4A). FSMC also produced these cytokines in similar manners, compared with BMMC. Similarly, LPS induced the secretion of MIP-1α and MIP-2 from both FSMC and BMMC in a dose-dependent manner (Fig. 4B). Thus, both FSMC and BMMC similarly produced proinflammatory cytokines and chemokines in response to a TLR4 activator. These results may reflect comparable expression levels of TLR4 by both FSMC and BMMC.

**Proinflammatory cytokine production by FSMC in response to TLR3, TLR7, and TLR9 activators**

Because we could confirm relatively specific mRNA expression of TLR3, TLR7, and TLR9 by FSMC, we decided to focus on the functional responsiveness to these three TLR. We tested the following TLR activators: 1) poly(I:C), a synthetic analog for viral dsRNA for a TLR3 activator; 2) R-848, an imidazoquinoline compound for a TLR7 activator; and 3) CpG ODN, an unmethylated synthetic ODN containing CpG motifs for a TLR9 activator. As shown in Fig. 5, A and B, FSMC produced both TNF-α and IL-6 in response to poly(I:C) and R-848 in a dose-dependent manner. In contrast, BMMC hardly responded to these activators, although marginal secretion was detected at the highest tested concentrations. Likewise, FSMC, but not BMMC, potently produced TNF-α and IL-6 in response to CpG ODN in a dose-dependent manner (Fig. 5C). In contrast, FSMC did not produce these cytokines in response to non-CpG ODN, indicating the specificity to CpG sequence motifs. Thus, FSMC have the capability to respond to TLR3, TLR7, and TLR9 activators, whereas BMMC only marginally respond to these activators.

**Chemokine production by FSMC in response to TLR3, TLR7, and TLR9 activators**

TLR-mediated signaling is known to stimulate many immune cells to produce a wide variety of chemokines, which attract various immune cells to the inflammation sites (53). Although we observed that LPS induced MIP-1α and MIP-2 production by both mast cells (Fig. 4), it remains undetermined whether mast cells produce chemokines in response to other TLR activators. Therefore, we examined whether FSMC and BMMC would produce chemokines in response to TLR3, TLR7, and TLR9 activators. As shown in Fig. 6, FSMC produced MIP-1α, MIP-2, and RANTES in response to poly(I:C), R-848, and CpG ODN in a dose-dependent manner, suggesting that FSMC have the potential to produce these chemokines by specific recognition of bacterial and viral nucleic acid components. In contrast, BMMC hardly responded to these activators, although these chemokines were slightly secreted at the highest tested concentrations.

**Effects of an endosomal acidification inhibitor on FSMC activation via TLR3, TLR4, TLR7, and TLR9 signaling**

Several recent studies have demonstrated that inhibitors of endosomal maturation (acidification), such as chloroquine and bafilomycin A1, inhibited TLR3-, TLR7-, and TLR9-mediated signaling
pathways, indicating that these signaling pathways require acidification and maturation of endosomes (51, 54, 55). Thus, we examined whether bafilomycin A1 would inhibit FSMC activation induced by TLR3, TLR7, and TLR9 activators. As shown in Fig. 7A, pretreatment of FSMC with bafilomycin A1 completely inhibited IL-6 production induced by poly(I:C), R-848, and CpG ODN to the baseline levels, without affecting cell viability. These results indicate that TLR3, TLR7, and TLR9 signaling pathways in FSMC require endosomal maturation. In contrast, the bafilomycin A1 pretreatment had no effect on LPS-induced IL-6 production (Fig. 7A), supporting a previous report that TLR4 signaling does not require the endosomal maturation (51). Likewise, MIP-2 production induced by poly(I:C), R-848, and CpG ODN was significantly inhibited by pretreatment with bafilomycin A1, whereas this pretreatment had no effect on LPS-induced MIP-2 production (Fig. 7B). Thus, TLR3-, TLR7-, and TLR9-mediated, but not TLR4-mediated signaling pathways require endosomal maturation in FSMC.

Effects of TLR activators on degranulation by mast cells

One of the functional hallmarks of mast cells is the degranulation-mediated immediate release of chemical mediators in response to FccRI aggregation and other stimuli (e.g., calcium ionophores). With respect to TLR-mediated mast cell degranulation, PGN (a TLR2 activator)-mediated mast cell degranulation remains controversial. Supajatura et al. (43) previously reported PGN-mediated degranulation by BMMC (43). Ikeda and Funaba (56), however, failed to induce degranulation by BMMC in response to PGN. In addition, the stimulation of mast cells with LPS reportedly failed to induce the degranulation while secreting cytokines (41, 42). It remains unknown whether other TLR signaling would induce mast cell degranulation. Therefore, we examined whether TLR3, TLR7, and TLR9 activators would induce degranulation by FSMC and BMMC with the \( \text{H}^+ \)-hexosaminidase assay. As shown in Fig. 8A, poly(I:C), R-848, and CpG ODN failed to induce degranulation by both FSMC and BMMC, whereas FccRI aggregation and ionomycin induced dose-dependent degranulation by both cell types. We next examined the effects of several TLR2 activators (PGN, zymosan, Pam\(_3\)Cys, and LTA) on degranulation. As shown in Fig. 8B, degranulation by both mast cells was undetectable in response to all the tested TLR2 activators. In contrast, ionomycin again induced degranulation by both cells.

Effects of TLR activators on IL-13 production by mast cells

Mast cells are known to produce IL-13 by FccRI aggregation and in response to other stimuli, including calcium ionophores (46, 57). In addition, BMMC are capable of producing IL-13 in response to LPS (42, 57). Thus, we next examined the effects of various TLR activators on IL-13 production by both mast cell types. As shown in Fig. 9A, poly(I:C), R-848, and CpG ODN failed to induce any
significant IL-13 production by both mast cell types, whereas imo-
mycin induced IL-13 production by both cell types. In contrast, 
PGN, LPS, and FcεRI aggregation potently induced IL-13 produc-
tion by both cells (Fig. 9B).

Discussion

Working with FSMC, which have characteristic features of CTMC, we have made two major findings in this study. First, we 
have observed that FSMC express TLR3, TLR7, and TLR9 mRNA, and produce proinflammatory cytokines and chemokines in response to these TLR activators without their degranulation. In contrast, BMMC, which retain immature mast cell phenotypes, only marginally express TLR3, TLR7, and TLR9 mRNA and fail to produce significant amounts of proinflammatory cytokines and 
chemokines in response to these TLR activators. To our knowl-
edge, our study is the first report documenting functional TLR3, 
TLR7, and TLR9 expression by mast cells. Second, PGN and LPS 
induce Th2 cytokine (i.e., IL-13) production by both mast cells, 
whereas the other TLR activators cannot induce this cytokine pro-
duction by either mast cell type.

As described in the introduction, mast cell-derived TNF-α plays 
crucial roles in the influx of neutrophils into sites infected with 
bacteria. Similarly, mast cell-derived MIP-2 has been reportedly 
produced in infected sites and caused the infiltration of neutrophils 
(58). Thus, mast cell-derived cytokines and chemokines are im-
portant mediators in host defense against bacteria. In this study, it 
was observed that FSMC produced cytokines (TNF-α and IL-6) 
and chemokines (MIP-2, MIP-1α, and RANTES) in response to
various TLR activators (poly(I:C), LPS, R-848, and CpG ODN) (Figs. 4–6). Because TLR3, TLR7, and TLR9 are now known to recognize nucleic acids derived from bacteria and viruses, these results suggest that mast cells secrete these mediators not only in response to bacteria-derived PAMPs, but also in response to virus-derived PAMPs. Although the involvement of mast cells in host defense against viral infection has not been evaluated in in vivo animal models, it is tempting to speculate that mast cells may be involved in innate immunity against a wide range of pathogens, including viruses.

Chemokines have been considered as important mediators linking the innate and acquired immune responses by attracting immune cells (e.g., DC and T cells) in acquired immunity (53). For example, MIP-1α and RANTES induce chemotaxis of immature DC and Th1 cells mediated by CCR5 (53, 59). We have found that FSMC potently produced chemokines (e.g., MIP-1α, RANTES, and MIP-2) in response to poly(I:C) and CpG ODN, indicating that bacterial and viral gene products are capable of activating mast cells to produce these chemokines through TLR signaling pathways. Therefore, it is conceivable that pathogens can activate

**FIGURE 8.** Effects of TLR activators on degranulation by mast cells. A, FSMC and BMMC were incubated for 30 min with the indicated TLR activators, or ionomycin at the indicated concentrations. Likewise, the cells pretreated for 18 h with mouse IgE anti-DNP mAb were stimulated for 30 min with the indicated concentrations of DNP-HSA. Culture supernatants were tested for degranulation by β-hexosaminidase release assay. Data are representative of three independent experiments, showing the means ± SD from triplicate cultures. B, FSMC and BMMC were incubated for 30 min with 100 µg/ml PGN, zymosan, Pam3Cys, LTA, or 1 µg/ml ionomycin, and then examined for degranulation, as described above.
tissue-resident mast cells (presumably together with the other resident cells) through TLR-mediated signaling to produce chemokines, which may, in turn, attract a variety of immune cells in acquired immune responses.

A wide range of chemical mediators (released by mast cell degranulation) and Th2 cytokines (secreted by mast cells) play important roles in type I allergic responses (60). Recent studies suggest that LPS or endotoxin is one of the aggravating factors in bronchial inflammation and hypersensitivity in asthma (61–64), and that mast cell-derived Th2 cytokines (e.g., IL-13) are involved at least in part in the pathogenesis of this disease (65–70). Indeed, LPS stimulates BMMC to produce IL-13 and to further enhance this cytokine production induced by FcεRI aggregation (57). This phenomenon was mediated by TLR4, as shown by the fact that LPS did not induce Th2 cytokine production from BMMC derived from TLR4 gene-deficient mice (C3H/HeJ and C57BL10ScCr) (57). Consistent with these observations, we found that PGN, LPS, and FcεRI aggregation induced IL-13 production by FSMC as well as by BMMC (Fig. 9). In contrast, the other tested TLR activators failed to induce this cytokine. These results show the contrasting impacts of TLR on cytokine production by mast cells. Further studies are needed to find whether the effects of TLR-mediated signals via a given pathogen on DC and tissue-resident mast cells cooperatively determine the type of acquired immune responses.

Zhu and Marshall (71) have demonstrated that CpG ODN induced both TNF-α and IL-6 production by BMMC. Their observations are inconsistent with our data showing the unresponsiveness of BMMC to CpG ODN. The reasons for this discrepancy remain to be determined. However, it may be explained by different culture conditions, because some stimuli (e.g., endothelin-1) trigger the degranulation by BMMC propagated in growth medium containing WEHI-3 cell-conditioned medium, but not by BMMC propagated in medium containing rIL-3 (72). It has been suggested that IL-4 existing in WEHI-3 cell-conditioned medium serves as a maturation factor for mast cells (72). Because their and our BMMC were propagated in the presence of WEHI-3 cell-conditioned medium or rIL-3, respectively, these different culture conditions may influence the expression levels of TLR9 and the responsiveness of BMMC to CpG ODN. Alternatively, a relatively higher concentration of CpG ODN (>20 μM) was used to examine the cytokine secretion from BMMC, whereas we tested the relatively lower concentrations (<1 μM). Therefore, FSMC may be more sensitive to respond to CpG ODN than BMMC.

Imidazoquinoline compounds (e.g., R-848 and imiquimod) have been reported to exhibit potent antiviral and antitumor activities (23, 73). In fact, imiquimod has been used for the treatment of genital warts (23, 73). A growing body of evidence indicates that imiquimod activates several immune cells (e.g., monocytes, macrophages, and DC) to produce a wide array of cytokines and chemokines (e.g., IFN-α, TNF-α, IL-12, MIP-1α, MIP-1β, and MCP) (22, 74–77). These factors, in turn, are thought to contribute to these antiviral and antitumor effects. Recently, Hemmi et al. (22) have discovered that imidazoquinoline compounds activate immune cells through the TLR7-mediated signaling pathway. We found in this study that FSMC potently produce proinflammatory cytokines and chemokines in response to R-848. Interestingly, R-848 was a stronger (4–10 times) inducer for these cytokines and chemokines than poly(I:C) and CpG ODN (Figs. 5 and 6). To our knowledge, this is the first report documenting the effects of imidazoquinoline compounds on mast cells. In addition to macrophages and DC, mast cells, thus, may be one of the target cells in antiviral and antitumor activities by imidazoquinoline compounds. We have also observed that R-848 did not induce degranulation and IL-13 production by FSMC. Recent observations have indicated that R-848 inhibited IgE production by peripheral mononuclear cells derived from nonallergic and allergic donors (78), and shifted allergen-specific CD4+ Th2 lymphocytes into IFN-γ-producing Th1 cells (79). These findings suggest that imidazoquinoline compounds may modulate immunity from Th2 to Th1 responses. Therefore, our observation that R-848 had no effect on degranulation and IL-13 production by FSMC with elaborating the other cytokines and chemokines, may provide another putative mechanism for Th1 responses induced by these compounds.

It is technically challenging to freshly isolate sufficient quantities of mast cells for in vitro investigations from murine adult skin because of limited number of mast cells in skin specimens and unhealthy cell conditions after repeated enzymatic treatments. For these reasons, functional differences between skin-derived mast cells and the other mast cell populations have not been well understood. With this regard, our culture system to obtain large numbers of FSMC in high purity enabled us to examine the expression and functions of TLR in one type of CTMC together with conventional BMMC. Although we found TLR4 and TLR9 expression by PMC (another CTMC), it will be essential to determine the complete TLR expression profiles of PMC or skin-associated mast cells in vivo to determine whether the profiles of FSMC would really represent those of CTMC or not. Nevertheless, we believe that FSMC will provide a useful tool to define the physiological roles of CTMC-associated TLR.

In summary, we demonstrated distinct TLR expression profiles between FSMC and BMMC. Reflecting the differences, we observed the contrasting impacts of TLR activators on these two mast cell types. Our data support the current concept that mast cells play important roles not only in allergic responses, but also in innate
immune responses through TLR-mediated signaling pathways. Moreover, upon bacterial and viral infections, mast cells may enhance local infiltration of a variety of immune cells by elaborating proinflammatory cytokines and chemokines, and may be involved in the transition of innate immune responses to acquired immune responses.

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References


