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Cutting Edge: Distinct Toll-Like Receptor 2 Activators Selectively Induce Different Classes of Mediator Production from Human Mast Cells1,2

Jeffrey D. McCurdy,* Timothy J. Olynych,* Lauren H. Maher,* and Jean S. Marshall3*†

Mast cells play a critical role in host defense against bacterial infection. Murine mast cells produce cytokines in response to bacterial peptidoglycan and LPS via Toll-like receptor (TLR) TLR2- and TLR4-dependent mechanisms. The expression of TLRs by human mast cells and responses to known TLR activators was examined. Human mast cells expressed mRNA for TLR1, TLR2, and TLR6 but not TLR4. Bacterial peptidoglycan and yeast zymosan were potent inducers of GM-CSF and IL-1β and also induced substantial short-term cysteinyl leukotriene generation. In contrast, a synthetic triacylated lipopeptide induced short-term degranulation but failed to induce cysteinyl leukotriene production. The TLR4 activator Escherichia coli LPS did not induce a GM-CSF, IL-1β leukotriene, or degranulation response. These data demonstrate highly selective production of different classes of mast cell mediators in response to distinct TLR activators of potential importance to the host response to bacterial or fungal pathogens. The Journal of Immunology, 2003, 170: 1625–1629.

Mast cells have long been known to contribute to allergic diseases and chronic inflammatory processes and, more recently, have been shown to play an important role in host defense against bacterial infections (1–4). Mast cells are well equipped to participate in these reactions based on their strategic tissue distribution and ability to selectively produce a wide range of proinflammatory mediators. Mast cells are a potent source of preformed, granule-associated mediators, newly synthesized lipid mediators, as well as a large number of cytokines and chemokines. Human mast cells produce the NF-κB-regulated cytokines, GM-CSF and IL-1β, which have been implicated in the mobilization, recruitment, and enhanced survival of effector cells, such as neutrophils, at the site of infections (5). Cysteinyl leukotriene production by mast cells is of particular interest because of their potent bronchoconstrictive and chemotactic effects (6).

Toll-like receptors (TLR)4 are a family of pattern recognition receptors known to play an important role in host defense (reviewed in Ref. 7): TLR2 is critical for responses to bacterial-derived lipopeptides (8–10) and peptidoglycan (PGN) (11, 12) as well as zymosan (13), the cell wall component of yeast, while TLR4 is the major signaling molecule for most types of LPS (7). Ligand specificity for a number of TLR2 activators is thought to require heterodimerization with additional TLR molecules, TLR1 and TLR6 (14, 15). Studies suggest that TLR2/TLR6 heterodimers mediate responses to PGN or zymosan, while TLR1/TLR2 heterodimers mediate responses to the synthetic tripalmitoyl lipopeptide Pam3Cys-Ser-(Lys)4 (Pam3Cys) in the mouse (14, 15). Distinct TLR molecules are capable of inducing differential cellular responses (16, 17). Rodent mast cells as well as murine mast cell lines produce several cytokines, including IL-6 and TNF-α, in response to activation with the TLR activators LPS and PGN (18–21). However, human mast cell responses to TLR activators are poorly defined.

In the current study, human mast cells are demonstrated to express a distinct profile of TLRs compared with murine mast cells and other leukocytes. Differences in TLR expression are reflected by selective mast cell stimulation by TLR activators. Furthermore, distinct TLR2 activators are shown to induce differential mediator production including, the novel selective induction of substantial cysteinyl leukotriene generation and cytokine production without evidence of classical degranulation.

Materials and Methods

Reagents

Escherichia coli LPS (serotype O55:B5), zymosan from Saccharomyces cerevisiae, and PGN, purified from Staphylococcus aureus, were purchased from Sigma (St. Louis, MO). S. aureus PGN contained <0.0025 ng/μl endotoxin as assessed using the Limulus amoebocyte lysate test from Sigma. Synthetic lipopeptide (Pam3Cys Ser-Lys4) was obtained from Prof. Dr. G. Jung University of Tubingen (Tubingen, Germany).

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7 Abbreviations used in this paper: TLR, Toll-like receptor; PGN, peptidoglycan; CBMC, cord blood-derived mast cell; MyD88, myeloid differentiation factor 88; BMMC, bone marrow-derived mast cell.
Mast cells

devoid of PGE2 for cells expressed c-kit

After a 20-min incubation, supernatants and pellet fractions were each analyzed with 1% FBS as a source of soluble CD14 and LPS-binding protein, were activated with the positive control A23187 (0.5 mM) or various TLR activators.

Cytokine analysis

IL-1β, IL-6, RANTES, and GM-CSF activation with various TLR activators were examined in supernatants harvested after 24 h, from CBMC cells following activation with various TLR activators. Cytokines were also analyzed by flow cytometry for the mast cell marker c-kit and the monocytic marker CD14. Less than 2% of the cells expressed CD14 (H11005). Cytokines were analyzed via "ELISA as previously described (22). Sensitivity of the IL-6 and IL-1β assay was 1.95 pg/ml, that of RANTES was 30 pg/ml, and that of GM-CSF was 3 pg/ml.

RT-PCR

Total RNA was isolated using TRIzol reagent (Life Technologies). RNA samples were treated with DNase I (Life Technologies). Primers used for PCR amplification of reverse-transcribed RNA samples for MD-2, myeloid differentiation factor 88 (MyD88), β-actin, TLR1, TLR2, TLR4, and TLR6 were purchased from Research Genetics (Huntsville, AL), and sequences were as follows: 1) human MD-2: sense, 5'-GGGACCTGATCCGATGCAAGT-3'; antisense, 5'-GGTGGTTTAGCAGTCTCTC-3'; 2) MyD88: sense, 5'-GGGGAGATGGACTTTGAGT-3'; antisense, 5'-GCCAAA GTGCTGATTTGAGT-3'; antisense, 5'-GCCAACAGCAAGCAAGACA-3'; 3) TLR1: sense, 5'-ATTCCATGCTCTTGAGG-3'; anti sense, 5'-GATCCACTAGCTGAGTG-3'; 4) TLR2: sense, 5'-GCCAAA GTGCTGATTTGAGT-3'; antisense, 5'-TGAGTGTCTCTCGCTCTG-3'; 5) TLR4: sense, 5'-TGATAGCTCTTCTCTTAAAG-3'; antisense, 5'-GAAATGGAGGCACCCCTTC-3'; 6) TLR6: sense, 5'-ATCGAAGAC TGACCAAGAGTCT-3'; antisense, 5'-GATGAGGACACAGCATCGTTG-3'. Thirty-four PCR cycles were used.

Short-term mediator release and β-hexosaminidase assay

CBMC (0.5 × 10^6/ml) in modified HEPES-Tyrode's buffer, supplemented with 1% FBS as a source of soluble CD14 and LPS-binding protein, were activated with the positive control A23187 (0.5 mM) or various TLR activators. After a 20-min incubation, supernatants and pellet fractions were each analyzed for β-hexosaminidase according to the method of Schwartz et al. (25).

Cysteinyl leukotriene assay

CBMC (1 × 10^6/ml) were incubated for a predetermined optimal time of 20 min at 37°C with activating agents or controls in RPMI 1640 medium supplemented with 1% FBS and 10 ng/ml stem cell factor. Supernatants were harvested by centrifugation, snap frozen, and later analyzed by an enzyme immunoassay using a commercial kit (Amersham, USA). Sensitivity of the assay was <30 pg/ml.

Immunohistochemistry

Nasal polyposis tissues were stored, after informed consent, from subjects requiring routine polypectomy. Only tissues from subjects who had not received topical steroid therapy in the 3 wk before surgery were used. Frozen tissue sections (5 μm) were stained with Alcian blue (pH 1.0). Endogenous peroxidase activity was quenched by treatment with 0.5% H₂O₂. Slides were then blocked with normal human serum and stained using anti-TLR2 polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) or normal goat IgG at the same dilution (DAKO, Carpinteria, CA). Ab binding was detected using biotinylated swine anti-goat Ab (Cedarlane Laboratories, Hornby, Ontario, Canada) and a commercial development system.

Statistics

Comparison of data sets was performed using ANOVA and Dunnett's post-test.

Results and Discussion

TLR mRNA expression by CBMC

It has been previously reported that murine mast cells express mRNA for a number of TLRs including: TLR2, TLR4, TLR6, and TLR8 (18, 20). To determine the human mast cell expression of TLRs, CBMC and the mast cell/basophil line KU812 were analyzed for TLR mRNA expression by RT-PCR. Prominent PCR products of the appropriate size were expressed for TLR1, TLR2, and TLR6 but not TLR4 by both CBMC and KU812 (Fig. 1A). In comparison, the human macrophage cell line U937 expressed bands for all TLRs examined, including TLR4. All cells examined expressed mRNA for the adapter molecules MyD88 and MD-2. Immunohistochemical analysis was used to assess the protein expression of TLR2 by human tissue mast cells. The majority of nasal polyp human mast cells, staining positive with Alcian blue, at low pH, also expressed low levels of TLR2 (Fig. 1, B and C). TLR2-positive mast cells were also observed in human lung tissue (data not shown). The staining pattern for TLR2 was consistent with surface expression on mast cells.

These data demonstrate that human mast cells express a unique profile of TLRs including TLR2 and TLR6, which may suggest a specialized role for in host defense. The lack of TLR4...
expression provides an additional marker to distinguish human mast cells from other myeloid cells.

**Selective stimulation of CBMC with TLR2 activators**

The responses of human mast cells to a number of classic TLR activators were examined. In initial experiments, CBMC (≥95% pure) were stimulated with various TLR activators including: *S. aureus* PGN or *E. coli* LPS. When stimulated with the TLR2 activator *S. aureus* PGN (11, 12) for 24 h, CBMC produced significant levels of GM-CSF (Fig. 2A) and IL-1β (Fig. 2B) in a dose-dependent manner. Levels of GM-CSF were 17- to 85-fold higher than unstimulated control cells when CBMC were stimulated with 10 µg/ml PGN (n = 15 CBMC donors). PGN induced substantially higher levels of GM-CSF than IgE-mediated activation (PGN, 5075 ± 1260% medium control n = 4 and IgE mediated, 350 ± 191% med control n = 4). These cytokines were not detected in sonicated CBMC samples or in the supernatant following 30-min activation with PGN, suggesting that CBMC do not contain or release substantial preformed stores (data not shown). Levels of GM-CSF remained low after a 6-h incubation (41 ± 4.9 pg/ml, n = 2) and increased in a time-dependent manner with 430 ± 39 pg/ml (n = 2) GM-CSF produced following a 12-h incubation. CBMC from six donors did not show a significant IL-6 response to PGN when considered as a group, although cells from some individual donors exhibited a marginal response. In contrast, production of RANTES was consistently enhanced following activation with PGN (mean, 542 ± 128 pg/ml following treatment with 10 µg/ml PGN compared with 163 ± 52 pg/ml unstimulated controls, n = 4).

Using conditions shown to be effective for TLR-mediated activation of murine bone marrow-derived mast cells (18), CBMC were activated with a high dose of the known TLR4 activator *E. coli* LPS (5 µg/ml). LPS treatment did not significantly increase GM-CSF (Fig. 2C) or IL-1β (Fig. 2D) production. The levels of cytokines produced following LPS challenge did not exceed a 2-fold increase compared with unstimulated control cells (n > 4; Fig. 2, C and D).

The selective responsiveness of CBMC to the TLR2 activator (PGN) but not to the TLR4 activator *E. coli* LPS is consistent with the TLR expression pattern of human mast cells (TLR2 but not TLR4). These findings are notably different from the rodent system whereby bone marrow-derived mast cells were shown to express mRNA for both TLR2 and TLR4 and to respond to both PGN and LPS (18, 20). These results suggest that studies of murine mast cell responses to pathogen products may not be good predictors of human mast cell function.

**Differential CBMC mediator production by TLR2 activators**

CBMC were activated with either the putative TLR2/TLR6 activator, PGN, and zymosan or the putative TLR2/TLR1 activator Pam3Cys and analyzed for the short-term release of classical preformed mediators, cytokine production, and cysteinyl leukotriene generation. Stimulation of CBMC for 24 h with zymosan resulted in the production of significant levels of GM-CSF (Fig. 3A) and IL-1β (Fig. 3B) in a dose-dependent manner. Significant cytokine production was observed with doses of zymosan as low as 0.01% (w/v) in all CBMC donors examined, with GM-CSF production at least 15-fold higher than in unstimulated control cells (n = 4). This profile of cytokines is consistent with that produced by CBMC following activation with PGN. Moreover, the levels of GM-CSF and IL-1β produced by CBMC were similar to those produced by freshly isolated peripheral blood leukocytes treated with these activators (data not shown). Stimulation of CBMC with Pam3Cys resulted in a marginal increase in GM-CSF (Fig. 3C) and IL-1β (Fig. 3D) at the highest dose of Pam3Cys examined (100 µg/ml).

CBMC were analyzed for cysteinyl leukotriene production after stimulation for 20 min (optimal) with TLR2 activators. The TLR2/TLR6 activators PGN and zymosan were potent inducers of cysteinyl leukotriene production whereas Pam3Cys was
not been previously described. Current models for TLR-mediated signaling do not include a clear mechanism by which leukotriene generation or degranulation would be initiated. A potential mechanism for leukotriene production may involve Rac-1-dependent activation of phosphatidylinositol 3-kinase which has recently been proposed as an important pathway in TLR2 signaling (27). The low yield of CBMC from individual donors and lack of GM-CSF and IL-1β responses to TLR activators in mast cell lines limits the ability to directly address these issues in this system.

 Mast cells are well recognized as an important source of leukotrienes in allergic disease. In a number of animal models and disease situations, it has been suggested that local leukotriene generation is dysregulated or may be enhanced by pathogen products under circumstances where there is minimal evidence of mast cell degranulation. LTC₄ and its breakdown products are known to be critical for the bronchoconstriction, edema, and mucous secretion observed in atopic asthma and to be a potent chemoattractant for a number of inflammatory cells as well as dendritic cells. The ability of human mast cells to produce cysteinyl leukotrienes in a degranulation-independent manner in response to TLR activators opens up new possibilities for the mechanisms by which infection exacerbates allergic asthma. The long-term production of RANTES, GM-CSF, and IL-1β by mast cells in response to PGN and zymosan may further enhance host responses to pathogen infection and contribute to the chronicity of inflammatory disease at mast cell-rich sites such as the skin and lung.

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References


