Biochemical and Biophysical Research Communications 391 (2010) 471–476

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Synergistic augmentation of inflammatory cytokine productions from murine mast cells by monomeric IgE and toll-like receptor ligands

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ARTICLE INFO

Article history:
Received 10 November 2009
Available online 13 November 2009

Keywords:
Mast cells
Allergy
IgE
Toll-like receptor
Rodent

ABSTRACT

Simultaneous activation of murine mast cells by monomeric IgE and toll-like receptor (TLR) ligands was examined. Inflammatory cytokine production elicited by the binding of IgE in the absence of antigen, was further enhanced by the addition of lipopolysaccharide (LPS) or peptidoglycan (PGN). Enhancement by LPS or PGN on cytokine production was mediated by TLR4 and TLR2, respectively, since TLR4- and TLR2-deficient mast cells did not show synergistic activation by monomeric IgE and LPS/PGN. Synergistic activation of mast cells was obtained via phosphorylation of several mitogen-activated protein kinases (MAPK). Furthermore, MAPK inhibitors, significantly attenuated the augmentation of inflammatory cytokine production by monomeric IgE and LPS or PGN. Altogether, these results suggest that simultaneous TLR activation of mast cells with IgE molecules, particularly highly cytokinergic (HC) IgE, might contribute to the exacerbation of allergic diseases associated with infection even in the absence of a specific antigen.

Introduction

Mast cells play a critical role in allergic inflammatory reactions and the host defense responses against certain bacterial and parasites [1–3]. These reactions are mediated by cross-linking IgE antibodies bound on high-affinity IgE receptors (FcεRI) on mast cells by multivalent antigens or direct recognition of pathogen-derived products via TLRs [4,5]. Activation of mast cells via FcεRI cross-linking results in the release and generation of inflammatory mediators, such as histamine, leukotriene C_4, PGD_2 and the production of a variety of cytokines [6]. Mast cells activated via TLRs, which recognize pathogen-associated molecular patterns (PAMPs) from various invading microbial pathogens [7], also lead to the production of pro-inflammatory cytokines with or without degranulation [4,5,8–11]. Although the amount of cytokines produced from mast cells by TLR ligands is not as large as that of released by IgE plus antigen, it has been reported that inflammatory cytokine production was synergistically augmented by the simultaneous activation of mast cells via FcεRI cross-linking and TLRs [12,13]. Two recent independent studies have shown that the sensitization of murine mast cells by monomeric IgE, which does not lead to cross-linking of FcεRI, induces prolonged survival of mast cells [14,15]. In addition, further studies have demonstrated that monomeric IgE molecules display a vast majority of function in mast cell activation, including proliferation [16], adhesion [17], migration [18], and the expression of cytokines [19]. We have shown previously that TLR4 ligand, LPS promoted the effect of monomeric IgE on prolonged mast cell survival in a growth factor-deprived condition [20]. Since infection by various pathogens and high levels of serum IgE, irrespective of the presence of specific antigen, often exacerbate allergic diseases, such as asthma and atopic dermatitis [21,22], we investigated whether synergistic activation of mast cells occurs by TLR ligands and monomeric IgE in the absence of specific antigens.

In this study, we show that the simultaneous activation of mast cells with IgE molecules and TLRs results in the synergistic augmentation of cytokine production via the activation of TLR- and several MAPK.

Materials and methods

Mice. C57BL6 mice were purchased from Japan SLC (Hamamatsu, Japan). TLR4-deficient (TLR4−/−) or TLR2-deficient (TLR2−/−) mice were originally provided by Dr. Shizuo Akira at Osaka University and were maintained in our animal facility [23,24]. All animal experiments were performed according to the approved guidelines of the Institutional Review Board of Juntendo University.

Generation of bone marrow-derived mast cells. Bone marrow-derived mast cells (BMMCs) were generated from the femoral bone marrow cells of mice and maintained in RPMI 1640 (Sigma–Al-

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doi:10.1016/j.bbrc.2009.11.082

drich, St. Louis, MO, USA) supplemented with 10% heat-inactivated FCS, 100 μM 2-ME, 10 μM MEM-nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% pokeweed mitogen-stimulated spleen-conditioned medium (PWM-SCM) as a source of mast cell growth factors, as previously described [5,8]. After 4 weeks of culture, more than 98% of cells were identifiable as mast cells, as determined by Toluidine blue staining and FACS analysis of cell-surface expressions of *c-kit* and FcεRI.

Cytokine measurement of stimulated BMMCs. BMMCs (2×10^6/ml) were stimulated with various concentrations of IgE (SPE7; Sigma–Aldrich, IgE3; BD Pharmingen) and LPS from *Escherichia coli* (serotype 0111:B4; Sigma–Aldrich) or PGN from *Staphylococcus aureus* (Fluka, Sigma–Aldrich) for 6 h. To remove the aggregated form of IgE, part of IgE (SPE7 and IgE3) was centrifuged at 20,400*g* for 30 min at 4 °C. For the stimulation of BMMCs with IgE and anti-IgE (BD Pharmingen), BMMCs were first incubated with 1 μg/ml of IgE (BD Pharmingen) for an hour at 4 °C, cells were washed with PBS to remove unbound-IgE, then stimulated with 0.5 μg/ml of anti-IgE (BD Pharmingen). The concentration of cytokines in supernatant was measured using ELISA kit according to the manufacturer's instruction (R&D Systems). For the MAPK inhibitor experiments, BMMCs were incubated with 10 μM PD98059, SB203580, U0126 (Calbiochem), SP600125 (Merk) or control SB20247, N′-methyl-1,9-pyrazoloanthrone (Merk, negative control for SP600125) for 30 min before stimulation with TLR ligands and IgE.

Statistical analysis. Statistical analysis was performed using Student's *t*-test or two-way ANOVA. *P* values less than 0.05 were considered significant.

Results

TLR ligands and monomeric IgE act in synergy to augment mast cell inflammatory cytokine production

Since it has been reported that several TLR ligands can augment the production of cytokines from IgE-sensitized and antigen-stimulated BMMCs [12,13], and recent reports suggest that just binding of monomeric IgE on FcεRI receptors on mast cells leads to production of inflammatory cytokines without specific antigen [14,15,19], we first examined whether monomeric IgE and TLR ligands can synergistically augment inflammatory cytokine production from mast cells. We used two monoclonal mouse IgE antibodies, highly cytokinergic (HC) SPE7 and poorly cytokinergic (PC) IgE3. As has been reported, high concentrations of SPE7 (>1 μg/ml) or TLR ligands alone significantly induced mast cell cytokine production: however, the combination of LPS (1 μg/ml) and PGN (100 μg/ml) with monomeric IgE synergistically enhanced inflammatory cytokine (IL-6, IL-13 and TNFα) production from mast cells (Fig. 1). The production of IL-4 was significantly induced by LPS or PGN. The combination of high concentration of SPE7 significantly augmented LPS, but not PGN-induced IL-4 productions. Significant production of other cytokines, such as IL-5 and CCL4 (eotaxin 2) was not observed by stimulation with LPS, PGN or combination with monomeric IgE. Although the amounts of cytokines were much less compared with SPE7, interestingly, PC IgE (clone IgE3) also lead to significant levels of cytokine production (IL-6 and IL-13) in the presence of LPS (1 μg/ml) but not PGN (100 μg/ml) (Figs. 1 and 2). The synergistic effects on cytokine production were more obvious in mast cells stimulated with LPS than in those stimulated with PGN because LPS (TLR4 ligand) is found to be more potent than PGN (TLR2 ligand) in stimulating cytokine production from murine BMMCs [5]. The synergistic cytokine-producing activity seemed not to due to the aggregation of prepared IgE, since the removal of aggregated-IgE by high-speed centrifugation did not affect these synergistic enhancements (Fig. 2), although the cytokine-producing activity observed by clone IgE3 or by SPE7 (10 μg/ml) was significantly reduced by high-speed centrifugation (Fig. 2). We have previously confirmed by HPLC that high-speed centrifugation was enough to remove the aggregated form of IgE (data not shown).

In similar experiments trying to observe mast cell degranulation using β-hexosaminidase and histamine measurement, we confirmed that the binding of monomeric SPE7 (>5 μg/ml) but not IgE3 slightly induced the degranulation of mast cells; neither LPS nor PGN induced mast cell degranulation (data not shown). Simultaneous activation of mast cells by monomeric IgE and PGN or LPS did not lead to further augmentation of mast cell degranulation (data not shown).

Synergistic augmentation of mast cell cytokine production by LPS and PGN is mediated by TLR4 and TLR2, respectively

Since we have previously reported that LPS or PGN activates murine mast cells via TLR4 or TLR2, respectively [5,8], we examined whether the synergistic augmentation of mast cell cytokine production by monomeric IgE and these TLR ligands was dependent on the respective TLR of mast cells. Although wild-type, TLR4- and TLR2-deficient BMMCs could equally produce cytokines by the addition of a high concentration of SPE7, TLR4-deficient BMMCs failed to show further enhancement of cytokine production stimulated by LPS (Fig. 3A). A similar failure in cytokine production was observed in TLR2-deficient BMMCs stimulated with PGN (Fig. 3B), suggesting that LPS or PGN synergistically enhance inflammatory cytokine production from mast cells in the presence of IgE molecules via TLR4 and TLR2, respectively.

MAPK pathways are responsible for synergistic inflammatory cytokine production of BMMCs stimulated by TLR ligands and monomeric IgE

Inflammatory cytokine production from mast cells is regulated by several protein kinases [6,25]. To investigate the molecular basis for the synergistic enhancement of cytokine production by TLR ligands and monomeric IgE, we investigated the MAPK pathways, which have been implicated in inflammatory cytokine production from mast cells stimulated with either via TLR receptors or monomeric IgE bound on FcεRI [5,16]. When we used specific MAPK inhibitors, the p38 MAP K inhibitor, SB203580, completely abrogated the responses to the monomeric IgE, TLR ligands (LPS and PGN), and to the combination of IgE and TLR ligands. Although the JNK inhibitor (SP600125), and Erk inhibitor (U0126) significantly attenuated the cytokine production mediated by monomeric IgE, they had minimal effects on the response to TLR ligands, and partially suppressed the response to the combination of monomeric IgE and TLR ligands (Fig. 4). Similar results were obtained in the response of other cytokines (TNFα and IL-13) to monomeric IgE and TLR ligands (data not shown).

Discussion

Mast cells are central effectors and regulatory cells in allergic reactions [26]. Although it is widely accepted that bacterial or viral infection occasionally worsens allergic inflammation, the mechanisms have not been clearly defined. A previous study demonstrated that TLRs, the receptors participating in the recognition of various pathogen-associated molecules, which are also expressed on mast cells, substantially enhanced antigen-induced cytokine production from mast cells [12,13]. Recent findings that mast cell activation, including survival, cytokine production, migration, degranulation, proliferation, and adhesion, occur by just binding of monomeric IgE to FcεRI in the absence of multivalent antigens [19], encouraged us to investigate whether TLR ligands also affect

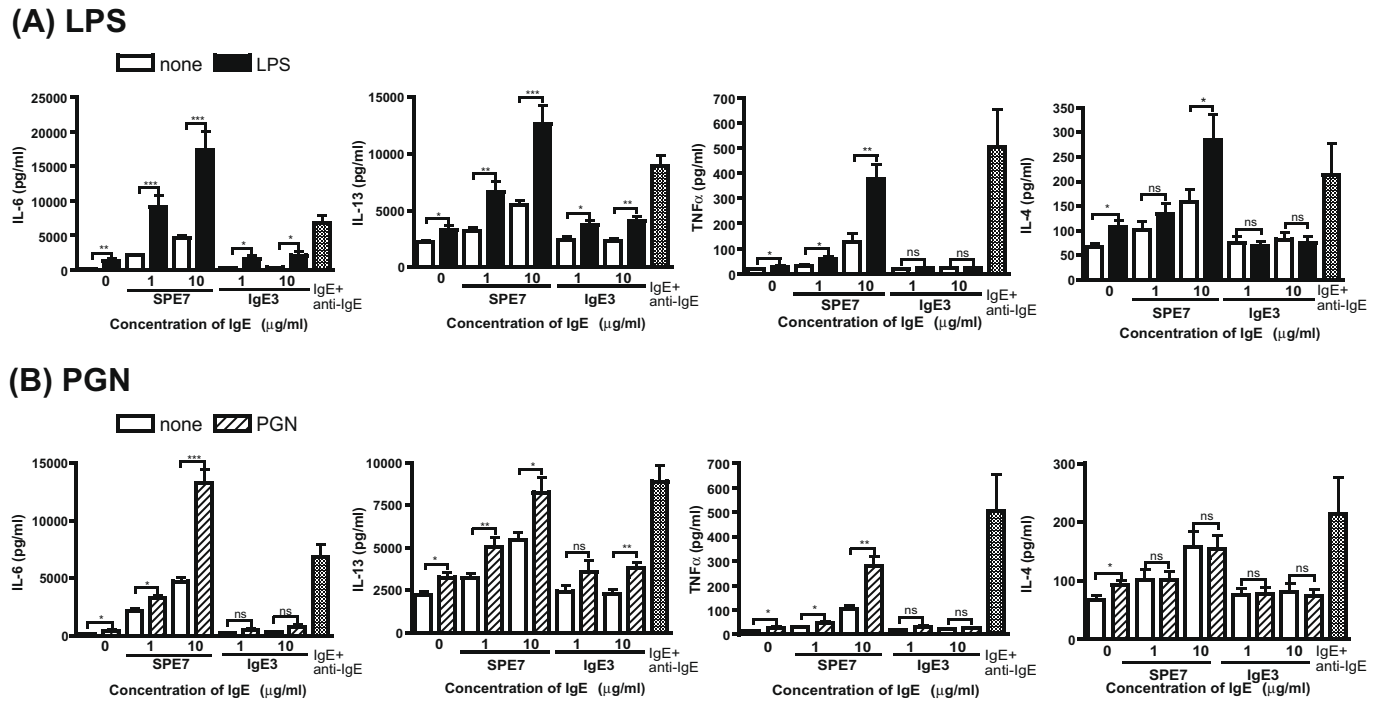

Fig. 1. TLR ligands synergistically augment inflammatory cytokine production from BMMC stimulated with monomeric IgE. BMMCs from C57BL/6 mice were simulated with various concentrations of monomeric IgE (SPE7 or IgE3, 1–10 μg/ml) and LPS (1 μg/ml) (A) or PGN (100 μg/ml) (B) for 6 h. The concentrations of IL-6, IL-13, TNFα and IL-4 in the supernatant were measured by ELISA. Cytokine production from BMMCs with IgE-sensitized and stimulated with anti-IgE was shown as positive control. Results show means ± SD of five to seven independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

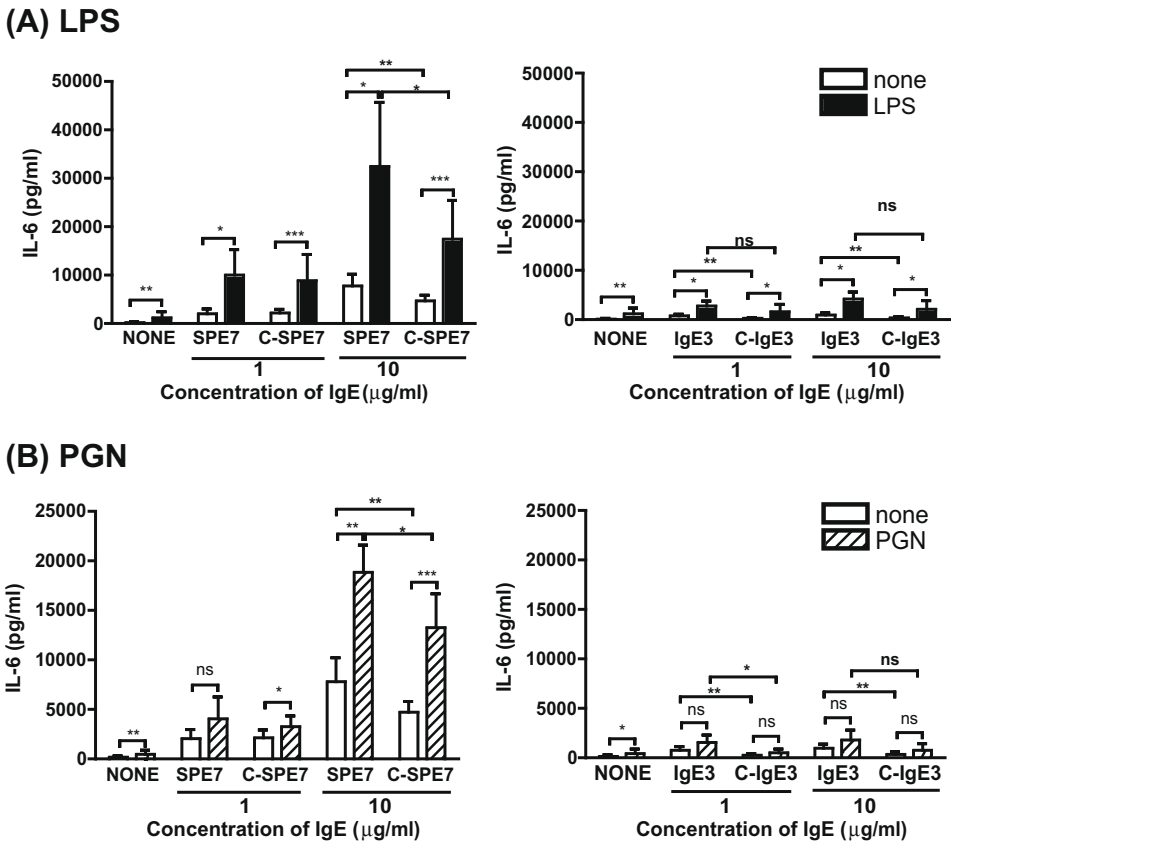

Fig. 2. Removal of aggregated form of IgE does not affect synergistic augmentation of inflammatory cytokine production by TLR ligands and monomeric IgE. BMMCs were stimulated as described in the legend of Fig. 1. To remove the aggregated form of IgE, part of IgE (SPE7 and IgE3) was used after centrifugation, as described in Materials and methods (indicated by C-). The concentration of IL-6 in the supernatant was measured by ELISA. Results show means ± SD of five to seven independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

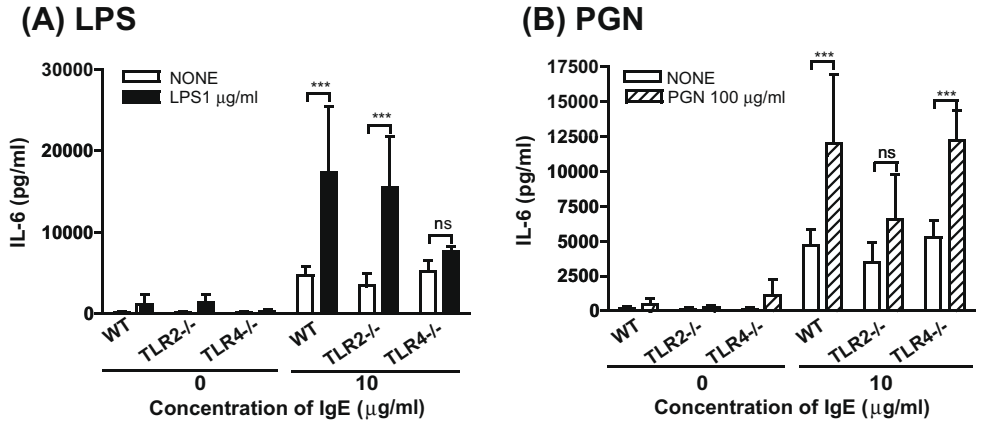

Fig. 3. Synergistic augmentation of mast cell cytokine productions by LPS and PGN is mediated by TLR4 or TLR2, respectively. (A) BMMCs from wild-type, TLR4- or TLR2-deficient mice were stimulated with indicated concentrations of monomeric IgE (SPE7, 10 μg/ml), LPS (1 μg/ml) or combination of both for 6 h. (B) BMMCs from wild-typed, TLR4- or TLR2-deficient mice were stimulated with indicated concentrations of monomeric IgE (SPE7, 10 μg/ml), PGN (100 μg/ml) or a combination of both for 6 h. The concentration of IL-6 in the supernatant was measured by ELISA. Results show means ± SD of five to eight independent experiments. $^{***}P < 0.001$.

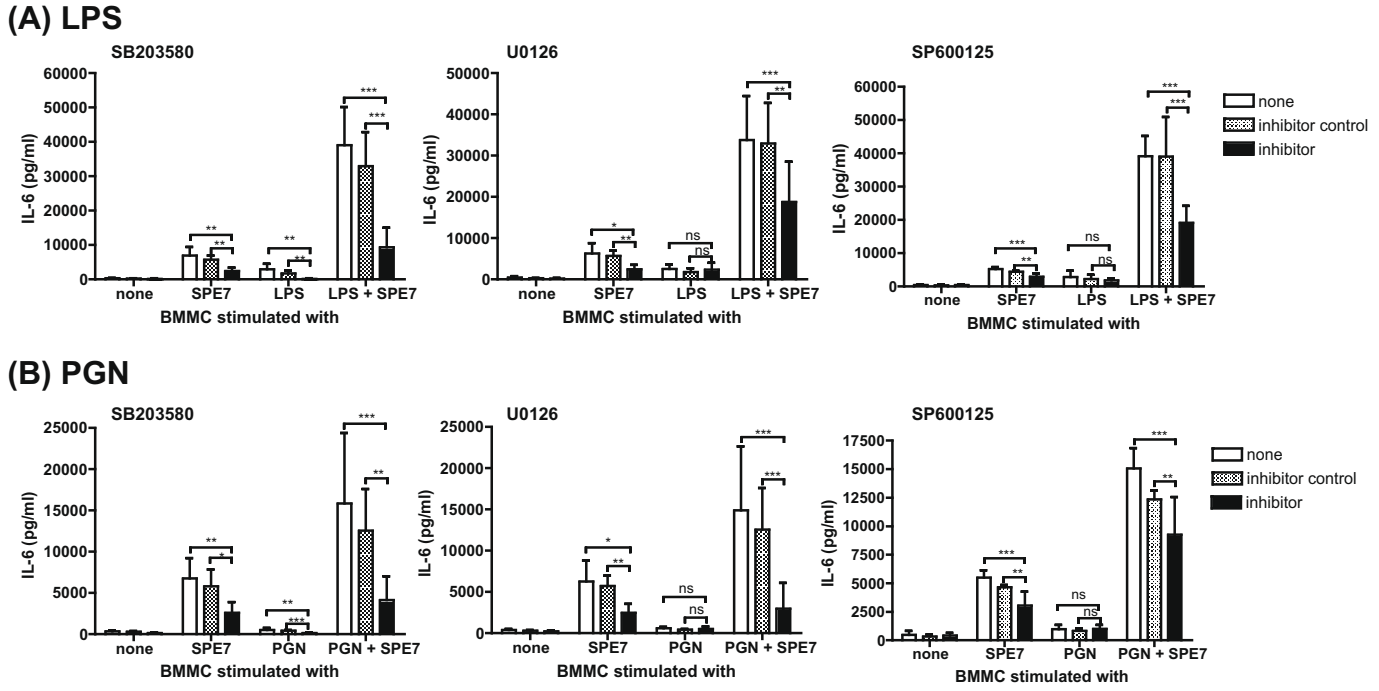

Fig. 4. MAPK pathways are responsible for the synergistic inflammatory cytokine productions by TLR ligands and monomeric IgE. BMMCs were incubated with 10 μM SB203580 (P38 inhibitor), U0126 (MEK1,2 inhibitor), SP600125 (JNK inhibitor) or control SB20247, N′-methyl-1,9-pyrazoloanthrone (negative control for SP600125) for 30 min before stimulation with (A) LPS (1 μg/ml), (B) PGN (100 μg/ml), and SPE7 (10 μg/ml) individually or in combination for 6 h. The concentration of IL-6 in the supernatant was measured by ELISA. Results show means ± SD of three to seven independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

these responses elicited by monomeric IgE in the absence of antigen. We have previously shown that TLR4 ligand, LPS, synergistically promoted mast cell survival with monomeric IgE [20]; thus, in this study, we evaluated the effects of TLR ligands on inflammatory cytokine production and degranulation of mast cells elicited by monomeric IgE.

Although monomeric IgE was found to induce a wide variety of mast cell activations, it has been controversial whether monomeric IgE responses are mediated by a similar signaling mechanism to antigen stimulation. Several reports suggest that signaling molecules are shared between the monomeric IgE response and the response induced by cross-linking with multivalent antigen; that is, weak but sustained tyrosine phosphorylation of several signaling molecules, which are intensively phosphorylated upon IgE plus antigen stimulation, were observed in mast cells stimulated with monomeric IgE [15,18]. In contrast, different roles of PKCβI and II in BMMC activated with monomeric IgE and IgE plus antigens have been reported [27–29]. Signals originating in TLR activation lead to several signaling pathways, such as NF-κB [30,31], AP-1, and MAPK pathways, including extracellular signaling-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 kinases, and the involvement of these three MAPK subtypes in the production of a number of cytokines, including IL-1, IL-6, IL-8, IL-10, and TNFα. In addition, Th1-associated cytokines such as IL-12 and IFNs, have been documented extensively in cells such as monocytes, macrophages, and B cells [30,31]; however, little is known about the combinatorial requirement for cytokine gene transcription in mast cells mediated by TLR. When cells were co-stimulated with TLR ligands and IgE in

combination with antigen, synergistic enhancement of the phosphorylation of JNK and the additive phosphorylation of P38 MAPK was notable [12]. We observed MAPK inhibitor, especially P38 inhibitor totally attenuated, and other inhibitors, such as JNK and Erk inhibitors, partially attenuated the synergistic augmentation of cytokine production from mast cells stimulated with monomeric IgE and TLR ligands (Fig. 4). These results suggest that MAPK pathways combination with other signaling components might contribute to the synergistic augmentation of cytokine production from mast cells stimulated with monomeric IgE and TLR ligands. In addition, other signaling components, activated by monomeric IgE, but not IgE plus antigen, should be examined to clarify the mechanisms, further.

IgE molecules that display the activation of mast cells in the absence of antigen have heterogeneity; for example, highly cytokinergic (HC) IgEs can induce survival, degranulation, proliferation, adhesion, migration, and cytokine production, in contrast, poorly cytokinergic (PC) IgEs do so inefficiently [16,19]. Even the mechanisms of survival-enhancing effects on mast cells by HC IgE and PC IgE were not the same, several lines of evidence, indicate that the binding of either HC or PC IgEs can result in FcεRI aggregation in the absence of the antigen [16,32,33]. We clearly observed synergistic activation of mast cell inflammatory cytokine production by TLRs with HC IgE molecules, SPE7. In contrast, PC IgE, IgE3 could not induce detectable levels of cytokine production by itself; however, the addition of LPS resulted in a low, but significant amount of cytokine production. Although the exact reason for these differences is not known, an intriguing possibility is that HC IgEs aggregate each other more easily than PC IgEs by binding the Ag recognition site of an epitope in their own structure or another cell surface component [32]. These results suggest that various pathogens activating several TLRs simultaneously may cause more severe inflammation when mast cells are sensitized by IgE rather than, not sensitized by IgE, or when serum IgE levels are high.

Even HC IgE, SPE7, caused slight degranulation of mast cells at higher concentrations (>5 μg/ml), LPS or PGN did not further augment this degranulation (data not shown). This result was consistent with a previous report by Qiao et al. [12], in which they simultaneously stimulated mast cells via IgE bound to mast cells and specific antigens with several TLR ligands, and found no enhancement of degranulation and arachidonic acid production.

A recent report has suggested that IgE molecules also affect the differentiation of mast cells by accelerating development, and by modifying their phenotype [34]. Mast cells cultured in the presence of HC IgE expressed more mouse mast cell protease (mMCP)-6 and -7, which were usually found to be low in BMMCs cultured with IL-3 alone. In addition, it has been reported that LPS and PGN differently affect the differentiation and phenotype of human mast cells by influencing numbers, cytokine and protease expression and composition [35], raising the possibility that these biological phenomena are also affected by co-stimulation with TLRs and IgE molecules. Although it is not known whether strongly receptor-aggregating HC IgEs similar to SPE7 are synthesized *in vivo* under some conditions, recent finding indicates that polyclonal mouse IgE molecules in sera from mice with atopic dermatitis could have activity to induce mast cell survival and cytokine production [36]. In addition, the levels of IgE present in serum have been reported to affect the immune response, such as contact hypersensitivity [37], and allergic airway inflammation [38], irrespective of their antigen specificity; thus, our present result may explain how bacteria infections contribute to worsening allergic diseases.

In summary, this study showed the synergistic augmentation of inflammatory cytokine production from mast cells by monomeric IgE and TLR ligands, LPS and PGN. These results indicate an important clue to understanding how infections affect chronic allergic diseases and contribute to the exaggeration of allergic inflammation in the absence of the irrespective antigen.

Conclusion

Monomeric IgE and TLR ligands, LPS and PGN induced the synergistic augmentation of inflammatory cytokine production from mast cells in the absence of the irrespective antigen, which might contribute to the exaggeration of allergic inflammation accompanying infection.

Acknowledgments

This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. The authors thank Dr. Shizuo Akira for the generous gift of TLR4- and TLR2-deficient mice. We also thank the members of the Atopy Research Center and the Department of Immunology for fruitful discussions and technical assistance, and Ms. Michiyo Matsumoto for secretarial assistance.

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