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Biochemical and Biophysical Research Communications 365 (2008) 137–142
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Monomeric IgE and lipopolysaccharide synergistically prevent mast-cell apoptosis

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Received 22 October 2007
Available online 31 October 2007

Abstract

The apoptosis of bone marrow-derived mast-cells (BMMCs) after growth factor withdrawal was significantly prevented by a high concentration of IgE in the absence of antigen, and further enhanced by the presence of Toll-like receptor4 (TLR4) ligand, lipopolysaccharide (LPS). The effect of LPS was mediated by TLR4, since TLR4-deficient BMMCs did not show synergistic effects with IgE. The neutralizing amount of anti-IL-3 did not reverse the anti-apoptotic effects of both IgE and combination with LPS. LPS treatment with monomeric IgE synergistically prevented the loss of mitochondrial membrane potentials and was associated with an enhanced expression of anti-apoptotic protein, Bcl-xL, or with a reduced expression of proapoptotic protein, Puma, and Bim, respectively. Altogether, these results suggest that LPS, in a TLR4-dependent manner, together with IgE, synergistically prevent mast-cell apoptosis and may contribute to regulate the tissue mast-cell number.

Keywords: Apoptosis; Bcl-family; BH-3 only proteins; LPS; IgE; Mast-cell

Mast cells are well-known effector cells not only in IgE-mediated allergic diseases but also in innate immunity by recognizing pathogen-derived products via Toll-like receptors (TLRs). They are derived from pluripotent hematopoietic stem cells in bone marrow and exit as committed precursors to complete their development in connective and mucosal tissues [1,2]. The number of mast cells in extravascular tissues are regulated by the balance between proliferation and cell death. Various factors regulate mast-cell viability, including growth factors, receptor signaling, and antibodies. The survival of murine mast cells is regulated by both stem cell factor (SCF) and interleukin 3 (IL-3) [3,4]. In the absence of these factors, mast cells undergo apoptosis. The activation status of mast cells also affects their survival. It has been reported that FcεRI-mediated mast-cell activation as well as a high concentration of monomeric IgE antibody prevent mast cell apoptosis in growth factor withdrawal conditions [5–7]. Two independent studies have shown that sensitization of murine mast cells by monomeric IgE, which does not lead to cross-linking of FcεRI, induces prolonged survival of mast cells [6,7]. This survival effect does not only appear to be because of the release of mediators by activated mast cells, since all monomeric IgE tested, irrespective of whether they were cytokinergic or not, were associated with anti-apoptotic activity [2,8]. Mast cells are also activated via TLRs, which recognize pathogen-associated molecular patterns (PAMPs) from various invading microbial pathogens [9],

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doi:10.1016/j.bbrc.2007.10.136

and lead to pro-inflammatory cytokine production [10,11]. Stimulation of TLR on mast-cells by LPS with interferon (IFN) γ, which leads to an increased expression of TLR4, has also been found to inhibit apoptosis of IL-3-deprived murine BMMCs [12].

Like other hematopoitic cells, fate of mast cells after loss of cytokine signaling appears to be regulated by Bcl-2 family of proteins, which may either be death antagonists (Bcl-2, Bcl-xl, Bcl-w, mcl-1, and A1) or death agonists (Bax, Bak, Bcl-xs, Bad, Bid, and Bik) [13]. SCF and IL-3 have been shown to upregulate Bcl-2 and Bcl-xL in mast-cells [14,15]. Also, the effect of LPS on the prevention of mast-cell apoptosis was reported to be associated with upregulation of Bcl-xL [12]. Recently, the roles of a third proapoptotic subgroup of the Bcl-2 family (Bad, Bim, and Puma), so-called BH3-only proteins, have been implicated in promoting apoptosis when overexpressed, probably by antagonizing prosurvival anti-apoptotic Bcl-2 family members [16], and Puma has been shown to play an essential role in growth factor-deprived mast-cell apoptosis [17]. Although it has been reported that IgE receptor aggregation by antigen resulted in the upregulation of both prosurvival Bcl-xL, A1 and proapoptotic Bim, and the balance between these signals may determine the fate of mast-cells [5,18,19], the effects of monomeric IgE on the expression of many members of the Bcl-2 family of proteins, including those such as Bcl-xL and Bad, were reported either to be unchanged or upregulated [6,7]. Also it is still unknown whether monomeric IgE or LPS affect the expression of BH-3 only proteins in growth factor-deprived mast-cell apoptosis. In this study, we first demonstrated the synergistic prevention of mast-apoptosis by LPS and monomeric IgE and those mechanisms by investigating various parameters, including the expression of BH-3 only proteins.

Methods

Mice. C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). TLR4-deficient (TLR4−/−) mice were originally provided by Dr. Shizuo Akira at Osaka University and were maintained in our animal facility [20,21]. All animal experiments were performed according to the approved manual of the Institutional Review Board of Juntendo University.

Generation of bone marrow-derived mast cells. Bone marrow-derived mast cells (BMMCs) were generated from the femoral bone marrow cells of mice and maintained in RPMI 1640 (Sigma–Aldrich, 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 [10,11]. 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.

FACS analysis of apoptotic cells. BMMCs (5×10^5 ml) were cultured in IL-3-deprived medium in the presence of various concentrations of IgE (SPE7, Sigma–Aldrich) and 1 μg/ml of LPS from *Escherichia coli* (serotype 0111:B4; Sigma–Aldrich) for the indicated time periods. For the neutralization experiment, anti-IL-3 (BD Biosciences, San Jose, CA) or isotype control rat IgG_1 (BD Biosciences) were added during incubation periods. Apoptosis of BMMCs was evaluated by FACS following staining with propidium iodide (PI, 5 μg/ml) and FITC-Annexin V (BD Biosciences).

Measurement of mitochondria membrane potentials. BMMCs (5×10^5 cells/ml) were cultured in IL-3-deprived medium as indicated above. Perturbation of mitochondrial membrane potential ($\Delta\psi m$) was monitored using MitoTracker® (Molecular Probes®, Invitrogen, Carlsbad, CA, USA). Briefly, cells were washed after incubation, and then stained with 200 nM of MitoTracker® (Molecular Probes®, In vitrogen, Carlsbad, CA, USA) for 30 min at 37 °C in the dark. Fluorescence intensity was measured using FACS Caliber.

Western blot analysis. BMMCs (2×10^6 cells/ml) were cultured in the presence of IgE or LPS in IL-3 (10 ng/ml)-deprived medium for 12 h. The cells were lysed in buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris–HCl (pH 7.5), and 1 mM EDTA) containing a protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA), and the lysates were subjected to 12.5% SDS–PAGE (Daiichi Pure Chemicals, Co., Ltd.). Immunoblotting using polyclonal antibodies to Bcl-xL, Puma, Bim (Cell Signaling Technology, Inc., Danvers, MA, USA) was performed according to the manufacturer's instructions. The membrane was developed with an enhanced chemiluminescence detection kit (GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA). An antibody to β-actin (Biolegend, San Diego, CA, USA) was used as a control to ensure that equal amounts of protein were loaded onto each lane.

RNA isolation and quantitative real-time PCR. Total RNA was extracted from BMMCs using Trizol reagent (BRL, Life Technologies, Rockville, MD), and first-strand cDNA was synthesized from 3 μg of total RNA using Superscript™ II (Invitrogen). Real-time PCR was performed by a 7500 real-time PCR System (Applied Biosystems, Branchburg, NJ) using the TaqMan Universal PCR Master Mix and primer/probe sets obtained from Applied Biosystems assays on demand (Applied Biosystems). Relative transcript levels of each sample were corrected by normalization based on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels. All real-time PCRs were performed in triplicate, and the changes in gene expression were expressed as fold-increases relative to cells in IL-3 containing medium.

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 and discussion

LPS and monomeric IgE act in synergy to prevent mast-cell apoptosis induced by growth factor withdrawal

Since it has been reported that both IgE and LPS can enhance the survival of BMMCs under growth factor withdrawal conditions, we first examined whether the combination of IgE and LPS can synergistically enhance mast-cell survival. As has been reported, high concentrations of IgE (>0.5 μg/ml) alone significantly prevented the mast-cell apoptosis induced by IL-3 withdrawal [6,7], however, the combination of LPS (1 μg/ml) with IgE, but not LPS alone, markedly augmented the effects of IgE on mast-cell survival (Fig. 1). This augmentation was evident at concentrations of IgE that by itself had minimal survival effects on BMMCs, therefore, IgE and LPS appeared to act in a synergistic manner or even potentiated one another at threshold concentrations. We could not observe significant apoptosis inhibitory effects by LPS alone, which might be because we did not stimulate mast cells with IFN-γ, which has been shown to increase the expression of TLR4 and therefore the responsiveness of mast cell to LPS [12].

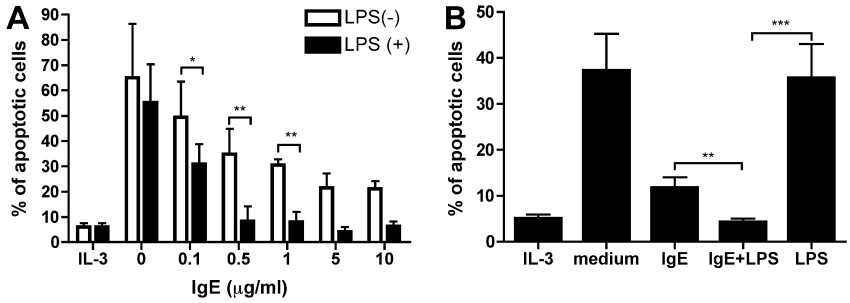

Fig. 1. LPS and monomeric IgE act in synergy to prevent mast-cell apoptosis induced by IL-3 withdrawal. (A) BMMCs from C57BL/6 mice were cultured in IL-3-deprived medium in the presence of various concentrations of monomeric IgE (SPE7, 0.1–10 μg/ml), LPS (1 μg/ml) or a combination of both. (B) BMMCs from C57BL/6 mice were cultured in IL-3-deprived medium in the presence of monomeric IgE (SPE7, 5 μg/ml), LPS (1 μg/ml) or a combination of both. The percent of apoptotic cells was determined at 48 h after IL-3 withdrawal by staining with FITC-conjugated annexin V and propidium iodide (PI) followed by flow cytometric analysis. Results show the means ± SD of five independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

TLR4, but not endogenous IL-3 production, mediates the synergistic prevention of mast-cell apoptosis by LPS and monomeric IgE

Since we have previously reported that LPS activates mast cells via TLR4 [10,11], we examined whether the synergistic prevention of mast-cell apoptosis by LPS combination with monomeric IgE was dependent on TLR4 of mast cells. Both BMMCs from TLR4-sufficient and -deficient mice underwent apoptosis by similar kinetics after IL-3-deprivation. Although monomeric IgE alone equally prevented the apoptosis of BMMCs from both phenotypes, the enhancement of mast-cell survival by the combination of IgE and LPS was observed only in TLR4-sufficient, but not in TLR4-deficient BMMCs (Fig. 2A). Although the activation of various immune cells via TLRs, leading to pro-inflammatory cytokines, is well documented, the mechanisms that affect cell survival via TLR activation are not well known. Reports have shown that various TLR ligands were effective at delaying spontaneous apoptosis of human polymorphonuclear neutrophils (PMN) by exerting anti-apoptotic effects by activation of NF-κB and PI3K [22]. Since we did not observe anti-apoptotic effect on mast cells by the addition of TLR2 ligand, PGN, even in the presence of IgE, activation of the NF-κB pathway alone cannot explain the prosurvival effect of TLR4 ligand on mast cells. We have also examined whether cytokine, especially IL-3, produced by BMMCs, was responsible for apoptosis prevention by IgE and LPS, since it has been reported that a rapid and large amount of autocrine IL-3 production is, at least in part, responsible for mast-cell survival by IgE in the absence of antigen [23], and stimulation of mast cells with LPS combined with IgE resulted in synergistic production of inflammatory cytokines [24]. The amount of anti-IL-3, which was enough to neutralize 1 ng/ml of IL-3, neither prevented anti-apoptotic effect by IgE nor combination with LPS (Fig. 2B). These results again support the idea that synergistic production of autocrine IL-3 did not play a crucial role in our mast-cell survival system induced by monomeric IgE and LPS. The discrepancy observed between our result and that of Kohno et al. [23], regarding the effect of monomeric IgE, might be due to differences of monoclonal IgE clones used (SPE7 vs H1-ε-26). Even both clones were reported to induce secretion of cytokines in the absence of antigen [8], we did not observe detectable levels (>5 pg/ml) of IL-3, but significant amounts of IL-6, TNF-α and IL-13 in our BMMC culture medium either incubated with IgE alone or combination with LPS for 6 h. Furthermore, culture condition of BMMCs might

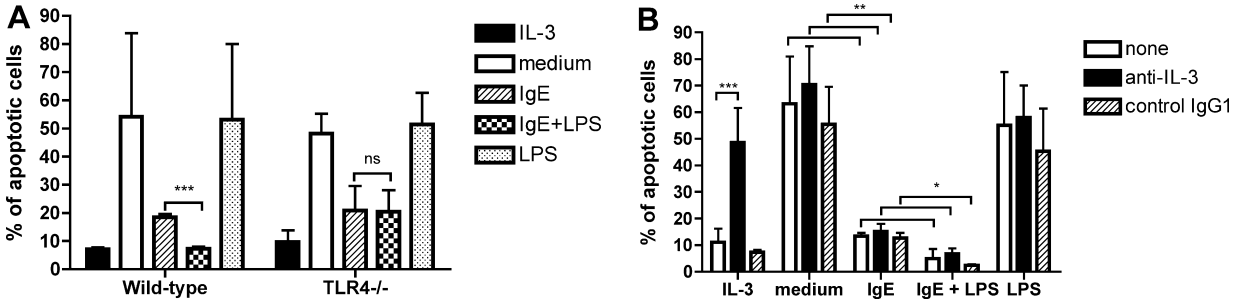

Fig. 2. TLR4, but not endogenous IL-3 production, mediates the synergistic prevention of mast-cell apoptosis by LPS and monomeric IgE. (A) BMMCs from wild-type or TLR4-deficient mice were cultured in IL-3-deprived medium in the presence of monomeric IgE (SPE7, 5 μg/ml), LPS (1 μg/ml) or a combination of both. (B) BMMCs from C57BL/6 mice were cultured in IL-3-deprived medium as described above in the presence of anti-IL-3 (25 μg/ml) or control rat IgG_1 (25 μg/ml). The percent of apoptotic cells was determined at 48 h as described in the legend of Fig. 1. Results show the means ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

affect the ability of mast cell to produce cytokines and responsibility to IL-3.

Loss of mitochondrial membrane transition potential ($\Delta\psi m$) is synergistically prevented by IgE and LPS

Mitochondrial permeability transition is an important step in the induction of cellular apoptosis and the loss of mitochondrial membrane potential ($\Delta\psi m$) is a characteristic of apoptosis. The withdrawal of IL-3 resulted in the loss of $\Delta\psi m$, which was partially prevented by monomeric IgE alone. The addition of LPS with IgE, markedly inhibited the loss of $\Delta\psi m$, to almost similar levels to BMMCs cultured in IL-3-containing medium (Fig. 3). Mitochondrial membrane potential changes are induced by binding of BH3-only proteins, such as Bim, Bid, and Puma, to Bcl-2 and Bcl-xL [25,26]. Thus, we next examined the roles of BH3-only proteins and anti-apoptotic Bcl-2 family members in the synergistic prevention of mast-cell apoptosis by monomeric IgE and LPS after growth factor-withdrawal conditions.

Synergistic prevention of mast-cell apoptotis by monomeric IgE and LPS is associated with the expression of anti-apoptotic Bcl-xL or proapoptotic Puma, or Bim, respectively

As shown in Fig. 4 and as reported previously [6,7,12], upon depletion of IL-3, the expression of anti-apoptotic protein, Bcl-xL, in mast cells was decreased. In contrast, the expressions of pro-apoptotic proteins, such as Puma, and Bim in BMMCs, were increased. Although the treatment of BMMCs with IgE alone significantly antagonized changes in the levels of these proteins, combination with LPS further enhanced the effect of IgE. Consistent with the results of cell viability, LPS alone hardly changed the levels of protein expression. Since transcriptional induction of BH3-only proteins in response to growth factor-deprivation is evident, we performed quantitative PCR to measure mRNA levels of Puma and Bim (Fig. 4B). Although mRNA levels of Puma were correlated with the result of Western blotting, LPS treatment in addition to IgE did not further prevent Bim induction. Among the BH3-only proapoptotic proteins, Bim has been reported to be essential for the normal regulation of apoptosis in numerous cell types, including mast cell [19,27–29]. Recently, roles of other BH3-only proapoptotic proteins, including Bad, Bid, Bmf, Noxa, and Puma, have been investigated in the apoptosis of mucosal-like mast cells and connective tissue-like mast cells. Puma was found to be critical for the induction of mast-cell death by both cytokine-deprivation and DNA-damaging agent [17]. We have also observed increased levels of Bim and Puma in BMMCs at both mRNA and protein levels after IL-3-deprivation. In addition, we clearly showed the inhibition of Puma and partly Bim expression by the addition of monomeric IgE as well as in combination with LPS. In contrast to our results, Bim and Puma appear not to play a critical role in the FcεRI-cross-linking-induced anti-apoptotic pathway since it has been reported that levels of Bim were upregulated and those of Puma were unchanged upon IgE-receptor cross-linking with antigen [17,19]. This might be a clear difference in anti-apoptotic signaling between monomeric IgE and IgE-receptor cross-linking with antigen. Since many factors, including culture conditions, may affect the expression levels of individual protein [30], further examinations would be required. Also, the involvement of other proteins, such as Bax and Bak is unlikely since cells lacking these proteins showed no signs of death when cultured in the absence of IL-3, as previously reported [31,32].

In summary, this study showed that the anti-apoptotic effect of monomeric IgE on BMMCs was further enhanced by the addition of LPS via TLR4. Furthermore, we demonstrated for the first time that Puma and Bim were involved in the prevention of mast-cell apoptosis by monomeric IgE and LPS following cytokine deprivation. These results suggest that pathogen-associated molecules together with monomeric IgE extend the mast-cell functional life span at the site of allergic inflammation accompanied by infection.

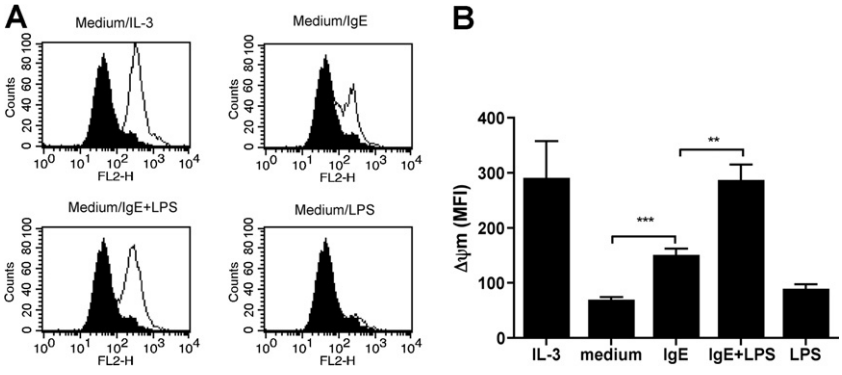

Fig. 3. Loss of mitochondrial membrane transition potential ($\Delta\psi m$) is synergistically inhibited by IgE and LPS. BMMCs were cultured as described above. Mitochondrial membrane transition potential ($\Delta\psi m$) was analyzed by flow cytometer at 48 h following staining with Mitotracker® orange CMTMR (200 nM) for 30 min at 37 °C. (A) Shaded line represents BMMCs in IL-3-deprived medium. Open line shows BMMCs incubated as indicated. Histogram shows a representative of three independent experiments. (B) Mean fluorescence intensity of $\Delta\psi m$ is shown. Results show the means ± SD of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

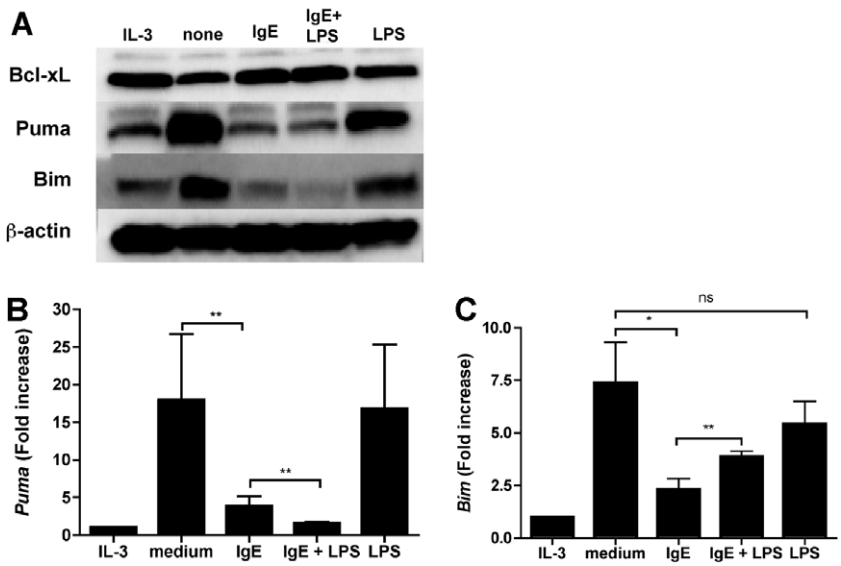

Fig. 4. Synergistic prevention of mast-cell apoptotis by monomeric IgE and LPS is associated with the expression of anti-apoptotic Bcl-xL or proapoptotic Puma, or Bim, respectively. (A) Cell lysates from BMMCs cultured as described above for 12 h were immunoblotted with an antibody to Bcl-xL, Puma, and Bim. Probing with an antibody to β-actin was used as a loading control. (B) Total RNA (3 μg) extracted from BMMCs cultured in same conditions for 3 h was used to analyze changes in Puma and Bim genes by Real-time PCR. Each bar shows the mean ± SD of three separate experiments performed in triplicate and is expressed as the values in fold-increases of gene expression of the above cells incubated in medium containing IL-3. $^{*}P < 0.05$, $^{**}P < 0.01$.

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-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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