Biochemical and Biophysical Research Communications 464 (2015) 949–955

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Acidic environment augments FcεRI-mediated production of IL-6 and IL-13 in mast cells

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ARTICLE INFO

Article history:
Received 10 July 2015
Accepted 15 July 2015
Available online 18 July 2015

Keywords:
Extracellular acidification
IL-6
IL-13
Interleukin
Mast cells
p38 MAPK

ABSTRACT

Although blood pH is maintained in a narrow range of around pH 7.4 in living organisms, inflammatory loci are characterized by acidic conditions. Mast cells tend to reside close to the surface of the body in areas such as the mucosa and skin where they may be exposed to exogenous acids, and they play an important role in immune responses. However, little is known about the effects of extracellular acidification on the functions of mast cell. Here, we found that extracellular acidification increased the dinitrophenyl-conjugated human serum albumin (DNP-HSA)-induced production of interleukin (IL)-6 and IL-13 in MC/9 cells or bone marrow-derived mouse mast cells sensitized with anti-DNP IgE. Extracellular acidification also inhibited migration of MC/9 cells toward DNP-HSA. In addition, acidic pH stimulated antigen-induced activation of p38 mitogen-activated protein kinase (MAPK) and protein kinase B (Akt). These findings suggest that extracellular acidification augmented antigen/IgE-induced and FcεRI-mediated production of IL-6 and IL-13 in mast cells, and that this was associated with the enhancement of p38 MAPK and Akt activation.

1. Introduction

Proton concentration or pH in human blood is strictly maintained at 7.4 ± 0.05. Imbalance of blood pH (e.g., lactic acidosis induced by sepsis) leads to life-threatening conditions for living organisms [1]. However, despite the typical maintenance of pH, inflammatory loci are usually associated with an acidic environment [2]. The pH of deaerated exhaled airway vapor condensate is also reportedly lower in patients with asthma than in healthy control subjects, and pH values correlate with the severity of patients' asthma [3]; thus, acidic pH apparently contributes to the pathogenesis of allergosis.

Bronchial asthma exhibits pathological characteristics such as the promotion of airway hyperresponsiveness (AHR) and airway mucus secretion, increasing concentrations of T-helper cell 2 (Th2) cytokines in bronchoalveolar fluid, and involvement of various immune cells including mast cells, eosinophils, dendritic cells (DCs), T-helper cells, and airway epithelial cells [4,5]. Previous studies have shown that the functions of several cell types are altered by changes in extracellular pH [6]. We have recently shown that proton-sensing receptors, including ovarian cancer G protein-coupled receptor 1 (OGR1) and T cell death-associated gene 8 (TDAG8), are involved in regulation of cell functions related to airway inflammation. Specifically, acidic pH attenuates proinflammatory cytokine production from mouse peritoneal macrophages through TDAG8 [7] and stimulates interleukin (IL)-6 and connective tissue growth factor production in human airway smooth muscle cells (ASMCs) through OGR1 [8,9]. Additionally, in

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http://dx.doi.org/10.1016/j.bbrc.2015.07.077
0006-291X/

an ovalbumin-induced asthma model, we found that OGR1-deficient mice are resistant to AHR and airway inflammation; this was associated with the remarkable inhibition of Th2 cytokine production via a change in DCs migration activity [10]. The actions of extracellular pH, mediated by OGR1 family G protein-coupled receptors (GPCRs), have also been reported by other research groups investigating ASMCs [11] and eosinophils [12].

Mast cells play a central role in allergy diseases and inflammation by producing chemical mediators, lipid mediators, and inflammatory cytokines [13]. Since mast cells tend to reside in areas close to the surface of the body such as the mucosa or skin, their exposure to exogenous acids is likely to be increased. It is possible, therefore, that protons are involved in the regulation of mast cell functions; however, little is currently known about the role played by extracellular acidification in these functions [14]. Hence, in the present study, we examined whether pH regulates mast cell functions such as cytokine production and migration.

2. Materials and methods

2.1. Cells and reagents

The MC/9 mouse mast cell clone, obtained from the American Type Culture Collection (Manassas, VA, USA), was maintained by passage in DMEM (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Biological Industries, Beit-Haemek, Israel), 50 μM 2-ME (Life Technologies, Grand Island, NY, USA), 5 ng/ml mouse rIL-3 (R&D systems, Minneapolis, MN, USA), 50 U/ml penicillin G sodium, and 50 μg/ml streptomycin sulfate (Invitrogen, Auckland, NZ) (complete medium). Mouse bone marrow-derived mast cells (BMMCs) obtained from the femurs of male C57BL/6 mice (see 2.2) was cultured in complete medium with 0.5 μg/ml amphotericin B. After 4 weeks of culture, we confirmed that >99% of cells showed $Fc\varepsilon RI^{+}$ CD117 (c-kit)$^{+}$ by using fluorescence activated cell sorting.

Mouse monoclonal anti-dinitrophenyl (DNP) IgE was obtained from Yamasa Corporation (Tokyo, Japan). DNP-conjugated human serum albumin (DNP-HSA) and 5-(*N*,*N*-dimethyl)-amiloride (DMA) were purchased from Sigma–Aldrich. SB203580, SB239063, and wortmannin were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany) and BIOMOL (Plymouth Meeting, PA, USA), respectively. LY294002 was obtained from Cell Signaling Technology (Danvers, MA, USA).

2.2. Animals

All animals were bred in the Institute of Animal Experimental Research of Gunma University, and animal procedures were performed in accordance with the guidelines of the Animal Care and Experimentation Committee of Gunma University. We have recently generated OGR1-deficient C57BL/6 mice [15] and G protein-coupled receptor 4 (GPR4)-deficient C57BL/6 mice. The preparation method of GPR4-deficient mice will be described in detail elsewhere. TDAG8-deficient C57BL/6 mice [7] were generously provided by Drs. Kyoji Horie and Junji Takeda of Osaka University, Dr. Takao Shimizu of University of Tokyo, and Dr. Satoshi Ishii of Akita University [16]. The respective GPCR-deficient mice and their littermates (wild-type mice) were used in experiments at 8 weeks of age.

2.3. Cytokine production assays

MC/9 cells or BMMCs ($1–5 \times 10^5$/ml) were cultured with 500 ng/ml anti-DNP IgE for 2 h. After washing with 0.1% BSA-DMEM, the cells (1×10^5/ml) were stimulated for 3 h with DNP-HSA (10 ng/ml). The levels of TNF-α, IL-6, and IL-13 protein in the supernatants were measured using a Duoset enzyme-linked immunosorbent assay (ELISA). Development kits were purchased from R&D systems.

2.4. Mast cell migration

MC/9 cell migration was quantified by using a modification of the Boyden chamber technique as previously reported [17]. The lower well was separated from the upper well by a 3-μm-pore polycarbonate filter (Chemotaxicell; Kurabou, Osaka, Japan). Mast cell movement was quantified by counting the number of mast cells in the lower well using a Fuchus Rosenthal calculator (Erma, Tokyo, Japan).

2.5. Cell death assay

The trypan blue exclusion test was used to determine the number of viable cells present in a cell population. The cells were covered with 0.4% trypan blue solution in PBS and 70% isopropanol and incubated for 3 min at room temperature. A drop of the trypan blue/cell suspension was placed on a hemacytometer and the cells were counted. The percentage of viable cells was calculated according to the total number of viable cells per ml of suspension [18].

2.6. Measurement of mRNAs

After stimulating MC/9 cells with 10 ng/ml DNP-HSA for 1 h, we measured cytokine mRNA expression. As for mRNA expression of proton-sensing GPCRs, MC/9 cells or BMMCs were used without any stimulation of antigen and antibody. Total RNA was prepared from the cells according to the manufacturer's instructions for RNAisoPlus (Takara, Japan). Quantitative real-time PCR (RT-qPCR) was performed according to [19] using hydrolysis probes of TaqMan technology (Applied Biosystems, Foster, CA, USA). The total RNA (5 μg) was treated with DNase I to remove possible trace of genomic DNA and subjected to the RT-qPCR. The expression level of the target mRNA was normalized to the relative ratio of the expression of glyceraldehydes 3-phosphate dehydrogenase (GAPDH) mRNA. The RT-qPCR assay was performed with 5 different RNA concentrations in each sample. We confirmed that the slope of cycle number vs. logarithm of RNA concentration was always the same for target gene and GAPDH. The RT–PCR probes of target genes were obtained from the selection of Taq-Man Assay-on-Demand Gene expression reagents (Applied Biosystems), i.e., TNF-α (Mm00443258_m1), IL-6 (Mm00446190_m1), IL-13 (Mm00434204_m1), GPR4 (Mm00558777), OGR1 (Mm01335272), TDAG8 (Mm00433695), and GAPDH (Mm99999915_g1).

2.7. Western blotting

Western blotting proceeded as previously described [8]. We used the primary antibodies specific to phosphorylated forms of p38 mitogen-activated protein kinase (MAPK) (Thr^{180}/Tyr^{182}), c-Jun N-terminal kinase (JNK) (Thr^{183}/Tyr^{185}), protein kinase B (Akt) (Thr^{308}), extracellular signal-regulated kinase (ERK) (Thr^{202}/Tyr^{204}), and antibodies for total forms (all from Cell Signaling Technology). Blots were scanned using a Molecular Imager FX system (Bio-Rad Laboratories, CA, USA). Densitometric analyses of bands were performed at non-saturation exposures using the software ImageJ (NIH, Bethesda, MD, USA). Values from phosphorylated proteins were normalized to their respective unphosphorylated total protein levels.

2.8. Statistical analysis

All values were expressed as the means ± SEM from the indicated number of experiments. For the results presented in Figs. 1A–C and G–K, 2B and C, and 4, the data were compared by using the Student's *t*-test with the Bonferroni correction for multiple comparisons. A value of $P < 0.05$, after adjustment for the number of comparisons, was considered statistically significant. In the data presented in Figs. 1D–F, and 3B and C, nonparametric analysis of variance was conducted using the Kruskal Wallis method to determine the significance among groups. $P < 0.05$ was again considered as statistically significant.

3. Results

3.1. Extracellular acidification stimulates IL-6 and IL-13 production and inhibits migration in mast cells

We first examined the effect of extracellular pH changes on FcεRI-mediated cytokine production in MC/9 cells. As shown in Fig. 1A–C, extracellular acidification did not induce any detectable cytokine production in the absence of DNP-HSA in anti-DNP IgE-sensitized MC/9 cells. Acidic pH, however, clearly enhanced DNP-HSA-induced production of IL-6 (Fig. 1B) and IL-13 (Fig. 1C) in the IgE-sensitized cells, which was associated with a significant increase in mRNA expression levels of IL-6 (Fig. 1E) and IL-13 (Fig. 1F). In contrast, extracellular acidic pH affected neither TNF-α protein (Fig. 1A) nor mRNA (Fig. 1D) expression. We observed similar results for acidification-induced enhancement of IL-6 and IL-13, but not TNF-α, production in BMMCs (Fig. 1G–I).

Mast cells have chemotactic abilities, and anti-DNP IgE-sensitized mast cells migrate toward DNP-HSA [17]. Therefore, we also investigated the effect of extracellular acidification on the migration capabilities of MC/9 cells using the Boyden chamber technique. Migration of MC/9 cells was significantly inhibited by an acidic pH (Fig. 1J). It is unlikely that the observed effects of pH change were due to changes in the viability of MC/9 cells because acidification of the extracellular medium had little effect on the viability of the cells according to the results of the trypan blue exclusion assay (Fig. 1K). These findings indicate that extracellular acidification regulates cytokine production and migration of mouse mast cells. In the following section, we characterize in detail the signaling mechanisms involved in the augmentation of cytokine production.

3.2. Involvement of p38 MAPK and Akt in the actions of acidic pH

We next examined the mechanisms underlying the regulation of cytokine production in mast cells by extracellular acidification. The production of IL-6 and IL-13 via FcεRI reportedly involves the MAPKs and PI3K/Akt signaling pathways [20]. As shown in Fig. 2A, stimulation of DNP-IgE-sensitized MC/9 cells with antigens resulted in marked activation of p38 MAPK, JNK, ERK, and Akt (as demonstrated by their phosphorylation). Although extracellular acidification did not appreciably affect the basal phosphorylation activity of the enzymes, it did significantly stimulate antigen-induced activation of p38 MAPK (Fig. 2B) and Akt (Fig. 2C). Conversely, neither JNK nor ERK activity was affected by the acidic pH (Fig. 2A). These results suggest that p38 MAPK and Akt are

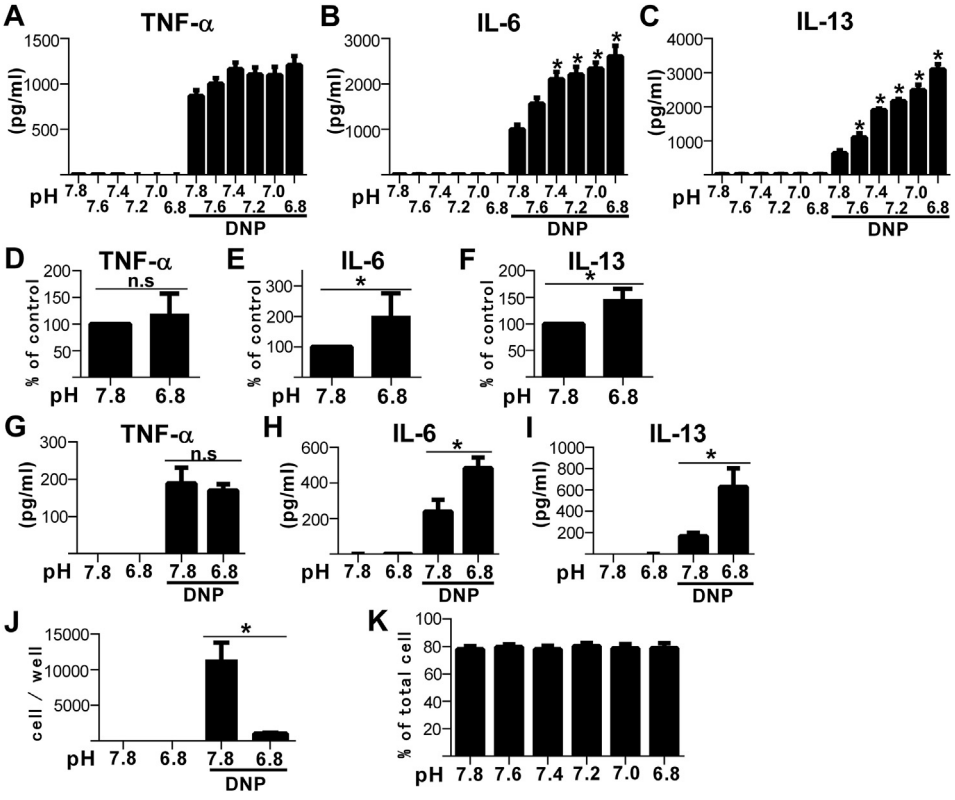

Fig. 1. Effect of extracellular acidification on cytokine production in mouse mast cells. (A–C) MC/9 cells sensitized with anti-DNP IgE were incubated for 3 h with or without 10 ng/ml DNP-HSA at the indicated pH. TNF-α (A), IL-6 (B), and IL-13 (C) in the supernatant were then measured using an ELISA. (D–F) Expression of TNF-α (D), IL-6 (E), and IL-13 (F) mRNA in MC/9 cells sensitized with anti-DNP IgE was examined after 1 h incubation with 10 ng/ml DNP-HSA at pH 7.8 or 6.8. (G–I) BMMCs sensitized with anti-DNP IgE were incubated for 3 h with or without 10 ng/ml DNP-HSA, and then TNF-α (G), IL-6 (H), and IL-13 (I) in the supernatant were measured using an ELISA. (J) Migration of MC/9 cells. The cells sensitized with anti-DNP IgE were placed in the upper wells and 10 ng/ml DNP-HSA was added to the lower wells. (K) The trypan blue exclusion test was used to determine the number of viable cells present in a cell population. *$P < 0.05$ compared with incubated cells at pH 7.8.

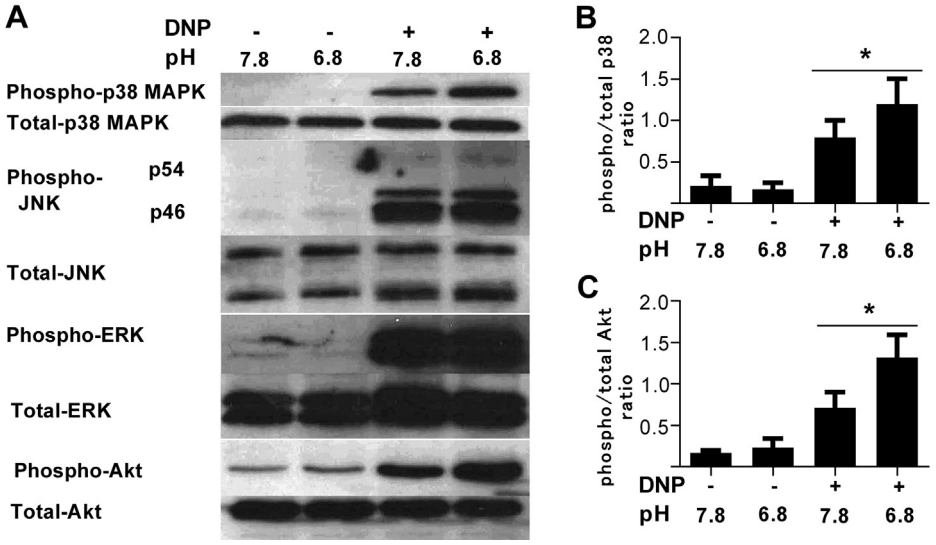

Fig. 2. Effects of acidic pH on MAPKs and Akt. MC/9 cells sensitized with anti-DNP IgE were stimulated with DNP-HSA. After 15 min, phosphorylated and total p38 MAPK, JNK, ERK, and Akt were determined with specific antibodies by using western blotting (A). The phosphorylation versus total activity for p38 MAPK (B) and Akt (C) was assessed using a densitometer. Results are the representatives (A) and the mean ± SEM of normalized values (B and C) obtained from at least three separate experiments. $^{*}P < 0.05$ compared with incubated cells at pH 7.8.

critical for the enhancement of IL-6 and IL-13 production.

The involvement of p38 MAPK and Akt was confirmed by using specific inhibitors. SB203580 and SB239063 (specific inhibitors of p38 MAPK) significantly suppressed extracellular acidification-induced production of IL-6 and IL-13 (Fig. 3A–D) in MC/9 cells. We used PI3K inhibitors to evaluate of the role of Akt because PI3K is known to regulate Akt activity. As expected, wortmannin and LY294002 (specific inhibitors of PI3K) significantly inhibited acidic pH-induced production of IL-6 and IL-13 (Fig. 3E–H) in MC/9 cells. In contrast, U0126, which is a specific inhibitor of MEK1 and MEK2,

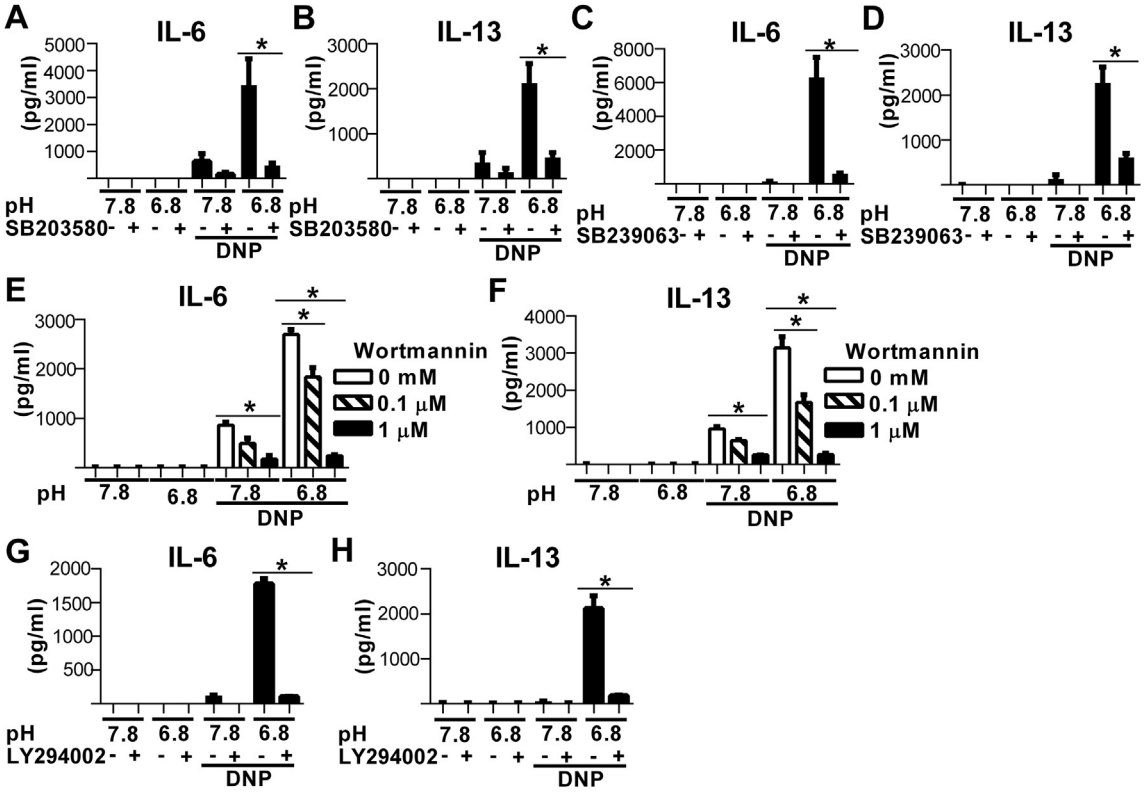

Fig. 3. Effects of p38 MAPK inhibitors or PI3K inhibitors on acidic pH-induced production of cytokine in MC/9 cells. MC/9 cells sensitized with anti-DNP IgE were incubated with 10 ng/ml DNP-HSA for 3 h in the presence or absence of SB203580 (10 μM; A, B), SB239063 (10 μM; C, D), wortmannin (at indicated doses; E, F), and LY294002 (10 μM; G, H). Subsequently, IL-6 (left) and IL-13 (right) were measured in the supernatant. The results are the mean ± SEM of three separate experiments. $^{*}P < 0.05$ compared with DNP-HSA stimulated cells (control cells).

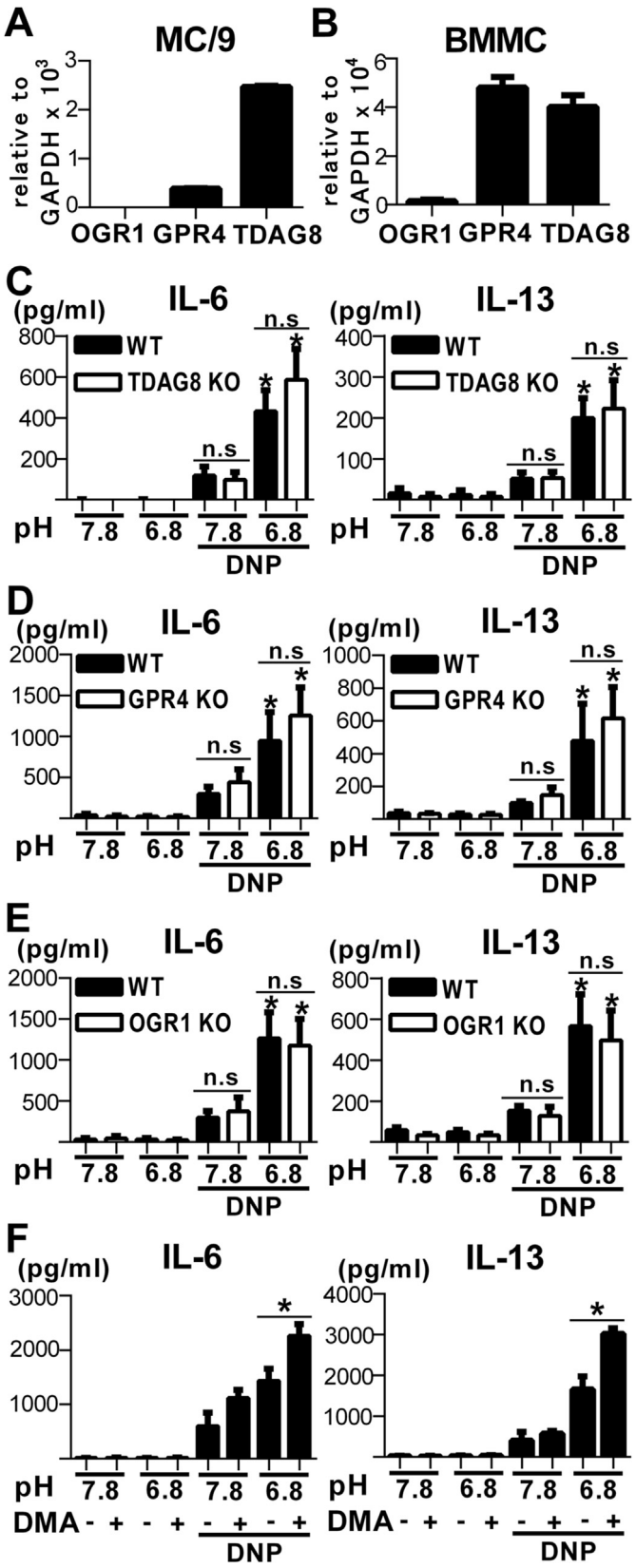

Fig. 4. Proton-sensing GPCR deficiency does not affect acidic pH-induced enhancement of cytokine production. (A, B) Expression of OGR1, GPR4, and TDAG8 mRNA in MC/9 cells (A) and BMMCs (B). (C–E) BMMCs sensitized with anti-DNP IgE from wild-type (WT) mice and those from TDAG8- (C), GPR4- (D), or OGR1-deficient (E) mice were incubated for 3 h with or without 10 ng/ml DNP-HSA. IL-6 (left) and IL-13 (right) were subsequently measured in the supernatant. $^*P < 0.05$ compared with incubated cells at pH 7.8. (F) Anti-DNP IgE-sensitized MC/9 cells were incubated with or without 10 ng/ml DNP-HSA for 3 h at pH 7.8 or 6.8 in the presence or absence of DMA, and IL-6 (left) and IL-13 (right) were then measured in the supernatant. The results represent the mean ± SEM of three separate experiments. $^*P < 0.05$ compared with DNP-HSA stimulated cells (control cells).

did not suppress cytokine production (Supplementary Fig. S1). These results suggest that p38 MAPK and Akt are part of the signaling pathways involved in augmentation of cytokine production by extracellular acidification in MC/9 cells.

3.3. Proton-sensing GPCRs are unlikely to be involved in production of cytokines in mast cells

Recent studies have reported that GPCRs, including TDAG8, GPR4, and OGR1, sense extracellular acidification through histidine residues [21–23]. Thus, these GPCRs can be stimulated under the conditions of pH 6–8. As shown in Fig. 4, TDAG8 and GPR4 mRNAs were expressed in MC/9 cells (Fig. 4A) and BMMCs (Fig. 4B); however, the expression of OGR1 mRNA was minimal or undetectable. The expression of TDAG8 mRNA in mast cells is well consistent with the previous results: TDAG8 has been shown to be expressed in bone marrow-derived cells and lymphoid tissues, including peripheral leukocytes, spleen, thymus, and lymph nodes [24]. Indeed, TDAG8 is expressed highly in microglia, macrophage, and dendritic cells, and moderately in MC/9 and BMMC. In neuron, its expression is not detected (Supplementary Table S1). As to GPR4, it is expressed universally in various cell types, including hematopoietic cells and neuron, except for macrophage. On the other hand, OGR1 is expressed in monocyte-derived cells among hematopoietic cells and neuron (Supplementary Table S1). In order to examine the role of proton-sensing GPCRs, we used receptor-deficient mouse BMMCs. Cells that were deficient in TDAG8 (Fig. 4C), GPR4 (Fig. 4D), and OGR1 (Fig. 4E) exhibited no appreciable effects related to the antigen-induced production of IL-6 and IL-13 cytokines or the enhancement by acidic pH of antigen-induced actions.

The role of GPCRs was further examined by using agents that modulated or mimicked G protein signaling. PTX (a G_o/G_i inhibitor), YM-254890 (a G_q inhibitor), and Y27632 (a Rho kinase inhibitor) had no effect on the responses of IL-6 and IL-13 to extracellular acidification in MC/9 cells (Supplementary Figs. S2–4). We also tested forskolin, which is an activator of adenylyl cyclase, because TDAG8 and GPR4 are coupled with the G_s/cAMP signaling pathway [6]. If cAMP mediated the observed acidification-induced effects, we would expect to find enhanced cytokine responses at neutral pH. However, forskolin inhibited rather than stimulated production of cytokines (Supplementary Fig. S5). These results suggest that the augmentation of cytokine production by MC/9 cells or BMMCs cannot be attributed to an association with known proton-sensing GPCRs.

Extracellular acidification usually causes intracellular acidification. Thus, we examined the possible role of intracellular acidification in MC/9 cells using DMA, which is a Na^+/H^+ exchanger inhibitor that can induce intracellular acidification and decrease intracellular pH in immune cells [25]. As shown in Fig. 4F, DMA increased the IgE-DNP-induced production of IL-6 and IL-13 in MC/9 cells. This result suggests that intracellular acidification might be involved in the enhancement of cytokine production in mast cells.

4. Discussion

In the present study, we reported for the first time that extracellular acidification regulates the cytokine production and migration activity of mast cells. The inflammatory locus in

respiratory infection is characterized by acidic conditions [26,27]. The depression of mast cell migration and enhancement of cytokine production under acidic conditions implies that mast cells may participate in host defense via assembly at the acidic inflammatory locus and in cytokine production. Hunt et al. [3] reported that the pH of exhaled breath condensation was lower in patients with acute asthma than in healthy volunteers and asthma patients treated with corticosteroids. Additionally, Neas et al. [28] found that the onset of asthma is increased by continual exposure to acidic fog. Thus, it is now recognized that airway acidification is involved in the pathogenesis of allergy disease. Since mast cells are pathologically important for asthma, clarification of the relationship between mast cell functions and pH could benefit the development of new medical treatments for asthma.

Acid-sensing ion channels (ASICs) and transient receptor potential vanilloid receptor subtype 1 (TRPV1), which are nociceptive receptors of temperature, acid, and pain, are known as receptors for extracellular protons [5]. Recent studies have shown that these receptors participate in the pathology of asthma [5]. Faisy et al. [29] reported that acidosis induces AHR and that pH-dependent hyperresponsiveness involves ASICs and TRPV1. Although the involvement of ASICs and TRPV1 in acidic pH-related effects was not investigated in the present study, our results suggest that these channels provide a minor contribution (if any at all) to cytokine responses in mast cells. We showed that DMA, which is an inhibitor of Na^+/H^+ exchanger and ASICs, augmented rather than attenuated acidic pH-induced cytokine production (Fig. 4F). We also observed that acidic pH did not affect basal and antigen-induced increases in intracellular Ca^{2+} concentration, which is known to increase with ASICs and/or TRPV1 stimulation (results not shown). Similarly, proton-sensing GPCRs are also unlikely to be involved in the observed cytokine responses to acidic pH because we found that deficiencies of TDAG8, GPR4, or OGR1 had little impact on acidic pH effects. On the basis of the results obtained from testing DMA (see Fig. 4F), we tentatively speculate that intracellular acidification might be involved in the extracellular acidic pH-induced effects reported here. However, further studies are necessary to clarify the mechanisms of proton sensing and signal transduction involved in cytokine responses in mast cells.

Although the molecular basis and events involved in proton-sensing and its early signaling pathways remain unknown, p38 MAPK and Akt may be convergent processes of both FcεRI and uncharacterized proton signaling in the augmentation of antigen/IgE-induced cytokine production under acidic pH conditions. At least three Src-family tyrosine kinase pathways are activated following antigen interactions with IgE via FcεRI in mast cells, i.e., the Lyn/Syk, Fyn, and Hck/Fgr/Yes pathways [30]. Among these, the Lyn/Syk and Fyn pathways are thought to be involved in p38 MAPK and Akt activation, which may function in the upstream signaling pathways of cytokine production and degranulation [20,30]. However, in the present study, extracellular acidification did not affect antigen-induced degranulation in BMMCs, which suggests that the contribution of the p38 MAPK and Akt signaling pathways to cytokine and degranulation responses may differ or that additional signaling events are involved in these functions (Supplementary Fig. S6).

In conclusion, here, extracellular acidification augmented antigen/IgE-induced and FcεRI-mediated IL-6 and IL-13 production in mast cells in association with the enhancement of p38 MAPK and Akt activation. Extracellular acidification also inhibited the migration of mast cells. The cytokine and migration responses observed following extracellular acidification in mast cells might contribute to the aggravation of inflammatory responses at inflammatory loci in allergic diseases (e.g., bronchial asthma and gastrointestinal anaphylaxis). Further characterization of proton targets and related signaling pathways in future research may provide new therapeutic options in the treatment of allergic diseases.

Conflict of interest

The authors have no financial conflicts of interest.

Acknowledgments

We are grateful to Drs. Kyoji Horie and Junji Takeda of Osaka University, Dr. Takao Shimizu of University of Tokyo, and Dr. Satoshi Ishii of Akita University for providing us with TDAG8-deficient mice. We are also thankful to Dr. Masatoshi Taniguchi of Astellas Co., Ltd. (Tsukuba, Japan) for providing YM-254890, and to Ms. Mutsumi Takano of Gunma University for technical assistance.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.07.077.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.077.

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