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Lactic acid suppresses MRGPRX2 mediated mast cell responses

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ABSTRACT

MAS related G-protein coupled receptor X2 (MRGPRX2) is a G-protein coupled receptor (GPCR) expressed in human mast cells that has been implicated to play an important role in causing pseudo-allergic reactions as well as exacerbating inflammation during asthma and other allergic diseases. Lactic acid, a byproduct of glucose metabolism, is abundantly present in inflamed tissues and has been shown to regulate functions of several immune cells. Because the endogenous ligands for MRGPRX2 (substance P and LL-37) are elevated during pathologic conditions, such as cancer and asthma, and given that lactic acid levels are also enhanced in these patients, we explored the role of lactic acid in regulating mast cells response via MRGPRX2 and MrgprB2, the mouse orthologue of the human receptor. We found that lactic acid suppressed both the early (Ca^{2+} mobilization and degranulation) and late (chemokine/cytokine release) phases of mast cell activation; this data was confirmed in LAD2, human skin and mouse peritoneal mast cells. In LAD2 cells, the reduction in degranulation and chemokine/cytokine production mediated by lactic acid was dependent on pH. In agreement with our *in vitro* studies, lactic acid also reduced passive systemic anaphylaxis to compound 48/80 (a known MRGPRX2/MrgprB2 ligand) and skin inflammation in a mouse model of rosacea that is dependent on MrgprB2 expression on skin mast cells. Our data thus suggest that lactic acid may serve to inhibit mast cell-mediated inflammation during asthma and reduce immune response during cancer by affecting mast cell activation through MRGPRX2.

1. Introduction

Inflammatory immune responses are critically mediated by mast cells. Activated mast cells degranulate in a piecemeal or anaphylactic fashion to transiently release inflammatory mediators from stored granules [1]. Principally, mast cells are known for rapid release of histamine, a vasodilator; however, mast cells also release a variety of effectors involved in recruitment of immune cells to the site of pathogenesis [2]. Stimulus-induced mast cell production of cytokines and subsequent recruitment of eosinophils, neutrophils, monocytes, T cells and dendritic cells present a lasting state of inflammation.

Mast cells express IgE receptors and are activated following cross-linking of these receptors by the IgE antibody and allergens [3]. While the IgE pathway in mast cells has been studied extensively in the context of allergy, other pathways operating through specific G-protein coupled receptors (GPCR) also activate mast cells [4–7]. MAS related G-protein coupled receptor X2 (MRGPRX2), a novel GPCR exclusively expressed on mast cell populations, has been identified as a potent activator of these cells [8–9]. Accordingly, MRGPRX2 has been implicated in chronic diseases such as rosacea [10], urticaria [11], atopic dermatitis [12], asthma [13] and rheumatoid arthritis [14]. Cationic amphiphilic molecules like compound 48/80 [8,15], neuropeptides such as substance P [16] and antimicrobial peptides such as LL-37 [17] have all been shown to stimulate MRGPRX2 and induce mast cell activation. Additionally, several FDA–approved drugs that induce anaphylaxis and allergy-like (pseudo-allergic) reactions have been shown to stimulate mast cell–MRGPRX2 and its murine orthologue, MrgprB2 [8,18]. Thus, MRGPRX2 plays a pivotal role in multiple inflammatory pathologies, making this receptor a target for reducing aberrant mast cell response.

Chronic inflammatory and pathologic conditions often present altered metabolism with an increase in lactic acid production. One such example is cancer, where cells shift to a glycolytic metabolism, and ferment lactic acid despite aerobic conditions, in a phenomenon known as the Warburg Effect [19]. Higher serum levels of lactate are also found in patients that have severe allergen- or exercise-induced asthma [20,21]. Lactic acid levels have also been linked to outcomes and/or

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severity in sepsis [22], and pulmonary embolism [23]. The effect of lactic acid on different inflammatory mediators has uncovered its potential role in immune modulation [24–26]. Increased amounts of lactic acid in the external milieu can inhibit glucose consumption and thereby reduce immune cell functions. Thus, it has been shown that lactic acid suppresses the inflammatory functions of macrophages, dendritic cells, and T cells. Furthermore, recent reports have shown that lactic acid inhibits IgE- [27], LPS- [28] and IL-33 [29]-induced mast cell activation. However, it is currently unknown if lactic acid regulates MRGPRX2 mediated responses in mast cells.

MRGPRX2 ligands are upregulated in the cancer microenvironment. Higher expression levels of substance P are found in colorectal [30] and breast cancer patients [31]. Similarly, LL-37 levels are enhanced in ovarian and lung cancer tissues [32]. Additionally, substance P [33] and LL-37 [34] are elevated in the lungs of asthmatic patients. Thus, it is possible that mast cells present in these tissues can be activated by these MRGPRX2 ligands to regulate the cancer and asthma disease progression. Since lactic acid is also present in these tissues, it is imperative to determine the role of this metabolite in regulating mast cell responses via the MRGPRX2 receptor. In the present study, we show that lactic acid treatment reduces intracellular Ca^{2+} mobilization and degranulation responses of human mast cells to the MRGPRX2 ligands: compound 48/80, substance P, LL-37 and *(R)*-ZINC-3573. A similar dose dependent reduction of cytokine/chemokine (CCL2 and interleukin (IL)-8) production was observed when mast cells were treated with lactic acid. In accordance with these *in vitro* findings, passive systemic anaphylaxis to compound 48/80 was severely attenuated in mice that were pre-exposed to lactic acid. Furthermore, lactic acid treatment significantly reduced skin inflammation in a mast cell-dependent murine rosacea model induced by LL-37. Collectively, our data highlight the role of lactic acid as an immunomodulatory metabolite of the mast cell pseudo-allergic receptor, MRGPRX2.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Media (DMEM), Iscove's Modified Dulbecco's Media (IMDM), penicillin, streptomycin and L-glutamine supplement were purchased from Corning Cellgro™ (Corning, NY, United States). Recombinant human stem cell factor (hSCF) and mouse stem cell factor (mSCF) was purchased from PeproTech (Rocky Hill, NJ, United States). Opti-MEM™, Stem-ProTM-34 SFM media, and TRIzol™ were purchased from Invitrogen (Carlsbad, CA, United States). Chemical reagents used in buffers, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO, United States). Compound 48/80, substance P (Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu) and *(R)*-ZINC-3573 were obtained from Sigma-Aldrich. Cathelicidin LL-37 (Leu-Leu-Gly-Asp-Phe-Phe-Arg-Lys-SerLys-Glu-Lys-Ile-Gly-Lys-Glu-Phe-Lys-Arg-Ile-Val-Gln-ArgIle-Lys-Asp-Phe-Leu-Arg-Asn-Leu-Val-Pro-Arg-Thr-Glu-Ser) was purchased from Tocris Bioscience (Minneapolis, MN, United States). Lactic acid and sodium lactate were purchased from Sigma-Aldrich as certified reference materials. Dilutions of lactic acid and sodium lactate were prepared in media, Siraganian (SIR) Buffer (118 mM NaCl, 5 mM KCl, 25 mM HEPES, 5.5 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% w/v bovine serum albumin), or phosphate buffered saline (PBS).

2.2. Mice

Balb/c and C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, United States). All mice were kept under specific pathogen-free conditions. All experiments had the approval of the Institutional Animal Care and Use Committee at Michigan State University. Both age- (6 weeks old) and gender-matched mice were used for experiments.

2.3. Cells

LAD2 mast cells: Human LAD2 mast cells were cultured in complete Stem-Pro-34 SFM medium containing penicillin (100 IU/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM) (PSG) supplemented with recombinant hSCF (100 ng/mL). Media was hemi-depleted once every week and cells were maintained at a concentration of 0.8×10^6 cells/mL.

Human embryonic kidney cells (HEK-293T) cells: HEK-293T cells were transfected with a MRGPRX2 expression plasmid using lipofectamine reagent (Invitrogen) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), PSG and puromycin (3 μg/ml) for selection of stable clones expressing high levels of MRGPRX2 receptor.

Human skin mast cells: As approved by the Internal Review Board at the University of South Carolina, mast cells were purified from fresh surgical specimens of human skin tissues that were purchased from the Cooperative Human Tissue Network (CHTN) of the National Cancer Institute [35]. The mast cells were grown in X-VIVO 15TM media (Lonza) supplemented with hSCF (100 ng/ml) for 8 weeks. Purity was assessed by flow cytometry staining for FcεRI expression with PE-labeled anti-human FcεRI antibody [clone AER-37 (CRA)] and mouse IgG2bk isotype control (BioLegend).

Mouse peritoneal mast cells: The peritoneal cavity of C57BL/6 mice was washed with RPMI media; cells were collected and cultured in RPMI media supplemented with 10% FBS, PSG and mSCF (10 ng/ml) for 3 weeks. Expansion of mast cells was confirmed by heterochromatic staining with Safranin O and Alcian Blue dyes.

2.4. Calcium mobilization

LAD2 cells and HEK-293T cells expressing MRGPRX2 (HEK-MRGPRX2) were washed and resuspended in 1 mL SIR buffer containing 0.1% BSA (SIR-BSA) supplemented with 6 μM Fluo-8 AM calcium dye (Abcam; Cambridge, United Kingdom) for 1 h at 37 °C and 5% CO_2. Cells were washed with SIR-BSA, resuspended in varying lactic acid concentrations and incubated for 30 mins at 37 °C and 5% CO_2. Before the assay, cells were washed with SIR-BSA and 100 μL (0.3×10^6 cells/mL) were plated per well of a 96-well black plate. Using the FlexStation ® 3 Flex-protocol, changes in fluorescence intensities (maximum - minimum values) were measured with the addition of 25 μL of 5X concentrations of compound 48/80, substance P, LL-37 or (*R*)- ZINC-3573. Excitation and emission wavelengths were 490 and 520 nm, respectively.

2.5. Degranulation assay

LAD2 cells, primary human skin-derived mast cells or mouse peritoneal mast cells were incubated for 30 min in concentrations of lactic acid or sodium lactate diluted in SIR-BSA. After incubation, cells were washed and resuspended to 20,000 (LAD2 and human skin mast cells) or 10,000 (PMCs) cells per 45 μL of SIR-BSA ($\sim 0.45 \times 10^6$ cells/mL) and plated at 45 μL per well of a 96-well plate. Cells were then stimulated with 5 μL of 10X concentrations of compound 48/80, substance P, LL-37 or (*R*)-ZINC-3573 for 30 mins. Cell-free supernatants (20 μL) were collected and incubated with equal volumes of 4 mM p-nitrophenyl-N-acetyl-β-D-glucosamine (PNAG, purchased from Sigma-Aldrich) for 1 h. The enzymatic assay was halted after 1 h by 5X volume of 0.1 M $NaHCO_3$/0.1 M Na_2Co_3 buffer. Absorbance was measured using FlexStation® 3 multi-mode plate reader (Molecular Devices; San Jose, CA, United States) at 405 nm. The total β-hexosaminidase content was measured by lysing 45 μL of cell suspension with 1 μL of 0.1% Triton X-100 and then incubating the supernatant of the lysed cells with PNAG for 1 h. Percent β-hexosaminidase release (degranulation) was calculated by first blanking each absorbance value with the absorbance of SIR-BSA buffer incubated with equal volume of PNAG and 5X volume of $NaHCO_3$/0.1 M Na_2Co_3 buffer. Blanked values were then divided by the total absorbance value measured from the lysed cell supernatant.

2.6. ELISA

LAD2 cells were washed twice in cytokine-deprived complete StemPro-34 SFM media, then 150 μL (1×10^6 cells/mL) were plated and incubated with different concentrations of lactic acid for 30 mins and stimulated with corresponding agonists for 6 h. Cells were centrifuged, and supernatants were collected. Cytokines/chemokines (IL-8 and CCL2) in the supernatants were quantified by ELISA (ELISA kits purchased from Invitrogen).

2.7. Western blotting

LAD2 cells (4×10^6 cells) were incubated with lactic acid (8.3 mM) for 30 min, washed stimulated with compound 48/80 (100 ng/ml) for different time intervals and lysed using radioimmunoprecipitation assay (RIPA) (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium-deoxycholate, 0.1% sodium dodecyl sulfate, 25 mM Tris [pH 8.0], 5 mM EDTA) buffer with protease inhibitor cocktail (Roche Applied Sciences; Mannheim, Germany). Twenty μg of protein was loaded in a 10% polyacrylamide gel for electrophoretic separation. Proteins were then transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked in 5% milk solution for 2 h, washed in Tris-buffered saline [pH 7.6] with 0.1% Tween-20 (TBST), then probed with primary antibodies (anti-phospho-p44/42 (ERK1/2), anti-p44/42, anti-phospho-AKT and anti-AKT). The following day, blots were washed in TBST and probed with LiCor IRDye® 680RD or IRDye® 800CW conjugated secondary antibodies for 2 h in the dark. Blots were imaged using LiCor Odyssey Imaging Systems (Lincoln, NE).

2.8. Passive systemic anaphylaxis (PSA)

C57BL/6J mice were first injected i.p with lactic acid (125 mg/kg, dissolved in PBS) or PBS alone. Sixteen hours later, mice received i.p. injections of compound 48/80 (3.75 mg/kg) to induce anaphylaxis. Core body temperature was measured using a rectal thermometer probe (Physitemp Instruments, Clifton, NJ).

2.9. Cathelicidin LL-37 induced Rosacea model

Balb/c mice received intraperitoneal (i.p.) injections of vehicle (PBS) or lactic acid (4 mg/kg) for 3 days. Intradermal injections (i.d.) of LL-37 (50 μL of 320 μM) was administered to the dorsal skin twice a day for 2 days while continuing the lactic acid treatment. Mice were euthanized and dorsal skin containing site of LL-37 injections was harvested. Dorsal skin was divided in two so that half could be snap-frozen in liquid N_2 for RNA analysis and half could be fixed in 10% formalin solution for H&E or Toluidine Blue staining. For epidermal thickness measurements, 10 random epidermal areas in H&E stained skin sections from each mouse were chosen and measured following the acquisition of images using a Nikon ® ECLIPSE 50i microscope equipped with a Lumenera ® Infinity 3 color camera. For assessing *in vivo* mast cell degranulation, skin tissues were stained with toluidine blue (0.1% in PBS, pH 2.3) and images were captured as described above. Degranulated mast cells (as determined by the staining intensity, appearance and/or location of the granules) were counted and expressed as percentage of total mast cells in the tissue sections [36].

2.10. Real-time PCR

Skin samples taken from mice were homogenized in liquid N_2 using a mortar and pestle. RNA was extracted using TRIzol™ reagent according to the manufacturer's protocol. RNA (2 μg) was transcribed to cDNA using the high capacity cDNA reverse transcription kit from Applied Biosystems (Foster City, CA, United States). RNA levels (*Ccl2, Il6, Tnf, Mmp9, Tpsab1* and *Cma1*) were quantified using gene expression assays with TaqMan™ Fast Advanced Master Mix and validated TaqMan™ probes obtained from Applied Biosystems.

2.11. Statistical analysis

Statistical analyses were performed using GraphPad PRISM software (San Diego, CA) and explained in the figure legends or the results sections. A *p*-value less than or equal to 0.05 was deemed to be significant.

3. Results

3.1. Lactic acid attenuates Ca^{2+} mobilization and degranulation in LAD2 human mast cells

Activation of LAD2 mast cells with the MRGPRX2 ligands such as compound 48/80, substance P and LL-37 results in early phase Ca^{2+} mobilization and degranulation[37]. To determine the role of lactic acid on Ca^{2+} mobilization, we first used HEK-MRGPRX2 cells and performed fluorometric Ca^{2+} assays. As reported previously [37], exposure to the MRGPRX2 ligands; compound 48/80, substance P, and LL-37 resulted in a substantial Ca^{2+} influx as shown by a rapid increase in fluorescence intensity measurements following ligand additions as compared to BSA treated cells. The changes in fluorescence intensity values ranged from 0 to 10 for BSA and 15–300 for the different ligands. (Fig. 1). We also used a recently identified MRGPRX2-specific synthetic ligand [(*R*)-ZINC-3573] [38] for our assays and as expected, (*R*)-ZINC-3573 induced robust intracellular Ca^{2+} mobilization in HEK-MRGPRX2 cells (changes in fluorescence intensity values ranged from 0 to 10 for BSA and 110–120 for (*R*)-ZINC-3573). As shown in Fig. 1A–D lactic acid treatment significantly reduced Ca^{2+} influx in HEK-MRGPRX2 cells in a dose-dependent manner to all the 4 ligands tested.

To directly test the effects of lactic acid in regulating MRGPRX2 responses in mast cells, we used the immortalized human mast cell line, LAD2. LAD2 cells endogenously express MRGPRX2 and respond to mast cell activators, providing a physiologically relevant *in vitro* model system. Substance P, compound 48/80, LL-37, and (*R*)-ZINC-3573 all induce Ca^{2+} mobilization in LAD2 cells through MRGPRX2. Consistent with our data with HEK-MRGPRX2 cells, pretreatment of LAD2 cells with lactic acid substantially reduced the intracellular Ca^{2+} mobilization response induced by the MRGPRX2 ligands (Fig. 2A–D).

Following Ca^{2+} influx, mast cells release preformed, high density granules containing inflammatory mediators, various proteases, and lysosomal hydrolases in a process defined as degranulation. β-hexosaminidase, a hydrolase found in the majority of mast cell granules [39], is rapidly released to the extracellular environment and its presence corresponds to the early phase of the mast cell response. Degranulation of MRGPRX2-stimulated LAD2 cells, as measured by β-hexosaminidase enzymatic assay, was significantly attenuated in a dose dependent manner following lactic acid pre-exposure (Fig. 2E–H).

3.2. Lactic acid suppresses cytokine/chemokine production following MRGPRX2 stimulation in LAD2 mast cells

Mast cell activation not only comprises of early events that include intracellular Ca^{2+} mobilization and degranulation but also includes a delayed phase that ultimately results in inflammatory chemokine/cytokine production and their release. To test whether lactic acid regulated the delayed response of mast cell activation, we exposed LAD2 cells to varying concentrations of lactic acid and assessed for chemokine and cytokine secretion by ELISA. We specifically chose to examine the production of CCL2 and IL-8 since LAD2 cells release these effectors upon MRGPRX2 stimulation [37]. LAD2 cells produced both CCL2 and IL-8 on stimulation with compound 48/80 (Fig. 3A and B) and substance P (Fig. 3C and D). Interestingly, lactic acid treatment inhibited the release of these inflammatory mediators.

The Ca^{2+} mobilization and degranulation assays in Figs. 1 and 2 were performed with lactic acid in Siraganian (SIR) buffer. As shown in

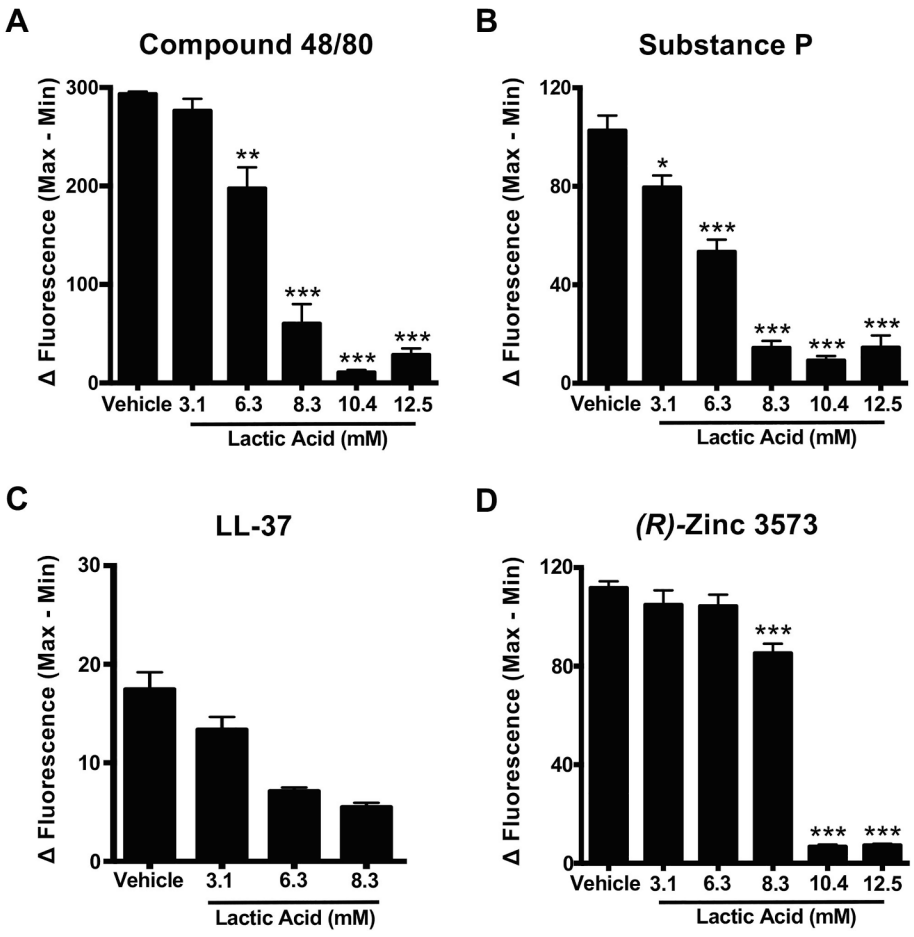

Fig. 1. *Intracellular Ca^{2+} mobilization induced by MRGPRX2 ligands is reduced by lactic acid in HEK 293T cells expressing MRGPRX2.* HEK-293T cells stably expressing the MRGPRX2 receptor were treated with vehicle (SIR buffer) or lactic acid (indicated concentrations) for 30 min. (A-D) Ca^{2+} mobilization assays were performed following incubation with (A) compound 48/80 (300 ng/ml), (B) substance P (100 nM), (C) LL-37 (0.5 μM) or (D) *(R)*-Zinc 3573 (0.5 μM). Data are plotted as the change in fluorescence intensity [minimum (Min) subtracted from maximum (Max) value] measurements. Results shown are mean ± S.E. of 3 independent experiments. Statistical significance was determined by unpaired Student's *t*-test and values from the lactic acid-treated group was compared with the vehicle group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Supplementary Fig. 1A, the pH values ranged between 6.8 and 5.7 for lactic acid concentration from 0 to 7.5 mM in SIR buffer. The pH does decrease to 4.4 and lower at higher concentrations (10–15 mM). However, the cell viability (determined using trypan blue dye exclusion) was ~ 75–80% in the presence of lactic acid (0–10 mM concentration) in the SIR buffer (Supplementary Fig. 1B) suggesting that LAD2 mast cells are moderately resistant to changes in extracellular pH. For the chemokine/cytokine production experiments (Fig. 3), lactic acid was dissolved in complete Stem-Pro-34 SFM media. In this case, the pH values of the media containing lactic acid (0–25 mM) ranged between 7.7–6.8 (Supplementary Fig. 1C). The cells were > 80% viable under these conditions (Supplementary Fig. 1D). Thus, the concentrations of lactic acid did affect the extracellular pH; however, the suppressive role of this metabolite on human mast cells was largely independent of its cytotoxic effects.

Previous reports have established that MRGPRX2-induced mast cell activation results in downstream signaling events that activates AKT and the MAP kinase, ERK1/2 [40]. These signaling pathways ultimately regulate mast cell degranulation and cytokine production [41]. Since lactic acid attenuated the functional responses of mast cells, we hypothesized that it likely affects upstream signaling events such as MAP kinase and AKT activation. Accordingly, we exposed LAD2 cells to compound 48/80 for different time intervals following exposure to lactic acid and analyzed MAP kinase and AKT activation by Western blotting. Robust phosphorylation of ERK1/2 and AKT was evident within 10 min following exposure to compound 48/80 (Fig. 3E). Surprisingly, ERK1/2 and AKT phosphorylation was not altered by treatment with lactic acid (Fig. 3E). Overall, our data demonstrates that lactic acid attenuates both the early and delayed stages of mast cell activation via MRGPRX2 independent of ERK1/2 and AKT activation.

3.3. MRGPRX2-mediated degranulation of primary skin-derived human mast cells is reduced by lactic acid

Connective tissue mast cells expressing the MRGPRX2 receptors include tissue resident, mature skin mast cells [11]. Primary human mast cells were cultured from the skin tissues of 4 donors and treated with or without lactic acid (8.3 mM) and exposed to MRGPRX2 ligands (compound 48/80, substance P, LL-37 and (*R*)-ZINC-3573) for degranulation assays (Fig. 4). We observed differences in percent degranulation for each ligand between donor samples. Four different forms of MRGPRX2 have been reported in humans [42] and the variation in degranulation between donors possibly reflects this finding. Regardless, lactic acid treatment reduced the degranulation of skin mast cells obtained from all donors to the MRGPRX2 agonists (Fig. 4).

3.4. The acidic moiety in lactic acid is responsible for its suppressive effects on MRGPRX2 responses in mast cells

Both degranulation and cytokine/chemokine production in LAD2 cells exposed to compound 48/80 or substance P was reduced in the presence of lactic acid. A recent report by Abebayehu et al., [27] showed that the acidic component in lactic acid was responsible for its inhibitory effects on mast cells following IgE/antigen stimulation and when lactic acid was replaced with sodium lactate the inhibitory effect was abolished. To assess whether a similar mechanism also regulated lactic acid effects on MRGPRX2-induced responses in mast cells, LAD2 cells were pretreated with equivalent concentrations of lactic acid and sodium lactate. Sodium lactate showed a modest effect on degranulation to compound 48/80 and substance P as compared to lactic acid; the reduction in degranulation by sodium lactate was not as robust as lactic

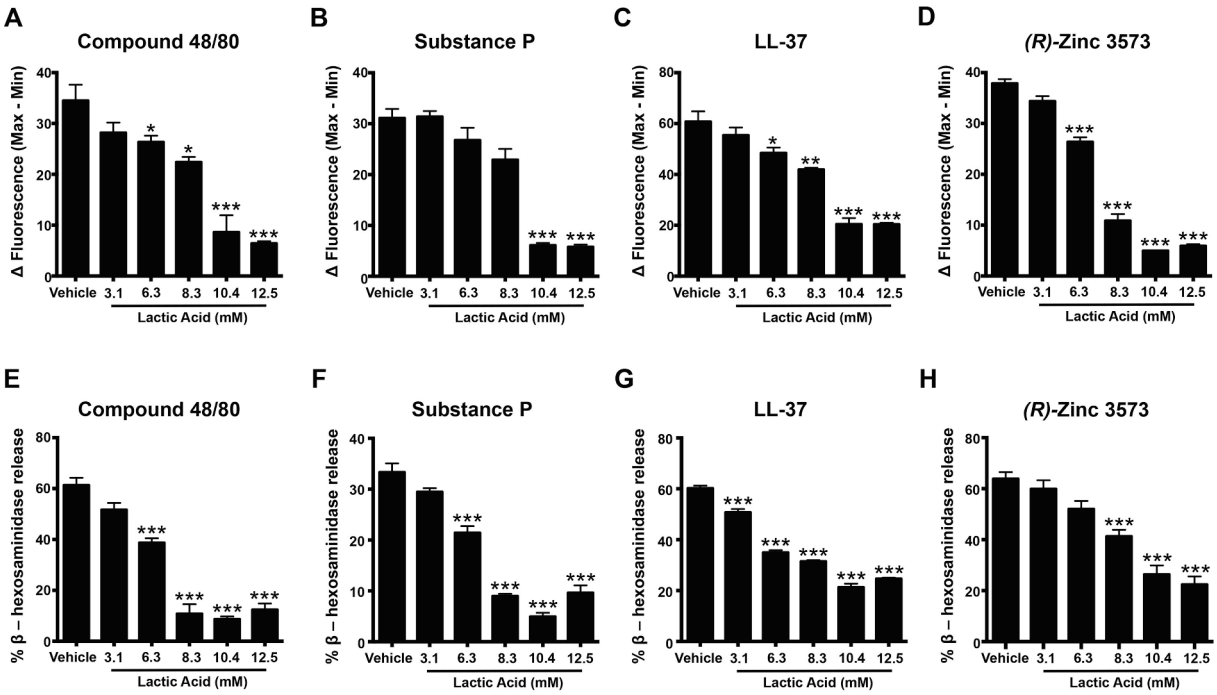

Fig. 2. *Lactic acid inhibits MRGPRX2-induced Ca^{2+} mobilization and degranulation in LAD2 mast cells.* (A-D) Intracellular Ca^{2+} mobilization and (E-H) degranulation in LAD2 human mast cells was determined following pre-incubation with vehicle (SIR buffer) or varying concentrations of lactic acid for 30 min. Cells were stimulated with the MRGPRX2 agonist (A) compound 48/80 (300 ng/ml), (B) substance P (300 nM), (C) LL-37 (3 μM) or (D) *(R)*-Zinc 3573 (2 μM) and changes in fluorescence intensities were recorded for 90 *sec*. Data are plotted as the change in fluorescence intensity values following ligand additions. Vehicle- or lactic acid-treated cells were exposed to (E) compound 48/80 (300 ng/ml), (F) substance P (300 nM), (G) LL-37 (3 μM) or (H) *(R)*-Zinc 3573 (2 μM) and degranulation was quantified by β-hexosaminidase release. Values are plotted as percentages of total cell lysate β-hexosaminidase content. Data shown are mean ± S.E. of 3 independent experiments. Statistical significance was determined by unpaired Student's *t*-test with values compared between the lactic acid- and vehicle-treated groups. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

acid (Fig. 5A and B).

We next tested whether sodium lactate also affected MRGPRX2-mediated CCL2 and IL-8 production in LAD2 cells. Cells were treated with matching concentrations of lactic acid or sodium lactate 30 min prior to compound 48/80 or substance P exposure and the supernatants were analyzed for the presence of CCL2 and IL-8 by ELISA. Consistent with previous results, lactic acid suppressed cytokine production and release in a dose-dependent manner for both the ligands (Fig. 6A–D). Surprisingly, sodium lactate did not reduce MRGPRX2-mediated CCL2 production in LAD2 cells (Fig. 6A and C). However, it did inhibit IL-8 production in LAD2 cells, albeit not as potently as lactic acid (Fig. 6B and D). Collectively, these data suggest that the acidic moiety of lactic acid was responsible for most of its suppressive effects.

3.5. *Lactic acid administration attenuates mast cell-dependent inflammation in vivo*

MrgprB2 is the mouse counterpart of human MRGPRX2 that is also activated by compound 48/80, substance P and LL-37 [8]. These ligands have been reported previously to induce pseudo-allergic phenotypes in animal models. Hence, our next goal was to test the role of lactic acid in regulating mast cell MrgprB2 responses *in vivo*. We adopted a previously described model of compound 48/80-induced systemic anaphylaxis that is dependent on MrgprB2 expression on mouse mast cells [8]. Control vehicle or lactic acid-treated mice were injected with compound 48/80 i.p. and core body temperature measurements were performed every 10 mins for 120 mins. We observed a drastic decrease in body temperature of control vehicle treated mice that received compound 48/80 (Fig. 7A). This response, however, was significantly reduced in the lactic acid-treated cohort. In a separate experiment we injected mice with lactic acid i.p. and isolated the peritoneal lavages at 10, 60 and 120 min after the injection. The pH values of the lavage supernatant ranged between 7.0 and 7.1 in for all samples. Our data thus suggests that lactic acid probably affects compound 48/80 mediated peritoneal mast cell activation resulting in decreased anaphylaxis reaction. To test this contention directly, we cultured peritoneal mast cells *ex vivo* and performed degranulation experiments following stimulation with compound 48/80, substance P and LL-37. As expected, peritoneal mast cells degranulated in the presence of the ligands, but this response was significantly reduced when the cells were pre-exposed to lactic acid (Fig. 7B–D).

We next investigated the effects of lactic acid in a more severe skin inflammation model of pseudo-allergic rosacea. The cathelicidin LL-37 is elevated in the skin tissues of human patients with rosacea, and consequently, it has been used for inducing the pathogenesis of experimental rosacea in rodents [43–44]. Specifically, Muto et al. [10], showed that the LL-37 injections in the skin causes rosacea like symptoms in mice that are dependent on the presence of mast cells. LL-37-induced Ca^{2+} mobilization and degranulation were attenuated by lactic acid in our experiments with human mast cells (Figs. 3 and 4). To directly test whether lactic acid inhibited mouse mast cell response *in vivo*, we pre-treated Balb/c mice with vehicle or lactic acid, followed by LL-37 administration in the dorsal skin. 72 h post exposure to LL-37, erythema and inflammation of the dorsal skin was observed. Skin samples at the site of injection were collected for histological staining and RNA analysis of inflammatory mast cell activation markers. Our data suggests that lactic acid treatment reduced epidermal thickness induced by LL-37 injections (Fig. 8A and B). Moreover, RNA analysis of inflammatory markers also corroborated with the attenuated skin inflammation in the lactic acid-treated cohort. Specifically, mRNA levels of mast cell inflammatory mediators such as CCL2, IL-6, TNF and MMP9 were significantly decreased in the presence of lactic acid (Fig. 8C). Interestingly, classical markers of mast cell activation, TPSAB1 (tryptase α-1

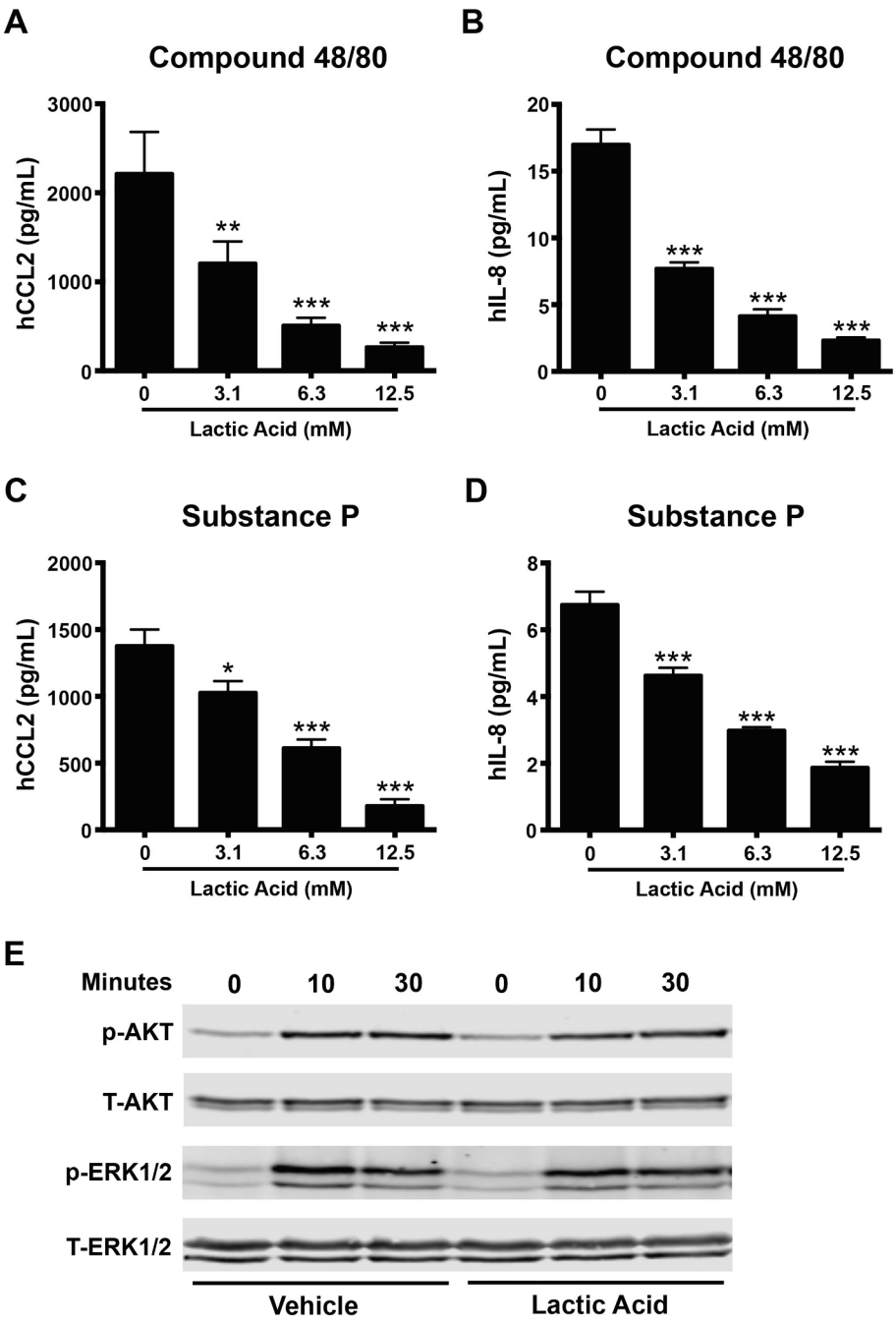

Fig. 3. *Lactic acid suppresses cytokine/chemokine production but does not affect ERK1/2 or AKT activation in LAD2 cells.* (A-F) LAD2 cells (0.15×10^6 cells/well) were exposed to vehicle (SIR buffer) or different concentrations of lactic acid for 30 min, washed and exposed to, (A, B) compound 48/80 (300 ng/ml) or (C, D) substance P (300 nM) for 6 h. Supernatants were collected and analyzed for (A, C) CCL2 and (B, D) IL-8 by ELISA. Data shown are mean ± S.E. of 3 independent experiments performed in triplicates. Statistical analysis was done using unpaired Student's *t*-test by comparing the vehicle- and lactic acid-treated groups. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. (E) LAD2 cells were treated with vehicle (SIR buffer) or lactic acid (8.3 mM) and then exposed compound 48/80 (300 ng/ml) for different time intervals. Western blotting was performed to detect phosphorylated ERK1/2 (p-ERK1/2) or AKT (p-AKT) proteins. The blots probed with ERK1/2 (T-ERK1/2) and AKT (*T*-AKT) antibodies for loading controls. Images of representative blots from 3 independent experiments are shown.

and tryptase β-1) and CMA1 (chymase) were also significantly reduced in the lactic acid treated group compared to the vehicle treated group (Fig. 8D). To directly test if lactic acid affected mast cell responses in this model, we enumerated the numbers of degranulated and non-degranulated mast cells in the skin tissues of mice (Fig. 8E and F). Dorsal skin sections were stained with toluidine blue, a cationic dye that binds to heparin found in mast cell granules. Degranulated mast cells and total mast cells in the sections were counted in a blinded manner manually with microscopy. Degranulated mast cells in the skin do not stain as densely as naive mast cells and appear with less defined boundaries. While there was no difference in the total numbers of mast cells between the vehicle and the lactic acid-treated cohorts of mice, the percentage of degranulated mast cells was significantly reduced in the lactic acid-exposed mice as compared to the control vehicle-treated group (Fig. 8E and F). Thus, these data demonstrate that lactic acid significantly reduced mast cell-induced inflammation associated with pseudo-allergic reactions *in vivo*.

4. Discussion

Dysfunctional inflammatory responses can present altered metabolic environments with the presence of anaerobic metabolites. Lactic acid, a prominent byproduct of anaerobic respiration, has been implicated in the progression of various immune-related pathologies and plays an important role in altering responses of different cell types. High levels of lactate coincide with the tumor microenvironment, tissue injury and healing, asthma and many other inflammatory processes [20–21,45]. The effects of lactic acid on inflammation and immunity are diverse and vary with the pathological condition and the cell types involved. In the current study, we investigated the role of lactic acid in modulating

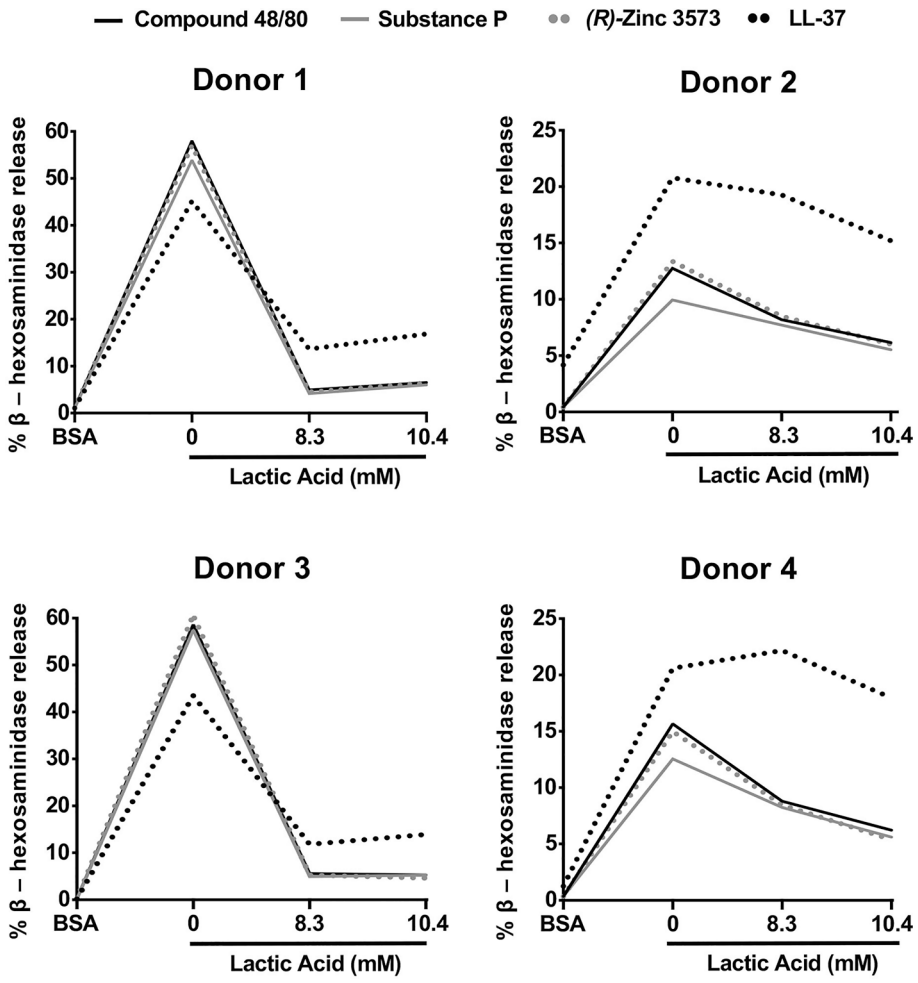

Fig. 4. *Lactic acid attenuates MRGPRX2-mediated degranulation in human skin mast cells.* Human skin-derived mast cells from 4 different donors were pre-treated with vehicle (SIR buffer) or lactic acid (8.3 mM and 10.4 mM) and exposed to compound 48/80 (1000 ng/ml), substance P (1 μM), LL-37 (5 μM) or *(R)*-Zinc 3573 (10 μM). Line graphs show degranulation of mast cells from different donors as estimated by β-hexosaminidase release in supernatants.

MRGPRX2-induced mast cell activation *in vitro* and *in vivo*. Our data reveals that lactic acid attenuated both the early (Ca^{2+} mobilization and degranulation) and late (MCP-1 and IL-8 production) phases of mast cell activation to several MRGPRX2 ligands. Consistent with these observations, lactic acid also suppressed passive systemic anaphylaxis to compound 48/80 and experimental rosacea mediated by LL-37 injections in animal models.

Classically, mast cells are associated with allergic reactions that are primarily induced the IgE-IgE receptor pathway. However, in addition to the IgE receptor, mast cells also express a diverse set of transmembrane receptors such as the toll like receptor (TLR4) and the receptor for IL-33 (ST2). Accordingly, previous reports have investigated the inhibitory effects of exogenous lactic acid on mast cell response through the IgE [27], ST2 [29], and TLR4 [28] receptors. Lactic acid acts as a negative regulator for mast cells when stimulated via these receptors. Mast cells also express a number of GPCRs that elicit robust degranulation and cytokine production to mediate inflammation. In particular, MRGPRX2, a promiscuous GPCR, has been established as a potent activator of mast cells in response to endogenous ligands (substance P and LL-37) [5] and clinically relevant, FDA-approved pharmacological agents such as fluoroquinolone antibiotics (ciprofloxacin), neuromuscular blocking agents (rocuronium), morphine, vancomycin etc [8]. Consequently, MRGPRX2 plays a pivotal role in injection site reactions and pseudo-allergy in humans. Some of the endogenous MRGPRX2 ligands are upregulated in pathological conditions. For example, substance P levels are increased in colorectal cancer [30], breast cancer [31] and asthma patients [33]. Additionally, ovarian cancer, lung cancer and lung tissues from asthmatic patients have enhanced levels of LL-37 [32,34]. Given that mast cells are also present in these tissues, it is possible that the inflammatory response is modulated by mast cell activation via the MRGPRX2 receptor. Our observation that lactic acid inhibits mast cell response may thus in part, explain the immune suppression occurring during cancer as a result of decreased mast cell activation.

Lactic acid produces low pH solutions that may affect cell viability resulting in decreased responses to the MRGPRX2 agonists. Our data with cytotoxicity assays showed that LAD2 mast cells survived in lactic acid concentrations up to 10 mM without any significant cell death. Additionally, the levels of lactate found in certain pathologies can reach a very high concentration of up to 40 mM [46,47]. All of our *in vitro* experiments were thus carried out at physiologically relevant lactic acid concentrations that showed no effect on mast cell viability. Furthermore, our data suggests that the primary effect of lactic acid was on the cells rather than the MRGPRX2 ligands because the cells were pre-treated with lactic acid and then cleaned with lactic acid-free buffers before ligand exposure for all experiments.

An interesting finding of our study was that lactic acid dose-dependently inhibited mast cell Ca^{2+} influx and degranulation for all the ligands tested despite the pharmacological differences between the agonists. Compound 48/80 is an organic polymer produced by the condensation of *N*-methyl-p-methoxyphenethylamine with formaldehyde. Substance P is a neuropeptide that comprises 11 amino acid

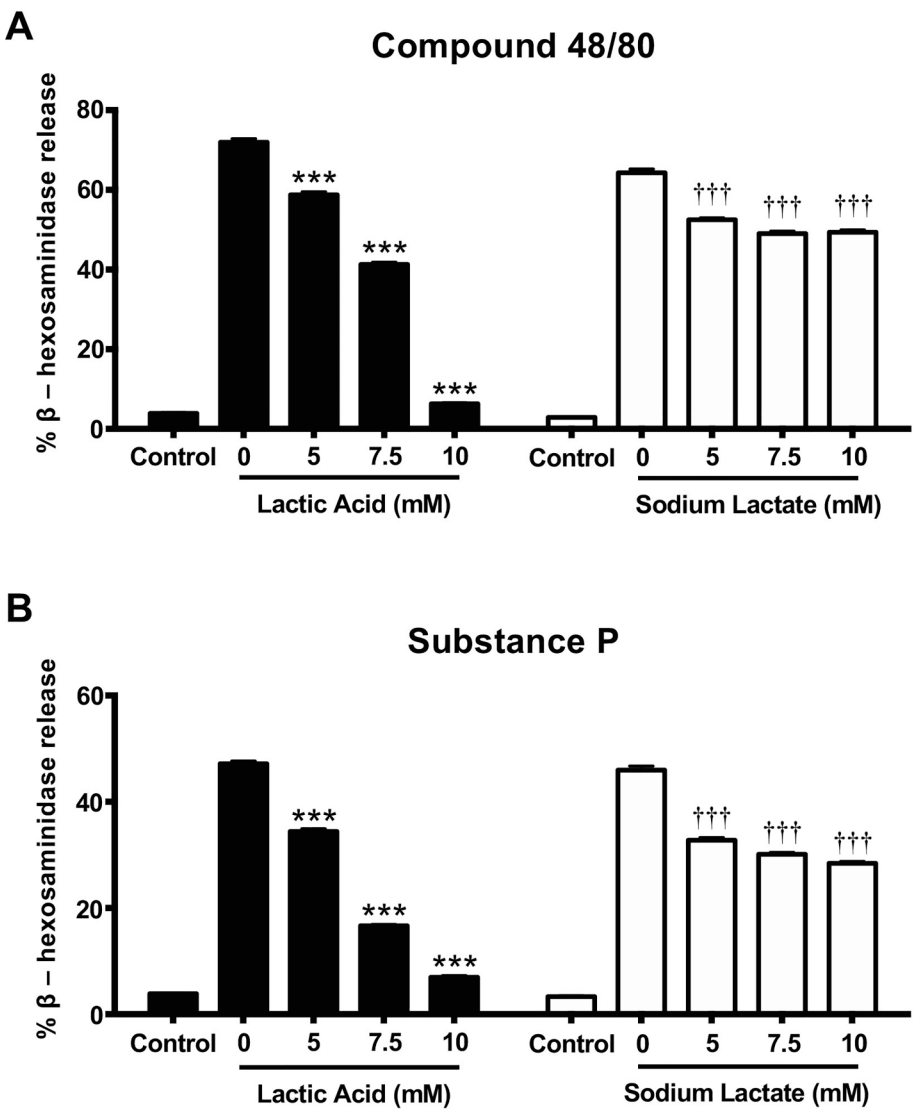

Fig. 5. *The acidic proton moeity in lactic acid contributes to its effects on degranulation of LAD2 cells to MRGPRX2 agonists*. LAD2 cells were exposed to vehicle (SIR buffer, control) or varying concentrations of lactic acid or sodium lactate for 30 mins. The cells were washed and stimulated with the MRGPRX2 agonist (A) compound 48/80 (300 ng/ml), (B) substance P (300 nM). Degranulation was quantified by β-hexosaminidase release. Values are plotted as percentages of total cell lysate β-hexosaminidase content. Data shown are mean ± S.E. of 3 independent experiments. Statistical significance was determined by unpaired Student's *t*-test with values. *** $p < 0.001$ indicates values compared between the lactic acid- and vehicle-treated groups. ††† $p < 0.001$ indicates values compared between the sodium lactate- and vehicle-treated groups.

residues and LL-37 is a C-terminal cleavage product of the human cathelicidin protein, hCAP18, comprising 37 amino acids. (*R*)-ZINC-3573 is a small synthetic organic compound that is a selective ligand for MRGPRX2 [38]. All of these ligands induce varying levels of mast cell activation; compound 48/80 is a more potent activator of mast cell MRGPRX2 as compared to LL-37 [40]. Regardless, lactic acid inhibits mast cell response induced by all of these ligands, suggesting that the effects of this metabolite are largely restricted to downstream receptor activation following ligand engagement. Our data that lactic acid reduced Ca^{2+} influx in HEK 293T cells expressing MRGPRX2 is consistent with our observations with LAD2 and primary human skin mast cells and further confirms that the inhibitory effect of lactic acid is specific for the MRGPRX2 receptor. Given that MRGPRX2/MrgprB2 stimulation of mast cells also induce histamine and tryptase secretion [48,49] along with the release of β-hexoseaminidase, it would be interesting to determine if lactic acid also affects the release of these mediators for future studies.

LL-37, an endogenous ligand for MRGPRX2 induces rosacea, a chronic skin inflammatory condition in mice [10,17]. Mice treated with lactic acid before sub-dermal exposure to LL-37 showed substantially lower symptoms of rosacea; notably, decreased *trans*-epidermal thickness and reduced expression of mast cell-derived pro-inflammatory cytokines (*Ccl2*, *Tnf* and *Il6*) in the skin tissue. In agreement with this, we also observed a significant attenuation of CCL2 and IL-8 production by human LAD2 cells in the presence of lactic acid in our *in vitro* assays. Interestingly, mRNA levels of *Cma1* and *Tpsab1*, markers of mast cell activity [50], were also significantly reduced implying that lactic acid specifically modulates skin mast cells. Skin sections stained with toluidine blue and assessed for mast cell degranulation further confirmed that suppressing LL-37-induced rosacea with lactic acid is at least in part, mast cell-dependent. In contrast to the rosacea model, the passive systemic anaphylaxis murine model is an acute model of inflammatory response. Mast cell activation and subsequent release of histamine and other mediators elicits vasodilation and increased vascular permeability resulting in a reduction in core body temperature. Mice treated with lactic acid 18 h before exposure to compound 48/80 showed significantly lower anaphylaxis reaction. This model relies on activating peritoneal mast cells, a connective tissue type that expresses MrgprB2, the mouse orthologue of human MRGPRX2. Accordingly, we also observed decreased degranulation of peritoneal mast cells in the presence of lactic acid to compound 48/80, substance P and LL-37.

Lactic acid has two chemical moieties that may play a role in suppression of mast cell response: an acidic proton and the lactate anion. MCT-1, a co-transporter expressed on all cell types brings both the lactate anion and the acidic proton into the cell [51]. The mode of action for suppressing mast cell activation by lactic acid could therefore be via one or both of chemical moieties it presents. Abebayehu et al., [27] recently reported that the acidic moiety in lactic acid was important for

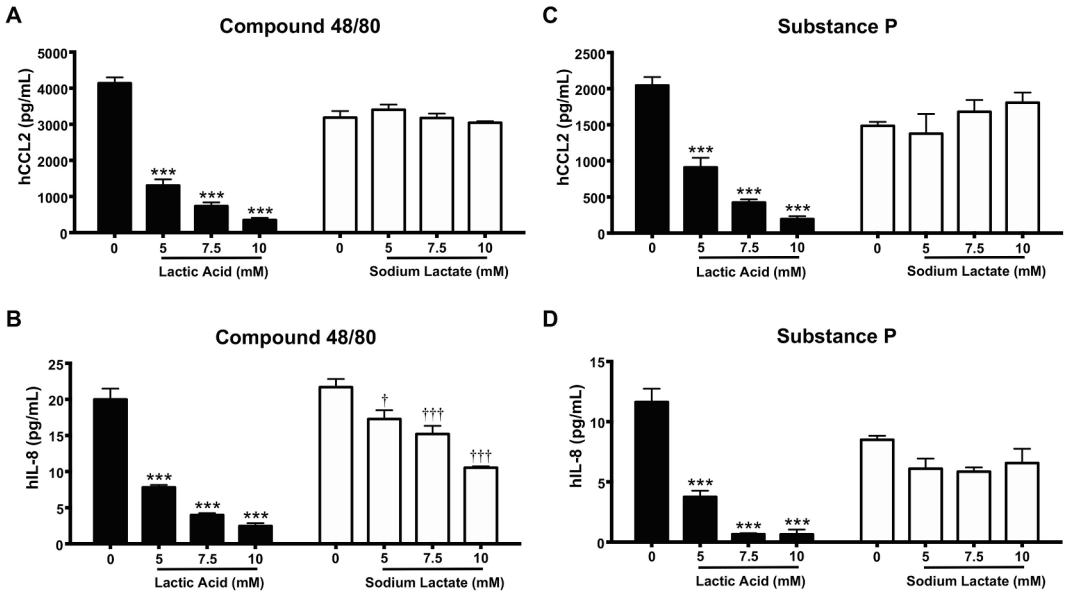

Fig. 6. *Sodium lactate has no effect on chemokine/cytokine production in LAD2 cells.* LAD2 cells were exposed to vehicle (SIR buffer, indicated by "0″) or different concentrations of lactic acid or sodium lactate, washed and exposed to, (A, B) compound 48/80 (300 ng/ml) or (C, D) substance P (300 nM) for 6 h. Supernatants were collected and analyzed for (A, C) CCL2 and (B, D) IL-8 by ELISA. Data shown are mean ± S.E. of 3 independent experiments performed in triplicates. Statistical analysis was done using unpaired Student's *t*-test by comparing the control vehicle- and lactic acid-treated groups. *** $p < 0.001$ indicates values compared between the lactic acid- and vehicle-treated groups. † $p < 0.05$ and ††† $p < 0.001$ indicates values compared between the sodium lactate- and vehicle-treated groups.

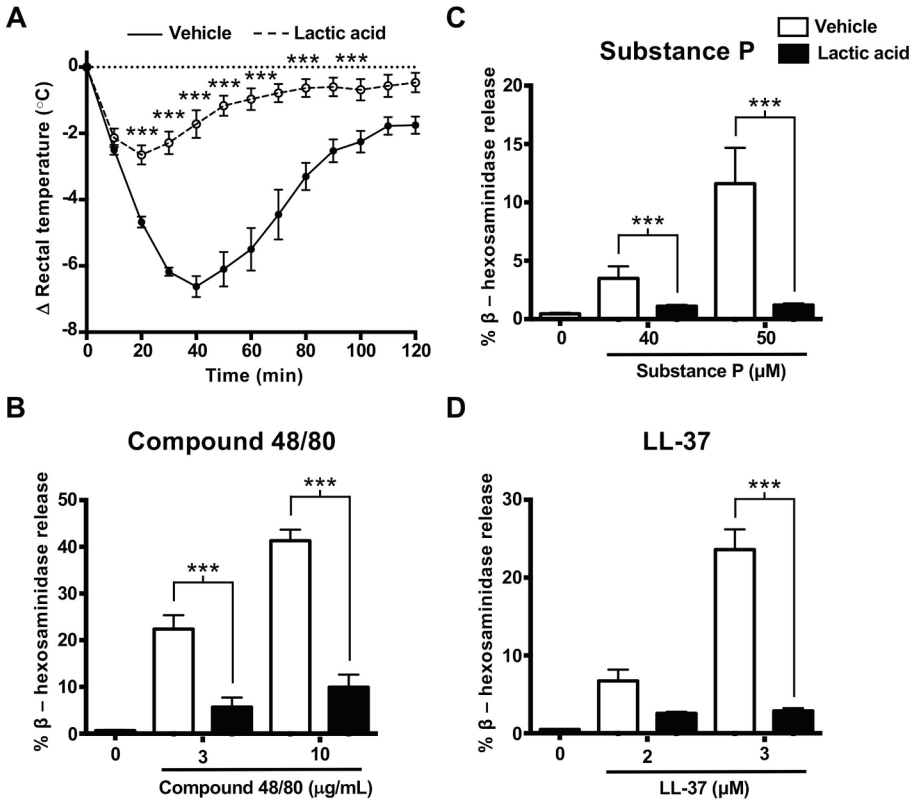

Fig. 7. *Lactic acid reduces systemic anaphylaxis to compound 48/80 in vivo.* (A) Vehicle- (PBS) or lactic acid- (125 mg/kg) treated C57BL/6 mice were exposed to compound 48/80, (C48/80, 3.75 mg/kg) and rectal temperature was measured at different time points. Line graph shows change in rectal temperature for the different groups of mice. Data shown are mean ± S.E. from 3 experiments (a total of n = 10 mice/group). Statistical significance was determined by Student's *t*-test. (B-D) Mouse peritoneal cells were exposed to vehicle (SIR buffer) or lactic acid (8.3 mM) and stimulated with the indicated concentrations of (B) compound 48/80, (C) substance P or (D) LL-37. Graphs shows degranulation of mast cells as estimated by β-hexosaminidase release in supernatants. Data shown are mean ± S.E. from 3 experiments (a total of n = 3–5 mice/group). Statistical significance was determined by unpaired Student's *t*-test. *** $p < 0.001$.

its suppressive effect on IgE-induced mast cell activation. To determine the effects of pH (acidic proton), we used equivalent concentrations of lactic acid and sodium lactate for *in vitro* degranulation and chemokine release assays. While sodium lactate did inhibit LAD2 degranulation via MRGPRX2, it was not as effective as lactic acid at same concentrations. This result suggests that the acidic proton residue plays a significant role, whereas the lactate anion is less important in inhibiting mast cell degranulation. Moreover, because of the absence of the acidic proton in sodium lactate, it is unlikely that lactate was transported into the cells through MCT-1, possibly suggesting that the relatively low inhibitory effect of the lactate anion was due its presence outside the cell or on the cell surface. In contrast, cytokine release was not affected by treatment

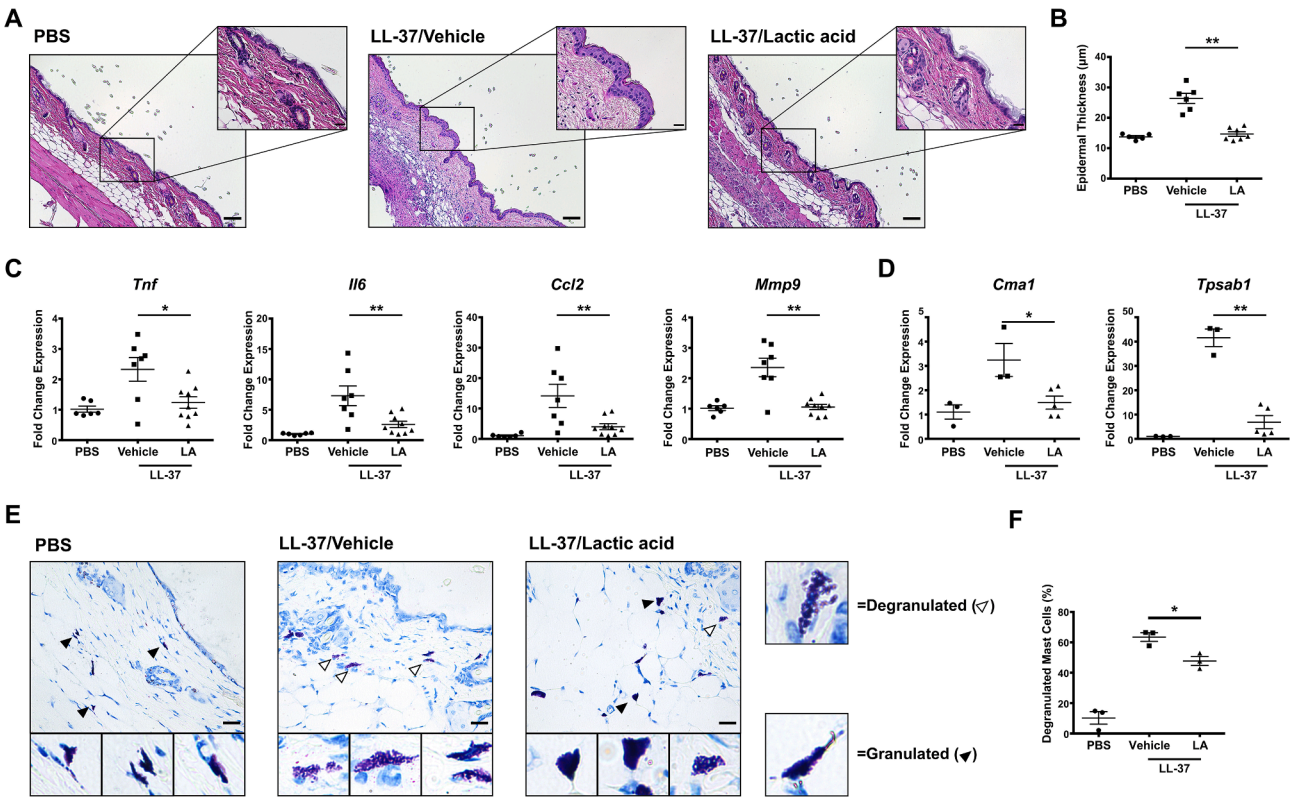

Fig. 8. *Lactic acid prevents the development of LL-37-induced rosacea in mice.* Vehicle- (LL-37/Vehicle) or lactic acid (LL-37/Lactic acid) treated Balb/c mice were injected with LL-37 into the dorsal skin twice daily for 2 consecutive days. Mice that only received PBS on the dorsal skin were used as control. (A) H&E stained skin sections of mice from different cohorts are shown. Scale bar = 100 μm. (B) Graph represents epidermal thickness of the H&E stained skin sections as measured by Image J. (C, D) mRNA expression of selected gene targets from the excised skin was analyzed by real-time PCR. Values are plotted as fold change ($2^{-\Delta\Delta Ct}$) normalized to GAPDH levels. (E) The paraffin embedded skin sections from different cohorts of mice were stained with toludine blue to detect mast cells. Representative pictures of the skin sections are shown. Bold closed arrowheads indicate intact mast cells whereas open arrowheads represent degranulated mast cells. The inset figure is an enlarged image of the mast cell(s) shown in the pictures. Scale bar = 100 μm. (F) Graph shows the percentage of degranulated mast cells in the skin tissue of different cohorts of mice. Data are mean ± S.E. from n = 3–9 mice/group. Statistical significance was determined by unpaired Student's *t*-test comparing the vehicle- vs lactic acid-treated groups. * $p < 0.05$ and ** $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with sodium lactate following stimulation with compound 48/80 and substance P. These data thus imply that the acidic proton is the main component in lactic acid that is necessary for its effects on mast cells and uptake of lactic acid is imperative for attenuation of mast cell response to MRGPRX2 ligands.

In an effort to identify the mechanism through which lactic acid inhibits mast cell MRGPRX2 responses, we determined phosphorylation of the MAP kinase ERK1/2, and AKT proteins. Both of these pathways are stimulated following intracellular Ca^{2+} mobilization by MRGPRX2 agonists and consequently regulate both the early and late phases of mast cell activation [41]. Western blotting analysis revealed no significant effects of lactic acid on activation of ERK1/2 or AKT, despite a clear reduction in mast cell degranulation and cytokine production to MRGPRX2 ligands. This observation is quite surprising given that, lactic acid inhibits the activation of MAP kinases following IgE [27] and IL-33 [29] stimulation. Currently it is unclear how this metabolite regulates MRGPRX2 activation. Because intracellular Ca^{2+} influx promotes mast cell degranulation, it is likely that the reduced degranulation observed when mast cells are treated with lactic acid in our experiments is actually due to the decreased Ca^{2+} mobilization occurring in these cells. Alternatively, it is also possible that lactic acid affects downstream signaling pathways such as activation of transcription factors like NFκB to affect mast cell responses. Whether these or other mechanisms regulate lactic acid effects on MRGPRX2 signaling is currently being investigated in our laboratory. In summary, our *in vivo* and *ex vivo* data suggests that lactic acid modulates both the early and late phases of mast cell activation following MRGPRX2 stimulation. Given that MRGPRX2 ligands such as substance P and LL-37 are upregulated in the cancer microenvironment [30–32] and that lactic acid levels are increased in cancerous tissues [19], the regulation of mast cell MRGPRX2 responses by lactic acid may provide a possible explanation for immune suppression observed during pathological conditions such as cancer.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2021.104422.

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