

IMPACT OF IL-33 ON PGD2 GENERATION BY ACTIVATED HUMAN CORD BLOOD-DERIVED MAST CELL: LACK OF EFFECT ON TRYPTASE RELEASE

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Mast cells are important not only in allergic reactions, but also in inflammation and are involved in a variety of responses including the immediate release of potent inflammatory mediators after activation by cross-linking of FcεRI molecules. Prostaglandin D2 (PGD2) is a major cyclooxygenase metabolite of arachidonic acid produced by mast cells and it is released following allergen challenge in allergic diseases. IL-33 is an inflammatory cytokine which is critically involved in the regulation of *in vitro* and *in vivo* cyclooxygenase production, providing a potential therapeutic target for inflammatory disorders. In this study, using human derived umbilical cord blood mast cells, we show that IL-33 (50 ng/ml), and calcium ionophore A 23187 (0.5 µg/ml), compound 48/80 (10⁻⁵ M) or anti-IgE (10 µg/ml), enhanced the production of PGD2 and this effect was inhibited by indomethacin. However, IL-33 was unable to induce tryptase release in these cells. These effects confirm the inflammatory property of IL-33 by stimulating PGD2 but not tryptase in human mast cells. The inhibitory effect of this new cytokine may have a potential therapeutic response in allergic and inflammatory diseases.

Cytokines are inflammatory and immune-regulatory proteins that play a central role in the pathogenesis of allergic diseases (1-9) and inflammation (10-14). However, some cytokines are anti-inflammatory such as IL-4, IL-10, IL-1 receptor antagonist (IL-1RA) and IL-37. Cytokines of the interleukin-1 (IL-1) family, such as IL-1 alpha/beta, IL-18 and IL-33, have also an important function in

host defence, immune regulation, and inflammation (15-20). The mediators released by mast cells, such as histamine, tryptase, serotonin and arachidonic acid products: PGD₂, LTC₄, LTD₄, LTE₄ induce, in general, vasodilation, bronchoconstriction, cellular chemotaxis and increased vascular permeability (21-25). Most of the eicosanoids generated by mast cells are either PGD₂ or LTC₄ (26-29).

Key words: IL-33, mast cells, inflammation, PGD2

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It has been reported that IL-1 β , IL-18 or IL-33 induced phosphorylation of Erk, p38 and JNK in naïve HDUCBMCs, and IL-33 or IL-1 β , but not IL-18, enhanced the survival of naïve HDUCBMCs (30-34). Prostaglandin D₂, the major cyclooxygenase metabolite generated from immunologically and non-immunologically stimulated mast cells, is thought to contribute to the pathogenesis of allergic diseases due to its various inflammatory effects (35-38).

The ability of mast cells to generate or release the vasoactive/spasmogenic mediators histamine, PGD₂, sulfidopeptide leukotrienes, platelet-activating factor and other factors is thought relevant to immediate broncho-spastic responses in association with mucus secretion and airway edema (39-41). Using human-derived umbilical cord blood mast cell (HDUCBMC), it is pertinent therefore, to evaluate the effect of IL-33 on PGD₂ generation in HDUCBMC cultures.

MATERIALS AND METHODS

Cells and materials

Reagents for cell culture were purchased from Sigma-Aldrich. HDUCBMC were cultured in Iscove's Modified Dulbecco's medium supplemented with 10% bovine calf serum, 1.2 mM monothioglycerol, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin either in 25 cm² tissue culture plates or in six-well tissue culture plates (Costar). Cells were plated at a density of 0.2 $\times 10^6$ per ml taken from the 3-day-old culture grown under the same conditions. Cells were grown in an incubator in a 5% CO₂ and 95% O₂ at 37°C.

Human derived cord blood mast cell isolation, culture and treatment.

Umbilical cord blood was collected in tubes containing 10 U/ml heparin and diluted 1:2 with Dulbecco's phosphate buffered saline (DPBS) from GIBCO BRL (Life Technologies, Grand Island, NY) containing 2 mM ethylenediamine-tetra acetic acid (Sigma). Non-phagocytic mononuclear cells were separated by density-gradient centrifugation using Lymphocyte Separation Medium (LSM). The isolation of hematopoietic stem cells (CD34+) was performed by positive selection of CD34+/AC133+ cells by magnetic associated cell sorting (MACS) using an AC133+ cell isolation kit (Miltenyi Biotec, Auburn, CA). CD133 expression is restricted to a subset of CD34 bright positive stem cells in human cord blood. Mast cells had been obtained by culturing cord blood mononuclear cells in the presence

of SCF, IL-6. CD34+ cells were suspended in Iscove's Modified Dulbecco's Medium (IMDM; GIBCO BRL), supplemented with 100 ng/ml rhSCF, 50 ng/ml IL-6, 10% fetal bovine serum, 5 $\times 10^{-5}$ M2-Mercaptoethanol, and 1% penicillin-streptomycin (GIBCO BRL) for 12 to 16 weeks. During this culture period, the cells were washed with DPBS every week and resuspended using fresh complete culture medium. Mast cell viability was determined by trypan blue (0.3%) exclusion and observed under light and electron microscope. For sensitization, HDUCBMCs were washed with DPBS and plain culture medium (without any growth factors) once in each and resuspended in serum-free complete culture medium, but without IL-6 supplementation. Cells (1 $\times 10^6$ cells/ml) were then incubated with human myeloma-IgE (2 μ g/ml/10⁶ cells) at 37°C for 48 h in 24-well Falcon cell culture plates. These sensitized HDUCBMCs were used in all the experiments and treated for 12 h with IL-33, A23187, 48/80 or anti-IgE.

Activation of HCBMC with anti-IgE

Anti-Human IgE (DAKO Rabbit anti-Human IgE, Specific for Epsilon-chains. Code number A 0094; Lot no. 069. Edition 05.07.00) was used in this study at 10 μ g/ml. For anti-IgE stimulation experiments, HDUCBMC (10⁶ cells/ml) were washed with Iscove's modified Dulbecco's medium and Tyrode's buffer, once in each, and passively sensitized by incubation with human myeloma IgE (2 μ g/ml/10⁶ cells; Chemicon Inc.) for 48 h in culture medium at 37°C. Cells were then washed two times and resuspended in fresh culture medium. Cells were stimulated with anti-IgE (Dako) at 15 μ g/ml in 96-well round bottom culture plates (1 $\times 10^6$ cells/ml in 200 μ l medium/ sample) for 12 h at 37°C in 5% CO₂.

Immunoassays for tryptase

Tryptase was measured in the supernatants and cell pellets by UniCAP Tryptase Fluoroenzyme-immunoassay System (Pharmacia Diagnostics AB, Uppsala, Sweden) as reported previously (Schwartz LB 1994). Briefly, anti-tryptase covalently coupled to ImmunoCAP® reacts with the tryptase in the specimen. After washing, enzyme-labeled antibodies against tryptase are added to form a complex. After incubation, unbound enzyme-anti-tryptase is washed away and the bound complex is then incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate is measured in the FluoroCount™ 96 microplate reader. The fluorescence is directly proportional to the concentration of tryptase in the sample. To evaluate the test results, the response for the samples is compared directly to the response of the standards. Tryptase was measured in the supernatants and cell pellets by fluoroenzyme-immunoassay (Pharmacia,

Uppsala, Sweden). These peptides were measured in the supernatant and pellet to calculate percent release.

Tryptase release assay: For tryptase measurements, HDUCBMC were sensitized with human myeloma IgE for 48 h in culture medium and were then stimulated for different periods of time in Tyrode's buffer (133 mM NaCl, 4 mM KCl, 0.64 mM KH_2PO_4 , 10 mM HEPES, 1 g/L glucose, 1 mM CaCl_2 , 0.6 mM MgCl_2 and 0.03% human serum albumin, pH 7.2). Results were expressed in ng/ 10^6 cells/ml.

Determination of cell viability

HDUCBMC were harvested, washed in PBS and centrifuged for 5 min at 400x g at room temperature. Cells were then resuspended in culture medium, 0.1% trypan blue solution was added for 5 min at room temperature and the cells were counted using a haemocytometer. Viability was 99%, expressed as percentage of cells which do not take up trypan blue.

Stimulation of HDUCBMC

Calcium ionophore A23187 (0.5 $\mu\text{g/ml}$), compound 48/80 (10^{-5} M), anti-IgE (10 mg/ml) or IL-33 (50 ng/ml) at different dilutions were made directly in HDUCBMC culture medium. In separate tubes, in each experiment, cells were exposed to the vehicle alone, to determine non-specific release. HDUCBMC (1×10^6 cells/ml) in a six well tissue culture dish were washed with culture medium containing 1 mg/ml bovine serum albumin (BSA) and without Ca^{++} to reduce spontaneous secretion. They were then sensitized in the same medium for 30 min at 37°C with 2 ml of mouse monoclonal anti-DNP IgE (500 ng/ml). After sensitization the cells were washed again and treated for 60 min at 37°C with 2 ml of DNP-BSA (10 ng/ml) in the same medium, but now supplemented with 0.5 mM calcium to permit secretion. Control samples without IgE were run simultaneously in the presence of 0.5 mM Ca^{++} and these values represented non-specific release. The cells were also activated for 12 h with calcium ionophore A23187 (0.5 $\mu\text{g/ml}$), compound 48/80 (10^{-5} M), anti-IgE (10 mg/ml) or IL-33 (50 ng/ml) and PGD₂ was calculated.

ELISA for PGD₂

All the experiments were performed with HDCBMC obtained over about 8 weeks. Briefly, the cells were cultured for 12 h with and without reagents to be tested with anti-IgE (10 mg/ml), 48/80 (10^{-5} M), A23187 (0.5 $\mu\text{g/ml}$), and IL-33 (50 ng/ml). After incubation, the cells were centrifuged at 4000 rpm. for 8 min. The supernatants were removed and stored at -20° C. The levels of the PGD₂ were determined by a PGD₂-specific ELISA. The kit is composed of 96 determinations and the assays were

performed according to the manufacturer's instructions. The ELISA reader was set at 405 nm absorbance and all the samples were read after 1 h after addition of stop solution. The wells were protected from light and incubated at room temperature.

The antiserum anti-PGD₂ (rabbit) was reconstituted in phosphate buffer containing BSA and sodium azide, as recommended by the manufacturer. PGD₂ alkaline phosphatase conjugate was supplied ready to use in bicarbonate buffer containing 0.1% BSA, magnesium chloride and sodium azide at pH 9.8 and stored at 4°C. PGD₂ was determined in cell-free supernatants with commercial ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's directions (sensitivity of the assay, 3 pg/ml).

Statistical analysis

All assays were performed in triplicate. The results were expressed as mean \pm SD. Data from three different experiments were combined and reported as the mean \pm SD. Student's *t*-test for independent means was used to provide statistical analyses ($p > 0.05$ was considered as not significant). Comparisons were made using ANOVA.

RESULTS

Prostaglandin D₂ secretion from purified HDUCBMC

The release of prostaglandin D₂, from preparations of purified HDCBMC was determined. The production of prostanoid PGD₂ was examined after mast cell activation. PGD₂ was the prostanoid produced after stimulation of human derived umbilical cord blood mast cells with the IL-33 (50 ng/ml), calcium ionophore A 23187 (0.5 $\mu\text{g/ml}$), compound 48/80 (10^{-5} M) or anti-IgE (10 $\mu\text{g/ml}$). The production of PGD₂ was apparent after 12 h (Table I) and was still detectble after 48 h (data not shown).

The principal cyclooxygenase products after 12 h was PGD₂; while other arachidonic acid compounds appeared if any in much lower concentrations (data not shown). Indomethacin (10^{-6} M) markedly inhibited the IL-33 (50 ng/ml), calcium ionophore A 23187 (0.5 $\mu\text{g/ml}$), compound 48/80 (10^{-5} M) or anti-IgE (10 $\mu\text{g/ml}$) induced release of PGD₂ from HDCBMC and completely suppressed evoked PGD₂ synthesis without evident effect on histamine release (data not shown), suggesting that the release of histamine in this model is not dependent on

Table I. PGD2 production from HDUCBMC (106 cells/ml) following the addition or not of IL-33 (50 ng/ml) and the corresponding samples treated with the calcium ionophore A23187 (0.5 µg/ml), anti-IgE or C48/80

HDUCBMC PGD2	ng/ml/10 ⁶ cells	p
Control	0.8 +/- 0.5	*
A23187 (0.5 µg/ml)	6.5 +/- 1.9	0.001
Anti-IgE (10 mg/ml)	3.7 +/- 0.4	0.001
C48/80 (10⁻⁵ M)	4.7 +/- 1.2	0.001
IL-33 (50 ng/ml)	2.8 +/- 0.6	0.005
Anti-IgE 10 mg/ml + Indo. 10⁻⁶ M	1.7 +/- 1.0	N.S.
A23187 (0.5 µg/ml) + Indo. 10⁻⁶ M	0.9 +/- 0.7	N.S.
C48/80 (10⁻⁵ M) + Indo. 10⁻⁶ M	1.2 +/- 0.8	N.S.
IL-33 (50 ng/ml) + Indo. 10⁻⁶ M	1.0 +/- 0.8	N.S.

The cells were cultured for 12 h at 37°C, 5% CO₂. P values (Student's t-test) are calculated by comparing treated HDUCBMC with untreated cells. The values ± S.D. are representative of three experiments in triplicate.

prostaglandin production. The ability of the cells to secrete PGD₂ appears as a cytochemical marker of the *in vitro* mast cells.

Tryptase released from HDUCBMC

HDUCBMCs have tryptase containing granules and receptors for IgE. In this study HDUCBMC were cultured with IL-33 (50 ng/ml) and a classic specific secretagogue anti-IgE, or a non-specific compounds such as calcium ionophore A23187 (0.5 µg/ml) or compound 48/80 (10⁻⁵ M). Table II shows in three representative experiments the release of tryptase following HDUCBMC treatment and incubated for 60 min with or without IL-33, C48/80, anti-IgE (10 µg/ml) and A23187. Anti-IgE, calcium ionophore A23187 (0.5 µg/ml) or compound 48/80 (10⁻⁵ M) strongly stimulated tryptase on HDUCBMC; while IL-33 was unable to do so.

DISCUSSION

Mast cells play a key role in the induction of allergic disorders, through the release of mediators including histamine, arachidonate products, proteases and several cytokines, which are found in relatively high quantities in these cells (42-46). When activated by diverse stimuli, mast cells mobilize arachidonic acid through cytosolic phospholipase A₂, and rapidly generate both prostaglandin D₂ (47-51). IL-33 induces biological effects on mast cells and T cells through its receptor IL-33Rβ-chain and may have pro-inflammatory potential effects similar to the cytokines from the same family (52-56). IL-33 is found to be highly expressed in high endothelial venules, tonsils, lymph nodes, and Peyer's patches, in vessels from inflamed tissues, in the intestine of patients with Crohn's disease, and in the synovium

Table II. Tryptase (ng/ml) release from HCBDMC (106 cells/ml) following the addition or not of IL-33 (50 ng/ml) and the corresponding samples treated with calcium ionophore (A23187) (0.5 µg/ml), C48/80 (10⁻⁵ M) or anti-IgE (10 µg/ml).

(pg/ml)		
TREATMENT	10 ⁶ CELLS	p values
Spontaneous	420±100	-
Anti-IgE (10 µg/ml)	610±110	0.005
IL-33 (50 ng/ml)	470.5±90	N.S.
A23187 (0.5 µg/ml)	700±115	0.005
C 48/80 (10 ⁻⁵ M)	680±8	0.005

The cells were cultured for 60 min. at 37°C, 5% CO₂. P values (Student's t-test) are calculated by comparing untreated HDUCBMC with anti-IgE, calcium ionophore (A23187), C48/80 or IL-33 treated cells. The values ± S.D. are representative of three experiments in triplicate.

of patients with rheumatoid arthritis (57-61). IL-33 is known to enhance experimental allergic inflammation by directly stimulation mast cells to produce inflammatory cytokines (62-65). Therefore, the mast cell is known as a conductor cell in allergic inflammation (66-67). Prostaglandin such as PGD₂ is the major prostanoid secreted from the activated mast cell and is an important mediator in the pathogenesis of allergy (68-73). However, the direct effect of IL-33 on PGD₂ generation by human mast cells is not known. Here, in this study, we found that IL-33 along with the physiological stimulator anti-IgE or with non-physiological compound A23187 or C48/80, strongly stimulated the production of PGD₂ in HDUCBMC. The stimulatory effect PGD₂ release was completely blocked by a non steroidal anti-inflammatory drug such as indomethacin. We also studied the effect of IL-33 on tryptase release by HDUCBMC and found that IL-33 did not increased the production of tryptase; while anti-IgE, A23187 or C48/80 had a strong stimulator effect.

These results suggest that IL-33 may play an important role in inflammatory diseases by activation PGD₂ an arachidonic acid product released from mast

cells. These results also indicated that IL-33 may be critically involved in regulation of cyclooxygenase production *in vitro* and probably *in vivo*, providing a potential therapeutic target for inflammatory disorders.

Our study shows that IL-33 activated HDUCBMCs can synthesize significant quantities of PGD₂ which may be important in anaphylactic reactions. Prostaglandin D₂ produced by mast cells in acute allergic reactions *in vivo* may contribute to the inflammatory reaction, either directly or in synergism with other mediators. The findings from these *in vitro* studies suggest that PGD₂ secreted from activated mast cells after IL-33 treatment, may be involved in the formation and/or maintenance of allergic inflammation through its dual receptor systems without involving the activation of tryptase system.

PGD₂ is the major cyclooxygenase product released from sensitized human lung and bronchoalveolar lavage mast cells, which possesses a myriad of biological actions relevant to the pathogenesis of asthma (74-79). Thus, modulation of the effects of products of arachidonic acid metabolism by IL-33 may provide a new and more specific treatment for asthma and allergy (80-85).

The localization of the arachidonic acid cascade to the secretory granule explains why the production of eicosanoids is so intimately tied to the process of granule exocytosis.

Asignificant number of therapeutic approaches for allergies have been designed based on antagonising specific mediators released from mast cells and on selectively inhibiting the activation of these cells (86-89). Here, since IL-33 is a strong stimulator of PGD₂ in human mast cells, the inhibition effect of this new cytokine may have a potential therapeutic response in allergic and inflammatory diseases.

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