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# Interleukin-6 potentiates FcεRI-induced $PGD_2$ biosynthesis and induces VEGF from human *in situ*-matured skin mast cells

Cody McHale[a], Zahraa Mohammed[a], Juline Deppen[b,1], Gregorio Gomez[a,*]

[a] Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, SC, USA
[b] Department of Biomedical Engineering, University of South Carolina School of Medicine, Columbia, SC, USA

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ABSTRACT

*Background:* Interleukin-6 is a gp130 utilizing cytokine that is consistently associated with allergic diseases like asthma and urticaria in humans where mast cells are known to play a critical role. However, the role of IL-6 in allergic disease in not known. IL-6 was reported to enhance degranulation of *in vitro*-derived mast cells, but the effect of IL-6 on mediator release from human *in situ*-matured tissue-isolated mast cells had not been reported.
*Methods:* Human mature mast cells were isolated and purified from normal skin tissue from different donors. The expression of surface-expressed IL-6 receptors was demonstrated by flow cytometry. The effect of IL-6 on FcεRI-induced degranulation, $PGD_2$ biosynthesis, and cytokine production was determined with β-hexosaminidase release assay, Western blotting, quantitative real-time PCR, and ELISA. The small molecule inhibitor of STAT-3, C188-9, was used to demonstrate STAT3 dependency.
*Results:* IL-6 significantly potentiated FcεRI-induced $PGD_2$ biosynthesis, but had no effect on degranulation. IL-6 also induced VEGF gene expression and protein secretion, and enhanced FcεRI-induced IL-8 production. Mechanistically, IL-6 enhanced FcεRI-induced COX-2 expression, PGD2 biosynthesis, and VEGF production in a STAT3 dependent manner.
*Conclusion:* Here, we demonstrate that IL-6 is a potentiator of FcεRI-induced $PGD_2$ biosynthesis, and can induce or enhance production of pro-angiogenesis factors VEGF and IL-8 from human *in situ*-matured skin mast cells.
*General significance:* These findings from this study indicate that IL-6 contributes to human allergic disease by enhancing the production of inflammatory $PGD_2$ from tissue-resident mast cells. Moreover, the data suggest a novel role for IL-6 in mast cell-mediated angiogenesis.

## 1. Introduction

Mature mast cells are tissue resident cells that are classically defined as the cell type responsible for allergic reactions, which are associated with crosslinking of the high affinity receptor for immunoglobulin E, FcεRI, by allergen [1]. The causative agents of allergies are pre-formed mediators like histamine and serine neutral proteases that are stored in cytoplasmic granules and released immediately after FcεRI crosslinking, and prostaglandins and leukotrienes that are rapidly biosynthesized from arachidonic acid. Mast cells also produce cytokines and chemokines that recruit other cell types and contribute to allergic inflammation. In addition to their role in allergy, mast cells are also involved in host defense against parasites and insect or reptile venom [2]. Mast cells have also been implicated in traditionally non-allergic diseases like diabetes [3,4] and various cancers [5,6].

Interleukin-6 (IL-6) is a pleiotropic cytokine involved in inflammation [7,8]. In humans, IL-6 levels are correlated with severity or pathogenesis of allergic diseases including asthma [9–12], urticaria [13,14], and mastocytosis [15,16] in which mast cells play a critical role. The exact role of IL-6 in allergic disease is not known, but IL-6 has been shown to modulate the development, survival, proliferation, adhesion, and chemokinesis of *in vitro*-derived mast cells [17–20]. In fact, IL-6 is routinely added to cultures of human cord blood CD34+ progenitors together with stem cell factor (SCF) to promote mast cell development *in vitro* [17,18,21,22]. Recently, it was shown that prolonged exposure to IL-6 could enhance FcεRI-induced degranulation of cord

*Abbreviations:* IgE, immunoglobulin E; $PGD_2$, prostaglandin $D_2$; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; CBMCs, cord blood-derived mast cells

* Corresponding author at: Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, SC 29208, USA.
*E-mail addresses:* mchalecc@email.sc.edu (C. McHale), Zahraa.Mohammed@uscmed.sc.edu (Z. Mohammed), juline.deppen@emory.edu (J. Deppen), gregorio.gomez@uscmed.sc.edu (G. Gomez).
[1] Present Address: The Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA, USA.

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blood-derived mast cells (CBMCs) [23]. Thus, IL-6 appears to contribute to allergic disease by regulating mast cell development and reactivity.

The IL-6 receptor (IL-6R) is comprised of the shared signaling subunit gp130 and the IL-6 binding subunit IL-6Rα [7,24,25]. Gp130 (130 kDa) is a signal transducing receptor subunit shared by members of the IL-6 family of cytokines that includes IL-6, IL-27, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, cardiotrophin 1, cardiotrophin-like cytokine, IL-11, and IL-35. IL-6Rα exists as transmembrane (80 kDa) and soluble (sIL-6Rα, 50–55 kDa) forms due to alternative splicing or proteolytic cleavage [26]. IL-6 can signal through membrane-bound IL-6Rα/gp130 complexes (classical signaling), or, alternatively, IL-6 can bind sIL-6Rα to form a soluble IL-6/sIL-6Rα complex that then binds to surface-expressed gp130 (trans-signaling). In this way, IL-6 can induce signaling in cells that do not express membrane-bound IL-6Rα on their surface. The expression of membrane-bound IL-6Rα and gp130 on *in vitro*-derived mast cells from human cord blood has been shown [17,23], but expression on human tissue-isolated mast cells had not been determined.

IL-6-induced gp130 signals JAK kinases [27] leading to phosphorylation and activation of Signal Transducer and Activator of Transcription 3 (STAT3), which has been implicated in mast cell degranulation and anaphylaxis [28–30]. STAT-3 induces transcription of Suppressor of Cytokine Signaling 3 (SOCS3), which serves to inhibit IL-6R signals [31] and regulate Th2-mediated allergic disease [32]. Although STAT3 has been implicated as positive regulator of degranulation, the effect of IL-6R classical signaling on mediator release from human *in situ*-matured mast cells had not been reported.

In this study, we determined that human skin mast cells constitutively express membrane-bound IL-6Rα and gp130 that can form functional receptors for IL-6, and investigate the effect of IL-6R classical signaling on FcεRI-induced effector function. Overall, the findings indicate that IL-6 contributes to allergic inflammation in humans by potentiating FcεRI-induced $PGD_2$ biosynthesis from mast cells, and suggests a role for IL-6 in mast cell-mediated angiogenesis.

## 2. Materials and methods

### 2.1. Isolation, purification, and culture of human skin mast cells

Human skin mast cells were isolated and purified from fresh surgical specimens of human skin tissue that were obtained from the Cooperative Human Tissue Network of the National Cancer Institute, as approved by the human studies Internal Review Board at University of South Carolina. The tissue was mechanically disrupted with surgical scissors and then digested 3 × 1 h at 37 °C with collagenase type II (Worthington Biochemical, Lakewood, NJ), hyaluronidase from bovine testes, and DNase I (Sigma-Aldrich, St. Louis, MO) in HBSS wash buffer (1X HBSS, 0.04% $NaHCO_3$, 1% fetal bovine serum, 1% HEPES, 0.1% $CaCl_2$) containing amphotericin B and Antibiotic/Antimycotic solution. After each digestion period, the samples were filtered through 40 μm nylon cell strainers. The filtered cells were collected by centrifugation, washed and re-suspended with wash buffer. After the final digestion, the collected cells were separated on Percoll by density centrifugation. The cells at the interface of buffer and Percoll layers were collected, washed and re-suspended at $5 \times 10^5$ cells/ml in serum-free X-VIVO 15™ media (Lonza, Walkersville, MD) containing recombinant human stem cell factor (SCF, 100 ng/ml) (PeproTech, Rocky Hill, NJ). The cells were transferred onto 24-well plates and maintained under standard culture conditions (37 °C, 5% $CO_2$) with weekly media changes. Purity was assessed by metachromatic staining with acidic toluidine blue, and by immunofluorescence staining for FcεRI expression with PE-labeled anti-human FcεRI antibody (clone AER-37 (CRA)) and mouse $IgG2b_k$ isotype control (BioLegend, San Diego, CA). Greater than 95% purity was achieved by 6 weeks of culture, and the mast cells were used thereafter.

### 2.2. IgE sensitization and FcεRI crosslinking

Mast cells ($10^6$ cells/ml) were incubated in X-VIVO 15™ media containing SCF (100 ng/ml) and 1 μg/ml chimeric human anti-NP IgE (human Fc + mouse Fab) (clone JW8/1) (AbD Serotec, Raleigh, NC) overnight at 37 °C, 5% $CO_2$. After washing to remove unbound IgE, the mast cells were re-suspended at $10^6$ cells/ml in X-VIVO 15™ media or Tyrode's buffer (135 mM NaCl, 1 mM $MgCl_2$, 20 mM Hepes, 5 mM KCl, 1.8 mM $CaCl_2$, 5.6 mM glucose; pH 7.4, 0.05% bovine serum albumin), and activated with the hapten 4-hydroxy-3-nitrophenylacetyl conjugated to bovine serum albumin at a 16:1 molar ratio (NP-BSA; Biosearch Technologies, Novato, CA) at the indicated concentration at 37 °C for the indicated amount of time.

### 2.3. $PGD_2$ and degranulation assays

Degranulation was measured by β-Hexosaminidase release assay. After sensitization with anti-NP IgE, the mast cells were washed and resuspended at $10^6$ cells/ml in Tyrode's buffer, pre-treated as describe in figure legends, and activated with NP-BSA for 30 min. After the activation period, mast cells and buffer were separated by centrifugation (2000 rpm × 5 min), and the pelleted cells were lysed with an equal volume of 1% Triton X-100. β-Hexosaminidase activity in supernatant and cell lysate was determined by the release of p-nitrophenol from substrate *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (pNAG; Sigma-Aldrich, St. Louis, MO) as described [33,34]. In a 96-well plate, 5 μl of supernatant or lysate were mixed with 45 μl of 4 mM *p*-Nitrophenyl *N*-acetyl-β-D-Glucosaminide (pNAG) in citric acid buffer (pH 4.5) and incubated for 1 h at 37 °C. The reaction was stopped with 150 μl of 0.2 M glycine, pH 10.7. Absorbance values at 405 nm were acquired with a BioTek Synergy HT microplate reader (BioTek, Winooski, VT). Percent β-hexosaminidase release was calculated from the absorbance values according to the formula: % β-hexosaminidase release = ((supernatant) / (supernatant + lysate)) × 100. $PGD_2$ in the supernatants was measured by commercial assay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol.

### 2.4. Quantitative real time PCR

Gene expression was determined by quantitative real time PCR. RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from total RNA with iScript cDNA Synthesis kit, and PCR was performed using iQ SYBR® Green Supermix (Bio-Rad, Hercules, CA). The kits were used according to the manufacturer's instructions. The PCR reaction mix was composed of 2 μl of cDNA, 1 μl each of sense and antisense primers (10 μM each) and 12.5 μl of iQ SYBR® Green Supermix in a final volume of 25 μl. A hot-start PCR protocol (95 °C × 5 min, (95 °C × 30 s, 55 °C × 30 s, 72 °C × 30 s) × 35 cycles, 95 °C × 1 min, 55 °C × 1 min) was performed on a CFX Connect Real Time PCR Detection System (Bio-Rad, Hercules, CA). Fold change in expression was determined by the $2^{\Delta\Delta Ct}$ method with β2 microglobulin (B2M) as the reference gene. The oligonucleotide primers used were (5′-3′; forward and reverse): VEGF (CCATGCAGATTATGCGGATCAAA and CACCAACGTACACGCTCCAG), SOCS3 (GCTCCAAGAGCGAGTACCAG and CTGTCGCGGATCAGAAA GGT), gp130 (TCT GGGAGTGCTGTTCTGCTT and TGTGCCTTGGAGGAG TGTGA), IL-6Rα (CTCATCTTTCTACAGACTACG and GACCATCCATGTT GTGAATG), COX-1 (TCTTGCTGTTCCTGCTCCTG and GTTGGAGCGCAC TGTGAGTA), COX-2 (ACTGCTCAACACCGGAATTT and CAAGGGAGTCG GGCAATCAT), and B2M (TGGGTTTCATCCATCCGACA and CTGCTTACA TGTCTCGATCCC).

### 2.5. Western blotting

$10^6$ mast cells were lysed with 0.1 ml Tris-Glycine SDS Sample Buffer (Life Technologies, Carlsbad, CA) containing 1% β-mercaptoethanol and 1 mM $Na_3VO_4$. Protein equivalents of