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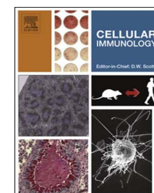

Research paper

# Notch signaling pathway regulates the growth and the expression of inflammatory cytokines in mouse basophils

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A R T I C L E I N F O

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Basophils (BAs) are the least common granulocytes of all leukocytes, but they play an important role in orchestrating of chronic allergic inflammation. The Notch signaling pathway is a highly conserved pathway that influences cell lineage decisions and differentiation during various stages of development. However, the relationship between Notch signaling and BA remains to be elucidate. Here, we report that several Notch signaling molecules were found to be expressed in BAs. γ-secretase inhibitor (GSI) treatment increase BAs apoptosis, and suppress BAs proliferation. Furthermore, GSI reduced BAs in the S phase, with a concomitant accumulation in G1 and G2 phases. In addition, GSI also significantly down-regulated mRNA levels of cytokines IL-4, IL-6 and IL-13 induced by A23187, and this effect was dependent on MAPK pathway. Finally, IL-6 inhibition was specifically associated with ERK and IL-13 with JNK. Therefore, Notch signaling regulates BA biological function, at least partially via the modulation of MAPK.

## 1. Introduction

Basophils (BAs) are a rare type of granulocyte that matures in the bone marrow and are subsequently released into the peripheral blood. In recent years, BAs have been gaining attention as critical to the pathogenesis of allergic inflammation, and many studies have demonstrated that BAs play a pivotal role in inflammatory diseases, such as allergic asthma. Dendritic cells (DC), for example, have been found to be dispensable for this disease process, whereas antigen presentation by BAs was necessary and sufficient for allergen-induced activation of Th2 responses *in vitro* and *in vivo* [1]. Recent studies have also revealed that BAs perform not only as antigen presenting cells (APCs) but also as effector cells that preferentially induce Th2 cells in response to complexes of antigen plus antigen specific IgE [2]. Furthermore, BAs have been considered as 'innate type 2 cells', which could lead the development of adaptive type 2 immunity by providing the initial IL-4 used by $CD4^+$ T cells in their differentiation to the Th2 phenotype [3].

The Notch signaling pathway is an important pathway that regulates development with high conservation across species [4]. In the hematopoietic tissues, Notch has been shown to influence cell lineage decisions and differentiation during various stages of development [5]. Specifically, Notch receptors and ligands are involved in the interaction between APCs and T cells. Furthermore, it has been demonstrated that several Notch signaling ligands are expressed on Mast cells [6], and that these molecules are involved in their proliferation [7,8]. BAs share several characteristics with tissue-resident Mast cells, including surface expression of the high-affinity IgE receptor (FcεRI) and the release of allergy-related chemical mediators such as histamine in response to various stimuli [9,10].

Th2 cytokines have been implicated in the pathogenesis of allergic asthma. IL-4, for example, plays a vital role in driving the differentiation of naive T cells into Th2 cells [11], and IL-13 induces eosinophils and Th2-recuiting chemokines. Transcriptional activation of cytokines in various types of APCs can be the result of induction by mitogen-activated protein kinases (MAPKs) [13]. MAPKs activated by various environmental stimuli, in fact, regulate the transcriptional activity of many genes involved in maintaining cellular homeostasis. There are three major groups of MAPKs in mammalian cells that are important regulatory proteins that transduce various extracellular signals into intracellular events: the extracellular signal-regulated protein kinase (ERK), c-Jun *N*-terminal kinase (JNK)/stress-activated protein kinase, and p38 subfamilies [12].

*Abbreviations:* APC, antigen presenting cells; BA, Basophil; CCK-8, cell counting kit-8; DC, dendritic cell; ERK, the extracellular signal-regulated protein kinase; GSI, γ-secretase inhibitor; IL, interleukin; JNK, c-Jun *N*-terminal kinase; MAPKs, mitogen-activated protein kinases; TGF-β, transforming growth factor-β; PBS, phosphate-buffered saline

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Whether Notch signaling and/or MAPKs regulate the antigen presentation function of BAs remains unclear. Here, expression of Notch signaling molecules was investigated in primary cultured mouse BAs. Furthermore, as these molecules may be involved in regulating the production of inflammatory mediators in asthma, a Notch inhibitor, γ-secretase inhibitor (GSI), was further evaluated as a potential therapeutic modality for the prevention of the onset and severity of allergic asthma. Our results demonstrated that GSI significantly inhibited increased production of inflammatory cytokines IL-6 and IL-13 induced in BAs, and that their expression was mediated through the modulation of MAPKs, JNK and ERK, respectively. Therefore, Notch signaling might play a central role in induction of allergic airway inflammation mediated BAs.

## 2. Materials and methods

### 2.1. Cells

Mice's bone morrow was flushed, and a single-cell suspension was prepared, followed by erythrolysis in ammonium chloride-potassium buffer. Bone marrow basophil (BMBA) were prepared by culturing BM cells in the presence of rIL-3 (300 pg/ml) for 1 week, followed by isolating the $CD49b^+$ cells using the IMag system with biotinylated anti-CD49b and streptavidin-conjugated magnetic particles (BD PharMingen, San Diego, CA, USA). FACS results demonstrated that the $CD117^-CD11c^-CD49b^+Fc\varepsilon RI^+$ cells were highly pure and viable basophils (Fig. S1).

### 2.2. RT-PCR

RNA was extracted with TRIzol reagent (Life Technologies), and cDNA was synthesized with reverse transcriptase (Fermentas; Vilnius, Lithuania) according to the manufacturer's instructions. RT-PCR was performed with the 2 × Taq Master Mix (KangWei, Beijing, China) on a thermocycler. Sequences of the primers used are listed in Supplementary Table S1. Amplified fragments were analyzed by electrophoresis on a 2% agarose gel.

### 2.3. Cell proliferation assay

Cells ($10^3$/well) were plated in 96-well plates in 200 μL of medium and incubated at 37 °C. Following treatment, reagent from the Cell Counting Kit-8 (CCK-8)10 μL (Dojindo) was added to wells and incubated for 2 h. The absorbance of the supernatant was measured at 450 nm. The cell viability curve was determined by plotting the mean value and standard deviation of the optical density. All experiments were done in 4 times.

### 2.4. Western blot analysis

Cells were lysed in phospho-lysis buffer (50 mM Tris-Cl, pH7.4, 150 mM NaCl, 1 mM MgCl2, 0.5% NP-40, 1 mg/mL bovine serum albumin [BSA], and 0.1 mM PMSF), and protein concentrations were determined with the BCA Protein Assay reagent (Pierce; Thermo Scientific; Rockford, IL, USA). Samples were separated by SDS-10% polyacrylmide gel electrophoresis, followed by Western blotting. Blots were incubated with the following primary antibodies: anti-Notch1 (ab-52627), anti-jag2 (ab-109627), pJNK123(ab59196), and JNK123 (ab124956; Abcam; Cambridge, MA, USA); anti-Notch3 (sc-5593), anti-delta4 (sc-28915) and phospho-erk (sc16982; Santa Cruz Biotechnology; Dallas, TX, USA); p38 (#4511), p38 (#9212), erk (#9102; Cell Signaling Technology; Boston, MA, USA). anti-rabbit-immunoglobulin G was used as secondary antibody.

### 2.5. FACS analysis

For cell cycle analysis, cells ($1 \times 10^6$) were fixed in 75% alcohol for 30 min at 4 °C, rinsed with cold PBS three times, and incubated at 37 °C for 30 min in 1 mL of PBS containing 40 μg propidium iodide (PI; Sigma; St. Louis, MO, USA) and 100 μg RNase A (Sigma). For apoptosis analysis, was detected with the Annexin V-FITC Apoptosis Detection Kit I (Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions. Cell suspensions were filtered through a nylon membrane and resuspended in FACS buffer for flow cytometry (PBS, 2% FCS, 0.05% $NaN_3$). All samples were analyzed for fluorescence staining and DNA content on the FACScalibur™ (BD Immunocytometry Systems; San Jose, CA, USA).

### 2.6. ELISA assays

BAs ($1 \times 10^6$ cells/mL) were seeded into 6-well plates. Supernatants from treated cells were screened for IL-4, IL-6, and IL-13 protein levels with a human ELISA kit according to the manufacturer's instructions (ELISA kits; Neobioscience; ShenZhen, China).

### 2.7. Real-Time PCR

Total RNA was extracted with TRIzol reagent (Life Technologies), and cDNA was synthesized with a reverse transcription kit (Toyobo; Osaka, Japan). Real-time PCR was performed with the SYBR Premix Ex Taq kit (Takara; Otsu, Japan) on the ABI PRISM 7300 (Applied Biosystems; Foster City, CA, USA). β-Actin was used as an internal control. Sequences of the primers used were the following: IL-4 Forward (F): 5′-ATGGGTCTCACCTCCCAACTGCT-3′, IL-4-Reverse (R): 5′-CGAACACTTTGAATATTTCTCTCTCCAACTGCT-3′; IL-6-F: 5′-ATGAACTCCTTCTCCACAAGCGC-3′, IL-6-R: 5′-GAAGAGCCCTCAGGCTGGACTG-3′; IL-13-F: 5′-CCACGGTCATTGCTCTCACTTGCC-3′, IL-13-R: 5′-CCTTGTGCGGGCAGAATCCGCTCA-3′; β-actin-F: 5′-TAGTTGCGTTACACCCTTTCTTG-3′, β-actin-R: 5′-TCACCTTCACCGTTCCAGTTT-3′.

## 3. Results

### 3.1. Expression of Notch-related genes in BAs

We primary cultured BAs for 7 days and enriched them by FACS according to $SSC^{low}CD117^-CD11c^-CD49b^+Fc\varepsilon RI^+$ (Fig. S1). Then, the expression of Notch related genes was examined in BAs. RT-PCR was performed with primers specifically targeting the mouse Notch1, Notch2, Notch3, Notch4, Jagged1 and Jagged2, Delta-like1, Delta-like3, Delta-like4 and Hes1 and 5, and β-actin as an internal control. The results revealed that Notch3, Notch4, and Delta-like 4 were highly expressed in BAs (Fig. 1A), while Notch2 was not detected. Two of the main Notch downstream genes, Hes1 and Hes5, were also highly expressed in BAs. Expression of Notch1, Notch3, Delta-like4, and Jagged2 in BAs was further confirmed by Western blot (Fig. 1B).

### 3.2. Notch signaling inhibitor (GSI) suppresses the growth of BAs

In order to investigate the role of Notch signaling in BAs growth, cells were exposed to an inhibitor of Notch signaling, GSI. The growth of BAs exposed to GSI, a γ-secretase inhibitor IX (Calbiochem, La Jolla, CA), which was used at the concentration of 75 μM/L, with DMSO as a control. was significantly decreased relative to control cells ($p < 0.05$, Fig. 2A). These data indicated that inhibition of Notch signaling suppressed the growth of mouse BAs *in vitro*.

### 3.3. GSI induces apoptosis and result in cell cycle arrest at the G1/G2 phase

Cell cycle parameters and apoptosis were examined by flow cytometry in order to identify mechanisms underlying the inhibition of BA

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