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Transcription factor RBP-J-mediated signaling regulates Basophil's immunoregulatory function in mouse asthma model

Short title: Regulation of BA's biological function by Notch receptors

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Key words: asthma; basophil; T cell; immunoregulation; OX40L

Abbreviations:

APC: antigen presenting cells

AHR: airway hyperreactivity

APTI: Airway pressure time index

BA: Basophil

BM: Bone marrow

BALF: Bronchoalveolar lavage fluid

HMI: Histological mucus index

rIL-3: recombinant interleukin 3

RBP-J: recombinant signal binding protein Jk

Summary

Basophils (BAs) play an important role in the promotion of aberrant Th2 immune response in asthma. It is not only the effective cell, but also modulates initiation of Th2 immune responses. We earlier demonstrated that Notch signaling regulates the biological function of BAs *in vitro*. However, whether this pathway plays the same role

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in vivo is not clear. The purpose of the present study was to investigate the effect of Notch signaling on BA's function in the regulation of allergic airway inflammation in a murine model of asthma. Bone marrow BAs were prepared by BM cell culture in the presence of rIL-3 (300 pg/mL) for 7 days, followed by isolation of the CD49b⁺ microbeads. The recombination signal binding protein J (RBP-J^{-/-}) BAs were co-cultured with T cells, and the supernatant and the T cell subtypes were examined. The results indicated disruption of the capacity of BAs for antigen presentation alongside an upregulation of the immunoregulatory function. This was possibly due to the low expression of OX40L in the RBP-J^{-/-} BAs. BAs were adoptively transferred to OVA-sensitized recipient mice, to establish an asthma model. Lung pathology, cytokine profiles of BALF, airway hyperactivity, and the absolute number of Th1/Th2 cells in lungs were determined. Overall, our results indicate that the RBP-J-mediated Notch signaling is critical for BA-dependent immunoregulation. Deficiency of RBP-J influences the immunoregulatory function of BAs, which include activation of T-cells and their differentiation into Th cell subtypes. Notch signaling pathway is a potential therapeutic target for BA-based immunotherapy against asthma.

Introduction

Although basophils (BA) account for < 0.5% of total leucocytes in circulation, their potent immunoregulatory functions such as induction of Th2 immunity were not known until recently [1]. Asthma represents a form of chronic inflammatory state in

the lower respiratory tract, which is characterized by largely reversible airflow obstruction and airway hyperresponsiveness. Excessive expression of T helper (Th) 2 cells is a key element believed to orchestrate chronic airway inflammation in allergic asthma [2]. BA-derived IL-4 has been shown to initiate the differentiation of naive CD4⁺ T cells into Th2 cells, both *in vitro* and *in vivo* [3]. Although BAs have been shown to act as *bona fide* APCs [4, 5], recent reports have questioned the absolute requirement of basophils in the initiation of Th2 responses [6, 7]. Therefore, whether basophils are key APCs in Th2 immunity and allergy continues to be a contested issue.

Notch signaling pathway regulates development with high conservation across species [8]; it has been shown to influence cell lineage decisions and differentiation during various stages of development [9]. In a previous study, we demonstrated the presence of several Notch receptors and ligands on primary cultured mouse BAs. Further, Notch was shown to regulate proliferation, apoptosis and immunoregulatory function of BAs *in vitro*. However, whether Notch signaling plays a similar role *in vivo* needs further research.

The expression of APC co-stimulatory molecules such as CD80/CD86 and MHC class II on BAs indicates [10] the ability of BAs for antigen uptake and processing. BAs exposed to IgEs antigens produce IL-4. In this study, we examined the level of co-stimulatory molecules in BAs of wild type and recombination signal binding protein J (*RBP-J*) knockout mice and compared their respective ability for antigen presentation.

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BAs were shown to be essential for the differentiation of naive CD4⁺ T cells into Th2 cells, both *in vitro* [11] and *in vivo* [12]. Recent studies suggest that OX40-OX40L interaction contributes to the induction of Th2 cells by APCs [13]. Furthermore, Notch signaling was shown to induce the expression of MHC-II and to upregulate the expression of OX40L on the surface of the mast cells. Moreover, BAs share several characteristics with tissue-resident mast cells, such as surface expression of the high-affinity IgE receptor, FcεRI, and the release of allergy-related inflammatory mediators such as histamine, in response to various stimuli [14,15]. In this study, we sought to investigate whether BA-induced differentiation of Th0 cells into Th2 cells is mediated *via* OX40-OX40L interaction in a mouse model of asthma, and assessed whether this effect was regulated by Notch signaling.

When Notch receptors are triggered by their ligands, the intracellular domain of Notch receptors is released and translocated into the nucleus where it associates with the DNA binding protein recombination signal binding protein-J_κ (RBP-J) [16]. This interaction was associated with cell differentiation, proliferation, and apoptosis. Therefore, it is believed that RBP-J mediates the transcriptional activation of all four Notch receptors [17]. Notch is an important regulator of T-helper cell differentiation, but its specific role is not well characterized. Delta-like family of ligands can promote naive T cells to differentiate into Th1, while jagged-2 has been shown to promote Th2 [18]. Many papers have reported that jagged1 stimulates APC-promoted proliferation of naive CD4⁺ T cells and their differentiation into Th2 cells producing IL-4, IL-5, IL-10,

and IL-13. However, it has been demonstrated that Th2 differentiation is unaffected by the jagged2 expression in dendritic cells (DC) [19]. Further, Worsley AG *et al*, found that jagged2 expression in DC is not essential for Th2 response induction *in vivo* [20]. Therefore, in this experiment, we examined the role of Notch ligands in the BA-induced Th2 differentiation.

We hypothesized that Notch signaling pathway plays an important role in the regulation of BA immunoregulation in an asthma model. In this study, we compared the effect of BA (RBP-J^{-/-}) and (RBP-J^{+/-}) in the regulation of Th1/2 differentiation, antigen presentation and regulation of airway inflammation in a mouse model of asthma and investigated the possible mechanisms.

Materials and Methods

Animals

Healthy female C57BL/6 mice (6-8 weeks of age) were purchased from the Experimental Animal Center at the Fourth Military Medical University (FMMU, Xi'an, China). RBP-J-floxed mice and Mx-Cre mice were purchased from Jackson library (Bar Harbor, ME). All mouse protocols were approved by the Animal Experiment Administration Committee at the FMMU.

Cell culture

Mice's bone marrow (BM) was flushed, and a single-cell suspension was prepared, followed by erythrolysis in ammonium chloride-potassium buffer. Bone marrow basophils (BMBA) were prepared from culture of BM cells in the presence of rIL-3 (300 pg/mL) for 1 week, followed by isolation of the CD49b⁺ cells using the IMag system with biotinylated anti-CD49b and streptavidin-conjugated magnetic particles (BD PharMingen, San Diego, CA, USA) [21]. Flow cytometry data analysis (FACS) results demonstrated that the CD117⁺CD11c⁺CD49b⁺FcεRI⁺ cells were highly pure and were viable BAs (Fig. S1). On culture, no difference was observed between the RBP-J knockout and control cells with respect to cell apoptosis and proliferation (Fig. S2).

Asthma models and study design

Mice were first sensitized with 100 µg OVA (Indoor Biotechnologies Inc., Charlottesville, VA) absorbed to 9% potassium alum (A1577; Sigma-Aldrich) *via* intraperitoneal injection on day 0 and 14. For depletion of BAs, on day 15, mice were immunized with intravenous administration of 100 µg MAR-1 antibody (eBioscience, San Diego, CA).

BMBAs from Mx-Cre×RBP-J^{-/-} and Mx-Cre×RBP-J^{+/+} mice were irradiated (10 Gy) and injected intranasally (dose: 1×10⁶ cells/mouse) into the sensitized mice on day 20.

Subsequent to this, all mice were challenged with OVA on days 21, 22, and 23. Mice were sacrificed 48 hr after the last challenge (Fig. 1).

Bronchoalveolar lavage fluid

In brief, 0.3 mL phosphate-buffered saline (PBS) was injected slowly into the trachea, and the bronchoalveolar lavage fluid (BALF) was centrifuged at 1200 g for 5 min. The pellets were resuspended in PBS for cellular analysis. The supernatants were stored at 4°C until further processing. Concentrations of IL-4, IL-5, IL-13, and IFN- γ in BALF were determined with ELISA kits (Neobioscience, China).

Mixed Lymphocytes Reaction

Purified naive CD4⁺ T cells were isolated from spleen of normal mice with use of CD4 magnetic beads (Miltenyi Biotec GmbH, Germany). Purity of CD4⁺ cells was above 90%. Naive T cells (5×10^5 /mL) were co-cultured with BAs (1×10^5 /mL) pretreated with IL-4 for 72 hrs. DNP-OVA were added at the beginning of the BA-T cell co-culture. After 72 hrs, the supernatants were collected for ELISA cytokine assays; T cells were collected for FACS analysis.

FACS analysis

In vitro, BAs were collected after 7 days of primary culture. Cells were stimulated with IL-4 and then were labeled with the following antibodies CD-80 (16-110A1), CD-86 (GL-1), and MHC-II (KH174) (BD PharMingen, San Diego, CA).

In vivo, after collection of BALF, lungs of mice in all groups were collected and minced. Cell suspensions were filtered with nylon membrane and resuspended in the FACS buffer (PBS, 2% FCS, 0.05% NaN₃). For surface staining, cells were labeled with PE-anti-mouse CD4 antibody (GK1.5, BD PharMingen, San Diego, CA), FITC-anti-mouse CD25 antibody (7D4, BD Pharmingen) and APC-anti-mouse OX40L antibody (sc-65282, Santa cruz). For staining of IL-4 and IFN- γ , a kit (Bioscience) containing monoclonal antibody (mAb) FKJ-16s was used according to the manufacturer's instruction. For analysis of intracellular markers, cells were stained with PE-Cyanine7-conjugated anti-mouse CD4 (clone GK1.5, eBioscience), fixed with IC fixation buffer, treated with permeabilization buffer and stained with IL-4 and IFN- γ Abs, according to the manufacturer's instructions (eBioscience). The samples were subjected to FACScan Flow Analyzer. The cells were analyzed by FACSCalibur™ flow cytometer (BD Immunocytometry Systems, San Jose, CA). Dead cells were excluded by propidium iodide (PI) gating. Data were analyzed by the Cell Quest software.

ELISA analysis

In vivo, BALF were centrifuged at 1200 g for 5 min and the supernatants were stored at 4°C for ELISA. *In vitro*, we collected the supernatants of primary cultured different groups of BAs (stimulated with IL-4 or PBS). The concentrations of IFN- γ , IL-4, IL-5, and IL-10 in BALF were determined with ELISA kits (Neobioscience, China). Total number of cells in BALF were counted with a hemocytometer.

Real-time PCR

As mentioned above, lung cell suspensions were collected and minced. In the *in vitro* studies, different groups of primary cultured BAs were isolated. Total RNA was extracted from Tregs with use of TRIzol reagent (Invitrogen, Carlsbad, CA), according to the protocols, and then was reverse transcribed with a kit (TOYOBO, Osaka, Japan). Real-time PCR was performed with a kit (SYBR Premix EX Taq, Takara) and the ABI PRISM 7300 real-time PCR equipment. β -actin was used as an internal control. Sequences of primers are listed in Table S1.

Lung histology

Lungs were fixed with 10% buffered formalin after BALF collection. Lung samples were embedded in paraffin and 4- μ m thick sections prepared. Sections were stained with H&E and Alcian blue-periodic acid Schiff (AB-PAS). The histological mucus index (HMI) was quantified by the formula as described elsewhere [22].

Determination of AHR

Mice were anesthetized, intubated and administered mechanical ventilation (120 breaths

/min; tidal volume: 0.2 mL). Subsequently, mice were paralyzed with decamethonium and injected intravenously with 50 mg/kg acetylcholine (Ach). Airway responsiveness was expressed as the airway pressure-time index (APTI).

Western blot

Mediastinal lymph nodes (MLN) of lung were collected, minced, and filtered through a nylon filter. BAs were separated by the IMag system with biotinylated anti-CD49b and streptavidin-conjugated magnetic particles. Homogenates were used for Western Blot analysis. Protein samples (20 µg/lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDSPAGE), and transferred on to nitrocellulose membranes (Sigma-Aldrich Co., St. Louis, MO, USA). After overnight blockade at 4 °C in a buffer containing PBS, 0.1% Tween 20, and 5% low fat milk powder, the resulting blots were incubated with the OX40L antibody (1:500) and β-actin antibody (1:1000), respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washes, the membranes were incubated with horseradish peroxidase conjugated secondary antibody (mouse anti-rabbit IgG, 1:5000; rabbit anti-goat IgG, 1:5000) (Santa Cruz Biotechnology) for 1 h at room temperature and processed for visualization by

enzyme-linked chemiluminescence. Relative intensities of the bands were quantified on densitometric analysis.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). Statistical analyses were performed by single-factor ANOVA for multiple groups and Tukey test for post hoc comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

Expression of Notch-related genes in primary cultured BAs

Firstly, we examined the expression of Notch related genes in primary cultured BAs.

We carried out q-PCR analyses with primers that specifically targeted the mouse Notch1, 2, 3, 4, jagged1 and 2, Delta-like 1, 3, 4 and Hes1 and Hes5, with β -actin as an internal control. The results revealed high expression levels of Notch 1, 3 and Delta-like 4 in BAs (Figure. 2), while Notch 2 was not detected. The two main Notch downstream genes, *Hes1* and *Hes 5*, were well expressed in BAs.

The effect of Notch signaling on the ability of antigen presentation of BAs

BAs constitutively express MHC class II, as well as co-stimulatory molecules such as CD80, CD86. FACS analysis was performed to study Notch mediation of antigen presentation of BAs. The results showed that the expressions of CD80 and CD86 co-stimulatory molecules were decreased in the RBP-J^{-/-} BAs as compared to that with the RBP-J^{+/-} BAs after IL-4 stimulation (Fig. 3). These results suggested that RBP-J^{-/-} BAs were not fully activated after stimulation.

Notch signaling inhibits immune regulation of BAs

We then performed ELISA assay to observe the Th2 cytokines secretion of BAs. For the initiation of Th2 cells, BAs (2×10^4 per well), and naïve CD4⁺ T cells (1×10^5 per well) were seeded on to 24-well plates in RPMI 1640 complete medium in the presence of rIL-2 (20 U/mL, R&D Systems), DNP-OVA (100 µg/mL, Biosearch Technologies, Novato) for 5 days. Then, we compared the cytokine levels between the two groups. Levels of IL-4, IL-5, and IL-13 in the supernatant were significantly lower than those in the RBP-J^{-/-} BAs (Fig. 4A) ($p < 0.05$).

RBP-J deficient BAs showed defects in the induction of Th2 cells

The mechanisms by which BA induce naïve Th0 cells to differentiate into Th1/2 cells are poorly understood. BAs from Mx-Cre×RBP-J^{-/-} or Mx-Cre×RBP-J^{+/-} and naïve Th0 cells were co-cultured with stimulation of IL-2 and DNP-OVA. The results showed that the percentage of CD4⁺IL-4⁺ Th2 cells was significantly decreased in RBP-J^{-/-} groups. Interestingly, we also found a remarkable increase in the ratio of CD4⁺ IFN-γ⁺ Th1 (Fig.5).

Notch signaling is involved in the initiation of Th2 immune responses and airway inflammation primed by BAs *in vivo*

RBP-J^{-/-} BAs recipients displayed significant reduction in inflammatory cellular infiltration, and mucus staining in lung tissue was attenuated as compared to that in the RBP-J^{-/-} group (Figure. 6A). The mice transferred with the RBP-J^{-/-} BAs exhibited significantly less airway inflammation than those in the RBP-J^{+/-} BAs group (Figure. 6B), indicated by HMI (Figure. 6C). Furthermore, we examined the capacity of RBP-J^{-/-} BAs to regulate airway hyperreactivity (AHR), as assessed by APTI (Figure. 6D). The positive control mice showed an elevation in ACh response as compared to that in the control group. RBP-J^{-/-} BAs significantly inhibited the ACh response.

The supernatants of BALF were collected to investigate the immune regulatory mechanisms of BA. The concentrations of IL-4, IL-5, IL-13, and IFN- γ were assayed by ELISA. The levels of IL-4 (Fig. 7A), IL-5 (Fig. 7B), and IL-13 (Fig. 7C) were increased and the levels of IFN- γ (Fig. 7D) were decreased significantly in the asthma group. In addition, BA RBP-J^{-/-} were more effective in decreasing the levels of IL-4, IL-5, and IL-13 than BA RBP-J^{+/+}. BA RBP-J^{-/-} showed a more potent immuno-regulatory effect.

Notch signaling regulates OX40L expression of BA *in vitro* and *in vivo*

Based on the above mentioned method among co-cultured BAs and T cells, we examined the mRNA and protein expressions of OX40L in BAs. The results showed a significant down-regulation in the RBP-J^{-/-} group (Figure 4B). *In vivo*, we set classic asthma models with Mx-Cre $\times$ RBP-J^{-/-} and Mx-Cre $\times$ RBP-J^{+/+} mice. The mice were sacrificed 48 hours after the last challenge. Mediastinal lymph nodes (MLN) of lung were collected, minced, and filtrated through a nylon filter. BAs were separated by the IMag system with biotinylated anti-CD49b and streptavidin-conjugated magnetic particles. The trend in mRNA and protein expressions of OX40L was similar to that in the *in vitro* studies (Figures 8A, B). These results indicated that Notch signaling might play an important role in the regulation of the ability of BAs to secrete cytokines, and that this effect may be mediated via downregulation of OX40L expression.

Discussion

Asthma is a chronic airway inflammatory disorder which is associated with an enhanced Th 2 response. As the BAs are the only APCs to stimulate Th2 cells *via* IL-4 production and presentation of peptide-MHC class II complexes, BAs are necessary and sufficient for the induction of Th2 cells *in vivo* and *in vitro* [23, 24]. However, the exact molecular mechanisms which regulate BAs' priming of Th2 are not clearly understood. Th1/2 cytokines are known to be essential for Th cell differentiation [25], and some signals from engagement of TNF family receptors and ligands contribute greatly to different aspects of T cell responses [25]. Therefore, understanding of the mechanisms which regulate priming of Th0 cells by BAs is essential for development of BA-based asthmatic therapies.

Notch signaling pathway is a highly conserved pathway across species and regulates development [4]. Notch receptors and ligands are involved in the interaction between APCs and T cells. Variable effects are produced by different Notch receptors and ligands. Delta-like proteins 1 and 4 were shown to promote differentiation of naive T cells into Th1; while in the pro-Th2/Th17 environment, the level of jagged-2 on the APC's surface is elevated [27]. In this study, we showed that the disruption of the transcription factor RBP-J, which is critical mediator in the canonical Notch signaling pathway, attenuated the BA-dependent Th2 differentiation and asthma pathological changes. Results of q-PCR and Western Blot showed high expressions of Notch 1, 3, 4

and Delta-like 1 in BAs, while Notch 2 was almost not detected. The two main Notch downstream genes, *Hes1* and *Hes5*, were well expressed in BAs. The findings imply an involvement of Notch in BA's function.

Constitutive expression of MHC class II, as well as co-stimulatory molecules such as CD40, CD80, and CD86 on BAs is well-documented [6], which suggests their role as potent APCs. Expression of MHC class II and CD80/86 suggests that BAs may also have the potential to induce Th2 cells. In this study, by co-stimulation of BAs with DNP-OVA and IL-2 *in vitro*, we found that RBP-J^{-/-} BAs had lower expressions of MHC molecules and the co-stimulatory molecules, CD80 and CD86. As APCs, the above molecules of BAs represented maturity. These findings implied the involvement of Notch in the maturation and activation of BAs. Asthma is characterized by Th2 bias. Th2 cytokines were elevated in asthma model and they play a role in airway hyperactivity, airway eosinophilia, and mucus hyper-secretion. ELISA and FACS analysis of *in vitro* cultured BAs and Th0 cells showed downregulation of IL-4, IL-5, and IL-13 of cell supernatants in RBP-J-deficient BAs group; moreover, Th2 cell number and ratio were both down-regulated. Natural BAs differentiate and undergo maturation in the bone marrow. The microenvironment for BA differentiation is very complex. In this experiment, we used rIL-3 to simulate the cell differentiation and sort cells with microbeads. Therefore, the *in vitro* generated cells can only partly simulate the biological characteristics of the natural BAs *in vivo*.

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Based on the classical mouse model of asthma, we used a novel BA transplantation method to establish a new kind of mice asthma model. Our results showed that mice who received BAs from RBP-J knockout mice exhibited weaker asthma responses. FACS analyses showed changes in the numbers and ratio of Th2 change in the recipient mice; Th1 indeed increased remarkably in mice who received BAs from RBP-J knockout mice. These results appear to implicate Notch-RBP-J signaling in the regulation of Th2-mediated asthmatic pathological changes in mice.

We have tried to unveil the potential molecular mechanisms that underlie Notch signaling-mediated differentiation and activation of BAs. OX40 plays an important role in the response of CD4⁺ T cells, including cell differentiation and survival [28]. OX40-OX40L interaction is involved in the Th2 responses driven by BAs *in vitro* [29]. Recent studies showed interaction of Notch signaling with OX40L in dendritic cells (DCs) and to result in mDC-driven Th2 responses and Treg expansion [30, 31]. Based on the above results, we hypothesize that Notch may also regulate the efficiency of Pro-Th2 of BA through OX40L, and our results suggest that interruption of RBP-J could reduce the mRNA and protein level of OX40L. Notch RBP-J signaling may be involved in exaggerated Th2 bias of asthma by up-regulating the expression of OX40L. Taken together, the Notch signaling pathway could affect the differentiation and the maturation of BAs. Our results suggest that interruption of Notch signaling pathway could impair BA-based pro-Th2 immunity through OX40L. Therefore, Notch signaling

might be a potential therapeutic target for BA-based anti-asthma immunotherapy. The other interactions between Notch and OX40L are subject to future investigation.

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The authors declare that they have no competing financial interests.

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Figure legends

Figure 1.

Protocol for BA-transferred mouse asthmatic model. C57BL/6 mice were sensitized by intraperitoneal administration of 100 µg of OVA complexed with Al(OH)₃ on day 0 and 14. On day 15, mice were immunized by intravenous administration of 100 µg MAR-1 antibody. Then, BMBAs from Mx-Cre×RBP-J^{-/-} or Mx-Cre×RBP-J^{+/-} mice were injected intranasally (1×10⁶ cells/mouse) into the sensitized mice on day 20. All mice

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were challenged with OVA on days 21, 22, 23. Mice were sacrificed 48 hr after the last challenge.

Figure 2.

Results of real time-PCR showing expression of Notch receptors and ligands in primary cultured mouse BAs. Total RNA was prepared from BMBAs and reverse-transcribed, and cDNA was used to amplify mouse Notch signaling pathway molecules. Mouse β -actin was used as an internal control.

Figure 3.

RBP-J-deficient BAs showed maturity defects upon IL-4 stimulation. (A) IL-4-stimulated BAs were analyzed by FACS using anti-CD80, CD86, and MHC II; (B) The absolute numbers of CD80, CD86, and MHC class II positive cells were compared between the two groups. Red line represents RBP-J^{+/+} group, while the green line represents RBP-J^{-/-} group. The result represent mean of six independent experiments. Bars represent mean $\pm$ S.D. (n=4). ** $P < 0.01$. *** $P < 0.001$.

Figure 4.

Cysteinyl leukotrienes levels in the supernatant of co-cultured cells. Naive T cells ($5 \times 10^5/\text{mL}$) were co-cultured with BAs ($1 \times 10^5/\text{mL}$) which were previously treated with IL-4 for 72 hrs. DNP-OVA were added at the beginning of the BA-T cell co-culture. (A) Decreased Th2 cytokines secretion of T-cells stimulated with RBP-J^{-/-} BAs; (B) RNA of BAs was collected, and the OX40L mRNA levels were determined by real-time PCR. The result represents six independent experiments. Bars represent mean $\pm$ S.D. (n=4). * $P < 0.05$. ** $P < 0.01$.

Figure 5.

RBP-J-deficient BAs displayed Th1-inducible phenotypes. Naive T cells ($5 \times 10^5/\text{mL}$) were co-cultured with BAs ($1 \times 10^5/\text{mL}$) which were previously treated with IL-4 for 72hrs. DNP-OVA was added at the beginning of the BA-T cell co-culture. A) Naïve T cells co-cultured with RBP^{+/-} or RBP^{-/-} BAs *in vitro*. After 72hrs, IL-4, and IFN- γ positive cells were analyzed by FACS; B) The positive percentage of IFN- γ and IL-4 cells were calculated and compared. The result represents 6 independent experiments. Bars represent mean $\pm$ S.D (n=4). * $P < 0.05$. ** $P < 0.01$.

Figure 6.

Adoptive transfer of BA RBP-J^{-/-} suppresses airway inflammation and mucus secretion *in vivo*. The mice were sensitized with OVA (100 µg, i.p.) absorbed with 9% potassium alum. BAs were transferred intranasally into the sensitized mice. Finally, the mice were challenged with OVA (100 µg intranasal). Results were assessed 48 h after the last challenge. (A) Goblet cells were stained with Alcian blue-periodic acid Schiff (AB-PAS); (B) Lungs were fixed in 10% buffered formalin, and lung sections were examined by H&E for signs of airway inflammation and pathological changes; (C) The histological mucus index (HMI) of each group is shown; (D) Airway hyperreactivity to methacholin. The result represents 3 independent experiments.

APTI = Airway pressure-time index. Bars represent mean ± S.D. (n=6).

P*<0.05, *P*<0.01, vs. control group; #*P*<0.05, ##: *P*<0.01, vs. Asthma model group; &*P*<0.05, &&*P*<0.01, vs. BA RBP-J^{-/+} group.

Figure 7.

BA RBP-J^{-/-} suppressed the production of Th2 cytokines. BALF were collected 48 hr after last challenge. Levels of Th1/2 and immunoregulatory cytokines in BALF (pg/mL) were assayed by ELISA. The levels of IL-4 (A), IL-5 (B), IL-13 (C), and IFN-γ (D) in BALF. There are four groups in the animal model experiments. a) control group; b) asthma model group; c) transfer BAs (RBP-J^{-/+}) group; d) transfer BAs (RBP-J^{-/-}) group. Bars represent mean ± S.D. (n=6). The result represents 3 independent experiments.

* $P < 0.05$, ** $P < 0.01$, vs. control group; # $P < 0.05$, ## $P < 0.01$, vs. Asthma model group; & $P < 0.05$, && $P < 0.01$, vs. BA RBP-J^{-/+} group.

Figure 8.

The interference of Notch signaling in BAs modulated the mRNA and protein expressions of OX40L. The mice were sacrificed 48 hours after the last challenge. Mediastinal lymph nodes (MLN) of lung were collected, minced, and filtrated through a nylon filter. BAs were separated by the IMag system with biotinylated anti-CD49b and streptavidin-conjugated magnetic particles. The mRNA and protein variation trend of OX40L were examined. (A) Two groups of BAs' RNA were collected, and the OX40L mRNA levels were determined by real-time PCR; (B) Western blotting was performed, and OX40L protein expression values were normalized to β -actin. The result represents 3 independent experiments. Bars represent mean $\pm$ S.D. (n=6). * $P < 0.05$.

Figure S1

Characterization of sorted BM-Bas. Gating strategy for sorting BMBA by FACS. FSC^{low} SSC^{low} $CD117^{-}CD11c^{-}CD49b^{+}Fc\epsilon RI^{+}$ cells were gated;

Figure S2

Effect of RBP-J deletion on *in vitro* cultured BAs. (A) Proliferation of BAs was determined with the CCK8 assay; (B) flow cytometric analysis was performed to detect apoptosis in RBP-J^{-/-} and RBP-J^{+/-} BAs with Annexin-V and PI; (C) Positive of Annexin-V and PI cells were counted, and were compared between the two groups. Bars represent mean $\pm$ S.D. (n=3). **P*<0.05; (D) Expressions of CD80, CD86, and MHC-II on the cultured BA were assessed by FACS; (E) Absolute number of MHC-II, CD80 and CD86 expression was compared between groups. The result represents 6 independent experiments.

Bars represent mean $\pm$ S.D. (n=4). **P*<0.05.















