VERSITA

Cent. Eur. J. Biol. • 8(11) • 2013 • 1102-1111
DOI: 10.2478/s11535-013-0231-8

Central European Journal of **Biology**

Hes-1-targeting siRNA inhibits the maturation of murine myeloid-derived dendritic cells

Research Article

Guoliang Chen[1], Chunyan Yu[1], Feiyue Xing[1,*], Pengtao You1, Jingfang Di[1], Shan Zeng[1], Jing Liu[2,*]

[1]*Institute of Tissue Transplantation and Immunology, Department of Immunobiology, Jinan University, 510632 Guangzhou, China*

[2]*Department of Stomatology, Jinan University, 510632 Guangzhou, China*

Received 04 February 2013; Accepted 24 June 2013

Abstract: Activation of Notch by Jagged-1 may plays a pivotal role in maturation of dendritic cells (DCs), but the mechanism has not been completely defined. In the present study, Hes-1 (Hairy/enhancer-of-split)-targeting siRNA was used to confirm a role of Jagged-1-Notch signaling pathway activation in maturation of murine bone marrow-derived DCs and to search for a target that plays a critical role. The results showed that compared with the control, lipopolysaccharide or Zymosan A groups, Jagged-1 (a soluble Jagged 1/Fc chimera protein) effectively increased expression of Hes-1 and Deltex-1 mRNA, which could be reversed by DAPT (2, 4-diamino-5-phenylthiazole), a specific inhibitor of the Notch signaling pathway. Hes-1-targeting siRNA could successfully down-regulate the endogenous Hes-1 expression in the DCs. Concurrently, a significant down-regulation of CD40, CD80, CD86 and MHC-II expressions on the surface of the DCs was found with the reduction of IL-12 yielded by the DCs. Our results demonstrate that Hes-1-targeting siRNA can inhibit the maturation of the DCs induced by Jagged-1, indicating Hes-1 may be an important target of Notch signaling mediating the maturation of DCs.

Keywords: *Jagged-1 • Hes-1 • siRNA • Dendritic cell • Maturation*

1. Introduction

Dendritic cells (DCs) are antigen-presenting cells with critically important functions in innate and adaptive immunity. Notch signaling plays an important role within hematopoietic and immune systems. Depending on cell types, Notch signaling can positively or negatively influence proliferation, differentiation, and apoptosis [1,2]. Current studies suggest that Notch-ligand interactions result in cleavage of the intracellular domain of Notch (NICD) and translocation of NICD to the nucleus where it interacts with the transcriptional repressor CSL(RBP-Jκ). Binding of NICD displaces co-repressor complexes, thereby activating transcription by promoters with CSL (CBF1, Su(H) and LAG-1) binding element [3-5].

Jagged-1, a single trans-membrane glycoprotein, is one of the major ligands for Notch receptors on the mammalian cell membrane, and expressed on many tissue cells, such as bone marrow, fetal liver stroma and thymus epithelium. It participates in controlling growth, developing numerous tissues and maintaining renewal and differentiation of normal haemopoietic stem cells [6]. Jagged-1 is also expressed highly on the surface of antigen presenting cells (APC), such as dendritic cells, B cells and macrophages. It can promote maturation of DCs [7] and Notch signaling can maintain and control differentiation of $CD8^-$ DCs [8], suggesting that Jagged-1-Notch signaling possibly plays an important part in an immune response.

Hes-1 (hairy enhancer of split-1) is known to code for a basic helix-loop-helix transcription factor [9]. Hes-1 protein binds to its own promoter and negatively regulates its activity by binding N-box domains located in the promoter region of Hes-1 [10-12]. Hes-1 transcriptional up-regulation is perhaps the best characterized function

* *E-mail: tfyxing@jnu.edu.cn; tjliu@jnu.edu.cn*

of intracellular Notch-1 activity. Hes-1 is moderately decreased in RBP-Jκ-deficient DCs, indicating that Hes-1 may play some roles in the differentiation and maturation of DCs [8]. In addition, activation of Notch signaling in hematopoietic progenitor cells promotes differentiation of conventional DCs *via* activation of a canonical Wingless pathway [13]. Therefore, in the present study Hes-1-targeting siRNA (Hes-1-siRNA) was employed to knockdown *Hes-1* gene in the Jagged-1-Notch signaling pathway in order to explore a role of Hes-1 in the maturation of DCs.

2. Experimental Procedures

2.1 Animals

Male Balb/c mice, purchased from the Guangdong Medical Animal Center (Guangzhou, China), were kept under specific pathogen-free conditions in our own facilities. Eight weeks old mice weighing 20.0±2.0 g were used. Animal experimental procedures were approved by the Animal Care and Use Committee of Guangdong Medical Animal Center.

2.2 Preparation of bone marrow cells

First, cervical cords in Balb/c mice were dislocated mechanically, then femurs and tibiae in mice were removed and purified from the surrounding muscle tissue and connective tissue under sterile conditions. Thereafter, intact bones were kept in 70% ethanol for 5 min for disinfection and washed with PBS. Then both ends were cut with scissors and the marrow was flushed with PBS using a syringe with a 0.45 mm diameter needle. Clusters within the marrow suspension were disintegrated vigorously by pipette. After being centrifuged at 300×*g* for 5 min, the cells were collected, re-suspended in PBS by adding red blood cell lysate for depletion of erythrocytes and incubated at 37°C for 5 min in the dark. Then they were washed with PBS and centrifuged at 300×*g* for 5 min 3 times. At last, the cells were harvested and suspended in RPMI1640 (Gibco BRL, USA) complete culture medium containing 10% (v/v) fetal bovine serum (FBS) (Gibco BRL, USA), 2 mmol L^{-1} L-glutamine, 10 μmol L^{-1} β-mercaptoethanol (Sigma-Aldrich), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, and adjusted to 2×10^9 L^{-1}.

2.3 Induction of bone marrow-derived DCs

The bone marrow cells above were seeded to a 6-well plate with 10^7 cells and the end volume of 1 ml per well. 0.25 mg L^{-1} of IL-4 and 5 mg L^{-1} of GM-CSF (PeproTech EC Ltd., London, UK) were added to stimulate the cells at the concentrations of 2.5 and 10 μg L^{-1}. The cells were cultured at 37°C in an incubator containing 5% CO_2 and all the medium with IL-4 and GM-CSF was renewed every other day until the 6th day. Then, the extracted total RNA, cytoplasmic protein and harvested cell culture supernatant were used for RT-PCR, Western blot and ELISA, respectively.

2.4 Isolation of bone marrow-derived DCs

The Mouse CD11c Positive Selection Kit (StemCell, Vancouver, BC, CA) was used for isolation of bone marrow-derived DCs. According to the manufacturer's instruction, the newly harvested bone marrow cells suspended in PBS were prepared at a density of $2x10^8$ in polystyrene tube with 1 ml volume. Then 50 μl of CD11c–PE labeling reagent was added into the cells, mixed, and laid aside at room temperature for 15 min. Then EasySep® PE Selection Cocktail at 100 μl mL^{-1} cells was mixed with the cells and incubated at room temperature for 15 min. Magnetic nanoparticles at 50 μl mL^{-1} cells were added and mixed by pipette vigorously 5 times and incubated at room temperature for 15 min. The cell suspension was supplemented to a total volume of 5.0 ml and gently mixed. Thereafter, the tube was put into the magnet and set for 5 min. The tube inside the magnet was inverted to pour off supernatant and the magnetically labeled cells left. Lastly, PBS was used to wash the cells 3 times, the tube was removed from the magnet and the cells were suspended for flow cytometry.

2.5 Cell culture and treatment

To determine whether or not Jagged-1 signaling pathway is activated, the DCs were treated with 1.0 mg L^{-1} of Jagged-1 (a soluble Jagged 1/Fc chimera protein) (R&D Systems, Minneapolis, MN, USA), 10 mmol L^{-1} of DAPT(N-[N-(3,5- difluorophenacetyl)-l-alanyl]-S-phenylglycinet-butyl ester) (Sigma-Aldrich, St. Louis, MO, USA) plus 1.0 mg L^{-1} of Jagged-1, 1.0 mg L^{-1} of lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA) and 5 μg L^{-1} of Zymosan A (Sigma-Aldrich, St. Louis, MO, USA), respectively. DAPT stimulation was performed 2 h before Jagged-1 was added. After treated for 24 h the total RNA was extracted for RT-PCR analysis.

To further confirm whether or not Hes-1 in Jagged-1 signaling pathway plays an important role in the differentiation and maturation of the DCs, the isolated CD11c positive cells without stimulus of rmIL-4 or rmGM-CSF were treated with or without Jagged-1 (1.0 mg L^{-1}), Jagged-1 (1.0 mg L^{-1}) plus Hes-1-siRNA (100 nmol L^{-1}), Hes-1-siRNA (100 nmol L^{-1}) and mutated Hes-1-siRNA (100 nmol L^{-1}) for 12, 24, 36, 48 and 72 h. Or the cells were treated with Hes-1-siRNA at the different concentrations of 30, 60, 100, 200 and 250 nmol L^{-1}

for 36 h. Then, cytoplasmic proteins were extracted for Western blot.

Simultaneously, the isolated CD11c positive cells without stimulus of rmIL-4 or rmGM-CSF were treated with or without Jagged-1 (1.0 mg L^{-1}), Jagged-1 (1.0 mg L^{-1}), Jagged-1 (1.0 mg L^{-1}) plus Hes-1-siRNA (100 nmol L^{-1}), Hes-1-siRNA (100 nmol L^{-1}) and mutated Hes-1-siRNA (100 nmol L^{-1}) for 36 h. The cells were used by the end of the cell culture for flow cytometry analysis and the supernatants of the 36 h-cultured cells were harvested for ELISA.

2.6 Hes-1-siRNA transfection

The sequence of Hes-1-siRNA was 5′-CGAGGUGACCCGCUUCCUGdTdT-3′ and the sequence of mutated Hes-1-siRNA was 5′-CGAGGUCACCCGGUUCCUGdTdT-3′ [14] and then synthesized by RiboBio Co., Ltd. (RiboBio, Guangzhou, China). 2 μL of SunBio Trans-EZ siRNA Transfection Agent (SunBio, Shanghai, China) plus 100 nmol L^{-1} of Hes-1-siRNA or 100 nmol L^{-1} of mutated Hes-1-siRNA was diluted respectively in Opti-MEM culture with a whole volume of 50 μL, then fully mixed and rested for 20 min at room temperature for formation of the transfection complex. Lastly, 100 μL of the complex was added gently into the cells.

2.7 Semiquantitative RT-PCR

As described above, total RNA from the treated DCs was extracted using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's protocols. RT-PCR was performed using OneStep RT-PCR Kit (QIAGEN GmbH, Hilden, Germany). The following primers were employed for a semiquantitative RT-PCR analysis. Hes-1: 5′-AGCACAGAAAGTCATCAAAGCC-3′ and 5′-TTCATGCACTCGCTGAAGCC-3′ with 454 bp of PCR product; Deltex: 5′-AAAGACATCGTCCTTGCC-3′ and 5′-CATTTATTTCTCCACCCAC-3′ with 352 bp of PCR product; β-actin: 5’ -AACAGTCCGCCTAGAAGCAC-3’ and 5’-CGTTGACATCCGTAA AGACC-3′ with 281 bp of PCR product [15]. RT-PCR was performed under the following conditions: 50°C/30 min for reverse transcription reaction, 95°C/15 min for activation of PCR, and then 30 cycles at 94°C 30 s for denaturation, 58°C 40 s for annealing and 72°C 1 min/cycle for extension. 8 μl of PCR product was run on a 2% agarose gel and stained with ethidium bromide. The gel image was performed on a FluorChem 8000 system with AlphaEaseFC software (Alpha Innotech, Santa Clara, CA, USA).

2.8 Western Blot

As described above, the cells were collected, and then lysed in 50 μl RIPA lysis solution (BioColors, Shanghai, China) supplemented with 0.5 μl PMSF on ice for 0.5 h. The lysate was harvested by centrifugation at 4°C for 20 min at 12,000 rpm. Each sample (20 μg) was separated on 5% stacking and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA), and transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with TBS containing 0.05% Tween 20 (TBST) and 5% non-fat milk at room temperature for 1 h. After washing 3 times with TBST for 5 min each time, the membranes were probed with anti-Hes-1 primary antibody (1:500) and anti-β-actin primary antibody (1:500) at room temperature for 2 h and washed 3 times with TBST for 5 min each time. Then the membranes were labeled with corresponding second antibodies marked with HRP. The bands were visualized by ECL according to the manufacturer's instruction. The band density was analyzed on a FluorChem 8000 system with AlphaEaseFC software (Alpha Innotech, Santa Clara, CA, USA).

2.9 Flow cytometry

As described above, the treated cells were collected and washed with PBS at 300×*g* for 5 min, and re-suspended in PBS containing10% fetal calf serum. Then they were stained with 1.0 μl anti-CD11c-FITC (0.5 μg/10^6 cells), 0.5 μl anti-CD40-APC (0.125 μg/10^6 cells), 0.3 μl anti-CD80-PE-CY5 (0.06 μg/10^6 cells), 0.5 μl anti-CD86-PE (0.125 μg/10^6 cells) or 0.5 μl anti-MHC-II-PE (0.125 μg/10^6 cells) (eBioscience, USA) in a 100 μl total volume, respectively. After mixing gently on a vortex machine, they were placed at 4.0°C in the dark for 30 min, then rinsed with PBS 2 times, and centrifuged at 300×*g* for 5 min. The expression level of these molecules on the surface of the cells was analyzed by flow cytometry (FAC-Scalibur, Becton Dickinson, USA). A total of 5×10^3 events were analyzed for each determination, and calculated by CellQuest software.

2.10 ELISA

As described above, the harvested supernatants were collected to test IL-12 using ELISA kit (Dakewe Biotech Company Limited, Shenzhen, China) according to the manufacturer's protocol. The absorbance of each well was read at 450 nm using a 680-type microplate reader (BIO-RAD, Berkeley, CA, USA). Each sample was tested in triplicate. The concentration was determined from a standard curve.

2.11 *In situ* immunofluorescence staining

Detection of *in situ* expression of Notch-1 on the above isolated DCs was performed using immunofluorescence staining. The DCs were fixed in cold methanol at 4°C

for 15 min and blocked with 5% BSA in PBS for 30 min. Afterwards, the cells were incubated with diluted (in blocking solution) rabbit anti-mouse Notch-1 antibody (Cell Signaling Technology, Inc. USA) at 4°C overnight. Subsequently, the cells were washed 3 times with PBS, and incubated with Alexa Fluor 488 conjugate anti-rabbit IgG (Cell Signaling Technology, Inc. USA) for 1 h at 37°C. Furthermore, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) for 10 min, and washed with PBS. Finally, the cells were examined under a Leica DMRA2 fluorescence microscope with a FW 4000 software (Leica, Germany).

2.12 Statistics

SPSS 10.0 software (SPSS Inc., IL, US) was used for statistical analysis. The results were expressed as means ±SD of three independent experiments. Individual comparisons were made by Student's t-test for paired data, and p-values less than 0.05 were considered to be statistically significant.

3. Results

3.1 Jagged-1 up-regulates the expression of both Hes-1 and Deltex-1 mRNA from bone marrow-derived DCs

Presence of Notch receptors on the surface of DCs had been verified before we explored whether Jagged-1 can activate downstream molecules of Notch signaling, and then levels of Hes-1 and Deltex-1 mRNA in Jagged-1-treated DCs were measured by semi-quantitative PCR, respectively. *In situ* expression of Notch-1 receptor on the surface of the separated DCs or induced DCs were clearly observed (Figure 1). As shown in the Figure 2a, Hes-1 mRNA expression was obviously up-regulated to approximately 6 fold of the control by Jagged-1 stimulus, which could be offset by DAPT. LPS slightly prompted the expression of Hes-1 mRNA, while Zymosan A showed little effect on the Hes-1mRNA. Apart from Hes-1 mRNA, the expression of Deltex-1 mRNA was also detected. As shown in the Figure 2b, Deltex-1mRNA expression was also highly up-regulated in Jagged-1-treated DCs, compared to the control, which could be entirely offset by DAPT as well. It was found that slight up-regulation of Deltex-1 mRNA expression in Zymosan A-treated DCs came out by addition of Zymosan A, much lower than that in Jagged-1-treated DCs. The results indicate that Jagged-1 can prompt both Hes-1 and Deltex-1 expression at their mRNA levels, suggesting that activation of Jagged-1-Notch signaling pathway occurs in the DCs, and Hes-1 and Deltex-1 might be key target molecules in formation of Jagged-1-treated DCs. LPS and Zymosan A may influence maturation of DCs mainly *via* other signaling pathways.

3.2. Hes-1 siRNA counteracts the action of Jagged-1 on Hes-1 protein expression in bone marrow-derived DCs

Since Jagged-1 could strikingly activate Hes-1 mRNA in bone marrow-derived DCs, in contrast to LPS and Zymosan A, we used Hes-1-siRNA to interfere with Hes-1 expression in DCs to further find evidence

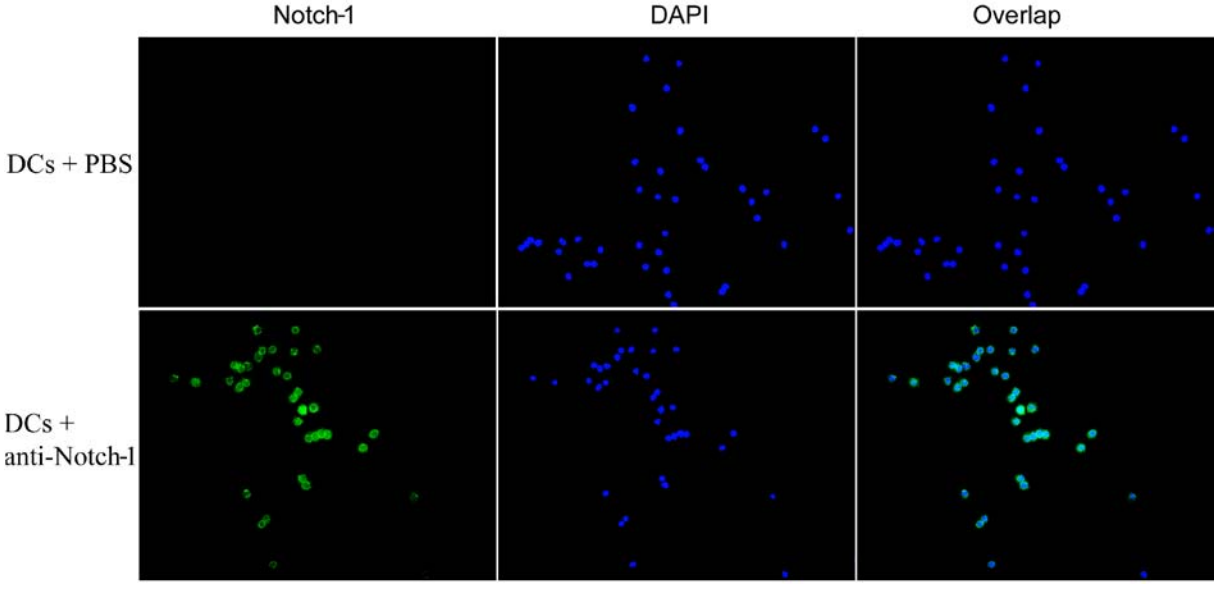

Figure 1. *In situ* expression of Notch-1 receptor on the surface of dendritic cells. The isolated DCs were fixed and stained with a monoclonal antibody specific for Notch-1or PBS, followed by incubation with Alexa Fluor 488 conjugate anti-rabbit IgG (x400, green). Furthermore, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) for nucleus staining (x400, blue).

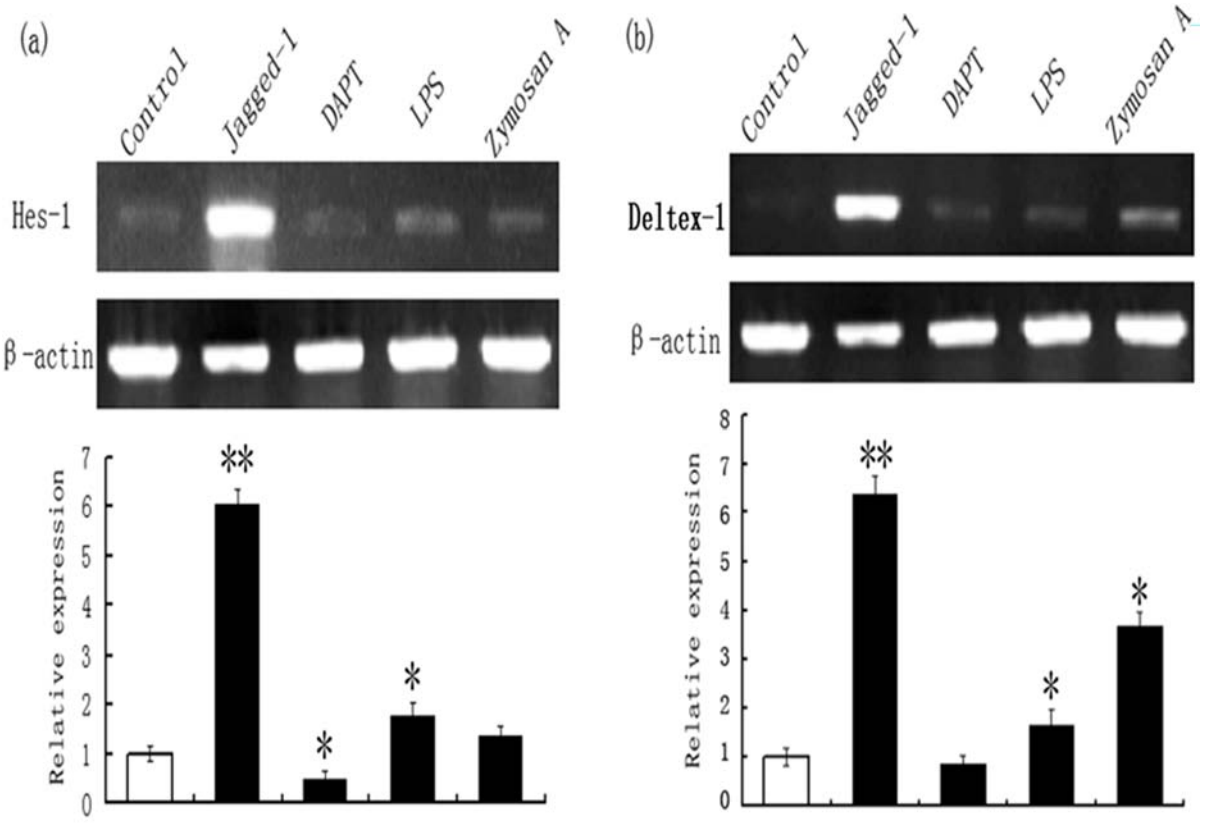

Figure 2. Effect of Jagged-1 on the expression of Hes-1 mRNA (a) and Deltex-1 mRNA (b). The bone marrow cells were treated with 5 mg L^{-1} of GM-CSF and 0.25 mg L^{-1} of IL-4 for 6 days, then stimulated with or without 1.0 mg L^{-1}Jagged-1, 10 mmol L^{-1} DAPT plus 1.0 mg L^{-1} Jagged-1, 1.0 mg L^{-1} LPS and 5 μg L^{-1} Zymosan A. The cells were collected and total RNA was extracted. Lastly, Hes-1 mRNA and Deltex-1mRNA were detected through RT-PCR. All of the results were repeated three times. The data are expressed as the means ±SD, *$P<0.05$ and **$P<0.01$ *vs* the corresponding control.

to support an important role of the activation of Jagged-1-Notch signaling pathway in Jagged-1-treated DCs. Therefore, the expression of Hes-1 protein in Jagged-1-treated DCs was measured by Western blot. The results showed that the expression level of Jagged-1 treated Hes-1 protein increased in DCs at the indicated time, which was inhibited 24 h post addition of Hes-1-siRNA. On the contrary, the action of Jagged-1 could not be significantly reversed by mutated Hes-1-siRNA (Figure 3a-e). Moreover, the level of Hes-1 in the DCs treated with Hes-1-siRNA was lower at 48 or 72 h compared to the control, suggesting the production of endogenous Hes-1 might be affected by application of Hes-1-siRNA. Simultaneously, different concentrations of Hes-1-siRNA were employed to treat DCs and it was revealed that the interfering effect of over 100 nmol L^{-1} Hes-1-siRNA was augmented markedly (Figure 3f). These data indicate that the enhancement of Hes-1 protein expression by Jagged-1 can be reversed with Hes-1 siRNA.

3.3 Hes-1-siRNA blocks the expression of co-stimulating molecules on bone marrow-derived DCs

As described above, Jagged-1 could promote Hes-1 expression, while Hes-1-siRNA could decrease Hes-1 expression. To further verify a potential role of the activated Jagged-1 signaling in the induction of DC maturation, CD11c positive cells were isolated from bone marrow cells in Balb/c mice, and then stimulated with or without 1.0 mg L^{-1} Jagged-1, 1.0 mg L^{-1} Jagged-1 plus 100 nmol L^{-1} Hes-1-siRNA, 100 nmol L^{-1} Hes-1-siRNA and 100 nmol L^{-1} of mutated Hes-1-siRNA for 36 h, respectively.Then, the cells were collected and stained for flow cytometry analysis. As shown in Figure 4, Jagged-1 could up-regulate remarkably the expression of CD40 (Figure 4a), CD80 (Figure 4b), CD86 (Figure 4c) and MHC-II (Figure 4d) molecules on the surface of DCs, compared with the control. But while siRNA target to Hes-1 was used together with Jagged-1 to treat the cells, the percentage of $CD40^+CD11c^+$ or $CD80^+CD11c^+$ or $CD86^+CD11c^+$ or $MHC\text{-}II^+CD11c^+$ cells decreased

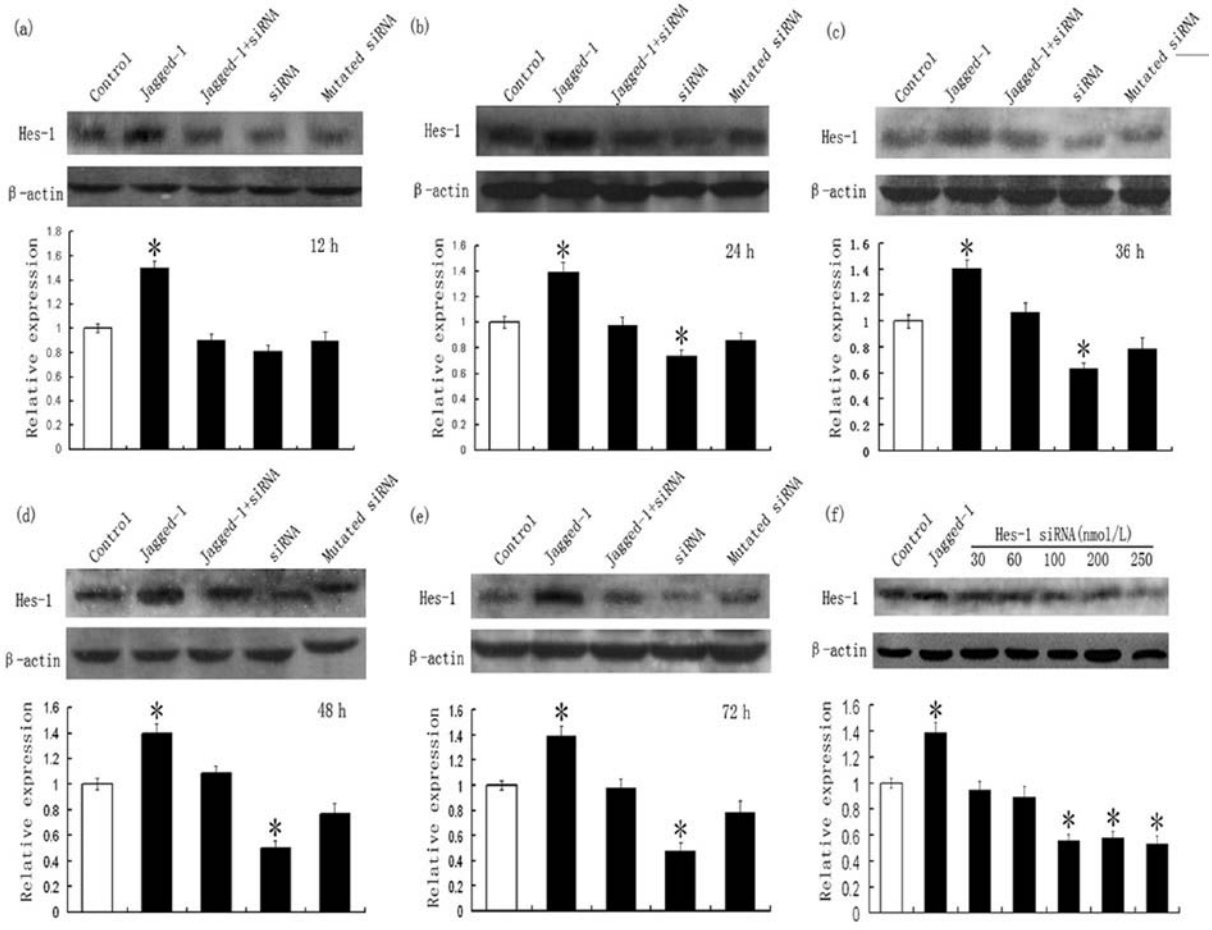

Figure 3. Effect of Hes-1-siRNA on the expression of Hes-1 from bone marrow-derived DCs. The bone marrow-derived DCs treated with 5 mg L^{-1} of GM-CSF and 0.25 mg L^{-1} of IL-4 for 6 days were first stimulated with or without 1.0 mg L^{-1} of Jagged-1, 1000 μg L^{-1} of Jagged-1 plus 100 nmol L^{-1} of Hes-1-siRNA, 100 nmol L^{-1} of Hes-1-siRNA and mutated Hes-1-siRNA at the dose of 100 nmol L^{-1} for 12 h (a), 24 h (b), 36 h (c), 48 h (d), 72 h (e), respectively. Then, the DCs were treated with or without 1.0 mg L^{-1} Jagged-1 and Hes-1-siRNA at the different doses of 30, 60, 100, 200 and 250 nmol L^{-1} for 36 h. Total protein was extracted from the cells using RIPA with PMSF (100:1). Hes-1 protein was detected the by Western blot. All of the results were repeated three times. The data are expressed as the means ±SD, $*P<0.05$ and $**P<0.01$ *vs* the corresponding control.

compared to Jagged-1 group. Interestingly, the cells treated by Hes-1-siRNA showed lower percentage of the foregoing double positive cells than the control possibly suggesting that endogenous activation or level of Hes-1 is indispensable for maintaining maturation of DCs as well. The cells treated with mutated Hes-1-siRNA showed neither a suppressing or promoting effect, similar to the control. These results provide further evidence that the activation of Jagged-1-Notch signaling may promote the maturation of the bone marrow derived DCs to an extent and Hes-1 may be a critical target in effect of Jagged-1-Notch signaling pathway on DC maturation.

3.4 Hes-1-siRNA inhibits the production of IL-12 by bone marrow-derived DCs

Interleukin-12 (IL-12) secreted by DCs is, at present, considered another mature marker of DCs in addition to co-stimulating molecules expressed on their surface [16]. To further assess the effect of siRNA target to Hes-1 on the maturation of DCs, we detected levels of IL-12 in the culture supernatant of the treated DCs through ELISA. Consistent with the change of DC phenotypes shown above, Jagged-1 stimulated the increase IL-12 production, appearing about 2 fold of the control ($P<0.01$), which could be offset by siRNA target to Hes-1, but the level of Jagged-1-increased IL-12 was still much lower than that of LPS-increased IL-12 ($P<0.01$). Compared with the control, Hes-1-siRNA also resulted substantially in down-regulation of IL-12, whereas mutated Hes-1-siRNA had no influence (Figure 5). Our results further supported that Hes-1 may be a critical target in effecting Jagged-1-Notch signaling pathways on DC maturation.

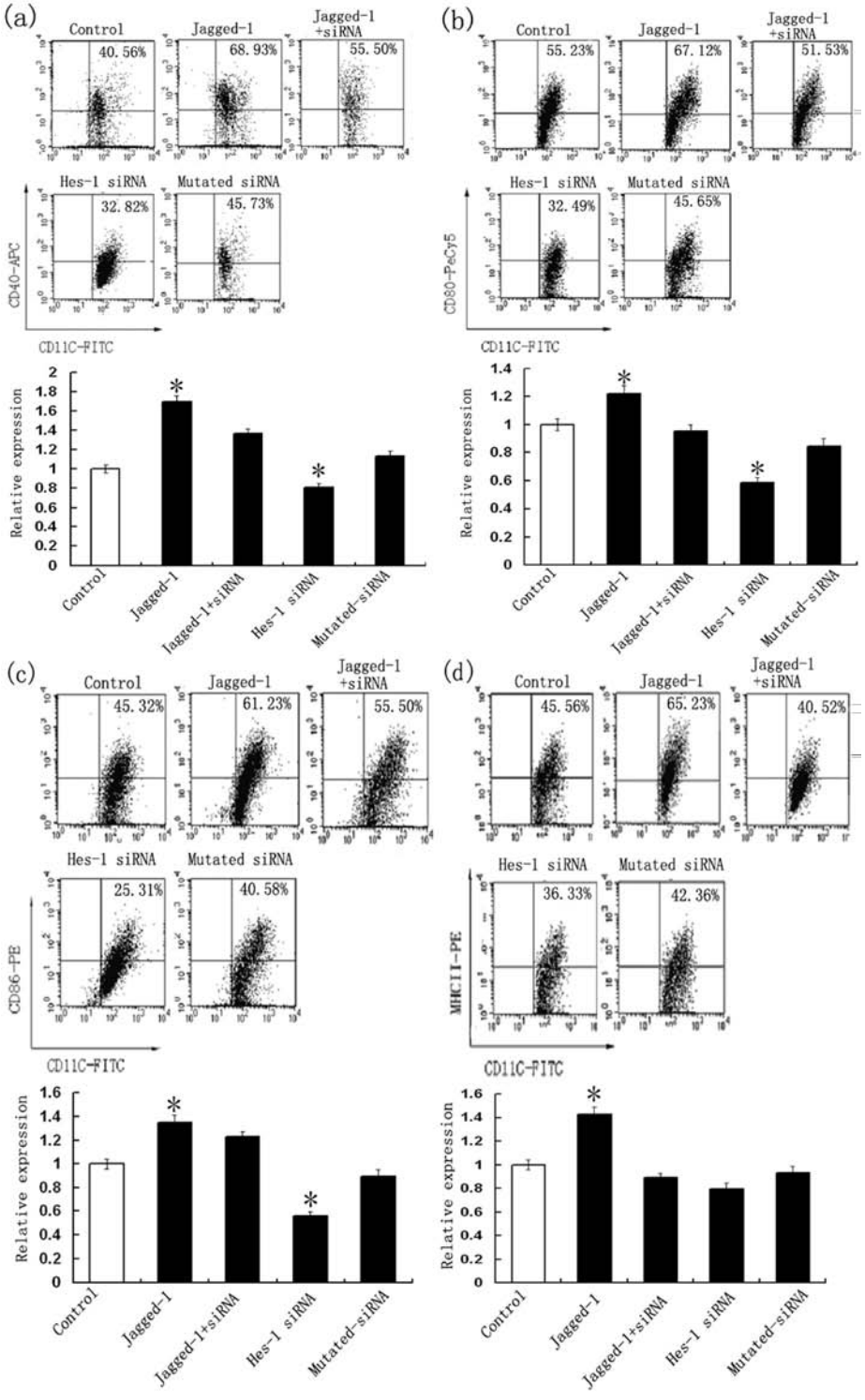

Figure 4. Effect of Hes-1siRNA on the maturation of bone marrow-derived DCs. The CD11c positive cells were isolated from bone marrow cells, then stimulated with or without 1.0 mg L^{-1} of Jagged-1, 1.0 mg L^{-1} of Jagged-1 plus 100 nmol L^{-1} of Hes-1-siRNA, 100 nmol L^{-1} of Hes-1-siRNA and 100 nmol L^{-1} of mutated Hes-1-siRNA for 36 h. After the cells were stained with 1.0 μl anti-CD11c-FITC (0.5 μg/10^6 cells) and meanwhile with 0.5 μl anti-CD40-APC (0.125 μg/10^6 cells), 0.3 μl anti-CD80-PECY5 (0.06 μg/10^6 cells), 0.5 μl anti-CD86-PE (0.125 μg/10^6 cells) or 0.5 μL anti-MHCII-PE (0.125 μg/10^6 cells) in a 100 μl total volume, respectively, the cells were detected through flow cytometry. All of the results were repeated three times. The data are expressed as the means $\pm$SD,*$P<0.05$ and **$P<0.01$ *vs* the corresponding control.

4. Discussion

All DCs are thought to arise from bone marrow precursors, divided into myeloid-derived DCs and lymphoid-related DCs. Myeloid-derived DCs are the most effective APCs for initiating native T cellresponses through intaking, processing and presenting antigens. Presently, it is universally accepted that CD11c serves as a relative specific differentiation molecule of DCs from murine and CD80 *etc*. as a maturation tagged molecule. DC differentiation is regulated *via* a network of soluble and cell-bound stroma. Since Jagged-1 expression was observed in endothelial cells [17,18], thymus [19], bone marrow stromal cells [20], and keratinocytes, Notch ligands like Jagged-1 expressed on adjacent bone marrow stromal cells may induce up-regulation of Notch down-stream target protein. Some studies stated that the levels of MHC-II, CD80, CD83 and CD86 molecule expression on the surface of DCs were up-regulated by co-culturing keratinocytes expressing Jagged-1 with immature DCs [21]. In this study, soluble Jagged-1 was directly used to induce differentiation and maturation of bone marrow-derived DCs in mice *in vitro*. The results show that Jagged-1 can promote, to some extent, bone marrow-derived DCs to differentiate and mature. This is executed through Hes-1 because the levels of its mRNA and protein expression are profoundly enhanced. Moreover, the level of IL-12 produced by the Jagged-1-induced DCs was concurrently augmented, further supporting the action of Jagged-1 in inducing the maturation of the DCs.

The mechanism to regulate the differentiation and maturation of DCs by Jagged-1-Notch signaling pathway remains poorly understood. The level of NF-κB transcriptional factor activity in Notch1$^{+/+}$ HPCs was 15 fold over the normal level, but the level of NF-κB activity in Notch1$^{-/-}$ HPCs was 1/4 of the normal level [22]. Additionally, NF-κB has been proposed to trans-activate Jagged-1 [23], indicating that NF-κB may play a prominent role in signaling cascades that lead to the differentiation and maturation of DCs induced by Jagged-1-Notch signaling. After hematopoietic progenitor cells (HPCs) were co-cultured with fibroblasts transfected with the expression vector containing Jagged-1 gene, c-promoter binding protein-1 (CBF-1), a transcriptional activator of Notch-1, was persistently elevated, as was proved by electrophoresis mobility gel shift assay, demonstrating that CBF-1 might also play a certain role in Jagged-1-Notch signaling. Another study reported that the expression of CXCR4 was down-regulated in RBP-J-deficient DCs and up-regulated in a γ-secretase-dependent manner when DCs were stimulated with Notch ligands. The forced

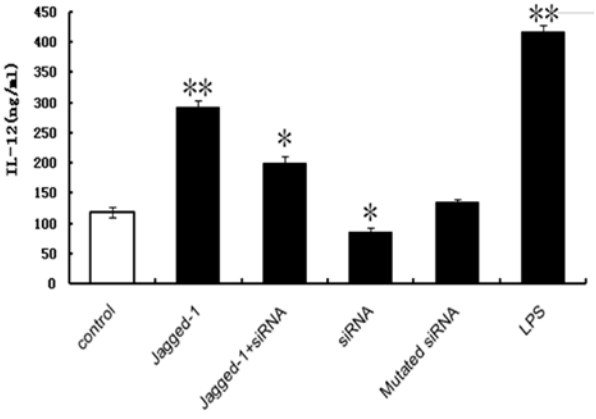

Figure 5. Effect of Hes-1 siRNA on the production of IL-12 in bone marrow-derived DCs. The bone marrow-derived cells were induced with 10 μg L^{-1} of GM-CSF and 2.5 μg L^{-1} of IL-4 for 6 days and then treated with or without 1.0 mg L^{-1} of Jagged-1, 1.0 mg L^{-1} of Jagged-1 plus 100 nmol L^{-1} of Hes-1-siRNA, 100 nmol L^{-1} of Hes-1-siRNA and 100 nmol L^{-1} of mutated Hes-1-siRNA for 36 h, the supernatant was collected to detect the IL-12 through ELISA. The data are expressed as the means ±SD,*P<0.05 and **P<0.01 *vs* the corresponding control.

expression of CXCR4 rescued RBP-J deficiency-induced block of DC differentiation. Furthermore, Notch triggering-induced DC differentiation might be blocked by a specific inhibitor of CXCR4. These findings show that the chemokine receptor CXCR4 is downstream to canonical Notch signaling, either directly or indirectly, and is responsible for Notch signaling-mediated differentiation transition of DCs [13].

It was found earlier that Hes-1 could directly inhibit the expression of proneural bHLH factors, such as Mash1, and thus inhibit neuronal differentiation [24]. In addition, Notch signaling through Hes-1 can also inhibit the differentiation of 3T3-L1 preadipocytes [25]. For this reason, in the current study Hes-1-targeting siRNA was utilized to investigate whether Jagged-1-Notch signaling functions *via* the Hes-1 molecule to regulate the maturation of the murine bone marrow-derived DCs. Our data revealed that Hes-1 expression could be obviously decreased by Hes-1-targeting siRNA, followed by inhibited maturation of the DCs, while Jagged-1 stimulus resulted in up-regulation of Hes-1 mRNA and protein, followed by the increased maturation of the DCs. Therefore, Hes-1 in a CSL-dependent route may exert its positive effect on phenotypic differentiation and maturation of myeloid DCs induced by Jagged-1-Notch activation pathway. DCs can direct helper T cell polarization. Th1-type response can be induced by CD8^{+}DCs *via* TLR and production of IL-12 [26]. Th1 response may also occur in the absence of IL-12

[27] *via* the Notch signaling-dependent pathway [28]. Our data presently indicates that Hes-1 deficiency decreases the product of IL-12 in the DCs, suggesting that the Jagged-1-Hes-1 signaling-educated DCs may also impact skewing of naïve $CD4^+$ T cells towards Th1 cells. The detailed mechanical process remains to be elucidated.

Taken together, controversy surrounds the differentiation of HPCs into DCs mediated by Notch signaling [21]. Our results demonstrate that Hes-1 may be an important target of Notch signaling mediating the maturation of DCs. Hence, we consider that this result is likely related to different Notch signals activated events by distinct Notch ligands, with different modalities of an identical ligand and with different Notch down-stream target proteins. Besides, direct utilization of a soluble Hes-1-siRNA and Jagged-1 to investigate DC differentiation and maturation may accurately control their doses correctly estimate its physiological, pharmacological and toxicological functions. The present observations not only present some theoretical basis on regulating the maturation of DCs, but also have potential clinical applications, meriting deeper exploration .

Acknowledgements

This project was supported by the National Natural Science Foundation of China (No 30971465, No 81172824, No 30471635) and “211” project grant.

References

[1] Artavanis-Tsakonas S., Rand M.D., Lake R.J., Notch signaling: cell fate control and signalintegration in development, Science, 1999, 284, 770-776

[2] Miele L., Osborne B., Arbiter of differentiation and death: Notch signaling meets apoptosis. J. Cell. Physiol., 1999, 181: 393-409

[3] Schroeter E.H., Kisslinger J.A., Kopan R., Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature, 1998, 393, 382-386

[4] Curry C.L., Reed L.L., Nickoloff B.J., Miele L., Foreman K.E., Notch-independent regulation of Hes-1 expression by c-Jun N-terminal kinase signaling in human endothelial cells. Lab. Invest., 2006, 86, 842-852

[5] Osborne B.A., Minter L.M., Notch signalling during peripheral T-cell activation and differentiation. Nat. Rev. Immunol., 2007, 7, 64-75

[6] Cheng P., Nefedova Y., Miele L., Osborne B.A., Gabrilovich D., Notch signaling is necessary but not sufficient for differentiation of dendritic cells. Blood, 2003, 102, 3980-3988

[7] Weijzen S., Velders M.P., Elmishad A.G., Bacon P.E., Panella J.R., Nickoloff B.J., et al., The Notch ligand Jagged-1 is able to induce maturation of monocyte-derived human dendritic dells. J. Immunol., 2002, 169, 4273-4278

[8] Caton M.L., Smith-Raska M.R., Reizis B., Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. J. Exp. Med., 2007, 204, 1653-1664

[9] Nakagawa O., McFadden D.G., Nakagawa M., Yanagisawa H., Hu T., Srivastava D., et al., Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of Notch signaling. Proc. Natl. Acad. Sci. USA, 2000, 97, 13655-13660

[10] de Souza N., Vallier L.G., Fares H., Greenwald I., SEL-2, the C. elegans neurobeachin/LRBA homolog, is a negative regulator of lin-12/Notch activity and affects endosomal traffic in polarized epithelial cells. Development., 2007, 134, 691-702

[11] Takebayashi K., Sasai Y., Sakai Y., Watanabe T., Nakanishi S., Kageyama R., Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor Hes-1. Negative autoregulation through the multiple N box elements. J. Biol. Chem., 1994, 269, 5150-5156

[12] Choi M.S., Yoo A.S., Greenwald I., Sel-11 and cdc-42, two negative modulators of LIN-12/Notch activity in C. elegans. PLoS One, 2010, 5, e11885

[13] Wang Y.-C., Hu X.-B., He F., Feng F., Wang L., Li W., et al., Lipopolysaccharide-induced Maturation of Bone Marrow-derived Dendritic Cells Is Regulated by Notch Signaling through the Up-regulation of CXCR4. J. Biol. Chem., 2009, 284, 15993-16003

[14] Kamakura S., Oishi K., Yoshimatsu T., Nakafuku M., Masuyama N., Gotoh Y., Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. Nat. Cell. Biol., 2004, 6, 547-554

[15] Choi J.W., Pampeno C., Vukmanovic S., Meruelo D., Characterization of the transcriptional expression of Notch-1 signaling pathway members, Deltex and Hes-1, in developing mouse thymocytes. Dev. Comp. Immunol., 2002, 26, 575-588

[16] Hovden A.O., Karlsen M., Jonsson R., Appel S., The bacterial preparation OK432 induces

IL-12p70 secretion in human dendritic cells in a TLR3 dependent manner. PLoS One., 2012, 7, e31217

[17] Lindner V., Booth C., Prudovsky I., Small D., Maciag T., Liaw L., Members of the Jagged/Notch gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell matrix and cell-cell interaction. Am. J. Pathol., 2001, 159, 875-883

[18] Villa N., Walker L., Lindsell C.E., Gasson J., Iruela-Arispe M.L., Weinmaster G., Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. Mech. Dev., 108, 2001, 108, 161-164

[19] Felli M.P., Maroder M., Mitsiadis T.A., Campese A.F., Bellavia D., Vacca A., et al., Expression pattern of notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: distinct ligand-receptor interactions in intrathymic T cell development. Int. Immunol., 1999, 11, 1017-1025

[20] Li L., Milner L.A., Deng Y., Iwata M., Banta A., Graf L., et al., The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. Immunity, 1998, 8, 43-55

[21] Cheng P., Nefedova Y., Corzo C.A., Gabrilovich D.I., Regulation of dendritic-cell differentiation by bone marrow stroma via different Notch ligands. Blood, 2007, 109, 507-515

[22] Cheng P., Zlobin A., Volgina V., Gottipati S., Osborne B., Simel E.J., et al., Notch-1 regulates NF-kappaB activity in hemopoietic progenitor cells. J. Immunol., 2001, 167, 4458-4467

[23] Bash J., Zong W.X., Banga S., Rivera A, Ballard D.W., Ron Y., et al., Rel/NF-kappaB can trigger the Notch signaling pathway by inducing the expression of Jagged1, a ligand for Notch receptors. EMBO J., 1999, 18, 2803-2811

[24] Kageyama R., Ohtsuka T., The Notch-Hes pathway in mammalian neural development. Cell. Res., 1999, 9, 179-188

[25] Ross D.A., Rao P.K., Kadesch T., Dual roles for the Notch target gene Hes-1 in the differentiation of 3T3-L1 preadipocytes. Mol. Cell. Biol., 2004, 24, 3505-3513

[26] Moser M., Murphy K.M., Dendritic cell regulation of TH1-TH2 development. Nat. Immunol., 2000, 1, 199-205

[27] Jankovic D., Kullberg M.C., Hieny S., Caspar P., Collazo C.M., Sher A., In the Absence of IL-12, CD4+ T Cell Responses to Intracellular Pathogens Fail to Default to a Th2 Pattern and Are Host Protective in an IL-10(-/-) Setting. Immunity, 2006, 16, 429-439

[28] Skokos D., Nussenzweig M.C., CD8- DCs induce IL-12-independent Th1 differentiation through Delta 4 Notch-like ligand in response to bacterial LPS. J. Exp. Med., 2007, 204, 1525-1531