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NF-κBp50 is Associated With DC-SIGN Expression Induced by IL-4 in THP-1 Cells*

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Abstract DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) is specific receptor on Dendritic cells, and plays a pivotal role on antigens presentation. Uptodate, the clear regulation mechanisms for DC-SIGN expression are not available. IL-4 is one of the most important cytokines inducing DC-SIGN production, while, NF-κB is an important transcription factor controlling signaling transduction. Both IL-4 and NF-κB are closely related to DC-SIGN regulation. NF-κB and IL-4 actions on DC-SIGN promoter activity, DC-SIGN expression as well as interactions between IL-4 and NF-κB were investigated in THP-1 cell. It was found that the mutation of NF-κB binding site in DC-SIGN promoter results in DC-SIGN promoter activity decrease about 50%. NF-κBp50 stimulates DC-SIGN expression in THP-1 cells. IL-4 upregulates DC-SIGN expression on THP-1 cells as well as NF-κB production. These data reveal that NF-κB is associated with IL-4 induced DC-SIGN expression.

Key words NF-κB p50, DC-SIGN, IL-4, THP-1 cell

Dendritic cells (DCs) play a critical role in initiating the immune response by virtue of their ability to capture and present antigens to T cells [1]. In this process, DC-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN), servering as receptors of many pathogens such as viruses, bacteria and protozoa, plays a pivotal roll on pathogens presentation and immune functions[2~6].

DC-SIGN is a kind of mannose specific C-type lectin about 44 ku. As the surface protein on DCs, DC-SIGN is encoded by its gene located on chromosome 19p13.2-3. *In vivo*, DC-SIGN is merely present on some types of DCs in dermal and mucosal tissues. PU.1 protein is responsible for basal and cell-specific expression of DC-SIGN in those tissues through *Ets* element in DC-SIGN promoter [7]. *In vitro* study, DC-SIGN production is IL-4 dependent, and can be induced on marrow stem cells or on THP-1 cell line if treated with IL-4 [8,9].

IL-4 is one of key cytokines for DC-SIGN production. Update, the exact mechanisms for DC-SIGN regulation by IL-4 are not available. Previous research suggests that IL-4 performs its activities by JAK-STAT pathway [10], yet, key processes that IL-4 regulates DC-SIGN expression need detailed study. Flores-Romo [11] suggests that IL-4 plays its regulatory function by inhibiting NF-κB expression, whereas, Shen[12] supports that IL-4 exerts its inducible transcriptional function *via* synergistic activations between STAT6 and NF-κB. Liu's study[13] confirms that DC-SIGN promoter is located from +251～+487 nucleotides on the 5' flank of DC-SIGN gene from start site, with an NF-κB binding site in this region. This research gives us a clue to explore what roles NF-κB plays on DC-SIGN expression. In our present study, we discuss the interactions between IL-4, NF-κB and DC-SIGN expression. We find that NF-κBp50 is closely associated with DC-SIGN expression induced by IL-4 in THP-1 cells.

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1 Material and methods

1.1 Cells

The acute monocytic leukemia cell line THP-1 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in RPMI 1 640 medium (Hyclone, USA) supplemented with 10% fetal calf serum (Gibico, USA), and maintained at 3×10^5 ～6×10^5 cells/ml. THP-1 differentiation was induced by treatment with IL-4 (1 000 U/ml, R&D systems, USA), either alone or in combination with PMA (10 μg/L, R&D systems, USA) as the method previously described [9]. In differentiation experiments, cells were routinely seeded at 5×10^5 cells/ml in culture dishes (Corning, USA) with no change of the culture medium after addition of the differentiation inducer (s). IL-4 was added into the cells 24 h after treatment with PMA and differentiation allowed to proceed for 48 h (PMA) or 24 h (IL-4). For subsequent analysis, differentiated THP-1 cells were collected from culture dishes by centrifugation and washed with PBS twicely. Then the obtained cell pellets were preserved on –80℃ for use.

1.2 Plasmids

DC-SIGN promoter gene was amplified by PCR and inserted into vector pGL3-basic (Promega,USA) at *Mlu* Ⅰ and *Bgl* Ⅱ endonucleases sites according previously described method[13] as a reporter plasmid. This constructed report plasmid (DC-SIGN promoter-pGL3-Basic, DSPGB) containing a DC-SIGN promoter and a fire fly luciferase gene encoding luciferase , could report activity of DC-SIGN promoter. Plasmid PGL3-Control was as a positive control with an SV40 promoter in plasmid circle. Plasmid pRL-TK (Promega) was used as an internal control, which had a Renilla luciferase gene. Plasmid pUNO-hNFκBp50 (Invogen,USA) encoding human NF-κBp50 was used to assay NF-κBp50 actions on DC-SIGN production. All plasmids used in this study were propagated in *Escherichia coli* Top10 and purified with Hipure plasmid midiprep Kit (Invitrogen, USA).

1.3 Mutagenesis of NF-κB binding site in DC-SIGN promoter region

According to DC-SIGN promoter sequence previously reported [13], we synthesized DC-SIGN promoter fragment with NF-κB binding site replaced by *Eco*R Ⅰ endonucleases sequence from commercial corporation , then inserted this fragment into plasmid pGL3-Basic to generate a plasmid DSPGBNF-κB.

1.4 Plasmids transfection and luciferase assay

20 μg of Plasmid pUNO-hNFkBp50 was transfected into 2×10^6 cells seeded in 10 cm culture dish to observe NFκBp50 actions on DC-SIGN production. As to luciferase assay, the cell were seeded into 96-well plate(Fallcon, USA)(0.5×10^4 cells/ well) in 100 μl of medium at one day before transfection, and maitained in medium without antibiotics, incubating at 37℃ in a humidified atmosphere of 95% air and 5% CO_2. On the day of transfection, 50 ng of pGL3-basic(negative control), 50 ng of DSPGB, 50 ng of DSPGBNF-κB$^-$ and 50 ng pGL3-Control vector (positive control), were mixed with 1ng of pRL-TK (Promega) (internal control) for transfection, respectively. Plasmid DNA was introduced into cells with Effectene (Qiagen, Germany). Twenty-four hours after transfection, cells were harvested and treated with a lysis solution (Promega), and 50 μl of the supernatant fraction was used for luciferase assay. Luciferase activities were assayed by using Bright-Glo™ Luciferase Assay System (Promega) or Dual-Luciferase Assay System (Promega) and measured at Orion Ⅱ microplate luminometer (Berthold Detection Systems, Germany). The experiments were repeated twice, and luciferase activities were determined from twelve independent samples for each assay.

1.5 RT-PCR

Total RNAs were abstracted from THP-1 cells mentioned above with Trizol(Invitrogen). 2 ng of total RNA were used for reverse-transcription to cDNA with RevertAid™ First Strand cDNA Synthesis Kits (Fermentas). Then 2 ng of cDNAs were used to

Table 1 PCR primers used in our research

	Primers(5'→3')	Product length/bp
DC-SIGN promoter	Sense ATC ATA CGC GTA TGA GTC CTT CTC CCT GTC Antisense TGT AAG ATC TGT CAC CCC ACT CTC CCC CAG	237
DC-SIGN	Sense AAC AAT CCA GGC AAG ACG 3 Antisense TGC TCA GGC AGG GTC AGT3'	625
β-Actin	Sense ATC ATG AAG TGT GAC GTG GAC A Antisense AAC CGA CTG CTG TCA CCT TCA	460

conduct PCR to detect expression of DC-SIGN. The primers used in our research were listed on Table 1.

1.6 Western-blot

Rabbit anti-hNF-κBp50 (Santa Cruz, USA) and mouse anti-hDC-SIGN (R&D systems) were used for immuno-bloting. Briefly, 2×10^6 cells were lysised and then the samples were resuspended in 2 × sample buffer. 20 μg of whole cell extracts were separated on SDS-PAGE, then transferred to PVDF (Millipore, USA) by electroblotting. Membrane was washed with PBS-T and then blocked for nonspecific protein with PBS containing 5% milk for 2 h at room temperature. The membrane was probed with first primary antibody at 4℃ overnight , then added with HRP labeled secondary antibody reacting for 2 h at room temperature. After washing, the protein-antibody complexes were visualized by incubating the membranes with the Supersignal West Fermto Western blotting detection system (Pierce,USA). The devices for Western-blot were from BIO-RAD (USA), and procedure was taken according to its protocol.

2 Results

2.1 Basic DC-SIGN promoter activity in THP-1 cell line

To observe IL-4 actions on DC-SIGN promoter activities, we first measured the basic activity of DC-SIGN promoter in THP-1 cell line. Plasmid pGL3-basic (negative control), pGL3-control (positive control), and DSPGB were transfected into THP-1 cells, respectively. At the same time, THP-1 cells without any plasmid introduction were set as blank. At the time of 24 h after transfection, luciferase activities in transfectants were measured. The result indicated the luciferase activities of pGL3-basic in THP-1 cells were closely near to background, whereas, DC-SIGN promoter had mean luciferase activities of 2430 in THP-1 cells. Compared with SV40 promoter in pGL3-control, DC-SIGN promoter induced a relatively weaker luciferase activity in THP-1 cells, (2430 vs. 32000)(Figure 1).

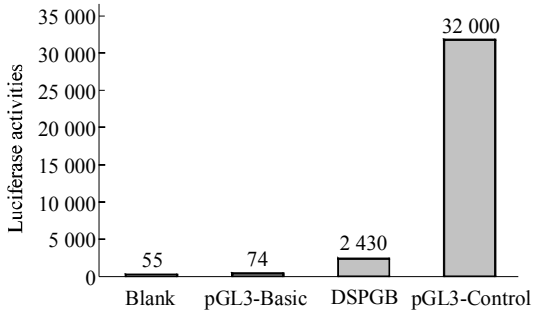

Fig. 1 The basic activity of DC-SIGN promoter in THP-1 cells

Luciferase activity induced by DC-SIGN promoter was 2430, which was significantly lower than pGL3-Control (32000). The luciferase of pGL-Basic(74) was very close to background(55).

2.2 Actions of IL-4 on DSPGB and DSPGBNF-κB⁻

In order to assess the IL-4 effects on DC-SIGN promoter activities, we generated two plasmids, DSPGB and DSPGBNF-κB⁻. The later plasmid is deficient of NF-κB binding site. We transfected THP-1 cells with 1ng pRL-TK along with 50 ng of DSPGB, 50 ng of DSPGBNF-κB⁻, 50 ng of pGL3-Basic, 50 ng of pGL3-Control , respectively. Plasmid pRL-TK gave an internal standard for diminishing the discrepancies of promoter activities resulted from cell different growth status. Our research indicated luciferase activities of DC-SIGN promoter were increased 4.4 times by IL-4 in THP-1 cells than those without IL-4 treatment (9 820/2 230=4.4). The increment of luciferase activity was enhanced by PMA (11 200 *vs* 9 820, Figure 2). PMA alone also slightly increased DC-SIGN promoter activity(2 850 *vs* 2 230).NF-κB site mutation within DC-SIGN promoter resulted in decline of DC-SIGN promoter activity nearly about 50% (2 230∶1 140), though the activities of DC-SIGN promoter without NF-κB binding site were also increased from 1 140 to 4 450 by IL-4. These results showed DC-SIGN transcription induced by IL-4 were probably associated with DC-SIGN promoter region. Deletion of NF-κB binding site in DC-SIGN promoter resulted in reduction of DC-SIGN promoter activities.

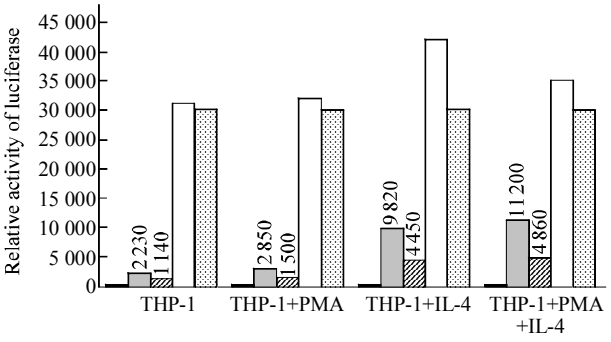

Fig. 2 Actions of IL-4 and NF-κB on DC-SIGN promoter activities

IL-4 increased DC-SIGN promoter activities about 4～5 times (9 820 vs 2 230). DSPGBNF-κB⁻ produced approximate 50% luciferease activities of DSPGB in THP-1 cells. ■ : pGL3-Basic; □: DSPGB; ▨: DSPGBNF-κB⁻; □:pGL3-Control; ⊠: pRL-TK.

2.3 NF-κBp50 effect on DC-SIGN expression

The luciferase assay revealed NF-κB was related to DC-SIGN promoter activities. So we transfected

NF-κBp50 expression plasmid pUNO-hNFκBp50 into THP-1 cells to observe whether NF-κBp50 could induce DC-SIGN expression on THP-1 cells or not. After 48 h of transfection, Western-blot assay was performed to observe NF-κBp50 action on DC-SIGN expression. The immunoblotting indicated that NF-κBp50 promote DC-SIGN production in THP-1 cells, suggesting the NF-κB was associated with DC-SIGN expression(Figure3) in THP-1 cells.

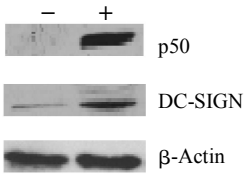

Fig. 3 NF-κBp50 enhanced DC-SIGN expression on THP-1 cells

2.4 IL-4 effects on expressions of NF-κB and DC-SIGN

IL-4 was one of the most important cytokines inducing DC-SIGN production on THP-1 cells. To determine whether IL-4 plays actions on NF-κB activities and DC-SIGN expression, we performed RT-PCR and immunoblotting to observe IL-4 actions on NF-κBp50 and DC-SIGN expressions in THP-1 cells. Results of RT-PCR and Western-blot suggested IL-4 increased the expression of DC-SIGN in THP-1 cells, and these increments were synergized by PMA. At the same time, NF-κB p50 expression was induced by IL-4, indicating NF-κB p50 expression was coordinated with DC-SIGN expression (Figure 4).

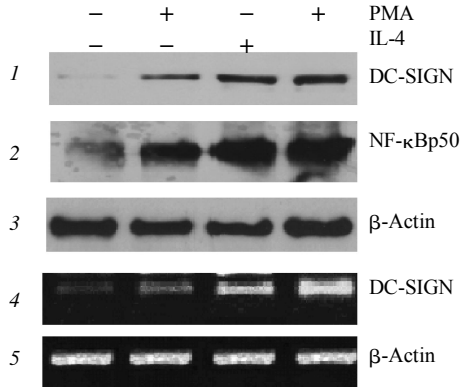

Fig. 4 IL-4 and PMA actions on DC-SIGN and NF-κBp50 in THP-1 cells

1,*2* and *3* were results of Western-blot, *4* and *5* were results of RT-PCR.

3 Discussion

NF-κB is an important transcriptional factor, controlling inflammation, cell survival, transformation, oncogenesis and signaling transduction. There are five members in the NF-κB family, namely, RelA (p65), RelB c-rel, NF-κB1/p50 and NF-κB2/p52. All the NF-κB proteins share a 300-residue N-terminal Rel homology domain that mediates the DNA binding, dimerization and nuclear targeting functions, as well as interaction with IκBs. Among the five NF-κB proteins, p65, c-Rel and RelB contain transcriptional activation domains, whereas p50 and p52 do not. The mature DNA-binding proteins p50 and p52 are generated by proteolytic processing of p105 and p100 precursors, respectively. Usually, in most cells, NF-κB homo- and heterodimers are associated with members of the family of inhibitor proteins called IκBs, remaining primarily in the cytoplasm as an inactive pool . Upon stimulation by different agents like IL-1, TNF-γ, CD40L, LPS, PMA or UV light, IκB molecules are rapidly phosphorylated, ubiquitinated, and degraded, allowing the NF-κB dimers to translocate from cytoplasm to the nucleus and regulate transcription through binding to NF-κB sites in gene promoter region [14,15]. NF-κB has a close association with DCs. Many pathogens recognize Toll Like Receptor (TLR) on DC surface and trigger the NF-κB expression, inducing DCs secret the cytokines and chemikines as well as DCs maturation [16,17], which lead to wide varieties of immune responses.

Gene promoter is one of the most important elements for controlling of gene expression, which is associated with the basic and specific gene expression in tissue. The specialized and limited expression of DC-SIGN *in vivo* reveals a unique regulation mechanism is probably responsible for DC-SIGN expression. In our present research , we isolate DC-SIGN promoter and insert it into reporter vector pGL3-Basic, trying find some clues for understanding of mechanisms of DC-SIGN expression regulation.

In our study , we construct two luciferase reporter plasmids, DC-SIGN-promoter-pGL3-Basic (DSPBB) and a mutant without NF-κB binding site (DSPGBNF-κB $^{-}$) , respectively. Two plasmids are transfected into THP-1 cells to examine DC-SIGN promoter activities and impacts of NF-κB binding site deletion on DC-SIGN promoter activities.

We find that deletion of NF-κB binding site

within DC-SIGN promoter region significantly decreases DC-SIGN promoter activity. At the same time, introduction of NF-κBp50 into THP-1 cell enhances DC-SIGN expression. These data suggest that NF-κBp50 plays an important role on DC-SIGN expression.

Our research also suggests, compared with SV40 promoter, DC-SIGN promoter is relatively weaker. DCSPGB only acts a low luciferase activity in THP-1 cell without IL-4 treatment, and this activity is increased significantly when IL-4 is added to cell culture system. Those data indicate that the regulatory function of IL-4 on DC-SIGN transcription is probably through associations with DC-SIGN promoter region. At the same time, deficiency of NF-κB site does not abolish DC-SIGN promoter activities completely. So, it is reasonable for us to conclude that regulation of DC-SIGN expression is also likely with other transcriptional binding sites, at least, DC-SIGN promoter activities were not solely dependent on NF-κB binding site..

At last, we examine the actions of IL-4 on NF-κBp50 during DC-SIGN expression induced by IL-4 in THP-1 cells. The results reveal IL-4 induces NF-κBp50 presentation in THP-1 cells. IL-4 combining with PMA displays a strongly co-stimulative response to NF-κBp50 expression. The presentation of NF-κBp50 in THP-1 cells is correlated to DC-SIGN expression, suggesting a potential relationship between DC-SIGN and NF-κB.

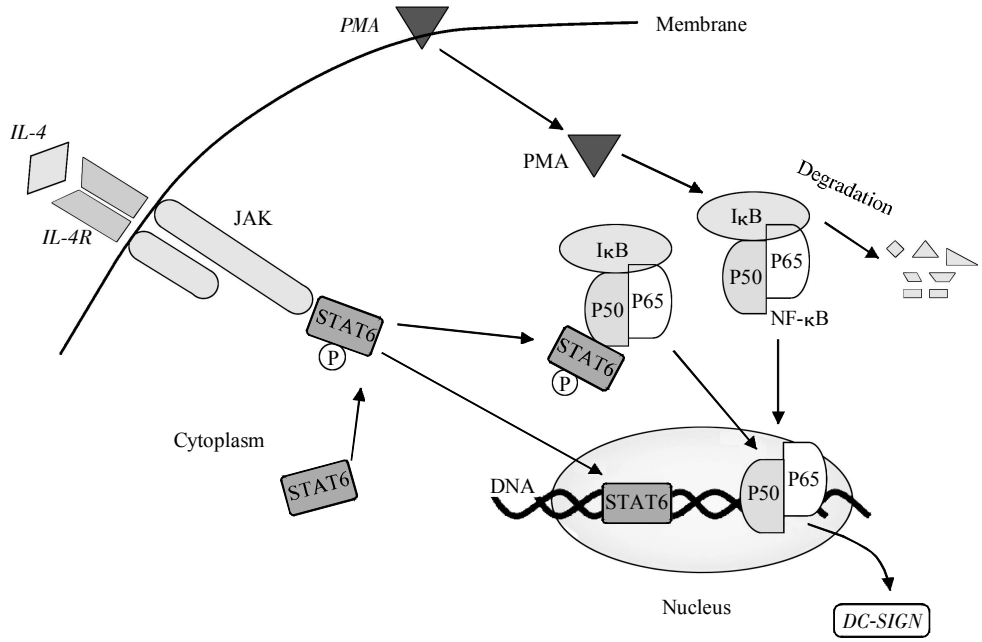

Fig. 5 The possible pathway IL-4 induces DC-SIGN production

NF-κB is induced by two ways: (1) STAT6 interacts with NF-κB complex. (2) IκB complex are degradated by PMA.

Previous data demonstrate that IL-4 biologic functions are mainly dependent on JAK-STAT6 signal pathway[10]. Our data suggest the possible pathways by which IL-4 induces DC-SIGN production are as follows: IL-4 stimulates STAT6 production by IL-4 receptor (IL-4R)-JAK-STAT6 pathway. The induced STAT5 interacts with NF-κB complex resulting in NF-κB activation. PMA directly acts on IκB complex, leading to IκB phosphorylation, ubiquitination and degradation. Thus, NF-κB is released and activated [14~15] (Figure 5). The activated NF-κB participates in DC-SIGN production.

In summary, we examine effects of NF-κB on DC-SIGN promoter activity and DC-SIGN expression in THP-1 cells on process of IL-4 induction. The results of our research indicate IL-4 inducing DC-SIGN is probably associated with NF-κB activities though interactions between STAT6 and NF-κB play important roles on DC-SIGN production. IL-4 leads to NF-κBp50 production in THP-1 cell during DC-SIGN expression. Presentation of NF-κBp50 in THP-1 cells enhances DC-SIGN expression. NF-κB deletion within DC-SIGN promoter reduced the DC-SIGN promoter activies about 50% . Those results revealed that NF-κBp50 is associated with DC-SIGN expression induced by IL-4 in THP-1 cells.

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NF-κBp50 参与 IL-4 在 THP-1 细胞中诱导 DC-SIGN 的表达 *

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摘要 树突状细胞表面特异的胞间黏附分子 3 捕获非整合素(DC-specific intercellular adhesion molecule-3-grabbing nonintegrin，DC-SIGN) 是树突状细胞表面特异的蛋白，在抗原呈递过程中起关键作用．这种特异性的表达和 DC-SIGN 的调节机制有关．到目前为止， DC-SIGN 表达调控的机制还不是很清楚．IL-4 是诱导 DC-SIGN 表达的最重要的细胞因子之一，而 NF-κB 是调控细胞信号转导的一个重要调控因子，两者都和 DC-SIGN 的表达调节相关．研究了 IL-4 和 NF-κB 对 DC-SIGN 启动子活性、对 DC-SIGN 表达的影响以及 IL-4 和 NF-κB 之间相互作用的关系．发现：DC-SIGN 启动子中 NF-κB 位点缺失可以使 DC-SIGN 启动子活性下降大约 50%， NF-κBp50 可以促进 DC-SIGN 在 THP-1 细胞的表达，IL- 在 THP-1 细胞诱导 DC-SIGN 表达的同时，也促进了 NF-κBp50 的表达．这些结果显示，在 THP-1 细胞中 NF-κBp50 参与 IL-4 诱导的 DC-SIGN 表达．

关键词 核转录因子 κBp50，树突状细胞表面特异的胞间黏附分子 3 捕获非整合素，白细胞介素 4，THP-1 细胞

学科分类号 Q

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