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RESEARCH ARTICLE

Cytokines mRNA in bone marrow-derived dendritic cells in asthmatic mouse

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Abstract By inducing and amplifying dendritic cells (DCs) derived from the bone marrow of asthma murine in vitro, cytokines mRNA were expressed, and the functions of DCs were investigated. Cells isolated from murine bone marrow have been cultured with rmGM-CSF and rmIL-4, and the expression of cytokines mRNA was determined by ribonuclease protection assay combined with multi-probe templates. Large numbers of DCs have been obtained from bone marrow, and they expressed interleukin-13 (IL-13), interleukin-9 (IL-9), and interleukin-3 (IL-3) mRNA. Moreover, the level of IL-13 mRNA and IL-9 mRNA expressed by DCs in asthmatic mice was significantly higher than those in the control groups ($P<0.05$). But, the level of IL-3 mRNA showed no discrepancy between the two groups ($P>0.05$). DCs are very important in the forming and developing of asthma, which implies that the therapy targeted at DCs will possibly become a new goal.

Keywords asthma, dendritic cell (DC), cytokine, interleukin-13 (IL-13), Interleukin-9 (IL-9)

1 Introduction

Asthma is a kind of chronic airway inflammation, characterized by airway hyperreactivity (AHR) and mucosae hypersecretion, and can induce intermittent airway damage. Large numbers of study results show that the occurrence of immune response to Th2 and hypersecretion of Th2-Class cytokine, such as IL-4，IL-5，IL-9，IL-13, may induce many inflammation symptoms such as the increase of airway eosinophilia, mucosae hypersecretion, AHR, synthesis of IgE, maturity, and activation of mastocyte. Dendritic cell (DC) is one of the most powerful antigen presenting cells (APC), it can recognize, uptake, process, and present antigen to T cell, then activate incipient T cell. More and more evidences show that stimulant and inhibitive signal by DC is the key factor to differentiate T cell and misregistration of Th1 / Th2 in antigen presentation process, and it is also the beginning of asthma induced by allergen.

The origin of DC is the multi-potent stem cell in marrow. Then it gradually migrates and differentiates to peripheral tissues during different developmental stages, and under the conditions of different microenvironment and cellular factors. Then it becomes the first immunizing defense in skin, gastrointestinal tract, airway, and lungs. Some experiments show that the number of DC increases quickly in the airway and its function strengthens following with the increasing of eosinophile granulocyte associated with Th2 cytokine in asthmatic attacks. All these DCs came from the peripheral blood (Lambrecht et al., 1998; Jahnsen et al., 2001). In this paper, in order to study the possible mechanism of action of DC in asthma, we induced DCs from marrow cells in asthmatic mouse and studied how many kinds of mRNA transcription of inflammatory cytokine gene were there in these DCs by using ribonuclease (RNase) protection analysis (RPA) of multiprobe template.

2 Materials and methods

2.1 Materials

BALB/C mouse (Shanghai Laboratory Animal Center of China Academy of Sciences, China, female, 4~6 weeks), ovalbumin (OVA，Grade Ⅱ, Sigma), RPMI1640 nutrient medium GIBCO), fetal calf serum (Hyclone), recombinant mouse interleukine-4 (rmIL-4) and recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-

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CSF) (R & D system), Trizol Reagent (GIBCO), RNase protection analysis kit Ⅲ (RPAIII) and T7 in vitro transcription kit (Ambion), multiprobe template (PharMingen), [α - ^{32}P]UTP (Beijing Furui Co.), and other reagents (analytical grade made in China).

2.2 Methods

2.2.1 Establishment of asthmatic mouse models (Chen et al., 2002)

Twenty mice, which were normally fed for 2 days, were randomly divided into two groups. The mice in the experimental group were all injected intraperitoneally with 0.1-ml suspension of OVA and $Al(OH)_3$ (including 10 mg OVA and 20 mg $Al(OH)_3$) at the first and eighth day respectively, and they were made to inhale 1% OVA ultrasonic nebulization (S-888E ultrasonic atomizer) for 2 weeks, after 14 days until they appear to show asthmatic response, which is characterized by shortness of breath, hyperspasmia of abdominal muscle, and dysphoria. Mice in the control group underwent the same method except ovalbumin was replaced with saline.

2.2.2 Cultivation of marrow-derived DCs

There were a few differences from the methods provided by Ding et al. (2003). Marrow cells from sacrificed mice (done by pulling the necks, striping the femoral bone and the shinning bone respectively) were washed out with Hank's solution and washed with PBS once. Then, we decomposed their erythrocyte with 0.83% Tris–NH_4CL solution, washed with RPMI1640 nutrient medium twice, adjusted cell concentration to 1×10^6 per mL, added in rmGM-CSF (1000 U/mL) and rmIL-4 (500 U/mL), and then they were inoculated in 24-hole plates. Then, we placed the plates in 37°C incubator with 5% CO_2 to incubate. On the third day, we inhausted most of the suspension cells by shaking the plates slightly. We then added them to equal amounts of nutrient medium and complement. Every other day, we replaced the semi nutrient medium, and on the seventh day we collected the slightly adherent suspension cells.

2.2.3 Extraction of total RNA

Total RNA of cells was extracted from 1×10^6 DCs which were cultivated for 7 days with Trizol reagent. The production and purity of total RNA were determined with UV spectrophotometer , $A_{260/280}$=1.9. The quality of RNA was determined by 1% agarose gel electrophoresis, the ratio of the 28S band area to the18S band area was close to 2:1 (Fig. 1).

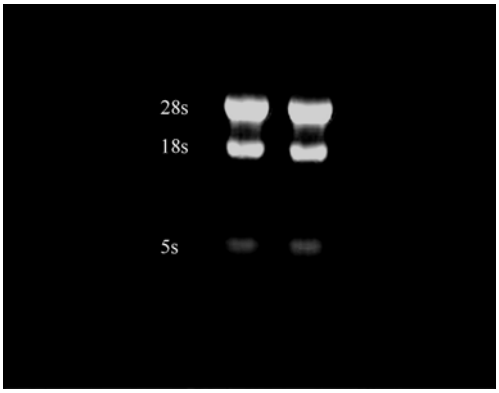


Fig. 1 1% Agarose electrophoretic analysis of RNA quality

2.2.4 Synthesis of cRNA probe

Multiprobe template contains the DNA which codes IL-4, IL-5, IL-10, IL-13, IL-9, IL-2, IL-3, and IFN-γ of mouse, IL-15 of human, house-keeping gene L32 and GAPDH. As a template, the DNA is transcribed to ^{32}P-marked cRNA probe in vitro with the presence of T7 RNA polymerase in accordance with the kit directions.

2.2.5 RNase protection analysis

In accordance with the kit directions, we added 2-μL marked cRNA probe (specific activity is 5×10^5 cpm/μL) to 10 and 20 μg total RNA, respectively, and then distributed the mixture sufficiently and centrifuged it transiently. The mixture was denatured for 5 minutes at 95°C and hybridized overnight at 56°C.While Yeast RNA and Mouse Control RNA were the control, we added proper RNase A/T1 to digestive buffer solution at the ratio of 1:1,000, then added 100 μL diluted RNase A/T1 to samples except to one sample of Yeast RNA control. All samples were incubated for 45 minutes at 30°C, then we added in 150 μL inactivated/precipitated solution which was one ingredient of the kit. It was left to stand for 30 minutes at −20°C and centrifuged for 30 minutes with 12,000×g at 4°C. We then removed the upper solution, dried the precipitate in air, added it in 8-μL gel loading buffer, and finally carried out 5% polyacrylamide/8mol/L urea/1×TBE denaturing gel electrophoresis. After that, we dried the gel and conducted radioautography for 3 days at −20°C.

2.3 Statistical analysis

The radioautographic photograph was scanned by computers; gray scale (density) of the signal analyzed by Scion Image analysis software. Relative abundance level can be represented by the relative gray scale ratio of target mRNA and mRNA of house-keeping gene L32 and GAPDH (Table 1), and the result was represented in the form of

mean±standard deviation ($\bar{x} \pm sd$). A *t*-test was conducted by using SPSS 10.0 software to show whether there was a significant difference in mRNA transcriptional level between model group and control ($P<0.05$).

3 Results

3.1 Cultivation of marrow-derived DCs

On the second day of culture, most of the cells grew adherently, and there were punctuated cell colonies. On the third day, we slightly shook the plates to remove suspended cells and continued to incubate the cells. After 2 days, it was obvious that cell colonies increased (Fig. 2). The bulk of the cells obviously increased and their shapes were irregular during the succeeding days. Meanwhile, 3–8 protuberances of typical dendrite appeared, and the cells gradually suspended in the solution and were moving away from the plate wall. On the 7th day, most of the cells were suspended in the solution while a few still slightly adhered to the wall and could suspend in the solution by threshing softly. We collected cells and observed them using an inverted microscope. The marrow-derived DCs in the asthmatic mouse and in the control mouse both had dendrites with varying sizes, while the difference in quantities was not significantly observed under the inverted microscope.

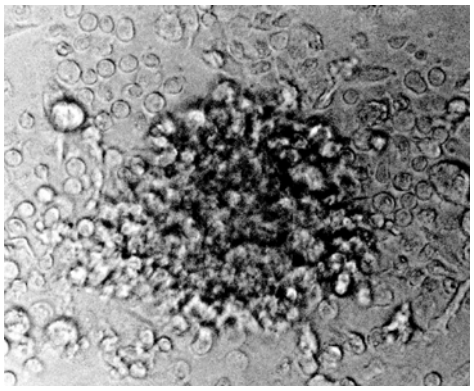
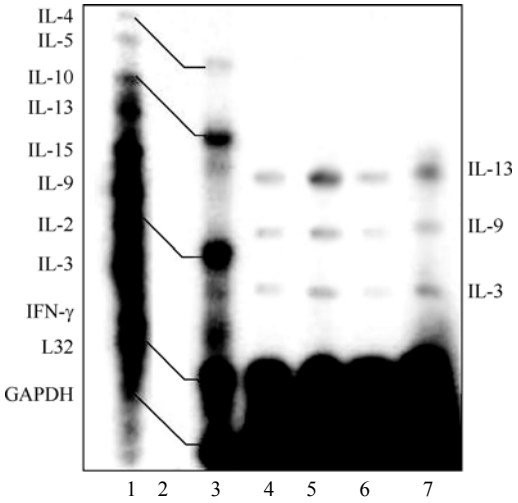


Fig. 2 Cell colonies of marrow-derived DCs in asthmatic mouse (400×)

3.2 RPA with multi-probe template

The result of the radioautography of multiprobe RPA shows that signals appeared. The signals were the zones which were "protected" after being bound, and the zones were located at 256 bp, 204 bp, and 163 bp. These positions corresponded to the places slightly below the bands for the IL-13, IL-9, and IL-3 probes. This meant that there were mRNA transcriptions of these three kinds of cytokine gene in marrow-derived DCs in both asthmatic mouse and control mouse (Fig. 3). Because Yeast RNA cannot combine with the cRNA probe of mouse, we used Yeast RNA control which was not digested with RNase to show all the probes. Therefore, Yeast RNA control digested with RNase could determine the activity of RNase as a negative control. As pointed out in the reference in the kit, there are gene transcriptions of IL-4, IL-10, IL-2, L32, and GAPDH in Mouse Control RNA. These means cRNA probe transcripted in vitro is effective. Transcription of house-keeping gene also proves that samples are unbroken. The protected zones migrate more quickly than probe zones, because the sequences of both ends of probes cannot combine with the mRNA of the samples, and are enzymolyzed and become shorter after binding. Therefore, the zones of the samples (the third to seventh lane from the left of electropherogram) appear slightly below the corresponding standard zones of probes (the first lane from the left of electropherogram).



Lane 1: probe; Lane 2: Yeast RNA; Lane 3: Mouse Control RNA; Lane 4: 10μg total RNA in DC of model mouse; Lane 5: 20μg total RNA in DC of model mouse; Lane 6: 10μg total RNA in DC of control mouse; Lane 7: 20 μg total RNA in DC of control mouse
Note: The position of Mouse Control RNA protected corresponding to location of probe IL-4, IL-10, IL-2, L32, GAPDH respectively is lower.

Fig. 3 mRNA transcription of cytokine in marrow-derived DCs between model group and control by RNase protection analysis

3.3 Signal gray scale analysis

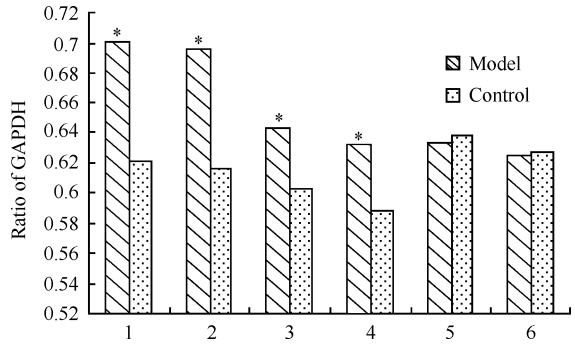
When molar concentrations of multiprobe template are the same, intensity of product signal depends on the amount of sample mRNA combining with ^{32}P-marked cRNA probe. When the signal is more intense, the amount of sample mRNA combining with ^{32}P-marked cRNA probe is larger, and there are more mRNA transcription for the corresponding gene. Therefore, the signal of 20 μg total RNA is more intense than that of 10 μg. Analysis of the radioautographic signal gray scale (density) showed that the amount of mRNA transcription in cytokine gene of mouse DCs between the two groups was different (Table 1 and Fig. 4). The amount of mRNA transcription in cytokine IL-13 and IL-9 gene of DCs in the model group was significantly more than that in the control ($P<0.05$). But there was no

Table 1 The comparison of mRNA relative level of cytokine gene between model group and control ($\bar{x} \pm s$)

Groups	IL-13		IL-9		IL-3	
	/L32	/GAPDH	/L32	/GAPDH	/L32	/GAPDH
Model	0.7007±0.1130*	0.6951±0.1102*	0.6430±0.0533*	0.6316±0.0543*	0.6341±0.0726	0.6245±0.0703
Control	0.6212±0.0330	0.6162±0.0530	0.6031±0.0318	0.5891±0.0348	0.6382±0.0769	0.6263±0.0850

Note: $n = 8$, comparing with control; $P < 0.05$

significant difference in the amount of mRNA transcription in cytokine IL-3 gene of DCs between the two groups ($P < 0.05$).



1: IL-13/L32; 2: IL-13/GAPDH; 3: IL-9/L32; 4: IL-9/GAPDH; 5: IL-3/L32; 6: IL-3/GAPDH; *$P < 0.05$

Fig. 4 The comparison of mRNA transcription level of cytokines gene between 2 groups

4 Discussion

Bronchial asthma (BA) is one kind of chronic airway inflammation with interactive multiple inflammatory cells and cytokines. Its immunity abnormality shows unbalance of Th1/Th2 cell ratio; shown by the increasing of Th2 cell number and the accentuation of Th2 cell function. DC is a kind of powerful antigen presenting cell (APC). It can construct a defensive network in the airway and lungs. Through the second signal provided by costimulatory molecules expressed on DC's surface, DC can efficiently and selectively activate antigenic specific incipient CD_4^+ Th cells, induce primary immune response, and secrete some solubility factors to induce Th0 cells which can differentiate toTh2 cells. At the same time, DCs can also express chemokine to attract activated Th2 cells in order to ensure activated effector cell staying in the airway and maintaining inflammatory reaction in the airway. The immune histochemical analysis results have proved that the amount of DCs in the airway and lungs of asthmatic mouse model is obviously larger than the control. In this experiment, we extracted bulk DC from marrow cells of mice and studied the gene transcription level in cytokine of mouse marrow-derived DCs in two groups at the mRNA level. The results show that the amount of genetic transcription in inflammatory cytokine IL-13 and IL-9 in the asthmatic group of mice is more than that of the control. This further proves that DCs are very important in the asthmatic etiopathogenesis, and it also suggests that therapy targeted to DCs will possibly become a new way in the future.

IL-13 is a kind of Th2 cytokine that can regulate many cellular immunity effects and can be generated from many kinds of cells. In alveolar irrigating solution, on mucous membrane of bronchus, and in peripheral blood mononuclear cell of allergic asthma patient, mRNA transcription of IL-13 can all be detected, and the amount of transcription is more than that of the control. This study also suggests that there is mRNA transcription of IL-13 gene in marrow-derived DCs, and the amount of transcription in the asthmatic group is more than that of the control group. The increase in mRNA transcription of IL-13 can aggravate asthmatic inflammation symptom in two ways: (1) It can induce epithelial cell expressive chemokine MIP-3α/CCL20 and further accelerate the recruit of immature DCs in the airway (Reibman et al., 2003) and maintain the expression of other Th2 cytokine on mucous membrane of bronchus; (2) It can promote the proliferation of B cells and induce the conversion of immunoglobulin class to Ig E, and finally increase the amount of IgE (Taube et al., 2003).

IL-13 has the same acceptor as IL-4 and can combine with the α-chain of the IL-4 acceptor (de Vries, 1998). IL-13 and IL-4 also use the same signal transduction system. But this experiment does not find IL-4 mRNA transcription in marrow-derived DCs, which suggests that IL-13 can take independent effect from IL-4 and play a leading part in the change of the pathobiology of asthma (Rissoan et al., 1999; Hershey, 2003). It was also reported that IL-13 can induce hyperreactivity of the airway independent of IL-4 and is the key factor in initiating allergen inducing $CD4^+$ T cell deviation (Wills-Karp et al., 1998). When using IL-13 mAb to treat mycete-sensitizing asthmatic mouse, IL-13 mAb can relieve AHR and inhibit subepithelial fibrosis of airway, collagen fibers deposition of lung tissue, and proliferation of goblet cell, which suggests that IL-13 mAb have a potential effect in treating asthma (Blease et al., 2001), and the effect may be realized by means of changing the gene transcription of IL-13 in DCs.

In this study, mRNA of IL-9 and IL-13 in DCs were transcribed at the same time, and the amount of transcription in experimental group is obviously more than that of control. This is in line with what the literatures have reported (Temann et al., 2002; Hershey, 2003). They found that IL-9 stimulates mucous generation through the same way IL-13 mediated in the allergic asthma model. IL-9 also is an important cytokine in anaphylactic disease. It can participate in soakage of eosinophil and hyperreaction of airway, promote generation of IgE, increase mucous secretion,

affect many kinds of cell in asthma (such as T cell, B cell, mastocyte and epithelium et al.), up-regulate mucus gene expression of epithelium of airway, induce translation of mucous cell, and increase mucous generations (Reader et al., 2003; Townsend et al., 2000; Whittaker et al., 2002). IL-9 induces proliferation of goblet cell and increases muramidase and mucosa of epithelium in restoring an injured epithelium in the airway (Vermeer et al., 2003). We suspect that inhibiting and obstructing the function of DC in the airway can be realized by lowering and blocking the generation of IL-9.

IL-3 is a kind of multiclone stimulating factor. It can affect most immature marrow ancestral cell, promote proliferation and differentiation of cell to mature cell type, and is important in hemopoiesis regulation. Now, there are two viewpoints to illustrate its effect in asthma: (1) It can promote the development and activation of eosinophil in marrow, and up-regulate the amount of eosinophil and mastocyte. But it can neither change the amount of lymphocyte and macrophage nor influence early or advanced stage symptoms of asthma (Du et al., 1999; Gregory et al., 2003); (2) IL-3 does not influence the increase of the amounts and the activity of allergen-stimulating eosinophil, and hyperreaction of the airway (Woolley et al., 1996). In this experiment, we found that mRNA transcription of IL-3 gene in DCs is present in both the asthmatic group and the control group, but no significant difference were found, possibly because they can promote the growth and differentiation of marrow ancestral cell. This proves the latter viewpoint in some ways. The reports about the expression of IL-3 mRNA in DC are less, and they need to be further studied.

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