

Brief report

Imiquimod attenuates airway inflammation and decreases the expression of thymus and activation regulated chemokine in allergic asthmatic mice

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Keywords: *asthma; Th₁/Th₂ cell; thymus and activation regulated chemokine; imiquimod*

Imiquimod is an imidazoquinoline derivative. Some studies have shown that imiquimod modulates the Th₁/Th₂ response by inducing the production of Th₁ cytokines IFN- γ and interleukin (IL)-12,¹ and by inhibiting Th₂ cytokines IL-4 and IL-5. These data suggest that imiquimod has therapeutic applications in atopic diseases such as allergic asthma that are associated with overexpression of Th₂ cytokines.

To determine the efficacy of imiquimod as a treatment for allergic asthma, we measured its effects on airway inflammation and its influence on the expression of macrophage derived chemokine (MDC); thymus and activation regulated chemokine (TARC) and CCR4 in murine lungs. Our hypothesis was that imiquimod treatment would significantly attenuate the development of airway dysfunction and decrease the Th₂ chemokine expression.

METHODS

Immunization and antigen challenge of mice

Male Balb/c mice (6 weeks – 8 weeks) were purchased from Shanghai Laboratory Animal Inc., China. Mice were divided into 3 groups: OVA challenge with imiquimod, OVA challenge, and control group, 10 mice in each group. On day 1 and day 7, all mice were actively immunized by intraperitoneal injection of 100 μ g ovalbumin (OVA) (Sigma Chemical Co., USA), adsorbed on 400 μ g alum in 0.2 ml 0.9% sterile saline. On day 14, two groups of mice were exposed to an aerosol of 1% OVA in 0.9% sterile saline for 30 minutes every day for 7 days. Mice in imiquimod group were also exposed to an aerosol of the 0.15% imiquimod suspension in 1% carboxymethylcellulose for 30 minutes before OVA challenge. Mice in control group were exposed to an aerosol of the 0.9% saline.

Evaluation of airway responsiveness

Airway responsiveness was measured 24 hours after the last OVA challenge. Mice were anaesthetized with 70 mg/kg pentobarbital sodium, tracheostomized and placed in a whole body, plethysmograph and ventilated via the intratracheal tube at 120 breaths/min and a tidal volume of 200 μ l. Methacholine aerosol was administered for 10 seconds (70 breaths/min, 400 μ l tidal volume) in increasing concentrations from 3.125 mg/ml to 50 mg/ml. Maximum values of the lung resistance (R_L) were taken.

Measurement of serum concentrations of MDC and TARC

After measurement of airway responsiveness, blood was drawn from the sinus cavernosus, serum was obtained by centrifugation at 1000 *g* for 10 minutes and kept at –70°C until tested. Serum MDC and TARC levels were determined with a commercial ELISA kit (R&D systems, Inc., USA). The limit of detection was 5 pg/ml for MDC and TARC.

Bronchoalveolar lavage

The airway lumina were washed twice with 0.5 ml of phosphate buffered saline (PBS). The bronchoalveolar lavage (BAL) fluid was immediately centrifuged (10 minutes, 4°C, 1000 *g*) and the upper fluid sample was retained for measurement of MDC, TARC, IL-4, and IFN- γ concentrations. The limit of detection was 20 pg/ml. Total cell numbers were counted with a haemocytometer. The cytospin slides

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of BAL cells were counted following Wright's staining. Differential cell counts were based on a count of at least 200 cells.

Lung histology

The lung were resected, fixed in 10% phosphate buffered formalin, embedded into paraffin, sectioned, stained with haematoxylin eosin solution and examined by light microscopy for histological changes.

Measurement of MDC and TARC protein by immunohistochemistry

MDC and TARC protein expressions in mouse lung tissue were determined with goat polyclonal MDC and TARC Ab (Santa Cruz,USA) using a goat SP kit (Zymed, USA). Control slides were stained with normal goat immunoglobulin (Ig) instead of primary Ab.

RT-PCR analysis

Total RNA was extracted from lung tissues by using TRIZOL reagent (Invivogen,USA) according to the manufacture's instruction. The purity and amount of RNA was assessed by spectrophotometry. One step RT-PCR kit (Clontech,USA) was used, first stand cDNA was synthesized and amplified from 1 µg of total RNA. The sequences of the primers used in the study are listed in Table 1. The amplified products were fractionated on 2.0% agarose gel for 30 minutes. The strap of amplified products was analyzed by Gel-Pro analyser 4.0 and the value A of each strap measured. The ratio of value A of aimed strap to that of GAPDH strap was taken as the measure of aimed mRNA.

Western blotting

Lung tissues were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100, 1% deoxycholic acid sodium, 0.1% SDS, 5 µg/ml aprotinin, 5 µg/ml leupeptin) at 4°C, vortexed, and centrifuged at 14 000 rpm at 4°C for 30 minutes and then 50 µg protein was subjected to SDS-PAGE. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membrane (Millipore,USA). The membrane was then blocked, probed with first Ab, then with appropriate secondary Ab conjugated to HRP and visualized with DAB.

Spleen cell isolation and flow cytometry

Two weeks after immunization, 15 mice were sacrificed by cervical dislocation, and spleens were removed. Spleen cells were excised, passed through a stainless steel mesh screen and washed three times in Hank's balanced salt solution. T cells were isolated by nylon wool depletion. T cells were diluted to 2×10^6 cells /ml with DMSO solution (containing 20 µg/ml imiquimod, 10 µM brefeldin A and 100 µg/ml OVA) and cultured for 24 hours at 37°C in an atmosphere of 5% CO₂. The Fc receptors on the mononuclear cells were blocked with an excess of purified murine Ig G then stained with PE conjugated CD₈ and fluorescein isothiocyanate (FITC)-CD₃ monoclonal antibody. Next, the cells were fixed and permeabilized in Cytotfix/Cytoperm (Caltag,USA). These permeabilized cells were washed twice with PBS, then stained with FITC labelled antimouse IFN-γ monoclonal antibody (Caltag,USA), and PE labelled antimouse IL-4 monoclonal antibody (Caltag,USA). Lastly, after two more washes with PBS, flow cytometry was performed using a FACScan (BD Co.,USA) and analyzed with Cell Quest software (BD Co.,USA).

Statistical analysis

The data are presented as mean ± standard error (SE). Statistical comparisons were performed by one way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Imiquimod attenuated Ag-induced airway hyperresponsiveness (AHR) in a murine model of asthma

Animals sensitized and challenged by OVA showed significantly higher expiratory resistance (Re) in response to methacholine compared with saline control animals. With the imiquimod treatment, the increase in inspiration resistance (Ri) was inhibited ($P < 0.05$), and the Ri was not significantly different from that in the control (saline injection and saline inhalation) after methacholine nebulization.

Total cell and differential cell counts in BAL fluid of each group mice

OVA challenge increased the numbers of lymphocytes, eosinophils and cells in total in BAL fluid; imiquimod reduced the numbers of lymphocytes,

Table 1. PCR primers used in this paper

Genes		Sequence (5'-3')	Annealling temperature(°C)	Size(bp)
MDC	Forward	TCG TAT GCA GGT CCC TAT GGT	58	206
	Reverse	TTA TGG AGT AGC TT CTT CAC		
TARC	Forward	CAG GAA GTT GGT GAG CTG GTA TA	58	331
	Reverse	TTG TGT TCG CCT GTA GTG CAT A		
CCR4	Forward	TCT ACA GCG GCA TCT TCT TCA T	58	199
	Reverse	CAG TAC GTG TGG TTG TGC TgT G		
IL-4	Forward	CAG AGC TAT TGA TGG GTC TC	62	398
	Reverse	TTC CAG GAA GTC TTT CAG TG		
IFN- γ	Forward	GGA TAT CTG GAG GAA CTG GC	58	309
	Reverse	CGA CTC C TT TTC CGC TTC CT		
STAT1	Forward	TGG GAG CAC GCT GCC TAT GAT GTC	60	600
	Reverse	CCT TCG CTT CCA CTC CAC GAG CTC		
STAT6	Forward	TCT CCA CGA GCT TCA CAT TG	60	319
	Reverse	CTG AGG CTG TTG TCG TTG AA		
GAPDH	Forward	AGT ATG ACT CCAC TCA CGG CAA	58	100
	Reverse	TCT CGC TCC TGG AAG ATG GT		

Table 2. BAL cells in immunized and challenged mice ($n = 10$, $10^6/L$, mean $\pm$ SE)

Groups	Total cells	Macrophages	Lymphocytes	Neutrophils	Eosinophils
OVA	8.75 $\pm$ 0.62	5.52 $\pm$ 0.43	0.51 $\pm$ 0.08	0.19 $\pm$ 0.04	2.62 $\pm$ 0.31
Imiquimod	4.12 $\pm$ 0.41*	2.68 $\pm$ 0.19*	0.08 $\pm$ 0.03*	0.46 $\pm$ 0.07*	0.36 $\pm$ 0.08*
Saline	1.82 $\pm$ 0.25*	1.64 $\pm$ 0.11*	0.06 $\pm$ 0.02*	0.09 $\pm$ 0.02*	0.01*

OVA: ovalbumin. * $P < 0.05$, OVA challenge animals vs OVA challenge with imiquimod pretreatment animals.

eosinophils and cells in total in BAL fluid, but increased the number of neutrophils (Table 2).

Effects of imiquimod on the levels of MDC and TARC in serum and BAL fluid

The effect of imiquimod on the levels of MDC and TARC in serum and BAL fluid was examined for 24 hours after antigen challenge to determine whether imiquimod was likely to mediate its effect on airway inflammation through the inhibition of MDC and TARC. The levels of MDC and TARC in BAL fluid were lower than 5 pg/ml. Imiquimod reduced serum TARC levels [(220 $\pm$ 24) – (163 $\pm$ 34)] pg/ml and IL-4 levels in BALfluid [(120 $\pm$ 25) – (65 $\pm$ 9)] pg/ml ($P < 0.05$).

Histological changes by imiquimod treatment

In accordance with the changes found in BAL fluid preparations, imiquimod reduced the number of cells infiltrating into the lung in response to OVA, most of which, by haematoxylin eosin staining, appeared to be mononuclear lymphocytes.

Effect of imiquimod on Th₂ type chemokine MDC and TARC expression in the lung

To determine whether imiquimod showed any effect on the expression of MDC and TARC in the lung, immunohistochemical staining was performed. In the OVA treated group, there was strong staining of MDC and TARC, mainly in bronchial epithelium and infiltrating cells. Vascular smooth cell also expressed MDC strongly. Imiquimod reduced the expression of MDC and TARC proteins in the lung.

Effect of imiquimod on TARC, MDC, CCR4, STAT1, STAT6 mRNA and STAT1, STAT6 protein levels in the lung

TARC, MDC and CCR4 mRNA were found in the lung tissues and were significantly higher in OVA challenged animals compared with controls (saline challenged animals) (Fig. 1). Imiquimod reduced the TARC, MDC and CCR4 mRNA levels in the lung tissues. Imiquimod inhibited STAT6 and IL-4 mRNA expression and increased IFN- γ mRNA expression in the lung tissues. STAT1 mRNA were significantly higher in OVA challenged mice compared with saline

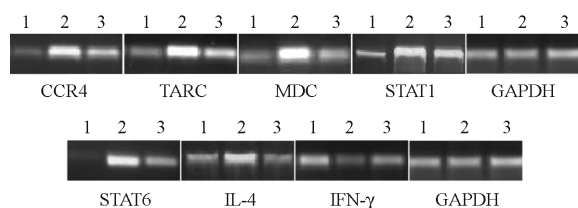


Fig 1. CCR4, TARC, MDC, STAT1, STAT6, IL-4 and IFN- γ mRNA expressions in lung tissues. lane 1: after saline challenge; lane 2: OVA challenge ; lane 3: OVA challenge with imiquimod pretreatment.

controls and imiquimod treated animals. In accordance with the RT-PCR results, imiquimod inhibited STAT6 protein expression but had no effect on STAT1 protein expression.

Detection of intracellular cytokine production in $CD_3^+ CD_8^-$ T cells treated with imiquimod by flow cytometric assay

We took IL-4 positive, CD_3 positive and CD_8 negative cells as Th_2 cells and IFN- γ positive, CD_3 positive and CD_8 negative cells as Th_1 cells. When T cells were incubated with DMSO, the percentages of Th_1 and Th_2 cells were low. The percentage of Th_2 cell increased nearly threefold following incubation with OVA, but prior treatment with 20 μ g/ml imiquimod reduced the number of Th_2 cells to base level. However, the numbers of Th_1 cells in both groups of treated cells remained unchanged (Fig. 2).

DISCUSSION

Specific immunotherapy has been approved by WHO as an active therapeutic approach for allergic diseases. It leads towards a Th_1 shift,² but specific immunotherapy is time consuming and expensive. Therefore, the search for new molecules that are capable of directing the immune system towards an increased Th_1 response began many years ago. It has been shown that resiquimod increases Th_1 activity but decreases Th_2 activity. We have shown in this study that imiquimod does inhibit OVA induced airway lymphocyte infiltration and AHR. An animal study revealed that imiquimod induces IFN- α and attenuates the development of airway dysfunction during acute viral illness in rats.³

To elucidate further the roles of imiquimod, MDC, TARC and CCR4 expression in the lung were analyzed. Recently, the important role of chemokines in the inflammatory network responsible for allergy and asthma has been discovered.⁴

Chemokines can directly cause cellular activation and inflammatory mediator release by eosinophils and basophils and promote the selective recruitment of different effector cell subsets, including Th_2 cells, in inflamed tissues. Investigations have revealed the existence of a number of lymphocyte directed chemo-

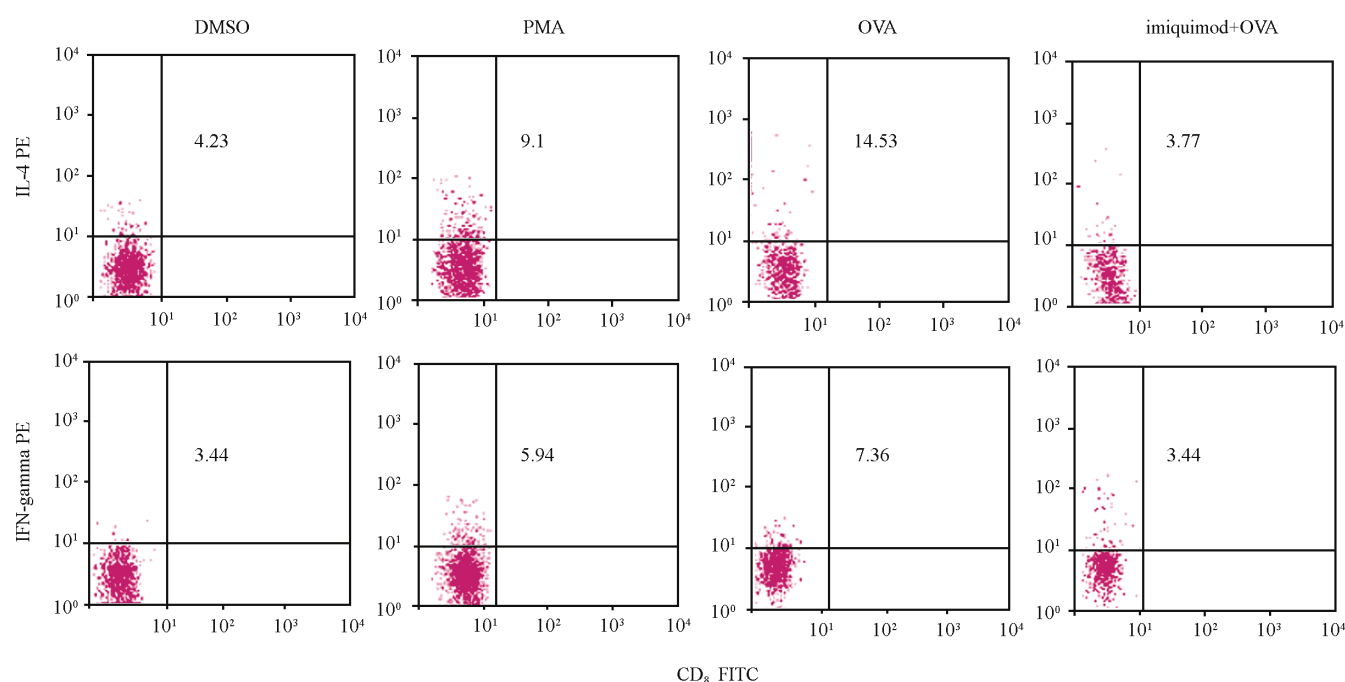


Fig 2. Intracellular IL-4 and IFN- γ production in $CD_3^+ CD_8^-$ T cells by flow cytometric assay.

kines and among these CC chemokines, TARC and MDC were identified as specific ligands for CCR4 and inducing chemotaxis of T cells (especially of the Th2 type CD4⁺ human T lymphocyte).⁵ Because imiquimod augments IFN- γ and inhibits IL-4 and IL-5 production in murine spleen cells, and Th₂ cytokines such as IL-4 and IL-13 increase the production of chemokines such as MDC and TARC;⁶ imiquimod could decrease MDC, TARC and CCR4 expressions on bronchial epithelium. In this study, TARC levels in sera of imiquimod treated mice were decreased to less than the base level. TARC and MDC mRNA expression in the lung were elevated in OVA challenged mice and were inhibited significantly by imiquimod pretreatment.

In vivo, T cells from STAT6-deficient mice are unable to differentiate into Th₂ cells in response to IL-4 or IL-13, and B cells from these mice cannot undergo class switching to IgE, suggesting that the STAT6 pathway is important for the allergic response.⁷ In fact, the number of pulmonary eosinophils, AHR and mucus production were decreased in STAT6^{-/-} mice, that were sensitized and challenged with OVA, compared with their wild type mice. MDC and TARC mRNA were undetectable in STAT6^{-/-} mice.⁸ In addition, some studies show that induction of IFN and stimulation of IFN responsive genes by imiquimod require active STAT1.⁹ We measured STAT6 and STAT1 proteins and mRNA expressions in the lung. STAT6 protein and mRNA expression in the lung were increased in OVA challenged mice and were inhibited significantly by imiquimod pretreatment. Thus, imiquimod attenuates airway inflammation and AHR may be through inhibiting IL-4/STAT6 signalling pathway. In contrast, imiquimod has no effect on STAT1 expression. That imiquimod increases the intracellular IFN- γ production may be through another signalling pathway.

The results of these studies in mice suggest that treatment with imiquimod modulates the development of airway inflammation and dysfunction by inhibiting the IL-4/STAT6 signalling pathway and increasing the expression of IFN.

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