· Original Article ·

Inhibitory effects of TLR7/8 ligand R-848 on IgE production of murine in vivo*

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Abstract: Objective To investigate whether R-848 could inhibit IgE production of mice immunized with OVA plus ALUM in vivo. **Methods** BALB/c mice were immunized s. c on the back with PBS, OVA, OVA plus R-848, OVA plus CpG, OVA plus ALUM, and OVA plus ALUM in R-848 or CpG every two weeks for three times. The blood from the mice was harvested after the last immunization, and the concentration of OVA specific or total IgE, IgG1 and IgG2a in the sera was determined; the splenocytes from the immunized mice were harvested and cultured with or without OVA for 3 days, and the concentration of IL-4 and IFNγ in the supernatants was determined. **Results** Firstly, we investigated that R-848 as Th1 adjuvant could enhance the production of OVA specific IgG2a in mice immunized with OVA. Secondly, further study showed that R-848 could inhibit the production of OVA specific IgE and total IgE, and promote the production of OVA specific IgG2a in the sera of mice immunized with OVA plus ALUM. Moreover, the results of ELISA showed that R-848 inhibiting IgE production was related to its reducing IL-4 production and enhancing IFNγ production. **Conclusion** R-848 could inhibit IgE production of mice immunized with OVA plus ALUM in vivo.

Key words: TLRs; R-848; IgE; IgG1; IgG2a

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IgE is a key component of asthma pathophysiology[1]. Increased IgE production is the strongest predisposing factor for the development of asthma. On cross-linking of receptor-bound IgE antibodies by allergens, basophils and mast cells released the potent chemical mediators stored inside their granules to initiate and propagate the immediate hypersensitivity reactions. Therefore, diminishing or reducing IgE in the sera is an effective therapy for allergic diseases. Omalizumab is a recombinant anti-IgE monoclonal antibody, and has been administrated to inhibit development of inflammatory cascade by reducing serum IgE levels in EU and USA[2]. Although this drug could reduces symptoms but could not cure the diseases.

R-848 (resiquimod, S-28463), TLR7/8 ligand, prevent allergen-induced airway hyperresponsiveness and eosinophilia in animal asthma model[3]. Early clinical trials demonstrated that administration of R-848 is safety. Moreover, R-848 has potential anti-viral and anti-tumor properties, and be a Th1 adjuvant[4-5]. Our published article investigated that R-848 could directly effect on mouse B cells to inhibit IgE production in vitro[6]. In this study, we further investigated whether R-848 could inhibit IgE production of mice immunized with OVA plus ALUM in vivo.

1 Materials and Methods

1.1 Mice Female BALB/c mice, 6-8 weeks old, were purchased from Sun Yat-sen University Animal Center (Guangzhou, China) and maintained in the animal care facility under pathogen-free conditions. All experiments were approved by our department's animal care and use committee.

1.2 Reagents RPMI 1640 medium, penicillin-streptomycin and 2-mercaptoethanol were purchased from GIBCO (Grand Island, NY, USA). OVA (OVA grade VI) and adjuvant aluminum solution (AL(OH)3) named as ALUM in the article were purchased from Sigma-Aldrich (St. Louis, MO). R-848 was a kind gift from 3M Pharmaceutical Corps (St. Paul, USA). CpG ODN 1826 (5′-TCC ATG ACG TTC CTG ACG TT-3′, named as CpG in the article) was synthesized by Shanghai Sangon Biological Engineering & Technology and Service Co., Ltd. (Shanghai, China).

1.3 Immunizations BALB/c mice were immunized s. c on the back with PBS, OVA (ovalbumin, 50 μg/mouse), OVA plus R-848(20 μg/mouse), OVA plus CpG (CpG ODN 1826, 20 μg/mouse), OVA plus ALUM (1.3 mg/mouse), and OVA plus ALUM in R-848 or CpG. Mice were immunized with the same regimen in total three times every two weeks.

1.4 Cell culture and measurement of cytokines in the culture supernatants by ELISA Spleens from individual mice were harvested one week after the last immunization. Single-cell suspensions of splenocytes were prepared and cultured with medium or OVA(100 μg/mL) for 3 days. Cell-free culture supernatants were collected, and levels of IFNγ and IL-4 were assessed by ELISA kit (BD PharMingen) according to the manufacturer's protocol. The lower limits of detection for IFNγ and IL-4 were 3.1 pg/mL and 15.6 pg/mL respectively.

1.5 Detection of OVA-specific Igs and total Ig isotypes in sera Sera were obtained from mice 1 week after the last immunization. ELISA plates (Greiner, Germany) were coated overnight with OVA (10 μg/mL), and blocked with buffer (PBS containing 10% fetal bovine serum) for 1 hour. Serum samples

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were added in serial dilutions and incubated for 2 hours at room temperature. Plates were washed, and HRP-conjugated anti-IgE, or HRP-conjugated anti-IgG1 (Bethyl Laboratories), or HRP-conjugated anti-IgG2a (Bethyl Laboratories) was added for 1 hour at room temperature. After washed, the plates were developed with tetramethylbenzidine (TMB) and hydrogen peroxide (BD Pharmingen, CA) and were read using Elx800 universal microplate reader (BIO-TEK, USA).

For detection of total Igs, the levels of total IgE, IgG1 and IgG2a in sera were determined by ELISA according to the manufacturer's protocols (IgE: BD PharMingen, detection range, 1.6-100ng/ml; IgG1 and IgG2a: Bethyl Laboratories, Montgomery, TX, USA, detection range, 3.9-250 ng/mL).

1.6 Statistical analysis　Statistical evaluation of differences between means of experimental groups was done by analysis of variance and a non-parametric two-tailed t-test. A p value of $<$ 0.05 was considered to be significant.

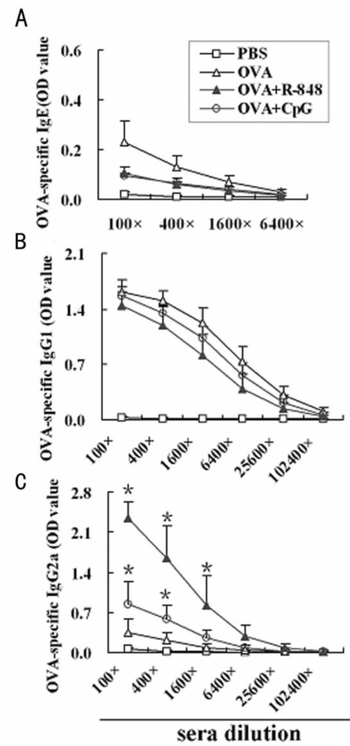
2 Results

2.1 R-848 as Th1 adjuvant could enhance the production of OVA specific IgG2a in mice immunized with OVA　As shown in Fig. 1, we can see that there was no significant change of OVA specific IgE or IgG1 in mice immunized with OVA plus R-848 compared with mice immunized with OVA alone. However, OVA specific IgG2a were markedly increased in mice immunized with OVA plus R-848 compared with mice immunized with OVA alone (Fig. 1C, $P<0.05$). The similar results were shown in mice immunized with OVA plus CpG. The above results showed that TLR7/8 ligand R-848 as Th1 adjuvant could enhance the production of OVA specific IgG2a in the sera of mice immunized with OVA.

2.2 R-848 could inhibit the production of IgE and enhance the production of OVA specific IgG2a in mice immunized with OVA plus ALUM　The Fig. 2A showed that mice immunized with OVA plus ALUM could produce markedly OVA specific IgE or total IgE in sera. However, R-848 or CpG could inhibit the production of OVA specific IgE and total IgE in the sera of mice when immunization with OVA plus ALUM (Fig. 2A and 2D, $P<0.05$).

In addition, there was no significant difference in the production of OVA specific IgG1 and total IgG1 of mice immunized with or without R-848 or CpG (Fig. 2B and 2E). Moreover, the concentration of OVA specific IgG2a significantly increased in the sera of mice immunized with OVA plus ALUM in R-848 (Fig. 2F, $P<0.05$, compared mice immunized with OVA plus ALUM). However, there was no significant difference in the production of total IgG2a by mice immunized with R-848 or without R-848 (Fig. 2C). The above results showed that R-848 could inhibit the production of IgE and enhance the production of OVA specific IgG2a of mice immunized with OVA plus ALUM.

2.3 The ratio of OVA specific IgG1/IgG2a of mice immunized with OVA plus ALUM in R-848 decreased　The ratio of antigen specific IgG1/IgG2a in sera could indicate the ratio of Th2/Th1 after immunization or infection, therefore, we calculated the ratio of OD value of OVA specific IgG1/IgG2a at sera dilution 400, and the results were showed in Fig. 3. Compared with PBS group, the ratio of mice immunized with OVA plus ALUM was higher, which indicated that mice immunized with OVA plus ALUM was inclined to produce OVA specific IgG1, and the immune response in the mice was Th2 dominant. When mice immunized with OVA plus ALUM in R-848 or CpG the ratio of OVA specific IgG1/IgG2a was significantly lower than that of mice immunized with OVA plus ALUM ($P<0.05$), which indicated that R-848 could promote Th1 immune response. We also got the ratio of OD value of OVA specific IgG1/IgG2a at sera dilution 6400, and the result was similar with sera dilution 400 (data not shown).



*: $P<0.05$, compared with OVA immunized group.

Fig. 1　R-848 could enhance the production of OVA specific IgG2a in the sera of mice immunized with OVA. The OVA specific IgE (A), IgG1 (B), and IgG2a (C) in the sera were determined by ELISA.

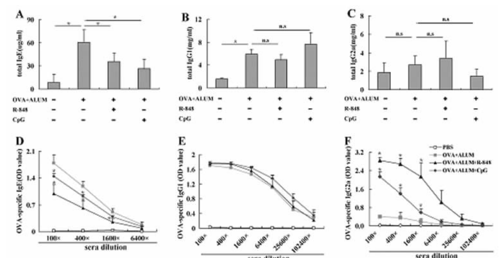
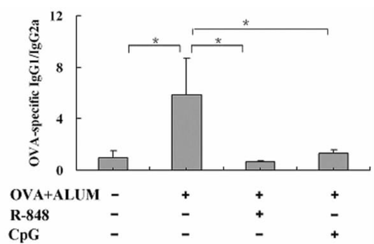


Fig. 2　R-848 could inhibit the production of OVA specific IgE and total IgE of mice immunized with OVA plus ALUM. *: $P<0.05$, compared with OVA plus ALUM immunized group. n. s: $P>0.05$.

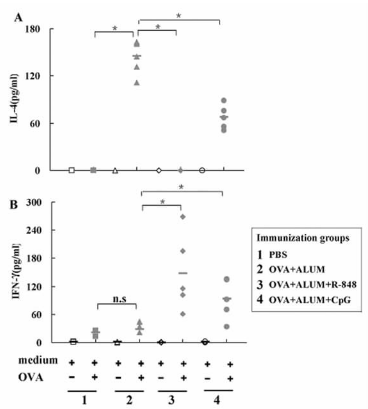
2.4 R-848 could inhibit OVA specific IL-4 production and en-

hance OVA specific IFNγ production　Compared with PBS group, splenocytes[7] from the mice immunized with OVA plus ALUM produced significantly IL-4 after incubation with OVA (Fig 4A, $P<0.05$). There was significant lower IL-4 production by splenocytes stimulated with OVA from mice immunized with OVA plus ALUM in R-848 or CpG, compared with OVA plus ALUM (Fig 4A, $P<0.05$). In contrast, there was markedly higher IFNγ production by splenocytes of mice immunized with OVA plus ALUM in R-848 after culturing with OVA (Fig. 4B, $P<0.05$). These results indicated that R-848 could inhibit OVA specific IL-4 production and promote OVA specific IFNγ production ex vivo. Above results suggested that R-848 inhibiting IgE production was related to its reducing IL-4 production and enhancing IFNγ production.



*: $P<0.05$.

Fig. 3　The ratio of OVA specific IgG1/IgG2a decreased in mice immunized with OVA plus ALUM in R-848.



*: $P<0.05$; n. s: $P>0.05$.

Fig. 4　Reduced IL-4 production and enhanced IFNγ production from OVA stimulated splenocytes in OVA plus ALUM in R-848 immunized mice. (A) IL-4; (B) IFNγ.

3　Discussion

According to the "hygiene hypothesis", increasing early exposure to microbial agents might protect against the development of allergy[7]. TLRs ligands act as the components or component analogs of pathogens to stimulate APC to initiate innate immune response and instruct the adaptive immune response. Various bacterial TLR ligands such as CpG have been shown to stimulate production of IL-12 and down-regulate Th2 response in animal models of allergic airway inflammation[8]. However, the capacity of viral TLR ligands such as R-848 to interfere with allergic sensitization or already established allergic airway inflammation is not well defined.

At first, we investigated that R-848 as Th1 adjuvant could enhance the production of OVA specific IgG2a in the sera of mice immunized with OVA. IgG2a is a Th1 type antibody, could predominate in antiviral antibody responses. Studies have shown that R-848 acts as Th1 adjuvant in a number of models in mice, rats, guinea pigs and monkeys[5,9]. However, most CpG-ODNs which activate mouse cells show only weak activation of human immune cells. Clinical trials with topically applied R-848 show the molecule to be well tolerated and capable of activation various aspects of the immune response.

Further study demonstrated that R-848 could inhibit IgE production, promote OVA specific IgG2a production, and do not change OVA specific IgG1 production of mice immunized with OVA plus ALUM. It has been demonstrated that TLR9 ligand CpG as Th1 adjuvant could inhibit IgE production in vivo and in vitro[8,10]. Moreover, R-848 during the sensitization phase prevented the production of OVA specific IgE and subsequently abolished all symptoms of experimental asthma including airway hyper-responsiveness and airway inflammation[3,11].

The immunologic basis of "hygiene hypothesis" is that the immune response of allergic diseases is dys-regulated Th1/Th2 cell response (dominant by Th2 cell response), and IL-4 produced by the Th2 cells could induce the isotype switch of IgE. IFNγ which is produced by Th1 cells could negatively regulate the production of IgE and promote isotype switch to IgG2a[1]. Next, we determined the production of Ag specific Th1 cytokine IFNγ and Th2 cytokine IL-4 ex vivo. The results showed that there was enhanced Ag specific IFNγ production and reduced Ag specific IL-4 production of splenocytes of mice immunized with OVA plus ALUM in R-848. These results suggested that R-848 could inhibit Th2 cell differentiation and promote the Th1 cell differentiation in vivo. Previous study showed that R-848 could induce a mount of IFNγ and IL-12 production in vitro and in vivo[10]. R-848 induced allergen-specific circulating T cells, including Th2 effectors, to produce IFNγ and even to lose the ability to produce IL-4, thus shifting their phenotype of cytokine production to Th0 or even Th1 cells[12]. In addition, our previous study demonstrated that R-848 could directly effect on mouse B cells to inhibit IgE production in vitro, and there are still not clear that the inhibitory effect of R-848 on IgE production in vivo was associated with the directly effect on B cell by R-848.

Taken together, we showed here that TLR7/8 ligand R-848 could inhibit the production of OVA specific IgE and total IgE, and promote the production of OVA specific IgG2a in mice immunized with OVA plus ALUM, which is associated with R-848 inducing naive $CD4^+$ T cells to differentiate towards Th1 cells. These results suggest that R-848 might be used to treat IgE-mediated allergic diseases.

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潜在的肿瘤血管抑制作用[11]。研究表明，Alphastatin能通过抑制VEGF通路和磷酸鞘氨醇(SIP-AKT)通路抑制肿瘤血管生成，对正常组织血管并没有抑制作用[12-13]。Alphastatin多肽能否抑制血管生成拟态，目前尚无相关研究。如果Alphastatin能同时抑制血管内皮依赖性血管和拟态血管，那它将是十分理想的血管抑制肽。

研究表明，Alphastatin多肽虽然不能抑制SHG44细胞的增殖，但是可以抑制其细胞迁移能力和管道形成能力，并且抑制作用与浓度呈明显量-效关系，提示Alphastatin不但能抑制血管内皮细胞的迁移和血管腔的形成，还可能具有抑制血管生成拟态的功能，并且对正常血管无明显破坏作用。Western blot检测表明Alphastatin不能直接作用于VEGF蛋白，但是可以抑制EphA2蛋白的磷酸化和MMP2蛋白的活化，减少层粘连蛋白5γ2切割片段γ2′和γ2x的形成，进而抑制血管生成拟态的形成。

综上所述，如果仅仅抑制血管内皮依赖性血管，拟态血管将发挥营养肿瘤的作用，不能达到治疗肿瘤的目的。Alphastatin不但能抑制血管内皮细胞依赖性血管，还能抑制血管生成拟态，可能是良好的血管抑制治疗药物，但是Alphastatin对SHG44血管生成拟态的抑制作用还需进一步的研究。

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