

TLR7/8 Ligand, R-848, Inhibits IgE Synthesis by Acting Directly on B Lymphocytes

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Abstract

TLRs are involved in the regulation of immune responses. R-848, a TLR7/8 ligand, has potent anti-viral and anti-tumour properties and has been used as a new immune response modifier for enhancing Th1 immune response. In this study, we found that R-848 significantly inhibited IgE synthesis from murine B cells at the single cell levels by anti-CD40 plus IL-4-stimulated splenocytes, in which R-848 acted on the early stage of B cell differentiation to modulate IgE synthesis. This inhibitory effect of R-848 on IgE synthesis was not isotype specific as it also inhibited IgG1 synthesis. Moreover, R-848 had no significant effect on the production of IgG2a by anti-CD40 plus IL-4-stimulated splenocytes. Further studies showed that R-848 markedly promoted murine B cell activation induced by anti-CD40 plus IL-4 by up-regulating the expression of B cell activation markers CD25, CD69 and co-stimulatory molecule CD80. In contrast, R-848 inhibited the proliferation and division of murine B cells in anti-CD40 plus IL-4-stimulated splenocytes. R-848 promoted the production of IFN- γ and IL-12 that were partially responsible for its inhibitory effect on IgE production by anti-CD40 plus IL-4 because the addition of anti-IFN- γ or anti-IL-12 mAbs to the cultures could significantly restore IgE production by splenocytes. Importantly, R-848 had a direct effect on purified B cells to inhibit IgE production induced by anti-CD40 plus IL-4. Taken together, these results demonstrate that R-848 markedly inhibits IgE synthesis, and suggest that R-848 could be used to treat allergic diseases.

Introduction

The increased prevalence of allergic diseases in recent years, especially in the industrialized countries, suggests the potential influence of improved hygiene and better infection control on the development of allergy. The basic tenet of 'the hygiene hypothesis' is that the immune system of the newborn infant is skewed toward Th2 cells and needs timely and appropriate environmental stimuli to create a balanced immune response [1, 2]. Based on the hypothesis, increasing exposure to microbial agents might protect against asthma. The role of viral infections and especially of viral TLR ligands remains to be clarified. Viral Toll-like receptor (TLR) ligands involve dsRNA and ssRNA that are recognized by TLR-3 or TLR-7, respectively [3].

R-848 (resiquimod, S-28463) is a low-molecular-weight compound of the imidazoquinoline family, and stimulates immune cells through the (TLR7)-MyD88-dependent pathway [4, 5] and TLR8 [6]. R-848 has been

previously shown to have potent anti-viral and anti-tumour properties via endogenous cytokine production [4, 7–9] and is a new immune response modifier with potential as a vaccine adjuvant for enhancing Th1 immune response [10–12]. Moreover, R-848 has been shown to possess potent B cell-activating properties, as it could induce immunoglobulin (Ig) secretion by B cells and promote B cell activation *in vitro* [13–15].

Immunoglobulin E (IgE) plays a crucial role in the pathogenesis of allergic diseases such as asthma, allergic rhinitis and atopic dermatitis, in which there is a polarization of T lymphocyte responses toward Th2 cells that produce cytokines including interleukin 4 (IL-4) and IL-5 [16, 17]. Dysregulated Th1/Th2 responses are thought to be central to the pathology of allergic diseases. Th1 cells produce IFN- γ , IL-2 and TNF- α , and induce cell-mediated immunity, whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13, and are associated with humoral immunity [18]. In mice, the production of selective Ig isotypes is known to be regulated by contrasting cytokines:

IL-4 induces IgE and IgG1 production [19], whereas IFN- γ induces IgG2a production [20]. Thus, rebalancing immune responses may be an effective strategy in developing therapeutic reagents for such diseases.

In our study, we demonstrated that R-848, a TLR7/8 ligand, inhibited IgE production by anti-CD40 plus IL-4-stimulated splenocytes. The inhibition of the production of Igs by R-848 was specific for IgE and IgG1 but not for IgG2a. The inhibitory effects of R-848 were likely due to a reduction in B cell division and promotion of IFN- γ and IL-12 cytokine production in splenocytes, and a direct effect on purified B cells to inhibit IgE production.

Materials and Methods

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.

Reagents. R-848 (S-28463, 4-amino-2-ethoxymethyl-*a,a*-dimethyl-1H-imidazo[4,5-c] quinoline-1-ethanol) was kindly provided by 3M Pharmaceuticals Corps (St Paul, MN, USA). Anti-mouse CD40, anti-IFN- γ (neutralizing), anti-IL-12 (p40/p70) (neutralizing), anti-CD25 PE, anti-CD69 FITC anti-CD80 PE, anti-CD23 PE and isotype-matched control mAbs were purchased from BD Pharmingen (San Diego, CA, USA). Recombinant mouse IL-4 was obtained from Peprotech (Rocky hill, NJ, USA). Anti-CD19 APC, anti-IgE PE and isotype-matched control mAbs were obtained from eBioscience (San Diego, CA, USA). RPMI 1640 medium, penicillin–streptomycin and 2-mercaptoethanol were purchased from Gibco (Grand Island, NY, USA).

Detection of Igs and cytokines by ELISA. The levels of IgE, IgG1 and IgG2a in the culture supernatants were determined by ELISA according to the manufacturer's protocols (IgE: BD Pharmingen, detection range, 1.6–100 ng/ml; IgG1 and IgG2a: Bethyl Laboratories, Montgomery, TX, USA, detection range, 3.9–250 ng/ml). For the detection of cytokines, splenocytes (2×10^6 cells/ml) were incubated, with or without R-848 (1 μ M), anti-CD40 (1 μ g/ml) plus IL-4 (10 ng/ml) or in anti-CD40 plus IL-4 with R-848 (1 μ M) for 3 days. The levels of IFN- γ , IL-12p40 and IL-12p70 in the supernatants were determined by ELISA according to the manufacturer's protocols (BD Pharmingen). The mouse IFN- γ ELISA (detection range: 3.13–200 pg/ml), the mouse IL-12p40 ELISA (detection range: 15.6–1000 pg/ml) and IL-12p70 ELISA (detection range: 62.5–4000 pg/ml) were used. The optical density was measured at 450 nm with an Immuno-reader (Bio-Tek ELX-800; Bio-Tek Instruments Inc., Winooski, VT, USA). All samples were run in triplicate.

Cell preparation, isolation and culture condition. Single-cell suspensions of splenocytes from Balb/c mice were

prepared and re-suspended in complete RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS, HyClone Laboratories, Logan, UT, USA), 100 U penicillin/ml, 100 μ g streptomycin/ml and 50 μ M 2-mercaptoethanol. CD19⁺ B cells were positively selected from mouse splenocytes with anti-CD19 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) on an LS or MS column (Miltenyi Biotec). The purity of B cells was 92–96% for the expression of CD19. Splenocytes (2×10^6 cells/ml) were incubated in 48- or 96-well flat-bottom culture plates, and purified B cells (1×10^6 cells/ml) were cultured with anti-CD40 (1 μ g/ml) plus IL-4 (50 ng/ml) at 37 °C in a humidified atmosphere containing 5% CO₂.

Cell surface and intracellular staining. For the surface staining, at the end of culture cells were harvested, washed twice with PBS, and Fc receptors were blocked with anti-CD16/CD32 Abs at 4 °C for 20 min, and stained with fluorescence labeled mAbs. All incubations were conducted on ice, and cells were washed twice with cold-staining buffer after each step (PBS containing 0.5% BSA and 0.1% NaN₃). Cells were collected on a FACSCalibur (BD Bioscience, Mountain View, CA, USA), and the data were analysed using FlowJo software (Tree Star, Ashland, OR, USA).

For the intracellular staining of IgE, splenocytes were cultured with medium, anti-CD40 plus IL-4 in the presence or absence of R-848 (1 μ M) for 7 days. The cells were collected, washed twice in cold PBS, then fixed with 4% paraformaldehyde, and re-suspended in permeabilization buffer (PBS containing 0.1% saponin (Fluka; Sigma, St Louis, MO, USA) and 0.5% BSA). After incubation at 4 °C for 2 h, anti-CD19 APC, anti-IgE PE or isotype-matched control mAbs was added, incubated for 30 min at 4 °C, washed with PBS, resuspended in cold-staining buffer and analysed by FACSCalibur (BD Bioscience). FACS data were analysed using FlowJo software.

Proliferation assays. The effect of R-848 on B cell proliferation was assessed by flow cytometric measurement of 5-bromo-20-deoxyuridine (BrdU) uptake according to the manufacturer's instructions. Briefly, splenocytes were cultured with anti-CD40 plus IL-4 in the presence or absence of R-848 (1 μ M) for 3 days. One hour before culture termination, BrdU was added to the cultures at a final concentration of 10 mM, and cells were collected, washed, re-suspended in staining buffer and stained with anti-CD19 APC mAbs. Cells were fixed and permeabilized, and DNase 100 ml was added for another 1 h at 37 °C. After washing, cells were re-suspended in 50 ml Perm/Wash Buffer and stained with FITC-labeled anti-BrdU mAbs. After incubation for 20 min at room temperature, cells were washed, re-suspended in staining buffer and analysed by flow cytometry. All the reagents used in these experiments were supplied by BrdU Flow Kit (BD Pharmingen).

To determine the effect of R-848 on B cell division, splenocytes were labeled with 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. A 5 mM stock solution of CFSE was prepared by dissolving in DMSO and stored at -20°C . Before labeling, cells were washed and re-suspended at 2×10^7 cells/ml in PBS containing 0.1% BSA (PBS/BSA). CFSE was then added into the cell suspensions at a final concentration of $2.5 \mu\text{M}$, and incubated for 10 min at 37°C . The cells were subsequently washed with complete RPMI 1640 medium, re-suspended and adjusted to a concentration of 2×10^6 cells/ml in complete RPMI 1640 medium and cultured with medium or anti-CD40 plus IL-4 in the presence or absence of R-848 ($1 \mu\text{M}$) for 3 days. Then cells were harvested and stained, and analysed by flow cytometry.

Statistical analysis. The significance of differences between two groups was evaluated using Student's *t*-test. *P* value of <0.05 was considered to be statistically significant.

Results

R-848 inhibits IgE production by anti-CD40 plus IL-4-stimulated splenocytes in a dose-dependent manner

To study the role of R-848 in the induction of IgE synthesis, splenocytes were stimulated with anti-CD40 plus IL-4 in the presence or absence of different concentrations of R-848 (1 nM – $10 \mu\text{M}$). Consistent with the previous reports [21, 22], the results showed that anti-CD40 plus IL-4 induced a significant amount of IgE production in three independent experiments (Table 1). Addition of R-848 to the cultures resulted in the suppression of IgE production in a dose-dependent manner (Table 1). When R-848 was used as low as 1 nM , there was no significant effect on IgE production ($P > 0.05$). The inhibition of IgE production reached the strongest when R-848 was used at $1 \mu\text{M}$ and the higher concentration of R-848 had

Table 1 TLR7/8 ligand, R-848, inhibits IgE production in a dose-dependent manner by anti-CD40 plus IL-4-stimulated splenocytes.

Culture conditions	IgE (ng/ml)		
	Exp 1	Exp 2	Exp 3
Medium	0.1 ± 0.0	0.7 ± 1.2	0.5 ± 0.2
anti-CD40 + IL-4	66.7 ± 4.2	67.9 ± 11.1	89.3 ± 11.1
+R-848 ($10 \mu\text{M}$)*	1.8 ± 0.7	1.0 ± 0.3	0.5 ± 0.3
+R-848 ($1 \mu\text{M}$)*	1.0 ± 0.4	1.2 ± 1.1	0.6 ± 0.2
+R-848(100 nM)*	2.0 ± 1.1	6.3 ± 1.0	0.3 ± 0.1
+R-848(10 nM)*	21.8 ± 4.6	40.7 ± 6.7	7.0 ± 2.5
+R-848(1 nM)	72.4 ± 5.2	45.7 ± 6.5	24.5 ± 10.7

The data are expressed as mean $\pm$ SD. * $P < 0.05$, in comparison to anti-CD40 plus IL-4-stimulated cells.

not further inhibition. Consistent with the previous study [21], neither anti-CD40 mAbs nor IL-4 alone could induce a detectable level of IgE synthesis (data not shown). We next examined the effect of R-848 on IgE production of B cells at the single-cell levels. When splenocytes were cultured with medium or anti-CD40 plus IL-4 in the presence or absence of R-848 for 7 days, IgE-positive B cells were significantly inhibited by R-848 (3.10%) compared with anti-CD40 plus IL-4-stimulated cells (7.24%; Fig. 1).

This inhibitory effect of R-848 was not specific for IgE because R-848 was also able to significantly inhibit the production of IgG1 in a dose-dependent manner (Fig. 2B). As shown in Fig. 2, neither R-848 alone nor IL-4 plus R-848 at different concentrations could induce IgE production, while R-848 or IL-4 plus R-848 could significantly induce the production of IgG1 in a dose-dependent manner. In addition, stimulation of splenocytes with anti-CD40 plus IL-4 in the presence or absence of R-848 had no effect on IgG2a production, whereas R-848 or IL-4 plus R-848 could enhance IgG2a synthesis in a dose-dependent manner (Fig. 2C).

To study whether R-848 could suppress the synthesis of Igs at different stages of B cell differentiation, splenocytes were stimulated with anti-CD40 plus IL-4, and R-848 was then added into the cultures at various time

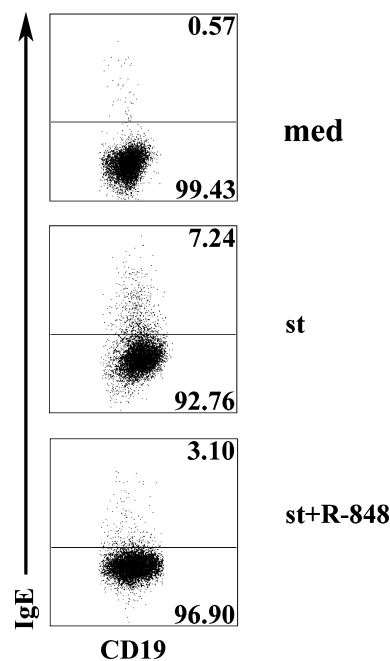


Figure 1 R-848 inhibits anti-CD40 plus IL-4-induced IgE production at the single cell levels. Splenocytes were cultured with medium (med), anti-CD40 plus IL-4(st) in the presence or absence of R-848 ($1 \mu\text{M}$) for 7 days. CD19⁺ B cells were first gated, and IgE⁺ cells were then analysed by FACS. The experiments were repeated twice with similar results. The numbers in the top right corner indicated the percentage of IgE⁺ B cells.

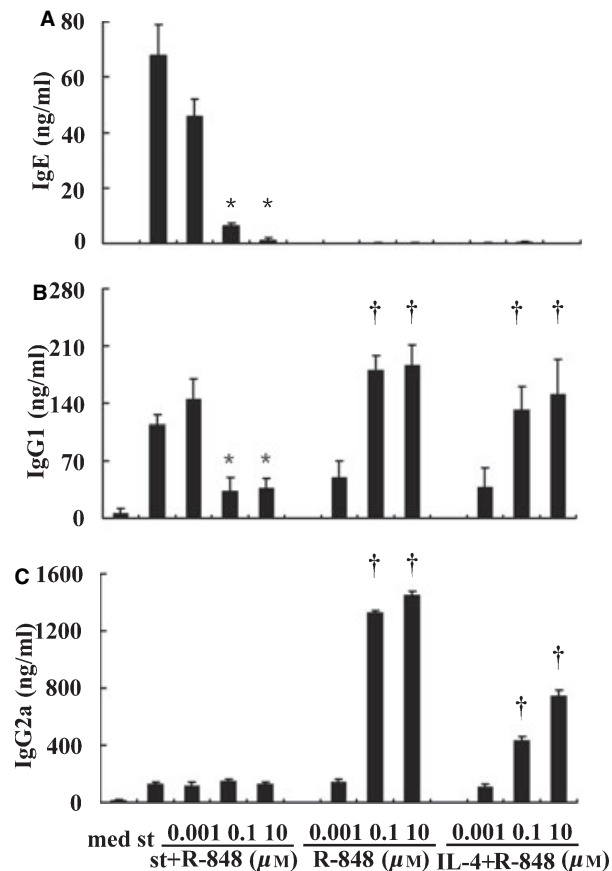


Figure 2 R-848 differentially regulates the production of IgE, IgG1 and IgG2a by splenocytes in a dose-dependent manner under different culture conditions. Splenocytes were cultured in different culture conditions, as indicated, in the presence or absence of R-848 (0.001, 0.1 and 10 μ M), respectively for 7 days. The levels of IgE (A), IgG1 (B) and IgG2a (C) in the culture supernatants were determined by ELISA. The experiments were repeated two times with similar results. The data are expressed as mean $\pm$ SD. Med, medium, st, anti-CD40 plus IL-4. * P < 0.05, in comparison to anti-CD40 + IL-4-stimulated cells. † P < 0.05, in comparison with medium.

points. As shown in Fig. 3A, B, in the early stages of culture, on day 0 to day 2, R-848 significantly inhibited the production of IgE (P < 0.05; Fig. 3A) and IgG1 (P < 0.05; Fig. 3B). After incubation for more than 2 days (on day 3 or 5), addition of R-848 had no marked effects on IgE and IgG1 synthesis (P > 0.05). Moreover, although R-848 slightly increased the production of IgG2a at different time points compared with controls, no statistical significance was observed (P > 0.05; Fig. 3C). These results suggest that R-848 acts on the early stages of B cell differentiation to inhibit IgE production.

R-848 promotes B cell activation and down-regulates CD23 expression

In search of possible mechanisms responsible for the observed inhibition of IgE synthesis, we analysed the

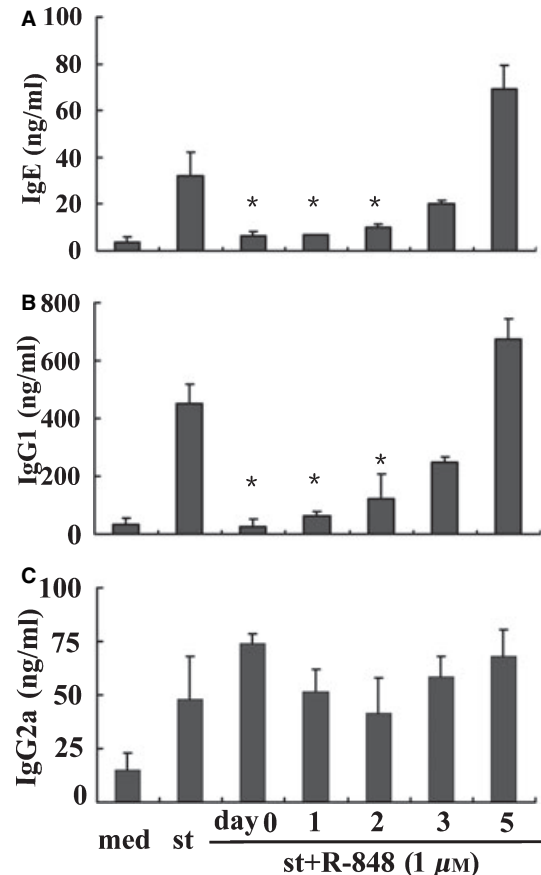


Figure 3 R-848 inhibits the production of IgE and IgG1 but not IgG2a in the early stage of culture of anti-CD40 plus IL-4-stimulated splenocytes. Splenocytes were incubated with medium or anti-CD40 plus IL-4. R-848 was then added into anti-CD40 plus IL-4-stimulated cultures on days 0, 1, 2, 3 and 5. After incubation for 7 days, the levels of IgE (A), IgG1 (B) and IgG2a (C) in the culture supernatants were determined by ELISA. The experiments were repeated two times with similar results. The data are expressed as mean $\pm$ SD. Med, medium, st, anti-CD40 plus IL-4. *, P < 0.05, in comparison to anti-CD40 plus IL-4-stimulated cells.

effect of R-848 on activation of B cells. The results showed that anti-CD40 plus IL-4 could markedly up-regulate the expression of B cell activation markers CD25 and CD69, co-stimulatory molecule CD80 compared with medium, and R-848 alone also enhanced those three molecule expression (data not shown). As shown in Fig. 4, R-848 markedly up-regulated the expression of B cell activation markers CD25 and CD69 as well as co-stimulatory molecule CD80 induced by anti-CD40 plus IL-4.

CD23, the low-affinity receptor for IgE, is expressed on B cells and is known for its pathophysiologic importance in type I hypersensitivity reactions and its capability to regulate IgE production. Membrane-bound CD23 on B cells could inhibit IgE production via a negative feedback loop [23, 24]. Therefore, we examined the role

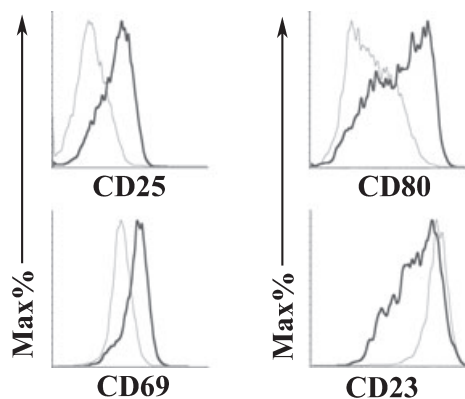


Figure 4 Effects of R-848 on B cell activation induced by anti-CD40 plus IL-4. Splenocytes were incubated with medium or anti-CD40 plus IL-4 in the presence or absence of R-848 (1 μ M) for 3 days for the expression of CD25, CD69 and CD80 or 2 days for CD23. The cells were harvested and stained with fluorescence labeled anti-CD19, anti-CD23, anti-CD25, anti-CD69 and anti-CD80 mAbs. CD19⁺ B cells were gated, and were then analysed for the expression of CD25, CD69, CD80 and CD23 by FACS. Dotted lines represent the anti-CD40 plus IL-4 cultures, and solid lines represent the anti-CD40 plus IL-4 cultures in the presence of R-848. The experiments were repeated four times with similar results.

of R-848 on membrane CD23 expression on B cells. Consistent with previous reports [25], stimulation of splenocytes with anti-CD40 plus IL-4 for 2 days resulted in a strong, significant up-regulation of the expression of CD23 compared with un-stimulated cells (data not shown). R-848 could down-regulate CD23 expression on B cells induced by anti-CD40 plus IL-4 (Fig. 4).

R-848 inhibits the proliferation and division of B cells from anti-CD40 plus IL-4-stimulated splenocytes

Previous studies demonstrated that the proliferation and division of B cells were related to Ig class switching and synthesis [26]. In order to study whether R-848 inhibited the proliferation and division mediated by anti-CD40 plus IL-4, BrdU incorporation (Fig. 5A) and CFSE intensity evaluating cell division (Fig. 5B) were performed. As for BrdU incorporation analysis, splenocytes were incubated with medium or anti-CD40 plus IL-4 in the presence or absence of R-848 for 3 days. At the end of stimulation, proliferation of B cells was assessed by flow cytometric evaluation of BrdU incorporation. The data showed that there was no BrdU incorporation in cells in medium alone (Fig. 5A). Stimulation with anti-CD40 plus IL-4 resulted in the proliferation of CD19⁺ B cells (23.50%). In contrast, addition of R-848 into anti-CD40 plus IL-4-stimulated cultures markedly inhibited the proliferation of CD19⁺ B cells (3.26%).

The effect of R-848 on B cell proliferation was also evaluated by CFSE intensity. CFSE-labeled splenocytes were incubated with anti-CD40 plus IL-4 in the presence

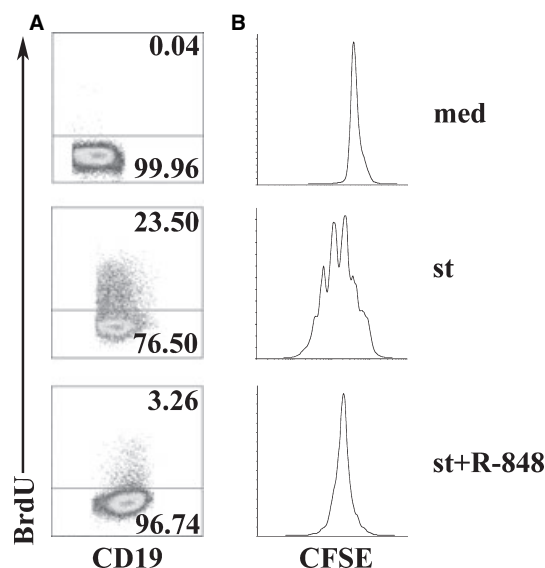


Figure 5 R-848 inhibits the proliferation and division of B cells in anti-CD40 plus IL-4-stimulated splenocytes. As for BrdU incorporation analysis (A), splenocytes were incubated with medium or anti-CD40 plus IL-4 in the presence or absence of R-848 for 3 days, and flow cytometric measurement of BrdU incorporation of the cultured cells was carried out according to the manufacture's instructions. CD19⁺ B cells were gated and analysed. The numbers in each quadrant were indicated as percentage. The experiments were repeated two times with similar results. To determine the effect of R-848 on B cell division (B), splenocytes were first labeled with CFSE and incubated with medium or anti-CD40 plus IL-4 in the presence or absence of R-848 for 3 days. The cells were harvested and stained with anti-CD19 APC mAbs. CD19⁺ B cells in splenocytes were gated, and then the division of cells was analysed by flow cytometry. The experiments were repeated three times with similar results. Med, medium; st, anti-CD40 plus IL-4.

or absence of R-848 for 3 days and the division of B cells was detected by the dilution of CFSE. As shown in Fig. 5B, there was no B cell division when splenocytes were cultured with medium alone. Stimulation with anti-CD40 plus IL-4 could promote some of B cell dividing one to four times (Fig. 5B). However, addition of R-848 to the cultures significantly inhibited B cell division. Majority of B cells divided once. We also observed that stimulation of splenocytes with R-848 alone for 3 days resulted in some of B cells dividing one to three times (data not shown).

Anti-IFN- γ or anti-IL-12 mAbs partially abolish R-848 mediated inhibition of IgE production

Previous studies reported that R-848 induced the production of IFN- γ and IL-12 by splenocytes [27]. These two cytokines were known to negatively regulate IgG1 and IgE synthesis [28, 29]. We next examined the production of these two cytokines in un-stimulated and in anti-CD40 plus IL-4-activated splenocytes in the presence or absence of R-848 after an incubation for 3 days.

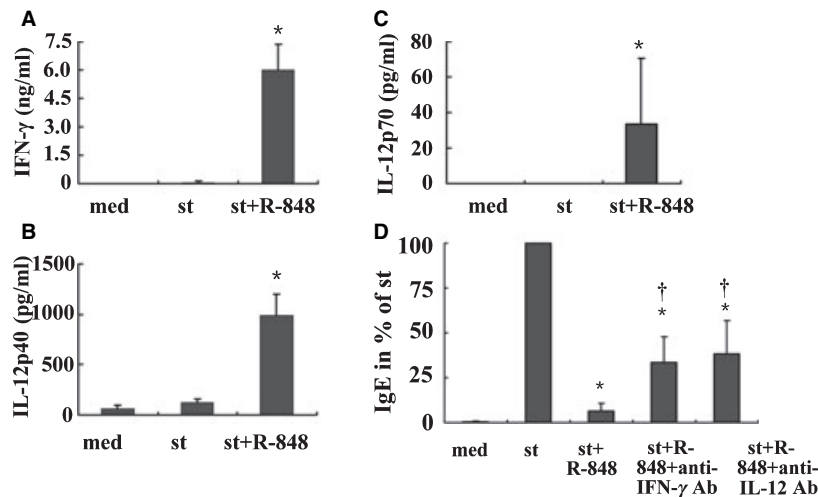


Figure 6 R-848 promotes the production of IFN- γ and IL-12 which were partially responsible for its inhibitory effect on IgE production by anti-CD40 plus IL-4. Splenocytes were incubated with medium, R-848 or anti-CD40 plus IL-4 in the presence or absence of R-848 (1 μ M) for 3 days. IFN- γ (A), IL-12p40 (B) and IL-12p70 (C) in the supernatants of the culture were determined by ELISA. (D) Splenocytes were incubated with medium, anti-CD40 plus IL-4 or with R-848 (1 μ M) in the presence or absence of neutralizing mAbs against IFN- γ (10 μ g/ml) or IL-12 (10 μ g/ml), respectively. The levels of IgE in the culture supernatants were determined by ELISA. The experiments were repeated three times with similar results. The data are expressed as mean $\pm$ SD. Med, medium, st, anti-CD40 plus IL-4. * P < 0.05, compared with anti-CD40 plus IL-4-stimulated cells. † P < 0.05, compared with anti-CD40 plus IL-4 plus R-848-stimulated cells.

As shown in Fig. 6A, there was a low level of IFN- γ production (70.3 ± 78.9 pg/ml) in anti-CD40 plus IL-4-stimulated splenocytes. However, addition of R-848 to the cultures markedly enhanced the production of IFN- γ (5977.3 ± 1360.4 pg/ml; P < 0.05, compared with anti-CD40 plus IL-4-stimulated cells). Moreover, consistent with previous studies [27], R-848 alone significantly induced the production of IFN- γ by splenocytes (data not shown). A similar effect was observed on the production of IL-12p40 and IL-12p70 (Fig. 6B,C). In order to determine whether IFN- γ or IL-12 was responsible for the inhibition of IgE synthesis by R-848, neutralizing mAbs to IFN- γ or IL-12 was added to anti-CD40 plus IL-4-stimulated splenocytes in the presence of R-848 (1 μ M). As shown in Fig. 6D, the inhibition of IgE synthesis induced by anti-CD40 plus IL-4-stimulated splenocytes by R-848 (P < 0.05, compared with anti-CD40 plus IL-4) was partially restored by the addition of neutralizing IFN- γ mAbs (P < 0.05, compared with anti-CD40 plus IL-4 plus R-848), or neutralizing IL-12 mAbs (P < 0.05, compared with anti-CD40 plus IL-4 plus R-848). However, addition of neutralizing IFN- γ or IL-12 to anti-CD40 plus IL-4 could only partially restore the inhibitory role of R-848 (P < 0.05, compared with anti-CD40 plus IL-4), which indicates that there are other factors or mechanisms to work together with these two cytokines to inhibit IgE production. In addition, there was no significant difference in IgE levels between neutralizing IFN- γ and neutralizing IL-12 in the anti-CD40 plus IL-4-stimulated cultures.

R-848 directly inhibits B cell-secreting IgE

R-848, the ligand of TLR7/8 that is expressed on murine splenic B cells, directly acted on B cells [30]. So we want to know whether R-848 act directly on B cells to regulate IgE synthesis induced by anti-CD40 plus IL-4. Purified B cells were prepared as described in Materials and Methods, and cultured with medium or anti-CD40 plus IL-4 in the presence or absence of R-848. As shown in Fig. 7, anti-CD40 plus IL-4-induced purified B cells to secrete IgE after incubation for 7 days. Addition of R-848 to the cultures could significantly inhibit IgE synthesis (P < 0.05).

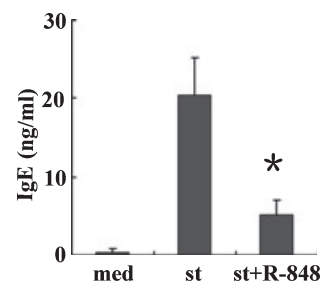


Figure 7 R-848 inhibits IgE production by purified B cells. Purified B cells were prepared as described in Materials and Methods, and cultured with medium, anti-CD40 plus IL-4 in the presence or absence of R-848 (1 μ M) for 7 days. The experiments were repeated three times with similar results. The data are expressed as mean $\pm$ SD. Med, medium, st, anti-CD40 plus IL-4. * P < 0.05, in comparison to anti-CD40 plus IL-4-stimulated cells.

Discussion

Toll-like receptors are pattern recognition receptors that trigger innate immunity, providing both immediate protective responses against pathogens and instructing the adaptive immune responses through the induction of dendritic cell recruitment and maturation. TLRs are involved in the control of antibody responses to T-dependent antigens in at least two ways. First, TLR signals induce dendritic cell maturation to promote Th cell differentiation that is required to provide help to B cells, primarily through CD40–CD40L interaction and cytokine secretion. Second, TLRs on B cells have direct effects on B cell activation and antibody production after ligation with TLR ligands [31]. In mice, TLRs have been shown to control the B cell response and in humans, TLR stimulation is required as a third signal required for activation of human naive B cells [32].

In the present study, we demonstrated that the TLR7/8 ligand, R-848, inhibited anti-CD40 plus IL-4-mediated IgE and IgG1 production in murine splenocytes in a dose-dependent manner. The inhibitory effects of R-848 on anti-CD40 plus IL-4-induced IgE synthesis by splenocytes appeared to be mediated by at least three different mechanisms. First, R-848 inhibited the proliferation and division of B cells in anti-CD40 plus IL-4-stimulated splenocytes. Second, R-848 promoted the production of IFN- γ and IL-12, which were partially responsible for the inhibition of IgE production. Third, R-848 had a direct effect on B cells to inhibit IgE production.

It was reported that R-848 inhibited anti-CD40 plus IL-4-mediated IgE production by human peripheral blood mononuclear cells from normal and allergic donors [33]. R-848 inhibited IgG1 production but had no effects on IgG2a production mediated by anti-CD40 plus IL-4-stimulated splenocytes, indicating that the inhibitory effect of R-848 was not due to a toxic effect on the cells. Meanwhile, we found that R-848 alone or IL-4 plus R-848 could induce both IgG1 and IgG2a production, whereas neither R-848 alone nor IL-4 plus R-848 at different concentrations could induce IgE production. An *in vivo* study showed that R-848 are effective at skewing immunity in the presence of Alum toward a Th1 Ab response (IgG2a) and away from a Th2 Ab response (IgE), and increasing IgG2a are associated with IL-12 and IFN- γ induction [10].

To study R-848 acting on which stages of B cell differentiation and to confirm whether R-848 could down-regulate ongoing IgE synthesis, splenocytes were stimulated with anti-CD40 plus IL-4, and R-848 was then added into the cultures at various time points. After incubation for 2 days, addition of R-848 had no marked effects on the synthesis of IgE ($P > 0.05$). Previous study showed that TLR9 ligand CpG could suppress IgE induc-

tion but fail to down-regulate ongoing IgE production *in vivo* [34]. This result indicates that R-848 might not have an inhibitory effect on developed IgE-producing B cells.

The proliferation and final maturation of peripheral B cells require a combination of signals first for progression of the resting B cell into an activated state, followed by isotype switching. We found that stimulation with R-848 alone or anti-CD40 plus IL-4 in the presence of R-848 resulted in up-regulation of B cell activation markers CD25 and CD69, and co-stimulatory molecule CD80. The similar effect of R-848 on the expression of co-stimulatory molecule CD86 was observed in a previous study [13]. Consistent with previous study in human [33], R-848 could down-regulate CD23 expression on B cells induced by anti-CD40 plus IL-4 in mice. These data indicated that R-848 inhibited IgE production neither through inhibition of B cell activation nor through regulation of CD23 expression.

Previous studies demonstrated that R-848 alone induced a significant increase in thymidine uptake in mouse splenocytes and purified B cells and promoted the proliferation of these cells [13]. In our study, we observed the similar role of R-848 in enhancing B cell proliferation. However, addition of R-848 to anti-CD40 plus IL-4-activated splenocytes resulted in the inhibition of B cell proliferation and division. Similar anti-proliferative effects of R-848 were obtained in human PBMCs [33]. B cell proliferation and class switching are linked to cell division numbers [26, 35]. As a result, inhibiting the division of B cells could indirectly decrease the number of switched cells. This may potentially be the mechanism by which R-848 mediated the inhibition of IgE production.

In the present study, we observed that R-848 alone could induce a mount of IFN- γ and IL-12 production. Similar results were observed in a previous study which had demonstrated that R-848 modulates Th responses by inducing the production of the Th1 cytokines, IFN- γ and IL-12 in mouse and human culture systems [27]. There were low levels of IFN- γ production in anti-CD40 plus IL-4-stimulated splenocytes. Addition of R-848 to anti-CD40 plus IL-4 cultures markedly enhanced the production of IFN- γ and IL-12. IFN- γ inhibits the development of Th2 cells and therefore inhibits the production of IL-4 and IL-13. IFN- γ also acts directly on B cells to repress C ϵ germline transcription and subsequently to down-regulate CSR (class switch recombination) to C ϵ [28]. And IL-12 markedly suppresses the IL-4-induced IgE production by human PBMC *in vitro* [29]. Consistent with the reports, anti-IFN- γ or anti-IL-12 mAbs partially abolish R-848-mediated inhibition of IgE production. In addition, an *in vitro* study in humans showed that inhibition of IgE production by R-848 was partially dependent on IFN- γ

in anti-CD40 plus IL-4-stimulated PBMCs from both non-allergic and allergic donors [33]. An *in vivo* study showed that R-848 during the sensitization phase prevented the production of OVA-specific IgE and IgG1 Abs and subsequently abolished all features of experimental asthma including airway hyper-responsiveness and allergic airway inflammation. Furthermore, administration of R-848 to animals with already established primary allergic responses revealed a markedly reduced secondary response following allergen aerosol re-challenges. R-848 has both preventive as well as suppressive effects on experimental asthma which is partially mediated by IL-12 [3].

Taken together, we show here that the TLR7/8 ligand, R-848, is a potent inhibitor of IgE production *in vitro*. The inhibition of Ig production by R-848 is specific for IgE and IgG1 but not for IgG2a in splenocytes, and the possible mechanisms are through the inhibition of B cell division, promoting IFN- γ and IL-12 production and directly acting on B cells to inhibit IgE synthesis. These results suggest that R-848 might be used to treat IgE-mediated allergic diseases.

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