

The novel synthetic immune response modifier R-848 (Resiquimod) shifts human allergen-specific CD4⁺ T_H2 lymphocytes into IFN- γ -producing cells

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Background: In experimental models, imidazoquinolines exhibit several immunomodulatory activities via Toll-like receptor signaling on cells of the innate immunity. **Objective:** The aim of our study was to investigate whether R-848 (Resiquimod), a small-molecular-weight synthetic compound belonging to the imidazoquinoline family and known for its ability to substantially delay the onset of recurrent genital herpes lesions in both animals and human beings, could influence, at least in vitro, the cytokine production profile of human hapten- or allergen-specific T cells. **Methods:** Ampicillin- and Der p 1-specific T-cell lines were derived from peripheral blood of allergic donors in the absence or presence of R-848 and assessed by flow cytometry at the single-cell level for their ability to produce IL-4 and/or IFN- γ . **Results:** R-848 induced both hapten- and allergen-specific circulating T cells, including T_H2 effectors, to produce IFN- γ and even to lose the ability to produce IL-4, thus shifting their phenotype of cytokine production to a type 0 (T_H0) or even T_H1 profile. This effect was associated with an increase in the production of IL-12, IFN- α , IL-18, TNF- α , IL-10, and IL-15 by CD14⁺ cells, as well as an increase in the proportions of IFN- γ -producing CD3-CD16⁺ (natural killer) cells. **Conclusions:** These results suggest that R-848, and probably other imidazoquinolines, might be used as adjuvants in view of novel allergen-specific immunotherapeutic regimens for the treatment of allergic disorders. (J Allergy Clin Immunol 2003;111:380-8.)

Key words: T_H1/T_H2, allergy, cytokines, immunomodulators

Abbreviations used

Amp: Ampicillin
CRTH2: Chemoattractant receptor for T_H2 cells
DC: Dendritic cell
Der p: *Dermatophagoides pteronyssinus*
NK: Natural killer
ODN: Oligodeoxynucleotide
PMA: Phorbol myristate acetate
Poly I:C: Polyinosinic:polycytidylic acid
rIL: Recombinant interleukin
TLR: Toll-like receptor

Atopic diseases are the result of type 2 T_H2-dominated responses to single or multiple environmental allergens.¹ Therefore, the development of biological agents or drugs able to shift the allergen-specific T_H2 response into a less polarized phenotype might be of great usefulness in the treatment of atopic disorders.² In vitro stimulation of circulating CD4⁺ T lymphocytes from atopic donors with the specific allergen in the presence of IFN- γ , IFN- α ,³ IL-12,⁴ polyinosinic:polycytidylic acid (Poly I:C),⁵ or appropriate phosphorothioate oligodeoxynucleotides (ODNs)⁶ or with ODN-conjugated allergens⁷ can promote the development of type 0 (T_H0) or even type 1 (T_H1) cells instead of T_H2 cells. More importantly, both IL-12 and ODNs not only favor the development of T_H0/T_H1 rather than T_H2 allergen-specific T cells but also promote the production of IFN- γ by already established allergen-specific memory T_H2 effectors.⁸

Imiquimod and the more potent analog Resiquimod (R-848), belonging to the family of imidazoquinolines, exhibit potent antiviral and antitumor activities in several experimental models.^{9,10} Recently, it has been reported that these compounds are able to induce the production of IL-12, IFN- γ , and IFN- α by murine and human mononuclear cells and to inhibit the production of both IL-4 and IL-5 by polyclonally stimulated T cells.¹¹

In this study, we investigated the in vitro effects of R-848 on the cytokine production profile of ampicillin-specific human T cells from β -lactam-sensitive donors, as well as of *Dermatophagoides pteronyssinus* (Der p) group 1-specific T cells from atopic Der p-sensitive donors. R-

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Supported by grants provided by Associazione Italiana Ricerca sul Cancro (AIRC), the Italian Ministry of Health (Project AIDS 2000), and European Community (Projects QLK3-CT2000-00270 and BIO4-98-0458).

Received for publication July 5, 2002; revised October 2, 2002; accepted for publication October 22, 2002.

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0091-6749/2003 \$30.00 + 0

doi:10.1067/mai.2003.102

848 inhibited the development of either ampicillin- or Der p 1-specific T cells into T_H2 cells and enabled established T_H2 effectors to produce IFN- γ . R-848 also induced the production of IL-12, IL-18, IFN- α , TNF- α , IL-10, and IL-15 by CD14⁺ cells, as well as the preferential expansion of CD16⁺ cells able to produce IFN- γ ; this indicates that the T_H1-switching effect on hapten- and allergen-specific T cells was related to the stimulatory activity on cells of the natural immunity, such as macrophages, dendritic cells (DCs), and natural killer (NK) cells. Thus, R-848 might be used as a powerful adjuvant for the specific immunotherapy of allergic disorders.

METHODS

Subjects

Blood samples used in this study were obtained from 7 informed adult ampicillin-sensitive donors and 4 Der p-sensitive atopic volunteers, in accordance with the ethical standards of the responsible regional committee on human experimentation.

Reagents

Ampicillin and Poly I:C were from Sigma Chemical Co (St Louis, Mo). Der p 1 allergen was kindly provided by Lofarma Allergeni (Milan, Italy). Phorbol myristate acetate (PMA), ionomycin, brefeldin A, and saponin were from Sigma. Recombinant interleukin (rIL) 2 was kindly provided by Eurocetus (Milan, Italy). IL-12 was from R&D Systems (Minneapolis, Minn). Anti-CRTH2 mAb was kindly donated by K. Nagata (R&D Center, BML Inc, Kawagoe, Saitama, Japan). Purified and fluorochrome-conjugated anti-CD3, anti-TCR $\alpha\beta$, anti-CD4, anti-CD8, anti-CD14, anti-CD16, anti-CD20, and anti-CD56 mAbs, as well as PE-conjugated anti-IL-4 (3010.211, IgG1) and FITC-conjugated anti-IFN- γ (25723.11, IgG2b) mAbs were purchased from Becton Dickinson (San Jose, Calif). Purified and fluorochrome-conjugated isotype control mAbs were purchased from Southern Biotechnology Associates (Birmingham, Ala).

R-848 (S28463) was kindly provided by 3M Pharmaceuticals (St Paul, Minn). The HCl salt was dissolved in pyrogen-free sterile water and stored as stock solution at 4°C for up to 4 months. Endotoxin levels in R-848, Der p 1, ampicillin, and Poly I:C were less than 0.003 EU/mL in the Lymulus Amoebocyte Assay (Bio Whittaker, Walkersville, Md).

Generation of antigen-specific short-term T-cell lines and allergen-specific T-cell clones

Ampicillin- and Der p 1-specific short-term T-cell lines were generated from PBMCs by stimulation with ampicillin (500 μ g/mL) or Der p 1 (10 μ g/mL) in the absence or presence of different concentrations of R-848 (20, 2, and 0.2 μ g/mL) or rIL-12 (2.5 ng/mL) for 6 days and subsequent expansion with rIL-2 (20 U/mL), as described previously.^{6,12} The specificity of short-term T-cell lines was assessed as described previously.^{6,12}

T-cell blasts from a Der p 1-specific short-term T-cell line obtained from one allergic donor were cloned under limiting dilution (0.3 cell/well), and the antigen specificity of T-cell clones was assessed as previously reported.¹²

Generation of antigen-specific short-term T-cell lines from purified CRTH2⁺ T cells

Positively selected CD4⁺CRTH2⁺ T cells (purity, >98%) obtained by high-gradient magnetic cell sorting (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)⁸ were stimulated with ampi-

cillin (500 μ g/mL) or Der p 1 (10 μ g/mL) and autologous highly purified CD14⁺ cells in the absence or presence of R-848 (2 μ g/mL) or IL-12 (2.5 ng/mL) for 6 days, then expanded with rIL-2 (20 U/mL). On day 14, T-cell blasts were stimulated for detection of intracellular IL-4 and IFN- γ synthesis, as described below.

Flow cytometric analysis of surface molecules and intracellular cytokine production

Surface molecules in T-cell blasts from short-term T-cell lines or clones were detected by incubation for 20 minutes with anti-CD3, anti-TCR $\alpha\beta$, anti-CD4, anti-CD8, anti-CD16, anti-CD20, and anti-CD56 or isotype control mAb. The cells were washed and analyzed by means of a FACSCalibur cytofluorimeter (Becton Dickinson) as described previously.⁸

Intracytofluorimetric analysis of IFN- γ and IL-4 synthesis at the single-cell level was performed as already described.^{8,12} Briefly, 1×10^6 T-cell blasts were stimulated with PMA (10 ng/mL) plus ionomycin (1 mmol/L) for 4 hours, the last 2 in the presence of brefeldin A (5 μ g/mL). After incubation, cells were fixed with formaldehyde, permeabilized with 0.5% saponin, incubated with the specific mAb, and analyzed on a FACSCalibur cytofluorimeter by means of CellQuest software (Becton Dickinson). The area of positivity was determined through use of an isotype-matched mAb. In all cytofluorimetric analyses, a total of 10^4 events, gated as CD3⁺CD4⁺TCR $\alpha\beta$ ⁺ or CD3⁺CD16⁺ cells, were acquired.

To evaluate the percentages of B and NK cells in specific T-cell lines, 2×10^5 cells were collected at the time of the cytokine assessment and stained with anti-CD3, anti-CD16, anti-CD20, anti-CD14, and anti-CD56 fluorochrome-conjugated mAbs (Becton-Dickinson) or isotype control mAbs; they were then washed and analyzed by flow cytometry through use of the FACScan system (Becton-Dickinson). A total of 10^4 cells for each sample were acquired.

Measurement of IL-4 and IFN- γ in supernatants of T-cell clones

We stimulated 10^6 /mL viable T-cell blasts for 36 hours with PMA (20 ng/mL) plus anti-CD3 mAb (UCHT1; BD Pharmingen, Franklin Lakes, NJ; 50 ng/mL). IL-4 (BD Pharmingen) and IFN- γ (Endogen, Woburn, Mass) concentrations were measured into cell-free supernatants by homemade ELISAs through use of commercial pairs of mAbs, as described previously.¹²

Production of cytokines by purified CD14⁺ cells

We cultured 1×10^6 /mL CD14⁺ cells positively selected by high-gradient magnetic cell sorting from PBMCs of 8 healthy subjects for 60 hours in the absence or presence of R-848 (2 μ g/mL), or Poly I:C (50 μ g/mL). Supernatants were then collected, centrifuged, and stored at -20°C. The amounts of IL-12 (Cytoscreen, Biosource Int, Camarillo, Calif), IL-18 (MBL, Nagoya, Japan), IFN- α (Cytoscreen), TNF- α (Cytoscreen), IL-10 (BD Pharmingen), and IL-15 (R&D Systems) were evaluated by commercial ELISAs.

For the analysis of IL-12 intracellular expression by CD14⁺ cells, 1×10^6 positively selected CD14⁺ cells from PBMCs of 3 healthy donors were cultured in the absence or presence of R-848 (2 μ g/mL) or Poly I:C (50 μ g/mL) for 2 hours, treated with brefeldin A (5 ng/mL) for 6 hours, and detached by EDTA 2 mmol/L in PBS pH 7.2. The cells were washed, fixed with formaldehyde, permeabilized, and incubated with the specific mAb, as described above.

Statistical analysis

Statistical analysis of the results was performed with the Student *t* test for paired data.

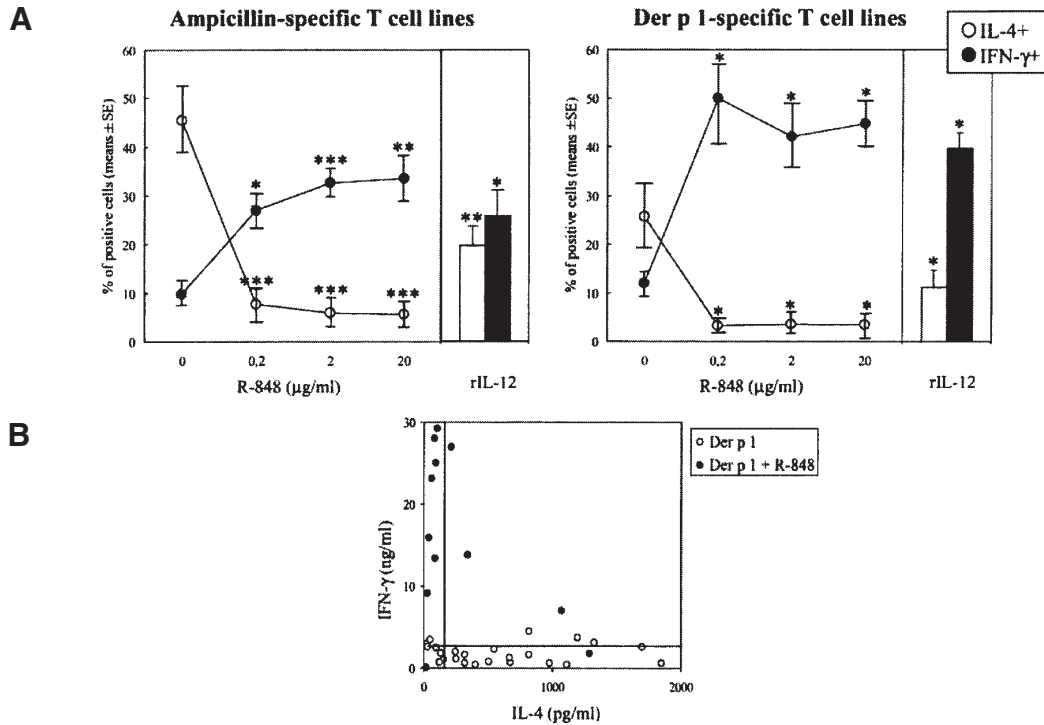


FIG 1. Production of IL-4 and IFN- γ by ampicillin- or Der p 1-specific short-term T-cell lines and by Der p 1-specific CD4⁺ T-cell clones. **A**, Ampicillin- and Der p 1-specific short-term T-cell lines were derived from 7 ampicillin-sensitive and 4 Der p 1-allergic donors in the absence or presence of rIL-12 or different concentrations of R-848. T-cell blasts were analyzed for the intracellular content of IL-4 and IFN- γ by flow cytometry, as described in the Methods section. The mean percentage ($\pm$ SE) of IL-4⁺ (white) and IFN- γ ⁺ (black) producing cells are reported. * $P < .05$; ** $P < .005$; *** $P < .0005$. **B**, T-cell clones generated from Der p 1-specific short-term T-cell lines, obtained in the absence (white circles) or presence (black circles) of R-848 (2 μ g/mL) from PBMCs of a single Der p-sensitive donor, were polyclonally stimulated, and IL-4 (x axis) and IFN- γ (y axis) production was assessed in cell-free supernatants by ELISA, as described in the Methods section. Lines represent the mean values ($\pm$ SDs) of cytokine concentrations produced by irradiated feeder cells alone in response to PMA plus anti-CD3 mAb.

RESULTS

Resiquimod is a powerful switching agent of ampicillin- and Der p 1-specific CD4⁺ T cells from the T_H2 to the T_H0/T_H1 profile

Ampicillin- and Der p 1-specific short-term T-cell lines were generated from 7 ampicillin-sensitive donors and 4 atopic Der p-sensitive subjects in the absence or presence of different concentrations of R-848, and their ability to produce IL-4 and IFN- γ in response to stimulation with PMA plus ionomycin was evaluated at the single-cell level by flow cytometry. As shown in Fig 1, A, the addition in culture of R-848 induced a strong decrease in the proportion of IL-4-producing CD4⁺TCR $\alpha\beta$ ⁺ T-cell blasts, with a parallel increase in the proportion of IFN- γ -producing cells in comparison with unconditioned cultures, the T_H1 switching effect of Resiquimod being even higher than that induced by IL-12. No differences in the percentage of cells producing both IL-4 and IFN- γ were

seen (data not shown). To provide direct evidence for the T_H1-switching activity of R-848 on the differentiation of allergen-specific T cells, Der p 1-specific T-cell lines from a single Der p-sensitive donor, derived in the absence or presence of R-848 (2 μ g/mL), were cloned under limiting dilution conditions and the obtained clones were examined for both antigen-specificity and the ability to produce IL-4 and IFN- γ . As shown in Fig 1, B, virtually all clones generated from the unconditioned culture produced large amounts of IL-4, but no IFN- γ , whereas the great majority of those generated from the parallel R-848-conditioned culture produced IFN- γ but not IL-4.

To establish whether R-848 only favored the differentiation of T helper cell precursors into T_H1/T_H0 cells instead of T_H2 cells or was even able to change the cytokine production potential of already established T_H2 effectors, CRTH2⁺CD4⁺ T cells were isolated from PBMCs of 3 subjects (1 Der p-allergic and 2 ampicillin-sensitive donors) and stimulated with the specific antigen in the absence or presence of R-848 or IL-12. As expected,

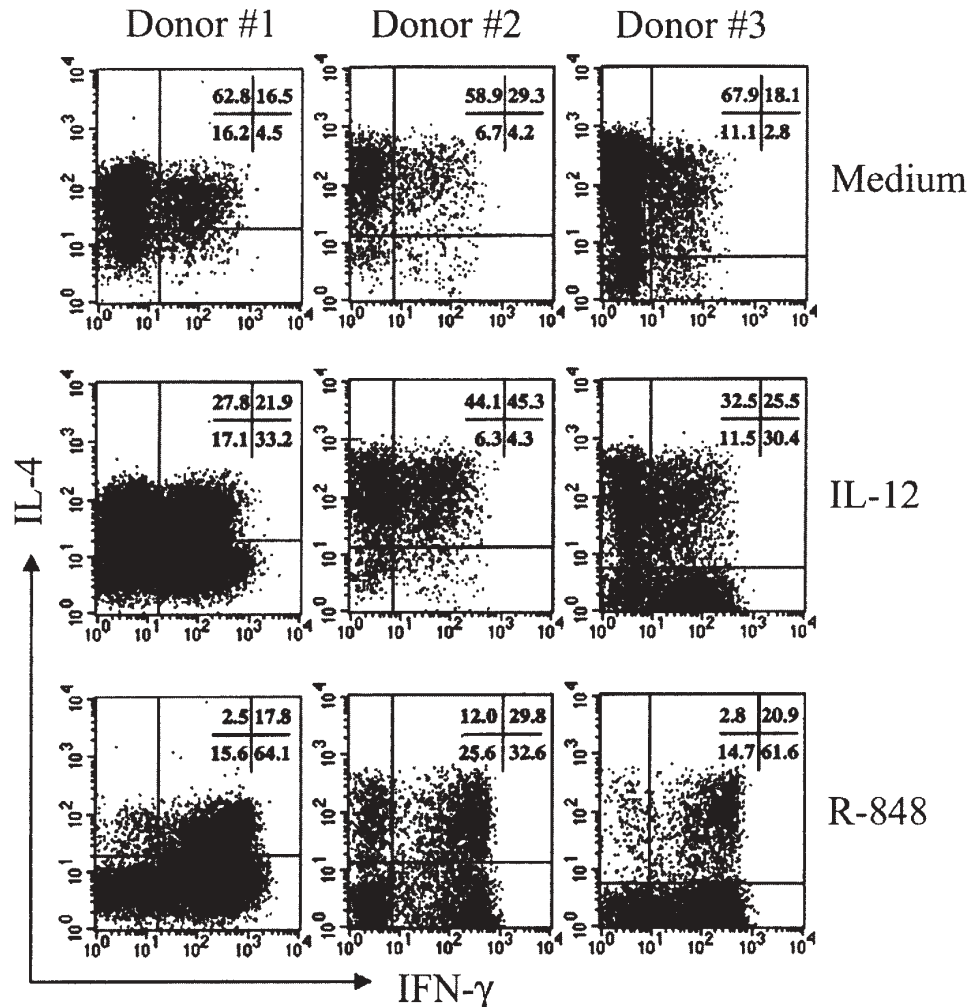


FIG 2. Induction of IFN- γ production by purified CD4⁺CRTH2⁺ T cells. CD4⁺CRTH2⁺ T cells were positively sorted from PBMCs of 2 ampicillin-sensitive subjects (*Donor #1* and *Donor #2*) and 1 atopic Der p-sensitive subject (*Donor #3*) and stimulated with the relevant antigen plus highly purified autologous CD14⁺ cells in the absence (*Medium*) or presence of rIL-12 or R-848 (2 μ g/mL). On day 14, intracellular synthesis of IL-4 and IFN- γ in response to 4 hours of stimulation with PMA plus ionomycin was assessed by flow cytometry, as described in the Methods section.

ed,⁸ the great majority of purified CD4⁺CRTH2⁺ T cells obtained in the absence of R-848 or rIL-12 developed into IL-4-producing, but not IFN- γ -producing, T-cell blasts. In contrast, the great majority of CRTH2⁺ cells derived from either IL-12- or R-848-conditioned cultures showed the ability to produce IFN- γ in addition to IL-4 or even to produce IFN- γ alone (Fig 2).

R-848 stimulates the production of IL-12, IL-18, IFN- α , TNF- α , IL-10, and IL-15 by CD14⁺ cells

To investigate the mechanism(s) by which R-848 exerted its effects on hapten- or allergen-specific T cells, purified CD14⁺ cells from PBMCs of 8 healthy donors

were cultured for 60 hours in the absence or presence of different concentrations of R-848. As shown in Fig 3, A, R-848 induced the production of large amounts of IL-12 by purified CD14⁺ cells, and this effect was related to the increase in the number of IL-12-producing CD14⁺ cells (Fig 3, B). The incubation of purified CD14⁺ cells with R-848 also resulted in the release of high concentrations of IFN- α , IL-18, TNF- α , and IL-10 as well as few picograms of IL-15, in comparison with unstimulated CD14⁺ cell cultures (Table I). The small IL-15 increase did not merely reflect an artifact, inasmuch as using semiquantitative RT-PCR enhanced IL-15 mRNA expression was detected in R-848-stimulated cultures in comparison with unstimulated CD14⁺ cells from the same donors (data not shown).

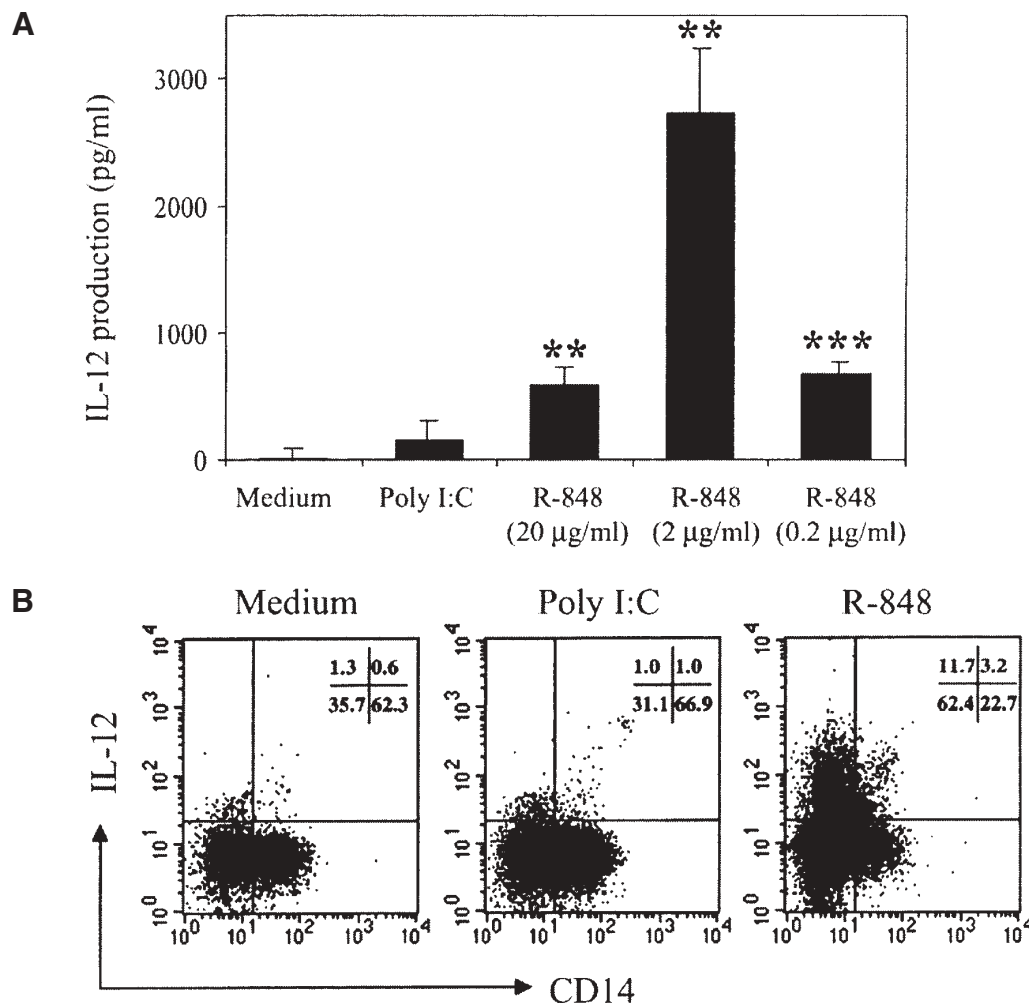


FIG 3. R-848 stimulates the production of large amounts of IL-12 by CD14⁺ cells. We stimulated 10^6 highly purified CD14⁺ cells, obtained from PBMCs of 8 donors by positive selection, for 60 hours in the absence or presence of different R-848 concentrations or, as a control, in the presence of Poly I:C (50 µg/mL). **A**, IL-12 p70 and p40 production was evaluated in cell-free supernatants by commercial ELISA. Columns represent mean $\pm$ SE values of IL-12 concentrations (pg/mL). ** $P < .008$; *** $P < .0001$. **B**, Purified CD14⁺ cells (1×10^6), either unstimulated (*Medium*) or stimulated with R-848 (2 µg/mL) or Poly I:C (50 µg/mL) for 6 hours (the last 2 in the presence of brefeldin), were assessed for intracellular expression of IL-12 by flow cytometry. One representative experiment is shown.

R-848 induces the expansion of IFN- γ -producing CD3-CD16⁺ cells in short-term T-cell lines

To establish whether R-848 might affect other cell types from innate immunity different from CD14⁺ cells, Der p 1-specific short-term T-cell lines were derived from 4 atopic Der p-sensitive donors. Significantly higher proportions of CD3-CD16⁺ cells, which also stained positively for CD56 (NK cells), were observed in R-848-conditioned cultures (2 µg/mL) in comparison with unconditioned cultures (Fig 4, A), whereas the proportions of CD3-CD20⁺ cells (B lymphocytes) in the 3 different cultures were comparable (Fig 4, B). More impor-

tantly, both IL-12- and R-848-conditioned cultures showed significantly higher proportions of CD3-CD16⁺ cells able to express intracellular IFN- γ in response to PMA plus ionomycin stimulation in comparison with unconditioned cultures (Fig 4, C and D). Similar results were also obtained when hapten-specific T-cell lines were analyzed (data not shown).

DISCUSSION

The demonstration that allergen-specific T_H2 responses play a fundamental role in the pathogenesis of allergy and asthma¹ provides the conceptual basis for the development of novel allergen-specific therapeutic strategies.²

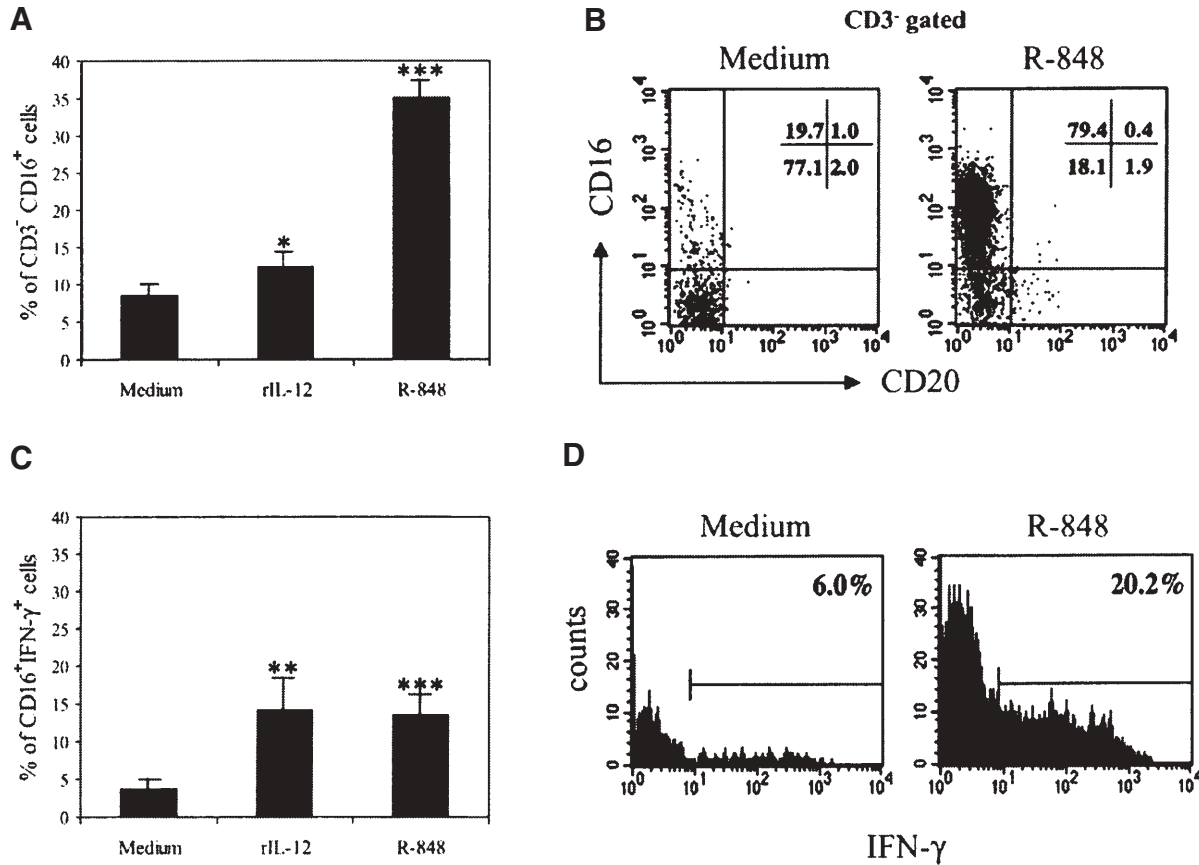


FIG 4. Expansion by R-848 of IFN- γ -producing CD3-CD16⁺ cells. PBMCs from 4 Der p-sensitive donors were stimulated with Der p 1 (10 μ g/mL) in the absence (*Medium*) or presence of IL-12 (2.5 ng/mL) or R-848 (2 μ g/mL) under the conditions described in Fig 1. On day 14, the proportions of CD3-CD16⁺ and CD3-CD20⁺ cells were assessed by flow cytometry. **A**, Proportions (mean $\pm$ SE values) of CD3-CD16⁺ cells. * P < .01; *** P < .0001. **B**, Expression of CD16 and CD20 by CD3⁺ cells in cultures established in the absence or presence of R-848, as detected in one representative experiment. **C**, Proportions (mean $\pm$ SE values) of CD3-CD16⁺ cells able to produce IFN- γ after stimulation with PMA plus ionomycin. ** P < .005; *** P < .0001. **D**, Production of IFN- γ by CD3-CD16⁺ cells in cultures established in the absence or presence of R-848, as detected in one representative experiment.

TABLE I. R-848 induces the production of cytokines by highly purified CD14⁺ cells

Cytokine	Mean ($\pm$ SE) of cytokine production in response to different stimuli* (pg/mL)				
	Medium	Poly I:C	R-848		
			20 μ g/mL	2 μ g/mL	0.2 μ g/mL
IFN- α	10 $\pm$ 0	130 $\pm$ 77	341 $\pm$ 147†	309 $\pm$ 116†	339 $\pm$ 152†
IL-18	73 $\pm$ 49	70 $\pm$ 60	416 $\pm$ 56†	348 $\pm$ 51‡	87 $\pm$ 27
IL-15	0 $\pm$ 0	4.2 $\pm$ 0.2§	5.7 $\pm$ 1.3†	7.0 $\pm$ 0.4§	4.4 $\pm$ 0.2§
TNF- α	41 $\pm$ 12	180 $\pm$ 18‡	2559 $\pm$ 140‡	4867 $\pm$ 784†	2472 $\pm$ 213§
IL-10	100 $\pm$ 0	4100 $\pm$ 600†	12650 $\pm$ 1300§	10780 $\pm$ 1100§	3468 $\pm$ 600†

*From 8 healthy volunteers, 1×10^6 /mL highly purified CD14⁺ cells were obtained by high-gradient magnetic cell sorting separation and stimulated for 60 hours in the absence or presence of polyinosinic:polycytidylic acid (*Poly I:C*; 50 μ g/mL) or R-848 at 3 different concentrations (20, 2, and 0.2 μ g/mL). The supernatants were collected and stored at -20°C until tested, and the cytokine content was determined by commercial ELISAs, as described in the Methods section.

† P < .05.

§ P < .005.

‡ P < .0005.

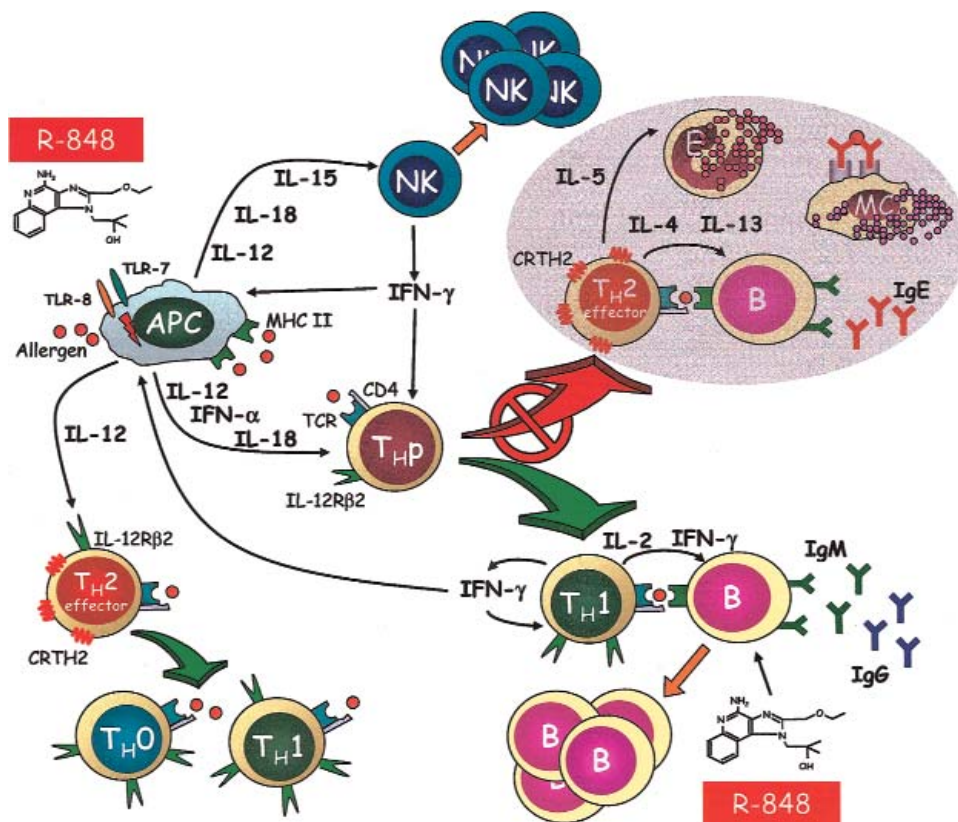


FIG 5. Possible mechanisms of action of R-848 for the modulation of allergen-specific T-cell responses. R-848 binding to TLR-7, and possibly to TLR-8, stimulates the professional antigen-presenting cells (APCs) to produce different T_H1 -inducing cytokines, such as IL-12, IFN- α , and IL-18. The presence of these cytokines in the microenvironment, where the allergen peptide is presented to T-helper cell precursors (T_{HP}), results in their polarization into the T_H1 , instead of the T_H2 , phenotype. IL-12, IL-15, and IL-18 secreted by APCs also promote the activation and proliferation of NK cells and the production of IFN- γ , which is responsible for the amplification of the T_H1 response. In addition, IL-12, produced by R-848-stimulated APCs, upregulates and stabilizes the expression of the $\beta 2$ chain of IL-12 receptor (*IL-12R β 2*) on the surface of the already established allergen-specific T_H2 cells (T_H2 effector), that can be identified and purified because of their expression of CRTH2. In the presence of IL-12, the T_H2 effector acquires the ability to produce IFN- γ in addition to IL-4, thus becoming a T_H0 cell, or can even lose the ability to produce IL-4, thus becoming a T_H1 cell. R-848 also enhances B-cell proliferation and stimulates the production of IgM and IgG through its direct interaction with B lymphocytes (B).

Some ODNs have recently been found to be able, at least in vitro, to induce a T_H1 switch of allergen-specific human T-cell responses.^{6,8} The bias of the specific immune response to a T_H1 -dominated cytokine pattern induced by synthetic phosphorothioate ODNs was due to their ability to stimulate the production of T_H1 -inducing cytokines, such as IL-12, interferons, and IL-18.^{6,8} However, the use of ODNs as adjuvants for successful specific immunotherapy of allergic disorders might not actually be suitable; this is because of not-fully-disclosed differences in the ODN motif(s) responsible for the T_H1 -switching effect in mice and human beings,^{6,13,14} as well as the harmful side effects of PS-ODNs observed in mice.¹⁵ Thus, the availability of novel adjuvants and safer immunomodulatory regimens for specific immunotherapy is a reason for hope.

Recently, Imiquimod, in the form of a 5% cream (Aldara), has been shown to display clinical efficacy against genital warts, genital herpes, and *Molluscum contagiosum*¹⁶ and has been proposed as a therapeutic agent in some dermatologic neoplasias.¹⁷ Moreover, its more powerful derivative Resiquimod (R-848) enhanced the clearance of *Mycobacterium bovis* BCG in infected mice,¹⁸ delayed the onset of recurrent genital herpes lesions (as a topical gel),¹⁹ and has been proposed as a novel treatment for *Leishmania major* infections in animals and human beings.²⁰ The effects of both Imiquimod and Resiquimod have been attributed to the functional but not phenotypic maturation of DCs,²¹ as well as to stimulation of the production of IFN- α , IL-12, IL-6, TNF- α , IL-8, monocyte chemotactic protein (MCP) 1, and macrophage inflammatory protein (MIP) 1 α .²²

R-848—upregulated macrophage genes have been recently identified by microarray analysis.²³ In a recent study, imidazoquinolines were ineffective in both MyD88- and TLR7-deficient mice,²⁴ which suggests that these compounds trigger the innate immunity through use of a highly conserved molecular and transcriptional pathway shared by bacterial products (LPS, lipopeptides, peptidoglycan, etc) and ODNs.²⁴⁻²⁶ In human beings, both TLR7 and TLR8 seem to be potential targets for interaction of R-848 with DCs.²⁷

The results of this study provide the first evidence that R-848 is able, at least in vitro, to shift the differentiation of allergen-specific human CD4⁺ T cells from a prevalent T_H2-like to a prevalent T_H1/T_H0-like profile of cytokine production. Inasmuch as adverse reactions to antibiotics are characterized by the expansion of highly polarized hapten-specific T_H2 cells in peripheral blood exerting a pathogenetic role¹² and provide an optimal in vitro model by which to assess the activity of different immunomodulatory agents as adjuvants, we have also investigated the ability of R-848 to shift the cytokine production profile of hapten-specific T cells, which show an even more polarized T_H2 phenotype than allergen-specific T cells. Independently of the stimulatory antigen used (ampicillin or Der p 1), our results clearly demonstrate that R-848 is a T_H1-skewing compound exhibiting an even more powerful effect than IL-12. In addition, and more importantly, R-848 enabled, at least in vitro, purified effector human T_H2 cells (CRTH2⁺) to produce IFN- γ . This finding suggests that Resiquimod did not actually abrogate the growth of specific T cells but did promote the development of allergen- and hapten-specific T cells into T_H1 effectors.

The T_H1-inducing effect of R-848 on hapten- and allergen-specific T cells appeared to be related to its powerful stimulatory activity on cells of the innate immunity (macrophages and NK cells), whereas no direct effect on T cells was observed (data not shown). In agreement with previously reported data,¹⁰ R-848 induced the activation of CD14⁺ cells, as indicated by the production of high levels of the T_H1-inducing cytokines IL-12, IL-18, and IFN- α , as well as other cytokines, including TNF- α , IL-10, and IL-15, some of these also representing important growth factors for NK cells. Moreover, R-848 induced a significant expansion of CD3-CD16⁺CD56⁺ (NK cells) able to produce high levels of IFN- γ in cultures from both hapten- and allergen-specific T-cell lines. Thus, the effects of R-848 on cells of the natural immunity, such as macrophages, NK cells, and DCs, likely account for its powerful T_H1-inducing capacity. A schema of the possible mechanisms of action of R-848 is depicted in Fig 5. It is of note that R-848 was even more powerful than Poly I:C and ODNs in inducing the switch of allergen-specific human CD4⁺ T cells into T_H0 or even T_H1 cells, as well as in stimulating the activity of both DCs and NK cells (data not shown). Although some T_H1-inducers and/or TLR ligands, such as ODNs, have been described as stimulating autoantibody production in experimental animals,²⁸ similar adverse effects

have not yet been reported by the use of imidazoquinolines. On the other side, in view of the powerful activities on the immune system by topical application of R-848,²⁹ the possibility of its use as adjuvant for local airway specific immunotherapy cannot be excluded. Thus, the injection or local administration of allergen mixed to, or modified by, the conjugation with an immune response modifier such as R-848, also in view of its apparently tolerable side effects,²⁹ might provide a valuable new immunotherapeutic strategy for the treatment of human allergic disorders.

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