

Ovalbumin (OVA) and *Mycobacterium tuberculosis* Bacilli Cooperatively Polarize Anti-OVA T-helper (Th) Cells toward a Th1-Dominant Phenotype and Ameliorate Murine Tracheal Eosinophilia

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A recent increase in allergic disorders has coincided with a decrease in infections, including tuberculosis. Although an inverse association between tuberculin responses and atopic disorders was reported, it was not known how T-helper (Th)1-biased immune responses to *Mycobacterium tuberculosis* influenced Th2-dominant responses to allergens. We examined whether *M. tuberculosis* could modulate ovalbumin (OVA)-induced eosinophilic inflammation in the murine trachea in a manner that transcended the barrier of antigen specificity. We found that CD4⁺ T cells primed with OVA in complete Freund's adjuvant (CFA) inhibited OVA-induced tracheal eosinophilia through interferon (IFN)- γ secretion. Immunization with an irrelevant antigen in CFA or with OVA in incomplete Freund's adjuvant failed to induce suppressor cells. *In vitro* experiments confirmed that both *M. tuberculosis* and OVA (as opposed to either one alone) were necessary to evoke polarized development toward a Th1-like phenotype through interleukin-12 secretion. These results indicate that exposure to an allergen along with *M. tuberculosis* switches development of allergen-specific T cells toward a Th1 phenotype, which, in turn, downregulates allergic manifestations in an antigen-specific manner. The possible implications of these results are discussed in the context of the causal relationship between a decrease in tuberculosis and an increase in allergic disorders. **Sano, K., K. Haneda, G. Tamura, and K. Shirato. 1999. Ovalbumin (OVA) and *Mycobacterium tuberculosis* bacilli cooperatively polarize anti-OVA T-helper (Th) cells toward a Th1-dominant phenotype and ameliorate murine tracheal eosinophilia. *Am. J. Respir. Cell Mol. Biol.* 20:1260–1267.**

Allergic disorders, such as bronchial asthma, atopic dermatitis, and allergic rhinitis, have been increasing rapidly in developed countries (1–4). Coincidentally in these areas, infections with *Mycobacterium tuberculosis* have been declining (5). A recent report has provided convincing evidence for an inverse association between tuberculin responses and atopic disorders (6).

T lymphocytes have been implicated in the pathogenesis of allergic disease (7). Moreover, it has been suggested that cytokines elaborated from type-2 CD4⁺ T-helper (Th2) cells, including interleukin (IL)-4 and IL-5, induce

immunoglobulin (Ig)E synthesis as well as activation and recruitment of eosinophils (8). Type-1 CD4⁺ T helper (Th1) cells are required for protection from infection by microorganisms, particularly those that grow in the cytoplasm of infected cells (9). Interferon (IFN)- γ secreted from Th1 cells activates macrophages, leading to the inhibition and killing of phagocytosed pathogenic microorganisms, which otherwise survive by evading digestive elimination in unactivated macrophages (10–12). It is interesting that these two types of CD4⁺ T cells act in a reciprocally inhibitory manner through the secretion of type-specific cytokines (8). The concurrence of an increase in allergic diseases mediated by Th2 cells and a decrease in infection with *M. tuberculosis* that activates Th1 cells, therefore appears to be more than a mere coincidence. The notion of a causal relation between these two trends is, however, challenged by the antigen-specific nature of the immune system; allergen-specific Th2 cells and *M. tuberculosis*-specific Th1 cells are not expected to mutually cross-regulate each other.

We examined the modulation by *M. tuberculosis* of eosinophilic inflammation evoked by a soluble allergen. *M. tuberculosis* switched the development of ovalbumin (OVA)-specific CD4⁺ T cells from a Th2 to a Th1 phenotype and inhibited airway eosinophilic responses. Essential to this skewing effect was the concurrent presence of

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Abbreviations: aluminum hydroxide, alum; antigen-presenting cell, APC; bacillus Calmette-Guerin, BCG; complete Freund's adjuvant, CFA; hen egg lysozyme, HEL; incomplete Freund's adjuvant, IFA; interferon, IFN; immunoglobulin, Ig; interleukin, IL; lymph node, LN; monoclonal antibody, mAb; ovalbumin, OVA; phosphate-buffered saline, PBS; T-cell receptor, TCR; transgenic, tg; T-helper, Th.

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M. tuberculosis with the allergen at the time of priming. In this article we report our findings and consider their possible implications in the context of the notion that a decrease in tuberculosis might be a causal factor in an increase in allergic disorders.

Materials and Methods

Animals and Immunization

BALB/c mice and BALB/c mice transgenic (tg) for T-cell receptor (TCR) specific for OVA (13) were bred in our animal facility and were used at 5 to 10 wk of age. For the induction of airway eosinophilic inflammation, BALB/c mice were immunized intraperitoneally with 10 μ g of OVA (grade V; Sigma Chemical Co., St. Louis, MO) precipitated with 4 mg of aluminum hydroxide (alum) in 200 μ l of phosphate-buffered saline (PBS) three times weekly. For one group of mice, the third dose consisted of 10 μ g of OVA emulsified in complete Freund's adjuvant (CFA) containing H37Ra *M. tuberculosis* (Difco Laboratories, Detroit, MI) rather than OVA/alum. For adoptive cell transfer and *in vitro* culture experiments, BALB/c mice received a footpad injection of 100 μ g of OVA emulsified in CFA or incomplete Freund's adjuvant (IFA; Difco) or of hen egg lysozyme (HEL; Wako Chemical Industries Ltd., Osaka, Japan) or PBS in CFA.

Antigen-Induced Eosinophilia in the Trachea

We have detailed our methods previously (14). In brief, 1 wk after the last intraperitoneal dose of OVA/alum, sensitized mice were lightly anesthetized with pentobarbital (Abbott Laboratories, North Chicago, IL), and 10 μ g of OVA in 10 μ l of PBS was instilled directly into the surgically exposed trachea. After 2 d, the excised trachea was fixed in 10% formalin and frozen in OCT compound (Miles Laboratories, Naperville, IL). Cryosections (7 μ m thick) of the frozen tissue were stained with Diff-Quik (International Reagents Corp., Kobe, Japan). The numbers of eosinophils infiltrating the submucosal tissue of the trachea were determined by light microscopy. The perimeter of the basement membrane of the trachea was measured with an MCID image analyzer (Imaging Research Inc., St. Catharines, ON, Canada). For every trachea, six to eight sections were used for counting, each of which was separated from the adjacent sections by more than 70 μ m. Results were converted to the number of eosinophils per 1-mm basement membrane length. Data were expressed as means $\pm$ standard error of the mean (SEM) for four to seven mice in each group. Each experiment was repeated at least twice. Student's *t* test was employed in the analysis of results.

Adoptive Transfer of Antigen-Primed Lymph Node Cells

Seven days after footpad immunization, 3×10^6 popliteal lymph node (LN) cells were injected intravenously into OVA/alum-sensitized BALB/c mice, either with or without fractionation. Between 1 and 2 h after cell transfer, the mice received an intratracheal dose of OVA as described below.

Fractionation of T Cells

Spleen or LN cells were preincubated with PBS, anti-CD4 (Gk1.5) (15), or anti-CD8 (3.155) (16) monoclonal antibodies (mAbs) for 1 h at 4°C. They were allowed to react with antimouse Ig (Caltag, South San Francisco, CA) immobilized on plastic plates (Falcon, Lincoln Park, NJ) for 1 h at 4°C, and nonadherent cells were recovered as T cells, CD8⁺ T cells, and CD4⁺ T cells, respectively. Both mAbs were used at a 1:1,000 dilution of the ascites form. Efficiency of depletion was determined by flow cytometry to be greater than 95%.

In Vivo Neutralization with mAbs

For *in vivo* treatment with mAbs, 30 μ l of the ascites form of neutralizing antibody to IFN- γ (R4-6A2; rat IgG₁) (17) or an isotype-matched control antibody that recognizes CD25 (PC61-5-3) (18) was administered intraperitoneally to OVA/alum-sensitized BALB/c mice at the time of cell transfer. These two hybridoma cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD), and ascites were raised in BALB/c nu/nu mice.

In Vitro Stimulation of BALB/c LN CD4⁺ T Cells

LN cells from BALB/c mice primed with OVA or HEL emulsified in CFA or IFA were enriched for CD4⁺ T cells as described above. Spleen cells from unimmunized BALB/c mice were treated with mitomycin C (50 μ g/ml; Wako) for 30 min and then used as antigen-presenting cells (APCs). After coculture of 10^5 CD4⁺ T cells and 2.5×10^5 APCs with 1 mg of OVA/ml for 2 d in 96-well plates, culture supernatants were assayed for IFN- γ and IL-4.

In Vitro Stimulation of Anti-OVA TCR tg T Cells with *M. tuberculosis*

Spleen cells (2.5×10^6) from unimmunized anti-OVA TCR tg mice were cultured with OVA (100 μ g/ml) in the presence or absence of heat-killed *M. tuberculosis* (1 μ g/ml; Aoyama B strain, kindly provided by Taeko Yokochi-Fukuda, University of Tokyo, Tokyo, Japan) in 12-well plates for 3 d. Anti-IL-12 mAb (Genzyme Co., Cambridge, MA), isotype-matched control mAb (rat IgG_{2a}; PharMingen, San Diego, CA), and recombinant murine IL-12 (Genzyme) were added at graded concentrations at the initiation of culture. The cells were cultured in fresh medium for another 3 d. Viable lymphocytes were recovered from the interface by Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) density-gradient centrifugation and were enriched for CD4⁺ T cells as described above. Next, 10^4 CD4⁺ tg T cells were cultured with 2×10^5 APCs in the presence or absence of OVA (1 mg/ml) in 96-well plates. Anti-CD4 or anti-CD8 mAb was added to cultures at a 1:3,000 dilution of ascites form. After 2 d, culture supernatants were assayed for IFN- γ and IL-4.

Assays for Cytokines

The concentrations of IFN- γ and IL-4 in culture supernatants were determined using enzyme-linked immunosorbent assay (ELISA) with paired mAbs to IFN- γ and IL-4 (PharMingen) according to the manufacturer's recommendations. Tetramethylbenzidine reagent (Kierkegaard &

Perry Laboratories, Gaithersburg, MD) was used for color development, and optical densities determined at 450 nm were converted to concentrations (nanograms per milliliter) according to the standard curve obtained with titrated concentrations of mouse IFN- γ (PharMingen) and IL-4 (Genzyme). Student's *t* test was employed in the analysis of results.

Results

Low Efficacy of CFA as an Adjuvant for Antigen-Induced Airway Eosinophilia

We first compared the efficacy of CFA and alum as adjuvants for the induction of antigen-induced tracheal eosinophilia. Tracheal eosinophilia was induced by intratracheal instillation of OVA into BALB/c mice sensitized with OVA (Figure 1). Mice immunized with three doses of OVA/alum developed 13.3 ± 2.3 eosinophils (mean $\pm$ SEM) in the trachea upon intratracheal instillation of OVA. In mice immunized with two doses of OVA/alum and one dose of OVA in *M. tuberculosis*-containing CFA, fewer eosinophils (3.8 ± 1.2) infiltrated the trachea. Thus, CFA was a less effective adjuvant than alum for the induction of eosinophilic inflammation.

Antigen-Specific and *M. tuberculosis*-Dependent Induction of Suppressor Cells

To examine whether immunization with OVA in CFA induced eosinophilia-inhibiting cells, we adoptively transferred regional LN cells draining OVA/CFA-injected footpads (Figure 2). Eosinophilic responses in the tracheal submucosa were severely impaired by adoptive transfer of OVA/CFA-primed LN cells (Figure 2, bar 2), indicating the induction of suppressor cells. *M. tuberculosis* in CFA was essential for the induction of the suppressor cells; LN cells primed with OVA in IFA, which differed from CFA

only in its lack of *M. tuberculosis*, failed to suppress eosinophilia (Figure 2, bar 3). The suppression was antigen-specific, because LN cells primed with PBS/CFA (data not shown) or with an irrelevant antigen HEL in CFA (Figure 2, bar 4) failed to suppress OVA-induced eosinophilic responses. Higher numbers of LN cells (10^7) from OVA/IFA- or HEL/CFA-primed BALB/c mice exerted no effect on eosinophilia (data not shown). Thus, immunization with a soluble antigen together with CFA induced suppressor cells whose inhibitory specificity was skewed toward allergic responses evoked by the same antigen as used for immunization.

CD4⁺ T Cells as the Active Suppressor Cells

We examined the phenotype of CFA-induced suppressor cells. The control response was inhibited by adoptive transfer of OVA/CFA-primed LN cells enriched for T cells (Figure 3, bar 2). A similar level of suppression was observed upon the transfer of a CD4⁺ T cell-enriched fraction (Figure 3, bar 3), whereas a T-cell population depleted of CD4⁺ cells failed to suppress eosinophilic inflammation (Figure 3, bar 4). These results indicate that the active suppressor cells were CD4⁺ T cells.

Neutralization of OVA/CFA-Induced Suppressor Cells by Anti-IFN- γ mAb

To examine whether IFN- γ was involved in the suppression of eosinophilia, we injected neutralizing anti-IFN- γ mAb intraperitoneally into OVA/alum-sensitized mice at the time of adoptive transfer of OVA/CFA-primed LN cells. The inhibition of OVA-induced eosinophilia was abrogated by neutralizing anti-IFN- γ mAb (Figure 4, bar 3), but not by an isotype-matched control antibody specific for CD25 (Figure 4, bar 4). Thus, IFN- γ elaborated from CFA/OVA-primed cells was the essential cytokine involved in the downregulation of eosinophilia.

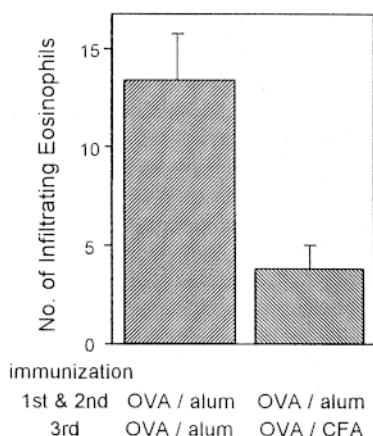


Figure 1. Low efficacy of CFA as an adjuvant for antigen-induced airway eosinophilia. BALB/c mice immunized with three doses of OVA/alum developed allergic airway eosinophilia upon intratracheal administration of OVA. The extent of eosinophilia was reduced when CFA was used in place of alum as the adjuvant for the third dose ($P < 0.02$). Experiments were repeated twice.

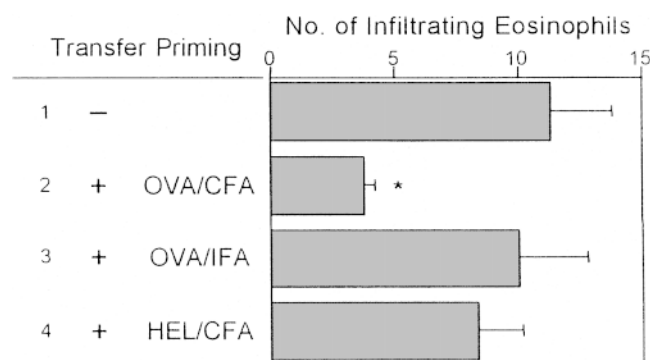


Figure 2. Antigen-specific and *M. tuberculosis*-dependent induction of suppressor cells. BALB/c mice sensitized with three doses of OVA/alum received an intravenous adoptive transfer of 3×10^6 LN cells from BALB/c mice primed with OVA/CFA, OVA/IFA, or HEL/CFA before intratracheal instillation of OVA. Note that *M. tuberculosis* present in CFA was essential for the induction of suppressor cells. The suppressor cells inhibited eosinophil responses in an antigen-specific manner. Experiments were repeated three times with similar results (* $P < 0.02$).

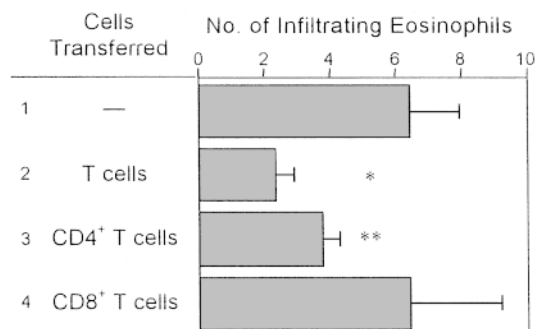


Figure 3. CD4⁺ T cells as the active suppressor cells in OVA-induced eosinophilia in the airway. OVA/CFA-primed LN cells (10⁷) enriched for T cells, CD4⁺ T cells, or CD8⁺ T cells were transferred intravenously to OVA/alum-sensitized BALB/c mice, which were then challenged intratracheally with OVA. Transferred LN cells failed to suppress the allergic airway eosinophilia when depleted of CD4⁺ T cells but not when depleted of CD8⁺ T cells. The data shown are representative of two separate experiments (**P* < 0.005; ***P* < 0.05).

M. tuberculosis-Induced Development of IFN- γ -secreting CD4⁺ T Cells

To substantiate the above findings, we examined IFN- γ and IL-4 production from LN CD4⁺ T cells of the BALB/c mice immunized with OVA in CFA or IFA (Figure 5). Cells produced low levels of cytokines when stimulated with low concentrations of OVA (< 1 to 10 μ g/ml). Cells from OVA/CFA-immunized mice produced IFN- γ in preference to IL-4 in response to higher doses of OVA (100 to 1,000 μ g/ml). Conversely, OVA/IFA-immunized cells produced high levels of IL-4 in comparison with IFN- γ . Thus, the presence of *M. tuberculosis* at the time of immunization with OVA caused a skewing toward predominantly IFN- γ -producing cells. Reverse transcriptase-polymerase chain reaction also demonstrated that, compared with OVA/IFA, OVA/CFA enhanced expression of IFN- γ and decreased expression of IL-4 in LN cells (data not shown). The LN cells primed with HEL/CFA produced lit-

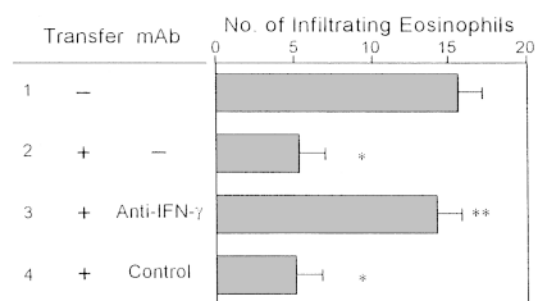


Figure 4. Neutralization of OVA/CFA-induced suppressor cells by anti-IFN- γ mAb. OVA/alum-sensitized BALB/c mice received an intraperitoneal injection of anti-IFN- γ mAb or an isotype-matched control mAb specific for CD25 at the time of intravenous transfer of OVA/CFA-induced suppressor cells. Suppressive activity of the transferred cells was neutralized by anti-IFN- γ mAb but not by the control mAb. Experiments were repeated twice (**P* < 0.002; ***P* < 0.002 compared with bar 2).

tle IFN- γ or IL-4 upon OVA stimulation; thus *M. tuberculosis* alone did not promote the development of IFN- γ -secreting CD4⁺ T cells.

Skewing the Development of CD4⁺ T Cells toward IFN- γ -Secreting Cells by OVA and *M. tuberculosis*

To verify the essential requirement for OVA and *M. tuberculosis* in the skewing of CD4⁺ T-cell development toward OVA-specific Th1 cells, we examined the differentiation of CD4⁺ T cells precultured in the presence of OVA and/or *M. tuberculosis* (Figure 6A). When CD4⁺ T cells from unimmunized anti-OVA TCR tg mice were precultured with OVA in the absence of *M. tuberculosis*, they exhibited Th2-dominant responses: high-level IL-4 and low-level IFN- γ production (Figure 6A, middle bar). In sharp contrast, the same tg cells stimulated in the presence of both OVA and *M. tuberculosis* showed predominantly Th1 differentiation, with high-level IFN- γ and low-level IL-4 production (Figure 6A, bottom bar). In the absence of OVA in restimulation cultures, levels of IFN- γ or IL-4 were < 0.2 ng/ml (data not shown). These results indicate that *M. tuberculosis* induces the biased development of CD4⁺ T cells toward a Th1 phenotype which otherwise defaulted to a Th2 response. CD4⁺ tg T cells precultured with *M. tuberculosis* but without OVA did not produce substantial levels of IFN- γ and IL-4 (Figure 6A, top bar). This finding offers further support for the requirement of the concomitant presence of both OVA and *M. tuberculosis* for the skewing toward OVA-specific Th1 cells. Both IFN- γ production and IL-4 production were completely

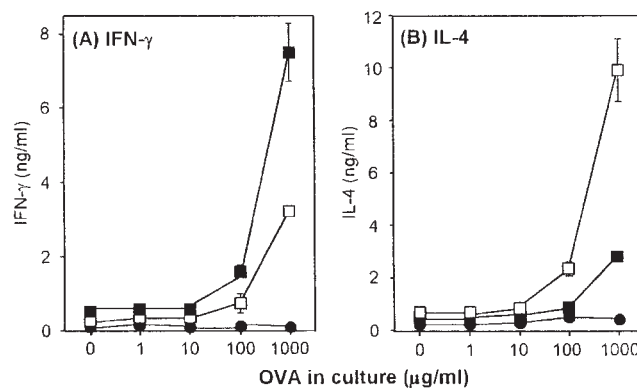
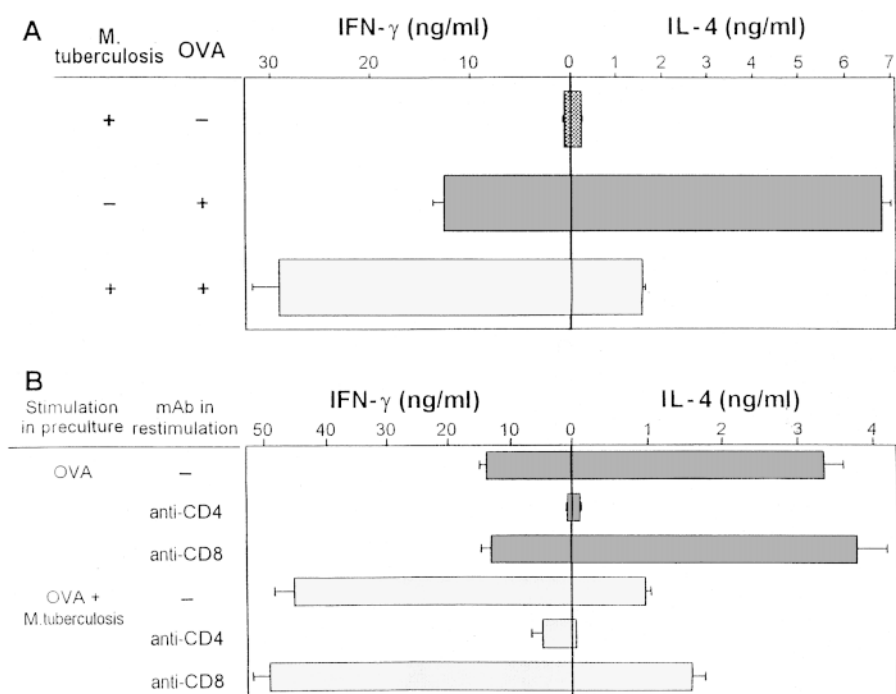


Figure 5. *M. tuberculosis*-induced development of IFN- γ -secreting CD4⁺ T cells. LN CD4⁺ T cells (10⁵) from BALB/c mice primed with OVA/CFA (filled squares), OVA/IFA (open squares), or HEL/CFA (filled circles) were cultured in the presence of graded concentrations of OVA and 2.5×10^5 mitomycin C-treated syngeneic spleen cells in 96-well plates. After 2 d, culture supernatants were assayed for IFN- γ (A) and IL-4 (B) by ELISA. CD4⁺ T cells primed with OVA in CFA (filled squares) and those primed with OVA in IFA (open squares) predominantly produced IFN- γ and IL-4, respectively, in response to *in vitro* antigen stimulation. Failure of HEL/CFA-primed CD4⁺ T cells (filled circles) to produce IFN- γ or IL-4 indicates that *M. tuberculosis*-induced skewing toward IFN- γ -secreting Th1 cells occurs in an antigen-specific manner. The data shown are representative of two separate experiments.

Figure 6. Skewing the development of CD4⁺ T cells toward IFN- γ -secreting cells by OVA and *M. tuberculosis*. (A) Spleen cells from unimmunized anti-OVA TCR tg mice were precultured with OVA (100 μ g/ml) and/or *M. tuberculosis* (1 μ g/ml) for 3 d and then in fresh medium for another 3 d. Recovered CD4⁺ T cells (10^4) were restimulated with or without OVA (1 mg/ml) in the presence of 2×10^5 APCs for 2 d in 96-well plates, and culture supernatants were assayed for IFN- γ and IL-4. Levels of both cytokines from any restimulation cultures without OVA were undetectable (data not shown). Stimulation of CD4⁺ T cells with OVA induced IL-4-secreting Th2-dominant responses, whereas concomitant stimulation with OVA and *M. tuberculosis* caused a switch to IFN- γ -secreting Th1-dominant responses. Stimulation with *M. tuberculosis* alone failed to cause polarization toward Th1 development; thus the coexistence of *M. tuberculosis* and OVA was essential for the induction of antigen-specific Th1 cells. (B) CD4⁺ tg T cells precultured with OVA or with OVA plus *M. tuberculosis* were restimulated with OVA. Anti-CD4 or anti-CD8 mAb was added at the initiation of the restimulation culture. The data shown are representative of three separate experiments.



inhibited by anti-CD4 mAb in restimulation cultures, whereas anti-CD8 mAb had no effect; these results confirmed that CD4⁺ but not CD8⁺ T cells produce these cytokines (Figure 6B).

IL-12-Mediated Switch toward Th1 Development in the Presence of *M. tuberculosis*

We examined whether IL-12 mediated a switch from Th2 to Th1 development. CD4⁺ tg T cells precultured in the presence of *M. tuberculosis* and OVA displayed a Th1-dominant phenotype (Figures 7A and 7B). When graded doses of anti-IL-12 mAb were included in precultures, the CD4⁺ T cells exhibited a Th2 phenotype upon restimulation with OVA. Isotype-matched control mAb had no effect. The effect of anti-IL-12 mAb on the bias toward Th2 development was reversed by excess amounts of IL-12 (Figures 7C and 7D): 1 ng of IL-12/ml neutralized the switching effect of anti-IL-12 mAb with reversion to Th1-dominant responses, which was almost comparable with control responses. These results indicate that IL-12 mediates a switch from a Th2 to a Th1 phenotype.

Discussion

This study was prompted by a recent increase in allergic disorders paralleled by a decrease in various infections, including tuberculosis. Recent reports have provided convincing evidence for an inverse association between tuberculin responses and atopic disorders and have suggested that exposure and response to *M. tuberculosis* inhibit atopic disorders (6). The issue of how Th1 cells induced by *M. tuberculosis* can regulate Th2 cells against distinct anti-

gens has not previously been addressed. We examined mechanisms by which *M. tuberculosis* might modulate OVA-induced eosinophilic inflammation in the murine trachea, transcending the barrier of antigen specificity.

In our experimental model, *M. tuberculosis* was indispensable in the induction of IFN- γ -producing anti-OVA Th1 cells. LN cells from mice immunized with OVA in CFA but not in IFA inhibited antigen-induced tracheal eosinophilia (Figure 2). The inhibitory activity of the transferred cells paralleled the production of IFN- γ from these cells, because LN cells from mice immunized with OVA/CFA but not OVA/IFA produced IFN- γ in preference to IL-4 (Figure 5). Anti-IFN- γ mAb reversed the inhibitory effect of the transferred cells on eosinophilic inflammation (Figure 4). Additional evidence came from an *in vitro* experiment in which IFN- γ -secreting Th1-like cells were found to be dominant in the presence but not the absence of *M. tuberculosis* bacilli (Figure 6). Thus, *M. tuberculosis* plays a causative role in a bias toward the development of a Th1-dominant phenotype among CD4⁺ T cells that otherwise default to a Th2-dominant phenotype.

Tuberculous infection per se favors the induction of a Th1 response to *M. tuberculosis* (19–22). Is *M. tuberculosis* sufficient for the induction of Th1-dominant responses to any antigens? Because antigen specificity is considered a basic characteristic of the immune system, a Th2 response to a certain antigen and a Th1 response to an irrelevant antigen are expected to occur independently. In fact, transfer of LN cells from OVA/CFA-immunized mice inhibited OVA-induced eosinophilic responses, whereas LN cells from PBS/CFA- or HEL/CFA-primed mice failed to do so (Figure 2). LN cells from mice immunized with

OVA/CFA but not HEL/CFA produced IFN- γ in response to OVA (Figure 5). CD4⁺ tg T cells cultured with *M. tuberculosis* alone did not manifest anti-OVA Th1 activities, whereas the concomitant presence of OVA and *M. tuberculosis* successfully induced OVA-specific Th1 cells (Figure 6). Thus, *M. tuberculosis* did not skew parallel and independent responses to irrelevant antigens toward a Th1-dominant phenotype, but instead selectively induced Th1 responses to a soluble antigen that was in close proximity to *M. tuberculosis*.

Our experiments reported in this paper revealed two possible mechanisms by which *M. tuberculosis* ameliorates Th2-mediated allergic responses. First, *M. tuberculosis* skewed the development of anti-OVA CD4⁺ T cells toward a Th1-dominant phenotype. OVA alone induced Th2-dominant responses *in vivo* and *in vitro* in the absence of *M. tuberculosis*, whereas the concomitant presence of *M. tuberculosis* skewed anti-OVA responses toward a

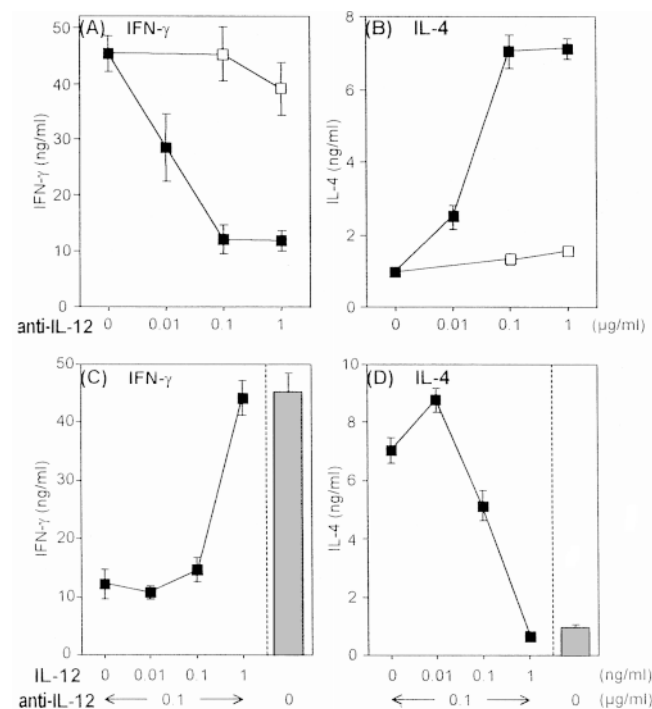


Figure 7. IL-12-mediated switch toward Th1 development in the presence of *M. tuberculosis*. (A, B) Spleen cells from unimmunized anti-OVA TCR tg mice were precultured with OVA (100 μ g/ml) and *M. tuberculosis* (1 μ g/ml) for 3 d, and then in fresh medium for another 3 d. Graded concentrations of anti-IL-12 mAb or isotype-matched control mAb were added at the initiation of cultures. Recovered CD4⁺ tg T cells (10^4) were cultured with 2×10^5 APCs and OVA (1 mg/ml) in 96-well plates for 2 d, and culture supernatants were assayed for IFN- γ (A) and IL-4 (B). (C, D) Graded concentrations of IL-12 were added to the precultures of tg spleen cells with OVA (100 μ g/ml), *M. tuberculosis* (1 μ g/ml), and anti-IL-12 mAb (0.1 μ g/ml). Bars represent cytokine production from CD4⁺ tg Th1 cells precultured with OVA (100 μ g/ml) and *M. tuberculosis* (1 μ g/ml). IL-12 neutralized the switching effect of anti-IL-12 mAb with a consequent reversion to a Th1-dominant response. Experiments were repeated twice with similar results.

Th1-dominant phenotype (Figures 2, 5, and 6), thereby leading to an increase in the ratio of Th1 cells to Th2 cells. Second, newly generated OVA-specific Th1 cells inhibited the function of preexisting Th2 cells mediating eosinophilic responses (Figures 2–4). Because the inhibition of Th2 cells by Th1 cells occurred in an antigen-specific manner (Figure 2), dysfunction of the immune response to other antigens, such as pathogenic microorganisms, could be avoided. IFN- γ delivered inhibitory signals from Th1 cells to Th2 cells (Figures 4 and 5) (23).

The skewing toward Th1-dominant anti-OVA responses caused by the presence of both *M. tuberculosis* and OVA was mediated by IL-12 (Figure 7). *In vivo* IL-12 treatment has been reported to abrogate antigen-induced pulmonary eosinophilia (24–26). However, signals other than IL-12 appear to be necessary for efficient *in vitro* Th1 development because *M. tuberculosis* in the absence of OVA failed to induce anti-OVA Th1 cells (Figure 6). We could not identify the cell sources secreting IL-12 in the present studies. IL-12 is known to be secreted from APCs such as dendritic cells and macrophages that phagocytose intracytoplasmic microorganisms (including *M. tuberculosis*) and to induce differentiation into Th1 cells (9, 27–30). In light of these findings, APCs that phagocytose, process, and present both *M. tuberculosis* and OVA are prime candidates for the cells responsible for IL-12 secretion. If *M. tuberculosis* and OVA were taken up by distinct APCs, IL-12 secreted from *M. tuberculosis*-laden APCs would not reach anti-OVA Th cells efficiently. One of the possible circumstances in which IL-12 would efficiently affect anti-OVA Th cells would be that in which OVA-specific Th cells recognized through TCR antigens on and were targeted to the IL-12-secreting APCs presenting *M. tuberculosis* and OVA. Phagocytosis and antigen presentation of both *M. tuberculosis* and OVA by the same or neighboring APC(s) are likely to occur when OVA is in temporal and spatial proximity to *M. tuberculosis*. Thus, the si-

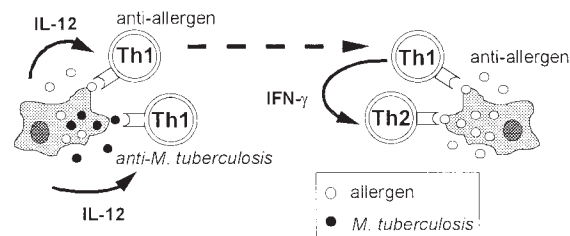


Figure 8. Hypothetical mechanisms for skewing of allergen-specific T cells toward a Th1 phenotype. Allergens and nearby *M. tuberculosis* cells tend to be engulfed, processed, and presented by the same or neighboring APC(s) to which both *M. tuberculosis*-specific and allergen-specific T cells are targeted. APCs engulfing *M. tuberculosis* elaborate IL-12, which facilitates differentiation into Th1 cells specific for allergens that otherwise default to a Th2 response. Essential to this effect of IL-12 is the proximity of allergen-specific T cells to APCs that phagocytose *M. tuberculosis* and secrete IL-12. Allergen-specific Th1 cells induced in this manner, in turn, inhibit preexisting Th2 cells with the same antigen specificity and thereby ameliorate Th2-mediated allergic manifestations. If *M. tuberculosis* were more prevalent and were repeatedly inhaled, the above scenario would be more likely.

multaneous presence of *M. tuberculosis* and OVA is an important factor in the facilitation of anti-OVA Th1 responses.

In view of the recent decrease in mycobacterial infections and the parallel increase in allergic disorders, the above findings could be interpreted as follows (Figure 8). Airway inhalation of allergens (e.g., house dust mites) favors the activation of Th2-dominant responses (31–34), whereas exposure to intracytoplasmic microorganisms (e.g., *M. tuberculosis*) tends to activate Th1-dominant responses (9, 19–22). If *M. tuberculosis* were more prevalent and were repeatedly inhaled, exposures to possible allergens and *M. tuberculosis* would be more likely to occur simultaneously. Allergens and nearby *M. tuberculosis* cells would tend to be engulfed, processed, and presented by the same or nearby APC(s) to which both *M. tuberculosis*-specific and allergen-specific T cells were targeted. APCs engulfing *M. tuberculosis* would elaborate IL-12, which would facilitate differentiation into Th1 cells specific for allergens as opposed to the usual default to a Th2 response (31–34). Essential to this effect of IL-12 would be the proximity of allergen-specific T cells to APCs that phagocytosed *M. tuberculosis* and secreted IL-12. Allergen-specific Th1 cells induced in this manner would in turn inhibit preexisting Th2 cells with the same antigen specificity and thereby ameliorate Th2-mediated allergic manifestations.

The skewing of allergen-specific Th cells toward Th1 responses required concomitant exposure to allergens and *M. tuberculosis*. More important, simultaneous inhalation of allergens and *M. tuberculosis* was not always necessary for regulation of Th2-mediated allergies. Even during Th2-mediated allergic responses, newly induced Th1 cells inhibited the preexisting Th2 cells. When *M. tuberculosis* was more prevalent, it was more likely that immune systems would encounter allergens and *M. tuberculosis* simultaneously and that the induction of allergen-specific Th1 cells would be followed by inhibition of Th2 cells. Thus, it is tempting to speculate that when *M. tuberculosis* was more prevalent, allergic reactions were more likely to be inhibited.

Recent clinical studies have dealt with the relation between tuberculous infection, bacillus Calmette-Guerin (BCG), and atopy. Shirakawa and colleagues reported an inverse association between tuberculin responses and atopic disorders, and concluded that exposure and response to *M. tuberculosis* might inhibit these disorders (6). Alm and coworkers investigated whether BCG vaccination against tuberculosis influenced the development of atopy and found that early BCG vaccination in children with atopic heredity did not affect the development of atopic diseases (35). Their study is in accord with our notion that *M. tuberculosis* given separately from allergen fails to cause a bias toward Th1 responses. More recently, Erb and associates demonstrated that BCG infection of the lung strongly inhibits the development of airway eosinophilia (36). They showed that the BCG infection-induced inhibition of airway eosinophilia was strongly inhibited in IFN- γ receptor-deficient mice. These results are in agreement with ours with respect to the inhibitory role of IFN- γ in *M. tuberculosis*-induced regulation of eosinophilia. They also showed that intranasal infection was superior to intraperitoneal or

subcutaneous infection in its ability to reduce airway eosinophilia, and they suggested that BCG should be administered directly into the lung. We reason that the efficient inhibition of lung eosinophilia was due to the simultaneous presence of BCG and OVA in the lung and not necessarily to the direct administration of BCG into the lung. In view of our observations, we speculate that subcutaneous immunization with BCG and allergens would efficiently induce allergen-specific Th1 cells, which in turn would inhibit allergic responses.

In the present study we inoculated *M. tuberculosis* via an intraperitoneal or subcutaneous approach. This approach is likely to be relevant to the ordinal infectious route of pulmonary tuberculosis with respect to how antigens are processed and presented to T cells by APC. Holt reported that the most potent APC in the lung was dendritic cells, which acquired the fully potent ability to stimulate T cells after migration to and activation in regional lymphoid tissues (37), and these features were shared with dendritic cells in tissues outside the respiratory tract (38). Thus, regardless of the route of *M. tuberculosis* entry, the ensuing processing and presentation to T cells of the bacilli would proceed in a similar manner.

CFA containing killed *M. tuberculosis* is commonly used for activation of CD4⁺ cells and production of antibodies, whereas *M. tuberculosis* is an intracellular pathogen and roles for CD8⁺ T cells in controlling tuberculous infection are also well demonstrated in mice infected with live bacilli (39–42). Because CD8⁺ T cells secrete Th1-like cytokines such as IFN- γ , these cells could be a potential regulator of Th2 cells. Failure of CD8⁺ T cells to exert inhibitory effects on tracheal eosinophilia (Figure 3) might reflect preferential activation of CD4⁺ T cells by killed *M. tuberculosis* inoculated as a form of CFA. The role of CD8⁺ T cells as a regulator of allergic responses needs further evaluation.

In summary, we examined the effect of *M. tuberculosis* on *in vivo* and *in vitro* Th2-mediated responses, and we discussed the mechanisms by which *M. tuberculosis* may ameliorate allergic diseases. We hope that these findings will be applicable to the development of antigen-specific vaccines useful in therapy for allergic disorders such as bronchial asthma.

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