

Vaccination with Heat-Killed *Listeria* as Adjuvant Reverses Established Allergen-Induced Airway Hyperreactivity and Inflammation: Role of CD8⁺ T Cells and IL-18¹

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Asthma is a respiratory disorder characterized by airway hyperreactivity (AHR) and inflammation and is associated with high serum IgE and overproduction of IL-4, IL-5, and IL-13 by allergen-specific Th2 cells. Our previous studies demonstrated that heat-killed *Listeria monocytogenes* (HKL) as an adjuvant in immunotherapy successfully reversed ongoing Ag-specific Th2-dominated responses toward Th1-dominated responses, but it was unclear if such immune modulation could reverse ongoing, established disease in target organs such as the lung. In this paper we show that a single dose of Ag plus HKL as adjuvant significantly reduced AHR in a murine model for asthma and reversed established AHR when given late after allergen sensitization. HKL as adjuvant also dramatically inhibited airway inflammation, eosinophilia, and mucus production, significantly reduced Ag-specific IgE and IL-4 production, and dramatically increased Ag-specific IFN- γ synthesis. The inhibitory effect of HKL on AHR depended on the presence of IL-12 and CD8⁺ T cells and was associated with an increase of IL-18 mRNA expression. Thus, our results demonstrate that HKL as an adjuvant for immunotherapy mediates immune deviation from a pathological Th2-dominated response toward a protective immune response in peripheral lymphoid tissues and in the lungs and may be clinically effective in the treatment of patients with established asthma and allergic disease. *The Journal of Immunology*, 2000, 164: 223–230.

Asthma, which affects 15 million individuals in the United States, is an atopic disorder characterized by airway hyperresponsiveness to a variety of specific and nonspecific stimuli, by chronic pulmonary inflammation with eosinophilia, and excessive mucus production. The pathology in asthma occurs as a consequence of increased production of IL-4, IL-5, and IL-13 by allergen-specific CD4⁺ Th2 cells (1, 2). In addition, the risk of developing asthma is directly related to elevated serum IgE (3, 4) and to the acquisition of immediate hypersensitivity to environmental allergens (5).

Although our understanding of the inflammatory process in asthma has improved remarkably over the past decade, the prevalence of asthma in industrialized countries has increased by almost 80% since 1980 (6). The specific causes for this increase in prevalence are not clear (7), but the rise in prevalence may be due in part to the absence of effective therapies that reverse the progression of, or cure, this disease. Currently available therapies, such as inhaled corticosteroids, anti-leukotrienes, or β_2 -agonists, focus rather on symptom relief, reduction or neutralization of effector molecules, and inflammatory mediators. This approach, al-

though effective for acute disease and for relieving symptoms, however, has limited long-term salutary effects because the environmental factors that cause and precipitate asthma are not eliminated, and patients redevelop symptoms of asthma when these medications are discontinued.

An alternative, more effective and long-lasting therapeutic approach for asthma therefore focuses on the development of vaccine strategies that alter the underlying immune response and convert or deviate detrimental allergic responses toward protective immune responses, thereby modifying the course of the disease. This immunotherapeutic strategy, however, which might cure asthma and reduce its prevalence, is feasible only if potent therapies are developed that reverse ongoing airway hyperreactivity (AHR)⁵ and reverse the ongoing allergic inflammatory process, which plays a critical role in the pathogenesis of asthma (5). Conventional allergen immunotherapy, while capable of reducing allergen-specific IL-4 production (8), requires multiple injections over several years time and is associated with frequent failure (9). Ag-specific methods of immunotherapy described up to now, for example using immunization with plasmids containing the cDNA for allergens (10–12), while effective in preventing the development of Th2-dominated immune responses, have not been shown to reverse ongoing AHR.

We recently demonstrated that employment of heat-killed *Listeria monocytogenes* (HKL) as an immunotherapeutic adjuvant during Ag immunotherapy successfully reversed ongoing Th2-dominated responses and induced an Ag-specific Th1-dominated response in mice (13). HKL, a potent stimulator of the innate immune system and a strong inducer of IL-12 and IFN- γ production, greatly reduced ongoing Ag-specific IL-4 production in lymph node (LN) cells and reduced Ag-specific IgE production. We now

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⁵ Abbreviations used in this paper: AHR, airway hyperreactivity; HKL, heat-killed *Listeria monocytogenes*; LN, lymph node; BAL, bronchoalveolar lavage.

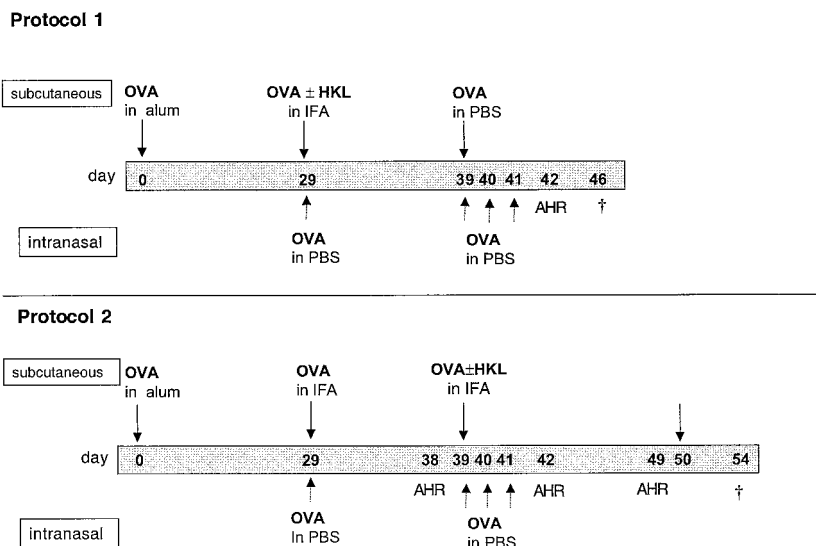


FIGURE 1. Immunization protocols. Protocol 1: BALB/c mice were primed in the footpads with OVA (50 μ g/mouse) adsorbed to 2 mg of alum ($\text{Al}[\text{OH}]_3$). Four weeks later (day 29) mice were injected in the footpads with 200 μ g OVA in IFA, or with 200 μ g OVA plus 10^8 HKL in IFA. Mice also received 50 μ g OVA in 50 μ l NaCl 0.9% intranasally on day 29. After an additional 10 days (day 39), all mice received a 100 μ g booster immunization of OVA in PBS in the footpads and were challenged with 50 μ g OVA in PBS intranasally on the same day and the 2 following days (day 40 and 41). One day after the last intranasal challenge with OVA, AHR was measured from conscious mice after inhalation of increasing concentrations of methacholine in a whole body (day 42). Mice were killed on day 46. Protocol 2: BALB/c mice were primed in the footpads with OVA (50 μ g/mouse) adsorbed to 2 mg of alum ($\text{Al}[\text{OH}]_3$). Four weeks later (day 29) mice were injected in the footpads with 200 μ g OVA in IFA. Mice received HKL (10^8 per mouse) mixed with OVA in IFA with the second boost (day 39) instead of the first boost. All mice were challenged with 50 μ g OVA in PBS intranasally on days 39, 40, and 41. AHR was measured 1 day before, and 3 days and 10 days after the injection of HKL. Mice received a final s.c. boost with OVA on day 50, and were killed on day 54.

show in a murine model of asthma that HKL as an adjuvant given once with Ag prevented the development of AHR and airway inflammation in OVA-immunized BALB/c mice and significantly reduced airway eosinophilia and mucus production. Moreover, when given late after allergen-sensitization, a single dose of HKL with Ag reversed established AHR and reduced airway inflammation. The inhibitory effect on AHR depended on the presence of IL-12 and on CD8^+ T cells, was associated with an increase of the IL-18 mRNA expression, and required close association between HKL and the Ag. Thus, our results demonstrate that HKL as an adjuvant very effectively promotes protective immune responses in the respiratory tract, and down-modulates ongoing Th2-dominated responses, indicating that HKL as an adjuvant for allergen immunotherapy may be clinically effective in the treatment of allergic asthma.

Materials and Methods

Animals

BALB/cByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The Stanford University Committee on Animal Welfare approved animal protocols used in this study.

Monoclonal Abs

mAbs were purified from ascites by ammonium sulfate precipitation and ion-exchange chromatography. We used the following hybridomas: R46A2 (anti-IFN- γ mAb), and 53.6.7 (anti- CD8^+) obtained from the American Type Culture Collection (Manassas, VA); XMG1.2 (anti-IFN- γ Ab), TRFK-4 and TRFK-5 (anti-IL-5 mAbs), generously provided by Dr. Tim Mosmann (University of Alberta, Edmonton, Canada); BVD4-1D11 and BVD6-24G2 (anti-IL-4 mAb), generously provided by M. Howard (DNAX Research Institute, Palo Alto, CA); and C17.8 (anti-IL12 mAb), generously provided by G. Trinchieri (Wistar Institute, Philadelphia, PA). Anti-38C13

Id mAb 4G10 (rat IgG2a) (14) was obtained from S. Levy (Stanford University, Stanford, CA) and was used as isotype control.

Immunizations

Protocol 1 (prevention of AHR). BALB/c mice were primed in the footpads with OVA (50 μ g/mouse) adsorbed to 2 mg of alum ($\text{Al}[\text{OH}]_3$) (Fig. 1). Four weeks later (day 29) mice were injected in the footpads with 200 μ g OVA in IFA or with 200 μ g OVA plus 10^8 HKL in IFA. Mice also received 50 μ g OVA in 50 μ l NaCl 0.9% intranasally on day 29. After an additional 10 days (day 39), all mice received a 100 μ g booster immunization of OVA in PBS in the footpads and were challenged with 50 μ g OVA in PBS intranasally on the same day and the 2 following days (days 40 and 41). One day after the last intranasal challenge with OVA, AHR was measured from conscious mice after inhalation of increasing concentrations of methacholine in a whole body plethysmograph (day 42). After an additional 4 days, mice were sacrificed with a lethal dose of phenobarbital (450 mg/kg), blood was taken, bronchoalveolar lavage (BAL) was performed, lungs were removed and fixed and lymphocytes were isolated from the draining LN for in vitro culture (day 46).

Protocol 2 (reversal of established AHR). In experiments designed to determine whether HKL as an adjuvant could reverse established AHR rather than inhibit the development of AHR, mice received HKL (10^8 per mouse) mixed with OVA in IFA with the second boost instead of the first boost (day 39 instead of day 29) (Fig. 1). AHR was measured 1 day before, and 3 days and 10 days after the injection of HKL. On day 50 mice received a final s.c. boost with OVA (50 μ g in PBS) and were sacrificed 4 days later for BAL and lung fixation.

To facilitate pulmonary aspiration during intranasal administration of Ag, mice were lightly anesthetized i.p. with 0.25 ml of ketamine (0.44 mg/ml)/xylazine (6.3 mg/ml) in normal saline. Seventy-five percent of the intranasally administered Ag can be subsequently detected in the lungs (15).

Treatment of mice with anti-cytokine Abs

BALB/c mice were injected i.p. with 1 mg of mAb C17.8 (for IL-12 depletion), mAb 53.6.7 (for CD8^+ depletion), or 4G10 (rat IgG2a control) in 0.5 ml PBS 1 day before, the day of, and 3 days following immunization with OVA in IFA or OVA in IFA with HKL, according to the immunization schedule of protocol 1.

Restimulation of LN cells in vitro

Draining LN were removed and depleted of resting B cells by adherence to goat anti-mouse Ig-coated plates. LN cells (5×10^5 cells/well) were restimulated in vitro with OVA in DMEM (Life Technologies, Grand Island, NY), which was supplemented as previously described (16), and contained 5×10^{-5} M 2-ME and 10% FCS (HyClone Laboratories, Logan, UT). Cells were cultured in 96-well microtiter plates in 150 μ l medium. Supernatants were harvested after 4 days for determination of IL-4 and IFN- γ levels. Cytokine content in each sample was measured in triplicate by ELISA.

Cytokine ELISA

ELISAs were performed as previously described (17). The Ab pairs used were as follows, listed by capture/biotinylated detection: IL-4, BVD4-1D11/BVD6-24G2; IFN- γ , R4-6A2/XMG1.2. Recombinant cytokine were used as standards, with curves generated in 1:2 dilutions from 500 to 39 pg/ml for IL-4, and from 20 to 0.156 ng/ml for IFN- γ .

Measurement of anti-OVA Ab isotypes

Mice were bled at the time of sacrifice and OVA-specific Ab was measured using a modified Ag-specific ELISA. For measurement of OVA specific IgG1 and IgG2a, plates were coated overnight with 5 μ g/ml OVA. After washing and blocking, serial diluted sera were added to the plates. Following overnight incubation, the plates were developed using HRP-conjugated goat anti-IgG subclass-specific Abs (Southern Biotechnology Associates, Birmingham, AL). These Abs are highly specific, are absorbed against competing subclasses, and have <1% cross-reactivity with competing subclasses (data not shown). After additional washing, OPD substrate was added, the plates developed, and the OD determined at 492 nm. Anti-OVA IgG1 mAb 6C1 and anti-OVA IgG2a mAb 3A11 (18) were used as standards for quantitation of each IgG subclass. Determination of OVA-specific IgE was performed by ELISA using rat anti-mouse IgE mAb EM95 (5.0 μ g/ml) to coat plates. After the samples were applied and incubated overnight, plates were washed and biotinylated OVA (10 μ g/ml) was added. Two hours later, plates were washed and HRP-conjugated streptavidin (Southern Biotechnology Associates) was added. Plates were developed with *o*-phenylenediamine (OPD) substrate and the OD determined at 492 nm. Sera from mice hyperimmunized with OVA in alum was used as standard for the OVA-specific IgE ELISA and was first standardized for IgE levels against an anti-OVA IgE mAb generously provided by E. Gelfand (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO) (19).

Preparation of HKL

HKL were prepared as described previously (13). A clinical isolate of *Listeria monocytogenes* (kindly provided by Dr. Lucy Tompkins and Barbara Allen, Stanford University) was grown in nutrient broth cultures (Difco, Detroit, MI) overnight at 37°C on a rotator. Cultures in log phase growth were harvested, centrifuged, and washed three times in PBS. The recovered bacteria were resuspended in PBS and incubated at 80°C for 1 h. After two additional washes in PBS, absence of viable colonies was confirmed by lack of growth on nutrient agar plates. Bacteria concentration was enumerated by comparing the absorbance of a serial dilution of HKL at 570 nm compared with a standard dilution of a known concentration of *Listeria* previously enumerated by counting the outgrowth of colonies from serial dilutions of bacteria plated on nutrient agar.

Preparation of splenic adherent cells

Spleen cells (5×10^6 /ml) were cultured in cDME medium in 24-well tissue culture plates for 2–3 h at 37°C. The nonadherent cells were removed by washing with warm DMEM + 10% FCS until visual inspection revealed a lack of lymphocytes (>98% of the cell population).

RNA isolation and IL-18 RT-PCR assay

Splenic adherent cells cultured with HKL (10^8 /ml) for 8, 16, or 24 h, and popliteal LN cells taken from mice 12 and 24 h after footpad injection of 10^8 HKL were analyzed for IL-18 mRNA expression. Cells were processed using Qiagen RNA isolation kits (Qiagen, Valencia, CA). Reverse transcription was performed with 200 ng of RNA, 2 μ g of oligo(dT) (Life Technologies) and 1 U of Superscript II Reverse transcriptase at 60°C for 60 min. Samples were stored at –20°C until further use.

Primers specific for β -actin and IL-18 (20) were synthesized at the Stanford PAN (Protein and Nucleic Acid) facility (Stanford, CA). cDNA (10 ng) was mixed with 10 $\times$ buffer, dNTPs (0.2 mM final), MgCl₂ (2.5 mM final), 5' and 3' primers, and Taq DNA polymerase (1 U/reaction; Life

Technologies) in a final volume of 25 μ l. PCR was performed in a DNA thermal cycler (MJ Research, Cambridge, MA) for 30 cycles, and products were visualized by electrophoresis. Data shown are representative of three experiments.

Measurement of airway responsiveness

Airway responsiveness was assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole body plethysmograph (model PLY 3211, Buxco Electronics, Troy, NY). Pulmonary airflow obstruction was measured by Penh using the following formula: $\text{Penh} = (\text{Te}/\text{RT} - 1) \times \text{PEF}/\text{PIF}$, where Penh = enhanced pause (dimensionless), Te = expiratory time, RT = relaxation time, PEF = peak expiratory flow (ml/s), and PIF = peak inspiratory flow (ml/s) (21). Enhanced pause (Penh), minute volume, tidal volume, and breathing frequency were obtained from chamber pressure, measured with a transducer (model TRD5100) connected to preamplifier modules (model MAX2270) and analyzed by system XA software (model SFT 1810, all from Buxco Electronics). Measurements of methacholine responsiveness were obtained by exposing mice for 2 min to NaCl 0.9% (Portable Ultrasonic, 5500D, DeVilbiss Health Care, Somerset, PA), followed by incremental doses (2.5–40 mg/ml) of aerosolized methacholine and monitoring Penh. This plethysmographic method for measuring AHR has been validated after direct comparison with airway measurements in intubated mice (21).

Collection of BAL fluid and lung histology

Animals were injected i.p. with a lethal dose of phenobarbital (450 mg/kg). The trachea was cannulated, the lung was then lavaged with 0.8 ml of PBS three times, and the fluid pooled. Cells in the lavage fluid were counted using a hemocytometer, and BAL cell differentials were determined on slide preparations stained with Hansel Stain (Lide Laboratories, Florissant, MO). At least 200 cells were differentiated by light microscopy based on conventional morphologic criteria. In some animals, no BAL was performed but lungs were removed, washed with PBS, fixed in 10% formalin, and stained with hematoxylin and eosin.

Results

HKL as an adjuvant inhibits the development of AHR in OVA-immunized BALB/c mice

We previously demonstrated that immunization of mice with *Listeria monocytogenes* as adjuvant successfully biased the development of Ag-specific cytokine synthesis toward Th1 cytokine production in both primary and secondary immune responses (13). Because the pathogenesis of asthma is tightly associated with Th2 cytokines and because Th1 cytokines may protect against asthma, we investigated the ability of HKL to inhibit the development of AHR in OVA-immunized BALB/c mice.

BALB/c mice were immunized s.c. with OVA adsorbed to alum, which provokes an OVA-specific Th2-dominated immune response. The mice were then boosted with OVA with or without HKL. Furthermore, to induce AHR, mice were also challenged with OVA intranasally (protocol 1, Fig. 1), following which AHR was measured in a whole body plethysmograph by challenge with increasing concentrations of methacholine. Fig. 2 demonstrates that immunization of BALB/c mice with OVA s.c. and intranasally resulted in the development of significant AHR. OVA-primed mice immunized with OVA plus HKL as an adjuvant at the time of the first boost showed dramatically reduced AHR, to levels as low as in control mice, indicating that HKL as an adjuvant inhibited the development of AHR in OVA-immunized BALB/c mice.

HKL as an adjuvant significantly reduces airway inflammation in OVA-immunized BALB/c mice

At the day of sacrifice, lung histology was examined after fixation and staining with hematoxylin and eosin. Lung sections from BALB/c mice that were immunized with OVA without HKL as an adjuvant showed significant airway inflammation with peribronchiolar and perivascular infiltrates, consisting of lymphocytes, eosinophils and some neutrophils (Fig. 3A). In contrast, lung sections from mice that were immunized with OVA and HKL as adjuvant

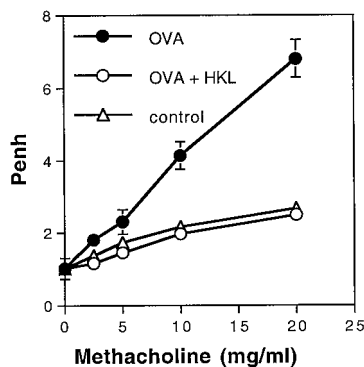


FIGURE 2. HKL as an adjuvant inhibits the development of AHR in OVA-immunized BALB/c mice. BALB/c mice were primed according to protocol 1 (Fig. 1). Control mice received OVA in alum in the footpads, but not intranasally. One day after the last intranasal challenge with OVA, AHR in response to increasing concentrations of methacholine was measured in conscious mice placed in a whole body plethysmograph. Data are expressed as Penh (mean $\pm$ SEM); $n \geq 10$ for each data point.

showed almost normal lung histology, with only marginal perivascular and peribronchiolar lymphocytic infiltrates (Fig. 3B). Thus, HKL as adjuvant during the immunization significantly reduced airway inflammation in OVA-immunized BALB/c mice.

HKL as an adjuvant significantly reduces the total cell number and eosinophilia in BAL fluid of OVA-immunized BALB/c mice

The histopathologic analysis was extended by examination of the cell numbers and types in the BAL fluid, which was harvested 5 days after the last intranasal challenge with OVA. The total number of cells recovered in the BAL fluid of BALB/c mice boosted with OVA plus HKL as an adjuvant was significantly lower than that in the control group (Fig. 4). Furthermore, vaccination with HKL as an adjuvant in the immunization protocol significantly reduced the proportion of eosinophils from 48% in the control group to 11% in HKL-treated mice (Fig. 4). These results demonstrate that HKL as an adjuvant significantly reduced the total cell number and the proportion of eosinophils recovered in the BAL fluid of OVA-immunized mice and confirm the results observed with lung histology.

HKL as an adjuvant elicits a Th1 like cytokine response

To determine whether the reduced AHR in mice immunized with HKL as adjuvant correlated with alteration of cytokine profiles in CD4⁺ T cells, mice were sacrificed 4 days after measurement of AHR. Draining LN were removed and LN cells were stimulated with OVA *in vitro*. Fig. 5 shows that cells from mice immunized with OVA s.c. and intranasally produced high levels of IL-4 and low levels of IFN- γ . In contrast, immunization with OVA plus HKL as adjuvant inhibited IL-4 production and greatly enhanced IFN- γ synthesis.

We also analyzed the isotype and subclass distribution of anti-OVA Ab responses in serum collected on day 46. Fig. 5 shows that vaccination with OVA plus HKL adjuvant greatly reduced anti-OVA IgE Ab responses and enhanced anti-OVA IgG2a Ab responses as compared with control mice immunized with OVA alone. Levels of anti-OVA IgG1 Ab were not significantly different in the two groups (711 ng/ml anti-OVA IgG1 in the group vaccinated with OVA plus HKL adjuvant vs 823 ng/ml IgG1 anti-OVA in the OVA/IFA control group).

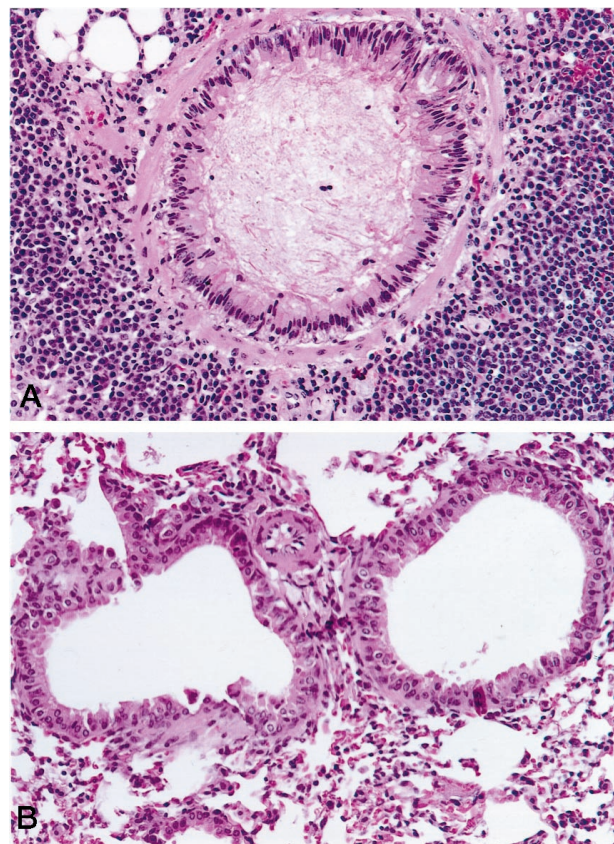


FIGURE 3. HKL as an adjuvant significantly reduces airway inflammation in OVA-immunized BALB/c mice. BALB/c mice were immunized with OVA, with or without HKL according to protocol 1 in Fig. 1. Lung tissue was fixed in formalin and stained with hematoxylin and eosin (H&E) at the day of sacrifice (day 46). A, Lung tissue from BALB/c mice after immunization with OVA revealed dense peribronchiolar mononuclear cell infiltrates consisting of lymphocytes, eosinophils, and some neutrophils. The bronchus lumen is filled with mucus. H&E, $\times 250$. B, Lung tissue from BALB/c mice after immunization with OVA plus HKL had only minimal lung disease with very few lymphocytes and almost no mucus production. H&E, $\times 250$.

HKL must be in close physical association with OVA to inhibit responses

To determine whether HKL had a generalized effect on immune responses or affected only responses to Ags in close physical association with it, mice were immunized with HKL and OVA in separate footpads. Fig. 6 shows that mice which received HKL and OVA together in the same footpad showed greatly reduced AHR, whereas mice which received the HKL in a different footpad from the OVA showed only minimal reduction in AHR. When HKL and OVA were injected separately in different footpads, the reduction of IL-4 and the increase of IFN- γ were about 50% of that when administered together (data not shown). Thus, the inhibition of AHR in OVA-primed mice was most efficient when HKL and the Ag were in close physical association with each other.

HKL as an adjuvant reverses established AHR in OVA-immunized BALB/c mice

To determine whether HKL as an adjuvant could reverse established AHR in addition to inhibiting the development of AHR, mice were boosted with OVA plus HKL on day 39, after the establishment of AHR (protocol 2, Fig. 1). Fig. 7A shows that AHR was present before administration of HKL, but 10 days after administration of HKL with OVA there was a significant reduction in

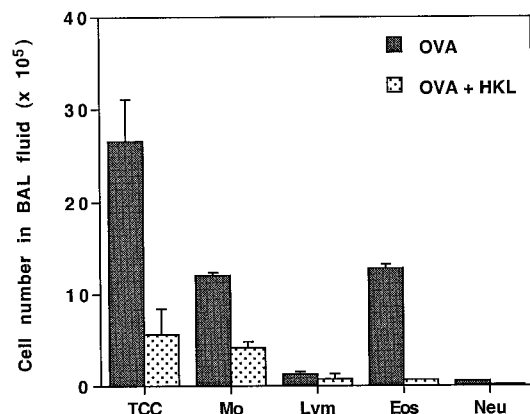


FIGURE 4. HKL as an adjuvant significantly reduces the total cell number and the relative number of eosinophils in BAL fluid of OVA-immunized BALB/c mice. BALB/c mice were immunized with OVA s.c. and intranasally according to protocol 1 in Fig. 1. BAL was performed 1 day after measurement of AHR (day 43) with three aliquots of 0.8 ml PBS per mouse. The relative number of different types of leukocytes (lung cell differentials) was determined from Hansel Stain slide preparations of BAL fluid. The data are expressed as mean $\pm$ SEM of each cell type in BAL fluid derived from differentials based on 200 cells ($n \geq 6$ for each group).

AHR. This protective effect with HKL could not be detected 3 days after the mice had received HKL, indicating that it required at least 10 days after administration of HKL to develop. Control mice that received OVA without HKL showed high AHR at all time points.

Analysis of the cytokine profiles of LN cells obtained on day 54 and stimulated with OVA *in vitro* showed that HKL increased OVA-specific IFN- γ production, and decreased OVA-specific IL4

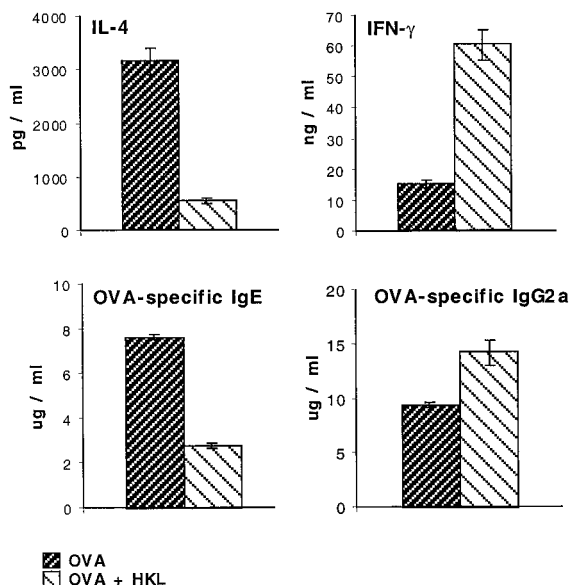


FIGURE 5. HKL as an adjuvant converts an established Th2- to a Th1-like cytokine response and inhibits the production of OVA-specific IgE. BALB/c mice were immunized with OVA s.c. and intranasally according to protocol 1 in Fig. 1. Mice were bled 7 days after the last s.c. injection, and LN cells were removed and cultured at 5×10^5 cells/well with 100 μ g/ml OVA. IL-4 and IFN- γ levels in supernatants were determined after 4 days by ELISA. Serum Ab levels were determined by ELISA. Data are the mean of triplicate cytokine determinations $\pm$ SD. Representative results from one of three experiments are presented.

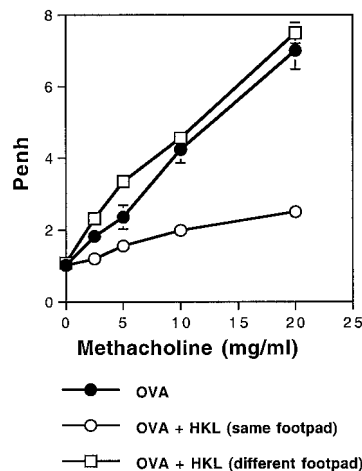


FIGURE 6. HKL is most efficient as adjuvant when mixed with the Ag before administration. Mice were immunized essentially as described in protocol 1 (Fig. 1). Four weeks after the initial footpad priming with 50 μ g OVA adsorbed to 2 mg alum, one group of mice received OVA in IFA and HKL in IFA in opposite footpads. Other groups of mice received OVA in IFA or OVA mixed with HKL in IFA in both footpads. All mice received a total of 50 μ g OVA. One day after the last intranasal challenge with OVA, AHR in response to increasing concentrations of methacholine was measured from conscious mice placed in a whole body plethysmograph. Data are expressed as Penh (mean $\pm$ SEM); $n \geq 6$ for each data point.

and IgE production (Fig. 7B). These results demonstrate that HKL as an adjuvant not only prevents the development of AHR when given during the earlier phase of the immunization protocol but also reverses established AHR and the cytokine profiles of CD4⁺ T cells.

The effect of HKL on the development of AHR is mediated by CD8⁺ T cells

To investigate the mechanism by which HKL affected OVA-specific responses, we administered blocking Ab to IL-12 or depleting Ab to CD8⁺ T cells during the immunization protocol. As expected, mice immunized with OVA had high airway reactivity, which was reduced by vaccination with OVA plus HKL in the presence of a control mAb (Fig. 8A). However, treatment with anti-CD8 mAb reversed the effect of HKL as adjuvant and restored OVA-induced AHR. Treatment with anti-IL-12 mAb partly eliminated the effect of HKL on AHR in OVA-immunized mice. In addition, the reduction by HKL of OVA-specific IgE levels was partially reversed by treatment with either anti-CD8 or anti-IL12 mAb (Fig. 8B). Furthermore, treatment of mice with anti-CD8 mAb partially reversed the effect of HKL on IFN- γ and IL-4 production. These results indicate that both CD8⁺ T cells and IL-12 play a role in the immunomodulatory effects of HKL on the AHR.

*HKL as an adjuvant increases IL-18-mRNA expression in spleen cells *in vitro* and *in vivo**

Because HKL as adjuvant strongly induced IFN- γ production (Fig. 4), we asked whether HKL also increased IL-18 production. We analyzed IL-18 mRNA levels by semiquantitative PCR analysis in splenic adherent cells cultured *in vitro* with HKL. Fig. 9A demonstrates that HKL induced a significant quantity of IL-18 mRNA expression after 8, 16, and 24 h of culture. Moreover, treatment of mice *in vivo* with OVA with HKL, but not with OVA alone, induced IL-18 mRNA expression in draining LN (Fig. 9B). The

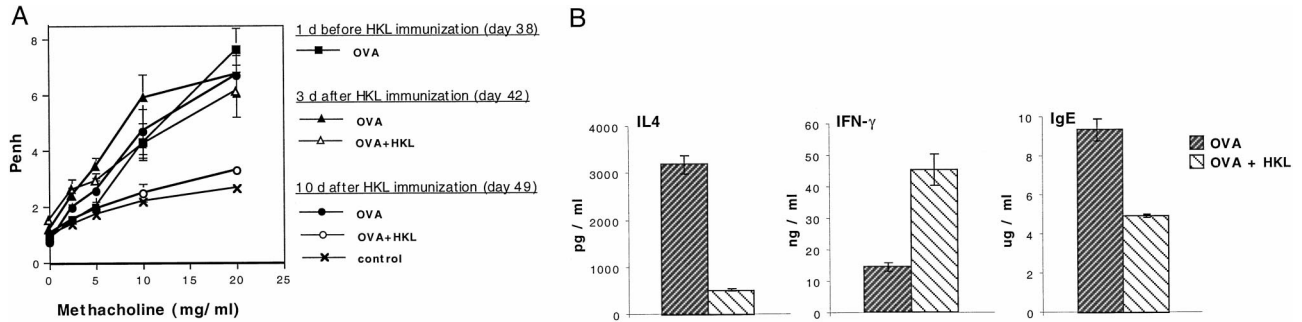


FIGURE 7. HKL as an adjuvant reverses established AHR in OVA-immunized BALB/c mice. **A**, To determine whether HKL as an adjuvant could reverse established AHR, BALB/c mice were immunized as shown in Fig. 1 (protocol 2). Mice received OVA in IFA or OVA mixed with HKL (10^8 per mouse) in IFA with the second boost instead of the first boost (day 39 instead of day 29). AHR in response to increasing concentrations of methacholine was measured 1 day before, and 3 days and 10 days after the injection of HKL from conscious mice placed in a whole body plethysmograph. Data are expressed as Penh (mean $\pm$ SEM); $n \geq 6$ for each data point. **B**, On day 50 mice received a final s.c. boost with OVA (50 μ g in PBS). Mice were bled 4 days later, and LN cells were removed and cultured at 5×10^5 cells/well with 100 μ g/ml OVA. IL-4 and IFN- γ levels in supernatants were determined after 4 days by ELISA. Serum Ab levels were determined by ELISA. Data are the mean of triplicate cytokine determinations $\pm$ SD. Representative results from one of three experiments are presented.

mRNA expression was detected 24 h but not at 12 h after immunization. These data indicate that the induction of IL-18 expression is associated with the immunomodulatory effects of HKL.

Discussion

In this study we demonstrated in a murine model of asthma that HKL as adjuvant very effectively inhibited AHR and airway inflammation. These effects were accompanied by the conversion of an Ag-specific Th2-dominated immune response into an Ag-specific Th1-like immune response and by a dramatic decrease of Ag-specific IgE. Moreover, HKL as adjuvant not only prevented, but also reversed ongoing, AHR and inflammation. Our observations demonstrate that HKL is a very promising adjuvant for the improvement of allergen immunotherapy, and suggest that patients with allergic asthma might benefit from such a therapeutic agent.

Asthma is characterized by the overproduction of the Th2 cytokines IL-4, IL-5, and IL-13, which initiate and sustain the allergic asthmatic inflammatory response by enhancing the production of IgE and the growth, differentiation, and recruitment of mast cells, basophils, and eosinophils (1). The Th2-driven inflammatory process may be a consequence of a relative insufficiency in IFN- γ

production because IFN- γ can inhibit the development of Th2 responses (22). In addition, clinical studies demonstrated that reduced IFN- γ secretion in neonates is associated with the subsequent development of atopy (23). Furthermore, a predisposition toward the overproduction of Th1 cytokines may protect against atopy, because patients with multiple sclerosis, rheumatoid arthritis, or infection with tuberculosis (conditions associated with increased production of Th1 cytokines) have a reduced predisposition toward the development of atopy (24–27). These studies together suggest that methods to enhance IFN- γ production might be clinically useful in the treatment of allergic asthma.

Indeed, immunotherapies and immune modulatory approaches that enhance Th1-dominated responses appear to be beneficial for allergic individuals (8, 28), and in animal models of allergic disease (10, 12, 29–33). Immunotherapies in these models, however, while effective in preventing the development of AHR, have not been shown to reverse established AHR. IL-12 administered intratracheally to mice has been shown to reverse Ag-induced AHR and inflammation in one study, but the effect of IL-12 has been controversial (29, 34, 35). In contrast, our current report describes an immunotherapy that is highly effective in reversing ongoing

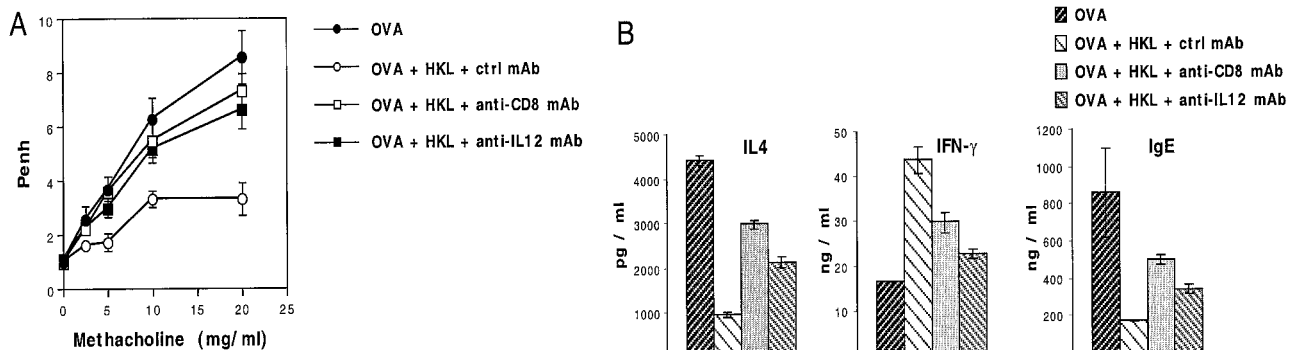


FIGURE 8. Inhibition of the development of AHR by HKL as adjuvant depends on IL-12 and CD8 $^{+}$ cells. **A**, BALB/c mice were immunized according to the immunization schedule of protocol 1 (see Fig. 1). Mice were injected i.p. with 1 mg of mAb C17.8 (for IL-12 depletion), mAb 53.6.7 (for CD8 $^{+}$ depletion), or 4G10 (rat IgG2a control) in 0.5 ml PBS 1 day before, the day of, and 3 days following immunization with OVA in IFA or OVA mixed with HKL (10^8 per mouse) in IFA. One day after the last intranasal challenge with OVA, AHR in response to increasing concentrations of methacholine was measured from conscious mice placed in a whole body plethysmograph. Data are expressed as Penh (mean $\pm$ SEM); $n \geq 6$ for each data point. **B**, Mice were bled 7 days after the last s.c. injection, and LN cells were removed and cultured at 5×10^5 cells/well with 100 μ g/ml OVA. IL-4 and IFN- γ levels in supernatants were determined after 4 days by ELISA. Serum Ab levels were determined by ELISA. Data are the mean of triplicate cytokine determinations $\pm$ SD. Representative results from one of three experiments are presented.

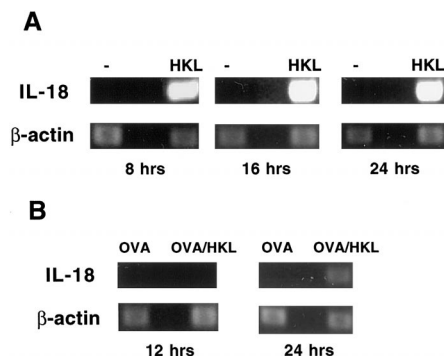


FIGURE 9. HKL as an adjuvant increases IL-18-mRNA expression in spleen cells *in vitro* as well as *in vivo*. Semiquantitative assessment of β -actin and IL-18 mRNA production by RT-PCR (30 cycles). *A*, Splenic adherent cells were cultured with HKL (10^8 /ml) for 6, 18, or 24 h and analyzed for IL-18 mRNA expression. *B*, Popliteal LN cells were isolated from BALB/c mice 12 and 24 h after footpad injection of OVA in CFA or OVA mixed with HKL (10^8 per mouse) in CFA and were analyzed for IL-18 mRNA expression.

AHR. This reversal of AHR with HKL as adjuvant was associated with a significant increase in IFN- γ production and a significant reduction of IL-4 production, in allergen-specific IgE production, and in IL-10 production (13). The reversal in AHR with HKL as an adjuvant required only one dose of the HKL plus Ag, and suggests that immunotherapy with HKL might be effective in patients with asthma, who by definition have ongoing AHR.

The potent capacity of HKL to reverse established AHR and inflammation might be due to the fact that HKL activates multiple immunological mechanisms. *Listeria monocytogenes* is a Gram-positive, facultative intracellular bacterium, which elicits a strong classical cell-mediated immune response, characterized by the presence of potent Ag-specific CD8 $^+$ killer cells (36, 37). The *L. monocytogenes* proteins listeriolysin O and p60 are processed through the MHC class I pathway and stimulate protective CD8 $^+$ CTL responses (38, 39). In our model system, CD8 $^+$ T cells induced by HKL plus Ag may play an important role in down modulating AHR, because treatment with anti-CD8 mAb reversed the inhibitory effect of HKL on AHR. The *Listeria* cell wall component lipoteichoic acid potently induces IL-12 production in macrophages (40, 41). The effect of IL-12 is thought to be due to the fact that it stimulates the production of IFN- γ by NK cells and T cells, which further enhances Th1 CD4 $^+$ T cell development, activates microbicidal activity of macrophages, and promotes the development of cell-mediated immune responses (42, 43). Moreover, our study demonstrated that HKL not only induces the production of IL-12 but also stimulates transcription of IL-18 mRNA and presumably production of IL-18 protein. IL-18 is a product of activated macrophages and Kupffer cells and is 10-fold more potent than IL-12 in driving the development of Th1 cytokine synthesis in naïve and memory T cells (44). IL-18 synergizes with IL-12 in inducing IFN- γ production (43), in inhibiting IgE production in B cells (45), and in promoting the differentiation of CD8 $^+$ T cells (46) and possibly CD8 $^+$ $\gamma\delta$ cells, which have been shown to inhibit AHR (47). Production of both IL-12 and IL-18 may be enhanced by the presence of CpG ISS motifs in *Listeria* DNA, which can induce IL-12 and IL-18 (48). However, we do not believe that the activity of *Listeria* as an adjuvant is due only to CpG ISS, as may be the case with killed mycobacteria (49), which appears to be less effective than HKL in inhibiting Th2-dominated immune responses (13, 32). Finally, recent studies in our laboratory suggest that heat killed *Listeria* also induces TGF- β production *in vivo*,

which may provide an additional mechanism that may down-regulate allergic inflammation and AHR (our unpublished observations). Thus, the effectiveness of HKL as an adjuvant may therefore depend on the induction of IL-12, IL-18, and IFN- γ production, and well as the induction of CD8 and Th1 cells.

The mechanism by which HKL as an adjuvant reverses established AHR and inflammation may also involve the conversion of OVA-specific CD4 $^+$ Th2 cells into Th1 cells, or the inhibition or attrition of Th2 effector cells over time while a protective immune response develops from uncommitted OVA-specific precursor cells. Because the cytokine profile of Th2 effector cells are relatively fixed, we speculate that attrition of Th2 effector cells as well as the induction of several types of Th2-inhibiting regulatory cells are involved in this process. We recently showed that localization of Ag-specific Th1 cells in the lungs caused airway inflammation and lung injury, and did not reduce AHR (50), suggesting that other types of cells in addition to Th1 cells (e.g., CD8 cells or CD4 Th3 cells) (51) are induced by HKL. The fact that CD8 cells and TGF- β production are induced by HKL supports this idea. The generation of such OVA-specific regulatory cells by HKL may enhance the development of a protective immune response, such that further exposure to allergen does not result in AHR and airway inflammation. The existence of such regulatory cells and the mechanisms by which they affect allergic inflammatory processes, and how long the effect of HKL persists are currently being investigated.

The attractiveness of *Listeria* as adjuvant therapy lies also in the fact that its immunomodulatory effects remain largely Ag specific. *Listeria* had minimal effect on AHR, IgE, and cytokine production unless the *Listeria* was administered in a mixture with the Ag. Thus *Listeria* did not induce a generalized enhancement of IFN- γ production in recipients, but rather induced a protective response that was Ag specific. The capacity to induce Ag-specific modulation is very important, because this specificity avoids nonspecific immune augmentation, which could result in the development of autoimmune diseases. Ag-specific therapy is feasible for the treatment of allergic rhinitis and allergic asthma, because the major offending allergens are virtually always identified. Thus, the use of *Listeria* as an adjuvant could greatly improve and refine conventional allergen immunotherapy (9), which currently requires multiple injections of soluble allergen over several years time and is associated with frequent failures. The safety issues of using *Listeria* in humans may not be of major concern because killed rather than live *Listeria* is effective for immune modulation. In addition, live *Listeria* is a relatively common organism, and is a pathogen primarily in immunosuppressed patients or in the setting of pregnancy.

In conclusion, we demonstrated that allergen immunotherapy with HKL as adjuvant greatly inhibited the development of AHR and airway inflammation. Immunotherapy with HKL as adjuvant reversed ongoing airway disease, and converted allergic inflammatory responses into protective immune responses. The effect involved multiple mechanisms, including the induction of IL-18 and CD8 $^+$ T cells, activation of the innate immune system and inhibition of Th2 cytokine production. Our results suggest that allergen immunotherapies with HKL as adjuvant for asthma may be feasible, and further studies in humans with HKL are warranted to determine the effectiveness of HKL in human disease.

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