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The Pulmonary Environment Promotes Th2 Cell Responses After Nasal-Pulmonary Immunization with Antigen Alone, but Th1 Responses Are Induced During Instances of Intense Immune Stimulation[1]

Harlan P. Jones,* Lisa M. Hodge,* Kohtaro Fujihashi,† Hiroshi Kiyono,†‡ Jerry R. McGhee,† and Jerry W. Simecka[2]*

The purpose of this study was to determine the nature of the CD4$^+$ Th cell responses induced after nasal-pulmonary immunization, especially those coinciding with previously described pulmonary inflammation associated with the use of the mucosal adjuvant, cholera toxin (CT). The major T cell population in the lungs of naive mice was CD4$^+$, and these cells were shown to be predominantly of Th2 type as in vitro polyclonal stimulation resulted in IL-4, but not IFN-γ, production. After nasal immunization with influenza Ag alone, Th2 cytokine mRNA (IL-4 and IL-5) levels were increased, whereas there was no change in Th1 cytokine (IL-2 and IFN-γ) mRNA expression. The use of the mucosal adjuvant, CT, markedly enhanced pulmonary Th2-type responses; however, there was also a Th1 component to the T cell response. Using in vitro Ag stimulation of pulmonary lymphocytes, influenza virus-specific cytokine production correlated with the mRNA cytokine results. Furthermore, there was a large increase in CD4$^+$ Th cell numbers in lungs after nasal immunization using CT, correlating with the pulmonary inflammatory infiltrate previously described. Coincidentally, both macrophage-inflammatory protein-1α (MIP-1α) and MIP-1β mRNA expression increased in the lungs after immunization with Ag plus CT, while only MIP-1β expression increased when mice were given influenza Ag alone. Our study suggests a mechanism to foster Th1 cell recruitment into the lung, which may impact on pulmonary immune responses. Thus, while Th2 cell responses may be prevalent in modulating mucosal immunity in the lungs, Th1 cell responses contribute to pulmonary defenses during instances of intense immune stimulation. ***The Journal of Immunology*****, 2001, 167: 4518–4526.**

Induction of both mucosal and systemic (circulating) immunity is optimal for protection and recovery from respiratory infections, and may be critical determinants in pulmonary disease. Ab responses in the circulation protect against pulmonary infections and disease (1–3). Presumably, IgG Abs cross the alveolar-capillary barrier into the alveoli, where they provide resistance to infection. Importantly, the airway epithelium is the initial site for many infections (e.g., *Mycoplasma* and influenza virus) in the upper and lower respiratory tracts. Mucosal IgA responses are involved in protection of these surfaces from infection and perhaps in recovery from disease (2, 4–9). For example, we previously showed that immune responses can contribute to the severity of pulmonary inflammatory responses in *Mycoplasma* respiratory disease, while at the same time preventing dissemination of the infection to extrapulmonary sites (10). Furthermore, immunity is central to the development of respiratory allergies and asthma (11, 12), in which infectious agents and environmental factors appear to affect the susceptibility or severity of these conditions (13–21). Thus, immunity can have both beneficial and detrimental effects on respiratory disease, and it is critical to understand the factors that influence immune responses that can impact the progression and prevention of respiratory diseases.

Nasal immunization is commonly used to induce immunity along the respiratory tract. This route of immunization results in mucosal IgA and systemic IgG Ab responses, which has the advantage of protecting the entire respiratory tract from respiratory infections (22–27). Importantly, nasal immunization also has the potential to elicit immunopathologic responses (28, 29). Nasal immunization can result in the generation of Ag-specific IgE Ab responses. Mucosal surfaces are associated with generation of IgE responses, which play essential roles in respiratory allergies and asthma (30). IgE-mediated responses may also contribute to the pathogenesis of infectious diseases, including *Mycoplasma* and viral pneumonias (13, 31, 32), and interestingly, infectious agents may actually increase the risk or severity of asthma (14, 16). Furthermore, the use of the mucosal adjuvant cholera toxin (CT)[3] resulted in significant enhancement of IgE Ab responses, with concomitant mucosal IgA and serum IgG Ab responses (28, 29). When CT is used as an adjuvant, there is a massive infiltration of

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[3] Abbreviations used in this paper: CT, cholera toxin; β_2m, β_2-microglobulin; DTH, delayed-type hypersensitivity; MIP, macrophage-inflammatory protein.

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mononuclear cells into the lungs. Thus, an understanding of the mechanisms for these responses will potentially provide insights into the induction of immunity during respiratory infections and diseases, as well as suggesting new approaches for mucosal vaccines development.

Due to their central role in immunity, alterations in the intensity or the type of $CD4^+$ Th cell responses can have a profound effect on respiratory disease. Th cells may be divided into two major functional populations, in which Th1 cells promote cell-mediated and inflammatory responses through their production of cytokines, such as IL-2 and IFN-γ (33). For example, Th1 responses are critical in controlling mycobacterial pulmonary infections (34–36). In contrast to Th1 cells, Th2 cells regulate Ab responses through secretion of IL-4, IL-5, IL-10, and IL-13 (33). In the gastrointestinal tract, Th2 cells are the dominant resident Th cell population (37, 38), and presumably, Th2 cells are critical along mucosal surfaces of the respiratory tract as well. The Th2 cytokines are intimately involved in the generation of mucosal IgA Ab responses (39). Importantly, IL-4 produced by Th2 cells also promotes the generation of IgE Ab responses (33, 40), and therefore, in addition to mucosal IgA responses, Th2 cells are major mediators of allergies and asthma (41–45). These responses appear to also contribute to the pathology of some respiratory infections (46–48). Past studies suggest that Th2 cell responses are often polarized due to cytokines promoting one response and inhibiting the other (49–51). However, recent studies suggest that Th1 cells may also contribute to the pathogenesis of the inflammatory responses associated with the Th2 cell-mediated disease, asthma (45). Thus, both Th cell subsets can be involved in the pathogenesis of inflammatory diseases of the respiratory tract. The purpose of the present studies was to assess Th cell subset responses in the lung after nasal immunization. Our results provide important insights into the mechanisms of pulmonary immunity in health and disease, and perhaps reveal new approaches for the development of mucosal vaccines and immunotherapy.

Materials and Methods

Mice

Viral-free female BALB/c mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). All mice were used between 8 and 12 wk of age. All mice were maintained in sterile microisolator cages, and given sterile water and food ad libitum. Before experimental manipulation, mice were anesthetized with an i.m. injection of ketamine-xylazine. For nasal-pulmonary immunization, mice were allowed to inhale 25 μl inoculum that was placed upon the nares (29, 52). This volume of inoculum results in the deposition of Ag in both nasal passages and lung (28).

Immunogens

Philippines influenza split virus vaccine (H3N2) was provided by M. W. Harmon, Connaught Laboratories (Swiftwater, PA). CT was purchased from List Biological Laboratories (Campbell, CA).

Cell isolation

Mononuclear cells were isolated from lungs, as previously described (32, 53, 54). Lungs were perfused with PBS without magnesium or calcium to minimize contamination of the final lung cell population with those from blood. The lungs were separated into individual lobes and finely minced. The tissues were suspended in RPMI 1640 (HyClone Laboratories, Logan, UT) medium containing 300 U/ml *Clostridium histolyticum* type I collagenase (Worthington Biochemical, Freehold, NJ), 50 U/ml DNase (Sigma, St. Louis, MO), 10% FBS (HyClone Laboratories), HEPES, and antibiotic/antimycotic solution (Life Technologies, Grand Island, NY). The tissues were incubated at 37°C while mixing on a Nutator (Fisher Scientific, Pittsburgh, PA) for 90–120 min. During the incubation period, the tissue was vigorously pipetted every 20 min. After incubation, the digestion mixture was passed through a 250-μm nylon mesh to remove undigested tissue. Mononuclear cells were purified from cell suspensions by density gradient centrifugation using Lympholyte M (Accurate Chemicals, Westbury, NY).

Spleen cells and lower respiratory (mediastinal and hilar) lymph nodes were isolated after centrifugation of cell suspensions, followed by red cell removal using ammonium chloride potassium lysis buffer, as previously described (55).

Immunofluorescent characterization of lymphocyte populations

Three-color immunofluorescent staining was performed to identify T cell populations using PE-labeled anti-murine CD3 mAb (KT3; Beckman Coulter, Fullerton, CA), PerCP-labeled anti-CD4 mAb (0.5 μg/ml, L3T4, RM4-5; BD PharMingen, San Diego, CA), and FITC-labeled anti-CD8 mAb (0.2 μg/ml, Lyt-2, 53-6.7; BD PharMingen). Briefly, 2×10^6 cells/tube were incubated with purified 2.4G2 mAb (BD PharMingen) for 5 min at 4°C to reduce nonspecific binding of FcII/III receptors before fluorescent Ab staining. The cells were incubated for 30 min at 4°C with 250 μl fluorescent Ab. The cells were washed in staining buffer (Mg^{2+}-free, Ca^{2+}-free PBS with 0.05% sodium azide, 1% FBS (HyClone Laboratories)) and fixed with 4% paraformaldehyde solution for 30 min, and the cells were then resuspended in staining buffer until analyzed.

The cells were analyzed using an EPICS XL-MCL flow cytometer (Beckman Coulter). Data collection was done using System 2 software (Beckman Coulter), with further analysis done using Expo 2 analysis software (Beckman Coulter). Lymphocyte gates and detector voltages were set using unstained (control) lung and splenic cells, and stained cell populations were seen as distinct peaks or clusters of cells. The proportion of each cell population was expressed as the percentage of the number of stained cells. To determine the total number of a specific lymphocyte population, their percentage was multiplied by the total number of lymphocytes isolated from a particular tissue.

In vitro depletion and isolation of T lymphocyte populations

A 100-μl aliquot of mouse anti-CD4 and/or anti-CD8 mAb-labeled magnetic beads (Dynabeads mouse CD4, mouse CD8; Dynal, Lake Success, NY) was added directly to lung and splenic cell suspensions at a final concentration of 1×10^7 cells/4×10^7 beads. Lymphoid cells were incubated at 4°C for 20 min on a rotator. After incubation, tubes containing lymphocytes were placed on a magnet (MPC magnet; Dynal) for 2 min, and both positive and negative fractions were collected for further studies. Confirmation of cell purity was determined using flow cytometry. Cell fractions with a depletion of >98% were used for subsequent experimentation.

Polyclonal stimulation of $CD3^+$ lymphocytes

A 50-μl aliquot of a 10 μg/ml concentration of purified anti-CD3 mAb (145-2C11; BD PharMingen) diluted in PBS was added to a sterile 96-well flat-bottom microtiter plate and incubated overnight at 4°C. In some experiments, anti-CD28 mAb (37.51; BD PharMingen) was added to the cell cultures at a concentration of 1.25 μg/ml. Plates were gently washed with sterile RPMI 1640 culture medium (HyClone Laboratories). Whole and purified lymphocyte fractions of lung and splenic mononuclear cells were placed in wells suspended in 100 μl culture media at a final concentration of 2×10^6 cells/ml and incubated for 4 days at 37°C and 5% CO_2 in air. Supernatants were collected and stored at −80°C until assayed for cytokine levels.

Cytokine assays

The levels of cytokine were measured by capture ELISA. Murine IL-4 levels were measured using OptEIA IL-4 ELISA set (BD PharMingen), while murine IFN-γ levels were determined using mouse IFN-γ MiniKit (Endogen, Woburn, MA). Easy-wash 96-well flat-bottom microtiter plates (BD Biosciences, Bedford, MA) were coated overnight at 4°C with 100 μl mAb specific for either murine IL-4 or IFN-γ diluted in 0.1 M Na_2HPO_4, pH 9. Plates were washed and blocked with 200 μl PBS/Tween 20 supplemented with 10% FBS for 30 min. Following a PBS/Tween 20 wash, 100 μl sample supernatants were placed into the appropriate wells and incubated overnight at 4°C. Plates were washed three times with PBS/0.05% Tween, and 100 μl biotinylated rat anti-mouse cytokine (IL-4 or IFN-γ) mAb was added to each well and incubated overnight at 4°C. To reveal the reaction, avidin-HRP and 3,3′5,5′-tetramethylbenzidine substrate (Moss, Pasadena, MD) were used. Plates were read using an MX80 plate reader (Dynatech, Chantilly, VA) at an absorbance of 630 nm. One hundred microliters of 0.25 M HCl were also added to the reaction as needed to increase the sensitivity of reaction, and read at an absorbance of 450 nm. Cytokine levels were determined by comparison with standard curves generated from murine recombinant cytokines (IL-4 and IFN-γ; BD PharMingen) after log/log quadratic linear regression analysis using Revelation 2.0 software (Dynatech).

RNA isolation from lungs

Total RNA was isolated from the entire lungs of mice using the Ultraspec-II RNA Isolation System (Biotecx Laboratories, Houston, TX), which is based on a previously described method (56). Briefly, the lungs were homogenized in the Ultraspec-II RNA reagent using a PRO 200 homogenizer (PRO Scientific, Monroe, CT). Chloroform was added to the homogenate and centrifuged at 12,000 × *g* (4°C) for 30 min. The RNA was precipitated by adding isopropanol to the aqueous phase and centrifuging the samples at 12,000 × *g* (4°C) for 10 min. The RNA pellet for each sample was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 5 min at 7,500 × *g* , and then resuspended in diethylpyrocarbonate-treated water. The concentration and quality of RNA in each of the samples were determined spectrophotometrically (GeneQuant II; Pharmacia Biotech, Piscataway, NJ) and by gel electrophoresis. The RNA samples were stored at −80°C until used.

Cytokine mRNA detection by RT-PCR

RT-PCR was performed using 100 ng RNA for each sample, as previously described (57). The sequences of the primers and the size of the resulting PCR fragments (in parentheses) for IL-2, IL-4, IL-5, IFN-γ, and the housekeeping gene, β_2-microglobulin (β_2mGL), are as follows (58): IL-2 (227 bp), 5′-GACACTTGTGCTCCTTGTCA and 5′-TCAATTCTGTGGCCT GCTTG; IL-4 (216 bp), 5′-TCGGCATTTTGAACGAGGTC and 5′-GAAAAGCCCGAAAGAGTCTC; IL-5 (201 bp), 5′-TCACCGAGCTCT GTTGACAA and 5′-CCACACTTCTCTTTTGGCG; IFN-γ (227 bp), 5′-GCTCGAGACAATGAACGCT and 5′-AAAGAGATAATCTGGCTCT GC; and β_2mGL (222 bp), 5′-TGACCGGCTTGTATGCTATC and 5′-CAGTGTGAGCCAGGATATAG. β-Chemokine primers used were as described in a previous study (59).

The increase in expression of cytokine mRNA after immunization was determined by the number of cycles of amplification that resulted in little or no PCR product for each cytokine in total lung RNA from sham-inoculated, control mice, as previously described (57, 59). For IFN-γ, IL-5, and β_2mGL, the samples were amplified for 30 cycles, and for IL-2 and IL-4, the samples were run for 35 cycles. The PCR products were separated on 1.8% agarose gels and stained with ethidium bromide. Gels were visualized using the α Image 2000 Documentation and Analysis System (Alpha Innotech, San Leandro, CA). The intensity of each band was determined using densitometry, and the relative cytokine mRNA reactions were compared after normalization to the housekeeping gene, β_2mGL.

Cytokine RNase protection assay

RiboQuant, Multiprobe Ribonuclease Protection Assay System (BD PharMingen), was used for T cell cytokine mRNA detection and quantitation. RNA probes specific for T cell-specific cytokine mRNAs (mCK-1 multiprobe template set; BD PharMingen) were synthesized using T7 RNA polymerase, GACU nucleotide pool, and [α-^{23}P]UTP (37°C for 1 h). The RNA probes were subsequently purified and quantitated for radiolabel incorporation. A 20-μg aliquot of total lung RNA was hybridized in solution to the radiolabeled probes for 16 h at 56°C. After hybridization, the samples were treated with RNase A to degrade unhybridized RNA probes, and the RNase A activity was subsequently inactivated using proteinase K, followed by phenol/choroform:isoamyl extraction to purify nucleic acids. The hybridized probes were resolved on a 5% polyacrylamide sequencing gel. The radiolabeled probes were visualized by exposure to x-ray film. Exposure times were varied to ensure visualization of probes and linearity of detection. The identity of the protected probes was verified using undigested probes, as m.w. markers, and comparing their sizes to expected m.w. For densitometry, x-ray films were analyzed using the α Image 2000 Documentation and Analysis System (Alpha Innotech). Probe intensities were compared between samples after normalization using the intensity of band produced by the housekeeping gene, L32. Yeast tRNA was used as a negative control to verify hybridization and digestion of radiolabeled antisense probes.

Ag-specific in vitro stimulation of mononuclear cells

Lymphoid cells were cultured in 96-well round-bottom microtiter plates in RPMI 1640 (HyClone Laboratories) supplemented with 5% FBS (HyClone Laboratories), HEPES, 10 U/ml rIL-2 (BD PharMingen), antibiotic/antimycotic solution (Life Technologies), and 50 μM 2-ME (Life Technologies). Lymphoid cells were stimulated at 37°C and 5% CO_2. Cells were stimulated with or without 2 μg/ml dialyzed influenza Ag in a final volume of 200 μl/well of culture media at a cell concentration of 2×10^6 cells/ml. Supernatants were collected 4 days later and stored at −80°C until assayed for cytokine levels.

Statistical analysis

Data were evaluated by ANOVA, followed by Fisher protected least significant difference multigroup comparison, Tukey's multigroup comparison, or Mann-Whitney *U* tests using Bonferroni-adjusted probabilities. These analyses were performed using the SYSTAT (SYSTAT, Evanston, IL) or StatView (SAS Institute, Cary, NC) computer programs. When appropriate, data were logarithmically transformed before statistical analysis, and confirmed by a demonstrated increase in power of the test after transformation of the data. A *p* value of less than or equal to 0.05 ($p \leq 0.05$) was considered statistically significant. If data were analyzed after logarithmic transformation, the antilog of the means and SEs of the transformed data was used to present the data, and are referred to as the geometric means ($x/\div$ SE).

Results

Resident pulmonary CD4$^+$ Th cells are predominantly of a Th2 type

To determine the resident lymphocyte environment in the lungs and spleens of naive mice, mononuclear cells were isolated from naive mice. Cells from lung and spleen were stained for CD3, CD4, and CD8 surface expression. The percentages of total CD3$^+$, CD3$^+$CD4$^+$, and CD3$^+$CD8$^+$ T cells were determined using flow cytometry. The major T cell subset in the lungs was CD3$^+$CD4$^+$ (73.4 ± 3.8% of total T cells), while CD3$^+$CD8$^+$ T cells represent approximately 20% of the entire pulmonary T cell population. A similar T cell distribution was seen in spleen.

To examine cytokine production by resident naive lung lymphocytes, we measured Th1 (IFN-γ) and Th2 (IL-4) cytokine production by isolated T cells after in vitro polyclonal activation.

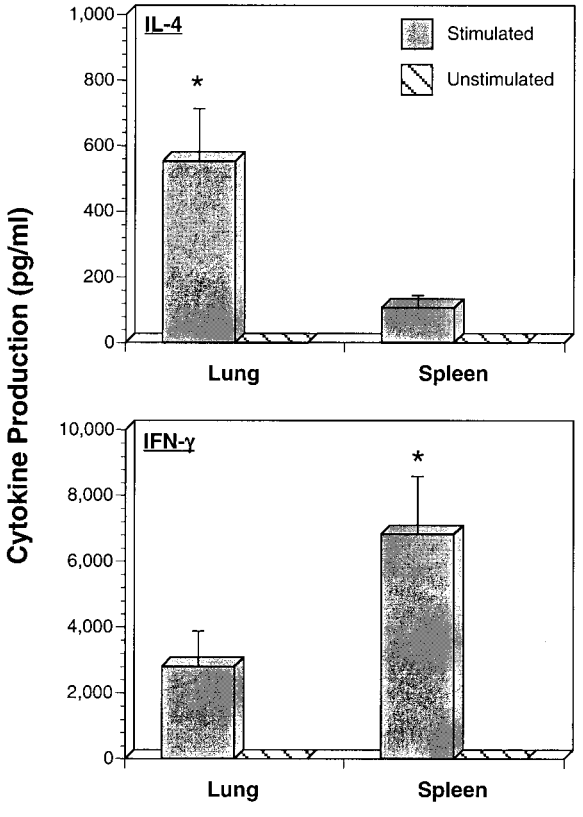

FIGURE 1. IL-4 and IFN-γ cytokine production by naive resident lung and splenic lymphocytes. Lung and spleen mononuclear cells, isolated from naive mice, were cultured in wells coated with (Stimulated) or without (Unstimulated) anti-CD3 mAb. After 4 days in culture, cytokine levels (picograms per milliliter) in culture supernatants were measured using ELISA. Vertical bars and error bars represent mean ± SE ($n = 3$). ∗, Significantly ($p \leq 0.05$) higher level of cytokines detected within culture supernatants.

Lung cells and splenocytes were collected from naive mice and cultured in wells coated with anti-CD3 mAb. After 4 days in culture, cytokine levels in culture supernatants were measured. Lung cells produced significantly higher amounts of IL-4 than did splenocytes (Fig. 1). In contrast, IFN-γ production was significantly higher in splenic cell cultures than in cells isolated from the lungs (Fig. 1).

To determine the contribution of $CD4^+$ and $CD8^+$ T cells to in vitro cytokine production, lung $CD4^+$ and/or $CD8^+$ T cell populations were depleted (>98% depletion) from naive whole lymphocyte populations. After depletion, lymphocytes were stimulated with anti-CD3 mAb, and the levels of cytokines in culture supernatants were determined (Fig. 2). Depletion of $CD4^+$ T cells markedly reduced IL-4 production by lung cell suspensions. On the other hand, depletion of $CD8^+$ T cells had no effect on IL-4 production by lung lymphocytes. In contrast, $CD4^+$ T cell depletion had no significant effect on IFN-γ levels when compared with unfractionated lung cells. Simultaneous depletion of both $CD4^+$ and $CD8^+$ T cell populations resulted in small amounts of IL-4 or IFN-γ cytokine production by lung cells. Similar results were obtained when anti-CD28 mAb was used with anti-CD3 mAb to stimulate naive T cells (data not shown).

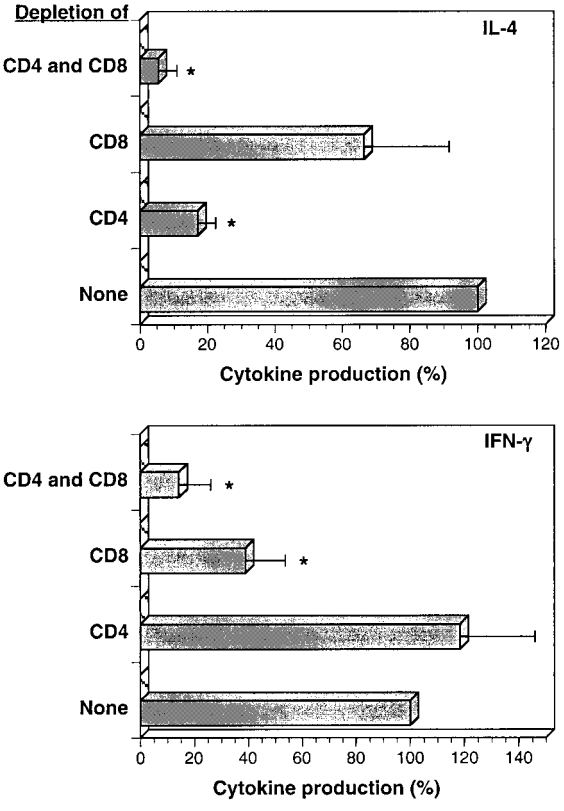

FIGURE 2. The contribution of lung $CD4^+$ and $CD8^+$ T cells to in vitro cytokine production. $CD4^+$ and/or $CD8^+$ lung lymphocyte populations were depleted (>98% depletion, as confirmed using flow cytometry) from naive whole lymphocyte populations. Lung cells were stimulated with (Stimulated, filled bar) or without (Unstimulated, hatched bar) anti-CD3 mAb, and the levels of IL-4 and IFN-γ cytokines in culture supernatants were determined using ELISA. Results are represented as the percentage of cytokine production detected within the culture supernatants of whole (undepleted (None)) lung cells. Vertical bars and error bars represent the mean ± SE ($n = 3$). *, Significant ($p \leq 0.05$) reduction in detectable levels of IL-4 and IFN-γ cytokine production from undepleted (None) lung cells.

Cytokine responses in lungs after nasal instillation of influenza Ag alone or in combination with CT

We next assessed $CD4^+$ Th cell cytokine mRNA expression in lungs after nasal-pulmonary immunization with influenza Ag and CT as adjuvant. Mice were given either Ag alone (7.5 μg) or with CT (0.1 μg) nasally on days 0 and 7. Three days after the second immunization, lung RNA was isolated, and Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-5) cytokine mRNA expression was determined by RT-PCR. After immunization with influenza Ag only, a significant induction of IL-4 and IL-5 mRNA expression was noted when compared with sham-immunized mice, but no significant increases in IL-2 or IFN-γ mRNA were seen (Fig. 3). In addition, using the adjuvant CT resulted in induction of both Th2 (IL-4 and IL-5) and Th1 (IL-2 and IFN-γ) cytokine mRNA expression ($p \leq 0.05$).

Using a quantitative RNase protection assay, the levels of Th cytokine mRNAs were further compared between mice immunized with Ag alone and those mice given Ag plus CT as adjuvant. The mRNAs encoding the Th2 cytokines, IL-4, IL-5, IL-6, IL-10, and IL-13, were significantly higher in lungs of mice given influenza vaccine with CT than in those given influenza Ag alone (Fig. 4). The levels of IL-4 mRNA were 25-fold higher in mice immunized with influenza Ag and CT than in those given Ag alone (Fig. 4*a*). The levels of IL-5, IL-6, IL-10, and IL-13 mRNA were 3- to 5-fold higher when CT was used as adjuvant (Fig. 4*b*). Similarly, IFN-γ and IL-2 mRNA levels were significantly higher with the inclusion of CT as adjuvant. The levels of IFN-γ mRNA were 3-fold, while IL-2 mRNA was 2-fold higher when CT was used when compared

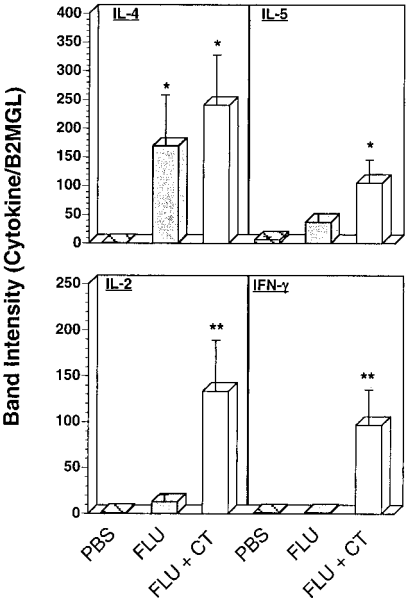

FIGURE 3. Th1 and Th2 cytokine mRNA expression after nasal immunization with influenza vaccine (FLU) alone or in combination with cholera toxin (FLU + CT). Total RNA was isolated from the lungs of mice 3 days after secondary nasal immunization (day 7) either with PBS (hatched bar), influenza vaccine alone (7.5 μg) (filled bar), or in combination with CT (0.1 μg) (open bar). Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-5) cytokine mRNA expression was examined in the lungs using RT-PCR. Relative differences in mRNA expression of cytokines between groups of mice were determined by the relative increase in the ratio of cytokine to the housekeeping gene β_2mGL. *, Significant ($p \leq 0.05$) difference in band intensity from PBS-treated mice; **, significant difference ($p \leq 0.05$) in band intensity from all other groups of mice. Results are based on the geometric mean ($x/\div$ SE, $n = 6$).

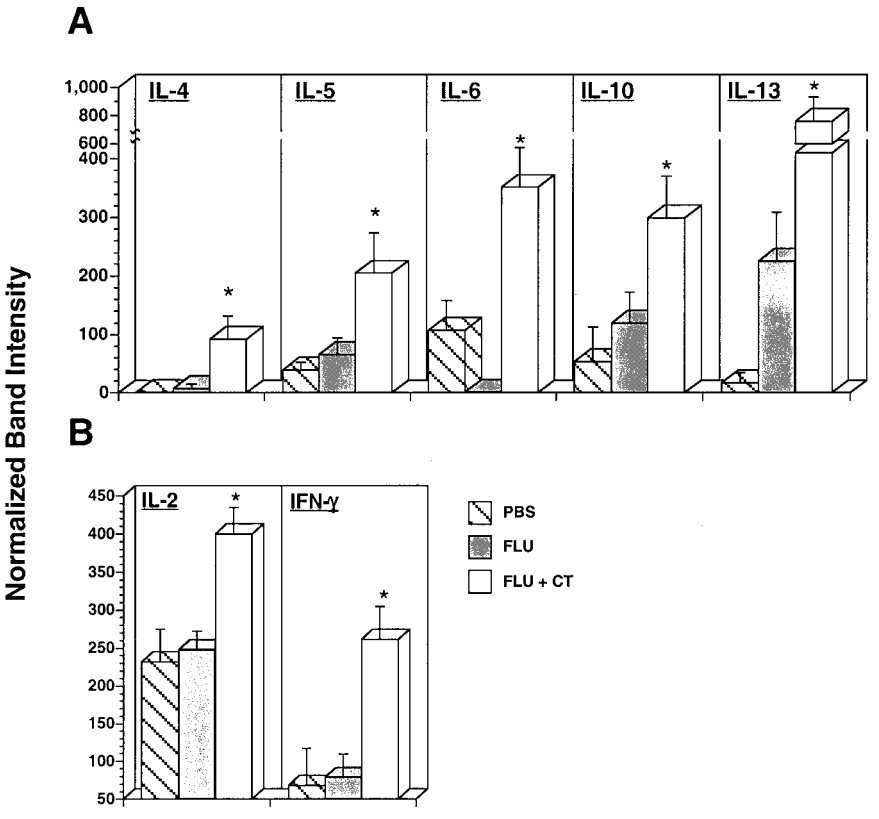

FIGURE 4. Th1 and Th2 cytokine mRNA expression after nasal immunization. Total RNA was isolated from the lungs of mice 3 days after secondary immunization either with PBS, influenza vaccine Ag (FLU), or vaccine Ag plus CT (FLU + CT). Relative differences in Th2 (*A*) and Th1 (*B*) mRNA expression of cytokines between groups of mice were determined by normalizing the cytokine band intensity with that of the housekeeping gene L32 for each sample. *, Significant ($p \leq 0.05$) difference in band intensity from PBS-treated mice. Results are based on the geometric mean ($x/\div$ SE, $n = 6$).

with mice immunized with influenza Ag only. Thus, although Th2-type cytokines were induced in lungs after immunization with Ag alone, the inclusion of CT also enhanced the expression of all the Th cytokine mRNAs tested.

Influenza-specific Th1 and Th2 cell responses in lungs of mice given CT nasally as adjuvant

To determine whether Ag-specific Th cell responses were generated, mice were given either Ag alone (7.5 μg) or with CT (0.1 μg) nasally on days 0 and 7. These dosages are consistent with our previous study (28). Three days after the second immunization, lung and spleen cells were isolated from these mice and stimulated with influenza Ag in vitro. Four days later, IL-4 and IFN-γ cytokines were measured in culture supernatants. IL-4 was detected in the supernatants from Ag-stimulated cultures isolated from mice immunized with Ag alone, whereas IFN-γ was not detected (Fig. 5). In contrast, lung and spleen cells from mice immunized with Ag plus CT produced not only IL-4, but also IFN-γ.

To analyze the contribution of $CD4^+$ T cells in T cell cytokine responses, $CD4^+$ and/or $CD8^+$ T cells were depleted from lung lymphocytes before in vitro stimulation with Ag. Depletion of $CD4^+$ T cells from lung lymphocytes collected from mice immunized with influenza Ag alone significantly reduced the levels of IL-4 from that of whole (undepleted) pulmonary cell isolates (Fig. 6). Depletion of $CD4^+$ T cells from lung cell isolates taken from mice immunized with CT resulted in a significant decrease in both IL-4 and IFN-γ. Depletion of lung $CD8^+$ T cells also significantly reduced ($p \leq 0.05$) IL-4 and IFN-γ levels when compared with Ag-stimulated whole cell cultures. The production of IL-4 and IFN-γ was diminished to almost undetectable levels in cultures depleted of both $CD4^+$ and $CD8^+$ T cells from immunized mice.

Nasal delivery of CT results in pulmonary $CD4^+$ T cell expansion

Our previous studies (28, 29) showed a marked increase in mononuclear cells in the lungs of mice given CT as a mucosal adjuvant. To determine whether Th cells are a major component of the mononuclear cell infiltrate, mice were given a primary and booster immunization with influenza Ag alone or in combination with CT. Three days later, lung cells were isolated and cell numbers were determined. The percentage and numbers of lung $CD4^+$ and $CD8^+$ T cell populations were determined by flow cytometry.

The greatest effect on $CD4^+$ T cells in lungs was seen when CT was included during nasal immunization. The numbers and percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ lung T cells from mice given Ag alone did not differ significantly from control mice. In contrast, there was a significant increase in the percentage of $CD3^+CD4^+$, but not $CD3^+CD8^+$, T cells isolated from lungs of mice immunized with Ag plus CT (Table I). Furthermore, total numbers of $CD3^+CD4^+$ T cells were significantly higher in the lungs after nasal immunization with the mucosal adjuvant CT, whereas the numbers of $CD3^+CD8^+$ T cells did not increase (Fig. 7). Thus, there was no major change in Th cells in lungs of mice after immunization with Ag alone; however, the inclusion of CT resulted in an overall increase in pulmonary Th cell numbers.

Nasal-pulmonary immunization induces expression of β-chemokine mRNA

We determined whether the increase in pulmonary cell numbers corresponded with increased β-chemokine expression, since these chemokines are chemotactic for T cells (60–62). Importantly, Th cell subsets differ in their responses to the β-chemokines, macrophage-inflammatory protein-1α (MIP-1α) and MIP-1β (63–65). Total RNA was isolated from lungs of mice 3 days after the second

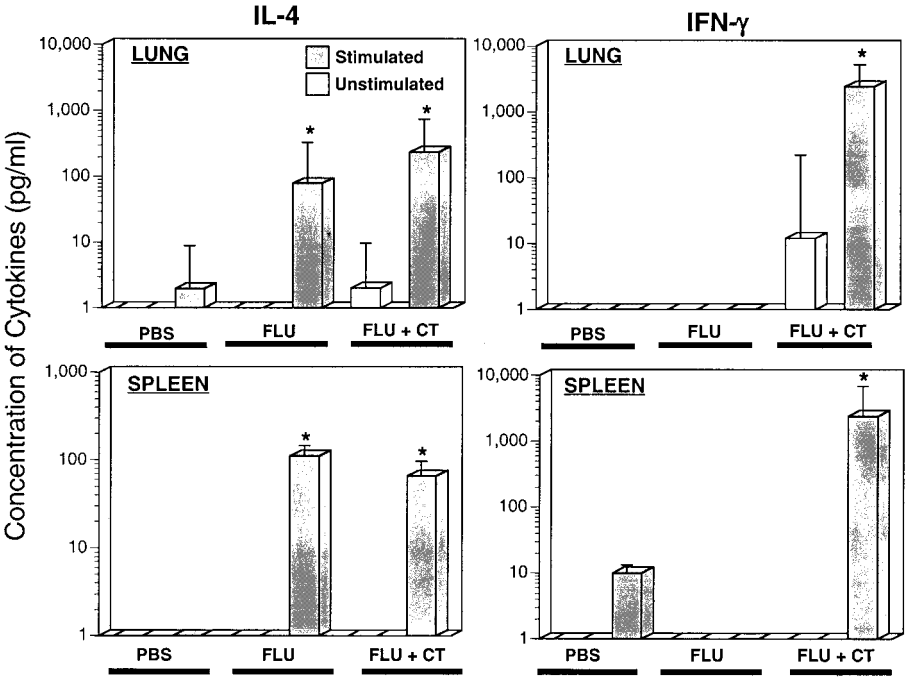

FIGURE 5. Ag-specific IL-4 and IFN-γ cytokine production in mice using CT. Mononuclear cells were isolated from the lungs and spleens of control and immunized mice. Lung and splenic lymphocytes were stimulated in vitro with or without influenza Ag. Four days later, culture supernatants were collected, and IL-4 and IFN-γ levels (picograms per milliliter) were measured using ELISA. Vertical bars represent the mean ± the SE ($n = 3$). ∗, Significant ($p \leq 0.05$) difference in cytokine levels from cells obtained from PBS-inoculated mice.

nasal-pulmonary immunization with influenza vaccine Ag alone or in combination with the mucosal adjuvant CT. The expression of MIP-1α, MIP-1β, monocyte chemoattractant protein-1, and RANTES mRNAs was analyzed by RT-PCR, as previously described (59). Significant induction of MIP-1β mRNA expression occurred in the lungs of immunized mice given Ag only or Ag plus CT; however, MIP-1β mRNA levels were significantly higher in mice immunized with Ag plus CT (Fig. 8). In contrast, increases in MIP-1α mRNA expression were significant only in mice receiving Ag plus CT. No differences in monocyte chemoattractant protein-1 and RANTES mRNA expression were found in the lungs of naive mice and any group of immunized mice.

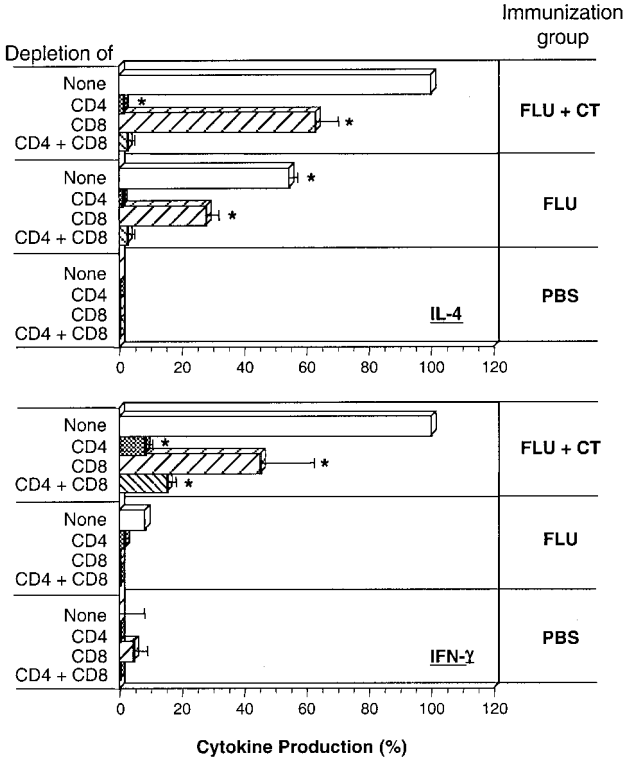

FIGURE 6. Ag-specific IL-4 and IFN-γ cytokine production after depletion of $CD4^+$ and/or $CD8^+$ lung cells collected from immunized mice. Lung cells from previously immunized mice were depleted of $CD4^+$ and/or $CD8^+$ T cells and placed in culture in the presence of influenza Ag. Four days later, levels of IL-4 and IFN-γ cytokines were evaluated using ELISA. Results are represented as the percentage of cytokine detected as compared with whole (undepleted (None)) cultures. Vertical bars and error bars represent the mean ± SE ($n = 2$). ∗, Significant ($p \leq 0.05$) reduction in detected cytokine levels as compared with whole (None) cell cultures.

Discussion

The purpose of the present study was to determine the Th cell subset responses generated in the lung after nasal-pulmonary immunization. In previous studies, we showed that nasal-pulmonary immunization resulted in the induction of Ag-specific IgE responses, and that use of CT markedly enhanced IgE Ab responses (28, 29). One also sees a massive infiltration of mononuclear cells into the lungs of BALB/c mice when CT is coadministered with Ag, but not when Ag is given alone. In preliminary studies, we also found a 2- to 3-fold increase in eosinophils in lung after nasal immunization with Ag plus CT. Based upon these past studies, we postulated that Th2 cell activity predominated after nasal-pulmonary immunization and contributed to the pulmonary inflammation

Table I. *T lymphocyte populations in pulmonary lymphocytes isolated from mice after nasal immunization*[a]

Group	% of $CD3^+$ Cells		CD4:CD8 Ratio[b]
	$CD3^+CD4^+$	$CD3^+CD8^+$	
PBS	69.3 (4.9)[c]	25.0 (2.6)	2.8:1 (0.45)
Influenza Ag alone	73.0 (2.4)	20.1 (2.9)	3.7:1 (0.63)
Influenza Ag + CT	80.2 (6.1)	13.1 (2.3)	6.3:1 (1.4)

[a] Mice were given PBS, influenza Ag alone, or influenza Ag plus CT nasally on days 0 and 7. Lung cells were isolated 3 days after the second immunization, and the cells were immunofluorescently stained and analyzed by flow cytometry ($n = 3$).
[b] The ratio of cells within tissues were based on the average percentage of cells from that tissue.
[c] Mean (SE) of percentage of fluorescently positive cells from the respective tissues.

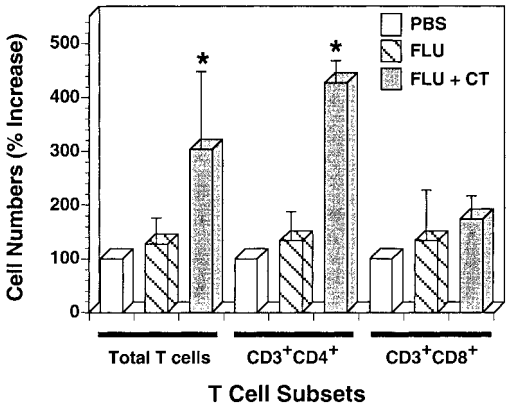

FIGURE 7. Numbers of $CD4^+$ T cells increase in the lungs after nasal immunization with CT. Mice were given a primary and secondary immunization with Ag (FLU) alone or with CT (FLU + CT). Three days later, lung lymphocytes were isolated, and cell numbers were determined. The numbers of total T cells ($CD3^+$) and $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell populations were determined using flow cytometry. Data represent the percentage of increase in the total number of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells based upon the percentage of $CD3^+$ cells compared with PBS (unimmunized) mice. Vertical bars represent mean ± SE ($n = 3$). *, Significant ($p \leq 0.05$) difference between immunized and unimmunized (PBS-inoculated) mice.

associated with the use of CT (29). This was supported by previous studies demonstrating preferential Th2 cell responses to parasites in the BALB/c mouse model (66, 67). Furthermore, a recent study (68) suggested that nasal infection with a parasite in a Th1-dominant mouse strain (C57BL/6N) resulted in pulmonary Th2 cell activation. However, studies have also shown that Th1-type responses can contribute to airway inflammation in a murine model of asthma with BALB/c mice (45). Thus, the goal of these studies was to determine the degree of pulmonary Th1 cell activation that occurs in a Th2-biased environment.

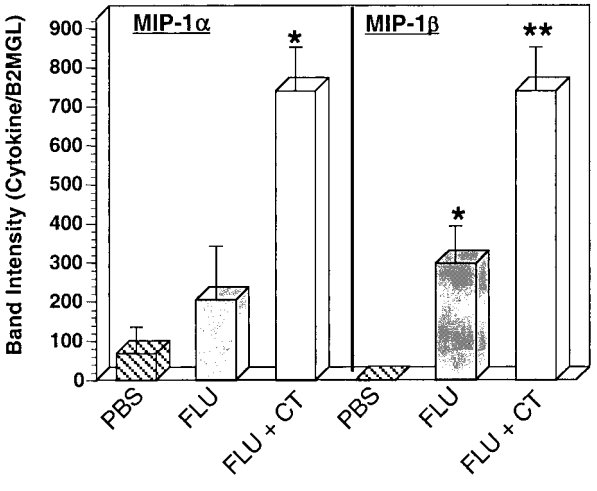

FIGURE 8. β-Chemokine mRNA expression after nasal immunization with CT. Three days after secondary immunization with PBS, influenza Ag alone, or influenza Ag in combination with CT, total RNA was isolated from mouse lungs. β-Chemokine mRNA expression was determined using RT-PCR. Relative differences in mRNA expression of cytokines between groups of mice were determined by comparing the ratio of cytokine mRNA with the housekeeping gene, β_2mGL, mRNA. Vertical bars represent mean ± SE ($n = 6$). *, Significant difference from PBS-treated mice; **, significantly ($p \leq 0.05$) higher band intensity than from all other groups of mice.

First, we noted that $CD4^+$ T cells were the major T cell population in the lungs of naive mice. After polyclonal stimulation of T cells using anti-CD3 Abs, the production of characteristic Th2 cytokine IL-4 was induced in culture. $CD4^+$ Th cells were the major source of IL-4 as their depletion eliminated production of this cytokine. $CD8^+$ T cells, not $CD4^+$ T cells, were the source of IFN-γ produced in response to polyclonal stimulation. In contrast to lung lymphoid cells, preliminary studies suggested that splenic Th cells produced both Th1 (IFN-γ) and Th2 (IL-4) cytokines after anti-CD3 mAb activation (data not shown) or after Con A stimulation (69), indicating a fundamental difference in $CD4^+$ Th cell populations residing in lungs and spleens. Thus, the resident $CD4^+$ T cell population in lungs is of Th2 type, and was consistent with the idea that humoral immunity, and in particular mucosal Abs, played a major role in protection from respiratory pathogens.

Influenza-specific Th2 cell responses in lungs were indeed generated after nasal-pulmonary immunization with influenza Ag only. The Th2 cytokine mRNAs, IL-4 and IL-5, were readily detected in the lungs of mice after nasal immunization with Ag alone. In contrast, increases in neither IFN-γ nor IL-2 cytokine mRNA expression were noted. In addition, IL-4 was found in culture supernatants of lung cells from immunized mice stimulated in vitro with Ag, and $CD4^+$ Th cells were the major source of IL-4 as their depletion eliminated IL-4 production. A similar IL-4 response was found in splenic lymphocytes, although the response from pulmonary lymphocytes was greater. It is now well established that IL-4 production is involved in mucosal immunity (39); in particular, IL-4 is an important factor in IgE production (70). In fact, our previous studies demonstrated that IgE responses are associated with nasal immunization (28, 29). Thus, influenza Ag introduced into the lung clearly generates almost solely Th2-type responses, reflecting the predominant resident Th2 environment.

The use of CT resulted in a significant enhancement of pulmonary $CD4^+$ Th2 cell responses; however, there was also a Th1 component to the response. Our previous studies demonstrated that inclusion of CT greatly enhanced both systemic and respiratory Ab responses to Ag (28), and the present study shows that CT also enhances T cell responses in the lungs and spleen. It is well established that CT enhances Th2-mediated responses after mucosal delivery (71). Indeed, Th2 cytokine (IL-4, IL-5, IL-6, IL-10, and IL-13) mRNA responses were greatly enhanced in the lungs of mice nasally immunized with influenza Ag and CT when compared with mice given Ag alone. The expression of Th1 cytokine (IL-2 and IFN-γ) mRNAs was also increased in the lungs of mice nasally immunized with Ag combined with CT. There was about a 25-fold greater expression of IL-4 mRNA in the lung after immunization with Ag plus CT as compared with either naive mice or those immunized with Ag alone. By comparison, the increase in IFN-γ mRNA expression due to CT was relatively modest (~5-fold). In addition, lung lymphoid cells isolated from mice immunized with influenza Ag plus CT secreted both Th1 and Th2 (IFN-γ and IL-4) cytokines after in vitro stimulation with Ag. We showed that Ag-specific $CD4^+$ Th cells were the major source of IL-4, as their depletion eliminated IL-4 production. However, $CD4^+$ Th cells also produced IFN-γ. $CD8^+$ T cells, however, accounted for ~50% of the IFN-γ produced, but $CD8^+$ T cells were not a source of IL-4. This is in contrast to other studies demonstrating little, if any, appreciable Th1 cell activation after oral CT given as a mucosal adjuvant (71). One possibility is that Th1-type responses are transient and are not seen at later time points. Preliminary results, however, demonstrate the presence of Th1-type responses in lungs at a later time point (data not shown). Furthermore, the activation of Th1 cells is consistent with our studies (29) and those by others (72) demonstrating a significant IgG2a

Ab response to Ags given nasally when CT was used as adjuvant. As the production of IgG2a is mediated by IFN-γ production, a product of Th1 cells (73), this supports the notion that nasal immunization with CT results in Th1 cell activation. As delayed-type hypersensitivity (DTH) reactions are mediated by Th1 cells (33), the generation of in vivo Th1 responses is also supported by preliminary studies demonstrating DTH responses against nasally inoculated Ag when CT is used (data not shown). Thus, our data show that CT, as anticipated, greatly enhances Th2 cell responses in the lung, but Th1 cell activation is also a significant component of the overall Th cell response.

The mechanisms through which CT enhances Th1 and Th2 cell responses in lungs are most likely linked to the development of immunopathologic reactions in the lung. These reactions are characterized by a massive infiltration of mononuclear cells around the pulmonary airways and vessels after nasal immunization using CT, but not Ag alone (28, 29). The present study demonstrated that there is an increase in the numbers of $CD4^+$ Th cells in lungs, suggesting that they are a major component of the inflammatory infiltrate. This is similar to previous studies demonstrating an increase in B cell responses in lungs due to the recruitment of extrapulmonary lymphocytes (28). The mechanisms through which lymphocyte populations are recruited are unknown; however, our results suggest that CT induces the production of chemotactic factors, such as β-chemokines, within the lungs. β-Chemokines are cytokines that are chemotactic for mononuclear cells (60–62). Importantly, Th cell subsets differ in their responses to the β-chemokines, MIP-1α and MIP-1β (65, 74). In fact, the inclusion of CT resulted in increased levels of both MIP-1α and MIP-1β mRNA, and the levels of MIP-1β were greater than in mice immunized with Ag alone. This is consistent with previous observations showing that MIP-1α and MIP-1β are preferentially chemotactic for Th1 cells, although Th2 cells respond to MIP-1α at a lower efficiency (65). This is also supported by differences in β-chemokine cell surface receptors on Th cell subsets (75, 76). β-Chemokines may also contribute to the inflammatory infiltrate seen as β-chemokines are involved in chronic inflammatory responses, such as DTH reactions (77). However, further studies are needed to confirm the role of β-chemokines in modulating Th cell responses and associated inflammatory reactions.

Overall, the resident Th cell populations in the lungs of BALB/c mice are of a Th2 type and reflect the type of immune responses generated to nasally delivered Ag. However, we found that Th1-type cell responses can be induced in the respiratory tract during instances of intense immune stimulation, as shown after nasal immunization with Ag plus CT. In another study, we have shown a similar shift in Th cell responses in *Mycoplasma* respiratory disease.[4] The Th1 cell responses were also shown to be present in other respiratory diseases, such as those due to influenza virus (78). Although pulmonary Th1 responses may have potentially beneficial effects, they can result in inflammatory responses along the airways and vasculature in lungs. These results have not only important implications for the use of nasal immunization to prevent respiratory disease, but they provide insight to the potential mechanisms of immunity involved in infectious and immunopathologic diseases of the lung. Using this immunization model, we will be able to elucidate the mechanisms critical to the development of Th cell responses and recruitment of cells to the lungs. Future studies can then determine whether these responses can be beneficially altered by treatment with recombinant cytokines or other stimulants that elicit IgE production or cellular recruitment, leading to vaccine/adjuvant combinations, which induce appropriate protective immune responses.

[4] H. P. Jones, L. Tabor, X. Sun, and J. W. Simecka. Depletion of $CD8^+$ T cells exacerbates $CD4^+$ Th cell-associated inflammatory lesions during murine mycoplasma respiratory disease. Submitted for publication.

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