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Induction and Inhibition of the Th2 Phenotype Spread: Implications for Childhood Asthma

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The interactions between genetic and environmental factors play a major role in the development of childhood asthma. We hypothesized that a pre-existing Th2/asthmatic response can promote Th2 responses to newly encountered Ags (i.e., phenotype spread). To test this hypothesis, we developed a mouse model in which the requirements for the induction and inhibition of phenotype spread to a clinically relevant neo-allergen (i.e., ragweed) were investigated. Our results indicate that 1) phenotype spread to the neo-allergen can be induced only within the first 8 h after a bronchial challenge with the first Ag (OVA); 2) Th2 differentiation of naive CD4+ T cells occurs in bronchial lymph nodes; 3) trafficking of naive CD4+ T cells to local lymph nodes and IL-4 produced by OVA-activated Th2 cells play essential roles in the differentiation of naive CD4+ T cells to Th2 cells; and 4) suppression of the production of chemokines involved in the homing of naive CD4+ T and Th2 cells to bronchial lymph nodes by a TLR9 agonist inhibited phenotype spread and abrogated the consequent development of experimental asthma. These findings provide a mechanistic insight into Th2 phenotype spread and offer an animal model for testing relevant immunomodulatory interventions. The Journal of Immunology, 2005, 174: 5864–5873.

Epidemiological evidence indicates that the key events that contribute to the development of allergic asthma occur in early childhood (1–3). An accelerated generation of Th2 memory responses against various aeroallergens also occurs during these early years (4, 5). The long term impact of these events involves multiple structural changes in lung parenchyma and airway tissues (airway remodeling) (6, 7). These alterations are largely irreversible and contribute to the significant surge in asthma morbidity and its related medical expenses in both developed and developing countries (5).

It is well established that the interactions between susceptibility genes and environmental factors, especially during childhood, play a major role in the development of allergic asthma (8, 9). Although this paradigm of genes-environment interaction (5) provides a general framework for the initial events that lead to asthma development, it ignores the important contribution of secondary microenvironmental alterations, e.g., cytokine milieu in the target organ, that are generated in a susceptible individual after these initial events have occurred.

Certain innate cytokines, such as IL-12 and IL-23, are known to affect the differentiation of naive CD4+ T cells toward a Th1 phenotype in vivo (10). In contrast, the roles of other innate cytokines in Th2 development have yet to be fully defined. Nevertheless, it is well established that IL-4, a Th2 cytokine, and IFN-γ, a Th1 cytokine, polarize naive CD4+ T cells toward a Th2 and a Th1 phenotype, respectively, under in vitro conditions (11, 12). Recent studies have demonstrated that a pre-existing memory-effector Th2 or Th1 immune response, via IL-4 or IFN-γ secretion, respectively, affects Th polarization to a codelivered neo-Ag in vivo (13–15).

Clinical data show that atopic patients sensitized to one allergen are quite likely to become further sensitized when exposed to new environmental allergens (16, 17). The existing Th2 phenotype to the first allergen facilitates subsequent priming of the naive CD4+ T cells to the neo-allergen (second allergen), termed Th2 phenotype spread. The present experiments were undertaken to develop an appropriate mouse model to study the conditions mandatory for the induction of Th2 phenotype spread and its impact on experimental asthma. Our data indicate that phenotype spread expands allergen-specific Th2 responses in a susceptible host and identify the cellular and molecular interactions that mediate this process. Furthermore, the mechanistic dissection of Th2 phenotype spread in our mouse model enabled us to design an immunomodulatory intervention that interferes with the development of Th2 phenotype spread and subsequent induction of experimental asthma and provides insight for the design of similar interventions that may be relevant for the inhibition of childhood asthma.

Materials and Methods

Animals

Wild-type (wt) BALB/c, DO11.10 (OVA TCR, MHC II-restricted) mice on the BALB/c background, SCID (BALB/c), IL-4R knockout (ko; BALB/c), C57BL/6, and lymphotoxin-α (Lta) ko (C57BL/6) mice were purchased from The Jackson Laboratory, OT-II (OVA TCR, MHC II-restricted) mice on the C57BL/6 background were a gift from Dr. W. Heath (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and

Department of Medicine, University of California at San Diego, La Jolla, CA 92033
Received for publication December 3, 2004. Accepted for publication February 27, 2005.

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1 This work is supported by National Institutes of Health Grants AI40682, AR057709, and HL79449.

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3 Abbreviations used in this paper: wt, wild type; AF-Dex, Alexa Fluor 647-dextran; AHR, airway hyper-reactivity; BALF, bronchial alveolar lavage fluid; BMDC, bone marrow-derived dendritic cell; i.n., intranasally; ISS-ODN, immunostimulatory oligodeoxynucleotide; ko, knockout; Lta, lymphotoxin-α; MDC, macrophage-derived chemokine; PTX, pertussis toxin; RW, ragweed extract; SIT, specific allergen immunotherapy; TARC, thymus- and activation-regulated chemokine; tg, transgenic; Th2CM, Th2 conditioned medium; PC200, concentration of methacholine required to increase the lung resistance by 200% from baseline; SLC, secondary lymphoid chemokine; ELC, EBV-induced molecule 1 ligand CC chemokine.
were bred at our animal facilities. IL-4R ko/D011.10 (BALB/c) were generated from their parental strains and were bred in our animal facilities. Mast cell-deficient (W/Wv) mice and their wt controls were obtained from The Jackson Laboratory. All animal procedures were performed following University of California-San Diego animal care guidelines.

Reagents and media

Immunostimulatory oligodeoxynucleotide (ISS-ODN; 5′-TGACTGTTGACGTTCCAGATGCA-3′) (18) was purchased from Trilink Technologies. OVA and pertussis toxin (PTX) were purchased from Sigma-Aldrich. Rag-weed extract (RW) was purchased from Greer Laboratories. Complete medium (RPMI 1640 (Irving Scientific) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, and 100 U/ml penicillin/100 μg/ml streptomycin) was used throughout the experiments. Endotoxin levels in the reagents were measured using the QCL1000 kit purchased from BioWhittaker. Reagents that contained <1 pg endotoxin/μg protein were used throughout the experiments.

Induction of experimental asthma

Induction of experimental asthma was performed as previously described (19). Briefly, mice were sensitized with OVA (20 μg) or RW (100 μg) adsorbed with 500 μg of alum by four weekly s.c. injections (Fig. 1A). Mice were challenged intranasally (i.n.) with OVA (20 μg) and/or RW (100 μg) on days 26 and 31 (first challenge). On days 45 and 52, mice were challenged i.n. with RW (100 μg; second challenge). In some experiments ISS-ODN was given 1 day before the first challenge (days 25 and 30), and these mice were challenged with RW (second challenge) on days 72 and 77. Twenty hours after the last challenge, airway responsiveness and airway resistance to methacholine were measured (20).

Compound 48/80 (1.2 mg/kg) was given daily for 4 days before the first challenge period as well as daily throughout the first challenge period for a total of 10 days (21, 22). Sodium cromoglycate (cromolyn; Sigma-Aldrich; 400 mg/kg) was i.p. injected 30 min before each the challenge of the first challenge period (23). Cimetidine (20 mg/kg), katancerin (20 mg/kg), or mepyramine (20 mg/kg) was given twice daily every day throughout the first challenge period (24). For the passive transfer model, OVA-hyperimmune serum, which was obtained from mice immunized with 20 μg of OVA/500 μg of alum four times, was transferred to the naive BALB/c mice. Serum obtained from a single naive or a single hyperimmunized mouse was transferred to a recipient mouse. The recipient mice were challenged with OVA and RW together (first challenge) and then challenged with RW alone (second challenge) as described above.

After the mice were killed, bronchial alveolar lavage fluid (BALF), lungs and spleen were recovered. The total number of cells and the differential count in BALF were determined as described previously (20). The level of each cytokine (IL-4, IL-5, and IL-13) in BALF was determined by ELISA (BD Pharmingen).

In the experiments in which colocalization of the Ag in lung APC was studied, mice were immunized by four weekly s.c. injection of 20 μg of OVA/500 μg of alum and challenged i.n. with 250 μg of FITC-OVA (Molecular Probes) and Alexa Fluor 647-dextran (AF-Dex; Molecular Probes), simultaneously or at various time intervals. Mice were killed 16 h after challenge with AF-Dex, and lungs and lymph nodes were harvested.

Preparation of OVA (OVA123-333)-specific Th2 cell lines

OVA-specific transgenic Th2 (tgTh2) cells derived from DO11.10 mice were prepared and characterized as described previously (20). To prepare tgOT-II Th2 cells, splenocytes from OT-II mice (2 × 10^9/ml) were cultured in complete medium with plate-bound anti-CD3 mAb (10 μg/ml; BD Pharmingen) and soluble anti-CD28 mAb (10 μg/ml; IL-4 (10 ng/ml; BD Pharmingen), anti-IL-12 mAB (C17.8; 5 μg/ml; BD Pharmingen), anti-IFN-γ (XMGI1.2; 5 μg/ml; BD Pharmingen), and IL-2 (10 ng/ml; BD Pharmingen) for 7 days. The cytokine profile in the supernatants of OVA-stimulated tgOT-II Th2 cells was high IL-4 (10 ± 0.1 ng/ml), IL-5 (2.1 ± 0.4 ng/ml), and IL-13 (33.1 ± 0.8 ng/ml) and low IFN-γ (3 ± 0.8 ng/ml).

Th2CM was prepared by culturing OVA-specific Th2 cells in complete medium for 48 h. The cell-free culture supernatant (Th2CM) was stored at −80°C until used in the experiments. Th2CM contained IL-4 (528 ± 16 ng/ml), IL-5 (129 ± 11 ng/ml), and IL-13 (213 ± 11 ng/ml) and was used at a final 1/50 dilution.

Adoptive transfer model

CD4+ T cells were isolated from splenocytes of BALB/c mice using anti-CD4 magnet beads (Miltenyi Biotec). CD4+ T cells (1.5 × 10^6) were i.v. transferred into SCID mice with or without in vitro-differentiated, OVA-specific tgTh2 cells (5 × 10^6) derived from DO11.10 mice (20). In the experiments in which the chemokine responsiveness of naive CD4+ T cells was blocked, isolated CD4+ T cells were treated with PTX (100 ng/ml) in complete medium for 2 h (25) and then washed extensively with complete medium before being co-transferred with tgTh2 cells. Mice were i.n. challenged with OVA (50 μg) and RW (100 μg) twice on day 1 after transfer, followed by two challenges with RW (100 μg) on days 19, 21, and 23 (Fig. 24). Twenty-four hours after the last challenge, airway hyper-reactivity (AHR) and eosinophil counts in BALF were assessed (20). To evaluate the trafficking of CD4+ T cells to the regional lymph nodes, naive CD4+ T cells were labeled with 1 μM CFSE (Molecular Probes) before transfer (26). Mice were challenged with OVA and RW one (Fig. 3) or three (see Fig. 6) times and were killed at the indicated time points.

In the experiments in which Lta ko mice were used, 5 × 10^6 tgTh2 cells derived from OT-II mice were transferred into C57BL/6 or Lta ko mice.
restimulated splenocytes from mice transferred with naive CD4+/H11001 cells and tgTh2 cells in lung after the second RW challenge. WtCD4 and tgTh2 cells were identified as CD4+/H11001 Cells were gated for CD4 and levels of cytokines in BALF. *

Mice were challenged i.n. with OVA and RW on day 1 (first challenge). All mice not receiving cell transfer served as the controls. Mice were i.n. challenged with OVA (100 μg) and RW (100 μg) daily for 5 days after the transfer, followed by two challenges with RW (100 μg) on days 19, 21, and 23 (Fig. 2A). Twenty-four hours after the last challenge AHR and eosinophil infiltration in BALF were assessed.

Culture of bone marrow-derived dendritic cells (BMDC) with naive DO11.10 tgCD4+/H11001 T cells

Myeloid BMDC were prepared from BALB/c or IL-4R ko mice as previously described (26). BMDC were incubated with OVA-derived peptide (aa 323–339) and then washed extensively with complete medium. Peptide-pulsed BMDC were cultured for 5 days with naive CD4+/H9262 T cells isolated from spleen of DO11.10 or IL-4R ko DO11.10 mice in complete medium containing Th2CM (1/50 dilution), Th2CM plus anti-IL-4 mAb (10 μg/ml), IL-4 (10 ng/ml), or IL-13 (10 ng/ml). The complete medium alone served as a control. In some experiments, BMDC were incubated with Th2CM (1/50 dilution) for 8 or 16 h before pulsing with peptide, washed, then incubated with DO11.10 CD4+/H11001 T cells. After incubation, DO11.10 cells were recovered, and 0.1 × 10⁶ recovered cells were restimulated with OVA peptide and irradiated splenocytes for 48 h. Another set of recovered DO11.10 cells was cultured with plate-bound anti-CD3 mAb (10 μg/ml; BD Pharmingen) and soluble anti-CD28 mAb (1 μg/ml) for 5 h. Monensin (2 μM) was added for the last 3 h. Cells were harvested and subjected to intracellular cytokine staining.

ELISA, FACS, and fluorescent microscopic analysis

BALF was collected after centrifugation, and the levels of cytokines (IL-5 and IL-13) in the supernatant were determined by ELISA (BD Pharmingen). Serum samples were collected before mice were killed, and RW-specific IgG1 and IgE were measured as previously described (27).

Single-cell suspensions from lungs, bronchial lymph nodes, and spleens were prepared by collagenase digestion as previously described (28). Cells were passed through a 10-μm pore size cell strainer and cultured with plate-bound anti-CD3 mAb (10 μg/ml) and soluble anti-CD28 mAb (1 μg/ml) for 5 h. monensin (2 μM) was added for the last 3 h. Cells were stained for surface CD3, CD4 (BD Pharmingen), and DO11.10 TCR (Caltag Laboratories) and were fixed with 2% paraformaldehyde. For intracellular IFN-γ and IL-4 staining, cells were permeabilized with CytoFix/CytoPerm (BD Pharmingen) and stained with FITC-conjugated anti-IFN-γ mAb or PE-conjugated anti-IL-4 mAb (BD Pharmingen). Stained cells were analyzed by FACS using a FACS Calibur flow cytometer (BD Bioscience Immunometry Systems). Naive CD4+/H9253 T and tgTh2 cells were identified as CD4+/H9262 DO11.10 and CD4+/H9262 DO11.10, respectively. Total cell number was calculated by multiplying the total number of live cells by the percentage of CD4+/H9262 DO11.10 or CD4+/H9262 DO11.10 cells in the cell suspension.

In some experiments, single-cell suspensions of lymph node cells from mice challenged with FITC-OVA and AF-Dex were stained for surface CD11c and analyzed by FACS. The total cell number of OVA+/H9262, Dex+/H9262, or OVA/Dex/Dex CD11c+/H9262 cells was calculated by multiplying the total number of live cells by the percentage of CD11c+/OVA, CD11c+/Dex, or CD11c+/OVA/Dex cells.

Quantitative expression of chemokine transcripts

Lymph node cells were prepared, and total RNA was isolated using TRIzol (Invitrogen Life Technologies). Real-time PCR was performed using ABI PRISM 7700 (Applied Biosystems). Primers were generated using Primer3 software (29) (www.genome.wi.mit.edu/genome_software/other/primer3.html). Specific primers for cyclophilin were used for standardization. For each sample, the levels of chemokine gene expression were normalized relative to its expression in mice transferred with naive CD4+/H11001 T cells and treated with PBS.

Statistical analysis

Data are expressed as the mean ± SEM and were compared using Student’s t test. A value of p < 0.05 was considered significant.

Results

Induction and characterization of phenotype spread in allergic mice

The experimental protocol for inducing phenotype spread is shown in Fig. 1A. BALB/c mice were first immunized by four weekly s.c. injections of OVA in alum. A control group was primed with RW in alum. On day 26, 5 days after the last s.c. OVA immunization, mice were challenged i.n. with either OVA or RW, or simultaneously with OVA and RW. After a resting period of 2 wk, the mice were challenged i.n. with RW alone. Mice primed and challenged with RW (group 1) developed experimental asthma, i.e., AHR (low concentration of methacholine required to increase the
lung resistance by 200% form baseline (PC200) and increased airway resistance as well as increased Th2-mediated lung inflammation (high eosinophil count and IL-5 and IL-13 levels in BALF; Fig. 1B). OVA-primed mice that were first challenged with OVA (group 2) as well as OVA-primed mice that were first challenged with RW (group 3) did not develop experimental asthma when challenged 2 wk later with RW. Only OVA-primed mice that received both OVA and RW during the first challenge (group 4) developed experimental asthma upon the second RW challenge (Fig. 1B). This group also developed high anti-RW IgE and IgG1 Abs as well as high RW-specific IL-5 and IL-13 responses (Fig. 1C).

IgE bound to mast cells, upon interaction with its specific Ag, triggers the release of granule products that induce Th2 development (30). To determine whether IgE bound to mast cells contributes to the induction of phenotype spread, we followed the protocol described above using mast cell-deficient mice (i.e., W/Wv) (30). Following this protocol we were able to induce Th2 phenotype spread in these mice (data not shown). To confirm the lack of involvement of mast cells in Th2 phenotype spread, we repeated the above experiments using adoptively transferred OVA-hyperimmune serum. The hyperimmune serum was transferred to naive BALB/c mice, which were then challenged with OVA and RW daily on days 1–5 (first challenge). The mice that did not receive tgTh2 cells served as controls. All mice were challenged with RW on days 18, 20, and 22 (second challenge). AHR (PC200), eosinophil counts, and levels of cytokines in BALF were determined.

**FIGURE 3.** Th2 phenotype spread depends on the trafficking of naive CD4\(^+\) T cells to regional lymph nodes. A, Protocol used in these experiments. CFSE-labeled naive CD4\(^+\) T cells were i.v. transferred into SCID mice with or without tgTh2 cells (DO11.10) on day 0. To inhibit the chemokine signaling of naive CD4\(^+\) T cells, in some experiments cells were treated with PTX in vitro, washed extensively, and transferred with tgTh2 cells. Mice were challenged with OVA and RW and were killed 16 and 36 h after the final challenge. B, FACS analysis of naive CD4\(^+\) T cells and tgTh2 cells in bronchial lymph nodes. Single-cell suspensions of lymph nodes and lungs were stained with mAbs to DO11.10 and CD4 and analyzed by FACS. Naive CD4\(^+\) T cells and tgTh2 cells were identified as CD4\(^+\) CFSE\(^-\) and CD4\(^+\) DO11.10\(^+\) cells, respectively. C, Total number of naive CD4\(^+\) T cells and tgTh2 cells in lymph nodes 16 h after the final challenge. Cell numbers were calculated by multiplying the total number of live cells by the percentage of positive cells in the cell suspension. D, Naive wtCD4\(^+\) T cells or PTX-treated wtCD4\(^+\) T cells were transferred to SCID mice with or without tgTh2 cells and challenged as described in Fig. 2. On day 24, AHR and eosinophil counts in BALF were assessed 24 h after the last challenge. Splenocytes were cultured with RW (200 \(\mu\)g/ml), and the levels of the secreted cytokines were determined by ELISA. E, In vitro-differentiated tgTh2 cells (OT II; \(5 \times 10^6\)) were transferred to C57BL/6 or Lta ko mice. Mice were challenged daily with OVA and RW on days 1–5 (first challenge). The mice that did not receive tgTh2 cells served as controls. All mice were challenged with RW on days 18, 20, and 22 (second challenge). AHR (PC200), eosinophil counts, and levels of cytokines in BALF were determined. * indicates P<0.05 compared with naive CD4\(^+\) T cell transfer group.
spread, the following treatments were applied during the first challenge; 1) compound 48/80 to deplete premade mast cell mediators (21, 22); 2) cromolyn, a mast cell stabilizer (23); and 3) various histamine receptor antagonists, including histamine type 1 or 2 receptor antagonists (mepramine and cimetidine, respectively), and serotonin type 2 antagonist (kantacancerin) (24). None of these treatments altered the response to RW challenge (second challenge; data not shown). Taken together, these data indicate that mast cells do not play a major role in the induction of Th2 phenotype spread.

Activated Th2 cells are sufficient for induction of Th2 phenotype spread

Recent studies demonstrated that a pre-existing Th immune response can modify the priming of naive Th cells toward new Ags (13, 15). Additional experiments were designed to address the role of activated Th2 cells in the induction of Th2 phenotype spread observed in the above-described experimental asthma model. For this purpose we used an SCID mouse adoptive transfer model (20, 32). These mice were adoptively transferred with in vitro-differentiated OVA-TCR tgTh2 cells (DO11.10) and naive wtCD4+ T cells (BALB/c) and thus lack OVA-specific Abs (Fig. 2). One day after cell transfer, mice were challenged twice simultaneously with OVA and RW, and after a 2-wk interval, they were challenged three times with RW (Fig. 2A). As shown in Fig. 2B, only mice adoptively transferred with both tgTh2 and naive CD4+ T cells developed experimental asthma upon RW challenge (i.e., low PC20, high eosinophil count, and high IL-5 and IL-13 levels in BALF). The recovery of wtCD4+ T cells in the lung was significantly greater in mice receiving both naive CD4+ T and tgTh2 cells than in mice receiving only one cell type (Fig. 2B). Additional evidence for Th2 phenotype spread was the 10-fold increased IL-4-producing wtCD4+ T cells recovered from the spleen (Fig. 2, D and E, respectively). Taken together, these results demonstrate that activated effector Th2 cells, and not mast cells, are sufficient for acquisition of a Th2 phenotype by naive CD4+ T cells under the conditions specified above.

Phenotype spread and subsequent experimental asthma depend on trafficking of naive CD4+ T cells to regional lymph nodes

To evaluate whether trafficking of naive CD4+ T cells to bronchial lymph nodes is mandatory for the induction of phenotype spread we labeled these cells with CFSE and treated them with PTX, an inhibitor of chemotaxis (Fig. 3A) (25). Treatment with PTX did not affect the in vitro proliferation, differentiation, or cytokine production of DO11.10 CD4+ T cells (data not shown). SCID mice were adoptively transferred with in vitro-differentiated tgTh2 and CFSE-labeled naive CD4+ T cells. One day after adoptive transfer, mice were challenged with OVA and RW, then evaluated 16 or 36 h later (Fig. 3A). PTX treatment abolished the trafficking of CFSE-labeled CD4+ T cells into bronchial lymph nodes (Fig. 3, B and C), but not their trafficking to lungs (data not shown). Mice adoptively transferred with PTX-treated CD4+ T cells did not develop AHR or show an increase in eosinophil count, and their RW-specific splenic Th2 response was abolished (Fig. 3D). The same experiment was repeated in Lta ko mice, which have underdeveloped lymph nodes (33, 34). As presented in Fig. 3E, these mice did not develop either AHR or show an increase in eosinophil count. Collectively, these data demonstrate that under the conditions specified above, the trafficking of naive CD4+ T cells to regional lymph nodes is mandatory for their differentiation to the Th2 phenotype, and their trafficking to bronchial lymph nodes is necessary for the induction of experimental asthma.

IL-4 produced by activated Th2 cells determines the phenotype of naive CD4+ T cells

To determine the role of Th2 cytokines secreted by activated Th2 cells in the acquisition of a Th2 phenotype by naive CD4+ T cells, naive CD4+ T cells (DO11.10) were cultured with BMDC (BALB/c) pulsed with MHC class II, OVA-derived peptide (aa 323–395) under the following conditions: 1) with the addition of conditioned medium generated by activated tgTh2 cells (DO11.10 and Th2CM), 2) with the addition of Th2CM and anti-IL-4 Abs, and 3) with the addition of rIL-4 or rIL-13. As controls, we used OVA peptide-pulsed BMDC cultured with OVA-specific tgTh1 or tgTh2 lines (DO11.10). After 5 days, CD4+ T cells (DO11.10) were isolated and incubated again with OVA peptide and freshly irradiated splenocytes (BALB/c), and the levels of various lymphokines were analyzed in the harvested supernatants. As shown in Fig. 4A, incubation of naive CD4+ T cells with Th2CM or rIL-4 resulted in the acquisition of a Th2 phenotype, i.e., OVA-specific induction of IL-4, IL-5, and IL-13. Addition of anti-IL-4 Abs to Th2CM abolished the development of the Th2 phenotype. In contrast, preincubation of BMDC with Th2CM (Pre-Th2CM) before their incubation with naive CD4+ T (DO11.10) did not affect the differentiation of naive CD4+ T cells toward a Th2 phenotype. As expected, the incubation of OVA peptide-pulsed irradiated splenocytes with tgTh1 or tgTh2 lines induced the secretion of IFN-γ and IL-4, respectively (Fig. 4A). The intracellular staining of IL-4 and IFN-γ in the various CD4+ T cell populations is shown in Fig. 4B. The incubation of OVA peptide-pulsed IL-4R ko BMDC (BALB/c) with tcgCD4+ T cells (DO11.10) and Th2CM resulted in their differentiation to Th2 cells, but when IL-4R ko tcgCD4+ T cells (derived from the intercross of IL-4R ko and DO11.10 mice) were incubated with wtBMDC and Th2CM, the naive tcgCD4+ T cells failed to differentiate to a Th2 phenotype and demonstrated a Th1 phenotype (Fig. 4, C and D). Taken together, these data indicate that the IL-4 derived from activated Th2 cells directly influences the Th2 differentiation of naive CD4+ T cells (i.e., phenotype spread), and that this differentiation is not mediated indirectly via the influence of IL-4 on BMDC.

Phenotype spread depends on the time interval between administration of first and second Ag

Our previous data indicate that the simultaneous administration of OVA and RW (i.e., at the first challenge) induces Th2 phenotype spread to RW (Figs. 1 and 2). To further explore the time requirements for the induction of phenotype spread, mice were primed as described in Fig. 1A and were challenged with OVA. RW was given simultaneously with or 8, 24, or 48 h after OVA challenge (Fig. 5A). OVA and RW given simultaneously or 8 h apart induced significant AHR and high eosinophil counts in BALF. In contrast, administration of RW 24 or 48 h after challenge with OVA did not induce experimental asthma (Fig. 5A).

To determine whether colocalization of both Ags in the same APCs is affected by the timing of Ag exposure, mice were immunized with OVA, as described in Fig. 1A, and were challenged i.n. with FITC-OVA and AF-Dex, simultaneously or 8, 24, or 48 h apart. Regional lymph nodes were collected after AF-Dex challenge, and CD11c+ cells were analyzed by FACS. As shown in Fig. 5B, 16 and 9.2% of the cells were positive for both OVA and Dex when the Ags were given simultaneously or 8 h apart, respectively. In contrast, <2% of the CD11c+ cells contained both Ags when the mice were inoculated with AF-Dex 24 and 48 h after FITC-OVA challenge (Fig. 5, B–D).
Administration of ISS-ODN prevents Th2 phenotype spread

TLR9 agonists (i.e., ISS-ODN, also known as CpG-ODN) are potent inhibitors of experimental asthma in rodents (35–37) and primates (38). This inhibition is transient and lasts for 6 wk (39). To determine whether ISS-ODN could prevent Th2 phenotype spread, we immunized mice as described in Fig. 1A, except that 50 μg of ISS-ODN was injected i.p. the day before i.n. OVA and RW challenge (Fig. 6A). When challenged with RW 2 wk after ISS-ODN administration, ISS-treated mice did not develop AHR to RW, and both their RW- and OVA-specific Th2 responses were suppressed (i.e., low RW- or OVA-specific IL-5 and IL-13 responses; data not shown). When challenged with RW 6 wk after ISS-ODN administration, ISS-treated mice did not develop AHR to RW and did not acquire a dominant RW-specific Th2 phenotype. However, the OVA-specific Th2 phenotype was not suppressed by ISS-ODN (Fig. 6B).

The effect of ISS-ODN on Th2 phenotype spread was also tested in the SCID adoptive transfer model (Fig. 6C). As shown in Fig. 6D, ISS-ODN treatment prevented trafficking of tgTh2 and wtCD4+T cells to regional lymph nodes (Fig. 6D). These data suggested that administration of ISS-ODN could affect chemokine production in bronchial lymph nodes. As shown in Fig. 6E, administration of ISS-ODN indeed inhibited the transcript levels of secondary lymphoid chemokine (SLC) (CCL21; 3.7-fold), EBV-induced molecule 1 ligand CC chemokine (ELC) (CCL19; 14-fold), which attract naive CD4+ T cells, thymus- and activation-regulated chemokine (TARC; CCL17; 3.5-fold), and macrophage-derived chemokine (MDC; CCL22; 7.4-fold), which attract Th2 cells. In contrast, administration of ISS-ODN enhanced the production of MIP-1α (CCL3; 1.8-fold) and MIP-1β (CCL5; 1.5-fold), which attract mainly monocytes. Taken together, these data indicate that ISS-ODN administration intervenes in the induction of Th2 phenotype spread mainly by inhibiting the trafficking of activated Th2 and naive CD4+T cells to regional lymph nodes.

Discussion

Our studies established an appropriate mouse model in which the in vivo conditions mandatory for induction of phenotype spread and subsequent induction of experimental asthma could be explored and verified. Our results underline the importance of phenotype spread in expanding the allergen-specific (Th2) repertoire in a susceptible host and potentially provide an experimental framework to study certain mechanistic aspects related to the development of childhood asthma and the nature of relevant immunomodulatory interventions.

The data presented in Fig. 3, indicate that the co-homing of already established Th2 cells and naive CD4+ T cells to bronchial lymph nodes, but not to lung, is a prerequisite for the differentiation of the naive CD4+ T cells toward a Th2 phenotype. Indeed, PTX treatment, which blocks chemokine signaling (40), abolished both the trafficking of naive CD4+ T cells to these lymph nodes and the development of RW-induced experimental asthma (Fig. 3). In contrast, the trafficking of these naive CD4+ T cells to lung was unaffected (data not shown). Similar results were obtained with Lta ko mice, which have a developmental defect of their lymph nodes (Fig. 3) (33, 34). These data indicate the crucial role of bronchial
lymph nodes in the inductive phase of phenotype spread and suggest that any immunomodulatory strategy aimed at blocking this process should target these secondary lymphoid organs. In this regard, the administration of ISS-ODN inhibited the homing of activated Th2 and naive CD4\(^{+}\) T cells to bronchial lymph nodes by suppressing the chemokine production essential for the trafficking of naive CD4\(^{+}\) T cells (e.g., SLC and ELC) and differentiated Th2 cells (e.g., TARC and MDC; Fig. 6). Thus, these results expand the already known antiasthmatic activities attributed to ISS-ODN at the effector phase, e.g., the induction of IDO (20), to include novel activities related to the inductive phase of experimental asthma.

Recent studies demonstrated that activated Th2 cells facilitated subsequent priming of naive T cells to new Ags (i.e., collateral priming), and that this priming is dependent on IL-4 production by the activated Th2 population (15). IL-4 is also known to be produced by mast cells and basophils (41–46). Our data indicated that IL-4 produced by activated Th2 cells is sufficient to expand the Th2 phenotype in the experimental system described in this study. The possibility that non-T, IL-4-producing cells contribute to the Th2 phenotype spread in other experimental systems has not been ruled out. Our data underscore the essential role of Th2-mediated IL-4 in the induction of phenotype spread (Fig. 3). These results strongly suggest that the IL-4 secreted by Ag (OVA)-activated Th2 cells directly influenced naive CD4\(^{+}\) T cells to acquire a Th2 phenotype and argue against any major contribution of IL-4-modified BMDC in the observed phenotype spread (47). Nevertheless, these DCs were an essential component for the induction of phenotype spread, because they copresented two different antigenic peptides to two different CD4\(^{+}\) T cell populations, i.e., activated Th2 cells and naive CD4\(^{+}\) T cells. Interestingly, we identified that the 8-h interval between the administration of the first and second Ag was the upper limit for both the in vivo and the in vitro experiments for the induction of phenotype spread (Fig. 5). This interval is most likely derived from the unique property of DCs to shut down their phagocytic machinery after their initial phagocytic event (i.e., the first Ag) (48). Thus, we favor a three-cell interaction model in which a DC functions mainly as a cellular scaffold that brings together memory-effector Th2 and naive CD4\(^{+}\) T cells via the copresentation of two different antigenic peptides. It is well established that when a memory-effector Th2 cell is activated, it rapidly secretes IL-4 (49). This IL-4 is then readily available to naive CD4\(^{+}\) T cells and influences their differentiation process. Due to

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\(^*\) indicates \(P<0.05\) compared with naive mice.

**FIGURE 5.** Th2 phenotype spread depends on the interval between administration of the first and the second Ag. A, Mice were immunized four times with OVA/alum and then i.n. challenged (first challenge) with OVA and RW, given simultaneously (0 h) or 8, 24, and 48 h apart. Mice were then challenged with RW as described in Fig. 1. Naive mice served as controls. Twenty-four hours after the last challenge, AHR and eosinophil counts in BALF were assessed. Data shown are the mean \(\pm\) SEM of four mice from one of four independent experiments that yielded similar results. *, \(p<0.05\) compared with naive mice. B–D, OVA/alum-immunized mice were i.n. challenged with FITC-OVA and AF-Dex, given simultaneously (0 h) or 8, 24, and 48 h apart. B, Single-cell suspensions of lymph nodes were stained for surface CD11c and analyzed by FACS. C, Total cell number of FITC-OVA\(^{+}\) and/or AF-Dex\(^{+}\) cells in the CD11c\(^{+}\) population in lymph nodes. D, Lymph node cells that contain FITC-OVA (green) and AF-Dex (red). The images were taken unfiltered (1, 5, and 9), with a green filter for FITC (2, 6 and 10), or with a red filter for AF647 (3, 7, and 11) (magnification, \(\times\) 400). The images were optically merged (4, 8 and 12). The yellow color represents colocalization of green and red fluorescence. Data shown are from one of two independent experiments that yielded similar results.
the substantial difference in time requirements between the activation of memory Th cells \((1/\text{H}11011\text{ h})\) (49) and the differentiation of naive CD4\(^+\) T cells \((1/\text{H}11011\text{ h})\) (50, 51), this model also suggests that several rounds of memory-effector Th2 cells are involved in the differentiation of a single naive CD4\(^+\) T cell. Hence, the naive CD4\(^+\) T cell, although stimulated via its TCR engaged with its corresponding peptide-loaded MHC class II on DC, is exposed to several rounds of IL-4 produced by the continuous activation of memory-effector Th2 cells. Each individual round of IL-4 further increases the chances of influencing CD4\(^+\) T cell to acquire a complete Th2 phenotype.

What are the implications of the induction and the inhibition of Th2 phenotype spread? Recent epidemiological studies have indicated that the key events essential for the development of persistent allergic asthma, such as contact with aeroallergens, occur in early childhood (1, 2, 52). There is also mounting evidence that the basis of Th2 memory against these allergens and the subsequent clinical outcome (e.g., the allergic march) are generated during these early years (53–55). Based on the results of the current study, we propose that the phenotype spread, or a similar mechanism, is responsible for the accelerated development of the allergic/asthmatic phenotype in a susceptible individual in early childhood. Early childhood therefore appears to be the appropriate window for therapeutic intervention (5). It is plausible that the inhibition of phenotype spread in these early years would have the best success in inhibiting the severity of childhood asthma and its persistence through adulthood. This hypothesis is nicely reinforced by recent epidemiological studies indicating that early exposure to certain microbial products, e.g., LPS (the ligand of TLR4), markedly decreases the risk of developing allergic asthma (56). It is believed that the microbial burden in childhood counter-regulates and limits the generation of an allergen-specific Th2 repertoire (57). Our experimental data (Fig. 6) strongly support these epidemiological observations. The administration of ISS-ODN, a synthetic form of bacterial DNA and the ligand of TLR9, inhibited the production of chemokines essential for the homing of naive CD4\(^+\) T cells and Th2 cells (i.e., SLC and ELC) and Th2 cells (i.e., TARC and MDC). Consequently, ISS-ODN administration inhibited Th2 phenotype spread and subsequent development of experimental asthma. Interestingly, a recent human trial with ISS-based immunotherapy against the same allergen (ragweed) documented the reduction of TARC and MDC in ISS-treated, but not in PBS-treated, patients, suggesting that similar mechanisms are operating in both ISS-treated mice and ISS-treated humans (58).

Accumulating evidence suggests that specific allergen immunotherapy (SIT; i.e., allergen desensitization) inhibits the allergen-induced Th2 response via the induction of regulatory T cells (59,
60). Recent studies have documented that SIT to an aeroallergen (e.g., house dust mite) given in early childhood resulted in an allergen-specific clinical improvement and prevented the onset of new sensitizations in these children (16, 61), including the reduction in the incidence of allergic asthma (62). We suggest that in this case, allergen-activated regulatory T cells recruited to the regional lymph nodes inhibited Th2 phenotype spread to other coinhabited allergens. We speculate that if the inhibition of phenotype spread by SIT to a seasonal allergen (e.g., cat dander), which provides a continuous population of activated regulatory T cells, might result in a more profound inhibition of phenotype spread and allergic asthma in early childhood.

In summary, the Th2 phenotype spread expands the sensitization to inhaled allergens in already Th2-primed and asthmatic host. This process occurs in regional lymph nodes and is mediated by IL-4 produced by activated Th2 cells. We propose that immunomodulatory interventions aimed at inhibiting phenotype spread during early childhood, e.g., administration of ISS-ODN (allergen modulatory interventions aimed at inhibiting phenotype spread and allergic asthma in early childhood.


cells determine development of allergen-induced eosinophilic airway inflammation in mice. J. Exp. Med. 189:553.


