IL-4 differentially regulates eotaxin and MCP-4 in lung epithelium and circulating mononuclear cells

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Eotaxin is a unique member of the C-C family of chemokines that selectively recruits eosinophils into the tissues of rodents and primates (9, 48, 52). The mechanism of this selective effect on eosinophils is believed to relate to its specificity for the CCR-3 chemokine receptor, which is expressed predominantly on eosinophils (11, 16, 27, 28, 47). In addition, eotaxin activates proinflammatory functions of eosinophils, such as production of reactive oxygen metabolites and upregulation of the integrin CD11b (13, 60). The ability of eotaxin to recruit and activate eosinophils implies that eotaxin is involved in the pathophysiology of diseases where eosinophils are present and can cause or contribute to relevant disease processes (32). MCP-4 is a C-C chemokine that has nucleotide sequences similar to those of eotaxin (4, 17, 20, 63). The binding of MCP-4 to both CCR-2B and CCR-3 receptors accounts for its potent chemotaxis for monocytes and eosinophils (17, 63). The role of MCP-4 in disease is less well defined,
but recent reports imply that, like eotaxin, it is involved in inflammatory cell recruitment in allergic disease (17, 56). The CCR-3 receptor has been found to be expressed not only by eosinophils but by basophils and Th2 lymphocytes that are also relevant to allergic inflammation (53, 64). These observations suggest that the CCR-3 ligands such as eotaxin and MCP-4 are also important in recruiting basophils and for establishing or amplifying the Th2 response in allergic airway inflammation.

We have reported that the proinflammatory cytokines IL-1β and tumor necrosis factor (TNF-α) induce eotaxin expression in human lung epithelial cells (34). Recent reports imply that mononuclear cells are also a source of eotaxin protein in the human airway (32, 48). Similarly, MCP-4 is induced by these cytokines in bronchial epithelial cells and mononuclear cells, and these cells express MCP-4 in the airways of patients with asthma and sinusitis (17, 31). We also reported that eotaxin is induced by TNF-α in a human monocytic cell line and peripheral blood monocytes (42). These observations suggest that these chemokines can be mobilized not only in lung epithelial cells but also in circulating mononuclear cells. However, the mechanisms by which the tissue chemokine gradients are established despite chemokine elaboration at both epithelial and vascular sites have not been elucidated.

The proallergic effects of the Th2-type cytokine IL-4, which are thought to be highly relevant to the pathogenesis of asthma, include the induction of iNOS by IFN-γ and the promotion of the clonal expansion of airway Th2 lymphocytes, which elaborate the eosinophil growth and differentiation factor IL-5 (58). IL-4 can also have anti-inflammatory effects by inhibiting the production of IL-1, IL-8, TNF-α, and interferon (IFN)-γ-inducible protein-10 in monocytic cells (12, 15, 33, 55). Furthermore, IL-4 has been shown to regulate IL-8 expression differently in cells of alternative types (55). We propose that this differential regulation facilitates the creation of chemokine gradients that promote eosinophil migration from the vascular space to the airway epithelium. To test this hypothesis, we studied differences in the effects of IL-4 on eotaxin and MCP-4 expression among human peripheral blood mononuclear cells (PBMCs), lower airway mononuclear cells obtained by bronchoalveolar lavage (BAL), and human lung epithelial cells.

**MATERIALS AND METHODS**

**PBMC culture.** PBMCs were isolated from the heparinized venous blood of 14 healthy volunteers and 8 platelet donors by density gradient centrifugation with Histopaque 1077 (Sigma, St. Louis, MO). PBMCs from the platelet donors were used for the experiments requiring a large number of PBMCs, e.g., time courses of stimulated PBMCs (n = 2), time courses of monocytes and lymphocytes (n = 2), effects of IL-4 on monocytes and lymphocytes (n = 2), and IFN-γ effects on PBMCs (n = 2) as described below. All of the other experiments were performed with PBMCs from the healthy volunteers. This procedure yielded ~1 × 10^8 PBMCs from 100 ml of venous blood from each volunteer and 0.5–1.5 × 10^6 cells from each donor, with a PBMC purity >98%. None of the subjects had allergic disease or peripheral blood eosinophilia (eosinophil percentages were <5%), and all gave written informed consent with the prior approval of the appropriate institutional review board. PBMCs from the volunteers and the platelet donors were cultured in RPMI 1640 medium with 10% heat-inactivated FBS on 10-cm culture plates (Falcon 3003; Becton Dickinson, Lincoln Park, NJ) at a concentration of 5 × 10^6 cells/ml. After a 2-h incubation, PBMCs from 10 volunteers were cultured for an additional 4 h in the presence or absence of IL-1β (10 ng/ml), IL-1β + IL-4 (10 ng/ml), or IL-1β + dexamethasone (1 μM). We studied the PBMC dose response by adding increasing doses of IL-4 (0.1–10 ng/ml) at the time of stimulation with IL-1β (10 ng/ml) and TNF-α (10 ng/ml) using the cells obtained from two volunteers. The cells were harvested after a 4-h stimulation with cytokitons after 2 h of incubation. Time-course studies used PBMCs harvested from the same platelet donors (n = 2) 1, 2, 4, 8, and 24 h after stimulation with IL-1β (10 ng/ml), TNF-α (10 ng/ml), IL-4 (10 ng/ml), or IL-1β + IL-4. Time-course experiments for unstimulated PBMCs were performed using two volunteers. These time-course experiments were initiated without a 2-h incubation on the culture plates. In experiments in which it was involved, dexamethasone was added 30 min before cell stimulation. In the monocyte and lymphocyte experiments, PBMCs from four platelet donors were cultured overnight in RPMI 1640 medium with 10% type AB human serum (Sigma). After nonadherent cells were removed, adherent cells were washed three times with PBS and designated as monocytes. Monocyte purity was judged to be >80% by microscopic examination. Lymphocytes were isolated from the nonadherent cell population by discontinuous density gradient centrifugation with Percoll (Sigma; see Ref. 23). Lymphocyte purity was determined, and subpopulations were identified by flow cytometry (FACScan; Becton Dickinson) with antibodies to human CD14, CD3, CD19, and CD56 (PharMingen, San Diego, CA). Lymphocyte purity was >98%, and the percentages of T cells, B cells, and NK cells were 75–89%, 5–12%, and 8–11%, respectively.

**BAL cell culture.** Bilateral BAL was performed in five healthy volunteers who did not overlap with those in PBMC experiments. The subjects were lifelong nonsmokers, had no positive allergic disease responses on a standard questionnaire, had normal pulmonary function and methacholine sensitivity, and did not demonstrate dermal sensitivity to a battery of 12 standard allergens. Prior written informed consent was obtained from all of the subjects. A bronchoscope was wedged in a segment of the right middle lobe or lingula. Sterile normal saline was instilled through the bronchoscope in 50-ml volumes to a total of 150 ml in the middle lobe and lingula. BAL fluid cells were cultured in RPMI 1640 medium with 10% FBS for 2 h on 10-cm culture plates at a concentration of 0.5–1.0 × 10^6 cells/ml. After a 2-h incubation, the cells were cultured for an additional 4 h in the presence or absence of IL-1β (10 ng/ml), IL-1β + IL-4 (10 ng/ml), IL-1β + dexamethasone (1 μM), or TNF-α (10 ng/ml). In experiments in which it was involved, dexamethasone was added 30 min before cell stimulation.

**Epithelial cell culture.** A549 cells, derived from a lung adenocarcinoma with the alveolar type II cell phenotype, were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in F-12-K medium with 10% FBS. Before stimulation with cytokines (24 h), the medium was exchanged for an identical formulation not containing FBS. BEAS-2B cells (a generous gift from C. Harris, National Cancer Institute, Bethesda, MD), a human bronchial epithelial cell line transformed by hybrid adenovirus SV-40, were cultured in DMEM-F-12 medium with 10%
Effects of IL-4 on eotaxin and MCP-4 expression. To investigate whether the presence of IFN-γ alters the effects of IL-4 on eotaxin mRNA expression, PBMCs, A549 cells, and SAEC were cultured in the presence or absence of IL-4 (10 ng/ml), TNF-α (10 ng/ml), IFN-γ (10 ng/ml), IL-1α + TNF-α, TNF-α + IFN-γ, TNF-α + IFN-γ + IL-4, or TNF-α + IFN-γ + dexamethasone (1 μM) for 6 h. PBMCs were obtained from two healthy volunteers and two platelet donors and were incubated for 2 h before stimulation with cytokines in RPMI 1640 medium with 10% FBS. BEAS-2B cells were cultured in the presence or absence of TNF-α (10 ng/ml), IFN-γ (100 ng/ml), TNF-α + IFN-γ, TNF-α + IFN-γ + IL-4 (10 ng/ml), or IL-1α + IFN-γ + dexamethasone (1 μM) for 4 h. PBMCs were obtained from two healthy volunteers and two platelet donors and were incubated for 2 h before stimulation with cytokines in RPMI 1640 medium with 10% FBS. BEAS-2B cells were cultured in the presence or absence of IL-4 (10 ng/ml) at a concentration of 5 × 10⁶ cells/ml after a 2-h incubation without stimulation. A549 cells were cultured in F-12-K medium with 10% FBS. Before stimulation (24 h), FBS was removed from the medium. The cells were cultured for an additional 24 h in the absence or presence of IL-4 (10 ng/ml), IL-1α (10 ng/ml), IL-1β + IL-4 (10 ng/ml), or IL-1α + dexamethasone (1 μM) at a concentration of 5 × 10⁶ cells/ml. FBS was added to each well. The eotaxin signal was detected at the site expected for a 1.3-kb transcript. The blots of eotaxin, MCP-4, IL-8, and GAPDH were exposed to BioMax films (Kodak, Rochester, NY) for 40, 40, 3, and 5 h, respectively. In each case, the eotaxin signal was detected at the site expected for a 0.8-kb transcript, MCP-4 at that expected for a 0.8-kb transcript, IL-8 at that expected for a 1.8-kb transcript, and GAPDH at that expected for a 1.3-kb transcript. ELISA for eotaxin, MCP-4, GM-CSF, IL-3, and IL-5. ELISA was performed with cell supernatant of PBMCs and A549 cells. PBMCs isolated from five control subjects whose PBMCs were used for RNA analysis were cultured in RPMI 1640 medium with 10% FBS on culture plates for 24 h in the absence or presence of IL-4 (10 ng/ml), IL-1α (10 ng/ml), IL-1β + IL-4 (10 ng/ml), or IL-1α + dexamethasone (1 μM) for 4 h. PBMCs were obtained from two healthy volunteers and two platelet donors and were incubated for 2 h before stimulation with cytokines in RPMI 1640 medium with 10% FBS. Before stimulation (24 h), FBS was removed from the culture medium. The cells were cultured for an additional 24 h in the absence or presence of IL-4 (10 ng/ml), IL-1α (10 ng/ml), IL-1β + IL-4 (10 ng/ml), or IL-1α + dexamethasone (1 μM). After culture, cell supernatant was collected for assay. ELISA for human eotaxin was performed as previously described (32, 34). Briefly, each well of a high-binding-efficiency 96-well plate was coated with 200 ng of a mouse anti-eotaxin monoclonal antibody, designated 2A12. The plate was blocked with a 3% solution of BSA (Sigma) in PBS with 0.02% sodium azide. After being washed with PBS, standards or samples were added; the plate was incubated for 2 h at room temperature in a humid environment and was washed again with PBS, and 50 μl of a rabbit anti-eotaxin polyclonal serum diluted 1:500 in blocking buffer were added to each well. The plate was washed with PBS after a 2-h room temperature incubation; 50 μl of a horseradish peroxidase-linked anti-rabbit IgG derived from goats (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were diluted 1:1,000 in blocking buffer and added to each well. After a 90-min room temperature incubation, the plate was developed by the 3,3′,5,5′-tetramethylbenzidine microwell-peroxidase substrate method according to the instructions of the manufacturer (Kirkegaard & Perry Laboratories). The antibodies reacted strongly to 100 ng of human eotaxin but did not react to 100 ng of human MCP-1, -2, -3, -4; macrophage inflammatory protein-1α or -1β; or RANTES. Under these conditions, this assay was sensitive to 15 pg/ml. The amount of eotaxin recovered from PBMC and A549 cell supernatants was calculated from the ELISA concentration and the cell numbers and is reported as the amount recovered per 10⁶ cells. ELISA for human MCP-4 was performed as follows (31). Briefly, each well of a high-binding-efficiency 96-well plate was coated with 100 ng of a mouse anti-MCP-4 monoclonal antibody, designated L1290. The plate was blocked with a 3% solution of BSA (Sigma) and goat serum (GIBCO BRL, Gaithersburg, MD) in PBS with 0.02% sodium azide. After a wash with PBS with 0.05% Triton X-100, standards or samples were added; the plate was incubated for 2 h at room temperature in a humid environment and was washed again with PBS, and 50 μl of a rabbit anti-MCP-4 polyclonal serum diluted 1:5,000 in blocking buffer were added to each well. The plate was washed with PBS after a 2-h room temperature incubation; 50 μl of a horseradish peroxidase-linked anti-rabbit IgG derived from goats (Kirkegaard & Perry Laboratories) were diluted 1:2,000 in blocking buffer and added to each well. After a 60-min room temperature incubation, the plate was devel-
Effects of IL-4 on stability of eotaxin mRNA in PBMCs and A549. To examine the effects of IL-4 on the stability of eotaxin mRNA, actinomycin D was added to PBMCs and A549 cells for inhibiting transcription (29). PBMCs isolated from four healthy volunteers were stimulated with IL-1β (10 ng/ml) for 8 h. Next, the culture medium was exchanged for IL-1β-free medium containing actinomycin D (10 μg/ml). The cells were harvested 1 and 4 h after the addition of actinomycin D in the presence or absence of IL-4 (10 ng/ml). A549 cells were stimulated with IL-1β (10 ng/ml) for 4 h. Next, the culture medium was exchanged for an IL-1β-free formulation containing actinomycin D (10 μg/ml). After incubation with or without IL-4 (10 ng/ml), the cells were harvested 1 and 4 h later (n = 4).

Statistics. Values for eotaxin, MCP-4, and IL-8 mRNA densities in PBMCs from the 10 healthy volunteers, in BAL fluid cells from the 5 control subjects, and in A549 cells from 5 different experiments are expressed as means ± SE as are values for eotaxin densities in PBMCs from the 4 control subjects in IFN-γ experiments. Eotaxin, MCP-4, GM-CSF, IL-3, and IL-5 protein levels in PBMCs and in A549 cells are also expressed as means ± SE (n = 5). These values were tested for normality and equal variance and were compared by ANOVA as appropriate. The Student-Newman-Keuls test was performed as a post hoc test. To compare the eotaxin mRNA signal with and without IL-4 in the experiments with actinomycin D, the unpaired t-test was used. A P value <0.05 was considered significant.

**Fig. 1.** Regulation of eotaxin, monocyte chemoattractant protein (MCP)-4, and interleukin (IL)-8 mRNA expression in peripheral blood mononuclear cells (PBMCs). A: representative series of Northern blots from 10 distinct subjects. The concentrations of IL-1β, IL-4, and dexamethasone (Dex) in the culture medium were 10 ng/ml, 10 ng/ml, and 1 μM, respectively. The cells were harvested 4 h after stimulation. The blots of eotaxin, MCP-4, IL-8, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were exposed to films for 40, 40, 3, and 5 h, respectively. B: mean ± SE values for the mRNA density ratio of eotaxin to GAPDH for each of the groups (n = 10). US, unstimulated. C: mean ± SE values for the mRNA density ratio of MCP-4 to GAPDH for each of the groups (n = 10). D: mean ± SE values for the mRNA density ratio of IL-8 to GAPDH (n = 10). *P < 0.01 and #P < 0.01 compared with the other 3 groups.
RESULTS

Regulation of eotaxin, MCP-4, and IL-8 mRNA expression in PBMCs. PBMCs from normal subjects expressed significantly more eotaxin mRNA after a 4-h incubation with IL-1β than did unstimulated cells (Fig. 1, A and B). When IL-4 was added at the time of IL-1β stimulation, eotaxin mRNA expression was suppressed to levels significantly lower than those observed after 4 h in unstimulated cells. When 1 μM dexamethasone was added 30 min before stimulation, IL-1β-induced eotaxin expression was reduced to levels equivalent to those of unstimulated cells. Similarly, MCP-4 mRNA expression in PBMCs was increased by IL-1β stimulation, and the addition of IL-4 decreased this expression (Fig. 1, A and C). As with its effects on eotaxin, dexamethasone diminished MCP-4 expression. IL-8 mRNA expression was detected in unstimulated PBMCs and enhanced by IL-1β (Fig. 1, A and D). Similar to our findings for eotaxin and MCP-4, IL-4 and dexamethasone suppressed IL-1β-induced IL-8 mRNA expression.

Maximal eotaxin mRNA was expressed 8 h after stimulation with TNF-α or IL-1β (Fig. 2A). Unstimulated PBMCs also had detectable eotaxin mRNA expression after culture for 4–8 h on the plates (Fig. 2A). The addition of IL-4 at the time of stimulation decreased the expression in cytokine-stimulated PBMCs at all time points studied and in unstimulated cells at 4 and 8 h after culture was started. IL-4 did not induce significant eotaxin mRNA except the faint expression detected at 24 h. Modest doses of IL-4 suppressed IL-1β- and TNF-α-induced eotaxin expression in PBMCs (Fig. 2B).

Regulation of eotaxin, MCP-4, and IL-8 mRNA expression in BAL fluid cells. BAL fluid cell differential counts for five normal subjects revealed 91.4 ± 2.3% alveolar macrophages and 8.6 ± 2.3% lymphocytes. No significant eotaxin or MCP-4 mRNA expression was detected in unstimulated cells at 4 and 8 h after culture was started. IL-4 did not induce significant eotaxin mRNA except the faint expression detected at 24 h. Modest doses of IL-4 suppressed IL-1β- and TNF-α-induced eotaxin expression in PBMCs.
clearly detected in BAL fluid cells in the absence or presence of stimulation with IL-1β or TNF-α (Fig. 3, A and B). Dexamethasone tended to inhibit IL-8 expression more efficiently than did IL-4.

Regulation of eotaxin, MCP-4, and IL-8 mRNA expression in epithelial cells. Unstimulated A549 cells did not express detectable eotaxin mRNA, but expression was detected after a 4-h incubation with IL-1β (Fig. 4, A and B). A549 cells faintly expressed eotaxin mRNA without stimulation when FBS was present in the culture medium (data not shown). When IL-4 was added at the time of IL-1β stimulation, eotaxin mRNA expression was not suppressed significantly. When 1 μM dexamethasone was added 30 min before stimulation, IL-1β-induced eotaxin expression was reduced significantly but was still greater than that in unstimulated cells. Similarly, MCP-4 mRNA expression in A549 cells was increased in the presence of IL-1β stimulation, but the addition of IL-4 only slightly decreased its expression (Fig. 4, A and C). In contrast, 1 μM dexamethasone significantly reduced MCP-4 expression. IL-8 mRNA expression was undetectable in unstimulated A549 cells but was induced by IL-1β (Fig. 4, A and D). Treatment of the cells with IL-4 had little effect on IL-1β-induced IL-8 expression, whereas 1 μM dexamethasone significantly reduced this expression to levels slightly greater than those in unstimulated cells. TNF-α induced more eotaxin mRNA expression than IL-1β in BEAS-2B cells, whereas eotaxin expression was undetectable in unstimulated cells in the presence or absence of IL-4 (Fig. 4E). IL-1β-induced eotaxin mRNA expression was enhanced in the presence of IL-4 but was significantly reduced by 1 μM dexamethasone. Costimulation of BEAS-2B cells with TNF-α and IL-1β further enhanced eotaxin expression, which was enhanced in the presence of IL-4 and suppressed by dexamethasone. IFN-γ slightly induced eotaxin and MCP-4 mRNA, which was enhanced in the presence of TNF-α in SAEC (Fig. 4F). Chemokine expression was slightly enhanced in the presence of IL-4 in the primary culture of human epithelial cells. Neither IL-1β, TNF-α, IL-4, nor IL-1β + TNF-α induced the chemokine expression in these cells. The pattern of chemokine expression observed in NHBE cells was similar to that in SAEC, but the expression was fainter than that in SAEC (data not shown). Because IL-1β did not induce significant chemokine expression in BEAS-2B cells and primary culture of epithelial cells, various combinations of cytokines were examined to induce sufficient chemokine expression in these cells. The effects of IL-4 on chemokine expression were then investigated in the presence of comparable signal.

Maximal eotaxin mRNA expression occurred 4 h after stimulation with IL-1β in A549 cells (Fig. 5A). The addition of IL-4 at the time of stimulation did not decrease expression at any time point studied. IL-4 alone did not induce significant eotaxin mRNA at any time points studied. Dose-response experiments of IL-4 demonstrated that IL-1β-induced eotaxin expression was not inhibited by IL-4 from 0.1 to 100 ng/ml (Fig. 5B). Maximal eotaxin mRNA expression occurred 8 h after stimulation with TNF-α and IFN-γ in SAEC (Fig. 5C). The addition of IL-4 at the time of stimulation increased eotaxin expression at 4–24 h after stimulation. Dose-response experiments of IL-4 demonstrated that cytokine-induced eotaxin expression was slightly enhanced by IL-4 from 0.1 to 100 ng/ml (Fig. 5D).

Fig. 3. Eotaxin, MCP-4, and IL-8 mRNA expression in bronchoalveolar lavage (BAL) fluid cells. A: 2 representative Northern blots from 5 control subjects. B: mean ± SE values for the mRNA density ratio of eotaxin (solid bars), MCP-4 (hatched bars), and IL-8 (open bars) to GAPDH (n = 5).
Effects of IFN-γ on eotaxin mRNA expression. IL-1β-induced eotaxin mRNA expression was slightly enhanced in the presence of IFN-γ in PBMCs, but the inhibitory effects of IL-4 and dexamethasone were not significantly affected by IFN-γ (Fig. 6A). IL-1β-induced eotaxin expression was also slightly enhanced by IFN-γ in A549 cells, and IFN-γ treatment did not induce these cells to respond to IL-4 (Fig. 6B). TNF-α-induced eotaxin mRNA expression was synergistically enhanced by IFN-γ in BEAS-2B cells, and this expression was slightly enhanced by IL-4 and inhibited by dexamethasone (Fig. 6C). A summary of the effects of cytokines on eotaxin mRNA expression in various cell types is presented in Table 1.

ELISA for eotaxin, MCP-4, GM-CSF, IL-3, and IL-5. Eotaxin protein was detected in cell supernatant of PBMCs after 24 h of culture without stimulation (Fig. 7A). The amount of eotaxin tended to increase in the presence of IL-1β. Eotaxin protein induced by IL-1β was decreased by the addition of IL-4 or dexamethasone. Eotaxin protein was also detectable in cell supernatant of A549 cells after 24 h of culture without stimulation (Fig. 7B). The amount of MCP-4 was markedly increased in the presence of IL-1β. MCP-4 induced by IL-1β was decreased by the addition of IL-4 or dexamethasone. MCP-4 protein was also detectable in cell supernatant of A549 cells after 24 h of culture without stimulation (Fig. 7D). IL-1β also increased the amount of MCP-4 in this cell
IL-1β-induced MCP-4 levels were unchanged by the addition of IL-4 but decreased in the presence of dexamethasone. Direct effects of IL-4 on MCP-4 protein expression were not demonstrated in either cell type. GM-CSF, IL-3, and IL-5 protein levels in cell supernatant of PBMCs and A549 cells are shown in Table 2. IL-1β increased GM-CSF protein expression in PBMCs. IL-1β-induced GM-CSF levels were decreased by IL-4 or dexamethasone in these cells. IL-1β markedly induced GM-CSF protein secretion from A549 cells, which was significantly decreased by the addition of IL-4 or dexamethasone. In contrast, IL-3 protein levels in supernatant of PBMCs were increased in the presence of IL-1β and IL-4. However, these effects were not demonstrated in A549 cells. There were no significant effects of IL-1β or IL-4 on IL-5 protein expression in either PBMCs or A549 cells.

Eotaxin mRNA and protein expression in monocytes and lymphocytes. We tested the IL-1β responsiveness of subpopulations of purified monocytes and lymphocytes (n = 2; Fig. 8, A and B). Both populations of cells were responsive to IL-1β but with different time courses. Purified monocytes demonstrated maximal eotaxin mRNA expression at 2–4 h, whereas purified lymphocytes demonstrated maximal expression at 48 h. IL-4 inhibited IL-1β-induced eotaxin mRNA expression in monocyte-rich (4 h after stimulation) and lymphocyte (24 h after stimulation) subpopulations (n = 2). Eotaxin immunoreactivity was detectable in IL-1β-stimulated monocytes but not lymphocytes 24 h after stimulation (Fig. 8C). IL-1β-induced eotaxin immunoreactivity in monocytes was inhibited by IL-4 (Fig. 8D). Eotaxin immunoreactivity was also detectable without stimulation and was enhanced by IL-1β in

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EFFECTS OF IL-4 ON EOTAXIN AND MCP-4 EXPRESSION

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Fig. 5. Time courses of IL-4 effects on cytokine-induced eotaxin mRNA expression and dose-response effects of IL-4 on this expression in A549 cells and SAEC. A: time courses of eotaxin mRNA expression after stimulation with IL-1β, IL-4, and IL-1β + IL-4. A representative series of Northern blots from 2 distinct experiments is presented. B: dose-response effects of IL-4. A representative Northern blot from 2 distinct subjects is presented. C: time courses of eotaxin mRNA expression after stimulation with TNF-α and IFN-γ in the absence or presence of IL-4. A representative series of Northern blots from 2 distinct experiments is presented. D: dose-response effects of IL-4. A representative Northern blot from 2 distinct subjects is presented.
PBMCs. IL-1β-induced immunoreactivity was decreased by the addition of dexamethasone in PBMCs. No significant staining was observed with the negative controls (data not shown).

**Effects of IL-4 on stability of eotaxin mRNA in PBMCs and A549 cells.** Eotaxin mRNA expression in PBMCs decreased after the inhibition of transcription by actinomycin D, but the effects of IL-4 on the stability of eotaxin mRNA were not demonstrated. Eotaxin mRNA expression in A549 cells gradually decreased by the addition of actinomycin D in the epithelial cell line, and there was no significant difference in the mRNA stability between the conditions with and without IL-4 (Fig. 9).

**DISCUSSION**

IL-4 markedly inhibited eotaxin and MCP-4 mRNA expression induced by IL-1β and TNF-α in human PBMCs but had little or positive effect on chemokine mRNA expression in human epithelial cells. IL-1β-induced eotaxin and MCP-4 protein production was also decreased in the presence of IL-4 in PBMCs, whereas IL-4 increased eotaxin protein production in A549 cells. In contrast, dexamethasone inhibited eotaxin and MCP-4 expression in both PBMCs and epithelial cells. Cytokine-induced expression of eotaxin and MCP-4 mRNA in lower airway mononuclear cells obtained by BAL was significantly less than that in PBMCs or epithelial cells, whereas BAL fluid cell expression of the neutrophil chemoattractant IL-8 was comparable to that in PBMCs and epithelial cells. These results suggest that eotaxin and MCP-4 are induced by proinflammatory cytokines in airway epithelial cells and PBMCs with far greater efficiency.

**Table 1. Summary of the effects of cytokines on eotaxin mRNA expression**

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<tr>
<th>Cytokine Combinations</th>
<th>PBMCs</th>
<th>A549</th>
<th>BEAS-2B</th>
<th>SAEC</th>
<th>BAL</th>
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<tr>
<td>IL-1β, TNF-α, IFN-γ</td>
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<td>IL-4</td>
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* IL, interleukin; TNF-α, tumor necrosis factor-α; IFN, interferon; SAEC, small airway epithelial cells; PBMC, peripheral blood mononuclear cells; BAL, bronchoalveolar lavage; ND, not done. *From Ref. 34.
than in BAL fluid cells; in the absence of modifying influences, this response would increase the chemokine concentrations both at the airway epithelium and in the circulation.

We have recently observed that plasma eotaxin levels were greater in patients with asthma than in normal subjects (43). With targeted disruption of the mouse eotaxin gene and neutralizing antibodies for mouse eotaxin, a role for eotaxin in regulating the number of circulating eosinophils and in recruiting eosinophils to the airways after antigen challenge has been demonstrated (21, 51). These observations are consistent with our concept that chemokines in circulation, partly produced by PBMCs, contribute to increasing the number of peripheral eosinophils, whereas eotaxin and MCP-4 production by epithelial cells contributes to airway eosinophil recruitment. Although eotaxin has been reported to be produced by various cell types, including epithelial cells, mononuclear cells, eosinophils, endothelial cells, fibroblasts, and smooth muscle cells (18, 19, 32, 34, 61), pathological investigations of the airway specimens obtained from asthmatics have indicated that epithelial cells and mononuclear cells are major sources of eotaxin in the airways (32, 70). Similarly, these cell types have suggested to be important sources of MCP-4 in the lungs of asthmatics (31). Our finding that IL-4 has divergent effects on eotaxin and MCP-4 expression in PBMCs and epithelial cells supports the proposition that greater concentrations of chemokines will be present at airway epithelial sites, where eosinophils are known to exert their pathophysiological effects, than in microcirculation of the lungs when IL-4 is available. This chemotactic gradient would facilitate eosinophil migration to the airways at sites of IL-4 production, a proposition consistent with studies demonstrating the coexistence of IL-4 and eosinophils in asthmatic airways (69). This notion is further supported by the finding that the CCR-3 ligands eotaxin and MCP-4 can recruit CCR-3-

### Table 2. GM-CSF, IL-3, and IL-5 protein levels in supernatant of PBMCs and A549 cells

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<th></th>
<th>US</th>
<th>IL-4 (10 ng/ml)</th>
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<th>IL-1 + Dex (1 μM)</th>
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<tr>
<td>GM-CSF</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>4.0 ± 0.7*</td>
<td>2.3 ± 1.0</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>IL-3</td>
<td>1.2 ± 0.9</td>
<td>2.7 ± 1.1</td>
<td>4.1 ± 1.1</td>
<td>8.4 ± 3.0†</td>
<td>2.0 ± 1.3</td>
</tr>
<tr>
<td>IL-5</td>
<td>3.4 ± 1.1</td>
<td>3.2 ± 1.0</td>
<td>2.1 ± 0.9</td>
<td>2.6 ± 0.5</td>
<td>1.9 ± 1.0</td>
</tr>
<tr>
<td><strong>A549 cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>1.5 ± 0.6</td>
<td>1.5 ± 0.6</td>
<td>169.2 ± 15.9*</td>
<td>43.3 ± 4.5‡</td>
<td>8.7 ± 2.5</td>
</tr>
<tr>
<td>IL-3</td>
<td>1.7 ± 1.5</td>
<td>2.3 ± 0.8</td>
<td>1.3 ± 0.9</td>
<td>1.8 ± 1.1</td>
<td>2.5 ± 1.2</td>
</tr>
<tr>
<td>IL-5</td>
<td>2.1 ± 1.5</td>
<td>5.7 ± 4.4</td>
<td>2.0 ± 1.9</td>
<td>3.2 ± 1.7</td>
<td>2.0 ± 2.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. Units are pg/10⁶ cells. US, unstimulated; GM-CSF, granulocyte-macrophage colony-stimulating factor; Dex, dexamethasone. †P < 0.01 compared with the other 4 groups; *P < 0.05 compared with US; †P < 0.01 compared with US, IL-4, and IL-1 + Dex.
expressing Th2 lymphocytes (53) that secrete IL-4 to sites where these chemokines are produced.

We demonstrated similar regulation of the eosinophil chemoattractants eotaxin and MCP-4 by proinflammatory cytokines and IL-4. Although IL-8 acts mainly on neutrophils, the pattern of regulation that we observed for IL-8 in PBMCs and airway epithelial cells was similar to that of eotaxin and MCP-4. These similarities are not surprising in light of the fact that neutrophils and eosinophils are known to be recruited to the airways early after segmental allergen challenge and are prominent in the airways of some patients with fatal and steroid-treated severe asthma (39, 57, 66).

The ability of BAL fluid cells to produce IL-8 is thought to be relevant to the pathogenesis of nonallergic diseases, including airborne bacterial infections, acute respiratory distress syndrome, and idiopathic pulmonary fibrosis, in which neutrophils accumulate in the alveolar spaces (6, 40, 41). GM-CSF, IL-3, and IL-5 are known to play a role in eosinophil inflammation in allergic diseases by promoting eosinophil growth and differentiation (62, 67). The pattern of regulation that we observed for GM-CSF, IL-3, and IL-5 in PBMCs and airway epithelial cells was different from that for eotaxin and MCP-4. GM-CSF protein was markedly induced by IL-1β in A549 cells, but the expression was significantly decreased in the presence of IL-4. IL-3 protein levels increased by the addition of IL-4 and IL-1β to PBMCs compared with unstimulated cells. IL-4 did not affect IL-5 expression in PBMCs and A549 cells. Although the physiological roles of differential regulation of these cytokines between the cell types in airway inflammation are to be elucidated, the regulatory pattern observed in eotaxin, MCP-4, and IL-8 may be characteristic of chemotactic cytokines (17, 18) and potentially promote the recruitment of inflammatory cells to the inflamed tissue.

Although Th2 lymphocyte activation is a central feature of asthma (50), a growing body of knowledge describes the complex interplay between Th2 and Th1 lymphocytes in inflamed airways (7, 14, 17, 46). It has recently been demonstrated that the prototypical Th1 cytokine IFN-γ can facilitate the production of eotaxin and MCP-4 in human epithelial and endothelial cells (7, 18, 34). We then studied the effects of IFN-γ on eotaxin mobilization in PBMCs and epithelial cells in the presence or absence of the Th2-derived cytokine IL-4. IFN-γ slightly enhanced cytokine-induced eotaxin mRNA expression in both epithelial cells and PBMCs but did not significantly alter the effect of IL-4 on eotaxin expression in either cell type. Our results also suggested the direct effects of IFN-γ on chemokine expression in primary cultures of human epithelial cells. In BEAS-2B cells, which are derived from the bronchial epithelium, TNF-α was a stronger inducer of eotaxin expression than IL-1β, whereas IL-1β induced
more eotaxin expression in A549 cells (34), which are derived from alveolar type II epithelial cells. In contrast, both proinflammatory cytokines induced comparable chemokine expression in PBMCs. Taken together, the combination of proinflammatory cytokines and IFN-γ efficiently induces chemokine expression in various cell types (Table 1). It may be of importance that differential effects of IL-4 on chemokine expression between epithelial cells and mononuclear cells were consistently observed despite the difference in the optimal combination of cytokines in each cell type.

The time courses of eotaxin mRNA expression after IL-1β stimulation differed between A549 cells and PBMCs. Expression in A549 cells was maximal 2–4 h after stimulation, whereas that in PBMCs was maximal at 8 h and declined gradually after the peak. It is interesting that the half-life of eotaxin mRNA was longer in A549 cells than that in PBMCs when transcription was inhibited, which was in contrast to the time courses of IL-1-induced eotaxin mRNA in A549 cells and PBMCs. These findings suggest that newly transcribed RNases or their repressive factors may be involved in the regulation of eotaxin mRNA induced by proinflammatory cytokines. The peak of cytokine-induced eotaxin mRNA expression in the primary epithelial cells was at 8 h after stimulation but declined more rapidly after the peak than in PBMCs. Time-course studies of cell subpopulations demonstrated that the monocyte-predominant fraction expressed eotaxin mRNA maximally 2–4 h after stimulation, similar to A549 cells, whereas expression was maximal at 8–48 h after stimulation of lymphocytes. Eotaxin protein was detected 24 h after IL-1β stimulation mainly in monocytes. Chemokines produced in this time frame would be present at the time eosinophils are recruited after allergic and nonallergic airway stimulation (36, 67). Eotaxin-mediated eosinophil recruitment to mouse skin was also observed within 8 h after antigen injection (59). In addition, eotaxin and MCP-4 were clearly detected in mononuclear cells and epithelial cells but not in lymphocytes in inflamed human airways (31, 32). However, a role for lymphocytes in airway eosinophilia is suggested by a recent study of the mouse model demonstrating that lymphocyte depletion reduced pulmonary eotaxin expression and airway eosinophilia (37). These findings suggest that lymphocytes may not be primary sources of eotaxin but may promote chemokine-associated airway eosinophilia by indirect mechanisms. Our data suggest that one of these mechanisms is the ability of Th2 lymphocyte-derived IL-4 to influence chemokine gradients.

Although chemokine mRNA expression was unchanged by the addition of IL-4 in A549 cells, the expression of various molecules in A549 and BEAS-2B cells is regulated by IL-4 (1, 26). A previous study (26) directly demonstrated IL-4 receptor gene expression in A549 cells. These observations suggest that effects of IL-4 on chemokine expression in epithelial cells may be mediated by IL-4 receptors, even in A549 cells. The differential expression of IL-4 receptors between the cell types may be one of the possible reasons for the differential effects of IL-4. Because cytokine-induced
eotaxin mRNA expression was decreased markedly by IL-4 in PBMCs, we examined the effects of IL-4 on the stability of eotaxin mRNA. However, no significant effects of IL-4 on the mRNA stability were demonstrated in either PBMCs or A549 cells. Recent studies demonstrated that nuclear factor (NF)-κB activation is important in transcriptional activation of the human eotaxin gene by the proinflammatory cytokines IL-1β and TNF-α (25, 38), and a signal transducer and activator of transcription (STAT)6 binding site, which is associated with the response to IL-4, overlapped the NF-κB consensus site of the promoter region of the eotaxin gene (38). These findings suggest that interaction between NF-κB and STAT6 can be related to the distinct effects of IL-4 in different cell types. Because the effects of IL-4 on translational processes were demonstrated (45), it is also possible that increased IL-1-induced eotaxin protein secretion by IL-4 was involved in its translational regulation despite the unchanged mRNA expression in A549 cells. Although mechanisms for the differential effects of IL-4 have not been elucidated fully, previous in vivo findings with IL-4 knockout mice were consistent with our in vitro results. Pulmonary eosinophilia and airway resistance were attenuated in IL-4 knockout mice in asthma models (10, 68). Reduced MCP-3 and eotaxin expression was reported in the lungs of IL-4 knockout mice in an antigen-elicited granuloma formation model (8).

In summary, we have demonstrated that eotaxin and MCP-4 production by circulating cells is inhibited by IL-4, whereas that by airway epithelial cells is resistant to or enhanced by IL-4. These results support our hypothesis that IL-4 can differentially affect chemotactant expression in diverse cell types and may facilitate the establishment of chemokine concentration gradients in inflamed tissues. These observations are most relevant to eosinophil recruitment at sites where Th2-derived IL-4 is present, such as the allergic airway.

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