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Cytokine-Stimulated Human Lung Alveolar Epithelial Cells Release Eotaxin-2 (CCL24) and Eotaxin-3 (CCL26)

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ABSTRACT

Asthma is a complex inflammatory disease characterized by a prolonged underlying airway inflammation resulting from cytokine-orchestrated signaling between many types of cells, including airway epithelial cells. Trafficking, recruitment, and activation of cells in airway disease are, in part, modulated by the newly discovered CC subfamily of chemokines, eotaxin (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26), which transduce signals by acting as agonists for the CCR3 receptor. The specific cytokine stimuli that modulate CCL24 and CCL26 release in airway epithelial cells remain poorly defined. Thus, human 549 alveolar type II epithelium-like cells were stimulated singly and with combinations of 1–100 ng/ml tumor necrosis-factor-α (TNF-α), interleukin-1β (IL-1β), and IL-4, cytokines known to be elevated in the airways of asthmatics. Release of CCL11, CCL24, and CCL26 was quantified by ELISA, and CCR3 receptors monitored by immunocytochemistry and FACS analysis. Results suggest that epithelial cells release CCL11 during the first 24 h of stimulation, in contrast to a significant increase in CCL24 and CCL26 release after 24–48 h of stimulation. Differential release of the eotaxins in response to cytokine combinations was noted. The alveolar type II epithelial cells were found to possess constitutive CCR3 receptors, which increased after proinflammatory cytokine stimulation. The airway epithelium CCR3 receptor/eotaxin ligand signal transduction system may be an important target for development of novel mechanism-based adjunctive therapies designed to interrupt the underlying chronic inflammation in allergic and inflammatory disorders.

INTRODUCTION

HEREDITARY AND ENVIRONMENTAL FACTORS, including allergies, viral infections, and irritants, are involved in the onset of asthma, a chronic inflammatory disease of the airways that affects an estimated 7% of the U.S. population.[1,2] Asthma is a complex inflammatory disease of the lung characterized by variable airflow obstruction, bronchial hyperresponsiveness, and airway inflammation.[3] It is well accepted that occupational or allergic asthma, respiratory distress syndrome, and the like are characterized by airway inflammation involving activation of mast cells and $CD4^+$ lymphocytes with a Th2 cytokine pattern, followed by infiltration of eosinophils. Results of experiments with airway epithelial cells strongly suggest that the airway epithelium not only functions as a physical barrier between the internal and external milieu of the lungs but also actively engages in communicating with cells of the immune and inflammatory systems.[4] It is, then, the cytokine-orchestrated signaling among all these cell types that creates a prolonged underlying airway inflammation in the pulmonary microenvironment.

Airway epithelial cells may be early targets of environmental stimuli and local cytokines, and they respond by synthesizing and releasing a variety of proinflammatory mediators. Evidence suggests that the altered airway epithelium thus becomes a continuous source of products, including leukocyte growth/survival-promoting cytokines, and chemokines.[5,6] Trafficking and recruitment of leukocytes in airway disease are modulated by lipid mediators, cytokines, and the CC subfamily of chemokines, which includes eotaxin-1 (CCL11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26). Eosinophil recruitment is one defining characteristic of allergic airway inflammation now thought to contribute to the pathogenesis of bronchial asthma and allergic rhinitis.[7] The eotaxins may also act cooperatively with other cytokines to modulate eosinophil activation and secretory responses as well as enhance effector functions of eosinophils.[8–10] Although the airway sources of eotaxin family members have not all been completely delineated, there is

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compelling evidence that the airway bronchial epithelium is a cytokine-stimulated source of CCL11.[11–14] Infection of airway epithelial cells with human rhinoviruses has been shown to upregulate CCL11 and CCL24 expression,[15] and influenza type A H3N2 infection reportedly activates epithelial cell expression of CCL11.[16]

CCL11, CCL24, and CCL26 are known to transduce signals by acting as agonists for the CCR3 receptor. Of importance to mechanistic studies involved in airway allergic and inflammatory responses is the discovery that eosinophils express high CCR3 levels.[17] This receptor may be involved in eosinophil differentiation, as it is upregulated by Th2 cytokines in $CD34^+$ hematopoietic progenitor cells.[18] CCR3 receptors are also expressed on basophils,[19] Th2 lymphocyte subsets,[20] human dendritic cells (DCs),[21] and human lung parenchyma mast cells.[22] Interestingly, constitutive expression of the CCR3 receptor has recently been detected on normal human bronchial epithelial cells[23] and A549 human alveolar type II epithelial cells.[24] In experiments using primary bronchial epithelial cells as well as cell lines, CCR3 expression was induced by tumor necrosis factor-α (TNF-α) and potentiated by the addition of interferon-γ (IFN-γ).[25] These results suggest that members of the eotaxin family may contribute to pathogenesis at sites of inflammation not only through CCR3-ligand signal transduction pathways in eosinophils but modulation of the airway epithelium through ligand-CCR3 receptor feedback mechanisms. Airway epithelial cells may serve as both effector and target cells for factors released into the pulmonary microenvironment.

The relative roles of the three eotaxins in cytokine-stimulated airway epithelial cells remain poorly defined, particularly with respect to CCL24 and CCL26. Thus, the current investigations were carried out to test the hypothesis that alveolar epithelial cells are a major source of pulmonary eotaxins and selectively respond to cytokine stimulation by release of CCL11, CCL24, CCL26 and modulation of their CCR3 cell surface receptors. The airway epithelium CCR3 chemokine receptor-ligand system may be an important target for development of novel mechanism-based adjunctive therapies designed to interrupt the underlying chronic inflammation in allergic and inflammatory disorders.

MATERIALS AND METHODS

Culture and stimulation of airway epithelial cells

Human A549 alveolar type II epithelium-like cells (ATCC CCL-185) were purchased from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. Trypsin-dispersed cells were suspended in fresh medium in new flasks or wells at 0.2×10^6 cells/ml or 1×10^6 cells/ml, respectively. Experiments were performed after subcultured cells had reached about 80% confluence. Viability of cells used for experiments was assessed by trypan blue exclusion and the LIVE/DEAD viability/cytotoxicity calcein AM/ethidium homodimer-1 fluorescence assay (Molecular Probes, Eugene, OR). Only populations of cells with viability >93% were used for experiments.

Cells were trypsinized, resuspended in complete culture medium, dispensed to 24-well plates at 1×10^6 cells/well, and allowed to attach and grow for 24 h. Prior to stimulation, cells were incubated in serum-free DMEM for 6 h, then stimulated in fresh serum-free DMEM with indicated concentrations and combinations of TNF-α, interleukin-1β (IL-1β), and IL-4 (Sigma-Aldrich Chemical Co., St. Louis, MO, and Atlanta Biologicals, Atlanta, GA). Cytokine stock solutions were prepared and handled as previously described.[8]

CCL11, CCL24, and CCL26 detection by specific ELISA

Following stimulation of A549 epithelial cells for 24 or 48 h with the indicated cytokines, supernatants were removed and centrifuged at 500*g* for 5 min at 4°C and immediately assessed for the presence of each of the eotaxins by specific ELISA (R & D Systems, Minneapolis, MN). These specific ELISAs recognize both natural and recombinant human CCL11, CCL24, and CCL26. No cross-reactivity of recombinant human CCL11, CCL24, and CCL26 was noted when the chemokines were tested at 100 ng/ml. Secreted eotaxins were quantified with a Power Wave X 340 microplate reader equipped with KC4 v3.0 PowerReports software (Bio-Tek Instruments, Winooski, VT).

Detection of CCR3 in airway epithelial cells by immunocytochemistry

Cells (1×10^4) were cultured in Labtek coverslip chambers for 24 h. Cells were washed three times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 min. Fixed cells were permeabilized with 0.2% Triton-X-100 for 5 min, blocked in 10% normal goat serum, then incubated overnight with or without 20 μg/ml rat monoclonal hCCR3 antibody, followed by 1-h incubation with 1 μg/ml Texas red-labeled goat antirat IgG (H+L). Slides were mounted with slow fade containing DAPI, and expression of CCR3 protein was visualized and captured by an Olympus fluorescent microscope (40× objective) fitted with an Olympus DP70 camera. Photographs were documented using Adobe Photoshop.

Detection of airway epithelial cell CCR3 receptors by flow cytometry

A549 airway epithelial cells were washed twice in PBS and resuspended in serum-free DMEM at 1×10^6 cells/ml and dispensed to 24-well plates. Cells were treated with 1–100 ng/ml indicated cytokines either alone or in combination and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 6 h. After incubation, cells were detached with 0.5 mM EDTA in PBS and centrifuged at 500*g* at 4°C for 5 min. Pellets were washed twice in cold FACSflow buffer (BD Biosciences, San Jose, CA) containing 2% FBS and resuspended to a final concentration of 5 x 10^6 cells/ml. Aliquots of cells were stained with 500 ng/ml biotinylated human recombinant eotaxin or the equivalent amount of negative control biotinylated soybean trypsin inhibitor for 60 min at 4°C, followed by 1 μg/ml fluorescein-conjugated avidin (Fluorokine flow cytometry reagents, R&D Systems) for 30 min in the dark at 4°C. After two washes in cold FACSflow buffer, stained cells were maintained at 4°C, then subjected to flow cytometry on a FACScalibur (BD Bio-

sciences). Data were analyzed using CellQuest software (BD Biosciences).[17]

Data handling and analysis

Unless otherwise stated, experiments were conducted in triplicate and repeated on at least three to four separate occasions (Western blots and flow cytometry experiments were performed in duplicate on several different occasions). Unless otherwise stated, all data are expressed as the mean ± SEM. When indicated, one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparisons posttest, was applied to experimental results to determine statistical significance ($p < 0.05$) between indicated groups.

RESULTS

A549 airway epithelial cells release CCL11

The cytokines TNF-α, IL-1β, and IL-4 were tested alone and in combination at 1, 10, and 100 ng/ml for their abilities to trigger release of CCL11 from serum-deprived A549 airway epithelial cells. Results after stimulation with all cytokine pairs for either 24 or 48 h are shown in Figure 1. Unstimulated cells did not release detectable amounts of CCL11. TNF-α with IL-1β stimulation caused a concentration-dependent release of 9–24 pg/10^6 cells at 24 h, and this did not change significantly when stimulation was extended to 48 h. In contrast, cells stimulated with IL-1β plus IL-4 released up to 45.6 pg/10^6 cells at 24 hr, and this significantly increased to a maximum of 173 pg/10^6 cells at the 100 ng/ml concentrations for 48 h. Costimulation of A549 airway epithelial cells with TNF-α and IL-4 at 1, 10, and 100 ng/ml caused a concentration-dependent increase in CCL11 release to a maximum of 101 and 111 pg/10^6 cells at 24 and 48 h, respectively. In data not shown, it was determined that cells stimulated with 1–100 ng/ml IL-1β released a maximum of 9.7 ± 3.49 pg/10^6cells/48 h. Cells stimulated with TNF-α released up to 16.1 ± 0.534 pg/10^6 cells/24 h and 27.7 ± 1.59 pg/10^6 cells/48 h. IL-4 as the sole stimulant elicited a concentration dependent increase of 1.23 ± 0.295–43.7 ± 2.05 pg/10^6 cells/24 h, and this did not significantly increase at 48 h. These results suggest that A549 alveolar type II epithelium-like cells are a source of CCL11. Costimulation with the Th2-type cytokine IL-4 and either IL-1β or TNF-α increased release of CCL11.

Alveolar type II epithelium-like cells are a source of CCL24 and CCL26

Investigations were carried out to explore the ability of these proinflammatory cytokines to induce release of the more re-

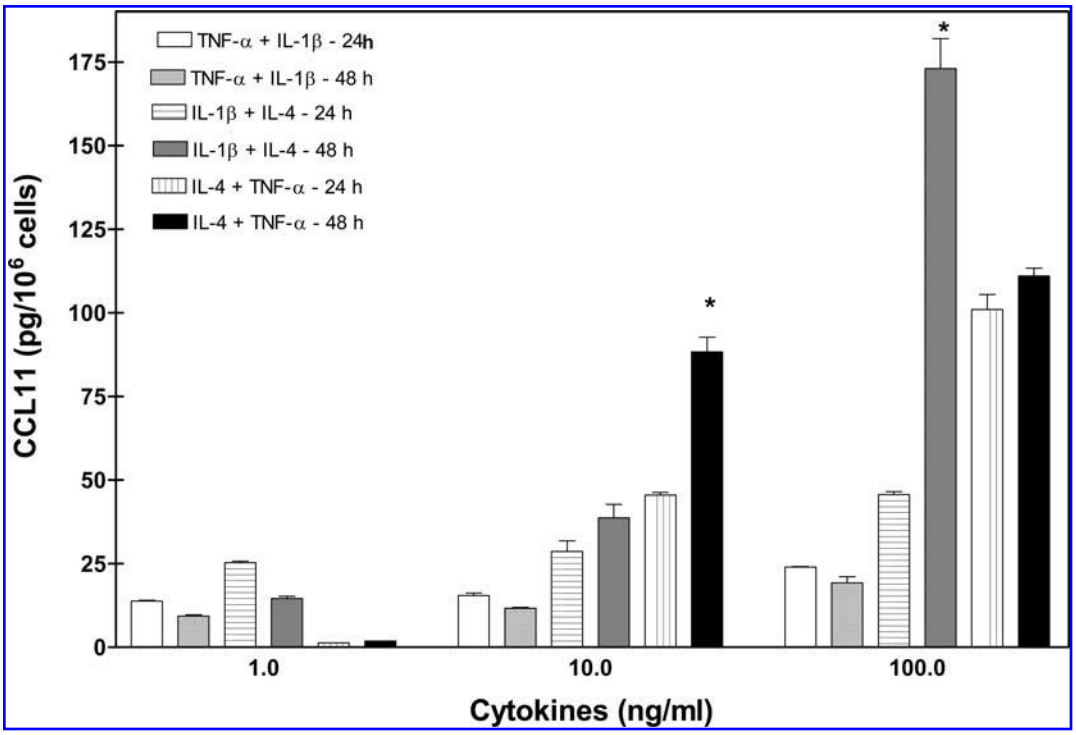

FIG. 1. The proinflammatory cytokines TNF-α, IL-1β, and IL-4 stimulate release of CCL11 from A549 alveolar type II epithelium-like cells. Cells were dispensed to 24-well plates (1×10^6 cells/well) and allowed to attach and grow overnight. Cells were then serum deprived for 6 h, then stimulated in fresh serum-deprived DMEM with 0, 1, 10, and 100 ng/ml of the indicated cytokine pairs for 24 or 48 h. Culture supernatants were collected and assessed for CCL11 by ELISA. Data shown are the mean ± SEM of three experiments, each conducted in triplicate. No detectable CCL11 was found in supernatants of unstimulated controls. ANOVA was followed by the Tukey-Kramer multiple comparisons posttest to determine significance between 24 and 48 h matched treatments. *48-h chemokine levels that differed significantly from 24-h matched treatments at $p < 0.05$.

cently described CCL24 and CCL26. TNF-α, IL-4, and IL-1β were tested alone or in combination at concentrations of 1–100 ng/ml in A549 cells under serum-free conditions for up to 48 h. These conditions were not cytotoxic, as determined by microscopic appearance, trypan blue exclusion, and LIVE/DEAD viability/cytotoxicity fluorescence assay results. Release of CCL24 from cells stimulated with each of the proinflammatory cytokines as the sole stimulating agent is depicted in

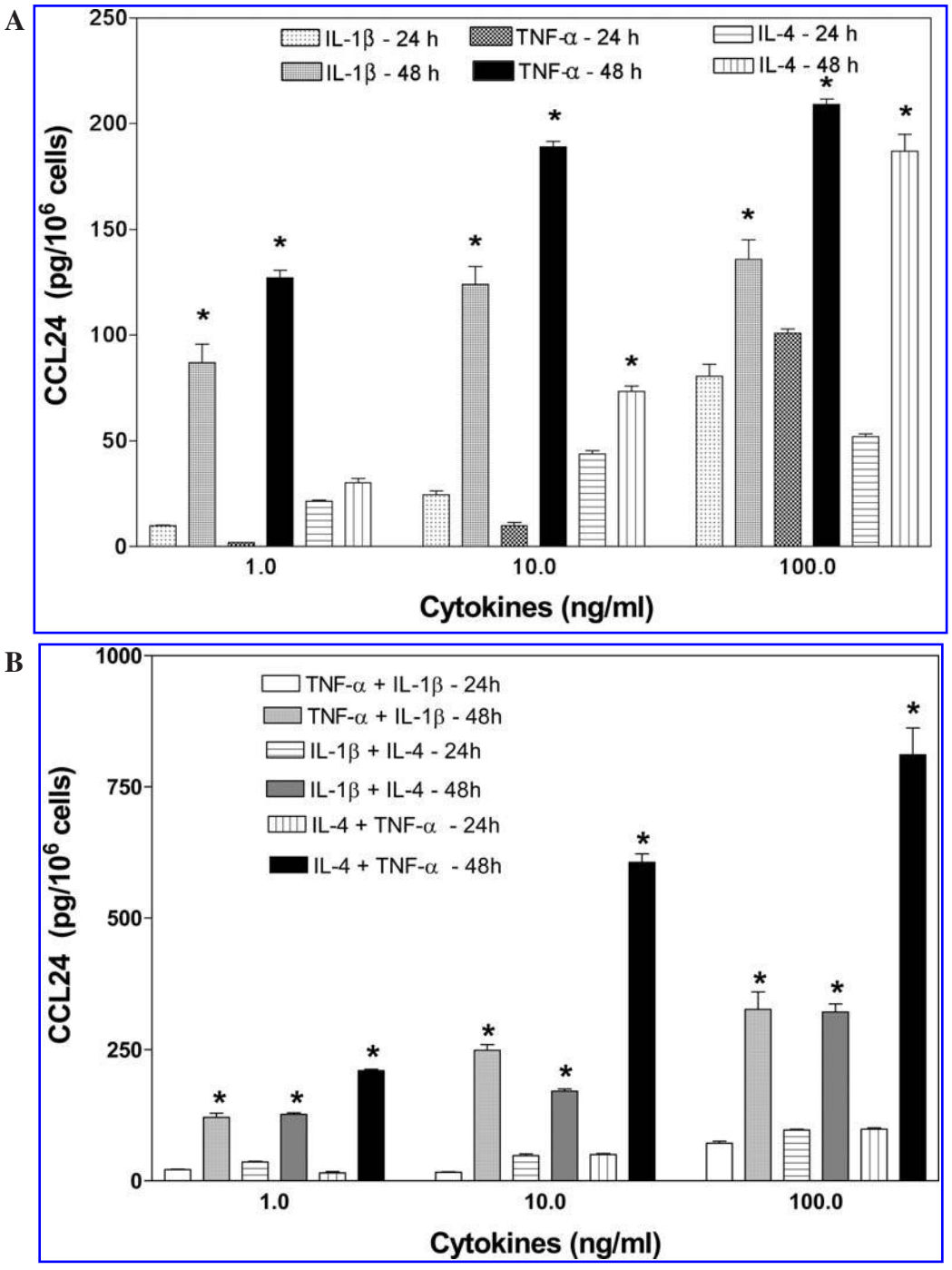

FIG. 2. A549 alveolar type II epithelium-like cells release CCL24 when stimulated with TNF-α, IL-1β, and IL-4 alone or in combination. Experiments were carried out as described in Figure 1. (**A**) Cells were stimulated with TNF-α, IL-1β, or IL-4 at the indicated concentrations for 24 or 48 h. No CCL24 was detected in supernatants of unstimulated controls. (**B**) Cells were stimulated with TNF-α + IL-1β, IL-1β + IL-4, or IL-4 + TNF-α at the indicated concentrations for 24 or 48 h. No CCL24 was detected in supernatants of unstimulated controls. Data shown are the mean ± SEM of three experiments, each conducted in triplicate. ANOVA was followed by the Tukey-Kramer multiple comparisons posttest to determine significance between 24 and 48 h matched treatments. *48-h chemokine levels that differed significantly from 24-h matched treatments at $p < 0.05$.

Figure 2A. ELISA results for detection of CCL24 released from A549 cells stimulated with IL-1β, TNF-α, or IL-4 caused concentration-dependent release up to 136 $\pm$ 9.11, 209 $\pm$ 2.67, and 187 $\pm$ 8.01 pg/10^6 cells in 48 h, respectively. In contrast to CCL11 release, the release of CCL24 from A549 cells was more robust and increased significantly during the 24–48-h stimulation interval. Results of stimulation with cytokine pairs are shown in Figure 2B. All the cytokine pair combinations sig-

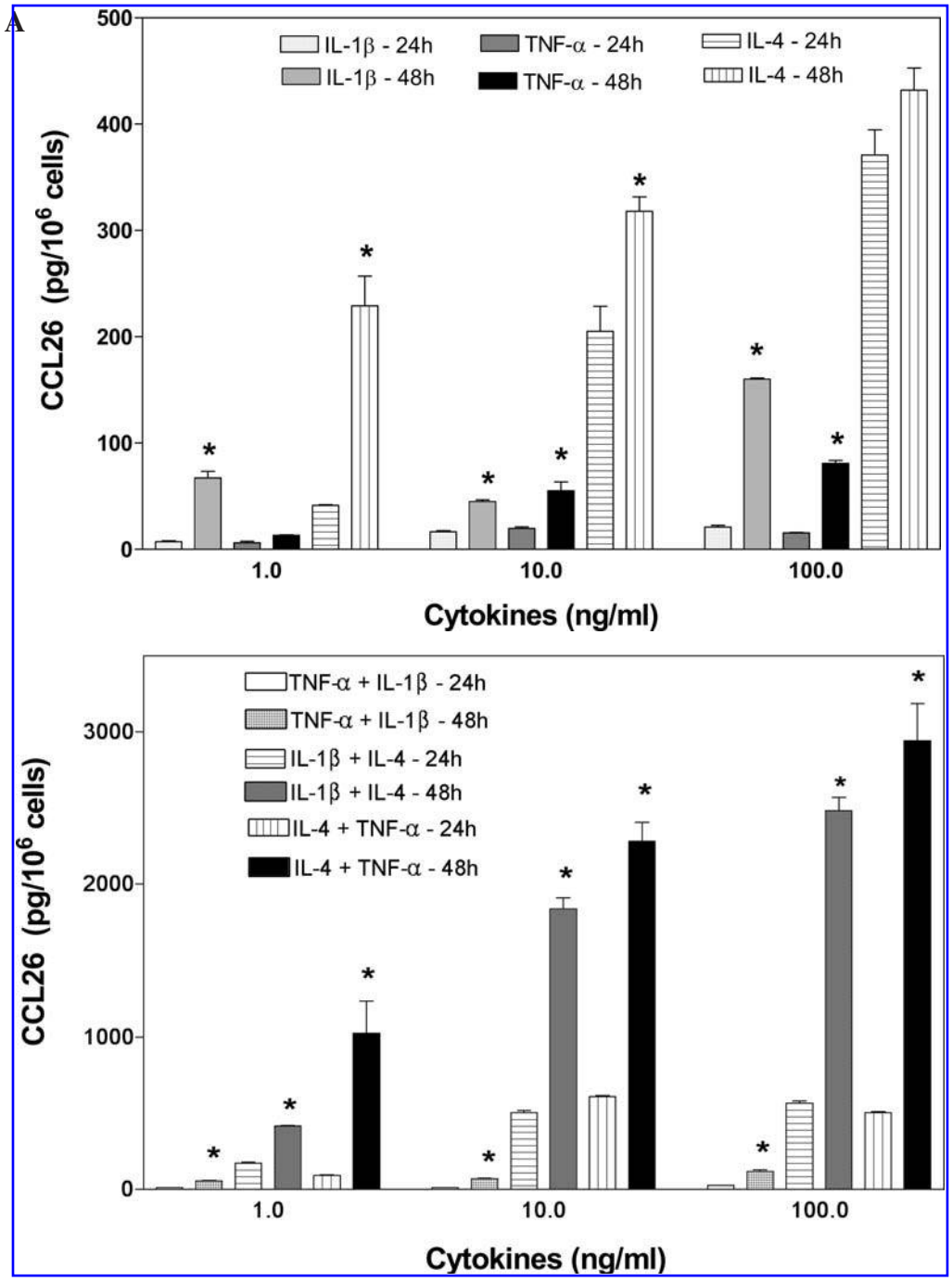

FIG. 3. A549 alveolar type II epithelium-like cells release CCL26 when stimulated with TNF-α, IL-1β, and IL-4 alone or in combination. Experiments were carried out as described in Figure 1. (**A**) Cells were stimulated with TNF-α, IL-1β, or IL-4 at the indicated concentrations for 24 or 48 h. No CCL26 was detected in supernatants of unstimulated controls. (**B**) Cells were stimulated with TNF-α + IL-1β, IL-1β + IL-4, or IL-4 + TNF-α at the indicated concentrations for 24 or 48 h. No CCL26 was detected in supernatants of unstimulated controls. Data shown are the mean $\pm$ SEM of three experiments, each conducted in triplicate. ANOVA was followed by the Tukey-Kramer multiple comparisons posttest to determine significance between 24 and 48 h matched treatments. *48-h chemokine levels that differed significantly from 24-h matched treatments at $p < 0.05$.

nificantly increased release of CCL24 in the 24–48-h interval of stimulation. At the 48-h treatment time, pairing of TNF-α with IL-4 increased CCL24 release by 4-fold over each of the cytokines alone.

Investigations with the most recently discovered of the eotaxins, CCL26, indicated yet another epithelial cell response pattern to proinflammatory cytokine stimulation. Results following stimulation with single cytokines are depicted in Figure 3A. Release of CCL26 in response to IL-1β was very similar to release of CCL24, whereas release of CCL26 in response to TNF-α was reminiscent of CCL11 release. In response to IL-4, the A549 alveolar type II cells released concentrations of CCL26 that exceeded those of CCL24 or CCL11 by 3-fold or 10-fold, respectively. In all cases, except TNF-α (1 ng/ml) and IL-4 (100 ng/ml), CCL26 release was significantly increased during the 24–48-h stimulation interval. Airway epithelial cells were then stimulated with all cytokine combinations (Fig. 3B). Treatments resulted in both concentration-dependent and time-dependent increases in CCL26 release. Chemokines released may differ depending on the stimulating agents. In contrast to results seen with CCL24, IL-1β and IL-4 together induced maximal amounts of 565 $\pm$ 14.4 pg/10^6 cells and 2.48 $\pm$ 0.091 ng/10^6 cells at 24 h and 48 h, respectively. TNF-α and IL-4 also acted to significantly increase release of CCL26 to a maximum of 2.94 $\pm$ 0.346 ng/10^6 cells at 48 h. Collectively, these results indicate that human airway epithelial cells are a major source of the eosinophil-selective chemokines CCL11, CCL24, and CCL26, whose releases are both stimulus specific and time specific.

Expression of CCR3 receptors is regulated by proinflammatory cytokines

The constitutive presence of CCR3 eotaxin receptor was documented by immunocytochemistry. Results are depicted in Figure 4. In stimulated human eosinophils, CCR3 receptor internalization, degradation, and reexpression have been reported, and large intracellular, rapidly mobilized CCR3 receptor pools have been found in human mast cells and dendritic cells (DCs). Thus, FACS analysis experiments were carried out to monitor effects of cytokine stimulation on cell surface CCR3 receptors in these alveolar epithelial cells. Results are shown in Figure 5. CCR3-specific staining revealed the presence of surface receptors in unstimulated alveolar type II epithelial cells (Fig. 5A). Concentration-dependent increases in cell surface CCR3 expression were noted after 6-h treatments with 1 or 10 ng/ml TNF-α with IL-1β (Fig. 5B), with no further increases noted at 100 ng/ml (data not shown). Receptor increases were also seen after treatment with 10 and 100 ng/ml IL-1β with IL-4 (Fig. 5C) or IL-4 with TNF-α (Fig. 5D). Collectively, these results suggest that alveolar type II epithelial cells possess constitutive CCR3 receptors and that stimulation with proinflammatory cytokines, including TNF-α, IL-1β, and IL-4, known to be elevated in the airways of asthmatic patients, induces release of CCL11, CCL24, and CCL26 as well as increased expression of the CCR3 receptor for which they act as specific agonists.

DISCUSSION

Interactions of chemokines with their respective receptors are emerging as important events in the selective recruitment, priming, and activation of leukocytes at sites of allergy and inflammation. Discovery of cellular sources of the chemokines continues. With respect to airway disease, the data suggest an active role for the airway epithelium in prolonging underlying airway inflammation. Findings of the present investigation suggest that human alveolar type II epithelium-like cells in culture are a source of cytokine-stimulated CCL11 and a rich source

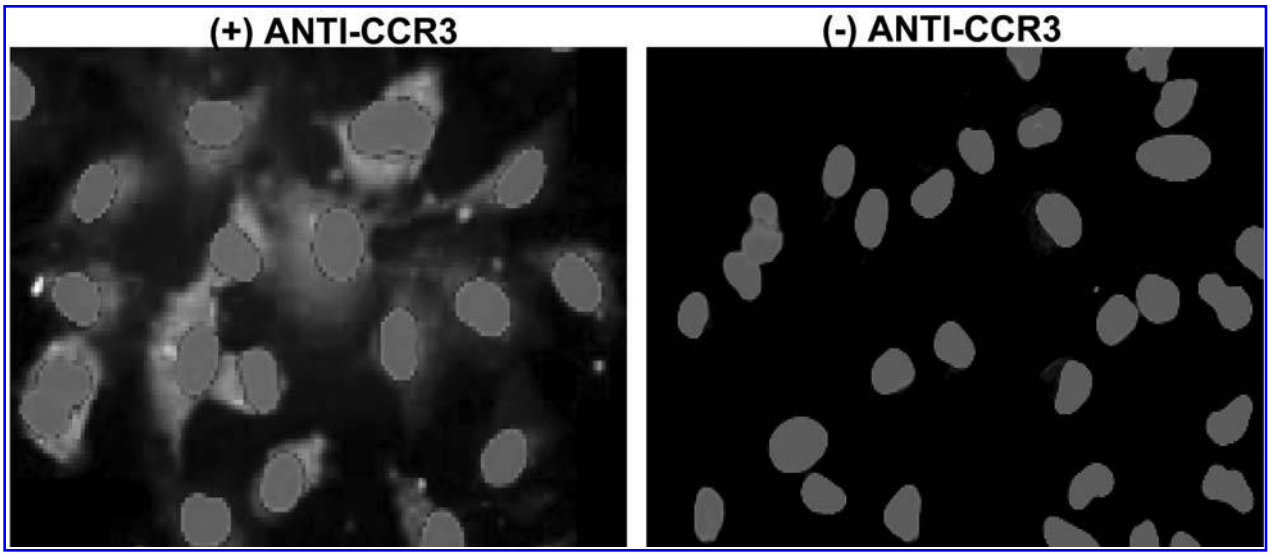

FIG. 4. CCR3 expression is detectable in unstimulated A549 airway epithelial cells. A549 airway epithelial cells were grown for 24 h on chambered coverslips, then fixed, permeabilized, and incubated overnight with (**left**) or without (**right**) anti-CCR3 antibody, followed by 1-h incubation with Texas red-labeled IgG. Slides were mounted with slow fade containing DAPI. Images were captured by on Olympus fluorescent microscope (40$\times$ objective) fitted with an Olympus DP70 camera. Photographs were documented using Adobe Photoshop.

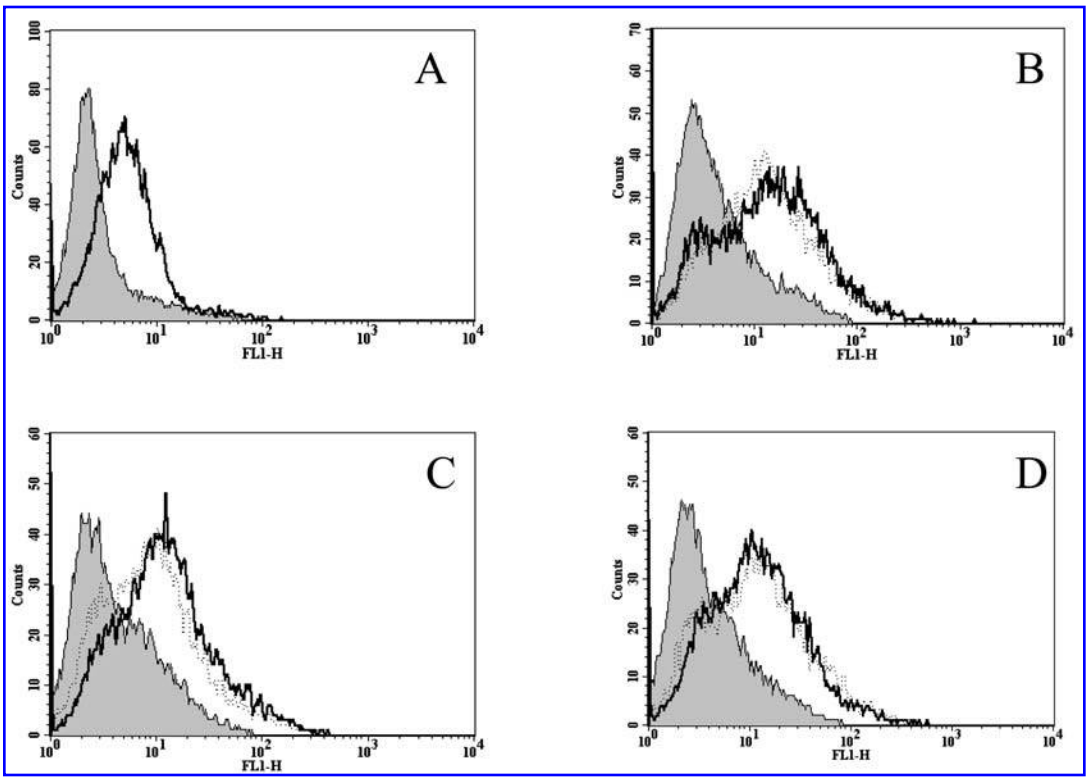

FIG. 5. Cell surface expression increases after stimulation with combinations of TNFα, IL-1β, and IL-4. A549 airway epithelial cells were treated with combinations of cytokines at 1–100 ng/ml for 6 h. Cells were washed, stained with human recombinant eotaxin and fluorescein-conjugated avidin, and washed twice, and expression of CCR3 was measured by FACS analysis. In all panels, the gray histogram is the negative control. Cytokine treatments were (**A**) unstimulated cells (solid line), (**B**) TNF-α + IL-1β at 1 ng/ml (dashed line), and 10 ng/ml (solid line), (**C**) IL-1β + IL-4 at 10 ng/ml (dashed line) and 100 ng/ml (solid line), (**D**) TNF-α + IL-4 at 10 ng/ml (dashed line) and 100 ng/ml (solid line). Histograms are representative of an experiment repeated with similar outcomes on at least four separate occasions.

of CCL24 and CCL26. TNF-α, IL-1β, and IL-4, alone and in combination, elicited specific alveolar airway epithelial cell responses as assessed by release of the three eotaxins. Differences in amounts, synergistic effects of the stimulating cytokines, and time courses of CCL11, CCL24, and CCL26 release were found. Constitutive expression of the CCR3 eotaxin receptor on the alveolar type II epithelial cells was also documented, as was the dynamic modulation of the receptor by treatment with the proinflammatory cytokines.

There is clinical correlative evidence between the presence of $CD4^+$ Th2 cells and eosinophils and disease severity in asthmatics. This suggests an integral role for Th2 cells as sources of mediators that orchestrate the underlying airway inflammation. IL-4, in particular, is produced at elevated levels in the asthmatic lung and is thought to be a central regulator of the hallmark features of the disease.[26] Resident pulmonary system cells, including parenchymal mast cells and alveolar macrophages, are also sources of TNF-α and IL-1β, which are known to be elevated in allergic inflammation.[27] A549 human alveolar type II epithelium-like cells, employed in these studies, are considered representative of alveolar epithelial cells and have been very instrumental in delineating mechanisms important in modulation of asthmatic responses.[28–30] Stimulation with TNF-α, IL-1β, and IL-4 each paired with the other two at 1–100 ng/ml caused a concentration-dependent release of CCL11 from the A549 epithelial cells (Fig. 1). This supports the recent findings that IL-1β increased eotaxin release from A549 cells and that release was further increased by costimulation of cells with IL-1β and IL-4. Interestingly, in BEAS-2B human hybrid adenovirus SV 40-transformed bronchial epithelium-like cells, TNF-α induces a more robust CCL11 expression response than in A549 alveolar epithelial cells. The converse is seen following IL-1β treatment.[10,11] IL-4 has been shown to potentiate TNF-α-induced expression of CCL11 both in BEAS-2B bronchial cells[31] and in these present experiments with A549 alveolar cells.

Having established CCL11 release, investigations were carried out to explore the possibility that the alveolar epithelium may be a source of the two recently discovered chemokines. Results indicate that when used as the sole stimulant, TNF-α, IL-1β, or IL-4 was capable of eliciting concentration-dependent and time course-dependent release of either CCL24 or CCL26 from the A549 epithelial cells (Figs. 2 and 3). Thus, human alveolar type II airway epithelial cells may be a stimulus-specific major source of the eosinophil-selective and eosinophil-active chemokines CCL24 and CCL26. Similar results were reported recently for bronchial epithelial lineage cells stimulated with IL-4 and IL-13 and

also demonstrated by immunohistochemistry in asthmatic airway tissues.[14]

Cytokine-specific release of CCL24 has been reported for several cell types, including IL-1β-stimulated human blood monocytes/macrophages.[32] Human dermal fibroblasts also constitutively express mRNA for CCL11, CCL24, and CCL26. When stimulated with IL-4 or IL-4 + TNF-α, a rank-ordered expression of CCL11 > CCL26 > CCL24 was observed.[33] IL-4 has been shown to be a potent inducer of CCL26 in human umbilical vein endothelial cells[34,35] and dermal fibroblasts.[33,36] In dermal fibroblasts and NCI-H727 lung epithelial cells, CCL26 mRNA was not detectable in unstimulated cells but was induced after stimulation with IL-4 and IL-13, which both acted with TNF-α to superinduce CCL26 mRNA. It was concluded that modulation of CCL26 mRNA expression by Th2 cytokines is different from that of the other two eotaxins further supporting a distinct biologic role for eotaxin-3.[36] Results of these present investigations support these reported results in alveolar epithelial cells. Collectively, these reports are important in elucidating the stimulating agents that govern expression of the newly discovered members of the eotaxin family, CCL24 and CCL26, whose roles in pathogenesis of allergic and inflammatory airway disease are not fully understood. Furthermore, the results suggest that Th2 cytokine modulation of each of the eotaxins is distinct. This may support distinct roles for CCL11, CCL24, and CCL26 and substantiate the central role of alveolar airway epithelial cells in amplifying allergic and inflammatory reactions.

Kinetics of CCL11 and CCL24 expression in the skin of allergen-challenged atopic patients were recently correlated with leukocyte infiltration. A clear relationship was demonstrated between early 6-h eosinophil infiltration and CCL11 expression and a later 24-h eosinophil infiltration with CCL24 expression. It was suggested that late phase eosinophil influx into areas of inflammation or allergic reactions may depend on chemokines induced early by activation of mast cells or later via recruited Th2 cells.[37] Kinetics of all eotaxin family members were recently explored in the bronchoalveolar lavage fluid (BAL) of asthmatic subjects. CCL11 and CCL24 gene expression was upregulated in these patients and increased after allergen challenge. In contrast, when compared with nonasthmatics, prechallenge CCL26 gene expression was not upregulated; however, 24 h after allergen challenge, CCL26 expression was dramatically enhanced, in some cases up to 100-fold.[38] These results suggest that CCL26 may be an important mediator for ongoing recruitment of inflammatory cells into sites of allergy or inflammation. In regard to the recently reported antagonistic activity of CCL26 at CCR1, CCR2, and CCR5, eotaxin-3 may also have a modulator role. CCL26 may be capable of polarizing cellular recruitment by attracting Th2 lymphocytes, eosinophils, and basophils via CCR3, while concomitantly blocking the recruitment of Th1 lymphocytes and monocytes via CCR1, CCR2, and CCR5.[39,40] Results of the present investigation with alveolar epithelial cells support these findings in both skin and BAL. These results further support work with asthmatic subjects by suggesting that the airway epithelium may be participating in the coordinated expression of several chemokines at different times after an allergic stimulus.

Signal transduction of all three eotaxins occurs through the CCR3 receptor, known to be expressed on cells including eosinophils,[17] basophils,[19] Th2 lymphocyte subsets,[20] human DCs,[21] human lung parenchyma mast cells,[22] and human bronchial epithelial cells.[23–25] Results of the present investigation indicate constitutive expression of the CCR3 receptor on human alveolar type II epithelial cells (Figs. 4 and 5). These results are in agreement with those reported for BEAS-2B bronchial epithelial cells, which also constitutively express CCR3.[25] Results of the present investigation indicate that TNF-α treatments also upregulate CCR3 alveolar epithelial cells. In contrast to the bronchial epithelial cells, however, alveolar cells were responsive to IL-4 alone and in combination with TNF-α.

CCR3 receptor modulation by cytokines has been reported recently for several cell types involved in inflammatory/allergic responses.[18,41,42] Having found that cytokine-stimulated alveolar type II epithelial cells express all three eotaxins and possess CCR3 receptors, we stimulated A549 epithelial cells with TNF-α, IL-1β, or IL-4 alone or in combination and explored receptor modulation. Flow cytometry results confirmed that CCR3 receptors were increased by cytokine exposure (Fig. 5). A variety of mechanisms, such as upregulation, downregulation, desensitization,[17] mobilization of intracellular receptor pools,[21,43] and internalization,[44,45] may contribute to receptor modulation during cytokine-induced activation of cells engaged in inflammatory responses. Data from the present investigation are consistent with the possibility that airway epithelial cells may also internalize or sequester CCR3 receptors, which may be rapidly mobilized and alter effector functions, causing orchestration and prolongation of aspects of airway inflammatory responses. Ongoing studies are designed to delineate the consequences of eotaxin treatment of airway epithelial cells on the fate of expressed and sequestered CCR3 receptors.

Collectively, these data support autocrine/paracrine CCR3-ligand interactions that contribute to the perpetuation of the underlying inflammation in pulmonary diseases, such as asthma. Parenchymal mast cells, alveolar macrophages, and T cells, colocalized in the airways, are paracrine sources of cytokines (IL-4, TNF-α, IL-1β) that stimulate cytokine/chemokine release from airway epithelial cells and upregulate CCR3 receptors. Extracellular release of CCL11 from the airway epithelium then signals trafficking and recruitment of eosinophils but may also interact in an autocrine fashion with the cell surface CCR3 receptors. Resident mast cells and recruited eosinophils serve as paracrine sources of CCL11. Autocrine/paracrine stimulation of the epithelial CCR3 receptors may signal synthesis and release of CCL24 and CCL26. The potential for further autocrine/paracrine activation of both eosinophils and the airway epithelium exists and may serve to amplify the inflammatory response.

In conclusion, results of these investigations have demonstrated that cytokine-stimulated alveolar type II epithelial cells are a pulmonary source of not only CCL11 but also of CL24 and CCL26 and that these cells possess the CCR3 receptors through which the eotaxins transduce intracellular messages. Results also suggest that stimulated alveolar type II epithelial cells may participate in coordinating expression of several chemokines at different times after an allergic stimulus. Cytokines have become a focus for development of novel anti-inflammatory and antiallergic therapeutic agents,[46–48] and these results support the contention that the eotaxin CCR3 pathway should be included as an attractive drug target.

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