

Induction of granulocyte-macrophage colony-stimulating factor by a new cytokine, ML-1 (IL-17F), via Raf I-MEK-ERK pathway

Mio Kawaguchi, MD,^{a,b} Fumio Kokubu, MD,^b Miho Odaka, MD,^b Shin Watanabe, MD,^b Shintaro Suzuki, MD,^b Koushi Ieki, MD,^b Satoshi Matsukura, MD,^b Masatsugu Kurokawa, MD,^b Mitsuru Adachi, MD,^b and Shau-Ku Huang, PhD^a

Baltimore, Md, and Tokyo, Japan

Background: ML-1 (IL-17F) is a recently discovered cytokine, and its function remains elusive. GM-CSF is a crucial cytokine for the maturation of various cell types and regulates allergic airway inflammation.

Objective: The functional effect of ML-1 in the expression of GM-CSF was investigated.

Methods: The levels of gene and protein expression in normal human bronchial epithelial cells (NHBEs) in the presence or absence of various kinase inhibitors or, in some cases, of a Raf1 dominant-negative mutant were determined by RT-PCR and ELISA, respectively. Western blotting was performed to investigate kinase activation.

Results: The results showed first that ML-1 induces, in a time-dependent and dose-dependent manner, the gene and protein expression for GM-CSF NHBEs, which are associated with activation of Raf1 and MAP kinase kinase (MEK) kinases. Selective MEK inhibitors, PD98059 and U0126, and Raf1 kinase inhibitor I significantly inhibited ML-1-induced GM-CSF production. Furthermore, overexpression of Raf1 dominant-negative mutants inhibited IL-17F-induced GM-CSF expression. The combination of PD98059 and Raf1 kinase inhibitor I completely blocked GM-CSF production, whereas 2 protein kinase C inhibitors, Ro-31-7549 and GF109203X, and a phosphatidylinositol 3-kinase inhibitor, LY294002, showed no inhibitory effect.

Conclusion: These findings suggest that ML-1 induces GM-CSF expression through the activation of the Raf1-MEK—extracellular signal-regulated kinase 1/2 pathway. (*J Allergy Clin Immunol* 2004;114:444-50.)

Key words: Bronchial epithelial cells, ERK1/2, GM-CSF, MAP kinase, Raf1

Airway allergic inflammation is characterized by activation and migration of various inflammatory cells, such as eosinophils, granulocytes, and macrophages. The

Abbreviations used

DMSO: Dimethyl sulfoxide
ERK: Extracellular signal-regulated kinase
MEK: MAP kinase kinase
NF- κ B: Nuclear factor κ B
NHBE: Normal human bronchial epithelial cell
PI3K: Phosphatidylinositol 3-kinase
PKC: Protein kinase C

accumulation of these cell types causes bronchoconstriction, lung damage, and fibrosis. Bronchial epithelial cells play an important role in the regulation of airway inflammation through their ability to express a wide range of proinflammatory cytokines, including GM-CSF.¹ Investigation of the regulatory pathway involving modulation of airway epithelium by cytokines is thus important to uncover the pathogenic mechanisms of airway inflammation.

GM-CSF plays a pivotal role in inflammatory and immunologic processes. Release of GM-CSF in the airway can mediate acute inflammatory responses as well as initiate and perpetuate local immune responses. Elevated levels of GM-CSF have been well demonstrated in bronchoalveolar lavage fluid, endobronchial biopsy, and sputum samples from asthmatics.²⁻⁴ Elevated levels of GM-CSF, mainly derived from epithelial cells, have been demonstrated to increase eosinophil activation and survival in asthmatics.⁵⁻⁷ Bronchial asthma is an inflammatory disease of the airways characterized by eosinophil infiltration of the lung and airway hyperreactivity. GM-CSF stimulates the recruitment and activation of eosinophils via β -integrin-mediated adhesion to epithelial and endothelial cells⁸ and prolongs eosinophil survival via inhibition of apoptosis.⁹⁻¹¹ Moreover, mouse models of asthma and diesel-induced hyperresponsiveness have demonstrated an association between epithelial cell-derived GM-CSF and airway hyperresponsiveness.^{12,13} These findings suggest that GM-CSF is 1 of the critical cytokines in the pathogenesis of allergic airway inflammation, and airway epithelium plays a pivotal role in causing and perpetuating airway inflammation via the induction of GM-CSF. However, the regulatory mechanisms of GM-CSF expression in airway epithelium have not been well understood.

From ^athe Asthma and Allergy Center, Johns Hopkins University, Baltimore; and ^bthe First Department of Internal Medicine, Showa University School of Medicine, Tokyo.

Supported in part by National Institutes of Health grant AI-40274 and an Astra Zeneca Asthma Research Award.

Received for publication December 23, 2003; revised March 3, 2004; accepted for publication March 15, 2004.

Reprint requests: Shau-Ku Huang, PhD, Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224-6801. E-mail: skhuang@jhmi.edu.

0091-6749/\$30.00

© 2004 American Academy of Allergy, Asthma and Immunology
doi:10.1016/j.jaci.2004.03.047

Recently, we and others independently discovered a novel cytokine, ML-1¹⁴ (IL-17F^{15,16}), belonging to the IL-17 gene family, but its function and signaling pathways remain to be defined. ML-1 is expressed in activated CD4⁺ T cells, basophils, and mast cells, 3 important cell types involved in allergic inflammation.¹⁴ Significantly, upregulated ML-1 gene expression is seen at sites of allergen challenge in the airways of asthmatics,¹⁴ but its involvement in pulmonary allergic inflammation is unclear. To gain further understanding of the function and signaling pathways of ML-1, the role of ML-1 in the expression of GM-CSF was investigated. In this article, we provide evidence that ML-1 is able to induce GM-CSF expression in bronchial epithelial cells, involving the activation of the Raf1–MAP kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) 1/2 signaling pathway.

METHODS

Cell culture and human recombinant ML-1

Normal human bronchial epithelial cells (NHBEs) were purchased from Clonetics (San Diego, Calif) and cultured in bronchial epithelial basal medium (Clonetics) containing 0.5 ng/mL human recombinant epidermal growth factor, 52 µg/mL bovine pituitary extract, 0.1 ng/mL retinoic acid, 0.5 µg/mL hydrocortisone, 5 µg/mL insulin, 10 µg/mL transferrin, 0.5 µg/mL epinephrine, 6.5 ng/mL tri-iodothyronine, 50 µg/mL gentamicin, and 50 µg/mL amphotericin-B (Clonetics). The cells were cultured for no more than 3 passages before the analysis. In the case of the experiments using Raf1 dominant-negative mutants, a bronchial epithelial cell line, BEAS-2B, was used instead of NHBE for efficient transfection and was cultured in Hanks' F12/Dulbecco modified Eagle medium (Biofluids, Rockville, Md) with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 ng/mL streptomycin (Life Technologies-BRL, Gaithersburg, Md). Human recombinant ML-1 was generated as described previously.¹⁴ Endotoxin levels were tested by using Kinetic-QCL Chromogenic Limulus amebocyte lysate (Bio Whittaker, Walkersville, Md). Endotoxin levels were undetectable. The cells were treated with ML-1 (10 and 100 ng/mL) for various periods.

Gene expression of GM-CSF

Total RNA was extracted by using RNeasy (Qiagen, Chatsworth, Calif) from 1×10^6 cells at 1, 2, 4, 12, and 24 hours after stimulation with 10 and 100 ng/mL ML-1. cDNAs were synthesized from 500 ng of total RNA in the presence of Moloney murine leukemia virus reverse transcriptase (1 U/reaction; Sigma, St Louis, Mo), oligo (dT) primer, and reaction buffer at 42°C for 90 minutes, followed by PCR. The sequences of PCR primers for GM-CSF were forward, 5'-GTGGCCTGCAGCATCTCT-3', and reverse, 5'-AAAGG-GGATGACAAGCAGAA-3'; and for G3PDH were forward, 5'-ACCACAGTCCATGCCATCAC-3', and reverse, 5'-TCCACC-ACCCTGTTGCTGTA-3'. The amplification reaction was performed for subsaturating cycles with denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds. The expected size for GM-CSF was 375 bp and for G3PDH was 450 bp. The PCR products were detected by ethidium bromide staining and quantified by video densitometry with Image 1.61 software (National Institutes of Health Public Software, National Institutes of Health, Bethesda, Md). The level of GM-CSF gene expression was quantified by calculating the ratio of densitometric readings of the band intensity for GM-CSF and G3PDH from the same cDNA sample. The values are expressed as means $\pm$ SDs

($n = 3$ experiments). In all cases, the induction of GM-CSF by ML-1 was clearly evident, allowing quantitative comparison by RT-PCR.

Protein levels of GM-CSF

GM-CSF protein levels in the supernatants and cell lysates of ML-1-stimulated cells were determined with a commercially available ELISA kit (Biosource, Camarillo, Calif) according to the manufacturer's instructions. Cell supernatants were harvested from cultures in the absence or presence of 10 or 100 ng/mL ML-1 at 2, 6, 12, 24, or 48 hours after stimulation. The amount of secreted GM-CSF was determined by the ELISA and expressed as the amount recovered per 10^6 cells. Cells corresponding to the supernatant samples described were lysed in 0.5 mL Nonidet P-40 lysis buffer (20 mmol/L Tris [pH 7.4], 4 mmol/L EDTA, 1 mmol/L phenylmethanesulfonyl fluoride, 100 mg/mL aprotinin, 200 mg/mL leupeptin, 50 mmol/L NaF, 5 mmol/L Na₄P₂O₇, and 1% Nonidet P-40 [all Sigma]). The GM-CSF concentration of cell lysate was reported as the amount recovered per 10^6 cells. The values are expressed as means $\pm$ SDs ($n = 6$ experiments).

ML-1-induced Raf1 and MEK activation and the effect of inhibitors

For analysis of activation of the Raf1-MEK-ERK1/2 pathway, NHBEs were treated with ML-1 (100 ng/mL) and in some cases with or without the treatment with a Raf1 kinase inhibitor I or the vehicle control (Me₂SO), for 1 hour. The final concentration of Me₂SO did not exceed 0.1% (vol/vol). After treatment and lysis of the cells, the cellular extracts (1×10^6 cell equivalents/lane) were suspended with an equal volume of 2 times loading buffer (0.1 mol/L Tris-HCl, pH 6.8; 4% SDS; 0.005% bromophenol blue; and 20% glycerol) containing 2-mercaptoethanol (0.7 mol/L), subjected to 4% to 20% Tris-glycine gel electrophoresis (NOVEX, San Diego, Calif), and probed with various antibodies: rabbit anti-MEK1/2 antibody, antiphospho-MEK1/2 antibody (Cell Signaling Technology, Beverly, Mass), anti-Raf1 antibody, and antiphospho-Raf1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), followed by incubation with peroxidase-linked donkey anti-rabbit Ig antibody (Amersham Biosciences, Piscataway, NJ) and visualized with enhanced chemiluminescence.

For analysis of the effects of kinase inhibitors, the cells were treated in the presence or absence of the following kinase inhibitors at varying doses: MEK1/2 inhibitors, PD98059 (2'-amino-3'-methoxyflavone; Calbiochem, La Jolla, Calif) and U0126 (1,4-diamino-2,3-dicyano-1,4-bis[o-aminophenylmercapto] butadiene; New England Bio Labs, Beverly, Mass); p38 inhibitor, SB202190 (4-[4-fluoro-phenyl]-2-[4-hydroxyphenyl]-5-[4-pyridyl] 1H-imidazole; Calbiochem); a Raf1 kinase inhibitor I (5-iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone; Calbiochem); a c-Jun N-terminal kinase inhibitor, SP600125 (Anthra [1,9-cd]pyrazol-6[2H]-one; Calbiochem); protein kinase C (PKC) inhibitors, Ro-31-7549 (2-[1-(3-aminopropyl) indol-3-yl]-3-[1-methylindol-3-yl] maleimide acetate; Calbiochem) and GF109203X (3-[1-(dimethylaminopropyl) indol-3-yl]-4-[indol-3-yl] maleimide, 3-[1-(3-[dimethylamino]propyl)-1H-indol-3-yl]-4-[1H-indol-3-yl]-1H-pyrrole-2,5-dione; Sigma); a phosphatidylinositol 3-kinase PI3K inhibitor, LY294002 (2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one; Calbiochem); and a vehicle control, dimethyl sulfoxide (DMSO; Me₂SO) for 1 hour before treatment with ML-1 (100 ng/mL). The cells and cell supernatants were harvested at 2 and 24 hours after stimulation for analyses of RT-PCR and ELISA, respectively. The final concentration of DMSO did not exceed 0.1% (vol/vol). GM-CSF gene expression and protein levels in the supernatants were determined as described. The values are expressed as means $\pm$ SDs ($n = 4$ experiments).

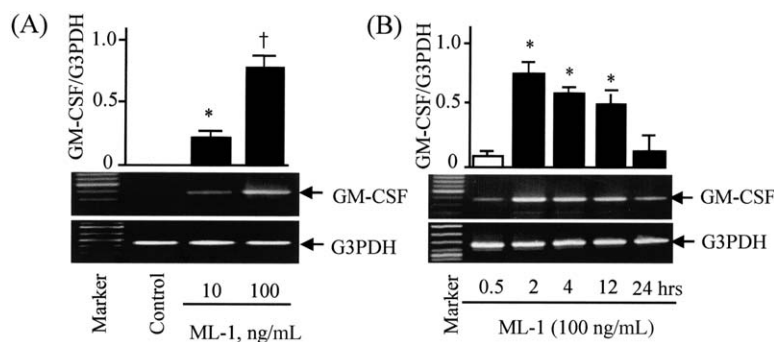


FIG 1. A, Induction of GM-CSF gene expression by different doses of ML-1. * $P < .05$ was considered significant versus control. † $P < .05$ was considered significant versus 10 ng/mL ML-1-stimulated cells. **B**, Time course study of GM-CSF gene expression. The values are expressed as means $\pm$ SDs ($n = 3$). * $P < .05$ was considered significant versus the intensity of 0.5-hour time point.

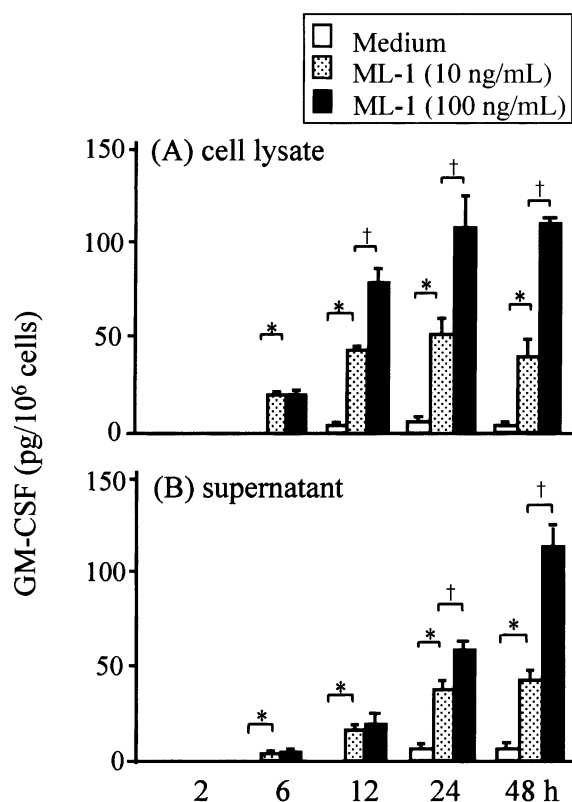


FIG 2. GM-CSF protein levels in lysates (**A**) and supernatants (**B**) of NHBs. The values are expressed as means $\pm$ SDs ($n = 6$). * $P < .05$ was considered significant versus control. † $P < .05$ was considered significant versus 10 ng/mL ML-1-stimulated cells.

Overexpression of Raf1 dominant-negative mutant in BEAS-2B cells

The plasmid encoding pCMV-RafS621A vector (dominant-negative mutant of Raf1) cloned into pCMV and a control vector were purchased from Clontech (Palo Alto, Calif). The plasmids were prepared by using the Qiagen plasmid DNA preparation kit. NHBs were difficult to use for transfection experiments. An epithelial cell line, BEAS-2B, was used instead. The cells were cultured on 100-mm plates and were transfected by an Effectene Reagent (Qiagen)

according to the manufacturer's instructions. The cells were selected with 500 ng/mL Geneticin (G418; Gibco/BRL, Grand Island, NY). After selection, the cells were seeded into 6-well culture plates. The cells were near confluent, and the cells and cell supernatants were then harvested at 2 and 24 hours after stimulation with 100 ng/mL ML-1 for analyses of RT-PCR and ELISA, respectively. GM-CSF gene expression and protein levels in the supernatants were determined as described. The values are expressed as means $\pm$ SDs ($n = 3$ experiments).

Data analysis

The statistical significance of differences was determined by ANOVA. The values are expressed as means $\pm$ SDs from independent experiments. Any difference with P values $< .05$ was considered. When ANOVA indicated a significant difference, the Scheffe F test was used to determine the difference between groups.

RESULTS

To examine whether ML-1 was able to induce GM-CSF expression, NHBs were stimulated with varying doses of ML-1, and the levels of gene expression and protein production in cell lysates and supernatants were analyzed. The results showed first that although GM-CSF gene expression was not seen in control culture, ML-1 induced, in a dose-dependent manner, the expression of GM-CSF at the 2-hour time point (Fig 1, *A* and *B*). In the time course experiments, ML-1-induced GM-CSF gene expression peaked at the 2-hour time point in ML-1 (100 ng/mL)-treated NHBs (Fig 1, *C* and *D*) and returned to baseline 24 hours after stimulation. To analyze the protein expression for GM-CSF, NHBs were cultured in the absence or presence of varying concentrations of ML-1 at 5 different time points. GM-CSF proteins were not detected in the absence of ML-1 in NHBs. The levels of GM-CSF in cell lysates were significantly increased 6 hours after stimulation with 10 and 100 ng/mL ML-1 and reached plateau at the 12-hour and 24-hour time points, respectively (Fig 2, *A*), whereas the levels of secreted GM-CSF were significantly elevated 24 hours after stimulation and were increased further at 48 hours (Fig 2, *B*).

Bronchial epithelial cells are exposed to numerous environmental stimuli, which can activate intracellular

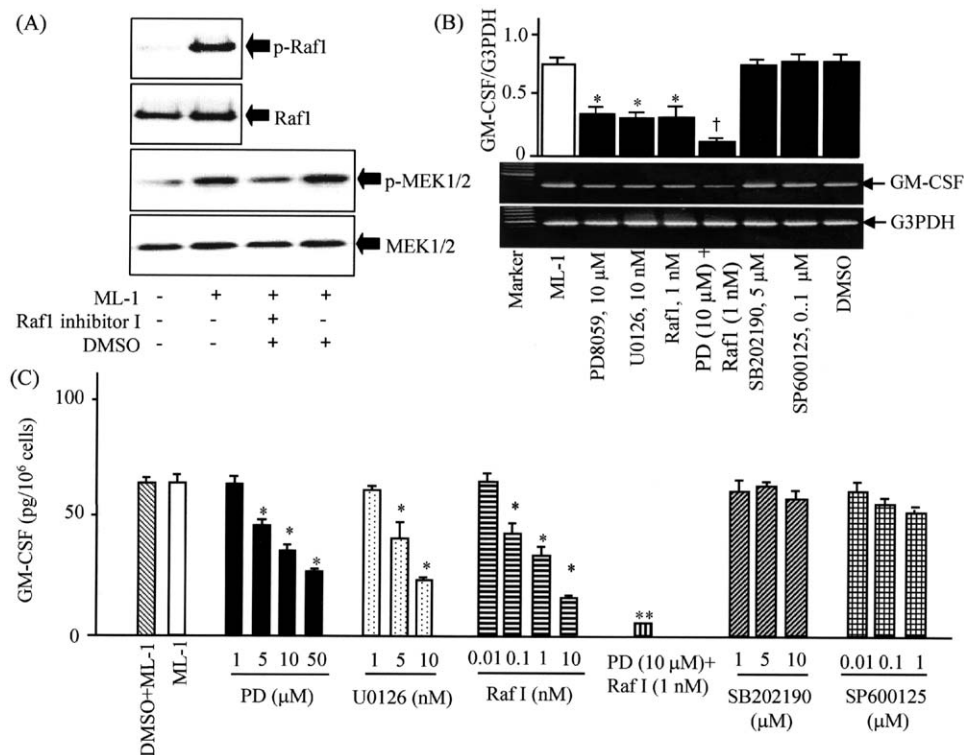


FIG 3. A, Western blotting analysis of Raf1 and MEK activation. The results shown are representative of 3 separate experiments. Effect of kinase inhibitors on GM-CSF gene **(B)** and protein **(C)** expression in NHBs. The values are expressed as means $\pm$ SDs ($n = 4$ experiments). * $P < .05$ was considered significant versus ML-1-stimulated cells. † $P < .05$ was considered significant versus single inhibitor.

signaling cascades, leading to upregulated gene expression and a wide range of responses, such as cytokine expression, proliferation, and apoptosis.¹⁷ The mitogen-activated protein kinase family is likely to be central to these processes because they are known to regulate intracellular signal transduction in response to many agonists, including growth factors, cytokines, hormones, oxidants, and environmental stress factors.¹⁸ To investigate ML-1-mediated signaling events leading to the induction of GM-CSF, the activation of the Raf1-MEK-ERK1/2 pathway was investigated. First, Western blotting analysis revealed activation of Raf1 and MEK1/2 in ML-1-stimulated cells (Fig 3, A), and preincubation of the cells with a Raf1 kinase inhibitor I (1 nmol/L) for 1 hour diminished the activation of MEK1/2 in comparison with that seen in the vehicle control culture (Fig 3, A). Furthermore, analysis of various kinase inhibitor effect on ML-1-induced GM-CSF expression showed that pretreatment of the cells for 1 hour with each of the selective MEK inhibitors, PD98059 (10 μ mol/L), U0126 (5 nmol/L), and Raf1 kinase inhibitor I (1 nmol/L), significantly decreased the levels of ML-1-induced GM-CSF gene expression in NHBs (Fig 3, B). As shown in Fig 3, C, 1 hour pretreatment of the cells with PD98059, U0126, and Raf1 kinase inhibitor I significantly attenuated, in a dose-dependent manner, the production of GM-CSF, whereas 1 hour pretreatment of the cells with vehicle alone (0.05% DMSO) did not affect GM-CSF release. In

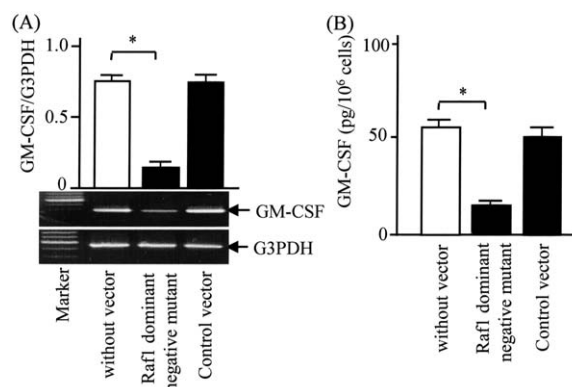


FIG 4. Effect of overexpression of Raf1 dominant-negative mutants on GM-CSF gene **(A)** and protein **(B)** expression in BEAS-2B cells. The values are expressed as means $\pm$ SDs ($n = 3$). * $P < .05$ was considered significant versus the intensity of ML-1-stimulated cells without vector.

addition, the protein levels of GM-CSF were unchanged in ML-1-treated cells in the presence of a p38 kinase inhibitor, SB202190, and a JNK inhibitor, SP600125, even at a dose of 10 μ mol/L and 1 μ mol/L, respectively (Fig 3, C). Although induction of GM-CSF is partially inhibited by PD98059, U0126, or Raf1 kinase inhibitor I even at relatively high doses (50 μ mol/L, 10 nmol/L, and 10 nmol/L, respectively), the combination of 10 μ mol/L

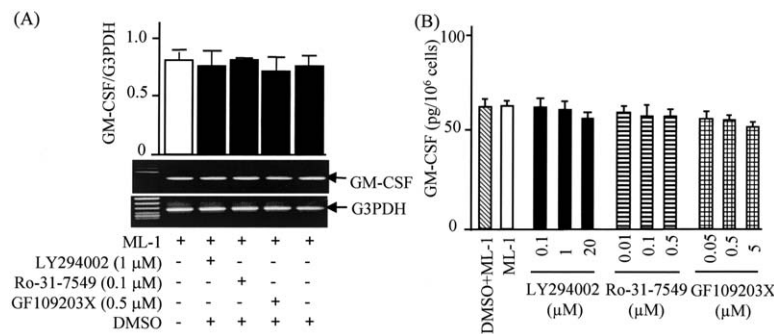


FIG 5. Effect of PD98059, LY294002, Ro-31-7549, and GF109203X on GM-CSF gene (A) and protein (B) expression in NHBs.

PD98059 and 1 nmol/L Raf1 kinase inhibitor I inhibited, to a greater degree, the production of GM-CSF in NHBs (Fig 3, B and C). The total number of cells and cell viability at the end of the culture period for each experiment did not differ among all culture conditions as determined by trypan blue exclusion assay, suggesting that the inhibition of ML-1-induced GM-CSF expression did not result from cytotoxicity.

The involvement of Raf1 kinase in ML-1-induced GM-CSF expression was further confirmed by the use of a Raf1 dominant-negative mutant with serine to alanine substitution at position 621. The results showed that overexpression of Raf1 dominant-negative mutants in BEAS-2B cells significantly inhibited ML-1-induced GM-CSF gene expression (Fig 4, A) and protein release (Fig 4, B), whereas the cells transfected with a control vector showed no significant change in the level of GM-CSF expression (Fig 4). These results suggest, therefore, the importance of the Raf1-MEK-ERK signaling pathway in ML-1-induced GM-CSF expression. It has been demonstrated that both PKC and PI3K are linked to the MAP kinase pathway, and that signaling from receptor tyrosine kinases to ERK1/2 is dependent on Ras and Raf1. Further, PKC has been shown to be a key activator of the Raf1/mitogen-activated protein kinase cascade at multiple steps that result in upregulation of ERK1/2. In our experiment, however, selective PKC inhibitors, Ro-31-7549 (0.1 μmol/L) and GF109203X (0.5 μmol/L), did not decrease the level of ML-1-induced GM-CSF gene expression in NHBs (Fig 5, A). Similarly, no significant inhibitory effect was seen on ML-1-induced GM-CSF protein production when the inhibitors were used (Fig 5, B). Moreover, a PI3K inhibitor, LY294002, showed no significant inhibition of ML-1-induced GM-CSF gene expression in NHBs (Fig 5, A) and protein production in NHBs (Fig 5, B) when LY294002 (0.1-20 μmol/L) was used.

DISCUSSION

In this report, we provide evidence that ML-1 (IL-17F) is able to induce GM-CSF expression in primary bronchial epithelial cells through the activation of the Raf1-MEK-

ERK1/2 pathway. ML-1 is derived from activated CD4⁺ T cells, basophils, and mast cells, which are important regulatory cells for allergic airway inflammation.¹⁴ The ability of ML-1 to induce epithelium-derived GM-CSF raises a strong possibility that ML-1-induced GM-CSF is involved in allergic inflammation. In our previous report, the ML-1 gene is upregulated in the bronchoalveolar lavage cells from asthmatic patients after segmental allergen challenge.¹⁴ The current study lends support for a role of ML-1 in the airway inflammatory responses via the induction of GM-CSF.

The results of the current study demonstrate the involvement of the Raf1-MEK-ERK signaling pathway in ML-1-induced GM-CSF expression. It is of interest to note that the induction of IL-6 and IL-8 by ML-1 is dependent on the activation of ERK1/2, but not p38 and JNK,¹⁹ suggesting that ML-1 is able to induce multiple cytokines via the same signaling pathway. Also, ERK1/2 may serve as a pivotal signaling molecule for IL-17 family members, such as IL-17A and ML-1 (IL-17F), and would be a potential therapeutic target for IL-17 family-associated airway diseases. Further, our pharmacologic studies showed that neither PKC nor PI3K is involved in ML-1-induced GM-CSF expression. This is in contrast to the finding that TNF-α induces GM-CSF expression via activation of PKC and subsequent activation of ERK1/2 kinases in NHBs.²⁰ It is noted also that a PKC inhibitor, Ro-31-7549, does not affect IL-17A-induced IL-6 and IL-8 expression in bronchial epithelial cells.²¹ Another PKC inhibitor, GF-109203X, also does not block diesel exhaust particle-induced GM-CSF expression.²² Furthermore, a selective PI3K inhibitor, LY294002, does not affect IL-17A-induced IL-6 and IL-8 in bronchial epithelial cells.²³ Our data also showed that LY294002 did not inhibit ML-1-induced GM-CSF expression. Activated Ras promotes cell survival in epithelial cells through activation of PI3K and Akt/PKB, and it is known that at high dose (20 μmol/L), LY294002 induces apoptosis.²⁴ In the current study, the cell number and viability were not altered in the presence of 20 μmol/L of LY294002 (data not shown). This is likely a result of different culture conditions and cell types used. However, we cannot fully exclude the involvement of other molecules, such as RalGDS and PLC-ε, because recent

reports have demonstrated that Ras is able to activate these signaling molecules besides Raf1.^{25,26} These results demonstrate, therefore, that multiple functional pathways exist in the regulation of GM-CSF expression.

To date, the upstream signaling pathway of Ras-Raf1-MEK-ERK1/2 induced by ML-1 is unclear. There have been few reports concerning the upstream signaling pathway of GM-CSF expression. However, the results of the current study suggest that Raf1 is predominantly associated with the activation of the MEK-ERK1/2 pathway. Therefore, we concluded that the Raf1-MEK-ERK1/2 pathway is a central upstream pathway of ML-1-induced GM-CSF expression in NHBs. Further study is needed to identify functionally the putative receptor for ML-1 and its proximal signaling pathways. Also, the downstream signaling pathway is currently unclear. GM-CSF is known to be regulated by a transcription factor, nuclear factor κ B (NF- κ B).²⁷ IL-17A is known to activate NF- κ B in chondrocytes and intestinal epithelial cells.²⁸ Because of high homology between IL-17A and ML-1, it is possible that ML-1 is able to activate NF- κ B in the downstream signaling pathway. However, we could not detect the activation of NF- κ B by ML-1 in NHBs (data not shown), suggesting an NF- κ B-independent pathway, for which further investigation is needed.

Bronchial epithelial cells are able to release GM-CSF in response to physiologic stimuli relevant for asthma, including dust mite proteolytic allergens, human rhinovirus 16, respiratory syncytial virus, and histamine.^{29,30} Furthermore, bronchial epithelial cells have been demonstrated to secrete GM-CSF in response to several cytokines, such as TNF- α , IL-1 β , IL-4, and IL-13, which are strongly involved in allergic response.^{31,32} A substantial amount of GM-CSF is produced in the airway epithelium after antigen challenge in asthmatic subjects, and GM-CSF appears to be the major cytokine responsible for eosinophil survival in the bronchoalveolar lavage fluid of symptomatic asthmatic subjects.³³ In addition, GM-CSF acts as a cofactor for superoxide production and degranulation^{33,34} and elicits multiple effects on other components of the immune systems, including dendritic cells, which initiate and perpetuate allergic inflammation.³⁵ Furthermore, GM-CSF also has multiple effects on neutrophil function, including increasing 5-lipoxygenase level, tyrosine phosphorylation, aggregation, superoxide anion production, degranulation, cytotoxicity, and adhesion to vascular endothelial cells.³⁶ Finally, GM-CSF stimulates monocyte survival and differentiation into mature macrophages, which are the largest cell population in the airways of patients with allergic inflammatory diseases.^{37,38} Hence, these data strongly suggest that ML-1 shows multiple biological activities via the induction of GM-CSF, adding to a growing list of cytokines involved in the regulation of allergic inflammation.

In conclusion, this study reports that ML-1 induces GM-CSF via the activation of the Raf1-MEK-ERK1/2 pathway. These results suggest that ML-1 is involved in allergic inflammation via the induction of GM-CSF. ML-1 may cause and perpetuate airway inflammation at multiple

steps. Furthermore, the control of the Raf1-MEK-ERK1/2 pathway is an attractive pharmacotherapeutic strategy for inhibition of ML-1-induced airway immunologic and inflammatory responses.

We thank Hiroko Takeuchi, Tomoko Shinbara, and Makoto Murakami for their excellent technical assistance.

REFERENCES

1. Polito AJ, Proud D. Epithelial cells as regulators of airway inflammation. *J Allergy Clin Immunol* 1998;102:714-8.
2. Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol* 1992;89:958-67.
3. Oh JW, Lee HB, Kim CR, Yum MK, Koh YJ, Moon SJ, Kang JO, Park IK. Analysis of induced sputum to examine the effects of inhaled corticosteroid on airway inflammation in children with asthma. *Ann Allergy Asthma Immunol* 1999;82:491-6.
4. Soloperto M, Mattoso VL, Fasoli A, Mattoli S. A bronchial epithelial cell-derived factor in asthma that promotes eosinophil activation and survival as GM-CSF. *Am J Physiol* 1991;260:L530-8.
5. Cox G, Ohtoshi T, Vancheri C, Denburg JA, Dolovich J, Gauldie J, Jordana M. Promotion of eosinophil survival by human bronchial epithelial cells and its modulation by steroids. *Am J Respir Cell Mol Biol* 1991;4:525-31.
6. Masuda T, Suda Y, Shimura S, Maruyama N, Aizawa T, Tamura G, Sasaki H, Takishima T. Airway epithelial cells enhance eosinophil survival. *Respiration* 1992;59:238-422.
7. Vignola AM, Chanez P, Chiappara G, Siena L, Merendino A, Reina C, Gagliardo R, Profita M, Bousquet J, Bonsignore G. Evaluation of apoptosis of eosinophils, macrophages, and T lymphocytes in mucosal biopsy specimens of patients with asthma and chronic bronchitis. *J Allergy Clin Immunol* 1999;103:563-73.
8. Jagels MA, Daffern PJ, Zuraw BL, Hugli TE. Mechanisms and regulation of polymorphonuclear leukocyte and eosinophil adherence to human airway epithelial cells. *Am J Respir Cell Mol Biol* 1991;21:418-27.
9. Gasson JC. Molecular physiology of granulocyte-macrophage colony-stimulating factor. *Blood* 1991;77:1131-45.
10. Lopez AF, Williamson J, Gamble JR, Begley CG, Harlan JM, Klebanoff SJ, Waltersdorff A, Wong G, Clark SC, Vadas MA. Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression, and survival. *J Clin Invest* 1986;78:1220-8.
11. Yousefi SK, Blaser H, Simon U. Activation of signaling pathways and prevention of apoptosis by cytokines in eosinophils. *Int Arch Allergy Immunol* 1997;112:9-12.
12. Yamashita N, Tashimo H, Ishida H, Kaneko F, Nakano J, Kato H, Hirai K, Horiuchi T, Ohta K. Attenuation of airway hyperresponsiveness in a murine asthma model by neutralization of granulocyte-macrophage colony-stimulating factor (GM-CSF). *Cell Immunol* 2002;219:92-7.
13. Lei XF, Ohkawara Y, Stampfli MR, Gauldie J, Croitoru K, Jordana M, Xing Z. Compartmentalized transgene expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in mouse lung enhances allergic airways inflammation. *Clin Exp Immunol* 1998;113:157-65.
14. Kawaguchi M, Onuchic LF, Li XD, Essayan DM, Schroeder J, Xiao HQ, Liu MC, Krishnaswamy G, Germino G, Huang SK. Identification of a novel cytokine, ML-1, and its expression in subjects with asthma. *J Immunol* 2001;167:4430-5.
15. Starnes T, Robertson MJ, Sledge G, Kelich S, Nakshatri H, Broxmeyer HE, Hromas R. IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J Immunol* 2001;167:4137-40.
16. Hymowitz SG, Filvaroff EH, Yin JP, Lee J, Cai L, Risser P, Maruoka M, Mao W, Foster J, Kelley RF, Pan G, Gurney AL, de Vos AM, Starovasnik MA. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J* 2001;20:5332-41.

17. Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. *Adv Cancer Res* 1998;74:49-139.
18. Brunet A, Pouyssegur J. Mammalian MAP kinase modules: how to transduce specific signals. *Essays Biochem* 1997;32:1-16.
19. Kawaguchi M, Onuchic LF, Huang SK. Extracellular signal-regulated kinase (ERK) 1/2 regulates interleukin (IL)-6 and 8 expression by a novel cytokine, ML-1. *J Biol Chem* 2002;277:15229-32.
20. Kawaguchi M, Kokubu F, Kuga H, Matsukura S, Hoshino H, Ieki K, Imai T, Adachi M, Huang SK. Modulation of bronchial epithelial cells by IL-17. *J Allergy Clin Immunol* 2001;108:804-9.
21. Yao Z, Spriggs MK, Derry JM, Strockbine L, Park LS, VandenBos T, Zappone JD, Painter SL, Armitage RJ. Molecular characterization of the human interleukin (IL)-17 receptor. *Cytokine* 1997;9:794-800.
22. Boland S, Bonvallot V, Fournier T, Baeza-Squiban A, Aubier M, Marano F. Mechanisms of GM-CSF increase by diesel exhaust particles in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L25-32.
23. Laan M, Lotvall J, Chung KF, Linden A. IL-17-induced cytokine release in human bronchial epithelial cells in vitro: role of mitogen-activated protein (MAP) kinases. *Br J Pharmacol* 2001;133:200-6.
24. Vojtek AB, Der CJ. Increasing complexity of the Ras signaling pathway. *J Biol Chem* 1998;273:19925-8.
25. Song C, Hu CD, Masago M, Kariyai K, Yamawaki-Kataoka Y, Shibatohe M, Wu D, Satoh T, Tataoka K. Regulation of a novel human phospholipase C, PLCepsilon, through membrane targeting by Ras. *J Biol Chem* 2001;276:2752-7.
26. Kim J, Sanders SP, Siekierski ES, Casolaro V, Proud D. Role of NF-kappa B in cytokine production induced from human airway epithelial cells by rhinovirus infection. *J Immunol* 2000;165:3384-92.
27. Shalo-Barak T, Quach J, Lotz M. Interleukin-17-induced gene expression in articular chondrocytes is associated with activation of mitogen-activated protein kinases and NF-kappaB. *J Biol Chem* 1998;273:27467-73.
28. Awane M, Andres PG, Li DJ, Reinecker HC. NF-kappa B-inducing kinase is a common mediator of IL-17-, TNF-alpha-, and IL-1 beta-induced chemokine promoter activation in intestinal epithelial cells. *J Immunol* 1999;162:5337-44.
29. Churchill L, Friedman B, Schleimer RP, Proud D. Production of granulocyte-macrophage colony-stimulating factor by cultured human tracheal epithelial cells. *Immunology* 1992;75:189-95.
30. Soukup JM, Becker S. Role of monocytes and eosinophils in human respiratory syncytial virus infection in vitro. *Clin Immunol* 2003;107:178-85.
31. Hashimoto S, Matsumoto K, Gon Y, Maruoka S, Kujime K, Hayashi S, Takeshita I, Horie T. p38 MAP kinase regulates TNF alpha-, IL-1 alpha- and PAF-induced RANTES and GM-CSF production by human bronchial epithelial cells. *Clin Exp Allergy* 2000;30:48-55.
32. Nakamura Y, Azuma M, Okano Y, Sano T, Takahashi T, Ohmoto Y, Sone S. Upregulatory effects of interleukin-4 and interleukin-13 but not interleukin-10 on granulocyte/macrophage colony-stimulating factor production by human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1996;15:680-7.
33. Park CS, Choi YS, Ki SY, Moon SH, Jeong SW, Uh ST, Kim YH. Granulocyte macrophage colony-stimulating factor is the main cytokine enhancing survival of eosinophils in asthmatic airways. *Eur Respir J* 1998;12:872-8.
34. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;392:245-52.
35. Khwaja A, Linch DC. Effects of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor on neutrophil formation and function. *Curr Opin Hematol* 1994;1:216-20.
36. Arm JP, Lee TH. The pathobiology of bronchial asthma. *Adv Immunol* 1992;51:323-82.
37. Young DA, Lowe LD, Clark SC. Comparison of the effects of IL-3, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor in supporting monocyte differentiation in culture: analysis of macrophage antibody-dependent cellular cytotoxicity. *J Immunol* 1990;145:607-15.
38. Puddicombe SM, Davies DE. The role of MAP kinases in intracellular signal transduction in bronchial epithelium. *Clin Exp Allergy* 2000;30:7-11.