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Oncostatin M Regulates Eotaxin Expression in Fibroblasts and Eosinophilic Inflammation in C57BL/6 Mice[1]

Carrie Langdon, Christine Kerr, Li Tong, and Carl D. Richards

Oncostatin M (OSM) is a member of the IL-6/LIF (or gp130) cytokine family, and its potential role in inflammation is supported by a number of activities identified in vitro. In this study, we investigate the action of murine OSM on expression of the CC chemokine eotaxin by fibroblasts in vitro and on mouse lung tissue in vivo. Recombinant murine OSM stimulated eotaxin protein production and mRNA levels in the NIH 3T3 fibroblast cell line. IL-6 could regulate a small induction of eotaxin in NIH 3T3 cells, but other IL-6/LIF cytokines (LIF, cardiotrophin-1 (CT-1)) had no effect. Cell signaling studies showed that murine OSM, LIF, IL-6, and CT-1 stimulated the tyrosine phosphorylation of STAT-3, suggesting STAT-3 activation is not sufficient for eotaxin induction in NIH 3T3 cells. OSM induced ERK-1,2 and p38 mitogen-activated protein kinase phosphorylation in NIH 3T3 cells, and inhibitors of ERK (PD98059) or p38 (SB203580) could partially reduce OSM-induced eotaxin production, suggesting partial dependence on mitogen-activated protein kinase signaling. OSM (but not LIF, IL-6, or CT-1) also induced eotaxin release by mouse lung fibroblast cultures derived from C57BL/6 mice. Overexpression of murine OSM in lungs of C57BL/6 mice using an adenovirus vector encoding murine OSM resulted in a vigorous inflammatory response by day 7 after intranasal administration, including marked extracellular matrix accumulation and eosinophil infiltration. Elevated levels of eotaxin mRNA in whole lung were detected at days 4 and 5. These data strongly support a role of OSM in lung inflammatory responses that involve eosinophil infiltration. ***The Journal of Immunology,*** **2003, 170: 548–555.**

Regulation of inflammatory processes involves the interaction between various cell types through direct cell contact or through soluble products, with the result being a broad spectrum of responses. Inflammation involving the infiltration of particular cells into local tissue sites is thought to be largely controlled by chemotactic factors including chemokines (reviewed in Refs. 1–3) with relative specificity for certain cell types. Eotaxins 1, 2, and 3 are members of the CC chemokines, and are recognized to play an important role in the recruitment of eosinophils to sites of inflammation (4–8). Eotaxin-1 binds to the CCR3 which is expressed on eosinophils and basophils and T cells (9, 10). Eotaxin-1 can be induced by cytokines important in inflammation including TNF, IL-1, and IL-4 in human bronchial epithelial cells (11–13), IL-4 in intestinal epithelial cells (14), TNF and IL-4 in human nasal and dermal fibroblasts (15–17), and TNF and IL-4 in human lung fibroblasts (18). Its expression can be supressed by IFN-γ or corticosteroids (19, 20). Although these mediators have been identified as effective regulators of eotaxin, it is likely that there are additional cytokines which participate in its induction in vitro and in vivo. IL-6 can be found at elevated levels in various inflammatory conditions, but regulation of eotaxin by IL-6 or its related family members has not yet been extensively studied.

The IL-6/LIF family of cytokines (also called gp130 cytokines) can regulate a variety of activities in vitro (reviewed in Refs. 21–23), and activate the Janus kinase (JAK)[3]/STAT and mitogen-activated protein (MAP) kinase signaling pathways (21, 23, 24). Previous studies have shown that one family member, oncostatin M (OSM), is prominent in its ability to regulate responses of connective tissue cells in vitro. We have shown that human OSM regulates the expression of chemokines and cytokines by fibroblasts in vitro, including induction of monocyte chemoattractant protein (MCP)-1 and IL-6 and suppression of IL-8, RANTES, and GM-CSF (25, 26). Human OSM binds the LIF-receptor complex (type I OSM receptor) and can also bind an OSM-specific receptor that is composed of gp130 and the OSM-specific β-receptor subunit (type II OSM receptor) (27). OSM stimulates the JAK/STAT pathway such that JAK kinases cause phosphorylation of STAT molecules leading to their dimerization and subsequent translocation into the nucleus to bind STAT-specific DNA-binding elements (28–30). Depending somewhat on cell types, OSM stimulation has also been reported to activate MAP kinases, in particular extracellular-regulated kinase (ERK)-1 and -2 (p42/p44) (31–34).

Murine OSM (muOSM) has been cloned as an immediate early gene whose expression is up-regulated in response to IL-2, IL-3, and erythropoietin (35). It stimulates hemopoietic cell proliferation and regulates gonadocyte and astrocyte differentiation (36–38), up-regulates liver acute phase protein synthesis by hepatocytes, and can induce tissue inhibitor of metalloproteinase 1 (TIMP-1), IL-6, and MCP-1 expression by mouse fibroblasts (39, 40). In contrast to the human cytokine, muOSM interacts with a specific mouse OSM receptor complex and does not bind the murine (mu) LIF receptor complex (41–43). In this study, we identify strong

Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

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[2] Address correspondence and reprint requests to Dr. Carl D. Richards, Head, Rheumatic Diseases, Faculty of Health Sciences, Center for Gene Theapeutics, Department of Pathology and Molecular Medicine, McMaster University, Room 4H18, 1200 Main Street West, Hamilton, Ontario L8N 3Z5 Canada. E-mail address: richards@mcmaster.ca

[3] Abbreviations used in this paper: JAK, Janus kinase; OSM, oncostatin M; MAP, mitogen-activated protein; MCP, monocyte chemoattractant protein; TIMP-1, tissue inhibitor of metalloproteinase 1; ERK, extracellular-regulated kinase; muOSM, murine OSM; mu, mouse; *Ct*, threshold cycle; Ad, adenovirus; MLF, mouse lung fibroblast; BAL, bronchoalveolar lavage; ECM, extracellular matrix; CT-1, cardiotrophin-1; PD, PD98059; SB, SB203580.

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regulation of eotaxin-1 expression by muOSM in vitro, and show marked induction of eosinophil infiltration upon overexpression of muOSM in mouse lungs in vivo.

Materials and Methods

Unless otherwise noted, all reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines

NIH 3T3 cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in DMEM containing 10% calf serum (Life Technologies, Gaithersburg, MD), 1% penicillin/streptomycin, and 1.5% Fungizone. Primary mouse lung fibroblasts (MLFs) were derived from explants of finely minced lung tissue from C57BL/6 mice (10- to 12-wk-old; Charles River Breeding Laboratories, Wilmington, MA), and were cultured in Earle's modified MEM (F-15) medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1.5% Fungizone, and 0.03% L-glutamine.

Cytokines and pharmacologic inhibitors

muOSM was a generous gift from Dr. T. Hara (Institute of Molecular and Cellular Biosciences, Toyko, Japan). muIL-6, muLIF, murine cardiotrophin-1 (muCT-1), muIL-1β, and pTGF-β were all purchased from R&D Systems (Minneapolis, MN). Murine epidermal growth factor (muEGF) was purchased from Sigma-Aldrich. PD98059 (PD) and SB203580 (SB) were purchased from Calbiochem (San Diego, CA).

ELISA

Subconfluent cultures (80–90%) were stimulated for 24 h in normal supplemented medium, supernatants were collected and stored at −20°C until analysis by ELISA. Murine eotaxin ELISA kits were purchased from R&D Systems, and used according to the manufacturer's protocol to measure eotaxin-1 levels in supernatants.

RNA purification and analysis by real-time RT-PCR (TaqMan)

Subconfluent fibroblast (80–90%) cultures were stimulated with the indicated cytokines in medium supplemented with 2% serum and incubated for 18–24 h. Total RNA was extracted from cultures or from tissues using TRIzol (Life Technologies) according to manufacturer's directions. Genomic DNA was removed using the Ambion DNA-free kit (Ambion, Austin, TX). RNA was reverse transcribed using the Ambion RETROscript kit (Ambion) using random decamers as primers to obtain cDNA.

Expression of mRNA for eotaxin was analyzed using real-time quantitative PCR (TaqMan). The forward and reverse primers along with the fluorescent probe were designed using PrimerExpress verson 1.5 software (Applied Biosystems, Foster City, CA). Two sets of primers and probes were used with similar results. For the first set the forward primer was 828-5′-TATCACCCTGACTGACCTGTAACTCA, the reverse was 912-5′-CACTTAAAGGCAGAGGCAGGTAA, and the probe was 855-5′-FAM-TGTAGACCAGGCTGACCTCAAACTCACAGA-TAMRA. For the second set the forward primer was 380-5′-CTGCTTGATTCCTTCTCTTTCCTAA, the reverse primer was 440-5′-GGAACTACATGAAGCCAAGTCCTT, and the probe was 406-5′-FAM-ACTGGTGCTGATATTCCCTCAGAGCACGT-TAMRA. The housekeeping gene was GAPDH. Primers and the VIC-labeled probe for GAPDH were obtained as predeveloped assay reagents from Applied Biosystems. TaqMan Universal PCR Master Mix (Applied Biosystems), a mixture containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs, a passive reference dye and optimized buffer components, was used as the source of the PCR reagents. The plated reaction mixture was placed in the ABI Prism 6700 Sequence Detection System (Applied Biosystems), which was operated by Sequence Detector version 1.7 software (Applied Biosystems).

Gene expression was quantitated relative to the expression of GAPDH. Since, in an optimized PCR, the number of copies of the target DNA is doubled with each cycle, the relative expression of the gene of interest vs GAPDH was calculated as $2^{-\Delta Ct}$, where ΔCT is the difference between the threshold cycle (*Ct*) for the gene of interest and the *Ct* for GAPDH. In each experiment, the value of the relative expression of the control sample (untreated) was given a value of one and the expression of the other treatments were plotted relative to this.

Western blot

NIH 3T3 cells were cultured and stimulated as above and lysed in RIPA buffer containing 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 0.003% aprotinin, and 1 mM sodium orthovanadate. Lysates were passed through a 21-gauge needle, incubated on ice for 30 min, centrifuged at 12,000 × *g* for 10 min, and frozen at −70°C. Protein concentration was determined using the Bio-Rad Protein assay (Bio-Rad, Hercules, CA), and equal amounts were loaded onto 8 or 10% SDS-PAGE gels. Proteins were transferred to Immobilon-NC membrane (Millipore, Bedford, MA). Western blots were blocked in PBS containing 5% low-fat milk powder, probed with Abs to STAT-3 and tyrosine-phosphorylated STAT-3, ERK1/2 and phosphorylated ERK1/2, p38 and phosphorylated p38, all purchased from NEB (Beverly, MA). Blots were washed, incubated with HRP-conjugated secondary Abs (Santa Cruz Biotechnology, Santa Cruz, CA), and visualized using an ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Adenovirus administration in vivo

Female C57BL/6 mice (10–12 wk old) were anesthetized by isofluorane and treated with either adenovirus (Ad) muOSM (expressing muOSM as previously characterized; Ref. 44) or Addl70 (control virus) via intranasal administration. Animals were sacrificed by dissecting the abdominal aorta and lungs were either stored in liquid nitrogen for RNA extraction, or were lavaged with PBS, then inflated and perfused with 10% buffered formalin for histological analysis. Lavage fluid was stored on ice, the cells were then pelleted, resuspended in HBSS, and cytospins were prepared using a Shandon Cytospin 3 centrifuge (Shandon, Pittsburgh, PA). Hema 3 stain (Fisher Scientific, Nepean, Ontario, Canada) was used to differentiate cell types. All procedures were approved by the Animal Research Ethics Board of McMaster University (Hamilton, Ontario, Canada).

Statistical analysis

Data were analyzed for statistical significance with one way analysis of variance using SigmaStat (SPSS, Chicago, IL).

Results

We examined the expression of eotaxin in the NIH 3T3 fibroblast cell line which has previously been shown to respond to muOSM (39). Fig. 1 shows that muOSM elevated eotaxin levels in 24-h conditioned medium in a dose-dependent fashion. Effective concentrations of OSM started at ~1 ng/ml and plateaued at 25–50 ng/ml. Levels at days 2 or 3 of stimulation were similar in trend (data not shown). To test the ability of other IL-6/LIF cytokines to regulate eotaxin production, NIH 3T3 cells were stimulated with 20 ng/ml of murine LIF, IL-6, or CT-1, 5 ng/ml IL-1β, or 1 ng/ml porcine TGF-β. Fig. 2*A* shows that neither LIF, CT-1, nor TGF-β were able to regulate eotaxin levels in cell supernatants. IL-6 at 20 ng/ml was able to elevate eotaxin levels significantly as did IL-1β,

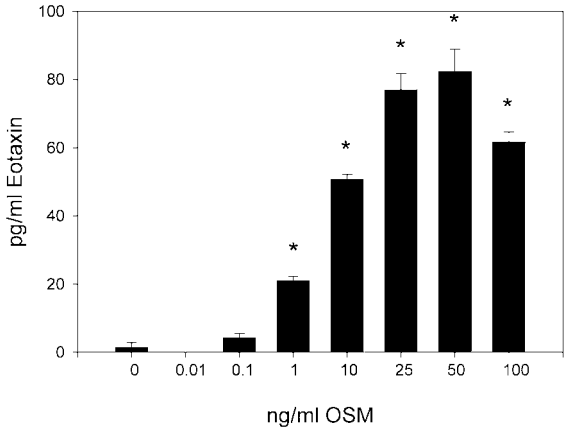

FIGURE 1. Dose response induction of eotaxin by muOSM in NIH 3T3 cells. NIH 3T3 cells were plated in 24-well plates and stimulated with increasing amounts of muOSM for 24 h in quadruplicate. Eotaxin in cell culture supernatants was measured by ELISA. Statistically significant (*, $p < 0.001$) elevation in levels of eotaxin were found at concentrations of muOSM as low as 1.0 ng/ml. Consistent with previous findings examining the production of other cytokines and chemokines, the optimal concentration of muOSM was 25–50 ng/ml.

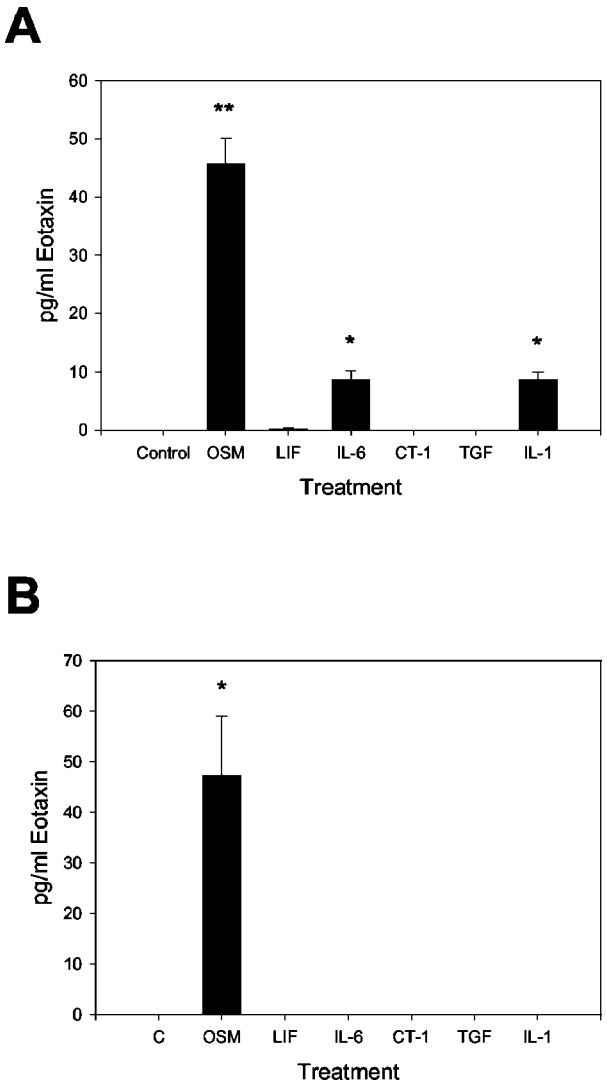

FIGURE 2. Regulation of eotaxin by gp130 cytokines in mouse fibroblasts. NIH 3T3 (*A*) or C57BL/6 MLF cells (*B*) were plated in 24-well plates. Cytokines were added in quadruplicate at the following concentrations: muOSM, LIF, IL-6, and CT-1 20 ng/ml, porcine TGF-β 1 ng/ml, murine IL-1β 5 ng/ml. After 24 h, supernatants were collected and assayed for eotaxin by ELISA. In NIH 3T3 cells (*A*), the levels of eotaxin induced by IL-6 and IL-1β were similar (*, $p < 0.05$), but muOSM produced a more robust induction of eotaxin (**, $p < 0.001$). In C57BL/6 cells (*B*), only muOSM induced eotaxin (*, $p < 0.001$).

but levels were considerably lower than in muOSM-stimulated NIH 3T3 cell culture supernatants. MLF cell lines were established from C57BL/6 mice to examine responses to OSM, and Fig. 2*B* shows that these cells also responded to muOSM with increased eotaxin release. None of LIF, IL-6, CT-1 (20 ng/ml), TGF-β (1 ng/ml), or IL-β (5 ng/ml) were able to induce an eotaxin response in these cells. These results in NIH 3T3 and C57BL/6 MLF were consistent in at least three separate experiments.

OSM receptor cell signal transduction systems include the JAK/STAT pathway and activation of MAP kinase pathways have also been shown to be activated by OSM depending on the cell types examined. To examine STAT activation in response to IL-6/LIF cytokines in NIH 3T3 cells, Western blots of total cell lysates were probed with an Ab specific for tyrosine-phosphorylated STAT3 and total STAT-3 protein. Previous studies have shown that 15 min after stimulation, STAT phosphorylation can be readily detected in responsive cells. As shown in Fig. 3*A*, markedly elevated levels of activated STAT-3 were induced by OSM, LIF, IL-6, and CT-1 stimulation, all to a similar degree compared with unstimulated cells. Probing of cell lysates at the same time point with specific Abs showed that OSM can also induce the phosphorylation of both ERK1/2 (p42/44) and p38 MAP kinases. Established pharmacological inhibitors were used to assess the effects of inhibiting these kinases (Fig. 3*B*). Preincubation of NIH 3T3 cells with SB or PD (to inhibit p38 MAP kinase or p42/44 MAP kinase, respectively) showed a significant reduction ($p < 0.001$) in the levels of eotaxin detected in 24-h cell culture supernatants.

Analysis of RNA levels using real-time RT-PCR (TaqMan) showed that, consistent with the results of protein assays, muOSM was a potent regulator of eotaxin levels in both NIH 3T3 and MLF cells when compared with the other cytokines tested (Fig. 4). When corrected for the levels of the housekeeping gene, GAPDH, muOSM induced a dramatic increase in the relative expression of eotaxin mRNA when compared with unstimulated cells. Relative fold induction was calculated using the $\Delta\Delta Ct$ formula (45). As was the case at the protein level, IL-6 induced a significant increase in eotaxin expression in NIH 3T3 but not MLF cells. However, the relative fold increase for IL-6 stimulation was significantly lower than that of muOSM. Similar to results for other cell types, IL-4 and IL-1β elevated eotaxin expression in NIH 3T3 cells (data not shown). Preincubation of NIH 3T3 cells with the MAP kinase inhibitors PD or SB significantly reduced the level of eotaxin mRNA by ~15 and 72%, respectively (Fig. 4*C*).

Previous work characterizing the AdmuOSM adenovirus vector and its effects in mouse lung tissue have shown that virus-encoded

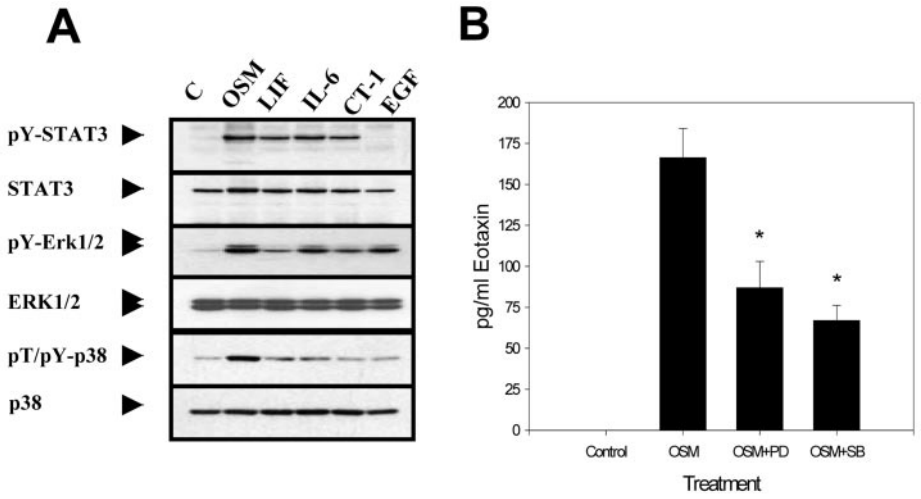

FIGURE 3. OSM induces MAP kinase signaling in NIH 3T3 cells. *A*, NIH 3T3 cells were cultured in serum-free conditions overnight, stimulated with 25 ng/ml of the indicated cytokines for 15 min, and total cell lysates were prepared (as in *Materials and Methods*). Western blots of the extracts were completed for STAT-3, ERK1/2, and p38 MAP kinases, as well as their tyrosine-phosphorylated forms (pY-STAT-3, pY-ERK1/2, and pT-pY p38), and visualized using an ECL detection system (as in *Materials and Methods*). *B*, NIH 3T3 cells were plated in 24-well plates and stimulated with with 25 ng/ml OSM for 24 h in quadruplicate. Cells were also pretreated for 1 h with vehicle (OSM), 20 μM PD (OSM + PD), or 10 μM SB (OSM + SB). Data are the mean ± SD.

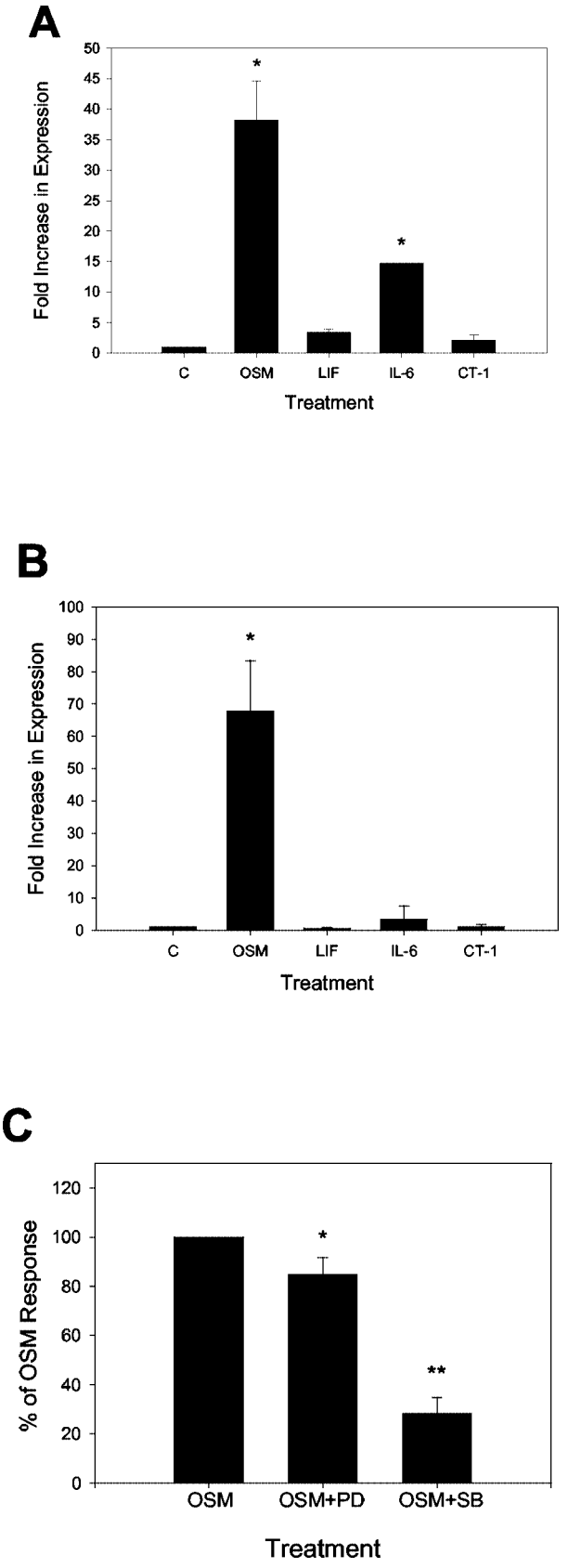

FIGURE 4. muOSM regulation of eotaxin mRNA. Cultures of NIH 3T3 (*A*) and C57BL/6 MLF cells (*B*) were stimulated with muOSM, LIF, IL-6, and CT-1 at 25 ng/ml. cDNA was prepared as described in *Materials and Methods* and analyzed for eotaxin expression using real-time quantitative PCR (TaqMan). Relative expression of eotaxin following cytokine stimulation was compared with unstimulated cells. muOSM but not LIF or CT-1 could induce substantial increases in eotaxin expression ($p < 0.001$). IL-6 also produced significant increases in eotaxin expression in NIH 3T3 cells. *C*, Cultures of NIH 3T3 cells were pretreated with vehicle (OSM), 20 μM PD (OSM/PD), or 10 μM SB (OSM/SB) and then stimulated with 25 ng/ml OSM. Relative expression of eotaxin following pretreatment was compared with that from cells stimulated with OSM using TaqMan. Pretreatment with either SB or PD was able to significantly inhibit eotaxin expression induced by OSM, but the percentage of inhibition of eotaxin mRNA synthesis by SB was more potent than that produced by PD (**, $p < 0.001$; *, $p < 0.05$).

muOSM mRNA (as assessed by Northern blot) is expressed at days 1 and 3, and less consistently, is still sustained at day 7 after administration (44). Major effects on lung histopathology were not seen within the first 3 days after administration. However, preliminary results showed that at later time points after administration, eosinophil numbers in lung tissues were markedly elevated in C57BL/6 mice. The lungs of C57BL/6 mice were examined histologically after intranasal administration of increasing doses of AdmuOSM or Addl70. At various times after administration, the animals were sacrificed, bronchoalveolar lavage (BAL) was performed, and the lungs were fixed and stained with H&E or Masson's trichrome stain (Fig. 5). Adenovirus produces focal infection of bronchial and alveolar epithelial cells, and to a more limited extent, infects alveolar macrophages, throughout the tracheobronchial tree (46). At the highest doses tested (AdmuOSM at 10^7 PFU/animal), profound effects were evident at day 7. Overexpression of muOSM produced an interstitial pneumonia characterized by pronounced infiltration of mononuclear cells in the lung parenchyma and alveolar spaces and dense accumulations in the peribronchial and perivascular areas. The infiltrating cell population included neutrophils, and most particularly, large numbers of eosinophils. Lungs showed obliteration of alveolar structure in some areas, and evidence of fibroblast proliferation and increased collagen deposition. BAL fluid from these mice showed a mean level of 35 pg/ml eotaxin in two separate experiments (data not shown). At day 14, the extent of infiltration was declining, but mononuclear cells and abundant eosinophils remained, as well as increased levels of extracellular matrix (ECM). By day 21, inflammation was clearly resolving and normal alveolar structure was returning. Remodeling of lung parenchyma was taking place to the extent that intensity of collagen staining was returning to normal. However, minor scarring persisted as long as 70 days post viral administration (data not shown). Control Ad vector (Addl70) did not produce such effects. Minor bronchial epithelium hyperplasia and occasional mild mononuclear cell infiltration were the only effects produced by Addl70. These findings are summarized semiquantitatively in Table I. Counts of cells in the BAL fluids also reflect a similar effect in that a marked increase in eosinophils was observed. This was consistent in all of four similar experiments with intranasal administration. Significant effects were evident at doses of AdmuOSM as low as 10^7 PFU/animal (Fig. 6). Addl70-treated mice did not show significant differences from untreated mice.

To pinpoint the onset of eotaxin expression in vivo, mice received AdmuOSM via intranasal administration and were sacrificed at various time points. Lungs were collected at days 3–6 and RNA was prepared. Results using real-time RT-PCR showed that expression of eotaxin occurred at days 4 and 5 postadministration, and by day 6 levels had declined to close to background (Table II). Eotaxin expression at days 4 and 5 is consistent with the observation of marked tissue eosinophilia at day 7.

Discussion

These results clearly demonstrate that muOSM can induce eotaxin expression in mouse fibroblasts at the protein and mRNA level.

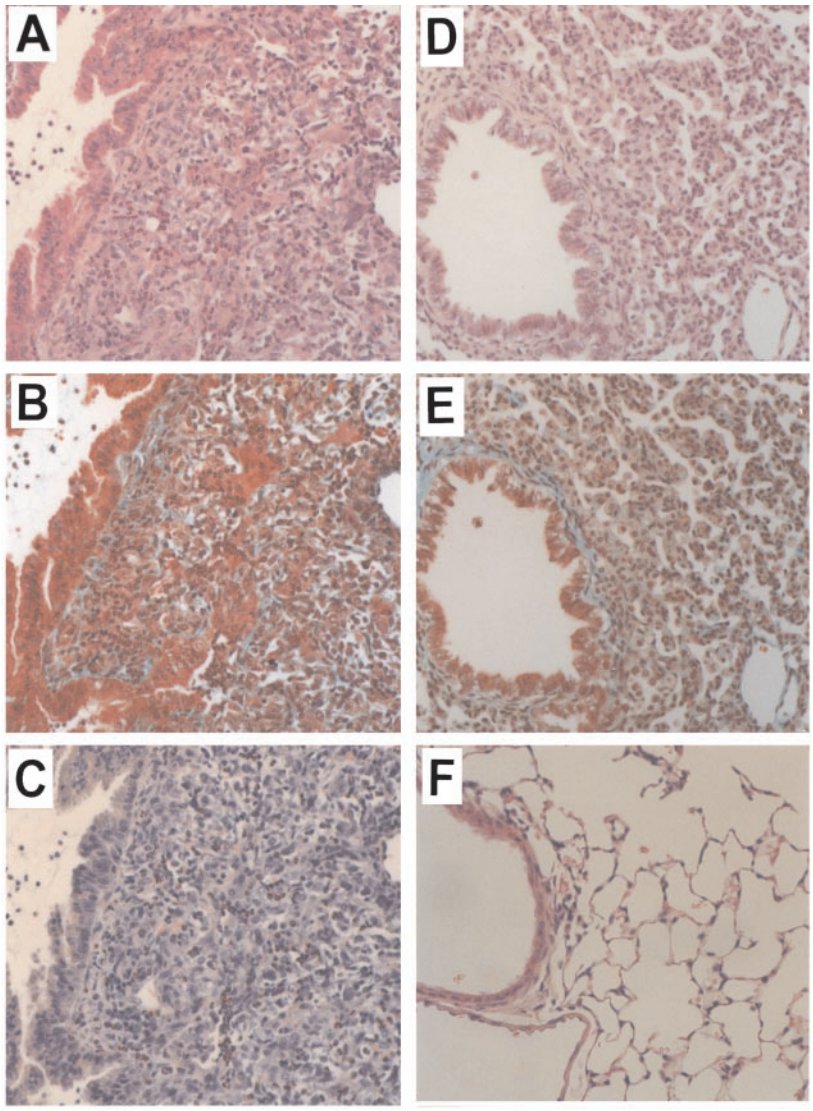

FIGURE 5. Adenovirus encoding muOSM induces eosinophilia in C57BL/6 mouse lung. C57BL/6 mice received 5×10^7 PFU AdmuOSM (*A–E*) or Addl70 (*F*) via intranasal administration and were sacrificed 7 (*A–C*) or 21 (*D* and *E*) days later. Lung tissues were stained with H&E (*A*, *D*, and *F*), Masson's trichrome (*B* and *E*), or Congo red (*C*). At day 7, lungs were densely infiltrated with mononuclear cells, eosinophils, and neutrophils. Masson's trichrome stain (*B*) illustrates the dense cellular infiltration and fibroblast proliferation (red) as well as the deposition of ECM, specifically collagen (green). Congo red shows the high concentration of eosinophils in the lung parenchyma as well as in the bronchial lumen (*C*). By day 21, inflammation was resolving and alveolar structure was regenerating (*D*), although some extracellular collagen deposition remained (*E*). Addl70 (*F*) did not produce significant alterations to lung architecture other than very minor mononuclear cell infiltration and epithelial hyperplasia.

The results of AdmuOSM administration in vivo in C57BL/6 mice show a marked elevation of eosinophils in lungs that is evident at days 7 and 14 after treatment, consistent with the effects of OSM on eotaxin in vitro. This is the first identification of this activity of OSM, and indicates a need to consider OSM in inflammatory responses that involve eosinophilic inflammation.

Our results also show that IL-6 family cytokines LIF and CT-1 cannot regulate eotaxin in vitro in NIH 3T3 cells despite being able to induce STAT-3 phosphorylation to a similar degree as OSM. This suggests that NIH 3T3 cells express receptors for LIF and CT-1 but that STAT-3 activation is not sufficient to enhance the expression of eotaxin in these cells. Thus, the signals induced by

Table I. *Histopathology in C57BL/6 mouse lung overexpressing muOSM*

		Inflammatory Parameters[a]					
Treatment[b]	Day	Mononuclear cells	Neutrophilia	Eosinophilia	Fibrosis/ hyperplasia	Granuloma formation	Collagen deposition
AdmuOSM	7	+++	++	+++	+++	++	++
	14	+++	+	++	+++	++	++
	21	++	−	+	++	+	+
Addl70	7	+	−	−	−	−	−
	14	++	−	−	−	−	−
	21	++	−	−	−	−	−

[a] Lung tissues were fixed with 10% buffered formalin, paraffin-embedded, sectioned, and stained with H&E, Congo red, Masson's trichrome, and Elastin von Giesen stain. Tissues were examined in a blinded manner for the presence and extent of mononuclear cell accumulation, neutrophilia, and granuloma formation (H&E); fibrosis/hyperplasia and collagen deposition (H&E, Masson's trichrome, and Elastin von Giesen); and eosinophilia (Congo red).

[b] C57BL/6 mice (minimum three mice per treatment three separate experiments) received 5×10^7 PFU AdmuOSM or Addl70 via intranasal administration and were sacrificed 7, 14, or 21 days later.

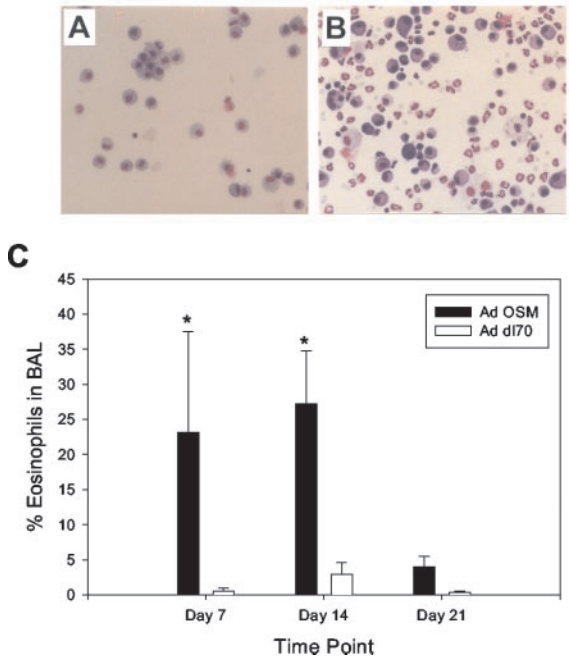

FIGURE 6. Eosinophilia in BAL fluid of AdmuOSM-treated C57BL/6 mice. Cells collected by cytospin from BAL fluid from animals treated with Addl70 (*A*) or AdmuOSM (*B*). The majority of cells in Addl70 BAL fluid are macrophages. In contrast, AdmuOSM BAL fluids show large numbers of eosinophils and neutrophils, as well as macrophages. *C*, C57BL/6 mice received 5×10^7 PFU AdmuOSM intranasally. Animals were sacrificed 7, 14, or 21 days later. Lungs were lavaged with PBS, and cells were collected, stained, and scored. Animals treated with AdmuOSM showed substantial and statistically significant increases in the number of eosinophils in BAL fluid at days 7 and 14, to a peak of 27.2% at day 14 (*, $p < 0.05$). Values returned close to normal by day 21. Mice treated with Addl70 showed no significant increases in eosinophils.

OSM in addition to STAT-3 must play a role in eotaxin regulation. It has recently been demonstrated that eotaxin transcription can be regulated by STAT-6 (activated by IL-4) and NF-κB (activated by TNF or IL-1) in epithelial cells (47). However, there has been no indication that OSM regulates STAT-6 or NF-κB, which suggests that other pathways can regulate eotaxin expression. Identification of activation of ERK1/2 and p38 by OSM (Fig. 3*A*) and of inhibition of eotaxin levels by pharmacological inhibitors (Fig. 3*B*) of these pathways indicates partial dependence on ERK1/2 and p38 of OSM-induced response in mouse fibroblasts. Furthermore, reduction in the level of eotaxin mRNA by inhibitors of ERK or p38 suggests that OSM signal transduction mediated by the MAP kinase pathway stimulates eotaxin expression at the transcription level. Although the concentrations of PD and SB used in this study are in keeping with the current literature, we cannot rule out possible nonspecific effects of these inhibitors. We have observed that the ERK inhibitor PD did not affect the tyrosine phosphorylation of p38, STAT-1, and STAT-3, nor did the p38 inhibitor SB alter the tyrosine phosphorylation of ERK, STAT-1, or STAT-3 (assessed by immunoblots, data not shown) in OSM-stimulated NIH 3T3 cells; however, they may potentially affect other signal transduction pathways that impinge upon the OSM-mediated induction of eotaxin. In other systems, OSM has also been reported to activate STAT-1 and -5 (30, 34) as well as JNK MAP kinase, phosphatidylinositol 3′-kinase, and src kinases (34, 48, 49). Further investigation is required to determine the participation of such pathways in regulating eotaxin.

Table II. *Administration of AdmuOSM regulates eotaxin mRNA in lung tissue of C57BL/6 mice*

Treatment[a]	Relative Increase in Eotaxin Expression[b]
PBS day 3	1.0
Addl70-3 day 3	0.4
AdmuOSM day 3	2.1
AdmuOSM day 4	3.4
AdmuOSM day 5	25.6
AdmuOSM day 6	5.2

[a] C57B1/6 mice received 5×10^7 PFU AdmuOSM, Addl70-3, or vehicle (PBS) via intranasal administration and were sacrificed 3–6 days later. Total RNA was prepared from lung tissue, reversed transcribed as described in *Materials and Methods* and cDNA analyzed for eotaxin expression using real-time quantitative PCR (TaqMan).

[b] Relative expression from lung tissue of mice that received AdmuOSM or Addl70-3 was compared to that from mice treated with PBS. Data represent a mean of two animals for each of the AdmuOSM treatments and one animal from the PBS and Addl70-3 groups. Levels of eotaxin expression from these tissues were examined individually by TaqMan a minimum of three times.

IL-6 stimulation resulted in a much lower but detectable increase in eotaxin protein and mRNA production in NIH 3T3 cells; however, IL-6 was not able to stimulate such responses in MLF. Our results of STAT activation show that LIF, IL-6, and CT-1 can induce STAT-3 in MLF (L. Tong and C. D. Richards, unpublished observations) indicating receptor activation by these cytokines; however, additional signals recruited by OSM must also play a role in the regulation of eotaxin in these fibroblasts. Previous work examining human lung fibroblasts was not able to detect regulation of eotaxin by IL-6 or IL-10 (18), which is consistent with our results in the mouse system. Collectively, these data suggest that OSM should be considered unique among the IL-6/LIF cytokines in stimulating eotaxin release by mouse fibroblasts, and supports the need for further investigation into regulation of eotaxin by OSM in human systems.

Previous results have demonstrated that OSM is able to induce TIMP-1 in a number of connective tissue cell types, suggesting a possible role in the regulation of ECM metabolism. The regulation of TIMP-1 by OSM reflects induction of eotaxin, in that OSM but not LIF or CT-1 was previously shown to elevate TIMP-1 mRNA in NIH 3T3 cells and mouse fibroblasts in vitro (39, 40), suggesting that STAT-3 activation is not sufficient to regulate TIMP-1. AdmuOSM treatment of mice elevated TIMP-1 RNA in vivo (44). The increase in matrix that appears evident by histology (Fig. 5) is consistent with this, although the control of ECM metabolism is a complex process involving ECM synthesis as well as ECM catabolism that in turn results from the net balance of matrix metalloproteinases and their inhibitors. Human OSM has been shown to elevate collagen synthesis in dermal fibroblasts (50); however, it is not yet known whether similar action is shown by muOSM in mouse fibroblasts.

Our results clearly indicate the potential of OSM to play a role in the regulation of cellular infiltration in inflammatory conditions. However, it is not yet clear that the levels of OSM that are present in our system of overexpression of OSM in vivo in mice have a direct relevance to the levels that might be found in other mouse models of lung disease, nor is it yet clear if these cytokine effects in mice have a direct correlation to human lung inflammatory conditions. Although similar signaling events have been described in human cells in response to OSM, the mouse system may show species-specific effects. In addition, since the effects of OSM in the lung are in context of an adenoviral infection, there may be other virus-induced events required for the effect in vivo in this system. The regulation of eotaxin, MCP-1, and TIMP-1 (39, 40, 44) in

vitro in NIH 3T3 cells and in MLFs could argue that overexpression of OSM is sufficient; however, there may be other factors in the mechanism of OSM-induced inflammation and pathology in vivo. Although further analysis is required, our results indicate that such investigation has merit.

Inflammation due to asthma is characterized by infiltration of eosinophils into lung tissue and the bronchoalveolar spaces, and the control of eosinophil infiltration is likely influenced in large part by the generation of chemotactic agents, particularly eotaxin, within lung tissue (9, 51). In addition to the regulation of eotaxin identified by our results in this study, other effects of OSM in vitro include induction of the proliferation of smooth muscle cells (52), and potential ECM modulation through influence on matrix metalloproteinase/TIMP balance, which are identified as characteristic of asthma-induced lung pathology. OSM may also potentially be involved in the pathogenesis of eosinophilic pneumonia, which involves chronic or recurrent eosinophilic infiltrates, and has been shown to be associated with increased levels of the chemokine MCP-1 in BAL fluids (53, 54) and suppression of Fas and apoptosis (55). Persistence of eosinophils in the AdmuOSM-treated mice may reflect similar mechanisms. We have shown previously that human OSM (on human cells) and mouse OSM (on mouse cells) are able to up-regulate the expression of MCP-1 by fibroblasts (26, 40). Eosinophilia has also been shown to be induced in the absence of eotaxin using eotaxin-deficient mice (56), likely through the action of RANTES, macrophage inflammatory protein-1α, MCP-5, and MCP-1 (57). Analysis of local expression of OSM in asthma and other pulmonary disorders such as eosinophilic pneumonia may lead to further information of the mechanisms of pathogenesis of these conditions.

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References

1. Baggiolini, M. 2001. Chemokines in pathology and medicine. *J. Intern. Med. 250:91.*
2. Gerard, C., and B. J. Rollins. 2001. Chemokines and disease. *Nat. Immunol. 2:108.*
3. Mackay, C. R. 2001. Chemokines: immunology's high impact factors. *Nat. Immunol. 2:95.*
4. Van Coillie, E., J. Van Damme, and G. Opdenakker. 1999. The MCP/eotaxin subfamily of CC chemokines. *Cytokine Growth Factor Rev. 10:61.*
5. Jose, P. J., D. A. Griffiths-Johnson, P. D. Collins, D. T. Walsh, R. Moqbel, N. F. Totty, O. Truong, J. J. Hsuan, and T. J. Williams. 1994. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J. Exp. Med. 179:881.*
6. Garcia-Zepeda, E. A., M. E. Rothenberg, R. T. Ownbey, J. Celestin, P. Leder, and A. D. Luster. 1996. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nat. Med. 2:449.*
7. Forssmann, U., M. Uguccioni, P. Loetscher, C. A. Dahinden, H. Langen, M. Thelen, and M. Baggiolini. 1997. Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. *J. Exp. Med. 185:2171.*
8. Shinkai, A., H. Yoshisue, M. Koike, E. Shoji, S. Nakagawa, A. Saito, T. Takeda, S. Imabeppu, Y. Kato, N. Hanai, et al. 1999. A novel human CC chemokine, eotaxin-3, which is expressed in IL-4-stimulated vascular endothelial cells, exhibits potent activity toward eosinophils. *J. Immunol. 163:1602.*
9. Teran, L. M. 2000. CCL chemokines and asthma. *Immunol. Today 21:235.*
10. Sallusto, F., C. R. Mackay, and A. Lanzavecchia. 1997. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science 277:2005.*
11. Jedrzkiewicz, S., H. Nakamura, E. S. Silverman, A. D. Luster, N. Mansharamani, K. H. In, G. Tamura, and C. M. Lilly. 2000. IL-1β induces eotaxin gene transcription in A549 airway epithelial cells through NF-κB. *Am. J. Physiol. Lung Cell Mol. Physiol. 279:L1058.*
12. Fujisawa, T., Y. Kato, J. Atsuta, A. Terada, K. Iguchi, H. Kamiya, H. Yamada, T. Nakajima, M. Miyamasu, and K. Hirai. 2000. Chemokine production by the BEAS-2B human bronchial epithelial cells: differential regulation of eotaxin, IL-8, and RANTES by TH2- and TH1- derived cytokines. *J. Allergy Clin. Immunol. 105:126.*
13. Nakamura, H., A. D. Luster, H. Tateno, S. Jedrzkiewicz, G. Tamura, K. J. Haley, E. A. Garcia-Zepeda, K. Yamaguchi, and C. M. Lilly. 2001. IL-4 differentially regulates eotaxin and MCP-4 in lung epithelium and circulating mononuclear cells. *Am. J. Physiol. Lung Cell Mol. Physiol. 281:L1288.*
14. Winsor, G. L., C. C. Waterhouse, R. L. MacLellan, and A. W. Stadnyk. 2000. Interleukin-4 and IFN-γ differentially stimulate macrophage chemoattractant protein-1 (MCP-1) and eotaxin production by intestinal epithelial cells. *J. Interferon Cytokine Res. 20:299.*
15. Terada, N., N. Hamano, T. Nomura, T. Numata, K. Hirai, T. Nakajima, H. Yamada, O. Yoshie, T. Ikeda-Ito, and A. Konno. 2000. Interleukin-13 and tumour necrosis factor-α synergistically induce eotaxin production in human nasal fibroblasts. *Clin. Exp. Allergy 30:348.*
16. Mochizuki, M., J. Bartels, A. I. Mallet, E. Christophers, and J. M. Schroder. 1998. IL-4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy. *J. Immunol. 160:60.*
17. Miyamasu, M., T. Nakajima, Y. Misaki, S. Izumi, N. Tsuno, T. Kasahara, K. Yamamoto, Y. Morita, and K. Hirai. 1999. Dermal fibroblasts represent a potent major source of human eotaxin: in vitro production and cytokine-mediated regulation. *Cytokine 11:751.*
18. Teran, L. M., M. Mochizuki, J. Bartels, E. L. Valencia, T. Nakajima, K. Hirai, and J. M. Schroder. 1999. Th1- and Th2-type cytokines regulate the expression and production of eotaxin and RANTES by human lung fibroblasts. *Am. J. Respir. Cell Mol. Biol. 20:777.*
19. Miyamasu, M., Y. Misaki, M. Yamaguchi, K. Yamamoto, Y. Morita, K. Matsushima, T. Nakajima, and K. Hirai. 2000. Regulation of human eotaxin generation by Th1-/Th2-derived cytokines. *Int. Arch. Allergy Immunol. 122 (Suppl. 1):54.*
20. Lilly, C. M., H. Nakamura, H. Kesselman, C. Nagler-Anderson, K. Asano, E. A. Garcia-Zepeda, M. E. Rothenberg, J. M. Drazen, and A. D. Luster. 1997. Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. *J. Clin. Invest 99:1767.*
21. Taga, T. 1997. The signal transducer gp130 is shared by interleukin-6 family of haematopoietic and neurotrophic cytokines. *Trends Mol. Med. 29:63.*
22. Baumann, H., and J. Gauldie. 1994. The acute phase response. *Immunol. Today 15:74.*
23. Kishimoto, T., S. Akira, M. Narazaki, and T. Taga. 1995. Interleukin-6 family of cytokines and gp130. *Blood 86:12243.*
24. Heinrich, P. C., I. Behrmann, G. Muller-Newen, F. Schaper, and L. Graeve. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J. 334:297.*
25. Richards, C. D., C. Langdon, F. Botelho, T. J. Brown, and A. Agro. 1996. Oncostatin M inhibits IL-1-induced expression of IL-8 and granulocyte-macrophage colony-stimulating factor by synovial and lung fibroblasts. *J. Immunol. 156:343.*
26. Langdon, C. M., J. Leith, F. Smith, and C. D. Richards. 1997. Oncostatin M stimulates monocyte chemoattractant protein-1- and interleukin-1-induced matrix metalloproteinaise-1 production by human synovial fibroblasts in vitro. *Arthritis Rheum. 40:2139.*
27. Mosley, B., C. Delmus, D. Friend, N. Boiani, B. Thoma, L. S. Park, and D. Cosman. 1996. Dual Oncostatin M (OSM) receptors. *J. Biol. Chem. 271: 32635.*
28. Taga, T. 1997. The signal transducer gp130 is shared by interleukin-6 family of haematopoietic and neurotrophic cytokines. *Ann. Med. 29:63.*
29. Botelho, F., D. Edwards, and C. D. Richards. 1998. Oncostatin M stimulates c-Fos to bind a transcriptionally responsive AP-1 element within the tissue inhibitor of metalloproteinase-1 promoter. *J. Biol. Chem. 273:5211.*
30. Auguste, P., C. Guillet, M. Fourcin, C. Olivier, and J. Veziers. 1997. Signaling of type II Oncostatin M receptor. *J. Biol. Chem. 272:15760.*
31. Faris, M., B. Endoli, N. Stahl, G. Yancopoulos, A. Nguyen, S. Wang, and A. E. Nel. 1996. Differential activation of the extracellular signal-regulated kinase, Jun kinase and Janus kinase-stat pathways by Oncostatin M and basic fibroblast growth factor in AIDS-derived Kaposi's sarcoma cells. *AIDS 10:369.*
32. Amaral, M. C., S. Miles, G. Kumar, and A. E. Nel. 1993. Oncostatin-M stimulates tyrosine protein phosphorylation in parallel with the activation of p42mapk/ERK-2 in Kaposi's cells evidence that this pathway is important in Kaposi's cell growth. *J. Clin. Invest. 92:848.*
33. Stancato, L. F., M. Sakatsume, M. David, P. Dent, F. Dong, E. F. Petricoin, J. J. Krolewski, O. Silvennoinen, P. Saharinen, J. Pierce, et al. 1997. β interferon and Oncostatin M activate Raf-1 and mitogen- activated protein kinase through a JAK1-dependent pathway. *Mol. Cell. Biol. 17:3833.*
34. Wang, Y., O. Robledo, E. Kinzie, F. Blanchard, C. D. Richards, A. Miyajima, and H. Baumann. 2000. Receptor subunit-specific action of Oncostatin M in hepatic cells and its modulation by leukemia inhibitory factor. *J. Biol. Chem. 275:25273.*
35. Yoshimura, A., M. Ichihara, I. Kinjyo, M. Moriyama, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, T. Hara, and A. Miyajima. 1996. Mouse Oncostatin M: an immediate early gene induced by multiple cytokines through the JAK-STAT5 pathway. *EMBO. J. 15:1055.*
36. Mukouyama, Y., T. Hara, M. Kim, H. Kogo, and A. Miyajima. 1998. In vitro expansion of murine multipotential hematopoietic progenitors from the embryonic aorta-gonad-mesonephros region. *Immunity 8:105.*
37. Hara, T., K. Tamura, M. de Miguel, Y.-S. Mukouyama, H. Kim, H. Kogo, P. J. Donovan, and A. Miyajima. 1998. Distinct roles of oncostatin M and leukemia inhibitory factor in the development of primordial germ cells and sertoli cells in mice. *Dev. Biol. 201:144.*
38. Yanagisawa, M., K. Nakashima, and T. Yaga. 2000. STAT3-mediated astrocyte differentiation from mouse fetal neuroepitheleal cells by mouse oncostatin M. *Neurosci. Lett. 269:169.*

39. Richards, C. D., C. Kerr, M. Tanaka, T. Hara, A. Miyajima, D. Pennica, F. Botelho, and C. M. Langdon. 1997. Regulation of tissue inhibitor of metalloproteinase-1 in fibroblasts and acute phase proteins in hepatocytes in vitro by mouse oncostatin M, cardiotrophin-1, and IL-6. *J. Immunol. 159:2431.*
40. Langdon, C., C. Kerr, M. Hassen, T. Hara, A. L. Arsenault, and C. D. Richards. 2000. Murine oncostatin M stimulates mouse synovial fibroblasts in vitro and induces inflammation and destruction in mouse joints in vivo. *Am. J. Pathol. 157:1187.*
41. Lindberg, R. A., T. S. C. Juan, A. A. Welcher, Y. Sun, R. Cupples, B. Guthrie, and F. A. Fletcher. 1998. Cloning and characterization of a specific receptor for mouse oncostatin M. *Mol. Cell. Biol. 18:3357.*
42. Ichihara, M., T. Hara, H. Kim, T. Murate, and A. Miyajima. 1997. Oncostatin M and leukemia inhibitory factor do not use the same functional receptor in mice. *Blood 90:165.*
43. Tanaka, M., T. Hara, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, and A. Miyajima. 1999. Reconstitution of the functional mouse Oncostatin M (OSM) receptor: molecular cloning of the mouse OSSM receptor B subunit. *Blood 93:804.*
44. Kerr, C., C. M. Langdon, F. Graham, J. Gauldie, T. Hara, and C. D. Richards. 1999. Adenovirus vector expressing mouse Oncostatin M induces acute phase proteins and TIMP-1 expression in vivo in mice. *J. Interferon Cytokine Res. 19:1195.*
45. Winer, J., C. K. Jung, I. Shackel, and P. M. Williams. 1999. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal. Biochem. 270:41.*
46. Xing, Z., Y. Ohkawara, M. Jordana, F. L. Graham, and J. Gauldie. 1996. Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J. Clin. Invest. 97:1102.*
47. Matsukura, S., C. Stellato, J. R. Plitt, C. Bickel, K. Miura, S. N. Georas, V. Casolaro, and R. P. Schleimer. 1999. Activation of eotaxin gene transcription by NF-κB and STAT6 in human airway epithelial cells. *J. Immunol. 163:6876.*
48. Soldi, R., A. Graziani, R. Benelli, D. Ghigo, A. Bosia, A. Albini, and F. Bussolino. 1994. Oncostatin M activates phosphatidylinositol-3-kinase in Kaposi's sarcoma cells. *Oncogene 9:2253.*
49. Schieven, G., J. Kallestad, J. Brown, J. Ledbetter, and P. Linsley. 1992. Oncostatin M induces tyrosine phosphorylation in endothelial cells and activation of p62 yes tyrosine kinase. *J. Immunol. 149:1676.*
50. Duncan, M. R., A. Hasan, and B. Berman. 1995. Oncostatin M stimulates collagen and glycosaminoglycan production by cultured normal dermal fibroblasts: insensitivity of scleroderma and keloidal fibroblasts. *J. Invest. Dermatol. 104:128.*
51. Lamkhioued, B., P. M. Renzi, S. Abi-Younes, E. A. Garcia-Zepada, Z. Allakhverdi, O. Ghaffar, M. D. Rothenberg, A. D. Luster, and Q. Hamid. 1997. Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J. Immunol. 159:4593.*
52. Grove, R. I., C. Eberhardt, S. Abid, C. Mazzucco, J. Liu, P. Kiener, G. Todaro, and M. Shoyab. 1993. Oncostatin M is a mitogen for rabbit vascular smooth muscle cells. *Proc. Natl. Acad. Sci. USA 90:823.*
53. Katoh, S., N. Matsumoto, K. Fukushima, H. Mukae, J. I. Kadota, S. Kohno, and S. Matsukura. 2000. Elevated chemokine levels in bronchoalveolar lavage fluid of patients with eosinophilic pneumonia. *J. Allergy Clin. Immunol. 106:730.*
54. Tateno, H., H. Nakamura, N. Minematsu, K. Amakawa, T. Terashima, S. Fujishima, A. D. Luster, C. M. Lilly, and K. Yamaguchi. 2001. Eotaxin and monocyte chemoattractant protein-1 in chronic eosinophilic pneumonia. *Eur. Respir. J. 17:962.*
55. Saita, N., T. Yamanaka, H. Kohrogi, M. Ando, and M. Hirashima. 2001. Apoptotic response of eosinophils in chronic eosinophilic pneumonia. *Eur. Respir. J. 17:190.*
56. Tomkinson, A., C. Duez, G. Cieslewicz, and E. W. Gelfand. 2001. Eotaxin-1-deficient mice develop airway eosinophilia and airway hyperresponsiveness. *Int. Arch Allergy Immunol. 126:119.*
57. Gonzalo, J., C. Lloyd, D. Wen, J. Albar, T. Wells, A. Proudfoot, A. Martinez, M. Dorf, T. Bjerke, A. Coyle, and J. Gutierrez-Ramos. 1998. The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J. Exp. Med. 188:157.*