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Oncostatin-M Up-Regulates VCAM-1 and Synergizes with IL-4 in Eotaxin Expression: Involvement of STAT6

Dominik K. Fritz, Christine Kerr, Li Tong, David Smyth, and Carl D. Richards

Oncostatin-M (OSM) is an IL-6/gp130 family member that can stimulate the eosinophil-selective CC chemokine eotaxin-1 in vitro and eosinophil accumulation in mouse lung in vivo. The adhesion molecule VCAM-1 and eotaxin have been implicated in extravasation and accumulation of eosinophils into tissue in animal models of asthma. In this study, we investigated the role of OSM in regulation of VCAM-1 expression, and STAT6 tyrosine 641 phosphorylation in murine fibroblasts. OSM induced VCAM-1 expression in C57BL/6 mouse lung fibroblasts (MLF) and NIH 3T3 fibroblasts at the protein and mRNA level in vitro. OSM also induced STAT6 Y641 phosphorylation in MLF and NIH 3T3 fibroblasts, an activity not observed with other IL-6/gp130 cytokine family members (IL-6, leukemia inhibitory factor, cardiotropin-1, and IL-11) nor in cells derived from STAT6−/− mice (STAT6−/− MLF). STAT6 was not essential for OSM-induced VCAM-1 or eotaxin-1 as assessed in STAT6−/− MLF. Combination of IL-4 and OSM synergistically enhanced eotaxin-1 expression in MLF. IL-4 induction and the IL-4/OSM synergistic induction of eotaxin-1 was abrogated in STAT6−/− MLF, however, regulation of IL-6 was similar in −/− or wild-type MLF. Induction of VCAM-1 by OSM was diminished by pharmacological inhibitors of PI3K (LY294002) but not inhibitors of ERK1/2 (PD98059) or p38 MAPK (SB203580). These data support the role of OSM in eosinophil accumulation into lung tissue through eotaxin-1 and VCAM-1 expression and the notion that OSM is able to induce unique signal transduction events through its receptor complex of OSMR β-chain and gp130. The Journal of Immunology, 2006, 176: 4352–4360.

Asthma is characterized by airway remodeling and airway inflammation, which subsequently leads to loss of lung function (1). A prominent feature of airway inflammation in asthmatic disease states is the infiltration of high numbers of eosinophils into the lung tissue (2). Eosinophils constitute a small population of circulating or tissue-resident leukocytes and the associated infiltration of these cells suggests the existence of molecular mechanisms that are responsible for their selective recruitment into tissues. Migration of eosinophils into lung tissue is a complex process orchestrated by key cytokines, chemokines, and adhesion receptor/molecule interactions.

The endothelial cell adhesion molecules P-selectin and VCAM-1 have been demonstrated to be important in eosinophil tethering, rolling, and firm adhesion on endothelium, respectively (3, 4). Eosinophils have been shown to express VLA-4 (αvβ6/CD49d/CD29 integrin) which binds VCAM-1 in vitro (5–8). The importance of these interactions has been demonstrated in studies where in vivo blockade of VCAM-1/VLA-4 inhibited OVA allergen-induced eosinophil infiltration into the lung (9, 10) and eotaxin-induced eosinophil adhesion and transendothelial migration into tissue (11, 12). IL-4 has been documented to selectively regulate the expression of VCAM-1 on human endothelial cells (13, 14) and to increase the adherence of eosinophils to endothelial cells in vitro in a VCAM-1-dependent manner (4). Other stromal cells have been shown to express VCAM-1 in response to IL-4 including corneal fibroblasts (15), synovial fibroblasts (16, 17), and lung fibroblasts (18–20). Induction of P-selectin on endothelial cells is also regulated by IL-4 (4, 13) in a STAT6-dependent manner (21). Interestingly, oncostatin-M (OSM) (22) has also been documented to induce the expression of P-selectin on endothelial cells (13). The ability of OSM or any other IL-6/gp130 cytokine family members to regulate the expression of VCAM-1 in lung-derived cells is to our knowledge not known.

The role of IL-6/gp130 cytokines in airway disease processes has yet to be comprehensively investigated. This family of cytokines includes OSM, IL-6, IL-11, leukemia inhibitory factor (LIF), cardiotropin-1 (CT-1), ciliary neurotrophic factor, and the recently identified cardiotrophin-like cytokine (also referred to as novel neutrophin-1/B cell-stimulating factor-3; Refs. 22–26). The potential role of OSM in the pathogenesis of asthma, particularly with respect to airway remodeling, has been suggested (27). We and others have shown that OSM can induce eotaxin-1 in mouse lung fibroblasts (MLF) (28) and lung smooth muscle cells (29). Moreover, we have demonstrated that transient high mouse OSM (mOSM) expression in the murine lung leads to airway remodeling and predominant eosinophil infiltration in vivo following intranasal adenovirus-mOSM administration (28). IL-4, a Th2 cytokine, has been implicated in the pathology of atopic disease states and in leukocyte infiltration, particularly eosinophil accumulation, into sites of inflammation in animal models of asthma (30, 31). These observations suggest that OSM is able to induce molecular events that overlap with IL-4 in producing features reminiscent of an asthmatic-like inflammation.

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3 Abbreviations used in this paper: OSM, oncostatin-M; LIF, leukemia inhibitory factor; CT-1, cardiotropin-1; MLF, mouse lung fibroblast, m, murine; rm, recombinant murine; pY, phosphotyrosine; wt, wild type; RIPPA, radioimmunoprecipitation; Ct, threshold cycle; TIMP-1, tissue inhibitor of matrix metalloproteinase-1.
The CC chemokine eotaxin family members eotaxin-1 (eotaxin), eotaxin-2, and eotaxin-3 are thought to be the most potent eosinophil-selective chemoattractants (32–34), particularly eotaxin-1, because they act through a single eotaxin-specific CC chemokine receptor, CCR3, which has been shown to be expressed on human (35, 36), guinea pig (35), and mouse (37) eosinophils. The importance of this chemokine in selective tissue eosinophilic infiltration has been demonstrated by injection of eotaxin into guinea pig skin (32) or into mouse peritoneum (12) and lung eosinophilia was demonstrated following exposure of guinea pig to aerosolized eotaxin (38). Increased content of eotaxin in bronchoalveolar lavage and the presence of high numbers of airway eosinophils have been found in asthmatic patients (39, 40). IL-4 has been documented to regulate the expression of eotaxin-1 (41), eotaxin-2 (42), and eotaxin-3 (34) in endothelial cells and fibroblasts in a STAT6-dependent manner (42–45). IL-4 signal transduction includes activation of STAT6 (46–50). Our laboratory has shown mOSM to regulate the expression of eotaxin-1 in vitro in MLF in a dose-dependent manner (28), however, the intracellular mechanisms associated with eotaxin expression by OSM are not clear, particularly with respect to STAT6.

In this study, we examine the regulatory role of mOSM in VCAM-1 expression and its potential in inducing STAT6 Y641 phosphorylation in vitro in mouse fibroblast cell lines, and show that mOSM is prominent among IL-6/gp130 cytokines in its ability to induce VCAM-1 up-regulation and STAT6 activation. We also demonstrate that STAT6 is not essential in mOSM-induced VCAM-1 expression, however, it is required for the IL-4 induction of eotaxin or the synergistic expression of eotaxin-1 induced in combination of mOSM and mIL-4 in vitro.

Materials and Methods

Cell lines

Primary wild-type (wt) or STAT6−/− C57BL/6 MEF cell lines were derived from explants of finely minced lung tissue from wt C57BL/6 mice (10–12 wk old; Charles River Laboratories) or STAT6−/− mice (10–12 wk old; provided by Drs. Z. Xing and Y. Wua, McMaster University, Ontario, Canada). MEF were cultured in Earle’s modified MEM (F-15) medium supplemented with 10% FBS, 1% penicillin-streptomycin, 1% l-glutamine, and 0.1% fungizone. NIH 3T3 cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% calf serum and incubated for 3 h at 37°C in 5.0% CO2 conditions. Subconfluent fibroblast cultures (75–85%) were stimulated and whole cell lysates were generated using RIPA lysis buffer. Cell culture media was removed and fibroblasts were washed once with cold 1× PBS. PBS was removed and a second RIPA lysis buffer was added. Fibroblasts were scraped using cell scrapers and whole cell lysates were incubated for 1 h on ice. Cell lysates were then vigorously sheared using a syringe with a 21-gauge needle, centrifuged at maximum speed (12,000 × g) at 4°C, and stored at −70°C. Protein concentration was determined using the Bio-Rad Protein assay (Bio-Rad). Equal amounts of protein were loaded onto 8% SDS-PAGE gels for electrophoresis. Proteins were transferred to Immobilon-NC membranes (Millipore). Western blots were blocked in 1× TBS containing 0.15% Tween 20 and 5.0% fat-free milk powder for 1 h at room temperature and probed with primary Ab overnight at 4°C. Membranes were washed, incubated with HRP-conjugated secondary Abs for 1 h at room temperature, and visualized using an ECL Western Blotting Analysis System (Amersham Pharmacia Biotech).

ELISA

Subconfluent cultures (75–85%) were stimulated for 24 h in 2% serum-supplemented medium; supernatants were collected and stored at −20°C until analysis by ELISA. Culture supernatant levels of mIL-6, eotaxin, mIL-13, and mIL-4 were measured using ELISA. Murine IL-6 and eotaxin DuoSet ELISA kits were purchased from R&D Systems. Sensitivity of mIL-6 and eotaxin DuoSet ELISA kits was 15 pg/ml, respectively. mIL-13 and mIL-4 Quantikine ELISA kits were purchased from R&D Systems. Sensitivity of mIL-13 and mIL-4 Quantikine ELISA kits was 1.5 pg/ml and sensitivity of ELISAs was conducted according to the manufacturer’s instructions.

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

Subconfluent fibroblast (75–85%) cultures were stimulated with the indicated cytokines in medium supplemented with 2% serum and incubated for 24 h. Total RNA was extracted from fibroblast cultures using TRIzol (In- tercell Life Technologies) according to manufacturer’s instructions.

Genomic DNA was removed using the Ambion DNA-free kit (Ambion). The RNA concentration was determined by OD260 reading on a spectrometer. RNA was reverse-transcribed using the Ambion RETROscript kit (Ambion) using random decamers as primers to obtain cDNA.

Expression of mRNA for murine eotaxin was analyzed using real-time quantitative PCR (TaqMan). The forward and reverse primers along with the fluorescent probe were designed using PrimerExpress version 1.5 software (Applied Biosystems). The forward primer was 5′-CTGCTTGTATTCCCTCAGAGCAGT-TAMRA and the reverse primer was 5′-GGAACTACATCCTTCTCTTCTTCTTAA, where 

$$C_{\text{t}} = \frac{-\log_{2} C_{\text{t}}}{C_{\text{t}} + K}$$ where

$$K = \frac{-\log_{2} C_{\text{t}}}{C_{\text{t}} + K}$$

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the fluorescent probe were designed using PrimerExpress version 1.5 software (Applied Biosystems). Expression of mRNA for murine eotaxin-1 was analyzed using real-time quantitative PCR (TaqMan). The housekeeping gene used was 18S RNA. Data were presented as the mean and SD of three separate reactions per RNA sample.

Cell lyses and immunoblots

Cell lysates were prepared using radioimmunoprecipitation (RIPA) lysis buffer (1× PBS pH 7.4), 1.0% igepal CA-630, 0.5% sodium deoxycholate (C12H7O5Na), 0.1% SDS, including inhibitors (1 mM PMSF, 1 mM sodium orthovanadate (Na3VO4), and 1 mg/ml aprotonin). Fibroblasts were preincubated with fresh medium supplemented with 0.10% DMEM media for NIH 3T3 or 10.0% FBS MEM (F-15) media for primary FBLN) overnight. Before cytokine stimulation, culture media was removed and replaced by fresh 2.0% serum-containing culture media and cells were incubated for 3 h at 37°C in 5.0% CO2 conditions. Subconfluent fibroblast cultures (75–85%) were stimulated and whole cell lysates were generated using RIPA lysis buffer with inhibitors. Cell culture media was removed and fibroblasts were washed once with cold 1× PBS. PBS was removed and a second RIPA lysis buffer was added. Fibroblasts were scraped using cell scrapers and whole cell lysates were incubated for 1 h on ice. Cell lysates were then vigorously sheared using a syringe with a 21-gauge needle, centrifuged at maximum speed (12,000 × g) at 4°C, and stored at −70°C. Protein concentration was determined using the Bio-Rad Protein assay (Bio-Rad). Equal amounts of protein were loaded onto 8% SDS-PAGE gels for electrophoresis. Proteins were transferred to Immobilon-NC membranes (Millipore). Western blots were blocked in 1× TBS containing 0.15% Tween 20 and 5.0% fat-free milk powder for 1 h at room temperature and probed with primary Ab overnight at 4°C. Membranes were washed, incubated with HRP-conjugated secondary Ab for 1 h at room temperature, and visualized using an ECL Western Blotting Analysis System (Amersham Pharmacia Biotech).

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Cytokines and other reagents

Stimulations were performed in vitro on fibroblast monolayers using recombinant mouse (rm) OSM, mIL-6, mIL12, rMCT-1, rM1L-11, rmIL-4, and rmTNFα purchased from R&D Systems. Pharmacological inhibitors PD98059 (PD), SB203580 (SB), and LY294002 (LY) were purchased from Calbiochem. In experiments involving pharmacological inhibitors, fibroblasts were pretreated with 25 μM PD98059 for 2.0 h, 10 μM SB203580 for 1.0 h, or 20 μM LY294002 for 0.25 h before cytokine stimulation. Primary Abs specific for phosphotyrosine (pY) 701 STAT1, pY705 STAT3, pY694 STAT5, pY641 STAT6, and STAT1 and STAT3 were purchased from Cell Signaling Technology (New England Biolabs). Primary Abs specific for STAT-5 (C-17) and STAT-6 (M-20), VCAM-1 (C-19), ERK1/2 (C-16), and actin (I-19) were purchased from Santa Cruz Biotechnology. Primary Abs against pY-STAT's, STAT-1, and STAT-3 were diluted 1/1000 in 5.0% BSA (Sigma-Aldrich). Primary Abs against STAT-5, STAT-6, VCAM-1, ERK1/2, and actin were diluted 1/1000 in 5.0% milk. For detection of primary pY-STATS, STATs, and ERK1/2 (C-16) Abs, secondary goat anti-mouse IgG HRP Ab was purchased from Sigma-Aldrich. Rabbit anti-goat IgG HRP Ab was diluted 1/2500 in 5.0% milk. For detection of primary VCAM-1 (C-19) and actin (I-19) Abs, rabbit anti-goat IgG HRP Ab was purchased from Sigma-Aldrich. Rabbit anti-goat IgG HRP Ab was diluted 1/2500 in 5.0% milk.
for 24 h. Total RNA was extracted and purified as mentioned above. The RNA concentration was determined by OD260 reading on a spectrophotometer. Twenty micrograms of RNA was loaded onto 1.2% formaldehyde agarose gels for electrophoresis, transferred to nylon membranes (Millipore) by capillary action using 20× SSC buffer, and then cross-linked by UV irradiation using methods published previously (51). Two antisense oligonucleotide VCAM-1 probes were synthesized (MOBIX, McMaster University) with a sequence of 5′-TCCAACAGTCTTGGTTCTGAT GTTCAGAT-3′ and 5′-TAAGCTGAAAGGTTCCTTGGAGAGATCATT-3′ that correspond to bp 951–980 and 2640–2669, respectively, of the mouse gene for VCAM-1 (52). Antisense oligonucleotide 18S ribosomal RNA probe was also synthesized (MOBIX, McMaster University) with a sequence of 5′-GCCGATGTTACGTCTCAGGATCAACACACG-3′ that corresponds to base pairs 151–180 of the mouse gene for 18S RNA (53). The probes were labeled with [γ-32P]ATP using T4 polynucleotide kinase (NEB). Northern blots were prehybridized with hybridization buffer containing 0.1 M NaCl, 50 mM PIPES (pH 6.8), 50 mM NaH2PO4 (pH 4.0), 1 mM EDTA (pH 8.0), and 5% SDS, and then hybridized with labeled probe overnight at 45°C. The membranes were washed with 4× SSC buffer containing 0.1% SDS and then with 0.1× SSC buffer containing 0.5% SDS two to three times at 42°C. Specific hybridization signals on the blots were measured using Molecular Dynamics PhosphorImager and analyzed using Typhoon Scanner Control version 5.0 software.

**VCAM-1 quantification**

Western blots were exposed by X-OMAT Scientific Films (Kodak) and images scanned at 300–400 dpi resolution. Band density per area (density intensity/mm²) was analyzed for both VCAM-1 and loading control (ERK1) using Quantity One-GelDoc version 4.1.0 software. Density levels for VCAM-1 were corrected and normalized using density values of the loading control (ERK1) and fold induction for VCAM-1 was quantified.

**Results**

**OSM induces VCAM-1 expression**

We first examined the ability of mOSM and other mIL-6/gp130 cytokine family members to regulate VCAM-1 expression on C57BL/6 MLF and NIH 3T3 fibroblasts. Fibroblasts were stimulated with 25.0 ng/ml mOSM for various time points for a total period of 48 h and Western blots showed a specific signal at the correct molecular mass of ~100 kDa. mOSM-induced VCAM-1 expression by MLF was detectable as early as 12 h (~3.1-fold) and increased by ~4.3- and ~5.2-fold at 24 and 48 h time points, respectively (Fig. 1A). Similar results were obtained in NIH 3T3 fibroblasts (Fig. 1B). Northern blot mRNA analysis revealed that mOSM increased VCAM-1 mRNA transcripts in fibroblasts following 24 h stimulation (Fig. 1C). In dose response experiments, expression of VCAM-1 was detectable at 2.5 ng/ml mOSM and reached a plateau at 10.0–25.0 ng/ml (Fig. 2A). To compare the effect of mOSM on VCAM-1 expression with other IL-6/gp130 cytokines, C57BL/6 MLF were stimulated with 25.0 ng/ml mIL-6, mILF, mCT-1, mIL-11, or mOSM for a period of 24 h. Fig. 2B shows that mOSM induced VCAM-1 expression to the greatest degree (~4.1-fold). mIL-4, which has been shown to regulate the expression of VCAM-1 in other studies (13–20), was also able to up-regulate VCAM-1 expression in our system (Fig. 2B) but to a lesser degree (~1.7-fold). These results were consistent in at least three separate MLF culture experiments.

**OSM induces STAT6 activation**

Other studies have shown that both OSM and IL-4 are able to induce common signal transduction pathways including insulin receptor substrate-1, PI3K, and src homology and collagen protein (54–57), and mediate expression of eotaxin (28, 41) and P-selectin (4, 13). IL-4 effects have been shown to be dependent on STAT6 activation (21, 43), however, OSM regulation of STAT6 has not previously been identified. Fig. 3A shows that mOSM induced STAT6 Y641 phosphorylation in a dose-dependent manner that was detectable at 2.5 ng/ml and plateaued at a concentration of 10.0 ng/ml. In comparison to IL-4 (known to stimulate STAT6 activation, Refs. 46–50), we found that mOSM induced STAT6 activation to a similar degree in MLF. Similar results were observed in at least three separate experiments and in NIH 3T3 fibroblasts (Fig. 3B).

The specific OSM receptor (OSMR) complex (in human system OSMR-type II), which uses the specific OSMR β-chain, has been shown to mediate distinct cellular responses in addition to those induced by other IL-6/gp130 cytokine family members (56–58). We tested the effects of other IL-6/gp130 cytokines on STAT activation in comparison to that induced by mOSM in C57BL/6 MLF (Fig. 4A). mOSM induced STAT1 (Y701), STAT3 (Y705), and STAT5 (Y694) phosphorylation as reported by others (57–59). Moreover, mOSM, but not mIL-6, mLIF, mCT-1, nor mIL-11, induced STAT6 Y641 phosphorylation. Similar results were obtained in NIH 3T3 fibroblasts (Fig. 4B). These results suggest that STAT6 protein activation by mOSM is mediated specifically by...
the OSMR β-chain among the IL-6/gp130 cytokine receptor complexes in this murine system. Both C57BL/6 MLF and NIH 3T3 fibroblast cell lines responded similarly to mIL-6, mLIF, and mL11 with respect to STAT3 Y705 phosphorylation, indicating the presence of functional receptors for these cytokines on these cells. mCT-1 responses were observed in NIH 3T3 but not MLF suggesting differences in CT-1 receptor expression in these two cell types.

OSM and IL-4 synergize in eotaxin expression that is STAT6 dependent

To assess the role of STAT6 in OSM-induced responses, we have developed STAT6-deficient lung fibroblast cell lines derived from STAT6−/− C57BL/6 mice, and data shown represents results with identical trends in at least two separate experiments. We have shown previously that mOSM is able to regulate eotaxin-1 in MLF (28) and eotaxin has been shown to be regulated by STAT6 in other systems (42–45). Thus, we investigated induction of eotaxin by mOSM in wt and STAT6−/− MLF. Twenty-four-hour stimulation with 25.0 ng/ml mOSM induced detectable increases in eotaxin-1 expression at the mRNA and protein level in both wt and STAT6−/− MLF (Fig. 5, A and C). Stimulation of wt MLF using 10.0 ng/ml mIL-4 induced eotaxin mRNA expression and protein production that was nondetectable in STAT6−/− MLF (Fig. 5, A and C). mRNA levels were slightly lower in STAT6−/− MLF than wt MLF in response to mOSM, although protein levels produced were similar. The reasons for this are not clear and to test for the possibility of altered kinetics of induction, time-course experiments in wt MLF indicated mOSM or mIL-4 induced eotaxin expression as early as 12 h (~6.0 pg/10⁴ cells for mOSM or ~12.5 pg/10⁴ cells for IL-4) with continued increase in eotaxin expression at 24 and 48 h (data not shown). mOSM-induced eotaxin protein responses at either 12, 24, or 48 h, were similar in wt vs STAT6−/− MLF. This supports the suggestion that STAT6-independent mechanisms regulate eotaxin or compensate in the absence of STAT6 in mOSM-induced MLF. However, eotaxin was not detected in STAT6−/− MLF at any of these time points after mIL-4 stimulation. These data indicate that STAT6 activation is not essential for mOSM-induced eotaxin expression, but is required for IL-4-mediated eotaxin induction in these cells.

Because various studies have demonstrated the ability of IL-4 to synergize with TNF-α or IL-4 in eotaxin expression in various cell lines (43, 45, 60–63), we examined the effects of mIL-4 on mOSM-induced eotaxin expression in both wt and STAT6−/− MLF. Combination of 25.0 ng/ml mOSM and 10.0 ng/ml mIL-4 had a synergistic effect on eotaxin mRNA expression and protein secretion that was completely abrogated in STAT6−/− MLF (Fig. 5, A and C). Time-course experiments in wt MLF indicated mOSM/mIL-4 induced eotaxin synergy as early as 12 h (means of ~80.0 pg/10⁴ cells) with continued increase in eotaxin expression at 24 h (~185.0 pg/10⁴ cells) and 48 h (~210.0 pg/10⁴ cells).

In addition, our studies have shown that neither mIL-1β nor mTNF-α induce eotaxin levels in MLF under these culture conditions (data not shown). mTNF-α or mIL-1 alone or in combination with mOSM did not induce eotaxin expression or synergy, respectively, in lung fibroblasts following 24 h stimulation (data not shown). Furthermore, mOSM did not induce detectable levels of IL-4 or IL-13 expression in lung fibroblasts following 24 h stimulation as detected by ELISA (sensitivity, 1.5 and 2.0 pg/ml) suggesting indirect effects of OSM through IL-1, TNF, IL-4, or IL-13 were unlikely.

We also compared the effects of mOSM on IL-6 expression in both wt and STAT6−/− MLF (Fig. 5, B and D) and show that STAT6−/− MLF respond similarly to wt MLF in IL-6 expression at both the mRNA and protein levels. This indicates a selective effect of STAT6 deficiency on eotaxin-1 but not IL-6 expression. To verify the absence of the STAT6-signaling cascade in STAT6−/− MLF, we assessed mOSM and mL11 mIL-4 induced responses with respect to STAT3 and STAT6 activation. Fig. 6 confirms the absence of STAT6 protein and STAT6 induction in STAT6−/− MLF but equivalent activation of STAT3 in both wt and STAT6−/− MLF.
STAT6 and PI3K in OSM induced VCAM-1

We then assessed VCAM-1 induction in wt and STAT6"/" MLF in response to mOSM and other cytokines. mTNF-α, mIL-4, or mOSM induced VCAM-1 protein by ~11.1-, 2.0-, and 3.6-fold of control, respectively (Fig. 7A). Combinations of these cytokines showed no detectable difference in effects on VCAM-1 from that seen in response to individual cytokines alone (Fig. 7A). Levels of VCAM-1 in STAT-6-deficient MLF were somewhat reduced compared with those observed in wt MLF, however, the ability of mOSM or mTNF-α to induce VCAM-1 was maintained (Fig. 7B). mTNF-α was found not to regulate STAT6 Y641 phosphorylation in our system (data not shown).

Since STAT6 was found to be dispensable in regulating the expression of VCAM-1 by mOSM, we examined the effects of inhibiting other signaling cascades using various pharmacological inhibitors in both wt and STAT6-deficient MLF. We have previously shown that mOSM induces ERK, p38 MAPK, and Akt activation in mouse fibroblasts and that ERK and p38 MAPK inhibitors decrease eotaxin and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) responses to mOSM (28, 64). We tested concentrations of inhibitors that we have shown established effects on MAPK, PI3K/Akt activation, and regulation of TIMP-1 or eotaxin-1 protein expression by MLF (28, 64). The ERK1/2 inhibitor (PD98059) and p38 MAPK inhibitor (SB203580) had no detectable effect in modulating mOSM-induced VCAM-1 expression in either the wt and STAT6"/" MLF, however, the PI3K inhibitor (LY294002) abrogated induction of VCAM expression by mOSM (Fig. 7, C and D). Supernatants of these experiments showed reduction in eotaxin levels by ELISA analysis (data not shown) as previously published (28). We have previously shown mOSM induced Akt phosphorylation is inhibited by LY294002 but not ERK1/2 or p38 MAPK (64), suggesting a role of the PI3K/Akt pathway in VCAM-1 protein induction by mOSM.

FIGURE 3. OSM and IL-4 induce STAT6 Y641 phosphorylation in fibroblasts. C57BL/6 MLF (A) and NIH 3T3 fibroblasts (B) were stimulated with increasing concentrations ng/ml of mOSM or mL-4 as indicated for 20 min, and whole cell lysates were generated (see Materials and Methods). Western blots were prepared and probed with specific Ab (see Materials and Methods).

FIGURE 4. Activation of STAT proteins by IL-6/ gp130 cytokines in fibroblasts. C57BL/6 MLF (A) and NIH 3T3 fibroblasts (B) were stimulated for 20 min with 25 ng/ml murine OSM, IL-6, LIF, CT-1, IL-11, and 10 ng/ml mIL-4 and whole cell lysates generated. pY-STAT and STAT Western blots were prepared as above (see Materials and Methods). mOSM induces STAT6 pY641 phosphorylation while other IL-6/gp130 cytokines tested did not.
Discussion
We have previously shown that mOSM overexpression leads to eosinophilic accumulation in the lung parenchyma, bronchial lumens, and bronchoalveolar lavage fluid in mice and that mOSM also induces eotaxin expression in MLF in vitro (28). In addition to eotaxin, we hypothesized that accumulation of eosinophils into the murine lung occurs by enhanced adhesion receptor/ligand interactions regulated by mOSM, and here we show that mOSM is able to regulate the expression of VCAM-1 in C57BL/6 MLF and NIH 3T3 fibroblasts at both the mRNA and protein level. This finding reveals a newly identified activity for mOSM on lung fibroblasts and indicates a potential additional mechanism in eosinophil accumulation in the lung parenchyma. VLA-4 (α4β7/CD49d/CD29 integrin), a receptor for VCAM-1, is expressed on eosinophils (5–8). VCAM-1/VLA-4 binding has been shown to be important in OVA allergen-induced eosinophil infiltration into the lung (9, 10) and eotaxin-induced eosinophil migration into tissue (11, 12). In addition to its cell adhesion activities, VCAM-1 was shown to augment eosinophil function (65–68) and survival (69). OSM was shown to regulate the expression of VCAM-1 in mesenchymal bone marrow stromal cells (70). VCAM-1 regulation by OSM in HUVECs was shown in a study by McIntyre and colleagues (71), but not in another study by McEver and colleagues (13). This suggests that OSM may contribute to eosinophil extravasation into lung tissue by enhancing rolling/tethering and adhesion along the endothelium through P-selectin and possibly VCAM-1 up-regulation (13, 71) and, on the basis of our results here, may also contribute to lung parenchymal fibroblast VCAM-1 facilitating cell migration and survival.

Eotaxin has been shown to act as an eosinophil-selective chemotactant in vivo (12, 32, 38), and expression of the CC chemokine eotaxin family members has been shown to occur in a STAT6-dependent manner following IL-4 stimulation (42–44). Studies have previously demonstrated that IL-4 and OSM induce overlapping signal transduction pathways (insulin receptor substrate-1, P13K, Ref. 54, and src homology and collagen protein, Ref. 55–57). IL-4 is known to signal through STAT6 (46–50) and OSM has been shown to induce STAT1, STAT3, and STAT5 (51, 57–59). We here show that mOSM is unique among IL-6/gp130 cytokine family members in activating STAT6 Y641 phosphorylation in fibroblasts, suggesting that STAT6 Y641 phosphorylation is selectively activated by the OSMR β-chain of the mOSMR complex (OSMR-type II in the human system; Refs. 72 and 73). This further suggests that roles of OSM in inflammation may overlap somewhat with those of IL-4, whereas the roles of other IL-6/gp130 cytokines do not.

Recent data (29) has shown that STAT3 mediates eotaxin regulation by OSM in human lung smooth muscle cells. Our data showing STAT6 to be dispensable for mOSM induction of eotaxin is consistent with function of STAT3 but not STAT6 in OSM regulation of eotaxin-1. In contrast, STAT6 was required for mIL-4 action or for mOSM/mIL-4 synergy in eotaxin regulation. Although the precise intracellular mechanism of the synergy is not

![FIGURE 5. Regulation of eotaxin (A and C) and IL-6 (B and D) expression in wt and STAT6−/− MLF. MLF were stimulated with 25 ng/ml mOSM or 10 ng/ml mIL-4 alone or in combination for 24 h in wt (■) and STAT6−/− MLF (□) (1.2 × 10^6 cells/well). RNA was generated and cDNA prepared as described in Materials and Methods and analyzed (A and B) by real-time quantitative PCR (TaqMan). Supernatants were analyzed by ELISA (C and D) as described in Materials and Methods. Concentrations correspond to −8.0 pg/10^6 cells in response to OSM, 14.0 pg/10^6 cells in response to IL-4 and 162 pg/10^6 cells in response to OSM/IL-4. One-way ANOVA was used to test significant difference in ELISA results. *, STAT6−/− MLF response to IL-4 or OSM/IL-4 in eotaxin levels is lower (p < 0.001) than wt cells. No significant differences were observed in eotaxin response to OSM alone, and IL-6 responses were similar in wt vs STAT6−/− cells.](http://www.jimmunol.org/content/173/5/4357/F5)

![FIGURE 6. Regulation of STAT activation in wt and STAT6−/− MLF. Fibroblasts were stimulated with 25.0 ng/ml mOSM or 10.0 ng/ml mIL-4 alone or in combination for 20 min and whole cell lysates generated. Western blots were probed with pY-STAT and STAT Abs for STAT3 and STAT6 as above.](http://www.jimmunol.org/content/173/5/4357/F6)
known, clearly STAT6 is one of the required cell responses for IL-4 action and therefore of OSM/IL-4 combination effects on these cells. This corroborates the importance of this STAT factor in regulation of eotaxin by IL-4 (43–45). Synergy induced by mIL-4/mOSM may be mediated by regulation of IL-4R expression by OSM as shown in smooth muscle cells (29). Alternatively, the enhanced eotaxin mRNA expression may be attributed to increased eotaxin mRNA stabilization involving mRNA-stabilizing proteins such as HuR (60), however, further analysis is required to examine these two possibilities in fibroblast populations. Clearly, OSM regulation of IL-6 is intact in STAT6+/−/− MLF (Fig. 5, B and D), indicating a selective role of STAT6 in eotaxin induction, but not IL-6.

Comparison with other IL-6/gp130 cytokine family members revealed mOSM to induce VCAM-1 up-regulation to a greater degree. IL-4 has been documented to selectively regulate the expression of this adhesion molecule on various human cells (13–17) including human lung fibroblasts (18–20) and to synergize with TNF-α in VCAM-1 expression on human endothelial cells, corneal fibroblasts, and synovioocytes (14, 15, 17). Combination of these cytokines had no effect on VCAM-1 expression on fibroblasts in the mouse system examined here. This may be due to subtle differences in the responses of lung fibroblasts vs fibroblasts from other tissues, or possibly due to differences between mouse (our studies) and human cell responses in vitro.

Expression of VCAM-1 in STAT6+/−/− MLF in response to mOSM or mTNF-α were partially reduced overall compared with wt MLF, suggesting involvement of STAT6 in regulation of VCAM-1 expression. This was not due to overall reduction in protein synthesis because IL-6 protein levels are not reduced in STAT6+/−/− cells (Fig. 5). However, STAT6 was not essential for the up-regulation of VCAM-1 by mOSM or mTNF-α (Fig. 5). STAT6 may participate in the regulation of VCAM-1 transcription, although presence of a typical binding sequence for STAT6 in the VCAM-1 promoter has not been identified (74, 75). STAT6 may participate through indirect actions on other transcription factors, RNA stability of protein translation, although this is not known. Studies have shown the importance of p42/p44 MAPK (ERK2/ERK1) and p38 MAPK signal transduction pathways in VCAM-1 expression (76, 77). Neither the ERK1/2 inhibitor (PD98059) nor the p38 MAPK inhibitor (SB203580) had any regulatory effect on mOSM inhibitor (SB203580) had any regulatory effect on VCAM-1 expression in wt and STAT6+/−/− MLF, whereas, we have shown previously that inhibition of these pathways resulted in reduced eotaxin-1 and TIMP-1 expression.

FIGURE 7. VCAM-1 expression in wt and STAT6+/−/− MLF. A, C57BL/6 MLF and (B) STAT6+/−/− MLF were stimulated with 25 ng/ml mOSM, 20 ng/ml mTNF-α, or 10 ng/ml mIL-4 alone or in combination for 24 h as in the Fig. 1 legend (see Materials and Methods). STAT6 appears not essential for VCAM-1 up-regulation by mOSM. Effects of pharmacological inhibitors on VCAM-1 expression were tested (C and D). wt C57BL/6 MLF (C) and STAT6−/− MLF (D) were stimulated with 25 ng/ml mOSM for 24 h in the absence (−) or presence of the indicated pharmacological inhibitors (25 μM PD98059, 10 μM SB203580, 20 μM LY294002), whole cell lysates generated and probed as above (see Materials and Methods). Pretreatment with the PI3K inhibitor (LY) reduced VCAM-1 expression induced by mOSM in both wt MLF and STAT6+/−/− MLF.
expression (28, 64). However, VCAM-1 expression was abrogated to basal levels when MLF were pretreated with the PI3K inhibitor (LY, LY294002) before mOSM treatment. These data suggest a specific role for the PI3K/Akt pathway in regulating VCAM-1 expression by mOSM in MLF. OSm induces PI3K/Akt (64) and LY was found to inhibit activation of Akt, but not OSM-induced activation ERK, p38 MAPK, STAT1, STAT3, or STAT5 indicating its specificity for Akt (and hence, PI3K (p110/ps5)) in mouse fibroblasts (54, 64).

In summary, we show that mOSM induces VCAM-1 through a PI3K/Akt-dependent pathway and although STAT6 is robustly activated by mOSM, STAT6 does not appear to be essential for mOSM induction of VCAM-1 or eotaxin. Furthermore, mOSM is unique compared with other IL-6/gp130 cytokines in engaging STAT6 signaling that is essential for mOSM synergy with mIL-4 in eotaxin, but not IL-6, mRNA expression and protein secretion in MLF. These results support a role of OSM, and suggest pathways by which this cytokine is mediating effects, in atopic disease states not only in the context of airway remodeling (27) but also in leukocyte homing and accumulation into sites of airway inflammation.

Disclosures

The authors have no financial conflict of interest.

References

17. Spain, J. P. 1999. The Journal of Immunology

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