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Induction of C-X-C chemokines, growth-related oncogene (GRO)α expression and ENA-78, by ML-1 (IL-17F) involves activation of Raf1-MEK-ERK1/2 pathway

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Abbreviations

ENA-78	epithelial-cell derived neutrophil activating protein-78
ERK1/2	extracellular signal-regulated kinase
GROα	growth-related oncogene α

HUVECs	human umbilical vein endothelial cells
NHBEs	normal human bronchial epithelial cells
MAPK	mitogen-activated protein kinase
MEK	MAP kinase kinase
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C

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Abstract

Neutrophil recruitment into the airway typifies pulmonary inflammation, and is regulated through chemokine network, in which two C-X-C chemokines play a critical role. Airway epithelial cells and vein endothelial cells are major cell sources of chemokines. ML-1 [interleukin (IL)-17F] is a recently discovered cytokine and its function still remains elusive. In this report, we investigated the functional effect of ML-1 in the expression of growth-related oncogene (GRO)α and epithelial-cell derived neutrophil activating protein (ENA)-78. The results showed first that ML-1 induces, in time- and dose-dependent manners, the gene and protein expressions for both chemokines in normal human bronchial epithelial cells (NHBEs) and human umbilical vein endothelial cells (HUVECs). Furthermore, selective MEK inhibitors, PD98059, U0126, and Raf1 kinase inhibitor I partially inhibited Ml-1-induced GROα and ENA-78 production. In contrast, the combination of PD98059 and Raf1 kinase inhibitor I completely abrogated the chemokine production, while a protein kinase C (PKC) inhibitor, Ro-31-7549, and a phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, did not affect their production. Taken together, these data indicates a role for Raf1-mitogen-activated protein kinase kinase (MEK) - extracellular signal-regulated kinase (ERK)1/2 pathway in ML-1 induced CXC chemokine expression, suggesting potential pharmacologic

targets for modulation.

Introduction

Airway epithelial and vein endothelial cells play a central role for airway inflammation, since these cells are able to activate inflammatory cells, such as neutrophils, via induction of cytokines, chemokines and adhesion molecules. Neutrophil recruitment and activation are characteristics of airway inflammatory diseases, such as chronic obstructive pulmonary disease (COPD), bronchial asthma and cystic fibrosis (Koller et al, 1995; Jatakanon et al, 1999; Betsuyaku et al, 1999). Neutrophil is a crucial cell type for causing and perpetuating airway inflammation. Many reports have suggested that C-X-C chemokines play an important role for their accumulation and activation into the airway. The C-X-C chemokines are classified into two subsets based on the presence or absence of specific amino acid sequences, Glu-Leu-Arg (ELR) (Baggiolini et al, 1997; Zlotnik and Yoshie, 2000). Although the ELR C-X-C chemokine, IL-8, is one of the important chemoattractants for neutrophils, neutralization of IL-8 activity resulted in only partial inhibition of neutrophil accumulation *in vivo* (Broaddus et al, 1994; Matsukawa et al, 1994; Matsukawa et al, 1998), suggesting the involvement of other ELR C-X-C chemokines, such as GROα and ENA-78. These two chemokines are also detected in the tissue and biological fluids of various human diseases, including acute respiratory distress syndrome, bacterial pneumonia, rheumatoid arthritis, psoriasis,

and bacterial meningitis, where abundant neutrophils are seen (Luster, 1998). However the mechanisms of GROα and ENA-78 production are not fully understood.

Recently, we and others have independently discovered a novel cytokine, ML-1 (Kawaguchi et al, 2001; or IL-17F, Starnes et al, 2001; Hymowitz et al, 2001), belonging to the IL-17 gene family (Kawaguchi et al, 2001), but its function and signaling pathways remain as yet to be defined. ML-1 is expressed in activated $CD4^+$ T cells, basophils, and mast cells, three important cell types involved in airway inflammation (Kawaguchi et al, 2001). We have previously shown that ML-1 is able to induce the expression of IL-6, IL-8 and (intercellular adhesion molecule (ICAM)-1 in bronchial epithelial cells (Kawaguchi et al, 2001), and that this activation process is mediated, in part, through the phosphorylation of ERK1/2, but not p38 and Jun-N-terminal kinase (JNK) (Kawaguchi et al, 2002). The importance of MAPKs in controlling cellular response to the environment and in regulating gene expression, cell growth and apoptosis has made them a priority for research related to many human diseases (English and Cobb, 2002). The ERK1/2, p38, and JNK pathways are all molecular target for drug development, and inhibitors of MAPKs will be one of the next group of drugs developed for the treatment of human diseases (Johnson and Lapadat, 2002).

To gain further understanding of the function and signaling pathways of ML-1, the role of ML-1 in the expression of two critical chemokines, GROα and ENA-78, was investigated. In this communication, we provide evidence that ML-1 is a potent inducer of GROα and ENA-78, involving the activation of the Raf1-MEK-ERK1/2 signaling pathway in NHBEs and HUVECs, and suggest that the Raf1-MEK-ERK1/2 pathway may be a potential target for pharmacotherapeutical intervention of ML-1-induced CXC chemokine expression in the airway inflammatory diseases.

Materials and Methods

Cell culture

NHBEs were purchased from Clonetics (San Diego, CA), and cultured in bronchial epithelial basal medium (Clonetics) containing 0.5 ng/ml human recombinant epidermal growth factor (EGF), 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 triiodothyronine, 50 μg/ml gentamicin, and 50 pg/ml amphotericin-B (Clonetics). HUVECs were obtained from Clonetics (San Diego, CA). The cells were cultured for no more than 3 passages prior to the analysis.

Generation of human recombinant ML-1

Human recombinant ML-1 was generated as described previously (Kawaguchi et al, 2001). The coding sequence of ML-1 was amplified by PCR and subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA) to generate a C-terminal His fusion gene. The vector pcDNA 3.1 was transfected into COS-7 cells by an Effectene Reagent (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. ML-1 was purified with affinity purification by Ni-NTA agarose beads (Qiagen) for His-tagged proteins. Then the concentration of ML-1 protein was quantified by Bradford assay

(BIO-RAD, Hercules, CA) and stored at –80 ^{o}C until used. Endotoxin levels were tested using Kinetic-QCL Chromogenic LAL (Bio Whittaker, Walkersville, MD). Endotoxin levels were undetectable. The cells were treated with ML-1 (10 and 100 ng/ml) for various time periods.

Gene expression of GROα and ENA-78

Total RNA was extracted using RNeasy (Qiagen, Chatsworth, CA) from 1x10^{6} cells at 0.5, 2, 4, 12 and 24 hrs after stimulation with 10 and 100 ng/ml of ML-1. cDNAs were synthesized from 500 ng of total RNA in the presence of MMLV reverse transcriptase (1U/reaction; Sigma, St. Louis, MO), oligo(dT) primer, and reaction buffer at 42 ^{o}C for 90 min, followed by PCR. The sequences of PCR primers for GROα: forward, 5'- CGCTCCTCTCACAGCCGCCA -3', reverse, 5'-AGGAACAGCCACCAGTGAGC-3'; ENA-78: forward, 5'-TGTGTTGAGAGAGCTGCGTTGCGTT-3', reverse, 5'-TCAGTTTTCCTTGTTTCCACC-3'; G3PDH: forward, 5'-ACCACAGTCCATGCCATCAC-3', reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. The amplification reaction was performed for 26 cycles with denaturation at 94 ^{o}C for 45 s, annealing at 53 ^{o}C for 45 s, and extension at 72 ^{o}C for 45 s. The expected size for GROα was 400 bp, for ENA-78 was 222 bp and for G3PDH was 450 bp. PCR products were detected by ethidium bromide staining, and quantified by video densitometry using

Image 1.61 software (NIH Public Software, National Institutes of Health, Bethesda, MD). The level of GROα and ENA-78 gene expression was quantified by calculating the ratio of densitometric readings of the band intensity for chemokines and G3PDH from the same cDNA sample. The values are expressed as mean ± SD (n=3 experiments).

Protein levels of GROα and ENA-78

GROα and ENA-78 protein levels in the supernatants and cell lysate of ML-1-stimulated cells were determined with a commercially available ELISA kit (Biosource, Camarillo, CA) according to the manufacturer's instruction. Cell supernatant was harvested from unstimulated or stimulated cultures with 10 and 100 ng/ml of ML-1 at 2, 6, 12, 24, or 48 hrs after stimulation. The amount of secreted GROα and ENA-78 was determined by the ELISA and expressed as the amount recovered per 10^6 cells. Cells corresponding to the supernatant samples described above were lysed into 0.5 ml NP-40 lysis buffer (20 mmol/L Tris [pH 7.4], 4 mmol/L EDTA, 1 mmol/L PMSF, 100 mg/mL aprotinin, 200 mg/mL leupeptin, 50 mmol/L NaF, 5 mmol/L $Na_4P_2O_7$, and 1% Nonidet P-40 [all purchased from Sigma]). The chemokine concentration of cell lysate was reported as the amount recovered per 10^6 cells. The values are expressed as mean ± SD (n=6 experiments).

Effect of inhibitors on the expression of GROα and ENA-78

For analysis of activation of the Raf1-MEK-ERK1/2 pathway, 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, CA) and U0126 (1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto) butadiene; New England Bio Labs, Beverly, MA); 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 PKC inhibitor, Ro-31-7549 (2-(1-(3-Aminopropyl) indol-3-yl)-3-(1-methylindol-3-yl) maleimide, acetate; Calbiochem); a PI3K inhibitor, LY294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; Calbiochem); a vehicle control, DMSO (Me_2SO) for 1 h before treatment with ML-1 (100 ng/ml) for 24 hrs. The final concentration of DMSO did not exceed 0.1% (v/v). GROα and ENA-78 protein levels in the supernatants were determined as described above. The values are expressed as mean ± SD (n=4 experiments).

Data analysis

The statistical significance of differences was determined by analysis of variance (ANOVA). The values are expressed as mean + SD from independent experiments. Any difference with p values less than 0.05 was considered. When ANOVA indicated a significant difference, the Scheffe F-test was used to determine the difference between groups.

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Results

To determine the functional role of ML-1 in the regulation of C-X-C chemokine expression, the mRNA expression of GROα and ENA-78 was examined. While detectable gene expression for both GROα and ENA-78 was found in control cells at 2 hr time point (Fig. 1A), the induction of chemokine gene expression was evident in both NHBEs and HUVECs (Fig. 1A and 1B), and ML-1-induced gene expression peaked at 2 hr time point and returned to baseline at 24-hr time point in ML-1 (100 ng/ml)-treated NHBEs (Figs. 1C and 1D). No increase of chemokine gene expression was seen in cells treated with a His-tag control protein (Positope, 10 or 100 ng/ml; Invitrogen, Carlsbad, CA; data not shown). A similar time course of gene expression in HUVECs was also found (data not shown).

To investigate the protein expression for both chemokines, NHBEs and HUVECs were cultured in the absence or presence of varying doses of ML-1 at five different time points. GROα and ENA-78 proteins were detected in the absence of ML-1 in NHBEs. Cell lysate GROα protein level were significantly increased 6 hrs after stimulation with 10 and 100 ng/ml of ML-1. Cell lysate ENA-78 protein level were significantly increased 12 hrs after stimulation with 10 and 100 ng/ml of ML-1.

They were significantly elevated at 24 and 48 hrs time points measured, and attained their highest level 24 hrs after stimulation (Fig. 2A). Cell supernatant GROα and ENA-78 levels were significantly elevated 24 hrs after stimulation and were increased further at 48 hrs (Fig. 2B). A similar pattern was observed in HUVECs, except for significant induction of GROα secretion was seen at 12-hr time point when HUVECs were stimulated with 100 ng/ml of ML-1 (Fig. 3A and 3B), suggesting that ML-1 is more potent in the induction of GROα expression when compared to the ENA-78 expression in both cell types.

We next investigated whether the activation of the Raf1-MEK-ERK1/2 pathway was necessary for the stimulation of chemokine production. As shown in Figs. 4 and 5, 1 hr pretreatment of selective MEK inhibitors, PD98059, U0126, and Raf1 kinase inhibitor I significantly attenuated, in a dose-dependent manner, the production of GROα and ENA-78 in HHBEs and HUVECs, respectively, while 1 hr pretreatment of the cells with vehicle alone (0.05% DMSO) did not affect the protein release. In addition, the protein levels of GROα and ENA-78 were unchanged in ML-1-treated cells in the presence of a p38 kinase inhibitor, SB202190, even at a dose of 10 μM (Figs. 4 and 5). While induction of GROα and ENA-78 is partially inhibited by PD98059, U0126, or Raf1 kinase inhibitor I even at relatively high dose (50 μM, 10 μM and 10 nM, respectively), the

combination with 10 μM of PD98059 and 1 nM of Raf1 kinase inhibitor I completely inhibited the production of these two chemokines in NHBEs and HUVECs.

We also investigated whether other signaling molecules, such as PKC and PI3K, are involved in upstream signaling pathway of the C-X-C chemokine expression. The results showed that no significant inhibitory effect on ML-1-induced chemokine expression in NHBEs and HUVECs was found when a selective PKC inhibitor, Ro-31-7549 (0.01-0.5 μM), or a PI3K inhibitor, LY294002 (0.1-20 μM), were used (Figs. 6A and 6B, respectively).

Discussion

GROα has been shown to be a potent neutrophil chemoattractant and activator *in vitro*, and this chemotactic activity is equivalent to that of IL-8 ((Balentien et al, 1990). Similarly, ENA-78 is as equally potent as IL-8 in inducing neutrophil chemotaxis, however, it is consistently less active in inducing the release of granules from neutrophils (Walz et al, 1991). Besides eosinophils, it is reported that neutrophils are also involved in the features of bronchial asthma, airway hyperreactivity, airway hypersecretion and airway wall remodeling (Molet et al, 2001). In addition, pulmonary neutrophilia has also been found in severe asthmatic airways, and at sits of allergen challenge in asthmatic subjects (Ordonez et al, 2000). Several inflammatory stimuli such as TNFα, lipopolysaccharide, diesel exhaust exposure and respiratory syncytial virus infection can induce GROα and/or ENA-78 (Lucas et al, 1995; Matsukawa et al, 1999; Salvi, 2000; Nasu et al, 2001; Zhang et al, 2001). The expression of GROα and ENA-78 has been found in several inflammatory models and diseases (Luster, 1998), strongly implicating its role in the pathogenesis of inflammation.

ML-1 is derived from activated $CD4^+$ T cells, basophils and mast cells, which are important

regulatory cells for the inflammation (Kawaguchi et al, 2001). It is thus a strong possibility that ML-1-induced GROα and ENA-78 are involved in neutrophilic inflammation. It is of interest to note that ML-1 induces C-X-C chemokines, but not C-C chemokines, such as eotaxin and RANTES, which are potent chemoattractants for eosinophil (data not shown), suggesting a selective role of ML-1 in neutrophil recruitment and activation. As a corollary, a recent study has suggested an *in vivo* role of human IL17F in recruiting neutrophils into the pulmonary mucosa in mice following adenoviral gene transfer (Hurst et al, 2002), further suggesting a potential role of ML-1 in the pathogenesis of neutrophilic inflammation.

Raf-1 is a MAP kinase kinase kinase (MAP3K), which functions downstream of the Ras family of membrane associated GTPases, and is able to activate the dual specificity protein kinases, MEK1 and MEK2, which in turn activate the serine/threonine specific protein kinases, ERK1 and ERK2 (English and Cobb, 2002). Our previous and current data demonstrated that ML-1-induced IL-8, GROα and ENA-78 production is dependent on the activation of ERK1/2, but not p38 and JNK in the MAPK signaling pathway (Kawaguchi et al, 2002). ERK1/2 is known to be involved in the regulation of cell proliferation and apoptosis. In this study, the inhibitors used did not affect both the cell number and viability (data not shown), suggesting an effect on gene and protein

expression.

Finally we used inhibitors for other signal molecules, such as PKC and PI3K, since several studies have demonstrated both PKC and PI3K are linked to MAP kinase pathway. PKC is a key activator of the Raf1/MAP kinase cascade at multiple steps. It is known that PKC can regulate Raf1 signaling through phosphorylation of Raf kinase inhibitory protein (Corbit et al, 2003), and also phosphorylates Raf1 at serine 499 (Kolch, 1993). On the other hand, Ras is likely to act through additional proteins besides Raf1. PI3K is a candidate Ras effector (Rodriguez-Viciana et al, 1997). Activation of PI3K by a variety of extracellular stimuli leads to the accumulation of the second messenger phosphatidylinositol 3, 4, 5-trisphosphate. Its final target is the serine/threonine kinase Akt/PKB. Activated Ras promotes cell survival in epithelial cell through activation of PI3K and Akt/PKB because at high dose, 20μM, of LY294002 induces apoptosis (Khwaja et al, 1997). In our study, however, LY294002 did not show any effect on the cell number and viability (data not shown). This is likely due to different stimuli and cell types used.

To date little is known about the upstream signaling pathway of GROα and ENA-78 expression. However, the results of current study suggest that Raf is predominantly associated with the

activation of MEK-ERK1/2 pathway. Therefore, we concluded that the Raf-MEK-ERK1/2 pathway is a central upstream pathway of ML-1 induced GROα and ENA-78 expression in NHBEs and HUVECs. In fact, MAP kinases are important molecules in the airway epithelial activation in response to various stimuli such as TNF-α, IL-1, diesel exhaust particles and influenza virus infection (Reibman et al, 2000; Hashimoto et al, 2000a and 2000b; Griego et al, 2000). Also, MAP kinases, including ERK1/2, are involved in cytokine signaling in HUVECs (May et al, 1998; Goebeler et al, 1999; Surapisitchat et al, 2001). On the other hand, the downstream signaling pathway is currently unclear. IL-17 is known to activate transcription factor, nuclear factor–kappa B (NF)-kB, in chondrocytes and intestinal epithelial cells (Shalo-Barak et al, 1998; Awane et al, 1999). Because of high homology between IL-17 and ML-1, it is possible that ML-1 is able to activate NF-kB in the downstream signaling pathway.

It is noted that a delay between the synthesis and release of both GROα and ENA-78 chemokines was observed. For example, the level of ML-1-induced GROα in cell lysate was noted at the 6 hr time point, while significant increase of GROα in the supernatants was seen at 24 hr after stimulation. The significance of this delay is at present unclear. It is noted, however, that a trend of increase for GROα secretion is seen at the 12 hr time point, although it did not reach statistical

significance. Additional time intervals between the 12 and 24 hr time points will be needed to identify its release kinetics. Of interest, we have also found previously a similar "delay" phenomenon for ML-1-induced IL-6 secretion (Kawaguchi et al, 2002). The "delay" in protein expression may be as a result of the required time frame for protein modification and/or transport, or alternatively, but not mutually exclusively, a faster synthesis/secretion kinetic require an additional factor induced by ML-1. It is also noted that chemokine gene expression is induced by ML-1 at the 2 hr time point, suggesting a direct effect of ML-1 on de novo synthesis of transcripts. However, until a ML-1-inducible factor, if it exists, is found, a possible secondary (or perhaps additive) effect of ML-1 on the induction of chemokine gene and protein expression cannot be ruled out in the current study.

In conclusion, this study reports that ML-1 induces C-X-C chemokines, GROα and ENA-78 via the activation of the Raf1-MEK-ERK1/2 pathway. These results suggest a potential role of ML-1 in the pathogenesis of the airway inflammatory diseases, such as COPD, bronchial asthma and bacterial pneumonia, and the Raf1-MEK-ERK1/2 pathway is a potential pharmacotherapeutical target for inhibition of ML-1-induced neutrophil recruitment and activation in the airway inflammatory diseases.

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Footnotes:

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Figure Legends

Figure 1. GROα and ENA-78 gene expression by different doses of ML-1. (A) NHBEs and HUVECs were treated with 10 and 100 ng/ml of ML-1 for 2 hrs. RT-PCR is performed as described in *Materials and Methods*. (B) The GROα/G3PDH and ENA-78 / G3PDH ratios were determined by densitometric analysis from the same cDNAs. *$p<0.05$ was considered significant vs control.

Time course study of GROα and ENA-78 gene expression. (C) NHBEs were treated with 100 ng/ml of ML-1 for 0.5-24 hrs. RT-PCR is performed as described in *Materials and Methods*. (D) The GROα/G3PDH and ENA-78 / G3PDH ratios were determined by densitometric analysis from the same cDNAs. The values are expressed as mean + SD (n=3 experiments). *$p<0.05$ was considered significant vs the intensity of 0.5 hr time point.

Figure 2. GROα and ENA-78 protein levels in NHBEs. NHBEs were treated with 10 and 100 ng/ml of ML-1 for 2-48 hrs. ELISA was performed as described in *Materials and Methods*. (A) GROα and ENA-78 protein was measured in NHBE cell lysates harvested at the indicated time points after stimulation with ML-1. The cells were lysed as described in *Materials and Methods*. (B) GROα and ENA-78 proteins were measured in the supernatants at the indicated time points stimulated with

ML-1. The values are expressed as mean + SD (n=6).*p<0.05 was considered significant vs control.

Figure 3. GROα and ENA-78 protein levels in HUVECs. HUVECs were treated with 10 and 100 ng/ml of ML-1 for 2-48 hrs. ELISA was performed as described in *Materials and Methods*. (A) GROα and ENA-78 protein was measured in HUVEC cell lysates harvested at the indicated time points after stimulation with ML-1. The cells were lysed as described in *Materials and Methods*. (B) GROα and ENA-78 proteins were measured at the indicated time points following stimulation with ML-1 in the supernatants. The values are expressed as mean + SD (n=6).*p<0.05 was considered significant vs control.

Figure 4. Effect of PD98059, U0126 and Raf1 kinase inhibitor I on GROα and ENA-78 expression in NHBEs. NHBEs were preincubated with 1-50 μM of PD98059 (PD), 1-10 μM of U0126, 0.01-10 nM of Raf1 kinase inhibitor I (Raf I), 1-10 μM of SB202190 or a combination of 10 μM PD and 1 nM Raf I for 1 hr, followed by stimulation with 100 ng/ml of ML-1 for 24 hrs. The results are expressed as the mean ± SD (n=4 experiments). *p<0.05 was considered significant vs ML-1- stimulated cells.

Figure 5. Effect of PD98059, U0126 and Raf1 kinase inhibitor I on GROα and ENA-78 expression in HUVECs. The cells were preincubated with various inhibitors same as described in Fig. 4 legend, followed by stimulation with 100 ng/ml of ML-1 for 24 hrs. The results are expressed as the mean ± SD (n=4 experiments). *$p<0.05$ was considered significant vs ML-1- stimulated cells.

Figure 6. Effect of Ro-31-7549 and LY294002 on GROα and ENA-78 expression. NHBEs and HUVECs were preincubated with 0.01-0.5 μM of Ro-31-7549 and 0.1-20 μM of LY294002 for 1 hr, followed by stimulation with 100 ng/ml of ML-1 for 24 hrs. The results are expressed as the mean ± SD (n=4). *$p<0.05$ was considered significant vs ML-1- stimulated cells.

Figure 1.

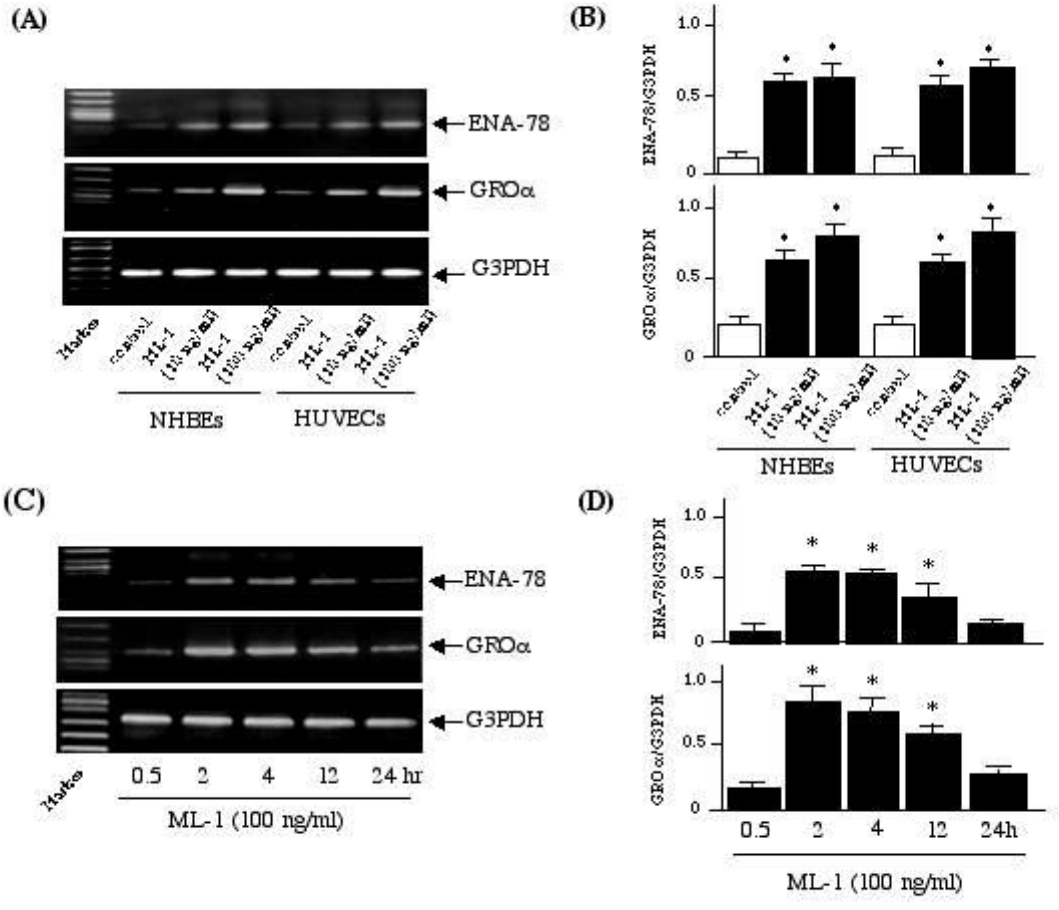

(A)
ENA-78
GROα
G3PDH
Marker
control
ML-1 (10 ng/ml)
ML-1 (100 ng/ml)
NHBEs
HUVECs
(B)
ENA-78/G3PDH
GROα/G3PDH
1.0
0.5
0
NHBEs
HUVECs
(C)
ENA-78
GROα
G3PDH
Marker
0.5
2
4
12
24 hr
ML-1 (100 ng/ml)
(D)
ENA-78/G3PDH
GROα/G3PDH
1.0
0.5
0
0.5
2
4
12
24h
ML-1 (100 ng/ml)

Figure 2.

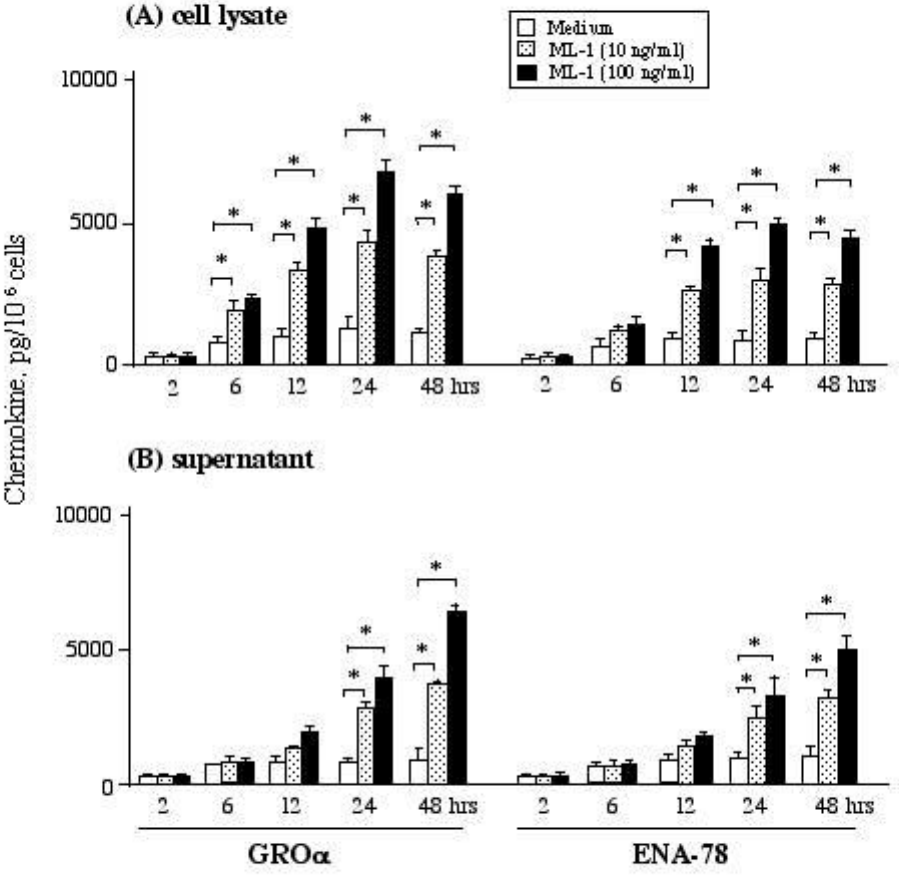

(A) cell lysate
Medium
ML-1 (10 ng/ml)
ML-1 (100 ng/ml)
Chemokine, pg/10 6 cells
10000
5000
0
2
6
12
24
48 hrs
(B) supernatant
GROα
ENA-78

Figure 3.

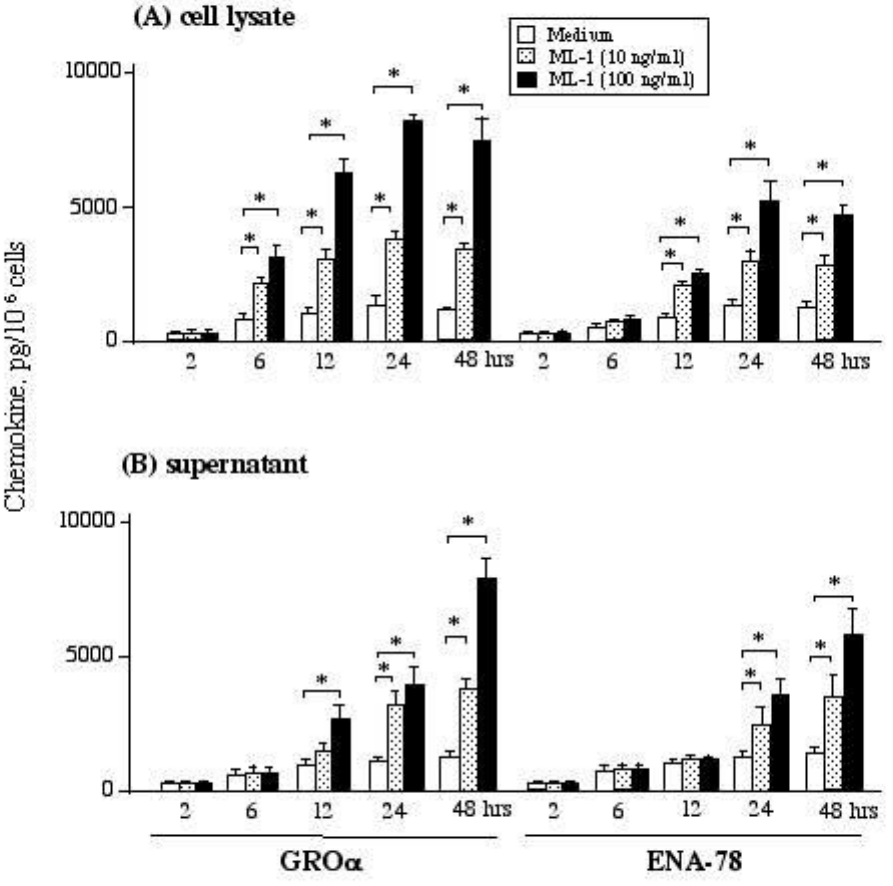

(A) cell lysate
Medium
ML-1 (10 ng/ml)
ML-1 (100 ng/ml)
Chemokine, pg/10 6 cells
10000
5000
0
2
6
12
24
48 hrs
(B) supernatant
GROα
ENA-78

Figure 4.

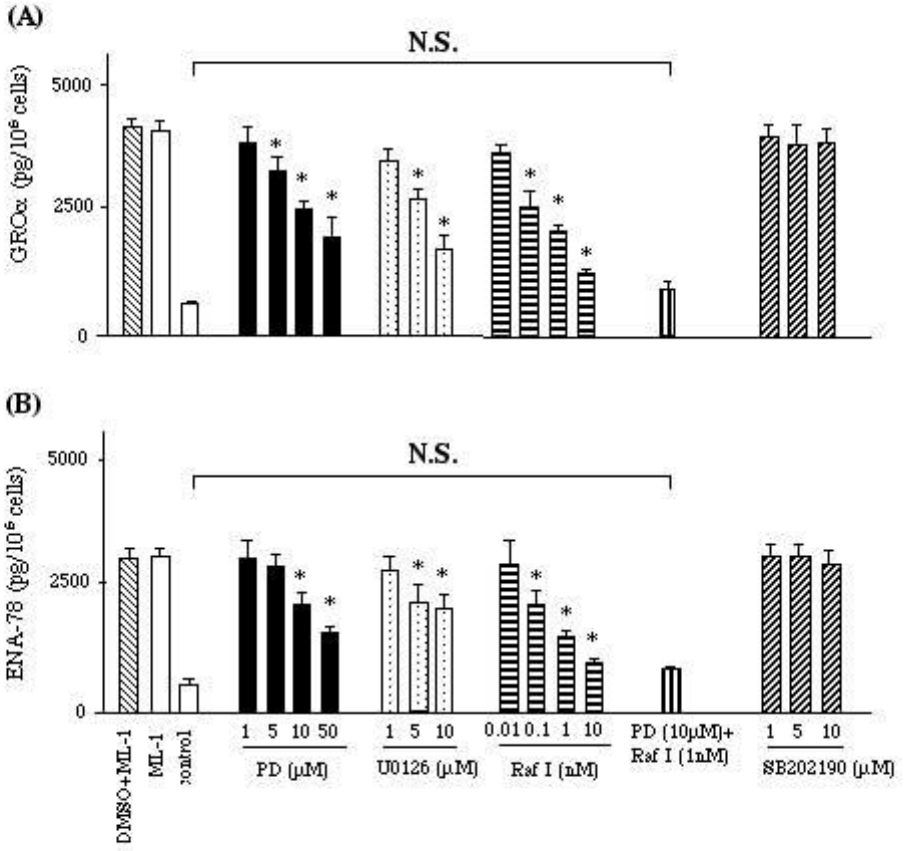

(A)
N.S.
GROα (pg/10⁶ cells)
5000
2500
0
(B)
N.S.
ENA-78 (pg/10⁶ cells)
5000
2500
0
DMSO+ML-1
ML-1
control
1 5 10 50
PD (μM)
1 5 10
U0126 (μM)
0.01 0.1 1 10
Raf I (nM)
PD (10μM)+ Raf I (1nM)
1 5 10
SB202190 (μM)

Figure 5.

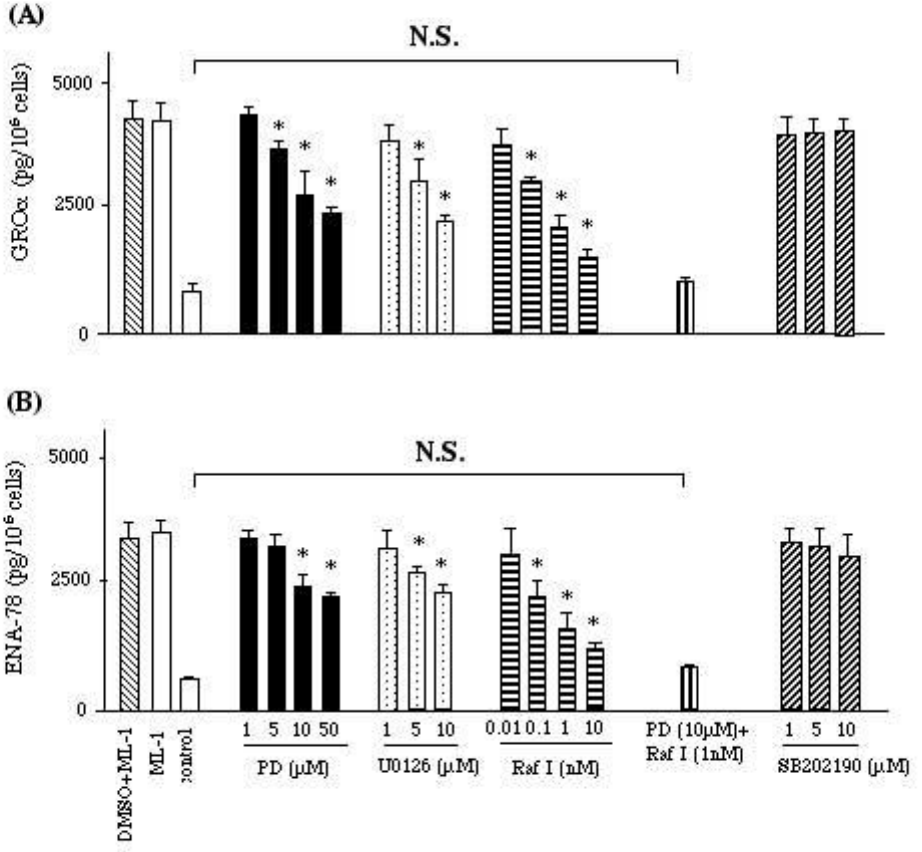
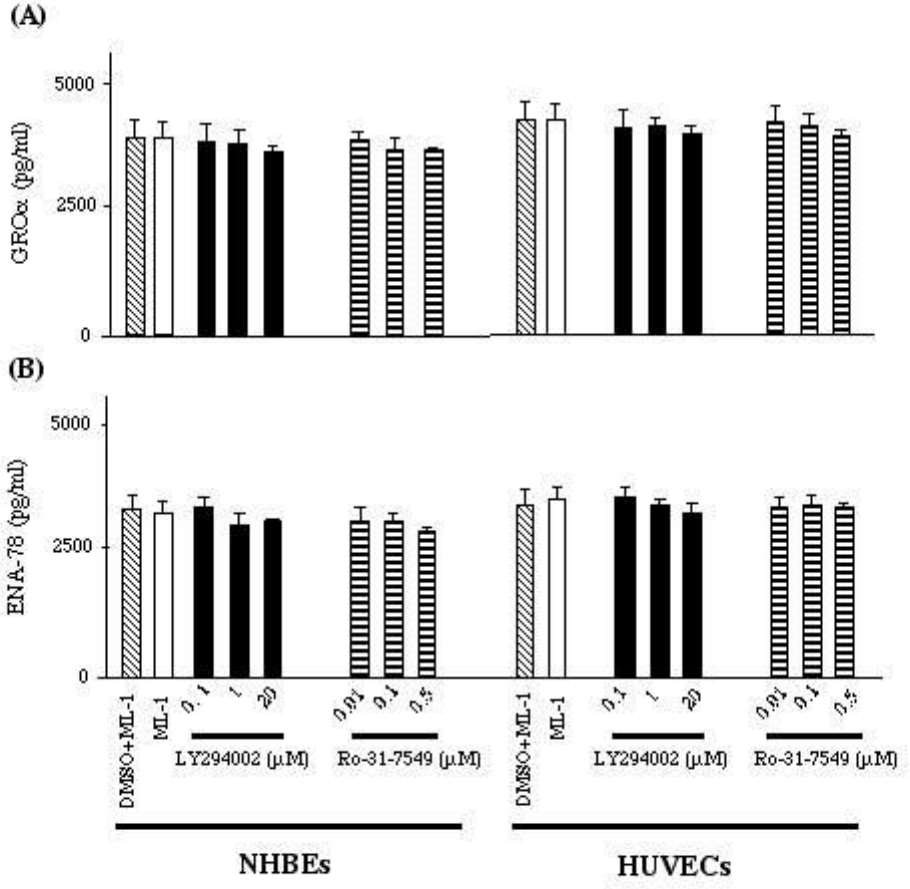

(A)
N.S.
GROα (pg/10⁶ cells)
5000
2500
0
(B)
N.S.
ENA-78 (pg/10⁶ cells)
5000
2500
0
DMSO+ML-1
ML-1
control
1 5 10 50
PD (μM)
1 5 10
U0126 (μM)
0.01 0.1 1 10
Raf I (nM)
PD (10μM)+ Raf I (1nM)
1 5 10
SB202190 (μM)

Figure 6.


(A)
GROα (pg/ml)
5000
2500
0
(B)
ENA-78 (pg/ml)
5000
2500
0
DMSO+ML-1
ML-1
0.1
1
20
0.01
0.1
0.5
LY294002 (μM)
Ro-31-7549 (μM)
NHBEs
HUVECs