Table 2 Summary of restraints and structural statistics

NOE distance restraints	
Total	2,289
$\|i-j\| = 0$	646
$\|i-j\| = 1$	603
$2 \le \|i-j\| < 5$	239
$\|i-j\| \ge 5$	801
Dihedral angle restraints	
ϕ	111
ψ	89
$\chi 1$	10
Hydrogen bonds[a]	50
Energies (kcal mol^{-1})[b]	
E_{total}	93.9 ± 0.85
E_{LJ}[c]	−590 ± 17
Maximum violation	
Distance (Å)	0.24
Angle (°)	0.94
R.m.s. deviation from ideal geometry	
Bonds (Å)	0.0011 ± 0.00006
Angles (°)	0.2831 ± 0.0023
Improper (°)	0.1193 ± 0.0054
Coordinate precision (residues 1–157)	
Backbone (Å)	0.940
Heavy (Å)	1.442
Coordinate precision (residues 2–33, 43–157)	
Backbone (Å)	0.570
Heavy (Å)	1.147
Ramachandran statistics (residues 1–157)	
Most favored (%)	78.5
Additionally (%)	18.9
Generously (%)	1.9
Disallowed (%)	0.7

Statistics are calculated for 20 of the lowest-energy structures of 100 calculated.

[a]Two distance restraints per hydrogen bond were used, providing a total of 100 restraints. [b]The default parameters and force constants of protein-allhdg.param and anneal.inp in CNS 1.1 were used for calculation. [c]The Lennard-Jones van der Waals energy was not used in the structure calculation.

A total of 50 mutant IL-18 proteins were prepared in the same manner as that of wild-type protein, in which each amino acid was replaced by alanine or glycine. Residues to be mutated were selected considering sequence alignments with IL-1 and IL-18 family members, comparison of the structures of IL-18, IL-1β (PDB entry 2I1B) and the IL-1β–IL-1RI complex (PDB entry 1ITB), and previously reported mutagenesis data for IL-1 molecules[22].

NMR spectroscopy. All NMR spectra were acquired at 303 K on a Bruker DMX500, DRX500 or DRX800 NMR spectrometer. For assignment of the ^{1}H, ^{13}C and ^{15}N resonances of the backbone and the side chains, a series of three-dimensional experiments were conducted[23]. The stereospecific assignment of methyl groups of the valine and leucine residues was carried out as described[24]. Distance restraints were obtained from ^{15}N-^{15}N-, ^{15}N-^{13}C- or ^{13}C-^{13}C-resolved 4D NOESY experiments with a mixing time of 100 ms (ref. 23).

Structure calculation. The obtained main chain resonance assignment indicated the presence of a minor conformation at or around the segment between strands S3 and S4, possibly as a result of *cis-trans* isomerization of the Ala42–Pro43 peptide bond. Differences of ^{1}H and ^{15}N chemical shifts between the major and minor conformations are relatively small. We obtained structure restraints for only the major conformation. Initially, structure calculation and NOE peak assignment were done in an iterative and manual manner using DYANA (version 1.5)[25]. Backbone torsion angle restraints were derived from $^{3}J_{HNH}\alpha$ of HNHA[23] and TALOS[26]. The torsion angles $\chi 1$ of Phe30, Phe47, Phe52, Phe83, Phe101, Phe102, Phe115, Phe124, Phe151, Tyr52 and His109 were estimated from $^{3}J_{C'C}$ and $^{3}J_{NC}$ coupling constants[27]. After determining the global fold manually, the CANDID algorithm was used to assign the remaining NOE peaks[28], yielding 2,289 meaningful NOE upper distance restraints. With these restraints, final structures were calculated using CNS (version 1.1)[29]. At this stage, hydrogen bond restraints from the slowly exchanging backbone amides were added as distance restraints of 2.8–3.4 Å for N–O and 1.8–2.4 Å for HN–O atom pairs, respectively. Non-stereospecifically assigned protons were treated as floating chirality and $\sum (r^{-6})^{-1/6}$ sum. A total of 100 structures were refined, and the 20 lowest-energy structures were analyzed using MOLMOL[30], AQUA and PROCHECK-NMR software[31] (Table 2).

IFN-γ induction and receptor binding assay. We prepared the following mutants of IL-18: Y1G, F2A, K4A, L5A, E6A, K8A, R13A, L15G, N16A, D17A, D23A, R27A, E31A, M33A, D35A, R39A, D40A, M51G, K53A, D54A, R58A, M60A, K67A, E69A, K70A, E77A, K79A, K84A, E85A, D90A, K93A, D94A, K96A, D98A, R104A, D110A, K112A, E121A, E128A, E130A, D132A, F134G, K135A, K139A, K140A, E141A, E143A, R147A, V153G and D157A. The wild-type and mutant IL-18 proteins were examined for their ability to induce IFN-γ production at 37 °C as described[32]. For each of the mutants that showed substantial reduction in activity, ^{15}N-labeled proteins were prepared and the ^{1}H-^{15}N-correlation spectrum at 30 °C was compared with that of the wild-type IL-18. All these mutants showed no evidence of mutation-induced disruptions of the native fold. The stability of the wild-type and mutant proteins, F2A, M33A, D35A, M60A, K79A and D98A, was further examined by measuring their circular dichroism spectra at 37 °C. Results suggest that the structural stability of these mutant proteins, except that of F2A, is similar to that of the wild-type protein at this temperature. Mutant F2A was omitted from the additional studies because of the possible destabilization of the structure.

In vitro affinities of the wild-type and mutant IL-18 for IL-18 receptors were measured at 25 °C by surface plasmon resonance experiments using BIACORE3000 (Pharmacia Biosensor AB). A specific binding surface was prepared by coupling the anti-human IgG Fc antibody (Rockland) to a CM5 sensor chip by the amine coupling method. Then, the recombinant IL-18Rα–Fc or IL-18Rβ–Fc chimera proteins (R&D Systems) were immobilized on chips. The coupling density was limited to 200 resonance units (RU). Samples of IL-18 were diluted in HBS–EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.0 mM EDTA, 0.005% (v/v) surfactant P-20). For the dissociation constant analysis of the wild-type and mutant IL-18 proteins to IL-18Rα, different amounts of the samples were injected over the sensor chip at 2 μl min^{-1} until the equilibrium phases were obtained. The sensor surface was regenerated by two 60-s pulses of 0.2 M glycine-HCl, pH 1.5. Dissociation constants were determined by Scatchard plot analysis. For evaluation of the trimeric complex–forming abilities, the IL-18 proteins were first passed over the sensor chips until the IL-18Rα binding sites were saturated, and then the same amount of the IL-18 proteins mixed with the IL-18Rβ protein (3.12–100 nM) was injected.

Modeling. Modeling of the IL-18 and IL-18Rα complex is based on the structure of IL-18 and the crystal structure of the IL-1β–IL-1RI complex (PDB entry 1ITB). The MOE (http://www.chemcomp.com) was used for model construction and structure verification. The coordinates of the NMR structure of IL-18 and the modeled structure of IL-18Rα were superimposed on the IL-1β–IL-1RI complex. The IL-18–IL-18Rα complex model was refined by the molecular dynamics calculation of 70 ps at 300 K with the AMBER 5.0 package (http://www.amber.ucsf.edu) using the all-atom force field. The final structure was additionally energy minimized.

Coordinates. Coordinates have been deposited in the Protein Data Bank (accession code 1J0S).

ACKNOWLEDGMENTS

We thank Y. Matsuo, T. Ikegami, T. Furuya and K. Kasahara for their technical assistance and K. Sukegawa and T. Fukao for critical reading of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 17 December 2002; accepted 13 August 2003
Published online at http://www.nature.com/naturestructuralbiology/

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Expression and Function of Toll-Like Receptors in Eosinophils: Activation by Toll-Like Receptor 7 Ligand[1]

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We investigated the expression of a panel of Toll-like receptors (TLRs) and their functions in human eosinophils. Eosinophils constitutively expressed TLR1, TLR4, TLR7, TLR9, and TLR10 mRNAs (TLR4 greater than TLR1, TLR7, TLR9, and TLR10 greater than TLR6). In contrast, neutrophils expressed a larger variety of TLR mRNAs (TLR1, TLR2, TLR4, TLR6, TLR8 greater than TLR5, TLR9, and TLR10 greater than TLR7). Although the expression levels in eosinophils were generally less prominent compared with those in neutrophils, eosinophils expressed a higher level of TLR7. Furthermore, among various TLR ligands (*S*-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-*N*-palmitoyl-Cys-Ser-(Lys)^4, poly(I:C), LPS, R-848, and CpG DNA), only R-848, a ligand of TLR7 and TLR8, regulated adhesion molecule (CD11b and L-selectin) expression, prolonged survival, and induced superoxide generation in eosinophils. Stimulation of eosinophils by R-848 led to p38 mitogen-activated protein kinase activation, and SB203580, a p38 mitogen-activated protein kinase inhibitor, almost completely attenuated R-848-induced superoxide generation. Although TLR8 mRNA expression was hardly detectable in freshly isolated eosinophils, mRNA expression of TLR8 as well as TLR7 was exclusively up-regulated by IFN-γ but not by either IL-4 or IL-5. The up-regulation of the TLRs by IFN-γ had potentially functional significance: the extent of R-848-induced modulation of adhesion molecule expression was significantly greater in cells treated with IFN-γ compared with untreated cells. Although the natural ligands for TLR7 and TLR8 have not yet been identified, our results suggest that eosinophil TLR7/8 systems represent a potentially important mechanism of a host-defensive role against viral infection and mechanism linking exacerbation of allergic inflammation and viral infection. *The Journal of Immunology,* **2003, 171: 3977–3982.**

Massive accumulation of eosinophils is a characteristic feature of inflammation associated with allergic diseases such as bronchial asthma and atopic dermatitis. Infiltrating eosinophils are strongly implicated in the pathogenesis of these disorders by virtue of their capacity to release an array of tissue-damaging mediators (1). Although Ag-mediated immune responses are central mechanisms directing eosinophilic inflammation, several lines of evidence have indicated that bacterial and/or viral infections also modulate allergic inflammation. For example, viral or bacterial infections often precede asthma exacerbation in both children and adults (2, 3). Exacerbation of atopic dermatitis is associated with infections by bacteria, such as *Staphylococcus aureus* (4). The link between infection and exacerbation of allergic diseases may consist of various pathways having multiple steps, but direct activation of eosinophils by microbe-derived molecules potentially represents one clear explanation of such mechanisms.

As functionally important receptors for recognition of conserved motifs in pathogens termed pathogen-associated molecular patterns (PAMPs)[3] (5), the Toll-like receptors (TLRs) have been identified in mammals. TLRs share the Toll/IL-1R homology domain, and 10 different human TLR proteins have been identified and cloned to date (6–10). Expression of TLR4 in eosinophils has been reported by several groups (11, 12), but the precise expression profiles and functions of other TLRs have remained largely unclear. In this study, we explored TLR expression and their functions in eosinophils, and we have shown that a TLR7 ligand, R-848 (13), is capable of activating eosinophils.

Materials and Methods

Reagents

The following reagents were purchased as indicated: *S*-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-*N*-palmitoyl-Cys-Ser-(Lys)^4 (Pam3CSK^4; EMC Microcollections, Tubingen, Germany); poly(I:C), LPS (*Escherichia coli*, 026:B6), cytochrome *c*, superoxide dismutase (Sigma-Aldrich, St. Louis, MO); PMA (Calbiochem, San Diego, CA); rIL-4, rIL-5 (PeproTech, Rocky Hill, NJ); rIFN-γ (Shionogi Pharmaceutical, Osaka, Japan), and SB203580 (Promega, Madison, WI). R-848 was synthesized in Pharmaceuticals and Biotechnology Laboratory, Japan Energy Corporation (Saitama, Japan). R-848 was dissolved in DMSO to yield a stock solution of 10 mM, and corresponding concentrations of DMSO was used as controls in every experimental setting. Phosphorothioate-stabilized CpG DNA 2006 (TCGT CGTTTTGTCGTTTTGTCGTT) was purchased from Hokkaido System Science (Sapporo, Japan).

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Received for publication April 24, 2003. Accepted for publication August 11, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants-in-aid from the Ministry of Health, Welfare and Labor of Japan, and the Ministry of Education, Science, Sports and Culture of Japan (to K.H.). H.N. is a Research Fellow of the Japan Society for the Promotion of Science.

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[3] Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; Pam3CSK^4, *S*-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-*N*-palmitoyl-Cys-Ser-(Lys)^4; MESF, molecules of equivalent soluble fluorochrome units; MAPK, mitogen-activated protein kinase.

0022-1767/03/$02.00

Cell preparation and culture conditions

Eosinophils were isolated by density centrifugation followed by negative selection using anti-CD16-bound micromagnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) from normal volunteers, as previously described (14). Neutrophils were purified by two-step density centrifugation, as previously described (15). Both cells were further purified by negative selection using anti-CD14-bound micromagnetic beads (Miltenyi Biotec). Anti-CD14-bound micromagnetic beads neither inhibited nor stimulated the LPS-induced IL-8 generation by neutrophils (data not shown). Cytospun preparations were stained with May-Gruenwald-Giemsa, and differential counts were determined. Purity of eosinophils and neutrophils were >99% and >98.6% ± 0.4%, respectively, and their viability was consistently >95%. In some experiments, PBMC purified by Ficoll-Paque (Pharmacia, Uppsala, Sweden) from venous blood were subjected to positive selection using anti-CD14-bound micromagnetic beads. Purity of $CD14^+$ monocytes was 98.2 ± 0.3%.

Unless otherwise stated, eosinophils and neutrophils were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS (Sigma-Aldrich) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO_2 in a total volume of 200 μl in flat-bottom 96-well culture plates (Iwaki Glass, Chiba, Japan).

Real-time quantitative PCR analysis of TLR mRNA

Total RNA was extracted from 3–10 × 10^5 cells using a Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany), and the first-strand cDNA was reverse-transcribed as previously described (14). The purity of eosinophil preparations was consistently >99.8%. Real-time PCR was performed as previously described (15). Briefly, the TLR gene-specific primers and FAM-labeled probes were purchased from Applied Biosystems (Foster City, CA) and their sequences are presented in Table I. Input cDNA was normalized using a β-actin primer/probe pair (Applied Biosystems) as an internal control gene. The standard curve was constructed with serial dilutions of specific PCR products, which were obtained by amplifying PBMC cDNA. The amplification efficacy ($E = 10^{-1/slope}$) was calculated from the slope of the standard curve (16), and the primer sets used had high efficacy of between 1.89 and 1.96. The correlation coefficient of the standard curve was always >0.997.

Flow cytometry

Flow cytometric analysis was performed as previously described (14). Briefly, purified cells were stained with FITC-conjugated anti-CD11b mAb (Bear 1; Beckman Coulter, Tokyo, Japan) or PE-conjugated anti-L-selectin mAb (DREG-56; eBioscience, San Diego, CA). To investigate TLR expression, cells were incubated with anti-TLR1 mAb (clone GD2.F4; eBioscience), anti-TLR2 mAb (clone TL2.1; eBioscience), or anti-TLR4 mAb (clone HTA125; eBioscience), followed by the staining with FITC-labeled goat $F(ab')_2$ against mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Stained cells were analyzed using EPICS XL SYSTEM II (Coulter, Miami, FL). At least 3000 cells were assessed to calculate the median value of fluorescence intensity. The median values of fluorescence intensity of cells were converted to the number of molecules of equivalent soluble fluorochrome units (MESF) using a Quantum Fluorescence kit (Sigma-Aldrich) on each day of an experiment, as previously described (17). Surface receptor levels expressed in MESF units were calculated using the following formula: ΔMESF = (MESF of cells stained with anti-adhesion molecule mAb) − (MESF of cells stained with isotype control IgG).

Quantification of cell survival

Analysis of cell survival was performed as previously described (14). Briefly, live cells were quantitatively determined by negative staining for annexin V and propidium iodide.

Superoxide generation

Generation of superoxide by eosinophils was measured by superoxide dismutase-inhibitable reduction of cytochrome *c*, as previously described with slight modification (18). In brief, purified cells were resuspended in HBSS (Life Technologies) with 0.1% gelatin and 0.1% BSA. The reaction wells of 96-well culture plates were measured for absorbance at 550 nm in an ELISA reader (Bio-Rad, Hercules, CA). Superoxide generation was calculated with an extinction coefficient of 21.1 × 10^3 $l \cdot mol^{-1} \cdot cm^{-1}$ for reduced cytochrome *c* at 550 nm and was expressed as nanomoles of cytochrome *c* reduced per 1 × 10^6 cells.

Western blot analysis

Purified eosinophils were lysed with ice-cold lysis buffer containing 1% Nonidet P-40, 25 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1 mM EDTA, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM leupeptin, and 1 mM PMSF. Whole-cell lysates were subjected to SDS-PAGE (10% polyacrylamide) and then electrically transferred to Hybond ECL membranes (Amersham, Chalfont St. Giles, U.K.). After blocking, the membranes were incubated with anti-p38 MAPK Ab or anti-phosphorylated-p38 MAPK Ab (Cell Signaling, Beverly, MA) for 2 h at room temperature. The membranes were then incubated with HRP-conjugated anti-rabbit IgG Ab (DAKO, Glostrup, Denmark) for 30 min. The reactive bands were visualized with an ECL system (Amersham).

Statistical analysis

All data are expressed as the mean ± SEM. Unless otherwise noted, the differences between values were analyzed by the one-way ANOVA test. When this test indicated a significant difference, Fisher's protected least significant difference test was used to compare individual groups.

Results

TLR mRNA expression in eosinophils

Previous studies demonstrated positive TLR4 mRNA and controversial TLR2 mRNA expression in eosinophils (11, 12), but expression of other TLR mRNAs has not been investigated to date. In our first series of experiments, by using real-time PCR, we quantified the expression levels of transcripts of TLR1–10 in eosinophils in comparison with neutrophils. As shown in Fig. 1, eosinophils expressed transcripts of TLR1, TLR7, TLR9, and TLR10 in addition to TLR4. Although weak expression of TLR6 mRNA was observed in eosinophils, expression of TLR2, TLR3, TLR5, and TLR8 mRNAs was virtually undetectable. Previous studies showed that neutrophils express TLR1, TLR2, and TLR4 (12, 19). In addition to these transcripts, we found apparent expression of mRNAs for TLR5, TLR6, TLR8, TLR9, and TLR10 in neutrophils. A low level of TLR7 mRNA was also detected. When the expression levels of TLRs were compared between eosinophil and

Table I. *Primers and probes used for real-time quantitative PCR*[a]

	Forward Primer	Probe	Reverse Primer
TLR1	TTCACAGTGTCTGGTACACGCAT	TGCCCATCCAAAATTAGCCCGTTCC	ACCGTGTCTGTTAAGAGATTATTGGA
TLR2	GAAGAATCCTCCAATCAGGCTTC	CTGTCTTGTGACCGCAATGGTATCTGCA	GCCCTGAGGGAATGGAGTTT
TLR3	GCCGCCAACTTCACAAGGTATA	AGCCAGAATTGTGCCAGAAACTTCCCA	AGCTCATTGTGCTGGAGGTTC
TLR4	AATCTAGAGCACTTGGACCTTTCC	TGCGGGTTCTACATCAAATGCCCCTAC	GGGTTCAGGGACAGGTCTAAAGA
TLR5	CCATAGATTTTTCCTCCAACCAAATA	CTCGAGCCCCTACAAGGGAAAACGCT	TCATACATTTTCCCCAGTCCACT
TLR6	CATCCTATTGTGAGTTTCAGGCAT	TCAATGATTTCAAGGCCCTGCCCA	GCTTCATAGCACTCAATCCCAAG
TLR7	GGAGGTATTCCCACGAACACC	CGAACCTCACCCTCACCATTAACCACAT	TGACCCCAGTGGAATAGGTACAC
TLR8	AAACTTGACCCAACTTCGATACCTAA	CCCTCAGGAAGATTAATGCTGCCTGGTT	GATCCAGCACCTTCAGATGAGG
TLR9	GGACCTCTGGTACTGCTTCCA	ACGATGCCTTCGTGGTCTTCGACAAA	AAGCTCGTTGTACACCCAGTCT
TLR10	AAGAAAGGTTCCCGCAGACTT	ACCCCAGCCACAACGACACTGGAT	TGTTATGGCATAGAATCAAAACTCTCA

[a] All sequences are presented in the 5′–3′ direction.

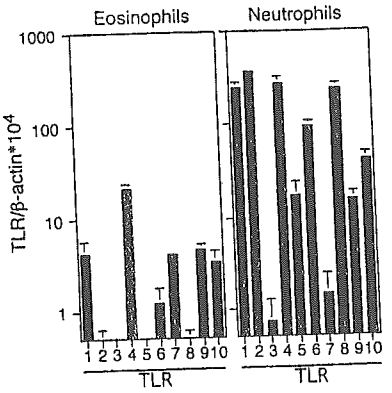

FIGURE 1. Quantitative analysis of TLR1–10 mRNA expression in eosinophils and neutrophils. The data are expressed as a ratio: copy number of TLR gene/copy number of β-actin gene ($n = 4$).

neutrophils, eosinophils generally expressed lower levels of TLR mRNA compared with neutrophils except for TLR7.

By using established Abs against TLRs, we studied the surface expression of TLR1, TLR2, and TLR4 on eosinophils. Consistent with a previous report by other researchers (12), our results of flow cytometry experiments demonstrated the absence of surface expression of TLR2 and TLR4 on eosinophils. We observed marginal TLR1 expression in one of three samples, but the expression was undetectable in the other samples ($n = 3$, data not shown).

Eosinophil activation by PAMPs

In the next series of experiments, we studied the functional properties of eosinophil TLRs. For this purpose, we investigated the effect of various PAMPs on several eosinophil functions, i.e., adhesion molecule expression, survival, and superoxide generation. As shown in Fig. 2, *A* and *B*, CD11b expression was up-regulated and L-selectin expression was conversely down-regulated by an antiviral reagent, R-848, a ligand of TLR7. On the other hand, other PAMPs, including Pam3CSK^4 (TLR2) (20), poly(I:C) (TLR3) (21), LPS (TLR4) (22) and CpG DNA (TLR9) (23, 24), exhibited no significant effects at all. Dose-dependent experiments revealed that 1 μM of R-848 was sufficient to affect the expression of CD11b and L-selectin in eosinophils (Fig. 2, *C* and *D*). In contrast, a higher dose of R-848 was required to show a significant effect on either CD11b or L-selectin expression in neutrophils.

The effect of PAMPs on eosinophil survival is exhibited in Fig. 3*A*. The results of survival enhancement were virtually identical with those of adhesion molecule expression. R-848 significantly prolonged in vitro eosinophil survival. Although LPS slightly enhanced eosinophil survival, the effect did not reach statistical significance. Other TLR ligands showed no significant effect at all. The dose-response curves of survival paralleled those of adhesion molecule expression (Fig. 3*B*). Although R-848 prolonged neutrophil survival, a higher dose of R-848 was required to exert a significant effect.

As shown in Fig. 4*A*, R-848 induced superoxide generation in eosinophils. Again, none of the other TLR ligands elicited significant superoxide production at all. Neutrophil superoxide generation was also induced by R-848, but the generation was retarded when compared in eosinophils; statistically significant generation was observed only after 180 min of incubation (Fig. 4*B*).

Activation of p38 MAPK in eosinophils stimulated by R-848

To verify that intracellular signaling pathways of eosinophils are actually activated by R-848, we investigated the phosphorylation of p38 MAPK, which has been demonstrated to be a hallmark of

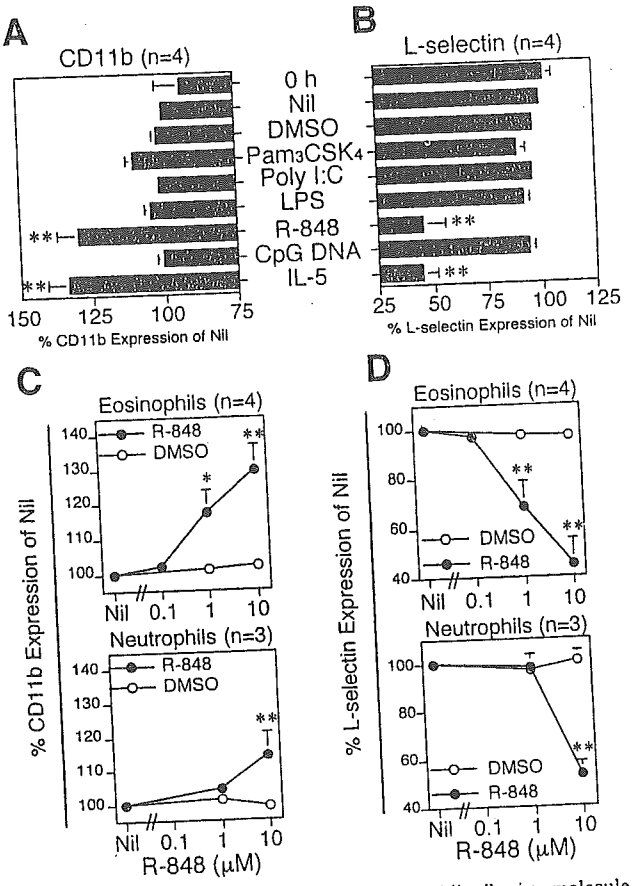

FIGURE 2. The effect of PAMPs on eosinophil adhesion molecule expression. CD11b (*A*) or L-selectin (*B*) expression was analyzed in eosinophils just after purification (0 h) or in cells incubated for 90 min with PAMPs as indicated. The data are expressed as the percentage of the calculated ΔMESF values of eosinophils cultured for 90 min without stimulus (Nil = 100%). The concentrations of reagents used were as follows: Pam3CSK^4 (10 μg/ml); poly(I:C) (50 μg/ml); LPS (10 μg/ml); R-848 (10 μM); CpG DNA (2 μM); IL-5 (1 nM); and DMSO (0.1%). Dose-dependent effect of R-848 on CD11b (*C*) or L-selectin (*D*) expression after 90-min incubation. **, $p < 0.01$, *, $p < 0.05$ vs value of Nil or DMSO (for R-848) (*A* and *B*), and vs value in cells treated with corresponding concentration of DMSO (*C* and *D*).

cellular activation via TLRs (25). As shown in Fig. 5*A*, stimulation of eosinophils with R-848 resulted in apparent phosphorylation of p38 MAPK after 15 min of stimulation. Furthermore, p38 MAPK functionally regulated the R-848-induced superoxide generation in eosinophils; a p38 MAPK inhibitor, SB203580, almost completely attenuated the superoxide generation in eosinophils (Fig. 5*B*).

Cytokine-mediated regulation of TLR mRNA expression in eosinophils

In the last series of experiments, we examined the modulation of eosinophil TLR expression by eosinophil-active cytokines of both Th1 and Th2 origin. Eosinophils were stimulated with single concentrations of cytokines based on our previous study of CXCR4 expression in eosinophils (14). As shown in Fig. 6*A*, expression of transcripts of TLR2 and TLR3 remained at low levels in eosinophils stimulated for 4 h with IL-4, IL-5, or IFN-γ. These cytokines also failed to induce significant changes in the expression level of TLR9. On the other hand, TLR4 mRNA expression was increased 2- to ~3-fold by IFN-γ with statistical significance. However, the most prominent change in expression was observed for both TLR7

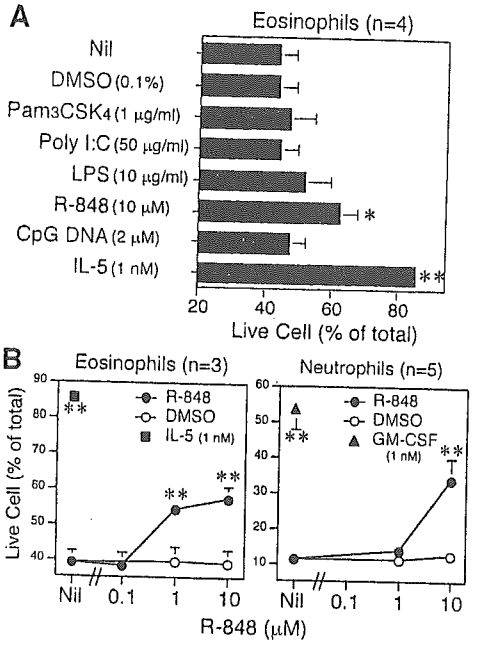

FIGURE 3. The effect of PAMPs on eosinophil survival. *A*, Eosinophils were cultured for 20 h in the presence or absence of PAMPs, and the viability was analyzed by flow cytometry. The data are expressed as the percentage of live cells of total inoculated cells. *B*, Dose-dependent effect of R-848 on survival of 20-h cultured eosinophils or 15-h cultured neutrophils. **, $p < 0.01$, *, $p < 0.05$ vs value of Nil or DMSO (for R-848) (*A*), and vs value in cells treated with corresponding concentration of DMSO (*B*).

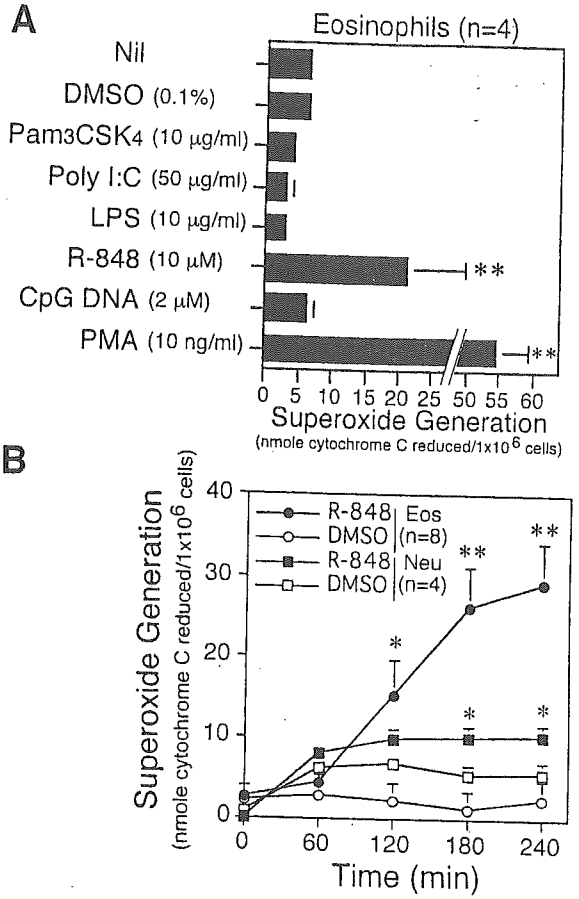

FIGURE 4. Effect of PAMPs on superoxide generation in eosinophils. *A*, The effect of PAMPs on superoxide generation in eosinophils after 180-min incubation. **, $p < 0.01$ vs value of Nil or DMSO (for R-848). *B*, The time course of superoxide generation. The difference between superoxide generation in R-848-treated (10 μM) and DMSO-treated (0.1%) cells at the same time point was tested by the paired *t* test (**, $p < 0.01$, *, $p < 0.05$).

and TLR8. IFN-γ exclusively and strongly (~10-fold) up-regulated the expression of TLR7 mRNA in eosinophils. Despite negligible expression in resting eosinophils, de novo expression of TLR8 at levels comparable to TLR7 was observed in IFN-γ-treated cells.

To determine whether the IFN-γ-induced up-regulation of the expression of TLR7 and TLR8 affects the biologic response to the ligand, we compared R-848-induced changes in adhesion molecules in cells cultured for 4 h in the presence and absence of IFN-γ. As shown in Fig. 6*B*, the extent of increase in CD11b expression as well as decrease in L-selectin expression was significantly greater in cells treated with IFN-γ compared with untreated cells, suggesting functional relevance of IFN-γ-mediated up-regulation of the expression of TLR7 and/or TLR8 in eosinophils.

Discussion

The expression profiles of TLRs as well as their functions have been increasingly clarified in various immune cells (26), but little information is available for eosinophils except for TLR4 (12). In the present study, we for the first time examined the mRNA expression of a panel of TLRs in eosinophils and found that eosinophils constitutively expressed mRNA of TLR1, TLR7, TLR9, and TLR10 in addition to TLR4. The expression profile in eosinophils was qualitatively and quantitatively different from that in neutrophils. In contrast to eosinophils, neutrophils expressed almost all TLR mRNAs. The ubiquitous expression profile in neutrophils may reflect their important role as first-line effector cells in host defense. Furthermore, eosinophil TLR mRNA expression was generally weak compared with in neutrophils. Eosinophils expressed ~10 and ~50 times lower levels of TLR4 and TLR1, respectively, compared with neutrophils.

The TLR4 ligand LPS represents the most extensively studied PAMP, but previous reports have shown conflicting results regarding the effects of LPS on human eosinophils. Initial studies indicated that LPS was able to activate eosinophils and prolong their life span (11, 27). However, more recent studies demonstrated that LPS was totally inactive when monocytes were depleted from eosinophil preparations (12, 28). The results of our present experiments using $CD14^+$ cell-depleted preparations showed that LPS did not affect any of the tested eosinophil functions, lending further support to the concept that LPS is totally inactive on eosinophils in the absence of monocytes. The absence of functional significance of LPS on eosinophils results from the lack of surface protein expression of TLR4 in these cells. Consistent with a previous report (12), we found no significant protein expression of TLR4 on the eosinophil surface, even though they expressed transcripts of TLR4. Thus, mRNA expression of TLRs does not always guarantee the expression of functional proteins. Furthermore, it is also likely that eosinophils have impaired machinery for transporting TLR4 proteins to the cell surface, such as MD-2 (29).

Expression of TLR transcripts in eosinophils was generally less prominent compared with neutrophils, but a higher level of TLR7 mRNA expression was observed in eosinophils. Because specific Abs for exploring the protein expression of TLR7 are not available at present, and mRNA expression does not definitively guarantee the expression of a functional protein, we should be careful to conclude that there is expression of TLR7 on the cell surface without any protein data. However, our results of functional studies strongly suggested the expression of functional TLR7 on eosinophils. Among various TLR ligands tested, only R-848, a ligand of

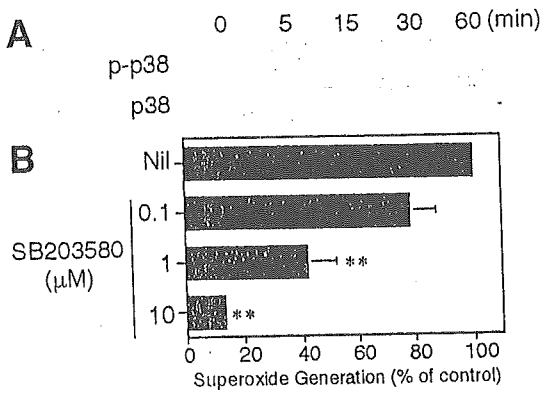

FIGURE 5. Activation of p38 MAPK in eosinophils stimulated with R-848. *A*, R-848 (30 μM) was added to eosinophils (1×10^6 cells per sample) for the indicated times, and Western blotting was performed using Abs against phosphorylated or nonphosphorylated forms of p38 MAPK. The data shown are representative of three independent experiments showing similar results. *B*, Eosinophils were preincubated with serially diluted SB203580 at 37°C for 10 min, further incubated for 180 min with R-848 (10 μM), and superoxide generation was determined. Data are indicated as the percentage of superoxide generation in eosinophils without pretreatment with SB203580 (Nil = 100%). **, $p < 0.01$ vs Nil.

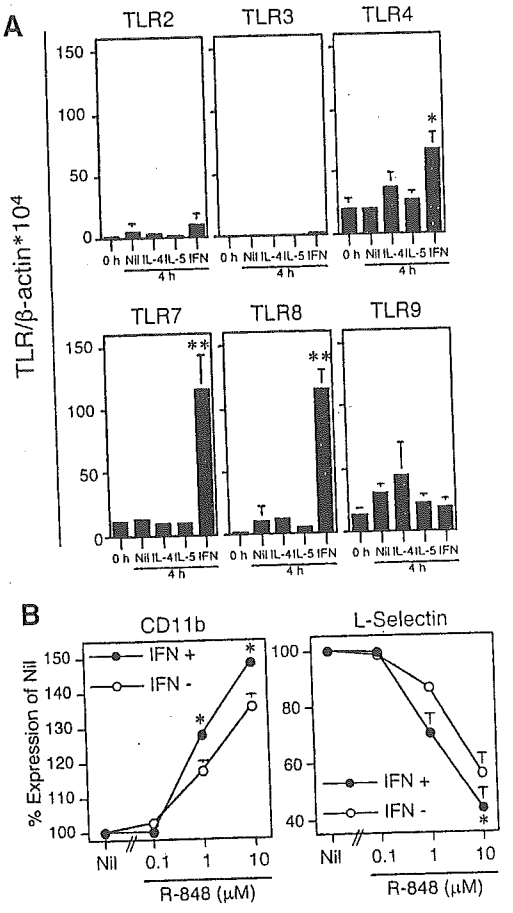

FIGURE 6. Modulation of expression and function of eosinophil TLR by cytokines. *A*, The level of TLR mRNA expression was determined by real-time PCR in eosinophils just after purification (0 h), or in cells cultured for 4 h with IL-4 (10 ng/ml), IL-5 (10 ng/ml), IFN-γ (300 U/ml) or medium alone (Nil). The data are expressed as a ratio: copy number of TLR gene/copy number of β-actin gene. **, $p < 0.01$, *, $p < 0.05$ vs value of Nil ($n = 3$). *B*, The levels of CD11b and L-selectin expression were analyzed in eosinophils cultured for 4 h in the presence or absence of IFN-γ followed by stimulation with R-848 for 90 min. The data are expressed as the percentage of the calculated ΔMESF values of eosinophils cultured without R-848 (Nil = 100%). The difference between the adhesion molecule expression in IFN-γ-treated (300 U/ml) or nontreated cells stimulated with the same concentration of R-848 was tested by the paired *t* test (*, $p < 0.05$, $n = 3$).

both TLR7 and TLR8 (13, 30), was active on freshly isolated eosinophils in modulation of adhesion molecule expression, prolongation of survival, and induction of superoxide generation. In addition, R-848 induced phosphorylation of p38 MAPK, known to be involved in signal transduction via various TLRs (25), while inhibition of p38 MAPK resulted in almost complete attenuation of R-848-induced superoxide generation in eosinophils. R-848 is a low molecular weight compound of the imidazoquinoline family with antiviral properties (31). The specificity of R-848 for human TLR7 and TLR8 has been confirmed by genetic complementation by several groups (13, 30, 32). Activation of HEK293 cells by R-848 was observed when cells were transfected with only human TLR7 or TLR8 but not with human TLR2, TLR3, or TLR9 (32). Although R-848 has been shown to activate both TLR7 and TLR8, TLR7 shows 10-fold higher sensitivity to R-848 (30). Furthermore, eosinophils constitutively expressed considerable levels of TLR7 mRNA, whereas TLR8 mRNA was hardly detectable in freshly isolated cells. Taken together, these findings strongly suggest that TLR7 is mainly responsible for R-848-induced activation of resting eosinophils. However, the fact that TLR8 mRNA expression became apparent during stimulation with IFN-γ suggests the possible involvement of TLR8 in eosinophil activation under certain conditions. Future development of selective blocking Abs or antagonists against TLR7 and TLR8 will clarify this issue.

The concentration of R-848 required for activation of eosinophils (1 μM) is high compared with the concentrations reported for other types of cells such as dendritic cells (0.01–0.1 μM) (33, 34). We suppose that the difference in the concentrations potentially results from differences in the expression levels of TLR7 among cell types. We observed that monocytes expressed ~15 times higher levels of TLR7 than eosinophils (59.13 ± 12.64 vs 4.13 ± 0.37, indicated as TLR7 mRNA copies/β-actin copies $\times 10^4$, $n = 4$). Several studies have shown that dendritic cells express even higher levels of TLR7 mRNA compared with monocytes (33, 35, 36). Although the precise differences in surface TLR7 protein levels among these cell types are not fully known, it could be reasonably stated that relatively higher concentrations of agonist are required to occupy enough receptor molecules in eosinophils with a low level of TLR7 expression.

The natural ligands of TLR7 and TLR8 have still not been identified, but they are strongly assumed to be either some viral compounds or endogenous molecules generated during viral infections. To date, several lines of evidence have revealed that eosinophils express binding sites for several viruses, such as ICAM-1 (37), but little is known regarding the direct activation of eosinophils by viruses or their products. In this context, TLR7/8 systems may represent unique and potentially important mechanisms of eosinophil activation, linking viral infection with exacerbation of allergic inflammation. In fact, expression of TLR7 and TLR8 was up-regulated exclusively by IFN-γ but not by either IL-4 or IL-5. Although we cannot fully exclude the possibility that modulation of CD11b expression could be dependent upon actions of IFN-γ after up-regulation of TLR7, the apparent up-regulation of TLR7/8 mRNA may support the biological relevance of TLR7/8 systems under conditions associated with viral infections.

In summary, we have comprehensively investigated expression of mRNAs of TLRs and their functions in eosinophils. TLR expression in eosinophils was generally less prominent compared

with neutrophils, but a higher level of TLR7 expression was observed in eosinophils. TLR7 expression as well as TLR8 expression was up-regulated by treatment with IFN-γ, and R-848, a ligand of both TLR7 and TLR8, exclusively activated eosinophil functions. Stimulation of eosinophils via TLR7 and/or TLR8 may be involved in the exacerbation of allergic inflammation during viral infection. Furthermore, because eosinophil-derived neurotoxin and eosinophil cationic protein potentially exert antiviral effects because of their strong RNase activities (38, 39), these receptors may also play a host-defensive role against viral infection.

Acknowledgments

We thank Chise Tamura and Masako Imanishi for their excellent technical assistance. Thanks are also extended to Sachiko Takeyama for valuable secretarial help.

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Differential regulation of eotaxin expression by IFN-γ in airway epithelial cells

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Background: Eotaxin is a chemokine that binds with high affinity and specificity to the chemokine receptor CCR3 and plays an important role in the pathogenesis of allergic disease.
Objective: We studied the regulation of eotaxin expression by the T_H1 cytokine IFN-γ and analyzed its molecular mechanisms.
Methods: Levels of eotaxin mRNA and protein expression in the airway epithelial cell line BEAS-2B were determined with RT-PCR and ELISA. Mechanisms of transcriptional regulation were assessed by means of electrophoretic mobility shift assays and luciferase assay with eotaxin promoter–luciferase reporter plasmids.
Results: Although IFN-γ did not directly induce the expression of eotaxin protein, it increased the induction by TNF-α when these cytokines were added simultaneously. In contrast, preincubation of cells with IFN-γ for 24 hours profoundly inhibited the production induced by TNF-α. IFN-γ did not influence the TNF-α–induced binding of nuclear factor κB to a DNA probe derived from the eotaxin promoter. IFN-γ did not increase the ability of TNF-α to activate the eotaxin promoter. Studies of eotaxin mRNA levels indicate that IFN-γ combined with TNF-α increased the expression of eotaxin mRNA. When cells were preincubated with IFN-γ, there was no inhibition of the appearance of eotaxin mRNA.
Conclusion: These studies demonstrate that IFN-γ enhances eotaxin expression when added in combination with TNF-α and profoundly inhibits eotaxin expression after preincubation. In both cases the available data indicate that the effect is mediated by a posttranscriptional mechanism. (J Allergy Clin Immunol 2003;111:1337-44.)

Key words: Eotaxin, IFN-γ, epithelial cells, asthma

Abbreviations used
EMSA: Electrophoretic mobility shift assay
γIRE: IFN-γ-responsive element
IRF: IFN regulatory factor
NF-κB: Nuclear factor κB
STAT: Signal transducer and activator of transcription

Asthma is a disease characterized by the infiltration of eosinophils and lymphocytes into airway epithelium and subsequent epithelial damage and tissue remodeling. T_H2 cells and their cytokine products play a crucial role in this process.[1,2] Eotaxin is a C-C chemokine that was identified by means of the purification of an eosinophil chemoattractant from bronchoalveolar lavage fluid of ovalbumin-sensitized and ovalbumin-challenged guinea pigs.[3] CCR3 is a specific receptor for eotaxin and is expressed on important cells in allergic disease, such as eosinophils, basophils, a subset of T_H2 cells, and mast cells.[4-8] Eotaxin has been reported to be highly expressed in the epithelium of asthmatic patients and might play an important role in the infiltration of these cells into the airways. Although many different cell types can express eotaxin, airway epithelial cells are a major cell source of eotaxin in the airway.[9,10]

Previous reports have demonstrated that TNF-α and IL-4 synergistically stimulate eotaxin expression in epithelial cells and fibroblasts.[11-14] A similar synergistic effect on the induction of eotaxin expression in airway epithelial cells has been observed with TNF-α and IL-13.[14] Upregulation of eotaxin expression by TNF-α and IL-4/IL-13 might be expected to occur in asthma because these cytokines are known to be produced in asthma and have been implicated in the pathogenesis of asthma. We have demonstrated that the eotaxin promoter has adjacent binding sites for nuclear factor κ B (NF-κB) and signal transducer and activator of transcription 6 (STAT-6), and these 2 transcription factors activate eotaxin transcription and subsequent mRNA-protein induction in response to TNF-α and IL-4/IL-13 stimulation, respectively.[13,14]

It has been reported that IFN-γ can potentiate eotaxin expression induced by TNF-α in airway epithelial cells.[12,15,16] On the other hand, recent reports showed that IFN-γ inhibits eotaxin expression in fibroblasts.[17,18]

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Supported by the Pollution-Related Health Damage Compensation and Prevention Association, AstraZeneca Asthma Research Foundation, and National Institutes of Health grants AI44885 and HL68546.
Received for publication October 21, 2002; revised February 9, 2003; accepted for publication March 4, 2003.
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0091-6749/2003 $30.00 + 0
doi:10.1067/mai.2003.1513

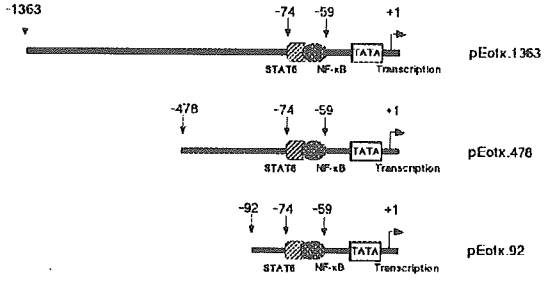


FIG 1. Structures of the eotaxin promoter–luciferase reporter plasmids containing 1363, 478, and 92 bp of the eotaxin promoter region (referred to as pEotx.1363, pEotx.478, and pEotx.92, respectively) are depicted.

Regulation of epithelial cell eotaxin expression by IFN-γ is likely to be relevant to various types of inflammation of the airways, and it is important to better understand this process.

In the present study we show that the effect of IFN-γ on eotaxin expression in airway epithelial cells is dependent on the timing of exposure to the cytokine: preincubation with IFN-γ profoundly inhibited eotaxin expression, whereas coincubation enhanced eotaxin expression when TNF-α was used as a stimulus. We also investigated the possible mechanisms of these effects and present evidence that the regulation of eotaxin transcription by IFN-γ occurs at the posttranscriptional level.

METHODS

Cell culture

BEAS-2B is a human airway epithelial cell line transformed with adenovirus 12-SV40 hybrid virus (a kind gift from Dr Curtis Harris).[19] BEAS-2B cells were cultured in Dulbecco modified Eagle medium/F12 with 10% FBS, 100 U/mL penicillin, and 100 ng/mL streptomycin (Invitrogen Corp, Tokyo, Japan) at 37°C with 5% CO_2 in humidified air.

Assay of eotaxin and RANTES protein release into the culture medium

Concentrations of eotaxin and RANTES in the collected culture medium were determined with a commercially available system for ELISA (R&D systems, Tokyo, Japan), as descried previously.[12] The limit of detection in the assay was 5 pg/mL for eotaxin and 8 pg/mL for RANTES.

Nuclear extraction

BEAS-2B cells were treated for 24 hours with or without IFN-γ (50 ng/mL) and then incubated with control medium, IFN-γ (50 ng/mL), or TNF-α (100 ng/mL) for 30 minutes. Then nuclear extracts were prepared on the basis of methods described previously.[13]

Electrophoretic mobility shift assays

DNA-protein binding assays were based on methods described previously.[13] Aliquots of 5 μg of nuclear extracts were incubated in 10 μL of total reaction volume containing 10 mmol/L Tris-HCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 10% glycerol, 0.1% Triton X-100, 50 μg/mL poly(dI-dC), 0.1 mg/mL BSA, and 50 mmol/L KCl (Invitrogen Corp) with phosphorous 32–labeled oligonucleotide probe at room temperature for 20 minutes with or without unlabeled oligonucleotide probe. In some experiments, antibodies against p50 (NF-κB1), p65 (Rel A), STAT-1, and IFN regulatory factor 1 (IRF-1; Santa Cruz Biotech, Tokyo, Japan) were incubated with the mixture for 30 minutes after incubation with labeled probe. The reaction products were analyzed by means of electrophoresis in a 5% polyacrylamide gel with 0.5× TBE buffer. The gels were dried and analyzed by means of autoradiography. The sequence of the oligonucleotide probe used in electrophoretic mobility shift assays (EMSAs) is 5′-GGCTTCCCTGGAATCTCCCACA-3′, including the binding sites for STAT-6 and NF-κB in the eotaxin promoter, as reported previously.[13]

Construction of eotaxin promoter–luciferase reporter plasmids

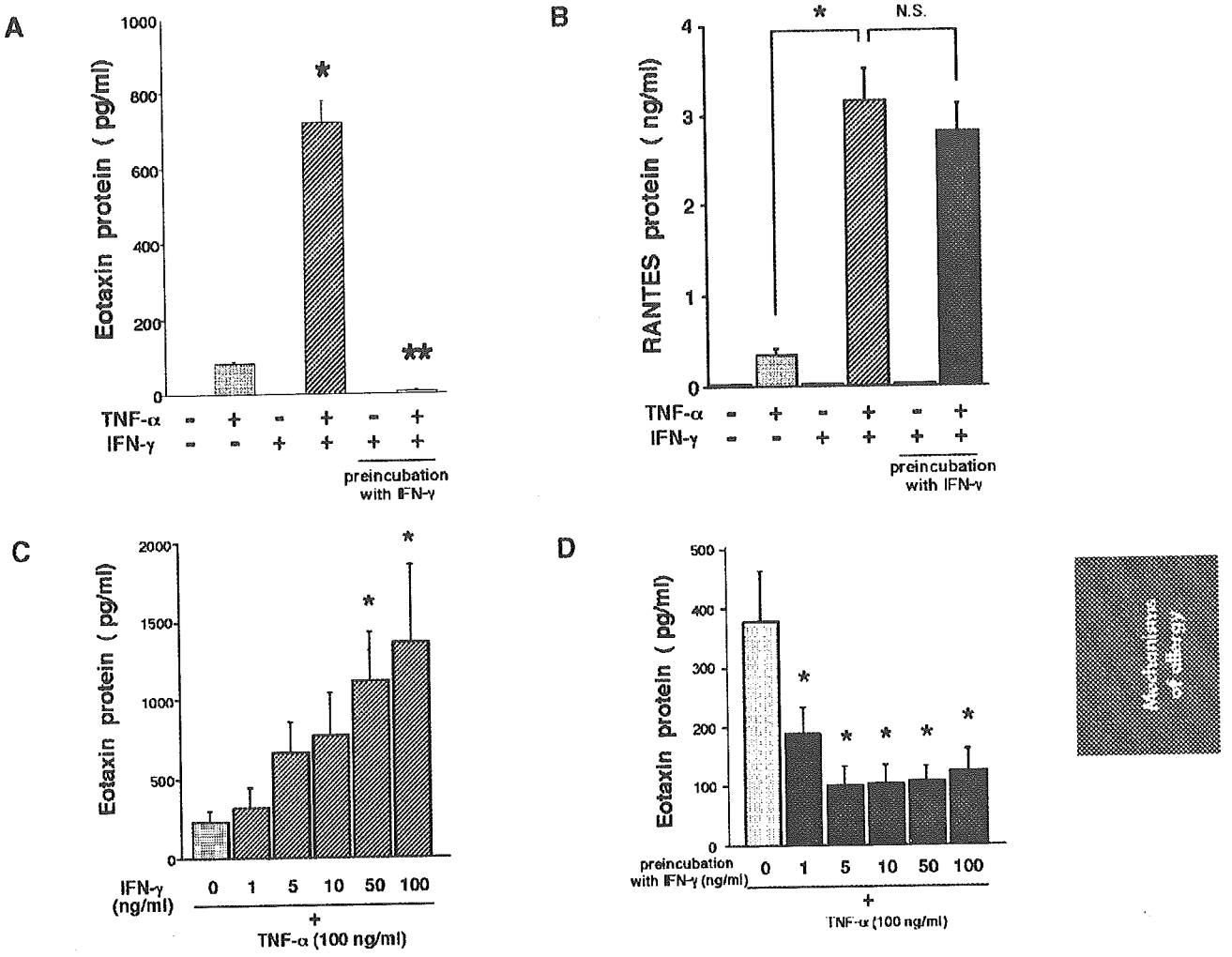
Methods of construction of eotaxin promoter-luciferase reporter plasmids have been described previously.[13] A 1363-bp fragment of the promoter region of the gene encoding eotaxin (site −1363 to −1) was amplified by means of PCR and ligated into *MluI* and *BglII* sites of a firefly-luciferase reporter pGL3-basic vector (Promega, Madison, Wis), and the construct is referred to as pEotx.1363. pEotx.478 and pEotx.92 were constructed by deleting portions of the 5′ end of the eotaxin promoter sequence of pEotx.1363 (Fig 1).

Transient transfections and luciferase assay

BEAS-2B cells were seeded into 6-well plates and allowed to grow to 50% to 70% confluence. Cells were transfected with 1 μg of reporter plasmids and 10 ng of a control renilla-luciferase vector pRL-TK (Promega, Tokyo, Japan) by using 3 μL of Fugene 6 transfection reagent (Roche Diagnostics, Tokyo, Japan) and incubated for 24 hours in 2 mL of medium. After incubation with or without IFN-γ (50 ng/mL) for 24 hours, additional stimuli were then added, and 6 hours later, cells were washed twice with Ca^{2+}- and Mg^{2+}-free PBS, solubilized by means of incubation in 500 μL of lysis buffer for 20 minutes, transferred to microtubes, and centrifuged to pellet cellular debris. The supernatants were stored at −80°C until luciferase activity was measured with the Dual-Luciferase Assay System (Promega) and a Gene-Light 55 luminometer (Microtech Nichion, Chiba, Japan). The firefly-luciferase activity of the eotaxin promoter–luciferase reporter plasmid was normalized by using renilla luciferase activity and calculated as fold induction compared with the control value.

Reverse transcriptase–polymerase chain reaction

Gene expression of eotaxin and β-actin was analyzed by means of RT-PCR, as previously described.[20] BEAS-2B cells were treated for 24 hours with or without IFN-γ (50 ng/mL) and then incubated with control medium, IFN-γ (50 ng/mL), or TNF-α (100 ng/mL) for 24 hours, and total RNA was extracted with ISOGEN reagent (Nippon-Gene, Tokyo, Japan). First-strand cDNAs were synthesized by using a cDNA extraction kit (Amersham Pharmacia Biotech, Tokyo, Japan). After cDNAs were denatured at 94°C for 5 minutes, the PCR was performed with 5 pmol each of the forward and reverse primers, 5 μL of cDNA, 0.6 U of Taq polymerase (Roche Diagnostics), and 2.5 μL of PCR reaction buffer (100 mmol/L Tris-HCl, 15 mmol/L $MgCl_2$, and 500 mmol/L KCl; Roche Diagnostics); distilled water was added to bring the reaction volume to 25 μL. The sequences of the primers for eotaxin were 5′-CCAACCACCTGCTGCTTTAACCTG-3′ (forward) and 5′-GCTTTGGAG TTGGAGATTT TTGG-3′ (reverse), and those for β-actin were 5′-GTGGGGCGCCCCAGGCACCA-3′ (forward) and 5′-CTCCTTAATGTCACGCACGATTTC-3′ (reverse). The amplification reaction was performed for 30 cycles, with denaturation at 94°C for 45 seconds, annealing at 57°C for 45 seconds, and extension at 72°C



for 1 minute (Perkin-Elmer Cetus, Norwalk, Conn). After incubation at 72°C for 10 minutes, PCR-amplified products were analyzed with 1.5% agarose gel electrophoresis and ethidium bromide staining, followed by visualization with a UV transilluminator. Results are shown as the ratio of eotaxin to β-actin densitometric units.

Statistical analysis

Analysis of data was performed by using Stat-View IV (Abacus Concept, Inc, Berkeley, Calif). Data are expressed as means ± SEM. Statistical differences were determined by means of ANOVA with Fisher protected least significant difference.

RESULTS

A small amount of eotaxin protein was detected in the medium of BEAS-2B stimulated with 100 ng/mL TNF-α (Fig 2, *A*). Alone, IFN-γ (50 ng/mL) did not induce detectable eotaxin production. The combination of TNF-α and IFN-γ synergistically stimulated eotaxin production ($P < .05$ vs either alone). Pretreatment with 50 ng/mL IFN-γ for 24 hours before TNF-α stimulation markedly inhibited expression of eotaxin production induced by TNF-α ($P < .05$ vs TNF-α alone). To confirm

FIG 2. Release of eotaxin (A, C, D, and E) or RANTES (B) protein into the medium by BEAS-2B cells. Concentrations of eotaxin or RANTES protein in the media are presented as the means ± SEM of a total of 6 independent experiments.

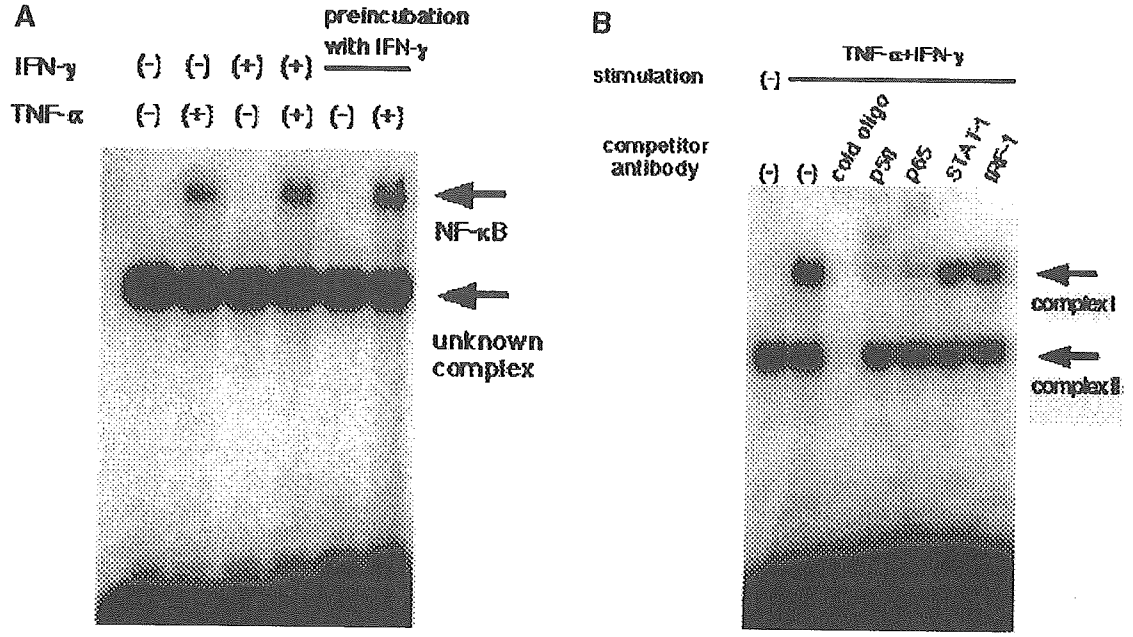


FIG 3. IFN-γ does not alter formation of a binding complex of NF-κB (p50/p65) with a DNA probe derived from the proximal region of the eotaxin promoter. A, TNF-α induced formation of a binding complex, which was not changed by costimulation or preincubation with IFN-γ. B, The binding complex was confirmed to consist of p50/p65 NF-κB family by using antibodies as indicated. Results are representative of 3 independent experiments.

that the inhibitory effect of preincubation with IFN-γ was not due to a generalized inhibition of cellular responses, we analyzed the effect of IFN-γ on RANTES production in BEAS-2B cells. RANTES protein production was increased by TNF-α, and this response was dramatically enhanced by IFN-γ (Fig 2, *B*). Preincubation with IFN-γ did not further increase RANTES expression nor did it inhibit the induction of RANTES by TNF-α. These data indicate that the effect of IFN-γ is distinct for the expression of each chemokine. We next confirmed the concentration and time-dependent effects of both activating and inhibitory effects of IFN-γ. The synergistic effect of IFN-γ on the expression of eotaxin protein induced by TNF-α (100 ng/mL) depended on the concentration of IFN-γ used (Fig 2, *C*). Likewise, the inhibition of eotaxin protein expression with preincubation was dependent on the concentration of IFN-γ (Fig 2, *D*). Interestingly, the inhibitory effects of IFN-γ required lower concentrations than the activating effects (Fig 2, *C* and *D*). The inhibitory effect of preincubation with IFN-γ (50 ng/mL) also depended on the time of preincubation, and a significant inhibitory effect was observed only with preincubation with IFN-γ for more than 12 hours (Fig 2, *E*).

Unfortunately, several laboratories, including our laboratories in Tokyo and Baltimore, as well as the laboratory of Luster et al in Boston (personal communication), have found that primary epithelial cell isolates make very little eotaxin. Because epithelial cells are well documented to express large amounts of eotaxin in vivo, we believe that this is a technical problem. It precludes repeating these studies in primary cells for the time being, however.

We next focused experiments on the regulation of eotaxin transcription by IFN-γ. First, we hypothesized that IFN-γ might influence the binding of NF-κB to the NF-κB binding site in the eotaxin promoter.[21] EMSAs with nuclear extracts from BEAS-2B cells stimulated with 100 ng/mL TNF-α resulted in formation of a strong binding complex (Fig 3, *A*). This binding complex was confirmed to be a heterodimer of p50 and p65 NF-κB Rel family members, as reported earlier.[13] Binding of NF-κB was not affected by means of either simultaneous stimulation or preincubation with IFN-γ. Formation of the complex in extracts from cells stimulated with the combination of IFN-γ and TNF-α was inhibited by an excess of unlabeled oligonucleotide and was supershifted by anti-p50 and anti-p65 NF-κB Rel family antibodies but was not inhibited by anti-STAT-1 or anti-IRF-1 antibodies (Fig 3, *B*). These experiments indicate that IFN-γ neither inhibits nor enhances the ability of TNF-α to activate NF-κB.

We next analyzed the effect of IFN-γ on the transcriptional activity of the eotaxin promoter. TNF-α (100 ng/mL) activated the eotaxin promoter–luciferase reporter plasmid pEotx.1363, as reported previously (2.4 ± 0.1–fold induction; Fig 4, *A*).[13] IFN-γ (50 ng/mL) alone did not affect the eotaxin promoter activity. Simultaneous stimulation with IFN-γ and TNF-α was no different than stimulation with TNF-α alone (2.1 ± 0.1–fold induction). Pretreatment with IFN-γ only modestly inhibited the activity induced by TNF-α (1.6 ± 0.2–fold induction). This pattern of regulation of eotaxin promoter activity was also observed with pEotx.478 (Fig 4, *B*). Interestingly, the inhibitory effect of preincubation with

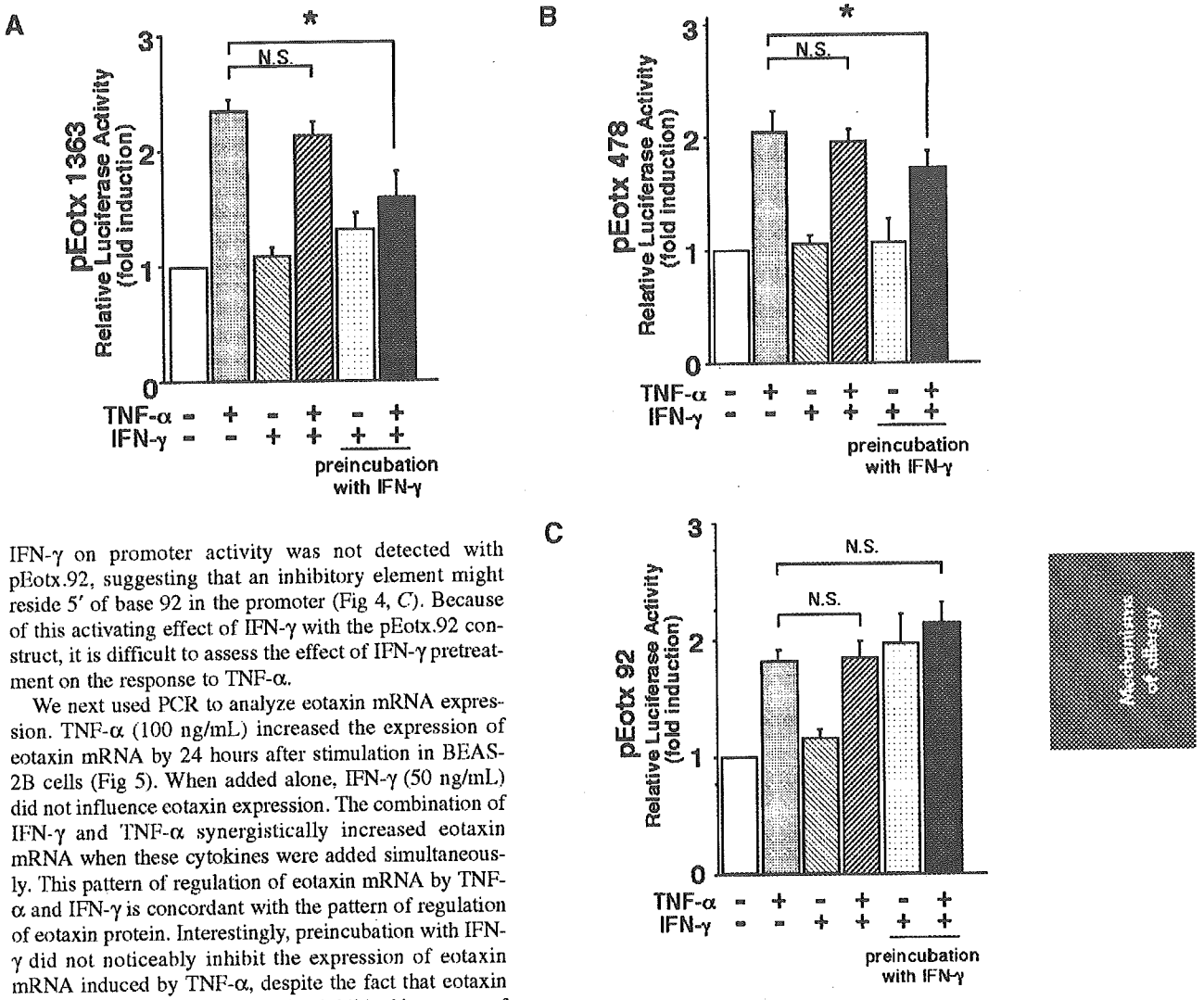


FIG 4. Effect of IFN-γ on eotaxin promoter activity. BEAS-2B cells were transfected with the eotaxin promoter–luciferase reporter plasmids shown in Fig 1 (pEotx.1363, pEotx.478, and pEotx.92) and the control vector pRL-TK. The relative luciferase activity was calculated as fold induction compared with the control value. The data are presented as the means ± SEM of a total of 3 independent experiments. *$P < .05$. A, pEotx.1363; B, pEotx.478; and C, pEotx.92.

IFN-γ on promoter activity was not detected with pEotx.92, suggesting that an inhibitory element might reside 5′ of base 92 in the promoter (Fig 4, *C*). Because of this activating effect of IFN-γ with the pEotx.92 construct, it is difficult to assess the effect of IFN-γ pretreatment on the response to TNF-α.

We next used PCR to analyze eotaxin mRNA expression. TNF-α (100 ng/mL) increased the expression of eotaxin mRNA by 24 hours after stimulation in BEAS-2B cells (Fig 5). When added alone, IFN-γ (50 ng/mL) did not influence eotaxin expression. The combination of IFN-γ and TNF-α synergistically increased eotaxin mRNA when these cytokines were added simultaneously. This pattern of regulation of eotaxin mRNA by TNF-α and IFN-γ is concordant with the pattern of regulation of eotaxin protein. Interestingly, preincubation with IFN-γ did not noticeably inhibit the expression of eotaxin mRNA induced by TNF-α, despite the fact that eotaxin protein production was remarkably inhibited by means of preincubation with IFN-γ (Fig 2, *A*). We confirmed that the level of amplified gene encoding eotaxin was dependent on the number of cycles, and the linearity of amplification was observed with 30 cycles (data not shown).

DISCUSSION

We demonstrate here that the T_H1 cytokine IFN-γ regulates expression of eotaxin in a time-dependent manner. Although IFN-γ alone did not induce the expression of eotaxin, simultaneous treatment with IFN-γ potentiated the expression of eotaxin protein induced by TNF-α, which is in agreement with previous reports.[12,15] Interestingly, preincubation with IFN-γ significantly inhibited the induction of eotaxin protein expression by TNF-α.

IFN-γ is a T_H1-type cytokine believed to play an important role in the regulation of allergic disease. Administration of T_H1-inducing stimuli suppresses T_H2 responses and manifestations of allergic disease, such as asthma.[2,22] However, it has been reported that IFN-γ might contribute to the generation of airway inflammation and acute deterioration of asthma.[23-25] Our results suggest that the chronic presence of IFN-γ could counteract the development of allergic inflammation. Our results also suggest that acute production of IFN-γ, as might occur during a viral infection, could enhance production of eotaxin and exacerbate allergic inflammation.

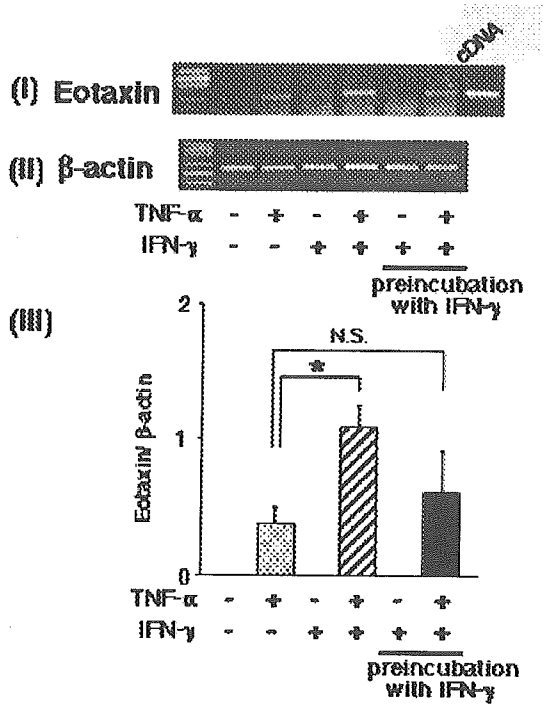


FIG 5. Effect of IFN-γ on eotaxin mRNA expression. RNA was extracted from BEAS-2B cells that had been treated for 24 hours with or without IFN-γ (50 ng/mL), followed by incubation with control medium or indicated cytokines for 24 hours. Extracted RNA was subjected to RT-PCR: *I*, eotaxin mRNA expression; *II*, β-actin mRNA expression; *III*, mean ± SEM of the densitometric analysis of eotaxin mRNA expression normalized with β-actin expression. The data are presented as a representative experiment (*I* and *II*) or as the mean ± SEM of a total of 3 independent experiments (*III*). $^*P < .05$.

Our studies indicate that IFN-γ can either potentiate or inhibit eotaxin induction, depending on the timing of exposure. IFN-γ is known to induce a wide variety of genes related to inflammation. In many cases STAT-1 and IRF-1 are pivotal transcription factors that mediate the response to IFN-γ. These transcription factors are known to cooperate with NF-κB, an important mediator of the response to TNF-α.[21,26] We first hypothesized that IFN-γ might activate STAT-1 or IRF-1 and that these transcription factors might bind to the eotaxin promoter region. However, we could not identify any putative binding sequences (TTCN3GAA for STAT-1 or tandem repeats of GAAA for IRF-1) in the eotaxin promoter spanning 1363 bp.[27] Hein et al[28] reported 3 kb of the sequence of the 5′ upstream region of the eotaxin gene. In our analysis of this sequence, there is one consensus sequence of TTCN3GAA in a site −1998 to −2006 bp of the upstream region of the eotaxin gene. We examined whether IFN-γ induces formation of a binding complex of STAT-1 with this region by using the DNA probe 5′-GGCATTCTTGGAAACCA-3′ derived from site −1994 to −2010 bp, but we could not detect a binding complex after stimulation with IFN-γ (data not shown).

The binding complex induced by the combination of TNF-α and IFN-γ with a DNA probe derived from the proximal region of the eotaxin promoter was confirmed to be a p50/p65 NF-κB heterodimer and was not altered by anti-STAT-1 (Fig 3, *B*). Members of the STAT family are known to bind to the DNA consensus sequence TTCN3GAA. As mentioned earlier, STAT-6 binds to the proximal region of the eotaxin promoter. However, the consensus sequence for this binding site is TTCN4GAA, which is reported to be a specific motif for only STAT-6 among this family.[29,30] Our findings that IFN-γ does not induce binding of STAT-1 to the proximal region of the eotaxin promoter is in agreement with the findings of these previous reports.

One possible mechanism of synergy between TNF-α and IFN-γ might be the activation of NF-κB or the upregulation of TNF receptors by IFN-γ.[21] In these studies TNF-α activated NF-κB and induced the binding of NF-κB to the DNA probe derived from eotaxin promoter, but IFN-γ did not alter formation of this binding complex. Hein et al[28] and Garcia-Zepeda et al[31] reported that multiple putative IFN-γ–responsive elements (γIREs) might exist in the eotaxin promoter. γIRE is known to mediate transcriptional activation of MHC class II by IFN-γ.[32] Although the eotaxin promoter–luciferase reporter plasmids pEotx.1363 and pEotx.478 contain numerous consensus binding sites for γIRE, IFN-γ did not induce promoter activity of these reporters or enhance activity induced by TNF-α in our studies, indicating that γIRE might be not involved in the activation of the eotaxin promoter. The reporter data suggest that IFN-γ does not increase transcription either alone or in combination with TNF-α, whereas the findings with RT-PCR suggest that IFN-γ increased steady state eotaxin mRNA levels in the presence of TNF-α. Together, these results suggest that IFN-γ either increases transcription through interactions with the endogenous promoter but not the reporter or that the effect is due to posttranscriptional influences of IFN-γ.

The pattern of regulation of chemokine expression by cytokines might differ among cell types and experimental systems. Regulation of eotaxin expression by cytokines appears to be similar in several cell types. Various investigators reported that IL-4/IL-13 and TNF-α stimulate eotaxin expression, and the combination of these cytokines acts in a synergistic manner in fibroblasts.[17,33] A similar synergism of TNF-α and IL-4/IL-13 on eotaxin expression has been observed in airway epithelial cells. We demonstrated that TNF-α activates NF-κB, whereas IL-4 and IL-13 activate STAT-6, and these 2 transcription factors activate eotaxin transcription.[13,14] Hoeck and Woisetschlager[33] recently demonstrated that NF-κB and STAT-6 similarly regulate eotaxin transcription in fibroblasts. These reports indicate that there is common role for these transcription factors in the regulation of eotaxin in both fibroblasts and epithelial cells. Teran et al[17] and Miyamasu et al[18] reported that IFN-γ inhibited eotaxin protein expression induced by

TNF-α in fibroblasts. Our results also indicate that IFN-γ is a powerful inhibitor of eotaxin expression. Preincubation with IFN-γ for 24 hours inhibited eotaxin protein expression in airway epithelial cells by greater than 95%. We hypothesized that IFN-γ might inhibit the formation of the NF-κB–DNA binding complex. Direct assay for formation of this complex with EMSAs showed that IFN-γ did not change the binding of NF-κB to the eotaxin promoter–derived DNA. Preincubation with IFN-γ only modestly inhibited eotaxin promoter activity induced by TNF-α. Studies using 5′-deleted eotaxin promoter–luciferase reporter plasmid indicate that the responsible region for this modest inhibitory effect might exist distal to 93 bp in the eotaxin promoter. This inhibitory effect of preincubation with IFN-γ on eotaxin transcription, as detected by means of PCR or with reporters, was modest when compared with the nearly complete inhibition of eotaxin protein production.

Recent studies indicate that IFN-γ can exert a repressive effect on target protein translation. Ben-Asouli et al[34] reported that IFN-γ downregulates its own translation through the double-stranded RNA-activated protein kinase PKR. Mazumder and Fox[35] demonstrated that incubation of cells with IFN-γ for 24 hours, but not for 8 hours, induced inhibition of translation of ceruloplasmin. They suggested that a factor distinct from PKR was synthesized by means of incubation of cells with IFN-γ for 24 hours, and this factor might suppress binding of the translational machinery to the 3′-untranslated region of ceruloplasmin mRNA. On the basis of the dissociation of effects of IFN-γ on eotaxin transcription (as detected on the basis of mRNA levels and reporters) and production of eotaxin protein (as detected on the basis of ELISA), it is reasonable to hypothesize that IFN-γ might decrease eotaxin protein synthesis through inhibition of eotaxin translation. Further studies focusing on the translation of eotaxin, including the role of PKR and other factors, are clearly needed to further test this hypothesis.

We thank Dr Tsutom Hirano, Dr Takeshi Kasama, Dr Steve N. Georas, Dr Vincenzo Casolaro, Mr James R. Plitt, and Ms Carol Bickel for excellent assistance and helpful discussions and Ms Mieko Mori, Ms Tomoko Akabane, Ms Eri Matsukura, and Ms Bonnie Hebden for skillful assistance.

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0022-3565/03/3073-1213–1220$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 307:1213–1220, 2003

Vol. 307, No. 3
56341/1110841
Printed in U.S.A.

Induction of C-X-C Chemokines, Growth-Related Oncogene α Expression, and Epithelial Cell-Derived Neutrophil-Activating Protein-78 by ML-1 (Interleukin-17F) Involves Activation of Raf1-Mitogen-Activated Protein Kinase Kinase-Extracellular Signal-Regulated Kinase 1/2 Pathway

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Received June 30, 2003; accepted September 5, 2003

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-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 and human umbilical vein endothelial cells. Furthermore, selective mitogen-activated protein kinase kinase (MEK) inhibitors 2′-amino-3′-methoxyflavone (PD98059), 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto) butadiene (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, whereas a protein kinase C inhibitor, 2-(1-(3-aminopropyl) indol-3-yl)-3-(1-methylindol-3-yl) maleimide, acetate (Ro-31-7549), and a phosphatidylinositol 3-kinase inhibitor, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), did not affect their production. Together, these data indicates a role for Raf1-MEK-extracellular signal-regulated kinase 1/2 pathway in ML-1 induced C-X-C chemokine expression, suggesting potential pharmacological targets for modulation.

Airway epithelial and vein endothelial cells play a central role for airway inflammation, because 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, bronchial asthma, and cystic fibrosis (Koller et al., 1995; Betsuyaku et al., 1999; Jatakanon 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, 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

This work was supported, in part, by National Institutes of Health Grant AI-40274 and Astra Zeneca Asthma Research Award.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.103.056341.

ABBREVIATIONS: ELR, Glu-Leu-Arg; IL, interleukin; GROα growth-related oncogene α; ENA-78, epithelial-cell derived neutrophil activating protein-78; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; NHBE, normal human bronchial epithelial; HUVEC, human umbilical vein endothelial cell; MEK, mitogen-activated protein kinase kinase; PCR, polymerase chain reaction; bp, base pair; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; PKC protein kinase C; PI3K phosphatidylinositol 3-kinase; DMSO, dimethyl sulfoxide.

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 (Hymowitz et al., 2001; Starnes 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-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 C-X-C 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, 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

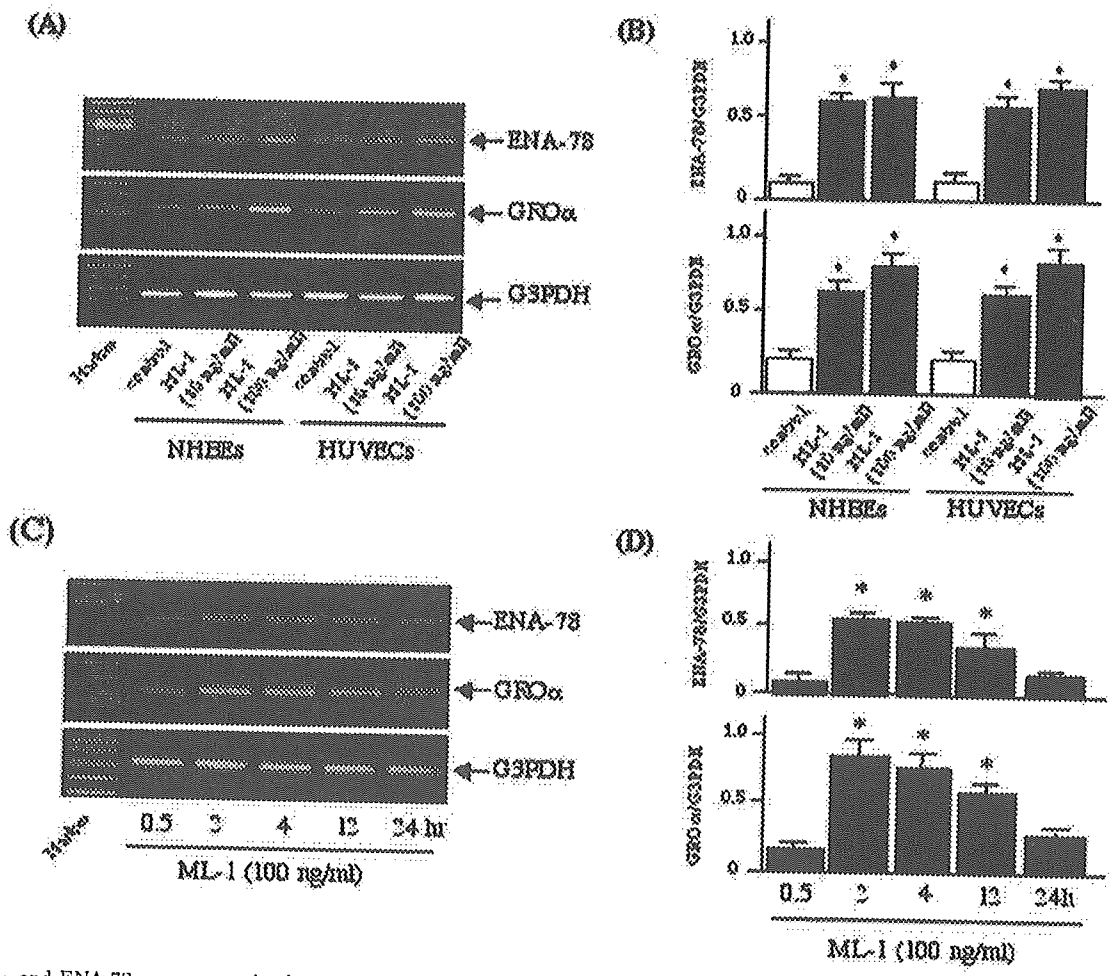

Fig. 1. GROα and ENA-78 gene expression by different doses of ML-1. A, NHBEs and HUVECs were treated with 10 and 100 ng/ml ML-1 for 2 h. Reverse transcription-PCR is performed as described under *Materials and Methods*. B, GROα/G3PDH and ENA-78/G3PDH ratios were determined by densitometric analysis from the same cDNAs. *, $p < 0.05$ was considered significant versus control. Time-course study of GROα and ENA-78 gene expression. C, NHBEs were treated with 100 ng/ml ML-1 for 0.5 to 24 h. Reverse transcription-PCR is performed as described under *Materials and Methods*. D, GROα/G3PDH and ENA-78/G3PDH ratios were determined by densitometric analysis from the same cDNAs. The values are expressed as mean + S.D. ($n = 3$ experiments). *, $p < 0.05$ was considered significant versus the intensity of 0.5-h time point.

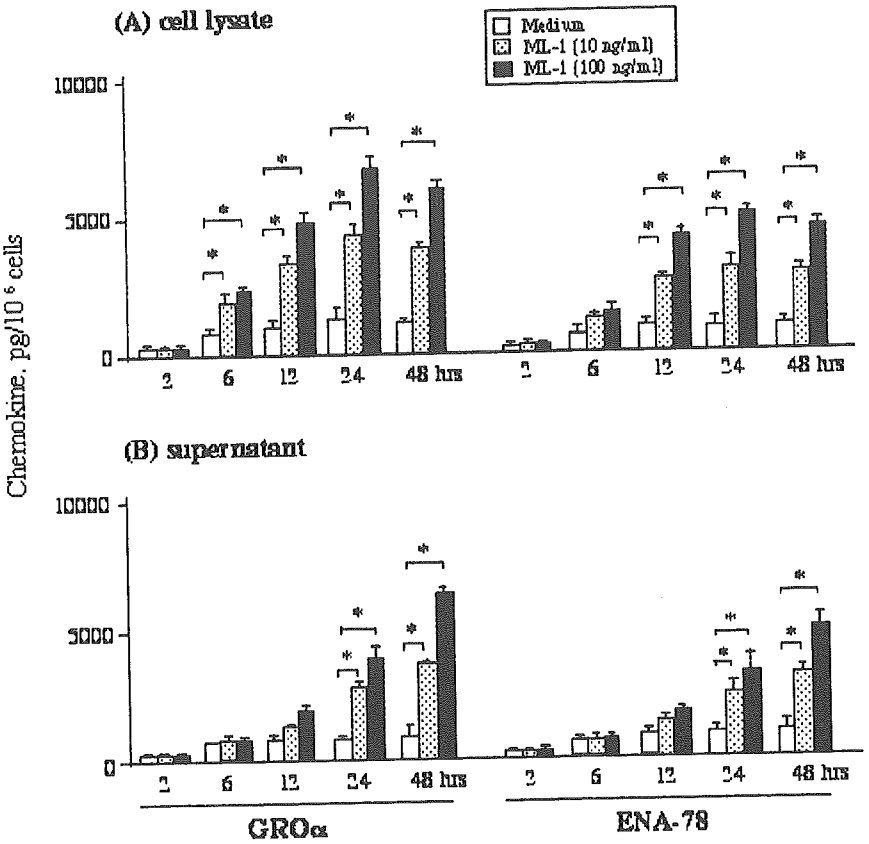

Fig. 2. GROα and ENA-78 protein levels in NHBEs. NHBEs were treated with 10 and 100 ng/ml ML-1 for 2 to 48 h. ELISA was performed as described under *Materials and Methods*. A, GROα and ENA-78 protein was measured in NHBE cell lysates harvested at the indicated time points after stimulation with ML-1. The cells were lysed as described under *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 + S.D. ($n = 6$). *, $p < 0.05$ was considered significant versus control.

Clonetics. The cells were cultured for no more than three passages before 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 polymerase chain reaction (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^{2+}-nitrilotriacetic acid 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°C until used. Endotoxin levels were tested using Kinetic-QCL chromogenic LAL (Cambrex Bio Science Walkersville, Inc., 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) from 1×10^6 cells at 0.5, 2, 4, 12, and 24 h after stimulation with 10 and 100 ng/ml ML-1. cDNAs were synthesized from 500 ng of total RNA in the presence of Moloney murine leukemia virus reverse transcriptase (1 U/reaction; Sigma-Aldrich, St. Louis, MO), oligo(dT) primer, and reaction buffer at 42°C for 90 min, followed by PCR. The sequences of PCR primers for GROα were as follows: forward, 5′-CGCTCCTCTCACAGCCGCCA-3′ and reverse, 5′-AGGAACAGCCACCAGTGAGC-3′; ENA-78, forward, 5′-TGTGTTGAGAGAGCTGCGTTGCGTT-3′ and reverse, 5′-TCAGTTTTCCTTGTTTCCACC-3′; and G3PDH, forward, 5′-ACCACAGTCCATGCCATCAC-3′ and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′. The amplification reaction was performed for 26 cycles with denaturation at 94°C for 45 s, annealing at 53°C for 45 s, and extension at 72°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 ± S.D. ($n = 3$ 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 International, Camarillo, CA) according to the manufacturer's instruction. Cell supernatant was harvested from unstimulated or stimulated cultures with 10 and 100 ng/ml ML-1 at 2, 6, 12, 24, or 48 h 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 Nonidet P-40 lysis buffer (20 mM Tris pH 7.4, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 100 mg/ml aprotinin, 200 mg/ml leupeptin, 50 mM NaF, 5 mM $Na_4P_2O_7$, and 1% Nonidet P-40; all purchased from Sigma-Aldrich). The chemokine concentration of cell lysate was reported as the amount recovered per 10^6 cells. The values are expressed as mean ± S.D. ($n = 6$ 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 2′-amino-3′-methoxyflavone (PD98059) (Calbiochem, La Jolla, CA) and 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto) butadiene (U0126) (New England Bio Labs, Beverly, MA); p38 inhibitor 4-(4-fluoro-phenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl) 1*H*-imidazole (SB202190) (Calbiochem); a Raf1 kinase inhibitor I (5-iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone (Calbiochem); a PKC inhibitor, 2-(1-(3-aminopropyl) indol-3-yl)-3-(1-methylindol-3-yl) maleimide, acetate (Ro-31-7549) (Calbiochem); a PI3K inhibitor, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) (Calbiochem); and

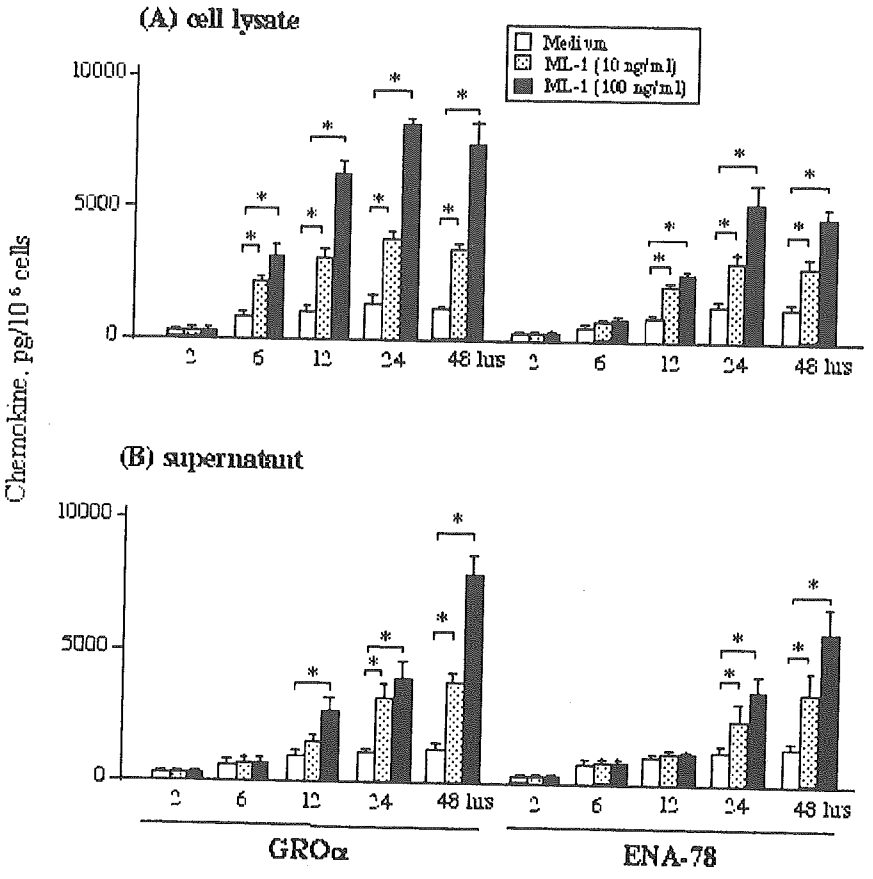

Fig. 3. GROα and ENA-78 protein levels in HUVECs. HUVECs were treated with 10 and 100 ng/ml ML-1 for 2 to 48 h. ELISA was performed as described under *Materials and Methods*. A, GROα and ENA-78 protein was measured in HUVEC cell lysates harvested at the indicated time points after stimulation with ML-1.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

a vehicle control, DMSO (Me_2SO) for 1 h before treatment with ML-1 (100 ng/ml) for 24 h. 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 ± S.D. (n = 4 experiments).

Data Analysis. The statistical significance of differences was determined by analysis of variance. The values are expressed as mean + S.D. from independent experiments. Any difference with p values less than 0.05 was considered. When analysis of variance indicated a significant difference, the Scheffe's F test was used to determine the difference between groups.

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. Although detectable gene expression for both GROα and ENA-78 was found in control cells at 2-h time point (Fig. 1A), the induction of chemokine gene expression was evident in both NHBEs and HUVECs (Fig. 1, A and B), and ML-1-induced gene expression peaked at 2-h time point and returned to baseline at 24-h time point in ML-1 (100 ng/ml)-treated NHBEs (Fig. 1, C and D). No increase of chemokine gene expression was seen in cells treated with a His-tag control protein (Positope, 10 or 100 ng/ml; Invitrogen; 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 h after stimulation with 10 and 100 ng/ml ML-1. Cell lysate ENA-78 protein level were significantly increased 12 h after stimulation with 10 and 100 ng/ml ML-1. They were significantly elevated at 24- and 48-h time points and attained their highest level 24 h after stimulation (Fig. 2A). Cell supernatant GROα and ENA-78 levels were significantly elevated 24 h after stimulation and were increased further at 48 h (Fig. 2B). A similar pattern was observed in HUVECs, except for significant induction of GROα secretion was seen at 12-h time point when HUVECs were stimulated with 100 ng/ml ML-1 (Fig. 3, A and B), suggesting that ML-1 is more potent in the induction of GROα expression compared with 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-h 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, whereas 1-h 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). Although induction of GROα and ENA-78 is partially inhibited by PD98059, U0126, or Raf1 kinase inhibitor I even at relatively high dose (50, 10, and 10 nM, respectively), the combination with 10 μM PD98059 and 1 nM Raf1 kinase inhibitor I completely inhib-