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Leukotriene B_4 Production in Human Mononuclear Phagocytes Is Modulated by Interleukin-4-Induced 15-Lipoxygenase

MIRELLA PROFITA, ANGELO SALA, LIBORIA SIENA, PETER M. HENSON, ROBERT C. MURPHY, ALESSANDRA PATERNO, ANNA BONANNO, LOREDANA RICCOBONO, ANGELA MIRABELLA, GIOVANNI BONSIGNORE, and ANTONIO M. VIGNOLA

Istituto di Fisiopatologia Respiratoria, Consiglio Nazionale delle Ricerche, Palermo, Italy (M.P., L.S., A.P., A.B., L.R., G.B.); Dipartimento di Scienze Famacologiche, Università di Milano, Milan, Italy (A.S.); National Jewish Medical and Research Center, Denver, Colorado (A.S., P.M.H., R.C.M.); and Istituto di Medicina Generale e Pneumologie, Università di Palermo, Palermo, Italy (A.M., A.M.V.).

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ABSTRACT

The aim of this study was to evaluate the consequences of interleukin (IL)-4-induced 15-lipoxygenase (15-LO) expression on leukotriene B_4 (LTB_4) synthesis in human monocytes. Human monocytes incubated for 24, 48, and 72 h with IL-4 (10 ng/ml) were stimulated with Ca^{2+}-ionophore A23187 (calcimycin; 5 μM) or opsonized zymosan. 15(*S*)-hydroxyeicosatetraenoic acid [15(*S*)-HETE], LTB_4, and arachidonic acid (AA) release were measured by high-performance liquid chromotography/radioimmunoassay, liquid chromotography/tandem mass spectrometry (LC/MS/MS), or gas chromatography/mass spectrometry. 15-LO activity was evaluated in AA-treated monocytes. 15-LO, 5-lipoxygenase (5-LO) and 5-LO activating protein (FLAP) expression were analyzed by reverse transcription-polymerase chain reaction. Neutrophil chemotactic activity was evaluated using a microtaxis chamber assay. A23187-induced synthesis of 15(*S*)-HETE was significantly increased after treatment with IL-4 (10 ng/ml) for 48 and 72 h ($p < 0.001$). Concomitant decrease of LTB_4 release was observed after 72 h of incubation with IL-4 ($p < 0.001$). LC/MS/MS analysis confirmed the production of 15(*S*)-HETE and the significant inhibition of LTB_4 synthesis in IL-4-treated monocyte after challenge with opsonized zymosan. IL-4 treatment induced 15-LO enzymatic activity as well as 15-LO mRNA, but did not affect either 5-LO or FLAP mRNA expression in monocytes. Supernatant from IL-4-treated monocytes showed significantly lower neutrophil chemotactic activity than controls. 15(*S*)-HETE significantly inhibited LTB_4 production induced by A23187-stimulated human monocytes without affecting AA release. IL-4-induced expression of 15-LO in monocytes caused a significant reduction of LTB_4 production. Whereas this effect did not reflect changes in 5-LO and FLAP mRNA expression, synthetic 15(*S*)-HETE was able to significantly inhibit the synthesis of LTB_4, without affecting AA release.

Human mononuclear phagocytes migrate from bone marrow to inflamed tissue via the peripheral blood system, and they differentiate into mature macrophages, these being the phagocytic cell of the lineage. Activated monocytes can promote the bronchial inflammatory response by releasing numerous mediators, including arachidonic acid metabolites (Ferreri et al., 1986; Demoly et al., 1994) that contribute to the development of inflammatory processes such as asthma (Rankin, 1989). Histological studies on asthmatic subjects have shown that in airways, macrophages are in close contact with inflammatory cells, particularly mast cells, basophils, and Th2 cells (Bradley et al., 1991); these cells are able to release several immunomodulatory cytokines that may affect macrophage functions and activities.

Among the cytokines potentially playing a role in the inflammatory response IL-4 is known to regulate the expression of 15-lipoxygenase as well as 15(*S*)-HETE production in human monocytes (Conrad et al., 1992). Bronchial allergen challenge in atopic asthmatic subjects has been found to yield a 30-fold increase in the concentrations of 15(*S*)-HETE recovered in bronchoalveolar lavage fluid (Murray et al., 1986); interestingly, 15(*S*)-HETE exerts several immunoregulatory functions that may be relevant in asthma and chronic bronchitis (Samuelsson et al., 1987).

Thus, 15(*S*)-HETE is a potent mucosecretagogue in the human airway (Marom et al., 1982) and has been reported to

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ABBREVIATIONS: IL, interleukin; 15-LO, 15-lipoxygenase; LTB_4, leukotriene B_4; 15(*S*)-HETE, hydroxyeicosatetraenoic acid; AA, arachidonic acid; LC/MS/MS, liquid chromatography/tandem mass spectrometry; RP, reversed-phase; HPLC, high-performance liquid chromatography; 5-LO, 5-lipoxygenase; FLAP, 5-LO-activating protein; RIA, radioimmunoassay; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; fMLP, formyl-methionyl-leucyl-phenylalanine; bp, base pairs; A23187, calcimycin; LY 223982, (*E*)-5-(3-carbobenzoyl)-2-((6-(4-methoxyphenyl)-5-hexenyl)oxy)benzenepropanoic acid; MK-886, 3-(1-(4-chlorobenzyl-3-*t*-butyl-thio-5-isopropylindol-2-yl)-2, 2-dimethylpropanoic acid.

possess chemotactic activity for neutrophils, contributing to the recruitment of these cells in the airways (Johnson et al., 1985; Kirsch et al., 1988). Furthermore, it can prolong the duration of airway obstruction during the early response to allergen challenge, augmenting the release of mediators from mast cells and their effects on airway smooth muscle (Lai et al., 1990). Bronchial epithelial cells from asthmatic subjects express more 15-LO immunoreactivity than cells obtained from normal subjects, and this enhanced expression appears to correlate with the clinical severity of the disease (Bradding et al., 1995).

Aside from its proinflammatory actions, 15(*S*)-HETE may also modulate the inflammatory response through the inhibition of leukotriene production in a variety of cell types (Vanderhoek et al., 1982; Profita et al., 2000a). Different studies have shown that steroid-dependent asthmatic patients generate 5(*S*),15(*S*)-diHETE and lipoxins from 15(*S*)-HETE, with a concomitant reduction of LTB_4 production (Chavis et al., 1998); recently it has been shown that FLAP, in addition to its role in leukotriene biosynthesis, may also function as a more general lipid-binding protein that significantly increases the metabolism of 15(*S*)-HETE by 5-lipoxygenase (Mancini et al., 1998).

The ability of IL-4 to induce the expression of 15-LO, and the potential effect of 15(*S*)-HETE on leukotriene biosynthesis prompted us to study the effect of IL-4 treatment of human monocytes on their ability to synthesize leukotrienes. In this study we provide evidence for a potential anti-inflammatory role of IL-4 in human monocytes through the enhanced expression of 15-LO and production of 15(*S*)-HETE, resulting in inhibition of the synthesis of the potent chemotactic factor LTB_4.

Materials and Methods

Reagents. Human recombinant interleukin-4 (IL-4) and polyclonal sheep anti-human IL-4 were obtained from Genzyme (Cambridge, MA). All solvents were HPLC grade and were obtained from Merck (Darmstadt, Germany). Cell culture media were from Invitrogen (Carlsbad, CA). Nutritive medium came from Whittaker (Veviers, Belgium) and fetal calf serum from Hyclone Laboratories (Logan, UT). 15(*S*)-HETE RIA kit was purchased from Advanced Magnetics (Cambridge, MA). LTB_4 RIA kit was purchased from Amersham-Pharmacia Biotec (Milan, Italy). Eicosanoid standards were from Cayman Chemical (Ann Arbor, MI). Reagents for PCR were from Promega (Madison, WI). Compound MK-886 was kindly provided by Dr. A.W. Ford-Hutchinson (Merck Frosst, Pointe Claire, PQ, Canada).

Purification and Culture of Monocytes. Mononuclear cells were isolated from buffy coats by density gradient centrifugation using Ficoll-Hypaque cushions (Conrad et al., 1992). The mononuclear cell band was removed and washed three times by centrifugation with PBS containing $CaCl_2$ and $MgCl_2$ at final concentrations of 0.5 and 1 mM, respectively (PBS buffer). After resuspension ($4–20 \times 10^6$ cells/ml) in RPMI 1640 + 10% heat-inactivated (56°C, 30 min) fetal calf serum + 1% penicillin-streptomycin solution + 1 mM *l*-glutamine (RPMI buffer), cells were allowed to adhere to 150-mm polystyrene tissue culture dishes for 2 h at 37°C. More than 96% of the adherent cells stained positive for nonspecific esterase, and the viability was >95%, as assessed by trypan blue exclusion.

After removal of nonadherent cells, the adherent monocytes were harvested by gentle scraping, washed, and resuspended at the concentration of 2×10^6 cells/ml in RPMI buffer. Monocytes were then incubated in 6-well plates for 24, 48, and 72 h with IL-4 at the concentration of 10 ng/ml, which in previous concentration-response experiments was found to be optimal for 15-LO expression. IL-4-treated monocytes cells were also incubated in the presence of a polyclonal sheep anti-human IL-4 antibody (5–40 μg/ml). At the end of the incubation time cells were recovered by gentle scraping, washed twice, and resuspended at the final concentration of 2×10^6 cells/ml in PBS buffer for stimulation of eicosanoid synthesis.

LTB_4 and 15(*S*)-HETE Production in Monocytes. IL-4-treated and untreated cells (2×10^6 cells/ml) were allowed to adhere to tissue culture plates for 2 h at 37°C, and were then incubated with A23187 to a final concentration of 5×10^{-6} M for 15 min. Alternatively monocytes were challenged with opsonized zymosan (100 particles per cell, 1 h at 37°C). Zymosan was opsonized by incubation with fresh human serum; after boiling 100 mg in 2 ml of PBS for 1 h, and washing twice with PBS, zymosan was resuspended in PBS (2 ml) and incubated with 6 ml of fresh human serum at 37°C for 20 min. After centrifugation and washing twice with PBS, opsonized zymosan was resuspended at a concentration of 10 mg/ml. Zymosan particle count was performed using an hemocytometer.

In separate experiments monocytes were incubated with different concentrations (10^{-5}, 10^{-6}, 10^{-7} M) of 15(*S*)-HETE for 15 min at 37°C prior to A23187 challenge. At the end of the incubation, the cell supernatants were harvested and, after the addition of the appropriate internal standard (prostaglandin B_2 for RIA/RP-HPLC, d_4-LTB_4 for LC/MS/MS, and d_8-AA for gas chromatography/mass spectrometry analysis) were stored under an argon atmosphere at −80°C. Radioimmunoassay (RIA) of 15(*S*)-HETE and LTB_4 was performed after RP-HPLC purification, using a gradient liquid chromatograph (System Gold apparatus, model 126; Beckman Coulter, Inc., Fullerton, CA), connected to a diode-array UV detector (model 168; Beckman Coulter, Inc.). The column was developed at a flow rate of 1 ml/min using a gradient from A (water/acetic acid, 100:0.1, v/v) to B (acetonitrile/acetic acid, 100:0.1, v/v). Solvent B was set to 20% for 2 min and increased to 100% over 18 min. Retention times of 15(*S*)-HETE and LTB_4 were checked daily using synthetic standards and were 21.7 ± 0.5 min and 17.2 ± 0.7 min, respectively. The fractions corresponding to 15(*S*)-HETE and LTB_4 were collected and evaporated to dryness under a stream of nitrogen, redissolved in RIA buffer and quantified using a specific RIA, which was performed according to the manufacturer protocol. Results were expressed as ng/2×10^6 cells.

To test if reduction of LTB_4 production may result from a shunt of AA released upon activation from 5-LO to the newly synthesized 15-LO, we performed experiments in which monocytes, after 72 h of incubation with or without IL-4, were challenged with A23187 in the presence of 30 μM AA. Arachidonic acid release by A23187-stimulated monocytes in the presence of exogenous 15(*S*)-HETE was evaluated by gas chromatography/mass spectrometry upon extraction and derivatization to the pentafluorobenzyl ester, using d_8-AA as an internal standard as previously published (Hadley et al., 1988).

Analysis of AA Metabolites by Electrospray Ionization-Tandem Mass Spectrometry. Arachidonic acid metabolites generated by monocytes stimulated with opsonized zymosan, and from selected experiments carried out with A23187 and exogenous 15(*S*)-HETE, were extracted on LC18 solid phase cartridges after addition of d_4-LTB_4 as an internal standard, and analyzed by electrospray ionization-tandem mass spectrometry. Aliquots of extracted samples (50 μl) were injected into a RP-HPLC column (Columbus 3 μm, 1 × 125 mm, Phenomenex, Torrance, CA) directly interfaced into the electrospray source of a triple quadrupole mass spectrometer (Sciex API-3000; Perkin Elmer Instruments, Norwalk, CT). The column was eluted at a flow rate of 50 μl min^{-1} using a linear gradient from 15 to 100% solvent B (A: water, 0.05% acetic acid, pH 5.7 with NH_3; B: AcCN/MeOH, 65:35) over 30 min, and arachidonic acid metabolites were detected by selected reaction monitoring, using collision-induced dissociation and specific transitions for the different metabolites (*m/z* 339 to 198 for d_4-LTB_4, 335 to 195 for LTB_4, 319 to 219 for 15(*S*)-HETE, and 335 to 201 for 5(*S*),15(*S*)-diHETE).

15-LO Activity and reverse transcription (RT)-polymerase chain reaction (PCR). To assess 15-LO activity in IL-4-treated and untreated samples, cells (2×10^6 cells/ml) were allowed to adhere to tissue culture plates for 2 h at 37°C, and were then incubated with 100 μM AA dissolved in ethanol (0.5% final concentration) for 20 min in PBS buffer. At the end of the incubation with AA, the cell supernatants were harvested and, after the addition of prostaglandin B_2 as an internal standard, were stored under an argon atmosphere at −80°C, and 15(*S*)-HETE analyzed as described above.

RT-PCR was performed on total cellular RNA, extracted from human monocytes (10^6 cells), according to the method of Chomczynski and Sacchi (1987), using RNAzol (Biotech Italia, Rome, Italy) according to the manufacturer's instructions. Briefly, RNAzol reagent was added to the cell pellet, and the cells were suspended. Chloroform was added to the homogenate and mixed thoroughly. After 5 min on ice, the suspension was centrifuged at 12,000*g* for 15 min at 4°C. The colorless upper aqueous phase was transferred to a fresh tube, an equal volume of isopropanol was added, mixed well, allowed to stand for 15 min at 4°C, and centrifuged at 12,000*g* for 15 min at 4°C. The RNA pellet was washed once with 75% ethanol, air-dried and dissolved in diethylpyrocarbonate (DEPC)-treated water (Sigma Chemical Co., St. Louis, MO). The extracted total RNA was quantified by measurement of absorbance at 260 nm with a spectrophotometer (DU-65; Beckman Coulter, Inc.). The integrity of the purified total RNA was determined by visualization of the 28S and 18S ribosomal RNA bands after electrophoresis of RNA aliquots on formaldehyde-agarose gel.

Two micrograms of total RNA were reverse transcribed to synthesize the first-strand cDNA, and a reverse transcription was carried out at 37°C for 1 h, in a reaction mixture (total volume, 25 μl) containing 250 ng of oligo(dT)12-18 primer, 10 mM dithiothreitol, 20 U of RNasin ribonuclease inhibitor, 0.5 mM each of dNTP, and 200 U of Moloney murine leukemia virus reverse transcriptase in supplied buffer (all from Invitrogen). The reaction mixture was heated to 98°C for 5 min to inactivate RT, and then cooled to 4°C. Aliquots of 10% from RT reaction were used as templates for PCR amplification.

The sequences of oligonucleotide primers used in PCR (Kaminski et al., 1996) are as follows: β-actin: sense 5′-ATTGGCAATGAGCGGTTCCG-3′, antisense 5′-CCGCCGATCCACACGGAGTA-3′; 15-LO: sense 5′- CAACGTCATTCTCTGTAGCC-3′, antisense 5′-CCATGTCAGAGACCAGCCCA-3′; 5-LO: sense external 5′-TTTGAGCTGCTGGATGGCAT-3′, antisense external 5′-GCACCCAGATTTTGGCCAAA-3′; 5-LO: sense internal 5′-GATGCCAACAAAACAGACCC-3′, antisense internal 5′-GCCAGTCGTATTTTGCATCC-3′; FLAP: sense external 5′-CACTTGCCTTTGAGCGGGTC-3′, antisense external 5′-AGGAAATGAGAAGTAGAGGG-3′; FLAP: sense internal 5′-ACACTGCCAACCAGAACTGT-3′, antisense internal 5′-AGATGGTGGTGGAGATCGTC-3′. The predicted sizes of amplification products were 281 bp in β-actin, 377 bp in 15-LO, 166 bp in 5-LO, and 298 bp in FLAP.

PCR amplification was carried out in a Perkin-Elmer model 2400 thermal cycler, in a 50-μl total volume, containing 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 1 U of *Taq* DNA polymerase, 0.2 mM each of dNTPs (Invitrogen), and 0.2 μmol/l each of oligonucleotide primers. β-actin was used as a positive control for the PCR reaction and a no template cDNA control was used as the negative control. PCR was performed for 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 35 s, and extending at 72°C for 45 s, followed by a last extension step at 72°C for 5 min to extend the partially amplified products. β-actin and 15-LO cDNAs were amplified using standard PCR (30 cycles). 5-LO and FLAP cDNAs were amplified using the nested PCR technique: after external amplification (30 cycles) a 10% aliquot was further amplified with internal primers for another 30 cycles. PCR products were analyzed by electrophoresis on ethidium bromide-stained 1.8% agarose gels. The gels were visualized under UV light exposure.

Purification of Neutrophils from Peripheral Blood and Neutrophil Chemotaxis Assay. Peripheral blood was obtained from normal subjects and neutrophils were prepared by dextran sedimentation and centrifugation over Ficoll cushions. Neutrophils were resuspended at a concentration of 1×10^6/ml in PBS buffer in the presence and absence of an LTB_4 receptor antagonist (LY 223982 10 μM; Eli Lilly, Basingstoke, UK), or 15(*S*)-HETE (1–100 μM), at 37°C. Chemotaxis was performed using a 48-well microchemotaxis chamber (Costar; Neuro Probe Inc., Cabin John, MD) as previously described (Profita et al., 2000a). Neutrophils were loaded into the upper well and the monocyte supernatant, or synthetic compound, were placed in the bottom chamber. The two wells were separated by a polycarbonate membrane filter with a pore size of 3 μm. Chamber was incubated at 37°C for 1 h. At the end of incubation, the filter was fixed, stained, and mounted on a glass microscope slide (observed at 400×). Migration was assessed by counting the number of cells that had migrated beyond a certain depth into the filter. Each experimental condition was performed in duplicate, and three to four fields were assessed for cell migration. Chemotactic activity of standard 15(*S*)-HETE, 5(*S*),15(*S*)-diHETE, and LTB_4 dissolved in PBS buffer was also evaluated.

Uptake and Metabolism of Radiolabeled 15(*S*)-HETE. Metabolism of 15(*S*)-HETE by monocytes (2×10^6 cells), was studied in cells grown for 72 h with and without IL-4 (10 ng/ml). Cells were then incubated with [^{3}H]15(*S*)-HETE (0.5 μCi/ml) for 20 min at 37°C, and stimulated with A23187 at a final concentration of 5×10^{-6} M for 15 min 37°C, in the presence or absence of MK-886. At the end of the incubation the cell-free medium was analyzed by RP-HPLC as described above. One-minute fractions were collected and radioactivity analyzed by liquid scintillation counting.

Statistical Analysis. Results are given as mean ± S.E. of n observations. Statistical analysis was performed by one-way analysis of variance. Mean data for individual experiments were also compared using Student's t test for paired or unpaired samples, as appropriate. A p value < 0.05 was considered statistically significant.

Results

Treatment with IL-4 for 24, 48, and 72 h significantly increased the amount of 15(*S*)-HETE observed after stimulation with the Ca^{2+} ionophore A23187, resulting in over a 10-fold increase after 72 h of incubation (Fig. 1A). Concomitant measurement of LTB_4 production showed a progressive decrease over IL-4 incubation time, reaching a minimum after 72 h, where LTB_4 production was less than 15% of that observed in the absence of IL-4 pretreatment (Fig. 1B). The effect of IL-4 was abolished upon pretreatment with a specific anti-IL-4 polyclonal antibody, and the production of 15(*S*)-HETE and LTB_4 after 72 h of incubation with IL-4 resulted in 2.1 ± 1 and 3.5 ± 1.1 ng/ml, respectively, compared with values of 22.6 ± 2.5 and 0.6 ± 0.1 ng/ml observed in samples treated with IL-4 in the absence of the anti-IL-4 polyclonal antibody.

To test if the effect of IL-4 on LTB_4 could be observed in the presence of a more physiological stimulus, control and IL-4-treated (72 h) monocytes were challenged with opsonized zymosan. LC/MS/MS analysis of supernatants showed the presence of 15(*S*)-HETE in IL-4-treated cells only (Fig. 2). As observed in A23187-activated monocytes, LTB_4 production in IL-4-treated monocytes was also inhibited (45 ± 11%, $n = 3$, $p = 0.05$; LTB_4 production in controls was 0.44 ± 0.24 ng/2 × 10^6 cells).

In agreement with data previously published, 15-LO activity, evaluated upon addition of excess arachidonic acid (Table 1), as well as 15-LO mRNA (Fig. 3) showed a significant increase in IL-4 treated monocytes compared with untreated cells. On the

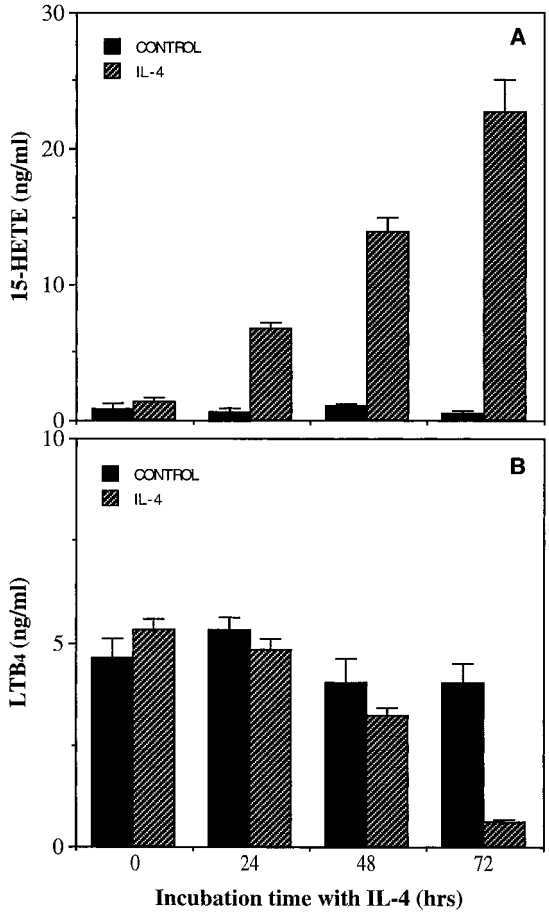

Fig. 1. Effects of IL-4 on 15(*S*)-HETE and LTB_4 production in human monocytes. The effects of IL-4 treatment on the production of 15(*S*)-HETE (A) and LTB_4 (B) were evaluated in normal human peripheral monocytes activated with A23187 (5 μM). Monocytes (2×10^6 cells/ml) were cultured in the presence or absence of IL-4 for 24, 48, and 72 h. 15(*S*)-HETE and LTB_4 were quantified in supernatants by specific RIAs after RP-HPLC separation as described under *Materials and Methods*. Data are expressed as mean ± S. E. of eight consecutive experiments. Statistical analysis was performed using Student's *t* test for unpaired samples.

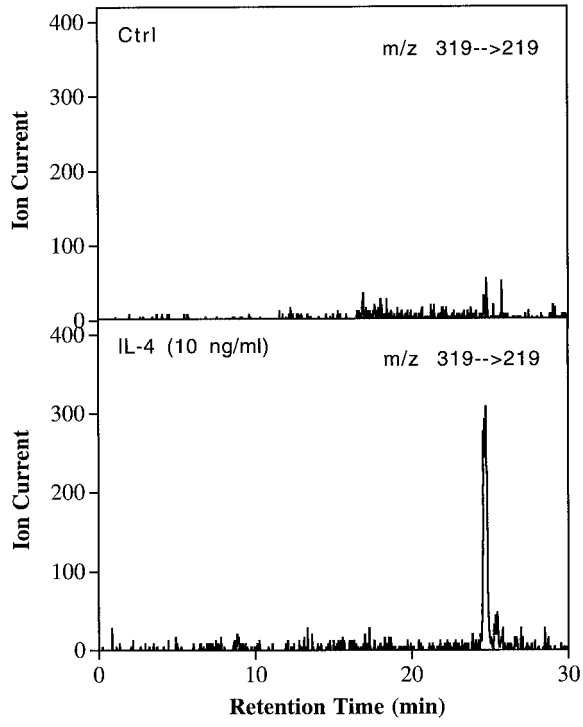

Fig. 2. LC/MS/MS analysis of supernatant from opsonized zymosan-activated human monocytes. Untreated (top panel) and IL-4 treated (bottom panel; 10 ng/ml, 72 h, 37°C) monocytes were challenged with opsonized zymosan (100 particles per cell, 1 h at 37°C). Supernatant was extracted and analyzed by LC/MS/MS as described under *Materials and Methods*. Negative ion chromatograms represent the specific transition from *m/z* 319 to *m/z* 219, resulting from the collision induced dissociation of the carboxylate anion of 15(*S*)-HETE.

TABLE 1

15-LO enzymatic activity in IL-4-treated monocytes

Monocytes (2×10^6/ml) treated with and without IL-4 (10 ng/ml) for 24, 48, and 72 h were incubated with AA (100 μM) for 15 min. 15(*S*)-HETE was quantified in supernatants by specific RIA after RP-HPLC separation as described under *Materials and Methods*. Results are expressed as mean ± S.E. of six consecutive experiments. Statistical analysis was performed using Student's *t* test for unpaired samples.

	15-HETE (ng/ml)	
	Control	AA (100 μM)
Baseline	0.2 ± 0.1	86 ± 0.5
IL-4 (24 h)	0.2 ± 0.1	134 ± 7*
IL-4 (48 h)	1.5 ± 0.2	566 ± 45**
IL-4 (72 h)	0.4 ± 0.2	745 ± 132**

* $p < 0.05$; ** $p < 0.01$ vs. baseline.

contrary, the decrease in LTB_4 production observed in IL-4 treated monocytes did not reflect decreased expression of mRNA for enzymes involved in its synthesis, such as 5-LO and FLAP (Fig. 3).

To verify if newly expressed 15-LO, which utilizes AA, may reduce LTB_4 biosynthesis simply by decreasing substrate availability, monocytes were challenged in the presence of excess substrate (30 μM AA). The results showed a significant decrease of LTB_4 in IL-4-treated monocytes compared with untreated cells (10 ± 3 and 98 ± 3 ng/ml, respectively; $n = 3$, $p < 0.01$).

We have previously shown that 15(*S*)-HETE is able to inhibit the synthesis of 5-LO metabolites in human neutrophils. To test if the product of arachidonic acid metabolism by 15-LO may be responsible for the observed inhibition of LTB_4 production, we tested the effect of 15(*S*)-HETE on A23187-induced formation of LTB_4. Preincubation of isolated monocytes with exogenous 15(*S*)-HETE prior to A23187 challenge resulted in a concentration-dependent inhibition of LTB_4 production, with an IC_{50} of 1 μM (Fig. 4). The observed inhibitory effect did not reflect a decreased availability of the substrate, as AA release was not reduced by pretreatment with 15(*S*)-HETE (31.5 ± 7.5 and 50.6 ± 11.5 ng/2 $\times$ 10^6 cells; control and 15(*S*)-HETE 10^{-5} M, respectively).

Incubation of radiolabeled 15(*S*)-HETE with human monocytes showed significant production of the double lipoxygenation product 5(*S*),15(*S*)-diHETE when cells were activated with A23187 (Fig. 5). Formation of 5(*S*),15(*S*)-diHETE required 5-LO activation and translocation as shown by complete inhibition of its synthesis by the FLAP inhibitor MK-886. LC/MS/MS analysis of monocytes preincubated with 15(*S*)-HETE, also showed the presence of the 5(*S*),15(*S*)-diHETE, as detected by the specific transition from *m/z* 335 to 201 (data not shown).

Given the potent neutrophil chemotactic activity of LTB_4, it was of interest to see if the treatment with IL-4 was able to significantly affect the biological activity of the substances

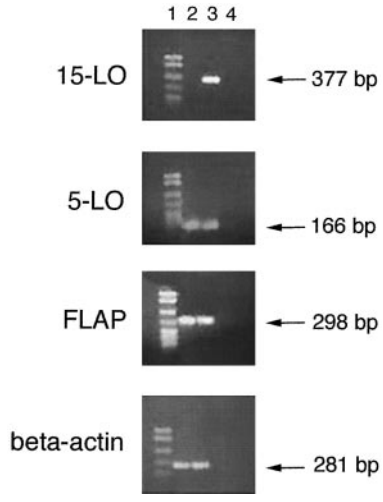

Fig. 3. Expression of lipoxygenases and FLAP mRNA in normal human peripheral monocytes cultured in the presence and absence of IL-4 for 72 h. Equal amounts of total RNA (2 ng) obtained from monocytes were reverse-transcribed into cDNA. 5-LO and FLAP were amplified with nested specific primers. Arrows indicate predicted sizes (bp) of amplification products. Number on the top of figure indicates the sample: lane 1, size marker (pBR322 DNA-Mps I Digest Biolabs, Beverly, MA); lane 2, monocytes without IL-4; lane 3, monocytes with IL-4 (10 ng/ml) for 72 h; lane 4, negative control (no template cDNA control).

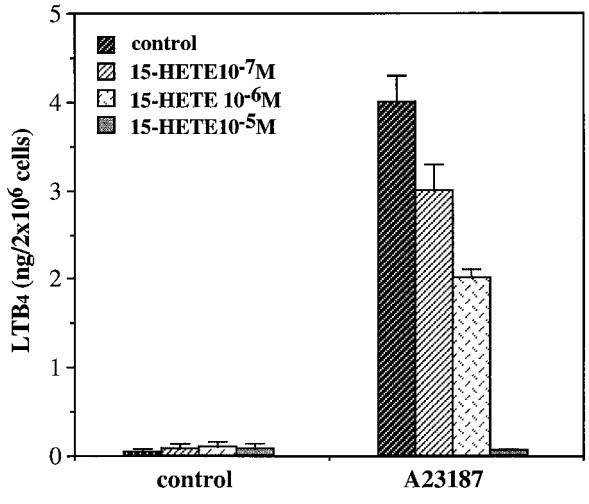

Fig. 4. Effect of exogenous 15(*S*)-HETE on LTB_4 production by human monocytes challenged with A23187. Monocytes (2×10^6 cells/ml) were preincubated with increasing concentrations of 15(*S*)-HETE and challenged with A23187 (5 μM) for 15 min. LTB_4 was quantified in supernatants by specific RIA after RP-HPLC separation as described under *Materials and Methods*. Results are expressed as mean ± S.E. of six consecutive experiments. Statistical analysis was performed using Student's *t* test for paired samples.

released by activated monocytes. We therefore evaluated the neutrophil chemotactic activity of supernatants obtained from control and IL-4-treated (72 h) monocytes after challenge with A23187. Neutrophil chemotaxis induced by the supernatants of A23187-activated monocytes was significantly lower in IL-4-treated monocytes (Fig. 6). To evaluate the specific contribution of LTB_4 to the overall chemotactic activity released by activated monocytes, we also tested their activity in the presence of the specific LTB_4 receptor antagonist LY 223982. Pretreatment with LY 223982 significantly inhibited the chemotactic activity of supernatants from control monocytes, pointing out that about 50% of the chemotactic activity released by activated monocytes was the result of the biological activity of LTB_4. A similar pretreatment did not have significant effects against the chemotactic activity of supernatants obtained from IL-4 treated cells (Fig. 6), suggesting that the reduced biological activity released by IL-4-treated monocytes upon A23187 activation well correlated with the decreased amounts of LTB_4 observed under these experimental conditions.

Given that significant neutrophil chemotactic activity has previously been reported for 15(*S*)-HETE in vivo (Johnson et al., 1985), we tested the in vitro chemotactic activity of 15(*S*)-HETE and 5(*S*),15(*S*)-diHETE. Both compounds, at the concentration of 1 μM, had an efficacy that was less than 50% of the effect observed for LTB_4 10 nM, tapering off at a higher concentration (Fig. 7); the lower efficacy and potency for 15-LO metabolites as chemotactic factors is well in agreement with the observed reduction of chemotactic activity observed in supernatants from the IL-4-treated monocyte, where a shift of arachidonic acid metabolism from LTB_4 to 15(*S*)-HETE was observed.

Discussion

The results of this study showed that IL-4 induced expression of 15-LO in human monocytes is accompanied by a significant inhibition of LTB_4 production upon challenge with either the Ca^{2+} ionophore A23187 or a physiologically relevant stimulus such as opsonized zymosan. Inhibition of LTB_4 synthesis upon treatment with IL-4 and activation of monocytes in the presence of excess AA, showed that the effect of IL-4 did not simply reflect the presence of newly expressed 15-LO, and therefore a potential decrease in substrate availability to 5-LO.

Treatment with IL-4 did not change the expression of mRNA for key enzymes involved in leukotriene biosynthesis, such as 5-LO and FLAP. Although we did not measure actual proteins or enzymatic activity, the results of the mRNAs expression suggest that the observed inhibition of LTB_4 synthesis did not result from a direct transcriptional effect of IL-4 on 5-LO and FLAP. Concerning the potential effect of IL-4 on the LTA_4 hydrolase, a recent paper reported that IL-4 may indeed increase the expression of this enzyme (Zaitsu et al., 2000). Exogenous 15(*S*)-HETE concentration-dependently inhibited the formation of LTB_4 by monocytes, without affecting the availability of the substrate AA, as evaluated measuring the AA released after A23187 challenge; this result suggests that 15(*S*)-HETE formed by IL-4-treated monocytes may be responsible for the observed inhibition of LTB_4 production.

IL-4 is a cytokine released by Th2 and mast cells (Bradding et al., 1995) that may play a major role in the pathogenesis of inflammatory lung disease (Robinson et al., 1992; Walker et al., 1992; Humbert et al., 1996). Besides its IgE-regulating effects (Del Prete et al., 1988; Pene et al., 1988), IL-4 is involved in the maturation of blood monocytes (Mosmann et al., 1986), as well as in the expression of cell membrane markers such as CD23 and class II major histocompatibility complex antigen on monocytes (de Velde et al., 1988). On the other hand, together with its potential proinflammatory activity, in normal subjects IL-4 has been found to be an inhibitor of mononuclear phagocyte functions in vitro and ex vivo (Hart et al., 1989; Yanagawa et al., 1991; Wong et al., 1992).

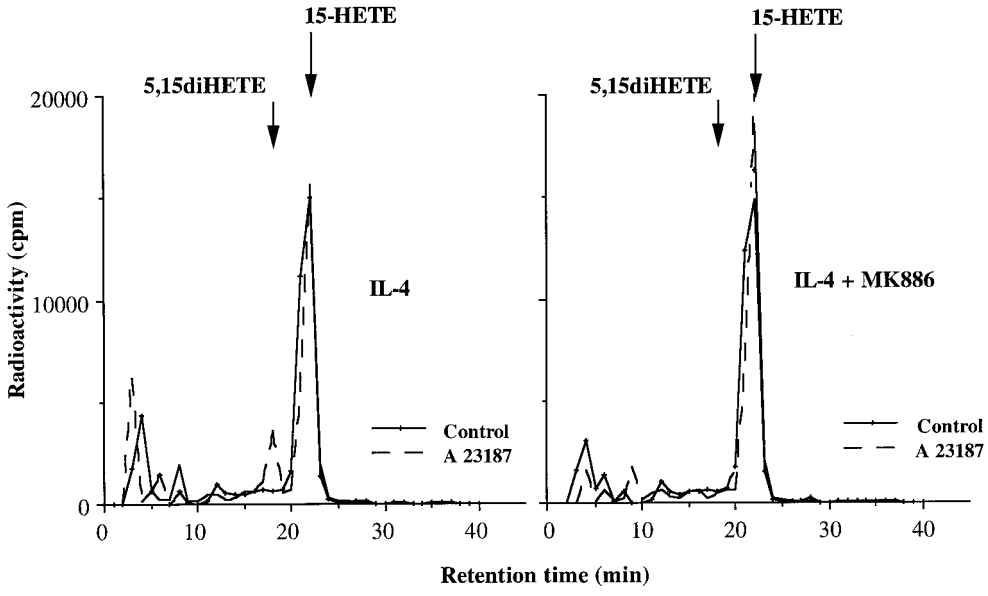

Fig. 5. Metabolism of radiolabeled 15(*S*)-HETE by human monocytes. Monocytes (2×10^6 cells/ml) were preincubated with radiolabeled 15(*S*)-HETE and challenged with A23187 (5 μM) for 15 min in the presence or absence of the FLAP inhibitor compound MK-886 (1 μM). Supernatants were extracted and analyzed by RP-HPLC as described under *Materials and Methods*. One-minute aliquots were collected and radioactivity was evaluated by liquid scintillation counting. Retention time of synthetic standard compounds are shown by arrows.

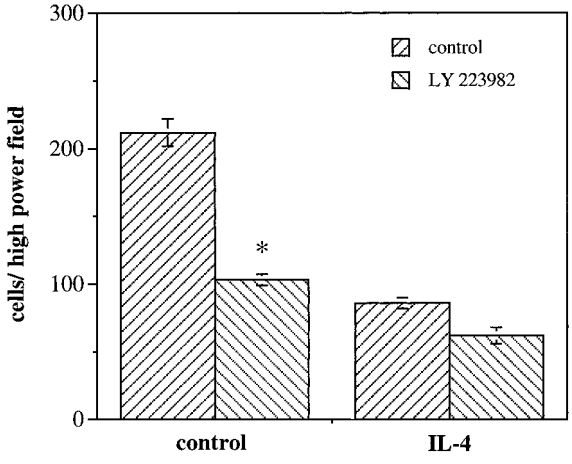

Fig. 6. Effect of IL-4 treatment on neutrophil chemotactic activity of supernatants from A23187-challenged monocytes. Chemotaxis was performed using a 48-well microchemotaxis chamber as described under *Materials and Methods*. Neutrophils were preincubated either in the absence or the presence of the LTB_4 receptor antagonist LY 223982 at the concentration of 10 μM. Results are expressed as mean ± S.E. of eight consecutive experiments. Statistical analysis was performed using Student's *t* test for unpaired samples.

This inhibition appeared to be mediated by transcriptional effects of IL-4 (Yanagawa et al., 1991) and was not due to a cytotoxic effect.

The potential for IL-4 to significantly change the profile of arachidonic acid metabolites from the production of leukotrienes to that of 15(*S*)-HETE raises important questions on the biological relevance of such an effect. 15(*S*)-HETE represents the major AA metabolite produced in lung homogenates (Hamberg et al., 1980), as well as in cultured human lung tissue obtained from both asthmatic and normal donors. It has been shown that when lung tissue obtained from asthmatics undergoes in vitro allergen challenge, 15(*S*)-HETE production is approximately 100 times greater than that of LTC_4 (Dahlen, 1983). Bronchial allergen challenge in atopic asthmatic subjects leads to a 30-fold increase of the concentrations of 15(*S*)-HETE recovered in bronchoalveolar lavage fluid (Murray et al., 1986). Once released, 15(*S*)-HETE can exert several immunoregulatory functions that may be relevant to the pathogenesis of asthma. 15(*S*)-HETE has been shown to be a potent mucosecretagogue in the human airway (Marom et al., 1982), and to possess chemotactic activity for neutrophils directly, contributing to the recruitment of these cells in the airways (Johnson et al., 1985). It has also been found that 15(*S*)-HETE can prolong the duration of airway obstruction during the early response, suggesting that it either augments the release of mediators from mast cells or potentiates the effects of other mediators on airway smooth muscle (Lai et al., 1990). On the other hand, it has been reported that 15(*S*)-HETE inhibits 5-lipoxygenase (Vanderhoek et al., 1980; Borgeat et al., 1983; Profita et al., 2000a), and incorporation of 15(*S*)-HETE into membrane phospholipids impairs the response of human polymorphonuclear neutrophil to inflammatory stimuli such as the formylated tripeptide fMLP (Brezinski and Serhan, 1990).

Recently we showed that 15(*S*)-HETE is present in high concentration in induced sputum obtained from asthmatics (Profita et al., 2000b), as well as that of chronic bronchitis patients (Profita et al., 2000a). Furthermore we showed that its concentration inversely correlates with the percentage of neutrophils recovered in induced sputum, and that 15(*S*)-HETE inhibits LTB_4 production in A23187, as well as in fMLP-activated human neutrophils (Profita et al., 2000a). Hence, the ability of IL-4 to increase the release of 15(*S*)-HETE by human monocytes/macrophages, and to concomitantly inhibit the production of a potent chemotactic factor such as LTB_4, may play an important role in the evolution of pulmonary inflammatory responses. In agreement with the expected effect of reduced production of LTB_4, supernatants from IL-4-treated monocytes showed a significantly lower chemotactic activity toward neutrophils. Neutrophil chemotaxis evaluation in the presence of an LTB_4-receptor antagonist showed that over 50% of the chemotactic activity of activated monocytes depends on the presence of the 5-LO metabolite LTB_4. This result suggests that neutrophilic influx following monocyte activation may be significantly inhibited by the expression of 15-LO induced by IL-4.

A recent paper raised the possibility that inhibition of 5-LO product formation by 15(*S*)-HETE in human neutrophils

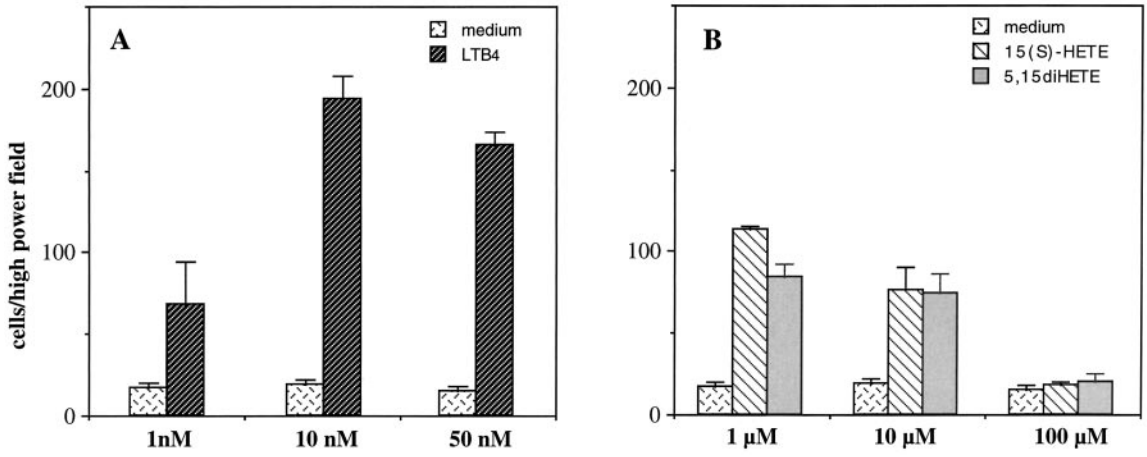

Fig. 7. Neutrophil chemotactic activity of LTB_4 (A), and 15(*S*)-HETE and 5(*S*),15(*S*)-diHETE (B). Neutrophil chemotactic activities of LTB_4 (1–50 nM), 15(*S*)-HETE, and 5(*S*),15(*S*)-diHETE (1–100 μM) were tested using a 48-well microchemotaxis chamber as described under *Materials and Methods*. Results are expressed as mean ± S.E. of six consecutive experiments.

(Petrich et al., 1996) may simply reflect that 15(*S*)-HETE is an alternative substrate to arachidonic acid for 5-LO. We tested the metabolism of radiolabeled 15(*S*)-HETE in monocytes and found that measurable formation of the dihydroxy-derivative 5(*S*),15(*S*)-diHETE could indeed be observed. Interestingly, as recently shown by Mancini and coworkers by selective expression of 5-LO and FLAP in Sf9 insect cells (Mancini et al., 1998), conversion of 15(*S*)-HETE by 5-LO was dependent on FLAP, and pretreatment with the FLAP inhibitor MK-886 completely abolished the conversion of radiolabeled 15(*S*)-HETE in monocytes. This evidence suggests that FLAP is responsible for appropriate handling of substrate, either AA of 15(*S*)-HETE, to 5-LO. Irrespective of the mechanism underlying the inhibition of LTB_4 synthesis (true inhibitor or alternative substrate) it is important to stress that IL-4-induced expression of 15-LO causes a significant shift in arachidonic acid metabolism from LTB_4 to 15(*S*)-HETE, and possibly minor amounts of 5(*S*),15(*S*)-diHETE. This shift appears to be biologically relevant as we found that 15(*S*)-HETE, as well as 5(*S*),15(*S*)-diHETE, both showed only a very modest neutrophil chemotactic activity (approximately 50% of the efficacy of LTB_4 at 100-fold higher concentration); furthermore this effect tapered off at higher concentrations. This modest chemotactic activity may be responsible for the results observed after in vivo administration of 15-HETE in dogs, in which enhanced influx of neutrophils as well as mast cells was observed (Johnson et al., 1985). On the other hand, the potential effect on the production of LTB_4 may contribute to the observed inhibitory effect of 15(*S*)-HETE on neutrophil responses in vitro (Brezinski and Serhan, 1990). These complex mechanisms may well play a role in the regulation of the resolution of the inflammatory response.

In conclusion, this study shows that in human monocytes IL-4 may directly regulate the production of arachidonic acid-derived lipid mediators, such as LTB_4 and 15(*S*)-HETE, therefore contributing to the evolution of inflammatory responses resulting in monocyte activation. The evidence provided in this study underscores a potential modulatory role for IL-4 in the inflammatory response, unveiling a novel anti-inflammatory activity of this cytokine.

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Address correspondence to: Dr. Angelo Sala, Center for Cardiopulmonary Pharmacology, Via Balzaretti 9, 20133 Milan, Italy. E-mail: angelo.sala@unimi.it