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Contrasting effect of interleukin-4 on the expression of cytosolic phospholipase A2, 5-lipoxygenase and 5-lipoxygenase-activating protein in peritoneal macrophages from control and ovalbumin-sensitized rats.

Abstract

Interleukin-4 (IL-4), which has been widely described as an anti-inflammatory cytokine, can also exert proinflammatory effects. In this study, we extend these

findings to demonstrate, in an allergic model, the dual effect of IL-4 on arachidonic acid (AA) metabolism in macrophages. In peritoneal macrophages from control rats (cPM), IL-4 had no effect on cPLA2 and 5-LO expression, but increased FLAP expression without affecting basal and A23187- or PMA-challenged arachidonic acid (AA) metabolism. In contrast, in peritoneal macrophages from ovalbumin-sensitized rats (sPM), IL-4 decreased cPLA2, 5-LO and FLAP expression and PMA-challenged eicosanoid production. A23187-challenged AA metabolism of sPM was not affected by IL-4 pretreatment. Thus, IL-4 acts differently on cPLA2, 5-LO and FLAP expression and AA metabolism in peritoneal macrophages depending on their resident or sensitization-induced differentiated status.

Full text

INTRODUCTION

Interleukin-4 (IL-4) is a Th2-derived cytokine involved in the modulation of inflammatory processes. It is considered as an anti-inflammatory cytokine because it inhibits the production of inflammatory cytokines such as IL-1beta and TNF-alpha in monocytes/macrophages [1-3], the expression of receptors such as CD14 and TNF receptors [4, 5], and it amplifies the expression of anti-inflammatory products such as the IL-1 receptor antagonist [6, 7]. IL-4 is also able to reduce arachidonic acid metabolism in activated cells, such as cells from inflammatory sites [8] or cells stimulated *in vitro* by proinflammatory cytokines (TNF-alpha, IL-1alpha) [9, 10] or infectious agents (LPS) [4, 8]. However, in unstimulated cells, IL-4 has been found to have proinflammatory effects. Studies have demonstrated that IL-4 can stimulate the release of inflammatory molecules such as leukotriene B4 in normal human monocytes [11]. This ability of IL-4 to have pro- and anti-inflammatory effects is not so surprising given that IL-13, another Th2-derived cytokine which shares receptor and signaling pathways with IL-4 [12, 13] also has, as demonstrated in our laboratory, anti-inflammatory effects (decrease in PMA-induced respiratory burst in human monocytes [14] and pro-inflammatory effects (induction of cytosolic phospholipase A_2 ($cPLA_2$) mRNA and protein expression and increase in zymosan-activated arachidonic acid (AA) metabolism in mouse peritoneal macrophages [15]).

The aim of this work was to study this dual IL-4 effect in a physiopathological model. Interleukin-4 is secreted during allergic reactions [16] in which

leukotrienes produced by various cells, notably macrophages, participate in the symptomatology of the reaction. This cytokine is considered to be the major cytokine involved in the sensitization phase of hypersensitivity reactions, by inducing IgE synthesis [17-20] and expression of CD23, a low-affinity receptor for IgEs, on monocytes/macrophages [21, 22]. We previously demonstrated in an allergic rat model that peritoneal macrophages from ovalbumin-sensitized animals secreted leukotrienes on *in vitro* ovalbumin challenge whereas peritoneal macrophages from control rats did not [23]. In the present study, we used this allergic rat model to compare the effect of IL-4 on leukotriene metabolism of macrophages from control or ovalbumin-sensitized rats.

Leukotrienes are lipoxygenated metabolites of arachidonic acid (AA), a 20-carbon fatty acid which is a constituent of cell membrane lipids. The first rate-limiting step of leukotriene synthesis is the release of free AA from membrane phospholipids (mostly from phosphatidylcholine but also from phosphatidylserine and phosphatidylinsitol [24]) by the AA-selective, 85 kDa cytosolic phospholipase A_{2alpha} ($cPLA_2$) [25-27]. The second rate-limiting enzymatic step of leukotriene synthesis is the conversion of free AA by 5-lipoxygenase (5-LO). The activation of 5-LO requires its translocation to the nuclear envelope [28, 29] where it can interact with an 18 kDa membrane protein termed five-lipoxygenase-activating protein (FLAP) [30, 31].

This study reports the contrasting effects of IL-4 on $cPLA_2$, 5-LO and FLAP expression and on basal and challenged AA metabolism of peritoneal macrophages from control and ovalbumin-sensitized rats.

MATERIALS AND METHODS

Animals and reagents

Male Brown-Norway rats (220-250 g) were purchased from Iffa-Credo (l'Arbresle, France) and housed under a 12-hour light/dark cycle in an air-conditioned room (25° C, 50% relative humidity), and under pathogen-free conditions.

Ovalbumin, PMA and A23187 were obtained from Sigma Chemical (La Verpillière, France). Rat interleukin-4 was purchased from Serotec (Oxford, England). Monoclonal anti-rat $cPLA_2$ antibody (4-4B-3C) was provided by Tebu (Le Perray en Yvelines, France). The polyclonal anti-5-LO and anti-FLAP antibodies were a

generous gift from Dr Ford-Hutchinson (Merck Frosst, Canada). They were respectively raised against human 5-LO and FLAP [32, 33] but can be used with rat enzymes [34, 35]. The secondary antibodies, the ECL kit, the [^{3}H]-labelled AA (specific activity 220 Ci/mmol) and the radiolabelled phospholipid, neutral lipid, prostaglandin and leukotriene standards were provided by Amersham (France). The culture media were prepared using Gibco products (Cergy-Pontoise, France). Protein concentrations were measured using a Bio-Rad assay kit supplied by Sigma (St-Quentin-Fallavier, France). All reagents and solvents were of analytical grade.

Active sensitization of animals

Active sensitization of rats against ovalbumin was achieved, as already reported [23], by two subcutaneous injections of 0.5 mg of ovalbumin and 100 mg of $Al(OH)_3$ in 0.5 ml NaCl 0.9%. Control rats received two subcutaneous injections of 0.5 ml NaCl 0.9%.

Preparation of peritoneal cells

Control and sensitized rats were anaesthetized and killed 14 days after the sensitization procedure. Peritoneal cells were harvested by peritoneal lavage with 100 ml 199 medium containing 20 IU/ml heparin, 100 IU/ml penicillin and 100 mug/ml streptomycin. After centrifugation at 800 x *g* for 10 min at 20° C, the cell pellet was resuspensed in Dulbecco's modified Eagle's medium containing 1% FCS, 100 IU/ml penicillin, 100 mug/ml streptomycin and 0.6 mM glutamine (DMEM-FCS).

Macrophage purification

Peritoneal cell suspensions from control and sensitized rats were seeded in Falcon multiwell plates (1.10^6 cells per well for studies of AA metabolism and 2.10^6 cells for $cPLA_2$, 5-LO and FLAP expression). Cells were left to adhere for two hours at 37° C in an atmosphere of 5% CO_2 in air. Then, they were gently rinsed twice with DMEM to remove non-adherent cells. As demonstrated by May-Gründwald-Giemsa staining, more than 98% of the adherent cells were macrophages (control macrophages (cPM) when the cell suspension was from control rats, and sensitized macrophages (sPM) when the cell suspension was

from actively sensitized rats). Macrophage viability was assessed by trypan blue dye exclusion. More than 98% of the macrophages were viable.

IL-4 treatment and [^{3}H]AA incorporation

Purified cPM and sPM were incubated in the presence or absence of rat IL-4 (1 ng/ml) for 8 or 24 hours in 500 mul DMEM, at 37° C in an atmosphere of 5% CO_2 in air. For the AA metabolism study, 1 muCi [^{3}H]AA was added to the culture medium in the wells at the same time as the cytokine.

Study of cPLA$_2$, 5-LO and FLAP expression

Macrophages were incubated for 8 or 24 hours with 1 ng/ml rat IL-4. Then, they were scraped off into 40 mul homogenization buffer containing 50 mM potassium phosphate, 100 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin and 60 mg/ml soybean trypsin inhibitor (pH 7.1). The total protein content of each sample was quantified by Bradford's method using a Bio-Rad Protein Assay Kit with bovine gamma-globulin as standard. Then, equal amount of total protein was separated by SDS-PAGE and transferred onto a nitrocellulose membrane, which was incubated with the anti-cPLA$_2$ antibody (1:100), the anti-5-LO antibody (1:3,000) or the anti-FLAP antibody (1:5,000) for 1 hour. Immune complexes were revealed by an anti-mouse IgG coupled to peroxidase at a 1:1,000 dilution (for cPLA$_2$ immunodetection) or with anti-rabbit IgG coupled to peroxidase at a 1:5,000 dilution (for 5-LO and FLAP immunodetection) and visualized by an ECL system. Bands were numerized using a scanner and Adobe Photoshop software, then quantified using the program Morphostar (IMSTAR, France).

Arachidonic acid metabolism study

To evaluate the AA metabolism of macrophages, both the process of [^{3}H]AA incorporation by macrophages and the ability to release free [^{3}H]AA and [^{3}H]metabolites by [^{3}H]labelled macrophages were studied. The study of the [^{3}H]AA incorporation process was performed at the end of the incorporation by scraping off the adherent macrophages in 800 mul distilled water in order to determine into which lipids the [^{3}H]AA had been incorporated. The ability of the [^{3}H]labelled macrophages to release free [^{3}H]AA and [^{3}H]metabolites was studied after removing the [^{3}H]AA incorporation medium and incubating the

labelled cells in 500 mul DMEM with or without stimulus (1 muM A23187 or 100 nM PMA) for a further hour. At the end of this hour, the supernatant and the 500 muL DMEM used to rinse each well were collected to determine [^{3}H]AA and [^{3}H]metabolite secretion in the culture medium.

Extraction and analysis of membrane lipids

The extraction of membrane phospholipids and neutral lipids used a technique initially described by Bligh and Dyer [36]. Briefly, cell lipids were extracted after adding a mixture of chloroform and methanol (water/chloroform/methanol; 1:1:1, v/v). The chloroform phase containing the lipids was evaporated to dryness under nitrogen. The residues were dissolved in 80 mul chloroform and applied to thin layer silica-gel plates (LK-6- DF Whatman) previously heated for 1 hour at 100° C. The plates were run up to 10 cm with chloroform/methanol/water/triethylamine (30:34:8:35, v/v) to separate the radioactive phospholipids (phosphatidylcholine, PC; phosphatidylserine, PS; phosphatidylethanolamine, PE; phosphatidylinositol, PI), and were dried. They were then run again in the same direction for a further 6 cm with petroleum ether/diethyl ether/acetic acid (50:50:1, v/v) to separate the cholesteryl esters (CE) and neutral lipids (monoacylglycerol, MG; diacylglycerol, DG, triacylglycerol, TG; fatty acid, FA). The radioactive metabolites were identified with a Berthold TLC Scanner using retention times and co-migration with authentic standards. The results are expressed in dpm/10^6 cells.

Extraction and analysis of free [^{3}H]AA and [^{3}H]metabolites secreted by macrophages

[^{3}H]labelled macrophage supernatants were centrifuged at 3,000 x *g* for 10 min, acidified to pH 5.0 with 1 M HCl and then loaded onto Sep-Pack C18 columns (Waters) previously activated by methanol and distilled water. The loading of pure methanol (5 ml) on the columns allowed the elution of free [^{3}H]AA and [^{3}H]metabolites (extraction rate of approximately 80%). The methanol was evaporated to dryness under nitrogen. The residue was dissolved in 80 mul methanol and applied to thin-layer silica-gel plates (LK-6-DF Whatman) previously heated for 1 hour at 100° C. The organic phase of ethyl acetate/water/isooctane/acetic acid (110:100:50:20, v/v) was used to separate the [^{3}H]products (6-keto-prostaglandin F_{1alpha}, 6k-PGF_{1alpha}; thromboxane B_2, TXB_2; prostaglandin E_2, PGE_2; prostaglandin F_{2alpha}, PGF_{2lpha}; prostaglandin D_2,

PGD_2; leukotrienes (leukotriene B_4, LTB_4; leukotriene C_4, LTC_4; leukotriene D_4, LTD_4), hydroxyeicosatetraenoic acids, HETEs and AA). The radioactive metabolites were identified with a Berthold TLC Scanner using retention times and co-migration with authentic standards.

Data analysis

SDS-PAGE gels were from a representative experiment composed of three separate experiments. A statistical analysis of gels from three independent experiments was performed. Data from numerization and quantification of bands were expressed as the percentage of the numerical value of the band of IL-4-treated macrophages relative to the numerical value of the band of untreated macrophages. These data were presented as mean ± standard error (SEM) of the three independent experiments. The effect of IL-4 was considered significant when the confidence interval of the data did not include the reference value 100.

Concerning the study of AA metabolism, results were expressed as the percentage of incorporated radioactivity and were the mean ± SEM of three independent experiments. Statistical analysis of the data was performed using a 2-way analysis of variance with 3 replications per experimental group (SAS software).

For all these statistical analysis, $P < 0.05$ was considered significant.

RESULTS

$cPLA_2$, 5-LO and FLAP expression in IL-4-treated cPM and sPM

Adherent cPM and sPM were cultured for 8 and 24 hours in the presence of IL-4 and their $cPLA_2$, 5-LO and FLAP content were analysed as described in Materials and Methods.

In cPM, expression of $cPLA_2$ and 5-LO in cells incubated for 8 or 24 hours in DMEM increased through-out the culture time. As shown in the gels representative of three independent experiments (Figure 1), and as demonstrated by the statistical analysis of data from the three independent experiments (Table 1), the presence of IL-4 in the culture medium of cPM did not

significantly modify the expression of $cPLA_2$ or 5-LO at any of the culture time points. In contrast, the expression of FLAP in cPM cultured without IL-4 decreased throughout the culture. The addition of IL-4 into the culture medium significantly increased the expression of FLAP in cPM at 24 hours (Figure 1, Table 1).

In sPM, expression of $cPLA_2$ decreased with culture time. The addition of IL-4 to the culture medium of sPM amplified this decrease at 8 and 24 hours (Figure 1, Table 1).

The expression of 5-LO in sPM did not vary significantly during the culture. The addition of IL-4 into the culture medium induced a dramatic decrease of the expression of 5-LO in sPM at each time point. At 24 hours, the expression of 5-LO was so inhibited by the cytokine that it became undetectable. The expression of FLAP in sPM increased with culture time, contrary to cPM. In sPM, IL-4 inhibited the expression of FLAP at 8 and 24 hours (Figure 1, Table 1).

Basal AA metabolism in IL-4-treated cPM and sPM

Adherent cPM and sPM were incubated in the presence of 1 muCi [^{3}H]AA ± 1 ng/ml IL-4 for 24 hours in DMEM-FCS. Analysis of the incorporation of [^{3}H]AA into membrane lipids at the end of the incorporation course showed that tritiated AA was principally incorporated into PC and PI/PE in control and IL-4-treated cPM and sPM (Figure 2). Two-way analysis of variance of data showed that, in cPM, IL-4 potentiated [^{3}H]AA incorporation into PC and PI/PE as compared to untreated macrophages (Figure 2). More [^{3}H]FA was also found in IL-4-treated cPM. Unlike in cPM, in sPM IL-4 induced an increase in AA incorporation into PS and did not modify the incorporation of [^{3}H]AA into neutral lipids (Figure 2).

To study basal eicosanoid secretion, prelabelled cPM and sPM were rinsed, then cultured, for a further hour, in DMEM alone. At the end of this hour of culture, [^{3}H]metabolites secreted in the culture medium were extracted, identified and measured as described in Materials and Methods. The [^{3}H]cyclooxygenated and [^{3}H]lipoxygenated metabolite production are shown in Figure 3. Two-way analysis of variance of these data shows, on one hand, no significant effect of IL-4 or sensitization, and on the other hand, no significant interaction between the sensitization and the IL-4 treatment. Thus, IL-4 treatment of rat peritoneal

macrophages, whether they are from control or sensitized animals, does not modify their basal AA metabolism.

A23187 and PMA-challenged AA mobilization from phospholipids and AA metabolism in IL-4-treated cPM and sPM

To study the effect of IL-4 on A23187- and PMA-stimulated cPM and sPM, prelabelled macrophages pretreated or not by IL-4 were rinsed then cultured, for a further hour, in DMEM containing 1 muM A23187 or 100 nM PMA. At the end of the stimulation period, [^{3}H]metabolites secreted in the culture medium were extracted, identified and measured as described in Materials and Methods.

As demonstrated by 2-way analysis of variance, A23187 and PMA significantly induced mobilization of [^{3}H]AA from phospholipids, and production of cyclooxygenated and lipoxygenated [^{3}H]metabolites in the culture medium in untreated and IL-4-treated cPM and sPM ($P = 0.0001$) (Figure 4). In both types of macrophages, PMA appeared to be a better stimulator of AA mobilization than A23187.

In cPM, IL-4 had no significant effect on [^{3}H]AA mobilization induced by A23187 or PMA. IL-4 did not modify the production of [^{3}H]cyclooxygenated or [^{3}H]lipoxygenated metabolites (Figure 4) and did not modify the release of free [^{3}H]AA in these cells (data not shown).Thus, no significant interaction between IL-4 treatment and PMA/A23187 challenge was found in cPM.

In contrast, IL-4 was found to exert a significant effect on [^{3}H]AA mobilization and [^{3}H]cyclooxygenated and [^{3}H]lipoxygenated metabolite production in sPM (respectively $P = 0.011$, $P = 0.0008$, $P = 0.0047$). Moreover, this IL-4 effect was found to significantly interact with PMA/A23187 stimulation. Indeed, IL-4 significantly decreased the [^{3}H]AA mobilization and the cyclooxygenated and lipoxygenated [^{3}H]metabolite production induced by PMA (Figure 4).

Detailed analysis of cyclooxygenated and lipoxygenated [^{3}H]metabolites secreted in the culture medium showed (Figure 5) that the significant interaction between IL-4 treatment and PMA challenge concerns the production of TXB_2, PGE_2, PGF_{2alpha} and PGD_2, LTC4/D4 and HETEs, indicating that IL-4 inhibits the production of these metabolites in sPM.

Cholesterol ester metabolism during A23187 and PMA challenge of cPM and sPM

In [^{3}H]labelled macrophages pretreated or not by IL-4, variations of [^{3}H]AA incorporation into CE during stimulation by PMA or A23187 were analysed by comparing the quantity of [^{3}H]CE at the end of the incorporation course and the quantity of [^{3}H]CE at the end of the last hour of stimulation in cPM and sPM.

In the absence of any agonist, the pool of [^{3}H]CE decreased in cPM and sPM during the last hour of culture (Figure 6). In contrast, on stimulation by PMA or A23187, whereas the pool of [^{3}H]phospholipids decreased (data not shown), the pool of [^{3}H]CE in cPM and sPM increased during the hour of stimulation (P = 0.0001 as demonstrated by the 2-way analysis of variance) (Figure 6). Interestingly, the magnitude of the increase of [^{3}H]CE induced by PMA and A23187 paralleled the magnitude of the eicosanoid production induced by these agonists. Thus, during PMA or A23187 stimulation, part of the [^{3}H]AA mobilized is redistributed into CE. We did not observe any AA reincorporation in other phospholipids (data not shown). In cPM, pretreatement by IL-4 did not modify this A23187- or PMA-induced [^{3}H]AA reincorporation into CE (Figure 6). In contrast, in sPM, IL-4 dramatically decreased the PMA-[^{3}H]AA reincorporation into CE (P = 0.0245).

DISCUSSION

The majority of studies reporting the effect of IL-4 on AA metabolism have been performed in activated cells. Thus, the inhibitory effects of the cytokine have been widely described both in cells from inflammatory sites [8] and in cells stimulated by inflammatory cytokines [9], LPS [4, 8] or A23187 [37]. Nevertheless, some papers described pro-inflammatory effects of IL-4 in resting cells. In human monocytes, IL-4 was found to induce LTB4 production [11]. As demonstrated by our group, IL-13, another Th2-derived cytokine which shares receptor and signaling pathways with IL-4 [12, 13], induces cPLA$_2$ mRNA and protein expression and increases AA mobilization induced by opsonized zymosan in murine peritoneal macrophages [15]. In the present study, we analysed the potential dual role of IL-4 using a rat model of the hypersensitivity reaction in which IL-4 is known to be strongly involved. We studied the effect of IL-4 on AA metabolism in peritoneal macrophages from control rats and

peritoneal macrophages from ovalbumin-sensitized rats in order to compare the effect of the cytokine according to the maturation status of the macrophages.

Analysis of $cPLA_2$, 5-LO and FLAP expression in both types of macrophages showed a different modulation of the expression of these enzymes during culture according to whether macrophages are from control or ovalbumin-sensitized rats. In our culture conditions, $cPLA_2$ expression decreased in sPM while it increased in cPM. Basal expression in $cPLA_2$ was found to be higher in sPM as compared to cPM (unpublished data). Thus, this culture-induced decrease of $cPLA_2$ in sPM could result from the lack of a stimulating agent present in sensitized rats and whose role is to maintain enhanced expression of $cPLA_2$ in peritoneal macrophages. This agent is not IL-4 because the addition of IL-4 to the culture medium of sPM did not counteract this inhibitory effect.

In sPM, 5-LO expression remained stable during culture whereas it increased in cPM. Finally, whereas FLAP expression decreased during culture in cPM, it increased in sPM. This difference of modulation of $cPLA_2$, 5-LO and FLAP expression during culture in cPM and sPM, could result from a difference of adhesion marker expression or from a difference of adhesion-induced signalling transduction in the two types of macrophages.

These observations confirm that ovalbumin-induced sensitization of rats provides a very interesting model of macrophages in different states of maturation, with control resident macrophages (cPM) and differentiated, but not activated, sensitized macrophages (sPM). Indeed, this model allowed us to show that IL-4 has contrasting effects on AA metabolism according to whether macrophages are from control or sensitized rats.

In cPM, IL-4 did not affect $cPLA_2$ and 5-LO expression but it increased FLAP expression of macrophages by opposing the inhibitory effect of culture. In contrast, in sPM, IL-4 decreased the expression of $cPLA_2$, 5-LO and FLAP. Inhibitory effects of IL-4 on the expression of the enzymes involved in AA metabolism have already been reported in stimulated cells. IL-4 inhibits $cPLA_2$ mRNA and protein expression in synovial cells from inflammatory sites [8]. It also inhibits the LPS-induced expression of cyclooxygenase-2 in human alveolar macrophages [4] and the LPS-induced expression of $cPLA_2$ in U. 937 cells [8]. However, the originality of our result is that the inhibitory effect of IL-4 occurred in unstimulated but sensitization-differentiated cells. This dual effect of IL-4

according the differentiation status of cells is supported by the study of Naïf *et al.* [38] who reported that IL-4 stimulates HIV RNA expression in monocytes whereas it inhibits it in monocyte-derived macrophages.

It remains to be elucidated how IL-4 decreases the expression of $cPLA_2$, 5-LO and FLAP in macrophages from sensitized rats. It is likely that the inhibitory action of IL-4 in these cells uses a signaling pathway that does not exist in macrophages from control rats. IL-4 could down-regulate the expression of these proteins transcriptionally or translationally. IL-4 has been described to inhibit c-fos and c-jun mRNA expression [39] whose proteins are potential activators of the transcriptional AP-1 box of the $cPLA_2$ gene promoter [40], and to inhibit NF-kappaB, whose binding site exists on the promoter of the $cPLA_2$ and 5-LO genes [40, 41].

Analysis of [^{3}H]AA metabolism in cPM and sPM showed that different phospholipids were involved in the increased AA incorporation according to whether IL-4-treated macrophages were from control or ovalbumin-sensitized rats. These results suggest that ovalbumin-sensitization induces in peritoneal macrophages modifications of the expression and/or activity of acyltransferases and of the other forms of PLA2s involved in AA incorporation.

IL-4 did not modify basal AA metabolism in either cPM or sPM, suggesting that neither the IL-4-increased FLAP expression observed in cPM nor the IL-4-decreased $cPLA_2$, 5-LO and FLAP expression observed in sPM have consequences on basal eicosanoid production in peritoneal macrophages. Similar observations have already been reported. Alveolar macrophages, as compared to monocytes, have greater amounts of 5-LO and FLAP but have the same basal eicosanoid production [42]. The present results differ from previous data which showed that IL-4 induced basal eicosanoid production in cPM and had no effect in sPM [23]. This difference could be attributed to the IL-4 used. M'Rini *et al.* [23] used human IL-4 to treat rat macrophages whereas, in this study, we used rat IL-4. The comparison of these two studies shows the importance of respecting species. The contrasting effects of IL-4 on AA metabolism in rat macrophages poses the question as to the effect of IL-4 in humans. It would be interesting to compare the effects of IL-4 in human monocytes from control and allergic patients.

When stimulated by A23187 or PMA, we observed AA mobilization in both cPM and sPM. In both types of macrophage, released AA was metabolized into cyclooxygenated and lipoxygenated metabolites but a position was also reincorporated into CE. The magnitude of AA reincorporation into CE correlates with the magnitude of AA metabolization into eicosanoids, suggesting that A23187 and PMA stimulate the activation of acyl-coenzyme A:cholesterol acyltransferase equally in cPM and sPM, and that the quantity of AA reincorporation into CE depends on the quantity of AA released by A23187 and PMA.

No difference in A23187- and PMA-induced eicosanoid production was observed between cPM and IL-4-treated cPM. Thus, the IL-4-induced FLAP expression does not turn cPM AA metabolism towards leukotriene secretion as expected. This result is inconsistent with the studies of Coffey *et al.* [43] who showed that an increase in FLAP expression accounts for the capacity for 5-LO metabolism of alveolar macrophages as compared to peripheral blood monocytes. 5-LO is predominantly nuclear in alveolar macrophages, contributing to the high leukotriene synthesis capacity of alveolar macrophages [44]. The discrepancy between our results and Coffey's could be explained by a difference of 5-LO localization in the cells. Thus, it will be interesting to study the localization of this enzyme in IL-4-treated cPM.

In sPM, IL-4, described to inhibit $cPLA_2$, 5-LO and FLAP expression, inhibits PMA-stimulated eicosanoid synthesis whereas it has no effect on A23187-stimulated eicosanoid synthesis. Taken together, these results suggest that AA mobilization is more dependent on the signaling pathway leading to $cPLA_2$ activation than on the $cPLA_2$ expression level. The results confirm that the A23187-induced signaling pathway, involving calcium mobilization, is a minor pathway as compared to the PKC pathway triggered by PMA [45]. They also show that IL-4 exerts different effects on these signaling pathways. Concerning the PKC pathway, IL-4 was found to inhibit PMA-induced AA metabolism only in sPM, suggesting that the PKC pathway is modified in peritoneal macrophages during ovalbumin immunization. It would be interesting to test in sPM, whether IL-4 inhibits the $cPLA_2$ phosphorylation induced by MAP-kinases and PKC.

The differential responses of cPM and sPM to IL-4 we observed in this study, could be linked to structural and/or functional modifications of IL-4 receptor, induced by active sensitization. Indeed, differential responses of human

monocytes and macrophages to IL-4 and IL-13 have been linked to reduced gammac chain and IL-13Ralpha1 mRNA levels of IL-4 and IL-13 receptors in monocyte-derived macrophages and to a significant reduction in the ability of IL-4 and IL-13 to activate STAT6 [46].

This work raises the question of the significance of the contrasting effects of IL-4 in hypersensitivity reactions. During the sensitization phase, IL-4 participates in the sensitization process by inducing the synthesis of allergen-specific IgEs [17-20] and by inducing the expression of CD23 on monocytes/macrophages [21, 22]. IL-4 could also, by its inductive action on FLAP expression in macrophages, prepare these cells to secrete leukotrienes in response to allergen challenge as we have already reported [23]. If this is the case, IL-4 would favor allergic inflammation. During allergic responses, IL-4, which is again secreted [16], could then act as an anti-inflammatory mediator by decreasing the expression of the enzymes involved in leukotriene production as we observed in this study.

CONCLUSION

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Figures

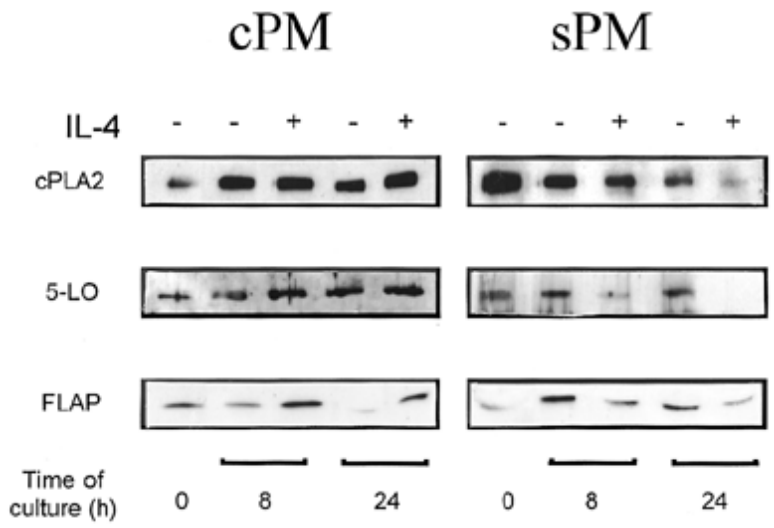


Figure 1

Effect of IL-4 on the expression of $cPLA_2$, 5-LO and FLAP in cPM and sPM.

Adherent cPM and sPM were cultured in DMEM-FCS ± IL-4 for 8 or 24 hours then lysed as described in Materials and Methods. Protein extracts were submitted to electrophoresis and immunoblot analysis. For each protein, a representation of three independent experiments is shown.

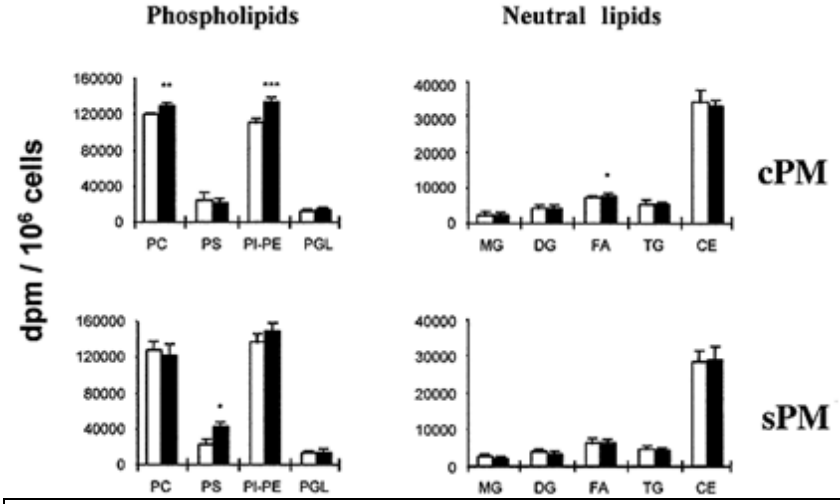


Figure 2

Effect of IL-4 on [^{3}H]AA incorporation in phospholipids and neutral lipids of cPM and sPM.

Adherent cPM and sPM were incubated in the presence of 1 muCi [^{3}H]AA for 24 hours in DMEM-FCS with (solid bars) or without (open bars) IL-4. At the end of the incorporation course, membrane lipids of cPM were extracted and identified as described in Materials and Methods. Results are expressed in dpm/10^6 cells and are means ± SEM of 3 experiments. Statistical analysis of data was performed using 2-way analysis of variance with 3 replications per experimental group. * (**, ***) $P < 0.05$ (0.01, 0.001) indicates a significant effect of IL-4.

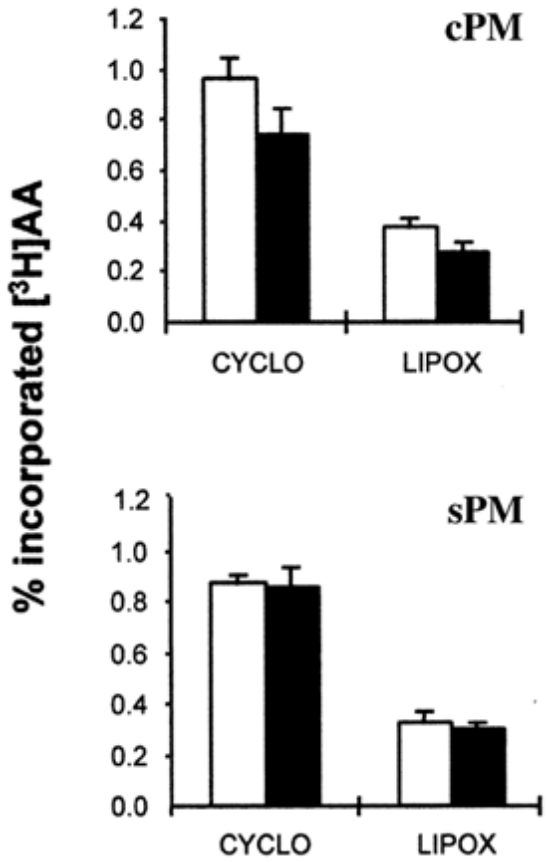


Figure 3

Effect of IL-4 on basal AA metabolism in cPM and sPM.

Adherent cPM and sPM were incubated in the presence of 1 muCi [^{3}H]AA for 24 hours in DMEM-FCS with (solid bars) or without (open bars) IL-4, then were rinsed, and cultured in DMEM alone for a further hour. At the end of this hour of culture, cyclooxygenated (CYCLO) and lipoxygenated (LIPOX) [^{3}H]metabolites secreted in the culture medium were extracted, identified then measured. Results are expressed as the percentage of incorporated [^{3}H]AA and are means ± SEM of three independent experiments. Statistical analysis of data was performed using a 2-way analysis of variance with 3 replications per experimental group.

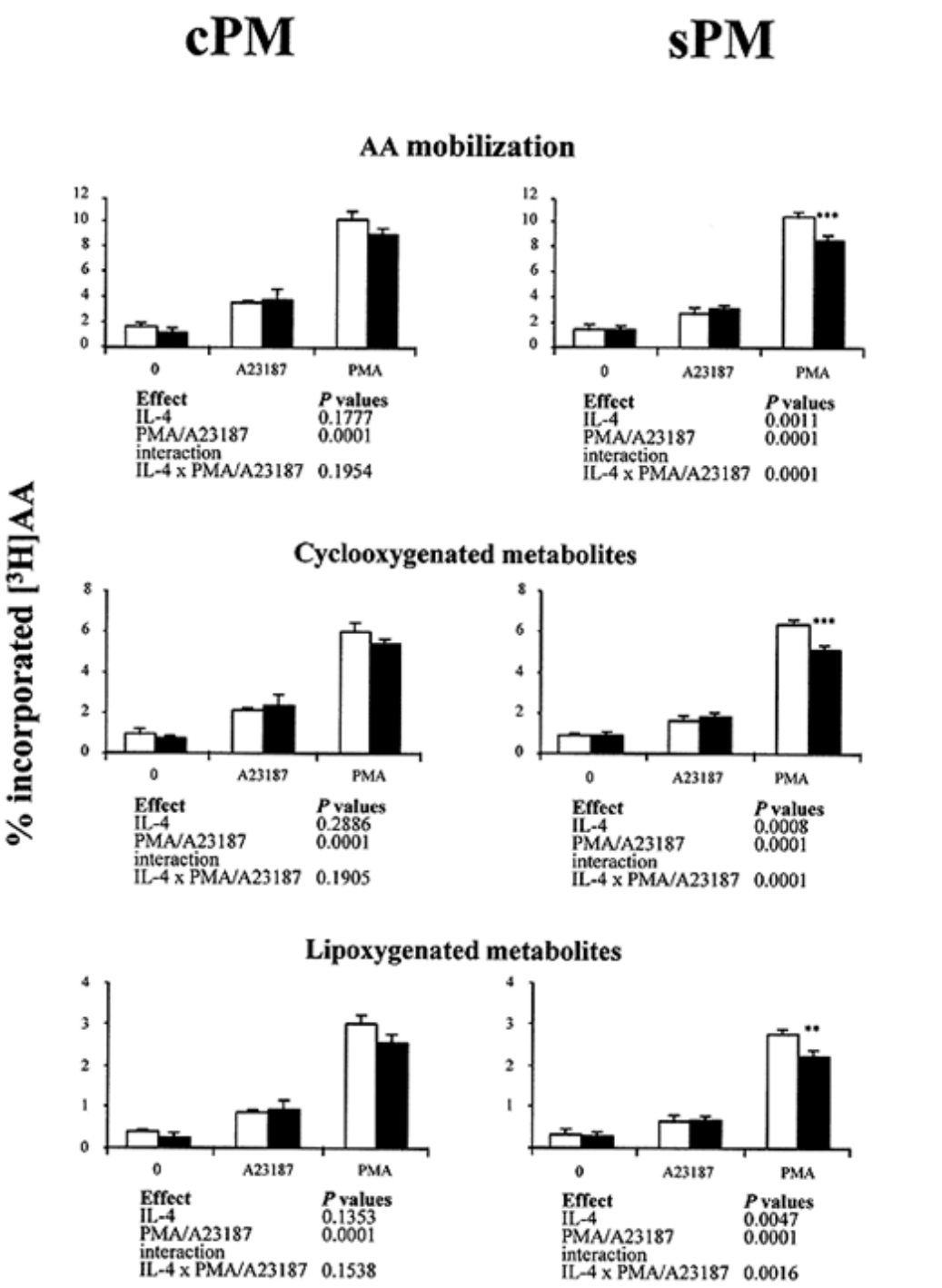
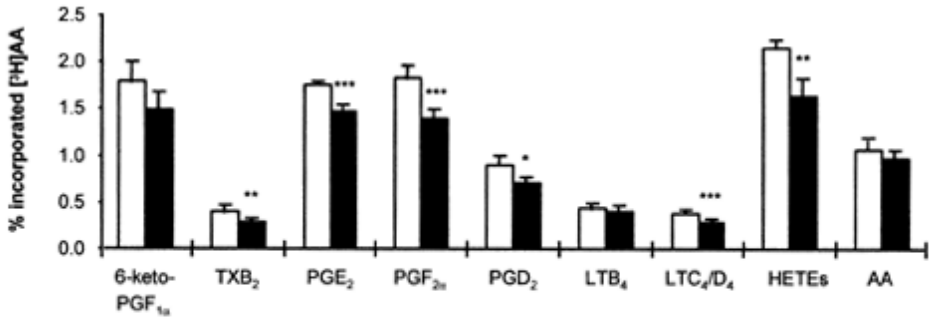


Figure 4

Effect of IL-4 on A23187- and PMA-challenged AA metabolism in cPM and sPM.

Adherent cPM and sPM were incubated in the presence of 1 muCi [^{3}H]AA for 24 hours in DMEM with (solid bars) or without (open bars) IL-4, then rinsed and cultured, for a further hour, in DMEM containing 1 muM A23187 or 100 nM

PMA. At the end of stimulation, cyclooxygenated and lipoxygenated [^{3}H]metabolites secreted in the culture medium were extracted, identified then measured. AA mobilization represents the quantity of [^{3}H]AA released from phospholipids. Results are expressed as the percentage of incorporated [^{3}H]AA and are means ± SEM of three independent experiments. Statistical results from 2-way analysis of variance with 3 replications per experimental group (*P* values) are also presented. *P* values below 0.05 (0.01; 0.001) indicate a significant effect at 0.05 (0.01; 0.001) level. On histograms, ** (***) *P* < 0.01 (0.001) indicates a significant interaction between IL-4 treatment and stimulation.



Effect	*P* values								
IL-4	0.3459	0.0384	0.0240	0.0004	0.0640	0.4270	0.0053	0.0089	0.9239
PMA	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
interaction IL-4 x PMA	0.1972	0.0017	0.0001	0.0005	0.0163	0.7530	0.0001	0.0046	0.1377

Figure 5

Detailed production of cyclooxygenated and lipoxygenated [3^H]metabolites in PMA-challenged sPM.

Adherent sPM were incubated in the presence of 1 muCi [^{3}H]AA for 24 hours in DMEM-FCS with (solid bars) or without (open bars) IL-4, then rinsed and cultured, for a further hour, in DMEM containing 100 nM PMA. At the end of stimulation, cyclooxygenated and lipoxygenated

[^{3}H]metabolites secreted in the culture medium were extracted, identified then measured. Results are expressed as the percentage of incorporated [^{3}H]AA and are means ± SEM of three independent experiments. Statistical results from 2-way analysis of variance with 3 replications per experimental group (*P* values) are also presented. *P* values below 0.05 (0.01; 0.001) indicate a significant effect at 0.05 (0.01, 0.001) level. On histograms, * (**; ***) $P < 0.05$ (0.01; 0.001) indicates a significant interaction between IL-4- and PMA-treatment.

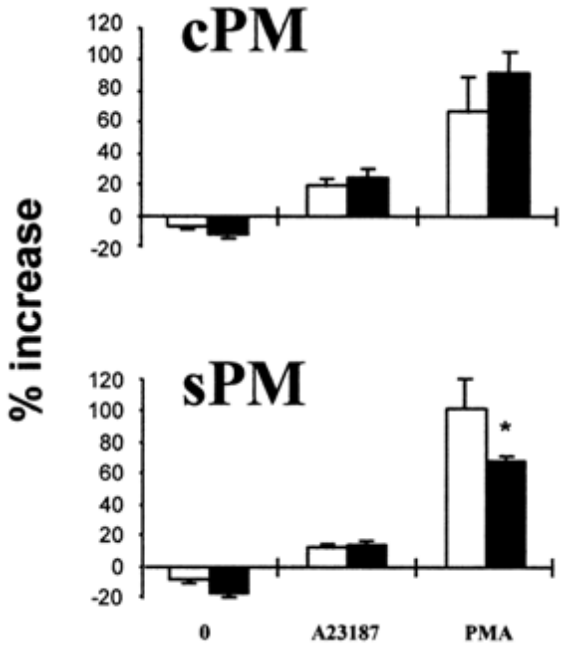


Figure 6

Effect of IL-4 on AA reincorporation during A23187 and PMA challenge of cPM and sPM.

Adherent cPM and sPM were incubated in the presence of 1 muCi [^{3}H]AA for 24 hours in DMEM-FCS with (solid bars) or without (open bars) IL-4, then rinsed and cultured, for a further hour, in DMEM containing 1 muM A23187 or 100 nM PMA. AA reincorporation into CE during the last hour of culture was assessed by comparing the [^{3}H]CE at the end

of the incorporation course and the [^{3}H]CE at the end of the last hour of stimulation. Results are expressed as the percentage increase between these values and are means ± SEM of three independent experiments. Statistical analysis of data was performed using 2-way analysis of variance with 3 replications per experimental group. * $P < 0.05$ indicates a significant difference as compared to macrophages not pretreated by IL-4.

Table 1
Effect of IL-4 on the expression of cPLA$_2$, 5-LO and FLAP in cPM and sPM

	cPM		sPM	
Time of IL-4 treatment	8 hours	24 hours	8 hours	24 hours
cPLA$_2$	82.45 ± 15.32	186.09 ± 64.37	64.57 ± 4.37 **	20.50 ± 5.50 **
5-LO	130.04 ± 139.59	84.64 ± 43.36	9.95 ± 6.22 **	7.23 ± 12.53 **
FLAP	231.36 ± 82.76	1075.8 ± 216.3 *	27.48 ± 17.03 *	48.96 ± 7.64 **

Data, from numerization and quantification of bands as described in Materials and Methods, are expressed as the percentage of the numerical value of IL-4-treated macrophages relative to the numerical value of the band of untreated macrophages. These data are presented as means ± SEM of three independent experiments. The effect of IL-4 was considered significant when the confidence interval of these data did not include the reference value 100. * (**) $P < 0.05$ (0.01) indicates a significant effect of IL-4.

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