

Interleukin-4 production by human alveolar macrophages

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Summary

Background IL-4 is a key factor for T helper type 2 (Th2) differentiation and Ig class switching to IgE and IgG₄ during the development of immune responses. IL-4 is produced by T cells, mast cells, basophils, and eosinophils. However, there is also evidence suggesting that rat alveolar macrophages (AMs) produce IL-4.

Objective Given the importance of AMs and Th2-related diseases in the lung, we investigated the production of IL-4 by human AMs.

Methods Human AMs were isolated from bronchoalveolar lavage, purified, and IL-4 production was investigated at mRNA and protein levels using real-time PCR, flow cytometry, immunocytochemistry, and ELISA. The presence of IL-4 was investigated in subjects with asthma or asymptomatic airway hyper-responsiveness, and in normal non-smokers.

Results IL-4 and IL-4δ2 (a splice variant found in other IL-4 producing cells) mRNAs were found in all these subjects, but IL-4 expression could not be correlated with a particular disease. Protein production was verified by immunocytochemistry and flow cytometry analysis demonstrating, respectively, up to 69% and 59% positive AMs, regardless of the subject condition. Furthermore, phorbol-12-myristate-13-acetate and calcium ionophore stimulated the release of IL-4 after 48 h treatment in the presence of anti-IL-4 receptor antibody.

Conclusion Our results show for the first time that IL-4 and IL-4δ2 mRNA are expressed and IL-4 protein produced and released by human AMs, suggesting a contribution of these cells in the modulation of Th2 immune response.

Keywords alveolar macrophages, IL-4, IL-4δ2, real time PCR

Submitted 1 September 2004; revised 2 February 2005; accepted 21 March 2005

Introduction

Since Mosmann et al. [1] discovered a dichotomy in T helper (Th) populations, IL-4 was shown to be a key factor in the development of Th2 cells [2]. IL-4 is important for the differentiation of naïve Th cells into mature Th2 type [3] and for Ig class switching to IgE and IgG₄ [4]. The production of this potent cytokine is restricted to few cell types, such as T cells, mast cells, basophils, and eosinophils [2, 5, 6], allowing an adequate control of its regulatory effect. Recently, the existence of a splice variant of IL-4, IL-4δ2, in which exon 2 is removed, was demonstrated [7–10]. This deletion does not result in a frameshift, suggesting that this spliced IL-4 mRNA may code for a truncated IL-4 protein [8], although the existence of this protein has never been shown *in vivo*. Interestingly, recombinant IL-4δ2 protein was shown to inhibit IL-4 activities on various cell types *in vitro* [11, 12], suggesting that IL-4 biological effect can be modulated by the production of IL-4δ2 splice variant.

Alveolar macrophages (AMs) represent the most numerous cells recovered in bronchoalveolar lavage (BAL) [13]. These

cells are able to produce both Th1 (TNF, IFN-γ, IL-12) and Th2 cytokines (IL-6, IL-10, IL-13) [14–17]. Moreover, we have previously demonstrated that AMs play an important role in the development of allergic immune response in the lung [18]. Adoptive transfer of AMs from an allergy-resistant rat into AM-depleted susceptible recipient 24 h before allergen challenge prevented airway hyper-responsiveness (AHR) development [19]. These observations, in addition to evidence showing the role of AMs in orienting the development of Th1 response [20], indicate that AMs may play a crucial role in the Th1/Th2 balance in the lung. Furthermore, there is some evidence suggesting that AMs produce IL-4. In a rat model of radiation-induced fibrosis, cells expressing and producing IL-4 were identified by *in situ* hybridization and associated to AMs by immunofluorescence with a macrophage-specific antigen [21]. Moreover, peripheral blood mononuclear cells (PBMC) and BAL cells have been shown to produce IL-4 and IL-4δ2 mRNAs [11], but the cell types expressing IL-4 mRNA were not identified. Thus, we investigated IL-4 production by purified human AMs and blood monocytes. IL-4 production by AMs could help to identify the initial event that leads to the dysregulation of Th2 response observed in asthmatic patients.

This study demonstrates for the first time that human AMs express IL-4 and IL-4δ2 mRNA. This mRNA expression

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correlates with IL-4 protein production and this, regardless of the subject condition: asthma, asymptomatic AHR, or normal. Blood monocytes also express IL-4, but to a lesser extent. Furthermore, treatment of AMs with phorbol-12-myristate-13-acetate (PMA) and calcium ionophore A23187 for 48 h stimulated the release of IL-4.

Methods

Isolation of cells from bronchoalveolar lavage

AMs were obtained from BAL of non-atopic normal subjects with no history of asthma or systemic diseases (methacholine PC₂₀ > 16 mg/mL), subjects with asymptomatic AHR but no history of asthma (methacholine PC₂₀ < 16 mg/mL) and subject with mild allergic asthma (methacholine PC₂₀ < 8 mg/mL) using only inhaled β_2 -agonist on demand. None used inhaled or systemic corticosteroids and all subjects were non-smoker. This study was approved by the Hospital Laval Human Ethic Committee and written consent was obtained from each subject before participation.

BAL fluids were centrifuged at $350 \times g$ for 10 min at 4 °C. For immunocytochemistry and flow cytometry analysis (FACS), cells were resuspended at 10^6 AMs/mL. For reverse transcription and amplification (RT-PCR) and ELISA, AMs were purified by adherence (2 h) giving a purity of >98% AMs [13]. Cell purity was verified using the Diff-Quik staining (VWR, Ville Mont-Royal, QC, Canada). In some experiments, AMs were purified from lymphocytes by negative selection using bead-conjugated anti-CD3 mAb (Miltenyl Biotec Inc., Auburn, CA, USA) and a magnetic cell sorter followed by adherence giving a purity of >99%.

Isolation of peripheral blood monocytes

Peripheral blood monocytes were isolated on a Ficoll-Paque gradient (Amersham Biosciences, Baie d'Urfé, QC, Canada), centrifuged for 20 min at $700 \times g$ at 4 °C [22]. Monocytes were further purified by adherence, as for AMs.

Reverse transcription and amplification

RNA was extracted from cells using the Trizol reagent according to manufacturer protocol (Invitrogen Canada Inc., Burlington, ON, Canada). Total RNA was quantified using RiboGreenTM RNA quantification reagent (Molecular Probes, Eugene, OR, USA). Reverse transcriptase was performed using 2 μ g RNA, 4 μ L First Strand Buffer 5 $\times$ (Invitrogen Canada Inc.), 1 μ L dNTP (20 μ M) (Invitrogen Canada Inc.), 200 units Moloney Mouse Leukemia Virus (MMLV) reverse transcriptase (Invitrogen Canada Inc.), 1 μ L oligo dT (2.5 μ g/ μ L) (Invitrogen Canada Inc.), filled to 20 μ L with RNase-free water (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada).

PCR reactions were performed using 1 μ L cDNA, 12.5 μ L Sybr Green mix 2 X (Qiagen Inc., Mississauga, ON, Canada), 1 μ M MgCl₂ (Promega Corporation, Madison, WI, USA), and 0.2 μ M each primer, filled to 25 μ L with molecular biology grade water (Sigma-Aldrich). Primers used were: human IL-4 sense: 5'-CGA GTT GAC CGT AAC AGA CAT-3' and antisense: 3'-CGT CTT TAG CCT TTC CAA GAA G-5'

(281 bp); human IL-4 δ 2 sense: 5'-CAG AGC AGA AGA ACA CAA CTG-3' and antisense: 3'-CGT CTT TAG CCT TTC CAA GAA G-5' (254 bp); human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense: 5'-AGT CAA CGG ATT TGG TCG-3' and antisense: 3'-TCT GGC TCC TGG AAG ATG-5' (220 bp). Primers were synthesized by Biosource International Inc. (Camarillo, CA, USA). Amplification parameters used were: 95 °C 15 min, (95 °C 30 s, 60 °C 30 s, 72 °C 30 s) 40 cycles, 72 °C 2 min on Smart Cycler 2 apparatus from Cepheid (Sunnyvale, CA, USA).

Intracellular protein detection using flow cytometry

Total BAL cells and adherence purified AMs were incubated with goat serum for 30 min at 37 °C, washed, and in some samples, cytofix/cytoperm (BDPharmingen, San Diego, CA, USA) was added for 20 min at 4 °C to permeabilize the cells. Cells were then transferred to siliconized tubes, washed, and incubated with phycoerythrin (PE)-labelled anti-human IL-4 (PE-rat IgG₁ κ) for 1 h at 4 °C in dark. Isotype control was also used with each sample (PE-rat IgG₁ κ) (BDPharmingen). Cells were washed twice before to be analysed using an EPICS[®] XL-MCLTM flow cytometer (Beckman-Coulter, Miami, FL, USA). Light source was an air-cooled 15 mW argon laser. Acquisition of fluorescence was gated by forward angle light scatter and side scatter using a data rate of less than 500 events per second. A minimum of 5000 events was evaluated.

Intracellular protein detection by immunocytochemistry

After fixation with paraformaldehyde 4% on ice for 10 min, cells were cytospun on a Cytospin 2 (Thermo Shandon Inc., Pittsburgh, PA, USA). Endogenous peroxidase was inhibited using peroxidase blocking solution 0.3% (5 mg NaN₃, 50 μ L H₂O₂ 30% and 5 mL Tris buffer saline (TBS) pH 7.5), for 30 min. Slides were washed with TBS three times, as between each following step and then blocked with phosphate-buffer saline 0.1 M/bovine serum albumin 1%, and 15 μ L goat serum during 30 min. A second blocking step was done with Protein Block (DakoCytomation, Mississauga, ON, Canada) before mouse IgG₁ anti-human IL-4 (Bender Medsystems, Vienna, Australia) was added for 1 h (isotype control: mouse IgG₁ (BDPharmingen)). The second antibody (rabbit anti-mouse) (DakoCytomation) was added to the sample for 1 h. Enzyme solution was prepared according to manufacturer protocol (horseradish peroxidase enzyme) (DakoCytomation) and incubated with the sample for 40 min. AEC substrate (DakoCytomation) was added for 15 min. Cells were counterstained with hematoxylin (DakoCytomation).

Enzyme-linked immunosorbent assay

Purified AMs were incubated with 1 μ g/mL PMA (Sigma-Aldrich Canada Ltd.) and 1 μ g/mL calcium ionophore A23187 (Sigma-Aldrich Canada Ltd.) for different periods of time (2, 4, and 6 days) in the presence or not of anti-IL-4 receptor (R & D systems, Minneapolis, MN, USA) and cytokine release was measured by ELISA with the Human IL-4 Quantikine HS ELISA Kit (R & D systems). The sensitivity of the assay was 0.13 pg/mL.

Statistical analysis

Data issued from immunocytochemistry, flow cytometry, and ELISA were analysed for statistical significance by analysis of variance (ANOVA), combined with Student's test for paired data, using StatView 5.0 (SAS Institute Inc., Cary, NC, USA) with a level of significance of 5%.

Results

Expression of interleukin-4

To investigate the expression of IL-4 mRNA, AMs from BAL were purified by adhesion. BAL contained $86.2 \pm 2.8\%$ AMs and $11.7 \pm 2.6\%$ lymphocytes before adhesion and $98.0 \pm 0.4\%$ AMs and $2.0 \pm 0.4\%$ lymphocytes after purification. There were $73.5 \pm 4.8\%$ AMs and $23.0 \pm 4.4\%$ lymphocytes in the non-adherent cell population (Fig. 1). RT-PCR was performed on adherent cell populations from BAL and human peripheral blood monocytes purified by adherence.

Real-time PCR was performed and Table 1 gives the amplification values as cycle threshold values (Ct), corresponding to cycle in which the beginning of log-linear amplification was detected. Ct values inversely correlate with the initial concentration of mRNA. All samples of AMs tested (five asthmatic, two asymptomatic AHR, and three normal subjects) expressed IL-4 mRNA, whereas IL-4 δ 2 was mainly expressed in AMs from asthmatic subjects. Furthermore, monocytes from four asthmatic subjects expressed both IL-4 and IL-4 δ 2 mRNA. When no amplification was observed because of failure in RNA extraction, RT or PCR, the notation ≥ 40 cycles was noted for the Ct value and used in further calculations.

In all samples, IL-4 had an earlier Ct than IL-4 δ 2, suggesting that IL-4 δ 2, the splice variant of IL-4, was less expressed than IL-4 itself. The mean IL-4 Ct value in normal

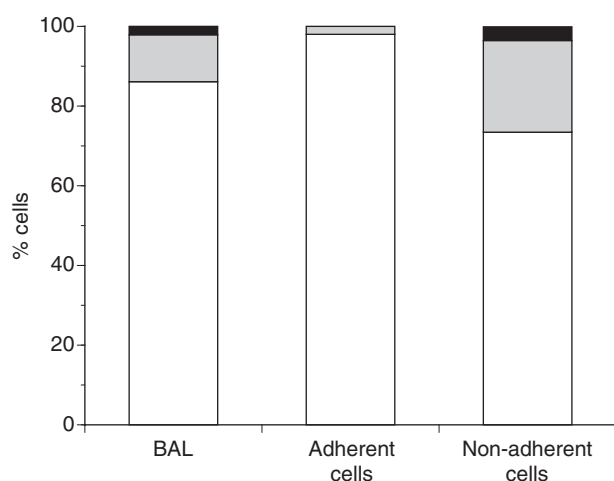


Fig. 1. Percentages of cells in bronchoalveolar lavage (BAL), before and after purification. Alveolar macrophages (AMs) were purified by adhesion and cell counts were made using the Diff Quik method. Percentages of AMs, lymphocytes and other cells in BAL, adherent and non-adherent populations are presented. White bars represent AMs, grey bars lymphocytes and black ones, other cells. Means $\pm$ SEM of seven experiments.

Table 1. Detection of GAPDH, IL-4, and IL-4 δ 2 mRNA from AMs and monocytes

Cells	Ct values			Subject
	GAPDH	IL-4	IL-4 δ 2	
AMs	16.15	30.91	35.34	Asthma
AMs	17.40	29.81	32.63	Asthma
AMs	15.95	28.87	36.40	Asthma
AMs	16.26	32.92	34.10	Asthma
AMs	18.70	33.32	34.86	Asthma
AMs	17.82	31.49	≥ 40	AAHR
AMs	17.30	35.08	≥ 40	AAHR
AMs	16.65	31.15	32.23	Normal
AMs	18.51	33.66	≥ 40	Normal
AMs	17.13	27.63	≥ 40	Normal
Monocytes	17.38	22.36	25.58	Asthma
Monocytes	16.30	26.96	28.78	Asthma
Monocytes	18.94	26.62	28.36	Asthma
Monocytes	16.04	23.11	26.12	Asthma

AAHR, asymptomatic airway hyper-responsiveness; AM, alveolar macrophages; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

subjects was 6.6 cycles lower than IL-4 δ 2 (Table 2). Given that each cycle doubles the amount of amplicons, the following equation, $2^{6.6 \text{ cycles}} = 97$, was used to calculate the difference in the expression of IL-4 and IL-4 δ 2 mRNA. Thus, there is 97-fold more IL-4 than IL-4 δ 2 mRNA in normal subject, 104-fold (6.7 cycles) in asymptomatic AHR, but only 11-fold (3.5 cycles) in asthmatics. To evaluate the amount of IL-4 and IL-4 δ 2 in normal, asymptomatic AHR, and asthmatic subjects, IL-4/GAPDH and IL-4 δ 2/GAPDH ratios were calculated (Table 2). According to these ratios, AMs from normal subjects had 4.7- and 1.8-fold more IL-4 mRNA than asymptomatic AHR and asthmatic subjects, respectively. However, asthmatic subjects expressed 5- and 7.1-fold more IL-4 δ 2 than normal and AHR subjects.

To confirm that IL-4 RNA was from AMs, lymphocytes were depleted using bead-conjugated anti-CD3 and magnetic

Table 2. Average of Ct values and differences in mRNA expression

	Mean Ct values		
	Normal	AAHR	Asthma
GAPDH	17.4	17.6	16.9
IL-4	30.8	33.3	31.2
IL-4 δ 2	37.4	40.0	34.7
Δ Ct: IL-4 δ 2 & IL-4			
Normal	6.6		IL-4/IL-4 δ 2 ratio 97
AAHR	6.7		104
Asthma	3.5		11
Δ Ct: IL-4 & GAPDH			
Normal	13.4		IL-4/GAPDH ratio 9.1×10^{-5}
AAHR	15.7		1.9×10^{-5}
Asthma	14.3		5.0×10^{-5}
Δ Ct: IL-4 δ 2 & GAPDH			
Normal	20.0		IL-4 δ 2/GAPDH ratio 1.0×10^{-6}
AAHR	22.4		0.7×10^{-6}
Asthma	17.8		5.0×10^{-6}

AAHR, asymptomatic airway hyper-responsiveness; Ct, cycle threshold; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

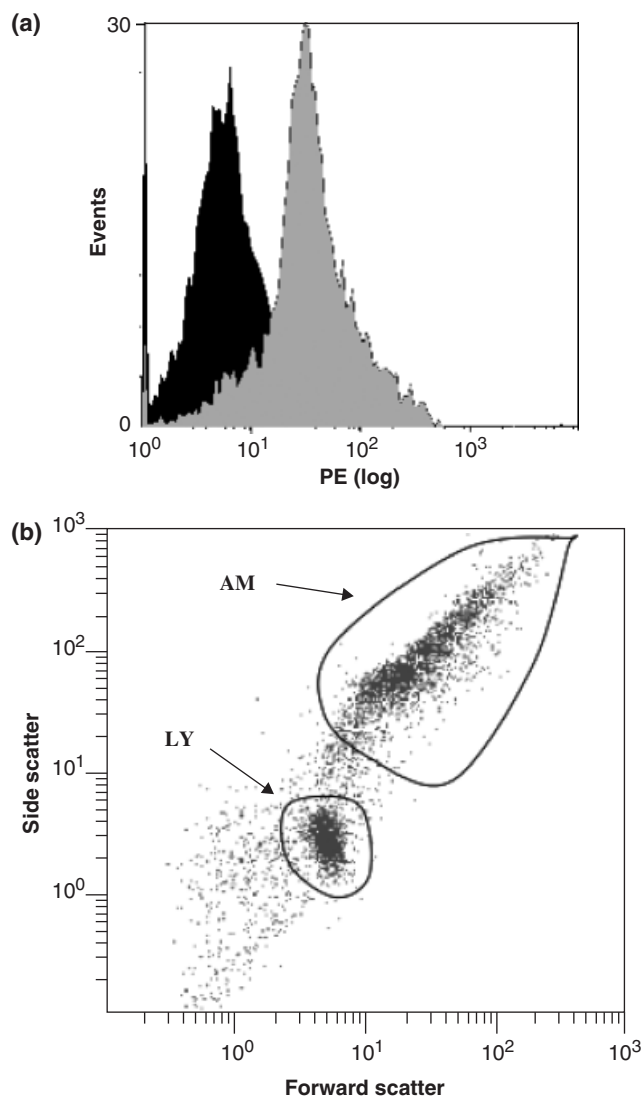


Fig. 2. Flow cytometry analysis of IL-4 protein expression. (a) Alveolar macrophages (AMs) from normal subject were permeabilized and incubated with phycoerythrin (PE) labelled anti-human IL-4. The black filled area represents fluorescence values obtained using the isotype control and the grey filled area represents fluorescence values obtained using anti-IL-4 antibody. Fluorescence values (FL1-H) are in log units on the x-axis and the cell counts are on the y-axis. This is one representative experiments out of seven. (b) AM and lymphocytes (LY) from unpurified bronchoalveolar lavage cells were defined based on light scatter profiles.

cell sorter before performing adherence. These purified AMs showed a Ct value of 34.7 for IL-4 and 35.7 for IL-4 δ 2, giving similar results than adherence purified AMs.

Protein production

To further characterize IL-4 production, protein expression was investigated by FACS (Fig. 2a) and immunocytochemistry. FACS analysis revealed that 59.2 ± 14.3 , 32.8 ± 7.0 , and $48.2 \pm 6.5\%$ AMs were positive for IL-4 in normal, asymptomatic AHR, and asthmatic subjects, respectively. There was no significant difference between groups.

To confirm that IL-4 $^{+}$ cells were AMs, unpurified BAL cells were analysed using forward and side scatter to identify cell populations (Fig. 2b). AM population contained 3.4%

CD3 $^{+}$ cells, whereas lymphocyte population contained 2% AM. Using this gating, $44.1 \pm 6.1\%$ AMs and $36.4 \pm 7.6\%$ lymphocytes were IL-4 $^{+}$. However, when the cells were not permeabilized, only $15.6 \pm 5.3\%$ AMs were IL-4 $^{+}$, whereas there were still $24.2 \pm 9.5\%$ IL-4 $^{+}$ lymphocytes, suggesting that AMs have mainly intracellular IL-4. The treatment of AMs with IL-4 (10 pg/mL) for 1 h increased the number of unpermeabilized IL-4 $^{+}$ AMs to $51.5 \pm 6\%$ without affecting permeabilized cells ($52.3 \pm 5.1\%$), suggesting that AMs do not internalize IL-4 bound to its receptor within this time.

Immunocytochemistry was performed to corroborate the observation made by flow cytometry. Two blinded observers counted a minimum of 300 AMs per slide and the average of the results was calculated. Figure 3 shows that more than 50% AMs were positive for IL-4, in all groups (57.7%, 57.3%, and 63.8% for AMs from normal, asymptomatic AHR, and asthmatic subjects, respectively). In contrast, very low percentages of blood monocytes were positive for IL-4 in all subjects (4.5%).

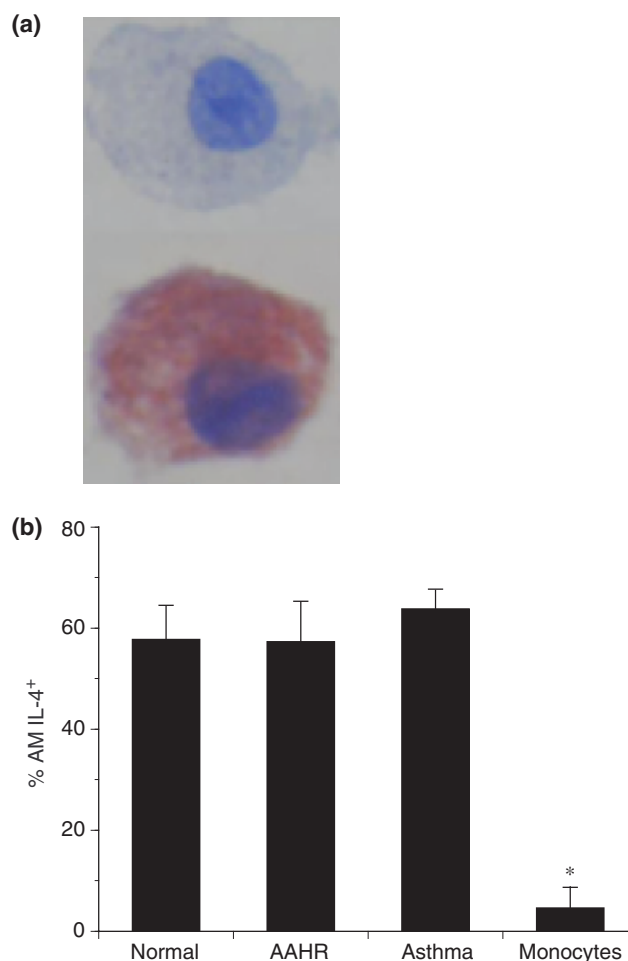


Fig. 3. Percentage of IL-4 $^{+}$ alveolar macrophages (AMs) using immunocytochemistry. AMs from normal, asthmatic, and asymptomatic AHR subjects produce significantly more IL-4 than monocytes (* $P < 0.0001$). Means $\pm$ SEM of seven to 18 experiments for AMs and four for blood monocytes. In overhead, negative AM from isotypic control (a) and IL-4 $^{+}$ AM (b).

Protein secretion

To investigate the release of IL-4, AMs were stimulated with PMA and calcium ionophore A23187 in presence or not of anti-IL-4 receptor antibody (5 µg/mL) to avoid the binding of IL-4 to the cells. Unstimulated AMs did not release IL-4 even in the presence of anti-IL-4 receptor antibody. However, AMs from normal and asthmatic subjects released significant amount of IL-4 (Figs. 4a and b, respectively) when stimulated with PMA and A23187 in the presence of anti-IL-4 receptor antibody. There was no significant difference between periods of incubation (2, 4, and 6 days) and between normal (1.81 ± 0.48 pg/ 10^6 AMs) and asthmatic subjects (1.58 ± 0.55 pg/ 10^6 AMs) because of variation between subjects. Given the low release of IL-4 and the small contamination of AMs by T lymphocytes, peripheral blood lymphocytes were purified and stimulated with PMA and A23187 for 4 days using the same number than AMs. In these conditions, lymphocytes released

more IL-4 (23.7 pg/mL) compared with AMs (2.4 ± 0.8 pg/mL). However, when the number of lymphocytes was reduced to the number found with AMs, only 0.2 pg/mL of IL-4 was released leaving around 2 pg produced by AM, suggesting that lymphocytes were not the source of IL-4 in our context.

Discussion

IL-4 is a pleiotropic cytokine that drives Th2 polarization and antagonizes the effects of Th1 cytokines [23]. Given the importance of IL-4 in the development of asthma [24, 25], it is crucial to know which cell types produce it. Thus, we investigated the production of IL-4 by human AMs and clearly showed that AMs express, synthesize, and release IL-4. More than 50% AMs expressed the protein regardless of subject groups (asthma, asymptomatic AHR, or normal).

This is the first report demonstrating production of IL-4 by human AMs. The comparatively low number of IL-4 mRNA copies in these cells may explain, in part, why this observation has not been reported before. This small number of copies may be because of short IL-4 mRNA half-life as reported in T cells [26]. Furthermore, IL-4 mRNA may be less stable in AMs as demonstrated in Th1 cells compared with Th2 cells [27]. It is important to mention that IL-4 mRNA was previously detected in rat AMs using *in situ* hybridization [21], a technique in which samples are rapidly fixed to prevent degradation. Thus, it is possible that many IL-4 mRNA copies are degraded during AM mRNA isolation. We therefore propose that IL-4 mRNA in human AMs is unstable, explaining that no previous observation of IL-4 expression in these cells has been reported.

The method used to purify AMs from BAL gave a purity of 98% AMs leaving around 2% contaminating lymphocytes. To confirm that mRNA was not from contaminating lymphocytes, these cells were depleted from BAL using anti-CD3 magnetic bead-conjugated antibody. The resulting purified BAL cell population was further purified by adherence. The IL-4 mRNA levels in this purified AM population was similar to adherence purified AMs, demonstrating that lymphocytes were not responsible for IL-4 mRNA expression.

IL-4 exists in two isoforms, IL-4 and IL-4δ2 [7, 8, 11], but the latter has not been thoroughly investigated. The alternative splicing of exon 2 does not cause a frameshift in mRNA [8], suggesting that a truncated protein may be produced, but no IL-4δ2 protein has yet been identified *in vivo*. However, recombinant IL-4δ2 proteins show IL-4 antagonist effects [11, 12]. Furthermore, there may be tissue specific expression in the lung as thymocytes and cells from BAL expressed higher levels of IL-4δ2 mRNA than IL-4 mRNA [11]. In contrast, we observed that AMs produced more IL-4 than IL-4δ2 (Table 2). This contradiction may be explained by the purity of the cells. Lymphocytes from BAL might produce more IL-4δ2, explaining the results obtained with unpurified BAL cells [11], whereas we used pure AMs (98%), which produce more IL-4 than IL-4δ2.

Seah et al. [9] reported that PBMCs from asthmatic patients express a ratio of IL-4/IL-4δ2 500-fold more elevated than control subjects. In contrast, the ratio of IL-4/IL-4δ2 was 8.8

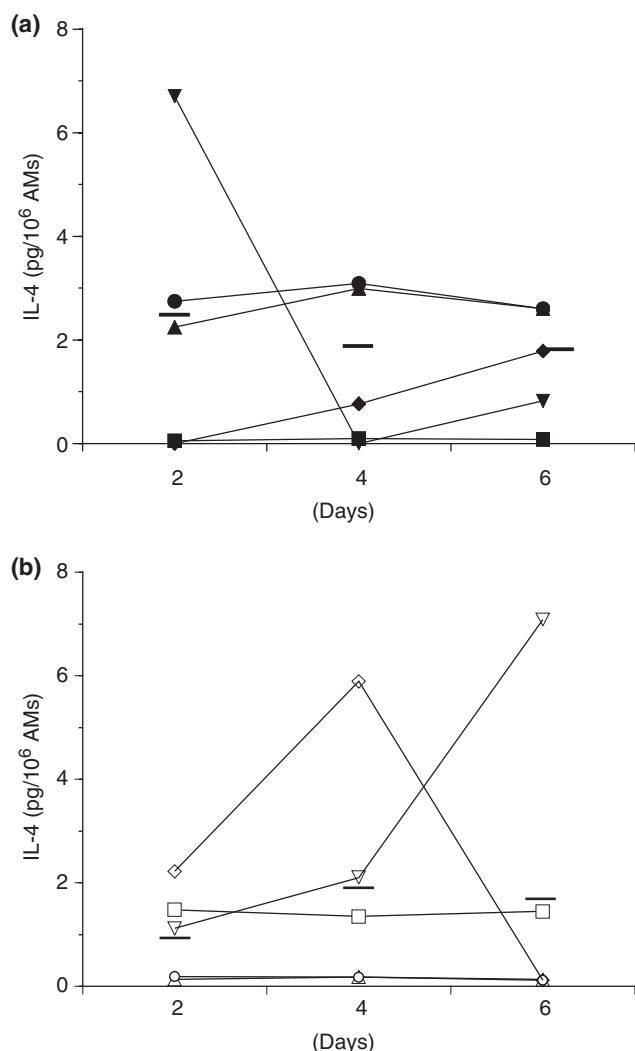


Fig. 4. Release of IL-4 by alveolar macrophages (AMs). AMs from normal (a) and asthmatic (b) subjects were stimulated with phorbol-12-myristate-13-acetate and calcium ionophore A23187 for different periods of time in the presence of anti-IL-4 receptor antibody. Each symbol represents different subject. No significant difference was observed between times of incubation and asthmatic and normal subjects. Bars represent the mean production for the given time.

times lower in AMs from asthmatic compared with normal subjects (Table 2), suggesting that the production of IL-4 may be differently modulated depending on cell types: monocytes, lymphocytes, and AMs. This difference may reflect the distinct roles of these cells in immune response. AMs may produce more IL-4 δ 2 to reduce the effect of IL-4 produced by other cell types in asthma. Thus, AMs from asthmatic subjects may contribute to limit the Th2 immune response.

Analysis in flow cytometry and experiments in immunocytochemistry performed on non-stimulated cells revealed that AMs constitutively produce IL-4. Although results from these two methods were similar, some differences were observed in percentages of positive cells. These differences may be imparted to the methods. Different antibodies were used for direct and indirect labelling in FACS and immunocytochemistry. However, both methods demonstrated consistent results in production of the cytokine, confirming that IL-4 mRNA was effectively translated into protein and located into cells identified as AMs. To confirm the origin of the observed IL-4, cytometry was also used with whole BAL cells. Of importance, gated AM population was shown to be IL-4⁺ in greater proportions than the identified population of lymphocytes. Therefore, AMs contain IL-4. However, we needed to show that IL-4 was not only membrane bound. To address this issue, non-permeabilized AMs were used and showed that less AMs were positive for IL-4 than in permeabilized conditions. This result clearly shows that the majority of IL-4⁺ AMs contain this IL-4 in cytoplasm and not only on membrane. The remaining question was in regard to the possible uptake of IL-4 produced by other cells. To assess this other possibility, we conducted experiments where AMs were incubated with IL-4. This incubation, that allowed AMs to uptake free IL-4, revealed that the membrane or receptor-bound IL-4 increased (non-permeabilized cells), but that permeabilized cells did not vary, showing that signal in permeabilized AMs is not dependent on uptake of external IL-4. Therefore, this FACS confirmed that AMs are IL-4⁺, a signal that is not dependent on membrane- or receptor-bound IL-4, neither on IL-4 uptake.

It is not clear what stimuli can initiate IL-4 secretion by AMs *in vivo*. PMA and calcium ionophore A23187 have been shown to stimulate the release of IL-4 by mast cells and T cells [28, 29]. Thus, we used these stimuli for different periods of time. Unstimulated cells did not release IL-4 and release of IL-4 was not always detected in stimulated AMs in the absence of anti-IL-4 receptor antibody. The use of IL-4 receptor blocking antibody during the stimulation enhanced the sensitivity of the assay as previously demonstrated [30, 31]. Interestingly, IL-4 production could not be attributed to the contaminating lymphocytes. As we showed, when the number of lymphocytes was equivalent of the one found in purified AM population, IL-4 production was ten times less than in purified AMs. This observation closes the demonstration that the contaminating lymphocytes are not the source of IL-4 in our experiments. However, further investigations are needed to better understand the mechanism of IL-4 release from AMs. This mechanism could necessitate the interaction with other cell types. It is important to emphasize that IL-4 is found at the level of mRNA expression, but also as a translated protein in the cytoplasm and furthermore detected as a secreted cytokine. The fact that IL-4 can be secreted also

confirms that IL-4 found in the cytoplasm is produced *de novo*, since endocytosed IL-4 would not be re-secreted. This new observation could be of prime importance as it shows that AMs may also be a primary source of Th2 cytokines in the lung. Many studies implicating co-cultures with AMs may need to be examined from a different point of view in regard to IL-4 production. Furthermore, we have previously suggested that there may be different populations of AMs [17], and IL-4 production may come from a subpopulation of AMs, AM-2.

IL-4 mRNA expression and protein production could not be linked to any of the conditions investigated: subjects with asthma, asymptomatic AHR, and normal non-smokers. Our results show that there is always more IL-4 mRNA than IL-4 δ 2. However, the role of differential expression of IL-4 and IL-4 δ 2 in different diseases needs further investigation. Our study demonstrated that human AMs constitutively produce IL-4 suggesting that AMs have an underestimated regulatory capacity and that their role in lung homeostasis need further investigation.

Acknowledgements

The authors would like to thank Dr Simon Martel for performing BAL, Luce Trépanier for volunteer recruitment, and Marc Veillette for technical assistance. This work was supported by the Réseau en Santé Respiratoire du Fonds de la Recherche en Santé du Québec (FRSQ) and by the Canadian Institutes of Health Research. E. Y. B. is a senior FRSQ scholar and P. P. has a studentship from the Réseau en Santé Respiratoire du FRSQ.

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