# Responsiveness of human neutrophils to interleukin-4: induction of cytoskeletal rearrangements, *de novo* protein synthesis and delay of apoptosis

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Interleukin-4 (IL-4) and IL-13 are cytokines that share many biological activities. We have previously demonstrated that IL-13 affects a number of neutrophil responses, and here we extend our observations to IL-4. We present, for the first time, direct evidence for the presence of functional IL-4 receptors on human neutrophils. We report that IL-4 induces RNA synthesis in a concentration-dependent manner and, based on observations of the induction of morphological cell shape changes and spreading onto glass, we demonstrate that IL-4 activates neutrophil cytoskeletal rearrangements. We further show that IL-4 is a potent activator of *de novo* protein synthesis in neutrophils, and we identify by microsequencing one of these proteins as the cytoskeletal protein actin. We were also able to demonstrate for the first time that actin is cleaved into at least two fragments of ~ 30 kDa (pI 5.4) and ~ 25 kDa (pI 5.0) in neutrophils. Finally, we report that IL-4 delays neutrophil apoptosis, as assessed by morphological observations from cytocentrifuge preparations, as well as by measurement of differences in staining by flow cytometry with both propidium iodide and Hoechst reagent. Taken together, we conclude that IL-4 is a more potent neutrophil agonist than previously believed. We discuss the possibility that the induction of the *de novo* synthesis of actin by IL-4 is related to the mechanism by which this cytokine delays apoptosis; in addition, the cleavage of this protein is likely to contribute to the apoptotic process.

## INTRODUCTION

Interleukin-4 (IL-4) is a major immunoregulatory molecule that plays a central role in Th2-mediated immunity [1]. It can enhance immune function within various leucocyte populations, and has been shown to augment the tumour cytolytic activity of eosinophils and lymphocytes [2–14]. Considerable clinical interest has been generated by this cytokine because of its potential use as an anti-cancer agent in humans. A number of clinical trials have been initiated, all of which are in phase I/II [15,16]. In view of this, the necessity of fully determining all of the effects of IL-4 on host cells cannot be neglected.

We recently reported that IL-13, a cytokine known to share many biological actions with IL-4, activates a number of functions in human neutrophils, including morphological cell shape changes, tyrosine phosphorylation, RNA and protein synthesis and IL-8 production [17]. To date, only classical neutrophil responses such as phagocytosis, the respiratory burst, chemotaxis and degranulation have been the subject of investigation with IL-4 [18,19]. Results from these studies led to the conclusion that IL-4 is a weak neutrophil agonist. However, Th2-mediated inflammation was found to cause local tissue inflammation in which neutrophil infiltration was prominent [20], and injection of IL-4 into humans caused significant neutrophilia [21]. Furthermore, it has been reported that IL-4 up-regulates the expression on this cell type of the type II IL-1 receptor [22], a receptor that acts as a decoy for IL-1. These latter findings clearly suggest that IL-4 may be a neutrophil agonist of greater physiological importance than previously thought.

Although there are no reported studies clearly identifying the presence of functional IL-4 receptors (IL-4Rs) on human neutrophils, it is important to note that neutrophils constitutively express the common $\gamma$ chain ($\gamma_c$) [23–26]. This chain was shown to be one component of the IL-4R, and was found to contribute to a 2–3-fold increase in the binding of this cytokine to its receptor [27,28]. The other known component of the IL-4R is the 140 kDa IL-4R$\alpha$ chain, now referred to as CDw124 [29]. Prior to the present study, no information on the presence or absence of this IL-4R component in human neutrophils could be found in the literature. In the present study, we report that neutrophils express functional IL-4Rs, and describe novel findings on the effects of IL-4 in human neutrophils.

## MATERIALS AND METHODS

### Neutrophil isolation and incubation conditions

Cells were isolated from the venous blood of healthy volunteers, as previously described [17,23,30–32], by centrifugation over Ficoll/Hypaque obtained from Pharmacia Biotech Inc. All cell suspensions contained fewer than 1 % monocytes, as determined by monoesterase staining. Cell viability, as monitored by the ability to exclude Trypan Blue, was greater than 95 % immediately after isolation and after 4, 12 and 24 h of incubation in the presence or absence of agonists. Unless otherwise specified, neutrophils were resuspended in RPMI medium supplemented with 1 % (v/v) human autologous serum.

### Agonists

Recombinant human IL-4 ($1 \times 10^7$ units/mg) was purchased from Genzyme (Cambridge, MA, U.S.A.), and recombinant human IL-2 ($22 \times 10^6$ units/mg) was provided by Cetus Corp. (Emeryville, CA, U.S.A.). Lipopolysaccharide (LPS; from

Abbreviations used: IL-4 (etc.), interleukin-4 (etc.); IL-4R, IL-4 receptor; GM-CSF, granulocyte/macrophage colony-stimulating factor; LPS, lipopolysaccharide; ICE, interleukin-1$\beta$-converting enzyme; Jak, Janus kinase; STAT, signal transducers and activators of transcription.

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*Escherichia coli* 0111.B4) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF; $9\times10^6$ units/mg) was a gift from the Genetics Intitute (Boston, MA, U.S.A.). For simplicity, the recombinant human forms of IL-2, IL-4 and GM-CSF will be referred to simply as IL-2, IL-4 and GM-CSF throughout the text.

### Flow cytometry

Freshly isolated neutrophils were preincubated with 20 % (v/v) autologous serum for 30 min in order to saturate Fc sites. After washing with neutral PBS, cells were incubated with specific anti-(human IL-4R$\alpha$) monoclonal antibody (anti-CDw124) from Genzyme [29] or buffer for 45 min, washed, and then incubated with FITC-labelled goat anti-mouse IgG F(ab′)$_2$ (Bio/Can Scientific, Missisauga, Ont., Canada) for 45 min. In order to prevent internalization, all steps were performed at 4 °C.

### RNA synthesis assay

This assay was performed by measuring the incorporation of [5-$^3$H]uridine (Amersham Corp., Oakville, Ont., Canada) into total RNA essentially as previously described [17,23,30–32]. Portions of 100 $\mu$l of a $5\times10^6$ cells/ml suspension were incubated in 96-well microtitre plates in the presence of 1 $\mu$Ci of [$^3$H]uridine plus agonists for 4 h (optimal time point) at 37 °C in 5 % $CO_2$. Following incubation, the cells were collected on to borosilicate glass-fibre paper by a multiple-cell culture harvester (Skatron Instruments Inc., Sterling, VA, U.S.A.), and sections of the filter corresponding to each microwell were then punched out and placed in scintillation counting vials in the presence of 4 ml of Aquasol-2 (Dupont NEN, Boston, MA, U.S.A.). All experiments were performed in triplicate.

### Microscopic observations of neutrophils

These were performed essentially as previously reported [17,30]. Cells ($5\times10^6$ cells/ml) were incubated at 37 °C in 5 % $CO_2$ in 96-well plates for up to 24 h in the presence of buffer, GM-CSF (65 ng/ml), IL-2 (1–4000 ng/ml) or IL-4 (1–1000 ng/ml). Morphological changes in cells were observed under light microscopy ($\times200$), and micrographs were taken using Kodak Tmax 100 ASA film for black-and-white prints. In parallel, cells were gently mixed in each well and aliquots of cells were taken and loaded on to a haemocytometer in order to evaluate the percentage of cells that had been activated (shape changes) by increasing concentrations of the agonist. This was evaluated by dividing the number of cells displaying shape changes by the total number of cells and multiplying by 100.

### Neutrophil spreading on to glass

The ability of neutrophils to spread on to glass may be used as a marker of neutrophil cytoskeletal activity, distinct from the induction of cell shape changes [33,34]. Cells ($5\times10^6$ cells/ml) were incubated in 24-well plates at 37 °C/5 % $CO_2$ in the presence or absence of LPS (1 $\mu$g/ml), IL-4 (10 ng/ml, the lowest concentration having a significant effect on RNA synthesis; see Figure 3) or the negative control IL-2 (45.5 ng/ml) for 12 h. The 12 h time point was chosen because it was previously reported to be optimal [17,30,33,34]. At 12 h, 10 $\mu$l of the cell suspension was loaded on to a haemocytometer and incubated for 5 min at 37 °C. Immediately after incubation, cells were examined under light microscopy and scored as spread or non-spread. The results were expressed as the percentage of spreading cells.

### Metabolic labelling of neutrophils, protein precipitation and two-dimensional gel electrophoresis

The metabolic labelling of neutrophils (200 $\mu$l; $50\times10^6$ cells/ml) was performed with [$^{35}$S]methionine and [$^{35}$S]cysteine (Amersham), both at 125 $\mu$Ci/$10^7$ cells. Cells were collected after 20 h of incubation under optimal conditions [32] as previously described [17,23,30–32] in the presence of protease inhibitors (aprotinin, 60 trypsin-inhibitory units/ml; PMSF, 1 mM; leupeptin, 0.5 $\mu$g/ml; EDTA, 200 $\mu$M). Protein precipitation was performed in Eppendorf tubes with a final concentration of 70 % ethanol for 1 h at −20 °C. After centrifugation, the pellet was solubilized with lysis buffer (9.5 M urea, 2 % Nonidet P-40 and 5 % $\beta$-mercaptoethanol), and 10 $\mu$l portions of each fraction were placed in scintillation-counting vials with 4 ml of Aquasol-2 in order to determine the amount of radiolabelled protein loaded for the migration.

High-resolution two-dimensional gel electrophoresis was performed with intracellular neutrophil proteins by the method of O'Farrell [35], using the Millipore Investigator 2D Electrophoresis System. This was carried out using lysates obtained from an equal number of cells ($5\times10^6$), since we are interested in the *de novo* synthesis of proteins, as we have previously documented [17,23,30–32]. First-dimension isoelectric focusing was performed using 2 % Ampholytes (1:4, v/v; pH ranges 4–8 and 3–10). Gels for the second dimension were 12 % polyacrylamide. The gels were dried and exposed for 3–5 days at −70 °C. Two-dimensional gel analysis of proteins was performed with a Bio-Image 110-S analyser (Millipore) using the 2D Gel Match Program. The two-dimensional gel analyser permitted us to compare and evaluate the intensity of each paired spot in order to confirm if the *de novo* synthesis of a particular protein is up- or down-regulated.

### Microsequencing of proteins

Following two-dimensional gel electrophoresis, proteins were transferred on to PVDF membranes, stained with Coomassie Blue, excised and microsequenced with an Applied Biosystems gas phase sequencer model 475A with on-line phenylthiohydantoin analysis and data collection, as previously reported [30]. All the chemicals and protocols were those recommended by the manufacturer.

### Assessment of neutrophil apoptosis by cytology and flow cytometry

Cytocentrifuge preparations of neutrophils were obtained and processed as previously described using a Cyto-tek® centrifuge (Miles Scientific) [17,30]. Cells were incubated in the presence or absence of IL-4 (10 or 500 ng/ml) or GM-CSF (65 ng/ml) for 24 h in RPMI-1640 containing 10 % autologous serum, and were stained with a Diff-Quick stain set (Baxter), according to the manufacturer's instructions. Cells were examined by light microscopy at $\times400$ final magnification, and apoptotic neutrophils were defined as cells containing one or more characteristic darkly stained pyknotic nuclei [17,30,33,34]. An ocular lens containing a $10\times10$ square grill was used in order to count at least five different fields (> 500 cells in total) for assessment of apoptotic cells. Experiments were performed with duplicate samples, and results were expressed as the percentage of cells in apoptosis.

We also evaluated apoptotic neutrophils by flow cytometry, by assessing differences in their staining with both propidium iodide and Hoechst reagents, essentially as previously described [30,36]. Cells were incubated as above. Following the 24 h incubation, aliquots of 350 $\mu$l of each cell suspension (representing

$\sim 2.5 \times 10^6$ cells, after 24 h) were washed twice with neutral PBS, followed by the addition of 100 $\mu$l of propidium iodide (from a 20 $\mu$g/ml solution) for 30 min on ice. Cells were then protected from light throughout the procedure. After this, cells were treated with 950 $\mu$l of 25 % ethanol and 50 $\mu$l of HO33342 (from a 112 $\mu$g/ml solution) and kept on ice for 12 h before performing FACS analysis (10000 events) using an EPIC 753 instrument (Coulter, Miami Lakes, FL, U.S.A.), as previously reported [30].

### IL-8 production

In contrast with many other investigators, we measured the IL-8 concentration not only in the external milieu but also in the intracellular fraction. The measurement of IL-8 production was determined using a commercially available ELISA kit (R&D Systems), essentially as previously described [17]. Freshly isolated human neutrophils were incubated in the presence or absence of IL-4 (10 ng/ml) at 37 °C in 5 % $CO_2$ for 20 h in a 24-well plate containing RPMI-1640 supplemented with 5 % (v/v) fetal calf serum. Both supernatants and cell lysates were harvested by centrifugation and stored at $-70$ °C before performing ELISA.

## RESULTS

### Detection of IL-4R$\alpha$ by flow cytometry

In previous studies, it was found that human neutrophils constitutively express the $\gamma_c$ chain [23–26]. In order to determine if neutrophils express the complete IL-4R, we studied IL-4R$\alpha$ (CDw124) expression on the surface of these cells using a specific anti-(human IL-4R$\alpha$) antibody and flow cytometry. As shown in Figure 1, we were able to detect the presence of this component on virtually all cells, since they all appeared to shift channel fluorescence intensity, with significantly different means of 111.2 $\pm$ 2.6 for IL-4R$\alpha$-positive cells and 43.4 $\pm$ 10 for negative controls ($P = 0.0028$, $n = 3$).

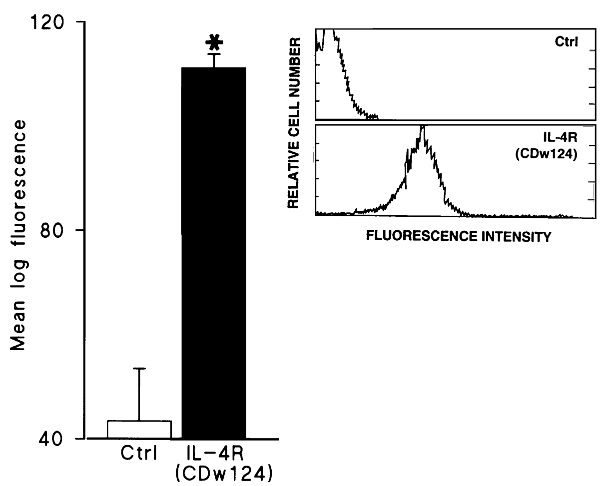


**Figure 1 Detection of the CDw124 component of the IL-4R on the surface of human neutrophils by flow cytometry**

Flow-cytometric analysis was performed with freshly isolated cells that had been incubated for 30 min with 20% (v/v) autologous serum in order to saturate the Fc sites. Cells were then incubated with buffer (Ctrl) or with anti-(human CDw124) monoclonal antibody, and then with FITC-conjugated goat anti-mouse IgG $F(ab')_2$, as described in the Materials and methods section. Results are means from three different donors. $*P < 0.05$ compared with control (Student's *t* test). Inset: typical results obtained with neutrophils from one donor; results on the *x* axis are expressed as mean log fluorescence intensity, and those on the *y* axis are the relative number of cells.

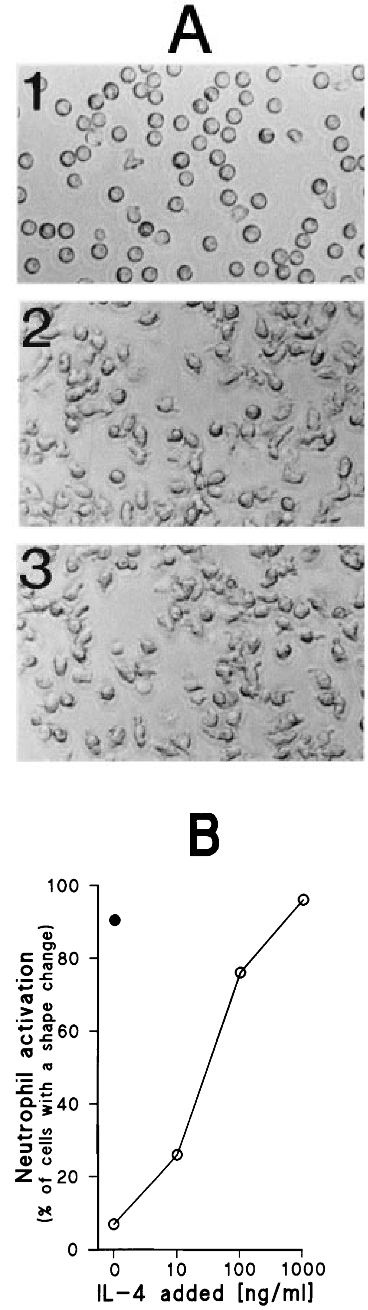


**Figure 2 IL-4 induces morphological changes in neutrophils *in vitro***

Cells ($5 \times 10^6$ cells/ml) were incubated in the presence or absence of agonists at 37 °C in 5% $CO_2$ for up to 24 h in 96-well plates. (**A**) Results obtained after 3 h of incubation (the time point we found to be optimal). Cells were incubated with buffer (panel 1), 65 ng/ml GM-CSF (panel 2) or 100 ng/ml IL-4 (panel 3). Magnification $\times 130$. (**B**) Dose–response curve of the morphological cell shape changes induced by IL-4 (○), representative of results using cells from five different donors. ●, GM-CSF-treated cells used as positive control [17,23,30].

### IL-4 is a potent activator of gene expression

We have frequently used the uridine uptake assay as an indicator of gene expression in order to obtain a first indication of whether or not a particular molecule is a neutrophil activator [12,23,30–32]. By this means, we have previously identified formyl-Met-Leu-Phe, GM-CSF and tumour necrosis factor-$\alpha$ [31,32], and

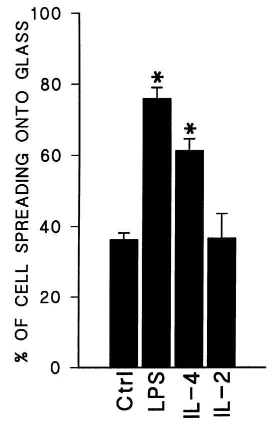


**Figure 3 IL-4 increases the spreading of neutrophils on to glass**

Cells ($5 \times 10^6$ cells/ml) isolated from the blood of healthy volunteers were incubated in the absence (Ctrl) or presence of agonists (LPS, 1 $\mu$g/ml; IL-4, 10 ng/ml; IL-2, 45 ng/ml), and the ability to spread on to glass was assessed as described in the Materials and methods section. The results are means ± S.E.M. from four different donors. *$P < 0.05$ compared with control (Student's *t* test). IL-2 was used as a negative control.

more recently IL-13 [17] and IL-15 [30], as neutrophil agonists with regard to their ability to activate gene expression. Here we show that uridine uptake was increased by IL-4 in a dose–response fashion ($n = 3$). The results obtained were 432 ± 52, 592 ± 192, 2318 ± 287, 2451 ± 378 and 2489 ± 338 c.p.m. for neutrophils incubated with 0, 1, 10, 100 and 1000 ng/ml IL-4 respectively. When compared with the agonist GM-CSF (uptake of 2202 ± 710 c.p.m. at 65 ng/ml), IL-4 appears to be a potent neutrophil agonist, since similar results to those with this dose of GM-CSF were obtained with only 10 ng/ml IL-4. Thus the 10 ng/ml dose was chosen for the other experiments in the present study.

### IL-4 induces morphological changes in neutrophils

The induction of morphological cell shape changes in neutrophils is a reflection of cytoskeletal activity. Since this function was observed to be activated by IL-13 [17], we incubated neutrophils with increasing concentrations of IL-4 (1–1000 ng/ml) and observed, over time, their morphology under light microscopy. As shown in Figure 2(A), unstimulated neutrophils remained spherical (panel 1), whereas GM-CSF-activated (panel 2) and IL-4-activated (panel 3) cells responded with morphological changes. Results illustrated in Figure 2(A) were obtained after 3 h of incubation, the time point observed to be optimal [17,30], with a concentration of 100 ng/ml IL-4, a concentration found to induce notable morphological cell shape changes in many of the cells (Figure 2B). When up to 4000 ng/ml IL-2 was used as a negative control in this assay ([17,30]; results not shown), morphological changes were not observed. Figure 2(B) shows that the induction of morphological cell shape changes by IL-4 is a dose-dependent response. Although percentages varied slightly between donors, a dose–response effect was always observed. Such results were never obtained with IL-2 (not shown).

### IL-4 enhances cell spreading of neutrophils on to glass

Another assay that reflects cytoskeletal activity is the ability of cells to adhere spontaneously to glass [30,33,34]. As illustrated in Figure 3, after 12 h of incubation (optimal time point [30,34,37]), we observed that the percentage of cells spreading on to glass was significantly increased by LPS (76.0 ± 3.0 %) or 10 ng/ml IL-4 (61.3 ± 3.3 %) compared with control (36.3 ± 1.9 %) and IL-2-treated (36.7 ± 6.9 %) cells. Our results obtained with LPS agree well with those previously published by others [33].

### Induction of neutrophil protein synthesis by IL-4

Neutrophils were incubated in the presence or absence of 10 ng/ml IL-4 for 20 h in the presence of [$^{35}$S]methionine and [$^{35}$S]cysteine. Cell viability remained greater than 95 % after the 20 h incubation period, as assessed by Trypan Blue exclusion. Before loading the gels, the radiolabelled proteins were precipitated from cell lysates with 70 % ethanol, and total counts (c.p.m.) of unstimulated and IL-4-stimulated cells were compared. Differences in c.p.m. between stimulated and unstimulated cells varied among donors ($n = 5$), but were always greater in the former (results not shown). The induction of *de novo* protein synthesis was analysed by two-dimensional gel electrophoresis and fluorography. It appears that the intensity of many spots was enhanced when the cells were stimulated with IL-4 when compared with control cells. This is in agreement with the potent effect of IL-4 on total RNA synthesis observed herein. The two-dimensional gel analyser (using a paired–matched spots program) revealed that 12.0 ± 5.9 % of paired–matched spots ($n = 3$) were more intense in unstimulated cells, indicating that IL-4 can also down-regulate the *de novo* synthesis of some proteins (results not shown).

### Identification of proteins by microsequencing

Three reproducible protein spots were selected for microsequencing experiments because they were easily detected on PVDF membranes following staining with Coomassie Blue (see the Materials and methods section). We first microsequenced a spot designated spot #1. By performing 29 cycles with this material, we observed a perfect match (100 % identity) with the human cytoskeletal protein actin. In the one-letter amino acid code, the sequence was: M-V-G-M-G-Q-K-D-S-Y-V-G-D-E-A-Q-S-K-R-G-I-L-T-L-K-Y-P-I-E. This sequence is common to both the non-muscle $\beta$-and $\gamma$-actin isoforms [38]. Two other spots, spot #2 and spot #3, were microsequenced; surprisingly, these spots were also identified as human actin. The number of cycles performed was seven and 27 for spots #2 and #3 respectively. Interestingly, it was recently reported that actin is cleaved by interleukin-1$\beta$-converting enzyme (ICE) into two fragments of ~ 30 kDa with different isoelectric points (by two-dimensional SDS/PAGE) and one fragment of ~ 14 kDa [37]. However, in the present study we did not microsequence a spot in the 14 kDa region. Densitometric analysis of the three spots revealed that the intensity was markedly increased by IL-4 (8.8 ± 3.8, 13.3 ± 4.0 and 5.7 ± 3.0 for spots #1, #2 and #3 respectively) when compared with that in control cells (3.0 ± 1.6, 4.6 ± 1.6 and 1.7 ± 0.8 respectively).

### IL-4 delays apoptosis of neutrophils

It has been demonstrated previously that various cytokines modulate apoptosis in neutrophils [30,33,34,39], but IL-13 does not [17]. We therefore examined whether IL-4 (at both 10 and 500 ng/ml) exerts such an effect on these cells. As shown in Figure 4, after a 24 h incubation, IL-4 treatment delayed apoptosis when compared with unstimulated cells: 26.1 ± 3.8 % (10 ng/ml IL-4), 20.0 ± 3.6 % (500 ng/ml IL-4) and 41.0 ± 5.8 % (control) cells were in apoptosis at this time ($n = 5$). When incubated with GM-CSF (known to delay neutrophil apoptosis), 10 ± 2.7 % of cells were in apoptosis (Figure 4). Of note is that

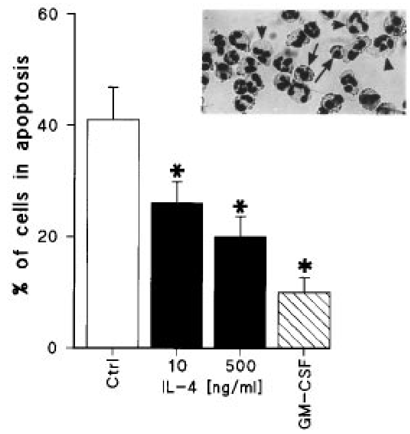


**Figure 4 IL-4 delays the apoptosis of neutrophils**

Cells were incubated in the absence (Ctrl) or presence of IL-4 (10 or 500 ng/ml) or GM-CSF (65 ng/ml) for 24 h, as described in the Materials and methods section. The inset illustrates a representative cytocentrifuge preparation of neutrophils used for evaluating the percentage of apoptotic cells. Arrows indicate neutrophils that have undergone apoptosis (note the characteristic pyknotic nuclei and cell shrinkage), and arrowheads indicate normal neutrophils. Results are means ± S.E.M. of duplicates from five different donors. $^{*}P < 0.05$ compared with control (Student's *t* test).

**Table 1 Confirmation using a flow-cytometric procedure that IL-4 delays neutrophil apoptosis**

The two methods were performed in parallel using neutrophils from two different donors. GM-CSF and IL-4 were present at 10 ng/ml and 65 ng/ml respectively.

| | | Cells in apoptosis (%) | |
|---|---|---|---|
| Method | Addition | Expt. 1 | Expt. 2 |
| Flow cytometry | None | 49 | 40 |
| | GM-CSF | 22 | 21 |
| | IL-4 | 30 | 25 |
| Cytospin | None | 38 | 36 |
| | GM-CSF | 15 | 18 |
| | IL-4 | 24 | 20 |

both GM-CSF- and IL-4-treated cells, as well as control cells, excluded Trypan Blue just before performing the assay (results not shown). This indicates that, under all conditions, no death by necrosis was observed. In a previous study it was reported that IL-4 had no effect on neutrophil apoptosis [39]. This discrepancy may be explained in part by differences in experimental culture conditions. Recently it was demonstrated that the number of neutrophils undergoing apotosis may vary according to the cell concentration used [40]. Cells cultured at $(1–5)\times 10^{6}$/ml were unaffected by IL-6; in contrast, IL-6 inhibited apoptosis in neutrophils cultured at $(10–20)\times 10^{6}$/ml [41]. In the present study, we cultured neutrophils at $10\times 10^{6}$ cells/ml, whereas a density of $1\times 10^{6}$ cells/ml was used in the previous study [36].

We also evaluated neutrophil apoptosis by flow cytometry. Our aim was to correlate the data obtained by microscopic observations from cytocentrifuge preparations with those obtained using another method. We selected the flow-cytometric procedure, which was performed essentially as described previously [30,36]. The results are shown in Table 1. A good correlation was observed between the two methods, as we have previously reported [30], leading us to conclude definitely that IL-4 delays neutrophil apoptosis.

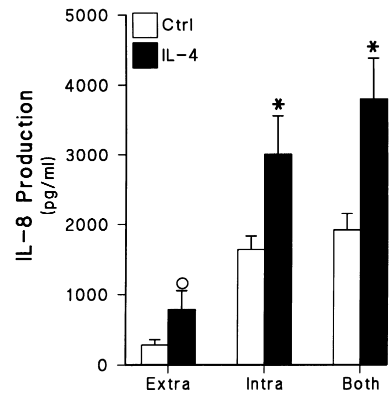


**Figure 5 IL-8 production is increased by IL-4**

The IL-8 concentration was measured in both the extracellular (Extra) and intracellular (Intra) fractions by ELISA 20 h after the addition of IL-4 (10 ng/ml) to neutrophil cultures, as described in the Materials and methods section. Results are means ± S.E.M. from seven different neutrophil donors. $^{*}P < 0.05$ compared with control by Student's *t* test; $\bigcirc P < 0.05$ compared with control by paired non-parametric Mann–Witney test (two-tailed). Ctrl, control.

### IL-4 increases IL-8 production in human neutrophils

Studies on IL-8 production were undertaken, since this cytokine plays a key role in the accumulation of leucocytes at sites of inflammation. In addition, IL-8 is a potent neutrophil chemoattractant, it induces degranulation of neutrophil-specific granules and, moreover, it is known to be produced by neutrophils. Since we had observed previously that IL-13 induces IL-8 production, we decided to examine the effect of IL-4 (known to share many biological activities with IL-13) on IL-8 production. Neutrophil IL-8 production was increased by IL-4 when compared with that in control cells (Figure 5). Overall, neutrophil IL-8 production was significantly increased by IL-4; on accumulating data from both the supernatant and cell lysate fractions, we observed that IL-8 production increased from 1921 ± 233 to 3800 ± 580 pg/ml upon stimulation with 10 ng/ml IL-4 (Figure 5). This represents an increase of almost 2-fold, greater than the increase of 1.6-fold observed on stimulation with IL-13 [17].

## DISCUSSION

IL-4 exerts a variety of biological activities on a large array of cell types by binding to a specific high-affinity receptor [1,2,12,14,27,28]. It is also known that cells responding to IL-4 express a relatively small number of receptors per cell [1,2]. The presence of the IL-4R on neutrophils has been proposed, since these cells were shown to respond to this cytokine both *in vitro* and *in vivo*. However, no direct evidence for the presence of functional IL-4Rs on human neutrophils was reported. In the present study we demonstrated by flow cytometry that the CDw124 component of the IL-4R (previously designated IL-4R$\alpha$) is present on virtually all neutrophils. The confirmation that human neutrophils also express the $\gamma_c$ chain [23–26], and the observation that these cells respond strongly to IL-4 (the present study), leads us to conclude that these cells express functional IL-4Rs.

We have performed experiments on the binding of IL-4 to human neutrophils by flow cytometry using freshly isolated cells incubated with biotinylated IL-4 followed by avidin/FITC reagent (kit from R&D Systems). Our preliminary results indicated that IL-4 binds effectively to the neutrophil surface. However, only about 30 % of cells were positive when compared with controls (D. Girard, R. Paquin and A. D. Beaulieu, unpublished work). The specificity of binding was confirmed by preincubating cells with an excess of unlabelled IL-4. We observed a marked inhibition ($\sim 80\,\%$) of binding of biotinylated IL-4 to the surface of neutrophils. It is important to mention that we always observed that the mean fluorescence intensity was greater by the order of 15 channels when using IL-4R-positive cells in comparison with IL-4R-negative cells. This is an important increase on a log scale. The fact that only 30 % of cells were considered positive is probably due to the limit of sensitivity of the assay, as we have observed previously in IL-2 binding studies [23].

Nevertheless, our results demonstrate that human neutrophils express enough IL-4R to respond functionally to this cytokine. This finding was demonstrated by investigating several neutrophil responses, such as: (i) RNA synthesis; (ii) morphological cell shape changes; (iii) spreading on to glass; (iv) *de novo* synthesis of several neutrophil proteins, of which one was found to be the cytoskeletal protein actin and two others were identified as fragments ($\sim$ 30 kDa, pI 5.4; $\sim$ 25 kDa, pI 5.0) of this protein; (v) apoptosis; and (vi) IL-8 production.

It is of interest that neutrophils treated with IL-4 were found to both retain cytoskeletal function and show delayed apoptosis. In a previous study, a direct relationship was observed between the occurrence of apoptosis and loss of cytoskeletal function in this same cell type [34]. Our results suggest that neutrophil apoptosis is delayed through the ability of IL-4 to increase the *de novo* synthesis of actin, allowing the cell to increase its actin pool. This is in agreement with a recent study showing that actin by itself can play an important role in delaying apoptosis [38]. Actin, in its intact form, is known to be an inhibitor of the activity of DNase I, an enzyme that plays a central role in the internucleosomal fragmentation of DNA that accompanies apoptosis. Actin was also found to be a substrate for the pro-apoptotic cysteine protease ICE in neuronal PC12 cells, and cleavage of actin by ICE led to a loss of the ability of actin to inhibit DNase I activity [37]. In our study, actin synthesis was found to be increased, but cleavage of this protein was also observed. Whether cleavage of actin is ICE-dependent in human neutrophils remains to be determined. Cleavage of actin could be central to how these cells eventually die by apoptosis.

The signalling events occurring after IL-4 binding to human neutrophils remain unknown. Since we had previously observed that IL-13 increases the tyrosine phosphorylation of some proteins, we have recently initiated similar experiments with IL-4. Preliminary results indicate that IL-4 induces tyrosine phosphorylation of more proteins than does IL-13, and to a greater intensity. More specifically, the phosphorylation of a protein with an apparent molecular mass of 120 kDa is strongly increased. To date, the only intracellular protein known to be associated with $\gamma_c$ is Janus kinase (Jak)-3 (molecular mass $\sim$ 120 kDa) [41]. Interestingly, the presence of some Jaks and STATs (signal transducers and activators of transcription) in human neutrophils is starting to be described in the literature. For example, it was found that GM-CSF stimulates Jak-2 and rapidly activates STAT-1 and STAT-3 in these cells [42]. Therefore it is plausible that IL-4 uses the Jak/STAT pathway in human neutrophils, since $\gamma_c$ is present in these cells. This, however, remains an unexplored area of research.

The various effects of IL-4 on neutrophils observed in the present study should alert us to the necessity of fully investigating IL-4–neutrophil interactions in the context of designing and analysing clinical trials, since both neutrophils and IL-4 play important roles in host responses. Presently, the use of IL-4 as a therapeutic agent in clinical conditions other than cancer is being contemplated. This cytokine down-regulates Th1 immunity *in vitro* and in animal model experiments, and its use in controlling T-cell-mediated autoimmune disease has been proposed [43]. Furthermore, IL-4 has potent monokine-suppressing activity, and its utilization as an anti-inflammatory agent in rheumatoid arthritis and inflammatory bowel disease is being considered [44]. However, neutrophils are known to exert mainly pro-inflammatory and tissue-damaging effects due to the rapid release into the extracellular milieu of oxidation compounds and host-defence proteins. How these reponses of neutrophils will manifest themselves during IL-4 therapy in humans will need to be closely monitored. Furthermore, the possibility that neutrophils play other functions in host reponses besides their well characterized functions in acute inflammation is starting to emerge. These cells were shown to have the ability to produce many different cytokines (including IL-8), and thus have the potential to influence other cells important in both humoral and cellular immune responses. Furthermore, although long considered to be short-lived cells, it is now well established that the life cycle of neutrophils may be considerably prolonged under the influence of a number of immunoregulatory molecules [21,30,33,34].

Our results extend the scope of the few previous studies on the effects of IL-4 on human neutrophils. The novel findings are that these cells clearly express functional IL-4Rs, and that IL-4 modulates RNA synthesis, cytoskeletal activity and actin synthesis, and delays apoptosis. Furthermore, because IL-4 markedly affects protein synthesis in neutrophils, it is likely to have a profound effect not only on this cell type but also on surrounding cells. Close monitoring of neutrophil responses will therefore be critical in ongoing and future clinical trials with IL-4, and could provide greater insight into the beneficial as well as the detrimental effects of this form of therapy in humans.

Taken together, our data indicate that, in human neutrophils, IL-4 is more potent than IL-13 in inducing various biological activities. This is not surprising, since similar results have been observed with various other cells [1,2,15]. However, we found a major different biological activity between these two cytokines: IL-4 modulates neutrophil apoptosis, whereas IL-13 does not.

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