Clin Exp Immunol 1995; 99:129–136

IL-4 induces neutrophilic maturation of HL-60 cells and activation of human peripheral blood neutrophils

L. A. BOBER, T. A. WATERS, C. C. PUGLIESE-SIVO, L. M. SULLIVAN, S. K. NARULA & M. J. GRACE *Department of Immunology, Schering-Plough Research Institute, Kenilworth, NJ, USA*

(Accepted for publication 8 September 1994)

SUMMARY

IL-4 is a T-helper cell derived cytokine that has effects on myelomonocytic cell maturation and activation. We have studied the effect of IL-4 on neutrophilic maturation using the cell line HL-60 and found that it has a profound effect on the maturation and activation of the cell line. The treatment of HL-60 cells with recombinant hu IL-4 (0·15 to 15·0 ng/ml) induced a shift in the percentage of HL-60 cells staining positive for chloroacetate esterase enzyme activity (indicating commitment to the neutrophilic lineage). IL-4 increased surface expression of the neutrophil-lineage antigen WEM G11, the complement receptors CR3 (CD11b) and CR1 (CD35), but not for the monocyte differentiation antigen CD14. IL-4 treated HL-60 cells demonstrated enhanced Fc- and complement-mediated phagocytic capacity and increased hexose-monophosphate shunt activity. In addition, IL-4 was capable of sustaining the neutrophil maturation of HL-60 cells that had been pre-treated for 24 h with DMSO. To investigate the effect of IL-4 on the mature neutrophil, we studied freshly isolated and rested human peripheral blood neutrophils. In the absence of other stimuli, neutrophils were induced by IL-4 to have significantly elevated phagocytic responses. The response was specific since treatment with anti-human IL-4 abolished phagocytic stimulation. Finally, IL-4 treatment also stimulated resting neutrophils to migrate toward zymosan-activated serum (ZAS) and human IL-5. The results demonstrate that IL-4 is a potent maturation factor for myelocytes to become neutrophils and that IL-4 can stimulate resting mature neutrophils.

Keywords differentiation HL-60 neutrophils IL-4

INTRODUCTION

The biological activity profile of IL-4 has been expanded from the original observation of the molecule as a B cell growth factor to that of an extremely pleiotropic molecule (for a review, see [1]). Human IL-4 is secreted by T cells, mast cells, and some B cell lines and has been reported to have a variety of effects on lymphoid-lineage cells (B and T cells) [2–5]. For monocyte lineage cells, IL-4 has been reported to activate resting monocytes [6]; induce MHC class II expression [7]; stimulate phagocytosis and killing of intracellular parasites [8] and *Candida albicans* [9]. IL-4 has also been shown to enhance the expression of the low affinity IgE receptor CD23, and the LPS-protein receptor CD14 on the promonocytic cell line U-937. At the molecular level, IL-4 has been shown to induce T cell secretion of GM-CSF and granulocyte colony-stimulating factor (G-CSF) [10] and to induce the expression of M-CSF and G-CSF [8], suggesting an indirect role for IL-4 on myelomonocytic differentiation *in vivo*. IL-4 has been demonstrated to be regulatory depending on the activation state of the monocyte. For instance, IL-4 was a suppressive molecule when cultured with LPS, GM-CSF, or IFN-γ stimulated monocytes. IL-4 treatment of stimulated human monocytes has also been reported to decrease surface expression for CD14 [11], and decrease the steady state mRNA message for IL-1β [12].

Despite these numerous observations as to the effect of IL-4 on monocytes, the literature on the effect of this cytokine on granulocyte-lineage cells is sparse. Neutrophils have been shown to express surface receptors for IL-4 [13]. IL-4 has been reported to enhance the respiratory burst in fMLP-treated human peripheral blood neutrophils, resulting in increased phagocytosis and bactericidal activity [14]. However, IL-4 has also been shown to suppress the steady-state mRNA message for IL-8 in LPS-stimulated human neutrophils. Our laboratory has previously demonstrated that the continuous low-level delivery of murine IL-4 to mice resulted in increased numbers of granulocyte lineage cells in the blood and spleen, and phagocytic activation [15]. In the present study, we examined the effect of human IL-4 on human neutrophil maturation and on resting neutrophil activation.

Correspondence: Dr Michael J. Grace, Department of Immunology, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA.

MATERIALS AND METHODS

Cell culture

The HL-60 cell line was grown from ATCC stock cultures (American Type Culture Collection, Rockville, MD) at a density of 2×10^5 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin G (100 U/ml) and streptomycin sulphate (100 μg/ml), and at 37°C with 5% CO_2. Media pH was adjusted to pH 7·1 prior to addition to cells by titration with sterile 10% sodium carbonate. HL-60 cells used in the experiments were determined to be free from mycoplasma contamination. Media used in the experiments was determined to be free from detectable LPS. Media and supplement reagents, including DMSO, were obtained from CellGro (Fisher Scientific, Springfield, NJ) and JRH Biosciences (Lenexa, KS). In experiments for phagocytosis and esterase activity, IL-4 (0·015–15 ng/ml) or 1·3% DMSO treatment of HL-60 cells was initiated on day 0, with fresh medium and supplement added on day 3. In experiments requiring 1·3% DMSO pre-treatment, cells were treated with DMSO 24 h prior to day 0, with IL-4 then being added on day 0. Cultures were terminated on day 6. Phagocytosis experiments studying the effect of IL-4 on mature human peripheral blood derived neutrophils only had an 18-h incubation with IL-4.

Cytokines and antibodies

Human *Escherichia coli*-derived recombinant IL-4 and NS-1-derived recombinant IL-5 were provided by Dr T. L. Nagabhushan (Schering-Plough Research Institute, Union, NJ). Human *E. coli*-derived recombinant IFN-γ (GIF-E, 4-137 a.a.) was a gift from Dr D. Lundell (Department of Immunology, Schering-Plough Research Institute). Human cell-derived IL-1β was purchased from Collaborative Research (Bedford, MA); granulocyte colony-stimulating factor (G-CSF) was purchased from Genzyme (Boston, MA). Anti-human IL-4 (25D2) was a kind gift from Dr J. Abrams (DNAX Research Institute, Palo Alto, CA).

Histochemical staining for cellular differentiation

HL-60 cells were drawn from incubation cultures, washed in Dulbecco's Modified PBS-Ca^{2+}-Mg^{2+} (DPBS), and centrifuged in triplicate onto glass slides on a low-speed cycle using a Cytospin (Shandon, Pittsburgh, PA). Specific staining for α-naphthyl esterase (ANE) and chloroacetate esterase (CAE) has been described previously [16]. Staining was accomplished using kits purchased from Sigma Chemical Co. (St Louis, MO). Each treatment of HL-60 cells was assayed in triplicate with one slide stained for ANE, one for CAE, and one stained using haematoxylin–eosin. Positive staining was measured by open-field counting using light microscopy; for each slide, triplicate counts were made on minimum 200-cell open fields. Cells were scored as staining positive for CAE if they contained red-purple staining on at least 50% of the cytoplasmic volume; cells were scored as staining positive for ANE if they contained brown-black staining on at least 50% of the cytoplasmic volume. Haematoxylin and eosin slides were used as check controls for CAE, and to control for cellular distortion from the centrifugation.

Cytometry

HL-60 cells were incubated in 2 ml of culture medium in sterile 17 × 100 mm polypropylene test tubes at a density of 2×10^5 cells/ml on day 0. The tubes were lightly capped to allow for air exchange and incubated at 37°C and 5% CO_2 until day 6 as described. Cells were split to 2×10^5 cells/ml and resupplemented with fresh medium on day 3. On day 6, the cells were removed from culture, washed with fresh culture medium (without cytokine), and Fc-receptors were blocked by a short incubation with 1 μg/ml heat-aggregated human IgG. The Fc-blocked cells were washed in DPBS, centrifuged to a pellet in labelling tubes, and resuspended in 50 μl of diluted specific antibody in ice-cold DPBS. Specific antibodies used were: 1 μg WEM G11 (Accurate Antibodies, Westbury, NY); 1 μg anti-CD11b (OKM-1) (Coulter, Hialeah, FL); 0·5 μg anti-CD14 (anti-Leu M3) and 0·1 μg anti-CD35 (anti-CR1) (Becton Dickinson Immunocytometry, San Jose, CA). All specific antibodies were mouse anti-human immunoglobulin. Cells and antibodies were incubated on ice for 30 min, washed with ice-cold DPBS, and reincubated with 100 μl (10 μg) of FITC-conjugated goat anti-mouse IgG1, IgG2b, or IgM for 30 min on ice. After incubation, the cells were washed with DPBS, resuspended in 1 ml PBS and samples were run on a FACScan (Becton Dickinson). Isotype controls for murine IgG1, IgG2b, and IgM, as well as second antibody controls were run for every IL-4 dilution. For each sample 10 000 events were collected in list mode using an open-gate; analysis gates were set so that >99% of isotypic and second antibody staining were excluded from the FL1 positive analysis gate. Data were analysed by LYSIS II software. The data were expressed as the percentage increase in cells with increased mean fluorescence intensity (MFI) for $FL1^+$ cells (treated with increasing levels of IL-4) above the percentage of cells having constitutive expression MFI level (medium alone). The mean percentage of constituent expression observed across six experiments was 25% WEM G11, 21% anti-CD14, 56% anti-CD11b, and 55% anti-CR-1.

Phagocytosis and hexose monophosphate-shunt assays

An assay for the enumeration of the ingestion of heat-killed baker's yeast (*Saccharomyces cerevisiae*) was used [17]. Briefly, 4×10^8 cells/ml of heat-killed yeast particles were opsonized with human serum prior to the addition of cytokine treated HL-60 cells. Opsonized yeast was co-incubated with HL-60 cells (4×10^7 cells: 1×10^6 cells) in a shaking water bath for 90 min. Incubation was terminated by transferring the tubes to an ice-bath. Following centrifugation, ingested yeasts were enumerated with haemocytometer light microscopy; ingested yeasts were discriminated from non-ingested yeast by prior staining with 0·4% trypan blue and 0·2% eosin Y. Cell counts were enumerated for both mean yeast particles per cell and the total percentage of phagocytic cells containing at least one complete yeast particle. Fields of 100 cells were counted in duplicate. The phagocytic index was derived by multiplying the mean particles/cell by the mean percentage phagocytic cells. Data were analysed for statistical validity using the unpaired Student's *t*-test in Statworks 1.2 and a MacIntosh IIci computer.

Hexose-monophosphate shunt (HMP) activity, a measure of the cellular energy required for the catabolism of ingested particles, was determined using an nitroblue-tetrazolium (NBT) reduction assay [18]. Briefly, cytokine-treated HL-60 cells were recovered from culture, washed in DPBS, and resuspended to 1×10^6 cells/ml in reaction medium and

aliquoted at 200 μl/well into wells of a 96-well culture plate. Reaction medium consisted of RPMI 1640 containing 2% fetal bovine serum. Cells were pulsed with 100 μl/well of a 20 μM phorbol myristate acid (PMA) solution in reaction medium followed by 100 μl/well of a 2 mg/ml NBT solution in reaction medium. The plates were incubated for 30 min at 37°C and 5% CO_2. The reaction was terminated by centrifuging the cells at low speed onto glass slides using a Cytospin. Cell preps were counter-stained lightly with DIFF-Quik (Fisher Scientific, Springfield, NJ) and enumerated using open-field light microscopy. Cellular fields were enumerated by scoring black-stained foramazan positive cells (> 50% staining in cytoplasm) in counting fields of 100; scores are the mean of triplicate field-counts. A modified technique was used to examine HMP activity in human peripheral blood neutrophils. Neutrophils were incubated in polypropylene test tubes for only 15 min; a final concentration of 0·06 μM PMA was used.

Isolation of human peripheral blood neutrophils

Venous blood was drawn from healthy human volunteers using EDTA-containing vacutainer tubes. The leucocytes were fractionated by gravity sedimentation over a 4% dextran/saline solution (T500, Pharmacia-LKB, Piscataway, NJ) followed by density gradient centrifugation of the washed supernatant fraction over a discontinuous gradient of 61% and 76% Percoll (Pharmacia-LKB). The granulocytes were recovered from the lower interface in the gradient, washed twice, and incubated in 17 × 100 mm polypropylene test tubes at 2×10^6 cells/ml in 2 ml of DMEM/F12 containing 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin and streptomycin. Cellular viability was checked 18 h post-isolation. Neutrophils were determined to be routinely 95% pure using gentian violet nuclear staining, and cellular viability after 18 h incubation was greater than 95% by trypan blue exclusion.

Chemotaxis

Human neutrophils were tested for their ability to migrate toward a panel of standard chemoattractants. Neutrophils were purified from venous blood drawn from a donor panel of six volunteers who had been pre-screened for neutrophil chemotactic response. This preparation contained 3% or less total eosinophils by specific staining. Neutrophils were incubated in DMEM/F12 culture medium containing IL-4 (0·1–15 ng/ml) for 2 h prior to chemotaxis. Following incubation, the cells were washed, resuspended at 2×10^6 cells/ml, and loaded into pre-charged Boyden chambers. The Boyden chambers were assembled with 3 μm nucleopore membranes that had been coated with 0·005% poly-L-lysine; the base chambers were charged with one each of the following chemoattractants: zymosan-activated serum (ZAS) (1:10 dilution); LTB_4 (3×10^{-7} M); PAF (2×10^{-5} M); hu recombinant IL-5 (100 ng/ml). Zymosan, LTB_4, and PAF were purchased from Sigma Chemical Co. (St Louis, MO). ZAS was prepared by incubating 15 mg zymosan/1·0 ml serum for 1 h at 37°C. The ZAS was heat-inactivated and clarified prior to aliquoting for storage at −70°C. After assembly, the chambers were incubated for 90 min at 37°C and 5% CO_2. Upon termination of migration, the chambers were disassembled and the filters were processed for staining with Wright–Giemsa to distinguish neutrophils from contaminating eosinophils and basophils. The chemoattractant side of the filters was then examined using light microscopy and the cellular migration enumerated by open-field counting of 10 individual fields using a Bioquant Image Analysis system (Bioquant, Nashville, TN).

RESULTS

The effect of hu IL-4 on the differentiation of the premyelocytic cell line HL-60 to an active phagocyte

The HL-60 cell line has been used as a model for the investigation of neutrophil cell function and as a tool to elucidate the various pathways of myelomonocytic cell differentiation. The HL-60 cell line is heterogeneous in commitment towards either the neutrophil or the monocyte and has been shown to be responsive to either one or the other pathway depending on stimulus [19]. Using a simple cytoplasmic staining technique for either α-naphthylesterase (monocytic) or chloroacetate esterase (neutrophilic), we examined HL-60 cytospin preparations for an effect of IL-4 on lineage commitment. HL-60 cells were incubated in RPMI culture medium supplemented with either IL-4 or controls for 7 days. HL-60 cells incubated in medium alone stained 25% ANE positive and 23% CAE positive, with the remaining cells staining negative for either of the esterases (Table 1). Treatment with IL-4 between 0·15 and 15 ng/ml doubled the percentage of cells staining positive for CAE (55–56%) and increased the number of cells staining positive for ANE (36–39%) (Table 1). The positive neutrophil commitment control, DMSO, increased CAE^+ staining cells to 52% while having no effect on the percentage of ANE^+ staining cells. The positive monocyte commitment control, IFN-γ, increased ANE^+ staining cells to 56% while increasing the percentage CAE^+ staining cells to 31% (Table 1). The increase in CAE-staining cells with IL-4 treatment appeared to arise partly from the already committed neutrophil lineage cells, about 25% in medium culture, and an additional 25% from cells that stained esterase negative in the absence of cytokine. The increase in the percentage of cells staining positive for ANE, while much smaller than that seen for CAE, was significant as well; this suggested that while IL-4 may preferentially

Table 1. IL-4 treatment increases the percentage of HL-60 cells staining positive for chloroacetate esterase

Treatment	Percent positive chloroacetate esterase	Percent positive alpha napthyl esterase
IL-4 (ng/ml)		
15·0	58 (5)	40 (1)
1·5	55 (3)	39 (4)
0·15	54 (5)	32 (3)
DMSO		
1·3%	52 (4)	19 (2)
IFN-γ		
50	31 (2)	56 (5)
Medium	25 (2)	23 (3)

HL-60 cells were incubated with cytokine, medium alone, or 1·3% DMSO for 7 days and then stained for cytoplasmic esterase activity. The data are presented as the mean percent positive staining cells (s.e.m.). The experiment is a single representative experiment from six.

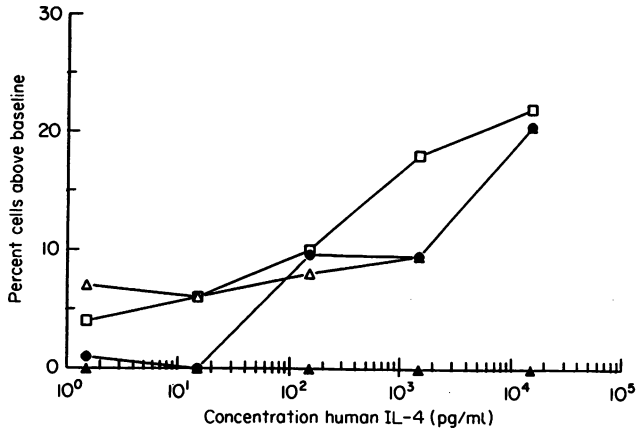

Fig. 1. The effect of human IL-4 on HL-60 surface expression of myeloid maturational antigens. HL-60 cells were incubated for 7 days with either IL-4 or in medium alone. After treatment the cells were washed free of IL-4 and labelled using specific MoAb for WEM G11 antigen (□), CD14 (▲), CR1 (CD35) (△), or CR3 (CD11b) (●). Labelling was detected using FITC-conjugated goat anti-murine immunoglobulins and fluorescence cytometry as described in Materials and Methods. Data are a single representative experiment from six.

push HL-60 cells to a neutrophilic lineage, some cells, possibly through synergy of another autocrine factor with IL-4, could clearly be pushed to a monocytic lineage. The response of the HL-60 cells in this assay was not dose-dependent but ceased with a threshold lower than 0·015 ng/ml. This same effect was observed with IFN-γ titrations (data not shown) on HL-60 ANE^+ staining and thus appeared to be a limitation of the assay.

IL-4 treatment of HL-60 cells also induced changes in the surface expression of antigens relevant to neutrophil maturation and phagocytosis. Seven day cultures of HL-60 cells incubated with IL-4 were examined by cytofluorometry using specific antibodies for the expression of WEM G11 antigen (associated with myeloid development), CR3 (CD11b), CR1 (CD35), and as a control, the monocyte lineage marker CD14. Baseline expression of the antigens was established with the medium only controls on day 6. The data in Fig. 1 summarize the results from the experiment by expressing any increase due to IL-4 incubation as an increase in the percentage of increased mean fluorescent intensity (MFI) above that of medium alone. In this experiment we were able to observed dose-dependent increases by IL-4 in the percentage of cells expressing increased WEM G11 antigen, CR3 (CD11b), and CR1 (CD35) above medium control (Fig. 1). However, we were not able to observe any IL-4 induced increase in the percentage of cells expressing the monocyte maturation antigen CD14. CD14 was inducible on the HL-60 cell line since CD14 cellular MFI was slightly increased with IL-4 treatment, and IFN-γ treatment induced a significant increase in the cellular MFI and percentage CD14 expression (data not shown). This experiment was repeated three times on consecutive passages of HL-60 cells with similar results observed.

Since the primary role of the neutrophil in the host is to aid in the attack and clearance of invasive pathogens by phagocytosis, we wanted to determine whether the IL-4 induced phenotypic maturation of HL-60 cells translated into a fully functional maturation. We modified a microscopic assay to help assess the effect of IL-4 treatment on the ability of the HL-60 cells to ingest heat-killed baker's yeast. In this assay, ingested yeast cells can be easily observed and enumerated under high power, and non-ingested yeast distinguished by staining with trypan blue and eosin. IL-4 treatment from 0·015 ng/ml to 15 ng/ml induced an increase in the HL-60 phagocytic capacity (Table 2). The effect of IL-4 above 0·15 ng/ml almost doubled the amount of yeast ingested per HL-60 cell (from 2·4 to 5·4) and the percentage of HL-60 cells ingesting at least one yeast cell (from 18% to 36%). When expressed as a phagocytic index, there was an IL-4 dose-dependent increase in the response of HL-60 cells. This increase was comparable to that seen with the positive control, DMSO. Parallel experiments were performed for the measurement of hexose-monophosphate shunt activity and for CAE staining (Table 2). Analysis of NBT percentage, a measure of HMP-shunt activity, revealed that HL-60 cells increased from 17% in medium alone to 35% or greater with at least 0·15 ng/ml IL-4 treatment (Table 2). These data also

Table 2. The effect of IL-4 on HL-60 phagocytosis and HMP-shunt

Cytokine	Particles/cell	Percent phagocytic	Phagocytic index	Percent† NBT	Percent * CAE
IL-4 (ng/ml)					
15·0	5·6 (1·1)‡	44 (4)‡	254 (51)‡	34 (3)‡	62 (3)‡
1·5	5·4 (1·3)‡	41 (4)‡	221 (31)‡	38 (4)‡	56 (6)‡
0·15	5·4 (1·5)‡	36 (3)‡	184 (38)‡	35 (5)‡	53 (3)‡
0·015	3·7 (1·1)	27 (5)	96 (22)	36 (4)‡	40 (2)‡
DMSO					
1·3%	3·6 (0·4)‡	71 (10)‡	259 (54)‡	92 (2)‡	56 (2)‡
Medium	2·4 (0·3)	18 (5)	42 (12)	17 (2)	21 (5)

HL-60 cells were incubated with cytokine, medium alone, or 1·3% DMSO for 7 days and then tested for phagocytic activity, HMP-shunt, and CAE staining. Refer to Materials and Methods for assay specifics. The data are presented as the mean (s.e.m.) from six separate experiments.

*Percent HL-60 cells staining positive for chloroacetate esterase activity.

†Percent HL-60 cells staining positive for NBT redution (foramazan).

‡$P < 0·05$ as determined by unpaired Student's *t*-test.

Table 3. DMSO pulse followed by IL-4 continues HL-60 development toward neutrophil lineage

Treatment	Particles/cell	Percent phagocytic	Phagocytic index	Percent† NBT	Percent* CAE
IL-4 (ng/ml)					
15·0	4·0 (0·5)‡	59 (10)‡	236 (15)‡	50 (0·3)‡	50 (0·2)
1·5	3·9 (1·0)‡	45 (3)‡	175 (31)‡	52 (2·0)‡	53 (0·7)
0·15	3·6 (1·0)‡	37 (8)	133 (41)	51 (2·0)‡	50 (0·4)
0·015	2·9 (0·5)	35 (1)	102 (20)	42 (3·0)	51 (0·2)
DMSO					
1·3%	3·4 (0·6)‡	58 (09)‡	189 (05)‡	100 (0·0)‡	70 (0·3)‡
Medium	2·2 (0·4)	20 (04)	42 (16)	24 (0·4)	32 (0·7)

HL-60 cells were incubated for 24 h in medium alone or 1·3% DMSO; after 24 h, the cells were washed free from DMSO and aliquoted to tubes containing either IL-4 or 1·3% DMSO. The incubation was continued for 6 days further, with cells being split and resupplemented on day 3. Data are presented as mean (s.e.m.) from six separate experiments.

*Percent HL-60 cells staining positive for chloroacetate esterase activity.

†Percent HL-60 cells staining positive for NBT reduction (foramazan).

‡$P < 0·05$ as determined by unpaired Student's t-test.

appear to correlate well with the increase in percentage observed for phagocytic response, implying that the phagocytic positive cells were the HMP-shunt active population. CAE staining of the HL-60 cells from these experiments was observed to be higher than the percentage of phagocytic active HMP-shunt activated cells (Table 2).

IL-4 was also observed to maintain the differentiation process of HL-60 cells that had been exposed to but were subsequently removed from DMSO (Table 3). If DMSO is removed from culture medium within the first 24 h, HL-60 cells regress back to an undifferentiated premyelocytic stage [20]. However, the exposure of HL-60 cells to IL-4 after the removal of DMSO in the first 24 h continued the differentiation process of the cells toward a mature functional granulocyte. As shown in Table 3, IL-4 treatment resulted in increased phagocytic function, HMP-shunt activation, and the percentage of CAE-staining cells, indistinguishable from a 7 day treatment with DMSO alone or IL-4 alone (Table 3).

Table 4. IL-4 enhances the phagocytic function of human peripheral blood neutrophils

Treatment	Particle/cell	% Phagocytic cells	Phagocytic index
Medium	2·9 (0·2)	19·8 (1·9)	63 (10)
IL-4 (ng/ml)			
15·0	4·2 (0·3)*	41·6 (3·0)	184 (21)*
1·5	4·0 (0·3)*	35·6 (2·5)*	143 (15)*
0·15	2·9 (0·2)	28·9 (4·2)*	82 (12)
GM-CSF (ng/ml)			
2·5	4·8 (0·3)*	55·4 (4·1)*	264 (29)*
IL-4 + 25D2†	2·9 (0·3)	20·9 (2·2)	66 (10)
IL-4 + Isotype	4·1	40·2	164

Human neutrophils were isolated from whole blood as described in Materials and Methods. Cells were rested and then treated for 18 h with cytokine or medium alone followed by assay for phagocytic response. Data are presented as the mean (s.e.m.) across a donor panel of six.

*$P < 0·05$ as determined by unpaired Student's t-test.

†15 ng/ml IL-4 and 10 μg/ml of 25D2 MoAb were co-incubated for 30 min at 37°C prior to phagocytic assay.

The effect of hu IL-4 on the activation of human peripheral blood neutrophils.

To extend our observations from the HL-60 cell line, we tested the effect of IL-4 treatment on mature human peripheral blood neutrophils prepared from normal healthy volunteers. The first experiments were designed to corroborate the phagocytic response. IL-4 treatment for 18 h (overnight) prior to assay resulted in a dose-dependent increase in the mean yeast particles/cell ingested, mean percentage of phagocytic neutrophils containing at least one yeast particle, and the derived phagocytic index (Table 4). The stimulation in phagocytic response was observed to be statistically significant ($P < 0·05$) at 1·5 and 15 ng/ml IL-4 treatment. This effect was specific for IL-4 since treatment with the anti-human IL-4 MoAb 25D2 abolished the phagocytic stimulation whereas the isotype control had no effect. This effect could not be explained simply by IL-4 inducing neutrophil survival since the 18 h viability measurements demonstrated no significant difference in the percent viable neutrophils between IL-4 treatment and the media control.

Neutrophil extravasation and migration toward sites of infection in the host is critical for the initiation of a competent cell-mediated immune response. To test the concept for the role of IL-4 in neutrophil localization, we examined the ability of IL-4 treatment on human neutrophils to influence chemotactic migration towards a panel of known chemoattractants and human IL-5. Human peripheral blood neutrophils were treated for 18 h with IL-4 prior to assay. After washing the cells to remove IL-4, the cells were placed in Boyden chambers containing in the lower chamber ZAS, LTB_4, PAF, or hu recombinant IL-5. Neutrophil migration across the nucleopore membrane was quantified using image analysis. The data in Table 5 are presented as chemotactic index (i.e. the x-fold stimulation of cytokine treated migration across the membrane compared with medium control migration). The neutrophil chemotaxis observed with IL-4 treatment toward ZAS was weak, compared with the GM-CSF control, but was statistically significant ($P < 0·05$) at 9 and 15 ng/ml IL-4. IL-4 treatment did not significantly induce neutrophil chemotaxis toward LTB_4 or PAF. Of interest was the effect of IL-4 treatment on neutrophil chemotaxis towards IL-5. Neutrophil migration towards IL-5

Table 5. IL-4 enhances the chemotactic migration of human peripheral blood neutrophils

Cytokine	Concentration (ng/ml)	Chemotactic index IL-5	ZAS	LTB4	PAF
IL-4	15·0	2·8 (1·2)*	1·3 (0·1)*	ND†	ND†
	9·0	3·0 (1·0)*	1·2 (0·1)*	1·1 (0·4)	1·1 (0·2)
	4·5	2·0 (0·7)*	1·3 (0·2)	1·0 (0·2)	1·0 (0·1)
	1·5	1·6 (0·3)*	1·2 (0·2)	1·3 (0·6)	0·9 (0·1)
	0·1	1·9 (0·9)	1·1 (0·2)	1·0 (0·3)	1·5 (0·5)
GM-CSF	1·0	3·6 (2·0)*	1·6 (0·2)*	1·7 (0·4)*	3·0 (1·0)*

Cytokine or medium-alone treated neutrophils (2 h) were placed in Boyden chambers and incubated for 90 min to assay for chemotaxis toward a panel of known chemoattractants or human recombinant IL-5.

The chemotactic index (CI) is derived from the equation CI = mean migrated cells per field (MMCPF) with cytokine treatment/MMCPF in medium alone. The MMCPF was derived from image analysis of 10 separate fields. The MMCPF for the chemotactic media control (i.e. no chemoattractant) was less than 10 cells/field regardless of whether the neutrophils were incubated in medium or IL-4. The media treatment control MMCPF with the chemoattractants was 131 + 21 (ZAS), 51 + 15 (IL-5), 152 + 21 (LTB_4), and 83 + 10 (PAF). $*P < 0{\cdot}05$ as determined by Wilcoxin rank-order analysis of the chemotactic indices derived from MMCPF data from three separate experiments.

†Data from three complete experiments were not available.

was significant ($P < 0{\cdot}05$) at all levels tested; GM-CSF was also a potent inducer of neutrophil migration towards IL-5.

DISCUSSION

This study demonstrates that human IL-4 plays a role in the maturation of immature myelocytes to become functionally competent phagocytes of the neutrophil lineage. In addition, we confirm and extend previous observations that IL-4 can activate fully mature human peripheral blood neutrophils. The neutrophil has long been considered a key component of cellular-mediated and non-specific immunity, i.e. a professional killer of bacteria and fungi, and heavily involved in acute inflammatory reactions, both beneficial and detrimental to the host. Only recently has the effect of a T cell cytokine regulatory network been studied on neutrophil responsiveness [21]. IL-4 has been suggested to support granulocytopoiesis either directly [22], or indirectly via other cytokines [23]; these haematopoietic studies have demonstrated that IL-4 can induce CFU-GM colony formation in human [24] and in mouse bone marrow stem cell cultures [22,23].

Our study has addressed the effect of IL-4 on the myeloid-committed pre-neutrophil–monocyte cell line HL-60. *In vitro* treatment with low levels of IL-4 (1·5–15 ng/ml) induced esterase negative HL-60 cells to stain positive for CAE. CAE conversion appeared to be a threshold event that proceeded fully functional maturation since CAE^+ staining cells were found at a higher percentage than the phagocytically active cells. Further support for phenotypic neutrophil maturation was derived from the increase observed in the percentage of cells expressing neutrophil antigen (WEM G11) and complement receptors. This increase in the percentage of surface antigen expression was dose-responsive for IL-4; and the increased surface antigen positive population correlated well (about 25%) with the increase in the commitment of esterase-negative staining cells compared with those staining positive for CAE.

Interestingly, IL-4 also appeared to increase the percentage of HL-60's staining positive for ANE, though the percentage increase was smaller than observed for CAE staining. However, IL-4 was very weak in inducing surface-antigen expression typical of monocytes, since there was no observed increase in the percentage of HL-60 cells expressing CD14. The effect of IL-4 on monocyte maturation and commitment is unclear from the current literature, but based on the potent effects of IL-4 on mature monocytes, should be investigated further.

IL-4 induced a neutrophil-like phenotype on the HL-60 cells and stimulated a fully functional phagocytic response from the cells. This stimulation was observed in both the yeast per cell measurement and the total percentage of phagocytic cells; this suggests that IL-4 induced both functional activation of mature cells as well as the differentiation of functionally immature cells. This further supports the concept that IL-4 is not only activating the HL-60 population that was CAE^+ prior to the addition of IL-4 to culture, but also activating a population of HL-60 cells that were staining esterase negative before treatment. However, the larger percentage of CAE staining cells in the phagocytosis experiments indicates that some of the CAE^+ HL-60 cells were not fully functional within the time frame of the experiments in this study. With the HMP-shunt measurements, we observed a good correlation of IL-4-induced phagocytosis and HMP-shunt activated cells.

A recent report by Vassiliadis and colleagues demonstrated that IL-4 induced HL-60 cells to express increased surface levels of MAC-1 (CD11b) and increased phagocytic response to latex beads [25]; these authors concluded that IL-4 resulted in the functional maturation of HL-60 cells to a monocyte lineage. We confirm their observations for the enhancement of CD11b and phagocytosis but clearly demonstrate that these endpoints result from neutrophilic maturation rather than monocytic. We observed a significant increase in CAE^+ ANE^- staining cells, which we regard as a biochemical neutrophil phenotype. Second, and most important, we regard the absence of CD14 on cells expressing the WEM G11 (neutrophilic) antigen as strong evidence for an immunological neutrophil phenotype. We have also observed that cultures of the IL-4-treated HL-60 cells appeared to contain higher percentages of cells with a metamyelocyte-like nucleus, and even a low percentage (5–8%) of cells with an early band-form-like nucleus (data not shown). It is important to note, however, that our data also demonstrate that monocyte lineage activation of HL-60 cells is possible, though to a lesser degree compared with myeloid lineage commitment in our studies. We conclude that IL-4 can induce HL-60 cells to commit to a neutrophil lineage that is fully capable of ingesting and catabolizing an invasive pathogen.

IL-4 has been reported to suppress differentiation of HL-60 cells induced by IL-1, IL-6, and TNF-α [26]. We feel that these results do not contradict our results, rather they serve to underscore the regulatory nature of the IL-4 molecule. Our experiments were performed on non-stimulated resting HL-60 cells. Thus, the effect of IL-4 on neutrophils is activation-state

dependent; and the effect directly correlates with the effect of IL-4 on resting monocytes *versus* LPS-stimulated monocytes [11,12].

To support our observations in the HL-60 cell line, we investigated the effect of IL-4 on mature human peripheral blood neutrophils. Our phagocytosis experiments confirm and expand the existing literature on neutrophil action by IL-4. We demonstrate that IL-4 induces fully competent activation of neutrophils for phagocytosis and HMP-shunt. Boey and colleagues [14] have only observed an IL-4 mediated increase in fMLP respiratory burst, analogous to our HMP-shunt observations; they did not look at the phagocytic response. However, the effect of IL-4 on neutrophils appears to depend on the activation state; IL-4 has been shown to down-regulate LPS-stimulated neutrophil production of IL-8 [27]. In our study, we used non-stimulated and rested human neutrophils.

An interesting observation is the effect of IL-4 on enhancing neutrophil chemotaxis toward IL-5. Like IL-4, IL-5 is a Th2 cell derived cytokine [28–30]. In addition mast cells have also been reported to produce both IL-4 and IL-5 at high levels, suggesting an important role in mediating allergic inflammation [31]. IL-5 promotes eosinophil activation and has a role in maintaining the presence of the cell in allergic responses [28, 32–34]. These data suggested an interrelationship between IL-4 and IL-5 in regulating neutrophil responses. IL-5 is not selective in its priming of eosinophil migration but can also prime neutrophils for migration [35]. Neutrophils participate in lung inflammation [32], airway hyperreactivity [33], and in the late phase reaction in asthma [36]. Our data support the hypothesis that IL-4 can induce neutrophil migration to another auto-inflammatory produced cytokine. There is a definite positive effect to IL-4 induced neutrophil migration as well. These data may partially explain the observed influx of eosinophils and neutrophils into rejecting tumours in mice that had been implanted with tumour cells transfected to produce IL-4 [37], and thus demonstrate a mechanism for driving cell-mediated, non-specific tumour cytotoxicity. A potential anti-tumour mechanism for IL-4 is further supported by the observation that IL-4 treated neutrophils migrated towards ZAS (C5a).

In summary, we have observed that IL-4 is an important molecule for the direct maturation of pre-myeloid cells to a neutrophil lineage and that IL-4 serves as a functional activator of mature neutrophils. These activities appear to correlate with and complement those reported for IL-4 on monocyte lineage cells. Together these data appear to help begin elucidate a picture for IL-4 as an important regulatory molecule in the process of the generation of cellular mediated immunity.

ACKNOWLEDGMENTS

The authors would like to thank Mr Bruce Bennett for his contributions to the HMP-shunt experiments and Ms Serafina Braun for her contributions to the phagocytosis experiments. The authors also thank Dr Jay Fine for his critical review and discussion and Ms Maria Carter for the preparation of this manuscript.

REFERENCES

1 The immunobiology of interleukin 4. In: O'Garra A, Spits H, eds. The 53rd Forum in Immunology, Res Immunol 1993; **144** (8).
2 Mosmann T, Bond M, Coffman R, Ohara J, Paul W. T cell and mast cell ines respond to B cell stimulatory factor. Proc Natl Acad Sci USA 1986; **83**:5654.
3 DeFrance T, Aubry J, Rousset F, Vanbervliet B, Bonnefoy J, Arai N, Takebe Y, Yokota T, Lee F, Arai K, De Vries J, Bancereau J. Human recombinant interleukin 4 induces Fcε receptors (CD23) on normal human B lymphocytes. J Exp Med 1987; **165**:1459–67.
4 Valle A, Zuber C, DeFrance T, Djossou O, De Rie M, Banchereau J. Activation of human B lymphocytes through CD40 and interleukin 4. Eur J Immunol 1989; **19**:1463.
5 Shapira S, Vercelli D, Jabara H, Fu S, Geha R. Molecular analysis of the induction of immunoglobulin E synthesis in human B cells by interleukin 4 and engagement of CD40 antigen. J Exp Med 1992; **175**:289.
6 Te Velde A, Klomp J, Yard B, De Vries J, Figdor C. Modulation of phenotypic and functional properties of human peripheral blood monocytes by IL-4. J Immunol 1988; **140**:1548.
7 Crawford R, Finbloom D, Ohara J, Paul W, Meltzer M. B cell stimulatory factor 1 (interleukin 4) activates macrophages for increased tumoricidal activity and expression of Ia antigens. J Immunol 1987; **139**:135.
8 Wirth J, Kierzenbaum J, Zlotnik A. Effects of IL-4 on macrophage functions: Increased uptake and killing of a protozoan parasite (*T. cruzi*). Immunol 1989; **66**:296.
9 Redmond H, Schuchter L, Shou J, Hofmann K, Leon P, Marodi L, Johnston R, Daly J. Interleukin 4 (IL-4) enhances macrophage microbicidal function. J Leukocyte Biol 1990; **48** (Suppl 1): 24.
10 Broxmeyer H, Lu L, Cooper S, Tushinski R, Mochizuki D, Rubin B, Gillis S, Williams D. Synergistic effects of purified recombinant human and murine B cell growth factor on colony formation *in vitro* by hematopoietic precursor cells. J Immunol 1988; **141**:3852.
11 Lauener R, Goyert S, Geha R, Vercelli D. Interleukin 4 down-regulates the expression of CD14 in normal human monoyctes. Eur J Immunol 1990; **20**:2375–81.
12 Geiger T, Arnold J, Rordorf C, Hann R, Vosbeck K. Interferon γ overcomes the glucocorticoid mediated and interleukin 4 mediated inhibition of interleukin 1β synthesis in human monocytes. Lymphokine Cytokine Res 1993; **12**:271–8.
13 Park L, Friend D, Sassenfeld H, Urdal D. Characterization of the human B cell stimulatory factor 1 receptor. J Exp Med 1987; **166**: 476.
14 Boey H, Rosenbaum R, Castracane J, Borish L. Interleukin 4 is a neutrophil activator. J Allergy Clin Immunol 1989; **83**:978.
15 Sullivan L, Bober L, Grace M, Braun S, Macosko H, Payvandi F, Pugliese-Sivo C, Narula S. The *in vivo* biological activity of murine IL-4 and the role of endogenous cytokines. Pathobiology 1994; **62**:59–72.
16 Yam L, Li C, Crosby W. Cytochemical identification of monocytes and granuloctyes. Am J Clin Path 1971; **55**:283.
17 Patterson-Delafield J, Lehrer R. A simple microscopic method for identifying phagocytic cells *in vitro*. J Immunol Meth 1977; **18**:377.
18 Baechner R, Nathan D. Quantitative nitroblue tetrazolium test in granulomatous disease. New Engl J Med 1968; **278**:971.
19 Lord J, Bunce C, Duncan R, Phillips I, Shennan K, Docherty K, Brown G. Changes in insulin receptor expression in HL-60 cells induced to differentiate towards neutrophils or monocytes. J Mol Endocrinol 1988; **1**:197–201.
20 Yen A, Brown D, Ishbaugh J. Precommitted states induced during HL-60 myeloid differentiation: possible similarities of retinoic acid and DMSO-induced early events. Exp Cell Res 1987; **173**:80–4.
21 Campbell P. The neutrophil, a professional killer of bacteria, may be controlled by T cells. Clin Exp Immunol 1990; **79**:141.
22 Kishi K, Ihle J, Urdal D, Ogawa M. Murine B cell stimulatory factor 1 (BSF-1)/interleukin 4 is a multilineage colony stimulating factor that acts directly on primitive hemopoietic progenitors. J Cell Physiol 1989; **139**:463.
23 Sonoda Y, Okuda T, Yokota S, Maekawa T, Shizumi Y, Nishigaki

H, Misawa S, Fuji H, Abe T. Actions of human interleukin 4/B cell stimulatory factor 1 on proliferation and differentiation of enriched hematopoietic progenitor cells in culture. Blood 1990; **75**:1615.

24 Noma T, Nakakubo H, Hama K, Honjo T. Multiple effects of human recombinant interleukin 4 on human myeloid monocyte cell lines. Immunol Lett 1989; **21**:323.

25 Vassiliadis S, Kyrpides N, Papamatheakis J. The role of IL-4 in human myeloid leukemia: stimulation of RNA synthesis and transduction of differentiation signals through an IL-4 receptor leads to functional and HLA positive HL-60 cells. Leuk Lymphoma 1992; **7**:235–42.

26 Yamashita U, Tanaka Y, Shirakawa F. Suppressive effect of interleukin 4 on the differentiation of M1 and HL-60 myeloid leukemic cells. J Leukocyte Biol 1993; **54**:133–7.

27 Wertheim W, Kunkel S, Standiford T, Burdick M, Becker F, Wilke C, Gilbert A, Strieter R. Regulation of neutrophil derived IL-8: the role of prostaglandin E_2, dexamethasone, and IL-4. J Immunol 1993: **151**:2166–75.

28 Lopez A, Sanderson C, Gamble J, Campbell H, Young I, Vadas M. Recombinant human interleukin 5 is a selective activator of human eosinophil function. J Exp Med 1988: **167**:219.

29 VanLeeuven B, Martinson M, Webb G, Young I: Molecular organization of the cytokine gene cluster involving the human IL-3, IL-4, IL-5, and GM-CSF genes on human chromosome 5. Blood 1989; **73**:1142.

30 Swain S, Bradley L, Croft M, Tonkonogy S, Atkins G, Weinberg A, Duncan D, Hedrick S, Dutton R, and Huston D. Helper T-cell subsets: phenotype, function, and the role of lymphokines in regulating their development. Immunol Rev 1991: **123**:115–44.

31 Moller A, Grabbe J, Czarnetski B. Mast cells and their mediators in immediate and delayed immune reactions. Skin Pharmacol 1991; **4** Suppl 1:56–63.

32 Morley J, Smith D. Lung inflammation and its significance for asthma therapy. Agents Actions 1989; **26**:31.

33 Larsen G, Bethel R, Irvin C, Martin R, Uchida D. Granulocytes and airway reactivity. Am Rev Respir Dis 1991; **143**:S64.

34 Kroegel C. The role of eosinophils in asthma. Lung 1990; **168**: (Suppl. 5–17).

35 Kapp A, Zeck-Kapp G. Interleukin 5 induced granulocytic action in atopic patients. Br J Dermatol 1991; **125**:108–15.

36 Hogg J, James A, Pare P. Evidence for inflammation in asthma. Am Rev Respir Dis 1991; **143**:S39.

37 Tepper R, Levinson D, Stanger B, Campos-Torres J, Abbas A, Leder P. IL-4 induces allergic like inflammatory disease and alters T cell development in transgenic mice. Cell 1990; **62**:457.