

Role of leukotrienes in the regulation of human granulocyte behaviour: dissociation between agonist-induced activation and retardation of apoptosis

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1 Since most inflammatory mediators that stimulate granulocyte responsiveness also delay apoptosis, it is often assumed that activation and longevity are causally related. Using isolated human peripheral blood neutrophils and eosinophils, we examined this association by exploiting the proinflammatory lipid mediators, the leukotrienes (LTs), and investigated granulocyte function and apoptosis.

2 LTB₄ induced elevation of intracellular free Ca²⁺ concentration ([Ca²⁺]_i), cell polarisation and retardation of neutrophil apoptosis, although the antiapoptotic effect occurred only at concentrations ≥300 nM. LTB₄-induced activation was attenuated by CP-105,696, a BLT1-specific antagonist suggesting classical LTB₄ receptor BLT1 involvement.

3 Despite demonstrating the presence of the neutrophil intracellular LTB₄ receptor peroxisome-proliferator activator receptor-α (PPARα) in neutrophils, the selective PPARα agonist WY-14,643 did not mimic LTB₄-induced pro-survival effects.

4 LTB₄-induced survival, however, also appeared to be mediated by BLT1 since CP-105,696 inhibited the LTB₄-mediated antiapoptotic effect. Furthermore, based on studies with CP-105,696 and 5-lipoxygenase inhibitors, lipopolysaccharide (LPS)-, granulocyte–macrophage colony-stimulating factor (GM-CSF)-, dexamethasone- and dibutyryl-cAMP (db-cAMP)-induced delay of neutrophil apoptosis did not involve autocrine production of LTB₄.

5 Although LTB₄ and LTD₄ induced human eosinophil [Ca²⁺]_i elevation and polarization, these LTs did not influence eosinophil apoptosis. Furthermore, LTB₄- and LTD₄-induced eosinophil activation was attenuated by CP-105,696 and the Cys-LT₁ receptor antagonist montelukast, respectively, highlighting specific receptor dependency.

6 Thus, mediator-triggered granulocyte activation and antiapoptotic pathways are distinct events that can be differentially regulated.

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Abbreviations: BLT, LTB₄ receptor; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; Cys-LT, cysteinyl leukotriene; db-cAMP, dibutyryl-cAMP; fMLP, formyl-leucyl-phenylalanine; GM-CSF, granulocyte/macrophage colony-stimulating factor; LPS, lipopolysaccharide; PAF, platelet-activating factor; PPAR, peroxisome-proliferator activator receptor

Introduction

Granulocytes are key effector cells of the immune system, where neutrophils provide first-line protection against bacterial and fungal invasion and eosinophils play an important role in mediating antiparasitic defences. Over-recruitment, inappropriate activation or dysregulated clearance of these cells likely contribute to the pathogenesis and propagation of a wide variety of inflammatory disorders. While neutrophil

products can cause tissue damage in inflammatory diseases such as chronic obstructive pulmonary disease and rheumatoid arthritis, inappropriate release of eosinophil products is associated with allergic disorders such as allergic rhinitis and asthma (Rossi & Haslett, 1998; Giembycz & Lindsay, 1999; Gompertz & Stockley, 2000). Apoptosis or programmed cell death is a critical process regulating the lifespan of inflammatory cells and the resolution phase of inflammation (Haslett, 1999; Ward *et al.*, 1999b; Lawrence *et al.*, 2002). Apoptosis is associated with the maintenance of plasma membrane integrity, downregulation of functional responsiveness (Whyte *et al.*, 1993b), and triggers apoptotic cell recognition

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and engulfment by phagocytic cells (Savill *et al.*, 1989; Stern *et al.*, 1992; Walsh *et al.*, 1999). Remarkably, the phagocytic clearance of these cells fails to excite an inflammatory response (Meagher *et al.*, 1992); indeed, ingestion of apoptotic granulocytes *in vitro* induces an anti-inflammatory cytokine and mediator profile in the phagocyte (Stern *et al.*, 1996; Fadok *et al.*, 1998; Liu *et al.*, 1999). Thus, this process provides an efficient and noninflammatory mechanism for the clearance of potentially toxic and unneeded cells from the inflamed site.

Neutrophils and eosinophils are derived from a common myeloid precursor, and when activated, exhibit similar responses (e.g., cell polarisation, chemotaxis, adhesion, degranulation and superoxide anion generation). Comparative studies of functional responses in these cells have contributed significantly to our understanding of granulocyte behaviour, including apoptosis. Of interest, despite their close haematopoietic origins, apoptosis of eosinophil and neutrophil granulocytes can be regulated in a selective manner. For example, glucocorticoids induce eosinophil apoptosis but retard this process in the neutrophil (Meagher *et al.*, 1996). Many of the proinflammatory mediators present at an inflamed site not only influence granulocyte responsiveness but also delay the rate of constitutive apoptosis. For example, lipopolysaccharide (LPS) and granulocyte/macrophage colony-stimulating factor (GM-CSF) have been shown to upregulate neutrophil function by priming for enhanced functional responsiveness to secretagogue agonists and also prolong cell longevity by delaying apoptosis (Colotta *et al.*, 1992; Lee *et al.*, 1993). A similar dual effect has been observed in eosinophils stimulated with IL-5 and GM-CSF with both cytokines enhancing eosinophil function and delaying apoptosis (Stern *et al.*, 1992; Walsh *et al.*, 1996). Thus, it is often perceived that a close and inevitable relation exists between granulocyte activation status and longevity; indeed, a large family of inflammatory mediators promote granulocyte function by initially inducing cell priming and subsequently by delaying the onset of constitutive apoptosis (Mecklenburgh *et al.*, 1999). In this study, we investigated this association by exploiting the archetypal proinflammatory lipid mediators, the leukotrienes (LTs).

The LTs are produced by the oxygenation of deacylated membrane-bound arachidonic acid through a reaction catalysed by 5-lipoxygenase (5-LO) and 5-lipoxygenase-activating protein (FLAP) to yield 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is converted to leukotriene A₄ (LTA₄), an unstable epoxide intermediate. In neutrophils, and other cells that contain LTB₄ synthase (e.g., alveolar macrophages), LTA₄ is converted to LTB₄ (Samuelsson *et al.*, 1987). Indeed, neutrophils when activated by a variety of stimuli (e.g., the calcium ionophore, A23187 and receptor-directed stimuli such as platelet-activating factor (PAF), formyl-leucyl-phenylalanine (fMLP) and GM-CSF) are capable of synthesising and releasing LTB₄ (Lin *et al.*, 1982; Samuelsson *et al.*, 1987; Dahinden *et al.*, 1988; Rossi *et al.*, 1993). LTB₄ has also been shown to be a powerful activator of neutrophil responsiveness causing cell polarisation, chemotaxis, adhesion, degranulation and reactive oxygen species generation (Ford-Hutchinson *et al.*, 1980; Rossi *et al.*, 1993). In addition, LTB₄ has been shown to influence neutrophil longevity by delaying apoptosis (Hebert *et al.*, 1996). LTB₄ is believed to elicit its effects by interacting with distinct seven transmembrane G-protein-coupled receptors, the classical BLT1 receptor (Yokomizo

et al., 1997) and the recently identified BLT2 receptor (Kamohara *et al.*, 2000; Serhan & Prescott, 2000; Yokomizo *et al.*, 2000). The other receptor type that binds LTB₄ that has received much recent attention is the peroxisome-proliferator activator receptor- α (PPAR α) (Devchand *et al.*, 1996). This receptor is a member of the PPAR group of transducer proteins belonging to the steroid/thyroid/retinoid receptor family, which regulate the expression of target genes by binding to PPAR response elements. PPAR α is primarily expressed in tissues that have high fatty acid catabolism, including the liver and the immune system (Devchand *et al.*, 1996).

In eosinophils, mast cells and other cells that contain LTC₄ synthase, LTA₄ is converted mainly to LTC₄, through conjugation with reduced glutathione. LTC₄ is exported from the cytosol to the extracellular environment and sequentially cleaved to form LTD₄ and the less potent LTE₄. These cysteinyl LTs are now well established as mediators of immediate hypersensitivity reactions, and are potent agonists of bronchoconstriction and vascular permeability and play a role in the persistent tissue eosinophilia that is characteristic of asthma (Leff, 2000). Cysteinyl LTs may influence various stages of the eosinophil life cycle including bone marrow differentiation, adhesion and margination; however, the effects of the cysteinyl LTs on the activation status and functional longevity of these cells are ill defined. In this study, we have examined the relation between granulocyte activation and apoptosis by comparing the effects of the LTs on neutrophil and eosinophil function and lifespan *in vitro*.

Methods

Neutrophil and eosinophil isolation

Neutrophils and eosinophils were isolated from the peripheral venous blood of healthy adult donors by dextran sedimentation followed by centrifugation through discontinuous PBS-Percoll gradients (Rossi *et al.*, 1998; Ward *et al.*, 1999a). Only neutrophil preparations with a purity of >98% were used. Eosinophils were separated from contaminating neutrophils using an immunomagnetic separation step with sheep anti-mouse IgG-Dynabeads (Dynabeads M-450, Dynal, Merseyside, U.K.) coated with the murine antineutrophil antibody 3G8 (anti-CD16; a gift from Dr J. Unkeless, Mount Sinai Medical School, New York, U.S.A.). Cells were mixed with washed 3G8-coated Dynabeads at a bead:neutrophil ratio of 3:1 on a rotary mixer at 4°C for 20 min, and the beads removed magnetically by two 3 min stationary magnetic contacts (Dynal Magnetic Particle Concentrator, MPC-1) to yield an eosinophil population of >98% purity. After harvesting and purification, cells were washed twice in PBS (without divalent cations) and once in PBS containing MgCl₂ and CaCl₂ before resuspending in appropriate buffer.

Culture of granulocytes

Neutrophils (5×10^6 cells ml⁻¹) and eosinophils (2.5×10^6 cells ml⁻¹) were resuspended in Iscove's MDM (Life Technologies, Paisley, U.K.) containing 50 U ml⁻¹ penicillin and 50 U ml⁻¹ streptomycin and supplemented with 10% autologous serum (or 0.1% BSA) and cultured in flat-bottomed 96-

well Falcon flexiwell plates (Becton-Dickinson, U.K.) at 37°C in a humidified 5% CO₂ atmosphere for the time periods indicated. The cells were cultured in the absence or presence of the reagents of interest as described in the figure legends and unless otherwise stated, all experiments were done at least three times with each treatment performed in triplicate.

Morphological assessment of granulocyte apoptosis

After culture, cells were gently resuspended and 100 µl harvested from each well, cytocentrifuged in a Shandon Cytospin II (Shandon, U.K.) and the resulting slide preparations air dried, fixed in methanol and stained with Diff-Quik™. Cell morphology was examined by × 100 objective oil immersion light microscopy to determine the proportion of cells with highly characteristic apoptotic morphology (Savill *et al.*, 1989; Stern *et al.*, 1992; Ward *et al.*, 1999a). For each condition examined, slides were prepared from triplicate incubations and after coding, a total of at least 500 granulocytes were counted over a minimum of five high power fields with the observer blinded to the assay conditions. The data were expressed as the mean per cent apoptosis ± s.e.m. Cell viability was determined in parallel by the ability of granulocytes to exclude the vital dye trypan blue assessed by light microscopy and, on occasion, by cellular incorporation of propidium iodide as assessed by flow cytometry (Ward *et al.*, 1999a). Under all conditions and treatments used, there was no loss of cell membrane integrity and cell loss was minimal.

Annexin V binding

A separate and independent assessment of apoptosis was performed by flow cytometry using FITC-labelled recombinant human annexin V that binds to phosphatidylserine exposed on the surface of apoptotic cells. Stock annexin V (Bender MedSystems, Vienna, Austria) was diluted 1 : 200 with binding buffer and then added (25 µl) to 75 µl of the recovered cell samples. Following a 10 min incubation at 4°C, these samples were fixed by the addition of 100 µl of 3% paraformaldehyde before analysis using an EPICS Profile II (Coulter Electronics, Luton, U.K.) or a FACSCalibur (Becton Dickinson, Oxford, U.K.) (Ward *et al.*, 1999a). The fluorescence from a minimum of 5000 cells was recorded.

Determination of cytosolic free calcium ion levels ([Ca²⁺]_i)

Freshly isolated granulocytes were washed (3 ×) in HBSS (Ca²⁺ and Mg²⁺ free) before being resuspended at 10⁷ ml⁻¹ in HBSS (Ca²⁺ and Mg²⁺ free) and incubated with fura-2/AM (final concentration 2 µM) for 30 min at 37°C (O'Flaherty & Rossi, 1993). The cells were then washed (2 ×) and left in HBSS (Ca²⁺ and Mg²⁺ free) for a further 10 min for optimal de-esterification of the fura-2-acetoxymethyl ester, before finally resuspending the granulocytes at 2 × 10⁶ cells ml⁻¹ in HBSS (containing Ca²⁺ and Mg²⁺). Changes in fluorescence following agonist addition were determined using a Perkin-Elmer LS50B fluorimeter, with dual wavelength excitation (340 and 380 nm) and emission at 510 nm. The fluorimeter has a fitted thermostated cuvette compartment and stirring attachment to ensure complete mixing of reagents at 37°C. Calibration of [Ca²⁺]_i was performed as described (O'Flaherty & Rossi, 1993).

Granulocyte shape change assay

Freshly isolated granulocytes (180 µl, 2 × 10⁶ cells ml⁻¹ in PBS containing CaCl₂ and MgCl₂) were equilibrated at 37°C in a shaking water bath for 15 min in the presence of buffer alone or the antagonist of interest. Agonists were then added to the required concentration and incubations continued for a further 15 min before the addition of 200 µl of 2.5% glutaraldehyde. Samples were analysed for shape change by flow cytometry (Coulter EPICS Profile II; Coulter Electronics, Luton, U.K.) (Kitchen *et al.*, 1996). Percentage shape change was calculated from the mean forward light scatter of each sample by gating on the nonshape-changed population.

Western blotting for PPARα

Cell samples (5 × 10⁶ cells ml⁻¹) were lysed (Ward *et al.*, 1999a, 2002; Fujihara *et al.*, 2002) and the whole cell lysates were run on a 9% SDS gel and after transfer to nitrocellulose membranes (Amersham Pharmacia Biotech U.K. Ltd), blocked by 5% milk protein before an overnight incubation with the polyclonal PPARα antibody, N/19 (SantaCruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) diluted 1 : 500. After washing, blots were incubated with HRP-conjugated antibiotin antibody diluted at 1 : 2500 and developed using standard ECL reagents.

Materials

Further specific materials were obtained as follows: LTB₄, LTC₄, LTD₄, and WY-14,643 (Biomol, Affinity Research Products, Mamhead, U.K.) and GM-CSF, IL-5 (R&D Systems Europe Ltd, Oxon, U.K.) and MK-886 (Calbiochem Ltd, Beeston, Nottingham, U.K.). CP-105,696 was a gift from Henry J. Showell, Pfizer Inc., Connecticut, U.S.A. Montelukast (1-(((1(R)-3-(2-(7-chloro-2-quinolinyl)-(E)-ethenyl)phenyl)(3-2-(1-hydroxy-1-methylethyl)phenyl)propyl)thio)methyl)cyclopropane) acetic acid sodium salt) was a gift from Dr Richard Tomiak, Merck, Sharp and Dohme Ltd. All other reagents were obtained from Sigma Chemical Co., Poole, U.K. and were of the highest purity.

Statistical analysis

The results are expressed as means ± s.e.m. of (*n*) the number of independent experiments each using cells isolated from separate donors with each treatment performed in triplicate, unless otherwise stated. Statistical analysis was performed by ANOVA with comparisons between groups made using the Newman-Kuels procedure. Differences were considered significant when *P* < 0.05.

Results

Effect of LTB₄ on human neutrophil apoptosis

Initially, we demonstrated that incubation of human neutrophils for 20 h with LTB₄ (1 µM) delayed the rate of constitutive apoptosis as assessed by morphological criteria (Figure 1a). In contrast, the cysteinyl LTs LTC₄ (1 µM) and LTD₄ (1 µM) had no effect on the constitutive rate of apoptosis in these cells

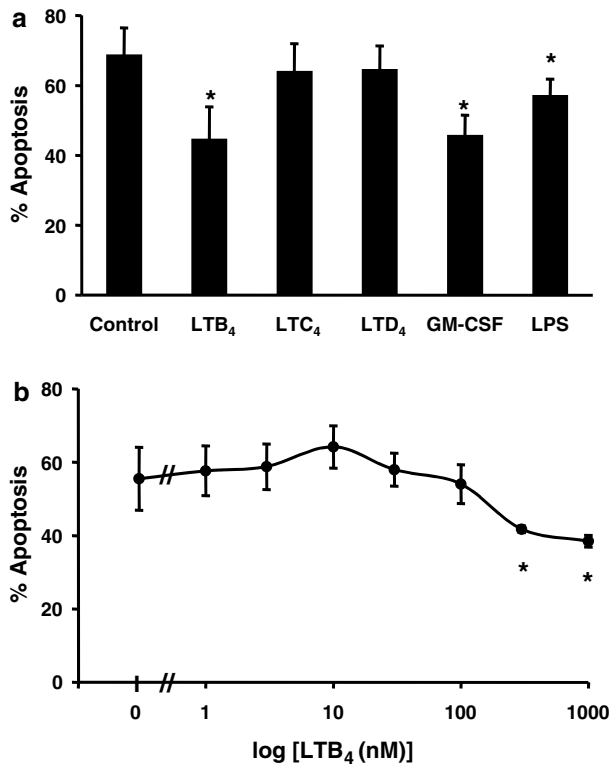


Figure 1 Effect of LTs on human neutrophil apoptosis. Neutrophils (5×10^6 cells ml^{-1}) were cultured in serum-supplemented Iscove's MDM alone (control) or in identical medium containing stimuli (final concentration). (a) LTB₄ ($1 \mu\text{M}$), LTC₄ ($1 \mu\text{M}$), LTD₄ ($1 \mu\text{M}$), GM-CSF (50 U ml^{-1}) or LPS ($1 \mu\text{g ml}^{-1}$) and (b) LTB₄ ($1 \text{ nM} - 1 \mu\text{M}$). Cells were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent the means \pm s.e.m. of three separate experiments, each performed in triplicate, where * represents $P < 0.05$ compared to control values.

(Figure 1a). The extent of the retardation of neutrophil apoptosis observed with LTB₄ was similar to that observed with human recombinant GM-CSF (50 U ml^{-1}) and LPS ($1 \mu\text{g ml}^{-1}$) (Figure 1a). Assessment of cell recovery (haemocytometer counts) and viability (trypan blue exclusion) demonstrated that the inflammatory mediators investigated had no effect on either of these parameters as compared to vehicle-treated controls (data not shown). Experiments performed to examine the concentration dependency of LTB₄-mediated neutrophil survival revealed that this response was only observed with relatively high concentrations of LTB₄ ($\geq 300 \text{ nM}$; Figure 1b). Accordingly, an LTB₄ concentration of $1 \mu\text{M}$ was selected for use in all further apoptosis studies.

Effect of LTB₄ on neutrophil $[\text{Ca}^{2+}]_i$ levels and cell polarisation

Signalling mechanisms such as changes in $[\text{Ca}^{2+}]_i$ are fundamental in regulating neutrophil activation and may play a key role regulating granulocyte apoptosis (Whyte *et al.*, 1993a). We therefore examined the effects of LTB₄ on $[\text{Ca}^{2+}]_i$ in human neutrophils and compared the responses to those induced by other agonists. $[\text{Ca}^{2+}]_i$ levels in freshly isolated neutrophils were measured using the Ca^{2+} -sensitive dye fura-2

following the addition of PAF (100 nM), LTB₄ (100 nM) or fMLP (100 nM) as indicated in Figure 2. The resting $[\text{Ca}^{2+}]_i$ levels recorded in these experiments ($< 100 \text{ nM}$) were consistent with those reported in previous studies in neutrophils (O'Flaherty & Rossi, 1993; Whyte *et al.*, 1993a). Addition of PAF, LTB₄ or fMLP induced a rapid rise in $[\text{Ca}^{2+}]_i$ to a peak of approximately 600 nM , before returning to control values (Figure 2). To investigate the mechanism of LTB₄-mediated elevation of $[\text{Ca}^{2+}]_i$, neutrophils were preincubated in the presence or absence of the specific BLT1 receptor antagonist CP-105,696 ((+)-1-(3S,4R)-[3-(4-phenyl-benzyl)-4-hydroxy-chroman-7-yl]-cyclopentane carboxylic acid; Showell *et al.*, 1995), prior to the addition of LTB₄, PAF or fMLP (100 nM). CP-105,696 ($1 \mu\text{M}$) had no effect on either the basal levels of $[\text{Ca}^{2+}]_i$, or on PAF- or fMLP-induced responses, but abrogated LTB₄-mediated elevation of $[\text{Ca}^{2+}]_i$ (Figure 2) indicating that this response is a specific, BLT1 receptor-mediated event. Moreover, the data suggest that fMLP- and PAF-induced elevation of $[\text{Ca}^{2+}]_i$ is not mediated by the autocrine production of LTB₄.

We next examined the effect of a range of LTB₄ concentrations on cell polarisation. Incubation of freshly isolated neutrophils with LTB₄ for 10 min induced a dramatic polarisation response as assessed by changes in forward scatter by flow cytometric analysis (compare Figure 3a and b). This response exhibited a concentration-dependency and was sensitive to much lower LTB₄ concentrations of 1 nM and above (Figure 3e). The cysteinyl LTs LTC₄ and LTD₄ failed to elicit a response at $1 \mu\text{M}$, a concentration that was maximal for LTB₄-induced shape change (Figure 3e). Preincubation of neutrophils (15 min at 37°C) with the BLT1 receptor antagonist CP-105,696 ($3 \mu\text{M}$) had no effect on basal shape change (compare Figure 3a and c) but abrogated the maximal ($1 \mu\text{M}$) LTB₄-induced shape change (compare Figure 3b and d) without influencing fMLP-induced polarisation (Figure 3f). Taken together, these data indicate that the rapid activation responses to LTB₄ observed in human neutrophils are BLT1 receptor-mediated and support the use of CP-105,696 as a potent and specific BLT1 receptor antagonist.

Effect of a BLT1 receptor antagonist on LTB₄-mediated survival of human neutrophils

As a separate assessment of apoptosis, we show that incubation of neutrophils with LTB₄ ($1 \mu\text{M}$) for 20 h delayed apoptosis as assessed by annexin V binding (Figure 4a and b). Initial preliminary experiments showed that the presence of autologous serum interfered with the ability of CP-105,696 to inhibit LTB₄-mediated retardation of neutrophil apoptosis (data not shown). We therefore cultured our cells in the presence of 0.1% BSA since delayed apoptosis induced by LTB₄ is not dependent on the presence of serum and neutrophils cultured for 20 h in the absence of any protein would invariably undergo secondary necrosis. Thus, to explore the receptor-dependency of LTB₄-mediated delay of neutrophil apoptosis, cells were cocultured in Iscove's containing 0.1% BSA with the BLT1 receptor antagonist CP-105,696 at a concentration shown in this study to completely antagonise maximal LTB₄-stimulated $[\text{Ca}^{2+}]_i$ elevation and shape change (Figures 2 and 3c). LTB₄-delayed neutrophil apoptosis was now almost totally inhibited by CP-105,696 (Figure 4c).

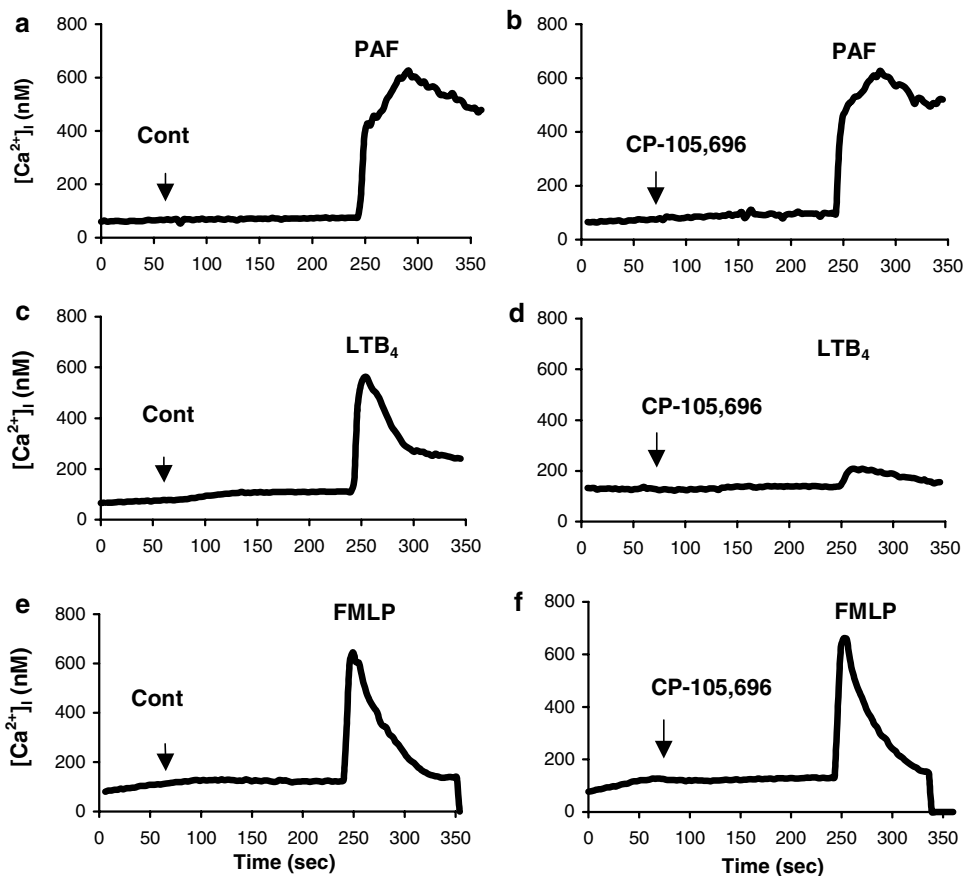


Figure 2 Measurement of cytosolic calcium levels in freshly isolated human neutrophils in response to agonist stimulation. Cytosolic calcium measurements (nM) in fura-2-loaded, neutrophils (2×10^6 cells ml^{-1}) over 350 s. Buffer alone (control, a,c,e) or CP-105,696 ($1 \mu M$; b,d,f) was added to the cell suspension followed by the sequential addition of PAF (100 nM; a, b), LTB_4 (100 nM; c, d), or FMLP (100 nM; e, f), after 245 s. The data show traces obtained from a typical experiment performed at least three times.

Effect of a BLT1 receptor antagonist and a FLAP inhibitor on GM-CSF-, LPS-, dexamethasone- and db-cAMP-mediated survival of human neutrophils

It has been suggested that the delay of neutrophil apoptosis by GM-CSF, LPS and dexamethasone is mediated by autocrine generation of LTB_4 (Lee *et al.*, 1999). To pursue this hypothesis in more detail, we examined: (i) whether the survival effects of LTB_4 and GM-CSF/LPS were additive; (ii) the effect of FLAP and 5-LO inhibitors on GM-CSF and LPS-mediated survival and (iii) whether the effects of these inflammatory mediators, dexamethasone and the cell-permeable analogue of cAMP, db-cAMP was modified by CP-105,696 at a concentration shown to block LTB_4 -mediated shape change, $[Ca^{2+}]_i$ transients and survival. Coculture experiments demonstrated that the antiapoptotic effects of LTB_4 and GM-CSF, and LTB_4 and LPS were in fact additive (Figure 5a), perhaps suggesting that these agents act via discrete rather than common signalling pathways. This latter observation however requires further investigation. Moreover, preincubation of neutrophils (30 min, $37^\circ C$) with the specific FLAP inhibitor (MK-886; $1 \mu M$), or the BLT1 antagonist CP-105,696 (1 and $3 \mu M$), had no influence on basal apoptosis *per se* and did not inhibit GM-CSF-, LPS-, dexamethasone- or db-cAMP-mediated suppression of apoptosis (Table 1, Figure 5b). Taken together, these data demonstrate that

GM-CSF and LPS are unlikely to mediate directly their survival signal in neutrophils via the generation and autocrine action of LTB_4 .

Role of PPAR α in LTB_4 -mediated survival of human neutrophils

It is now well recognised that LTB_4 is a potent activating ligand for PPAR α (Devchand *et al.*, 1996). In these studies, we have detected PPAR α (molecular weight 55 kDa) in the cytosol of freshly isolated human neutrophils and eosinophils as well as monocytes and monocyte-derived macrophages by Western blotting (Figure 6a). To determine whether the LTB_4 -stimulated survival signal in human neutrophils is mediated via this receptor, we exploited the synthetic PPAR-activating ligand pirinixic acid (WY-14643), which has been shown to be selective for PPAR α (Yu *et al.*, 1995). Culture of neutrophils for 20 h with a wide range of concentrations of WY-14643 (1–300 μM) had no influence on the rate of constitutive apoptosis in these cells (Figure 6b). Therefore, although PPAR α is present in human neutrophils, stimulation of this receptor does not mimic LTB_4 -mediated retardation of neutrophil death suggesting that the survival effect of LTB_4 may not be mediated via activation of this receptor.

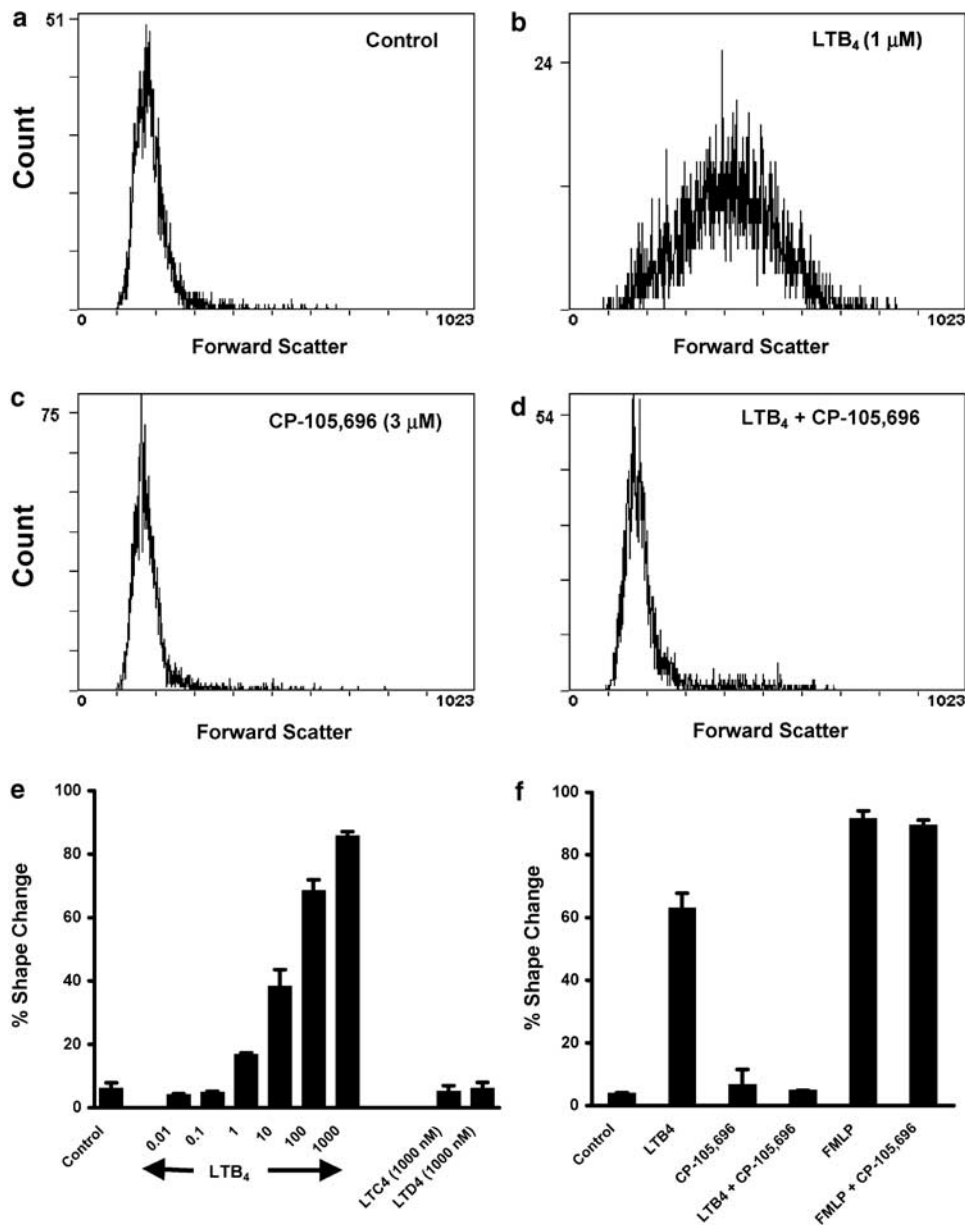


Figure 3 Effect of LTB₄ on shape change in human neutrophils. Neutrophils (2×10^6 cells ml⁻¹ in 180 μ l PBS with CaCl₂ and MgCl₂) were equilibrated for 15 min at 37°C, then preincubated with 10 μ l CP-105,696 (3 μ M) or vehicle prior to stimulation for 10 min with 10 μ l LTB₄ (1 μ M), or vehicle. (a) represents control, (b) LTB₄, (c) CP-105,696 and (d) LTB₄ plus CP-105,696. (e) Shows the concentration – response for LTB₄-induced shape change where neutrophils (2×10^6 cells ml⁻¹ in 190 μ l PBS with CaCl₂ and MgCl₂) were equilibrated for 15 min at 37°C, then incubated with 10 μ l buffer alone (control), LTB₄ (0.01 nM – 1 μ M), LTC₄ (1 μ M) or LTD₄ (1 μ M) for 10 min. (f) Shows the specificity of CP-105,696 for BLT₁-mediated shape change where neutrophils (2×10^6 cells ml⁻¹ in 180 μ l PBS with CaCl₂ and MgCl₂) were equilibrated for 15 min at 37°C, then incubated with 10 μ l buffer alone (control), or CP-105,696 (3 μ M) prior to stimulation for 10 min with 10 μ l LTB₄ (1 μ M), fMLP (100 nM), or buffer alone. All reactions were terminated by addition of 200 μ l 2.5% glutaraldehyde. (a – d) Show representative flow cytometry (EPICS Profile II), *x*-axis: mean forward light scatter (FS); *y*-axis: relative cell number. (e) and (f) show per cent shape change as analysed by flow cytometry as detailed in Methods. Data represent means \pm s.e.m. of one representative experiment of three, each performed in triplicate.

Effects of LTs on eosinophil [Ca²⁺]_i levels

The effects of LTs on [Ca²⁺]_i in freshly isolated human eosinophils were measured using fura-2 in a similar set of experiments as described for neutrophils. The rise in eosinophil [Ca²⁺]_i in response to LTB₄ (100 nM; Figure 7a), was abrogated by preincubation with the BLT₁ receptor antagonist CP-105,696 (data not shown). While LTC₄ had no effect on

[Ca²⁺]_i (Figure 7a), incubation with LTD₄ induced a rapid and transient rise in [Ca²⁺]_i (Figure 7a and b). Moreover, LTD₄-mediated flux in [Ca²⁺]_i was blocked by preincubation with the Cys-LT₁ receptor antagonist montelukast (1 μ M) (Figure 7b), which had no influence on resting [Ca²⁺]_i or on LTB₄ or PAF-mediated responses (Figure 7b). Interestingly, there was no evidence of heterologous desensitisation with regard to the [Ca²⁺]_i responses induced by LTB₄, LTD₄ and PAF (see

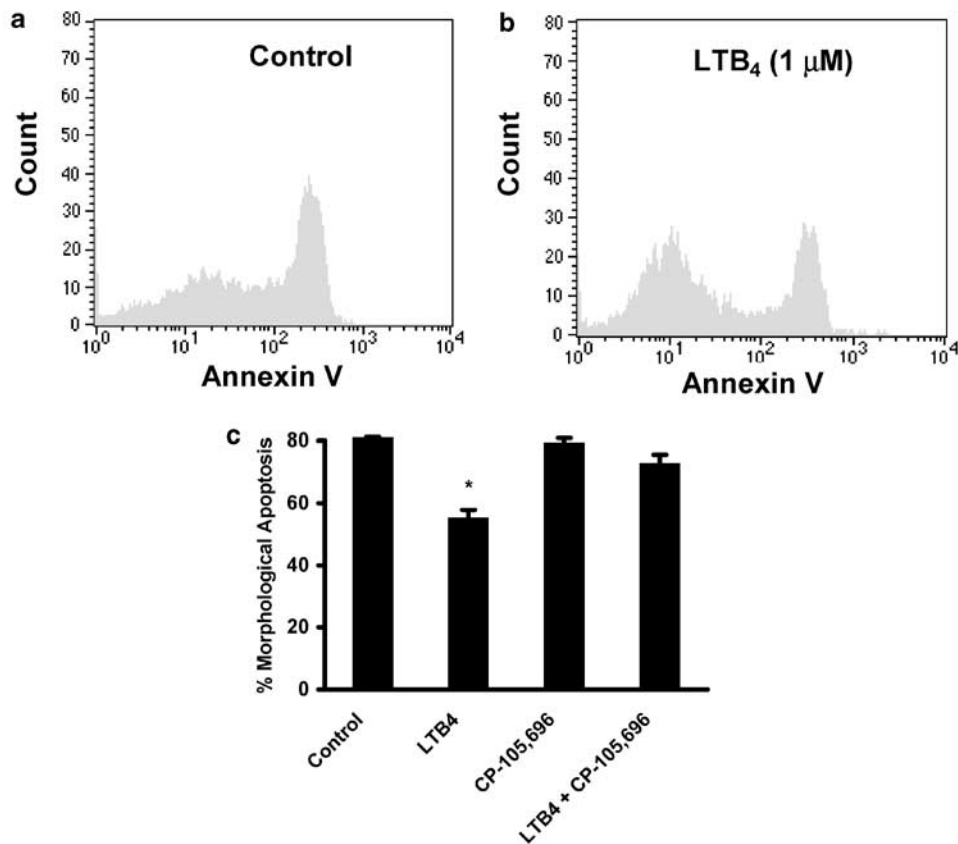


Figure 4 Effect of BLT1 receptor antagonist CP-105,696 on LTB₄-mediated retardation of human neutrophil apoptosis. Neutrophils (5×10^6 cells ml⁻¹) were cultured in serum-supplemented Iscove's MDM alone (control, a) or in the presence of LTB₄ ($1 \mu\text{M}$, b). Neutrophils were resuspended following 20 h in culture and incubated with FITC-labelled recombinant human Annexin V to determine phosphatidylserine expression. The cells were then fixed and analysed using an EPICS Profile II. Mean fluorescence values are shown for a minimum of 5000 cells for each condition. (c) Neutrophils (5×10^6 cells ml⁻¹) were preincubated (37°C , 15 min) in serum-supplemented Iscove's MDM containing BSA (0.1%) and treated with LTB₄ ($1 \mu\text{M}$) with or without CP-105,696 ($3 \mu\text{M}$). Samples were harvested to assess for morphological features of apoptosis; data represent the means \pm s.e.m. of three independent experiments performed in triplicate, where * represents $P < 0.05$ compared to control values.

Figure 7 and data not shown). This supports the existence of a specific Cys-LT₁-receptor mediated LTD₄-induced flux in [Ca^{2+}]_i in human eosinophils.

Effect of LTs on eosinophil apoptosis

In these studies, we sought to determine the potential role of LTB₄ and the cysteinyl LTs (LTC₄ and LTD₄) in modulating eosinophil apoptosis. In initial experiments, we examined the effects of LTB₄, LTC₄, and LTD₄ ($1 \mu\text{M}$) on eosinophil longevity at 20 h, when the constitutive rate of apoptosis in these cells was still relatively very low ($17.0 \pm 1.2\%$; Figure 8). At this time point, we were unable to detect any significant effects of the LTs on eosinophil apoptosis. The cys-LT₁ receptor antagonist montelukast ($10 \mu\text{M}$) was also unable to modulate the constitutive rate of eosinophil apoptosis, either on its own, or following coculture with the LTs (Figure 8). The responsiveness of these cells to stimuli that have been previously reported to modulate apoptosis was confirmed by the delay of apoptosis by IL-5 (50 ng ml^{-1}) (Stern *et al.*, 1992). In experiments where cells were harvested following longer periods in culture, where the rates of constitutive apoptosis recorded were much higher (40 h: $30.3 \pm 1.9\%$; 60 h: $70.7 \pm 1.2\%$), we were still unable to detect any influence of LTB₄, LTC₄, LTD₄ or montelukast on eosinophil apoptosis

(data not shown). Similar results were also obtained at these time points using LTs at 10 and 300 nM, and montelukast at $3 \mu\text{M}$ (data not shown). Thus, in these studies, neither LTB₄ nor the cysteinyl LTs, LTC₄ and LTD₄ had any influence on the rate of eosinophil apoptosis at the various concentrations and time points investigated.

Discussion

In this study, we have examined the effects of the proinflammatory lipid mediators, the LTs, on granulocyte activation and survival. LTB₄ is a potent stimulus of rapid activation events in neutrophils (i.e., [Ca^{2+}]_i fluxes and shape change). For example, LTB₄-stimulated cell polarisation occurred at concentrations as low as 1 nM, with an EC₅₀ of $\cong 10 \text{ nM}$. In addition, LTB₄-induced [Ca^{2+}]_i elevation and shape change were attenuated by the specific BLT1 receptor antagonist CP-105,696 whereas these responses induced by PAF and fMLP appear to be mediated by non-LTB₄ receptor-dependent mechanisms.

To investigate LTB₄-mediated survival of human neutrophils, we examined the potential role of specific receptor-dependent mechanisms. Our initial studies using CP-105,696 also suggested that LTB₄-induced retardation of this response

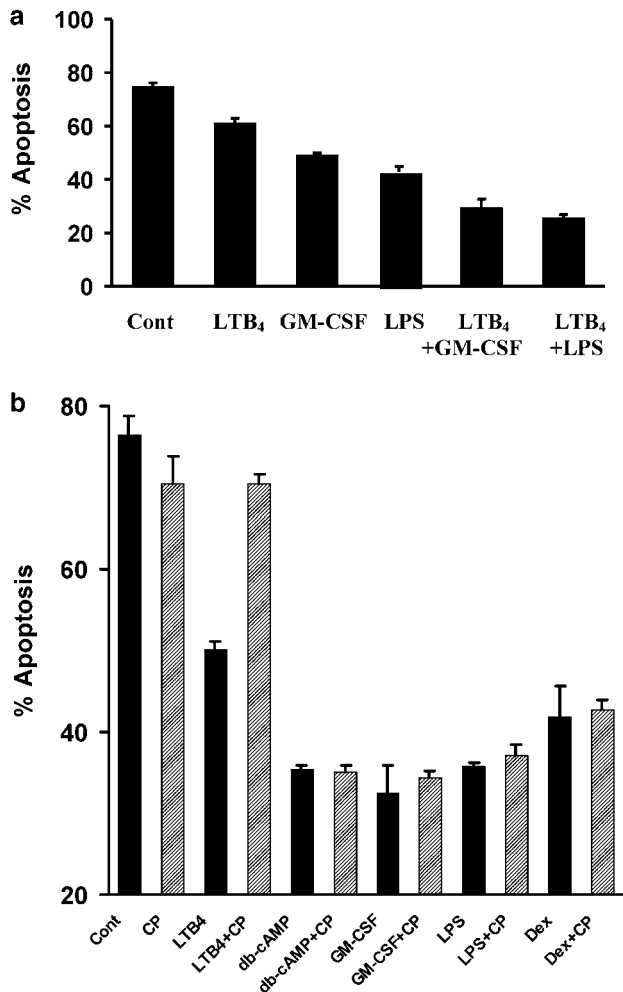


Figure 5 (a) Effect of coculture with GM-CSF and LPS on LTB₄-mediated retardation of human neutrophil apoptosis. Neutrophils (5×10^6 cells ml⁻¹) were incubated in serum-supplemented Iscove's MDM alone or in identical medium containing LTB₄ ($1 \mu\text{M}$), GM-CSF (50 U ml^{-1}), LPS ($1 \mu\text{g ml}^{-1}$), LTB₄ ($1 \mu\text{M}$) plus GM-CSF (50 U ml^{-1}) or LTB₄ ($1 \mu\text{M}$) plus LPS ($1 \mu\text{g ml}^{-1}$). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent the means \pm s.e.m. of one representative experiment of three, each performed in triplicate. (b) Effect of CP-105,696 on LTB₄-, db-cAMP-, GM-CSF-, LPS- and dexamethasone-mediated retardation of human neutrophil apoptosis. Neutrophils (5×10^6 cells ml⁻¹) were pre-incubated (37°C , 15 min) in BSA (0.1%)-supplemented Iscove's MDM alone (control; black bars) or in identical medium containing CP-105,696 at final concentrations of $3 \mu\text{M}$ (hatched bars) prior to cell culture in the medium alone (control), LTB₄ ($1 \mu\text{M}$), db-cAMP (0.2 mM), GM-CSF (50 U ml^{-1}), LPS ($1 \mu\text{g ml}^{-1}$) or dexamethasone ($1 \mu\text{M}$). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent the means \pm s.e.m. of triplicate samples from an individual experiment from at least 3 separate experiments.

appears to be mediated via the BLT1 receptor. This conclusion is supported by our studies investigating the now recognised LTB₄ receptor PPAR α . For this, we initially probed for the presence of the receptor by Western blotting analysis and show for the first time that PPAR α (molecular weight 55 kDa) is present in whole-cell lysates of freshly isolated human neutrophils and eosinophils, as well as monocytes and

Table 1 Effect of the FLAP inhibitor MK-886 on LTB₄, GM-CSF and LPS-mediated retardation of human neutrophil apoptosis

Treatment	Control	MK-866
Control	72.5 \pm 5.0	72.0 \pm 5.8
LTB ₄	59.1 \pm 3.3	58.9 \pm 3.7
GM-CSF	50.9 \pm 2.7	51.8 \pm 3.7
LPS	51.1 \pm 9.2	49.3 \pm 8.0

Human neutrophils (5×10^6 cells ml⁻¹) were preincubated (37°C , 30 min) in serum supplemented Iscove's MDM alone or in identical medium containing MK-886 ($1 \mu\text{M}$) prior to cell culture in the medium alone (control), LTB₄ ($1 \mu\text{M}$), GM-CSF (50 U ml^{-1}) or LPS ($1 \mu\text{g ml}^{-1}$). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis (expressed as percentage of apoptotic cells). Data represent the means \pm s.e.m. of three separate experiments, each performed in triplicate.

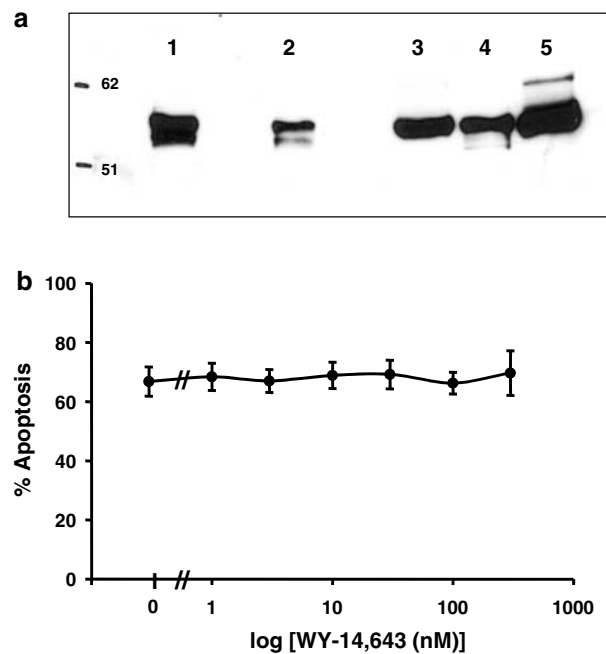


Figure 6 Detection of PPAR α in freshly isolated human leukocytes (a). Leukocyte cytosolic samples ($5 - 10 \times 10^6$ cells in $100 \mu\text{l}$ of lysis buffer as detailed in the Methods section) were run on a 9% acrylamide gel and probed for human PPAR α . Lanes represent: (1) fresh neutrophils (10×10^6), (2) fresh neutrophils (5×10^6), (3) fresh eosinophils (10×10^6), (4) monocytes (10×10^6), (5) monocyte-derived macrophages (10×10^6). Effect of WY-14,643, a synthetic PPAR α -activating ligand, on human neutrophil apoptosis (b). Human neutrophils (5×10^6 cells ml⁻¹) were incubated in serum-supplemented Iscove's MDM alone (control) or in identical medium containing a range of concentrations of WY-14643 ($1 - 300 \mu\text{M}$). Cells were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent the means \pm s.e.m. of three separate experiments, each performed in triplicate.

monocyte-derived macrophages. Despite the presence of PPAR α protein in freshly isolated neutrophils, culture of these cells for 20 h with a wide range of concentrations of the synthetic and selective PPAR α -activating ligand pirinixic acid (WY-14643) ($1 - 300 \mu\text{M}$) had no influence on the rate of constitutive apoptosis in these cells. The higher concentrations of WY-14643 used in our study are by far in excess of that used ($10 \mu\text{M}$) to enhance interferon- γ plus TNF- α -induced apoptosis

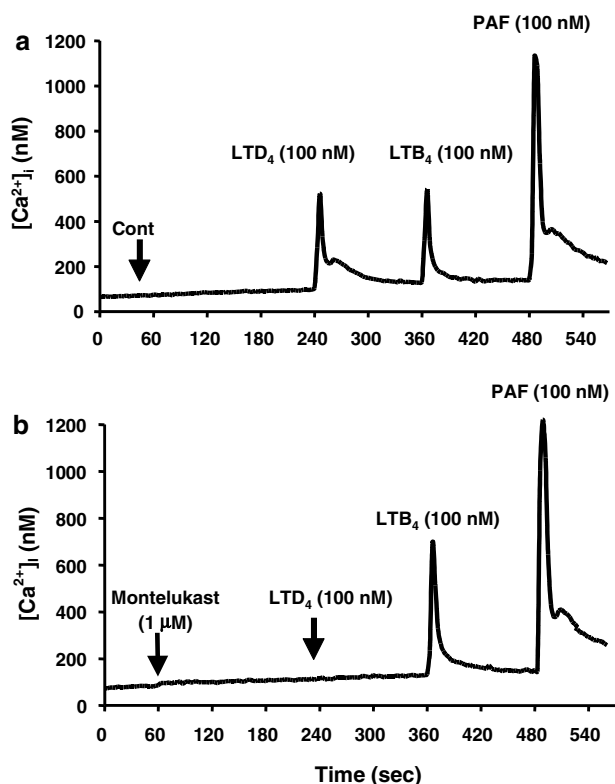


Figure 7 Effect of the Cys-LT₁ receptor antagonist montelukast on agonist-induced calcium fluxes in human eosinophils. Cytosolic calcium measurements (nM) in fura-2-loaded eosinophils (2×10^6 cells ml^{-1} in HBSS without phenol red, with CaCl_2 and MgCl_2) over 540 s. Buffer alone (control, a) or montelukast ($1 \mu\text{M}$, b) was added to the cell suspension after 60 s, followed by the sequential addition of LTD₄ (100 nM), LTB₄ (100 nM) and PAF (100 nM) after 245, 365 and 485 s, respectively. The data show traces obtained from a typical experiment performed at least three times.

in isolated human differentiated macrophages (Chinetti *et al.*, 1998). Of particular relevance to our studies is the recent characterisation and cloning of a novel cell surface-bound G-protein-coupled receptor for LTB₄, designated BLT2 (Yokomizo *et al.*, 2000; Kamohara *et al.*, 2000). This receptor binds LTB₄ with a K_d value of 23 nM compared with 1.1 nM for BLT1, but still efficiently transduces intracellular signalling. In contrast to BLT1, which is expressed predominantly in leucocytes, BLT2 is expressed more ubiquitously with a large amount of BLT2 mRNA detected in human spleen as well as in peripheral blood leucocytes (Kamohara *et al.*, 2000; Yokomizo *et al.*, 2000). Yokomizo *et al.* (2000) also revealed that several BLT1 antagonists failed to inhibit LTB₄ binding to BLT2 demonstrating that BLT2 is pharmacologically distinct and may mediate cellular functions in tissues other than leucocytes. Further work is required to investigate the potential role of this receptor in LTB₄-induced neutrophil function and, in particular, apoptosis. This is especially important given that there is evidence suggesting that LTB₄ may mediate retardation of neutrophil apoptosis via cell surface receptor signalling events distinct from those involved in neutrophil degranulation and respiratory burst (Hebert *et al.*, 1996). Taken together, however, our results suggest that the BLT1, and not the PPAR α receptor, is the likely receptor responsible for the retardation of neutrophil apoptosis induced by LTB₄.

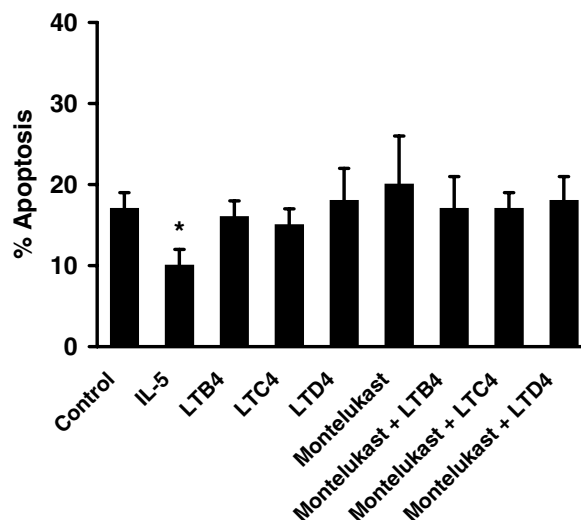


Figure 8 Effect of LTs (LTB₄, LTC₄, LTD₄) and montelukast on human eosinophil apoptosis at 20 h *in vitro*. Eosinophils (2.5×10^6 cells ml^{-1}) were cultured in serum-supplemented Iscove's MDM alone (control) or in identical medium containing: final concentration LTB₄ ($1 \mu\text{M}$), LTC₄ ($1 \mu\text{M}$), LTD₄ ($1 \mu\text{M}$), montelukast ($10 \mu\text{M}$) or IL-5 (50 ng ml^{-1}). Coculture conditions were also prepared with LTB₄, LTC₄, LTD₄ ($1 \mu\text{M}$) in the presence of montelukast ($10 \mu\text{M}$). Eosinophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent the means \pm s.e.m. of three independent experiments, each performed in triplicate, where * represents $P < 0.05$ compared to control values.

We have also demonstrated, using the specific FLAP antagonist MK-886, that GM-CSF- and LPS-mediated survival in neutrophils does not appear to be mediated via the generation and autocrine effects of LTB₄. These data conflict with a recent report (Lee *et al.*, 1999) that proposed that the delay of neutrophil apoptosis observed with GM-CSF, LPS and dexamethasone could be attributed to the generation of LTB₄. These latter observations are supported by a recent report showing that BLT1 receptor expression is upregulated by dexamethasone and that this effect may be one of the mechanisms through which glucocorticoids can prolong neutrophil survival. Indeed, dexamethasone-induced survival may be mediated by LTB₄ as determined by use of an LTB₄ receptor antagonist (Stankova *et al.*, 2002). Difference between these studies and our observations may be because of differences in isolation procedures, activation/priming status of the cells, culture conditions or reagents and inhibitors used. Our studies however indicate that, whatever the precise mechanism utilised by LTB₄ to mediate its suppression of neutrophil apoptosis, it is unlikely that dexamethasone, LPS, GM-CSF or db-cAMP delay apoptosis by this mechanism since blockade of LTB₄ action by CP-105,696 and/or LTB₄ synthesis by the FLAP inhibitor do not abrogate the effects of these agents.

It has been assumed previously that a close relation exists between neutrophil activation and survival in that agents which serve to prime neutrophils for enhanced functional responsiveness to secretagogue signals or directly activate these cells also increase functional longevity by delaying the onset of constitutive apoptosis. We have shown that while LTD₄, and to a lesser extent LTB₄, were potent stimuli for elevation of $[\text{Ca}^{2+}]_i$ in freshly isolated human eosinophils, neither LTB₄,

nor the cysteinyl LTs LTC₄ and LTD₄ had any influence on the basal rate of apoptosis. Although it has been reported that cysteinyl LTs can delay apoptosis of eosinophils isolated from asthmatic patients (Lee *et al.*, 2000), our data suggest that the LTs, despite elevating [Ca²⁺]_i, do not prolong survival of eosinophils isolated from normal or mildly atopic individuals. Furthermore, analyses of the documented effects of various agonists on granulocyte activation reveals that there may be no unifying hypothesis to correlate these two events, that is that the mechanisms leading to granulocyte activation do not appear to be critical for delaying granulocyte apoptosis. This is supported by the observation that TNF α , a potent neutrophil priming agent, induces neutrophil apoptosis at early time points (<8 h) while delaying this process at later times (<20 h) (Murray *et al.*, 1997). Moreover, while nitric oxide (NO) donors induce neutrophil apoptosis (Blaylock *et al.*, 1998; Fortenberry *et al.*, 1998; Ward *et al.*, 2000; Taylor *et al.*, 2001), these reagents also have the capacity to downregulate other functions such as shape change and chemotaxis (Ward *et al.*, 2000). Interestingly, the ultimate biological effect of NO on apoptosis and function may depend on the source and

concentration of NO, and the cell under investigation (Taylor *et al.*, 2003). In addition, neutrophils exposed to agents that elevate cAMP are rendered less responsive to activation but exhibit a greater longevity (Rossi *et al.*, 1995; Martin *et al.*, 2001). This is further supported by our demonstration that preincubation of neutrophils with PAF (1 μ M) prior to stimulation with fMLP (100 nM), a protocol that results in a dramatic upregulation of fMLP-induced superoxide anion release (Ward *et al.*, 2000), has no effect on neutrophil apoptosis following 6 and 20 h in culture (data not shown). These data offer the possibility that more rational and selective anti-inflammatory therapies may be designed and further underline the importance of elucidating and understanding the mechanisms of activation and signalling in response to local LT generation and other inflammatory agents present at sites of inflammation.

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