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Biological Effects of Leukotriene E₄ on Eosinophils

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Abstract

Studies demonstrate the existence of novel receptors for cysteinyl leukotrienes (CysLTs) that are responsive to leukotriene (LT) E₄ and might be pathogenic in aspirin-exacerbated respiratory disease (AERD) asthma. Given the eosinophilic infiltration in this disorder, we investigated eosinophil expression of P2Y₁₂ and gpr99 and their capacity to respond to LTE₄. Receptor transcript expression was investigated via quantitative PCR and surface protein expression via flow cytometry. We investigated LTE₄ influences on eosinophils including Ca²⁺ flux, cAMP induction, modulation of adhesion molecule expression, apoptosis and degranulation. Eosinophils displayed both transcript and surface protein expression of P2Y₁₂ and gpr99. We could not find evidence of LTE₄ activation of eosinophils, however, LTE₄ induced cAMP expression, and preincubation of eosinophils with LTE₄ inhibited degranulation. Even though eosinophils are an important source of CysLTs in AERD, eosinophils are not themselves the pro-inflammatory biological target and, in contrast, LTE₄ via cAMP primarily elicits anti-inflammatory responses.

Keywords

aspirin-exacerbated respiratory disease; cysteinyl leukotrienes; eosinophils; calcium; cAMP

Introduction

Early pharmacological-based studies suggested the existence of at least just two receptors for cysteinyl leukotrienes (CysLTs) and these speculations were later confirmed with the cloning of the CysLT1 and 2 receptors [1, 2]. The CysLT1 receptor is expressed prominently on airway smooth muscle cells, mediates bronchospasm, and is the target of

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current leukotriene receptor antagonists. It is characterized by high affinity for leukotriene (LT)₄, approximately 10-fold higher than that for LTC₄. In contrast, the CysLT₂ receptor is not thought to mediate bronchospasm, has a unique pattern of distribution, and is characterized by equivalent affinity for LTC₄ and LTD₄ not thought to be important in asthma and allergic inflammation. Neither of these receptors binds LTE₄ with clinically-relevant affinity (the biology of these receptors is widely reviewed elsewhere [3]). The existence of additional receptors has been the subject of much speculation along with the expectation that the type 3 (or 4) receptor would primarily recognize LTE₄ as its ligand. LTE₄ was historically recognized as a stable, readily detectable, metabolite that could be used to define the prior generation of CysLTs, but, given its inability to bind either CysLT₁ or CysLT₂ receptors at pharmacologically relevant concentrations, which was dismissed as being functionally unimportant. This dismissal negated the obvious point that, being the stable metabolite, it is the one CysLT that is consistently detected, is present at the highest concentrations, and, as such, can mediate the most prolonged effects. LTE₄ concentrations are, for example, particularly elevated in severe asthma, acute exacerbations of asthma, and aspirin-exacerbated respiratory disease (AERD) [4–8]. The importance of LTE₄ is supported by studies showing that LTE₄, more so than LTD₄ and C₄, mediates sustained constriction of tracheal explants [9]. Similarly, cells of the LAD2 mast cell line demonstrate enhanced responsiveness to LTE₄ with regards to its ability to induced prostaglandin (Pg)_{D2} secretion [10] and Th2 cells also demonstrated enhanced responsiveness to LTE₄ with regard to cytokine secretion [11]. And, in contrast to predictions derived from the its affinity data derived for the CysLT₁ and 2 receptors, LTE₄ is *comparably* potent as LTC₄ and D₄ in eliciting wheal and flare responses [12]. LTE₄ mediates enhanced bronchoconstriction [13–15] and eosinophil influx into the airway [16, 17] and enhanced responsiveness to LTE₄ over other CysLTs is particularly prominent in AERD [14, 15]. Together, these observations suggest that LTE₄ may mediate potent pro-inflammatory influences in asthma and allergic diseases by acting through a previously unappreciated receptor, and – importantly – a receptor *not* targeted by the current generation of leukotriene receptor antagonists.

The generation of the CysLT₁ and 2 receptor double knockout mouse confirmed the existence of pro-inflammatory CysLT-mediated pathways independent of these two receptors [18] having ligand specificity for LTE₄. Using *in silico* modeling, it was predicted that LTE₄ could bind to the purinergic receptor P2Y₁₂ and Chinese Hamster Ovary cells transfected with P2Y₁₂ gained the capacity to respond to LTE₄ [19]. However, more recent evidence suggested that LTE₄ does not bind directly to P2Y₁₂ receptors, but instead acts as a co-receptor [19]. As such, P2Y₁₂ antagonism with siRNAs or the selective antagonist MesAMP blocked the ability of LTE₄ to induce ERK phosphorylation, as well as secretion of PgD₂ and MIP-1β by mast cells [10, 20]. Most recently, gpr99 has also been identified as a direct LTE₄-specific receptor and mice lacking CysLT₁, CysLT₂, and gpr99 receptors lose all ability to respond to all CysLTs, including LTE₄ [21].

Previously, LTC₄ and LTD₄, acting through CysLT₁ and possibly CysLT₂ receptors were shown to influence eosinophil differentiation [22], survival [23], chemotaxis [24, 25], adhesion [26], and degranulation [27]. As noted, however, human LTE₄ inhalation challenges – but not challenges with LTC₄ or LTD₄ – induce bronchospasm associated with

eosinophil influx into the airways [16, 17] and this is particularly prominent in AERD. We therefore speculated hypothesized that functional LTE_4 receptors would be expressed on eosinophils. These studies were performed to investigate the expression of P2Y_{12} and gpr99 and activities of LTE_4 on eosinophils.

Materials and Methods

Subjects

Subjects were enrolled with informed consent under a protocol approved by the human investigations committee of the University of Virginia and included healthy controls without allergies and asthmatics. None of the asthmatic subjects had taken systemic steroids within 1 month.

P2Y_{12} and gpr99 Expression

Eosinophils were isolated from peripheral blood via sequential application of ficoll-hypaque density centrifugation and dextran sedimentation. Eosinophils were enriched (>95% pure) via negative selection using magnetic affinity purification as the CD16 negative population. P2Y_{12} and gpr99 transcript expression was evaluated by quantitative PCR (qPCR) and protein expression via flow cytometry.

Flow Cytometry

Eosinophils were co-labeled with PE-conjugated anti-CCR3 (BD Bioscience, San Jose, CA) and either anti-human P2Y_{12} (MBL International, Woburn, MA), anti-human OXGR1/gpr99 (Lifespan Biosciences, Seattle, WA) or isotype control antibodies, each conjugated to alexa488 (Zenon Probe, Invitrogen, Grand Island, NY). Flow cytometry was performed on a Becton Dickinson FACSCalibur machine with the flow cytometer gated to collect data on the CCR3^+ population. Data were analyzed as percent of the CCR3 cells expressing each of the receptors (in comparison to control antibody binding). Additional studies were performed with the presence of a platelet-specific antibody, PE conjugated anti- CD61 (BD Bioscience), to evaluate the presence of adherent platelets on the eosinophils.

qPCR

Total RNA was extracted from cells using TRI® reagent (Sigma, St Louis, MO). Conversion of mRNA to cDNA was performed using a Taqman Reverse Transcription kit (Roche, Branchburg, NJ). PCR was performed with iQ sybr-green supermix (Bio- Rad), cDNA, and P2Y_{12} , gpr99 , or β -actin primers (SABiosciences; Frederick, MD). Data were analyzed as the comparative CT of the P2Y_{12} transcripts in comparison to β -actin as described [28–30], where CT = threshold cycle gene of interest-threshold cycle housekeeping gene.

Degranulation

We evaluated the ability of LTE_4 to induce degranulation, assessed as secretion of eosinophil derived neurotoxin (EDN) and CCL5 (RANTES). Eosinophils were primed with GM-CSF (10 ng/ml for 15 min; BD Bioscience) and then exposed to LTE_4 (10^{-8} and 10^{-6}

M) for 30 min. Secreted EDN (sensitivity >0.62 µg/ml) and CCL5 (sensitivity >2.0 pg/ml) were quantified using commercial EIAs (MBL and R&D Systems Quantikine, Minneapolis, MN, respectively). Additional experiments were performed to assess the ability of LTE₄ to modulate eosinophil degranulation. Eosinophils were pretreated with LTE₄ (10⁻⁶ M and GM-CSF) for 15 min and then activated with lysine aspirin (10 mM; Sanofi-Synthelabo), formyl methionine leucine phenylalanine (fMLP; 5×10⁻⁷ M), or the combination of phorbol myristate acetate (PMA; 100 ng/ml) and calcium ionophore (A23187; 2 µg/ml; all from Fischer Scientific).

Adhesion Molecule Modulation by LTE₄

Expression of adhesion molecules on eosinophils was evaluated by flow cytometry for expression of β1 (CD29), β2 (CD11b), and α4 integrin (CD49d; all from BD Bioscience) with and without pretreatment with LTE₄ (10⁻⁶ M) for 1 hr.

Apoptosis

We investigated the ability of LTE₄ to prolong eosinophil survival in serum-reduced (0.1%) conditions for 48 hrs. Apoptosis was detected by flow cytometry as annexin V positive cells and viability as propidium iodide (PI) exclusion (Sigma Aldrich). Further experiments were performed in the additional presence of the CysLT synthesis inhibitor MK886 (10 µM; Cayman Chemical Company, Ann Arbor, MI).

cAMP Secretion and Ca²⁺ Flux

cAMP was quantified in eosinophil supernatants by EIA (R&D Systems, Minneapolis, MN) after exposure to various concentrations of LTE₄. We examined the ability of LTE₄ to induce calcium fluxes, using the FLIPR assay kit (Molecular Devices, Sunnyvale, CA). Reagent buffer supplemented with probenecid (2.5 mM) was applied and cells were stimulated with LTE₄ (250 nM - 2 µM; Cayman). Cytoplasmic [Ca²⁺] was determined in triplicate at an extinction wavelength of 485 nm and an emission wavelength of 525 nm using a Flexstation fluorescence spectrophotometer.

Statistical Analyses

Data were contrasted between unstimulated and stimulated cells by Wilcoxon Rank Sum for nonparametric data analyses or paired t-test for parametric data using GraphPad Prism 6.

Results

P2Y₁₂ and gpr99 Flow Cytometry

Eosinophils were obtained from representative healthy non-allergic, non-asthmatic controls and asthmatic subjects (n=5 each) and percent cell surface receptor expression was evaluated by flow cytometry (online supplement, figure 1S). P2Y₁₂ was modestly expressed on eosinophils from control subjects (47.4±7.7) and this was not insignificantly upregulated different in asthma (54.3±7.3)(Table I). To assure that the observed P2Y₁₂ binding was not driven by eosinophil-adherent platelets P2Y₁₂ is also expressed on platelets where it is responsible for ADP-dependent aggregation and recent studies have demonstrated adherence

of platelets to eosinophils, especially in eosinophils obtained from subjects with AERD [31], w We therefore also performed additional flow cytometry studies with the platelet-specific marker (CD61) and confirmed that although platelet adherence to eosinophils could be identified, the expression of P2Y₁₂ could not be explained by platelet adherence alone (data not shown). Gpr99 was also modestly expressed on eosinophils from control subjects (44.2±11.2) and a non-significant trend for decreased expression in asthma was observed (26.9±7.9).

P2Y₁₂ and gpr99 qPCR

Eosinophils demonstrated P2Y₁₂ and gpr99 transcripts via qPCR (Table II) and, as with surface receptor expression, no differences were observed between control and asthmatic subjects.

Degranulation (EDN/CCL5)

For our initial functional studies, we evaluated the ability of LTE₄ to induce degranulation, assessed as secretion of EDN and CCL5 (RANTES). EDN and CCL5 are contained within distinct intracellular granules and their secretion is differentially regulated. No evidence for degranulation secretion of CCL5 was observed (data from 10 subjects are displayed in Table III) including amongst the eosinophils obtained from asthmatic subjects (n=4). Surprisingly, the addition of LTE₄ was associated with significant (p<0.05) inhibition of the spontaneous release of EDN.

Adhesion molecule modulation by LTE₄

Enriched eosinophils were evaluated by flow cytometry for expression of β1 (CD29), β2 (CD11b), and α4 (CD49d) integrins with and without pretreatment with LTE (10⁻⁶ 4 M for 1 hr). These data (n=4) were analyzed as a percent of cells expressing each receptor (online supplement figure 2S). No evidence for modulation of cell surface adhesion molecule expression was observed (Table IV).

Apoptosis

We investigated the ability of LTE₄ to prolong eosinophil survival under serum-reduced (0.1%) conditions. After 48 hrs apoptosis and cell viability were analyzed by flow cytometry as annexin V positive cells and propidium iodide (PI) exclusion, respectively. The mean of 10 experiments is displayed in Table V. As expected, eosinophils from the asthmatic cohort displayed increased inherent resistance to apoptosis and cell death (data not shown). However, nNo evidence for an influence of LTE₄ on either annexin V expression or cell viability was observed including in a subgroup of subjects with asthma (n=8) although, as expected, eosinophils from the asthmatic cohort displayed increased inherent resistance to apoptosis and cell death (data not shown). We speculated hypothesized that our failure to identify a robust LTE₄-induced effect could reflect the activity of endogenously-derived CysLTs behaving in an autologous fashion. We therefore performed additional experiments incubating eosinophils with the CysLT synthesis inhibitor MK886 for 48 hrs prior to performing these studies. Again, no influence on apoptosis or PI exclusion was observed after addition of LTE₄ (data not shown).

cAMP Secretion and Ca²⁺ Flux

We investigated the coupling of P2Y₁₂ and gpr99 to their physiological second messenger, specifically cAMP generation, in eosinophils exposed to LTE₄. Exposure of eosinophils to LTE₄ consistently induced production of cAMP in a dose-dependent fashion (Figure 1). To assess the possible existence of additional, eosinophil-activating pathways, we also evaluated Ca²⁺ flux. Ca²⁺ flux studies were performed with 3 control and 3 asthmatic subjects and representative data from one subject are displayed in Figure 2. These studies failed to elicit a Ca²⁺ signal with LTE₄ across a wide range of concentrations in either cohort.

LTE₄ modulation of Eosinophil Degranulation

These last observations led us to consider that, at least amongst circulating eosinophils, the primary function of LTE₄ might be to *inhibit* eosinophil function. We therefore investigated the ability of LTE₄ to modulate degranulation of eosinophils using control, lysine aspirin, fMLP, and PMA/ionomycin as stimuli. While no inhibition of degranulation was detected using lysine aspirin or PMA/ionomycin, modest but statistically significant inhibition of fMLP-mediated degranulation was observed (Figure 3; $p < 0.01$).

Discussion and Conclusions

Asthma and in particular AERD is characterized by eosinophilic infiltration of the upper and lower airways [30, 32]. These patients are uniquely susceptible to the pro-inflammatory exacerbating influences of LTE₄ [9, 10, 12–17]. Our previous studies and those of others demonstrated expression of both CysLT1 and CysLT2 receptors on eosinophils [33], however, the observations regarding the distinctive sensitivity of AERD asthmatic patients to LTE₄ is inconsistent with the known binding affinities of either of these receptors, suggesting the involvement of a distinct unappreciated receptor. The evidence suggesting the existence of an additional receptor(s) displaying unique affinity for LTE₄ was initially confirmed by data regarding the continuing sensitivity to CysLTs displayed by the CysLT1/CysLT2 receptor double knockout mouse [18]. One candidate receptor is the purinergic receptor P2Y₁₂, a receptor previously defined by its affinity for ADP and its role in platelet aggregation [10, 18–20]. The most compelling evidence for this being involved in LTE₄ signaling was derived from the ability of the P2Y₁₂ antagonist clopidogrel to block LTE₄-mediated inflammation in mice. However, subsequent evidence suggests the P2Y₁₂ may not directly bind LTE₄, but instead primarily function as a co-receptor [20]. More recently, the existence of a specific LTE₄ receptor has been identified as gpr99 [18, 21]. We investigated LTE₄-mediated effects on eosinophils from control and asthmatic subjects.

We initially investigated the expression of P2Y₁₂ and gpr99 on eosinophils and confirmed their presence both via cell surface protein expression (Table I and figure 1S) and as mRNA transcripts (Table II). In comparison to the CysLT1 and CysLT2 receptors, mRNA expression of P2Y₁₂ was slightly higher on eosinophils, while expression of gpr99 was approximately 2–4 times the level of these other two receptors [28]. Eosinophils contain granules including those that contain toxic cationic peptides and distinct granules that secrete preformed cytokines and chemokines. Therefore, we initially evaluated LTE₄-

mediated degranulation of EDN and CCL5 and, surprisingly, failed to demonstrate an effect (Table III). These studies, in fact, suggested inhibition of basal degranulation. It should be noted that this aspect of our study contrasts with the reported ability of LTE₄ to act on extruded eosinophil granules to induce degranulation [34, 35]. In these studies, eosinophil cationic protein was released from enriched isolated eosinophil granules in response to LTE₄ in a process that was inhibited by the P2Y₁₂ antagonist MRS2395. These intact membrane-bound eosinophil granules have been recognized in the extracellular tissue of many eosinophilic inflammatory disorders such as asthma [36] and, in AERD, this likely extends to the sinusitis/nasal polyp tissue. While contributing to expression of eosinophil granule-derived peptides in AERD, such a limited LTE₄-mediated process cannot explain the recruitment and survival of eosinophils in AERD tissue or the influx of eosinophils that occurs after LTE₄ inhalation challenges [16, 17].

Eosinophil activation is associated with increased expression and activation of integrins, which allows them to assume a “hyperadhesive” phenotype [37]. This includes induction (or upregulation) of α4β1 (VLA4 or CD49d/CD29), αDβ2 (αD/CD11b), and αMβ2 (αM/CD11b) integrins (and the conversion of β2 integrin into an activated conformation) [37]. Again, however, LTE₄ did not increase eosinophil adhesion molecule expression (Table IV). We also performed preliminary studies using an antibody for the αD integrin (gift of Dr. Bruce Bochner) and to the activated conformation of β2 integrin and these results have also been negative.

Eosinophils are normally short-lived cells after their egress from the marrow, but in the presence of various anti-apoptotic factors, classically the cytokines IL-5 and GM-CSF, they can survive for days. The ability of LTE₄ to prolong eosinophil survival was studied and, again, demonstrated negative results (Table V).

Together, these paradoxical results led us to speculate that the LTE₄ receptors expressed on eosinophils may exist in an uncoupled state. We assessed the modulation by LTE₄ of cAMP expression, reflecting the Gs-mediated pathways characteristic of both gpr99 and P2Y₁₂. LTE₄ did drive the generation of cAMP (Figure 1). Increased cAMP, however, is more generally associated with *anti*-inflammatory effects and, along with the inhibition of spontaneous degranulation observed (Table III) led us to study the ability of LTE₄ to inhibit induced degranulation (Figure 3). While inhibition was not observed with lysine aspirin or PMA/ionomycin, using the more physiological stimulus, fMLP, significant inhibition was achieved. The negative results for Ca²⁺ flux (Figure 2) argue against expression of additional LTE₄ receptors on eosinophils that might drive cellular activation.

These studies do not mitigate the unique importance of LTE₄ to driving both the daily symptoms of asthma and, amongst AERD subjects, the severity of reactions after aspirin ingestion, but argue that, in this context, eosinophils are not the primary target of the LTE₄. Physiologically, eosinophils are the most prominent source of the constitutively high levels of CysLTs observed in AERD and the surge in LTE₄ observed after aspirin (or other non-steroidal anti-inflammatory drug) ingestion [38]. While being the *source* of the LTE₄, eosinophils are apparently not the *target* of this mediator. Our studies are consistent with the

recently published work that also failed to demonstrate an LTE_4 response through P2Y_{12} in various cell models despite being responsive to known P2Y_{12} agonists [39].

LTE_4 inhalation challenges in asthmatics induce eosinophilic responses [14, 16, 17]. From the current studies, it can be discerned that this is not driven by a *direct* influence of the LTE_4 on the eosinophils themselves. Eosinophilia reflects a combination of influences on eosinophilopoiesis, eosinophilic recruitment, and eosinophil survival. For example, a pro-inflammatory influence of LTE_4 on eosinophil progenitors ($\text{CD34}^+\text{IL-5R}\alpha^+$ cells) remains plausible. Endothelial cells uniquely express CysLT_2 receptors and respond to CysLTs with enhanced expression of adhesion molecules that could selectively promote eosinophil recruitment [40, 41]. Influences of LTE_4 on epithelial, smooth muscle, and other cells capable of generating eosinophil chemotaxins remain plausible. And finally, CysLT receptors are expressed on T helper cells [28] where they could enhance secretion of pro-eosinophilic cytokines [11].

Given the ability of clopidogrel to inhibit LTE_4 -mediated inflammation in a murine model, we were particularly surprised by our results, especially after demonstrating the expression by eosinophils of P2Y_{12} (Table I) [20]. Some of the effects in the murine challenge may have been driven by the established involvement of LTE_4 in activating mast cells in a P2Y_{12} -dependent fashion. It is important to note, however, that the inflammation in the LTE_4 challenge model was attenuated after platelet depletion. It is intriguing that these investigators have more recently published data regarding the aggregation of platelets to eosinophils in AERD and provided evidence that transcellular lipid transfer may underlie much of the surge in CysLT synthesis observed after aspirin ingestion [31]. As such, it is plausible that clopidogrel may have functioned in their earlier studies – and, by extension, could function in asthmatic subjects – in part by attenuating platelet-eosinophil aggregation and the associated synergistic synthesis of CysLTs .

In summary, it does not appear that eosinophils themselves are a target of LTE_4 in AERD. The upregulated expression of P2Y_{12} we describe here may be relevant as a mechanism contributing to platelet-eosinophil aggregation and secondarily to CysLT synthesis, but does not appear to render those eosinophils sensitive to LTE_4 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AERD	aspirin-exacerbated respiratory disease
CysLT	cysteinyl leukotriene

EDN	eosinophil derived neurotoxin
EIA	enzyme immunoassay
LT	leukotriene
PI	propidium iodide
Pg	prostaglandin
qPCR	quantitative PCR
SI	stimulation index

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Figure 1.

LTE₄-mediated cAMP production in eosinophils. Eosinophils were exposed to a range of concentrations of LTE₄ and supernatants collected. cAMP was quantified via EIA. * p<0.05, **p<0.02

Figure 2.

LTE₄-mediated Ca⁺² flux in eosinophils. Eosinophils were incubated at 37°C for 1 hr with FLIPR® reagent and subsequently stimulated with a range of concentrations of LTE₄ or, as a positive control, with ATP (300 μM). Intracellular calcium was fluorescently determined using an extinction coefficient of 485 nm and an emission wavelength of 525 nm. Data are expressed in random relative fluorescence units and the displayed data represent the mean of triplicate data derived from a representative subject with AERD.

Figure 3.

Modulation of eosinophil degranulation by LTE₄. Eosinophils were pretreated with LTE₄ (and GM-CSF) for 15 min and subsequently activated via exposure to lysine aspirin, fMLP, or PMA/ionomycin. Supernatants were collected and EDN quantified via EIA. * p<0.01

Table IEosinophil Expression of P2Y₁₂ and gpr99

	Control	Asthma
P2Y ₁₂	47.4±7.7%	54.3±7.3%
gpr99	44.2±11.2%	26.9±7.9%

Table IIEosinophil Expression of P2Y₁₂ and gpr99 Transcripts

	P2Y ₁₂	gGprp99
CT	-3.53±0.72	-0.39±0.16
2 CT	0.09	0.76

Data represent a comparison of the gene to the house-keeping gene β -actin.

Table IIIEosinophil Degranulation by LTE_4

	Control	LTE_4 (10^{-8})	LTE_4 (10^{-6})
EDN (npg/ml)	158.6 $\pm$ 27.0	101.2 $\pm$ 27.2	90.6 $\pm$ 19.1 *
CCL5 (pg/ml)	190.3 $\pm$ 61.6	206.8 $\pm$ 60.2	208.1 $\pm$ 46.0

Data represent mean $\pm$ SEM of 10 results.

*
p<0.05 compared to control.

Table IVModulation of eosinophil adhesion molecule expression by LTE₄

CD11b		CD29		CD49d	
Control	+LTE ₄	Control	+LTE ₄	Control	+LTE ₄
32.5±7.9	31.4±6.8	12.3±2.1	11.6±1.7	17.2±2.5	15.6±2.3

* Data represent % of eosinophils expressing each integrin.

Table VModulation of apoptosis by LTE_4

	Control	$\text{LTE}_4\ 10^{-8}$	$\text{LTE}_4\ 10^{-6}$
Annexin V	58.3±7.4	66.0±6.7	58.6±7.9
PI negative	50.4±8.2	42.3±7.2	45.6±7.8

* Data represent % of eosinophils expressing annexin V or taking up the vital dye PI, respectively.