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Enhancement of neutrophil function by the bronchial epithelium stimulated by EGF

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ABSTRACT

The bronchial epithelium is an important physical barrier that also regulates physiological processes including leukocyte trafficking. We aimed to elucidate the mechanisms whereby the bronchial epithelium, stimulated by epidermal growth factor (EGF) as part of a response to acute or chronic injury, could activate and chemoattract human neutrophils.

Sub-confluent 16HBE cells were stimulated with EGF to mimic what happens *in vivo* after injury. The effect of the resulting conditioned media (EGF-CM) was compared with that of basal conditioned media (basal-CM) in respect of neutrophil activation and chemotaxis. Such findings were then confirmed using primary bronchial epithelial cells (PBECs) from healthy volunteers.

EGF-CM from 16HBE cells caused increased expression of CD11b/CD66b and CD62L loss on neutrophils when compared to basal-CM. EGF-CM contained significant neutrophil chemotactic activity involving GM-CSF and IL-8 that was potentiated by LTB_4. This was dependent on neutrophil PI3K activation and Akt phosphorylation, with partial regulation by PLD, but not mTOR. Consistent with these observations, EGF-CM derived from PBECs displayed increased chemotactic activity.

Our results suggest that the enhanced chemotactic activity of the EGF-conditioned epithelium can enhance neutrophil-mediated immunity during acute injury, while during continued injury and repair, as in chronic asthma, this could contribute to persistent neutrophilic inflammation.

Key words: bronchial epithelium, chemotaxis, epidermal growth factor, inflammation, lung, neutrophils.

Abbreviations:

basal-CM, basal epithelium conditioned medium; EGF, epidermal growth factor; EGF-CM, EGF-treated conditioned medium; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GM-CSF, granulocyte macrophage colony stimulating factor; HIMO, 1-L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate; LTB_4, leukotriene B_4; mTOR, mammalian Target Of Rapamycin; PI3K, phosphatidylinositol 3-kinase; PLD, phospholipase D; PtdOH, phosphatidic acid; 5-LO, 5-lipoxygenase; TGF-β2, transforming growth factor-β2; TNF-α, tumour necrosis factor α.

Introduction

Neutrophil-mediated inflammation is the subject of extensive research [1]. Influx of neutrophils into tissues begins by rolling mediated via surface CD62L (L-selectin) interacting with complementary ligands on endothelial cells and is followed by firm neutrophil adhesion to the endothelium using β_2 integrins (CD11a and CD11b) and trans-migration. Upon activation, neutrophils shed CD62L and increase surface expression of CD11b [2, 3] and CD66b [4], accompanied by degranulation, respiratory burst [5, 6] and release of a range of pro-inflammatory agents [1, 5, 7]. Neutrophil-mediated inflammation occurs during acute infection, in acute respiratory distress syndrome [8] and in chronic diseases, such as chronic obstructive pulmonary disease (COPD) [9]. Increasingly, neutrophils are being implicated in asthma [10], a disorder long viewed as an eosinophilic disease [11]. Thus, emerging evidence points to their role during infectious exacerbations and in severe chronic asthma [12-14], with raised numbers observed in bronchoalveolar lavage (BAL), induced sputum and both bronchial and transbronchial biopsies [7, 12, 15] and increased concentrations of neutrophil-active mediators IL-8 [13, 16], LTB_4 [16, 17], GM-CSF [18, 19] and TNF-α [18, 20] in BAL.

The mechanisms regulating the accumulation and activation of neutrophils in the airways remain poorly understood but are increasingly thought to involve the bronchial epithelium and growth factors such as epidermal growth factor (EGF) which acts via the EGF receptor (EGFR), a member of the ErbB family (c-erbB1, HER1; c-erbB2, HER2, neu; c-erbB3, HER3; and c-erbB4, HER4) [21, 22]. In addition to its role in epithelial regeneration and repair [22], the EGFR pathway is believed to contribute to lung inflammation [23, 24]. We have previously identified extensive immunostaining for EGFR in the asthmatic bronchial epithelium which increases with disease severity and correlates with IL-8 expression [25]. These data are consistent with observations that activation of the EGFR pathway causes IL-8 secretion [24, 26, 27] and provide a mechanism for neutrophil migration. EGFR-mediated mechanisms have also been implicated in bronchial epithelial repair in asthma [22, 28], augmenting the

inflammatory potential of virus-infected bronchial epithelial cells [29] and modulating the effects of cigarette smoke [26]. Our own studies [24], [30], and those of others [31] have shown that primary bronchial epithelial cell cultures derived from asthmatic patients release neutrophil-active mediators IL-8, GM-CSF and TNF-α. We have also shown that EGF induces a corticosteroid-insensitive increase in IL-8 gene and protein expression by bronchial epithelial cells [24, 25] and have demonstrated a strong correlation between epithelial EGFR expression and both epithelial IL-8 immunostaining and neutrophil numbers [24].

The functional significance of the augmented production of pro-inflammatory mediators, and their relative roles in driving neutrophilic inflammation remain uncharacterised. Furthermore, little is known about the intracellular neutrophil signaling pathway(s) involved in the communication between the epithelium and neutrophils. In this study, we hypothesized that exposure of bronchial epithelial cells to EGF induces pro-inflammatory factors which promote activation and recruitment of neutrophils and that this activation involves PI3K and its down-stream pathway. In health, EGFRs are localized on the basolateral surfaces of epithelial cells where they are inaccessible to ligands in the airway lining fluid. However, damage to the barrier allows ligand penetration and subsequent EGFR activation. To test our hypothesis, we first used the 16HBE 14o- human bronchial epithelial cell line stimulated by EGF to mimic the activation that occurs when the epithelial barrier is damaged. Studies have shown that 16HBE cells display morphological, functional and permeability properties resembling those of the primary bronchial epithelium [32-34]. We examined whether the epithelium switched into repair mode by EGF could modulate neutrophil function by studying the ability of conditioned media from EGF-stimulated epithelium (EGF-CM) to activate and chemoattract neutrophils and its capacity to trigger signaling pathways in neutrophils. After showing major effects of EGFR on the way 16HBE cells modulate neutrophil function, we repeated the chemotaxis experiments using bronchial epithelial cells (PBECs) from healthy non-atopic individuals as a source of conditioned media to confirm that the

observations seen in the cell line were reflective of the function of primary cells derived from volunteer subjects.

Materials and Methods

Epithelial Cell culture

16HBE 14o– epithelial cells and fresh primary bronchial epithelial cells (PBECs), obtained by bronchial brushings from healthy nonatopic control subjects, were grown as previously reported [22, 35]. 16HBE cells were cultured in T75 flasks in minimal essential medium (MEM) with Earle's salts (Gibco BRL, Paisley, U.K.) supplemented with 10% (v/v) FCS (TCS Biologicals, Buckingham, U.K.), 2 mM L-glutamine (Gibco), 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco) whereas primary PBECs were grown in hormonally supplemented bronchial epithelial growth medium (BEGM; Clonetics, San Diego, California) at 37^{o}C in a humidified atmosphere of 5% CO_2 (v/v) in air. Following sequential passages of epithelial cells, approximately 4 x 10^5 cells were seeded into 24-well culture plates and grown to 70% confluency. The subconfluent cell monolayers were then rendered quiescent for 24 h in serum-free medium (SFM) (RPMI 1640 without phenol red containing 1% (v/v) BSA, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin for 16HBE cells and Bronchial Epithelial Basal Medium (BEBM; Clonetics) containing 1% of insulin, transferrin, and sodium selenite (ITS) media supplement plus 0.3% v/v BSA for PBECs). To mimic the repairing and resting phenotypes, subconfluent epithelial cell cultures were incubated for 24 h with EGF (10 ng/ml, 24 h) or SFM, respectively, and generated EGF-conditioned media (EGF-CM) and basal-conditioned media (basal-CM) were harvested by centrifugation. Secretion of transforming growth factor-β2 (TGF-β2) was increased significantly by EGF (Fig. D1 Online Depository) from 16HBE cells, consistent with the notion that EGF-induced repair causes release of epithelium-derived profibrogenic factors [22]. The 16HBE epithelium cell CM were also analysed for GM-CSF, IL-8, LTB_4 and TNF-α levels using commercial ELISA kits (R&D Systems, Abingdon, U.K.).

Analysis of neutrophil activation induced by the EGF-stimulated epithelium

Peripheral venous blood was obtained from normal healthy donors and neutrophils purified as previously detailed [36, 37] using dextran sedimentation and discontinuous plasma-Percoll gradients (GE Healthcare Ltd, Buckinghamshire, U.K.) with minor modifications. After separation, neutrophils were washed with 50% platelet-poor plasma (PPP)-PBS solution, followed by Dulbecco's PBS without or with Ca^{2+} and Mg^{2+}. This procedure routinely yielded, >96% pure, unprimed neutrophils, with <0.5% mononuclear cell contamination and >98% viability, as assessed by trypan blue exclusion.

In order to assess the effects of the epithelium on the expression of cell surface markers of neutrophil activation freshly purified neutrophils (5 x 10^5 cells per condition) were suspended in PBS with Ca^{2+} and Mg^{2+} and incubated with epithelial CM at 37°C for 1, 5, 15, 30, 60 min and 2 h. PBS or 10 μM fMLP were used as negative and positive controls, respectively. Reactions were quenched by adding 1 ml of ice-cold PBS followed by immersion of tubes in ice. Cells were then washed in PBS and incubated for 15 min on ice with 10% autologous serum to block non-specific binding sites. Specific staining was then performed with fluorochrome-coupled anti-CD16, -CD14, -CD62L, -CD11b, -CD66b (all purchased from BD Biosciences, Oxford, U.K.) using FACS staining buffer (FSB) ([PBS/2% heat-inactivated FCS/0.09% sodium azide]) for 30 min on ice. Unbound antibody (Ab) was removed by washing twice in FSB and cells analysed on a FACSCalibur using Cell Quest software (both from BD Biosciences, Oxford, U.K.). Neutrophils were identified by their forward and side scatter characteristics and all gated cells were CD16+/CD14−. Dead cells were excluded by 7-amino-actinomycin D (7AAD), the staining of which was routinely <5%.

To assess activation further, neutrophils were stimulated with CM for 30 min, the optimal time-point based on the time-course study using flow cytometry, and assessed for secretion of.

myeloperoxidase (MPO) determined by the 3,3-dimethoxybenzidine method [37, 38]. Cells (10^6) were first suspended in PBS and then incubated with SFM, EGF, basal-CM or EGF-CM at 37^{o}C. Reactions were terminated by placing the plates on ice for 5 min before centrifugation (200 *g*, 10 min, 4^{o}C). Supernatants were incubated with phosphate buffer (pH 6.2), 3, 3-dimethoxybenzidine (0.2 mg/ml), and H_2O_2 (90 nM) for 30 min at 37^{o}C and reactions terminated by adding 2 mM sodium azide prior to spectrophotometric detection of coloured products (460 nm) (Jenway 6505 UV/VIS spectrophotometer, Jenway, Felsted, U.K.). The amount of MPO released was expressed as percentage of the maximal MPO secretion obtained after stimulation with fMLP [10 μM].

Assessment of neutrophil chemotactic activity generated in epithelial culture

The ability of EGF-stimulated epithelial cells to generate chemoattractants for neutrophils was first assessed using the 16HBE and then confirmed in primary epithelial cells from healthy volunteers. The chemotactic activity was measured in a fluorescence-based chemotaxis chamber assay using calcein-labelled neutrophils as responder cells. Purified neutrophils were resuspended in fresh chemotaxis loading buffer (Hanks' balanced salt solution that contained 10 mM HEPES [pH 7.4] and supplemented with 10% autologous serum) at a concentration of 10 x 10^6 cells/ml, then incubated for 45 min at 37°C with 5 μM Calcein AM (Molecular Probes Europe BV, Leiden, Netherlands). Thirty one μl of basal-CM, EGF-CM or SFM were loaded into the bottom wells of the 96-well plate chemotaxis chamber (ChemoTX© System, Neuroprobe). For inhibition studies, putative inhibitors (all from Calbiochem, Merck Biosciences Ltd, Nottingham, U.K., except for CP-105696, a kind gift from Dr. Mike Yeadon, Pfizer Ltd., Kent, U.K.) were aliquoted at equal concentration to both the upper and lower wells. Subsequently, the polycarbonate filter (pore size 3 μm) was placed over the bottom chamber. Neutrophils were washed with Chemotaxis Buffer to eliminate non-incorporated Calcein AM and loaded (30 μl, 3×10^5 cells/ml) on top of the filter directly over each sample well in triplicate.

After allowing cells to migrate at 37°C (5% CO_2 v/v) for 60 min, the non-migrated cells were gently aspirated and 40 μl of detachment buffer (PBS supplemented with 20 mM EDTA and 0.3% BSA v/v) applied on top of each well and incubated for 30 min at 4°C. Residual neutrophils remaining on top of the filter were flushed carefully with PBS and the plate centrifuged for 8 min at 120 *g* to collect any cells that had partially migrated through the filter. The ChemoTX© System was disassembled gently and the bottom plates read in a Bio-Tek FLX 800 Microplate Fluorescence reader using 485 nm and 530 nm as the excitation and emission wavelengths, respectively. Fluorescence of migrated neutrophils was extrapolated from the standard curve constructed using known numbers of fluorescently labelled cells. The number of cells migrating in the absence of chemoattractant was subtracted to determine chemoattractant-mediated chemotaxis.

Analysis of signaling via Akt by Western blotting

Neutrophils were exposed for 5 min to basal-CM and EGF-CM from 16HBE cultures and fMLP [10 μM] and SFM or EGF as controls. Whole-cell protein lysates were then prepared from the cell pellets by solubilising in lysis buffer (20 mM Tris-HCl, pH 7.8, 138 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1% Nonidet P-40 and 1% Triton X-100) supplemented with a Protease Inhibitor Cocktail (all reagents purchased from Sigma Aldrich, Poole, U.K.). Protein samples (50 μg of protein per condition) were denatured, separated by SDS/PAGE, and transferred overnight onto nitrocellulose membrane by using the Mini-PROTEAN 3 apparatus (Bio-Rad Ltd., Watford, U.K.). Following the blocking step using PBS with 5% skimmed milk and 0.1% Tween 20 (Sigma, Poole, U.K.) for 1 h, the membranes were incubated with primary Ab at room temperature for 1 h with anti-phospho-Akt (Ser^{473} specific) monoclonal Ab (1:1000 dilution; Cell Signaling Technology). After sequential washing, the membranes were incubated for 1 hr with the secondary Ab, a goat anti-mouse IgG conjugated to horseradish peroxidase (1:2500 dilution of stock, obtained from Sigma). The immunoblotted

membranes were visualised using Pierce ECL Western Blotting Substrate (Pierce, Tattenhall, Cheshire, U.K.). For reprobing, membranes were stripped (in buffer containing 62.5 mM Tris, 2% SDS, and 0.8% (v/v) β-mercaptoethanol) for 45 min at 55^{o}C, followed by extensive washing before reblocking and reprobing with either anti-total-Akt polyclonal Ab (1:1000 dilution; Cell Signaling Technology) or anti-α-tubulin (1:5000 dilution; Sigma).

Statistical analysis

All values are expressed as mean ± SEM from *n* separate experiments. Data were analysed using the Student's *t* test with the statistical PRISM software (GraphPad) and were considered statistically significant when $P<0.05$.

Results

Effects of epithelial culture-CM on neutrophil activation

Control conditions, i.e. SFM and basal-CM, or EGF alone (data not shown) did not significantly alter the proportion of neutrophils expressing CD11b and CD66b until 120 min (Fig. 1, *a* and *b*). Incubation of neutrophils in EGF-CM markedly upregulated CD11b and CD66b at 30 min ($P<0.001$) (Fig. 1, *a* and *b*). In parallel, CD62L expression reduced markedly within 5 min of exposure to both basal-CM or EGF-CM (Fig. 1*c*) and followed a similar trend over 2 hr. While the rate of CD62L shedding was similar for basal-CM and EGF-CM, this was significantly different throughout the time-course when compared to control conditions. EGF-CM also induced a significant increase in MPO release ($P=0.0065$). A further minor stimulatory effect on MPO release was observed when EGF-CM-treated neutrophils were compared with basal-CM-treated cells, but this difference was not statistically significant (Online Depository data, Fig. D2).

Neutrophil chemotactic activity detected in epithelial culture-CM

Both basal-CM and EGF-CM from 16HBE cells induced significant ($P<0.0001$) neutrophil migration (Fig. 2*a*), with the effect of EGF-CM being significantly ($P=0.0043$) greater than basal-CM, while EGF alone had no effect (Fig. 2*a*). Whereas dose-response curves of chemotactic responses to individual chemoattractants are typically bell-shaped, assessment of responses to three dilutions of EGF-CM (1:10, 1:5 and 1:1) showed that a 1:1 dilution of the original EGF-CM induced a maximum migratory response (Fig. 2*b*). Therefore, this dilution was chosen for all subsequent experiments. Using primary bronchial epithelial cells grown from bronchial brushings obtained from healthy volunteers the effect of EGF on neutrophil chemotactic activity was shown to be similar, although both basal- and EGF-CM had a slightly less potent effect (Fig. 2*c*).

Signaling pathways involved in the regulation of neutrophil chemotaxis induced by an EGF-CM

Based on previous studies [39, 40], we hypothesized that the observed enhanced chemotactic response to EGF-CM involved activation of PI3K and its downstream signaling pathways in migrating neutrophils. Pre-treatment of neutrophils with the PI3K inhibitor, wortmannin (100 nM, 30 min), prior to exposure to EGF-CM obtained with 16HBE cells reduced chemotaxis to levels achieved with basal-CM, with no effect on basal-CM-induced migration (Fig. 3*a*). We then investigated the role of Akt, a well characterised PI3K-dependent protein kinase known to regulate various neutrophil functions [41, 42]. While the selective Akt inhibitor, 1L-6-hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecylcarbonate (HIMO, 10 μM, 30 min) [43] had no effect on basal-CM-mediated chemotaxis, it reduced significantly the EGF-CM-induced migration down to levels seen with basal-CM (Fig. 3*b*). Western blotting using a monoclonal antibody (mAb) specific for endogenously phosphorylated Akt (Ser^{473} specific) produced a distinct band of ~60 kDa that corresponded to the predicted size of phosphorylated Akt (Fig. 3*c*), with the intensity being greater in EGF-CM-stimulated cells when compared with basal-CM-treated neutrophils (Fig. 3*c*).

Since phospholipase D (PLD) is essential for the hydrolysis of phosphatidylcholine into phosphatidic acid (PtdOH) and subsequent participation in neutrophil function [37], we further hypothesized that the PLD enzymatic pathway may also regulate neutrophil migration induced by EGF-conditioned epithelium. The primary alcohol, butan-1-ol (0.5% v/v), which participates in a transphosphatidylation reaction by diverting specific PLD-derived PtdOH into a more stable metabolite whilst being a poor substrate for subsequent PLD-driven signaling cascades, caused significant inhibition, while, the control alcohol, butan-2-ol (0.5% v/v) had no effect (Fig. 4*a*).

Finally, we examined whether EGF-CM-mediated neutrophil chemotaxis occurs via specific members of the PI3K-related kinase (PIKK) family, most notably, mammalian Target Of Rapamycin

(mTOR), a target substrate of the PI3K/Akt signaling pathway [44]. Using rapamycin, which selectively inactivates mTOR, we showed that chemotaxis induced by either EGF-CM or basal-CM was largely unaffected by a maximal inhibitory concentration (100 nM) of this lipophilic macrolide (Fig. 4*b*).

Characterisation of neutrophil-active factors released by epithelial cells

Initial time-course analysis of CM from 16HBE cells showed that IL-8 release peaked at 24 h (Fig. 5*a*). Analysis of the same time-point for the other mediators showed that, by comparison with basal-CM, EGF-conditioned epithelial cells also secreted greater quantities of GM-CSF (334.8 ± 116.83 pg/ml vs. 85.72 ± 13.4 pg/ml), TNF-α (54.17 ± 11.07 pg/ml vs. 26.07 ± 1.9 pg/ml, $P<0.05$) and IL-8 (341.24 ± 48.49 pg/ml vs. 151.5 ± 27.29 pg/ml, $P<0.001$) (Fig. 5, *b-d*). In contrast, the EGF-conditioned epithelium did not produce any more LTB_4 than the control epithelium (40.29 ± 3.81 pg/ml in basal-CM and 33.11 ± 4.9 pg/ml in EGF-CM) (Fig. 5*e*).

The roles of the individual neutrophil chemotaxins were further studied using neutralising antibodies and blocking agents. In preliminary experiments, chemotactic dose-responses were obtained for TNF-α, GM-CSF, IL-8 and LTB_4 (Online Depository data, Fig. D3, *a-d*), and the effects of neutralising mAbs against TNF-α and GM-CSF, and the selective antagonists for the IL-8 receptor, CXCR2 (SB-225002) and the LTB_4 receptor, BLT1 (CP-105696) were assessed (Online Depository data, Fig. D3, *e-h*). Using optimal concentrations of blocking agents, the chemotactic activity in basal-CM could not be significantly inhibited by any antagonist or neutralising mAb in isolation. The activity was partially, but not significantly, reduced only when either the selective BLT1 receptor antagonist or the CXCR2 antagonist were combined with mAbs for TNF-α or GM-CSF (Fig. 6*a*). EGF-CM-mediated chemotaxis could be attenuated partially close to basal levels when the CXCR2 (45.2% inhibition compared with control neutrophils) and BLT1 receptor (51.2% inhibition) antagonists, or the

anti-GM-CSF mAb (56.3% inhibition) were applied individually. Anti-TNF-α mAb alone was ineffective (Fig. 6*b*). Complete abrogation of neutrophil chemotaxis caused by EGF-CM down to below basal levels (present in basal-CM) was achieved by combining anti-GM-CSF mAb with either the CXCR2 or BLT1 antagonists alone or together (Fig. 6*b*). Interestingly, the co-application of anti-TNF-α mAb to either anti-GM-CSF mAb or the CXCR2 and BLT1 antagonists partially reversed some of the inhibitory effects achieved with these blocking agents alone (Fig. 6*b*).

To confirm further that the chemotactic activity present in EGF-CM involves LTB_4, 16HBE cells were cultured in the presence of the 5-lipoxygenase-activating protein (FLAP) inhibitor, MK-886 (1 μM) for 30 min prior to EGF stimulation. The increase in chemotaxis towards EGF-CM was significantly attenuated by MK-886 to levels seen with basal-CM (Fig. 7*a*), consistent with its effect on blocking 5-lipoxygenase (5-LO)−mediated LTs, suggesting that LTs participated in the generation of the chemotactic activity.

Having observed that upon pre-treatment of neutrophils with CP-105696 neutrophil migration to EGF-CM was substantially reduced, we questioned whether this was due to the BLT1 antagonist blocking the chemotactic effects of LTB_4 generated by migrating neutrophils upon their initial exposure to EGF-CM. Indeed, 5-LO−initiated pathways and their products, including LTB_4, have been reported previously to be markedly increased in neutrophils during their migration in a murine air pouch model of inflammation [45]. Therefore, we used the FLAP inhibitor, MK-886 (1 μM, 30 min), to analyse whether neutrophil migration is mediated via autocrine release of LTB_4 upon exposure to basal-CM or EGF-CM. Neutrophil migration in response to both CM after pre-treatment with MK-886 remained unaltered (Fig. 7*b*). MK-886 was also ineffective in diminishing neutrophil chemotaxis towards IL-8 and LTB_4 (Fig. 7*b*), excluding the possibility of an autocrine process involving LTB_4 and suggesting that steady-state levels of LTB_4 can synergise with other critical mediators in EGF-CM.

Discussion

Previous studies have shown that, when stimulated by EGF, the epithelium produces mediators which activate neutrophils [24, 26, 27], but there has been no direct evidence to link these mediators with activation and chemotaxis of neutrophils and to quantify their relative contribution. We present here evidence that, while a resting epithelium can influence, to an extent, neutrophil behaviour, these responses are considerably enhanced when epithelial cells are stimulated by EGF. This setting reflects a response to any type of acute or chronic epithelial damage that compromises the physical integrity of the epithelial barrier allowing exposure of EGFRs on the basolateral surface of the epithelium to ligands present in the airway lining fluid. We have shown that neutrophil chemotactic responses to EGF-CM are regulated through PI3K and downstream signaling pathways involving Akt and PLD but not mTOR. This work also shows that the activated epithelium possesses a greater secretory potential as it releases larger quantities of the neutrophil-active mediators, GM-CSF, IL-8 and TNF-α. Consequently, the inhibition of their activity resulted in complete abrogation of the additional chemotactic activity that the EGF-conditioned epithelium produces over and above levels seen with a resting epithelium. Finally, we have also demonstrated that while LTB_4 release by the activated epithelium is not raised relative to the basal epithelium, it acts to potentiate the chemotactic responses of the other pro-inflammatory mediators present in EGF-CM. Taken together, the results of this study show that the epithelium has a significant potential to enhance neutrophil function when driven into a repair mode, a phenotype that is seen after acute (e.g. viral infection) or chronic lung injury (e.g. asthma).

This study shows for the first time that the activated epithelium can up-regulate CD11b and CD66b, and reduce CD62L expression on neutrophils. Neutrophils utilise such processes in the acute setting when enhanced neutrophil function is beneficial at a time when the epithelial barrier is

compromised. Following restoration of the epithelial barrier, EGF in the mucosal lining fluid is unable to stimulate basolaterally localized EGFRs in the pseudostratified columnar bronchial epithelium. If, however the insult is excessive and/or chronic and does not allow complete repair, EGF-mediated epithelial activation will persist, causing sustained neutrophil activation with the potential to cause further tissue damage [5, 7, 10, 46]. A pivotal role of EGF in repair processes has been demonstrated in inflammatory bowel diseases [47, 48], renal injury [49], psoriasis [47, 50] and corneal damage [47, 51]. While these disorders also have a significant neutrophilic component, the association between EGF-mediated epithelial repair and neutrophil activation and recruitment has not been explored in detail. Hence, the demonstration of EGF modulatory effects in primary human bronchial epithelial cells herein complements and extends our previous observations [24, 25] and further implicates interaction between the repairing bronchial epithelium and neutrophils as a mechanism of neutrophil-mediated tissue damage.

In agreement with previous reports of neutrophil migration induced by various chemoattractants [39, 40], we have demonstrated a role for PI3K signaling in the activation and migration of neutrophils induced by an EGF-conditioned epithelium. Likewise, EGF-CM-mediated neutrophil chemotaxis also involved enhanced activity of Akt down-stream of PI3K.and the PLD signaling pathway, the latter finding being consistent with the report by Lehman and colleagues (2006) [52]. However, selective inhibition of mTOR did not affect the migratory responses to either basal-CM or EGF-CM, which is surprising given the reports by Gomez-Cambronero (2003) [44] that neutrophil migration induced by GM-CSF is dependent on mTOR. This apparent discrepancy may arise because of regulation of neutrophil chemotaxis occurring via an mTOR component that is resistant to rapamycin treatment. This premise is based on observations in various mammalian cell types that mTOR can remain functionally active even after rapamycin treatment [53, 54]. This large protein kinase exists in two distinct complexes: one complex contains mTOR, GßL and raptor, and can be inhibited by rapamycin [55], while the other contains mTOR, GßL and rictor, and is rapamycin-insensitive [53, 55]. The cellular

function of the latter complex is only beginning to be unravelled [53], and our study did not address the question of whether the rictor-mTOR complex regulates the observed rapamycin-insensitive chemotaxis in response to basal-CM and EGF-CM.

In this study, we have found that epithelial cells increase GM-CSF, IL-8 and TNF-α production when stimulated by EGF. Using selective inhibitors we were able to dissect the relative contributions of these epithelium-derived factors and to show synergy between individual mediators. We have reported previously that neutrophilic chemotaxins, like IL-8, are markedly upregulated in the altered epithelial phenotype in asthma [24]. A more recent study [27] has shown that the EGFR-pathway is involved in stimulating IL-8 production in airway epithelial cells via a novel signaling cascade involving TACE activation by dual oxidase 1 (Duox1). However, this chemokine is unlikely to be solely responsible for driving epithelial-mediated neutrophil migration as our data clearly show that when the selective antagonist for the CXCR2 receptor (SB-225002) was applied individually, it failed to completely abrogate neutrophil chemotaxis evoked by EGF-CM (see Fig. 6*b*). Furthermore, EGF significantly upregulated secretion of GM-CSF and the observed chemotactic responses were sensitive to both the neutralising anti-GM-CSF mAb and the selective BLT1 antagonist, suggesting that these two mediators have independent effects in the mediation of EGF-CM-driven chemotaxis.

Bronchial epithelial cells have been previously shown to generate biologically-active LTB_4 [56, 57]. A recent study has shown that both 16HBE cells and PBECs constitutively express 5-LO, FLAP and LTA_4 hydrolase at the mRNA and protein levels [58]. As detected by ELISA and confirmed by Reversed-Phase HPLC purification, the amounts of LTB_4 released over 6 h by stimulated 16HBE cells (119 pg per 10^6 cells) were essentially similar to LTB_4 levels of 30 pg per 10^5 cells detected at 24 h in the current study. Both the recent data [58] and the current study show that LTB_4 can be significantly reduced by pre-treatment with the specific FLAP inhibitor MK-886, confirming the authenticity of the measured LTB_4. Our current work further demonstrates bioactivity of the LTB_4 released by 16HBE cells because selective blockade of the BLT1 receptors using CP-105,696 significantly reduced EGF-

CM-mediated chemotaxis and this leukotriene was also able to potentiate the effects of the other chemotactic factors present in EGF-CM (see Fig. 6*b*). Of note, the subnanomolar concentrations of LTB_4 present in EGF-CM (about 30 pg/ml, as detected by ELISA; see Fig. 5*e*) were at an adequate level to induce chemotaxis of human neutrophils [59]. Comparison of the results obtained with basal-CM and those with EGF-CM showed no differences in amounts of LTB_4 release, indicating that EGF is not capable of modifying the spontaneous release of LTB_4 from human bronchial epithelial cells described above, at least in 24 h cultures.

In summary, this is the first in-depth elucidation of direct modulatory effects of an EGF-conditioned epithelium on human neutrophil activation and migration. We speculate that while EGF has the capacity to enhance neutrophilic innate immunogenicity during acute lung damage (e.g. viral injury), an aberration of these responses may be pathological in the context of chronic diseases (e.g. severe neutrophilic, forms of asthma) where bronchial epithelial damage is a common feature.

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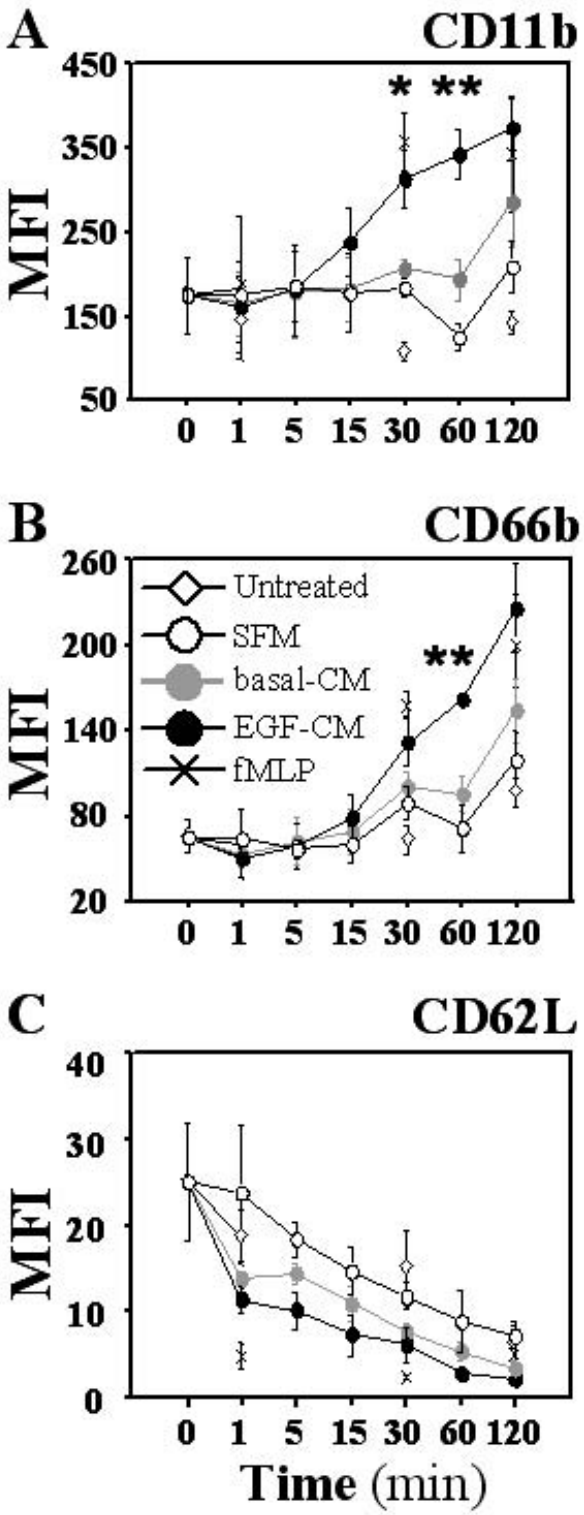
Figure Legends

FIGURE 1. Time-course study of EGF-CM effects on neutrophil activation marker expression. Purified neutrophils were treated with SFM (O), basal-CM (●) or EGF-CM (●), while PBS (◇) and fMLP (10 μM) were used as negative and positive controls, respectively. Expression of neutrophil surface molecules was determined by flow cytometric analyses as described in Methods. ** $P<0.01$ compared to basal-CM-treated neutrophils. Data represent mean ± SEM of four independent experiments performed using neutrophils purified from blood of four different donors.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10
0
0 1 5 15 30 60 120
Time (min)

FIGURE 1

FIGURE 2. Chemotaxis induced by EGF-CM and basal-CM. Calcein-loaded neutrophils were exposed to either SFM (■), EGF (■), basal-CM (□) or EGF-CM (■) (all at 1:1 dilution). While EGF alone induced no chemotactic activity, EGF-CM contained greater activity than basal-CM (***a***). ***P*<0.01 vs. neutrophils exposed to basal-CM alone, and $^{\dagger\dagger\dagger}P$<0.001 compared to SFM-treated neutrophils. The epithelium-derived chemotactic activity acted in a concentration-dependent manner, with a maximal effect at 1:1 dilution (***b***). ****P*<0.001 vs. neutrophils treated with basal-CM. EGF-CM from human PBECs contained greater activity than basal-CM (***c***). ***P*<0.01 vs. neutrophils exposed to basal-CM alone. Data are shown as mean ± SEM of experiments performed in triplicate using neutrophils from five different donors and PBECs were obtained from bronchial brushings of six healthy control subjects.

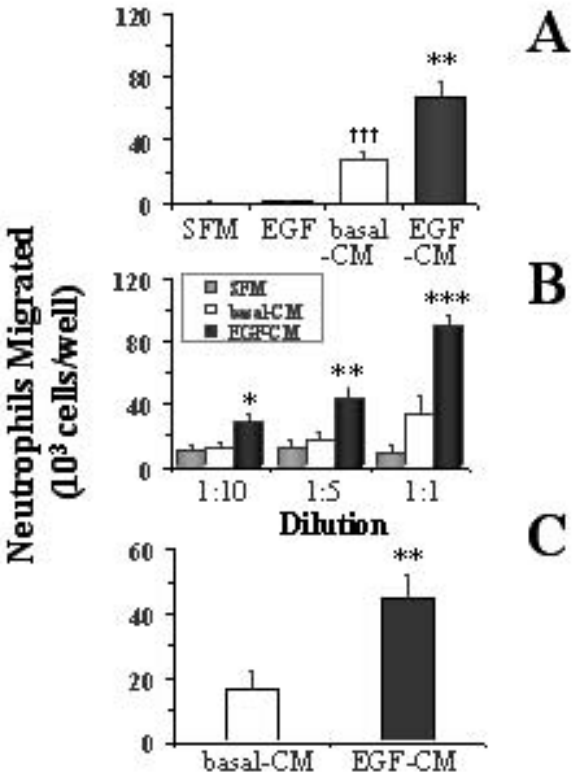

FIGURE 2

FIGURE 3. Effect of the PI3K inhibitor wortmannin (***a***) or Akt inhibitor, HIMO (***b***) neutrophil migration induced by EGF-CM or basal-CM. Calcein-loaded neutrophils were pre-treated with either buffer (open bars), or wortmannin (100 nM, filled bars) (***a***) or HIMO (10 μM, filled bars) (***b***) for 30 min before exposure of cells to SFM, basal-CM or EGF-CM. ****P*<0.001 compared to untreated controls. Results are shown as means ± SEM of seven and five independent experiments performed in triplicate, respectively.

Western blot of whole-neutrophil lysates showing that EGF-CM stimulation induces Akt phosphorylation. Purified neutrophils were stimulated with SFM, EGF, basal-CM, EGF-CM or fMLP

(10 μM) as a positive control at 37°C for 5 min, and then lysed (***c***). Equal amounts of protein from neutrophil lysates (50 μg of lysate/lane) were run on 10% SDS-PAGE, transferred to PVDF and phosphorylated Akt (p-Akt) on Ser^{473} visualised by Western blotting (***c***). α-tubulin expression is shown as a loading control (***c***). A representative blot of three independent experiments is presented.

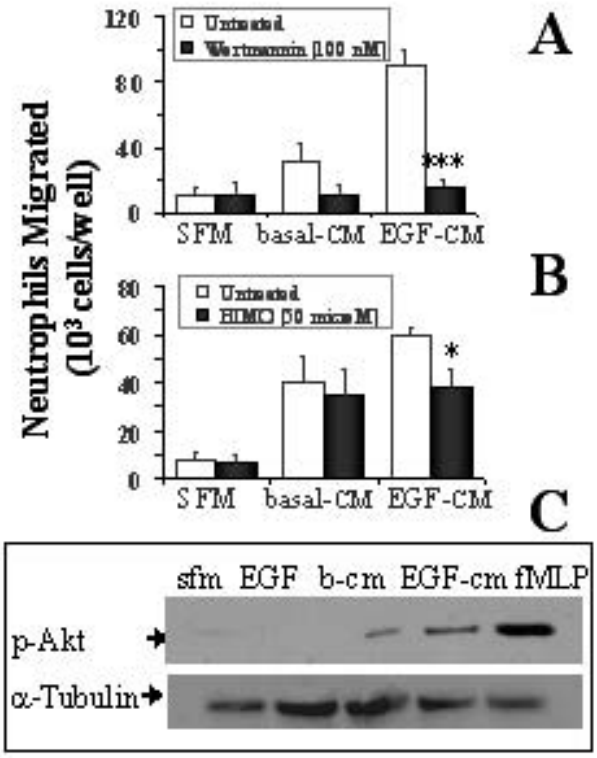

FIGURE 3

FIGURE 4. Effect of the modulators of PLD or the mTOR inhibitor, rapamycin, on basal-CM and EGF-CM-triggered neutrophil migration. Calcein-loaded neutrophils (10 x 10^6 cells/ml) were pre-treated with either buffer (open bars), butan-1-ol (0.5% v/v, filled bars), or butan-2-ol (0.5% v/v, grey bars) (***a***) or rapamycin (100 nM, filled bars) (***b***) for 30 min before exposure of cells to SFM, basal-CM

or EGF-CM for 60 min at 37°C. The number of chemoattractant-mediated neutrophils was extrapolated as detailed in *Methods*. **P*<0.05 compared to untreated controls. Results are shown as mean ± SEM of four and six independent experiments performed in triplicate, respectively.

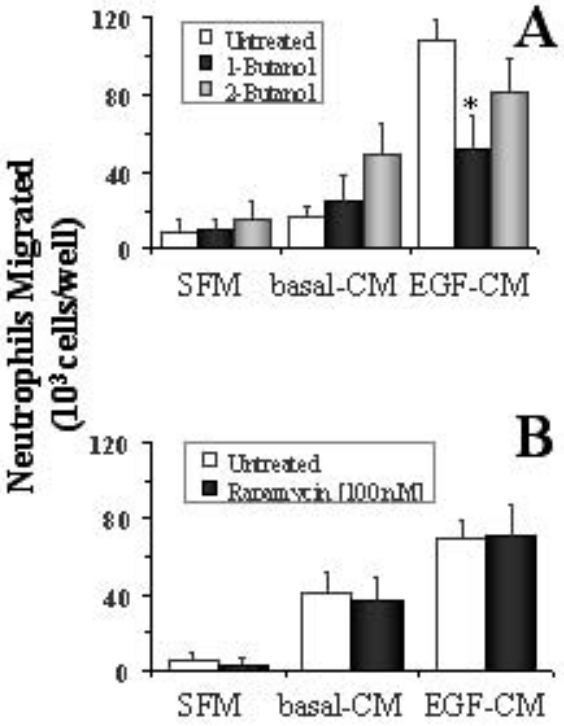

FIGURE 4

FIGURE 5. Treatment of 16HBE cells with EGF upregulates epithelium-derived mediator release. Time-course of IL-8 production measured by ELISA in CM harvested at the indicated time-points after stimulation of 16HBE cells with SFM (grey bars) or EGF (10 ng/ml, filled bars) (***a***). The CM were also analysed for GM-CSF (***b***), IL-8 (***c***) TNF-α (***d***) and LTB_4 (***e***) secretion at the 24 h time-point. Apart from LTB_4, the levels all mediators detected in EGF-CM were considerably higher than from SFM treated cells (basal-CM). ****P*<0.001 versus SFM-treated controls. All values are shown as mean ± SEM of four to nine separate experiments, each performed in duplicate.

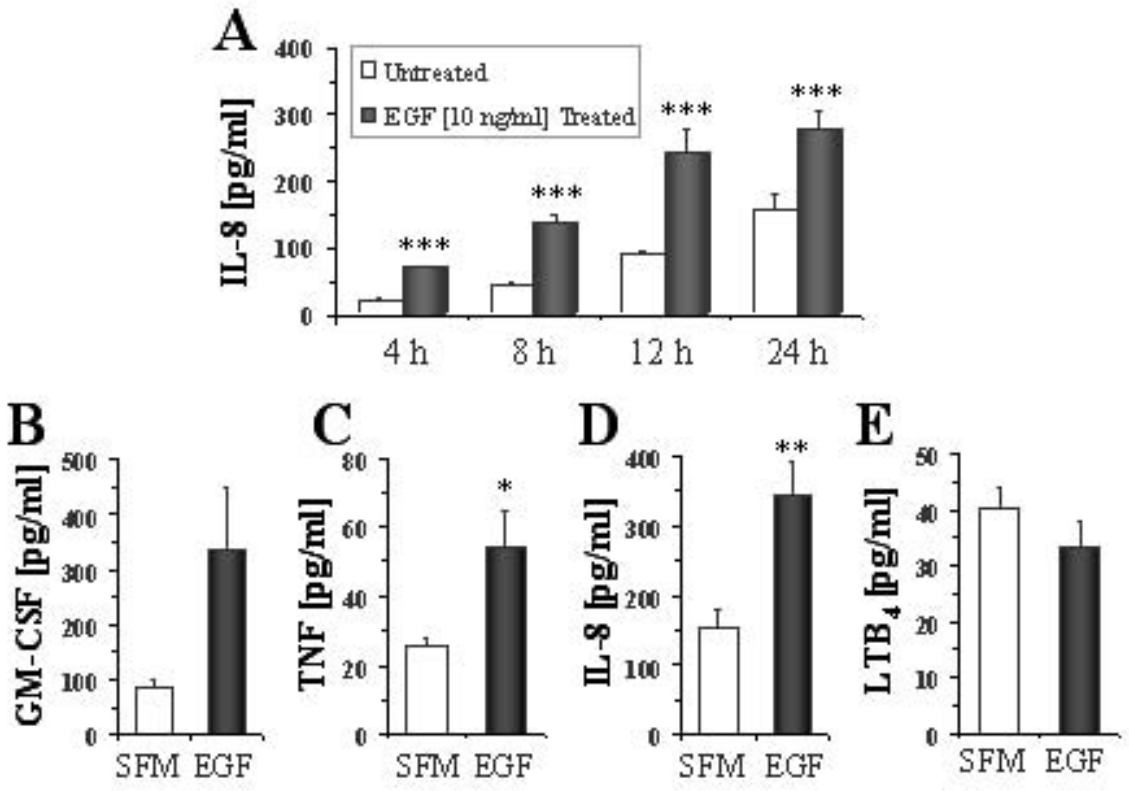

FIGURE 5

FIGURE 6. Neutralisation/blockade of epithelial-derived mediator activity abrogates neutrophil migration. Administration of the BLT1 antagonist, CP-105696 (10 μM) or the CXCR2 antagonist SB-225002 (100 nM) along with the anti–TNF-α mAb (100 μg/ml) or anti–GM-CSF mAb (100 μg/ml) respectively, partially attenuated basal-CM-mediated chemotaxis compared to untreated basal-CM control (grey bar) (***a***). Complete abrogation of the chemotactic activity exerted by EGF-CM (filled bar) on neutrophils was achieved down to basal levels (dashed line) with the combination of anti-GM-CSF mAb with SB-225002 and/or CP-105696 (***b***). ****P*<0.001 compared to untreated EGF-CM control. Data are shown as mean ± SEM of five independent experiments performed in triplicate using peripheral blood neutrophils isolated from different donors.

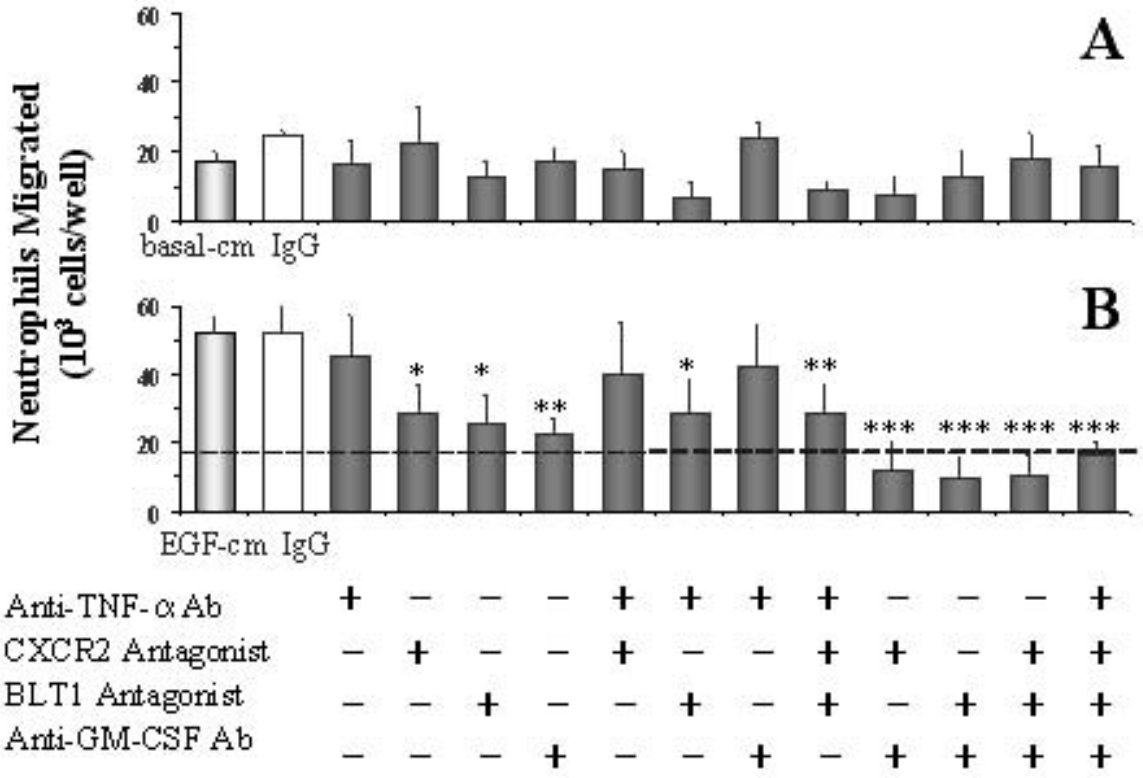

FIGURE 6

FIGURE 7. Inhibition of leukotriene synthesis in reparative 16HBE cells using the FLAP inhibitor, MK-886, attenuates EGF-CM-induced neutrophil migration. 16HBE cells were pre-treated with either buffer (open bars) or MK-886 (1 μM, filled bars) for 30 min before stimulation with SFM or EGF (10 ng/ml) for 24 h at 37°C. CM was collected from epithelial cultures then the chemotaxis assessed in calcein-loaded neutrophils (10 x 10^6 cells/ml). ***P<0.001 compared with untreated controls (***a***). Autocrine secretion of LTB_4 does not regulate EGF-CM-mediated chemotaxis as inhibition of FLAP in neutrophils using MK-886 does not affect the migratory responses. Calcein-loaded neutrophils (10 x 10^6 cells/ml) were pretreated with buffer (open bars) or MK-886 (1 μM, filled bars) for 30 min before exposure of cells to basal-CM, EGF-CM or IL-8 (10 nM), or LTB_4 (100 nM) for 60 min at 37°C.

Results are means ± SEM of four independent experiments performed in triplicate using neutrophils purified from blood of different donors (***b***).

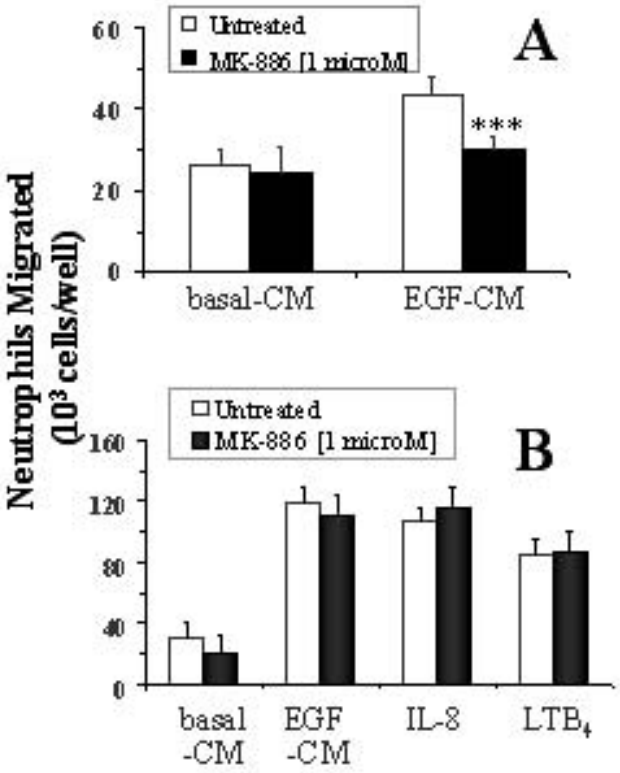

FIGURE 7

References

1. Nathan C. Points of control in inflammation. *Nature* 2002: 420(6917): 846-852.
2. Kishimoto TK, Jutila MA, Berg EL, Butcher EC. Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science* 1989: 245(4923): 1238-1241.
3. Condliffe AM, Chilvers ER, Haslett C, Dransfield I. Priming differentially regulates neutrophil adhesion molecule expression/function. *Immunology* 1996: 89(1): 105-111.
4. Stocks SC, Ruchaud-Sparagano MH, Kerr MA, Grunert F, Haslett C, Dransfield I. CD66: role in the regulation of neutrophil effector function. *European journal of immunology* 1996: 26(12): 2924-2932.

5. MacNee W, Selby C. New perspectives on basic mechanisms in lung disease. 2. Neutrophil traffic in the lungs: role of haemodynamics, cell adhesion, and deformability. *Thorax* 1993: 48(1): 79-88.

6. Berton G, Yan SR, Fumagalli L, Lowell CA. Neutrophil activation by adhesion: mechanisms and pathophysiological implications. *International journal of clinical & laboratory research* 1996: 26(3): 160-177.

7. Louis R, Lau LC, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *American journal of respiratory and critical care medicine* 2000: 161(1): 9-16.

8. MacNee W. Pulmonary neutrophil kinetics. *Clin Phys Physiol Meas* 1990: 11 Suppl A: 133-139.

9. Barnes PJ. Mechanisms in COPD: differences from asthma. *Chest* 2000: 117(2 Suppl): 10S-14S.

10. Louis R, Djukanovic R. Is the neutrophil a worthy target in severe asthma and chronic obstructive pulmonary disease? *Clin Exp Allergy* 2006: 36(5): 563-567.

11. Barnes PJ. A new approach to the treatment of asthma. *The New England journal of medicine* 1989: 321(22): 1517-1527.

12. Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. *American journal of respiratory and critical care medicine* 1997: 156(3 Pt 1): 737-743.

13. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *American journal of respiratory and critical care medicine* 1999: 160(5 Pt 1): 1532-1539.

14. Carroll N, Carello S, Cooke C, James A. Airway structure and inflammatory cells in fatal attacks of asthma. *Eur Respir J* 1996: 9(4): 709-715.

15. Teran LM, Campos MG, Begishvilli BT, Schroder JM, Djukanovic R, Shute JK, Church MK, Holgate ST, Davies DE. Identification of neutrophil chemotactic factors in bronchoalveolar lavage fluid of asthmatic patients. *Clin Exp Allergy* 1997: 27((4)): 396-405.

16. Vachier I, Bonnans C, Chavis C, Farce M, Godard P, Bousquet J, Chanez P. Severe asthma is associated with a loss of LX4, an endogenous anti-inflammatory compound. *Journal of Allergy and Clinical Immunology* 2005: 115(1): 55-60.

17. Wenzel SE, Larsen GL, Johnston K, Voelkel NF, Westcott JY. Elevated levels of leukotriene C4 in bronchoalveolar lavage fluid from atopic asthmatics after endobronchial allergen challenge. *The American review of respiratory disease* 1990: 142(1): 112-119.

18. Bodey KJ, Semper AE, Redington AE, Madden J, Teran LM, Holgate ST, Frew AJ. Cytokine profiles of BAL T cells and T-cell clones obtained from human asthmatic airways after local allergen challenge. *Allergy* 1999: 54(10): 1083-1093.

19. Cotter TP, Hood PP, Costello JF, Sampson AP. Exposure to systemic prednisolone for 4 hours reduces ex vivo synthesis of GM-CSF by bronchoalveolar lavage cells and blood mononuclear cells of mild allergic asthmatics. *Clin Exp Allergy* 1999: 29(12): 1655-1662.

20. Howarth PH, Babu KS, Arshad HS, Lau L, Buckley M, McConnell W, Beckett P, Al Ali M, Chauhan A, Wilson SJ, Reynolds A, Davies DE, Holgate ST. Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. *Thorax* 2005: 60(12): 1012-1018.

21. Davies DE, Polosa R, Puddicombe SM, Richter A, Holgate ST. The epidermal growth factor receptor and its ligand family: their potential role in repair and remodelling in asthma. *Allergy* 1999: 54(8): 771-783.

22. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, Davies DE. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *Faseb J* 2000: 14(10): 1362-1374.

23. Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel PR, Takeyama K, Tam DC, Nadel JA. IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. *American journal of physiology* 2001: 280(1): L134-140.

24. Hamilton LM, Torres-Lozano C, Puddicombe SM, Richter A, Kimber I, Dearman RJ, Vrugt B, Aalbers R, Holgate ST, Djukanovic R, Wilson SJ, Davies DE. The role of the epidermal growth factor receptor in sustaining neutrophil inflammation in severe asthma. *Clin Exp Allergy* 2003: 33(2): 233-240.

25. Hamilton LM, Puddicombe SM, Dearman RJ, Kimber I, Sandstrom T, Wallin A, Howarth PH, Holgate ST, Wilson SJ, Davies DE. Altered protein tyrosine phosphorylation in asthmatic bronchial epithelium. *Eur Respir J* 2005: 25(6): 978-985.

26. Richter A, O'Donnell RA, Powell RM, Sanders MW, Holgate ST, Djukanovic R, Davies DE. Autocrine ligands for the epidermal growth factor receptor mediate interleukin-8 release from bronchial epithelial cells in response to cigarette smoke. *Am J Respir Cell Mol Biol* 2002: 27(1): 85-90.

27. Nakanaga T, Nadel JA, Ueki IF, Koff JL, Shao MX. Regulation of interleukin-8 via an airway epithelial signaling cascade. *Am J Physiol Lung Cell Mol Physiol* 2007: 292(5):L1289-96

28. Vignola AM, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, la Rocca AM, Bellia V, Bonsignore G, Bousquet J. Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *American journal of respiratory and critical care medicine* 1997: 156(2 Pt 1): 591-599.

29. Monick MM, Cameron K, Staber J, Powers LS, Yarovinsky TO, Koland JG, Hunninghake GW. Activation of the epidermal growth factor receptor by respiratory syncytial virus results in increased inflammation and delayed apoptosis. *The Journal of biological chemistry* 2005: 280(3): 2147-2158.

30. Lordan JL, Bucchieri F, Richter A, Konstantinidis A, Holloway JW, Thornber M, Puddicombe SM, Buchanan D, Wilson SJ, Djukanovic R, Holgate ST, Davies DE. Cooperative effects of Th2 cytokines and allergen on normal and asthmatic bronchial epithelial cells. *J Immunol* 2002: 169(1): 407-414.

31. Cox G, Gauldie J, Jordana M. Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. *Am J Respir Cell Mol Biol* 1992: 7(5): 507-513.

32. Cozens AL, Yezzi MJ, Kunzelmann K, Ohrui T, Chin L, Eng K, Finkbeiner WE, Widdicombe JH, Gruenert DC. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1994: 10(1): 38-47.

33. Ehrhardt C, Kneuer C, Fiegel J, Hanes J, Schaefer UF, Kim KJ, Lehr CM. Influence of apical fluid volume on the development of functional intercellular junctions in the human epithelial cell line 16HBE14o-: implications for the use of this cell line as an in vitro model for bronchial drug absorption studies. *Cell and tissue research* 2002: 308(3): 391-400.

34. Manford F, Tronde A, Jeppsson A-B, Patel N, Johansson F, Forbes B. Drug permeability in 16HBE14o- airway cell layers correlates with absorption from the isolated perfused rat lung. *European Journal of Pharmaceutical Sciences* 2005: 26(5): 414-420.

35. Bucchieri F, Puddicombe SM, Lordan JL, Richter A, Buchanan D, Wilson SJ, Ward J, Zummo G, Howarth PH, Djukanovic R, Holgate ST, Davies DE. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am J Respir Cell Mol Biol* 2002: 27(2): 179-185.

36. Haslett C, Guthrie LA, Kopaniak MM, Johnston RB, Jr., Henson PM. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *The American journal of pathology* 1985: 119(1): 101-110.

37. Cadwallader KA, Uddin M, Condliffe AM, Cowburn AS, White JF, Skepper JN, Ktistakis NT, Chilvers ER. Effect of priming on activation and localization of phospholipase D-1 in human neutrophils. *European journal of biochemistry / FEBS* 2004: 271(13): 2755-2764.

38. Henson PM, Zanolari B, Schwartzman NA, Hong SR. Intracellular control of human neutrophil secretion. I. C5a-induced stimulus-specific desensitization and the effects of cytochalasin B. *J Immunol* 1978: 121(3): 851-855.

39. Sasaki T, Irie-Sasaki J, Jones RG, Oliveira-dos-Santos AJ, Stanford WL, Bolon B, Wakeham A, Itie A, Bouchard D, Kozieradzki I, Joza N, Mak TW, Ohashi PS, Suzuki A, Penninger JM. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* 2000: 287(5455): 1040-1046.

40. Knall C, Worthen GS, Johnson GL. Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. *Proceedings of the National Academy of Sciences of the United States of America* 1997: 94(7): 3052-3057.

41. Hannigan M, Zhan L, Li Z, Ai Y, Wu D, Huang CK. Neutrophils lacking phosphoinositide 3-kinase gamma show loss of directionality during N-formyl-Met-Leu-Phe-induced chemotaxis. *Proceedings of the National Academy of Sciences of the United States of America* 2002: 99(6): 3603-3608.

42. Klein JB, Rane MJ, Scherzer JA, Coxon PY, Kettritz R, Mathiesen JM, Buridi A, McLeish KR. Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways. *J Immunol* 2000: 164(8): 4286-4291.

43. Hu Y, Qiao L, Wang S, Rong SB, Meuillet EJ, Berggren M, Gallegos A, Powis G, Kozikowski AP. 3-(Hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt, and cancer cell growth. *Journal of medicinal chemistry* 2000: 43(16): 3045-3051.

44. Gomez-Cambronero J. Rapamycin inhibits GM-CSF-induced neutrophil migration. *FEBS Letters* 2003: 550(1-3): 94-100.

45. Levy BD, Clish CB, Schmidt B, Gronert K, Serhan CN. Lipid mediator class switching during acute inflammation: signals in resolution. *Nat Immunol* 2001: 2(7): 612-619.

46. Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med* 1989 320((6)): 365-376.

47. Schultz G, Rotatori DS, Clark W. EGF and TGF-alpha in wound healing and repair. *Journal of cellular biochemistry* 1991: 45(4): 346-352.

48. Beck PL, Podolsky DK. Growth factors in inflammatory bowel disease. *Inflammatory bowel diseases* 1999: 5(1): 44-60.

49. Strutz F, Neilson EG. New insights into mechanisms of fibrosis in immune renal injury. *Springer seminars in immunopathology* 2003: 24(4): 459-476.

50. Tomic-Canic M, Komine M, Freedberg IM, Blumenberg M. Epidermal signal transduction and transcription factor activation in activated keratinocytes. *Journal of Dermatological Science* 1998: 17(3): 167-181.

51. Joyce NC, Joyce SJ, Powell SM, Meklir B. EGF and PGE2: effects on corneal endothelial cell migration and monolayer spreading during wound repair in vitro. *Current eye research* 1995: 14(7): 601-609.

52. Lehman N, Di Fulvio M, McCray N, Campos I, Tabatabaian F, Gomez-Cambronero J. Phagocyte cell migration is mediated by phospholipases PLD1 and PLD2. *Blood* 2006: 108(10): 3564-3572.

53. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* 2004: 14(14): 1296-1302.

54. Constantinou C, Clemens MJ. Regulation of the phosphorylation and integrity of protein synthesis initiation factor eIF4GI and the translational repressor 4E-BP1 by p53. *Oncogene* 2005: 24(30): 4839-4850.

55. Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN. Two TOR Complexes, Only One of which Is Rapamycin Sensitive, Have Distinct Roles in Cell Growth Control. *Molecular Cell* 2002: 10(3): 457-468.

56. Koyama S, Rennard SI, Robbins RA. Bradykinin stimulates bronchial epithelial cells to release neutrophil and monocyte chemotactic activity. *The American journal of physiology* 1995: 269(1 Pt 1): L38-44.

57. Behera AK, Kumar M, Matsuse H, Lockey RF, Mohapatra SS. Respiratory syncytial virus induces the expression of 5-lipoxygenase and endothelin-1 in bronchial epithelial cells. *Biochemical and biophysical research communications* 1998: 251(3): 704-709.

58. Jame AJ, Lackie PM, Cazaly AM, Sayers I, Penrose JF, Holgate ST, Sampson AP. Human bronchial epithelial cells express an active and inducible biosynthetic pathway for leukotrienes B4 and C4. *Clin Exp Allergy* 2007: 37(6): 880-892.

59. Ford-Hutchinson AW, Bray MA, Doig MV, Shipley ME, Smith MJ. Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature* 1980: 286(5770): 264-265.