

Adhesion molecules involved in hepxilin A₃-mediated neutrophil transepithelial migration

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Introduction

A consequence of the innate inflammatory response at mucosal surfaces is the infiltration of neutrophils (PMNs) from the bloodstream to the epithelium, which separates the lumen from the underlying tissue [1–3]. PMNs travel across this epithelial barrier into the lumen in order to confront colonizing pathogenic bacteria [1–3]. The mechanisms underlying the movement of PMNs from the bloodstream to sites of infection are complex and involve numerous adhesion molecules, cytokines and chemoattractants that function to direct PMNs through endothelial barriers, basements membranes, extracellular matrix and epithelial barriers [2,3]. Our group has recently established a discreet role for the PMN chemoattractant, hepxilin A₃ (HXA₃) in the process of PMN recruitment [4,5].

Summary

A common feature underlying active states of inflammation is the migration of neutrophils (PMNs) from the circulation and across a number of tissue barriers in response to chemoattractant stimuli. Although our group has recently established a discreet role for the PMN chemoattractant, hepxilin A₃ (HXA₃) in the process of PMN recruitment, very little is known regarding the interaction of HXA₃ with PMNs. To characterize further the event of HXA₃-induced PMN transepithelial migration, we sought to determine the adhesion molecules required for migration across different epithelial surfaces (T84 intestinal and A549 airway cells) relative to two well-studied PMN chemoattractants, formyl-methionyl-leucyl-phenylalanine (fMLP) and leukotriene B₄ (LTB₄). Our findings reveal that the adhesion interaction profile of PMN transepithelial migration in response to HXA₃ differs from the adhesion interaction profile exhibited by the structurally related eicosanoid LTB₄. Furthermore, unique to PMN transepithelial migration induced by gradients of HXA₃ was the critical dependency of all four major surface adhesion molecules examined (i.e. CD18, CD47, CD44 and CD55). Our results suggest that the particular chemoattractant gradient imposed, as well as the type of epithelial cell monolayer, each plays a role in determining the adhesion molecules involved in transepithelial migration. Given the complexities of these interactions, our findings are important to consider with respect to adhesion molecules that may be targeted for potential drug development.

Keywords: adhesion molecules, hepxilin A₃, neutrophils, *Pseudomonas aeruginosa*, *Salmonella typhimurium*

HXA₃ is an eicosanoid derived from the 12-lipoxygenase pathway, which is produced by epithelial cells and secreted from the apical surface resulting in the guidance of PMNs across the epithelial monolayer from the basolateral to the apical side [4,5]. Infection of epithelial cells with pathogenic bacteria stimulates HXA₃ production and secretion and subsequent PMN transmigration, a phenomenon that has been observed in both lung and intestinal epithelial cells [4,5]. This is supported *in vivo* by an inhibitor of 12-lipoxygenase, the enzyme required for HXA₃ synthesis, resulting in a dramatic reduction in PMN infiltration in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*)-infected human intestinal xenograft tissue [4].

The adhesion molecules involved in the migration of PMNs across epithelial barriers has been investigated using primarily N-formyl-methionyl-leucyl-phenylalanine (fMLP)

as a chemoattractant [6,7]. Currently, very little is known regarding the interaction between HXA₃ and PMNs. Unlike other PMN chemoattractants, including fMLP and leukotriene B₄ (LTB₄), HXA₃ acts as a pure PMN chemoattractant as it does not activate PMNs to release reactive oxygen intermediates or cause the release of secondary granule content [4,8]. Many PMN chemoattractants have also demonstrated the capability to modulate the expression of certain adhesion molecules [9]. For example, fMLP has been shown to cause an increase in expression of CD18/CD11b on the surface of PMNs [9]. These studies have revealed that not only does CD18/CD11b play a critical role in mediating PMN transmigration [6,7] but CD44, CD47 and CD55 also play defined roles [10–13]. More recently, Blake *et al.* demonstrated that, although PMN migration across T84 monolayers in response to an imposed gradient of fMLP is dependent upon CD18/CD11b, PMNs are capable of migrating across T84 monolayers in a CD18-independent manner in response to gradients of alternative chemoattractants such as interleukin (IL)-8, complement component (C5a) and LTB₄ [14]. This intriguing observation implies a versatility by which PMNs utilize distinct adhesion molecules depending upon the chemoattractant gradient applied during transepithelial migration. Modulation of PMN surface adhesion molecules in response to HXA₃ has yet to be explored.

In this study, we have characterized further aspects of HXA₃-mediated PMN migration across epithelial surfaces in comparison with other known PMN chemoattractants, including fMLP and LTB₄. Our studies suggest that both the type of epithelial cell monolayer and the particular chemoattractant gradient imposed each play a role in dictating the adhesion molecules involved in transepithelial PMN migration. Interestingly, the adhesion interaction profile of PMN transepithelial migration in response to HXA₃ differs from the profile exhibited by the structurally related eicosanoid LTB₄.

Materials and methods

Epithelial cell culture

The A549 cell line was derived originally through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. These cells display properties of type II alveolar epithelial cells and were maintained in Ham's F-12K medium with 2 mM L-glutamine 1.5 g/l NaHCO₃, 10% fetal bovine serum and 100 U of Penn/Strep [5]. T84 intestinal epithelial cells (passages 46–66) were grown in a 1:1 mixture of Dulbecco–Vogt modified Eagle's medium and Ham's F-12 nutrient mixture supplemented with 15 mM HEPES (pH 7.5), 14 mM NaHCO₃, 40 µg/ml penicillin, 8 µg/ml streptomycin, 8 µg/ml ampicillin and 6% fetal bovine serum [15]. Polarized monolayers of A549 and T84 cells were grown on the underside of 0.33 cm² collagen-coated polycarbonate Transwell filters (5 µm pore size) to

study PMN migration in the physiological basolateral to apical direction, as described previously [4,5,15]. A549 cells form barriers when grown on permeable filters, but do not develop transepithelial electrical resistance (TEER) [5]. TEER of approximately 800–1500 Ω cm² was reached in all T84 monolayers used in this study.

Measurement of [³H]-inulin flux

Monolayers of A549 and T84 were grown on Transwells for 4–18 days and were washed twice in Hanks's balanced salt solution (HBSS) before the addition of 100 µl of 2.5 µCi/ml [³H]-inulin (PerkinElmer, Boston, MA, USA) in HBSS to the top well (basolateral side) and 600 µl of HBSS to the bottom well (apical side). After 3 h, 10 µl was sampled from the apical chamber and added to 3 ml scintillation fluid and counts per minute were measured using a Beckman Coulter scintillation counter. Data are presented in triplicate as a percentage of the amount of counts added to the top chamber [16]. For measurement of complete equilibration of radioactivity in the absence of a barrier, collagen-coated Transwells without cells were used.

Isolation of human PMN for transmigration assays

Human peripheral PMN were isolated from anti-coagulated 10% acid citrate dextrose (pH 4) and human whole blood collected by venipuncture from normal donors of both sexes [15]. The buffy coat was separated by centrifugation at 600 g at room temperature (RT). The plasma and mononuclear cells were removed by aspiration, and the majority of erythrocytes were removed by a 2% gelatin sedimentation technique. Residual erythrocytes were lysed in cold NH₄Cl lysis buffer. This technique allows for the rapid isolation of functionally active PMN (> 95% as detected by trypan blue dye exclusion) at greater than 90% purity [4,5,15]. The PMN were resuspended in HBSS (without Ca²⁺ and Mg²⁺ and supplemented with 10 mM HEPES, pH 7.4; Sigma Chemical Co., St Louis, MO, USA) (HBSS⁻) at a concentration of 5 × 10⁷/ml.

PMN transmigration assay

The PMN transmigration assay using inverted cell culture monolayers of polarized cells has been described [4,5,15]. For monolayers infected with bacteria, the apical surface of A549 monolayers was exposed for 1 h to 1.5 × 10⁶ *Pseudomonas aeruginosa* (PA01)/monolayer [5]. Alternatively, the apical surface of T84 monolayers was exposed to 2 × 10⁸ *S. typhimurium* for 1 h [4,15]. After infection, monolayers were washed three times and PMNs (1 × 10⁶) were applied to the basolateral chamber. PMNs were allowed to migrate to the apical side for 2 h at 37°C. For the analysis of individual chemoattractants, various doses of HXA₃, LTB₄ and fMLP were placed into the apical chamber of uninfected epithelial

monolayers while PMNs were placed into the basolateral chamber. PMNs were quantified by the myeloperoxidase assay [15]. Data are displayed as a representative experiment that was performed at least three times with the mean \pm standard deviation (s.d.) of at least three independent monolayers/condition calculated for each experiment. HXA₃ was obtained from Biomol (Plymouth Meeting, PA, USA) at a concentration of 50 μ g/ml in ethanol. For experiments with T84 monolayers where high concentrations of HXA₃ are needed to observe PMN transmigration, ethanol was evaporated to 5–10 μ l in siliconized tubes using a Speed Vac and resuspended in HBSS. LTB₄ and fMLP were both obtained from Sigma-Aldrich (St Louis, MO, USA).

Monoclonal antibodies

To study the role of PMN surface receptors on transmigration, the following blocking antibodies were used: anti-CD18 (clone MHM23, 0.5 mg/ml; Dako Cytomation, Carpinteria, CA, USA), anti-CD44 (clone IM7, 1 mg/ml; BD Biosciences, San Jose, CA, USA), anti-CD47 (clone C5D5, 0.95 mg/ml; a gift from C. Parkos, Emory University, GA, USA) and anti-CD55 (clone BRIC 216, 1 mg/ml; Serotec, Raleigh, NC, USA). Irrelevant isotype-matched antibodies (IgG1 and IgG2b), which cover the isotype specificity of the blocking antibodies used in this study, were purchased from BD Biosciences. The IgG1 isotype served as a control for antibodies against CD18, CD55, and CD47, while the IgG2b isotype served as a control for the antibody against CD44. For every 1×10^6 PMNs, 4 μ l of antibody solution was added and the resultant mixture was incubated in siliconized tubes for 20 min at room temperature. At the end of the incubation period, the cell–antibody mixtures were added directly to the basolateral chamber for migration.

Fluorescence activated cell sorter (FACS) analysis

Whole blood was drawn from healthy human volunteers using heparin vacutainers (BD Biosciences) and used immediately. The various chemoattractants fMLP, LTB₄, HXA₃, and ethanol (negative control) were diluted with 250 μ l of HBSS (with Ca²⁺) to the appropriate concentrations, then mixed with 250 μ l of whole blood. The blood–chemoattractant solutions were incubated in a 37°C water bath for 30 min. The stimulated blood cells were then fixed with equal volumes (500 μ l) of 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in phosphate-buffered saline (without Ca²⁺) for at least 15 min on ice. The majority of red blood cells were removed by lysis using cold NH₄Cl lysis buffer for 10 min on ice, then centrifuged to remove supernatant and resuspended in 1 ml HBSS. Various cell surface receptors (CD18, CD44, CD47, CD55 and isotype control) were labelled by the addition of 10 μ l of fluorescein isothiocyanate-labelled anti-human antibodies (BD Biosciences) to each 200 μ l of cell suspension and

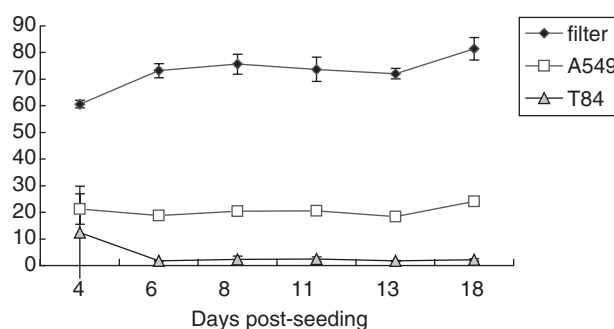


Fig. 1. Percentage [³H]-inulin flux across Transwell filters. Transwell filters alone (black diamonds) or epithelial cells grown on Transwell filters for various days, A549 (white squares) or T84 (grey triangles) were washed with Hanks's balanced salt solution (HBSS) and 100 μ l (2.5 μ C/ml) in HBSS was added to the basolateral surface on the day depicted (days 4–18). After 3 h, radioactivity was sampled from the apical chamber. Data are presented as percentage [³H]-inulin flux by calculating the number of counts per minute (cpm) divided by the total number of cpm added, taking into account the different volumes in the apical (600 μ l) and basolateral (100 μ l) chambers. Based on the respective volumes of each chamber, 85.7% represent complete equilibration of percentage [³H]-inulin. Each data point represents three independent Transwell filters and is depicted as mean \pm standard deviation.

incubated at 4°C for 15 min. The cell suspensions were then analysed with a FACScan flow cytometer (Becton Dickinson).

FACS data analyses were performed using WinMDI software (Scripps Institute, La Jolla, CA, USA; <http://facs.scripps.edu>). Whole blood was used in order to minimize activation of PMNs and gating was used to single out the PMN populations. PMN populations were identified based on front- and side-scatter clustering and the corresponding mean fluorescent intensities (MFI) were measured. Percentage change in cell surface receptor expression was obtained by comparing the stimulated population MFI against the ethanol negative control.

Results

It has been shown previously that T84 cells form polarized barriers with high levels of transepithelial electrical resistance (TEER) when grown on Transwells [4,6,15]. These monolayers are extremely selective to the passage of small molecules such as proteins, dextrans and radioisotope tracers [17]. Conversely, A549 cells do not form measurable TEER, but do form barriers that are selective to flux of proteins such as horseradish peroxidase (HRP) [5]. Before initiating chemoattractant gradient experiments, we investigated the relative barrier function of T84 cells compared to A549 cells by measuring the percentage of [³H]-inulin flux across the monolayers over a 3-h period. As shown in Fig. 1, monolayers were tested for the ability to restrict the movement of [³H]-inulin added basolaterally. The percentage of

[³H]-inulin flux that occurs across the filter alone represents complete equilibration of [³H]-inulin added basolaterally with day-to-day experimental variability. By day 6, both A549 cells and T84 cells form stable barriers. T84 monolayers are highly restrictive to [³H]-inulin flux and significantly more effective than A549 cell monolayers at maintaining a strict barrier (Fig. 1). However, A549 cell monolayers are capable of restricting free equilibration of [³H]-inulin, as significantly less [³H]-inulin crosses to the apical well when an A549 cell barrier is present compared with no barrier (filter alone). Both T84 and A549 monolayers maintained barrier function for as long as 18 days post-seeding (Fig. 1).

Having established the relative barrier properties between T84 intestinal epithelial monolayers and A549 lung epithelial monolayers, we next sought to determine the concentration gradient of HXA₃ required to stimulate PMN transmigration across T84 and A549 monolayers, respectively. For comparison, we also examined the concentration gradient leading to PMN transmigration of the well-studied PMN chemoattractant LTB₄. LTB₄, like HXA₃, is an eicosanoid produced from an arachidonic acid precursor; however, LTB₄ is produced through the 5-lipoxygenase enzymatic pathway as opposed to HXA₃, which is produced via the 12-lipoxygenase pathway, giving each molecule structural distinctions [18,19]. Moreover, while HXA₃ can be produced from epithelial cells in response to bacterial stimuli [4,5], LTB₄ is produced primarily from leucocytes in response to inflammatory mediators [20], suggesting functional distinctions *in vivo* as well.

Various concentrations of HXA₃ were applied to the apical chamber of A549 monolayers and the number of PMNs migrating from the basolateral to the apical side were measured. A significant increase in the number of migrated PMNs began to occur at approximately 50 ng/ml, which is consistent with our previous findings (Fig. 2a) [5]. As concentrations of HXA₃ added to the apical chamber increase, we observed a dose-dependent increase in PMN transmigration. We observed the same trend in PMN migration across T84 monolayers, although significant PMN transmigration did not occur until at least 200 ng/ml was present (Fig. 2a). This is consistent with the observation that T84 monolayers form a more selective barrier than A549 monolayers.

PMN transmigration in response to LTB₄ occurs at significantly lower concentrations when compared with HXA₃ (Fig. 2b). PMNs migrated across A549 cells to a significant extent in response to less than 1 ng/ml of LTB₄ added to the apical chamber (Fig. 2b). Migration occurred in a dose-dependent fashion until approximately 10 ng/ml, in which a plateau of transmigration was achieved and persisted up to 1 µg/ml LTB₄. Again, a similar trend was observed in T84 cells, except that significant transmigration did not occur until at least 10 ng/ml LTB₄ was added. Also, the overall number of PMNs that migrated across A549 monolayers was

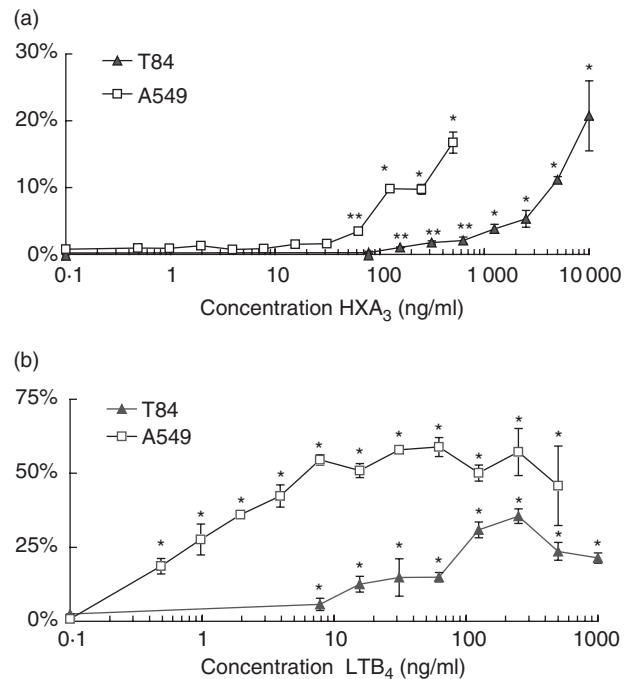


Fig. 2. Dose curve for chemoattraction of neutrophils (PMNs) by eicosanoids. (a) Various doses of hepxilin A₃ are added to the apical chamber at the same time that 1×10^6 PMNs are added to the basolateral chamber. (b) Various doses of leukotriene B₄ are added to the apical chamber at the same time that 1×10^6 PMNs are added to the basolateral chamber. PMN migration is represented as the percentage of PMNs that migrate fully across epithelial monolayers (A549: white squares or T84: black triangles) of the total number of PMNs added. Experiments were performed multiple times with a representative experiment depicted herein. Each data point represents three independent Transwell filters and is depicted as mean \pm standard deviation. Significant difference between groups (compared to the 0.1 ng/ml concentration) was assessed using Student's *t*-test. **P*-values < 0.01; ***P*-values < 0.05.

much higher than across T84 cells at all concentrations of LTB₄ tested.

Next we sought to determine the contribution of adhesion molecules in the process of PMN migration across T84 monolayers and A549 monolayers in response to various chemoattractants. We have shown previously that *S. typhimurium* induces PMN transmigration across T84 cells by stimulating the production of HXA₃ [4]. Monoclonal antibodies are capable of blocking PMN cell surface molecules; CD18, CD44, CD47 and CD55 all exerted some degree of inhibition of *S. typhimurium*-induced PMN transmigration when compared with isotype controls (Fig. 3). Blocking antibodies to CD18 and CD55 potently blocked fMLP-induced PMN migration across T84 cells and, consistent with previous findings [7,10,11], antibodies to CD47 blocked PMN transmigration at least by 50%. However, antibodies to CD44 did not have any effect on fMLP-induced PMN transmigration in our studies. Migration of PMNs

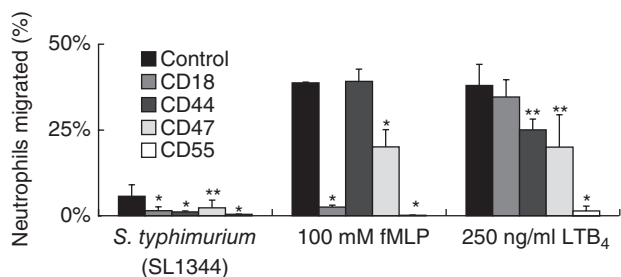


Fig. 3. Effects of interference with blocking antibodies of adhesive surface molecules on neutrophil (PMN) transepithelial migration. Antibodies were mixed with PMNs before addition to the basolateral well of T84 cells. Leukotriene B₄ or formyl-methionyl-leucyl-phenylalanine were added to the apical chamber at the same time that PMNs with antibodies (blocking and isotype-matched controls) were added to the basolateral chamber. For epithelial cells treated with pathogens, T84 were preinfected with wild-type *Salmonella typhimurium* (SL1344) for 1 h before addition of PMNs. Experiments were performed multiple times with a representative experiment depicted herein. Each data point represents three independent Transwell filters and is depicted as mean \pm standard deviation. Significant difference between groups was assessed using Student's *t*-test. **P*-values < 0.01; ***P*-values < 0.05.

across T84 monolayers in response to an LTB₄ gradient demonstrated an altogether distinct pattern from *S. typhimurium* and fMLP-induced PMN transmigration (Fig. 3). Anti-CD18 monoclonal antibodies had no effect on PMN transmigration, anti-CD44 and anti-CD47 exerted a slight effect and anti-CD55 resulted in almost complete inhibition of LTB₄-induced PMN transmigration. Taken together, these results further support the hypothesis that adhesion molecules utilized by PMNs to migrate across epithelial cells differ depending upon the chemoattractant involved.

Next we examined PMN transmigration using monolayers of the lung epithelial cell A549. We have shown previously that *P. aeruginosa* causes PMN migration across A549 monolayers through the production and secretion of HXA₃ in a mechanism that shares similarities with *Salmonella*-induced PMN migration across T84 monolayers [5]. Consistent with our establishment of HXA₃ as the chemoattractant underlying *Pseudomonas*-induced PMN transmigration, we observed an identical pattern of antibody inhibition in *Pseudomonas*-infected monolayers and uninfected monolayers receiving a gradient of HXA₃ (Fig. 4). Both cases resulted in significantly reduced migration when blocking antibodies to CD18, CD44, CD47 and CD55 were compared to the addition of isotype control. All four blocking antibodies also significantly inhibited transmigration in response to fMLP, when PMNs were preincubated with each before addition to the basolateral chamber of A549 monolayers. When a gradient of LTB₄ was established across A549 cells, anti-CD44, anti-CD47 and anti-CD55 significantly blocked PMN transmigration, but anti-CD18 did not appear to interfere with transmigration. Data obtained from various chemoattractants

inducing PMN migration across A549 monolayers supports the notion that PMN cell surface molecules responsible for transmigration vary depending upon the chemoattractant applied. PMN migration across airway epithelial monolayers was more dependent upon CD44 and CD47 in comparison to intestinal epithelial monolayers when responding to imposed gradients of fMLP and LTB₄. This suggests that the type of epithelial cell monolayer also plays an important role in determining which cell surface molecules operate during transmigration.

Because the anti-CD55 blocked PMN transepithelial migration to every stimulus examined across both epithelial cell lines, we next sought to determine whether PMN treatment with anti-CD55 simply immobilizes PMNs, irrespective of the effects on interactions with epithelial cells. This was an important consideration, as PMNs express CD55 and there is some suggestion that this receptor may be important in allowing PMN to survive and function at inflammatory sites where there is a rapid complement turnover [21]. Therefore, we investigated the extent to which the CD55 antibodies were capable of blocking migration of PMN across acellular (bare) collagen-coated polycarbonate Transwell filters in response co-imposed gradients of fMLP, HXA₃ and LTB₄. A dose-response was first performed in order to establish the optimal concentration of each chemoattractant needed to induce PMN migration across acellular monolayers (data not shown). After establishing these effective chemoattractant concentrations [fMLP (1 μ M); HXA₃ (5 ng/ml); LTB₄ (25 ng/ml)] we next determined whether anti-CD55 prevented PMNs from

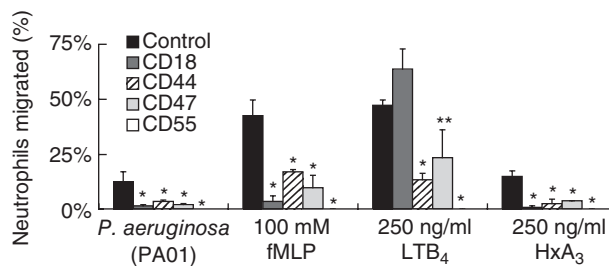


Fig. 4. Effects of interference with blocking antibodies of adhesive surface molecules on neutrophil (PMN) transepithelial migration. Antibodies were mixed with PMNs before addition to the basolateral well of A549 cells. Leukotriene B₄, formyl-methionyl-leucyl-phenylalanine or hepxilin A₃ were added to the apical chamber at the same time that PMNs with antibodies (blocking and isotype-matched controls) were added to the basolateral chamber. For epithelial cells treated with pathogens, A549 cells were preinfected with *Pseudomonas aeruginosa* (PA01) for 1 h before addition of PMNs. Experiments were performed multiple times with a representative experiment depicted herein. Each data point represents three independent Transwell filters and is depicted as mean \pm standard deviation. Experiments were performed multiple times with a representative experiment depicted herein. Significant difference between groups was assessed using Student's *t*-test. **P*-values < 0.01; ***P*-values < 0.05.

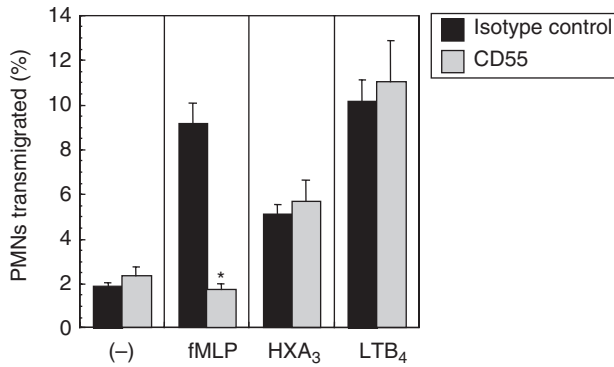


Fig. 5. Relative contribution of neutrophil (PMN) CD55 to PMN migration. Collagen-coated permeable supports preincubated in media overnight were used in transmigration assays using the same protocol as described above for Figs 3 and 4. Migration is shown in the absence of any chemotactic stimuli ((-)) or the presence of a formyl-methionyl-leucyl-phenylalanine (1 μ M), hepxilin A₃ (5 ng/ml) or leukotriene B₄ (25 ng/ml) Transwell gradient. Transmigration in the presence of an isotype-matched control antibody (IgG1) is compared to that in the presence of the CD55 antibody. Bars represent the mean \pm standard deviation of three monolayers for each condition. One of three experiments performed showing the same result. Significant difference between groups was assessed using Student's *t*-test. **P*-values < 0.01.

migrating across acellular filters in response to various stimuli. As shown in Fig. 5, PMN migration across acellular filters in response to HXA₃ and LTB₄ is not affected by the addition of anti-CD55 antibodies, demonstrating that anti-CD55 treatment does not simply obstruct the movement of PMN. This is not the case with fMLP-induced migration across acellular filters, which is blocked completely by anti-CD55 antibodies. This result suggests that the anti-CD55 blocking effect of PMN transepithelial migration in response to fMLP is acting in a manner independent of PMN epithelial contacts.

Chemoattractants have been shown to stimulate increased surface expression of adhesion molecules on PMNs [9]. Previous studies have demonstrated that fMLP can up-regulate CD18 on freshly isolated PMNs [9]. Using whole blood from healthy volunteers (see Materials and methods) we confirm this observation, but fail to see any increase in surface expression of CD44, CD47 and CD55 (Fig. 6). In fact, there appears to be a slight decrease in expression of these surface molecules upon fMLP stimulation. LTB₄ also causes an increase in CD18 expression, without affecting expression of any of the other surface molecules tested (Fig. 6). In contrast, we did not observe any change in expression of any of the surface adhesion molecules CD18, CD44, CD47 and CD55 when PMNs were exposed to HXA₃. This result is consistent with the notion that HXA₃, unlike other PMN chemoattractants, does not activate various PMN responses despite having the ability to serve as a PMN chemoattractant [4,8].

Discussion

The primary objective of PMNs is to phagocytose and destroy pathogenic microorganisms, and thus represent an important effector of the acute inflammatory response [1]. Therefore, PMNs must be able to migrate from out of the blood circulation and across a number of tissue barriers in response to mucosal stimuli. This is the case in many of the major organ systems where mucosal surface infections are common, including the respiratory, urinary and gastrointestinal tracts. We have demonstrated recently that the eicosanoid HXA₃ is secreted from the apical surface of the epithelial barrier and establishes a chemotactic gradient required for guidance of PMNs across both model intestinal and airway epithelium, representing the final step in PMN recruitment to the mucosal lumen [4,5]. A complex series of adhesive and de-adhesive events can be envisaged to allow successful migration of PMNs through the paracellular spaces of the epithelia, along the basolateral surface of epithelial cells, before reaching the apical milieu [11]. As a critical role for HXA₃ in PMN-mediated events has been

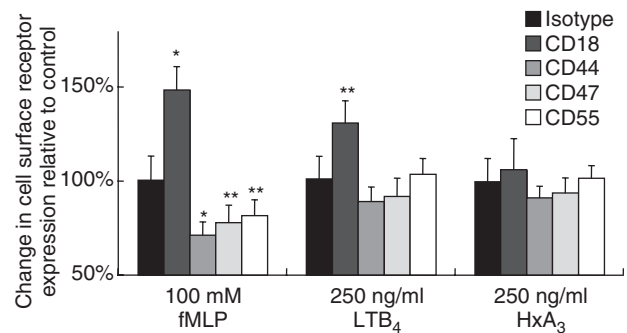


Fig. 6. Neutrophils (PMN) expression of various surface adhesion molecules in response to treatment with chemoattractants. Whole blood was mixed for 30 min with leukotriene B₄, formyl-methionyl-leucyl-phenylalanine, hepxilin A₃ or vehicle control (ethanol), followed by lysis of red blood cells. Blood preparations were then incubated with antibodies to surface adhesion molecules (anti-CD18, anti-CD44, anti-CD47, anti-CD55 and isotype control) and incubated subsequently with fluorescein isothiocyanate-labelled secondary antibodies for detection and quantification by fluorescence activated cell sorter analysis with gating on the PMN subpopulation. Irrelevant isotype-matched antibodies were used as controls. The IgG1 isotype served as a control for antibodies against CD18, CD55 and Cd47, while the IgG2b isotype served as a control for the antibody against CD44. Values are reported as percentage of results obtained from vehicle control-treated whole blood in combination with each antibody. Experiments were performed multiple times with a representative experiment depicted herein. Each data point represents three independent samples per condition and displayed as mean \pm standard deviation (s.d.). The standard deviation of the three values is determined before setting the average value at 100%. The percentage s.d. was used subsequently to calculate the s.d. of the 100%. Significant difference between groups was assessed using Student's *t*-test. **P*-values < 0.01; ***P*-values < 0.05.

identified for both intestinal and pulmonary inflammation [4,5], in the current study we sought to characterize the adhesion molecules fundamental to HXA₃-mediated PMN transepithelial migration.

The present understanding of PMN–epithelial interactions during transmigration is based largely on studies using imposed gradients to fMLP and involves several integrated steps [11]. As the initial step at the subepithelium, PMNs adhere to the basolateral surface of epithelial cells via the PMN adhesion molecule CD11b/CD18, where it interacts with epithelial counter receptors that probably includes multiple fucosylated glycoproteins (and perhaps other undefined ligands) [6,7]. Next, during the migration step, interactions between epithelial CD47 and PMN signal regulatory protein alpha (SIRP α) appear to enhance the rate of PMN transepithelial migration [10]. Adhesive interactions of PMN with epithelial intercellular tight junction proteins (i.e. JAM-C) are also likely to occur [7]. Lastly, after migration across the apical junctional complex, PMNs reach the lumen and contact the apical epithelial membrane. At this surface, apically expressed intercellular adhesion molecule 1 (ICAM-1) is an accessible ligand for CD11b/CD18 and is thought to serve as a foothold for PMNs on the luminal surface [22]. These elegant studies initially identified CD11b/CD18 as the principal adhesion molecule because all migration to fMLP across model intestinal epithelia can be blocked with anti-CD11b or anti-CD-18 (β_2 integrin) antibodies. However, recent lines of evidence indicate that PMNs may use CD18-independent mechanisms to cross the intestinal epithelium depending upon the chemoattractant encountered [14]. For example, whereas essentially all migration to fMLP across model intestinal epithelia was prevented by anti-CD18 antibody, significant migration to C5a, IL-8 and LTB₄ persisted despite the presence of anti-CD18 antibody, indicating that PMNs are capable of CD18-independent PMN migration [14].

Such studies have established the emerging concept that PMNs utilize distinct adhesion molecules during transepithelial migration, depending upon the chemoattractant gradient imposed. Our data substantiate this concept as we found that the adhesion interaction profile of PMN transepithelial migration in response to HXA₃ differs from the adhesion interaction profile exhibited by the structurally related eicosanoid LTB₄. This finding underscores the fact that although HXA₃ and LTB₄ may be structurally similar and are both potent PMN chemoattractants, they each most probably serve fundamentally different roles in the PMN recruitment process. As PMNs must follow both endogenous and bacterial chemoattractant signals to arrive at the site of infection, PMNs must undergo a decision-making process requiring both end-target and intermediary endogenous chemoattractants [23]. In this regard, HXA₃ would be considered an end-target chemoattractant, as it emanates from the site of infection to establish a chemotactic gradient that guides PMNs across mucosal surfaces characterizing

the final step in PMN recruitment to the mucosal lumen. On the other hand, LTB₄ would be considered an intermediary endogenous chemoattractant, as this PMN chemoattractant is encountered en route to sites of infection. For example, LTB₄ is produced from leucocytes and macrophages, as well as mast cells [24], where it induces the adhesion and activation of leucocytes on the endothelium, allowing them to bind to and cross into the tissue [20]. Based upon this model, end-target chemoattractants not only dominate over intermediary chemoattractants but are also predominately CD11b/CD18-dependent [23]. This would be consistent with our findings as HXA₃, but not LTB₄, was found to be CD18-dependent. It has also been determined that end-target and intermediary PMN chemoattractants induce different intracellular signalling pathways in PMNs [23], which we hypothesize may be linked to the differences noted in the adhesion interactive profiles occurring in response to each distinct chemoattractant [4,15]. In fact, HXA₃ activates PMN intracellular Ca²⁺ release but fails to cause the release of reactive oxygen intermediates and also fails to cause PMNs to discharge their primary and secondary granule content [4,15]. Furthermore, in this study we have shown that HXA₃ treatment of PMNs does not lead to an increase in surface expression of CD18 (Fig. 6). Thus, unlike LTB₄, which activates PMNs to release granule content and to up-regulate CD18 expression [8,15], HXA₃ is considered a 'pure chemoattractant'. Evaluation of the roles of both HXA₃ and LTB₄ in a more complex *in vivo* model will be necessary to understand fully the discreet roles of each of these eicosanoids in PMN recruitment. Nevertheless, our present study provides further evidence that distinct PMN chemoattractants promote PMN migration through epithelial tissues by eliciting different adhesion mechanisms.

While PMN migration across intestinal epithelial cell monolayers has served traditionally as the model for establishing the current paradigm for the study of PMN–epithelial interactions, another important implication of our findings is that both the type of epithelial cell monolayer, in addition to the particular chemoattractant gradient imposed, also plays an important role in the adhesion molecules involved in transepithelial migration. Interestingly, we found that PMN transepithelial migration across airway epithelial monolayers was more dependent upon CD44 and CD47 in response to imposed gradients of fMLP and LTB₄. A study investigating monocyte migration through the alveolar epithelial barrier found that monocyte migration across A549 monolayers depended largely upon CD11b/CD18 and CD47, and also required the additional engagement of β_1 integrins for optimal migration [25]. Further studies will be necessary to clarify the function of both CD47 and CD44 in the interaction of airway epithelial cells and PMNs in a more detailed fashion. Although we did not investigate ICAM-1 interactions with PMN, it has been shown that ICAM-1 is expressed on type I and type II pneumocytes and is

up-regulated in the presence of proinflammatory cytokines [26,27]. Characterization of ICAM-1 involvement in PMN migration across the airway epithelium in response to HXA₃ will be an important future extension of this work.

We have shown previously that HXA₃ is not only secreted from the apical surface of human intestinal or lung epithelial cells stimulated with pathogenic *S. typhimurium* or *P. aeruginosa*, respectively, but HXA₃ is also both necessary and sufficient for promotion of bacterial-induced PMN transepithelial migration [4,5]. Consistent with these observations is our finding that the adhesion molecules involved during PMN transepithelial migration to exogenously added HXA₃ were identical to the profile of adhesive interactions characteristic of bacterial-induced PMN transepithelial migration. Unique to PMN transepithelial migration induced by gradients of HXA₃, in particular, was the involvement of all four major surface adhesion molecules examined (i.e. CD18, CD47, CD44 and CD55) in both airway and intestinal cell monolayers. However, the specific roles of each adhesion molecule during HXA₃-induced PMN transepithelial migration has yet to be explored.

Anti-CD55 antibodies blocked PMN transepithelial migration induced by all stimuli examined in this study. CD55 (decay accelerating factor) has been shown to function as a key anti-adhesive surface glycoprotein that regulates the rate of PMN migration across the apical epithelial membrane by promoting the release of PMN from the apical surface of the epithelium after successful breach of cell-to-cell junctions [12]. We observed that PMN migration across collagen-coated acellular Transwell filters in response to gradients of HXA₃ and LTB₄ was unaffected by anti-CD55 antibody treatment. This finding is consistent with a role for CD55 in PMN epithelial interactions. Interestingly, anti-CD55 antibody treatment did, however, interfere with fMLP-induced PMN migration across acellular filters, suggesting a differential role for CD55 during PMN transmigration in response to fMLP *versus* the eicosanoids HXA₃ and LTB₄.

In conclusion, the findings reported here support the hypothesis that adhesion molecules utilized by PMNs to migrate across epithelial cell monolayers are different depending upon the chemoattractant and the type of epithelial monolayer involved. Moreover, our study provides new information regarding the adhesion molecules fundamental to HXA₃-mediated PMN transepithelial migration. A key observation was that the adhesion interaction profile of PMN transepithelial migration in response to HXA₃ differs from the profile exhibited by the structurally related eicosanoid LTB₄. Thus, as mucosal surfaces lined by epithelial cells (i.e. airway and intestine) offer a potential direct target for therapy, given that these surfaces are directly available for non-invasive administration of therapeutic compounds, our findings will be important to consider when determining specific adhesion molecules to exploit for anti-inflammatory drug development.

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