RESEARCH PAPER

Role of neutrophil elastase in LTB$_4$-induced neutrophil transmigration *in vivo* assessed with a specific inhibitor and neutrophil elastase deficient mice

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Background and purpose: The serine protease neutrophil elastase (NE) appears to regulate inflammatory responses at multiple levels but its role in leukocyte transmigration *in vivo* remains unclear. The present study aimed to address this issue by using both an NE inhibitor (ONO-5046) and NE deficient (NE$^{-/-}$) mice.

Experimental approach: A number of inflammatory mediators (LTB$_4$, KC and PAF) were investigated *in vitro* for their ability to stimulate the release and the surface expression of NE by neutrophils. In addition, the role of NE in leukocyte migration elicited by topical LTB$_4$ was investigated *in vivo* in mouse cremasteric venules as observed by intravital microscopy.

Key results: Amongst the mediators tested *in vitro*, LTB$_4$ was found to be a highly potent and efficacious inducer of NE cell surface expression on murine neutrophils. Furthermore, in wild-type mice (WT), LTB$_4$-induced leukocyte transmigration was reduced by intravenous ONO-5046 (66% inhibition), an effect that appeared to occur at the level of the perivascular basement membrane. Interestingly, LTB$_4$-induced responses were normal in NE$^{-/-}$ mice and, while ONO-5046 had no inhibitory effect in these animals, the broad-spectrum serine protease inhibitor aprotinin suppressed leukocyte transmigration in both WT and NE$^{-/-}$ mice.

Conclusions and implications: The findings demonstrate the potent ability of LTB$_4$ to induce cell-surface expression of NE and provide evidence for the involvement of NE in LTB$_4$-induced neutrophil transmigration *in vivo*. The results also suggest the existence of compensatory mechanisms in NE$^{-/-}$ mice, highlighting the added value of investigating pharmacological blockers in parallel with genetic deletion.

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Introduction

Neutrophil elastase (NE) is a 30 kD neutral serine protease stored in an active form in the azurophil granules of neutrophils. The potential substrates of NE include most components of the extracellular matrix (for example, collagen, fibronectin and laminin) as well as a wide range of other proteins such as cytokines, clotting factors, adhe-

sion molecules and components of the complement cascade (Lee and Downey, 2001). In addition, NE has been shown to regulate the activity of certain enzymes such as activation of matrix metalloproteinase-9 (gelatinase B) from its pro-isoform (Delclaux *et al.*, 1996). As a result of this broad substrate specificity and the ability of this enzyme to cause tissue damage, NE has been implicated in the pathogenesis of numerous inflammatory conditions (Weiss, 1989) and indeed it is frequently used as both a predictor and an indicator of inflammatory disease severity (for example, Groeneveld *et al.*, 1995; Orem *et al.*, 1997; Smith *et al.*, 2000). Specifically, NE has been found in elevated levels in synovial fluid and/or blood in rheumatoid arthritis (Elsaid *et al.*, 2003) and under conditions of ischemia/reperfusion injury (Vila

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et al., 1999). Furthermore, NE is found at high levels in bronchoalveolar lavage of patients with inflammatory lung disorders such as chronic obstructive pulmonary disease, acute lung injury, cystic fibrosis and adult respiratory distress syndrome, disorders in which it has been strongly implicated (Lee and Downey, 2001; Shapiro and Ingenito, 2005).

Considering the destructive nature of NE, a pathological role for this enzyme under inflammatory conditions where it is released inappropriately, in excess and/or in a prolonged manner, is entirely comprehensible (Lee and Downey, 2001; Shapiro and Ingenito, 2005). However, since NE is stored within neutrophil granules at high concentrations (estimated at ~5 mM), it is also entirely appropriate to consider a physiological role for this enzyme in host defence (Barrick *et al.*, 1999; Shapiro, 2002). In this context, there is much evidence for the antibactericidal activity of NE (Gabay and Almeida, 1993), a concept for which direct support has been obtained from studies using NE-deficient mice (Belaaouaj *et al.*, 1998; Tkalcevic *et al.*, 2000). There is also evidence to suggest that NE may play a critical role in regulating the bioavailability of inflammatory cytokines and chemokines in inflammation (Bank and Ansorge, 2001; Adkison *et al.*, 2002; Young *et al.*, 2004), thus contributing to the onset and/or termination of an inflammatory response. NE may also have additional roles in the process of wound healing (Barrick *et al.*, 1999) but a controversial possibility relates to the potential role of NE in mediating leukocyte migration, specifically migration through venular walls, that is, through endothelial cells and/or its associated perivascular basement membrane (BM). In support of this, NE inhibitors have been shown to suppress neutrophil migration through BM-like structures (Delclaux *et al.*, 1996; Delacourt *et al.*, 2002) and to degrade or penetrate components of the venular BM (Heck *et al.*, 1990; Steadman *et al.*, 1993; Wang *et al.*, 2005, 2006). Refuting a role for NE in leukocyte transmigration there are the findings of Huber and Weiss indicating that neutrophil migration through endothelial cell BM is not inhibited by a range of protease inhibitors (Huber and Weiss, 1989). Similar negative results were obtained in other studies employing NE inhibitors and investigating neutrophil migration through cultured endothelial cells or venular walls (Furie *et al.*, 1987; Rosengren and Arfors, 1990; Mackarel *et al.*, 1999). Furthermore, NE-deficient neutrophils exhibit a normal transendothelial cell migration response *in vitro* (Allport *et al.*, 2002) and NE-deficient mice exhibit a largely normal profile of neutrophil transmigration *in vivo* (Belaaouaj *et al.*, 1998; Tkalcevic *et al.*, 2000; Hirche *et al.*, 2004).

Despite the discrepancies detailed above, there remains a plausible possibility that NE may play a role in regulating neutrophil migration through venular walls. Of relevance, neutrophils can be stimulated *in vitro* to express NE on their cell surface, conditions under which the enzyme has been shown to remain active and resistant to endogenous inhibitors (Owen *et al.*, 1995, 1997) and neutrophils migrating through endothelial cell junctions *in vitro* and transmigrated neutrophils *in vivo* have been shown to express NE on their cell surface (Cepinskas *et al.*, 1999; Wang *et al.*, 2005). In addition and of direct relevance, we have recently found that a specific NE inhibitor suppresses neutrophil migration through IL-1β-stimulated cremasteric

venules at the level of the basement membrane (Wang *et al.*, 2005), an effect that appears to be related to the ability of NE to facilitate migration of neutrophils through vulnerable regions within the venular BM (Wang *et al.*, 2006). To extend these findings, the present study addressed the role of NE in neutrophil migration induced by topical LTB$_4$ in the mouse cremaster muscle, as observed by intravital microscopy and investigated using both an NE inhibitor and NE-deficient mice. LTB$_4$ was chosen as the stimulus under investigation since in initial studies this chemoattractant was found to be a potent and efficacious stimulus in inducing cell surface expression of NE on mouse neutrophils and in eliciting neutrophil transmigration *in vivo*. Collectively the findings of the present study provide evidence for the potent ability of LTB$_4$ to induce cell surface expression of NE on murine neutrophils and for a specific NE inhibitor to suppress LTB$_4$-induced neutrophil migration through the BM, though no such defect was seen in NE-deficient mice, possibly due to existence of compensatory mechanisms in the latter.

Materials and methods

Animals

Twenty to twenty-five gram male wild-type (WT) C57BL/6 mice (Harlan Olac, Bicester, UK) were purchased at least 2 weeks in advance of being employed in the studies. Mice deficient in neutrophil elastase (NE$^{-/-}$), generated by targeted gene disruption as detailed previously (Belaaouaj *et al.*, 1998) were a gift from Professor S Shapiro (Harvard Medical School, Boston, MA, USA) and were backcrossed onto a C57BL/6 background for six generations before their use. All experiments on animals have been reviewed and approved by the local ethical review panel of Imperial College, London, and by the UK Home Office.

Quantification of NE cell surface expression/release *in vitro*

Neutrophils were isolated from the bone marrow of WT and NE$^{-/-}$ mice as detailed previously (Young *et al.*, 2004; Wang *et al.*, 2005). Briefly, femurs from two mice per group were removed and flushed with sterile Hank's balanced salt solution. Neutrophils were purified using a discontinuous Percoll gradient and then washed before being resuspended in modified PBS containing 0.25% bovine serum albumin (BSA), 5 mM glucose, 1 mM Ca^{2+}/Mg^{2+}. The cell suspension purity was assessed by Kimura's stain and found to be typically >80% (contaminating cells consisting of mononuclear cells). For performing the assays, pooled neutrophils were added to BSA-coated (1 μg ml^{-1}) 96-well plates (5 × 10^5 neutrophils per well) and were allowed to adhere for 15 min at 37°C before addition of stimuli. LTB$_4$, PAF, the chemokine KC (CXCL1; considered to be a murine homologue of human IL-8/CXCL8) or modified PBS (control) was added to neutrophils and the plates were then incubated at 37°C for a further 30 min period. The plates were then centrifuged to allow for removal of supernatants for subsequent analysis of released elastase. The pelleted cells were then fixed using 3% paraformaldehyde/0.5% glutaraldehyde and finally resuspended in Tris buffer (0.2 M Tris base, 0.15 M NaCl, 0.02 M CaCl$_2$ at pH8.5) for assay of cell surface NE as described

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previously (Owen et al., 1995; Young et al., 2004). Enzyme activity was assayed using a fluorogenic substrate specific for elastase, methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethyl coumarin (MeoSuc-Ala-Ala-Pro-Val-AFC), as described by Owen et al. (1995). This substrate has previously been shown not to access intracellular stores of NE and hence, using fixed intact cells (to retain cell-associated NE), this substrate can be used to quantify cell-bound enzyme activity. Briefly, fixed intact cells, cell lysates (obtained following addition of 1% of Triton X-100 to cell pellets) or purified human NE (used to establish a standard curve) were incubated with 400 μM substrate in 0.2 M tris(hydroxymethyl)-aminomethane (pH 8.5) containing 0.15 M NaCl and 0.02 M CaCl$_2$. After incubation at 37°C for 25 min in the dark, the level of liberated 7-amino-4-trifluoromethyl coumarin was quantified in a fluorescent plate reader (Millipore, Watford, UK) using excitation 409 nm and emission 530 nm. A standard curve, incorporated into each experiment, was established using commercially obtained purified human NE and was used to represent the results in terms of murine neutrophil enzyme activity equivalent to the activity detected in a known concentration (ng ml^{-1}) of purified human NE.

Chemotaxis assay

Purified neutrophils (25 μl of 4×10^6 cells ml^{-1} solution) were placed on the top compartment of 3 μm diameter pore filtered chemotaxis chambers (NeuroProbe, Gaithersburg, MD, USA). The bottom wells contained 28 μl of PBS supplemented with 0.25% of BSA, 1 mM of Ca^{2+}/Mg^{2+} and 5 mM of glucose in the presence or the absence of a range of concentrations of LTB$_4$, PAF or KC. The chambers were then incubated at 37°C in a humidified atmosphere (5% CO$_2$) for 4 h. Supernatant from the upper chambers (containing the non-transmigrated cells) was removed, the plates spun and the lower chamber supernatants harvested to count the number of migrated cells by microscopy using a Neubauer hematocytometer. Results are expressed as the number of transmigrated cells/chemotaxis well.

Intravital microscopy of murine cremasteric venules

Intravital microscopy was used to observe leukocyte responses within mouse cremasteric venules as previously described (Thompson et al., 2001). Briefly, after induction of anaesthesia with ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) by intraperitoneal injection, the carotid and jugular vein were cannulated for measurement of mean arterial pressure and the administration of drugs, respectively. Animals were maintained at 37°C on a custom-built heated Perspex microscope stage and the cremaster muscle was surgically exteriorized. The tissue was kept warm and moist throughout each experiment by superfusion of warmed Tyrode's balanced salt solution and leukocyte-endothelial cell interactions were observed on an upright fixed-stage microscope (Axioscop FS, Carl Zeiss, Welwyn Garden City, UK) fitted with water immersion objectives. Basal readings of leukocyte firm adhesion and transmigration were then quantified for 15 min at 5 min intervals, while the tissue was superfused with Tyrode's balanced salt

solution. At time 0, LTB$_4$ (10^{-8} M or 10^{-7} M), PAF (10^{-7} M) or KC (10^{-8} M) was added to the superfusion buffer and readings taken at 10–15 min intervals for 60 min. Some mice received the elastase inhibitor, ONO-5046 (Kawabata et al., 1991), the serine protease inhibitor, aprotinin, or the vehicle, saline, by a cannula inserted into the jugular vein. Owing to the short plasma half-life of these drugs, both were administered first as a bolus (50 mg kg^{-1} 200 μl^{-1} for ONO-5046; 100 000 KIU kg^{-1} 200 μl^{-1} for aprotinin) at $t = -10$ min and then as a continuous infusion (50 mg kg^{-1} h^{-1} for ONO-5046; 100 000 KIU kg^{-1} h^{-1} for aprotinin) until the end of the experiment. The dosing regimes employed were selected based on advice from manufacturers/suppliers and also based on previous experiments from our group (Wang et al., 2005, 2006). Firmly adherent leukocytes were considered as those remaining stationary for at least 30 s within a given 100 μm vessel segment. Transmigrated leukocytes were quantified as those in the extravascular tissue within 50 μm of the 100 μm vessel segments quantified. In each animal, all parameters were quantified in several vessel segments (4–5), within multiple venules (3–5) and averaged.

Confocal microscopy

Cremaster muscles were dissected away from mice, fixed in 4% paraformaldehyde overnight and immunostained for components of venular walls to localize position of leukocytes, as observed by confocal microscopy, as detailed previously (Wang et al., 2005). Briefly, following a blocking/permeabilization step in PBS supplemented with 20% FCS serum and 0.5% Triton X-100 for 1 h at room temperature, tissues were incubated with primary antibodies against the endothelial cell basement membrane component laminin 10 (anti-laminin α5 chain polyclonal antibody 405, gift from Professor LM Sorokin, University Waldeyerstrasse, Muenster, Germany) (Sorokin et al., 1997) and the cytosolic neutrophil-specific protein MRP-14 (rat anti-mouse MRP-14 clone 2B10, gift from Dr Nancy Hogg, Cancer Research UK, London, UK) (Newton and Hogg, 1998) at room temperature, overnight. The samples were then incubated with goat anti-rabbit and goat anti-rat secondary antibodies directly conjugated to Alexa Fluor 488 and 633, respectively (Invitrogen, Paisley, UK) at room temperature, for 3 h. Following washes in PBS and a second blocking step in PBS supplemented with 20% FCS for 2 h at room temperature, a third immunostaining step was added using APC-conjugated primary antibody against the endothelial junctional molecule PECAM-1 (rat anti-mouse PECAM-1 clone Mec13.3, BD-Pharmingen, Oxford, UK) for 3 h. In all studies, appropriate control antibodies were used in parallel with the specific primary antibodies. Samples were viewed using a Zeiss LSM 5 PASCAL confocal laser-scanning microscope to detect the position of leukocytes in the vessel wall of immunostained tissues.

Statistical analysis

All results are expressed as mean $\pm$ s.e.m. Statistical significance was assessed by one-way analysis of variance with Neuman–Keuls multiple comparison test. Where two

variables were analysed a Student's *t*-test was used. $P < 0.05$ was considered significant.

Reagents

The following reagents were obtained commercially: ketamine (Ketalar, Parke-Davis, Eastleigh, UK), xylazine (Rompun, Bayer, Bury St Edmunds, UK), methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethyl coumarin (MeO-Suc-Ala-Ala-Pro-Val-AFC) (Enzyme Systems Products, Livermore, CA, USA), human NE (Merck Biosciences, Nottingham, UK). All other general reagents were purchased from Sigma-Aldrich (Poole, UK). The synthetic and specific NE inhibitor ONO-5046 (Sivelestat) (Kawabata *et al.*, 1991) was a kind gift from ONO Pharmaceuticals (Osaka, Japan).

Results

LTB$_4$ is a potent inducer of murine neutrophil cell surface expression of NE *in vitro*

For NE to participate in leukocyte migration it must be mobilized from its intracellular azurophil granule stores to the cell surface or released locally into the extracellular environment in response to appropriate stimuli. To investigate the ability of a number of neutrophil chemoattractants to induce NE release and/or cell surface NE expression, an *in vitro* assay for detection of murine NE activity was developed as described previously (Young *et al.*, 2004). The fluorogenic NE-specific substrate employed, methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethyl coumarin (MeOSuc-Ala-Ala-Pro-Val-AFC), has previously been shown not to access intracellular stores of NE (Owen *et al.*, 1995), and hence when using fixed intact cells (to retain cell-associated NE), the substrate can be used to quantify cell surface activity by quantifying the level of liberated 7-amino-4-trifluoromethyl coumarin. As this assay was originally developed for detection of human NE activity (Owen *et al.*, 1995), its specificity for detection of murine NE activity was confirmed by the absence of detectable activity in lysates of neutrophils from NE-deficient mice and inhibition of activity by a specific NE inhibitor, ONO-5046 (Figure 1a and b).

The agonists KC (CXCL1), PAF and LTB$_4$ were investigated for their ability to stimulate NE release/cell surface expression using murine neutrophils, purified from bone marrow. Figure 2a shows that with respect to both NE release and cell surface expression, LTB$_4$ was more potent and efficacious than the other stimuli tested. Furthermore, LTB$_4$ appeared to be more effective at eliciting cell surface expression of NE as opposed to inducing NE release and indeed in some studies, LTB$_4$ was found to induce a small level of cell-associated NE at concentrations of less than 10^{-12} M (data not shown). Interestingly, LTB$_4$ was also found to be the more potent and efficacious stimulus when tested in a chemotaxis assay (Figure 2b). Specifically, LTB$_4$ caused significant leukocyte migration through Boyden chamber filters at all concentrations investigated in the range of 10^{-9}–10^{-7} M, while a significant response to KC was only noted at 10^{-8} and 10^{-7} M and PAF failed to elicit a detectable chemotaxis response within the concentrations tested (10^{-10}–10^{-7} M).
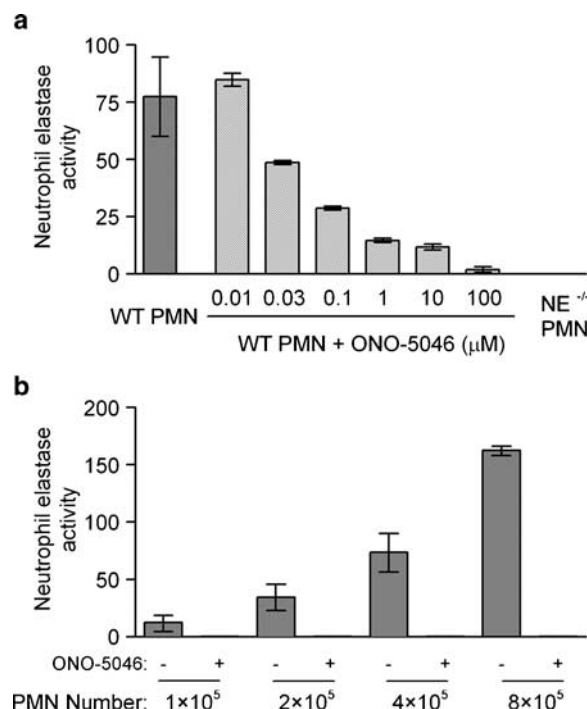

Figure 1 Characterization of an *in vitro* assay for detection of murine neutrophil elastase (NE) activity. Neutrophils from wild-type (WT) and NE$^{-/-}$ mice were purified from bone marrow as described in Materials and methods, seeded onto bovine serum albumin (BSA)-coated plates and lysed with 1% Triton X-100. Following centrifugation, supernatants were harvested and incubated for 25 min at 37°C with 400 μM of a fluorogenic NE-specific substrate (MeOsuc-Ala-Ala-Pro-Val-AFC). Murine NE activity was detected using a fluorescent plate reader (excitation: 405 nm, emission: 530 nm) and compared with a standard curve established using commercially available purified human NE. In some experiments, ONO-5046 (Sivelestat), a specific NE inhibitor, was added to the wells at the concentrations indicated. Results are expressed as murine NE activity equivalent to the activity detected from ng ml^{-1} of purified human NE. (**a**) The total amount of murine NE activity expressed by 4×10^5 lysed neutrophils from WT, ONO-5046-treated WT and NE$^{-/-}$ mice. (**b**) The increasing amount of NE activity relative to the number of neutrophils lysed, in the absence or presence of the NE inhibitor ONO-5046. Graphs shown are representative of $n = 3$ experiments (two mice used per experiment).

LTB$_4$ is more effective than PAF and KC in inducing leukocyte transmigration through mouse cremasteric venules *in vivo*

Following on from the above *in vitro* results, we next sought to compare the abilities of LTB$_4$, PAF and KC in inducing leukocyte migration *in vivo*. The *in vivo* model employed was leukocyte migration through mouse cremasteric venules, as induced by topical stimuli and observed by intravital microscopy. Topical application of LTB$_4$ or PAF (both at 10^{-7} M) induced significant and comparable leukocyte firm adhesion responses as compared to the small levels detected following topical application of Tyrode solution (Figure 3a). Interestingly, in the same studies, LTB$_4$-induced leukocyte transmigration was more pronounced than the transmigration response observed following topical PAF (Figure 3a). Similarly, while topical LTB$_4$ and KC (10^{-8} M used for both stimuli due to limited availability of the chemokine KC) induced equivalent levels of leukocyte firm adhesion, LTB$_4$

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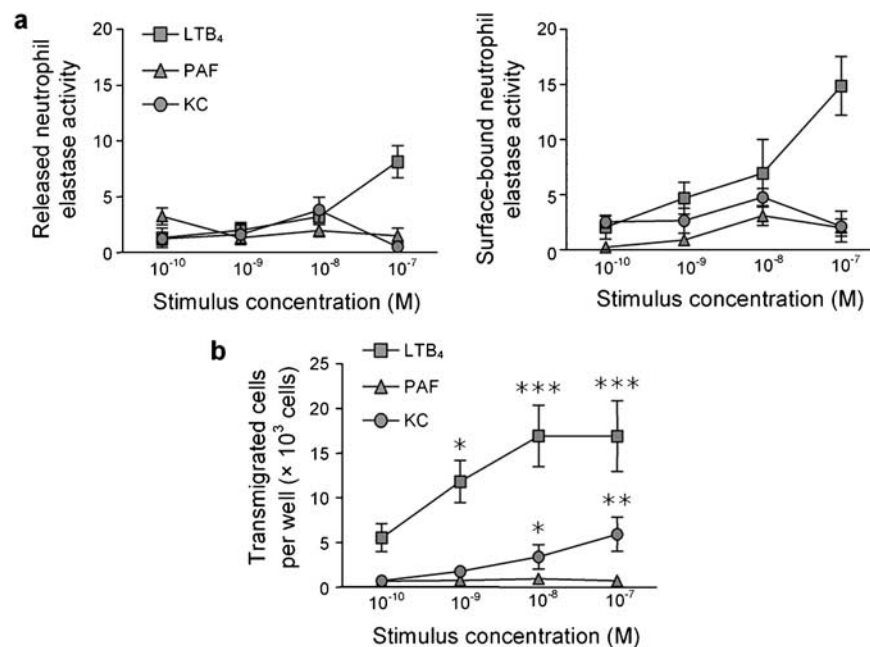RE Young *et al*

Figure 2 Effect of soluble chemoattractants on cell surface expression and release of NE and on chemotaxis *in vitro* of murine neutrophils. Neutrophils were isolated from wild-type mouse bone marrow and differential counts performed to enable addition of 5×10^5 neutrophils per well to bovine serum albumin (BSA)-coated 96-well plates as detailed in Materials and methods. Cells were incubated in plates for 15 min before addition of LTB$_4$, PAF and KC (10^{-10}–10^{-7} M) for 30 min. Plates were spun and supernatants collected for assay of NE activity before the pelleted cells were fixed. Cells or supernatants were then incubated for 25 min at 37°C with 400 μM of the NE-specific fluorogenic substrate. NE activity was detected using a fluorescent plate reader and compared with a standard curve created using purified human NE as detailed in Materials and methods. (**a**) Stimulated released (left-hand graph) and cell surface expression of NE (right-hand graph). All data have been corrected for the small level of NE activity from unstimulated samples (3.61 and 3.20, released and cell surface NE, respectively). Results are from $n = 6$–8 separate experiments. Significant released NE activity above basal levels were detected for LTB$_4$ at 10^{-8} M ($P<0.01$) and 10^{-7} M ($P<0.001$) and for PAF at 10^{-8} M ($P<0.05$). Significant cell surface NE expression above basal levels were detected for LTB$_4$ at 10^{-8} M ($P<0.01$) and 10^{-7} M ($P<0.001$) and for both PAF and KC at 10^{-8} M ($P<0.01$). For clarity, significant differences are not indicated on graphs by asterisks. (**b**) Stimulated murine neutrophil chemotactic responses. Bone marrow neutrophils (1×10^5 cells) were placed in the top wells of 3 μm filter 96-transwell NeuroProbe chemotaxis chambers and increasing concentrations of the chemoattractants LTB$_4$, PAF and KC (or medium alone as control) were added to the bottom wells. The chambers were then incubated for 4 h at 37°C after which the number of cells in the bottom wells was counted as detailed in Materials and methods. Results are from $n = 3$–10 separate experiments and all data have been corrected for the responses seen in control chambers (548 cells per well). Significant statistical differences from control samples are indicated by asterisks, *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

again elicited a significantly greater leukocyte transmigration response (Figure 3b).

Leukocyte transmigration through LTB$_4$-stimulated cremasteric venules is inhibited in mice treated with a specific NE inhibitor but not in NE-deficient mice

Since LTB$_4$ was found to be a highly potent inducer of surface expression of NE and inducer of leukocyte transmigration *in vivo*, we next investigated the potential role of NE in LTB$_4$-induced leukocyte migration through mouse cremasteric venules using a specific NE inhibitor ONO-5046 and NE-deficient mice. In these studies, leukocyte transmigration was elicited by topical 10^{-7} M LTB$_4$.

Treatment of WT mice with ONO-5046 (50 mg kg^{-1} loading dose; 50 mg kg^{-1} h^{-1} infusion) had no inhibitory effect on LTB$_4$-induced leukocyte adhesion, but caused significant inhibition of LTB$_4$-elicited leukocyte transmigration with a 66% inhibition at 60 min (Figure 4a). In contrast to the effects seen with the NE inhibitor in WT mice, no significant inhibition in leukocyte responses induced by LTB$_4$ was seen in NE$^{-/-}$ mice as compared to WT animals (Figure 4b).

NE-deficient mice appear to exhibit compensatory mechanisms in regulation of leukocyte transmigration

To extend the above findings, we initially sought to investigate the potential specificity of the ONO-5046 compound as an NE inhibitor through its testing in NE$^{-/-}$ mice. Interestingly, in contrast to the inhibitory effects seen in WT animals, ONO-5046 had no inhibitory effects on LTB$_4$-induced leukocyte transmigration in NE$^{-/-}$ mice, indicating the specificity of the compound in the present model (Figure 5). Since the lack of effect of the NE inhibitor suggested the existence of potential compensatory mechanisms in the NE-deficient animals, this was further investigated by testing the effect of the broad spectrum serine protease inhibitor aprotinin in both WT and NE$^{-/-}$ mice. For this purpose, using a dosing protocol extrapolated from that previously used in a rat model (Asimakopoulos *et al.*, 2000; Pruefer *et al.*, 2002), aprotinin (loading dose: 100 000 KIU kg^{-1} and infusion: 100 000 KIU kg^{-1} h^{-1}) was administered in the same manner as ONO-5046. In contrast to the findings with ONO-5046, aprotinin inhibited LTB$_4$-induced leukocyte transmigration (Figure 5), but not firm adhesion (not shown), in both WT and NE$^{-/-}$ mice with an inhibition of 59.6 and 79.2%, respectively. Collectively these
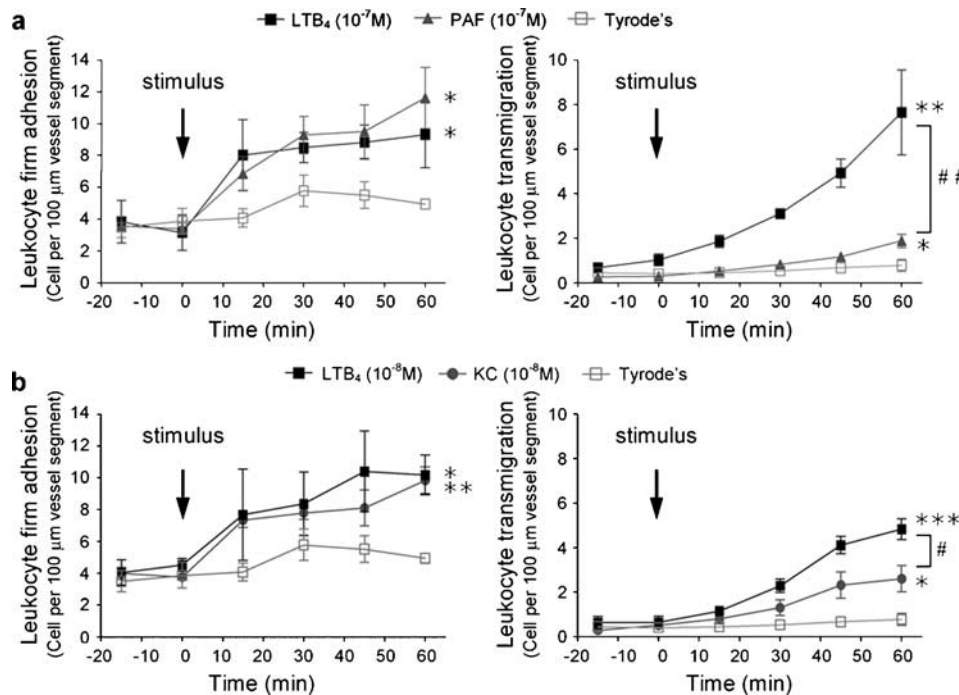
Figure 3 Effect of topical LTB$_4$, PAF and KC on leukocyte responses in mouse cremasteric venules *in vivo*. Leukocyte firm adhesion (left-hand panels) and transmigration (right-hand panels) in mouse cremasteric venules in response to topical stimuli were investigated by intravital microscopy. The mouse cremaster muscle was surgically exteriorized, superfused with Tyrode's solution and basal leukocyte responses were quantified for 20 min, as detailed in Materials and methods. (**a**) LTB$_4$ and PAF (both at 10^{-7} M) were applied topically at $t = 0$ min to the exteriorized cremaster muscles and leukocyte responses were quantified at regular intervals for 60 min. (**b**) LTB$_4$ and KC (both at 10^{-8} M) were applied topically at $t = 0$ min to the exteriorized cremaster muscles and leukocyte responses were quantified at regular intervals for 60 min. Statistically significant differences between Tyrode's and stimulus-treated groups are shown by asterisks; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Additional statistical differences are indicated by lines and hatched symbols, #$P < 0.05$, ##$P < 0.01$ ($n = 4$ mice per group).

results suggest that as employed, the NE inhibitor used in the present studies, ONO-5046, is a specific NE inhibitor and that the NE-deficient mice appear to exhibit compensatory mechanisms through involvement of alternative serine proteases in the process of leukocyte transmigration elicited by topical LTB$_4$.

The NE inhibitor ONO-5046 inhibits LTB$_4$-induced neutrophil transmigration at the level of the perivascular BM

In a final series of experiments to delineate the stage in leukocyte transmigration at which the NE inhibitor ONO-5046 inhibited neutrophil transmigration, selected cremaster muscle tissues were further analysed by confocal microscopy. For this purpose, tissues were immunofluorescently stained with antibodies against PECAM-1 (as a marker for endothelial cells), laminin 10 (as a marker for venular basement membrane) and MRP-14 (as a marker for neutrophils) before being observed using a confocal microscope (Figure 6a), as detailed previously (Wang *et al.*, 2005). Analysis of 1 μm optical sections running through whole intact venules from at least four random sections per tissue indicated an increased number of leukocytes within venular walls (that is, between endothelial cells and the venular basement membrane) in tissues from animals treated with ONO-5046 (56.8% increase; Figure 6b). Since suppression of LTB$_4$-induced leukocyte transmigration by the NE inhibitor ONO-5046 was associated with an increase in number

of leukocytes trapped in the venular wall, the findings suggest that treatment of mice with the NE inhibitor can suppress neutrophil transmigration at the level of the perivascular BM.

Discussion and conclusions

The role of leukocyte proteases in leukocyte transmigration remains a contentious issue (Shapiro, 2002; Yadav *et al.*, 2003). Previous studies from our group have provided evidence to indicate a role for the serine protease NE in regulation of neutrophil transmigration in response to zymosan particles and IL-1β (Young *et al.*, 2004; Wang *et al.*, 2005, 2006), though the mechanisms through which NE mediated these responses appeared very different. With respect to zymosan particles, NE played a role in generation of endogenous inflammatory mediators and regulation of phagocytosis of zymosan particles (Young *et al.*, 2004). With respect to neutrophil transmigration elicited by IL-1β, NE appeared to mediate migration of neutrophils through the venular basement membrane (Wang *et al.*, 2005, 2006). To extend these studies further, we have now investigated the role of NE in regulation of leukocyte transmigration as elicited by the potent chemoattractant LTB$_4$ (Ford-Hutchinson *et al.*, 1980; Bray *et al.*, 1981). LTB$_4$ was chosen as the inflammatory mediator to be investigated as initial *in vitro* studies indicated its potent ability to induce cell
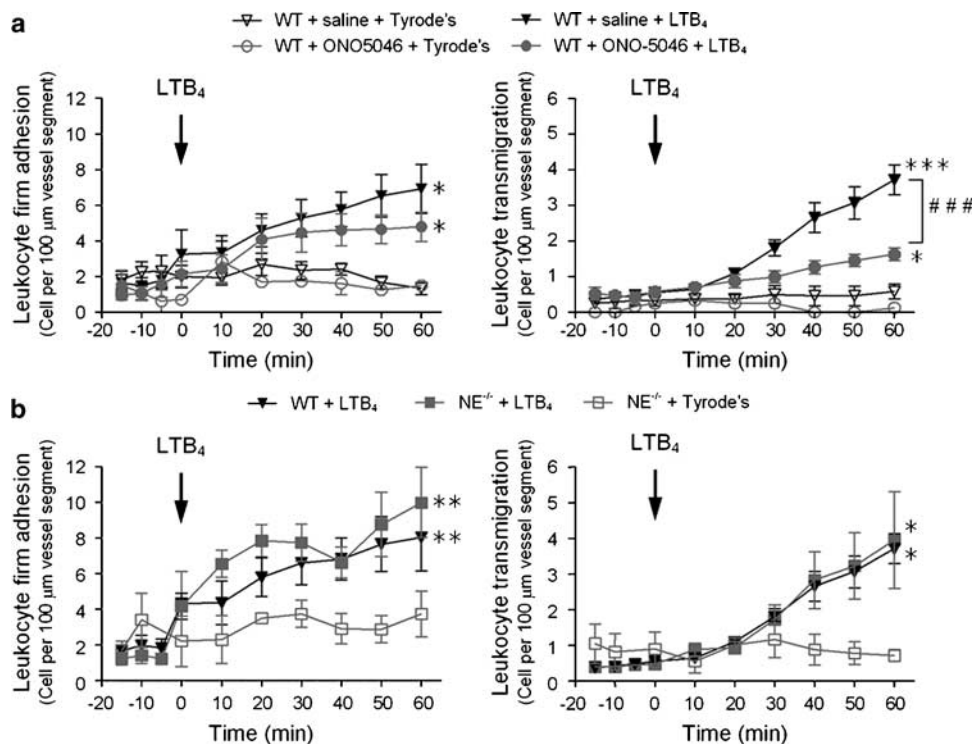
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Figure 4 Effect of pharmacological inhibition and genetic deletion of neutrophil elastase (NE) on LTB$_4$-induced leukocyte responses in mouse cremasteric venules. The mouse cremaster muscle was surgically exteriorized for investigations by intravital microscopy and leukocyte firm adhesion and transmigration were quantified following topical application of LTB$_4$ (10^{-7} M), as detailed in Materials and methods. (**a**) Mice were treated intravenous with a bolus injection followed by a continuous infusion of ONO-5046 (Sivelestat), a specific NE inhibitor (50 mg kg^{-1} h^{-1}) or with saline as control, before topical application of LTB$_4$. (**b**) Leukocyte responses elicited by topical LTB$_4$ in wild-type (WT) and NE$^{-/-}$ mice were compared. Results are from $n = 5$–10 mice per group. Statistically significant differences between Tyrode's- and LTB$_4$-treated groups are shown by asterisks; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Additional statistical differences are indicated by lines and hatched symbols, $^{\#\#\#}P < 0.001$.

surface expression of NE on murine neutrophils. Furthermore, LTB$_4$ was found to be a highly efficacious stimulus at eliciting neutrophil migration through mouse cremasteric venules as observed in real-time by intravital microscopy. This response was selectively suppressed at the level of transmigration (apparently at the level of the venular basement membrane) by a specific NE inhibitor. Interestingly, no such defect was noted in NE-deficient mice and evidence was obtained to suggest the existence of compensatory mechanisms in these animals. Collectively, the findings demonstrate the potent ability of LTB$_4$ to induce cell surface expression of NE on neutrophils and provide further evidence to suggest a role for NE in regulation of neutrophil migration *in vivo*.

NE is a serine protease that is stored within azurophil granules of neutrophils at high concentrations (Shapiro, 2002). Traditionally, enzymes of this class are believed to contribute primarily to intracellular degradation of microorganisms within phagolysosomes, serine proteases can also exert a wide range of extracellular proteolytic activities (Pham, 2006). For this to occur, enzymes such as NE need to be mobilized to the cell surface or released into the extracellular environment. With respect to the latter, released enzymes can be rapidly inhibited by plasma protease inhibitors present in the extracellular environment to protect against proteolytic tissue damage to the host

(Owen and Campbell, 1999). In contrast, cell-associated enzymes appear to be protected from extracellular inhibitors (Owen and Campbell, 1999) and hence could contribute to the regulation of inflammatory and immune responses through degradation, regulation of activity and/or bioavailability of specific proteins (Pham, 2006). In this context, Owen *et al*. (1995, 1997) have previously demonstrated that inflammatory stimuli, such as PAF, fMLP and TNFα, can induce cell surface expression of NE on human neutrophils *in vitro*. Since we hypothesized that cell surface-associated NE may play a role in regulating neutrophil transmigration through venular walls, we first sought to investigate the ability of certain chemoattractants in inducing cell surface expression of the enzyme. For this purpose, we adapted an assay developed previously by Owen *et al*. (1995) for measuring cell surface-associated NE activity on human neutrophils for quantification of released/cell surface-associated murine NE. The specificity of the assay was confirmed by the lack of activity in lysates of NE-deficient murine neutrophils and also in lysates of WT neutrophils treated with the specific NE inhibitor ONO-5046 (Sivelestat) (Kawabata *et al*., 1991). Using this assay, NE activity, both released and cell associated, following stimulation of bone marrow-derived murine neutrophils with LTB$_4$, PAF and KC (CXCL1) was quantified. The findings indicated that PAF- and KC-stimulated cells exhibited very low levels of NE release/cell
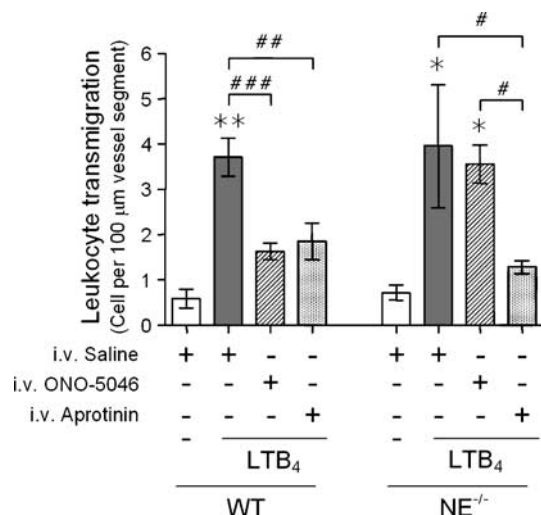
Figure 5 Effect of aprotinin on LTB$_4$-induced leukocyte responses in cremasteric venules of wild-type (WT) and neutrophil elastase (NE$^{-/-}$) mice. The cremaster muscle of WT and NE$^{-/-}$ mice was surgically exteriorized for analysis of LTB$_4$-induced leukocyte transmigration by intravital microscopy as detailed in Materials and methods. Mice were pretreated with either intravenous saline (control), the NE inhibitor ONO-5046 or the broad-spectrum serine protease inhibitor aprotinin (see Materials and methods for dosing regime) before the topical application of LTB$_4$ (10^{-7} M). Control mice were treated with topical Tyrode's solution. The graph shows leukocyte transmigration responses quantified at 60 min after application of LTB$_4$. Data are from $n = 6$ mice per group. Statistically significant differences between Tyrode's- and LTB$_4$-treated mice are shown by asterisks; *$P < 0.05$ and **$P < 0.01$. Additional statistical comparisons are indicated by lines and by hatched symbols; #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$.

surface expression and were also very weak at eliciting murine neutrophil chemotaxis. In contrast, in line with its potent chemotactic properties, LTB$_4$ was a very potent inducer of cell surface expression of NE for murine neutrophils (significant responses detected at 10^{-9} and 10^{-8} M, respectively). Of interest, at all concentrations that induced degranulation, LTB$_4$ was more effective at inducing cell surface expression of NE as opposed to the release of the enzyme. To our knowledge this is the first report on the potent ability of LTB$_4$ to induce cell surface expression of NE on murine neutrophils. Since there is evidence to show that transmigrating neutrophils express NE on their cell surface in vitro (Cepinskas et al., 1999) and in vivo (Wang et al., 2005), we next investigated the role of NE in LTB$_4$-induced neutrophil transmigration in vivo.

LTB$_4$-induced neutrophil transmigration in vivo was investigated in the murine cremaster muscle as observed directly by intravital microscopy and the functional role of NE in this response was investigated using both a selective NE inhibitor, ONO-5046 (Sivelestat) and NE-deficient mice. Topical application of LTB$_4$ (10^{-7} M) induced rapid leukocyte firm adhesion and transmigration (significant at 10–20 min and 20–40 min after application of LTB$_4$, respectively) within mouse cremasteric venules. Treatment of WT mice with the NE-inhibitor had no effect on LTB$_4$-induced leukocyte firm adhesion but significantly suppressed the associated leukocyte transmigration response (66% inhibition at 60 min after
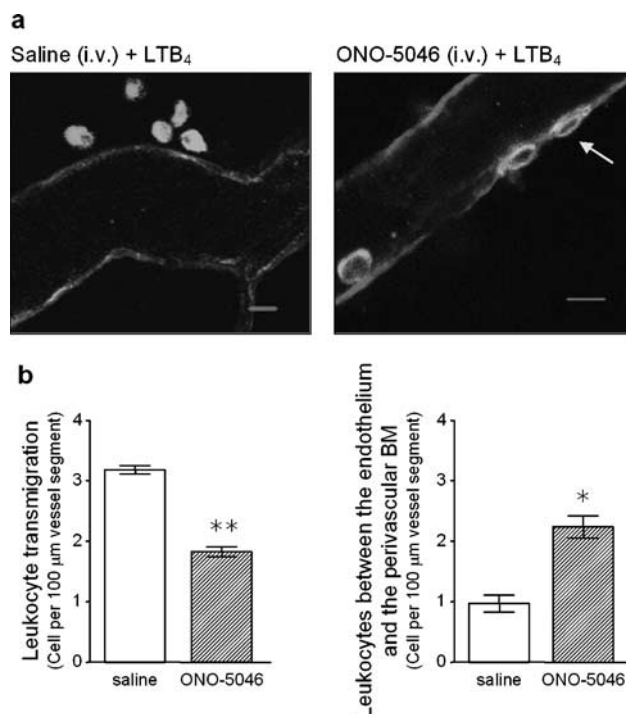

Figure 6 Analysis of the site of arrest of leukocytes in mice treated with the neutrophil elastase (NE) inhibitor. Cremaster muscles from wild-type (WT) mice treated with intravenous saline (control) or with the NE inhibitor ONO-5046 (see Materials and methods for dosing regime) and stimulated with topical LTB$_4$ (10^{-7} M) were dissected away from mice for immunostaining and analysis by confocal microscopy. Briefly, tissues were fixed in paraformaldehyde and immunostained overnight by using primary antibodies directed against mouse laminin 10 (as a marker for the endothelial cell basement membrane; red), PECAM-1 (as a marker for endothelial cells; green) and MRP14 (as a marker for neutrophils; blue), as detailed in Materials and methods. Samples were analysed by confocal microscopy as detailed in Materials and methods. (**a**) The figure shows representative images of postcapillary venules from saline-treated (left) or ONO-5046-treated mice (right) captured by confocal microscopy. While the image from the saline-treated mouse shows several leukocytes in the extravascular tissue, the tissue from ONO-5046-treated mouse shows clear evidence of leukocytes trapped within the vessel wall. (**b**) The graphs show quantification of leukocytes transmigrated into the surrounding tissue (left-hand graph) or trapped within the vessel wall (right-hand graph), as observed by confocal microscopy. Leukocyte transmigration (left panel) was quantified as number of cells in the tissue within 50 μm of the vessel wall across a 100 μm vessel segment. Within the same vessel segments, the number of leukocytes trapped between the basement membrane and the endothelial cells (right panel), following LTB$_4$ stimulation (60 min), was quantified. Results represent a mean of 5–8 tissues analysed and a significant difference between saline- and ONO-5046-treated groups is indicated by asterisks; *$P < 0.05$ and **$P < 0.01$. (For colour figure see online version.)

application of LTB$_4$). In contrast, no defect in leukocyte transmigration (or adhesion) was noted in NE-deficient animals. To ensure that the NE inhibitor employed was specific for NE in our model, the effect of ONO-5046 was also tested in the NE-deficient mice and no inhibitory effects noted, strongly suggesting its specificity for NE in the present model. Collectively, the inhibitory effect of ONO-5046 on LTB$_4$-induced neutrophil transmigration in WT but not NE-deficient mice and the normal leukocyte transmigra-

tion response observed in NE-deficient animals suggest (i) a role for NE in LTB$_4$-induced transmigration and (ii) the NE-deficient mice exhibit compensatory mechanisms. The former is further supported by the fact that stimuli that were weak at inducing cell surface expression of NE on mouse neutrophils (PAF and KC) also elicited lower levels of neutrophil transmigration *in vivo*. Evidence for the latter possibility was obtained in studies showing that the broad-spectrum serine protease inhibitor aprotinin suppressed LTB$_4$-induced leukocyte transmigration in both WT- and NE-deficient mice. The normal leukocyte migration response observed in NE-deficient animals in the present study is in line with previous work using this mouse strain where no defect in leukocyte migration was noted in numerous inflammatory models, for example neutrophil migration was elicited by bacterial infections (Belaaouaj *et al*., 1998), the non-specific inflammatory stimulus thioglycollate (Tkalcevic *et al*., 2000) and the cytokines IL-1β and TNFα (Young *et al*., 2004). Overall such findings suggest that NE does not play a critical role in mediating neutrophil transmigration in certain inflammatory models and that its functional role maybe compensated for by other molecules/mechanisms. Nonetheless, our findings do indicate that selective inhibition of NE does lead to suppression of neutrophil transmigration and so in a final series of experiments the precise site of arrest of the neutrophils under conditions of NE blockade was investigated.

To analyse the site of arrest of LTB$_4$-stimulated emigrating neutrophils under conditions of NE inhibition, cremasteric tissues from control and ONO-5046-treated mice were immunostained with markers for endothelium, venular laminin and neutrophils and analysed by confocal microscopy. The results demonstrated that in ONO-5046-treated animals, suppression of neutrophil transmigration into the extravascular tissue was directly in line with an increase in the number of neutrophils trapped within the venular wall, suggesting a blockade at the level of the perivascular basement membrane. These results are in agreement with our previous findings indicating a role of NE in IL-1β-induced neutrophil migration through the basement membrane of cremasteric venules (Wang *et al*., 2005, 2006). Specifically, our previous results suggested that NE regulates neutrophil migration through permissive regions within certain components of the endothelial cell basement membrane (for example, laminin 10 and collagen IV), possibly via a contribution to the remodelling of these regions during the transmigration response (Wang *et al*., 2006). Although the precise mechanism by which NE may regulate such an event remains unclear, it is potentially possible that a similar phenomenon may contribute to the process of neutrophil transmigration as elicited by LTB$_4$ in the present investigation. Of relevance, numerous *in vitro* studies have previously demonstrated the ability of this enzyme to degrade components of basement membranes (Heck *et al*., 1990; Delclaux *et al*., 1996; Delacourt *et al*., 2002; Shapiro, 2002; Wang *et al*., 2006). The broad substrate specificity and biological functions of NE does, however, strongly suggest that alternative modes of action of NE, for example, regulation of cell surface receptors, adhesion molecules and other enzymes as well as regulatory effects

on intracellular signalling and cytoskeletal rearrangement (Pham, 2006), may also play a role in NE-dependent neutrophil transmigration.

In summary, the findings show that LTB$_4$ is a potent inducer of cell surface expression of NE on murine neutrophils and that NE plays a role in mediating LTB$_4$-induced neutrophil transmigration through the venular wall at the level of the endothelial cell basement membrane. While the mechanism through which NE regulates neutrophil transmigration remains unclear, the findings of this study highlight the need for further investigations into this unresolved aspect of leukocyte biology. Furthermore, the findings contribute to the growing list of functional discrepancies observed when studying molecules under conditions of pharmacological blockade as compared to genetic deletion.

Acknowledgements

Conflict of interest

The authors state no conflict of interest.

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