

CD44 cross-linking induces protein kinase C-regulated migration of human T lymphocytes

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Abstract

The cell surface receptor CD44 is widely implicated in leukocyte migration to inflammatory sites. In this study, the responses of human T cells following cross-linking of CD44 were examined. We demonstrate that engagement of CD44 using immobilized mAbs or hyaluronan-enriched extracellular matrix lattices induces active migration in T lymphocytes accompanied by cycles of cytoskeletal rearrangement and cell polarization. We have investigated the functional impact and subcellular localization of protein kinase C (PKC) isoenzymes, β and δ , previously shown by our group to be involved in active T cell locomotion induced by leukocyte function-associated antigen-1 (LFA-1) integrin receptors. PKC β was associated with the centrosome and the microtubule-rich tail of the polarized cell and PKC δ was predominantly located about the region of the microtubule organizing center. A selective pharmacological inhibitor of classical PKC isoforms, Gö6976, suppressed lymphocyte polarization and migration following CD44 ligation. Selective targeting of PKC δ using the pharmacological inhibitor rottlerin or a pseudosubstrate-blocking peptide reduced CD44-activated cell migration but did not completely ablate it. Our data demonstrate that ligation of CD44 induces phenotypic changes, cytoskeletal rearrangements and redistribution of PKC isoforms β and δ , resulting in cell migration, as previously described for the cell surface receptor, LFA-1. This suggests potential convergence of intracellular signaling pathways induced via CD44 and LFA-1 integrin.

Introduction

Efficient operation of the adaptive immune system requires the continuous migration of T lymphocytes from circulation and across the endothelium into tissue and back to blood again, either at lymphoid organs or at sites of inflammation. This process is characterized by initial tethering of the lymphocyte to the surface of specialized endothelial cells (1), which is thought to be mediated largely by selectin interaction with their carbohydrate ligands expressed on the endothelium surface, although other lymphocyte receptors have been implicated in this process (2–4). This slows lymphocyte flow and cells roll along the surface of the endothelium until chemokines activate molecules of the integrin family on T cells, leading to lymphocyte arrest and subsequent diapedesis across the endothelium. Here, we examine T cell migration induced by cross-linking of the CD44 receptor, a glycoprotein previously ascribed a role in lymphocyte homing and inflammation (2, 5–7).

CD44 is a broadly distributed transmembrane glycoprotein that is involved in a number of normal processes, such as regulation of growth, survival, differentiation and motility, whereas its altered expression or dysfunction may contribute to malignancy; for example, expression of CD44 variants can mean a poor prognosis in colorectal carcinomas [reviewed in Ponta *et al.* (8)]. This receptor has also been associated with several functions of the normal immune system. Expression is increased following antigen stimulation of the TCR (9), and CD44 is reported to be co-stimulatory for T cell activation (10). The role of CD44 in lymphocyte homing to sites of inflammation was clearly demonstrated by Camp *et al.* (5), who showed that antibody-induced loss of CD44 slowed the onset of a cutaneous delayed-type hypersensitivity response following antigen challenge in a murine model, although migration to lymphoid sites was normal. The CD44-negative, unactivated

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lymphocytes migrate identically to cells from control mice, but following challenge, the response is altered, with lymphocyte extravasation to inflammatory sites delayed. Similarly, a study using murine CD44^{-/-} T cells confirmed that receptor expression was not required for normal lymph node homing but the migration of CD44-deficient cells into areas of inflammation was delayed (11). A role for CD44 in the pathogenesis of inflammatory disease has been implicated by a number of studies. The level of circulating T cells expressing activated CD44 was found to be elevated in patients with autoimmune diseases, such as systemic lupus erythematosus and arthritis (12). Administration of anti-CD44 antibodies inhibited inflammation in murine models of inflammatory bowel disease, collagen (CL)- and proteoglycan-induced arthritis and cutaneous inflammation (13), and has led to interest in CD44 as a target for possible therapeutic intervention or as a marker of autoimmune disease activity. The principal ligand for CD44 is hyaluronan (HA), a broadly distributed glycosaminoglycan, whose expression on endothelial cells is inducible by pro-inflammatory cytokines and mediates CD44-dependent rolling during inflammation (7, 14). Although not all ligands are identified, CD44 is known to interact with the extracellular matrix (ECM) components, CL, laminin and fibronectin, as well as osteopontin and serglycin (15).

Protein kinase C (PKC) enzymes are a family of serine-threonine kinases that are divided into three classes (i) classical (α , β I, β II, γ), (ii) novel (δ , ϵ , η , θ) and (iii) atypical (ζ , ι/λ) and are responsible for signal transduction in many systems (16). PKC signaling has been implicated in CD44 function. For example, the binding of CD44 to its native ligand HA is altered following phorbol myristate acetate (PMA) treatment (17) and CD44 acts as a substrate for PKC phosphorylation (18). Importantly, a recent study describes the complex regulation of phosphorylation and dephosphorylation of the cytosolic domain of CD44 by PKC activation, a process that allows the dissociation of the CD44-ezrin complex and controls directional migration (19). In addition, PKC β I (identified here as PKC β) has been shown to be crucially important for leukocyte function-associated antigen-1 (LFA-1)-mediated locomotion (20). Therefore, we investigated the role of PKC isoforms in mediating the migratory response of T cells following CD44 ligation. Using HUT-78 cells, a T lymphoma cell line and peripheral blood T lymphocytes (PBTs), we were able to demonstrate active migration following CD44 cross-linking. The PKC isoforms, β and δ , were found to be associated with the microtubule cytoskeleton in cells displaying a migratory, polarized phenotype. The use of pharmacological inhibitors and pseudosubstrate-blocking peptides allowed the function of these PKC isoforms in CD44-induced migration to be investigated and established a critical role for PKC β in this event.

Methods

Cells and cell culture

In most experiments, the human T lymphoma cell line HUT-78 (American Type Culture Collection, Manassas, VA, USA) was used. Where indicated, normal human PBTs were isolated

using a Ficoll-Hypaque density gradient. Before PBTs were used for experiments they were activated (21) by pre-treatment with 25 ng ml⁻¹ PMA (Sigma, St. Louis, MO, USA) or 500 U ml⁻¹ of purified protein derivative (PPD) prepared from *Mycobacterium tuberculosis* (Medeva Pharma Ltd, Leatherhead, UK) for 72 h at 37°C. Cells were maintained using CO₂-Independent Media (Gibco BRL, Rockville, MD, USA) supplemented with 10% FCS, 1% penicillin and 1% streptomycin at 37°C.

T lymphocyte migration

Lymphocyte migration studies for 2-D analysis and video recording were performed using Lab-Tek eight-well chambered slides (permanox plastic or borosilicate) or 96-well plates (Nalge Nunc Intl., Naperville, IL, USA). The surfaces were pre-coated with goat anti-mouse Ig (Dako, Bucks, UK) and subsequently incubated with cross-linking mAbs. Antibodies to CD44 used were D2.1 (22), J173 (Immunotech, Marseille, France), L3D1 (a gift from M. B. Omary) or F10-44-2 (Serotech, Oxford, UK). Other antibodies used were an anti-LFA-1 mAb, SPV-L7, an anti-intracellular adhesion molecule-1 (ICAM-1) mAb, Mem111 (Sanbio, Uden, The Netherlands) and an anti-CD3 mAb, OKT3 (American Type Culture Collection). As a control, wells were coated with poly L-lysine or cells were seeded onto positively charged slides (cells adhered to this surface without activation). Cells were seeded into chambers at a density of 2×10^4 cells per well in 200 μ l of warmed media. Studies were carried out using the selective PKC δ inhibitor, rottlerin [inhibitory concentration 50% (IC₅₀) = 3–6 μ M] (Alexis Corp., San Diego, CA, USA) and a selective classical PKC inhibitor Gö6976 (IC₅₀ = 7.9 nM) (Calbiochem, San Diego, CA, USA). The PKC δ and ϵ -specific inhibitory peptides and control peptide were a kind gift of Daria Mochly-Rosen (Stanford University, CA, USA). Cells were pre-treated with inhibitors at the indicated concentration for 30 min at 37°C before being seeded onto immobilized cross-linking antibodies. Cells were fixed 4 h after incubation with immobilized antibody in pre-coated chambers (when activation was maximal).

The assays utilizing T cell migration in 3-D matrices were performed in the wells of flat-bottomed 96-well plates pre-filled with ECM gel (Sigma) alone or enriched with either of two distinctive molecular weight hyaluronic acid variants mixed with the ECM gel at the final concentration of 1 mg ml⁻¹. High-molecular weight (1.76×10^6 Da) HA was from Lifecore Biomedical, Inc., Chaska, MN, whereas HA fragments (200 kDa) were purchased from ICN (Valeant) Pharmaceuticals (Costa Mesa, CA, USA). T cells were seeded on the surface of polymerized gels and allowed to migrate into the gel in the presence or absence of PKC inhibitors. The assays were stopped after 60 min by addition in the wells of 4% formaldehyde at ambient temperature for 30 min. The wells were then washed in PBS, cells stained with acridine orange solution and the number of cells migrated into the gels analyzed on the confocal UltraVIEW Live Cell Imager (Perkin Elmer, Cambridge, UK) using $\times 20$ dry long working distance lens. At least five randomly chosen microscopic fields taken at the equivalently positioned Z-plane for experiment and control wells were included in the analysis. For the high-resolution analysis of the morphology of the migrating cells,

the assays were set up in a similar manner, but using eight-well chambered borosilicate coverslips (Nunc) instead of 96-well plates.

Deformation index studies

In order to study cell morphology, the activated lymphocytes were fixed using 3.7% PFA. Images from five randomly selected fields of view were recorded for analysis. The fixed cells were scored using the following formula for the deformation index (DI)

$$\text{deformation index} = \frac{\text{elongation index}}{\text{circularity index}}$$

where

$$\text{elongation index} = \frac{\text{major ellipse diameter}}{\text{minor ellipse diameter}}$$

and

$$\text{circularity index} = 4 \times \pi \times (\text{area}/(\text{perimeter})^2).$$

This index provides a stronger measure of the degree of cell polarization in comparison to elongation index and circularity alone. Typically values of 1–3 are assigned to cells that were non-migratory, i.e. cell shape nearing circular. Those cells that display a polarized phenotype (cell body and trailing uropod) and therefore a higher degree of deformation had higher DI values (>5) (Volkov *et al.*, manuscript in preparation). On average, >40 randomly chosen cells were scored for each set of conditions. Analysis was performed using the NIH Image software (Scion Corporation, Frederick, MD, USA).

Time-lapse video microscopy

Cell migration was monitored on a Nikon TE-300 inverted microscope equipped with a JVC TK-C1380 CCD camera. Sequential image frames were digitized and cell migration was evaluated by the distance traveled by the cell centroid over the entire observation period (indicated in the figure legends for each study). Between 40 and 50 cells were recorded and analyzed per observation field.

Immunofluorescence staining

Prior to fixation for immunofluorescence analysis, the unbound cells were washed from slides with warm PBS, and attached cells were fixed in acetone at -20°C . Isoform-specific PKC antibodies were used to determine the subcellular localization of PKC δ (Research and Diagnostic Antibodies, Berkeley, CA, USA) and PKC β (I) (Sigma). These were visualized using a FITC-labeled secondary antibody (Dako). The microtubule cytoskeleton was examined using a mAb raised against α -tubulin and detected using a tetramethylrhodamine isothiocyanate (TRITC)-labeled secondary antibody (Sigma). Fluorescently labeled cells were examined using an oil immersion $\times 100$ lens on a Nikon TE3000 inverted microscope attached to UltraVIEW Live Cell Imager (Perkin Elmer) confocal workstation.

Statistics

The significance of differences observed in the various assays was evaluated by the Mann–Whitney test, an analysis of non-paired, non-parametric measurements.

Results

Cross-linking CD44 induces a polarized, locomotion-associated phenotype in T lymphocytes

In this study the response of both HUT-78, a T lymphoma cell line, and PBTLs following ligation of their CD44 receptor was examined using a series of immobilized CD44 mAbs. The antibodies used were D2.1, J173, L3D1 and F10-44-2. F10-44-2 has been reported to up-regulate HA-binding ability of activated T cells whereas J173 was found to inhibit HA adhesion in these activated cells (23). When the CD44 receptor was cross-linked in HUT-78, the cells developed a polarized morphology (Fig. 1), characterized by a leading cell body and a trailing process, in agreement with data previously reported by Kelleher *et al.* (21). All antibodies used induced a migratory phenotype. A similar polarized phenotype was seen in LFA-1-activated lymphocytes (Fig. 1) and these morphological changes were demonstrated to correlate with active lymphocyte locomotion (20). Cross-linking of CD3, ICAM-1, and other adhesion and signaling receptors expressed on the surface of T cells did not induce these morphological changes (Fig. 1). Cells adhered to the substrate following cross-linking of CD3, but they did not develop a polarized morphology. A similar response was seen with the ligation of ICAM-1. Resting PBTLs did not display any morphological changes in response to ligation of either CD44 or LFA-1, but required pre-activation with a PKC activator such as the phorbol ester PMA or antigen stimulation for 72 h. Pre-exposure of T cells to the tuberculin antigen, PPD, resulted in the formation of a locomotory phenotype following CD44 cross-linking in a discrete significant population of antigen-responsive cells, similar to PKC-activating agents.

Using time-lapse video microscopy, we verified that the locomotory phenotype displayed by CD44-activated cells was accompanied by active migration. Measuring the displacement of the cell centroids over a 60-min observation period gave clear evidence that both HUT-78 cells and PBTLs triggered through cross-linking of CD44 traveled at a similar rate to that of cells activated through cross-linking of LFA-1. Mean velocity traveled by both LFA-1 and CD44-activated HUT-78 cells were similar at $14.7 \mu\text{m h}^{-1}$ (mean value of peak velocity $-76.65 \mu\text{m h}^{-1}$) and $14.4 \mu\text{m h}^{-1}$ (mean value of peak velocity $-52.94 \mu\text{m h}^{-1}$), respectively, while PBTLs were migrating at $15.0 \mu\text{m h}^{-1}$ (mean value of peak velocity $-56.7 \mu\text{m h}^{-1}$) and $12.0 \mu\text{m h}^{-1}$ (mean value of peak velocity $-39.7 \mu\text{m h}^{-1}$) following ligation of LFA-1 and CD44, respectively (Fig. 2), a slow and apparently random mode of locomotion.

CD44-dependent T cell migration in 3-D ECM lattices

Following cross-linking of CD44 in the activated PBTLs, the cells developed a polarized morphology, with the response most pronounced in D2.1 activated cells (Fig. 1). However,

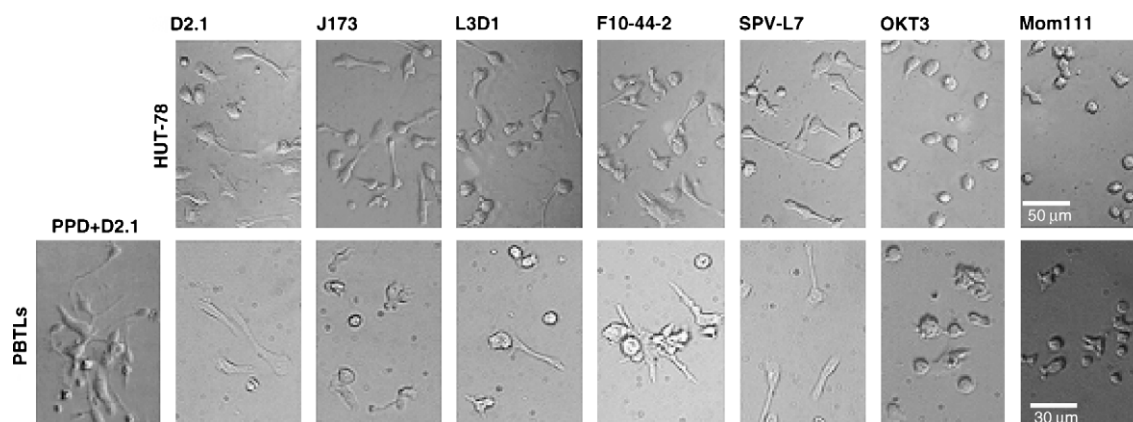


Fig. 1. Phenotypic changes in HUT-78 T lymphoma cells and PBTls induced by CD44 antibodies. HUT-78 cells and PBTls (activated for 72 h using PMA or antigen-stimulated with PPD as described in Methods) developed a polarized morphology following cross-linking of CD44 using the mAbs D2.1, J173, L3D1 and F10-44-2. Panel PPD + D2.1 shows antigen-specific T cells acquiring a locomotory behavior on anti-CD44 D2.1 mAbs. Similar phenotypic changes were seen with cells activated by cross-linking LFA-1, using SPV-L7. Cross-linking of CD3 using OKT3 and ICAM-1 using Mem111 did not induce the same morphological changes. Resting PBTls (not activated via antibody or PMA pre-treatment) remain static and non-polarized (data not shown).

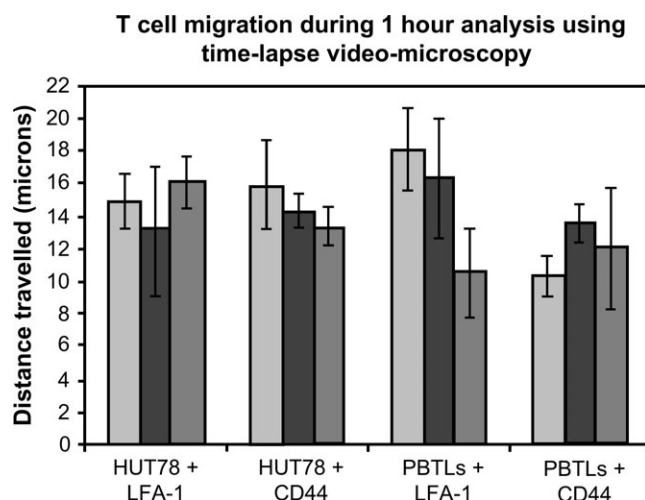


Fig. 2. Active T lymphocyte migration following ligation of CD44. The average distance traveled by HUT-78 cells and PBTls activated by ligation of either CD44 or LFA-1 over 1-h intervals measured using time-lapse video microscopy. Cell locomotion was triggered via the CD44 pathway using immobilized D2.1 and by LFA-1 cross-linking with immobilized SPV-L7. Bars reflect mean values $\pm$ SEM obtained in three independent studies (light gray, dark gray and hatched bars, respectively).

when HUT-78 and PBTl responses to immobilized purified HA (both high and intermediate molecular weight hyaluronic acid) were examined, and few cells (~15%) developed the firm adhesion and polarized morphology that was evident in cells activated by antibody cross-linking (data not shown). We therefore more closely addressed the physiological equivalents of the observed phenomena by investigation of T cell migratory characteristics in 3-D ECM lattices enriched with natural CD44 ligands (Fig. 3). As seen from the chart, incorporation of the high-molecular weight HA in the ECM significantly enhanced T cell migratory potential in comparison to the ECM alone and matrix enriched with HA fragments (Fig. 3a). Corresponding XYZ confocal planes (Fig. 3b and c)

clearly illustrate a substantially deeper penetration of the T cells into the ECM lattice incorporating high-molecular weight HA. High-resolution analysis of individual cell morphology under similar conditions (Fig. 3d and e) revealed that T cells acquired locomotion-associated phenotypical changes similar to those registered on the motility-inducing CD44 antibodies and described above (see also corresponding supplementary movie 1, available at *International Immunology Online*).

Translocation of PKC isoforms to the microtubule cytoskeleton in response to CD44-induced migration

We next examined the distribution of the PKC isoforms following ligation of CD44. Resting HUT-78 stained for PKC β showed a diffuse staining pattern with discrete cytoplasmic localization and partial association with the microtubule cytoskeleton. Following activation of CD44 by receptor cross-linking, the microtubule cytoskeleton becomes reorganized with the microtubule organizing center (MTOC) located to the rear of the migrating cell body with a microtubule-rich trailing process. There was a redistribution of PKC β to the region of the MTOC and along the microtubule-based trailing process in the CD44-activated HUT-78 (Fig. 4). Examination of PKC δ localization in resting HUT-78 cells showed a diffuse cytoplasmic staining with an association with the MTOC. In the migrating T cells, PKC δ was found localized to a position adjacent to the MTOC (Fig. 5) and also is present as uninvolved cytoplasmic pool in the cell body.

Suppression of PKC activity attenuates CD44-induced T cell motility

In order to further investigate the role of the above-indicated PKC isoforms, selective inhibitory strategies were implicated. As PKC β translocation was observed following CD44 ligation, we used Gö6976 to examine the functional consequences of classical PKC isoform inhibition. HUT-78 cells were pre-treated with Gö6976 for 30 min before activation on immobilized cross-linking antibody. Untreated CD44-activated cells

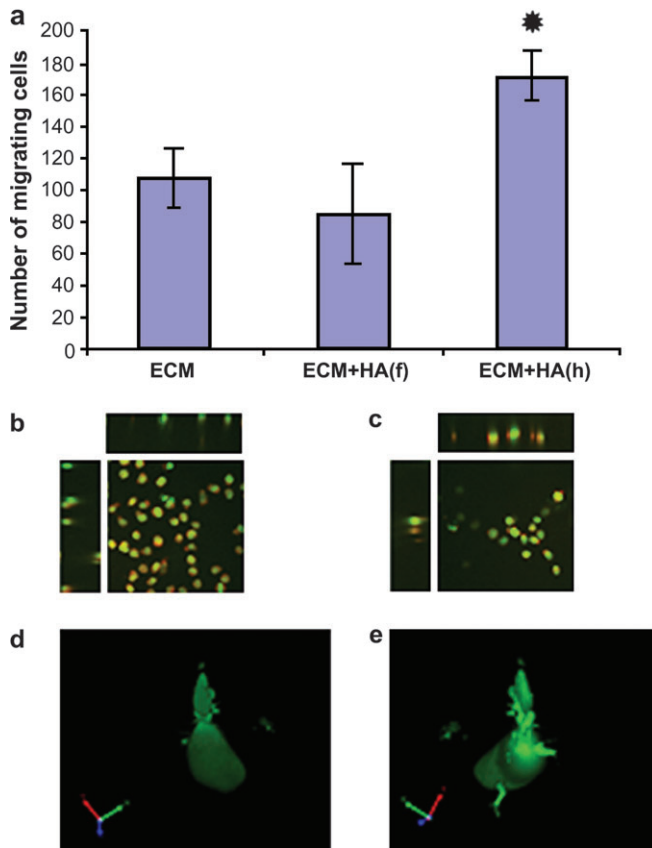


Fig. 3. CD44-dependent T cell migration in 3-D ECM lattices. Quantitative evaluation of HUT-78 T cell migration into 3-D ECM gels enriched with HA variants (a). The number of cells migrating into the ECM is significantly increased after incorporation of high-molecular weight HA into the gel (ECM + HA(h)) compared with the ECM alone and ECM including HA fragments (ECM + HA(f)). Parts (b) and (c) below the corresponding columns show equivalently positioned confocal XYZ-projections taken between 20–80 μ m below the gel surface (middle panels) and corresponding XZ and YZ-projections (left and top panels). The cells migrating into ECM + HA(h) are penetrating much deeper inside the gel than those exposed to ECM + HA(f). T cells migrating into the HA-enriched ECM gels display a characteristic polarized phenotype typical of locomotory cells (d) and (e). The panels show a representative 3-D reconstructed image of a T cell migrating into the gel rotated 170° around the Y-axis (see also corresponding supplementary movie 1, available at *International Immunology* Online). * Indicates $P < 0.05$ in comparison to ECM and ECM + HA(f).

developed a polarized and elongated morphology (Fig. 6a), but when pre-incubated with Gö6976, HUT-78 cells on immobilized anti-CD44 remained adherent, but no longer acquired this morphology (Fig. 6b). Similar results were seen with anti-LFA-1 (Fig. 6d and e). Using the DI as a quantitative descriptor of cell morphology, the development of a motile phenotype was inhibited by Gö6976 in a dose-dependent manner (Fig. 6g) when cells were activated by CD44 cross-linking. LFA-1-activated cells respond to Gö6976 pre-treatment in a comparable manner (Fig. 6h).

Further work was carried out utilizing time-lapse video microscopy to determine if the failure of cells to develop a migratory phenotype was also accompanied by the loss of active locomotion. Following Gö6976 pre-treatment, HUT-78

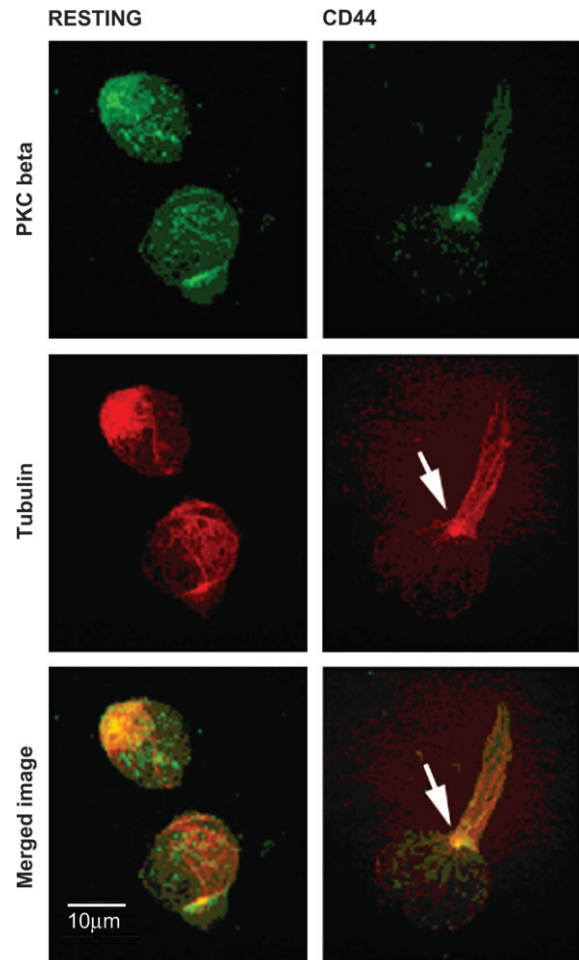


Fig. 4. PKC β localization in CD44-activated HUT-78 cells. Resting (left vertical panel) shows HUT-78 on poly L-lysine coated slides. CD44 (right panel)—migrating HUT-78 activated by CD44 cross-linking using immobilized D2.1. Resting and CD44-triggered cells were stained for PKC β (green) and tubulin (red). Merged image, identical microscopic fields illustrating PKC β and tubulin co-localization (yellow–orange overlay colour). Note PKC β association with the microtubule-rich tail of the migrating cell.

cells showed significantly decreased migration in response to either LFA-1 or CD44 activation (Fig. 6i). Additional evidence for the role of PKC β in CD44-induced migration was obtained by analyzing the response of K4 cells to CD44 ligation. K4 cells are a HUT-78 clone deficient in PKC β (20, 24). These cells failed to migrate successfully following cross-linking of CD44 (Fig. 6c, f and i).

Gö6976 inhibited CD44-stimulated cytoskeletal reorganization and cells did not form microtubule-rich tails. There was no significant development of a polarized phenotype, but the MTOC was located in the position adjacent to the nucleus typical of motile cells. In these cells, there was a clear association of PKC β at the area of the MTOC, but not with the microtubules. Rather, this isoform remained in a diffuse cytosolic location (Fig. 7). In Gö6976-treated cells, CD44 cross-linking did not induce redistribution of PKC δ and it remained concentrated in discrete spots in the region of the MTOC similar to the resting cells (Fig. 8).

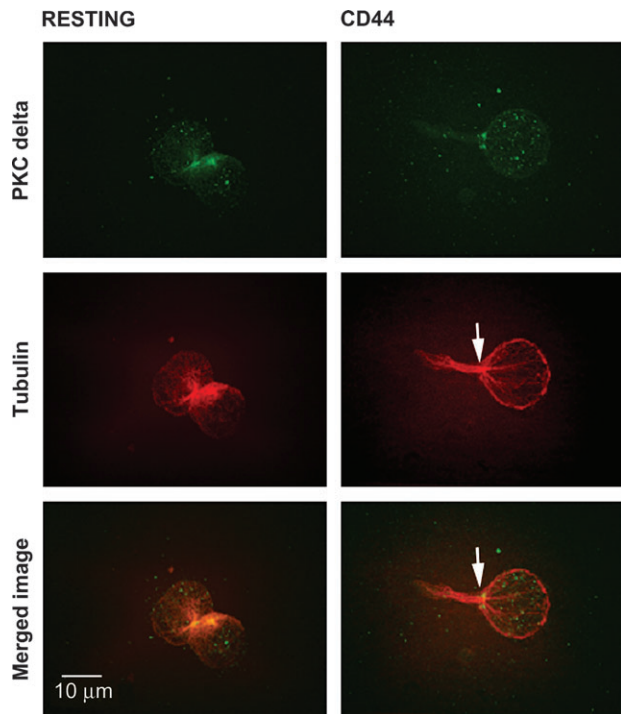


Fig. 5. PKC δ localization in CD44-activated HUT-78 cells. Resting (left vertical panel)—HUT-78 on negatively charged slides. CD44 (right panel)—migrating HUT-78 activated by CD44 cross-linking using immobilized D2.1. Resting and CD44-triggered cells were stained for PKC δ (green) and tubulin (red). Merged image, identical microscopic fields illustrating PKC δ and tubulin co-localization (yellow–orange overlay). Arrows here and below indicate the positions of the MTOC in migrating cells.

We subsequently used an isoform-specific PKC inhibitor rottlerin to examine the role of PKC δ in T cell migration. HUT-78 cells were pre-treated with rottlerin, at concentrations of 1, 5 and 10 μ M, for 30 min before activation through either CD44 or LFA-1. On morphological examination cells appeared flattened, and frequently produced multiple thin cytoplasmic projections reflecting that the adhesion *per se* was not distorted (Fig. 9b and d). The DI did not show significant alteration in response to rottlerin pre-treatment (Fig. 9e and f) and time-lapse video revealed migration to be reduced but not completely ablated (Fig. 9g).

HUT-78 cells treated with rottlerin prior to cross-linking of CD44 were subsequently examined to determine the localization of PKC β and PKC δ . As can be seen in Figs 7 and 8, the extended processes are tubulin-rich and are supported by well-defined microtubule structures. While PKC β remained associated about the area of the MTOC, it was not fully associated with the microtubule-rich projections (Fig. 7). In the resting cell, localization of PKC δ at the MTOC appears diffuse. As can be seen in Fig. 8, in rottlerin-treated cells PKC δ displayed a granular pattern and were not as tightly associated with the MTOC as commonly seen in the untreated migrating T cell (Fig. 5).

In order to more clearly delineate the impact of PKC δ on T cell locomotory properties, we further extended the studies in a 3-D model described above utilizing HA-enriched ECM in

the presence of cell-permeable isoform-specific pseudosubstrate PKC inhibitory peptides (Fig. 10). The PKC δ -specific peptide significantly decreased T cell migration into the 3-D ECM lattices incorporating high-molecular weight HA in comparison to the ECM–HA alone and control peptide. The peptide blocking another functionally distinctive novel PKC ϵ isoform did not significantly affect cell motility. These findings clearly demonstrate the requirement of intact PKC δ function for T cell locomotion in a physiological microenvironment.

Discussion

This study was designed to examine the response of human T cells to ligation of the adhesion receptor, CD44. Following activation of CD44, these cells acquired a polarized migratory phenotype, which we subsequently confirmed to be associated with active T cell migration. Further investigation revealed a role for the PKC isoforms β and δ in this migratory response following receptor triggering. Using a series of immobilized mAbs to cross-link CD44, we observed in human lymphocytes and HUT-78 cells a human T lymphoma cell line, the development of an elongated morphology with leading edge, cell body and posterior trailing cytoplasmic projections (Fig. 1). We did not observe an equivalent percentage of polarized T cells in response to purified HA immobilized on a planar substrate. However, incorporation of HA into 3-D ECM lattices significantly enhanced T cell polarization and migration. HA is abundantly expressed in many tissues and is now known to play a role in the regulation of cell motility, invasion and proliferation (25). Although HA is the principle ligand for CD44, the failure of HA to elicit the same magnitude of response in T cells as cross-linking antibodies in a 2-D model may be due to a number of reasons. The activation state, glycosylation and density of CD44 will influence its response to HA ligation (9, 17, 26, 27). In addition, under near-physiological conditions mimicked by a 3-D ECM lattice, HA is presented to the lymphocyte CD44 receptor in a more complex spatially organized form. In a real *in vivo* situation HA might be also immobilized and spatially oriented by a counter-receptor on the endothelial cell surface (28).

Using time-lapsed video microscopy, we have demonstrated active HUT-78 and human T lymphocyte migration in response to cross-linking of CD44 using immobilized mAbs. These CD44-activated T cells develop a similar morphology and migrate at a similar velocity as those cells activated by ligation of LFA-1 (Fig. 2). The relevance of this slow cell migration to the *in vivo* microenvironment is discussed by Volkov *et al.* (20). A recent study by Katakai *et al.* (29) demonstrated that the transendothelial migration of T_H1 cells across a murine endothelial cell line could be blocked using antibodies to CD44 (as well as LFA-1 and ICAM-1). Cross-linking of CD44 on the surface of these murine T_H1 cells resulted in the development of an elongated polarized morphology, which was similar to the phenotype seen in this study (29). T cells develop a similar polarized morphology following cross-linking of LFA-1 (using both recombinant ICAM-1 and a motility-inducing anti-LFA-1 antibody), which was earlier demonstrated to be associated with active migration (20). During our study of T cell migration, Jurkat, a CD44-negative T leukemic cell line, did not produce

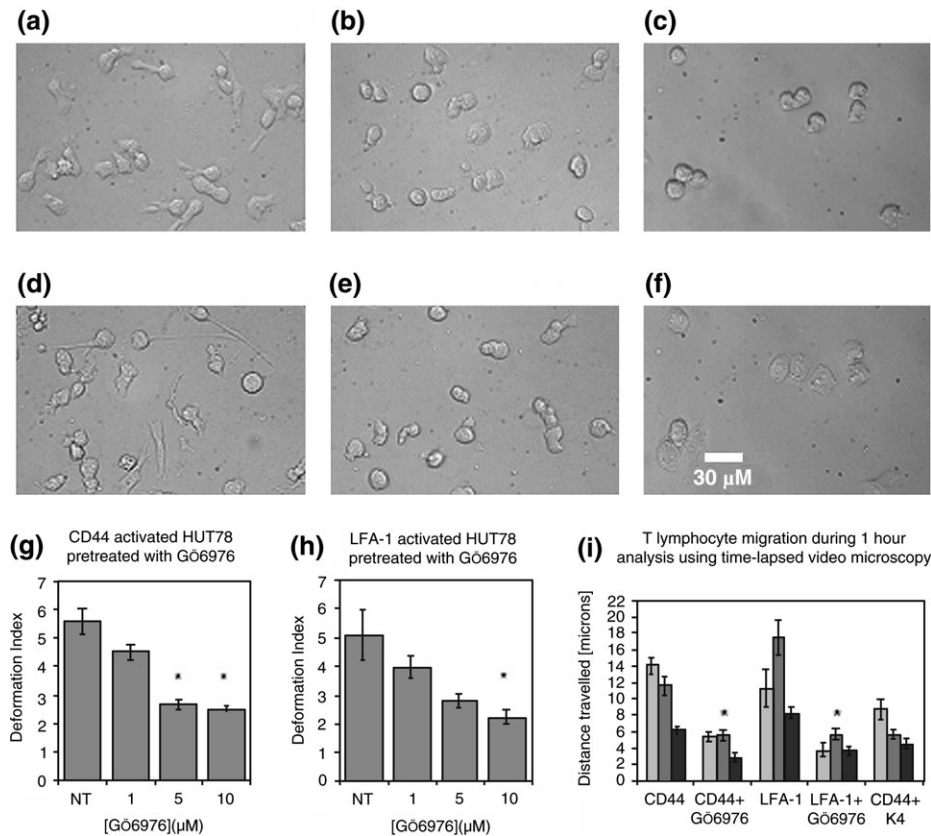


Fig. 6. HUT-78 migration-associated characteristics in response to CD44 ligation following inhibition of classical PKC isozymes. Typical polarized morphology of HUT-78 cells following ligation of CD44 (a) and LFA-1 (d). Cells pre-treated with 10 μ M Gö6976 display a rounded morphology when incubated with immobilized anti-CD44 (b) and anti-LFA-1 (e). Also shown is the typical morphology of CD44-activated K4 cells at $t = 0$ and $t = 60$ min following incubation with immobilized D2.1 [(c) and (f), respectively]. Dose-response migration inhibition by Gö6976 in cells activated via immobilized anti-CD44 (g) and anti-LFA-1 (h) (as determined by measurement of the deformation index). (i) Distance traveled during the 1-h recording interval was measured for cells pre-treated with 10 μ M Gö6976 or dimethyl sulfoxide (DMSO) control followed by receptor cross-linking using SPV-L7 (anti-LFA-1) or D2.1 (anti-CD44) (* indicates $P < 0.05$ reduction in migration relative to control). Also shown is distance traveled by K4 cells following cross-linking of CD44.

a migratory response to LFA-1 cross-linking. This finding may further underline the co-operative interaction between CD44 and LFA-1 in the induction of a T cell migration.

Several sets of evidence have stressed the importance of CD44 in T lymphocyte migration to sites of inflammation. There is increased expression of functionally active CD44 on T lymphocytes in the peripheral blood of human patients with active autoimmune diseases (12). Expression of HA on endothelial cells is up-regulated in response to stimulation by pro-inflammatory cytokines (7, 14). This has served to build up a model for the role of CD44 in inflammatory conditions, with the initial activation of CD44 on antigen-specific T cells within the lymph node, subsequent mobilization of these cells into the periphery and finally CD44-dependent migration to inflammatory sites. Here, we have evidence that CD44 ligation signals the firm adhesion, polarization and repeated cycles of cytoskeletal reorganization required for the controlled migration of human T cells during extravasation to inflammatory sites.

Our data demonstrate both the dependence of CD44-induced T cell migration on the functional PKC status and isoform-specific intracellular redistribution of PKC enzymes

accompanying active cell locomotion. A number of studies have implicated PKC signaling in the migratory response. The importance of PKC β in mediating LFA-1-induced T cell migration was demonstrated by Volkov *et al.* (20) and highlighted the critical role of PKC β association with the microtubule cytoskeleton. Inhibition of classical PKC isoforms significantly decreased HUT-78 migration in response to CD44 ligation (Fig. 6i). When localization of PKC β and δ was examined these PKC isoforms remained associated about the area of the MTOC when pre-treated with Gö6976 and rottlerin (Figs 7 and 8). The importance of PKC β activity is further strengthened by the lack of a migratory phenotype in K4 cells, a HUT-78 clone deficient in PKC β , following CD44 cross-linking (Fig. 6).

When treated with rottlerin prior to CD44 activation, cells adhered and flattened to the immobilized substrate. Cells frequently developed multiple cytoplasmic projections, but there was no significant alteration of DI by rottlerin pre-treatment. Examination of cell migration using time-lapse video microscopy indicated that without PKC δ activity, HUT-78 migration in response to CD44 ligation was reduced without complete inhibition. However, in a more physiologically

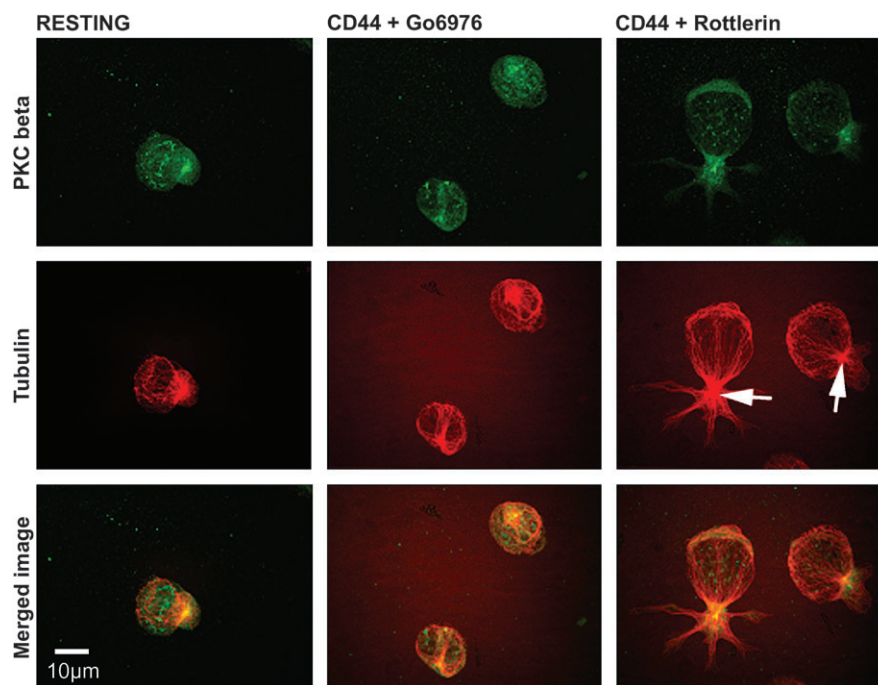


Fig. 7. Localization of the PKC β isoform in Gö6976 and rottlerin pre-treated HUT-78 cells following CD44 cross-linking. Resting (left panel), HUT-78 on negatively charged slides. CD44 + Gö6976 (middle panel), 10 μ M Gö6976 pre-treated HUT-78 cells incubated on immobilized D2.1. CD44 + Rottlerin (right panel), 5 μ M rottlerin pre-treated HUT-78 cells incubated with immobilized D2.1. PKC β (green) and α -tubulin (red) staining of the same cells. Merged image, identical microscopic fields illustrating PKC β and tubulin co-localization (yellow/orange overlay).

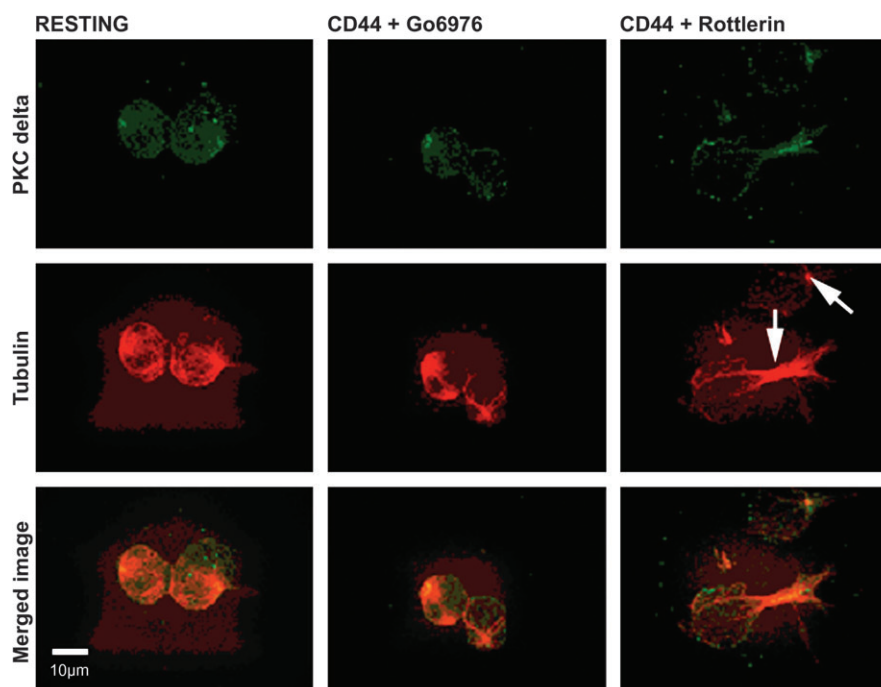


Fig. 8. Localization of the PKC δ isoform in Gö6976 and rottlerin pre-treated HUT-78 cells following CD44 cross-linking. Resting (left panel), HUT-78 on negatively charged slides. CD44 + Gö6976 (middle panel), 10 μ M Gö6976 pre-treated HUT-78 cells incubated on immobilized D2.1. CD44 + Rottlerin (right panel), 5 μ M rottlerin pre-treated HUT-78 cells incubated with immobilized D2.1. PKC δ (green) and α -tubulin (red) staining of the same cells. Merged image, identical microscopic fields illustrating PKC δ and tubulin co-localization (yellow–orange overlay).

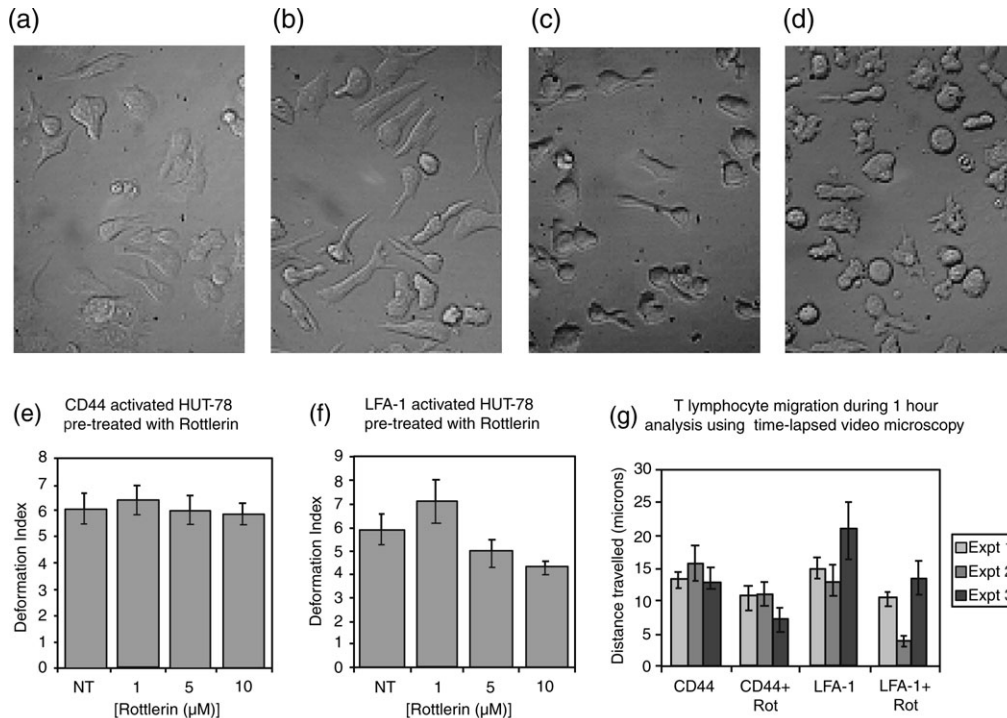


Fig. 9. HUT-78 migration-associated characteristics in response to CD44 ligation following inhibition of PKC δ . Typical polarized morphology of HUT-78 cells following ligation of CD44 (a) and LFA-1 (c). Cells pre-treated with 5 μ M rottlerin followed by incubation with immobilized anti-CD44 (b) and anti-LFA-1 (d). Rottlerin dose-response curve followed by incubation with immobilized anti-CD44 (e) and anti-LFA-1 (f) (as determined by measurement of the deformation index). Distance travelled during the 1-h recording interval was measured for cells pre-treated with 5 μ M rottlerin or dimethyl sulfoxide (DMSO) control followed by receptor cross-linking using SPV-L7 (anti-LFA-1) or D2.1 (anti-CD44) (a,b indicates $P < 0.05$ reduction in migration relative to control).

relevant model of cell migration in HA-enriched 3-D ECM lattices, PKC- δ -specific pseudosubstrate inhibitory peptide clearly reduced T cell migratory capacity thereby highlighting the involvement of PKC- δ in this process.

The finding that T cells activated via CD44-HA interaction or by CD44 antibody cross-linking acquired active motility perfectly correlates to the unique positioning of this receptor, spanning the extracellular environment with subcellular structures and underlying cytoskeleton via its cytoplasmic domain. Receptor association with the cytoskeleton is important in the transduction of the "outside in" signals and altering the phosphorylation status of the cytoplasmic domain is often a feature of cell migration and adhesion (19, 30, 31). Phosphorylation of the cytoplasmic domain of CD44 was required for migration in fibroblast and melanoma cells on a HA substrate, and phosphorylation mutants could no longer migrate on HA but could still bind HA (30). Also, PKC activation alters CD44 phosphorylation and its association with the cytoskeletal linker protein, ezrin. This dynamic association and dissociation of CD44 and ezrin influences directional migration (19).

It is interesting to note that the phenotypic changes accompanying T cell migration, cytoskeletal rearrangements and the response to PKC β and δ inhibition are evidently similar in both CD44 and LFA-1-activated (32) HUT-78 cells, indicating that these receptors may operate through converging or shared signal transduction pathways in the

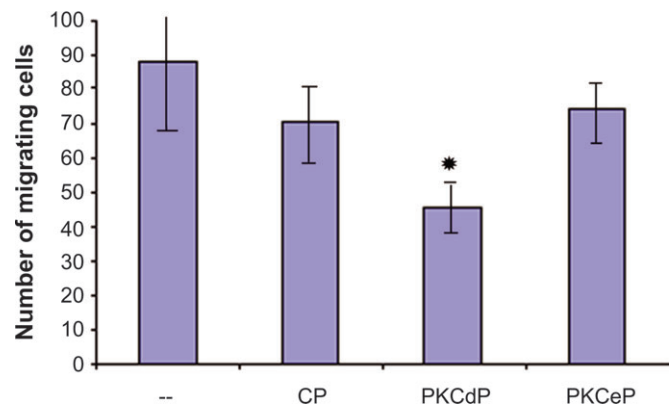


Fig. 10. HUT-78 migration into HA-enriched 3-D ECM lattices following isoform-specific PKC inhibition. HUT-78 cell migration into ECM gels enriched with high-molecular weight HA was quantified in the presence or absence of the pseudosubstrate PKC inhibitory peptides. Left column, untreated cells; CP, control peptide; PKCdP, PKC-delta inhibitory peptide; PKCeP, PKC-epsilon inhibitory peptide. PKC-delta inhibition significantly reduces cell migration reduced in comparison to control, PKC-epsilon peptide and intact cells (* indicates $P < 0.05$).

migratory response. These findings underscore the importance of CD44 in the inflammatory response and may have significant implications for the understanding of the mechanisms underlying cell migration in inflammatory processes

and might contribute towards the development of novel approaches for its targeted regulation.

Supplementary data

High-resolution 3-D reconstruction of the HUT-78 T lymphoma cell migrating into the ECM enriched with high-molecular weight HA. Cells display a characteristic polarized phenotype typical of locomotory cells with a clearly defined leading edge and trailing tail projection (uropod). The image is rotated to provide a 360° view around the Y-axis.

Supplementary data are available at *International Immunology* Online.

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Abbreviations

CL	collagen
DI	deformation index
ECM	extracellular matrix
HA	hyaluronan
IC ₅₀	inhibitory concentration 50%
LFA-1	leukocyte function-associated antigen-1
MTOC	microtubule organizing center
PBTLs	peripheral blood T lymphocytes
PKC	protein kinase C
PMA	phorbol myristate acetate
PPD	purified protein derivative

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