A Multidisciplinary Approach to the Study of T Cell Migration

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ABSTRACT: **Active T cell locomotion depends on efficient repeated cycles of integrin receptor/ligand interactions mediating cell adhesion and detachment, intracellular signaling cascades orchestrating posttranslation modifications of interacting proteins, dynamic reassembly of participating cytoskeletal elements, and structural support of associated scaffolding molecules. Using an integrated approach based on novel cutting edge technologies of live cell imaging, cell transfection, proteomics, and nanotechnology, we provide here a detailed characterisation of crucial mechanisms involved in LFA-1 integrin-mediated T cell migration. Polarization and phenotypic changes associated with LFA-1–triggered T cell locomotion is largely dependent on the intact functioning of the microtubule cytoskeleton. Experiments utilizing 4-D (3-D over time) confocal live imaging of T cells, microinjected with fully functional constructs encoding protein kinase C beta (PKC-β) isoenzyme tagged with enhanced green fluorescent protein (GFP), elucidate that LFA-1–induced activation is associated with translocation of PKC-β to sites associated with centrosomes and tubulin cytoskeleton in locomotory T lymphocytes. We also provide here a characterization of a novel microfluidics-based multichannel platform enabling detailed analysis of leukocyte adhesion and migration under regulated shear stress conditions. Using precision machined surfaces, we demonstrate that the substrate topography can influence the motile response of the two different T cell types in different ways, and this can be quantified in terms of specified motility parameters. Finally, using an original *in situ* immunoprecipitation method, in which LFA-1 antibodies are utilized to induce intracellular association of proteins in the cytoskeletal/signaling complex, we demonstrate that this complex includes a number of structural and signaling proteins, which have been identified by 2-D electrophoresis and MALDI-TOF protein sequencing.**

KEYWORDS: **T cells; migration; nanotechnology; cell signaling**

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INTRODUCTION

An effective immune system requires that leukocytes can continuously migrate from the circulation across the endothelium into tissue and interchange between the bloodstream and the lymphatics. In doing so they can "patrol" the body for antigen and enter sites of inflammation. This process is characterized by initial tethering of the leukocyte to the surface of specialized endothelial cells, which is thought to be mediated largely by selectin interaction with their carbohydrate ligands expressed on the endothelium surface. This slows leukocyte flow, and cells roll along the surface of the endothelium. Chemokines, secreted by cells within the tissue and immobilized on the endothelial surface, activate molecules of the integrin family on the rolling cells, leading to leukocyte arrest and subsequent diapedesis across the endothelium. The process of diapedesis is facilitated by polarization of the cell, which crawls and squeezes between endothelial cells. Leukocytes polarize both in terms of shape and subcellular expression of receptors, adhesion and signaling molecules, and cytoskeletal elements. Actin, which is distributed throughout the lymphocyte, concentrates at the leading edge, as do the chemokine receptors. Microtubules distribute to the trailing edge or uropod.

Integrins are a widely expressed family of non-covalently linked α and β subunits that mediate cell–cell and cell–extracellular matrix contacts. The integrins on circulating leukocytes bind minimally to their ligands, but their adhesive capacity can be increased by stimulation of intracellular signaling pathways–for example, through the T cell receptor or selectin molecules, by chemokines, or by the phorbol ester PMA *in vitro*. This mechanism of activation is termed *inside-out signaling*. Integrins may also be activated by divalent cations such as Mg^{2+} and Mn^{2+} or special stimulatory anti-integrin monoclonal antibodies. Once activated, the integrin signals back into the cell, upon ligand binding; this is called *outside-in signaling*. Active $\beta1$ and $\beta2$ integrins play an important role in extravasation of leukocytes from the circulation to sites of inflammation and in the homing of lymphocytes to secondary lymphoid organs.

LFA-1–MEDIATED LOCOMOTION OF ACTIVATED T CELLS

We have developed an *in vitro* model of T lymphocyte migration. In this system, activated human peripheral blood T lymphocytes (PBTL) or the HuT78 T cell line migrate when incubated with either immobilized recombinant ICAM-1-Fc fusion protein (the natural ligand for the leukocyte function-associated antigen-1, LFA-1) or a triggering antibody to the α_L-chain of the LFA-1 molecule itself.[1] T lymphocytes stimulated through the LFA-1 molecule adopt a locomotion-associated phenotype (FIG. 1). With this polarized or migratory phenotype the cell acquires a long uropod or "tail," which can be several times the actual diameter of the cell body. In addition, cells undergo net body translocation and migrate on the substrate as demonstrated by time-lapse microscopy. At the same time the LFA-1 integrin molecule itself redistributes on the cell surface to cluster at a location surrounding the "neck" of the trailing uropod at a site adjacent to the centrosome. This cell surface localization of LFA-1 was visualized in live migrating cells following incubation of cells in the presence of polystyrene beads coated with an antibody to LFA-1 by phase con-

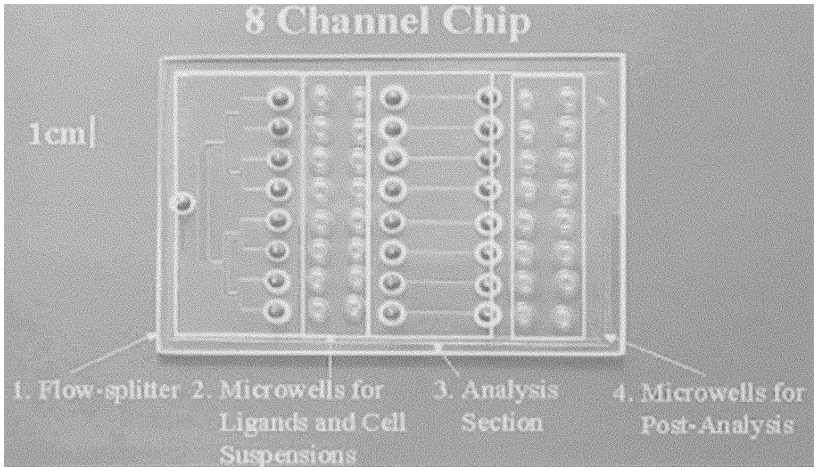

FIGURE 1. Eight-channel microfluidic biochip, comprising four distinct sections for the preparation of proteins/ligands, cell suspensions, subsequent injection into the 8 parallel microfluidic channels, and collection in the microwells for further analysis.

trast microscopy. Pretreatment with phorbol ester is necessary for adhesion and the development of cytoskeletal rearrangements on LFA-1 cross-linking in PBTL, but not in Hut78. The acquisition of this phenotype is directly associated with active cell body translocation, a process involving the formation of leading lamellae, translocation of the nucleus (nucleokinesis), and extension of the trailing uropod. Contraction of the uropod is accompanied by rear release of membrane integrins (deaggregated) on the substrate and reclustering of LFA-1 integrin molecules around the neck of the cell adjacent to the centrosome. This crawling locomotion involves repeated cycles of cytoskeletal assembly and disassembly, a process largely regulated by phosphorylation. Upon further analysis of the polarized cells in this model, it was demonstrated that the microtubule cytoskeleton undergoes a dramatic rearrangement and is distributed primarily in the trailing uropod of migrating cells.

PROTEIN KINASE Cβ EXPRESSION IS ESSENTIAL FOR LFA-1–STIMULATED T CELL MIGRATION

Protein kinase C (PKC) is a family of serine/threonine kinases involved in intracellular signal transduction in a variety of cell types. We investigated the subcellular distribution of PKCβ and -δ in resting and LFA-1–stimulated T cells by immunofluorescence microscopy. In T cells triggered via cross-linking of the LFA-1 integrin, two PKC isoenzymes, PKCβI and -δ, translocated from a primarily cytosolic distribution in resting cells to associate with the centrosome and the microtubule cytoskeleton in migrating cells.[2] Specifically, in resting cells, PKCδ displayed a diffuse granular cytoplasmic pattern with distinct spots at the centrosomes of mitotic cells. In migrating HuT78, this isoenzyme localized to a compact spot adjacent to

the centrosome in all cells with a reduction in the cytosolic distribution. PKCβI also demonstrated a diffuse cytosolic distribution in resting cells with a more granular distribution and translocation to centrosome and microtubules in the uropods of locomotory cells (in contrast to PKCδ, which did not associate with the microtubule-rich uropod).

LFA-1–mediated locomotion (but not adhesion) of Hut78 cells was sensitive to both the broad-spectrum PKC inhibitor staurosporine and the the classical (α/β/γ) PKC inhibitor Go6976, indicating a specific involvement of PKCα and/or -β (human T cells do not express PKCβ) in the migratory process. Furthermore, a more selective PKCβ inhibitor, LY379196, effectively blocked T cell polarization and migration.

We used a PKCβ-deficient clone, K-4, derived from the parental Hut78 line, to confirm that this PKC isoenzyme plays a critical role in LFA-1–stimulated migration. These cells adhere and spread but fail to polarize and form a microtubule-rich uropod typical of the parent Hut78 cells on cross-linking of the LFA-1 molecule, providing strong evidence that PKCβ plays an important role in the regulation of the tubulin-based cytoskeleton and motility. (The actin cytoskeleton appears to be functional in these cells, which generated multiple filopodia upon integrin cross-linking). We restored the ability of K-4 cells to acquire a locomotory-associated phenotype in response to LFA-1–induced activation by microinjecting them with a plasmid expressing PKCβI and EGFP (PKCβ–EGFP). Injection of a vector containing EGFP alone did not induce changes in cell morphology. This acquired migratory phenotype, upon expression of PKCβ-EGFP in K-4 cells, was indeed associated with changes in their locomotory behavior, and migration of cells could be tracked by labeling (by microinjection) the nuclei of PKCβ-EGFP–expressing cells with Texas Red–labeled dextran and tracing migratory cells by repositioning of colored nuclei. These studies also demonstrated a relationship between level of PKCβI expression and cell polarization. The level of PKCβ-EGFP expression was evaluated by mean fluorescence intensity, and it was demonstrated that high fluorescence was detected in cells with the most extended uropods, whereas cells displaying little or no polarity had low levels of fluorescence. Interestingly, as the uropod retracted during the forward motion of cells, a decrease in PKCβ protein expression in the uropod (as detected by fluorescence intensity) preceded contraction of the projection before a new locomotory cycle. The specificity of PKCβI in its association with the microtubule cytoskeleton and the migratory process is underlined by the fact that in locomotory T cells other PKC isoenzymes either do not undergo significant translocation or, as is the case for PKCδ, demonstrate a pattern associated with the centrosome but not with the microtubule cytoskeleton.

A MULTIDISCIPLINARY APPROACH TO THE STUDY OF T CELL MIGRATION

While the elucidation of PKCβI as a key signaling enzyme required for LFA-1–stimulated T cell migration is important, it is clear that we still have some way to go to specifically characterize the precise molecular mechanism of the migratory process in an *in vivo* setting. The complete intracellular signaling pathways including the identification of signaling complex components, their interaction with mem-

brane receptors, and the cytoskeleton and downstream signaling molecules remain to be determined. In addition, because shear forces and flow dynamics in the microvascular environment will naturally have a crucial impact on leukocyte behavior, it is absolutely necessary that the molecular mechanisms of lymphocyte migration be studied under conditions that closely simulate this environment. As such, novel technologies and a multidisciplinary approach hold the key to the success of further studies. This approach would combine the fields of biophysics and bioengineering, which could provide precision-machined surfaces and microenvironments with state-of-the art microfluidic technology. These facilities, when used together with traditional and innovative biochemical techniques, will greatly facilitate and, indeed, accelerate the characterization of the migratory process.

MICROFLUIDICS MULTICHANNEL SYSTEM

We have developed a miniaturized microfluidics enabling platform to simulate individual or combinatorial factors affecting lymphocyte behavior in human capillaries.[3] In contrast to conventional glass capillary flow systems, our microfluidic enabling platform in combination with high-resolution digital microscopy enabled us to monitor the behavior of single cells and to design experiments that yield a wider range of quantitative data. An understanding of the mechanisms of lymphocyte migration is critical for the development of novel strategies for the treatment of inflammatory diseases. Key factors affecting this process are blood flow parameters (blood rheology), cell receptors' interaction with extracellularly presented ligands, and additional stimuli of various kinds including distant messengers of inflammation (i.e., chemokines and cytokines). We can model these significant events in our microfluidics enabling platform through the use of appropriate immobilized ligands (either separate or in combination) for lymphocyte surface receptors as substrate coating agents in the biochips. Also, precision regulated blood flow conditions may be achieved by altering the sheer stress coefficient in the biochip assays. In addition, inflammatory microenvironmental conditions may be simulated through the use of chemokines. Taken together this technology enables us to study living cell behavior in a model closely related to the physiological situation *in vivo*. An example of an 8-channel microfluidics biochip is shown in FIGURE 1.

LYMPHOCYTE MIGRATION ON PRECISION-MACHINED SURFACES

In these studies, *in vitro* analysis of cell migration was carried out to observe and quantify the differences in locomotion of two types of lymphoid cells on flat and grooved surfaces.[4] A number of parameters was included for quantitative evaluation of cell motile response, such as the speed and persistence of motion, direction of migration (diffusion), and migratory tracks alignment. The cells studied were peripheral blood T lymphocytes (PBTL) and a malignant T lymphoma cell line (HUT78). It was found that, on the grooved pattern, the lymphoma HUT78 cells were more diffusive in their migration than the T lymphocyte PBTL cells; whereas on the flat surface, the T lymphocyte PBTL cells had a more diffusive response than the T lymphoma HUT78 cells (TABLE 1). It was also found that malignant T lymphoma

TABLE 1. Motilityy of Hut78 and PBTL cells on unconstrained and micropatterned surfacces coated with motility-triggering anti-LFA-1 antibodies

	Individual cell		Cell population			
					χ-test	
Cell type	Speed (μm/min)	Persistence (minutes)	Diffusion coefficient (μm^2/minute)	% Motile Cells	χ^2 value	*P* value
On unconstrained surface						
Hut78	2.39 ± 0.502	28.9 ± 2.74	170.92 ± 23.51	65.9 ± 8.3	38.607	0.991
PBTL	3.69 ± 0.701	20.8 ± 2.56	297.17 ± 25.30	75.8 ± 9.1	36.458	0.960
On micropatterned surface						
Hut78	3.61 ± 0.532	22.3 ± 2.39	309.65 ± 29.59	76.5 ± 10.3	9.530	1.000
PBTL	3.21 ± 0.702	24.3 ± 2.41	262.51 ± 29.45	74.4 ± 11.3	14.469	1.000

NOTE: Values are mean and standard errors in the mean (standard deviation for % motile cells) based on a population of 30 cells for 60 minutes' tracking.

cells are significantly slower and less diffusive when exposed to a plane substrate than when subjected to a grooved substrate. On the contrary, PBTL cells showed only a moderate drop in cell speed and diffusion on the grooved compared to the flat substrate. PBTL cells did, however, show a general alignment to the direction of the grooves, whereas HUT78 did not. Therefore, we have found that the surface topography can influence the motile response of the two different T cell types in different ways, and this can be quantified in terms of specified motility parameters.

MAJOR CYTOSKELETAL AND SIGNALING COMPONENTS OF LFA-–ASSOCIATED COMPLEX: *IN SITU* IMMUNOPRECIPITATION TECHNIQUE

This technique was developed (a) to investigate if a signaling complex is associated with the cross-linked LFA-1 molecule in migrating T cells and (b) to identify the components and functional significance of the complex. For the *in situ* immunoprecipitation, T cells were activated with anti-LFA-1 immobilized on culture flasks. Cells incubated at 37°C developed a locomotory phenotype with extended microtubule-rich uropods, while those at 4°C adhered to the flasks but did not polarize or migrate.[2] Following lysis of the cells with a "microtubule-friendly" buffer, the remaining LFA-1–associated complexes anchored by the antibody were scraped from the surface and analyzed by polyacrylamide gel electrophoresis (PAGE). This demonstrated the specific formation of a multicomponent protein complex in migrating cells. Using a candidate approach and Western blot analysis, we have demonstrated that PKCβ and tubulin are enriched in this complex. In contrast, these proteins were not present in high quantities in cells in which LFA-1 was cross-linked at 4°C and where levels of the LFA-1 molecule itself remained unchanged. Currently we are taking a more broad proteomic approach to identify proteins that associate with the

LFA-1 signaling complex and are subjecting the *in situ* immune precipitates to 2-dimensional gel electrophoresis to be followed by mass spectrometric analysis.

CONCLUDING REMARKS

Efficient, informative studies into the mechanisms of lymphocyte locomotion require nowadays a complex multidisciplinary approach enabling a researcher to dissect the impact of individual stimuli propagating from the multifactorial influence of extracellular microenvironment and intracellular signaling pathways. The emerging technological platforms outlined here and likely to be widely used in future studies include microfluidics biochips, precision-engineered artificial surfaces, live cell imaging with functionally intact fluorescent beacons, and novel techniques in high-throughput proteomics.

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