Characterization of the migration of lung and blood T cells in response CXCL12 in a three-dimensional matrix

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Summary

The ability of T cells to microlocalize within tissues, such as the lung, is crucial for immune surveillance and increased T-cell infiltration is a feature of many inflammatory lung conditions. T-cell migration has mainly been studied in two-dimensional assays. Using three-dimensional collagen gels to mimic the extracellular matrix of lung tissue, we have characterized the migration of T lymphocytes isolated from peripheral blood (PBT) and lung (LT) in response to interleukin-2 (IL-2) and CXCL12. Freshly isolated PBT and LT showed a low degree of migration (blood 4.0 ± 1.3% and lung 4.1 ± 1.7%). Twenty-four hours of culture increased the percentage of migrating PBT and LT (blood 17.5 ± 2.9% and lung 17.7 ± 3.8%). The IL-2 stimulation modestly increased migration of PBT after 6 days (32.3 ± 6.0%), but had no effect on the migration of LT (25.5 ± 3.2%). Twenty-four hours of stimulation with anti-CD3/CD28 caused a small but significant increase in the migration of PBT (to 36.4 ± 5.8%). In a directional three-dimensional assay, CXCL12 failed to induce migration of fresh PBT or LT. Twenty-four hours of culture, which increased CXCR4 expression of PBT 3.6-fold, significantly increased the migration of PBT in response to CXCL12. Migration of PBT to CXCL12 was blocked by pertussis toxin, but not by the phosphoinositide 3-kinase inhibitor wortmannin. Twenty-four-hour cultured LT did not respond to CXCL12. CD3/CD28-stimulation inhibited CXCL12-mediated migration of PBT. These results suggest that the migration pattern of PBT is distinct from that of LT.

Keywords: chemokine; CXCL12; lung; migration; T cell

Introduction

T lymphocytes play a central role in the immune system as both effectors and regulators of the immune response. Throughout the lifespan of a T cell, the ability to migrate to and within lymphoid organs is necessary for survival and function. The lung harbours a large pool of T cells, which reside in the bronchial lamina propria, the alveolar walls and the interstitium.¹ Lung T cells are phenotypically different from blood T cells, a majority being memory T cells expressing the activation markers CD69, CD103 and CD49a.² Lung-resident T cells are involved in the defence against the large number of inhaled pathogens. Hence, recruitment to, and microlocalization within, the lung is crucial for immune surveillance. However, T-cell infiltration is also a feature of many inflammatory lung conditions such as asthma and sarcoidosis.³,⁴ T cells re-circulate from blood to tissue in an organ-specific manner with a bias for memory cells to return to the organ where they encountered their cognate antigen, a process termed lymphocyte homing.³ This process is controlled by a combination of adhesion and chemokine receptors on specific T-cell subsets binding to their ligands, which are expressed on post-capillary venule endothelium in an organ-specific manner. Whereas naive cells circulate only from blood to lymph nodes in a random fashion, memory cells can also enter other organs, particularly of mucosal origin, where they accumulate in
large numbers. The extent to which these cells immediately enter the tissue migrate to the lymph nodes, and then to the blood, and the extent to which they become resident in the tissue, is not known. However, a significant proportion of tissue T cells (unlike blood cells) express receptors such as CD49a, which are only expressed some weeks after activation, suggesting that these T cells at least are resident for a significant period. These resident memory T cells form a pool of antigen-specific cells that are strategically located for a rapid response to the antigen against which they are targeted. Although a great deal is known about the steps controlling migration of T cells from the blood into the tissue, very little is known about the signals that control compartmentalization in tissue. One problem in understanding this process is that to study tissue-specific migration you should be using tissue T cells, which are not readily available.

\textit{In vitro}, T cells have been shown to migrate in response to a variety of stimuli, such as T-cell receptor (TCR) activation, growth factors and chemokines. Like other leukocytes, T cells migrate in an amoeboid fashion, which means that a small protrusion forms a leading edge at the front of the cell; the cell body containing the nucleus is in the middle and an elongated uropod is at the rear. For this tightly regulated cell polarization to occur, the molecular processes in the front of the cell must be compartmentalized. This is thought to be achieved and maintained in part through the action of Rho-family guanine triphosphate (GTP)ases.

Most studies investigating T-cell migration have been conducted in two-dimensional (2D) assays (collagen or fibronectin-coated microscope slides or Boyden chambers), but the mechanisms used for migration within the three-dimensional (3D) environment of the lung tissue are likely to be different. For example, leukocyte migration over a 2D surface requires integrin adhesion, whereas rapid migration in a 3D matrix is integrin-independent and the cell migrates forward by pushing and squeezing itself forward between the matrix fibres.

Collagen is one of the main components of the extracellular matrix and intravital microscopy has shown that leukocytes have a similar migration pattern in 3D collagen gels to that \textit{in vivo} in peripheral tissues. We have therefore used collagen gels to mimic the extracellular matrix of the lung to characterize the migration of human T lymphocytes isolated from peripheral blood and lung in response to the T-cell growth factor interleukin-2 (IL-2) and the chemokine CXCL12. CXCL12 is a potent T-cell chemokine. It has one known activating receptor, CXCR4, which is expressed on almost all T cells. CXCL12 is also expressed constitutively in the lung, making it a suitable mediator to study chemokine-induced migration of T cells in a 3D collagen matrix environment. Although CXCL12 is not considered an inflammatory chemokine, a mouse model of allergic airway disease found a role for CXCL12 in the development of lung inflammation. Hoshino et al. found increased expression of CXCL12 in asthmatic patients compared with normal controls. CXCL12 is also expressed constitutively in the lung. There are no previous studies investigating the migration of human T cells in a 3D assay. However, we have developed a method to purify sufficient numbers of viable lung T cells to investigate lung T-cell migration.

In this study, we found that 24-hr cultured blood T cells migrated significantly towards CXCL12 in a dose-dependent manner, whereas lung T cells and blood T cells stimulated with CD3/CD28 beads did not. The \(G_{\text{z}}\) inhibitor pertussis toxin, but not the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin, blocked this migration.

\section*{Materials and methods}

\subsection*{Cells}

All patients and healthy volunteers gave written informed consent and the study was approved by the Leicestershire Research Ethics Committee. CD3\(^+\) T cells were purified from peripheral blood from healthy volunteers, using Histopaque 1077 (Sigma, Sigma-Aldrich Company LTD, Poole, Dorset, UK) and immunomagnetic sorting for CD3 (Miltenyi Biotech, Surrey, UK) according to the manufacturer’s instructions. Lung T cells were purified from lung resection tissue obtained from patients undergoing surgery for carcinoma at the Glenfield Hospital in Leicester, UK, according to a previously described method. Briefly, the lung specimen was finely chopped and filtered through gauze. T cells were then purified from the resulting filtrate using a discontinuous 40/70\% Percoll gradient and positive selection of CD3\(^+\) cells by immunomagnetic sorting. The viability of the purified cells was > 95\%. The purity of the T cells used in the migration assays was over 95\% for blood and between 80 and 95\% for lung T cells. The contaminating cells in the lung T-cell preparations were macrophages, which could be easily excluded from the analysis of migration because of their size.

\subsection*{Migration in 3D collagen gels}

The T cells (at \(5 \times 10^6\) cells/ml) were incorporated into collagen gels and filmed using OPENLAB software (Improvision, Coventry, UK) and an inverted microscope as previously described. \(^{15}\) T-cell migration was analysed using VOLOSOFT software (Improvision). Cells that were tracked for less than 60 seconds and duplicate tracks of the same cell were excluded from analysis. A migration index was calculated as the percentage of tracks that had a total displacement from the point of origin of more than 10 \(\mu\)m. A threshold of 10 \(\mu\)m was chosen because this avoided...
counting cells that moved on the spot and the percentage of migrating cells was used rather than distance migrated because many cells during the course of the experiment migrated into the gel and were lost from view. The velocity of migration was also recorded for some experiments.

**Stimuli:** CD3/CD28-conjugated Dynal beads (Invitrogen, Paisley, UK) were used according to the manufacturer’s instructions. Interleukin-2 (R&D Systems, Oxford, UK) was used at 4 ng/ml for 24 hr, 48 hr and 5–6 days at 37°C.

In the directional migration assay, the gel chamber was filled to 75% with the cell–collagen mixture, which was allowed to polymerize. CXCL12 (R&D Systems) was then added suspended in phosphate-buffered saline at one side of the gel, immediately before the chamber was sealed and the edge of the gel closest to the chemokine was filmed.

**Inhibitors:** Cells were pre-treated with inhibitors before being added to the collagen. Pertussis toxin (100 ng/ml; Sigma), 2 hr at 37°C. Wortmannin (50 nm; MERCK Chemicals, Nottingham, UK) and Diprotin A (50 nm; Sigma) 30 min at 37°C. The viability of the cells, as determined with trypan blue, was not altered by treatment with any of the inhibitors.

**Flow cytometry**

**Antibodies:** CXCR4 (10 μ/ml) (R&D Systems), fluorescein isothiocyanate-conjugated polyclonal rabbit anti-mouse (1:10; Dako Cytomation, Cambridge, UK), phycoerythrin-conjugated CD26 (1:200; Invitrogen) phycoerythrin-Cy5.5-conjugated CD3 (1:200; Caltag) and phycoerythrin-conjugated CD3 (1:200; BD Biosciences, Oxford, UK). Cells were stained as previously described and acquired in a FACSCanto flow cytometer (BD Biosciences). The results were analysed by FCSDEVA software (BD Biosciences).

**Western blotting**

Blood T cells cultured for 24 hr were exposed to the following conditions: (i) untreated, (ii) 100 ng/ml of CXCL12 for 30 min, (iii) pre-treatment 0-0025% of dimethyl sulphoxide for 30 min + 100 ng/ml for 30 min or (iv) pre-treatment 50 nm wortmannin + 100 ng/ml CXCL12 for 30 min. The cells were pelleted and lysed in Laemml buffer (Sigma) diluted 1:2 in distilled water. The lysates were boiled for 5 min and run on a 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis gel. The proteins were then electro-transferred to Immobilon P transfer membranes (Millipore, Watford, UK). After blocking for 1 hr in 5% milk, the membranes were probed with rabbit anti-human Phospho-Akt antibody (1:1000) (Cell Signaling Technology, Danvers, MA) in 5% bovine serum albumin washed, followed by 1 μg/ml of horseradish peroxidase-conjugated polyclonal swine anti-rabbit-horseradish peroxidase antibody in 5% milk. For detection, enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK), was used. Total Akt was used as a loading control, using the same staining protocol on stripped membranes.

**Statistical analysis**

To test differences between two groups, Student’s t-test was used, whereas one-way analysis of variance (ANOVA) and Bonferroni’s post test were used to test differences among three or more groups.

**Results**

**Freshly isolated T cells do not migrate spontaneously**

When freshly isolated T cells from blood or lung were incorporated into 3D collagen gels in a non-gradient assay in the absence of any chemotactic signal, only a very few cells migrated (blood 4.0 ± 1.3%, n = 14; lung 4.1 ± 1.7%, n = 15). After 24-hr culture in medium there was a significant increase in migration of both lung and blood T cells although still only a minority of cells demonstrated migratory behaviour (blood 17.5 ± 2.9%, P < 0.05; lung 17.7 ± 3.8%, P < 0.001, n = 15) (Fig. 1a,b). Similar results were obtained when the cells were cultured in serum-free medium (data not shown). There were no significant differences between the lung and blood T cells in the percentage of migrating cells or in the velocity of migration (7.45 ± 0.9 μm/min versus 8.06 ± 1.9 μm/min, P = 0.40). Cells migrated in a non-directional manner (Movie S1).

**IL-2 stimulation**

Stimulation of peripheral blood T cells with IL-2 resulted in a small increase in migration, which only reached significance compared with spontaneous migration after between 5–6 days in culture (17.7 ± 5.0% versus 23.2 ± 6.0%, P < 0.05, n = 5) (Fig. 2a). In contrast stimulation of lung T cells had no effect on migration compared with control at any time-point (28.3 ± 5.2% versus 25.5 ± 3.2%, n = 8) (Fig. 2b).

**T-cell migration in response to CXCL12**

To investigate the migration of T cells towards a chemokine in the 3D collagen matrix, we exposed the T cells to CXCL12 in a modification of the non-directional assay in which the chemokine was placed on one side of the gel.
and allowed to diffuse into the gel so creating a chemotactic gradient. Freshly isolated blood T cells were unable to migrate towards CXCL12, (4.5 ± 2.2% versus 6.2 ± 2.1%, n = 4). After 24 hr of culture, which resulted in a 3.6-fold up-regulation of CXCR4 (data not shown), a large proportion of blood T cells migrated in response to CXCL12 with an optimal concentration of 100 ng/ml (67.3 ± 5.30%, n = 4; Fig. 3a). Blood T cells responded to CXCL12 by migrating in a directional manner towards the chemoattractant gradient, as seen in a vector diagram of the pattern of movement of the individual cells (Fig. 3b and Movie S2). Neither freshly isolated nor 24-hr cultured lung T cells demonstrated significant migration in response to 100 ng/ml of CXCL12 (1.63 ± 0.8 versus 2.73 ± 1.6%, n = 3 and 11.75 ± 3.2 versus 21.24 ± 6.0%, P = 0.22, n = 4, respectively) (data not shown).

**Stimulation with CD3/CD28**

Stimulation of blood T cells with CD3/CD28 beads for 24 hr caused a significant although modest increase in random migration in the non-gradient assay (36.4 ± 5.8%, versus 18.37 ± 3.1%, P < 0.05, n = 10; Fig. 4). We then investigated the effect of CXCL12 in a
We therefore investigated if the enzymatic activity of this protein was the reason why lung T cells were constitutively unresponsive to CXCL12 and why CD3/CD28 stimulation rendered the blood T cells unresponsive to CXCL12. The majority of lung (75.3 ± 6.7%, n = 4) and blood (77.7% n = 1 with CD3/CD28 activation and 75.6% n = 2 without activation) T cells expressed CD26. However, inhibition of CD26 activity by Diprotin A did not increase the migration in response to CXCL12 of either lung T cells or activated blood T cells (data not shown).

**Pertussis toxin, but not wortmannin, inhibits CXCL12-induced migration**

Migration in response to CXCL12 in the gradient assay was completely blocked when 24-hr cultured blood T cells were pre-treated with pertussis toxin, confirming that the Gi subunit of the G-protein coupled receptor is involved in CXCL12-mediated migration in our model (Fig. 5a). In contrast, inhibition of PI3K activity with wortmannin had no effect of CXCL12-induced migration of blood T cells (Fig. 5b), although Western blotting showed that the PI3K product phospho-Akt increased after stimulation with CXCL12 and that this was blocked when the cells had been treated with wortmannin before stimulation (Fig. 5c).

**Discussion**

T-cell trafficking through the lung is important for immune surveillance and host defence but inappropriate
T-cell activation is associated with a number of inflammatory lung diseases. The factors controlling migration of lung T cells in lung tissue are therefore of considerable interest. Lung T cells are quite distinct from peripheral blood T cells in particular in terms of adhesion, activation and chemokine receptor expression. The migratory behaviour of tissue-derived T cells is therefore more relevant to the physiology of T-cell migration in tissue than that of peripheral blood T cells but the migratory behaviour of human tissue-derived T cells is rarely studied. This is the first study we are aware of that has investigated the migratory pattern of lung T cells or indeed tissue T cells from any source in an assay of migration that attempts to model the tissue matrix environment. We have demonstrated that unlike blood T cells, lung T cells were unable to respond to the growth factor and chemokine-activating stimuli IL-2 and CXCL12. In addition, we demonstrated that migration of peripheral blood T cells to CXCL12 was inhibited by activation through the TCR. Migration to the chemokine was pertussis toxin dependent but independent of PI3K. These data are consistent with a model in which tissue T cells which encounter cognate antigen become unresponsive to migration stimuli and so become resident in the tissue for prolonged periods.

Immediately after isolation, both lung and blood T cells failed to migrate either spontaneously or in response to CXCL12 (Fig. 1 and data not shown). The cause of the lack of responsiveness of freshly isolated T cells and whether this has any in vivo significance is not clear but may be simply the result of an effect of being kept at 4°C for several hours during the purification procedure. However, in one experiment using the Boyden chamber, freshly isolated blood cells migrated to CXCL12, although migration was less than cells cultured overnight in medium (40% versus 99%). This suggests that as well as an effect of the isolation procedure, cells were less able to migrate in the 3D environment compared with the 2D environment. The reasons for this are not clear, although they may be associated with the relative unimportance of adhesion receptors in the 3D assay. After 24 hr in culture, both lung and T cells migrated spontaneously to a similar degree with up 40% of cells demonstrating random migration. This did not appear to be the result of factors in fetal calf serum because spontaneous migration also occurred after culture in serum-free media.

Eosinophils do not migrate spontaneously in the 3D collagen assay but show vigorous random migration in response to the growth factor granulocyte–macrophage colony-stimulating factor after only a few minutes of stimulation. We therefore hypothesized that IL-2, an archetypal T-cell growth factor, would have the same effect. However, IL-2 at a universally established effective dose had only a modest effect on the migration of blood T cells compared with spontaneous migration, which was only significant after 5 days and there was no effect at all on lung T-cell migration. Similar data to purified blood CD3 cells were obtained with peripheral blood mononuclear cells obtained without magnetic bead purification (data not shown) suggesting that the response to IL-2 was not influenced by the method of purification. Similarly another activating stimulus, CD3/CD28, had only a modest effect on blood T-cell migration. It was not possible to stimulate lung T cells through CD3 because they were purified using positive selection with anti-CD3 antibody. In contrast to IL-2 a robust response was seen with CXCL12. CXCL12 is a potent T-cell chemokine and its receptor is expressed by almost all T cells, making it a good candidate for comparing the response of blood and lung T cells to chemokines in our system. CXCL12 is also constitutively expressed in the lung. CXCL12 had a marked and consistent chemotactic effect on blood T cells after 24 hr in culture. The difference between the response of freshly isolated and 24-hr cultures of blood T cells may be the result of a marked up-regulation of expression of the receptor for CXCL12, CXCR4. The expression of CXCR4 on freshly purified blood cells was the same as expression on the T cell before purification, showing that the receptor was not shed or down-regulated during purification (data not shown). Lung T cells that highly expressed CXCR4 did not respond effectively to CXCL12 even after overnight culture.

We cannot exclude the possibility that the differences between the lung and blood T cells were the result of either differences in the source of these cells or the method of purification. We were unable for logistical reasons to obtain matched samples and the lung cells were from patients undergoing resection for lung cancer whereas the blood cells were from healthy volunteers. However, patients undergoing lung resection are generally healthy with only minimal smoking-related airways disease and early-stage malignancy and it seems unlikely that their blood cells would behave in a fundamentally different way to those from healthy volunteers. The purification methods were similar involving immunomagnetic selection although selection was positive for the lung T cells. However, the beads used are not thought to stimulate T-cell activation.

In vivo T cells are likely to be exposed to both activating and migratory stimuli. We therefore investigated the response of blood T cells stimulated with both anti-CD3/CD28-conjugated beads for 24 hr and CXCL12. Perhaps surprisingly, stimulation with anti-CD3/CD28 rendered the cells unresponsive to CXCL12. One explanation for this result may be that the activation status of the T cells affected their response to chemokines. A majority of lung T cells are activated effector/memory T cells. A cross-talk between the TCR and CXCR4 has been described by Peacock et al., who showed that TCR-stimulation reduced chemotaxis to CXCL12 and conversely that CXCL12...
inhibited TCR-mediated signalling pathways. The two signalling pathways were shown to converge at protein kinase C or phospholipase C/β3, which phosphorylated CXCR4, triggering its desensitization for CXCL12. This cross-talk might explain the lack of migration of the in vitro-activated blood T cells to CXCL12 and could possibly also affect the migration of lung T cells.

We investigated the possibility that up-regulation of the dipeptidase CD26 on activated T cells might destroy the CXCL12 gradient by proteolytic cleavage. However, flow cytometry of CD26 showed no difference in the CD26 expression between unstimulated blood T cells, CD3/CD28-stimulated blood T cells and lung T cells. Nor did inhibition of CD26 using Diprotin A restore chemotaxis of lung T cells or activated blood T cells to CXCL12 suggesting that CD26 is not responsible for the inhibition of chemotactic responses to CXCL12 by activated T cells in our 3D migration assay.

It is not clear how the signal mediated by CXCL12 is transduced from CXCR4 to the migratory machinery of T cells migrating within a three-dimensional environment. As expected, the chemotactic migration of blood T cells to a CXCL12 gradient was completely inhibited by pertussis toxin, which confirms that G proteins containing the Gαi subunits are crucial for chemokine-mediated migration in our 3D collagen gel model. Chemokine receptor engagement triggers the exchange of GDP to GTP on the Gα subunit, which causes the heterotrimeric G protein to disassemble into the Gβγ subunits. These then propagate effects downstream of the receptor, ultimately leading to polarization of the cell and subsequent migration towards the chemokine.19 A fairly shallow chemokine gradient of < 10% difference in concentration between front and back of the cell can still cause directional movement. This is thought to be possible because of gradient amplifying Rho GTPases. Rac and Cdc42 at the front of the cell activate Wiskott-Aldrich syndrome protein (WASP) and WASP-family verprolin homologous protein, respectively, which in turn mediate actin polymerization through the Arp2/3 complex.20,21 Rac is thought to be involved in a positive feedback loop with PI3K/phosphatidyl inositol 3,4,5-triphosphate (PIP3),22,23 whereas Rho is more active at the feedback loop with PI3K/phosphatidyl inositol 3,4,5-triphosphate at the rear and so amplifies the polarization.25 For this reason, PI3K is considered a central molecule in chemotactic migration. It was therefore surprising to find that inhibiting PI3K with wortmannin had no effect on blood T-cell migration to CXCL12. The same lot of the inhibitor was efficient in inhibiting neutrophil migration to CXCL8 in the same 3D gradient assay (manuscript in preparation). We also showed that up-regulation of the PI3K product phospho-Akt was inhibited when the cells had been pre-treated with wortmannin, confirming the effect of the inhibitor. It is interesting that although PI3K is clearly activated by CXCL12, it is dispensable for the migration towards this chemokine in 3D collagen gels. It has been suggested that DOCK2, which has been shown to be essential for the chemotaxis of T cells to CXCL12 in a transwell chemotaxis assay, can act as a Rac guanine nucleotide exchange factor independently of PI3K.26,27 The activity of DOCK2 might explain why blood T cells were able to migrate in response to the CXCL12 gradient although PI3K was inhibited. More work is needed to dissect the signalling pathway of CXCL12-induced migration.

When we compared the gene expression profile of lung and blood T cells in a 6000 gene microarray, we found that Rac2 was consistently more expressed by T cells from the blood, whereas the Rho was preferentially expressed by lung T cells. The balance between Rac and Rho has been shown to be important for cell migration.28 Treatment of a brain cancer cell line with an inhibitor which caused down-regulation of Rac and up-regulation of Rho led to a significant decrease in mobility.29 It is therefore possible that the Rho/Rac imbalance present in lung T cells could have influenced their migration within the collagen matrix.

In summary, we demonstrated that in a three-dimensional migration model, T cells derived from lung tissue have a migratory phenotype distinct from that of blood T cells, particularly in terms of their response to chemotactic signals. We also found that chemotaxis of blood T cells to CXCL12 in this type of assay is independent of PI3K activity.

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 Supporting Information

Additional Supporting Information may be found in the online version of this article:

 Movie S1. Representative time-lapse movie of the spontaneous migration of 24-h cultured peripheral blood T cells in a three-dimensional collagen matrix.

 Movie S2. Representative time-lapse movie showing the directional migration within a 3D collagen matrix of 24-h cultured T cells from peripheral blood in response to a gradient of CXCL12. 100 ng/ml of the chemokine was added to the right side of the polymerized gel immediately before filming.

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