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Epithelial Rho-GTPases and the trans-epithelial migration of lymphocytes

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Abstract

Tissue injury and inflammation leads to leukocyte recruitment from the blood stream into the inflamed organ. Leukocytes in excessive numbers and over prolonged periods can cause tissue damage, and so it is important that the trafficking of leukocytes is regulated. Although much attention has focussed on leukocyte recruitment much less is known of about the resolution of inflammation. Hollow organs, such as the lung and the gut, are unique in that tissue accumulation of leukocytes is determined by recruitment of leukocytes from the blood; survival of tissue leukocytes; and migration of leukocytes from the interstitial space, either to the lymphatics or into the lumen of the organ, so called egression (Sheppard, 2002). The physiological importance of egression was demonstrated by Corry and colleagues who showed that preventing egression of peribronchial leukocytes in a murine model of bronchial inflammation was fatal (Corry et al., 2004; Corry et al., 2002). This has led to an interest in the molecular mechanisms underlying egression from the lung. We have used a human bronchial cell line, 16HBE14⁰⁻, in vitro to analyze trans-epithelial migration and to investigate the role of Rho-GTPases in this process. Here we describe methods to establish monolayers of bronchial epithelial cells either the correct way up or inverted on TranswellTM filters, we describe an assay of trans-epithelial migration of primary human T lymphocytes across this monolayer. We show how this system can be used to dissect out the molecular events that are required for successful egression. In particular, pre-treatment of either the lymphocytes or the epithelium with blocking antibodies against cell surface receptors or with cell permeable inhibitors directed against signaling molecules allows an analysis of the individual roles played by the T lymphocytes and the epithelial monolayer.

II. Introduction

1. Trans-epithelial migration in the lung, gut and other hollow organs

The lung epithelium provides an extensive surface area, in direct contact with the outside environment. This is essential for effective gas exchange but leaves the lung uniquely susceptible to damage or infection by inhaled allergens and pathogens, and may explain why lung disease is the single greatest cause of death worldwide (WHO, 2003). Because of this threat there is a need for constant immune surveillance, and lymphocytes traffic through the lung continuously, with rapid recruitment of T lymphocytes when foreign antigens are recognised. Th1/Tc1 effector T lymphocytes play an essential role in the immune response against infectious diseases and may be distinguished from Th2/ Tc2 cells by a higher expression of CCR5 and CXCR3 and an enhanced response to the ligands for these receptors (Bonecchi et al., 1998; D'Ambrosio et al., 1998; Moser and Loetscher, 2001). Although essential to fight infection, excessive or prolonged infiltration of the lung by effector Th1/ Tc1 cells may underlie pathology of diseases as diverse as influenza

(Humphreys et al., 2003; Hussell et al., 2004) and tuberculosis (Guyot-Revol et al., 2006), and non infectious lung diseases, such as chronic obstructive pulmonary disease (COPD) (Grumelli et al., 2004), a common debilitating inflammatory diseases of the lung caused by tobacco smoke and other inhaled pollutants. An understanding of the patho-physiology behind these disease underlies much of respiratory medicine; tuberculosis is responsible for >1.5 million deaths a year, influenza may occur in devastating epidemics, and COPD is predicted to become the 3rd most common cause of death worldwide by 2020. Much of the damage and death caused by these diseases has been shown to be due to tissue destruction in association with excessive leukocyte recruitment. It is therefore essential that, during the immune response to these diseases, effector T cell movement into the lung is regulated and that accumulated T cells are promptly cleared when the immediate threat is over.

Although much is know of how effector T lymphocytes enter the lung, the clearance of these cells from inflamed tissue during the resolution phase has been less well studied The chemokine receptor CCR7 directs the migration of CCR7⁺ effector and memory lymphocytes from peripheral tissues (via afferent lymphatics) to the lymph-nodes (Bromley et al., 2005; Debes et al., 2005), but the factors, if any, that determine the exit of CCR7⁻ effector and memory T cells from peripheral non-lymphoid tissues such as the lung remain unknown. It has been assumed that most infiltrating leukocytes either undergo necrosis or apoptosis at the site of inflammation, ignoring a potentially very important exit pathway across the bronchial epithelial barrier. Migrating leukocytes, having crossed the epithelial barrier, into the airway would be carried on the mucociliary escalator to the pharynx for removal. Such egression or luminal clearance from the lung has been described for eosinophils and neutrophils (Erjefalt et al., 2004; Uller et al., 2001), and there is recent compelling evidence that trans-epithelial migration of all classes of leukocyte plays a key protective role in experimental murine models of bronchial inflammation (Corry et al., 2004; Corry et al., 2002).

Despite the potential importance of lymphocyte egression from the lung across the bronchial epithelium, this process has not been well studied, in stark contrast to the extensive research into trans-endothelial migration. There are critical differences in migration across the endothelial and epithelial barriers, the most obvious being that the epithelium forms a much 'tighter' barrier than that formed by the endothelium and maintenance of this barrier is essential: in addition, leukocyte recruitment across epithelium occurs in a basal-to-apical direction, opposite to that across endothelium.

2. Role of Rho-GTPases in TEpM

Epithelia form a barrier between the organism and the outside world. All epithelia are polarised into an apical (luminal) compartment and a baso-lateral compartment, which are separated by the apical junctional complex (AJC). The AJC consists of the apical tight junction and the more basal adherens junction and desmosomes. Rho-GTPases have been shown to be involved in the formation and stabilisation of intercellular junctions (Etienne-Manneville and Hall, 2002; Van Aelst and Symons, 2002). It is assumed that the majority of leukocytes that cross a tight epithelial barrier will move between adjacent epithelial cells and across the tight inter-epithelial junctions. It is thought that the leukocytes will have to transiently disrupt the junctions to allow their passage across. It therefore seems highly likely that the Rho-family of GTP-ases and the actin cytoskeleton will be involved in tight junction regulation during leukocyte trans-epithelial migration.

III. Culture of bronchial epithelial cells

We screened several bronchial epithelial cell lines to find one that was suitable for this assay. The 16HBE14⁰⁻ is frequently used in studies that are looking at tight junctions, and

has been shown by others to make structural and functional tight junctions (Wan et al., 2000; Wan et al., 1999).

1. Materials

The SV40 transformed human bronchial epithelial cell line, 16HBE14o- (Gruenert et al., 1988), was a kind gift from Dieter Gruenert. BSA, EDTA, EGTA, polyvinylpyrrolidone and human fibronectin are from Sigma-Aldrich (Gillingham, UK); modified Eagle medium (MEM) is from Gibco, Invitrogen Corporation (Paisley, UK). PureCol is from Nutacon BV (Leimuiden, The Netherlands). Fetal bovine serum (Mycoplex™) is from PAA Laboratories (Yeovil, UK). Cotton wool tips are purchased at a local drug-store and autoclaved before use (Boots, Nottingham, UK).

2. Methods

16HBE14⁰- cells are best cultured on flasks or membranes that have been pre-coated overnight at 4°C with 30 µg/ml PureCol, 100 µg/ml BSA and 10 µg/ml fibronectin in PBS-A (we call this coating mix). To coat the flasks we add the coating mix at 100 µl/cm², and for filter membranes we coat only the side of the filter on which we want the cells to grow. We add 300 µl/cm² to coat the inner membrane or 1200 µl/cm² in the outer well to coat the underside. After incubation overnight, the coating mix is removed and the flasks or membranes are rinsed once with PBS-A. The 16HBE14⁰- cells grow well in MEM with 10% fetal bovine serum at 37 °C with 5% CO₂. Flasks of cells should be passaged (see below) when the cells are 80% confluent. If monolayers are left for a long period, they will overgrow and cells may pile up on each other.

2.1 Culture of 16HBE14o- cells as monolayers on filters—For the trans-epithelial migration assay 16HBE14⁰ cells are passaged under gentle trypsinization. The monolayer of cells is washed in PBS-A to remove inhibitors of trypsin and then released from the plastic in 0.02% trypsin/ 0.0016% EDTA with 0.02% EGTA and 1% polyvinylpyrrolidone solution in hepes buffered saline. The cells take several minutes to be released under these conditions. After release, the cells are spun down for 5 min. at 1,000 rpm, and re-suspended in fresh culture medium. Then 100 µl of cells at 1×10^6 /ml are plated on 8 µm pore size, 6.5 mm diameter polycarbonate filters in 24-well Transwell chambers (Costar, Corning) and grown for 10-11 days. During this time epithelial cells move across the filter to grow on both sides. The monolayers are checked for impermeability to fluid by the ability to maintain a fluid level difference between inner and outer wells. The inner well is filled right up with medium and the outer well has 600 µl of medium so that there is a fluid level difference between the two. The monolayers are then returned to the incubator for 24 h. After this time the fluid level in the inner wells should be unchanged. If the inner well fluid level falls, the monolayers are not yet ready and should be left for another 24 h before retesting. We find that after 10-12 days most of the monolayers are tight. Cells are then removed from one side of the monolayer with a cotton wool tip to leave a monolayer on either the top or bottom of the filter. To do this the wells are emptied and washed with PBS-A, and 100 µl of fresh PBS-A is pipetted into the inner well. Then, one end of a cotton wool tip is gently pushed against the filter and moved in two full circular motions, to remove adherent cells from that side of the filter. Any non-adherent cells are then removed by a further rinse with PBS-A and the monolayers are put back in the incubator with 300 µl of medium in the inner well and 400 µl of medium in the outer well. After four hours if a fluid level difference is maintained then an air liquid interface is introduced for the bronchial epithelial cells. This involves having medium only on the basolateral side of the monolayer and leaving the apical surface with no medium. This air liquid interface is thought to help the tight junctions to reform more quickly. The monolayers are left in culture for a further 24 h before recording their TER and permeability to FITC-dextran 40 kDa (see below).

IV. Measurement of TER and permeability to FITC-dextran 40 KDa

1. Materials

EVOM™ Epithelial Volt-ohmmeter was purchased from World Precision Instruments Ltd (Stevenage, UK). FITC-dextran 40 kDa is from Sigma-Aldrich, SPECTRAMax GEMINI XS Microplate Spectrofluorometer was from Molecular Devices Ltd (Wokingham, UK). Hanks balanced salt solution (HBSS) is from Gibco Invitrogen Corporation.

2. Methods

Trans-epithelial electrical resistance (TER) is measured using a volt-ohmmeter. Confluent monolayers are washed and placed in HBSS to equilibrate for 30 min at 37°C. The voltage across the monolayer is recorded and expressed as ohm.cm^2 . The voltage across a filter with no epithelial cells is taken as the background reading and may be subtracted from other readings. To measure FITC-dextran permeability, the FITC-dextran is dissolved in water at 50 mg/ml and diluted 1:200 in RPMI, and briefly sonicated, prior to use. Permeability to FITC-dextran is measured by washing the monolayers into RPMI/ 0.5% BSA, and taking a filter without epithelial cells as a control. After washing, the monolayers and filters are placed in an empty 24 well plate with 600 μl of RPMI/ 0.5% BSA in each well. Then, 100 μl of FITC-Dextran, molecular weight 40 kDa, is added to the upper chamber. The plate is incubated at 37°C. After 30 min, 100 μl of medium is withdrawn from the lower chamber and put in an empty well of a 96 well plate, 100 μl of medium alone is added to an empty well of the 96 well plate to assess background fluorescence. Fluorescence of the medium in the 96 well plate is measured on a SPECTRAMax GEMINI XS Microplate Spectrofluorometer (Molecular Devices; Ex: 485 λ ; Em. 538 λ). Fluorescence is linear for the concentration of FITC-dextran used and the permeability of the monolayer is expressed as % permeability of the filter alone.

IV. Culture of primary human T lymphocytes

1. Materials

T cells are obtained as a buffy coat preparation from UK National Blood Services (Tooting, London UK). Lymphoprep® is from Pharmacia Diagnostics AB (Uppsala, Sweden); RPMI 1640 is obtained from Gibco, Invitrogen Corporation. Phytohaemagglutinin (PHA) is from Murex Diagnostics (Dartford, UK). Interleukin (IL)-2 is from Euro Cetus UK Ltd., (Harefield, UK)

2. Methods

Peripheral blood mononuclear cells are prepared from single donor leukocyte buffy coats by centrifugation through Lymphoprep®. T cells are expanded from this population by culturing in RPMI 1640 plus 10% FCS in the presence of PHA at 1 $\mu\text{g/ml}$ for 72 h as previously described (Dransfield et al., 1992). Mononuclear cells attach to the plastic flask during culture and can be removed after 72 h. Cells are washed and maintained for 1-2 weeks in medium supplemented with 20 ng/ml recombinant IL-2. The cells, are used between days 10 and 14, and are 99% CD3+ population, containing 65% CD8+ and 35% CD4+ cells. The population is negative for the natural killer cell marker CD56. The lymphocytes are of a Th1/Tc1 effector phenotype expressing CXCR3, with varying levels of CCR5 and CCR7. These T cells will undergo chemotaxis in response to various chemokines including CCL5, CXCL9, 10 and 11 (data not shown).

V. Development of an assay for lymphocyte transepithelial migration

1. Materials

Transwell polycarbonate filters are from Corning/Costar (Corning, NY, USA). SonicSeal™ 4-well slide (permanox™ pastic) are purchased from Nalge Nunc (Rochester, NY, USA). Cell tracker green (CMFDA) is obtained from Molecular Probes, Invitrogen Corporation. Chemokines are from Peptrotech EC (London, UK). Rabbit anti-human ZO-1 antibody (Z-R1) is from Zymed Laboratories (San Francisco, USA). Cytochalasin D and Y27632 are from Sigma Aldrich, UK; Wiskostatin is from Calbiochem (Merck Chemicals Ltd., Nottingham, UK). Chemokines and cytokines are purchased from Peptrotech. Formaldehyde 16% (w/v) is purchased from TAAB Laboratories (Reading, UK); Triton X-100 is purchased from Sigma-Aldrich; Mouse blocking monoclonal antibody '38' against LFA-1 was a kind gift from Nancy Hogg (CRUK, London, UK). DakoCytomation fluorescent mounting medium is from Dako North America, Inc. (Carpenteria, USA).

2. Methods

2.1. Chemotaxis assays—Chemotaxis of T cells in response to various chemokines is investigated in 24-well Transwell chambers using 5 µm pore size, 6.5 mm diameter polycarbonate filters. First, T cells are labelled with 50 nM cell tracker green (CMFDA). T cells are washed twice into RPMI alone, and then resuspended at 2×10^6 / ml, and 50 nM CMDA. The T cells are incubated at 37°C and 5% CO₂ for 30 min. Labelled T cells are then washed in RPMI and re-suspended in RPMI/ 0.5% BSA at 2×10^6 / ml. Chemokine is placed in the lower chamber in 600 µl of RPMI/ 0.5% BSA and then 100 µl of T cells is added to the upper chamber. After 90 min. migrating T cells are retrieved from the lower chamber and counted as FL1 positive cells recorded in a set time (30 secs.) by fluorescence activated cell scanning (FACS). A sample well in which 100 µl of T cells is added directly to an outer well containing 600 µl of medium is also counted and taken as the 'total cells added'. Chemotactic index is expressed as number of T cells migrating as % of total cells added.

2.2. Trans-epithelial migration assays—To establish an assay of trans-epithelial migration we first established a polarised epithelial monolayer of the bronchial epithelial cell line, 16HBE14⁰⁻, cultured either as standard or as inverted monolayers on Transwell™ filters to allow access to both apical and basal surfaces (Fig. 1a). The presence of tight junctions was confirmed using a combination of fluorescence microscopy for the junctional component, zona occludens-1, (ZO-1; Fig. 1a), combined with measurements of TER using a volt-ohmmeter and quantification of permeability to 40kDa FITC-dextran. We find that the 16HBE14⁰⁻ cell line provides an accurate representation of human bronchial epithelium as seen *in vivo*; the cells polarise and form tight junctions. The 16HBE14⁰⁻ monolayers are fluid impermeable with TER >250 Ω.cm² and with permeability to 40kDa F-D of <0.5% of the filter alone. There is no difference in tight junctions between the standard or inverted monolayers.

Only those monolayers with a TER of 200 ohm. cm² and a permeability of <0.5% of filter alone are used in our trans-epithelial migration assays. Epithelial monolayers are washed extensively to remove dextran and then rinsed with RPMI/ 0.5% BSA and placed in an empty 24 well plate. At this point, 100 µl of labelled T cells at 4×10^6 / ml are added to the monolayers, and the inserts are transferred to new wells of a 24-well plate containing 0.6 ml of RPMI/ 0.5% BSA and the indicated chemokine in each outer well. The Transwells are then incubated at 37°C in 5% CO₂. After 90 min, T cells, which have migrated through the epithelial monolayer into the lower chambers, are recovered. FL1 positive cells are counted on FACS and the percentage of migrated cells is calculated as above.

Although very few (<5%) of total T cells added to the inner well move across an uncoated filter or a monolayer of 16HBE14⁰ cells in the absence of a chemotactic gradient, 10-60 % of the T cells will move across both the filter (chemotaxis) or the epithelial monolayer (trans-epithelial migration) in response to a chemotactic gradient. The variation occurs because T cells for each experiment are from individual donors. To check that this is true chemotactic movement, the chemotactic gradient can be abolished, by placing equal concentrations of chemokine in the upper and lower well. Under these conditions both chemotaxis and trans-epithelial migration should be prevented. Likewise, the specificity of the chemotaxis can be examined by the ability of blocking monoclonal antibody (mAb) against the appropriate chemokine receptor to prevent both chemotaxis and trans-epithelial migration. In this case the T cells are washed and then pre-incubated for 30 min. on ice with the appropriate blocking monoclonal antibody. The T cells are then washed and resuspended in RPMI/ 0.5% BSA before being used in a chemotaxis or trans-epithelial migration assay.

2.3 Time-lapse digital microscopy of trans-epithelial migration—Leukocyte/epithelial cell interactions can be visualized in real time to allow further insight into the route taken by migrating T cells. The trans-epithelial migration assay is set up as above. To look at basal-to-apical migration, a filter with T cells in the inner well and epithelial cells growing on the underside of the filter, is moved into a SonicSeal™ 4-well slide. Medium containing a chemoattractant is placed in the outer well. Microscopy is performed with time-lapse photography. Nomarski differential interference contrast images are captured every 30sec and analyzed on quick time movies using ImageJ software. The time course for trans-epithelial migration is slower than that for chemotaxis, and cells continue to migrate for several hours after the beginning of the assay whilst the majority of chemotaxis has occurred within 90 min. The video microscope is focused on the basal surface of the monolayer with the filter above the plane of focus so that the holes in the filters appear out of focus (Fig. 2; open black arrows). This video tracking allows us to see where the T cells (Fig. 2: white arrows) interact with the epithelium, how they move along the epithelium and preferential areas of trans-epithelial migration (Fig. 2; closed black arrows show migrated T cells).

2.4 Effect of inhibition of Rho-GTPase effectors on trans-epithelial migration—This model for trans-epithelial migration is very amenable to manipulation using antibodies against surface molecules on the T cell or the epithelial monolayer, or the use of inhibitors on the T cell or the epithelial monolayer. When using antibodies, we find it best to titrate the antibodies on the T cells or the epithelium, using FACS, to find the optimal concentration of antibody to use to block all the cell surface receptors. T cells or epithelium can be preincubated with the appropriate antibody for 30 min. at 37°C before washing twice in RPMI/0.5% BSA and performing the assay as normal. One advantage of the Transwell™ system is that antibodies can be added to either the apical or the basal surface of the epithelium to look for differential effects. The use of blocking antibodies allows a dissection of the cell surface molecules involved in the different stages of trans-epithelial migration.

As well as manipulation with blocking antibodies, the system can be used to examine the effects of inhibitors on either the T cells or the epithelium. We have used this system to examine the role of the epithelial Rho-GTPases during the trans-epithelial migration of T cells. The epithelial monolayer is washed and then incubated in various concentrations of a given inhibitor for various times at 37°C and 5% CO₂. The inhibitor is then washed out extensively, we wash 3 times and place the filters into a new 24-well plate. In order to be able to interpret the results of inhibitor assays, it is very important to document the effect of the inhibitors on the monolayer and epithelial junctions. To do this we measure the TER and epithelial permeability before treatment, during incubation with the inhibitors and after washing out the inhibitors. We then fix some of the monolayers and look at the effect on the monolayer and epithelial junctions (see section 2.5 below for staining details). Concurrently

with these experiments we also carry out trans-epithelial migration assays with unfixed but treated epithelial monolayers as above. These assays are very good for screening an effect of an inhibitor but further detailed studies will be required depending on the specific findings. If we identify a blocking antibody or inhibitor that affects trans-epithelial migration, then we have found it helpful to look in detail at effect on the migrating cells using confocal microscopy.

To perform such confocal experiments, we carry out trans-epithelial migration assays with and without the relevant blocking monoclonal antibody or inhibitor and at various time-points we wash off the non adherent T cells and fix the epithelial monolayers. The fixed monolayers can then be stained for analysis using confocal microscopy. This approach allows us to identify at which point in the process of trans-epithelial migration the T cells are inhibited and is described in detail below.

2.5 Confocal microscopy of epithelial monolayers with migrating T lymphocytes

—For confocal studies trans-epithelial migration is performed as above, but the T cells are unlabelled. At the indicated time the monolayers are washed briefly in warmed RPMI/ 0.5% BSA to remove non-adherent T cells and then fixed in 4% formaldehyde/ PBS-A for 20 min. at room temperature. Monolayers are permeabilized for detection of intracellular antigens, such as ZO-1, by incubation with 0.4% triton-X 100 for 10 min at room temperature. Antibody stainings can be performed whilst the filters are still in the wells. If the antibody is only available in small amounts then the wells can be inverted and 50-100 μ l of antibody/ PBS-A can be placed on the upturned undersurface. The filters must be kept in a humid chamber during the incubation to prevent them drying out. Alternatively the filters can be cut from the well prior to staining using a scalpel blade and taking care to remember which way up the epithelial cells are orientated. Subsequent antibody binding steps can be carried out by inverting the filters epithelial side down onto 50-100 μ l of antibody on parafilm™. Depending on the antibody we usually leave the primary antibody on for 30 min. at room temperature and then wash three times in PBS-A. We then use an appropriate fluorescent coupled secondary antibody and incubate for 30 min. at room temperature in the dark. We then wash four times with PBS-A before mounting the filters, epithelial cells down on 12 μ l of DakoCytomation fluorescent mounting medium on a clean microscope slide. A further 12 μ l of DakoCytomation fluorescent mounting medium is placed on top of the filter and this is then covered with a circular coverslip. The slides are left in the dark at room temperature overnight for the mountant to set. T cells can be well visualized by labelling with mAb against cell surface molecules that allow the lymphocytes to be distinguished from the epithelial cells, for example anti-CD3, followed with a fluorophore conjugated goat anti-mouse secondary. In contrast, epithelial junctions can be visualized with rabbit anti-ZO-1 and a goat anti-rabbit secondary antibody that has been conjugated to a different fluorophore. We have successfully performed confocal microscopy on such specimens, using a Zeiss laser-scanning microscope LSM 510 equipped with a 60X oil immersion objective (Karl Zeiss, Oberkochen, Germany). Images are collected as horizontal sections taken at intervals through whole cell volumes and can be compiled for display from a projection of the complete Z-series, or as a Y-Z display using ImageJ software. This allows a very careful analysis of the spatial distribution of junctional proteins and lymphocyte cell surface molecules during the multi-step process of egression.

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Figure 1. Assay for transepithelial migration of human T cells across a confluent monolayer of human bronchial epithelial cells

A. Human bronchial epithelial cells (HBE14⁰⁻) can be grown on either side of a costar® transwell filter, and polarise with the TJ marker ZO-1 correctly positioned; ZO-1 is in red on the Z series and ZX series, the porous filter is stained green. B. T cell migration can be measured in the apical-to-basal direction (epithelial cells grown on the topside of the filter) and in the more physiological basal-to-apical direction (epithelial cells are grown on the underside of the filter).

Figure 2. Live cell imaging and fixed cell analysis of the different stages of trans-epithelial migration

(A-D) Time course of video imaging of T lymphocytes during trans-epithelial migration. (A) White arrows mark T cells on the basal surface of the epithelium. Open black arrows mark the 8 μ M pores in the filter that are out of focus and through which the T cells have already passed. Two of these pores have been labelled '1' and '2' in white. (B) The epithelial junctions can be seen and one section is marked with small black arrows. (C-D) T cells that have successfully undergone trans-epithelial migration move out of the plane of focus and are labelled with closed black arrows. Scale bar is 10 μ m.