Epithelial ICAM-1 and ICAM-2 regulate the egression of human T cells across the bronchial epithelium

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ABSTRACT   Egression of inflammatory cells from the lung interstitium into the airway lumen is critical for the resolution of inflammation, but the underlying mechanisms of this egression are unclear. Here, we use an *in vitro* system, in which human T cells migrate across a bronchial epithelial monolayer, to investigate the molecules involved. We show that although inhibition of T-cell LFA-1 blocks egression by 75 ± 5.6% ($P<0.0001$), inhibition of the LFA-1-ligand ICAM-1 on the epithelium only inhibits by 52.7 ± 0.06% ($P=0.0001$). We, therefore, looked for other epithelial ligands for LFA-1 and demonstrate that ICAM-2, but not ICAM-3, is expressed on the bronchial epithelium. Blocking ICAM-2 inhibits egression by 50.95 ± 10.79% ($P=0.04$), and blocking both ICAM-1 and ICAM-2 inhibits egression by 69.6 ± 5.2% ($P<0.0001$). Inhibition of LFA-1/ICAM-1 and ICAM-2 interactions on the basolateral epithelium does not prevent egressing T cells from adhering, polarizing, or moving over the basal epithelium, but it does prevent their recognition of the interepithelial junctions. In conclusion, we show that egression of T cells involves three distinct sequential steps: adhesion, junctional recognition, and diapedesis; we further demonstrate that ICAM-2 is expressed on the bronchial epithelium and, together with ICAM-1, has an essential function in the clearance of T cells from the lung.—Porter, J. C., Hall, A. Epithelial ICAM-1 and ICAM-2 regulate the egression of human T cells across the bronchial epithelium. *FASEB J.* 23, 000–000 (2009)

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THE LUNG EPITHELIUM is exposed to infectious and physical agents over a large surface area, and it depends on trafficking leukocytes to neutralize potential danger; however, excessive numbers of interstitial leukocytes may result in airway hyper-responsiveness, as seen in asthma and chronic obstructive pulmonary disease (1–4). Because the lung is a hollow epithelial organ, the overall accumulation of leukocytes within the interstitium of the lung is determined by several factors: the recruitment of leukocytes from the blood, the survival of tissue leukocytes, and the migration of leukocytes

from the interstitial space, either to the lymphatics (5, 6) or egression into the airway lumen to be removed on the mucociliary escalator and finally expectorated (7). Although much is known about the movement of leukocytes into the lung, much less is known about their exit. In animal models of airway disease, the prevention of leukocyte egression has been shown to be deleterious, resulting in prolonged hyper-responsiveness (7, 8).

We have previously established an *in vitro* model of lymphocyte egression, in which primary human T lymphocytes move across a bronchial epithelial monolayer from the basal to the apical surface, equivalent to moving from the interstitium of the lung into the airway lumen, in response to the T-cell chemoattractant CXCL11 (9). The T cells are cultured in IL-2 and have a Th1-like phenotype and express CXCR3 (9). The advantages of our system are that we use an exclusive T-cell chemoattractant that we have demonstrated to be produced in a polarized manner by the bronchial epithelium in health and disease and that does not disrupt the monolayer or alter adhesion molecule expression and distribution (9). In addition, we can dissect out the individual steps required for successful egression; in this way, we are able to distinguish events at the basal surface of the epithelium from diapedesis, in which the T cell moves across the tight junctions between adjacent epithelial cells.

There has been very little previous work on the mechanisms of lymphocyte egression across the bronchial epithelium (9–11). We and others have shown that lymphocyte egression requires an interaction between the integrin LFA-1 on the T cell and ICAM-1 on the epithelial cell (9, 11). However, we found no difference in the numbers of T cells adherent to the basal epithelium when LFA-1/ICAM-1 interactions were blocked, suggesting that LFA-1 regulates a postadhesion step in egression (9).

Here, we further analyze the mechanisms of egression and show that the movement of human T cells across an intact bronchial epithelial barrier is a multi-

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step process, comparable to the movement of T cells across the vascular endothelium. We identify at least three distinct steps in this process: *1*) adhesion of T cells to the basal surface of the epithelium; *2*) T-cell recognition of the epithelial junctions, which requires LFA-1 on the T cell and ICAM-1 and ICAM-2 on the epithelial cells; and *3*) diapedesis of the T cells across the epithelial tight junctions, which depends, in part, on the epithelial coxsackie-adenovirus receptor (CAR).

MATERIALS AND METHODS

Antibodies and reagents

Monoclonal antibodies (mAbs) 38 (LFA-1-blocking), 15.2 (ICAM-1-blocking) were gifts from Nancy Hogg (Cancer Research UK, London, UK). CBR-IC2/2 (ICAM-2-blocking) was purchased from Serotec, MorphoSys (Kidlington, UK). P5D2 (β1-subunit function-blocking antibody) was a gift from Fiona Watt (Cancer Research UK). mAb 2T6 (CAR blocking) was purchased from U.S. Biological (Swampscott, MA, USA). Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies were from Molecular Probes (Carslbad, CA, USA). The inhibitors cytochalasin D, wiskostatin, NSC23766, and Y27632 came from Sigma-Aldrich (Poole, UK); chemokines and cytokines were purchased from Peprotech (London, UK).

Cell culture

Primary human bronchial epithelial cells (NHBE; Clonetics, Cambrex BioScience, Wokingham, UK) were cultured on flasks and membranes that had been precoated overnight at 4°C with 10% human placental collagen (Sigma). The medium used for growing NHBE cells was bronchial epithelial cell basal medium (BEBM; Clonetics). NHBE cells were used at passage 2–4. The SV40 transformed human bronchial epithelial cell line 16HBE14o⁻ (16HBE) was a gift from Dr. Dieter C. Gruenert (California Pacific Medical Center, San Francisco, CA, USA). 16HBE cells were cultured in Dulbecco modified Eagle medium (DMEM) plus 10% fetal calf serum (FCS) on flasks or membranes that had been precoated overnight at 4°C with 30 μg/ml Vitrogen (Cohesion, Palo Alto, CA, USA), 100 μg/ml BSA (Sigma), and 10 μg/ml fibronectin (Sigma) in PBSA.

Confocal microscopy epithelial monolayer

16HBE cells (100 μl of cells at 1×10^6/ml) were plated on 8-μm-pore size, 6.5-mm-diameter polycarbonate filters in 24-well Transwell chambers (Costar; Corning, NY, USA) and grown for 7–9 days. During this time, epithelial cells moved across the filter to grow on both sides of the filter. The monolayers were checked for their impermeability to fluid by their ability to maintain a fluid level difference between inner and outer wells. Cells were removed from one side of the monolayer with a cotton wool tip to leave a monolayer on the underside of the filter. At the indicated time, the monolayers were washed and fixed with 4% formaldehyde for 20 min at room temperature. Monolayers were visualized with mouse anti-ICAM-1 and anti-ICAM-2 and a goat anti-mouse secondary labeled with Alexa-488. The monolayers were then briefly permeabilized with 0.2% Triton X-100 for 10 min at RT before addition of TRITC-phalloidin at 250 ng/ml for 10 min at RT. Confocal microscopy was performed using a Zeiss LSM

510 laser-scanning microscope equipped with an ×60 oil-immersion objective (Carl Zeiss, Oberkochen, Germany). Images were collected as horizontal sections taken at intervals through whole cell volumes and were compiled for display from a projection of the complete Z series, as a Y-Z display using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). Because the holes in the filters take up fluorescence, the sections that pass through the filter are easily identified, and the basal (next to the filter) and apical (farthest from the filter) surfaces can be distinguished (data not shown). Images were taken at 0.1-μm intervals and projected as Z series.

Preparation of lymphocytes

Peripheral blood mononuclear cells were prepared from single-donor leukocyte buffy coats by centrifugation through Lymphoprep (Pharmacia Diagnostics AB, Uppsala, Sweden). T cells were expanded from this population by culturing in RPMI 1640 plus 10% FCS (GibcoLife Technologies, Paisley, UK) in the presence of phytohaemagglutinin (Murex Diagnostics, Dartford, UK) at 1 μg/ml for 72 h, as described previously (12). Cells were washed and maintained for 1–2 wk in medium supplemented with 20 ng/ml recombinant IL-2 (Euro Cetus UK, Harefield, UK). The T cells, which were used between days 10 and 14, were a 99% CD3+ population, containing 65% CD8+ and 35% CD4+ cells. The population was negative for the natural killer cell marker CD56. The T cells express LFA-1, VLA-4, and CXCR3 (9).

Transepithelial migration assay

16HBE cells were grown as a monolayer on the underside of the filter as described above. The monolayers were left in culture for a further 24 h before recording their permeability to FITC-dextran 40 kDa (see above). Only monolayers with permeability of <0.5% of filter alone were used in subsequent assays. Monolayers were washed extensively to remove dextran. Unactivated epithelial monolayers were used to prevent junctional disruption (9). T cells and/or epithelial monolayers were preincubated with antibodies for 30 min before beginning the assay. When inhibitors were being used, the epithelial monolayers were incubated with the inhibitors for the stated time before extensively washing 5 times in RPMI/0.5%BSA. The epithelial monolayers were rinsed with RPMI/0.5% BSA and 100 μl of labeled T cells at 4×10^6/ml were added to the monolayers, and the inserts were transferred to new wells (lower chambers) of a 24-well plate containing 0.6 ml of RPMI/0.5% BSA and the indicated chemokine. The transwells were then incubated at 37 C in 5% CO_2. After 90 min, T cells, which had migrated through the epithelial monolayer into the lower chambers, were recovered. FL1-positive cells were counted on FACS, and the percentage of migrated cells was calculated as above.

Flow cytometry of bronchial epithelial cells

16HBE and primary human bronchial epithelial cells were passaged under gentle trypsinization in 0.02% trypsin/0.0016% EDTA with 0.02% EGTA and 1% polyvinylpyrrolidine solution (Sigma-Aldrich) in HEPES-buffered saline (polyvinylpyrrolidine-EGTA-trypsin). After release, the cells were spun down for 5 min. at 1000 rpm, and they were allowed to recover at 37 C for 1 h in modified essential medium (MEM) with 10% FCS. Following recovery, cells were washed and 2×10^5 cells in 50 μl RPMI/0.1% BSA were added to flexiwell plate wells (Dynex Technologies, Ashford, UK), with appropriate concentrations of primary antibodies.

After 60 min incubation on ice, epithelial cells were washed 3 times and incubated with an appropriate secondary antibody on ice for 30 min, before washing 3 times. Propidium iodide (10 µg/ml) was added so that FL2-positive dead cells could be gated out. Fluorescence was detected using a FACScan flow cytometer (Becton Dickinson, Oxford, UK).

Confocal microscopy of T cells and epithelium during transepithelial migration

An assay of transepithelial migration was performed as above, but using unlabeled T cells, and with blocking mAbs as appropriate. At the indicated time, the monolayers were washed and fixed with 4% formaldehyde for 20 min at room temperature. Monolayers to be permeabilized were incubated with 0.4% triton-X 100 for 10 min at room temperature. Once transepithelial migration had been stopped and the monolayers had been fixed and permeabilized, the T cells were labeled with anti-LFA mAb 38 followed by a goat anti-mouse secondary, and junctions were visualized with rabbit anti-ZO-1 and a goat anti-rabbit secondary. Some samples were stained with TRITC-phalloidin at 250 ng/ml for 10 min at room temperature. Confocal microscopy was performed using a Zeiss LSM 510 laser-scanning microscope equipped with an ×60 oil-immersion objective. Images were collected as horizontal sections were taken at intervals through whole cell volumes and were compiled for display from a projection of the complete Z series, as a Y-Z display using ImageJ software. Because the holes in the filters take up fluorescence, the sections that pass through the filter are easily identified, and the basal (next to the filter) and apical (furthest from the filter) surfaces can be distinguished (data not shown). In addition, staining of ZO-1 in the epithelial tight junctions allowed us to distinguish basal epithelium and migrating T cells that had not yet crossed the tight junctions from apical epithelium and migrating T cells that had crossed the tight junctions (data not shown). In some situations, images were taken at 0.1- to 0.2-µm intervals extending 1 µm basal to the ZO-1 staining and for 1 µm apical to the ZO-1 staining and then projected as a composite attack of images. In addition the ZO-1 staining from the same series has been analyzed frame by frame, and the composite was imported into Adobe Illustrator (Adobe Systems, San Jose, CA, USA) and recreated as an overlay in orange to show the junctions without the confusion of the background staining. Monolayers were viewed under identical fluorescence and camera exposure to allow comparison between conditions.

Epithelial permeability assay

Transepithelial electrical resistance (TER) was measured using a volt ohmmeter. Confluent monolayers were washed and placed in HBSS to equilibrate for 30 min at 37°C. The voltage across the monolayer was recorded and expressed as Ω/cm^2.

Statistical analysis

Data are expressed as means $\pm$ SD from one experiment performed in triplicate and are representative of at least 3 independent experiments. For pooled experiments, at least 3 independent experiments were pooled. Data are presented as means $\pm$ SE. Data were anyalzed using paired or unpaired Student's t tests as appropriate. A value of $P < 0.05$ was taken as significant.

RESULTS

ICAM-1 expressed on the basal, but not on the apical, surface of the bronchial epithelium is required for LFA-1-dependent transepithelial migration of T cells

We have previously shown that leukocytes will egress across the bronchial epithelium in an apical-to-basal direction in response to CXCL11 (9). We have demonstrated the polarized secretion of CXCL11 in response to inflammatory stimuli and that human T-cell LFA-1 interacts with epithelial ICAM-1 during such egression (9). However, immunohistochemistry studies have detected very little, if any, ICAM-1 expressed on the basal surface of alveolar (13), bronchial (11, 14), and intestinal epithelia (15). To determine the polarized distribution of ICAM-1, an immortalized human bronchial epithelial cell line (16HBE) was cultured on 8-µm polycarbonate filters for 7–9 days. Under these conditions, a fully polarized monolayer (**Fig. 1***A*) can be established as described previously (9). After presentation of CXCL11 to the apical side of the monolayer, cells were fixed and stained and ICAM-1 distribution visualized by confocal microscopy. As shown in Fig. 1, ICAM-1 was expressed on the apical surface of the epithelial monolayer (Fig. 1*D, E, G*) and, to a lesser extent, on the lateral surfaces basal to the tight junction (Fig. 1*C*), but there was very little ICAM-1 on the basal surface (Fig. 1*B, G*).

To determine whether the migrating T cells bound to ICAM-1 on the apical or basal side of the tight junctions, we used a transepithelial migration assay. Primary human T cells migrate across a tight bronchial epithelial monolayer in response to a chemoattractant (9), and blocking mAbs to ICAM-1 were added to either the basal (interstitial) or the apical (luminal) side of the tight junctions. The antibodies were unable to cross the tight junctions and did not alter junctional integrity (data not shown). Only antibodies added to the basal surface blocked migration (Fig. 1*H*; $P=0.0001$). In contrast, antibodies against ICAM-1 added to the apical surface of the monolayer, increased the number of transmigrated cells (Fig. 1*H*; $P=0.047$). Therefore, T cells bind ICAM-1 at the basolateral surfaces of the intercellular junctions of epithelial cells before negotiating the tight junctions.

Egression requires the interaction of T cells with ICAM-1 and ICAM-2 on the bronchial epithelium

Although blocking basolateral ICAM-1 inhibited transepithelial migration, the effect was not as complete as blocking leukocyte LFA-1 (Fig. 1*H*; $P=0.004$), pointing to another epithelial ligand for LFA-1. As well as ICAM-1, LFA-1 can also bind ICAM-2 and ICAM-3, but the expression of ICAM-2 and ICAM-3 is thought to be restricted to hematopoietic cells and endothelium; neither has been found on bronchial epithelium. However, FACS analysis showed that ICAM-2 is expressed by the human bronchial epithelial cell line, 16HBE cells (**Fig. 2***A*), and by primary human lung epithelial cells (Fig. 2*B*), though these cells do not express ICAM-3 (Fig. 2*B*). Expression of ICAM-1 is increased by CXCL11 (9), but we found no change
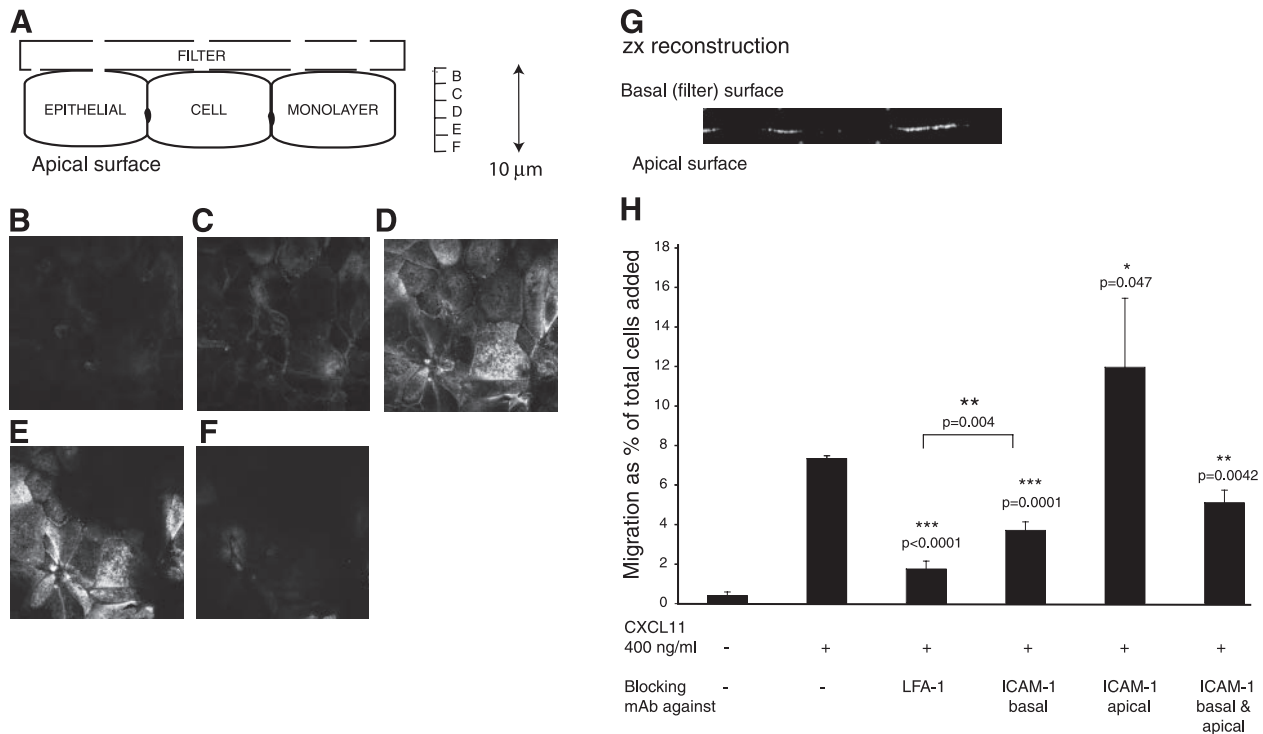
Figure 1. Expression and functional activity of ICAM-1 on human bronchial epithelium. *A–F*) Laser-scanning confocal microscopy (LSCM) was used to image the distribution of ICAM-1 on a CXCL11-stimulated bronchial epithelial monolayer. MAb to ICAM-1 was presented on both sides of the monolayer. The cartoon demonstrates the position of the serial Z series (*A*); projected Z series are shown from basal (*B*), middle (*C*), apical (*D, E*), and luminal epithelium (*F*). *G*) ZX reconstruction. One representative experiment of 3 is shown. *H*) Epithelial monolayers were preincubated with blocking mAb to ICAM-1 on the apical and/or basal side of the monolayer before washing and performing transepithelial migration assays, as described previously (9). For blocking control, T cells were preincubated with mAb against LFA-1 before transepithelial migration. Data are expressed as means ± SD of triplicates, 1 representative experiment of 3 is shown. NS, not significant ($P \geq 0.05$). **$P < 0.005$, ***$P < 0.0005$; 2-tailed Student's t test.

in the levels of ICAM-2 under the same conditions (data not shown).

We next examined the distribution of ICAM-2 on the bronchial epithelium. Confocal microscopy showed that expression of ICAM-2 was found mainly on the apical and lateral surfaces on the bronchial epithelial cells (Fig. 2*D′*–H′), similar to that of ICAM-1. We investigated the role of ICAM-2 in egression and found that, as for ICAM-1, blocking ICAM-2 on the basal surface inhibited transepithelial migration (**Fig. 3*A***; $P=0.04$). The effects of blocking both ICAM-1 and ICAM-2 on the basal side of the bronchial epithelium (Fig. 3*A*; $P=0.004$) were quantitatively similar to the inhibition seen when LFA-1 on the T cells was blocked (Fig. 3*A*; $P=0.21$). Thus, we identified ICAM-1 and ICAM-2 as the ligands for LFA-1 on the bronchial epithelium. We conclude that egression requires interaction of T-cell LFA-1 with either ICAM-1 or ICAM-2 on the basolateral surfaces of the bronchial epithelium.

Blocking LFA-1/ICAM-1 and LFA-1/ICAM-2 does not prevent migration of T cells across the basal surface of the epithelium toward the intercellular junctions

Investigations of transendothelial migration have established a critical β2-integrin-dependent postadhesion step termed "locomotion" (16, 17), during which leukocytes move to the intercellular junctions. We, therefore, investigated whether T cells were still able to move to the junctions between bronchial epithelial cells when LFA-1/ICAM-1 and ICAM-2 interactions were blocked. We used confocal microscopy to examine the number and polarity of the T cells on the basal surface of the epithelium, which is a function of T-cell migration across the filter, motility between the filter and the basal epithelium, and speed of diapedesis to the apical side. We have previously shown that in the absence of CXCL11, control T cells adhere to the basal surface of the epithelium but are relatively unpolarized, but in the presence of CXCL11, on the apical side of the epithelium, many of the T cells become polarized and move to the apical surface of the epithelium (9). This diapedesis, to the apical side, takes several hours, and during the first hour of the assay, T cells move over the basal epithelium, but very few T cells cross to the apical epithelium until after 90 min (9). We therefore used confocal microscopy to examine the T cells in the first hour of the assay as they migrated over the epithelium. Figure 3*B–F* shows the basal surface of the epithelial monolayer, which was fixed 1 h after the addition of T cells. In the absence of CXCL11, control T cells adhere to the basal surface (Fig. 3*B*), similar to the findings in the presence of CXCL11 (Fig. 3*C*). However, examin-
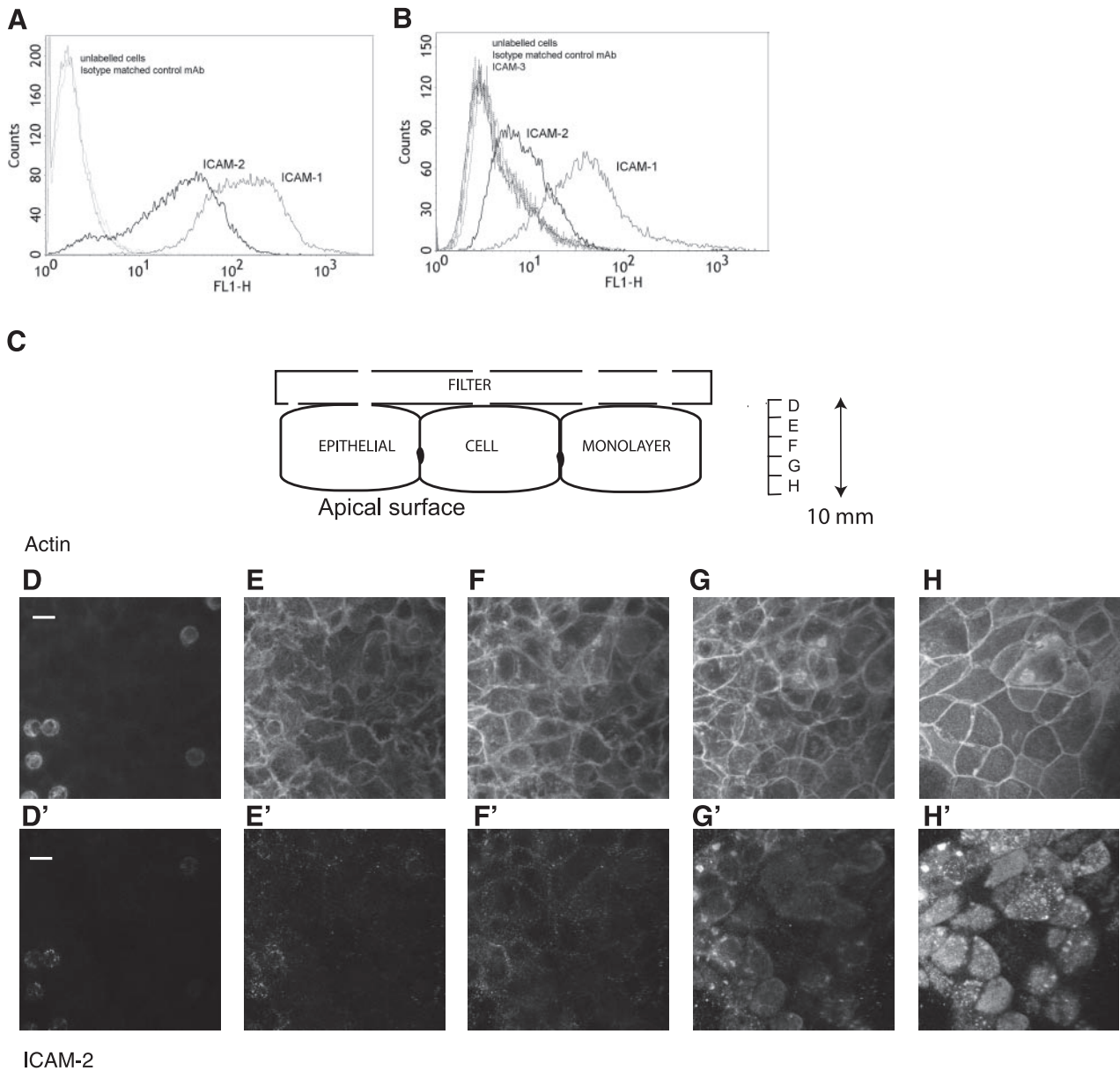
Figure 2. ICAM-1 and ICAM-2, but not ICAM-3 expression on bronchial epithelial cells. *A, B*) FACS analysis of ICAM-1 and ICAM-2 expression on 16HBE cells (*A*) and primary human bronchial epithelial cells (*B*). Peaks are labeled to their right and show unlabeled epithelial cells, isotype-matched control (anti-CD3), ICAM-1, ICAM-2, and ICAM-3. One experiment representative of 3 similar experiments is shown. LSCM was used to image ICAM-2 staining of a bronchial epithelial monolayer grown on a filter. (MAb to ICAM-2 was presented on both sides of the monolayer). *C*) The cartoon demonstrates the position of the serial Z series. *D–H′*) Monolayers are stained for actin (*D–H*) and ICAM-2 (*D′–H′*). Projected Z series of 5 images taken at 0.1-µm intervals are shown for basal (*D, D′*), middle (*E, E′–F, F′*), apical (*G, G′*), and luminal epithelium (*H, H′*). One representative experiment of 3 is shown.

ing larger numbers of cells confirmed our previous findings that in the presence of CXCL11, the T cells are more polarized, with a leading edge and a trailing uropod (9) (data not shown). In the presence of a β1-integrin-blocking antibody, very few T cells were able to cross the filter and the extracellular matrix, as previously demonstrated (9). For this reason, only a small number of T cells could be seen on the basal surface of the epithelium (data not shown), but these cells were polarized, with a leading edge (Fig. 3*D*). When LFA-1 was blocked, T cells remained adherent between the filter and the basal surface of the epithe-

lium and were polarized, with a trailing uropod. Many of the cells were unusually elongated in comparison to control cells, and they had moved over the basal surface of the epithelium, passing over several junctions (Fig. 3*E*). The results were similar when ICAM-1 was blocked (Fig. 3*F*). In summary, although inhibition of LFA-1/ICAM-1 and ICAM-2 prevented T cells from crossing the epithelium, it did not inhibit their adhesion, polarization, or migration over the basal surface of the epithelium to reach the intercellular junctions; in fact, some of the cells appeared to have passed over several junctions.
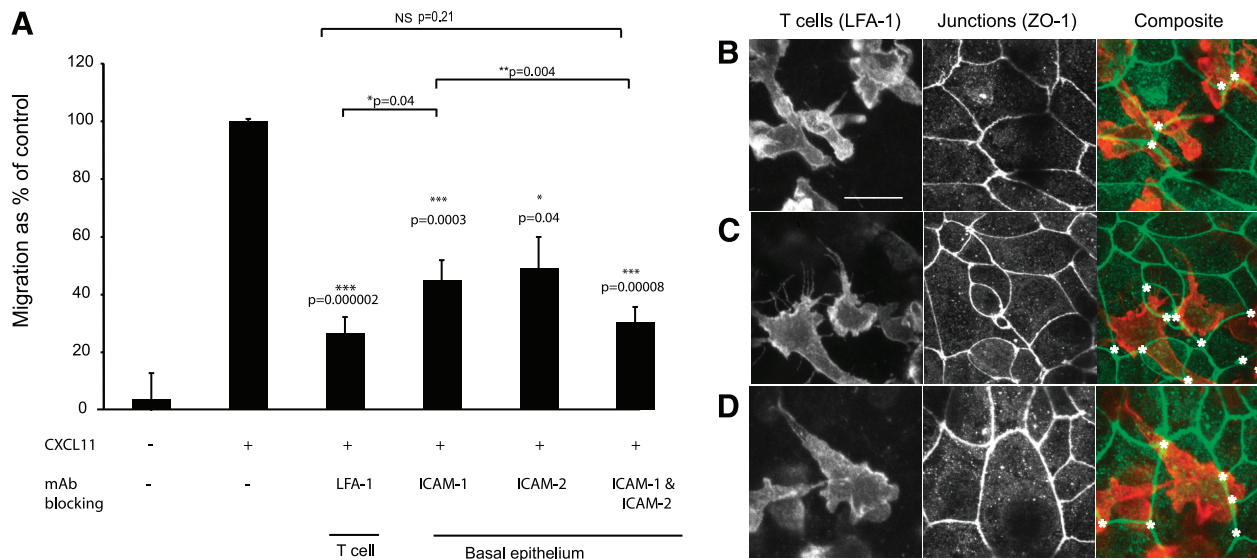
Figure 3. LFA-1 binds ICAM-1 and ICAM-2 on the basal surface of bronchial epithelium during T-cell egression. *A)* Epithelial monolayers were preincubated with blocking mAb to ICAM-1 and/or ICAM-2 on the basal side of the monolayer before washing and perfoming transepithelial migration assay as described (9). T cells were preincubated with mAb against LFA-1 before transepithelial migration. Data are expressed as percentage migration of control, in which the control is maximal migration seen in response to CXCL11 with no antibody. Data are means ± SE from 3 separate experiments performed in duplicate. NS, not significant ($P \geq 0.05$). *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$; paired Student's *t* test. *B–D)* LSCM images of the basal surface of the epithelium 1 h into a transepithelial migration assay to show migrating T cells (labeled with anti-LFA-1; red), on the epithelium (epithelial junctions labeled with anti-ZO-1; green). *B)* Control; no CXCL11. *C–F)* CXCL11; T cells preincubated with no antibody (*C*), blocking mAb to β1 integrin (*D*), and blocking mAb to LFA-1 (*E*); and bronchial epithelial monolayers preincubated with blocking mAb against ICAM-1 (*F*). Images show 1 representative area from an experiment analyzing 10 similar areas. Scale bar = 10 μm. Asterisks mark tricellular junctions in the vicinity of the T cell.

A role for the epithelial junctional coxsackie-adenovirus receptor during diapedesis

It has previously been reported that CAR is expressed at tight junctions in epithelial cells (18). We first confirmed the expression of CAR in bronchial epithelial cells and found it colocalized with the tight junction marker ZO-1 (data not shown). Preincubation of the bronchial epithelial monolayer with a blocking antibody against CAR inhibited transepithelial migration of T cells by 40% (**Fig. 4A**; $P=0.0019$). Confocal microscopy at 1 h showed that when CAR was blocked, T cells were still polarized and able to move along the basal epithelium (Fig. 4*B*). Pretreatment of the epithelial monolayer with cytochalasin D leading to disruption of cell junctions did not significantly increase the baseline transepithelial migration (Fig. 4*C*; $P=0.7$), but it did rescue transepithelial migration inhibited by an anti-CAR antibody (Fig. 4*C*; $P=0.0005$). Cytochalasin D did not rescue the inhibition of transepithelial migration caused by blocking of LFA-1 (Fig. 4*C*; $P=0.93$), indicating that the CAR-dependent and LFA-1-dependent steps are distinct. We conclude that there are at least 3 distinct steps in the movement of T cells across 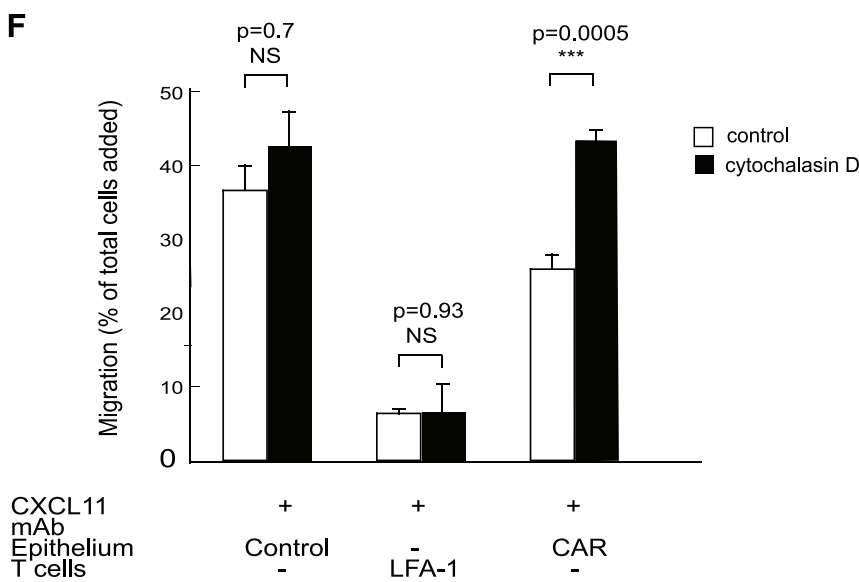the epithelium: adhesion, LFA-1-dependent movement into the lateral intercellular space to locate the junctions, and CAR-dependent diapedesis.

The Rho-kinase inhibitor Y27632 and the N-WASp inhibitor wiskostatin disrupt epithelial barrier function but have opposite effects on T-cell transepithelial migration

In an attempt to understand these steps in T-cell egression further, we turned our attention to the epithelial cell. In models of leukocyte extravasation, the engagement and cross linking of endothelial ICAM-1 by adherent lymphocytes triggers Rho GTPase, which may reorganize junctions in preparation for diapedesis (19, 20). We therefore examined epithelial signaling pathways downstream of T-cell adhesion and the role of the Rho family GTPases-Rho, Rac, and Cdc42. Inhibition of Rac with the small molecule NSC23766 (21) had no effect on the permeability of the epithelial monolayer as measured by TER (data not shown), whereas inhibition of the Rho/Rho kinase pathway with the inhibitor Y27632 (22) or inhibition of the Cdc42/N-WASp pathway with wiskostatin (23) caused an increase in epithelial junctional permeability comparable to that seen
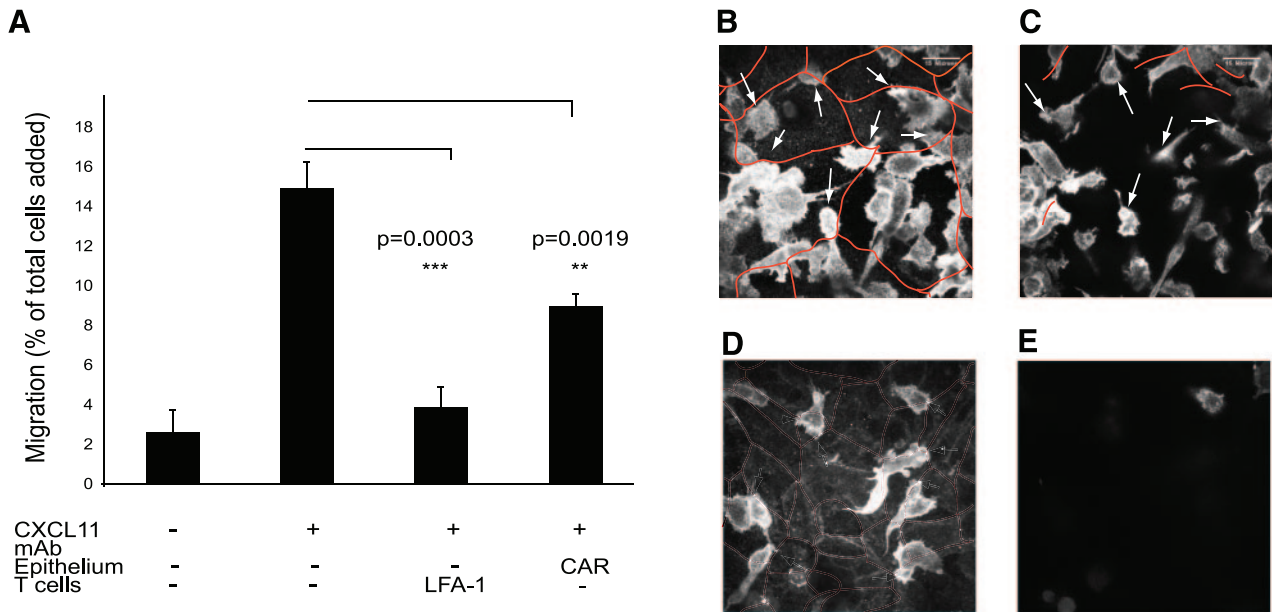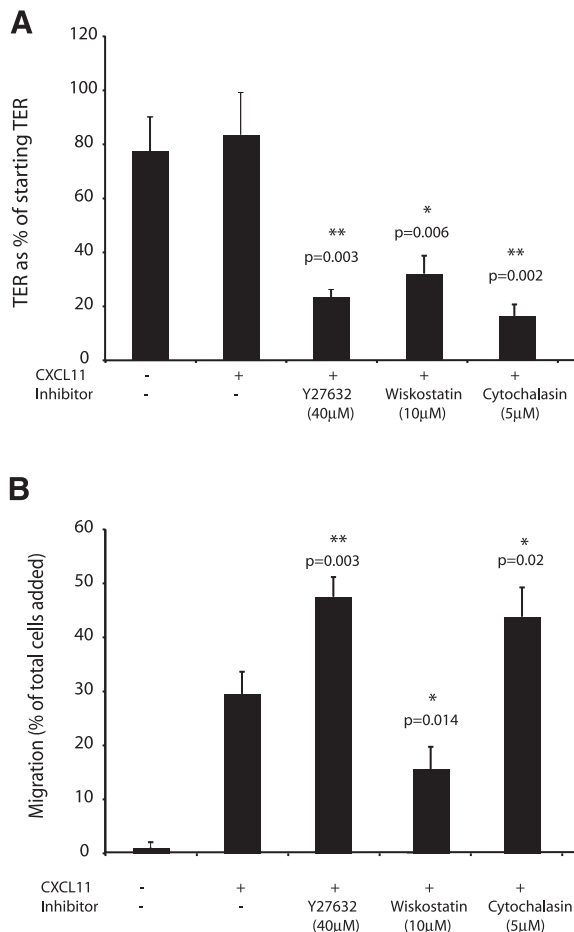
Figure 4. Role of CAR in transepithelial migration. *A*) Transepithelial migrations assays were performed as described. Preincubation of T cells with a blocking mAb against LFA-1, or of the epithelial monolayer with a blocking mAb against CAR, caused a significant reduction in transepithelial migration. *B–E*) LSCM images of basal (*B, D*) and apical (*C, E*) epithelium 1 h into a transepithelial migration assay, following preincubation of the epithelial monolayer with an isotype-matched control mAb (*B, C*) or a blocking mAb against CAR (*D, E*). Images were taken at 0.2-μm intervals extending 1 μm basal to the ZO-1 staining (*B, D*) and 1 μm apical to the ZO-1 staining (*C, E*) and then projected as a composite. In addition, the ZO-1 staining from the same series was analyzed frame by frame, and the composite was imported into Adobe Illlustrator and recreated as an overlay in orange to show the tight junctions. Scale bar = 15 μm. *F*) Epithelial cell monolayers were untreated (white bars) or pretreated with 5 μM cytochalasin D (black bars) and/or a control mAb or a blocking mAb to CAR. Monolayers were washed and transepithelial migration assays were performed as described above. T cells were untreated or treated with a blocking mAb to LFA-1. Data are means ± SD of triplicates; 1 representative experiment of 3 is shown. NS, not significant ($P \geq 0.05$). **$P < 0.005$, ***$P < 0.0005$; 2-tailed Student's *t* test.

with cytochalasin D (**Fig. 5A**; $P < 0.01$ for all 3 conditions). However, although inhibition of epithelial Rho kinase increased transepithelial migration to a similar extent as pretreatment of the epithelium with cytochalasin D (Fig. 5*B*; $P < 0.02$), pretreatment with wiskostatin inhibited transepithelial migration (Fig. 5*B*; $P = 0.014$). The effect of wiskostatin was dose dependent, although at high doses (50 μM), the epithelial monolayer became disrupted, and at 100 μM, transepithelial migration increased (data not shown), indicating that wiskostatin was not inhibiting the migration of T cells, but was acting on the epithelial cells. Chlorinated and unhalogenated analogues of wiskostatin that do not inhibit N-WASp had no effect (data not shown).

When we examined the T cells on the basal epithelium (**Fig. 6A–D**) we found that, even after pretreatment of the epithelium with wiskostatin (Fig. 6*D*), T cells were able to cross the filter and interact with the basal surface of the epithelium, as we had already observed for both control T cells in the absence (Fig. 6*A*) or presence (Fig. 6*B*) of CXCL11, or T cells treated with LFA-1-blocking antibody (Fig. 6*C*). Quantification of T cells adherent to the basal surface of the epithelium confirmed no difference in the number of adherent T cells, compared to control, either when LFA-1 on the T cells was blocked (Fig. 6*E*; $P = 0.88$, not significant) or when the epithelium was pretreated with wiskostatin (Fig. 6*E*; $P = 0.13$, not significant). In addi-

A

B

Figure 5. Contribution of Rho-GTPase pathways to epithelial junction integrity and to transepithelial migration. *A*) TER was measured before and after incubation and washout of inhibitors and is expressed as a percentage of control TER. Preincubation of the monolayer with cytochalasin D was used as a control for junctional disruption; Inhibition of Rho-kinase (Y27632) or N-WASp (wiskostatin) decreased TER. *B*) Epithelial monolayers were preincubated with inhibitors at doses shown. After 2 h, the inhibitors were extensively washed out, and transepithelial migration was measured. Rho-kinase (Y27632) and cytochalasin D caused an increase, and wisko-statin caused a decrease in transepithelial migration. Data are means ± sd of triplicates; 1 representative experiment of 3 is shown. NS, not significant ($P{\geq}0.05$). *$P < 0.05$, **$P < 0.005$ *vs.* control; 2-tailed Student's t test.

tion, blocking LFA-1 on the T cells or pretreatment of the epithelium with wiskostatin had no effect on the ability of adherent cells to polarize and elongate along the basal epithelium. Of interest, there was a small but significant increase in the number of basally adherent T cells in the absence of CXCL11 (Fig. 6*E*, $P=0.004$).

Wiskostatin treatment of the epithelium-inhibited T-cell egression at a step that took place after adhesion but before diapedesis, and it did so despite loosening the tight junctions. This inhibition was indistinguishable from that caused by inhibiting LFA-1/ICAM-1 and ICAM-2 binding. We therefore investigated the effect of wiskostatin on epithelial ICAM-1 expression. Epithelial monolayers were left untreated (**Fig. 7*A–C, J***) or were treated with 2 μm cytochalasin D (Fig. 7*D–F, K*) or 50 μm

wiskostatin (Fig. 7*G–I, L*) for 2 h, before washing out the inhibitor, fixing the monolayers, and staining them with a mAb directed against ICAM-1. Confocal images were

E
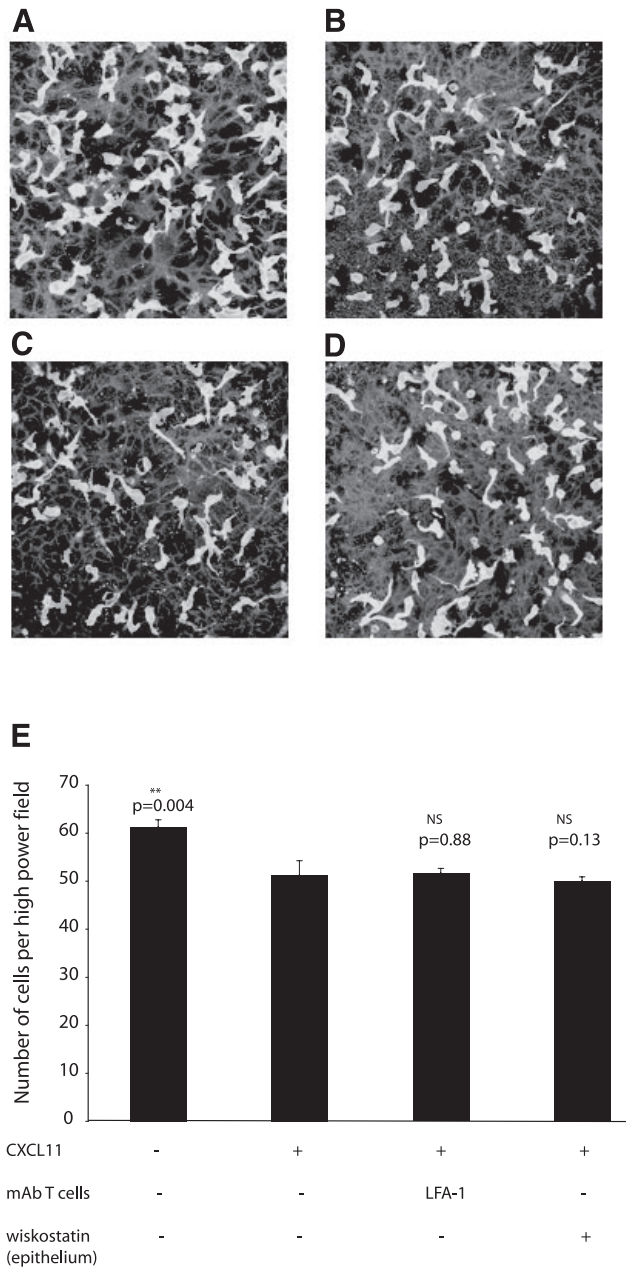
Figure 6. Effect of wiskostatin on T-cell polarization and adhesion to the basal surface of epithelium. *A–D*) Basal surface of the epithelium was analyzed 1 h into a transepithelial migration assay to visualize the relation of migrating T cells (labeled with anti-LFA-1), and epithelium (labeled with phalloidin). Scale bar = 10 μm. Images show representative high-power fields with no CXCL11 (*A*), CXCL11 (*B–D*), and T cells preincubated with an LFA-1 integrin blocking mAb (*C*). *D*) Epithelium preincubated with 20 μM wiskostatin. One representative of 6 high-power fields from 1 experiment of 3 is shown. *E*) Quantification of T cells adherent to the basal surface of the epithelium. For each condition, cells were counted from 6 high-power fields. Data are means ± sd; 1 representative experiment of 3 is shown. NS, not significant ($P{\geq}0.5$).**$P < 0.01$; 2-tailed Student's t test.
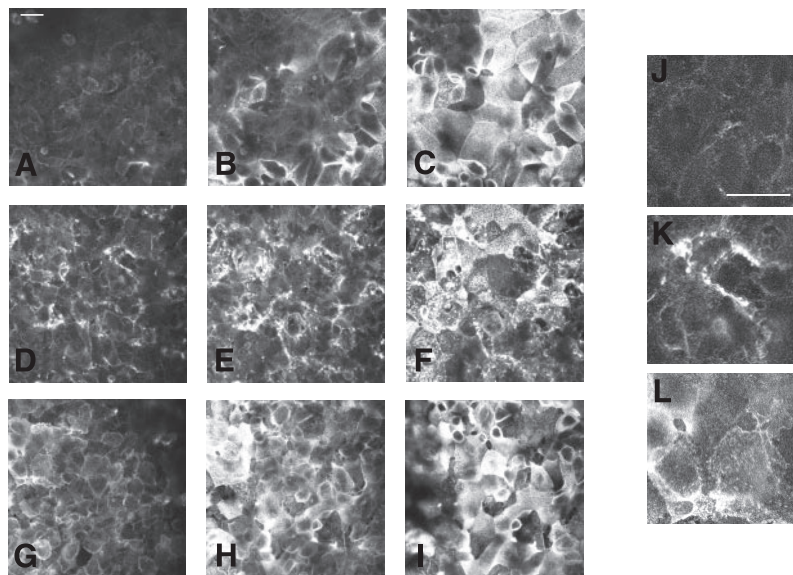
Figure 7. Effect of cytochalasin D and wiskostatin on the redistribution of epithelial ICAM-1. Epithelial monolayers were left untreated (*A–C, J*), or treated with 2 μm cytochalasin D (*D–F, K*) or 50 μm wiskostatin (*G–I, L*) for 2 h, before washing out the inhibitor, fixing the monolayers, and staining on both sides of the monolayer with a mAb against ICAM-1. Panels show confocal images, collected under identical conditions and settings, of 2.5-μm stacks from different regions of the epithelium: basal (*A, D, G, J–L*), middle (*B, E, H*), and apical (*C, F, I*). Panels *J–L* show higher magnifications of representative areas of panels *A, D,* and *G,* respectively. One representative experiment of 3 is shown. Scale bars = 10 μm.

taken of 2.5-μm stacks from the three different regions of the epithelium; basal (Fig. 7*A, D, G, J–L*), middle (Fig. 7*B, E, H*), and apical (Fig. 7*C, F, I*). In the control monolayers, the majority of the ICAM-1 was seen on the apical surface (Fig. 7*C*) and in the epithelial cell-cell contact areas (Fig. 7*A–C*). Treatment with cytochalasin D caused clustering of the ICAM-1 at cell-cell contacts (Fig. 7*D–F*) but did not dramatically increase the amount of ICAM-1 seen on the basal surface (Fig. 7*D, K*). Wiskostatin treatment increased the amount of ICAM-1 seen at the cell-cell junctions (Fig. 7*G–I*) but also on the basal surface of the epithelium (Fig. 7*L*). Thus, wiskostatin caused a redistribution of ICAM-1 on the bronchial epithelium, resulting in expression of ICAM-1 on both the basal and apical epithelial surfaces, which might disrupt the normal ICAM-1 gradient.

DISCUSSION

This study demonstrates that egression of human T cells from the lung across bronchial epithelium is a multistep process. In particular, we describe an LFA-1-dependent event during which T cells bind ICAM-1 and ICAM-2 on the basolateral epithelium, before crossing the junctions. We present evidence that the ICAM-1 and ICAM-2, together with a chemoattractant, direct the T cells to the lateral interepithelial cell space. When LFA-1 is blocked, the T cells move over the basal epithelium but do not appear to recognize the intercellular junctions and therefore do not cross them. In addition, we show an essential role of epithelial CAR for successful transepithelial migration.

Although transepithelial migration is inhibited when ICAM-1 on the basal epithelium is blocked, the inhibition is less than when LFA-1 on the T cells is blocked, suggesting other ligands for LFA-1. ICAM-2 and ICAM-3 were thought to be restricted to endothelial and hematopoietic cells, but RNA for ICAM-2 has been shown in human bronchial epithelium (24). Here, we demonstrate that both primary and immortalized human airway cells express ICAM-2. Moreover, we show

that inhibition of both ICAM-1 and ICAM-2 has the same effect as inhibition of LFA-1, demonstrating, for the first time, a functional role for ICAM-2 in the transepithelial migration of leukocytes. These findings highlight a completely new role for ICAM-2 as a molecule involved in the resolution of inflammation and may explain the paradoxical findings of worsening asthma in ICAM-2-deficient mice (2). Our findings also emphasize the potential importance of differential tissue regulation of molecules, such as ICAM-1 and ICAM-2, which are involved in both the recruitment and clearance of leukocytes from epithelial organs.

Interestingly, blocking mAbs against ICAM-1 or ICAM-2 are only effective when placed on the basal side of the intercellular junctions, implying that, although LFA-1/ICAM-1 and ICAM-2 interactions are not required for adhesion to the basal surface of the epithelium, they are required in a postadhesion step prior to diapedesis. There are technical difficulties in the study of egression: T cells migrate across the epithelium in a basal to apical direction, and there are optical limitations imposed by imaging through the monolayer (9). For this reason, we have used confocal microscopy to visualize and quantify T-cell adhesion to the basal epithelium prior to transepithelial migration. We show that when LFA-1 is blocked, the T cells are still able to adhere, polarize, and elongate along the epithelium, but they are elongated and appear to cross several intercellular junctions, without migrating across them. So that although egressing T cells do not require ICAM-1/2 for adhesion to the basal epithelium, they do require the limited amount of ICAM-1/2 at the interepithelial space to guide them.

The LFA-1-dependent postadhesion step, whereby T cells move into the subjunctional epithelial intercellular space, resembles locomotion (16, 17). Schenkel reported that inhibition of monocyte integrin CD18 during transendothelial migration resulted in pirouetting cells, unable to recognize ligand on the apical endothelium; in contrast, inhibition of endothelial ICAM-1 and ICAM-2 resulted in elongated monocytes

crossing many junctions on the apical endothelium, leading him to conclude that there were other unidentified endothelial ligands for CD18 (17). In contrast, in our epithelial assay, inhibition of LFA-1 (the only CD18 integrin expressed on these T cells) did not prevent lymphocytes becoming polarized and elongated. This suggests that integrin/ligand pairings other than LFA-1/ICAM-1 and ICAM-2 are involved in adhesion and migration over the basal epithelium (which is unsurprising given the relative paucity of ICAM-1/2 on the basal epithelium compared to the apical endothelium), but that LFA-1/ICAM-1 and ICAM-2 are required to guide T cells into the lateral intercellular space so that they can contact the tight junction. The dramatic effect on egression seen when blocking either ICAM-1 or ICAM-2, but leaving the other ligand functional suggests that even subtle disruptions of the ICAM-1/2 gradient may prevent egression. This may be particularly important in the epithelium where the cells are tall and columnar; endothelial cells are flatter and the tight junctions between them may be relatively more accessible.

We show that CAR is important for the movement of T cells across the bronchial epithelium when tight junctions are intact. CAR is a tight junction protein, which is used by luminal coxsackie and adenoviruses to enter the lung (25) or by basal viruses to disrupt tight junctions (26). The normal physiological function of CAR was unclear until it was recently shown to be involved in neutrophil migration across the colonic epithelium (27). The inhibition of T-cell egression seen when CAR is blocked in the bronchial epithelium can be overcome when the epithelial junctions are disrupted by pretreatment of the epithelium with cytochalasin D. Under these conditions, mAbs that block LFA-1 are still able to inhibit transepithelial migration. This is evidence that the LFA-1-dependent step is distinct from diapedesis and takes place before it. The finding that blocking CAR only partially inhibits egression suggests that other CAR-independent pathways may be involved. Leukocytes migrate across endothelial monolayers *via* both transcellular and paracellular passages (28–31) and it is possible that similar pathways exist across an epithelial monolayer.

The surprising observation that, in response to a given concentration of CXCL11, a similar number of T cells migrate across a filter as migrate across a tight epithelial monolayer, suggests that the epithelium plays an active part in the migration. In examining the role of Rho, Rac, and Cdc42 GTPases in the bronchial epithelial cells, we find that ICAM-1 cross-linking does not induce junctional rearrangements or permeability changes, but inhibition of Rho-kinase causes partial disruption of the tight junctions and an increase in transepithelial migration, similar to that seen with cytochalasin D treatment. A novel finding is that wiskostatin, an inhibitor of the Cdc42 effector N-WASp, disrupts the epithelial cell junctions but paradoxically causes an inhibition of transepithelial migration. N-WASp is a signaling molecule that, when bound by active Cdc42 is able to initiate Arp2/3-dependent cortical actin polymerization (32), including that involved in the formation of filopodia, lamellipodia and tight junctions, membrane trafficking, and the intracytoplas-

mic movement of various pathogens [reviewed by Takenawa and Suetsugu (33)].

Because inhibition of epithelial N-WASp and inhibition of lymphocyte LFA-1 appear to inhibit T cells at the same stage in their egression, we examined the effect of wiskostatin on epithelial ICAM-1 distribution. We find a redistribution of ICAM-1 with disruption of the normal basal-to-lateral gradient. This suggests that the effect of wiskostatin may be due, in part, to disruption of the haptotactic gradient of ICAMs. Cytochalasin D disrupts tight junctions to the same extent as wiskostatin, but it has a different effect on ICAM-1 distribution, suggesting that the effect of wiskostatin on ICAM-1 is not wholly attributable to junctional disruption and a resultant lateral mobility of membrane proteins.

After successful egression, T cells down-regulate LFA-1 on arrival in the airway (34), allowing the cells to detach from the epithelial cell surface and be removed on the mucociliary escalator (35). An increase in the detachment rate probably accounts for the apparent increase in transepithelial migration seen when the mAb against ICAM-1 is presented apically. These findings emphasize the importance not just of overall levels of adhesion molecules but also of their distribution.

Although our study investigates the egression of human T cells, we believe that these results may extend to other leukocytes. The advantages of our system are that we use an exclusive T-cell chemoattractant that we have demonstrated to be produced in a polarized manner by the bronchial epithelium in health and disease, and that does not disrupt the monolayer or alter adhesion molecule expression and distribution (9). In addition, in contrast to myeloid cells, primary human T cells express no or very low levels of the promiscuous β2 integrin MAC-1 (αMβ2;CD18:CD11b), and the results involving LFA-1/ICAM-1 are therefore easier to interpret (36). The use of lymphocytes also avoids the artifactual disruption of endothelial and epithelial monolayers by granulocytic cells, which has been described [reviewed by Zen and Parkos (37)].

In conclusion, we have identified key steps in the CXCL11-induced egression of T cells across a bronchial epithelial monolayer and have shown that the egression is a multistep process, analogous to that across the endothelium. We describe an essential postadhesion step that requires LFA-1 binding to ICAM-1/2 expressed on the lateral surface of the epithelial cells, basal to the tight junctions. This interaction results in a movement of T cells into the lateral intercellular space and positioned for subsequent diapedesis. The T cells then interact with CAR and probably other junctional proteins to allow movement across the tight junctions. The T cells finally deadhere from the apical ICAMs, and the released cells are eventually cleared from the airway by expectoration. [Fj]

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