The border-crossing behavior of eosinophils and neutrophils in the lung
Zuurberg, A.E.M.

Citation for published version (APA): Zuurbier, A. E. M. (2001). The border-crossing behavior of eosinophils and neutrophils in the lung
CHAPTER IV

Sequential Migration of Neutrophils across Monolayers of Endothelial and Epithelial Cells

*Journal of Leukocyte Biology, 2000, 68:529-537*
Migration of Neutrophils across Endothelial and Epithelial Monolayers
Sequential Migration of Neutrophils across Monolayers of Endothelial and Epithelial Cells


Frederik P.J. Mul and Astrid E.M. Zuurbier contributed equally to this work

Central Laboratory of the Netherlands Blood Transfusion Service (CLB) and Laboratory for Experimental and Clinical Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
Division of Cell Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands
Dept. of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands

In the course of granulocyte-dominated inflammation, granulocytes migrate across the endothelium and epithelium of the lung and cause severe tissue damage. To study this process in more detail, we developed a bi-layer transmigration model comprised of primary human endothelial and lung epithelial cells, simultaneously cultured on opposite sides of Transwell filters. Electron microscopical analysis showed that both the morphology of the cells and the expression of junctional proteins remained unaltered, and that matrix components were deposited onto the filter. Intriguingly, neutrophil migration was more efficient across the bi-layers than across single epithelial monolayers, and did not differ from migration across single endothelial monolayers. Co-culture experiments showed that endothelial cells stimulated epithelial cells to release IL-6 and that epithelial cells enhanced release of IL-8 from endothelial cells. Together these data reveal bi-directional signaling and enhanced neutrophil migration in a transmigration model of primary human epithelial and endothelial cells.

Introduction

In lung inflammation and asthmatic reactions, circulating leukocytes, primarily neutrophils and eosinophils, migrate across the lung endothelium and the lung epithelium into the airway lumen. The process of transmigration initially involves selectin-mediated rolling of the granulocytes on the endothelial cell surface and is followed by firm, integrin-mediated adhesion to and passage across the endothelial cell layer. Subsequently, the granulocytes migrate through the interstitial matrix and across the epithelium into the lung lumen (1-5). Concomitant activation of the granulocytes and release of oxygen radicals and toxic proteins (e.g. eosinophil cationic protein or elastase) cause damage to the lung tissue.

Activation of endothelial cells by inflammatory stimuli promotes leukocyte infiltration through increased cellular adhesion-molecule expression, increased vascular permeability and production of chemoattractants (1; 6-12). The molecular basis underlying transendothelial migration has been well described (9; 10; 13; 14). Granulocyte transmigration is triggered by various types of chemoattractant, such as chemokines, e.g. Interleukin (IL)-8, lipid mediators, e.g. PAF, bacteria-derived peptides, e.g. fMLP and complement fragments, e.g. C5a (15-17). Migration of granulocytes is inhibited by antibodies that either block ligand binding of granulocyte integrins (9; 18; 19), integrin-associated proteins such as CD47, or Ig-like adhesion molecules such as CD31 (20-25).
Migration of Neutrophils across Endothelial and Epithelial Monolayers

Similar to the endothelium, the epithelium also plays an important role in leukocyte infiltration at sites of inflammation. However, whereas a large number of molecules are implicated in the control of transendothelial migration, only few molecules are known to be involved in the transmigration across epithelial monolayers. These include the leukocyte \( \beta_2 \)-integrin CD11b/CD18 and the glycoprotein CD47 (15; 26; 27). The epithelial ligand for the \( \beta_2 \)-integrin has not yet been firmly established (27), although adhesion of eosinophils to human bronchial epithelium was recently described to depend on CD18/ICAM-1 interaction (28). These results are in line with earlier reports of up-regulation of cellular adhesion molecules, such as ICAM-1, on activated epithelial cells (29-32).

In addition, activated epithelial cells release a variety of proinflammatory mediators, chemokines and lipid mediators that may all modulate leukocyte infiltration (15; 26; 33-35). For instance, bronchial epithelial cells of asthmatic patients have been shown to produce increased levels of IL-1\( \beta \), IL-6, IL-8, granulocyte/macrophage-colony stimulating factor and IL-16 (35). Finally, the orientation of the epithelial monolayer is essential to allow efficient \textit{in vitro} transmigration. The physiologically relevant basolateral-to-apical migration of leukocytes is much more efficient than migration in the opposite direction, implicating the polarity of the epithelium as an additional regulatory factor of leukocyte transmigration (26).

Despite the large body of knowledge on migration across monolayers of endothelial or epithelial cells, possible interactions between these cell types and the resulting modulation of leukocyte transmigration have not been thoroughly studied. We have therefore developed a transmigration model in which we simultaneously culture monolayers of primary human lung epithelial cells and human umbilical cord vascular endothelial cells (HUVECs) on opposite sides of Transwell filters. We have characterized this model with respect to morphology and chemoattractant-induced transmigration of granulocytes, and compared the results with those obtained with single endothelial or epithelial cell monolayers. The main results of this study reveal a paracrine interaction between the endothelial and epithelial monolayers resulting in increased release of cytokines and chemokines and enhanced transmigration of neutrophils.

Materials and Methods

Reagents

PAF, C5a, fMLP and isoproterenol were from Sigma Chemical Co. (St. Louis, MO). Recombinant human (rh) IL-8 was purchased from PeproTech (Rocky Hill, NJ) and bFGF from Boehringer Mannheim (Mannheim, Germany). Monoclonal antibody IB4 (CD18, IgG1) (36) was isolated from the supernatant of the hybridoma by precipitation with 50% saturated ammonium sulphate and subsequent protein-A affinity chromatography. CD31 antibody (IgG1: monoclonal antibody HEC170) (37; 38) was also isolated from hybridoma supernatants as described above. CD14 antibody (IgG1; monoclonal antibody 8G3), HSA and fibronectin were obtained from the CLB (Amsterdam, The Netherlands). Fluorescent secondary antibodies were from Dako (Glostrup, Denmark). Vitrogen was obtained from Cohesion (Palo Alto, CA). Calcein-AM and FITC-dextran 3000 was from Molecular Probes (Eugene, OR). RPMI was from Gibco (Breda, The Netherlands).

Granulocyte isolation

Blood was obtained from healthy volunteers. Granulocytes were isolated from a buffy coat of 500 ml of blood by density gradient centrifugation over isotonic Percoll (Pharmacia, Uppsala, Sweden) (39). After lysis of the erythrocytes in the pellet fraction with cold lysis buffer (155 mM NH\(_4\)Cl, 10 mM KHCO\(_3\) and
0.1 mM EDTA, pH 7.4), the granulocytes (> 95% neutrophils) were washed in PBS and resuspended in HEPES medium (132 mM NaCl, 6.0 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgSO₄, 1.2 mM KH₂PO₄, 20 mM HEPES, 5.5 mM glucose and 0.5% (w/v) HSA, pH 7.4). This fraction is hereafter referred to as neutrophils.

**Cell culture and experimental models**

**Endothelial cells.** The human papilloma virus-immortalized HUVEC cell line (40) or freshly isolated, primary HUVECs (41) were cultured in HUVEC medium (RPMI 1640 supplemented with 10% (v/v) heat-inactivated human serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 1 ng/ml bFGF) in culture flasks coated with 1 mg/ml fibronectin. The doubling time of the HUVECs was approximately 48 hours. At confluence, cell suspensions were obtained by trypsin/EDTA treatment. The 2nd-4th passages of the primary HUVECs were used for sub-culturing on fibronectin-coated polycarbonate Transwell filters (3.0 µm pore size, 12 mm diameter; Costar, Cambridge, MA). 150,000 HUVECs (in 0.5 ml culture medium) were added to the upper compartment and the Transwells were cultured for another 4 days to obtain confluent HUVEC monolayers.

**Epithelial cells.** The human lung adenocarcinoma-derived cell line H292 (American Type Culture Collection CRL 1848) (42) was grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated human serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine, in uncoated culture flasks. The doubling time of the H292 cells was approximately 24 hours. At confluence, cell suspensions were obtained by trypsin/EDTA treatment. The 4th-30th passages of H292 cells were used for sub-culturing on the bottom side of Transwell filters according to Parkos et al (43) with minor modifications (26). In brief, a sterile polyoxymethylene polyacetal collar, with an inner diameter equal to the outer diameter of the Transwell insert and with a height of 13 mm, was tightly fixed to the bottom of the Transwell insert. Subsequently, 80,000 H292 cells (in a volume of 0.5 ml culture medium) were allowed to attach for 18 hours (5% CO₂, 37°C). Thereafter, the collars were removed, and the Transwell inserts were placed upright in 12-well culture dishes and incubated for 5 days.

**Primary epithelial cells.** Subcultures of primary human bronchial epithelial cells were obtained from bronchial tissues with macroscopically normal appearance from patients undergoing lobectomy or pneumectomy for lung cancer. The cells were cultured in serum-free keratinocyte medium (Keratinocyte-SFM, Gibco) with 1 mM isoproterenol (44) in culture flasks coated with 10 µg/ml fibronectin, 30 µg/ml vitrogen and 10 µg/ml BSA. After the monolayers had reached confluence, cell suspensions were obtained by mild trypsin/EDTA treatment (Gibco). The detached cells were washed once in PBS containing Soybean trypsin inhibitor type-II (Sigma) before seeding. The 3rd-4th passages of the bronchial epithelial cells were used for culturing at the bottom side of Transwell filters. The inverted monolayers were created as described above for H292 cells, except that approximately 200,000 bronchial epithelial cells were added to coated Transwell filters, and cultured in 50% keratinocyte medium and 50% RPMI 1640 supplemented with 2.5% HSA and 2 mM glutamine (final CaCl₂ concentration 0.5 mM)(50/50 medium).

**Bi-layer model.** The epithelial cells (75,000 cells/well) were allowed to adhere to the bottom side of the Transwell filters as described above. After 1 day (H292) or 5-7 days (primary cells), the top side of the filters was coated with 1 mg/ml fibronectin and 150,000 HUVECs were seeded. The bi-layers comprised
of cell line cells were cultured in HUVEC medium, and the bi-layers comprised of primary cells were cultured in 50/50 medium. The bi-layers were cultured for another 4 days to allow formation of confluent monolayers of epithelial and endothelial cells. To confirm confluence, the cells at either side of the Transwell filters were labeled by adding 4 μg/ml calcein-AM (45) in HEPES medium to the lower or upper compartment of the Transwell system. The filters were washed after 15 minutes, mounted on glass slides and inspected by fluorescence microscopy (Dialux, Leitz, Germany). Alternatively, cells on the filters were stained by May-Grünwald/Giemsa. The different monolayers were consistently found to reach confluence within the time frame of culture.

Co-culture model. The primary epithelial cells (75,000 cells/well) were cultured for 5-7 days in serum-free keratinocyte medium with 1 mM isoproterenol on the bottom of culture plates coated with 10 μg/ml fibronectin, 30 μg/ml vitrogen and 10 μg/ml BSA. Primary HUVEC (150,000 cells/well) were seeded on fibronectin-coated Transwell inserts and cultured in HUVEC medium for 4 days. The monolayers were subsequently washed and the inserts were placed in the wells with epithelial cells cultured on the bottom. The co-culture was cultured in 50/50 medium for another day. The two cell types were subsequently separated, washed and cultured separately in 50/50 medium for another day.

Electron microscopy
Transwell filters, with endothelial cells on the top of the Transwell filter and epithelial cells on the bottom side, were fixed with 2.5% glutaraldehyde (v/v) in 0.1 M cacodylate buffer (pH 7.2) for 1 hr and post-fixed in 1% (w/v) osmium tetroxide in the same buffer for 1 hr. The filters were subsequently block-stained with uranyl acetate, dehydrated and embedded in LX-112. Thin sections were stained with uranyl acetate and lead citrate and examined with a CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Calcein-AM labeling and transmigration
The endothelial and epithelial cell line monolayers and bi-layers were cultured in HUVEC medium, and the primary endothelial and epithelial monolayers and bi-layers in 50/50 medium. Fresh medium was added to the Transwells 4 hours before the start of the assay. The Transwells were washed twice with HEPES medium just before the start of the experiment. Neutrophils (10^7/ml) were labeled with 4 g/ml calcein-AM in HEPES medium for 45 min at 37°C prior to the start of the transmigration assay (45). After labeling, the cells were washed twice and resuspended in HEPES medium (final cell concentration 10^6/ml). Where indicated, neutrophils or monolayers of endothelial or epithelial cells were pretreated for 15 min with 10 μg/ml antibody to β2-integrins, CD31 or to CD14 as a control, followed by washing of the cells with HEPES medium. Calcein-labeled neutrophils (0.5x10^6 cells) were placed in the upper compartment, and chemoattractants were placed in the lower compartment. The chemoattractant concentrations in the lower compartment were PAF, 100 nM; fMLP, 10 nM; IL-8, 10 nM; C5a, 10 nM. The Transwells were incubated for 35 min at 37°C.

To quantify transmigration, cells in the upper and lower compartments and cells attached to the filter were separately lysed in lysis buffer (PBS supplemented with 0.1% (v/v) Tween-20, 0.2% (w/v) hexadecyl-trimethyl-ammoniumbromide (Sigma), 0.2% (w/v) BSA and 20 mM EDTA). The amount of fluorescence in each of these compartments was measured in a spectrofluorimeter (Model RF-540, Shimadzu Corporation, Kyoto, Japan; EX 485 nm; EM 525 nm) and related to the fluorescence of the total input (set at 100%).
IL-6 and IL-8 quantification
The concentration of IL-6 and IL-8 in the supernatants, collected from both the upper and lower compartments of the different transmigration and co-culture models, was determined by ELISA (CLB) according to the manufacturer's instructions. The absorption was measured in a Multiscan Multisoft microplate reader (Labsystems Oy, Helsinki, Finland) at 450 nm.

Statistical analysis
Results were expressed as the mean ± SEM of at least 3 independent experiments, performed with cells from different donors. Results were analyzed with the Student's paired t-test (indicated in the legend of the figures) (46). Two-tailed P values were calculated, and P values exceeding 0.05 were considered not significant.

Results
Morphological characterization of the bi-layer model
We here describe a transmigration model in which sequential migration of neutrophils across human umbilical cord vascular endothelial cells (HUVEC) and human lung epithelial cells was studied (Fig. 1a). The results obtained with this model were compared to those obtained with either single endothelial or epithelial monolayers. The proper formation of confluent monolayers of endothelial and epithelial cells on the same filter required careful titration of the number of cells that was seeded. This was especially true for epithelial cells, which showed a tendency to penetrate the pores of the Transwell filters when seeded at high density.

The electron microscopy studies showed that the endothelial as well as the epithelial cells displayed a normal morphology and well-developed cell-cell contacts, and they revealed the striking difference in thickness between the two cell layers (Fig. 1b). On the basal surface of the epithelial cells hemidesmosomes were formed (Fig. 2) and filamentous structures could be seen between the plasma membrane and the filter, suggesting a basement membrane-like deposition (Fig. 2). Finally, neutrophils migrating through the intercellular junctions of the epithelial cells could also be visualized (Fig. 2).

Immunocytochemical staining for CD31 of the bi-layers, composed of the immortalized endothelial cells and the H292 cell-line epithelial cells, confirmed the localization of CD31 at the cellular junctions of the endothelial cells (47; 48). The staining was restricted to the cells on the topside of the Transwell filter, i.e. the endothelial cells (data not shown). Similarly, staining of the bi-layers for E-cadherin revealed E-cadherin expression at intercellular junctions of the epithelial cells, present exclusively on the bottom side of the filter (data not shown).
Migration of Neutrophils across Endothelial and Epithelial Monolayers

**Figure 1.** Schematic and morphological representation of the bi-layer model. (a) Schematic representation of the bi-layer model, comprised of endothelial and epithelial cells cultured on the top, respectively, the bottom of a porous polycarbonate membrane. (b) Morphological characterization of the bi-layer model comprised of immortalized HUVEC and H292 epithelial cells. Transmission electron micrograph of a Transwell filter carrying the two monolayers. Indicated in the photomicrograph are the filter (F), the thin monolayer of endothelial cells (en) and, underneath the filter, the relatively thick monolayer of epithelial cell (ep). The epithelial cells are cuboidal and occasionally grow through the pores (p) of the filter. Bar represents 1 um.

**Figure 2.** Morphological characterization of the bi-layer model comprised of immortalized HUVEC and H292 epithelial cells. (a) High magnification of a contact area of an epithelial cell with the filter (F) showing hemidesmosomes (large arrows) characterized by two electron dense plaques (small arrows) separated by an electron-lucent space. Extracellular matrix deposition, represented by the fine filaments (arrowheads), can be detected in the region between the plasma membrane and the filters. Bar represents 0.5 um. (b) An area of the epithelial cell layer (ep) is shown where a neutrophil (n), migrating through the epithelial cell-cell junction, can be seen. Arrows indicate neutrophil granules. Bar represents 1 um.
Having established the bi-layer model, we tested whether the characteristics of neutrophil migration across the bi-layers were different from those of migration across single monolayers. Transmigration of neutrophils across single epithelial monolayers, as induced by a series of chemoattractants, was lower when compared to migration across single endothelial monolayers (Fig. 3a). This may be due to the relatively thick and compact epithelial monolayer (Fig. 1b), being a more difficult barrier to cross. Interestingly, the percentage of neutrophils that migrated across the bi-layers equaled the percentage of cells that migrated across single endothelial monolayers, despite the presence of the additional epithelial monolayer. This was particularly evident for PAF, C5a and IL-8. FMLP already induced a relatively high migration of neutrophils across epithelial monolayers, and the migration across the bi-layer did not differ significantly from the migration in the other models.

The results obtained with the cell line cells were then compared to those obtained with primary endothelial and primary lung epithelial cells. Here, migration across single epithelial monolayers was equally efficient as migration across single endothelial monolayers (Fig. 3b). This result correlated with the higher basal permeability of primary epithelial cells, when compared to the H292 cells (data not shown). Moreover, these primary cells appeared less cuboidal and more flattened than the H292 cells and may thus represent less of a barrier for the migrating neutrophils (data not shown). Importantly, similar to

---

**Figure 3. Neutrophil transmigration in the different models.** Migration of neutrophils across endothelial monolayers cultured on the top of the filters (open bars), epithelial monolayers cultured on the bottom of the filter (hatched bars), and bi-layers (filled bars) (a) Transmigration across HUV-C cell line and H292 epithelial monolayers and bi-layers cultured in HUVEC medium was measured towards medium alone or in response to various chemotactic stimuli. Data are mean ± SEM of 3-8 independent experiments using cells from different donors. Student’s paired t-test was performed to compare transmigration across the bi-layers with migration across the epithelial monolayers. ★: P < 0.05. (b) Transmigration across primary endothelial monolayers, primary lung epithelial monolayers and bi-layers cultured in 50/50 medium was determined as in a. Data are mean ± SEM of 3-6 independent experiments. Student’s paired t-test was performed to compare transmigration across the bi-layers with migration across the epithelial monolayers. ★: P < 0.05.
neutrophil migration across cell line monolayers and bi-layers, the migration towards PAF, C5a and IL-8 was significantly higher across the primary bi-layers than across primary epithelial monolayers, and again the migration towards tMLP was not significantly elevated across the primary bi-layers (Fig. 3b). These results show that neutrophil migration across bi-layers of endothelial and epithelial cells, either cell line cells or primary cells, is increased in comparison to the migration across single epithelial monolayers.

The relative increase in neutrophil migration across the bi-layer, as compared to the migration across single epithelial monolayers, was not simply due to increased adhesion. The percentage of neutrophils associated to endothelial monolayers cultured on filters with a pore size which does not allow passage of neutrophils (0.45 μm), was similar in the absence or presence of epithelial cells on the bottom of the filter (data not shown). The interaction of neutrophils with the extracellular matrix deposited by endothelial cells onto the filter did not seem to play a role either, because the presence of a deposited HUVEC matrix or fibronectin coating onto the topside of the filter did not enhance subsequent transepithelial migration (data not shown).

Role of β-integrins and CD31 in neutrophil transmigration

Neutrophil transmigration in the three different models was almost completely CD18 dependent, since CD18-blocking antibodies inhibited more than 95% of the transmigration across endothelial and epithelial monolayers and across bi-layers (Fig. 4). Pretreatment of the endothelial cells with a blocking antibody to CD31 inhibited transmigration of neutrophils across the bi-layers and across single HUVEC monolayers for 64% and 47%, respectively. The CD31 antibody did not block migration across single epithelial monolayers, which is explained by the absence of CD31 on epithelial cells. These data show that the formation of the bi-layer does not significantly alter the role of prototypic adhesion molecules in neutrophil adhesion and transmigration. When neutrophils were pre-treated with the CD31 antibody, transmigration was inhibited and adhesion to the endothelial monolayers was increased (data not shown), possibly due to CD31-mediated activation of β1 and/or β2-integrins on the surface of neutrophils (22, 49).

Figure 4. Role for β-integrins and CD31 in neutrophil transmigration. PAF-induced neutrophil transmigration was measured across monolayers of HUVEC cell line (open bars), H292 epithelial cells (hatched bars), and the bi-layers (filled bars) after pretreatment of neutrophils with an antibody to CD18 (monoclonal antibody IB4) or after pretreatment of endothelial and epithelial cells with an antibody to CD31 (monoclonal antibody HEC 170). Results are represented as percentage of inhibition of neutrophil migration in the presence of an irrelevant antibody to CD14. Data are mean of, respectively, 2 and 4 independent experiments.
Role of IL-1β, IL-6 and IL-8

Endothelial cells and, in particular, epithelial cells are known to produce cytokines and chemokines spontaneously, as well as after stimulation with inflammatory cytokines. It is known that IL-1β is produced by endothelial and epithelial cells and that it activates either cell type, resulting in increased expression of adhesion molecules and production of various cytokines and chemokines, e.g. IL-6 and IL-8 (6; 15; 19; 26).

In an initial effort to address the notion that these agents are involved in the efficient transmigration of neutrophils across the bi-layers, we measured the concentration of IL-1β, IL-6 and IL-8 in the culture supernatant by means of ELISA. IL-1β was found in the supernatant of primary epithelial cells, whereas in the supernatant of primary endothelial cells hardly any IL-1β was detected (Fig 5a). Measurement of the IL-6 concentration revealed that the supernatant of monolayers of primary endothelial cells, H292 epithelial cells and primary epithelial cells contained hardly any IL-6. Yet, the IL-6 level in the supernatant of the primary bi-layers was significantly higher (Fig. 6). Moreover, a substantial amount of IL-6 was detected in the supernatant of primary epithelial cells that had previously been co-cultured with primary endothelial cells, whereas the IL-6 level in the endothelial supernatant was low with or

**Figure 5.** Concentration of interleukins in the supernatant of primary endothelial or primary epithelial cells, with or without preceding co-culture with the other cell type. The cells were first cultured for one day on filters with or without the other cell type on the bottom of the Transwell culture plate. Thereafter, the cells were refreshed, separated when indicated and incubated for another day. Open bars, culture for one day without co-culture with the other cell type. Hatched bars, culture for one day after preceding co-culture with the other cell type. Black bars, co-culture for one day after preceding co-culture. The IL-1β (a), IL-6 (b) and IL-8 (c) concentration of the supernatants was measured by means of ELISA. The data are mean ± SEM of 3 independent experiments.
Migration of Neutrophils across Endothelial and Epithelial Monolayers

without previous co-culture with epithelial cells (Fig. 5b). In contrast, the supernatant of H292 epithelial cells did not contain more IL-6 after co-culture with primary endothelial cells (data not shown). Thus, the augmented IL-6 level in the supernatant of primary bi-layers appears to be due to increased IL-6 production by epithelial cells in response to soluble factors secreted by endothelial cells.

IL-8 was also hardly detectable in the supernatant of endothelial cell monolayers, and the concentration of IL-8 in the supernatant of bi-layers of primary cells was increased significantly in comparison to the concentration detected in monolayers of endothelial and epithelial cells. However, in contrast to IL-6, IL-8 was found in considerable amounts in the supernatant of primary epithelial cell monolayers (Fig. 6). This was not only true for primary epithelial cells (6.8 ng IL-8/ml), but also for H292 epithelial cells (3.5 ng IL-8/ml). Moreover, the IL-8 level in the supernatant of primary epithelial cells that had previously been co-cultured with primary endothelial cells was not elevated, whereas augmented IL-8 levels were detected in the supernatant of primary endothelial cells that had previously been co-cultured with primary epithelial cells (Fig. 5c). Thus, the augmented IL-8 level in the supernatant of bi-layers appears to be the result of both the spontaneous epithelial IL-8 production and the stimulated endothelial IL-8 production. Together, these data show that co-culture of endothelial and epithelial cells results in increased production and/or release of cytokines and chemokines, e.g. IL-6 and IL-8.

![Figure 6. Concentration of IL-6 (open bars) and IL-8 (filled bars) in the supernatant of primary endothelial or primary epithelial cells separately, or in combination, when cultured as a bi-layer. The IL-6 and IL-8 concentrations of the supernatants after two days of culture was measured by means of an ELISA. The IL-6 data are mean ± SEM of 4 independent experiments. The IL-8 data are mean ± SEM of 5 independent experiments. Student’s paired t-test was performed to compare the concentration of IL-6 or IL-8 in the supernatant of bi-layers and epithelial monolayers. *: P <0.05.](image)

Discussion

Previous studies on in vitro leukocyte transmigration have relied on models that consisted of a single monolayer of endothelial, epithelial, mesothelial or even (transfected) fibroblast cells, cultured on porous membranes. These models have provided important experimental evidence for the role of cell-adhesion molecules, cytokines, and chemokine receptors in leukocyte transmigration (9; 10; 23; 26; 50). However, transmigration across a particular monolayer of endothelial cells is followed in vivo by passage through the basement membrane and contact with a second cell type, e.g. epithelial cells in the lung or stromal cells in the bone marrow. In an attempt to mimic this complex in vivo situation, we developed an experimental model to investigate granulocyte migration in the context of lung inflammatory disorders, using monolayers of endothelial and lung epithelial cells, both primary cells and cell lines, cultured on opposite sides of the same Transwell filter.
The characterization of this bi-layer model by electron microscopy and immunocytochemistry showed that cellular morphology, distribution of the junctional proteins CD31 and E-cadherin, and the integrity of the endothelial and epithelial monolayers was unaltered in the bi-layer model. In addition, the bi-layer barrier was found to be less permeable to 3 kD FITC-conjugated Dextran than the single endothelial and epithelial monolayers (data not shown).

Epithelial cells were found to grow into pores (size 3 μm) of the filters. This phenomenon has also been described for endothelial cells by Mackarcl et al. (51), and is likely to be a general phenomenon. We found no indications for specialized structures or basement membranes at the zones of contact between endothelial and epithelial cells. Moreover, the molecular basis of neutrophil migration in the bi-layer models appeared to be unaltered, i.e. the migration was largely mediated by β1-integrins and CD31. Yet, the transmigration of neutrophils in the bi-layer model was more efficient than in the single epithelial monolayer model, and equaled the migration in the single endothelial monolayer models. Several mechanisms may be implicated in this phenomenon.

We tested whether the endothelial monolayer in the context of the bi-layer model would represent a more adhesive surface for neutrophils, as compared to a single endothelial monolayer. However, neutrophil adhesion to the endothelium was not different in the bi-layer model, indicating that increased adhesion does not occur. Currently, we cannot exclude that qualitative changes in neutrophil adhesion, i.e. the use of additional types of adhesion molecules other than β1-integrins or CD31, play an important role in the migration in the bi-layer model. Future research will therefore include the analysis of the adhesion molecule repertoire on the endothelial cells in the absence or presence of epithelial cells.

Transendothelial migration may enhance leukocyte motility, thus facilitating subsequent passage across an epithelial monolayer. Such effects may, for example, result from the interaction with endothelial CD31, as CD31-mediated interactions have recently been shown to stimulate the rate of integrin-supported neutrophil migration (52). The interaction with the extracellular matrix at the basal side of the endothelial cells could enhance subsequent neutrophil transepithelial migration as well. However, the presence of a matrix deposited by endothelial cells did not alter the migration of neutrophils across the epithelial monolayer.

Finally, the endothelial and epithelial cells may influence each other such that neutrophil transmigration across both monolayers is enhanced, i.e. the epithelial cells may secrete soluble factors that promote neutrophil transepithelial migration, and, vice versa, endothelial cells may secrete factors that promote migration across epithelial monolayers. Our results indeed show that transendothelial migration is increased when epithelial cells are co-cultured on the bottom of the Transwell culture plate for two days, and that transepithelial migration is increased when endothelial cells are co-cultured. Thus, physical contact between these cell types is not required for the increase in transmigration. Instead a paracrine interaction between the epithelial and the endothelial cells seems to be implicated in the increase of neutrophil transmigration.

Our present results further support this idea, i.e. co-culture of endothelial and epithelial cells dramatically increases the release of particular cytokines, an as yet undescribed phenomenon. For instance, the concentration of IL-6 was significantly and synergistically increased when primary endothelial and epithelial cells were co-cultured, due to stimulated epithelial IL-6 production. IL-6 enhances survival of neutrophils in vitro (53) and has been shown to decrease cell-cell associations of carcinoma cells (54). In addition, IL-8, a potent neutrophil chemoattractant that has been described to be secreted by epithelial and stimulated endothelial cells (35), was found to be significantly and synergistically increased in the bi-layer model as a result of spontaneous epithelial IL-8 production and
Migration of Neutrophils across Endothelial and Epithelial Monolayers

stimulated endothelial IL-8 production. In general, we found that the primary lung epithelial cells produce more IL-6 and IL-8 than did the H292 cell line cells, an effect that may also contribute to the relatively efficient migration across primary versus H292 epithelial monolayers.

IL-8 release induced by IL-1 has previously been shown to promote migration of neutrophils across monolayers of endothelial and lung epithelial cells (55; 56). In parallel, our data suggest that in the bi-layer model epithelial-derived IL-1β induces IL-8 production by endothelial cells. Co-culture with epithelial cells increases the endothelial IL-8 production, whereas the spontaneous IL-8 production of epithelial cells is unaffected by co-culture with endothelial cells. In addition, epithelial cells spontaneously produce high levels of IL-1β, in contrast to endothelial cells, which hardly produced any IL-1β. Moreover, antibodies to IL-1β prevented almost completely the production of IL-8 in epithelial monolayer and bi-layer cultures (data not shown). These data show that IL-1β regulates epithelial IL-8 secretion via an autocrine loop. Whether epithelial cell-derived IL-1β is indeed the initiating factor in the stimulated neutrophil migration across the bi-layers remains to be established.

The enhanced release of IL-6 and IL-8 may contribute to the increase in neutrophil migration across the bi-layer. This may occur as a result of enhanced chemotaxis, but may also involve cytokine-mediated changes in the endothelial or epithelial monolayers. Regardless the mechanism involved, a strong chemotactic stimulus was still required for neutrophil transmigration, as the spontaneous migration across the bi-layers remained as low as across the single monolayers.

Recently, a similar transmigration model for neutrophils was described, combining HUVECs with the A549 lung epithelial cell line (57). Although the experimental set-up of this work is similar to ours, these studies did not address the relative role for adhesion molecules in the migration or reveal any paracrine communication, involving cytokines or chemokines, between epithelial and endothelial cells. Moreover, in this model the migration across the bi-layers was not increased, when compared to migration across the individual monolayers. The differences with our findings may be related to the alveolar, rather than bronchial, origin of A549 tumor line, and these cells may behave differently with respect to production and release of soluble mediators.

In conclusion, our current results with the bi-layer model provide new insights in the molecular basis of neutrophil transmigration. This model adds new aspects to the research on leukocyte migration in lung inflammatory disorders by providing an extra level of complexity, i.e. the 'cross-talk' between monolayers of different cell types and the concomitant effects on leukocyte passage. These interactions between endothelial and epithelial cells are likely to be relevant for inflammatory disorders in the lung, and may also play a role in other tissues where endothelial and epithelial cell linings are in close proximity.

Footnote
This study was financially supported by the Netherlands Asthma Foundation (grant no. 32.96.43).
References


Migration of Neutrophils across Endothelial and Epithelial Monolayers