

Effects of inflammatory cytokines on the permeability of human lung microvascular endothelial cell monolayers and differential eosinophil transmigration

Julie B. Sedgwick, PhD,^a Indu Menon, MS,^a James E. Gern, MD,^b and William W. Busse, MD^a *Madison, Wis*

Background: Rhinovirus (RV) infections can result in asthma exacerbations in both adults and children. Respiratory epithelium, the primary site of RV replication, responds to the viral infection by generating a variety of cytokines and chemokines capable of promoting airway inflammation and hence might increase asthma severity. Some of these mediators might also affect the permeability of underlying vascular endothelium.

Objective: We hypothesized that RV infections can promote airway inflammation and thus asthma by enhancing local vascular permeability.

Methods: Confluent human lung microvascular endothelial cell (HMVEC-L) monolayers were used as an in vitro model of vascular endothelium to determine whether cytokines associated with RV-induced infections are capable of modulating endothelial cell permeability as measured by means of transendothelial electrical resistance. Recombinant cytokines and chemokines were added to confluent HMVEC-L monolayers cultured on Transwell filters, and permeability was measured as decreased electrical resistance over time. Eosinophil transendothelial migration was assessed under the same experimental conditions.

Results: TNF- α , IL-1 β , and IFN- γ significantly increased HMVEC-L permeability. In contrast, GM-CSF, G-CSF, IL-8, IL-6, and RANTES had no effect. Although incubation of HMVEC-L monolayers with either TNF- α or IL-1 β promoted eosinophil migration, IFN- γ had no effect, indicating that enhanced permeability alone was not sufficient for eosinophil infiltration.

Conclusion: Select cytokines, generated in response to RV infection, can increase vascular permeability and might provide a mechanism by which RV infection can lead to edema, cellular infiltration, and inflammation and thus compromised airflow. (*J Allergy Clin Immunol* 2002;110:752-6.)

Key words: Permeability, transendothelial electrical resistance, rhinovirus, eosinophils, transmigration, asthma, cytokines, adhesion

Abbreviations used

HMVEC-L: Human lung microvascular endothelial cell

RV: Rhinovirus

TEER: Transendothelial electrical resistance

Although rhinovirus (RV) respiratory infections are the most frequent cause of asthma exacerbations, the pathologic mechanisms involved in the development of asthma obstruction and inflammation are incompletely understood. It has been suggested that asthma associated with RV infections is due to a host immunologic response to RV rather than to direct pathophysiologic changes stemming from the infection.¹ The hallmarks of RV infection of the upper airway are increased nasal secretions and congestion as a result of leakage of plasma proteins from the vascular tissue of the nasal mucosa.² By using albumin and IgG levels as markers of serum transudation, it has been shown that increased nasal permeability is strongly correlated with the appearance of maximum cold symptoms.² Moreover, there is evidence that RV also infects the lower airways and increases lung microvasculature permeability.^{3,4} The development of bronchial obstruction in viral-induced episodes of asthma is likely to be multifactorial. Presently, the mechanisms underlying these changes in pulmonary function have been focused largely on enhanced airway inflammation and greater bronchial hyperresponsiveness. It is also possible that bronchial mucosa edema occurs during viral respiratory infection and contributes to airway narrowing.

The mechanisms by which viral infections affect endothelial integrity are as yet not fully defined. RV infection has been shown to result in local infiltration of neutrophils and production of various mediators, kinins, and cytokines (IL-6, IL-8, TNF- α , IFN- α , RANTES, GM-CSF, and IL-1) in the upper airways.^{1,5,6} Experiments conducted with human umbilical vascular endothelial cells and other nonpulmonary cells indicate that proinflammatory cytokines (TNF- α , IFN- γ , IL-1 β , and IL-6), also produced by RV-infected epithelial and inflammatory cells, affect the barrier function of endothelial cells.⁷⁻⁹ Because endothelial cells from different sites, tissues, and species can respond differently to inflamma-

From ^athe Division of Allergy and Immunology, Department of Medicine, and ^bthe Department of Pediatrics, University of Wisconsin, Madison.

Supported by National Institutes of Health grant HL-60993.

Received for publication February 7, 2002; revised July 10, 2002, and July 23, 2002; accepted for publication July 24, 2002.

Reprint requests: Julie B. Sedgwick, PhD, CSC-3244 H6/355 Allergy, 600 Highland Ave, Madison, WI 53792.

© 2002 Mosby, Inc. All rights reserved.

0091-6749/2002 \$35.00 + 0 1/83/128581

doi:10.1067/mai.2002.128581

tory stimuli or have variable susceptibility to injury,¹⁰ data obtained from a nonpulmonary or macrovascular endothelium source might not adequately represent cells from lung sites. Therefore human lung-derived microvascular endothelial cells (HMVEC-Ls) were used to test the hypothesis that cytokines associated with RV respiratory infections disrupt the endothelial cell barrier and that these changes are reflected in alterations in transendothelial electrical resistance (TEER).

METHODS

Reagents

Percoll was purchased from Amersham Pharmacia (Franklin Lakes, NJ). HBSS, PBS, newborn calf serum, FCS, and penicillin-streptomycin were obtained from Life Technologies (Grand Island, NY). Recombinant human IL-1 β , TNF- α , IL-6, GM-CSF, IL-8, and RANTES were purchased from R&D Systems (Minneapolis, Minn). IFN- γ was obtained from Promega (Madison, Wis). All other reagents were purchased from Sigma Chemical Co (St Louis, Mo) unless stated otherwise.

Endothelial cell culture

HMVEC-Ls were purchased from BioWhittaker (Walkerville, Md) as isolates from individual donors and cryopreserved at passage 4. The cells were characterized for the expression of acetylated low-density lipoprotein receptors, von Willebrand factor VIII, and platelet endothelial cell adhesion molecule. HMVEC-Ls were seeded onto collagen-coated tissue culture flasks (Biocoat; BD Labware, NJ) and grown at 37°C in a humidified atmosphere of 5% CO₂. EGM-2MV growth medium (Biowhitaker) consisted of basal medium supplemented with human recombinant epidermal growth factor (10 ng/mL), hydrocortisone (1 μ g/mL), gentamicin (50 μ g/mL), amphotericin B (50 μ g/mL), bovine brain extract (3 μ g/mL), and FBS (5% vol/vol) and was supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin. The medium was changed every 2 to 3 days, and cells were passaged when they reached 90% confluency (3-5 days); the cultures were used between 5 and 10 passages. Polycarbonate Transwell inserts (24-well tissue culture plates; Corning Costar Co, Cambridge, Mass) were pretreated with 10 μ g/mL plasma fibronectin for 1 hour at 37°C and seeded with 1×10^5 HMVEC-Ls. The wells were incubated at 37°C and 5% CO₂ for 2 to 3 days to develop confluence before TEER assessment.

Eosinophil isolation

Subjects ranged from 18 to 55 years of age and had allergic rhinitis or mild allergic asthma. Immediate hypersensitivity was confirmed by at least one positive skin reaction (>3 mm) with the prick-puncture technique to extracts of common allergens, such as ragweed, house dust mite, grass and tree pollen, or cat and dog dander. Except for as-needed inhaled β -agonists, subjects were taking no medication at the time of blood donation. Informed consent was obtained before subject participation, and the study was approved by the University of Wisconsin Human Subjects Committee.

Eosinophils were isolated by means of Percoll density gradient centrifugation and negative selection with anti-CD16 magnetic beads (Miltenyi Biotech, Auburn, Calif), as described previously.¹¹ Cell purity and viability was greater than 95%, with neutrophils as the major contaminating cell.

Measurement of TEER

At the initiation of each experiment, fresh medium was added to both Transwell chambers (0.4- or 8- μ m pore; 0.25 mL in the upper chamber and 0.75 mL in the lower chamber). Cytokines and other

treatments were added to the chambers as indicated. Resistance across the endothelial cell monolayers was measured with an epithelial volt-ohmmeter and STX-2 electrodes (EVOM; World Precision Instruments, Sarasota, Fla). Electrical resistance was determined over 0 to 24 hours for each monolayer-cytokine condition. TEER values (in ohms) for the negative control of an uncoated filter were subtracted from each monolayer measurement. TEER measurements were multiplied by the membrane area to give the resistance in ohms times area units ($\Omega \times \text{cm}^2$). A decrease in TEER represented disruption of endothelial cell-cell junctions and increased permeability, and increased TEER signaled increased monolayer integrity. Data are expressed as the percentage of basal TEER obtained by dividing values obtained at each time point by the initial TEER value.

Eosinophil adhesion

Eosinophil adhesion to HMVEC-Ls was measured as the eosinophil peroxidase activity of adherent cells, as previously described.¹² HMVEC-Ls were cultured to confluence (2-3 days) on fibronectin-coated 96-well tissue culture plates and then pretreated with cytokines for 4 to 24 hours. Eosinophils ($1 \times 10^4/100 \mu\text{L}$) were added to each well and incubated for 60 minutes, and nonadherent cells were rigorously washed off with warm HBSS. Eosinophil adhesion was calculated as the percentage of eosinophil peroxidase activity on the basis of the activity in the total cells added.

Eosinophil transendothelial migration

Eosinophil migration through HMVEC-L monolayers was assayed as described previously.¹² HMVEC-L monolayers on Transwell filters (8- μ m pore) were confirmed for confluency by means of TEER and then pretreated with medium (control), TNF- α (100 pmol/L), IL-1 β (100 pmol/L), IFN- γ (100 pmol/L), or IL-6 (100 ng/mL) for 6 hours at 37°C. The monolayers were washed, and 100 μ L of 3 to 5×10^6 eosinophils/mL in RPMI supplemented with 5% FCS and penicillin-streptomycin was placed above the monolayer. The lower chamber contained medium in the absence or presence of 100 nmol/L platelet-activating factor (PAF), as a chemotactic agent. After incubation at 37°C for 3 hours, the migrated cells were counted in duplicate with a hemacytometer, and the percentage of migration was determined.

Data analysis

Data are presented as means $\pm$ SEM, and group-specific differences were analyzed by using 1-way ANOVA (SigmaStat; SASS, Chicago, Ill). A *P* value of less than .05 was considered significant.

RESULTS

Electrical resistance of endothelial monolayers

The mean electrical resistance of confluent, unstimulated HMVEC-L monolayers (filter and medium control subtracted) varied between 13.4 and 26.5 $\Omega \times \text{cm}^2$. These values were similar to TEER values reported for human umbilical vascular endothelial cell monolayers.⁵ Monolayers were treated with 5 μ g/mL cytochalasin D (Sigma), a potent reagent capable of disorganizing the actin cytoskeleton,¹³ to validate the HMVEC-L monolayers as an in vitro model system for testing endothelial cell permeability and to compare HMVEC-L responses with those obtained with other endothelial cell types. Under these conditions, electrical resistance decreased to less than 10% of basal TEER level within 2 hours (Fig 1).

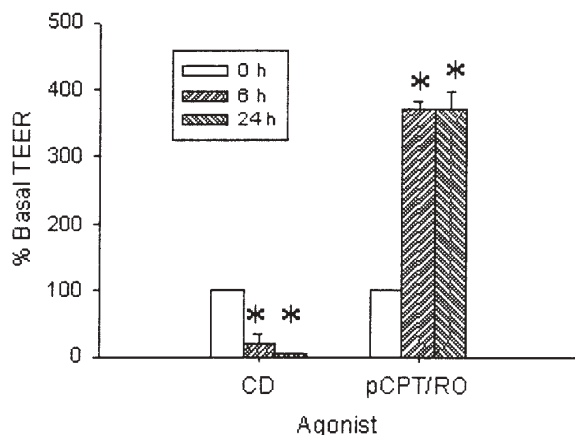


FIG 1. Effect of cyclic AMP and cytochalasin D on HMVEC-L TEER. HMVEC-L monolayers were incubated with the cAMP analogue CPT-cAMP (pCPT; 750 μ mol/L) and the phosphodiesterase inhibitor RO20-1724 (RO; 35 μ mol/L) or cytochalasin D (5 μ g/mL) for 24 hours, and TEER values were monitored. Values are expressed as the percentage of basal TEER (pCPT/RO, $n = 3$; cytochalasin D, $n = 8$). * $P < .001$ versus 0 hours.

In addition, the combination of a cyclic AMP analog (CPT-cAMP) and a phosphodiesterase inhibitor (RO20-1724), which increase cAMP levels and membrane integrity,¹³ produced a rapid increase (>3-fold) in electrical resistance over values noted with monolayers treated with medium only, paralleling results obtained with bovine endothelial cells.¹⁴ Continued incubation of the monolayers with these agonists for up to 24 hours had no further effect on endothelial permeability.

Cytokine effect on HMVEC-L permeability

The proinflammatory cytokines TNF- α , IL-1 β , and IFN- γ significantly decreased the baseline TEER of HMVEC-L monolayers when compared with untreated monolayers. The decrease in TEER was significant within 6 hours of incubation with the cytokines, was sustained over 24 hours (Fig 2, A), and was concentration dependent (Fig 2, B). Additional experiments demonstrated that IL-6, G-CSF, GM-CSF, IL-8, RANTES, and IFN- α did not affect the electrical resistance of the HMVEC-L monolayers up to 24 hours in culture (data not shown). These cytokines were tested at multiple doses (0.1, 1, 10, and 100 ng/mL) and in combination to identify possible synergy; no changes in TEER were observed (data not shown).

Eosinophil adhesion and transendothelial migration

HMVEC-L monolayers were incubated with medium, TNF- α (100 pmol/L), IL-1 β (100 pmol/L), IFN- γ (100 pmol/L), or IL-6 (100 ng/mL) for 4 to 24 hours to determine whether cytokines that induced monolayer permeability (decreased TEER) were accompanied by changes in eosinophil adhesion to the endothelial cells and subsequent transmigration. TNF- α and IL-1 β significantly

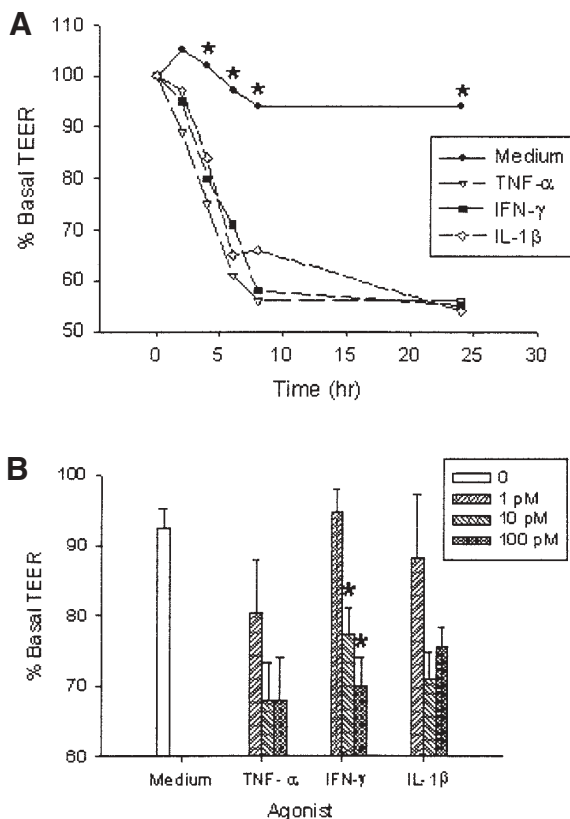


FIG 2. Effect of cytokines on HMVEC-L permeability. **A**, Kinetics. Confluent HMVEC-L monolayers were incubated with medium, TNF- α (100 pmol/L), IL-1 β (100 pmol/L), or IFN- γ (100 pmol/L). Values are means $\pm$ SEM ($n = 6$). * $P < .05$ versus all 3 cytokines (by means of ANOVA for repeated measurements). Error bars are not included in the figure for clarity. **B**, Dose response. Increasing concentrations of cytokines were incubated with HMVEC-L monolayers for 8 hours, and TEER was determined ($n = 3$). * $P < .05$ versus no cytokine added.

enhanced eosinophil adhesion ($33.1\% \pm 4.9\%$ and $34.8\% \pm 6.5\%$ adhesion, respectively, vs $7.2\% \pm 1.4\%$ for the medium control; $P < .05$; Fig 3). In contrast, neither IFN- γ nor IL-6 promoted eosinophil adhesion under the conditions tested. Eosinophil adhesion to HMVEC-Ls was optimal when the monolayers were preincubated with the cytokines for 6 hours; additional incubation (up to 24 hours) did not significantly change adhesion.

Monolayers were pretreated for 6 hours with medium, TNF- α (100 pmol/L), IL-1 β (100 pmol/L), IFN- γ (100 pmol/L), or IL-6 (100 ng/mL; reported to promote endothelial permeability¹⁵) to determine whether cytokine-enhanced permeability of HMVEC-L monolayers promoted eosinophil transmigration. In the presence of 100 nmol/L PAF, both TNF- α and IL-1 β promoted significant transmigration compared with untreated monolayers ($41.7\% \pm 10.3\%$ and $30.6\% \pm 4.7\%$ migration vs $7.4\% \pm 2.7\%$, respectively; $P < .05$; Fig 4). In contrast, neither IFN- γ nor IL-6 enhanced eosinophil migration.

DISCUSSION

Pivotal to the development of inflammation, modulation of vascular endothelial permeability can facilitate increased passage of macromolecules and circulating cells from the circulation to tissues. To understand how RV and other respiratory virus infections affect vascular permeability, we tested the hypothesis that specific cytokines produced in response to viral infections regulate the barrier properties of lung microvasculature. Eosinophils are an important component of asthmatic inflammation exacerbated by RV infection, and changes in endothelial permeability might be a mechanism of enhanced eosinophil adhesion and transmigration to the airway. Because the biologic responses of endothelial cells can vary by site or tissue,¹⁶⁻¹⁹ we used lung-derived endothelial cells in these experiments.

Previous studies have established that nonlung vascular endothelial permeability can be altered by TNF- α , IFN- γ , and IL-1 β .⁷⁻⁹ Our results show that TNF- α , IFN- γ , and IL-1 β , which can all be induced by RV infection,^{1,3,4} increased HMVEC-L monolayer permeability, as measured by means of TEER. However, other cytokines and chemokines generated during viral infections had no effect. Reports on the effect of IL-6 on endothelial permeability have been conflicting^{15,20}; we found that IL-6 had no effect on HMVEC-L permeability. Experimental RV16 infection increased bronchoalveolar lavage levels of TNF- α to 1 to 8 ng/mL in healthy control subjects²¹; infected patients with allergic rhinitis who were then challenged with allergen by means of segmental bronchoprovocation demonstrated further increases. These levels are in the range of our in vitro TNF- α doses (100 pmol/L: 1.7 ng/mL).

A likely consequence of increased permeability of the microvasculature in response to virus-induced cytokines is an increase in exudation of plasma proteins from the circulation into the airway tissues. In the upper airway this could represent a mechanism for nasal congestion and rhinorrhea during RV-induced colds. In addition, RV infections have been shown to be associated with increased inflammation in lower airway biopsy specimens,²² and there is evidence that RV infections can affect lower airway inflammation³ and function.²³ Therefore cytokines might produce similar effects in the lower airways and result in an increase in airway edema and secretions to further airway obstruction and, in some individuals, lead to asthma exacerbations. There is clinical evidence that vascular permeability, as indicated by increases in serum proteins in respiratory secretions, is increased in viral infections and asthma. Levels of sputum albumin are increased during natural acute respiratory viral infections in healthy subjects and further increased in infected patients with asthma.^{4,23} Moreover, sputum albumin concentrations are also increased in patients with moderate and severe asthma, even in the absence of respiratory viral infection, are inversely related to measures of airway obstruction, and correlate with sputum eosinophil and eosinophil cationic protein lev-

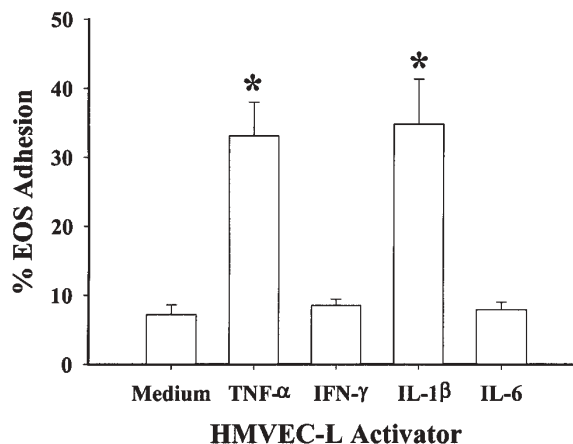


FIG 3. Eosinophil adhesion to cytokine-activated HMVEC-Ls. Confluent HMVEC-L monolayers were incubated for 6 hours with medium, TNF- α (100 pmol/L), IFN- γ (100 pmol/L), IL-1 β (100 pmol/L), or IL-6 (100 ng/mL), and eosinophil adhesion was then determined (n = 4). *P < .05 versus medium.

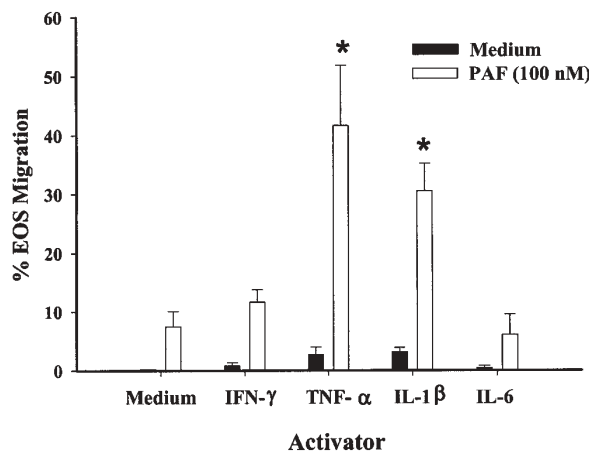


FIG 4. Effect of cytokine pretreatment of HMVEC-L monolayers on eosinophil transendothelial migration. One hundred microliters (3.5×10^6 cells/mL) of eosinophils was cultured for 3 hours with HMVEC-L monolayers pretreated (6 hours) with TNF- α (100 pmol/L), IL-1 β (100 pmol/L), IFN- γ (100 pmol/L), IL-6 (100 ng/mL), or medium alone. PAF (100 nmol/L) was added to the bottom chamber as a chemoattractant (n = 3). *P < .05 versus medium.

els.^{24,25} Therefore mediators that promote lung vascular permeability during an RV infection might also potentiate eosinophil airway infiltration.

In addition to altering endothelial permeability, TNF- α , IFN- γ , and IL-1 β increase the expression of adhesion molecules, such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, on endothelial cells and promote recruitment of leukocytes.^{12,26} Our results demonstrated that in vitro activation of HMVEC-L monolayers by TNF- α or IL-1 β under conditions that promote permeability enhanced eosinophil adhesion, as well as transmigration, in response to a chemotactic agent. Even though IFN- γ also increased endothelial per-

meability, it did not promote eosinophil adhesion of migration. The apparent lack of correlation between permeability and eosinophil transmigration by IFN- γ suggests that these cells do not simply pass through the endothelium when its permeability is enhanced but rather that the cells require signals that capture (adhesion molecules) and guide them (chemotactic signal) to their destination. For example, unlike TNF- α and IL-1 β , IFN- γ did not enhance HMVEC-L expression of either intercellular adhesion molecule 1 or vascular cell adhesion molecule 1 (data not shown). Thus lung microvascular endothelium does not act as a passive sieve through which cells and molecules permeate. Instead, multiple factors appear to differentially regulate the efflux of inflammatory cells.

In summary, our data demonstrate that specific cytokines produced in response to RV and other respiratory viral infections can elicit permeability of lung-derived microvascular endothelial cells; however, this alone is not a sufficient signal for eosinophil transmigration. These findings might have clinical relevance both to upper and lower airway consequences of viral infections and suggest that the maintenance and restoration of the endothelial cell-cell barrier might be an important pharmacologic target to limit the inflammatory response associated with respiratory illnesses and subsequent asthma exacerbations. Because there are multiple mediators and cells that can modulate endothelial permeability, the identification of a common underlying mechanism regulating the cell cytoskeletal structure is an important goal, and such an approach might be helpful in regulating the inflammatory reaction and asthma associated with cold.

We thank Kristyn Jansen for help with eosinophil isolation.

REFERENCES

- Gern JE, Busse WW. Association of rhinovirus infections with asthma. *Clin Microbiol Rev* 1999;12:9-18.
- Igarashi Y, Skoner DP, Doyle WJ, White MV, Fireman P, Kaliner MA. Analysis of nasal secretions during experimental rhinovirus upper respiratory infections. *J Allergy Clin Immunol* 1993;92:722-31.
- Fraenkel DJ, Bardin PG, Sanderson G, Lampe F, Johnson SL, Holgate ST. Lower airways inflammation during rhinovirus colds in normal and asthmatic subjects. *Am J Respir Crit Care Med* 1995;151:879-86.
- Pizzichini MMM, Pizzichini E, Efthimiadis A, Chauhan AJ, Johnston SL, Hussack P, et al. Asthma and natural colds. Inflammatory indices in induced sputum: a feasibility study. *Am J Respir Crit Care Med* 1998;158:1178-84.
- Subauste MC, Jacoby DB, Richards SM, Proud D. Infection of a human respiratory epithelial cell line with rhinovirus: induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. *J Clin Invest* 1995;96:549-57.
- Griego SD, Weston CB, Adams JL, Tal-Singer R, Dillon SB. Role of p38 mitogen-activated protein kinase in rhinovirus-induced cytokine production by bronchial epithelial cells. *J Immunol* 2000;165:5211-20.
- Marcus BC, Wyble CW, Hynes KL, Gewertz BL. Cytokine-induced increases in endothelial permeability occur after adhesion molecule expression. *Surgery* 1996;120:411-6.
- Beynon HL, Haskard DO, Davies KA, Haroutunian R, Walport MJ. Combinations of low concentrations of cytokines and acute agonists synergize in increasing the permeability of endothelial monolayers. *Clin Exp Immunol* 1993;91:314-9.
- Carley WW, Niedbala MJ, Gerritsen ME. Isolation, cultivation, and partial characterization of microvascular endothelium derived from human lung. *Am J Respir Cell Mol Biol* 1992;7:620-30.
- Murphy HS, Bakopoulos N, Dame MK, Varani J, Ward PA. Heterogeneity of vascular endothelial cells: differences in susceptibility to neutrophil-mediated injury. *Microvasc Res* 1998;56:203-11.
- Hansel TT, de Vries IJM, Iff T, Rihs S, Wandzilak M, Betz S, et al. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J Immunol Methods* 1991;145:105-10.
- Yamamoto H, Sedgwick JB, Busse WW. Differential regulation of eosinophil adhesion and transmigration by pulmonary microvascular endothelial cells. *J Immunol* 1998;161:971-7.
- Rubin LL, Hall DE, Porter S, Barbu K, Cannon C, Horner HC, et al. A cell culture model of the blood-brain barrier. *J Cell Biol* 1991;115:1725-35.
- Adamson RH, Liu B, Fry GN, Rubin LL, Curry FE. Microvascular permeability and number of tight junctions are modulated by cAMP. *Am J Physiol* 1998;274:H1885-94.
- Gaffney AB, Keenan AK. Modulation of human endothelial cell permeability by combinations of the cytokines interleukin-1 α/β , tumor necrosis factor- α and interferon- γ . *Immunopharmacology* 1993;25:1-9.
- Beekhuizen H, Furth RV. Growth characteristics of cultured human macrovascular venous and arterial and microvascular endothelial cells. *J Vasc Res* 1994;31:230-9.
- Beilke MA. Vascular endothelium in immunology and infectious disease. *Rev Infect Dis* 1994;11:273-83.
- Craig LE. Endothelial cells from diverse tissues exhibit differences in growth and morphology. *Microvasc Res* 1998;55:65-76.
- Moore TM, Chetham PM, Kelly JJ, Stevens T. Signal transduction and regulation of lung endothelial cell permeability. Interaction between calcium and cAMP. *Am J Physiol* 1998;275:L203-22.
- Ali MH, Schlidt SA, Chandel NS, Hynes KL, Schumacker PT, Gerrert BL. Endothelial permeability and IL-6 production during hypoxia: role of ROS in signal transduction. *Am J Physiol* 1999;277:L1057-65.
- Calhoun WJ, Dick EC, Schwartz LB, Busse WW. A common cold virus, rhinovirus 16, potentiates airway inflammation after segmental antigen bronchoprovocation in allergic subjects. 1994;94:2200-8.
- Folkerts G, Busse WW, Nijkamp FP, Sorkness R, Gern JE. Virus induced airway hyperresponsiveness and asthma. *Am J Respir Crit Care Med* 1998;157:1708-20.
- Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma. *J Allergy Clin Immunol* 1995;95:843-52.
- Louis R, Lau LCK, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *Am J Respir Crit Care Med* 2000;161:9-16.
- Pizzichini E, Pizzichini MM, Efthimiadis A, Evans S, Morris MM, Squillace D, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996;154:308-17.
- Worthylake RA, Burridge K. Leukocyte transendothelial migration: orchestrating the underlying molecular machinery. *Curr Opin Cell Biol* 2001;13:569-77.