

# Chemokine transport across human vascular endothelial cells

## Journal Item

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**Short Title:** Chemokine transport across endothelium

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**List of abbreviations**

CCL2 = MCP-1 = Macrophage chemotactic protein 1
CXCL10 = IP-10 = Interferon gamma inducible protein 10
CXCL11 = ITAC = Interferon-inducible T cell α-chemoattractant
DMEM = Dulbecco's modification of Eagle's medium
DMVEC = Dermal microvascular endothelial cells
ECs = Endothelial cells
EGM = Endothelial growth medium
HBSS = Hanks balanced salt solution
LMVEC = Lung microvascular endothelial cells
SVEC = Saphenous vein endothelial cells

# Abstract

Leukocyte migration across vascular endothelium is mediated by chemokines that are either synthesised by the endothelium or transferred across the endothelium from the tissue. The mechanism of transfer of two chemokines, CXCL10 (IP-10) and CCL2 (MCP-1), was compared across dermal and lung microvessel endothelium and saphenous vein endothelium. The rate of transfer depended on both the type of endothelium and the chemokine. The permeability coefficient (Pe) for CCL2 movement across saphenous vein was twice the value for dermal endothelium and four-times that for lung endothelium. In contrast, the Pe value for CXCL10 was lower for saphenous vein endothelium than the other endothelia. The differences in transfer rate between endothelia was not related to variation in paracellular permeability using a paracellular tracer, inulin, and immuno-electron microscopy showed that CXCL10 was transferred from the basal membrane in a vesicular compartment, before distribution to the apical membrane. Although all three endothelia expressed high levels of the receptor for CXCL10 (CXCR3), the transfer was not readily saturable and did not appear to be receptor-dependent. After 30 minutes, the chemokine started to be reinternalised from the apical membrane in clathrin-coated vesicles. The data suggests a model for chemokine transcytosis, with a separate pathway for clearance of the apical surface.



**Keywords**:
Chemokines, chemokine receptors, endothelial cells, permeability, transcytosis.

# Introduction

During inflammation or as part of normal immune surveillance, leukocytes migrate into different tissues of the body. The populations of leukocytes that are found in each condition vary greatly and are initially determined according to which cells migrate across the vascular endothelium of each tissue (Butcher, et al., 1999). The specific combination of molecular signals acting on the circulating cells determines whether they are stimulated to migrate and this depends on the inflammatory stimulus, the cells present in each tissue and the characteristics of the local endothelium. The multistep model of egression of leukocytes from the circulation into an inflamed tissue states that migration is principally mediated by the expression of chemokines on the luminal surface of the endothelium, but also requires appropriate adhesion molecules of the selectin and CAM families (Springer, 1994, Baggiolini, 2001). Endothelium itself is a major source of chemokines and the profile of chemokine expression depends on the tissue of origin of the endothelium. This is true both in resting cells or following stimulation with the inflammatory cytokines, TNFα and IFNγ (Hillyer et al., 2003). Many cell types located within the tissues can also produce chemokines and to elicit leukocyte migration from the blood these chemokines would need to reach the luminal surface of the endothelium and be presented on the apical surface (Rot, 1992). Many studies have assumed that the chemokines that are expressed on the endothelial surface would be a faithful reflection of those produced within the tissues. However this assumption has not been tested – transfer of chemokines across the endothelium could be selective for some chemokines and not others. Likewise luminal expression of chemokines will depend on the profile of endothelial glycosaminoglycans which bind chemokines (Middleton et al., 2002) and the rate of chemokine clearance from the cell surface. We have previously shown that the surface expression of different chemokines varies between endothelia and is dependent on the glycosaminoglycans they express (Hillyer and Male, 2005). This paper addresses the question of whether the phenotype of the endothelium affects chemokine transport.

Theoretically there are several ways in which a chemokine can be transported using: 1) transcellular transport via an endocytic compartment 2) paracellular diffusion between the endothelial cells or 3) movement in the plane of the cell membrane, while associated with cell surface molecules (McIntosh et al., 2002). It has been generally assumed that chemokines reach the circulation from the inflamed tissue by diffusion through the gaps between the endothelial cells (Ebnet et al., 1996) although this route is not available for endothelial barriers with continuous tight junctions. Moreover Middleton and colleagues (2002) have reported that endothelial cells actively transfer chemokines to their luminal surfaces attached to macromolecules, specifically glycosaminoglycans, or the Duffy antigen receptor for chemokines (DARC). Furthermore, CXCL8 (IL-8) has been reported to be internalized from the abluminal surface of the endothelial cells, transported transcellularly via plasmalemmal vesicles, and released onto the luminal membrane where it appeared located preferentially on tips of membrane protrusions (Middleton et al., 1997, Rot et al., 1996). Despite this, many questions remain unanswered. For example, we do not know whether all chemokines are transfered with equal efficiency across endothelia, or whether the transfer varies between tissues. Nor do we know if cytokine-activated endothelia transfer chemokines more quickly or in greater quantity, than resting endothelia. Several chemokine receptors have been identified on endothelia (Murdoch et al., 1999): their functions are generally undefined, but one possibility is that they mediate transcytosis.

The aim of this study was to investigate the rate and mechanisms of chemokine transfer across different endothelia. Following our previous results, we selected two different chemokines, CXCL10 and CCL2, which are not synthesised by resting endothelia in significant amounts (Hillyer et al., 2003). We examined transfer across primary human endothelial cultures from dermis, lung and saphenous vein, and used immunoelectron microscopy to elucidate the route by which chemokines are transferred to the luminal surface of the cells.

# Materials and methods

## Cell culture

Dermal and lung endothelial cells (DMVEC and LMVEC respectively) were purchased from Clonetics/Biowhittaker (Wokingham, Berks, UK) and expanded according to the manufacturer's recommendation in EGM2 supplemented medium. Saphenous vein endothelium (SVEC) was prepared by collagenase digestion of surgical resections obtained with informed consent from patients at Milton Keynes Hospital (Marin et al., 2001). Endothelial cells (ECs) were cultured until confluence, passaged using trypsin and were used at passage 5-8 in the assays. For a detailed description of the culture conditions see Hillyer et al., 2003. Table 1 summarises the endogenous production of chemokines CXCL10 and CCL2 in the conditions used in these assays as measured by ELISA.

## Permeability measurements

ECs were grown to confluence on transwell clear filters (Costar, Cambridge, MA). The polyester membranes (12mm diameter, 0.4μ pore size) were coated for one hour with 1mg/ml collagen type I (Sigma, UK) and fixed by exposure to ammonia vapour for 20 minutes. Membranes were then washed 4 times with HBSS. Cells were seeded onto the membrane at 1.5 $x10^5$ cells per filter and 0.5ml of medium was added to the upper compartment and 1.5ml to the lower compartment of the transwell plate. Cells were grown until confluence and rested for 24h. Some cultures were then activated with 25ng/ml TNFα for 24h. Cytokine-containing medium was removed and all cultures were washed before assay with HBSS.

The integrity of the monolayers was assessed before the assays by measuring trans-endothelial resistance using an EndOhm chamber (World Precision Instruments, Hertfordshire, UK). The value represents the resistance ($Ohm/cm^2$) to direct current flowing perpendicular to the endothelial monolayer. Actual EC resistance was calculated by subtracting the resistance determined in the absence of ECs from that in their presence. The resistance of all of the monolayers varied between 6.5-7.5 $Ohm/cm^2$ and no change was observed after 24 hours TNFα treatment. However the resistance of the endothelia was decreased at the end of the 60 minute transfer assays, by 0-2 $Ohm/cm^2$.

The integrity of monolayers grown in these conditions was also confirmed by staining cells on the filters with trypan blue and viewing by light microscopy, although it was not technically possible to carry out light microscopy and radioligand transport studies on the same filters.

Inulin transcellular exchange was measured to verify the integrity of the endothelial cells monolayer and to assess variations in paracellular transport (Ek et al., 2001, Salvetti et al., 2001). Cells were washed with HBSS and incubated with 0.6mg/ml inulin in DMEM supplemented with BSA 0.1% (Sigma, UK), which had been filtered before use (0.22μ). In each experiment, 1.5mls inulin solution was added to the well below the filter and 0.5ml DMEM/BSA was placed in the upper compartment. Samples were taken from the upper compartment at 5, 15, 25, 35 and 45 minutes and the transfer of fluoresceinated tracer was measured using a multiwell fluorimetric plate reader (Wallac Victor 1420).

**Chemokine transport and competition binding experiments**

Endothelial cultures on transwells were prepared as indicated above and the chemokine transport experiments were performed in a similar way to the experiments with fluoresceinated tracers except for the substitution of $^{125}$I-radiolabelled chemokine in the lower compartment. The initial chemokine concentration in the lower compartment was 1.25ng/ml and the specific activity was approximately 2000Ci/mmole for each chemokine ($^{125}$I-IP-10 or $^{125}$I-MCP-1 (Amersham, UK)). In competition experiments, 100ng/ml of unlabelled chemokine was also included in the lower compartment. Samples of 40μl were removed from the upper chamber, at 5, 15, 25, 35, 45 and 60 minutes and read on a gamma counter (1470 automatic gamma counter Perkin Elmer, Life Sciences, UK).

Chemokine permeability experiments were performed on at least three separate occasions for each endothelial cell type. The permeability coefficients (Pe) were calculated according to the method of Dehouck and colleagues (1992) for each endothelial cell type in each experiment and the Pe values from all the experiments were combined and compared in figure 2.

Pe is calculated as follows: The cleared volume of radioactive tracer (radioactivity in the upper chamber in cpm/initial radioactivity in the lower chamber in cpm/μl) was plotted against time. The slopes of the curves were fitted using linear least squares regression to give the rate of clearance (or permeability x surface area, PS) in μl/min, where $m_e$ is the slope of the curve corresponding to the PS value of endothelial cell monolayers on filters and $m_f$ is the slope of the curve corresponding to the PS of the filter alone. Pe of each endothelial monolayer was then calculated using the following formulae:

$1/PS = 1/m_e - 1/m_f$

$Pe = PS/S$

Where PS (clearance) is the permeability surface area product, and S is the surface area of the filter. The permeability values (Pe) are expressed as microlitres cleared per minute per $cm^2$ membrane.

Differences in Pe values between the endothelia and the effects of TNFα pretreatment (25ng/ml for 24hours applied to the apical surface) were assessed using ANOVA, followed by a post-hoc Newman Keuls, multiple comparison test.

**Immunoelectron microscopy**

All cell types were washed once with PBS and with 0.01% glutaraldehyde/ 4% paraformaldehyde in 0.1M phosphate buffer (PB) for 1h at RT. The filters were then treated with 1% sodium borohydride in 0.1M PB for 30 minutes and freeze-thawed through liquid freon and then liquid nitrogen. After blocking non-specific binding for 30 minutes in 0.5% bovine serum albumin (BSA) in 0.1M tris-buffered saline (TBS), the filters were incubated for 48h at $4^oC$ in a 1:100 dilution of goat anti-IP-10 (R&D systems) in 0.1% BSA in TBS or a 1:10 dilution of mouse IgG anti-CXCR3 (R&D systems).

For IP-10 immunoperoxidase labelling, the secondary antibody was a 1:200 dilution of biotinylated donkey anti-goat IgG (Jackson ImmunoResearch) followed by a 1:200 dilution of avidin-biotin complex of the Elite kit (Vector) for 30 minutes at RT and then visualised with the chromogen diaminobenzidene.

For silver-enhanced immunogold labelling of CXCR3, the incubation with primary antibody was followed by a 1:50 dilution of anti-mouse IgG coupled to 1nm gold (British BioCell International, BBI) at 1:50 for 2h at RT. The bound gold particles were fixed with 2% glutaraldehyde, washed in 0.2M citrate buffer before silver enhancement (BBI) of 6-8 minutes. The filters were post-fixed in 2% osmium tetroxide in PB, dehydrated through a graded series of ethanols then propylene oxide before finally flat-embedding in Epon between two sheets of Aclar. Ultrathin sections were collected on 300 mesh copper grids, counter-stained with uranyl acetate and lead citrate before examination in a JEM 1010 electron microscope.

**FACS analysis of chemokine receptors**

Endothelial cells were detached from their culture wells using trypsin/EDTA and fixed in 4% paraformaldehyde diluted in phosphate-buffered isotonic saline (PBS), for 10 minutes on ice and permeabilised for one minute using 0.1% Triton X-100 in PBS and were then washed in PBS and blocked using 10% normal goat serum/0.1mg/ml rabbit IgG in PBS for 30 mins on ice. 1 x $10^5$ cells were taken per microfuge tube in a volume of 25µl. FITC-conjugated, anti-chemokine receptor antibody was added to each tube and incubated at 4˚C for 1 hour. CXCR3, and CCR2 were detected by the addition of 10µl antibody as supplied (R & D systems). The cells were washed with PBS and then resuspended in 500µl PBS, before analysis using a Becton Dickinson FACSCalibur. 10,000 events were analysed per chemokine receptor. The results shown are one representative of at least three experiments, giving similar results.

# Results

**Selective transfer of chemokines across endothelial monolayers**

We examined the rate of transfer of the chemokines CXCL10 (IP-10) and CCL2 (MCP-1) across lung, dermal and saphenous vein EC monolayers from the basal to the apical suface over a period of 60 minutes expressed as cleared volume from the lower chamber (Figure 1). Note that the volume cleared of tracer is much higher for filters with no cells than those with endothelial cell monolayers, indicating that all three endothelia constituted a barrier to the free diffusion of the chemokine from the lower to the upper compartment. This figure shows one representative experiment using all three different endothelial cell types, and with triplicate determinations (ie 3 separate monolayers) of each endothelial cell type.
The results indicated that the rate of $^{125}$I-CXCL10 transport was slower across SVEC than the microvascular endothelium whereas the transfer of $^{125}$I-CCL2 was higher across SVEC than the other endothelia. The slopes of the curves, or clearance for each chemokine, were used to calculate the permeability coefficient (Pe) for each endothelium and the combined results from all experiments are shown in figure 2. Transfer of CCL2 was significantly faster across SVECs than LMVECs, with DMVECs having intermediate rates. Conversely, transfer of CXCL10 was significantly slower across SVECs than across LMVECs. The absolute Pe values for CXCL10 transfer were in general lower than the values for CCL2, and this was particularly marked for SVECs. (The rate of CXCL10 transfer across collagen-coated filters lacking endothelium was also lower than for CCL2, which may reflect the fact that CXCL10 binds much more strongly to collagen than CCL2.) Note also that the endogenous production of chemokines by cells in these experiments was always much lower than the exogenous concentrations used in the transport assays (Table 1).

In order to determine whether chemokine transfer is enhanced by inflammatory cytokines, cells were treated for 24 hours before the assay with 25ng/ml TNFα. Figure 2 compares the permeability coefficient of CCL2 and CXCL10 on treated and untreated cells. Pretreatment of SVEC and DMVECs with TNFα did not alter the rate of chemokine transfer. LMVECs pretreated for 24h with TNFα did

show an increase in the mean Pe value of approximately 40% in comparison with untreated cells, but this was not significant ($p>0.05$).

The data imply that there are differences between endothelia in the rate at which chemokines are transferred but that this also depends on the type of chemokine.

We considered the possibility that receptor-mediated uptake could account for the differences in chemokine transfer by the endothelia. FACS analysis showed that the cells strongly expressed CXCR3, the sole receptor for CXCL10 (Figure 3). CCR2, the primary receptor for CCL2, was not detected on lung or saphenous vein endothelium, although weak expression was present on dermal endothelium (Figure 3). The results show that SVECs which have the lowest transfer rate of CXCL10, have the highest expression of CXCR3, while DMVECs, which have the highest transfer of CXCL10, had comparatively low CXCR3 expression. These observations do not support the theory that specific chemokine receptors are involved in transcytosis of the chemokines, or account for the differences between the endothelia.

**Route of chemokine transfer**

We considered two possible routes of chemokine transfer, the paracellular route, either free in solution or associated with cell surface glycoproteins, and the transcellular route. In order to test whether there was any variation in the paracellular route of transfer we measured the permeability of endothelial monolayers to inulin-FITC. This tracer was chosen as a paracellular marker because it does not cross cell membranes (Kazakoff et al., 1995). The results showed that the amount of inulin transferred across each endothelial monolayer increased linearly up to 35 minutes and that the permeability was similar for each endothelial cell type (Figure 4). This observation suggests that the differences between endothelia seen in chemokine transfer (Figs 1 and 2) cannot be accounted for by differences in paracellular diffusion.

To gain some insight into whether the transport mechanism was chemokine-specific we attempted to inhibit the transfer of radiolabelled chemokine (1.25ng/ml) with an

80x excess of unlabelled chemokine (100ng/ml). Chemokine transfer was partly inhibited in some experiments (<20%), but the effect was not consistently significant. (Note that the concentration of chemokine used here is sufficient to block binding to high affinity chemokine receptors, but cannot block binding to the endothelial proteoglycans, which are present at higher levels, and to which binding is of lower affinity. We have used up to 1000ng/ml chemokine without saturating surface proteoglycans on these endothelia.)

To identify the route of chemokine transfer, we first carried out immuno-electron microscopy to localise CXCL10 at different times following addition of chemokine to the lower chamber. Immuno-electron microscopy using peroxidase labelling revealed that CXCL10 is detectable on the basal membrane of the dermal endothelium within 1 minute of application (Figure 5a) and is rapidly transferred to the apical membrane. The micrograph in figure 5a shows a section of endothelium that immediately overlies a membrane pore. It is notable that the CXCL10 is present only in a column that overlies the pore, which suggests that transfer is occurring across the cell by transcytosis. From 5 to 25 minutes we observed less CXCL10 labeling on the basal membrane of the cell but some focal labelling located in vesicles, through the cytoplasm of the cells . At this time the chemokine is observed distributed along the apical membrane (Figure 5b). This pattern of staining, with CXCL10 located suceessively at the basal membrane, within the cell and at the luminal membrane was consistently seen with all three endothelia. Staining on lateral membranes or around intercellular junctions was not seen.

CXCR3 identified by immunogold labelling was almost entirely confined to the apical membrane of the cells and was not associated with the vesicular transcytosis compartments (Figure 5c). This observation again suggests that CXCR3 is not involved in the transcytosis of CXCL10 from the basal to the luminal membrane. The candidate vesicles for transfer of the chemokine are caveolae and clathrin-coated vesicles (CCV). Using electron microscopy, it was not possible to identify vesicles in dermal endothelium, but lung endothelial cells were seen to contain vesicles of ~100nm diameter, associated with both the basal and apical membranes (Figures 6a and 6b). In TNFα- treated lung endothelium, at 60 minutes, larger

vesicles associated with CXCL10 were present at the apical membrane (Figure 6c); these structures, with electron dense membranes, are suggestive of CCVs. The electron micrographs suggest that chemokines are transferred rapidly across the cell from the basal to the apical membrane, within a vesicular compartment and may then be reinternalised at a later stage (>30min) via CCV from the luminal membrane. Transcytosis of CXCL10 appears not to occur via the specific chemokine receptor, CXCR3, although it is possible that this receptor is involved in reinternalisation of CXCL10 from the apical membrane.

## Discussion

Chemokines have been convincingly implicated in the induction of leukocyte emigration during inflammation. It has been assumed that chemokines would either diffuse through the intercellular gaps between ECs or be transferred laterally in the plasma membrane by attachment to membrane glycoproteins or be transcytosed across the endothelium to be presented at the cell surface (Middleton et al., 2002). However it remains unclear if the process previously described for CXCL8 transport across EC can be extrapolated to other chemokines (Middleton et al., 1997). In this report we have examined the transport of two chemokines across human ECs derived from different vascular beds.

Our results clearly show that there are significant differences in the transport of CXCL10 compared with CCL2 across endothelia. Although the rate of transfer of CCL2 was somewhat greater than that of CXCL10 across lung and dermal endothelia, saphenous vein endothelium showed a particularly high transfer of CCL2 and low transfer of CXCL10. This suggests that some element(s) in the transport system varies between the endothelia, and this manifests itself as a selective difference in chemokine transfer. The transfer rate is not related to the level of expression of specific chemokine receptors (CXCR3, CCR2), which suggests that receptor-mediated transport is not the explanation. However, we have previously shown that dermal and lung microvessels bind CXCL10 at a significantly higher level than does saphenous vein endothelium (Hillyer and Male, 2005). It is possible therefore that the low rate of transfer of CXCL10 by saphenous

vein endothelium is due to a low uptake on cell surface glycosaminoglycans, prior to transcytosis. The differences between transfer rates of the different chemokines, could therefore be accounted for by the different chemokine binding properties of the endothelia which relates to the heparan-sulphate proteoglycan (HSPG) binding specificities of the chemokines (Witt and Lander, 1994, Lortat-Jacob et al., 2002) and the expression of particular sets of HSPG by each endothelium.

The functional role of the numerous specific endothelial chemokine receptors still remains undetermined. The electron microscopy did however demonstrate that CXCR3 is exclusively at the apical membrane and the receptor is therefore a candidate for mediating clearance of the apical membrane of excess chemokines, ie endocytosis, rather than transcytosis. Our observation of CXCL10 clearance into clathrin-coated vesicles, is conceptually similar to the agonist induced internalisation of chemokine receptors, including CCR5, into clathrin-coated vesicles (Signoret et al., 2005, Venkatesan et al., 2003).

Other studies have shown that inflammatory cytokines including TNFα can enhance permeability of some endothelia, and the number of vesicular profiles seen on endothelia at sites of inflammation in vivo, is often increased. We therefore looked for the possibility of enhanced chemokine transfer in response to TNFα. Interestingly, an increased transfer was only seen with the lung endothelium, corresponding to ~40% increase with CXCL10, which was not statistically significant. The increase of endothelial permeability in response to TNFα is often ascribed to an increase in paracellular permeability. However these results indicate that any increased transfer of CXCL10 is more probably due to enhanced transcytosis, either as a result of enhanced chemokine binding to the cell surface of cytokine-treated cells and/or increased vesicular traffic across cytokine-activated endothelium.

One other study has considered the possibility that chemokine transcytosis is receptor mediated. Dzenko and colleagues (2001) described CCR2 on mouse brain endothelium and proposed that transcytosis of CCL2 could be mediated by the receptor. Their experiments showed that endocytosis was CCR2-mediated, but it

was uncertain whether this was the initial stage of transcytosis, or internalisation as part of a mechanism to clear the cell surface. It is also notable in our study that 100ng/ml of cold chemokine gave only a small reduction in transcytosis. This level of chemokine is sufficient to block high affinity binding to chemokine receptors, but we have found that chemokine binding to glycosaminoglycans does not saturate even at 1000ng/ml. This is related to the high level of glycosaminoglycans on the endothelial surface and the relatively low affinity of binding of chemokines to them. As it was not technically possible to saturate the surface glycosaminoglycans with unlabelled chemokine, it was not possible to establish whether chemokine binding to the cell surface proteoglycans is required for transcytosis, or whether fluid-phase movement can account for the level of chemokine transfer.

The rapid transfer rate of the chemokines accords with other descriptions of transcytosis (Schnitzer, 2001). Indeed Mundy and colleagues (2002) have shown, using GFP-coupled caveolin, that caveolae can move at rates of up to 2μm/second, attached to microtubules. This is sufficiently fast to account for the rapid transfer across the endothelium, which occurs in less than 5 minutes. Originally the finding that most endothelial caveolae were associated with the basal or apical membranes lead to the proposal that caveolae were static structures. The current view is that the paucity of caveolae within the cytoplasm reflects the fact that once they have detached from parts of the actin cytoskeleton underlying the cell membranes, their passage along microtubules is very fast (McIntosh et al., 2002).

Our immuno-electron microscopy studies clearly indicated a progressive movement of CXCL10 from the abluminal-to-luminal side of the endothelial cell. At the early time points (1min) CXCL10 could be detected binding to the abluminal membrane of the EC whereas after 25minutes most of the labelling was located on the luminal side of the cell with less CXCL10 located in the cytoplasm. In this work we saw only small numbers of caveolae within the cells or associated with the basal and apical surfaces. This may be related to the in vitro culture system. In the absence of shear force, the numbers of caveolae seen in vitro, are generally much less than seen on comparable endothelia in vivo (Rizzo et al., 1998, Schnitzer, 2001). Over the last decades the significance of transendothelial transport by the caveolae

system has been debated and there is considerable data to indicate a role in the transport of macromolecules (McIntosh et al., 2002). In vitro, it appears that the transport of chemokines involves active shuttling of vesicles across the cells. In vivo, we would anticipate that the capacity of endothelium to transfer chemokines is much more important, because of the higher level of transcytosis, related to the metabolic demands of the tissue.

Finally the importance of transendothelial transfer of chemokines during inflammation, must be placed in the context of endothelial chemokine production. These endothelia are capable of producing significant quantities of chemokines (Hillyer et al, 2003). Although production of CXCL10 and CCL2 in response to inflammatory cytokines is generally moderate or comparable to production by cells from the tissues, endothelial production of (eg) CXCL8 is very high, and likely to exceed production by tissue cells. Thus the migration of cells such as neutrophils and macrophages in response to CXCL8 is principally controlled by endothelium, whereas the migration of T cells, mediated by CXCL10, may equally be induced by chemokines released in the tissues which have been transported across the endothelium.

This report demonstrates the importance of chemokine transfer and presentation by endothelial cells and its potential contribution to the control of leukocyte traffic. Tissue-specific variations in endothelial chemokine synthesis and presentation have been documented previously. This report shows that endothelia from different tissues selectively transfer particular chemokines which further emphasizes the importance of the endothelium in controlling local inflammatory responses.

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**Table 1 Production of chemokines by endothelia: pg/hour/cm$^2$.**

| | CCL2[1] | | CXCL10[1] | |
|---|---|---|---|---|
| **Endothelium** | **Resting** | **TNFα [2]** | **Resting** | **TNFα[2]** |
| Dermal microvessel | 0 | 158 | 0 | 32 |
| Lung microvessel | 0 | 333 | 0 | 18 |
| Saphenous vein | 6.7 | 76 | 0 | 0 |

1. Chemokine production measured by capture ELISA in the supernatant of confluent endothelial cell monolayers (1cm$^2$) after 24 hours in culture.
2. Cultures were stimulated with 25ng/ml human TNFα, for the 24hour culture period.

**Figure 1.**

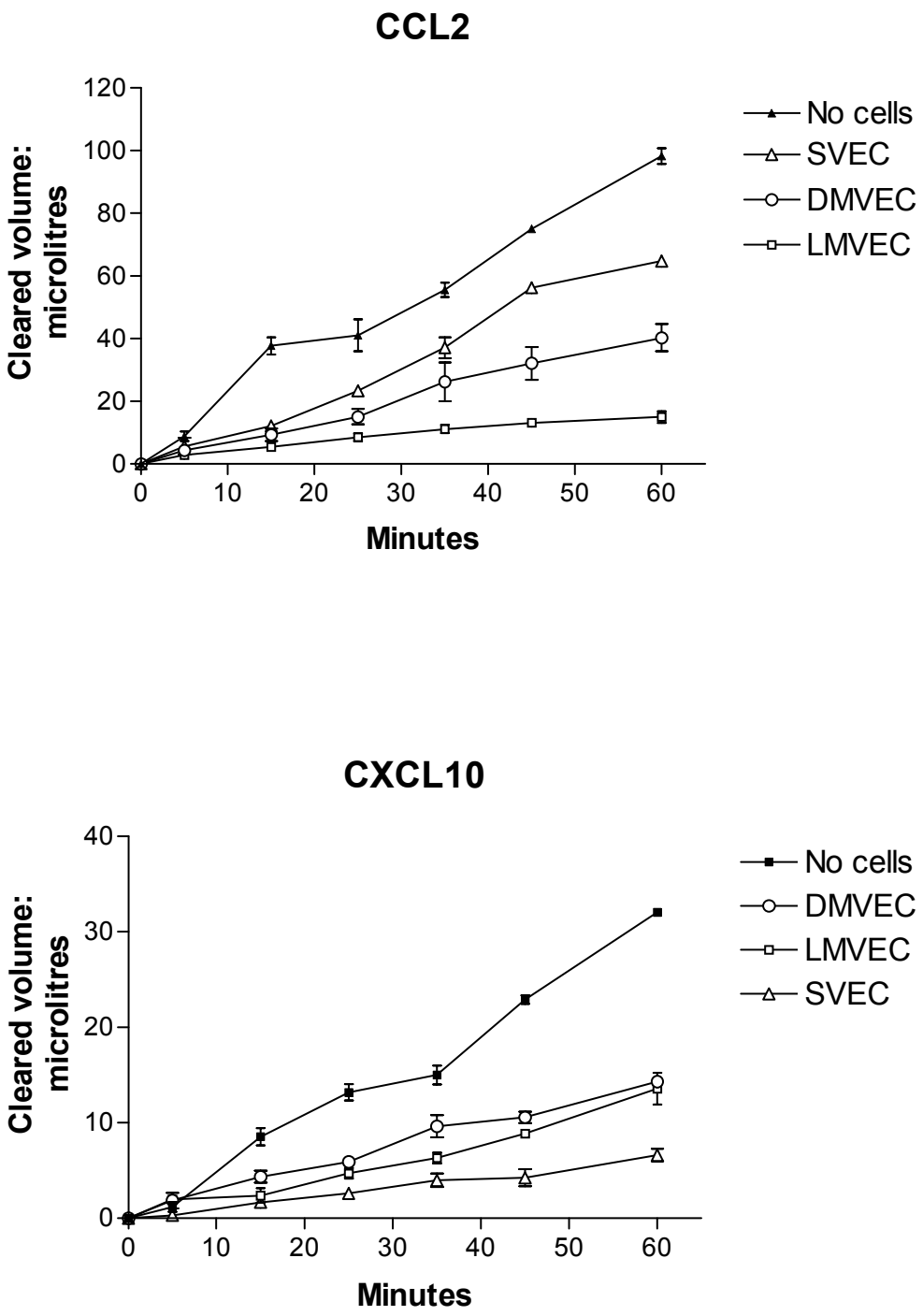

CCL2
Cleared volume: microlitres
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Minutes
No cells
SVEC
DMVEC
LMVEC
CXCL10
Cleared volume: microlitres
40
30
20
10
0
0
10
20
30
40
50
60
Minutes
No cells
DMVEC
LMVEC
SVEC

**Figure 2.**

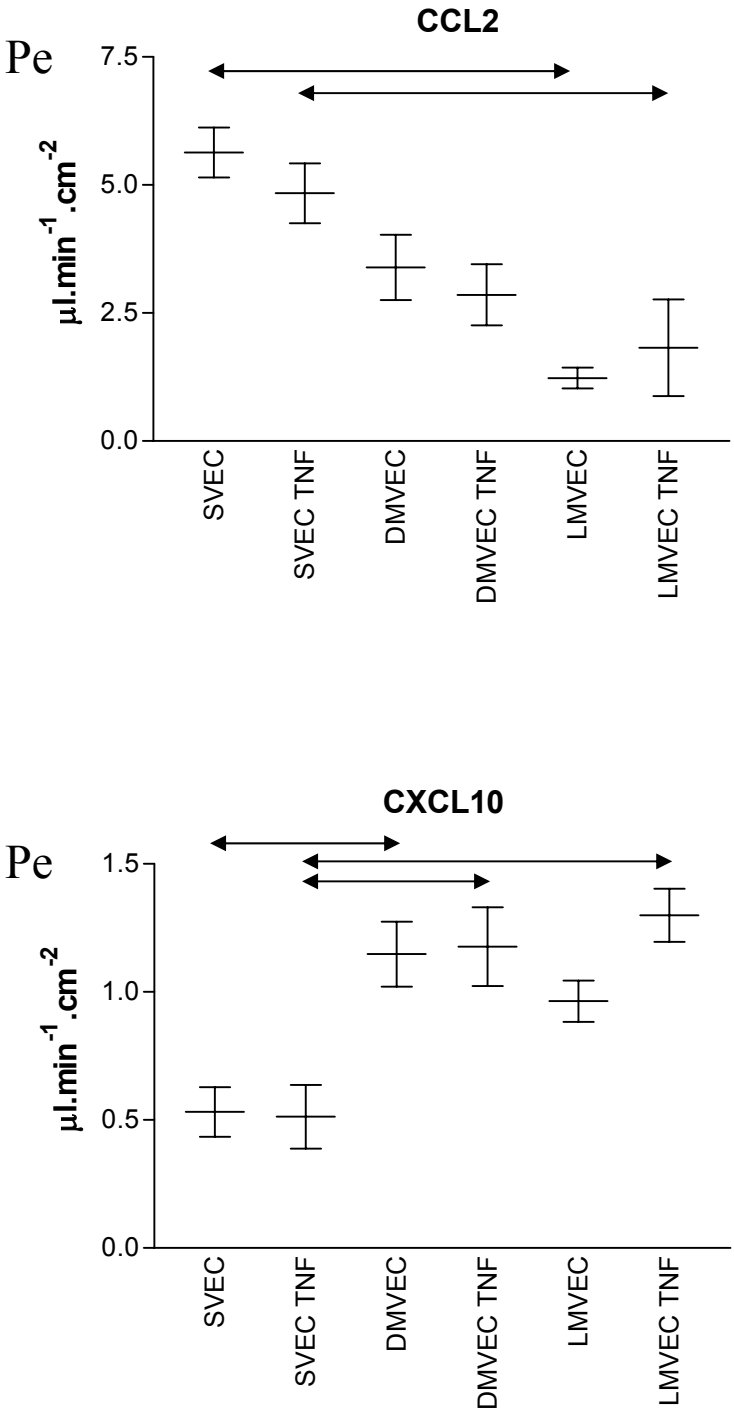

CCL2
Pe
7.5
5.0
2.5
0.0
µl.min-1.cm-2
SVEC
SVEC TNF
DMVEC
DMVEC TNF
LMVEC
LMVEC TNF
CXCL10
Pe
1.5
1.0
0.5
0.0
µl.min-1.cm-2
SVEC
SVEC TNF
DMVEC
DMVEC TNF
LMVEC
LMVEC TNF

**Figure 3.**

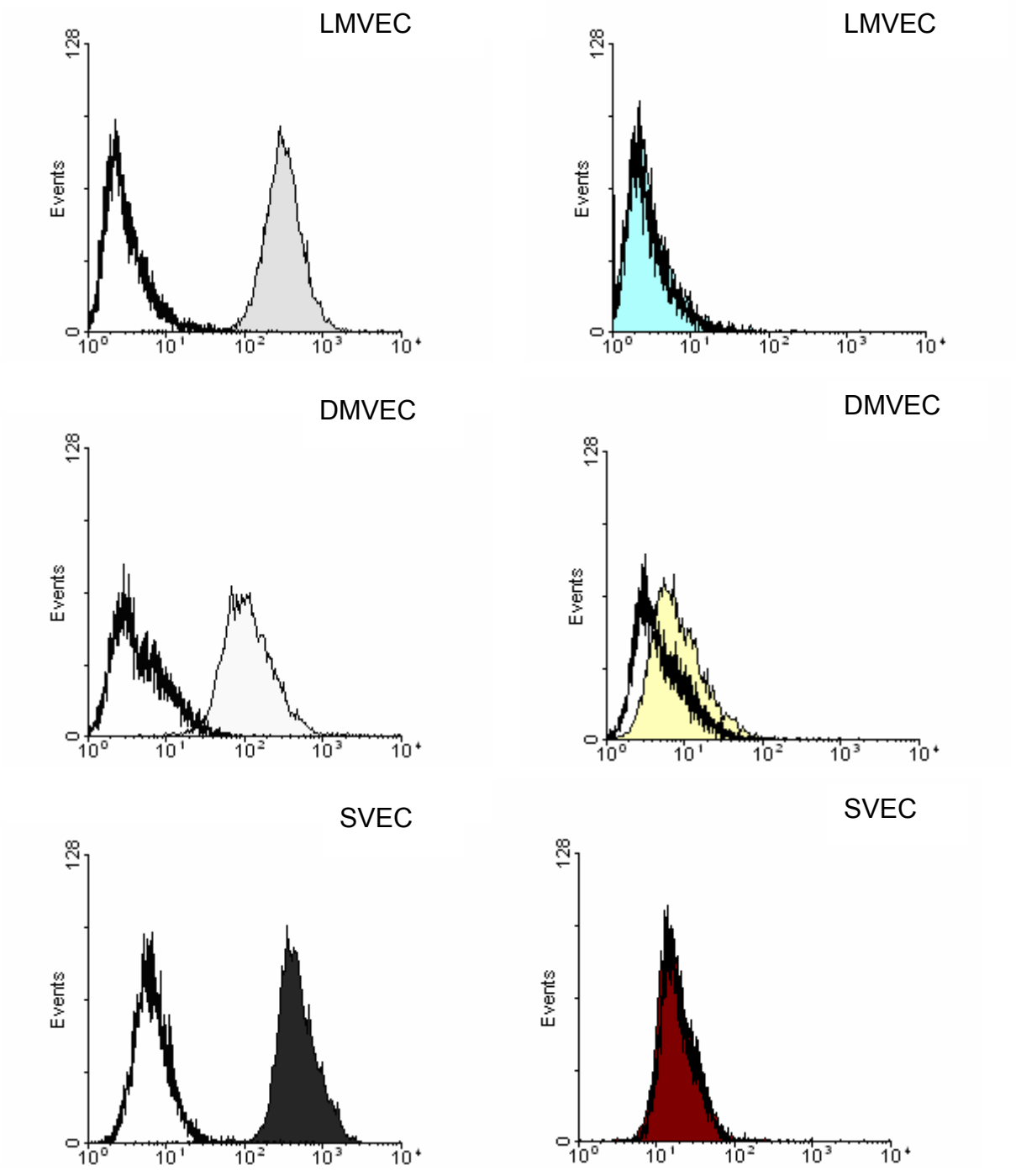

LMVEC
LMVEC
DMVEC
DMVEC
SVEC
SVEC
Events
128
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
CXCR3: Fluorescence
CCR2: Fluorescence

**Figure 4**

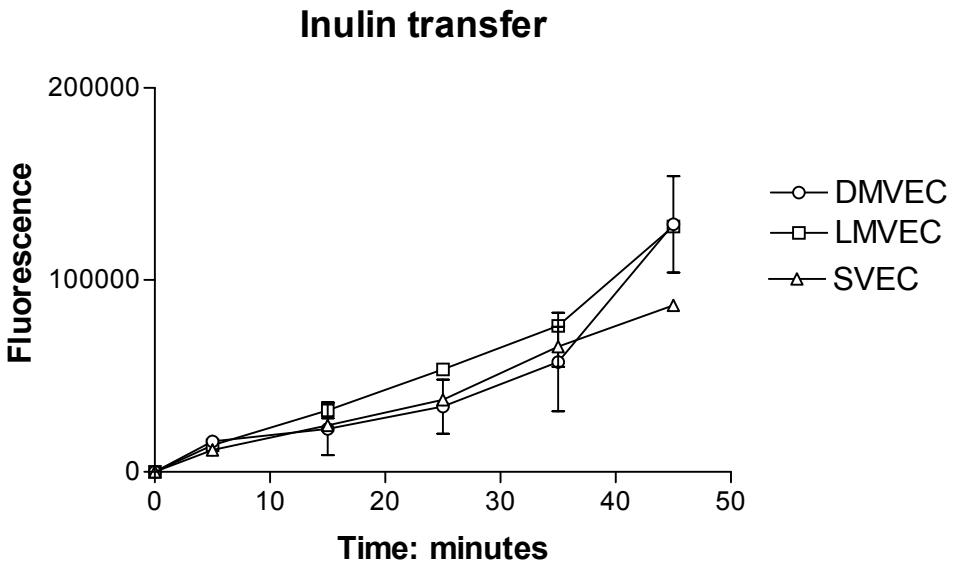
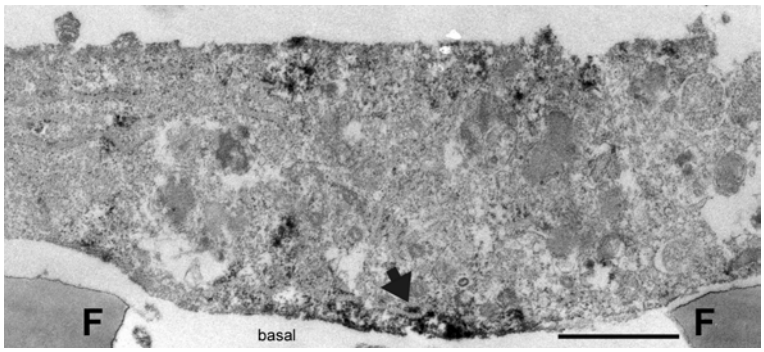
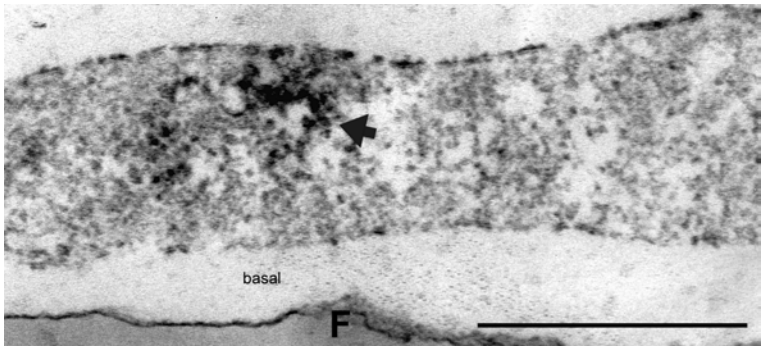

Inulin transfer
200000
100000
0
Fluorescence
0
10
20
30
40
50
Time: minutes
DMVEC
LMVEC
SVEC

**Figure 5**

**5a**




**5b**




**5c**

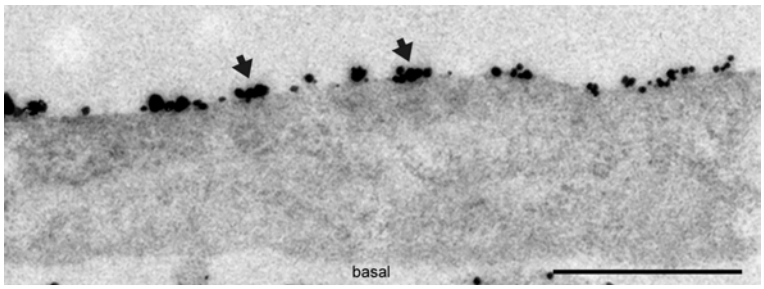
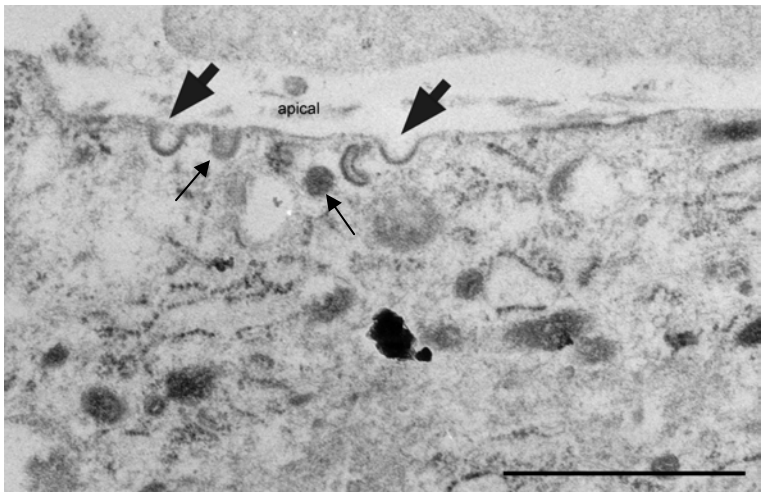
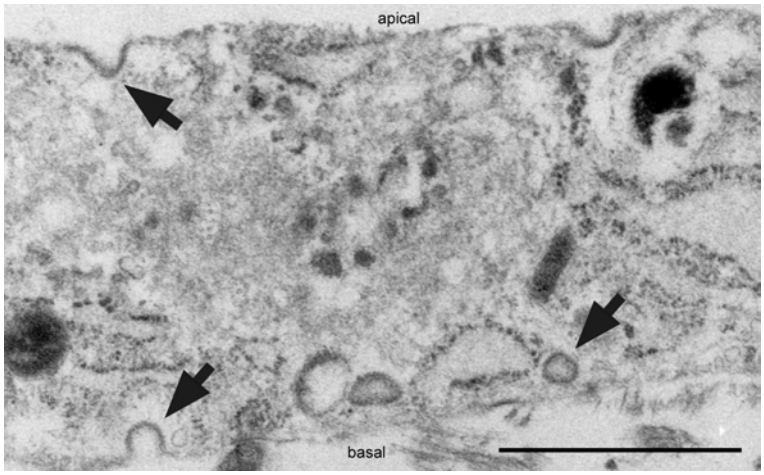


**Figure 6**

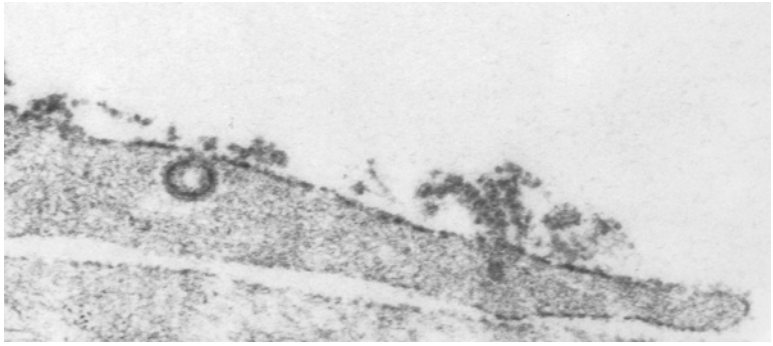
**6a**




**6b**




**6c**



**Legends**

**Fig. 1: Transfer of chemokines across endothelia**.

The rate of transfer of radiolabelled CCL2 and CXCL10 across monolayers of lung (LMVEC), dermal (DMVEC) and saphenous vein endothelium (SVEC), over one hour, compared with the rate of diffusion across blank collagen-coated filters. Values are calculated as cleared volumes, and each value represents mean ±SD from triplicate cultures.

**Fig. 2: Transfer rate of chemokines across endothelia.**

The rate of transfer of CXCL10 and CCL2 across untreated and TNFα-treated endothelia (25ng/ml for 24h) is expressed as the permeability coefficient (Pe), with combined data from three experiments, and triplicate cultures in each experiment. Values represent the 95% confidence interval of the mean. The data was analysed by ANOVA ($p<0.05$ for both chemokines) and post-hoc Newman Keuls test to determine whether there were differences between endothelia, or between treated and untreated cells. The double-ended arrows indicate significant differences between those cell types ($p<0.05$).

**Fig. 3: Expression of chemokine receptors for CXCL10 and CCL2 on endothelia.**

The expression of receptors CXCR3 and CCR2 was measured by FACS analysis on lung, dermal and saphenous vein endothelia (LMVEC, DMVEC, SVEC), shown as filled histograms, compared with isotype-matched control antibodies (open histograms).

**Fig. 4: Para-cellular transport of inulin across endothelial monolayers.**

The results show transfer of Inulin across resting endothelial monolayers over 45 minutes, as determined by fluorescence in the upper chamber (arbitrary units).

**Fig. 5: Localisation of CXCL10 and CXCR3.**

**5a.** Dermal endothelium pretreated with TNFα , and for 1minute prior to fixation with CXCL10 at the basal membrane. Immunoperoxidase staining for CXCL10 identifies chemokine at the basal membrane (arrow) and in a column extending to the apical membrane. The micrograph shows a region of the cell that overlies a pore in the membrane filter (F). Bar = 1micron

**5b.** Dermal endothelium pretreated with TNFα and for 25minutes prior to fixation with CXCL10 at the basal membrane. Immunoperoxidase staining for CXCL10 identifies intracellular chemokine and at the apical membrane (arrows). Bar = 0.5micron

**5c.** Dermal endothelium, pretreated with TNFα and for 25minutes prior to fixation with CXCL10 at the basal membrane. Silver-enhanced immunogold staining shows CXCR3 to be wholly confined to the apical membrane (arrows). Bar = 0.5micron.

**Fig. 6: Vesicular compartments in lung endothelium**.

**6a, 6b.** Lung endothelium, treated 25 minutes prior to fixation with CXCXL10 at the basal surface, and immunoperoxidase stained for CXCL10. Clusters of vesicles (possibly caveolae) are seen at both apical and basal membranes (arrowed). Labelled intracellular vesicles (small arrows) are associated with the cluster at the apical membrane. Bars = 1micron

**6c.** Lung endothelium pretreated with TNFα and then for 60 minutes before fixation with CXCL10 at the basal membrane, stained by immunoperoxidase for CXCL10. CXCL10 is located at the apical surface and appears to be reinternalised into a vesicle with an electron-dense rim (possibly a clathrin-coated vesicle). Arrows indicate immunoperoxidase product.