

Interleukin-4 increases the permeability of human endothelial cells in culture

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Summary

Background IL-4 plays a key role in the induction of allergic inflammation, but its role as an effector molecule is less well-established. Although some observations suggest that IL-4 may mediate increased vascular permeability, which is a characteristic feature of allergic inflammation, evidence for a direct effect on endothelial cell permeability is lacking.

Objective To determine the effect of human IL-4 on the albumin permeability of cultured human endothelial cells.

Methods Human umbilical vein endothelial cells were cultured on permeable membranes and the albumin permeability of endothelial monolayers was measured with and without exposure to recombinant human IL-4. Endothelial cells were exposed to various concentrations of IL-4 (0.001–100 U/mL), for various durations (6–24 h), either in the presence or absence of anti-IL-4 antibody. Recovery of endothelial barrier function following exposure to IL-4 was also examined.

Results IL-4 induced a dose-dependent, reversible increase in endothelial permeability to albumin. Low concentrations of IL-4 (1 U/mL) induced a significant increase in endothelial permeability ($P = 0.004$). IL-4-mediated endothelial leak occurred rapidly, within 6 h of exposure.

Conclusions IL-4 has the capacity to induce vascular leak by a direct effect on cultured endothelial cells, suggesting a potential effector role for IL-4 in the pathogenesis of vascular leak in allergic diseases.

Keywords endothelium, HUVEC, IL-4, permeability, vascular leak

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Introduction

IL-4 is a pleotropic cytokine that plays a central role in the pathogenesis of allergic inflammation. During induction of allergic inflammatory responses, IL-4 stimulates growth and/or differentiation of Th2 cells, IgE-secreting B cells and mast cells [1]. IL-4 also plays a crucial role in recruitment of circulating effector cells, especially eosinophils, through induction of vascular cell adhesion molecule-1 (VCAM-1) on vascular endothelium [2]. Activated mast cells and Th2 cells are both sources of IL-4, and the autocrine and anti-apoptotic effects of IL-4 are likely to be important in sustaining the inflammatory response [3]. IL-4 has a less defined role as an effector molecule during established allergic inflammation, but may contribute to smooth muscle contractile responses, mucus overproduction and stimulation of fibroblast growth, and secretion of extracellular matrix proteins [4].

Microvascular leakage, predominantly from post-capillary venules, is an important consequence of allergic inflammation [5, 6]. This has largely been attributed to the action of soluble

vasoactive mediators released by activated mast cells, such as histamine, leukotrienes and TNF- α [7]. However, Th2 cytokines are also key components of allergic inflammation and IL-4 is a well-established activator of endothelial cells as shown by changes in morphology, expression of adhesion molecules such as VCAM-1 and P-selectin, and production of cytokines, particularly IL-6 and IL-8 [8–12]. Vascular leak was a common adverse effect of IL-4 therapy in human cancer patients, although the mechanism has not been established [13–16]. We have recently developed a sensitive method for measuring permeability of endothelial cells [17] and use this to show that IL-4 reversibly increases vascular endothelial cell permeability in a dose-dependent manner, which may have important implications for the pathogenesis of asthma and other allergic disorders.

Materials and methods

Reagents

Purified recombinant human IL-4 (specific activity 1.8×10^7 U/mg) was provided by the Sterling Drug Company Inc. (Malvern, PA, USA). TNF- α (specific activity 4×10^7 IU/mg), was purchased from R&D Systems (Abingdon, Oxon, UK). Tissue culture media MCDB131 was purchased from Life

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Technologies (Paisley, UK). Fetal calf serum (FCS) was purchased from Globepharm (Esher, Surrey, UK). Rabbit polyclonal antiserum against IL-4 and normal rabbit serum were a gift from Dr R. Armitage (Immunex, Seattle, WA, USA). Horseradish peroxidase (HRP)-conjugated and unconjugated rabbit anti-human albumin polyclonal antibodies were purchased from Dako (Ely, Cambs., UK). 3,3',5,5'-Tetramethylbenzidine chromogenic substrate and bovine serum albumin were purchased from Sigma (Poole, Dorset, UK).

Measurement of endothelial cell permeability

Human umbilical vein endothelial cells (HUVEC) were prepared and cultured in MCDB131 medium on Transwell Clear cell culture inserts (Corning, Schiphol-Rijk, the Netherlands) as previously described [17]. In this system, the HUVEC monolayer is cultured on a permeable membrane that separates a luminal compartment (inside the insert) from a sub-luminal compartment (the well of the tissue culture plate). After 11 days, the monolayers were exposed to IL-4 at different concentrations and times as indicated. Experiments were performed in triplicate for each condition. HUVEC monolayer permeability was then measured by transendothelial albumin flux from luminal to subluminal compartment, as previously described [17].

Albumin ELISA

ELISA plates ('medium bind', Corning) were incubated with 100 µL of 2 µg/mL rabbit anti-human albumin polyclonal antibody in buffer A ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.35 g/L, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 1.34 g/L, NaCl 8.47 g/L, pH 7.2) overnight at 4 °C. Plates were then washed three times in buffer B ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.35 g/L, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 1.34 g/L, NaCl 29.22 g/L, Tween 20 1 mL/L, pH 7.2). Standards (serial dilutions of human serum containing 0.5–500 µg/mL of albumin) and samples, diluted in buffer B, were added and incubated at room temperature for 2 h before a further three washes. One-hundred microlitres of 0.15 µg/mL HRP-conjugated rabbit anti-human albumin in buffer B was then added for a further hour at room temperature. Plates were washed a final three times before addition of 100 µL TMB substrate. Plates were stopped by addition of 100 µL 2 M H_2SO_4 and read at 450 nm. Concentrations of albumin in samples were determined from the standard curve using Dynatech Revelation software (Dynatech Labs, Chantilly, VA, USA).

Calculation of endothelial permeability

The permeability co-efficients of the endothelial monolayers, corrected for the contribution of the membrane, were calculated as described previously [17]. In order to take account of the biological variability in baseline permeability co-efficient of HUVECs cultured from different donors [17], the effects of IL-4 stimulation were calculated as ratios of stimulated to unstimulated permeability co-efficients using cells from a single donor. These ratios were then $\log_{10}$ transformed in order to enable normal distribution statistics to be used. Sample means were compared to 0 (indicating no change in permeability) using a one-sample *t*-test.

Results

IL-4 increases endothelial cell permeability

The baseline permeability co-efficient for albumin on unstimulated endothelial cells in our model was $1-4 \times 10^{-7}$ cm/s. This value is consistent with our previous report and is presently the lowest permeability value that has been described for cultured human endothelial cells [17]. To test the effect of IL-4 on endothelial cell permeability, serial dilutions of recombinant human IL-4 were added to the luminal culture chamber containing the HUVEC monolayer for 24 h before measuring the transendothelial albumin flux. Recombinant human TNF- α , which is a well-established cytokine mediator of vascular leak [18], was included as a positive control. IL-4 induced a dose-dependent increase in endothelial monolayer permeability (Fig. 1). A statistically significant increase in permeability was obtained by treatment of the endothelial cells with IL-4 at concentrations of 1 U/mL ($P = 0.004$) or greater. At higher concentrations of IL-4 (10–100 U/mL), the permeability increase was similar to that obtained with 100 U/mL of TNF- α (Fig. 1). Inclusion of a polyclonal anti-IL4 antibody completely abrogated the increase in permeability, showing that the effect was mediated by IL-4 (Fig. 2).

Kinetics and recovery of IL-4-induced vascular leak

The timing of leakage may be critical during an allergic inflammatory response. To investigate the kinetics of IL-4-induced leakage, the endothelial cell monolayers were stimulated with 10 U/mL IL-4 for 6, 15 and 24 h before measuring the transendothelial albumin flux. There was no change in baseline permeability of unstimulated endothelial cell monolayers over this time-course. An increase in permeability over baseline was detected at the earliest time point of 6 h and maximum levels were reached by 15 h (Fig. 3).

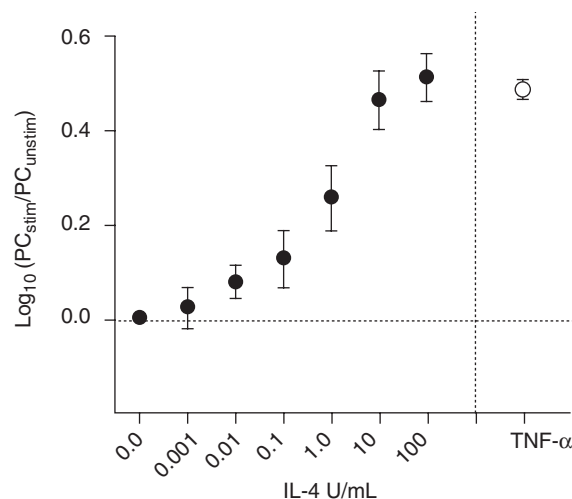


Fig. 1. Permeability of endothelial cell monolayers stimulated with IL-4. Endothelial cell monolayers grown in Transwell cultures were incubated with IL-4 at concentrations 0.001–100 U/mL (●) or with TNF- α at 100 U/mL (○) for 24 h. Transendothelial flux of albumin was then measured and the change in permeability expressed as the $\log_{10}$ ratio of the permeability co-efficient (PC) of stimulated endothelium to unstimulated endothelium. Each point is the mean of not less than 4 experiments $\pm$ 1 SEM. A value of 0 indicates no change in permeability of stimulated monolayers.

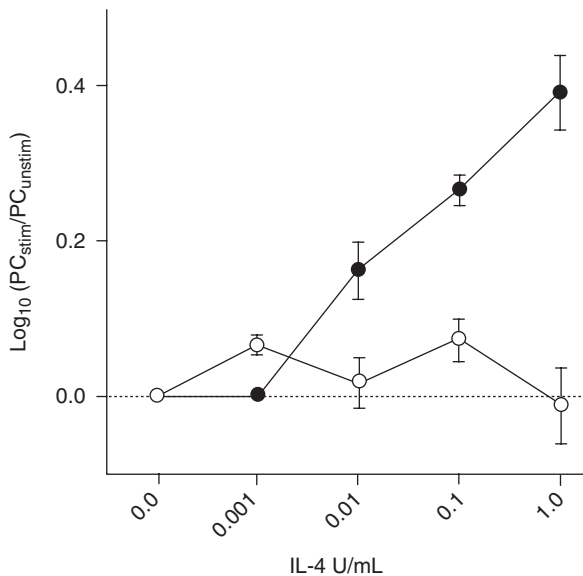


Fig. 2. Permeability of endothelial cell monolayers stimulated with IL-4 in the presence and absence of anti-IL-4 antibody. Endothelial cell monolayers grown in Transwell cultures were incubated with IL-4 at concentrations from 0.001 to 1 U/mL for 24 h in the presence of rabbit polyclonal anti-IL-4 antibody (○) or normal rabbit serum (●). Transendothelial flux of albumin was then measured and the change in permeability expressed as the log₁₀ ratio of the permeability co-efficient (PC) of stimulated endothelium to unstimulated endothelium. Each point is the mean of not less than three experiments ± 1 SEM. A value of 0 indicates no change in permeability of stimulated monolayers.

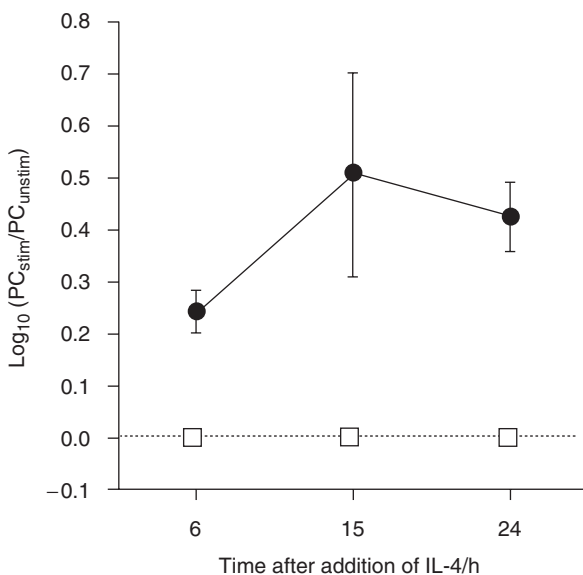


Fig. 3. Kinetics of IL-4-induced vascular leak. Endothelial cell monolayers grown in Transwell cultures were incubated with media alone (□) or media containing IL-4 at 10 U/mL (●) for 6, 15 and 24 h. Transendothelial flux of albumin was then measured and the change in permeability expressed as the log₁₀ ratio of the permeability co-efficient (PC) of stimulated endothelium to unstimulated endothelium. Each point is the mean of not less than three experiments ± 1 SEM. A value of 0 indicates no change in permeability of monolayers.

To examine recovery, the monolayers that had been stimulated with 10 U/mL of IL-4 for 6, 15 or 24 h were washed and cultured further in the absence of IL-4. After allowing the monolayers to recover for 24 and 108 h, transendothelial

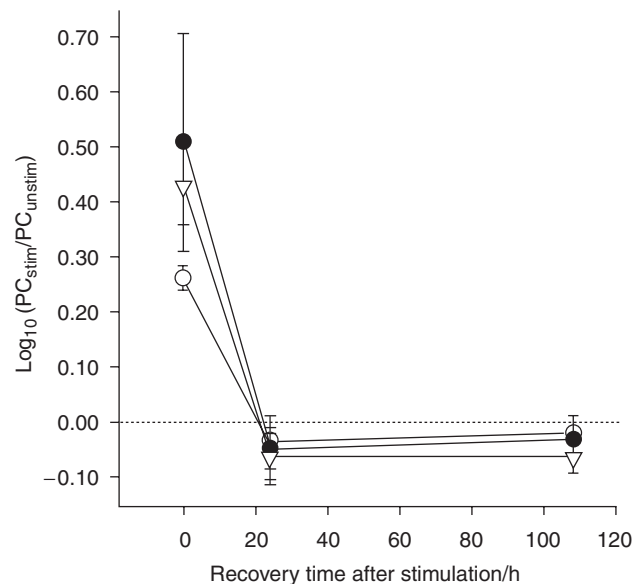


Fig. 4. Kinetics of recovery after IL-4-induced vascular leak. Endothelial cell monolayers grown in Transwell cultures were incubated with IL-4 at 10 U/mL for 6 (○), 15 (●) and 24 (▽) h. Transendothelial flux of albumin was measured, and the cells were then washed and allowed to recover in medium without IL-4 for 24 and 108 h. Changes in permeability were expressed as the log₁₀ ratio of the permeability co-efficient (PC) of stimulated endothelium to unstimulated endothelium. Each point is the mean of not less than three experiments ± 1 SEM. A value of 0 indicates no change in permeability of stimulated monolayers.

albumin flux was measured again. During the recovery period, there was no change in baseline permeability of cell monolayers that had not previously been exposed to IL-4 (data not shown). Permeability of cell monolayers exposed to IL-4 returned to baseline levels within 24 h after removal of the IL-4 (Fig. 4).

Discussion

We show here that recombinant human IL-4 induces a dose-dependent, reversible increase in human endothelial cell monolayer permeability. This effect was completely blocked by anti-IL-4 antibody, confirming that the increased permeability was mediated by IL-4. Time-course experiments demonstrated that IL-4-mediated increases in permeability occurred rapidly, within 6 h, suggesting that this is a direct effect of IL-4 rather than an indirect effect via a second mediator produced by endothelial cells. It has been shown previously that endothelial cells express the type II IL-4 receptor comprised of the IL-4R α and IL-13R (but not the common γ -chain found in the type I receptor) and thus have the capacity to respond directly to IL-4 stimulation [8, 12]. In our experiments, HUVECs stimulated for 24 h with IL-4 expressed high levels of VCAM-1 but not intercellular cell adhesion molecule-1 (ICAM-1) or E-selectin expression (data not shown), which are induced by TNF- α [19]. This pattern of adhesion molecule expression is characteristic of IL-4 stimulation and implies direct stimulation by IL-4 rather than indirect stimulation by other cytokines such as TNF- α [19, 20]. Furthermore, TNF- α in culture supernatants after stimulation of HUVECs with IL-4 for 24 h was below the limit of

detection (<15 pg/mL) by ELISA (data not shown). This is in keeping with our previous results showing that TNF- α and other cytokines that have the capacity to increase endothelial permeability, such as IL-1, are not secreted by IL-4 stimulated human endothelial cells [19].

Two previous studies have investigated the effect of IL-4 on endothelial monolayer permeability, both with negative results [21, 22]. Although IL-4 alone had no effect, Beynon et al. [22] showed that it acted synergistically with both TNF- α and IFN- γ to increase vascular leak but the mechanism was not defined. The differences between these findings and our results, which show that exposure to IL-4 alone increases endothelial permeability, most likely reflect significant advances in our model system when compared with previous studies. Our model has been characterized morphologically by electron microscopy and optimized functionally, so that it is currently the closest approximation to the post-capillary venule [17]. Most significantly, baseline permeability in our model is approximately 1 log lower than for previously reported cultured endothelial barriers [23], resulting in greatly increased sensitivity. Instead of using artificial tracer molecules to determine transendothelial flux, we assess changes in endothelial permeability by measuring transendothelial flux of native human albumin, which is the most physiologically relevant molecule. Taken together, these features make our model well-suited to studies of endothelial permeability changes in response to inflammatory mediators such as IL-4.

Our study suggests a potential role for IL-4 as an effector molecule in allergic inflammation, acting along with other soluble mediators to activate vascular endothelium and increase vascular permeability. The effects of IL-4 on endothelial cell permeability were observed at doses equal to or lower than reported for T cell and B cell responses *in vitro*, suggesting that IL-4 is a potent mediator of endothelial cell permeability at biologically relevant concentrations. IL-4 is well-established as one of the key mediators during the induction of allergic responses, but its role as an effector molecule in both acute and established allergic inflammation in humans is less well-defined. The number of IL-4-positive mast cells has been associated with the extent of the immediate allergic weal reaction in the skin of atopic individuals, but although a direct local effect of IL-4 was suggested it was not proven [24]. In a recent study using a mouse model of anaphylaxis, IL-4 was found to exacerbate vascular leak, most probably through a direct effect on vascular endothelium [25]. The predominant effect appeared to be enhanced sensitivity to other vasoactive mediators, although a direct effect on endothelial cells was not excluded. In humans, intravenous infusion of IL-4 has also been observed to cause severe vascular leak [13–16]. Our data using a HUVEC monolayer imply that IL-4 has the capacity to increase endothelial permeability directly, but do not absolutely exclude involvement of other mediators.

Regardless of the precise mechanism(s) by which IL-4 promotes vascular leak, this may be a significant factor in the pathogenesis of allergic inflammation. The importance of IL-4-mediated vascular leak in the pathogenesis of human allergic diseases awaits further study, but our *in vitro* data add further credence to the idea that anti-IL-4 strategies may be a rational component of therapy. In a recent clinical trial, the IL-4 antagonist sIL-4R (soluble IL-4 receptor) was found to have

a beneficial effect in patients with moderate persistent asthma who were withdrawing from inhaled corticosteroids [26]. It is plausible that sIL-4R acts predominantly by targeting multiple pathways of inflammation at the key central point of Th2 differentiation, which is uniquely dependent on IL-4. However, in an earlier phase I study, sIL-4R was observed to have a beneficial effect on pulmonary function as early as 2 h after administration [27], implying that it is acting in addition by blockade of a direct effector function of IL-4. Our study suggests that IL-4 may contribute to the oedema of the early and/or late phase allergic response and that inhibition of IL-4-mediated increases in vascular permeability may be at least a component of the immediate therapeutic effect of sIL-4R.

In conclusion, this *in vitro* study shows for the first time that IL-4 has the capacity to induce vascular leak by a direct effect on endothelial cells. This may be an important mechanism in the pathogenesis of allergic inflammation, and further studies are warranted to define the contribution of IL-4-induced vascular leak to the pathogenesis of human allergic diseases.

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