Immune-mediated podocyte injury and the idiopathic nephrotic syndrome
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Chapter 2

Interleukin-4 and interleukin-13 act on glomerular visceral epithelial cells

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Interleukin-4 and interleukin-13 act on glomerular visceral epithelial cells

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In minimal change nephrotic syndrome (MCNS), proteinuria is associated with structural changes of the glomerular visceral epithelial cells (GVEC). The occurrence of MCNS has been associated with T-helper2 lymphocyte-dependent conditions. To examine a direct role for Type2 cytokines in GVEC injury, the expression of interleukin (IL)-4/IL-13 receptors by GVEC and direct effects of IL-4 and IL-13 on GVEC were studied. Reverse transcription-PCR showed that isolated human and rat glomeruli and cultured human and rat GVEC expressed mRNA for IL-4Ra, IL-13Ra1 and IL-13Ra2. Protein expression of IL-4Ra and IL-13Ra2 by GVEC in human kidney biopsies and by cultured human GVEC was detected by immunohistochemistry. Western blotting demonstrated phosphorylation of STAT6 in cultured GVEC upon incubation with IL-4 or IL-13. This indicated signal transduction via the heterodimeric receptor complex IL-4R2, which is composed of the IL-4Ra and the IL-13Ra1. Direct effects on GVEC function were examined in monolayer experiments. IL-4 and IL-13 dose-dependently decreased transepithelial electrical resistance of monolayers of rat GVEC to approximately 30% and 40% of baseline values, respectively. The transepithelial electrical resistance decrease was associated with a significant increase in short-circuit current, whereas no changes were observed in the transmonolayer flux of the macromolecules horseradish peroxidase (molecular weight, 44 kDa) and 14C-mannitol (molecular weight, 182 Da). No changes in cell structure were observed with electron microscopy. It is concluded that by binding to specific IL-4/IL-13 receptors, IL-4 and IL-13 can exert specific effects on GVEC function, which could be of pathogenetic relevance for glomerular injury in MCNS.

Minimal change nephrotic syndrome (MCNS) is a common cause of nephrotic syndrome in children. In the renal biopsies of these patients, structural alterations are restricted to the glomerular visceral epithelial cells (GVEC). GVEC are highly specialized cells with a complex arborized structure of interdigitating foot processes, which contribute to the specific characteristics of the glomerular filtration barrier. In MCNS, foot process retraction and effacement are associated with the occurrence of proteinuria. The pathogenesis of these changes in GVEC structure and their relation to the development of proteinuria are as yet unknown.

MCNS can be treated successfully with immunosuppressive drugs, and the involvement of immunological processes in the pathogenesis of MCNS has been suggested [1]. Several groups have described a factor with permeability-enhancing properties in the supernatant of stimulated peripheral blood mononuclear cells (PBMC) from patients with MCNS. After infusion, this supernatant induced proteinuria and foot process retraction in rats [2-4]. Other investigators have focused on the cytokine profile of T lymphocytes of patients with MCNS. Recently, it was shown that in patients with MCNS T lymphocytes produce more
interleukin (IL)-13 than healthy controls and patients in remission [5, 6].

IL-13 is a pleiotropic cytokine that, like IL-4, is produced by appropriately stimulated CD4+ T-helper2 (Th2) lymphocytes. Activation of Th2 lymphocytes plays a key role in IgE-mediated inflammation, as in atopic syndromes and in immune responses to parasites and fungi. A possible role for Type2 cytokines in the induction of proteinuria in MCNS is supported by clinical observations, such as the association of MCNS with atopy, and the apparent induction of MCNS by allergic events in some patients [7]. In earlier studies, we showed that IL-4 can exert specific effects on rat GVEC in vitro [8]. IL-4 and IL-13 have overlapping effector profiles [9]. This overlap is at least partially due to the shared use of a heterodimeric IL-4/IL-13 receptor complex, the IL-4R2. This receptor complex is composed of the IL-4 binding IL-4Ra chain and the IL-13 binding IL-13Ra1 chain. Alternatively, the IL-4Ra chain can be associated with the common γ-chain (y-chain) to form the heterodimeric IL-4R1, which signals only after binding of IL-4 [10]. Another IL-13-binding chain, the IL-13Ra2 chain, is believed to bind IL-13 with high affinity without subsequent signaling [11]. There is now growing evidence that in asthma IL-13 functions as a key mediator independently of IL-4 [12, 13]. Correspondingly, it was shown that in MCNS, T lymphocytes are prone to produce IL-13 rather than IL-4 [5, 6].

Whether the observed increased production of IL-13 by T lymphocytes of patients with MCNS plays a role in the pathogenesis of this disease is as yet unclear. We hypothesized that IL-13 may play an important role in the development of proteinuria, by exerting direct effects on GVEC. To address this hypothesis, we studied effector functions of IL-4 and IL-13 on rat GVEC in vitro. Furthermore, we examined the expression of IL-4 and IL-13 receptors on human and rat GVEC, both in vitro and in vivo. We show that IL-4 and IL-13 act directly on GVEC in vitro, probably by binding to a functional IL-4R2, which is likely to be expressed by GVEC in vivo.

Materials and Methods

Reagents

Recombinant human IL-4 and human IL-13 were purchased from Pharma Biotechnologie (Hannover, Germany). Recombinant rat IL-4 was obtained from Serotec (Oxford, UK). Hybridoma cells producing a blocking monoclonal antibody directed against rat IL-4 (OX 81, mouse IgG1) were kindly provided by Dr. D. Mason (Oxford, UK). Hybridoma cells producing a control monoclonal antibody TS2/9.1.4.3 (TS2, anti-human CD58, mouse IgG1) were obtained from the American Type Culture Collection (Rockville, MD). OX81 and TS2 were purified from hybridoma culture supernatants by protein A chromatography [14]. Recombinant rat IL-13 and blocking antibody anti-rat IL-13 (polyclonal rabbit IgG) were prepared as described previously [15]. Normal rabbit serum (Dako, Glostrup, Denmark) was used as control polyclonal rabbit antibodies. Monoclonal antibody to human IL-4Ra (MAB230, mouse IgG2a) was purchased from R&D Systems (Abingdon, UK). Monoclonal antibody to human IL-13Ra2 (B-D13, mouse IgG1) was previously described [16]. Polyclonal antibody to phosphorylated STAT6 (Phospho-Stat6 [Tyr641], rabbit IgG) was purchased from New England Biolabs (Beverly, MA). Monoclonal antibody to STAT6 (SC-1689, mouse IgG1) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells

Established lines of rat GVEC [17], human GVEC [18], and rat mesangial cells [19] were used. Cells were cultured at 37°C in plastic flasks in a humidified 5% CO2/95% air incubator. Rat GVEC were grown on a type I collagen gel (Vitrogen 100, Collagen Corp., Palo Alto, CA); 0.2% collagenase type IA (330 U/mg, Sigma, St. Louis, MO) in Hanks’ balanced salt solution (Life Technologies, Breda, The Netherlands) was used to detach cells before each passage. Human GVEC and rat mesangial cells were grown on plastic; trypsin (DIFCO Laboratories, Detroit, MI) was used to detach cells. The GVEC culture medium contained 45% Dulbecco’s modified Eagle’s medium (ICN, Costa Mesa, CA), 45% HAM-F10 (Life Technologies), 1% K1 hormone mix (5 µg/mL insulin, 25 ng/mL prostaglandin E1, 0.5 nM T3, 10 nM sodium selenite, 5 µg/mL transferrin, 50 nM hydrocortisone [all ingredients from Sigma] in Hanks’ balanced salt solution), 1% insulin (Sigma), and 5% Nu serum (Becton Dickinson, Bedford, MA). Mesangial cell culture medium was composed of 95% Dulbecco’s modified Eagle’s medium and
5% fetal calf serum (Life Technologies). Culture media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (both from Life Technologies).

Isolation of glomeruli
To obtain human glomerular tissue, kidneys were used that had been resected because of chronic renal allograft rejection (n = 2), acute renal allograft rejection (n = 1) or recurrent pyelonephritis (n = 1). From each kidney, 20 glomeruli were microdissected from a 10-μm-thick cryostat section with a Positioning Ablation Laser Microscope (P.A.L.M., Mikrolaser Technologie, Bernried, Germany). Rat glomeruli were isolated under sterile and RNase-free conditions from normal Brown Norway rats by differential sieving and centrifugation of minced kidney cortices.

cDNA synthesis
Total RNA was isolated from cultured human and rat GVEC, from cultured rat mesangial cells and from isolated rat glomeruli, using Trizol Ragent (Life Technologies) according to the manufacturer's instructions. From isolated human glomerular tissue, cDNA was synthesized without prior RNA isolation. For cDNA synthesis, either 10 μg of isolated RNA or the isolated human glomerular tissue was incubated with 5 nmol of Pd(N)6 primer (Pharmacia Biotech, Roosendaal, The Netherlands) for 10 min at 65°C. After cooling on ice, the reverse transcription (RT)-reaction mixture was added to a final volume of 50 μL, containing 400 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies), 8 mmol/L dithiothreitol (DTT), 1 mmol/L of each dNTP, 1 × first-strand buffer (50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L MgCl2) and 60 U of RNase inhibitor (Boehringer Mannheim, Almere, The Netherlands). The reaction was performed for 1 h at 37°C. Subsequently, the reverse transcriptase was inactivated by heating the sample for 10 min at 95°C.

Polymerase chain reactions (PCR)
Species-specific primers for synaptopodin, γc-chain, IL-4Ra, IL-13Ra1 and IL-13Ra2 were designed on the basis of sequences available from the Entrez sequence databank from National Center for Biotechnology Information and synthesized by Pharmacia Biotech. The sequences of the primers are listed in Table 1. In each PCR, 2 μL of the cDNA reaction mixture was used or, for nested PCR, 5 μL of mixture containing the initial PCR product. Water was used as a control to exclude contamination. The PCR mixture contained 1 x Taq buffer (20 mmol/L Tris-HCl, 50 mmol/L KCl, pH 8.4), 0.2 mmol/L of each dNTP, 2.5 mmol/L MgCl2, 2 U of Taq polymerase (Life Technologies), and 0.5 μmol/L of each primer. Thirty cycles of amplification, or 35 cycles for nested PCR, were performed in the thermal cycler (PTC-100, M.J. Research Inc, Watertown, MA), i.e., successively 60 s at 95°C, 60 s at 58°C, and 60 s at 72°C. The reaction was completed during 7 min at 72°C. PCR products were analyzed in 10-μL samples on a 1.5% agarose gel (Sigma) containing ethidium bromide.

Amplification of contaminating genomic DNA during the RT-PCR procedures was excluded by PCR on isolated genomic human and rat DNA, which did not yield transcripts of the size expected for the target mRNA.

Sequencing of PCR products
PCR products were isolated from the PCR mixture with a QIA-Quick kit (Qiagen, Hilden, Germany) and were sequenced using an ABI sequencer (Perkin Elmer Corp., Norwalk, CT) with a dye-terminator cycle-sequencing kit (Perkin Elmer Corp.) according to the manufacturer's instructions.

Immunohistochemistry
The protein expression of IL-4Ra and IL-13Ra2 was analyzed by immunohistochemistry on cryostat sections of human renal tissue and on cultured human GVEC. The human renal tissue was derived from histologically normal parts of kidneys that had been resected because of a Grawitz tumor (n = 3) and from 33 renal biopsy specimens from patients with the following diagnoses: MCNS (n = 8), chronic renal allograft rejection (n = 3), acute renal allograft rejection (n = 3), membranous glomerulopathy (n = 3), diabetes mellitus nephropathy (n = 3), focal and segmental glomerulosclerosis (n = 3), IgA nephropathy (n = 4), tubulointerstitial nephritis (n = 4) and Wegener's vasculitis (n = 2).
**Table 1. Sequences of amplification primers.**

<table>
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<tr>
<th>mRNA</th>
<th>Species</th>
<th>Sense</th>
<th>Antisense</th>
<th>Size (bp)</th>
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<td>Synaptopodin</td>
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<td>TCCCCAAAATGTCTCTCT</td>
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<tr>
<td></td>
<td></td>
<td>GAGGAATGCAGCACTGCTGA*</td>
<td>GCGGGACATTATGTTCTCT*</td>
<td>780</td>
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<tr>
<td>γ-chain</td>
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<td>CCCAGAGGTCAGTGTTTTG</td>
<td>CACTGATTGCAGTCAGTCAGC</td>
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<tr>
<td></td>
<td>Mouse</td>
<td>GAGGTTCAGTGCTTTTGTTT</td>
<td>CAGCTTCGATCTCCTGGT</td>
<td>419</td>
</tr>
<tr>
<td>IL-4Ra</td>
<td>Human</td>
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<td>TAATGTGAGAGAGGAGAAGG</td>
<td>332</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
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<td>GAAATCCAGACTTCAGG</td>
<td>522</td>
</tr>
<tr>
<td>IL-13Ra1</td>
<td>Human</td>
<td>ATGCCTCCGACACTAATAC</td>
<td>CGGAAGTATTAAAGGCACTAT</td>
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<tr>
<td></td>
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<td>GTGGAGATCGAGGTTACA*</td>
<td>258</td>
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<tr>
<td>IL-13Ra2</td>
<td>Human</td>
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<td>CACCATGCGAGTTGCCCAA</td>
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<td></td>
<td>Rat</td>
<td>CTGATCTCAGAGCAGGAG*</td>
<td>CCAAGCCCTCATACAGGTA</td>
<td>468</td>
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</tbody>
</table>

Non-fixed, 4-μm-thick sections of human renal tissue and acetone-fixed human GVEC, which had been cultured on glass slides, were preincubated with 10% normal goat serum (Sera Lab, Sussex, UK), followed by incubation with the primary antibodies overnight at 4°C. Appropriate dilutions in phosphate-buffered saline (PBS) were determined for each antibody. Endogenous peroxidase activity was blocked by incubation with 0.1% NaN₃ and 0.03% H₂O₂ (both from Sigma) in PBS. Subsequently, antibody binding was detected with isotype-specific horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig antibodies (Southern Biotechnology Associates, Birmingham, AL); 10% normal human ABO-serum (CLB, Amsterdam, The Netherlands) was added to the secondary antibody to inhibit possible cross-reactivity to human immunoglobulins. Peroxidase activity was detected with 3-amino-9-ethylcarbozole (Sigma) and 0.03% H₂O₂. Negative controls were performed by replacing the first-step antibody by incubation buffer only or by isotype-matched and species-matched monoclonal antibodies that do not bind to human tissue (Dako).

**Monolayer experiments**

Because the rat GVEC line forms continuous monolayers, whereas the human GVEC line does not, we used rat GVEC for monolayer experiments. Cells were seeded on Transwell porous filters with a pore diameter of 0.4 μm and a growth area of 0.33 cm² (from Costar, Badhoevedorp, The Netherlands) at a density of 150,000 cells/cm². The inner and outer chambers contained 0.25 and 1.2 mL of medium, respectively. For most experiments, the cells were seeded in the inner chamber, on the top of the filter. In this situation, the inner and outer chambers represented the apical and basolateral compartments, respectively. In some experiments, cells were grown on the lower side of the filter instead, and the inner and outer chambers represented the basolateral and the apical compartments, respectively. Cytokines were added...
to the apical compartment, to the basolateral compartment or to both compartments.

Transepithelial electrical resistance (TER) was measured with a Millipore apparatus. After reaching TER values > 2.5 kΩ×cm², the confluent monolayers were treated as detailed below. For short-circuit current (Isc) measurements, confluent GVEC monolayers were mounted in an Ussing chamber and bathed at 37°C with incubation medium containing (in μM) 140 NaCl, 2 KCl, 1 K2HPO4, 1 KH2PO4, 1 MgCl2, 1 CaCl2, 5 glucose, 5 L-alanine, and 10 Hepes-Tris, pH 7.4. The solutions bathing the monolayers were connected via agar bridges and Ag-AgCl electrodes to a voltage-clamp current amplifier (Physiological Instruments, San Diego, CA); the Isc was recorded and was used as an estimate of transcellular ion transport.

To measure the transmonolayer flux of macromolecules, in some experiments 14C-mannitol (0.25 μCi/ml) or HRP (10 μg/ml) (both from Sigma) was added to the medium in the basolateral chamber, 2 d after addition of the cytokines to the basolateral chamber. Subsequently, 14C-mannitol radioactivity or HRP enzyme activity, respectively, was measured in both chambers. 14C-mannitol radioactivity was determined by liquid scintillation. HRP activity was determined using orthophenylene-diamine (Eastman Kodak Co., Rochester, NY) and H2O2 as substrates; the absorbance at 492 nm was quantified by a Bio-Rad microplate reader.

To measure cell death, the lactate dehydrogenase (LDH) release from the GVEC monolayers was determined. Three days after incubation with the cytokines, samples were taken from both the apical and the basolateral chambers, and from the cells after detergent extraction in a total volume of 150 μL with 2% Triton X-100. LDH was measured by spectrophotometry on a Hitachi 747 (Hitachi, Ltd., Tokyo, Japan) at 37°C with lactate as the substrate.

To study the ultrastructure of the rat GVEC monolayers, GVEC cultured on the Transwell filters were fixed with 2.5% glutaraldehyde. Filters and cells were cut and embedded in Epon (LADD Research Industries Inc., Williston, VT). The integrity of the monolayers was studied by electron microscopy of ultrathin sections, with special attention to cell-cell and cell-filter adhesion. Monolayers that had been basolaterally exposed to IL-4 or to IL-13 for 3 d were compared to untreated monolayers of the same age.

**Western blot analysis of STAT6 phosphorylation**

Phosphorylation of STAT6 on the Tyr641 residue in cultured human and rat GVEC was determined by Western blotting. Human GVEC grown on plastic were incubated with human IL-4 or IL-13 (both 100 ng/ml). Rat GVEC were seeded onto large Transwell porous filters (pore diameter 0.4 μm, growth area 4.7 cm², Costar). The apical and basolateral compartments contained 2.0 and 3.0 mL of culture medium, respectively. When TER values of approx. 4 kΩ×cm² were reached, GVEC monolayers were incubated with rat IL-4 (10 ng/ml) or rat IL-13 (1 U/ml) in the basolateral compartments.

After incubation with the indicated cytokines for 0, 10, 20, 30, or 60 min, cells were lysed with sample buffer, containing 100 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0.001% bromphenol blue and 100 mM 2-mercaptoethanol. Proteins in cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and were blotted onto nitrocellulose paper. Staining with Ponceau S confirmed effective protein transfer. Blocking was carried out with 5% milk powder (Nutricia, Zoetermeer, The Netherlands) in Tris-buffered saline containing 0.1% Tween 20 (Sigma) (TBSTM) for 1 h at room temperature. The nitrocellulose membranes were then incubated overnight at 4°C with rabbit anti-Phospho-STAT6 antibody; incubation with PBS was used as a negative control. After extensive washing with TBSTM, the membranes were incubated with HRP-conjugated mouse-anti rabbit IgG (Dako) for 1 h. Binding was visualized using a chemiluminescence detection system (ECL, Amersham, Buckinghamshire, United Kingdom) and analyzed with a Lumi Imager F1 Workstation (Roche Molecular Biochemicals, Basel, Switzerland).

Subsequently, to remove primary and secondary antibodies, membranes were incubated in stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris-HCl, pH 6.7) at 50°C for 30 min. After washing and blocking in TBSTM for 1 h at room temperature, membranes were reprobed with anti-STAT6 antibody. Immunodetection was performed as described above, except that HRP-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates) was used as the secondary antibody.
**Statistical analysis**

Values are expressed as means ± SD when appropriate. One-way ANOVA tests were performed to analyze differences in $I_{SC}$, in transmonolayer flux of $^{14}$C-mannitol or HRP, and in LDH release between control cultures and cultures incubated with IL-4 or IL-13. Differences were considered to be statistically significant at $P < 0.05$.

**Results**

**Expression of IL-4/IL-13 receptors**

Specific transcripts for mRNA of IL-4Ra, IL-13Ra1 and IL-13Ra2 were detected by RT-PCR in the isolated human glomeruli of all patients studied, in isolated rat glomeruli, in both human and rat GVEC lines, and in rat mesangial cells. Messenger RNA for the γc-chain was detected only in isolated rat glomeruli and in rat mesangial cells (Figure 1). In isolated human and rat glomeruli and in both GVEC lines, expression of mRNA for synaptopodin was shown. In addition to identification of the RT-PCR products by their expected size, unequivocal proof of the authenticity of the amplicons was obtained by sequencing, which yielded results identical to the published sequences.

The expression of the IL-4Ra chain and the IL-13Ra2 chain on human GVEC was further demonstrated at the protein level by immunohistochemistry. In human renal biopsies from patients with various kidney diseases (see Materials and Methods), we found glomerular staining with the respective monoclonal antibodies to these receptor chains, as shown in Figure 2. GVEC expressed both IL-4Ra and IL-13Ra2 in all cases studied, whereas expression of these proteins by mesangial cells and by glomerular endothelium was variable. This limited study did not reveal

![Figure 1](image1)

**Figure 1.** RT-PCR analysis of mRNA for the GVEC marker synaptopodin (syn) and the IL-4/IL-13 receptor components: common gamma chain (γc), IL-4Ra, IL-13Ra1 and IL-13Ra2. DNA size markers are indicated on the left (M). A. The product of the PCR on isolated human glomeruli is shown in the first lane, and the product of the PCR on the cultured human GVEC in the second lane. B. The product of the PCR on isolated rat glomeruli is shown in the first lane, the product of the PCR on cultured rat GVEC in the second lane, and on cultured rat mesangial cells in the third lane.
typical staining patterns that could be correlated to the diagnostic groups. Anti-IL-4Rα as well as anti-IL-13Rα2 also specifically bound to human GVEC cultured on glass slides (not shown).

**STAT6 phosphorylation**

Because binding of the IL-4R2 both by IL-4 and by IL-13 is known to be able to induce signal transduction via STAT6 [20], we examined STAT6 phosphorylation in cultured rat and human GVEC by Western blotting with an antibody specific for an epitope containing the phosphorylated Tyr641 residue of STAT6. In untreated cells, no STAT6 phosphorylated at Tyr641 was detectable (Figure 3). In human GVEC, phosphorylated STAT6 was detected as a protein of approximately 100 kDa within 10 min of incubation with IL-4 (Figure 3) and was also detected at 30 and 60 min of incubation with IL-4 (not shown). In rat GVEC cultured on porous filters, phosphorylated STAT6 was only detected after 30 and 60 min of incubation with IL-4 (Figure 3). Upon incubation with IL-13, Tyr641 phosphorylation of STAT6 was detected in rat GVEC, but not in human GVEC. Reprobing the membranes with an antibody against STAT6 revealed that total STAT6 was equally present in both treated and untreated samples. After incubation with IL-4 or IL-13, an additional protein (with an apparent molecular weight of approximately 85 kDa) was detected in rat GVEC with the antibody against phosphorylated STAT6, but not with the antibody against total STAT6 (Figure 3).

**Transepithelial electrical resistance (TER)**

Within 3 d from seeding onto filters, rat GVEC formed confluent monolayers with TER values of 2.5 kΩ×cm², rising to a maximum of 6.0 kΩ×cm² after 5 d. IL-4 and IL-13, when added to the basolateral compartment of the cell monolayer at day 0, caused a strong decrease in TER (Figure 4). After addition of these cytokines, the TER dropped within 1 d and was maximally decreased after 2 to 3 d, reaching values of 1.0 to 1.5 kΩ×cm². Administration of the cytokines to the apical compartment of the Transwells did not affect TER (Figure 5), regardless of whether the apical compartment was represented by the inner chamber or by the outer chamber, indicating that this specificity was not due to differences in *volumina* (not shown). The effect of IL-4 and IL-13 was dose-dependent and could be specifically blocked by OX81 (monoclonal anti-IL-4 antibodies, 0.1 ng/mL) and by anti-IL-13 (rabbit polyclonal antibodies, 1/100 dilution), respectively, but not by species-matched and isotype-matched non-specific antibodies in comparable dilutions (Figure 6).

**Short-circuit current (I_{SC})**

Transcellular ion transport across GVEC monolayers was measured as I_{SC}. After incubation of the monolayers with IL-4 or IL-13 for 3 d, the I_{SC} was significantly increased (Figure 7).
IL-4 and IL-13 act on GVEC

**Figure 3.** STAT6 phosphorylation is induced in GVEC by IL-4 and IL-13. Western blot analysis was performed on lysates from human GVEC (panel A) and rat GVEC (panel B) that were either untreated (control) or treated with IL-4 or IL-13 for up to 60 min. Blots were incubated with an antibody against phosphorylated STAT6 and then reprobed with an antibody against total STAT6.

**Monolayer permeability to macromolecules**

To assess whether the decrease in TER induced by IL-4 and IL-13 was paralleled by an increase in monolayer permeability to macromolecules, we quantified the transmonolayer flux of two organic compounds of different molecular size. 14C-Mannitol (molecular weight, 182 Da) and HRP (molecular weight, 44 kDa) were added to the basolateral compartments of monolayers that had been pretreated with cytokines for 2 d. Subsequently, the appearance of these compounds in the apical compartment was measured after 24 and 48 h. As shown in Table 2, pretreatment of the GVEC with IL-4 or IL-13 did not increase monolayer permeability to these macromolecules.

**Figure 4.** IL-4 and IL-13 induce a strong decrease in TER across GVEC monolayers. Rat GVEC were seeded on day -3 and were basolaterally exposed to IL-4 (10 ng/mL) and IL-13 (1U/mL) from day 0. Values are the means of one experiment, run in triplicate, and are representative of at least five independent experiments.

**Figure 5.** Specificity of TER decrease induced by IL-13 on GVEC. GVEC were seeded on day -3 and were exposed to IL-13 (1 U/mL) in the apical, the basolateral or both compartments from day 0. Values are the means of one experiment, run in triplicate, and are representative of three independent experiments.
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Figure 6. Dose response and specificity of the TER decrease induced by IL-4 and IL-13. Confluent GVEC were basolaterally exposed to IL-4 or IL-13 in various concentrations. After three days, TER was measured and expressed as percentage relative to TER value of untreated control monolayers of the same age. At maximally effective doses of IL-4 or IL-13, the blocking effects were examined either of OX81 (anti-IL-4) or TS2 (control antibodies), or of anti-IL-13 or normal rabbit serum (NRS, control antibodies), respectively. Values are the means of three independent experiments, run in triplicate.

Electron Microscopic Findings

The ultrastructure of rat GVEC monolayers was studied by electron microscopy. No obvious structural differences were observed between control monolayers and monolayers that had been treated with IL-4 or IL-13 for 3 d.

Cytotoxicity

LDH activity in the apical supernatant was not significantly different for GVEC cultures incubated with cytokine for 3 d compared to controls of the same age (266 ± 63 U/L after IL-4, 224 ± 19 U/L after IL-13 and 224 ± 43 U/L in controls, n = 8). In the basolateral medium, LDH activity was negligible and not different among the groups. Intracellular LDH content in the cytokine-treated cells was significantly higher than in controls (751 ± 97 U/L after IL-4, 726 ± 121 U/L after IL-13 and 248 ± 87 U/L in controls, P < 0.001). Taken together, the release of LDH into the supernatants from cytokine-treated cells was similar to that from controls, indicating that the decrease in TER induced by IL-4 and IL-13 was not due to cytotoxicity.

Discussion

Our study shows that the Type2 cytokines IL-4 and IL-13 have effects on GVEC in culture, which may be of interest to our understanding of the pathogenesis of glomerular injury leading to proteinuria. Many studies have suggested an association between MCNS and atopy. The association of proteinuria and atopy was first described in 1959 by Hardwicke et al., who reported seasonal relapses of MCNS in an adult with pollen allergy [21]). Since then, isolated reports on the development of a nephrotic syndrome following allergic reactions to inhaled allergens [22-25], insect stings [26, 27], vaccinations [28, 29] and food [24, 30, 31] have been published. Furthermore, the incidence of atopy in patients with MCNS has been suggested to be higher than in healthy subjects [32-37], ranging from 17-40% in MCNS patients versus 10-23% in age-matched controls. In atopy, Th2-mediated inflammation plays a key role. When activated, Th2 lymphocytes secrete IL-4, IL-5, IL-6, IL-10 and IL-13. Both IL-4 and IL-13 can direct
synthesis of IgE by B lymphocytes. In atopic syndromes such as asthma, atopic dermatitis and allergic rhinitis, exaggerated production of IL-4, IL-13 and IgE is well-documented [12, 13, 38-40]. It has been shown that in patients with MCNS, serum levels of IgE are often elevated [5, 32, 35, 41-45], although this is controversial [37, 46]. Other studies have demonstrated in vitro increased mitogen-stimulated production of IL-1 [47], IL-2 [47, 48], IL-4 [48], and TNF-α [49] in PBMC from patients with MCNS. To circumvent artefacts induced by stimulation with mitogens, more recently the unstimulated production of cytokines by T lymphocytes of patients with MCNS was studied and increased expression and production of IL-13 were found [5, 6]. These investigators suggested that circulating IL-13 may act on monocytes by triggering the production of vascular permeability factor, which in turn would increase glomerular permeability [6]. We hypothesized that IL-4 and IL-13 may directly act on GVEC. To address this hypothesis, we first studied the expression of the specific IL-4/IL-13 receptors on GVEC. At the mRNA level, we showed that rat and human GVEC express the two components of the IL-4R2, as well as the IL-13Rα2. In isolated rat and human glomeruli, the same pattern of expression was detected. Only in rat glomeruli did we find expression of mRNA for the γc-chain. This mRNA is probably derived from passenger cells or from mesangial cells rather than from GVEC, since cultured rat mesangial cells expressed γc-chain mRNA. At the protein level, immunohistochemistry revealed expression of IL-4Rα and IL-13Rα2 by GVEC in human glomeruli and by human GVEC in culture. Because no specific antibodies to IL-13Rα1 are as yet available, we could not study protein expression of this receptor chain.

IL-4 and IL-13 are known to activate STAT6 by binding to the IL-4R2 in human colon carcinoma cell lines [20] and in HUVEC [50]. In rat GVEC in vitro, we showed induction of STAT6 phosphorylation both by IL-4 and by IL-13, indicating that these cells express a functional IL-4R2. In the human GVEC line, STAT6 phosphorylation was only observed upon incubation with IL-4, which, in the absence of the γc-chain, is probably the result of binding to a functional IL-4R2. The lack of STAT6 phosphorylation in cultured human GVEC in the presence of IL-13 might be due to overexpression of the high-affinity receptor IL-13Rα2, which does not signal after binding, as has been described for cultured human synovial fibroblasts [11].

Functioning of the IL-4R2 on rat GVEC was further evidenced by the induction of specific changes in cell function both by IL-4 and by IL-13. To study these direct effects of IL-4 and IL-13 on GVEC function, we measured TER over a monolayer of rat GVEC in a Transwell system. TER is a commonly used estimate of epithelial cell function, representing at least three characteristics of an epithelial monolayer: monolayer permeability, transcellular ion transport, and cell structure. We showed that IL-4 and IL-13 specifically decreased the TER of a monolayer of rat GVEC. This decrease was associated with an increase in Isc, suggesting an increase in transcellular ion transport, but it was not accompanied by increased monolayer permeability to macromolecules, or by cell death. Electron microscopic examination of the GVEC monolayers revealed no gross structural changes induced by IL-4 or IL-13. Of course, TER, Isc, and monolayer permeability in vitro cannot be translated to glomerular permeability in vivo. Furthermore, these rat GVEC in culture do not form foot processes. Therefore, the relevance of our findings on GVEC function and on GVEC structure in vitro with respect to induction of proteinuria and foot process effacement warrants further investigation in models in vivo.

In conclusion, we show that IL-4 and IL-13 exerted specific effects on GVEC in vitro, most likely by binding to the IL-4R2, which may also be expressed by GVEC in vivo. In MCNS, circulating IL-13 produced by triggered T lymphocytes [5, 6] could directly act on GVEC by binding to the IL-4R2, and thus possibly play a role in the pathogenesis of the specific changes in GVEC structure and glomerular permeability observed in these patients.

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