Chemoattraction of T cells expressing CCR5, CXCR3 and CX3CR1 by proximal tubular epithelial cell chemokines

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Abstract

Background. Chemokines produced by resident renal cells promote the infiltration of leukocyte subsets. We have analysed the chemotactic responses of CD3+ peripheral blood lymphocytes (PBLs) to factors secreted by proximal tubular epithelial cells (PTEC), assessing the role of chemokines and chemokine receptors in this process.

Methods. By FACS we analysed expression of the chemokine receptors CCR5, CXCR3, CX3CR1, CCR2, CXCR1 and CXCR2 on both freshly isolated and activated PBLs. Using Boyden chambers we studied the chemotactic activity of supernatant from resting and cytokine-stimulated (TNF-α and IFN-γ) PTEC towards PBLs. Soluble recombinant chemokines and blocking antibodies were used to study the role of individual chemokine receptors. Chemokine secretion by PTEC was analysed by ELISA.

Results. Only a small proportion of freshly isolated cells expressed the chemokine receptors and there was low grade chemotaxis of these cells towards cytokine-stimulated PTEC supernatant compared with unstimulated PTEC supernatant. After activation, 84% of PBLs expressed CCR5, 90% expressed CXCR3 and 19% expressed CX3CR1. There remained low expression levels of CXCR1, CXCR2 and CCR2. Activated PBLs showed strong chemotactic responses to supernatant from cytokine-stimulated PTEC compared with unstimulated PTEC (P < 0.001). Chemotaxis of these cells was inhibited by blocking CCR5, CXCR3 and CX3CR1 by 69%, 71% and 29% respectively, with complete inhibition following combined blockade. ELISA showed high levels of the chemokine RANTES/CCL5 (for CCR5) and IP-10/CXCL10 (for CXCR3) in cytokine-stimulated PTEC supernatant.

Conclusions. Chemokines produced by cytokine-activated PTEC promote the selective recruitment of activated T cells via the receptors, CCR5, CXCR3 and CX3CR1. These receptors may be amenable to therapeutic manipulation in renal inflammation.

Keywords: CCR5; CXCR3; CX3CR1; chemokines; renal inflammation; proximal tubular epithelial cells; T cells

Introduction

In human renal inflammatory diseases CD3+ T cells traffic to interstitial and tubular sites. Many of these cells are of an activated memory phenotype [1,2] and have a central role in directing injury. The trafficking of subsets of circulating T cells to tissue sites requires the generation of a chemotactic gradient by locally expressed chemokines [3–5]. A number of members of the CC and CXC chemokine subfamilies and the CX3C chemokine fractalkine CX3CL1 ligate receptors that are expressed on circulating T cells. However, there is substantial functional redundancy in chemokine expression at inflammatory sites and the major control on selective recruitment of circulating leukocytes is restricted chemokine receptor expression on leukocyte subsets [6]. This expression varies between different types of leukocyte and as the phenotype of a leukocyte develops [7,8].

Recent studies have shown that the chemokine receptors CCR5 and CXCR3 are of particular importance in the recruitment of T cells to inflammatory sites [9]. Furthermore, the presence of CCR5+ cells have been reported in the human kidney during inflammation [10–12]. CCR5 is expressed on activated and memory T cells, which have been found to respond to the chemokines RANTES/CCL5, MIP-1α/CCL3 and MIP-1β/CCL4 in chemotactic assays [13–15]. CXCR3 is expressed predominantly on activated T cells [16].

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and virtually all of these cells are also CCR5+ [9]. Recent studies in receptor knockout mice have identified CXCR3 as the most important chemokine receptor in the development of allograft rejection [17]. CXCR3 is ligated by the non-ELR CXC chemokines IP-10/CXCL10, Mig/CXCL9 and I-Tac/CXCL11 [18–20]. Recently we have demonstrated a potential role for the in situ production of fractalkine/CX3CL1, including proximal tubular epithelial cell (PTEC)—derived fractalkine, in monocellular cell recruitment during human renal inflammation [21]. Fractalkine/CX3CL1 ligates CX3CR1, which is expressed on a proportion of activated T cells [22]. PTEC are a rich source of chemokines and several studies have demonstrated their inducibility after stimulation by pro-inflammatory cytokines [23–26]. Further studies have shown in situ expression of chemokines by PTEC in disease states [27–32]. Whether all the chemokines expressed by PTEC have functional roles during T cell recruitment to the tubulointerstitium remains to be determined.

In these studies we have demonstrated that RANTES/CCL5, IP-10/CXCL3 and fractalkine/CX3CL1 are chemotactic for activated peripheral blood lymphocytes (PBLs). Enzyme linked immunosorbent assays (ELISAs) were used to identify the expression of RANTES/CCL5 (for CCR5), IP-10/CXCL10 (for CXCR3), MCP-1/CCL2 (for CCR2) and IL-8/CXCL8 (for CXCR1 and CXCR2) in PTEC supernatant. FACS analysis was used to demonstrate variable chemokine receptor expression on freshly isolated and IL-2 activated PBLs. The role of individual chemokine receptors in promoting chemotaxis in activated T cells towards PTEC was determined using blocking monoclonal antibodies and soluble recombinant human fractalkine/CX3CL1 (srHufractalkine).

**Subjects and methods**

**Isolation and activation of peripheral blood lymphocytes**

Venous blood from normal human volunteers was collected into heparin and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Hypaque centrifugation (Amersham Pharmacia Biotech, Amersham, UK) at 400 g for 30 min. PBMCs were washed in phosphate-buffered saline and added to fibronectin coated Petri dishes (fibronectin 1 µg/cm², New York Blood Center, USA) for adherence depletion of monocytes. Non-adherent cells (PBMCs) were isolated and activated PBLs. Twenty-five microlitres of PBLs (1 × 10⁶ cells/ml) were incubated for 30 min with 2 µl monoclonal mouse anti-human antibodies (a gift from Millenium Pharmaceuticals, Cambridge, MA, USA) against CCR5 (2D7, IgG1 isotype, 1 mg/ml), CXCR3 (1C6, IgG1 isotype, 0.9 mg/ml), CXCR1 (5A12, IgG2a isotype, 0.9 mg/ml), CXCR2 (6C6, IgG1 isotype, 0.9 mg/ml) or 3 µl antibody against CCR2 (5A11, IgG1 isotype, 0.8 mg/ml) or their isotype matched controls. Cells were then washed three times in phosphate-buffered saline containing 0.1% BSA and 0.1% azide (FACS wash buffer), and incubated for 30 min with FITC conjugated anti-mouse IgG. For CX3CR1 expression 1 × 10⁶ cells were resuspended in 4 nM fractalkine/CX3CL1 conjugated to secreted alkaline phosphatase fusion protein (FKN-SEAP) or control SEAP (CON-SEAP) (a gift from Millenium Pharmaceuticals) and incubated for 60 min at room temperature, washed with FACS wash buffer and incubated with 50 µl of sheep anti-human alkaline phosphatase (Binding Site, Birmingham, UK) (1:10) for 60 min. After washing, cells were incubated with 50 µl donkey anti-sheep/goat IgG FITC (Binding Site) (1:10) for 60 min. Cells were then washed again and fixed in 2% paraformaldehyde. Unless otherwise stated all incubations were performed at 4°C.

In order to determine the phenotype of activated PBLs obtained following CD3 and IL-2 treatment, the cells were stained for CD3, CD25, CD45RA and CD45RO. Monoclonal anti-human CD3 FITC conjugated antibody (Dako, Cambridge, UK) was used at dilution of 1:10 and cells were incubated for 30 min. Cells were then washed and fixed in 2% paraformaldehyde. Anti-human CD25 antibody (Dako) was used at a concentration of 1:50 and cells were incubated for 45 min. Cells were washed, incubated with a secondary FITC conjugated anti-mouse IgG antibody (Sigma Ltd, UK) for 45 min and then fixed. The CD45RO (memory) and CD45RA (naive) subsets were also analysed in both freshly isolated and activated PBLs. Twenty-five microlitres of PBLs (1 × 10⁶ cells/ml) were incubated for 30 min with 1 µl of mouse anti-human CD45RO FITC (Dako) or CD45RA RPE (Beckham Coulter, Bedfordshire, UK). Cells were then washed and fixed.

**Cultures of renal proximal tubule epithelial cells**

PTEC were cultured and characterized as previously described [33]. A density of 1.5 × 10⁶ cells in 25 cm² tissue culture flasks were treated with 10 ng/ml (110 U/ml) TNF-α and 200 U/ml IFN-γ, in 3 ml of PTEC culture medium, either singly or in combination. These concentrations of cytokines have been shown by ourselves and others
[23, 25, 26, 34, 35] to stimulate significant production of RANTES/CCL5 (by TNF-α and IFN-γ), IP-10/CXCL10 (by IFN-γ and TNF-α), MCP-1/CCL2 (by TNF-α and IFN-γ), IL-8/CXCL8 (by TNF-α) and fractalkine/CX3CL1 (by TNF-α) from PTEC. Supernatants were harvested at fixed time points for use in chemotactic assays and assessment by ELISAs for RANTES/CCL5, IP-10/CXCL10, MCP-1/CCL2, and IL-8/CXCL8.

Chemotaxis of PBLs

Chemotactic activity of the supernatants of unstimulated or cytokine-stimulated PTEC towards resting and activated PBLs was studied using 48-well modified Boyden chamber assays. Twenty-five microlitres of supernatant were assayed in triplicate for chemotactic assays and assessment by ELISAs for RANTES/CCL5 and IP-10/CXCL10, or for 1 h for MCP-1/CCL2. For IL-8/CXCL8, a peroxidase-conjugated donkey anti-goat IgG (Binding Site) (in phosphate-buffered saline containing 10% dried skimmed milk powder) was added at a dilution of 1:1000. For RANTES/CCL5, MCP-1/CCL2 and IP-10/CXCL10, plates were developed with tetramethylbenzidine and the reaction was stopped using 50 μl of 20% H2SO4 per well. The absorbance was then measured at 450 nm using a Multiskan bichromatic analyser (Labsystems, Helsinki, Finland). For IL-8/CXCL8, 5 μl of 30% H2O2 was added to 25 ml of 0.4 mg/ml fibronectin and stained with Diff Quick solution. Migrated cells were counted using light microscopy. Five high-power fields (×400) were counted per well. In control experiments PBLs were (i) chemoattract by srHuRANTES/CCL5 at a concentration of 10 ng/ml, srHuIP-10/CXCL10 at 100 ng/ml and srHufractalkine/CX3CL1 at 250 ng/ml (preliminary experiments had identified these concentrations of chemokines as optimal for maximal chemotaxis) or (ii) inhibited by pre-treatment of PBLs with 1 μg/ml pertussis toxin at 37°C for 1 h which prevents signalling through G-protein receptors and abolishes chemokine derived chemotactic signals. For antibody blocking experiments, cells were pre-incubated at 4°C for 30 min with the anti chemokine receptor antibodies CCR5, CXCR3, CXCR1 and CXCR2 or their isotype-matched irrelevant controls at concentrations of 5 μg/ml, at which the antibodies effectively block their target receptor [9, 36, 37]. A CXCR1 blocking antibody is not commercially available, but as srHufractalkine/CX3CL1 is the only ligand for this receptor, cells were pre-incubated with fractalkine/CX3CL1 at 1 μg/ml at 4°C for 30 min before loading onto the Boyden chamber to block chemotaxis through CX3CR1. A blocking antibody for CCR2 was not available.

ELISAs

PTEC supernatants were assayed in triplicate for chemokines. Ninety-six-well plates (Nunc, Paisley, UK) were coated overnight at 4°C with 100 μl of optimized concentrations of monoclonal anti-human RANTES/CCL5 (at 2 μg/ml), MCP-1/CCL2 (at 1.25 μg/ml), IL-8/CXCL8 (at 2 μg/ml) or IP-10/CXCL10 (at 2 μg/ml) (R & D Systems, Abingdon, UK) in a carbonate buffer (0.03 M Na2CO3, pH 9.6). The plates were blocked for 1 h with 1% BSA for all ELISAs except IL-8/CXCL8, where plates were blocked for 1 h with 10% dried skimmed milk powder in phosphate-buffered saline (200 μl per well). Supernatant was added at 100 μl per well. Recombinant human RANTES/CCL5, MCP-1/CCL2, IP-10/CXCL10 or IL-8/CXCL8 (R & D Systems) were used at concentrations ranging from 16 pg/ml to 1000 pg/ml to standardize the ELISAs. The standards were diluted in phosphate-buffered saline, 0.1% BSA and 0.05% between 20. After 1 h RANTES/CCL5 (5 ng/ml), MCP-1/CCL2 (100 ng/ml) or IP-10/CXCR10 (50 ng/ml) biotinylated goat anti-human chemokine antibodies (in phosphate-buffered saline, 0.1% BSA and 0.05% between 20) or 2 μg/ml IL-8 goat polyclonal anti-human chemokine IL-8/CXCL8 antibody (R & D Systems) (in phosphate-buffered saline containing 10% dried skimmed milk powder) was added. After a further 1 h, Streptavidin-HRP conjugate (1:5000) (in phosphate-buffered saline, 0.1% BSA and 0.05% between 20) was added either for 30 min for RANTES/CCL5 and IP-10/CXCL10, or for 1 h for MCP-1/CCL2. For IL-8/CXCL8, a peroxidase-conjugated donkey anti-goat IgG (Binding Site) (in phosphate-buffered saline containing 10% dried milk powder) was added at a dilution of 1:1000. For RANTES/CCL5, MCP-1/CCL2 and IP-10/CXCL10, plates were developed with tetramethylbenzidine and the reaction was stopped using 50 μl of 20% H2SO4 per well. The absorbance was then measured at 450 nm using a Multiskan bichromatic analyser (Labsystems, Helsinki, Finland). For IL-8/CXCL8, 5 μl of 30% H2O2 was added to 25 ml of 0.4 mg/ml o-phenylenediamine hydrochloride in citrate buffer (0.1 M citric acid solution made up to pH 5.0 using K2HPO4), and this was then added to the plate. The reaction was stopped after 15 min using 20% H2SO4 (50 μl per well) and the absorbance measured at 492 nm. The assays were performed at 37°C until the addition of H2O2 and subsequent steps were performed at room temperature. All ELISAs were capable of detecting 20 pg/ml of chemokine, and variation between tests was less than 10%.

Statistical analysis

Differences between samples were analysed by a two-tailed unpaired Student’s t-test. Kruskal–Wallis analysis of variance was used to determine variance between groups of samples. The level of significance was set at P<0.05.

Results

Expression of chemokine receptors and cell surface markers by PBLs

Flow cytometry was used to analyse the cell surface expression of chemokine receptors on freshly isolated PBLs and activated PBLs. Three separate donors were studied. The T cell marker CD3 was expressed in 75±3.1% (mean±SEM) of freshly isolated PBLs and in 96.7±0.3% of activated PBLs (Figures 1d and 2). For freshly isolated PBLs, 15.3±1.9% expressed CCR5 and 21.5±10% expressed CXCR3 (Figures 1a and 2). Following activation, 84±2.1% of PBLs were CCR5 positive and 90.7±0.9% were CXCR3 positive (Figures 1a and 2). This data is consistent with that reported by Qin and colleagues [9], who showed a major amplification in cell surface expression of CCR5 and CXCR3 on activation of PBLs by CD3 and IL-2. Less than 15% of freshly isolated, and 5% of activated, PBLs expressed CXCR1 or CXCR2 (Figures 1a and 2). Fewer than 2% of freshly isolated, and 7% of activated, PBLs expressed CCR2 (Figures 1a and 2). The efficacy of antibodies to CXCR1 and CXCR2 was confirmed
by demonstrating binding of both antibodies to 97% of freshly isolated neutrophils (Figure 1b). The efficacy of the anti-CCR2 antibody was confirmed in experiments using THP-1 cells, where the majority of cells were shown to express the receptor (Figure 1b). For CX3CR1, $3 \pm 0.01\%$ of freshly isolated and $19 \pm 0.1\%$ of activated cells expressed the receptor as determined using a fusion protein (Figure 1c). The efficacy of the FKN-SEAP construct was confirmed in experiments using NK cells where 70% of cells expressed the receptor [35]. On further analysis of T cell subsets, 34% of freshly isolated and 76% of activated PBLs were of a memory (CD45RO+) phenotype and activation was confirmed by CD25 positivity of virtually all CD3 and IL-2 treated PBLs (Figure 1d).

Migration of PBLs to PTEC supernatant

Preliminary experiments showed chemoattraction of activated PBLs to srHuRANTES/CCL5 (maximal at 10–100 ng/ml), srHuIP-10/CXCL10 (maximal at 100 ng/ml) and srHufractalkine/CX3CL1 (maximal at 250 ng/ml) when compared with transmigration medium alone. There was no significant chemotaxis to srHuMCP-1/CCL2 at concentrations of 10–250 ng/ml, consistent with the little CCR2 expression on these cells. A checkerboard analysis showing the relative contribution of chemokinetic activity to overall chemotaxis is presented in Table 1. For subsequent experiments the chemokinesis of PBLs has been subtracted from the chemotactic index. Freshly isolated PBLs showed chemotaxis to srHuRANTES/CCL5 (7.95 ± 0.53 cells/hpf), srHuIP-10/CXCL10 (8.41 ± 0.5 cells/hpf), srHufractalkine/CX3CL1 (7.9 ± 0.33 cells/hpf) and all three chemokines in combination (11.93 ± 0.76 cells/hpf). There was little chemoattraction to unstimulated PTEC supernatant (0.65 ± 0.04 cells/hpf) and low grade but significant chemoattraction to supernatant from PTEC stimulated by TNF-α and IFN-γ for 24 h (11.25 ± 0.33 cells/hpf) (Figure 3a). Recombinant chemokines, both singly and in combination, induced significant levels of migration of activated PBLs (srHuRANTES/CCL5, 42.89 ± 2.28 cells/hpf; srHuIP-10/CXCL10, 55.24 ± 2.75 cells/hpf; srHufractalkine/CX3CL1, 36.49 ± 1.56 cells/hpf; combined chemokines, 55.91 ± 2.6 cells/hpf). Similarly, activated PBLs showed significant migration to TNF-α plus IFN-γ stimulated PTEC supernatant (87.54 ± 6.67 cells/hpf) when compared with

![Fig. 1.](image-url) (a) Histograms of immunofluorescent staining of single cell suspensions of freshly isolated and activated PBLs with anti-CCR5, anti-CXCR3, anti-CCR2, anti-CXCR1 and anti-CXCR2 monoclonal antibodies. Histograms are compared with isotype control MoAb (IgG1 or IgG2a; only IgG1 is shown as the two antibodies behaved similarly).
Fig. 1. (b) Histograms of immunofluorescent staining of single cell suspensions of freshly isolated neutrophils with CXCR1 and CXCR2 and THP-1 cells with CCR2. Histograms are compared with isotype controls for each antibody; (c) Histograms showing CX3CR1 expression on freshly isolated (thin line) and activated (thick line) PBLs using FKN-SEAP; CON-SEAP fusion protein is shaded; (d) Histograms of immunofluorescent staining of single cell suspensions of freshly isolated and activated PBLs with CD3, CD25, CD45RO and CD45RA. Surface marker expression on freshly isolated cells is shown as thin line, activated cells as thick line and negative control shaded. Results are of one representative of three experiments.
unstimulated PTEC supernatant. There was low grade chemoattraction to unstimulated PTEC supernatant (4.6 ± 0.34 cells/hpf) (Figure 3b). In separate experiments pertussis toxin abolished chemotactic activity suggesting it was mediated via G-protein linked receptors.

The chemoattractant potential of supernatants obtained following treatment of PTEC with TNF-α and/or IFN-γ over 8–72 h was monitored. Chemotraction was induced in response to TNF-α plus IFN-γ stimulated supernatant obtained at 8 h with a maximal response to supernatants at 24 h (Figure 4).

There was no significant chemotaxis to supernatant from PTEC stimulated by either TNF-α or IFN-γ at 24 h. However, chemotactic responses were present to supernatant harvested after 48 and 72 h of stimulation with either TNF-α or IFN-γ. The kinetics of

Fig. 2. A comparison of chemokine receptor expression on PBLs freshly isolated (A) and after IL-2 activation (B) as determined by flow cytometry. Single cell suspensions were stained with mouse anti-human MoAbs against CD3 and a panel of antibodies against different chemokine receptors. Results represent mean ± SEM of three separate experiments. CX3CR1 data is given in text as expression was determined using a different methodology.

Table 1. Checkerboard representation of migration of PBLs in Boyden chambers

<table>
<thead>
<tr>
<th></th>
<th>Chemokinesis</th>
<th>Unstimulated PTEC supernatant</th>
<th>TNF-α and TNF-γ stimulated PTEC supernatant</th>
<th>RANTES/IP10/fractalkine combined</th>
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<tbody>
<tr>
<td>Freshly isolated PBLs</td>
<td>3.5 ± 0.27</td>
<td>4.2 ± 0.31</td>
<td>14.8 ± 0.68</td>
<td>15.49 ± 1.03</td>
</tr>
<tr>
<td>Activated PBLs</td>
<td>9.51 ± 1.12</td>
<td>14.06 ± 1.46</td>
<td>96.07 ± 7.79</td>
<td>65.4 ± 3.72</td>
</tr>
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Values represent the number of cells/high power field. Chemokinesis represents the random motion of PBLs towards PTEC culture medium. The values for migration towards unstimulated or cytokine-stimulated PTEC supernatant or towards the chemokines RANTES/CCL5, IP-10/CXCL10 and fractalkine/CX3CL1, includes chemokinesis.
chemoattraction are of interest since, although chemokine production was increased further by 48 and 72 h (see Figures 6–9), no further enhancement of migration was observed. This suggests chemokine receptor saturation and receptor internalization leading to desensitization of the cells to further increases in chemokine levels; this phenomenon has previously been reported by others [38–40].

Inhibition of CCR5, CXCR3 and CX3CL1 mediated chemotaxis

To analyse the relative activities of chemokines for supporting migration of PBLs, monoclonal antibodies with blocking activity against chemokine receptors and srHufractalkine/CX3CL1 were used. These studies were performed using supernatant from PTEC stimulated for 24 h with both TNF-α and IFN-γ. Pre-treatment of activated PBLs with anti-CCR5 resulted in a 69% reduction in chemotaxis, with anti-CXCR3 a 71% reduction, and 29% with srHufractalkine/CX3CL1 (Figure 5). When CCR5, CXCR3 and srHufractalkine/CX3CL1 were combined, chemotaxis was completely blocked. In separate experiments isotype control antibodies did not inhibit chemotaxis. Blocking CCR5 and CXCR3 in combination had no significant effect over either receptor blocked alone (data not shown). Blocking antibodies against CXCR1 and CXCR2 did not reduce chemotaxis when compared to isotype-matched controls (data not shown).

Expression of chemokines by cytokine stimulated PTEC

The levels of chemokines active against CCR5 (RANTES/CCL5) and CXCR3 (IP-10/CXCL10) in supernatants from cytokine-stimulated PTEC were quantified by ELISA. We also analysed expression of IL-8/CXCL8 and MCP-1/CCL2. An ELISA against srfractalkine/CX3CL was not available. Results are expressed as mean ± SEM. Each experiment was repeated at least three times.

RANTES/CCL5 (Figure 6). There were modest levels of RANTES/CCL5 in the supernatant of PTEC treated singly with TNF-α or IFN-γ. This was first detectable over background at 24 h, with maximal levels of 0.773 ± 0.072 ng/ml on TNF-α stimulation and 0.883 ± 0.037 ng/ml on IFN-γ stimulation, respectively, at 72 h (Figure 6 inset). There was massive synergistic induction of RANTES/CCL5 production by TNF-α and IFN-γ in combination. This was first detectable at 8 h, with maximal levels at 48–72 h that were over 80-fold those produced on treatment with single cytokines (at > 60 pg/ml).

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There were low levels of IP-10 uCXCL10, maximal at 72 h (0.307 ± 0.026 ng/ml), in TNF-α treated PTEC. IFN-γ stimulates over 6 ng/ml IP-10, maximal at 48 h. The combination of TNF-α and IFN-γ stimulated large amounts of IP-10 maximal at 72 h. Results are expressed as mean ± SEM.

**IP-10/CXCL10** (Figure 7). There were low levels of IP-10/CXCL10, maximal at 72 h (0.307 ± 0.026 ng/ml), in TNF-α treated PTEC. IFN-γ induced maximal production of IP-10/CXCL10 at 48 h with 6.95 ± 0.12 ng/ml. The combination of TNF-α and IFN-γ was synergistic, IP-10/CXCL10 being first detectable at 2 h, with maximal levels of 89.26 ± 6.93 ng/ml at 24 h.
the relative importance of different components of the chemokine and chemokine receptor hierarchy in T cell recruitment in renal inflammation. Information from such *in vitro* studies is necessary to guide tests *in vivo* to confirm or refute the relative importance of individual chemokines. We show that activated CD3+ lymphocytes, which express the chemokine receptors CCR5, CXCR3 and CX3CR1, are highly responsive to chemotactic factors produced by PTEC, which in turn have been stimulated by proinflammatory cytokines. Others have previously shown a requirement for both T cell receptor engagement and then ligation of the high affinity IL-2 receptor for full T cell activation [9]. We show that virtually all these cells expressed the activation marker CD25 (the α chain of the IL-2 receptor) and the majority was CD45RO+ (memory phenotype) [9]. Where studied, this is the dominant T cell phenotype *in situ* in human renal inflammation [2,42]. In blocking studies we show that chemotaxis of these cells is mainly attributable to ligation of CCR5 and CXCR3. Ligation of CX3CR1 has an additional effect. There are several candidate chemokines that may promote this process: RANTES/CCL2 and IP-10/ CXCL10, which were present in large amounts in the supernatants from activated PTEC that we used in these studies; and fractalkine/CX3CL1, which we have recently shown expressed by PTEC in human renal inflammation [21].

The relevance of these studies, using cells activated in culture to express this chemokine receptor phenotype, to disease processes has been demonstrated by Segerer and colleagues [12] who showed the presence of CCR5 on infiltrating cells in renal biopsy material from patients with various renal diseases. There was a strong correlation between CCR5 expressing cells and CD3+ T cells; in certain disease states up to 90% of T cells were CCR5+ [12]. Other studies have also demonstrated the presence of CCR5-expressing cells in human renal disease [10,11]. Although a role for CXCR3 has not been previously studied in human renal inflammation, Qin and colleagues [9] have shown that all activated T cells that express CCR5 are also CXCR3+, indicating that these cells may be chemotacted through either receptor. They also demonstrated that virtually all T cells at sites of non-renal human inflammation having prominent mononuclear cell infiltrates were CXCR3+. The recent studies of Hancock [17] using chemokine receptor knockout mice, showed that CXCR3 is the most important chemokine receptor for the development of acute allograft rejection. Loetscher and colleagues [43] have previously shown that up to 40% of freshly isolated T cells express CXCR3, but these cells only migrate towards IP-10/CXCL10 after activation. In our studies just over 20% of freshly isolated T cells expressed CXCR3 and 15% expressed CCR5, but on activation by anti-CD3 antibody and IL-2 approximately 90% of cells expressed both receptors. Furthermore, others have shown that there is little chemotraction of unstimulated lymphocytes by chemokines active against CCR5 [13–15]. As recent studies have indicated

**Discussion**

In renal inflammation, infiltrating lymphocytes accumulate at tubular and interstitial sites, and many are activated [2,41]. The processes that drive this recruitment are not well characterized. Although previous investigations have addressed the individual chemokines selected here, this study has begun to analyse

MCP-1/CCL2 (Figure 8). Both TNF-α (44.1 ± 6.15 ng/ml, maximal at 72 h) and IFN-γ (6.38 ± 1.99 ng/ml, maximal at 48 h) stimulated PTEC production of IL-8/CXCL8 and this production was significantly inhibited by the use of IFN-γ. IFN-γ treatment alone did not stimulate IL-8/CXCL8 production by PTEC. Results are expressed as mean ± SEM.

IL-8/CXCL8 (Figure 9). TNF-α stimulated 6.62 ± 0.22 ng/ml IL-8, maximal at 72 h. There was no induction of IL-8/CXCL8 by IFN-γ, and IFN-γ downregulated TNF-α-induced IL-8/CXCL8 production (3.16 ± 0.01 ng/ml at 72 h).
a role for fractalkine/CX3CL1 in mononuclear cell recruitment during renal inflammation, and CX3CR1 is expressed on a subpopulation of activated T cells, we also studied this chemokine/receptor pair. We showed that sRHufractalkine/CX3CL1 has a significant chemotactic effect on activated PBLs, and blocking studies showed that targeting CCR5, CXCR3 and CX3CR1 together completely inhibits chemotaxis to cytokine-activated PTEC supernatant.

We have shown little expression of the MCP-1/CCL2 receptor, CCR2, on unstimulated or activated cells, and that MCP-1/CCL2 is not chemotactic for the cell population studied. Others have reported that CCR2 is variably expressed on T cells, with low expression on peripheral blood T cells, and some expansion of expression on anti-CD3 upregulation [44]. Although MCP-1/CCL2 is expressed in the kidney during inflammation [31,45], there have been no studies to date on a role for CCR2 in human renal disease. However, recent in vitro analyses indicate the major role for PTEC derived MCP-1/CCL2 may be for monocyte recruitment [26]. There is little expression of the IL-8/CXCL8 receptors CXCR1 and CXCR2 on freshly isolated or activated T cells so, despite local expression of IL-8/CXCL8 in human renal inflammation [32], this chemokine is unlikely to have a central role in directing T cell infiltration.

The presence of chemokines that bind CCR5 has been identified in human renal disease. RANTES is expressed by PTEC, endothelial cells and infiltrating cells in human renal inflammation [27,31,46]. There are less data available for MIP-1α/CCL3 and MIP-1β/CCL4, although expression by PTEC of mRNA for both chemokines [31] and MIP-1α/CCL3 protein [47] has been demonstrated in human glomerulonephritis. Fractalkine/CX3CL1 is expressed by endothelial cells and PTEC in cases of acute allograft rejection and ANCA-associated glomerulonephritis [21]. The expression of the CXCR3 directed chemokines IP-10/CXCL10, Mig/CXCL9 or I-Tac/CXCL11 has not been studied to date in biopsies from patients with renal inflammation, although analyses of tissue expression from other sites indicate an important role for these chemokines in directing T cell trafficking [9].

The heavy production of RANTES/CCL5 [31], MCP-1/CCL2 [23] and IL-8/CXCL8 [24] by PTEC has been demonstrated in earlier studies, and recently Kuroiwa and colleagues [26] have shown that activated T cell/PTEC co-culture induced production of high levels of IP-10/CXCL10 from PTEC. At the concentrations of TNF-α and IFN-γ used in the present studies we show that (i) heavy production of RANTES/CCL5 is dependent on both TNF-α and IFN-γ, (ii) although IP-10/CXCL10 synthesis is IFN-γ dependent, with the addition of TNF-α there is an 80-fold increase in synthesis, (iii) TNF-α stimulated MCP-1/CCL2 production is increased by the addition of IFN-γ and (iv) IFN-γ downregulates TNF-α stimulation of IL-8/CXCL8 production. A similar observation has been made for IL-8/CXCL8 expression by biliary epithelial cells [48]. This ability of IFN-γ to downregulate some TNF-α-induced chemokine production indicates that chemokine expression is under tight regulatory control by cytokines and that IFN-γ may also play a role in switching the cellular response from neutrophil to mononuclear cell rich.

In conclusion, we have shown that supernatants from cytokine-stimulated PTEC are chemotactic towards CD3+ lymphocytes with an activated and memory phenotype. The majority of this effect is mediated by the chemokine receptors CCR5 and CXCR3, with an additional role for CX3CR1. The candidate chemokines that direct this process may include RANTES/CCL5, IP-10/CXCL10 and fractalkine/CX3CL1. Our studies suggest a hierarchy of chemokine receptors and their ligation by chemokines during T cell recruitment in renal inflammation. The potential clinical significance of these interactions has been shown in animal models of renal disease where blocking a specific chemokine receptor abrogates leukocyte infiltration [49,50]. Further studies are now required to determine the efficacy of chemokine receptor blockade in the treatment of renal disease.

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