MIP-3α/CCL20 in Renal Transplantation and Its Possible Involvement as Dendritic Cell Chemoattractant in Allograft Rejection

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Graft-infiltrating dendritic cells (DC) and alloreactive T lymphocytes play a critical role in renal allograft rejection. Renal proximal tubular epithelial cells (TEC) are considered as active players in the attraction of leukocytes during renal inflammatory responses. Macrophage inflammatory protein (MIP)-3α/CCL20 is a major chemokine expressed by epithelial cells that attracts immature DC. In the present study, we present evidence that also the transplanted kidney can be a major source of MIP-3α/CCL20. Renal transplant recipients with rejection showed significantly increased excretion of urinary MIP-3α/CCL20 that correlated with transplant function. The tubular staining for MIP-3α/CCL20 in renal biopsies of patients with rejection as well as *in vitro* studies with primary human TEC indicated that TEC might be responsible for the increased urinary MIP-3α/CCL20. Furthermore, MIP-3α/CCL20 produced by activated TEC was highly potent in the attraction of CD1a+CD34+-derived DC precursors. These data suggest a role for MIP-3α/CCL20 in amplification of the immune response during renal allograft rejection by attraction of CCR6+ inflammatory cells, which may include DC, to the site of inflammation.

Key words: Chemokines, dendritic cells, epithelial cells, kidney transplantation, rejection

Introduction

Renal allograft rejection is an inflammatory process characterized by cellular infiltration and tissue destruction in which graft-infiltrating dendritic cells (DC) and alloreactive T lymphocytes play a critical role (1,2). DC are professional antigen presenting cells that function as sentinels of the immune system (3). DC are the passenger leukocytes that appear to play a critical role in the immune response to allografts, as promoters of rejection and as mediators of tolerance (2,4). In case of direct allo-antigen recognition, donor-derived DC migrate to lymphoid organs to activate resting T cells. For the indirect pathway of allo-antigen recognition, infiltrating recipient-derived DC capture the allo-antigen and present it to recipient T cells (2,5). During the indirect response, recipient DC precursors or immature DC infiltrate the kidney as part of the early inflammatory response that follows the surgical procedure or other danger signals, such as ischemia (6–8).

Although the signals that regulate the complex traffic pattern of DC are incompletely understood, chemokines seem to play an important role in this process (9). These chemokines are secreted by activated leukocytes themselves and by activated resident tissue cells, including endothelial and epithelial cells. It has been suggested that proximal tubular epithelial cells (TEC) of the kidney play an active role in the attraction of leukocytes during renal inflammatory responses, such as rejection (10,11). Upon activation with inflammatory stimuli, such as CD40L or pro-inflammatory cytokines, TEC can secrete a wide variety of chemokines and cytokines, which are responsible for the attraction and activation of infiltrating T cells, monocytes and DC.

Several chemokines have been reported to attract immature DC (12), but it seems that macrophage inflammatory protein (MIP)-3α/CCL20 is the main chemokine expressed by epithelial cells that attracts immature DC (13,14). MIP-3α/CCL20 is chemotactic for memory lymphocytes and CD1a+ dendritic cells (15–18) via binding to CCR6, which is not shared by any other known chemokine. *In vivo* expression of MIP-3α/CCL20 is rather restricted to mucosal and inflamed epithelium (15,19,20) as observed in inflamed epithelial surfaces of tonsil (18) and inflamed skin from patients with atopic dermatitis (21) and psoriasis (22). In addition, also infiltrating mononuclear cells have been shown to express MIP-3α/CCL20 (23,24).

Since it has been demonstrated that urine can be a good source for monitoring kidney abnormalities (25–28) we

determined MIP-3α/CCL20 in daily collected sera and urines of renal transplant recipients to identify whether MIP-3α/CCL20 plays a role in renal allograft rejection. Our data show that levels of MIP-3α/CCL20 found in urine significantly correlate with renal function as determined by serum creatinine concentration. Migration experiments and immunohistochemical analysis of renal biopsies suggested that TEC may have an active role in the attraction of inflammatory cells such as DC, via the expression of MIP-3α/CCL20.

Materials and Methods

Study design

Between October 1996 and October 1997, 80 consecutive patients gave informed consent and participated in a study with prospective collection of daily urinary and blood samples up to 3 months after transplantation (Table 1). Initially, standard immunosuppressive regimen after kidney transplantation was dual therapy including prednisolone (start 20 mg/day followed by tapering of 2.5 mg every fortnight until maintenance dose of 10 mg/day) and cyclosporine microemulsion (CsA). CsA was administered intravenously in a daily dose of 3 mg/kg via continuous infusion for the first 24–48 h, starting at the onset of surgery. The initial oral dose was 5 mg/kg b.i.d with subsequent dose adjustments to reach trough target levels of 300 µg/L in the first 3 months. When mycophenolate mofetil (MMF) became available in the Netherlands, immunosuppressive treatment consisted of prednisolone, CsA and MMF (triple therapy). MMF was given in a fixed dose of 2 g/day. None of the kidney transplant recipients received prophylactic treatment with poly- or monoclonal antibodies. Clinically, an acute rejection episode was defined by a rise in serum creatinine of 10% or more over baseline on at least two consecutive days in the absence of another explanation. An acute rejection episode was confirmed by biopsy unless contraindicated. Biopsies were analyzed and categorized according to the Banff '97 working classification of renal allograft pathology. Acute rejection episodes were treated with 1 g methylprednisolone (Solu-Medrol®; Pharmacy, LUMC, Leiden, the Neitherlands) intravenously for three consecutive days. Rejection was considered steroid-resistant if no stabilization or improvement occurred within 10 days after treatment with Solu-Medrol. Recurrent or steroid-resistant acute rejection episodes were treated with rabbit antithymocyte globulin (ATG) for 10 days guided by CD3 counts in peripheral blood. Overall 32% of the rejections required treatment with ATG.

Collection and storage of serum and urine samples

For the present evaluation we selected the samples of recipients of a first cadaveric kidney transplant with a percentage panel reactive antibodies un-

Table 1: Patient characteristics

Renal transplant recipients	n = 80
Simultaneous pancreas/kidney	n = 12
Single kidney	n = 68
Deceased donor	n = 60
Living (un)related donor	n = 8
Mean age (year)	49 ± 13
% female / male	40/60
Dual / Triple therapy (n)	25/43
≥1 rejection episode	n = 40
Dual therapy	n = 17
1st episode post-Tx (days)	12 ± 6
Triple therapy	n = 23
1st episode post-Tx (days)	21 ± 18

der 50%, who received the triple immunosuppressive regimen and who did not experience delayed graft function. We selected seven rejectors (R), which presented steroid-resistant rejection and seven adjacent nonrejectors (NR). Rejectors experienced their (first) biopsy-proven rejection episode at 13 ± 7 days after transplantation.

Morning urinary specimens were collected during the first 3 months post-transplantation (odd days). During admission the serum samples were taken daily and in the outpatients clinic once or twice weekly. Urinary samples were also collected from 10 healthy nontransplanted individuals. Urine (n = 625) and serum (n = 331) samples were centrifuged and stored at −20°C until analysis.

Detection of chemokines by enzyme-linked immunosorbent assays (ELISAs)

A sandwich ELISA consisting of mouse-anti-human MIP-3α/CCL20 (319F6; Schering-Plough, Dardilly, France) and peroxidase-labeled mouse-anti-human MIP-3α/CCL20 (206D9; Schering-Plough) was used for the detection of human MIP-3α/CCL20 in serum and TEC supernatants (18). Recombinant human MIP-3α/CCL20 (rMIP-3α/CCL20, R&D, Abington, UK) served as a standard (detection limit: 10 pg/mL). TMB substrate conversion was determined at 650 nm (Bio Kinetics Reader EL-312e).

Sandwich ELISAs to detect MIP-3α/CCL20 in urines were performed using the mouse-anti-human MIP3α/CCL20 antibodies 307H9 and 308D11 (Schering-Plough). Recombinant MIP-3α/CCL20 diluted in pooled urines of healthy controls served as a standard (detection limit: 20 pg/mL).

Measurement of RANTES/CCL5 in culture supernatants has been described previously (29).

Immunohistochemical staining for MIP-3α /CCL20, CCR6 and BDCA-1

Intragraft MIP-3α/CCL20 expression was investigated in paraffin-embedded 4 µm human tissue sections derived from kidneys technically not suitable for clinical transplantation (Eurotransplant, Leiden, The Netherlands; n = 5), histologically normal parts of kidneys that had been resected because of Grawitz tumors (n = 4) or percutaneous biopsies of renal allografts with moderate interstitial rejection (n = 6). Expression of CCR6 and BDCA-1 were determined in frozen 4 µm tissue sections of human kidneys technically not suitable for clinical transplantation (Eurotransplant; CCR6: n = 3), and percutaneous biopsies of renal allografts with interstitial rejection (CCR6: n = 7; BDCA-1: n = 5). Due to material restrains, MIP-3α/CCL20 and CCR6 could not be determined in tissue of the same patients.

Detection of MIP-3α/CCL20 with mouse-anti-human MIP-3α/CCL20 (5 µg/mL MAB360, R&D systems) in paraffin tissue was performed as described before (23). Antibody binding was detected with MouseEnvision-HRP (DAKO, Glostrup, Denmark) followed by VECTOR® NovaRED™ (Brunschwig, Amsterdam, The Netherlands) detection.

Prior staining for CCR6 or BDCA-1, sections were fixed with acetone and endogenous peroxidase activity was blocked with 0.1% H_2O_2 and 0.1% NaN_3 for 30 min at RT. Slides were blocked with PBS-1% BSA-5% heat-inactivated normal human serum (NHS) and subsequently incubated with mouse-anti-human CCR6 (MAB195 R&D systems) or mouse-anti-human BDCA-1 (Miltenyi Biotec GmBH, Bergish Gladbach, Germany) in PBS-1% BSA-1% NHS. Antibody binding was detected with HRP-labeled goat-anti-mouse Ig (DAKO) in PBS-1% BSA-1% NHS followed by incubation with Tyramide-FITC in tyramide buffer (NEN™ Life Science Products, Dordrecht, the Neitherlands), HRP-conjugated rabbit-anti-FITC (DAKO) and development with VECTOR® NovaRED™ (Brunschwig).

Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany), dehydrated and mounted with imsol (Klinipath, Duiven, The Netherlands). To test the specificity, the primary antibody was omitted or replaced by mouse IgG.

Cell cultures

Tubular epithelial cells: Primary human TEC were cultured from cortical tissue of human kidneys not suitable for transplantation because of anatomical reasons or from pre-transplant biopsies as described before (29). For passage of the cultures, cells were harvested by trypsinization with 0.02% (w/v) EDTA/0.05% (w/v) trypsin (Sigma St. Louis, MO). Cells were used between passage 2 and 9 of culture.

L cells: Mouse fibroblast L cells, stable transfected with human CD40L (L-CD40L), have been described previously (29). Nontransfected L cells (L-Orient) were used as negative control. L cells were irradiated at 80 Gy before use.

CD34$^+$-derived DC: CD34$^+$ hematopoietic progenitor cells were isolated from umbilical cord blood samples and used in cultures to generate immature DC as described before (30).

TEC activation experiments

TEC were stimulated in RPMI containing 10% heat-inactivated FCS and P/S in 48-well plates (Costar, Cambridge, MA) at a density of 1×10^5 TEC/mL. Irradiated L cells were added in a 1:1 ratio. Stimulation experiments were performed using the following reagents: IL-1α (1 ng/mL, Genzyme, Uden, The Netherlands), IL-4 (5 ng/mL, Peprotech, Rocky Hill, NJ), IFN-γ (50 ng/mL, Peprotech), TNFα (500 U/mL, Peprotech), IL-1 receptor antagonist (IL-1RA, 120 ng/mL; kindly provided by Dr. A. Steinkasserer, University of Oxford, Oxford, UK) and neutralizing anti-CD40L antibodies (LL48, 10 μg/mL, Schering-Plough).

TEC activation for the generation of supernatants required for migration assays was performed in RPMI containing 2% heat-inactivated FCS and P/S. It was not possible to use supernatants of TEC stimulated with CD40L transfected L cells, since mouse L cells produce an unknown factor that influences the migration of human CD34$^+$-derived DC (data not shown).

Migration assay

Migration of CD34$^+$-derived DC was investigated using Transwell (5 μm pore, Costar). Six hundred microliter of rMIP-3α/CCL20 (500 ng/mL) or serial dilutions of TEC supernatants were added to 24-well plates (Costar). Day-6 CD34$^+$-derived DC precursors ($3–5 \times 10^5$/well) were added to transwell inserts and incubated for 1 h at 37°C. Transmigrated cells were stained for FACS analysis to count and to differentiate between CD1a$^+$ and CD14$^+$ DC precursors. Results are expressed as index of migration, which represents the ratio between the number of migrated cells upon rMIP-3α/CCL20 or TEC supernatant and the number of cells migrated upon control medium.

Statistical analysis

MIP-3α/CCL20 present in patients sera and urine were analyzed for statistical significance with GraphPad Prism 4.01 software (GraphPad Software, Inc., San Diego, CA) using analysis of variance (ANOVA) followed by unpaired Student's t-tests. Spearman correlation coefficient was calculated to investigate statistical relations.

Concentration of chemokines in TEC cultures is presented as mean $\pm$ SD from representative experiments. Differences in MIP-3α/CCL20 production between stimulated and nonstimulated TEC cultures were statistically tested using Student's t-test for paired samples of the composite experiments. p-values were considered statistically significant when p was below 0.05.

Results

MIP-3α/CCL20 concentration in urine correlates with renal function

Urine and serum samples of renal transplant recipients were analyzed for the presence of MIP-3α/CCL20. Urine MIP-3α/CCL20 concentrations were determined in 615 urine samples of 14 patients (seven rejectors, seven non-rejectors), serum MIP-3α/CCL20 concentrations were investigated in nine patients (five rejectors; four nonrejectors). Serum MIP-3α/CCL20 concentrations did not significantly differ between rejectors and nonrejectors (NR: 26 $\pm$ 3 pg/mL, R: 31 $\pm$ 2 pg/mL; Figure 1A) and were comparable with serum concentrations observed in healthy individuals (31). Levels of urinary MIP-3α/CCL20 levels varied greatly among the transplant recipients, ranging from ≤20 pg/mL to 3280 pg/mL, whereas no MIP-3α/CCL20 could be detected in healthy individuals (Figure 1B).

The concentrations of MIP-3α/CCL20 found in urine during the first 3 months following transplantation significantly differed between the two groups (Figure 1B). Immediately after transplantation (days 1–7), 80% of renal transplant recipients showed elevated urinary MIP-3α/CCL20 levels that did not differ between nonrejectors and rejectors (NR: 560 $\pm$ 169 pg/mL; R: 458±97 pg/mL, p = 0.59), and declined when renal function improved (Figure 2). Especially after the first week post-transplantation, rejectors showed ≥2-fold more MIP-3α/CCL20 in urine than nonrejectors (R: 188 $\pm$ 29 pg/mL; NR: 66 $\pm$ 11 pg/mL). The level of urine MIP-3α/CCL20 significantly correlated with the level of serum MIP-3α/CCL20 (Figure 1C) as well as with renal function as determined by serum creatinine levels (Figure 1D). No significant correlation could be observed between serum MIP-3α/CCL20 and renal function (Figure 1E).

Among the rejectors, seven patients underwent in total eight acute rejection episodes. In seven of eight rejection episodes, urine MIP-3α/CCL20 concentrations increased during rejection (Figure 2A; peak values: 34–3280 pg/mL). Also serum MIP-3α/CCL20 concentrations increased during four of six rejection episodes (Figure 2B). During clinically stable periods with normal renal function, patients demonstrated continuously low levels of urine MIP-3α/CCL20 concentrations (Figure 2C).

MIP3α/CCL20 expression is increased in renal tissue with rejection and can be responsible for the presence of CCR6$^+$ infiltrating cells

Then, paraffin-embedded renal tissue was examined for the expression of MIP-3α/CCL20. In all cases of rejection (six of six), expression of MIP-3α/CCL20 was found in many tubuli (Figure 3A, B). Although leukocytes positively staining for MIP-3α/CCL20 were also observed in biopsies
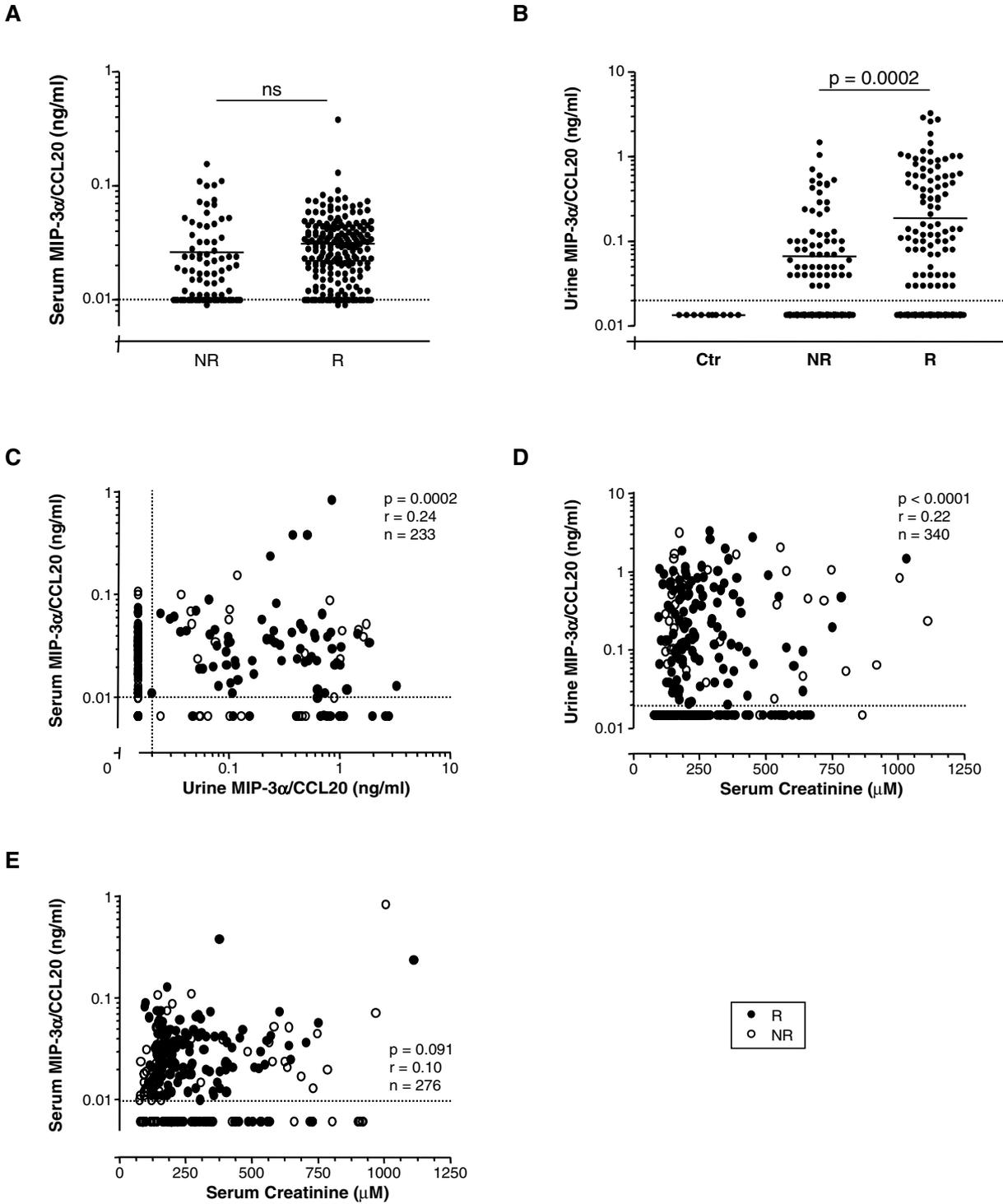
Figure 1: Urinary MIP-3α /CCL20 levels correlate with renal graft function. AB. Serum (A) and urine (B) MIP3α/CCL20 concentrations were determined in patients with (R) or without rejection (NR) episodes in the first 3 months post-transplantation. All odd days were included except for the first week after transplantation. MIP-3α/CCL20 concentrations were also determined in healthy controls (n = 10). Data were analyzed using ANOVA and Student's t-tests for unpaired samples. CDE. Urine MIP-3α/CCL20, serum MIP-3α/CCL20 and serum creatinine were determined in patients with (R; ●) or without rejection (NR; ○) episodes during 3 months post-transplantation. Spearman correlation coefficients were determined of the overall patient group to calculate statistical relations.
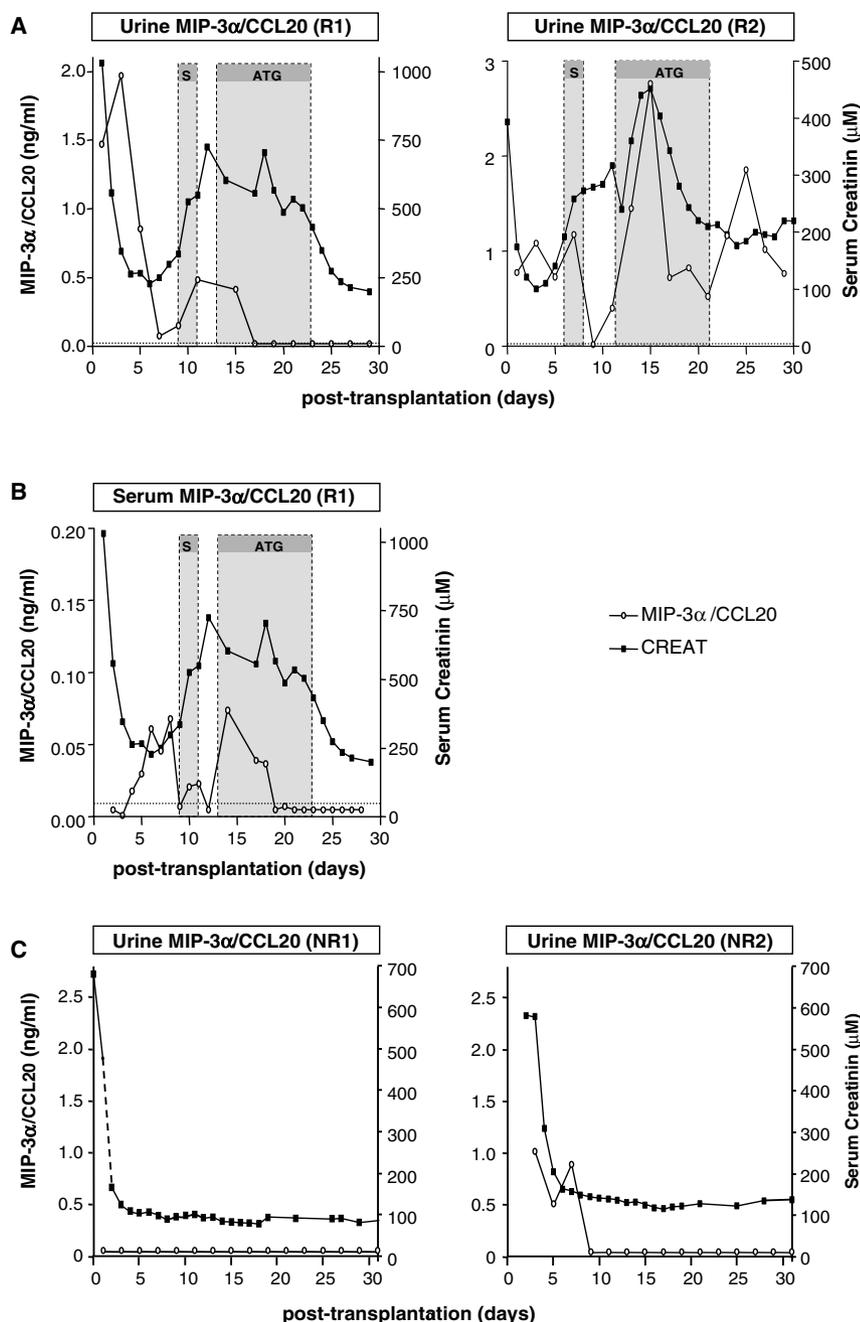
Figure 2: MIP-3α/CCL20 in serum and urine of renal transplant recipients. Urinary as well as serum MIP-3α/CCL20 levels and serum creatinine of two representative renal transplant recipients with biopsy-confirmed acute rejection episodes (R1 and R2)(AB) and two representative renal transplant recipients with stable graft function (NR1 and NR2)(C). Rejection treatment with Solu-Medrol® (S) and ATG are indicated by the gray bars.

with extensive cellular infiltration (four of six), staining was predominantly localized to renal tubular epithelium. Detection of MIP-3α/CCL20 in control renal tissue varied between negative and very weak tubular staining (Figure 3C) (Table 2).

The presence of MIP-3α/CCL20 during renal rejection episodes may predict infiltration of CCR6^{+} cells. Therefore, the presence of CCR6^{+} cells was investigated in frozen tissue sections with or without rejection. CCR6 could not be observed on control renal tissue (zero of three; data not shown). In five of seven cases with rejection, CCR6 expression was detected on part of the infiltrating cells (Figure 3D). TEC were negative, and a few CCR6^{+} cells could be observed within glomeruli.

Since it has been shown that MIP-3α/CCL20 is the main chemokine expressed by epithelial cells that attracts immature DC, we questioned whether DC are present within the infiltrate. The expression of BDCA-1/CD1c as myeloid DC marker was assessed in renal tissue with rejection and was found to be positive in all cases
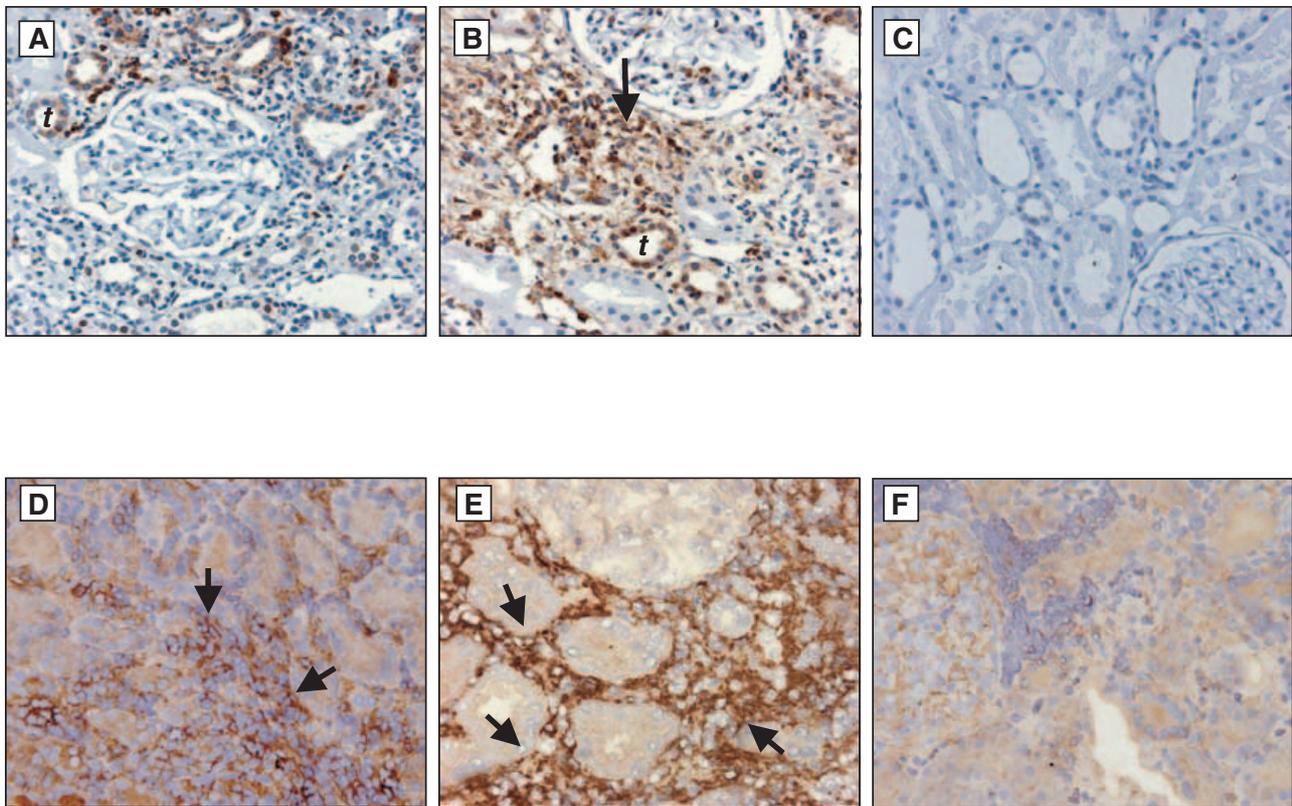
Figure 3: Expression of MIP-3α /CCL20, CCR6 and BDCA-1 in renal tissue during rejection. Sections of renal biopsies with rejection (AB) and normal renal tissue (C) were investigated for the expression of MIP-3α/CCL20 as described in *Materials and Methods*. Rejection biopsies showed tubular MIP-3α/CCL20 expression (indicated by a *'t'*) (AB), but also sometimes infiltrating cells (arrow) stained positive for the chemokine (B). Normal kidneys demonstrated weak or negative (C) staining for MIP-3α/CCL20. CCR6 (D) and BDCA-1 (E) expression by infiltrating cells (arrows) in renal biopsies with rejection. Renal biopsies with rejection stained with mouse IgG were negative (F).

Table 2: MIP-3α/CCL20 expression in human kidney sections

Tissue	Banff grading	Staining Tubuli	Staining Infiltrate	Infiltrate present
C 1[a]	Normal	−	−	N
C 2[a]	Normal	±	−	N
C 3[a]	Normal	−	−	N
C 4[a]	Normal	−	−	N
C 5[a]	Normal	±	−	N
C 6[b]	Normal	±	−	N
C 7[b]	Normal	±	−	N
C 8[b]	Normal	−	−	N
C 9[b]	Normal	−	−	N
R 1	IA	+	−	Y
R 2	IA	+	+	Y
R 3	IB	+	+	Y
R 4	IB	+	+	Y
R 5	IIA	+	−	Y
R 6	IIB	++	++	Y

C = control renal tissue (n = 9); [a]Normal kidneys not suitable for transplantation (n = 5); [b]Normal parts of tumor kidney (n = 4); R = renal tissue with rejection (n = 6).

(Figure 3E). Sections stained with mouse IgG were negative (Figure 3F).

Regulation of MIP3α /CCL20 production by TEC in vitro

Since renal tubuli expressed MIP-3α/CCL20 *in situ*, the regulation of MIP-3α/CCL20 production by TEC *in vitro* was investigated in detail. Nonstimulated TEC produced little amounts of the chemokine. Stimulation of TEC with CD40L significantly increased the production of MIP-3α/CCL20 by TEC over baseline levels and the combination with IL-1α even synergistically increased the production of MIP-3α/CCL20 (Figure 4). Experiments using IL-1RA completely prevented IL-1α-induced MIP-3α/CCL20 productions (Figure 4A). Similarly, neutralizing anti-CD40L antibodies completely blocked the CD40L-induced effects, which was not observed with control antibodies (Figure 4B).

Titration of IL-1α revealed that MIP-3α/CCL20 production by TEC is regulated in a dose-dependent manner, which reached a plateau when using 5 ng/mL IL-1α (Figure 5A). Combined stimulation of TEC showed that both TNFα and CD40L in combination with IL-1α synergistically
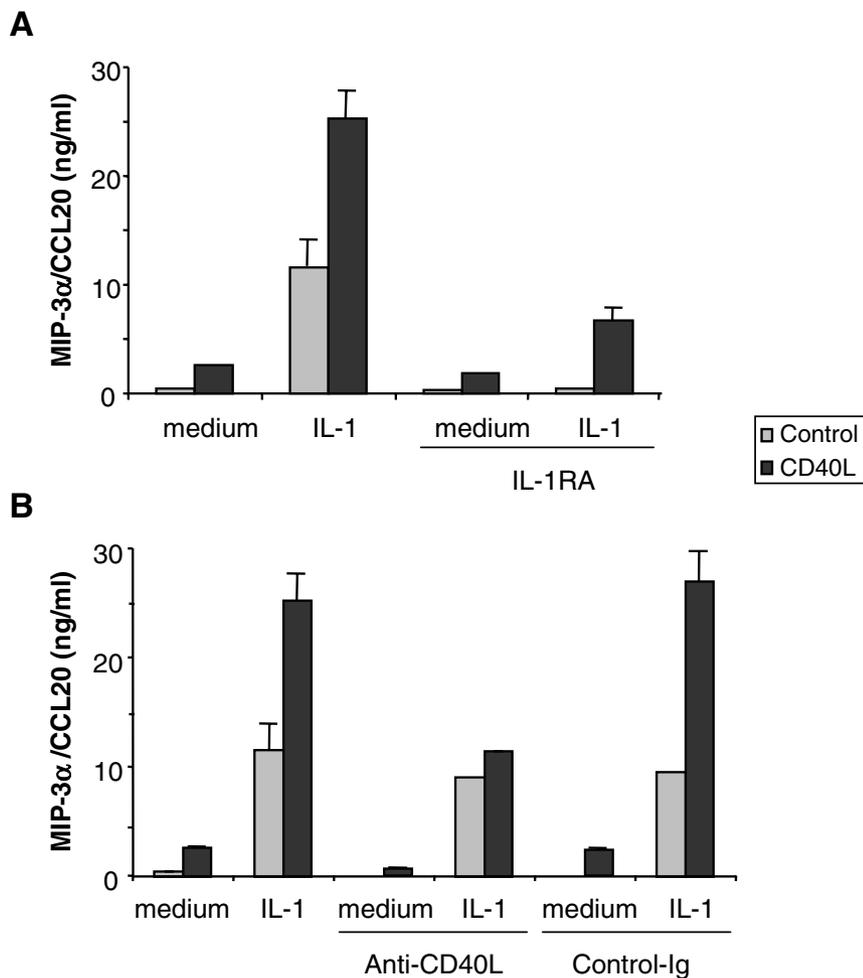
A

B

Figure 4: IL-1α and CD40L synergistically increase the production of MIP-3α/CCL20 by TEC. Forty-eight-well plates were used for cocultures of TEC with either L-Orient or L-CD40L in the presence of IL-1α. IL-1RA (A) and LL48 (B) were used for neutralizing the effects of IL-1α and CD40L, respectively. After 48 h of incubation, supernatants were harvested and analyzed for the concentration of MIP-3α/CCL20. Mean (±SD) MIP-3α/CCL20 productions presented are derived from duplicate cultures. Data shown are representative for three independent experiments with different primary TEC lines.

increased the production of MIP-3α/CCL20, which was also dose-dependently influenced by IL-1α. Kinetic studies showed that IL-1α- and CD40L-induced MIP-3α/CCL20 production increased in time, which reached its maximum between 72–96 h of incubation (Figure 5B). In contrast, both IFNγ and IL-4 showed clear inhibitory effects on IL-1- and CD40L-induced MIP-3α/CCL20 production by TEC (Figure 6A, B). Interestingly, these combinations were previously shown to increase the production of the chemokine RANTES/CCL5 (32) as confirmed in the same supernatants used for MIP-3α/CCL20 measurements (Figure 6C, D).

In conclusion, these results show the unique regulation of MIP-3α/CCL20 by TEC upon culture in the presence of different cytokines and CD40L.

Attraction of CD1a+ CD34-derived DC by TEC supernatants is dependent on MIP-3α/CCL20

We analyzed the ability of MIP-3α/CCL20 produced by activated TEC to attract human DC. Addition of supernatant of nonstimulated TEC cultures to the lower part of a transwell system clearly induced migration of both CD14+ and CD1a+ DC precursors (Table 3, Figure 7A) suggest-

ing that TEC constitutively produce chemotactic factors *in vitro* that induce migration of CD34+-derived DC precursors. Supernatants derived from TEC stimulated with either IL-1α or TNFα dose dependently increased the migration of CD1a+ DC precursors as compared with non-stimulated TEC, but these conditions were less potent than the conditions with supernatants derived from TEC stimulated with both IL-1α and TNFα (Table 3, Figure 7B). Interestingly, using supernatant of activated TEC instead of resting TEC did not further increase the migration of CD14+ DC precursors (Table 3). Control conditions in which IL-1α, TNFα or IL-1α/TNFα were added to the lower part of the transwell system did not show any effect on the migration of DC (data not shown).

Both the migration of CD1a+ cells in response to recombinant MIP-3α/CCL20 and the migration in response to supernatant derived from activated TEC were strongly reduced by the use of anti-MIP-3α/CCL20 antibodies, but not by control antibodies (Figure 7C). The migration in response to supernatant derived from resting TEC was not affected by either anti-MIP3α/CCL20 or control antibodies. These results show that MIP-3α/CCL20 produced by TEC is

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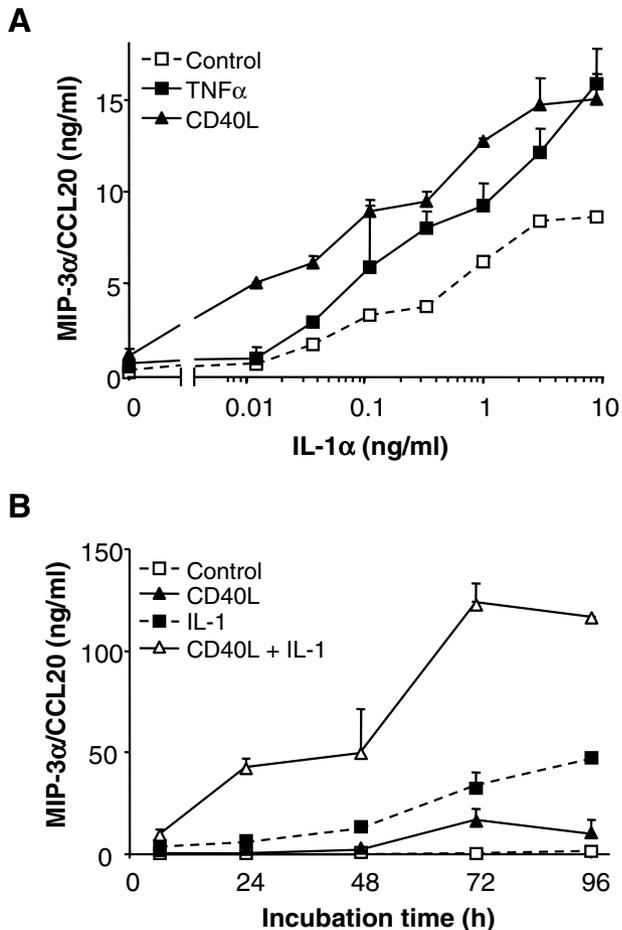

B

Figure 5: IL-1α and either CD40L or TNFα synergistically enhance MIP-3α /CCL20 production by TEC in a dose- and time-dependent manner. A. Forty-eight-well plates were used for co-cultures of TEC with L-Orient, either with or without TNFα (500 U/mL), or L-CD40L in the presence of different concentrations IL-1α. After 48 h of incubation, supernatants were harvested and analyzed for the concentration of MIP-3α/CCL20. Mean (±SD) MIP-3α/CCL20 productions presented are derived from duplicate cultures. Data shown are representative for two independent experiments with different primary TEC lines. B. Forty-eight-well plates were used for cocultures of TEC with either L-Orient or L-CD40L in the presence of IL-1α. Supernatants were harvested at different time points and analyzed for the concentration of MIP-3α/CCL20. Mean (±SD) MIP-3α/CCL20 productions presented are derived from duplicate cultures. Data shown are representative for four independent experiments with different primary TEC lines.

biologically active and can be considered the chemokine responsible for the migration of CD1a^{+} DC induced by activated TEC.

Discussion

Leukocyte trafficking from peripheral blood into the tubulo-interstitial area of the kidney is a hallmark of almost any

kind of renal inflammatory disease, including allograft rejection (1,11). Rejection is triggered by presentation of allo-antigens to recipient T cells that initiates the expansion and mobilization of a complex cellular immune response. Increasing evidence emphasizes the contribution of the indirect pathway of antigen recognition to the induction of allograft rejection (5,33). This means that infiltrating recipient DC precursors or immature DC capture allo-antigen and present it as mature DC to recipient T cells. The regulation of DC trafficking involves the participation of selective chemokines. MIP-3α/CCL20 is a chemokine that is mainly expressed in inflamed epithelium *in vivo* and that can strongly attract immature DC to the site of inflammation (12). Previous studies showed the expression of MIP-3α/CCL20 in appendix, tonsil, liver, placenta, thymus, lymph nodes, skin, lung and small intestine (13,18–20,22,34). The present study demonstrates for the first time that both *in vitro* and *in vivo* TEC can be a major source of MIP-3α/CCL20 during renal allograft rejection and that urine of renal transplant recipients contain elevated levels of MIP-3α/CCL20 during rejection episodes.

Urinary MIP-3α/CCL20 levels significantly correlated with renal function as assessed by serum creatinine. Elevated urinary MIP-3α/CCL20 levels found during the first week after transplantation in both patient groups probably demonstrates the early aspecific response following the surgical procedure that may lead to the attraction of leukocytes (6,35). Interestingly, renal transplant recipients with rejection episodes showed significantly increased urinary MIP-3α/CCL20 concentrations within the first 3 months after transplantation compared to patients without rejection episodes. Both immunohistochemical stainings of renal biopsies as well as our *in vitro* studies with primary human TEC demonstrated that TEC might be responsible for the increased urinary MIP-3α/CCL20 levels during rejection episodes. Urinary MIP-3α/CCL20 peak levels did not precede increased serum creatinine levels. Although, it was found that in clinically stable periods MIP-3α/CCL20 could not be detected in patient urines, some patients demonstrated MIP-3α/CCL20 in the absence of renal allograft rejection, which could correspond to other inflammatory responses such as urinary tract and CMV infections. However, elevated MIP-3α/CCL20 levels did not precede rejection episodes and hence the present study does not indicate that MIP-3α/CCL20 expression induced by pro-inflammatory signals other than rejection triggers renal allograft rejection. Thus, MIP-3α/CCL20 cannot be superior to creatinine as marker for the occurrence of renal allograft rejection, but it may be a helpful chemokine to monitor local inflammation.

In vitro activation of TEC with IL-1, TNFα or CD40L to mimic the *in vivo* situation during renal inflammation (36,37) increased the production of MIP-3α/CCL20. Like synoviocytes, the major source of MIP-3α/CCL20 in rheumatoid arthritis (38), TEC reduced their MIP-3α/CCL20 production in the presence of IL-4. Also IFNγ inhibited the

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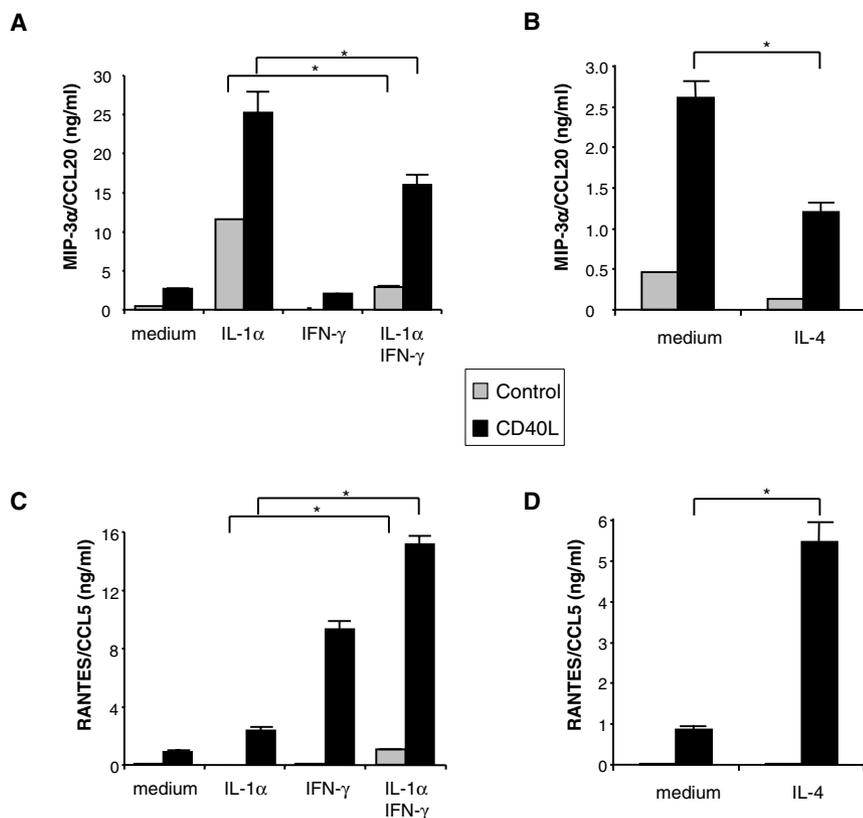

B

C

D

Figure 6: Production of RANTES/ CCL5 and MIP-3α /CCL20 by TEC is differently regulated by IFNγ and IL-4. Forty-eight-well plates were used for cocultures of TEC with either L-Orient or L-CD40L in the presence of IL-1α (1 ng/mL), IFNγ (50 ng/mL), IL-1α/IFNγ or IL-4 (5 ng/mL). After 48 h of incubation, supernatants were harvested and analyzed for the concentration of MIP-3α/CCL20 (AB) and RANTES/CCL5 (CD). Mean (±SD) chemokine productions presented are derived from duplicate cultures. Data shown are representative for four independent experiments with different primary TEC lines. *Significant decrease (p < 0.05).

Table 3: TEC supernatants induce migration of day-6 CD34^{+}-derived DC subsets

		Migration index		MIP-3α/CCL20 (ng/mL)
		CD1a^{+}	CD14^{+}	
	Medium	1.0	1.0	<0.01
TEC stimulus	Medium	3.4 (1.4)	7.6 (2.4)	1.4 (0.1)
	IL-1α	7.0 (2.1)	6.3 (0.2)	9.8 (0.4)
	TNFα	6.9 (0.8)	5.9 (0.6)	7.1 (0.2)
	IL-1α + TNFα	10.6 (2.0)	5.4 (1.0)	53.8 (2.3)

Transwell migration of day-6 CD34^{+}-derived DC precursors in response to medium or supernatants (1:2 dilution) derived from TEC cultured in medium, IL-1α, TNFα or the combination IL-1α + TNFα. Migrated cells were harvested and stained for CD14 and CD1a and subsequently analyzed and counted using flow cytometry. Results are expressed as mean (SD) index of migration (= ratio between number of migrated cells upon TEC supernatant and cells migrated upon normal medium) of two independent experiments showing the effect of TEC supernatant on the migration of CD1a^{+} and CD14^{+} cells. The mean (SD) production of MIP-3α/CCL20 by TEC was determined by specific ELISA.

MIP-3α/CCL20 production by TEC, which is in contrast to the stimulatory effect of IFNγ on keratinocytes, the major source of MIP-3α/CCL20 and responsible for the attraction of DC in inflammatory skin diseases (21,22). Furthermore, IL-4 and IFNγ enhanced the production of RANTES/CCL5 by the same TEC demonstrating that the signals delivered by infiltrating cells to resident cells are distinct for the regulation of different chemokines as well as different cell types.

Supernatant of *in vitro* activated TEC was highly potent in the attraction of both CD14^{+}- and CD1a^{+} CD34^{+}-derived

DC precursors. The migration of CD1a^{+} DC precursors was strongly inhibited by neutralizing MIP-3α/CCL20 antibodies showing that TEC produce biologically active MIP-3α/CCL20, which at the same time seems to be the major CD1a^{+} DC attractant produced by TEC.

Although MIP-3α/CCL20 appears as the most powerful chemokine in inducing migration of immature CD34^{+}-derived DC (9,14,20,39), the DC population responding to MIP-3α/CCL20 *in vivo* has not yet been fully identified. Langerhans cells or their direct precursors represent potential target populations, whereas immature CD11c^{+} blood

Figure 7: MIP-3α /CCL20 produced by TEC specifically attracts CD1a+ DC precursors. A. Transwell migration of day-6 CD34+-derived DC precursors in response to medium, or supernatants (1:2 dilution) from either resting (medium) or activated (IL-1α + TNFα) TEC is shown. Migrated cells were harvested and stained for CD14 and CD1a and subsequently analyzed and counted using flow cytometry. Since flow cytometric analysis was performed with fixed time points, the number of dots present in the right panel of the figure directly corresponds with the number of migrated cells as described in *Materials and Methods*. Numbers shown in the dotplots represent the percentage of CD1a+ or CD14+ cells within the migrated cell population. B. Transwell migration of CD1a+ day-6 CD34+-derived DC precursors in response to supernatants derived from TEC cultured in the presence of medium, IL-1α, TNFα or the combination IL-1α + TNFα. Supernatants were used in 1:2 (50%), 1:5 (20%) and 1:10 (10%) dilutions. Migration indices were calculated as described in materials and methods. C. Transwell migration of CD1a+ day-6 CD34+-derived DC precursors in response to rMIP-3α/CCL20 (500 ng/mL) or supernatants (1:2 dilution) derived from either resting (medium) or activated (IL-1α + TNFα) TEC in the presence of mouse-anti-human MIP-3α/CCL20 (10 μg/mL 206D9), control mouse-IgG1 (10 μg/mL) or medium alone. Migration indices shown in B and C are representative for three independent experiments with different primary TEC lines.
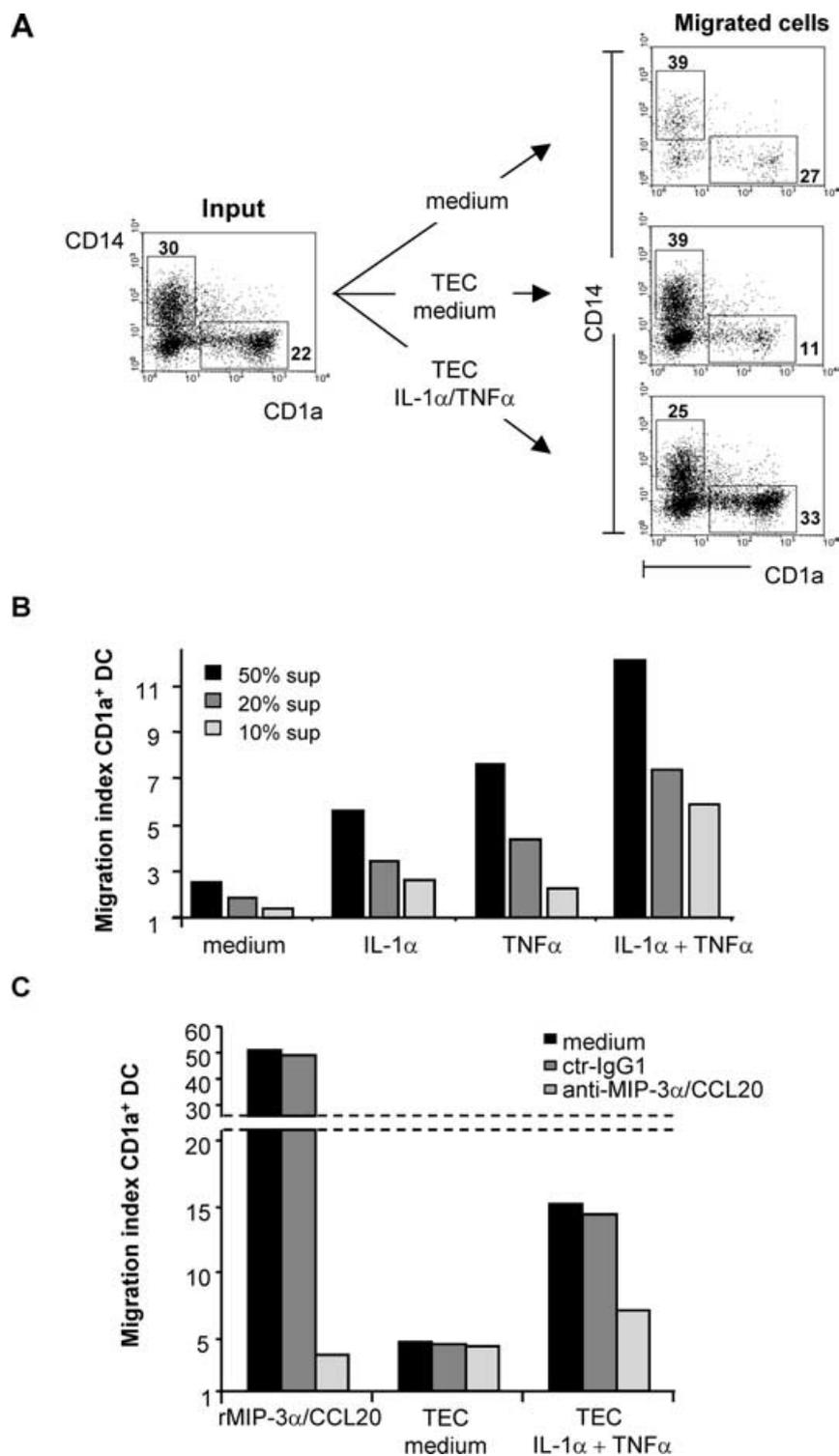
DC and monocyte-derived DC are unresponsive in *in vitro* migration assays. However, the latter cell type does respond to MIP-3α/CCL20 in the presence of TGF-β1 (40), which is known to be expressed during renal allograft rejection (41).

It has been shown that MIP-3α/CCL20 attracts mostly immature DC (42), but in accordance with the expression of the only known MIP-3α/CCL20 receptor CCR6, CD4+ T cells, B cells, NK cells and neutrophils (43–47) can also be attracted by MIP-3α/CCL20 to the inflammatory site.

Inflammatory cells present in biopsies of kidneys with rejection represent among other cell types, such as T cells, also many BDCA-1/CD1c^{+} DC, which are closely related to CD1a^{+} DC (48–50). The infiltrate contains CCR6^{+} cells and BDCA-1/CD1c^{+} cells in about similar numbers and locations. However, due to technical restrictions colocalization studies could not be performed and therefore we can only speculate that at least part of the CCR6^{+} leukocytes are DC. Whether CCR6^{+} graft-infiltrating cells are DC or other cell types will be part of future research. Because the stainings for MIP-3α/CCL20 and CCR6 had to be performed on paraffin-embedded tissue and cryosections, respectively, it was impossible to link the amount of MIP-3α/CCL20 expression to the amount of infiltrating CCR6^{+} cells. Although RANTES/CCL5 appears to be the major chemokine responsible for the accumulation of CD4^{+} T cells (51,52), the chemotactic effect of MIP-3α/CCL20 on T cells might contribute to the damage of the transplanted graft. Studies using transplantation models with disrupted chemokine receptors, receptor antagonists and blocking antireceptor antibodies have already shown the therapeutic significance of the interference with chemokine receptors and their ligands (53). It has been shown that CCR6-deficient mice have impaired cellular and humoral immune responses (54,55), but whether this might prolong allograft survival remains to be elucidated.

In conclusion, our observations suggest that MIP-3α/CCL20 can play a role in the amplification of the immune response during renal allograft rejection. Given the large number of chemokines coexpressed in inflamed renal tissue and the diverse biological actions of MIP-3α/CCL20 on other cell types next to DC, a more detailed analysis of the type of cells specifically attracted by MIP-3α/CCL20 and their role in the initiation and amplification of renal allograft rejection is required before directed interference with MIP-3α/CCL20-induced DC infiltration can be considered as a therapeutic option.

Acknowledgments

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