Interleukin-17 and CD40-Ligand Synergistically Enhance Cytokine and Chemokine Production by Renal Epithelial Cells

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Abstract. Renal allograft rejection is characterized by an influx of inflammatory cells. Interaction between infiltrating T cells and resident parenchymal cells might play an important role in the ongoing inflammatory response. The present study demonstrates that CD40L, a product of activated T cells, is locally expressed in kidneys undergoing rejection. Furthermore, during rejection, CD40 expression not only is present on most graft infiltrating cells but also is increased on resident tubular epithelial cells (TEC). To obtain more detailed insight in the consequences of T cell/TEC interaction, we analyzed the production of chemokines, including interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) and regulated upon activation, normal T cell expressed and secreted (RANTES), and the production of IL-6 by cultured human primary TEC in response to activation with CD40L *in vitro*. In addition, we studied the interaction with IL-17, a T-cell–specific cytokine previously demonstrated to be present during renal allograft rejection. The results, obtained by enzyme-linked immunosorbent assay, indicate that simultaneous activation of TEC with IL-17 and CD40L synergistically enhances production of IL-6 (2.1-fold higher than sum of single stimulations) and the chemokines IL-8 (15-fold) and RANTES (5.8-fold) as demonstrated by statistical analysis ($P < 0.05$), whereas effects on MCP-1 (1.4-fold) are additive. Part of the synergy can be explained by increased CD40 expression on TEC upon IL-17 stimulation. The synergy is not unique for TEC, because similar responses were found with human synoviocytes and a foreskin fibroblast cell line (FS4). Stimulation of TEC with CD40L results in activation of NF-κB and induction of cytokine production by IL-17 and CD40L is prevented by addition of the NF-κB inhibitor pyrrolidine dithiocarbamate. These data suggest an important role for T cells in renal allograft rejection by acting on parenchymal cells via both soluble mediators and direct cellular contact.

Renal allograft rejection is characterized by cellular infiltration and tissue destruction, in which graft infiltrating alloreactive T lymphocytes play a critical role (1). Cytokines, secreted by infiltrating activated T cells, contribute to the initiation and maintenance of this inflammatory response. In accordance, elevated levels of different cytokines have been demonstrated during rejection episodes (2,3). It has been suggested that resident parenchymal cells, such as tubular epithelial cells (TEC), play an active role in renal inflammation. TEC are a potential source of various inflammatory mediators, including complement components and cytokines. More important, both *in vitro* and *in vivo* TEC can produce a wide variety of chemokines, including interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), regulated upon activation, normal T cell expressed and secreted (RANTES), and ENA-78

(4,5). Because most of these chemokines are specific for a certain cell type, the ratio and concentration of the different chemokines produced will determine the composition of the cellular infiltrate (6). Therefore, it is important to acquire insight in the production and regulation of chemokines and cytokines at the local site and which signals are responsible for the link between specific immune responses and nonspecific inflammatory responses.

In recent years, it has become clear that CD40-CD40L interactions are potentially involved in the regulation of inflammatory responses. CD40 expression and function has been studied extensively on B lymphocytes and other antigen-presenting cells (monocytes and dendritic cells) (7). In addition, CD40 is functionally expressed on endothelial cells, fibroblasts, and epithelial cells (8). *In vitro* activation of these cells results in an increased inflammatory potential, such as expression of adhesion molecules and production of various cytokines. Concerning the kidney, TEC express CD40, and *in vitro* activation increases chemokine production (4). In addition, co-activation with IL-4 specifically increases the CD40L-induced RANTES production (9). Blockade of the CD40-CD40L pathway prevents chronic inflammation in various animal models, including glomerulonephritis (7,10). Moreover, treatment with anti-CD40L antibodies interferes with acute and chronic allograft rejection of different organs, including the kidney (11–13).

Recently, an IL-17 antagonist (mIL-17R:Fc fusion protein) has been presented as an additional tool to prolong allograft survival (14). IL-17 is a proinflammatory cytokine that is specifically produced by activated T cells (15–17). IL-17–producing cells were demonstrated in rejected human kidney allografts, either by immunofluorescence staining (18) or by reverse transcriptase-PCR (RT-PCR) analysis (19). The biologic activities of IL-17 are mostly restricted to the activation of nonhematopoietic cells. IL-17 stimulates the production of IL-6 and IL-8 by foreskin fibroblasts and synoviocytes, granulocyte colony-stimulating factor and prostaglandin E_2 by synoviocytes, and IL-6 by epithelial and endothelial cells (15). Moreover, *in vitro* activation of renal TEC with IL-17 increases the production of IL-6, IL-8, and MCP-1 (18).

It was the purpose of the present study to explore the hypothesis that infiltrating T cells contribute to the renal inflammatory process by activating resident TEC. This study focuses on the interaction between CD40L/CD40 and IL-17/IL-17R, because (*1*) IL-17 and CD40L both are able to stimulate TEC, (*2*) IL-17 and CD40L are produced or expressed by activated T cells, and (*3*) neutralization of IL-17 or CD40L interferes with allograft rejection. We demonstrated that during renal allograft rejection, local expression of both CD40 and CD40L is strongly increased. *In vitro*, combined stimulation of TEC with IL-17 and CD40L results in a strong synergistic stimulation of chemokine and cytokine production, underlining the potential role of activated T cells in orchestrating the local inflammatory response.

Materials and Methods
Immunofluorescence Staining

The following human renal tissues were used: two normal kidneys not suitable for transplantation, the normal part of two tumor kidneys, three different pretransplant biopsies, five percutaneous biopsies of renal allografts with rejection, and three renal transplantectomies with rejection. Details of the patient material have been given in a previous study (18).

Frozen tissue sections of 4 μm were fixed for 10 min in water-free acetone at room temperature. After washing and blocking endogenous peroxide with 0.03% H_2O_2, sections were incubated with 20 μg/ml mAb89 (msIgG1 anti-human CD40), 20 μg/ml LL56 (msIgG2a anti-human CD40L), or 20 μg/ml total msIgG in incubation buffer, containing 0.1 M Tris/HCl, 0.15 M NaCl with 0.5% Boehringer blocking reagent (Boehringer Mannheim, Mannheim, Germany) for 45 min. After sections were washed with phosphate-buffered saline (PBS), they were incubated in 1:50 diluted horseradish peroxidase-labeled rabbit anti-mouse Ig (DAKO, Glostrup, Denmark) in incubation buffer with 10% normal human serum for 30 min. Slides were washed with PBS/0.05% Tween 20 (pH 7.4; OPG Farma, Utrecht, The Netherlands), followed by washing in PBS, and incubated with 0.5 mg/ml tyramide-fluos (NEN-Dupont, Dordrecht, The Netherlands) in 0.2 M Tris/HCl, 10 mM imidazole (pH 8.8; Sigma Chemical Co., St. Louis, MO) with 0.01% H_2O_2, for 30 min (20). After slides were washed in PBS/Tween and PBS, they were mounted with 0.1% (wt/vol) P-phenylenediamine (Sigma) in glycerol. Photographs were taken using Kodak TX-400 films (San Diego, CA) on a Leitz microscope (Weesp, The Netherlands) with a 4 mmBG 38 + 5 mmBG 12 filter for FITC.

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 pretransplant biopsies (4). TEC monolayers were cultured on a matrix of collagen type I (Sigma) and heat-inactivated fetal calf serum (FCS; Gibco Life Technologies/Life Technologies Inc., Gaithersburg, MD), in a selective medium, consisting of a 1:1 ratio of Dulbecco's modified Eagle's medium and Ham F12 (both from Seromed Biochem KG, Berlin, Germany), supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), tri-iodothyronine (40 pg/ml), and epidermal growth factor (10 ng/ml) (all from Sigma). Morphologic appearance and immunofluorescence staining confirmed specific outgrowth of TEC (4). Cells were used between passages 2 and 9 of culture.

Foreskin Fibroblasts. The foreskin fibroblast cell line FS4 (21) was cultured in Iscove's modified Dulbecco's medium (IMDM) (Life Technologies), 10% heat-inactivated FCS, and penicillin/streptomycin.

Synoviocytes. Primary synoviocytes were cultured from the synovial tissue of patients with rheumatoid arthritis undergoing total or partial knee or elbow surgery (22). Synoviocytes were cultured in IMDM, 20% heat-inactivated FCS, and penicillin/streptomycin. Primary synoviocytes were used between passages 2 and 7 of culture.

L Cells. Mouse fibroblast L cells, stable transfected with human CD40L (L-CD40L) and parental nontransfected L cells (L-Orient), were cultured in IMDM supplemented with 10% heat-inactivated FCS and penicillin/streptomycin (23). For passage of the different cell cultures, cells were harvested by trypsinization (0.02% [wt/vol] ethylenediaminetetraacetate/0.05% [wt/vol] trypsin in PBS; Sigma).

Culture and Activation Experiments

For induction of cytokine production, the cells were trypsinized and seeded in a final concentration of 5×10^4 (TEC), 4×10^4 (FS4), or 3×10^4 (synoviocytes) cells/well in 48-well plates. CD40 activation was achieved by incubation with a chimeric protein containing the extracellular domains of mouse CD8α and human CD40L (CD8αCD40L) (23). The original construct was recloned into an expression vector containing the hygromycin resistance gene, and stably transfected Chinese hamster ovary cells producing the chimeric protein were generated. Alternatively, TEC were cocultured with irradiated (80 Gy) CD40L-transfected L cells in a 1:1 ratio (4). Recombinant human IL-17 (50 ng/ml, unless indicated otherwise) was used for activation (15). Neutralizing antibodies against CD40L (LL48; IgG1) or IL-17 (mAb5; IgG1) were used at 10 μg/ml. Pyrrolidine dithiocarbamate (PDTC; Sigma) was used as an inhibitor of NF-κB activation.

Determination of Cytokines and Chemokines

The concentration of cytokines and chemokines in supernatants of cultured cells was measured by enzyme-linked immunosorbent assay (ELISA). Details on the measurement of IL-6, IL-8, MCP-1, and RANTES have been described in previous reports (4,18).

Fluorescence-Activated Cell Sorter Analysis

For fluorescence-activated cell sorter (FACS) experiments, cells were harvested by brief trypsinization to prevent proteolysis of surface receptors. After the cells were washed with FACS buffer (1% BSA, 1% decomplemented normal human serum, and 0.02% sodium azide in PBS), 2×10^5 cells were incubated with 10 μg/ml mAb89 (αCD40) or 10 μg/ml mAb M203 (αIL-17R; a kind gift of Dr. M.

Kubin, Immunex, Seattle, WA) (24). After incubation for 45 min at 4°C, the cells were washed twice with FACS buffer and subsequently incubated with goat anti-mouse Ig-PE (DAKO) for 30 min at 4°C. Finally, the cells were washed, fixed with 1% paraformaldehyde, and assessed for fluorescence using a FACScan. Data analysis was performed using LYSIS-II software (Becton Dickinson, Mountain View, CA). Receptor expression was calculated by the ratio of mean fluorescence intensity (MFI), *i.e.*, the quotient of MFI with and without specific antibody.

Electrophoretic Mobility Shift Assay

TEC were stimulated with IL-17 and/or CD8αCD40L for 1 h. Nuclear extracts were prepared according to the protocol described by Gobin *et al.* (25). For each nuclear extract, 3 μg of protein was tested for NF-κB–binding activity using two different NF-κB–binding probes: a ^{32}P-labeled NF-κB consensus oligo (5′-AGT TGA GGG GAC TTT CCC AGG C; Promega, Madison, WI) and a ^{32}P-labeled oligo containing the κB-site from human leukocyte antigen-A (HLA-A) (5′-GTG GGG ATT CCC CAC TGC A) (25). Competition experiments were performed using cold κB-probe from HLA-A and a cold divergent κB-probe from HLA-B, which is unable to bind NF-κB (25). For the supershift assays, the following antibodies were used: anti-p50 Ab (sc-114), anti-p65 Ab (sc-109), anti-c-Rel Ab (sc-71), and the irrelevant Ab anti-mouse-IRF-1 Ab (sc-640), all from Santa Cruz Biotechnology (Santa Cruz, CA). Each Ab (1 μg) was added to the nuclear extract and probe mixture and incubated for 1 h at 4°C. Samples were run on a 6% polyacrylamide gel in 0.25× Tris-borate/ethylenediaminetetraacetate buffer and analyzed by autoradiography.

Statistical Analyses

The production of chemokines and cytokines is presented as mean concentration ± SD from representative experiments. To quantitatively express synergy between IL-17 and CD40L, we defined an index of synergy, which was statistically tested using the Wilcoxon matched pairs signed-ranks test. An index of synergy >1 and $P <$ 0.05 was considered significantly synergistic.

$$\text{Index of synergy} = \frac{\text{cytokine production}_{(\text{IL-17} + \text{CD40L})}}{\text{cytokine production}_{(\text{IL-17})} + \text{cytokine production}_{(\text{CD40L})}}$$

Differences in receptor expression between stimulated and nonstimulated cultures were statistically tested using *t* test for paired samples ($P <$ 0.05).

Results

Expression of CD40 and CD40L in Renal Tissue

In a previous study, we demonstrated the expression of IL-17 in renal allografts undergoing rejection (18). Using the same biopsies, we investigated the distribution of CD40 and CD40L. In 4 of 7 cases, cryosections of normal human kidneys showed a relatively weak staining for CD40 of a limited number of tubuli (Figure 1a, Table 1). In contrast, in all cases of rejection, an intense tubular staining for the CD40 antigen at sites of interstitial infiltrate was observed (Figure 1, b and c). In addition, a strong staining for CD40 was seen of the majority of graft infiltrating cells. In all cases, no detectable staining was observed with control antibodies.

CD40L expression was not detectable in any of the control tissues (0 of 7) but was clearly positive in 6 of 8 tissues derived from patients with ongoing rejection (Figure 2, Table 1). In contrast with the staining for CD40, expression of CD40L was completely restricted to graft infiltrating cells, presumably T cells, as suggested by parallel staining for CD4$^+$ T cells.
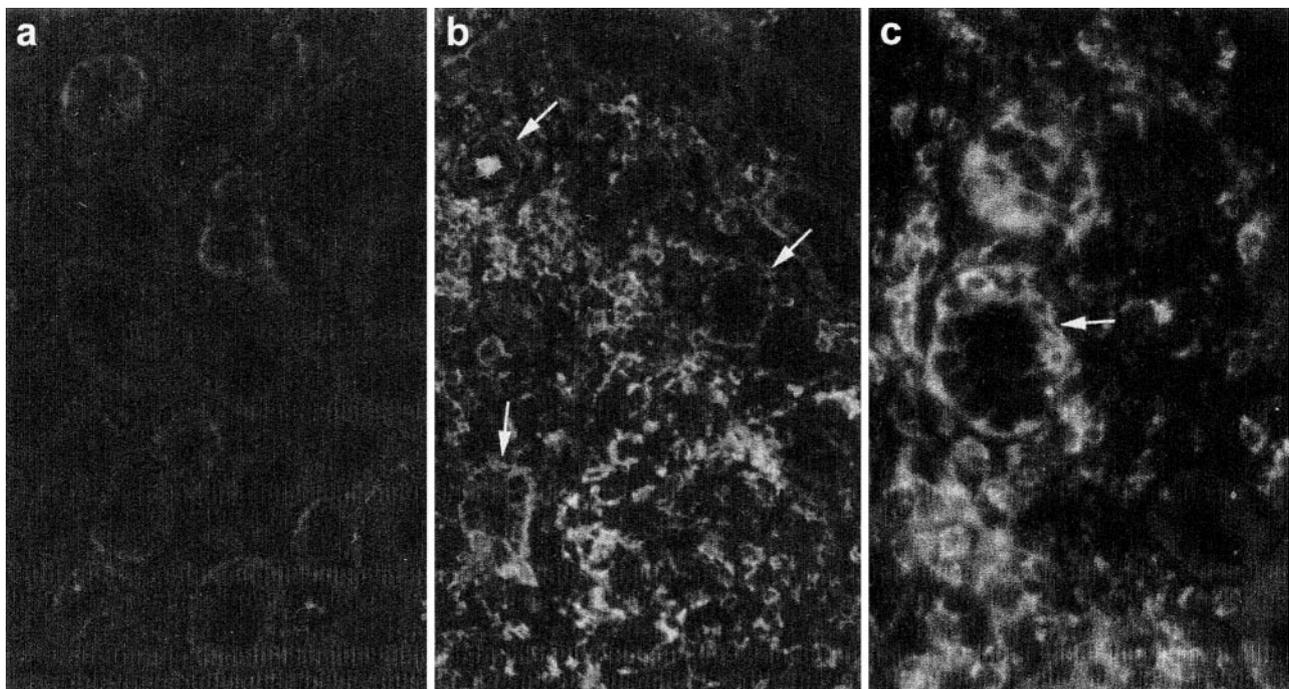

Figure 1. Expression of CD40 protein in renal biopsies. Cryosections were stained for CD40 expression by the tyramide-fluos method. Shown are stainings with anti-CD40 antibody mAb89 of a biopsy of a normal kidney (a) or biopsies with signs of rejection (b, c). Magnifications: 250× in a and b; 400× in c. Arrows indicate the strong staining of CD40 on tubuli. Stainings with control IgG were negative.

Table 1. Immunofluorescence staining of CD40 and CD40L in renal tissue[a]

	Banff Grading	CD40 Tubules	CD40 Infiltrate	CD40L Tubules	CD40L Infiltrate
Control					
1[b]	normal	−	n.i.	−	n.i.
2[b]	normal	±	n.i.	−	n.i.
3[c]	normal	−	n.i.	−	n.i.
4[c]	normal	±	n.i.	−	n.i.
5[d]	normal	±	n.i.	−	n.i.
6[d]	normal	−	n.i.	−	n.i.
7[d]	normal	±	n.i.	−	n.i.
Patient					
1[e]	I A	+	+	−	+
2[e]	II A	+	+	−	+
3[e]	II A	±	+	−	−
4[e]	I B	+	++	−	++
5[e]	I A	++	+	−	−
6[f]	II B	+	+	−	+
7[f]	I B	+	+	−	+
8[f]	II B	++	+	−	+

[a] Degree of staining of the tubuli and infiltrating cells were analyzed semiquantitatively, according to the following scale: −, no positive cells; ±, few positive cells; +, positive cells; ++, many positive cells; n.i., no infiltrate present.
[b] Cryosections prepared from normal kidneys not suitable for transplantation ($n = 2$).
[c] Cryosections prepared from normal parts of tumor kidney ($n = 2$).
[d] Cryosections prepared from pretransplant biopsies ($n = 3$).
[e] Cryosections prepared from posttransplantation biopsies ($n = 5$).
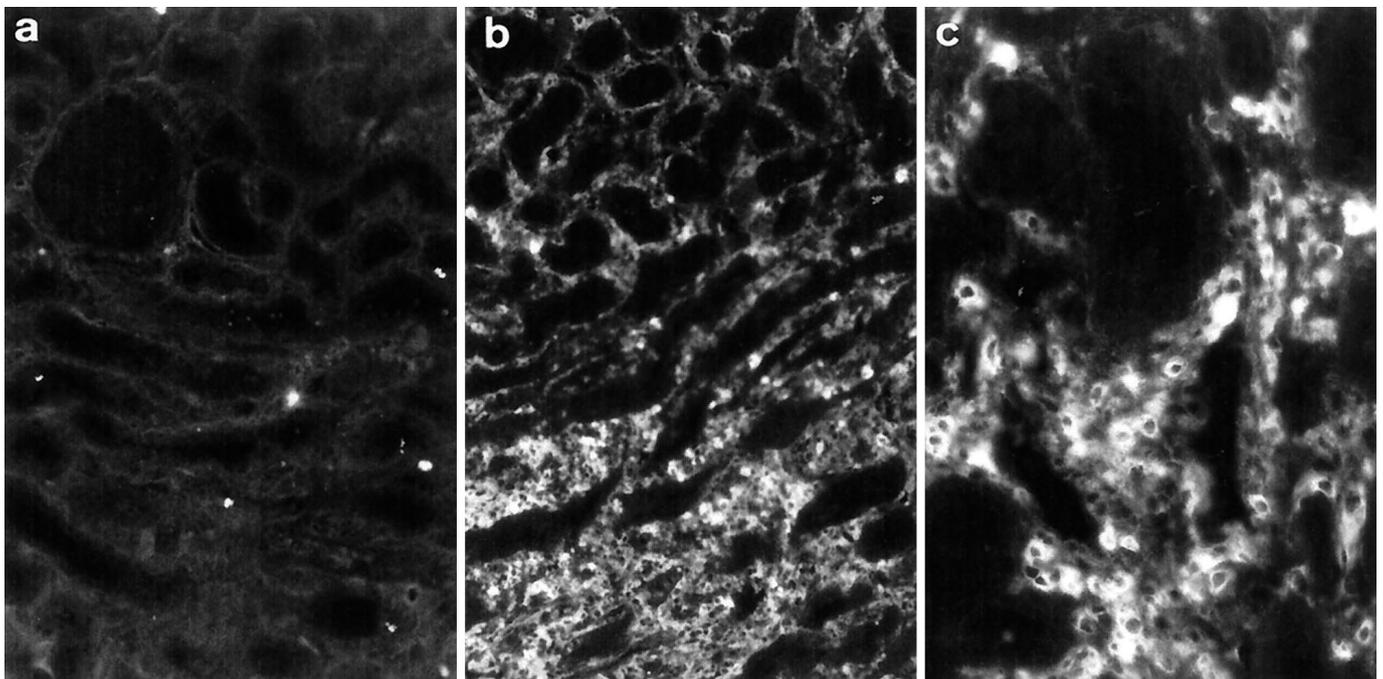[f] Cryosections prepared from transplantectomy specimens ($n = 3$).

Figure 2. Expression of CD40L protein in renal biopsies. Cryosections were stained for CD40L expression by the tyramide-fluos method. Shown are stainings of a rejection biopsy with control IgG (a) or anti-CD40L antibody LL56 (b and c). Magnifications: 250× in a and b; 400× in c).

IL-17 and CD40L Synergistically Enhance IL-6 Production by TEC

In the results above and in other studies, it was observed that TEC and T cells are frequently in close proximity during allograft rejection (26). Because the T-cell products IL-17 and CD40L both are expressed during renal allograft rejection, a possible cooperation between IL-17 and CD40L was investigated. Activation of TEC with either IL-17- or CD40L-transfected L cells (L-CD40L) increased IL-6 production over baseline levels (Figure 3). Stimulation of TEC with a combination of IL-17 and CD40L resulted in synergistic effects on IL-6

Figure 3. IL-17 and CD40L synergistically enhance IL-6 production by tubular epithelial cells (TEC). TEC (5×10^4) were cultured with either L-Orient or L-CD40L and stimulated with IL-17 (50 ng/ml). Cultures were supplemented with blocking anti–IL-17 (mAb5; 10 μg/ml), anti-CD40L (LL48; 10 μg/ml), or isotype-matched control antibodies. After 4 d, supernatants were harvested and tested for IL-6 production by specific ELISA. Data indicated show the mean production ($\pm$ SD) of duplicate cultures.

production. The mean index of synergy of nine independent experiments was statistically significant ($MIS_{IL-6} = 2.08$; range, 1.06 to 4.03; $P = 0.008$). Neutralizing antibodies against either CD40L or IL-17 significantly inhibited IL-6 production induced by the combination of CD40L and IL-17 (Figure 3).

The Effect of IL-17 and CD40L Interaction on the Production of Chemokines by TEC

The production of chemokines at the site of inflammation is a central event in the regulation of interstitial infiltration. Therefore, we investigated the effect of IL-17 and CD40L on the production of chemokines by TEC, including IL-8, MCP-1, and RANTES. Production of both IL-8 and MCP-1 was increased by single stimulation with either IL-17 or CD40L but was enhanced more strongly after combined stimulation (Figure 4, A and B). Titration of both IL-17 and CD40L showed that IL-8 production was upregulated in a dose-dependent fashion (Figure 5A). As demonstrated for surface-bound CD40L (L-CD40L; Figure 4A), this soluble chimeric protein also showed synergy with IL-17 on the production of IL-8 by TEC. Synergistic effects on IL-8 production were found over a wide range of IL-17 (1.2 to 100 ng/ml) and CD8αCD40L (1:20 to 1:5) concentrations (Figure 5A). Addition of increasing concentrations of IL-17 to CD40L-stimulated TEC also caused a strong dose-dependent enhancement of MCP-1 production, which was blocked by addition of neutralizing anti–IL-17 antibodies (Figure 5B). Statistical analysis revealed that the co-stimulatory effect of IL-17 and CD40L on the production of IL-8 was significantly synergistic, whereas the co-stimulatory effect on the production of MCP-1 was additive (Table 2).

Production of RANTES was not detectable in supernatant of nonstimulated or IL-17–stimulated TEC (Figure 4C). Even after a 5-d culture period, IL-17 did not induce detectable RANTES production, either by ELISA (Figure 6) or by RT-PCR analysis (data not shown). In accordance with previous
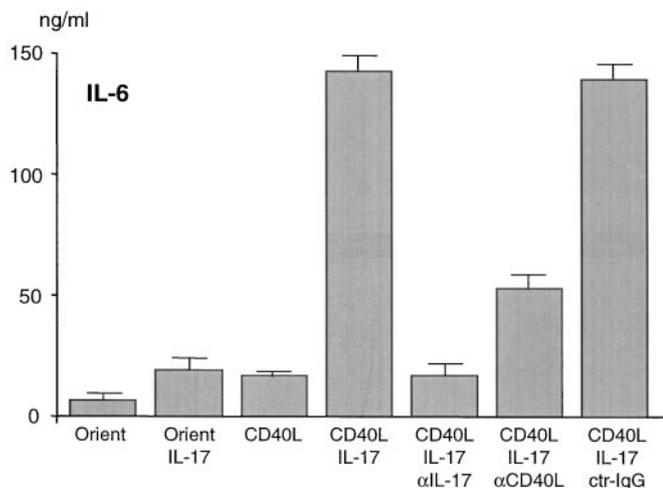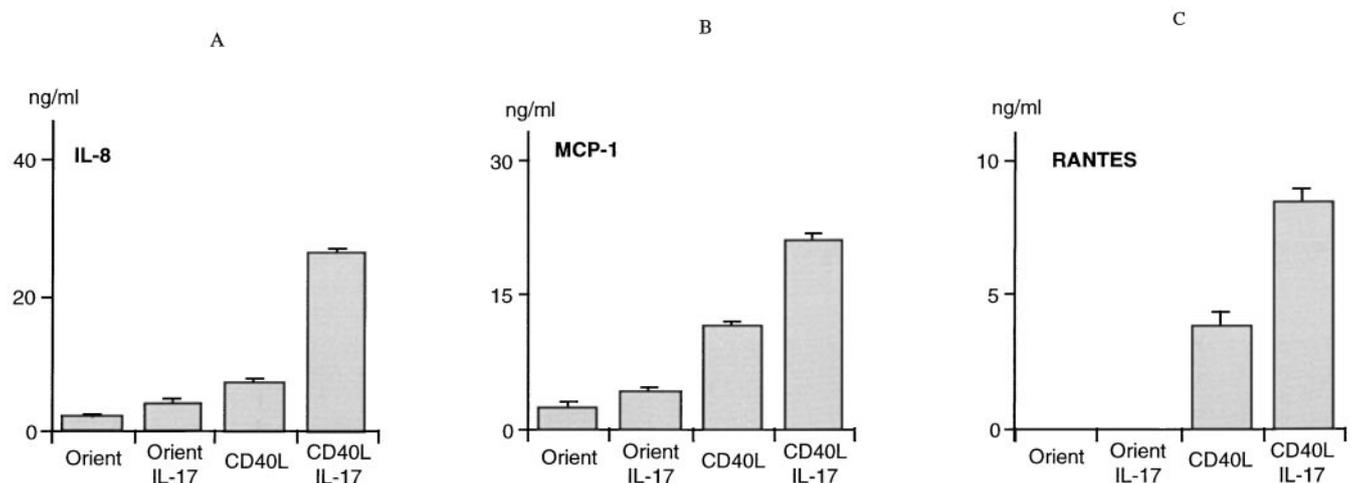

Figure 4. IL-17 and CD40L synergistically enhance chemokine production by TEC. TEC were cultured with medium, IL-17 (50 ng/ml), CD40L, or the combination. After 4 d, supernatants were harvested and tested for IL-8 (A), monocyte chemoattractant protein-1 (MCP-1; B), and regulated upon activation, normal T cell expressed and secreted (RANTES; C) by specific ELISA. Shown is the mean production ($\pm$ SD) of duplicate cultures.
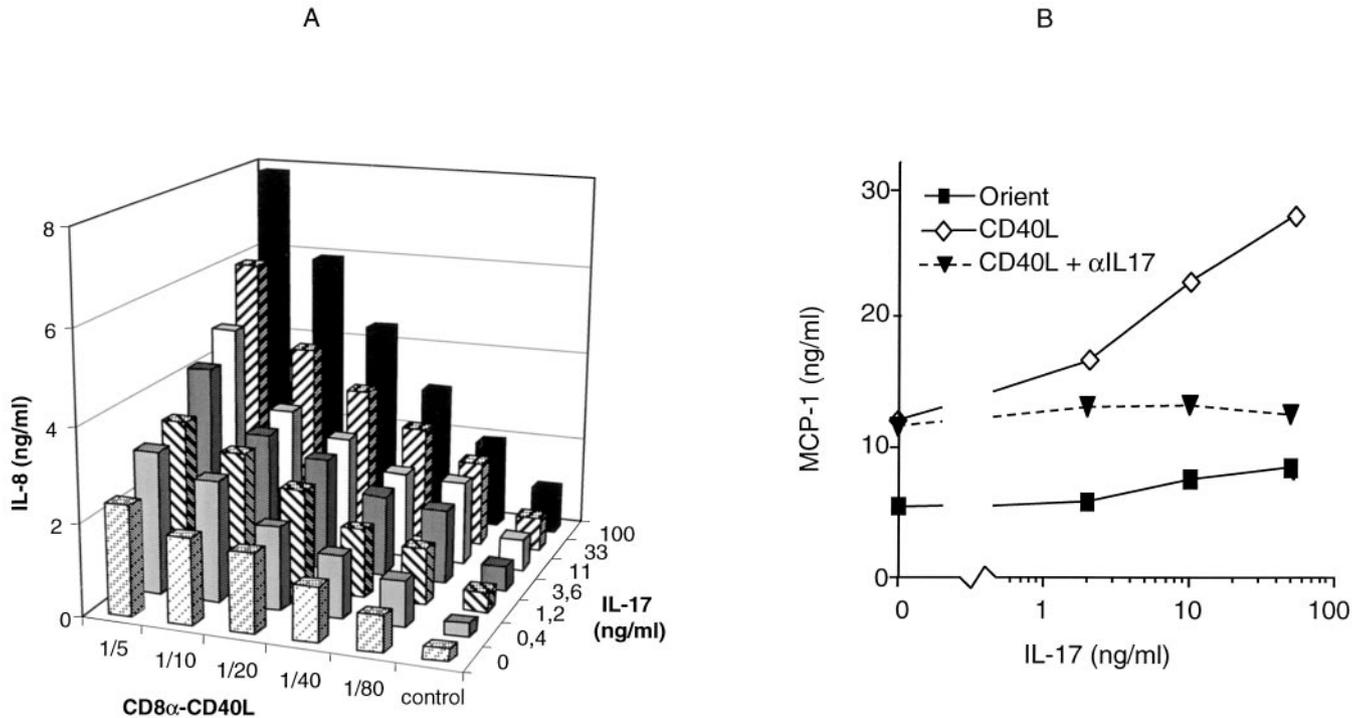
A

B

Figure 5. Dose-dependent increase in IL-8 and MCP-1 production by TEC. (A) TEC were stimulated with increasing concentrations of soluble CD8αCD40L together with increasing concentrations of IL-17. Supernatants were taken after 72 h of stimulation and tested for IL-8 production. Shown is the mean production (± SD) of duplicate cultures. (B) TEC were cultured in the presence of L-Orient or L-CD40L cells with increasing doses of IL-17. Cultures were supplemented with blocking anti—IL-17 antibody (mAb5; 10 μg/ml). Supernatants were taken after 3 d of culture and tested for MCP-1 production. Shown are the mean production (± SD) of duplicate cultures.

Table 2. Mean indices of synergy for the production of cytokines and chemokines after combined stimulation with IL-17 and CD40L[a]

	IL-6			IL-8			MCP-1			RANTES		
	n	Mean	P	n	Mean	P	n	Mean	P	n	Mean	P
TEC	9	2.08	0.008	9	14.95	0.008	8	1.40	0.124	9	5.82	0.008
FS4	8	3.30	0.012	8	2.97	0.012	7	1.98	0.018	4	ND	ND
Synoviocytes	4	1.23	0.043	4	2.19	0.043	4	0.85	0.144	4	ND	ND

[a] IL, interleukin; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated upon activation, normal T cell expressed and secreted; TEC, tubular epithelial cells; ND, not detectable. TEC, FS4, and synoviocytes were stimulated with IL-17, CD40L, or the combination and tested for their production of IL-6, IL-8, MCP-1, and RANTES. For each experiment, the index of synergy was determined as described in the Materials and Methods section. Shown are the number of independent experiments (*n*), the mean index of synergy, and *P* values derived from the Wilcoxon matched pairs signed-ranks test.

studies, we found that CD40 activation did induce RANTES production (Figures 4C and 6). Combined treatment with CD40L demonstrated that IL-17 synergistically increased CD40L-induced RANTES production at all time points (Figure 6, Table 2).

Synergistic Effects of IL-17 and CD40L Are Not Specific for Epithelial Cells

To investigate whether the synergy between IL-17 and CD40L is specific for renal TEC, we studied two different

fibroblast populations. Stimulation of primary synoviocytes with either IL-17 or CD40L increased the production of IL-6 and IL-8 (Figure 7), and combined treatment showed a significant synergy between IL-17 and CD40L (Table 2). Similarly, simultaneous stimulation with IL-17 and CD40L resulted in dose- and time-dependent synergistic effects on IL-6 and IL-8 production by the foreskin fibroblast cell line FS4 (Table 2, Figure 8). Co-stimulation showed a synergistic effect on the production of MCP-1 by FS4, which was not observed with synoviocytes. For both fibroblast populations, no significant increase in RANTES production was found in culture supernatants after either single or simultaneous stimulation (Table 2).
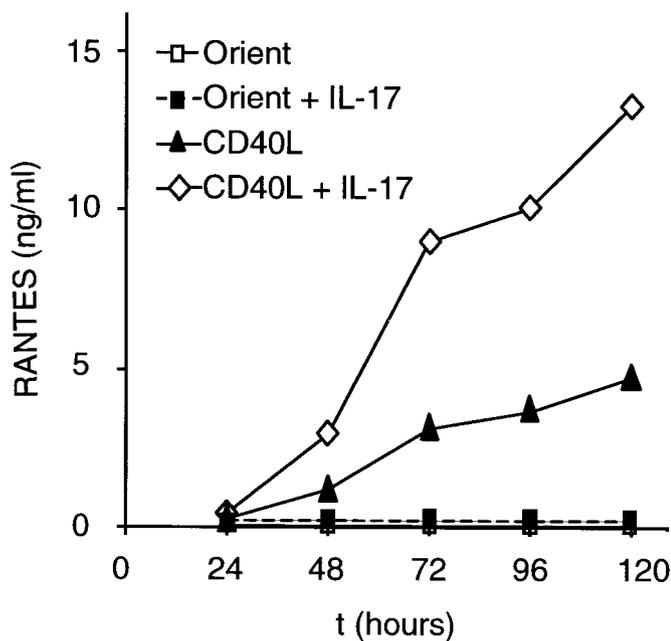
Figure 6. Time-dependent synergistic enhancement of RANTES production by TEC. TEC were cultured with L-Orient or L-CD40L and stimulated with IL-17 (50 ng/ml). Supernatants were harvested at different time points during a 5-d culture period and tested for RANTES production. Data indicated are representative of three experiments, and shown is the mean production (± SD) of duplicate cultures.

Regulation of IL-17 Receptor and CD40 Expression

Specific regulation of IL-17R and CD40 expression might contribute to the observed synergistic effect on cytokine production. FACS analysis using specific monoclonal antibodies demonstrated a homogeneous expression of CD40 and IL-17R on TEC, synoviocytes, and FS4 (Figure 9A). Activation with CD8αCD40L did not affect IL-17R expression on any of these cell types. Also, IL-17 treatment of synoviocytes and FS4 did not alter surface expression of CD40. However, activation of TEC with IL-17 resulted in a twofold increase in CD40 expression (Figure 9B). In 10 independent experiments, IL-17 increased the ratio mean fluorescence (CD40 staining over control staining) from 4.5 (range, 1.2 to 8.7) to 9.2 (range, 3.8 to 14.9).

IL-17- and CD40L-Induced Cytokine and Chemokine Production Is NF-κB Dependent

Electrophoretic mobility shift assays were performed to investigate NF-κB activation. Nonstimulated TEC showed low NF-κB–binding activity, which was not significantly influenced by IL-17. CD40L activation strongly increased NF-κB binding, which was not further increased by co-stimulation with IL-17 (Figure 10A). The specificity of the binding was tested by competition with cold κB probes. Binding of the complex was prevented when using the cold κB probe containing the homologous sequence, but was not affected with a cold divergent κB probe (κB-mut; Figure 10B). Supershift analysis with antisera specific for p50, p65, and c-Rel determined the nuclear proteins present in the bound complex (Figure 10C). The upper band of the complex was shown to contain p50 and p65, because this band shifted with both anti-p50 and anti-p65 antibodies. This protein/DNA complex probably represents the p50/p65 heterodimer. The intense lower band showed a significant reduction in intensity by addition of anti-p50 antibody, most likely representing the p50/p50 homodimer. No marked difference was observed by using anti-c-Rel antibody. No supershift was obtained with an irrelevant antibody.

The involvement of NF-κB in CD40L- and IL-17–induced cytokine production was investigated using PDTC, an inhibitor of NF-κB activation. Two-h preincubation of TEC with 3 to 30 μM PDTC and subsequent activation with IL-17, CD40L, or the combination showed a dose-dependent inhibition of IL-6 production (Figure 11A). Complete inhibition was found by addition of 30 μM PDTC to the cultures. Similar inhibition was obtained for IL-8 and RANTES production by TEC (data not shown) and IL-6 (Figure 11B) and IL-8 production by synoviocytes and FS4 (data not shown).
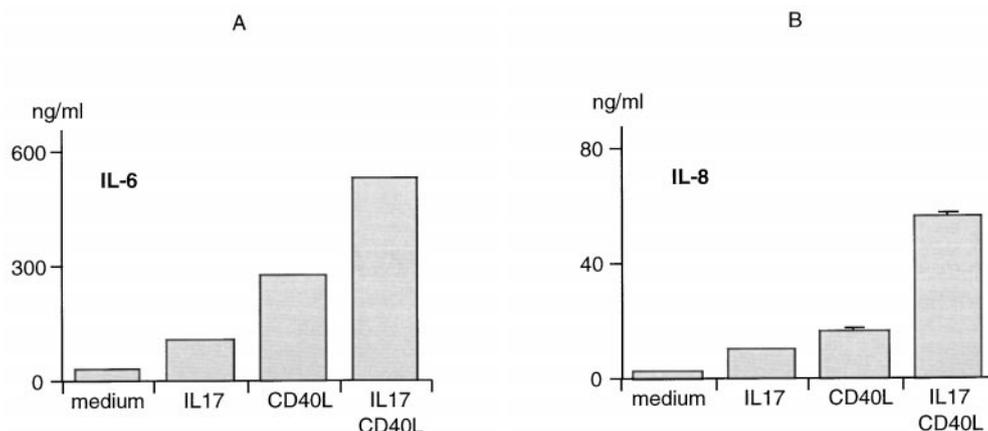

Figure 7. IL-17 and CD40L synergistically enhance IL-6 and IL-8 production by synoviocytes. Synoviocytes were cultured with medium, IL-17 (50 ng/ml), CD8αCD40L, or the combination. After 4 d, supernatants were harvested and tested for IL-6 (A) and IL-8 (B) by specific ELISA. Shown is the mean production (± SD) of duplicate cultures.
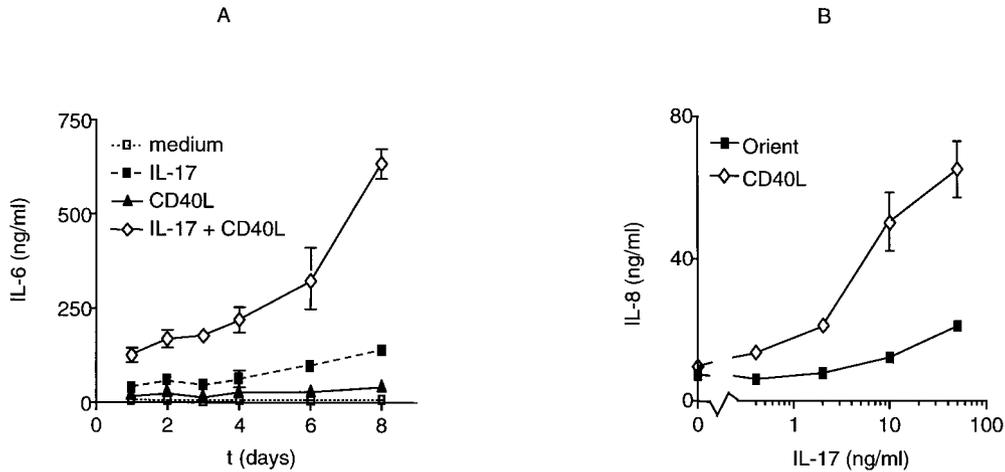
Figure 8. Time- and dose-dependent increase in IL-6 and IL-8 production by FS4. (A) FS4 were cultured with medium, IL-17 (50 ng/ml), CD8αCD40L, or the combination. Supernatants were harvested at different time points during an 8-d culture period and tested for IL-6 production. Shown are the mean production (± SD) of duplicate cultures. (B) FS4 were cultured with L-Orient or L-CD40L, in the presence of increasing concentrations of IL-17. Supernatants were taken after 4 d of culture and tested for IL-8 production. Shown are the mean production (± SD) of duplicate cultures.

Discussion

The present study demonstrates an important role for CD40L and IL-17 in the regulation of chemokine production by renal TEC. Combined stimulation leads to synergistic effects on IL-6, IL-8, and RANTES production and additive effects on the production of MCP-1. We previously demonstrated the presence of IL-17 in inflamed renal tissue (18), which is now extended with the demonstration of an increased expression of CD40 and CD40L in biopsies of patients with rejection. Because both IL-17 and CD40L are products of activated T cells, they represent one of the links between allospecific immune responses and nonspecific inflammation.

Normal kidneys show expression of CD40, which is restricted to a limited number of proximal and distal tubuli and some endothelial cells (4,27,28). In the present study, we demonstrated that tubular expression of CD40 is strongly increased during allograft rejection. This means that not only CD40L is an inducible T-cell activation marker, but also that expression of the CD40 receptor is subject to regulation upon local inflammation. In accordance, we found that proinflammatory cytokines, such as IL-1 (29) and IL-17, can increase CD40 expression in TEC. Although in normal renal tissue TEC express low CD40, cultured TEC shows a strong homogeneous CD40 surface expression, suggesting that other, as yet unidentified signals might also regulate CD40 expression.

Immunofluorescence staining showed that during rejection, both CD40 and CD40L are broadly expressed on graft-infiltrating lymphocytes. As shown by others, we also found that CD40L was exclusively expressed on infiltrating cells (Figure 2) (28). In addition, we were not able to detect CD40L expression by cultured TEC using FACS or RT-PCR analysis (data not shown). However, it has been suggested that during chronic rejection, CD40L might also be expressed on TEC (30). The expression of CD40L in renal biopsies was performed on the same biopsies as used for the detection of IL-17 (18). In
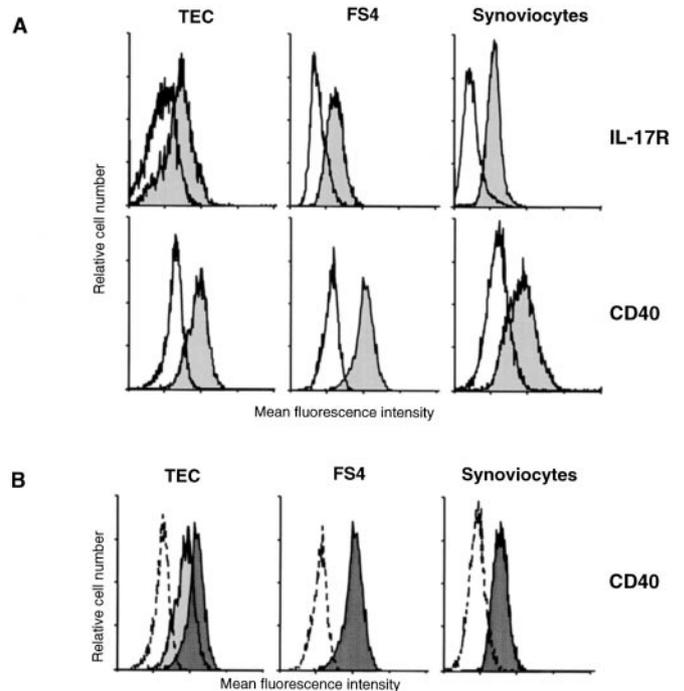

Figure 9. Expression of IL-17R and CD40 on TEC, FS4, and synoviocytes. (A) Surface expression of CD40 and IL-17R on cultured TEC, FS4, and synoviocytes. Filled histograms represent expression of IL-17R and CD40, using M203 and mAb89, respectively. Open histograms represent basal fluorescence level after staining with only goat anti-mouse–phycoerythrin. (B) TEC, FS4, and synoviocytes were stimulated for 48 h with IL-17 (50 ng/ml) and subsequently stained for CD40 (mAb89). The light histograms represent receptor expression on nonstimulated cells, whereas the dark histograms represent the receptor expression on activated cells. Background fluorescence levels are represented by the stippled histograms. Upregulation of CD40 expression on TEC is statistically significant (P < 0.05).
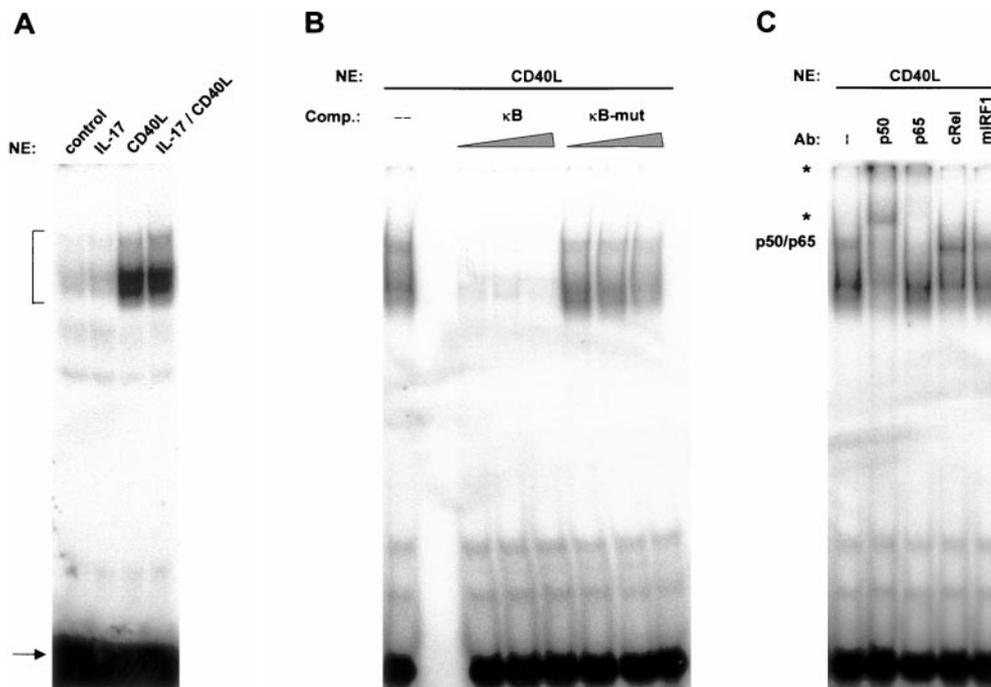
Figure 10. Activation of NF-κB/Rel transcription factors in TEC. (A) TEC were incubated with medium, IL-17 (50 ng/ml), CD8αCD40L, or the combination for 1 h. Nuclear extracts were analyzed by electrophoretic mobility shift assays (EMSA) using the NF-κB-consensus probe, as described in the Materials and Methods section. The specific complex of proteins bound to the DNA is indicated by a bracket, whereas the free probe is indicated by an arrow. (B) TEC were incubated with CD8αCD40L for 1 h. Nuclear extracts were analyzed by EMSA using the HLA-A κB probe. Cold κB-probe containing the homologous sequence (κB) or the mutated κB-probe (κB-mut) was added in 25-, 50-, and 100-fold excess as competitor. (C) TEC were incubated with CD8αCD40L for 1 h. Nuclear extracts were analyzed by EMSA using the HLA-A κB-probe. For supershift analysis, anti-p50, anti-p65, anti-c-Rel, and the irrelevant anti-mouse-IRF-1 Abs were used. Supershifted complexes are indicated by an asterisk. No supershift was obtained with an irrelevant Ab (anti-mIRF1).
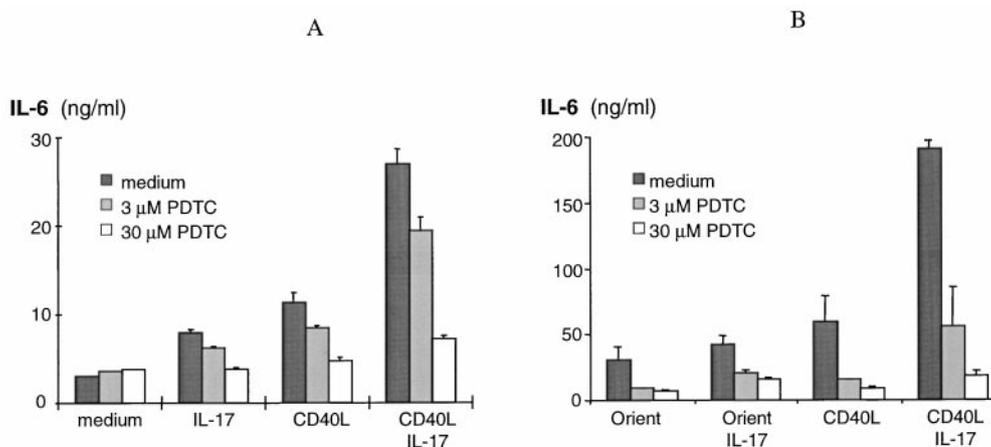

Figure 11. Addition of pyrrolidine dithiocarbamate (PDTC) blocks IL-17 and CD40L induced cytokine production. TEC (A) and synoviocytes (B) were incubated with medium, 3 μM or 30 μM PDTC. After 2 h of incubation, medium, IL-17 (50 ng/ml), CD8αCD40L, or the combination of IL-17 and CD8αCD40L were added to the TEC, and L-Orient or L-CD40L with or without IL-17 (50 ng/ml) was added to the synoviocytes. After 48 h, supernatants were harvested and tested for IL-6 by specific ELISA. Shown is the mean production (± SD) of duplicate cultures, which is representative for three independent experiments.

consecutive sections of these biopsies, IL-17 and CD40L expression overlapped the area of infiltrating cells and paralleled the staining for CD4+ T cells (data not shown). Local expression of IL-17 and CD40L within the kidney will favor the activation of IL-17R- and CD40-expressing cells, which might

include renal TEC, endothelial cells, and graft-infiltrating monocytes, B cells and T cells, all of which can contribute to the rejection process. Analysis of CD40L expression, at either the protein or the mRNA level, was shown to correlate with renal allograft rejection (28,30), and a similar situation has

been described for cardiac allograft rejection (31). However, expression of CD40L is not specific for rejection. This is best illustrated by the observation of increased CD40L expression in lupus nephritis and other renal diseases (27), in which auto-antigens rather than allo-antigens are the driving force of T cell activation.

The increased MCP-1 production and the synergistically enhanced IL-8 and RANTES production by TEC in response to IL-17 and CD40L emphasize the important role for these T-cell products during rejection episodes. An elevated production of chemokines leads to further attraction of inflammatory cells (6) and hence an amplification of the initial (allo)antigen-specific response. It is thought that the rejection process is predominantly driven by a T_h1 immune response (32). Expression of CD40L on activated T cells is not restricted to a specific T_h type and can be induced in T_h0, T_h1, and T_h2 cells (33). Because of its high chemotactic capacity on T cells, especially T_h1 cells, the production of RANTES plays an important role in the rejection process (5,26,34,35). Activation via CD40 seems to be a critical step in the production of RANTES by TEC. Previous reports have demonstrated that both IL-1 (29) and IL-4 (9) synergize with CD40L in the enhancement of RANTES production by TEC. IL-1 is mainly produced by monocytes and macrophages (36), and IL-4 is a T_h2-derived cytokine (37). In contrast, production of IL-17 is specific for activated T cells, predominantly of the T_h1 type, but not by T_h2 cells (17). Therefore, the combination of IL-17 and CD40L might play a prominent role in the T_h1-mediated renal allograft response.

It will be interesting to test the combined inhibition of IL-17 and CD40L in a model of renal transplantation, because individual blocking has already demonstrated prolongation of graft survival in different experimental models (12,14). Although CD40 activation is critical for upregulation of B7 molecules and for further T-cell activation and cytokine production, prevention of chemokine production by TEC might be part of the observed effects of anti-CD40L treatment.

IL-17–induced CD40 upregulation on TEC might partially explain the mechanism of synergy between IL-17 and CD40L. Because synergistic activation of fibroblasts was independent of IL-17–induced CD40 upregulation, it is most likely that other mechanisms, such as the convergence of intracellular pathways, also contribute to this process. In accordance with previous studies using other epithelial cells and fibroblasts (38,39), we showed an increased NF-κB binding activity after CD40L activation of TEC. We did not observe a further increase in NF-κB activity by co-stimulation of TEC with IL-17 and CD40L. However, induction of IL-6 and IL-8 production was completely prevented by an inhibitor of NF-κB. This supports the role of NF-κB as a pivotal transcription factor in chronic inflammation (40). These data suggest that part of the action of corticosteroids, which are used in posttransplantation therapy and block NF-κB activation (41), is via the inhibition of resident epithelial cells.

We showed that combination of IL-17 and CD40L synergistically activates fibroblasts. Although little information is available on human renal fibroblasts, it is evident that they

participate in the pathologic changes of renal inflammation and chronic allograft rejection (42). More is known about the role of synoviocytes in rheumatoid arthritis or lung fibroblasts in chronic lung inflammation and fibrosis. Expression of IL-17 and CD40L has been demonstrated in rheumatoid arthritis synovium supernatants (43) and in synovial biopsies (44). Administration of soluble CD40L in the lung was demonstrated to induce pulmonary inflammation (45), whereas treatment with anti-CD40L or use of CD40L-knockout mice prevented the development of lung inflammation and fibrosis (46,47). In the skin, keratinocytes are responsive to both CD40L (48) and IL-17 (49). Therefore, local expression of CD40/CD40L and cooperation with IL-17 most likely is not restricted to the kidney but is operational in different organs.

In conclusion, the T-cell products IL-17 and CD40L, which both are expressed during renal allograft rejection, synergistically increase cytokine and chemokine production by renal epithelial cells and fibroblasts. IL-17 and CD40L might potentially explain the link between (allo)specific immune reactivity and nonspecific inflammation. This study extends the recent findings that interaction between cells of the hematopoietic system (especially T cells) and nonhematopoietic cells might play an important role in the regulation of inflammation.

Acknowledgments

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