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Gene transfer of RANTES elicits autoimmune renal injury in MRL-*Fas*^{lpr} mice

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Infiltrating macrophages and T cells are instrumental in autoimmune kidney destruction of MRL-*Fas*^{lpr} mice. We report that the β-chemokine RANTES, a chemoattractant for macrophages and T cells, is up-regulated in the MRL-*Fas*^{lpr} kidney prior to injury, but not normal kidneys (MRL-++, C3H-++) and increases with progressive injury. Furthermore, we establish an association between RANTES expression in the kidney and renal damage using a gene transfer approach. Tubular epithelial cells genetically modified to secrete RANTES infused under the renal capsule incites interstitial nephritis in MRL-*Fas*^{lpr}, but not MRL-++ or C3H-++ mice. RANTES recruits predominantly macrophages (Mø) and CD4$^+$ and CD8$^+$ T cells. In contrast, gene transfer of CSF-1, another molecule up-regulated simultaneously with RANTES in MRL-*Fas*^{lpr} kidneys, promotes the influx of Mø, CD4$^+$ T cells and the unique double-negative (DN) T cells (CD4$^-$,CD8$^-$), which are prominent in diseased MRL-*Fas*^{lpr} kidneys. Thus, RANTES and CSF-1 recruit distinct T cell populations into the MRL-*Fas*^{lpr} kidney. In addition, delivery of RANTES and CSF-1 into the kidney of MRL-*Fas*^{lpr} mice causes an additive increase in pathology. We suggest that the complementary recruitment of T cell populations by RANTES (CD4, CD8) and CSF-1 (CD4, DN) promotes autoimmune nephritis in MRL-*Fas*^{lpr} mice.

The MRL-*Fas*^{lpr} mouse is a particularly appealing model of human systemic lupus erythematosus, since renal disease is rapid, predictable and regulated by a single gene mutation [1]. Nephritis in MRL-*Fas*^{lpr} mice is complex, with glomerular, perivascular, and interstitial components, and the rapid disease progression is fatal (50% mortality at 6 months of age) [2]. By contrast, renal disease in congenic MRL-++ mice lacking the *Fas*^{lpr} mutation is indolent, and is not evident until the second year of life. Thus, the *Fas*^{lpr} mutation accelerates renal disease in the predisposed autoimmune MRL strain [2]. Fas mediates apoptosis of autoreactive T cells in the periphery. Mutation of this gene (*Fas*^{lpr}) results in the accumulation of a unique subset of "double negative" (DN) T cells (CD4$^-$, CD8$^-$, B220$^+$) in MRL-*Fas*^{lpr} mice [1]. Macrophage (Mø) and T cell infiltration is a prominent component of the renal pathology in MRL-*Fas*^{lpr} mice [3, 4]. Multiple T cell subsets are present in renal lesions; equal numbers of CD4$^+$ and DN T cells accumulate in perivascular, glomerular, and interstitial lesions, while fewer CD8$^+$ T cells accumulate, predominantly in the interstitium. However, the molecules responsible for recruiting and activating these inflammatory cells in the kidney have not been clearly defined.

MRL-*Fas*^{lpr} mice have multiple cytokine abnormalities prior to the onset of autoimmune injury that are thought to promote disease progression. We and others have reported up-regulation of colony stimulating factor-1 (CSF-1), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, transforming growth factor-B (TGF-β), and IL-6 in MRL-*Fas*^{lpr} mice [3–8]. To define the importance of a single cytokine in the pathogenesis of kidney disease, ideally the cytokine should be expressed in a selected cell type in a specific location in the mature kidney. Gene transfer strategies that tailor cytokine expression in selected sites in the kidney have been limited by the lack of tissue specific promoters, and short-lived and variable expression of the transferred molecules [9–11]. To overcome these obtacles, we devised an *ex vivo* gene transfer system using retrovirally modified tubular epithelial cells (TEC) as a carrier cell to deliver cytokines into the kidney [12]. Syngeneic tubular epithelial cells, genetically engineered *ex vivo* to express a cytokine, are infused under the renal capsule, where they remain localized and provide stable, sustained delivery of cytokine into the kidney. Using this gene transfer approach, we established that CSF-1, but not TNF-α or IL-6, elicits renal injury in MRL-*Fas*^{lpr} mice [12–15]. In spontaneous disease, CSF-1 is up-regulated in MRL-*Fas*^{lpr}, but not congenic MRL-++ mice, normal strains or other strains with the *Fas*^{lpr} mutation, well in advance of renal disease, and increases in proportion to the severity of kidney damage [3, 16]. Our finding that introduction of CSF-1 initiates renal damage in MRL-*Fas*^{lpr} mice is supported by compelling evidence from experiments in which single nephritic MRL-*Fas*^{lpr} kidneys transplanted into MRL-++ mice resulted in a loss of CSF-1 expression, the disappearance of Mø and the reversal of renal lesions [17]. Taken together, we conclude that CSF-1 is responsible for attracting and fostering Mø and T cells in the MRL-*Fas*^{lpr} kidney. This prompted us to determine whether other molecules in addition to CSF-1 contributed to the accumulation of Mø and T cells in MRL-*Fas*^{lpr} kidneys.

The migration of leukocytes into an inflammatory site requires the generation of a chemotactic gradient by cells of the extravascular compartment. The β-chemokine, RANTES (Regulated upon Activation, Normal T cell Expressed and Eecreted), is chemotactic for monocytes and T cells, and induces cellular activation of these leukocytes [18–20]. RANTES is expressed by

[1] See *Editorial* by Danoff, p. 1808

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renal mesangial and tubular epithelial cells (TEC) [21–23], and by T lymphocytes *in vitro* [24]. In addition, RANTES can promote the adhesion of invading leukocytes to matrix components of glomeruli [25]. Thus, the release of RANTES by intrinsic renal cells may initiate the influx of leukocytes into the kidney and promote autoimmune inflammatory renal injury. Therefore, we tested the hypothesis that RANTES initiates Mø and T cell recruitment into the MRL-*Fas*^{lpr} kidney. We now report that RANTES is expressed in the kidney of MRL-*Fas*^{lpr} mice prior to the onset of autoimmune renal injury. Using a retroviral gene transfer strategy, the consequence of RANTES expression in the recruitment and propagation of inflammatory cells was evaluated in the MRL-*Fas*^{lpr} kidney. RANTES fostered the accumulation of a distinct subset of T cells ($CD4^+ > CD8^+ \gg$DN), and initiates renal damage. By contrast, CSF-1, which is simultaneously up-regulated, promoted the influx of DN and $CD4^+$ T cells, but few $CD8^+$ T cells. Thus, we propose that RANTES and CSF-1 are complementary in promoting autoimmune nephritis by their recruitment of Mø and different T cell subsets into the MRL-*Fas*^{lpr} kidney.

METHODS

Mice

MRL/MpJ-++ (MRL-++), MRL/MpJ-*Fas*^{lpr}/*Fas*^{lpr} (MRL-*Fas*^{lpr}) and C3H-HeJ (C3H-++) mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) or bred in our pathogen-free animal facility.

Retrovirus-mediated cytokine gene transfer into cultured tubular epithelial cells

Tubular epithelial cells (TEC) derived from MRL-*Fas*^{lpr}, MRL-++ and C3H-++ mice one to two months of age were isolated as previously described [26]. Briefly, kidneys were removed and renal cortices were minced, dispersed in collagenase solution, and passed through a series of steel sieves (250, 150, 75 and 38 μm). Cells were collected, resuspended in modified K1 medium (50% DMEM, 50% Ham's F12, 10% FCS, 100 U/ml each of penicillin and streptomycin, 10 mM HEPES), and incubated for one hour at 37°C. Non-adherent cells were transfered to Primaria tissue culture plates (Falcon Labware) and grown to confluence. Prior to retroviral infection, TEC were trypsinized and plated at 1×10^6 cells/10-cm plate for 18 hours.

CRIP packaging cell lines producing helper-free recombinant retroviruses carrying cytokine genes were generated as previously described [27]. Briefly, the DNA sequence encoding RANTES was subcloned into the MoMuLV-based MFG vector containing the long terminal repeat viral promoter and the ψ sequence necessary for viral RNA packaging. The MFG vector carrying the RANTES gene was transfected into a 3T3 packaging cell line (CRIP) containing the proviral sequences necessary for encapsidation of the recombinant viral RNA. The resulting virus-producing cell line elaborates recombinant retroviruses that stably transfer the recombinant viral genome containing the RANTES gene upon infection of a suitable host cell. Producer cells were grown in DMEM complete medium (10% FCS, 100 U/ml each of penicillin and streptomycin, 10 mM HEPES) to subconfluence (5×10^6 cells/10 cm dish), replenished with 8 ml of fresh medium and incubated for 18 hours. The virus-containing cell culture supernatant was harvested, filtered through a 0.45 μm membrane, and applied to TEC cultures as previously described [12]. Following retroviral infection, TEC were replenished with K1 medium, and grown to confluence. RANTES gene transfer into TEC was verified and quantitated by Northern analysis as described above. The retroviral transfer of the CSF-1 and TNF-α gene into TEC has been previously established in our laboratory and characterized in detail [12–14]. Genetically modified cytokine producing TEC retain the ability to produce cytokine *in vitro* for at least 10 months, and upon implantation, persist under the renal capsule for up to 28 days [12]. Prior to infusion under the renal capsule, production of CSF-1 by transduced TEC was verified by bone marrow proliferation assay, and production of TNF-α was verified by ELISA assay (Endogen, Woburn, MA, USA) as previously described [12, 14]. In this manuscript, we abbreviate TEC transduced with the RANTES, CSF-1, and TNF-α genes as RANTES-TEC, CSF-1-TEC, or TNF-TEC, respectively.

Northern analysis

RNA was extracted and Northern blot analyses were performed as previously described [3]. Total RNA was isolated from cultured TEC or dissected renal cortices using RNAzol B (Tel-Test Inc., Friendswood, TX, USA), which is a modification of the guanidium thiocyanate-phenol-chloroform method [28]. Total RNA (20 μg) was electrophoresed through a 1% agarose-formaldehyde gel, blotted to nylon membrane and hybridized in 50% formamide with ^{32}P-labeled nick-translated probes at 42°C. Hybridized membranes were washed in 2 × SSC, 0.1% SDS at room temperature and 0.2 × SSC, 0.1% SDS at 60°C. Autoradiography was performed using a PhosphoImager (Molecular Dynamics Corp., Sunnyvale, CA, USA). The RANTES probe was kindly provided by Dr. E. Neilson (University of Pennsylvania Medical Center, PA, USA) and consisted of a 450 kb fragment of the PBS KS plasmid containing the cDNA. Blots were reprobed with β-actin (Pst-1 fragment of pBA-1) as an internal control for quantity and integrity of RNA. Densitometry analysis was performed to quantitate mRNA expression.

Immunocytochemical evaluation of tubular epithelial cells

TEC were cultured in chamber slides (2×10^4 cells/well) overnight and treated with 5 μM monensin (Boehringer Mannheim) for three hours prior to staining. TEC were fixed in acetone and incubated for 30 minutes with 2.5% goat serum, followed by goat anti-human RANTES Ab (20 μg/ml; 1 hr; R & D Systems), which is weakly cross-reactive for mouse RANTES. Cells were rinsed three times with phosphate buffered saline (PBS) and immunoreactivity was visualized by incubating with rabbit anti-goat IgG-FITC conjugated Ab for one hour.

Western analysis

Cell culture supernatants (48 hr) were collected from 5×10^6 MRL-*Fas*^{lpr} and C3H-++ RANTES-TEC or uninfected TEC and concentrated fourfold using centricon concentrators-3 (Amicon, Beverly, MA, USA). Samples were heat denatured in the presence of Laemmli sample buffer containing 5% 2-mercaptoethanol and electrophoresed on a 15% linear gradient SDS-PAGE gel. Proteins were transfered to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) in the presence of Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) using a wet electrophoretic transfer apparatus. Membranes were blocked in TBS-T (Tris buffered saline/0.1% Tween-20)

containing 5% (wt/vol) non-fat dry milk for one hour, and then incubated in fresh buffer containing rabbit anti-murine RANTES Ab (1:2000) for one hour. Membranes were washed in TBS-T and incubated with peroxidase conjugated anti-rabbit Ab (1:20000) in TBST-T/5% dry milk. Immunoreactive proteins were visualized using the enhanced chemiluminescence detection system (Dupont NEN, Boston, MA, USA) and exposed to X-ray film.

Infusion of tubular epithelial cell under the renal capsule

Using our gene transfer strategy, we have previously established that delivery of CSF-1 under the renal capsule incites an accumulation of Mø and T cells in the kidney of MRL-*Fas*^{lpr} mice six weeks of age or older, but not in younger mice [12]. Therefore, we delivered RANTES into the kidney of mice that were 6 to 8 weeks of age at the time of TEC implantation to study the impact of RANTES overexpression on the kidney. We placed RANTES-TEC, CSF-1-TEC, or uninfected TEC under the renal capsule of syngeneic MRL-*Fas*^{lpr}, MRL-++ or C3H-++ mice as previously described [12]. We anesthetized mice with sodium pentobarbital and ether, and exposed the left kidney through a flank incision. A cell suspension of 1×10^6 TEC in 50 μl of HBSS was infused under the renal capsule, and the peritoneum and skin were sutured. Mice were sacrificed 7, 14 or 28 days post-implantation (post-Ix) and the implanted kidney and control contralateral kidney were removed for histological evaluation.

To establish the combined effect of CSF-1 and RANTES on the MRL-*Fas*^{lpr} kidney, we placed equal numbers of (1×10^6) RANTES-TEC and CSF-1-TEC, or RANTES-TEC and uninfected TEC, or CSF-1-TEC and uninfected TEC under the renal capsule of MRL-*Fas*^{lpr} mice as described above. Mice were sacrificed at 28 days post-Ix and the implanted kidney and control contralateral kidney were removed for histological evaluation.

Histological evaluation

Kidneys were halved and fixed in 10% phosphate-buffered formalin for paraffin-embedded sections or snap-frozen in OCT compound (Miles, Naperville, IL, USA). Paraffin-embedded tissue sections were stained by the hematoxylin and eosin (H & E) method and evaluated by light microscopy. Sequential frozen sections (6 μ) were taken every 250 μ and stained with toluidine blue to identify the area of largest cell accumulation in the implant site, as previously described [12–14]. The mean subcapsular cell accumulation in TEC implanted kidneys was evaluated by counting the cell layers in the area of accumulation under the capsule. Intrarenal lesions emanating from the subcapsular implant were scored according to the depth of infiltration into the kidney: 0 = none, 1 = cortex, 2 = inner cortex, 3 = medulla. To evaluate T cell and Mø accumulation in the kidney, we stained sequentially sectioned tissue specimens by the immunoperoxidase technique using purified rat anti-mouse Abs to F4/80 Ag (Mø), CD4 (T cell), CD8 (T cell), and CD21/35 (B cell) and the B220 determinant (B cell, DN T cell) (Pharmingen, San Diego, CA, USA) as previously described [12–15]. We identified the unique DN T cells (CD4$^-$,CD8$^-$, CD21/35$^-$, B220$^+$) by examining sequential sections. In brief, endogenous peroxidase (0.6% H_2O_2, 0.2% sodium azide; 10 min), and avidin and biotin (Avidin/Biotin blocking kit; Vector Laboratories, Burlingame, CA, USA) were blocked prior to incubating the tissue sections with primary Abs (5 μg/ml, 2 hr). Sections were washed in TBS/BSA 0.1% and incubated with biotinylated goat anti-rat IgG (mouse adsorbed) for one hour. We exposed the tissue sections to avidin-peroxidase complex (Vector Elite Kit; Vector Laboratories) for one hour, followed by 3-3′-diamino-benzidine (0.5 mg/ml in TBS containing 0.02% H_2O_2) for a chromogenic reaction and counterstained with methyl green/alcian blue. Specificity controls included replacement of primary Ab with normal rat IgG. Immunohistochemical staining was analyzed by counting the number of F4/80$^+$, CD4$^+$, CD8$^+$, or CD21/35$^-$B220$^+$ in more than six random fields (×40) in the induced lesion. These analyses were performed independently by two investigators in a blinded fashion. Statistical analyses were performed using the Mann-Whitney μ test.

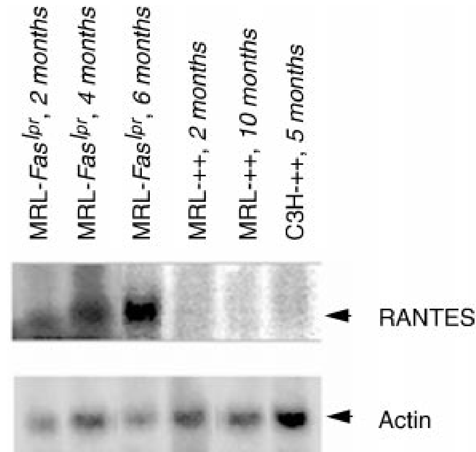

Fig. 1. RANTES mRNA is expressed in MRL-*Fas*^{lpr} kidneys. Total RNA was extracted from kidney cortex of MRL-*Fas*^{lpr} mice 2, 4, and 6 months of age (lanes 1, 2, 3), congenic MRL-++ mice 2 and 10 months of age (lanes 4, 5), and normal C3H-++ mice five months of age (lane 6). A total of 20 μg of RNA was electrophoresed, blotted and hybridized with a DNA probe for mouse RANTES and reprobed with β-actin. Each time point reflects the pooled kidney cortex RNA from 3 mice. Results are representative of 2 separate experiments.

RESULTS

RANTES mRNA is expressed in MRL-*Fas*^{lpr}kidneys

By Northern analysis, we determined that RANTES is expressed in the MRL-*Fas*^{lpr} kidney prior to overt renal injury, and increases with advancing renal pathology (Fig. 1). RANTES mRNA is expressed in MRL-*Fas*^{lpr} kidney cortex as early as two months of age, and is highly up-regulated in MRL-*Fas*^{lpr} kidney cortex at six months of age during fulminant renal disease. In contrast, RANTES mRNA is absent in mice with normal kidneys, including congenic MRL-++ (2 and 10 months of age) and C3H-++ (5 months of age) strains. This pattern of RANTES up-regulation in the MRL-*Fas*^{lpr} kidney parallels that previously described for CSF-1 and TNF-α [3–5].

RANTES incites renal damage in autoimmune MRL-*Fas*^{lpr} mice

Primary renal TEC were retrovirally transduced with the RANTES gene sequence, to create a cellular vehicle for the constitutive delivery of RANTES into the kidney. We verified that the transduced TEC expressed RANTES in culture (Fig. 2). Both MRL-*Fas*^{lpr} and C3H-++ TEC constitutively express high levels of RANTES mRNA after retroviral gene transfer (Fig. 2A). By contrast, uninfected MRL-*Fas*^{lpr} and C3H-++ TEC do not

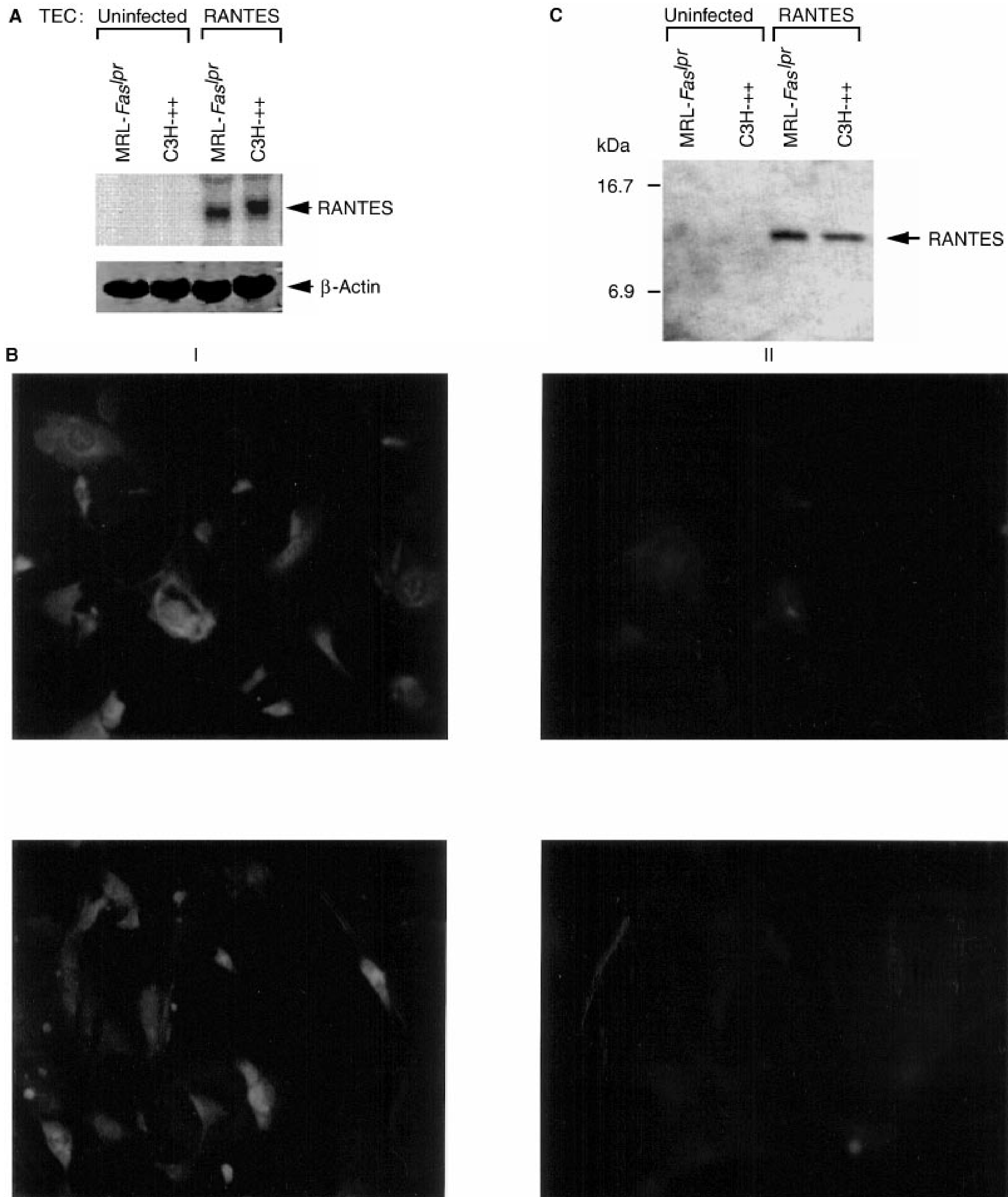

Fig. 2. Primary culture tubular epithelial cells (TEC) retrovirally transduced with the RANTES gene sequence constitutively express RANTES. (*A*) Northern analysis demonstrating expression of RANTES mRNA by transduced RANTES-TEC but not uninfected TEC. Total RNA (10 μg) from uninfected (lanes 1, 2) or RANTES-TEC (lanes 3, 4) derived from MRL-*Fas*^{lpr} or normal C3H-++ mice was hybridized with a DNA probe for mouse RANTES, and reprobed with β-actin. (*B*) Immunohistochemical staining documenting the expression of RANTES by transduced RANTES-TEC, but not uninfected TEC. RANTES-TEC (panel I) and uninfected TEC (panel II) from MRL-*Fas*^{lpr} (top) or C3H-++ (bottom) were incubated with goat anti-human RANTES-Ab and immunoreactivity was visualized with a rabbit anti-goat IgG-FITC conjugated Ab (magnification ×400). (*C*) Western analysis verifying secretion of RANTES by transduced RANTES-TEC, but not uninfected TEC. Cell culture supernatants from MRL-*Fas*^{lpr} and C3H-++ RANTES-TEC or uninfected TEC were subjected to Western analysis and RANTES protein was detected using a rabbit anti-murine RANTES Ab as described in the **Methods** section.

express endogenous RANTES mRNA. RANTES protein expression by transduced TEC was confirmed by staining for the presence of RANTES by immunohistochemistry (Fig. 2B). RANTES was detected in the cytoplasm of > 90% of MRL-*Fas*^{lpr} and C3H-++ RANTES-TEC, but not in uninfected TEC of either strain. Furthermore, MRL-*Fas*^{lpr} and C3H-++ RANTES-TEC secreted similar levels of RANTES into the culture media as determined by Western analysis (Fig. 2C). These data confirm that the RANTES gene was successfully integrated into the TEC genome resulting in constitutive expression, and that uninfected TEC do not express RANTES.

To determine whether RANTES expression in the kidney incites renal damage, RANTES-TEC was infused under the renal capsule of syngeneic MRL-*Fas*^{lpr,} MRL-++, or C3H-++ mice at two months of age, and examined kidney pathology at 7, 14 and 28 days post-implantation (Ix). RANTES incites an accumulation of mononuclear cells under the renal capsule in MRL-*Fas*^{lpr} mice, which infiltrate into the cortex forming a lesion, as early as seven days post-Ix (Fig. 3). By 14 days post-Ix, these invading cells expand into the inner cortex of the MRL-*Fas*^{lpr} kidney. In contrast, RANTES-TEC only induced a cortical lesion in one of four congenic MRL-++ mice at this time point. By 28 days post-Ix, a massive accumulation of mononuclear cells is apparent under the renal capsule in MRL-*Fas*^{lpr} mice, which infiltrate into the kidney cortex and cause extensive interstitial lesions (Figs. 3 and 4A). By comparison, renal injury is not noted in similarly manipulated C3H-++ mice; only a modest accumulation of cells is noted under the renal capsule at 28 days post-Ix, and these cells do not invade the kidney cortex (Fig. 4B). As we have previously reported [12–15], infusion of uninfected TEC under the renal capsule does not incite renal injury in MRL-*Fas*^{lpr}, MRL-++, or C3H-++ mice (Fig. 4C). Furthermore, placing RANTES-TEC under the renal capsule of MRL-*Fas*^{lpr}, MRL-++, or C3H-++ mice for 28 days does not foster the accumulation of leukocytes or incite renal damage in the unmanipulated contralateral kidney (data not shown). We have excluded the possibility that the renal damage incited by RANTES-TEC in MRL-*Fas*^{lpr} kidney is a result of retroviral transduction of the TEC. TEC transduced with the TNF-α gene placed under the renal capsule of MRL-*Fas*^{lpr} mice elicits only an accumulation of inflammatory cells which remain localized in the subcapsular space, but do not infiltrate into the cortex, similar to the response to infusion of uninfected TEC (Fig. 4D). Taken together, these data indicate that expression of RANTES in the kidney of autoimmune MRL-*Fas*^{lpr} mice, but not normal mice, specifically incites inflammatory renal damage.

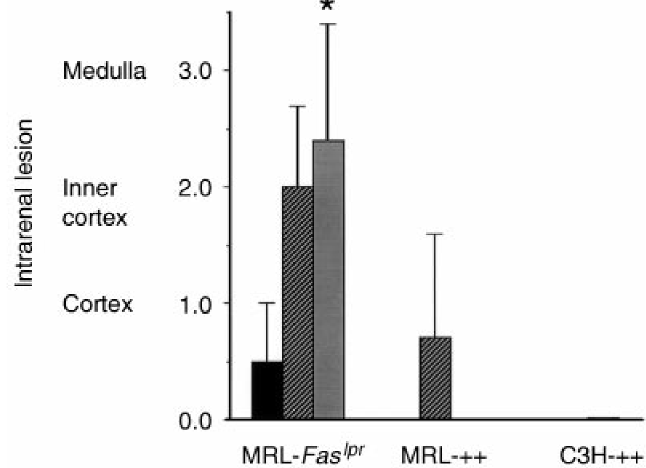

Fig. 3. RANTES incites progressive renal injury. The progression of renal injury induced by RANTES-TEC in MRL-*Fas*^{lpr}, MRL-++ and C3H-++ mice was determined at 7 (■), 14 (▨), and 28 (▩) days post-Ix. Intrarenal lesions emanating from the subcapsular implant were scored according to the depth of infiltration into the kidney; 0 = none, 1 = cortex, 2 = inner cortex, 3 = medulla. Values are expressed as the mean ± SE; $N \geq 3$ mice per group. * $P < 0.05$.

RANTES incites an accumulation of CD4^{+} > CD8^{+} ≫ DN T cells, and few Mø in MRL-*Fas*^{lpr} kidneys

To identify the inflammatory cell phenotypes recruited by RANTES in the MRL-*Fas*^{lpr} kidney, the accumulation of CD4^{+}, CD8^{+}, or DN T cells (CD4^{-}, CD8^{-}, CD21/35^{-}B220^{+}) and Mø (F4/80) was evaluated in the incited lesion at 7 and 28 days post-Ix. At seven days post-Ix, RANTES incited an accumulation of predominantly Mø and CD4^{+} T cells in the induced lesion (Table 1). By 28 days post-Ix, the balance of infiltrating cells recruited by RANTES had shifted to predominantly CD4^{+} T cells, lesser numbers of CD8^{+} T cells and Mø, but few DN T cells (Table 1 and Fig. 5). Furthermore, RANTES induced a prominent accumulation of CD4^{+} T cells in the interstitium and surrounding glomeruli in MRL-*Fas*^{lpr} kidneys, that was absent in normal kidneys (Fig. 6).

Since CSF-1 is up-regulated in MRL-*Fas*^{lpr} mice prior to renal injury [3, 4], in a similar time frame as RANTES, we compared the inflammatory T cell subsets recruited by CSF-1 and RANTES using our gene transfer strategy at 7 and 28 days post-Ix. These time points were selected to reflect the early and late events incited by RANTES and CSF-1. At seven days post-Ix, CSF-1-TEC induced a prominent accumulation of Mø, but unlike RANTES, induced an equal accumulation of CD4^{+}, CD8^{+}, and DN T cells (Table 1). By 28 days post-Ix, the difference in the attraction of T cell subsets by RANTES and CSF-1 was even more pronounced. RANTES elicited predominantly CD4^{+} T cells and fewer CD8^{+} T cells, while DN T cells were notably absent. In contrast, CSF-1 elicited equal numbers of CD4^{+} and DN T cells, and no CD8^{+} cells (Table 1). It should be noted that we did not detect CD21/35^{+} cells in the CSF-1 induced kidney lesion, but by comparison, CD21/35^{+} cells were abundant in the normal spleen control. Thus, RANTES and CSF-1 both initially attract Mø, however, these cytokines foster distinct and complementary T cell subsets in the MRL-*Fas*^{lpr} kidney.

Combining CSF-1-TEC and RANTES-TEC under the renal capsule causes an additive increase in renal damage

Since expression of both CSF-1 and RANTES increases in the kidneys of MRL-*Fas*^{lpr} mice prior to overt renal injury [3, 4], and these cytokines recruit complementary inflammatory T cell subsets, we investigated whether CSF-1 and RANTES interact to accelerate renal damage. CSF-1-TEC and RANTES-TEC were implanted together under the renal capsule of MRL-*Fas*^{lpr} mice and evaluated for renal damage at 28 days post-Ix. The simultaneous expression of CSF-1 and RANTES in the MRL-*Fas*^{lpr} kidney resulted in an additive increase in the size of cortical lesions emanating from the subcapsular implant site, as compared

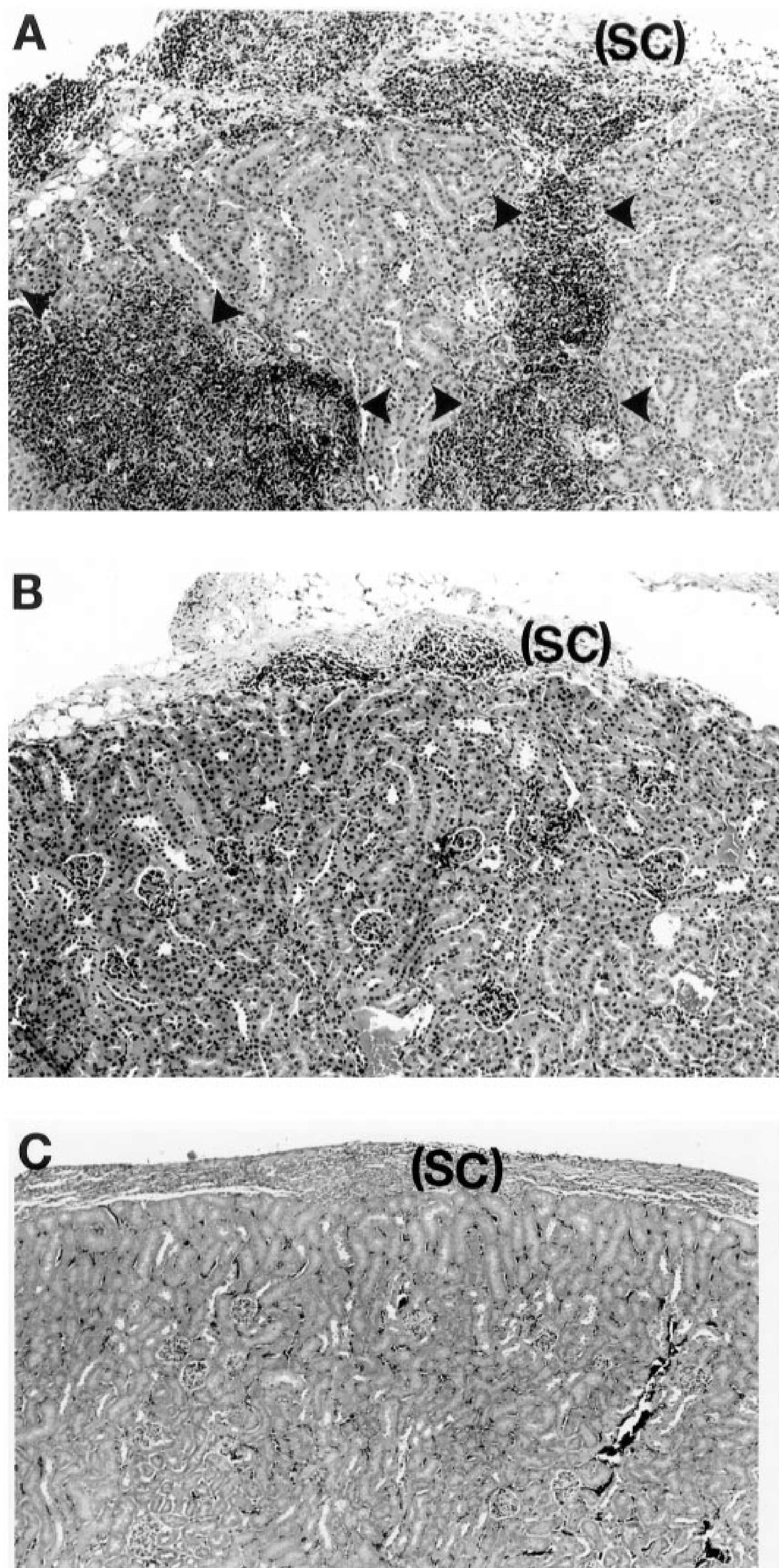

Fig. 4. RANTES incites renal injury in autoimmune MRL-*Fas*^{lpr}, but not in C3H-++ mice with normal kidneys. RANTES-TEC incite a massive accumulation of mononuclear cells under the renal capsule (SC; subcapsule) that actively infiltrated into the kidney cortex (arrows) causing large interstitial lesions in autoimmune MRL-*Fas*^{lpr} mice (*A*). By contrast, RANTES-TEC placed under the renal capsule of normal C3H-++ mice, induce a modest accumulation of cells at 28 days post-Ix (*B*). Uninfected TEC (*C*) or TNF-TEC (*D*) placed under the renal capsule of MRL-*Fas*^{lpr} mice do not incite a renal lesion (paraffin-embedded tissue sections, hematoxylin and eosin stained; magnification ×400).

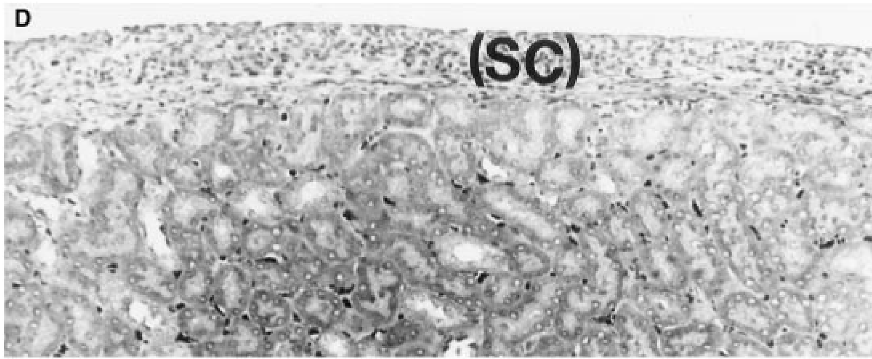

Fig. 4. *Continued.*

with kidneys implanted with RANTES-TEC or CSF-1-TEC alone (Table 2). This implies that RANTES and CSF-1 independently incite renal damage, but do not interact to synergistically enhance tissue injury.

Table 1. RANTES and CSF-1 promote the accumulation of different T cell subsets in the kidney MRL-*Fas*^{lpr} mice

	Cell accumulation within lesion post-Ix			
	7 days		28 days	
Phenotype	RANTES	CSF-1	RANTES	CSF-1
Macrophage (F4/80)	136 ± 14	210 ± 30	253 ± 9	175 ± 38
	(53.1%)	(68.2%)	(11.5%)	(10.5%)
T cells				
$CD4^+$	70 ± 16[a]	33 ± 5	1157 ± 24[a]	700 ± 154
	(27.3%)	(10.7%)	(52.4%)	(42.0%)
$CD8^+$	48 ± 9[a]	29 ± 4	631 ± 13[a]	55 ± 13
	(18.8%)	(9.4%)	(28.5%)	(3.3%)
DN[b]	2 ± 1[a]	36 ± 7	168 ± 3[a]	735 ± 162
	(0.8%)	(11.7%)	(7.6%)	(44.1%)

Recipients were MRL-*Fas*^{lpr} mice, 8 weeks of age; $N = 3$; 1×10^6 TEC implanted. Abbreviations are: CSF-1, colony stimulating factor-1; Ix, implantation.

Cell phenotypes in intrarenal lesions are reported as the mean ± SE of F4/80, B220, CD4 or CD8 positive cells in 6 fields of ×40.

[a] $P < 0.05$, as compared to CSF-1

[b] DN = $CD4^-$, $CD8^-$, $B220^+$

DISCUSSION

Increasing evidence suggests that RANTES is instrumental in renal inflammation. In support of this concept, RANTES is detected in several forms of renal injury. For example, RANTES gene expression has been detected in human and rat renal allograft rejection [29, 30], and an induced form of anti-GBM nephritis in the rat [23, 31]. This study investigated the importance of RANTES in autoimmune kidney destruction. We now report that RANTES is expressed in the kidney cortices of autoimmune MRL-*Fas*^{lpr} mice prior to renal injury and increases with the severity of nephritis. Furthermore, a connection between RANTES expression in the kidney and renal damage using an *ex vivo* gene transfer strategy is established. RANTES secreting TEC infused under the renal capsule of pre-diseased MRL-*Fas*^{lpr} mice elicit an intrarenal lesion extending from the implanted subcapsular site into the cortex, which is absent in similarly manipulated MRL-++ or C3H-++ mice with normal kidneys. Furthermore, RANTES promotes the influx and propagation of Mø and specific T cell subsets, $CD4^+ > CD8^+ >$ DN T cells, into the MRL-*Fas*^{lpr} kidney. Together, these data establish that RANTES expression in the kidney of autoimmune MRL-*Fas*^{lpr} mice promotes the recruitment and accumulation of Mø and $CD4^+$ and $CD8^+$ T cells, and in turn, incites renal damage.

RANTES expression in the kidney requires a suitable genetic background (MRL) and a contributing molecular factor (*Fas*^{lpr} mutation) to incite renal pathology. This finding parallels our previous observation that CSF-1, up-regulated in the MRL-*Fas*^{lpr} kidney, incites renal damage in mice with the *Fas*^{lpr} mutation, but not in normal strains [12]. It is important to appreciate that the capacity of RANTES and CSF-1 to elicit renal pathology is specific and not related to retroviral genes. Using the same gene transfer system, IL-6 and TNF-α did not elicit renal pathology [14, 15]. Thus, renal pathology in response to RANTES and CSF-1 delivery into the kidney is a specific consequence of the properties of these molecules to recruit and activate autoreactive inflammatory cells. We speculate that RANTES fails to elicit renal injury in normal C3H-++ mice since autoreactive T cells are not available to target the kidney. It should be noted that MRL-++ mice are pre-disposed to autoimmune renal disease, which is manifested in the second year of life [2]. Since autoimmune MRL background genes are required for renal injury, it is not surprising that in our study, renal damage was initiated in only a small percentage of MRL-++ kidneys implanted with RANTES-TEC. This would suggest that in the presence of RANTES, other molecules required to incite renal damage may be available, at least in some MRL-++ mice, well in advance of renal injury.

To evaluate the early and more distal impact of RANTES expression on kidney pathology, mice were examined at 7 to 28 days following implantation of cytokine-TEC. It should be noted that renal pathology in MRL-*Fas*^{lpr} mice examined following delivery of RANTES for 28 days is minimal. We previously established that a small increase in Mø and the beginning of perivascular infiltration of T cells can be detected [12]. Our prior studies indicate that CSF-1-TEC deliver high sustained levels of CSF-1 and remain localized in the subcapsular implant site for 28 days post-Ix [12]. Since in our system, the implanted TEC delivers RANTES locally into a single kidney, renal pathology is restricted to the area proximal to the implant site and survival is not compromised. Even in studies in which TEC deliver GM-CSF into the kidney for three months, although the lesion continued to expand, there was insufficient amount of renal damage to be fatal [12]. This is not surprising and suggests that a more diffuse

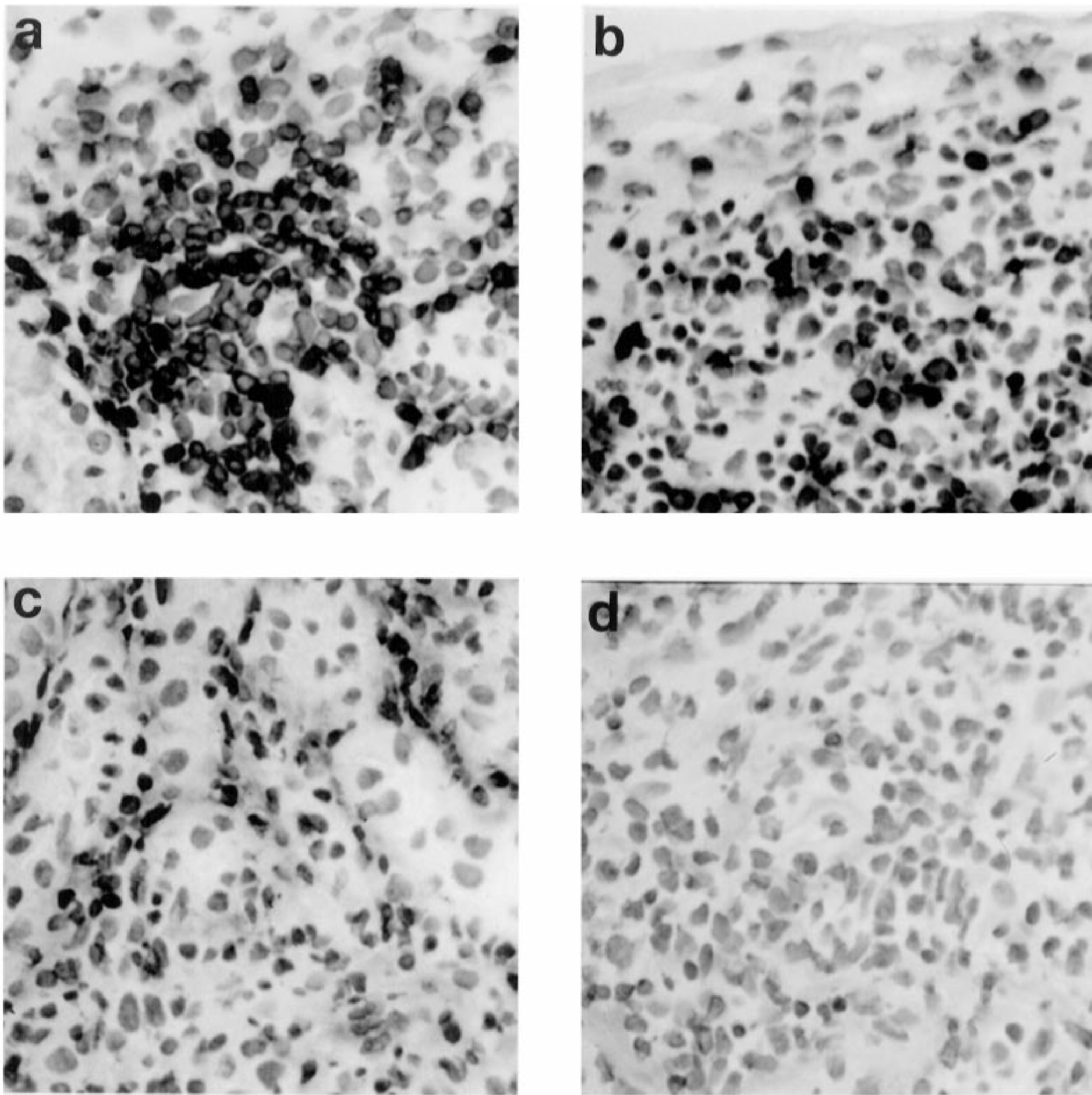

Fig. 5. RANTES incites an accumulation of CD4$^+$ > CD8$^+$ > DN T cells in MRL-*Fas*^{lpr} kidneys. RANTES-TEC placed under the renal capsule of MRL-*Fas*^{lpr} mice, induce a predominant accumulation of CD4$^+$ cells (dark staining) (*a*), and CD8$^+$ cells (*b*) within the interstitial lesion at 28 days post-implantation (Ix). In contrast to CD4$^+$ and CD8$^+$ cells, Mø accumulate along the periphery of the lesion (*c*), and few DN T cells are observed within the lesion (*d*) (frozen tissue sections; magnification ×1000 for a and b; ×800 for c and d).

delivery of these cytokines is required to cause the loss of renal function.

The interaction of the *Fas*^{lpr} mutation and the MRL background results in a rapid and fatal spontaneous renal injury characterized by the infiltration of Mø and multiple T cell subsets into the kidney [2]. *Fas* mediates apoptosis of autoreactive T cells in the periphery, and mutation of this gene results in the accumulation of a unique subset of DN T cells [1]. These DN T cells accumulate in equal numbers with CD4$^+$ T cells within perivascular and interstitial lesions, while CD8$^+$ T cells are fewer in number [32]. Thus, it is notable that our gene transfer study demonstrates that RANTES recruits substantial numbers of CD4$^+$ and CD8$^+$ T cells into the MRL-*Fas*^{lpr} kidney. This suggests that another molecule(s) present in the MRL-*Fas*^{lpr} kidney is responsible for recruiting and fostering the accumulation of DN T cells.

We have previously documented that the Mø growth factor CSF-1 is expressed in the MRL-*Fas*^{lpr} kidney prior to renal injury, beginning at four weeks of age, in glomeruli and TEC, and expression increases with disease severity [3, 4]. Thus, CSF-1 and RANTES are up-regulated in the MRL-*Fas*^{lpr} kidney at a similar time point. Therefore, we compared the individual contributions

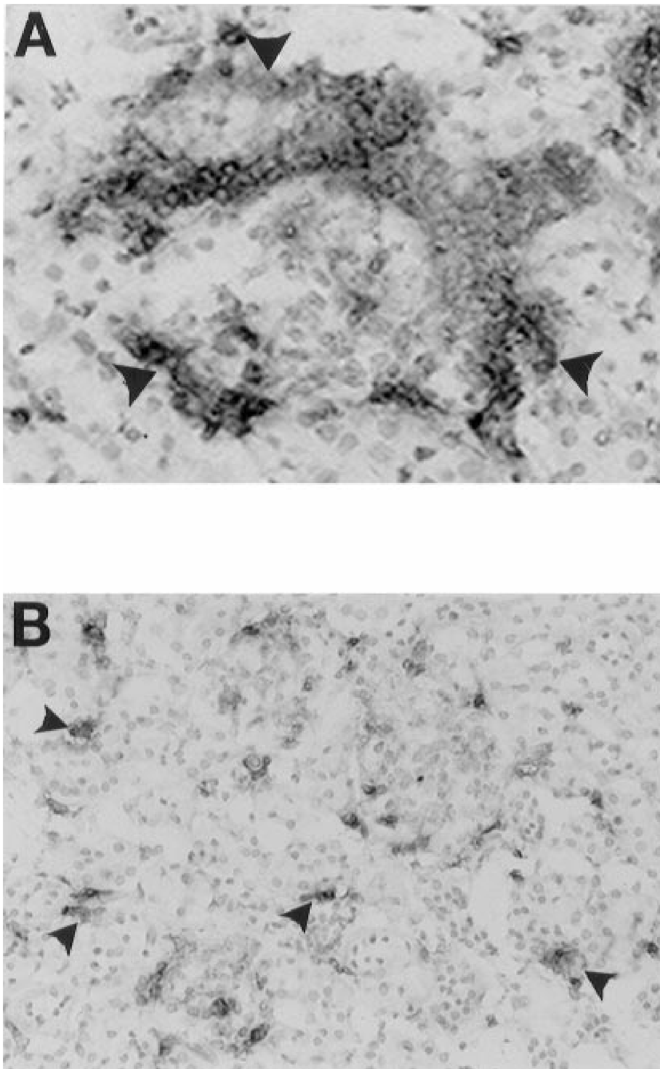

Fig. 6. RANTES incites an interstitial and periglomerular accumulation of CD4$^+$ T cells in MRL-*Fas*^{lpr} kidneys. RANTES-TEC placed under the renal capsule of MRL-*Fas*^{lpr} mice, induce a prominent accumulation of CD4$^+$ T cells (dark staining; arrows) outside the induced lesion, surrounding glomeruli (*A*), and within the interstitium (*B*) at 28 days post-implantation (Ix) (frozen tissue sections; magnification A ×1200 and B ×400).

of CSF-1 and RANTES in the recruitment of inflammatory cells, and assessed their combined impact on renal pathology in MRL-*Fas*^{lpr} mice. While both RANTES and CSF-1 promote an initial influx of Mø, they differ significantly in their attraction and propagation of T cell subsets: CSF-1 promotes the accumulation of CD4$^+$ and DN T cells, while RANTES promotes the accumulation of CD4$^+$ and CD8$^+$ T cells in the kidney of MRL-*Fas*^{lpr} mice. Since renal disease consists of CD4$^+$, CD8$^+$, DN T cells in MRL-*Fas*^{lpr} mice, it is likely that both RANTES and CSF-1 contribute to the progression of autoimmune renal injury.

Since CSF-1 and RANTES recruit distinct and complementary T cell subsets into the MRL-*Fas*^{lpr} kidney, we evaluated the impact of dual delivery of CSF-1 and RANTES on renal pathology in MRL-*Fas*^{lpr} mice. We would predict that the simultaneous recruitment of Mø, CD4$^+$ and CD8$^+$ T cells by RANTES, and Mø, CD4$^+$ and DN T cells by CSF-1, would create an explosive environment for inflammatory renal injury. However, we were surprised to determine that expression of both CSF-1 and RANTES in the MRL-*Fas*^{lpr} kidney resulted in an additive, not synergistic, increase of renal damage. Thus, unlike TNF-α, which we have previously shown to interact with CSF-1 to potently increase renal injury [14], RANTES mediates renal damage independently of CSF-1. Therefore, we conclude that CSF-1 and RANTES recruit distinct T cell subsets, which together constitute the total T cell populations that accumulate in spontaneous renal lesions of MRL-*Fas*^{lpr} mice, but these cytokines do not interact to enhance the infiltration of leukocytes into the kidney.

Table 2. Introduction of CSF-1 and RANTES into the kidney additively increases renal injury in MRL-*Fas*^{lpr} mice

Strain	*N*	TEC Ix[a]	Intrarenal lesion[b]
MRL-*Fas*^{lpr}	4	RANTES + CSF-1	1.1 ± 0.9
	3	RANTES + Uninf.	0.8 ± 0.3
	3	CSF-1 + Uninf.	0.3 ± 0.2
	3	Uninf.	0 ± 0

Values represent mean ± SE.
[a] RANTES + CSF-1: 1×10^6 TEC each; RANTES or CSF-1 alone: $1 \times 10^6 + 1 \times 10^6$ uninfected TEC to equalize *N* of cells. Mice were sacrificed at 28 days post-implantation (Ix) and the lesion extending from the implant site was quantified by [b] Grade of infiltration: 0 = none, 1 = outer cortex, 2 = inner cortex, 3 = outer medulla

The chemoattraction of inflammatory cells into a site of tissue damage such as the kidney is mediated by a cascade of molecular events, including endothelial cell activation, the expression of adhesion molecules, leukocyte-endothelial adhesive interactions, diapedesis and the migration of leukocytes beyond vascular barriers [33, 34]. There are several possible mechanisms whereby RANTES may facilitate T cell and Mø accumulation in the MRL-*Fas*^{lpr} kidney and incite renal damage, including: (*1*) direct chemoattraction [18], and/or (*2*) cellular activation [35, 36], and/or (*3*) cytokine induction. The probability of each mechanism is discussed below.

The recruitment of leukocytes from the circulation into the kidney via chemoattraction requires the maintenance of a stable chemokine gradient [33]. In addition, the leukocytes must overcome a forceful hydrostatic barrier. RANTES is expressed by renal mesangial and tubular epithelial cells (TEC), and by infiltrating T lymphocytes [21–24]. Furthermore, RANTES binds to proteoglycans on the surface of capillary endothelial cells, and thus provides a strong, constant chemokine gradient whereby leukocytes are attracted [37]. Following the recruitment of leukocytes, RANTES strengthens adhesive interactions between endothelial cells and T cells/Mø by up-regulating ICAM-1, ICAM-3, CD43, CD44 and CD11b/c [38, 39], and by enhancing adhesion of T cells to the extracellular matrix [25]. Thus, RANTES expression in the MRL-*Fas*^{lpr} kidney both initiates migration of leukocytes to this site and fosters leukocyte accumulation by enhancing the adhesion of the recruited cells.

In addition to directing chemotaxis of leukocytes, RANTES is a potent cellular activator of both T cells and Mø, and can induce cytokine production [18]. Activation of Mø by RANTES results in the release of a large number of monokines such as CSF-1, TNF-α, IL-1β, and IL-6 in the kidney [40]. It should be noted that each of these molecules are detectable in the MRL-*Fas*^{lpr} kidney

undergoing autoimmune destruction [3–8]. In addition, the activation of T cells by RANTES results in an autocrine up-regulation of RANTES [24], further compounding this inflammatory response. The initiation of a cytokine cascade, as a result of RANTES activation of MØ and T cells, leads to the additional recruitment of leukocytes and amplification of tissue damage. Thus, we propose that expression of RANTES in the MRL-*Fas*^{lpr} kidney prior to the onset of disease directs the extravascular migration of leukocytes and primes these cells to initiate tissue damage. Furthermore, the unleashing of this inflammatory reaction results in the continual increase in RANTES in the MRL-*Fas*^{lpr} kidney, and is in turn responsible for driving kidney destruction.

The cause of RANTES up-regulation in the MRL-*Fas*^{lpr} kidney is unknown. Multiple cytokine abnormalities in MRL-*Fas*^{lpr} mice may be responsible for an increase in RANTES in the kidney. For example, CSF-1, TNF-α, TGF-β and IL-1β are up-regulated in the kidney, and increases with progressive renal damage [3–8]. Since both TNF-α and IL-1β induce secretion of RANTES by mesangial cells, it is possible that dysregulation of TNF-α and IL-1β in the MRL-*Fas*^{lpr} kidney promotes the production of RANTES [20, 41]. Conversely, RANTES expression may precede CSF-1, TNF-α, and IL-1β, since activation of MØ by RANTES induces the release of these pro-inflammatory cytokines [40]. Thus, it is unclear whether RANTES is the primary molecular defect, or whether another cytokine is responsible for its induction. Taken together, the simultaneous dysregulation of RANTES, CSF-1, TNF-α, and IL-1β in the MRL-*Fas*^{lpr} kidney creates a volatile inflammatory environment; however, it is unclear which molecule(s) initiates this cascade.

Even though whether or not RANTES is induced by a cytokine(s) in the MRL-*Fas*^{lpr} kidney is yet unknown, it is clear that MRL genes which regulate MØ and cytokine abnormalities promote autoimmune kidney destruction. For example, MØ of MRL mice (++, *Fas*^{lpr}) hyperrespond to CSF-1 and TNF-α, resulting in rapid proliferation and accumulation of MØ in the kidney, and renal injury [13, 14]. We propose that the simultaneous expression RANTES, CSF-1, TNF-α, and IL-1β, and the unique responses of MRL-*Fas*^{lpr} MØ to CSF-1 and TNF-α, drive the fulminant renal injury characteristic of the MRL-*Fas*^{lpr} strain.

In conclusion, we report that RANTES is expressed in the kidney of MRL-*Fas*^{lpr} mice prior to onset of disease and increases with progressive nephritis. Using a retroviral gene transfer strategy, we establish that RANTES elicits autoimmune renal damage. Together this suggests that intrarenal RANTES in MRL-*Fas*^{lpr} mice is associated with autoimmune renal disease. In addition, we report that RANTES is responsible for the accumulation of CD4^{+} and CD8^{+}, but few DN T cells in the MRL-*Fas*^{lpr} kidney. Since we have established that CD4^{+} T cells are required to incite renal injury in MRL-*Fas*^{lpr} mice (manuscript in preparation), we predict that strategies that mask the expression of RANTES would be therapeutic and obviate autoimmune renal injury. Further studies with mouse Ab or antagonist molecules that would block intrarenal RANTES would be informative, however, these reagents are presently unavailable.

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APPENDIX

Abbreviations used in this article are: CSF-1, colony stimulating factor-1; DN, double negative; IL, interleukin; IX, implantation; MØ, macrophage; PBS, phosphate buffered saline; RANTES, Regulated upon Activation, Normal T cell Expressed and Secreted; TEC, tubular epithelial cells; TGFβ, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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