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Biphasic increase in circulating and renal TNF-α in MRL-*lpr* mice with differing regulatory mechanisms

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Biphasic increase in circulating and renal TNF-α in MRL-*lpr* mice with differing regulatory mechanisms. Tumor necrosis factor (TNF)-α contributes to expansion of lymphocytes in neonatal mice and can accelerate renal injury. T cells induced by the *lpr* gene promote renal injury. However, the *lpr* gene alone is insufficient to cause renal damage, since MRL-*lpr*, but not C3H-*lpr* mice develop lupus nephritis. In this study, we examined the temporal expression of TNF-α in the kidney and circulation of mice (MRL and C3H) with the *lpr* gene and their congenic counterparts (++). We measured a bioactive TNF-α using L929 cells and tissue expression with an avidin-biotin immunoperoxidase method. A biphasic increase in circulating TNF-α in MRL-*lpr* mice was detected. There was an initial peak in neonatal mice (703 ± 208 pg/ml) which normalized by two months of age (87 ± 13 pg/ml) and reascended proportional to the severity of renal injury (non-proteinuric 570 ± 87, proteinuric; 1255 ± 135 pg/ml). In addition, there was only a single peak in neonatal C3H-*lpr* mice (1270 ± 318 pg/ml) with a nadir by six weeks of age (434 ± 52 pg/ml). In contrast, serum TNF-α was low in MRL-++ and C3H-++ mice (80 ± 3 and 95 ± 30 pg/ml), respectively. TNF-α expression in kidneys paralleled the serum pattern in MRL-*lpr* mice. Enhanced TNF-α expression was restricted to tubular epithelial cells (TEC) in neonatal MRL-*lpr* and C3H-*lpr* mice, and not detected in congenics. In adult mice, intrarenal TNF-α expression was more ubiquitous and was detected in glomeruli, vascular smooth muscle and perivascular infiltrating cells as well as TEC. In addition, TNF-α expression intensified in the kidneys in proportion to the severity of proteinuria. TNF-α was absent in age matched C3H-*lpr*, C3H-++ and MRL-++ mice. Additional studies indicated that: (1) neither MRL-*lpr* or C3H-++ TEC constitutively secreted substantial amounts of TNF-α and required a cytokine stimulation; and (2) the clearance of TNF-α via TNF-α receptors was similar in MRL-*lpr*, MRL-++ and C3H-++ mice, suggesting the increase of serum TNF-α was not a result of a defect in clearance. Thus, these results indicate two distinct mechanisms of TNF-α regulation in MRL-*lpr* mice: (1) neonatal up-regulation related to the *lpr* gene; and (2) an increase in mature mice proportional to the severity of lupus nephritis.

Enhanced expression of cytokines contribute to the initiation and progression of the autoimmune tissue injury [1, 2]. An increase in multiple cytokines within the kidney of mice with autoimmune renal disease raised the possibility that cytokine expression promoted renal injury. Our previous studies established that an increase in the tumor necrosis factor (TNF)-α and interleukin (IL)-1 in the kidneys of autoimmune MRL-*lpr* mice was detrimental, since these cytokines increased in proportion to progressive tissue damage and delivery of either into autoimmune prone mice contributed to the acceleration of progressive renal damage [3]. The concept that an increase in cytokines may be instrumental in autoimmune renal disease is not limited to the MRL-*lpr* strain; numerous reports in other autoimmune mice (NZB × NZW F1 hybrid) and patients with systemic lupus erythematosus (SLE) document increases in several cytokines including, not only TNF-α and IL-1, but IL-6 and interferon (IFN)-γ [4–7].

The interaction of the *lpr* gene and the MRL strain background causes autoimmune renal disease. Neither the *lpr* gene alone nor the MRL background causes the fulminant autoimmune renal injury in MRL-*lpr* mice [8, 9]. Loss of renal function in MRL-*lpr* mice is obvious by three to four months of age, and progresses rapidly resulting in death by five to six months of age [8]. By comparison, congenic MRL-++ mice, lacking the *lpr* gene, express only a mild, indolent renal injury in the second year of life, while other strains with the *lpr* gene share abnormal serological features and hematopoietic cells characteristic of MRL-*lpr* mice, but are not compromised by kidney injury [9]. Therefore, using several strains with the *lpr* mutation allows us to determine whether a change in cytokine expression is related to the presence of *lpr* alone exclusive of renal injury.

While the increased expression of cytokines in the kidney during progressive tissue damage can be attributed to a chronic inflammatory process, the detection of cytokines in neonatal mice suggests the possibility that cytokine dysregulation may be central in the initiation of autoimmune tissue destruction. We previously established that newborn MRL-*lpr* mice express heightened levels of circulating macrophage stimulating factor-1 (CSF-1), while others report an elevation of serum IL-6 several weeks after birth [5, 10]. Furthermore, we have detected increased amounts of CSF-1 transcripts in the kidneys of MRL-*lpr* mice at one month of age, well in advance of renal injury [10, 11].

In this study we have investigated the temporal expression of TNF-α in the serum and in the kidney of MRL-*lpr* mice and identify the intrarenal cells expressing this cytokine beginning in neonates and extending throughout the progression of renal disease. Since the genetic and molecular analyses of the mouse *Fas* gene indicated that *Fas* is encoded by the gene at the locus of the mouse mutant *lpr*, and since the *Fas* antigen and ligand belong

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to the TNF receptor and TNF family, the relationship between TNF-α and the presence of *lpr* was also examined [12–16]. We now report that there is a biphasic increase in TNF-α in the serum and kidney of MRL-*lpr* mice with differing regulatory mechanisms; the increase in the second phase is proportional to the degree of renal damage. In addition, we establish that the increase in circulating TNF-α was not caused by a defect in the clearance of this cytokine prior to renal injury, and that up-regulation of the expression of TNF-α in TEC isolated from normal or MRL-*lpr* mice is similar and requires a stimulus to increase production.

Methods

Mice

Female MRL/MpJ-*lpr/lpr* (MRL-*lpr*), MRL/MpJ-+/+ (MRL-++), C3H/Fej and C3H/Hej (C3H-++), C3H/Hej-*lpr/lpr* (C3H-*lpr*) and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred and maintained in our facilities. Sera samples were stored at −70°C until TNF-α was measured.

Reagents

Tissue culture media were from Gibco (Grand Island, NY, USA) and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant mouse TNF-α was provided from Genentech (South San Francisco, CA, USA) and recombinant human TNF-α was purchased from Genzyme (Cambridge, MA, USA). Rabbit anti-mouse TNF-α antibody was a gift from Dr. A. Cerami (Picower Institute for Medical Research, Manhasset, NY, USA [17]). Antibody specificity for TNF-α was confirmed by Western blot analysis [18] and by the neutralization of L929 cell bioassay.

TNF-α L929 cell bioassay

We determined TNF-α activity in the sera and culture supernatants by a standard cytotoxicity assay using L929 mouse fibroblasts [American Type Culture Collection (ATCC), Rockville, Maryland, USA] [19]. Briefly, we plated 5 to 7 × 10^4 L929 cells/well into 96-well flat bottom plates (Costar, Cambridge, MA, USA), then added the sera or supernatants to wells containing an equal volume of RPMI 1640 media with 10 μg/ml of actinomycin D. These cultured supernatants were added undiluted or diluted at 1:2, 1:4, and 1:8. The initial serum samples were diluted 1:10 (or 20 μl/well). We incubated the plates for 24 hours at 37°C in a humidified chamber with 5% CO_2, then removed the media, and added 0.1% crystal violet in methanol to each well for 20 minutes. After rinsing with distilled water, we added 100 μl of 0.5% SDS to each well. Each sample was analyzed in triplicate. The standard curve was generated by using serial dilutions of murine recombinant TNF-α (100 to 0.4 pg of TNF-α/well/200 μl or 500 to 2 pg/ml). TNF-α activity was neutralized with anti-TNF-α antibody (100 ng/ml). Optical densities were read on a 96-well microtiter plate reader at 570 nm (Vmax kinetic microplate reader, Molecular Devices Corp., Menio Park, CA, USA). The lowest detectable level was 10 pg/ml.

Immunohistochemical detection of TNF-α

TNF-α was localized in tissue sections using immunohistochemical techniques. We fixed tissues in 10% phosphate-buffered formalin, embedded in paraffin and detected TNF-α with an immunoperoxidase technique. Briefly, we blocked endogenous peroxidase activity with 0.6% hydrogen peroxide and 0.2% sodium azide for 10 minutes, and then washed the tissue sections several times with 0.01 M Tris, pH 7.4, 0.85% NaCl (TBS) containing 0.1% bovine serum albumin (BSA) followed by permeabilization with bovine testicular hyaluronidase (1 mg/ml in 0.1 M sodium acetate, pH 5.5) for 30 minutes in a humidified chamber at 37°C. To block endogenous avidin and biotin we used an Avidin/Biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were then incubated with anti-murine TNF-α antibody (1:150 dilution) in a humidified chamber for two hours at room temperature, washed extensively and exposed to biotinylated goat anti-rabbit IgG antibody for one followed by avidin-peroxidase complex using the Vectastain ABC Elite Kit (Vector Laboratories). Sections were then exposed to 3-3′-diaminobenzidine (0.5 mg/ml in TBA containing 0.015% H_2O_2) for a chromogenic reaction and counterstained with methyl green-alcian blue. Specificity controls included the replacement of primary antibody with normal rabbit IgG and the incubation with the anti-murine TNF-α antibody neutralized with a 20-fold molar excess of TNF-α (Genentech, South San Francisco, CA, USA). Sections were also stained for alkaline phosphatase to identify the brush border of proximal TEC using the Vector Red ALP reagent kit (Vector Laboratory).

Cell culture and cell lines

Renal TEC lines derived from C3H/Fej mice (CS3.1) and MRL-*lpr* mice (M3.1) were transfected with antigen defective SV40 and characterized as previously described [20]. TEC were cultured in K1 medium (1:1 mixture of DMEM4.5 and Ham's F12 medium), containing 5% fetal calf serum (FCS), 1% penicillin and streptomycin and hormonal additives. We obtained P388D1 (p388) cells from the ATCC, and these cells were cultured in RPMI1640 with 10% FCS and 1% penicillin and streptomycin.

Primary TEC cultures from MRL-*lpr* and C3H/Fej mice were prepared beginning at three months of age by sequential sieving techniques as previously reported [20]. Primary TEC cultures from each kidney were plated on four collagen-coated 100 mm dishes (day 0), the medium was exchanged on day 5 and the cells were transferred onto collagen-coated 24 well plates (Falcon, 1 × 10^5/well) three days later.

Preparation of conditioned medium

All cell lines and primary cells were grown in 24 well plates seeded at a density of 1 × 10^5/well for 24 hours, in K1 media. After the cells were confluent, we replaced K1 media with DMEM1.0 media without FCS three times during the next 24 hours to synchronize the cell cycle at the G_0 stage. Finally, DMEM1.0 or DMEM1.0 containing LPS (1 μg/ml), IL-1α (100 ng/ml) or CSF-1 (10 ng/ml) was added, and the supernatants were collected after 24 hours and stored at −70°C.

In vivo clearance of exogenous recombinant murine and human TNF-α

Since mouse TNF-R1 binds to both mouse and human TNF, while mouse TNF-R2 binds exclusively to mouse TNF [21], we can determine whether the TNF-α clearance is related to the 55 kDa receptor (TNF-R1) and/or the 77 kDa receptor (TNF-R2). Therefore, either recombinant mouse (250 ng/mouse) or human TNF-α

(500 ng/mouse) was i.p. injected into C3H-++, MRL-++ and MRL-*lpr* mice two months of age (N = 5/group). In preliminary study, several doses of mouse or human TNF-α were injected to determine the adequate dose for the clearance study. Mice two months of age were chosen for injection since serum TNF-α levels before injection and renal function were similar in three strains. Mice were bled at 30, 60, 120 and 180 minutes later. Bioactive TNF-α levels were measured by a L929 assay.

Statistics

We performed statistical analysis using Statview SE+ (Abacus Concepts, Berkeley, CA, USA), by comparing the differences between groups using a one way ANOVA, unpaired t-test, Wilcoxon single-rank sum test, Fisher's exact test, or Pearson's correlation coefficient for the analysis of parametric and non-parametric data. All results were expressed as mean ± SEM.

Results

*Circulating TNF-α was increased biphasically in MRL-*lpr *as compared to MRL-++ and C3H-++ mice*

There was a biphasic increase in bioactive TNF-α levels in the serum of MRL-*lpr* mice as compared to MRL-++ and C3H-++ mice (Fig. 1). Circulating levels of TNF-α initially peaked in neonatal mice (day 7), normalized by two months of age, and then reascended continually and steadily throughout the remaining lifespan. A comparison of serum TNF-α in MRL-*lpr* mice two to six months of age indicated that mice that became proteinuric had substantially higher serum TNF-α levels as compared to non-proteinuric mice (Fig. 1A). In fact, although there was a modest increase in serum TNF-α in neonatal MRL-++ (4 times less than MRL-*lpr*), this increase was transient and circulating TNF-α in MRL-++ was barely detectable after this initial rise. A comparison of serum TNF-α levels of MRL-++ at six months of age with several normal strains age and sex matched, including C3H/Fej, C3H/Hej (Fig. 1B) and BALB/c indicated they were similar (80 ± 3 pg/ml, 95 ± 23 pg/ml, 134 ± 22 pg/ml, respectively; N = 5/group). There was a positive correlation between the amount of serum TNF-α and the degree of proteinuria (r = 0.84, $P < 0.001$, N = 22). Serum TNF-α detection was specific since L929 cell cytotoxicity was completely neutralized with anti-TNF-α antibody.

Elevated serum TNF-α in neonates is associated with the presence of lpr

Circulating TNF-α levels was higher in C3H-*lpr* mice as compared with age- and sex-matched normal C3H/Fej or C3H/Hej mice. Circulating TNF-α levels in C3H-*lpr* mice peaked in neonatal mice (day 7), declined to a nadir by six weeks of age and never reascended (Fig 1B). However, detectable levels of serum TNF-α in C3H-*lpr* mice were three to five times higher than C3H mice lacking the lpr gene. The serum TNF-α in C3H/Fej and C3H/Hej remained minimal and stable from one week through six months of age (Fig. 1B). Since there was no difference between serum TNF-α levels in C3H/Fej and C3H/Hej, we refer to these mice as C3H-++.

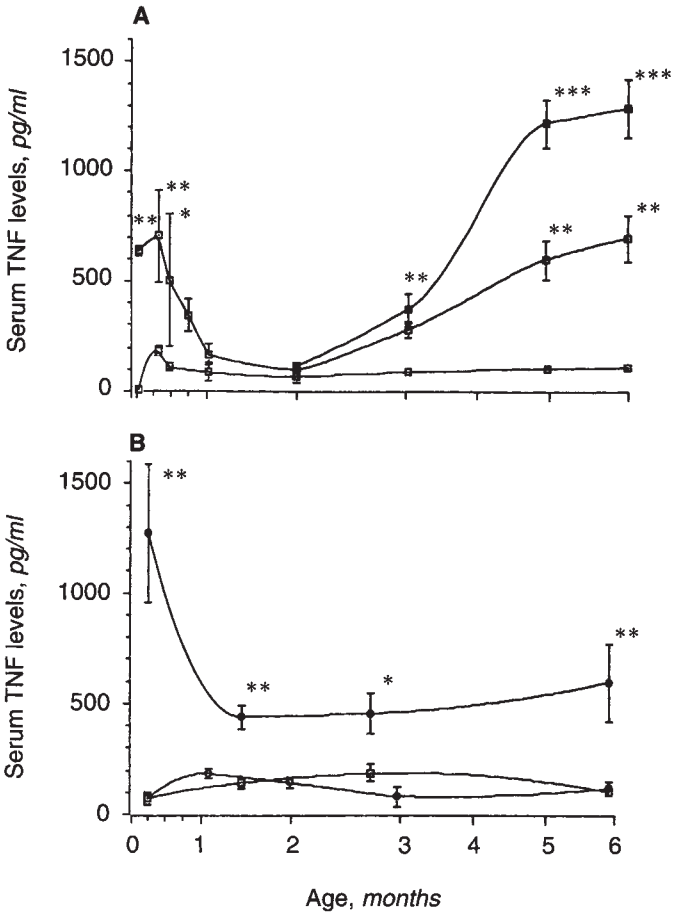

Fig. 1. *Serum TNF-α was increased in MRL-*lpr *and C3H-*lpr *mice as compared to congenic strains.* We determined TNF-α activity using a standard L929 mouse fibroblasts cytotoxicity assay. **A.** There was a biphasic increase in circulating TNF-α in MRL-*lpr* mice. TNF-α was compared in proteinuric and non-proteinuric MRL-*lpr* mice beginning at 5 months of age. There was a marked elevation of circulating TNF-α in proteinuric MRL-*lpr* mice. Values are expressed as x ± SEM proteinuric MRL-*lpr* (■, N = 6), non-proteinuric MRL-*lpr* (⊡, N = 3–9) and MRL-++ (□, N = 3–5), (MRL-*lpr* proteinuric and non-proteinuric vs. MRL-++), $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$. **B.** Serum TNF-α levels were elevated in neonatal C3H-*lpr* mice as compared to normal C3H/Fej and C3H/Hej mice. C3H-*lpr* (N = 3–5), C3H/Hej (N = 4), and C3H/Fej (N = 3–5). C3H-*lpr* vs. C3H/Fej and C3H/Hej, $^*P < 0.01$, $^{**}P < 0.001$.

*Renal TNF-α expression is enhanced in neonatal MRL-*lpr *and C3H-*lpr *mice as compared with MRL-++ and C3H-++ mice exclusively in TEC*

We investigated the temporal pattern of TNF-α expression in the kidneys of neonatal MRL-*lpr*, MRL-++, C3H-*lpr* and C3H-++ mice. There was a greater expression of TNF-α in cortical and medullary TEC in MRL-*lpr* and C3H-*lpr* mice during the first week of life as compared to their congenic counterparts (Fig. 2, Table 1). The increase TNF-α was higher in the cortex than medulla. Since the brush border are poorly developed in neonatal mice, we were unable to detect the alkaline phosphatase reaction product. Increased TNF-α expression was transient in TEC of MRL-*lpr* mice, and decreased dramatically by the second week of life (Table 1). This enhanced expression of TNF-α in neonates was limited to the TEC, since the level of TNF-α in the

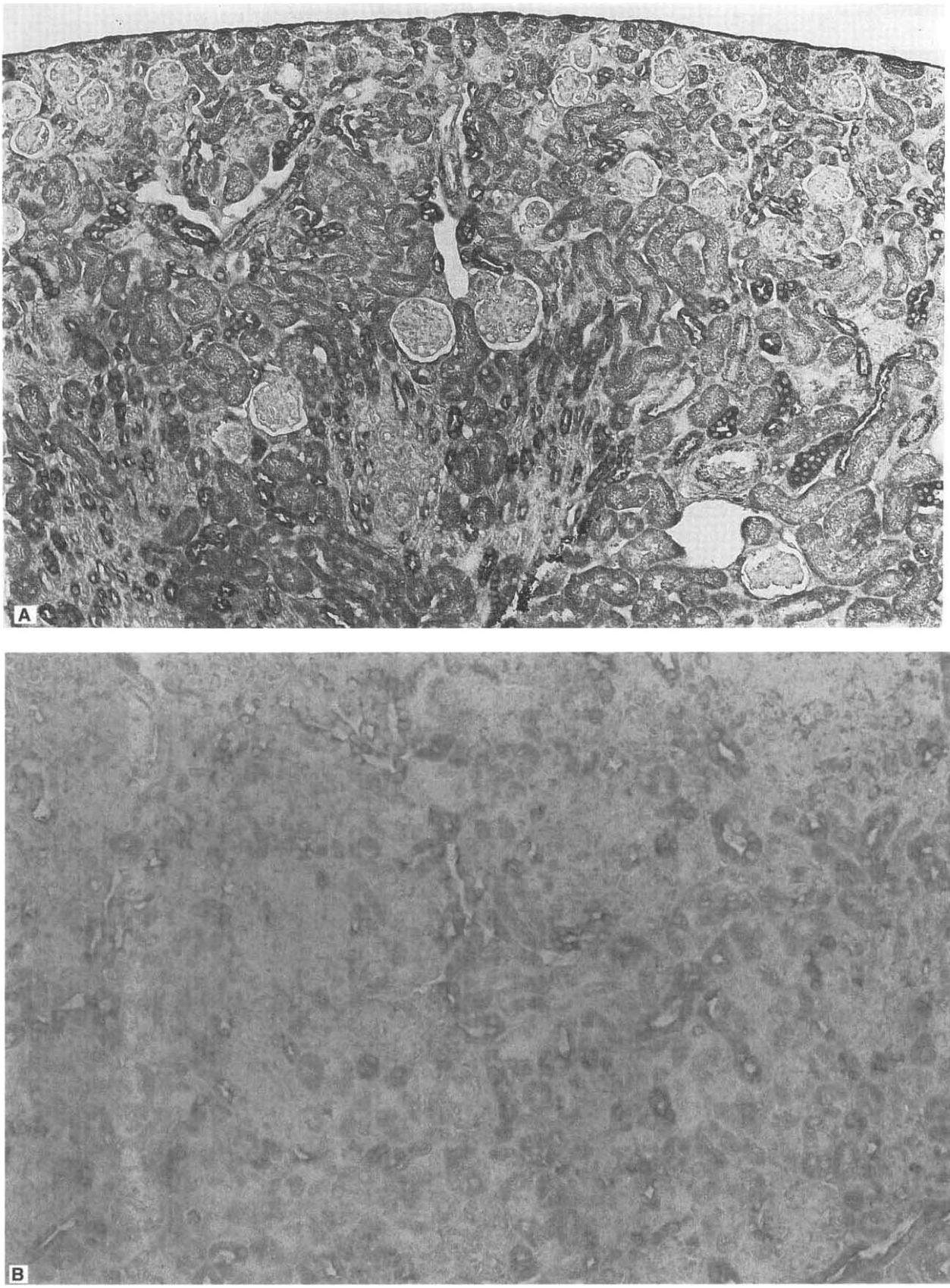

Fig. 2. *Localization of TNF-α in the kidney in neonatal and adult MRL-*lpr *mice.* TNF-α was detected by the avidin-biotin immunoperoxidase method using polyclonal rabbit anti-murine TNF-α antibody. **A.** An increase in TNF-α was detected in cortical TEC (3+) from MRL-*lpr* mice (5 days). (× 100) **B.** TNF-α was barely detectable in TEC from MRL-++ mice (7 days). (× 250) **C.** Proteinuric MRL-*lpr* mice (6 months) expressed TNF-α in the cortical TEC (Grade = 3+), glomeruli (Grade = 2), and perivascular mononuclear cell infiltrates (arrow, Grade = 3+ focal). (× 500) **Insert** upper right. In this glomerulus TNF-α is detected in epithelial cells (arrows) and mesangial cells (× 1000). **D.** TNF-α is barely detectable in C3H/Fej mice (5 months) (× 250).

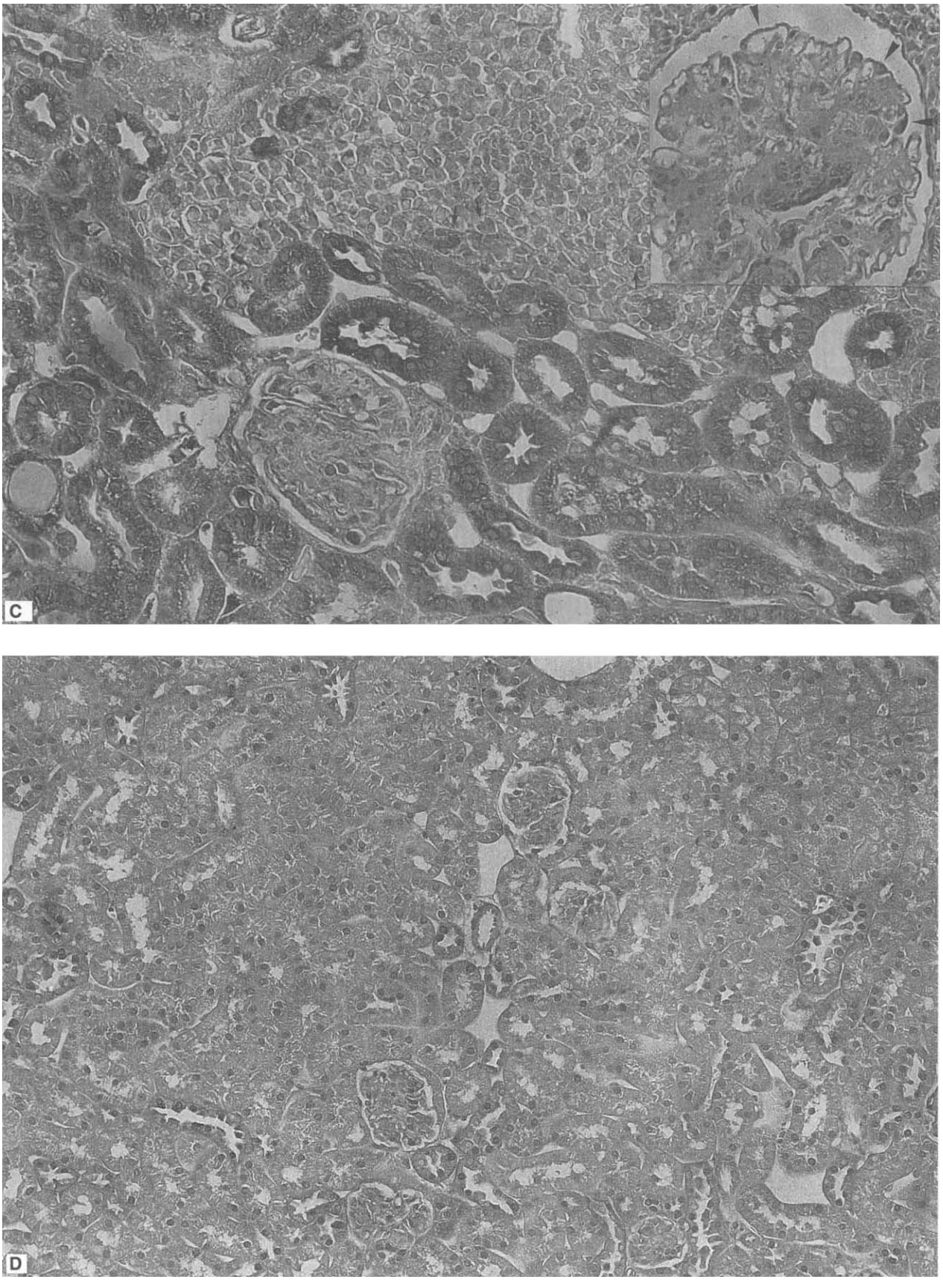

Fig. 2. *Continued.*

Table 1. Enhanced TNF-α in the kidneys of neonatal MRL-*lpr* and C3H-*lpr* mice

Strain	Age *day*	*N*	TEC Cortex[a]	TEC Medulla
MRL-*lpr*	1–3	2	2.5(F)	1.2
	4–7	5	3.0[b]	0.8
	10–14	8	1.5	0.4
MRL-++	1–3	5	1.0(F)	0.5
	4–7	4	0.8(F)	0.5
	10–14	2	0.5	0.0
C3H-*lpr*	4–7	3	2.5(F)[c]	1.5
C3H-++	4–7	5	0.5(F)	0.5

Paraffin embedded kidneys were stained for the presence of TNF-α.
[a] Grade = 0, not detectable; 1, weak; 2, moderate; 3, intense. Abbreviation F is focal. Values = x ± SEM; all SEM were below 0.5.
[b] MRL-*lpr* (4–7 days) vs. MRL-++ and C3H-++, $P < 0.05$
[c] C3H-*lpr* (4–7 days) vs. C3H-++, $P < 0.05$

Table 2. Renal TNF-α expression increases with progresive renal injury in MRL-*lpr* mice

Mouse Strain	Age	n	Up[a]	TEC[b]	Glom	PVI	VSMC
MRL-lpr	2 weeks	6	0.5	1.5(F)	0	0	1.0
	6 weeks	6	0.5	1.7(F)	0.5	0	1.5
	3 months	4	1.2	2.5	1.0	1.0	1.5
	4 months	5	2.2	2.5[c]	1.5[c]	2.0[c]	1.7
	>5 months	4	3.5	3.0[c]	2.0[c]	3.0[c]	3.0[c]
MRL-++	6 weeks	7	0.5	1.3(F)	0.5	0	1.3
	>4 months	4	1.0	1.0	0.8	0	1.7
C3H-lpr	6 weeks	3	0.5	1.0(F)	0.5	0	1.0
	>4 months	2	1.0	1.5	0.5	0	1.5
C3H-++	6 weeks	5	0.5	0.5(F)	0.5	0	1.0
	>4 months	3	1.0	0.5	0.5	0	1.0

Paraffin embedded kidneys stained for the presence of TNF-α. Values = x ± SEM, all SEM were below 0.5. Abbreviations are F, focal; Glom, glomeruli; PVI, perivascular infiltrates; VSMC, vascular smooth muscle cells.
[a] Urinary protein, 0.5 = trace, 1 = 30 mg/dl, 2 = 100 mg/dl, 3 = 300 mg/dl, 4 = ≥2,000 mg/dl (value ≥2 are pathological proteinuria)
[b] Grade = 0, not detectable; 1, weak; 2, moderate; 3, intense
[c] MRL-*lpr* (4–5 months) vs. MRL-++, C3H-*lpr*, and C3H-++ (>4 months), $P < 0.05$

glomerular mesangial cells and vascular smooth muscle cells was similar in MRL-*lpr*, MRL-++, C3H-*lpr* and C3H-++ mice.

*Enhanced renal TNF-α expression increases with progressive renal injury in MRL-*lpr *mice*

The kidneys of MRL-*lpr* mice with proteinuria displayed a more diffuse and intense TNF-α as compared to MRL-*lpr* mice with normal urinary protein levels and age and sex matched MRL-++ as C3H-++ mice (Table 2). This increased expression was particularly notable in the TEC, glomeruli, vascular smooth muscle and perivascular infiltrating cells (Table 2). The majority (50 to 60%) of TEC which expressed TNF-α were positive for alkaline phosphatase reaction. By comparison, renal TNF-α expression was similar in MRL-++, C3H-*lpr* and C3H-++ mice. In addition, we detected greater TNF-α expression in TEC surrounded by mononuclear cellular infiltrates as compared to TEC in areas without these infiltrates (Fig. 2, Table 3). Furthermore a large proportion (30 to 50%) of these perivascular mononuclear

Table 3. TNF-α expression on TEC is enhanced by mononuclear cell infiltrates in MRL-*lpr*

TEC[a]	MNC	MRL-*lpr* age *months* 4	MRL-*lpr* age *months* 5	C3H-*lpr* 5
+	+	2.6 ± 0.2[c]	2.8 ± 0.2	—[d]
+	−	1.5 ± 0.2	1.7 ± 0.2	1.5 ± 0.2
Background[b]		0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.1

Paraffin embedded kidneys were stained for the presence of TNF-α.
Abbreviation MNC is mononuclear cell infiltrates. MRL-*lpr* 4 or 5 months: MNC (+) vs. MNC (−) $P < 0.05$.
[a] TEC were evaluated in 10 separate areas ± MNC
[b] Normal rabbit IgG
[c] Grade = 0, not detectable; 1, weak; 2, moderate; 3, intense
[d] No MNC infiltrates
Values = x ± SEM.

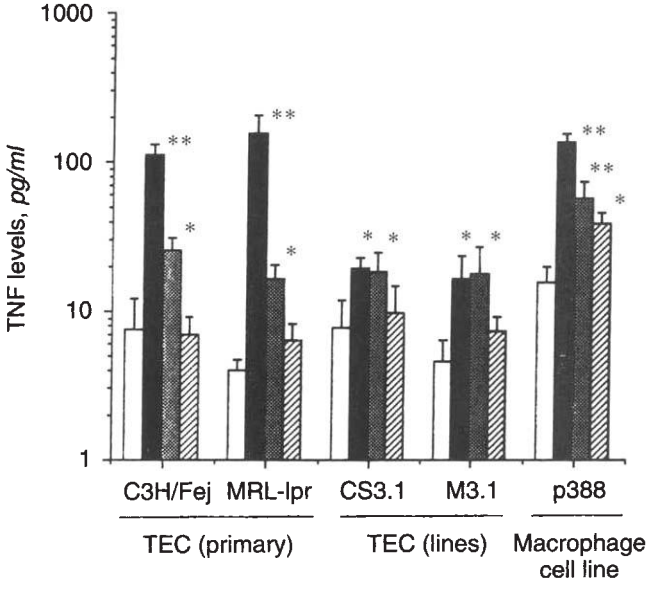

Fig. 3. *TNF-α production in primary cultured TECs and TEC cell lines (M3.1 and CS3.1) derived from MRL-*lpr *and C3H-++ mice.* Confluent TECs and p388 cells (1×10^5/well) were stimulated for 24 hours with LPS (1 μg/ml, ■), IL-1α (100 ng/ml, ▩) and CSF-1 (10 ng/ml, ▨). Results are means of triplicate samples and representative of two experiments. Stimulated vs. unstimulated cells are $P < 0.05$ and $P < 0.01$. Constitutive production of TNF-α by TEC was similar in primary cultured and TEC lines derived from MRL-*lpr* or C3H-++ mice. TNF-α production was increased in TEC by stimulating with LPS or IL-1α. TNF-α production was similarly inducible in MRL-*lpr* and C3H-++ mice. In contrast, CSF-1 did not induce TNF-α in TEC. As a control, the macrophage cell line P388D1 was stimulated with LPS, IL-1α or CSF-1 and produced increased amounts of TNF-α.

infiltrates also expressed TNF-α. TNF-α expression was also detected by this procedure in the majority of mononuclear cells in the enlarged spleens of MRL-*lpr* and C3H-*lpr* mice.

*TEC derived from MRL-*lpr *and C3H-++ mice secrete similar amounts of TNF-α*

TNF-α in the supernatants of primary cultured TEC from MRL-*lpr* mice (4 ± 1 pg/ml) or C3H-++ mice (8 ± 5 pg/ml) three months of age were similar (Fig. 3). TNF-α production was readily

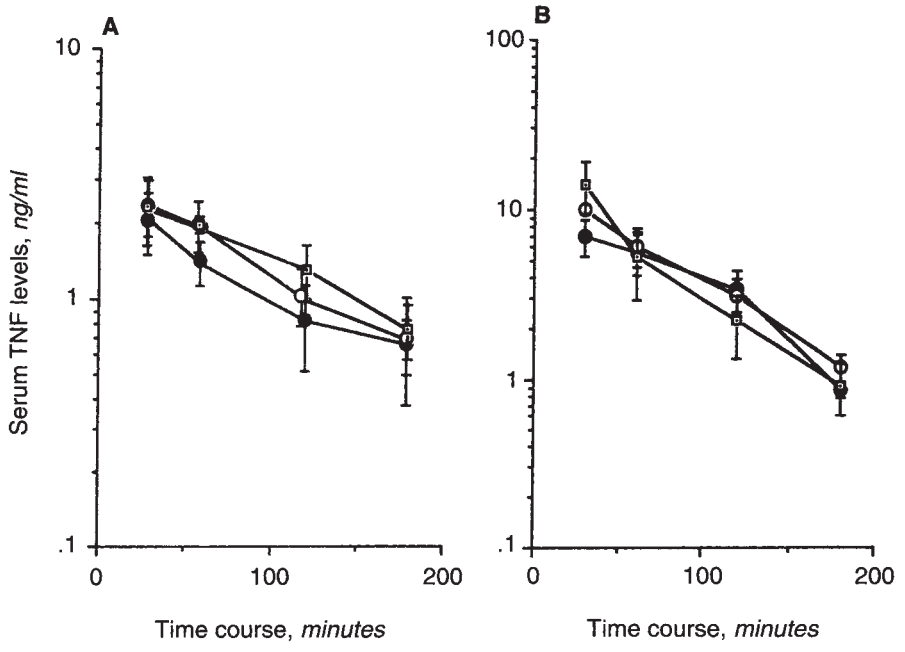

Fig. 4. *The clearance of exogenous murine and human TNF-α was similar in MRL-lpr (●), MRL-++ (○) and C3H-++ (⊡) mice.* To investigate the ability of TNF-R1 and TNF-R2 to remove TNF-α from the circulation, we injected **(A)** recombinant mouse (250 ng) or, **(B)** recombinant human (500 ng) TNF-α (i.p.) into C3H-++, MRL-++ and MRL-*lpr* mice of 2 months of age. The clearance rate of mouse and human TNF-α was similar in MRL-*lpr*, MRL-++ or C3H-++ mice. Values = x ± SEM (N = 5, at each point).

increased with either LPS, IL-1α, but not CSF-1 and similar in the MRL-*lpr* and C3H-++ strains. In addition, spontaneous and stimulated TNF-α production was similar in TEC cell lines (M3.1 and CS3.1) and primary cultured TEC. The macrophage cell line, P388D1, was used as a positive control.

*Clearance of exogenous murine and human TNF-α is similar in MRL-*lpr*, MRL-++ and C3H-++ mice*

The levels of bioactive TNF-α after injection and the time required to remove mouse and human recombinant TNF-α were virtually identical in MRL-*lpr*, MRL-++ and C3H-++ mice (Fig. 4 A, B). The calculated clearance time ($=t_{1/2}$) was 80 to 94 minutes and 40 to 51 minutes for mouse and human TNF-α, respectively. Thus, since the clearance of TNF-α via TNF receptors (TNF-R1 and TNF-R2) was similar in MRL-*lpr*, MRL-++ and C3H-++ mice, enhanced serum TNF-α does not appear to be a consequence of a defect in the clearance.

Discussion

In this study we reported a biphasic increase in TNF-α in the circulation and kidney of autoimmune MRL-*lpr* mice. The first phase increase detected in neonatal mice is transient, disappears after several weeks and is associated with the presence of the *lpr* gene. In contrast, the second phase increase is related to the severity of renal tissue injury. In neonates up-regulated TNF-α expression is restricted to TEC, while TNF-α expression in mice with progressive lupus nephritis is more broadly and abundantly expressed to include TEC, vascular smooth muscle cells, mononuclear infiltrating cells, glomerular epithelial cells and cells in the mesangial area. It is of interest that the temporal expression of TNF-α during each phase in the kidney and circulation are similar.

The increase in TNF-α in the kidney and circulation during the second phase can be explained by evolving renal injury. Our previous studies established an increase in TNF-α proportional to the progression of renal injury in MRL-*lpr* mice [3], identified a secreted and a membrane bound form of TNF-α following the stimulation of TEC in normal kidneys [18, 20], and reported that macrophages accumulating in the glomeruli of mice with lupus nephritis expressed high levels of TNF-α transcripts [11]. We have now identified which intrarenal cells are responsible for secreting TNF-α during progressive increase of this cytokine in the kidney and circulation proportional to the increase in severity of renal injury. Since we previously established that renal injury can be accelerated by administering TNF-α systemically once renal injury is initiated, we would predict that the persistent exposure of this cytokine via the circulation or intrarenally is detrimental to the kidney [4]. Enhanced expression of cytokines during renal injury is not limited to TNF-α; we have reported an increase in CSF-1, TGF-β and IL-1 in the kidney as well as an abundance of CSF-1 in the circulation [3, 10–11, 19]. We have determined that the kidney is the major source of circulating CSF-1 since transplanting a MRL-*lpr* kidney with mild nephritis into a bilateral nephrectomized congenic strain (MRL-++) causes an increase in CSF-1 in the circulation of non-autoimmune recipient. We would predict that during progressive renal destruction that the circulation is a reservoir for many cytokines that are being pumped out of this damaged tissue undergoing autoimmune attack. In support on this concept, it is interesting to note that the temporal expression of increased TNF-α in the kidney and circulation are similar. We are currently measuring elevations in the circulation of many of the cytokines known to increase within the kidney during murine lupus nephritis and investigating whether the kidney alone is the source responsible for an increase of these cytokines in the serum.

Increasing evidence supports the concept of a regulatory defect for cytokines in MRL-*lpr* mice. In fact, over a decade ago several of the original studies investigating mechanisms of autoimmune injury in this strain reported a defect in the production of IL-2 [22]. More recent findings note enhanced TNF-α and IL-1β gene expression [3], an increase in the production of TNF-α in Kupffer

cells of MRL-*lpr* mice [23] and an increase of TNF-α transcription and production in T cells in mice with the *lpr* mutation [24, 25]. To identify the primary cytokine defect and distinguish it from a secondary response, it is important to identify cytokine expression during the earliest stages of development. Therefore, we have investigated the production of cytokines beginning in newborn mice. We have previously established an increase in serum CSF-1 in the circulation of MRL-*lpr* neonates [10]. We now document a similar increase in TNF-α, not only in the circulation, but also expressed by TEC in the kidney. This temporal expression of peak TNF-α levels is similar in the circulation and kidney. While increased CSF-1 expression in neonates is occurs in MRL-*lpr* but not other strains with the *lpr* mutation and, therefore, is not linked to the *lpr* gene, TNF-α is overexpressed in neonates in strains bearing *lpr*. At least in adult MRL-*lpr* kidneys, CSF-1 is not constitutively overexpressed, but dependent on a stimulus, such as TNF-α, to induce this cytokine. This is based on experiments in which a transplanted MRL-*lpr* kidney placed into a bilaterally nephrectomized MRL-++ recipient loses the capacity to increase serum CSF-1 after several weeks, indicating a circulating stimulant in the MRL-*lpr* mouse is required for persistent production of this cytokine (manuscript in preparation). In support of this concept, TNF-α is not constitutively overexpressed in TEC derived from MRL-*lpr* mice three months of age, suggesting that TNF-α may also be up-regulated in response to at least another stimulant in adult mice. Since CSF-1 cannot induce TNF-α but TNF-α induces CSF-1, it is unlikely that upregulation of CSF-1 precedes TNF-α. It is, therefore, more plausible that overexpression of TNF-α may be attributed to a primary regulatory defect. This concept is bolstered by the association of neonatal expression of TNF-α with the presence of the *lpr* mutation. It should also be noted that in neonates TNF-α expression is mild and diffusely expressed in TEC, while this expression is further increased in TEC surrounded by infiltrating mononuclear cells in adult MRL-*lpr* mice. It is possible that there is a primary regulatory defect which is influenced by cytokines released by the mononuclear cells invading the kidney. Future studies will test the hypothesis that there is a regulatory defect in TNF-α which is responsible for inducing lupus nephritis.

Since an increase in circulating levels of TNF-α could be caused by overproduction and/or impaired removal, we explored the possibility that MRL-*lpr* mice had a defective clearance of this cytokine. Bioactive TNF-α is rapidly removed from circulation in a two step process; first the molecule is biologically inactivated by binding to the circulating soluble form of TNF-R and then the TNF-TNF-R complex is cleared from circulation [26]. Recent studies indicate that the removal of TNF-TNF-R is dependent on the kidney, since following bilateral nephrectomy of normal mice TNF-TNF-R complex cannot be cleared from circulation [26]. We evaluated the removal of TNF-α by injecting either mouse or human TNF-α into mice at two months of age. We selected this age since circulating TNF-α is at its nadir in MRL-*lpr* mice and would not obscure evaluation of the clearance rates, and because this is a period prior to overt renal injury. We can determine whether the TNF-α clearance is related to the 55 kDa receptor (TNF-R1) and/or the 77 kDa receptor (TNF-R2), since mouse TNF-R1 binds to both mouse and human TNF, while mouse TNF-R2 binds exclusively to mouse TNF [21]. Since either form of TNF-α had a similar clearance rate in MRL-*lpr*, congenic MRL-++ or C3H/Fej strains, we conclude that the clearance of TNF-α is not impaired in these adult mice prior to the loss of renal function.

Lymphoid abnormalities, a central feature of mice with the *lpr* mutation, may be directly introduced by overexpression of TNF-α. This is based on studies reporting that anti-mouse TNF-α antibodies initiated during pregnancy or at the birth, result in a marked atrophy of the thymus, spleen, lymph nodes, lymphopenia and impaired development of T and B cell peripheral lymphoid structures [27]. It is of interest that all strains with the *lpr* gene have a prominent lymphoid proliferation consisting of a unique T cell which accumulate predominantly in the spleen, lymph nodes and kidney [10, 24, 25, 28, 29]. Thus, an overproduction of TNF-α may cause the expansion of these lymphoid cells. In addition, the Fas antigen is absent in strains with the *lpr* mutation and may be responsible for failure to delete this unique type of T cell [30]. Since the Fas antigen belongs to the same TNF-α receptor family [12, 13, 16], it will be important to clarify the relationship between the inability to express this antigen and overexpression of TNF-α. Further experiments will also be directed towards understanding the importance of an increase in TNF-α in neonates in this autoimmune strain with lymphocyte abnormalities.

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