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Retinoic acid treatment protects MRL/lpr lupus mice from the development of glomerular disease

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Retinoic acid treatment protects MRL/lpr lupus mice from the development of glomerular disease.

Background. Retinoic acid (tRA) is an active metabolite of vitamin A with potent anti-inflammatory properties. We analyzed the effects of tRA on the development of lupus nephritis in MRL/lpr mice.

Methods. MRL/lpr mice received chow supplemented with vehicle or tRA (daily 10 mg/kg) from 8 to 14 weeks until their sacrifice. MRL/wt mice served as an additional control.

Results. tRA-treated MRL/lpr mice showed reduced lymphoadenopathy and splenomegaly as compared to vehicle-treated controls. Treatment reduced proteinuria to almost basal levels. Plasma IgG and anti-DNA antibodies increased comparably in both vehicle and tRA-treated mice. Vehicle-treated mice showed characteristic renal lesions. In contrast tRA-treated mice showed almost normal glomerular histology with a pronounced reduction in endocapillary cell proliferation. T-cell and macrophage infiltrates were reduced after tRA treatment within glomeruli and interstitium as compared to vehicle-treated animals. In spite of this, immune complex and complement deposition were comparable in both groups. Adoptively transferred T cells from vehicle-treated to tRA-treated MRL/lpr mice did not induce renal lesions or proteinuria. These beneficial effects of tRA treatment were associated with reduced renal expression of chemokines and inflammatory cytokines. Surprisingly, renal transforming growth factor-β (TGF-β) mRNA levels of tRA-treated mice were elevated, possibly indicating that TGF-β acts as an anti-inflammatory signal in this lupus model.

Conclusion. tRA treatment reduces lymphoproliferation and glomerulonephritis in MRL/lpr mice. This occurs in spite of unaltered anti-DNA titers and glomerular immune complex deposition, and cannot be overcome by T-cell transfer from nephritic MRL/lpr mice.

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Key words: lupus nephritis, retinoic acid, MRL/lpr mice, autoimmunity, inflammation.

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Treatment with retinoic acid (tRA) derivates has been reported to ameliorate renal disease in a variety of experimental models [1–9]. tRA is a natural active derivate of vitamin A that regulates a broad range of biologic processes through specific nuclear receptors [retinoic acid receptors (RAR-α, RAR-β, and RAR-γ or RXR-α, RXR-β, and RXR-γ)], which are structurally and functionally related to glucocorticoid receptors [10]. tRA is physiologically essential for embryonic development, organogenesis, and organ functions [11,12]. Among other actions, tRA has anti-inflammatory effects, reducing the infiltration of leukocytes (mononuclear and polymorphonuclear cells) into sites of inflammation. Reduction of the expression of monocyte chemoattractant protein-1 (CCL2/MCP-1) and other inflammatory cytokines by tRA has been demonstrated both in vitro [13,14] and in vivo [2, 6, 9]. It has been postulated that part of the anti-inflammatory effect of tRA may be due to enhanced transforming growth factor-β (TGF-β) generation [15,16]. TGF-β is a multifactorial cytokine that has major anti-inflammatory actions but also contributes to chronic fibrosing precesses [17,18]. In addition, tRA is a potent antiproliferative agent for different cell types, including renal glomerular cells [19,20], promotes cell differentiation [10], modulates extracellular matrix remodeling [1], and regulates apoptotic pathways in vitro [10, 21]. Dietary deficiency of vitamin A has been reported to affect innate and acquired immune response in humans and rodents [22,23]. In vitro incubation of naive Th0 cells with tRA leads to a Th2-type response by RAR-mediated mechanism [22]. In vivo, tRA seems to regulate

cellular immune response, since vitamin A deficiency leads to a shift from a Th2-type to a Th1-type response by increasing interferon-γ (IFN-γ) and interleukin (IL)-12 mRNA synthesis while IL-4 and IL-5 production is decreased [23]. These effects are reversed by supplementation with retinoids [23].

The anti-inflammatory and antiproliferative effects of tRA have been examined in several types of kidney diseases in animal models. In the rat model of Thy 1-1 mesangioproliferative glomerulonephritis tRA reduces mesangial expansion, glomerular damage, and proteinuria [2,3]. tRA also promoted the recovery of podocyte function and structure and proteinuria in puromycin aminonucleoside-induced nephrosis in the rat [4,5]. tRA has also beneficial effects on tubulointerstitial damage in a rat model of unilateral ureteral obstruction (UUO) model, an effect which is associated with reduced expression of matrix components [8]. Finally, tRA significantly ameliorated functional, vascular, glomerular, and tubulointerstitial lesions in a rat model of acute renal graft rejection [7]. Recently, Kinoshita et al [6] showed that tRA treatment reduced autoimmune-mediated kidney damage and increased survival of NZB/W F1 lupus mice. The authors attributed this beneficial effect of tRA in this mouse strain to decreased IFN-γ and IL-2 expression and a consequent shift of the immune response toward a Th2 phenotype.

In the present study we examined the effects of tRA on the development of lupus-like renal disease in the murine model of MRL/lpr mice. These animals carry a mutation producing a nonfunctional fas protein, and, as a consequence, apoptosis is almost completely abolished [24,25], resulting in lymphoproliferation and the accelerated development of autoimmunity [26]. The renal disease of MRL/lpr mice is initially characterized by the glomerular deposition of immune complexes, binding and activation of complement and the development of proliferative glomerulonephritis [27, 28]. In the late course of the disease terminal renal dysfunction leads to death of the mice [28]. In a previous study we have demonstrated the changes of different chemokines and proinflammatory cytokines in the development of the nephritis in MRL/lpr mice [27].

In the present study we examined the effects of tRA treatment on the development of nephritis in MRL/lpr mice. Our results show that treatment with tRA has protective effects on the lupus-like glomerular injury in MRL/lpr mice and is associated with a reduction of renal chemokine and cytokine expression. These effects occur in spite of unaltered or even enhanced generation of autoantibodies and immune complex deposition within the glomeruli. As TGF-β mRNA levels are elevated, we propose that TGF-β may contribute to the tRA-induced suppression of the renal inflammatory response.

Although tRA has some side effects, these are less severe than those observed with classical steroid or other immunosuppressive therapies. This fact makes tRA a promising drug, which at present is mainly used in the treatment of acute promyelocytic leukemia and other neoplasic disorders [10] as well as in different inflammatory skin diseases [29].

METHODS

Reagents and antibodies

The following antibodies were used: fluorescein isothiocyanate (FITC)-conjugated rat antimouse IgG antibody (Dako, Glostrup, Denmark), FITC-conjugated goat antimouse complement C3 IgG fraction (ICN, Aurora, OH, USA) and peroxidase-conjugated goat antirabbit Ig secondary antibody (Dako); rabbit antimouse Ki67 antiserum (Dianova, Hamburg, Germany); rat antimouse monocyte/macrophage monoclonal antibody (ER-HR3) (Acris, Bad Nauheim, Germany) and rat antimouse CD3 antibody (Serotec, Oxford, UK). Kits were used for the quantification of mouse albumin [enzyme-linked immunosorbent assay (ELISA) (Bethyl Laboratories, TX, USA) and IgG [enzyme immunosorbent assay (EIA) (BD Pharmingen, Dan Diego, CA, USA)].

All reagents for the anti-DNA ELISA were from Boehringer Mannheim, except the mouse monoclonal antisingle- and double-stranded DNA antibody used as a standard (Chemicon International, Temecula, CA, USA). Reagents for the detection of creatinine were from Merck Diagnostika (Darmstadt, Germany). Radiolabeled [α-^{32}P] uridine triphosphate (UTP) (3000 Ci/mmol) for RNase Protection Assay was from NEN (Boston, MA, USA). The multiprobe RNase protection assay (RPA) template set mCK5 lacking the interferon-producing protein-10 (IP-10) probe was from Pharmingen (San Diego, CA, USA). tRA was from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents or solvents (analysis grade) were from Merck (Darmstadt, Germany).

Experimental protocol

MRL/MpJ-*Tnfrsf6*^{lpr} (MRL/lpr) and MRL/MpJ-*Tnfrsf6*$^{+}$ (MRL/wt) mouse lines were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred and housed under specific pathogen-free conditions and a 12-hour light and dark cycle, with free access to water. The access to food was limited to achieve a nearly complete intake. All experimental procedures were performed according to German law after authorization by local governmental authorities. At the age of 8 weeks, virgin female animals were randomly distributed into four different groups and fed with standard mouse autocleavable chow (Sniff; Soest, Germany) supplemented

with tRA (10 mg/kg body weight) or control diet. Supplemented chow pellets were prepared daily using a 1.68 mg/mL ethanolic tRA stock solution as previously described [5], and the real intake was assessed by weighing the remaining food daily. Mice showing irregular intake and those who took less than 80% of the mean dose were rejected from further analysis. For each group, 8 to 12 mice were finally analyzed.

Blood and spot urine samples were obtained from each mouse at week 8 (before the beginning of the treatment), as well as at the end of the treatment as previously described [27]. Albuminuria, antidouble-stranded DNA antibodies, total circulating IgG levels were determined using standard analytic protocols in accordance with previously described protocols [30].

After 6 weeks of treatment, mice were euthanized by cervical dislocation. One kidney was used exclusively for isolation of total RNA, using a standard protocol [31]. The other kidney was removed and split into two halves and used for histologic and immunohistologic analysis. The first half was fixed in 4% neutral buffered formaldehyde for 24 hours and then paraffin embedded. The other half of the renal tissue was embedded in tissue freezing medium (Jung; Leica Instruments, Wetzlar, Germany), snap-frozen in liquid nitrogen, and stored at −80°C until used for immunohistochemical studies.

Light microscopy

Sections of 4 μm thickness from paraffin-embedded renal tissue were routinely stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and silver methenamine stains. A renal pathologist blinded for the treatment performed the histopathologic analysis as previously described [27]. Glomerular lesions were semiquantitatively graded from 0 to 3+ for hypercellularity, mesangial matrix expansion, and necrosis (0, absence; 1, mild; 2, moderate; and 3, severe), as well as for the percentage of sclerotic glomeruli and presence of crescents (0, less than 10%; 1, 10% to 25%; 2, 25% to 40%, and 3, more than 40%). A glomerular score was obtained from the sum of each parameter. Tubulointerstitial lesions were also graded from 0 to 3+ for peritubular and pericapillar mononuclear cell infiltrate, tubular damage, interstitial fibrosis and vasculitis. A tubulointersitial score was also defined as the sum of all parameter. Finally, perivascular lymphoproliferative mononuclear cell infiltrate was graded from 0 (absence) to 3+ (maximal intensity).

Immunohistochemistry

Studies were performed on paraffin-embeded sections or cryosections and processed as described above, using standard techniques [32].

Direct immunofluorescence studies were performed on 5 μm, ether/ethanol-fixed, cryostat sections from three independent mice by using FITC-conjugated rabbit antimouse IgG antibody and goat antimouse complement C3 [30].

CD3, ER-HR3, and Ki67-positive cells were characterized and quantified on 4 μm thick paraffin sections from renal tissue [30]. Peroxidase-conjugated goat antirabbit or goat antirat IgG secondary antibody was always developed with diaminobenzidine as chromogen and counterstained with hemalaun. Respective isotype IgG control served as negative controls. For quantification, positively stained cells were counted within 20 glomeruli or within 10 high power fields (630×) of tubulointerstitial tissue from four independent mice per group and expressed as cells per glomerulus or high power field.

Renal mRNA expression

Chemokine expression was analyzed by a commercial RPA as previously described [33]. Twenty micrograms of total RNA from each sample was used. The RNase-protected probes were separated on 5% denaturing polyacrylamide gels and analyzed by phosphorimaging (Storm 840 PhosphorImager; Molecular Dynamics, Sunnyvale, CA, USA). Bands were quantified using ImageQuant software (Molecular Dynamics, Eugene, OR, USA).

Adoptive T-cell transfer

T cells were isolated from the enlarged kidney-draining lymph nodes of a proteinuric, 14 weeks old, untreated MRL/lpr mouse. The lymph node was aseptically extracted and a cell dispersion generated in RPMI medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (RPMI + 10% PS) by several gentle strokes in a potter-homogenizer using a loose pestle. After removing coarse debris by decantation, the remaining cell suspension was collected, centrifuged for 5 minutes at 600*g*, washed in RPMI + 10% PS and cell number determined using a Neubauer chamber. Two $\times 10^7$ cells were stained with PKH26 fluorescence dye according to the manufacturer's instructions (Sigma Chemical Co.). After two washings, cells were resuspended in isotonic saline to a final concentration of 1.5×10^7 cells/mL and 200 μL of the suspension were transferred by jugular vein injection into age-matched MRL/lpr mice treated with tRA for 6 weeks. Urine from these mice was collected twice a week starting at day 8 before T-cell transfer until their sacrifice 11 days after transfer. In addition to the histopathologic analysis, cryosections of spleen, lymph nodes, and kidneys were analyzed by fluorescence microscopy to assess the T-cell migration to these target organs.

Table 1. Effect of retinoic acid treatment (tRA) treatment on clinical parameters in MRL/lpr mice

	Vehicle	tRA
Body weight *g*	36.6 ± 4.1	28.1 ± 1.3[a]
Lymph node weight *mg*	265 ± 109	125 ± 50[a]
Spleen weight *mg*	433 ± 48	248 ± 51[a]
White blood cell counts ×*1000/μL*	6.0 ± 1.2	6.9 ± 3.6

Values are mean ± SD of six to nine animals for each group at the age of 14 weeks.
[a] $P < 0.05$ vs. vehicle-treated animals.

Statistical analyses

Data were expressed as mean ± standard deviation (SD) and analyzed for statistical significance with either the unpaired two-way analysis of variance (ANOVA) and *t* test with Bonferroni correction (for parametric data) or the Kruskal-Wallis and Mann-Whitney *U* test (for nonparametric data) as needed. The null hypothesis was rejected at $P < 0.05$.

RESULTS

Body and organ weights

Treatment with tRA for 6 weeks resulted in a slightly lower body weight as compared with vehicle-treated MRL/lpr mice at 14 weeks of age (Table 1). This was accompanied by visibly reduced lymphoadenopathy, as well as a reduction in the spleen and lymph node weight. Treatment had no effect on total peripheral white blood cell counts (Table 1).

Biochemical parameters

Plasma IgG (Fig. 1A) and anti-DNA (Fig. 1B) antibody levels were determined at 8 and 14 weeks of age from vehicle and tRA-treated MRL/lpr and MRL/wt mice. MRL/lpr mice treated for 6 weeks from 8 weeks of age with either vehicle (IgG 89.6 ± 57.6 mg/mL; anti-DNA antibodies 2.1 ± 0.9 μg/mL) or tRA (IgG 155.8 ± 49.9 mg/mL; anti-DNA antibodies 2.79 ± 1.35 μg/mL) showed comparably increased antibody concentrations as compared to 8-week-old MRL/lpr mice (IgG 32.3 ± 27.4 mg/mL; anti-DNA antibodies 0.4 ± 0.3 μg/mL) or 14-week-old MRL/wt mice (IgG 36.1 ± 14.7 mg/mL; anti-DNA antibodies 0,4 ± 0.1 μg/mL). No statistical differences in antibody levels between vehicle and tRA-treated MRL/lpr mice were found (Fig. 1A and B)

As expected vehicle treated MRL/lpr mice developed albuminuria at the age of 14 weeks (47.9 ± 33.7 μg albumin/mg creatinine), as compared to either 8-week-old MRL/lpr mice (0.9 ± 0.4 μg albumin/mg creatinine) or 14-week-old MRL/wt mice (0.6 ± 0.2 μg albumin/mg creatinine). Development of proteinuria was almost completely prevented by tRA treatment (3.1 ± 4.4 μg albumin/mg creatinine), with urinary albumin excretion close to basal levels (Fig. 1C).

Histopathologic and immunohistochemical findings

At the age of 8 weeks no histologic signs of renal lesions were apparent in any of the groups. At week 14 of age, vehicle-treated MRL/lpr mice showed well-established renal alterations, mostly in glomerular but also in tubulointerstitial compartments (Fig. 2C). Glomerular lesions were characterized at this time point by enlarged, hypercellular glomeruli with increased numbers of both resident cells and infiltrating leukocytes, as well as mesangial matrix expansion (Fig. 2I). Interstitial lesions included the presence of peritubular and pericapillar mononuclear cell infiltrates, which were distributed focally and irregularly throughout the cortex of the affected kidneys. As in human lupus, these interstitial lesions are very mild in MRL/lpr mice at the age of 14 weeks. By contrast to the mild interstitial lesions, the presence of large accumulations of lymphoid cells located in the medulla and around big renal vessels was noted in the 14-week-old MRL/lpr mice. This is a hallmark of the lymphoproliferative state of the MRL/lpr mice and does not represent real tubulointerstitial inflammation.

After 6 weeks of treatment with tRA, 14-week-old MRL/lpr mice showed reduced glomerular damage (Fig. 2D), characterized by both reduced glomerular hypercellularity and mesangial matrix expansion as compared to vehicle-treated animals (Fig. 2C). Glomerular IgG immune complex and complement C3 deposition were present to a comparable extent in both tRA-treated and untreated mice (Fig. 2E to H).

As shown in Figure 2J, tRA treatment was associated with a dramatic reduction of endocapillary cell proliferation as assessed by staining for the cell-proliferation marker Ki67 (vehicle 2.97 ± 0.24 Ki67+ cells/glomerulus; tRA 0.13 ± 0.03; $P < 0.05$) and a reduction in the overall glomerular damage score (Fig. 2I). In addition, tRA treatment markedly decreased the number of glomerular infiltrating T cells (vehicle 0.38 ± 0.06 CD3+ cells/glomerulus; tRA 0.13 ± 0.03; $P < 0.05$) and macrophages (vehicle 0.93 ± 0.12 ER-HR3+ cells/glomerulus; tRA 0.13 ± 0.04; $P < 0.05$) (Fig. 3). No necrotic, sclerotic, or crescentic lesions were found.

The prominent perivascular lymphoproliferative lesions typical of MRL/lpr mice were not affected by tRA treatment (not shown). However, in the cortical tubulointerstitial compartment treatment with tRA did markedly reduce the CD3+ lymphocytic (vehicle 9.0 ± 0.2 cells/high power field (630×); tRA 2.3 ± 0.2; $P < 0.05$) and ER-HR3+ mononuclear cell infiltrate (vehicle 4.7 ± 0.7; tRA 1.6 ± 0.3; $P < 0.05$) (Fig. 3) present at 14 weeks as compared to vehicle-treated MLR/lpr mice.

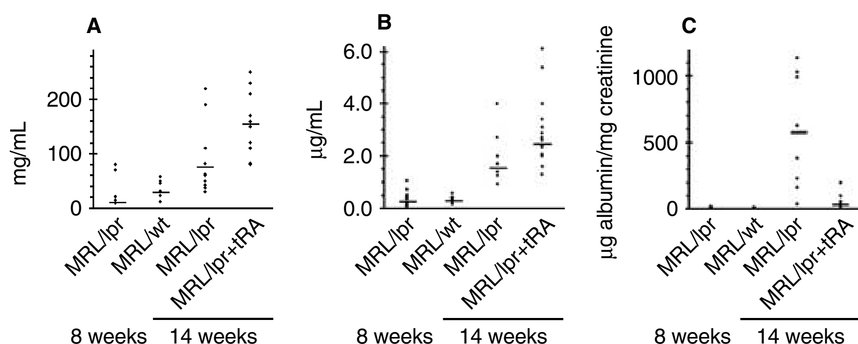


Fig. 1. Effect of retinoic acid treatment (tRA) treatment on biochemical findings in MRL/wt and MRL/lpr mice. (*A*) Total plasma IgG levels, detected by enzyme-linked immunosorbent assay (ELISA) and expressed in mg/mL. IgG levels were significantly increased in both 14-week-old vehicle- or tRA-treated MRL/lpr mice as compared to 8-week-old MRL/lpr mice or 14-week-old MRL/wt mice ($P < 0.05$). The elevated IgG levels of vehicle and tRA treatment groups were not statistically different. (*B*) Total plasma anti-DNA antibody levels, detected by ELISA and expressed in µg/mL. Anti-DNA antibody levels were significantly increased in both 14-week-old vehicle- or tRA-treated MRL/lpr mice as compared to 8-week-old MRL/lpr mice or 14-week-old MRL/wt mice ($P < 0.05$). Anti-DNA antibodies in mice from vehicle and tRA treatment groups were not statistically different. (*C*) Albuminuria, detected by ELISA, expressed as µg albumin per mg urinary creatinine. At the age of 14 weeks, vehicle-treated animals showed significantly increased albuminuria as compared to both 8-week-old MRL/lpr mice or 14-week-old MRL/wt mice ($P < 0.05$). tRA treatment reduced albuminuria to almost basal levels ($P < 0.05$).

Renal mRNA expression of proinflammatory cytokines and chemokines

Changes in the Th2/Th1 balance with changed expression of cytokines such as IL-12, IL-10, and IFN-γ and of chemokines is supposed to alter the course of experimental lupus nephritis [27, 34–37]. We therefore analyzed the expression of proinflammatory cytokines and chemokines in total kidney RNA by RPA (Fig. 4) and compared them to vehicle-treated MRL/lpr mice (set at 100). In the tRA-treated animals, renal mRNA expression was reduced for IL-1α (16 ± 5 vs. 100 ± 46 in untreated MRL/lpr mice; $P < 0.05$), IL-1β (25 ± 12 vs. 100 ± 39; $P < 0.05$), INF-γ–inducible factor (43 ± 13 vs. 100 ± 22; $P < 0.05$), and IL-12p35 (22 ± 9 vs. 100 ± 48; $P < 0.05$) as well as IL-12p40 (23 ± 12 vs. 100 ± 63, $P < 0.05$). A nonstatistically significant reduction for IL-10 mRNA (44 ± 18 vs. 100 ± 63) was also observed. No differences in the mRNA levels of IL-1Ra and IFN-γ were observed. Treatment with tRA also significantly reduced the renal expression of CCL5/RANTES (30 ± 7 vs. 100 ± 12 in vehicle-treated mice; $P < 0.05$), CCL4/MIP-1β (45 ± 30 vs. 100 ± 11; $P < 0.05$), CCL3/MIP-1α (20 ± 9 vs. 100 ± 15; $P < 0.01$), and CCL2/MCP-1 (49 ± 14 vs. 100 ± 24; $P < 0.05$). CXCL1/MIP-2α showed the same tendency but did not reach statistical significance (54 ± 31 vs. 100 ± 18). TGF-β is a multifunctional cytokine with important roles in suppressing autoimmune reactions and in fibrosing processes [17,18]. We therefore also determined renal TGF-β mRNA levels in vehicle and tRA-treated MRL/lpr mice. Surprisingly, TGF-β mRNA levels in kidneys were somewhat elevated by tRA-treatment (179 ± 26 vs. 100 ± 14; $P < 0.05$).

Effect of adoptive T-cell transfer from untreated to tRA-treated MRL/lpr mice

Our results with tRA treatment showed no effect on one of the key events of immune-complex nephritis (i.e., the glomerular immune complex and complement deposition), yet the glomerular disease was markedly attenuated. In order to analyze whether this might be due to differences in T-cell activation we performed T-cell transfer. T cells from renal lymph nodes from vehicle-treated, nephritic and proteinuric mice (age 14 weeks) were transferred to tRA-treated mice (tRA + transfer) to see if this could then trigger the nephritis. The transferred T cells, which were fluorescently labeled with PKH26 dye prior to transfer, could be detected within the lymph nodes and the spleen, but not in the renal tissue of the tRA-treated mice (not shown). As shown in Figure 5, tRA-treated mice receiving 10^7 T cells from 14-week-old, vehicle-treated MRL/lpr mice did not develop proteinuria (Fig. 5C) or glomerulonephritis as judged by glomerular damage score (Fig. 5D) within 11 days post-transfer ($N = 7$). Furthermore stopping the tRA treatment at the day 0 did not result in the development of glomerular disease or proteinuria during the next 11 days in the mice previously treated with tRA and without T-cell transfer (tRA + transfer) (Fig. 5A).

DISCUSSION

In the present work, we have studied the effects of the administration of tRA on the development of renal damage in MRL/lpr mice. As compared with vehicle treatment, mice treated for 6 weeks with tRA

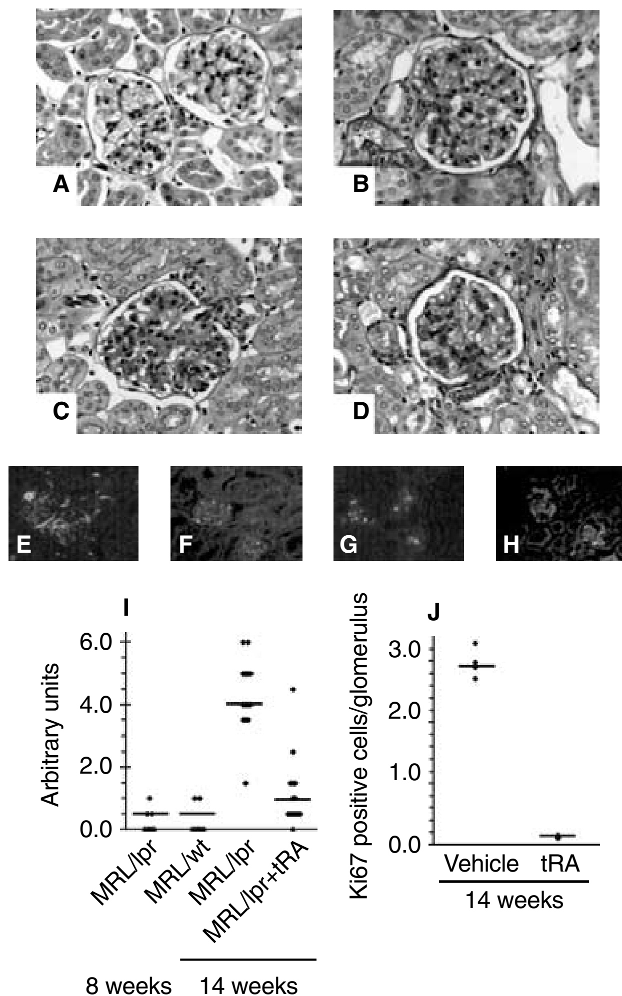


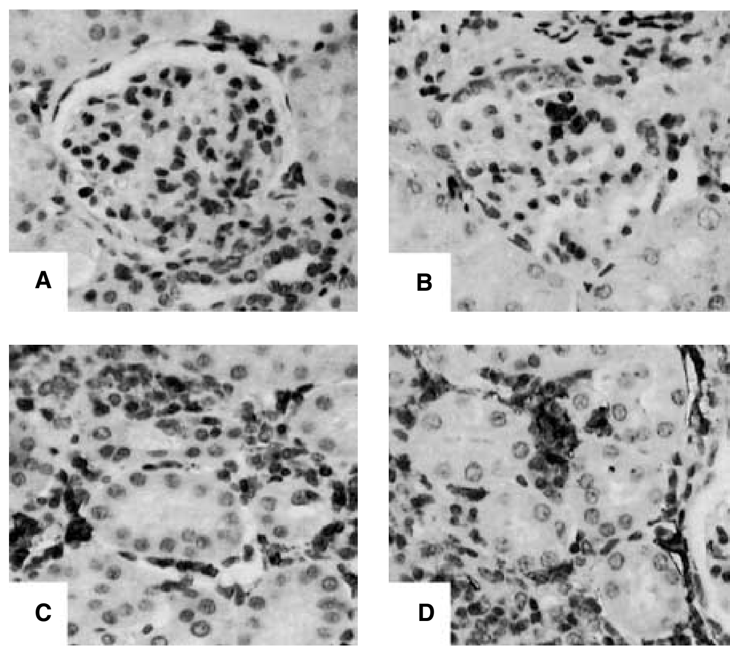
Fig. 2. Effect of retinoic acid treatment (tRA) treatment on glomerular and tubulointerstitial damage in the MRL/lpr or MRL/wt mice. Light microscopy (400×) showing periodic acid-Schiff (PAS)-stained renal tissue from representative mice of the following experimental groups: (*A*) vehicle-treated MRL/wt at 14 weeks of age, (*B*) 8-week-old MRL/lpr, (*C*) 14-week-old vehicle-treated MRL/lpr, or (*D*) 14-week-old tRA-treated MRL/lpr mouse. The prominent glomerular hypercelularity and mesangial matrix expansion observed in 14-week-old vehicle-treated MRL/lpr mice (C) is significantly prevented by 6 weeks of tRA treatment (D). Fluorescence microcopy (100×) showing immunofluorescence staining in vehicle-treated MRL/lpr mice for complement C3 (*E*) or for IgG (*F*) and in tRA-treated MRL/lpr for complement C3 (*G*) or for IgG (*H*). (*I*) Semiquantitative analysis of glomerular damage index using the glomerular hypercellularity, mesangial matrix expansion, and leukocyte exudate as injury parameters. The glomerular damage index was significantly reduced in tRA-treated MRL/lpr mice as compared to vehicle treated controls ($P < 0.05$). No statistical differences were found between ML at 8 weeks of age or 14-week-old Mw and the tRA-treated MRL/lpr mice. (*J*) Quantitative analysis of Ki67-positive cells, counted in 50 glomeruli of kidney sections from four animals per groups. The number of proliferating endocapillary cells was significantly reduced in in tRA-treated MRL/lpr mice as compared to vehicle-treated controls ($P < 0.05$). ML, MRL/lpr; Mw, MRL/wt.

showed, at the age of 14 weeks, (1) reduced lymphoproliferation with smaller spleen and lymph nodes; (2) failure to develop proteinuria and glomerular damage with reduced endocapillar cell proliferation and reduced lymphocyte and macrophage cell infiltrate, in spite of (3) unchanged serum levels of IgG and AntiDNA antibodies and unchanged glomerular immune complex deposition and complement activation; (4) the reduced glomerular injury by tRA was associated with reduced expression of cytokines, except for TGF-β, which even increased; (5) renal chemokine expression was reduced by tRA; and (6) transfer of T cells from vehicle-treated mice did not cause disease in tRA-treated mice.

At the beginning of treatment at the age of 8 weeks no signs of palpable lymphoadenopathy, systemic autoimmunity, proteinuria or renal morphologic alterations were evident. During the next 6 weeks vehicle-treated mice developed splenomegaly and lymphoadenopathy, which was markedly attenuated by treatment with tRA. The mechanism by which MRL/lpr mice exacerbate autoantibody production is through the escape from fas-mediated apoptosis of autoreactive lymphocytes [28]. A possible mechanism for reduced splenomegaly and lymphoadenopathy in tRA-treated mice could be the previously described increased, non-fas–dependent, proapoptotic effect of tRA [38]. This explanation cannot be ruled out, though we found no direct evidence to support it. Spleens and lymph nodes of tRA-treated mice showed histologically similar amounts of apoptotic cells as vehicle-treated controls (data not shown). Most likely tRA reduces general lymphoproliferation, perhaps by causing maturation of lymphocytes in the primary lymphoid tissues. These effects would be similar to tRA therapeutic effects in leukemia [10]. In any case, the multitude of genes regulated by tRA does not allow assignment of a single mechanism specific for the antiproliferative effects observed.

Treatment with tRA prevented the development of albuminuria and morphologic glomerular alterations so that renal histology and function remained comparable to 8-week-old MRL/lpr mice or 14-week-old, non-nephritic MRL/wt mice. The reduction of glomerular lesions by tRA included reduced hypercellularity, reduced mesangial matrix expansion, and reduced T-cell and macrophage infiltration with concomitant absence of proteinuria.

tRA has been described to successfully inhibit the development of inflammatory renal disease in a rat model of Thy 1-1 mesangioproliferative glomerulonephritis [2,3], in puromycin aminonucleoside-induced nephrosis in the rat [4,5] and recently in NZB/W F1 systemic lupus erythematosus (SLE) mice [6], unilateral ureteral ligated rats [8], and in a rat model of acute renal allograft rejection [7]. In all these studies tRA treatment was associated with decreased inflammatory cell infiltrate and, in general, with decreased expression of proinflammatory cytokines and growth factors. Our present observations of reduced glomerular hypercellularity after tRA treatment are in keeping with these reports. We could assign the



	CD3		ER-HR3	
	Vehicle	tRA	Vehicle	tRA
Intraglomerular infiltrates	0.38 ± 0.06	0.1 ± 0.03*	0.93 ± 0.12	0.13 ± 0.04*
Tubulointertitial infiltrates	9.03 ± 0.21	2.28 ± 0.24*	4.67 ± 0.63	1.58 ± 0.33*

Fig. 3. Effect of retinoic acid treatment (tRA) treatment on renal mononuclear cell infiltration. Light microscopy (630×) showing representative immunostaining for glomerular (*A*) and peritubular (*C*) CD3+ T cells and for glomerular (*B*) and peritubular (*D*) ER-HR3+ macrophages of representative renal tissue from 14-week-old mice treated for 6 weeks with vehicle. As shown in Table 1 the quantitative analysis shows a significant reduction in the glomerular and peritubular CD3+ T cells and ER-HR3+ macrophages by tRA as compared to vehicle-treated MRL/lpr mice. For each mouse the mean of 20 glomeruli or 10 high power fields of interstitial tissue (630×) were determined. The numbers express means ± SD of three animals per group. $^*P < 0.05$ vs. vehicle-treated animals.

reduced glomerular cellularity to a combination of decreased proliferation of glomerular cells, as assessed by Ki67 staining, and reduced cell infiltration. The antiproliferative effect of tRA on renal and nonrenal cells is supported by previous observations [19,20]. Wagner et al described glomerular antiproliferative effects of tRA in a rat model of mesangioproliferative nephritis [2,3]. A possible mechanistic explanation for the reduced glomerular cell proliferation could be that the reduced leukocyte infiltration observed within the glomeruli of tRA-treated mice may result in reduced glomerular production of mediators of inflammation and growth factors.

It is generally accepted that the generation of autoantibodies and the subsequent glomerular deposition of immune complex and complement activation is the major trigger for the pathogenic process in lupus nephritis in humans [39] and in MRL/lpr mice [40,41]. Surprisingly, tRA treatment neither reduced the levels of autoantibodies nor altered the amount or the pattern of glomerular immune complex and complement deposition. This argues against the possibility of reduced immune complex deposition and complement activation as an explanation for the observed protective effect of tRA. Our observations are somewhat discrepant from the recently reported decrease in immune complex deposition induced by tRA treatment in the NZB/W F1 model of lupus nephritis [6]. At present the reasons for this discrepancy remain unclear but may relate to differences in the pathobiology of the renal disease between both models [26, 42]. A number of reports do, however, support our observation that immune complex nephritis can be improved or prevented in mice by experimental maneuvers in spite of unaltered autoantibody production and immune complex deposition. For example, uncoupling of the signal transduction via Fcγ receptors [43], or lack of inflammatory cytokines or chemokines such as IFN-γ [36], IL-12 [35], or CCL2/MCP-1 [37] reduces the glomerulonephritis of lupus models in spite of unaltered glomerular immune complex deposition. Based on these reports and our results we conclude that in the case of the MRL/lpr lupus nephritis autoantibody production, immune complex and complement deposition is not sufficient to promote leukocyte infiltration and the subsequent glomerular inflammation.

As cytokines and chemokines play a role in immune mediated glomerulonephritis [27] we examined their renal expression as potential mechanisms for the beneficial effects of tRA administration. Furthermore, reduced expression of different cytokines such as tumor necrosis factor-α (TNF-α), IL-1β, IFN-γ, platelet-derived growth

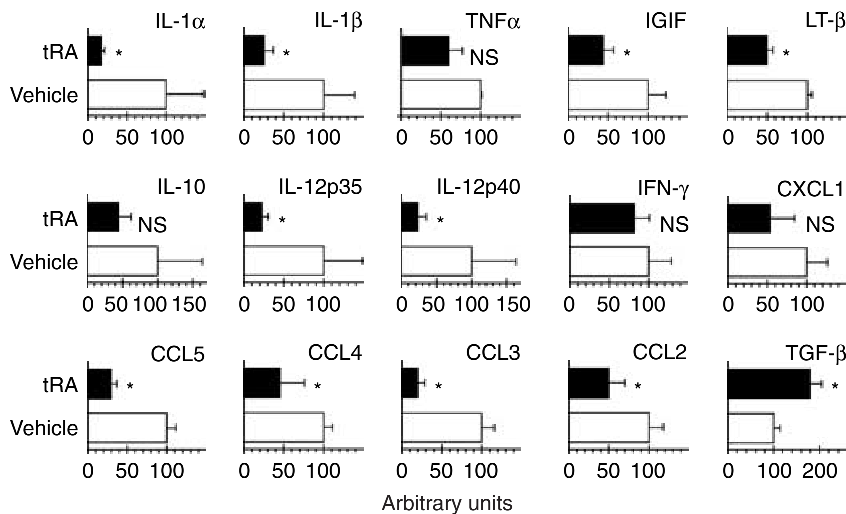


Fig. 4. Effect of retinoic acid treatment (tRA) treatment on renal expression of cytokines and chemokines in the MRL/lpr mice. Expression of cytokines and chemokines in kidney RNA from vehicle- and tRA-treated mice at 14 weeks of age as assessed by RNase protection assay (RPA). Values are arbitrary units expressing the densitometric ratio between a given gene and glyceraldehyde-3-phophate dehydrogenase (GAPDH). The mean of the vehicle-treatment group is set as 100. Results are means ± SD from three to four mice per group. $^*P < 0.05$ vs. vehicle-treated mice.

factor (PDGF), or CCL2/MCP-1 was noted after tRA treatment in models of anti-glomerular basement membrane (GBM) [9], puromycin [5] or lupus nephritis [6]. Our present observations of reduced cytokine expression are in keeping with those observations. Of special interest are the reduced mRNA levels for IL-12 that we observed. It has been previously described that IL-12 expression is reduced by tRA in macrophages [14]. In the model of pristan-induced lupus nephritis mice deficient for IL-12p35 showed no signs of proliferative glomerulonephritis or proteinuria [35] in spite of unchanged levels of nephritogenic autoantibodies, immune complex deposits and complement activation. Similarly, in MRL/lpr mice deficient for IL12p40 the glomerular pathology was improved while peripheral antibody levels were unchanged and only a slight reduction of immune complex deposition has been reported [44]. In our present results the renal expression of IL-12p35 and IL-12p40 was reduced by 80% by tRA treatment in MRL/lpr mice. The down-regulation of IL-12 by tRA could help to explain why the renal disease was reduced by tRA in spite of the elevated serum levels of IgG and anti-DNA antibodies and immune complex deposition. Furthermore IL-12 can drive the expression of IFN-γ [45], another cytokine which appears to play a key role in the development of glomerulonephritis in NZB/W F1 [46] and MRL/lpr mice [36, 47]. While tRA has been reported to inhibit the expression of IFN-γ in NZB/W F1 mice [6], in our model the renal expression of IFN-γ was not altered by tRA. Thus, a change in IFN-γ appears an unlikely contributing factor for the beneficial effect of tRA treatment in MRL/lpr nephritis. On the other hand, the reduction of the proinflammatory cytokines IL-1α, IL-1β, and TNF-α together with the suppression of IL-12 could contribute to the reduced renal inflammation in MRL/lpr mice treated with tRA [27]. Suppressed IL-1 and IL-12 expression, together with the unreduced peripheral IgG titers, point toward a possible shift of the immune response to a predominant Th2 phenotype as a possible mechanism contributing to the reduced renal inflammatory response.

In a previous study, we have shown that several chemokines are up-regulated early during the development of nephritis in MRL/lpr mice [27]. The significance of CCL2/MCP-1 expression was evaluated by Tesch et al [37] in CCL2/MCP-1–deficient MRL/lpr mice. The lack of CCL2/MCP-1 markedly decreased macrophage and T-cell infiltration, glomerular damage, proteinuria, and prolonged survival as compared with wild-type littermates in spite of unchanged circulating immune complex levels and glomerular deposition of immune complex [37]. Furthermore a CCL2/MCP-1 antagonist also improves the renal disease of MRL/lpr mice [48]. Our finding that tRA administration also reduces renal chemokine expression, including CCL2/MCP-1, is therefore of interest. This reduction in local chemokine generation could help to explain the decreased number of infiltrating T cells and macrophages that we observed after tRA administration. It is unlikely that reduced expression of chemokine receptors on the surface of leukocyte contributes to this effect as we found no changes in the CCR2 and CCR5 surface expression on peripheral blood leukocytes by flow cytometric analysis from tRA treated MRL/lpr mice as compared to vehicle-treated controls (data not shown).

Immune complex deposition and complement C3 are both factors, which induce glomerular chemokine synthesis [49]. Since tRA reduces the expression of chemokines and cytokines in spite of unaltered immune complex deposition and complement activation, the question is, how could tRA treatment suppress chemokine production in kidneys of MRL/lpr mice? A hypothetical factor could be the enhanced generation of TGF-β in response to tRA, which could reduce renal cytokine and chemokine expression. TGF-β is a multifactorial

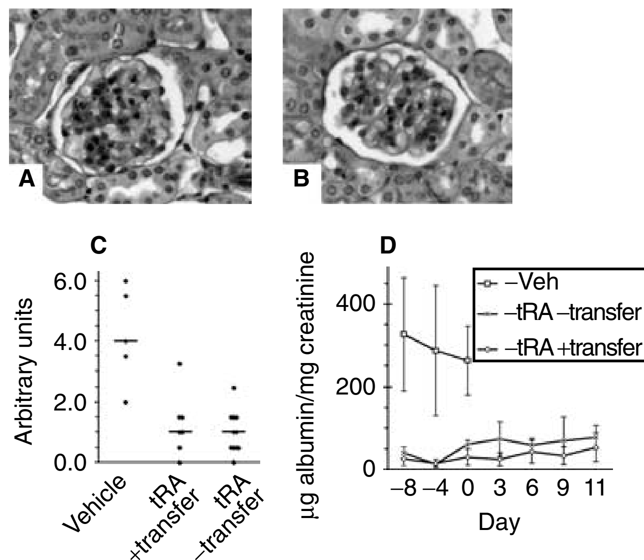


Fig. 5. Effect of adoptive T-cell transfer from untreated to retinoic acid treatment (tRA)-treated MRL/lpr mice on glomerular pathology and proteinuria. Light microscopy (400×) showing periodic acid-Schiff (PAS)-stained renal tissue from representative mice from tRA-treated mouse 11 days after receiving 10^7 T cells from 14-week-old proteinuric vehicle-treated mouse (*A*) or from tRA-treated mouse without T-cell transfer (*B*). No difference is apparent. Adoptive T-cell transfer had no effect on the development of either renal damage as evaluated by the glomerular score (*C*) or albuminuria (*D*). The glomerular injury score was determined at day 11 after transfer except for the vehicle group, where the evaluation was performed on the day of transfer. Albuminuria was determined twice a week starting 8 days before transfer (day 0) until sacrifice at day 11 post-transfer. No increase in proteinuria was observed in tRA-treated mice, irrespective of whether they were transferred (tRA + transfer) or not (tRA transfer). Albuminuria and glomerular score values are means ± SD of five to ten mice per group.

cytokine which exerts, depending on the overall conditions, either beneficial effects during inflammation, by acting as an anti-inflammatory, proapoptotic, and antiproliferative cytokine, or detrimental effects, by acting as a profibrotic cytokine [17,18]. In this context, it is of special interest that genetic disruption of the TGF-β gene results in the development of severe autoimmunity [50,51], whereas TGF-β overexpression can improve Th1-mediated inflammation, inhibit collagen synthesis, and reduce pulmonary fibrosis in an IL-10 dependent manner [52]. Finally, TGF-β is directly able to reduce chemokine production in cultured glomerular mesangial cells [53]. The increase in renal mRNA expression of TGF-β that we observed after tRA treatment could point toward a positive effect of TGF-β on the autoimmune disease of MRL/lpr mice, possibly by a direct effect of tRA on TGF-β generation as reported by Glick et al [15,16]. Thus induction of TGF-β by tRA in MRL/lpr lupus nephritis could reduce the expression of proinflammatory cytokines and chemokines and thereby improve the renal disease process in spite of unaltered glomerular immune complex and complement deposition. This hypothesis could be considered to be in conflict with the observations that tRA down-regulate the expression of TGFβ in anti-Thy 1-1- [1] and anti-GBM glomerulonephritis [9] and in unilateral ureter ligation in the rat [8]. These apparent discrepancies might relate to the different pathogenic mechanisms between the above models and the autoimmune SLE-like disease of MRL/lpr mice. Beneficial effects of TGF-β in an autoimmune setting are well supported by the literature [15,16, 50,51]. On the other hand, detrimental effects of TGF-β in chronic fibrotic disease are equally well documented [1, 8,9], illustrating the two sides of TGF-β in disease process [18]. Support for such dual effects of TGF-β comes from a recent report on different outcomes with different degrees of TGF-β blockade in puromycin nephrosis [54]. The hypothesis of beneficial effects of TGF-β as an anti-inflammatory cytokine in autoimmune glomerulonephritis has to be further analyzed in the future.

The discrepancy between the unaltered antibody levels and glomerular deposition of immune complex on the one hand and the lack of glomerular cell infitration and proliferation on the other hand led us to consider that tRA treatment had altered the phenotype of the lymphocytes. We therefore performed adoptive T-cell transfer from nephritic, proteinuric, vehicle-treated 14-week-old mice into age-matched tRA-treated mice. If the tRA treatment had altered the phenotype of T cells the passive transfer could have resulted in glomerular inflammation. In principle, with this type of experiments effects mediated by T cells can be transferred as shown, for example, for anti-GBM nephritis [55]. However, the transfer experiments did not support the notion of tRA-mediated alteration of T-cell phenotype as a key feature for the glomerular protection. This leaves us with the notion of a locally altered inflammatory milieu contributing to the beneficial effects of tRA treatment in MRL/lpr mice. Such a beneficial effect could also involve decreased infiltration of activated T cells because of reduced chemokine generation.

Taken together, our results suggest that tRA treatment protects MRL/lpr from the development of immune complex–mediated glomerulonephritis by influencing the expression patterns of mediators of inflammation. The beneficial effect may relate to the coordinated down-modulation of chemokines such as CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES and of the proinflammatory cytokines IL-1α, IL-1β, leukotriene β, IL-12, or TNF-α. As retinoids are already in use for oncologic and dermatologic diseases, its potential therapeutic value in SLE should be evaluated.

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