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Original Article

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All-trans-retinoic acid aggravates cryoglobulin-associated membranoproliferative glomerulonephritis in mice

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Abstract

Background. Transgenic (tg) mice overexpressing thymic stromal lymphopoietin (TSLP) develop mixed cryoglobulinaemia with renal disease closely resembling human cryoglobulinaemic membranoproliferative glomerulonephritis (MPGN), as well as systemic inflammation involving lung, liver and skin as a result of cryoglobulin deposits. We assessed the effect of all-trans-retinoic acid (ATRA), a powerful anti-inflammatory agent, on this model of cryoglobulinaemic MPGN.

Methods. Groups of male TSLP tg mice and wild-type controls were treated with either ATRA (20 mg/kg) or vehicle 3 times weekly by intraperitoneal injection for 4 or 8 weeks, when mice were then sacrificed. Routine histology and immunohistochemistry for collagen IV, α-smooth muscle actin, Mac-2 and Ki67 were performed. Immunoglobulin levels were measured by enzyme-linked immunosorbent assay.

Results. ATRA unexpectedly exacerbated renal injury in TSLP tg mice with increased glomerular extracellular matrix, mesangial cell activation, glomerular cell proliferation, glomerular macrophage influx and immune complex deposition. Systemic injuries involving liver and lung, and the amount of circulating cryoglobulins were all worsened by ATRA treatment. Furthermore, ATRA resulted in increased IgG1 and IgM levels, the main components of the cryoglobulins in TSLP tg mice, and a manifestation of an enhanced Th2 immune response.

Conclusions. ATRA is not protective but instead aggravates cryoglobulinaemic MPGN and its systemic manifestations in TSLP tg mice. We speculate these findings may be due to augmented production of pathogenic immunoglobulins and/or an enhanced systemic Th2 response. Although disappointing, our results also suggest caution in the application of retinoid therapy to human disease based on the largely positive animal data reported to date.

Keywords: cryoglobulinaemia; membranoproliferative glomerulonephritis (MPGN); retinoic acid; thymic stromal lymphopoietin (TSLP)

Introduction

Transgenic (tg) mice overexpressing thymic stromal lymphopoietin (TSLP) develop mixed cryoglobulinaemia with renal disease closely resembling human cryoglobulinaemic membranoproliferative glomerulonephritis (MPGN) [1]. The MPGN is characterized by glomerular macrophage infiltration, mesangial cell proliferation and mesangial matrix expansion and deposition of immune complexes containing cryoglobulins in capillary loops. There is concurrent systemic inflammation involving lung, liver and skin as a result of cryoglobulin deposits. The predictability and early development of the disease makes these mice particularly well suited to study specific interventions in the development of this disease.

Retinoids, naturally active derivatives of vitamin A, are characterized by their capacity to bind and activate retinoid nuclear receptors. These include retinoic acid receptors (RARs) and/or retinoid X receptors (RXRs), each having three isotypes, α, β and γ. Mesangial cells, vascular smooth muscle and endothelial cells express different types of retinoid receptors. In these cells, retinoids have been shown to inhibit the expression of factors involved in renal diseases such as transforming growth factor-β1 (TGF-β1), endothelin-1, platelet-derived growth factor (PDGF) and nitric oxide [2]. In addition, retinoids exert anti-inflammatory effects and attenuate the production of inflammatory mediators such as tumour necrosis factor-α (TNF-α). Accordingly, retinoids have been tested and shown to have efficacy in reducing various renal disease manifestations in animal models of lupus nephritis,

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mesangial proliferative glomerulonephritis, puromycin aminonucleoside nephrosis and anti-glomerular basement membrane (GBM) antibody-induced crescentic glomerulonephritis [3], and are currently being evaluated as a therapeutic for human focal and segmental glomerulonephritis in a clinical trial sponsored by the U.S. National Institutes of Health.

Therefore, the present study was designed to determine whether all-trans-retinoic acid (ATRA), a carboxylic acid form of vitamin A and its major metabolite, might reduce glomerular macrophage infiltration as well as systemic inflammation, and improve murine cryoglobulinaemic MPGN. Unexpectedly, we found that ATRA caused aggravation of the cryoglobulinaemia and its systemic manifestations in this model. Our studies provide a cautionary note for the use of retinoids testing as an approach to treating glomerulonephritis.

Materials and methods

Experimental protocol

The experimental protocol for this study was reviewed and approved by the Animal Care Committee of the University of Washington in Seattle. Mice tg for TSLP have been previously described in detail [1]. The animals were housed in the animal care facility of the University of Washington under standardized specific pathogen-free conditions (25°C, 50% humidity, 12 h dark/light cycle) with free access to food and water. After backcrossing for more than eight generations to a C57Bl6 background, male TSLP tg animals were mated with WT C57Bl6 females. At 3 weeks of age mice were weaned and genotyped as described previously [1].

Male TSLP tg and WT mice were randomly assigned into four groups: treated TSLP tg mice, untreated TSLP tg mice, treated WT mice, untreated WT mice. Treated groups received ATRA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in corn oil three times weekly by intraperitoneal injection. Untreated groups received an equal volume of corn oil as a vehicle control. Treatment was started after weaning at day 21 and was continued for either 4 or 8 weeks. An initial pilot study was conducted with TSLP tg or WT mice treated with ATRA at 5 mg/kg ($n=3$), 10 mg/kg ($n=2$) or 20 mg/kg ($n=3$) for 4 weeks. For the expanded study, ATRA was administered at 20 mg/kg and 5–7 mice were finally analysed. Body weight was examined weekly. Spot urine samples were collected from each mouse 1 day before sacrifice. At the end of the study mice were anesthetized, blood was collected from the retro-orbiotal sinus and organs were collected. Renal tissue was snap frozen in liquid nitrogen or stored in either 10% neutral buffered formalin, methyl Carnoy's solution (60% methanol, 30% chloroform, 10% acetic acid) or half-strength Karnovsky's solution (1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 mol/l Na cacodylate buffer, pH 7.0) for later use.

Tissue preparation and histological staining

Tissue fixed in formalin and methyl Carnoy's solution were embedded in paraffin using routine protocols. Paraffin-embedded materials were sectioned at 4 μm for routine staining with haematoxylin and eosin (H&E), periodic-acid Schiff (PAS) and immunohistochemistry. Two micrometre thick sections were used for periodic-acid methenamine silver stains. Immunofluorescence was performed on snap frozen kidneys, sectioned at 6 μm and fixed in ice-cold acetone for 10 min.

Immunohistochemistry

For immunohistochemical staining, histological sections of paraffin-embedded tissue were deparaffinized in xylene and rehydrated in graded ethanol. When required, antigen retrieval was performed by steam heating the tissue sections in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA). Endogenous peroxidases were blocked in 3% hydrogen peroxide, and endogenous biotin was blocked using the Avidin/Biotin blocking kit (Vector Laboratories). The slides were then incubated with the primary antibody diluted in 1% bovine serum albumin (BSA) in phosphate-buffered saline solution (PBS) overnight at 4°C. After that the sections were washed with PBS and immersed in the appropriate biotinylated secondary antibody, again diluted in PBS with 1% BSA. Signal amplification was performed using the ABC-Elite Reagent (Vector Laboratories). The 3,3′-diaminobenzidine with nickel enhancement was used as chromogen, which results in a black colour product. The slides were counterstained with methyl green, dehydrated and then coverslipped. A rat Mac-2 antibody (Cederlane; Ontario, Canada) was used to detect macrophages. A mouse-adsorbed anti-rat antibody (Vector Laboratories) was utilized as secondary antibody. For the detection of mesangial cell activation and smooth muscle-like transformation, a α-smooth muscle actin (αSMA) antibody (clone 1A4; Dako, Carpenteria, CA, USA) was used. In this case, the secondary antibody was an anti-IgG2a antibody (Pharmingen, San Diego, CA, USA). Cellular proliferation was assessed using a mouse monoclonal Ki67 antibody (Pharmingen) with the Dako animal Research kit. A goat polyclonal antihuman collagen IV antibody (Southern Biotechnology, Birmingham, CA, USA) was used for the staining of extracellular matrix. A biotinylated rabbit anti-goat antibody was applied as secondary antibody (Vector Laboratories).

Immunofluorescence

Frozen sections were rehydrated in phosphate-buffered saline solution (PBS), blocked with normal rabbit serum and then incubated with fluorescein-conjugated antibodies against IgM, IgG, IgA and complement factor C3 (all from Cappel Pharmaceuticals, Aurora, OH, USA), coverslipped with Vectashield mounting medium (Vector Laboratories), and viewed with a Zeiss fluorescence microscope, as previously described [1].

Laboratory data

Urine samples were evaluated for proteinuria by determining the albumin/creatinine ratio. Albuminuria was measured using the Albuwell Kit (Exocell, Inc., Philadelphia, PA, USA) mouse albumin enzyme-linked immunosorbent assay

(ELISA) and creatinine using the Creatinine Companion (Exocell) according to the protocols of the manufacturer. Sera were analysed by ELISA for total IgG1, IgG2a and IgM. For total serum IgG1, IgG2a and IgM plates were coated overnight with 2 μg/ml goat anti-mouse Ig (H+L; Southern Biotechnology Associates, Inc.), blocked with PBS/1% BSA and incubated sequentially with serial dilutions of mouse sera, 2 μg/ml alkaline phosphatase (AP)-conjugated rat anti-mouse IgG1/IgG2a/IgM (1:1000; Southern Biotechnology Associates, Inc.), and 1 mg/ml of the AP substrate DNP phosphate (DNPP; Sigma-Aldrich). Plates were read on a microplate autoreader at 405 nm, and concentrations were calculated by plotting against standard curves generated from purified IgG1, IgG2a and IgM (Southern Biotechnology Associates, Inc.).

Analytical methods and statistics

Glomerular cellularity was assessed in tissue sections stained with haematoxylin and eosin. Fifteen random glomerular cross-sections were photographed with a digital camera (Olympus DP11; Olympus America, Melville, NY, USA). Pictures were imported into the Image Pro Plus Software (Media Cybernetics, Silver Spring, MD, USA) and glomerular cellularity and glomerular tuft area were quantified. The same method was used to quantify extracellular matrix in PAM-stained slides and in sections stained for collagen type IV, MAC-2 and αSMA by immunohistochemistry. Serum samples were stored at 4°C for more than 5 days and cryocrit was photographed and quantified using this software. The number of Ki-67-positive cells was assessed by counting positive cells in 50 random glomerular cross-sections. The fluorescence intensity of cryosections stained for immunoglobulins and complement was assessed by describing the fluorescence intensity on a scale from 0 (negative) to 3 (strong staining) as previously described [1]. All morphometric analyses were performed by an examiner blinded as to the origin of the sample. The liver and lung sections were scored blindly on a scale of 0–4 for inflammation: 0—no inflammation, 1—mild, 2—moderate, 3—severe, 4—very severe.

Statistical differences between two groups were analysed by the unpaired Student's *t*-test (parametric data) or the Mann–Whitney U-test (non-parametric data) and differences between multiple groups of data were assessed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test. Correlation analyses were performed using Pearson's coefficient (parametric data) or Spearman's coefficient (non-parametric data). Data were recorded as the mean ± SEM and values of $P < 0.05$ were considered significant. All analysis was accomplished using the software in InStat program (Version 3.0; Intuitive Software for Science, San Diego, CA, USA).

Results

Pilot study

All samples from the TSLP tg mice in the pilot study contained visible cryoprecipitates. At all doses of ATRA administered (5, 10 and 20 mg/kg BW), the relative serum cryocrit volume was increased (1.4-fold, 1.4-fold and 1.6-fold, respectively) compared with vehicle-treated TSLP tg mice. Histological examination of kidneys from ATRA-treated TSLP tg mice revealed increased cryoglobulin deposition and mesangial matrix accumulation, assessed by morphometry of collagen IV deposition, at all 3 doses, compared with vehicle-treated TSLP tg mice (TSLP control 17.5 ± 0.4%, TSLP + 5 mg/kg ATRA 20.2 ± 1.2%, TSLP + 10 mg/kg ATRA 19.8 ± 0.8% and TSLP + 20 mg/kg ATRA 24.6 ± 0.6% of glomerular tuft area positive for collagen IV). Body weights of ATRA-treated mice at sacrifice did not differ significantly from controls (TSLP control 21.8 + 0.6 g, TSLP + 5 mg/kg ATRA 21.3 + 1.0 g, TSLP + 10 mg/kg ATRA 17.7 + 2.4 g and TSLP + 20 mg/kg 19.1 + 1.9 g). Wild-type littermates that received ATRA at these doses showed no signs of renal toxicity and no adverse effects on their general health, including no weight loss, dermatitis or other signs of distress. As the lower doses did not result in discernable improvement of disease after 4 weeks of treatment, we chose to treat mice with 20 mg/kg of ATRA for the expanded study.

ATRA increased cryoglobulin production in TSLP tg mice

All samples from ATRA-treated TSLP tg mice and 10 of 11 samples from vehicle-treated TSLP tg mice contained visible cryoprecipitates. ATRA increased the total volume of cryoprecipitates 1.67- and 1.71-fold after 4 and 8 weeks treatment evaluated by cryocrit in TSLP tg mice, respectively (Figure 1).

ATRA aggravated renal pathology in TSLP tg mice

Vehicle-treated male TSLP tg mice demonstrated typical features of the previously described cryoglobulinaemic MPGN [1]. Glomerular lesions were characterized by increased glomerular size, an absolute increase in the cell number per glomerulus, an increase of glomerular extracellular matrix and immune complex deposition in the mesangial regions, in the capillary walls and as large aggregates in the capillary lumina. No histological differences were detected between vehicle-treated and ATRA-treated WT mice, both of which had normal appearing kidneys. Unexpectedly, the renal pathology in TSLP tg mice was aggravated after treatment with ATRA. In periodic-acid methenamine silver-stained histological sections, an increase in glomerular tuft size was apparent and the percentage of glomerular tuft area occupied by silver-stained matrix in TSLP tg mice was significantly increased after treatment with ATRA at 4 ($P < 0.05$) and 8 weeks ($P < 0.01$) (Table 1; Figure 2). A second measure of glomerular matrix expansion was accumulation of collagen type IV. Collagen type IV, assessed by the percentage of glomerular tuft area occupied by collagen type IV expressing matrix,

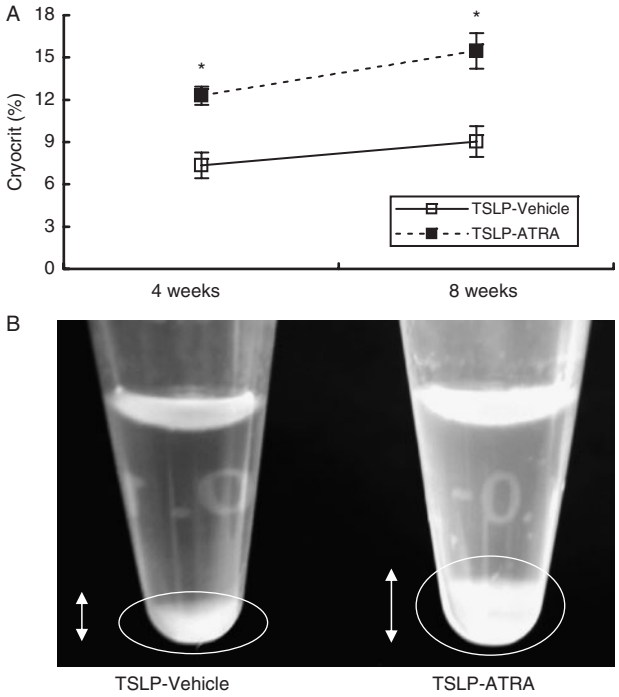

Fig. 1. ATRA increases cryoglobulin levels in TSLP tg mice. (**A**) Compared with vehicle-treated TSLP tg mice, a significant increase of circulating cryoglobulin levels, measured as a cryocrit, was observed in ATRA-treated TSLP tg mice at each time point. Data are mean ± SEM. $^{*}P < 0.001$ *vs* vehicle-treated TSLP tg mice. (**B**) A representative cryoglobulin precipitate from vehicle-treated TSLP tg mice and ATRA-treated TSLP tg mice at 8 weeks treatment is shown. The sera from vehicle-treated TSLP tg mice demonstrate visible cryoprecipitates that were increased after treatment with ATRA. TSLP, thymic stromal lymphopoietin; ATRA: all-trans-retinoic acid.

is significantly increased in control TSLP tg mice compared with WT mice at 4 ($P < 0.001$) and 8 weeks ($P < 0.01$). Treatment with ATRA significantly increased glomerular collagen type IV in TSLP tg mice at 4 ($P < 0.001$) but not 8 weeks (Table 1; Figure 3). Treatment with ATRA had a variable effect on glomerular cell number. In H&E-stained tissue sections, the cell number per glomerular cross-section was significantly increased in control TSLP tg mice compared with WT mice at 4 weeks treatment ($P < 0.05$), but this difference, while persistent, was no longer statistically significant after 8 weeks of treatment (Table 1; Figure 2). ATRA-treated TSLP tg mice demonstrated a trend towards further increase in glomerular cell number compared with that in control TSLP tg mice, but this trend did not achieve statistical significance (Table 1; Figure 2). We examined glomerular cell proliferation more specifically by counting number of cells expressing the proliferation marker Ki-67 per 50 glomerular cross-sections. Although the number of cells expressing Ki-67 in control TSLP tg mice did not differ significantly compared with WT mice after 4 weeks treatment, the number was increased 2.20-fold by the treatment with ATRA ($P < 0.001$). On the other hand, after 8 weeks treatment, cells expressing Ki-67 exhibited a trend towards an increase in vehicle-treated TSLP tg mice compared with WT mice, but treatment with ATRA did not significantly affect the increase in this cell number (Table 1).

As shown in Figure 3, mesangial cell activation, assessed by the percentage of glomerular tuft area

Table 1. Morphometric data

	WT-Vehicle	WT-ATRA	TSLP-Vehicle	TSLP-ATRA
Glomerular size (mm^2)				
4 weeks	2790.73 ± 117.47	3130.74 ± 178.73	3477.44 ± 163.96	3656.50 ± 109.88
8 weeks	3176.63 ± 46.08	3110.77 ± 67.96	3585.83 ± 138.63	4006.94 ± 219.04†
%GTA occupied by silver-stained matrix				
4 weeks	9.41 ± 0.74	8.18 ± 0.51	12.11 ± 0.98	16.67 ± 1.31*†††
8 weeks	8.54 ± 0.60	9.10 ± 0.44	10.95 ± 0.76	17.36 ± 1.83**†††
Cell number/glomerular cross-section				
4 weeks	32.97 ± 1.04	34.43 ± 0.58	38.36 ± 1.52†	42.74 ± 1.59
8 weeks	34.37 ± 0.92	33.40 ± 0.40	37.95 ± 1.72	39.67 ± 0.78
%GTA occupied by collagen type IV stained matrix				
4 weeks	11.23 ± 0.99	13.92 ± 0.56	17.54 ± 0.49†††	24.61 ± 0.71***†††
8 weeks	11.41 ± 0.68	10.84 ± 0.28	19.32 ± 1.10††	23.12 ± 1.72†††
%GTA occupied by αSMA-expressing cells				
4 weeks	0.30 ± 0.04	0.55 ± 0.13	2.03 ± 0.52	6.06 ± 2.14†
8 weeks	0.31 ± 0.09	0.46 ± 0.09	2.21 ± 0.27	4.89 ± 1.09†
GTA occupied by Mac-2-expressing cells (μm^2)				
4 weeks	12.24 ± 3.15	21.41 ± 1.40	62.08 ± 17.64	176.69 ± 38.05**†††
8 weeks	11.22 ± 1.23	12.39 ± 1.25	71.92 ± 31.89††	140.12 ± 17.73††
Ki-67-expressing cells/50 glomerular cross-sections				
4 weeks	28.80 ± 4.21	35.25 ± 5.17	31.75 ± 3.20	72.00 ± 7.38***††
8 weeks	21.67 ± 2.12	22.00 ± 3.71	42.20 ± 15.06	49.50 ± 24.89

Morphometric analysis of kidney sections from vehicle-treated WT mice, ATRA-treated WT mice, vehicle-treated TSLP tg mice and ATRA-treated TSLP tg mice.
Data are mean ± SEM.
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* vehicle-treated TSLP tg mice.
$^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ vs WT mice receiving the same treatment.
GTA, glomerular tuft area; αSMA, α-smooth muscle actin; WT, wild-type; TSLP, thymic stromal lymphopoietin; ATRA, all-trans-retinoic acid.

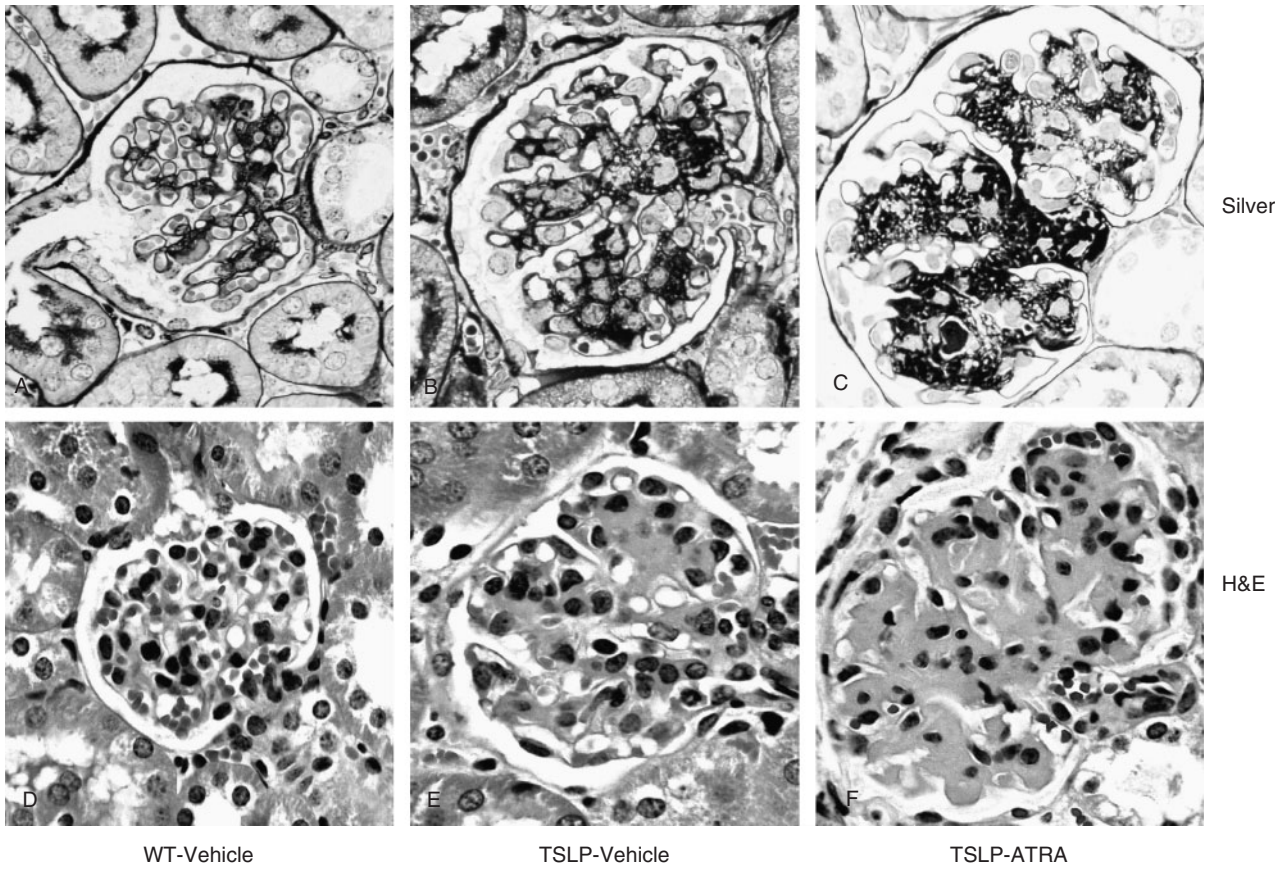

Fig. 2. ATRA exacerbates glomerulonephritis in TSLP transgenic mice. Light microscopic features of the glomerular lesions. Representative glomeruli stained with silver methenamine (A–C) and H&E (D–F) of a vehicle-treated WT mouse (A, D), a vehicle-treated TSLP tg mouse (B, E) and an ATRA-treated TSLP tg mouse (C, F) at 8 weeks treatment. TSLP tg mice treated with ATRA develop progressive matrix accumulation. Overall cell number is increased in the glomerulus shown in F, compared with that shown in E, but overall statistically significant differences between the two groups were not observed. All original magnifications: 400×. WT, wild-type; TSLP, thymic stromal lymphopoietin; ATRA, all-trans-retinoic acid; H&E, haematoxylin and eosin.

occupied by αSMA-expressing cells demonstrated a trend towards greater activation in TSLP tg mice compared with WT mice. In ATRA-treated TSLP tg mice, αSMA expression tended to be higher than in vehicle-treated TSLP tg mice, but this difference did not achieve statistical significance (Table 1). There was a significant increase of glomerular macrophage infiltration in TSLP tg mice compared with WT mice as assessed by glomerular tuft area occupied by Mac-2-positive cells at 8 weeks treatment ($P < 0.01$). Glomerular Mac-2^+ macrophages in TSLP tg mice were increased 2.85-fold ($P < 0.01$) and 1.95-fold (*NS*) after 4 and 8 weeks treatment with ATRA compared with vehicle treatment, respectively (Table 1; Figure 3).

There was significantly increased albumin excretion in control TSLP tg mice, measured by albumin/creatinine ratios compared with WT mice (Table 2). TSLP tg mice treated with ATRA developed increased proteinuria compared with vehicle treated controls, but the differences were not statistically significant (Table 2). Albumin excretion in WT mice was not affected significantly by treatment with ATRA.

Effects of ATRA on glomerular immunoglobulins and complement deposition in TSLP tg mice

Vehicle-treated TSLP tg mice showed markedly increased deposition of immunoglobulins and complement component C3 in the glomeruli compared with WT mice at each time point (IgG: 4 weeks: 0.33 ± 0.18 *vs* 2.25 ± 0.15, $P < 0.01$; 8 weeks: 0.83 ± 0.31 *vs* 2.80 ± 0.12, $P < 0.01$; IgM: 4 weeks: 0.83 ± 0.09 *vs* 2.42 ± 0.13, $P < 0.01$; 8 weeks: 1.17 ± 0.21 *vs* 2.40 ± 0.19, $P < 0.05$; IgA: 4 weeks: 0.83 ± 0.09 *vs* 1.50 ± 0.19, $P < 0.05$; 8 weeks: 0.67 ± 0.31 *vs* 2.10 ± 0.24, $P < 0.05$; C3: 4 weeks: 0.17 ± 0.14 *vs* 1.83 ± 0.41, $P < 0.05$; 8 weeks: 0.50 ± 0.22 *vs* 1.88 ± 0.38, $P < 0.05$, vehicle-treated WT mice *vs* vehicle-treated TSLP tg mice, respectively). No significant differences were seen in the amount of deposited glomerular immunoglobulin and C3 deposition between vehicle and ATRA treatment in TSLP tg mice (data not shown).

ATRA aggravated systemic injuries in TSLP tg mice

In concert with the accelerated glomerular injury, ATRA aggravated the accumulation of leucocytes in

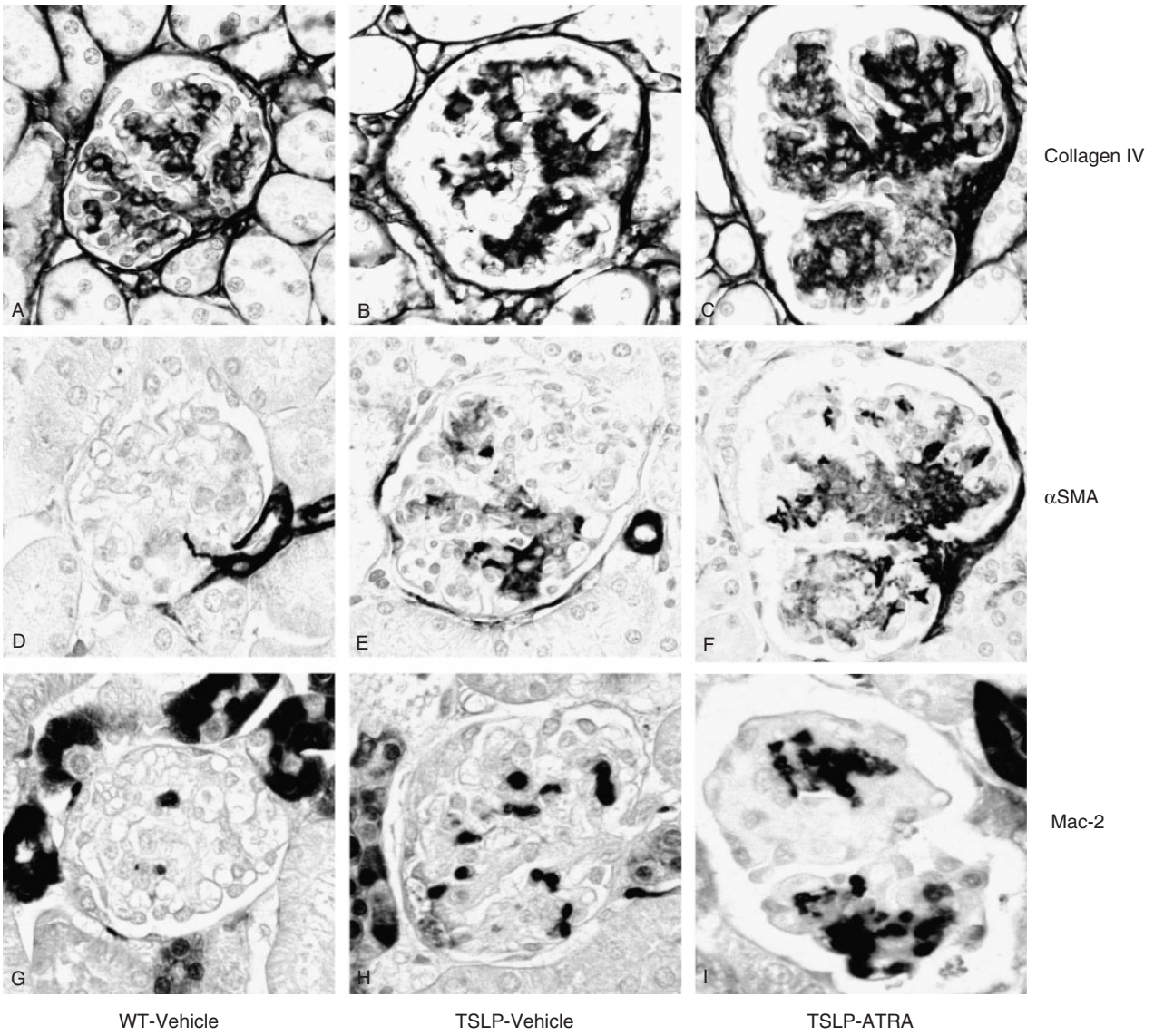

Fig. 3. ATRA exacerbates multiple features of glomerulonephritis in TSLP transgenic mice. Representative glomeruli stained for collagen type IV (**A–C**), αSMA (**D–F**) and Mac-2 (**G–I**) of a vehicle-treated WT mouse (A,D,G), a vehicle-treated TSLP transgenic mouse (B,E,H) and an ATRA-treated TSLP tg mouse (C,F,I) at 8 weeks treatment. ATRA exacerbates renal injury in TSLP tg mice with increased glomerular matrix deposition (collagen type IV), mesangial activation (αSMA expression) and glomerular macrophage influx. All original magnifications: 400×. WT, wild-type; TSLP, thymic stromal lymphopoietin; ATRA, all-trans-retinoic acid; αSMA A, α-smooth muscle actin.

Table 2. Effects of ATRA on albuminuria of the study groups

	WT-Vehicle	WT-ATRA	TSLP-Vehicle	TSLP-ATRA
Albuminuria (μg albumin/mg creatinine)				
4 weeks	15.15 ± 3.23	9.72 ± 4.17	40.59 ± 13.24	92.04 ± 42.52
8 weeks	15.05 ± 1.17	16.26 ± 2.48	44.05 ± 8.45	57.80 ± 11.72

Data are mean ± SEM.
WT, wild-type; ATRA, all-trans-retinoic acid; TSLP, thymic stromal lymphopoietin.

the liver compared with vehicle-treated TSLP tg mice at each time point (data not shown). Vehicle-treated TSLP tg mice showed mild and moderate lung involvement with mixed peribronchial and perivascular leucocyte infiltration at 4 and 8 weeks treatment, respectively (Figure 4). Treatment with ATRA exacerbated the leucocyte infiltration in lungs in TSLP tg mice at each time point (Figure 4).

A

Group	Weeks of treatment	
	4	8
TSLP-Vehicle	1.60 ± 0.25	2.20 ± 0.37
TSLP-ATRA	1.83 ± 0.48	2.57 ± 0.52

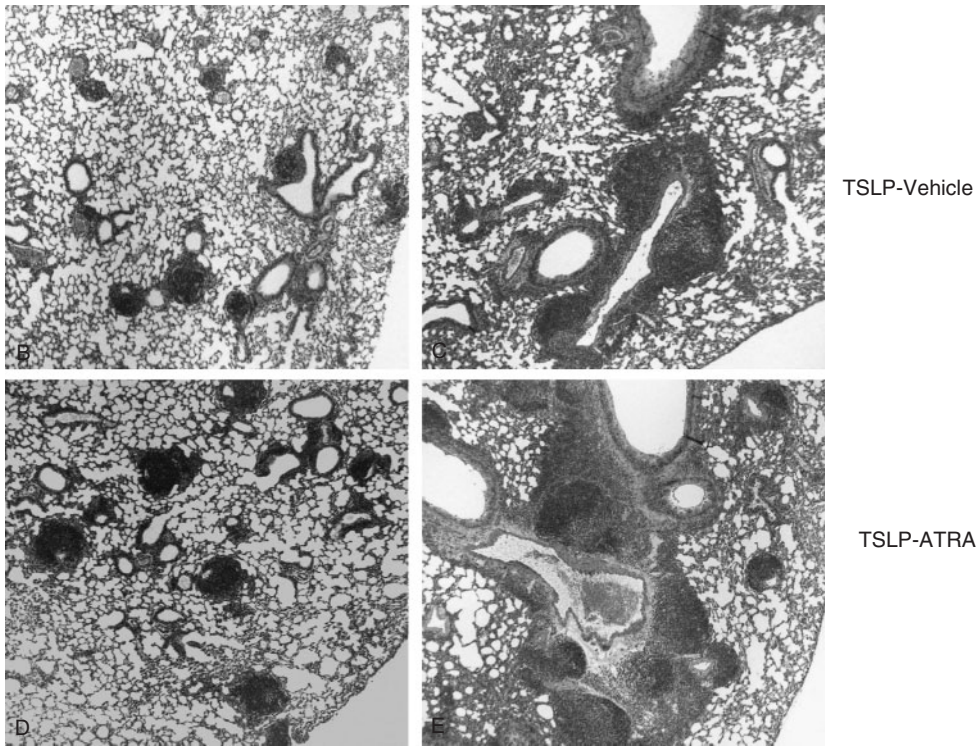

Fig. 4. ATRA exacerbates systemic organ inflammation, including in the lung. Semiquantitative scoring of the severity of pulmonary inflammation scored on a scale of 0–4+ (**A**). Data are mean ± SEM. Representative lung sections stained with H&E of a vehicle-treated TSLP tg mouse (**B**,**C**) and an ATRA-treated TSLP tg mouse (**D**,**E**) at 4 weeks (B,D) and 8 weeks (C,E) treatment. All original magnifications: 40×. H&E, haematoxylin and eosin; TSLP, thymic stromal lymphopoietin; ATRA, all-trans-retinoic acid.

Affected organs of TSLP tg mice including lung, liver, spleen and thymus (1) were significantly increased in their relative weight compared with WT mice (Table 3). Treatment with ATRA further increased the relative weights of kidney and spleen in TSLP tg mice compared with vehicle treatment (Table 3). Organ weights in WT mice were not affected significantly by treatment with ATRA.

Effect of ATRA on T helper cells type 1 (Th1) and type 2 (Th2) associated immunoglobulin isotypes in TSLP tg mice

Serum levels of IgG1, IgG2a and IgM were markedly increased in TSLP tg mice compared with those of WT mice (Figure 5A–C). Treatment with ATRA resulted in further increased concentrations of IgG1 and IgM of TSLP tg mice, but did not affect IgG2a concentrations in TSLP tg mice (Figure 5A–C). In vehicle-treated TSLP tg mice, IgG1/IgG2a immunoglobulin ratios were increased compared with WT mice, and this was more pronounced at 8 weeks (Figure 5D). ATRA further enhanced the characteristic Th2 immune response in TSLP tg mice as indicated by increases in IgG1/IgG2a immunoglobulin ratios (Figure 5D). Treatment with ATRA also shifted the immunoglobulin ratios in WT mice towards a Th2 phenotype as shown in Figure 2D.

The renal, liver and lung injury in TSLP tg mice is strongly associated with serum IgG1 levels

In control TSLP tg mice, there were strong correlations between the αSMA expression, collagen type IV

Table 3. Effects of ATRA on relative organ weights of the study groups

Organ	WT-Vehicle	WT-ATRA	TSLP-Vehicle	TSLP-ATRA
Heart weight/BW				
4 weeks	6.15 ± 0.34	6.51 ± 0.28	5.42 ± 0.25	5.94 ± 0.42
8 weeks	5.54 ± 0.17	6.51 ± 0.52	5.47 ± 0.09	6.66 ± 0.58
Kidney weight/BW				
4 weeks	12.88 ± 0.51	15.01 ± 0.96	12.12 ± 0.31	13.16 ± 0.66
8 weeks	12.17 ± 0.27	12.01 ± 0.63	11.95 ± 0.41	14.00 ± 0.44*†
Lung weight/BW				
4 weeks	6.58 ± 0.22	7.10 ± 0.24	10.57 ± 0.82†	11.41 ± 1.29††
8 weeks	6.39 ± 0.25	6.68 ± 0.51	10.14 ± 1.38	14.26 ± 2.31††
Liver weight/BW				
4 weeks	64.55 ± 3.01	58.05 ± 4.74	67.73 ± 1.93	75.08 ± 3.24†
8 weeks	57.08 ± 3.73	60.84 ± 1.54	64.10 ± 2.53	74.46 ± 3.55†
Spleen weight/BW				
4 weeks	3.68 ± 0.43	5.13 ± 0.70	14.55 ± 1.16†††	22.24 ± 1.90**†††
8 weeks	3.38 ± 0.20	3.83 ± 0.18	11.73 ± 2.07††	19.35 ± 1.85**†††
Thymus weight/BW				
4 weeks	2.84 ± 0.27	2.93 ± 0.19	8.37 ± 0.66†††	8.26 ± 0.92†††
8 weeks	2.82 ± 0.12	2.53 ± 0.29	6.11 ± 0.61†††	5.19 ± 0.43†††

Relative organ data are expressed as milligrams organ weight/grams body weight.
Data are mean ± SEM.
*$P < 0.05$, **$P < 0.01$ *vs* vehicle-treated TSLP tg mice.
†$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ *vs* WT mice receiving the same treatment.
WT, wild-type; ATRA, all-trans-retinoic acid; TSLP, thymic stromal lymphopoietin.

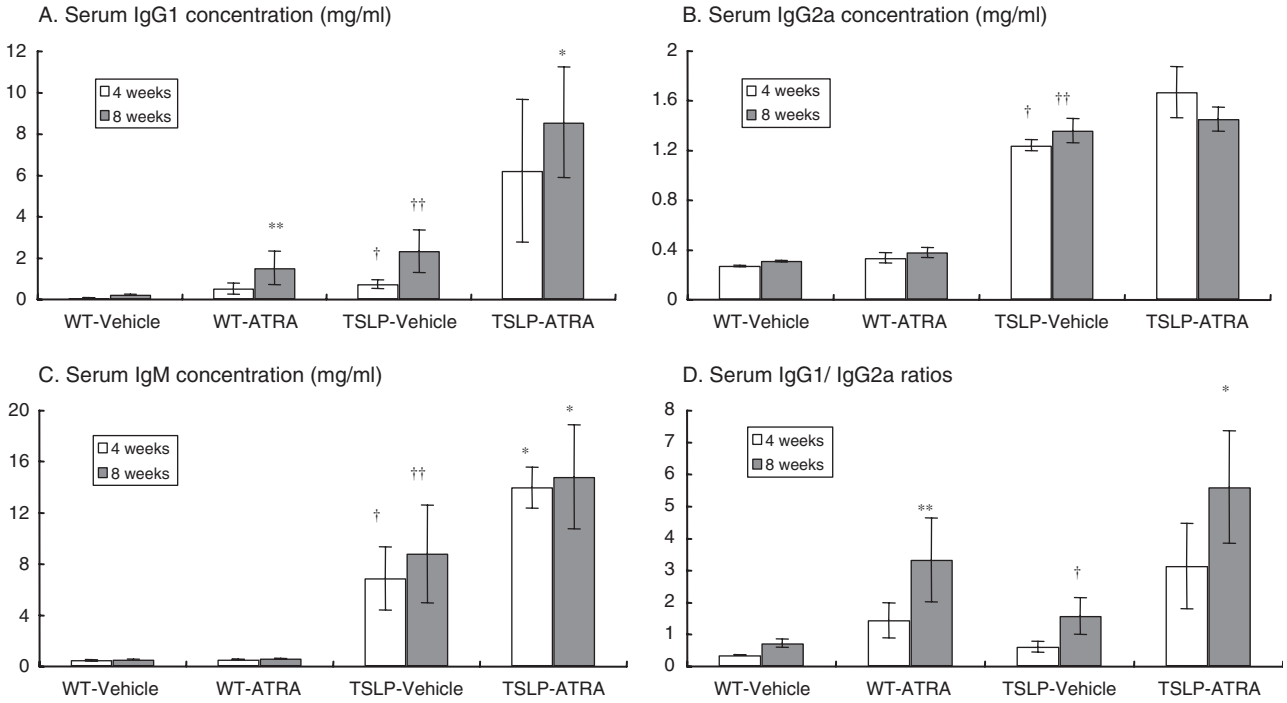

Fig. 5. Effect of ATRA on serum immunoglobulin levels and IgG1/IgG2a immunoglobulin ratios of the study groups. (**A**) Serum IgG1, IgG2a and IgM levels were determined by ELISA at 4 and 8 weeks treatment. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$ *vs* mice of the same genotype with vehicle injection. †$P < 0.05$, ††P < 0.01 *vs* vehicle-treated WT mice. (**B**) The preferentially increased IgG1 levels in ATRA-treated TSLP tg mice results in an increased IgG1/IgG2a ratio, most characteristic of a Th2 immune response, at 4 and 8 weeks. WT, wild-type; TSLP, thymic stromal lymphopoietin; ATRA, all-trans-retinoic acid.

accumulation and Mac-2 expression with serum IgG1 levels at 8 but not 4 weeks treatment (Table 4). In ATRA-treated TSLP tg mice, there were strong correlations between the αSMA expression and collagen type IV accumulation with serum IgG1 levels at 8 weeks treatment, while there was a strong correlation between the αSMA expression and Mac-2 expression with serum IgG1 levels at 4 weeks treatment. Combined, these data indicate that serum IgG1 levels were associated with the progression of renal injury in

Table 4. Correlation of characteristics in TSLP tg mice with serum IgG1 levels

	4 weeks		8 weeks	
	TSLP-Vehicle	TSLP-ATRA	TSLP-Vehicle	TSLP-ATRA
Renal injury				
% GTA occupied by α-SMA-expressed cells	0.13 (NS)	0.95 (0.0035)	0.97 (0.0073)	0.86 (0.012)
% GTA occupied by collagen IV-expressed matrix	−0.38 (NS)	−0.04 (NS)	0.97 (0.0065)	0.85 (0.0153)
GTA occupied by Mac-2-possitive cells	−0.12 (NS)	0.90 (0.015)	0.99 (0.0002)	−0.29 (NS)
Systemic injuries				
Liver injury	−0.48 (NS)	0.97 (0.0012)	0.88 (0.048)	0.84 (0.0358)
Lung injury	−0.29 (NS)	0.89 (NS)	0.95 (0.0167)	0.92 (0.0067)
Relative organ weights				
Kidney	−0.49 (NS)	0.81 (0.049)	0.55 (NS)	0.43 (NS)
Liver	0.18 (NS)	0.92 (0.0099)	0.75 (NS)	0.18 (NS)
Lung	−0.04 (NS)	0.88 (0.021)	0.93 (0.0208)	0.95 (0.0013)
Spleen	−0.02 (NS)	0.74 (0.0936)	0.91 (0.0309)	0.73 (NS)

Data are *r*-value based on Pearson's correlation coefficient; (*P*-value).
GTA, glomerular tuft area; αSMA, α-smooth muscle actin; TSLP, thymic stromal lymphopoietin; ATRA, all-trans-retinoic acid; NS, not significant.

TSLP tg mice, especially at an advanced stage (Table 4). After 8 weeks of ATRA treatment, a correlation of the severity of liver and lung injury and serum IgG1 levels was observed, similar to the renal injury (Table 4).

This was further quantified by measures of the weights of the affected organs as well as spleen as a measure of expansion of the lymphoid tissues. In control TSLP tg mice, the relative weights of the lung and spleen significantly correlated with serum IgG1 levels after 8 but not 4 weeks of treatment. In ATRA-treated TSLP tg mice, there was a strong correlation between the relative weight of the lung with serum IgG1 levels, and a weaker correlation, albeit not statistically significant, between the relative weight of the spleen with serum IgG1 levels at each time point. The increased relative weights of the kidney and liver significantly correlated with serum IgG1 levels in ATRA-treated TSLP tg mice after 4 weeks of treatment. These observations suggest that the affected organ weights, especially the lung and spleen weights in TSLP tg mice, are closely associated with serum IgG1 levels (Table 4).

Discussion

In the present study, we have shown that the administration of ATRA in cryoglobulinaemic TSLP tg mice results in a marked deterioration of the systemic injuries seen in these mice. This unexpected outcome was associated with (i) increased circulating cryoglobulins as revealed by serum cryoglobulin precipitates; (ii) altered circulating immunoglobulins with increased IgG1; and (iii) increased systemic inflammatory injuries involving lung and liver, two notable sites of injury in this model of cryoglobulinaemia. In the kidney, ATRA treatment resulted in increased glomerular size, glomerular extracellular matrix, glomerular immune complex deposition, glomerular fibrosis, glomerular cell activation, glomerular macrophage infiltration and glomerular cell proliferation. All of these untoward findings were unexpected. Based on the well-characterized anti-inflammatory activities of ATRA, we expected that treatment with ATRA might have beneficial effects on the course of the disease, with improvement in renal function and histopathology and/or prolonging survival of the diseased mice. Numerous previous studies have demonstrated a benefit of ATRA therapy in various kinds of experimental models of kidney disease, further supporting this hypothesis [3,4–8]. The dose of ATRA utilized in these studies ranged from daily administration of 10 mg/kg BW [4,5] or 15 mg/kg BW [6] to thrice weekly injections of 5 mg/kg BW [7] or 25 mg/kg BW [8], and are in the same range that was used in the present study (20 mg/kg BW three times weekly). These studies have, in aggregate, shown that the beneficial effects of ATRA therapy are the results of modified expression of a multitude of cytokines and chemokines, including IFN-γ, IL-2, IL-10, TNF-α and MCP-1 leading to a reduction of inflammatory events. Interestingly, recent studies suggest that the renoprotective effects of ATRA may be at least in part due to activities specifically affecting podocytes [9,10]. Bek *et al*. [9] found that Stra13, a retinoic acid-inducible gene, might play an important protective role against oxidative stress in podocytes. Vaughan *et al*. [10] demonstrated that ATRA induced podocyte process formation, enabled podocytes to maintain a differentiated phenotype and reduced podocyte proliferation *in vitro*, and prevented the loss of nephrin and podocin expression *in vivo* in an experimental glomerulonephritis model induced by anti-glomerular basement antibodies. These experimental studies, in aggregate, have been so compelling that there is a currently active clinical trial to use retinoids as a therapy for focal segmental glomerulonephritis that is sponsored by the U.S. National Institutes of Health.

However, a detrimental effect of ATRA on kidney has also been reported previously *in vitro* [11] and *in vivo* [12]. Alique *et al*. [11] demonstrated that ATRA

exacerbated glycated albumin effects on intracellular oxidation and the expression of the molecules involved in leucocyte infiltration in cultured human mesangial cells. Moulder *et al.* [12] reported that ATRA had detrimental effects on radiation nephropathy and they speculated that it might be the result of stimulation of renal cell proliferation or inhibition of renal nitric oxide activity. In humans, ATRA is usually well tolerated, but adverse effects on the kidney have been observed in patients treated with ATRA for acute promyelocytic leucaemia (APL), who may develop acute renal failure [13]. The pathophysiology of renal failure associated with ATRA is still poorly understood, but proposed mechanisms are thought to involve changes in cytokine secretion [13].

We believe a likely explanation for the detrimental effects of ATRA treatment observed in TSLP tg mice might be an immunomodulatory effect of ATRA on B cell function. ATRA treatment augmented serum IgG1 and IgM levels, the main components of cryoglobulins in TSLP tg mice. Increased serum IgG1 levels in ATRA-treated mice had strong correlations with increased cryoglobulin levels, which in turn provides a likely basis for the exacerbated renal and systemic injuries. The direct effects of retinoids on B cells have been reported. Despite the report that retinol inhibited the proliferation and differentiation of primary human B cells [14], van Bennekum *et al.* [15] have shown that the proliferative response of splenocytes to B cell mitogens is decreased in cells isolated from vitamin A-deficient rats. More specific effects of retinoids on mature B-cell populations are largely unknown. Recent findings have suggested that ATRA can affect the TSLP signalling pathway. Li *et al.* [16] demonstrated that application of an ATRA receptor γ –selective agonist (BM961) on mouse ear skin led to a significant increase of TSLP transcripts, and topical application of ATRA resulted in a similar induction. They also demonstrated that expression of TSLP is rapidly induced in keratinocytes by selective ablation of RXRα and RXRβ in adult mouse epidermal keratinocytes (RXR$\alpha\beta^{ep-/-}$ mice), a model of atopic dermatitis [17]. Taken together, these findings indicate that ATRA might have a direct effect as well as an indirect effect on B cells via increasing systemic TSLP transcripts, thereby augmenting the altered immunoglobulin profile characteristic of TSLP tg mice.

The paradigm of Th1 and Th2 immune responses is well established and plays a crucial role in autoimmune diseases. Vitamin A deficiency causes strong regulatory T cell imbalance with excessive Th1 cell interferon (IFN)-γ synthesis and diminished Th2 cell development and function [18]. On the other hand, ATRA inhibits IFN-γ stimulatory activity of antigen presenting cells, enhances Th2 cell differentiation and inhibits Th1 cell IFN-γ synthesis [18,19]. These findings are further supported by the findings of Kinoshita *et al.* [20] that ATRA dramatically suppressed Th1 cytokines IFN-γ and IL-2, but not the Th2 cytokine IL-4 in a murine model of systemic lupus. Thus, vitamin A deficiency may bias the immune response towards a Th1 direction, whereas ATRA may bias the response in a Th2 direction. Consistent with these findings, our data indicates that immune modulation by ATRA is associated with a shift towards a Th2 immunoglobulin phenotype. During B-cell differentiation, the presence of Th1 or Th2 cytokines determines the switch to different immunoglobulin isotypes. For instance, in mice a Th1 profile is associated with the preferential production of IgG2a while a Th2 profile favours IgG1 and IgE [21]. In our studies, ATRA augmented the Th2-dependent serum IgG1 levels but did not affect the Th1-dependent serum IgG2a levels in both TSLP tg mice and WT mice. This suggests that Th-2 switching was induced in response to ATRA. Other studies examining immunological alterations in TSLP tg mice have reported dominance of the Th2 response and have shown that TSLP is an important factor necessary and sufficient for the initiation of allergic inflammation [17,22–25]. Taken together, these data suggest that Th2 dominance, known to be involved in TSLP tg mice, was further enhanced by the administration of ATRA, resulting in worsening of disease manifestations in multiple organs. Indeed, the higher IgG1/IgG2a ratios, a marker of Th2 polarization, already present in TSLP tg mice, were substantially increased by treatment with ATRA. These Th2-dependent IgG1 levels were tightly correlated with disease manifestations in ATRA-treated TSLP tg mice.

Our results demonstrate that ATRA is not protective but instead aggravates cryoglobulinaemic MPGN and its systemic manifestations. These findings may be due to the altered immunoglobulin profile and/or enhanced systemic Th2 response. Previously reported evidence as well as our present data point to a role for Th1/Th2 balance in mediating the manifestations of disease in cryoglobulinaemic TSLP tg mice. Although disappointing, our results also suggest caution in the application of retinoid therapy to human disease based on the largely positive animal data reported to date.

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Conflict of interest statement. None declared.

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