RESEARCH ARTICLE

Mechanisms of immune complex-mediated experimental glomerulonephritis: possible role of the balance between endogenous TNF and soluble TNF receptor type 2

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ABSTRACT. In an experimental model of immune-complex-mediated glomerulonephritis, mice excreted increased levels of urinary protein starting three days after the induction. Mice lacking the TNF receptor type 2 (TNFR2) were protected from early proteinuria and enhanced mortality. Analysis of the molecular basis of the mechanisms of glomerulonephritis revealed that naïve mice continuously excrete soluble TNF-neutralizing TNFR2 in urine. Mice kept in a specific pathogen-free environment did not go on to develop early proteinuria or enhanced mortality, following induction of glomerulonephritis. TNFR2-deficient mice were protected from early proteinuria and enhanced mortality only when housed conventionally. Mice producing human TNFR2 that can be activated by mouse TNF, in addition to mouse TNFR2, did not demonstrate enhanced susceptibility to the lethal effects of glomerulonephritis, indicating that pro-inflammatory signalling via TNFR2 does not account for a sensitizing effect. Finally, we suggest that the protective effect seen in mice lacking TNFR2 results rather from environment-induced attenuation by low dose bacterial endotoxins than from missing pro-inflammatory signalling via the TNFR2.

Key words: tumor necrosis factor, p75TNF receptor, TNFR2, kidney, inflammation, tolerance

Tumor necrosis factor (TNF) is a central inflammatory cytokine and an important mediator of inflammatory tissue damage (reviewed in [1]). It is released within minutes of stimulation with tiny amounts of inflammatory agents such as LPS. Hence, regulation of expression as well as the biological availability of TNF are of critical importance to the immune system and are thus tightly controlled. One main regulatory protein is TNF receptor type 2 (TNFR2), which can be cleaved off the cell surface and neutralizes TNF, thus acting as a soluble TNF scavenger.

Many experimental and clinical studies support a critical role for TNF in the pathogenesis of acute and chronic renal disease (reviewed in [2, 3]). Previous data by Vielhauer *et al*. involving a model of immune complex-mediated glomerulonephritis (GN) showed that TNFR1-mediated effects are critical for the development of GN, while TNFR2-deficient mice were found to be completely protected from GN [4]. TNFR2 expression on intrinsic cells, but not on leukocytes, was identified as critical for the development of GN, leading to the conclusion that TNFR2 has an essential, pro-inflammatory function in GN.

We used an experimental model of immune complex-induced GN that involved injecting rabbit antibodies directed against glomerular basement membrane (GBM) proteins into mice previously immunized with rabbit Ig [5]. This model has also been used in previous studies by Vielhauer *et al*. [4]. We analyzed the impact of the environment on the protective mechanism seen in TNFR2-deficient mice. To this end, mice were housed either under specific pathogen-free (SPF) conditions, with presumably low levels of inflammatory factors in the environment, or in an open access, conventional animal facility with presumably higher levels of these factors. We show here that TNFR2-deficient mice were protected from early proteinuria and enhanced mortality only when housed conventionally. TNFR2-over-expressing transgenic mice showed neither increased protein levels in the urine early after induction of GN, nor enhanced mortality. Since naïve mice continuously excrete soluble TNFR2 in the urine, these findings indicate that the protective effects seen in mice lacking TNFR2 are rather based on an environmental-induced attenuation by low-dose bacterial endotoxins than on absent, pro-inflammatory signaling via the TNFR2.

METHODS

Mice

C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). TNFR2-deficient mice (Tnfrsf1b) [6] were purchased from The Jackson Laboratory (Bar Harbour, Maine, USA). Deficiency of TNFR2 expression was verified by PCR. Mice transgenic for the human TNFR2 (hTNFR2tg) were generated as described in detail below. Confirmed hTNFR2tg founder mice were crossed

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with C57BL/6 mice for more than five generations; non-transgenic littermates were used as controls. Mice were housed in the animal facility of the University of Regensburg and handled in accordance with institutional guidelines. Two different types of housing were used:
– SPF-like conditions, with strict hygienic control and regulations for access and inward transfer of materials
– conventional housing with open access conditions.
All experiments were performed in compliance with the federal guidelines for animal experimentation.

Plasmid construction and generation of hTNFR2tg mice

To generate hTNFR2tg mice, full length hTNFR2-cDNA was cloned into *Hind* III and *Xho* I sites of an expression vector driven by the human ubiquitin C promoter [7]. The sequence of the cloned hTNFR2 construct was confirmed by sequencing. The transgenic hTNFR2 fragment was released by *Nde* I/*Kpn* I digestion of the vector and microinjected into pronuclei of fertilized oocytes prepared from FvB mice using standard protocols. The offspring were screened for transgene integration by DNA extraction from tail biopsies. The purified DNA was analyzed by Southern blot analysis using a vector-specific fragment as probe. Confirmed founders were back-crossed to a C57BL/6 background and transgene transmission in the offspring was tested by the polymerase chain reaction (PCR), as detailed below.

PCR genotyping

For PCR analysis, DNA was obtained from tail biopsies by phenol/chloroform extraction. Genomic DNA was analysed using the following primers: for TNFR2-deficient mice: 5'-GCG CAT CGC CTT CTA TCG CC-3' and 5'-CCT CTC ATG CTG TCC CGG ATT-3'. For specific amplification, 35 cycles were performed: 94°C for 45 s, annealing temperature 56 °C for 30 s and 72 °C for 60 s. For hTNFR2tg mice: 5'-CCT GCA TCG TGA ACG TCT GTAGC-3' and 5'-GTC CAA GGT TCC GTT CGC GCG-3'. For specific amplification, 35 cycles were performed: 94°C for 45 s, annealing temperature 62 °C for 30 s and 72 °C for 30 s. Aliquots of the samples were analysed by electrophoresis on 1.2% agarose gel and visualized by ethidium bromide staining.

Induction of GN and collection of urine, serum, and kidneys

Nephrotoxic serum was prepared by immunizing rabbits with mouse glomerular basement membrane (GBM), as described previously [5]. Briefly, Chinchilla rabbits were immunized with a preparation of homogenized glomeruli isolated from mouse kidneys (kindly provided by R. Witzgall, Regensburg, Germany) to generate nephrotoxic serum containing rabbit anti-mouse GBM antibodies. Male, 7-18 week-old mice were immunized subcutaneously with rabbit IgG (0.2 mg, Jackson Immuno Research, Suffolk, UK) in complete Freund's adjuvant (Sigma Aldrich, Deisenhofen, Germany). Six days later, mice were injected intravenously with 0.25 mL of the nephrotoxic serum. Urine samples were collected from the mice once or twice a day prior to immunization, and up to day 15 following injection of the nephrotoxic serum. Mice were killed by cervical dislocation on day 15 and both kidneys were removed, paraffin-fixed, sectioned and stained with both hematoxylin & eosin, and PAS.

Quantification of protein and creatinine in urine and serum

Protein concentrations in urine were measured according to the method of Bradford using albumin as standard (BCA Protein Assay Kit, Thermo Scientific, Schwerte, Germany). Urine samples were diluted in PBS. Creatinine concentrations were measured in urine and serum in order to determine glomerular damage. The assay was carried out in accordance with the QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, USA). Urine and serum sample measurements were performed twice.

Quantification of TNF and TNFR2 concentrations in urine and serum

Quantifications of mouse TNF, soluble mouse TNFR2, and soluble human TNFR2 in urine and serum were carried out using the respective ELISA Kits (R&D Systems, Wiesbaden, Germany).

Statistics

Two-way ANOVA analysis with the Bonferroni *post hoc* test was used in experiments with two or more experimental groups. For survival curves, the log-rank (Mantel-Cox) test was used. $p<0.05$ was accepted as significantly different. All statistics were performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, USA).

RESULTS

Development of GN in TNFR2-deficient mice

First, we tested mice housed under open access conditions for their susceptibility to GN. Groups of wild type and TNFR2-deficient mice were subjected to the nephropathogenic protocol by injection of the nephrotoxic rabbit anti-mouse GBM serum six days after immunization with rabbit IgG. In accordance with the findings by Vielhauer *et al*., TNFR2-deficient mice were less affected by this treatment and showed less proteinuria in the so-called heterological early phase after injection of the nephrotoxic serum (*figure 1A*) [4]. Also, survival 14 days after induction of GN was clearly better in the group of TNFR2-deficient mice (six out of eight mice survived) compared to the wild type mice (one out of six mice survived) (*figure 1B*). Creatinine concentrations in urine did not differ between the two mouse lines and were constant on day 7 and 15 after induction with the nephrotoxic serum (data not shown). The ratios of protein to creatinine values in urine were similarly increased on day 15 in both mouse lines.
We also examined the concentration of soluble mouse TNFR2 in the urine of wild type mice treated according to the protocol for induction of GN. Surprisingly, even naïve mice secreted between 10 and 20 ng of soluble TNFR2 per mL in the urine (*figure 1C*). This concentration was enhanced immediately after immunization with rabbit IgG (day 1), as well as after injection of the nephrotoxic serum (day 7), and fluctuated to a higher level during the course

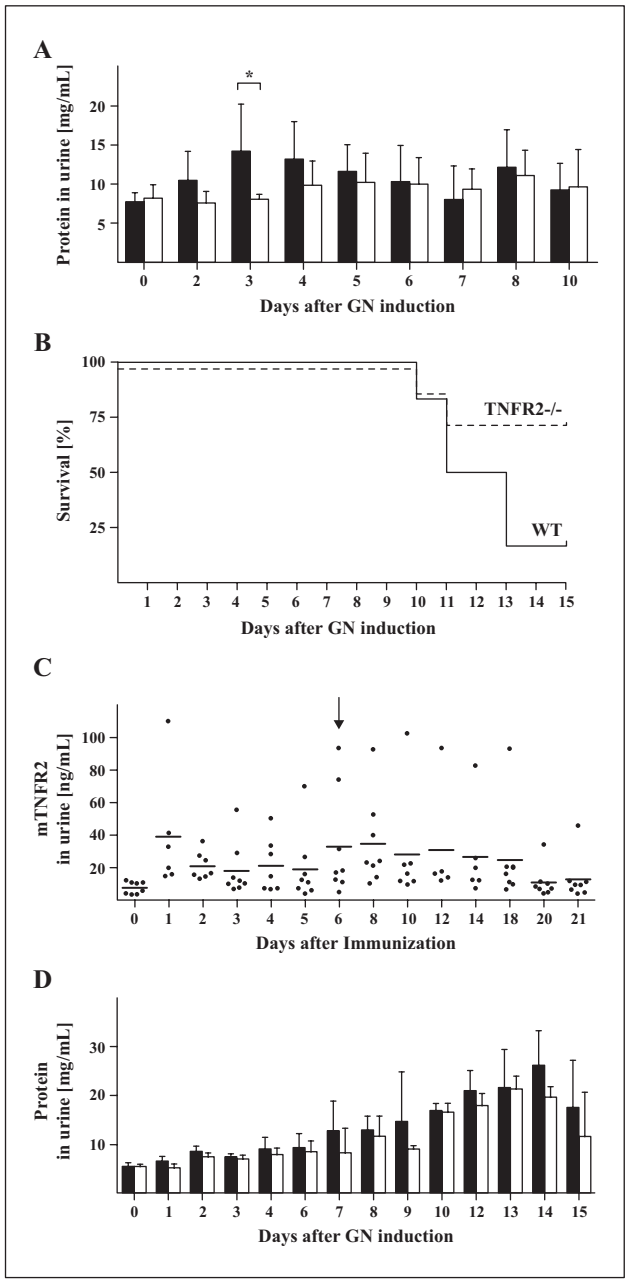

Figure 1
Development of proteinuria in TNFR2-deficient mice.
A) GN was induced in wild type (n=6, black bars) and TNFR2-deficient (n=8, white bars) mice. The mice had been transferred to an animal facility with open access, four weeks before the experiment. Proteinuria was determined for 10 days after injection of the nephrotoxic serum. Data are shown as mean with standard deviation as error bars; * indicates a statistical difference between the groups on the respective day (two-way ANOVA with a Bonferroni post-test). **B)** Survival curves for the experiment described in (A). The survival rates of mice from the two mouse lines were not significantly different (p=0.1 in the log-rank (Mantel-Cox) test). **C)** Release of soluble TNFR2 in urine from mice housed under open access conditions. All mice were immunized with rabbit IgG, according to the protocol for induction of GN, on day 0. The concentrations of TNFR2 in urine from mice before (day 0) and after immunization with rabbit Ig were determined. Mice were challenged with the nephrotoxic rabbit serum on day 6 (black arrow). Each symbol represents one mouse. Horizontal lines represent means. (D) Development of proteinuria in wild type (n=7, black bars) and TNFR2-deficient mice (n=7, white bars) under SPF-like conditions. Proteinuria was determined for 15 days after injection of the nephrotoxic serum. The proteinuria was statistically significantly different in TNFR2-deficient mice compared to control wild type mice ($p<0.05$ in two-way ANOVA). Data are shown as mean with standard deviation as error bars.

of the experiment. After immunization, increased soluble TNFR2 levels in urine were also observed for more than three weeks when there had been no challenge with nephrotoxic serum (data not shown).

Environmental effects (low-dose LPS exposure) on GN induction

To assay the impact of the environment on the observed effects, we analyzed mice born, raised, and kept under SPF-like conditions. The development of proteinuria in these mice was very similar that seen in TNFR2-deficient mice and wild-type mice, with a continuous increase of protein secreted in the urine from day 6 after induction of GN (*figure 1D*). Even though the difference in proteinuria between TNFR2-deficient and wild type mice was calculated as statistically significant, the actual differences in protein concentrations were very small and probably not biologically relevant. The creatinine concentrations in urine did not differ in the two mouse lines on day 7 and 15 (data not shown). Also, no difference was observed in survival of TNFR2-deficient mice under these conditions (six out of seven wild type mice survived and all seven TNFR2-deficient mice survived). These results were reflected in histological studies where the degree of tissue pathology correlated in individual mice with the severity of proteinuria at later time points. Mice with protein levels (<12 mg/mL) on day 15 did not show glomerular histological changes in contrast to mice with severe proteinuria (>27 mg/mL). Glomerular damage typical of crescentic or mesango-proliferative glomerulonephritis, such as a large crescent-shaped zone enclosing the glomerular capillaries due to massive extra-capillary hyper-cellularity, clustering of cells in capillaries, and the Bowman's capsule showing signs of hypercellularity and broadening (as shown in *figure 3D*) was seen.

Development of GN in hTNFR2-transgenic mice

Mouse TNF is perfectly able to interact with the human TNFR2. Therefore, if pro-inflammatory activation and signalling through the membrane receptor TNFR2 contributes to kidney damage, the over-expression of human TNFR2 would be expected to sensitize the organism to the development of GN. Several, independent mouse lines showing constitutive expression of the human TNFR2 gene under the influence of the promoter of ubiquitin C (*figure 2A*), were generated. Identification of transgenic founder mice and transmission of the hTNFR2 transgene were verified by Southern blot and PCR analysis (*figure 2B*-C). Transgenic mice from one of the mouse lines were tested for secretion of soluble human TNFR2 in the urine. As expected and in contrast to their non-transgenic littermates, all transgenic mice excreted soluble hTNFR2 (12.1 ± 3.7 ng/mL) in the urine (*figure 2D*). As already shown above for wild type mice, after having been subjected to the nephropathogenic protocol, all transgenic mice also excreted increased levels of soluble mouse TNFR2 (18.1 ± 3.1 ng/mL) in the urine at concentrations similar to those seen in their non-transgenic littermates (15.8 ± 6.5 ng/mL) (*figure 3A*). The concentrations of soluble mouse TNFR2 in serum on day 15 after induction

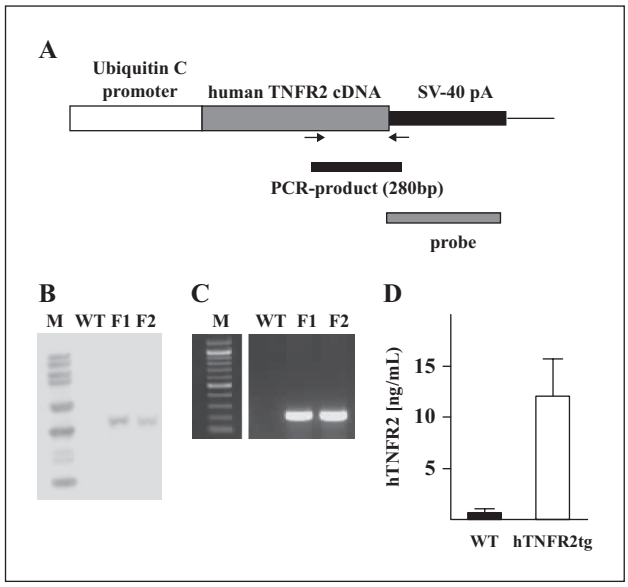

Figure 2
Generation of hTNFR2 transgenic mice (hTNFR2tg).
A) Schematic representation of the transgenic construct. The probe used for Southern blot analysis and the localization of primers and size of amplified PCR product are indicated. Genomic DNA isolated from hTNFR2tg mice (F1, F2) and wild type littermates (WT) were analyzed by Southern blot **(B)** and PCR analysis **(C)**. M: molecular weight marker. **D)** Concentrations of human TNFR2 were determined in urine from naïve hTNFR2tg mice (n=43, white bar) and their wild type littermates (WT, n=51, black bar). Data are mean with standard deviation as error bars.

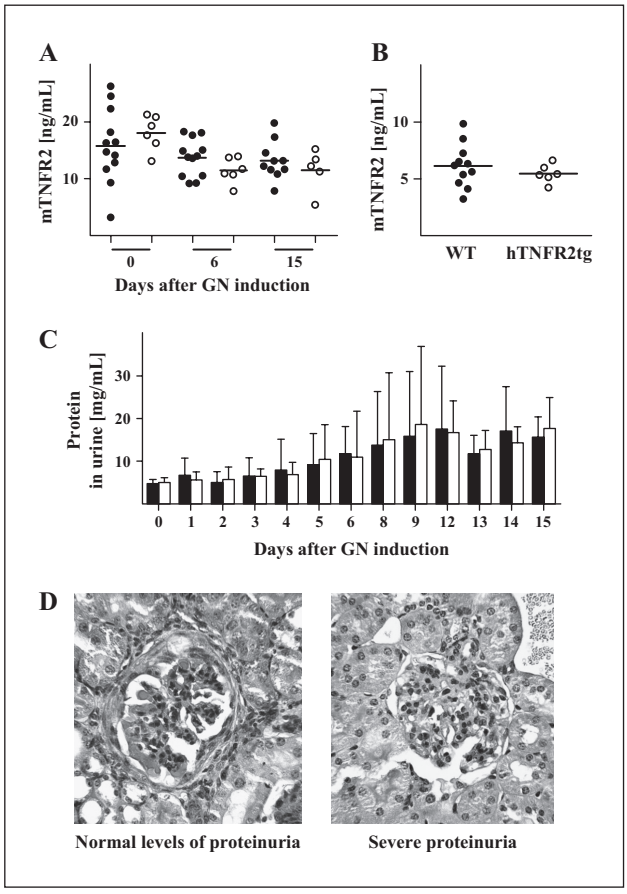

Figure 3
Development of proteinuria in hTNFR2tg mice.
A) Mouse TNFR2 was determined in urine from naïve mice (day 0), and on days 6 and 15 after the induction of GN in hTNFR2tg mice (open symbols) and their wild type littermates (closed symbols). Each symbol represents one mouse, horizontal lines represent means. **B)** Mouse TNFR2 was determined in serum on day 15 after induction of GN in wild type littermates (closed symbols) and hTNFR2tg mice (open symbols). Each symbol represents one mouse, horizontal lines represent means. **C)** GN was induced in hTNFR2 transgenic mice (n=11, white bars) and their wild type littermates (n=13, black bars). Proteinuria was determined for 15 days after injection of the nephrotoxic serum. Data are shown as mean with standard deviation as error bars. **D)** Representative pictures of PAS-stained kidney sections from mice, 15 days after induction of GN with proteinuria in the normal range (left) and severe proteinuria (right).

of GN was lower than in the urine, and were similar in both the transgenic mice and their non-transgenic littermates (*figure 3B*). Similarly, the levels of human TNFR2 in urine (31±26 ng/mL) and in serum (26±16 ng/mL) were enhanced on day 15 after induction.

No difference in proteinuria was observed when hTNFR2 transgenic mice were compared to their non-transgenic littermates during the entire course of GN (*figure 3C*). A continuous rise in protein concentration was observed after day 4 of GN induction. There was no difference in survival, with three (one on day 9 and two on day 12) of the 13 wild type and two (one on day 11 and one on day 12) of the 11 transgenic mice had died by day 15. Creatinine concentrations in urine did not differ between transgenic mice and their non-transgenic littermates, and were constant when measured on days 6 and 15 during GN. In both mouse groups, the ratios of the protein and creatinine values in urine were similarly increased on days 6 and 15 after induction of GN compared to mice before injection of the nephrotoxic serum. In both mouse groups, *post-mortem* histological examination of individual mice revealed the same correlation between the severity of proteinuria at day 15 and the extent of the glomerular lesions typical of crescentic or mesango-proliferative glomerulonephritis as seen in the wild type and TNFR2-deficient mice (*figure 3D*).

DISCUSSION

In the classical model of immune complex-induced GN [5] characterized by accumulation of inflammatory cells in the glomerulus and capillary damage followed by crescent formation, it was found that TNF activation of TNFR1 plays an important role in the initiation of GN, while not affecting the deposition of immune complexes in the glomeruli (reviewed in [2]). We studied the impact of TNFR2 on the induction of GN in more detail and found that TNFR2-deficient mice were protected, in particular, from the early development of proteinuria and from mortality, when they were kept in a conventional animal facility, confirming previously described protection of TNFR2-deficient mice from GN [4]. Surprisingly, no major difference in the course of proteinuria after induction of GN was seen between these mouse lines when the mice were kept under SPF-like conditions.

In the study of Vielhauer *et al*. TNFR2-deficient mice were completely protected from GN at all time points despite an intact humoral immune response [4]. TNFR2 expression by intrinsic cells, but not by leukocytes, was important for the development of GN indicating a contributing, pro-inflammatory function of TNFR2 in non-hematopoietic cells. However, in our study, expression of human TNFR2, in addition to mouse TNFR2, in transgenic mice did not change the course of GN compared to non-transgenic

littermates. This demonstrates that ubiquitous overexpression of the human TNFR2, which is perfectly capable of being activated by mouse TNF [8], did not confer enhanced susceptibility and further, does not support a direct, pro-inflammatory function of TNFR2.

On the other hand, anti-inflammatory functions of the interaction of TNF with presumably TNFR2 have been shown by TNF supporting expansion of regulatory T cells and a correlation of high TNFR2 expression with a strong, suppressive function of regulatory T cells [9, 10]. Besides signalling through TNFR2, the role of soluble TNFR2 also needs to be considered for its anti-inflammatory functions. Soluble TNFR2 acts as an inhibitor of TNF and, as such, has found its way into the clinic as anti-inflammatory therapy. In addition, soluble TNFR2 might play another, more indirect anti-inflammatory role: TNF can induce cross-tolerance to LPS *in vivo* [11] as well as *in vitro* [12], and thus modulate immune reactions in an anti-inflammatory way [13]. TNF blockade induced autoimmune symptoms in some patients indicating that TNF is not only an important mediator of inflammation, but that it has also an additional function in down-regulating adaptive immune responses [14]. Some of the immunosuppressive effects of TNF might be explained by attenuated T cell receptor signalling following chronic exposure to TNF [15]. Considering the fact that TNFR2-deficient mice lack soluble TNFR2, it could be expected that each inflammatory challenge in such mice induces endogenous TNF that is not mitigated by the TNF-scavenging soluble TNFR2 and, thus, may lead to TNF tolerance. A comparison of the immunological phenotype of myeloid cells from TNFR2-deficient mice demonstrating a reduced inflammatory response to further stimulation supports this notion (manuscript in preparation).

The observation of soluble mouse TNFR2 in urine and serum (data not shown) from naïve mice was surprising since the expression of TNFR2 is considered to be strongly regulated by stimulation [1]. The higher concentration of soluble TNFR2 in urine compared with serum demonstrated continuous production of soluble TNFR2 and excretion by the kidney. Continuous exposure to low-dose bacterial endotoxin and other inflammatory stimuli from the environment might account for this TNFR2 production. In a recent publication by Liu *et al*. [16], it was reported that the expression of soluble TNFR2, after stimulation, is mainly derived from non-bone marrow cells. This fits the observation that TNFR2 on intrinsic cells is responsible for the protection from GN [4]. Our finding that the observed protection of TNFR2-deficient mice from GN was lost in respect to the early proteinuria when the animals were raised and kept under SPF-like conditions might therefore be due to reduced tolerizing stimuli in an environment that was efficiently controlled for infective agents.

In conclusion, our data show that TNFR2 is important in the development of nephropathology. TNFR2 has at least two functions:

– as a membrane receptor for TNF, TNFR2 transduces signals; this remains to be analyzed in detail.

– the extracellular domain of TNFR2 acts as a soluble TNF inhibitor, dampening the acute inflammatory effects of TNF and preventing TNF-mediated tolerance so that the organism can react adequately to a subsequent, pathogenic challenge.

In the model of immune complex-mediated GN, TNFR2-deficient mice are protected by induction of TNF-mediated tolerance rather than by the lack of any signalling via TNFR2. It is the attenuated reactivity of the host to inflammation that reduces the severity of and mortality from GN in TNFR2-deficient mice.

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