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Spontaneous Recovery from Early Glomerular Inflammation Is Associated with Resistance to Anti-GBM Glomerulonephritis: Tolerance and Autoimmune Tissue Injury

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Abstract

Different susceptibility to anti-GBM glomerulonephritis (GN) among animal strains has been reported. Using our rat model for T cell-mediated anti-GBM GN, this study initiated an investigation on the mechanism related with GN-susceptibility. Anti-GBM GN was induced either through immunization with the nephritogenic T cell epitope pCol(28-40) from Col4 α 3NC1 or through the transfer of specific T cells. WKY rats were highly susceptible to GN while immuno-compatible LEW rats were GN-resistant. GN-resistance in LEW rats was not associated immune response to pCol(28-40). First, both strains mounted a Th1 T cell response to pCol(28-40) with identical specificities; transfer of T cells from LEW to WKY rats induced glomerular injury. Second, co-transfer of antibody from WKY to LEW failed to induce GN. Time-course studies revealed that LEW rats did develop T cell-mediated inflammation in glomeruli at early stages similar to WKY rats, as evidenced by histopathology, proteinuria, CD4⁺ T cell infiltration in glomeruli, and glomerular expression of inflammatory molecules. However, glomerular inflammation in LEW rats was transient followed by a full recovery. Thus, GN-resistance in LEW rats was due to its ability to contain early T cell-mediated autoimmune glomerular damage. Our model may reveal a potential tolerance mechanism after autoimmune tissue damage has been initiated.

Keywords

autoimmune susceptibility; inflammation; kidney; T cell; tolerance

1. Introduction

Anti-GBM glomerulonephritis (GN), or Goodpasture Syndrome, was one of the earliest recognized human autoimmune diseases (1,2). Collagen IV α 3 chain NC domain (Col4 α 3NC) has been identified to be the responsible autoantigen (3,4). Later studies further demonstrated that immunization with Col4 α 3NC induced anti-GBM GN in many animal models (5).

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Differences in susceptibility to anti-GBM-GN or other type of GN has been reported among animal strains. For example, Wistar Kyoto (WKY) rats are highly susceptible to not only anti-GBM GN but also other types of GN such as nephritoxin (NT) GN, while Lewis (LEW) rats are resistant (6,7). Recently, polymorphic expression of gene *Fcγr3* has been linked to susceptibility to NT GN (7). The same group also reported that the susceptibility may be linked to both kidney and myeloid cells (8). Those findings may help to understand mechanisms behind the susceptibility to anti-GBM GN as well.

On the other hand, many animal models used whole Col4α3 as an immunogen. Thus, it is difficult to clarify which immune compartments, T cell or antibody, or others contribute to the pathogenesis of anti-GBM GN. T cell mediated cellular immunity has long been suspected to be potentially the most important mediators of GN (9). Contributions of T cells to GN have been investigated in animal models either lacking T cells or with interrupted B7/CD28 co-stimulation pathway (10-12). However, it is not clear in those models whether T cells merely acted as helper cells in a T-dependent antibody response to renal autoantigens, or directly participated in glomerular damage. In order to precisely determine the role of T cells in anti-GBM GN, we have developed a rat model, in which the disease is induced by immunization with a well-characterized T cell epitope pCol(28-40) derived from Col4α3 or by transfer of Col4α3-specific T cells (13-14). We also showed that anti-GBM GN and pulmonary hemorrhage can be induced even by bacterial peptides which mimic pCol(28-40) (15). Thus, antigen specific CD4⁺ T cells *per se* are able to initiate glomerular injury. We further demonstrated that the production of diverse anti-GBM antibodies is a consequence of B cell epitope spreading initiated by a single T cell epitope (16). Thus, anti-GBM antibodies, which are produced only after glomerular damage, are not associated with disease severity.

In this paper, we first demonstrated that WKY and LEW rats were immuno-compatible. Like in other GN models, LEW rats are resistant to GN in our model. GN-resistance in LEW was not associated with Th type or specificity of T cell response. A rapid recovery from T cell-mediated glomerular inflammation at an early stage by an unknown mechanism probably was responsible for the GN-resistance in LEW. As immune responses and inflammatory cells can be precisely determined and analyzed, our model may provide an additional tool for investigation of the cellular or molecular mechanism in GN-resistance.

2. Materials and methods

2.1. Peptide preparation

Peptides were synthesized on an automatic peptide synthesizer, AMS 422 (Gilson, Middleton, WI) using Fmoc chemistry. Peptides were purified by reverse phase C18 column on a preparative HPLC (Water, Millford, MA). Purified peptides were analyzed by HPLC for purity and mass spectrometry for the correct sequence. Peptides, exceeding 95% purity, were dissolved in milli-Q water at a 1mM concentration, and used for immunization or other investigative purposes.

2.2. GN induction and evaluation

Female WKT or LEW rats (4-6 weeks of age) were purchased from Harlan (Indianapolis, IN). The rats were maintained in the animal facility at the University of Texas, Houston Health Science Center and allowed to acclimate for a minimum of three days. WKY/LEW F1 was bred in the same animal facility and used for disease induction at 6-8 weeks. Rats were immunized with peptide (0.125 μmol) emulsified in CFA, in one hind footpad and at the base of tail. Rats immunized with CFA alone, or with a 13-mer irrelevant peptide, namely J peptide (NSSSSQFQIHGPR), served as controls. All experimental procedures involving animals in

the present study have been reviewed and approved by the institutional Animal Welfare Committee.

GN was evaluated by albuminuria and renal histopathology. Random urine samples were monitored daily by Multistix (Bayer, Pittsburgh, PA). Urine albumin was semi-quantitated by 12% SDS-PAGE (2µl urine/lane) using BSA as a standard. The experimental animals were sacrificed around 40 days post immunization or as indicated. Kidney tissues fixed in Bouin's solution were used for H & E staining. Glomeruli with crescentic lesions, hypercellularity, or no injury were counted. A glomerular injury score was calculated as [(number of crescentic glomeruli × 100)+(number of hypercellular glomeruli × 50)] ÷ total glomerular number (14). Portion of the kidney tissues were snap-frozen in liquid nitrogen for direct immunofluorescence staining.

2.3. Lymphocyte proliferation assay (LPA) and mixed lymphocyte reaction (MLR)

Generation of antigen specific T cells lines and LPA was carried out as previously described (13,14). Briefly, lymphocytes were prepared from immunized rats and CD3⁺ T cells were isolated with a T cell enrichment column (RTCC, R&D, Minneapolis, MN). The purity of isolated T cells was determined by flow cytometry analyses (FACSCalibur, Becton Dickinson, San Jose, CA) after staining with anti-CD4-PE and anti-rat IgM/GFITC Abs (Pharmingen, San Diego, CA). T cells and irradiated syngeneic thymocytes (1:1) were cultured in 96-well plates at 4×10^5 cells/well in 200 µl of complete T cell medium. If a T cell line was used, 2×10^4 T cells were added to each well. Various concentrations of peptide (0.3 to 30 µM) were added to each well in triplicate, and PPD was used as positive control. The cells were incubated at 37°C in a humidified, 5% CO₂ atmosphere for 72 hours, pulsed with [³H]-thymidine (0.5 µCi/well) for 18 hours (ICN, Costa Mesa, CA), and harvested onto glass fiber filters using a semiautomatic cell harvester (Skatron Instruments, Sterling, VA). The incorporated radioactivity was measured by a liquid scintillation counter (Beckman, Fullerton, CA). The results were expressed as Δcpm (mean triplicate cpm with antigen minus mean triplicate cpm without antigen).

For MLR, splenocytes (SPC) were isolated from two strains to be tested. Whole SPCs from one strain were used as stimulator cells, and enriched T cells from SPC of another strain as responder cells. The stimulator cells were irradiated and cultured with responder cells of various numbers in 200 µl of complete T cell medium (10^5 stimulator cells v.s. 0.5 to 2×10^5 responder cells/well) in 96-well round-bottom plates, and ³H-thymidine incorporation was assessed for the last 18 hours.

2.4. RT-PCR detection of cytokines

One million T cells (either enriched from lymph node cells of immunized rats or from T cell lines) at resting or harvested after stimulation with pCol(28-40) for three days were used to isolate total RNA by a kit from Ambion (Austin, TX). cDNA was synthesized using a RT-PCR kit (Sigma, St. Louis, MO). PCR was carried out to detect expression of IL-4 and INF-γ followed a published method (14). PCR products were visualized by ethidium bromide staining after RAGE electrophoresis.

2.5. Detection of antibodies

Blood samples were taken from tail veins. The sera were kept at -80°C until use. A previously described ELISA was applied to detect antibody to peptides (17). Briefly, plates were coated with 50 µl peptide (10 µM) in a carbonate buffer (pH9.5). Serially diluted serum (200 to 10,000) was added to each well in duplicate. The bound antibodies were detected by HRPO labeled goat anti-rat IgG (1:10,000, Southern Biotechnology, Birmingham, AL) using O-

phenoldiamine (0.25 mg/ml) as the substrate. The plates were read on an ELISA reader (Molecular Devices, Sunnyvale, CA) at 490 nm.

For detecting anti-GBM antibodies deposited to GBM, direct immunofluorescence was applied. Frozen kidney sections of the immunized rats were incubated with FITC-labeled anti-rat IgG antibody (1:50, Southern Biotechnology, Birmingham, AL). For detection of circulation antibody to native GBM antigens, indirect immunofluorescence was carried out. Undiluted serum was incubated with frozen sections of SCID mouse kidney, followed by FITC-labeled goat anti-rat IgG. A rat monoclonal antibody, SR-13, that reacts with Col4 α 3NC1 was used as a positive control. The sections were viewed using a fluorescence microscope (BH-2, Olympus).

2.6. Allogeneic skin grafting

Allogeneic skin grafting performed following a standard method (18). Briefly, the donor (female) was sacrificed, shaved, and the tail skin was swabbed with 70% ethanol. Circle full-thickness skin grafts ($\sim\Phi$ 0.6 cm) were prepared from the trunk skin of donors and kept in cold PBS. Graft beds ($\sim\Phi$ 0.8 cm) were prepared on the right lateral thoracic wall of the recipient rats (female). The graft was fixed to the graft bed with sutures of 5-0 silk thread and covered with protective tape. The first inspection was conducted 7 days postgrafting, followed by daily inspection. Grafts were considered as rejected at the time of complete sloughing or when they formed a dry scar.

2.7. Flow cytometry on glomeruli-infiltrating leukocytes

After kidneys were perfused with PBS, glomeruli were isolated to a high purity as previously described (16) and digested with a mixture of collagenase D and Accutase at room temperature for 30 minutes with gentle stir. The released leukocytes were collected by centrifugation and dead cells removed by Ficoll density centrifugation. The cells were stained by PE-labeled anti-CD4 antibody (OX4) and FITC labeled anti-CD8 α antibody (OX8). The stained cells were analyzed by flow cytometry.

2.8. DNA microarray detection of inflammatory molecule expression

Glomeruli were isolated to a high purity as previously described (16). Total glomerular RNA was isolated with a kit following manufacturer's instructions (RNAqueous kit, Ambion), treated with DNase I to eliminate genomic DNA, and used for reverse transcription (RT). Complementary RNA was synthesized in the presence of biotinylated-UTP using cDNA as template (TrueLabeling-AMP 2.0, SuperArray). After purification with ArrayGrade Cleanup Kit (SuperArray), cRNA was hybridized to a microarray for rat inflammatory molecules following the manufacturer's instruction (kit ORN-011, SuperArray). Chemiluminescent Detection Kit, provided by SuperArray, was utilized to detect bound cRNA.

3. Results

3.1. LEW and WKY are immuno-compatible

LEW and WKY rats were from the same ancestor outbred Wistar rats. The MHC molecule of LEW has been well characterized to be RT-1^l haplotype. We have cloned MHC class II molecules of WKY (class II: RT-1 α and β chain, RT-1D α and β chain), which are identical to LEW rats (17). We recently cloned MHC class I molecules of WKY (RT-1A1, GeneBank bankit1001544 EU040040). Comparison with various haplotypes, RT-1A1 of WKY was closest to that of LEW; there were only one unmatched nucleotide out of the cloned 939 bp between the two, which result in only one amino acid residue substitution (Leu₂₉₁ to Pro). Those results suggested that the two strains were immuno-compatible. The compatibility

between the two was further confirmed by the following experiments. First, mixed lymphocyte reaction (MLR), a sensitive way to test compatibility of MHC between two strains/individuals, was performed (Fig. 1A). MLR between WKY and LEW was at a low level as that between LEW individuals. In contrast, an extremely strong MLR was observed between LEW and Sprague-Dawley (SD)(RT1^b). Second, skin graft experiments were performed. The tail skin of LEW grafted to WKT rats were able to survive as intact graft for longer than 28 days, and completely rejected by approximately 42 days (Fig. 1B). In contrast, LEW skin graft to SD rats was completely rejected by 8-10 days post grafting.

3.2. LEW and WKY rats mount similar immune response to nephritogenic T cell epitope pCol (28-40)

We next compared the T cell response to pCol(28-40) between LEW and WKY rats. We have shown previously that pCol(28-40) is restricted by RT-1B^I, and thus a similar T cell response between the two were expected (17). First, LEW rats mounted as strong a T cell response to pCol(28-40) as that in WKY after immunization with pCol(28-40) (Fig. 2A). Since the two strains have identical RT-1B, T cell lines generated from the immunized LEW rats responded well to APC from WKY and *vice versa* (Fig. 2B). Second, the T cell lines from the two strains were tested for their specificity in recognition of pCol(28-40). The T cells were stimulated with a set of pCol(28-40) peptides with single substitution (Fig. 2C). The responsiveness of LEW T cell lines to the substituted peptides was almost identical to that of WKY, suggesting a similarity in specific recognition of the T cell epitope pCol(28-40) by the two strains. Third, cytokine expression in the T cells after activation was investigated. A predominant INF- γ expression was also observed in the both strains (Fig. 2D)(13). On the other hand, IL-4 expression was also undetectable in LEW. Thus, both showed a Th1 dominant response. Fourth, pCol(28-40)-specific T cell line was generated from immunized LEW and was transferred into five naïve WKY. Glomerular damage and significant proteinuria were observed in the recipients (Figs. 2E and F). Glomerular injury was characterized by hypercellularity. Thus, pCol(28-40) specific T cells in LEW were pathogenic. In contrast, T cell lines generated from WKY rats failed to transfer any glomerular damage in LEW.

We also compared antibody response between the two strains. LEW rats produced circulation antibodies to peptidic B cell epitope nest in pCol(28-40)(Fig. 3A)(16). There were no significant differences in the antibody titer between the two strains. Specificity of antibody in LEW and WKY rats was compared using a set of pCol(28-40) with a single amino acid residue substitution (Fig. 3B). Antibody from LEW showed a reactivity pattern similar to that of WKY rats. GBM-bound IgG antibody was not detected in the both strains before day 25. WKY rats began to show GBM-bound IgG after day 25. It was predictable that B cell epitope spreading would not occur in the immunized LEW rats due to lack of tissue damage. Thus, LEW rats failed to produce antibodies to diverse native GBM antigens as seen in WKY rats, as evidenced by absence of linear binding of IgG to GBM in any immunized LEW rats (Fig. 3C). Circulating antibody to native GBM was not detected in the either strains (Fig. 3D).

3.3. LEW rats are resistant to GN induction

GN development in the immunized LEW rats was investigated. LEW rats were sacrificed at day 40 post immunization and their kidneys were removed for renal pathology. None of the fifteen LEW rats showed any sign of glomerular injury despite their T/B cell response to pCol (28-40) being identical to those of WKY (Fig. 4). As mentioned before, direct immunofluorescence demonstrated that LEW rats also failed to produce any anti-GBM antibody in the form of GBM-bound IgG (Fig. 3C). This is not surprising because B cell epitope spreading would never occur without glomerular damage (16). In conclusion, LEW rats were resistant to GN induction despite having a MHC and T cell response to pCol(28-40) identical to that seen in WKY rats. These results suggest that anti-GBM GN susceptibility in WKY is

not associated with MHC type, or Th type and specificity of T cell response. Since LEW rats were not able to initiate B cell epitope spreading due to lack of glomerular injury, it raised the question whether the circulating antibody from WKY rats might enhance the severity of GN in LEW. Sera from diseased WKY at day 20 and 25 after immunization was transferred into immunized LEW rats. However, we still failed to find any signs of glomerular injury or inflammation at day 35.

3.4. LEW develops T cell-mediated glomerular inflammation similar to WKY but is able to recover at an early stage

We next investigated possible mechanism which leads to GN-resistance in LEW. First, a detailed time-course study on renal pathology was conducted. Similar to WKY rats, a infiltration of leukocytes into the glomeruli of the immunized LEW was observed at approximately 20-25 days after immunization (Fig. 5A). GBM-bound IgG was not detected in the any rats of both strains. Morphologically, the infiltrating leukocytes were lymphocytes and macrophages. Affected glomeruli were slightly enlarged, suggesting an intra-glomerular inflammation similar to that seen in WKY. Unlike WKY, the leukocytes in LEW vanished from the glomeruli after day 25. At day 30, the glomeruli returned to almost normal size (Fig. 5A). Transient glomerular inflammation had temporally damaged the GBM. This was supported by observation of a transient elevation in urine albumin (albuminuria, 100 to 1000 mg/dl) in 70% of the immunized LEW, coincident with lymphocyte infiltration (Fig. 5B). Thus, LEW rats were not resistant to lymphocyte invasion, but rather, they were able to rapidly recover from tissue damage by an unknown mechanism.

In order to confirm transient inflammation in the glomeruli in LEW, two additional experiments were carried out. To insure accuracy of the following experiments, the kidneys were first perfused with culture medium to eliminate circulating leukocytes, and glomeruli were then isolated to a high purity for either leukocyte isolation or RNA isolation. First, isolated glomerular infiltrating leukocytes from the immunized rats were analyzed by flow cytometry after staining with anti-CD4 and anti-CD8 antibodies. A CD4⁺ population, obviously CD4⁺ T cells, of a significant size was observed in both WKY and LEW as early as 15 days post immunization (Fig. 5). This CD4⁺ population had an increased size in both strains at day 20. However, the CD4⁺ population disappeared in LEW after Day 30. In contrast, WKY showed a greatly expanded CD4⁺ population at the same time point. We also detected a CD8⁺ population among infiltrating leukocytes in both LEW and WKY (Fig. 5B). However, the nature of this leukocyte population was not determined in this study. Second, we examined expression of inflammatory molecules in the glomeruli after immunization with pCol(28-40) in both LEW and WKY at day 20 and day 30 (Fig. 6). A DNA microarray was used to detect expression of 120 inflammatory molecules including cytokines, chemokines, and adhesion molecules. Comparison of LEW with WKY showed that glomeruli of the two strains expressed a similar set of inflammatory molecules at an early stage (day 20) when T cells infiltrated glomeruli (Fig. 7). However, dramatic differences were seen at or after Day 30. In WKY, in addition to these earlier expressed molecules, numerous other genes such as $IL-1\beta$ and MCP-1 began to express at high levels (unpublished data, J. Robertson et al.). In contrast, LEW ceased to express the molecules, which had been detected, and the expression of new genes was not observed. These results suggest again that a similar T cell-mediated intra-glomerular inflammation has occurred at an early stage in LEW, but the inflammation is inhibited later by some unknown mechanisms.

3.5. LEW/WKY F1 is resistant to GN induction

LEW/WKY F1 was bred and their susceptibility to GN induction was examined. Among 11 tested F1 offspring, only one developed very mild glomerulonephritis characterized by

leukocyte infiltration without crescent formation. All others did not show any sign of glomerular inflammation (Fig. 4). Their immune response to pCol(28-40) including T cell and antibody response were similar to WKY (data not shown). Thus, GN susceptibility associated genes or loci are recessive.

4. Discussion

Our study showed that although LEW and WKY rats were immuno-compatible and mounted a similar immune response to the nephritogenic T cell epitope pCol(28-40), WKY rats were highly GN-susceptible while LEW rats were GN-resistant. In fact, resistance to autoimmune disease induction is common in animal models for autoimmune diseases, including other anti-GBM GN models (6,7,19-21). A previous study reported that glomerular injury was not observed in LEW rats at 28 day after immunization with recombinant Col4 α 3 (6). Difference in susceptibility to NT GN in the two strain rats has also been reported, although NT GN is not an autoimmune disease model (7). A careful observation in our study revealed that the GN-resistance in LEW is due to rapid recovery from T cell-mediated glomerular inflammation at an early stage. Although variation in GN-resistance among strains is well known, an early glomerular injury by a T cell mechanism in the resistant strain has not been reported or analyzed in detail.

Many peripheral tolerance mechanisms, such as regulatory T cells and the danger signal theory, have been described in both animal models and humans (22-27). Most, if not all, of those mechanisms prevent autoimmune diseases by manipulation of naïve self-reactive T cells. Thus, naïve T cells may become apoptotic, or differentiate into Th2 type or regulatory T cells. For example, potential regulatory T cells in human anti-GBM GN have been studied (28). As a result, naïve T cells have no opportunity to be properly activated to become a pathogenic effector cell. Autoimmune diseases are prevented *de novo*. Thus, a balance between autoaggressive and protective T cell response prevents occurrence of autoimmune diseases (29). Those tolerance mechanisms, however unlikely, can explain the spontaneous recovery from an early stage of GN in LEW in our model.

What is the potential mechanism that leads to a full recovery from autoimmune tissue damage in LEW rats? *First*, it is unlikely that the GN resistance in LEW rats is associated with Th type or specificity of T cell response. Different susceptibility to EAE induction in WKY and LEW rats has been linked to a Th2 dominant response in WKY (30). However, we have previously demonstrated that a Th1 dominant T cell response caused GN in WKY (13). We further showed in this study that GN-resistant LEW rats also mounted a Th1 dominant response to pCol(28-40). Repertoire-dependent immunopathology has been described (31). However, T cells in both strains displayed a similar specificity in their recognition of nephritogenic T cell epitope pCol(28-40) in our model. In addition, the experiment that transferred LEW T cells caused glomerular damage in WKY rats strongly suggests that T cells from LEW rats were potentially nephritogenic. Thus, the GN resistance is not associated with the T cells or T cell response. *Second*, as LEW rats did not produce anti-GBM antibody, is it possible that the GN resistance in LEW may be due to its inability to produce the antibody? A previous study also showed that both antibodies and T cells may be involved in the pathogenesis (32). However, we have previously demonstrated that anti-GBM antibody is a consequence of T cell-mediated glomerular damage through B cell epitope spreading (16). Production of anti-GBM antibody in WKY is much later than glomerular damage and showed no correlation with GN severity (17). In fact, there were no antibody bound to GBM in both WKY and LEW when T cell mediated glomerular inflammation occurred. Other group has reported similar findings in their model (6). For example, immunization of whole Col4 α 3 failed to induce antibody to native GBM in LEW rats. Although anti-GBM antibody transferred into LEW rats bound to host's GBM, it fails to induce any glomerular injury (6). Clearly, absence of anti-GBM antibody in

LEW is unlikely responsible for its GN-resistance, but rather it suggests that uncontrolled autoimmune tissue damage as seen in WKY rats in either ours or other models is a prerequisite for B cell epitope spreading. Therefore, other factors beyond T cell and/or B cell response to nephritogenic T cell epitope must be responsible for different GN susceptibility between the two strains in our model. There are several potential explanations. Different antigen processing and presentation in the target tissue has been linked to the susceptibility to autoimmune diseases (33). However, multiple experiments in the present study suggest that it may not be the case in our model. A transient inflammation in glomeruli of LEW rats, which was mediated by pathogenic T cells and similar to the early glomerular inflammation in WKY, was constantly verified by multiple experiments. Those results indicate that activated GBM-specific T cells in both strain were able to initiate and amplify glomerular inflammation. Thus, the endogenous GBM antigens must have been properly processed and presented to the activated T cells in both LEW and WKY rats.

It is well known that FcγR plays a critical role in pathogenesis of lupus GN. Recently, copy number polymorphisms in FcγR3 have been linked to NT GN in a rat model (7). Those previous studies suggest that both autoantibody and FcγR expressing inflammatory cells such as macrophages must be required for the glomerular injury. However, we have showed that anti-GBM-GN in our model is induced by a T cell mechanism, but not anti-GBM antibody. Thus, it is still questionable whether the resistance in LEW in our model is also associated with polymorphisms of FcγR3. In fact, we have analyzed FcγR3 expression level on the surface of infiltrating macrophages, and found no difference between the two strains. Rather, we recently identified a special infiltrating leukocyte population in LEW, coincident with the recovery of glomerular inflammation (unpublished data, J. Wu et al.). However, it remains unclear whether this population was associated with GN resistance. Further investigation is required. It has been shown that multiple genetic factors beyond tolerance mechanisms may contribute to different phenotypes of lupus GN in a mouse model (34,35). It will be interesting to see whether those factors also play roles in GN-resistance in our model. In addition, local environment and innate immunity may also play very important role in pathogenesis. For example, inhibition of metalloproteinase inhibited T cell's attack on β-cell in a diabetes model (36,37).

WKY rats are highly susceptible to several types of GN (6,7,38,39). Thus, discovery of a new mechanism in WKY may contribute to our understanding of mechanisms of glomerular injury in other types of GN as well. We are currently investigating the GN resistance mechanism at cellular and molecular levels by comparison between WKY and LEW rats. Our model has several advantages which will greatly facilitate our investigation of the mechanism for GN resistance. *First*, immune response and GN progression can be precisely determined or manipulated because of the well characterized nephritogenic T cell epitope pCol(28-40). *Second*, we have established a unique method to isolate a large quantity of glomerular infiltrating leukocytes for cellular immunology studies both *in vivo* or *in vitro* (40)(Fig. 7). Only a few, if any, existing autoimmune disease models have this advantage. *Third*, because LEW and WKY rats are immuno-compatible, it is possible to design special experiments such as cell transfer or construction of chimeras. The above advantages have made our model a unique tool for the study of post tissue injury tolerance mechanism, especially at cellular immunology level.

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References

1. Couser WG. Glomerulonephritis. *Lancet* 1999;353:1509–1515. [PubMed: 10232333]
2. Steblay RW. Glomerulonephritis induced in sheep by injection of heterologous GBM and Freund's complete adjuvant. *J. Exp. Med* 1962;116:253–258. [PubMed: 13916466]
3. Wieslander J, Bygren P, Heinegard D. Isolation of the specific glomerular basement membrane antigen involved in Goodpasture syndrome. *Proc. Natl. Acad. Sci. USA* 1984;81:1544–1548. [PubMed: 6324201]
4. Neilson EG, Kalluri R, Sun S, Gunwar MJ, Danoff T, Mariyama M, Myers JC, Reeders ST, Hudson BG. Specificity of Goodpasture autoantibodies for the recombinant noncollagenous domains of human type IV collagen. *J. Biol. Chem* 1993;268:8402–8405. [PubMed: 8473281]
5. Kalluri R, Gattone VH Jr, Noelken ME, Hudson BG. The $\alpha 3(\text{IV})$ chain of type IV collagen induces autoimmune Goodpasture syndrome. *Proc. Natl. Acad. Sci. USA* 1994;91:6201–6205. [PubMed: 8016138]
6. Reynolds J, Albouainain A, Duda MA, Evans DJ, Pusey CD. Strain susceptibility to active induction and passive transfer of experimental autoimmune glomerulonephritis in the rat. *Nephrol. Dial. Transplant* 2006;21:3389–3397. [PubMed: 16998215]
7. Aitman TJ, Dong R, Vyse TJ, Norsworthy PJ, Johnson MD, Smith J, Mangion J, Robertson-Lowe C, Marshall AJ, Petretto E, Hodges MD, Bhargal G, Patel SG, Sheehan-Rooney K, Duda M, Cook PR, Evans DJ, Domin J, Flint J, Boyle JJ, Pusey CD, Cook HT. Copy number polymorphism in *Fcgr3* predisposes to glomerulonephritis in rats and humans. *Nature* 2006;439:851–855. [PubMed: 16482158]
8. Smith J, Lai PC, Behmoaras J, Roufosse C, Bhargal G, McDaid JP, Aitman T, Tam FW, Pusey CD, Cook HT. Genes Expressed by Both Mesangial Cells and Bone Marrow–Derived Cells Underlie Genetic Susceptibility to Crescentic Glomerulonephritis in the Rat. *J. Am. Soc. Nephrol* 2007;18:1816–1823. [PubMed: 17475818]
9. Rovin BH, Schreiner GF. Cell-mediated immunity in glomerular disease. *Annu. Rev. Med* 1991;42:25–33. [PubMed: 2035970]
10. Tipping PG, Huang XR, Qi M, Van GY, Tang WW. Crescentic glomerulonephritis in CD4- and CD8-deficient mice. *Am. J. Pathol* 1998;152:1541–1548. [PubMed: 9626058]
11. Reynolds J, Tom F, Chandraker A, Smith J, Karkar AM, Cross J, Peach R, Sayegh MH, Pusey CD. CD28-B7 blockage prevents the development of experimental autoimmune glomerulonephritis. *J. Clin. Invest* 2000;105:643–646. [PubMed: 10712436]
12. Bolton WK, Tucker FL, Sturgill BC. New avian model of experimental glomerulonephritis consistent with mediation by cellular immunity. *J. Clin. Invest* 1984;73:1263–1276. [PubMed: 6585368]
13. Wu J, Hicks J, Borillo J, Glass WF II, Lou YH. CD4+ T cells specific to a glomerular basement membrane antigen mediate glomerulonephritis. *J. Clin. Invest* 2002;109:517–524. [PubMed: 11854324]
14. Wu J, Borillo J, Glass WF II, Hicks J, Ou CN, Lou YH. T cell epitope of glomerular basement membrane antigen induces severe glomerulonephritis. *Kidney Int* 2003;64:1292–1301. [PubMed: 12969147]
15. Arends J, Wu J, Borillo J, Troung L, Zhou C, Vigneswaran N, Lou YH. T cell epitope mimicry in anti-glomerular basement membrane disease. *J. Immunol* 2006;176:1251–1258.
16. Wu J, Arends J, Borillo J, Zhou C, Merszei J, McMahon J, Lou YH. A self T cell epitope induces Autoantibody response: mechanism for production of antibodies to diverse glomerular basement membrane antigens. *J. Immunol* 2004;172:4567–4574. [PubMed: 15034074]
17. Robertson J, Wu J, Arends J, Glass W II, Southwood S, Sette A, Lou YH. Characterization of the T cell epitope that causes anti-glomerular basement membrane glomerulonephritis. *Kidney Int* 2005;68:1061–1070. [PubMed: 16105036]
18. Numata M. Skin graft and kidney transplantation in rats. *Res. Exp. Med* 1986;186:443–448.
19. Olsson T, Dahlman I, Wallstrom E, Weissert R, Piehl F. Genetics of rat neuroinflammation. *J. Neuroimmunol* 2000;107:191–200. [PubMed: 10854656]

20. Sun B, Sun SH, Chan CC, Caspi RR. Evaluation of *in vivo* cytokine expression in EAU-susceptible and resistant rats: a role for IL-10 in resistance? *Exp. Eye Res* 2000;70:493–502. [PubMed: 10865998]
21. Reynolds J, Cook PR, Ryan JJ, Norsworthy PJ, Glazier AM, Duda MA, Evans DJ, Aitman TJ, Pusey CD. Segregation of experimental autoimmune glomerulonephritis as a complex genetic trait and exclusion of Col4a3 as a candidate gene. *Exp Nephrol* 2002;10:402–407. [PubMed: 12381925]
22. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol* 1995;155:1151–1164. [PubMed: 7636184]
23. Matzinger P. Tolerance, danger, and the extended family. *Annu. Rev. Immunol* 1994;12:991–1045. [PubMed: 8011301]
24. Linsley PS, Ledbetter J. The role of the CD28 receptor during T cell responses to antigen. *Ann. Rev. Immunol* 1993;11:191–212. [PubMed: 8386518]
25. Heath WR, Carbone FR. Cross-presentation, dendritic cells, tolerance and immunity. *Ann. Rev. Immunol* 2001;19:47–64. [PubMed: 11244030]
26. Alvarado-Sanchez B, Hernandez-Castro B, Portales-Perez D, Baranda L, Layseca-Espinosa E, Abud-Mendoza C, Cubillas-Tejeda AC, Gonzalez-Amaro R. Regulatory T cells in patients with systemic lupus erythematosus. *J. Autoimmun* 2006;27:110–108. [PubMed: 16890406]
27. Ban Y, Tozaki T, Tobe T, Ban Y, Jacobson EM, Concepcion ES, Tomer Y. The regulatory T cell gene FOXP3 and genetic susceptibility to thyroid autoimmunity: an association analysis in Caucasian and Japanese cohorts. *J. Autoimmun* 2007;28:201–207. [PubMed: 17418529]
28. Salama AD, Chaudhry AN, Holthaus KA, Mosley K, Kalluri R, Sayegh MH, Lechler RI, Pusey CD, Lightstone L. Regulation by CD25+ lymphocytes of autoantigen-specific T-cell responses in Goodpasture's (anti-GBM) disease. *Kidney Int* 2003;64:1685–1694. [PubMed: 14531801]
29. Abbas AK, Lohr J, Knoechel B. Balancing autoaggressive and protective T cell responses. *J. Autoimmun* 2007;28:59–61. [PubMed: 17363216]
30. Stevens DB, Gold DP, Sercarz EE, Moudgil KD. The Wistar Kyoto (RT1(l)) rat is resistant to myelin basic protein-induced experimental autoimmune encephalomyelitis: comparison with the susceptible Lewis (RT1(l)) strain with regard to the MBP-directed CD4+ T cell repertoire and its regulation. *J. Neuroimmunol* 2002;126:25–36. [PubMed: 12020954]
31. Milner J, Ward J, Keane-Myers A, Min B, Paul WE. Repertoire-dependent immunopathology. *J. Autoimmun.* 2007in press
32. Dean EG, Wilson GRA, Li M, Edgton KL, O'Sullivan KM, Hudson BG, Holdsworth SR, Kitching AR. Experimental autoimmune Goodpasture's disease: a pathogenetic role for both effector cells and antibody in injury. *Kidney Int* 2005;67:566–575. [PubMed: 15673304]
33. Anderton SM, Viner NJ, Matharu P, Lowrey PA, Wraith DC. Influence of a dominant cryptic epitope on autoimmune T cell tolerance. *Nature Immunology* 2002;3:175–181. [PubMed: 11812995]
34. Gilkeson GS, Ruiz P, Pritchard AJ, Pisetsky DS. Genetic control of inflammatory arthritis and glomerulonephritis in congenic lpr mice and their F1 hybrids. *J. Autoimmun* 1991;4:595–606. [PubMed: 1777011]
35. Wandstrat A, Wakeland E. The genetics of complex autoimmune diseases: non-MHC susceptibility genes. *Nature Immunol* 2001;2:802–809. [PubMed: 11526390]
36. Woods CC, Sundar K, Tessler C, Lebsack TW, Grainger L, Nielsen A, Bleich D, DeLuca D. Tissue inhibitor of metalloproteinase-2 inhibits T-cell infiltration and preserves pancreatic beta-cell function in an *in vitro* type 1 diabetes mellitus model. *J. Autoimmun* 2006;27:28–37. [PubMed: 16765565]
37. Lang KS, Burrow A, Kurrer M, Lang PA, Recher M. The role of the innate immune response in autoimmune disease. *J Autoimmun.* 2007in press
38. Tam F, Smith J, Morel D, Karkar A, Thompson E, Cook H, Pusey DC. Development of scarring and renal failure in a rat model of crescentic glomerulonephritis. *Nephrol. Dial. Transplant* 1999;14:1658–1666. [PubMed: 10435873]
39. Little MA, Smyth CL, Yadav R, Ambrose L, Cook HT, Nourshargh S, Pusey CD. Antineutrophil cytoplasm antibodies directed against myeloperoxidase augment leukocyte-microvascular interactions *in vivo*. *Blood* 2005;106:2050–2058. [PubMed: 15933057]

40. Robertson J, Wu J, Arends J, Zhou C, McMahon J, Torres L, Lou YH. Activation of GBM-specific B cells in the renal draining lymph node following T cell mediated glomerular injury. *J. Am. Soc. Nephrol* 2005;16:3256–3263. [PubMed: 16162812]

Figure 1. LEW and WKY rats are immuno-compatible

A. Mixed lymphocyte reactions between rat strains as indicated; LEW vs LEW serves as negative control, while LEW vs SD a positive control; the responsiveness is expressed as ^3H -thymidin incorporation. **B.** Skin allograft rejection; days of rejection are counted from day of grafting until the graft is completely rejected; *inset* shows a LEW skin graft in WKY at 4 weeks.

Figure 2. LEW rats mount a pathogenic T cell response to pCol(28-40) similar to WKY rats
A. T cell response to pCol(28-40) in LEW (●) and WKY rats (○) after immunization; the responsiveness of T cells is expressed as ^{3} H-thymidin incorporation. **B.** A T cell line from LEW or WKY rats responds to pCol(28-40) at the presence of thymocytes from WKY rats. **C.** A T cell line from LEW (■) responds to a set of pCol(28-40) with single substitution in almost identical pattern to those of WKY (□). **D.** RT-PCR detection of IL-4 or INF- γ in a T cell line from LEW rats after stimulation with pCol(28-40). **E.** Glomerular injury in pCol(28-40)-specific T cell recipients as indicated; severity of glomerular injury is expressed as glomerular injury score. **F.** Proteinuria in pCol(28-40)-specific T cell recipients as indicated; transfer of WKY's T cells to WKY recipient serves as a positive control; albumin is indicated by an arrow.

Figure 3. Antibody response in LEW rats immunized with pCol(28-40)

A. Circulating antibody activity to pCol(28-40) from immunized LEW or WKY rats; sera from CFA immunized WKY were used as a negative control; **B.** Reactivity of circulating antibody from immunized WKY or LEW to a set of pCol(28-40) with single substitution. Antibody activities are expressed as normalized OD. **C.** Indirect immunofluorescence shows absence of anti-GBM antibody in circulation of LEW and WKY rats; SR-13 serves as a positive control. **D.** Direct immunofluorescence shows absence of GBM-bound IgG in an immunized LEW rat.

Figure 4. Renal histopathology of LEW or WKY rats after immunization with pCol(28-40)

A. Kidney section of an immunized LEW rats at 40 days post immunization shows normal glomeruli. **B.** Kidney section from WKY shows glomeruli with fibrous crescent. **C.** A group of kidney sections under high magnification from the immunized rats as indicated; lymphocyte infiltration is notable in LEW at day 20 (arrows); crescent lesion (arrow heads) is shown in glomerulus of WKY at day 30.

Figure 5. Transient glomerular inflammation in LEW rats after GN induction with pCol(28-40)

A. Summary of renal pathology of different animal groups; severity of glomerulonephritis is expressed as glomerular injury score (0-100). **B.** Summary of albuminuria in the immunized rats at an early or later stage as indicated; albuminuria is expressed as mg/dl.

Figure 6. Flow cytometry detection of a transient CD4⁺ T cell-mediated glomerular inflammation in LEW rats after GN induction with pCol(28-40)
Leukocytes were isolated from glomeruli of WKY or LEW rats at indicated times and stained with anti-CD4 and anti-CD8 antibodies.

Figure 7. Glomeruli of LEW rats express inflammatory molecules similar to those of WKY rats at an early stage after GN-induction

Glomerular expression of inflammatory molecules as indicated. Only one representative array is shown. Arrows indicate the same set of genes, which includes Bmp2, caspase-1, IL-4R α , MIP-2 α , Frag1, TGF- β 1i4, IL-1R1.