DNA Vaccination Against Specific Pathogenic TCRs Reduces Proteinuria in Active Heymann Nephritis by Inducing Specific Autoantibodies

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We have previously identified potential pathogenic T cells within glomeruli that use TCR encoding Vβ5, Vβ7, and Vβ13 in combination with Jβ2.6 in Heymann nephritis (HN), a rat autoimmune disease model of human membranous nephritis. Vaccination of Lewis rats with naked DNA encoding these pathogenic TCRs significantly protected against HN. Proteinuria was reduced at 6, 8, 10, and 12 wk after immunization with Fx1A (p < 0.001). Glomerular infiltrates of macrophages and CD8+ T cells (p < 0.005) and glomerular IFN-γ mRNA expression (p < 0.001) were also significantly decreased. DNA vaccination (DV) causes a loss of clonality of T cells in the HN glomeruli. T lymphocytes with surface binding of Abs were found in DNA vaccinated rats. These CD3+/H11545 have shown that blocking CD4 (5). However, studies on the role of T cells in the etiology of HN have shown that blocking CD4+ T cells by using anti-CD4 mAb before immunization totally prevents proteinuria, glomerular Ig deposition, and mononuclear cell infiltrates in the kidney in this model (6). More recently, studies illustrate that have shown the appearance of activated T cells, principally Th1 and cytotoxic effector T cells and macrophages within the glomeruli at 8 wk, is coincidental with the development of proteinuria (7). This result implies a role for T cells, and more specifically CTLs, may mediate glomerular injury.

TCR CDR3 spectratyping is a powerful tool for identifying autoimmune disease-inducing T cells whose mechanism of action is poorly understood (9). We have recently demonstrated clonal overexpansion of T cells using TCR encoding Vβ5, Vβ7, and Vβ13 in combination with Jβ2.6 (10) within glomeruli in this HN model. This was done using TCR spectratyping analysis and subsequent CDR3 region sequencing of spectratype-derived T cell clones. These overexpressed clones potentially represent pathogenic T cells. Vaccination with DNA encoding the Vβ region of an autoreactive TCR has been shown to provide an effective means to prevent autoimmune diseases such as EAE by generating suppressor T cells (11). This study tested the effect of DNA vaccination targeting specific TCR Vβ chains in HN. We sought to confirm the pathogenic nature of the subset of T cells bearing this restricted TCR repertoire and investigate the therapeutic use of DNA vaccination in HN. The results of these studies are outlined below.

Materials and Methods

Experimental animals and induction of active HN

Inbred male Lewis rats were obtained from the Animal Resources Centre in Perth, Australia. Rats weighing 180–200 g at the age of 6 wk were used in all experiments. Outbred male Sprague-Dawley rats were used for producing Fx1A. Fx1A was prepared as described (12, 13). Each group of six Lewis rats was immunized s.c. into each of their hind footpads with 100 μl Fx1A emulsified in CFA, which contained a total of 15 mg of Fx1A, 1 mg Mycobacterium tuberculosis H37Rv (Difco, Detroit, MI), 100 μl IFA (Sigma-Aldrich, St. Louis, MO), and 100 ml of PBS. The control rats were immunized with the appropriate emulsion prepared without Fx1A.

DNA vaccination

RNA was isolated from the HN kidney as described (10) and reverse transcribed into cDNA. cDNA was amplified by PCR with primers specific for either Vβ5 (5′-AAGATGTCCTGGGTGCTCGGT-3′), Vβ7 (5′-AG CATGATCTGGATCGAAGACCCCAGATC-3′), or Vβ13 (5′-AGAA TGACTGGGTACCCAGTCCC-3′) and a reverse Jβ2.6 primer (5′- CGTGCATAAAAACCGTGAGCTTGGTGC-3′). cDNA was amplified with a Vβ8.2 primer as described (9) and a β2 microglobulin primer to generate a control vaccine. All forward primers were designed to include an in-frame ATG.
The mAbs used were W3/25 for CD4 diaminobenzidine tetrahydrochloride to produce a dark brown end-product. Trypsin inhibitor in PBS. The slides were then washed in PBS for 15 min. Glomeruli were isolated as previously described (10). Isolated glomeruli were enzymatically permeabilized for immunoperoxidase staining by incubating at 37°C with a solution of 0.1 mg/ml collagenase D and 2 mg/ml DNA was performed using the Mega prep kit (Qiagen, Hilden, Germany). For DNA vaccination, animals were pretreated with 0.75% bupivacaine (1 μg/bodyweight; Sigma-Aldrich) by injection into the tibialis anterior muscle one week before the first vaccination. This is known to enhance the efficiency of DNA vaccination. DNA (300 μg) was injected into the same site three times at weekly intervals. Two weeks after the last vaccination, rats were challenged with Fx1A in CFA.

Autoantibody determination
Anti-Fx1A Ab titers (total Ig) were determined by ELISA as described (6). Triplicate sample ODs were read at 405 nm, corrected for a control sample of known strongly positive serum OD. IgG subclass were assessed by the same method using alkaline phosphatase-conjugated mouse mAb to rat IgG1 and IgG2a (BD PharMingen, San Diego, CA).

Urinary protein estimation
Twenty-four hour urine samples were collected in metabolic cages at 4, 6, 8, 10, and 12 wk post-Fx1A/CFA inoculation. Urine protein concentrations were determined by colorimetric assay (Bio-Rad, Hercules, CA).

Immunoperoxidase staining
Glomeruli were isolated as previously described (10). Isolated glomeruli were enzymatically permeabilized for immunoperoxidase staining by incubation at 37°C with a solution of 0.1 mg/ml collagenase D and 2 mg/ml trypsin inhibitor in PBS. The slides were then washed in PBS for 15 min and stained with mAbs, followed by HRP-labeled goat anti-mouse IgG (BD PharMingen, San Diego, CA). The slides were incubated with 3,3-diaminobenzidine tetrahydrochloride to produce a dark brown end-product. The mAbs used were W3/25 for CD4+ T cells and some macrophages, MRCOx-8 for CD8+ T cells, ED-1 for macrophages and some dendritic cells, and MRCOx-12, which recognizes rat IgG-L chains (all purchased from Serotec, Oxford, UK). Stained cells in each isolated glomerular specimen were counted in individual glomeruli throughout all focal planes and the results were expressed as cells per glomerulus with 50–100 glomeruli counted per sample.

RT-PCR for TCR Vβ repertoire
RNA was extracted from isolated glomeruli as described (10) and reverse transcribed to cDNA (14). Oligonucleotide primers used for the rat TCR Vβ1–20 were those described (15). The PCR profile consisted of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 40 cycles in a DNA thermal cycler (PerkinElmer, Wellesley, MA). Aliquots (1 ul) of the PCR products were re-amplified for six cycles with a nested 5’-FAM-labeled Cβ primer.

CDR3 spectratyping
Each labeled PCR product was loaded on POP4 sequencing gels together with size standard (Genescan-500 ROX) on an Applied Biosystems (Foster City, CA) 373A DNA sequencer. Data were processed using the Genescan Analysis 2.1 Software (Applied Biosystems) which records the fluorescence intensity in each peak.

Semiquantitive RT-PCR for cytokine genes
Semiquantitative RT-PCR for cytokine genes was performed as previously described (14). Briefly, oligonucleotide primers used for rat cytokine genes IFN-γ and IL-10 were those described (7, 16, 17). GAPDH primers served as the internal control. The PCR profile used was one minute each at 95, 55, and 72°C for 30 cycles for GAPDH, 35 or 40 cycles for IFN-γ, and 40 cycles for IL-10. RT-PCR fragments of cytokine genes were ethidium bromide stained on a 2% agarose gel. Signals were quantitated by a Molecular Analyst (Bio-Rad).

Flow cytometry analysis and cell sorting
Mononuclear cells extracted from blood were stained with goat anti-rat IgG Ab conjugated with FITC (Zymed Laboratories, San Francisco, CA) and
anti-rat CD3 mAb (clone G4.18) conjugated with PE (BD PharMingen San Diego, CA), or with anti-rat IgG conjugated with PE and anti-rat CD8a (clone OX 8), or anti-rat CD4 (clone OX35) conjugated with FITC (BD PharMingen). All samples were analyzed on a FACScan analyser (BD Biosciences, Mountain View, CA). IgG/H11001 CD3/H11001 cells were then sorted using a FACSCaliber (BD Biosciences).

**Statistical analysis**

Urine protein estimations, anti-Fx1A Ab levels for each group were expressed as mean ± SD. Comparison between groups and among the groups was made by ANOVA. Paired Student t tests were used to determine whether changes in the levels of cytokine gene expression were significantly different. A value of p < 0.05 was considered significant.

**Results**

**DNA vaccination protects against HN**

Rats were divided into four groups: group 1 (DV): DV with TCR Vβ5, 7, and 13 in HN; group 2 (DV control): DNA vaccination with TCR Vβ8 used as a control vaccine in HN; group 3 (HN): HN; group 4 (CFA): immunization with CFA only as a HN control. DNA vaccination encoding TCR Vβ5, Vβ7, and Vβ13 significantly reduced proteinuria at 6, 8, 10, and 12 wk post Fx1A immunization (Fig. 1) compared with HN and DV controls. There was no significant difference in proteinuria between the HN and DV control groups. Control rats immunized with only CFA never developed proteinuria.

Examination of isolated glomeruli from HN rats at 12 wk demonstrated significant infiltrates of macrophages (ED1), CD8+ and CD4+ T cells in the glomeruli as compared with CFA control rats (Fig. 2). Macrophage and CD8+ T cell infiltrates in glomeruli in the DV group were significantly less than in the HN and DV control groups (p < 0.005). There were no significant differences in staining for CD4+ T cells and deposition of Ig in glomeruli across DV, HN, and DV control groups (Fig. 2).

**DNA vaccination reduces IFN-γ mRNA expression in HN glomeruli**

To test whether DNA vaccination alters T cell function in the glomeruli, we examined cytokine profiles in isolated glomeruli at 12 wk post-Fx1A/CFA immunization by semiquantitative RT-PCR. The results demonstrated lower mRNA expression for IFN-γ (p < 0.01) in the DV group when compared with HN and DV controls (Fig. 3) with levels not different from CFA controls.

**DNA vaccination reduces the clonality of T cells response in HN glomeruli**

CDR3 spectratyping is a well described method used as a measure of oligoclonality of T cells. A normal splenic sample of a single Vβ family gives a Gaussian distribution of 6–11 peaks each separated by three nucleotides. Oligoclonal T cells give fewer peaks
in a restricted distribution. Single clones give a single peak. Our previous study demonstrated oligoclonal expansion of T cells in HN glomeruli (10) using CDR3 spectratyping and subsequent sequencing of the CDR3 regions of spectratype-derived T cell clones. To test whether DNA vaccination would change a pattern of TCR CDR3 spectratypes in glomeruli, we examine CDR3 spectratypes of TCR Vβ9, 5, 7, and 13 in glomeruli in DNA vaccinated rats. HN glomeruli showed marked restriction with a single peak in CDR3 spectratypes of Vβ9, 5, 7, and 13 as we previously reported (10). The glomeruli isolated from DNA vaccinated rats showed diverse CDR3 lengths with more peaks in their CDR3 spectratypes (Fig. 4) which matched the CFA control glomeruli. This result indicates that DNA vaccination may reduce the expansion of pathogenic T cell clones in the glomeruli, revealing underlying background TCR diversity.

DNA vaccination has no significant effect on anti-Fx1A Abs
There was no difference in the level of serum anti-Fx1A Abs or in the levels of IgG1 in DV, HN, and DV control groups; however, IgG2a was suppressed by DNA vaccination early in the course of HN (Fig. 5).

DNA vaccination induces specific autoantibodies
We analyzed the mechanism underlying the protection induced by DNA vaccination. To test whether DNA vaccination with TCR Vβ5, 7, and 13 would delete T cells expressing Vβ5, 7, and 13 by producing autoantibodies, we analyzed the TCR Vβ repertoire by RT-PCR in DNA vaccinated rats. We found that DNA vaccinated rats still expressed TCR Vβ5, 7, and 13 in both peripheral blood and isolated glomeruli. No significant changes in TCR repertoire were found in peripheral blood between DNA vaccinated and unvaccinated rats. We then asked whether DNA vaccination produces autoantibodies that bind the TCR. We sought to identify these T cells to see whether they were deleted or functionally impaired. We stained mononuclear cells from DNA vaccinated and unvaccinated rats with PE conjugated anti-CD3 Ab and FITC-conjugated anti-IgG. A small population of CD3+ IgG+ T cells were found in DNA vaccinated rats, in both control TCR vaccination and pathogenic TCR vaccination, but not in unvaccinated HN rats (Fig. 6). We also found that those CD3+ IgG+ cells were CD8+ T cells by staining mononuclear cells from DNA vaccinated and unvaccinated rats with FITC-conjugated anti-CD8 Ab and PE-conjugated anti-rat IgG (Fig. 7). Furthermore, on analysis of their TCR Vβ repertoire, sorted CD3+ IgG+ T cells almost exclusively expressed TCR Vβ5 and Vβ13, two of the three Vβ families encoded in the DNA vaccination compared with diverse TCR repertoire seen in sorted CD3+ T cells (Fig. 8). Analysis of cytokine mRNA expression in the CD3+IgG+ T cells showed that IL-10 and IFN-γ mRNA were not detected (Fig. 9). These results suggest that TCR DNA vaccination produced specific autoantibodies that bound to the TCRs encoded by the DNA vaccination, resulting in activation blockade of these T cells.

Discussion
In Ag driven organ-specific autoimmune diseases, it is possible to define a restriction in the TCRs used by pathogenic T cells. This restriction is seen in diseases such as experimental autoimmune

![FIGURE 6. Identification of IgG+CD3+ cells in peripheral lymphocytes in DNA vaccination rats by FACS. Freshly isolated lymphocytes from HN rats without DV (HN), rats with pathogenic TCR DV, and control TCR DV were stained with anti rat IgG (FITC) and anti-rat CD3 (PE). The number in brackets represent the percentage of IgG+CD3+ cells among total CD3+ T cells.](http://www.jimmunol.org/)

![FIGURE 7. Identification of IgG+CD8+ T cells in peripheral lymphocytes in DNA vaccination rats by FACS. Freshly isolated lymphocytes from HN rats without DV (HN) and rats with DV were stained with anti rat IgG (PE) and anti-rat CD8 (FITC) or anti-rat CD4 (FITC).](http://www.jimmunol.org/)
encephalomyelitis (EAE). In EAE, encephalitogenic T cell lines and clones are used mainly as restricted TCR Vβ 8.2 genes (18) (19). HN is induced in Lewis rats by immunization with a renal tubular Ag (RAT/Fx1A) or megalin (gp330). Recent studies suggest that CD8+ CTLs play a key role in causing glomerular injury in HN (8). We have identified HN-inducing TCRs by direct analysis of glomerular infiltrating T cells and have demonstrated that T cells within glomeruli in HN use T cell receptors encoding Vβ5, Vβ7, and Vβ13 in combination with Jβ2.6 (10). In this study, immunotherapy using TCR-based DNA vaccines against these three Vβ families significantly reduced the severity of disease in HN, strongly suggesting that T cells bearing TCR Vβ5, 7, and 13 are pathogenic and mediate glomerular injury. These findings support the evidence of clonality of TCR seen in HN previously reflecting expansion of pathogenic T cells (10).

In HN, previous studies by Penny et al. (7) demonstrated that the onset of proteinuria is associated with glomerular infiltration of CD8+ Tc1 cells and macrophages. Furthermore, permanent depletion of these CD8+ T cells prevents the onset of proteinuria, suggesting that Tc1 cytotoxic cells may mediate glomerular injury (8). A recent study has shown that IL-4 therapy prevents the development of proteinuria in active HN by inhibition of cytotoxic CD8+ T cells (20). In this study of IL-4 therapy, HN rats treated with rIL-4 had reduced infiltrating CD8+ cells in glomeruli and reduced mRNA level for IFN-γ but unchanged CD4+ T cell infiltration in glomeruli compared with HN controls. In our study, DNA vaccination reduced the glomerular infiltrates of CD8+ T cells and macrophages, but accumulation of glomerular CD4+ T cells was similar to HN controls. IFN-γ mRNA expression was significantly reduced in glomeruli of DNA vaccinated rats compared with HN rats, consistent with reduced infiltrates of CD8+ T cells, and suggesting that the major source of IFN-γ in HN is CD8+ T cells. Furthermore, CDR3 spectratype patterns in glomeruli with DNA vaccinated rats were similar to those seen in CFA controls, compared with an oligoclonal restricted TCR repertoire in HN glomeruli. This supports that specific DNA vaccination reduced the oligoclonal pathogenic T cells in glomeruli exposing the underlying diverse pattern of TCR repertoires normally found. Taken together, these results suggest that DNA vaccination suppressed the cytotoxic CD8+ T cell response, which is necessary for induction of proteinuria in this model (8). As mentioned above, IL-4 treatment inhibits CD8 T cells while leaving CD4 T cell infiltrates unchanged in HN but significantly reducing proteinuria (20). It is possible that suppression of CD8+ T cell activation in HN glomeruli by specific DNA vaccination results in a reduced number of CD8+ T cells infiltrating the glomeruli but leaves CD4+ T cells unchanged.

We have demonstrated that TCR DNA vaccination produced specific Abs to the TCRs that the DNA vaccination encoded. The Ab coated wells were seen using flow cytometry. The small number of cells coated with Abs possibly reflects the combination of the three Vβ families with Jβ2.6, that is one of 12 possible Jβs. Thus, Abs are generated to only the pathogenic subsets of the T cell

**FIGURE 8.** TCR repertoire analysis of sorted IgG+CD3+ T cells from peripheral lymphocytes in rats with DNA vaccination with Vβ5, 7, and 13. These CD3+/IgG+ T cells expressed Vβ5 and Vβ13 that the DV encoded. TCR gene usage of the sorted IgG+CD3+ T cells from three individual animals in the top three panels restricted to Vβ5 and Vβ13 in contrast to the diversity of Vβ genes seen in the sorted CD3+ T cells in the bottom panel. Lanes 1–20 represent each TCR Vβ family and the last lane represents a negative control for RT-PCR.

**FIGURE 9.** Expression of cytokine mRNA by sorted IgG+CD3+ T cells from peripheral lymphocytes in rats with DNA vaccination with Vβ5, 7, and 13 by semiquantitative RT-PCR. IFN-γ and IL-10 were not detected in the sorted IgG+CD3+ T cells. GAPDH gene served as an internal control. Lanes 1–3 represent PCR products from sorted IgG+CD3+ T cells from peripheral lymphocytes in vaccinated rats (n = 3). Lanes 4 and 5 represent RT-PCR products from ConA stimulated T cells and Th2 cell lines we described previously, which served as positive controls for cytokine genes. Lane 6 represents a negative control for RT-PCR.
population. These specific Abs did not lead to the depletion of Vβ5, Vβ7, and Vβ13 T cells. These results are consistent with the observations made in transplantation models and the EAE model (11). Anti-TCR Vβ13-specific DNA vaccination prolonged heart allograft survival in adult rats (21) and DNA vaccination against an immunoregulatory CD8 + T cell clone bearing a Vβ8.1β2.7 TCR abolished DST-induced allograft tolerance without the depletion of the clone (22). In the EAE model, encephalitogenic T cell lines and clones mainly used a restricted TCR gene Vβ8.2 (18) (19). Vaccination with DNA encoding the Vβ8.2 region of an autoreactive TCR protected mice from EAE (11). This protection was attributed to a shift from a Th1 to Th2 cytokine profile in the Vβ8.2 target cells rather than due to their depletion. However, suppressive effects were obtained from other studies with no signs of a Th2 bias (23), suggesting that Th2 deviation is not essential for the protective effect of DNA vaccination. We have examined the cytokine profile in HN glomeruli. DNA vaccination reduced glomerular IFN-γ mRNA expression but did not elevate levels of Th2 cytokine mRNA. We also analyzed the cytokine profile of CD3 + IgG2a cells from DNA vaccinated rats. CD3 + IgG2a cells, which were CD8 + T cells, had little IFN-γ and IL-10 mRNA expression. It is interesting to note that CD8 + T cells were significantly decreased in number in the glomeruli of the DNA vaccinated rats. We speculate that autoantibodies produced following DNA vaccination bind specific TCRs and block the activation of these T cells. Our results suggest that this specifically targeted pathogenic subset of the T cell population either did not infiltrate the glomeruli or failed to expand and were unable to express pathogenic cytokines. This would appear to demonstrate the specific suppressive effects of DNA vaccination. The interaction of the TCR with Ag in the context of MHC has been greatly advanced by the recent crystallographic evidence of TCR structure and mutational analysis of TCR activation. It is possible that Abs binding to the Vβ-chain can directly limit the normal activation of these T cells preventing clonal expansion and cytokine production (24). Furthermore, data on the effect of Vβ Abs in blocking CD8 function has been shown in antitumor specific CTL clones, which is consistent with our data (25).

In conclusion, we have shown that immunotherapy with TCR-based DNA vaccination protects against HN. This is based on our previous identification of pathogenic Vβ TCRs by TCR CDR3 spectratyping analysis and subsequent CDR3 region sequencing of spectratype-derived T cell clones. The mechanism for this appears to be the induction of Vβ specific Abs. DNA vaccination targeting the T cell receptors of pathogenic T cells is an attractive therapeutic alternative to broad immunosuppression in the treatment of glomerulonephritis.

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References