DNA Vaccination with CCL2 DNA Modified by the Addition of an Adjuvant Epitope Protects against “Nonimmune” Toxic Renal Injury

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CC-chemokine-encoding DNA vaccine has been reported to be capable of inducing immunologic memory to corresponding pathogenic self CC-chemokines in animal models of autoimmune disease. This study investigated whether introduction of a foreign T helper epitope into monocyte chemoattractant protein 1 (CCL2) DNA vaccine could boost its immunogenicity by inducing strong neutralizing autoantibody against the pathogenic chemokine CCL2 sufficiently to be protective in a classically nonimmune model of disease, Adriamycin nephropathy (AN). Modification of the CCL2 DNA vaccine by replacing a surface loop region of CCL2 sequence with tetanus toxoid T helper epitope P30 elicited a strong self-specific CCL2 autoantibody production, as well as an IFN-γ-producing T cell cellular response. The increased immunogenicity of modified CCL2 DNA vaccination but not unmodified CCL2 DNA vaccination was protective against functional and structural renal injury in rat AN. The protective effect of the modified CCL2 DNA vaccine was associated with blockade of glomerular and interstitial macrophage recruitment by neutralizing autoantibody against CCL2, which plays a critical role in eliciting renal injury in AN. Therefore, modification with a foreign T helper epitope breaks self-tolerance by inducing a cellular and humoral response against self-protein and provides a strategy to increase the potency of DNA vaccination sufficiently to afford protection in toxin-induced chronic renal disease.

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driamycin nephropathy (AN) is an experimental analogue of human focal glomerular sclerosis, characterized by severe nephrotic syndrome, focal segmental glomerulosclerosis, tubular atrophy and prominent infiltration of macrophages and CD4* and CD8* T cells (1–5). AN is a toxic response to Adriamycin (ADR) that can occur in the absence of cognate immune responses. Overt chronic proteinuria develops shortly after ADR administration, and glomerulosclerosis and tubulointerstitial injury become obvious by week 4 (2,6). CC-chemokines, particularly CCL2, play a crucial role in attracting mononuclear inflammatory cells to the interstitium and glomerulus and in modulating interactions between resident and inflammatory cells and consequent renal injury (7–10).

Several pieces of evidence have demonstrated the importance of CCL2 in causing renal interstitial and glomerular inflammation. We showed previously that NF-κB–dependent CCL2 transcription in rat renal cortex was increased 1 wk after ADR administration and peaked at week 2 (11), coincident with the peak of interstitial macrophage infiltration (3,6). In vitro studies revealed that CCL2 expression in rat proximal tubular epithelial cells was induced by LPS and by albumin and transferrin (12,13). Deletion of CCL2 dramatically reduced macrophage and T cell recruitment to kidney and consequent tubular injury (10). Blockade of chemokines and their receptors using specific antibody (Ab) has been shown to be only partially effective or ineffective (14,15) in models of renal disease, which might be a consequence of development of host Ab to the therapeutic Ab after repeated administration.

DNA vaccination represents a novel immunization strategy that uses in vivo antigen expression to induce both humoral and cellular immune responses. DNA vaccines encoding CC-chemokines have been reported to be capable of breaking host immune tolerance to pathogenic chemokines that are produced under disease conditions such as experimental autoimmune encephalomyelitis and adjuvant arthritis (16,17). The advantages of DNA vaccination in these autoimmune diseases are its long-lasting protective immunity against target antigen and its ability to break immune tolerance to pathologic self-proteins.
However, the potency of DNA vaccination remains a challenge to its application (18,19). We demonstrated recently that DNA vaccination using vaccines encoding both CCL2 and CCL5 is protective against renal injury in AN (20). Blockade of CCL2 alone using dominant negative mutant CCL2 interference has been shown to be sufficient to attenuate renal injury induced by protein-overload proteinuria (21) and prevent renal fibrosis (8). Nevertheless, redundancy of chemokine networks suggests that more than one chemokine is likely to be operative and that targeting a single chemokine by DNA vaccination may not be capable of affording protection. In this study, modification of wild-type CCL2 gene by insertion of a foreign T helper epitope, P30 from tetanus toxoid, into surface loop region to enhance the potency of the DNA vaccine was tested for its efficacy in the classically nonimmune model, AN.

DNA is known to activate antigen-presenting cells through the Toll receptors. However the mechanism of DNA vaccination in inducing responses against self-antigens is still not clear. Adjuvants including CpG motifs, complement factors, and co-stimulatory molecules have been incorporated into naked DNA vaccines to enhance efficacy. As compared with viral vaccine strategies, naked DNA is restricted in its actions, which may be of benefit in not inducing pathogenic autoreactivity. We have previously shown that DNA vaccination with the addition of CpG adjuvant can induce inhibition of TCR Vβ by production of antibodies against the Vβ (22). Here we show that the addition of P30 induces a cellular as well as a humoral response that is sufficient to protect against CCL2-mediated macrophage-driven tissue injury.

Materials and Methods

Construction and Modification of the CCL2 DNA Vaccine and Vaccination

Wild-type rat CCL2 cDNA was amplified by reverse transcription–PCR (RT-PCR) from RNA extracts from kidney of AN rats using the following primers: CCL2 sense 5'-caactatgcaggtctctgtc-3' and antisense 5'-atcacattccaaatcacactag-3'. Rat CCL2 gene was modified by replacing a surface loop region 109 to 138 (37 to 46 amino acids) by P30 tetanus toxoid helper epitope sequence FNNFTVSFWLRVPKVSASHLE using a surface loop region 109 to 138 (37 to 46 amino acids) by P30 tetanus toxoid helper epitope sequence FNNFTVSFWLRVPKVSASHLE using overlap primer extension PCR. Rat CCL2 primers in combination with overlapping primers were used for this modification. The overlapping primers were as follows: Overlap primer 1 (antisense) GACCTTGGGCACGCCGAGCGGCGTAAGCTGACGGTGTTGGAatgactctctctgctgag and overlap primer 2 (sense) TGGCTGGCGGT-GGCCAAGGTCACGCGGACCTGGAGatgagtcggctggagaacta.

CCL2 5' primer and overlap primer 1 were used to amplify the first fragment of modified CCL2 gene, whereas overlap primer 2 and CCL2 3' primer were used to amplify the second fragment of modified CCL2. The first fragment annealed to the second fragment at overlap region, then the whole modified CCL2 gene was extended further and amplified with 5' and 3' CCL2 primers. The modified CCL2 PCR product was cloned into pTarget vector (Promega, Madison, WI) to make the modified CCL2 DNA vaccine. The modified CCL2 (ΔCCL2) vaccine sequence was verified by DNA sequencing after cloning. Plasmid DNA was prepared in large-scale using Qiagen Plasmid Mega Kit (Qiagen, Hilden, Germany).

In Vitro Expression of Modified CCL2 Gene and Three-Dimensional Prediction of Modified CCL2 Protein

HeLa cells, which do not express CCL2, were transiently transfected with plasmid DNA of pTarget vector, pTarget/CCL2, and pTarget/ΔCCL2 using lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cell lysates were immunoblotted with rabbit anti-rat CCL2 (Chemicon, Temecula, CA), followed by goat anti-rabbit IgG:horseradish peroxidase, and detected with a chemiluminescent ECL detection system (Chemicon). The three-dimensional structure of modified rat CCL2 protein was predicted and compared with wild-type using SWISS-MODEL online (http://swissmodel.expasy.org/).

DNA Vaccination and Induction of AN

Male Wistar rats that were approximately 4 to 5 wk of age and weighed 90 to 110 g were purchased from the Australian Research Council and maintained under standard sterile conditions in the Department of Animal Care at Westmead Hospital. Experiments were carried out in accordance with protocols approved by the Animal Ethics Committee of Sydney West Area Health Service. Animals were divided randomly into five groups: Normal control (n = 4), ADR control (n = 6), vector control (n = 6), unmodified CCL2 vaccine (n = 6), and modified CCL2 vaccine (n = 7). Rats were pretreated with 0.75% bupivacaine (1 μl/g body wt; Sigma, St. Louis, MO) by intramuscular injection into tibialis anterior muscle 1 wk before plasmid DNA vaccination. Plasmid DNA (300 μg) was injected weekly on four occasions into the same site as bupivacaine. One week after the fourth DNA vaccination, AN was induced by a single tail-vein injection of ADR (5 mg/kg; David Bull Labs, Victoria, Australia; Figure 1).

Evaluation of Antichemokine Ab Titer in Sera of DNA-Vaccinated Rats

A direct ELISA assay as described (16) was used to determine the anti-CCL2 or anti-CCL3 (MIP-1α) Ab titer in sera from DNA-vaccinated rats. Recombinant rat CCL2 and CCL3 were coated onto 96-well ELISA plates (Titertek, Horsham, PA) at a concentration of 50 ng/well in 100

Figure 1. Experimental protocol for assessment of DNA vaccination.
were embedded in OCT compound, fixed with acetone at 4°C for 10 min, and immersed in 0.3% H2O2 for 10 min to eliminate endogenous peroxidase and blocked with Background Buster (AXELL, Westbury, NY) at 10 min each to minimize background and nonspecific Ab binding. Sections were incubated individually with mAb against macrophages (CD68+) and CD8+ and CD4+ T cells (Serotec, Oxford, UK) for 90 min, followed by goat anti-mouse IgG:horseradish peroxidase-conjugated secondary Ab (Serotec) and visualized by addition of freshly prepared 3,3-diaminobenzidine tetrahydrochloride. The number of macrophages and CD8+ and CD4+ T cells was quantified in 10 nonoverlapping cortical fields (×400), expressed as number of cells per ×400 field per animal.

**Detection of CCL2 Expression in AN Kidney**

The level of CCL2 mRNA expressed in glomeruli and tubules of AN rats was analyzed by Laser Capture Microdissection (PALM; Microlaser Technologies AG, Bernried, Germany) of OCT (Tissue-Tek, Sakura Rinetek, Torrance, CA) frozen tissue sections and RT-PCR. OCT-embedded frozen kidney cortex was cut in 10-μm sections and mounted on DEPC-pretreated glass slide, fixed in 70% ethanol and briefly stained in Mayers Hematoxylin, then laser captured and measured into lysis buffer (Absolutely RNA Nanoprep Kit; Stratagene, La Jolla, CA) under ×200 magnification. Total RNA from microdissected glomerular or tubular samples was isolated using the Absolutely RNA Nanoprep Kit. RT-PCR was performed using Superscript III CellsDirect cDNA Synthesis Kit (Invitrogen), then amplified for CCL2 with primers described previously and glyceraldehyde-3-phosphate dehydrogenase with the following primers: Sense 5’-aaggggtatgcctggtg-3’ and antisense 5’-tggggcctgagttgggatgg-3’. The level of CCL2 expression was quantified by the densitometric ratio of CCL2/glyceraldehyde-3-phosphate dehydrogenase.

**Renal Function**

Blood and 16-h urine samples were collected weekly after ADR administration. Urine protein concentrations were determined by colorimetric assay (Bio-Rad). Blood and urine creatinine were determined as described previously (6). Creatinine clearance was calculated as creatinine excretion divided by serum creatinine concentration.

**Histopathology**

Four weeks after ADR administration, animals were killed for tissue collection. A coronal slice of kidney from each animal was fixed in 10% neutral-buffered formalin for 24 h and then dehydrated in graded alcohols and embedded in paraffin. Tissues were cut at 5 μm and stained with periodic acid-Schiff and hematoxylin. Glomerulosclerosis, tubular atrophy, and interstitial expansion were measured using a quantitative method (5). Digital images of cortical sections were analyzed using image software (Image J; National Institutes of Health, Bethesda, MD). Glomerular capillary tuft was outlined and computed as a measure of total glomerular area. Glomerulosclerosis was quantified as percentage of periodic acid-Schiff–positive staining area in total glomerular area of the same glomerulus. The mean of 20 randomly selected glomeruli was determined for each section. Tubular atrophy was defined by low cell height with absence of brush border. The degree of interstitial expansion was determined by relative interstitial volume. The mean value of five cortical fields was determined for each section. Random cortical fields were viewed by an observer who was blinded to group identifications at a magnification of ×200. The areas for morphometric analysis were anatomically identical for each section and positioned before microscopic visualization.

**Immunohistochemistry**

Frozen sections were cut at 6 μm from cortical slices of kidneys that were embedded in OCT compound, fixed with acetone at 4°C for 10 min, and immersed in 0.3% H2O2 for 10 min to eliminate endogenous peroxidase and blocked with Background Buster (AXELL, Westbury, NY) at 10 min each to minimize background and nonspecific Ab presentation. Anti-CCL2 Ab binding. Sections were incubated individually with mAb against macrophages (CD68+) and CD8+ and CD4+ T cells (Serotec, Oxford, UK) for 90 min, followed by goat anti-mouse IgG:horseradish peroxidase-conjugated secondary Ab (Serotec) and visualized by addition of freshly prepared 3,3-diaminobenzidine tetrahydrochloride. The number of macrophages and CD8+ and CD4+ T cells was quantified in 10 nonoverlapping cortical fields (×400), expressed as number of cells per ×400 field per animal.

**Enzyme-Linked Immunospot Assay**

Enzyme-linked immunospot (ELISpot) assay was performed according to Youssef’s method (16) with modification. Rat rCCL2 (10 ng/ml; Chemicon) in RPMI 1640 was added to lower wells of six-well plates with or without preincubation with the sera of DNA-vaccinated rats. Monocytes that were prepared by Lymphoprep (Axia-Shield PoC AS, Oslo, Norway) from rat splenocytes were seeded onto upper wells (insert with polycarbonate membrane of 8 μm pore size; Nunc, Roskilde, Denmark) at 4 × 106 cells/ml in RPMI 1640 enriched with 1% IBA and incubated at 37°C, 5% CO2 for 24 h. The detection Ab, biotin-conjugated mouse anti-r IFN-γ (Biosource, Camarillo, CA; 25 ng/well), was added, then kept at 4°C overnight, incubated with streptavidin alkaline phosphatase (Becton Dickinson, San Jose, CA) at room temperature for 2 h, and then developed with BCIP/NBT kit (Bio-Rad). Spots were counted under a dissecting microscope. The results are expressed as number of spot-forming cells/106 DLN cells.

**In Vitro Chemotaxis Assay**

The chemotaxis assay was performed according to Youssef’s method (16) with modification. Rat rCCL2 (10 ng/ml; Chemicon) in RPMI 1640 was added to lower wells of six-well plates with or without preincubation with the sera of DNA-vaccinated rats. Monocytes that were prepared by Lymphoprep (Axia-Shield PoC AS, Oslo, Norway) from rat splenocytes were seeded onto upper wells (insert with polycarbonate membrane of 8 μm pore size; Nunc, Roskilde, Denmark) at 4 × 106 cells/ml in RPMI 1640 enriched with 1% BSA and incubated at 37°C, 5% CO2 for 2 h. Cells that migrated to lower wells were harvested by trypsinization, and the total number of migrated cells in each well was counted by hemocytometer. The results are expressed as total number of migrated cells after subtracting the number of cells in control wells without rCCL2.

**Statistical Analyses**

Data are presented as mean ± SEM. Significance of differences was examined using one-way multiple range ANOVA test by *post hoc* Fisher PLSD analysis to compare means among groups. *P* < 0.05 was considered significant.

**Results**

**Modification of Wild-Type CCL2 DNA Vaccine to Express a Hybrid Protein In Vitro that Could be Recognized by Anti-CCL2 Ab**

DNA sequencing and three-dimensional structure prediction revealed that modification of the CCL2 DNA vaccine replaced a surface loop region of the wild-type CCL2 with foreign T helper epitope P30 while maintaining its natural folding and presenting the P30 peptide at surface loop region (Figure 2) to induce an autoimmune response against the self-antigen rat CCL2. In vitro expression demonstrated that modified CCL2 DNA vaccine expressed a hybrid protein with a slightly higher
molecular weight (15 kD) than the wild type (14 kD) and could be recognized by anti-rat CCL2 Ab by Western blot (Figure 3).

Production of Autoantibodies against CCL2 and CCL3 by Vaccination with Modified CCL2 DNA

Self-specific Ab against CCL2 was produced in sera of rats after vaccination with modified CCL2 DNA as well as unmodified CCL2 DNA. However, the Ab titer in the sera of rats that were vaccinated with modified CCL2 was significantly higher than that of rats that were vaccinated with unmodified CCL2 at week 2 of AN (Figure 4A), the time of peak interstitial macrophage infiltration. High-level autoantibody was sustained throughout the disease course until week 4 (Figure 4A). Ab titer of normal rats that were vaccinated with modified CCL2 was significantly lower than that of AN rats that were vaccinated with modified or unmodified CCL2 but not different from that of AN rats that were vaccinated with vector control and normal control.

As cross-reactivity of Ab against CC-chemokine DNA vaccine toward homologous CC-chemokines has been reported elsewhere (17), anti-sera from rats that were vaccinated with modified CCL2 was tested also for Ab titer to CCL3 and CCL5 (RANTES). Ab titer to rCCL3 was significantly higher in rats that were vaccinated with modified CCL2 as well as unmodified CCL2 compared with that of the vector control group at weeks 2 and 4 (Figure 4B) but not so for rCCL5 (data not shown).

Glomerular and Tubular CCL2 Expression in AN

CCL2 expression was detected in both glomeruli and tubules that were isolated by laser capture microdissection. Significantly higher levels of CCL2 expression were detected in tubules 1 wk after ADR compared with normal control (Figure 5). The high level of CCL2 expression in tubules was also observed at weeks 2, 3, and 4. Compared with tubular expression, glomerular expression of CCL2 was much lower but significantly increased from week 1 to week 4 after ADR in comparison with its normal control (Figure 5).

Protection against Kidney Injury in AN by DNA Vaccination with Modified CCL2 Vaccine

Rats that were vaccinated with modified CCL2 DNA vaccine developed much less severe renal structural injury at week 4 of AN. Glomerular and tubular damage was only mild in rats that were vaccinated with modified CCL2 DNA, in comparison with very severe glomerular and tubular injury in rats from ADR control, vector control, and unmodified CCL2 vaccine groups (Figure 6). Morphometric analysis (Table 1) showed significantly less glomerular injury with respect to total glomerular area, percentage of glomerulosclerosis, and number of nuclei per glomerulus in rats that were vaccinated with modified CCL2 compared with that of rats that were vaccinated with vector control or unmodified CCL2. Tubular cell height—but not tubular diameter—and interstitial volume were also significantly less in the modified CCL2 group compared with unmodified CCL2 and vector control groups.
Renal function also was protected significantly by vaccination with modified CCL2 vaccine. Creatinine clearance was significantly higher in rats that were vaccinated with modified CCL2 than that of ADR control, vector control, and unmodified CCL2 vaccine groups at weeks 2 and 4 (Figure 7) but not different from normal controls. There were no statistical differences in creatinine clearance among groups at weeks 1 and 3, although the mean value of the modified CCL2 group was higher than all other groups that received ADR.

Urine protein/creatinine ratio was lower in rats that received modified CCL2 vaccination than in groups that received vector control and ADR only at week 3 and all other ADR groups at week 4 (Figure 8).

Protection of AN Was Associated with Reduced Glomerular and Interstitial Macrophage Infiltration

Immunohistochemical staining of inflammatory cells revealed that modified CCL2 DNA vaccination significantly increased CCL2 expression in diseased kidney of AN rats that were isolated by microdissection. Values presented in the graph are densitometric ratios of CCL2 to glyceraldehyde-3-phosphate dehydrogenase control from three independent experiments. *P < 0.05 and **P < 0.01 versus normal control.

Renal function also was protected significantly by vaccination with modified CCL2 vaccine. Creatinine clearance was significantly higher in rats that were vaccinated with modified CCL2 than that of ADR control, vector control, and unmodified CCL2 vaccine groups at weeks 2 and 4 (Figure 7) but not different from normal controls. There were no statistical differences in creatinine clearance among groups at weeks 1 and 3, although the mean value of the modified CCL2 group was higher than all other groups that received ADR. Urine protein/creatinine ratio was lower in rats that received modified CCL2 vaccination than in groups that received vector control and ADR only at week 3 and all other ADR groups at week 4 (Figure 8).
reduced glomerular macrophage recruitment compared with unmodified CCL2 DNA vaccine, vector control, and ADR control. There was an even greater reduction in interstitial macrophage infiltration (Figure 9). There were no differences among unmodified CCL2, vector control, and ADR control groups. There was no difference in numbers of interstitial CD8⁺ and CD4⁺ T cells among the groups (Figure 9).

Table 1. Morphometric quantification of histologic changes in AN rats 4 wk after Adriamycin

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<thead>
<tr>
<th></th>
<th>Normal Group 1; n = 4</th>
<th>Vector Control Group 2; n = 6</th>
<th>CCL2 Group 3; n = 6</th>
<th>ΔCCL2 Group 4; n = 7</th>
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<tr>
<td><strong>Glomerular changes</strong></td>
<td></td>
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<tr>
<td>glomerular area (μm²)</td>
<td>4281.4 ± 328.2</td>
<td>1652.0 ± 312.4b</td>
<td>1883.0 ± 558.1b</td>
<td>4647.3 ± 175.1c,d</td>
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<tr>
<td>glomerulosclerosis (%)</td>
<td>0.0 ± 0.0</td>
<td>39.8 ± 7.7b</td>
<td>35.0 ± 1.2b</td>
<td>12.9 ± 5.0c,d</td>
</tr>
<tr>
<td>no. of nuclei/glomerular area</td>
<td>55.0 ± 4.8</td>
<td>26.8 ± 3.4b</td>
<td>32.5 ± 1.7b</td>
<td>49.0 ± 2.9e</td>
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<tr>
<td><strong>Tubular changes</strong></td>
<td></td>
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<tr>
<td>tubular diameter (μm)</td>
<td>46.6 ± 3.1</td>
<td>75.9 ± 5.8f</td>
<td>71.4 ± 7.3f</td>
<td>65.9 ± 4.3f</td>
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<tr>
<td>tubular cell height (μm)</td>
<td>15.4 ± 0.6</td>
<td>5.9 ± 0.9b</td>
<td>4.8 ± 0.6b</td>
<td>11.2 ± 1.2b,e</td>
</tr>
<tr>
<td>interstitial volume (%)</td>
<td>1.5 ± 0.4</td>
<td>20.9 ± 1.1b</td>
<td>18.6 ± 3.2b</td>
<td>3.7 ± 2.0e</td>
</tr>
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*AN, Adriamycin nephropathy; ΔCCL2 = modified CCL2.

bP < 0.01 versus group 1.
cP < 0.05 versus group 3.
dP < 0.01 versus group 2.
eP < 0.01 versus groups 2 and 3.
fP < 0.05 versus to group 1.

Figure 6. Protection against renal structural injury in AN rats by vaccination with modified CCL2 DNA vaccine. Kidney tissues that were harvested 4 wk after ADR administration were analyzed under periodic acid-Schiff stain. Representative histology of normal control (A), rats that were vaccinated with vector control (B), rats that were vaccinated with modified CCL2 DNA (C), and rats that were vaccinated with unmodified CCL2 DNA (D).

Increased IFN-γ-Producing T Cells from DLN after Modified CCL2 DNA Vaccination

To assess the cellular response of modified CCL2 DNA vaccine in comparison with unmodified vaccine and vector control, the numbers of IFN-γ-producing T cells from DLN of vaccinated normal rats were assessed by ELISpot assay. Rats that were vaccinated with modified CCL2 DNA had more T cells from DLN.
that produced IFN-γ when stimulated with 100 ng/ml rCCL2 in vitro than rats that were vaccinated with unmodified CCL2 or vector control rats. The number of IFN-γ-producing T cells of rats that were vaccinated with unmodified CCL2 vaccine was not different from that of vector control (Figure 10).

Antisera Neutralization of Chemotactic Effect of CCL2 toward Monocytes In Vitro

To explore the mechanism whereby modified CCL2 DNA vaccination reduced glomerular and interstitial macrophages, the effect of antisera was tested in vitro for its blocking effect against CCL2-induced monocyte chemotaxis. The total number of migrated monocytes was significantly lower under antisera of rats that were vaccinated with modified CCL2 than under antisera from rats that were vaccinated with unmodified CCL2 DNA or vector control or with rCCL2 only control wells (Figure 11).

Discussion

DNA vaccination is a new approach in which genes that encode target antigens are delivered and expressed in vivo within host cells. Although underlying mechanisms to explain both humoral and cellular immune responses to DNA vaccination are not fully understood, the presence of stimulatory CpG motifs within the expression plasmid backbone of bacterial origin is thought to act as adjuvant for stimulation of specific host immune response toward the antigen encoded by the
DNA vaccine (23–25). Although DNA vaccines have shown broad promise in models of infectious diseases, cancer, and autoimmune diseases, low potency remains a hurdle to its clinical application (26). Approaches to increase potency of DNA vaccine include improved vaccine delivery and increased immunogenicity of expressed antigens by the manipulation of their encoding genes. The foreign T helper epitope has been reported to break B cell tolerance to the highly conserved self-protein ubiquitin when expressed as a hybrid protein with ubiquitin (27,28). P30 from tetanus toxoid was also reported to bypass immunologic tolerance in a vaccine against IL-5 (29). T cell help has been considered crucial in breaking B cell tolerance to permit development of autoantibodies against self-proteins (29,30). In this study, we sought to enhance immunogenicity of DNA vaccine against self-protein CCL2 by modification of its gene. Modification of the CCL2 gene, by insertion of a foreign T helper epitope P30 in the surface loop region, enhanced production of autoantibody against native CCL2 protein, as well as the number of IFN-γ-producing T cells from DLN.Modified CCL2 hybrid protein was designed to maintain the tertiary structure of native CCL2 as much as possible, to induce cross-reactive antibodies against self CCL2 protein.

Using this DNA vaccination strategy, we demonstrated that modified CCL2 DNA vaccination is protective against both renal functional and structural injury in AN. Given the concurrence of elevated CCL2 expression with macrophage infiltration and renal damage in AN (3,11) and the widely known role of CCL2 in recruitment and activation of macrophages, it is likely that CCL2 is responsible for recruitment and activation of macrophages to the interstitium and glomerulus and consequent renal injury. The pathogenic role of CCL2 has been proved in other models of renal disease by the use of neutralizing antibodies against CCL2 (14,31) and by blockade of CCL2/CCR2 using mutant CCL2 gene therapy (21,32). Consistent with these findings, our study showed that blockade of CCL2 by autoantibodies that are produced through DNA vaccination with modified CCL2 protected the chemokine-mediated renal injury of AN. This contention is strongly supported by reduced interstitial and glomerular macrophage infiltration in rats that were vaccinated with modified CCL2, high titers of anti-CCL2 autoantibodies in their sera, and the neutralizing effect of the anti-sera in blocking CCL2-mediated monocyte chemotaxis in vitro.

Glomerular expression of CCL2 has been reported in other models of kidney disease to be responsible for recruitment of macrophages, which in turn are responsible for triggering production of inflammatory cytokines and other chemokines that lead to tissue injury and consequent glomerulosclerosis (10,14,33,34). In this study, we demonstrated that although its expression was predominantly tubular, CCL2 was also expressed by glomeruli in AN. Blockade of CCL2-induced macrophage recruitment and activation within glomeruli may well explain the protection of glomerulosclerosis, as well as tubulointerstitial injury, by modified CCL2 DNA vaccination. Consistent with this effect on glomerulosclerosis, proteinuria was also reduced by vaccination with modified CCL2 DNA. Proteinuria itself may be injurious to glomerular and tubular epithelium (35–38) and lead to local upregulation of chemokines, particularly CCL2 (13,37,39).

Only modified CCL2 DNA vaccine but not unmodified CCL2 DNA vaccine was found to be protective against both functional and structural renal injury in AN, and anti-CCL2 Ab titers and number of IFN-γ-producing T cells from DLN were greater with modified than unmodified DNA vaccination. Thus, the potency of unmodified DNA vaccine, with the adjuvant effect of stimulatory CpG motif within backbone to enhance its immunogenicity, was insufficient to block the effect of CCL2, whereas its modification with foreign T helper epitope P30 to greatly enhance its immunogenicity rendered it sufficiently potent to block the effect of CCL2. It is interesting that, in vaccinated rats, not only the titer of anti-CCL2 antibody was significantly increased but also that of anti-CCL3. This may be due to the structural similarities of these chemokines leading to cross-reactivity. As multiple chemokines such as CCL2, CCL3, and CCL5 have been reported to be implicated in glomerular and tubulointerstitial inflammation (40), particularly in rat AN (11,20), it is possible that the additional inhibitory effect against closely related chemokines such as CCL3 in this study may have helped to overcome redundancy and provide greater clinical efficacy.

Another important finding in this study was that the anti-CCL2 autoantibody level in normal rats that were vaccinated with modified CCL2 was much lower than in vaccinated rats with AN and not different from that of vector control vaccination, indicating the disease specificity of autoantibody production and education of host immune system against the pathogenic self-protein. This interesting feature of DNA vaccination responses that are limited to inflamed tissues is intriguing and suggests the possibility that innate danger signals may play a role in priming the autoantibody responses while limiting these in noninflamed tissues. The presence of cellular immunity of a Th1 type found in the DLN with the use of the modified vaccine.

Figure 11. Blockade of monocyte chemotaxis by sera of DNA-vaccinated rats, as assessed by total number of cells that migrated to the lower chamber through an 8-μM membrane in response to rCCL2. Values are means ± SEM of three different serum samples. **P < 0.01 versus unmodified CCL2 and vector control.
was shown by the presence of IFN-γ-producing T cells from DLN of vaccinated rats, specific for rCCL2. These IFN-γ-producing T cells include both CD4+ and CD8+ T cells (41,42). CD4+ IFN-γ-producing T cells represent helper and memory T cells that assist B cell antibody production and long-lasting memory, whereas CD8+ IFN-γ-producing T cells are effector or cytotoxic T cells whose role in modified CCL2 DNA vaccination requires further investigation.

**Conclusion**

Modification of CCL2 DNA vaccine by the addition of P30 enhanced its immunogenicity, leading to a novel cellular response and an increased humoral response to CCL2 chemokine. The effect of the inhibition of this crucial pathway is shown by the inhibition of interstitial and glomerular macrophage infiltration and consequent renal injury in a toxin-induced model of chronic renal disease.

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**References**