Short Term Administration of Costimulatory Blockade and Cyclophosphamide Induces Remission of Systemic Lupus Erythematosus Nephritis in NZB/W F<sub>1</sub> Mice by a Mechanism Downstream of Renal Immune Complex Deposition

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Short Term Administration of Costimulatory Blockade and Cyclophosphamide Induces Remission of Systemic Lupus Erythematosus Nephritis in NZB/W F₁ Mice by a Mechanism Downstream of Renal Immune Complex Deposition¹

Lena Schiffer,²* Jayashree Sinha,²* Xiaobo Wang,* Weiqing Huang,* Gero von Gonsdorff,* Mario Schiffer,* Michael P. Madaio,‡ and Anne Davidson³*†

NZB/W F₁ mice with established nephritis were treated with a single dose of cyclophosphamide with or without a 2-wk course of murine CTLA4Ig, either alone or in combination with anti-CD154. Sixty to 80% of treated mice entered remission, and remission could be reinduced following relapse. A decrease in the frequency of anti-DNA-producing B cells and activated T cells was observed in treated mice, but this effect lasted only 3–6 wk, while remissions were sustained for up to 20 wk. Light microscopy of the kidneys of mice in remission revealed less glomerular inflammation, less tubular damage, and less infiltration of inflammatory cells. By immunofluorescence, however, IgG and C3 staining of glomeruli was no different in treated mice vs controls. Since chemokines and their receptors play an important role in inflammatory cell infiltration of affected organs in autoimmune diseases, we examined chemokine expression in the kidneys. Decreases in the expression of inflammatory cytokines and chemokines were evident in mice in the early stages of remission, but these differences were no longer present in late remission. Increased expression of CXCL13 was detected in the inflammatory infiltrates of the control NZB/NZW mice. Strikingly, we could not detect any CXCL13 in the kidneys of the treated group even in late remission. These findings suggest that costimulatory blockade together with cyclophosphamide influence the activation state of renal CD11c-positive cells and therefore lead to less B and T cell infiltration and nephritis. The Journal of Immunology, 2003, 171: 489–497.

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2 L.S. and J.S. are equal contributors to this work.

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* Abbreviations used in this paper: SLE, systemic lupus erythematosus; CTX, cyclophosphamide; MCF, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; TCA-3, T cell activation gene-3 chemokine.

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Materials and Methods

Mice and therapeutic regimens

Fully murine CTLA4Ig consisting of the extracellular domain of CTLA4 fused to the CH2-CH3 domains of murine IgG2a mutated so that it no longer binds FcRs was obtained from the serum of SCID mice injected with a prokaryotic expression plasmid expressing adenovirus. This virus induces serum levels of CTLA4Ig in the range of 5–20 mg/ml and has previously been described in detail (7). Hamster anti-CD154 (MR1) was purified from the supernatant of a high expressing cell line (a gift from S. Kalled, Biogen, Cambridge, MA) by protein A chromatography.

NZB/NZW F1 females were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a conventional animal housing facility throughout the experiment (7). Mice were followed weekly from the age of 20 wk. Urine was tested for proteinuria by dipstick (Multistick; Fisher Scientific, Pittsburgh, PA) every week. Once fixed proteinuria of >300 mg/dl had appeared on two occasions 2 days apart, mice were randomized to treatment with a single CTX dose of 750 mg/m2 (~21 mg/kg) and a course of 250 μg of anti-CD154 either alone (dual therapy, 13 mice) or in combination with 250 μg of anti-CD154 (triple therapy, 19 mice), each given i.p. six times over a 2-wk period as previously described (10). Initially the mice were randomized equally, but because preliminary results showed that only six of 13 mice in the dual therapy group entered durable remission (>5 wk compared with 11 of 13 in the triple therapy group), six additional mice were added to the triple therapy group. Control mice received either CTX alone (one mouse for every four treated mice, total of seven mice), CTLA4Ig/anti-CD154 without CTX (11 mice), or no treatment (30 mice). Because CTLA4Ig/anti-CD154 was not therapeutically beneficial, we did not treat with CTLA4Ig alone. Mice in the double or triple therapy groups that entered remission (proteinuria, <30 mg/dl for 3 or more wk) and subsequently developed >300 mg/dl proteinuria again (15 mice) were retreated with a course of triple therapy (Fig. 1). Triple therapy was used as the retreatment because the mice treated with double therapy appeared to relapse sooner than those treated with initial triple therapy.

We analyzed the effects of treatment on splenic lymphocytes and kidney at intervals after treatment. Some mice underwent survival splenectomy, and some were sacrificed at intervals after treatment for analysis of both spleens and kidneys. To determine the final outcome of treatment on survival, a cohort was also followed until death (Table I).

Anti-dsDNA Abs

ELISA plates (BD Biosciences, Franklin Lakes, NJ) were coated with 100 μl of 100 μg/ml salmon sperm DNA made double-stranded by passage through a 45-μm pore size filter (USA Scientific, Ocala, FL). After drying, the plates were blocked and then incubated sequentially for 1 h at 37°C with a 1/10,000 dilution of serum, followed by peroxidase-conjugated F(ab’2) goat anti-mouse IgG1, anti-IgG2a (1/5000; Accurate Antibodies, Westbury, NY), or alkaline phosphatase-conjugated anti-IgG3 in PBS/1% BSA for 1 h at 37°C and then either ABTS substrate (Kirkegaard & Perry, Gaithersburg, MD) or 5-bromo-4-chloro-3-indolyl-phosphate (Sigma-Aldrich) in AMP buffer (0.75 mM MgCl, 0.01% Triton X, and 9.58% 2-amino-methyl-1-propanol, pH 10.25). Plates were then developed with 1 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Sigma-Aldrich) in AMP buffer (0.75 mM MgCl, 0.01% Triton X, and 9.58% 2-amino-methyl-1-propanol, pH 10.25). Spots were counted using a dissecting microscope. The total numbers of Ig-secreting cells were measured the same way using anti-mouse IgGs (Cappel, Westchester, PA) to coat the plates.

Generation of hybridomas

Hybridomas were generated from spleen cells of the same mice described above by standard techniques, using NSO cells as the fusion partner. Hybridomas were screened for anti-dsDNA activity by ELISA as described above. Positive hybridomas were then isolated using specific peroxidase-conjugated secondary Abs for IgM and IgG (Cappel).

Analysis of spleen lymphoid cell subsets

Spleen cells harvested from mice in remission at 3–6 or >10 wk after a single course of double or triple therapy were analyzed by flow cytometry for B and T cell markers using Abs to CD4 (Caltag, Burlingame, CA), CD8 (Caltag), and CD19 (BD PharMingen, San Diego, CA). The presence of activated CD4 cells was determined by double staining with FITC-anti-CD4 and PE-anti-CD69 (BD PharMingen). The presence of naive and activated/memory CD4 cells was determined by triple staining with FITC-anti-CD4, anti-IgG1 (BD PharMingen), and PE-anti-CD62L (BD PharMingen). Spleen dendritic cells were identified using PE-anti-CD11c and FITC-anti-CD11b (BD PharMingen). Myeloid (CD11c+/CD11b−), lymphoid (CD11c−/CD11b+), and blood-derived (CD11c+/CD11b+) dendritic cells were identified according to previously published methods (12).

For further analysis of spleen dendritic cells, fluorescent staining of cryosections was used. Five-micrometer cryosections were stained with FITC-anti-mouse CD11c and PE-anti-mouse CD45R/B220 (BD PharMingen) in 10% normal goat serum-FC5/PBS for 1 h at room temperature. Images were captured using a digital CCD camera system (Diagnostic Instruments, Sterling Heights, MI) connected to a Nikon microscope (Melville, NY).

Examination of kidneys

Kidneys were harvested from mice in remission 3–6 or >10 wk after a single course of double or triple therapy. Four or five mice were examined in each group. One-half of a kidney was fixed by overnight immersion in 10% formaldehyde and paraffin-embedded. The other half was snap-frozen in methyl butane and embedded in OCT (Miles, Elkhart, IN) for cryosections.

To determine the extent of renal damage, sections were stained with H&E and periodic acid-Schiff and scored for pathologic changes according to two different methods (13, 14) by two blinded observers (M.P.M. and T.S.F.). For further analysis of cryosections, sections of the kidneys were hydrated and microwaved three times for 3 min each time in sodium acetate buffer, pH 6.0. After blocking of endogenous peroxidase activity in methanolic H2O2 (0.3%), the sections were immunoperoxidase-labeled using Vectastain ABC kit and the avidin biotin blocking kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s protocol. Peroxidase-conjugated anti-mouse C3 (Cappel) staining for 1 h at room temperature was followed by diaminobenzidine substrate development (Vector Laboratories). Goat anti-mouse CXCCL13 polyclonal Ab (Genzyme, Cambridge, MA) was applied at 4°C overnight. For signal enhancement the tyramide signal amplification kit (NEN Life Science, Boston MA) was used according to the manufacturer’s protocol.

For analysis of Ig deposition and for identification of infiltrating lymphocytes, fluorescent staining of cryosections was used. Five-micrometer
cryosections were stained with FITC-anti-mouse IgG (Cooper Biomedical, Westchester, PA) in 10% normal goat serum-FCS/PBS, PE-anti-mouse CD4 (BD PharMingen), PE-anti-protein CD45R/B220 (BD PharMingen), FITC-anti mouse CD4 (BD PharMingen), FITC-anti-mouse CD3 (BD PharMingen), or FITC-anti-mouse CD11c (BD PharMingen) for 1 h at room temperature. Images were captured using a digital CCD camera system (Diagnostic Instruments), connected to a Nikon microscope.

RNase protection assay

The second kidney from each of the mice described above was snap-frozen and immediately homogenized in TRIzol reagent (Life Technologies, Grand Island, NY). RNA was extracted according to the manufacturer’s instructions. RNase protection assay was performed using both the mCK-5 kit and the mCK-3 kit (BD PharMingen), according to the manufacturer’s instructions for the following chemokines and cytokines: monocyte chemotactic protein 1 (MCP-1), T cell activation gene-3 chemokine (TCA-3), macrophage inflammatory protein 2 (MIP-2), MIP-1α, MIP-1β, RANTES (mCK-5), TNF-α, lymphotxin-β, IFN-β, IFN-γ, TGF-β, and macrophage migration inhibitory factor (mCK-3) using 20 μg of total RNA for each assay. The assay was performed twice with reproducible results. Control kidney cells were obtained from 12–14 and 34–38 wk untreated NZB/W F1 mice.

Results

Clinical outcomes

Neither CTX alone nor combined CTLA4Ig/anti-CD154 was effective at inducing remission in proteinuric NZB/W F1 mice. In contrast, complete remission of proteinuria (<30 mg/dl) occurred within 2–3 wk of treatment in 60–80% of mice given either CTX/CTLA4Ig (double) or CTX/CTLA4Ig/anti-CD154 (triple) therapy. The average time to relapse was longer in the triple therapy than in the double therapy group, with statistically significant differences at wk 6–8 after treatment (p < 0.05). Importantly, once relapse occurred, remission could be induced in 50% of the mice with a second course of triple therapy (Fig. 1 and Table I). The life span of mice treated with either double or triple therapy was markedly prolonged, and 50% of the mice were still alive 6 mo after treatment (Fig. 2 and Table I). Serum Abs to dsDNA fluctuated during the first few weeks after treatment, but by 4 wk after treatment were the same as pretreatment levels (not shown).

Effect of treatment on autoreactive B cells

Treatment with CTX and costimulatory blockade resulted in a decrease in spleen size. Three weeks after treatment the spleen cell number in treated mice was lower than that in the untreated control group (2.1 ± 0.42 × 107 in untreated mice vs 5.2 ± 2.5 × 107 in triple treated mice (p < 0.001) and 8.6 ± 3.5 × 107 in double treated mice (p < 0.02)). The difference in spleen cell number from mice treated with CTX alone (1.4 ± 0.8 × 107) was only significant for the triple treated mice (p < 0.03). ELISPOT assays were performed at intervals to determine the effect of treatment on the frequency of Ig- and autoantibody-producing splenic B cells. There was a significant decrease in the frequency of IgG anti-dsDNA-producing B cells only in triple treated mice (p < 0.002 compared with untreated aged controls). However, by 5–6 wk after treatment the frequency of IgG anti-dsDNA-producing B cells was no different in spleens from triple treated mice from that in untreated controls, although the spleens were still decreased in size (7.6 ± 5.2 × 107, Fig. 3). Mice that were retreated with triple therapy and studied 3 or 5 wk after treatment were indistinguishable from those examined after the first treatment course (not shown).

Table I. Time to relapse and death in treated and control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Treated</th>
<th>No. in Remissiona</th>
<th>Time to Relapse of &gt;300 mg/dl Proteinuriab</th>
<th>Time to Deathc</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX alone</td>
<td>7</td>
<td>2</td>
<td>18, &gt;4</td>
<td>26, 10.7, 6.6, 3</td>
</tr>
<tr>
<td>CTLA4Ig/anti-CD154</td>
<td>11</td>
<td>3</td>
<td>&gt;4, &gt;4.4</td>
<td>15, 11, 10, 7.5, 5.4.4.3</td>
</tr>
<tr>
<td>CTX/CTLA4Ig (double therapy)</td>
<td>13</td>
<td>10</td>
<td>37.34, 13, &gt;10, &gt;10, &gt;5.5, 3.3.3</td>
<td>45, 45, 43, 43, 39, 29, 21, 16, 7.4</td>
</tr>
<tr>
<td>Triple therapy retreatment of double therapy group</td>
<td>19</td>
<td>16</td>
<td>19,16, &gt;15, &gt;14,12, 12 &gt;10,10,9, &gt;8, &gt;8, &gt;8, &gt;8, &gt;8, &gt;5, &gt;5, &gt;3</td>
<td>41,41,29,28,19,18,8,3</td>
</tr>
<tr>
<td>CTX/CTLA4Ig/anti-CD154 (Triple therapy)</td>
<td>6</td>
<td>3</td>
<td>&gt;14, &gt;10,6</td>
<td></td>
</tr>
</tbody>
</table>

b Remission defined as 30 mg/dl of proteinuria or less maintained for at least 3 wk.

c Mice that were still in remission but were sacrificed for kidney analysis or that died during surgery are indicated by a > sign. Time is shown in weeks.

d Time to death (in weeks) only for mice that were followed until natural death. All mice in the study are included in the survival plot shown in Fig. 2.

FIGURE 2. Overall survival of all treated mice compared with controls. The average age of onset of 300 mg/dl proteinuria is shown in parentheses. Survival is shown as time after initiation of treatment. Mice in both treated groups had a markedly prolonged survival compared with controls (p < 0.001).

FIGURE 3. Frequency of total IgM- and IgG-producing cells per 10⁷ spleen cells and of IgM and IgG anti-dsDNA-producing B cells per 10⁷ spleen cells measured by ELISPOT at intervals after treatment. Four to eight mice were examined in each group. There was no effect of treatment on IgM anti-dsDNA-producing B cells. The only significant decrease in the frequency of IgG anti-dsDNA-secreting B cells was observed in mice treated with triple therapy 3 wk after treatment (p < 0.002 compared with CTX alone), but this effect was no longer evident 5 wk after treatment.
To determine whether the cells detected by ELISPOT were sufficiently activated to generate spontaneous hybridomas, we performed spleen cell fusions on control and treated mice and screened the hybridomas for anti-dsDNA activity. In untreated mice 6% of hybridomas were positive for dsDNA binding, and 80% of these were of the IgG isotype. Treatment with a single dose of CTX alone resulted in a significant decrease in the frequency of hybridomas recovered 3–5 wk after treatment ($p < 0.003$). Among the treated groups, the only statistically significant difference from CTX treatment alone was observed in spleens obtained from mice 3 wk after triple therapy ($p = 0.0001$; Fig. 4). With the exception of the triple therapy group at 3 wk in which insufficient positive hybridomas were retrieved for analysis, none of the treatment protocols altered the ratio of IgM/IgG anti-dsDNA-producing hybridomas, suggesting that there was no effect on class switching.

**Spleen lymphoid cell subsets**

To determine whether the activated phenotype of splenic lymphocytes could be altered by treatment, flow cytometry was performed on spleens of double or triple therapy treated mice that were still in remission 3–5 or >10 wk after a single course of therapy. The mice in early remission, whether induced by double or triple therapy, had a significant alteration in the frequency of activated T cells compared with 34–38 wk untreated nephritic NZB/W F$_1$ controls. This was manifest by a reduction in the frequency of CD4 cells that were CD69$^+$, a reversal of the CD4/CD8 ratio back to that of young prenephritic mice, and a reversal of the accumulation of CD4 T cells bearing the activated/memory CD44$^+$/CD62L$^-$ markers (Fig. 5). The phenotype of these mice was similar to that of 22–25 wk NZB/W F$_1$ mice. In contrast, all mice in remission >10 wk after therapy had reverted to the activated phenotype of untreated controls. Two further mice examined at 7 wk after treatment had the phenotype of untreated controls (not shown). No alteration in spleen cell phenotype was observed in mice that did not enter remission (not shown). Similar findings were observed upon examination of spleen dendritic cells. A marked increase in spleen CD11c$^+$/CD11b$^+$ cells was observed in 34–38 wk nephritic NZB/W F$_1$ mice compared with 18 wk NZB/W F$_1$ controls ($6.2 \pm 2.7$ vs $1.5 \pm 0.5%$; $p = 0.002$). Immunohistochemical staining showed that this accumulation occurred adjacent to B cell follicles (Fig. 6). Following treatment, the percentage of CD11c-positive cells decreased in the spleen in mice in early remission, particularly in those mice treated with triple therapy ($3.3 \pm 0.7%$; $p = 0.03$), but was not significantly different from that in nephritic controls in late remission. Mice treated with cytoxan alone did not display this decrease in CD11c$^+$/CD11b$^+$ cells.

**Treatment with combined CTX/costimulatory blockade decreases renal damage and lymphocytic infiltrates**

Kidneys were obtained from mice in remission at various times after treatment with either a single course of double or triple therapy. Light microscopy of kidney sections from control nephritic mice revealed severe glomerulonephritis and interstitial inflammation. The mice also had extensive proteinaceous tubular casts, consistent with their proteinuria. Similar findings were observed in mice treated with CTX alone or with CTLA4Ig/anti-CD154 that did not enter remission (not shown). Nephritic NZB/W F$_1$ mice also had extensive periglomerular and perivascular mononuclear cell infiltrates. Kidney sections from mice in early remission (<6 wk after treatment), whether induced by double or triple therapy, revealed almost complete reversal of renal pathology, with almost complete absence of glomerular proliferation, interstitial infiltrates, and casts. Small pericycical infiltrates were still evident in some of these mice. Mice in late remission (>10 wk after treatment) showed variable reaccumulation of interstitial, perivascular, and pericycical infiltrates, but less glomerular proliferation and less tubular atrophy and casts than untreated controls (Fig. 7, A–D). Scores for renal inflammation, lymphocytic infiltrates, and tubular atrophy are shown in Fig. 8.
To determine whether immune deposits were still present in the kidneys, sections were stained with anti-IgG and anti-C3. No glomerular deposition of immune complexes was observed in 22–25 wk prenephritic mice; however by 32 wk intense staining was present. There was no difference in glomerular deposition of IgG and C3 between nephritic controls and treated mice in either early or late remission (Fig. 7, E–H).

The lymphocytic infiltrates in nephritic mice contained abundant B cells, T cells, and macrophages in a disorganized pattern (Fig. 9, A and B). Lymphocytes were not detected in the interstitium or the perivascular or periglomerular regions of kidneys of mice in early remission, although small collections were present in the pericalyceal areas. These collections contained B and T cells, but had either absent or decreased numbers of CD11c-positive cells compared with controls (Fig. 9, C–E, G, and H). In late remission, CD11c-positive cells were present, but decreased in frequency compared with controls (Fig. 9F).

The expression of chemokines in the kidney was also reduced by therapy. Young NZB/W F1 mice had low chemokine expression not different from that in BALB/c mice (not shown). Nephritic NZB/W F1 mice had an increase in the expression of inflammatory chemokines, which was modulated by treatment; 3–6 wk after combined treatment there was a significant decrease in MCP-1, MIP-2, and TCA-3 expression. In contrast, RANTES, MIP-1α, and MIP-1β were not altered. Particularly noteworthy however, kidneys from mice in remission examined >10 wk after treatment had similar chemokine expression as their nephritic counterparts, with the exception of a continued decrease in TCA-3 expression (Fig. 10A).

Because CD11c-positive cells appeared sensitive to treatment with CTX/costimulatory blockade, we wondered whether their function was also altered. It has recently been reported that CD11c-positive cells with abundant expression of the B cell attracting chemokine CXCL13 accumulate in multiple organs of NZB/W F1 mice. Aberrant expression of CXCL13 is sufficient to induce the formation of ectopic lymphoid tissue in nonlymphoid organs (15) and may be responsible for the accumulation of B cells and activated T cells that express the CXCL13 receptor CXCR5 (16). We therefore stained the kidneys described above from control and treated mice for CXCL13. Kidneys from nephritic mice contained many large cells within the lymphoid aggregates that stained strongly for cytoplasmic CXCL13. As previously reported,
dritic cells from NZB/W F1 mice can be induced to express CXCL13 upon exposure to inflammatory cytokines, including TNF-α and IL-1, but not IFN-α. Furthermore, CXCL13 expression is normally regulated by lymphotoxin-α1β2. Since these cytokines can be expressed in inflamed tissue, we were interested in whether the kidneys of mice in remission expressed these cytokines. Young mice expressed low levels of inflammatory cytokines, and these increased in older nephritic mice. The levels of most cytokines tested decreased during early remission, but returned to pretreatment levels by 10 wk after therapy (Fig. 10B). Thus, the absence of CXCL13 expression by cells in the kidneys of mice in late remission does not appear to be due to an absence of expression of cytokines known to regulate CXCL13 expression, such as TNF-α or lymphotoxin-α1β2.

Discussion

Numerous studies in NZB/W F1 mice and other mouse models of SLE have dealt with treatments that prevent or postpone disease, but few have described means of remission induction, which more closely corresponds to the treatment of human disease. In both NZB/W F1 mice and MRL/lpr mice some investigators have been able to show that CTX given at intervals in high dose can stabilize established disease (18, 19). This finding together with the demonstration that CTX therapy decreases the frequency of autoreactive B cells for a period of only 1–4 wk even in prenephritic mice pointed the way to current protocols for treatment of human SLE nephritis with glucocorticoids and monthly CTX (20). However, CTX therapy is limited by its serious and long-term toxicity, and as many as 50% of patients with nephritis relapse in the 2 years following cessation of therapy (1).

It may be possible to take advantage of the inhibition of autoreactive B cell expansion afforded by treatment with CTX while minimizing the exposure to its toxic effects by the simultaneous use of agents that block costimulation. Daikh and Wofsy (11) have demonstrated that several different multidose regimens of CTX in association with CD28 blockade using CTLA4Ig induced disease remission in nephritic NZB/W F1 mice, whereas no resolution of proteinuria occurred with either agent alone. In this study we found that a single CTX dose of 20 mg/kg (equivalent to a dose in humans of 750 mg/m²) in association with a 2-wk course of either CTLA4Ig (double therapy) or CTLA4Ig and anti-CD154 (triple therapy) induced a high rate of disease remission in NZB/W F1 mice with established nephritis, whereas CTX alone, CTLA4 alone

FIGURE 9. Immunohistochemistry of kidneys from control and treated mice. Results are representative of four mice in each group. A and B, Periglomerular infiltrate from a control mouse double stained with PE-anti-B220 and FITC-anti-CD11c (A) or FITC-anti-CD4 (B). All three cell types are present in a disorganized structure. C–E, Staining of perivascular infiltrates from a control mouse (C) and two early remission mice 6 wk after treatment (D and E). The mouse in D has no CD11c-positive cells in the infiltrate. F, Perivascular infiltrate of a mouse in late remission stained with PE-anti-B220 and FITC-anti-CD11c shows reaccumulation of CD11c-positive cells. G and H, Double staining of sections from the same mice as those shown in D and E with PE-anti-B220 and FITC-anti-CD4. Many B and T cells are present in the lymphoid aggregates. Magnification, ×100.

endothelium also stained strongly for CXCL13 (17). In contrast, we were unable to detect any CXCL13 staining in mice in either early or late remission on endothelial cells or within the lymphoid aggregates that were still present in the pericycual regions, even when CD11c-positive cells were present (Fig. 6, I and J).

It has previously been demonstrated that peripheral blood dendritic cells from NZB/W F1 mice can be induced to express CXCL13 upon exposure to inflammatory cytokines, including TNF-α and IL-1, but not IFN-α. Furthermore, CXCL13 expression is normally regulated by lymphotoxin-α1β2. Since these cytokines can be expressed in inflamed tissue, we were interested in whether the kidneys of mice in remission expressed these cytokines. Young mice expressed low levels of inflammatory cytokines, and these increased in older nephritic mice. The levels of most cytokines tested decreased during early remission, but returned to pretreatment levels by 10 wk after therapy (Fig. 10B). Thus, the absence of CXCL13 expression by cells in the kidneys of mice in late remission does not appear to be due to an absence of expression of cytokines known to regulate CXCL13 expression, such as TNF-α or lymphotoxin-α1β2.

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FIGURE 10. A, An RNase protection assay was performed for an inflammatory chemokine set on kidney RNA from five mice at 34–38 wk (nephritic mice) and mice that were still in complete remission (proteinuria <30 mg/dl) either 3–6 wk (five mice) or >10 wk (four mice) after triple therapy. These were compared with kidneys from three young NZB/W F1 mice at 12–14 wk of age. Young mice had significantly less expression of all six chemokines than aged controls. Mice in early remission had significantly lower expression of MCP-1 (p < 0.02), TCA-3 (p < 0.01), and MIP-2 (p < 0.03) compared with aged controls. With the exception of TCA-3 (CCL1) (p < 0.01), there was no decrease in the inflammatory chemokines in the late remission group. B, The same assay was performed using an inflammatory cytokine set. Young mice had significantly lower expression of all cytokines, except for macrophage migration inhibitory factor, than aged untreated controls (p < 0.05). Mice in early remission had significantly lower expression of TNF-α (p < 0.02), IFN-β (p < 0.02), IFN-γ (p < 0.02), and TGF-β3 (p < 0.01) compared with aged untreated controls, but there was no significant difference between mice in late remission and aged controls.
(11), or the combination of CTLA4 and anti-CD154 did not have the same efficacy. Furthermore, treatment with triple therapy induced a second remission in mice whose nephritis relapsed after the initial induced remission. Treated mice had a markedly increased life span compared with untreated mice or mice treated with CTX alone or in combination CTLA4lg/anti-CD154.

Renal disease in SLE is attributed to inflammation induced by the glomerular deposition of autoantibodies, and in the NZB/W F1 mouse nephritis is usually associated with elevated serum levels of Abs to dsDNA (1). By 28 wk of age nearly all NZB/W F1 mice have developed high serum titers of anti-dsDNA Abs that continue to increase until the onset of nephritis, at which time they fall as a result of either tissue deposition or elimination in the urine (21). In mice treated with costimulatory blockade as preventive therapy, the emergence of anti-dsDNA Abs is delayed, and no immune complexes deposit in the kidneys (7). In this remission induction study we found a decrease in the frequency of autoreactive B cells in the spleens of mice treated with CTX, both with and without costimulatory blockade in the first month after therapy, compared with age-matched untreated mice. However, a decrease in the frequency of splenic autoreactive B cells beyond that observed with CTX alone occurred only in the mice given triple therapy. Despite the observed effects on splenic autoreactive B cells, anti-dsDNA Abs did not decrease in most of the triply treated mice, suggesting that the autoantibodies present in serum of mice in early remission derive from cells outside the spleen, most likely from mature plasma cells in bone marrow that are costimulation independent (22) and evidently invulnerable to CTX. By 5–6 wk following combination treatment, autoreactive B cells had reaccumulated in the spleens. At this time the frequency of anti-dsDNA-binding B cells detected by ELISPOT analysis was no different from that in untreated controls, and the recovery rate of autoreactive hybridomas was no different from that in mice treated with CTX alone that did not respond to treatment.

Suppression of autoreactive B cells is evidently not the only effect of CTX in combination with costimulatory blockade. Mice in early remission induced by either double or triple therapy showed a striking reduction in the frequency of activated T cells to a level similar to that of young NZB/W F1 mice. This was not observed in mice treated with CTX alone or in our previous study of mice that were treated with CTLA4lg and anti-CD154 prevention therapy and retreated at the time of onset of proteinuria (10) and examined 6 wk later. By 7 wk after combination treatment the phenotype of splenic T cells had reverted to that of untreated controls, and even in mice that remained in clinical remission. These findings show that activation of splenic B and T lymphocytes is not sufficient to induce the immediate return of renal disease. Treatment was also associated with a temporary decrease in the frequency of dendritic cells in the spleens, particularly those bearing both CD11c and CD11b markers.

Histologic examination of the kidneys of mice in both early and late remission revealed that Ig and complement deposition were present in the kidney in amounts similar to those found in untreated mice. However, inflammation, tissue damage, and periglomerular and perivascular lymphocytic infiltrates were markedly reduced in mice in remission compared with untreated controls. The finding of immune complex deposition that does not lead to renal damage has been reported in SLE-prone mice deficient in FcR γ-chain (23) or in MCP-1 (24) and in mice treated with caspase inhibitors, total lymphoid irradiation, or very high doses of anti-CD154 Abs (25). These findings are consistent with a disease model in which immune complex deposition in the kidney triggers a cascade of inflammatory events mediated by engagement of FcRs on renal cells or circulating monocytes, followed by up-regulation of renal and monocyte-derived inflammatory chemokines; transmigration of inflammatory cells into the renal parenchyma; release of damaged tissue, inflammatory cytokines, and mediators that amplify the process; and, finally, irreversible renal damage.

Chemokines of renal cell origin appear to be responsible for the early influx of inflammatory cells into the kidneys of lupus-prone mice, since they appear several weeks earlier than lymphoid cell-derived inflammatory cytokines (26, 27). We found decreased levels of several of these inflammatory chemokines in mice in early remission. Consistent with this finding, on immunohistologic study treated mice were found to have few lymphocytes, confined to pericyelial areas, in contrast to the many B cells, T cells, and CD11c-positive cells in the interstitium and periglomerular areas of nephritic mice. However, with the exception of TCA-3 (CCL1), chemokine expression in mice remaining in remission 10 wk after treatment was similar to that in nephritic mice, indicating that regulation of these chemokines was not responsible for long-term maintenance of disease remission. The kidneys of nephritic NZB/W F1 mice contain large numbers of CD11c+ myeloid dendritic cells that express high levels of CXCL13, a potent attractant of activated B and T cells (16). Ectopic expression of CXCL13 induces the development of lymphoid structures outside lymph nodes via a pathway that is dependent on lymphotoxin-α1β2, a predominantly B cell-derived cytokine (28). Abnormal CXCL13 expression has been observed in autoimmune diseases in which lymphoid neogenesis occurs in target organs (29, 30), and once present, CXCL13 can function to up-regulate lymphotoxin-α1β2 expression in an amplification loop. The small inflammatory infiltrates in the kidneys of mice in early remission contained few CD11c+ myeloid dendritic cells. CD11c+ cells were found in late remission, although they were still not as abundant as those observed in untreated controls. However, we found no CXCL13 in the kidneys of treated mice in remission, even at late stages when lymphoid aggregates containing CD11c+ cells were accumulating. CXCL13 expression and function are influenced by several molecules, including Ox-40, lymphotoxin-α1β2, and lymphotoxin-α1 (28, 31). Blockade of the lymphotoxin-β receptor has been shown to cause dissolution of lymphoid structures and beneficial clinical effects in the NOD mouse in which extensive lymphocytic infiltration of the pancreas causes irreversible damage (32). In addition, it has recently been demonstrated that Ox-40 ligand and TNF receptors are overexpressed in the kidneys of patients with proliferative nephritis (33). Circulating CD11c+ dendritic cells do not express CXCL13, but they can be induced to do so upon activation (34, 35). The absence of CXCL13 in the CD11c+ cells in kidneys of mice in remission might therefore be due to the lack of sufficient activation signals either from the kidney environment or intrinsic to the dendritic cells themselves. To begin to approach this question we performed an RNAse protection assay on kidney RNA for a set of inflammatory cytokines, including TNF-α and lymphotoxin-β. It has previously been shown that circulating myeloid dendritic cells in the peripheral blood of NZB/W F1 mice respond to inflammatory chemokines and can be induced to up-regulate CXCL13 by TNF-α and IL-1, but not by IFN-α (35). We found that in late remission the expression of all cytokines examined, including TNF-α and lymphotoxin-β, was no different from that in untreated nephritic controls, suggesting that the absence of CXCL13 expression by CD11c-positive cells in the kidney may be due to an intrinsic alteration of the responses of these cells to inflammatory stimuli. We further propose that in the absence of CXCL13 expression in the kidneys of treated mice, the size of the T and B cell infiltrates may be limited, resulting in decreased release of inflammatory cytokines and less renal damage.
The mechanism by which costimulatory blockade might affect chemokine expression, particularly by dendritic cells, is currently not known, but other studies provide supporting evidence that costimulatory molecules play a role in inflammation and cell trafficking apart from their known function in T and B cell activation (36–43). The single dose of CTX added to the regimen of costimulatory blockade is essential to the remission process. CTX appears to be responsible for the initial elimination of activated cells in the spleen. Additional experiments will be necessary to determine whether CTX kills activated cells present in the kidneys in addition to its effects on the spleen or whether it potentiates the effects of costimulatory blockade on chemokine production or the functional status of dendritic cells.

The results of these studies may have clinical implications. First, the findings presented here suggest that in established disease, the combination of CTX and costimulatory blockade may induce a temporary reversion of the activated splenic B and T cell phenotype to a more naive phenotype, probably by a mechanism of preferential deletion of activated or dividing cells. This could render the disease process susceptible to further modulation with less toxic regimens, such as CTLA4Ig alone, that are effective in disease prevention. Second, our findings represent further confirmation that high titers of serum anti-dsDNA Abs and renal deposition of immune complexes can be dissociated from subsequent renal damage. Third, our study suggests that established anti-dsDNA-secreting cells are relatively resistant to both costimulatory blockade and the dose of CTX used in our study. These cells are presumably plasma cells, since initial information from open-label clinical trials has shown that a substantial population of anti-dsDNA-secreting cells may be resistant to both costimulatory blockade with anti-CD154 (44) and anti-CD20 treatment in humans (45). Our previous studies in the NZB/W F1 model have shown that autoantibody-producing B cells are susceptible to costimulatory blockade earlier in disease before nephritis occurs (21). Thus, serologic remissions resulting in delay of nephritis onset can be achieved with less aggressive therapy early in disease. Once nephritis has developed, a variety of therapies designed to interrupt the tissue-damaging inflammatory cascade that follows immune complex deposition may be useful adjuncts to immunosuppression. The availability of this model of remission induction that high titers of serum antibody reveals an antibody-independent role for B cells in murine lupus. J. Exp. Med. 189:1639.


