Anti-CD40 Ligand Antibody Treatment of SNF\textsubscript{1} Mice with Established Nephritis: Preservation of Kidney Function

Susan L. Kalled, Anne H. Cutler, Syamal K. Datta and David W. Thomas

*J Immunol* 1998; 160:2158-2165;
http://www.jimmunol.org/content/160/5/2158

---

**References**  This article cites 46 articles, 29 of which you can access for free at: http://www.jimmunol.org/content/160/5/2158.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Anti-CD40 Ligand Antibody Treatment of SNF<sub>1</sub> Mice with Established Nephritis: Preservation of Kidney Function<sup>1</sup>

Susan L. Kalled,* Anne H. Cutler,* Syamal K. Datta,† and David W. Thomas*<sup>1</sup>

Prior studies have demonstrated that treatment of young, prenephritic lupus-prone mice with Ab directed against CD40 ligand (CD40L) prolongs survival and decreases the incidence of severe nephritis. In this report, we show that for (SWR × NZB)<sub>F<sub>1</sub></sub> (SNF<sub>1</sub>) animals with established lupus nephritis, long-term treatment with anti-CD40L beginning at either 5.5 or 7 mo of age prolonged survival and decreased the incidence of severe nephritis. “Older” mice were chosen for these studies to more closely resemble the clinical presentation of patients with established renal disease. We show that age at the start of treatment, which typically correlates with severity of disease, is an important factor when determining an efficacious therapeutic protocol since animals that began treatment at 7 mo of age required a more aggressive treatment protocol than animals at 5.5 mo of age. Remarkably, several anti-CD40L-treated animals beginning treatment at age 5.5 mo demonstrated a decline in proteinuria, as opposed to increasing proteinuria levels seen in hamster IgG (HIg)-treated controls, and histologic examination of kidneys from anti-CD40L-treated mice revealed dramatically diminished inflammation, sclerosis/fibrosis, and vasculitis, in marked contrast to the massive inflammation and kidney destruction observed in control animals that received hamster IgG. Spleens from anti-CD40L-treated mice also exhibited markedly reduced inflammation and fibrosis compared with controls. Together, these results show that treatment of older, nephritic SNF<sub>1</sub> animals with long-term anti-CD40L immunotherapy significantly prolongs survival, reduces the severity of nephritis, and diminishes associated inflammation, vasculitis, and fibrosis. The Journal of Immunology, 1998, 160: 2158–2165.

Systemic lupus erythematosus (SLE)<sup>3</sup> is a spontaneously arising autoimmune disease with a female predominance and is characterized by the production of a variety of pathogenic anti-nuclear autoantibodies (1). In lupus nephritis, kidney damage is mediated by both cellular and humoral immune mechanisms, including the formation of immune complexes that deposit in kidney glomeruli and activate the complement cascade resulting in glomerulonephritis. It has previously been established that the production of anti-nuclear autoantibodies in both human and mouse SLE is driven by cognate interactions between select populations of autoimmune Th cells and B cells (2–4). Autoantibody-inducing Th cells have been cloned from (SWR × NZB)<sub>F<sub>1</sub></sub> (SNF<sub>1</sub>) mice with lupus nephritis as well as from nephritic patients with SLE (5–9), and such clones from the SNF<sub>1</sub> model rapidly induce immune-deposit glomerulonephritis when transferred into young preautoimmune mice. In the absence of these Th cells, the autoantibody-producing B cells are not sustained and presumably undergo apoptosis.

Critical to the production of Ab against T-dependent Ags is the interaction between CD40L on Th cells and its receptor, CD40, on the cognate B cell. This interaction is essential for germinal center formation, B cell proliferation and differentiation, isotype switching, and generation of B cell memory (reviewed in 10). CD40-CD40L interaction is also important for T cell activation since T cells require costimulatory signals through molecules that are up-regulated upon CD40-CD40L engagement. CD40-CD40L interaction has been shown to be important for several experimentally induced autoimmune diseases, such as collagen-induced arthritis (11), experimental allergic encephalomyelitis (EAE) (12), oophoritis (13), as well as graft-vs-host disease (14, 15), since induction of all of these diseases can be blocked with anti-CD40L treatment at the time of Ag administration. In addition, CD40-CD40L interaction appears to be critical for the production of pathogenic autoantibodies in spontaneous murine lupus. Blocking this interaction, even briefly (for 1 wk) in young, prenephritic SNF<sub>1</sub> lupus animals with anti-CD40L therapy produced unexpected long-term benefit, such as increased survival and diminished incidence of severe nephritis at 12 mo of age (16). Similar results were seen in the NZB/NZW lupus model with long-term anti-CD40L therapy (6 mo) (17).

Given the results from these numerous studies, there has been much speculation as to the potential usefulness of a mAb directed against the human CD40L molecule in treatment of autoimmune disease. What is lacking is data that show efficacy of anti-CD40L Ab in established renal disease, both moderate and severe. With this in mind, we designed studies using SNF<sub>1</sub> mice that would resemble the stage of disease with which patients with established lupus nephritis could present for initial diagnosis and treatment. In this study, we report the effects of anti-CD40L therapy on animals that began treatment at 5.5 or 7 mo of age, receiving an initial regimen of anti-CD40L Ab for several weeks, followed by monthly dosing for the duration of the study. Anti-CD40L immunotherapy resulted in prolonged survival, decreased autoantibody levels, and diminished proteinuria, indicating an arrest of established disease. Anti-CD40L-treated mice also exhibited reduced renal inflammation, cellular proliferation, vasculitis, and sclerosis/fibrosis, as well as diminished inflammation and fibrosis in the

*Department of Immunology, Biogen Inc., Cambridge, MA 02142; †Department of Medicine, Microbiology-Immunology, and Multipurpose Arthritis Center, Northwestern University Medical School, Chicago, IL 60611

Received for publication July 29, 1997. Accepted for publication November 7, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 S.K.D. is supported by grants from the National Institutes of Health (RO1-AI41985 and AR39157) and The Arthritis Foundation.

2 Address correspondence and reprint requests to Dr. Susan L. Kalled, Department of Immunology, Biogen Inc., 14 Cambridge Center, Cambridge, MA 02142.

3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; CD40L, CD40 ligand; SNF<sub>1</sub>, (SWR × NZB)<sub>F<sub>1</sub></sub>; HIg, hamster IgG; H&E, hematoxylin-eosin.
spleen. Lastly, in contrast to 5.5 month-old mice, 7 month-old animals beginning treatment for the first time required more frequent dosing of anti-CD40L in the first 12 wk to establish efficacy, most likely because of components of advanced disease that are not currently understood.

Materials and Methods

Mice

SWR and NZB mice were purchased from The Jackson Laboratory (Bar Harbor, ME). (SWR × NZB)F1 (SNF1) hybrids were bred in the animal facility at Biogen under conventional barrier conditions. Female SNF1 mice were used for all studies.

Antibodies

The MR1 hybridoma (18), which produces Armenian hamster anti-mouse CD40L Ab, was purchased from the American Type Culture Collection (Rockville, MD). The hybridoma HA4/8-3.1, an Armenian hamster IgG mAb specific for keyhole limpet hemocyanin, was kindly provided by Dr. Donna Mendrick (Human Genome Sciences Inc., Rockville, MD). Both mAbs were purified from culture supernatant on a protein A Fast Flow column (Pharmacia Biotech, Piscataway, NJ).

Treatment protocols

All injections were given i.p. Each study consisted of a control group that received HA4/8-3.1 and a treated group that received anti-CD40L mAb. Animals received in the first week 250 μg of Ab on days 1, 3, and 5, then a single dose of 500 μg of mAb once per wk for either 6 or 12 wk as indicated in the text, followed by a single injection of 500 μg monthly until death of the animal or termination of the study. Studies began when animals were either 5.5 mo or 7 mo of age.

ELISA assays

For total Ig and anti-CD40L ELISAs, ELISA plates (Corning Glass Works, Corning, NY) were coated overnight at 4°C with 5 μg/ml of goat anti-mouse IgG+IgM (Jackson ImmunoResearch, West Grove, PA) and anti-CD40L, respectively. After blocking, serial serum dilutions were added, followed by the detection Ab, biotin-conjugated donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch), and streptavidin-horseradish peroxidase (SA-HRP) reagent (Southern Biotech, Birmingham, AL). The developing reaction was stopped by adding 2N sulfuric acid. Plates were read at an OD of 450 nm, and a standard curve was generated using known quantities of purified whole mouse Ig (Jackson ImmunoResearch). Anti-ssDNA and anti-dsDNA ELISAs were performed using NUNC-Immuno Plate MaxiSorp plates (NUNC A/S, Denmark). Plates were coated overnight at 4°C first with 100 μg/ml methylated BSA (Calbiochem Corp., La Jolla, CA), then with 50 μg/ml grade I calf thymus DNA (Sigma, St. Louis, MO). The calf thymus DNA was sheared by sonication and then digested with S1 nuclease before use. For the anti-ssDNA assay, the DNA was boiled for 10 min and chilled on ice before use. After blocking, serial dilutions of serum samples were added and incubated at room temperature for 2 h. Autoantibodies were detected with goat anti-mouse IgG-AP (Sigma) and developed with 4-nitrophenyl phosphate (Sigma) in 1 M diethanolamine buffer. Plates were read at an OD of 405 nm, and standard curves were obtained by using known quantities of anti-DNA mAb 205, which is specific for both ss- and dsDNA (2).

Assessment of renal disease

The urine of each mouse was monitored weekly with Albustix (Bayer Corp., Terrytown, NY) to measure proteinuria. Proteinuria level is scored as follows: 0.5, 15 to 30 mg/dl; 1, 30 mg/dl; 2, 100 mg/dl; 3, 300 mg/dl; 4, >2000 mg/dl.

The overall score for histopathologic grading of lupus nephritis is described elsewhere (19, 20) and was based on glomerular, interstitial, and tubular changes. The grades 0 to 4+ are based on percent involvement of the structure being examined (i.e., glomeruli, vessels, etc.). Kidneys without lesions were graded as “0,” and all tissue samples were coded and read blind.

Immunohistochemistry

Kidneys and spleens were fixed in 10% buffered formalin and embedded in paraffin. Five-millimeter cryostat sections were baked at 55°C, deparaffinized, hydrated in ethanol, and stained with hematoxylin-eosin (H&E) for histologic examination or used for immunohistochemical staining. Briefly, sections were incubated first with a mAb that detects a cytoplasmic protein specific to reticular fibroblasts, ER-TR7 (S erotec, Oxford, UK) for 30 min at room temperature, washed with PBS, incubated with mouse anti-rat IgG (H&L) F(ab)2, for 30 min at room temperature (Jackson ImmunoResearch, West Grove, PA), and visualized using the substrate 3,3’-diaminobenzidine (DAB) (Vector Laboratories, Inc., Burlingame, CA). Sections were counterstained with a 25% Wright-Giemsa (Fisher Diagnostic s, Pittsburgh, PA) solution. Endogenous peroxidase activity was blocked using 2% hydrogen peroxide in methanol for 20 min before staining with the primary Ab. Photographs were taken on a Zeiss Axioplan photomicroscope at magnifications of ×100 and ×400.

Statistical analysis

Survival curves were estimated by life-table methodology, and groups were compared by the Wilcoxon test (21). The proportion of mice with ≥3+ (≥300 mg/dl) proteinuria was analyzed by a χ2 test. Histopathologic renal scores were analyzed by a Wilcoxon two-sample test. Comparison of autoantibody levels was analyzed by Student’s t test.

Results

Long-term anti-CD40L therapy beginning at 5.5 mo of age significantly prolongs survival of SNF1 mice

Seven SNF1 mice, beginning at 5.5 mo of age, were treated in the first week with 250 μg of either anti-CD40L mAb or control hamster IgG on days 1, 3, and 5 followed by a weekly injection of 500 μg for 5 consecutive wk, then monthly injections of 500 μg until death of the animal or termination of the study. By 10 mo of age (4.5 mo after the start of treatment), 6 of 7 (85.7%) control animals had died, whereas no anti-CD40L-treated animals had died (Fig. 1A). By 13 mo of age (7.5 mo after start of treatment) no control animals remained alive, yet all anti-CD40L-treated animals were alive. In fact, these animals appeared healthy up to 15.5 mo of age when the study was terminated and all animals, except one, were euthanized for histopathology (one mouse died during a kidney biopsy at ~13 mo of age and was not included in the survival timepoints of Figure 1A or statistics beyond 13 mo). As Hlg-treated controls became moribund, the animals were euthanized and their organs removed for histology. Overall, anti-CD40L-treated mice demonstrated a survival rate significantly different (p < 0.001) from Hlg-treated controls.

Long-term anti-CD40L therapy beginning at age 5.5 mo significantly inhibits development of severe nephritis, renal vasculitis, and fibrosis

Consistent with the prolonged survival effect of anti-CD40L therapy described above, this treatment also significantly inhibited the development of severe nephritis, defined as a proteinuria level of ≥3+ (p < 0.001 at all timepoints). As seen in Figure 1B, only 1 of 7 anti-CD40L-treated animals developed ≥3+ proteinuria by 13 mo of age whereas controls rapidly developed 4+ proteinuria within 1 mo after treatment began (although 2 animals had 4+ proteinuria when the study began due to a random assignment of groups). Remarkably, the proteinuria levels of 6 of 7 (85.7%) anti-CD40L-treated mice declined. This decline began as early as 3 mo after the start of treatment in some cases and as late as 6 mo in others.

Since anti-CD40L immunotherapy resulted in a decline in proteinuria, we asked if the severity of glomerulonephritis was also reduced as compared with controls. For this purpose, H&E-stained kidney tissue sections were read and scored blind to assess renal morphology and pathology. An example of normal kidney structure is seen in Figure 2a, a kidney section from an SWR female mouse, the normal parent of the (SWR × NZB) cross. Glomeruli are numerous, distinct with patent capillaries, normal cellularity, and architecture, and tubules are compact and of normal shape. In comparison, kidney sections from Hlg-treated SNF1 mice (Fig. 2b)
exhibited severe disruption of kidney architecture, lesions involving all glomeruli, massive perivascular lymphoid accumulations, and tubular atrophy or dilation with proteinaceous casts. The glomeruli in these animals were enlarged and exhibited hypercellularity with crescents, hyaline deposits effacing capillary loops, thickening of capillary loops, basement membrane as well as mesangial thickening, and significant glomerular sclerosis. In stark contrast to the Hlg-treated animals, tissue sections from anti-CD40L-treated animals (Fig. 2c) revealed that, in general, the overall structural integrity of the kidneys was intact. Anti-CD40L-treated animals at age 15.5 mo had no to moderate (0 to 2+) glomerulonephritis, except one mouse that developed 2 to 3+ disease, and only 3 animals exhibited rare sclerotic glomeruli. Mouse CLR, which died at ~13 mo of age due to complications from a kidney biopsy, exhibited severe disruption of kidney architecture, lesions involving all glomeruli, massive perivascular lymphoid accumulations, and tubular atrophy or dilation with proteinaceous casts. The glomeruli in these animals were enlarged and exhibited hypercellularity with crescents, hyaline deposits effacing capillary loops, thickening of capillary loops, basement membrane as well as mesangial thickening, and significant glomerular sclerosis. In stark contrast to the Hlg-treated animals, tissue sections from anti-CD40L-treated animals (Fig. 2c) revealed that, in general, the overall structural integrity of the kidneys was intact. Anti-CD40L-treated animals at age 15.5 mo had no to moderate (0 to 2+) glomerulonephritis, except one mouse that developed 2 to 3+ disease, and only 3 animals exhibited rare sclerotic glomeruli. Mouse CLR, which died at ~13 mo of age due to complications from a kidney biopsy, had no obvious sign of glomerulonephritis in the biopsied tissue (Table I). Furthermore, most animals had no or only mild interstitial infiltration of mononuclear cells, although 2 of 7 animals had moderate infiltration. A comparison of the overall renal histopathologic scores for anti-CD40L-treated mice and the controls shows a significant difference in severity of glomerulonephritis by the Wilcoxon two-sample test (p < 0.01).

Consequences of interstitial infiltration include the activation of a variety of cell types and release of cytokines/growth factors resulting in fibrosis (22, 23), an overproduction of extracellular matrix components, and proliferation of normally quiescent cells, such as fibroblasts, which can lead to irreversible tissue damage. The presence of sclerosis/fibrosis was assessed by histologic examination of H&E-stained kidney sections (Table I), and comparison of the difference in incidence and severity of sclerosis/fibrosis between anti-CD40L-treated and control mice was found to be significant (p < 0.01). To further examine the effect of anti-CD40L therapy on the development of fibrosis, kidney tissue sections were analyzed with the mAb ER-TR7. This mAb recognizes reticular fibroblasts and stains the connective tissue of many organs. (Note: this Ab stains normal connective tissue strongly on frozen tissue sections. The tissue sections used in this report have been embedded in paraffin, resulting in a barely detectable staining, except in instances of damaged and fibrotic tissue.) In a kidney tissue section of the normal parent, SWR, no staining is detected in the kidney cortex (Fig. 2d). In Hlg-treated animals, however, this Ab stained intensely the areas surrounding dilated tubules, interstitial infiltrate, and sclerotic glomeruli (Fig. 2e). Tissue sections of kidneys from anti-CD40L-treated animals exhibited no ER-TR7 staining above what was seen for controls (Fig. 2f). Additionally, examination of H&E-stained kidney sections revealed that, compared with Hlg-treated mice that developed severe vasculitis, the severity of vasculitis in anti-CD40L-treated animals was significantly diminished (p < 0.01; Table I).

Long-term anti-CD40L therapy beginning at age 5.5 mo reduces splenic inflammation and inhibits development of fibrosis

Since animals in this lupus model develop splenomegaly, splenic tissue sections were examined to determine whether anti-CD40L therapy had any effect on inflammation/proliferation in the spleen, where hyperproliferation of autoantibody-producing B cells occurs. Normal splenic architecture can be seen in an H&E-stained
tissue section of the normal parent, SWR, (Fig. 3a), where areas of red pulp and lymphocyte-containing white pulp are clearly discernible. The spleens of Hlg-treated SNF1 mice, however, often had such severe hyperplasia, accompanied by hyaline degeneration of the central follicular arterioles, that the typical H&E staining pattern distinguishing red and white pulp was completely disrupted and obscured, and there appeared to be a loss of white pulp altogether (Fig. 3b). There was also evidence of splenic necrosis in some animals. Splenic tissue sections from animals receiving anti-CD40L therapy revealed a marked expansion of the white pulp due to an increased number of follicles and expansion of what appeared to be dendritic-like cells. Nevertheless, no areas of necrosis were obvious, and inflammation and lymphoid proliferation were markedly reduced. In addition, anti-CD40L-treated mice exhibited...
only rare, mild incidences of hyaline degeneration of central arterioles (Fig. 3c).

Splenomegaly is also characterized by fibrosis. To examine the extent of fibrosis in anti-CD40L-treated and control mice, splenic tissue sections were analyzed with the mAb, ER-TR7. In normal spleen this Ab stained very faintly within the red pulp only, the area containing splenic connective tissue (Fig. 3d). In the spleens of Hlg-treated mice with active lupus, however, there was extensive staining with ER-TR7 not only in the red pulp, but extending into the white pulp as well, which appeared characteristic of periarterial fibrosis (Fig. 3e). In contrast, in sections from anti-CD40L-treated mice, there was typical faint staining in the red pulp, as seen in normal mice, and rare occasions of staining extending into the white pulp, thus indicating either no or only mild fibrosis in these animals (Fig. 3f).

**SNF1 lupus-prone mice beginning immunotherapy at 7 mo of age need a more aggressive anti-CD40L treatment protocol than 5.5 month-old animals**

Since long-term immunotherapy with anti-CD40L proved so successful with animals that began treatment at 5.5 mo of age, this identical treatment regimen was repeated with older animals, 7 mo old, that typically have higher proteinuria levels as well as autoantibody titers. This treatment protocol, however, did not have any beneficial effect in older mice; anti-CD40L-treated animals developed severe nephritis and died at the same rate as controls (data not shown). We reasoned that more frequent anti-CD40L dosing early in therapy might be necessary given that the autoimmune response in these older animals is likely more robust than in younger mice. Therefore, 10 animals were given an extended weekly dosing regimen of 500 μg of either anti-CD40L or Hlg for 12 consecutive wk followed by monthly injections of 500 μg/dose. This aggressive therapy significantly increased the survival rate of anti-CD40L-treated mice compared with controls (p = 0.05 by Wilcoxon test). At 10 mo of age (3 mo after start of therapy), only 20% of controls were alive compared with 80% of treated animals. At 13.5 mo of age the survival rate was 0% and 40% for control and anti-CD40L-treated animals, respectively, and at age 15.5 mo when the study was terminated two anti-CD40L-treated mice remained alive and appeared healthy (Fig. 4A).

The development/persistence of severe nephritis was assessed on a weekly basis by determining proteinuria levels; severe nephritis is defined as a proteinuria grade of ≥3+ (≥300 mg/dl). The effect of anti-CD40L therapy on proteinuria levels in the 7 month-old mice was not as dramatic as that seen in animals that began treatment at age 5.5 mo, probably because 6 of 10 animals in the anti-CD40L-treated group had severe nephritis before the start of treatment. Regardless, when compared with Hlg-treated animals (5 of 10 had severe nephritis before treatment), the proportion of anti-CD40L-treated mice with ≥3+ proteinuria differed significantly (p < 0.001) from controls at each timepoint analyzed except at 7.5, 8.5, and 10 mo (Fig. 4B). Furthermore, two anti-CD40L-treated animals exhibited a decline in proteinuria that remained at the diminished level for several mo (Fig. 4B).

**Long-term anti-CD40L therapy reduces autoantibody production in SNF1 mice**

Autoantibodies, a hallmark of human SLE and the SNF1 lupus model, are seen in increasing amounts as SNF1 female mice age. After reaching a peak level, which in our colony is seen at approximately age 9.5 mo, detectable serum titers drop dramatically, probably due to immune complex deposition in the kidneys and other tissues. Serum levels of anti-ssDNA and anti-dsDNA autoantibodies were determined at regular intervals, and, regardless of whether mice began anti-CD40L treatment at 5.5 or 7 mo of age, anti-CD40L therapy resulted in an overall reduction in the mean value of anti-ssDNA and anti-dsDNA autoantibody detected when compared with Hlg-treated controls (Fig. 5). Most animals that began immunotherapy at age 5.5 mo had detectable autoantibody levels and, whereas the Hlg-treated controls developed increased titers until age 9.5 mo, the mean values for anti-CD40L-treated mice remained low and in some cases declined. These differences were significant at the timepoints indicated in Figure 5 (p < 0.05 at 8.5 mo for both anti-ssDNA and anti-dsDNA). Mice that began treatment at age 7 mo also had detectable autoantibody titers at the start of therapy; however, the mean values of anti-ssDNA and anti-dsDNA autoantibodies for anti-CD40L-treated mice remained low or declined compared with controls, which continued to rise until approximately age 9.5 mo. These values differed significantly at certain timepoints (at 8.5 mo, p = 0.06 for anti-ssDNA and p < 0.05 for anti-dsDNA; at 9.5 mo, p < 0.05 for anti-dsDNA). Statistical analysis was not done for those timepoints where there were fewer than 2 control mice alive.
Although it was expected that anti-CD40L treatment would serve to inhibit Ab responses in general, including anti-anti-CD40L Ab, animals in both studies did, in fact, develop such a response. For those mice that began anti-CD40L treatment at age 5.5 mo, three animals exhibited serum anti-anti-CD40L titers at 9.5 and 11.5 mo, and one animal exhibited titers at 11.5 and 14.5 mo (Table II). The response, however, was not persistent and the sporadic nature did not appear to adversely affect the animals since there was no parallel increase in proteinuria (Fig. 1B), and only 50% of mice had a correlative rise in autoantibody titers (Fig. 5). On the other hand, several animals that did not begin anti-CD40L treatment until age 7 mo developed an anti-anti-CD40L response within 2 mo of therapy, and it was persistent (Table III). Two of eight surviving animals 1.5 mo after the start of therapy (age 8.5 mo) had developed an anti-anti-CD40L response, and this number increased to four of eight surviving mice at age 9.5 mo. For mice beginning immunotherapy at age 7 mo, there was a strong correlation between the animal having severe nephritis at the start of therapy, development of an anti-anti-CD40L response, and a lack of long-term survival.

Discussion
This work provides the first evidence that inhibiting the CD40-CD40L costimulatory pathway with an anti-CD40L mAb has a

Table II. Anti-anti-CD40L response in mice that began treatment at age 5.5 months

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Age in Months</th>
<th>8.5</th>
<th>9.5</th>
<th>10.5</th>
<th>11.5</th>
<th>12.5</th>
<th>13.5</th>
<th>14.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a “−” indicates no detectable response; “+” indicates a detectable response.

Table III. Anti-anti-CD40L response in mice that began treatment at age 7 months

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Age in Months</th>
<th>8.5</th>
<th>9.5</th>
<th>11.5</th>
<th>12.5</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>XIDR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIDN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIDLR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIER</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIEL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIELR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIFR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIFN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a “−” indicates no detectable response; “+” indicates a detectable response.

*ND, not done since mouse died due to a biopsy complication at ~13 mo of age.

*a “−” indicates no detectable response; “+” indicates a detectable response.

*b “O” indicates the mouse died before this timepoint.
beneficial therapeutic effect in animals with established lupus nephritis. The impact of long-term anti-CD40L therapy on nephritic 5.5-month-old SNF1 mice with proteinuria was a stabilization followed by a decline in proteinuria in six of seven animals, a significantly increased survival rate at age 15.5 mo, and decline in the incidence of severe glomerulonephritis, vasculitis, and fibrosis. These results show that anti-CD40L can function to arrest established renal disease and are striking, given that in this model SNF1 females die of severe nephritis usually by age 12 mo (16, 24).

The hallmarks of interfering with the CD40-CD40L pathway, inhibiting Ig isotype switch and Ab production, have been demonstrated many times in various disease models, primarily, however, in a fashion that inhibited the onset of a humoral immune response. In general most SNF1 animals, without therapeutic intervention, developed significant autoantibody titers as they aged, reaching a peak before disappearance from the periphery due to immune complex deposition in the kidneys and other tissues. Animals that received anti-CD40L immunotherapy, however, maintained consistently low autoantibody titers when compared with controls, often falling below baseline, which was determined just before the start of therapy. Nevertheless, an anti-anti-CD40L response in animals that began treatment at age 7 mo developed rather quickly and was persistent, unlike what was observed in mice that received treatment earlier, at age 5.5 mo, where such a response was sporadic among a few mice and apparently without consequence to disease progression. It is possible that with advanced disease select B cells no longer require a costimulatory signal through CD40, or they may function in a T-independent manner (25), making inhibition of the CD40-CD40L pathway inconsequential. Indeed, CD40L knockout/pr mice can produce some autoantibodies and develop a markedly delayed and mild form of lupus (26).

Additionally, for mice not receiving anti-CD40L therapy until age 7 mo, increased dosing was necessary to establish efficacy. It was clear that animals with ≥ 3+ nephritis just before dosing did not benefit from treatment, most likely because of pre-existing kidney damage that was too severe and irreversible. Possible explanations for the need of a more aggressive anti-CD40L treatment regimen in 7-month-old animals are an increased level of CD40L expression on Th cells and a greater number of cells expressing CD40L. Studies have documented the disregulated expression of CD40L on Th cells in SNF1 mice (16) as well as in lupus patients (27), whereby autoreactive Th cells of lupus express an abnormally high level of CD40L, including T cells taken directly from patients without further in vitro stimulation. CD40L has also been shown to be expressed on normal human B cells when stimulated in vitro and, surprisingly, B cells from lupus patients have been found to exhibit endogenous hyperexpression of CD40L, reaching the level expressed by activated Th cells (27, 28). (It should be noted that we have examined both freshly isolated and mitogen-stimulated purified B cells from SNF1 mice at various ages and have been unable to convincingly detect CD40L either by flow cytometry or RT-PCR (data not shown)). Interestingly, previous examination of freshly isolated PBMCs from lupus patients has shown that patients with the highest level of CD40L expression had active or end stage renal disease (28). Lastly, with advanced disease, it is possible that non-T, CD40L-bearing cells, as found in humans, such as stimulated NK cells (29), vascular endothelial cells, smooth muscle cells, and macrophages (30) may interact with and activate CD40L cells, resulting in an expanded pool of stimulated lymphoid and non-lymphoid cells.

Together the above data indicate that interrupting CD40-CD40L interaction not only blocks the initiation and maintenance of the pathogenic immune response, particularly the humoral arm of the response, but is also beneficial during the effector phase of disease when CD40-CD40L interaction takes place in a T/non-B cell or non-T/non-B cell setting. Indeed, the presence of CD40 on vascular endothelial cells (31, 32) and on a variety of parenchymal and nonparenchymal cells in the normal human kidney has already been established (33). Interestingly, in patients with lupus nephritis, CD40 expression is markedly increased in the kidney along with the presence of infiltrating CD40L+ mononuclear cells (33). Because CD40-mediated signals can induce secretion of proinflammatory cytokines by monocytes, dendritic cells, and fibroblasts (34–37), it has been suggested that CD40L+ mononuclear cells may interact with CD40+ renal target cells to induce or enhance proinflammatory molecules that contribute to renal inflammation and damage (33). van Kooten et al. (38) have recently demonstrated that cross-linking CD40 on human proximal tubular epithelial cells leads to the production of chemokines IL-8, monocyte chemoattractant protein (MCP)-1, and RANTES, known inflammatory mediators that may contribute to the pathway leading to tissue damage and fibrosis. RANTES may be of particular importance since it is a known chemoattractant for T cells (39), and IL-2 and IFN-γ produced by activated T cells can directly activate human proximal tubular epithelial cells (40–42), thus providing a positive feedback loop for interstitial infiltration. In fact, Lloyd et al. (43) have used a nephrototoxic serum animal model to show that MCP-1 is indeed involved in glomerular crescent formation and interstitial fibrosis and together with RANTES plays a role in the inflammatory phase of crescentic nephritis. The ability of anti-CD40L immunotherapy to inhibit the development of fibrosis is of special significance given that there is a correlation between the degree of interstitial fibrosis and incidence of chronic renal failure in patients with glomerular diseases (44, 45). Current studies are underway to examine CD40, CD40L, and chemokine expression in SNF1 mice treated with anti-CD40L vs controls.

It has been suggested that anti-CD40L may not be effective after establishment of disease, particularly for Th1-mediated autoimmune diseases (46). Our report demonstrates that long-term immunotherapy with an anti-CD40L mAb provides significant therapeutic benefit to nephritic, autoimmune SNF1 female mice, a lupus model in which the nephritogenic autoantibodies are Th1-dependent (47). Cytokines from CD40L-expressing Th2 cells have also been shown to be necessary for survival of autoimmune B cells and disease progression in lupus (48, 49); thus, our data suggest that preventing CD40-CD40L interaction has consequences for both the Th1 and Th2 subpopulations of T cells. Furthermore, we present the longest survival of SNF1 females ever reported, which is extraordinary at age 15.5 mo. At this age these animals continued to appear in good health with no obvious signs of infection or complications, which is particularly remarkable given that they are autoimmune-prone and were housed in a conventional facility. Importantly, there was no effect on total serum Ig levels (data not shown) indicating there was no general immunosuppression. These data suggest that long-term immunotherapy with anti-CD40L in people may not result in significant detrimental consequences, and overall the data lend promise to the potential use of anti-CD40L immunotherapy to treat human SLE and, possibly, other autoimmune diseases as well.

Acknowledgments
We thank Joseph Amatucci for production and purification of the Ha4/8-3.1 mAb, Konrad Miatowski and Janine Ferrant for production and purification, respectively, of the anti-CD40L mAb, Arthur McAllister for statistical analysis, Dr. Fabienne Mackay for useful suggestions and technical advice regarding immunohistochemistry, and Drs. Yen-Ming Hsu and...
References


