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Depletion of Folate-Receptor-Positive Macrophages Leads to Alleviation of Symptoms and Prolonged Survival in Two Murine Models of Systemic Lupus Erythematosus

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Received May 11, 2007; Revised Manuscript Received August 8, 2007; Accepted August 9, 2007

Abstract: Systemic lupus erythematosus (SLE) is an autoimmune disease involving deposition of immune complexes in normal tissues and the consequent accumulation of immune cells and tissue injury. Activated macrophages are thought to contribute to disease pathogenesis by releasing inflammatory mediators that both cause direct tissue damage and attract other immune cells that augment inflammation. Previous studies in animal models of rheumatoid arthritis have shown that activated macrophages express a folate receptor that can be targeted with folate-linked haptens, leading to (1) marking of the activated macrophages with highly immunogenic haptens, (2) recognition of the marked cells by Fc receptor-expressing immune cells, and (3) destruction of the antibody-coated macrophages by the body's own immune system. Here we demonstrate that the same folate–hapten-targeted immunotherapy can greatly suppress symptoms of SLE in two animal models of the disease, resulting in reduced immune complex deposition, diminished damage to normal tissues, and prolonged animal survival.

Keywords: Folate; immunotherapy; macrophage; lupus; SLE; folate receptor

Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disorder characterized by autoantibody production, formation of immune complexes with self-antigens, and deposition of these complexes on normal tissues, resulting in inflammation and eventual damage.[1] Although the stimulus for autoantibody production is still a matter of debate, reduced clearance of apoptotic cells by macrophages has been proposed to constitute a contributing factor.[2] Because a major site of immune complex deposition resides in the kidneys, ~40% of SLE patients eventually succumb to kidney failure or glomerulonephritis.[3] Damage to the heart, liver, muscle, joints, and spleen is also observed to cause morbidity and mortality associated with the disease. Current therapies, including corticosteroids and cyclophosphamide, are aimed at suppressing activated immune cells; however, these treatments are often ineffective, and prolonged use of such drugs can be accompanied by unacceptable toxicities. Clearly, research into less toxic, more potent therapies for SLE is warranted.

Although macrophages were not originally considered a major contributor to SLE, recent studies have demonstrated that macrophages indeed play a prominent role in the pathogenesis of the disease.[1,4–10] Studies by Jin et al., for example, show that patients suffering from SLE have

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10.1021/mp0700615 CCC: $37.00
Published on Web 09/12/2007

increased levels of neopterin and interferon-γ in their serum (i.e., accepted markers of activated macrophage involvement) and levels of these markers correlate positively with disease activity. Data from the same laboratory also suggest that macrophages become activated during development of SLE.[2] Ikezumi et al.[11] have further shown that activated macrophages are abundantly present in biopsies of kidney tissues from patients with SLE, yet they are absent from kidneys of healthy individuals. Moreover, mice with elevated numbers of circulating marrow-derived macrophage progenitor cells spontaneously develop an SLE phenotype,[12] and inhibition of macrophage infiltration into the kidneys with a fractalkine/CX3CL1 antagonist prevents development of disease symptoms.[13] Taken together, these data argue that infiltration of activated macrophages constitutes a prominent step in the pathogenesis of SLE.

Activated macrophages, but not quiescent macrophages (or other immune cells), have recently been shown to express the β form of the folate receptor (FR-β).[14–16] Because previous studies have demonstrated that folate can target attached drugs to FR-expressing cells,[17–24] identification of FR-β on activated macrophages has opened an opportunity to deliver drugs specifically to activated macrophages without exposing healthy tissues to the same therapy. Although most effort in the field of folate targeting has focused on delivery of cytotoxic agents to FR-expressing cancer cells,[24–27] results from our laboratory have established considerable success in treating both cancer and rheumatoid arthritis with a folate–hapten-targeted immunotherapy.[17,22,25] In this strategy, animals are first immunized against a small immunogenic hapten such as fluorescein in order to generate high titers of anti-fluorescein antibodies. The animal is then treated with folate-linked fluorescein, allowing the folate to carry the attached immunogenic fluorescein to FR on the surfaces of the activated macrophages. Once the activated macrophages are decorated with large numbers of the immunogenic hapten, the macrophages become recognized by anti-fluorescein

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antibodies, leading to macrophage removal, much like an antibody-coated virus or bacterium.

Two mouse models of SLE have served prominently in the molecular characterization and preclinical evaluation of therapies for SLE. These include the MRL/MpJ*Tnfrsf6*^{lpr} mouse and the NXBW/F1 mouse.[28–32] In this paper, we show that folate–hapten immunotherapy alleviates the SLE-related symptoms in both lupus-prone mouse models, leading to significant prolongation of survival in both cases. We suggest that this therapy warrants further evaluation as a possible approach for treatment of SLE in humans.

Experimental Details

Materials. Potassium carbonate, Tween-20, bovine serum albumin (BSA), gelatin, SigmaFast OPD tablets and anti-mouse IgG–horseradish peroxidase (HRP) were all purchased from Sigma (St. Louis, MO). Keyhole limpet hemocyanin (KLH) was procured from ICN Biomedicals (Irvine, CA), while aminofluorescein and fluorescein isothiocyanate (FITC) were purchased from Molecular Probes (Carlsbad, CA). PD-10 desalting columns were obtained from Amersham Biosciences (Piscataway, NJ) and TiterMax Gold was purchased from CytRx Corporation (Norcross, Georgia). A BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL). EC20, folate–FITC, GPI-0100, and alum were all generously provided by Endocyte, Inc. (West Lafayette, IN). Female NXBW/F1 mice and MRL/MpJ*Tnfrsf6*^{lpr} breeder pairs were both purchased from Jackson Laboratories. The mice were bred in the Purdue Animal Facility and 5 week old MRL/MpJ*Tnfrsf6*^{lpr} female mice were used for the described studies. The Purdue Animal Care and Use Committee approved all studies.

Induction of Anti-FITC Antibodies. To induce anti-fluorescein isothiocyanate (FITC) antibodies, keyhole limpet hemocyanin (KLH) was first coupled to FITC. KLH–FITC was then emulsified with one of three adjuvants: TiterMax Gold, alum, or GPI-0100.

The procedure for the preparation of KLH–FITC was as described previously.[17,22] Briefly, 10 mg of KLH was dissolved in 0.5 mL of water and added to 2 mg of FITC. The pH was adjusted to 9.5 using 50 mg/mL K_2CO_3, and the solution was stirred overnight at 37 °C before purification of the conjugated KLH–FITC on a PD-10 column. The concentration of KLH–FITC was measured with a BCA kit using the manufacturer's instructions.

Animals were immunized subcutaneously (s.c.) on their backs with 100 μL of an emulsion containing equal volumes of KLH–FITC solution (50 μg/mouse) and adjuvant. Animals that were immunized with KLH–FITC/TiterMax Gold received the s.c. injection on days 0 and 28. Animals that were immunized with either KLH–FITC/alum or KLH–FITC/GPI-0100 received injections on days 0, 14, and 28.

Measurement of Anti-FITC Antibody Titer. On day 38, animals were bled via the paraorbital vein under anesthesia. The blood was first allowed to clot at 37 °C and then stored overnight at 4 °C. The clotted blood was centrifuged at high speed in a microcentrifuge, and serum was collected and stored at 4 °C. Anti-FITC antibody titer was analyzed by enzyme-linked immunosorbent assay (ELISA), as described previously with few modifications.[22] Briefly, 96-well ELISA plates were coated with 2 μg/well BSA–FITC (prepared similar to KLH–FITC) overnight at 4 °C. Wells were washed three times with phosphate-buffered saline–Tween 20 (PBS-T; PBS, pH 7.4, with 0.05% Tween-20) and coated with 100 μL of PBS-T containing 0.2% gelatin for 1 h at 37 °C. Wells were washed again with PBS-T, and serum samples were added to the wells in 2-fold serial dilutions prior to incubation for 1 h at 37°. After washing with PBS-T, each well received 100 μL of 1:2500 dilution of anti-mouse IgG–HRP antibody solution (diluted in PBS, pH 7.4). Wells were washed with water, and 150 μL of HRP substrate (SigmaFast OPD tablets; prepared according to manufacturer's instructions) was added to each well, and the plate was incubated for 30 min at room temperature in the dark. The enzymatic reaction was stopped by addition of 37.5 μL of 3 M HCl, and the plate was read at 490 nm on a microplate reader. The results were plotted as average optical density vs log serum dilution.

Folate-Targeted Immunotherapy. Since commercial rodent chows contain excessive amounts of folic acid, all animals were placed on a folate-deficient diet for 2 weeks prior to study to allow serum folate to reach physiological levels. Folate–hapten-targeted immunotherapy (FHTI) was then initiated by immunizing the animals against the hapten, fluorescein (FITC), by injection of KLH–FITC emulsified in one of three adjuvants: TiterMax Gold, alum, or GPI-0100. The first immunization occurred at ~5 weeks of age and treatment with folate–FITC began at approximately 10 weeks of age. All animals were weighed on a weekly basis to measure lupus-related weight loss.

Analysis of Bone Erosion. To determine whether FHTI decreased bone and cartilage degradation, radiographs of limbs were taken at the end of each study, as described previously.[17] Lateral radiographic projections were taken of the tarsus of each mouse. Radiographs were taken with direct exposure (1:1) on un-screen KODAK X-OMAT TL film (Kodak, Rochester, NY) using a Faxitron X-ray system with a 0.5-mm focal spot and beryllium window (Faxitron X-ray

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Corporation, Wheeling, IL). The radiographs were graded numerically according to severity as follows: increased soft tissue volume (0–5), narrowing or widening of joint spaces (0–5), subluxation (0–3), subchondral erosion (0–3), periosteal reaction (0–4), osteolysis (0–4), and degenerative joint changes (0–3). A board-certified veterinarian without knowledge of the assigned treatment groups scored all radiographs.

Analysis of Spleen Size. Spleen enlargement is a common indicator for systemic inflammation.[33] At the end of each study, the spleen was harvested and weighed.

Analysis of White Blood Cell Counts and Blood Urea Nitrogen Levels. Blood samples were taken from mice by cardiac puncture and submitted to the Purdue Clinical Pathology Laboratory for analysis of white blood cells and blood urea nitrogen levels.

Necropsy and Immunohistochemical Analyses. Mice that were treated with FHTI as described above were sacrificed at 20 weeks for necropsy, and kidneys were resected for immunohistochemical analysis. Kidneys were fixed in 10% formalin for 24 h, transferred to 70% ethanol, embedded in paraffin, and sectioned on a microtome. Deposition of immune complexes in cortical tubules was determined by staining tissue sections with anti-mouse IgG labeled with HRP (Serotec, Inc., Raleigh, NC). All mice were submitted to Purdue Animal Disease and Diagnostic Lab for necropsy analysis. Tissues were resected, sectioned, and processed for hematoxylin and eosin staining. Tissues were analyzed by board-certified veterinarians for damage, lupus-related abnormalities, and immune cell infiltration.

Results

Lymphadenopathy in FHTI-Treated MRL/MpJ*Tnfrsf6^{lpr}* Mice. An important clinical measure of disease progression in MRL/MpJ*Tnsfrf6^{lpr}* mice is lymphadenopathy. This swelling of the lymph nodes is reflective of increased immune activity. The incidence of superficial lymphadenopathy did not differ among the treated and untreated groups. However, lymphadenopathy showed a delayed onset in FHTI-treated mice when compared with untreated control mice (data not shown).

Body and Organ Weights of FHTI-Treated MRL/MpJ*Tnsfrf6^{lpr}* Mice. Average body weight increased initially in all groups; however, as the disease progressed, only FHTI-treated groups continued to gain weight, while untreated animals rapidly lost weight, especially in their final weeks (Table 1). In contrast, spleen weight increased in untreated mice but remained relatively normal in treated mice (Table 1). Because spleen weight often increases during chronic inflammation, the larger spleens in the untreated animals suggest sustained systemic inflammation.

Blood Analyses in FHTI-Treated MRL/MpJ*Tnsfrf6^{lpr}* Mice. Whole blood was collected when mice were 20 weeks old and analyzed for white blood cell counts and blood urea nitrogen (BUN). There was no significant difference between treated and untreated groups in white cell numbers; however, treated animals had lower levels of BUN (Table 1). Because elevated BUN is a diagnostic factor for malfunction of the kidneys, these data suggest that FHTI can protect lupus-prone mice from autoimmune-related damage to the kidneys.

Table 1. Effect of Folate-Targeted Immunotherapy on Clinical Parameters in MRL/MpJ*Tnsfrf6^{lpra}*

	untreated	FHTI-treated
body weight (g)	24 ± 2.45	36 ± 3.04
spleen weight (mg)	433 ± 79	246 ± 11
white blood cell counts (×1000/μL)	6.9 ± 3.6	7.34 ± 1.2
blood urea nitrogen (mg/dL)	53 ± 8.7	32 ± 3.4

[a] Mice were fed folate-deficient chow, immunized against TiterMax/KLH–FITC and then treated with folate–FITC 7×/week. They were sacrificed at the age of 20 weeks and analyzed for the parameters listed. White blood cell counts and blood urea nitrogen levels were analyzed by the Purdue University Clinical Pathology Lab.

Radiographic Analysis of the Joints of FHTI-Treated MRL/MpJ*Tnsfrf6^{lpr}* Mice. Polyarthritis occurs in a fraction of MRL/MpJ*Tnsfrf6^{lpr}* mice, with a penetrance of ~15–25%.[34,35] Affected mice are reported to develop swelling in the hind feet and lower legs. In our study, mice were sacrificed, and paws were processed and scored for bone and cartilage degradation. Radiographs indicated no significant difference in damage to joints between treated and untreated mice at 20 weeks of age (data not shown).

Kidney IgG Deposition in FHTI-Treated MRL/MpJ*Tnsfrf6^{lpr}* Mice. Antibody deposition is characteristic of lupus-related glomerulonephritis. As described above, two groups of animals were immunized against TiterMax/KLH–FITC, and one group was further treated with 600 nmol/kg folate–FITC 7×/week to promote elimination of their FR-expressing activated macrophages. Autoantibody deposition in the kidneys was analyzed by direct immunohistochemical staining of kidney sections of both groups of animals using an HRP-labeled anti-mouse IgG antibody. Higher levels of autologous IgG were detected in the kidneys of untreated than those of FHTI-treated mice (Figure 1).

Survival of FHTI-Treated MRL/MpJ*Tnsfrf6^{lpr}* mice. Female MRL/MpJ*Tnsfrf6^{lpr}* mice gradually develop clinical nephritis and eventually succumb to the disease by 4–7 months of age. To determine whether FHTI might enhance survival of lupus-prone mice, animals were immunized with TiterMax/KLH–FITC and treated as described above to promote elimination of their activated macrophages. Analysis

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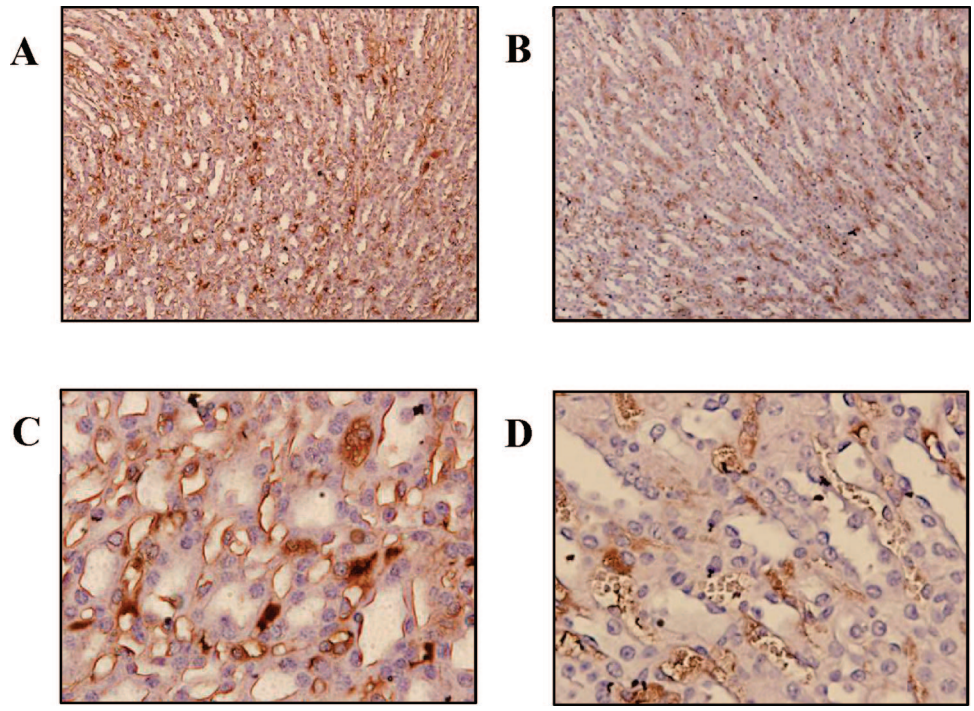

Figure 1. Analysis of IgG deposition in MRL/MpJ*Tnsfrf6*^{lpr} mice. Mice were immunized against TiterMax/KLH–FITC and subsequently treated with either PBS (A, C) or 600 nmol/kg folate–FITC 7×/week (B, D). Animals were sacrificed at 20 weeks of age, and kidneys were extracted and processed in paraffin for direct immunohistochemical analysis using anti-IgG HRP antibody. Slides were studied at 10× (A, B) and 40× (C, D) magnification.

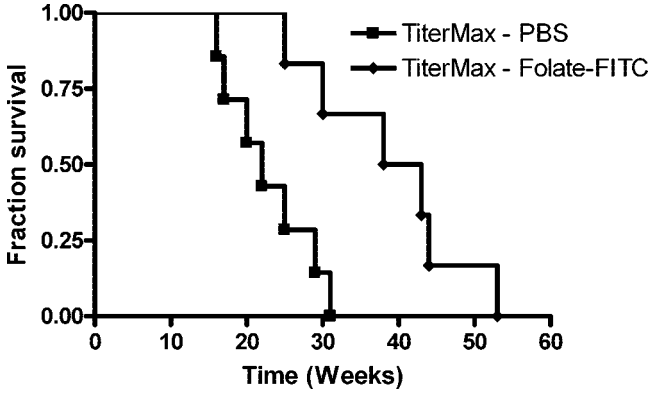

Figure 2. Kaplan–Meier analysis assesses the extension in lifespan in FHTI-treated MRL/MpJ*Tnsfrf6*^{lpr} mice. Animals were immunized against TiterMax/KLH–FITC and treated with either PBS or 600 nmol/kg folate–FITC 7×/week (log rank test, $P = 0.0057$).

of animal survival (Figure 2) indicates that treatment with FHTI is indeed capable of significantly prolonging the lifespan of lupus prone mice (25–53 weeks versus 16–31 weeks for untreated mice, log rank test, $P = 0.0057$), suggesting that activated macrophages contribute to the accelerated mortality of MRL/MpJ*Tnsfrf6*^{lpr} mice.

Visual Assessment of the Effects of FHTI Therapy on MRL/MpJ*Tnsfrf6*^{lpr} Mice. MRL/MpJ*Tnsfrf6*^{lpr} mice were observed visually during the course of FHTI treatment for any indications of a decline in health. Mice treated with FHTI were seen to be much more active and responsive to stimuli than untreated mice. FHTI-treated mice also developed dramatically fewer spontaneous skin lesions or sores than untreated mice. This assessment was confirmed by pathologists independent of our laboratory. In their detailed report, they state that the untreated mice suffered from bloody and scarred lesions on the skin, whereas FHTI-treated mice did not. Finally, treated mice continued to gain weight in contrast to untreated mice, which lost weight during the course of the study (Figure 3).

Pathology. All mice involved in the survival study were submitted at death for necropsy to the Purdue Animal Disease Diagnostic Laboratory. Untreated mice were diagnosed with membranoproliferative glomerulonephritis, tubular epithelial degeneration and necrosis, hepatocellular epithelial degeneration and necrosis, and mild myocardial degeneration. Furthermore, massive granulocyte and macrophage infiltration was seen in the affected tissues. In stark contrast, FHTI-treated mice displayed no signs of nephritis, tissue necrosis, heart defects, nor granulocyte and macrophage infiltration. Instead, pathological examination indicated that the treated mice likely died from excessive lymphoproliferation. That is, lymph nodes were profoundly enlarged and elevated lymphocyte infiltration was seen in multiple organs of the body.

Survival of FHTI-Treated NZBW/F1 Mice. NZBW/F1 mice constitute an alternative model of SLE. The average lifespan for female NZBW/F1 mice ranges from 8 to 12 months. In this study, female NZBW/F1 mice were immunized with TiterMax/KLH–FITC and subsequently treated daily with either folate–FITC or PBS. Although one treated mouse died a few weeks (at 83 weeks of age) after the last

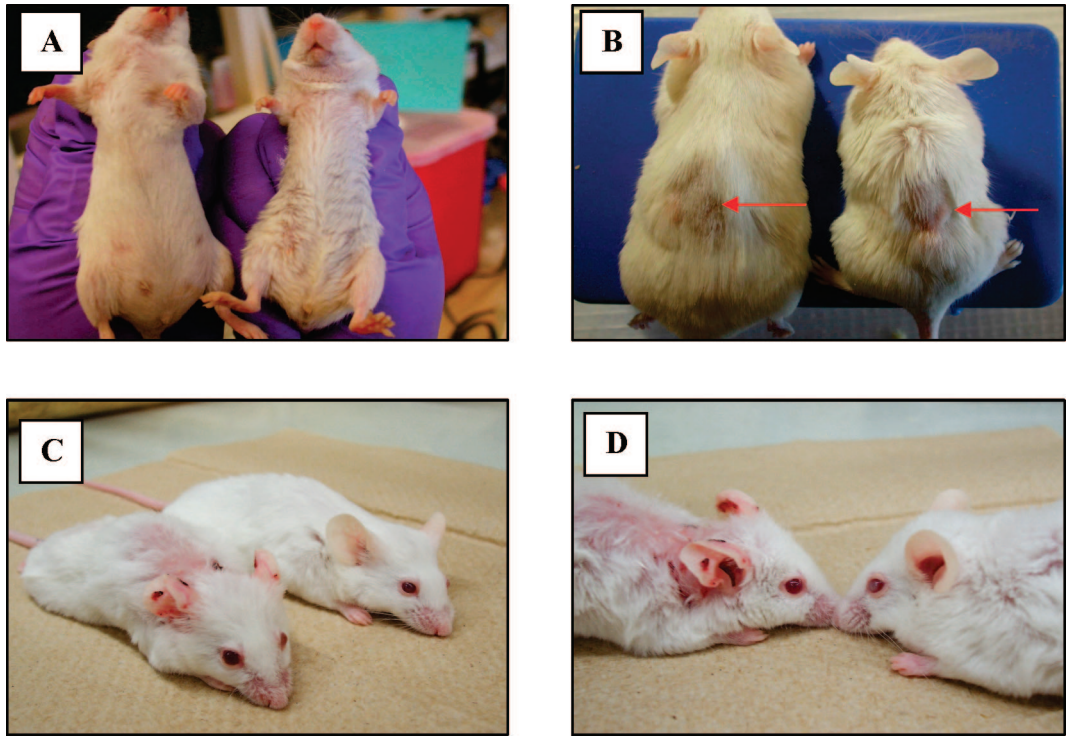

Figure 3. Pictures of FHTI-treated and untreated MRL/MpJ*Tnsfrf6*^{lpr} mice. Animals were immunized against TiterMax/KLH–FITC and treated with either PBS or folate–FITC as described in the Experimental Details. FHTI-treated mice are on the left in A and B, whereas they are on the right in C and D. Arrows indicate sites of immunization.

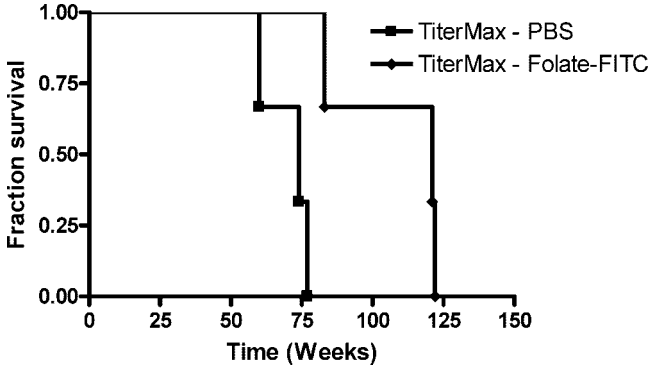

Figure 4. Kaplan–Meier analysis of lifespan of FHTI-treated and untreated NZBW/F1 mice. Animals were immunized against TiterMax/KLH–FITC and treated with either PBS or 600 nmol/kg folate–FITC 7×/week (log rank test, $P = 0.0246$).

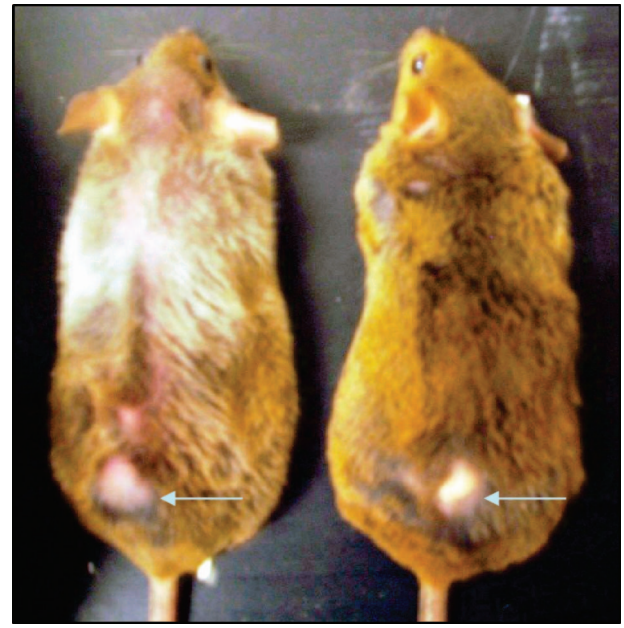

Figure 5. Pictures of FHTI-treated and untreated NZBW/F1 mice. An untreated (PBS-treated) mouse is on the left and an FHTI-treated mouse is on the right. Arrows indicate sites of immunization.

of the untreated mice had died (60–77 weeks of age), the remaining FHTI-treated mice survived much longer than untreated animals (121–122 weeks, Figure 4, log rank test, $P = 0.0246$). Similar to FHTI-treated MRL/MpJ*Tnsfrf6*^{lpr} mice, FHTI-treated NZBW/F1 mice also responded much more sensitively to stimuli, had fewer skin lesions, and exhibited a smoother coat than untreated NZBW/F1 mice (Figure 5).

Effect of Adjuvant on Folate-Targeted Hapten Immunotherapy. Different vaccine adjuvants are known to exert different polarizing effects on the immune system. For example, GPI-0100 has been shown to shift the immune system strongly towards a Th1 response, and alum shifts the immune system towards Th2,[34] while TiterMax induces a mixed Th1/Th2 response.[35] Unfortunately, unlike many other autoimmune diseases, SLE has a complex immune profile consisting of both Th1 and Th2 characteristics, making it difficult to predict which shift in immune profile would be

more beneficial for treatment of SLE.[36–38] In order to compare the abilities of the aforementioned adjuvants to facilitate folate-targeted hapten immunotherapy, NZBW/F1 and MRL/MpJ*Tnsfrf6*^{lpr} mice were immunized with each of the above adjuvants and examined for extension of lifespan in response to FHTI. The longest extension of lifespan was observed upon vaccination with TiterMax, with both alum and GPI-0100 affording only a few additional weeks of survival beyond the untreated controls (data not shown). These data suggest that biasing the immune system towards either a solely Th1 or Th2 response may not be beneficial to immunotherapy of SLE.

Discussion

The MRL/MpJ*Tnsfrf6*^{lpr} mouse is a well-established murine model of SLE characterized by glomerulonephritis with immune complex deposition, vasculitis, splenomegaly, lymphadenopathy, and autoantibody production. Macrophages are notably abundant in the kidneys of these mice, and they mediate renal destruction by enhanced production of such inflammatory cytokines as IFN-γ, TNF-α, and reactive oxygen species.[4] In view of the tissue damage that can be caused by these mediators of inflammation, it stood to reason that strategies that prevent macrophage recruitment, proliferation, and activation in MRL/MpJ*Tnsfrf6*^{lpr} mice will alleviate disease symptoms and tissue destruction.

Previous reports from our laboratory and others have already shown that activated macrophages express a cell surface receptor for folic acid[14,15] that can enable the FR-expressing macrophages to be targeted with folate-linked conjugates.[15,17] In some studies, this targeting ability was exploited to deliver folate-linked radioaimaging agents selectively to areas of inflammation, allowing their visualization by γ-scintigraphy.[15] In other studies, the same targeting ability was used to deliver folate-linked haptens to the inflamed joints of two rodent models of rheumatoid arthritis, mediating elimination of their activated macrophages and alleviating symptoms of their disease.[17] Based on this success, the studies described above were undertaken to investigate whether the same therapy might reduce symptoms in another model of a macrophage-mediated disease, namely, systemic lupus erythematosus (SLE). Although FHTI was first administered when mice were 10 weeks of age and had already begun developing SLE, FHTI was still capable of abolishing all histological evidence of glomerulonephritis and inflammatory damage to other tissues. In addition, histology showed that the tissue infiltration of macrophages widely seen in untreated mice was noticeably absent in treated animals. Animal survival was also significantly prolonged, and normal mouse behavior was sustained for the duration of the therapy.

The pathology in MRL/MpJ*Tnsfrf6*^{lpr} mice is caused by the *lpr* transgene that encodes a defective Fas molecule, resulting in faulty lymphocyte apoptosis and uncontrolled lymphoproliferation. In females, lymphadenopathy begins by 3 months of age, and by the end of life, lymph nodes can reach 100 times their normal size. The proliferation of $CD4^+$ T lymphocytes is thought to drive predisposed B cells to elaborate an array of autoantibodies against normal cellular antigens. Production of these autoantibodies can lead to formation of immune complexes with the cognate antigens, and subsequent accumulation of these immune complexes in organs such as the kidneys can trigger the influx of Fc receptor-expressing immune cells (e.g., macrophages, neutrophils, and NK cells), which in turn cause tissue damage and inflammation. Recognition of the immune complexes by macrophages is presumably important, because the consequent activation of the macrophages will induce their release of inflammatory cytokines, chemokines, and reactive oxygen species, which in turn can sustain and exacerbate the inflammation.[4] Progression of these and related events in the kidneys, joints, liver, and heart of affected animals is thought to result in glomerulonephritis, arthritis, hepatocellular necrosis, and myocardial degeneration, respectively. However, prevention of these sequelae in the MRL/MpJ*Tnsfrf6*^{lpr} mice was not predicted to lead to a healthy phenotype because their lymphocytes were still expected to remain nonresponsive to apoptotic signals.[39] Thus, their uncontrolled proliferation was still anticipated to cause their eventual death, that is, as seen in the FHTI-treated mice.

Finally, it should also be noted that FHTI was capable of extending the survival of lupus-prone NZBW/F1 mice by almost 10 months. Because this mouse strain is not characterized by an underlying lymphoproliferative disorder, they appeared to die of natural causes.

Acknowledgment. We thank Endocyte, Inc for funding this research and Dr. Rick Widmer, DVM (Department of Veterinary Sciences, Purdue University), for his time and assistance in interpreting all radiographs.

Supporting Information Available: Graph showing the effect of immunotherapy on body weight. This material is available free of charge via the Internet at http://pubs.acs.org.

MP0700615

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