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PAPER

Immunomodulatory effects of H.P. Acthar Gel on B cell development in the NZB/W F1 mouse model of systemic lupus erythematosus

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H.P. Acthar Gel® (Acthar) is a highly purified repository gel preparation of adrenocorticotropic hormone ($ACTH_{1\text{-}39}$), a melanocortin peptide that can bind and activate specific receptors expressed on a range of systemic lupus erythematosus (SLE)-relevant target cells and tissues. This study was performed to evaluate the effects of Acthar in a mouse model of SLE, using an F1 hybrid of the New Zealand Black and New Zealand White strains (NZB/W F1). Twenty-eight week old NZB/W F1 mice with established autoimmune disease were treated with Acthar, Placebo Gel (Placebo), or prednisolone and monitored for 19 weeks. Outcomes assessed included disease severity (severe proteinuria, $\geq 20\%$ body weight loss, or prostration), measurement of serial serum autoantibody titers, terminal spleen immunophenotyping, and evaluation of renal histopathology. Acthar treatment was linked with evidence of altered B cell differentiation and development, manifested by a significant reduction in splenic B cell follicular and germinal center cells, and decreased levels of circulating total and anti-double-stranded DNA (IgM, IgG, and IgG2a) autoantibodies as compared with Placebo. Additionally, Acthar treatment resulted in a significant decrease of proteinuria, reduced renal lymphocyte infiltration, and attenuation of glomerular immune complex deposition. These data suggest that Acthar diminished pathogenic autoimmune responses in the spleen, peripheral blood, and kidney of NZB/W F1 mice. This is the first preclinical evidence demonstrating Acthar's potential immunomodulatory activity and efficacy in a murine model of systemic lupus erythematosus. *Lupus* (2014) **0,** 1–11.

Key words: Systemic lupus erythematosus; NZB/W F1; melanocortin peptides; ACTH; Acthar Gel; B cells

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by diverse clinical manifestations, a relapsing–remitting course, and the production of anti-nuclear autoantibodies.[1] Symptoms of SLE are caused by inflammation and tissue damage secondary to immune complex deposition in the microvasculature of multiple organs and tissues.[1,2] Animal and human studies indicate that the pathophysiology of SLE may involve defects in B cell tolerance and homeostasis, with subsequent autoantibody production.[3–6] Advances in the understanding of the role of B cell survival and differentiation in the pathophysiology of SLE contributed to the recent FDA approval of anti-B lymphocyte stimulator (BLyS) antibody (belimumab),[7] which is thought to have efficacy as a treatment for SLE by reducing pathological increases in B lymphocytes.

H.P. Acthar Gel® (Acthar) is a highly purified preparation of full length adrenocorticotropic hormone ($ACTH_{1\text{-}39}$), derived from porcine pituitary and formulated into a repository gel for prolonged release. Historically, the clinical efficacy of Acthar was thought to be due to its ability to stimulate endogenous corticosteroid production by the adrenal gland. More recently it has been demonstrated that $ACTH_{1\text{-}39}$, the principal component of Acthar, binds to and activates all five known melanocortin receptors (MC1R to MC5R), not just MC2R

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(the primary receptor mediating steroidogenesis in the adrenal cortex[8]). Acthar, like other melanocortin peptides, may therefore produce anti-inflammatory and immunomodulatory effects by directly activating MCRs expressed on SLE disease-relevant organs, tissues, and immune cells (e.g. B and T cells, macrophages, and dendritic cells).[9–11] Previous studies demonstrate that ACTH and other melanocortin peptides inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity and suppress pro-inflammatory cytokine production (e.g. interleukin-1 (IL-1), IL-6, IL-8, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-2, and IL-17) and cell adhesion molecule expression (e.g. ICAM-1).[9] Melanocortin peptides may also promote immunosuppression by increasing the expansion of regulatory T cells (Tregs), upregulating anti-inflammatory cytokines (e.g. IL-10), and/or mediating inhibitory effects on MCP-1 expression.[9,12,13] Additional preclinical data suggest that melanocortin peptides reduce podocyte and renal tubular cell apoptosis, tubulointerstitial fibrosis, oxidative stress, and inflammatory cell infiltration in the kidney,[14–16] with published evidence generated from both animal studies and in patients with nephrotic renal diseases supporting a role for Acthar in the treatment of proteinuria.[11,17,18]

Historical reports support clinical efficacy of ACTH in SLE,[19,20] and Acthar is FDA approved for use during an exacerbation or as maintenance therapy in selected cases of SLE. However, the specific immunomodulatory actions of Acthar have not been previously investigated in preclinical models of systemic autoimmune disease. The present study was conducted in order to evaluate the efficacy and to begin to explore the potential mechanisms of action of Acthar in a murine model of SLE using an F1 hybrid of the New Zealand Black and New Zealand White strains (NZB/W F1). NZB/W F1 mice spontaneously develop SLE-like disease manifestations over time so that by the age of five to six months they display splenomegaly, elevated serum titers of IgG and anti-double-stranded (ds) DNA IgG (especially isotype switched IgG2a),[21] proteinuria, and immune-mediated glomerulonephritis.[22,23] Furthermore, these mice develop altered tolerance checkpoints, including hyperactivation and positive selection of autoreactive B cells from the follicular compartment to germinal centers (GCs).[22] Historical data demonstrate that these disease manifestations of autoimmunity are unique to the NZB/W F1 hybrid, as they are not seen in other inbred mouse strains[24] and the NZB and NZW parental strains show only limited autoimmunity.[23] Potential beneficial effects of a 19-week course of Acthar were evaluated in NZB/W F1 mice with established autoimmunity. Disease assessments included serial in-life measurement of body weight, autoantibody levels, and proteinuria. Terminal endpoints included quantification of splenic B, T, and dendritic cell (DC) populations and renal histopathology. The data presented support a significant role for Acthar in attenuating disease progression and severity in this murine model of SLE.

Methods

Animals

Female NZB/W F1 mice (The Jackson Laboratory, Bar Harbor, ME, USA) were group housed in semi-rigid mouse isolators in an AAALAC-accredited conventional animal facility, and maintained in accordance with the guidelines of the BRM Institutional Animal Care and Use Committee.

Protocol

At 28 weeks of age, mice with moderate proteinuria ($1-2+$, equivalent to 30–100 mg/dl) were assigned to one of three treatment groups ($n=10$/group) to achieve an equal mean proteinuria score representing established disease. Treatment began with Acthar (160 U/kg) or an equivalent volume of Placebo Gel (Placebo; Questcor Pharmaceuticals, Hayward, CA, USA) administered subcutaneously (s.c.) every other day, or with prednisolone (5 mg/kg s.c.; Solu-Delta-Cortef, Pfizer, New York, NY, USA) given for six days each week. This dose of prednisolone was previously reported to attenuate disease in NZB/W F1 mice.[25] Treatment was continued until animals reached 46 weeks of age unless pre-defined criteria necessitating early removal from the study were met. At the end of the treatment period mice were sacrificed by thoracotomy and rapid exsanguination under isoflurane anesthesia (1–4%, to effect).

In-life measurements

Body weight was measured at least once weekly (up to three times weekly if proteinuria was $\geq 3+$). Serum samples for measurement of autoantibody titers were obtained from 28-week old mice prior to the initiation of treatment, and every two weeks thereafter until study completion.

Flow cytometry

After euthanasia, spleens were gently crushed using microscope slides. Red blood cells were lysed with ACK Lysis Buffer (Lonza, Allendale, NJ, USA); lysates were washed with RPMI 1640, and passed through a 70 μm nylon filter. Cells were counted, Fc receptors were blocked with TruStainfcX (Biolegend, San Diego, CA, USA), and then cells were stained in three- or four-color panels in Cell Staining Buffer (Biolegend, San Diego, CA, USA) on wet ice. Cells were fixed with Cytofix (BD Biosciences, San Jose, CA, USA) on wet ice, then washed once and resuspended in Cell Staining Buffer prior to acquisition. The following specific $CD19^+$ B cell subsets were analyzed: activated ($CD20^+CD69^+$), immature ($CD21^{lo}CD23^{lo}$), T1 transitional cells ($IgM^{hi}IgD^{lo}$), T2 transitional cells ($IgM^{lo}IgD^{hi}$), follicular ($CD21^{int}CD23^{hi}$), marginal zone (MZ; $CD21^{hi}CD23^{lo}$), GCs ($GL\text{-}7^+$), and plasma cells ($CD20^-CD138^+$). Other cell populations analyzed included: T cells ($CD3^+$), T helper cells ($CD3^+CD4^+$), activated T helper cells ($CD3^+CD4^+CD69^+$), macrophages ($CD11b^+$) and dendritic cells ($CD11c^+$). Gating and analysis were performed using FlowJo v7.6.5 software (Treestar, Inc., Ashland, OR, USA).

Autoantibody measurements

Serum titers of total and anti-dsDNA IgG, IgG2a, and IgM were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits from Alpha Diagnostics International (ADI, San Antonio, TX, USA). Assays were performed as per kit manuals in duplicate.

Renal endpoints

Semi-quantitative assessment of proteinuria was determined every two weeks with Uristix (Siemens Healthcare, Tarrytown, NY, USA). If a measurement of $\geq 3+$ was observed (equivalent to approximately 300 mg/dl), an additional test was performed the following week. One kidney was formalin-fixed and paraffin-embedded, then sectioned and stained with hematoxylin and eosin (H&E). H&E-stained slides were scored for glomerulonephropathy, dilated tubules, degenerate tubules, and lymphocyte aggregates by an independent pathologist blinded to the treatment groups and disease status of the mice. All scoring was based on a 0–5 system (with 0 = normal, 1 = least discernible or slight, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe). Representative photomicrographs were viewed on a Nikon Eclipse E400 microscope (Nikon Instruments, Inc., Melville, NY, USA) and captured using a SPOT Insight Color digital camera and SPOT v5.0 software (SPOT Imaging Solutions, Sterling Heights, MI, USA). The contralateral kidney was flash frozen in optimal cutting temperature (OCT) media, then cryosections (6 μm) were used for immunofluorescence staining. Glomerular IgG and C3 deposition were evaluated using Alexa-488-conjugated anti-mouse IgG (Life Technologies, Grand Island, NY, USA) or FITC-conjugated anti-mouse complement C3 (MP Biomedicals, LLC, Santa Ana, CA, USA) antibodies, respectively. Glomerular staining for IgG and C3 was scored by a blinded pathologist using a semi-quantitative scale based on signal present (with 0 = no signal, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = intense). Representative images were captured using a Nikon Eclipse 80i microscope attached to a Prior Lumen 200 Fluorescent Illumination System with a Nikon DXM 1200C camera.

Criteria for early study termination

Animals met criteria for removal from the study with early euthanasia if they displayed one of the following: $\geq 3+$ proteinuria on two consecutive measurements, $\geq 20\%$ body weight loss, or prostration.

Statistical analyses

All analyses were performed using Prism v6 (Graphpad Software, San Diego, CA, USA). Measurements repeated over time (percent of mice meeting early study termination criteria, body weight, antibody levels, and proteinuria) were analyzed for statistical significance using the Friedman test, followed by Dunn's multiple comparison post-test if significance was identified in the primary comparison. Measurements made at a single time point (splenocyte subsets, semi-quantitative histopathology scores) were analyzed using Kruskal–Wallis, followed by Dunn's multiple comparison post-test if significance was identified in the primary comparison. Statistical significance was set at $p \leq 0.05$.

Results

Acthar treatment prevented disease severity and progression in NZB/W F1 mice

Disease severity and/or progression were significantly attenuated in Acthar-treated NZB/W F1

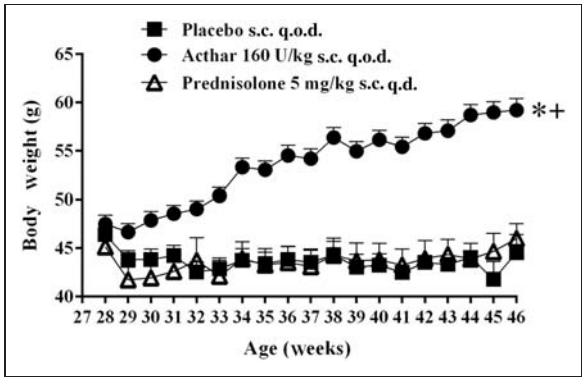

Figure 1 Effects of Acthar on body weight. Comparison of body weight between Acthar- ($n=10$), Placebo- ($n=10$), and prednisolone- ($n=10$) treated animals. Body weights were measured at least once weekly from 28 weeks up to 46 weeks of age. Body weight increased over time in Acthar-treated mice, while both Placebo- ($p \leq 0.0001$) and prednisolone- ($p \leq 0.0001$) treated animals failed to gain weight during the 19-week treatment phase. Values shown are the mean ± SEM.
*Denotes significant differences compared with Placebo.
$^{+}$Denotes significant differences compared with prednisolone.

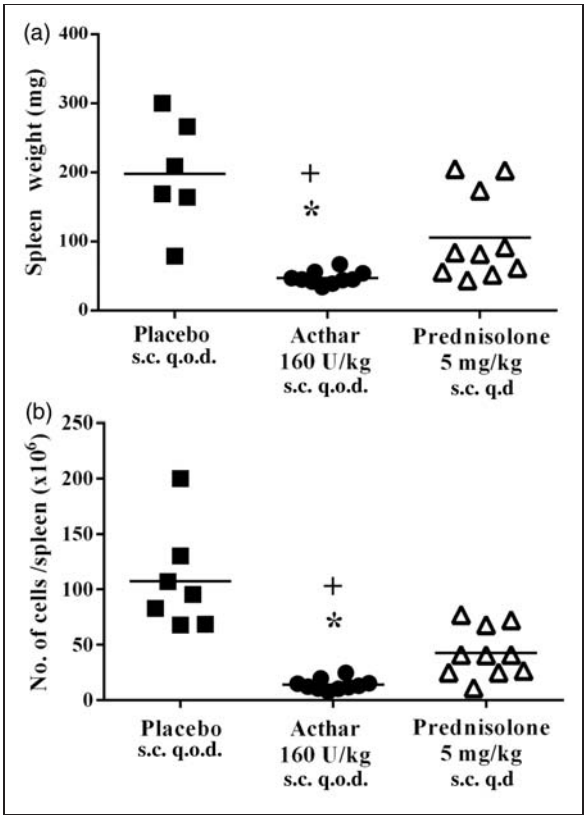

Figure 2 Effects of Acthar on splenomegaly. (a) Splenomegaly, estimated by measurement of spleen weight, was significantly reduced in Acthar-treated mice as compared with Placebo- ($p \leq 0.001$) and prednisolone- ($p \leq 0.05$) treated animals. (b) Spleen total cell number was significantly diminished in Acthar-treated animals as compared with Placebo- ($p \leq 0.0001$) and prednisolone-treated mice. Data are presented as a scatter plot of values from individual mice with the mean value represented by a horizontal line ($n=10$/group).
*Denotes significant differences compared with Placebo.
$^{+}$Denotes significant differences compared with prednisolone.

mice. While 80% of Placebo-treated mice developed severe proteinuria requiring early termination from the study and 20% of mice receiving prednisolone met early termination criteria (including one mouse that developed hindlimb paralysis from a spine fracture and another with ≥ 20% body weight loss), all of the Acthar-treated mice survived the 19-week treatment period ($p \leq 0.0001$ Acthar versus Placebo). Shown in Figure 1, the body weight of Acthar-treated mice increased throughout the study, while Placebo- and prednisolone-treated animals failed to gain weight during the 19-week treatment period ($p \leq 0.0001$ Acthar versus Placebo; $p \leq 0.0001$ Acthar versus prednisolone).

Acthar diminished splenomegaly and activated and differentiated B and T cell subsets in the spleen

As shown in Figure 2(a), spleen weights were significantly lower in Acthar-treated mice as compared with Placebo- ($p \leq 0.001$) and prednisolone- ($p \leq 0.05$) treated animals. The reductions in spleen weight corresponded with significantly lower total spleen cell counts in Acthar- versus Placebo- and prednisolone-treated mice ($p \leq 0.0001$ and $p \leq 0.05$ Acthar versus Placebo and prednisolone respectively; Figure 2(b)). Consistent with the decrease in total spleen cell counts, spleens from Acthar-treated mice had a lower absolute number of splenic CD19^{+} B cells at all developmental stages (activated, immature, T1 and T2 transitional, follicular, MZ, GC, and plasma cells) compared with Placebo-treated mice ($p \leq 0.001$), whereas absolute numbers of only five of these CD19^{+} B cell subsets (activated, T1 and T2 transitional, follicular and MZ) were reduced in prednisolone-treated animals ($p \leq 0.05$ versus Placebo; data not shown).

In addition, shown in Table 1, the frequency of immature and T1 CD19^{+} B cells as a proportion of total splenic B cells was significantly increased in Acthar-treated mice when compared with Placebo ($p \leq 0.01$), while the frequency of follicular (Placebo versus Acthar, $p \leq 0.001$) and GC CD19^{+} B cells was reduced by Acthar treatment (Placebo versus Acthar, $p \leq 0.01$). Furthermore, compared with Placebo, Acthar treatment resulted in a significant increase in MZ CD19^{+} B cell frequency ($p \leq 0.05$; Table 1).

Table 1 Flow cytometric analysis of spleens reported as percent frequency of cells

Cell populations	*Placebo (n = 7)*	*Acthar (n = 10)*	*Prednisolone (n = 10)*
Lymphocytes	81.8 ± 1.5	80.1 ± 1.2	81.1 ± 1.9
B cells ($CD19^+$)	57.7 ± 3.1	47.8 ± 1.8	53.4 ± 5.7
Activated B cells ($CD20^+CD69^+$)	4.7 ± 0.7	3.2 ± 0.2	2.3 ± 0.3[a]
Immature B cells ($CD21^{lo}CD23^{lo}$)	20.9 ± 2.1	35.5 ± 3.3[a]	32.9 ± 6.5
T1 transitional B cells ($IgM^{hi}IgD^{lo}$)	15.6 ± 2.7	35.3 ± 4.0[a,b]	20.4 ± 2.8
T2 transitional B cells ($IgM^{hi}IgD^{hi}$)	22.3 ± 1.9	22.0 ± 1.7	21.4 ± 2.5
Follicular B cells ($CD21^{int}CD23^{hi}$)	58.8 ± 1.5	35.7 ± 3.5[c]	41.7 ± 5.1[d]
Marginal zone B cells ($CD21^{hi}CD23^{lo}$)	16.4 ± 2.3	25.4 ± 2.1[d]	20.5 ± 2.6
B cell germinal center ($GL\text{-}7^+$)	22.7 ± 6.4	9.5 ± 0.5[a]	11.7 ± 2.5[d]
Plasma cells ($CD20^-CD138^+$)	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.2
T cells ($CD3^+$)	38.0 ± 3.5	43.0 ± 1.8	34.5 ± 4.7
T helper B cells (Th, $CD4^+$)	74.8 ± 3.1	48.4 ± 5.0[a]	64.4 ± 2.7
Activated Th cells ($CD69^+$)	36.5 ± 4.3	29.9 ± 2.9	27.9 ± 3.2
Macrophages ($CD11b^+$)	11.8 ± 1.9	8.3 ± 1.2	8.5 ± 1.0
Dendritic cells ($CD11c^+$)	7.8 ± 2.4	2.9 ± 0.3[d]	4.4 ± 1.1

Values presented represent mean ± SEM. Absolute numbers of cells were utilized to calculate frequency of specific splenocyte subsets. Percent of B cells, T cells, macrophages, and dendritic cells were calculated as a fraction of total lymphocyte number. Percent of specific B and T cell subsets were calculated as a fraction of total number of B and T lymphocytes respectively.
[a] $p \leq 0.01$ compared with Placebo-treated mice.
[b] $p \leq 0.05$ compared with prednisolone-treated mice.
[c] $p \leq 0.001$ compared with Placebo-treated mice.
[d] $p \leq 0.05$ compared with Placebo-treated mice.

T cell frequency was similar across all treatment groups, although splenic $CD4^+$ T frequency ($p \leq 0.01$) was markedly lower in Acthar-treated mice as compared with Placebo-treated animals (Table 1). Decreased frequency of DCs was also observed in Acthar-treated mice compared with Placebo-treated mice ($p \leq 0.05$; Table 1).

Acthar prevented the progressive increase of circulating autoantibodies

Serum total and anti-dsDNA immunoglobulin titers were similar across groups prior to the initiation of treatment at 28 weeks. Shown in Figure 3(a), (c), and (e), Acthar significantly prevented the increase of total serum IgG, IgG2a, and IgM seen in Placebo-treated mice ($p \leq 0.01$). Similarly, total IgG and IgG2a were significantly lower in Acthar-treated animals when compared with prednisolone treatment ($p \leq 0.05$). Serum dsDNA autoantibodies increased progressively throughout the 19-week treatment period in Placebo- and prednisolone-treated animals, while increases were not observed in Acthar-treated mice ($p \leq 0.001$ for anti-dsDNA-IgG, anti-dsDNA-IgG2a, and anti-dsDNA-IgM in Acthar- versus Placebo-treated animals; Figure 3(b), (d) and (f)).

Acthar improved renal outcomes in NZB/W F1 mice

Acthar significantly prevented the development of severe proteinuria during the 19-week treatment period (Figure 4(a); $p \leq 0.05$ versus Placebo), while prednisolone had no statistically significant effect on this endpoint. None of the Acthar-treated mice developed severe proteinuria (score $\geq 3+$), whereas eight out of 10 Placebo-treated mice were removed throughout the 19-week treatment phase because they displayed severe proteinuria on two consecutive measurements one week apart. Histologic assessment suggested the protective effects of Acthar on proteinuria were associated with evidence of reduced renal inflammation and glomerular pathology. Renal lymphocyte aggregates were significantly reduced in Acthar-treated mice ($p \leq 0.05$ versus Placebo), with trends for reduced glomerulonephropathy, renal tubular dilation, and renal tubular degeneration histopathology scores (Figure 4(b)). In contrast, prednisolone did not significantly alter any of these renal outcome measures. In addition, while both Acthar ($p \leq 0.01$) and prednisolone ($p \leq 0.05$) significantly attenuated glomerular IgG staining when compared with Placebo-treated animals, only Acthar significantly reduced glomerular C3 staining ($p \leq 0.01$) (Figure 4(c)). Taken together, these data suggest that Acthar minimized the progressive renal damage seen in Placebo-treated NZB/W F1 mice.

Discussion

The pathophysiology of SLE is thought to involve defects in B cell tolerance checkpoints. When tolerance checkpoints are active, negative selection (deletion, editing, or anergy) reduces autoreactive B cells.[3–5] This process encompasses the entire differentiation pathway from immature B cells (in the bone marrow) to mature B cells (in the peripheral lymphoid organs), as well as autoantibody production.[3–6,26] Because Acthar and other melanocortin peptides may suppress inflammation and modulate autoimmunity,[9,10,27,28] the present study was performed to evaluate the efficacy of Acthar in a well-established murine model of SLE.

Data demonstrating increased frequency of immature and T1 B cells in Acthar-treated, but not Placebo- or prednisolone-treated NZB/W F1 mice, suggest that Acthar halted the differentiation

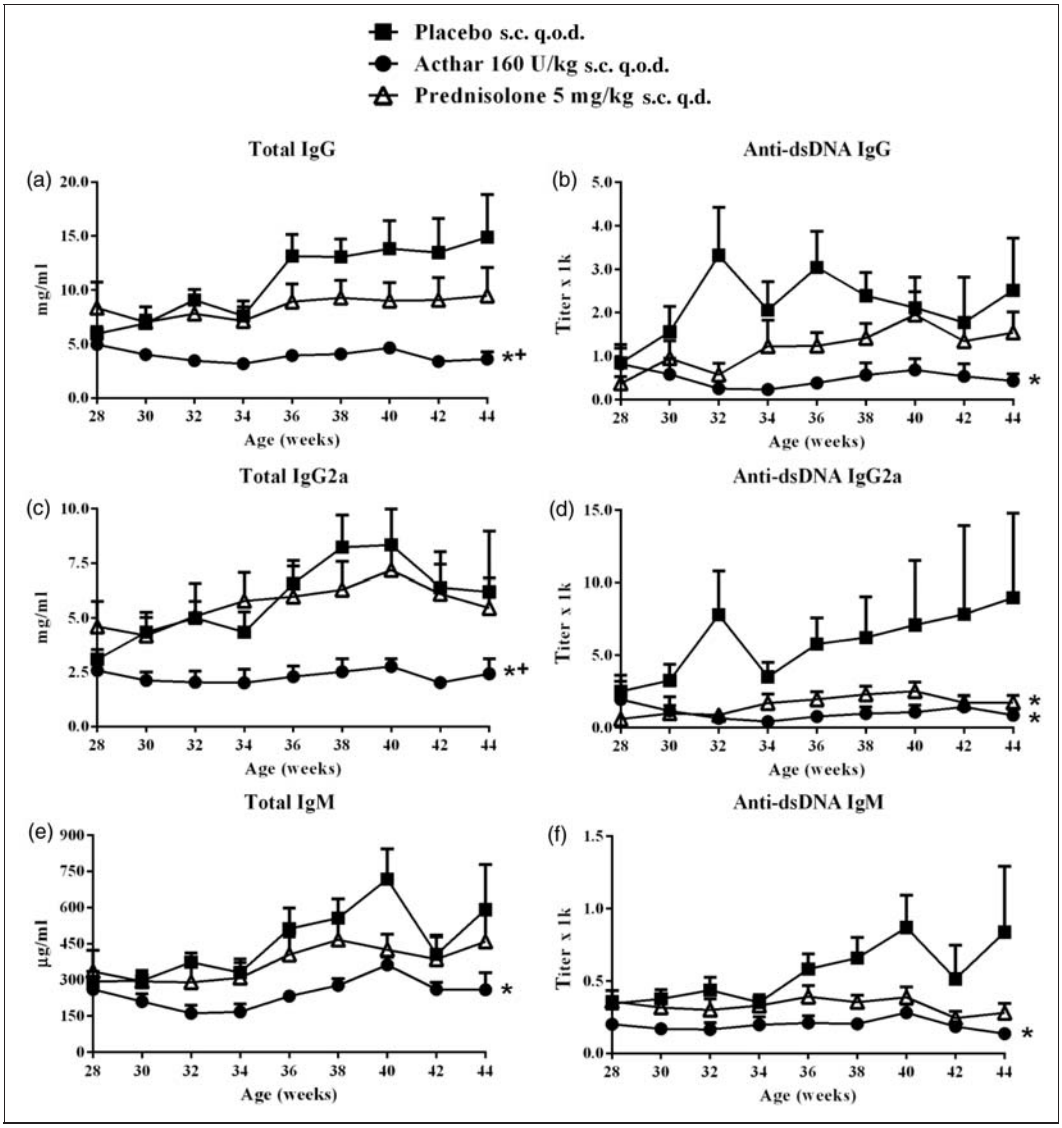

Figure 3 Effects of Acthar on serum immunoglobulins and autoantibodies. Serum levels of total IgG, IgG2a and IgM and anti-dsDNA IgG, IgG2a and IgM were measured at 28 weeks of age (prior to initiation of treatment) and every two weeks thereafter until study completion. Acthar treatment significantly prevented the increase in total serum immunoglobulins as compared with both Placebo (IgG, Panel (a); IgG2a, Panel (c); IgM, Panel (e), $p \leq 0.01$) and prednisolone (IgG, Panel (a); IgG2a, Panel (c), $p \leq 0.05$) treatment groups. Acthar treatment also significantly inhibited the increase of circulating anti-dsDNA autoantibodies (IgG, Panel (b); IgG2a, Panel (d); IgM, Panel (f)) compared with Placebo ($p \leq 0.001$) over the 19-week treatment period. In contrast, only anti-dsDNA IgG2a (Panel (d)) was attenuated by prednisolone treatment. Values shown are the mean ± SEM ($n = 10$/group).
*Denotes significant differences compared with Placebo.
$^{+}$Denotes significant differences compared with prednisolone.

of autoreactive B cells and prevented their progression from the T1 to the T2, follicular, and GC states.[29,30] Previously published *in vitro* studies suggest that NF-κB signaling is required for B cell differentiation into T2 and follicular cells.[31] Prior reports suggest that ACTH inhibits NF-κB signaling,[28,31,32] suggesting a potential mechanism by which Acthar might attenuate B cell differentiation in this model. Acthar could also inhibit B cell differentiation by inhibiting the NF-κB-regulated expression of the B-cell activating factor (BAFF) receptor.[33] Published literature suggests that blockade of BAFF-mediated signaling in NZB/W F1 mice results in increased immature and T1 cell populations, as was seen with Acthar treatment.[29,30] Potential Acthar-mediated effects on BAFF signaling are supported by evidence that serum BAFF/BLyS levels were significantly

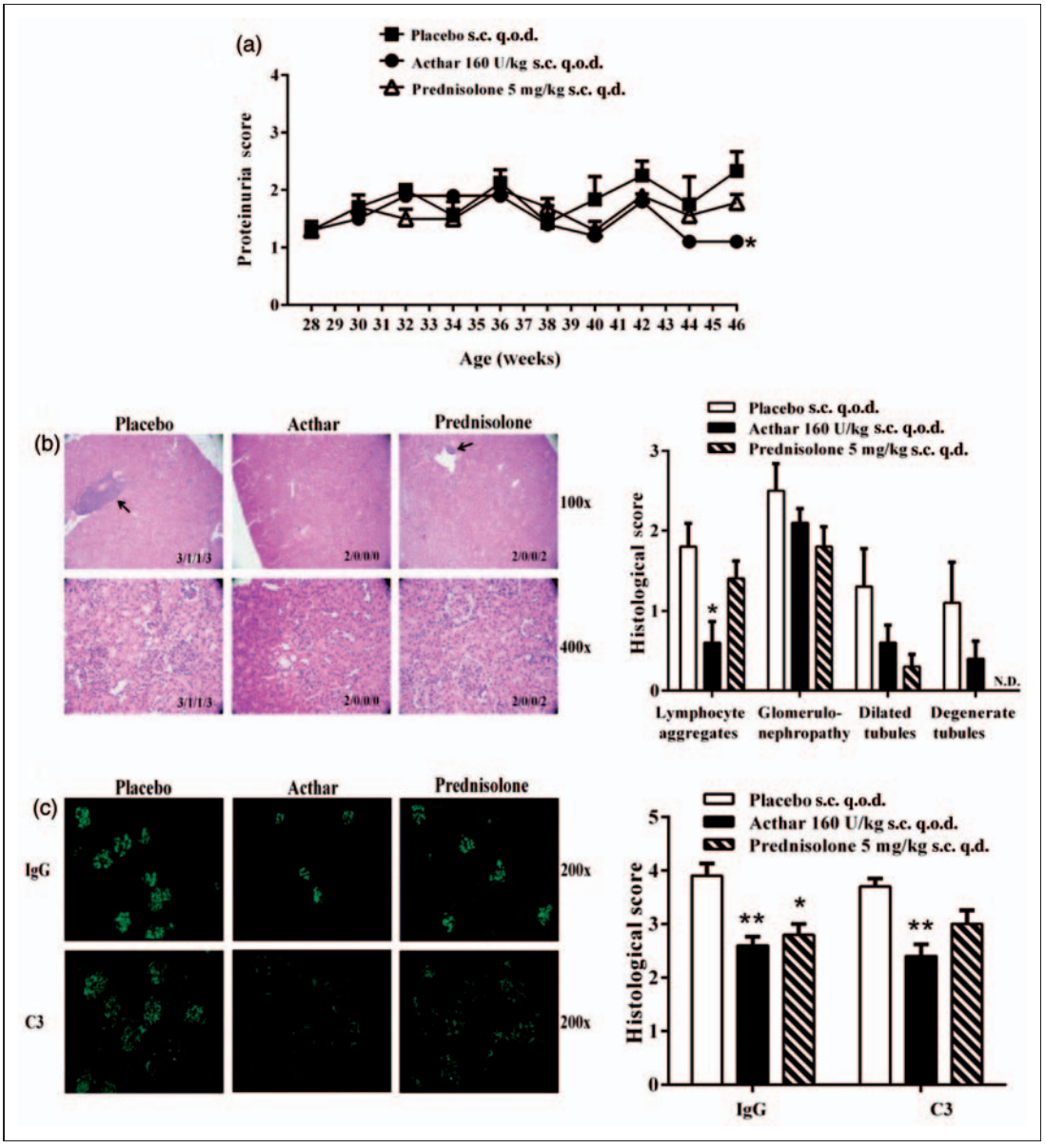

Figure 4 Effects of Acthar on renal endpoints. (a) Proteinuria worsened during the 19-week treatment period in Placebo-treated NZB/W F1 mice, while Acthar-treated animals developed significantly less severe proteinuria over time ($p \leq 0.05$ versus Placebo). Prednisolone did not significantly attenuate proteinuria progression. Values represent mean ± SEM for proteinuria score ($n = 10$/group). (b) Histopathological scoring of kidneys ($n = 10$/group) was performed at 46 weeks of age unless early study termination was necessary. Left panel: representative images for hematoxylin and eosin (H&E)-stained kidney sections for each treatment group. The numbers in black text on each panel denote the semi-quantitative scores for the representative image (glomerulonephropathy/dilated tubules/degenerate tubules/and lymphocyte aggregates). Arrows denote areas of lymphocyte aggregates. Right panel: average semi-quantitative histopathology scores (values represent mean ± SEM). Two H&E-stained kidney sections per animal were scored for glomerulonephropathy, dilated tubules, degenerate tubules, and lymphocyte aggregates using a 0–5 scoring system. Acthar significantly reduced lymphocyte aggregates ($p \leq 0.05$) while non-significant trends of reduced kidney disease severity were seen in all other scored categories compared with Placebo. (c) Scoring of immunohistochemical staining of kidneys for glomerular IgG and C3 deposition ($n = 10$/group) was performed at 46 weeks of age unless early study termination was necessary. Left panel: representative images for glomerular anti-IgG and anti-C3 immunofluorescence staining. Right panel: average semi-quantitative immunofluorescence scores (values represent mean ± SEM). For analysis of immune complex deposition, two fresh frozen kidney sections per animal were stained with anti-IgG or anti-C3 antibodies. Semi-quantitative scoring (0–5) was performed. Acthar treatment significantly attenuated both glomerular IgG ($p \leq 0.01$) and C3 ($p \leq 0.01$) deposition as compared with Placebo. In contrast, prednisolone therapy reduced glomerular IgG deposition ($p \leq 0.05$ versus Placebo) but did not significantly reduce C3 immunofluorescence staining.
N.D.: not detectable

reduced in patients receiving Acthar therapy for opsoclonus myoclonus syndrome.[9,34] The importance of an increased proportion of T1 cells is relevant for treatment of SLE, as sustained clinical remission in patients with SLE was associated with a repopulation of transitional B cells following B cell depletion therapy with rituximab, whereas a rapid repopulation of memory B cells predicted a poor outcome of disease.[35]

Most transitional B cells differentiate either into MZ B cells or follicular B cells,[36] and studies suggest that when B cell maturation is blocked, cells are channeled into the MZ compartment.[37] The increase in MZ B cells seen in Acthar-treated mice therefore suggests a funneling of transitional B cells to the MZ instead of the autoreactive follicular compartment of the spleen.[26] Sequestration and positive selection of autoreactive B cells into the MZ compartment has been recognized in recent studies not only as a tolerance checkpoint,[5,26] but also as a potential mechanism for preventing autoimmunity, leading to decreased SLE propensity in mice.[35,38]

SLE B-cell hyper-responsiveness is correlated with spontaneous GC formation,[1] a process that was suppressed by Acthar treatment in this study. Consistent with the observed increases in immature T1 and MZ B cells, Acthar therapy resulted in a 12% decrease of GC formation in NZB/W F1 mice with already established autoimmune disease. These effects were not seen with prednisolone therapy, and previous reports suggest that other B cell targeted therapies had less robust effects on GC formation in NZB/W F1 mice.[30,39–41] GC B cells further differentiate and undergo clonal expansion, B cell Ig heavy-chain class-switching recombination, and differentiation into long-lived plasma cells generating an autoantibody response.[1,26,42] Although the decrease in GC formation was not correlated with a significant decrease in splenic plasma cell frequency in Acthar-treated animals, this finding is not unexpected as most antibody secreting plasma cells would be expected to localize to the periphery.

In addition to modifying splenic B cell populations and diminishing B cell autoreactivity, Acthar treatment correspondingly prevented progressive disease assessed by measurement of circulating autoantibodies. Titers of total IgG, IgG2a, and IgM were lower in NZB/W F1 mice receiving Acthar, indicating a dampening of the humoral immune response in this model. These observations are supported by previous evidence confirming that ACTH not only binds to B cells, but also significantly attenuates antigen-induced immunoglobulin secretion by B cells.[43,44] In addition, reduced levels of circulating anti-dsDNA IgG, IgG2a, and IgM suggest suppression of autoreactive B cells, which could have attenuated disease severity or progression.[45–47] Treatment with other clinically efficacious therapies has not been associated with such marked effects on circulating autoantibody titers in SLE murine models. For example, BAFF inhibition therapy in NZB/W F1 mice only modestly delayed the increase in total IgM and ds-DNA IgM in this model, and had no significant effects on total IgG or ds-DNA IgG levels.[29,30] Similarly, B cell depletion therapy was not linked with a change in circulating autoantibodies in mice.[6,41] Even the combination of B cell depletion therapy with BAFF blockade in NZB/W F1 mice did not significantly decrease serum autoantibodies.[41]

It has been reported that T cells also play an important role in the autoimmunity that develops in NZB/W F1 mice, as these mice have increasing numbers of $CD4^+$ T cells as they age,[29] and treatment with anti-$CD4^+$ T cell antibody prevented autoimmunity in these mice in association with decreased peripheral $CD4^+$ T cell counts, serum ds-DNA autoantibody titers, and proteinuria.[22,48] Acthar-treatment was associated with a similar significant inhibition of $CD4^+$ T cell frequency and serum dsDNA autoantibody titers that usually accompany aging in this model.[29] $CD4^+$ T cell stimulation is needed for the differentiation of follicular B cells into GC B cells, suggesting that the decreased T cell frequency could be an important modifier of the autoimmune response in these animals.[22,26,42] In comparison, prior studies evaluating the effects of B cell depletion therapy and inhibition of BAFF in NZB/W F1 mice did not demonstrate a similar reduction in the frequency of $CD4^+$ T helper cells.[29,30,41]

The beneficial effects of Acthar treatment were not limited to spleen cell immunophenotyping and reduction of circulating autoantibodies, as Acthar also prevented the development of severe proteinuria in these animals. Severe proteinuria is a measure of glomerulonephritis development and disease severity in NZB/W F1 animals, and has been attributed to increased serum autoantibodies that lead to immune complex deposition, which drives local inflammatory responses and cellular infiltration that lead to tissue damage.[45,47,49] The beneficial effects of Acthar on proteinuria progression may therefore be explained by alterations in several potential mechanistic pathways. First, Acthar reduced glomerular IgG and C3 deposition. Prior studies have demonstrated an association between reduced autoantibodies and improved

proteinuria,[29,46] while other investigations suggest that attenuation of proteinuria progression requires a reduction in both circulating autoantibodies and glomerular IgG deposition.[45] Interestingly, while both prednisolone and Acthar reduced glomerular IgG, only Acthar also significantly reduced circulating autoantibodies and the development of severe proteinuria. Acthar, but not prednisolone, also significantly attenuated glomerular C3 immunostaining. In conjunction with reduced circulating autoantibodies, decreased glomerular C3 deposition has previously been linked with reduced proteinuria in NZB/W F1 mice.[45,47]

Alternatively, prevention of progressive proteinuria might be due to reduced lymphocyte aggregates, as decreased renal lymphocyte infiltration has been associated with disease remission in other mouse models of SLE.[49,50] Another possibility is that the observed improvements in proteinuria might indirectly result from a reduction in the frequency and absolute number of splenic DCs seen in Acthar-treated mice. DCs that migrate into the kidneys of NZB/W F1 mice during active disease can secrete chemokines that attract inflammatory cells, including B and T cells, resulting in local upregulation of inflammatory mediators and renal

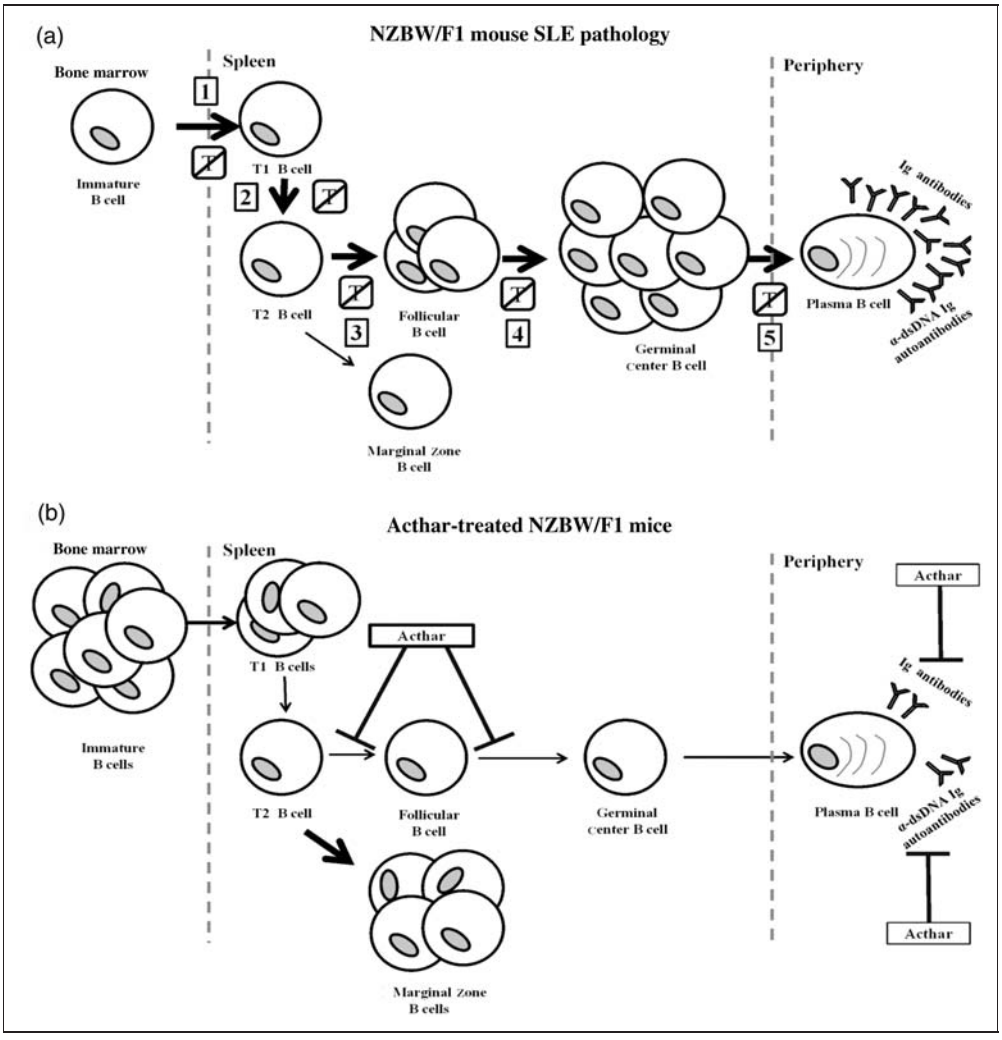

Figure 5 Summary of potential points of Acthar efficacy on systemic lupus erythematosus (SLE) pathology in NZB/W F1 mice. (a) Schematic diagram representing the breakdown of tolerance mechanisms that are thought to contribute to SLE pathology in NZB/W F1 mice. Tolerance checkpoint breakdown is denoted by ⊠ at the following places: 1) maturation of immature B cells to T1 transitional cells and migration from the bone marrow to the spleen; 2) maturation from a T1 transitional B cell to mature T2 transitional B cell; 3) maturation from the T2 transitional cells to MZ or autoreactive follicular B cells; 4) differentiation from autoreactive follicular B cells to clonal expansion in the germinal center; 5) differentiation into long lived plasma cells that secrete autoantibodies into the periphery. (b) Representative schematic diagram demonstrating the observed effects of Acthar at these tolerance checkpoints in NZB/W F1 mice, suggesting multiple points along this pathway at which Acthar may act to restore B cell tolerance and inhibit autoantibody production.

damage.[29,30,46] Finally, Acthar could prevent progression of proteinuria in the NZB/W F1 model via direct effects on podocytes, as prior evidence demonstrates MCR expression on podocytes, as well as protective effects of this drug on proteinuria in both animal and human nephrotic disease.[11,16,18]

Of note, in these experiments, Acthar treatment was not associated with any identified adverse events. In contrast, prednisolone-treated NZB/W F1 mice failed to gain weight throughout the treatment period, and were more likely to require early termination from the study. Notably, one prednisolone-treated animal was removed from study early due to hindlimb paralysis from a spinal fracture, a known complication of glucocorticoid therapy.[51] Although detailed dose–response relationships were not evaluated, differences between Acthar- and prednisolone-treated animals for efficacy outcomes suggest that Acthar and exogenous corticosteroids could modulate inflammation by differing mechanistic pathways.[10,16,27,28]

In summary, the results of the current study highlight that Acthar has profound immunomodulatory activity in NZB/W F1 mice, impacting B cell development, circulating autoantibody titers and renal immune complex deposition, while also attenuating the severity of proteinuria. The B-cell mediated pathophysiology of autoimmunity in these mice is summarized in Figure 5(a), and the multiple tolerance checkpoints present throughout B cell differentiation are identified. Briefly, in NZB/W F1 mice, B cells progress through the tolerance checkpoints freely, resulting in an increased population of autoreactive follicular and GC B cells, which then lead to high levels of autoantibodies in the periphery. As summarized in Figure 5(b), Acthar treatment may restore tolerance checkpoint activity, as demonstrated by increased immature, transitional, and MZ B cell populations, and decreased autoreactive follicular and GC B cells. Decreased autoreactive B cell populations would be predicted to decrease autoantibodies in the periphery, with attenuation of target organ damage. Taken together, these data suggest that Acthar is likely to be an efficacious treatment alternative for patients with SLE, and may have broader implications for the potential of Acthar as a treatment for other autoimmune diseases.

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Conflict of interest statement

CG is an employee of Biomedical Research Models, Inc., the commercial research organization contracted (more than $10,000) to perform the animal experiments. DAD, LO, PMB, DY, SJ are employees of Questcor Pharmaceuticals, Inc. (more than $10,000) and hold stock or stock options (more than $10,000) in Questcor Pharmaceuticals, Inc.

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