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Decrease in Glomerulonephritis and Th1-Associated Autoantibody Production After Progesterone Treatment in NZB/NZW Mice

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Objective. **While estrogen treatment exacerbates disease in models of systemic lupus erythematosus (SLE), the effects of progesterone are unclear. This study was undertaken to assess the effects of continuous progesterone treatment on autoantibody production and spontaneous glomerulonephritis (GN) in a mouse model of SLE.**

Methods. **Female (NZB × NZW)F_1 (NZB/NZW) mice were treated with vehicle, 2 mg of depot medroxyprogesterone acetate (DMPA), or 10 mg of DMPA every 6 weeks. Survival, proteinuria, and serum anti–double-stranded DNA (anti-dsDNA) levels were monitored. At 39 weeks of age, kidneys were analyzed for abnormalities and glomerular accumulation of IgG subclasses and C3. Spleen leukocyte subsets were also analyzed.**

Results. **DMPA treatment reduced mortality in a dose-dependent manner in association with reduced proteinuria and glomerular damage. High-dose DMPA treatment resulted in a reduction of total serum IgG and IgG2a anti-dsDNA antibody levels, whereas IgG1 anti-dsDNA antibody levels were modestly increased. High-dose DMPA reduced glomerular accumulation of IgG1, IgG2a, IgG3, and complement, while low-dose DMPA decreased glomerular IgG2a and IgG3 levels compared with vehicle treatment.**

Conclusion. **Our findings indicate that treatment of premorbid female NZB/NZW mice with DMPA reduces mortality and attenuates spontaneous GN, likely through multiple mechanisms, including altered ratios of protective Th2-related IgG antibodies versus nephritogenic Th1-related IgG autoantibodies. Thus, estrogen and progesterone may have disparate effects on lupus autoimmunity, lending new significance to observed hormonal imbalances in patients with SLE. These data also suggest that treatment of SLE patients with DMPA may have therapeutic benefit.**

Female hormones such as sex steroids are thought to influence disease development in human systemic lupus erythematosus (SLE) because 9 of 10 patients are female, and disease incidence increases rapidly after menarche and falls after menopause. Sex hormones in animals and patients with SLE can influence disease development and activity, respectively (1–3). However, the mechanisms linking sex steroids such as estrogen and progesterone with lupus autoimmunity remain obscure (1).

Many studies have demonstrated the importance of Toll-like receptor (TLR) signaling and type I interferons (IFNs) (IFNα and IFNβ) in the pathogenesis of murine and human SLE (4,5), and we have recently shown that treatment of mice with progesterone can block TLR-induced pathways of IFNα induction (6). Moreover, IFNα, as well as IFNγ, appears to be particularly important in the development of human and murine lupus glomerulonephritis (GN), in part through increased production of highly nephritogenic Th1-related autoantibodies. Interestingly, estrogen treatment of lupus-prone mice causes increased mortality and GN in association with selectively increased serum Th1-induced autoantibody levels (7), perhaps through direct effects on B cells (8) and induction of IFNγ (1).

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Progesterone treatment of mice with lupus can have protective effects, despite increases in serum autoantibody levels (9,10). This apparent inconsistency remains unexplained. However, progesterone treatment of mice can suppress Th1-related antibody production, perhaps through suppression of IFNα and interleukin-12 (IL-12) (6) and direct effects on Th1 cell development (11). This is important with regard to human SLE, in which there is increased estrogen activity (12) along with low progesterone levels (13,14), and in which overexpression of Th1-associated cytokines or IFNα is associated with the development of GN (15–18).

Herein we show that continuous treatment of lupus-prone female (NZB $\times$ NZW)F_1 (NZB/NZW) mice with a commonly used form of progesterone birth control, depot medroxyprogesterone acetate (DMPA) (Depo-Provera; Pfizer, New York, NY), prevents mortality and GN, likely via decreased production and glomerular binding of nephritogenic Th1-related autoantibodies, increased production and binding of protective Th2-related autoantibodies, and antiinflammatory effects in kidneys.

MATERIALS AND METHODS

Mice and hormone treatments. Six-week-old female NZB/NZW mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a specific pathogen–free facility in barrier cages. Beginning at 6 weeks of age, mice (n = 8 per group) received either 2 mg or 10 mg of DMPA diluted to 200 μl in vehicle or an equal volume of vehicle alone subcutaneously every 6 weeks until the end of the study (when mice were age 39 weeks). The vehicle was compounded in a sterile manner based on DMPA carrier components described in the Depo-Provera product information sheet (19).

Measurement of proteinuria. Urine protein was measure by dipstick (Multistix 9; Bayer, Bury St. Edmunds, UK) and quantified as negative (0 mg/dl), trace (30 mg/dl), 1+ (100 mg/dl), 2+ (300 mg/dl) or 3+ (>2,000 mg/dl), according to the recommendations of the manufacturer.

Renal histology and immunohistochemistry. At age 39 weeks, surviving mice were killed and kidneys were isolated. Left kidneys were preserved in 10% formalin and embedded in paraffin. Right kidneys were snap-frozen in liquid nitrogen and TissueTek OCT compound (Sakura Finetek, Torrance, CA) and stored at −70°C. Histopathologic assessments were performed on 4-μm sections of paraffin-embedded, formalin-fixed kidneys stained with hematoxylin and eosin (H&E) or methenamine–silver and trichrome. A total of 25 sequential glomeruli from the superior, middle, and inferior cortices of each kidney (stained with H&E) were scored for damage by an evaluator (GCH) who was blinded with regard to experimental group, on a scale of 0–3+, where 0 = normal, 1+ = segmental hyaline deposits, 2+ = global hyaline deposition, and 3+ = global hyaline deposition plus fibrinoid necrosis or sclerosis. The number of nuclei detected by H&E staining in each glomerulus was also recorded. Kidney cryosections (4 μm) were fixed in ice-cold acetone, washed with phosphate buffered saline, blocked with normal goat serum, and then incubated with fluorescein-conjugated goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-mouse IgG or C3 (Cappel, West Chester, PA), IgG1, IgG2a, or IgG3 (Santa Cruz Biotechnology). Images were obtained using a Nikon Eclipse E600 microscope with a QImaging Retigia EX CCD camera. Relative fluorescence intensity of 25 sequential glomeruli from each section was scored using a scale of 0–3+, where 0 = no apparent staining compared with isotype control and 3+ = maximum staining intensity for a particular IgG subclass among all sections. For Mac-2 staining, paraffin-embedded sections were incubated sequentially with normal horse serum, rat anti-mouse Mac-2 antibody (Cedarlane, Burlington, NC), and horseradish peroxidase–conjugated rat anti-mouse polymer components (Biocare, Concord, CA). The immunoreaction was visualized using the NovaRed kit (Vector, Burlingame, CA). Morphometric analysis of Mac-2–stained sections was performed on 50–100 glomeruli per animal, as previously described (20).

Detection of serum antibodies. Serum anti-DNA IgG and IgM levels were quantified by sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (21). Briefly, polystyrene microtiter plates were coated with calf thymus DNA (Sigma-Aldrich, Bornem, Belgium) overnight at 4°C. After blocking of the plates with 1% bovine serum albumin, test sera were added and incubated at a 1:100–1:2,000 dilution, washed, and reacted with enzyme-conjugated goat anti-mouse IgM, IgG, IgG1, IgG2a, or IgG3 (Sigma-Aldrich and The Jackson Laboratory). The reactions were developed with enzyme substrate, and optical density was determined. For all serum anti–double-stranded DNA (anti-dsDNA) antibody assays, the results were expressed in arbitrary units relative to a standard positive control of pooled sera from 9-month-old MRL/*lpr* mice, where 1,000 units/ml represents equivalent binding. Total serum Ig and subclass levels were quantified by sandwich ELISA as previously described (22).

Isolation of splenocytes and flow cytometry. Spleens were treated with Liberase Blendzyme 2 (Roche, Indianapolis, IN) and Gey's lysis buffer to yield total splenocytes for staining. Cells were stained with fluorescein-conjugated anti-mouse CD11c, CD3, and CD19 (PharMingen, San Diego, CA) and phycoerythrin-conjugated anti-mouse CD40, CD86, programmed death ligand 1 (PDL-1), CD69, or isotype controls (PharMingen) and analyzed on a BD FACScan.

Statistical analysis. Log rank analysis and dose–trend of survival curves and *P* values (unpaired *t*-tests with Welch's correction, single tailed) were generated using GraphPad Prism software, version 5.

RESULTS

Prevention of death and proteinuria in NZB/NZW mice treated with DMPA. To examine the effects of continuous progesterone exposure on spontaneous development of lupus, we injected 6-week-old premorbid female NZB/NZW mice with vehicle, 2 mg of DMPA, or 10 mg of DMPA subcutaneously every 6 weeks. We chose the 2 mg DMPA dose because it is sufficient to suppress anti–herpes simplex virus type 2

immunity and TLR-induced IFNα production in mice (6,23). Based on the results of a prior study (24), we estimated that treatment with 10 mg of DMPA would yield sustained serum medroxyprogesterone levels similar to serum progesterone levels in late human pregnancy (100–200 ng/ml). Similar levels (40–70 ng/ml) are found in patients receiving medroxyprogesterone for recurrent breast cancer (25,26). Therefore, the DMPA doses used in our study likely resulted in physiologically and pharmacologically relevant serum concentrations.

NZB/NZW mice treated with vehicle alone exhibited the expected frequency of mortality (10,27,28); approximately three-quarters of the animals had died by 39 weeks (Figure 1A). In contrast, all of the mice treated with 10 mg of DMPA were alive at 39 weeks, as were the majority of the mice treated with 2 mg of DMPA.

Immune complex (IC) GN and its sequelae are thought to be the main causes of death in female NZB/NZW mice (27). Proteinuria is a measure of GN activity, and in female NZB/NZW mice, its development is closely followed by death (28). To investigate how DMPA treatment might prolong survival, we collected urine at various time points and measured protein content by dipstick. Scores (0–3+) were converted to minimum protein concentrations. At 31, 32, and 34 weeks, DMPA-treated groups showed lower levels of proteinuria than did the vehicle-treated group, which was significant for the DMPA 2 mg group at 34 weeks and for the DMPA 10 mg group at 31 and 34 weeks (Figure 1B). Taken together, these findings suggest that DMPA prevented death by attenuating spontaneous GN in these animals.

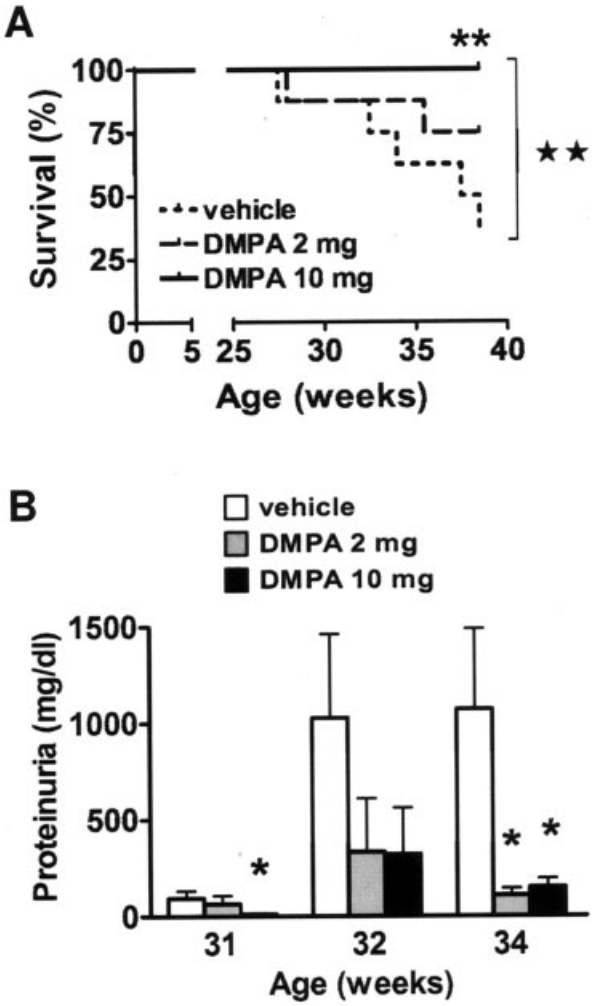

Figure 1. Prevention of death and reduction of proteinuria in (NZB $\times$ NZW)F_1 (NZB/NZW) mice by continuous treatment with depot medroxyprogesterone acetate (DMPA). Prenephritic (6-week-old) female NZB/NZW mice were treated with vehicle, 2 mg of DMPA, or 10 mg of DMPA every 6 weeks. **A**, Survival over 39 weeks. $** = P < 0.01$ versus vehicle, by log rank analysis; ★★ = P for trend $<$ 0.01 by log rank analysis. **B**, Level of proteinuria at indicated ages. Bars show the mean and SEM of multiple determinations in 6–8 animals per time point per treatment group. $* = P < 0.05$ versus vehicle.

Attenuation of development of GN and kidney damage in NZB/NZW mice after DMPA treatment. To evaluate the effects of DMPA on the development of GN, all surviving mice were killed at 39 weeks, and kidney sections were examined by light and fluorescence microscopy. Glomeruli from the vehicle-treated group showed the acute and chronic proliferative GN and structural damage expected in mice of this age, including hyaline "wire loop" lesions, fibrinoid necrosis, cellular crescents, mesangial proliferation, and mesangiolysis (27,28) (Figure 2A). In contrast, many glomeruli from the DMPA 10 mg group showed either normal or mildly abnormal histology, while those from the DMPA 2 mg group showed an intermediate degree of abnormality.

To quantify these changes, we performed blinded scoring of the following key features indicative of late and severe GN in these mice: deposition of proteinaceous hyaline material (which includes IC deposits), fibrinoid necrosis, and glomerulosclerosis (27–29). Together, these features were scored as "glomerular damage." DMPA treatment significantly reduced glomerular damage in a dose-dependent manner (Figure 2B). To examine a feature of early GN in these mice (28), we measured glomerular cellularity (nuclei per glomerulus). Glomerular cellularity was decreased in the DMPA 10 mg group compared with the vehicle group but was increased in the DMPA 2 mg group (Figure 2C). The onset of GN and proteinuria in NZB/NZW mice is marked by renal infiltration of activated macrophages (30). Therefore, we measured glomerular infiltration of

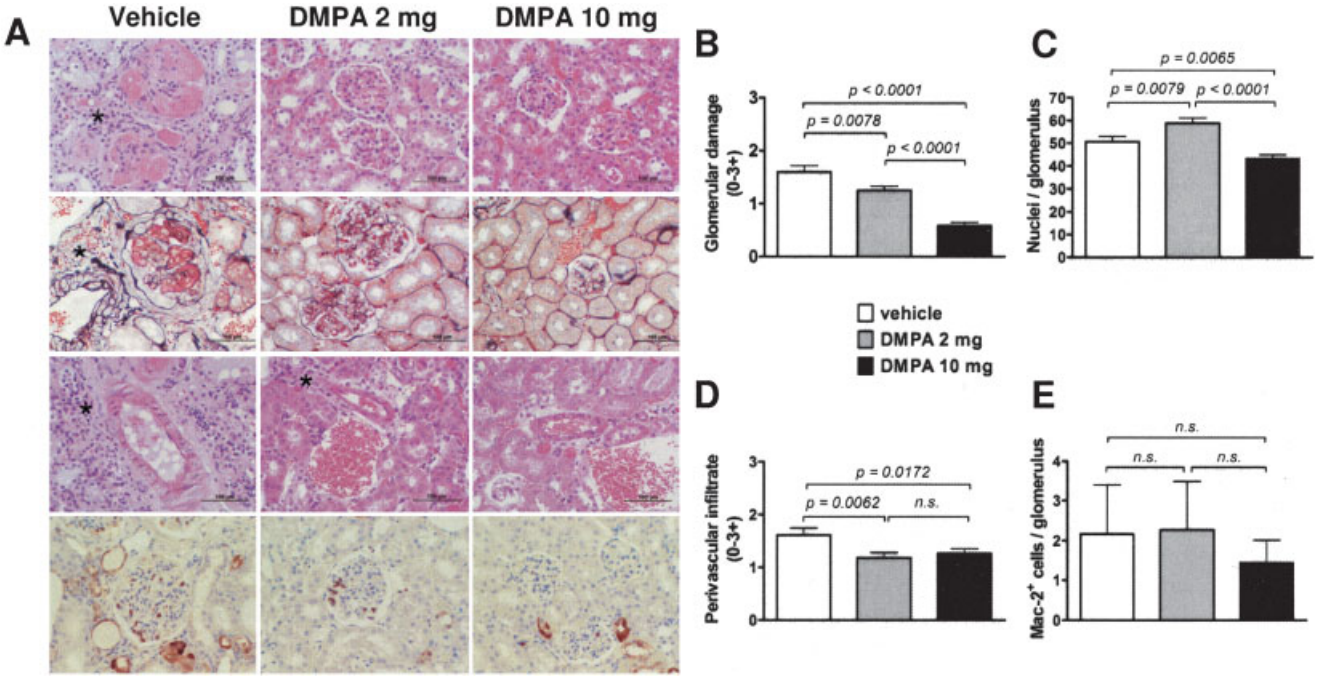

Figure 2. Amelioration of glomerular disease in NZB/NZW mice after DMPA treatment. **A**, Representative hematoxylin and eosin (H&E)–stained glomeruli (row 1), methenamine–silver and trichrome–stained glomeruli (row 2), H&E-stained renal arteries (row 3), and Mac-2–stained glomeruli (row 4). Asterisks show mononuclear cell infiltrate. Bars = 100 mm. (Magnification × 40 in row 4.) **B**, Glomerular damage score. **C**, Glomerular cellularity score. **D**, Perivascular infiltrate score. **E**, Number of Mac-2–positive cells per glomerulus. Extraglomerular Mac-2 staining represents expression of galectin 3 antigen in renal tubules. Bars show the mean and SEM of multiple determinations in 3–8 animals per treatment group. NS = not significant (see Figure 1 for other definitions).

Mac-2–positive macrophages. While the average number of Mac-2–positive cells per glomerulus was lower in the DMPA 10 mg group compared with the vehicle and DMPA 2 mg groups, the difference was not statistically significant (Figure 2E). Perivascular inflammatory cell infiltration is a pathologic finding in kidneys of NZB/NZW mice that does not necessarily correlate with GN (27,28). DMPA treatment significantly decreased periarterial leukocyte infiltration (Figure 2D), indicating extraglomerular hormone effects.

Reduction in glomerular complement activation but not total IgG deposition after DMPA treatment. In lupus nephritis, ICs accumulate in glomerular basement membranes, where they are thought to initiate inflammation by activating complement and/or signaling activatory Fcγ receptors (FcγR) (31–34). To understand how DMPA might decrease GN, we assessed glomerular IgG binding by immunofluorescence microscopy. Global IgG staining (entire glomerulus) was noted in nearly all glomeruli from all mice (Figure 3A). Blinded scoring of total IgG staining intensity revealed increased IgG staining in the DMPA 2 mg group compared with the vehicle group but no difference between the DMPA 10 mg group and the vehicle group (Figure 3B). This indicated that at 39 weeks, the amount of total IgG present in the glomeruli was not correlated with damage (Figure 2B). To assess in situ activation of complement, we stained sections for C3 (Figure 3A). Blinded scoring revealed a significant decrease in average C3 staining in the DMPA 10 mg group compared with the control group (Figure 3C). However, similar to findings with IgG staining, there was increased C3 staining in the DMPA 2 mg group compared with the vehicle group, despite decreased damage observed on light microscopy. This suggested that modulation of complement activation at 39 weeks alone was insufficient to explain the effect of DMPA on GN in these animals.

Selective decrease in production and glomerular binding of Th1-related antibodies and modest increase in serum Th2-related anti-dsDNA levels after DMPA treatment. In NZB/NZW mice, the emergence of serum anti-dsDNA IgG is closely followed by GN, proteinuria, and death (28). Consistent with previous observations (35,36), total serum anti-dsDNA IgG levels peaked in control animals at 30 weeks and decreased thereafter (Figure 4A). The DMPA 10 mg group showed significantly lower anti-dsDNA IgG levels compared with the control group at 32 and 34 weeks. In contrast, the

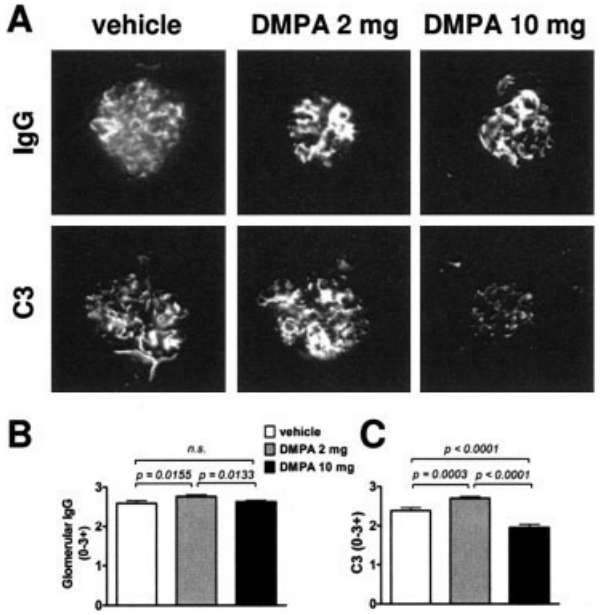

Figure 3. Effects of DMPA treatment on glomerular IgG and C3 staining in kidneys from 39-week-old NZB/NZW mice. **A**, Representative immunofluorescence staining for IgG and C3 (100-msec exposure). **B** and **C**, Aggregate scores for glomerular IgG (**B**) and glomerular C3 (**C**). Bars show the mean and SEM of multiple determinations in 3–8 animals per treatment group. NS = not significant (see Figure 1 for other definitions).

DMPA 2 mg group showed increased levels of serum IgG anti-dsDNA at 34 weeks, although peak anti-dsDNA IgG levels were delayed compared with those in control animals.

In mice, Th1-related anti-dsDNA antibodies are thought to be nephritogenic because they activate stimulatory FcγR (IgG2a) and complement (IgG3) more efficiently than does Th2-related IgG1, which may be protective by preferentially stimulating inhibitory FcγR (31). Since progesterone treatment in mice selectively suppresses Th1-induced antibody production (37), we investigated whether DMPA treatment affected levels of anti-dsDNA IgG subclasses. We quantified serum IgG, IgG1, IgG2a, and IgG3 anti-dsDNA levels from mice at 32 and 34 weeks, before differences in survival were significant. DMPA-treated mice showed increased IgG1 anti-dsDNA levels at 32 weeks (DMPA 2 mg group only) and 34 weeks, but these differences were not statistically significant (Figure 4B). At 34 weeks, the DMPA 10 mg group showed significantly lower levels of IgG2a anti-dsDNA (Figure 4C), suggesting that selective inhibition of Th1-related anti-dsDNA IgG2a at the 10-mg dose accounted for at least some of the differences in total anti-dsDNA IgG. There was a statistically significant increase in IgG3 anti-dsDNA levels in the DMPA 2 mg group, but not in the DMPA 10 mg group, compared with control animals (Figure 4D). Findings of recent studies (21,31) support the notion that increased ratios

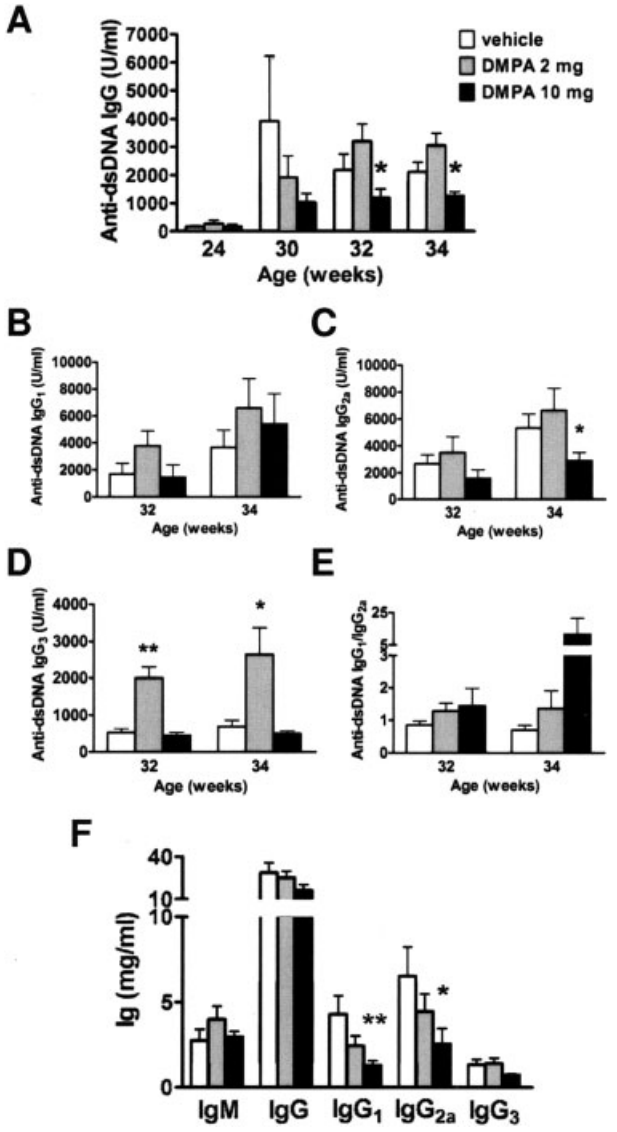

Figure 4. Effects of DMPA treatment on total IgG and IgM concentrations and on serum IgG, IgG1, IgG2a, and IgG3 anti–double-stranded DNA (anti-dsDNA) levels in NZB/NZW mice. Serum anti-dsDNA Ig levels were determined at the ages shown. **A**, Total IgG level. **B**, IgG1 level. **C**, IgG2a level. **D**, IgG3 level. Bars show the mean and SEM arbitrary units based on pooled sera from aged mice with lupus. **E**, Ratio of anti-dsDNA IgG1 to IgG2a in sera. Bars show the mean and SEM. **F**, Total serum IgM, IgG, and IgG subclass concentrations in sera from 34-week-old mice. Bars show the mean and SEM of multiple determinations in 5–8 animals per treatment group. * = $P < 0.05$; ** = $P < 0.01$, versus vehicle. See Figure 1 for other definitions.

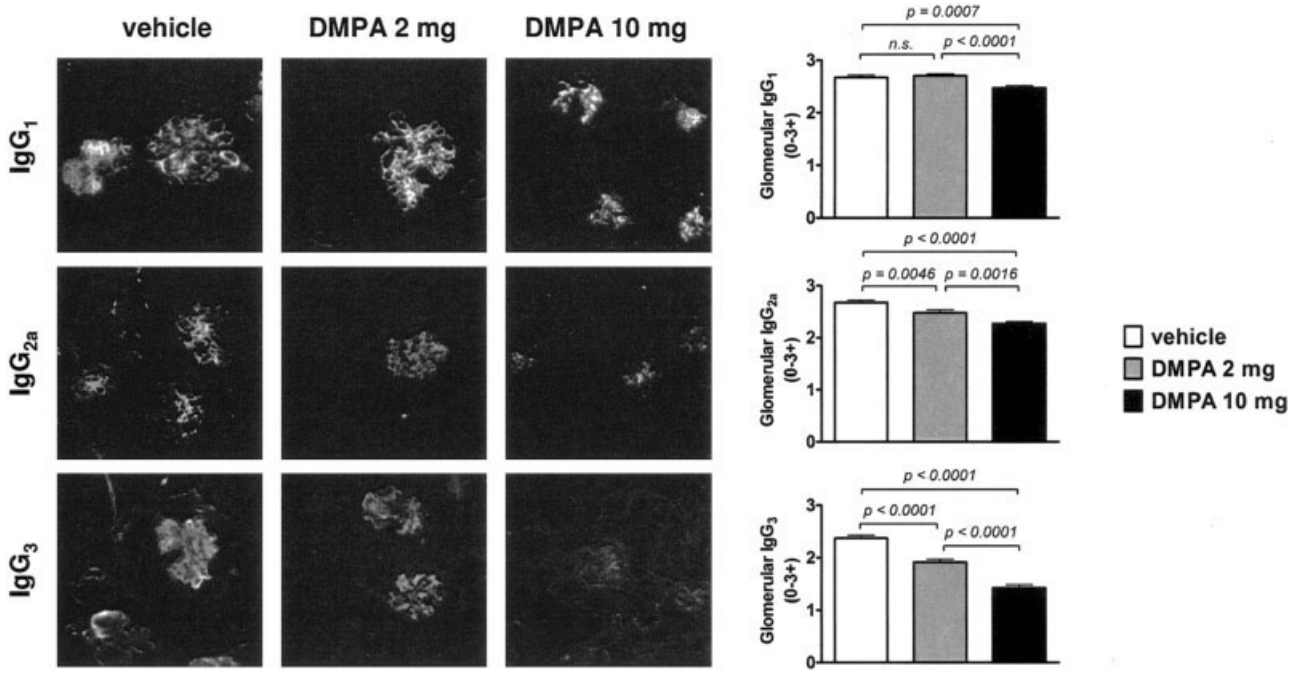

Figure 5. Predominant decrease in IgG2a and IgG3 binding in glomeruli of NZB/NZW mice. **Left**, Representative images showing glomerular staining for various IgG subclasses. Images were obtained using a 60-msec exposure for IgG1 and IgG2a and a 700-msec exposure for IgG3 (magnification × 40). **Right**, Aggregate scores for staining of various IgG subclasses in each treatment group. Bars show the mean and SEM. NS = not significant (see Figure 1 for other definitions).

of IgG1 to IgG2a autoantibodies may limit the extent of IC-mediated GN. We therefore calculated ratios of serum anti-dsDNA IgG1 to IgG2a in each animal and compared treatment groups. DMPA treatment increased the ratio of IgG1 to IgG2a in a dose-dependent manner, although these differences did not reach statistical significance (Figure 4E).

IgM anti-dsDNA antibodies may also protect against nephritis in NZB/NZW mice, by unknown mechanisms (38,39). Thus, we measured serum anti-dsDNA IgM at 32 and 34 weeks. The mice treated with 10 mg of DMPA had lower levels of IgM anti-dsDNA antibodies than did the mice treated with vehicle or 2 mg of DMPA, but the differences were not statistically significant (data not shown).

To assess whether DMPA effects were specific to anti-dsDNA responses, we measured concentrations of total IgM, IgG, and IgG subclasses in sera at 34 weeks (Figure 4F), when significant differences in anti-dsDNA IgG, IgG2a, and IgG3 levels were observed. Similar to anti-dsDNA IgG2a, the level of total serum IgG2a in the DMPA 10 mg group was significantly reduced. In contrast, in the DMPA 2 mg group, no consistent relationship between alterations in levels of serum total versus anti-dsDNA IgM, IgG, or IgG subclass was observed. In view of the inhibitory effects of IgG1, it is interesting to note that, despite a reduction in total serum IgG1, IgG1 anti-dsDNA levels actually increased, although the increase did not reach statistical significance.

To assess whether DMPA treatment also affected glomerular accumulation of IgG subclasses, we stained kidney sections for IgG1, IgG2a, and IgG3. Unlike serum IgG1 anti-dsDNA levels, glomerular IgG1 staining was significantly reduced in mice treated with 10 mg of DMPA (Figure 5). Consistent with lower serum IgG2a anti-dsDNA levels, glomerular IgG2a staining was also decreased in mice treated with 10 mg of DMPA. Despite undiminished serum anti-dsDNA IgG2a in the DMPA 2 mg group, glomerular IgG2a staining was reduced in this treatment group compared with the control group. Similarly, IgG3 staining was markedly diminished, in a dose-dependent manner, by DMPA treatment. Taken together, these data indicate that treatment with DMPA predominantly decreased glomerular accumulation of pathogenetic Th1-related IgG2a and IgG3 subclasses, whereas Th2-associated IgG1 was altered to a lesser degree.

Reduction of the expression of CD86 on dendritic cells (DCs) by DMPA. To identify which cell types DMPA might act on in order to modulate autoimmune

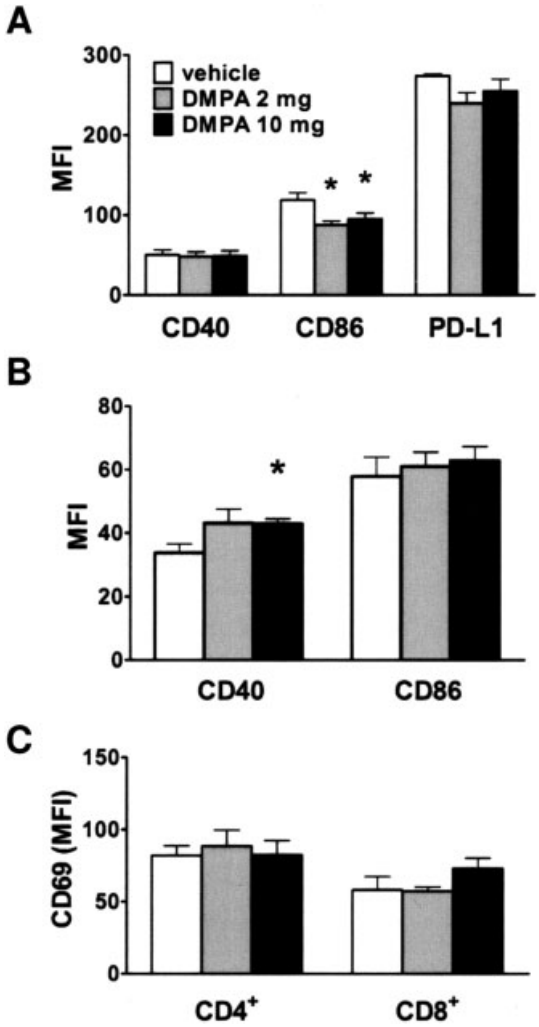

Figure 6. Effects of DMPA treatment on splenocyte activation in NZB/NZW mice. **A**, Expression of CD40, CD86, and programmed death ligand 1 (PDL-1) on spleen CD11c+ myeloid dendritic cells. **B**, CD40 and CD86 expression on spleen CD19+ B cells. **C**, CD69 expression on spleen CD3+CD4+ and CD3+CD8+ T cells. Bars show the mean and SEM fold increase in mean fluorescence intensity (MFI) compared with isotype staining control in 3–8 animals per treatment group. $* = P < 0.05$ versus vehicle. See Figure 1 for other definitions.

responses, we assessed activation of spleen leukocytes. Although there was a trend toward decreased numbers of spleen myeloid DCs, plasmacytoid DCs, T cells, and B cells in the DMPA 10 mg group compared with the control group, the differences were not statistically significant (data not shown). When cell activation markers were assessed, spleen CD11c+ DCs from treated mice showed significantly lower expression of the marker CD86 than did CD11c+ DCs from control mice; no differences were seen in the levels of the costimulatory receptor CD40 or the inhibitory molecule PDL-1 (Figure 6A). These findings are consistent with our previously published observations of impaired CD86 up-regulation in spleen CD11c+ DCs in virally infected mice treated with DMPA (6).

DMPA is known to directly affect B cell function (40); therefore, we also examined B cell phenotypes. B cells from the DMPA 10 mg group showed significantly higher expression of CD40 (Figure 6B), a molecule important for B cell isotype class switching and germinal center formation (41). Unlike the finding with DCs (Figure 6A), there was no difference between treatment groups in B cell expression of CD86 (Figure 6B). Finally, there were no differences in the expression of the activation marker CD69 on either CD4+ or CD8+ spleen T cells (Figure 6C). These data suggest that in this model, DMPA may modulate autoimmune responses in part through its effects on DCs.

DISCUSSION

Progesterone has antiinflammatory and immunosuppressive properties and is believed to play an important role in antiviral immunity and maternal–fetal tolerance (1,42). In models of immunity, progesterone treatment can suppress production of Th1-related subclasses and increase production of Th2-related subclasses (38,40). How progesterone does this is unclear but may involve direct effects on T cells and B cells or suppression of DC-derived cytokines important in Th1 immunity, such as IFNα and IL-12 (1,42).

Female sex hormones are strongly implicated in the pathogenesis of SLE. While estrogen has been studied in SLE models and to a lesser degree in SLE patients, far less attention has been paid to progesterone. Mounting evidence suggests that estrogen and progesterone have disparate, sometimes opposing, effects on inflammation, immunity, and autoimmunity (1). Closer examination of the effects of progesterone on lupus autoimmunity can help identify female-specific factors in pathogenesis and identify novel approaches to disease prevention and therapy. The data presented here extend the findings of previous experiments involving progesterone-treated NZB/NZW mice, offer insight into hormonal regulation of lupus autoimmunity, and provide a rationale for the therapeutic use of DMPA and other forms of progesterone in SLE patients.

The prolonged survival time observed in our experiments differs from the results of 2 previous studies of progesterone treatment in female NZB/NZW mice, which showed increased autoantibody levels despite small effects on survival (9,10). Several factors could

account for this difference. In the present study, we provided continuous hormone treatment at a cumulative dose of up to 6 times greater than the one-time dose used in the previous studies. Furthermore, progesterone treatment increased mortality in mice that had been subjected to ovariectomy (10) but had at least some protective effects in intact mice (ref. 9 and present report); thus, ovarian factors may be necessary for progesterone to have a protective effect. For example, ovarian estrogen could, as in reproductive tissue, regulate progesterone receptor (PR) expression in immune cells (43).

In our study, DMPA treatment limited anti-dsDNA IgG responses in the DMPA 10 group but appeared to only delay them in the DMPA 2 mg group. In the DMPA 10 mg group, there was a decrease in both anti-dsDNA IgG and total IgG levels (which was not statistically significant), suggesting that suppression of IgG production was not specific to anti-dsDNA responses. Increased levels of protective serum anti-dsDNA IgM did not appear to contribute to the effects of DMPA on GN, since anti-dsDNA IgM levels were unchanged or slightly decreased by hormone treatment. Overall, these data suggest that DMPA treatment can both delay and limit pathogenetic autoimmune responses in NZB/NZW mice.

In mice, IgG2a signals activatory FcγRIV, while IgG1 preferentially engages inhibitory FcγRIIb (31). IgG3 is the most potent subclass for activating complement, possibly through self aggregation (34). Interestingly, treatment of NZB/NZW mice with exogenous estrogen receptor ligands increases mortality and GN in association with selective increases in Th1-related serum IgG2a and IgG3 anti-dsDNA levels (7,44). In contrast, progesterone treatment of mice suppresses Th1-related cytokines (6) and antibody responses (37) while enhancing Th2-related responses (40), likely through direct actions on T cells (11), B cells (40), and DCs (1). Consistent with the results of those studies, we observed markedly reduced GN in association with predominant suppression of serum IgG2a anti-dsDNA levels in the DMPA 10 mg group. Interestingly, at 34 weeks, serum anti-dsDNA IgG1 levels were maintained or slightly elevated despite decreased total serum IgG1 levels, suggesting that DMPA treatment specifically affected autoimmune responses involving the IgG1 subclass. Though not statistically significant, analysis of ratios of serum anti-dsDNA IgG1 to IgG2a from individual mice yielded a consistent pattern of regulation. Taken together, these data suggest that DMPA treatment may limit GN by increasing the ratio of protective IgG1 autoantibodies to nephritogenic IgG2a autoantibodies. It is unknown whether the inhibitory effects of DMPA on macrophage FcγR expression (45) are specific to activatory versus inhibitory subtypes, but combined down-regulation of activatory FcγR and suppression of IgG2a could greatly limit inflammation and damage induced by ICs.

Taken together, our results and previously published data (1) suggest that progesterone and estrogen have opposing effects on Th-related anti-dsDNA responses. Consequently, the high ratios of estrogen to progesterone observed in SLE patients could influence the development of nephritis by altering the relative abundance of nephritogenic versus protective autoantibodies, and progesterone supplementation or DMPA treatment may reverse this tendency.

DMPA treatment had marked effects on renal pathology. Glomerular damage was significantly reduced, in a dose-dependent manner, as was extraglomerular inflammation. IgG2a comprises nearly 70% of the IgG eluted from kidneys of aged female NZB/NZW mice (46) and therefore likely represents the major pathogenetic subclass. Accordingly, absolute reduction of glomerular IgG2a binding represents a likely mechanism of protection against GN in DMPA-treated mice. Similar to the increased ratio of anti-dsDNA IgG1 to IgG2a in sera of DMPA-treated mice, IgG1 binding in glomeruli from both treatment groups appeared to be diminished to a lesser degree than did IgG2a binding; there was a dose-dependent trend toward increased glomerular IgG1/IgG2a binding, though the differences were not statistically significant (data not shown).

The importance of decreased glomerular IgG3 binding in DMPA-treated mice is unclear. IgG3 efficiently activates complement, so it was not surprising that C3 staining was decreased in the DMPA 10 mg group, in which IgG3 binding was also clearly decreased. However, C3 staining was significantly increased in the DMPA 2 mg group. Increased complement activation in this group could be due to binding of unmeasured complement-activating IgG2b, which accounts for up to 20% of renal IgG in aged NZB/NZW mice (46), or due to direct effects of DMPA at this dose on complement levels or activity. In any case, these data provide evidence against the notion that decreased complement activation is a sufficient mechanism to explain DMPA amelioration of GN in these experiments.

Decreased periarterial inflammation indicated that DMPA had antiinflammatory effects beyond the glomerulus, although how this relates to survival remains unclear. DMPA 10 mg also reduced glomerular macrophage infiltration, although this reduction was not statistically significant. Nevertheless, these results suggest

that DMPA might affect inflammatory cell recruitment, an effect that would be enhanced by the known ability of DMPA to decrease macrophage FcγR expression and therefore IC engagement in vivo (45).

DMPA may have indirect effects on autoimmunity via suppression of endogenous estrogen levels. In humans, DMPA treatment causes hypoestrogenemia (47). In NZB/NZW mice, estrogen treatment increases autoimmunity and Th1-related anti-dsDNA antibodies. However, reduction of endogenous estrogen levels by ovariectomy does not alter spontaneous disease in NZB/NZW mice (36). So, while suppression of endogenous estrogen cannot be the sole mechanism of disease reduction in our experiments, increased ratios of progesterone versus estrogen may be an important factor in determining autoantibody responses, GN, and survival.

The cellular targets of DMPA in this model are unknown. PRs are expressed by several immune cell types, including T cells, B cells, natural killer cells, and DCs, all of which may contribute to lupus autoimmunity (1). As mentioned above, progesterone may act directly on T cells to suppress Th1 differentiation and functions, so characterizing Th cell cytokine production in this model is an important line of future investigation. DMPA also enhances total and Th2-induced antibody responses in vivo, likely involving direct effects on B cells (40). DCs are important in both T-dependent and T-independent antibody responses, so direct hormone actions on DCs could contribute to the effects of DMPA on autoimmunity in NZB/NZW mice. Consistent with this, spleen DCs from DMPA-treated mice expressed significantly lower levels of surface CD86 than did DCs from vehicle-treated mice (Figure 6). In mice that do not have lupus, DMPA impairs TLR-7–induced IFNα production by plasmacytoid DCs (6), one of the pathways that is aberrantly activated by ICs in human lupus, as well as virus-induced up-regulation of CD86 on spleen DCs. Whether progesterone also regulates this pathway in NZB/NZW mice remains to be determined.

Increased CD40 expression on spleen B cells was observed in the DMPA 10 mg group. This observation may be unrelated to the beneficial effects of high-dose progesterone, or, since CD40 expression on B cells is important for induction of Th2-associated antibodies (48), increased CD40 expression could be related to continued expression of IgG1 anti-dsDNA antibodies. Determining direct and indirect effects of DMPA on the functions of B cells, T cells, and DCs, particularly hormone effects on TLR signaling and abnormally high IFNα activity recently observed in NZB/NZW mice (49), is an important future goal for understanding how endogenous and exogenous forms of progesterone regulate lupus autoimmunity.

In summary, our findings indicate that treatment of premorbid female NZB/NZW mice with DMPA prolongs survival and attenuates GN in older mice through several mechanisms, including suppression of nephritogenic Th1-related IgG subclasses, enhancement of protective Th2-induced subclasses, and, likely, local antiinflammatory effects. This contrasts starkly with the effects of estrogen treatment in these same mice. Thus, the relative contributions of estrogen versus progesterone may influence lupus disease development, particularly GN, lending new significance to hormonal abnormalities observed in SLE patients. Moreover, these data suggest that treatment of SLE patients with DMPA, a widely used, safe, and available agent, may have therapeutic benefit.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Hughes had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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