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Research Article

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Genetic Dissection of SLE Pathogenesis

Sle1 on Murine Chromosome 1 Leads to a Selective Loss of Tolerance to H2A/H2B/DNA Subnucleosomes

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Abstract

One of the hallmarks of SLE is the loss of tolerance to chromatin. The genes and mechanisms that trigger this loss of tolerance remain unknown. Our genetic studies in the NZM2410 lupus strain have implicated genomic intervals on chromosomes 1 (*Sle1*), 4 (*Sle2*), and 7 (*Sle3*) as conferring strong lupus susceptibility. Interestingly, B6 mice that are congenic for *Sle1* (B6.NZMc1) have elevated IgG antichromatin Abs. This study explores the antinuclear antibody fine specificities and underlying cellular defects in these mice.

On the B6 background, *Sle1* by itself is sufficient to generate a robust, spontaneous antichromatin Ab response, staining Hep-2 nuclei homogeneously, and reacting primarily with H2A/H2B/DNA subnucleosomes. This targeted immune response peaks at 7–9 mo of age, affects both sexes with equally high penetrance (> 75%), and interestingly, does not "spread" to other subnucleosomal chromatin components. *Sle1* also leads to an expanded pool of histone-reactive T cells, which may have a role in driving the anti-H2A/H2B/DNA B cells. However, these mice do not exhibit any generalized immunological defects or quantitative aberrations in lymphocyte apoptosis. We hypothesize that *Sle1* may lead to the presentation of chromatin in an immunogenic fashion, or directly impact tolerance of chromatin-specific B cells. (*J. Clin. Invest.* 1998. 101:1362–1372.) Key words: autoimmunity • anti-DNA • chromatin • lupus • nucleosomes

Introduction

Several pathogenic mechanisms have been described to act in concert to generate SLE (lupus) (for reviews see references 1 and 2). One of the hallmarks of this disease, both in humans and mice, is the loss of tolerance to nuclear antigens. The dominant presence of serum autoantibodies against the exposed, conformational epitopes on chromatin strongly suggests that the pathogenic immune response in lupus is driven by chromatin (3–6). The demonstration of T cells specific for chromatin components that can drive the antinuclear antibody (ANA)[1] producing B cells in both murine (7, 8) and human (9, 10) lupus further illustrates how loss of B cell and T cell tolerance to chromatin constitutes a central feature of lupus.

The genes and mechanisms that trigger the loss of tolerance to chromatin remain unknown. Recent gene mapping studies using microsatellite markers have identified at least nine different loci that confer lupus susceptibility (for reviews see references 11 and 12). Our genetic studies of lupus have focused on the NZM2410 strain, an acutely lupus-prone strain derived from a cross between NZB and NZW. In this strain, genomic intervals on chromosomes 1, 4, and 7 are strongly linked to lupus nephritis (13). Notably, in this backcross analysis, *Sle1* on telomeric chromosome 1 (with peak linkage at D1MIT15) is strongly associated with glomerulonephritis (χ^2 = 36.7; $P < 10^{-6}$; LOD score = 10.12). To elucidate the mechanisms by which *Sle1* confers lupus susceptibility and to facilitate the eventual identification of the culprit genes within this interval, a 37-cM NZM2410-derived chromosome 1 interval has been backcrossed successfully onto the normal, C57BL/6 background (14). Initial characterization of these congenic mice, B6.NZMc1, revealed that they have elevated total serum IgG and Abs to chromatin and double-stranded DNA (dsDNA), but not to two control antigens, DNP-Keyhole limpet hemocyanin (KLH) and thyroglobulin (15). Despite the presence of serum ANAs, these mice are relatively healthy, with < 20% of them exhibiting mild (defined as having < 10% of the glomeruli being affected) glomerulonephritis.

This study explores the fine specificity of the ANAs in these mice and the associated underlying cellular defects by comparing a large cohort of B6.NZMc1 mice to age-matched B6 mice. On the B6 background, *Sle1* by itself is sufficient to generate a strong, spontaneous, humoral ANA response, staining Hep-2 nuclei homogeneously, and reacting primarily with H2A/H2B/DNA subnucleosomes. This targeted immune response peaks at 7–9 mo of age, affects both sexes with equally high penetrance (> 75%), and interestingly, does not "spread" to other subnucleosomal components on chromatin. *Sle1* also leads to an expanded pool of histone-reactive T cells, which may have a role, in vivo, in driving the anti-H2A/H2B/DNA B cells. However, these mice do not exhibit any generalized immunological defects or quantitative aberrations in lymphocyte apoptosis. We hypothesize that *Sle1* may lead to the presentation of chromatin in an immunogenic fashion or directly impact tolerance of chromatin-specific B cells.

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1. *Abbreviations used in this paper:* ANA, antinuclear antibodies; APC, antigen-presenting cell; dsDNA, double-stranded DNA; KLH, Keyhole limpet hemocyanin; L.E., lupus erythematosus; PI, propidium iodide.

Methods

Mice. C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred in our animal colony. The derivation of the B6 congenic mice bearing NZM2410-derived lupus-susceptibility intervals, including the B6.NZMc4 and B6.NZMc1 mice, has been detailed previously (14). B6 or B6.NZMc4 mice (which are congenic for *Sle2* but do not differ from B6 mice with respect to the phenotypes described in this communication) were used as experimental controls. B6.NZMc1 mice are C57BL/6 mice congenic for a 37-cM interval representing a 95% confidence interval flanking *Sle1* on murine chromosome 1, with termini at D1MIT101 and D1MIT155 (13). The entire *Sle1* interval is derived from the NZW parent of the NZM2410 strain. Although it is possible that more than one gene within this interval may be contributing to the observed phenotypes, *Sle1* is described in this communication as if it were a single locus. All mice used for this study were bred and housed under identical conditions at the University of Florida Department of Animal Resources mouse colony. Since sex did not affect the phenotypes detailed here, both male and female mice were used for the experiments. Nevertheless, for the in vivo antigen challenge experiments, age- and sex-matched mice were used.

Immunofluorescence assay. Immunofluorescence assay for ANAs was performed as described previously (16). Briefly, Hep-2 cell coated slides (T Cell Diagnostics, Cambridge, MA) were incubated with B6, B6.NZMc1, or NZM2410 sera at dilutions of 1:15, 1:40, and twofold dilutions thereof, till the end point, for 30 min, washed and developed with a 1:100 dilution of FITC-coupled goat anti–mouse IgG (γ-chain specific; Sigma Chemical Co., St. Louis, MO), and then viewed with a fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) under ultraviolet illumination. The titer (i.e., the highest dilution that still gives positive nuclear staining) and pattern of immunofluorescence were recorded.

Cell preparation. Splenocytes were depleted of red blood cells using 0.83% NH_4Cl, and single-cell suspensions were prepared. Splenic antigen-presenting cells (APCs) were prepared as described before (7), using pretitrated amounts of anti-Thy1.2 (Accurate Chemicals, New York) and rabbit complement (Accurate Chemicals) to lyse T cells, and then irradiated at 1,000 rads before use. These cells were typically 85–95% positive for the B cell marker, B220. To prepare B-enriched splenocytes for helper coculture assays, anti-CD4 and anti-CD8 (PharMingen, San Diego, CA) were used in addition to anti-Thy1.2, for T cell depletion. Splenic T cells were prepared as described (7). In brief, red cell–depleted splenocytes were loaded onto nylon wool columns (Robbins Scientific, Sunnyvale, CA) and incubated at 37°C for 45 min. Nonadherent cells were washed through, and then incubated with pretitrated amounts of anti–I-A^b (clone K25-8.7; Accurate Chemicals) and anti-CD24 (PharMingen) on ice for 45 min. Ab-bound cells were lysed with rabbit complement (Accurate Chemicals), yielding T cells with > 90% purity. Prepared single-cell suspensions were counted and used for flow cytometric analysis or culture, as described below.

Flow cytometry. FACS® was performed as described previously (17). All primary Abs were obtained from PharMingen and were used at pretitrated dilutions. Briefly, cells were first blocked with staining medium (PBS, 5% horse serum, 0.05% azide) containing 10% normal rabbit serum. Cells were then stained on ice with optimal amounts of FITC, phycoerythrin, or biotin-conjugated primary mAbs diluted in staining medium for 30 min. After two washes, biotin-conjugated Abs were revealed using streptavidin-phycoerythrin (GIBCO BRL, Gaithersburg, MD) or streptavidin-Quantum red (Sigma Chemical Co.). Cell staining was analyzed using a FACScan® (Becton Dickinson, San Jose, CA). Dead cells were excluded on the basis of scatter characteristics and propidium iodide (PI) uptake, and 10,000 events were acquired per sample.

Proliferation and antigen response assays. $F(ab')_2$ goat anti–mouse IgM (Cappel, Durham, NC) and LPS (Sigma Chemical Co.) were used for B cell stimulation, at concentrations from 0.1 to 100 μg/ml. For the T cell antigen response assays, splenic T cells (5×10^5/well) were cultured with irradiated, syngeneic splenic APCs (5×10^5/well) for 72 h in 200-μl vol cultures, in serum-free HL-1 medium (HyCor Biomedicals, Irvine, CA) with or without added antigens. dsDNA (Sigma Chemical Co.), dissolved in PBS, and filtered through cellulose acetate before use), a control nonnuclear antigen, thyroglobulin (Sigma Chemical Co.), histones H1, H2A, H2B, H3, and H4 (all from Boehringer Mannheim, Indianapolis, IN), and chromatin (prepared as described in reference 7) were added directly to the culture wells at an optimal concentration of 1 μg/ml, as described before (7). In some assays, splenic APCs were first preincubated with antigens at l μg/ml, washed, and then cocultured with splenic T cells. T cell response was assessed either by assaying the extent of proliferation, using tritiated thymidine incorporation (ICN Biomedicals, Costa Mesa, CA), over the last 18 h of culture, or by measuring the amount of IL-2, IL-4, or IFN-γ secreted 48 h after culture, by ELISA.

IL ELISAs. IL produced in culture was assayed by ELISA, as described previously (8). The following reagents were purchased from PharMingen: anti–IL-2, anti–IL-4, anti–IFN-γ, biotinylated anti–IL-2, biotinylated anti–IL-4, biotinylated anti–IFN-γ, and recombinant IL-2, IL-4, and IFN-γ standards. Briefly, capture anti-IL (-2, -4 or IFN-γ) coated Immulon-I plates (Dynatech, Chantilly, VA) were blocked, and then incubated for 2 h with culture supernatants (diluted 1:2), or serial dilutions of the recombinant IL standard. Captured IL was detected using biotin-coupled anti-IL, avidin-alkaline phosphatase (1:10,000; Sigma Chemical Co.) and pNPP substrate (Sigma Chemical Co.). Optical densities were converted to nanograms per milliliter using the derived standard curve.

Helper assays. The ability of splenic T cells to drive B cell autoantibody production in vitro was assessed by coculture assays, as described before (17). Briefly 0.5×10^6 splenic T cells were cocultured with 0.5×10^6 B cells in 200-μl culture volumes of HL-1 medium, at 37°C. Supernatants were harvested 7 d after culture, diluted 1:8 with ELISA sample buffer, and assayed for anti-H2A/H2B/DNA Ab levels by ELISA, as described below.

ELISA. The anti-dsDNA, antichromatin, and antihistone ELISAs were carried out as described before (7). Antisubnucleosome Abs were detected as described (18, 19), with some modifications. In brief, mBSA-precoated Immulon-II (Dynatech) plates were coated overnight with 50 μg/ml dsDNA dissolved in PBS and filtered through cellulose acetate before use (Sigma Chemical Co.), and then postcoated with 10 μg/ml of either histone H1, a 1:1 mixture of histones H2A:H2B, a 1:1 mixture of histones H3:H4, or total histones (which is a mixture of all histones), overnight at 4°C. All histones were purchased from Boehringer Mannheim. "H2A/H2B/DNA" refers to dsDNA postcoated with a mixture of histones H2A and H2B, with a similar nomenclature being adopted for the other subnucleosomes. These different nucleosomal antigens (free histones, dsDNA, H2A/H2B/DNA, H3/H4/DNA, H1/DNA, and free histone/DNA) were coated onto serial wells of the same ELISA plates, permitting the relative reactivities of any given serum to these different epitopes to be compared. The concentrations of antigens used in these ELISAs have been shown to be sufficient to saturate all available binding sites (18). After blocking with PBS/3% BSA/0.1% gelatin/3 mM EDTA, 1:100 dilutions of the test sera were incubated for 2 h at room temperature. Bound IgG was detected using alkaline phosphatase–conjugated anti–mouse IgG (γ-chain specific; Jackson ImmunoResearch, West Grove, PA) and pNPP substrate. Raw optical densities were converted to units per milliliter, using a standard curve derived from an NZM2410-positive control serum, arbitrarily setting the reactivity of a 1:100 dilution of this serum to 100 U/ml. This control serum showed equally strong reactivities (optical densities) to all six tested antigens, again allowing the relative reactivities of the test sera to the different antigens to be compared with each other. Penetrance was defined as the percentage of mice at each age group exhibiting serum ANA levels at least two standard deviations above the mean ANA levels in B6 mice, as determined for a panel of 11 7–9-mo-old B6 mice.

Anti-DNP Abs in the sera of DNP-KLH immunized mice were assayed by reacting 1:100 dilutions of the test sera on DNP-BSA (Calbiochem, La Jolla, CA) coated Immulon-II plates (Dynatech), and revealing any bound Abs using alkaline phosphatase–conjugated anti–mouse IgM or anti–mouse IgG, and pNPP substrate.

Absorption ELISA. Sera exhibiting reactivity to chromatin, histones/DNA complex, or H2A/H2B/DNA subnucleosomes were subjected to cross-absorption studies, performed as described previously (6), to determine if the observed anti-H2A/H2B/DNA reactivity was indeed due to the presence of anti-dsDNA or antihistone Abs in the serum, and if the antichromatin Abs in B6.NZMc1 mice recognized epitopes other than those present on the H2A/H2B/DNA subnucleosomes. Briefly, 1:100 dilutions of the test sera were serially incubated four times (1 h each) on fresh antigen-coated ELISA wells, and then reacted on ELISA wells coated with the second antigen, in parallel with unabsorbed serum controls. The ELISA was developed as described above. The percentage of antigen reactivity retained after absorption, relative to that of the unabsorbed serum, was calculated for each individual serum sample. Control assays show that total histone-coated wells have the ability to absorb away at least 84% of the reactivity due to a control antihistone Ab (Boehringer Mannheim), and likewise, dsDNA-coated wells absorb away at least 77% of the reactivity due to a control anti-dsDNA Ab (Boehringer Mannheim).

Apoptosis assays. Splenocytes, splenic B cells, or splenic T cells were purified as described above, and cultured at 37°C for 24 h. Aliquots of this culture were assayed at 2-, 6-, and 24-h time points for the percentage of apoptotic cells in culture, using PI, as described previously (20). Briefly, the cell pellets were resuspended in hypotonic PI solution (3.4 mM sodium citrate, 50 μg/ml PI, 0.1% Triton X-100, 1 mM Tris, 0.1 mM EDTA), and analyzed by FACS®, using logarithmic scales. Apoptotic nuclei were distinguished by their hypodiploid DNA content and scatter characteristics, after excluding the debris.

ELISA to measure free chromatin. The amount of cell-free chromatin in sera and culture supernatant was measured with a sandwich ELISA using mouse mAbs directed against DNA and histones, respectively. All reagents were purchased from Boehringer Mannheim and used following the manufacturer's instructions. Briefly, Immulon-I plates were coated with antihistone Ab and then blocked. Sera diluted 1:100, or cell-free splenic culture supernatants diluted 1:20, were incubated on these plates for 90 min at room temperature. Culture supernatants used in these assays were harvested 6 h after culture of 5×10^6 splenocytes/ml in PBS. After washing, the plates were incubated with peroxidase coupled anti-DNA Ab, and finally developed with ABTS substrate. The amount of free chromatin was determined from their OD_{405} values, using a standard curve constructed using serial dilutions of chromatin, prepared as described before (7).

In vivo assays. 2-mo-old B6 or B6.NZMc1 mice (three mice per group) were injected intraperitoneally with DNP-KLH (100 μg/mouse; Calbiochem) in complete Freund's adjuvant, and boosted 2 wk later with 100 μg/mouse DNP-KLH in incomplete Freund's adjuvant. The mice were killed 5 d later. Immunized mice were assessed for serum antihapten titers (preimmune and postchallenge) and for splenic germinal centers by immunohistochemistry.

Statistics. Data obtained for the B6.NZMc1 mice were compared with those from the B6 mice using the Student's *t* test.

Results

Homogeneous nuclear staining. In contrast to B6 sera, sera from 6–9-mo-old B6.NZMc1 mice ($n = 20$) showed positive homogeneous nuclear staining of Hep-2 nuclei (Fig. 1), with titers typically ranging from 1:320 to 1:1280, with a geometric mean of 1:784. Positive nuclear staining of Hep-2 nuclei correlated closely with the presence of antichromatin and anti-H2A/H2B/DNA autoantibodies (see below). Sera from younger (1–3-mo-old) B6.NZMc1 mice typically did not stain nuclei positively. Unlike the NZM2410 sera, B6.NZMc1 sera do not stain any cytoplasmic antigens.

Selective reactivity to H2A/H2B/DNA subnucleosomes. Our earlier work documented that sera from B6.NZMc1 mice bound strongly to chromatin, but not to several nonnuclear antigens, including thyroglobulin, myosin, DNP-KLH, and ovalbumin (reference 15 and Mohan, C., unpublished observations). Also, the immunofluorescence assay described above demonstrates that B6.NZMc1 sera react strongly to nuclear but not cytoplasmic antigens. Since studies in murine and human lupus have demonstrated how the antinuclear antigenic fine specificities in lupus evolve over time (5, 6, 21), we wanted to define the subnucleosomal structures that the ANAs in B6.NZMc1 sera preferentially recognize. Sera from B6, B6.NZMc1, and NZM2410 mice were monitored for reactivity to the following six subnucleosomal structures, over a spectrum of ages: free histones, free dsDNA, H2A/H2B/DNA complex, H3/H4/DNA complex, H1/DNA complex, and total histone/DNA complex, as detailed in Methods.

As shown in Fig. 2 *A*, the parental NZM2410 strain typically exhibits high titers of IgG ANAs directed against all six nuclear antigens. The mean (±1 standard deviation) levels in 5–8-mo-old NZM2410 mice ($n = 9$) are 18.9±10.9 U/ml antihistone Abs, 22.1±13.4 U/ml anti-dsDNA Abs, 48.9±22.3 U/ml anti-H2A/H2B/DNA Abs, 39±21.7 U/ml anti-H3/H4/DNA Abs, 53.2±17.4 U/ml anti-H1/DNA Abs, and 50.8±20.2 U/ml antihistone/DNA Abs. Typically, the NZM2410 mice exhibit maximal titers of ANAs at this age, in which period also > 80% of these mice succumb to lupus nephritis (22).

Sera from B6.NZMc1 congenic mice exhibit strong reactiv-

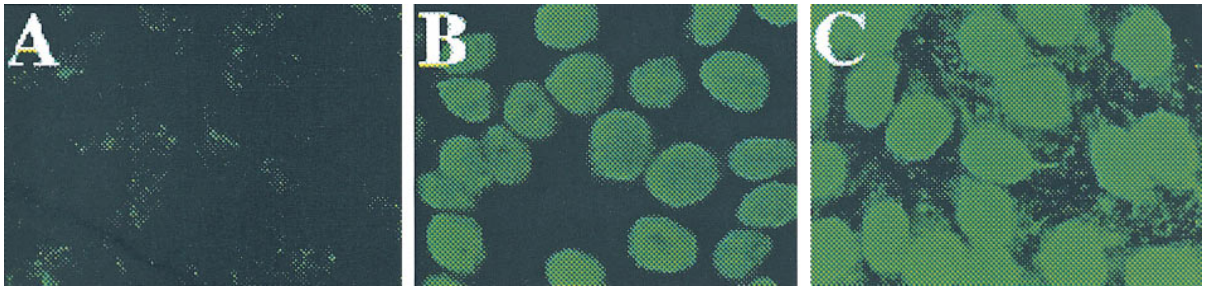

Figure 1. Immunofluorescence staining of Hep-2 cells. Sera from 6–9-mo-old B6 mice (*A*), B6.NZMc1 mice (*B*), and NZM2410 mice (*C*) were reacted with Hep-2 cells. Bound IgG was revealed using FITC-coupled anti–mouse IgG. Shown staining patterns (at a dilution of 1:15) are representative of other age-matched B6 ($n = 10$), B6.NZMc1 ($n = 20$), and NZM2410 ($n = 4$) sera. The actual titers are detailed in the text. ×60.

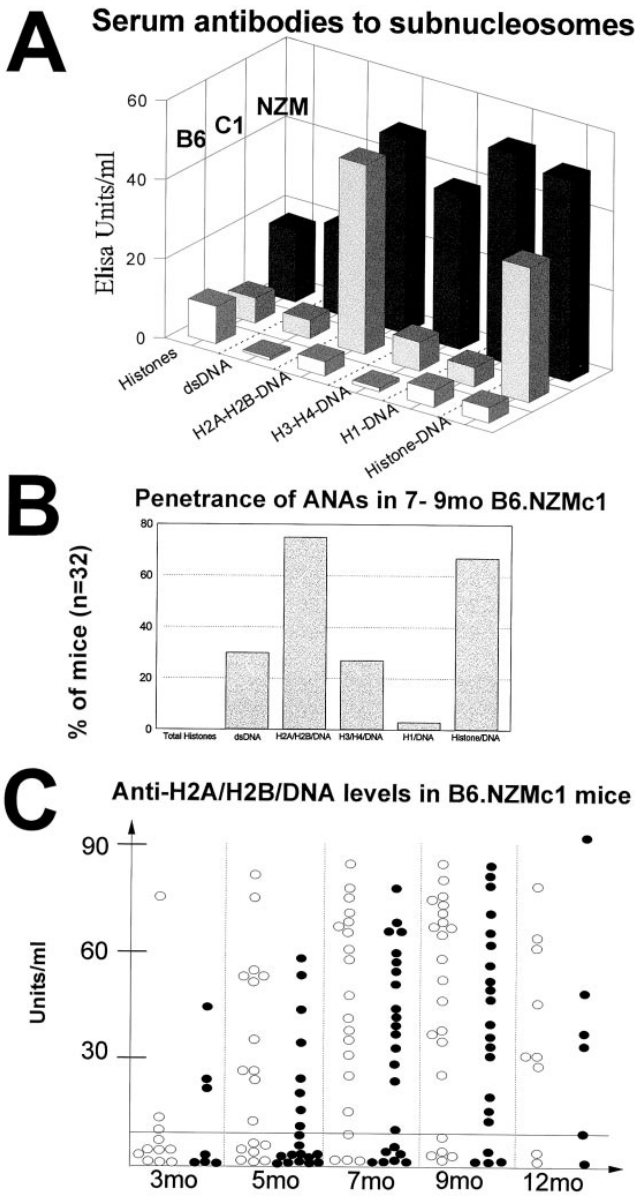

Figure 2. Antisubnucleosome Ab profile in B6.NZMc1 mice. (*A*) Sera from a panel of 7–9-mo-old B6 mice (*white bars*, $n = 11$), 7–9-mo-old B6.NZMc1 mice (*gray bars*, $n = 32$), and 5–8-mo-old NZM2410 mice (*black bars*, $n = 9$) were assayed by ELISA for their serum levels of ANAs directed against histones, dsDNA, H2A/H2B/DNA, H3/H4/DNA, H1/DNA, and histone/DNA complex, as described in Methods. Shown are the mean ANA levels. The standard errors are detailed in Results. (*B*) B6.NZMc1 sera with ANA levels in excess of two standard deviations above the mean level found in a panel of 11 B6 mice were scored as positive. This figure depicts the penetrance (percentage of mice that scored positive) for each of the six ANAs, at the age of 7–9 mo. (*C*) Female (*open dots*, $n = 9$–22) and male (*closed dots*, $n = 6$–23) B6.NZMc1 mice at various ages were assayed for the serum levels of anti-H2A/H2B/DNA Abs. The dotted line represents the cutoff that was used to define which sera were positive. This value represents two standard deviations above the mean anti-H2A/H2B/DNA Ab level in a panel of 11 7–9-mo-old B6 mice.

ity to histone/DNA complex (33.9±27.1 U/ml) and H2A/H2B/DNA subnucleosomes (47.8±27 U/ml), with penetrance approaching 80% (Fig. 2, *A* and *B*). The latter figures represent the mean (±1 standard deviation) levels in 7–9-mo B6.NZMc1 mice ($n = 32$). The levels of these ANAs are not significantly different from the NZM2410 levels, but are significantly higher than the levels in B6 mice ($P < 0.0001$). B6 mice were typically negative for these ANAs even past the age of 12 mo (data not shown). As depicted in Fig. 2 *C*, serum anti-H2A/H2B/DNA is detectable as early as 5 mo of age, peaks at 7–9 mo of age, and wanes thereafter, with a similar pattern for antihistone/DNA Abs (not shown). Females acquire this phenotype somewhat earlier than males: the penetrance of this phenotype in 5-mo-old females is significantly higher than in age-matched males (61 vs. 40%, $n = 18$–20; $P < 0.04$). However, by 7–9 mo of age, both males and females attain equally high penetrance and serum levels (Fig. 2 *C*).

In contrast, compared with the NZM2410 strain, B6.NZMc1 mice exhibit 6–10-fold lower levels of antihistone Abs (6.2±4.7 U/ml, $P < 0.005$), anti-dsDNA Abs (4.7±12.3 U/ml, $P < 0.002$), anti-H3/H4/DNA Abs (7.1±13.4 U/ml, $P < 0.001$), and anti-H1/DNA Abs (4.7±5.1 U/ml, $P < 0.001$). Nevertheless, B6.NZMc1 serum levels of these Abs are still significantly higher than those of B6 controls. Owing to the low-grade seroreactivity, the calculated penetrance appears to be sensitive to the actual reactivities recorded in B6 controls. Thus, though we had earlier reported that as high as 60–80% of B6.NZMc1 sera exhibit anti-dsDNA Abs (15), in this study only $\sim$30% of the 7–9-mo-old B6.NZMc1 mice studied exhibit anti-H3/H4/DNA and anti-dsDNA Ab levels significantly higher than the B6 controls, as depicted in Fig. 2 *B*. Thus, in contrast to the strong, highly penetrant reactivity to H2A/H2B/DNA subnucleosomes, *Sle1* leads, at best, to a low-grade humoral responsiveness to other subnucleosomal structures, with a varying penetrance.

It can also be surmised from Fig. 2 *A* that several B6.NZMc1 sera that are strongly positive for anti-H2A/H2B/DNA do not show any significant reactivity to dsDNA or free histones, suggesting that these sera preferentially target unique epitopes generated only when dsDNA and histones are put together. This is confirmed by the cross-absorption studies depicted in Fig. 3 which demonstrate that preabsorption of these sera with dsDNA or histones does not significantly reduce the reactivity to H2A/H2B/DNA subnucleosomes: B6.NZMc1 sera still exhibit $\sim$ 68% (SEM = 3.9%; $n = 15$ sera) of their H2A/H2B/DNA reactivity after removing anti-dsDNA Abs, and $\sim$ 85% (SEM = 6.9%; $n = 15$ sera) of their H2A/H2B/DNA reactivity after removing antihistone Abs. As a control, preabsorption with H2A/H2B/DNA subnucleosomes reduces H2A/H2B/DNA reactivity to significantly lower levels (mean = 18%; SEM = 1.5%; $n = 15$ sera; $P < 0.0005$). In addition, it appears that the antichromatin, antihistone/DNA, and anti-H2A/H2B/DNA Abs in B6.NZMc1 sera recognize the same epitope(s), since preabsorption with any one of these three antigens shuts down the reactivity to the other two (Fig. 3 *B* and data not shown). In summary, *Sle1* by itself is sufficient to drive the production of high titers of antichromatin ANAs primarily targeting conformational determinants on H2A/H2B/DNA subnucleosomes, while sparing other determinants.

B6.NZMc1 mice have activated T cells. B6.NZMc1 mice do not develop any splenomegaly or lymphadenopathy. FACS®

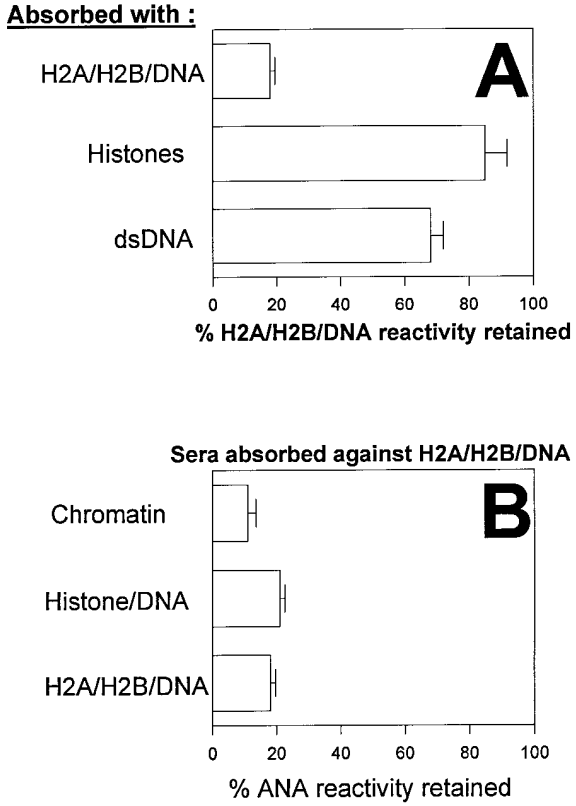

Figure 3. The relationship of anti-H2A/H2B/DNA Abs to the other ANAs. (*A*) B6.NZMc1 sera that were positive for anti-H2A/H2B/DNA Abs were absorbed either with H2A/H2B/DNA complex or with free histones, or with dsDNA, and then assayed for their anti-H2A/H2B/DNA reactivity by ELISA, in parallel with unabsorbed serum controls. The extent of H2A/H2B/DNA reactivity retained after absorption with the antigens was expressed as a percentage of H2A/H2B/DNA reactivity exhibited by the unabsorbed control. The bars represent the mean percentage (±SEM) recorded for a panel of 15 7–9-mo-old B6.NZMc1 sera. (*B*) The same panel of 7–9-mo-old B6.NZMc1 sera were first absorbed against H2A/H2B/DNA complex, and then assayed for their reactivity to chromatin, histone/DNA complex, or H2A/H2B/DNA subnucleosomes by ELISA, in parallel with unabsorbed controls. The shown percentage of retained seroreactivity represents the mean (±SEM) values obtained for the panel of 15 sera.

analysis of their marrow, thymi, spleens, lymph nodes, and peritoneal cavities reveals that they do not differ from B6 controls in the numbers or percentages of B1 or B2 cells, CD4 or CD8 T cells, or macrophages. However, as shown in Fig. 4, beginning at ~ 3 mo of age, B6.NZMc1 spleens and lymph nodes exhibit increasing numbers of $CD4^+$ T cells bearing the activation marker CD69. Whereas ~ 12% (range = 8.8–13.9%; n = 11) of 3–6-mo-old B6 or B6.NZMc4 splenic T cells express CD69, significantly more age-matched B6.NZMc1 mice (mean = 18.4%; range = 9.1–29.2%; n = 8; $P < 0.02$) express this activation marker. Likewise, 12-mo-old B6.NZMc1 mice had significantly higher levels of splenic $CD69^+$ CD4 T cells (mean = 33.5%; range = 19.4–50.5%; n = 6; $P < 0.03$), compared with age-matched B6 or B6.NZMc4 mice (mean = 20.5%; range = 14.5–32.4%; n = 18). Although a similar pattern was observed with CD25 expression, the levels did not reach statistical significance. Also, no significant difference was noted in the activation status of B6.NZMc1 B cells or $CD8^+$ T cells, compared with B6 lymphocytes (data not shown).

B6.NZMc1 T cells respond to core histones. Histone and chromatin specific T cell response has been demonstrated previously in murine and human lupus (7–10). To determine if the activated $CD4^+$ T cells seen in B6.NZMc1 mice are also chromatin/histone specific, splenic or lymph node $CD4^+$ T cells from B6.NZMc1 mice of varying ages were monitored for their proliferative and IL production response to chromatin, histones, and two control antigens, dsDNA and thyroglobulin. B6 and B6.NZMc1 splenic T cells did not show any appreciable proliferative response to dsDNA or thyroglobulin. They had a mean (±1 standard deviation) stimulation index of 1.13 (±0.25). These data were obtained from six independent proliferation assays. This serves as a background against which to gauge the proliferative response to the tested nuclear antigens. As shown in Fig. 5, B6.NZMc1 splenic T cells show significantly stronger proliferation to the core histones, H2A ($P < 0.003$), H2B ($P < 0.03$), H3 ($P < 0.03$), and H4 ($P < 0.02$), compared with age-matched B6 mice (n = 9–11 mice, for each strain). B6.NZMc1 splenic T cells also showed a similar level of proliferative response to chromatin and histone/DNA complex (data not shown). A similar pattern of response was seen with B6.NZMc1 lymph node T cells (not shown).

In addition, compared with age-matched B6 mice, B6.NZMc1 T cells also secrete significant amounts of IFN-γ in

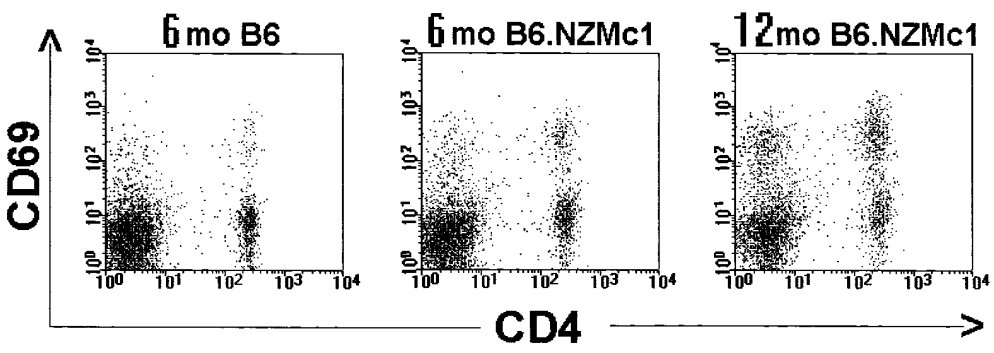

Figure 4. Enhanced CD69 expression by B6.NZMc1 splenic $CD4^+$ T cells. The dot plots depict the CD69 expression levels on splenic $CD4^+$ cells from 6-mo-old B6 mice (*left*), 6-mo-old B6.NZMc1 mice (*middle*), and 12-mo-old B6.NZMc1 mice (*right*). The mean percentages of $CD4^+$ T cells expressing CD69 are 12 and 20.5% in 6- or 12-mo-old control mice (B6 or B6.NZMc4), respectively, and 18.4 and 33.5% in 6- or 12-mo-old B6.NZMc1 mice, respectively, as detailed in Results.

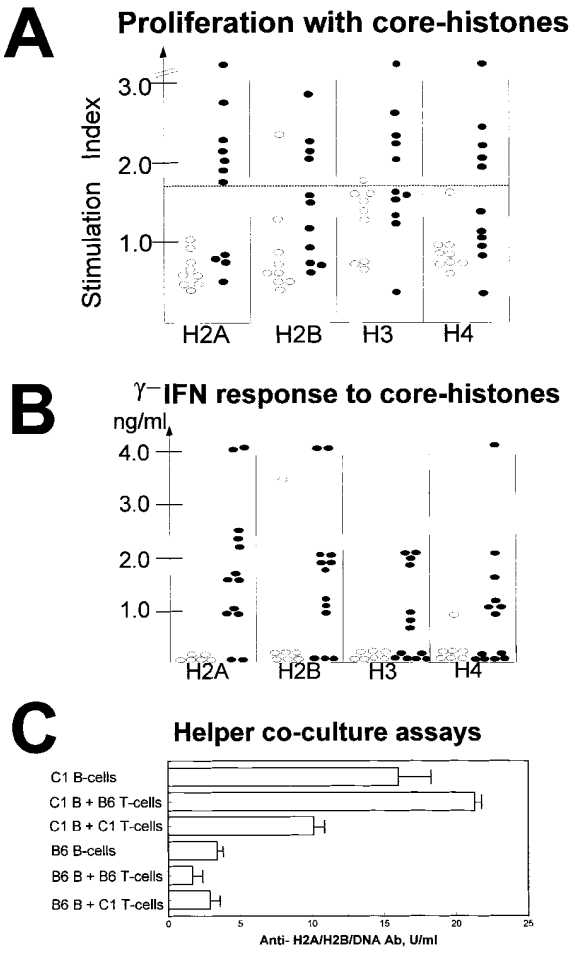

Figure 5. Chromatin/histone reactive T cells in B6.NZMc1 mice. (*A*) Splenic T cells from 3–7-mo-old B6 mice (*open dots*, $n = 9$) or B6.NZMc1 mice (*closed dots*, $n = 11$) were cocultured with irradiated, syngeneic splenic T-depleted APCs, with 1 μg/ml of histones H2A, H2B, H3, H4, dsDNA, or thyroglobulin. Simulation index represents the cpm values in stimulated cultures divided by the cpm values in antigen-free cultures. The dotted line represents the mean (±2 standard deviations) stimulation index recorded from culture wells stimulated with the control antigens, dsDNA, or thyroglobulin, obtained from six independent proliferation assays. Unstimulated cultures typically had backgrounds of 1,000–2,000 cpm. Each dot represents the mean of duplicate or triplicate cultures. (*B*) Splenic T cells from 3–7-mo-old B6 mice (*open dots*, $n = 7$) or B6.NZMc1 mice (*closed dots*, $n = 13$) were cocultured with irradiated, syngeneic splenic T-depleted APCs, with 1 μg/ml of histones H2A, H2B, H3, or H4. The amount of IFN-γ secreted 48 h after culture was assayed by ELISA. Each dot represents the mean of duplicate or triplicate cultures. (*C*) Splenic T cells from an anti-H2A/H2B/DNA seropositive B6.NZMc1 mouse (or a seronegative B6 mouse) were cocultured with splenic B cells from B6 or B6.NZMc1 mice, and 7-d postculture supernatants were assayed for anti-H2A/H2B/DNA Ab levels by ELISA. Each bar represents the mean (±SEM) of triplicate cultures. The experiment shown is representative of three independent experiments involving B cells from strongly or mildly seropositive B6.NZMc1 mice.

response to the core histones H2A ($P < 0.02$), H2B ($P < 0.05$), H3 ($P < 0.005$), and H4 ($P < 0.02$), as detailed in Fig. 5 *B*, and to chromatin and histone/DNA complex (data not shown). The amount of IFN-γ secreted by B6 and B6.NZMc1 T cells in response to the control antigens dsDNA and thyroglobulin is not significantly above the background levels in unstimulated cultures (< 0.1 ng/ml). Although the responding T cells also secrete IL-2 in culture, the levels and penetrance were not as strong as that of their IFN-γ response. B6.NZMc1 and B6 T cells secreted little or undetectable amounts of IL-4 in culture, even in response to anti-CD3 stimulation. In general, the proliferative and IL secretion response to the histones was most prominent in seropositive mice, with little or no response seen in 1–3-mo-old seronegative mice (data not shown).

Helper coculture assays. To determine if the histone/chromatin reactive T cells had the ability to "drive" syngeneic B cells to produce IgG ANAs in vitro, splenic T cells from 3–7-mo-old seropositive B6.NZMc1 mice that did secrete IL in response to chromatin/histones were cocultured with self or syngeneic B cells for 7 d, to assay ANAs secreted into culture. As shown in Fig. 5 *C*, in all instances, B cells from these mice, cultured alone, secreted maximal amounts of IgG anti-H2A/H2B/DNA Abs, which could not be "driven" any further by B6.NZMc1 T cells. Coculturing T cells (or purified $CD4^+$ T cells) from these mice with B cells from strongly or mildly seropositive B6.NZMc1 mice also yielded similar results (data not shown, see Discussion).

B6.NZMc1 mice show no generalized immune dysfunction or hyperactivity. Thus far, the B cell and T cell responses of B6.NZMc1 mice appear to be targeting chromatin. To confirm that *Sle1* does not lead to generalized immune hyperactivity, the in vitro response of B6.NZMc1 B cells and T cells to stimuli, and their in vivo response to challenge with a nonnuclear antigen, DNP-KLH, were assessed. B6.NZMc1 mice do not differ significantly from B6 mice in their splenic B cell proliferative response to LPS or $F(ab')_2$ anti-IgM, their splenic T cell proliferative response to anti-CD3 or IL-2 (data not shown), or in their in vivo IgM and IgG antihapten response upon an-

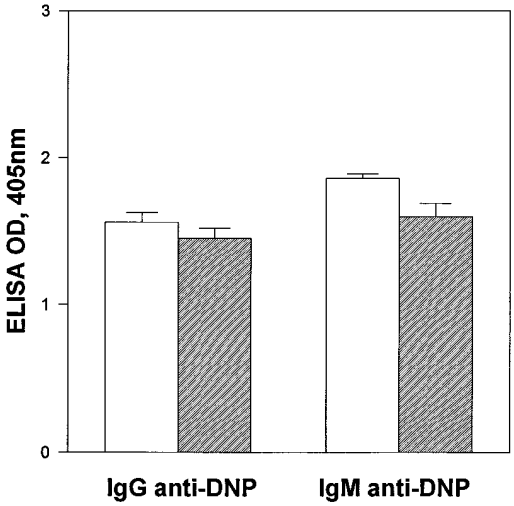

Figure 6. Humoral immune response after challenge with DNP-KLH. 2-mo-old B6 (*shaded bars*) or B6.NZMc1 mice (*open bars*) immunized and boosted with DNP-KLH in adjuvant were monitored for their serum IgM and IgG antihapten levels, 5 d after challenge. The bars represent the mean (±SEM) antihapten levels recorded for three mice, for each experimental group. Both B6 and B6.NZMc1 preimmune sera typically do not react with this hapten (ODs < 0.1, data not shown).

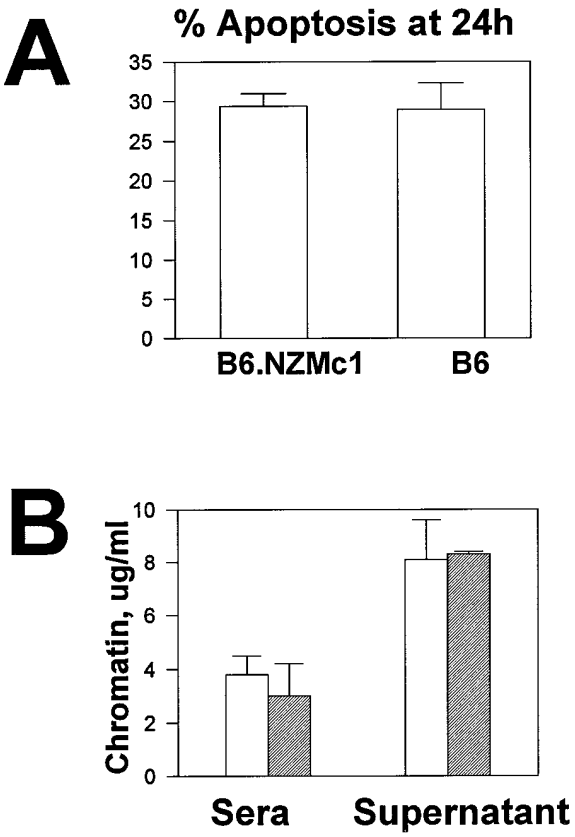

Figure 7. B6 and B6.NZMc1 mice exhibit similar levels of apoptosis and cell-free chromatin. (*A*) Splenic B cells from 3–7-mo-old B6 (n = 10) or B6.NZMc1 (n = 8) mice were cultured and then assayed for the percentage of apoptosis, at various time points after culture. The shown bars represent the mean (±SEM) percentage of apoptosis 24 h after culture. (*B*) Sera or splenic 6-h culture supernatants were assayed for levels of cell-free chromatin by ELISA, as detailed in Methods. Shown are the mean (±SEM) levels of free chromatin in the sera and culture supernatant of 3–7-mo-old B6 or B6.NZMc4 mice (n = 3–6, *shaded bars*), and B6.NZMc1 mice (n = 3–6, *open bars*), and these data are representative of three independent experiments.

tigenic challenge with DNP-KLH (Fig. 6). Both B6 and B6.NZMc1 spleens displayed germinal centers at time of killing, which were similar in frequency and appearance, as determined by immunohistochemistry (data not shown).

B6.NZMc1 mice have normal rates of apoptosis and normal levels of free chromatin in their serum and culture supernatant. Since B6.NZMc1 mice show selective, spontaneous autoimmunization with chromatin, we wished to determine if *Sle1* escalates the generation (or the steady-state levels) of chromatin, which might then be presented in an immunogenic fashion, leading to a break in tolerance to chromatin. To accomplish this, we determined the rates of spontaneous and anti-IgM induced apoptosis of splenic B cells, and the levels of free chromatin (presumably arising from apoptosing cells) in their sera and splenic culture supernatant, as detailed in Methods.

As shown in Fig. 7 *A*, B6.NZMc1 splenic B cells had similar rates of spontaneous apoptosis as B6 mice. There was also no significant difference in the rates of anti-IgM–induced splenic B cell apoptosis or spontaneous splenic T cell apoptosis (data not shown). Similar results were obtained when apoptosis was assessed using annexin binding or the TUNEL technique (using reagents from R & D Systems, Minneapolis, MN, and Boehringer Mannheim, data not shown). Finally, the levels of cell-free chromatin in the sera and 6-h splenic culture supernatant of B6.NZMc1 mice did not significantly differ from those of B6 (Fig. 7 *B*). Thus, *Sle1* does not appear to cause any increased chromatin generation or impaired chromatin clearance, in vivo, as determined by the steady-state levels of chromatin in the sera and culture supernatants of these mice.

Discussion

In 1948, Hargraves and colleagues discovered and described the "lupus erythematosus" (L.E.) factor as a pathognomonic feature of SLE (23). Studies in the 1950s established that the L.E. factor is indeed an Ab directed against nuclear nucleoprotein (3, 24, 25). Succeeding studies by several investigators documented the presence of ANAs in lupus, reacting strongly with chromatin but not with isolated histones or DNA (4, 5, 26–29).

Further analysis of these antinucleosome or antichromatin ANAs was facilitated by the use of different components of chromatin as ELISA substrates. Chromatin is composed of a repeating array of nucleosomes (30). Within a nucleosome, two each of the four core histones are organized to form an $(H2A/H2B/H3/H4)_2$ octamer with $\sim$ 200 bp of DNA wrapped around it, with histone H1 binding to the outside of this core. Micrococcal nuclease digestion and treatment with 3 M urea dissociate nucleosomes into subnucleosomal particles, consisting of H1 with 60–70 bp of DNA, H2A/H2B with 50–60 bp of DNA, and H3/H4 with 70–80 bp of DNA (30, 31). Interestingly, these subnucleosome complexes with DNA can also be reconstituted in vitro to form complexes that still retain their antigenicity and conformation (32–34). Such complexes reconstituted on solid phase have indeed been very useful in dissecting out the fine specificities of ANAs in lupus (5, 6, 19).

By seromonitoring BXSB and MRL-lpr lupus mice over a spectrum of ages, Burlingame and Rubin found that the initial ANAs that appear in these mice are IgG ANAs, directed preferentially against H2A/H2B/DNA subnucleosomes, with little immunoreactivity to the other subnucleosomes in chromatin (6). However, as these mice aged, they exhibited ANAs with specificities also involving H3/H4/DNA, H1/DNA, free histones, and DNA. Such "spreading" of the ANA response has also been confirmed in other murine studies (21) and in lupus patients (5, 28). This study was carried out to determine if the fine specificities of the antichromatin Abs triggered by *Sle1* evolved with a similar pattern.

B6.NZMc1 sera strongly stain Hep-2 nuclei in a homogeneous fashion, as is typical of lupus sera (35, 36). As shown in Figs. 2 and 3, the antichromatin response in these mice is targeted solely against the H2A/H2B/DNA subnucleosomal complex. Since the latter complex has epitopes that are most accessible in native chromatin (37), *Sle1* most likely leads to autoimmunization with native chromatin. Surprisingly however, unlike what is seen in murine and human lupus, these mice do not go on to develop a robust ANA response against other subnucleosomal structures.

The pathogenic significance of antinucleosome or antihistone/DNA Abs in lupus has been well studied and recently re-

viewed (38). Abs with this specificity have been shown to bind glomeruli avidly, through antigenic bridges (39, 40). In some patients, the presence of these Abs has also been shown to correlate well with nephritis (5, 28). Indeed, such ANAs have been eluted from nephritic kidneys isolated from lupus mice (21). Collectively, these studies assign a pathogenic role for antinucleosome Abs. However, although B6.NZMc1 mice exhibit high levels of these ANAs, < 20% of these mice show any evidence of nephritis (which is mild) or renal Ig deposits (15). This model demonstrates that the mere presence of IgG anti-H2A/H2B/DNA Abs may not be sufficient to ensure renal deposition and subsequent nephritis. Thus, although *Sle1* may be sufficient to trigger loss of tolerance to chromatin, and a strong antichromatin immune response, other lupus susceptibility loci (not present in the B6 background) may be instrumental in rendering these Abs pathogenic, possibly by affecting the isotype, avidity, or clearance of the ANAs, or in tuning renal sensitivity to Ab-mediated damage. Interestingly, the presence of Abs to H2A/H2B and H2A/H2B/DNA, with little or no renal pathology, is also the hallmark of lupus induced by procainamide and other drugs (41–43). It is possible that such drugs may also impinge on the same pathogenic pathways triggered by *Sle1*, leading to autoimmunization with chromatin.

The crucial role of chromatin/histone specific T cells in "driving" autoimmune B cells to make ANAs has been well documented (7–10). It was thus not surprising that B6.NZMc1 T cells respond to chromatin and histones. However, this response does not appear to be as robust as that described in lupus-prone strains (7). This could be due to the fact that other genes in these strains may also be impinging on this pathogenic mechanism. The proliferative response of B6.NZMc1 T cells to these nuclear antigens is not simply due to a mitogenic effect of these antigens because (*a*) a similar response is not seen with B6 T cells; (*b*) the proliferative response to chromatin/histones is accompanied by strong IFN-γ secretion (Fig. 5 *B*); and (*c*) a similar response is seen when B6.NZMc1 T cells were cocultured with antigen-pulsed splenic APCs, i.e., without the continuous presence of antigens in culture (data not shown). Despite the fact that *Sle1* is entirely NZW derived, the NZW strain does not exhibit any IgG antihistone/DNA Abs or T cell reactivity to core histones (Mohan, C., and L. Morel, unpublished observations). We now know that this is due to the presence of "suppressor loci" in the NZW genome that epistatically modulate the potential effects of *Sle1* (Morel, L., unpublished observation). Despite being activated and chromatin-reactive, we could not demonstrate any helper function for these T cells in coculture assays with self or syngeneic B cells (Fig. 5 *C*). It is possible that the B6.NZMc1 B cells were already well differentiated, having been triggered by self T cells in vivo, such that no further help was possible in vitro. Experiments are in progress to determine if increasing the T cell/B cell coculture ratio from 1:1 (as performed in this study) to 5–10: 1 will uncover any helper function for B6.NZMc1 T cells. On the other hand, one cannot exclude a more autonomous, T-independent role for *Sle1* bearing B cells in producing antichromatin Abs, as discussed below. Interestingly, such T-independent secretion of antichromatin Abs by autoimmune B cells in vitro has been described previously in the MRL/lpr murine model (44).

It was also intriguing that T cell tolerance to all core histones was lost, whereas B cell tolerance was lost predominantly to H2A/H2B/DNA subnucleosomes. Thus, the presence of T cells that can recognize various core histones does not seem to be sufficient to trigger loss of B cell tolerance to these other subnucleosomal determinants. In considering the fine differences in antigen specificity to which B cell versus T cell tolerance is lost in this congenic model, the following possibilities need to be entertained. As the H2A/H2B/DNA complex is the most exposed determinant on chromatin, it is likely that B cells with this specificity will preferentially be expanded, as tolerance to chromatin is breached. Other subnucleosomal components may never get adequately exposed in this model, and thus B cells specific for these other determinants may never get expanded, even if cognate T cell help were available. Secondly, despite their modest response to core histones, B6.NZMc1 T cells may not be good helpers and therefore may not be very efficient in expanding B cells with other subnucleosomal specificities, and/or in mutating the anti-H2A/H2B/DNA Abs to become more broadly cross-reactive variants. Importantly, additional lupus susceptibility gene(s) may be necessary for these events to occur. To explore this possibility, studies are in progress with B6.NZM mice bicongenic for *Sle1* and a second lupus susceptibility interval (*Sle2, Sle3* or the *H2* locus).

The findings detailed above suggest that *Sle1* leads to a selective loss of B and T cell tolerance to chromatin. In contrast to B6.NZMc4 mice congenic for *Sle2* (45), B6.NZMc1 mice do not show any expansion of B1 cells, and exhibit normal proliferative responses when stimulated with anti-IgM, LPS, IL-2, and anti-CD3 (data not shown). Furthermore, in response to challenge with a T-dependent antigen, DNP-KLH, B6.NZMc1 and B6 mice exhibit an equally strong IgM and IgG antihapten response and normal germinal center formation. By all these parameters, B6.NZMc1 mice seem to have an otherwise normal immune system, except that tolerance to chromatin is breached.

The puzzle as to what gene *Sle1* actually encodes is intimately tied to the question of how *Sle1* leads to a selective breakdown in tolerance to chromatin. Since the B6.NZMc1 immune system thus far appears to be normal, one potential underlying mechanism may relate to how self chromatin is exposed to the immune system. Thus, *Sle1* might lead to the production of chromatin that is aberrant in quantity (increased production or reduced clearance) or in quality (thus generating novel/cryptic epitopes). Aberrant rates of apoptosis (46–49) and increased levels of free circulating chromatin (50–53) have been reported in murine and human lupus. It was thus important to see if *Sle1* led to any of these scenarios. By hypotonic lysis and PI incorporation, by annexin binding, or nick-labeling, and by light microscopic morphology, B6.NZMc1 mice do not appear to have any aberrant B cell or T cell apoptosis (Fig. 7 *A* and data not shown). Given that *Sle1* is entirely NZW derived, this observation is consistent with the finding that NZW lymphocytes have normal rates of apoptosis (reference 49 and unpublished observations). Furthermore, these congenic mice do not differ from B6 mice in their steady-state levels of cell-free chromatin in their sera and culture supernatants (Fig. 7 *B*). The nuclear antigens that are commonly targeted in lupus have been documented to reside in localized intracellular blebs within apoptosing cells (54); although apoptosing lymphocytes from B6.NZMc1 and B6 mice have similar light microscopic morphology, we cannot yet exclude any subtle differences in the distribution or antigenic makeup of these apoptotic blebs. Thus, it is conceivable that *Sle1* might prima-

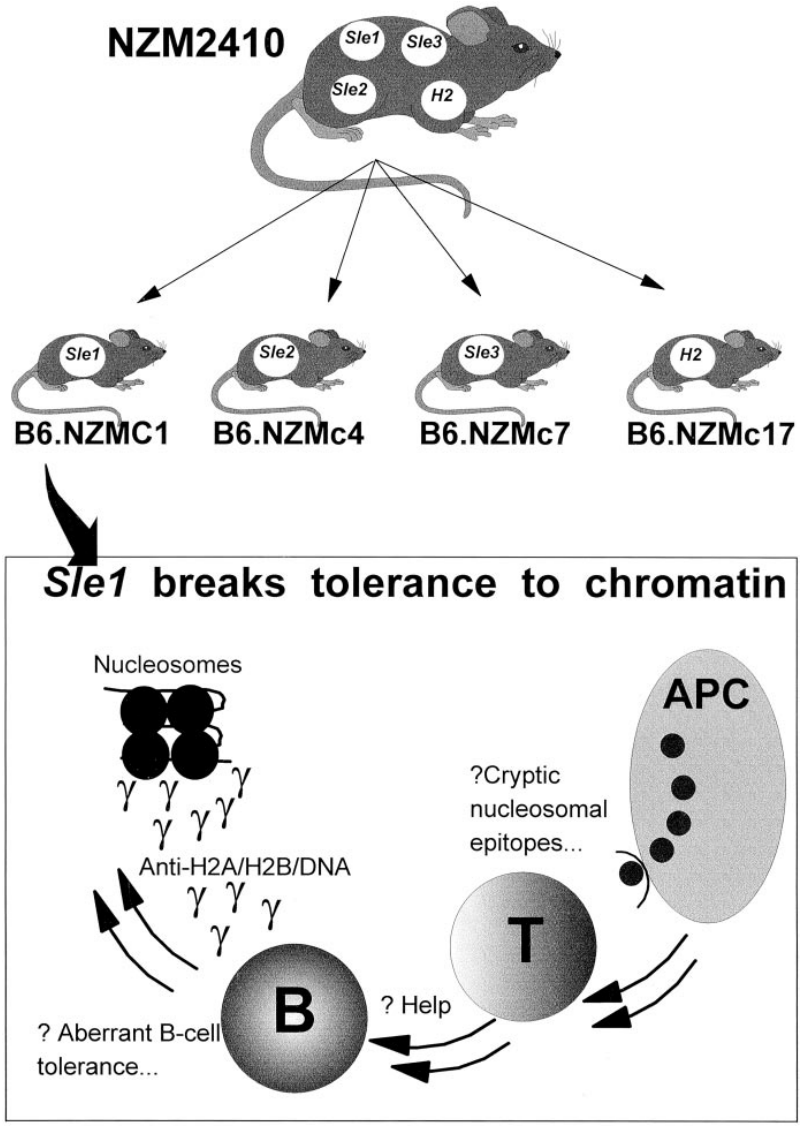

Figure 8. Genetic dissection of SLE pathogenesis. *Sle1* leads to a selective loss of tolerance to chromatin. In the NZM2410 strain, genomic intervals on chromosomes 1, 4, 7, and 17 are strongly linked to lupus nephritis (13). These intervals have been successfully backcrossed onto the normal, B6 background (14). In contrast to the B6.NZMc4, B6.NZMc7, and B6.$H2^z$ congenics, B6.NZMc1 mice develop high levels of antichromatin Abs (15, 44). This study shows that these mice suffer a profound breach in B cell and T cell tolerance to chromatin, despite an otherwise normal immune system. It is not presently clear how *Sle1* triggers autoimmunization with chromatin. One hypothesis is that *Sle1* might facilitate the efficient presentation of cryptic nucleosomal epitopes, and this could break T cell tolerance to this antigen. Though these chromatin-specific T cells might have a potential role in helping the ANA-producing B cells, this could not be demonstrated in this study. Alternatively, *Sle1* may be directly impacting B cell tolerance, perhaps by affecting allelic exclusion, receptor editing, B cell anergy, or other unknown mechanisms, leading to ANA formation.

rily be affecting the presentation of self-chromatin in an immunogenic fashion, so as to trigger otherwise ignorant or quiescent antichromatin/histone T cells, that then can "drive" B cell IgG ANA production.

In addition to viewing loss of T cell tolerance to chromatin as the central or initiating event triggered by *Sle1*, we also have to consider the possibility that *Sle1* might directly be impacting B cell tolerance to chromatin (Fig. 8). With this alternative, we would have to propose that *Sle1* might lead to a T-independent, intrinsic B cell abnormality that allows these B cells to make IgG ANAs, as has been demonstrated in the case of *lpr* and NZW B cells (44, 55, 56). Since B6.NZMc1 mice bear the NZW allele of *Sle1,* it is interesting to note that recent pre–B cell transfer studies have demonstrated that NZW B cells are also intrinsically abnormal, being able to make IgG ANAs upon transfer (57). With this alternative, however, it is more puzzling to fathom why the immune response should be targeting just chromatin. It could very well turn out that chromatin is but one member of a suite of self-antigens (e.g., nuclear antigens with repeating determinants) against which *Sle1* triggers loss of tolerance. Indeed, our recent allotype-marked bone marrow transfer studies indicate that *Sle1* bearing B cells may intrinsically be more prone to generate IgG ANAs, independent of whether the T cells bear *Sle1* or not (Sobel, E., C. Mohan, L. Morel, J. Schiffenbauer, and E.K. Wakeland, manuscript submitted for publication).

In addition to our mapping study, other genetic mapping studies in related murine models of lupus have also highlighted the significance of this chromosome 1 locus (for reviews see references 11 and 12). Interestingly, a more recent mapping study in human lupus has also localized a human chromosome 1 interval (syntenic to murine *Sle1*) that is linked to high serum IgG antichromatin ANAs (58). Collectively, these studies advance *Sle1* on murine chromosome 1 as a major player orchestrating selective loss of B cell and T cell tolerance to chromatin. This is in sharp contrast to the more generalized (not nuclear antigen restricted) immunophenotypes that *Sle2* and *Sle3* lead to (15, 45). At present, none of the immunologically relevant genes in the *Sle1* interval readily accounts for the B6.NZMc1 phenotype. Coding region sequencing has also shown that CTLA4, SAP, *cfh*, and FasL (four candidate genes

within the interval) are unlikely to be the culprit genes (reference 15 and unpublished observations). However, the process of generating congenic recombinants and screening them for anti-H2A/H2B/DNA Abs will allow us to progressively narrow the *Sle1* interval, so as to permit positional cloning of the causative gene. Until then, one thing seems certain: Enshrouded in the *Sle1* interval is the quintessential lupus gene responsible for the "L.E." phenomenon Hargraves discovered 50 years ago.

Acknowledgments

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