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Cutting Edge: A Role for CD1 in the Pathogenesis of Lupus in NZB/NZW Mice¹

Defu Zeng, Mi-Kyeong Lee, James Tung, Andrea Brendolan, and Samuel Strober²

Since anti-CD1 TCR transgenic T cells can activate syngeneic B cells via CD1 to secrete IgM and IgG and induce lupus in BALB/c mice, we studied the role of CD1 in the pathogenesis of lupus in NZB/NZW mice. Approximately 20% of B cells from the spleens of NZB/NZW mice expressed high levels of CD1 (CD1^{high} B cells). The latter subset spontaneously produced large amounts of IgM anti-dsDNA Abs *in vitro* that was up to 25-fold higher than that of residual CD1^{int/low} B cells. T cells in the NZB/NZW spleen proliferated vigorously to the CD1-transfected A20 B cell line, but not to the parent line. Treatment of NZB/NZW mice with anti-CD1 mAbs ameliorated the development of lupus. These results suggest that the CD1^{high} B cells and their progeny are a major source of autoantibody production, and activation of B cells via CD1 may play an important role in the pathogenesis of lupus. *The Journal of Immunology*, 2000, 164: 5000–5004.

Systemic lupus erythematosus is an autoimmune disease with a variety of anti-protein and non-protein autoantibodies that cause injury to multiple organ systems including the kidneys and CNS (1). In the hereditary lupus of NZB/NZW mice, cationic IgG2a anti-dsDNA Abs are pathogenic and contribute to immune complex glomerulonephritis (2, 3). The transition of autoantibody secretion from IgM to IgG in NZB/NZW mice occurs at the age of about 6 mo, and T cells play an important role in regulating the IgG autoantibody production (4, 5). Proposed mechanisms of T cell help for anti-dsDNA Ab secretion include T cell recognition of DNA-associated protein Ags such as histones (6, 7) and recognition of anti-DNA Ab-derived peptides in the context of class II MHC (8, 9).

CD1 is a nonpolymorphic, class I MHC-like, non-MHC encoded molecule that associates noncovalently with β_2 -microglobu-

lin (10). CD1 molecules have been demonstrated to be Ag-presenting molecules for glycolipid and hydrophobic peptides (11–13). All murine B cells express CD1 on the cell surface (14), and there is a subset of splenic B cells that expresses high levels of CD1 (CD1^{high} B cells) and accounts for about 20% of total splenic B cells. CD1^{high} B cells in nonautoimmune mice have been demonstrated to be predominantly marginal zone B cells (15–17). We previously showed that BALB/c anti-CD1 TCR transgenic T cells that secrete high levels of IFN- γ and IL-10, but low levels of IL-4, can activate syngeneic B cells *in vitro* to secrete IgM and IgG by cross-linking CD1, and the transgenic T cells induced lupus in BALB/c nu/nu recipients with high levels of serum anti-dsDNA Abs and glomerulonephritis (18). This indicates that T and B cell interactions via CD1 can play an important role in the pathogenesis of lupus (18).

In the current study, the role of CD1 in the development of lupus in NZB/NZW mice was examined. The results show that IgM⁺CD1^{high} B cells from the NZB/NZW spleen spontaneously secreted IgM and IgM anti-dsDNA autoantibodies at levels 5- to 25-fold higher than CD1^{int/low} B cells. Anti-CD1 T cells were present in the spleen of NZB/NZW mice also. *In vivo* anti-CD1 mAb treatment reduced the peak levels of serum IgG and IgG anti-dsDNA Abs, delayed the onset of proteinuria, and prolonged the survival period. Our results suggest that CD1 is expressed on the precursors of IgM and IgG autoantibody-secreting B cells and that the interaction between the CD1^{high} B cells and anti-CD1 T cells may play an important role in the pathogenesis of hereditary lupus in NZB/NZW mice.

Materials and Methods

Mice

C57BL/6 female mice were obtained from the Department of Comparative Medicine, Stanford University breeding facility. NZB/NZW female mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

mAbs, immunofluorescent staining, flow cytometric analysis, and sorting

Single-cell suspensions of spleen cells or bone marrow cells obtained from the femur and tibia were prepared and stained with mAbs as described previously (19–22). Stainings were performed in the presence of anti-CD16/32 (2.4G2; PharMingen, San Diego, CA) at saturation to block Fc γ RII/III receptors, and propidium iodide (Sigma, St. Louis, MO) was added to staining reagents to exclude dead cells. Erythrocytes were excluded by light scatter gating. FACS analysis and sorting were performed with a FACS Vantage (Becton Dickinson, Mountain View, CA), and data were analyzed using FlowJo software (Becton Dickinson) (21). The purity of sorted cells was >98%. The following conjugated mAbs were used for staining: FITC- and PE-anti-B220 (RA3-6B2), FITC-anti-IgM (R6-60.2),

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PE-anti-CD1(1B1), biotinylated anti-CD1 (1B1), PE-streptavidin purchased from PharMingen. Biotinylated anti-CD1 (3C11) was purified and conjugated as described previously (18).

In vitro secretion of IgM and IgG

Sorted splenic T and/or B cells were incubated in 96-well flat-bottom plastic plates in complete RPMI 1640 medium with 10% FBS for 1–5 days at 37°C in 5% CO₂. At the end of the culture period, supernatants were harvested and the concentrations of IgM and IgG were measured with the ELISA using affinity-purified goat anti-mouse heavy chain-specific Abs as described below.

ELISA of total IgM and IgG and anti-dsDNA IgM and IgG

Measurements of total IgM and IgG in culture supernatants or sera were performed using an ELISA with goat anti-mouse IgM plus IgG (H + L chain) Abs (Southern Biotechnology Associates, Birmingham, AL) to capture mouse IgM and IgG, and alkaline phosphatase-labeled affinity-purified goat Abs specific for mouse Ig isotypes (Southern Biotechnology Associates) for detection as described previously (18).

IgM and IgG anti-dsDNA Abs were captured using deproteinized calf thymus DNA as described previously (9), and ELISA was performed as described above. Anti-dsDNA titers are expressed as units per milliliter using a reference-positive standard of pooled serum from 6- to 7-mo-old NZB/NZW mice. A 1:100 dilution of this standard serum was arbitrarily assigned a value of 100 U/ml.

In vitro proliferative responses

Sorted splenic T cells (Thy1.2⁺B220⁻) were incubated (1×10^5 cells/well) together with graded numbers ($1\text{--}25 \times 10^3$ cells/well) of irradiated (5000 rad) stimulator cells. The latter were either the A20 B cell line derived from BALB/c mice or CD1-transfected A20 B cells obtained from Dr. M. Kronenberg (La Jolla Institute of Allergy and Immunology, La Jolla, CA) (23). Cells were cultured in 10% FCS complete RPMI 1640 medium in 96-well round-bottom plastic plates for 72 h at 37°C in 5% CO₂. [³H]Thymidine (1 μCi/well) was added 24 h before cells were harvested. [³H]Thymidine (New England Nuclear, Boston, MA) incorporation was measured in a liquid scintillation counter (Betaplate; Wallac, Turku, Finland). All assays were performed in triplicate wells with responder or stimulator cells alone or together.

In vivo treatment of anti-CD1 and control mAbs

Anti-CD1 mAb (rat IgG2b) was purified from the hybridoma 1B1 (a gift from Dr. M. Kronenberg) and anti-human lymphoma-Id mAb (rat IgG1) was purified from hybridoma R9A9 (a gift from Dr. R. Levy, Stanford University). Hybridoma supernatants were purified using recombinant protein G-agarose columns (Life Technologies, Grand Island, NY), and eluates were injected i.p. into NZB/NZW mice. Proteinuria was measured on a 1–4+ scale using a colorimetric assay for albumin (Albustix; Miles, Elkhart, IN). Mice were considered to have proteinuria if at least two consecutive urine samples were $\geq 2+$ (100 mg/dl) (18). Serum levels of IgM and IgG and anti-dsDNA were measured with the ELISA as described above.

Results

Splenic CD1^{high} B cells in NZB/NZW and C57BL/6 mice

We compared the percentage of CD1^{high} B cells in the spleen of lupus-prone NZB/NZW and nonautoimmune C57BL/6 mice at 3 and 6 mo of age. As shown in Fig. 1, A and B, the spleen of C57BL/6 mice contained a subset of CD1^{high} B cells, which accounted for about 7% of live nucleated spleen cells and about 20% of total spleen B cells, as judged by staining for CD1 receptors (using 3C11 and 1B1 mAbs) vs B220 receptors. The C57BL/6 CD1^{high} B cells were almost all IgM⁺ cells when anti-IgM mAb was used instead of anti-B220 mAb (Fig. 1C). The percentage of CD1^{high} B cells in the spleen of 6-mo-old NZB/NZW mice with nephritis (proteinuria, $\geq 3+$) was similar to that of the age- and sex-matched C57BL/6 mice. Almost all NZB/NZW CD1^{high} B cells were IgM⁺ (Fig. 1, D–F). The percentages of CD1^{high} B cells in the spleen of C57BL/6 (Fig. 1, G–I) and NZB/NZW (Fig. 1, J–L) mice at 3 mo were similar also.

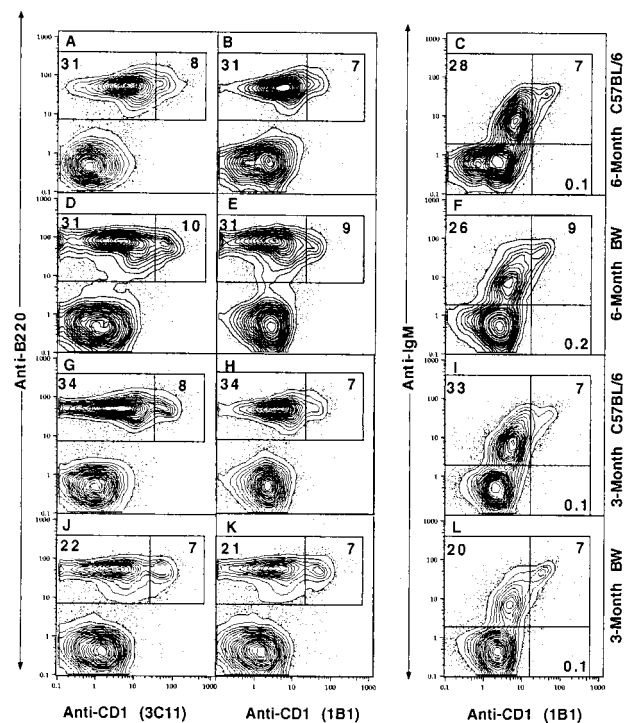


FIGURE 1. Two-color flow cytometric analysis of CD1 expression on splenic B cells. Spleen cells from 6-mo-old C57BL/6 mice (A–C), 6-mo-old NZB/NZW mice with proteinuria (D–F), 3-mo-old C57BL/6 mice (G–I), and 3-mo-old NZB/NZW mice (J–L) were stained with FITC anti-B220 or FITC anti-IgM vs anti-CD1-biotin (3C11 or 1B1) and counterstained with PE streptavidin. A subset of B220⁺CD1^{high} or IgM⁺CD1^{high} B cells is enclosed in the right box or upper right box in each panel, and the percentage of CD1^{high} B cells among live nucleated cells is shown for each box. The IgM⁻CD1^{high} B cells are enclosed in the lower right box in some panels. Each panel is representative of at least four replicate experiments.

NZB/NZW splenic CD1^{high} B cells are a major source of spontaneous IgM secretion

Splenic B cells from 6-mo-old NZB/NZW mice spontaneously secrete large amounts of autoantibodies *in vitro* (4, 24). To examine the role of CD1^{high} B cells in secretion of autoantibodies, B cells from 3- and 6-mo-old NZB/NZW mice without proteinuria or 6-mo-old mice with proteinuria were studied. The highest percentage of B220⁺ B cells in the spleen was found in the 6-mo-old mice without proteinuria (data not shown). An example of the studies of splenic B cells from a group of the latter mice is shown in Fig. 2. Cells were sorted into B220⁺CD1^{high}, B220⁺CD1^{int}, and B220⁺CD1^{low} populations using a nonactivating anti-CD1 mAb (1B1) (Fig. 2A). The cells of each population were cultured (5×10^5 cells/well) *in vitro* with or without syngeneic cocultured T cells (1.25×10^5) for 5 days. Thereafter, the supernatants were assayed in duplicate for the concentrations of total IgM and IgG and IgM and IgG anti-dsDNA Abs. As shown in Fig. 2, B and C, CD1^{high} B cells produced large amounts IgM (about 14 μg/ml) and IgM anti-dsDNA Abs (about 30 U/ml) even without T cells in coculture. This was five times higher than that secreted by CD1^{int} B cells ($p < 0.001$, two-tail Student's *t* test) and 25 times higher than that secreted by CD1^{low} B cells ($p < 0.001$, two-tail Student's *t* test). Add-back of syngeneic T cells significantly enhanced the IgM and IgM anti-dsDNA Ab secretion by the CD1^{high} B cells ($p < 0.01$, two-tail Student's *t* test). IgG secretion (< 100 ng/ml) by the three subsets of B cells was too low to be compared. These results indicate that the IgM autoantibodies spontaneously secreted

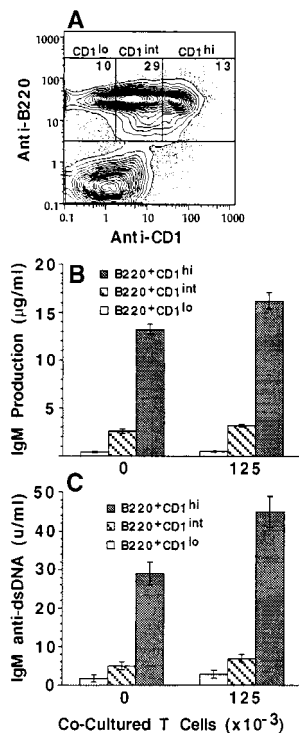


FIGURE 2. Spontaneous secretion of IgM Abs by CD1^{high} B cells. *A*, Gates for spleen cells from 6-mo-old NZB/NZW mice without proteinuria after staining with anti-B220-FITC vs anti-CD1 (1B1)-PE and sorting into B220⁺CD1^{low}, B220⁺CD1^{int}, and B220⁺CD1^{high} subsets. The percentage of cells among live nucleated cells is shown for each gate. *B* and *C*, Concentration of IgM and IgM anti-dsDNA Abs, respectively, in culture supernatants of each subset (5×10^5 cells/well) with or without syngeneic T cells (1.25×10^5 cells/well). Data are the means \pm SE of six cultures from two experiments.

by splenic B cells of 6-mo-old NZB/NZW mice without proteinuria are predominantly derived from the CD1^{high} B cell population. Studies using 6-mo-old NZB/NZW mice with proteinuria and 3-mo-old mice without proteinuria also showed that the CD1^{low} B cells secreted little IgM autoantibody, and most was secreted by CD1^{int} and CD1^{high} B cells (data not shown). In addition, sorted C57BL/6 CD1^{high} B cells secreted about 40 times less IgM (mean \pm SE, 0.3 ± 0.1 μ g/ml) as compared with that secreted by NZB/NZW CD1^{high} B cells (mean \pm SE, 14 ± 0.5 μ g/ml).

Glomerulonephritis and proteinuria are associated with spontaneous secretion of IgG anti-dsDNA Ab by splenic B cells (4). Splenic CD1^{high} B cells from 6-mo-old NZB/NZW mice with proteinuria were almost all IgM⁺, and few, if any, were IgM⁻ (Fig. 1*F*). We tested whether both IgM and IgG autoantibodies were secreted by IgM⁺ B cells as compared with all B220⁺ B cells from these mice. As shown in Fig. 3, while sorted B220⁺ cells produced large amounts (about 5.5 μ g/ml) of both IgM and IgG, IgM⁺ cells produced large amounts (about 5.2 μ g/ml) of IgM, but <0.5 μ g/ml of IgG. Sorted residual B220⁺IgM⁻ cells were the source of IgG (data not shown). There were insufficient CD1^{high}IgM⁻ spleen cells to assay for spontaneous IgG secretion (Fig. 1*F*).

Anti-CD1 T cells in the spleen of NZB/NZW mice

To determine whether anti-CD1 T cells were present in the spleen of 3-mo-old NZB/NZW mice, sorted splenic T (Thy1⁺) cells were obtained from the latter mice and incubated with either the CD1-transfected A20 (A20/CD1) B cell line derived from BALB/c

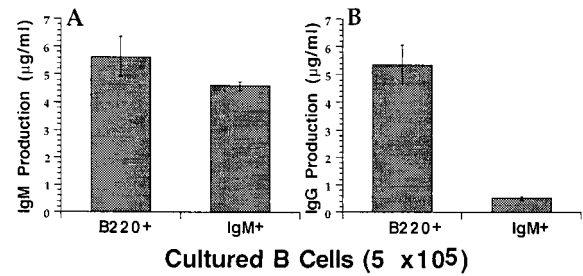


FIGURE 3. Spontaneous secretion of IgM and IgG by IgM⁺ and B220⁺ B cells. *A* and *B*, respectively, the IgM and IgG production by sorted splenic B220⁺ and IgM⁺ B cells (5×10^5 cells/well) from 6-mo-old NZB/NZW mice with proteinuria ($\geq 3+$). Data are the means \pm SE of six cultures from two experiments.

mice or the nontransfected A20 B cell line. As shown in Fig. 4, the sorted T cells proliferated vigorously in response to stimulation by A20/CD1 cells, but not to the A20 cells (Fig. 4*C*). A20 cells did not express CD1, but A20/CD1 cells expressed high levels of CD1 (Fig. 4, *A* and *B*). T cells from the spleens of C57BL/6 mice were not tested in this proliferation assay, because A20 cells (H-2^d) and C57BL/6 (H-2^b) T cells are MHC mismatched, whereas NZB/NZW (H-2^{d/z}) T cells are not stimulated to proliferate by the shared H-2^d MHC of the BALB/c-derived cell line. In addition, the proliferation of the NZB/NZW T cells to A20/CD1 cells was about 2-fold higher than that of BALB/c (H-2^d) T cells (data not shown).

In vivo anti-CD1 mAb treatment suppresses the development of lupus

Since anti-CD1 T cells were found in the spleen of NZB/NZW mice and CD1^{high} B cells secreted IgM autoantibodies, it was possible that the T cells stimulated the secretion of IgM autoantibodies via CD1. Thereafter, the stimulated B cells could switch the autoantibody isotype from IgM to IgG associated with the development of disease. To interfere with B cell signaling via CD1 in a preliminary study, groups of 8-wk-old NZB/NZW mice were injected i.p. five times over a 30-day period with 250 μ g/mouse rat IgG anti-CD1 (1B1) mAb or control irrelevant rat IgG mAb (days

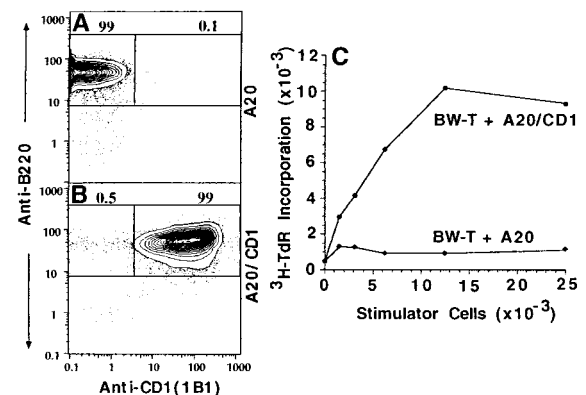


FIGURE 4. Proliferation of T cells in response to stimulation by CD1-transfected A20 cells. *A* and *B*, the expression of CD1 on A20 cells, a B cell lymphoma line, and CD1-transfected A20 cells (A20/CD1) by staining the cells with anti-B220 vs anti-CD1 mAbs. *C*, The proliferation of sorted splenic T (Thy1.2⁺) cells (1×10^5 /well) from 3-mo-old NZB/NZW mice cocultured with the irradiated (5000 rad) graded numbers of A20 or A20/CD1 cells as measured with [³H]thymidine incorporation. Each panel is representative of three replicate experiments.

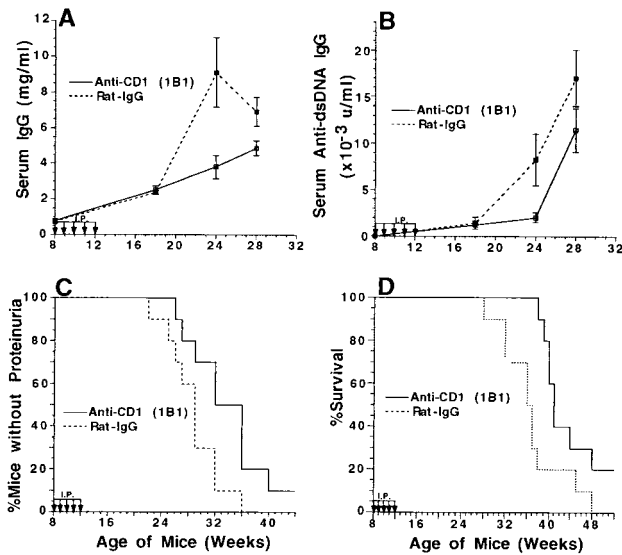


FIGURE 5. Amelioration of lupus by in vivo anti-CD1 mAb treatment. Groups of 8-wk-old NZB/NZW mice were given five i.p. injections of anti-CD1 mAb or control rat IgG at a dose of 250 $\mu\text{g}/\text{mouse}$ over a period of 30 days (days 0, 3, 5, 15, and 30). Thereafter, the mice were monitored and serum levels of IgG, anti-dsDNA IgG, proteinuria, and survival are shown in A–D, respectively. There were 10 mice in each group. Arrows show time points of injections.

0, 3, 5, 15, and 30). Thereafter, the mice were monitored for levels of serum IgG and IgG anti-dsDNA, proteinuria, and survival (Fig. 5). The anti-CD1 mAb was rat IgG2b, a complement-binding isotype, and the control mAb was rat IgG1, a noncomplement-binding isotype (25, 26). The control isotype was unlikely to worsen renal disease by the deposition of aggregate-complement complexes in the glomeruli.

As compared with the control group, anti-CD1 treatment significantly reduced the peak levels of serum IgG and IgG anti-dsDNA autoantibodies (Fig. 5, A and B). At the age of 24 wk, the mean serum IgG level (about 3000 $\mu\text{g}/\text{ml}$) of mice given anti-CD1 mAbs was three times lower than that (9000 $\mu\text{g}/\text{ml}$) of the mice given control rat IgG ($p < 0.01$, two-tail Student's t test). The mean serum IgG anti-dsDNA Ab level at the same time point (about 1000 U/ml) was eight times lower than that (8000 U/ml) of mice given control rat IgG ($p < 0.01$, two-tail Student's t test). More than 4 wk later, the differences in the mean levels of serum IgG and IgG anti-dsDNA Abs of the two groups were reduced, but still remained significantly different ($p < 0.05$, two-tail Student's t test). The serum levels of IgM and IgM anti-dsDNA Ab of the two groups increased during the observation period; however, the mean levels were not significantly different at all time points ($p > 0.4$, data not shown). Bleeding for serum samples was not done after 28 wk of age due to the increasing morbidity and mortality after that time point, especially in the control group. The brief course of anti-CD1 mAb treatment also delayed the onset of proteinuria and prolonged the survival of the mice by 4–8 wk (Fig. 5, C and D). The differences in the onset of proteinuria ($p < 0.05$, log rank test) and in survival ($p < 0.01$, log rank test) were both significant. Thus, anti-CD1 mAb treatment delayed disease progression by at least the duration (4 wk) of the treatment period in this small preliminary study.

To determine whether administration of anti-CD1 mAb depleted CD1^{high} B cells, a single injection of 250 $\mu\text{g}/\text{mouse}$ was given to three mice, and 1 wk later, the spleen cells were assayed for the

expression of CD1 on B cells. As reported previously (27), the staining pattern of CD1 vs B220 receptors and the absolute number of CD1^{high} B cells was unchanged after the administration of the 1B1 mAb (data not shown). Thus, the anti-CD1 mAb neither down-regulated CD1 expression on B cells nor depleted CD1^{high} B cells.

Discussion

It is still not clear how T cells provide help for B cells secreting autoantibodies directed to a wide variety of nonprotein Ags in lupus-prone NZB/NZW mice. One of the possible mechanisms is T cell recognition of the CD1 molecule associated with endogenous ligands on the surface of B cells, since adoptive transfer of anti-CD1 transgenic T cells into syngeneic hosts can induce anti-dsDNA Ab production and lupus (18). In the current study, CD1^{high} B cells in the spleen of NZB/NZW mice were found to be the predominant source of in vitro spontaneous secretion of IgM autoantibodies as compared with CD1^{int} or CD1^{low} B cells. However, those $\text{IgM}^+ \text{CD1}^{\text{high}}$ B cells were not the source of IgG autoantibodies, since $\text{IgM}^- \text{B220}^+$ cells mediated IgG secretion. The latter cells may have been derived from the former during the isotype switching to IgG with down-regulation of surface IgM.

CD1^{high} B cells in the spleen of nonautoimmune C57BL/6 mice are predominantly $\text{CD21}^{\text{high}}$ marginal zone B cells (15–17), and they spontaneously secreted little IgM in vitro. Although the CD1^{high} B cells in the spleen of C57BL/6 mice had little contribution from CD5^+ B (B-1) cells (15), our recent studies indicate that splenic NZB/NZW CD1^{high} B cells between ages 3 and 6 mo have a markedly increased contribution from CD5^+ B (B-1) cells, and these $\text{CD1}^{\text{high}} \text{CD5}^+$ B cells are responsible for the majority of spontaneous IgM secretion (J. Tung, N. Baumgarth, L. S. Herzenberg, and S. Strober, manuscript in preparation). This is consistent with our previous studies showing that CD5^+ B cells in the spleen of NZB/NZW mice are the predominant source of spontaneous IgM secretion (24).

The presence of anti-CD1 T cells in the spleen of NZB/NZW mice was shown by experiments in which T cells from the NZB/NZW spleen proliferated vigorously to CD1-transfected A20 B cells, but not to the parental nontransfected A20 B cells. Anti-CD1 mAb treatment was administered in vivo to interfere with the interaction between anti-CD1 T cells and CD1^{high} B cells. The administration of the anti-CD1 mAb markedly reduced the peak levels of serum IgG and IgG anti-dsDNA Ab, but did not reduce the levels of serum IgM and IgM anti-dsDNA Ab. These results suggest that IgM anti-dsDNA Ab-secreting CD1^{high} B cells may be the precursors of IgG anti-dsDNA Ab-secreting B cells, and the interaction of anti-CD1 T cells and the CD1^{high} B cells via CD1 may play an important role in the isotype switch of anti-dsDNA Abs from IgM to IgG. This notion is consistent with the report that Th1-like anti-CD1 TCR transgenic T cells induced lupus with high levels of serum anti-dsDNA IgG2a (18). It is of interest that IL-4-producing NK T cells that express the invariant $V_{\alpha}14J_{\alpha}281$ TCR are selectively reduced in NZB/NZW mice (28). Our recent studies found that NK T cells in the bone marrow of NZB/NZW mice produced large amounts of IFN- γ but small amounts of IL-4 as compared with that of C57BL/6 mice (D. Zeng and S. Strober, manuscript in preparation). This indicates that there may be an intrinsic cytokine abnormality in anti-CD1 T cells in NZB/NZW mice that augments the autoantibody isotype switch from IgM to IgG2a.

The mechanisms of in vivo anti-CD1 mAb amelioration of the development of lupus are not clear as yet. The administration of anti-CD1 mAb did not reduce the serum levels of IgM and IgM

anti-dsDNA Ab, and did not deplete the CD1^{high} B cells in the current study and in a previous report (27). It is most likely that the effect is due to blocking the interaction between anti-CD1 T cells and the CD1 ligand on CD1^{high} B cells and possibly on CD1^{high} macrophages and dendritic cells, thereby blocking the isotype switch of autoantibody from IgM to IgG. In conclusion, our results suggest that the interaction between anti-CD1 T cells and CD1^{high} B cells may play an important role in the development of hereditary lupus in NZB/NZW mice.

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