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MECHANISMS OF DRUG-INDUCED LUPUS

III. Sex-Specific Differences in T Cell Homing May Explain Increased Disease Severity in Female Mice

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Objective. **To determine if sex-specific differences in lymphocyte trafficking could contribute to increased disease severity in female mice.**

Methods. **A lupus-like disease was induced by injecting male and female mice with procainamide-treated T cell clones. Trafficking was examined by labeling the injected cells with ^{51}Cr or 5-chloromethylfluorescein diacetate.**

Results. **Females developed more autoimmune liver disease and greater titers of anti-DNA antibodies than did males, and 2–7 times more cells accumulated in the female spleens. Splenectomy prevented the development of autoantibodies and renal and liver disease. Oophorectomy decreased the splenic homing, autoantibody titer, and liver disease severity, to levels found in males.**

Conclusion. **T cells traffic differently to the spleen in male and female mice, and the spleen appears to be essential in the disease process. This suggests that differences in T cell homing could contribute to sex-specific disease severity in this murine model, and also possibly in human disease.**

Systemic lupus erythematosus is an idiopathic autoimmune disease that primarily affects women (1). Extensive studies have implicated female sex hormones in disease susceptibility, suggesting that estrogen-dependent effects such as increased cell-mediated immunity and increased antibody responses play a role in disease severity (2,3). However, despite intensive investigation, the mechanisms causing the increased immune responsiveness and sex-specific disease manifestations are unknown.

Our group has described a novel lupus model in which D10 cells, a cloned murine antigen-specific T helper 2 line, are made autoreactive by treatment with DNA methylation inhibitors such as procainamide (Pca), then given in adoptive transfer to nonirradiated syngeneic recipients (AKR mouse strain). The autoreactive cells interact with other cells of the immune system, lysing macrophages and inducing B cell differentiation (4–6). The recipients subsequently develop an immune complex glomerulonephritis, pulmonary alveolitis, a liver disease resembling primary biliary cirrhosis, and autoantibodies to single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) (4), by a mechanism resembling that of chronic graft-versus-host disease (7,8). A lupus-like disease can also be induced using polyclonal DBA/2 CD4+ T cells and syngeneic recipients (5), indicating that induction of autoimmunity by this mechanism is not unique to D10 cells or to the AKR strain.

The autoreactivity that is induced in vitro and lupus-like disease that develops in vivo can be directly attributed to the overexpression of lymphocyte function–associated antigen 1 (LFA-1), which is caused by the drug treatment (8). LFA-1 is an adhesion molecule that binds intracellular adhesion molecule 1 (ICAM-1) on other cell types, including endothelial cells (9). A recent study demonstrated that estrogens can increase expression of endothelial adhesion molecules, including E-selectin, ICAM-1, and vascular cell adhesion molecule

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1 (10), and others have shown that endothelial cells in different tissues can express different surface proteins (11). This raises the intriguing possibility that estrogens may affect T cell trafficking patterns by altering endothelial adhesion molecule expression in an organ-specific manner.

We sought to determine whether sex-specific differences in T cell trafficking could affect the severity of disease manifestations in this murine lupus model. We used the D10 model to correlate sex-specific disease manifestations with differences in T cell homing between male and female recipients. The results indicated a crucial role for splenic homing in this model, and demonstrated a sex-specific difference in the number of T cells accumulating in the spleen, which can explain the differences in disease severity between male and female mice. These results support the very interesting concept that T cells can traffic differently to lymphoid tissues in males and females. Our findings raise the possibility that this difference in trafficking can contribute to sex-specific disease manifestations.

MATERIALS AND METHODS

Mice. Six-to-8-week-old female and male AKR mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in a pathogen-free environment provided by the Unit for Laboratory Animal Medicine at the University of Michigan. Splenectomy, oophorectomy, and orchiectomy were performed on adult (6–8-week-old) mice by personnel at Jackson Laboratories. These mice were allowed to recover for at least 4 weeks before use in adoptive transfer protocols. All mice were killed by 8 months of age, and samples were extracted for histologic analysis and measurement of autoantibodies.

Cells and cell culture. D10.G4.1 cells (12) were obtained from American Type Culture Collection (Rockville, MD) and cultured as previously described (4,5). D10 cells are conalbumin specific, express interleukin-4 (IL-4), IL-6, and IL-10, and do not express Fas ligand or perforin. Because of a report that indicated that the D10 line may contain an autoreactive subset (13), the cells were subcloned by limiting dilution at 0.2 cells/well, and a nonautoreactive subclone was selected for use in these studies. Where indicated, 50 μM Pca (Sigma, St. Louis, MO) was added 1 day after restimulation, and the cells were used for adoptive transfer or homing studies 6 days later.

Cell labeling. D10 cells were labeled with ^{51}Cr by culturing 10^7 cells with 100 μCi [^{51}Cr]-$Na_2Cr_2O_7$ (New England Nuclear, Boston, MA) in 1 ml at 37°C for 1 hour. The cells were then washed 3 times with RPMI 1640 (Gibco, Grand Island, NY). Other D10 cells were labeled with 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes, Eugene, OR) using the protocol provided by the manufacturer.

Adoptive transfer. For homing experiments, 5×10^6 ^{51}Cr- or CMFDA-labeled D10 cells were injected intravenously via the tail vein, and the mice were killed 24 or 48 hours later. For disease induction, 6 intravenous injections of 5×10^6 drug-treated or untreated D10 cells were administered in 2-week intervals to the mice in groups of 5. Four weeks after the last injection, these mice were killed.

Lymphocyte homing. ^{51}Cr-labeled cells in various tissues were detected by first weighing the tissue samples and then measuring gamma emissions using a Gamma Trac 1191 gamma spectrometer (Tracor Analytic, Elk Grove Village, IL). CMFDA-labeled D10 cells were detected by staining splenocytes with phycoerythrin (PE)–conjugated anti-CD4 (PharMingen, San Diego, CA), then enumerating cells expressing both CD4 and CMFDA with a Coulter (Hialeah, FL) ELITE flow cytometer, analyzing at least 10^6 cells per sample.

Cytofluorography. Untreated or Pca-treated D10 cells were reacted with fluorescein isothiocyanate (FITC)–conjugated antibodies to MEL-14, as previously described (14). These cells were then analyzed with a Coulter ELITE flow cytometer.

Histologic analysis. Tissues were prepared and stained with hematoxylin and eosin as previously described (4,5). Direct immunofluorescence studies were performed on frozen sections of the liver, lung, and kidney, as previously described (4,5), using FITC-conjugated goat anti-mouse Ig (GAM-FITC; Sigma). Indirect immunofluorescence was performed by reacting frozen sections of normal liver and lung with 1:20 and 1:80 dilutions of mouse sera, followed by washing and staining with GAM-FITC, as was done for direct immunofluorescence.

Autoantibody assays. Total serum IgG and IgM concentrations as well as anti-ssDNA and anti-dsDNA antibodies were measured by enzyme-linked immunosorbent assay (ELISA), using previously described protocols (4,5,8) and peroxidase-conjugated polyvalent or γ-chain–specific antibodies from Sigma. Positive controls included pooled serum from $\geq$6-month-old female New Zealand black $\times$ New Zealand white (NZB $\times$ NZW) mice, similar to that used in previous experiments (4,5,8). All determinations were performed in quadruplicate.

Statistical analysis. Differences between groups were analyzed using Student's 2-tailed t-test or chi-square analysis with Yates' correction.

RESULTS

Effects of Pca-treated D10 cells in male and female mice. Initial experiments compared the effects of untreated and Pca-treated D10 cells in male and female syngeneic (AKR) mice. Each group received 6 intravenous injections of treated or untreated cells. Four weeks after the last injection, the mice were killed and examined for serologic and histologic evidence of autoimmunity. Figures 1A and B compare the total anti-ssDNA and anti-dsDNA antibody responses in the 4 groups. Male and female mice receiving untreated cells produced no significant amounts of either autoantibody. Female mice receiving Pca-treated cells produced signif-

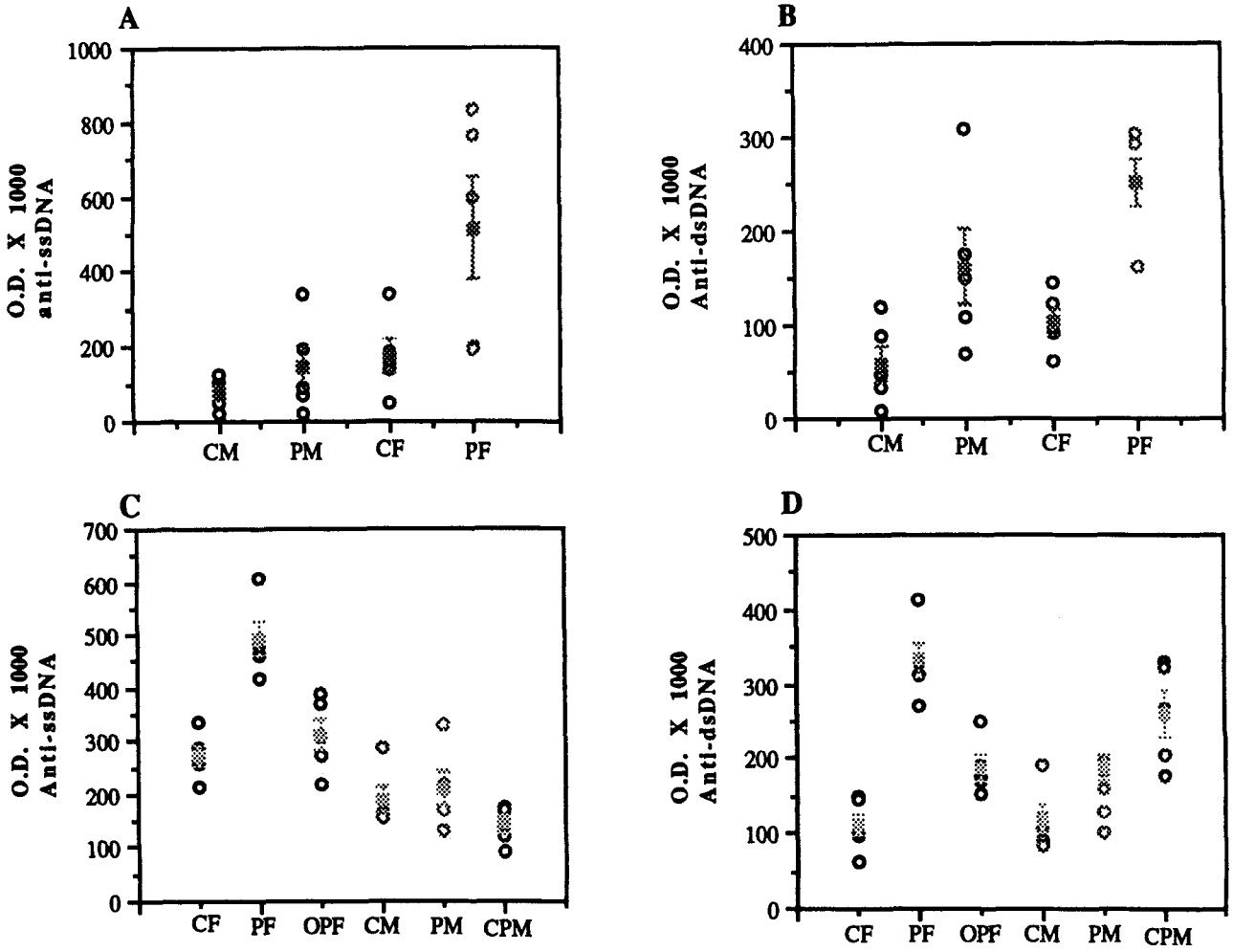

Figure 1. Anti-DNA antibody responses in male and female mice. **A,** Sera from male mice receiving untreated D10 cells (CM), male mice receiving procainamide (Pca)–treated cells (PM), female mice receiving untreated cells (CF), or female mice receiving Pca-treated cells (PF) were tested for antibodies to single-stranded DNA (anti-ssDNA) by enzyme-linked immunosorbent assay (ELISA). **B,** Sera from the same mice as in **A** were tested for anti–double-stranded DNA (anti-dsDNA) antibodies by ELISA. **C,** Sera from female mice receiving untreated D10 cells, female mice receiving Pca-treated cells, oophorectomized mice receiving Pca-treated cells (OPF), male mice receiving untreated D10 cells, male mice receiving Pca-treated cells, or castrated male mice receiving Pca-treated cells (CPM) were tested for anti-ssDNA antibodies by ELISA. **D,** Sera from the same mice as in **C** were tested for anti-dsDNA antibodies by ELISA. Each circle represents the mean of quadruplicate determinations on serum from a single mouse. Squares and error bars show the mean ± SEM values for each group of mice. O.D. = optical density.

icantly more anti-ssDNA antibodies than did males receiving the same cells ($P < 0.05$). A similar trend was observed for titers of anti-dsDNA antibodies, whereby female mice produced higher titers than were found in 4 of the 5 male mice tested. These results were confirmed in second experiments for both anti-ssDNA and anti-dsDNA (Figures 1C and D; $P < 0.001$ for male versus female mice receiving Pca-treated cells).

Using an IgG-specific ELISA, the anti-ssDNA and anti-dsDNA responses in female mice receiving untreated D10 cells were 0.202 ± 0.030 and 0.238 ± 0.031 (mean ± SEM optical density [OD] units), respectively, while females receiving Pca-treated cells mounted a greater response (anti-ssDNA 0.327 ± 0.042, $P < 0.05$; anti-dsDNA 0.502 ± 0.073, $P < 0.01$). In contrast, no significant increase in the IgG anti-ssDNA or anti-dsDNA responses was seen in the male mice after receiving Pca-treated cells (anti-ssDNA 0.136 ± 0.017 versus 0.143 ± 0.028; anti-dsDNA 0.056 ± 0.032 versus 0.093 ± 0.029). The difference between the male and

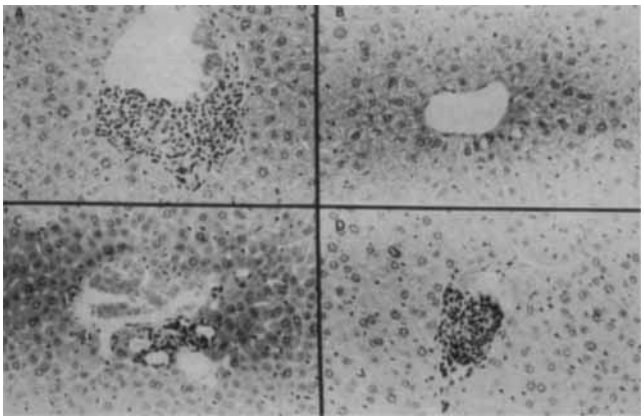

Figure 2. Histologic analysis of liver specimens from male and female mice. **A,** Representative liver section from a female mouse receiving procainamide (Pca)–treated D10 cells, showing lymphocytic periductal inflammation. **B,** Representative liver section from a male mouse receiving Pca-treated cells. The liver appears normal. **C,** Representative liver section from an oophorectomized female mouse receiving Pca-treated cells, showing very mild periductal inflammation. **D,** Representative liver section from an orchiectomized male mouse, showing a lymphocytic inflammatory infiltrate adjacent to a bile duct. (Hematoxylin and eosin stained, original magnification × 200.)

female IgG anti-ssDNA responses was significant ($P < 0.01$), as were the IgG anti-dsDNA sex-specific responses ($P < 0.001$). Quantitation of total IgG and IgM by ELISA showed no significant differences between the mice receiving treated cells and those receiving untreated cells, as reported previously (4,5,8), indicating that the differences in autoantibody titers were not a result of polyclonal B cell activation.

Histologic analysis revealed both renal and pulmonary disease in male and female mice (previously reported in ref. 4). However, although periductal inflammation was observed in female mice receiving Pca-treated cells (Figure 2A), but not in female mice receiving untreated D10 cells (previously reported in ref. 4), none of the male mice developed liver disease (Figure 2B) ($P < 0.005$). To exclude the possibility that the male mice mounted an antibody response to as-yet-unidentified determinants on the D10 cells, which would result in elimination of the transferred cells and subsequent diminished effect, evidence for anti-D10 antibodies was sought. Sera from the 4 groups were diluted 1:100 and then allowed to bind D10 cells. Bound Ig was detected using FITC-conjugated anti-mouse Ig, and quantitated using flow cytometry. No difference in staining of the D10 cells was found between male and female mice. Together, these results demonstrated that D10 cells cause significantly higher-titer anti-DNA antibodies and greater frequency of autoimmune liver disease in female mice compared with male mice.

Gonadal effects in this system were examined by comparing serologic and histologic evidence of autoimmunity in normal and oophorectomized females, and in normal and orchiectomized males. Figures 1C and D show the differences in anti-ssDNA and anti-dsDNA antibody responses in normal mice receiving untreated or Pca-treated D10 cells compared with neutered mice receiving Pca-treated D10 cells. Oophorectomy resulted in a significant decrease in the total anti-ssDNA response in female mice receiving Pca-treated cells ($P < 0.005$). Moreover, a small but statistically insignificant decrease in titer was seen in castrated versus normal male mice receiving Pca-treated cells. Similar results were seen for the anti-dsDNA response in female mice receiving Pca-treated cells ($P < 0.001$, control versus oophorectomized mice). The oophorectomized mice receiving Pca-treated cells did not mount significant IgG responses to ssDNA or dsDNA relative to controls (data not shown). However, castrated males produced greater total Ig and IgG-specific anti-dsDNA responses than did controls ($P < 0.02$ and $P < 0.01$, respectively), whereas no change was observed in the anti-ssDNA response. Earlier reports have indicated that antibody responses to dsDNA appear earlier than do responses to other nucleic acid antigens in castrated male NZB × NZW mice (15), thus suggesting an explanation for our findings.

Histologic evaluation demonstrated pulmonary disease in neutered males and females, essentially identical to that seen in intact animals. Mild renal disease was also seen in both groups. However, in oophorectomized females, the severity of the liver disease markedly decreased (Figure 2C), while periductal inflammation was seen in 4 of the 5 castrated males (Figure 2D). These results indicate that oophorectomy yields significantly decreased autoantibody responses, to levels approximating those seen in males. Moreover, oophorectomy decreases the severity of hepatic disease in mice, while orchiectomy may increase it.

Homing of untreated and Pca-treated D10 cells in male and female mice. We next analyzed whether differences in homing could be correlated with these sex-specific differences in disease manifestations. It was important to first determine if Pca alters expression of MEL-14, a T cell homing molecule that targets lymphocytes to peripheral lymph nodes (16). In a previous study using flow cytometry, D10 cells expressed no detectable MEL-14, consistent with its down-regulation in "memory" T cells (16). In the present study, the treatment of D10 cells with Pca, using schedules and concentrations

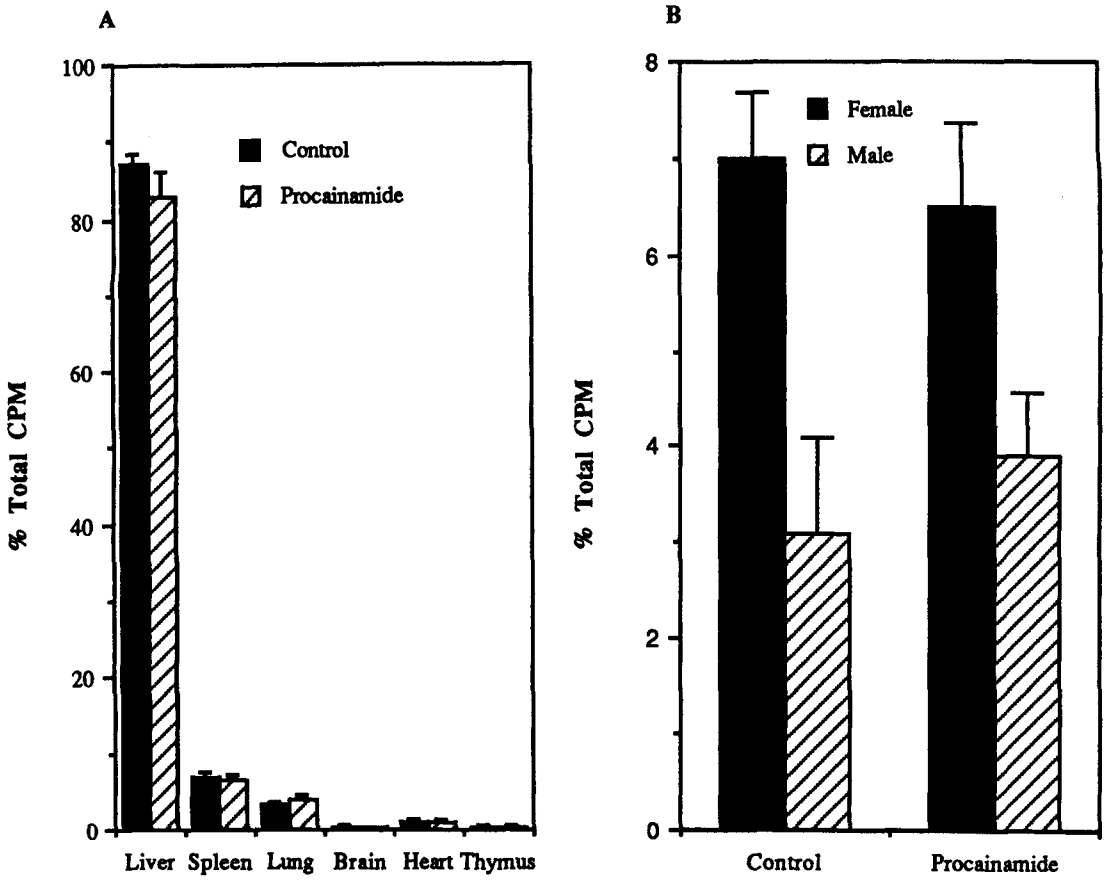

Figure 3. Homing of ^{51}Cr-labeled D10 cells. **A,** Female mice were injected intravenously with 5×10^6 ^{51}Cr-labeled untreated D10 cells or procainamide (Pca)–treated D10 cells. The indicated organs were removed 24 hours later and ^{51}Cr uptake was determined by measuring gamma emissions. Total counts per minute from the organs shown were added together, and results for each organ are expressed as a percentage of this sum. Bars show the mean and SEM percentage for 8 mice receiving untreated cells or 9 mice receiving treated cells. **B,** Female (n = 8–9 per group) or male (n = 5 per group) mice received injections of untreated or Pca-treated D10 cells, and homing to the liver, spleen, lung, brain, heart, and thymus was measured as described in **A.** Bars show the mean and SEM percentage of labeled cells homing to the spleen.

known to induce autoimmunity in vivo, did not induce expression of MEL-14, indicating that altered expression of this molecule does not contribute to autoimmunity in this system.

The effect of Pca treatment on D10 tissue distribution was then determined. Female AKR mice were injected with 5×10^6 ^{51}Cr-labeled untreated or Pca-treated D10 cells as before, then killed 24 or 48 hours later. The liver, spleen, lungs, brain, heart, and thymus were removed as whole organs, and representative samples of mesenteric lymph nodes, salivary glands, skin, and muscle were obtained. All tissue samples were weighed, and ^{51}Cr content was determined by measuring gamma emissions. Overall, at 24 hours, no more than 1% of the injected radioactivity could be detected in mice receiving treated or untreated cells. This decreased to 0.6% at 48 hours, suggesting that relatively few cells are needed to induce autoimmunity. Figure 3A compares the homing of untreated D10 cells (n = 8) and Pca-treated cells (n = 9) in female AKR mice at 24 hours. There was no difference in homing of treated and untreated cells in any of the organs studied. Overall, the liver retained the greatest amount of radioactivity, and less, but still significant, amounts were retained by the spleen and lung. No significant radioactivity accumulated in the brain, heart, or thymus. Similarly, no significant amount of radioactivity could be detected in the lymph nodes, muscle, skin, or salivary gland samples.

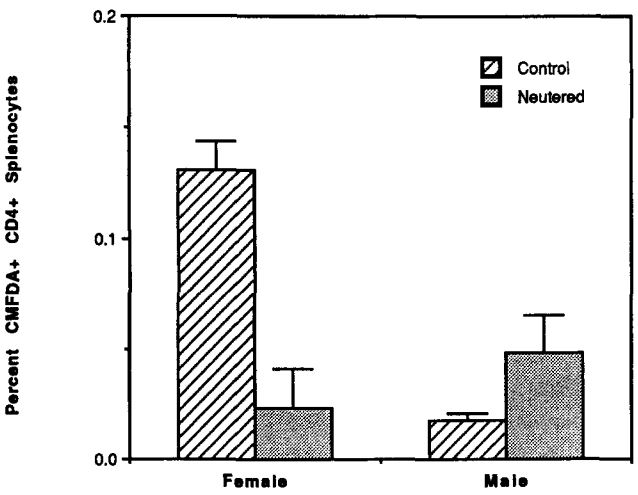

Figure 4. Splenic homing of 5×10^6 5-chloromethylfluorescein diacetate (CMFDA)–labeled D10 cells injected into intact (control) or neutered female and male mice. Twenty-four hours later, the splenocytes were isolated and stained with phycoerythrin-conjugated anti-CD4, then CMFDA+ and CD4+ cells were analyzed by flow cytometry. Results were calculated as (the number of cells bearing both markers divided by the total number of CD4+ cells) × 100. Bars show the mean and SEM percentage of splenic homing.

Essentially identical results were observed at 48 hours (4 mice per group), with similar tissue distribution and again no difference between treated and untreated cells.

The same studies were performed in males, using 5 mice per group, and performed in parallel with female controls. Overall, the same amount of radioactivity was retained as in female mice, and again no difference in homing was seen between untreated and Pca-treated cells. Uptake by the liver, lung, brain, heart, thymus, lymph nodes, salivary glands, skin, and muscle was also identical to that in the female mice. However, approximately twice as much radioactivity accumulated in the spleens of female mice compared with male mice. Figure 3B shows the difference in radioactivity taken up by the spleens of female and male mice that had received either treated ($P < 0.05$) or untreated ($P < 0.01$) D10 cells.

The difference in radioactivity taken up by the spleens of male and female mice could be due to differences in D10 trafficking, larger spleens in the female mice, or increased uptake of released ^{51}Cr by female splenic phagocytic cells. The possibility that larger spleen size in female mice contributed to the difference was excluded by weighing spleens from both male and female mice. There was no significant difference in splenic weight between male and female mice (mean ± SEM 87 ± 4 mg in 10 males versus 91 ± 5 mg in 18 females).

To determine if the radioactivity represented labeled T cells, splenic homing studies were repeated using CMFDA-labeled cells. First, female AKR mice were injected with 5×10^6 CMFDA-labeled control or Pca-treated D10 cells. Twenty-four hours later, the mice were killed, splenocytes were isolated and stained with anti-CD4–PE, and cytofluorographic analysis was performed to quantitate cells expressing both CD4 and CMFDA. Again, no significant difference in splenic homing was observed between treated and untreated cells (n = 3 mice/group). These studies were then performed in 8 male and 10 female mice, using untreated D10 cells. In female mice, CMFDA-labeled cells represented 0.131 ± 0.013% of the CD4+ splenocytes, while in male mice, they represented 0.018 ± 0.003% ($P < 0.001$), a 7-fold difference. This difference could not be attributed to differences in the total percentage of splenic CD4+ T cells in these animals (mean ± SEM 50.9 ± 4.0% in males versus 49.3 ± 2.9% in females). These results confirm those obtained using ^{51}Cr, indicating that more D10 cells accumulate in the spleens of female mice compared with male mice. Attempts were also made to detect CMFDA-labeled cells in sections from the livers, lungs, and spleens by fluorescence microscopy. No fluorescent cells were detected, possibly due to the low number of cells surviving injection.

The effect of male and female castration on splenic homing was examined by comparing the results in 5 orchiectomized male mice and 4 oophorectomized female mice with those in normal controls (Figure 4). Castration produced a small, but statistically insignificant, increase in male splenic homing. However, splenic homing in oophorectomized females was significantly ($P < 0.001$) less than in intact females, and not significantly different from that in males. This suggests that female sex steroids may influence splenic T cell homing.

Importance of splenic homing. Although a reproducible and statistically significant difference in splenic accumulation could be seen between normal male and female mice, the relevance of splenic homing to autoimmunity was uncertain. The importance of splenic homing was determined by comparing disease severity in splenectomized mice with that in control mice. Intravenous injections of 5×10^6 untreated or Pca-treated D10 cells were administered to control or splenectomized female AKR mice using protocols identical to those described above. In Figure 5, titers of autoantibodies to ssDNA and dsDNA are compared among the 4 groups of mice. Figure 5A shows the results of the anti-ssDNA

assay. Splenectomized mice receiving Pca-treated cells produced anti-ssDNA antibodies in amounts similar to those in controls, and produced significantly ($P < 0.05$) less than did intact mice receiving Pca-treated cells. IgG-specific ELISAs gave similar results for splenectomized mice (mean ± SEM OD units 0.170 ± 0.016 in those receiving untreated cells versus 0.193 ± 0.043 in those receiving Pca-treated cells).

Figure 5B shows sera from the same mice analyzed for anti-dsDNA autoantibody responses. Again, control mice receiving Pca-treated cells produced significantly ($P < 0.02$) greater amounts of anti-dsDNA than did splenectomized mice. Similarly, IgG-specific assays demonstrated a significant decrease in the anti-dsDNA response in splenectomized mice (mean ± SEM OD units 0.502 ± 0.073 in intact mice versus 0.227 ± 0.061 in splenectomized mice; $P < 0.02$). To exclude the possibility that the differences in autoantibody titers were due to polyclonal increases in Ig, levels of total serum IgG and IgM were again measured in all 4 groups. No significant differences were found, thus ruling out this possibility.

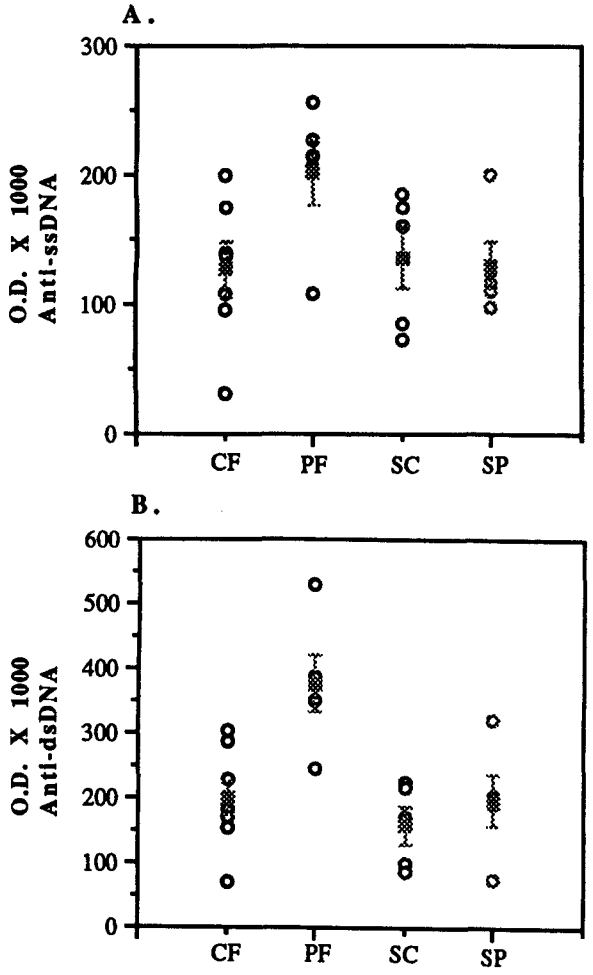

Figure 5. Anti-DNA antibody responses in splenectomized mice. Sera were obtained from female mice receiving untreated D10 cells (CF) or procainamide (Pca)–treated cells (PF), or from splenectomized female mice receiving untreated D10 cells (SC) or Pca-treated cells (SP). **A,** Sera were analyzed for anti–single-stranded DNA (anti-ssDNA) antibodies by enzyme-linked immunosorbent assay as in Figure 1. **B,** The same sera were analyzed for anti–double-stranded DNA (anti-dsDNA) antibodies as in Figure 1. Each circle represents the mean of quadruplicate determinations on serum from a single mouse. Squares and error bars show the mean ± SEM values for each group of mice. O.D. = optical density.

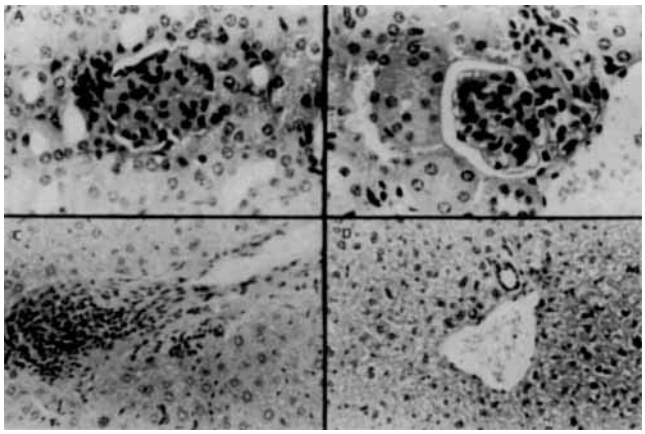

Figure 6. Histologic analysis of kidney and liver specimens from control and splenectomized mice. **A,** Glomerulus from a control mouse receiving procainamide (Pca)–treated cells. Karyorrhectic debris and increased mesangial matrix with obliteration of capillary loops are seen (hematoxylin and eosin [H & E] stained, original magnification × 375). **B,** Representative glomerulus from a splenectomized mouse receiving Pca-treated cells. The glomerulus appears normal (H & E stained, original magnification × 375). **C,** Liver section from a normal female mouse receiving Pca-treated D10 cells, showing periductal inflammation (H & E stained, original magnification × 200). **D,** Representative liver section from a splenectomized mouse receiving Pca-treated cells, showing normal histologic features (H & E stained, original magnification × 200).

Figure 6 shows representative sections from the kidneys and livers of control and splenectomized mice. In Figure 6A, karyorrhectic debris and increased mesangial matrix with obliteration of capillary loops can be seen in a section from a control mouse receiving Pca-treated cells. In other sections, hypercellularity was observed. In Figure 6B, the glomerulus appears to be normal in a splenectomized mouse receiving Pca-treated cells. In Figure 6C, autoimmune liver disease is apparent in a normal female mouse after administration of Pca-treated D10 cells. In contrast, Figure 6D shows a

normal-appearing liver sample from a splenectomized mouse receiving the same cells. Among all 5 of the splenectomized mice receiving Pca-treated cells, none developed either liver or kidney disease, while all 5 of the controls developed lesions in both of these organs after receiving Pca-treated cells ($P < 0.005$).

In contrast to the kidney and liver manifestations, no changes were observed in the pulmonary disease. Since no anti-DNA antibodies, renal disease, or liver disease developed in splenectomized mice, it is possible that autoantibodies contributed to the kidney and liver disease but splenectomy prevented their generation. Frozen sections of the liver, lung, and kidneys of intact female mice receiving Pca-treated cells were therefore examined for immune complex deposition, by staining with GAM-FITC. While immune complexes could be readily observed in the kidney (previously reported in ref. 4), no immune complex deposition was detected in the liver and lung. Indirect immunofluorescence experiments, in which sera from female mice with liver and lung disease were used to stain frozen liver and lung sections from normal female AKR mice, also failed to demonstrate the presence of autoantibodies. Together, these results suggest that the spleen is essential to the autoantibody response and to the severity of kidney and liver disease, but not to pulmonary disease. Moreover, these results show that neither the lung disease nor the liver disease appeared to involve autoantibodies.

DISCUSSION

These experiments have allowed us to make several novel observations regarding the relationship between sex, autoimmune disease, and T cell homing. The initial experiments characterized the sex-specific disease manifestations of this system. The female recipients developed higher titers of autoantibodies, which decreased following oophorectomy. Similar results have been reported in NZB × NZW mice (17), in which T cells were also important for disease development (18). However, although the level at which sex affects the immune response in the genetic models is uncertain, the use of a defined signal in the present model suggests that a major effect of sex is at the level of the T cell–host interaction, since the stimulus was identical in both male and female mice.

There was also a sex-specific effect on liver disease. The liver disease in this model most closely resembles human primary biliary cirrhosis, with bile duct proliferation and an intense inflammatory infiltrate (4). This manifestation had a striking female predilection, with all of the female and none of the male mice demonstrating abnormalities. The liver disease was ameliorated by oophorectomy, and orchiectomy increased the degree of liver damage. This suggests that female sex steroids may augment, and male sex steroids suppress, this manifestation. Further studies examining the role of exogenous sex steroids in this model may shed additional light on the pathogenesis of this lesion, as will studies characterizing the infiltrating cells. Interestingly, more than 95% of patients with primary biliary cirrhosis are women, and some have positive antinuclear antibody (19), suggesting that this may be a useful model for studying this sex-specific disease in humans.

In contrast to the autoantibody and liver test results, the pulmonary disease induced by drug-treated cells had no sex predilection, and was not modified by neutering or splenectomy. This suggests that pulmonary disease is independent of sex steroids and does not require homing to lymphoid tissue. It is possible that the autoreactive cells, injected intravenously and retained by the lung, contributed directly to the pulmonary lesions. Future studies using cells with a transgenic marker may permit homing studies of longer duration, to examine this hypothesis.

Homing studies demonstrated a sex-specific difference in T cell uptake by the spleen, which, to the best of our knowledge, has not been previously reported. The initial studies used ^{51}Cr-labeled cells, and demonstrated no significant differences in the homing of treated or untreated cells. The majority of the radioactivity was found in the liver, and lesser amounts were found in the lung and spleen. This distribution is consistent with that seen in earlier studies using the same approach (20), and suggests that Pca treatment has no significant effect on T cell trafficking to these organs. Comparison of trafficking in male and female mice, however, demonstrated that more cells accumulate in the spleens of female mice than in those of males. This was shown using both ^{51}Cr and CMFDA, and was observed with both untreated and Pca-treated cells. Moreover, neutering the females decreased splenic homing, suggesting that estrogens may play a role in this phenomenon. Together, these results support the contention that lymphoid homing of T cells is different in males and females, and that female sex steroids may contribute to this difference, possibly because of an effect of estrogens on endothelial adhesion molecule expression, as previously shown (10). Further experiments using exogenous administration of estrogens and measuring trafficking and endothelial adhesion molecule expression in neutered and control mice would help test this possibility.

We also determined the significance of splenic homing by comparing disease manifestations in normal mice with those in splenectomized mice. Splenectomy abrogated the autoantibody response, and splenectomized mice did not develop renal or liver disease. This indicates that the spleen is crucial for these disease manifestations, and suggests that the spleen is the site where D10 cells interact with other cells of the immune system, lysing macrophages and secreting cytokines such as IL-4 and IL-6 (4–6). Combined with other splenic T cells, this appears to result in the development of anti-DNA antibodies and other disease manifestations, by mechanisms similar to those producing chronic graft-versus-host disease (7,8). This is consistent with the findings of other studies, in which the spleen and splenic lymphocytes have been implicated in the development of lupus-like autoimmune diseases, although earlier splenectomy was required in these models (21–23). We have also previously observed that IgM anti-DNA antibodies appear first, and isotype switching to IgG occurs ~4 weeks later (ref. 5 and unpublished results), further supporting a role for T cells in the development of these autoantibodies. The lack of glomerulonephritis in the splenectomized group may be attributed to the absence of autoantibodies, since this manifestation is associated with immune complex deposition (4,5). Moreover, others, in addition to our group, have shown the importance of anti-DNA antibodies in the pathogenesis of lupus nephritis (24–26).

However, the pathogenesis of the liver disease in this model appears to be different. We could find no evidence for liver autoantibodies, either by staining for Ig deposition, or by indirect immunofluorescence using sera from animals with liver disease and using male and female liver tissue from healthy animals. The observation that the spleen is required for the liver disease manifestations also suggests that cells homing to the liver are probably not directly responsible for the autoimmune lesions. It is possible that the D10 cells trigger the development of other autoreactive lymphocytes in the spleen, which, in turn, contributes to the liver abnormalities.

In summary, homing to the spleen appears to be important for the development of autoantibodies, renal disease, and liver lesions. Significantly more T cells accumulate in the spleens of female mice than in those of males, and the degree of uptake varies directly with the severity of the autoantibody response and liver disease. Finally, oophorectomy results in fewer cells accumulating in the spleen, and in milder disease. Together, these results suggest that the female-specific increase in disease severity may be due to greater numbers of autoreactive D10 cells homing to the spleens of female mice. The results also suggest that autoreactive D10 cells cause autoimmunity by several mechanisms. Homing studies of longer duration, detailed characterization of the cells infiltrating the liver and lung, and studies on the effector mechanisms will be necessary to clarify the process by which autoreactive D10 cells cause tissue lesions in this system.

Other interpretations have been considered. It is possible that trauma and stress associated with oophorectomy or splenectomy may have decreased the severity of some manifestations. However, 3 observations weaken this possibility. The surgery was performed at least 5 months prior to killing the mice. The immunologic effects of surgery are seen shortly after the operation and are self-limited (27). It is unlikely that nonspecific effects of surgery, performed 5 or more months prior to studying the mice, would completely eliminate the autoantibody response, as was observed. Furthermore, other investigators have included sham-splenectomized mice as controls in studies on the effects of splenectomy, and have demonstrated little effect of the control operation on Ig levels and antibody responses (28). Finally, those same studies have demonstrated that splenectomized mice are capable of mounting an antibody response by bone marrow B cells (28). Therefore, the lack of an autoantibody response in the splenectomized mice described in the present report provides further evidence that the spleen, rather than other sites such as the bone marrow, is where the autoreactive D10 cells interact with other cells of the host's immune system.

Another interpretation of these results is that cells killed during the injection process contribute to the autoantibody response by providing a source of chromatin as antigen. However, similar numbers of treated and untreated cells survived the injection, and only the autoreactive cells caused autoimmunity. Furthermore, we have reported that heat-killed autoreactive cells do not trigger autoimmunity (5). For these reasons, it seems likely that viable autoreactive cells are responsible for the disease manifestations observed.

These results thus support the contention that splenic homing of autoreactive cells can trigger an autoimmune disease in this system, and that there is a sex-specific difference in the number of cells homing to the spleen, which can explain the increased severity in female mice. This may have relevance to the striking sex-specific predilection that lupus has for women. It should also be noted that dramatic improvement in human lupus, including increased survival, has been

reported following splenectomy (29,30). Sex-specific homing of autoreactive T cells to splenic or peripheral lymphoid tissue in humans could play a role similar to the spleen in this murine model. Studies comparing endothelial adhesion molecule expression in lymphoid tissue from men and women would help confirm this hypothesis.

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REFERENCES

1. Hochberg MC: The Epidemiology of Systemic Lupus Erythematosus. Philadelphia, Lea & Febiger, 1993
2. Cutolo M, Sulli A, Seriolo B, Accardo S, Masi AT: Estrogens, the immune response and autoimmunity. Clin Exp Rheumatol 13:217–226, 1995
3. Grossman CJ: Interactions between the gonadal steroids and the immune system. Science 227:257–260, 1985
4. Yung RL, Quddus J, Chrisp CE, Johnson KJ, Richardson BC: Mechanisms of drug induced lupus. I. Cloned Th2 cells modified with DNA methylation inhibitors in vitro cause autoimmunity in vivo. J Immunol 154:3025–3035, 1995
5. Quddus J, Johnson KJ, Gavalchin J, Amento EP, Warren JS, Chrisp C, Yung RL, Richardson BC: Treating activated CD4+ T cells with either of two distinct DNA methyltransferase inhibitors, 5-azacytidine or procainamide, is sufficient to induce a lupus-like disease in syngeneic mice. J Clin Invest 92:38–53, 1993
6. Richardson BC, Liebling MR, Hudson JL: CD4+ cells treated with DNA methylation inhibitors induce autologous B cell differentiation. Clin Immunol Immunopathol 55:368–381, 1990
7. Pals S, Radaszkiewicz T, Roozendaal L, Gleichmann E: Chronic progressive polyarthritis and other symptoms of collagen vascular disease induced by graft-vs-host reaction. J Immunol 134:1475–1482, 1985
8. Yung R, Powers D, Johnson K, Amento E, Carr D, Laing T, Yang J, Chang S, Hemati N, Richardson BC: Mechanisms of drug-induced lupus. II. T cells overexpressing lymphocyte function-associated antigen 1 become autoreactive and cause a lupus-like disease in syngeneic recipients. J Clin Invest 97:2866–2871, 1996
9. Stoolman LM: Adhesion molecules controlling lymphocyte migration. Cell 56:907–910, 1989
10. Cid MC, Kleinman HK, Grant DS, Schnaper HW, Fauci AS, Hoffman GS: Estradiol enhances leukocyte binding to tumor necrosis factor (TNF)-stimulated endothelial cells via an increase in TNF-induced adhesion molecules E-selectin, intercellular adhesion molecule type 1, and vascular cell adhesion molecule type 1. J Clin Invest 93:17–25, 1994
11. Gumkowski F, Kaminska G, Kaminski M, Morrissey LW, Auerbach R: Heterogeneity of mouse vascular endothelium: in vitro studies of lymphatic, large blood vessel and microvascular endothelial cells. Blood Vessels 24:11–23, 1987
12. Kaye J, Porcelli S, Tite J, Jones B, Janeway CA Jr: Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. J Exp Med 158:836–856, 1983
13. Saizawa MK, Hug E, Haque S, Portoles P, Suzuki S, Eichmann K: Autoreactivity of low but not of high CD4 variants of an antigen-specific, I-A-restricted mouse T cell clone. J Immunol 148:702–709, 1992
14. Richardson B, Kahn L, Lovett EJ, Hudson J: Effect of an inhibitor of DNA methylation on T cells. I. 5-azacytidine induces T4 expression on T8+ T cells. J Immunol 137:35–39, 1986
15. Roubinian JR, Papoian R, Talal N: Androgenic hormones modulate autoantibody responses and improve survival in murine lupus. J Clin Invest 59:1066–1070, 1977
16. Pober JS, Cotran RS: Immunologic interactions of T lymphocytes with vascular endothelium. Adv Immunol 50:261–302, 1991
17. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK: Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F_1 mice. J Exp Med 147:1568–1583, 1978
18. Wofsy D, Seaman WE: Reversal of advanced murine lupus in NZB/NZW F1 mice by treatment with monoclonal antibody to L3T4. J Immunol 138:3247–3253, 1987
19. Michieletti P, Wanless IR, Katz A, Scheuer PJ, Yeaman SJ, Bassendine MF, Palmer JM, Heathcote EJ: Antimitochondrial antibody negative primary biliary cirrhosis: a distinct syndrome of autoimmune cholangitis. Hepatology 18:10–15, 1993
20. Jung LKL, Good RA, Fernandes GA: Studies on lymphocyte homing in autoimmune prone NZB mice. Immunol Invest 15:11–23, 1986
21. Smathers PA, Santoro TJ, Chused TM, Reeves JP, Steinberg AD: Studies of lymphoproliferation in MRL-lpr/lpr mice. J Immunol 133:1955–1961, 1984
22. Burnet FM, Holmes MC: The natural history of the NZB/NZW F_1 hybrid mouse: a laboratory model of systemic lupus erythematosus. Aust Ann Med 14:185–191, 1965
23. Roubinian JR, Papioan R, Sawada S, Talal N: Immunological regulation of spontaneous antibodies to DNA and RNA. III. Early effects of neonatal thymectomy and splenectomy. Immunology 33:399–406, 1977
24. Swanson PC, Yung RL, Blatt NB, Eagan MA, Norris JN, Richardson BC, Johnson KJ, Glick GD: Ligand recognition by anti-DNA autoantibodies. II. Genetic analysis and pathogenicity. J Clin Invest 97:1748–1760, 1996
25. Koffler D, Schur PH, Kunkel HG: Immunological studies concerning the nephritis of systemic lupus erythematosus. J Exp Med 126:607–623, 1967
26. Lambert PH, Dixon FJ: Pathogenesis of the glomerulonephritis of NZB/W mice. J Exp Med 127:507–521, 1968
27. Strausser HR, Fiore RP, Belisle EH: Alterations in Immune Function with Age, Sex Hormones, and Stress. New York, Marcel Dekker, 1984
28. Rozing J, Brons NHC, Benner R: Effects of splenectomy on the humoral immune system: a study in neonatally and adult splenectomized mice. Immunology 34:909–917, 1978
29. Best WR, Darling DR: A critical look at the splenectomy S.L.E. controversy. Med Clin North Am 46:19–47, 1962
30. Gruenberg JC, van Slyck EJ, Abraham JP: Splenectomy in systemic lupus erythematosus. Am Surg 52:366–370, 1986