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Increased Severity of Murine Lupus in Female Mice Is Due to Enhanced Expansion of Pathogenic T Cells

Thomas J. Lang,2* Phuong Nguyen,* John C. Papadimitriou, † and Charles S. Via*

A strong female predominance is a well-recognized feature of human lupus. The mechanism by which sex influences disease expression and severity is not fully understood. To address this question, we used the parent-into-F1 (p→F1) model of chronic graft-vers-host disease (cGVHD) in which lupus-like humoral autoimmunity and renal disease are induced in normal F1 mice. An advantage of this model is that the pathogenic T cells driving disease (donor strain) can be studied separately from nonspecifically activated T cells (host strain). We observed that lupus-like disease using female donor and host mice (f→F cGVHD) is characterized by more severe long-term disease (glomerulonephritis) than with male donor and host (m→M cGVHD). Interestingly, differences in disease parameters could be seen at 2 wk after parental cell transfer, as evidenced by a 2- to 3-fold greater engraftment of donor CD4+ T cells in f→F cGVHD mice, which persisted throughout disease course. Enhanced engraftment of donor CD4+ T cells in f→F cGVHD mice was not due to differences in splenic homing, alloreactive precursor frequency, initial proliferation rates, or apoptotic rates, but rather to sustained high proliferation rates during wk 2 of disease compared with m→M cGVHD mice. Crossover studies (m→F, f→M) demonstrated that enhanced donor CD4+ T cell proliferation and engraftment segregate with the sex of the host. These results demonstrate that the sex of the recipient can influence the expansion of pathogenic T cells, thus increasing long-term the burden of autoreactive T cells and resulting in greater disease severity. The Journal of Immunology, 2003, 171: 5795–5801.

The mechanisms by which sex influences disease expression in murine lupus are not fully understood. A major difficulty in the study of spontaneous murine lupus models such as NZB/W mice is the inability to identify and study the pathogenic T cells that drive disease expression. To this end, we have used an induced murine model of lupus, the parent-into-F1 (p→F1) model of chronic graft-vers-host disease (cGVHD) in which the pathogenic T cells driving disease expression can be identified and studied separately from nonspecifically activated T cells. In this model, normal F1 mice develop a lupus-like disease following the i.v. transfer of homozygous parental strain CD4+ T cells (16). Previously, it has been reported that multiple transfers of parental strain donor cells result in lupus-like renal disease that is more severe in females (17). In the present study, we demonstrate that a single transfer of parental DBA splenocytes into BDF1 mice results in lupus-like renal disease that is more severe in females (17). In the present study, we demonstrate that a single transfer of parental DBA splenocytes into BDF1 mice results in lupus-like renal disease that is more severe in females, and that the increased disease severity is mediated by enhanced donor CD4+ T cell expansion in female hosts during the second week of disease.

Materials and Methods

Mice

Six- to 8-wk-old female and male DBA/2J (DBA) (H-2^d) and B6D2F1 (BDF1) (H-2^bd) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Induction of cGVHD

Single cell suspensions of DBA splenocytes were prepared, and the number of CD4+ T cells was quantitated by flow cytometry before injection. For all experiments, cGVHD was induced by injecting unfractionated splenocytes containing 10–12 × 10^6 DBA CD4+ T cells into the tail vein of recipient BDF1 mice. Control mice were age- and sex-matched uninjected F1 mice. These controls are phenotypically identical with syngeneic controls (F1→F1), as neither develops features of cGVHD, such as renal disease (18).

Assessment of glomerulonephritis in cGVHD

Mice were sacrificed 10 wk after parental cell transfer, and kidneys were fixed in phosphate-buffered Formalin, paraffin embedded, and stained routinely with H&E. All slides were scored blindly by a renal pathologist.

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3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; 5-bromo-2'-deoxyuridine; cGVHD, chronic graft-vs-host disease.

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Glomerular capillary membrane thickness and crescents were semiquantitatively graded using the following scale: 0 = normal; 1+ = mild; 2+ = moderate; 3+ = severe. A glomerular cellular score was also determined in which perivascular cell infiltrates and mesangial cell numbers for each group were quantitated by counting these features in 10 fields per section and calculating the group mean ± SEM (19). Proteinuria was measured by urine dipstick and reported on a scale of 0–4+ (Albustix; Bayer, Elkhart, IN).

Flow cytometric analysis

Single cell suspensions of splenocytes were first incubated with anti-murine FcγR mAb 2.4G2 (20), then stained with saturating concentration of either FITC-conjugated, PE-conjugated, or biotin-conjugated anti-CD4, anti-CD8, anti-B220, and anti-H-2Kb (BD PharMingen, San Diego, CA). Three-color flow cytometry was performed using a FACScan flow cytometer (BD Immunocytometry Division, San Jose, CA.). Lymphocytes were gated based on forward and side scatter and analyzed using CellQuest acquisition and analysis software. Ten thousand events were collected per sample. Donor CD4⁺ and CD8⁺ T cells were identified as staining positively for CD4 or CD8 and negatively for H-2Kb, whereas host T cells stain positively for H-2Kb.

Serologic assays

Serum was tested by ELISA for the presence of anti-ssDNA IgG Abs, as described (21). Briefly, microtiter plates were coated with heat-denatured salmon sperm DNA, blocked with 2% BSA-PBS, and incubated with serial dilutions of mouse serum beginning at a dilution of 1/40. Wells were washed and incubated with anti-mouse IgG alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, AL). OD was determined at 405 nm. MRL/lpr sera was assayed as a standard, and arbitrary units were calculated assuming that serum diluted 1/40 contains 100 U anti-ssDNA Ab/ml.

Splenic homing, proliferation, and alloreactive precursor frequency of donor CD4⁺ T cells

CFSE (Molecular Probes, Eugene, OR) labeling of donor splenocytes and analysis of donor cell proliferation by flow cytometry were performed, as previously described (22, 23). For homing studies, mice were sacrificed at 24 h after transfer of CFSE-labeled donor cells. For donor CD4⁺ T cell proliferation and alloreactive precursor frequency studies, mice were sacrificed at 72 h after parental cell transfer.

5-bromo-2’-bromodeoxyuridine (BrdU) incorporation

On days 7, 10, or 14 after donor cell transfer, mice received two doses of 0.8 mg BrdU i.p. separated by a 2-h interval. Two hours after the second dose, mice were sacrificed and splenocytes were stained with anti-CD4 PE and anti-H-2Kb CyChrome (BD PharMingen). BrdU-positive cells were identified using BrdU Flow Kit (BD PharMingen). Proliferating donor T cells were defined as staining positively for BrdU and CD4, but negatively for H-2Kb.

Determination of apoptotic donor CD4⁺ T cells

Splenocytes from cGVHD mice at day 7 or 10 were stained for CD4 and H-2Kb, as described above, then stained by either TUNEL (Apo-Direct), annexin V (Apo-annexin V), or anti-activated caspase 3 Ab (all from BD PharMingen), according to the manufacturer’s instructions.

In vitro MLR

MLRs were performed in round-bottom 96-well microtiter plates. Normal male and female DBA responders were plated at 4 × 10⁵ splenocytes per well. Stimulators consisted of day 7 m→M or f→F cGVHD splenocytes irradiated with 3000 rad and plated at 1 × 10³ or 2 × 10³ cells/well. Cells were pulsed with [³H]thymidine 12 h before harvesting at either 48 or 60 h.

Statistical analysis

Data were examined for normality and equal variance (Kolmogorov-Smirnov). If satisfactory, groups were compared by two-tailed Student’s t test.

Results

A single injection of 80 × 10⁶ DBA splenocytes results in more severe lupus-like disease in f→F than m→M cGVHD

Previous work has reported that four sequential injections of 30 × 10⁶ DBA splenocytes administered every 3 days into BDF₁ mice resulted in lupus-like renal disease that is more severe in females than in males (17). Lupus-like renal disease can also be induced in BDF₁ mice following a single injection of 80–100 × 10⁶ DBA splenocytes (19, 24). To determine whether lupus-like cGVHD induced by a single dose of parental spleen cells is more severe in females, we compared disease outcome seen when both donors and hosts were female (f→F cGVHD) with that when both donors and...
hosts were male (m→M cGVHD). Renal histology performed at 10 wk after donor cell transfer (Fig. 1) demonstrates that f→F cGVHD mice exhibit more severe glomerulonephritis (Fig. 1, C and D) compared with either m→M cGVHD mice (Fig. 1B), normal female F1 mice (Fig. 1A), or normal male F1 mice (data not shown). Specifically, f→F cGVHD mice exhibited thickened capillary loops, mesangial proliferation, sclerosis, and glomerular enlargement. Severity of hyalinosis and crescent formation in f→F cGVHD ranged from 1+ to 3+, while in m→M cGVHD, it was 0 to 1+. Moreover, f→F cGVHD kidneys exhibited greater glomerular cellularity (normal male or female F1 = 25 ± 3 cells/glomerulus; f→F = 40 ± 3.7 cells/glomerulus; m→M = 28 ± 1 cells/glomerulus; p < 0.05, f→F vs m→M). Only a female glomerulus is shown in Fig. 1, as histologic scoring of normal male and female glomeruli did not differ.

Other measures of lupus-like disease were also more severe in f→F cGVHD mice compared with m→M cGVHD mice, such as a greater degree of proteinuria (p < 0.05 at 4 and 8 wk) (Fig. 2A), and higher serum levels of anti-ssDNA Ab (Fig. 2B) (p < 0.05 at 6 and 8 wk). In the same group of animals, visible ascites was present in 3 of 5 f→F cGVHD mice and 0 of 5 m→M cGVHD mice at 8 wk (data not shown).

**Increased disease severity in f→F cGVHD mice is associated with increased engraftment of donor CD4+ T cells and expansion of host B cells at 2 wk after donor cell transfer**

F→F cGVHD exhibit an increase in serum anti-ssDNA Ab compared with m→M cGVHD mice as early as 4 wk after disease induction (Fig. 2B). Although the presence of anti-dsDNA Ab is more specific for SLE renal disease in both humans and mice, anti-dsDNA Ab in cGVHD mice are typically not detected until ≥4 wk of cGVHD and occur in as few as 40% of cGVHD mice (25). The explanation for this delay is not clear and may reflect more complex processes such as epitope spreading or stochastic events. By contrast, increased anti-ssDNA Ab is a reliable marker of alloantigen-driven, polyclonal B cell hyperactivity and is observed in nearly 100% of cGVHD mice as early as 10 days after disease induction (26). Moreover, for donor inocula ≤10⁷ cells, the level of anti-ssDNA Ab correlates with the magnitude of the allogeneic T cell response in that the injection of greater numbers of donor T cells results in higher levels of IgG anti-ssDNA Ab at 2 wk (24). To determine whether the elevated anti-ssDNA in f→F cGVHD mice reflect greater T and/or B cell expansion, flow cytometric analysis of donor and host lymphocyte subsets was performed 2 and 10 wk after disease induction. At 2 wk, both m→M and f→F cGVHD mice exhibit the characteristic increase in host B cells compared with control F1 mice (Table I); however, the increase in splenic B cell numbers is significantly greater for females (140% increase over control F1) than for males (60% increase over control F1) (p < 0.01, f→F vs m→M). A more striking difference was seen for donor CD4+ T cell engraftment with f→F cGVHD mice exhibiting ~2- to 3-fold greater engraftment of donor CD4+ T cells compared with m→M cGVHD mice, despite the injection of equal numbers of CD4+ T cells. Because cGVHD is a CD4+ T cell-driven disease (16, 26, 27), these results suggest that enhanced expansion of B cells and autoantibody production in f→F cGVHD mice is a consequence of enhanced CD4+ T cell engraftment and expansion (24, 28). Moreover, the increased numbers of donor CD4+ T cells and host B cells seen for f→F cGVHD relative to m→M cGVHD are still observed at 10 wk of disease, indicating that early differences in engraftment and expansion of donor cells are long lasting.

**Enhanced engraftment of donor CD4+ T cells in f→F cGVHD is not due to increased splenic homing**

Sex-dependent differences in T cell homing have been previously reported in an animal model of drug-induced SLE (29). To determine whether differential splenic homing of donor cells in cGVHD contributed to enhanced donor CD4+ T cell engraftment in f→F cGVHD, the presence of donor T cells in host spleens was determined within 24 h after the injection of unfractionated CFSE-labeled splenocytes containing equivalent numbers of donor CD4+ T cells.

**Table I. Expansion of donor CD4+ T cell and host B cells is greater in f→F vs m→M cGVHD mice at both 2 and 10 wk of disease**

<table>
<thead>
<tr>
<th>Group</th>
<th>Donor T Cell</th>
<th>Host B220+</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal F1 female</td>
<td>ND ⁹</td>
<td>ND ⁹</td>
</tr>
<tr>
<td>f→F cGVHD</td>
<td>8.0 ± 0.6 ⁸</td>
<td>0.6 ± 0.3 ⁵</td>
</tr>
<tr>
<td>Normal F1 male</td>
<td>ND ⁹</td>
<td>ND ⁹</td>
</tr>
<tr>
<td>m→M cGVHD</td>
<td>2.5 ± 0.5 ⁸</td>
<td>0.3 ± 0.05 ⁵</td>
</tr>
<tr>
<td>10 wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal F1 female</td>
<td>ND ⁹</td>
<td>ND ⁹</td>
</tr>
<tr>
<td>f→F cGVHD</td>
<td>1.6 ± 0.3 ⁸</td>
<td>0.2 ± 0.03 ⁵</td>
</tr>
<tr>
<td>Normal F1 male</td>
<td>ND ⁹</td>
<td>ND ⁹</td>
</tr>
<tr>
<td>m→M cGVHD</td>
<td>0.6 ± 0.1 ⁰</td>
<td>0.2 ± 0.08 ⁰</td>
</tr>
</tbody>
</table>

* cGVHD was used as described, and mice were sacrificed at either week 2 or 10. Splenic lymphocyte subsets were determined by flow cytometry, and values are shown as group mean ± SEM × 10⁶ (n = 5 mice/group). Similar results were seen in a separate independent experiment for each time point.

ND, Not detectable above background.

"p < 0.01; f→F vs m→M cGVHD."
T cells (12 × 10⁶/mouse). CFSE-stained female and male donor CD4⁺ T cells display a single peak of fluorescence, indicating that donor cells have not undergone cell division at 24 h after cell transfer (Fig. 3, A and B). Thus, the number of donor cells in both groups at 24 h postinjection reflects splenic homing alone and does not differ significantly between f→F (1.96 ± 0.15 × 10⁶) and m→M (2.06 ± 0.10 × 10⁶) cGVHD mice (Fig. 3C).

Enhanced engraftment of donor CD4⁺ T cells in f→F cGVHD is not due to increased frequency of alloreactive precursors

To address whether sex differences in alloreactive precursor frequency contribute to differences in donor CD4⁺ T cell engraftment, CFSE-labeled donor splenocytes were injected into F₁ mice, and the donor CD4⁺ T cells were examined at 3–4 days postinjection by flow cytometry (23). Representative donor CD4⁺ T cell CFSE-staining profiles at 72 h postinjection are shown in Fig. 4, A and B, for a single f→F and m→M cGVHD mouse, respectively. There was no significant difference in the calculated DBA CD4⁺ anti-F₁ precursor frequency between males and females (Fig. 4C) (males = 3.1 ± 0.3%; female = 3.7 ± 0.5%; p = 0.11; n = 6/group).

Enhanced engraftment of donor CD4⁺ T cells in f→F cGVHD is due to greater proliferation during the second week of disease

CFSE-labeled donor cells were used to determine whether differential proliferation rates could account for the enhanced donor CD4⁺ T cell engraftment in f→F cGVHD (23). At 72 h, donor CD4⁺ T cells in both m→M and f→F cGVHD mice have undergone a maximum of five cell divisions, indicating similar rates of division during this time period (Fig. 4, A and B). Moreover, the rate of cell division, as reflected in the percentage of total donor CD4⁺ T cells that have undergone one, two, three, four, or five cell divisions, did not differ significantly between m→M and f→F cGVHD (Fig. 4D).

Because CFSE staining is not useful for determining proliferation rates beyond 4–5 days of disease, BrdU pulse labeling was used to estimate proliferative rates at days 7, 10, and 14 after parental cell transfer. Increased donor CD4⁺ T cell engraftment in f→F cGVHD is not detected at day 7, but by day 10 a >2-fold
increase is seen compared with m→M cGVHD (Table II). Additionally, both the percentage and absolute number of donor CD4+ T cells incorporating BrdU were significantly greater at days 7 and 10 in f→F compared with m→M cGVHD. However, by day 14, BrdU incorporation in f→F cGVHD declines to a level comparable to m→M cGVHD. Thus, the increased engraftment of donor CD4+ T cells observed at day 14 for f→F cGVHD (Tables I and II) can be seen as early as day 10 after parental cell transfer and reflects the prolonged female donor CD4+ T cell proliferation during wk 2 of disease.

To determine whether the above differences in proliferation reflected reduced rates of apoptosis in f→F cGVHD mice during the second week of disease, donor CD4+ T cell apoptosis was assessed at 7 and 10 days after cGVHD induction. No statistically significant differences in apoptosis of donor CD4+ T cells tested directly ex vivo could be detected at day 7 (Fig. 5A) or 10 (Fig. 5B). Staining for activated caspase 3 also showed no difference at day 7 (data not shown).

Enhanced donor CD4+ T cell expansion is dependent on the sex of the host

To determine whether enhanced donor CD4+ T cell expansion segregates with the sex of the donor or the host, female and male donor splenocytes were injected into either the same or the opposite sex BDF1 hosts. Donor CD4+ T cell engraftment in f→F cGVHD is significantly greater at 2 wk of disease compared with m→M cGVHD (Fig. 6), consistent with the data in Tables I and II. Of note, engraftment of donor CD4+ T cells in m→F cGVHD mice is significantly increased compared with m→M cGVHD (p < 0.001), and did not differ significantly from that of f→F cGVHD (p = NS). In contrast, donor CD4+ T cell engraftment in f→M cGVHD was significantly reduced compared with f→F cGVHD (p < 0.001), and was comparable to that seen in m→M cGVHD (p = NS). In a separate experiment, BrdU incorporation into female donor CD4+ T cells at day 10 was significantly increased when injected into female hosts as compared with male hosts (f→F = 11.1% ± 1.1 vs f→M = 6.7 ± 1.0; p < 0.05). These results indicate that it is the sex of the host that influences donor CD4+ T cell proliferation and engraftment in cGVHD.
**Enhanced T cell proliferation is not due to enhanced host APC function ex vivo**

Enhanced T cell proliferation is not due to enhanced host APC function ex vivo. It is the foregoing sex-dependent differences in T cell proliferation reflect differences in the ability of host APCs (including B cells) to drive donor CD4+ T cell proliferation. Based on the data presented in Table II demonstrating that differential donor T cell proliferation is not observed until day 7 of disease, we tested the APC function of day 7 cGVHD splenocytes in a MLR. Splenocytes from either f→F or m→M cGVHD mice were obtained at day 7 of disease and used as stimulators for naïve parental male or female DBA responder cells in an in vitro MLR. There was no significant difference in the incorporation of [3H]thymidine by responder DBA T cells (male or female) stimulated with either male or female cGVHD splenocytes (data not shown).

**Discussion**

In the present study, we demonstrate that lupus-like disease induced using female donor and host mice is more severe than that seen using male donor and host mice. Long-term outcome variables such as proteinuria and renal pathology are worse in f→F than in m→M cGVHD. Importantly, differences in disease severity can be observed as early as 2 wk after parental cell transfer. Serum anti-ssDNA Ab titers, donor CD4+ T cell engraftment, and host B cell expansion are significantly increased in f→F cGVHD mice compared with m→M cGVHD mice and reflect greater donor CD4+ T cell proliferation during the second week of disease in f→F cGVHD. Specifically, donor CD4+ T cell homing, alloreactive precursor frequency, and proliferation during the first week after cell transfer do not differ between m→M and f→F cGVHD mice. However, during the second week of disease, donor CD4+ T cell proliferation declines in m→M cGVHD mice, but is sustained in f→F cGVHD mice. The relatively longer period of proliferation in f→F cGVHD mice results in a 2- to 3-fold greater engraftment of donor CD4+ T cells by the end of the second week of disease compared with m→M cGVHD mice. This difference in the absolute number of donor CD4+ T cells is maintained throughout the observed disease course. Thus, relative differences in the duration of donor CD4+ T cell proliferation early in disease have long-term effects on the total burden of persisting pathogenic T cells, which in turn influence long-term disease severity.

Crossover experiments (m→F and f→M) clearly demonstrate that enhanced donor CD4+ T cell proliferation and engraftment are determined by the sex of the host. The exact nature of the host mechanism(s) that promotes donor CD4+ T cell expansion in female hosts is not understood at present and is most likely complex. Our results demonstrate that differential donor CD4+ T cell expansion is not seen until after day 7 of disease argue that the initial host APC function (DC and macrophages) is equivalent. However, it has been previously shown that by day 7 of disease, host B cells are significantly activated in cGVHD mice (30). The APC function of activated host B cells could contribute to the differential expansion of donor CD4+ T cells during wk 2 of disease. However, in experiments designed to compare the relative capacity of day 7 cGVHD splenocytes to drive proliferation of naïve T cells, we were unable to demonstrate a difference between f→F and m→M cGVHD day 7 splenocytes in vitro.

The dependence of enhanced donor CD4+ T cell proliferation on the sex of the host suggests a role for sex-specific host-derived factors, such as sex hormones (i.e., estrogen, androgen, and prolactin). Sex hormones have been previously studied for their ability to modulate not only normal immune responses, but also autoimmune responses (6, 7). Specifically, lupus-like disease in NZB/W mice is significantly worse in females as measured by autoantibody titers, glomerulonephritis, and mortality (9–11). Castration causes acceleration of disease (13), while treatment of ovariectomized females with androgen reduced disease severity (11). Additionally, estrogen treatment of mice transgenic for an anti-DNA IgH inhibits development of tolerance and enhances bcl-2 expression in naive B cells (31). Prolactin also accelerates disease in NZB/W mice (32) and promotes expansion of Ag-specific T cells (33). Similarly, sex hormones are most likely important in the cGVHD model of lupus, as ovariectomy has been reported to reduce both autoantibody levels and proteinuria (34). The exact mechanisms by which sex hormones influence disease expression in murine lupus are not fully explained. The potential immunomodulatory effects of sex hormones in cGVHD are complex, and further studies are required to understand their role in modulating donor CD4+ T cell expansion in the cGVHD model.

In conclusion, we have demonstrated that long-term lupus-like disease in the p→F1 model is significantly influenced by the sex of the host. Moreover, increased disease severity in female cGVHD mice is due to a greater long-term burden of pathogenic CD4+ T cells that becomes apparent as early as 7–10 days of disease. These results suggest that strategies to reduce T cell expansion early in the course of human lupus may have long-term therapeutic effects.

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