Apopotosis and Altered Dendritic Cell Homeostasis in Lupus Nephritis Are Limited by Anti-CD154 Treatment

Susan L. Kalled, Anne H. Cutler and Linda C. Burkly

*J Immunol* 2001; 167:1740-1747; doi: 10.4049/jimmunol.167.3.1740

http://www.jimmunol.org/content/167/3/1740
Apoptosis and Altered Dendritic Cell Homeostasis in Lupus Nephritis Are Limited by Anti-CD154 Treatment

Susan L. Kalled, Anne H. Cutler, and Linda C. Burkly

Autoimmunity results from a failure in central and/or peripheral tolerance; however, the events that initiate and maintain this dysfunction remain unclear. To better understand the mediators involved in autoimmunity, we investigated the cellular mechanisms maintaining disease in the (SWR × NZB)F1 (SNF1) mouse model of systemic lupus erythematosus. Previously, we have shown that autoimmunity in this model is dependent on CD40-CD154 interactions. Herein, our studies reveal that the severity of disease in SNF1 mice correlates with a marked increase in the frequency of apoptotic splenocytes, including a higher proportion of apoptotic dendritic cells (DC) in vivo. In addition, we demonstrate a significant disease-related increase in the absolute number of splenic CD11chi DC. The increased DC number appears to be attributable to DC proliferation and enhanced migration to the spleen, most likely induced by elevated splenic expression of secondary lymphoid chemokine. Importantly, these imbalances in apoptosis, secondary lymphoid chemokine expression, and DC homeostasis were reduced or normalized by anti-CD154 treatment. Thus, our data demonstrate CD154-dependent regulation of apoptosis and DC homeostasis in mice with lupus-like autoimmune disease. We suggest that these mechanisms comprise an autostimulatory loop, maintaining the cascade of autoimmunity by DC presentation of self-Ags derived from apoptotic cells and CD154-mediated costimulation.

1 Address correspondence and reprint requests to Dr. Susan L. Kalled, Biogen, 12 Cambridge Center, Cambridge, MA 02142. E-mail address: susan_kalled@biogen.com

2 Abbreviations used in this paper: FasL, Fas ligand; DC, dendritic cell; SLC, secondary lymphoid chemokine; SLE, systemic lupus erythematosus; Hlg, hamster Ig, BrdU, 5-bromo-2′-deoxyuridine.
To gain further understanding of the mechanisms underlying the promotion of autoimmunity and the loss of peripheral tolerance, we have investigated the cellular mechanisms maintaining autoimmunity in the SNF1 mouse model of SLE. SNF1 mice have been studied extensively and shown to resemble human SLE with nephritis occurring spontaneously in female mice (18). Previously, we have shown that autoimmunity in the SNF1 model is dependent on CD40/CD154 interactions (19). In this report, we reveal for the first time that the severity of autoimmune disease in the SNF1 model correlates with an increased frequency of splenic apoptosis and elevated numbers of splenic DC in vivo. The increase in DC numbers appears to be attributable to DC proliferation and enhanced DC migration associated with elevated splenic expression of secondary lymphoid chemokine (SLC). Importantly, the elevated levels of apoptotic cells, DC, and SLC were CD154 dependent. We discuss how these linked imbalances may form an auto-stimulatory loop that maintains autoimmunity.

Materials and Methods

Mice and mAb treatment

SWR and NZB mice were purchased from The Jackson Laboratory (Bar Harbor, ME). SNF hybrids were bred in the animal facility at Biogen under conventional barrier conditions. Female SNF2 mice were used for all studies.

Mice undergoing mAb treatment received a single dose of 500 μg of anti-CD154 mAb (MR1; Ref. 20) or Armenian hamster IgG (HlgG) mAb Ha4/8-3.1, kindly provided by Dr. Donna Mendrick (Human Genome Sciences, Rockville, MD), once weekly for 6 wk, followed by a single injection of 500 μg monthly until age 11–12 mo. Mice began treatment at age 5.5 mo, at which time they exhibited moderate nephritis unless indicated otherwise. Assessment of renal disease was performed as described elsewhere (19).

Immunohistochemistry

Paraffin-embedded splenic tissue sections were used for H&E staining and for assessing apoptosis by TUNEL assay with an ApopTag kit (Intergen, Purchase, NY) according to the manufacturer’s recommendations, except that 1 μl of TdT was used and sections were counterstained with Nuclear Fast Red (Rowley Biochemical Institute, Danvers, MA). Frozen splenic tissue sections were fixed in acetone, air dried, and blocked for endogenous peroxidase with 0.09% H2O2 in methanol for 10 min. Sections were washed with TBS/0.5%/Tween 20 three times and then blocked in TBS/0.25%/BSA/0.5% normal rabbit and goat sera plus Fc Block (BD Pharmingen, San Diego, CA) for 1 h. Chemokine SLC was detected with biotinylated anti-m6Ckine (R&D Systems, Minneapolis, MN) followed by the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma, St. Louis MO). Sections were counterstained with 0.05% Giemsa (Fluka, Buchs, Switzerland). B cells and DC were detected with anti-B220-FITC and anti-CD11c-biotin followed by streptavidin-PE, and sorted for CD11chigh cells on a MoFlo Cytometer (Cytometry, Ft. Collins, CO). Sorted DC were labeled with the cell-tracing reagent CFSE using the Vybrant CFDA SE Tracer kit (Molecular Probes, Eugene, OR) according to the manufacturer’s recommendations. CFSE becomes fluorescent on cleavage in live cells, allowing visualization under UV light. Healthy (ages 2–2.5 mo) and nephritic (ages 10–11.5 mo) SNF1 mice, received 0.4–0.6 × 106 labeled DC i.v. from healthy or nephritic donors. After 22 h, spleens were harvested, snap frozen, and frozen sections were examined under UV light to determine the presence of donor DC. To compare the number of migrating DC among experimental groups, DC from six fields of view at ×100 magnification were counted and totaled for each spleen section.

DC enrichment

Spleens were digested with collagenase type IV (Sigma) and erythrocytes were lysed with an ammonium chloride solution. The CD11chigh DC population was enriched with MACS CD11c microbeads and magnetic separation column (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s recommendations, except that the cell adherence step was eliminated. DC-enriched populations consisted of 23–54% DC, defined as CD11chigh.

Flow cytometry

Enriched DC and the splenocytes obtained from the flow-through of the DC enrichment process (DC-depleted splenocyte fraction) were used for determination of cell populations undergoing apoptosis. mAbs directed against CD19, CD4, CD8a, Ly6-GGR-1, and CD11c (BD Pharmingen) were used to identify B cells, T cells, granulocytes, and DC, respectively. Anti-Annexin VFITC (BD Pharmingen) and 7-amino-actinomycin D (1 mg/ml; Calbiochem, La Jolla, CA) uptake were used to identify apoptotic cells.

Phenotypic characterization of enriched CD11chigh DC was performed with the following mAbs purchased from BD PharMingen: anti-CD11c-biotin followed by streptavidin-CyChrome, anti-CD8a-FTTC, and PE-conjugated anti-MHC class II, CD40, CD80 (B7.1), CD86 (B7.2), CD13, CD95 (Fas), and CD54 (ICAM-1). Cells were incubated with 10 μg/ml Fc Block (BD Pharmingen) before addition of specific mAbs to inhibit FcR binding. A total of 50,000–100,000 events were collected on a FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed with CellQuest software (BD Biosciences).

Proliferation analysis

To assess splenocyte proliferation, mice received i.v. 1.5 mg/g body weight of 5-bromo-2′-deoxyuridine (BrdU; Sigma) in PBS. Mice were sacrificed 1.5 h later, and splenic DC were enriched as described above. CD11c+ DC were assessed for BrdU incorporation by anti-BrdU-FTTC or a control mouse IgG1-FTTC mAb (BD Pharmingen) and analyzed by flow cytometry.

DC migration studies

To obtain a pure population of splenic DC, pooled donor splenocytes from 21 to 25 healthy or nephritic SNF1 mice were subjected to DC enrichment with anti-CD11c-coated magnetic beads as described above, visualized with anti-CD11c-biotin followed by streptavidin-PE, and sorted for CD11chigh cells on a MoFlo Cytometer (Cytometry, Ft. Collins, CO). Sorted DC were labeled with the cell-tracing reagent CFSE using the Vybrant CFDA SE Tracer kit (Molecular Probes, Eugene, OR) according to the manufacturer’s recommendations. CFSE becomes fluorescent on cleavage in live cells, allowing visualization under UV light. Healthy (ages 2–2.5 mo) and nephritic (ages 10–11.5 mo) SNF1 mice, received 0.4–0.6 × 106 labeled DC i.v. from healthy or nephritic donors. After 22 h, spleens were harvested, snap frozen, and frozen sections were examined under UV light to determine the presence of donor DC. To compare the number of migrating DC among experimental groups, DC from six fields of view at ×100 magnification were counted and totaled for each spleen section.

Results

Increased splenocyte apoptosis in SNF1 mice correlates with disease severity and is reduced by anti-CD154 treatment

Based on the finding that PBMCs from patients with SLE are more susceptible to apoptosis in culture (3, 4), we asked whether nephritic SNF1 mice exhibit increased levels of programmed cell death in vivo. Splenic tissue sections from untreated SNF1 mice at various stages of disease, prenephritic (age 2 mo), moderately nephritic (age 5.5 mo) and severely nephritic (age 10 or 11 mo), and a normal SWR parental control (age 11 mo) were used for TUNEL analysis. Relatively few apoptotic cells were detected in the splenic white pulp of prenephritic SNF1 and SWR control animals (Fig. 1). However, we found that the frequency of TUNEL+ cells in the splenic white pulp progressively increased in SNF1 mice with advancing age and disease severity (Fig. 1). Interestingly, 11-mo-old SNF1 animals treated with a blocking anti-CD154 mAb starting at age 5.5 mo displayed improved survival of splenocytes as compared with control HlgG-treated or untreated mice (Fig. 1), in addition to increased survival and inhibition of disease progression (Ref. 19 and data not shown). When splenocytes from the control HlgG-treated animals were further examined by immunofluorescent staining and flow cytometry, we found a marked increase in the percentage (7-fold) and number (3.4 × 106 cells) of apoptotic DC in the 11-mo-old control HlgG-treated SNF1 mice as compared with the prenephritic animals (0.08 ± 0.01 × 106 cells), and smaller increases in the percentage and number of apoptotic B cells (3-fold percent increase; 24.5 ± 106 cells in HlgG-treated and 5.75 ± 0.15 × 106 cells in prenephritic mice), T cells (3.5-fold percent increase; 2.4 ± 106 cells in HlgG-treated and 0.6 ± 0.1 × 106 cells in prenephritic mice), and granulocytes (3.6-fold percent increase; 0.8 ± 106 cells in HlgG-treated and 0.07 ± 0.01 × 106 cells in prenephritic mice; Fig. 2). Subset analysis of splenocytes...
Increased number of splenic DC in nephritic SNF<sub>1</sub> mice correlates with disease severity and is reduced by anti-CD154 treatment

The increased frequency of apoptotic splenocytes in nephritic SNF<sub>1</sub> mice suggested a source of Ag for presentation to self-reactive lymphocytes. Thus, we further characterized the status of DC in these animals. Severely nephritic animals (ages 10–12 mo) clearly exhibited splenomegaly (average spleen weight, 1.2 ± 0.4 g, n = 7) as compared with 2- to 3-mo-old prenephritic SNF<sub>1</sub> mice and 18-mo-old BALB/c mice (average spleen weight, 0.12 ± 0.03 g, n = 6 and 0.13 ± 0.01 g, n = 3, respectively). Splenic hyperplasia in nephritic SNF<sub>1</sub> mice was also evidenced by total splenocyte number, which increased from a mean of 78 ± 4.5 × 10<sup>6</sup> at age 2 mo to 239 ± 48 × 10<sup>6</sup> at age 11.5 mo, a 3.1-fold difference. Histologic changes in the splenic architecture also were observed with increasing spleen size, age, and disease severity (Fig. 3A). At age 2 mo, typical areas of white pulp were visible surrounded by red pulp. However, by age 5.5 mo, the white pulp was markedly expanded, and by age 11 mo, the white pulp was so enlarged that there were few or no discernible areas demarcating white and red pulp. In fact, the spleens of some mice appeared to consist predominantly of white pulp. Changes within the white pulp were further elucidated by immunofluorescent staining for B cells and DC (Fig. 3B). Anti-B220 staining was used to discriminate the B cell areas from the central T cell areas and showed a normal pattern in healthy, prenephritic SNF<sub>1</sub> mice. The DC pattern also was normal, with DC located either within the T cell zone or in the marginal zone bridging channels. However, there was a progressive increase in size of the white pulp, including a striking increase in CD11c<sup>+</sup> staining by age 5.5 mo. In addition, by age 11 mo, the normal localization of cells in the white pulp was lost. The apparent detection of some B220<sup>+</sup>CD11c<sup>+</sup> cells (appearing yellow) may reflect the colocalization of B cells and DC in the diseased tissue or the appearance of a subpopulation of CD11c<sup>+</sup> B cells (21). Notably, spleens from 11-mo-old mice treated with anti-CD154 beginning at age 5.5 mo were reduced in size (average spleen weight, 0.37 ± 0.1 g, n = 5) and exhibited smaller, more organized white pulp areas as compared with 11-mo-old untreated or HlgG-treated SNF<sub>1</sub> mice, or age-matched parental SWR mice (Fig. 3C).

To definitively quantify and characterize the disease-related increase in the DC population, splenic CD11c<sup>hi</sup> DC were enriched from SNF<sub>1</sub> mice at various stages of disease and quantified in terms of absolute number and percentage of splenocytes by flow cytometry. Subset analysis was used to distinguish changes in the CD11c<sup>hi</sup>CD8α<sup>−</sup> and CD11c<sup>hi</sup>CD8α<sup>+</sup> DC populations. As expected in healthy, prenephritic SNF<sub>1</sub> mice, the CD11c<sup>hi</sup>CD8α<sup>−</sup> and CD11c<sup>hi</sup>CD8α<sup>+</sup> DC subsets represented 1 and 0.28% of

![Image 1](http://www.jimmunol.org/)

**FIGURE 1.** Splenocyte apoptosis in SNF<sub>1</sub> mice. Splenic tissue sections were analyzed for apoptotic activity by TUNEL assay as described in Materials and Methods. Untreated SNF<sub>1</sub> mice aged 2, 5.5, and 11.5 mo (top), 11.5-mo-old SNF<sub>1</sub> mice that received anti-CD154 mAb or control HlgG since age 3.5 mo, and 11.5-mo-old SWR parental control mice (bottom) were screened for TUNEL-positive cells (brown). Images were acquired at ×100 magnification and are representative of results from 3 to 13 individual animals per experimental group.

**FIGURE 2.** Frequency of apoptotic cells in spleen cell subsets of SNF<sub>1</sub> mice. CD11c<sup>+</sup>-enriched DC and the DC-depleted splenocyte fraction were screened for apoptotic activity using pooled spleens (n = 6–11) from prenephritic (age 2 mo), and 11-mo-old anti-CD154-treated and control HlgG-treated SNF<sub>1</sub> mice. Apoptotic cells were annexin V<sup>+</sup> 7-amino-actinomycin D<sup>−</sup>, as determined by flow cytometry. Gates were set on CD11c<sup>hi</sup>, B220<sup>+</sup>, CD4<sup>+</sup>/CD8<sup>+</sup> (in combination), and GR-1<sup>+</sup> cells to segregate DC, B cells, T cells, and granulocytes, respectively. Data shown are the mean percent and standard deviation of apoptotic cells within each subset from two independent experiments, except for the HlgG-treated controls where a single experiment was performed.
total splenocytes, respectively. We found a progressive increase with age and disease severity in the percentage of DC (Fig. 4). Interestingly, we also found that the total DC number increased from a mean of \(1.2 \pm 0.1 \times 10^6\) in prenephritic mice to \(8.7 \pm 1.5 \times 10^6\) in nephritic animals. This observation was disease-related, as normal BALB/c mice did not show an increase in either total splenocyte or DC number with age (data not shown). Furthermore, the percentage and number of splenic DC were markedly reduced to normal or near normal levels by anti-CD154 treatment (Fig. 4).

Thus, our results demonstrate that the disease-related splenic hyperplasia in SNF mice is characterized by cellular expansion of the white pulp, including a significant increase in the absolute number and proportion of DC that is CD154 pathway dependent.

Phenotypic analysis of DC from nephritic SNF mice

Given the increased number of DC in the spleens of nephritic mice, we asked if their phenotype was altered when compared with DC from healthy animals. Mature splenic DC in normal mice are CD11c<sup>hi</sup> and MHC class II<sup>hi</sup>. These cells also express CD13 (22) and costimulatory molecules CD40, CD80, and CD86 (16), which are up-regulated with capture of Ag. Enriched splenic DC subsets, CD11c<sup>hi</sup>CD8α<sup>-</sup> and CD11c<sup>hi</sup>CD8α<sup>+</sup>, from SNF mice aged 2.5 and 11–12 mo were analyzed by flow cytometry to determine their surface phenotype. The CD8α<sup>-</sup> DC subpopulation in both healthy and nephritic SNF mice exhibited phenotypic markers as described previously (16, 22), except that DC from nephritic mice had slightly diminished CD95 expression as compared with the same subpopulation from healthy animals (Fig. 5B). This CD95 decrease was inhibited by long-term anti-CD154 treatment. The CD8α<sup>-</sup> DC subpopulation from nephritic mice also displayed subtle changes, with decreased CD40, CD13, and CD54 expression and slightly increased CD95 expression as compared with DC from prenephritic animals (Fig. 5A). The decreases in CD40, CD13, and CD54 were inhibited by long-term anti-CD154

FIGURE 4. Quantitation of splenic DC in SNF mice. CD11c<sup>+</sup> splenic DC were enriched and subjected to flow cytometry with anti-CD11c and anti-CD8α mAbs to visualize the individual DC subsets as described in Materials and Methods. Shown are the means of at least three experiments per age group, each experiment consisting of DC derived from a pool of two to six spleens. The percent and absolute numbers of each DC subset were significantly reduced, or approached significance, in SNF mice that received anti-CD154 treatment beginning at age 5.5 mo (indicated by *). Values of \(p\) are shown and were determined by Student’s \(t\) test.

FIGURE 3. Histologic analysis of spleens from SNF mice. A, H&E-stained splenic tissue sections taken from untreated SNF mice at 2, 5.5, and 11 mo of age. B, Splenic tissue sections from untreated SNF mice at 2, 5.5, and 11 mo were incubated with anti-B220 (green) and anti-CD11c (red) to visualize B cells and DC, respectively. C, Splenic tissue sections from 11-mo-old SNF mice treated since age 5.5 mo with either anti-CD154 or HIgG control, and from an untreated 11-mo-old normal parental control, SWR, were incubated with anti-B220 (green) and anti-CD11c (red) to visualize B cells and DC, respectively. All images were acquired at \(\times 100\) magnification and are representative of 3–13 individual animals per experimental group.
FIGURE 5. Phenotype of DC from the spleens of untreated 2-mo-old prenephritic and 11-mo-old nephritic SNF1 mice, and 11-mo-old SNF1 mice that were treated with anti-CD154 since age 5.5 mo. Splenic DC were enriched with anti-CD11c-coated magnetic beads. DC were gated as CD11c<sup>high</sup>CD8α<sup>-</sup> (A) and CD11c<sup>high</sup>CD8α<sup>+</sup> (B) to segregate the subpopulations, which then were analyzed for surface markers (shaded histograms) as indicated. Data shown are representative results from two to four experiments per group, with each group consisting of cells from two to six pooled spleens. Open histograms represent isotype controls.

Splenic DC from nephritic SNF<sub>1</sub> mice exhibit increased proliferation

Nephritic SNF<sub>1</sub> mice clearly have an elevated number of splenic DC. However, the mechanism for this increase is unknown. One possibility is a proliferative expansion of DC. To test this, prenephritic and nephritic SNF<sub>1</sub> mice were administered BrdU i.v. and possibility is a proliferative expansion of DC. To test this, prenephritic and nephritic SNF<sub>1</sub> mice were administered BrdU i.v. and were analyzed for BrdU incorporation by flow cytometry. DC from nephritic SNF<sub>1</sub> mice exhibited BrdU incorporation (Fig. 6, bottom left), whereas DC from prenephritic mice had little or no detectable BrdU incorporation (Fig. 6, top left). Thus, in contrast to splenic DC from healthy animals, the DC from nephritic SNF<sub>1</sub> mice are actively proliferating. Interestingly, long-term anti-CD154 mAb treatment did not effect the proliferative status of DC from nephritic mice because 11-mo-old SNF<sub>1</sub> mice that received anti-CD154 mAb continuously from age 5.5 mo had an anti-BrdU staining profile (Fig. 6, bottom right) that was comparable to that of control HlgG-treated (Fig. 6, top right) and untreated mice (data not shown).

Enhanced DC migration to the spleens of nephritic SNF<sub>1</sub> mice

The increased proliferative activity of splenic DC in nephritic SNF<sub>1</sub> mice cannot be the sole mechanism for the elevated number of DC because anti-CD154 mAb treatment, which resulted in near normalization of the splenic DC number, had no antiproliferative effect. Therefore, we hypothesized that in nephritic mice there is enhanced DC migration to the spleen. This could be attributable to the splenic microenvironment of nephritic animals and/or factors intrinsic to their DC. To test this hypothesis, we compared the ability of purified CD11c<sup>high</sup> splenic DC from healthy SNF<sub>1</sub> mice (prenephritic DC) and nephritic SNF<sub>1</sub> mice (nephritic DC) labeled with CFSE to migrate to the spleen after injection of each population into both healthy and nephritic SNF<sub>1</sub> animals. Migration to recipient spleens was visualized by fluorescence microscopy. Very few “prenephritic” (12 ± 6) and “nephritic” (11 ± 0) DC were detected in the spleens of prenephritic SNF<sub>1</sub> recipients (Fig. 7, A and C). In contrast, numerous prenephritic (100 ± 10) DC and nephritic (87 ± 12) DC were clearly visible in the spleens of nephritic recipients (Fig. 7, B and D). Thus, migration of DC to...
spleens of nephritic mice is ~9-fold greater than to spleens of healthy animals. Splenic tissue sections from these mice were also stained for B220<sup>+</sup> B cells to provide orientation within the spleen, and this revealed that the vast majority of labeled DC were localized within the splenic white pulp (data not shown). These data suggest that nephritic SNF<sub>1</sub> mice exhibit a unique splenic microenvironment that promotes DC recruitment.

**Increased splenic expression of chemokine SLC in nephritic SNF<sub>1</sub> mice is reduced by anti-CD154 treatment**

Given that the splenic microenvironment of nephritic mice appears to foster DC recruitment to the spleen, we speculated that the levels of DC homing chemokines such as SLC and ELC (15) might be elevated in the spleens of these animals. Expression of SLC, produced by splenic stromal cells within the T cell-rich area of the white pulp and by DC, was assessed immunohistochemically in prenephritic mice, in 10- to 11-mo-old nephritic mice, and in 12-mo-old SNF<sub>1</sub> mice treated with anti-CD154 mAb from age 5.5 mo. Our studies reveal increased SLC expression in the T cell areas of spleens from nephritic SNF<sub>1</sub> mice as compared with healthy, prenephritic animals (Fig. 8, top). Interestingly, 12-mo-old SNF<sub>1</sub> mice that received long-term anti-CD154 mAb treatment had levels of splenic SLC that resembled more closely those in prenephritic mice (Fig. 8, bottom). Thus, our data demonstrate that SLC expression is elevated in spleens of diseased SNF<sub>1</sub> animals and that these abnormally high SLC levels are dependent on CD154. Furthermore, they suggest that anti-CD154 mAb treatment reduces splenic DC numbers in nephritic animals, at least in part, by reducing expression of the DC homing chemokine, SLC.

**Discussion**

We aimed to better understand the mechanisms involved in driving and maintaining autoimmunity using a mouse model of SLE. The pathogenesis of SLE is not currently understood but most likely results from defects in one or more peripheral rather than central regulatory mechanisms, (1, 23, 24). Efforts to identify autoantigen in SLE have shown that self-Ag derived from apoptotic cells can be presented by DC to activated T cells in vitro (11–13) and that apoptotic cells can be immunogenic in vivo (9), leading to autoantibody production. However, there have been no in vivo data establishing an association between apoptotic cells and spontaneous autoimmune disease. Herein, we provide such evidence by showing a markedly increased frequency of apoptotic cells in the spleens of SNF<sub>1</sub> mice, as well as a progressive increase in the

**FIGURE 7.** Adoptive transfer studies to assess in vivo migration of splenic DC. Purified CD11c<sup>high</sup> DC were obtained from healthy and nephritic SNF<sub>1</sub> mice and labeled with CFSE as described in Materials and Methods. DC pooled from healthy SNF<sub>1</sub> mice were adoptively transferred to healthy (A) and nephritic (B) recipient SNF<sub>1</sub> mice, and DC pooled from nephritic SNF<sub>1</sub> mice were adoptively transferred to healthy (C) and nephritic (D) recipient SNF<sub>1</sub> mice. Green signals indicate DC that have migrated to the spleen of the recipient animal. Data shown are representative of results observed with from two to three recipients per group. Images were acquired under UV microscopy at ×100 magnification.

**FIGURE 8.** Splenic SLC expression in SNF<sub>1</sub> mice. Frozen spleen sections from prenephritic (n = 5; ages 2–2.5 mo), nephritic (n = 4; ages 10–11 mo), and anti-CD154-treated (n = 4; age 12 mo) SNF<sub>1</sub> mice were stained with an anti-SLC-specific mAb. Each image represents a separate animal, and images for the prenephritic and nephritic mice are representative of that entire group. Images were acquired at ×50 magnification.
absolute number, proportion, proliferation, and migration of splenic DC with advancing age and disease severity. We previously demonstrated that disease progression in the SNF1 model is CD154 dependent (19). Our current data now establish immunologic checkpoints that are directly or indirectly regulated by CD154, namely, splenocyte apoptosis, SLC production, and DC migration, and suggest that dysregulation at these checkpoints promotes autoimmunity.

The increased frequency of apoptosis in the spleens of nephritic SNF1 animals supports the hypothesis that the increased apoptosis observed in lupus patient PMBC in vitro occurs in vivo. Increased apoptosis in vivo may reflect an intrinsic defect in cells that predisposes them to die at a higher rate and/or a defect in apoptotic cell clearance. Our studies in SNF1 mice reveal a marked increase in the proportion of apoptotic DC and a smaller increase in the proportion of apoptotic B cells, T cells, and granulocytes in nephritic animals. Thus, it is unlikely that this increased apoptosis is attributable to a generic defect in cell death machinery. Rather, given the immune activation in this model as evidenced by splenic hyperplasia and our observation that white pulp expansion and elevated apoptosis are dependent on CD154, we speculate that the CD154 pathway may play a role in peripheral tolerance in this lupus-like disease setting.

Regardless of the mechanism, our results establish for the first time that splenic DC are recognized as key players in central tolerance in the thymus, and subtypes of DC may play a role in peripheral tolerance (36). However, DC also are recognized for their critical role in initiating robust adaptive immune responses to exogenous as well as endogenous Ag. Importantly, our studies revealed a 7.3-fold increase in the number of DC in the spleens of nephritic SNF1 mice, with the greatest increase (8.3-fold) occurring within the CD8alpha- subset. Approximately 50% of these DC have a mature phenotype. The importance of the preferential increase in CD8alpha+ DC is unclear because both the CD8alpha+ and CD8alpha- DC subsets have been found to possess near equivalent capability for Ag uptake in vivo (37). However, it has been suggested that each DC subset regulates T cell responses differently, and recent data indicate that CD8alpha- DC induce a Th2-type response, whereas CD8alpha+ DC induce a Th1-type response (37–39). In addition, CD8alpha- DC were found recently to exclusively express two newly identified genes, CIRE and FIRE (37). As the function of these and other novel DC-specific gene products are elucidated, an understanding of the roles of the different DC subsets in maintaining autoimmunity will emerge.

It was apparent from our results that the elevated splenic DC number in nephritic mice may result, in part, from proliferation. However, the ability of anti-CD154 treatment to significantly reduce DC number without affecting their proliferation and our adoptive transfer studies suggest that the CD154-dependent increase was likely regulated at the level of migration to the spleen. Thus, we have demonstrated that the splenic microenvironment of nephritic animals uniquely promotes recruitment of DC derived from healthy or nephritic animals, whereas we were unable to detect a difference in trafficking ability intrinsic to the DC. We have further shown increased expression of SLC in splenic tissue from nephritic animals that was CD154 dependent. It is difficult to discern whether the CD154-dependent increase in SLC expression is attributable to the increased cellularity of the spleen or whether CD154-mediated signals also increase SLC production on a per cell basis. Additional studies are required to quantify the differences in SLC expression and better address the ability of CD154 to directly regulate SLC production. Nevertheless, our results suggest that splenic DC migration in autoimmune SNF1 mice is regulated by CD154, potentially through its regulation of SLC. A link to SLC may also be important because chemokines, in addition to directing cell migration, can provide positional cues directing the localization of different cell types within lymphoid organs. This concept is supported by precedence with another TNF family ligand, LTβ, which provides critical cell positioning cues in the spleen through its regulation of chemokines BLC, SLC, and ELC (40). Thus, we speculate that the disorganization observed in the splenic white pulp of SNF1 mice, which was prevented by anti-CD154 treatment, may be mediated by a CD154/chemokine axis.

Our findings reported here, along with previous studies demonstrating hyperexpression of CD154 in lupus settings, show that three critical immune system components are altered in lupus-like autoimmune disease: 1) an abundant source of self-Ag, 2) an abundance of APC, and 3) an elevated level of CD154-mediated stimulatory signals. Although these results do not speak to disease initiation, we show that imbalances in these components are ultimately linked to each other and correlate with disease progression. As such, they have the capacity to form a stimulatory loop that perpetuates self-reactivity in vivo. Of course, we cannot rule out that other costimulatory molecules are also an integral part of this progression.

In summary, our studies delineate peripheral mechanisms likely underlying persistent autoimmunity in a mouse model of SLE. We demonstrated altered regulation of apoptosis and DC homeostasis that appear to be CD154 pathway dependent. To determine whether these observations are directly linked to CD154 or indirectly linked through activation of additional costimulatory pathways and/or chemokine or cytokine production requires further investigation because any mechanism which interferes with pathogenic T cell, B cell, or APC function may be effective in inhibiting disease progression. These findings may be relevant to the development of therapeutic strategies for the treatment of autoimmune disorders.

Acknowledgments
We thank Konrad Miatowski and Janine Ferrant for production and purification, respectively, of the anti-CD154 mAb, Joseph Amatucci for production and purification of the HAa87-3.1 mAb, and Drs. Fabienne
References


