Defective Stromal Remodeling and Neutrophil Extracellular Traps in Lymphoid Tissues Favor the Transition from Autoimmunity to Lymphoma

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ABSTRACT
Altered expression of matricellular proteins can become pathogenic in the presence of persistent perturbations in tissue homeostasis. Here, we show that autoimmunity associated with Fas mutation was exacerbated and transitioned to lymphomagenesis in the absence of SPARC (secreted protein acidic rich in cysteine). The absence of SPARC resulted in defective collagen assembly, with uneven compartmentalization of lymphoid and myeloid populations within secondary lymphoid organs (SLO), and faulty delivery of inhibitory signals from the extracellular matrix. These conditions promoted aberrant interactions between neutrophil extracellular traps and CD5+B cells, which underwent malignant transformation due to defective apoptosis under the pressure of neutrophil-derived trophic factors and NF-κB activation. Furthermore, this model of defective stromal remodeling during lymphomagenesis correlates with human lymphomas arising in a SPARC-defective environment, which is prototypical of CD5+B-cell chronic lymphocytic leukemia (CLL).

SIGNIFICANCE: These results reveal the importance of stromal remodeling in SLO to accommodate autoimmune lymphoproliferation while preventing lymphomagenesis. Our findings reveal a link between SPARC, collagen deposition, and the engagement of the immune-inhibitory receptor LAIR-1 on neutrophils, neutrophil cell death via NETosis, and the stimulation of CD5+B-cell proliferation. Moreover, we show that SPARC deficiency promotes CD5+B-cell lymphomagenesis and is correlated with CLL in humans. Cancer Discov; 4(1); 110–29. ©2013 AACR.

See related commentary by Brekken, p. 25.

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INTRODUCTION

Chronic inflammation and autoimmune disorders are associated with a high risk of lymphoid malignancies. Indeed, more than 10% of lymphoid neoplasms arise in the setting of an autoimmune diathesis (1, 2). Prototypical examples of such an association include mucosa-associated lymphoid tissue (MALT) lymphomas of the salivary glands and enteropathy-associated T-cell lymphomas arising in patients with Sjogren’s syndrome or celiac disease, respectively (2, 3). Malignant lymphoid clones of the ocular adnexa and gastric MALT lymphomas associated with *Chlamydia psittaci* and *Helicobacter pylori* infection, respectively, frequently express immunoglobulin VH genes that are typically found in rheumatoid arthritis, systemic lupus erythematosus (SLE), and other autoimmune disorders (4–6). Moreover, signs of lymphoid clone selection driven by autoantigens can be recognized in the B-cell receptor (BCR) configuration of chronic lymphocytic leukemia (CLL), splenic marginal zone lymphoma (MZL), and mantle cell lymphoma (MCL), none of which has been causally related to any specific infective, inflammatory, or autoimmune disease (7–9). Despite the numerous associations described above, the key factors driving the transition from an inflammatory/autoimmune background to malignant lymphoproliferation have not been identified (10–12).

Upon the induction of immune responses (either physiologic or autoimmune), lymphoid tissues undergo dynamic changes in the number, distribution, and phenotype of immune cell populations, which require substantial and concomitant remodeling of tissue architecture (e.g., secondary follicle formation and marginal zone expansion; ref. 13). These modifications normally occur within the fringes of the functionally compartmentalized stroma of secondary lymphoid organs (SLO), which are composed of cellular elements [e.g., follicular dendritic cells (FDC) and reticular cells] and extracellular matrix (ECM) that together contribute to the regulation of trafficking and activation of immune cells (14).

We hypothesized that biasing the stromal microenvironment through the defective expression of a matricellular protein is sufficient to drive malignant transformation in the presence of a lymphoproliferative stimulus, such as that occurring under autoimmune conditions. To this end, we crossed autoimmunity-prone *Fas*-mutant *lpr/lpr* mice with mice lacking the matricellular protein SPARC (secreted protein acidic rich in cysteine, also known as osteonectin; ref. 15). SPARC is a nonstructural pleiotropic matricellular protein that is a key regulator of tissue stroma remodeling in physiologic conditions and pathologic settings, including cancer and parenchymal fibrosis (16). SPARC also plays a role in the profound changes that occur in the tissue architecture of solid and hematologic neoplasms (17, 18). SPARC produced by innate immune cells influences the cross-talk between cancer cells and the ECM at the invasive edge of primary tumors and regulates proinflammatory TNF production in response to tissue damage (16). Moreover, bone marrow stroma–derived SPARC was reported to regulate the expansion of myeloid cell populations under myeloproliferative stress (18). In SLO, SPARC is a determinant of correct germinal center assembly and lymphocyte trafficking during adaptive immune responses (19). Accordingly, the transcriptional profile of the stroma of SLO responding to infection shows high SPARC expression (20).

Here, we show that *Sparc* deficiency exacerbated the autoimmune phenotype of *Fas*-mutant *lpr/lpr* mice, promoting overt lymphomagenesis. Moreover, the altered stromal organization within SLO stemming from *Sparc* deficiency promoted the redistribution and pathogenic interaction of CD5+ B cells and neutrophils, which can provide B-cell growth factors and autoantigens via neutrophil extracellular traps (NET).

RESULTS

Exacerbated Autoimmunity and Lymphoproliferation in *Fas*-Mutant *lpr/lpr* Mice Lacking the *Sparc* Gene

Stromal cells within SLO are highly specialized and directly contribute to immune cell homeostasis, differentiation, and responsiveness. In SLO, FDCs produce SPARC, which is necessary for proper dendrite branching (19) and to ensure the accurate regulation of lymphoid cell networking within follicles. To study the effect of defective SLO stromal responses under an autoimmune lymphoproliferative stimulus [ *Fas* mutation (21) in *lpr/lpr* mice (22)], we assessed the expansion of autoreactive lymphoid populations and the production of autoantibodies to nuclear antigens (i.e., ANA) in a *Sparc*−/− background. In addition, the onset and extent of lymphoproliferation and autoimmunity in *lpr/lpr/Sparc*−/− double-mutant mice were compared with those of *lpr/lpr/Sparc*+/− counterparts.

The double-mutant mice displayed an accelerated induction of autoantibodies in comparison with *lpr/lpr/Sparc*+/− mice (Fig. 1A) and developed overt lymphoproliferation and diffuse lymphadenopathy at 12 weeks of age (Fig. 1B). At sacrifice, *lpr/lpr/Sparc*−/− mice demonstrated a higher degree of splenomegaly, with spleen weights significantly exceeding those of the *lpr/lpr/Sparc*+/− controls (Fig. 1C), and a marked effacement of the splenic architecture characterized by diffuse expansion of medium-sized lymphoid cells, elements with plasmacytoid morphology, and a concomitant loss of the clear-cut delimitation between the lymphoid and myeloid parenchyma (Fig. 1D). These characteristics were not observed in the SLO of *lpr/lpr/Sparc*+/− mice, whose splenic architecture was conserved with minimal expansion of the white pulp. Similar to the spleen, the lymph nodes of *lpr/lpr/Sparc*−/− mice also showed expansion of medium-sized lymphoid elements, which diffusely effaced the architecture (Fig. 1D). Consistently, significantly higher bronchus-associated lymphoid tissue (BALT) proliferation was observed in *lpr/lpr/Sparc*−/− mice compared with *lpr/lpr/Sparc*+/− mice, confirming that the absence of SPARC exacerbated autoimmunity (Supplementary Fig. S1A). Moreover, the relevance of the exacerbated phenotype observed in *lpr/lpr/Sparc*−/− mice on a BALB/c background was strengthened, as the severity of autoimmunity related to *Fas*−/− mutation is strain-dependent and reduced in BALB/c mice compared with the original MRL background (23).
Exacerbated autoimmunity and lymphoproliferation in Fas-mutant lpr/lpr/Sparc mice lacking Sparc. A, quantification of anti-ANA, anti-ssDNA, and anti-dsDNA antibody levels in lpr/lpr/Sparc−/−, lpr/lpr/Sparc+/−, and control Sparc−/− and Sparc+/+ mice. Sera from individual mice from 1 to 10 months of age were assayed for immunoglobulin G (IgG) autoantibodies. Data are expressed as U/mL of IgG and plotted as a scattergram, with the antibody titer of each mouse versus its relative age (weeks) to show the time at which autoantibodies developed in each group (left). Regardless of the age of mice, collective data show that the levels of IgG anti-ssDNA or anti-dsDNA were significantly increased in lpr/lpr/Sparc−/− compared with lpr/lpr/Sparc+/− mice (**, Student t test) compared with lpr/lpr/Sparc−/− mice (n=25/strain) D, histopathologic analysis of spleen and lymph node samples from lpr/lpr/Sparc−/− and lpr/lpr/Sparc+/− mice showing the marked effacement of the spleen and lymph node architecture of lpr/lpr/Sparc−/− mice by medium-sized lymphoid cells and plasmacytoid elements. In the spleens of lpr/lpr/Sparc−/− mice, diffuse lymphoid expansion caused a loss of demarcation between the white pulp (black arrows) and red pulp (green arrows), characteristic of lpr/lpr/Sparc−/− spleens. Similarly, the lymph node architecture was characterized by the diffuse expansion of medium-sized lymphoid cells and elements with plasmacytic morphology. Scale bars, 50 μm. (continued on following page)

Malignant CD5− B-Cell Lymphoproliferation Develops in lpr/lpr/Sparc+− Mice and Can Be Transplanted into Nude Recipients

To better characterize the increased lymphoproliferation observed in lpr/lpr/Sparc−/− mice compared with lpr/lpr/Sparc+/− mice, SLO were analyzed by fluorescence-activated cell sorting (FACS) and immunofluorescence using a cocktail of monoclonal antibodies (mAb) to discern various B- and T-cell populations (24). We found that both strains displayed a similar increase in the amount of B220+CD3+CD4−CD8− double-negative T cells, which are commonly present in autoimmune mice (25). However, the onset of this population was earlier in the Sparc−/− background, as was the induction of autoantibodies (not shown). The most striking difference between the lpr/lpr/Sparc+− and lpr/lpr/Sparc−/− lymphoproliferative phenotype was detected in the B-cell compartment (see Supplementary Fig. S1B and SIC for gating strategy), consisting of a significant increase in the number of CD19+CD43−CD5−IgM+ B cells, which ultimately become CD5− B cells (Fig. 1E–G). The analysis of Ig gene rearrangement demonstrated that these expanded B-cell populations were clonal or oligoclonal. The sharing of identical VDJ rearrangements in the lymph nodes and spleen of the same animals was indicative of malignant clones (Supplementary Fig. S1D).

To confirm whether the enhanced CD5− B-cell proliferation in lpr/lpr/Sparc−/− mice was malignant, spleen and lymph nodes were minced and transplanted subcutaneously into athymic nude mice. Grafts from lpr/lpr/Sparc−/− mice, but not age-matched lpr/lpr/Sparc+− mice, generated subcutaneous tumors displaying diffuse proliferation of lymphoid elements that were similar in morphology to those subverting the SLO architecture of donor mice (Fig. 1H); moreover, these tumors disseminated to the spleen and lymph nodes of recipient mice, contributing to their enlargement (Fig. 1I). Indeed, a population of CD5− B cells was enriched in the SLO of mice receiving lpr/lpr/Sparc−/−, but not lpr/lpr/Sparc+−, grafts (Fig. J and K).
Biased Stroma in the Autoimmunity–Lymphoma Transition

Stromal and Hemopoietic Splenic Remodeling Characterizes the lpr/lpr Autoimmune Phenotype and Is Deregulated by Sparc Deficiency

The spleen consists of a plethora of innate and adaptive immune cells and has a highly organized microarchitecture that allows for the compartmentalization of these different cell types. Through ordered remodeling, this structure enables proper and selective interactions among cell types upon the induction of an immune response; accordingly, defective remodeling of the splenic stromal microenvironment impairs immune responses to infections (26). Thus, we evaluated whether the autoimmunity induced by Fas deficiency was associated with remodeling of the splenic architecture and whether SPARC played a role in this process. Spleens were stained with mAb against CD3, B220, collagen type I (Supplementary Fig. S2A) and type IV (Fig. 2A), CD5, and CD169 (Fig. 2B) and were analyzed by confocal microscopy. At steady-state conditions, collagen type I was mainly restricted to follicular B-cell areas of the white pulp in spleens from Sparc+/+ and Sparc–/– mice, whereas collagen type IV was enriched in the marginal zone and the red pulp. In autoimmune lpr/lpr/Sparc–/– mice, a population of autoreactive CD3+/B220+ T cells proliferated and localized within B- and T-cell areas, expanding the follicular area without changing the normal follicular architecture (Fig. 2A). In contrast, defective collagen remodeling was expected in Sparc-deficient mice in response to stress (15, 16), similar to what occurs in Fas-deficient lpr/lpr/Sparc–/– mice, where the need to accommodate the exacerbated proliferation of CD3+/B220+ double positive T cells is not supported by proper collagen rearrangement (Supplementary Fig. S2B). Indeed, the spleens of lpr/lpr/Sparc–/– double-mutant mice showed diffuse effacement of the red and white pulp architecture with the redistribution of splenocytes and altered compartmentalization (Fig. 2A). This condition provided an opportunity for unique cell–cell interactions that are otherwise impossible in intact spleen and lymph nodes. Indeed, the markedly defective collagen architecture of lpr/lpr/Sparc–/– mice resulted in

Figure 1. (Continued) E, fraction of follicular B (FoB) cells, marginal zone B (MZB) cells, and CD5+ B cells in the spleens of lpr/lpr/Sparc+/+, lpr/lpr/Sparc–/–, and control Sparc+/+ mice. Lpr/lpr/Sparc–/– mice showed increased MZB cells compared with wild-type Sparc+/+ mice. In lpr/lpr/Sparc–/– mice, contraction of MZB cells was associated with the expansion of CD5+ B cells. FoB cells remained largely unchanged (†, P < 0.05; Student t test). F, analysis of IgM and CD5 expression in cell suspensions obtained from the spleens of lpr/lpr/Sparc+/+, lpr/lpr/Sparc–/–, and control Sparc+/+ mice. Representative plots are shown. G, confocal microscopy analysis of the spleens of lpr/lpr/Sparc+/+ and lpr/lpr/Sparc–/– mice revealed the accumulation of CD5+/CD19 double-positive cells in the spleens of lpr/lpr/Sparc–/– mice but not lpr/lpr/Sparc+/+ mice. Scale bars, 20 μm. H, histopathologic analysis of the SLO of lpr/lpr/Sparc–/– mice and trocar-recipient nude mice indicated that the lymphoid parenchyma was effaced by the diffuse proliferation of small- to medium-sized cells in both conditions, which displayed the same cytomorphologic features (i.e., high nuclear/cytoplasmic ratio, margination chromatin, and prominent nucleoli) and a diffuse pattern of infiltration. Scale bars, 20 μm. I, spleen and lymph node enlargement in nude mice receiving trocar pieces from lpr/lpr/Sparc+/+ but not lpr/lpr/Sparc–/– mice. J, frequency of CD5+ B cells in the lymph nodes from nude mice that were transplanted with trocar pieces from the spleens of lpr/lpr/Sparc+/+ or lpr/lpr/Sparc–/– mice. Nude mice receiving lpr/lpr/Sparc+/+ trocar pieces showed significantly increased expansion of CD5+ B cells (CD19+/CD3+/CD43+) in the lymph nodes. A representative experiment from three total experiments performed with 7 recipient mice is shown (*, P < 0.05; Student t test). K, analysis of IgM and CD5 expression in spleen cell suspensions from nude mice receiving trocar pieces from lpr/lpr/Sparc+/+ and lpr/lpr/Sparc–/– mice.
disorganization of the stromal boundaries between the white pulp follicles and the red pulp, as visualized by stromal CD10 (Supplementary Fig. S3A) and macrophage CD169 staining (Fig. 2B). The impact of such stromal cell redistribution was demonstrated by diffuse lymphocyte proliferation in lpr/lpr/Sparc−/− spleens, which was otherwise restricted to the follicular area in lpr/lpr/Sparc+/+ spleens (Supplementary Fig. S3A). In concert with its role in remodeling the splenic parenchyma in response to immune stimulation, SPARC expression was confined to the scattered stromal elements of B-cell areas in Sparc−/− mice, whereas it extended into T cell-rich areas in spleens from lpr/lpr/Sparc−/− mice (Fig. 2C). Similar changes in lymphoid and stromal tissues were observed in the peripheral lymph nodes. These data suggest that autoimmunity promotes the remodeling of SLO microarchitecture, which involves SPARC.

Considering that SPARC endows antiproliferative functions (15), we compared the proliferation of splenic CD5+ B cells isolated from lpr/lpr/Sparc−/−, lpr/lpr/Sparc+/+, and control mice. The Sparc−/− background did not increase the basal or lipopolysaccharide (LPS)-induced proliferation of splenic CD5+ B cells (Supplementary Fig. S3B), confirming that microenvironmental changes, rather than the intrinsic Sparc deficiency of CD5+ B cells, were responsible for the enhanced proliferation observed in lpr/lpr/Sparc−/− mice. Taken together, these results emphasize the importance of SPARC in stromal remodeling of lymphoid organs associated with autoimmunity and delivering proliferative signals to lymphoid populations.

In the Absence of SPARC, CD5+ B Cells and Myeloid Cells Form Noncanonical, Unrestrained Contacts

We hypothesized that subversion of splenic architecture permits the establishment of noncanonical interactions between autoreactive B cells and cellular partners, providing a survival advantage through NF-κB. Within the spleen parenchyma, myeloid cells populating the red pulp have been shown to assist B cells in a T cell–independent fashion by releasing trophic and prosurvival factors (27). Furthermore, our recent findings linked stromal SPARC deficiency to myeloid cell proliferation in the bone marrow (18), and the observed myeloid cell skewing in the presence of autoimmunity (28) suggested that myeloid cell expansion and localization may be altered in lpr/lpr/Sparc−/−. Combining FACS, immunohistochemistry (IHC), and immunofluorescence analyses, we observed increases in spleen myeloid cells and myeloid precursors (LK, GMP) in Fas-mutant mice, and these populations were further expanded in the absence of SPARC, confirming that the Sparc−/− background is permissive for myeloproliferative signals (Fig. 2D). Notably, triple-marker immunofluorescence analysis detected clusters of Ly6G+ neutrophils within the lymphoid splenic parenchyma of lpr/lpr/Sparc−/− mice at the edge of residual red pulp foci in close contact with expanded CD19+CD5+ B cells (Fig. 2E).

**Figure 2.** Stromal and hematopoietic spleen remodeling in Fas-mutant mice. A, confocal microscopy analysis of spleens from control Sparc+/+ and Sparc−/− mice and Fas-mutant lpr/lpr/Sparc+/+ and lpr/lpr/Sparc−/− mice were stained with the following mAbs: anti-B220 (green), anti-CD3 (red), and anti-collagen type IV (blue). CD3+ and B220+ lymphocytes were confined to the follicular structures of the spleen in Sparc+/+ and Sparc−/− mice. In autoimmune lpr/lpr/Sparc+/+ mice, the expanding double-positive B220+CD5+ cells (arrows) enlarged the splenic follicles, whose structures remain defined, and the different cell types were separated and compartmentalized. In contrast, a loss of compartmentalization was observed in lpr/lpr/Sparc−/− spleens, where B and T lymphocytes as double-positive B220+CD3+ cells were interspersed. Scale bars, 10 μm. B, confocal microscopy analysis of spleens from control Sparc+/+ and Sparc−/− mice and Fas-mutant lpr/lpr/Sparc+/+ and lpr/lpr/Sparc−/− mice were stained with the following mAbs: anti-B220 (green), anti-CD3 (blue), and -CD169 (red). CD169+ cells were enriched in lpr/lpr/Sparc−/− mice. In these mice, CD169+ cells were also mixed with B and T lymphocytes. In control Sparc+/+ and mice and lpr/lpr/Sparc−/− mice, CD169+ cells remained confined to the marginal zone area of the splenic follicles (arrows). Scale bars, 20 μm. C, immunohistochemical analysis of SPARC expression in the spleens from Sparc+/+ and lpr/lpr/Sparc−/− mice showed that SPARC was expressed by scattered stromal elements of the B-cell areas of Sparc−/− mice, and SPARC staining also extended to the T-cell-rich areas of the spleens in lpr/lpr/Sparc−/− mice. Scale bars, 20 μm. D, flow cytometry analysis of spleen cell suspension was performed on control Sparc+/+ and Sparc−/− mice and Fas-mutant lpr/lpr/Sparc+/+ and lpr/lpr/Sparc−/− mice to evaluate splenic granulocytes and precursors (LK, GMP). Myeloid progenitors were identified on the basis of the expression of CD34 and CD16/CD32 within the gate of Lin-CD117+ cells. At different stages of maturation, granulocytes were identified according to their surface expression of the CD11b and GR-1 markers. The total number of GMPs was obtained by multiplying the fraction of LK cells by the total number of spleen cells. The total number of granulocytes was obtained from the fraction of each population multiplied by the fraction of CD5+ cells and then the total number of spleen cells (P < 0.05; Student t test). Overall, lpr/lpr/Sparc−/− spleens were characterized by the increased expansion of myeloid precursors and granulocytes compared with lpr/lpr/Sparc+/+ mice. Scale bars, 20 μm. E, confocal microscopy analysis of spleens from control Sparc+/+ and Sparc−/− mice and Fas-mutant lpr/lpr/Sparc+/+ and lpr/lpr/Sparc−/− mice stained with mAbs to GR-1, CD19, and CD5 showed granulocyte enrichment in the spleens of lpr/lpr/Sparc−/− mice compared with lpr/lpr/Sparc+/+ mice. In lpr/lpr/Sparc+/+ spleens, GR-1+ cells (green) made contact with CD5+ B cells (CD19+CD5+ double-positive, arrows). F, BAFF production evaluated by ELISA assay in splenic granulocytes from lpr/lpr/Sparc+/+ and lpr/lpr/Sparc−/− mice. Granulocytes were obtained by magnetic bead separation and seeded onto a 96-well plate. Their supernatants were harvested 24 hours after cell culture (P < 0.05; Student t test). G, flow cytometry analysis of IL-21 production by the same splenic granulocytes used above. The representative image shows increased production of IL-21 by granulocytes from lpr/lpr/Sparc−/− mice compared with lpr/lpr/Sparc+/+ or normal mice (P < 0.05; Student t test). H, in situ immunohistochemical analysis of IL-21 expression in paraffin-embedded spleen sections showed increased IL-21 expression and production in granulocytes (arrows) from the spleens of lpr/lpr/Sparc−/− mice. Scale bars, 20 μm, right; 50 μm, left.
Biased Stroma in the Autoimmunity–Lymphoma Transition

**Figure A**
- B220/CD3/Col-IV
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**Figure B**
- B220/CD169/CD8
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**Figure C**
- Sparc^+/+
- 
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**Figure D**
- LK
- GMP
- Total number of spleen cells
- Total number of spleen cells
- Total number of spleen cells
- Total number of spleen cells
- Total number of spleen cells
- 

**Figure E**
- Ly6G/CD19/CD5
- 
- 
- 

**Figure F**
- BAFF
- % of positive cells
- 
- 

**Figure G**
- IL-21
- % of positive cells
- 
- 

**Figure H**
- IL-21
- 

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**Figure 3.** Increased NET formation and anti-neutrophil cytoplasmic antibody (ANCA)-associated autoimmunity in lpr/lpr/Sparc−/− mice. A, representative immunofluorescence analysis revealed more efficient NETosis in spleen-purified polymorphonuclear leukocytes (PMN) from 3 representative lpr/lpr/Sparc−/− mice (#10, 20, and 30) compared with lpr/lpr/Sparc+/+ mice (#1, 2, and 3). PMNs were purified from the spleen by magnetic bead separation and seeded onto poly-d-lysine–coated glass for 16 hours. SYTOX green DNA dye was then added to PMNs after fixation with 4% paraformaldehyde. Scale bars, 20 μm. B, in situ detection of NETs was performed by staining the spleens of Fas-mutant mice with a mAb against histones (red) and evaluating the presence of extracellular DNA. The concomitant use of mAbs to CD19 (green) and CD5 (blue) allowed for the evaluation of NET contact with B cells. Histone staining (pan histone) was retained within the nuclei of cells from the spleens of lpr/lpr/Sparc+/+ mice (arrowheads). In contrast, in lpr/lpr/Sparc−/− spleens, the expression of histones was extracellular, suggestive of NETs (arrows), with few spleen cells retaining nuclear localization of histones (arrowheads). Scale bars, 20 μm, top; 10 μm, bottom. C, immunohistochemical analysis of frozen spleen sections demonstrated increased expression of myeloperoxidase (MPO) in lpr/lpr/Sparc−/− spleens compared with lpr/lpr/Sparc+/+ spleens. Hematoxylin and eosin (H&E) staining in serial sections showed concomitant loss of normal spleen structure in lpr/lpr/Sparc−/− mice, as the follicles were not recognizable. D, serum levels of anti-MPO–ANCA in Fas-mutant mice revealed increased production of MPO autoantibodies in lpr/lpr/Sparc−/− mice (*, P < 0.05; Student t test). E, histopathologic grading of the renal damage in lpr/lpr/Sparc−/− and lpr/lpr/Sparc+/+ mice (n = 7/group) (**, P < 0.01; ****, P < 0.0001; one-way ANOVA with posttest Dunn correction). Data represent the mean ± SD. F, H&E staining revealed parenchymal damage with vascular and periglomerular neutrophil infiltration (arrows), resembling vasculitis, in the kidneys from lpr/lpr/Sparc−/− mice. Neutrophil infiltration was strongly reduced in lpr/lpr/Sparc+/+ mice. Notably, renal histopathology analysis from the same mice (#1, 2, and 3 for lpr/lpr/Sparc−/− mice and #10, 20, and 30 for lpr/lpr/Sparc−/− mice) tested for spontaneous neutrophil NETosis (A) demonstrated a strong correlation between increased renal vasculitis and NETosis in lpr/lpr/Sparc−/− mice. Scale bars, 20 μm.
in contact with B cells in lpr/lpr/Sparc−/− mice compared with lpr/lpr/Sparc+/+ mice (Fig. 3B).

NETotic cell death, which occurs in activated neutrophils, represents a strong inflammatory stimulus and is directly pathogenic for anti-neutrophil cytoplasmic antibody (ANCA) development and systemic small-vessel vasculitis (SvV; ref. 29). In addition, NETosis requires myeloperoxidase (MPO) expression (30), which was increased in the spleens of lpr/lpr/Sparc−/− mice with NET-like extracellular structures (Fig. 3C). Accordingly, these mice developed significantly higher MPO-ANCA titers (Fig. 3D) and vasculitic tissue damage (Fig. 3E).

The similarities between MZB cells and CD5−/− naïve CD5+Sparc+ B cells initially committed by a weak BCR signal, activate NF-κB and the sensing of danger signals via Toll-like receptors (TLR), both of which converge on NF-κB activation (33). Accordingly, NF-κB activation in human CD5−/− naïve CD5+B cells is dependent on BCR and microenvironmental signals (32). In our mouse models, Western blot analysis measuring phosphorylation in lpr/lpr/Sparc−/− mice with NET-like extracellular structures (Fig. 3C).

NETs Stimulate CD5+ B-Cell Proliferation via NF-κB

To test whether neutrophils support the in vitro proliferation of CD5+ B cells depending on their Sparc or Fas genotype, naïve CD5+ B cells isolated from Sparc+/+, Sparc−/−, lpr/lpr/Sparc+/+, or lpr/lpr/Sparc−/− neutrophils. In the absence of in vitro stimuli, neutrophils from both Sparc+/+ and Sparc−/− mice were unable to stimulate CD5+ B-cell proliferation. In contrast, under the same culture conditions, CD5+ B-cell proliferation was stimulated by lpr/lpr/Sparc−/− neutrophils and was further enhanced by the addition of lpr/lpr/Sparc+/+ neutrophils (Fig. 4A). Thus, the extent of CD5+ B-cell proliferation correlated with the increase in neutrophils undergoing spontaneous NETosis in lpr/lpr/Sparc−/− mice. In addition, the functional link between NETosis and CD5+ B-cell proliferation was independent of the Fas or Sparc genotype; neutrophils from wild-type (WT) spleens were able to stimulate CD5+ B-cell proliferation following induction of NETosis with PMA (ref. 29; Fig. 4B). The addition of DNase, which destroys NETs by digesting their DNA threads, abolished neutrophil-induced B-cell proliferation (Fig. 4B). The importance of NETotic cell death was further emphasized by the fact that neither apoptotic nor necrotic neutrophils promoted CD5+ B-cell proliferation in vitro (Fig. 4B).

B-cell proliferation under physiologic or pathologic conditions (31, 32) depends on antigenic recognition via BCR and the sensing of danger signals via Toll-like receptors (TLR), both of which converge on NF-κB activation. Innate B cells, including marginal zone B (MZB) cells, which are initially committed by a weak BCR signal, activate NF-κB in response to stromal factors such as BAFF and Notch to provide signals for cell-fate decisions (33) and Ig switching (27). The similarities between MZB cells and CD5+ B cells suggest a common requirement for stroma-dependent NF-κB activation (34). Accordingly, NF-κB activation in human CD5+ B CLL is dependent on BCR and microenvironmental signals (32). In our mouse models, Western blot analysis measuring BCR signaling and NF-κB activation in splenic B cells showed comparable ZAP70/syk phosphorylation but increased IκBα phosphorylation in lpr/lpr/Sparc−/− mice compared with lpr/lpr/Sparc+/+ counterparts (Fig. 4C).

To test whether NETs drive the microenvironmental stimulation of NF-κB, p65 or IκBα phosphorylation was evaluated by flow cytometry. The ratio of p-p65/p65 and p-IκBα/IκBα was increased and IκBα was reduced, indicating that NETs induced NF-κB activation to a greater extent than naive, apoptotic, or necrotic polymorphonuclear leukocytes (PMN; Fig. 4D and E and Supplementary Fig. S6B), in line with its proteasomal degradation in stimulated cells. Thus, NETs are capable of stimulating CD5+ B-cell proliferation via NF-κB activation.

In our in vitro model, NF-κB activation in CD5+ B cells likely resulted from microenvironmental stimuli including BAFF, IL-21, auto-antigens, NETs, and TLR agonists. The additive effect of NETs and TLR ligands was tested in vitro by stimulating CD5+ B cells with CpG for 48 hours and then exposing these cells to NETs for 24 hours. NET addition significantly enhanced the CpG-induced proliferation of CD5+ B cells under conditions in which NETs alone (24 hours culture) were unable to induce B-cell proliferation (Fig. 4F).

Increased NETosis in lpr/lpr/Sparc−/− Mice Depends on Reduced Inhibitory Signals from the ECM

We next sought to determine whether lpr/lpr/Sparc−/− neutrophils were more prone to NETosis than their lpr/lpr/Sparc+/+ counterparts. In response to an inflammatory state, the full activation of NETosis requires cells to avoid apoptosis, and this condition can occur due to Fas deficiency or hyperexpression of antiapoptotic genes. Moreover, the higher rate of NETosis in lpr/lpr/Sparc−/− mice compared with lpr/lpr/Sparc+/+ mice underscores the role of SPARC in ECM organization, neutrophil activation, and NETosis. These events could result from the exacerbated inflammation observed in response to SPARC deficiency (16), together with the uncontrolled induction of NETosis due to defective ECM inhibition.

Considering the tight association between SLE, NETosis, and the activation of IFN pathways (35, 36), we investigated the gene expression profile (GEP) of neutrophils isolated ex vivo from lpr/lpr/Sparc+/+ and lpr/lpr/Sparc−/− spleens. Neutrophils from lpr/lpr/Sparc−/− mice showed significantly increased expression of several genes related to the IFN signature, including IL-18, IL-1β, IFI1, STAT1, IFI30, IFI47, FGFRT, DUSP9, and Ly6E, among others (Supplementary Fig. S5A). The differential expression of some of these key genes was validated by real-time PCR (RT-PCR), indicating that lpr/lpr/Sparc−/− neutrophils received more robust priming for NETosis in vivo as a result of apoptosis resistance, as suggested by Xaf-1 downregulation (Supplementary Fig. S5B).

Within lpr/lpr/Sparc−/− spleens, neutrophils localize in collagen-rich areas and make contact with the collagen type IV fibers produced by CD29+ mesenchymal stromal cells (Fig. 4G and H). In addition, lpr/lpr/Sparc−/− mice were characterized by reduced collagen deposition, though the number of CD29+ cells remained unchanged or increased (Fig. 4H). This result is in agreement with published data showing that Sparc−/− cells are defective in the assembly of collagen type IV fibers (37), a condition that promotes cataract formation in Sparc−/− mice (38). Moreover, collagens deliver inhibitory signals to different cells of the immune system, including neutrophils. LAIR-1 is the main inhibitory receptor that binds collagens (39), and is abundantly expressed on activated neutrophils. LAIR-1 binds to fibibr-forming collagens and transmembrane collagens, including collagen type IV (40). We found that the expression of this inhibitory receptor in lpr/lpr mutant spleens was restricted to the PMN subset and reduced in lpr/lpr/Sparc−/− mice compared with lpr/lpr/Sparc+/+.
NETosis is regulated by ECM molecules and promotes CD5⁺ B-cell proliferation through NF-κB activation. A, naïve CD5⁺ B cells were purified from the spleens of WT mice, labeled with the vital dye CFSE, and cocultured with spleen-purified PMNs from Ipr/Ipr/Sparc−/− and lpr/lpr/Sparc+/+ mice. The proliferation of CD5⁺ B cells was evaluated as the dilution of CFSE dye 3 days later, and the data were expressed as the fold change of proliferation normalized to the basal proliferation induced by naïve Sparc+/+ and Sparc−/− PMNs. B, CD5⁺ B cells were purified from the spleens of BALB/c mice, labeled with the vital dye CFSE, and cocultured with spleen-purified PMNs in the presence or absence of PMA to induce NET extrusion. Where indicated, DNAse (100 U/mL) was added to destroy NET formation. In the same experiment, CD5⁺ B cells were cocultured with necrotic or apoptotic PMNs obtained by freeze–thawing or incubation of naïve PMNs with FAS mAb, respectively. CD5⁺ B-cell proliferation was evaluated at 16 hours. C, the activation of SYK and NF-κB in Ipr/Ipr/Sparc−/− and lpr/lpr/Sparc+/+ mice was evaluated by Western blot analysis of the extracts of splenic-purified CD19⁺ cells. Relative quantitative analysis showed increased phosphorylation of JkBa, which was characteristic of CD19⁺ cells from lpr/lpr/Sparc−/− spleens. D and E, activation of NF-κB in CD5⁺ B cells cocultured with NETs. CD5⁺ B cells were purified from the spleens of WT mice and cocultured with apoptotic, necrotic, or NETotic PMNs obtained via treatment with FAS mAb, freeze–thawing, or PMA treatment, respectively. Where indicated, DNAse was added to destroy NET formation. NF-κB activation was evaluated by flow cytometry and expressed as the ratio of phospho-p65 and total p65 or phospho-IκBα and total IκBα (*, P < 0.05; **, P < 0.01; Student t test). F, CFSE-labeled splenic CD5⁺ B cells were treated with Cpg for 48 hours, and PMA-treated PMNs (NETotic PMNs) were added for an additional 24 hours. CD5⁺ B-cell proliferation was evaluated at day 3. Representative plots are shown. NETotic PMNs boosted Cpg-induced CD5⁺ B-cell proliferation in a setting where NETs alone were ineffective at 24 hours. G, confocal microscopy analysis of GR-1, CD5, and collagen IV demonstrated that GR-1⁺ cells made contact with collagen type IV fibers, which appeared faint in Ipr/lpr/Sparc−/− mice. In lpr/lpr/Sparc−/− mice, GR-1⁺ cells were enriched and made contact with CD5⁺ B cells. Scale bars, 20 μm. H, confocal microscopy analysis of CD29 and collagen IV showed that mesenchymal CD29⁺ cells colocalized with collagen type IV fibers in the spleens of Ipr/lpr/Sparc−/− mice. In the spleens of Ipr/lpr/Sparc−/− mice, this colocalization was strongly reduced. Scale bars, 20 μm. [continued on following page]

To test whether ECM composition directly influences NETosis, freshly isolated PMNs from lpr/lpr/Sparc−/− mice that spontaneously underwent NETosis were seeded onto a collagen matrix or poly-γ-Lysine (a permissive substrate for NET). Collagen matrix, but not poly-γ-Lysine, significantly inhibited NETosis in PMNs from lpr/lpr/Sparc−/− mice (Fig. 4K and L), as well as in naïve spleen-derived neutrophils that form NETs under PMA stimulation (Supplementary...
Biased Stroma in the Autoimmunity–Lymphoma Transition

**RESEARCH ARTICLE**

**Figure 4.** (Continued) I, FACS analysis of LAIR-1 was performed on splenocytes, and the histograms show the mean fluorescence intensity (MFI) of LAIR-1 within the gate of Ly6G+ cells. Data are expressed as the mean ± SD (*, P < 0.05; Student t test). J, confocal microscopy analysis of LAIR-1 revealed its expression in myeloid cells (inset) in the spleens of lpr/lpr/Sparc−/− mice. Scale bars, 20 μm. K, splenic PMNs from lpr/lpr/Sparc−/− mice were seeded onto collagen matrix and demonstrated reduced NET formation. PMNs purified from the spleens of 3 different mice were seeded onto poly-γ-lysine or collagen-coated glass, and the spontaneous NE Tosis occurring in PMNs from lpr/lpr/Sparc−/− mice was strongly reduced in the presence of collagen. Scale bars, 20 μm. L, NET formation was quantified by measuring the nuclear area of 200 cells, which was plotted against the percentage of SYTOX-positive cells corresponding to a given nuclear area. PMNs undergoing NETosis demonstrated a broad range of nuclear areas, whereas apoptotic neutrophils showed smaller nuclear areas that were less distributed and composed within a narrow peak. M, confocal microscopy analysis of NET formation by LAIR-1–silenced granulocytes. Granulocytes obtained from LAIR-1−/− neutrophils as an alternative to reducing LAIR-1 in vivo conditions of lpr/lpr/Sparc−/− neutrophils. Similar to PMA, IFNγ + C5a also promoted NET formation on poly-γ-lysine, but not collagen, unless LAIR-1 was silenced in neutrophils (Fig. 4M and N and Supplementary Fig. S6H). These results indicate that unrestrained NET formation could be stimulated by inhibiting LAIR-1 in Sparc−/− neutrophils as an alternative to reducing its ligand (collagen), as occurs in Sparc−/− mice. Of note, although PMA stimulation induced the highest expression of LAIR-1 and the most efficient NETosis, this stimulus has no...
SPARC Is Variously Modulated in Human Lymphomagenesis and Its Expression Correlates with ECM Organization and the Inflammatory Profile

Having shown that the exacerbated immune stimulation and altered SLO stromal architecture associated with SPARC deficiency creates conditions that favor the transformation of B-cell subsets, we next searched for human correlates of this model.

To investigate whether the spectrum of human B-cell lymphomagenesis includes entities with a SPARC-defective environment, SPARC expression was analyzed in a series of human B-cell non-Hodgkin lymphomas (B-NHL) by GEP. SPARC expression varied among the different B-NHLs, with the highest expression in diffuse large B-cell lymphomas (DLBCL) and MZL and the lowest expression in CLL. In particular, SPARC downregulation in CLL was statistically significant compared with each NHL histotype and reactive counterparts and remains merely an artificial boost for in vitro assays.

We further investigated whether distinct gene expression signatures could be identified in B-NHLs, according to SPARC expression. Indeed, B-NHLs could be divided into two groups according to the up- or downregulation of SPARC and its correlated genes (Fig. 6A–D). The biologic processes overrepresented in the SPARC-related signature comprised cell communication, immune and inflammatory response, ECM remodeling, and cell proliferation and death (Fig. 6B and Supplementary Table S1). According to our mouse model showing the impact of Sparc deficiency on the stromal control of myeloid cell activation and inflammation, we focused on the expression of genes belonging to the inflammatory/immune pathways, and found a correlation between SPARC expression and the enrichment of genes regulating inflammation (Fig. 6D and Supplementary Tables S1 and S2). Among the genes whose expression was negatively affected by SPARC downregulation were those coding for key regulators of apoptotic cell death, such as FAS, TNFRSF1A, and ADA, and for key mediators of apoptotic cell removal, such as the complement recognition factors C1Q, C1R, CIS, C3, and receptor CRII and markers of macrophage scavenger activity, including COLEC12 and CD14 (Supplementary Table S2).

Moreover, genes encoding key regulators of inflammation, such as IDO1 and CLU, mirrored SPARC expression, whereas suppressors of regulatory TGF-β1 signaling, such as SKI, were inversely correlated with SPARC. Furthermore, an inverse association was observed between SPARC expression and the ROR1 and BCL10 genes involved in CLL clone proliferation and survival via NF-κB and WNT5 activation, consistent with SPARC downregulation matching the pathogenic background of CLL.

Regulation of LAIR-1 engagement as a candidate mechanism for the enhanced NETosis in the lpr/lpr/Sparc−/− model prompted us to investigate LAIR-1 expression in B-NHL cases that were subdivided according to SPARC expression. LAIR-1 mRNA expression was significantly higher in B-NHL cases with a SPARC-high signature compared with those in the SPARC-low group (P < 0.0001; Fig. 6E). In the B-NHL setting, physiologic processes that were altered according to LAIR-1 expression included immune and defense responses, in agreement with the function of LAIR-1 in controlling immune cell activation in response to stimuli that perturb homeostasis (Fig. 6F). Corroborating the mRNA data, LAIR-1 expression was reduced in the microenvironment of B-NHL cases with low SPARC and collagen expression, such as CLL, compared with B-NHLs with higher SPARC and collagen ECM, such as DLBCL (Fig. 6G). Accordingly, a high frequency of NETotic PMN figures was detected in the form of extracellular MPO threads in CLL infiltrates, whereas the MPO-expressing PMN population of DLBCL infiltrates rarely showed signs of NET formation (Fig. 6H). Taken together, these results indicate that modulating SPARC expression in the microenvironment of human B-cell lymphomas correlates with features of defective apoptotic cell death and impaired clearance, sustaining myeloid cell activation, NET formation, and autoimmunity.

Finally, we investigated whether alterations in the stromal architecture of lymphoid tissues, which in our in vivo model were deeply influenced by SPARC, represent early events during human B-cell lymphomagenesis. To this end, we focused on “early” B-cell follicular lymphoma cases (i.e., in situ follicular lymphoma and partial follicular lymphoma involvement cases) characterized by the concomitant presence of reactive and transformed lymphoid follicles within the same lymphoid tissue (46). Within these samples, reactive and transformed follicles were identified by their differential expression of BCL-2, which selectively marked
Biased Stroma in the Autoimmunity–Lymphoma Transition

**Figure 5.** SPARC expression in human B-cell lymphoid malignancies.  
A, analysis of SPARC gene expression in tissue samples from human B-NHL and reactive lymphoid hyperplasia revealed markedly reduced expression of SPARC in CLL compared with other histotypes (left). SPARC gene expression analysis in purified neoplastic and control B-cell populations was comparable among various B-cell populations (right).  
B, representative immunohistochemical analysis of SPARC expression in stromal cells was performed using computer-assisted software analysis (see Methods) in one case each of DLBCL and CLL. The output represents the SPARC-positive area normalized for overall cellularity (nuclear area).  
C, quantification of SPARC expression in a TMA sample of B-NHL and control cases, as described in B (*, P < 0.05; one-way ANOVA).  
D, representative histochemical (Gomori and Masson’s trichrome staining) and IHC (Coll-I, Coll-IV, and SPARC) characterization of ECM deposition in prototypical cases of B-NHL and reactive lymphoid hyperplasia revealed changes in ECM remodeling as a result of SPARC expression. Notably, in CLL, the expression of all ECM proteins, including SPARC, was reduced compared with B-NHL. Scale bars, 20 μm.
Figure 6. Differential gene expression according to SPARC levels in B-NHLs. A, the box plot represents the distribution of gene expression in B-NHL samples divided into two groups based on SPARC gene expression (50th percentile high vs. 50th percentile low). On the y-axis, normalized gene expression intensity values are reported. In the box plots, horizontal bars represent the median expression values, central rectangles span the interquartile range, and red bars constitute outlier values. SPARC-high samples demonstrated greater overall gene expression distribution than SPARC-low samples. B, pie chart representation of the gene ontology biologic process categories for genes differentially expressed in B-NHL samples grouped according to SPARC gene expression (50th percentile high vs. 50th percentile low). C, hierarchical clustering of B-NHL samples according to the expression of selected inflammatory genes that were differentially expressed according to SPARC gene expression (50th percentile high vs. 50th percentile low). In the heatmap, each row represents a gene, and each column represents a sample. The color scale illustrates the relative expression level of a gene across all samples; red represents the expression level above the mean, and blue represents the expression level below the mean. Analysis of genes involved in the regulation of inflammation clearly revealed two different groups according to SPARC gene expression (50th percentile high in red vs. 50th percentile low in blue). D, the scatterplot shows the distribution of selected inflammatory genes that were differentially expressed in B-NHLs according to SPARC gene expression (50th percentile high vs. 50th percentile low). Each gene is colored according to its functional role in inflammation. Red lines indicate expression values = 0 and define four quadrants according to gene expression levels. The considered genes were significantly associated with a specific quadrant based on their biologic function ($\chi^2; P = 0.05$). E, the box plot represents the distribution of LAIR-1 gene expression in B-NHL samples divided into two groups based on SPARC gene expression (50th percentile high vs. 50th percentile low). On the y-axis, normalized gene expression intensity values are reported. In the box plots, horizontal bars represent median expression values, central rectangles span the interquartile range, and red bars constitute outlier values. SPARC-high samples showed a LAIR-1 gene expression distribution that was greater than that observed in SPARC-low samples. F, pie chart representation of gene ontology biologic process categories for genes coregulated with LAIR-1 in B-NHL samples according to SPARC gene expression (50th percentile high vs. 50th percentile low). G, in situ LAIR-1 expression analysis revealed that LAIR-1 was decreased within the microenvironment of CLL cases, as characterized by low SPARC and collagen expression compared with DLBCL cases, which consistently displayed higher SPARC and collagen ECM levels. Scale bars, 50 μm. H, in situ MPO expression revealed the presence of extracellular MPO, suggestive of NET formation in CLL cases. Scale bars, 20 μm, top; 10 μm, bottom.
Biased Stroma in the Autoimmunity–Lymphoma Transition

RESEARCH ARTICLE

transformed germinal centers (Fig. 7A). The proliferative fraction of reactive and transformed follicles was comparable, although slightly higher, in reactive follicles (Fig. 7B). Notably, within transformed follicles, Ki-67 marked lymphoid cells and spindle-shaped nuclei of stromal elements that were not marked in reactive follicles (Fig. 7C), suggesting that active reshaping of the stromal architecture is an integral part of early B-cell lymphomagenesis. Accordingly, the FDC mesenchymal architecture, which formed a dense and uniform meshwork in reactive follicles, appeared irregular and focally dispersed in transformed follicles (Fig. 7D).

Moreover, these subtle changes in the stromal architecture of transformed follicles were consistently associated with altered SPARC expression. Indeed, SPARC was expressed by germinal center–associated macrophages and FDCs in reactive follicles, whereas its expression was redistributed to scattered mesenchymal elements and lymphoid cells in lymphomatous follicles (Fig. 7E). These data indicate that altered stromal features and SPARC expression may accompany common events that occur early in B-cell transformation, including the imbalance between proliferation and apoptosis in expanding B-cell clones.

Figure 7. Stromal remodeling in early-stage follicular B-cell lymphomas. A, BCL-2 immunostaining was used to define reactive follicles (RF) and transformed follicles (TF) in early B-cell lymphomas. Reactive follicles were generally negative for BCL-2, whereas transformed follicles were positive for BCL-2 immunostaining. Scale bars, 40 μm, top; 20 μm, bottom. B and C, Ki-67 immunostaining revealed no difference in the frequency of Ki-67+ cells in reactive and transformed follicles. However, in transformed follicles, Ki-67 was expressed by spindle-shape stromal elements (black arrows). Scale bars in B, 20 μm, top; 10 μm, bottom. Scale bars in C, 20 μm. D, CD23 IHC identified a dense or irregular and dispersed FDC network in reactive versus transformed follicles. E, IHC analysis of SPARC expression revealed that SPARC was expressed by germinal center–associated macrophages and FDCs (red arrows) in reactive follicles and that SPARC expression was redistributed to scattered mesenchymal elements (black arrows) and lymphoid cells (green arrows) in lymphomatous follicles.
DISCUSSION

In this study, we show that alterations in stromal ECM components are involved in the dynamics linking chronic antigenic stimulation to lymphoma development. Deregulation of the immune system is a common aspect of autoimmune and lymphoproliferative disorders, and the fact that lymphomas can arise from a background of autoimmunity, persistent infection, or inflammation, suggests the existence of a common mechanism breachng immune cell homeostasis in these pathologic conditions. We identified stromal ECM modifications as a potential common denominator for this event and focused on the matricellular protein SPARC as a key regulator of relevant stromal changes. The end targets of ECM regulation in SLO are the lymphoid populations, as stromal cells support the survival of different subsets of T and B cells, providing prototypical homeostatic factors and functioning as antigen-presenting cells (47). During immune responses, SLO undergo profound architectural changes that are driven by stromal cells and involve the synthesis of ECM molecules, including matricellular proteins and collagens, which are somewhat unexpectedly implicated in the response outcome. Accordingly, we recently demonstrated that FDCs produce SPARC to promote germinal center formation and the development of experimental autoimmune encephalomyelitis (EAE). In this model, the absence of SPARC was associated with defective collagen deposition, impaired FDC network formation, Th17 induction, and EAE onset (19).

In contrast to our observations in the EAE setting, in Fas-mutant lpr/lpr mice, the absence of SPARC and consequent defective collagen remodeling resulted in excess immune stimulation and inflammation, which exacerbated autoimmunity and B-cell transformation. Explanations for these differences likely lie in the different homeostatic roles exerted by SPARC-competent ECM in SLO in these distinct pathologic conditions; in the EAE model, correct ECM remodeling favors FDC branching and promotes Th17-mediated immune responses, whereas in the lpr model, ECM deposition limits the unrestrained expansion of autoreactive B cells that control myeloid cell infiltration and activation.

Lymphoid and myeloid cell activation and function are regulated by immunoregulatory receptors that prevent unwanted inflammation and autoimmunity. ECM collagenas participate in immune cell regulation through the specific collagen-binding receptor LAIR-1. LAIR-1 belongs to a class of inhibitory receptors containing immunoreceptor tyrosine-based inhibitory motifs, and their expression characterizes almost all cell types in the immune system (48). Collagen engagement of LAIR-1 renders immune cells refractory to concomitant activating signals. In vivo, LAIR-1 has been shown to inhibit contact hypersensitivity (CHS) by acting on antigen-presenting cells and T and B lymphocytes (49). Similar to LAIR−/− mice, Sparc−/− mice display an exacerbated CHS response (50). In addition to immune cell activation, LAIR-1 controls the differentiation of immune cells, particularly neutrophils. Indeed, LAIR-1 expression is regulated throughout neutrophil differentiation, being highly expressed in CD34+ precursors, reduced in circulating blood neutrophils, and reexpressed in TNF/G-CSF activated neutrophils (39). Moreover, neutrophil activation and death are intimately linked. To avoid the excessive accumulation of neutrophils that may induce tissue damage (51) and contribute to autoimmunity (52), activated neutrophils must be promptly eliminated. However, the involvement of the LAIR-1 receptor and ECM ligands in neutrophil activation and death remains unexplored. Although neutrophil death via NETosis can be immunogenic, fostering B-cell activation and triggering autoimmune (29, 35), under physiologic conditions, NETosis remains confined and harmless. Thus, certain regulatory mechanisms must be lost to induce an imbalance between NETosis and other forms of neutrophil death (e.g., apoptosis), leading to chronic B-cell stimulation and autoimmunity. We recently demonstrated that the impaired neutrophil apoptosis and clearance observed in Fas-mutant lpr/lpr mice increased NET formation among spleen PMNs to induce SVV, a NET-related autoimmune disease (29). Here, we have shown that MPO autoantibody levels and the severity of SVV in lpr/lpr mice increased in the absence of Sparc; in particular, the incidence of SVV increased from 20–30% to nearly 60% in lpr/lpr mice in the presence and absence of Sparc, respectively. These data are consistent with the increased rate of NETosis in lpr/lpr/Sparc−/− mice, both in vivo and in vitro.

Consistent with these data, GEP analysis of splenic neutrophils showed enrichment of transcripts belonging to the IFN pathway, such as IRF1, Stat1, IL-18, and IFI30, in lpr/lpr/Sparc−/− mice. This result indicated that these neutrophils received a more conspicuous IFN stimulation in vivo, which has been shown to act as a priming factor for NETosis (36). This event might depend on the presence of increased IFN-producing cells in the splenic microenvironment of lpr/lpr/Sparc−/− mice or a lack of LAIR-1–mediated inhibitory signals. Accordingly, LAIR-1 expression was reduced in lpr/lpr/Sparc−/− neutrophils, and these cells were unable to limit cytokine activation in response to IFNs (53). On the basis of these data, we hypothesize that reduced inhibitory signals from the ECM and defective apoptosis are sufficient to induce the transition from autoimmunity to lymphomagenesis in a two-hit model, which would explain why Lair1-deficient mice do not show autoimmunity and why not all Fas mutants (human and mouse) develop lymphoma.

To provide auto-antigens for B-cell stimulation, NETs must contact B cells and professional antigen-presenting cells within lymphoid tissues. This contact is regulated by the compartmentalization provided by the stromal architecture of SLO, which is molded in an attempt to preserve homeostasis, even under conditions of aberrant immune stimulation (such as in the lpr setting). Herein, we demonstrated that the altered stromal ECM architecture of SLO in the absence of SPARC facilitated myeloid cell accumulation within lymphoid areas of SLO in response to autoimmunity-prone stimuli. This event favored unrestrained contact between lymphoid populations and neutrophils undergoing NETosis. Moreover, the transformation of CD5+ B cells in lpr/lpr/Sparc−/− mice is indicative of the selective susceptibility of innate B cells to such a transforming environment. The autoimmunity-lymphomagenesis transition in our in vivo model, in the context of an altered lymphoid stromal environment, prompted the search for correlates in human pathology. In particular, we observed that the above-mentioned modifications in the

CANCER DISCOVERY  JANUARY 2014 www.aacrjournals.org 124

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stromal architecture of SLO were tightly associated with human lymphomagenesis, occurring early upon malignant clone establishment. Such changes consisted of the proliferation and disarrangement of SLO mesenchymal components that affect the compartmentalization of lymphoid and myeloid (e.g., germinal center–associated macrophages) cells, and these changes involve variations in SPARC expression. When we investigated SPARC expression at the mRNA and protein levels in different histotypes of human B-NHLs, we found that it was consistently downregulated in human CLL, whereas a considerable degree of intercase variation was observed in other histotypes. The fact that SPARC expression was reduced in CLL was particularly noteworthy in light of the phenotypic similarities between the CD5+ leukemic/lymphomatous CLL clones and the CD5+ malignant B-cell populations expanded in lpr/lpr/Sparc−/− mice. Moreover, lpr/lpr/Sparc−/− mice showed NF-κB activation as well as BCR activation, which are characteristic of CLL clones, indicating that environment-mediated signals triggered NF-κB independent of intrinsic BCR activity (32). Underscoring the importance of microenvironmental control in CLL progression, the expression of LAIR-1 in transformed CD5+ B cells is progressively lost with increasing CLL clone aggressiveness (54). Unfortunately, the lack of LAIR-1 expression in murine B cells (48) did not allow us to directly test its role in our model. However, LAIR-1 is also expressed by the myeloid component of human lymphoma, and it is lost in CLL, but not DLBCL, in accordance with SPARC expression. These data suggest that the ECM influence on myeloid bystanders plays a role in CLL progression, as we demonstrated in lpr/lpr/Sparc−/− mice.

In human B-NHLs, stromal elements accounted for the differences in SPARC expression, as demonstrated by comparative GEP analysis of whole lymphoma sections and purified neoplastic cells and SPARC immunolocalization in stromal cells, including macrophages, fibroblasts, and FDCs. GEP analysis indicated that the best correlates of SPARC expression were key ECM components, including collagen type I, IV, VI, and XII, and fibronectin. This finding indicates that stromal expression of SPARC is part of the overall stromal and ECM remodeling that characterizes different lymphoid neoplasms. Recently, SPARC was reported to be one of the primary genes involved in determining stromal signatures in human B-cell lymphomas, particularly in DLBCLs (55). In these lymphomas, SPARC expression in bystander cells within the lymphoma stromal microenvironment, specifically macrophages, demonstrated prognostic significance, as a high SPARC stromal content independently predicts a more favorable disease outcome. Thus, a biologic prognostic scoring system that includes stromal SPARC assessment could be used. However, the biologic explanation underlying the favorable influence of a SPARC-rich lymphoma environment remains unclear. Here, we show that different GEP signatures were present in B-NHL cases according to their SPARC expression; SPARC-high and -low groups differed in the molecular programs related to tissue stromal and ECM remodeling, cell proliferation, cell death, cell–cell interactions, cell–stroma interactions and, most interestingly, those involving immune responses, inflammation, and autoimmunity. In particular, while many genes representative of effective innate and adaptive immune responses were overexpressed in the SPARC-high group, the SPARC-low group displayed downregulation of key genes involved in apoptotic cell death, scavenging of dead cell debris, and mediation of anti-inflammatory signals, all of which limit the establishment of excessive inflammatory and autoimmune conditions. Moreover, this finding is in accordance with the in vivo evidence that a SPARC-deficient stromal environment is associated with an enhanced autoimmune phenotype in lpr/lpr mice and with the enrichment of activated myeloid cells prone to immunogenic cell death, including NETosis. Indeed, we found a high frequency of NETotic PMN figures in the form of extracellular MPO threads in CLL, which were rarely detected in DLBCL cases showing high SPARC expression. This finding can be extended to human classic Hodgkin lymphoma, in which myeloid cells participate in regulating the composition of the reactive milieu, and cases characterized by defective SPARC stromal expression also display interactions between macrophages and NETs loaded with MPO (Supplementary Fig. S7).

In summary, this study demonstrates that stromal SPARC limits the magnitude of inflammatory stimuli in the setting of lymphoproliferation, which reconciles the apparent contrasting evidence that high numbers of SPARC-expressing macrophages have a positive influence, whereas dense macrophage infiltrates have an unfavorable effect, on human B-cell lymphomas. Thus, the contribution of stromal elements to the microenvironment of arising lymphoid clones may reflect the quality of ECM signals. Indeed, the presence of myeloid and stromal cells may play diverse roles in the establishment and expansion of autoimmune and/or lymphomatous clones in relation to the presence of normal or biased ECM milieu (Supplementary Fig. S8). In conclusion, our study indicates that the stroma regulates lymphomagenesis and that myeloid cells represent an emerging connection between autoimmunity and hematologic cancer.

**METHODS**

**Animals**

BALB/cAnNCrl mice of 8 to 10 weeks of age were purchased from Charles River Laboratories. CnCr.129S(B6)-Sparc−/− mice were developed in our institute by crossing B6;129-Sparc−/− mice (provided by Dr. Chin Howe, Wistar Institute, Philadelphia, PA) with BALB/cAnNCrl mice for 12 generations before intercrossing (17).

MRL/MpJ-Fas−/−] mice were originally purchased from The Jackson Laboratory and crossed with BALB/cAnNCrl mice for at least 12 generations. CnCr-Fas−/− mice were crossed with CnCr.129S(B6)-Sparc−/− mice and then were intercrossed. CnCr-Fas−/− mice, CnCr-Fas−/− Sparc−/− mice (referred to as lpr/lpr/Sparc−/− and lpr/lpr/Sparc−/− mice), and control mice were maintained in pathogen-free conditions in our animal facility. Immunodeficient CnCr.Foam−/− mice were maintained and crossed at the Fondazione IRCCS Istituto Nazionale dei Tumori (Milan, Italy). The institutional ethics committee for animal use authorized all animal experiments.

**Evaluation of Autoimmunity and Lymphoproliferation in Fas−/− Mice**

Swelling of SLO was evaluated by caliper measurement, whereas histopathologic and immunophenotypic analyses were performed according to the criteria for lymphoid neoplasm classification (56).
The expansion of tertiary lymphoid organs, such as the BALT, was also evaluated. The severity of autoimmune damage to renal and pulmonary parenchymal tissue was assessed by histopathology, and ELISA was used to measure the presence of nuclear autoantibodies in the sera [double-stranded DNA (dsDNA), ssDNA, ANA, and ribonucleoprotein (RNP)] as previously reported (29).

**Histopathology and IHC**

Immunohistochemical analysis for SPARC expression was performed on a TMA from 37 cases of follicular lymphoma, 19 cases of MCL, 31 cases of BL, 37 cases of DLBCL, 21 cases of MZL, and 9 cases of reactive follicular hyperplasia collected from the archives of the Hematopathology Unit of the Department of Hematology and Oncological Sciences, University of Bologna (Bologna, Italy), and the Human Pathology Section, Department of Health Sciences, University of Palermo (Palermo, Italy). All of the cases included in the study were revised according to the 2008 WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues for TMA construction. Each donor tissue block used for TMA preparation was punched twice to minimize issues with tissue heterogeneity. Quantitative analysis of SPARC immunohistochemical expression was determined in four nonoverlapping high-power fields from each TMA spot (×400 magnification) collected under a Leica DMD108 digital microscope using Leica Application Suite imaging software and analyzed using an adapted version of the Immuno-ratio plugin for NIH’s ImageJ Open Source image analysis software. This analysis allowed for evaluation of the SPARC immunostained area normalized for tissuecellularity. Outputs of the automated image analysis relative to the eight microphotographs obtained from the two TMA spots of each case were averaged to obtain the SPARC expression value for that case. Routine hematoxylin and eosin (H&E), Masson’s trichrome, and Gomori’s silver staining were performed as previously described (18).

IHC was performed as previously described (18) using the streptavidin-biotin–peroxidase complex method with the following primary antibodies: mouse anti-human SPARC (clone ON1-1; Takara); mouse anti-human BCL-2 (Novoceastra); mouse anti-human CD23 (Novoceastra); mouse anti-human K-67 (clone Mb-1; Dako Cytomation); anti-collagen I (Acris Antibodies); and anti-collagen IV (Millipore). Aminoethyl-carbazole (Dako Cytomation) or 3,3′-diaminobenzidine was used as the chromogenic substrate. Negative control staining was performed using mouse, rabbit, or goat immune sera instead of the primary antibodies.

The severity of renal damage in Fas−mutant mice was assessed by histopathologic analysis. A total of 7 mice were analyzed and scored using a modified version of the NIH (Bethesda, MD) semiquantitative scoring system for human lupus nephritis, as described previously (29).

**Confocal Microscopy**

Two consecutive rounds of single-marker immunostaining were used for double marker immunofluorescence analysis of human Hodgkin lymphoma samples (n = 6; three nodular sclerosis and three mixed cellularity). The slides were sequentially incubated with mouse anti-human MPO (Novocastra), which was revealed by staining with Alexa Fluor 488–conjugated goat anti-mouse secondary antibody (Invitrogen Molecular Probes), and mouse anti-human CD68 (Novoceastra), as revealed by Alexa Fluor 568–conjugated goat anti-mouse secondary antibody (Invitrogen Molecular Probes). The following primary mAbs were used for triple immunofluorescence on mouse tissues: rat anti-mouse CD19; alkaline phosphatase (APC)-or biotin-conjugated rat anti-mouse CD5; phycoerythrin (PE)-conjugated rat anti-mouse CD3; Alexa Fluor 488–conjugated rat anti-mouse CD45R; rat anti-mouse GR-1; and rat anti-mouse CD169. In addition, polyclonal antibodies against histones, collagen type I, and collagen type IV were used. All mAbs were purchased from eBioscience and all polyclonal antibodies were obtained from Chemicon. Sections were analyzed with a Microradiance 2000 (Bio-Rad Laboratories) confocal microscope equipped with Ar (488 nm), HeNe (543 nm), and red laser diode (638 nm) lasers. Confocal images (512 × 512 pixels) were obtained using a 20×, 0.5 NA Plan Fluor DIC or 60×, 1.4 NA oil immersion lens and analyzed using ImagePro 7.0.1 software. Immersion oil (ISO 8036) was purchased from Merck.

**ELISA Measurement of Serum Autoantibodies**

Quantitative determination of MPO-ANCA [immunoglobulin G (IgG)] levels was performed with a kit from Cusabio (CSB-E08675m), and dsDNA, ssDNA, and ANA antibodies were measured using kits from Alpha Diagnostic (#5110, 5210, and 5310, respectively).

**Western Blot Analysis**

Cell lysate preparation and Western blot analysis were carried out as previously described (16). The following antibodies were used for immunoreactions: polyclonal rabbit anti-SYK, anti–p-ZAP70/SYK, and anti-κBε/p-κBε (Cell Signaling Technology) and anti-actin (Sigma). Anti-goat and anti-rabbit secondary antibodies were purchased from Zymed (Invitrogen). The Quantity One (4.6.6) program (Bio-Rad) was used for densitometric analyses.

**Flow Cytometry**

The frequency and distribution of myeloid progenitors (Lin−cKit+; LK), including common myeloid progenitors (CMP), granulocyte-macrophage progenitor (GMP), and megakaryocytic/erythroid progenitor (MEP), were determined by staining spleen cell suspensions with a pool of antibodies for lineage (Lin)-positive markers, including CD3, CD11b, CD45R, Ly6G, CD4, CD8, and Ter-119, and stem cell and progenitor cell markers, including CD117, CD34, and CD16/CD32. Progenitors were identified according to their expression of CD34 and CD16/CD32 within the gate of Lin−CD117+ cells (18). Spleens were also evaluated for granulocyte enrichment by staining spleen suspensions with mAbs against CD11b, Ly6G, and CD45, followed by FACS analysis. To characterize B-cell subsets, cell suspensions of spleen or lymph node cells were stained with the following antibodies: anti-CD3, -CD19, -IgM, -IgD, -CD21/35, -CD93, -CD43, -CD15, and -CD23. All antibodies were purchased from eBioscience. Samples were acquired on an LSR II (BD).

**PMN Purification and Treatment**

To purify PMNs from the spleen, we used a magnetic bead separation protocol (Neutrophil Isolation Kit; Miltenyi Biotec). To evaluate spontaneous NETosis, naïve PMNs were seeded onto poly-l-lysine- or collagen-coated glass for 16 hours in the presence of the DNA dye SYTOX green (Invitrogen) but in the absence of any other stimuli. Sixteen hours later, the cells were fixed with 2% paraformaldehyde (PFA) and evaluated under a microscope. To induce NETosis, naïve PMNs were seeded onto poly-l-lysine-coated glass and treated with PMA (from 20 nmol/L in Iscove’s Modified Dulbecco’s Medium (IMDM) with 2% fetal calf serum (FCS), as previously described (29). To induce apoptosis and necrosis, neutrophils freshly isolated from the spleen were incubated with an anti-Fas/CD95 agonistic mAb (from BD Biosciences). Necrosis was induced in naïve PMNs via four cycles of freezing (liquid nitrogen) and thawing (37°C), leading to complete fragmentation of the cells (≤1% intact cells remaining). To assess apoptosis and necrosis after treatment, cells were stained with fluorescein isothiocyanate (FITC)–anti-mouse GR-1 and PerCoy5.5 anti-mouse CD11b, APC-conjugated annexin V, and 7-Aminoactinomycin D (7-AAD) (all from eBioscience) according to the manufacturer’s instructions. To evaluate BAFF production, spleen-purified PMNs were seeded onto
Biased Stroma in the Autoimmunity–Lymphoma Transition

96-well plates for 48 hours at a concentration of 1 × 10^6/mL. The supernatants were collected and an ELISA assay was used to quantify BAFF levels (R&D). PMNs silenced for LAIR-1 were obtained from Lin^- cells transfected with three specific siRNA sequences or a control scrambled sequence (Ambion), using 0.5 μg of each siRNA per 1 × 10^6 cells, following the manufacturer’s recommendations. Lin^- cells were incubated for 24 hours in the presence of SCF (20 ng/mL) + G-CSF (40 ng/mL), then transfected with siRNA in the presence of INTERFERin (PoliPlus) and kept in culture for 3 days. Cells were then harvested, monitored for Ly6G (Supplementary Fig. S6B) and LAIR-1 expression (Supplementary Fig. S6F and S6G) by FACS, and tested for NETosis by seeding them onto poly-o-lysine or collagen type IV for 3 hours in the presence of PMA or IFNy + C5a, according to previously described methods (36).

**CD5^+** Cell–PMN Co-culture for Immunization Experiments

CD5^+ cells were purified from the spleens of naive mice via magnetic bead separation after the depletion of CD3^- cells. The purity of CD5^- cells obtained was measured by FACS analysis based on the expression of surface immunoglobulin M (IgM) and CD45R. To evaluate the effect of NETs on CD5^+ cell proliferation, PMNs isolated from the spleen were seeded onto a 24-well plate, allowed to adhere and treated with PMA (10 ng/mL) for 4 hours in the presence or absence of DNAse (100 U/mL). Then, PMNs were gently washed and added to carboxyfluorescein succinimidy ester (CFSE)-labeled CD5^- cells (1:1 ratio). Three days later, CD5^- cells were analyzed for cell division, as measured by discrete peaks of decreasing fluorescence of CFSE in NET-stimulated cells or a single, bright CFSE peak in nonstimulated, undivided cells. To evaluate NF-κB activation, CD5^- cells were cocultured with NETotic, apoptotic, or necrotic PMNs for 2 hours.

For intracellular staining of p65, p-p65, IκBα, and p-IκBα, we used the following protocol. Cells were fixed with 2% PFA, washed, and permeabilized with 0.25% saponin buffer for 5 minutes. Primary and secondary mAbs were added in the same buffer for 15 minutes each. All antibodies used to detect NF-κB activation were purchased from Cell Signaling Technology and were used according to the manufacturer’s instructions.

**GEP of Human Lymphomas**

Gene expression analysis was performed on datasets previously generated with Affymetrix microarrays, which are available in the Gene Expression Omnibus (GEO) database (GSE12193, GSE14253, GSE16455, GSE2350, GSE24080, GSE26673, GSE4732); these samples correspond to 134 B-NHLs and 25 samples of normal B lymphocytes. GSE16455, GSE2350, GSE24080, GSE26673, GSE4732); these samples generated with Affymetrix microarrays, which are available in the Microarray Expression Database (http://www.ncbi.nlm.nih.gov/geo/).

The DAVID/EASE software was used to establish whether specific gene expression; specifically, we included all the cases belonging to the greater than 50th percentile of SPARC expression in the “SPARC-high” group. All the cases falling into the less than 50th percentile were defined as the “SPARC-low” group. Therefore, differential expression analysis comparing cases with higher or lower SPARC gene expression was performed using a two-tailed Student t test (P < 0.05, multiple testing correction, according to Benjamini–Hochberg), and a fold change greater than 2 was selected as the basic statistical criterion. The DAVID/EASE software was used to establish whether specific cell functions and biologic processes, defined according to gene ontology (59), were significantly represented among the deregulated genes (60).

Because the involvement of SPARC in inflammation regulation was particularly relevant to our investigation, we selected genes involved in such processes for further analyses. Specifically, we used hierarchical clustering (based on the Pearson correlation distance and average linkage method) and the χ^2 test (P < 0.05) to investigate whether genes directly or inversely related to inflammation were significantly associated with SPARC expression (Supplementary Table S2).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Sangaletti, C. Tripodo, C. Vitali, P. Portararo, P. Pinciroli, F. Puligoi, P.P. Pecalunga

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Guarinotta

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Defective Stromal Remodeling and Neutrophil Extracellular Traps in Lymphoid Tissues Favor the Transition from Autoimmunity to Lymphoma

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