Defective stromal remodeling and neutrophil extracellular traps in lymphoid tissues favor the transition from autoimmunity to lymphoma.

Running title. Biased stroma in the autoimmunity-lymphoma transition

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The authors disclose no potential conflicts of interest

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LIST OF ABBREVIATIONS

ANCA= anti-neutrophil cytoplasmic antibodies
BCR= B cell receptor
CLL= chronic lymphocytic leukemia
DLBCL= diffuse large B cell lymphoma
ECM= extracellular matrix
GC= germinal center
FDC= follicular dendritic cell
GEP= gene expression profile
LN= lymph node
MPO= myeloperoxidase
MZB= marginal zone B cell
NET= neutrophil extracellular traps
NHL= non-Hodgkin lymphoma
PMA= phorbol 12-myristate 13-acetate
PMN= polymorphonuclear leukocyte
SLO= secondary lymphoid organs
SVV= small vessel vasculitis
TLR= Toll-like receptor
WB= western blot
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. 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 ECM. These conditions promoted aberrant interactions between neutrophil extracellular traps (NET) 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.
INTRODUCTION

Chronic inflammation and autoimmune disorders are associated with a high risk of lymphoid malignancies. Indeed, greater 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 coeliac disease, respectively (2, 3). Malignant lymphoid clones of the ocular adnexa and gastric MALT lymphomas associated with *Chlamydophila psittaci* and *Helicobacter pylori* infection, respectively, frequently express immunoglobulin VH genes that are typically found in rheumatoid arthritis, systemic lupus erythematosus 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 physiological 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) (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) (15). SPARC is a non-structural pleiotropic matricellular protein that is a key regulator of tissue stroma remodeling in physiological conditions and
pathological 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 hematological neoplasms (17) (18). SPARC produced by innate immune cells influences the crosstalk between cancer cells and the ECM at the invasive edge of primary tumors, and regulates pro-inflammatory 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 (GC) 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 auto-antigens 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 to those of lpr/lpr/Sparc+/- counterparts.

The double-mutant mice displayed an accelerated induction of autoantibodies in comparison to 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 (LN) 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 to 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 lpr mutation is strain-dependent and reduced in BALB/c mice compared to the original MRL background (23).
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 to lpr/lpr/Sparc+/+ mice, SLO were analyzed by flow cytometry (FACS) and immunofluorescence (IF) using a cocktail of 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 (DN) 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 Figs. S1B and S1C 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 LN and spleen of the same animals was indicative of malignant clones (Supplemental Fig. S1D).

To confirm whether the enhanced CD5+ B cell proliferation in lpr/lpr/Sparc−/− mice was malignant, spleen and LN 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 LN 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. 1J-K).

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 (16) (15), similar to what occurs in Fas-deficient lpr/lpr/Sparc−/− mice, where the need to accommodate the exacerbated proliferation of CD3+B220+ DP 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 LN. Indeed, the markedly defective collagen architecture of lpr/lpr/Sparc−/− mice resulted in 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, while 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 LN. These data suggest that autoimmunity promotes the remodeling of SLO microarchitecture, which involves SPARC.

Considering that SPARC endows anti-proliferative 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 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 non-canonical, unrestrained contacts.

We hypothesized that subversion of splenic architecture permits the establishment of non-canonical interactions between auto-reactive 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 pro-survival 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 IF 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 IF analysis detected clusters of Ly-6G+ 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).

Splenic neutrophils of lpr/lpr/Sparc−/− mice promote B-cell factor production and are prone to NET formation.

The increase in Ly6G+ neutrophils contacting CD5+ B cells in lpr/lpr/Sparc−/− mice may affect the survival, expansion and activation of B cells. Indeed, production of the prototypical B-cell activating factor (BAFF) and IL-21 was higher in neutrophils purified from the spleens of lpr/lpr/Sparc−/− compared to lpr/lpr/Sparc+/− mice (Fig. 2F).
2F-H). In addition, freshly isolated lpr/lpr/Sparc<sup>−/−</sup> neutrophils were more prone to extrude NETs when seeded onto poly-D-lysine-coated glass in the absence of any other stimulus (i.e., phorbol 12-myristate 13-acetate; PMA), indicating that their priming toward NETosis had already occurred in vivo (Fig. 3A and Supplementary Fig. S4A). Accordingly, confocal microscopic analysis of spleen sections detected additional NETs in contact with B cells in lpr/lpr/Sparc<sup>+/−</sup> mice compared to lpr/lpr/Sparc<sup>+/+</sup> 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) (29). In addition, NETosis requires myeloperoxidase (MPO) expression (30), which was increased in the spleens of lpr/lpr/Sparc<sup>+/−</sup> 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-F) compared to lpr/lpr/Sparc<sup>+/+</sup> mice.

**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<sup>+/+</sup> spleens were co-cultured with Sparc<sup>+/−</sup>, Sparc<sup>−/−</sup>, lpr/lpr/Sparc<sup>+/−</sup> or lpr/lpr/Sparc<sup>−/−</sup> neutrophils. In the absence of in vitro stimuli, neutrophils from both Sparc<sup>+/+</sup> and Sparc<sup>−/−</sup> 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<sup>+/−</sup> neutrophils and was further enhanced by the addition of lpr/lpr/Sparc<sup>−/−</sup> neutrophils (Fig. 4A). Thus, the extent of CD5+ B cell proliferation correlated with the increase in neutrophils undergoing spontaneous NETosis in lpr/lpr/Sparc<sup>−/−</sup> mice. In addition, the functional link between NETosis and CD5+ B cell proliferation was independent of the Fas or Sparc genotype; neutrophils from WT spleens were able to stimulate CD5+ B cell proliferation following induction of NETosis with PMA (29) (Fig. 4B). 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 the 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 (WB) 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 to 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 naïve, apoptotic or necrotic polymorphonuclear leukocytes (PMNs) (Fig. 4D-E and Supplementary Fig. S6B), in line with its proteosomal degradation in stimulated cells. Thus, NETs are capable of stimulating CD5+ B-cell proliferation via NF-κB activation.

In our in vivo 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 h and then exposing these cells to NETs for 24 h. NET addition significantly enhanced the CpG-induced proliferation of CD5+ B cells under conditions in which NETs alone (24 h 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 to 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 interferon (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 IL18, IL1β, IRF1, STAT1, IFI30 and IFI47, FCGRT, DUSP3 and Ly6E, among others (Supplementary Fig. S5A). The differential expression of some of these key genes was validated by 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 down-regulation (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-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 fibril-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 to lpr/lpr/Sparc$^{+/-}$ mice (Fig. 4I-J). These data suggest that in the absence of Sparc, the expression of the immune inhibitory receptor LAIR-1 and its putative substrates are reduced, unleashing immune cell activation and NETosis. This hypothesis is also supported by our GEP analysis, which showed the up-regulation of genes belonging to the IFN signature in lpr/lpr/Sparc$^{-/-}$ neutrophils, and by previous data showing that IFNs are
potent NET inducers (29, 36).

To test whether ECM composition directly influences NETosis, freshly isolated PMNs from lpr/lpr/Sparc<sup>−/−</sup> mice that spontaneously underwent NETosis were seeded onto a collagen matrix or poly-D-lysine (a permissive substrate for NET). Collagen matrix, but not poly-D-lysine, significantly inhibited NETosis in PMNs from lpr/lpr/Sparc<sup>−/−</sup> mice (Fig. 4K-L), as well as in naïve spleen-derived neutrophils that form NETs under PMA stimulation (Supplementary Fig. S6A-B). Furthermore, collagen matrix, but not poly-D-lysine, significantly inhibited PMA-triggered PMN activation, as determined by reactive oxygen species production (Supplementary Fig. S6C-D). These data demonstrate that ECM molecules negatively regulate neutrophil activation and NETosis and that their deregulation may exacerbate autoimmunity.

Next, to directly link LAIR-1 engagement to NETosis, we produced neutrophils lacking LAIR-1 expression. BM-derived Lin- cells were differentiated into neutrophils with stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) and transfected with 3 siRNA sequences targeting LAIR-1 or a scrambled sequence (Supplementary Fig. S6 E-G). Granulocytes obtained from transfected Lin-cells were then seeded onto poly-D-lysine or collagen matrix and stimulated with PMA or IFN<sub>γ</sub>+C5a; the latter stimulus was used to mimic the in vivo conditions of lpr/lpr/Sparc<sup>−/−</sup> neutrophils. Similar to PMA, IFN<sub>γ</sub>+C5a also promoted NET formation on poly-D-Lysine, but not collagen, unless LAIR-1 was silenced in neutrophils (Fig. 4M-N and Supplementary Fig. S6H). These results indicate that unrestrained NET formation could be stimulated by inhibiting LAIR-1 in Sparc<sup>+/+</sup> neutrophils as an alternative to reducing its ligand (collagen), as occurs in Sparc<sup>−/−</sup> mice. Of note, although PMA stimulation induced the highest expression of LAIR-1 and the most efficient NETosis, this stimulus has no in vivo counterpart and remains merely an artificial boost for in vitro assays.

*SPARC is variably 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 lymphoid malignancies includes entities with a SPARC-defective environment, SPARC expression was analyzed in a series of human non-Hodgkin’s B cell lymphomas (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 to each NHL histotype and reactive lymphoid hyperplasia (Fig. 5A, left panel). This finding is particularly relevant given the similarities between the B-cell lymphoproliferation observed \textit{in lpr/lpr/Sparc$^{-/-}$} mice, human CLL and murine CLL models in terms of the dominant immunophenotype (CD19+, CD5+ and CD43+) and NF-$\kappa$B activation (3, 41, 42). The striking differences in SPARC expression among the various NHLs were predominantly microenvironment-intrinsic, as GEP analysis of purified B cell clones revealed less conspicuous variations (Fig. 5A, right panel). To validate the GEP results, we performed IHC analysis to assess SPARC expression on tissue microarrays (TMA) from 37 cases of follicular lymphoma (FL), 19 cases of MCL, 31 cases of Burkitt’s lymphoma (BF), 37 cases of CLL, 22 cases of DLBCL, 21 cases of MZL and 9 cases of reactive follicular hyperplasia. The fraction of SPARC$^{+}$ cells paralleled the GEP expression data, with high expression in DLBCL, BL and FL cases and very low expression in CLL (Fig. 5B-C). In cases with prominent SPARC expression, SLO stromal remodeling was associated with diffuse and dense reticulin and collagen ECM deposition, consistent with the role of SPARC in ECM remodeling. In contrast, lymphomas with low or absent SPARC expression, such as CLL, demonstrated SLO architecture effacement by neoplastic lymphoid cells, which occurred in the absence of significant ECM organization (Fig. 5D). These results indicated that SPARC is modulated alongside SLO remodeling in human lymphomas and that a SPARC-defective environment characterizes specific B NHL histotypes, such as CLL, strengthening the relevance of our \textit{in vivo} model.

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 down-regulation of SPARC and its correlated genes (Fig. 6A-D). The biological programs over-represented in the SPARC-related signature comprised cell communication, immune and inflammatory response, ECM remodeling and cell proliferation and death (Fig. 6B and
Supplemental 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 Supplemental Tables S1-S2). Among the genes whose expression was negatively affected by SPARC down-regulation 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, C1s, C3 and receptor CR2 (CD21) and markers of macrophage scavenger activity, including COLEC12 and CD14 (Supplemental Table S2). Notably, defects in the expression of these genes have been consistently associated with deregulated immune responses, overly inflammatory states and systemic autoimmunity (43-45). 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. Deregulation of LAIR-1 engagement as a candidate mechanism for the enhanced NETosis in the lpr/lpr/Sparc-/- model prompted us to investigate LAIR1 expression in NHL cases that were subdivided according to SPARC expression. LAIR1 mRNA expression was significantly higher in NHL cases with a SPARC-high signature compared to those in the SPARC-low group (p<0.0001) (Fig. 6E). In the NHL setting, physiological processes that were altered according to LAIR1 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 NHL cases with low SPARC and collagen expression, such as CLL, compared to 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.
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 FL and partial FL 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 transformed GCs (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 GC-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.

**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 breaching immune cell homeostasis in these pathological 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 GC 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 pathological 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 collagens 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 (ITIMs), 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 re-expressed 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 autoimmunity (29, 35), under physiological 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, IL18 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). Based on 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 2-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 correlatives in human pathology. In particular, we observed that the above-mentioned modifications in the 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 impact the compartmentalization of lymphoid and myeloid (e.g., GC-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 cell NHLs, we found that it was consistently downregulated in human CLL, whereas a considerable degree of inter-case 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 among 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 biological prognostic scoring system that includes stromal SPARC assessment could be employed. However, the biological 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 down-regulation 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 classical Hodgkin’s 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 hematological cancer.
MATERIALS AND METHODS

Animals. BALB/cAnNCrl mice at 8-10 weeks of age were purchased from Charles River Laboratories (Calco, Italy). CNCr.129S(B6)–Sparc<sup>tm1Hwe</sup> mice were developed in our institute by crossing B6;129-Sparc<sup>tm1Hwe</sup> mice (provided by Dr. Chin Howe; Wistar Institute, Philadelphia, PA, USA) with BALB/cAnNCrl mice for 12 generations prior to intercrossing (17).

MRL/MpJ-Faslpr/J mice were originally purchased at the Jackson Laboratory, and crossed with BALB/cAnNCrl mice for at least for 12 generations. CNCr-Fas<sup>lpr</sup> mice were crossed with CNCr.129S(B6)–Sparc<sup>tm1Hwe</sup> mice, and then were intercrossed. CNCr-Fas<sup>lpr</sup> mice, CNCr-Fas<sup>lpr</sup> Sparc<sup>tm1Hwe</sup> mice (referred to as lpr/lpr/Sparc<sup>+/+</sup> and lpr/lpr/Sparc<sup>−/−</sup> mice) and control mice were maintained in pathogen-free conditions in our animal facility. Immunodeficient CNCr-Foxn1<sup>nu</sup> mice were maintained and crossed at the Fondazione IRCCS Istituto Nazionale dei Tumori. The institutional ethics committee for animal use authorized all animal experiments.

Evaluation of autoimmunity and lymphoproliferation in Faslpr mice. Swelling of SLO was evaluated by caliper measurement, whereas histopathological 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 (ds-DNA, ss-DNA, ANA and RNP) as previously reported (29).

Histopathology and IHC. IHC analysis for SPARC expression was performed on a tissue microarray (TMA) from 37 cases of FL, 19 cases of MCL, 31 cases of Burkitt’s lymphoma, 37 cases of CLL, 22 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 and Human Pathology Section, Department of Health Sciences, University of 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 overcome issues with tissue heterogeneity. Quantitative analysis of SPARC immunohistochemical expression was determined in 4 non-overlapping high-power fields from each TMA spot (HPFs, x400 magnification) collected under a Leica DMD108 digital microscope using LAS 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 tissue cellularity. 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 (Abs): mouse anti-human SPARC (clone ON1-1, Takara); mouse anti-human Bcl2 (Novocastra); mouse anti-human CD23 (Novocastra); mouse anti-human Ki-67 (clone Mib-1, Dako Cytomation, Denmark); anti-collagen I (Acris Antibodies) and anti-collagen IV (Millipore). Aminooethyl-carbazole (AEC) (Dako Cytomation, Denmark) or 3-3’-Diaminobenzidine (DAB) was used as the chromogenic substrate. Negative control staining was performed using mouse, rabbit or goat immune sera instead of the primary Abs.

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 National Institutes of Health 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 IF analysis of human Hodgkin’s Lymphoma samples (n=6, 3 nodular sclerosis and 3 mixed cellularity). The slides were sequentially incubated with mouse anti human MPO (Novocastra), which was revealed by staining with Alexa 488-conjugated goat anti mouse secondary Ab (Invitrogen Molecular Probes), and mouse anti-human CD68 (Novocastra), as revealed by Alexa 568-conjugated goat anti-mouse secondary Ab (Invitrogen Molecular Probes). The following primary monoclonal Abs (mAb) were used for double and triple IF on
mouse tissues: rat anti-mouse CD19; APC- or biotin-conjugated rat anti-mouse CD5; PE-conjugated rat anti-mouse CD3; Alexa-488-conjugated rat anti-mouse CD45R; rat anti-mouse GR-1 and rat anti-mouse CD169. In addition, polyclonal Abs against pan histones, collagen type I and collagen type IV were used. All mAbs were purchased from e-Biosciences, and all polyclonal Abs 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 Flouor 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 (IgG) levels was performed with a kit from Cusabio (CSB-E08676m), and dsDNA, ssDNA and ANA Abs were measured using kits from Alpha Diagnostic (#5110, 5210, and 5310, respectively).

**WB analysis.** Cell lysate preparation and WB blot analysis were carried out as previously described (16). The following Abs were used for immunoreactions: polyclonal rabbit anti-Syk, anti Ph-ZAP70/syk and anti IkB-α/Ph-IkBα (Cell Signaling) and anti-actin (Sigma). Anti-goat and anti-rabbit secondary Abs were purchased from Zymed (Invitrogen). The Quantity One (4.6.6) program (Biorad) 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 Abs for lineage (Lin)-positive markers, including CD3, CD11b, CD45R, Ly-6G, CD4, CD8 and Ter-119, and stem cell and progenitor cell markers, including CD117, CD34 and CD16/32. Progenitors were identified according to their expression of CD34 and CD16/32 within the gate of Lin-CD117+ cells (18). Spleens were also evaluated for granulocyte enrichment by staining spleen suspensions with mAbs against CD11b, Ly-6G and CD45, followed by FACS analysis. To characterize B cell subsets, cell suspensions of spleen or lymph node cells were stained with the following Abs: anti-CD3, -CD19, -IgM, -IgD, -
CD21/35, -CD93, -CD43, -CD5 and -CD23. All Abs 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, from Miltenyi). To evaluate spontaneous NETosis in the spleen, PMNs isolated from Fas-mutant mice were seeded onto poly-D-lysine-coated or collagen-coated glass for 16 h 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, naive PMNs were seeded onto poly-D-lysine-coated glass and treated with PMA (from 20 nM) in IMDM 2% FCS, as previously described (29). To induce apoptosis or necrosis, neutrophils freshly isolated from the spleen were incubated with anti-Fas/CD95 agonistic mAb (from BD Biosciences). Necrosis was induced in naive PMNs via 4 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 FITC-anti-mouse GR-1 and PercPey5.5 anti-mouse CD11b, APC-conjugated annexin V and 7-AAD (all from e-Bioscience) according to the manufacturer's instructions. To evaluate Baff production, spleen-purified PMNs were seeded onto 96-well plates for 48 h at a concentration of 1x10⁶/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-negative cells transfected with 3 specific siRNA sequences or a control scrambled sequence (Ambion), using 0.5 μg of each siRNA per 1×10⁶ cells, following the manufacturer’s recommendations. Lin-negative cells were incubated for 24 h in the presence of SCF (20 ng/ml) +G-CSF (40 ng/ml) then transfected with siRNA in presence of Interferin (PoliPlus) and kept in culture for 3 days. Cells were then harvested, monitored for Ly6G (Supplementary Fig. S6E) and LAIR-1 expression (Supplementary Fig. S6F-G) by FACS and tested for NETosis by seeding them onto poly-D-lysine or collagen type IV for 3 h in the presence of PMA or IFNγ+C5a, according to previously described methods (36).

**CD5+ B cell-PMN co-culture for immunization experiments.** CD5+ B cells were purified from the spleens of naïve mice via magnetic bead separation after the depletion of CD3+ cells. The purity of CD5+ B cells obtained was measured by
FACS analysis based on the expression of surface IgM and CD45R. To evaluate the effect of NETs on CD5+ B cells 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 h in presence or absence of DNAse (100 U/ml). Then, PMNs were gently washed and added to CFSE-labeled CD5+ B cells (1:1 ratio). Three days later, CD5+ B 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 non-stimulated, undivided cells. To evaluate NF-κB activation, CD5+ B cells were co-cultured with NETotic, apoptotic or necrotic PMNs for 2 h. 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 min. Primary and secondary mAbs were added in the same buffer for 15 min each. All Abs used to detect NF-κB activation were purchased from Cell Signaling and used according to the manufacturer’s instructions.

**GEP of human lymphomas.**

Gene expression analysis was performed on data sets previously generated with Affymetrix microarrays, which are available in the GEO database (GSE12195; GSE12453; GSE16455; GSE2350; GSE24080; GSE26673; GSE4732); these samples correspond to 134 B-non Hodgkin lymphomas and 25 samples of normal B-lymphocytes. Specifically, we included the following cancer types: 40 FL, 14 MCL, 10 CLL, 14 multiple myelomas (MM), 36 Burkitt lymphomas, 20 DLBCL, 10 GC cell samples, 5 naïve (N) cell samples, 5 memory (M) cell samples and 5 plasma cell (PC) samples (Supplementary Table S3). Details on these cases have been previously reported [PMID: 15778709; 16760443; 18794340; 19412164; 20124476; 20676074; 21245480; 18492688].

Gene expression analysis was carried out as previously reported, using GeneSpring GX 12 (Agilent, USA) (57, 58) according to MIAMI guidelines. Briefly, for supervised analysis, we divided the B-NHL cases into 2 groups according to SPARC gene expression; specifically, we included all cases belonging to the higher 50° percentile of SPARC expression in the “SPARC-high” group. All of the cases falling in lower 50° percentile were defined as the “SPARC-low” group. Thereafter, differential expression analysis comparing cases with higher or lower SPARC gene expression was performed using a two-tailed Student’s 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 biological 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 chi-square 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
The authors declare no competing financial interests.

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Acknowledgments
The authors thank Ivano Arioli for assistance with mouse experiments and Prof. Stefano Pileri for critically reading the manuscript.

Grant Support
This work was supported by the Association for International Cancer Research (AICR UK, grant n° 11-0595), the Associazione Italiana per la Ricerca sul Cancro (AIRC: Program Innovative Tools for Cancer Risk Assessment and Diagnosis, 5 per mille n°12162 to CP and MPC; Investigator Grants n° 10137 to MPC and My First Grant n° 12810 to SS) and the Italian Ministry of Health.

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Figure 1. Exacerbated autoimmunity and lymphoproliferation in Fas-mutant lpr/lpr mice lacking Sparc. A. Quantification of anti-ANA, anti-ssDNA and anti-ds-DNA Ab levels in lpr/lpr/Sparc\textsuperscript{+/-}, lpr/lpr/Sparc\textsuperscript{-/-} and control Sparc\textsuperscript{+/-} and Sparc\textsuperscript{-/-} mice. Sera from individual mice from 1 to 10 months of age were assayed for IgG autoantibodies. Data are expressed as U/ml of IgG and plotted as a scattergram, with the Ab titer of each mouse vs. its relative age (wks) to show the time at which autoantibodies developed in each group (left column). Regardless the age of mice, collective data shows that the levels of IgG anti-ssDNA or anti-dsDNA were significantly increased in lpr/lpr/Sparc\textsuperscript{-/-} compared to lpr/lpr/Sparc\textsuperscript{+/-} mice (p<0.05) (right column). B. LN swelling in lpr/lpr/Sparc\textsuperscript{+/-} and lpr/lpr/Sparc\textsuperscript{-/-} mice. The onset of LN swelling was accelerated in lpr/lpr/Sparc\textsuperscript{-/-} compared to lpr/lpr/Sparc\textsuperscript{+/-} mice. (n=25/strain). C. Comparison of spleen weight at 6 months of age. The spleen weight of lpr/lpr/Sparc\textsuperscript{-/-} mice was significantly increased (p<0.001) compared to lpr/lpr/Sparc\textsuperscript{+/-} mice. (n=25/strain). D. Histopathological analysis of spleen and LN samples from lpr/lpr/Sparc\textsuperscript{+/-} and lpr/lpr/Sparc\textsuperscript{-/-} mice showing the marked effacement of the spleen and LN architecture of lpr/lpr/Sparc\textsuperscript{-/-} mice by medium-sized lymphoid cells and plasmacytoid elements. In the spleens of lpr/lpr/Sparc\textsuperscript{-/-} 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\textsuperscript{+/-} spleens. Similarly, the LN architecture was characterized by the diffuse expansion of medium-sized lymphoid cells and elements with plasmacytic morphology. Scale bars: 50 \mu m. E. Fraction of follicular B (FoB) cells, MZB cells and CD5\textsuperscript{+} B cells in the spleens of lpr/lpr/Sparc\textsuperscript{+/-}, lpr/lpr/Sparc\textsuperscript{-/-} and control Sparc\textsuperscript{+/-} and Sparc\textsuperscript{-/-} mice. Lpr/lpr/Sparc\textsuperscript{+/-} mice showed increased MZB cells compared to wt Sparc\textsuperscript{+/-} mice. In lpr/lpr/Sparc\textsuperscript{-/-} mice, contraction of MZB cells was associated with the expansion of CD5\textsuperscript{+} B cells. FoB cells remained largely unchanged. F. Analysis of IgM and CD5 expression in cell suspensions obtained from the spleens of lpr/lpr/Sparc\textsuperscript{+/-} and lpr/lpr/Sparc\textsuperscript{-/-} mice. Representative plots are shown. G. Confocal microscopy analysis of the spleens of lpr/lpr/Sparc\textsuperscript{+/-} and lpr/lpr/Sparc\textsuperscript{-/-} mice revealed the accumulation of CD5/CD19 double-positive cells in the spleens of lpr/lpr/Sparc\textsuperscript{-/-} mice but not lpr/lpr/Sparc\textsuperscript{+/-} mice. Scale bars: 20 \mu m. H. Histopathological analysis
of the SLO of lpr/lpr/Sparc<sup>−/−</sup> 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 cytomorphological features (*i.e.*, high nuclear/ cytoplasmic ratio, marginated chromatin and prominent nucleoli) and a diffuse pattern of infiltration. Scale bars: 20 μm. **I.** Spleen and LN enlargement in nude mice receiving trocar pieces from lpr/lpr/Sparc<sup>−/−</sup> but not lpr/lpr/Sparc<sup>+/+</sup> mice. **J.** Frequency of CD5<sup>+</sup> B cells in the LN from nude mice that were transplanted with trocar pieces from the spleens of lpr/lpr/Sparc<sup>+/+</sup> or lpr/lpr/Sparc<sup>−/−</sup> mice. Nude mice receiving lpr/lpr/Sparc<sup>−/−</sup> trocar pieces showed significantly increased expansion of CD5<sup>+</sup> B cells (CD19<sup>+</sup>CD5<sup>+</sup>CD43<sup>+</sup>IgM+) in the LN. A representative experiment from of 3 total experiments performed with 7 recipient mice is shown (p<0.05). **K.** Analysis of IgM and CD5 expression in spleen cell suspensions from nude mice receiving trocar pieces from lpr/lpr/Sparc<sup>+/+</sup> and lpr/lpr/Sparc<sup>−/−</sup> mice.

**Figure 2. Stromal and hematopoietic spleen remodeling in Fas-mutant mice.** **A.** Confocal microscopy analysis of spleens from control Sparc<sup>+/+</sup> and Sparc<sup>−/−</sup> mice and Fas-mutant lpr/lpr/Sparc<sup>+/+</sup> and lpr/lpr/Sparc<sup>−/−</sup> mice were stained with the following mAbs: anti-B220 (green), -CD3 (red) and -collagen type IV (blue). CD3<sup>+</sup> and B220<sup>+</sup> lymphocytes were confined to the follicular structures of the spleen in Sparc<sup>+/+</sup> and Sparc<sup>−/−</sup> mice. In autoimmune lpr/lpr/Sparc<sup>−/−</sup> mice, the expanding double-positive B220<sup>+</sup>CD3<sup>+</sup> 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<sup>−/−</sup> spleens, where B and T lymphocytes as well as double-positive B220<sup>+</sup>CD3<sup>+</sup> cells were interspersed. Scale bars: 10 μm. **B.** Confocal microscopy analysis of spleens from control Sparc<sup>+/+</sup> and Sparc<sup>−/−</sup> mice and Fas-mutant lpr/lpr/Sparc<sup>+/+</sup> and lpr/lpr/Sparc<sup>−/−</sup> mice were stained with the following mAbs: anti-B220 (green), -CD3 (blue) and -CD169 (red). CD169<sup>+</sup> cells were enriched in lpr/lpr/Sparc<sup>−/−</sup> mice. In these mice, CD169<sup>+</sup> cells were also mixed with B and T lymphocytes. In control Sparc<sup>+/+</sup> and Sparc<sup>−/−</sup> mice and lpr/lpr/Sparc<sup>+/+</sup> mice, CD169<sup>+</sup> cells remained confined to the marginal zone area of the splenic follicles (arrows). Scale bars: 20 μm. **C.** IHC analysis of SPARC expression in the spleens from Sparc<sup>+/+</sup> and lpr/lpr/Sparc<sup>+/+</sup> 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 based on the expression of CD34 and CD16/32 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 CD45+ cells and then the total number of spleen cells (*p<0.05, Student’s t-test). Overall, lpr/lpr/Sparc−/− spleens were characterized by the increased expansion of myeloid precursors and granulocytes compared to 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 to lpr/lpr/Sparc+/+ mice. In lpr/lpr/Sparc−/− spleens, GR-1+ cells (green) made contact with CD5+ B cells (CD19+CD5+ double positive, arrow). 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 h after cell culture (*p<0.05, Student’s 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 granulocyte from lpr/lpr/Sparc−/− mice compared to lpr/lpr/Sparc+/+ or normal mice. H. In situ IHC 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 panels; 50 μm, left panels.

Figure 3. Increased NET formation and ANCA-associated autoimmunity in lpr/lpr/Sparc−/− mice. A. Representative IF analysis revealed more efficient NETosis in spleen-purified PMNs from 3 representative lpr/lpr/Sparc−/− mice (#10, 20, 30) compared to lpr/lpr/Sparc+/+ mice (#1, 2, 3). PMNs were purified from the spleen by
magnetic bead separation and seeded onto poly-D-lysine-coated glass for 16 h. SYTOX green DNA dye was then added to PMNs after fixation with 4% PFA. 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, upper panels; 10 μm, lower panels. C. IHC analysis of frozen spleen sections demonstrated increased expression of MPO in lpr/lpr/Sparc−/− spleens compared to lpr/lpr/Sparc+/+ spleens. H&E staining in serial sections showed concomitant loss of normal spleen structure in lpr/lpr/Sparc−/− mice, as the follicles were not recognizable. In lpr/lpr/Sparc−/− mice, MPO was detectable in NET-like extracellular structures (arrows). D. Serum levels of anti-MPO-ANCA in Fas-mutant mice revealed increased production of MPO autoantibodies in lpr/lpr/Sparc−/− mice. E. Histopathological grading of the renal damage in lpr/lpr/Sparc+/+ and lpr/lpr/Sparc−/− mice (n=7/group); **p<0.01 (1-way ANOVA with posttest Dunn correction); ***p<.001. 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, #3 for lpr/lpr/Sparc+/+ mice and #10, #20, #30 for lpr/lpr/Sparc−/− mice) tested for spontaneous neutrophil NETosis (panel A) demonstrated a strong correlation between increased renal vasculitis and NETosis in lpr/lpr/Sparc−/− mice. Scale bars: 20 μm.

Figure 4. NETosis is regulated by ECM molecules and promotes CD5+ B cell proliferation through NF-kB activation. A. Naïve CD5+ B cells were purified from the spleens of WT mice, labeled with the vital dye CFSE and co-cultured with spleen-purified PMNs from lpr/lpr/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<sup>+</sup>/− and Sparc<sup>−/−</sup> PMNs. B. CD5<sup>+</sup> B cells were purified from the spleens of BALB/c mice, labeled with the vital dye CFSE and co-cultured 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<sup>+</sup> B cells were co-cultured with necrotic or apoptotic PMNs obtained by freeze-thawing or incubation of naïve PMNs with Fas mAb, respectively. CD5<sup>+</sup> B cell proliferation was evaluated at 16 h. C. The activation of Syk and NF-κB in lpr/lpr/Sparc<sup>+</sup>/− and lpr/lpr/Sparc<sup>−/−</sup> mice was evaluated by WB analysis of the extracts of splenic-purified CD19<sup>+</sup> cells. Relative quantitative analysis showed increased phosphorylation of ikBα, which was characteristic of CD19<sup>+</sup> cells from lpr/lpr/Sparc<sup>−/−</sup> spleens. D- E. Activation of NF-κB in CD5<sup>+</sup> B cells co-cultured with NETs. CD5<sup>+</sup> B cells were purified from the spleens of WT mice and co-cultured 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-ikBα and total ikBα (*p<0.05; **p<0.01, Student’s t-test). F. CFSE-labeled splenic CD5<sup>+</sup> B cells were treated with CpG for 48 h, and PMA-treated PMNs (NETotic PMNs) were added for an additional 24 h. CD5<sup>+</sup> B cell proliferation was evaluated at day 3. Representative plots are shown. NETotic PMNs boosted CpG-induced CD5<sup>+</sup> B-cell proliferation in a setting where NETs alone were ineffective at 24 h. G. Confocal microscopy analysis of GR-1, CD5 and collagen IV demonstrated that GR-1<sup>+</sup> cells made contact with collagen type IV fibers, which appeared faint in lpr/lpr/Sparc<sup>−/−</sup> mice. In lpr/lpr/Sparc<sup>−/−</sup> mice, GR-1<sup>+</sup> cells were enriched and made contact with CD5<sup>+</sup> B cells. Scale bars: 20 μm. H. Confocal microscopy analysis of CD29 and collagen IV showed that mesenchymal CD29<sup>+</sup> cells co-localized with collagen type IV fibers in the spleens of lpr/lpr/Sparc<sup>−/−</sup> mice. In the spleens of lpr/lpr/Sparc<sup>−/−</sup> mice, this co-localization was strongly reduced. Scale bars: 20 μm. I. FACS analysis of LAIR-1 was performed on splenocytes, and the histograms show the expression (MFI) of LAIR-1 within the gate of Ly6G<sup>+</sup> cells. Data are expressed as the mean ± SD (p<0.05). J. Confocal microscopy analysis of LAIR-I revealed its expression in myeloid cells (inset) in the spleens of lpr/lpr/Sparc<sup>−/−</sup> and its reduction in the spleens of lpr/lpr/Sparc<sup>−/−</sup> mice. Scale bars: 20 μm. K. Splenic PMNs from lpr/lpr/Sparc<sup>−/−</sup> mice were seeded onto...
collagen matrix and demonstrated reduced NET formation. PMNs purified from the spleens of 3 different mice were seeded onto poly-D-lysine or collagen-coated glass, and the spontaneous NETosis 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 were seeded onto collagen type IV matrix and stimulated with IFNγ+C5a or PMA, and NET formation was compared in LAIR-1-siRNA-transfected and control-transfected granulocytes. Scale bars: 10 μm. N. NET formation was quantified by measuring the nuclear area of 200 cells/sample. Additional siRNA data are shown in Supplementary Fig. S6F-H).

**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 to other histotypes (left panel). Sparc gene expression analysis in purified neoplastic and control B cell populations was comparable among various B cell populations (right panel). B. Representative IHC analysis of SPARC expression in stromal cells was performed using computer-assisted software analysis (see Material and Methods) in 1 case each of DLCBL 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 panel B. 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 to 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 (50° percentile high vs. 50° 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 biological process categories for genes differentially expressed in B-NHL samples grouped according to SPARC gene expression (50° percentile high vs. 50° 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 (50° percentile high vs. 50° 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 (50° percentile high in red vs. 50° percentile low in blue). D. The scatter plot shows the distribution of selected inflammatory genes that were differentially expressed in B-NHLs according to SPARC gene expression (50° percentile high vs. 50° percentile low). Each gene is colored according to its functional role in inflammation. Red lines indicate expression values = 0 and define 4 quadrants according to gene expression levels. The considered genes were significantly associated with a specific quadrant based on their biological function (chi-square, p=0.05). E. The box plot represents the distribution of LAIR1 gene expression in B-NHL samples divided in two groups based on SPARC gene expression (50° percentile high vs. 50° 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 LAIR1 gene expression distribution that was greater than that observed in SPARC-low samples. F. Pie chart representation of gene ontology biological process categories for genes co-regulated with LAIR1 in B-NHL samples according to SPARC gene expression (50° percentile high vs. 50° 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 to 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, upper panels; 10 μm, lower panels.

**Figure 7. Stromal remodeling in early stage follicular B cell lymphomas.** **A.** Bcl-2 immunostaining was used to define reactive and transformed follicles 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, upper panels; 20 μm, lower panels. **B-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, upper panels; 10 μm, lower panels. Scale bars in C: 20 μm. **D.** CD23 IHC identified a dense or irregular and dispersed FDC network in reactive vs. transformed follicles. **E.** IHC analysis of SPARC expression revealed that SPARC was expressed by GC-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.
Fig. 2

A

B

C

D

E

F

G

H

Il-6G/CD19/CD5

IL-21

SPARC

Author Manuscript Published OnlineFirst on November 4, 2013; DOI: 10.1158/2159-8290.CD-13-0276

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Fig. 5

A. Whole tissue and purified lymphoid cells showing box plots for SPARC gene expression values.

B. Comparison of original and segmented images for DLBCL and CLL, with labeled percentages of SPARC+ cells: 9.1% and 1.4%, respectively.

C. Bar graph showing percentage of SPARC+ cells on TMA for different cell types, with a significant difference indicated by an asterisk (*) for one of the groups.

D. Images of tissue sections stained for Gomori, Trichrome, Coll. I, Coll. IV, and Sparc, highlighting different markers and cellular structures.
Fig. 6

(A) Box plots showing normalized intensity values for SPARC High and SPARC Low.

(B) Pie chart depicting various biological processes.

(C) Heatmap illustrating gene expression patterns.

(D) Scatter plot showing gene expression analysis.

(E) Box plot for LAIR1 expression.

(F) Pie chart highlighting different biological processes.

(G) Immunohistochemical images of CLL and DLBCL in spleen and lymph node.

(H) Immunohistochemical images of melanoma.
Defective stromal remodeling and neutrophil extracellular traps in lymphoid tissues favor the transition from autoimmunity to lymphoma

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_Cancer Discovery_ Published OnlineFirst November 4, 2013.

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