SIGLEC-G deficiency increases susceptibility to develop B-cell lymphoproliferative disorders

Giorgia Simonetti,[1,2]* Maria Teresa Sabrina Bertilaccio,[1]* Tania Veliz Rodriguez,[1] Benedetta Apollonio,[1] Antonis Dagklis,[1] Martina Rocchi,[3] Anna Innocenzi,[3] Stefano Casola,[4] Thomas H. Winkler,[5] Lars Nitschke,[6] Maurilio Ponzoni,[3,7] Federico Caligaris-Cappio,[1,7,8] and Paolo Ghia[1,7,8]

[1]Division of Molecular Oncology, San Raffaele Scientific Institute, Milano, Italy; [2]Università degli Studi di Milano-Bicocca, Milano, Italy; [3]Pathology Unit, Unit of Lymphoid Malignancies, San Raffaele Scientific Institute, Milano, Italy; [4]The Institute of Molecular Oncology (IFOM) of the Italian Foundation for Cancer Research (FIRC), Milano, Italy; [5]Department of Biology, Nikolaus Fiebiger Center for Molecular Medicine, Friedrich Alexander University Erlangen-Nuremberg, Erlangen, Germany; [6]Department of Biology, Friedrich Alexander University Erlangen-Nuremberg, Erlangen, Germany; [7]Clinical Unit of Lymphoid Malignancies, Department of Oncology, San Raffaele Scientific Institute, Milano, Italy; and [8]Vita-Salute San Raffaele University, Milano, Italy

**GS and MTSB contributed equally to this work.*

ABSTRACT

The sialic-acid-binding immunoglobulin-like lectin SIGLEC-G is a negative regulator of B-cell receptor-mediated calcium signaling. Its deficiency leads to reduced turnover and increased proliferation and survival of murine B-1a cells. *Siglecg*$^{-/-}$ mice show a premature expansion of polyclonal CD5^{+} B cells in the spleen and the peritoneal cavity. Here we studied the fate of B lymphocytes in *Siglecg*$^{-/-}$ mice over time. We demonstrate that in aging animals SIGLEC-G deficiency promotes progressive accumulation of monoclonal B lymphocytes and increases the susceptibility to develop B-cell lymphoproliferative disorders. Lymphoid tumors arising in aged *Siglecg*$^{-/-}$ mice are monoclonal and histologically heterogeneous as they include diffuse large B-cell lymphoma, follicular lymphoma, and medium-to-large B-cell monomorphic lymphoma but surprisingly not chronic lymphocytic leukemia. The tumors express high levels of BCL-2 and are transplantable. In keeping with these findings we have also observed a remarkable down-regulation of the human ortholog SIGLEC10 in human B-cell lymphoma and leukemia cell lines. Taken together, these observations indicate that the down-regulation of negative B-cell receptor regulators such as SIGLEC-G/SIGLEC10 may represent another mechanism relevant to the pathogenesis of B-cell lymphomas.

Introduction

The murine sialic-acid-binding immunoglobulin-like lectin G (SIGLEC-G) is a type 1 cell membrane receptor expressed primarily on B lymphocytes and dendritic cells and involved in maintaining B-cell tolerance and homeostasis.[1-7] In human B lymphocytes a similar function is exerted by the ortholog, SIGLEC10, a member of the CD33-related family of SIGLECs.[8,9] Both SIGLEC-G and SIGLEC10 are also able to suppress Toll-like receptor-mediated activation of dendritic cells, thereby dampening the pro-inflammatory response induced by danger-associated molecular patterns.[10]

SIGLEC-G is expressed throughout the B-cell lineage including both CD5^{+} B-1a and the conventional CD5^{-} B-2 cells.[1] In peritoneal B220^{low}CD5^{+} B-1 cells it exerts negative control on B-cell receptor (BCR)-mediated calcium signaling and B-cell expansion. *Siglecg*$^{-/-}$ mice at the age of 8- to 12-weeks show an enlarged IgM^{+}CD5^{+} polyclonal B-cell population in the spleen and especially in the peritoneal cavity.[1,11] *Siglecg*$^{-/-}$ peritoneal B-1a cells injected into chimeric mice have both an increased rate of proliferation and an improved capacity for survival as compared to wild-type B-1a cells.[2] In addition, *Siglecg*$^{-/-}$ mice have a higher titer of serum IgM and with age have an increased production of autoantibodies,[1] although they do not develop an overt autoimmune disease.[1,3]

B-1 cells are the predominant B-cell population in the peritoneal and pleural cavities, represent a small percentage of the splenic B-cell compartment, which is dominated by B-2 cells, and are commonly increased in mice prone to autoimmune disorders, such as New Zealand Black (NZB) and New Zealand Black x New Zealand White (NZB/W) mice.[12] They also account for the malignant B-cell expansions observed in several lymphoma/leukemia mouse models such as chronic lymphocytic leukemia (CLL) *Eμ-TCL1* transgenic,[13] *13q14-minimal deleted region* (*MDR*)$^{-/-}$ [14] and *common deleted region* (*CDR*) conditional knock-out[15] mice and the diffuse large B-cell lymphoma (DLBCL) model *Eμ-BRD2* transgenic.[16] These mice develop a B-cell hyperplasia of the CD5^{+} lineage in the peritoneal cavity that evolves into a frank B-cell leukemia/lymphoma with variable penetrance (from 10% annually in *Eμ-BRD2* mice to 100% in *Eμ-TCL1* mice) and at different ages (from 7 months in *Eμ-BRD2* mice to 12-18 months in CLL mouse models). Such heterogeneous evolution indicates that the expansion and progression of malig-

 doi:10.3324/haematol.2013.100230
The online version of this article has a Supplementary Appendix.
Manuscript received on November 5, 2013. Manuscript accepted on May 19, 2014.
Correspondence: caligaris.federico@hsr.it

nant clones are explained by the interplay of the transforming genetic lesions and the cellular and molecular interactions that occur in the tumor microenvironment.[17-20] Within this general context, stimulation through the BCR and other receptors involved in the immune response appears pivotal to activate and modulate selective intracellular pathways that lead to increased malignant cell proliferation and survival.[21,22] It may, therefore, be asked whether there is any role for negative regulators of BCR stimulation in the pathogenesis of B-cell lymphomas.

The phenotype of *Siglecg*$^{-/-}$ mice prompted us to investigate the B-cell expansions that occur in aging *Siglecg*$^{-/-}$ mice. We herein demonstrate that over time the lack of the inhibitory receptor SIGLEC-G favors the development of B-cell lymphoproliferative disorders which are monoclonal, transplantable and histologically heterogeneous. They include DLBCL, follicular lymphoma, medium-to-large B-cell monomorphic lymphoma and atypical lymphoproliferations, but surprisingly not CLL. In addition we have observed that SIGLEC10, too, is commonly down-regulated in human B-cell lymphoma/leukemia cell lines. These findings indicate that the down-regulation of negative regulators of BCR signaling might represent another mechanism relevant to the pathogenesis of B-cell lymphoproliferative disorders.

Methods

Mice

Siglecg$^{-/-}$ mice (deriving from BALB/c ES cells) have been generated previously.[1] Wild-type BALB/c mice were supplied by Charles River Laboratories. All mice were housed and bred in a specific pathogen-free animal facility, treated in accordance with the USA Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Ethical Committee at San Raffaele Scientific Institute. The transplantation protocol is described in the *Online Supplementary Methods*.

Murine organ and cell preparation

Siglecg$^{-/-}$ and BALB/c control mice were sacrificed at 5, 10, 16 and 20 (-or more) months of age. Organs (spleen, lymph nodes, femora, kidneys, lungs, liver) were collected. Cells from spleen, peritoneal exudate and peripheral blood were analyzed by flow cytometry, as described in the *Online Supplementary Methods*. Calcium levels in murine spleen cells were measured as described in the *Online Supplementary Methods*.

Histopathology and immunohistochemistry

Collected tissues were fixed in 4% formalin for 12 h, then embedded in paraffin. Five-micron thick sections were cut and stained with hematoxylin and eosin. Microscopic specimens were blindly evaluated by a hemato-pathologist (MP). The protocol and reagents used for immunohistochemical studies are reported in the *Online Supplementary Methods*.

IGHV-D-J gene rearrangement analysis

For analysis of *immunoglobulin heavy chain gene* (*IGHV*) rearrangements, splenic CD19$^+$ cells were isolated using the EasySep Mouse B-Cell Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada). Genomic DNA was extracted from bone marrow, peritoneal exudate (in 1 case from mesenteric lymph nodes) and CD19$^+$-enriched spleen cells according to the QIAmp® DNA mini kit (QIAGEN, Düsseldorf, Germany) protocol and from whole blood samples using a Maxwell® 16 DNA Purification Kit and Maxwell® Instrument (Promega Corporation, Madison, WI, USA). The protocol and primers are reported in the *Online Supplementary Methods*.

Cells

Cell lines were obtained from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DMSZ, Braunschweig, Germany) and American Type Culture Collection (ATCC, Washington, DC, USA). Primary samples were obtained from healthy donors and CLL patients after informed consent as approved by the Institutional Ethical Committee (TONSIL-Prot-2012 and VIVI-CLL, respectively) of San Raffaele Scientific Institute (Milan, Italy) in accordance with the Declaration of Helsinki. Protocols and reagents used for cell culture and purification and for quantitative reverse-transcription polymerase chain reaction and western blot analysis are reported in the *Online Supplementary Methods*.

Statistical analysis

Statistical analysis was performed using the Student *t*-test. Data are expressed as the mean value ± standard deviation and P values <0.05 are considered statistically significant. The incidence of B-cell lymphoproliferative disorders was evaluated using a two-sided Fisher test. Survival curves were compared using the log-rank test.

Results

Siglecg$^{-/-}$ mice develop a progressive expansion of B cells

Given the previous report of an expansion of B-1a cells in 2- to 3-month old *Siglecg*$^{-/-}$ mice,[1] we performed a detailed analysis of B lineage cells in *Siglecg*$^{-/-}$ mice over time. Animals and age-matched controls were sacrificed at 5, 10 and 16 months of age and flow cytometric analysis of CD19$^+$ cells and CD19$^+$CD5$^+$ cells was carried out in samples from spleen, peripheral blood and peritoneal exudate. The percentages of CD19$^+$CD5$^+$ B cells in different tissues of 5-month old *Siglecg*$^{-/-}$ mice were higher than those in age-matched wild-type animals (Figure 1A and *Online Supplementary Table S1*). At 10 months, this difference was no longer evident in the spleen, although we detected a significant increase in both relative and absolute numbers of total CD19$^+$ splenocytes (Figure 1B and *Online Supplementary Tables S1* and *S2*).

The histopathological evaluation of spleen, lymph nodes, bone marrow, lung, kidney and liver showed a normal architecture of lymphoid and extralymphoid organs at 5 months of age (*data not shown*). A focal expansion of the splenic white pulp area due to accumulation of B220$^+$ B lymphocytes was occasionally observed at 10 and 16 months of age (*data not shown*).

Polymerase chain reaction analysis of *IGH* gene rearrangements revealed a polyclonal pattern of B-cell rearrangements in the spleen, peripheral blood and peritoneal exudate up to 16 months of age. In only one case, a clonal rearrangement was detected in the spleen and peripheral blood of a 16-month old SIGLEC-G-deficient mouse, whose histopathological analysis disclosed signs of an atypical lymphoproliferative process (see Figure 2B for a representative case of atypical lymphoproliferative process).

We also analyzed T lineage cells over time, although T-

cell numbers were reportedly stable in 2- to 3-month old *Siglecg*$^{-/-}$ mice.[1] In accordance with this previous report, the percentage of splenic CD3$^+$ T cells was unmodified at 5, 10 and 16 months of age. However, when we compared T-cell subsets in the spleen of *Siglecg*$^{-/-}$ and age-matched control mice, we observed a progressive age-related increase in the percentage of CD4$^+$ T cells (Figure 1C) along with a concomitant decrease of CD8$^+$ T cells (Figure 1D).

Aged Siglecg$^{-/-}$ *mice develop B-cell lymphoproliferative disorders*

To determine whether SIGLEC-G deficiency affected the overall survival of animals, we analyzed a group of *Siglecg*$^{-/-}$ mice and compared them to wild-type mice. The absence of the inhibitory receptor significantly shortened the animals' life span (P=0.02, Figure 2A).

To investigate whether B-cell expansions could account for the reduced survival of *Siglecg*$^{-/-}$ mice, we assessed the histopathology of the lymphoid tissues of *Siglecg*$^{-/-}$ mice at the age of 20 months or beyond (aged *Siglecg*$^{-/-}$ mice). Seventeen of 20 (85%) aged *Siglecg*$^{-/-}$ mice had a lymphoproliferative disorder that involved spleen and lymph nodes, but generally spared the bone marrow. According to the histopathological analysis, lymphoproliferations arising in the absence of SIGLEC-G were classified as DLBCL, follicular lymphoma, medium-to-large B-cell monomorphic lymphoma or atypical lymphoproliferative process.

In 13/17 (76%) affected animals we observed a progressive expansion of the splenic white pulp up to an effacement of the tissue architecture. Microscopic and immunohistochemical examination revealed that the expanded white pulp areas were composed of small B lymphocytes admixed with a variable prevalence of large blast-like cells that gave rise to a spectrum of B220$^+$ B-cell malignancies (Table 1; Figure 2B), including DLBCL (6/17, 35%), follicular lymphoma (4/17, 23.5%) and medium-to-large B-cell monomorphic lymphoma (3/17, 18%). No expression of CD5 antigen was detected in splenic B lymphocytes. In addition to the B-cell marker B220, the tumor cells expressed BCL-2, but showed weak (in follicular lymphoma) or no immunoreactivity for BCL-6 (Figure 2B). Variable levels of immunoreactivity for MUM1 were observed in the different tumor types, while Ki-67 was generally highly expressed and diffusely distributed [mostly in DLBCL and medium-to-large B-cell monomorphic lymphoma cases as compared to the negative control, in which the expression of Ki-67 was restricted to the germinal center (GC)-area, Figure 2B]. Multiple small foci of neoplastic B lymphocytes were also observed in the liver, lung and kidney, where small cells were predominant (*data not shown*).

In 4/17 (23.5%) affected *Siglecg*$^{-/-}$ mice, the histological pattern was consistent with an atypical lymphoproliferative process, characterized by discrete amounts of large B220$^+$ B cells (also negative for the expression of CD5) which were not confluent nor formed solid sheets thus lacking the conventional morphological features that

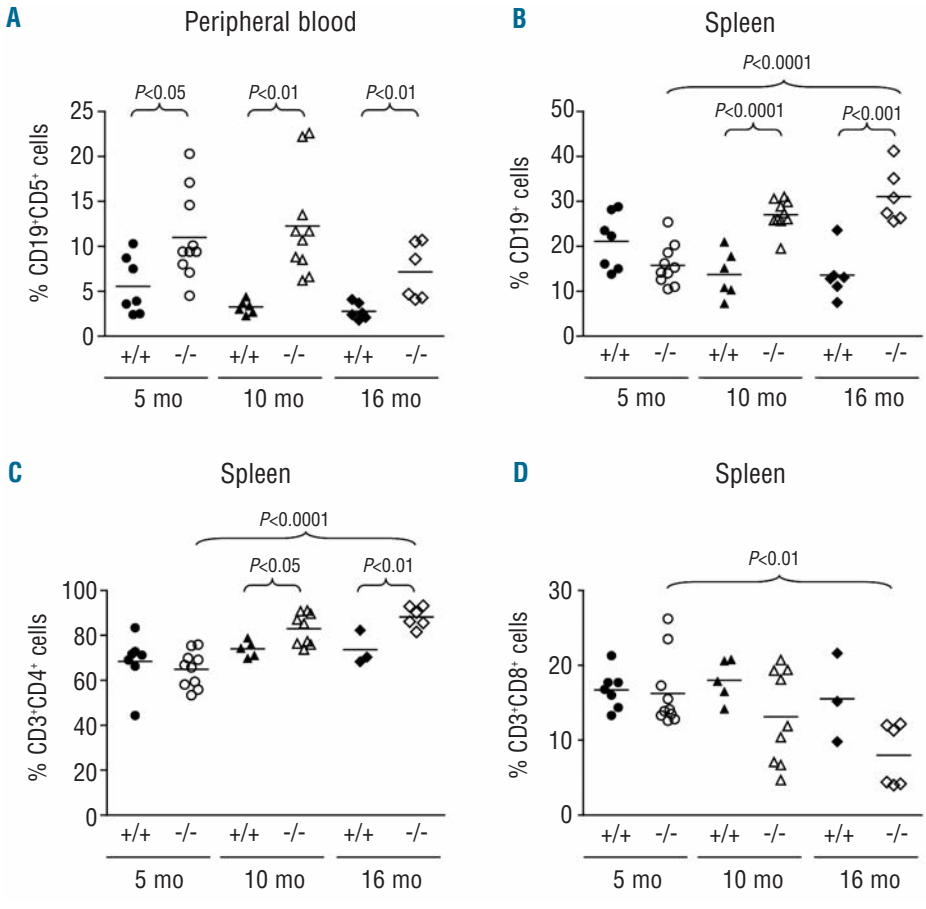

Figure 1. Evolution of B-1a, total B cells and T lineage cells in *Siglecg*$^{-/-}$ mice. (A-B) Flow cytometric analysis of peripheral blood and splenic B cells of 5-, 10- and 16-month old *Siglecg*$^{-/-}$ mice and age-matched control mice (n=6-10). Accumulation of (A) B-1a cells (CD19$^+$CD5$^+$) in the peripheral blood and of (B) total B cells (CD19$^+$) within the spleen of *Siglecg*$^{-/-}$ mice over time. (C-D) Cells from 5-, 10- and 16-month old *Siglecg*$^{-/-}$ mice and age-matched control mice (n=3-10) were analyzed by flow cytometry to define the percentage of (C) CD3$^+$CD4$^+$ and (D) CD3$^+$CD8$^+$ T-cell populations. Each symbol in the dot plots represents one mouse and the horizontal line indicates the mean value (+/+: wild-type, −/−: *Siglecg*$^{-/-}$ mice). Statistical significance was analyzed by the *t*-test.

allow the diagnosis of overt lymphoma (Figure 2B). At immunohistochemical examination these cells showed low expression of BCL-2 and Ki-67 and no immunoreactivity for BCL-6 or MUM1 (Figure 2B). Overall, only 3/20 (15%) aged *Siglecg*$^{-/-}$ mice displayed a normal architecture of the lymphoid organs.

To determine the clonality of the expanded B-cell populations, we analyzed the *IGH* gene rearrangements using a nested polymerase chain reaction approach followed by DNA sequencing, which showed the presence of monoclonal rearrangements in 16/17 affected *Siglecg*$^{-/-}$ mice (Table 1), confirming the clonal origin of the B-cell populations. The rearrangements were detected mainly in splenic and/or peripheral blood samples (only in a few cases in the bone marrow). Peritoneal exudates and lymph nodes were not analyzed (except for the mesenteric lymph node of *Siglecg*$^{-/-}$ mouse #330, which was analyzed because of unavailability of DNA from the spleen). The monoclonal rearrangements carried genes belonging to the *IGHV1*, *IGHV5* and *IGHV14* gene subgroups. Most *IGHV* genes were mutated, consistent with a GC or post-GC B-cell origin of the lymphoproliferations (Table 1).

As expected,[23,24] aged wild-type mice also developed spontaneous B-cell lymphoproliferative disorders, but at a

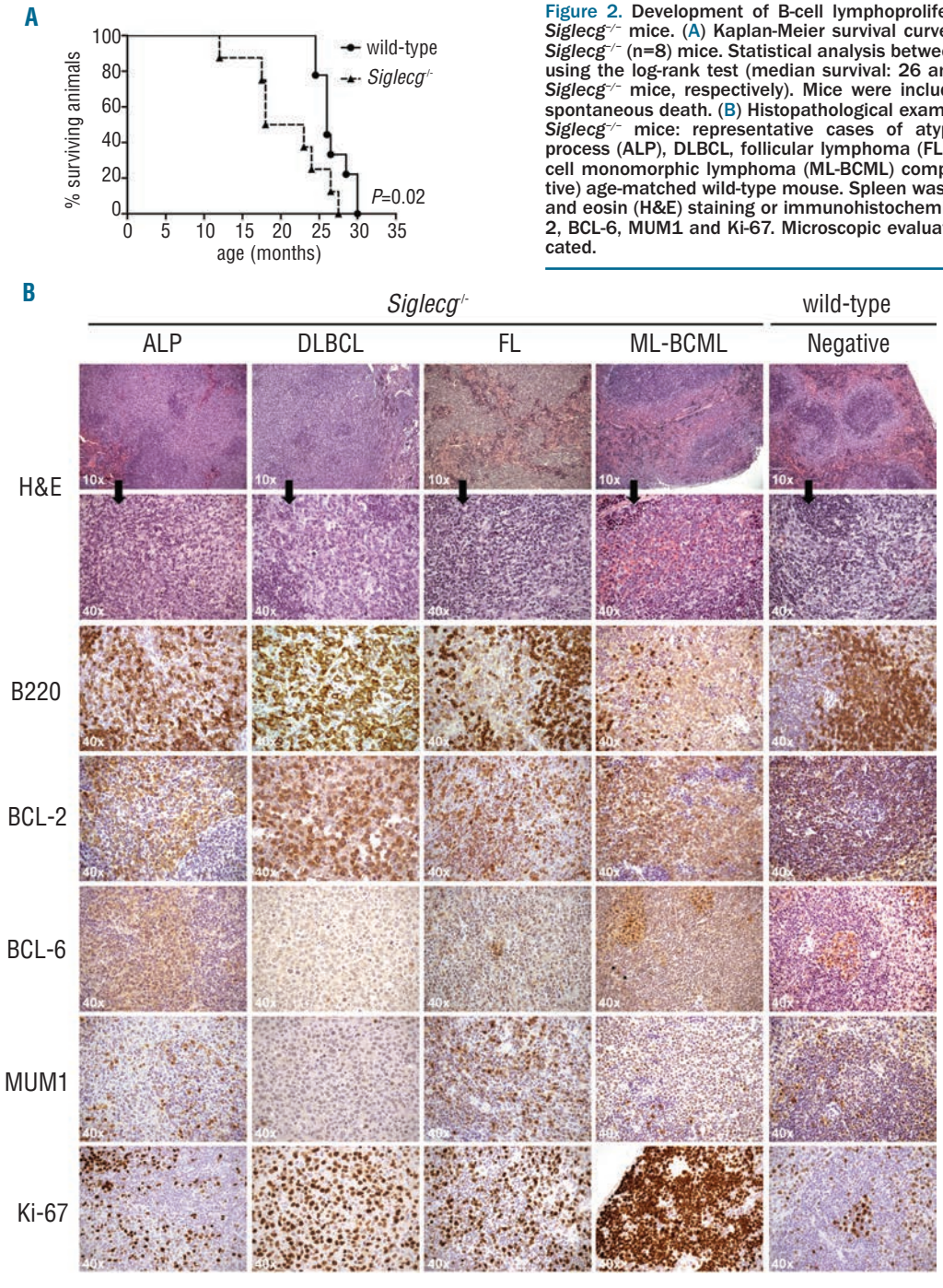

Figure 2. **Development of B-cell lymphoproliferative disorders in aged *Siglecg*$^{-/-}$ mice. (A) Kaplan-Meier survival curves of wild-type (n=9) and *Siglecg*$^{-/-}$ (n=8) mice. Statistical analysis between groups was performed using the log-rank test (median survival: 26 and 20.5 for wild-type and *Siglecg*$^{-/-}$ mice, respectively). Mice were included in the analysis after spontaneous death. (B) Histopathological examination of spleen of aged *Siglecg*$^{-/-}$ mice: representative cases of atypical lymphoproliferative process (ALP), DLBCL, follicular lymphoma (FL) and medium-to-large B-cell monomorphic lymphoma (ML-BCML) compared to a healthy (negative) age-matched wild-type mouse. Spleen was analyzed by hematoxylin and eosin (H&E) staining or immunohistochemical stains for B220, BCL-2, BCL-6, MUM1 and Ki-67. Microscopic evaluation: 10× or 40×, as indicated.**

significantly lower rate, being observed in 8/21 (38%) cases (P=0.004). The histopathological evaluation was consistent with the diagnosis of DLBCL in two cases and follicular lymphoma in three cases. Three aged wild-type mice displayed histological features of an atypical lymphoproliferative process. The remaining 13/21 (62%) control mice did not show any sign of disease (negative, Figure 2B).

As SIGLEC-G is a negative regulator of BCR-mediated calcium signaling[1] and its deficiency has been associated with up-regulation of the calcium-regulated transcription factor NFATc1 in peritoneal B-1a cells,[2] we analyzed both basal calcium levels and NFATc1 protein expression in splenic B cells of aged wild-type and SIGLEC-G-deficient mice (the latter showing a histopathological pattern consistent with the development of CD5$^-$ B cell lymphoproliferative disorders). We observed higher basal levels of intracellular calcium (Figure 3A) and up-regulation of NFATc1 expression (Figure 3B) in *Siglecg*$^{-/-}$ B cells from mice with lymphoproliferative disorders as compared to wild-type B cells.

B-cell lymphoproliferative disorders arising in aged Siglecg$^{-/-}$ mice are transplantable

To assess the tumorigenic potential of the expanded B-cell populations observed in aged *Siglecg*$^{-/-}$ mice, we conducted transplantation experiments of B cells purified from the spleen of four *Siglecg*$^{-/-}$ mice carrying a lymphoma or an atypical lymphoproliferation (#83, 84, 93, 96; Table 1 and *Online Supplementary Table S3*). Ten million B cells were injected intraperitoneally into syngeneic BALB/c mice and the appearance and expansion of a clonal CD19$^+$ population in the peripheral blood was monitored by monthly blood drawing. In the mice transplanted from three of four *Siglecg*$^{-/-}$ donors (#83, 84, 96; *Online Supplementary Table S3*), flow cytometric analysis revealed a progressive increase of circulating small and large CD19$^+$ cells, which phenotypically resembled donor cells (*data not shown*). Monoclonal *IGHV* gene rearrangements became detectable in the peripheral blood of the recipient mice 3 months after transplantation (*Online Supplementary Table S3*). Sequence analysis confirmed the identity with the clone from the donor mice. All transplanted animals were sacrificed when they developed relevant signs/symptoms of illness (6 to 19 months after transplantation; Figure 4A and *Online Supplementary Table S3*). The engraftment was also confirmed by histopathological and immunohistochemical evaluation which revealed a subversion of the tissue organization in the spleen (Figure 4B) and lymph nodes due to the presence of atypical, intermediate- to large-sized B lymphocytes.

As a control, total B cells purified from the spleen of an aged *Siglecg*$^{-/-}$ mouse without any evidence of disease (#95; Table 1) were injected into BALB/c recipient mice. Monoclonal disease was not detected in either the peripheral blood or in the tissues.

To assess whether *Siglecg*$^{-/-}$ B cells may be serially transplantable, BALB/c mice were challenged with 10^7 peritoneal cells (n=3 recipient mice) or splenic B cells (n=2 recipient mice) from a BALB/c mouse primary transplant (animal #1 from *Siglecg*$^{-/-}$ #84, follicular lymphoma, Table

Table 1. Biological, molecular and histological features of B-cell lymphoproliferative disorders arising in *Siglecg*$^{-/-}$ mice.

Mouse	Diagnosis	Tissue with clonal rearrangement	*IGHV* gene	% of identity to germline (n. of mutations)	*IGHD* gene	*IGHJ* gene	Transplant experiments
26	ML-BCML	pb,sp,bm	14-3*02	100 (0)	4-1*01	2*01	
27	negative	pb					
39	FL	sp	1S81*02	96.9 (9)	na	4*01	
42	DLBCL	pb,sp	1-7*01	98.3 (5)	1-1*01	4*01	
44	ML-BCML	pb,sp	5-17*02	100 (0)	2-13*01	3*01	
47	negative						
70	ALP	pb		99.7 (1)	6-1*01	2*01	
83	FL	pb,sp	5-12*02	91.3 (25)	2-3*01	3*01	I
84	FL	pb,sp	1S22*01	99.0 (3)	1-1*01	3*01	I , II
93	DLBCL	nd					I
95	negative						I
96	ALP	pb	5-12*-1*01	98.3 (5)	1-1*02	4*01	I
105	ALP	sp	14*3*02	98.3 (5)	2-4*01	3*01	
107	ML-BCML	sp	5-6*01	99.0 (3)	2-3*01	2*03	
113	DLBCL	sp	5-09*03	100 (0)	1-3*01	2*01	
135	ALP	pb†	5-09*02	99.7 (1)	2-1*01	1*03	
319	FL	pb,sp,bm	1S81*02	96.9 (9)	4-1*02	3*01	
329	DLBCL	sp	1-63*02	95.1 (14)	2-3*01	3*01	
330	DLBCL	pb,mln†	5-17*02	99.3 (2)	1-1*01	1*01	
331	DLBCL	pb,sp	1-14*01	96.9 (9)	1-1*01	3*01	

†*sp not available; ML-BCML: medium-to-large B-cell monomorphic lymphoma; FL: follicular lymphoma; ALP: atypical lymphoproliferative process; sp: spleen; bm: bone marrow; mln: mesenteric lymph nodes; nd: not detected; na: no* IGHD *gene could be assigned.*

1 and *Online Supplementary Table S3*). At 40 days, clonal *IGH* gene rearrangements and flow cytometric analysis revealed an expansion of B cells in the peripheral blood of all the recipient mice that died 4-7 months after transplantation (Figure 4A). In all mice, histopathological and immunohistochemical evaluation showed a massive expansion of large B cells in both lymphoid and extralymphoid compartments (*data not shown*).

SIGLEC10 is down-regulated in human B-cell lymphomas and leukemias

As these findings demonstrate that SIGLEC-G deficiency facilitates the development of B-cell lymphoproliferative disorders in the mouse, we investigated SIGLEC10 expression in human B lymphoid malignancies. We analyzed B-cell lymphoma and leukemia cell lines representing mature B cells at different stages of differentiation (GC DLBCL: DHL-4, DHL-16, H2, RL; Burkitt lymphoma: Raji, Ramos, Jijoye, Daudi; CLL: MEC1, MEC2, JVM-2, JVM-3; hairy cell leukemia: HC-1). Quantitative reverse transcriptase polymerase chain reaction analysis revealed a drastic reduction in the mRNA level of SIGLEC10 in all cell lines as compared to those in GC and total B cells purified from tonsils and total B cells from the peripheral blood of healthy donors (Figure 5A). At a protein level, very little if any SIGLEC10 expression was detectable in most cell lines [SIGLEC10 expression was only observed in ABC DLBCL cell lines (31.6%, 63.9% and 82.9% of Toledo, Riva and DHL-2, respectively; *data not shown*)], while SIGLEC10 protein was highly expressed in B cells obtained from healthy donors (Figure 5B), being present in both $CD5^+$ and $CD5^-$ B cells (from tonsils; *data not shown*) and in the vast majority of primary CLL samples (from both peripheral blood and bone marrow) analyzed (16/18; *data not shown*).

Discussion

SIGLEC-G, the murine ortholog of human SIGLEC10, is a negative regulator of BCR-mediated calcium signaling whose disruption leads to an early (2-3 months of age) expansion of $CD5^+$ B-1 cells in the spleen and peritoneal cavity.[1,11] The possibility that the accumulation of *Siglecg*$^{-/-}$

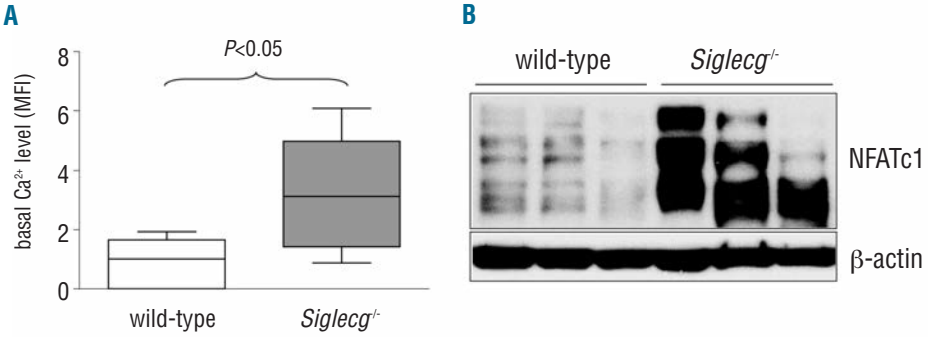

Figure 3. Enhanced calcium signaling in B lymphocytes from aged *Siglecg*$^{-/-}$ mice that developed B-cell lymphoproliferations. (A) Basal levels of intracellular calcium in B cells (gated as $CD19^+$) from aged *Siglecg*$^{-/-}$ (n=6) and wild-type (n=5) mice. Data are expressed as a whisker plot. Statistical significance was analyzed by the *t*-test. (B) Extracts from B cells of aged *Siglecg*$^{-/-}$ and wild-type mice were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblotting with NFATc1-specific antibody or β-actin-specific control antibody (three representative samples for each genotype are shown).

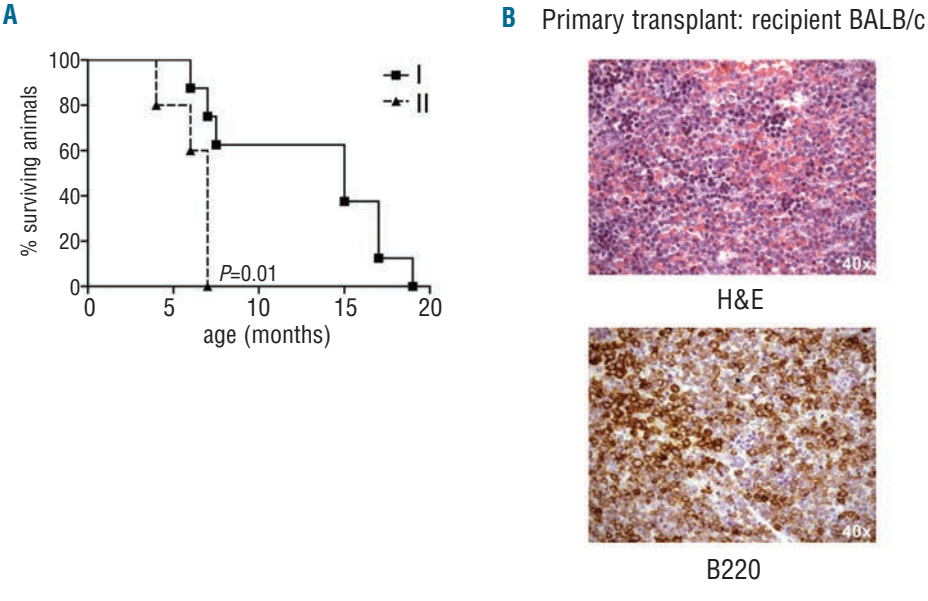

Figure 4. Engraftment of *Siglecg*$^{-/-}$ B-cell lymphoproliferative disorders. (A) Kaplan-Meier survival curves of mice that received *Siglecg*$^{-/-}$ B-cell lymphoproliferation and lymphoma transplants. Mice were included in the analysis after spontaneous death or when killed because of signs/symptoms of illness. Squares and triangles indicate the primary (I) and secondary (II) transplants, respectively. Statistical analysis between groups was performed using the log-rank test (median survival: 15 months for primary transplant, 7 months for secondary transplant). (B) Histopathological examination of spleen of BALB/c mice injected with *Siglecg*$^{-/-}$ B cells (primary transplant, one representative animal): hematoxylin and eosin (H&E) staining and immunohistochemical stain for B220; microscopic evaluation 40×.

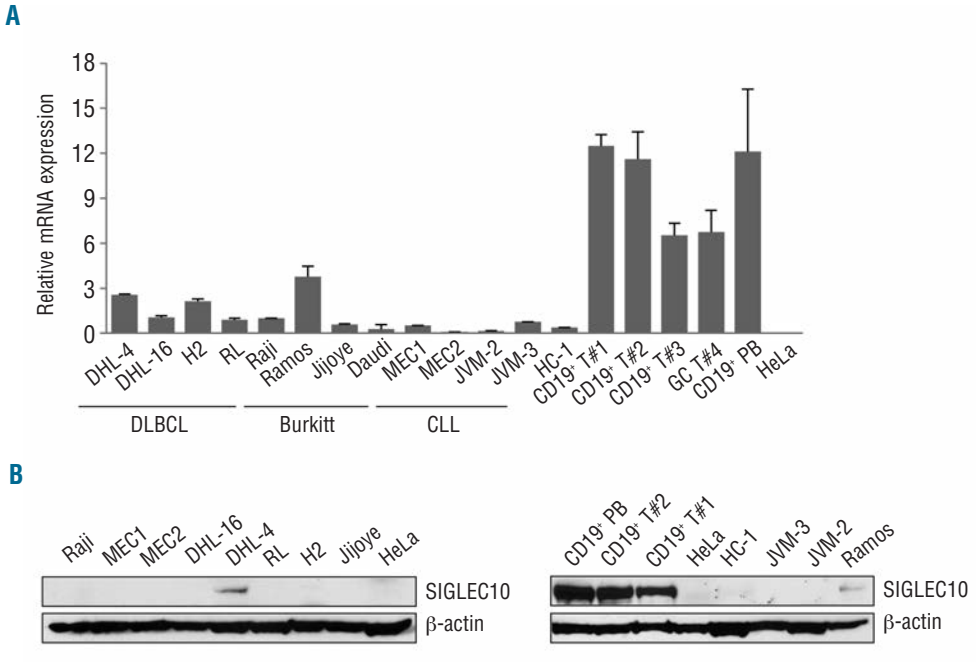

Figure 5. Downregulation of SIGLEC10 expression in human B-cell lymphoma and leukemia cell lines. (A-B) Human B-cell lymphoma/leukemia cell lines, primary B cells and GC B cells purified from tonsils (T) and primary B cells isolated from peripheral blood (PB) of healthy donors were analyzed for the expression of SIGLEC10. (A) Relative mRNA expression of SIGLEC10. The expression of SIGLEC10 was normalized to GAPDH and the results presented as a ratio between normalized SIGLEC10 expression in the target samples and in Raji cells (calibrator sample). Data shown are the average of two independent experiments ± standard deviation. (B) Western blot analysis of SIGLEC10 and β-actin expression on extracts from cell lines and primary cells.

B-1a cells, the major source of natural antibodies, might lead to an autoimmune disease has been ruled out.[1,3] We aimed to investigate whether the *Siglecg*$^{-/-}$ phenotype might favor a monoclonal expansion or even the development of an overt lymphoid malignancy.

The natural history of aging *Siglecg*$^{-/-}$ mice revealed an initial increase of CD5^{+} B cells in several tissues followed over time by an expansion of polyclonal CD5^{-} B cells which ultimately became monoclonal. In over 76% of the animals ≥ 20 months of age a wide range of different histologically evident lymphoproliferations emerged, including follicular lymphoma and DLBCL, but not CLL as one would have expected considering the initial expansion of CD5^{+} B-1 cells in younger mice. Of note, although *Siglecg*$^{-/-}$ mice initially show a B-1 cell phenotype, SIGLEC-G-deficiency affects both CD5^{+} and CD5^{-} splenic B cells and indeed SIGLEC-G can also suppress B-2 cell responses, as shown by overexpression in cell lines[1] or by high affinity SIGLEC-G ligands.[7] Furthermore, deficiency of SIGLEC-G can lead to an earlier onset of autoimmune diseases, involving earlier generation of IgG autoantibodies, without contribution of B-1 cells.[25] A plausible interpretation of the CD5^{-} phenotype of the lymphoproliferations arising in *Siglecg*$^{-/-}$ mice must take into account that CD5, too, negatively regulates antigen receptor-mediated growth signals in B-1 cells.[26-28] One may, therefore, speculate that the absence of CD5 expression in the B-cell disorders arising in *Siglecg*$^{-/-}$ mice may synergize with SIGLEC-G deficiency and favor enhanced B-cell proliferation and resistance to apoptosis.[2,26] The loss of inhibitory signals would allow B lymphocytes to undergo an unabated antigenic stimulation through the BCR, which is in line with the observed increase in the basal level of intracellular calcium that may involved in the development of B-cell lymphoproliferative disorders in *Siglecg*$^{-/-}$ mice. The impaired ability to dampen calcium signaling may be associated with NFATc1 up-regulation in the splenic B-cell compartment of aged SIGLEC-G-deficient mice, as has been previously suggested for peritoneal B-1a cells of younger mice.[2] NFATc1 up-regulation has been observed in both CD5^{+} and CD5^{-} B cells from *Siglecg*$^{-/-}$ mice, suggesting a general effect of SIGLEC-G-deficiency on the B-cell transcriptional program (*data not shown*). The heterogeneity of the lymphoma histology observed might reflect the different B-cell developmental or maturation stages (GC or post-GC) on which such stimulation acts in different animals. Alternatively, the different histotypes could be due to the progressive accumulation of different driver genetic aberrations, as suggested by the long latency of tumor onset. Genome-wide studies will allow dissection of the genetic differences and peculiarities of each type of B-cell lymphoma arising in *Siglecg*$^{-/-}$ animals.

The monoclonal nature of these B-cell lymphoproliferative disorders was confirmed by sequencing the *IGH* gene rearrangement in virtually all cases. As for the human diagnostic counterpart, the method of detection used here for the BALB/c mouse strain, although reliable in around 90% of the cases, does not allow amplification of each and every potential *IG* gene rearrangement, likely explaining the single case in which a monoclonal rearrangement was not found. The lack of an analysis of lymph nodes may be another explanation for why a clone was not detected in that mouse. The *IGH* rearrangements carried by *Siglecg*$^{-/-}$ malignant clones show similarities with the ones detected in *Siglecg*$^{-/-}$ B-1 cells.[2] This is possible evidence that most tumors originate from B-1 cells[29] that have lost CD5 expression following unabated antigen stimulation.[27] Although SIGLEC-G deficiency affects both CD5^{+} and CD5^{-} splenic B cells, the histological features lend support to the hypothesis that *Siglecg*$^{-/-}$ B-cell lymphoproliferative disorders do in fact originate from CD5^{-} B-2 cells. The presence of monoclonal *IGH* gene rearrangements carrying somatic hypermutations suggests that the disease originates from cells that have transited through the GC.

These possibilities may be taken to explain (although incompletely) why no cases of CLL were observed in aged *Siglecg*$^{-/-}$ mice, as this has also been suggested to originate from transitional/marginal zone B cells.[30]

Besides histology, grading and the presence of monoclonality, the malignant nature of the observed lymphoproliferations was confirmed by the tumorigenic capacity of *Siglecg*$^{-/-}$ B lymphocytes in transplantation experiments. The successful transplant in immunocompetent BALB/c recipient mice indicates that SIGLEC-G deficiency helps to confer a cell-intrinsic capacity of engraftment in a normal microenvironment, although other factors (likely extrinsic) seem to be necessary to support the development of a frank disease, as suggested by the relatively long latency time. In addition, the transplanted B cells show selective growth and/or a survival advantage over the recipient immune system, in agreement with the results of previous adoptive transfer experiments of *Siglecg*$^{-/-}$ and wild-type B-1a cells.[2] Taken together, these *in vivo* results highlight a possible role of SIGLEC-G as a guardian of B-cell behavior that would normally protect against successful B-cell lymphomagenesis. In keeping with this hypothesis, down-regulation of SIGLEC-G expression has been reported in a mouse model of non-Hodgkin lymphoma originating from the disruption of the steroid and xenobiotic receptor SXR[31] and we observed a lack of the expected band on a western blot analysis in aged wild-type mice that spontaneously developed atypical B-cell lymphoproliferation or follicular lymphoma (*data not shown*). Even more importantly, we have observed that most human B-cell lymphoma/leukemia cell lines show a significant reduction of SIGLEC10, the human counterpart of SIGLEC-G. Little information on SIGLEC10 expression in primary cases is available in the literature, mostly because of the relatively recent discovery of the gene[8] and the consequent lack of specific probes in profiling arrays. Moreover, different sources of control cells have been used and some controversial results have been obtained.[32,33] The levels of SIGLEC10 expression have been reported to be reduced, compared to those in normal B cells, in CD5^{+} and CD5^{-} B-cell malignancies, including Burkitt lymphoma,[33] DLBCL (in one out of two studies),[32] and CLL[34] but not in follicular lymphoma.[32,33] Future analyses specifically addressing the expression of SIGLEC10 protein will clarify the issues. In a preliminary series of 18 primary CLL cases, we observed readily detectable protein levels in 16/18 samples (*data not shown*). Finally, missense mutations in the *SIGLEC10* gene have been identified in ABC DLBCL by next-generation sequencing approaches;[35] such mutations may alter the conformation and/or function of the protein. Although these findings deserve further validation and investigation, they reasonably suggest that SIGLEC10 down-regulation or loss of function, releasing the brake for B-cell proliferation/activation and promoting B lymphocyte survival,[1,2] might be a common event favoring the development of human B-cell lymphoproliferative disorders.

Future studies in this novel B-cell lymphoma model will help to define the signaling pathways, microenvironmental stimuli and genetic alterations that synergize with the absence of SIGLEC-G, driving the development of the observed spectrum of B-cell malignancies, which include DLBCL, medium to large B-cell lymphoma and follicular lymphoma, but not CLL, in mice. Moreover, a better understanding of the molecular mechanisms potentially leading to the down-regulation and/or inactivation of the gene in lymphomagenesis will favor the design of novel therapeutic strategies for human B-cell lymphomas.

Acknowledgments

The authors would like to thank Cristina Scielzo and Michela Frenquelli for helpful suggestions and discussion, Arianna Vino, Eliana Di Cairano and Federica Barbaglio for technical support, and Gianluca Gaidano for materials. This research was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC) [Investigator grant (to PG) and Special Program Molecular Clinical Oncology 5 per mille #9965], Milano, Italy; Ricerca Finalizzata 2010, Ministero della Salute, Roma, Italy; PRIN, MIUR, Roma, Italy; and the CLL Global Research Foundation, Houston, TX, USA.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Hoffmann A, Kerr S, Jellusova J, Zhang J, Weisel F, Wellmann U, et al. Siglec-G is a B1 cell-inhibitory receptor that controls expansion and calcium signaling of the B1 cell population. Nat Immunol. 2007;8(7):695-704.
2. Jellusova J, Duber S, Guckel E, Binder CJ, Weiss S, Voll R, et al. Siglec-G regulates B1 cell survival and selection. J Immunol. 2010;185(6):3277-84.
3. Jellusova J, Wellmann U, Amann K, Winkler TH, Nitschke L. CD22 x Siglec-G double-deficient mice have massively increased B1 cell numbers and develop systemic autoimmunity. J Immunol. 2010;184(7):3618-27.
4. Duong BH, Tian H, Ota T, Completo G, Han S, Vela JL, et al. Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance in vivo. J Exp Med. 2010;207(1):173-87.
5. Nitschke L. CD22 and Siglec-G: B-cell inhibitory receptors with distinct functions. Immunol Rev. 2009;230(1):128-43.
6. Poe JC, Tedder TF. CD22 and Siglec-G in B cell function and tolerance. Trends Immunol. 2012;33(8):413-20.
7. Pfrengle F, Macauley MS, Kawasaki N, Paulson JC. Copresentation of antigen and ligands of Siglec-G induces B cell tolerance independent of CD22. J Immunol. 2013;191(4):1724-31.
8. Munday J, Kerr S, Ni J, Cornish AL, Zhang JQ, Nicoll G, et al. Identification, characterization and leucocyte expression of Siglec-10, a novel human sialic acid-binding receptor. Biochem J. 2001;355(Pt 2):489-97.
9. Liu Y, Chen GY, Zheng P. CD24-Siglec G/10 discriminates danger- from pathogen-associated molecular patterns. Trends Immunol. 2009;30(12):557-61.
10. Chen GY, Tang J, Zheng P, Liu Y. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. Science. 2009;323(5922):1722-5.
11. Ding C, Liu Y, Wang Y, Park BK, Wang CY, Zheng P, et al. Siglecg limits the size of B1a B cell lineage by down-regulating NFkappaB activation. PLoS One. 2007;2(10):e997.
12. Hayakawa K, Hardy RR, Parks DR, Herzenberg LA. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. J Exp Med. 1983;157(1):202-18.
13. Bichi R, Shinton SA, Martin ES, Koval A, Calin GA, Cesari R, et al. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. Proc Natl Acad Sci USA. 2002;99(10):6955-60.
14. Klein U, Lia M, Crespo M, Siegel R, Shen Q, Mo T, et al. The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. Cancer Cell. 2010;17(1):28-40.
15. Lia M, Carette A, Tang H, Shen Q, Mo T, Bhagat G, et al. Functional dissection of the chromosome 13q14 tumor-suppressor locus using transgenic mouse lines. Blood. 2012;119(13):2981-90.
16. Greenwald RJ, Tumang JR, Sinha A, Currier

N, Cardiff RD, Rothstein TL, et al. E mu-BRD2 transgenic mice develop B-cell lymphoma and leukemia. Blood. 2004;103(4):1475-84.
17. Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. Nat Rev Cancer. 2005;5(4):251-62.
18. Gribben J, Rosenwald A, Gascoyne R, Lenz G. Targeting the microenvironment. Leuk Lymphoma. 2010;51 (Suppl 1):34-40.
19. Lenz G, Wright G, Dave SS, Xiao W, Powell J, Zhao H, et al. Stromal gene signatures in large-B-cell lymphomas. N Engl J Med. 2008;359(22):2313-23.
20. Kridel R, Sehn LH, Gascoyne RD. Pathogenesis of follicular lymphoma. J Clin Invest. 2012;122(10):3424-31.
21. Caligaris-Cappio F. Role of the microenvironment in chronic lymphocytic leukaemia. Br J Haematol. 2003;123(3):380-8.
22. Davis RE, Ngo VN, Lenz G, Tolar P, Young RM, Romesser PB, et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature. 2010;463(7277):88-92.
23. LeMaoult J, Delassus S, Dyall R, Nikolic-Zugic J, Kourilsky P, Weksler ME. Clonal expansions of B lymphocytes in old mice. J Immunol. 1997;159(8):3866-74.
24. Szabo P, Li F, Mathew J, Lillvis J, Weksler ME. Evolution of B-cell clonal expansions with age. Cell Immunol. 2004;231(1-2):158-67.
25. Bokers S, Urbat A, Daniel C, Amann K, Smith KG, Espeli M, et al. Siglec-G deficiency leads to more severe collagen-induced arthritis and earlier onset of lupus-like symptoms in MRL/lpr mice. J Immunol. 2014;192(7):2994-3002.
26. Bikah G, Carey J, Ciallella JR, Tarakhovsky A, Bondada S. CD5-mediated negative regulation of antigen receptor-induced growth signals in B-1 B cells. Science. 1996;274(5294):1906-9.
27. Sen G, Bikah G, Venkataraman C, Bondada S. Negative regulation of antigen receptor-mediated signaling by constitutive association of CD5 with the SHP-1 protein tyrosine phosphatase in B-1 B cells. Eur J Immunol. 1999;29(10):3319-28.
28. Ochi H, Watanabe T. Negative regulation of B cell receptor-mediated signaling in B-1 cells through CD5 and Ly49 co-receptors via Lyn kinase activity. Int Immunol. 2000;12(10):1417-23.
29. Holodick NE, Repetny K, Zhong X, Rothstein TL. Adult BM generates CD5+ B1 cells containing abundant N-region additions. Eur J Immunol. 2009;39(9):2383-94.
30. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med. 2005;352(8):804-15.
31. Casey SC, Nelson EL, Turco GM, Janes MR, Fruman DA, Blumberg B. B-1 cell lymphoma in mice lacking the steroid and xenobiotic receptor, SXR. Mol Endocrinol. 2011;25(6):933-43.
32. Compagno M, Lim WK, Grunn A, Nandula SV, Brahmachary M, Shen Q, et al. Mutations of multiple genes cause deregulation of NF-kappa B in diffuse large B-cell lymphoma. Nature. 2009;459(7247):717-U124.
33. Brune V, Tiacci E, Pfeil I, Doring C, Eckerle S, van Noesel CJM, et al. Origin and pathogenesis of nodular lymphocyte-predominant Hodgkin lymphoma as revealed by global gene expression analysis. J Exp Med. 2008;205(10):2251-68.
34. Seifert M, Sellmann L, Bloehdorn J, Wein F, Stilgenbauer S, Durig J, et al. Cellular origin and pathophysiology of chronic lymphocytic leukemia. J Exp Med. 2012;209(12):2183-98.
35. Zhang J, Grubor V, Love CL, Banerjee A, Richards KL, Mieczkowski PA, et al. Genetic heterogeneity of diffuse large B-cell lymphoma. Proc Natl Acad Sci USA. 2013;110(4):1398-403.