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.

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Correspondence: caligaris.federico@hsr.it

Supplementary Material

Supplementary Methods

Flow cytometric analysis

Single-cell suspensions were first incubated in an ammonium chloride solution lysis buffer (NH_4Cl 0.15 M, $KHCO_3$ 10 mM, Na_2EDTA 0.1 mM, pH 7.2–7.4) to lyse red cells and then stained after blocking fragment crystallizable (Fc) receptors (1). The following antibodies were used: anti-mouse CD19 (PE-Cy7), anti-mouse CD5 (APC), anti-mouse CD3 (APC), anti-mouse CD4 (PE), anti-mouse CD8 (FITC) (from BD Biosciences, San Jose, CA, USA), anti-human CD19 (ECD), anti-human CD5 (Pe-Cy7) (from Beckman Coulter, Hialeah, FL, USA), anti-human SIGLEC10 (PE, BioLegend, San Diego, CA, USA). Intracellular staining for NFATc1 was performed using Intraprep permeabilization Reagent (Beckman Coulter) according to manufacturer's instructions and NFATc1 antibody (PE, BioLegend). The samples were analyzed using Cytomics FC500 (Beckman Coulter).

Immunohistochemistry

For immunohistochemical (IHC) studies sections were de-paraffinized, rehydrated and stained as previously described (1). The following antibodies were used: anti-mouse B220 (RA3-6B2, ABD Serotec, Raleigh, NC, USA), anti-Ki-67 (SP6, Neo Markers, Fremont, CA, USA), anti-mouse BCL-6 (Rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse BCL-2 (C21, Santa Cruz Biotechnology), anti-mouse IRF4 (MUM1p, Santa Cruz Biotechnology). The following secondary antibodies and reagents were used: biotin-conjugated anti-rat IgG (Vector Laboratories, Burlingame,

CA, USA), peroxidase-streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), rabbit-on-rodent HRP-polymer (Biocare Medical, Concord, CA, USA), anti-mouse Dako Envision+System-HRP labelled polymer (Dako, Glostrup, Denmark). Slides were incubated with 3,3′-diaminobenizidine tetrahydrochloride (DAB, Thermo Scientific, Waltham, MA, USA) and counterstained with Mayer-Hematoxylin. After dehydratation in ethanol and xylene, slides were permanently mounted in Eukitt (Bio-Optica, Milano, Italy). Images were acquired at an Axioscop 40 microscope (Zeiss, Oberkochen, Germany) by an Axiocam MRC camera (Zeiss), Axovision release 4.6 software (Zeiss).

IGHV-D-J gene rearrangement analysis

DNA was subjected to PCR (Taq DNA polymerase, Roche Applied Science, Basel, Switzerland) using IGHV1a,9 (CAGGTCCAGCTGCAGCAGTCTGG) (BALB/c strain-specific gene primer) and IGHJintron (TGAGGCTCTGAGATCCCTAGACAG) gene primers and products were run on a 7.5% PAGE (PROTOCOL 1). This protocol amplifies 4 distinct IG heavy chain derived products of different molecular weight (reflecting IGHV-D-J1, IGHV-D-J2, IGHV-D-J3, and IGHV-D-J4 rearrangements) in polyclonal B cell populations, with loss of some bands in oligoclonal or monoclonal populations (1). When a pattern of oligo/monoclonality was observed, the IGHV-D-J rearrangements were amplified with a nested PCR approach (PROTOCOL 2) and directly sequenced, as described (1). The following strain-specific forward primers were used in the nested PCR: IGHV1a,9, IGHV1b (CAGGTCCAACTGCAGCAGCCTGG), IGHV1c,3 (GAGGTCCAGCTTCAGCAGTCAGG), IGHV5,10 (GAGGTGC/AAGCTGGTG

GA/GTCTGG). Using these primers, almost 90% of all possible rearrangements for the BALB/c mouse strain are detectable. All primer sets were provided by Primm srl (Milano, Italy). For the analysis of somatic mutations in the monoclonal IGHV gene rearrangements, immunoglobulin sequence data were analyzed using the IMGT® databases and the IMGT/V-QUEST tool (http://www.imgt.org).

Calcium level measurement

Murine spleen cells ($5x10^6$) were incubated with anti-mouse CD19 (PE-Cy7) antibody. For measurement of basal calcium level, cells were loaded with 0.02% (vol/vol) of Pluronic F-127 (Sigma-Aldrich, St Louis, MO, USA) and with the calcium indicator Fluo-3 acetoxymethyl (AM) ester (Molecular Probes, Life Technologies, Carlsbad, CA, USA) alone or in combination with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) AM intracellular calcium chelator (Molecular Probes, Life Technologies) for 30 minutes at 37°C. Cells were then washed, resuspended at $5x10^6$ cells/mL in complete RPMI medium and pre-warmed at 37°C for 5 minutes before acquisition. Data were collected on Cytomics FC500 (Beckman Coulter) flow cytometer and analyzed with FlowJo software (TreeStar, Ashland, OR, USA). The basal calcium level was calculated as difference between the mean fluorescence intensity (MFI) of the Fluo and BAPTA signals in $CD19^+$ B cells.

Transplantation

BALB/c mice were injected intraperitoneally with 0.1 mL saline containing 10^7 cells. The cells were obtained from the spleen ($CD19^+$-enriched by using the EasySep Mouse B

Cell Enrichment kit - StemCell Technologies) of *Siglecg*$^{-/-}$ mice (in the case of first transplants) or from either the spleen (same as above) or the peritoneal exudate (containing 80% CD19^{+} cells) of a BALB/c animal previously engrafted with *Siglecg*$^{-/-}$ B cells (in the case of secondary transplant). Animals were assessed for weight and bled once a month in order to monitor the expansion of a clonal CD19^{+} population. The engraftment was assessed based on the IGH gene rearrangement and the flow cytometric analysis. Mice were sacrificed when they developed relevant signs/symptoms of illness (≥20% of weight loss and/or limb paralysis and/or abdominal enlargement) and tissues (PB, peritoneal exudate, spleen, lymph nodes and femurs) were collected and prepared for flow cytometry, histopathological, IHC and IGH gene rearrangement analysis. After sacrifice, the engraftment was confirmed by histopathological analysis.

Cells

The following cell lines were used and cultured in medium supplemented with 15 µg/mL gentamicin (Sigma-Aldrich): DHL-4, DHL-16, Raji, Ramos, Jijoye, Daudi, MEC1, MEC2 (2), JVM-2, JVM-3, Riva, Toledo and DHL-2 cells were cultured in RPMI 1640 medium (Invitrogen, Life Technologies) supplemented with 10% fetal bovine serum (FBS); H2, RL and HC-1 cells were cultured in RPMI 1640 medium supplemented with 20% FBS; HeLa cells were cultured in DMEM medium (Invitrogen, Life Technologies) supplemented with 10% FBS. For B-cell enrichment of peripheral blood and tonsil samples, depletion of NK, T lymphocytes, and monocytes was performed through column separation (Miltenyi Biotec, Auburn, CA, USA). Purity of preparations, once

checked by flow cytometry, was above 90%. For isolation of GC B cells, tonsil cells were stained with anti-IgD FITC (Southern Biotech, Birmingham, AL, USA), anti-CD19 PE and anti-CD38 PC5 (Beckman Coulter) and FACS-sorted ($CD19^{+}IgD^{-}CD38^{+}$) on a High Speed Sorter MoFLo (Dako).

Quantitative reverse-transcription PCR (qRT-PCR)

RNA was isolated from cell lines and primary samples with Trizol (Invitrogen, Life Technologies) and reverse transcribed (cDNA Synthesis kit, Fermentas, Thermo Scientific). qRT-PCR was carried out on the 7900HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies) using the Sybr Green PCR Master Mix (Applied Biosystems) and the following primers: SIGLEC10fw (TGGCTCAGAAGCGGA ATC), SIGLEC10rev (CCTCATTGGAACTTGACTTCTGC), GAPDHfw (GAAGGTGAAG GTCGGAGTC), GAPDHrev (GAAGATGGTGATGGGATTTC). The analysis of output data was performed with the SDS2 software (Applied Biosystems). To quantify the mRNA levels, the expression of SIGLEC10 was normalized to the GAPDH housekeeping gene. Raji cell line was used as calibrator sample and HeLa cell lines as negative control (3).

Cell lysis and Western blot analysis

Cells were lysed with ice-cold RIPA Buffer (Sigma-Aldrich). Proteins (about 30 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then electron-transferred onto nitrocellulose membranes and incubated with the following primary antibodies: mouse anti-SIGLEC10 (R&D systems, Minneapolis, MN,

USA), mouse anti-NFATc1 (7A6, Santa Cruz Biotechnology) and goat anti-SIGLEC-G (E-14, Santa Cruz Biotechnology). Immunoreactivity was revealed by incubation with goat anti-mouse IgG (Upstate Biotechnology, Bar Harbor, ME, USA) or donkey anti-goat IgG (Santa Cruz Biotechnology) secondary antibody conjugated with horseradish peroxidase (HRP). HRP-labeled anti-β-actin antibody (Sigma-Aldrich) was used for Western blot analysis.

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Supplementary Tables

Supplementary Table S1. Frequency of CD19$^+$ and CD19$^+$CD5$^+$ B lymphocytes in *Siglecg*$^{-/-}$ mice.

	Age (mo)	Mice (n)	% of CD19$^+$ cells			% of CD19$^+$CD5$^+$ cells (among B cells)		
			SP	**PB**	**PER**	**SP**	**PB**	**PER**
Wild-type	5	7	21.1 ± 6.2	38.2 ± 4.9	68.0 ± 9.9	3.7 ± 1.5	5.6 ± 3.2	31.4 ± 6.3
Siglecg$^{-/-}$	5	10	15.8 ± 4.6	43.4 ± 6.7	76.6 ± 6.7*	6.6 ± 1.9**	11.0 ± 4.9*	29.4 ± 8.9
Wild-type	10	6	13.7 ± 5.2	48.8 ± 4.8	72.6 ± 6.4	11.3 ± 5.1	3.3 ± 0.8	9.0 ± 4.0
Siglecg$^{-/-}$	10	10	24.2 ± 7.4**	41.3 ± 6.0*	75.3 ± 7.0	13.8 ± 9.9	12.3 ± 5.8**	30.6 ± 8.5***
Wild-type	16	6	13.6 ± 5.4	40.7 ± 11.6	62.6 ± 32.4	5.1 ± 4.9	2.8 ± 0.9	10.5 ± 5.5
Siglecg$^{-/-}$	16	6	31.1 ± 6.1***	47.7 ± 5.3	81.0 ± 4.0	6.3 ± 1.2	7.2 ± 3.1**	16.1 ± 5.3

SP: spleen; PER: peritoneal exudate; *p<0.05, **p<0.01, ***p<0.001.

Supplementary Table S2. CD19$^+$ and CD19$^+$CD5$^+$ B cells in the spleen of *Siglecg*$^{-/-}$ mice: absolute numbers.

	Age (mo)	Mice (n)	**N. of CD19$^+$ cells†**	**N. of CD19$^+$CD5$^+$ cells†**
Wild-type	5	7	20.9 ± 6.2	0.7 ± 0.3
Siglecg$^{-/-}$	5	10	31.3 ± 7.9*	1.6 ± 0.5**
Wild-type	10	6	25.8 ± 12.8	3.2 ± 2.2
Siglecg$^{-/-}$	10	6	44.0 ± 10.9*	6.7 ± 5.9
Wild-type	16	4	25.0 ± 10.2	1.5 ± 1.1
Siglecg$^{-/-}$	16	6	49.6 ± 11.8**	3.1 ± 1.1

† values are $\times 10^6$;

*p<0.05, **p<0.01.

Supplementary Table S3. Primary transplant: engrafted mice.

Mouse (donor)	Mouse (recipient)	Diagnosis of donor mouse	Engraftment (months from transplant)[†]	Sac/death (months from transplant)
Siglecg$^{-/-}$ #83	BALB/c #3	FL	7	15
	BALB/c #5	FL	14	17
Siglecg$^{-/-}$ #84	BALB/c #1	FL	3	7
	BALB/c #2	FL	3	6
	BALB/c #4	FL	7	17
Siglecg$^{-/-}$ #96	BALB/c #8	ALP	nd	19
	BALB/c #9	ALP	nd	7.5
	BALB/c #10	ALP	nd	15

[†] based on detection of the IGHV gene rearrangement of the donor mouse;

nd, not determined.