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Siglec-G Regulates B1 Cell Survival and Selection

Julia Jellusova,* Sandra Düber,† Eva Gückel,‡ Christoph J. Binder,§*,‡ Siegfried Weiss,† Reinhard Voll,‡ and Lars Nitschke*‌

Siglec-G is a negative regulator of BCR-mediated signaling in B1a cells. This population of B cells is highly increased in Siglec-G-deficient mice, but the mechanism of this expansion is not known so far. In this study, we demonstrate that Siglec-G–deficient mice show a lower level of spontaneous apoptosis and a prolonged life span. Mechanistically, the lower apoptosis could result from higher expression levels of the transcription factor NFATc1 in Siglec-G–deficient B1a cells. Interestingly, Siglec-G–deficient B1a cells display an altered BCR repertoire compared with wild-type B1a cells. As the BCR repertoire and the VDJ composition of Igs of Siglec-G–deficient B1a cells resembles more the Abs produced by adult bone marrow-derived B cells rather than canonical fetal liver-derived B1a cells, this suggests that the selection into the B1a cell population is altered in Siglec-G–deficient mice. *The Journal of Immunology, 2010, 185: 000–000.

Siglec-G, a member of the family of CD33-related Siglecs, functions as a negative regulator of BCR-mediated signaling. Siglec-G is expressed throughout the B cell lineage, with the highest expression levels in B1a cells (1–3). Although Siglec-G is a potent inhibitor of BCR-induced Ca2+ signaling when overexpressed in a B cell line, in mice it seems to play a dominant role only in B1a cells and not in conventional B2 cells. This has been shown with the aid of Siglec-G–deficient mice, which show increased calcium signaling in B1a cells, but not in B2 cells, upon anti-IgM stimulation. Further, Siglec-G–deficient mice show a highly enlarged population of B1 cells, increased numbers of IgM-producing cells in bone marrow and spleen, paralleled by higher preimmune IgM titers in serum (2).

B1 cells are thought to be responsible for most of the natural Abs in normal unimmunized mice. These Abs are mostly of the IgM isotype, and their titers are largely independent of exogenous antigenic stimulation. B1 cells are also important for the immune response to thymus-independent Ags (4). Upon Ag encounter, they quickly migrate to lymphoid organs (e.g., the spleen) to become Ab-producing cells (5). Natural Abs and B1 cells crucially contribute to the first-line defense against microbial infections in mice, acting before an adaptive immune response by conventional B2 cells is possible.

Apart from their functional specialization, B1 cells can be distinguished from the more abundant B2 cells by their surface markers, because they are CD23+ CD43+, B220lo, and IgMhi. Several B1 cell subpopulations have been defined, such as peritoneal B1a (CD5+, Mac1+) and B1b (CD5−, Mac1+) cells and splenic B1 (CD5+, Mac1−) cells. The early immune response of B1a cells is favored by their localization in the peritoneal cavity, where they are among the first cells to encounter pathogens from the gut. In addition, their BCR repertoire is skewed toward polyreactive, weakly self-reactive Igs, which can bind many common bacterial structures; ~5% of all B1a cells produce Igs, which can bind phosphocholine (PC) an Ag present as part of a phospholipid in cell membranes and exposed on a variety of Gram-negative and Gram-positive bacteria. Natural PC-binding Abs are crucial for defense against *Streptococcus pneumoniae* and are dominated in young BALB/c mice by Abs of the T15 idiotype (6). Another significant component of the B1 cell Ab repertoire constitutes Abs specific for phosphatidylcholine (PC), a phospholipid commonly found in cellular membranes. These Abs were shown to be protective against systemic bacterial infections (7, 8). Both specificities are thought to be exclusive for the B1a cell compartment.

Natural Abs have also been proposed to possess “housekeeping functions,” as they can bind to self Ags, which are exposed during apoptosis and are involved in clearing cellular debris. This is particularly important for oxidized Ags such as oxidized low-density lipoprotein (LDL), which is involved in atherosclerosis development (9). It has been proposed that the bias in the B1a cell Ab repertoire is a result of positive selection of B cells into this compartment. Direct evidence of positive selection has been provided for Thy-1–specific B1a cells, as B1a cells with BCRs against Thy-1 are not found in mice deficient for this molecule (10).

In contrast to conventional B2 cells, most B1a cells in the mouse are thought to be derived from precursors in fetal liver rather than from adult bone marrow. They are believed to maintain their cell numbers in adult mice by longevity and homeostatic proliferation. The preferential development of B1a cells from fetal liver might be the product of a restricted junctional diversity in this organ due to the lack of TdT expression and nonrandom VH usage caused by the homology-directed recombination mechanism (11, 12). In addition, a B1 cell progenitor was identified (Lin− CD45Rlo-neg. CD19+); this progenitor can be frequently found in the fetal liver but is less abundant in adult bone marrow (13). However, it remains controversial whether all B1a cells derive from this progenitor. There is also some evidence that common lymphoid progenitors can give rise to B1 cell progenitors, suggesting a possible shared ancestry between B1 and B2 cells (14). In accordance with the hypothesis that precursor B cells are selected to the B1a cell compartment is the observation that the development and/or main-
tenance of B1a cells is affected by the strength of the BCR signal. Genetically modified mice with decreased BCR signaling often fail to produce normal numbers of B1a cells, whereas various mutated mice with enhanced BCR signaling often have an enlarged B1a cell compartment (4). This is similar to the situation in Siglec-G–deficient mice.

The B1a cell expansion in Siglec-G–deficient mice is cell autonomous, occurs as soon as B1a cells appear in juvenile mice, and proceeds gradually until the B1a cell compartment is fully developed (2). Adult Siglec-G–deficient BALB/c mice show, on average, an 8-fold increased B1a cell population in the peritoneal cavity. The mechanism of this expansion remains enigmatic to this date because Siglec-G–deficient B1a cells show a lower proliferation, as determined by BrdU incorporation. In this paper, we show that Siglec-G–deficient B1a cells survive longer in vitro and become the dominant population when injected in competition with wild-type B1a cells into Rag1-deficient mice. Siglec-G–deficient B1a cells upregulate the transcription factor NFATc1 and exhibit an altered BCR repertoire that might indicate an altered selection of B cells into the B1a cell compartment.

Materials and Methods

Mice

Experiments were performed with adult (>8 wk) age-matched BALB/c (wild-type) and SiglecG−/− mice, which are on a pure BALB/c background (2). Rag1−/− mice (BALB/c background) for adoptive transfer experiments were kindly provided by Dr. André Gessner (Institute of Clinical Microbiology, Immunology and Hygiene, University Hospital Erlangen, Erlangen, Germany). CD45.1 mice were also on a pure BALB/c background.

Flow cytometry

The following fluorochrome-conjugated Abs and reagents were used: Anti-IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), anti-CD19, anti-B220 (eBioscience, San Diego, CA), anti-CD45.2 (BD Biosciences, San Jose, CA), anti-CD5 (eBioscience/BD Biosciences), anti-κ (BD
Biosciences), anti-Î±-lambdA.2.3 (BD Biosciences), Fe-block (clone: 2G2, our hybridoma), PC-BSA-FITC (Biosearch Technologies, Novato, CA), and Pic liposomes, carrying FITC, were prepared as described (15).

Cell sorting and T cell depletion
Peritoneal cavity cells were negatively sorted with anti-CD90-beads and anti-CD23-PE (eBioscience)/anti-PE Abs coupled to magnetic beads (Miltenyi Biotec, Auburn, CA) and positively sorted with anti-CD5-beads (Miltenyi Biotec) to obtain B1a cells. The achieved purity was 90%. Erythrocyte-depleted splenic cells were incubated with anti-CD4, -CD8, and -CD90 Abs (our hybridoma); T cells were lysed with baby rabbit complement (Cedarlane, Burlington, NC).

In vitro apoptosis test
Purified B1a cells from peritoneal cavity or splenic B cells obtained by T cell depletion were cultured in 5% FCS RPMI 1640 medium supplemented with 1 x nonessential amino acids (Life Technologies, Rockville, MD), 1 mM sodium pyruvate (Life Technologies), 100 U/ml Pen-Strep (Life Technologies), 1.2 mM L-Glutamine (Life Technologies), and 50 Î¼M 2-ME (Life Technologies). Each day, samples were extracellularly stained for CD5 and B220, fixed with 2% paraformaldehyde in PBS for 10 min at room temperature, permeabilized by incubating overnight in 1 ml 100% methanol in PBS, and intracellularly stained with 10 Î¼g DAPI/ml PBS + 0.1% Triton-X. Cells with subG1 DNA content were considered apoptotic.

Adoptive transfer
A 1:1 mixture of purified wild-type (CD45.1) and Siglecg -/-/- (CD45.2 -/-) B1a cells (10^6 cells in total) was i.p. injected into Rag1 -/-/- mice. Mice were sacrificed 4 wk or 6 wk later. Recovered IgM +/CD5 + cells were analyzed for CD45.2 expression. To determine the cell division rate, B1a cells were loaded for 5 min with 5 Î¼M CFSE in PBS + 0.1% BSA + 0.5% Na azide, washed, and incubated for 30 min in 10% FCS RPMI 1640 prior to adoptive transfer. A mouse sacrificed 1 d after transfer was used as a control for the initial CFSE labeling. To calculate the cell division rate, mean fluorescence intensities (MFIs) for CFSE at certain time points were determined and log2 values calculated. The log2 MFI (CFSE) of input cells subtracted by the log2 MFI (CFSE) after 4 wk or 6 wk gave the number of cell divisions.

Western blot analysis and EMSA
Purified B1a cells were lysed with Nonidet P-40 lysis buffer, and proteins separated by SDS-PAGE were transferred to nitrocellulose. The filters were blocked with 5% nonfat milk powder in 0.05% Tween-PBS and incubated with anti-Bcl-2 or anti-NFATc1, respectively. Ab binding was visualized using HRP-conjugated secondary Abs and ECL. To define the intensities of Bcl-2 in total lysates of peritoneal B1a cells were measured by Western blot.

RNA preparation and real-time PCR
Total RNA was prepared from sorted B1a cells, using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions, followed by RNA precipitation with isopropanol and SeeDNA (Amersham Biosciences/GE Healthcare, Piscataway, NJ). For cDNA synthesis, 500 Î¼g RNA was reverse transcribed using oligo(d-T)18 (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). Obtained cDNA was used for amplification in a real-time PCR. PCR conditions were as follows: 95°C for 15 min; 40 x (95°C 1 s, 60°C 1 min.), 95°C 1 s, 60°C 30 s, 95°C 15 s. The following primer pairs were used: A20: 5'-GACCAGCCGATCCGCAAGGG-GG-3' and 5'-GGACAGTTGGTGTCCTCACATTT-3'; IkBa: 5'-TGAAGGACGAGGATGAGCC-3' and 5'-TTCCGTGATGATGGGCAAGTG-3'; Bcl2: 5'-ATGCGGTCTGTTGACATATGCC-3' and 5'-GATGACCCAG-AGGCTAGTCGC-3'; b-actin: 5'-GGCTGTATCTCCCCCATCTG-3' and 5'-CCAGTTGGTAAAGCTGCATG-3'.

Relative levels of mRNA were calculated as follows: ÏCT = (CP value of respective mRNA – CP value of actin mRNA); ÏΔCT = (ΔCT value of respective mRNA – mean from all wild-type ΔCT values); relative level of target mRNA = 2 – ΔΔCT.

Single-cell sortting, amplification of VÎ¼DJÎ¼ Î¼-chain transcripts, and sequencing
Cell sorting, VÎ¼DJÎ¼ amplification, and sequence analysis were performed as described previously (18, 19). In short, VÎ¼DJÎ¼ transcripts from FACS-sorted single peritoneal IgM +/CD5 + cells were amplified in a two-round PCR. The primers VÎ¼s (5'-GAGGTGCAGCTGCTGAGATTCG-3') and CÎ¼1 (5'-ATGGCCACAGCTTCTATCGA-3') were used in the first round, and VÎ¼s and CÎ¼2 (5'-CATGGGAGAAGACTGA-3') in the second round. PCR conditions were as follows: first round—50°C 45 min; 95°C 15 min; 15 x (94°C 20 s, 50°C 40 s, 72°C 40 s); 72°C 15 min; 8°C 10 s; second round—94°C 20 s, 50°C 40 s, 72°C 40 s; 72°C 15 min; 8°C 10 s.

Figure 2: The expression of NFATc1 is increased in Siglecg -/-/- B1a cells. A, NF-Î¼B DNA binding activity was measured by EMSA. Nuclear extracts (3.5 Î¼g/sample) of purified peritoneal B1a cells and splenic B cells were incubated with an IDty/700 fluorescence dye-labeled double-stranded oligonucleotide containing a consensus Î¼B site and subjected to EMSA. The slower migrating band resulting from an NF-Î¼B–containing complex is shown. As a loading control, Oct1 EMASs were performed (not shown). The numbers below give the DNA binding ratio of NF-Î¼B bands divided by Oct1 bands. A representative result of three independent experiments is shown. B, Relative levels of A20, IÎ¼B, and Bcl-2 mRNA isolated from peritoneal B1a cells were determined by RT-PCR. Four independent samples per group were measured in triplicates. Actin was used as internal control. Data are presented as means +SD. *p < 0.05, C, Expression levels of NFATc1 and Bcl-2 in total lysates of peritoneal B1a cells were measured by Western blot. Actin was used as loading control. Numbers below images indicate sum marized ratios of NFATc1 or Bcl-2 band intensities to the band intensity of the loading control from five and four independent experiments, respectively, presented as mean +SD. **p < 0.005.
second round—95°C 15 min; 35 × (94°C 20 s, 50°C 40 s, 72°C 40 s); 72°C 10 min.; 8°C 10 s. PCR products were gel purified and sequenced.

ELISA
Specific serum Ab titers to respective Ag were measured by ELISA, as described previously (20). Ab titers were measured at a 1:100 dilution; malondialdehyde-modified LDL and malonacetaldehyde-modified BSA specific titers at 1:500; and total IgM at 1:35,000.

Software and statistical analysis
CellQuest Pro (BD Biosciences) was used for flow cytometry data analysis, Prism software (GraphPad, San Diego, CA) for statistical evaluation, and ImageJ for quantifying Western blot bands (Rasband, National Institutes of Health, Bethesda, MD). The significance of observed differences was estimated by unpaired t test for metric data or Fisher’s exact test for nominal data.

Results
To study the mechanism for the large expansion of the B1a cell population in Siglec-G–deficient mice, we first tested whether Siglec-G–deficient B1a cells show better survival in vitro by an apoptosis assay. Isolated B1a cells from the peritoneal cavity and splenic B cells were cultured for several days in medium without any stimulation or cytokines. Aliquots were assayed at certain time points for spontaneous apoptosis. Generally, B1a cells from the peritoneal cavity survived longer than did splenic B2 cells and showed a lower percentage of apoptosis during cell culture. Interestingly, at each time point a higher percentage of apoptotic cells could be detected in the wild-type B1a samples than in the Siglec-G–deficient B1a cell samples (Fig. 1A). In addition, the absolute cell numbers of living cells, as determined by counting after trypan blue staining, decreased more slowly during cell culture in Siglec-G–deficient B1a samples than in wild-type (not shown). In contrast, splenic B cells of Siglec-G–deficient and control mice died at a similar apoptosis rate (Fig. 1A). This finding shows that Siglec-G–deficient B1a cells survive better in vitro than do wild-type B1a cells. To assess the life span of Siglec-G–deficient B1a cells in vivo, Rag1-deficient mice were injected i.p. with an equal mixture (50–50%) of Siglec-G–deficient B1a cells and wild-type B1a cells. The mice were sacrificed 4 wk or 6 wk later to determine the composition of the recovered B cell population. After 4 wk the recovered B1a cells (CD5+IgM+) from the peritoneal cavity consisted, on average, of 70% Siglec-G–deficient and of 30% wild-type cells (Fig. 1B). This difference was statistically significant. Similarly, after 6 wk, a comparable higher percentage of Siglec-G–deficient B1a cells could be recovered (Fig. 1B). Only a few B1a cells were found in spleen or blood. Nevertheless, cells showed, on average, a composition similar to that of the population from the peritoneal cavity (not shown).

As the higher number of the recovered Siglec−/− B1a cells after transfer of B1a cell mixtures could be caused by a higher proliferation rate of the Siglec−/− B1a cells rather than prolonged life span, additional adoptive transfer experiments with CFSE-labeled cells were performed. Analysis of recipient Rag−/− mice 4 wk or 6 wk after transfer showed that most of the recovered B1a cells appeared to be the offspring of cycling cells, as judged by their low CFSE levels. From the mean CFSE levels, we calculated that wild-type B1a cells had gone, on average, through 6.9 cell divisions after 4 wk, and 7.7 divisions after 6 wk, respectively. There was a tendency for a slightly higher cell division rate of Siglec-G–deficient B1a cells compared with wild-type, with 7.7 cell divisions after 4 wk and 8.5 cell divisions after 6 wk, respectively (Fig. 1C). Nevertheless, the absolute numbers of recovered B1a cells did not significantly increase over this time, indicating that the composition of the recovered B cell population was probably a product of both proliferation and survival properties of the injected cells.

To determine the molecular mechanism of the B1 cell population expansion in Siglec-G–deficient mice, we analyzed known survival factors for B lymphocytes. The Rel/NF-κB family of transcription factors has been shown to regulate both proliferation and survival of B cells (21, 22). NF-κB signaling seems to play an essential role in B1a cells, because mice bearing targeted mutations in subunits of the NF-κB complexes display a selective reduction of B1a cells (22). The high accumulation of B1a cells in Siglec-G–deficient mice has been proposed to result from a higher activation of NF-κB (1). To verify this, we performed EMSA assays with a fluorescence-labeled double-stranded oligonucleotide containing a consensus NF-κB binding sequence to measure NF-κB DNA-binding activity in nuclear extracts from sorted B1a cells. Samples from Siglec-G–deficient B1a cells and wild-type B1a cells showed a comparable binding of activated nuclear NF-κB complexes to the consensus NF-κB probe. This NF-κB probe can bind all types of NF-κB complexes. The fact that NF-κB-probe complexes run equally in
the PAGE indicates very similar composition of the complexes (Fig. 2A). Further, we studied the transcription of A20 and IκBα genes. A20 and IκBα are both direct targets of NF-κB (23, 24); therefore, we would expect to find elevated levels of A20 and IκBα transcripts if the NF-κB activity was increased. The amplification of both IκBα and A20 transcripts from B1a cells by real time RT-PCR showed no difference between the Siglec-G−deficient and wild-type mice (Fig. 2B). Because this suggests a normal activity of NF-κB, we shifted our attention to other targets. Bcl-2 is an anti-apoptotic protein that can prevent apoptosis when it is overexpressed in B cells (25). We analyzed Bcl-2 expression in B1a cells of Siglecg−/− and control mice by real-time RT-PCR and Western blot. The relative levels of Bcl-2 mRNA were significantly increased, but no differences in Bcl-2 expression could be detected by Western blot (Fig. 2B, 2C). Another transcription factor that seems to play a crucial role in the development of B1a cells is NFATc1 (also designated NFAT2 or NFATc) (26). Interestingly, we found increased levels of NFATc1 in total lysates from Siglec-G−deficient B1a cells by Western blot (Fig. 2C).

The large expansion of the B1a cell population in Siglecg−/− mice may also be explained by an altered selection into this pool of cells. Normal B1a cells show a remarkably stable BCR repertoire from mouse to mouse, with a preference for certain specificities that are not typical for splenic B2 cells (4). Therefore, we studied the BCR repertoire of Siglec-G−deficient B1a cells and compared it with that of wild-type B1a cells. First, we determined the frequency of Ptc- and PC-binding cells by FACS stainings. BCRs for the distinct specificities of Ptc and PC are typically found on subpopulations of B1a cells (7, 8). Representative results from such stainings are shown in Fig. 3; ~7% of all wild-type B1a cells of the peritoneal cavity bound Ptc, and 5% bound PC. In contrast, we found a significantly decreased percentage of Ptc-binding cells of Siglecg−/− B1a cells compared with the wild type. Similarly, we also found PC-binding B1a cells of Siglec-G−deficient mice with a significantly lower frequency (Fig. 3). We also determined the IgM repertoire of secreted Abs in Siglec-G−/− mice, as the natural Abs of the IgM class are largely produced by B1 cells. As we reported previously (2), Siglec-G−deficient mice have more IgM-producing cells in bone marrow and in the spleen, which is reflected by elevated serum IgM levels. Therefore, it was not surprising that the serum levels of most Ag-specific IgM Abs, which were previously shown to be derived from B1 cells, were also elevated (Fig. 4). To analyze the IgM repertoire in both types of mice, the ratio of Ag-specific IgM to total IgM was calculated for studying whether the BCR repertoire was skewed toward certain specificities. This analysis showed normal relative levels of PC-specific IgM and IgM bound by AB1-2 (T15 idiotype IgM for oxidation-specific Ags are increased in Siglecg−/− mice. Levels of Ag-specific IgM of typical B1 cell specificities were determined by ELISA from wild-type and Siglecg−/− mice and are shown in the upper panel as arbitrary units. Ratios of Ag-specific IgM to total IgM are presented in the lower panel. *p < 0.05; **p < 0.005; ***p < 0.001.

FIGURE 5. The k/λ ratio is increased in Siglecg−/− B1 cells. k chain and λ chain expression was analyzed on peritoneal B1a (CD5−, B220−) and B2 + B1b (CD5−, B220+) cells, splenic B1 (CD5−, B220−), and B2 (CD5−, B220+) cells and bone marrow B (IgM+, B220+) cells. The upper panel shows representative flow cytometric analysis of k and λ chain expression on peritoneal B1a and splenic B1 cells. Percentage of k or λ chain positive cells, respectively, are given within the gates. k/λ chain expression ratio is depicted on the upper right of each plot. Summarized ratios of k to λ chain expression are presented as histograms +SD.
directed against PC). The dextran-specific IgM was reduced. Surprisingly, the levels of IgM specific for typical oxidation-specific epitopes as they occur during atherogenesis (malondialdehyde-modified LDL, malonacetaldehyde-modified BSA, Cu^{2+}-oxidized LDL, and 4-hydroxynonenal-modified BSA) were disproportionately increased in Siglec-G–deficient mice (Fig. 4). Thus, overall, a skewed repertoire of secreted natural IgM was detected in Siglec-G−/− mice. Although Abs such as anti-PC or anti-dextran were relatively normal, the frequencies of Abs against oxidized self Ags were increased.

In a broader BCR repertoire analysis of Siglec-G−/− B1 cells, the expression pattern of κ and λ Ig L chains was analyzed. The ratio of κ to λ chain expression can be indicative of selection processes of B cells in the bone marrow by a process called receptor editing (27). Interestingly, a changed κ/λ ratio was detected in Siglec-G−/− B1a cells of the peritoneum and B1 cells of the spleen, but not in bone marrow B cells or conventional B2 cells of the spleen (Fig. 5). The lower κ/λ ratio of Siglec-G−/− B1 cells reflects a relatively higher λ Ig expression in these cells. To allow conclusions on the usage of specific IgH chains from individual B1a cells, we performed single-cell RT-PCR with FACS-sorted B1a cells (CD5+, IgM+) isolated from peritoneum of adult Siglec-G–deficient and wild-type (BALB/c) mice. The murine H chain locus contains ~200 VH gene segments that can be divided into 14 families according to their nucleotide sequence similarities. We chose a promiscuous VH region primer to amplify all VH genes. A total of 97 sequences from wild-type and 73 sequences from Siglec-G–deficient single B1a cells were analyzed. In the pool of sequences from Siglec-G–deficient mice, 9 of 73 rearrangements were not productive, whereas only 2 of 97 sequences from wild-type mice contained stop codons in their CDR3 region (Supplemental Fig. 1). The pattern of VH-D-JH gene family usage was significantly different in the Siglec-G–deficient B1a cells compared with wild-type. Siglec-G–deficient B1a cells showed a decreased usage of DSP, decreased usage of JH1, and increased usage of JH3. VH12 and VH11 segments were completely missing in the pool of sequences from Siglec-G–deficient B1a cells (Fig. 6.). Canonical VH sequences known to encode for PtC specificities were independently recovered from separate wild-type B1a cells, but not from Siglec-G–deficient mice (Supplemental Fig. 1). Lack of appearance of PtC-specific IgH chains in Siglec-G−/− B1a cells was consistent with lack of expression of VH11 and diminished use of VH2 and VH12. PtC-binding Igs in BALB/c mice nearly exclusively contain one of these three VH segments (28, 29).

**FIGURE 6.** The VDJ IgH gene segment usage is altered in Siglec-G−/− B1a cells. A. Diagrams display patterns of VH family, D element, and J element usage of Siglec-G−/− (right) and wild-type (left) B1a cells, as determined by single-cell RT-PCR and sequencing. Sequences that could not be unambiguously assigned to a n-gene family are termed unknown. B. Comparison of N region addition in VDJ junctions of wild-type and Siglec-G−/− B1a cells. Significant changes in the Siglec-G−/− repertoire are indicated by asterisks. *p < 0.05; **p < 0.005; ***p < 0.001.
Further, a significantly higher percentage of the amplified IgH chains from wild-type mice, compared with Siglec-G–deficient B1a cells, contained noncoded (N) nucleotides. The lack of N nucleotides in many VDJ IgH junctions is a typical characteristic of normal, fetal liver-derived B1 cells.

**Discussion**

In view of the results presented in this paper, we speculate that the prolonged life span of Siglec-G–deficient B1a cells might, to a large extent, account for the increased numbers of these cells seen in Siglec-G–deficient mice. Despite the fact that NF-κB plays an important role in B1a cells and contrary to published data (1), we did not observe an effect of the Siglec-G deficiency on the activation of this transcription factor. We believe that the discrepancies between our findings and the results published by Ding et al. (1) lie in the experimental setup because we chose to study purified B1a cells, whereas the experiments by Ding et al. (1) were mostly performed with lysates from total unseparated cell populations from peritoneal washouts. Thus the altered composition of the populations in the peritoneal fluid from Siglec-G–deficient mice might explain the difference seen in the activity of NF-κB in samples from Siglec-G–deficient mice and wild-type mice. In addition, we cannot exclude the possibility that the genetic background plays a role because the mice used in our experiments were generated on pure BALB/c background, whereas those used in the other study were C57BL/6 (1).

Instead of NF-κB, we propose that the higher expression of NFATc1 and possibly Bcl-2 might play a direct role in the expansion of the B1a cell population in Siglec-G–deficient mice. NFATc1 is known to induce apoptosis resistance in lymphocytes [e.g., activated T cells (30)]. NFATc1 was shown to play a crucial role in the development of B1 cells. but not in B2 cells. Adaptively transferred fetal liver cells from NFATc1-deficient embryos were reported to be inefficient in repopulating the B1a cell compartment (26). Therefore an increased expression of NFATc1 in Siglec-G–deficient B1a cells might account for the prolonged life span of these cells. NFATc1 is activated by calcineurin—a calcium-dependent phosphatase. The expression of the short isoform NFATc1/A can be enhanced in an autoregulatory manner owing to binding of NFATc1 to the promoter region P1 (31). As Siglec-G–deficient B1a cells show strongly increased BCR-mediated calcium signaling, this suggests that increased tonic signaling in B1a cells due to the loss of Siglec-G might explain the elevated NFATc1 levels in Siglec-G–deficient B1a cells.

Studies of V_{H4}D_{H4} rearrangements in B1a, B1b, and conventional B cells provide evidence that the pattern of VDJ gene segment usage is different in each of these B cell subpopulations (32, 33). V_{H}12 and V_{H}11 families are used dominantly by B1 cells, consistent with the finding that PtC-binding cells belong exclusively to the compartment of B1 cells. Further, the usage of N insertions is less abundant in B1a cells than in B2 cells. Even though B1a cells use N region insertions less frequently, they show average length of CD3 regions comparable to what is found in B2 cells. As they do not gain length by insertion of N nucleotides, they must acquire it by other means. They use the long J_{H}1 element (19 potential CDR3 nucleotides) more frequently and the short J_{H}2 and J_{H}3 (14 nucleotides) less frequently than do B2 cells (33). In terms of N nucleotide insertion and V_{H}11/V_{H}12 usage, B1a cells derived from adult bone marrow resemble B2 cells rather than normal B1a cells (34, 35). Siglec-G–deficient B1a cells show a markedly altered BCR repertoire. They show a diminished usage of V_{H}11 and V_{H}12 genes paralleled by a decreased percentage of PtC-binding cells. Siglec^{−/−} B1a cells use the JH1 element less frequently and the JH3 element more frequently than do wild-type B1a cells and show an increased use of N insertions. They also display a decreased percentage of PC-binding cells. This indicates that canonical fetal liver-derived B1a cells are outnumbered in Siglec-G–deficient mice by B1 cells of other origin. As repeating sequences were only rarely found in Siglec-G–deficient mice, we can exclude the possibility that the increased B1a cells originate from an expansion of few individual clones. Therefore, we speculate that a higher proportion of the B1a cells in Siglec-G–deficient mice might be derived from adult bone marrow and/or that B cells not designed to become B1a cells are selected into this compartment owing to enhanced BCR signaling. The ELISA experiments revealed disproportionately increased titers of malondialdehyde-modified LDL−/−, malonatealdehyde-modified BSA−/−, Cu^{2+}-oxidized LDL−/−, and 4-hydroxynonenal-modified BSA−/− specific IgM in Siglec-G–deficient mice. Igs of these specificities are among the natural IgM and accumulate during the progression of atherosclerosis (9), but it is not clear whether they are produced exclusively by fetal liver-derived B1a cells. The population of B1b cells could also contribute to these IgM specificities. Siglec-G–deficient B1a cells share several features with normal B1a cells: the surface phenotype (CD5+, Mac1−, CD43−, B220lo), longevity in vitro, and production of natural Abs. However, they differ in respect to other features like the BCR repertoire. It will be interesting to study the functionality of these cells in infection models or mouse models for atherosclerosis.

In summary, in this study we demonstrate that Siglec-G–deficient B1a cells have a prolonged life span, produce increased amounts of NFATc1, and show a changed BCR repertoire in comparison with wild-type B1a cells. The characteristics of the changes in the BCR repertoire of Siglec^{−/−} B1a cells suggest a different selection, possibly from bone marrow precursors.

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**Disclosures**

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**References**


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