**SiglecG Limits the Size of B1a B Cell Lineage by Down-Regulating NFκB Activation**

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**Background.** B1 B cells are believed to be a unique lineage with a distinct developmental pathway, function and activation requirement. How this lineage is genetically determined remained largely obscure. **Methods and Principal Findings.** Using the SiglecG-deficient mice with a knockin of green-fluorescent protein encoding sequence, we show here that, although the SiglecG gene is broadly expressed at high levels in all stages and/or lineages of B cells tested and at lower levels in other lineages, its deletion selectively expanded the B1a B cell lineages, including the frequency of the B1 cell progenitor in the bone marrow and the number of B1a cells in the peritoneal cavity, by postnatal expansion. The expansion of B1a B cells in the peritoneal correlated with enhanced activation of NFκB and was ablated by an IKK inhibitor. **Conclusion and Significance.** Our data revealed a critical role for Siglec G-NFκB pathway in regulating B1a B cell lineage. These data lead to a novel model of B1a lineage development that explains a large array of genetic data in this field.

Citation: Ding C, Liu Y, Wang Y, Park BK, Wang C-Y, et al (2007) SiglecG Limits the Size of B1a B Cell Lineage by Down-Regulating NFκB Activation. PLoS ONE 2(10): e997. doi:10.1371/journal.pone.0000997

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**INTRODUCTION**

An important choice a B cell makes is whether to become B1a B cells that produce poly-reactive natural IgM, B1b cells that make adaptive IgM response, or conventional B2 B cells that primarily mediate T-dependent adaptive immunity [1,2,3]. While extensive studies have revealed critical roles for the B cell receptor (BCR) recognition, such as its self-reactivity, in determining the lineage choices [4,5], accumulating evidence indicate that the size of B1 cells are likely regulated by other factors. For instance, naturally occurring mutations of Ptpn6 which encodes Shp1 leads to dramatic increase of the B1a B cell compartment [6]. Recent studies using lineage-specific deletion of the Ptpn6 gene demonstrated a B cell-intrinsic function of SHP1 in limiting the size of the B1a B cells [7]. On the other hand, targeted mutations of C-Rel and NFκB1, two components of NFκB complex, leads to a significant signaling pathways are used by cell surface molecular interactions to regulate the size of B1 B cell lineage is largely unknown. In particular, targeted mutation of genes encoding several potential membrane proteins that can associate with SHP1, including those of CD72 and CD22, did not substantially impact the development of B1 B cell lineage [9,10,11,12].

Of particular interest are members of the sialic acid-binding, immunoglobulin-like lectin family, or Siglec, many of which have ITIM Motifs for association with SHP1 [13]. Several members of the families, including Siglec 10, the human orthologue of Siglec G, have been demonstrated to be associated with SHP1 [14,15]. While targeted mutation of Sialoadhesin reduced IgM level, this mutation does not appear to affect the B1 B cell lineage [16]. Siglec G and its human orthologue Siglec 10 appear to have several alternative splicing variants [17,18] [14,15]. All variants, however, shared the same cytoplasmic domain with the ITIM motif capable of interacting with SHP-1, which was known to be a negative regulator for B1B cell lineages. Since the function and expression of Siglec G have not been characterized in detail, we produced Siglec G-deficient mice with a knockin of GFP open-reading frame to report its expression. Our results revealed that Siglec G is expressed at high levels at essentially all stages of B cell development and significant but reduced levels in other cell lineages. Importantly, targeted mutation of the Siglec G gene dramatically expanded the B1a B cell lineages postnatally, by increasing NFκB activation. Our data revealed Siglec G is a molecular checkpoint that limits the postnatal expansion of B1a B cells by repressing NFκB activation.

**RESULTS**

GFP-knock-in mice reveal wide-spread expression of SiglecG in multiple lineages

Previous studies using flow cytometry have suggested that Siglec 10 may be expressed in subsets of human leukocytes including eosinophils, monocytes and a minor population of natural killer-like cells [18]. Because of the alternative splicing, it is unclear...
whether these data reflect specific alternative spliced variants. To investigate the cell lineage in which the Siglec locus is active while studying the function of the Siglec in vivo, we replaced essentially all coding exons of the Siglec sequence with a GFP coding sequence, as diagramed in Supplemental Fig. S1A. The genomic DNA clones were isolated from C57BL/6j DNA library for homologous recombination in the ES cells of C57BL/6 origin. The neomycin-resistant ES cell clones were screened for homologous recombination in the ES cells of C57BL/6 origin. DNA clones were isolated from C57BL/6j DNA library for sequence, as diagramed in Supplemental Fig. S1A. The genomic sequence is shown in Fig. S1C.

Since the GFP cDNA is fused into exon 2 of the Siglec gene, its expression is controlled by the regulatory elements of the Siglec locus. We therefore used the expression of GFP to report transcription of Siglec. The gene dose effects are measured by comparing the +/+ and −/− littersmates.

The hematopoietic cells in the bone marrow were identified as FLSK subset, which are Lin− Flk2− Sca1+ and c-Ki6+[19]. The size of the HSC does not differ among the littersmates of the three genotypes (Table 1). Nevertheless, the locus is found in the HSC, albeit at lower levels (Fig. 1A). The Pre-Pro/Pro B cells, as defined by IgM− B220+CD43+ [20,21], expressed higher levels of the Siglec transcripts (Fig. 1B). Among the spleen cells, the B cells (B220+) expressed the highest levels of Siglec (Fig. 1C). However, significant levels were also detected in DC (CD11c+), myeloid cells (CD11b+), and to a less extent, T cells (CD3+) (Fig. 1C). We further analyzed the GFP levels among the major known B cell subsets. As shown in Fig. 1D, essentially all known subsets expressed high levels of Siglec. Interestingly, heterogeneity of GFP levels was found at all subsets of hematopoietic cells studied, starting at the HSC.

Taken together, our data demonstrated that the Siglec locus is transcribed in essentially all the major cell types in the hematopoietic cell types analyzed, although B cells, as a group, seem to have the highest levels.

### Targeted mutation of Siglec leads to preferential expansion of B1a B cells in the peritoneal cavity

Given the wide-spread expression of Siglec, we determined whether targeted mutation of the gene affects the development of different compartments of the hematopoietic cells by flow cytometry. As shown in table 1, the compositions of the major hematopoietic subsets, including HSC, T cells, myeloid cells, dendritic cells and different stages of B cells, were comparable between the WT and the mutant mice. Surprisingly, we have observed a 5-fold increase in the size of B1a cells in the peritoneal, although the number of B1b subset was unchanged (Fig 2A). The number of B1a cells in the spleen, however, did not differ in adult mice (Fig. 2B lower panel and table 1), although a two fold expansion of spleen B1 B cells can be observed at two weeks (Fig. 2B, upper panel).

Corresponding to the expansion of the B1a cells, the levels of serum IgM increased by 5-10 fold (Fig. 2C). With exception of IgG1, which is reduced by approximately 2-fold, the levels of other Ig isotypes was unaffected by Siglec deletion. To determine whether deletion of Siglec affects adaptive B cell responses, we immunized the WT and Siglec-deficient mice with OVA and determined the antibody levels at one and two weeks after immunization. As shown in Fig. 2D, the antibody response was essentially unaffected by the Siglec deletion.

### Table 1. Selective expansion of B1a cells in the peritoneal of the Siglec-deficient mice.

| cell population      | % of cells | siglec−/−   | siglec+/+   |
|----------------------|------------|-------------|-------------|
| Bone marrow          |            |             |             |
| HSC (FLSK) cells     | 0.085 ± 0.05 | 0.094 ± 0.04 |             |
| Prepro/Pro B (CD43+ B220+IgM−) | 2.4 ± 0.30 | 2.81 ± 0.46 |             |
| PreB (fl. D) (CD43+ B220+ IgMlow IgD−) | 2.4± ± 0.39 | 2.15 ± 0.25 |             |
| New B (CD43+ B220+ IgM− IgD+) | 1.36 ± 0.22 | 1.22 ± 0.22 |             |
| Mature B (CD43+ B220+ IgM+ IgD+) | 1.07 ± 0.11 | 1.30 ± 0.19 |             |
| Dendritic cells (CD11c+) | 1.23 ± 0.09 | 1.19 ± 0.10 |             |
| cell number (× 10^6) |            |             |             |
| Spleen               |            |             |             |
| Total B (B220+)      | 42.45 ± 8.55 | 43.99 ± 10.50 |             |
| FO B (B220+CD23+CD21+) | 29.49 ± 3.62 | 32.16 ± 2.66 |             |
| MZ B (B220+CD23−CD21−) | 3.22 ± 0.60 | 2.55 ± 0.55 |             |
| T1 (B220+AA4.1+CD23−IgM+) | 6.07 ± 2.88 | 4.55 ± 1.98 |             |
| T2 (B220+AA4.1+CD23+IgM+) | 4.05 ± 1.77 | 4.89 ± 2.12 |             |
| B-1a (B220+CD43+CD5−) | 1.97 ± 0.36 | 2.12 ± 0.39 |             |
| Macrophages (CD11b+) | 4.97 ± 2.02 | 5.55 ± 1.99 |             |
| Dendritic cells (CD11c+) | 1.32 ± 0.22 | 1.16 ± 0.33 |             |
| T cells (CD3+)       | 23.02 ± 6.99 | 27.60 ± 8.60 |             |
| Peritoneal cavity    |            |             |             |
| B-1a (B220+CD11b+CD5−) | 2.41 ± 0.51 | 0.46 ± 0.16 |             |
| B-1b (B220+CD11b−CD5−) | 1.07 ± 0.25 | 1.25 ± 0.33 |             |
| T cells (CD3+)       | 0.97 ± 0.11 | 1.11 ± 0.29 |             |

*The cell subsets were phenotyped as described [27]. For BM HSC cells, N = 3. For spleen and peritoneal cavity T cells, N = 6. For all other cells, N = 12. doi:10.1371/journal.pone.0000997.t001
Postnatal expansion of B1a progenitors in the Siglec-/- mice

It is generally believed that the B1a progenitors give rise to mature B1a cells during fetal development and that self-renewal of the B1a cells after birth explains the long-life persistence of the B1a lineage [3]. More recently, a low number of B1a progenitor cells were identified in mature bone marrow [22]. In order to explain the drastic difference in the number of B1a cells in the peritoneal cavity, we carried out BrdU labeling to determine the proliferation of the B1a cells. As shown in Fig. S2, at two weeks, when a significant difference was observed in the % of B1a B cells found in the spleen, the rate of BrdU incorporation was the same in the two groups. Conversely, at 10 weeks, when no difference in the B1a subsets was observed in the spleen, there appeared to be a higher rate of BrdU incorporation in the siglec-deficient mice. Furthermore, in the peritoneal cavity, where a dramatic difference of the number of B1a cells were found, the rate of BrdU incorporation was essentially identical. Thus, while our data did not rule out the possibility that Siglec may regulate B1a proliferation in specific locations, such function does not account for the differential accumulation of B1a cells in the Siglec-deficient mice. Likewise, the % of Annexin V+ cells was also comparable (Fig. S3). Thus, neither increased self-renewal nor decreased apoptosis of mature B1a cells explain the expansion of the B1a compartment.

To explore the possibility that increased number of B1a cells may be due to the expansion of the B1 progenitors, we compared the number of the progenitors in the fetal liver and adult bone marrow. The Lin-Flt2-Sca-1-C-Kit+ hematopoietic stem cells (HSC), while the right panel depicts the GFP intensity of the gated HSC. B. Expression of the Siglec locus in the pre-Pro/Pro B cells. As in A, except that the IgM CD43+ B220lo cells were depicted. C. Transcription of the Siglec locus among splenic B cells (B220+), myeloid cells (CD11b+), dendritic cells (CD11c+) and T cells (CD3+). The top panel shows summary data, while the lower panels show representative FACS profiles. D. The Siglec locus is actively transcribed at all stage/lineages of B cells, as in C. Data shown in this Figure represents 2 mice per group, and has been repeated twice. doi:10.1371/journal.pone.0000997.g001

Figure 1. Transcription of the Siglec locus as determined by the expression of GFP which was used to replace the coding sequence of Siglec. Red, WT; green heterozygous; blue, homozygous. A. Low levels of GFP expression among the hematopoietic cells. The left panel shows the profiles of Lin-Flt2-Sca-1-C-Kit+ hematopoietic stem cells (HSC), while the right panel depicts the GFP intensity of the gated HSC. B. Expression of the Siglec locus in the pre-Pro/Pro B cells. As in A, except that the IgM CD43+ B220lo cells were depicted. C. Transcription of the Siglec locus among splenic B cells (B220+), myeloid cells (CD11b+), dendritic cells (CD11c+) and T cells (CD3+). The top panel shows summary data, while the lower panels show representative FACS profiles. D. The Siglec locus is actively transcribed at all stage/lineages of B cells, as in C. Data shown in this Figure represents 2 mice per group, and has been repeated twice.
littermates (Fig. 3C). In either compartment, the B2 progenitors were not affected by siglecg mutation (Fig. 3A–C).

To determine whether the adult bone marrow from the siglecg-deficient mice have increased tendency to reconstitute the B1a compartment, we mixed equal numbers of total bone marrow cells from Siglecg<sup>+/+</sup> (CD45.1) and the Siglecg<sup>2/2</sup> (CD45.2) mice, and adoptively transferred them into lethally irradiated WT or Siglecg<sup>2/2</sup> host. Unlike the mice with B cell-specific deletion of Ptpn6<sup>[7]</sup>, no expansion of B1a cells was found in the bone marrow was found in the Siglecg<sup>2/2</sup> mice (Fig. 4A), therefore no effort was made to delete the B1a cells from bone marrow. Thirteen weeks after bone marrow transplantation, the bone marrow, peritoneal cavity and spleen were harvested and analyzed by flow cytometry. As shown in Fig. 4A, regardless of the recipient genotypes, the Siglecg<sup>2/2</sup> bone marrow were more efficient in reconstituting the B cell compartment, including Pre-ProB/Pro and Pre/new B cells, and the total number of B cells. In contrast, bone marrows from the two genotypes were comparably efficient in reconstituting the myeloid compartment.

Importantly, in the peritoneal cavity, the number of Siglecg<sup>2/2</sup> B1a cells was 2–3 fold more abundant than that from the WT bone marrow, while the number total B cells were not significantly different (Fig. 4A lower panel and Fig. 4B). In addition to the difference in the total number, an interesting difference in the cell surface markers was also observed. Thus, the majority of the B1a cells derived from WT bone marrow expressed high CD5, essentially all of the B1a cells from the mutant bone marrow exhibited low levels of CD5. The increased number of progenitor cells in the adult bone marrow, but not in fetal liver, and over-presentation of mutant B1a cells in the peritoneal cavity strongly suggests that the expansion of the B1a cells in the peritoneal cavity of the Siglecg<sup>2/2</sup> mice was due to postnatal expansion of the B1a cells...
cells, perhaps due to increased frequency of B1a progenitor cells. Interestingly, unlike the peritoneal cavity, the number of B1a cells in the spleen is mainly derived from the WT donor (Fig. S5). Such phenotype is reminiscent of a recently reported phenotype associated with SHP-1 mutation in the B cell compartment [7].

**Activation of NFκB is essential for the expansion of the B1a compartment in the peritoneal cavity of the Siglec^−/− mice**

We compared ex vivo WT and Siglec-deficient peritoneal cavity cells for activation of a number of signal transduction pathways, including phosphorylation of Akt, Stat 3, Stat 5, P38, Erk and JNK. Our extensive analyses fail to reveal a significant difference between the WT and mutant cells (Data not shown). Since mutation of Ptpn6, which associate with Siglec orthologue Siglec 10, enhanced NFκB activation and expanded B1a compartment [6], and since mice with targeted mutation of components in NFκB pathway show selective reduction of B1a B cell compartment [8], we focused on the potential impact of Siglec deletion on NFκB activation. As shown in Fig. 5A, ex vivo peritoneal lavage from Siglec-deficient mice show greatly increased IκB phosphorylation in the cytosol and nuclear accumulation of P65, as demonstrated by Western blot (Fig. 5A). This corresponds to greater binding to NFκB probe as revealed by mobility shift assays (Fig. 5B). Furthermore, the increased NFκB function was revealed by luciferase assay when the peritoneal cells from WT and Siglec-deficient mice were compared (Fig. 5C).

The increased IκB phosphorylation indicated enhanced IKK activity in the B1a cells from the Siglec^−/− peritoneal cavity. To determine whether the activation of NFκB is essential for the expansion of the B1a compartment, we treated the 5-days old Siglec-deficient mice with IκB kinase complex (IKK) inhibitor VI, which we have demonstrated to work efficiently in the mice [23]. As shown in Fig. 6A, consecutive treatment resulted in greatly decreased IκB phosphorylation and nuclear P65 accumulation. Importantly, the percentage and number of B1a cells in the peritoneal cavity was reduced by 3–4 folds in the treated group. In contrast, despite effective inhibition of IKK, the number of B1a cells in the spleen was unaffected by the inhibitor (Fig. 6B).
results reveal that activation of the NFκB pathway is essential for selective expansion of the B1a cells in the peritoneal cavity of the Siglec-2−/− mice.

**DISCUSSION**

A long-standing observation in immunology is the preferential accumulation of B1a B cells in the peritoneal cavity. Given the importance of the B1a B cells in innate immunity and autoimmune diseases [1,2,3], the mechanisms that determine the size of B1a B cell subsets in the locality are of great interest. Using mice with targeted mutation of the Siglec gene, we demonstrate that Siglec controls B1 B cell expansion in the peritoneal cavity by repressing NFκB activation.

**Siglecg controls a checkpoint for peritoneal B1a B cell expansion**

We have observed mice with targeted mutation of Siglecg that had a 5–10 fold increase in serum IgM, while those of most other Ig isotypes were roughly normal. In search for a cellular basis for the over-production of IgM, we uncovered the dramatic expansion of the B1 B cells in the peritoneal cavity. Of the two subsets of B1 B cells in the cavity, the expansion is limited to the CD11b+ B1a subsets. Moreover, while the expansion of the B1a B cells in the peritoneal cavity were observed throughout the study, the expansion of the B1 B cells in the spleen is only transient, observable at 2 weeks but not 10 week.

Since no reagent is currently available to study the protein expression for mouse Siglecg, we used a GFP knockin transgenic line to determine the expression of Siglecg. Our data indicated that while cells among B cell lineage had the highest levels of Siglecg expression, essentially all major subsets of hematopoietic cells examined, including T cells, DC, and monocytes show active transcription of the locus. Despite the wide-spread expression of the gene, the impact of Siglecg deletion appear to be limited to the B1a compartments in the peritoneal cavity, although a smaller expansion of the B1a cells in the spleen can be observed at 2 weeks of age.

While our manuscript was in preparation, Hoffmann et al. [24] reported a Siglec-deficient mouse prepared with ES cells from the BALB/c. While they observed expansion of B1a cells in the spleen...
and peritoneal cavity, the B1a expansion observed in our study is more selective. The difference in background genes between the two strains of mice may account for the difference.

Postnatal expansion of B1a progenitors and expansion of B1 B cells in the Siglecg-deficient mice

While the B1a B cells are generally believed to have been produced from progenitors in fetal liver [3], recent studies identified B1a progenitors in adult bone marrow [22] [25]. Therefore, the expansion of the B1a B cell compartment can theoretically be attributed to either increased fetal production or postnatal production. Our analysis of adult progenitor cells in the bone marrow and fetal livers indicated a substantial increase in the B1a B progenitors in the bone marrow, but not in the fetal or new born liver. Consistent with the increased B1a B progenitor cells, the Siglecg-deficient bone marrow are more efficient in constituting the B1a B cells. These data, together with the fact that neither increased proliferation nor decreased apoptosis explains the expansion of B1a B cells in the peritoneal cavity, supports the notion that postnatal expansion of the B1a compartment is responsible for the expansion of B1a cells in the Siglecg−/− mice. Therefore, our data demonstrate a novel function of Siglecg in regulating postnatal expansion of the B1a compartment. It remains to be determined as to whether the expansion is intrinsic to genetic defects in the precursor cells, or due to Siglecg defects in other cell types.

It is worth pointing out that although the number of progenitors for B2 cells were not increased, the siglecg-deficient bone marrow was more efficient in reconstituting B cells than the WT bone marrow when they were mixed with WT bone marrow cells. These data suggest that in addition to the number of B1 B progenitors, Siglecg exerts a general repressive effect on the differentiation of the B cell compartment. Such requirement, however, seems to be reflected in competition with progenitor cells from the WT host, but not with other cell lineage, as the relative number of B cells are the same when the Siglecg-deficient mice were analyzed.

**Siglecg is a negative regulator of NFκB activity in peritoneal B cells: similarity with Shp1**

Our comparison between WT and Siglecg-deficient ex vivo peritoneal lavage revealed that activation of a number of signaling pathways, including AKT, STAT, Erk, P38 and JNK was not affected by the mutation (data not shown). In contrast, a profound activation of NFκB was observed in the peritoneal lavages from the Siglecg-deficient mice in comparison to that from WT mice, as indicated by increased phosphorylation of IκB, nuclear localization of P65, increasing mobility shift of the NFκB probe and increased promoter activity when the NFκB reporter constructs were used to transfect the peritoneal lavage cells. Since the activation correlates with the IκB phosphorylation, it is likely that the conical NFκB pathway is being activated. Moreover, since the IKK is responsible for IκB phosphorylation, we used the IKK inhibitor VI to block NFκB activation in order to study the significance of this activation in B1a cell expansion in the peritoneal cavity. Our data clearly demonstrated that activation of NFκB is responsible for the expansion of the B1a cells in the peritoneal cavity. Recently, Hoffmann et al. reported that mutant B cells display increased Ca2+ signaling in responses to several stimuli in vitro [24]. Since it is unclear if these stimuli are responsible for the B1a expansion in vivo, the significance of the enhanced Ca2+ response remains to be demonstrated.

Interestingly, despite the significant effect of the IKK inhibitor for spleen cells, no increase in the spleen B1a B cells were observed. These data clearly indicated that expansion of B1a B cells in the peritoneal cavity uses a mechanism that is not employed to maintain the number of spleen B1a B cells. The selective expansion of B1a B cells from the mutant peritoneal cavity suggests that the expansion requires both unique checkpoint in the specific cell lineage and specific stimuli in the peritoneal cavity. Moreover, we have observed no effect of IKK inhibitor on BrdU incorporation in B1a cells (data not shown). However, since our data also indicated that the expansion cannot be demonstrated by BrdU incorporation (Fig. S2), the cellular basis for the increase remains to be determined.

Recently, Rajewsky and colleagues demonstrated that targeted mutation of Shp1 in the B cell lineage causes expansion of B1a B cells in the peritoneal cavity [7]. Remarkable similarity can be found between the germline mutation of the Siglecg and B-cell specific deletion of Pipαb. Firstly, in terms of lineage size, the impact of mutation is limited within B1a B cells in the peritoneal cavity. Secondly, the expansion of B1a subsets is due to the postnatal expansion of B1a cells. Thirdly, in bone marrow chimeras...
mice, the mutant bone marrow shows a remarkable advantage in reconstituting the peritoneal B1a B cells, while displaying a disadvantage in constituting spleen B1a B cell compartment. These similarities in functional defects raise the possibility that the gene has a similar function within B cell compartment. Since Siglec10, the Siglec G orthologue in human, has been shown to be associated with Shp1[14,15], the simplest hypothesis is that Shp1 works down-stream of Siglec G in limiting the controlled expansion of B1a subset. This hypothesis has the potential to unify observations made from mice with genetic defects of Siglecg, Ptpn6, and NFκB1 and C-rel. Since mutation of Siglecg increases the titer of anti-DNA antibodies [24] and since deletion of Shp1 in B cells causes autoimmune diseases [7], the balance of the Siglecg signaling will likely be important in the proper tuning of the B1a function in innate immunity against infection vs. autoimmune side effect. Given recent advances in pharmaceutical targeting of the NFκB pathway [23], our study suggests a new approach in selective tuning of innate immunity and autoimmunity.

MATERIALS AND METHODS
Generation of Siglecg<sup>−/−</sup>GFP<sup>+/+</sup> mice using C57BL/6 ES cells

The production of the Siglecg<sup>−/−</sup>GFP<sup>+/+</sup> mice is carried out by the Ingenious Research Laboratory, Inc. (Long Island, New York) under a research contract. Detail information is provided by Fig. S1. Briefly, a ~9.8 kb region used to construct the targeting vector was first sub cloned from a positively identified BAC clone using a homologous recombination-based technique. The region was designed such that the short homology arm (SA) extends 1.8 kb 3' to exon 11. The long homology arm (LA) ends in exon 2 just before the ATG and is 8.8 kb long. The GFP/Neo cassette (Neo is flanked by both loxP and FRT sites) is inserted before the ATG of Exon 2 and replaces 5 kb of the gene sequence including exons 3–11.

Ten micrograms of the targeting vector was linearized by AscI and then transfectednow to electroporation of iTL C57/Bl6 embryonic stem cells. After selection in G418, surviving clones were expanded for PCR analysis to identify recombinant ES clones. Primers, A1, 2 and 3 were designed downstream (3') to the short homology arm (SA) inside the region used to create the targeting construct. This amplifies a band of 1.7 kb. As shown in Fig. S1B, Individual clone was screened with A1/N1, A2/N1 and A3/N1 primers. ES clone #361 was identified as a recombinant clone and injected into BALB/c blastocytes. The chimera mice were bred to B6 mice to obtain F1 mice, which were intercrossed to generate Siglecg<sup>−/−</sup>GFP<sup>+/+</sup> mice, and their WT and heterozygous littermates.

Figure 6. IKK inhibitor treatment decreased B-1a cells in peritoneal cavity but not spleen. (A). Efficacy of the treatment in reducing IKK activity among total spleen cells as revealed by IκBa phosphorylation in the cytosol (upper panel) P65 accumulation in the nuclei (lower panel) of spleen cells from treated or control mice. B. The impact of inhibiting the IKK on peritoneal B1a subsets. Data shown are means±SEM involving 5–6 mice per group. doi:10.1371/journal.pone.0000997.g006
Isotype specific ELISA
Serum immunoglobulin titers were determined by enzyme-linked immunosorbent assay (ELISA) using BD Falcon plates (BD biosciences) coated with goat anti-mouse immunoglobulin (2 µg/ml; Southern Biotechnologies, Birmingham, AL) diluted in sodium bicarbonate buffer (pH 8.2). Standard curves of each Ig isotype were generated with purified mouse IgM, IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnologies). Plates were blocked with 1% non-fat milk in PBS for 1 h at room temperature, and sera were diluted in blocking buffer (1% non-fat milk in PBS) (IgM and IgG1, 1:10,000; IgG2a, IgG2b and IgG3, 1:5,000) and allowed to bind to the plate for 2 h at room temperature. Horseradish isotype-specific antibodies (Southern Biotechnologies) were then added after washing with 0.05% Tween 20 in PBS and then substrate Sigma Fast OPD (Sigma Aldrich, St. Louis, MO). Optical densities at 490 nm were measured with a fluorescence plate reader (SpectroMax 190, Molecular Devices, Toronto, Canada).

Flow cytometry
Single-cell suspensions were prepared from tibial bone marrow, spleen and peritoneal cavity from mice euthanized with CO2. Cells were suspended in RPMI 1640. Prior to staining, red cells in spleens were lysed (BD Pharm Lyse, BD biosciences). Cells were then suspended in PBS with 2% FBS and 1% sodium azide for staining. Cells were surface stained for 30 minutes at 4°C and intracellular staining of BrdU is done following manufacturer (BD Pharmingen) provided protocol. Cells were then suspended in staining buffer and transferred for flow cytometry (BD LSR II, BD biosciences). Analysis of the flow cytometry data was done with Flowjo 7.2. Antibodies used were either purchased from BD Pharmingen (La Jolla, CA): B220, APC-Cy7 streptavidin, c-kit, Sca-1, CD21, CD5, CD19, and Lin markers, CD3, Gr-1, CD11b, Pharmingen (La Jolla, CA); CD24, CD44, CD49b, CD138, CD90, CD11c, and CD11b, from Biolegend, and CD11b, CD11c, CD49b, from BD Pharmingen. Cells were then stained for 30 minutes at 4°C and intracellular staining of BrdU is done following manufacturer (BD Pharmingen) provided protocol. Cells were then suspended in staining buffer and transferred for flow cytometry (BD LSR II, BD biosciences). Analysis of the flow cytometry data was done with Flowjo 7.2. Antibodies used were either purchased from BD Pharmingen (La Jolla, CA): B220, APC-Cy7 streptavidin, c-kit, Sca-1, CD21, CD5, CD19, and Lin markers, CD3, Gr-1, CD11b, Pharmingen (La Jolla, CA); CD24, CD44, CD49b, CD138, CD90, CD11c, and CD11b, from Biolegend, and CD11b, CD11c, CD49b, from BD Pharmingen.

Western blot
Cells were lysed by regular lysis buffer containing 1% Triton-X100, 1:100 diluted protein and phosphatase cocktails (both from Sigma). Nuclear and cytoplasmic proteins were isolated by a nuclear and cytoplasmic protein extraction kit (Pierce Biotechnology, Rockford, IL). Antibodies for western-blot are phospho-antibodies of p-AKT, p-GSK3α/β, p-p38, p-p42/44, p-p-AKT, p-p-Stat3, and p-Stat-5, all from Cell Signaling, from the ratio (2.5×10^6 each) and were injected into lethally irradiated (1100 RAD) WT or Siglec-G−/− mice. The bone marrow, spleen, lymph node and peritoneal lavages were harvested and analyzed by flow cytometry.

Statistics
The statistical significances of observed differences were analyzed by student t-tests. *, 0.05>P>0.01; **, 0.01>P>0.001; ***, P<0.001.

SUPPORTING INFORMATION

Figure S1

Generation of SiglecG−/− mice that expressed green fluorescence protein (GFP) under the control of SiglecG regulatory sequence. A. Diagram of construct (top), siglecG genomic structure (middle) and the recombinant knock-out/in allele (bottom). Top), diagram of the knock-out/in construct. LA, long arm that ended before the SiglecG coding sequence in exon 2, SA, short arm consisting of 1.8 kb intron 2 sequence. GFP coding sequence is linked to exon 2 with its own stop codon and polyadenylation sites. Neo sequence is transcribed from the opposite direction. Middle, genomic structure, shaded area indicates coding sequence from exon 2 to 12. Bottom, the structure of the knock-in allele.

The primers used are marked. B. Verification of homologous recombination by PCR. Data shown were for clone 361, which was used to generate the knockout/in mouse. Integration of the construction is confirmed by AT1/N1 primer pair, while integration is confirmed by A1/N1, A2/N1 and A3/N1 pairs.

NFκB activity reporter assay
Peritoneal cells extracted from WT and the SiglecG−/− mice were mixed at a 1:1 ratio (2.5×10^6 each) and were injected into lethally irradiated (1100 RAD) WT or SiglecG−/− mice. The bone marrow, spleen, lymph node and peritoneal lavages were harvested at 13 weeks after irradiation and analyzed by flow cytometry.

In vivo treatment with IKK inhibitor

Bone marrow chimera
Bone marrow cells from WT and the Siglec−/− mice were treated with 0.01 mg of IKK inhibitor VI (Calbiochem, San Diego, CA) intra-peritoneally twice daily for 8 days and spleen and peritoneal cavity cells were harvested for analysis.

In vivo treatment with IKK inhibitor

5-day old siglecG−/− mice were treated with 0.01 mg of IKK inhibitor VI (Calbiochem, San Diego, CA) intra-peritoneally twice daily for 8 days and spleen and peritoneal cavity cells were harvested for analysis.

Figure S2

Peritoneal proliferation of mature B1a cells does not explain the expansion of B1a compartment in the SiglecG-deficient mice. A. Proliferation of splenic B1 B cells of 2- (top) and 10- (bottom)-weeks old mice. B. Proliferation of peritoneal B1a B cells from 10 week old mice. Two or 10 weeks-old SiglecG−/− and SiglecG+/+ mice were injected (ip) with 1 mg of BrdU 27 and 5 hours before sacrifice. Spleen and peritoneal B-1 proliferation was used as internal control for monitoring transfection efficiency.

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is analyzed by BrdU incorporation. The gates used were shown in the left. Profiles of BrdU incorporation is shown in the middle, while the summary data are shown in the right. Data shown are representative of 3 independent experiments.

**Figure S3** Siglec-g does not control apoptosis of the B1B cells. Data shown are histograms depicting annexin V staining of ex vivo splenic (A) or peritoneal B1a B cells of 10-week old mice. The gates applied are the same as Fig. S2.

**Figure S4** No increase of B1a B cells in bone marrow in the Siglec-g/− mice.

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