SIGLEC-G IS A NEGATIVE REGULATOR OF NF-κB ACTIVATION
AND HAS PIVOTAL ROLES IN B-1 CELL DEVELOPMENT AND
RESISTANCE TO SEPSIS

DISSERTATION

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ABSTRACT

The Siglescs (sialic acid binding immunoglobulin-like Lectins) are I-type lectins which bind to sialic acid bearing molecules and convey a wide array of information in the immune system. Siglec-G, the mouse homologue of human siglec-10, is a member of this family. Using a Siglec-G knock out GFP knock-in model, we have found Siglec-G to be expressed on a variety of hematopoietic cells, with high levels on B cells and moderate levels on myeloid cells. Siglec-G deficient mice exhibit a dramatically expanded B-1a population in the peritoneal cavity. Blood IgM level is five folds higher in Siglec-G deficient mice. The enlarged peritoneal B-1a compartment results from post-natal expansion as the B-1a precursor cells have higher frequencies in adult Siglec-G knock out mice bone marrow but not in fetal liver. Bone marrow chimera studies showed that Siglec-G deficient bone marrow cells have competitive advantage in reconstituting peripheral B cell populations in the peritoneal cavity, but not in the spleen.

We have also found Siglec-G plays a key role in sepsis introduced by cecal ligation and puncture (CLP). Compared with wild type controls, Siglec-G−/− mice are more susceptible to CLP. Siglec-G−/− mice also showed more severe
bacteremia and systemic damages, which led to accelerated deaths. Proinflammatory cytokines, especially TNF-α and IL-6 are significantly elevated in Siglec-G-/- mice post-CLP.

Siglec-G-deficient peritoneal lavage cells contain more nuclear p65 and total phosphorylated p65 evidenced by western blot and ELISA, respectively. Gel shift assay also demonstrated higher accumulation of nuclear p50/p65. Therefore, Siglec-G works as a negative regulator of NF-κB. By using IKK inhibitor to block the NF-κB pathway, we blocked the expansion of peritoneal B-1a cell at early stage postnatally. Thus, Siglec-G controls the expansion of peritoneal B-1a cells by repressing NF-κB. Likewise, inhibition of NF-κB activation resulted in dramatically reduced proinflammatory cytokine productions and significantly improved survival rate of Siglec-G mutant mice. Taken together, our data demonstrated that Siglec-G is a novel negative regulator for NF-κB activation and controls B-1a B cell expansion and host resistance to sepsis.
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Dedicated to Rui and my family
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CHAPTER 1

INTRODUCTION

1.1 SIGLEC

1.1.1 Introduction

In the vertebrates, major classes of macromolecules are proteins, nucleic acids, lipids and polysaccharides (also called glycans). Biological functions depend on the interactions among these molecules. The precise modulation of the biologic system relies on the diversity and specificity of macromolecular interactions. Protein-protein interactions, such as antigen-antibody and TCR/BCR bindings have been extensively studied to possess both diversity and specificity. However, glycan-protein and glycan-glycan interactions have been far less explored, although their interactions are also crucial in the biological processes.

Carbohydrate-binding proteins can be generally divided into two classes: the lectins [1] and sulfated glycosaminoglycan-binding proteins [2]. Lectins can also be subdivided into C-type, P-type, I-type, etc, based on the characteristics of glycan-recognition domains [1]. Immunoglobulin superfamily (IgSF) molecules
are I-type lectins and serve as recognition molecules in the immune system.
Possessing the immunoglobulin (Ig) domain with combinatorial diversity, IgSF molecules virtually bind to an infinite number of structures on cell surface [3-5], just as the diversity and specificity achieved by antibody, TCR and BCR. A subfamily of IgSF, called SIGLEC (sialic acid binding immunoglobulin-like lectins), is I-type lectins which bind to sialic acid bearing molecules and transmits a wide array of information in the vertebrates [6, 7].

1.1.2 Two sub-families of SIGLEC

To date, there are 13 members of Siglecs discovered in human and 8 members in mouse. Siglecs can be divided into two groups: an evolutionarily conserved group consisting of Sialoadhesin (Sn), CD22 and Myelin-associated glycoprotein (MAG) in both primates and rodents; and a group of CD33/CD33-related Siglecs which are comprised of CD33, Siglec-5, 6, 7, 8, 9, 10, 11, 14 in primates and CD33, Siglec-E, F, G, H in rodent [8].

Sialoadhesin (also known as Siglec-1 and CD169), CD22 (also known as Siglec-2), and MAG (also known as Siglec-4) have orthologs in multiple species, are highly conserved domain structures, and are cell type restricted, with Sn on macrophages, CD22 on B cells and MAG on glial cells. In comparison, the CD33-
related Siglecs are less evolutionarily conserved. Clear-cut orthologs are difficult
to assign between human and mouse as there are 9 CD33-related Siglec
discovered in human but only 5 in mouse. In contrast with the Sn/CD22/MAG
group, CD33-related Siglecs are poorly conserved, and they are expressed on
variable cells in the immune system [Table 1.1]

1.1.3  Structural features

All Siglecs are type 1 trans-membrane proteins containing extracellular
domains with one or two N-terminal V-set Ig domain, followed by variable
numbers of C2-set Ig domains, ranging from 1 in CD33 to 16 in Sialoadhesin [9-
11]. Most Siglecs have two or more intracellular tyrosine-based signaling motifs
[12]. The cytoplasmic tails of CD33-related Siglecs contain one conserved
immunoreceptor tyrosine-based inhibitory motif (ITIM) and one ITIM-like motif
[13].

1.1.4  Cell-type specific expressions

The Sn/CD22/MAG group expression is conserved among multiple
species. In both human and mouse, Sn is expressed on macrophages, CD22 on B
cells and MAG on oligodendrocytes and Schwann cells, suggesting discrete
functions [6]. The expressions of CD33-related Siglecs are poorly conserved among mammalian species studied so far, are variable in the immune system. For example, in human, CD33 is expressed on myeloid progenitors while in the mouse, CD33 is found on neutrophils [7]. The human and mouse Siglec orthologs and their expressions are summarized in Table 1.2.

1.1.5 **Cis and Trans ligands**

Siglecs, as their names suggest, bind to sialic acid (Sia)-bearing structures. The potential ligands for Siglecs: sialylated glycans are extremely abundant in the mammalian system. Functionally, the Sia-Siglec bindings have been suggested to be important for cell adhesion and signaling [14]. Due to the high density of sialylated structures on the cell surface, the Sia can be so overwhelming that Siglecs are “masked” by Sia on the same cell surface [15]. This indicates that Siglecs can convey information to the same cells (cis) they are expressed. In spite of the seemingly important cis interaction, Siglecs also bind to Sia-containing structures on other cell surfaces and transmit information in trans fashion [15]. For example, CD22 on B cells can redistribute and bind to CD22 ligands expressed by other cells at the sites of contact [16], suggesting cis interaction with Sia could downregulate but does not prevent trans binding to its ligands and
bindings of Siglecs to their \textit{trans} ligands occur dynamically in the presence of \textit{cis} ligands [16].

\subsection*{1.1.6 Intracellular signaling}

Most of Siglecs have immunoreceptor tyrosine-based motif (ITIM) or ITIM-like motifs in their cytoplasmic tails, with the exceptions of Sn, MAG and Siglec-14 in human and Sn, MAG, CD33 and Siglec-H in mouse, which have very short intracellular domains [17]. The cytoplasmic ITIM and ITIM-like motifs are generally believed to be have inhibitory functions and counteract with receptors with immunoreceptor tyrosine-based activation motifs (ITAM) [17]. ITIM, once phosphorylated by a \textit{Src} family tyrosine kinase, can recruit \textit{Src} homology region 2 (SH2) domain-containing phosphatase SHP-1 and SHP-2, as well as the SH2-domain-containing inositol polyphosphate 5-phosphatase (SHIP) [18, 19].

\subsection*{1.1.7 Biological functions in human and mouse}

To study the functions of Siglecs, a number of genetically manipulated mice were generated. For example, Sialoadhesin knock out mice demonstrated subtle changes in B and T cell populations and reduced immunoglobulin M level
[20], resistance to experimental autoimmune uveoretinitis [21], and amelioration of myelin degeneration and axonopathic in the central nervous system in proteolipid protein (PLP)-overexpressing mice [22]. Deficiency of CD22 has been shown to affect B cells restrictively [23, 24], with phenotypes consistent with enhanced BCR signaling. Studies of MAG-deficient mice confirmed that it plays an important role in the myelin formation and maintenance [25]. With the CD33-related Siglecs, CD33-null mice did not exhibit overt phenotype [7]. This may be because mouse CD33 lacks the cytoplasmic ITIM. Knock out of Siglec-F exhibited hypersensitivity/allergy in mouse [26].

Besides the genetically manipulated mice phenotypes, a number of functions of Siglecs have also been found and proposed. Sialoadhesin and CD33-related Siglecs are found primarily on cells of the innate immune system suggest that is where their primary functions lie. The fact that most Siglecs, especially the CD33-related Siglecs possess the cytosolic ITIM and ITIM-like domains explains their inhibitory function of cellular functions, that is, down-regulation of innate immune cell reactivity. In keeping with this notion, siRNA studies of CD33 on human monocytes have demonstrated the “suppressor activity” of hCD33 [27]. In analyzing the probable functions of Siglecs, it is worth considering that the Sia-containing molecules can be either on the same cell surface (cis) or on other cell
surfaces (*trans*), thus making possible cell-cell interactions among Siglec-positive cells and among Siglec-positive and Siglec-negative cells.

A number of other functions of Siglecs include inhibition of cell growth of hCD33 in acute myelocytic leukemic (AML) cells [28], inhibition of monocytes cytokine release by of hCD33 [27], induction of apoptosis in eosinophils of Siglec-8 [29], inhibition of antibody-directed cytotoxicity [30] and cytotoxicity to GD3-expressing targets [31], etc. Except for CD22, whose functions are more clearly defined, the functions of the Siglecs remain largely unknown.

1.2 B-1 B cells

1.2.1 B-1 cells, their location and surface markers

Peripheral B lymphocytes can be broadly divided into three subsets: follicular, marginal zone and B-1 B cells. Originally identified as CD5⁺, B-1 cells comprise 1-5% of peripheral B cells and reside in a variety of tissues. The majority of B-1 cells are found in body cavity serosa, including peritoneal and pleural cavities [32]. And a smaller numbers of B-1 cells are in the spleen [33]. Most of the publications on B-1 cells have been focused on peritoneal B-1 cells due to their large number and ease of access. Functionally, B-1 cells are involved
in secretion of natural antibodies (IgM), pathogen host defense, autoimmunity and leukemia [34-36].

B-1 cells are distinct from conventional B cells (also called B-2 cells) by their surface markers. Over 20 years ago, CD5 has been identified as a surface marker for a subset of B cells, later found to be B-1 cells [37], although further work with that B cell population revealed some cells, especially those in the peritoneal cavity lacked CD5 expression [38]. In general, B-1 cells are CD45R(B220)lo, IgMhi, IgDlo, CD43+ and CD23lo, in contrast to conventional B cells that are CD45R(B220)hi, IgMhi/lo, IgDhi, CD43- and CD23hi [39]. Interestingly, peritoneal B-1 cells also express myeloid marker CD11b [40] and can be further subdivide into CD5+ B-1a and CD5- B-1b cells [41, 42].

1.2.2 B-1 cell development and progenitors

During the embryonic life, fetal liver is the main site for hematopoiesis, including B cell production. After birth, subsequent hematopoiesis takes place in bone marrow and lasts throughout adult life. Numerous cell transfer studies have demonstrated that adult bone marrow and fetal liver have different capacities in reconstituting B-1 and/or B-2 cells. Bone marrow transfer studies have shown that adult bone marrow is very inefficient in generating B-1 cells. On the contrary,
many cells in fetal liver produce B-1 cells [43-45]. These studies suggest B-1 and B-2 cells arise from different progenitors.

Although the majority of B-1 cells are derived from fetal liver progenitors, the adult bone marrow still retains the capability to generate B-1 cells [46, 47]. Recently, Encarnacion, et al described the phenotypes of B-1 and B-2 precursors in adult bone marrow [48]. They demonstrated AA4.1+Lin-CD45Rlo/negCD19+ cells to be able to efficiently generate functional sIgMhiCD11b+CD5+ B-1a cells or sIgMhiCD11b+CD5- B-1b cells, but not sIgMhiCD11b- B-2 B cells by bone marrow transplantation studies. And the adult bone marrow B-2 precursors mainly reside in the population characterized by AA4.1+Lin-CD45R+CD19- [48].

Development and maintenance of B-1 cells depends on B cell receptor (BCR) signaling, whose mutation leads to depletion of B-1 cell compartment, particularly B-1a cells, but with intact B-2 cells [39, 49]. Similarly, knockouts of molecules involved in BCR signaling also resulted in loss of B-1 cell population. These BCR-associated molecules include CD45 [50], BtK [51], BLNK [52], and PLC-γ [53]. In contrast, deletion of the intracellular negative regulator SHP-1 leads to massive expansion of B-1 cell [54]. However, deletion of another B cell negative regulator, CD22 does not have any effect on B-1 population [55].
1.2.3 Functions of B-1 B cells

The primary discovered function of B-1 cells is their role in antibody secretion (mainly IgM), resulting in bacterial clearance and autoimmunity [39, 56]. Antibody repertoire produced by B-1 cells tends to be more restricted than that produced by B-2 cells [57]. Furthermore, at the times of adaptive response, with the T helper cells, B-2 cells undergo somatic hypermutations of immunoglobulin genes. In contrast, B-1 cells are largely responsible for innate immune response, express germline immunoglobulin genes, and respond to T-independent antigens [58, 59]. In response to bacterial infection, B-1 cells rapidly migrate to spleen where they divide and differentiate into IgM-secreting plasma cells [60]. Natural antibodies produced by B-1 cells have low affinity and are polyreactive to bacterial pathogens [61]. This polyreactivity also raises the possibility of B-1 antibodies react with self antigens. And this autoreactivity has been proposed to play a role in autoimmunity [35, 62, 63]. Natural antibodies also constitute the first line of defense against bacterial pathogens. Antibodies (natural antibodies) secreted by B-1a cells, consisting mainly of IgM, constitute the first line of defense against bacterial pathogens by reacting with the polysaccharide antigens commonly found on bacterial surface. In contrast, B-1b cells produce antibodies
upon induction and provide long term protection and the ultimate clearance of the pathogens [41].

Besides their role in natural antibody production, host defense and autoimmunity, B-1 cells are also related to CD5⁺ B cell lymphomas and leukemias, both in mouse and human [64, 65]. In fact, CD5 was first characterized on B cells due to its presence on B cell chronic lymphocytic leukemia [66]. In mouse, B-cell lymphomas have been shown to bear a restricted set of BCRs that are commonly found on B-1 cells [66, 67].

1.2.4 B-1 B cell, natural antibodies and sepsis

Natural antibodies are the product of the long-lived, self-renewing B-1 cells that are enriched in the peritoneal cavity. Natural antibodies are the product of germline Ig gene expression, without undergoing somatic hypermutation as in adaptive antibody response. Predominantly IgM, natural antibodies react with both with self antigens such as phosphatidylcholine, a ubiquitous self antigen [68] and Thy-1 [69] and foreign antigens including influenza [70] and bacterial antigens [70, 71]. Restricted in their repertoire, natural antibodies possess broad reactivity to self and non-self antigens and protect against pathogens.
unencountered by the host previously [72]. Thus, natural antibodies provide protection between the onset of infection and adaptive immune response [73, 74].

Sepsis, as described in more detail in the following section, is a severe acute bacterial infection. Given the facts that natural antibodies (IgM) potentially protect the host from bacterial invasions, it is natural to postulate high titers of natural antibodies are protective in sepsis. However, our data showed that with Siglec-G mutation, high levels of natural antibodies did not yield any survival advantage in sepsis.

1.3 Sepsis and cecal ligation and puncture

1.3.1 Introduction

Sepsis is a major challenge in medicine, mainly developing from traumatic injuries. Sepsis cases were 450,000 in 1990 and deaths from sepsis were more than 100,000 in 1990 [75]. Sepsis results from acute infection and can lead to acute and long-term damages to the hosts, such as immunosuppression, and pulmonary complications [76]. Common septic symptoms include fever, anorexia, weakness and lethargy. Sepsis also leads to septic shock, which is a lethal hypotensive condition, resulted from overt inflammatory responses. In septic shock, peripheral resistance decreases due to vascular dilations due to local
inflammation; cardiac output is on the high end as a compensatory feedback to maintain blood pressure and tissue perfusion [77-79].

The model of cecal ligation and puncture (CLP) in rodents has been used extensively to investigate the clinical settings of sepsis and septic shock. This model is produced by ligating below the ileocecal valve followed by needle puncture of the cecum. CLP results in a hypotensive and hypometabolic state that may eventually leads to deaths in experiment rodents [80]. Peritonitis resulted from CLP is a relevant model for clinical sepsis and blood cultures are positive for enteric organisms after CLP [80].

1.3.2 Inflammatory cytokines are associated with CLP lethality

CLP has been extensively used as an animal model of experimental sepsis. Besides survival analysis, the cytokine expression has been studied widely. Proinflammatory cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, interferon (IFN)–γ, and chemokines as monocytes chemotactic protein (MCP)-1 have been suggested to be associated with the initiation of sepsis [76]. Proinflammatory cytokines IL-6 and TNF-α have been shown to increase following CLP [81-83]. It has been reported that high levels of IL-6 is strongly
correlated with survival after CLP and blockade of IL-6 results in increased survival following CLP [82]. Similarly, if TNF-α is inhibited by TNF-α antibodies, the mortality decreases [84, 85].

1.3.3 Factors controlling survival in CLP

The survival after CLP is strongly associated with several factors during the procedure. The length of the cecum ligated is a major determinant of the mortality [86]. Other factors include the size of the needle used for the puncture, the number of punctures, fluid resuscitation, and antibiotic treatment [87]. Besides the procedure itself, survival in CLP has been shown to positively correlate with inflammatory cytokine levels, such as TNF-α and IL-6 [87].

1.4 NF-κB and its roles in B-1 B cell development and sepsis

1.4.1 NF-κB signaling

The nuclear factor kappaB (NF-κB) transcription factors have been shown to be involved in the regulation of numerous genes in response to infection or stress environment. The term NF-κB refers to a family of five structurally related and evolutionarily conserved proteins: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and
its precursor p105), and NF-κB2 (p52 and its precursor p100). NF-κB factors lie mainly in the cytoplasm of cells and enter the nucleus upon activation to convey signals [88-90]. Under resting state, NF-κB factors form dimmers, either homodimers or heterodimers, interact with inhibitory proteins of NF-κB (IκB) and remain in the cytoplasm. Activation of NF-κB usually requires the degradation of IκB factors, which are inhibitory factors closely associated with NF-κB [91, 92]. The IκB factors function by masking the nuclear localization sequence (NLS) of the Rel homology domain of p50/p65 heterodimer and retain its cytoplasmic presence and prevent its entry into the nucleus. Degradation of IκB factors is accomplished by activation of IκB kinase (IKK) complex, which is a converging point for both the canonical and non-canonical NF-κB activation pathways [93-96]. And inhibition of NF-κB can be achieved by IKK inhibition through IKK inhibitors [97-99].

1.4.2 NF-κB signaling during B-1 B cell development

Development and maintenance of B-1 cells depends on BCR signaling and it has been shown that BCR/BtK/PLC-γ pathway is required for the development of B-1 cells [100, 101]. BtK is required for the development and survival of B-1
cells and Btk mutation leads to loss of B-1 cells [51]. Deletion of PLC-γ resulted in loss of B-1 cells [53]. One downstream pathway of BCR/Btk/PLC-γ signaling is the activation of PKC-β and subsequent activation of NF-κB. This pathway is required for BCR-induced B cell proliferation and the generation of B-1 cells [102, 103]. It has been suggested that PKC-β is recruited to lipid rafts where IkB kinase complex is also anchored and PKC-β somehow contributes to the activation of IkB [104].

Evidence that NF-kB activation is critical in B-1 cell development comes from gene deletion studies. During B-1 cell development, canonical NF-kB pathways are mediated by BCR-mediated signaling. Deficiencies of canonical pathway adaptor proteins, such as B-cell-leukemia/lymphoma-10 (Bcl-10) [105], mucosa-associated lymphoid tissue-1 (Malt-1) [106] and caspase recruitment domain family 11 (Card11) [107] have been shown to result in a dramatic reduction of B-1 cells, to a lesser extent a reduction of MZ B cells and negligible effect on FO B cells.
1.4.3 NF-κB signaling during inflammation and sepsis

NF-κB is regarded as an important regulator of inflammation. It plays critical roles in the initiation and amplification of inflammation, especially in innate immune response [108-110]. By responding to proinflammatory stimuli such as IL-1β and TNF-α, NF-κB activated the transcription of many proinflammatory and anti-apoptotic genes. In vivo functions of NF-κB are relatively difficult to study due to the embryonic lethality of NF-κB-deficient mice. However, in vitro, NF-κB has been shown to mediate inflammatory response in that its ablation leads to dramatic dampening of inflammatory mediator productions in different cell types such as macrophages, endothelial cells and lymphocytes [111, 112]. Moreover, NF-κB has been shown to regulate the transcription of more than 150 genes supposedly involved in inflammation [113].

As mentioned above, sepsis, and its murine model CLP is an acute inflammatory response. NF-κB activation has been shown to play a critical role in the sepsis (CLP). Upon activation by bacterial components, NF-κB activates the expressions of inflammatory cytokines such as TNF-α, IL-1β, IL-6, IFN-γ, MCP-1 and IL-12 [114, 115]. Activations of these inflammatory cytokine expressions result in fever and other symptoms observed during sepsis. NF-κB activation also
results in the expression of tissue factor plasminogen-activator inhibitor type-1, inducible nitric oxide synthase, cyclooxygenase-2, and adhesion molecules such as ICAM-1 and E-selectin, all of which play important roles in sepsis [116-119].

1.4.4 Siglec, SHP-1/2 and NF-κB

It has shown that the SH2 domain of SHP-1 and SHP-2 are associated with protein tyrosine phosphatase (PTP). The PTP-SHP-1 is a negative regulator of NF-κB activation [120-122]. PTP-SHP-1 deficient mice exhibited higher NF-κB activation and high percentage of early death due to inflammatory disorders [120]. However, PTP-SHP-2 has been suggested to be a positive regulator of NF-κB in that the abilities of TNF-α and IL-1 to activate NF-κB were impaired in SHP-2 knock out mice [123].

In our later discussions, Siglec-G has been shown to be a negative regulator of NF-κB and plays an important role in B-1 development and host immunity against bacterial pathogens. It will be of great interest to elucidate the molecular mechanisms of Siglec-G inhibition on NF-κB, the interaction with SHP-1 and SHP-2, and the bias between SHP-1 and SHP-2.
1.5 Purpose of study

Given the wide distribution in the immune system, Siglecs have long been proposed to play important roles in immunity, especially innate immunity. Our purpose is to delineate the characteristics of Siglec-G, a newly discovered murine Siglec. Since the attainment of the Siglec-G knock out GFP knock-in mouse model, we have conducted exploratory research on the expressions and functions of Siglec-G. In their sequence of being asked and addressed, questions are listed below.

1.5.1 Where is Siglec-G expressed?

This question was addressed by GFP analysis using Siglec-G+/−GFP+/− mice. We detected broad expressions of Siglec-G in the hematopoietic system, but not in the non-hematopoietic system. Both in the bone marrow and peripheral lymphoid organs, Siglec-G is highly expressed by B cells.

1.5.2 Is there any B cell phenotypic change?

B cells express high levels of Siglec-G. Since the primary function of B cells is to secret antibodies, we analyzed the serum immunoglobulins by ELISA and discovered high titers (5-fold increase) of IgM in Siglec-G+/− mice. The elevated IgM (natural antibodies) is due to expanded peritoneal B-1a population.
1.5.3 What roles do natural antibodies play during pathogen infections?

Natural antibodies are expected to provide protection against bacterial infections. Using a murine sepsis model, cecal ligation and puncture, we compared the survival rate of Siglec-G-deficient mice with wild type controls. Surprisingly, Siglec-G−/− mice exhibited a significantly reduced survival rate in comparison to Siglec-G+/+ controls.

1.5.4 Mechanisms involved in the B-1a/IgM and sepsis susceptibility phenotypes.

We performed extensive research on the underlying mechanisms exerted by Siglec-G and found that Siglec-G is a natural suppressor of NF-κB. The dependence of Siglec-G on NF-κB activation was evidenced by the facts that the increased peritoneal B-1a and susceptibility to sepsis was reversed upon NF-κB inhibition.
**Table 1.1 Comparison of the two major groups of Siglecs**

<table>
<thead>
<tr>
<th></th>
<th>Sn/CD22/MAG group</th>
<th>CD33-related Siglecs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Members</strong></td>
<td>Sialoadhesin</td>
<td>CD33</td>
</tr>
<tr>
<td></td>
<td>CD22</td>
<td>Siglec-5, 6, 7, 8, 9, 10, 11, 14 in human</td>
</tr>
<tr>
<td></td>
<td>Myelin-associated glycoprotein</td>
<td>Siglec-E, F, G, H in mouse</td>
</tr>
<tr>
<td><strong>Binding specificity</strong></td>
<td>Highly conserved</td>
<td>Poorly conserved</td>
</tr>
<tr>
<td><strong>Cell-specific expression</strong></td>
<td>Highly conserved</td>
<td>Highly variable</td>
</tr>
</tbody>
</table>
Table 1.2 Probable orthologs of human and murine Siglecs and their expressions

<table>
<thead>
<tr>
<th>Human Name</th>
<th>Human Expression</th>
<th>Mouse Name</th>
<th>Mouse Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sn/Siglec-1</td>
<td>Mac</td>
<td>Sn/Siglec-1</td>
<td>Mac</td>
</tr>
<tr>
<td>CD22/Siglec-2</td>
<td>B</td>
<td>CD22/Siglec-2</td>
<td>B</td>
</tr>
<tr>
<td>MAG/Siglec-4</td>
<td>OligoD, Schw</td>
<td>MAG/Siglec-4</td>
<td>OligoD, Schw</td>
</tr>
<tr>
<td>CD33/Siglec-3</td>
<td>MyP, Mo</td>
<td>CD33/Siglec-3</td>
<td>N</td>
</tr>
<tr>
<td>Siglec-5</td>
<td>N, Mo</td>
<td>Siglec-F</td>
<td>Eo</td>
</tr>
<tr>
<td>Siglec-6</td>
<td>Troph</td>
<td>Siglec-E</td>
<td>N, Mo, cDC</td>
</tr>
<tr>
<td>Siglec-7</td>
<td>NK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Siglec-8</td>
<td>Eo</td>
<td>Siglec-G</td>
<td>B, Mac, Mo, DC</td>
</tr>
<tr>
<td>Siglec-9</td>
<td>Mo, N, cDC</td>
<td>Siglec-E</td>
<td>N, Mo, cDC</td>
</tr>
<tr>
<td>Siglec-10</td>
<td>B</td>
<td>Siglec-G</td>
<td>B, Mac, Mo, DC</td>
</tr>
<tr>
<td>Siglec-11</td>
<td>Mac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Siglec-14</td>
<td>ND</td>
<td>Siglec-H</td>
<td>pDC</td>
</tr>
</tbody>
</table>

B, B cells; cDC: conventional dendritic cells; DC, dendritic cell; Eo, eosinophils; Mac, macrophages; Mo, monocytes; MyP, myeloid progenitors; N, neutrophils; ND, not determined; NK, natural killer cells; OligoD, oligodendrocytes; pDCs, plasmacytoid dendritic cells; Schw, Schwann cells; Troph, trophoblasts.
CHAPTER 2

Expression of Siglec-G in the Mouse

2.1 Abstract

Since Siglec-G is expressed at various isoforms and since anti-Siglec-G antibodies were not available commercially, we used mice in which the GFP cDNA was knocked into Siglec-G locus to analyze activation of the gene. Using the GFP as a surrogate marker, we detected activation of the Siglec-G locus on a subset of hematopoietic stem cells and all stages of B-lineage cells starting from the Pre-pro/Pro B cells in the bone marrow. In the periphery, Siglec-G is abundantly expressed on B cells. Dendritic cells and myeloid cells also express Siglec-G, though at a lower level than that on B cells. Siglec-G expression levels, as indicated by GFP intensity, are similarly high on all subsets of B cells in the periphery.
2.2 Introduction

Siglec-Gs are expressed on a wide variety of cells in both the hematopoietic system and non-hematopoietic system. In the human, Sialoadhesin is expressed on macrophages, CD22 on B cells, myelin associated protein (MAG) on oligodendrocytes and Schwann cells, Siglec-5 on neutrophils and monocytes, Siglec-7 on NK cells, Siglec-8 on eosinophils, Siglec-9 on monocytes, neutrophils and conventional dendritic cells, and siglec-11 on macrophages [12]. In the mouse, sialoadhesin is expressed on macrophages, CD22 on B cells, MAG on oligodendrocytes, and Schwann cells, CD33 on neutrophils, Siglec-E on neutrophils, monocytes and conventional dendritic cells, and Siglec-H on plasmacytoid dendritic cells [12].

We constructed a GFP knock-in mouse in which the Siglec-G exons 3 to 11 were replaced with a GFP-encoding sequence as diagramed in Fig. 2.1A. Since the expression of GFP is under the control of Siglec-G promoter and regulating elements, the GFP levels can be used as an indicator for activation of the Siglec-G locus. In addition, by comparing the +/- and +/- littermates for the levels of GFP among different subsets, we were able to determine whether both alleles are co-dominantly expressed. Furthermore, use of heterozygous mice will allow us to discern whether the gene is essential for the development and survival of given
cell types. The data presented here in demonstrated a wide expression in the hematopoietic system.

2.3 Materials and methods

Antibodies and flow cytometry

Single cell suspension of bone marrow, spleen, peripheral blood, and lymph nodes were prepared and first blocked with anti-FcR (2.4G2) to eliminate Fe-mediated non-specific bindings. For cell surface staining, samples were incubated with antibodies on ice for 30 minutes in staining buffer (1x PBS with 2% FCS and 0.05% sodium azide) and analyzed by flow cytometry. The CD3, CD11b, CD11c, B220, CD5, and lineage markers were purchased from BD pharminigen; Flt-2, Sca-1, CD43, CD21 and CD23 were purchased from eBiosciences. All samples were analyzed by a FACS Caliber II (BD Biosciences).

2.4 Results

2.4.1 Generation of the GFP knock-in mouse.

The production of the Siglec-G\(^{-/-}\) GFP\(^{+/+}\) mice is carried out by the Ingenious Research Laboratory, Inc. (Long Island, New York) under a research contract. Detail information is provided by Fig 2.1. 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 (Fig 2.1A).

Ten micrograms of the targeting vector was linearized by Ascl and then transfected by electroporation of iTL C57BL/6 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) outside the region used to generate the targeting construct. PCR reactions using A1, 2 or 3 with the N1 primer at the 5′ end of the Neo cassette amplify 2.0, 2.0 and 2.1kb fragments respectively. The control PCR reaction was done using AT1 and AT2, which is at the 5′ end of the SA inside the region used to create the targeting construct. This amplifies a band of 1.7 kb. As shown in Fig. 2.1B, 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 blastcytes. The chimera mice were bred to B6 mice to obtain F1 mice, which were intercrossed to generate Siglec-G\(^{-/+}\)GFP\(^{+/+}\) mice, and their WT and heterozygous littermates (Fig 2.1C).

### 2.4.2 Siglec-G is expressed widely in the hematopoietic system

Since the knock-in GFP is regulated by the Siglec-G promoter and regulatory elements, we used GFP expression to indicate the Siglec-G expression. Gene dose effect was compared between Siglec-G \(+/-\) and \(-/-\) littermates.

A subset of the hematopoietic stem cells, defined by Lin\(^{+}\)Flt2\(^{-}\)Sca-1\(^+\)cKit\(^+\) (Lin: CD3, B220, CD11b, Ter119, and Gr-1), was found to express Siglec-G (Fig 2.2A). High level expression of Siglec-G was detected on B lineage cells, beginning with bone marrow Pre-pro/Pro B cells (IgM\(\text{B220}^{lo}\)CD43\(^+\)) (Fig 2.2B). In the periphery, all B cells express high levels of Siglec-G. Dendritic cells (CD11c\(^+\)) and myeloid cells (CD11b\(^+\)) also express Siglec-G, albeit at a lower level (Fig 2.2C). Further analysis of Siglec-G levels on subsets of B cells (follicular, marginal zone, B-1a and B-1b cells), did not show any difference in Siglec-G expression (Fig 2.2D).
2.5 Discussion

Siglecs are expressed on a wide variety of cells. Using a GFP knock-in mouse model, in which the GFP expression is driven by Siglec-G promoter and regulatory sequences, we determined the expression of Siglec-G in the mouse. High expression of Siglec-G is found on B cells and all subsets of B cells (FO, MZ, B-1a and B-1b) express similar levels of Siglec-G. Albeit at lower levels, CD11b+ myeloid cells and CD11c+ dendritic cells also express Siglec-G. Interestingly, heterogeneity of Siglec-G expression is found in all cell populations. A portion of Siglec-G^{+/−} T cells seem to transcribe Siglec-G/GFP while Siglec-G^{+/−} T cells do not seem to have the transcript.

Siglec-G is restrictively transcribed in the hematopoietic system. Its expression can be traced to the heterogeneous FLSK cells (Lin^-Flt2^-Sca^-1+Kit^+), the hematopoietic stem cells. High expression starts at the stage of Pre-pro/Pro B cells (IgM^-B220^-CD43^-) in the bone marrow and continuously throughout all developmental stages of B cells. Although it is not clear that Siglec-G is expressed on early stages of myeloid or erythroid precursors in the bone marrow, Siglec-G is expressed by a subset of myeloid lineage cells (CD11b^+ and CD11c^+) in the periphery. Siglec-G expression is absent on neutrophils (Gr-1^+) or natural killer cells (NK1.1^+). It is interesting that although Siglec-G^{+/−} T cells do not seem
to transcribe Siglec-G, about 5% Siglec-G<sup>−/−</sup> T cells turn the transcription of GFP on in the spleen.
Figure 2.1

A

B

C

1.0 kb

1.0 kb

0.5 kb
Figure 2.1. Generation of Siglec-G\(^{-/-}\) mice that express green fluorescence protein (GFP) under the control of Siglec-G regulatory sequences. A.

Diagram of construct (top), Siglec-G 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 Siglec-G 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 mice. Integration of the construction is confirmed by AT1/N1 primer pair, while integration is confirmed by A1/N1, A2/N1 and A3/N1 pairs. The AT1/AT2 pair was the positive control for PCR. The primer sequences are: Primers used: A1: agctaagcacatgtgatggcta, A2: aggtgaataagtataggcccggc, A3: tgtgtgacctcaaggttgctc, AT1: tcagagtctcacttaccaetec, AT2: tattcagaggagttgtgge, N1: tgcgaggccagggcactttgtgagc. C. Genotyping of the F2 mice using primers that identify WT, heterozygous and homozygous knockout/in mice. Primers used: Siglec-G-F: tcccaagctcatgatgca, Siglec-G-R: atgttgtctctggaccagg, GFP-F:
Figure 2.2

A

(gated on LinFlt)

(FLSK)

C-Kit

% of max

GFP

Sca-1

B

(gated on IgM-)

(Pre-pro/Pro B cells)

B220

% of max

GFP

CD43

(Continued on page 34)
Figure 2.2 (continued)
Figure 2.2. Expression of the Siglec-G locus as determined by the expression of GFP which was used to replace the coding sequence of Siglec-G. 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-G 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-G 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-G 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.
Chapter 3

Siglec-G Limits the Size of Peritoneal B-1a Population by Suppressing NF-κB

3.1 Abstract

Siglec-G is widely expressed in the hematopoietic system. However, the major components of the hematopoietic system, including HSC, T cells, myeloid cells, dendritic cells and different developmental stages of B cells, were comparable between the wild type and mutant mice. Interestingly, we observed a 5-fold increase in the population of B-1a cells in the mutant peritoneal cavity. The B-1b population in the peritoneal cavity and B-1a population in the spleen did not shown any difference between the WT and mutant. Consistently, there is also a 5-fold increase of serum IgM levels in the Siglec-G mutant mice. And the serum IgG1 is reduced by approximately 50% in Siglec-G−/− mice. Siglec-G mutation did not seem to affect antigen-specific immune response as Siglec-G-deficient mice developed comparable titers of antibodies against chicken ovalbumin immunization.
The higher number of B-1a cells in the Siglec-G<sup>-/-</sup> peritoneal cavity result from postnatal preferential expansion of B-1 B cell precursors in bone marrow as fetal liver analysis did not exhibit difference in B-1 B cell precursors in Siglec-G mutant mice, while in adult bone marrow, significantly higher frequencies of B-1 precursors were found in Siglec-G mutant mice. Moreover, Siglec-G<sup>-/-</sup> bone marrow, when mixed with WT bone marrow, had a competitive advantage in reconstituting the bone marrow and peritoneal B cell populations, but not splenic B cells.

The expansion of Siglec-G<sup>-/-</sup> peritoneal B-1a compartment depends on NF-κB activation. When NF-κB activation was inhibited during early postnatal stage, peritoneal B-1a population in Siglec-G mutant mice demonstrated 4-5 fold reduction. Moreover, in naïve Siglec-G<sup>-/-</sup> peritoneal cells, there is an accumulation of nuclear phosphorylated p65 and total cellular phosphorylated p65 as demonstrated by western blotting and ELISA assays respectively. Electromobility shift assay also showed higher degree of accumulation of nuclear p50/p65 dimer in naïve Siglec-G<sup>-/-</sup> peritoneal lavage cells compared with Siglec-G<sup>+/+</sup>.
3.2 Introduction

The majority of B cells in the peripheral lymphoid tissues are generated by bone marrow and referred to as B2 cells, the conventional B cells. B-1 cells account for approximately 5% of the total B cells in mice and found in multiple lymphoid tissues, including the pleural and peritoneal cavities, the spleen and the intestine [124]. Similar to conventional B2 cells, B-1 cells also express common B cell surface antigens such as CD19 and B220 [125]. B-1 cells in the pleural and peritoneal cavities can be identified by their surface markers CD11b^+IgM^hiIgD^low and can be further divided into B-1a cells (CD5^+) and B-1b cells (CD5^-).

B-1a cells spontaneously secret IgM, known as natural antibodies, which constitute the first line of defense against certain bacterial pathogens with polysaccharide antigens, such as Streptococcus pneumoniae. In contrast, B-1b cells produce antibodies upon induction and provide long term protection and the ultimate clearance of the pathogens [41].

Several studies have indicated that precursor cells from fetal liver during mid-gestation preferentially develop into B-1 cells [126-128]. It has been suggested that the initial wave of B cell development in the embryo is biased towards B-1 cells [129]. Recently, B-1 lineage precursors have been identified in adult mouse bone marrow to be AA4.1^+Lin^-CD19^-CD45R^-low [48]. When tested
in vivo by transplantation into severe combined immunodeficient (SCID) mice, AA4.1^Lin^CD19^-^CD45R^-low cells purified from adult bone marrow reconstituted CD5^+^CD11b^-^IgM^-^IgD^- B-1a cells and CD5^-^CD11b^-^IgM^-^IgD^- B-1b cells in the peritoneal cavity of the recipient mice [48].

3.3. Materials and Methods

BrdU incorporation and measurement

Mice were injected i.p. with nucleotide analog bromodeoxyuridine (BrdU; 1 mg/mouse in 100 μl PBS) 24 hr and 3 hr before sacrifice. Splenocytes and peritoneal lavage cells were prepared and BrdU incorporation was detected by flow cytometry with a BrdU flow kit in conjunction with other cell surface markers, as described by the manufacturer (BD Biosciences, La Jolla, CA).

Bone Marrow Chimera

Bone marrow cells were isolated from 6-8 week old Siglec-G +/+ and +/- mice, and mixed at 1:1 ratio with 2.5 million cells from each donor. The mixed bone marrow cells were then intravenously transferred to Siglec-G +/+ and +/- mice, which received 1100 rad γ-irradiation one day before the transplantation.
Chicken ovalbumin immunizations

Four milligrams of Chicken ovalbumin (Sigma Aldrich, St. Louis, MO) were dissolved in MilliQ water, adjusted to 1 ml and emulsified 1:1 (vol: vol) in Freund’s complete adjuvant. 100 µl of each emulsion was injected in the hind footpads. 7 days after first immunization, same dose of OVA (200 ug per mouse) emulsified with incomplete Freund’s adjuvant was injected in the hind footpad. Blood was harvest before first injection, before second injection and 7 days after second injection.

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 Biotechnology, 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 Biotechnology). Plates were blocked with 1% non-fat milk in PBS for 1 hour at room temperature, and serum samples 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 hours at room temperature.
Horseradish peroxidase-conjugated isotype-specific antibodies (Southern Biotechnology) 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).

**Western blot**

Cells were lysed by regular lysis buffer containing 1% Triton-X100, 1:100 diluted, protein and phosphatase cocktails (both from Sigma-Aldrich). Nuclear and cytoplasmic proteins were isolated by a nuclear and cytoplasmic protein extraction kit (Pierce Biotechnology, Rockford, IL). Antibodies for western-blot are phosho-antibodies of p-AKT, p-GSK3β, p-p38, p-p42/44, p-JNK, p-IκB, p-p65, p-Stat3, and p-Stat-5, all from Cell Signaling (Davers, MA), and corresponding control antibodies of GSK3α/β, IκBα, from Cell Signaling (Davers, MA), and those specific for p-65, and Sp1 from Santa Cruz Biotechnology (Santa Cruz, CA).

**Electrophoresis mobility shift assay**

Approximately 10 µg aliquots of cell nuclear extracts were pre-incubated with 1 µg of poly(dI-dc) in binding buffer (10 mM Tris [pH 7.7], 50 mM NaCl,
20% glycerol, 1 mM dithiothreitol [DTT], 0.5 mM EDTA) for 10 min at room temperature. Approximately $1.5 \times 10^4$ cpm of $^{32}$P-labeled DNA probes were then added and the reaction proceeded for 30 min. The specificity of the binding was further confirmed by anti-P65 or anti-P50 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA)). The sequence for the probe was 5′-AGTTGAGGGGACTTTCCCAGGC-3′. The sequence for the probe was 5′-AGTTGAGGGGACTTTCCCAGGC-3′. The complexes were resolved on a 5% polyacrylamide gel in Tris-glycine buffer consisting of 25 mM Tris, 190 mM glycine and 1 mM EDTA at room temperature. The gel was dried at 80°C for 60 min and exposed to an X-ray film.

**NF-κB activity reporter assay**

Peritoneal cells extracted from WT and the Siglecg−/− mice were cultured in RPMI medium containing 10% FBS for overnight. Next day, the cells were transfected with NF-kB reporter plasmid of NF-κB-TA-luc (Clontech, San Diego, CA) by using Lipofectamine 2000 in 10% FBS Opti-MEM medium for 16 hours, and recovered in fresh complete RPMI medium for additional 12 to 16 hours. Plasmid Renilla luciferase (PRLSV40) was used as internal control for monitoring transfection efficiency.
**In vivo treatment with IKK inhibitor**

5-day old Siglec-G$^{-/-}$ 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.

**ELISA of NF-κB p65**

Five million spleen cells or peritoneal lavage cells were sonicated on ice in cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing 1 mM PMSF. Soluble proteins of the supernatant were analyzed by sandwich ELISA detecting phosphorylated NF-κB subunit p65 at serine 536 (PathScan, Cell Signaling Technology, Danvers, MA), according to the manufacturer’s recommendations. This assay includes application of capture antibody mouse anti-p65 (8F8) and detection antibody rabbit anti-phospho-p65 (93H1), recognized by HRP-linked anti-rabbit IgG.

**Statistics**

Data are expressed as mean ± SEM unless stated otherwise and statistical evaluation was done with student T test. *: 0.05>P>0.01; **: 0.01>P>0.001; ***: P<0.001.
3.3 Results

3.3.1 Siglec-G deficient mice exhibit higher peritoneal B-1a cell number and elevated serum IgM level.

Given the wide-spread expression of Siglec-G, 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 3.1, 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 Siglec-G WT and the mutant mice. Surprisingly, we observed a 5-fold increase in the size of B-1a cells in the peritoneal cavity, though the number of B-1b subset was unchanged (Fig. 3.1A). The number of B-1a cells in the 8-10 week old mice spleen, however, did not differ (Fig. 3.1B lower panel and Table 3.1), though a two-fold increase of spleen B-1 B cells can be observed at two weeks (Fig. 3.1B, upper panel).

Corresponding to the expansion of the peritoneal B-1a cells, the levels of serum IgM increased by 5 folds (Fig. 3.1C). With exception of IgG1, which is reduced by approximately 50%, the levels of other immunoglobulin isotypes were not affected by Siglec-G deletion. To determine whether deletion of Siglec-G affects adaptive B cell responses, we immunized the Siglec-G+/+ and Siglec-G−/−
mice with OVA and determined the anti-OVA levels at one and two weeks after immunization. As shown in Fig. 3.1D, the antibody response was essentially unaffected by the Siglec-G deletion.

### 3.3.2 Postnatal expansion of B-1a progenitors in the Siglec-G−/− mice

It is generally believed that the B-1a progenitors give rise to mature B-1a cells during fetal development and that self-renewal of the B-1a cells after birth explains the long-life persistence of the B-1a lineage [58]. More recently, a low number of B-1a progenitor cells were identified in mature bone marrow [48]. In order to explain the drastic difference in the number of B-1a cells in the peritoneal cavity, we carried out BrdU labeling to determine the proliferation of the B-1a cells. As shown in Fig. 3.2A, at two weeks, when a significant difference was observed in the percentage of B-1a 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 B-1a subsets was observed in the spleen, there appeared to be a higher rate of BrdU incorporation in the Siglec-G-deficient mice. Furthermore, in the peritoneal cavity, where a dramatic difference of the number of B-1a cells was found, the rate of BrdU incorporation was essentially identical (Fig 3.2B). Thus, while our data did not rule out the possibility that Siglec-G may regulate B-1a
proliferation in specific locations, such function does not account for the differential accumulation of B-1a cells in the Siglec-G-deficient mice. Likewise, the percentage of Annexin V+ cells was also comparable (Fig. 3.2C). Thus, neither increased self-renewal nor decreased apoptosis of mature B-1a cells explain the expansion of the B-1a compartment.

To explore the possibility that increased number of B-1a cells may be due to the expansion of the B-1 progenitors, we compared the number of the progenitors in the fetal liver and adult bone marrow. The Lin−AA4.1+ fetal liver and bone marrow mononuclear cells can be further divided into 4 subsets by their expression of B220 and CD19. Among them, CD19+ B220lo−neg subsets are considered the B-1 progenitors, while the CD19−B220+ subsets are regarded as the B-2 progenitors [48]. As shown in Fig. 3.3A, the number of B-1a progenitors in the E16.5–E17 fetal livers was unaffected by Siglec-G mutation. Likewise, the number of B-1a progenitor in the newborn livers was also similar between WT and mutant mice (Fig. 3.3B). In contrast, the number of B-1 progenitors was expanded by 4-fold in the bone marrow of adult Siglec-G-deficient mice, in comparison to what was found in the WT littermates (Fig. 3.3C). In either compartment, the B-2 progenitors were not affected by Siglec-G mutation (Fig. 3.3A-C).
To determine whether the adult bone marrow from the Siglec-G deficient mice have increased tendency to reconstitute the B-1a compartment, we mixed equal numbers of total bone marrow cells from Siglec-G$^{+/+}$ (CD45.1) and the Siglec-G$^{-/-}$ (CD45.2) mice, and adoptively transferred them into lethally irradiated WT or Siglec-G$^{-/-}$ host. Unlike the mice with B cell-specific deletion of Ptpn6 [54], no expansion of B-1a cells was found in the bone marrow was found in the Siglec-G$^{-/-}$ mice (Fig. 3.4A), therefore no effort was made to delete the B-1a 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. 3.4B, regardless of the recipient genotypes, the Siglec-G$^{-/-}$ bone marrow were more efficient in reconstituting the B cell compartment, including Pre-Pro/Pro B 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 Siglec-G$^{-/-}$ B-1a cells was 2–3 folds more abundant than that from the WT bone marrow, while the numbers of total B cells were not significantly different (Fig. 3.4B middle panel and Fig. 3.4C). 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 B-1a cells derived from WT bone marrow expressed high CD5, essentially all of the B-1a 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 B-1a cells in the peritoneal cavity strongly suggests that the expansion of the B-1a cells in the peritoneal cavity of the Siglec-G−/− mice was due to postnatal expansion of the B-1a cells, perhaps due to increased frequency of B-1a progenitor cells. Interestingly, unlike the peritoneal cavity, the number of B-1a cells in the spleen is mainly derived from the WT donor (Fig. 3.4B lower panel). Such phenotype is reminiscent of a recently reported phenotype associated with SHP-1 mutation in the B cell compartment [54].

3.3.3 Activation of NF-κB is essential for the B-1a population expansion in the peritoneal cavity of Siglec-G−/− mice.

We compared ex vivo WT and Siglec-G-deficient peritoneal cavity lavage cells for activation of a number of signal transduction pathways, including phosphorylation of Akt, Stat3, Stat5, P38, Erk and JNK. Our extensive analyses failed to reveal a significant difference between the WT and mutant cells (Data
not shown). Since mutation of Ptpn6, which associates with Siglec-G orthologue Siglec 10, enhanced NF-κB activation and expanded B-1a compartment [51], and since mice with targeted mutation of components in NF-κB pathway show selective reduction of B-1a B cell compartment [51], we focused on the potential impact of Siglec-G deletion on NF-κB activation. As shown in Fig. 3.5A, ex vivo peritoneal lavage cells from Siglec-G-deficient mice show greatly increased IκBα phosphorylation in the cytosol and nuclear accumulation of p65, as demonstrated by Western blot (Fig. 3.5A) as well as total phosphorylated p65, as evidenced by ELISA (Fig. 3.5B). This corresponds to greater binding to NF-κB probe as revealed by mobility shift assays (Fig. 3.5C). Furthermore, the increased NF-κB function was revealed by luciferase assay when the peritoneal cells from WT and Siglec-G-deficient mice were compared (Fig. 3.5D).

The increased IκBα phosphorylation indicated enhanced IKK activity in the B-1a cells from the Siglec-G−/− peritoneal cavity. To determine whether the activation of NF-κB is essential for the expansion of the B-1a compartment, we treated the 5-day-old Siglec-G-deficient mice with IκB kinase complex (IKK) inhibitor VI, which we have demonstrated to work efficiently in the mice [130]. As shown in Fig. 3.6A, consecutive treatment resulted in greatly decreased IκBα phosphorylation and nuclear p65 accumulation. Importantly, the percentage and
number of B-1a 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 B-1a cells in the spleen was unaffected by the inhibitor (Fig. 3.6B). These results reveal that activation of the NF-κB pathway is essential for selective expansion of the B-1a cells in the peritoneal cavity of the Siglec-G$^{-/-}$ mice.

3.4 Discussion

A long-standing observation in immunology is the preferential accumulation of B-1a B cell in the peritoneal cavity. Given the importance of the B-1a B cells in innate immunity and autoimmune diseases [131], the mechanisms that determine the size of B-1a B cell subsets in the locality are of great interest. Using mice with targeted mutation of the Siglec-G gene, we demonstrate that Siglec-G controls B-1a cell expansion in the peritoneal cavity by repressing NF-κB activation.

**Siglec-G controls a checkpoint for peritoneal B-1a B cell expansion**

We have observed mice with targeted mutation of Siglec-G that had a 5-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 B-1a cells in the peritoneal cavity. Of the two subsets of B-1 B cells in the cavity, the expansion is limited to the B220⁺CD11b⁺CD5⁺ B-1a subset. Moreover, while the expansion of the B-1a cells in the peritoneal cavity was observed throughout the study, the expansion of the B-1 B cells in the spleen is only transient, observable at 2 weeks but not 10 weeks.

Since no reagent is currently available to study the protein expression for mouse Siglec-G, we used a GFP knock-in transgenic line to determine the expression of Siglec-G. Our data indicated that while cells among B cell lineage had the highest levels of Siglec-G expression, essentially all major subsets of hematopoietic cells examined, including T cells, dendritic cells, and monocytes/macrophages show active transcription of the locus. Despite the widespread expression of the gene, the impact of Siglec-G deletion appear to be limited to the B-1a B cells in the peritoneal cavity, although a smaller expansion of the B-1a B cells in the spleen can be observed at 2 weeks of age.

While our manuscript was in preparation, Hoffmann et al [132] reported a Siglec-G-deficient mouse prepared with ES cells from the BALB/c. While they
observed expansion of B-1a cells in the spleen and peritoneal cavity, the B-1a 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 B-1a progenitors and expansion of B-1a B cells in the Siglec-G-deficient mice**

While the B-1a B cells are generally believed to have been produced from progenitors in fetal liver [126], recent studies identified B-1a progenitors in adult bone marrow [126]. Therefore, the expansion of the B-1a 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 B-1a B progenitors in the bone marrow, but not in the fetal or new born liver. Consistent with the increased B-1a B progenitor cells, the Siglec-G-deficient bone marrow are more efficient in constituting the B-1a B cells. These data, together with the fact that neither increased proliferation nor decreased apoptosis explains the expansion of B-1a B cells in the peritoneal cavity, supports the notion that postnatal expansion of the B-1a compartment is responsible for the expansion of B-1a cells in the Siglec-G−/− mice. Therefore, our data demonstrate a novel function of Siglec-G in
regulating postnatal expansion of the B-1a compartment. It remains to be
determined as to whether the expansion is intrinsic to genetic defects in the
precursor cells, or due to Siglec-G defects in other cell types.

It is worth pointing out that although the number of progenitors for B2
cells were not increased, the Siglec-G-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 B-1
B progenitors, Siglec-G 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 Siglec-G-
deficient mice were analyzed.

**Siglec-G is a negative regulator of NF-κB activity in peritoneal B cells:**

**Similarity with SHP-1**

Our comparison between WT and Siglec-G-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 NF-κB was observed in the peritoneal lavages from the Siglec-G-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 NF-κB probe and increased promoter activity when the NF-κB reporter constructs were used to transfect the peritoneal lavage cells. Since the activation correlates with the IκB phosphorylation, it is likely that the conical NF-κB pathway is being activated. Moreover, since the IKK is responsible for IκB phosphorylation, we used the IKK inhibitor VI to block NF-κB activation in order to study the significance of this activation in B-1a cell expansion in the peritoneal cavity. Our data clearly demonstrated that activation of NF-κB is responsible for the expansion of the B-1a cells in the peritoneal cavity. Recently, Hoffmann et al reported that mutant B cells display increased Ca\(^{2+}\) signaling in responses to several stimuli \textit{in vitro} [132]. Since it is unclear if these stimuli are responsible for the B-1a expansion \textit{in vivo}, the significance of the enhanced Ca\(^{2+}\) response remains to be demonstrated.

Interestingly, despite the significant effect of the IKK inhibitor for spleen cells, no increase in the spleen B-1a B cells were observed. These data clearly indicated that expansion of B-1a B cells in the peritoneal cavity uses a mechanism
that is not employed to maintain the number of spleen B-1a B cells. The selective expansion of B-1a 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 B-1a cells (data not shown). However, since our data also indicated that the expansion cannot be demonstrated by BrdU incorporation, the cellular basis for the increase remains to be determined.

Recently, Rajewsky and colleagues demonstrated that targeted mutation of SHP-1 in the B cell lineage causes expansion of B-1a B cells in the peritoneal cavity [54]. Remarkable similarity can be found between the germline mutation of the Siglec-G and B-cell specific deletion of *Ptpn6*. Firstly, in terms of lineage size, the impact of mutation is limited within B-1a B cells in the peritoneal cavity. Secondly, the expansion of B-1a subsets is due to the postnatal expansion of B-1a cells. Thirdly, in bone marrow chimera mice, the mutant bone marrow shows a remarkable advantage in reconstituting the peritoneal B-1a B cells, while displaying a disadvantage in constituting spleen B-1a B cell compartment. These similarities in functional defects raise the possibility that the gene has a similar function within B cell compartment. Since Siglec-10, the Siglec G orthologue in
human, has been shown to be associated with Shp1 [133], the simplest hypothesis is that SHP-1 works down-stream of Siglec-G in limiting the controlled expansion of B-1a subset. This hypothesis has the potential to unify observations made from mice with genetic defects of Siglec-G, Ptpn6, and NF-κB-1 and C-rel. Since mutation of Siglec-G increases the titer of anti-DNA antibodies [132] and since deletion of SHP-1 in B cells causes autoimmune diseases [54], the balance of the Siglec-G signaling will likely be important in the proper tuning of the B-1a function in innate immunity against infection vs. autoimmune side effect. Given recent advances in pharmaceutical targeting of the NF-κB pathway [130], our study suggests a new approach in selective tuning of innate immunity and autoimmunity.
Figure 3.1

A

(Gated on B220*)

Siglec-G -/-  Siglec-G +/+  

CD5  CD11b

B

Siglec-G -/-  Siglec-G +/+  

2 week  10 week

Siglec-G -/-  Siglec-G +/+  

B220

(Continued on page 58)
Figure 3.1 (continued)

C

\[ \text{µg/ml} \times \]

\[ \begin{align*} &\text{Siglec-g-/-} \\ &\text{Siglec-g+/-} \end{align*} \]

<table>
<thead>
<tr>
<th>IgM</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG2b</th>
<th>IgG3</th>
</tr>
</thead>
<tbody>
<tr>
<td>***</td>
<td>**</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D

\[ \text{OD @ 490 nm} \times \]

\[ \begin{align*} &\text{anti-OVA IgM} \\ &\text{anti-OVA IgG} \end{align*} \]

<table>
<thead>
<tr>
<th>Days</th>
<th>0</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD @ 490 nm</td>
<td>0.0</td>
<td>0.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Figure 3.1. Selective expansion of peritoneal cavity B-1a cells in mice with targeted mutation of the Siglec-G gene. A. Expansion of B-1a, but not B-1b population in the peritoneal cavity. The top panel shows the profile of B cells among peritoneal lavages of 10 weeks old mutant and age-matched WT controls, while the lower panel shows summary data involving 10 mice per group. This experiment has been repeated 5 times involving a total of 10–12 mice per group. B. Expansion of splenic B-1 cells in 2-week-old but not 10-week-old Siglec-G-deficient mice. Data of 2-week-old mice were summarized from 4-6 mice per group. Data of 10-week-old mice were summarized from 25-30 mice per group. C. Serum concentration of different isotypes of Ig, note substantial increase of IgM and decrease of IgG1 in the mutant mice. Data shown in the lower panels are Mean ± SEM, with 20 mice per group. D. Adaptive B cell response in WT and the Siglec-G-deficient mice. The WT and Siglec-G-deficient mice were immunized with 200 µg of OVA in complete Freund’s adjuvants (CFA) at day 0, boosted with the same dose of OVA at day 7. Sera were collected at day 1, 7 and 14 for measurement of anti-OVA antibodies by ELISA using the OVA-coated plate. Data shown are means ± SEM of optical density, n = 6. Sera were used at 1:500 dilutions for the ELISA.
Figure 3.2

A

B

C

**Figure 3.2**

A

B

C
Figure 3.2. Preferential proliferation of mature B-1a cells does not explain the expansion of B-1a compartment in the Siglec-G-deficient mice. A. Proliferation of splenic B-1 B cells of 2 (top) and 10 (bottom) weeks old mice. B. Proliferation of peritoneal B-1a B cells from 10 week old mice. Two or ten weeks-old Siglec-G$^{−/−}$ and Siglec-G$^{+/+}$ mice were injected (ip) with 1 mg of BrdU 27 and 3 hours before sacrifice. Spleen and peritoneal B-1 a proliferation 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. C. Data depicting annexin V staining of ex vivo splenic or peritoneal B-1a B cells of 10-week old mice. Data shown are representative of 3 independent experiments.
Figure 3.3

A. siglecG^+/+ vs. siglecG^+-

B. siglecG^+/+ vs. siglecG^+-

C. siglecG^+/+ vs. siglecG^+-

Fetal liver day 17.

Postnatal day 1 liver.

BM 10-week.
Figure 3.3. Postnatal expansion of B-1a but not B2 progenitor cells. A.

Progenitor cells from fetal liver. B. Progenitor cells from new born liver cells. C. Percentage of B-1 (CD19⁺B220lo) and B2 (CD19⁻B220hi). Data shown in A involve 2 WT and 2 mutant mice per group. Data shown in B involve 3 WT and 4 mutant mice per group. Similar levels were found in 12 heterozygous littermates. C. As in A, except 10 week old bone marrow were analyzed. N = 7.
Figure 3.4

A

(Continued on page 65)
Figure 3.4 (continued)
**Figure 3.4 (continued)**

C

- **Siglec-G−/− recipients**
- **Siglec-G+/+ recipients**

D

- **Siglec-G−/− recipients**
- **Siglec-G+/+ recipients**
Figure 3.4. Siglec-G-deficient bone marrow cells have competitive advantages in reconstituting bone marrow B cell lineage and peritoneal B-1a subsets in lethally irradiated recipients. The WT (CD45.1) and Siglec-G⁻/⁻ (CD45.2) bone marrow cells were mixed at a 1:1 ratio and used to reconstitute the lethally irradiated mice, 13 weeks later, the mice were sacrificed for analysis. The bone marrow and peritoneal lavages were analyzed by flow cytometry for reconstitution by WT and mutant bone marrow cells. A. No increase of B-1a B cells in bone marrow in the Siglec-G⁻/⁻ mice. Bone marrow cells were analyzed by flow cytometry before transplantation. B. Summary data showing the contribution of WT (open bars) and mutant bone marrow cells (filled bars). The cell subsets in bone marrow include total B cells (B220⁺), Pre-pro/Pro B (CD43⁺B220loIgM⁻) and pre/new B (CD43⁻B220⁺IgMlow-highIgDlow). The lower panel summarizes the total B cells and B-1a B cells in the peritoneal lavages. Data shown are Mean ± SEM, N = 5. C. Representative profiles depicting the relative contribution of WT (CD45.1) and Siglec-G-deficient (CD45.2) cells to the B-1a compartment. D. Representative profiles depicting distinct profile of WT and Siglec-G-deficient B-1a cells.
Figure 3.5

A

Cytosolic

WT KO

- p-IκBα
- β-actin

Nuclear

- p65
- Sp1

B

NF-κB P-p65 ELISA

OD @ 450 nm

0.0

0.2

0.4

0.6

0.8

1.0

Siglec-G KO

WT

PerC

Spl

C

@p50 @p65 Siglec-G

- + - -

+ - - -

+/- +/+ +/+ -/

D

Relative luciferase activity

0 2 4 6 8 10 12 14 16

WT KO

0.0

0.2

0.4

0.6

0.8

1.0

+/- +/+ +/+ -/

Nonspecific

free probe
Figure 3.5. Siglec-G⁻/⁻ peritoneal cavity lavage cells have increased NF-κB activity. A. Western blot analysis of cytosolic total and phosphorylated IkBα (upper panels) and nuclear P65, using the amounts of SP1 as loading control (lower panels). B. ELISA analysis of phosphorylated NF-κB factor p65 (both nuclear and cytosolic). C. EMSA analysis of nuclear NF-κB activity. Nuclear lysates from WT (+/+ ) or knockout (-/-) peritoneal lavages were prepared and tested for retardation of ³²P NF-κB probe. The specificity of the gel retardation was confirmed by super-shift with anti-p50 or p65 antibodies. D. Luciferase activity of peritoneal cells from WT and knockout mice. Data shown in this figure have been repeated twice.
Figure 3.6

A

B

Figure 3.6

A

B

---

70
**Figure 3.6.** 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 IκBα phosphorylation in the cytosol (upper panel) and p65 accumulation in the nuclei (lower panel) of spleen cells from treated (IKK inhibitor +) or control (IKK inhibitor -) mice. B. The impact of inhibiting the IKK on peritoneal B-1a subsets. Data shown are mean ± SEM involving 5–6 mice per group and have been repeated twice.
Table 3.1. Selective expansion of B-1a cells in the peritoneal of the Siglec-G-deficient mice.

<table>
<thead>
<tr>
<th>cell population</th>
<th>% of cells</th>
<th>Siglec-G−/−</th>
<th>Siglec-G+/+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bone marrow</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC (FLSK) cells</td>
<td></td>
<td>0.085 ± 0.05</td>
<td>0.094 ± 0.04</td>
</tr>
<tr>
<td>Prepro/Pro B (CD43−B220+IgM+)</td>
<td></td>
<td>2.45 ± 0.30</td>
<td>2.81 ± 0.46</td>
</tr>
<tr>
<td>PreB (fr. D)</td>
<td></td>
<td>2.43 ± 0.39</td>
<td>2.15 ± 0.25</td>
</tr>
<tr>
<td>New B (CD43−B220+IgMhiIgDlow)</td>
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<td>1.36 ± 0.22</td>
<td>1.22 ± 0.22</td>
</tr>
<tr>
<td>Mature B (CD43−B220+IgMhiIgDhi)</td>
<td></td>
<td>1.07 ± 0.11</td>
<td>1.30 ± 0.19</td>
</tr>
<tr>
<td>Dendritic cells (CD11c−)</td>
<td></td>
<td>1.23 ± 0.09</td>
<td>1.19 ± 0.10</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total B (B220+)</td>
<td></td>
<td>42.45 ± 8.55</td>
<td>43.99 ± 10.50</td>
</tr>
<tr>
<td>FO B (B220−CD23hiCD21hi)</td>
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<td>29.49 ± 3.62</td>
<td>32.16 ± 2.66</td>
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<tr>
<td>MZ B (B220−CD23lowCD2hi)</td>
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<td>3.22 ± 0.60</td>
<td>2.55 ± 0.55</td>
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<tr>
<td>T1 (B220−AA4.1−CD23lowIgMhi)</td>
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<td>6.07 ± 2.88</td>
<td>4.55 ± 1.98</td>
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<tr>
<td>T2 (B220−AA4.1−CD23hiIgMhi)</td>
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<td>4.05 ± 1.77</td>
<td>4.89 ± 2.12</td>
</tr>
<tr>
<td>B-1a (B220°CD43°CD5+)</td>
<td></td>
<td>1.97 ± 0.36</td>
<td>2.12 ± 0.39</td>
</tr>
<tr>
<td>Macrophages (CD11b+)</td>
<td></td>
<td>4.97 ± 2.02</td>
<td>5.55 ± 1.99</td>
</tr>
<tr>
<td>Dendritic cells (CD11c+)</td>
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<td>1.32 ± 0.22</td>
<td>1.16 ± 0.33</td>
</tr>
<tr>
<td>T cells (CD3+)</td>
<td></td>
<td>23.02 ± 6.99</td>
<td>27.60 ± 8.60</td>
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<tr>
<td><strong>Peritoneal cavity</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>B-1a (B220°CD11b°CD5+)</td>
<td></td>
<td>2.41 ± 0.51</td>
<td>0.46 ± 0.16 ***</td>
</tr>
<tr>
<td>B-1b (B220°CD11b−CD5−)</td>
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<td>1.07 ± 0.25</td>
<td>1.25 ± 0.33</td>
</tr>
<tr>
<td>T cells (CD3+)</td>
<td></td>
<td>0.97 ± 0.11</td>
<td>1.11 ± 0.29</td>
</tr>
</tbody>
</table>

The cell subset were phenotyped as described [134]. For BM HSC cells, N=3. For spleen and peritoneal cavity T cells, N=6. For all other cells, N=12.
Chapter 4

Siglec-G Confers Resistance to CLP-Induced Sepsis by Suppressing NF-κB

4.1 Abstract

Natural antibodies, predominantly IgM, constitute the first line of defense against polysaccharide-bearing bacterial pathogens. Despite the increased blood IgM levels, Siglec-G-deficient mice are more susceptible to peritoneal bacterial infections, introduced by cecal ligation and puncture (CLP), a peritonitis model for sepsis. Blood and peritoneal lavage fluid cultures yielded more bacterial colonies than wild type controls. We also detected dramatically increased proinflammatory cytokines, including TNF-α, IL-1β and IL-6, and chemokine MCP-1 in the blood of Siglec-G<sup>−/−</sup> mice received CLP. More severe hepatic damages were also evident in Siglec-G<sup>−/−</sup> mice, as demonstrated by elevated Aspartate Aminotransferase. Consistently, in vitro, co-culture of peritoneal cavity macrophages and enteric organisms also showed Siglec-G<sup>−/−</sup> macrophages had
impaired killing capacity. Moreover, Siglec-G\textsuperscript{−/−} macrophages produced significantly more TNF-α, IL-6 and IL-1β than the Siglec-G\textsuperscript{+/+} macrophages \textit{in vitro}.

Susceptibility of Siglec-G\textsuperscript{−/−} mice to CLP is dependent on NF-κB activation in that inhibition of NF-κB reversed the survival disadvantage of Siglec-G\textsuperscript{−/−} mice in CLP. Moreover, blockage of NF-κB signaling \textit{in vitro} blocked the TNF-α and IL-6 secretions of peritoneal macrophages.

4.2 Introduction

Cecal ligation and puncture (CLP) has been used extensively to investigate sepsis and septic shock. This model is produced by ligating below the ileocecal valve followed by needle puncture of the cecum. CLP results in a hypodynamic and hypometabolic state, which eventually leads to deaths in experiment animals. Peritonitis resulted from CLP is a relevant model for clinical sepsis, which is a common complication of traumatic injury, with a worldwide prevalence of 5 millions cases a year [135]. Sepsis is an acute infection in which the host is toxic. Common symptoms of sepsis include fever, anorexia, weakness and lethargy.
Sepsis often leads to septic shock, which is a collapse of the cardiovascular system.

CLP has been extensively used as an animal model of experimental sepsis. Blood cultures are positive for enteric organisms after CLP. The survival after CLP is associated with several factors during the procedure: the length of the cecum ligated, the size of the needle used for the puncture, the number of punctures, fluid resuscitation, and antibiotic treatment [86].

Proinflammatory cytokines have been implicated in the survival post-CLP. Two main proinflammatory cytokines, IL-6 and TNF-α, have been shown to increase following CLP [81, 82]. It has been reported that high levels of IL-6 is strongly correlated with survival after CLP and blockade of IL-6 results in increased survival following CLP [82]. Similarly, if TNF-α is inhibited by TNF-α antibodies, the mortality decreases [84].

NF-κB activation plays a critical role in the sepsis/CLP. Upon activation by bacterial components, NF-κB activates the expressions of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IFN-γ, MCP-1 and IL-12 [114, 115]. Activations of these proinflammatory cytokine expressions result in fever and other symptoms observed during sepsis. NF-κB activation also results in the
expression of tissue factor plasminogen-activator inhibitor type-1, inducible nitric oxide synthase, cyclooxygenase-2, and adhesion molecules such as ICAM-1 and E-selectin, all of which play important roles in sepsis [116-119].

Naïve siglec-G−/− mice peritoneal lavage cells have activated NF-κB compared with wild type and the NF-κB activation can be blocked with IKK inhibitor. Thus we hypothesize that increased susceptibility of siglec-G−/− mice to CLP is due to activation of NF-κB, which leads to enhanced inflammatory cytokine expression.

4.3 Materials and Methods

Cecal ligation and puncture

Mice were anesthetized with an i.p. injection of 2 mg of ketamine (Abbott Laboratories, Chicago, IL) and 150 ug of xylazine (LLoyd Laboratories, Shenandoah, IA). Under sterile surgical conditions, a 1-cm incision left to midline was made to the lower abdomen, and the cecum was exposed. The cecum was partially ligated at its base with a surgical knot and punctured nine times with 21-gauge needle. The cecum was returned to the peritoneal cavity, and the abdominal incision was closed using surgical staples. Mice were rehydrated with 1 ml saline.
subcutaneously and placed on a heating pad until they recovered from anesthetic. At 6, 24 and 72 h after surgery, CLP mice were bled and sera were used for cytokine analyses. Peritoneal lavage was performed with 9 ml of cold sterile saline. Peritoneal lavage cells were stained with Diff-Quik solutions (Dade Behring, Dudingen, Switzerland).

**Survival studies following CLP**

The survival study was performed to determine the effect of the presence of Siglec-G on survival in CLP-induced sepsis animal model. WT and Siglec-G−/− mice were subjected to CLP surgery in the absence or presence of the treatment with antibiotic INVANZ (Ertapenem) i.p. injected at 75 mg/kg (Merck & Co, Whitehouse station, NJ) 6 h after surgery and every 24 h until 3 days post-CLP. Survival was monitored for 7 days following surgery. Data were analyzed and graphed by Graphpad Software.

**Peritoneal macrophage collection and in vitro stimulation**

Total peritoneal cells were harvested by peritoneal lavage with 9 ml cold sterile saline from WT or Siglec-G−/− mice in naïve status. The cells were thoroughly washed with saline and counted in a hemocytometer. Cells were suspended in RPMI 1640 containing 10% FCS and penicillin/streptomycin and placed in 24-well culture plates and incubated in 37°C incubator with 5% CO2 for
48 hours. *In vitro* bactericidal activities of the cells were determined by a classical colony forming unit (CFU) assay. In brief, cells (5 × 10^5/well) were infected with 1.7 × 10^5 CFU of enteric organisms recovered from C57BL/6 mice cecum. After 4 h of culture in a 5% CO2 incubator, plates were placed at ~80°C for 30 min, and cells were lysed by thawing. This did not affect bacteria viability. The lysates were serially diluted, plated on LB agar plates supplemented with LB broth, and incubated overnight at 37°C, and the aerobic colonies were counted.

**Determination of CFU**

Ten microliters samples of peritoneal lavage fluids and 30 ul of peripheral blood from each mouse were serially diluted with sterile saline. Then, 100 ul of each dilution was plated on LB agar plates supplemented with LB broth and incubated overnight at 37°C, after which the number of aerobic colonies was counted. Data were expressed as CFU/ul.

**Cytokine ELISA**

Concentrations of TNF-α and IL-1β were measured in cell-free peritoneal lavage fluid, serum, and cell culture supernatants using a standardized sandwich ELISA technique. Briefly, 96-well plates (BD Falcon) were coated with 5 µg/ml
capture antibody in coating buffer overnight at 4°C and washed with PBS containing 0.05% Tween 20. All antibodies used for ELISA were purchased from BD Biosciences. Nonspecific binding sites were blocked with 2% non-fat milk in PBS for 60 min at room temperature. Plates were rinsed three times with wash buffer and cell-free supernatants or sera samples were loaded and incubated for 2 hrs at room temperature. After three washings, a biotinylated detection monoclonal antibody was added for 60 min at room temperature. The plates were washed again and peroxidase-conjugated streptavidin was added to the well for 60 min at room temperature. Plates were washed, and after the addition of chromogen substrate (BD Biosciences), OD readings were measured at 450 nm using an ELISA plate reader. Recombinant murine cytokines were used to generate the standard curves from which the concentrations present in the samples were calculated. The limit of detection of the assays was 10 pg/ml.

Cytometric bead array immunoassay

Cytometric bead assay (CBA) kit was purchased from Pharningen, San Diego, CA and detects one of the six cytokines (IFN-γ, TNF-α, IL-6, MCP-1, IL-1 or IL-10). Six standard curves (standard ranging from 12.5 to 5000 pg/ml) were obtained from one set of calibrators and six results were obtained on one test.
sample. For each sample of peritoneal lavage, sera and the standard mixture of cytokines, 30 μl of sample or standard of cytokine were added to the mixture of 30 μl of each antibody–bead reagent and antibody–PE detector. The mixture (90 μl) was incubated in darkness at room temperature for 120 min and then washed before data acquisition with flow cytometry.

**NF-κB inhibition in CLP**

Mice were injected with 20 ug of IKK inhibitor VI (Calbiochem) 1 hour before and 6 hours after CLP. IKK inhibitor (1 mg) was first dissolved in 200 ul of DMSO and then adjusted to a concentration of 100 ug/ml in PBS.

**4.4 Results**

**4.4.1 Siglec-G deficiency increases susceptibility to CLP in mouse.**

In initial studies, 90% mortality was observed in Siglec-G/- mice without antibiotic treatment as early as 3 days post-CLP (Fig. 4.1A). However, wild type mice survived significantly longer in CLP (p < 0.01). Clearly, the absence of Siglec-G provided a survival disadvantage compared with Siglec-G wild type mice. Antibiotic treatment for 3 days following CLP attenuated the severity of CLP and increased survival rates both in the WT and Siglec-G+/− groups (Fig.
4.1B). However, with antibiotic treatment, WT mice were still more resistant to CLP-induced mortality compared to Siglec-G deficient mice ($p < 0.01$). At day 7 after CLP, 70% of the WT mice were alive compared with 10% of the Siglec-G-/- mice at this time (Fig. 4.1B). The survivors were long-term at this point.

The microbial peritonitis induced by CLP is associated with an amplified inflammatory response, in which the local and systemic expression of many cytokines and chemokines are augmented [136]. We compared the cytokine profiles in blood between WT and Siglec-G-/- mice after CLP-induced peritonitis. Compared to WT mice, Siglec-G-/- mice produced a significantly higher amount of inflammatory cytokines, including IL-10, IL-6, MCP-1, IL-1β, and TNF-α 24 hours after CLP (Fig 4.2A). Kinetically, we analyzed TNF-α and IL-1β, and the levels of cytokines peaked at 24 hours post-CLP and gradually decreased (Fig. 4.2B).

Hepatic perfusion is impaired during the course of CLP/sepsis [137, 138]. As a result of impaired hepatic perfusion and cytokines like TNF-α, hepatocellular damages occur. Damages to the hepatocytes are reflected by increased levels of hepatic enzyme serum levels, such as Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT). And liver enzyme
levels often correlate the severity of liver damage [139-142]. At 24 hours post-CLP, Siglec-G deficient mice had significantly higher levels of AST (Fig. 4.3), indicating more severe liver damages and more severe sepsis.

Lethality from CLP is closely linked with bacterial burden [143, 144]. Post-CLP mice are usually positive for blood cultures of enteric organisms. Peritoneal lavage after sacrifice also contains enteric bacteria and the amount of bacteria in the lavage indicates the phagocytic and killing activities of in the peritoneal cavity. Siglec-G-deficient mice have impaired bacterial clearance demonstrated by their higher loads of bacteria in both blood and peritoneal lavage. Fig. 4.4A shows 24 hours after CLP, about two folds more viable bacteria were present in the Siglec-G−/− blood than WT controls. Seventy-two hours post CLP peritoneal lavage culture also showed a two-fold higher CFU in Siglec-G−/− mice (Fig. 4.4B).

CLP permits the leakage of polymicrobial flora in the peritoneal cavity, which leads to a local inflammatory response directed at eliminating bacteria from the peritoneal cavity [145]. Peritoneal macrophages phagocytize the bacteria and eventually enhance killing, while the recruitment of polymorphonuclear (PMN) cells indicate the severity of acute inflammatory response. Seventy-two hours
post-CLP, we found significantly higher frequencies of PMN and lower frequencies of macrophages in Siglec-G−/− peritoneum compared with wild type (Fig. 4.5).

Phagocytes, such as neutrophils and macrophages, are cells responsible for clearing bacteria [146, 147]. In order to examine the bactericidal activities of peritoneal lavage cells, we conducted experiments to collect peritoneal cells and cultured with live bacteria recovered from wild type C57BL/6 cecum. As shown in Fig. 4.6A, Siglec-G−/− peritoneal macrophages are less capable to kill the bacteria than WT controls. In addition, higher levels of TNF-α and IL-1β were detected in Siglec-G−/− lavage cells and enteric bacteria co-culture supernatants (Fig. 4.6B).

IKK inhibitor VI (Calbiochem) has previously shown to inhibit NF-κB activation in adult mice [130]. We treated 8-week old Siglec-G +/+ and −/− mice with IKK inhibitor VI 1 hour before and 6 hours after CLP with a dose of 20 ug per mouse. The treatment resulted in efficient inhibition of NF-κB activation, indicated by dramatically reduced phosphorylated p65 levels of Splenocytes (Fig. 4.7F). Inhibition of NF-κB improved survival of Siglec-G−/− mice in CLP (Fig.
4.7A) with similar survival rates were observed between SiglecG-/- and +/+ mice (Fig. 4.7B). Consistently, treatment of IKK inhibitor (IKKi) suppressed the expressions of proinflammatory cytokines, especially IL-10, IL-6 and TNF-α in both Siglec-G WT (Fig. 4.7C left panel) and KO (Fig. 4.7C right panel) mice 24 hours post CLP. Moreover, compared with the dramatic difference between proinflammatory cytokines (IL-10, IL-6, MCP-1 and TNF-α) levels without IKK inhibitor, IKK inhibitor treated Siglec-G WT and KO mice did not exhibit difference in their blood TNF-α levels, and the difference of IL-6 concentration was dramatically reduced (Fig. 4.7D). The survival rate of Siglec-G-/- mice in CLP was dramatically higher with IKK inhibitor treatment (Fig. 4.7D left panel) whereas the IKK inhibitor treatment did not exert a difference in Siglec-G WT survivals (Fig. 4.7D right panel). In addition, we also found IKK inhibitor to be able to suppress TNF-α and IL-6 in vitro PLF macrophages-bacteria co-culture (Fig 4.7E). All these results indicate NF-κB activation is an essential mechanism of the increased susceptibility of Siglec-G-/- mice to CLP.
4.5 Discussion

Sepsis is an acute infection in which common symptoms include fever, anorexia, weakness and lethargy. Sepsis often leads to septic shock, which is a collapse of the circulatory system. The model of cecal ligation and puncture in rodents has been used extensively to investigate the clinical settings of sepsis and septic shock.

Siglec-G-deficient mice, in spite of their high levels of blood IgM, are more susceptible to experimental sepsis induced by CLP. Peritoneal lavages and blood collected after CLP yielded more live bacteria than in Siglec-G\textsuperscript{+/+} mice. Proinflammatory cytokines, such as TNF-\(\alpha\), IL-6 and MCP-1 are significantly elevated in Siglec-G\textsuperscript{−/−} mice blood. Post-CLP SiglecG\textsuperscript{−/−} mice also have more severe liver damages, indicated by higher levels of aspartate transaminase. NF-\(\kappa\)B is critical in controlling many proinflammatory cytokine expressions, including TNF-\(\alpha\), IL-1\(\beta\), IL-6, IFN-\(\gamma\), MCP-1 and IL-12. Proinflammatory cytokines, especially TNF-\(\alpha\) and IL-6 have been closely linked to the survival in CLP. NF-\(\kappa\)B activity is enhanced by Siglec-G deficiency in naïve mice. Thus we hypothesize that the susceptibility of Siglec-G-deficient mice is due to activated
NF-κB, which leads to activated expressions of proinflammatory cytokines. This hypothesis is supported by the reversal of survival disadvantage of Siglec-G-/- mice when NF-kB activation was inhibited by IKK inhibitor. With NF-κB inhibition, serum levels of key cytokines as TNF-α and IL-6 were dramatically reduced in Siglec-G mice.

Several lines of evidence suggest the key role of NF-κB activation in sepsis. Studies have demonstrated that NF-κB inhibitors protect animal from CLP-induced sepsis [117, 148-150]. IL-10 protects mice from lethal endotoxemia [151], and recombinant human protein C protects patients from severe sepsis [152] are found to effect by inhibiting NF-κB activation. IL-10 has been proven to be a potent inhibitor of NF-κB [153, 154]. Surprisingly, high level of IL-10 is found in Siglec-G-/- mice serum post-CLP, while the high-level of IL-10 does not protect Siglec-G-/- mice from CLP.

Besides controlling inflammatory cytokine expressions, NF-κB also controls other components that are important in sepsis. These include the expression of tissue factor plasminogen-activator inhibitor type-1, inducible nitric oxide synthase, cyclooxygenase-2, and adhesion molecules such as ICAM-1 and
E-selectin. It is plausible to hypothesize that Siglec-G also influence these pathways to affect septic susceptibility in Siglec-G-deficient mice.
Figure 4.1

(A) 100

80

60

40

20

0

0

1

2

3

4

Days

Percent survival

Siglec-g/-

WT

p=0.0048

(B) 100

80

60

40

20

0

0

2

4

6

8

Days

Percent survival

Siglec-g/-

WT

p=0.0073
Figure 4.1. Survival study during CLP-induced severe sepsis. WT and Siglec-G−/− mice were subjected to CLP surgery, either in the absence (A) or in presence (B) of antibiotics treatment. Survival was monitored for 3 or 7 days following surgery. Data are representative of 2 similar experiments. Each group: n = 10.
Figure 4.2

A

IFN-γ

IL-10

P=0.0091

P=0.238

P=0.0041

P=0.028

IL-12p70

IL-6

P=0.398

P=0.0091

MCP-1

TNF-a

P=0.0018

P=0.0041

(Continued on page 91)
Figure 4.2 (continued)

B

**TNF-α**

<table>
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**IL-1**

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Figure 4.2. Proinflammatory cytokines profiles in WT and Siglec-G−/− mice post-CLP. A. IFN-γ, IL-10, IL-12p70, IL-6, MCP-1 and TNF-α profiles in the blood 24 hours post-CLP were analyzed by cytometric bead assay (n=5). B. Kinetic studies of TNF-α and IL-1β in the blood post-CLP by ELISA. n=10. Data are represented as Mean ± SEM. Data shown are representative of two independent experiments.
Figure 4.3

Figure 4.3. **More severe hepatic damages in Siglec-G−/− mice.** Higher levels of aspartate aminotransferase (AST) in the sera of Siglec-G deficient mice 24 hr post CLP. Data are represented as Mean ± SEM. N=10
Figure 4.4

A

24hr Post-CLP Blood CFU

CFU per ml blood

P < 0.05

SigecG KO B6 WT

B

72hr Post-CLP PLF CFU

CFU per ml PLF

P < 0.05

SigecG KO B6 WT
Figure 4.4. Bacterial load in the peripheral blood and peritoneum. Siglec-G -/- and +/+ mice received 9-puncture CLP. Twenty-four hours later, mice were bled retro-orbitally and blood samples were collected. Seventy-two hours later, mice were euthanized and peritoneal lavage fluid was obtained. 30 ul of blood (A) and 10 ul of peritoneal lavage fluid (B) was serially diluted and plated on LB agar plates with LB broth, and the CFU was determined. Data are represented as Mean ± SEM.
Figure 4.5

Peritoneal lavage cells

A

WT

Siglec-G KO

B

72hr Post-CLP PerC Mo/N

<table>
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<th>Siglec-g KO</th>
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<td>[Graph Data]</td>
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</table>

P<0.05
Figure 4.5. Siglec-G deficient mice demonstrated less peritoneal macrophages and more polymorphonuclear cells post-CLP. A. Peritoneal lavage cells from Siglec-G^{+/+} (left) and Siglec-G^{-/-} (right) mice. B. Significantly lower numbers of macrophages and higher numbers of PMN cells were found in Siglec-G^{-/-} mice peritoneal lavage. Data are represented as Mean ± SEM.
Figure 4.6

Figure 4.6. Impaired bactericidal capacity of Siglec-G/- peritoneal macrophages and increased proinflammatory cytokine secretion.

Macrophages were collected from WT and Siglec-G/- mice, as described in Materials and Methods. A, Cells (5 x 10⁵) were infected with bacteria (1.7 x 10⁵ CFU) and the killing activity was examined, as determined by CFU assay. B, TNF-α and IL-1β in the culture supernatants were analyzed by ELISA in the culture medium. Data are represented as Mean ± SEM. Data are representative of two independent similar experiments.
Figure 4.7

(A)  

(B)  

(Continued on page 100)
Figure 4.7 (continued)

C

Siglec-G WT

\( \square \) IKK (-)

\( \bullet \) IKK (+)

Siglec-G KO

\( \square \) IKK (-)

\( \bullet \) IKK (+)

D

IKK Inhibitor (-)

\( \square \) Siglec-G WT

\( \bullet \) Siglec-G KO

IKK Inhibitor (+)

\( \square \) Siglec-G WT

\( \bullet \) Siglec-G KO

(Continued on page 101)
Figure 4.7 (continued)

E

Inhibition of TNF-a and IL-6 by IKK Inhibitor

F

OD @ 450 nm

Siglec-G KO

WT

Spl (CLP)

Spl (CLP + Ikki)
Figure 4.7. IKK inhibitor reduced proinflammatory cytokines and prolongs survival in Siglec-G-deficient mice. A. IKK inhibitor treatment resulted in significantly improved survival in Siglec-G-deficient mice. B. Similar survival rates were achieved between Siglec-G WT and KO with IKK inhibitor treatment. C & D. IKK inhibitor treatment dramatically reduced IL-10, IL-6 and TNF-α levels in the blood collected 24 hr after CLP in both Siglec-G WT and KO. E. In vitro suppression of TNF-α and IL-6 in co-culture of peritoneal lavage cells and enteric bacteria. (IKK inhibitor concentration: 10 ug/ml). n=5. F. NF-κB phosphorylated p65 ELISA showed inhibition of NF-κB activation with IKK inhibitor. Data shown are Mean ± SEM. Data are pooled from two similar independent experiments. (For IKKi +, n=15, for IKK -, n=10).
Chapter 5

Concluding Remarks

In the immune system, cell surfaces are equipped with a wealth of glycans, which can be recognized by glycan-binding proteins. The Siglecs are a family of proteins that bind to sialic acids. Siglecs are thought to involve in cell-cell interactions and play important roles in immunity, especially innate immunity. To date, there are 13 Siglecs discovered in human and 8 in mouse. Structurally, with the exception of mouse CD33 and Siglec-H, all murine Siglecs have one ITIM and one ITIM-like motif in their cytoplasmic portions. The ITIM motifs are believed to transmit inhibitory signals counteracting with activating signals conducted by ITAM motifs [8].

Siglec-G is a newly discovered member of the Siglec family. Using a Siglec-G knock out GFP knock in mouse model, we detected broad expression of Siglec-G in the hematopoietic system. Siglec-G is highly expressed on B cells,
moderately on monocytes/macrophages and dendritic cells and weakly on T cells. Siglec-G expression is turned on in the hematopoietic stem cells (Lin-Flt2-Sca1+cKit+), with high level of transcript detected in Pre-Pro/Pro B cells. Despite of the wide expressions in the hematopoietic system, major populations, including HSC, Pre-pro/Pro B, Pre/New B, FO B, MZ B are not affected by Siglec-G mutation. The only B cell population that is changed by the Siglec-G deficiency is the peritoneal B-1a cells (B220⁺CD11b⁺CD5⁺). There is a 5-fold increase of peritoneal B-1a compartment in Siglec-G⁻/⁻ mice compared with WT. Concomitantly, serum IgM is elevated by 5-fold in Siglec-G-deficient mice. It is generally believed that a significant portion of serum IgM is natural antibodies, predominantly secreted by resident B-1 cells in the body cavity serosa, the pleural and peritoneal cavities.

Natural antibodies constitute the first line of defense against many bacterial pathogens, especially those bear polysaccharide antigens [70, 71]. We hypothesized that high titers of natural antibodies in Siglec-G-deficient mice are protective in bacterial infections, introduced by cecal ligation and puncture (CLP), a murine model for clinical sepsis. However, in spite of the high levels of natural antibodies, Siglec-G-deficient mice were significantly more susceptible to CLP.
The reason was partly that natural antibodies are only one of the many components involved in innate immunity. Proinflammatory cytokines have been found to be associated with survival in CLP. Particularly, high levels of TNF-α and IL-6 have been strongly correlated with high mortality in CLP [81, 82]. In keeping with this notion, concentrations of TNF-α and IL-6, as well as other proinflammatory cytokines, such as IL-10 and chemokine MCP-1 were found to be significantly higher in Siglec-G-deficient mice in CLP. Thus the high mortality observed in Siglec-G-deficient mice during CLP can be explained by the overproduction of proinflammatory cytokines by Siglec-G-deficient immune cells, such as macrophages.

Mechanistically, Siglec-G is a natural suppressor of NF-κB activation. In naïve state, Siglec-G-deficient peritoneal lavage cells contain more nuclear p65 and total phosphorylated p65 evidenced by western blot and ELISA, respectively. Gel shift assay also demonstrated higher accumulation of nuclear p50/p65.

During B-1a cell development, canonical NF-kB pathways are mediated by BCR-mediated signaling. Deficiencies of canonical pathway adaptor proteins, such as B-cell-leukemia/lymphoma-10 (Bcl-10) [105], mucosa-associated lymphoid tissue-1 (Malt-1) [106] and caspase recruitment domain family 11
(Card11) [107] have been shown to result in a dramatic reduction of B-1 cells, to a lesser extent a reduction of MZ B cells and negligible effect on FO B cells. Inhibition of NF-κB activation during early postnatal days resulted in a dramatic reduction of peritoneal B-1a cells. Taken together, our Siglec-G knock out model clearly show an over-activation of NF-κB, which leads to expanded B-1a population in the peritoneal cavity.

During sepsis, NF-κB is activated by bacteria, bacterial products and proinflammatory cytokines released during sepsis. Several lines of evidence indicate the important role of NF-κB activation in sepsis. In the mouse model of sepsis, cecal ligation and puncture, molecules that have been proved to protect mice from lethality or to improve survival exert their protective effect by inhibiting NF-κB activation [151, 155-157]. NF-κB activation initiates the transcriptions of multiple proinflammatory cytokines, including TNF-α and IL-6, which are associated with the survival in CLP [158]. Studies have shown that in vivo inhibition of NF-kB activation reduced mRNA and protein expressions of multiple proinflammatory cytokines during sepsis [158-160]. In our studies using the Siglec-G-deficient mice, we found when NF-κB activation is inhibited by a potent inhibitor IKK inhibitor VI (Calbiochem), the survival of Siglec-G-deficient
mice achieved a similar rate as with Siglec-G wild type controls. In addition, levels of TNF-α and IL-6 were significantly reduced with NF-κB inhibition in Siglec-G-deficient mice during CLP. In summary, the high mortality rate of Siglec-G-deficient mice to CLP is dependent on activation of NF-κB, which is a natural target of Siglec-G.

Although extensive efforts have been put on the elucidations of B-1 cell development and function, there are still many key questions yet to be answered. What is mechanism of B-1 cell self-maintenance and renewal? How important are natural antibodies in providing protection against bacterial infections? Our studies of Siglec-G certainly shed light on our understandings of B-1 cell development and function. Our studies have clearly shown Siglec-G down-regulates NF-κB specifically in peritoneum, leading to an enrichment of peritoneal B-1a cells, exerting no effect on splenic B-1a cell population in adulthood. Our studies have also shown that despite the elevated levels of natural antibodies, SiglecG-deficient mice are more susceptible of CLP-induced sepsis. Additional studies are needed to determine whether the protective effect was obscured by defective innate resistance. More importantly, more work is needed to elucidate how Siglec-G represses NF-κB activity and what factors are responsible for loss of resistance in
the Siglec-G-deficient mice. Regardless of what mechanism is responsible for Siglec-G-mediated resistance, identification of a molecular checkpoint such as Siglec-G may provides an important therapeutic target for the treatment of sepsis and for understanding genetic basis of the resistance to sepsis.
BIBLIOGRAPHY


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