Molecular and Cellular Approaches to Immune Regulation by Sialic acid-binding Lectins

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免疫制御機構に関する細胞工学的研究

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Contents

Chapter 1

General introduction

Introduction

Three nutrients important for the livings are protein, fat and sugar. It has been thought for many years that the sugar has only role as the energy source of us. However, significant development of molecular cell biology makes clear that the molecular mechanisms of various phenomena in our body are controlled by the 'sugar'-chain. For example, sugar-chains exist in the cell membranes as glycoproteins and glycolipids, and are participating in vital life phenomena, such as hormone adjustment, immune regulation, differentiation, interaction or recognition between cells, and fertilization. Moreover, it is known that sugar-chains function as receptors for pathogenic bacteria, viruses, and lymphocytes (Fig. 1). Therefore, now, sugar-chain is called as a 3rd chain essential for the livings in addition to chains of the nucleic acids and proteins as it is participating in various physiological functions.

Sialic acids reside in terminals of the sugar-chains of many glycoproteins and glycolipids especially in higher animals. In this outward exposed position, sialic acids contribute significantly to the structural and functional properties of these molecules on cell surfaces via regulating cellular and molecular interactions. For example, sialic acid is involved in the first step of the leukocyte-endothelial cell interaction called

rolling that proceed the adhesion of leukocytes and is crucial in the regulation of the immune responses during infection or in recirculation of lymphocytes to lymphatic organs [1]. In this thesis, I tried to make clear the regulatory roles of sialic acid and their binding lectins in immune responses.

In Chapter 2, I focused on signaling roles of L-selectin, one such lectin, in the activation of T lymphocytes. Rolling (Fig. 2) is mediated by transient interactions of L-selectins on leukocytes with their sialic acid-containing glycoprotein-ligands on endothelial cells under certain situations [2]. Mice deficient in L-selectin, which binds 6-sulfo sialyl-Le^X, is suffer from a lack of T cell homing and local inflammation [3], indicating essential roles for L-selectin in this type of immune regulation. Although the cytosolic domain of L-selectin is very short (Fig. 3), engagement of L-selectin induces the activation of various signaling molecules [4-9]. However, there is less information available on the involvement of L-selectin in signal transduction in primary T lymphocytes.

The activation of T cells requires two types of signals. One is antigen-dependent and is mediated by the T cell receptor-CD3 complex which recognizes antigen bound to MHC. The other is antigen-independent costimulation that is provided by soluble factors and/or surface ligands on antigen-presenting cells to interacting with their counterreceptors on the T cells. Although anti-L-selectin antibody alone does not induce T cell proliferation, it enhances the proliferation of mouse T cells in mixed lymphocyte reaction [12] or of human peripheral blood lymphocytes under anti-T cell receptor antibody stimulation [13]. Therefore, L-selectin is a candidate for a costimulatory pathway, although precise mechanism for proliferation remains to be

elucidated. Here, I show in Chapter 2, the study that the costimulatory activity of L-selectin for primary murine T cells using anti L-selectin monoclonal antibody, MEL14.

Innate immunity functions as a pathogen sensor and contributes to the eradication of pathogens and the establishment of adaptive immunity. A group of transmembrane proteins, Toll-like receptors (TLRs), recognize a range of chemicals produced by bacteria, viruses, fungi and protozoa to initiate first defense against pathogens (Fig. 4) [14,15]. Each TLR recognize specific structure as pattern-recognition receptors. The importance of TLRs in such pattern-recognition has been demonstrated by gene-targeted mice: lack of single TLR sometimes leads to complete abrogation of immune response against several types of pathogens. The activation and regulation of TLR-initiated immune responses are studied extensively. TLRs recognized their ligands in a form of heterodimer or homodimer, which in turn initiate a wide variety of intracellular signaling including MAP kinases and NF-κB by kinds of adapter proteins. The existence of adapters and heterodimers might explain the differences the specificity of immune responses initiated by each specific pathogen, though much is remained for elucidated for the specificity. In addition to the complexity within TLR family, its signal is reported to be regulated by various signals. These include cytokines such as IFN-γ, TGF-β and others as well as hormones. In fact, recent system biological study indicated that TLR signaling is affected by most other stimuli in monocytes/macrophages, which apparently more frequent than other combinations of stimuli.

Siglecs are sialic acid-recognizing Ig-superfamily lectins prominently expressed in

immune cells (Fig. 5 and 6) [16,17]. CD33-related Siglecs are a subset thought to down-regulated innate immune cell activation via cytosolic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Fig. 7). These ITIM recruit protein phosphatases, Src homology region 2 domain-containing phosphatases (SHPs) SHP-1 and SHP-2, which limit activation pathways stimulated by tyrosine kinases.

Despite the rarity of sialic acid in lower organisms, it is striking that many known pathogens have independently evolved the capacity to synthesize sialic acid or capture it from their hosts (Table 1). In several cases, expression of sialic acid has been shown to be essential for paghogenicity and survival within the host. The presence of sialic acid on these pathogens is likely to be important for host mimicry, prevention of complement activation, attenuation of antibody production and non specific charge-repulsion effects [18]. Interesting possibility is that sialic residues on pathogens interact with inhibitory molecule such as Siglec. This might seem an attractive hypothesis, but one of the unknown important points is whether Siglec modulate signaling of pathogen-receptor, such as TLR. Therefore, the study in Chapter 3 and 4, I focused on the immune-inhibitory activity of Siglecs on the activation of TLRs.

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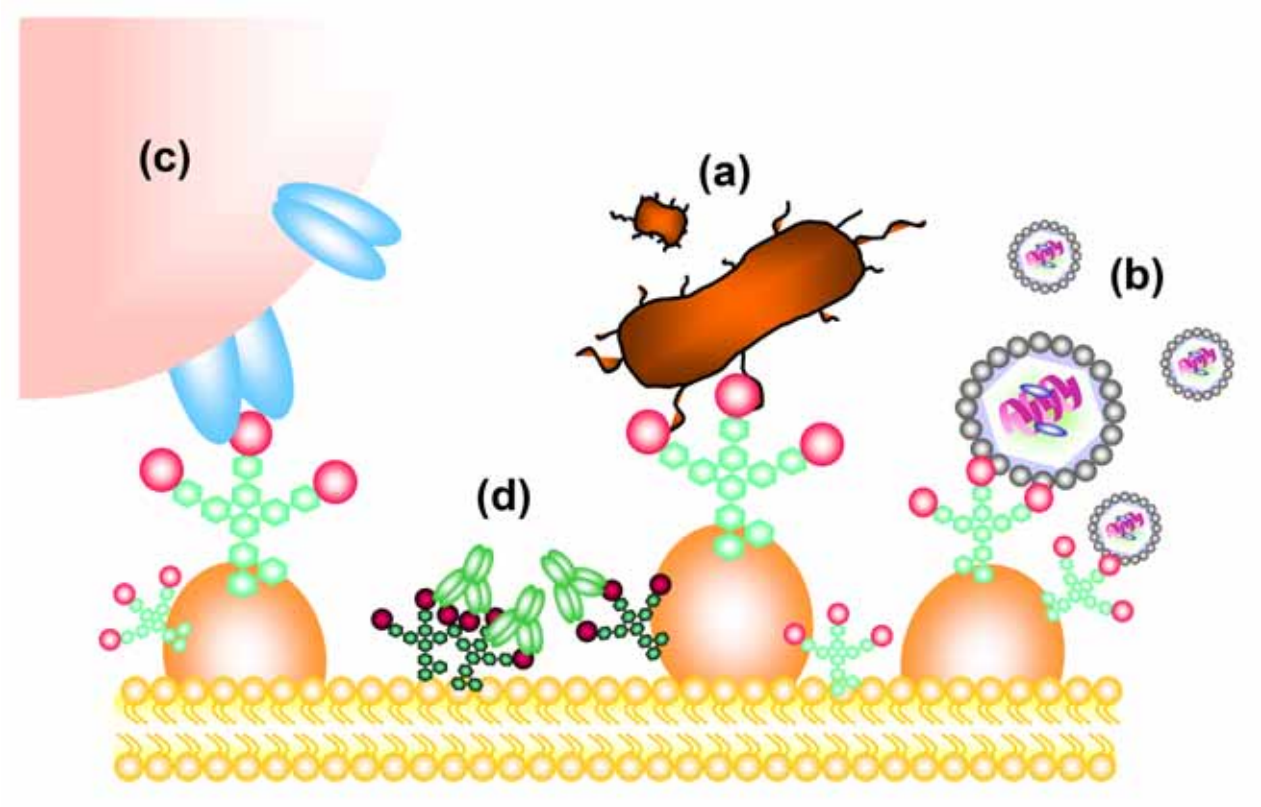

Figure. 1. Examples of the roles of cell surface sugar-chains in mediating molecular recognition events. (a) Cell surface sugar-chains are exploited by bacterial pathogens for adhesion and colonization. (b) Viral pathogens also exploit cell surface sugar-chain before invasion. (c) Cells can interact through the multivalent binding of sugar-chain on glycoproteins to receptors on cognate cells. (d) Glycolipids, including tumor-associated antigens, present sugar-chains that can be recognized by antibodies. Glycoprotein-based tumor antigens, such as mucin-like molecules on epithelial-derived cancers, also serve as immunogenic epitopes for antibody binding.

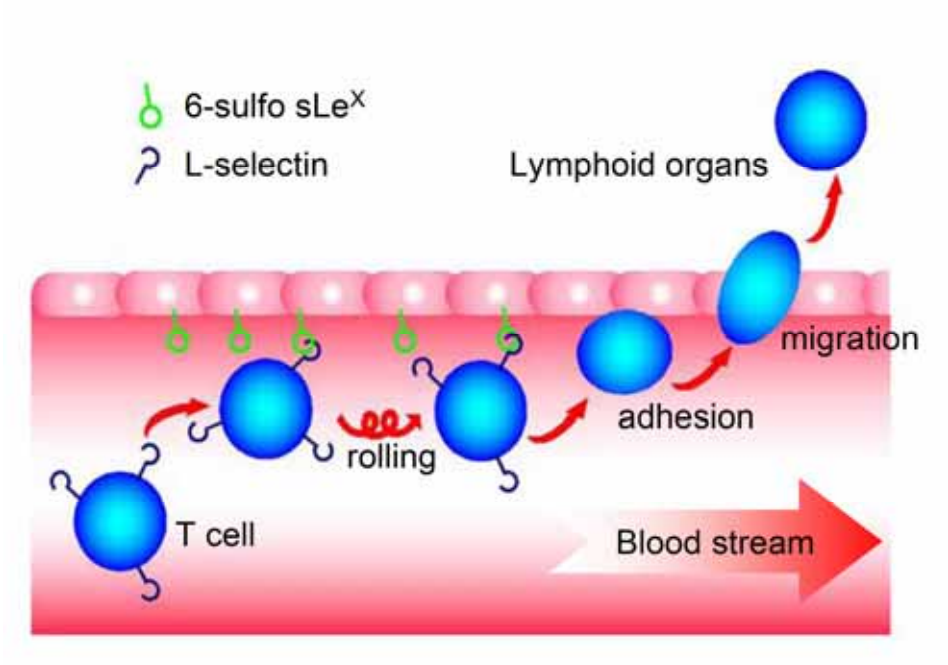

Figure. 2. L-selectin as a rolling receptor on T cells. L-selectin has an important role in controlling immunity as a rolling receptor on T cells, mediating the first step in the leukocyte-endothelial cell interaction. Partial deletion of the intracellular domain of L-selectin prevents leukocyte rolling without affecting ligand recognition, indicating that only binding of L-selectin to its ligand is not sufficient for leukocyte rolling.

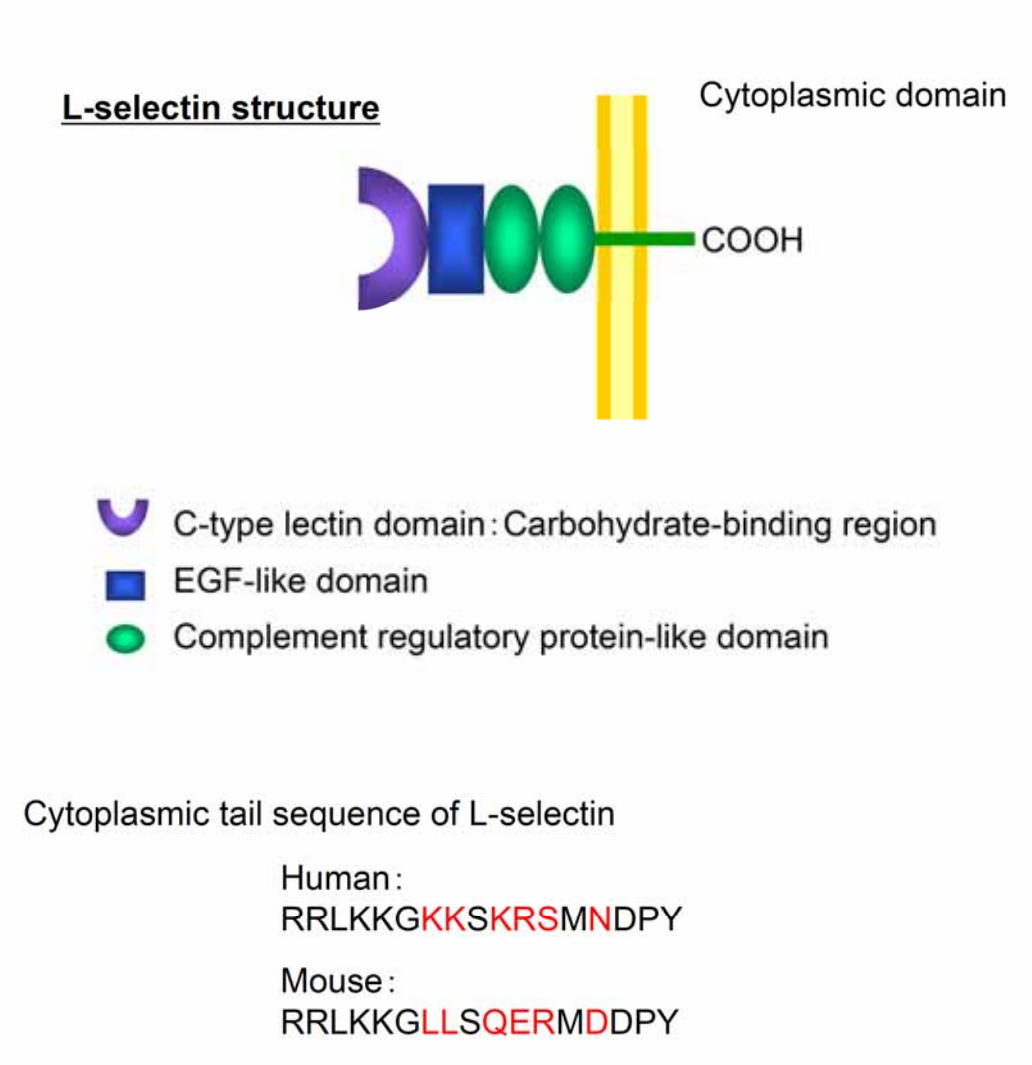

Figure. 3. The structure of L-selectin.

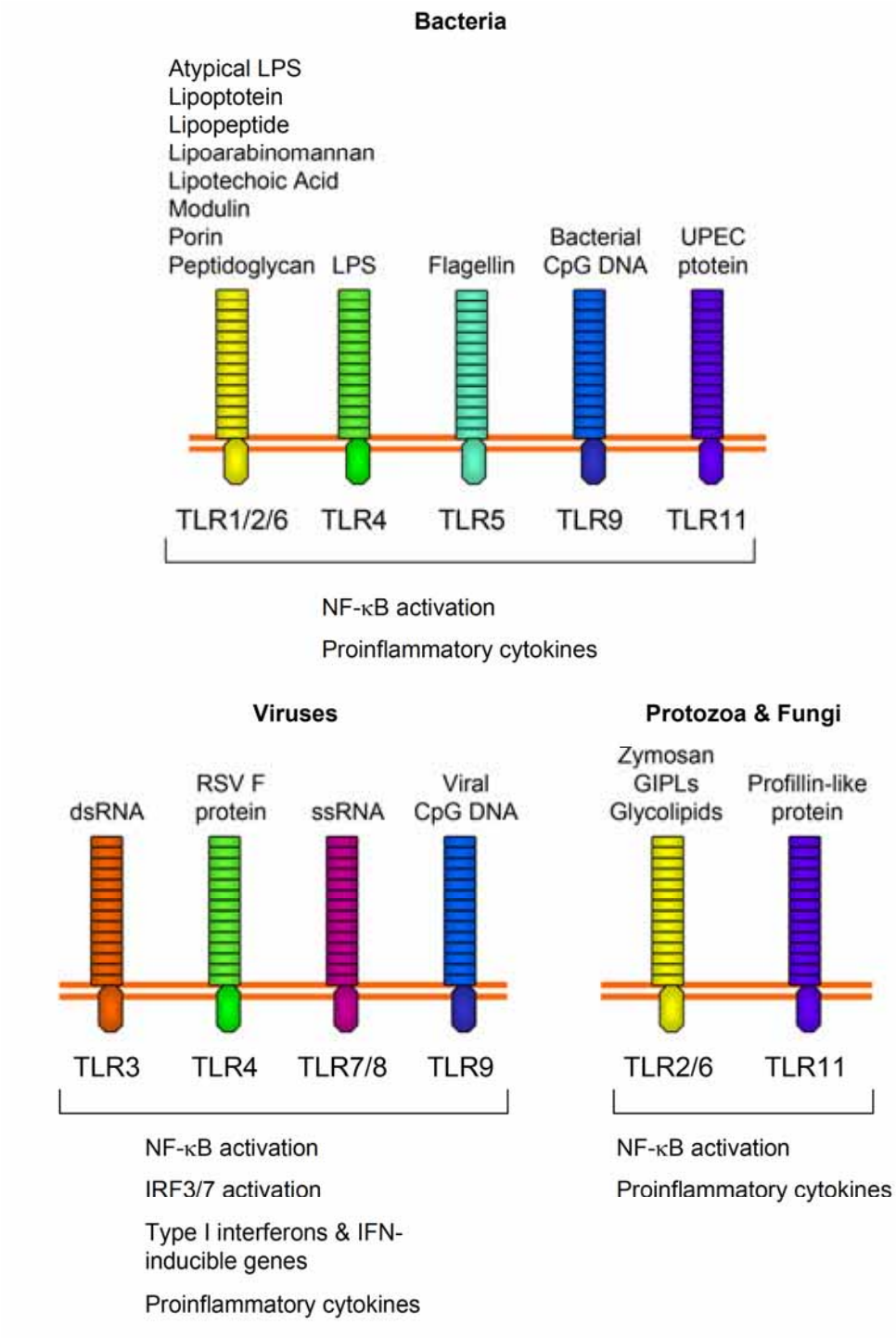

Figure. 4. TLR ligand specificities and immune responses.

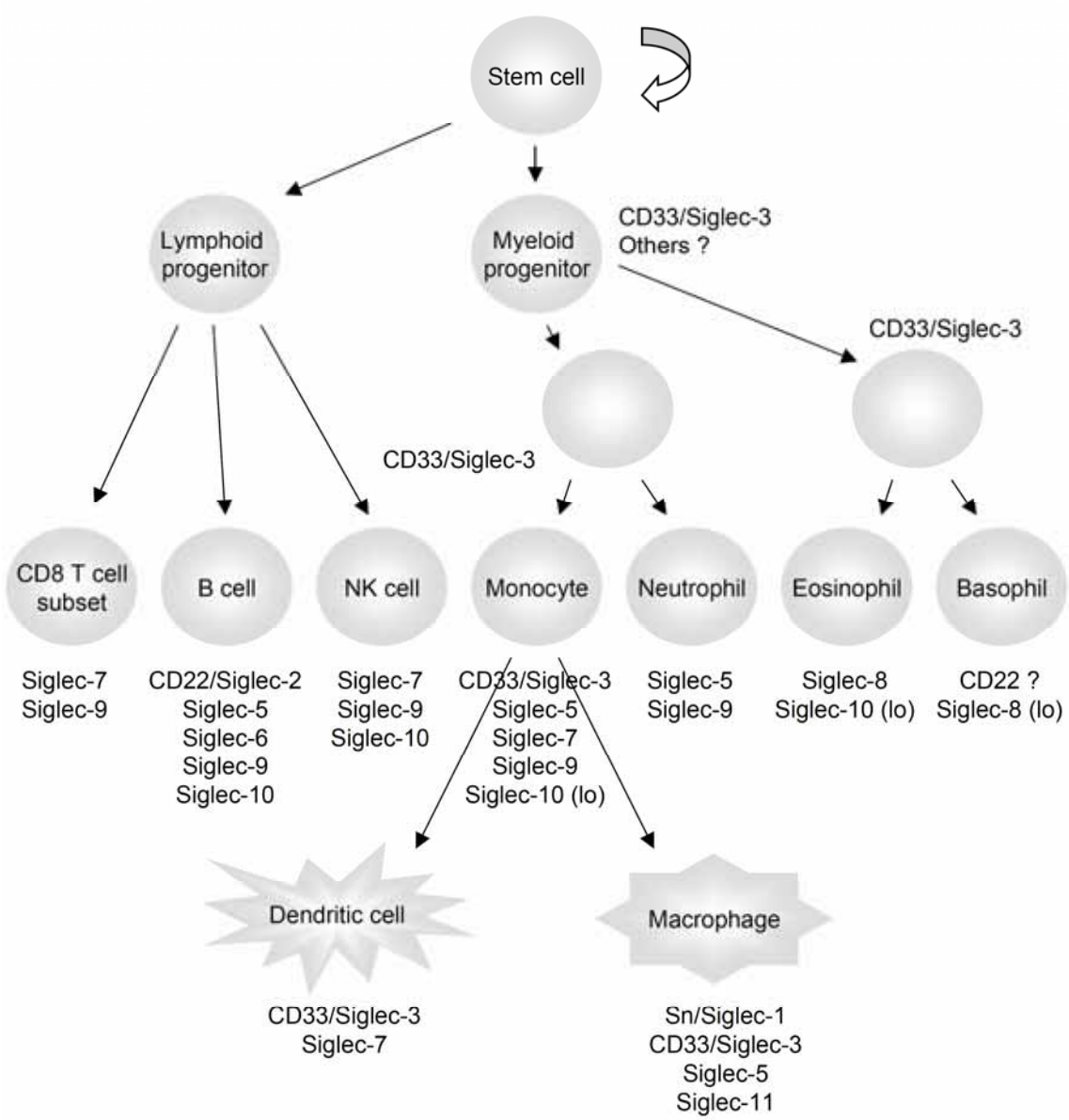

Figure. 5. Cell-type specific expression of Siglecs in the hematopoietic and immune cells of humans.

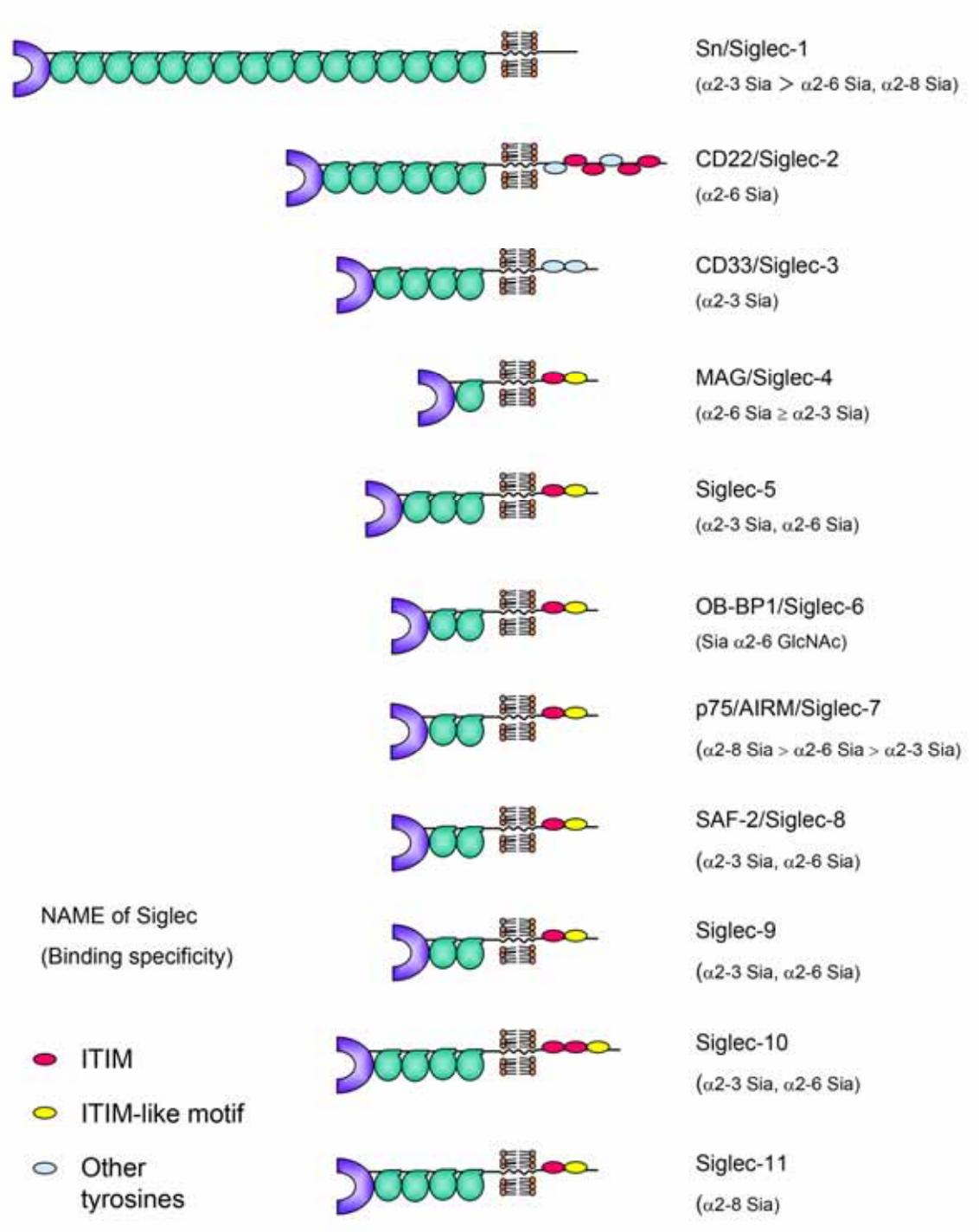

Figure. 6. Glycan-binding specificities of human Siglecs.

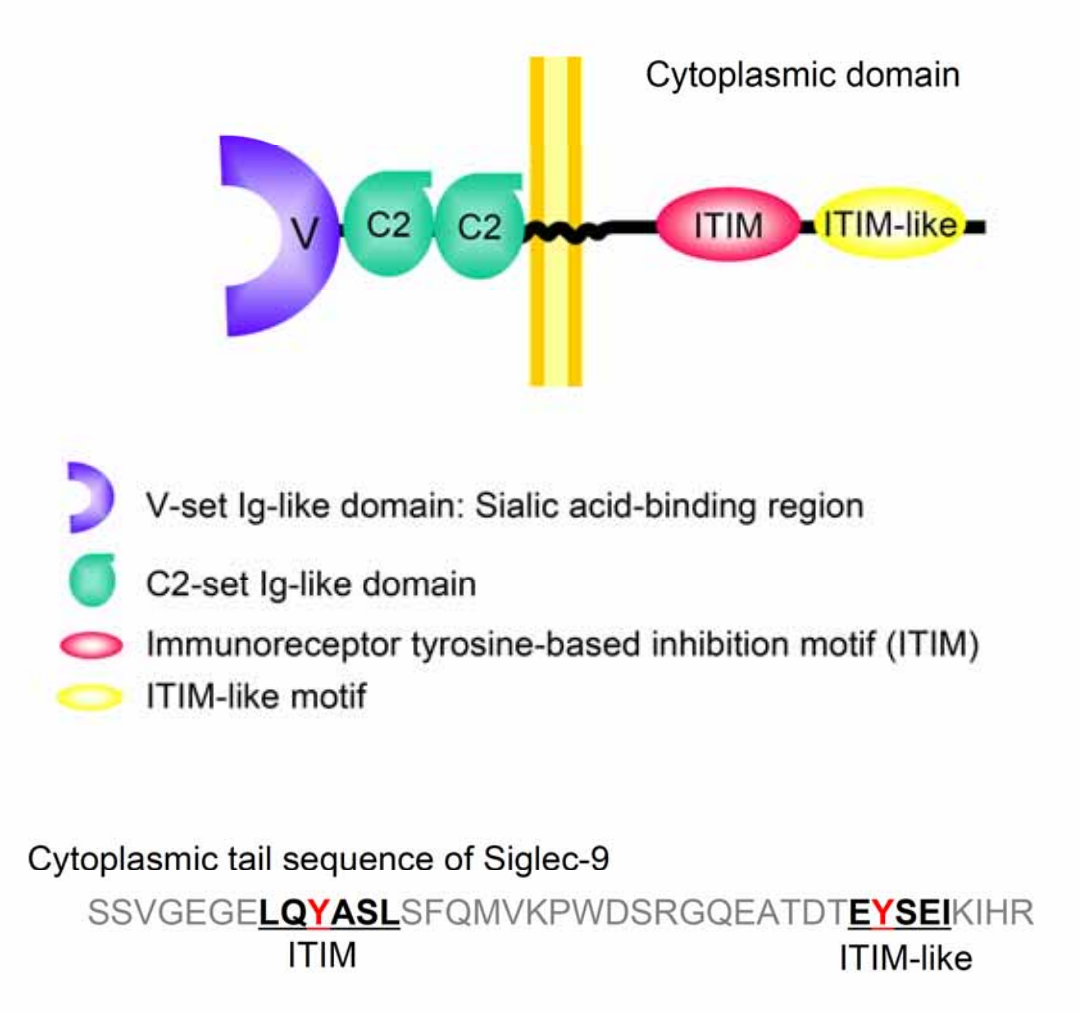

Figure. 7. The structure of Siglec-9.

Table 1. Sialic acid expression by pathogens and the associated disease [18]

Sia synthesized by the pathogen:

Neisseria meningitidis groups B and C (meningitis and septicaemia),
Haemophilus influenzae (infant meningitis and respiratory infection),
Haemophilus ducreyi (chancroid, a venereal disease),
Escherichia coli groups K1, K92 etc. (neonatal meningitis and septicaemia),
Pasturella hemolytica (bovine bronchitis),
Streptococcus group B (neonatal septicaemia).

Sia transferred from host glycoconjugates by trans-sialidase:

Trypanosoma cruzi (Chagas' disease),
Corynebacterium diphtheriae (diptheria).

Sia captured from host cytidine monophosphate-Sia by surface sialyltransferase:

Neisseria gonorrhoea (gonorrhoea),
Neisseria meningitidis group A (meningitis and septicaemia).

Source of Sia unknown:

Sporotrichium schenkii (fungal skin infection),
Cryptococcus neoformans (meningitis),
Candida albicans (mucosal infections),
Campylobacter jejuni (diarrhoea, Guillian–Barre syndrome).

Chapter 2

Costimulation of T cell proliferation by anti-L-selectin antibody is associated with the reduction of a cdk inhibitor p27

Introduction

L-selectin has an important role in controlling immunity as a rolling receptor on T cells, mediating the first step in leukocyte-endothelial cell interaction [1]. Mice deficient in L-selectin suffer from a lack of T cell homing and local inflammation [2], indicating vital roles for L-selectin in this class of immune regulation. Furthermore, it is suggested that L-selectin plays a fundamental role as a signal transduction molecule. Although the cytosolic domain of L-selectin is very short, the engagement of L-selectin induces the activation of various signaling molecules, including mitogen-activated protein kinases such as extracellular signal-regulated protein kinase (ERK) [3,4], c-Jun NH_2-terminal kinase [5] and p38 [6], small G proteins Ras [3] and Rac [3,7], calcium influx [8] and ceramide release [9]. A link to biologically relevant responses to such activation has been shown in neutrophils in which the generation of superoxide and the expression of cytokines such as IL-8 and tumor necrosis factor-α were induced by anti-L-selectin monoclonal antibody or ligand saccharides [8]. These findings were primarily made using human neutrophils and the leukemic Jurkat T cell line. Less information is available on the involvement of L-selectin in signal transduction in

primary T lymphocytes.

The optimal activation of T cells requires at least two types of signals: antigen-dependent signals through the T cell receptor (TCR)-CD3 complex and antigen-independent costimulation [10]. The essential role of interaction between CD28 on T cells and its ligands, CD80 and CD86, on antigen-presenting cells as a costimulatory pathway has been demonstrated by the finding that T cell responses are severely impaired in CD28-disrupted mice [11,12]. However, detectable T cell responses remained in these mice, suggesting that other costimulatory pathways are also physiologically relevant. To date, various molecules have been shown to have costimulatory activity: cytokines such as IL-1, IL-6 and IL-12 or antibodies to cell surface molecules such as CD2, CD5, CD9, CD11a (component of LFA-1), CD27, CD29, CD43, CD44, CD47, CD134 (OX40), CD137 (4-1BB), CDw150 (SLAM) and ICOS enhance T cell activation under given conditions [13]. In addition, L-selectin is a potential candidate for another costimulator, since anti-L-selectin antibody enhanced the proliferation of mouse T cells in a mixed lymphocyte reaction [14], and human peripheral blood lymphocytes stimulated with anti-T cell receptor antibody [15]. Furthermore, L-selectin was present in lipid rafts [16]. which are regions of the plasma membrane that possess a high content of cholesterol and sphingolipid and function as platforms for proteins in signal transduction. Here, we demonstrate the costimulatory activity of L-selectin in murine T cells using a monoclonal antibody.

Materials and Methods

Reagents

Staphylococcal enterotoxin B (SEB), a bacterial superantigen, was purchased from Toxin Technology (Sarasota, FL). Anti-CD3 antibody 145-2C11 [17] and anti-L-selectin antibody MEL14 [18] were purified from ascites with a POROS Protein G plastic column (NGK Insulators, Nagoya, Japan). Anti-CD28 antibody 37.51, which stimulates T cells [19], was purchased from BD PharMingen (San Diego, CA). Control rat IgG2a was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-labeled anti-IL-2 receptor α chain antibody 7D4 and control rat IgM were obtained from PharMingen and Santa Cruz Biotechnology, respectively.

T cell proliferation assay

Male BALB/c mice were purchased from Slc Japan (Hamamatsu, Japan). Lymph node cells from non-primed mice were isolated as previously described.[20] Whole lymph node cells (1×10^5) were cultured in microtiter plates in 200 μl of RPMI1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 100 U/ml of penicillin G, 100 μg/ml of streptomycin and 10% fetal calf serum (Biowittaker, Walkersville, MD) for 3 days, and the incorporation of ^{3}H-thymidine (18.5 kBq/well) in the last 16 h of culture was measured. At the initiation of culture, 2.5 μg/ml of SEB was added for stimulation. Soluble antibody was added at a concentration of 5 μg/ml.

To examine the direct effects of antibodies on T cells, purified T cells were stimulated with immobilized antibodies. T cells were negatively purified from whole

lymph node cells using a column packed with Nylon Fiber (Wako Chemicals, Osaka, Japan) as recommended by the manufacturer. Approximately 25% of the lymph node cells was collected as the T cell fraction, whose purity was confirmed by flowcytometry: the T cell fraction contained less than 5% surface Ig^+ cells and MHC class II^+ cells (data not shown). To immobilize antibodies, plates were coated with 50 μl of anti-CD3 antibody solution at a concentration of 2 μg/ml for 60 min, then control rat IgG2a and MEL14 antibodies were added to the wells at a final concentration of 5 μg/ml and incubated for a further 60 min. After the wells had been washed with phosphate-buffered saline, 1×10^5 cells were seeded for stimulation. In some experiments, cells were stimulated with soluble antibody at a concentration of 5 μg/ml.

To stimulate T cells with antibodies bound on beads, 2×10^7 of Protein G Magnetic Beads (2.8 μm in diameter, Dynal, Oslo, Norway) were incubated with 40 μl of 100 μg/ml each of a single or combination of antibodies for 60 min.

Reverse transcription-polymerase chain reaction (RT-PCR)

T cells (1×10^6) were cultured in 1 ml of medium in 24-well plates for 24 h. The plates were coated with antibodies similarly to microtiter plates except that 2 μg/ml of anti-CD28 antibody was also used. Total RNA was extracted and reverse-transcribed. cDNA was then amplified by real-time PCR (LightCycler, Roche Diagnostics, Mannheim, Germany) using SYBR Green dye for detection. Real-time PCR was performed in 20 μl reaction mixtures containing 2 μl of LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics), 500 nM of each primer, 0.5 μl of $MgCl_2$ solution (25 mM) and 2 μl of sample or plasmid DNA. To generate standard curves, plasmid vectors into which amplified fragments had been cloned were included.

LightCycler amplification involved a first denaturation at 95 ˚C for 60 s, followed by amplification of the target DNA for 45 cycles (95 ˚C for 15 s, 60 ˚C for 30 s, 72 ˚C for 30 s). The amount of each gene was first determined with LightCycler Software version 3.5 (Roche Diagnostics) based on the standard curve using corresponding plasmid, then expression levels were normalized by calculating the ratio of the mRNA of interest to the amount of GAPDH mRNA. The primers used were mouse IL-2 forward (5'-GCTCTACAGCGGAAGCACA-3') and reverse (5'-TCCTCAGAAAGTCCACCACAGT-3'), mouse IL-2 receptor α chain forward (5'-GTCTGTATGACCCACCCGAG-3') and reverse (5'-GTGAGCACAAATGTCTCCGTC-3'), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward (5'-CTACCCCCAATGTGTCCGTC-3') and reverse (5'-GCTGTTGAAGTCGCAGGAGAC-3').

Western blotting

Antibodies to phospho-p44/42 MAPK (#9101) and cyclin-dependent kinase (cdk) inhibitor p27 (#sc-528) were purchased from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology, respectively. To detect phosphorylation of ERK, cells (1×10^6) were stimulated for 120 min in 0.2 ml of medium in 24-well plates, and whole cell lysates were subjected to Western blot analysis. To assess p27, T cells were stimulated in 1 ml of medium in 24-well plates for 24 h. Ten micrograms of protein were loaded onto a 7.5 % polyacrylamide gel and transferred to PVDF membrane, which was sequentially incubated with 3% bovine serum albumin, primary antibody specific to each protein, peroxidase-conjugated anti-rabbit IgG antibody (#7074, Cell Signaling Technology). The proteins were visualized with Western Lightning^{TM}

Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA).

Results

Anti-L-selectin antibody enhances the proliferation of whole lymph node cells induced by a bacterial superantigen

The expression level of L-selectin in lymph node cells was evaluated by flowcytometry. L-selectin was expressed in 67% of whole lymph node cells (Fig. 1a). In the T cell fraction, approximately 70% of T cells expressed L-selectin (Fig. 1b), consistent with previous reports [21].

To assess whether anti-L-selectin antibody enhances the proliferation induced by bacterial superantigens, lymph node cells were stimulated *in vitro* with MEL14 in the presence of SEB. The soluble form of MEL14 significantly enhanced the proliferation, while control rat IgG had no effect (Fig. 2). MEL14 alone induced the aggregation of lymphocytes (data not shown) through the activation of signaling pathways [22]. but did not induce any proliferation (data not shown). These results suggest that L-selectin stimulates T cells as a costimulator.

Crosslinking L-selectin enhances the T cell proliferation induced by anti-CD3 antibody

To assess the direct action of MEL14 on T cells, the effects of the antibody on purified T cells were examined using antibodies immobilized on culture dishes. T cells proliferated in response to immobilized anti-CD3 antibody alone, and immobilized but not soluble MEL14 enhanced this proliferation (Fig. 3a). Neither soluble nor

immobilized MEL14 alone induced T cell proliferation.

To exclude the possibility that the activation of contaminated accessory cells by MEL14 indirectly enhanced T cell proliferation, cells were stimulated with antibodies immobilized on beads. As shown in Figure 3b, beads coated with a mixture of anti-CD3 antibody and MEL14 effectively stimulated T cell proliferation, while beads coated with anti-CD3 antibody alone induced only weak proliferation. This shows that stimulation with beads gave similar effects to that by antibodies immobilized on plates when both anti-CD3 antibody and MEL14 were immobilized on the same particles. On the other hand, when beads that had been separately coated with each antibody were mixed and used for stimulation, T cells exhibited proliferation to a similar magnitude as that induced by anti-CD3 antibody-coated beads alone, although MEL14-coated beads were added at the same time. The results indicate that anti-CD3 antibody and MEL14 are effectively in synergy to stimulate T cell proliferation only when immobilized on the same beads, excluding the possibility that MEL14 firstly acts on contaminated cells and indirectly stimulates T cell proliferation.

We also examined whether MEL14 enhanced T cell proliferation induced by both anti-CD3 and anti-CD28 antibodies. T cell proliferation stimulated by anti-CD3 antibody in combination with anti-CD28 antibody was significantly enhanced by immobilized MEL14 (Fig. 3c). Such additive effects on CD28 were reported for other costimulatory molecules such as CD2, CD5, CD9, CD11a, CD29 and CD44 [23].

Anti-L-selectin antibody does not enhance the expression of IL-2

It is possible that the enhanced proliferation of T cells is due to the enhanced

production of growth factors such as IL-2. When stimulated with anti-CD3 antibody alone, T cells expressed IL-2 weakly as judged by RT-PCR analysis (Fig. 4a). MEL14 did not increase the expression of IL-2 in anti-CD3 antibody-stimulated cells. On the other hand, anti-CD28 antibody strongly induced IL-2 expression in the presence of anti-CD3 antibody, as reported previously [19]. The amount of IL-2 in the culture supernatant was also measured by ELISA (Fig. 4b). Significant IL-2 production was detected when T cells were stimulated with anti-CD3 and anti-CD28 antibodies simultaneously while IL-2 was not produced on stimulation with anti-CD3 antibody and MEL14. These results indicate that signals mediated by CD28 enhance IL-2 production while those mediated by L-selectin do not. The enhancement of T cell proliferation without IL-2 enhancement is a common characteristic of other costimulatory molecules such as CD2, CD44 and CD11a [23].

The expression of IL-2 receptor was then examined. RT-PCR analysis demonstrated that the expression of IL-2 receptor α-chain was weakly induced by anti-CD3 antibody alone and the expression was enhanced by anti-CD28 antibody and MEL14 (Fig. 4a). Flowcytometric analysis revealed that the cell surface expression was similarly induced by this stimulation (Fig. 4c). These results show that both anti-CD28 antibody and MEL14 enhance IL-2 receptor expression.

Anti-L-selectin antibody reduces the amount of cdk inhibitor p27 in T cells

In general, various proteins, including cyclins, cdks and cdk inhibitors, regulate cell proliferation. The reduction of a cdk inhibitor, p27 protein, is considered a key regulatory mechanism for T cell proliferation [24-26]. The reduction of p27 is closely

linked to IL-2-driven proliferation [24], TCR/CD3 plus CD28-induced cell-cycle progression [25], and is specifically blocked in unresponsive T cells [26]. We therefore analyzed the amount of p27 under various conditions. Unstimulated T cells contained a large amount of p27 (Fig. 5). Stimulation with anti-CD3 antibody alone slightly reduced the p27 level. This is consistent with the finding that modest cell proliferation was induced, as shown in Figure 3. When T cells were stimulated with anti-CD3 antibody and MEL14, the p27 level decreased. The combination of anti-CD3 and anti-CD28 antibodies also reduced the amount of p27, consistent with a previous report [25]. These results suggest that the enhancement effect on T cell proliferation by MEL14 is mediated by a reduction in the level of cdk inhibitor p27.

Anti-L-selectin antibody stimulates ERK in murine T cells

The role of ERK in the reduction of p27 level has been demonstrated in several cell types; for example, the inhibition of MEK, a kinase upstream of ERK, prevents both a reduction in the p27 level and cell proliferation [27,28]. We therefore examined the activation of ERK in T cells stimulated with a combination of antibodies. Anti-CD3 antibody weakly stimulated ERK (Fig. 6), consistent with previous reports [27,29]. When T cells were costimulated with immobilized MEL14 in the presence of anti-CD3 antibody, strong ERK activation was induced compared with that evoked by anti-CD3 antibody alone, suggesting the possible role of ERK in L-selectin-mediated T cell costimulation. Anti-CD28 antibody similarly enhanced anti-CD3 antibody-induced ERK activation, as reported [30]. Thus, both L-selectin and CD28 enhance ERK activation induced by CD3.

Discussion

In this study, we showed that L-selectin had a costimulatory effect on T cells when stimulated with the specific antibody. The magnitude of the enhancing activity was similar to that of anti-CD28 antibody in our experimental condition, but was 3-5 fold weaker at saturated concentration of the antibodies (data not shown). It has been reported that unstimulated virgin T cells express L-selectin at a higher level, and that the expression level drops just after stimulation and recovers within 48 h, followed by gradual decrease over a week [31]. These results suggest that L-selectin has biological effects as a costimulatory molecule on virgin T cells during or just after stimulation in addition to its well-known role as a homing receptor.

L-selectin ligands are expressed on endothelial cells [32]. T cells circulating in the bloodstream interact with ligands during the rolling step of the homing and inflammatory processes to move across the endothelial layer [1]. *In vitro* studies clearly show that endothelial cells express MHC class II molecules and process soluble antigens to stimulate T cells [33,34], suggesting that the vascular surface is one possible site for T cell stimulation. Since endothelial cells do not express the ligands for CD28 even after exposure to various stimuli [35], another costimulatory mechanism may operate at the surface of blood vessels. There are several candidates for costimulators acting on virgin T cells through an interaction with endothelial cells [13]. Among them, CD2 on T cells and LFA-3 on endothelial cells are the most likely candidates, since blocking their interaction abrogates approximately half of the total costimulatory activity of endothelial cells toward human primary $CD4^+$ T cells [34]. Our results suggest that L-selectin is another candidate costimulator in addition to CD2/LFA-3.

It is reported that L-selectin ligands are expressed on some dendritic cells in the paracortex of lymph nodes, a zone enriched in T cells [32], suggesting that lymph nodes are a second site where L-selectin interacts with its ligands. Dendritic cells, one of the professional antigen-presenting cells, express high levels of CD80 and CD86 as ligands of CD28 [10]. However, this does not exclude the possibility that L-selectin has some additional roles in T cell activation in lymph nodes, because T cell proliferation induced by SEB, in which CD28 was activated with its natural ligand on antigen-presenting cells, was enhanced by MEL14 (Fig. 2).

We have provided some insight into how anti-L-selectin antibody enhances T cell proliferation. The expression of IL-2, which has strong activity to reduce p27 level [24], was not enhanced by MEL14 (Fig. 4), in clear contrast to the effect of anti-CD28 antibody. However, costimulation with MEL14 significantly reduced the p27 level (Fig. 5), suggesting that the activity of MEL14 to enhance T cell proliferation is mediated by a reduction in the level of p27. There exists an IL-2-independent pathway to reduce the p27 level in T cells via proteasome-dependent degradation [25], although the upstream signaling pathway(s) remains to be elucidated. The reduction of p27 induced by MEL14 plus anti-CD3 antibody is possibly mediated by this pathway. Our finding that the optimal activation of ERK requires both TCR/CD3 and costimulation via L-selectin suggests a possible role for the MEK/ERK pathway in this IL-2-independent reduction of p27. It is reported that crosslinking raft-resident molecules enhanced T cell proliferation via aggregation of lipid rafts [36]. Since L-selectin was present in lipid rafts [16], it is likely that L-selectin alters the localization of signaling molecules including TCR complex, as is reported for some non-CD28 costimulators [37]. Further investigation, including localization of signaling molecules,

will reveal the underlying mechanism of costimulation that regulates immune responses.

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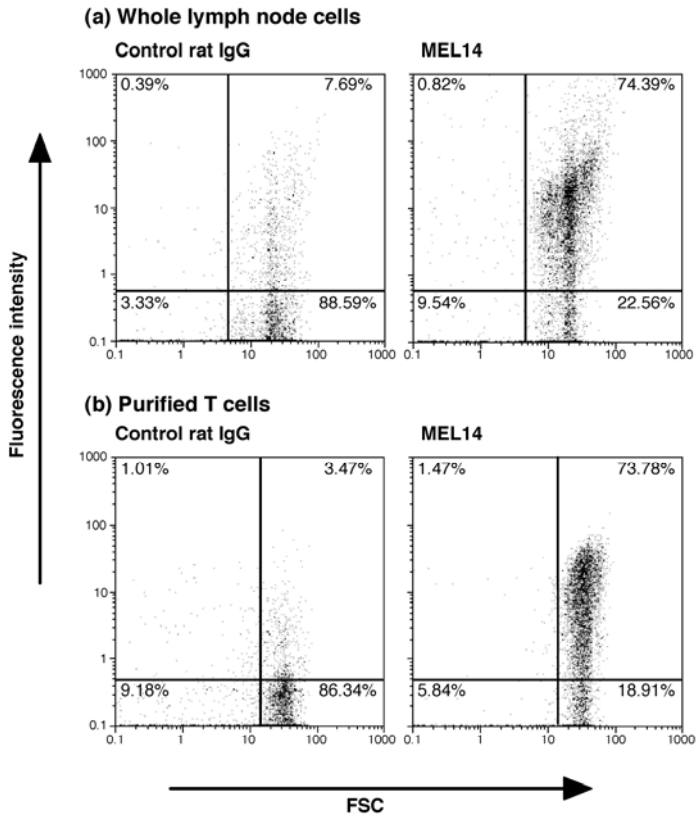


Figure 1. Lymph node T cells expressed L-selectin. Lymph node cells were indirectly stained with MEL14 or control rat IgG2a. (a) Whole lymph node cells. (b) Purified T cells. FSC, forward scatter.

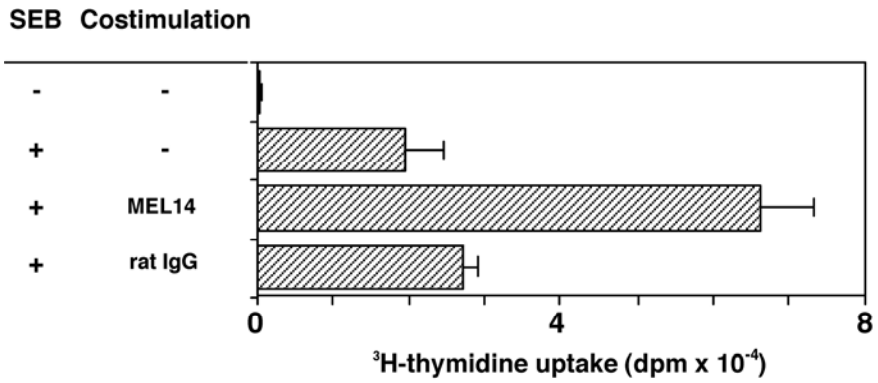
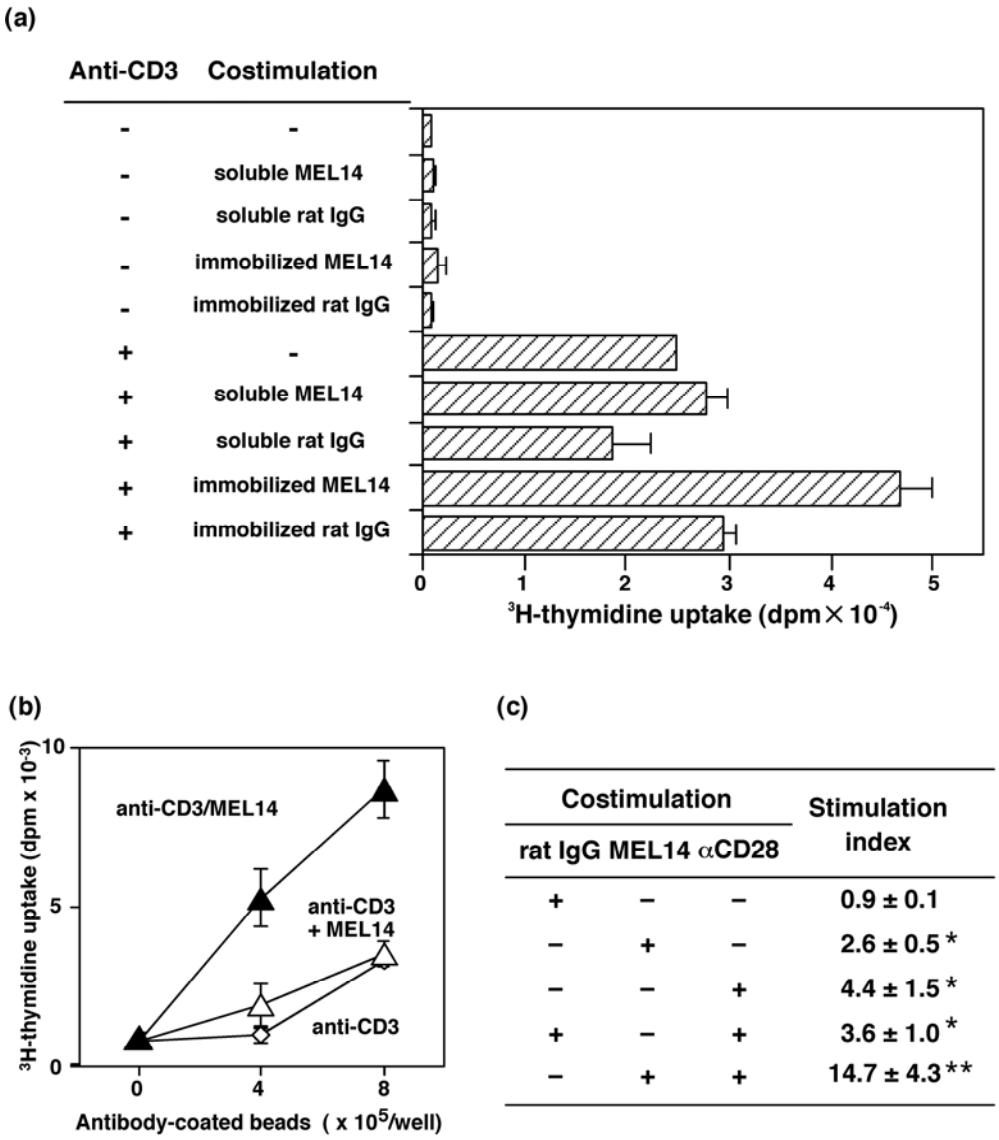


Figure 2. The proliferation of lymph node cells induced by SEB was enhanced by MEL14. Lymph node cells were stimulated with each antibody in the presence of SEB.



(c)

Costimulation			Stimulation index
rat IgG	MEL14	αCD28	
+	−	−	0.9 ± 0.1
−	+	−	2.6 ± 0.5 *
−	−	+	4.4 ± 1.5 *
+	−	+	3.6 ± 1.0 *
−	+	+	14.7 ± 4.3 **

Figure 3. The proliferation of purified T cells induced by immobilized anti-CD3 antibody was enhanced by MEL14. (a) Anti-CD3 antibody-induced proliferation was enhanced by immobilized but not soluble MEL14. Antibodies were immobilized on the microtiter plates to stimulate T cells. (b) MEL14 enhanced proliferation when it was immobilized on the same beads simultaneously with anti-CD3 antibody but not when these antibodies were immobilized on separate particles and these particles were mixed. Cells were stimulated with anti-CD3-coated beads (open diamonds), anti-CD3-coated beads plus MEL-14-coated beads (open triangles) or beads coated with anti-CD3 and MEL14 antibodies simultaneously (closed triangles). An equal number of beads was mixed and added to the wells. (c) MEL14 enhanced the proliferation that

was induced by anti-CD3 and anti-CD28 antibodies. Cells were stimulated with plate-coated antibodies as in (a), and 2 μg/ml of anti-CD28 antibody was used. Stimulation index was calculated by taking the ratio of mean thymidine uptake in each stimulating condition to mean thymidine uptake stimulated by anti-CD3 antibody alone. Data are expressed as mean ± standard error from three independent experiments and compared by Student's *t* test. *, $p < 0.05$ versus anti-CD3 plus rat IgG antibodies; **, $p < 0.05$ versus anti-CD3 plus rat IgG, anti-CD3 plus MEL14, anti-CD3 plus anti-CD28, anti-CD3 plus rat IgG plus anti-CD28 antibodies.

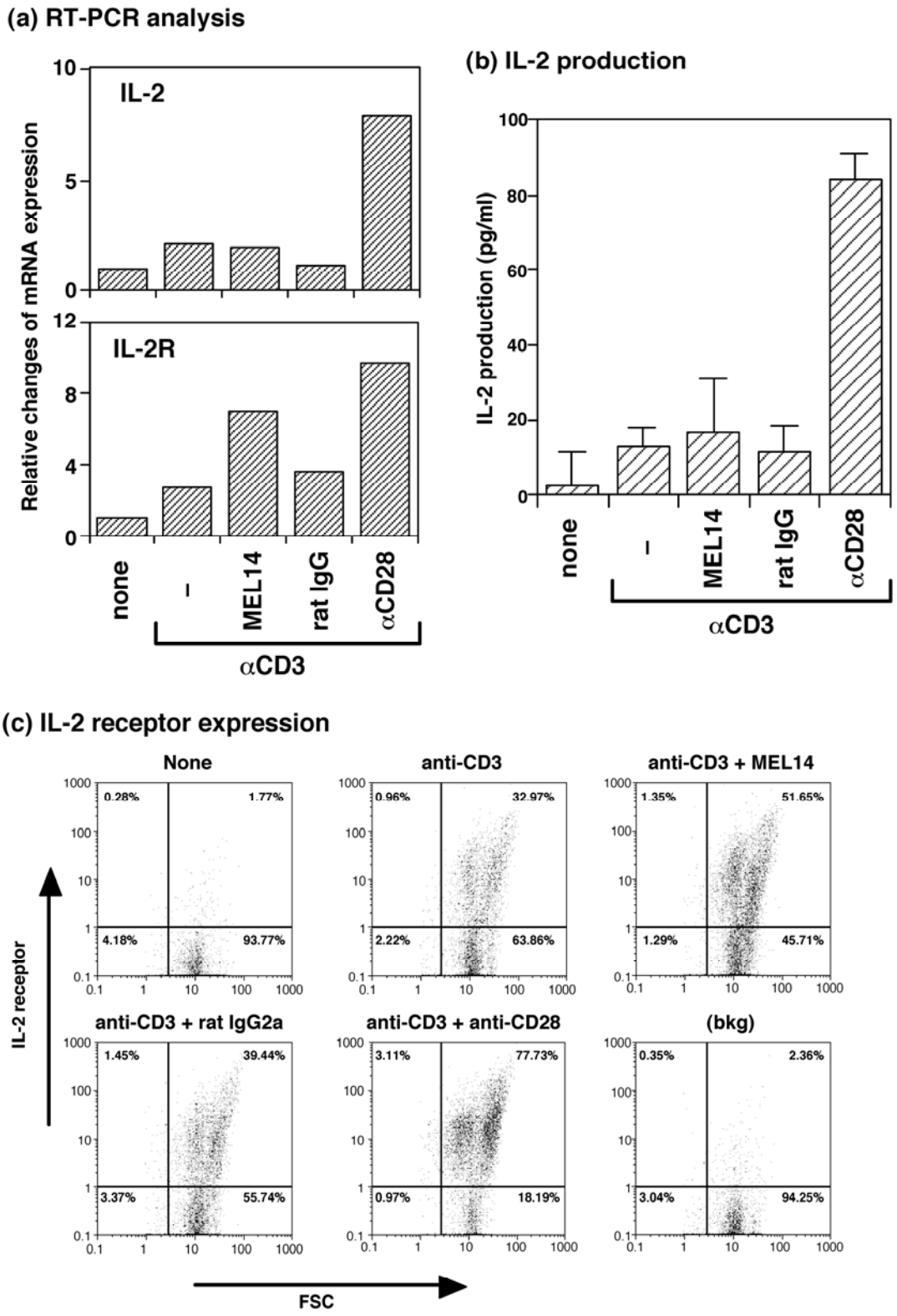


Figure 4. MEL14 did not enhance the expression of IL-2. Purified T cells were

stimulated with immobilized antibodies. (a) Cells were stimulated with immobilized antibodies for 24 h and subjected to RT-PCR analysis. Expression levels were normalized by GAPDH. (b) IL-2 production in the supernatants. Cells were cultured for 48 h and the amount of IL-2 in the supernatants was measured by ELISA (Quantikine M, R & D Systems, Minneapolis, MN). (c) Surface expression of IL-2 receptor. Cells were stained with either FITC-7D4 or control FITC-rat IgM (shown as bkg) after 48 h of culture.

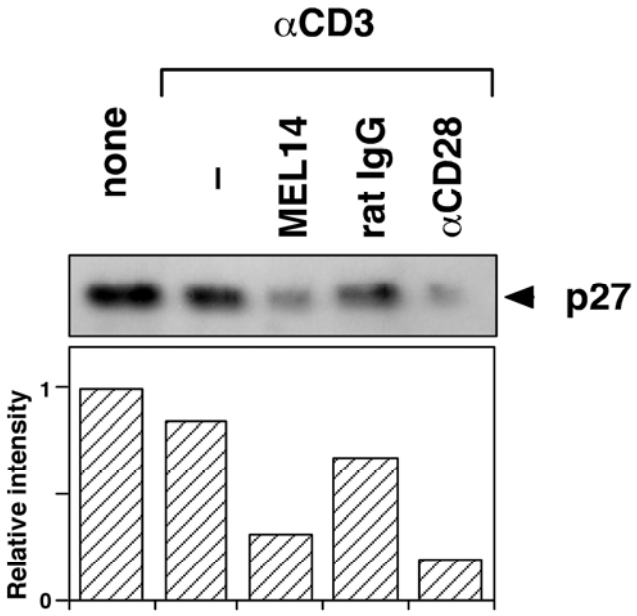


Figure 5. MEL14 reduced the amount of cdk inhibitor p27. Densitometric analysis was performed using the public domain NIH Image program (US National Institutes of Health).

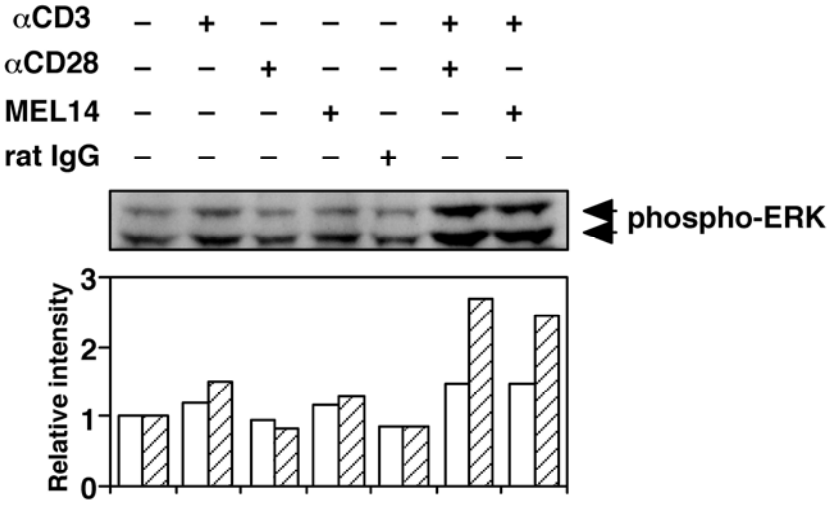


Figure 6. MEL14 enhanced the activation of ERK induced by anti-CD3 antibody. Relative intensities of p42 (open bar) and p44 (hatched bar) are shown at the bottom.

Chapter 3

Siglec-9 enhances IL-10 production in macrophages via tyrosine-based motifs

Introduction

Sialic acids on cell surface may contribute to the down-regulation of the immune system [1], which may be partly mediated by specific lectins. Siglecs are sialic acid-binding immunoglobulin-like lectins that are predominantly expressed by immune cells [2,3]. Members of CD33-related Siglecs down-regulate both innate and acquired immune responses, possibly via cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). These ITIMs recruit tyrosine phosphatases such as Src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2, which suppress tyrosine kinase-dependent signals activated by immunoreceptor tyrosine-based activation motif (ITAM)-containing B cell and Fcγ receptors [4].

Siglec-9 can interact with the sialic acid moiety and inhibits tyrosine kinase-based activation induced by the T cell, Fcγ or Fcε receptor [5-7]. Examples of immune cells that express Siglec-9 are monocytes/macrophages, which mediate innate immunity (the first defense against pathogens) and initiate acquired immunity via the stimulation of T cells. It is well established that macrophages are activated by a group of transmembrane proteins called Toll-like receptors (TLRs), which are essential for host defense, since they initiate innate immunity [8,9]. Upon stimulation of TLR,

macrophages produce large amounts of proinflammatory cytokines to elicit protective immunity against infection. Furthermore, anti-inflammatory cytokines such as interleukin (IL)-10 may be up-regulated by TLR stimulation following acute responses to suppress excess proinflammatory cytokine production, which is harmful to the host [10]. TLRs activate a wide variety of intracellular signaling that involves serine/threonine kinases; however, crosstalk between TLR signaling and ITAM/ITIM-based stimuli may also be operative [11,12]. Thus, we examined whether Siglec-9 modulates immune responses upon activation of macrophages by the ligands that stimulate TLR. We report that Siglec-9 reduces proinflammatory cytokine production but strongly increases IL-10 production.

Materials and Methods

Reagents

Peptidoglycan (PGN) from *Staphylococcus aureus*, lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 and poly(I:C) were purchased from Sigma-Aldrich (St Louis, MO, USA). All DNAs including CpG DNA (5′-TCCATGACGTTCCTGACGTT-3′ [13]) were obtained from Hokkaido System Sciences (Hokkaido, Japan). Anti-human goat antibodies to Siglec-5 and -9 were purchased from R&D Systems (Minneapolis, MN, USA). Neutralizing anti-IL-10 antibody JES5-2A5 was purchased from BioLegend (San Diego, CA, USA). Goat normal IgG was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Construction of plasmids

cDNA for human Siglec-9 (ResGen) was obtained from Invitrogen (Carlsbad, CA, USA), and the coding region was cloned into the pcDNA4/TO/*myc*-His A vector (Invitrogen) (designated as pcDNA4Sig9). Point mutations converting tyrosine residues ($Tyr^{433, 456}$) to phenylalanine for destruction of tyrosine-based signaling motifs were achieved by polymerase chain reaction (PCR) as described previously [14] (pcDNA4Sig9YFYF). Siglec-5 was amplified from a human lymph node cDNA library (Gibco) and cloned into pcDNA4 (pcDNA4Sig5). All primers used are listed in Supplementary data.

Establishment of stable transformants

The mouse macrophage cell line RAW264 was obtained from Riken BioResource

Center (Tsukuba, Japan) and was maintained in RPMI1640 containing 10% heat-inactivated fetal calf serum, 0.03% L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin G and 100 μg/ml streptomycin. Cells were transfected with the vectors using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol, and selected in the presence of 250 μg/ml Zeocin (Invitrogen). Cells that expressed Siglec-9 were sorted by an EPICS ALTRA cell sorter (Beckman-Coulter, Fullerton, CA, USA) and used in all experiments without a cloning procedure to avoid differences among clones. Cells transfected with the empty vector were used as the control cells. The human macrophage cell line THP-1 was obtained form Riken BioResource Center. Cells were transfected with the vectors by electroporation and Siglec-9-expressing cells were sorted in a similar manner.

Analysis of cellular activities

For stimulation experiments, cells (4×10^4) were cultured in microtiter plates in 100 μl of medium with various reagents for the indicated periods. The amounts of tumor necrosis factor (TNF)-α and IL-10 in the culture supernatant were measured using an OptEIA enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Diego, CA, USA). The levels of mRNA were estimated by real-time reverse transcription (RT)-PCR with LightCycler (Roche Diagnostics, Mannheim, Germany) [15]. Expression levels were normalized with the amount of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Results

Siglec-9 inhibits the production of TNF-α stimulated with LPS, PGN, CpG DNA and poly(I:C) via ITIM-like motifs in RAW264 cells

RAW264 macrophage cells have been widely used for studying TLR signaling. We confirmed similar levels of expression between wild and mutated Siglec-9 by flow cytometric analysis (Fig. 1a). RT-PCR analysis revealed that endogenous Siglecs and several genes required for TLR-mediated activation were not significantly different between the control and Siglec-9-transfected cells (Supplementary Table a and b).

Macrophages produce a large amount of the potent proinflammatory cytokine TNF-α upon stimulation of TLRs [8,9]. RAW264 cells were stimulated with LPS for TLR4 or PGN for TLR2 and the amount of TNF-α in the culture supernatant was determined by ELISA. Fig. 1b and c show the time and ligand-concentration dependence of TNF-α production. Cells expressing Siglec-9 produced low levels of TNF-α upon stimulation with LPS or PGN compared with the control RAW264 cells. Then, the effects of Siglec-9 on the production of several cytokines including TNF-α were also examined by the estimation of mRNA by real-time RT-PCR (Fig. 1d). The amounts of mRNA encoding TNF-α and another proinflammatory cytokine, IL-6, were significantly reduced by Siglec-9 when the cells were stimulated with either LPS or PGN. The expression of IFN-β induced by LPS was also reduced by Siglec-9. These observations indicated that Siglec-9 reduced several cytokines that were produced upon stimulation by LPS or PGN.

As with most CD33-related Siglecs, Siglec-9 has two conserved tyrosine-based

motifs (consensus ITIM and ITIM-like motifs) in the cytoplasmic region around amino acids 433 and 456. We therefore analyzed the inhibitory effects of a mutant Siglec-9 that had mutations at tyrosine residues in both ITIM-like motifs (Siglec-9YFYF). TNF-α production by cells expressing Siglec-9YFYF was not reduced compared with the control cells (Fig. 1b and c). The reduction of other proinflammatory cytokine expression was not observed in Siglec-9YFYF-expressing cells (Fig. 1d). These results suggested that ITIM-based signaling was involved in this inhibition.

Since CD33-related Siglecs can also function as endocytic receptors [2,3], we examined whether Siglec-9 affected the activation by the ligands recognized in the endosome. As shown in Fig. 1, CpG DNA for TLR9 and poly(I:C) for TLR3 induced TNF-α; however, the level of induction was relatively low compared with that by LPS or PGN. Similarly to the stimulation by LPS or PGN, the production of TNF-α was also down-regulated in Siglec-9-expressing cells; the reduction was not observed in Siglec-9YFYF-expressing cells. These results indicated that Siglec-9 inhibited TNF-α production induced by CpG DNA or poly(I:C).

Since IL-10 inhibits the production of several cytokines in macrophages [10], it is possible that the produced IL-10 can reduce TNF-α production in Siglec-9-expressing RAW264 cells in an autocrine manner. However, anti-IL-10 antibody (5 μg/ml) did not affected the TNF-α production induced by LPS (data not shown), suggesting that the inhibition of the production of proinflammatory cytokines by Siglec-9 in RAW264 cells was independent of the activity of IL-10.

Siglec-9 enhances the production of anti-inflammatory cytokine IL-10 in RAW264 cells

Macrophages produce the anti-inflammatory cytokine IL-10 as a relatively late event when stimulated with TLR ligands [10]. When the control cells were stimulated with LPS (0.5 μg/ml) or PGN (25 μg/ml), IL-10 production was observed after a 24-h stimulation (Fig. 2a). On the other hand, the amount of IL-10 produced in the culture supernatant of Siglec-9-expressing cells increased in the earlier phase, i.e., 4–8 h following stimulation. For various concentrations of ligands, IL-10 production in Siglec-9 cells was much higher than the control cells (Fig. 2b). Enhancement was also observed by real-time RT-PCR: more than 10 cycles of additional amplification were required to detect IL-10 in the control cells compared with Siglec-9-expressing cells (not shown). These results demonstrated that Siglec-9 enhanced the production of the anti-inflammatory cytokine IL-10 induced by LPS or PGN.

We then analyzed the IL-10 production in cells with Siglec-9YFYF that lacked both ITIM-like motifs. IL-10 production by cells expressing Siglec-9YFYF was almost similar to that by the control cells (Fig. 2a and b). The results showed that Siglec-9 required ITIM-like motifs in the cytoplasmic tail for the enhancement of IL-10 production induced by LPS or PGN.

CpG DNA (25 μg/ml) efficiently induced IL-10 compared with LPS and PGN (Fig. 2). Nonetheless, the production in Siglec-9-expressing cells at 24 h was approximately 2.5 times that of the control cells. By the stimulation of poly(I:C), the control cells produced little IL-10; however, Siglec-9-expressing cells produced certain amounts of the cytokine. The enhancement was not observed in Siglec-9YFYF-expressing cells. Thus, these results suggested that Siglec-9 enhanced IL-10 production induced by several TLRs.

Siglec-5 enhances IL-10 production while inhibiting TNF-α production in RAW264 cells

The effect of Siglec-5, another Siglec expressed by macrophages, on the cytokine production was examined. Flow cytometric analysis confirmed the expression of Siglec-5 in transfected cells (Fig. 3a). Siglec-5-expressing RAW264 cells produced copious IL-10 following stimulation with LPS, PGN, CpG DNA or poly(I:C) (Fig. 3b). On the other hand, TNF-α production of Siglec-5-expressing cells was low by these stimuli compared with that of the control cells (Fig. 3c). The results demonstrated that Siglec-5 enhanced IL-10 production while inhibiting TNF-α production, as observed with Siglec-9.

Siglec-9 enhances IL-10 production via ITIM-like motifs in THP-1 cells

To assess the enhancement of IL-10 by Siglec-9 in other cell lines, the human macrophage cell line THP-1 was stably transfected with the expression vector for Siglec-9. As shown in Fig. 4a, the expression of transfected Siglec-9 and Siglec-9YFYF in THP-1 cells was confirmed by flow cytometry. While THP-1 expressed several endogenous Siglecs, mRNA for Siglec-9 was not detected in control THP-1 cells (Supplementary Table c). Being different from RAW264 cells, control THP-1 cells produced certain level of IL-10 into the supernatant upon stimulation with LPS or PGN (Fig. 4b). In spite of this, the production of IL-10 was enhanced almost 2.5-4 times in Siglec-9-expressing cells. IL-10 production by cells expressing

Siglec-9YFYF was almost similar to that by the control cells. Real-time RT-PCR supported these results (Fig. 4c). Totally, Siglec-9 enhanced IL-10 production induced by LPS or PGN in THP-1 cells, as observed in RAW264 cells.

Discussion

In this study, we first show that the expression of Siglec-9 in the macrophage cell line RAW264 reduces the production of proinflammatory cytokines induced by LPS or PGN. Furthermore, Siglec-9 enhances anti-inflammatory cytokine IL-10 production. Until date, the function of CD33-related Siglecs has been reported with regard to the inhibition of the activation of immune responses via dampening of the activation signals or induction of apoptosis [5-7,16-24]. Our results suggest an additional pathway for Siglecs modulating immune responses. Given that IL-10 is one of only a few cytokines to have potent immune inhibitory capacity [10], the enhancement of IL-10 together with the reduction of proinflammatory cytokines suggests the important roles of Siglecs as anti-inflammatory regulators. Furthermore, acquired immunity might also be regulated since IL-10 induces long-term antigen-specific unresponsive state of T cells [25]. Thus, it is possible that regulation of IL-10 production by Siglecs and sialic acid-containing ligands work as fail-safe machinery to prevent autoimmunity toward self-proteins with sialic acids. This possibility remains to be verified.

Mouse splenic macrophages abundantly express CD33 and Siglec-E (data not shown). Since mouse CD33 lacks ITIMs in the cytoplasmic tail [2], it is likely that the signaling effects of Siglecs in mouse macrophages are mainly achieved by Siglec-E, which has the close similarity to human Siglec-9. In RAW264 cells, however, the expression level of Siglec-E was observed to be only 5% that of splenic macrophages (data not shown). For THP-1, as reported for several human cell lines [26], the expression of Siglec-9 was quite low. In this regard, RAW264 and THP-1 cells are suitable for analyzing the function of Siglecs by exogenous expression. In the present

study, we show that Siglec-5 as well as Siglec-9 could modulate cytokine production in a similar manner. This fact and other previous reports [6,7] suggest that different Siglec species are partly redundant and have an overlapping function, although the structures of sialic acid ligands are not identical. In this regard, it is noteworthy that THP-1 cells express CD33, which has two ITIM-like motifs in human [2]. It is possible that CD33 might cause a relatively higher expression of IL-10 in control THP-1 cells. Nonetheless, we reproducibly observed the enhancement of IL-10 production by Siglec-9, supporting the view that Siglecs regulated IL-10 production in human cells. The relative contribution of each Siglec to overall IL-10 production in primary cells should be determined further.

Siglecs inhibit tyrosine kinase-dependent signals activated by ITAM-containing receptors, such as T cell, B cell, Fcγ and Fcε receptors, mainly via the recruitment of SHP-1 [2,3]. Although the importance of tyrosine kinases has long been debated, several molecular bases for the role of tyrosine phosphorylation in TLR signaling have been reported recently, such as tyrosine phosphorylation of MyD88, Mal/TIRAP and TLRs [27-31]. Furthermore, SHP-1 can inhibit LPS-induced TNF-α and iNOS production in RAW264.7 cells [32], and SHP-2 inhibits cytokine production induced by LPS and poly(I:C) [33]. Together, these observations support the involvement of Siglecs and protein tyrosine phosphatases in the regulation of TLR signals. In fact, our results suggest that Siglec-9 modulates the TLR signal by ITIM-dependent mechanisms. However, we cannot rule out the possibility that the ligands used in this study modulated a signal cascade(s) other than TLRs. Further study is necessary to explore the precise mechanisms of Siglecs in modulating TLR signaling.

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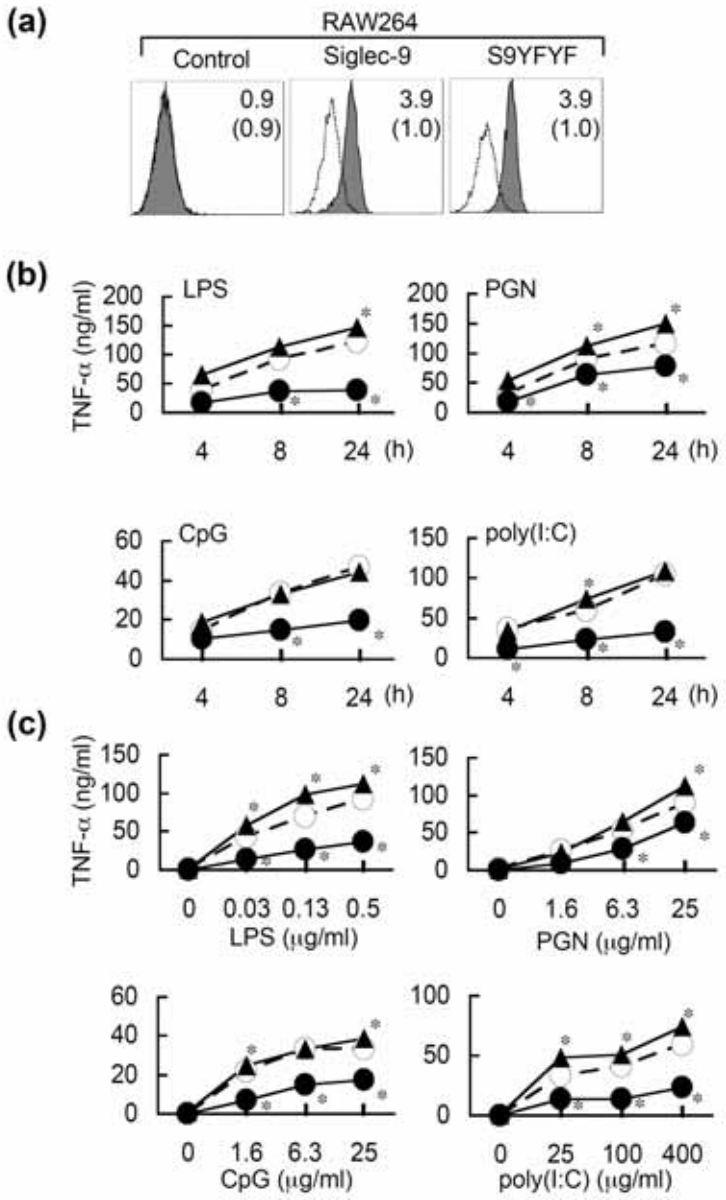

(a)
RAW264
Control
Siglec-9
S9YFYF
0.9
(0.9)
3.9
(1.0)
3.9
(1.0)
(b)
TNF-α (ng/ml)
LPS
PGN
CpG
poly(I:C)
4
8
24 (h)
(c)
LPS (μg/ml)
PGN (μg/ml)
CpG (μg/ml)
poly(I:C) (μg/ml)

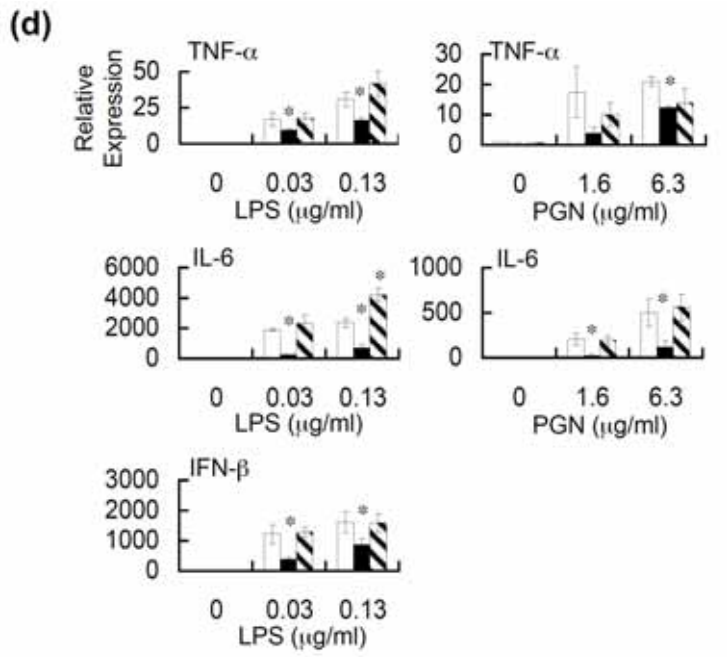


Figure 1. Siglec-9 reduces the production of proinflammatory cytokines via tyrosine residues. (a) RAW264 cells transfected with empty vector (control), pcDNA4Sig9 (Siglec-9) and pcDNA4Sig9YFYF (S9YFYF) were indirectly stained with control (normal goat IgG, *open histogram*) or anti-Siglec-9 (*filled histogram*) antibodies and analyzed by flow cytometry. Mean fluorescence intensity (MFI) level is shown for each histogram; control MFI level is shown in parenthesis. (b) Time-course analysis of TNF-α production by the cells stimulated with 0.5 μg/ml of LPS, 25 μg/ml of PGN, 25 μg/ml of CpG DNA or 400 μg/ml of poly(I:C). Control (○), Siglec-9-expressing (●) and Siglec-9YFYF-expressing (▲) cells were stimulated for 4, 8 or 24 h, and the amount of TNF-α produced in the culture supernatant was measured by ELISA. (c) Cells were stimulated with various concentrations of LPS, PGN, CpG DNA and poly(I:C) for 8 h. (d) The amounts of mRNA of TNF-α, IL-6 and IFN-β were quantified by real-time RT-PCR after stimulation with LPS or PGN for 4 h. Control (□), Siglec-9-expressing (■) and Siglec-9YFYF-expressing (▨) cells. One representative experiment out of three to seven is shown. ∗, $p < 0.05$ relative to control by Student's *t*-test.

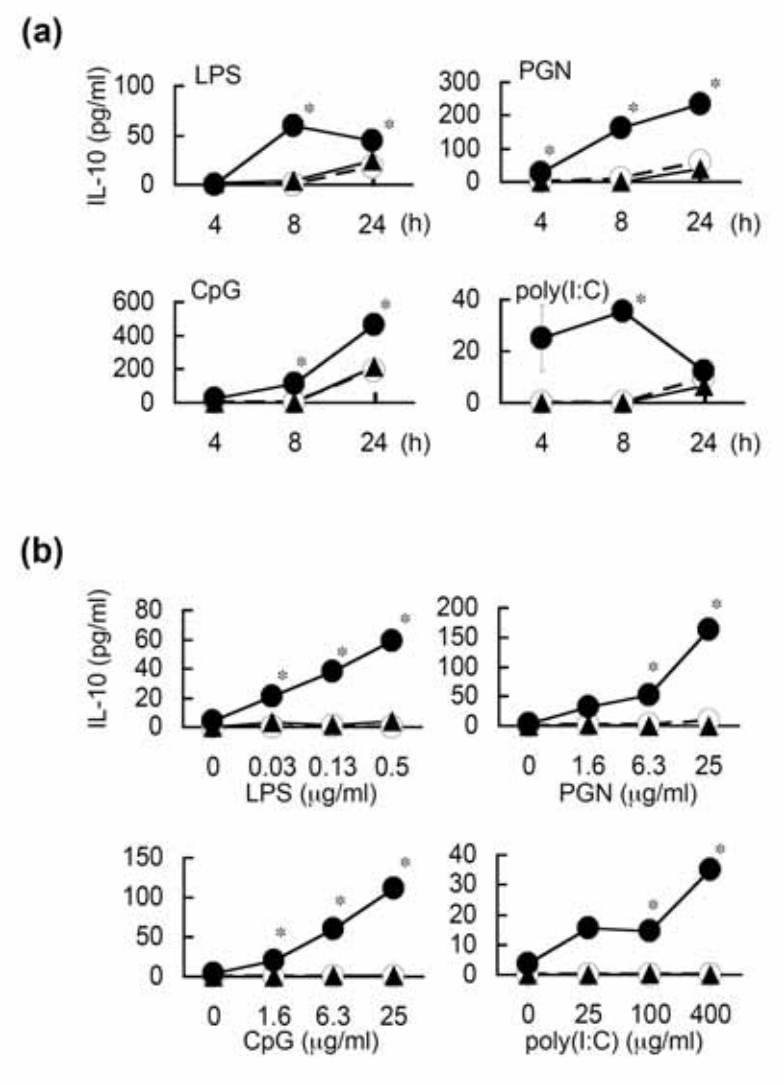


Figure 2. Siglec-9 enhances IL-10 production following stimulation with TLR ligand via tyrosine residues. (a) Time-course analysis of IL-10 production in cells stimulated with 0.5 μg/ml of LPS, 25 μg/ml of PGN, 25 μg/ml of CpG DNA or 400 μg/ml of poly(I:C). IL-10 in the culture supernatant was measured by ELISA. Symbols are as in Fig. 1. (b) Cells were stimulated with ligands for 8 h and IL-10 was measured. One representative experiment out of three to seven is shown. ∗, $p < 0.05$ relative to control by Student's *t*-test.

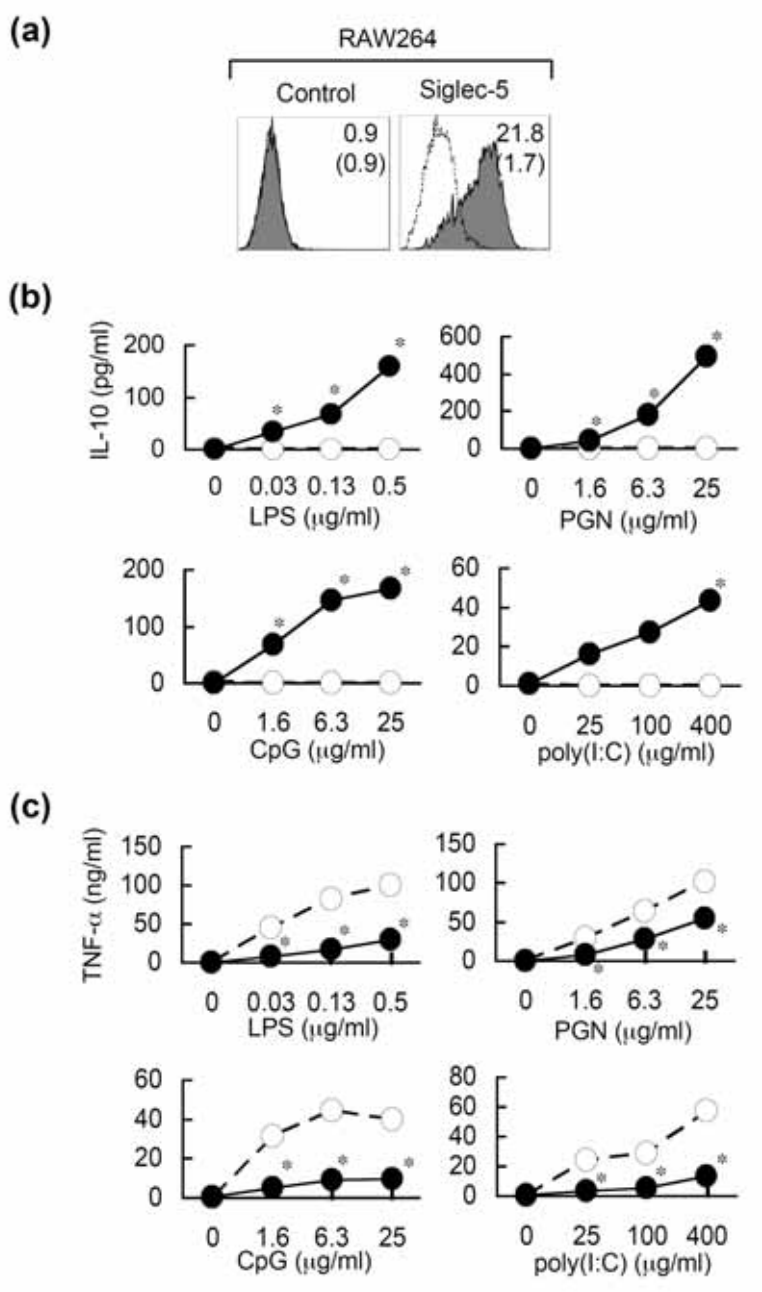


Figure 3. Siglec-5 enhances IL-10 production while reducing TNF-α production. (a) RAW264 cells transfected with empty vector (control) and pcDNA4Sig5 (Siglec-5) were indirectly stained with control (normal goat IgG, *open histogram*) or anti-Siglec-5 (*filled histogram*) antibodies and analyzed by flow cytometry. (b, c) Control (○) and Siglec-5-expressing (●) cells were stimulated for 8 h as in Fig. 1, and the amount of IL-10 (b) or TNF-α (c) in culture supernatant was measured by ELISA. One representative experiment out of two to three is shown. *, $p < 0.05$ relative to control by Student's *t*-test.

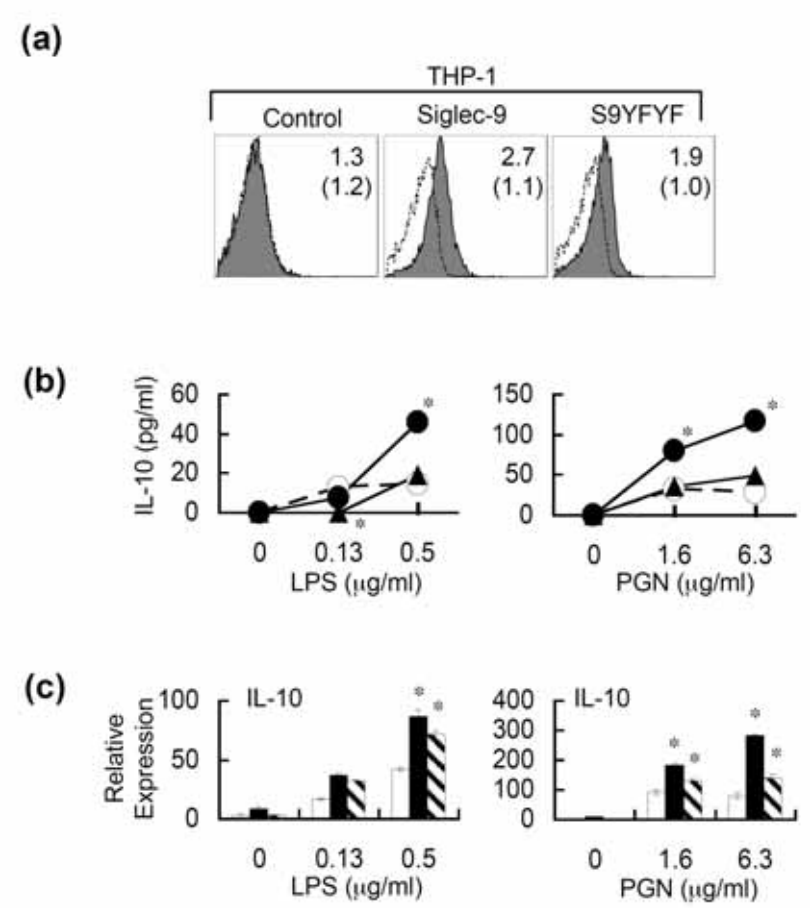


Figure 4. Siglec-9 enhances the IL-10 production in THP-1 cells. (a) THP-1 cells transfected with control vector (control), pcDNA4Sig9 (Siglec-9) and pcDNA4Sig9YFYF (S9YFYF) were indirectly stained with control (normal goat IgG, *open histogram*) or anti-Siglec-9 (*filled histogram*) antibodies and analyzed by flow cytometry. (b) Cells were stimulated for 8 h as in Fig. 1, and the amount of IL-10 in culture supernatant was measured by ELISA. (c) Cells were stimulated with LPS or PGN for 8 h, and IL-10 mRNA was quantified by real-time RT-PCR. Symbols are as in Fig. 1. One representative experiment out of three is shown. ∗, $p < 0.05$ relative to control by Student's *t*-test.

Supplementary Data

Primers used for the construction of pcDNA4Sig9:

5′-CATggtaccATGGGGCTGCTGCTGCTGCTGCC-3′

5′-CATctcgagTCATCTGTGGATCTTGATCTCCG-3′

(lower case letters indicate *Kpn* I and *Xho* I sites added for cloning)

Primers used for site-directed mutagenesis:

ITIM :

forward: 5′-GGAGAGCTCCAGTTTGCATCCCTCAGCTTCC-3′

reverse: 5′-GGAAGCTGAGGGATGCAAACTGGAGCTCTCC-3′

ITIM-like motifs :

forward: 5′-GGCCACTGACACCGAGTTCTCGGAGATCAAGATCC-3′

reverse: 5′-GGATCTTGATCTCCGAGAACTCGGTGTCAGTGGCC-3′

(Underlined letters indicate mutated nucleotides)

Primers used for the construction of pcDNA4Sig5:

5′-GCCaagcttGAGACATGCTGCCCCTGCTG-3′

3′-CGGtctagaGCCAGGACTGAACTCTGGGCA-5′

(lower case letters indicate *Hin*d III and *Xba* I sites added for cloning)

Primers for real-time RT-PCR of TLRs and TLR-related proteins:

Mouse (m)TLR2 forward: 5′-TGCGTTACATCTTGGAACTGTCG-3′

reverse: 5′-TCTACTGTGATTCGCTTCACCTTCT-3′

mTLR4 forward: 5′-ATTCAAGACCAAGCCTTTCAGGG-3′

reverse: 5′-TCAACCGATGGACGTGTAAACC-3′

mCD14 forward: 5′-TGCCTGGCTCGCAGAACT-3′

reverse: 5′-CCGCTGGGCGTCTCC-3′

mMyD88 forward: 5′-GTGGTTGTTTCTGACGATTATCTACAGA-3′

reverse: 5′-GATGAACCGCAGGATACTGGG-3′

Primers for real-time RT-PCR of cytokines:

mTNF-α forward: 5′-CAAAATTCGAGTGACAAGCCTG-3′

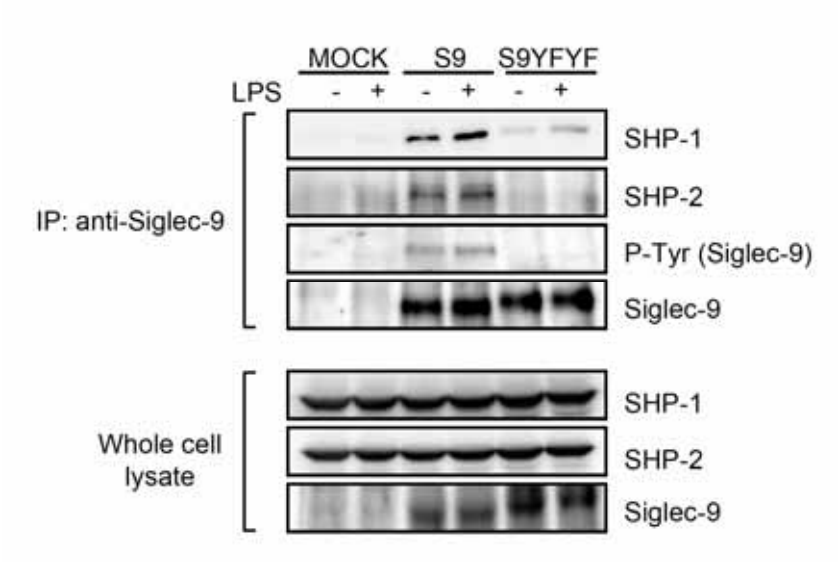
reverse: 5′-ACAAGGTACAACCCATCGGC-3′

mIL-6 forward: 5′-GAGGATACCACTCCCAACAGACC-3′

reverse: 5′-AAGTGCATCATCGTTGTTCATACA-3′
mIL-10 forward: 5′-AGCCGGGAAGACAATAACTGC-3′
reverse: 5′-AACCCAAGTAACCCTTAAAGTCCTG-3′
mIFN-β forward: 5′-GACGGAGAAGATGCAGAAGAGTTAC-3′
reverse: 5′-CAACAATAGTCTCATTCCACCCAG-3′
mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
forward: 5′-CTACCCCCAATGTGTCCGTC-3′
reverse: 5′-GCTGTTGAAGTCGCAGGAGAC-3′
Human (h)IL-10 forward: 5′-GCAACCTGCCTAACATGCTTCG-3′
reverse: 5′-ACCCAGGTAACCCTTAAAGTCCTCC-3′
hGAPDH forward: 5′-TCCCTCCAAAATCAAGTGGGG-3′
reverse: 5′-GTCCTTCCACGATACCAAAGTTGTC-3′

Primers for real-time RT-PCR of Siglecs:

mCD33 forward: 5′-GGTCAAAGTTCAGTGCCTTCCTC-3′
reverse: 5′-TTGAGCATTTAAAACCCCAAAGC-3′
mSiglec-E forward: 5′-CTCAGTGCTAACCATCACCCCT-3′
reverse: 5′-ATGACACGTTTAGACGGGTGG-3′
mSiglec-F forward: 5′-CGATTCTATCTCATGGGGAAGG-3′
reverse: 5′-GTTTGGTAGAATTGCCAGACACC-3′
mSiglec-G forward: 5′-GAAAATGGCTTAACCGTTCCC-3′
reverse: 5′-TCCGGAAGAAATAGTTTGTTGAGTC-3′
mSiglec-H forward: 5′-AAGGGTGACAACGGTTCTTACTTC-3′
reverse: 5′-AGCACATCACATTGGTAGGACG-3′
hCD33 forward: 5′-TGTGCATGTGACAGACTTGACCC-3′
reverse: 5′-TTATGAGCACCGAGGAGTGAGTAGTC-3′
hSiglec-5 forward: 5′-TGTCAGATGAAACGCCAAGGAG-3′
reverse: 5′-CAGGACCGGAAGGTATGAGGTG-3′
hSiglec-7 forward: 5′-GGCCTGTATCAGGAGTGTTGCT-3′
reverse: 5′-GCAGCCAGGCCATGGTG-3′
hSiglec-9 forward: 5′-GGGTGCTGGAGCTGCCTT-3′
reverse: 5′-GTCACTCCTGATGTGGCTTTGC-3′



Supplementary Figure 1. Siglec-9 binds with SHP-1 and SHP-2 via tyrosine residues in RAW264 cells. Control, Siglec-9, and Siglec-9YFYF-expressing cells were stimulated with 2 μg/ml of LPS for 10 min. Upper panel, cell lysates were immunoprecipitated with anti-Siglec-9 antibody and probed with the indicated antibodies; lower panel, whole cell lysates were analyzed.

Supplementary Table

(a)

Expression of endogenous genes required for TLR signaling in RAW264 cells
(% TLR or TLR-related protein mRNA/GAPDH mRNA)

Cell		TLR2	TLR4	CD14	MyD88
RAW264	Control	6.6×10^{-2}	3.8×10^{-2}	3.0	0.14
	Siglec-9	10×10^{-2}	6.4×10^{-2}	1.9	0.15
	S9YFYF	6.9×10^{-2}	7.1×10^{-2}	2.8	0.15

(b)

Expression of endogenous Siglecs in RAW264 cells
(% Siglec mRNA/GAPDH mRNA)

Cell		CD33	Siglec-E	Siglec-F	Siglec-G	Siglec-H
RAW264	Control	0.22	0.80×10^{-2}	ND *	ND *	ND *
	Siglec-9	0.28	1.4×10^{-2}	ND *	ND *	ND *
	S9YFYF	0.20	1.1×10^{-2}	ND *	ND *	ND *

*, Not detected after 50 cycles of amplification (less than 10^{-5}).

(c)

Expression of endogenous Siglecs in THP-1 cells
(% Siglec mRNA/GAPDH mRNA)

Cell		CD33	Siglec-5	Siglec-7	Siglec-9
THP-1	Control	0.28	3.9×10^{-4}	5.4×10^{-2}	ND *
	Siglec-9	0.46	7.7×10^{-4}	3.1×10^{-2}	1.1 **
	S9YFYF	0.37	4.2×10^{-4}	6.8×10^{-2}	0.98 **

*, Not detected after 50 cycles of amplification (less than 10^{-5}).

**, $p < 0.01$ relative to control by paired *t*-test.

Chapter 4

Siglec-9 translocated to microdomain upon TLR stimulation of macrophages to IL-10 production by its sialic acid-binding activity

Introduction

Recent works have demonstrated that some microbial pathogens exploit cholesterol-enriched lipid microdomains as essential docking sites to enter host cells [1,2]. These microdomains, also known as lipid rafts, are localized regions with elevated cholesterol and glycosphingolipid content that can be found on the plasma- and endosomal-membrane of eukaryotic cells [3-5]. Furthermore, lipid rafts contain numerous sialic acids. In fact, GM1, sialic acid-containing ganglioside, is one of the widely used lipid raft marker. Accumulating evidences suggest that lipid rafts play a key role in signal transduction through clustering of a broad array of cell surface receptors in different cell types [4,6,7].

The Toll-like receptor (TLR) family plays an instructive role in innate immune responses against microbial pathogens, as well as the subsequent induction of adaptive immune responses [8,9]. TLR recognize specific molecular patterns found in a broad range of microbial pathogens such as bacteria and viruses, triggering inflammatory and antiviral responses and dendritic cell maturation, which result in the eradication of invading pathogens. During the pathogen recognition by TLRs, the importance of

lipid raft formation has been reported. For example, TLR2 on the apical surface of 16HBE and 1HAEo airway epithelial cells [10], TLR4 on MonoMac-6 monocytes [11], TLR7 on RAW264.7 macrophages [12] are resides in lipid rafts fraction.

In previous Chapter, we demonstrate that macrophages expressing Siglec-9 produce less pro-inflammatory cytokines, TNF-α, IL-6, and IFNβ, upon stimulation through TLRs. In contrast, production of immunosuppressive IL-10 was enhanced following stimulation via TLRs. Thus in this Chapter, we assessed whether Siglec-9 resides and modulates TLR signaling in the lipid rafts.

Materials and Methods

Reagents and antibodies

Peptideglycan (PGN) from *Staphylococcus aureus* was from Wako. Biotin-labelled B subunit of choleara toxin was purchased from Wako Pure Chemicals (Osaka, Japan). Cholesterol Oxidase form *Cellulomonas sp.* was from Sigma-aldrich (St Lois). Sialidase derived from *Streptococcus sp.* was from Seikagaku Corporation. Anti-human Siglec-9 antibody (goat polyclonal antibody; AF1139) was purchased from R&D systems. Mouse monoclonal antibody toward phospho-Tyr (PY-20; sc-508), and rabbit polyclonal antibody toward Flotillin-1 (H-104, sc-25506), CD46 (H-294, sc-9098), Src homology region 2 domain-containing phosphatase (SHP)-1 (C-19; sc-287), SHP-2 (C-18; sc-280), and TLR2 (H-175; sc-10739) were purchased from Sant Cruz Biotechnology (Sant Cruz, CA, USA). OptEIA set for IL-10 was from BD PharMingen.

Construction of plasmids

cDNA for human Siglec-9 (ResGen) was obtained from Invitrogen. A point mutation of Siglec-9 converting an arginine residue (Arg^{120}) to alanine for deactivation of sialic acid recognition was obtained by PCR using (5'-GGGGAGATACTTCTTTGCTATGGAGAAAGGAAG-3') and (5'-CTTCCTTTCTCCATAGCAAAGAAGTATCTCCCC-3') as a set of primers. Point mutations converting tyrosine residues ($Tyr^{433, 456}$) to phenylalanine for deletion of tyrosine-based signaling motifs were also obtained by PCR using sets of primers described previously (Chapter 3). Then, the PCR products were cloned into the

pcDNA4/TO/*myc*-His A vector (Invitrogen). Individual clones were verified and purified with QIAfilter™ Midi Cartridge (QIAGEN Sciences, USA).

Cells and cultures

RAW264 macrophage cell line was obtained form Riken BioResource Center (Tsukuba, Japan) and maintained in RPMI1640 containing 10% heat-inactivated fetal calf serum, 0.03% L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin G, and 100 μg/ml streptomycin. RAW264 cells were transfected with each plasmid using lipofection reagent LF2000 (Invitrogen) as manufacturer's recommendation and selected in the presence of 250 μg/ml Zeocin (Invitrogen). Clones were maintained with the presence of Zeocin, characterized by flowcytometric analysis and western blotting for the expression of Siglec-9. 293FT (Invitrogen) cells and CHO-K1 cells were maintained in DMEM and F12, respectively. Both media contain 10% heat-inactivated fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin.

Western blotting

Equal proportions of each sample were separated by SDS-PAGE and transferred onto a polyvinlydifluoride (PVDF) membrane, followed by incubation with 1.5% skimmed milk for blocking. The membrane was proved with the appropriate dilution of primary antibody for 2 h, followed by peroxidase-conjugated secondly antibody for 1 h at room temperature. The proteins were visualized with Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA) according to the manufacturer's instructions and analyzed with a Luminescent image analyzer LAS-3000 mini (FUJIFILM, Japan). The intensities of the detected bands were

determined by Science Lab 2001 Image Gauge Ver. 4.0 (FUJIFILM, Japan).

Immuno-precipitation

Sample cells were lysed by lysis buffer for 1 h on ice. The lysis buffer contains 1% Triton X-100, inhibitors (protease inhibitors; 20 μg/ml Aprotinin and 100 μg/ml Phenylmethylsulfonylfluoride (PMSF), phosphatase inhibitors; 1 mM Na_3VO_4, 10 μM Sodium molybdate, and 10 mM NaF) based on TNE buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA). Whole cells and nuclei were removed by centrifugation at 10,000 × *g* for 20 min. Two-microliter of Dynabeads® ProteinG (DYNAL BIOTECH, Norway) were added to the lysate and gently mixed for overnight at 4ºC for pre-clearing. Then Dynabeads® were removed by Dynal MPC® (DYNAL BIOTECH, Norway). The supernatant was gently mixed with 0.5 μg of antibody for 2 h at 4ºC. Four-microliter of new Dynabeads® ProteinG was added to the mixture and incubated 1 h at 4ºC. Then Dynabeads® were washed two times with lysis buffer and three times with TNE buffer. Proteins bound to the Dynabeads® were separated by SDS-PAGE and detected by western blotting.

Isolation of lipid rafts

RAW264 cells (2×10^8) were lysed in 1.5 ml of lysis buffer for 1 h on ice. Whole cells and nuclei were removed by centrifugation at 10,000 × *g* for 20 min. Total volume of the sample were adjusted to 1.75 ml by TNE buffer and mixed with an equal volume of 85% sucrose in TNE. The sample was placed in the bottom of a centrifuge tube, and overlaid with 6 ml of 30% sucrose and 3.5 ml of 5% sucrose in TNE buffer; total 13 ml. The sample was centrifuged at 217,000 × *g* for 18 h at 4ºC and

1ml-fractions were gently collected from the top of the gradient. Each fraction was washed and concentrated by centrifugation using Amicon® Ultra (MILLIPORE, USA). For the stimulation with PGN, cells were incubated with 50 μg/ml of PGN for 3-30 min at 37ºC.

Confocal microscopy and immuno-fluorescence studies

Cultured cells were fixed by 1% formaldehyde for 10 min at room temperature, and were washed with PBS. Primary antibodies containing medium were added for 1 h at room temperature followed by three 5-minute PBS washes. Then, fluorescence isothiocyanate (FITC)- or Rhodamine B isothiocyanate (RITC)-conjugated secondary antibodies were added for 1 h and cells were washed three times.

Bimolecular fluorescence complementation (BiFC) analysis

The BiFC fragments of YFP truncated at residue 155 (YN—N-terminal residues 1-154; and YC—C-terminal residues 155-238) were selected according to Hu and Kerppola [13]. The expression vectors for BiFC analysis was based on p3×FLAG-CMV™-14 expression vector (SIGMA, USA). Siglec-9 or mutated-Siglec-9 was cloned into p3×FLAG-CMV™-14 vector using *Hind*III site and YN using *Bam*HI site (p3×FLAG-CMV-14/S9-YN). Siglec-9 was amplified by PCR using (5'-CATAAGCTTATTATGGGGCTGCTGCTGCTGCTGCC-3') and (5'-CATAAGCTTTCTGTGGATCTTGATCTCCGAGT-3') as a set of primers. For YN cloning, (5'-CATGGATCCATGGTGAGCAAGGGCGAG-3') and (5'-CATGGATCCCATGATATAGACGTTGTGGCTGTTG-3') was used as a set of primers. Similarly, TLR2 was cloned into p3×FLAG-CMV™-14 vector using *Hind*III

site and YC using *Bam*HI site (p3×FLAG-CMV-14/TLR2-YC), using (5'-CATAAGCTTACCATGGGGCCACATACTTTGTGGATGGTGTG-3') and (5'-CATAAGCTTGGACTTTATCGCAGCTCTCAGAT-3') for TLR2 cloning, or (5'-CATGGATCCGCCGACAAGCAGAAGAACG-3') and (5'-CATGGATCCGTACAGCTCGTCCATGCCG-3') for YC cloning. The peptide sequence AAANSSIDLISVPVDSR, encoded between *Hind*III and *Bam*HI sites by the multiple cloning sites of the p3×FLAG-CMV™-14 vector, used as a linker in BiFC experiments as recommended by Kerppola. CHO-K1 cells were transfected with the expression vectors using lipofection reagent LF2000 (Invitrogen™), and 48 h after transfection, the equal level of expression of the fusion proteins were checked by western blotting using anti-FLAG® pAb (Product No.F7425, SIGMA, USA). For detection of fluorescence, 24 h after transfectants were seeded on 35 mm glass based dish (Glass 12∅; IWAKI, Japan) and 48 h after transfection cells were stimulated by 100 μg/ml PGN for 15 min at 37ºC. After stimulation, cells were fixed with 4% paraformaldehyde for 10 min at room temperature followed by two PBS washes. Cells were analyzed by fluorescence microscope BZ-8000 (KEYENCE, Japan).

Analysis of cellular activities

For stimulation experiments, cells (4×10^4) were cultured in microtiter plates in 100 μl of serum-free medium with 66 mU/ml of sialidase or 1 U/ml of cholesterol oxidase for 30 min, followed by the stimulation with LPS or PGN for 8 h in 10% of heat-inactivated fetal calf serum containing medium. The amount of IL-10 in the culture supernatant was measured by ELISA.

Results

IL-10 production by Siglec-9 is attenuated by sialidase pretreatment or lipid raft destruction by cholesterol oxidase in RAW264 cells

IL-10 production upon various TLR ligands is enhanced by Siglec-9, a sialic acid-binding lectin, as shown in Chapter 3. We therefore analyzed the effect of sialidase on the IL-10 production in RAW264 cells. Control or Siglec-9-expressing cells were treated by 40 or 160 mU/ml of sialidase for 30 min. Then, the cells were stimulated for 8 h with LPS for TLR4 and PGN for TLR2, and the amount of IL-10 in the culture supernatant was determined by ELISA. Figure 1a and b show the ligand-concentration dependence of IL-10 production. Although the production in control cells was not affected by these treatments, the amount of IL-10 production in cells expressing Siglec-9 was reduced by pretreatment of salidase in dose-dependent manner. This observation indicates that IL-10 production by Siglec-9 was dependent on the binding between sialic acid and Siglec-9.

We next assessed whether lipid raft destruction affects the production of IL-10 by Siglec-9 expression. We tested the effect of cholesterol oxidase, which converts cholesterol to cholestenone and, therefore, disorganizes the composition of the lipid microdomain. Preincubation of the cells with cholesterol oxidase attenuated the amounts of IL-10 production in Siglec-9-expressing cells (Fig. 1c). This result suggests that lipid rafts are important for the production of IL-10 by Siglec-9 in RAW264 cells.

Establishment of RAW264 cells expressing ligand binding mutant of Siglec-9

To assess the role(s) of the sialic acid-binding domain on the function of Siglec-9, we established the ligand binding mutant of Siglec-9 which lacks the conserved Arg in the sialic acid-binding site by substitution of Arg with Ala at amino acids 120 (S9RA), as described in Materials and Methods. We confirmed that S9RA was impossible to bind sialic acid using chicken erythrocytes (Fig. 2a). RAW264 cells were transfected with the S9RA-expressing vector, and expression of S9RA was verified by flowcytometry and Western blotting (Fig. 2b and c). The expression levels of Siglec-9 and S9YFYF were also confirmed similarly.

Siglec-9 resides in lipid rafts via its lectin activity

As shown in Figure 1c, IL-10 production by Siglec-9 was attenuated by lipid raft destruction. To assess whether Siglec-9 resides in lipid rafts, we next isolated lipid raft by sucrose-density ultra-centrifugation, as described in the Materials and Methods, from Siglec-9- or mutated Siglec-9-expressing RAW264 cells. As shown in Figure 3a, ganglioside GM1- and Flotillin-1 (lipid raft markers)-enriched, and CD46 (non lipid raft marker)-negative lipid raft were detected in fraction 3 to 5. These fractions were mixed and used as the lipid raft fraction in all experiments. Using this fraction, the Western blotting in Figure 3b indicated that Siglec-9 and S9YFYF were distributed in the lipid raft fraction, whereas S9RA was not detected. These results suggest that Siglec-9 resides in lipid rafts via its sialic acid-binding activity.

To further demonstrate the association of Siglec-9 with lipid rafts, we also

performed fluorescence microscopy analysis in RAW264 cells and analyzed the immunostaining of Siglec-9 before and after GM1 cross-linking induced by the cholera toxin subunit B (CTxB)-Biotin/Avidin-FITC. As seen in Figure 3c, in non-crosslinked conditions (Control) Siglec-9 and GM1 were evenly distributed, whereas the GM1-containig patches formed after cross-linking and Siglec-9 was highly enriched in the patches. These results are consistent with the biochemical data and further demonstrate the association of Siglec-9 with membrane lipid rafts.

Further accumulation of Siglec-9 in lipid rafts upon PGN stimulation

To determine whether lipid rafts are important for Siglec-9 function in TLR2 signaling, Siglec-9- and mutated Siglec-9 expressing cells were stimulated with PGN and subjected to sucrose density ultra-centrifugation to isolate lipid raft fraction. Western blotting in Figure 4a showed that TLR2 was concentrated in lipid raft after PGN stimulation, as previously reported [10]. Although presence of Siglec-9 in lipid rafts seemed to be constitutively (Fig. 3b), further accumulation in lipid raft was observed after PGN stimulation (Fig. 4a). Interestingly, phospharylated tyrosine of Siglec-9 and SHP-2 were increased especially in lipid raft fraction after PGN stimulation (Fig. 4a and b). Similarly, Siglec-9 was copatched with GM1 ganglioside, a lipid raft marker, after PGN stimulation in the immunostaining experiment (Fig. 4c). Taken together, these results demonstrate that PGN stimulation induced further localization of Siglec-9 to the raft in RAW264 cells.

We next examined time-course experiments in RAW264 cells. We stimulated the cells with PGN for 3 to 30 min, and then fractionated samples were blotted with

anti-TLR2 and anti-Siglec-9 antibodies. Figure 5 shows that in RAW264 cells, TLR2 and Siglec-9 were localized as similar time course into lipid rafts in 10 min after PGN stimulation. And within 30 min after PGN stimulation, TLR2 and Siglec-9 were gradually dissociated from the lipid raft fraction. These results suggested that time-course of association after PGN stimulation between lipid rafts and Siglec-9 or TLR2 had simultaneity. It is of note that tyrosine phosphorylation of Siglec-9 was also detected in the same time-course. Taken together, these results strongly suggest that Siglec-9 may modulate the TLR2 signaling in lipid raft.

Siglec-9 interacts with protein tyrosine phosphatases, but unlikely associates with TLR2

Since the simultaneous residence of Siglec-9 and TLR2 in lipid raft was observed, we next analyzed the interaction of Siglec-9 with TLR2. After 3 to 30 min stimulation with PGN, the cell lysates of Siglec-9- or mutated Siglec-9-expressing RAW264 cells were subjected to immunoprecipitation as described in the Materials and Methods. As shown in Figure 6a using anti-Siglec-9 antibody, Siglec-9 coprecipitated with tyrosine phosphatase SHP-1 and -2, however, TLR2 was not detected. We also detected tyrosine phosphorylation of Siglec-9 peaked at 3min after PGN stimulation. On the contrary, S9YFYF did not coprecipitated with these phosphatases, suggesting that SHP-1 and -2 may interact cytoplasmic tyrosine motifs of Siglec-9. Furthermore, we found that tyrosine phosphorylation of S9RA and interaction of SHP-1 and -2 to S9RA were only partial, compared with Siglec-9. These results indicate that not only cytoplasmic tyrosine motifs, but lectin activity of Siglec-9 is important for effective

tyrosine phosphorylation and interaction with tyrosine phosphatases.

We further assessed whether Siglec-9 interact with TLR2 using Bi-molecular fluorescence complementation (BiFC) analysis. The BiFC approach is based on the formation of a fluorescent complex when two fragments of a fluorescent protein, YFP, are brought together by an interaction between proteins fused to the YFP fragments. The approach can be used for the analysis of interactions between many types of proteins and does not require information about the structures of the interacting partners. Thus, we fused YN, N-terminal of YFP, to Siglec-9 (Siglec-9-YN) and YC, C-terminal of YFP, to TLR2 (TLR2-YC). CHO-K1 cells were transfected with Siglec-9-YN and TLR2-YC expression vectors, and fluorescence of YFP was detected after PGN stimulation (Fig. 6b). The intensity of the fluorescence shown in Figure 6c suggests that Siglec-9 and TLR2 interaction was increased after PGN stimulation, but the increase of fluorescence was not significant.

Dynamics on the membrane and phosphorylation of cytoplasmic tyrosine of Siglec-9 are attenuated by sialidase pretreatment

We next assessed the effects of sialidase treatment. As in Figure 5a and 6a, lipid raft localization and coprecipitation of Siglec-9 after sialidase pretreatment were evaluated using Siglec-9-expressing RAW264 cells. Western blotting of lipid raft and non raft fractions shown in Figure 7a suggested that the translocation of Siglec-9 to lipid raft after PGN stimulation was dramatically decreased by sialidase pretreatment. Similarly, tyrosine phosphorylation of Siglec-9 was not increased not only lipid raft fraction, but in the whole cell lysate (Fig. 7a and b) in sialidase-treated cells.

Furthermore, Siglec-9 coprecipitated with tyrosine phosphatase SHP-1 and -2, but the amounts of precipitation were partial (Fig.7b), like S9RA cells shown in Figure 6a, compared with sialidase non-treated cells (not shown). Taken together, these results suggest that sialidase attenuates Siglec-9 dynamics and recruitment of tyrosine phosphatases via its tyrosine phosphorylation after PGN stimulation.

Discussion

In this Chapter, biochemical fractionations of cellular membranes and fluorescent-microscope studies suggest that Siglec-9 reside in microdomains on RAW264 macrophage cells in unstimulated condition. Following PGN stimulation to TLR2, Siglec-9 and TLR2 are recruited to the potentially site of PGN ligation within the lipid rafts. The PGN signaling machinery concentrates in the small receptor islands, lipid rafts, to form a 'transducing' complex and regulates the PGN-mediated signaling. This result suggests that Siglec-9 is involved in the complex. This colocalization is indicated by both the molecular analysis and the microscopic observation of submicron level might be involved in the modulating activity of Siglec-9 toward the cytokine production by RAW264.

The significance of Siglec-clustering has been reported in previously [14]. This study demonstrated that cell surface cross-linking of Siglec-3 results in its tyrosine phosphorylation and recruitment of both SHP-1 and SHP-2 in THP-1 monocyte. Furthermore, this cross-linking induces rapid and transient tyrosine phosphorylation of Siglec-3, appeared within 1 min and decreased to basal levels within 10 min. These results are consistent with our results that Siglec-9 is concentrated in lipid rafts and its tyrosine(s) is phosphorylated within 3 min, and it is gradually dissociated from lipid raft within 30 min after PGN stimulation. Furthermore, the amount of SHP-2 in the lipid rafts is increased simultaneously. These results suggest that Siglec-clustering, probably in lipid rafts, has crucial role for its tyrosine phosphorylation and recruitment of SHP-1 and SHP-2.

As shown in Figure 4a and 5, SHP-2 translocates in lipid raft in Siglec-9-expressing

cells upon PGN stimulation. SHP-2 is reported that it is critical for IL-1-induced phosphorylation of PLCγ1 and thereby enhances IL-1-induced Ca^{2+} influx and ERK activation [15,16]. Thus, we examined whether Ca^{2+} influx is important for IL-10 production by Siglec-9 upon TLR stimulation. Addition of EGTA, one of the selective chelator for Ca^{2+}, reduced IL-10 production by Siglec-9 in dose dependen fashion (data not shown). These results support the hypothesis that SHP-2 recruited by Siglec-9 to lipid rafts may have role(s) for IL-10 production via enhancement of Ca^{2+} influx, but this possibility remains to be verified.

It is generally accepted that the phosphorylation of the tyrosine residue in ITIM leads to the recruitment of SH2-containing phosphatases including the polyinositol 5'-phosphate phosphatase SHIP as well as protein tyrosine phosphatases SHP-1 and SHP-2 [17]. SHIP is specifically expressed in hemopoietic cells and antagonizes the effects of PI3K by hydrolyzing the 5' phosphate of its product phosphatidylinositol-3,4,5-triphospate. Interestingly, SHIP becomes tyrosine phosphorylated upon LPS stimulation in RAW264.7 cells, and translocats to lipid rafts along with IRAK [18]. Since there are no published studies about the interaction between Sigelc and SHIP, we assessed whether Siglec-9 coprecipitate with SHIP, but no precipitation was detected (data not shown). However, it is certainly deserves further investigation about possibility that SHIP play a role in the regulation of cytokine production by Siglec.

Insight in the cell surface organization of sialic acid-binding lectins such as Siglec will contribute to the development of novel strategies of immune regulations by sialic acid.

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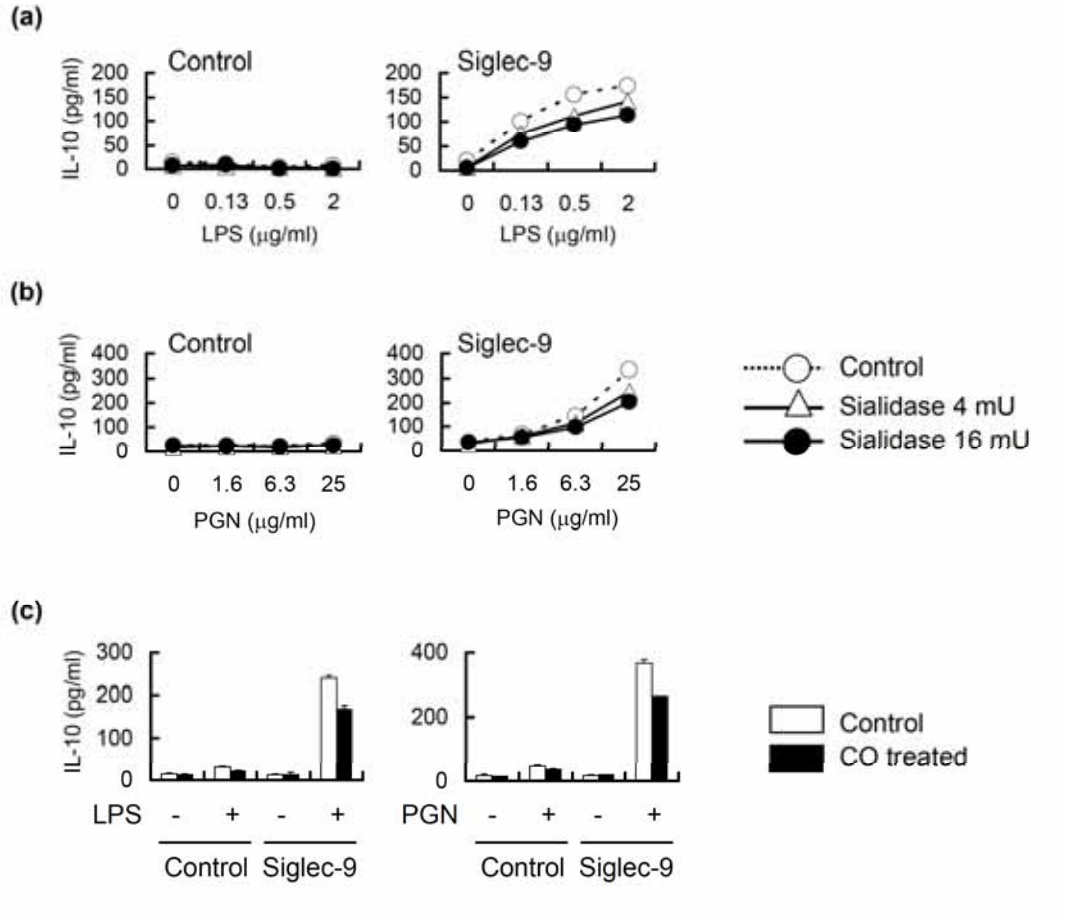


Figure 1. IL-10 production by Siglec-9 is attenuated by sialidase pretreatment or lipid raft destruction by cholesterol oxidase. (a, b) Control or Siglec-9-expressing RAW264 cells were treated by indicated concentration of sialidase in serum-free medium for 30 min, followed by the stimulation with LPS (a) or PGN (b) for 8 h. (c) Similarly, the cells were treated by 1 U/ml of cholesterol oxidase (CO) for 30 min in serum-free medium, and stimulated with 2 μg/ml of LPS or 25 μg/ml of PGN for 8 h. IL-10 in each supernatant was quantified by ELISA assay.

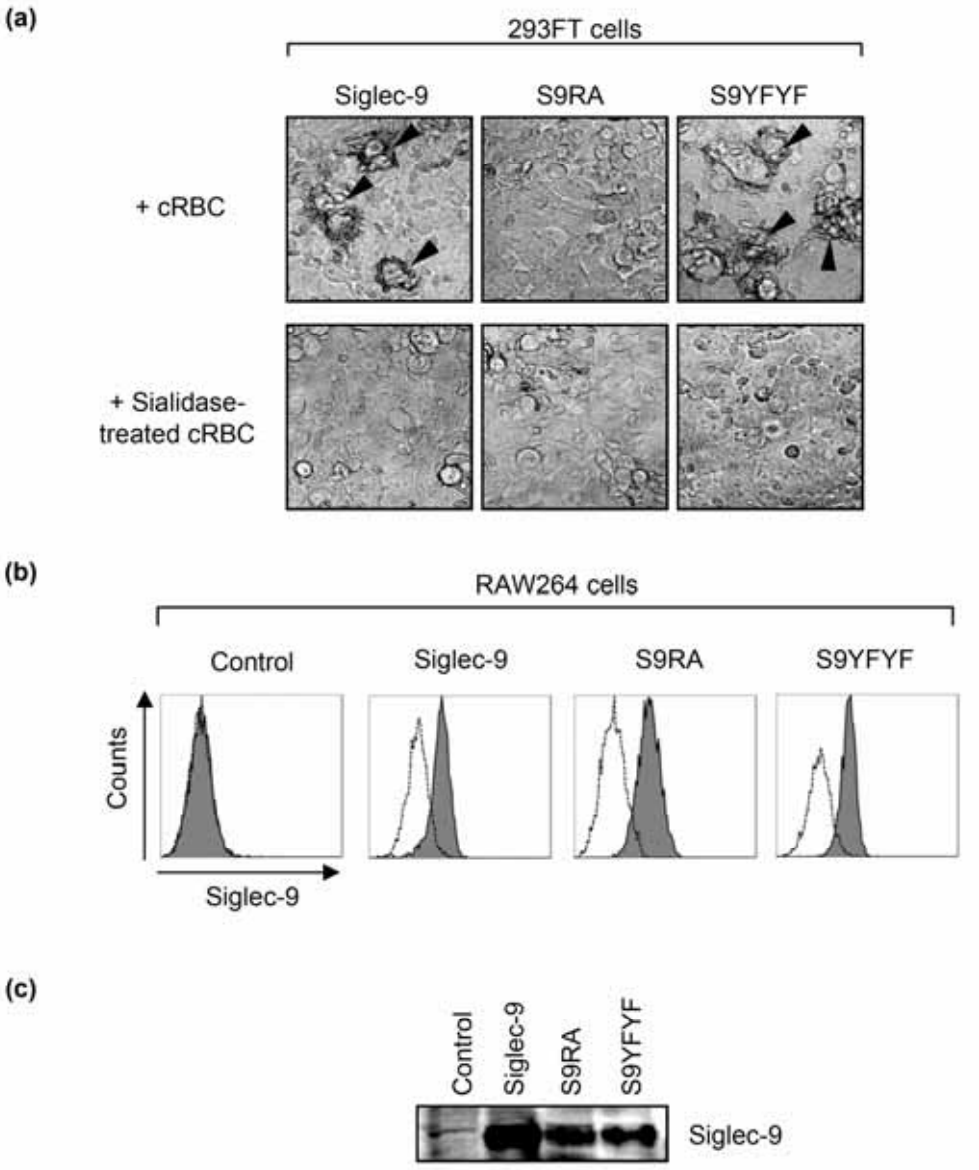


Figure 2. Stable expression of Siglec-9 and mutated-Siglec-9 in RAW264 cells. (a) Chicken erythrocytes (cRBCs) were rosetting by Siglec-9 and Siglec-9Y433FY456F (S9YFYF), but Siglec-9R120A (S9RA)-transfected 293FT cells (upper). However, no resetting was observed by using cRBCs treated by sialidase (lower). The rosettes of the erythrocytes are indicated by arrows. (b) RAW264 cells stably expressing Siglec-9, Siglec-9R120A, and Siglec-9Y433FY456F were prepared by transfection with the expression vector, and control cells were transfected with empty vector as described in Materials and Methods. Flowcytometry was used to evaluate surface expression of Siglec-9 or each mutated-Siglec-9 by using Siglec-9-specific polyclonal antibody and secondary FITC-conjugated antibody. (c) Whole cell lysates were analyzed by Western blotting.

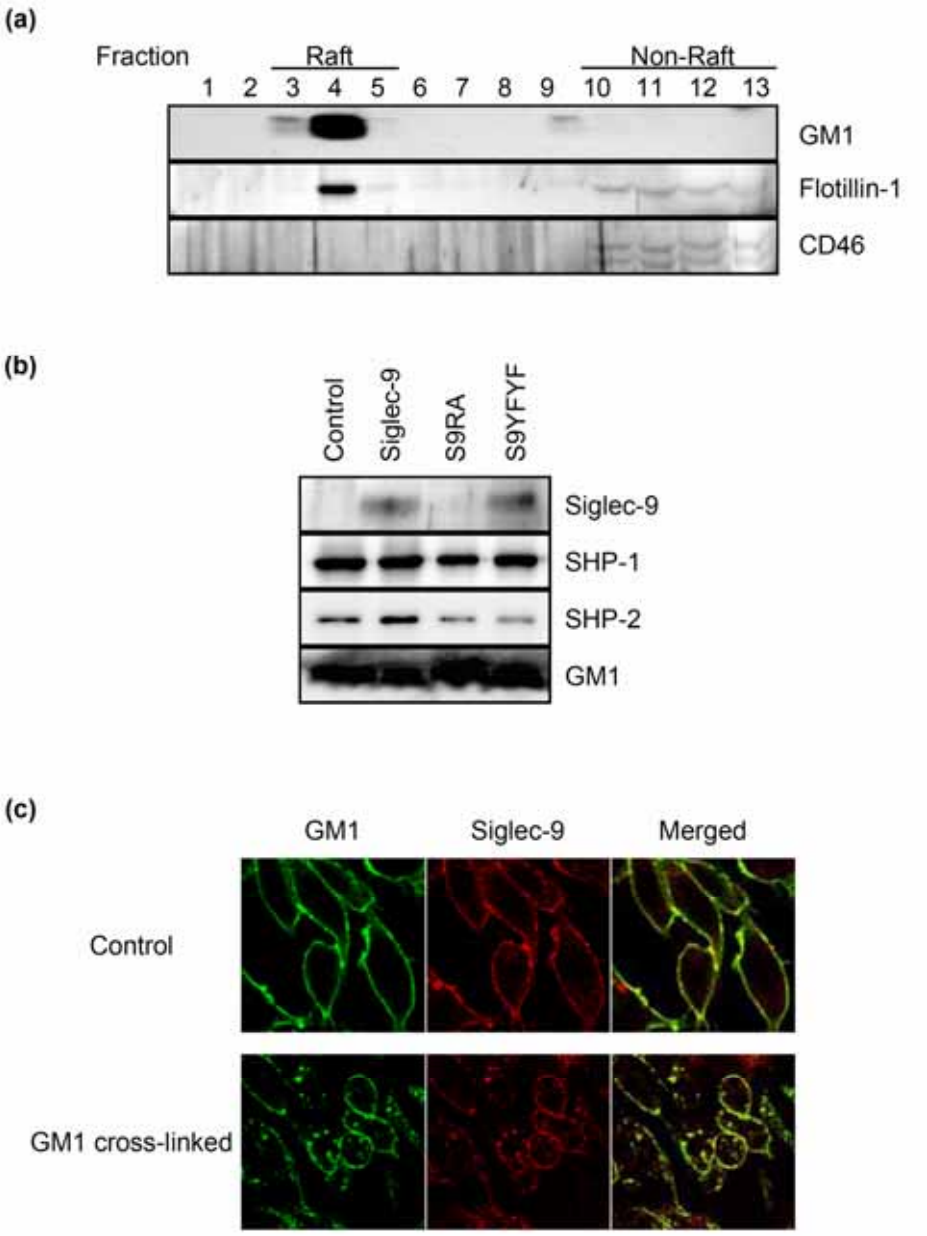


Figure 3. Siglec-9 is localized in lipid raft by its sialic acid-binding capacity. (a) RAW264 cells were lysed in 1% Triton X-100. After sucrose gradient ultra-centrifugation, fractions were collected from the top to the bottom and numbered 1 to 13. Each fraction was separated by SDS-PAGE and blotted with CTxB for GM1 detection, anti-Flotillin-1 antibody, and anti-CD46 antibody. GM1 and Flotillin-1 were used as lipid raft markers, and CD46 was used as a non-lipid raft marker. According to GM1, Flotillin-1, and CD46 distributions, fraction 3 to 5 and fraction 10 to 13 were corresponds to lipid raft and non-lipid raft fractions, respectively. (b) The lipid raft

samples from each clone were analyzed by Western blotting with indicated antibodies. (c) Siglec-9-expressing RAW264 cells were cross-linked by incubation with or with out CTxB-Biotin for 5 min, followed by avidin for 10 min. After fixation by 4% paraformaldehyde, Siglec-9 and GM1 were stained by anti-Siglec-9 antibody or CTxB, followed by each secondary RITC- or FITC-conjugated antibody, respectively. Fluorescence was analyzed by confocal microscopy.

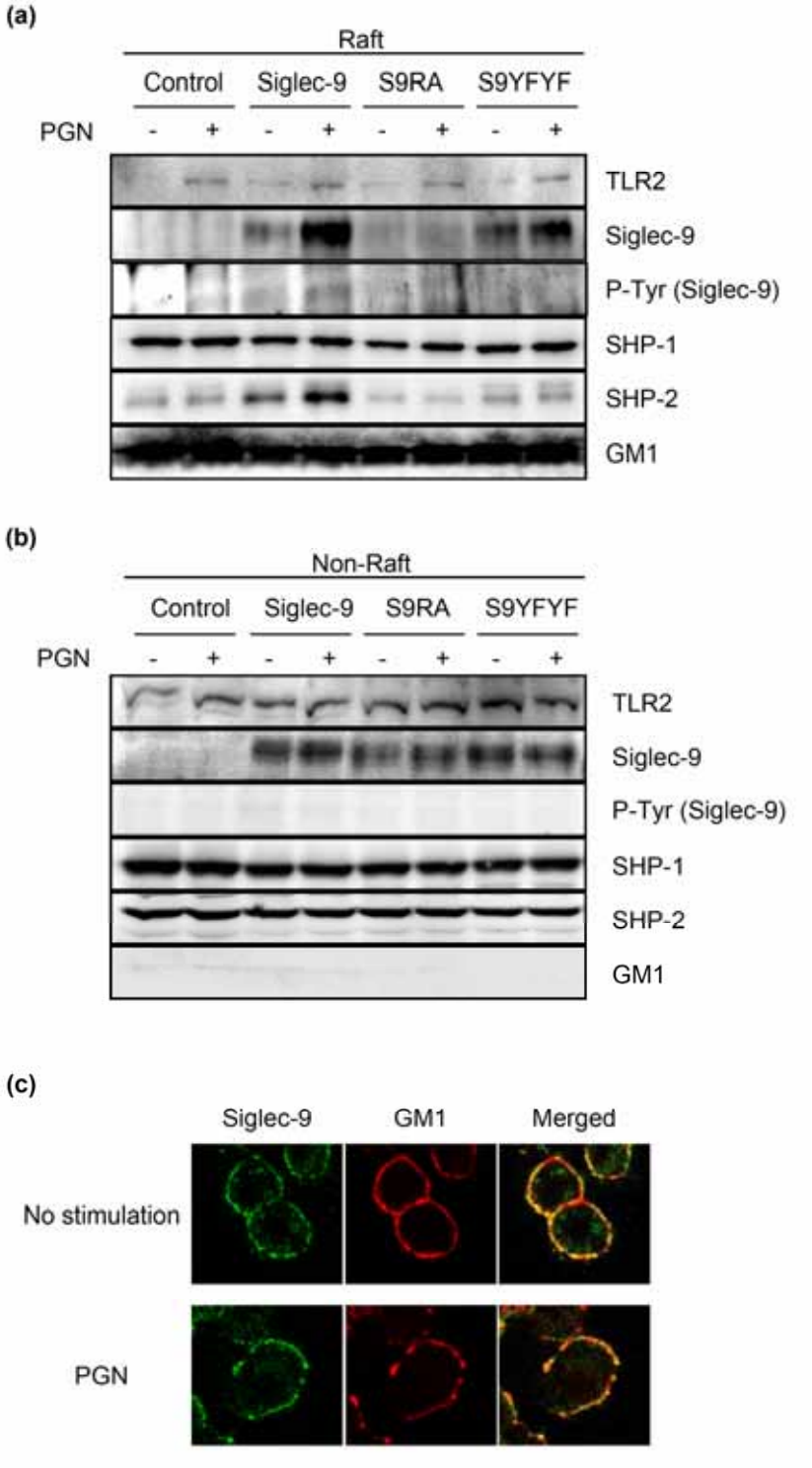
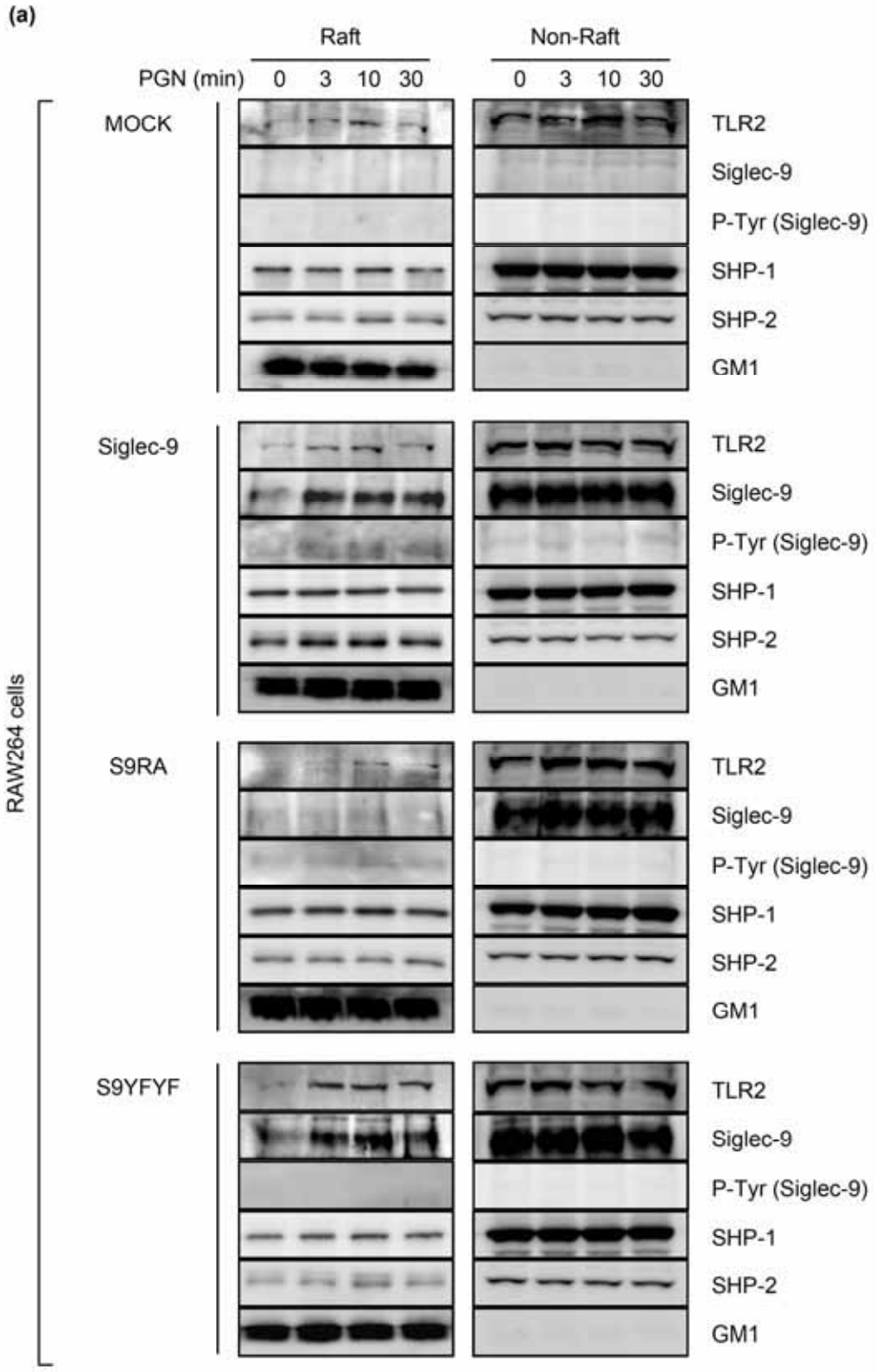


Figure 4. Siglec-9 is concentrated in lipid raft upon PGN stimulation. Siglec-9- or mutated Siglec-9-expressing RAW264 clones were incubated with or without 50

μg/ml of PGN for 10 min, and then lysed in 1% Triton X-100. After sucrose gradient ultra-centrifugation, lipid raft (a) and non-lipid raft (b) samples from each clone were analyzed by Western blotting with indicated antibodies. (c) Siglec-9-transfected RAW264 cells were stimulated by 50 μg/ml PGN for 10 min and fixed with 4% paraformaldehyde. Siglec-9 and GM1 were stained as Figure 3c. Fluorescence was analyzed by confocal microscopy.


(a)
Raft
Non-Raft
PGN (min)
0 3 10 30
0 3 10 30
RAW264 cells
MOCK
Siglec-9
S9RA
S9YFYF
TLR2
Siglec-9
P-Tyr (Siglec-9)
SHP-1
SHP-2
GM1

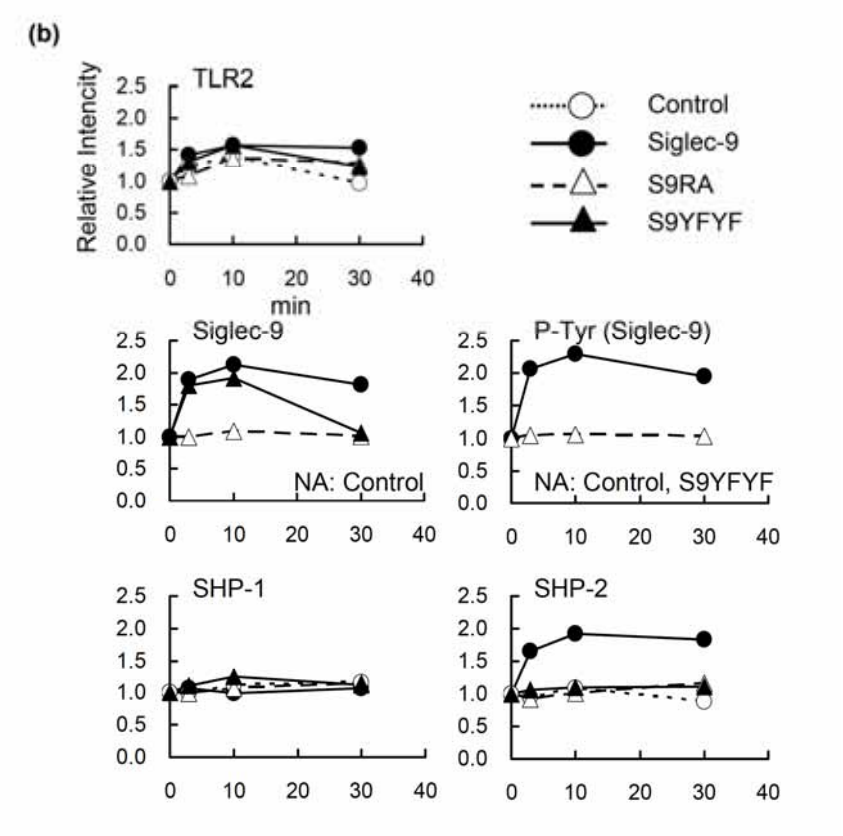


Figure 5. Dynamic localization of Siglec-9 with lipid raft in RAW264 cells upon PGN stimulation. (a) Siglec-9- or mutated Siglec-9-expressing RAW264 cells were stimulated with 50 μg/ml of PGN for 0, 3, 10, and 30 min, and then lipid raft fractions were isolated as Figure 3. Lipid raft and non-lipid raft samples were analyzed by Western blotting with indicated antibodies. (b) The band-intensities of Western blotting in each lipid raft samples were measured by Science Lab 2001 Image Gauge Ver. 4.0 (FUJIFILM, Japan). Relative intensities were shown on the average ratio in two or three independent experiments. NA: not applicable.

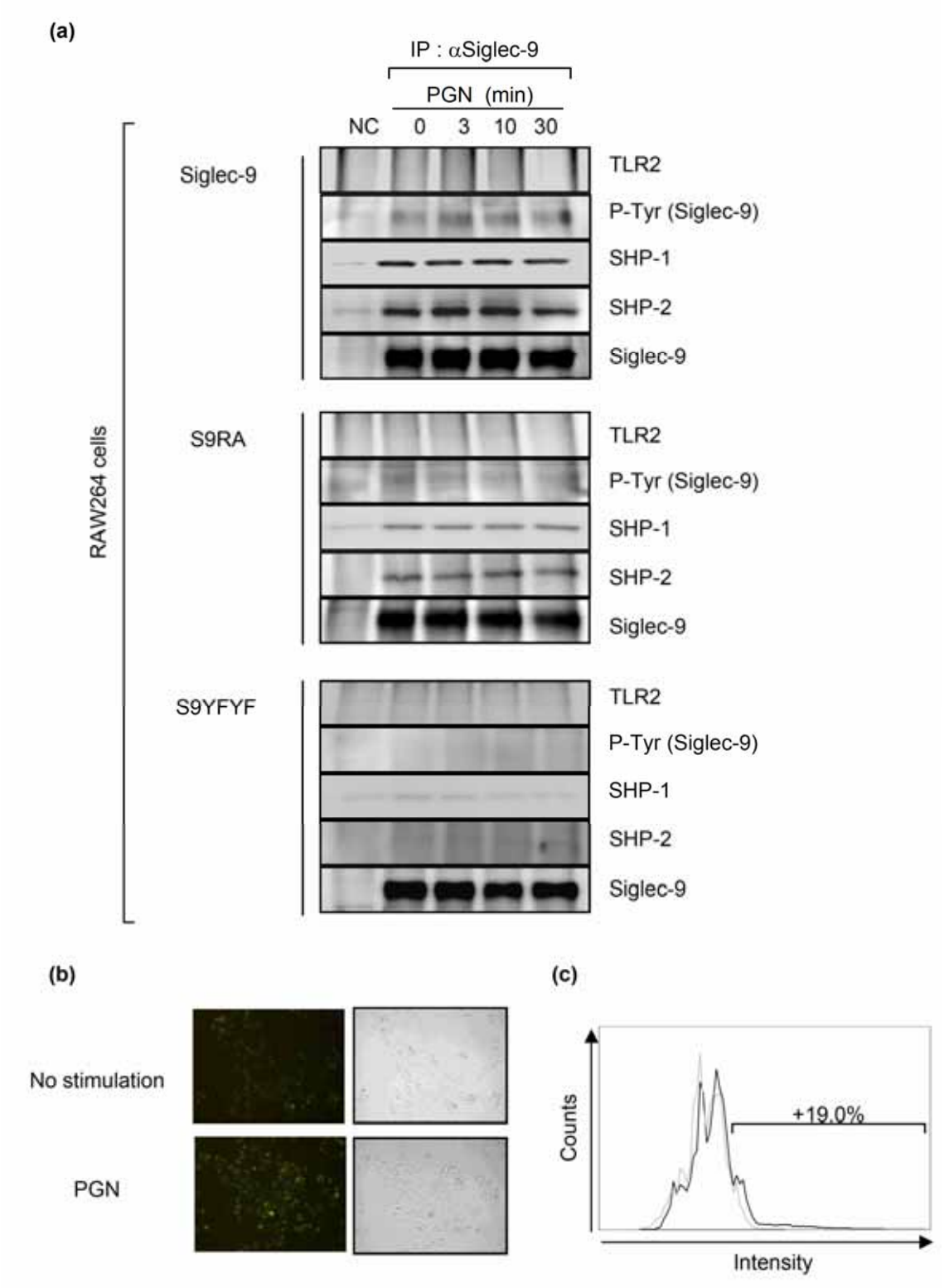


Figure 6. Siglec-9 interacts with protein tyrosine phosphatases, but unlikely associates with TLR2. (a) Siglec-9- or mutated Siglec-9-expressing RAW264 cells

were stimulated by 50 μg/ml PGN for the time indicated. Cells were lysed in 1% Triton X-100 and subjected to immuno-precipitation (IP) with anti-Siglec-9 mAb, followed by cross-link to the protein G on Dynabeads. Co-precipitated proteins were detected by indicated antibodies by Western blotting. (b) Interaction of Siglec-9 and TLR2 was detected by Bi-molecular fluorescence complementation (BiFC) analysis. The BiFC fragments of YFP, which truncated at residue 155, YN—N-terminal residues 1-154 and YC—C-terminal residues 155-238, were fused to C-terminal of Siglec-9 (Siglec-9-YN) and TLR2 (TLR2-YC), respectively. CHO-K1 cells were transfected with Siglec-9-YN and TLR2-YC expression vectors. For detection of fluorescence, 48 h after transfected cells were stimulated by 100 μg/ml PGN for 10 min at 37ºC. After stimulation, cells were fixed with 4% paraformaldehyde for 10 min. Fluorescence of the cells was analyzed by BZ-8000 (KEYENCE, Japan). (c) The fluorescent intensity of BiFC analysis was quantified by BZ-8000. The intensity of the cells stimulated with (black line) or without (gray line) PGN were shown in the histogram. The indicated number was % of the cells that increased the fluorescent intensity after PGN stimulation.

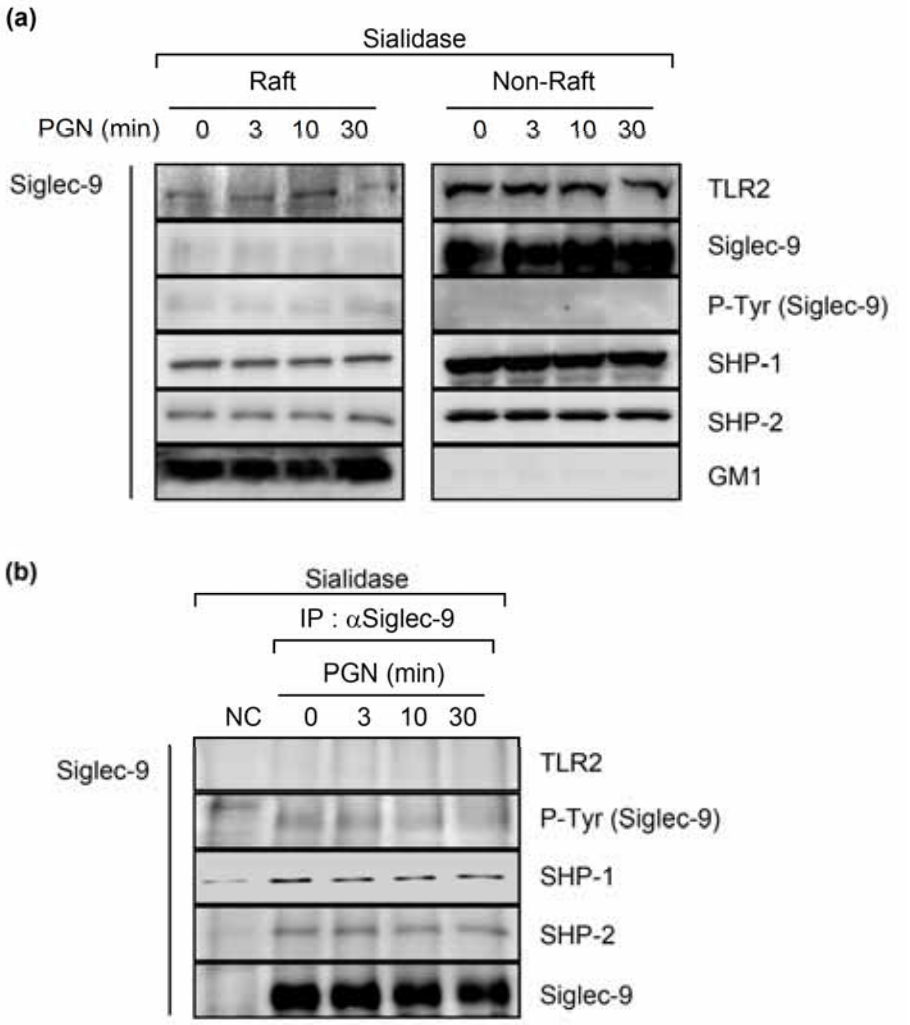


Figure 7. Dynamics on the membrane and phosphorylation of cytoplasmic tyrosine of Siglec-9 are attenuated by sialidase pretreatment. Siglec-9-expressing RAW264 cells were subjected to sialidase pretreatment for 1 h at 37 ºC followed by 50 μg/ml of PGN stimulation. (a) After sucrose gradient ultra-centrifugation, lipid raft and non-lipid raft samples from each clone were analyzed by Western blotting with indicated antibodies as Figure 5. (b) The cell lysates were subjected to IP with anti-Siglec-9 antibody, followed by cross-link to the protein G on Dynabeads. Co-precipitated proteins were detected by indicated antibodies by Western blotting as Figure 6.

Concluding Remarks

The conclusions obtained in each Chapter are summarized as follows:

Chapter 2: Costimulation of T cell proliferation by anti-L-selectin antibody is associated with the reduction of a cdk inhibitor p27

L-selectin, which binds 6-sulfo sialyl-Le^X, is one of the sialic acid binding lectin. In this Chapter, we show that anti-L-selectin antibody MEL-14 enhances proliferation of primary mouse unprimed-T cells with anti-CD3 antibody. An IL-2 independent signaling pathway involving phospho-ERK and the reduction of cdk inhibitor p27 was shown with quantitative analysis of soluble IL-2 and IL-2 receptor. The proliferation of T cell has crucial roles for effective immune responses. Therefore, the findings in this chapter suggest that sialic acid containing sugar-chain, 6-sulfo sialyl-Le^X, regulates inflammation via L-selectin.

Chapter 3: Siglec-9 enhances IL-10 production in macrophages via tyrosine-based motifs

Using the mouse macrophage cell line RAW264, we have shown in this Chapter that Siglec-9 enhances the production of anti-inflammatory cytokine IL-10, while it suppressed those of pro-inflammatory cytokines induced by various TLR ligands. Similar enhancement of IL-10 production is observed when the human monocytic cell line THP-1 is used, a finding that implies that the effect of Siglec-9 expression on the cytokine production is not limited to the RAW264 cell line. We also report that expression of Siglec-5, another Siglec expressed on myeloid cells, induced a similar

change of cytokine profile as Siglec-9 does. These results indicate that Siglec is the anti-inflammatory regulator on innate immune cells.

Chapter 4: Siglec-9 translocated to microdomain upon TLR stimulation of macrophages to IL-10 production by its sialic acid-binding activity

In this Chapter, we first show that the Siglec-9 expressing on the macrophage cell line RAW264 resides in lipid rafts using biochemical techniques. We also isolated lipid rafts before and after stimulation of PGN, the ligand of TLR2, and found that Siglec-9 is dynamically concentrated in lipid rafts after PGN stimulation. Furthermore, lipid rafts residence of Siglec-9 is abolished by the mutation in sialic acid binding domain, or attenuated by sialidase pretreatment to the cells. In addition, IL-10 production by Siglec-9 is depressed in sialidase- or cholesterol oxidase-, which disorganizes the lipid rafts, treated cells. These our findings suggest the importance of sialic acid-binding ability of Siglec-9 to reside in lipid rafts and to modulate cytokine production.

Finally, this paper contributes to provide new insights into immune regulation by sialic acid-containing sugar-chains via its binding lectins; L-selectin and Siglecs. However, the biological roles of them still remain unknown. Therefore, detailed investigations are necessary to further analysis: interaction of sialic acid-binding lectins with their counter-receptor(s), as well as to elucidate the resulting signals controlling their function(s). I hope that the study shown in this paper will act as a stimulus for further research that will help to elucidate not only the biological roles of sialic acid-binding lectins, but also the significance of sialic acid in immune regulation.

Publication List

Publications for this thesis

Munetoshi Ando, Wenjie Tu, Ken-ichi Nishijima, and Shinji Iijima

Siglec-9 enhances IL-10 production in macrophages via tyrosine-based motifs

(Submitting)

Munetoshi Ando, Ken-ichi Nishijima, Shusuke Sano, Aiko Hayashi-Ozawa, Yoshinori Kinoshita, and Shinji Iijima

Immune regulation by sialoglycoconjugate-binding lectins

Animal Cell Technology: Basic & Applied Aspects 14 (2006) 353-360

Ken-ichi Nishijima, **Munetoshi Ando**, Shusuke Sano, Aiko Hayashi-Ozawa, Yoshinori Kinoshita, and Shinji Iijima

Costimulation of T-cell proliferation by anti-L-selectin antibody is associated with the reduction of a cdk inhibitor p27

Immunology 116 (2005) 347-353

Other publications

Mahboob Morshed, Shusuke Sano, Daisuke Nishimiya, **Munetoshi Ando**, Ken-ichi Nishijima, and Shinji Iijima

Chicken ovalbumin promoter is demethylated upon expression in the regions

specifically involved in estrogen-responsiveness

Bioscience, Biotechnology, and Biochemistry 70 (2006) 1438-1446

Mahboob Morshed, **Munetoshi Ando**, Junko Yamamoto, Akitsu Hotta, Hidenori Kaneoka, Jun Kojima, Ken-ichi Nishijima, Masamichi Kamihira and Shinji Iijima

YY1 binds to regulatory element of chicken lysozyme and ovalbumin promoters

Cytotechnology 52 (2006) 159-170

Presentations at international conferences

Munetoshi Ando, Ken-ichi Nishijima, and Shinji Iijima

L-selectin regulates T cell proliferation as a costimulator

20th IUBMB International Congress of Biochemistry and Molecular Biology and 11th FAOBMB Congress, 3P-C-189, Kyoto, Japan, Jun. 2006

Ken-ichi Nishijima, **Munetoshi Ando**, Takeshi Hashimoto, and Shinji Iijima

Siglec-9 inhibits innate immunity

Sialoglycoscience 2006, Fifth International Conference, OP-1-6, Mishima, Japan, Aug. 2006

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