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Prohibitins function as endogenous ligands for Siglec-9 and negatively regulate TCR signaling upon ligation

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ABSTRACT

Previously we demonstrated that prohibitin-1 and -2 (prohibitins) were expressed on the surface of T cell leukemia cell lines and activated T lymphocytes. In the present study, we found that prohibitins play a role as counter receptors for Siglec-9 expressed on macrophages and dendritic cells. Siglec-9 bound to prohibitins in a sialic acid-independent manner. Mutated Siglec-9 with Arg^{120} changed to Ala lost the binding activity, suggesting a specific ionic peptide–peptide interaction. Phosphorylation of ERK1/2 in Jurkat cells on treatment with anti-CD3 antibody immobilized beads was markedly diminished on treatment with anti-CD3 antibody and Siglec-9 co-immobilized beads, indicating that engagement of prohibitins with Siglec-9 inhibits ERK1/2 phosphorylation. Phosphorylation of c-Raf was also reduced, maybe due to inhibition of the c-Raf-prohibitin interaction by Siglec-9 ligation. In parallel with inhibition of the ERK cascade, IL-2 production was markedly decreased in Jurkat cells. Thus, this interaction may be a useful immunotherapeutic target.

1. Introduction

Physiological interaction between T lymphocytes and antigen-presenting cells is required for an appropriate response to antigenic stimulation. Stimulating and inhibitory signals presented by dendritic cells during antigen presentation are integrated by T lymphocytes and determine the final outcome of T lymphocyte activation. Interactions of T lymphocytes with endogenous lectins such as selectins, Siglecs, and galectins regulate T lymphocyte functions such as trafficking, intracellular signaling and apoptosis [1]. Siglecs comprise a family of sialic acid-binding immunoglobulin-like lectins expressed on hematopoietic and immune cells. They mediate signaling as well as cell-to-cell interactions [2,3]. CD22 (Siglec-2), which is a member of the Siglec family expressed in B lymphocytes, was originally found to be a cell adhesion molecule that mediates cell-to-cell communication by binding to ligands in trans on other immune cells [4]. In mixed lymphocyte co-stimulatory assays, antibodies that inhibit CD22 binding to its ligand also dampened T cell activation [5]. In this context, we postulated that other Siglec family members expressed on antigen-presenting cells such as macrophages and dendritic cells might play similar roles in cell-to-cell interactions during the antigen presentation. Siglec-9 is expressed on the surface of macrophages and dendritic cells [6]. We found that soluble recombinant Siglec-9 could bind to T cell leukemia cell lines, and identified the Siglec-9 binding proteins as prohibitins (PHBs). Recent studies showed that PHBs are present in multiple cellular compartments and possess diverse functions [7]. Recently, we also found that PHBs are expressed on the surface of T cell leukemia cell lines and T lymphocytes stimulated with PMA/ionomycin [8]. In the present study, we found that Siglec-9 bound to PHBs in a sialic acid-independent manner, and co-ligation of CD3 and PHBs using anti-CD3 antibody and Siglec-9 co-immobilized beads resulted in a dramatic decrease in ERK1/2 phosphorylation, leading to reduction of IL-2 production in Jurkat cells. Modulation of PHB function by Siglec-9 ligation may promise to be a powerful option for manipulation of immune responses.

2. Materials and methods

2.1. Cells

T cell leukemia cell lines, Molt-3 and Jurkat cells, were obtained from ATCC, and cultured in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (Cell Culture Bioscience), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. CHO-KI cells were obtained from ATCC, and cultured in OPTI-MEM supplemented with 2% fetal calf serum (Cell Culture Bioscience), 50 IU/ml penicillin, and 50 μg/ml streptomycin.

2.2. Preparation of recombinant Siglec-9

FLAG-tagged soluble Siglec-9 (WT-hsSiglec-9-FLAG) was prepared as described previously [9]. Mutated Siglec-9 with Arg^{120}

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changed to Ala (RA) and V-set domain deleted Siglec-9 (ΔV) were prepared as follows. These mutated and deleted Siglec-9 expression vectors were constructed from p3xFLAG-sSiglec-9 by PCR using pairs of primers, 5′-GCTATGGAGAAAGGAAGTATAAAATGG-3′ and 5′-AAAGAAGTATCTCCCCGCATCAC-3′ (RA), 5′-TTGACCCA-CAGGCCCAAC-3′ and 5′-TGTCTGTCCTTCCGCCCTC-3′ (ΔV), followed by ligation of the PCR products. The resulting constructs were transfected into CHO-KI cells using FuGENE HD transfection reagent (Promega) and recombinant proteins were obtained from the conditioned medium. WT-, RA-, and ΔV-hsSiglec-9-agaroses were prepared by incubation of WT-, RA-, and ΔV-hsSiglec-9-FLAG with anti-FLAG-agarose (SIGMA) at 4 °C overnight, respectively.

2.3. Flow cytometry

Expression of Siglec-9 binding proteins on the surface of T cell leukemia cell lines was analyzed by flow cytometry using WT-hsSiglec-9-FLAG and FITC-conjugated mouse anti-FLAG antibody successively.

2.4. Preparation of Siglec-9-immobilized resin

FLAG-tagged Siglec-9-immobilized agarose (WT-hsSiglec-9-agarose) was prepared by incubation of WT-hsSiglec-9-FLAG (4 μg protein) with anti-FLAG-agarose, the amount of which is capable of binding to 6 μg FLAG peptide, at 4 °C for 3 h. RA- and ΔV-hsSiglec-9-agarose were prepared similarly. Fc-tagged Siglec-9-immobilized beads (Siglec-9-beads) were prepared by incubation of protein A-conjugated magnetic beads (protein A-beads, Millipore), the amounts of which are capable of binding to 6.25 μg rabbit IgG, with 5 μg Fc-tagged Siglec-9 (R&D) at 4 °C for 3 h.

2.5. Detection of Siglec-9 binding proteins on the surface of T cell leukemia cell lines

Cell surface proteins of T cell leukemia cell lines were labeled with biotin using EZ-Link sulfo-NHS biotin (Thermo Scientific) according to the manufacturer's instructions. After washing with PBS containing 0.1 M glycine, the cells were solubilized with lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, and 1% protease inhibitor cocktail (Nacalai Tesque)). WT-hsSiglec-9-agarose was added to the cell lysate (1 mg proteins), followed by stirring at 4 °C overnight. After washing with the lysis buffer, the precipitated proteins were subjected to SDS–PAGE and Western blotting. After blocking with PBS containing 5% BSA, the membranes were treated with streptavidin-HRP (Invitrogen). The bands were visualized using an ECL system (GE Healthcare).

2.6. Immunochemical identification of Siglec-9 binding proteins

Molt-3 cells were solubilized with the lysis buffer. WT-hsSiglec-9-agarose and Siglec-9-beads were added to the cell lysates (100 μg protein), followed by stirring at 4 °C overnight. After washing with lysis buffer, the precipitated proteins were subjected to SDS–PAGE and Western blotting. After blocking with PBS containing 5% BSA, the membranes were treated with mouse anti-PHB1 antibody (Neo Markers, clone II-14-10) and rabbit anti-PHB2 antibody (BETHYL), and successively with HRP-conjugated anti-mouse IgG antibody and HRP-conjugated anti-rabbit IgG antibody (Invitrogen), respectively. The bands were visualized as described above.

2.7. Treatment of Molt-3 cells with neuraminidase or sodium metaperiodate

Molt-3 cells (5×10^6 cells) were treated with 200 mU/ml neuraminidase (*Arthrobacter ureafaciens*, nacalai tesque) at 37 °C for 1 h or with 2 mM $NaIO_4$ on ice for 30 min in PBS.

2.8. Binding of mutated and deleted Siglec-9 to PHBs

Lysates of Molt-3 cells were incubated with WT-hsSiglec-9-agarose, RA-hsSiglec-9-agarose, or ΔV-hsSiglec-9-agarose, and stirred at 4 °C overnight. A control experiment was performed using anti-FLAG-agarose. Detection of PHBs was performed as described above, and the FLAG-tag was also detected with HRP-conjugated anti-FLAG antibody.

2.9. Preparation of artificial antigen-presenting beads

Siglec-9 and/or anti-CD3 antibody-immobilized beads were prepared as follows. Anti-CD3 antibody-immobilized beads (anti-CD3-beads) and Siglec-9-immobilized beads (Siglec-9-beads) were prepared by incubation of protein A-beads, the amounts of which are capable of binding to 6.25 μg rabbit IgG, with mouse anti-human CD3 antibody (0.5 μg protein, Millipore, clone UCHT1) and with Fc-tagged Siglec-9 (1 μg protein, R&D) at 4 °C for 3 h, respectively. Fc-tagged Siglec-9 and anti-CD3 antibody co-immobilized beads (Siglec-9-anti-CD3-beads) were prepared by incubation of the same amounts of protein A-beads as described above with Fc-tagged Siglec-9 (1 μg protein) and anti-CD3 antibody (0.5 μg protein) at 4 °C for 3 h. Throughout the experiments using Siglec-9 and/or anti-CD3 antibody-immobilized beads, T cells were treated with constant amounts of anti-CD3 antibody-immobilized beads. This amount of anti-CD3 antibody is enough for maximum stimulation of Jurkat cells (5×10^4 cells) based on the level of ERK1/2 phosphorylation.

2.10. Treatment of Jurkat cells with anti-CD3 antibody and Siglec-9

Jurkat cells (5×10^4 cells) were treated with anti-CD3-beads to induce TCR signaling. When the effects of Siglec-9 were examined, Jurkat cells were treated with anti-CD3 antibody and Siglec-9 under three different conditions using Siglec-9-anti-CD3-beads, Siglec-9-beads and anti-CD3-beads, or soluble Siglec-9 and anti-CD3-beads, respectively. Jurkat cells (5×10^4 cells) were treated with anti-CD3-beads, or Siglec-9-anti-CD3-beads as described above at 37 °C for 3–60 min. The cell lysates were subjected to SDS–PAGE and Western blotting. After blocking with PBS containing 5% BSA, the membranes were treated with mouse anti-phosphorylated ERK1/2 antibody (CST) and rabbit anti-ERK1/2 antibody (CST), followed by detection with HRP-conjugated anti-mouse IgG antibody and HRP-conjugated anti-rabbit IgG antibody (Invitrogen), respectively. The bands were visualized as described above and their intensities were determined with Image J software.

2.11. Transfection of siRNAs

To reduce PHB1 expression by RNA interference, Jurkat cells (2×10^5 cells) were cultured in 6-well plates and siRNA specific for PHB1 or luciferase (control) was transfected using Lipofectamine RNAiMAX (Invitrogen). The following siRNAs were used in this study, (1) luciferase specific siRNA: 5′-AACUUACGCUGAGUA-CUUCGA-3′ and 5′-UCGAAGUACUCAGCGUAAGUU-3′, (2) PHB1 specific siRNA mixture: 5′-CUUCAGAUCUCUAAUUAGU-3′ and 5′-ACUAAUUAGAGAUCUGAAG-3′, 5′-GAGCACAGGUGUCUGCCUA-3′ and 5′-UAGGCAGACACCUGUGCUC-3′, 5′-CAGGUGAGCGACGACC UUA-3′ and 5′-UAAGGUCGUCGCUCACCUG-3′.

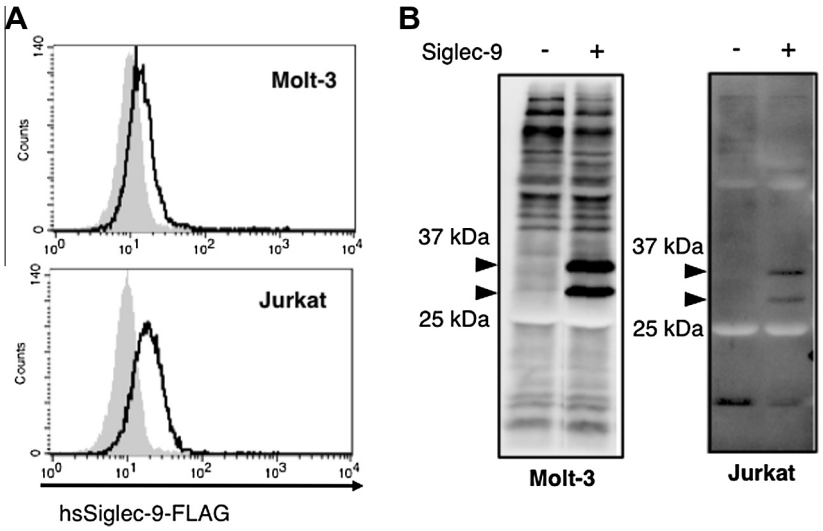

Fig. 1. Analysis of Siglec-9 binding proteins on the surface of T cell leukemia cell lines. (A) T cell leukemia cell lines, Molt-3 and Jurkat cells, were incubated with WT-hsSiglec-9-FLAG (solid line) or without WT-hsSiglec-9-FLAG (control, filled histogram), successively with FITC conjugated anti-FLAG antibody, and analyzed by flow cytometry. (B) Cell surface proteins on Molt-3 cells were labeled with biotin as described under Section 2. Anti-FLAG-agarose (Siglec-9: −) or WT-hsSiglec-9-agarose (Siglec-9: +) was added to the cell lysates, and precipitated proteins were analyzed as described under Section 2.

After 3 days, the cells were used for analysis of ERK signaling as described above.

2.12. Measurement of cytokine level

Jurkat cells (1×10^5 cells) were cultured in the presence of anti-CD3-beads or Siglec-9-anti-CD3-beads at 37 °C for 48 h and the level of IL-2 in the conditioned medium was determined using a Human IL-2 ELISA Kit (eBioscience) according to the manufacturer's instructions.

2.13. Statistical analysis

Student's *t* test was used to determine the significance of differences between sample means, $p < 0.05$ being considered significant.

3. Results and discussion

3.1. Identification of Siglec-9 binding protein on the surface of T cell leukemia cell lines

First, we examined the expression of Siglec-9 binding proteins on the surface of T cell leukemia cell lines, Molt-3 and Jurkat cells, using FLAG-tagged recombinant Siglec-9. Because Siglec-9 is expressed on the surface of macrophages and dendritic cells [6], and exhibits relatively wide sugar specificity, binding of Siglec-9 to the cell surface of T cell leukemia cell lines was examined by flow cytometry. Siglec-9 bound to the cell surface of these cells (Fig. 1A). To detect the cell surface binding protein for Siglec-9, cell surface proteins were labeled with biotin, and WT-hsSiglec-9-agarose was added to cell lysates of T cell leukemia cell lines. The precipitated proteins were subjected to SDS–PAGE, followed by Western blotting and detection of biotin-labeled proteins. Two proteins with molecular masses of about 28 and 31 kDa (arrow heads) were commonly detected (Fig. 1B). To identify these proteins, lysates of Molt-3 cells were subjected to affinity chromatography on WT-hsSiglec-9-agarose, and the eluted proteins were subjected to SDS–PAGE, followed by silver staining (Supplementary Fig. 1A). The protein bands corresponding to molecular weights of 28 and 31 kDa were cut from the gel, digested with trypsin, and subjected to PMF analysis (Supplementary Fig. 1B and Table 1). The analyzed set of peptide sequences coincided well with parts of the PHB1 and 2 protein sequences.

3.2. Binding of Siglec-9 to PHBs

Siglec-9 binding proteins were prepared using both Fc-tagged Siglec-9 and FLAG-tagged Siglec-9. The protein bands with molecular weights of 28 and 31 kDa were commonly detected, indicating that Siglec-9 could bind to PHBs irrespective of valency and tag form (Fig. 2A). Since PHB1 has two putative N-glycosylation sites [10], Siglec-9 may bind to the carbohydrate moiety of PHB1. To cleave sialic acid residues, Molt-3 cells were treated with neuraminidase or sodium metaperiodate as described under Materials and methods. It is generally known that binding of Siglecs to sialoglycans is abolished by neuraminidase or mild metaperiodate treatment [11]. WT-hsSiglec-9-agarose was added to the lysates, and the precipitated proteins were analyzed as described above. PHB1 and 2 were precipitated by WT-hsSiglec-9-agarose even after treatment with neuraminidase or sodium metaperiodate, indicating that sialic acid is not responsible for the interaction between Siglec-9 and PHBs (Fig. 2B). It has been reported that Siglec-5, -6, and -9 bind to cell wall-anchored β protein [12], leptin [13], and vascular adhesion protein-1 [14], respectively, in a sialic acid-independent manner. Next, we prepared mutated and deleted forms of recombinant Siglec-9. RA-hsSiglec-9 is a mutated recombinant Siglec-9 with Arg^{120} changed to Ala, which is an essential amino acid for the interaction with sialic acid [11]. ΔV-hsSiglec-9 is a recombinant Siglec-9 with the V-set domain deleted, this domain being an essential portion including Arg^{120} for the binding to sialoglycans. WT-, RA-, or ΔV-hsSiglec-9-agarose was added to the lysates of Molt-3 cells, and the precipitated proteins were analyzed as described above. Unexpectedly, the binding activities of RA- and ΔV-hsSiglec-9 were completely abolished (Fig. 2C, lanes (c) and (d)), indicating that the Arg^{120} residue and V-set domain were essential for the binding as well as that of Siglec-9 to sialoglycans. It is speculated that the anionic charge in the peptide portion of PHBs may be essential for the interaction with the Arg residue of Siglec-9. The molecular surface of PHB1 presents a mixed arrangement of electropositive and electronegative patches [15]. These electronegative

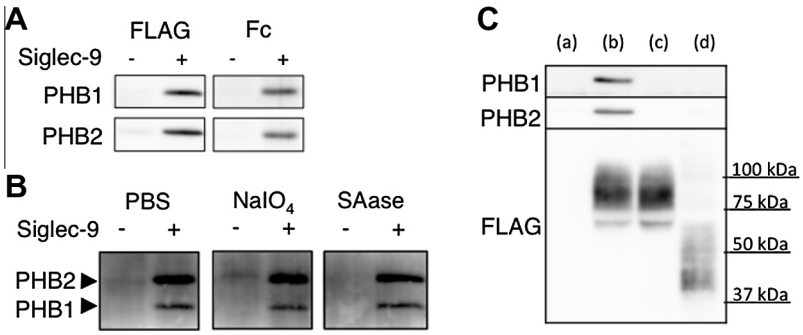

Fig. 2. Characteristics of the interaction between Siglec-9 and PHBs. (A) Siglec-9-binding proteins were precipitated from lysates of Molt-3 cells using WT-hsSiglec-9-agrose or Siglec-9-beads. The precipitates were subjected to SDS–PAGE and Western blotting, followed by detection of PHB1 and 2. (B) Molt-3 cells were treated with neuraminidase (SAase) or $NaIO_4$ as described under Section 2. Cell surface proteins on Molt-3 cells were labeled with biotin, and WT-hsSiglec-9-agarose was added to the cell lysates. Siglec-9 binding proteins were analyzed as described under Section 2. (C) Siglec-9 binding proteins were precipitated from lysates of Molt-3 cells using anti-FLAG-agarose (a), WT-hsSiglec-9-agarose (b), RA-hsSiglec-9-agarose (c), and ΔV-hsSiglec-9-agarose (d). The precipitates were subjected to SDS–PAGE and Western blotting, followed by detection with anti-PHB1, 2, and anti-FLAG antibodies.

patches may be responsible for the binding to the Arg residue of Siglec-9.

3.3. Down-modulation of TCR signaling by interaction of Siglec-9 with PHBs in Jurkat cells

Previously, we demonstrated that PHB1 and 2 are expressed on the surface of Jurkat cells and their distribution was not uniform, but patchy on the cell surface, and that PHB1 and 2 were completely co-localized with CD3 [8], suggesting that PHBs are located in the lipid rafts including CD3-TCR complexes, and relevant to TCR signaling. Since Jurkat cells possess TCR signaling system, we examined TCR-mediated signaling using Jurkat cells. The ERK family has been found to be required for stimulation of IL-2 transcription [16,17]. Therefore, we examined the phosphorylation of ERK1/2 by engagement of CD3 with anti-CD3 antibody immobilized artificial antigen-presenting beads (anti-CD3-beads). At 30 min after stimulation, cell lysates were subjected to SDS–PAGE, followed by Western blotting. Phosphorylated and total ERK1/2 were detected on a PVDF membrane. Phosphorylation of ERK1/2 was markedly elevated by treatment with anti-CD3-beads (Fig. 3A(a)). Next, we examined the effect of engagement of PHBs with Siglec-9 on the ERK1/2 phosphorylation. Generally, the effect of accessory molecules on TCR-mediated signaling seems to be dependent on their spatial distribution in relation to TCR. Thus, Jurkat cells were treated with anti-CD3 antibody and Siglec-9 under three different conditions, as follows. Jurkat cells were treated with Siglec-9-anti-CD3-beads (Fig. 3A(b)), Siglec-9-beads and anti-CD3-beads (Fig. 3A(c)), or anti-CD3-beads in the presence of soluble Siglec-9 (Fig. 3A(d)). Phosphorylation of ERK1/2 was examined as described above. A prominent inhibitory effect of Siglec-9 was detected only when the cells were treated with Siglec-9-anti-CD3 beads. A slight or negligible effect of Siglec-9 was detected under the other conditions. To determine whether or not the binding level of Siglecs as to PHBs are related with their inhibitory effect on ERK1/2 phosphorylation, similar experiments involving Siglec-5 and -7, which are also expressed in macrophages and dendritic cells, were performed. Although these Siglecs possess similar sugar binding specificity to that of Siglec-9, Siglec-5 and -7 bound slightly to PHBs (Supplementary Fig. 2A). In parallel with the binding level, these Siglec-beads co-immobilized with anti-CD3 antibody hardly inhibited the phosphorylation of ERK1/2 (Supplementary Fig. 2B) suggesting that the interaction of Siglecs with PHBs is essential to inhibit TCR signaling. Next, we investigated the time course of ERK1/2 phosphorylation after treatment with anti-CD3-beads or Siglec-9-anti-CD3-beads. Phosphorylation of ERK1/2 started at 10 min after stimulation with anti-CD3 antibody and peaked at 30 min, and was clearly inhibited by Siglec-9. At 30 min, about 80% and 70% of the phosphorylated ERK1 and ERK2, respectively, were reduced (Fig. 3B and C), indicating that interaction of Siglec-9 with PHBs may interfere with the ERK cascade. Rajalingam et al. demonstrated that PHB1 is required for Ras mediated Raf activation in epithelial cancer cells [18]. Therefore, we next examined the phosphorylation of c-Raf in Jurkat cells treated with anti-CD3-beads or Siglec-9-anti-CD3-beads as described above. Elevated phosphorylation of c-Raf was detected at 10 min after stimulation. Siglec-9 reduced the level of c-Raf phosphorylation to about 60% at 30 min compared with that in a control experiment (Supplementary Fig. 3A and B). Blocking of the ERK cascade is expected to lead in down-regulation of IL-2 production. Jurkat cells were cultured for 2 days in the presence of anti-CD3-beads or Siglec-9-anti-CD3-beads. The level of IL-2 in the conditioned medium was determined by ELISA. As expected, production of IL-2 was markedly inhibited by Siglec-9 (Fig. 3D).

3.4. TCR signaling in PHB1 down-modulated Jurkat cells

To further confirm the regulatory function of PHBs, we tried to reduce the expression of PHB1 in Jurkat cells by PHB1 specific siRNA treatment. Jurkat cells were treated with siRNA specific for PHB1 or luciferase (control) as described under Materials and methods. Expression of PHB1 was reduced to about 40% of that in control siRNA-treated cells (Fig. 4A). PHB1 or luciferase specific siRNA transfected Jurkat cells were treated with anti-CD3-beads or Siglec-9-anti-CD3-beads and phosphorylated ERK1/2 was detected as described above. The level of phosphorylated ERK1/2 was not reduced as much as that in the control cells by Siglec-9 ligation, which may be due to reduced expression of PHB1 (Fig. 4B and C). However, unexpectedly the level of phosphorylated ERK1/2 in PHB1 specific siRNA-transfected cells was similar to that in the control cells (Fig. 4B, lanes 1 and 4). ERK1/2 is phosphorylated in T lymphocytes by stimulation with anti-CD3 antibody before PHBs are expressed on the cell surface [19], indicating that ERK1/2 could be phosphorylated through TCR signaling without PHBs expressed on the cell surface. There is a possibility that B-Raf, which has been revealed not to bind to PHBs [18], may participate in the TCR signaling pathway before PHBs are expressed on the cell surface, maybe forming the Ras-B-Raf-MEK-ERK route. Rajalingam et al. proposed that PHB1 might play a role in the plasma membrane scaffold that ensures the Ras-c-Raf interaction [18], this being consistent with the report that there is functional redundancy between c-Raf and B-Raf [20]. Stomatin-

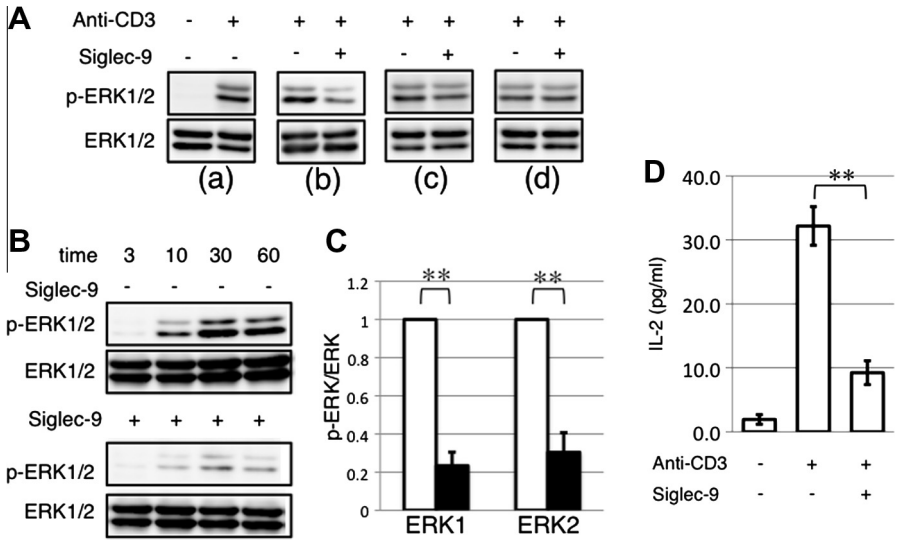

Fig. 3. Inhibition of TCR-mediated signaling and IL-2 production by Siglec-9. (A) Jurkat cells (5×10^4 cells) were stimulated with anti-CD3-beads at 37 °C for 30 min (a). The effect of Siglec-9 was examined as follows. Jurkat cells were incubated at 37 °C for 30 min in the presence of Siglec-9-anti-CD3-beads (b), Siglec-9-beads and anti-CD3-beads (c), or soluble Siglec-9 and anti-CD3-beads (d). Cell lysates were subjected to SDS–PAGE and Western blotting, followed by detection of phosphorylated ERK1/2 (p-ERK1/2) and ERK1/2. (B) Jurkat cells (5×10^4 cells) were treated with anti-CD3-beads (Siglec-9: −) or Siglec-9-anti-CD3-beads (Siglec-9: +) at 37 °C for 3–60 min. p-ERK1/2 and ERK1/2 were detected as described above and representative data are shown ($n = 3$). (C) The levels of p-ERK1/2 and ERK1/2 obtained from cells treated with anti-CD3-beads (control, opened bars) or Siglec-9-anti-CD3-beads (closed bars) as in B were compared. Histogram shows the relative intensities of p-ERK1/2/ERK1/2. The value for the control experiment was taken as 1. Data are expressed as means ± SD ($n = 3$, $^{**}p < 0.01$). (D) Jurkat cells (1×10^5 cells) were cultured in the presence of anti-CD3-beads or Siglec-9-anti-CD3-beads at 37 °C for 48 h, and then the level of IL-2 in conditioned medium was measured. Histogram shows the level of IL-2 in conditioned medium of Jurkat cells. Data are expressed as means ± SD, ($n = 4$, $^{**}p < 0.01$).

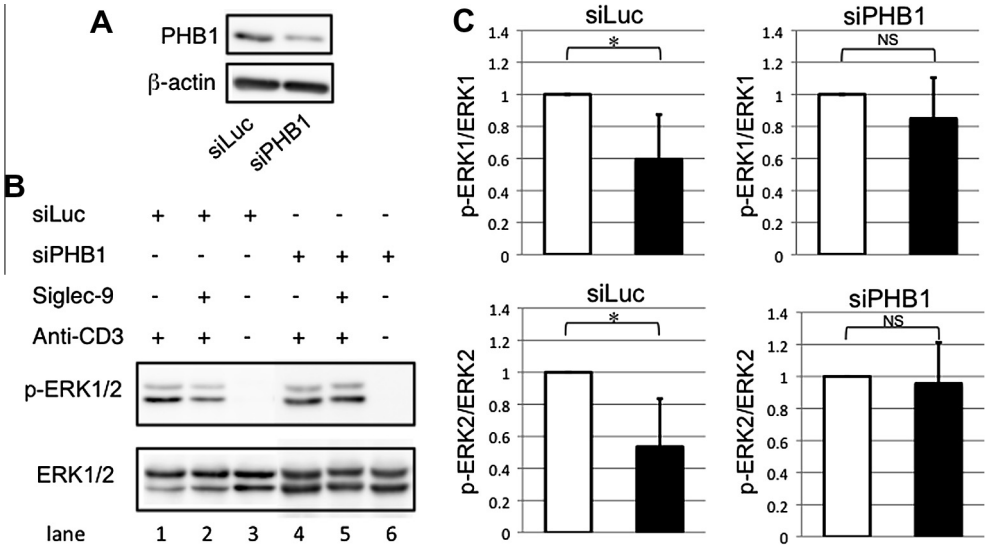

Fig. 4. Effect of PHB1 knockdown on Siglec-9 mediated inhibition of ERK1/2 phosphorylation. Jurkat cells were treated with siRNA specific for luciferase (control, siLuc cells) or PHB1 (siPHB1 cells) as described under Section 2. (A) Lysates of siLuc and siPHB1 cells were subjected to SDS–PAGE and Western blotting, followed by detection of PHB1 and β-actin. (B) siLuc and siPHB1 cells (5×10^4 cells) were treated with anti-CD3-beads or Siglec-9-anti-CD3-beads at 37 °C for 30 min, and phosphorylated ERK1/2 (p-ERK1/2) and ERK1/2 were detected as described under Section 2. Representative data are shown ($n = 4$). (C) The levels of p-ERK1/2 and ERK1/2 obtained from siLuc and siPHB cells treated with anti-CD3-beads (control, opened bars) or Siglec-9-anti-CD3-beads (closed bars) as in B were compared. Histogram shows the relative intensities of p-ERK1/2/ERK1/2. The value for the control experiment was taken as 1. Data are expressed as means ± SD ($n = 4$, $^{*}p < 0.05$, NS; not significant).

like protein-2, which is a member of the SPFH superfamily including PHBs and which constitutes a membrane-associated complex including PHBs at T cell immunological synapse, is an important player in T cell activation by ensuring sustained TCR signaling [21]. Thus, we speculate that after activation of T lymphocytes, PHBs may be incorporated into the TCR signaling pathway, forming the Ras-(PHB)-c-Raf-MEK-ERK route, and that PHBs may affect the signaling effectiveness. Therefore, silencing of PHB1 did not have a critical effect on TCR signaling. However, in Jurkat cells expressing PHBs on their cell surface, anti-PHB antibodies and Siglec-9 effectively inhibited anti-CD3 antibody-induced TCR signaling, resulting in suppression of ERK1/2 phosphorylation. Conformational change of PHB itself and/or PHB-containing membrane complex caused by ligation with anti-PHB antibodies and Siglec-9 may disturb the ordered arrangement of signaling molecules involved from Ras to ERK in the plasma membrane, maybe leading to significant inhibition of TCR signaling. These issues are currently under investigation.

IL-2 production is one of the most critical events of ERK-mediated T cell activation. IL-2 production is markedly reduced in parallel with the reduction of ERK1/2 phosphorylation by Siglec-9 treatment. We need to make sure that Siglec-9 expressed on the surface of antigen-presenting cells actually interacts with PHBs on the surface of T lymphocytes. A study along these lines is currently under investigation. Recently, we also reported that treatment of Jurkat cells with anti-CD3 antibody and anti-PHBs antibodies co-immobilized beads markedly reduced the phosphorylation of ERK1/2 [8]. Thus, the ability to modulate the activated T cell response by treatment with Siglec-9 or anti-PHB1 and 2 antibodies indicates that PHBs may be a potentially useful target for immune regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.085.

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